Norwegian University of Life Sciences
Faculty of Environmental Sciences
and Natural Resource Management

Philosophiae Doctor (PhD)
Thesis 2019:10

Silver nanomaterials in aquatic systems – linking uptake and effects in biota to exposure characterization

Sølvnanopartikler i akvatiske system – opptak og effekter i biota koblet til eksponeringskarakterisering

Merethe Kleiven

Silver nanomaterials in aquatic systems – linking uptake and effects in biota to exposure characterization

Sølvnanopartikler i akvatiske system – opptak og effekter i biota koblet til eksponeringskarakterisering

Philosophiae Doctor (PhD) Thesis

Merethe Kleiven

Norwegian University of Life Sciences
Faculty of Environmental Sciences and Natural Resource Management

Ås 2018

Thesis number 2019:10
ISSN 1894-6402
ISBN 978-82-575-1527-0

PhD SUPERVISORS

Professor Deborah H. Oughton
Faculty of Environmental Sciences and Natural Resource Management
Center for Environmental Radioactivity (CERAD)
Norwegian University of Life Sciences (NMBU)
P.O. Box 5003, N-1432 Ås, Norway
E-mail: deborah.oughton@nmbu.no

Professor Bjørn Olav Rosseland
Faculty of Environmental Sciences and Natural Resource Management, NMBU
Center for Environmental Radioactivity (CERAD)
Norwegian University of Life Sciences (NMBU)
P.O. Box 5003, N-1432 Ås, Norway
E-mail: bjorn.rosseland@nmbu.no

Dr. Hans-Christian Teien
Faculty of Environmental Sciences and Natural Resource Management, NMBU
Center for Environmental Radioactivity (CERAD)
Norwegian University of Life Sciences (NMBU)
P.O. Box 5003, N-1432 Ås, Norway
E-mail: hans-christian.teien@nmbu.no

Professor Knut Erik Tollefsen
Norwegian institute for water research (NIVA)
Gaustadalléen 21, NO-0349 Oslo, Norway

Faculty of Environmental Sciences and Natural Resource Management, NMBU
Norwegian University of Life Sciences (NMBU)
P.O. Box 5003, N-1432 Ås, Norway
E-mail: knut.erik.tollefsen@niva.no

Professor Brit Salbu
Faculty of Environmental Sciences and Natural Resource Management, NMBU
Center for Environmental Radioactivity (CERAD)
Norwegian University of Life Sciences (NMBU)
P.O. Box 5003, N-1432 Ås, Norway
E-mail: brit.salbu@nmbu.no

Senior scientist Erik Joner
Division of Environment and Natural resources
Norwegian Institute of Bioeconomy Research
P.O. Box 115, 1431 Ås, Norway
E-mail: erik.joner@nibio.no

EVALUATION COMMITTEE

Professor Richard Handy
University of Plymouth, School of Biological & Marine Sciences
Devon PL4 8AA, United Kingdom
E-mail: R.Handy@plymouth.ac.uk

Dr. Julia Farkas
SINTEF Ocean AS, Environment and New Resources
POBox 4762, Torgarden, 7465 Trondheim, Norway
E-mail: julia.farkas@sintef.no

Dr. Ole Christian Lind
Faculty of Environmental Sciences and Natural Resource Management, NMBU
P.O. Box 5003 NMBU, 1432 Ås, Norway
E-mail: ole-christian.lind@nmbu.no

ACKNOWLEDGEMENT

This PhD was mainly financed by a grant from the Norwegian University of Life Sciences (NMBU), with additional support from the Research Council of Norway through the projects NorNANoREG (grant number 239199) and NANOCHARM (grant number 221391). The research work was carried out at the facilities of NMBU, as well as the Norwegian Institute for Water Research (NIVA).

My supervisors during this PhD have been Prof. Deborah H. Oughton (NMBU, main supervisor), Prof. Bjørn Olav Rosseland (NMBU), Dr. Hans-Christian Teien (NMBU), Prof. II Knut Erik Tollefsen (NIVA, NMBU), Prof. Brit Salbu (NMBU) and Dr. Erik Joner (NIBIO). I would like to thank them all for their valuable contribution and support during this PhD research work, especially towards the end, critically reviewing my thesis. Thank you Bjørn Olav for always taking the time for a chat, your good humor and lovely Christmas dinners, Knut Erik for opening the doors to NIVA and all your feedback on papers and thesis, and Hans-Christian for our nice discussions – they were worth the wait outside your office ☺. Last but not least, special thanks to you Deborah for your support, constructive critic, positivity and endless patience and belief in your forever-lasting PhD student!

During these years as a PhD student I've been given the opportunity to work with many lovely people that has made this journey possible. Thank you to my colleagues at NMBU for your assistance during experiments, support and nine o'clock morning meetings, discussing everything from science and politics to the important topic of "Friday cake". Together with the love of my second child, I discovered my love for a good cup of coffee! Thank you my dear friends Yevgeniya, Lisa, Erica and Maria for our lovely coffee breaks, moral support and laughter, Claire, Dadou, Lisa and Erica for opening your homes and letting me stay with you from time to time this last year, it has been a joy and highly appreciated!

I also wish to extend my gratitude to co-authors, old and new colleagues and friends: Anicke, Lene, Cato, Anders, Marit, You, Pablo, Yet, Frøydis, Lene, Hilde, Karl-Andreas, Mirian, Dag, Julian and Ailbhe.

Finally, I want to thank my family: my love Ylva for her support, understanding and patience, and our lovely kids Eivor & Anker for putting things into perspective 💙

Merethe Kleiven, Ås, June 2018

CONTENTS

SUMMARY

The potential environmental impacts of engineered nanomaterials (ENMs) have received increased attention over the last decades. While the benefits of the development and use of ENMs are numerous (e.g., improved medical diagnostics, energy saving, improved environmental monitoring and remediation), there is also a risk of environmental release and potential negative effects to biota.

Due to the well-known antibacterial properties of silver, Ag ENMs are amongst the most frequently used ENMs on the market and can be found in, for example, medical applications (e.g., wound dressings, surface coatings of medical devices) and consumer products (e.g., cosmetics, cloths, cleaning agents, and food additives). Silver is known to be highly toxic to aquatic organisms, and the toxicity is usually ascribed to the dissolved species of Ag. The toxicity of Ag ENMs has been extensively studied, however, linking the observed toxicity to exposure characteristics is not always possible due the lack of exposure characterization. Given the tendency of ENMs to aggregate and be removed from the water column by sorption to organisms and sediments, which may in turn be taken up by sediment dwelling organisms, exposure routes to aquatic organisms can include both waterborne and dietary sources.

The overarching aim of this PhD research project has been to increase the understanding of the ways in which nanoparticle properties, and in turn their behaviour in toxicity testing media, influence accumulation and toxicity. To explore these issues, a range of experiments involving four different species (*Caenorhabditis elegans*, *Raphidocelis subcapitata*, *Salmo salar* and *Salmo trutta*) have been designed to test four interlinked hypothesis:

1. **Changes in Ag ion and Ag nanoparticle speciation will cause a time dependent change in the nanoparticle/colloidal fraction in test media exposure solutions.**
2. **Variation in the size fractions of Ag ion and Ag nanoparticles in test media will result in different bioavailability and bioaccumulation in test organisms.**
3. **Diet can be a significant route of silver uptake from Ag nanoparticles in fish.**
4. **Exposure to Ag nanoparticles can cause a nanospecific toxicity component.**

Experiments were carried out using $AgNO_3$ as well as a suite of nanomaterials (uncoated Ag NPs, citrate stabilized Ag NPs, a commercial nanosilver suspension Mesosilver, and standard reference materials NM300K, and NM302). The four species studied cover organisms used in standard toxicity tests (*C. elegans* and *R. subcapitata*), as well as environmentally and economically relevant species (*Salmo salar* and *Salmo trutta*).

Studies of the change in size distribution of both Ag ions and Ag NP in toxicity test media showed a change in size fractions, towards the larger particle sizes, with time in all waterborne exposures. Common for all $AgNO_3$ exposures were the higher concentrations of dissolved Ag species (<3 or 10 kDa) relative to the Ag NP exposures. For example, in the highest concentration of $AgNO_3$ in the exposure of *R. subcapitata*, the initial concentration of dissolved Ag was 24 µg Ag L^{-1} (98 % of total Ag concentrations), while for Mesosilver and NM300K Ag NPs the concentration the dissolved Ag fraction was 34 % (17 µg Ag L^{-1}) and 1 % (0.3 µg Ag L^{-1}), respectively. The aggregation continued, in all exposure suspensions, throughout the exposure period resulting in a decrease in the NP fraction (defined as > 3 kDa and < 220 nm) of between 13 and 98 % from T = 0 to the end of the exposure, in the Ag NP exposures. For $AgNO_3$, the picture was more complicated, with reductions in the dissolved fraction, combined with aggregation of colloids to larger particles, often leading to a transient increase in colloidal fraction.

For waterborne exposures, comparison of the size fractionation data with bioaccumulation in the different test organisms showed that Ag concentrations in both fish and *C. elegans* exposed to $AgNO_3$ were higher than after Ag NP exposures. This difference in accumulation of Ag could be correlated with the higher concentration of dissolved Ag species present in the $AgNO_3$ exposures relative to the Ag NP exposures. For NM300K exposures to fish, an absence of dissolved Ag species in exposure media resulted in a lack of systemic uptake of Ag.

In the dietary exposure of fish, both $AgNO_3$ and Ag NP exposures resulted in accumulation of Ag in liver. For two out of the three Ag NPs tested, the Ag concentrations in liver were similar to the levels after exposure to $AgNO_3$ (e.g., mean ± s.d; 1.2 ± 0.4 µg Ag/g dry weight and 1.9 ± 0.7 µg Ag/g dry weight, for NM300K and $AgNO_3$, respectively), although the Ag NP showed a much lower uptake than $AgNO_3$ from waterborne exposures. Thus, silver nanoparticles show a potential for dietary uptake and accumulation, however, no negative effects were detected in fish after dietary exposure.

Silver nitrate induced toxicity at lower exposure concentrations than any of the Ag NPs tested, across all organisms. The toxicity of the Ag species was in the order of $AgNO_3$≥ Mesosilver > NM300K > NM3002. The freshwater algae *R. subcapitata* being the most sensitive (the EC_{50} values for growth inhibition after 72 h exposure to $AgNO_3$ was 7.09 (95 % CI: 3.83-10.52) µg Ag L^{-1}), and the nematode *C. elegans* the least sensitive with EC values for 96 h growth one order of magnitude higher than for the algae.

The results provided two lines of evidence that the toxicity observed in the Mesosilver and NM300K Ag NPs exposures could not be explained by the presence of dissolved Ag species (<10

kDa) alone, but rather a nanospecific toxicity or a combination of the two. Comparison of growth inhibition to the dissolved fractions of ions in the NP exposures, showed that for both Mesosilver and NM300K, the growth inhibition was much larger than that seen for $AgNO_3$ groups with similar concentrations of dissolved Ag. Also, the general trend seen in algae growth inhibition over time (i.e., reduced effect on growth over time) was in line with the size fractionation results showing reduced concentrations of in the dissolved Ag and colloidal/NP Ag over time and an increased particulate matter >220 nm (Table 4).

To conclude, aggregation was the net dominant process, resulting in an decrease in NP (> 3 kDa and < 220 nm) and dissolved Ag fractions (< 3 or 10 kDa) and an increase in larger particulate matter (> 220 nm) with time. In the waterborne exposures accumulation, bioavailability and toxicity were linked to the presence of dissolved Ag species in the exposure. Since the results of the present research suggest that acute exposures to Ag NPs are not more toxic than $AgNO_3$, existing risk assessment criteria are unlikely to underestimate the environmental hazards of Ag NP. However, the evidence of an Ag NP specific component for algae toxicity, combined with the affinity of algae for absorption of Ag NP, means that care should be taken in extrapolating this conclusion to chronic exposures.

SAMMENDRAG

Menneskeskapte nanomaterialer (ENMs) har vært i søkelyset de siste tiårene på grunn av anvendelsen i industri, teknologi og ikke minst i forbrukerprodukter. Det er mange potensielle fordeler ved utvikling og bruk av ENMs (f.eks. bedret medisinsk diagnostikk, energisparing, forbedret miljøovervåkning og - sanering), men det er også en risiko for utslipp til miljøet og negative effekter i biota. Sølv (Ag) er kjent for sine antibakterielle egenskaper, og av denne grunn er Ag-nanopartikler (Ag NPs) blant de mest anvendte ENMs på markedet. Ag NPs anvendes blant annet innen medisin (f.eks. sårforbinding, overflatebehandling av medisinsk utstyr) og forbrukerprodukter (f.eks. kosmetikk, klær, rengjøringsmidler og tilsetningsstoffer i mat). Sølv er kjent for sin toksisitet overfor akvatiske organismer, og toksisiteten tilskrives vanligvis sølvioner (Ag(I)). Mange studier har tatt for seg opptak og toksisitet av Ag NPs, men relasjonen mellom observert toksisitet og eksponering er ikke alltid tydelig på grunn av manglende karakterisering av nanopartiklene og deres transformeringsprodukter. Grunnet nanopartiklers tendens til å forme aggregater og sorberes til sedimenter, noe som kan føre til opptak i bunndyr, er både vann og diett mulige eksponeringsruter.

Det overordnede målet i denne doktorgraden har vært økt forståelse av hvordan nanopartiklers iboende egenskaper, samt deres oppførsel i testløsning/medium, påvirker akkumulering og toksisitet i organismer. Dette ble undersøkt gjennom en rekke forsøk som involverte fire ulike arter (*Caenorhabditis elegans*, *Raphidocelis subcapitata*, *Salmo salar* (Atlantisk laks) og *Salmo trutta* (brunørret)) og ble designet til å teste fire sammenflettede hypoteser:

1. Endringer i spesiering av Ag tilstede i $AgNO_3$ og Ag NPs eksponeringene, vil føre til endringer i forekomsten av nanopartikulært/kolloidalt Ag (definert som > 3 eller 10 kDa og < 220 nm) i eksponeringene over tid.
2. Variasjon i forekomsten av løste Ag-komplekser (< 3 eller 10 kda) og nanopartikulært/kolloidalt Ag i test media vil resultere i forskjeller i biotilgjengelighet og akkumulering i testorganismene.
3. Diett kan være en signifikant kilde til opptak av sølv fra Ag NPs i fisk.
4. Eksponering til Ag NPs kan føre til en nanospesifikk toksisitet.

Disse hypotesene ble testet ved bruk av $AgNO_3$, samt en eller flere nanomaterialer (Ag NPs uten overflatebehandling, sitratstabilisert Ag NPs, en kommersiell nanosølvløsning (Mesosilver), og NM300K og NM302, som begge er Ag NPs standard referansematerialer). De fire artene som ble brukt i forsøk dekker organismer som er vanlige å bruke i standardiserte toksisitetstester (*C. elegans*, *R. subcapitata*), samt økologisk og økonomisk relevante arter *(S. salar* og *S. trutta*).

Resultatene viste en endring i størrelsesfraksjonene av Ag (som reflekterer en endring i spesiering) tilstede i testmedium over tid i alle eksponeringene, med en forskyvning mot større partikler (> 220 nm). Felles for alle $AgNO_3$ eksponeringene var høy konsentrasjon av løst Ag (< 3 eller 10 kDa) i forhold til i eksponeringene med Ag NPs. For eksempel i forsøket med *R. subcapitata*, var konsentrasjonen av løst Ag i begynnelsen av $AgNO_3$-eksponeringen 24 µg Ag L^{-1} (98 % av total Ag-konsentrasjon), mens den i eksponeringen med Ag NPs var henholdsvis 34 % (17 µg Ag L^{-1}) og 1 % (0.3 µg Ag L^{-1}) for Mesosilver og NM300K. Aggregering førte til en reduksjon på mellom 13 og 98 % av den nanopartikulære/kolloidale fraksjonen av Ag over tid i eksponeringsløsningene med Ag NPs. For $AgNO_3$ var bildet mer komplisert, med reduksjon i den løste fraksjonen av Ag, kombinert med aggregering av kolloider til større partikler, noe som i en overgangsfase ofte førte til en økt kolloidal fraksjon.

Ag-konsentrasjonen i fisk ved vannekspонering til $AgNO_3$ var høyere enn ved eksponering til Ag NPs. Denne forskjellen i bioakkumulering av Ag var korrelert med den høyere konsentrasjonen av løst Ag (< 3 eller 10 kDa) i $AgNO_3$-eksponeringen sammenlignet med Ag NPs-eksponeringen. Det mest ekstreme eksemplet var NM300K Ag NPs, hvor det ikke ble påvist systemisk bioakkumulering av Ag, noe som kunne kobles til fraværet av løst Ag i eksponeringsmediet.

Ved dietteksponering av fisk, førte både $AgNO_3$ og Ag NPs til akkumulering av Ag i lever. To av de tre Ag NPs som ble testet førte til akkumulering av Ag til samme nivåer som ved eksponering til $AgNO_3$ (f. eks., gjennomsnitt ± standard avvik; 1.2 ± 0.4 µg Ag/g tørrvekt og 1.9 ± 0.7 µg Ag/g tørrvekt for henholdsvis NM300K og $AgNO_3$). På tross av dokumentert opptak ble det ikke påvist toksisitet.

Toksisitet ble indusert av $AgNO_3$ ved lavere Ag-konsentrasjoner enn noen av de testede Ag NPs, uavhengig av organisme. Toksisiteten av de ulike formene for Ag kunne rangeres $AgNO_3$ ≥ Mesosilver > NM300K > NM302. Ferskvannsalgen *R. subcapitata* var den mest sensitive organismen (EC_{50}-verdier for veksthemning etter 72 t eksponering til $AgNO_3$ var 7.09 (95 % CI: 3.83-10.52) µg Ag L^{-1}), mens *C. elegans* var den minst sensitive med EC_{50}-verdier for 96 h vekst en størrelsesorden høyere enn for algen. I alle forsøkene, uavhengig av organisme, ble det observert en endring i størrelsesfraksjonene av Ag over tid. Aggregering var den netto dominerende prosessen, noe som resulterte i reduksjon i både nanopartikulært/kolloidalt (> 3/10 kDa og < 220 nm) og løst (< 3/10 kDa) Ag, samt en økning i større Ag-partikler (> 220 nm) over tid. Akkumulering, biotilgjengelighet og toksisitet ved vannekspонering til sølv kunne kobles til konsentrasjonen av løst Ag i eksponeringsmedium. I tillegg indikerte resultatene fra algestudiet en nanospesifikk komponent i toksisiteten av Mesosilver.

LIST OF PAPERS

Paper I. Characterizing NM300K Silver Nanoparticles Behavior, Uptake and Toxicity in *Caenorhabditis elegans* (KLEIVEN, M., ROSSBACH, L. M., GALLEGO-URREA, J. A., BREDE, D. A., OUGHTON, D. H. & COUTRIS, C. 2018. Characterizing NM300K Silver Nanoparticles Behavior, Uptake and Toxicity in *Caenorhabditis elegans*. *Environmental toxicology and chemistry*. DOI: 10.1002/etc.4144)

Paper II. Growth inhibition in *Raphidocelis subcapitata* – evidence of nanospecific toxicity of silver nanoparticles (KLEIVEN, M., MACKEN, A., OUGHTON, D.H. Under revision for Chemosphere)

Paper III. Route of exposure has a major impact on uptake of silver nanoparticles in Atlantic salmon (*Salmo salar*) (KLEIVEN, M., ROSSELAND, B.O., TEIEN, H-C., JONER, E., OUGHTON, D.H. 2018. Route of exposure has a major impact on uptake of silver nanoparticles in Atlantic salmon (*Salmo salar*). Environmental toxicology and chemistry. DOI: 10.1002/etc.4251)

Paper IV. Characterization of bioconcentration and toxicity of Ag ions and the Ag nanoparticle NM300K in brown trout (*Salmo trutta* L.) (KLEIVEN, M., HULTMAN, M.T., ROSSELAND, LEBED, P., TOLLEFSEN, K.E., TEIEN, H-C.)

DEFINITIONS AND ABBREVIATIONS

Ag(I)	Monovalent Ag ion
ADME	Absorption, distribution, metabolism, excretion. An abbreviation used to describe the disposition of a compound in the body/organism.
Agglomerates	Collection of weakly bound particles or aggregates where the resulting external surface area is similar to sum of the surface areas of the individual components.
Aggregates	Irreversible attachment of parent particles strongly bound or fused resulting in a reduction in surface area.
ANOVA	Analysis of variance
Bax	Bcl2 associated x protein, gene associated with apoptosis
Bioaccumulation	The increase in concentration of the test substance in or on an organism relative to the concentration of the test substance in the surrounding medium.
Bioavailability	the ability of a substance to interact with the biosystem of an organism
BLM	Biotic ligand model
Casp6a	Caspase 6A, gene associated with apoptosis
Colloid	Homogenous, amorphous substance dispersed throughout another substance and ranging in size from 1 to 1000 nm. In this PhD research it is defined as the Ag size fraction < 220 nm >3 kDa and includes the ENMS.
Da	Dalton, atomic mass unit
DLVO theory	Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, addressing colloidal stability considering the two opposing forces of electrostatic repulsion and Van-der Waals attraction.
DOM	Dissolved organic material
EC_x	The concentration of the test substance that results in a x % (e.g. 50 %) reduction in an effect measurement within a stated exposure period, e.g., 50 % reduction in algae growth rate after 96 h exposure period.

ENMs	Engineered nanomaterials; nanomaterials intentionally designed and produced to form new materials and products with unique properties, and may include individual nanoparticles (NP), nanocomposites, and materials composed of NPs having at least one dimension of 1-100 nm and display novel properties. See definition in Introduction.
FFF	Field flow fractionation
GST	Glutathione S transferase, gene associated with oxidative stress responses.
GR	Glutathione reductase, gene associated with oxidative stress responses.
GPx3	Glutathione peroxidase x3, gene associated with oxidative stress responses.
ICP-MS	Inductively coupled plasma mass spectrometry
LCA	Life-cycle assessment, a technique to assess the environmental impact of a product including all steps from raw material extraction to disposal and recycling.
LC50	Lethal concentration 50 % is a statistically derived concentration at which 50 % of the test organisms will be expected to die.
LMM	Low molecular mass
LOEC	The lowest tested concentration at which a substance is observed to have a statistical significant effect ($p < 0.05$) in comparison with control, within a given exposure time.
Mesosilver	A commercial colloidal Ag suspension, and one of the Ag ENMS included in this PhD research.
MoA	Mode of action
MT	Metallothionein
NaI detector	Sodium Iodine detector used for detection of gamma radiation
NIVA	Norwegian Institute for Water Research
NMBU	Norwegian University of Life Sciences
NM300K	A silver OECD reference nanomaterial
NM302	A silver OECD reference nanomaterial
NOEC	No observed effect concentration, the test concentration immediately below the LOEC which, when compared with the control, has no statistically significant effect ($p<0.05$), within a given exposure time.

NOM	Natural organic matter
NP	Nanoparticle
NTA	Nanoparticle Tracking Analysis
OECD	Organization for Economic Cooperation and Development
Particulate Ag	Particulate Ag is in this PhD research defined to be > 220 nm.
qPCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
spICP-MS	Single particle ICP-MS
TEM	Transmission electron microscope
TOC	Total organic material
Total dissolved silver	Dissolved Ag complexes with unknown speciation < 3 or 10 kDa

1. INTRODUCTION

Organisms are exposed to a range of environmental stressors at any time. These stressors can be factors associated with environmental conditions such as water quality (e.g., pH, salinity), or substances (e.g., radionuclides, organic pollutants) occurring naturally in the environment or as a result of anthropogenic activities. Nanomaterials are one such class of substances, and can be divided into three categories: natural, incidental and engineered nanomaterials (ENMs). Natural nanomaterials are non-processed nanoscale materials occurring in the environment, for example, natural colloids which are widespread nanoscale structures (1 nm to 1 μm in size) present in most aquatic systems (Figure 1). Other examples are clay and volcanic ash (Handy et al., 2011, Lead et al., 2018). Incidental or anthropogenic nanomaterials are unintentionally generated and released into the environment by human activities, for example, through mining, oil drilling, and combustion. Engineered nanomaterials are intentionally designed and produced to form new materials and products with unique properties (Handy and Shaw, 2007, Crane et al., 2008). At nanoscales, characteristics such as optical properties, conductivity, mechanical strength, and chemical reactivity start to change, and can differ drastically from that of their respective macro- and micron-sized materials (Klaine et al., 2012). The number of particles per unit mass or volume, as well as the surface area increases substantially in a nanomaterial compared to their bulk materials. The relative increase in surface atoms (in terms of the % of total number of atoms), results in an increase in chemical reactivity, making surface-related properties and the particle surface an essential feature of ENMs (Christian et al., 2008). The distinct physio-chemical properties are what makes ENMs suited for a range of applications, and why nanotechnology has had substantial growth the last years.

The idea behind nanotechnology is widely attributed to a 1959 talk given by Richard Feynman "*There's plenty of room at the bottom: An invitation to enter a new field of physics*" where he predicted that atoms one day in the future could be manipulated at the level of individual atoms to create materials with new, unique properties (Feynman, 1960). Since then nanotechnology has become a field of extensive research as well as industrial development (Navarro et al., 2008a, Gottschalk et al., 2015), and has been referred to as "*an emerging, cross-disciplinary technology designed to create and synthesize new materials at the nanoscale to generate innovative or altered material properties*" (Warheit, 2018). Nanotechnology, as a relatively young technology, has experienced a rapid growth. According to Navarro et al. (2008a) the global annual production of ENMs in 2004 was in the range of 10^3 tons, and expected to increase to 10^5 tons by 2010. The global investment in nanotechnologies was around $10 billion in 2005, and by 2011-2015 expected to increase to $1 trillion. Although the area of nanotechnology

research and development will not continue to grow exponentially, the annual production of ENMs has continued to increase over the last decade and is worth a considerable amount of dollars on the global market (Navarro et al., 2008a, Baalousha et al., 2016).

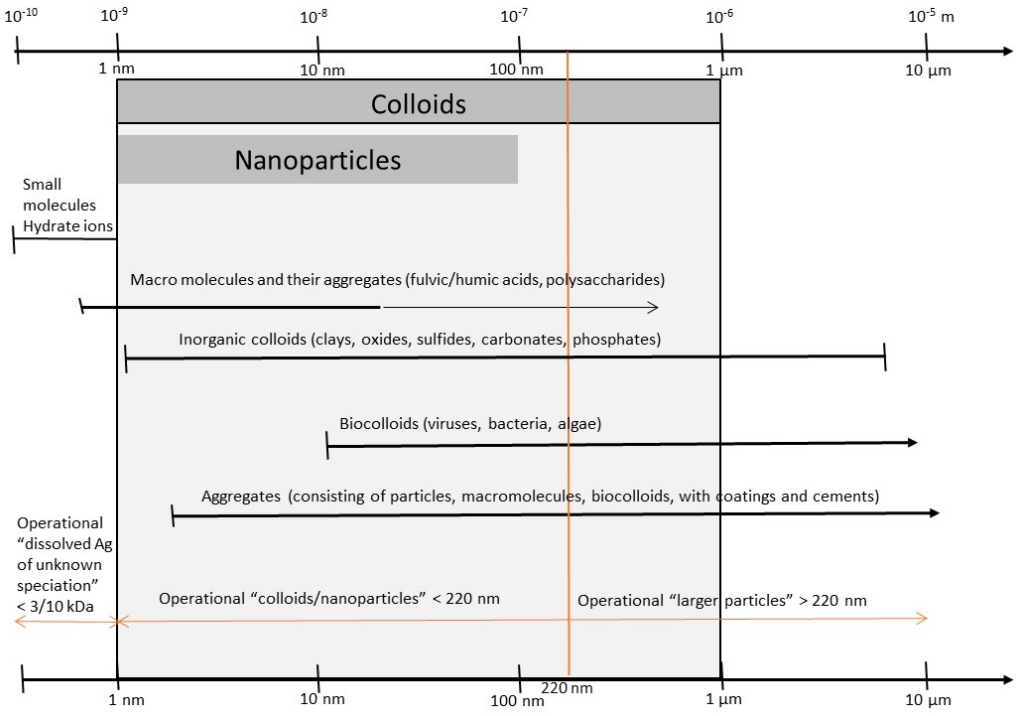


Figure 1. Size range and typical representation of natural colloids and nanoparticles. Modified figure from Christian et al. (2008). The operationally defined cut-off used in this PhD research is shown in orange, representing filtration at 200 nm and either 3 or 10 kDa.

The development of a wide range of nanomaterials and products has resulted in the need for additional ways of categorizing and defining the materials, beyond that of "natural, incidental, and engineered". Engineered nanomaterials are often further categorized according to composition, of which the main categories are: carbon-based (e.g., fullerenes and carbon nanotubes), ceramics, semiconductor (e.g., quantum dots), polymeric (normally organic-based NPs), lipid base, nanocomposites, nanohybrids, and metal (e.g., Ag ENMs, metal oxides). Within each of these groups and single chemical compounds, numerous materials exist with variations in, for example size, shape, surface charge, and coatings. Over the last years an extensive effort to come up with a sensible definition of nanomaterials has resulted in numerous definitions given by bodies ranging from governments to industry and standards organizations. Definitions exist not only for regulatory frameworks, but also for advisory purposes (Boverhof et al., 2015).

The International Organization for Standardization (ISO) defines a nanomaterial as a "*material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale*", where "nanoscale" is defined as the size range from approximately 1-100 nm (ISO, 2015). In addition to the largely arbitrary 100 nm mark, which has little theoretical or environmental significance, definitions based only on size have been criticized for being insufficient from a risk assessment point of view, because they fail to consider other factors that would be important to assess a nanomaterial's need for additional regulatory review (Maynard, 2011, Bleeker et al., 2013, Boverhof et al., 2015). The definition by the European Commission (2011) includes additional factors to the 1-100 nm size cutoff: "*A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm – 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 % to 50 %*". Boverhof and coauthors (2015) carried out a comparative assessment of different existing definitions of nanomaterials, and found large inconsistencies. Size was the only factor common to all definitions, however, there were differences in the defined size range. There were also inconsistencies in the way additional parameters (e.g., aggregation, solubility, novel properties) were addressed in the definitions. They concluded that, in addition to particle size, the following factors should also be considered in a definition of nanomaterials: a distributional threshold, size-dependent properties, the state of agglomeration/deagglomeration and aggregation/disaggregation, and that it should only include intentionally manufactured nanomaterials (ENMs). Others, like Maynard (2011), have raised the question of whether a limiting definition is the way to go. He further suggests that regulatory bodies rather work with a list of nanomaterial characteristics that trigger action at certain values, and that these trigger points need to be flexible enough to be able to incorporate new scientific knowledge, thus creating more adaptive regulatory frameworks.

There are clearly a number of positive aspects of the new materials developed and their applications, including potential environmental benefits such as energy saving, monitoring or remediation, as well as applications within medicine. But these applications also carry a risk from environmental release of ENMs or their transformation products, and the possibility of effects on biota. This rendering a need for a cost-benefit analysis for society for the specific material and uses. Risk assessment is a mandatory procedure to ensure safe use of all new substances, including ENMs. However, large variability between and within categories of ENMs and the difficulties of exposure assessment make this a challenging endeavor.

The underlying premise of this thesis is that more information on exposure characterization and exposure routes is required in order to understand and predict nanomaterial fate and behavior in the environment as well as their potential to induce toxicity. Due to the well-known antibacterial properties of silver (Ag), Ag ENMs are amongst the most commonly used nanomaterials in consumer products. And in part because of the well-established toxicity of ionic Ag to aquatic organisms, the environmental release of Ag ENMs and their potential toxicity to organisms has received great attention over the last decade. The overall goal of this PhD has been to determine whether the source of Ag (dissolved Ag species vs Ag ENMs) lead to differences in accumulation and toxicity to organisms, and whether these potential differences can be linked to observed variations in exposure characteristics and route of exposure.

1.1 Silver nanomaterials

Silver is a transition metal, naturally occurring as the isotopes ^{107}Ag and ^{109}Ag. It has the highest electric and thermal conductivity of any metal and the lowest contact resistance (Wood et al., 2012). Silver has been known for its antibacterial properties for centuries, which is also the reason why Ag ENMs are among the most frequently used nanomaterials on the market (Vance et al., 2015). Although the majority of Ag nano-containing products (e.g., cosmetics, cloths, cleaning agents, food additives) on the market are associated with its antimicrobial activity, Ag ENMs are also used in for example microelectronics and medical imaging due to their high electrical and thermal conductivity, catalytic activity, and optical properties (Fabrega et al., 2011, Echavarri-Bravo et al., 2017).

As a consequence of the expanding nanotechnology industry and the continuous introduction of new "nanoproducts", some release into the environment is inevitable. However, there is still limited information available on the manufacture, use and release of nanomaterials, and in what amounts. Generally, the production volumes in terms of numbers of ENMs given in the literature are associated with large uncertainties, and discharge of ENMs to the environment is generally difficult to prove and poorly understood (Giese et al., 2018, Lead et al., 2018)

Although release of Ag ENMs from consumer products (e.g., Ag ENMs from textiles) has been shown experimentally (Benn and Westerhoff, 2008, Benn et al., 2010), there are few studies documenting actual environmental release. Kaegi and coauthors (2010) reported a high initial leaching of Ag ENMs (<15 nm) from painted facades (145 µg Ag L^{-1}), with more than 30 % of the Ag ENMs released to the environment during the first year. According to Giese et al. (2018) the concentration of Ag ENMs (including both naturally occurring and ENMs) have been identified

and quantified to be 0.74 ng L^{-1} (Range; 0.0-6.2 ng L^{-1}) in surface waters within the Bavarian survey network of watercourses (Maier et al. 2016, Maier and Wegenke 2017 cited in Giese et al. 2018). The lack of concrete evidence of release reflects challenges in identification and quantification of ENMs in environmental samples (as well as other complex matrices such as media for ecotoxicity tests, soil and biological samples) due to the complexity of the samples, in combination with relatively low concentration and the difficulty of separating them from naturally occurring nanomaterials. For comprehensive reviews on characterization of ENMs, see Hassellöv and Kaegi (2009) and Von der Kammer et al. (2012).

Measurement of ENMs, environmental concentrations of ENMS are often estimated by modelling. For Ag ENMs, the production volume has been reported to be < 1000 tons per year (Giese et al., 2018), with estimated environmental concentrations in freshwater in the range of pg to ng L^{-1} and an expected 2-6 times increase towards 2050. In freshwater sediments, concentrations in the range of pg kg^{-1} have been quoted for 2017, and may reach mg kg^{-1} levels by 2050, depending on the assumptions of the modeling (e.g., degradation of ENMs in nature or not) (Giese et al., 2018). Considering the large uncertainties related to the input data (e.g., production volumes and transformation of the nanomaterials) the output of these models are also associated with large uncertainties.

As a result of the extensive research on the toxicity of nanomaterials conducted over the last 20 years or so, we now know that many nanomaterials are bioavailable and can induce toxic responses in a range of different organisms (see for example reviews by Klaine et al. (2008), Handy (2012) and Levard et al. (2012)). Silver in its ionic form is known for its high toxicity to aquatic organisms because it is compatible with membrane-transporters used for cellular transport of for example Na^+ and Cu^+ (Wood et al., 1999). Also Ag ENMs are reported to be among the nanomaterials that induce the highest toxicity, reported for organisms at all trophic levels: bacteria (Fabrega et al., 2009, Echavarri-Bravo et al., 2017), algae and invertebrates (Ribeiro et al., 2015a, Sørensen and Baun, 2015), and fish (Chae et al., 2009, Farmen et al., 2012, Bruneau et al., 2016). However, whether the toxicity is caused by the Ag nanomaterial itself (nano-specific), by the dissolved fraction or a combination of the two is one of the main questions to be answered in nanotoxicology. To address this question, we need to consider in more detail the behavior of silver and silver nanomaterials in the environment, their characterization, bioavailability and toxicity.

1.2 Silver and silver nanomaterials in the aquatic environment

Silver in pristine natural waters occurs in pg to low ng L^{-1} levels and is found in the < 0.45 µm size fraction (i.e., not retained in the 0.45 filter). Later a more precise characterization has indicated Ag largely to be in the colloidal fraction (> 10 kDa < 0.45 µm) (Adams and Kramer, 1999). It is established that free Ag ions (Ag^+) are among the most toxic of metals and cause acute toxicity in freshwater organisms. However, given the high affinity of Ag to interact with inorganic (e.g., chloride, sulfur, thiosulfate) and organic (e.g., NOM) ligands (Hiriart-Baer et al., 2006), free Ag ions most likely do not occur to any significant level in natural waters. The speciation in natural waters is dominated by complexation to sulfide, dissolved organic material, chloride and particles (Wood et al. 2002). This is in contrast to regulatory tests where $AgNO_3$ is used in simplified, synthetic exposure media, which results in a test where free Ag ions actually are available and thus the main inducer of the toxicity observed. This may result in an overestimation of Ag toxicity in natural freshwaters.

Following the release into the environment, ENMs will interact with environmental components, resulting in a transformation of the ENM (Lowry et al., 2012). The transformation processes can be divided into physical (e.g., aggregation, agglomeration, sedimentation), chemical (e.g., dissolution, redox reactions) and biologically mediated, and both these processes and the transformation products, will depend on the environment that they enter as well as the starting material. The different transformation processes will likely alter the physico-chemical properties of the ENMs, resulting in a transformation product or aged ENM that can be very different from the original material (Selck et al., 2016). Environmental transformation of ENMs can alter their mobility, bioavailability and toxicity. The same factors (e.g., presence of inorganic and organic ligands, ionic strength, pH) influencing the speciation of Ag in natural waters are believed to also influence the fate and behavior of Ag ENMs in the environment (Conine and Frost, 2017). For example, sulfidation of Ag ENMs has been reported to reduce toxicity in algae (Liu et al., 2012).

The Derjaguin-Landau-Verwey-Overbeek (DLVO) theory is used to explain the stability of colloids in suspension. It considers the two opposing forces of electrostatic repulsion and Van-der Waals attraction to explain how some colloidal systems “collapses” through coagulation/aggregation, while others remain stable colloidal suspensions (Verwey and Overbeek, 1948). This theory is also used to explain the stability of nanoparticles in suspension.

In metal ENM toxicity research, including Ag, the most frequent characterization of the tested nanomaterials are the primary particle size and shape usually obtained by transmission electron microscopy (TEM) or scanning electron microscopy (SEM), hydrodynamic diameter

obtained by dynamic light scattering (DLS), and relative surface charge/zeta potential, in addition to total Ag concentrations at the beginning of the toxicity test. Although the characterization of the initial nanomaterial is important, recognition that changes in speciation will occur over time calls for more emphasis to be placed on the exposure characterization. Characterizing ENMs can be a challenging task since it often requires facilities that are both advanced, costly and not always available. Different techniques and tools are available for characterization of pristine particles and in simple aqueous media (e.g., Transmission electron microscopy (TEM), Dynamic light scattering (DLS), Field-flow Fractionation (FFF), Single Particle Inductively Coupled Mass Spectrometry (spICP-Ms), Nanoparticle Tracking Analysis (NTA), synchrotron techniques), of which some have become the gold standard of characterization of pristine particles and stock suspensions (e.g., TEM and DLS). Relatively cheap, simple, *in situ* filtration techniques (e.g., ultrafiltration or cross-flow ultrafiltration) can be used to separate the different size fractions of a metal, in combination with measurements of total metal concentrations in each size fraction (by the means of e.g., ICP-MS or ICP-OES) in order to follow changes in metal size fractions throughout an exposure. Thus, obtaining more information on the transformation of ENMs and its influence on bioavailability and toxicity.

The importance of speciation of metals in inducing toxicity to aquatic organisms is a generally accepted fact and led to the development of the biotic ligand model (BLM). The BLM is a modeling tool used in aquatic toxicology that was developed to explain and predict how water chemistry affect the toxicity of metals to aquatic organisms (Paquin et al., 2002). It aims to predict the interactions of metals with the biotic ligand, which is the site where the metal binding would result in a toxic effect. The model also considers the competition to these active sites from other toxic metals as well as cations. A BLM for acute Ag toxicity has been established for fish (Wood et al., 2012), Daphnia magna, and algae. Depending on the mode of action of metal ENMs (toxicity through dissolution of ions vs. nanospecific toxicity), this model could also be useful in nano-metal toxicology.

1.2.1 Bioavailability and accumulation

Accumulation of a substance, for example a metal such as Ag, is a direct measure of its retention and often also its bioavailability to an organism. The processes of accumulation, bioavailability and toxicity are important to understand when analyzing risks of any pollutant, including ENMs. Exposure is a prerequisite for toxicity to organisms, and absorption is a prerequisite for accumulation. However, bioavailability, and resultant toxicity, can arise without the need for uptake across a cell membrane, for example due to interaction and deposition at the gill surface.

Uptake of a compound can be via passive diffusion through semipermeable membranes, or through active transport via macromolecular carriers in the membrane. Trace elements, like for example, Ag mainly enter the cells through active transport (Ratte, 1999). One of the main questions when it comes to metal ENMs is whether the ENMs themselves are the cause of observed toxicity, or their dissolution product. In addition to a secondary uptake of Ag ions released from the Ag ENMs, diffusion, endocytosis and vesicular transport are also suggested as mechanisms of uptake (Moore, 2006, Handy et al., 2008b) and will depend on the given ENMs size, surface properties, as well as the organism of which the interaction occur. Other factors impacting on uptake include the concentration of Ag ENMs in the surrounding environment (water, sediments, food), the properties of the Ag ENM and their transformation products, the composition and properties of the environment, route of exposure, and the biology and ecology of the organism (Fabrega et al., 2011). For example, in freshwater fish, Ag can be taken up at gills as free Ag ions, as the neutral complex $AgCl_0$, as silver thiosulfate complexes ($Ag(S_2O_3)_n$), and last as silver sulfide complexes (probably Ag_2S) (Wood et al., 2012). Free Ag ions are by far the form with the highest uptake, followed by $AgCl_0$. Silver ions enter the cells via the Na^+ channels on the branchial epithelia ionocytes in competition with Na^+. In addition, transport via the Cu^+-transporter Ctr 1 is another suggested uptake pathway due to the free Ag ions' high affinity to this transporter. The neutral $AgCl_0$ complex shows a considerable lower uptake into the gills than the free Ag ions, and is believed to be taken up though diffusion (Wood et al., 2012). Also in freshwater algae have been hypothesized to occur via facilitated cation transport, maybe through a Cu^+-transporter. However, Hiriart-Baer and coauthors (2006) also suggest uptake of Ag-thiosulfate complexes via membrane-bound sulfate transporters. In freshwater the association of Ag with algae has been observed to be different between Ag species as well as between algae species (Lee et al., 2005, Hiriart-Baer et al., 2006). Aggregation of the nanoparticles can, for example, change the route of exposure from water to sediment (or food) by removal of the particles from the water column, and thus changing the potential exposure and toxicity from pelagic to benthic organisms (Selck et al., 2016). For freshwater fish this could change the route of exposure from waterborne, i.e. gills, to dietary. Although less extensively researched, Ag has been reported to be bioavailable and accumulate in organisms also after dietary exposure to Ag ions and Ag NPs (Galvez et al., 1996, Lacave et al., 2017).

The accumulation is further influenced by the distribution, metabolism and excretion. After absorption into an organism the compound will be distributed within the organism such as, for example, to specific cell compartments in an algae or via the circulatory system to specific excretory organs (e.g., liver) in a fish species. As for any pollutant, accumulation of a given ENM can vary between different organs, however, knowledge about transfer to internal target organs

for small invertebrate species as well as algae is limited, simply due to their small size, which makes dissection for organ/organelle quantification difficult. Thus, most studies of organ distribution of ENMs in aquatic organisms have been conducted on fish (Lead et al., 2018).

Regardless of the chemical form in which Ag enters the cells (free Ag ion, $AgCl_0$, $Ag(S_2O_3)_n$, Ag_2S), it will quickly be bound up by chloride, sulfide, and biomolecules like glutathione, proteins, and amino acids. The target organs for Ag in freshwater fish have been reported to be liver, gills, gut and kidney, with the liver as the organ with the highest Ag concentration. For fish, hepatic excretion through bile and renal excretion through urine (not in freshwater fish) are the two main routes of excretion. Generally, the target organs for ENMs seem to be the same as for their equivalent traditional chemicals. Thus, for Ag ENMs highest Ag concentration would be expected to be found in liver, given an initial absorption of the Ag ENM or their dissolution product. Of the two excretion routes (biliary vs urine), hepatic excretion is the most likely pathway for nanomaterials since it depends on exocytosis and vesicular transport (size of vesicles approx. 200 nm), while the renal excretion involves filtration of the blood in the vertebrate kidney with a molecular mass cut-off around 60 kDa (approx. 2 nm), which is too small for most nanoparticles to be accumulated in the kidney (Handy et al., 2008b).

A main challenge when assessing accumulation of metal ENMs is verifying uptake of the ENMs themselves. In most studies, the accumulation of Ag in organs or tissues following exposure to Ag ENMs is assessed by digestion of the sample, and measuring total Ag using inductively coupled plasma mass spectrometry or similar techniques. Such measurements of total concentrations cannot distinguish between uptake of Ag ions released from the surface of the Ag ENM or direct uptake of ENMs. The presence of Ag ENM in tissue of both *C. elegans* (identified by CytoViva/hyperspectral imaging-based analysis) (Meyer et al., 2010) and zebrafish embryos (TEM and electron-dispersive x-ray analysis (EDS) (Asharani et al., 2008). Also in brown trout gill tissue areas of high electron density, assumed to be Ag ENMs, have been detected (Scown et al., 2010). Even though no EDS analysis was conducted to identify these electron dense areas as Ag, it is a plausible explanation. However, the formation of nanoscale particles after entrance to the cell cannot be discounted.

1.2.2 Toxicity

Toxicity is expressed as an effect threshold where the observed effects for a given endpoint (e.g., growth, reproduction, and mortality) are statistically significant (Ratte, 1999). How toxicity is expressed depends on, for example, if it is acute or chronic exposure. In acute tests, LC_{50} is commonly determined, which is the concentration causing 50 % mortality. In other tests, effect concentrations (e.g., EC_{50}) are determined; reflecting the concentration that elicits an estimated 50 % effect for a given endpoint. Also no-observed-effect concentrations (NOEC) are commonly presented in literature, although they have been criticized because of the high dependence on test circumstances like replication, concentration span and variability (Hoekstra and Van Ewijk, 1993). In chronic exposures, sub-lethal effects on endpoints like growth, fertility and reproduction are common.

Over the last decades extensive research efforts have been conducted on hazard identification of nanomaterials, and toxicity have been shown for a range of nanomaterials and organisms (Navarro et al., 2008a, Lead et al., 2018). The toxicity of dissolved species of Ag is well-known for both algae, invertebrates and fish (Wood et al., 1996, Ratte, 1999, Hogstrand et al., 2003), with no observed effect concentrations (NOEC) values in the range of ng L^{-1} for *Daphnia* spp (Bielmyer et al., 2002), µg to mg L^{-1} range for marine and freshwater algae, and LC10 values as low as 0.8 µg Ag ions L^{-1} for some sensitive freshwater species of fish (Fabrega et al., 2011). Accumulation and/or toxicity of Ag after Ag NP exposure have also been reported for a range of organisms: bacteria (Fabrega et al., 2009, Echavarri-Bravo et al., 2017), algae and invertebrates (Ribeiro et al., 2015a, Sørensen and Baun, 2015), and fish (Chae et al., 2009, Bruneau et al., 2016). Despite the extensive research effort on hazard identification of ENMs, and specifically on Ag ENMs, there are still uncertainties regarding toxicity mechanisms. Studies have reported Ag ions released from the surface of the Ag ENM to be the driving force behind the observed toxicity, while others ascribe the toxicity to the nanoparticles themselves or a combination of the two (Fabrega et al., 2011, Sendra et al., 2017). Which mechanism is the dominant one, will depend on several factors such as, for example, the physico-chemical properties of the specific Ag ENM, exposure conditions, and the test organism. The mechanisms behind Ag NP-specific toxicity are reported to be generation of reactive oxygen species (ROS) at the surface of the nanoparticle, resulting in oxidative stress (Carlson et al., 2008, Lim et al., 2012, Ribeiro et al., 2015b).

For *C. elegans*, image analysis has shown Ag ENM uptake to occur predominately via ingestion and that the Ag ENMs are taken up by intestinal cells (Meyer et al., 2010). Silver is reported to cause impairment of growth, fertility and reproduction (Meyer et al., 2010, Yang et al., 2012).

The toxicity of Ag towards freshwater algae are often ascribed to the presence of free Ag ions (Ratte, 1999, Ribeiro et al., 2015a). However, Lee and coauthors (2005) found difference between species of freshwater algae. For *R. subcapitata*, an algae shown to exhibit slow short term Ag uptake rates, the toxicity was determined by the free Ag ion concentration. While for *Chlamydomonas reinhardtii*, an algae with fast short-term silver uptake rates, the toxicity was not only determined by free Ag^+ concentrations, but rather dissolved Ag species including $AgCl_n^{(1-n)+}$. In addition to the standardized endpoint growth inhibition, other effects like reduction in carbon uptake in algae cells, penetration of cell wall and membranes, impairment of photosynthesis, as well as oxidative stress cause by reactive oxygen species.

The acute toxicity of Ag towards freshwater fish species is well documented, and is mainly ascribed to the free Ag ion. According to Wood et al. (2012) the 96 h LC_{50} values are typically found in the 2-30 µg L^{-1} range for many fish species. As freshwater fish are hypertonic to their surrounding water, water enters their body by osmosis. Thus, freshwater fish need to absorb ions (e.g., Na^+, Cl^-) by active transport at the gills to replace the losses that occur across the branchial and body surface. This means the sites of active transport of Na^+ are possible active sites also for Ag^+.

The mechanism behind acute toxicity to Ag ions is well-known and can be divided into two steps. First a quick (within the first two hours of exposure to Ag^+) inhibition of the carbonic anhydrase activity in the gills and a more or less total blockade of Cl^- uptake. Carbonic anhydrase is an enzyme that catalyzes the hydration of CO_2 producing the acid (H^+) and basic (HCO_3^-) ions with which the Na^+ and Cl^- uptake are exchanged at the apical surface (Evans et al., 2005). This approximately 30 % inhibition of the carbonic anhydrase activity occurs much quicker and long before the second step of the Na^+/K^+ ATPase activity inhibition and reduced/blocked Na^+ uptake. The mechanism of Na^+/K^+ ATPase inhibition does not involve a direct completion between Ag^+ and Na^+, but rather with Mg^{2+}. Two atoms of Ag^+ bind the activation site for Mg^{2+}, thus hindering the active uptake of Na.

For Ag ENMs, the general picture is a lower observed toxicity compared to exposures to free Ag ions ($AgNO_3$). It has been frequently hypothesized that the toxicity of metal ENMs is mainly caused by the release of ions followed by a “free ion” or low molecular mass metal species induced toxicity (Notter et al., 2014). If this is the case, the toxicity mechanisms would largely be expected to be the same in Ag ENM and $AgNO_3$ exposures. However, nanospecific toxicity or more often in combination with “free ion toxicity”, have also been reported (Scown et al., 2010, Bruneau et al., 2016). The mechanisms behind nanospecific toxicity is often reported to be oxidative stress induced by the formation of reactive oxygen specis (ROS) at the surface of the ENMs (Carlson et al., 2008).

The fact that accumulation of a compound is not necessary a prerequisite of toxicity is important to keep in mind. Adsorption to active sites at the membrane like, for example, Na^+/K^+ ATPase where free Ag ions binds active sites leading to severe problems with osmoregulation due to inhibited uptake of Na^+ and Cl^-. Also in algae adsorption of a compound to the cell wall or membrane could cause shading effects, thus inhibiting the photosynthetic activity leading to growth inhibition (Ribeiro et al., 2015a).

1.3 Challenges

To conclude, the uptake and especially toxicity after exposure to Ag ENMs have been reported in numerous studies. However, the majority of the studies have not determined whether or not this toxicity was nanospecific. This reflects in part the challenges associated with exposure characterization of ENM exposures. Characterization of the primary particles or particle suspensions in MQ water is not sufficient to answer the main question of whether any reported toxicity is caused by the metal ENMs or by ions released as a result of dissolution. Knowledge about the exposure characteristics over time, in the different exposure media, in the presence of test organisms, and with different routes of exposures, is important to improve the understanding and prediction of the fate and behavior of ENMs in the environment as well as their potential to induce toxicity.

2. AIMS OF THE RESEARCH

The overarching aim of the research is to increase the understanding of the ways in which nanoparticle properties, and in turn their behaviour in toxicity testing media, influence toxicity. Claims of a nano-specific toxicity need to demonstrate that organisms were actually exposed to nanoparticles during tests. Likewise, there is a need to differentiate whether observed discrepancies between ion and NP toxicity reflect differences due to "bioavailability" (in its most general meaning) or differences due to mechanisms. A range of experiments involving four different species (*Caenorhabditis elegans*, *Raphidocelis subcapitata*, *Salmo salar* and *Salmo trutta*) have been designed to test four interlinked hypothesis (schematic overview in Figure 2).

1. **Changes in Ag ion and Ag NP speciation will cause a time dependent change in the NP fraction in test media exposure solutions.**

This hypothesis was tested by following the size distribution of both ions and NP in all test media used in toxicity studies as a function of time, for both Ag ion and Ag NP exposures. Although the test media had different chemical compositions, which would be expected to influence speciation and transformation, these parameters were not systematically tested by controlled change of concentrations. Tests were carried out under normal exposure conditions, and time was the key variable studied across all tests.

2. **Variation in the size fractions of Ag ion and Ag NPs in test media will result in different bioavailability and bioaccumulation in test organisms.**

This was tested by using size fractionation data to compare the availability of different Ag NPs and Ag ions to different test organisms. Exposure experiments were largely carried out under acute conditions, and accumulation would be expected to be lower than equilibrium concentrations reached after chronic exposure. Relative accumulation would also vary if Ag ions and NPs have different uptake and release kinetics, however, the assumption is that the general trends seen under acute conditions should be sufficient to give insight into differences in availability. Furthermore the, studies allowed a comparison of general trends for accumulation of different NPs (and thus NP properties) between organisms.

3. Diet can be a significant route of silver uptake from Ag NPs in fish

Waterborne exposure is in general the most frequently tested route of exposure for aquatic organisms, including fish. However, transformation processes of NPs in the environment will under many conditions result in aggregation, subsequent sedimentation and removal from the water column. For example, the *Chironomide* larvae which feed in the sediments, is an important food source for brown trout and can be a direct link between sediment concentrated pollutants and dietary exposure to fish. The importance of dietary exposure was tested by comparing the uptake of Ag-ions and Ag NPs from waterborne and diet in two different fish species.

4. Exposure to Ag NPs can cause a nanospecific component of toxicity

This hypothesis was tested by comparing toxicity data under controlled analysis of the variability in size fractionation in exposure media, as well as the exposure route. In order to identify possible nanospecific toxicity, differences in response between Ag ions and Ag NPs were compared to the fraction existing as dissolved ions in the exposure media.

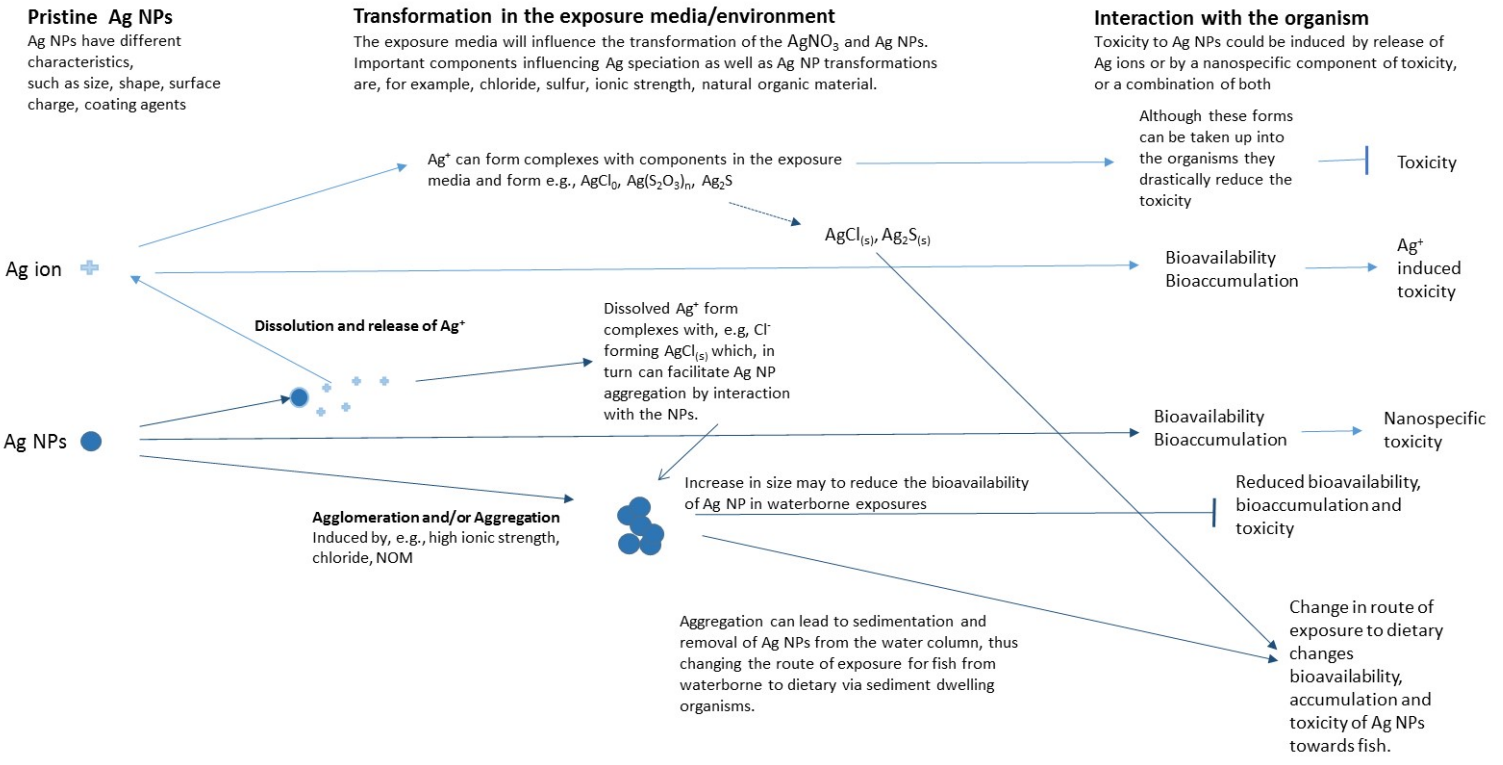


Figure 2. A schematic overview of the hypotheses of this PhD research.

3. METHODOLOGICAL ASPECTS

To test the above hypotheses, laboratory studies involving four different species (*Caenorhabditis elegans*, *Raphidocelis subcapitata*, *Salmo salar* and *Salmo trutta*), one or more Ag nanomaterials with comparison to Ag ions ($AgNO_3$), as well as waterborne and dietary exposures were conducted (Table 1). Since the research project was funded by different collaborative EU and national projects, the selection of organisms and ENMs was partly dictated by those research programs. However, common to all experiments, a thorough exposure characterization was performed in the attempt to link the observed effects of the Ag compounds to exposure characteristics.

3.1 Choice of organisms

Different organisms have different sensitivity towards pollutants, including Ag and Ag ENMs (for comprehensive reviews see Ratte (1999), Klaine et al. (2008), and Handy (2012)). The environmental conditions housing the organisms will transform the ENMs in different ways depending on a range of factors (e.g., pH, temperature, O_2, inorganic and organic ligands etc.), and so will the organisms themselves (e.g., secretion of mucus). The organisms of choice in this PhD research cover a range in both sensitivity towards pollutants as well as their relevance to standardized toxicity testing and the environment. An overview of the main characteristics of the media used for each organisms is given in Table 2.

Caenorhabditis elegans, a nematode living in soil pore water, have been extensively studied and was the first multicellular organism to have its whole genome sequenced. The extensive knowledge about this species physiology and biology, and its short lifecycle (96 h at 20 °C) allows for toxicity testing covering all development stages as well as across generations, making this a commonly used species in standardized toxicity testing (Hunt, 2017). Traditionally *C. elegans* toxicity testing has been conducted on agar plates bedded with *E. coli*, or rather high ionic strength media like M9 and K+ (media with high ionic strength and chloride concentrations). Due to the known effects high ionic strength and high chloride levels on Ag ENM stability, the media used in the current research was US EPA moderately hard reconstituted water (MHRW) (United States Environmental Protection Agency, 2002). This media was chosen as a low ionic strength media (relative to the M9 and K+ media) and also for being a more environmental relevant media for testing of ENMs and their effects on *C. elegans* (discussed in, e.g., Tyne et al. (2013)). Although the MHRW has relative low chloride concentration, the high concentration of sulfur as well as the high ionic strength (compared to

the media used for the other organisms tested in this work) would likely influence the speciation of Ag and the processes of aggregation and dissolution of Ag ENMs.

Raphidocelis subcapitata, is a freshwater green microalgae with a widespread distribution in aquatic freshwater systems. It is commonly used as a bioindicator species, as well as in standardized toxicity testing. Primary producers, play an essential role in aquatic ecosystems (Ribeiro et al., 2015a, Wang et al., 2016), and alterations in these communities as a consequence of pollutants are likely to also influence species in higher tropic levels and potentially whole ecosystems (Ribeiro et al., 2015a).

Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) both belong to the Salmonidae family. Salmonids are generally known to be sensitive towards contaminants and hence suitable for toxicity testing at environmentally relevant concentrations. The toxicity of the free Ag ions to these species are well documented and the underlying mechanisms understood. Fish species are also generally well suited for organ distribution studies simply due to their size allowing for dissection of organs. Impacts on these species can have a high environmental consequence, number one species for recreational fishing and they are also economically important species due to their use in aquaculture.

3.2 Ag materials

The Ag ENMs included in this PhD research covered a range in physicochemical characteristics (e.g., size, shape, coating, and surface charge) as well as OECD representative Ag nanomaterials and a consumer product (Table 1, Table 3, Figure 3).

Silver nitrate, $AgNO_3$ (p.a. quality, Sigma-Aldrich) was included in all experiments conducted in this PhD research as a source of dissolved Ag, and as a reference for dissolved Ag toxicity.

Mesosilver (Purest Colloids, Inc, Westampton, NJ, USA) is a commercial colloidal product advertised as a food additive and a cosmetic product (skin conditioner) and produced the same way as nanosilver in washing machines. According to the manufacturer the particle size is 0.65 nm, the surface area is 104.7 cm^2/mL, and it is purchased as a 20 mg L^{-1} colloidal suspension. Mesosilver has previously been reported to induce toxicity in both *C. elegans* (Ellegaard-Jensen et al., 2012), marine algae (Echavarri-Bravo et al., 2017) and Atlantic salmon (Farmen et al., 2012). This Ag ENM is tested in Paper II.

NM300K is an OECD representative Ag nanomaterial and provided by Joint Research Center Reference Nanomaterial Repository (Ispra, Italy). To date the NM300K ENMs have been used in

a range of studies undertaking investigations into speciation, characterization as well as toxicity studies (Voelker et al., 2015, Köser et al., 2017). Inclusion of NM300K material in the research should help comparison with previous and future studies, as well as contributing to providing a database on this potential reference material. It is supplied as aqueous suspensions dispersed in 4 % Polyoxyetylene Glycerol Trioleate and Tween 20, with a total Ag content of 10.16 % (w/w). According to the material information sheet, these are spherical Ag nanoparticles where 90 % of the particles are < 20 nm. This Ag ENM is tested in Paper I, III and IV.

NM302 is, as NM300K, an OECD representative Ag nanomaterial and also provided by Joint Research Center Reference Nanomaterial Repository. In addition to being an OECD representative Ag nanomaterial, its rod shape allows to investigate the effect of shape on toxicity. This nanomaterial is dispersed in the additives rheology modifiers (≤ 2 weight %), polymers and surfactants (≤ 1 weight %) and has a total Ag content of 7.4 weight %. It mainly consists of Ag rods and according to the material information sheet these rods are 100 to 200 nm thick and 5 to 10 μm long. This Ag ENM is tested in Paper II.

Uncoated Ag NP (QSI-nano silver, Quantum Sphere), is available for purchase as a powder and according to the product profile data sheet they have an average particle size between 20 and 40 nm and a specific surface area of 15-25 m^2/g. Previous studies conducted in by our group has measured this Ag ENM to be 20 nm (Oughton et al., 2008, Coutris et al., 2012). Due to the relative small size of this ENM, the fact that they are uncoated, and their suitability to neutron activation given they are delivered as a powder this ENM was included in the dietary exposure using radiolabeled Ag sources (Paper III).

Citrate stabilized Ag NP**s** were synthesized by sodium borohydride reduction according to Doty et al. (2005) (details can be found in Paper III), and used as a suspension. Citrate stabilized Ag NPs are one of the more frequently applied Ag NPs in ecotoxicity testing as a representative of an electrochemically stabilized nanomaterial. The citrate keeps the ENMs relatively dispersed and is a dispersing agent without any toxicity of its own. This ENM is tested in Paper III.

Table 1. Overview of the experiments conducted within this PhD research work.

Species	Source of Ag	Exposure concentrations#	Route of exposure	Exposure media	Endpoints	Characterization techniques
C. elegans	$AgNO_3$ NM300K Ag NPs NM302 Ag NPs	0.1 - 4 mg Ag L^{-1} 0.1 - 4 mg Ag L^{-1} 0.1 - 4 mg Ag L^{-1}	Combined waterborne and dietary	US EPA moderately hard reconstituted water(MHRW)	Accumulation Growth inhibition Fertility Reproduction	TEM DLS Size-fractionation* ICP-MS
R. subcapitata	$AgNO_3$ Mesosilver NM300K Ag NPs NM302 Ag NPs	0.3 - 25 µg Ag L^{-1} 5.4 - 53 µg Ag L^{-1} 2 - 24 µg Ag L^{-1} 0.26-25.6 mg Ag L^{-1} (nominal)	Waterborne	OECD 201	Growth inhibition	TEM DLS Size-fractionation* ICP-MS
Salmo salar	Radiolabeled: $AgNO_3$ Uncoated Ag NPs Citrate stabilized Ag NPs	Waterborne/dietary 3.0±0.4 µg Ag L^{-1}/0.6 mg Ag kg^{-1} fish[a] - / 0.6 mg Ag kg^{-1} fish[a] 3.0±1.2 µg Ag L^{-1}/0.6 mg Ag kg^{-1} fish[a]	Waterborne Dietary	US EPA very soft reconstituted water (VSRW)	Accumulation and organ distribution	TEM DLS Size-fractionation* ICP-MS
Salmo trutta	$AgNO_3$ NM300K Ag NPs	Waterborne: 2.0, 4.8, and 9.6 µg Ag L^{-1} Dietary: 15±2 µg Ag kg^{-1} fish[b] Waterborne: 1.6, 4.4, and 7.6 µg Ag L^{-1} Dietary: 11±1 µg Ag kg^{-1} fish[b]	Waterborne Dietary	Local drinking water	Organ accumulation Physiological effects Gene expression	TEM DLS Size-fractionation* ICP-MS spICP-Ms

* The size fractionation separated the total Ag concentration into size fractions > 220 nm, <220nm>3/10 kDa, < 3/10 kDa, by filtration through a 0.22 µm membrane syringe filter (Millipore) (not conducted in Paper I) and either hollow-fiber cross-flow ultrafiltration (Pall Microzoa Hollow Fiber Module) (Paper III and Paper IV) or with ultracentrifugation filter (Amicon Ultra-15 centrifugal filters) (Paper I and II) both with a molecular mass cut off of 3 or 10 kDa.

[a] Concentration of fish food was 3 kBq/g food, which was approximately 60 µg Ag/g food, and administered as a one-time dose 1 % of the fish body weight.

[b] The fish food was contaminated with either 17 ± 1 µg Ag/g food added as $AgNO_3$, or 12 ± 0.9 µg Ag/g food added as NM300K Ag NPs, daily fed 2 % of body weight and fed for 4 days.

Exposure concentration ranges were selected according to available toxicity data for *C. elegans* and *R. subcapitata*, while for the fish species these were selected to be non-lethal.

Table 2. The chemical parameters for the four different media used in the four experimental set-ups in this PhD research.

		Concentration of key components in exposure media (mg L^{-1})						pH	Conductivity	Ionic strength
		Mg	Ca	Cl	K	Na	S		µS/cm	mM
C. elegans [#]	US EPA MHRW: $NaHCO_3$, $CaSO_4$ $2H_2O$, $MgSO_4$, KCl	12	14	2	2	26	27	(7.4-7.8)[a]	306	4.9[¤]
*R. subcapitata**	OECD TG 201: $NaHCO_3$, NH_4Cl, $CaCl_2$, $MgSO_4$	2.8±0.1	4.7±0.1	17.8	0.6	13.5	1.9±0.1	7.9±0.1	NM	1.7
Salmo salar [#]	US EPA VSRW: $NaHCO_3$, $CaSO_4$ $2H_2O$, $MgSO_4$, KCl	1.5	1.7	0.2	0.3	3.2	3.4	6.8±0.2 (6.4-6.8)[a]	44±1	0.70[¤]
Salmo trutta	Local drinking water	0.4±0.03	4.5±0.3	2.3±0.2	0.4±0.1	3.9±0.3	1.3±0.1	7.1±0.2	47±2	0.75[¤]

[#] Concentrations calculated from the added main components given in the United States Environmental Protection Agency 2002

[¤] Ionic strength (mM) calculated from the Conductivity (µS/cm) according to Ionic strength (mol/L) = $1.6\ 10^{-5}$ × EC (µS/cm) (aqion, 2018.)

* Cl, K and Na concentrations are calculated from the main media components given in the ISO 8692

[a] pH range given in the protocol from United States Environmental Protection Agency 2002

NM = Not measured

3.3 Characterization of Ag materials

Characterization of the pristine nanoparticles and stock suspensions (in MQ) is considered good practice within nanotoxicology/ecotoxicology offering a reference point to compare against the biological media and other research. Therefore, the primary particle size, primary particle shape and the presence of aggregates, of all tested Ag ENMs, were determined with transmission electron microscopy (TEM) prior to addition to the exposure media. The hydrodynamic diameter was determined with dynamic light scattering (DLS) prior to addition to exposure media, so was zeta potential as a measure of relative surface charge. Characterization of the Ag ENMs during exposure in the exposure media and in the presence of the test organisms are also important for the understanding of transformation processes of the nanomaterials, as well as the mechanisms of accumulation and toxicity. The organisms can influence the behavior of the ENMs due to interactions with exudates/mucus or changes in media characteristics such as pH. However, exposure characterization can be challenging due to the complexity of the exposure and even more so in environmental samples. Colloids, other than the nanomaterials, already present in the exposure media giving a particle background masking the detection of the ENMs by techniques like NTA and DLS. There is no "perfect" technique of characterization, thus a combination of several are usually applied to obtain as complete a picture as possible. In this PhD research, TEM and DLS of stock suspensions were combined with nanoparticle tracking analysis (NTA) (Paper I), DLS (Paper I and Paper II), and size fractionation techniques of exposure suspensions (all papers). Size fractionation was performed on all exposure solutions, to separate the total Ag concentration into size fractions (> 220 nm, < 220 nm>3/10 kDa, and < 3/10 kDa). This was obtained by filtration through a 0.22 µm membrane syringe filter (Millipore) and either hollow-fiber cross-flow ultrafiltration (Pall Microzoa Hollow Fiber Module) (Paper III and Paper IV), or with ultracentrifugation filter (Amicon Ultra-15 centrifugal filters) (Paper I and II), both with a molecular mass cut off of 3 or 10 kDa. All analytical methods have potential problems, for example, the drying ENMs in suspension during sample preparation for TEM or massive dilution of a sample before analyzing it with spICP-MS. The greatest pitfalls using membrane filtration is related to clogging of the filter and thus changing the actual pore diameter of the filter producing filtrates with undefined size. A problem which increases with humic concentrations as well as the size and concentration of the ENM. With trace elements in natural samples, as well as at low exposure concentrations, adsorption to, for example the plastic filter containers could be a problem. However, the waters/media used in the current research work were not natural waters with

potential high humic levels, but rather controlled synthetically produced water/media. Thus, the concentration of the ENMs and the degree of aggregation is what could cause clogging of the filters. The exposure concentrations in the different experiment was however low (μg Ag L^{-1}), with exception of the *C. elegans* exposures where the exposure concentrations were in the low mg Ag L^{-1} range (Table 1). To avoid adsorption to the ultrafiltration tubes, they were conditioned before use. The use of cross-flow ultrafiltration utilizing a hollow fiber is a filtration method minimizing the problems with clogging due to the high tangential flow rate, the cross flow filter flow is relatively low (Salbu et al., 1985).

All ICP-MS related measurements as well as the NTA were conducted either by technical staff or by co-authors of the articles, and not by the PhD candidate personally.

Table 3. Size characteristics of the Ag nanomaterials (in stock suspensions) used in this research, as measured by transmission electron microscopy (TEM) (primary particle size), dynamic light scattering (hydrodynamic diameter: Z-averaged and by Number mean). The polydispersity index (PDI) given as a measure of the degree of polydisperisty in the sample ranging from 0-1, 1 indicating a highly polydisperse sample. Zeta potential (mV) included as a measure of surface charge.

			Hydrodynamic diameter ¤			
	Nominal Ag concentration in stock suspension	Primary particle size* (nm)	Z-average (nm)	Number mean (nm)	PDI	Zeta potential (mV)
NM300K	2.56 g L^{-1}	16±5 (N=383)	73.9±0.8	31±15	0.272±0.005	-4.5
Mesosilver NPs	0.020 g L^{-1}	11±3 (N= 425)	38.3±0.3	1.0±0.4	0.6±0.04	-39±2
NM302	2.56 g L^{-1}	176±41 (N=30)	NA	NA	NA	0.7
# Citrate Ag NPs	153 mg L^{-1}	4±1.6 (N=2180) 196±96 (N=147)	12.6±0.2	1.0±0.5	0.59±0.01	-40±2
Uncoated Ag NPs	2.56 g L^{-1}	34±20.8 (N=53)	195±4	27±27	0.50±0.01	-34.5

NA = Not applicable
# The two particle sizes given in the TEM results, reflects two separate size populations present in the sample.
* Primary particle size obtained by Transmission electron microscopy
¤ Hydrodynamic diameter obtained by dynamic light scattering. Z-average which is defined as the harmonic intensity averaged particle diameter (ref ISO 13321 and ISO 22412), the number mean particle size calculation from the intensity based measured results, and weighed according to particle concentration/number.

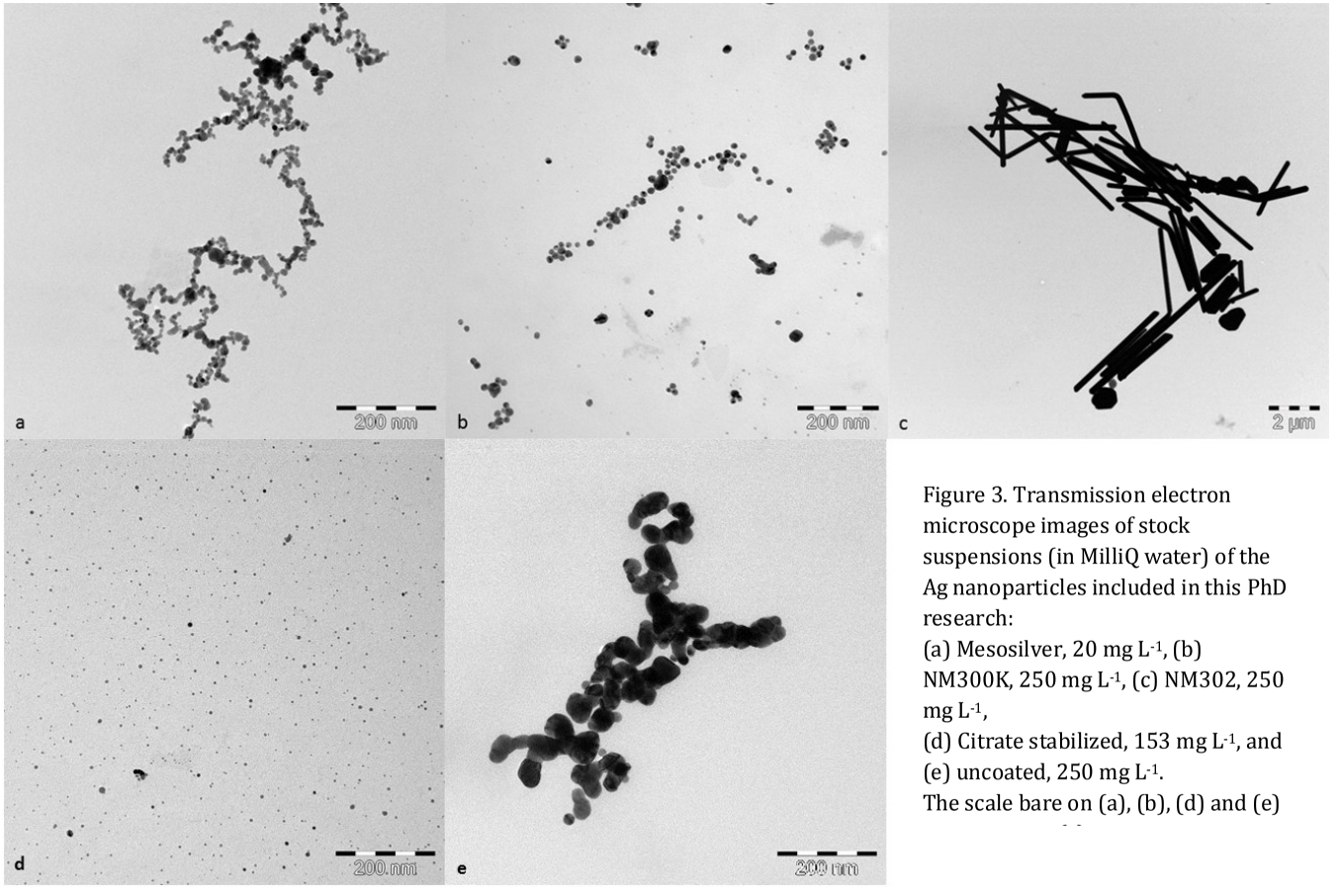


Figure 3. Transmission electron microscope images of stock suspensions (in MilliQ water) of the Ag nanoparticles included in this PhD research:
(a) Mesosilver, 20 mg L^{-1}, (b) NM300K, 250 mg L^{-1}, (c) NM302, 250 mg L^{-1},
(d) Citrate stabilized, 153 mg L^{-1}, and (e) uncoated, 250 mg L^{-1}.
The scale bare on (a), (b), (d) and (e)

3.4 Experimental designs

The experiments with *C. elegans* (Paper I) and *Salmo salar* (Paper III) were conducted at NMBU, while the waterborne and dietary exposures of *Salmo trutta* (Paper IV) were conducted at the Solbergstrand Marine Research station of NIVA, the algae experiment (Paper II) and the qPCR analysis of Paper IV were conducted at NIVA Oslo main laboratory.

3.4.1 *Caenorhabditis elegans*

In this study the hypothesis that changes in Ag ion and Ag NP speciation will cause time dependent changes in the NP fraction in the test media (hypothesis 1), which subsequently would influence the bioavailability (hypothesis 2) and toxicity (hypothesis 4), were addressed.

The relative short lifecycle of *C. elegans* allowed the study of growth from the first larval stage (L1) to full grown, reproducing adults, as well as effects on fertility and reproduction within the same toxicity test. The *C. elegans* (wild-type nematodes N2 Bristol, Caenorhabditis Genetic center, Minneapolis, USA) were chronically exposed to 0.1-4 mg Ag L^{-1} NM300K Ag NPs, NM302 and silver nitrate ($AgNO_3$) following the ISO 10872 guideline (ISO, 2010) with some modifications, mainly the change of exposure media to U.S. EPA MHRW (United States Environmental Protection Agency, 2002). The experiments were carried out as standard 96 h, at 20 °C, in the dark, in 24-well culture plates, three replicates and gently shaken to ensure sufficient oxygenation. Separate exposure plates were set up allowing the characterization of exposure suspension at time 0, 20 and 96 h by DLS and size fractionation followed by ICP-MS measurements.

This chronic exposure of *C. elegans* allows for investigation of adverse effects on growth, fertility and reproduction. These are major processes in an organism and effects on these would reflect the individual organism's ability to counteract the negative consequences of the toxic compound, and their ability to maintain homeostasis. Effects on reproduction could potentially lead to effects at the population level. All endpoints were assessed using a stereomicroscope (Leica M205C) equipped with a camera , and pictures were analyzed using an open source image processing program ImageJ (https://imagej.net) or the Leica software (LAS vs 4.4.0). The main endpoints were determined according to the ISO 10872 guideline (ISO, 2010). Growth was assessed by measuring the individual nematodes' body length, and the replicate growth, defined as the difference of the mean measured body length and mean measured body length of 40 individuals (L1) at the beginning and end of the

test. To assess fertility, the nematodes are scored as gravid (≥ 1 egg) or not, and % fertility calculated as the number of gravid exposed test organisms by the total number of recovered exposed test organisms. Reproduction is determined as the number of offspring per test organism, and was assessed by counting the number of offspring in each replicate. In addition to the standardized toxicity test, an additional experiment was conducted for determination of the potential uptake of NM300K Ag NPs, dissolved Ag ($AgNO_3$) and/or transformation products by the nematodes (details in Paper I).

3.4.2 *Raphidocelis subcapitata*

In this study the hypothesis that changes in Ag ion and Ag NP speciation will cause time dependent changes in the NP fraction in the test media (hypothesis 1) was addressed. The aim to link these changes to observed differences in toxicity, and identification of possible nanospecific component (hypothesis 4) was also investigated. The freshwater algae *R. subcapitata* (NIVA strain CHL 1 from The Norwegian Culture Collection of Algae, NORCCA, owned by NIVA, Oslo, Norway) was exposed to three different Ag NPs (NM300K, NM302, and Mesosilver (M-Ag NPs)) and $AgNO_3$ (Table 1). The tests were conducted according to the OECD 201 (OECD, 2011), with slight modifications to the growth media modified to be Fe-EDTA free. The toxicity endpoint investigated was growth inhibition as a response to exposure to different silver nanomaterials (NM300K, Mesosilver, NM302) and silver nitrate. The growth/growth inhibition was quantified by measuring the algal biomass as a function of time (72 h exposure). This was conducted by chlorophyll-a-extraction in accordance to the method specified in (Mayer et al., 1997). In addition to characterization of nanoparticle stock suspensions, exposure characterization was conducted at 0, 24, 48, and 72 h, coinciding with the measurements of algal growth every 24 h (details in Paper II).

3.4.3 Salmonids

Common for the studies with salmonids (*Salmo salar and Salmo trutta*), the hypothesis addressing time dependent changes in Ag ion and Ag NPs size fractions in the media (hypothesis 1), subsequently influencing the bioavailability and bioaccumulation of the Ag compounds (hypothesis 2) were investigated. Also hypothesis 3 addressing dietary exposure as a potential route of exposure in fish was tested in both fish experiments. In the exposure of *Salmo trutta*, the fourth hypothesis addressing the nanospecific toxicity component was also investigated.

In freshwater fish the gill is the organ for gas exchange, but also vital for homeostasis with its role in for example ion regulation and excretion of nitrogenous waste (Evans et al., 2005). It is also the main target organ for waterborne exposure to Ag as well as Ag NPs in freshwater (Handy et al., 2011). Thus, to obtain information of waterborne exposure Ag accumulation in gill was determined, in both fish studies (Paper III and IV), and included Ag accumulated in mucus and Ag adsorbed/absorbed on/in gill cells. Liver has been reported to be the main organ for accumulation and excretion in fish (Wood et al., 2012), and was therefore also sampled for measurements of accumulation of Ag in both fish studies, together with the kidney (Paper III and IV).

3.4.3.1 Salmo salar

Juvenile Atlantic salmon from the fish laboratory at NMBU were exposed, through water and diet, to different sources of Ag as radiolabeled $AgNO_3$ ($^{110m}AgNO_3$), citrate-stabilized ^{110m}Ag nanoparticles (C-Ag NPs), and uncoated ^{110m}Ag nanopowder (U-Ag NPs, only in dietary exposure) (Table 1) (Paper III). The waterborne exposure was conducted in static set up, while the dietary exposure was conducted by a one-time dose of contaminated feed (1 % of body weight) distributed by force feeding. Fish (24 fish in each treatment, 6 sacrificed at each sampling time point) were sacrificed after 3, 7, 24 and 48 h of exposure, and brain, spleen, heart, liver, bile, kidney, intestine and gut content were sampled according to a standard manual (Rosseland et al., 2001). To assess the uptake of ^{110m}Ag (originating from different sources of Ag: $^{110m}AgNO_3$ C-Ag NPs or U-Ag NPs), in the sampled tissue a NaI detector was applied. The total waterborne Ag concentration was measured along with size fractionation to obtain information of Ag speciation. The *in situ* size fractionation was performed at 0, 3, 7, 24 and 48 h, coinciding with the time points for fish sampling (details in Paper III).

Due to the use of radioactive Ag in this experiment, a flow through system was never an option. Thus, force feeding rather than conventional feeding was chosen to reduce the risk of waterborne transfer of dissolved Ag from non-eaten food added to the water and thereby also ensuring optimal

water quality. All fish were handled in accordance with the Norwegian Welfare Act and research animal legislation, and the experiments was approved in advance by the Norwegian Animal Research Authority (License number: 8059).

3.4.3.2 Salmo trutta

Juvenile brown trout (yearlings) originating from a local wild brown trout strain in Aurskog-Høland (Norway), were exposed to Ag-ions ($AgNO_3$) or Ag nanoparticles (NM300K Ag NPs) through waterborne or dietary exposure (Table 1). The waterborne exposure was conducted in a semi-static setup, with partial exposure renewal every 24 h, and given uncontaminated food once a day (1 % of body weight). The fish was exposed to 2. 5 and 10 µg Ag L^{-1}. The dietary exposure was conducted in a flow-through system, the fish were fed twice a day (in total 2 % of body weight) with food contaminated either with $AgNO_3$ or NM300K Ag NPs. The fish were exposed to a total dose of 15 ± 2 and 11 ± 1 µg Ag kg^{-1} fish for $AgNO_3$ and NM300K, respectively. After 96 h of exposure the fish were sacrificed, blood was sampled for plasma ion analysis, and organs were sampled for determination of Ag accumulation (liver, gill and kidney) and gene expression (qPCR analysis of liver tissue). The blood physiology of exposed brown trout were analyzed using a I-STAT® portable clinical analyzer (Abbott Point of Care Inc., Princeton, NJ, USA), which have proven suitable for fish blood samples to obtain rapid (*in situ*) results for several parameters (e.g., Na, Cl, and glucose) (Kroglund et al., 2007). In addition, a glucose meter (FreeStyle Lite, Blood Glucose Monitoring Systme, Abbot, East Windsor, USA), was used to measure blood glucose levels as a measure of stress response in fish. The genetic biomarkers were applied to study the systemic toxic responses by conducting a qPCR analysis on liver samples of brown trout exposed to Ag-ions ($AgNO_3$) and NM300K Ag NPs (Paper IV and unpublished material). As Ag nanomaterials have been reported to induce oxidative stress after generation of reactive oxygen species (Farmen et al., 2012), and oxidative stress may lead to activation of apoptotic pathways, the selected genes were associated with oxidative stress (Glutathione reductase, GR; Glutathione peroxidase x3, GPx3; Glutathione S transferase, GST), apoptosis (Caspase 6A, *Casp6a*; Bcl2 associated x protein, *Bax*), and metallothionein (MT). For detailed description of the experiment, see Paper IV.

All fish were handled in accordance with the Norwegian Welfare Act and research animal legislation, and the experiments was approved in advance by the Norwegian Animal Research Authority (License number: 2015/163472).

4. RESULTS

In this chapter a brief presentation of the results are given, for a more comprehensive presentation please see the papers presented at the end of this thesis.

4.1 Exposure characterization

In all waterborne exposures, regardless of organism (hence different medias, Table 2), the general findings were a decrease in total Ag concentration with time (generally higher for Ag NPs than for $AgNO_3$), higher concentration of dissolved Ag species (< 3 and 10 kDa) in $AgNO_3$ than in Ag NP exposures at all times, and a shift in Ag size fractions towards larger particulate matter (> 220 nm) over time, in both Ag NPs and $AgNO_3$ exposures (Table 4). The combination of these effects meant that the NP fraction in exposures also changed with time, decreasing by between 13 and 98 % from T=0 to the end of exposure, for Ag-NP. For $AgNO_3$, the picture was more complicated, with reductions in the dissolved fraction, combined with aggregation of colloids to larger particles, often leading to a transient increase in colloidal fraction (see *R. subcapita* and *S. trutta* data in Table 4).

Measured total concentrations were also less than nominal concentrations and decreased over the exposure period. In the NM302 Ag NP algae exposure (Paper II) the measured concentrations were far from the nominal, ranging from 79-250 µg Ag L^{-1} (at maximum 30 % at the highest concentration), and sedimentation of the NPs could be observed in the exposure vessels. The same behavior of the NM302 Ag rods was also observed in the exposures of *C. elegans* (unpublished results).

Common for all $AgNO_3$ exposures was a higher presence of dissolved Ag species (< 3/10 kDa) relative to Ag NPs under the same experimental conditions (Table 4). In the highest exposure concentration of $AgNO_3$ (25 µg Ag L^{-1}) in the algae experiment, as much as 98 % (24 µg Ag L^{-1}) of the total measured Ag was present as dissolved Ag species (< 3/10 kDa) at the beginning of the exposure. After 72 h dissolved Ag species (< 3/10 kDa) was only detected in the two highest exposure concentrations (12 and 1 µg L^{-1} in the 25 and 8 µg Ag L^{-1} exposures, respectively) (Table 4). In the other three experiments the initial dissolved Ag fraction varied between 10 and 90 % of total measured Ag concentrations depending on media, but also depending on total Ag

concentration within experiments (Table 4). In all experiments the dissolved Ag fraction decreased with time, while the presence of Ag particles increased.

Comparing different Ag ENM, the Mesosilver was the most stable Ag NP over time, with dissolved Ag and colloidal/ NP fractions (as % of total Ag) relatively constant at around 30 %. This corresponded to approximately 11 and 18 μg L^{-1} of dissolved Ag species in the two highest exposure concentrations, 35 and 53 μg Ag L^{-1}, respectively (Table 4 and Paper II Figure 1). The NM300K Ag NPs were far less stable in both the algae (Paper II) and the waterborne exposure to brown trout (Paper IV) and with no or negligible levels of dissolved Ag (< 3/10 kDa). Approximately 100 and 80 % of the Ag was defined as colloids/NPs (< 220 nm, > 3/10 kDa) at time zero in the brown trout and algae exposures, respectively (Figure 1 in Paper III and Figure 1 in Paper II). Within the first 24 h, the colloids/NP had transformed into larger particulate matter (>220 nm), accounting for approximately 50 % of total Ag concentration in both brown trout and algae experiment (Table 4). In the algae experiment, the aggregation continued throughout the exposure period resulting in on average 87 % of total Ag concentration being found as larger particulate matter (> 220 nm) after 72 h. The citrate stabilized Ag NPs in the exposure of Atlantic salmon (Paper III), also transformed into larger particulate matter with time (Table 4, Figure 2 in Paper III). The < 220 nm size fraction (colloids/NPs and dissolved Ag species) decreased from 68 to 23 % of total Ag concentration, respectively. The NM302 Ag NPs showed the same pattern in both *C. elegans (unpublished material)* and *R. subcapitata* studies. The particles did not stay suspended in the water column, but formed large clusters which quickly settled at the bottom of the exposure vessels. The NM302 Ag NPs formed large aggregates immediately after addition to the exposure media, and were not further characterized with size fractionation.

4.2 Silver concentration in organs/organisms

Accumulation of Ag in organisms after exposure to either $AgNO_3$ or Ag NPs was studied in the experiments with *C. elegans* (Paper I), Atlantic salmon (Paper III) and brown trout (Paper IV), which all showed the tested Ag compounds to be bioavailable through either waterborne or dietary exposure, or both.

The exposure of *C. elegans* to $AgNO_3$ and NM300K Ag NPs resulted in higher measured Ag concentration in nematodes exposed to NM300K Ag NPs than $AgNO_3$, prior to depuration (Table 4 in Paper I). However, after 2 hrs depuration the residual concentration of Ag was two times higher

in the $AgNO_3$ exposed nematodes (7 ± 2 vs 15 ± 3 ng mg^{-1} wet weight for NM300K Ag NPs and $AgNO_3$, respectively).

In all waterborne exposure to either Atlantic salmon (Paper III) or brown trout (Paper IV), the Ag concentrations in gill, liver and kidney were always higher in $AgNO_3$ exposures compared to Ag NP exposures. Gill concentrations of ^{110m}Ag in Atlantic salmon after exposure to $AgNO_3$ had already at the first sampling time point (3 h) reached the highest concentration, after which a slight reduction was seen over the total experimental period of 48 h (Figure 3 Paper III). In the liver, however, results showed a steady increase in ^{110m}Ag concentration over time, still increasing at the end of the 48 h experimental period. The same trends were observed for the citrate stabilized ^{110m}Ag NPs tested in this experiment, however, generally at 30 to 50 % lower Ag concentrations. In the exposure of brown trout (Paper IV), Ag concentrations in gill, liver and kidney increased with exposure concentrations after exposure to $AgNO_3$ (Figure 2 in Paper IV). Liver reached the highest Ag concentrations (in the range 5-20 μg g^{-1} dry weight, dependent on exposure concentration), being approximately 3 and 10 times higher than in kidney and gills, respectively. The NM300K Ag NPs also tested in the waterborne exposure of brown trout (Paper IV) showed no significant systemic uptake (liver and kidney) over the 96 h period of exposure (with renewal of exposure every 24 h). However, low levels (0.05 ± 0.02 and 0.08 ± 0.04 μg g^{-1} dry weight in the 5 and 10 μg Ag L^{-1} exposures) of Ag (Paper IV, Figure 2) were detected in/on gill tissue.

Table 4. Total Ag concentrations (µg L^{-1}) and size fractions in % of total Ag, in the different performed waterborne Ag exposures and organisms. The size fractionation separated the total Ag concentration into size fractions > 220 nm, <220nm>3/10 kDa, < 3/10 kDa, by filtration through a 0.22 µm membrane syringe filter (Millipore) and either hollow-fiber cross-flow ultrafiltration (Pall Microzoa Hollow Fiber Module) (Paper III and Paper IV) or with ultracentrifugation filter (Amicon Ultra-15 centrifugal filters) (Paper I and II) both with a molecular mass cut off of 3 or 10 kDa. In the *C. elegans* study, only total and < 3 kDa Ag concentrations were obtained.

			0h				24 h				48 h				72 h				96 h
				Size fractions as % of total Ag				Size fractions as % of total Ag,				Size fractions as % of total Ag,				Size fractions as % of total			
	Species	Nominal Ag concnetrations µg L^{-1}	Total Ag µg L^{-1}	Particulate >220 nm	Colloidal	<3 or10 kDa	Total Ag µg L^{-1}	Particulate >220 nm	Colloidal	<3 or10 kDa	Total Ag µg L^{-1}	Particulate >220 nm	Colloidal	<3 or10 kDa	Total Ag µg L^{-1}	Particulate >220 nm	Colloidal	<3 or10 kDa	Total Ag µg L^{-1}
$AgNO_3$	*C.elegans*	100	95	-	-	86	67	-	-	0	-	-	-	-	-	-	-	-	57
		500	518	-	-	23	423	-	-	0	-	-	-	-	-	-	-	-	427
		2000	2027	-	-	90	1960	-	-	0	-	-	-	-	-	-	-	-	2045
	R. subcapitata	0.32	0.3	44	21	34	0.1	77	18	4	0.1	83	10	7	0.1	83	13	5	-
		1	0.7	24	19	56	0.3	65	34	2	0.3	86	13	2	0.4	96	3	1	-
		3.2	2.4	21	4	75	0.7	69	30	1	0.8	88	11	1	1.6	96	4	0	-
		10	8.1	19	4	77	4.2	67	24	9	6.3	79	7	14	4.2	78	7	15	-
		32	24.6	0	4	98	21.0	24	29	48	21.0	48	18	35	22.5	31	17	52	-
	Salmo salar	6	3.1±0.1	18	22	59	2.1	52	48	0	2.8±1.9	60	40	0					-
	Salmo trutta	2	1,9	27	72	1	1.6	26	74	0	-	-	-	-	-	-	-	-	-
		5	4.8	34	56	10	4.2	37	63	0	-	-	-	-	-	-	-	-	-
		10	10.0	23	48	30	9.3	47	52	1	-	-	-	-	-	-	-	-	-
NM300K	*C.elegans*	100	81	-	-	4	79	-	-	0	-	-	-	-	-	-	-	-	72
		500	397	-	-	6	387	-	-	0	-	-	-	-	-	-	-	-	317
		2000	1555	-	-	4	1614	-	-	0	-	-	-	-	-	-	-	-	1584
	R.subcapitata	2.56	2.0	0	99	1	0.7	47	51	2	0.6	69	30	1	1.3	78	22	0	-
		4.6	3.7	0	100	0	1.7	55	44	1	1.1	87	12	1	2.1	97	2	0	-
		8.3	7.4	0	100	0	3.9	64	20	16	1.1	64	34	2	2.8	93	7	0	-
		14.9	13.8	0	100	0	6.6	55	30	15	5.1	84	14	2	7.3	84	8	8	-
		25.6	24.0	0	99	1	11.0	37	50	13	25.0	89	9	2	11.0	81	11	7	-
	Salmo trutta	2	1,7	22	78	0	1.4	53	47	0	-	-	-	-	-	-	-	-	-
		5	4.7	23	77	0	3.9	42	58	0	-	-	-	-	-	-	-	-	-
		10	8.7	25	74	1	6.9	43	57	0	-	-	-	-	-	-	-	-	-
Mesosilver	*R.subcapitata*	5	5.4	3	67	30	5.1	39	48	12	4.7	60	36	4	2.5	56	43	0	-
		10	10.7	6	59	35	9.6	24	54	22	8.8	32	47	22	8.2	47	37	16	-
		18	19.0	5	61	34	17.0	18	58	24	16.0	25	51	24	16	27	54	19	-
		32	35.0	23	46	31	30.0	17	47	37	31.0	19	42	39	26.7	16	40	44	-
		50	52.7	15	51	34	50.0	24	44	32	48.0	27	38	35	47.7	22	39	38	-
Citrate stabilized Ag NPs	*Salmo salar*	6	4.6±2.7	32	60	8	1.9±0.0	61	39	0	2.50±1.6	77	23	0	-	-	-	-	-
NM302	*C.elegans* *R.subcapitata*	The NM302 Ag NPs did not stay in suspension, but formed clusters that sedimented.																	

In the dietary exposures of both Atlantic salmon and brown trout, the Ag concentrations in gill, liver and kidney were not significantly different after exposure to $AgNO_3$ and Ag NPs, with the exception of the uncoated ^{110m}Ag NPs included in the exposure of Atlantic salmon (Paper III). The measured liver concentration/activity of ^{110m}Ag after 48 h exposure to the uncoated ^{110m}Ag NPs was 20 % lower than in the $AgNO_3$ and citrate stabilized ^{110m}Ag NP exposed fish (Paper III). In the exposure of brown trout low, but significant levels of Ag were measured in liver after dietary exposure to $AgNO_3$ and NM300K Ag NPs (1.9 ± 0.7 and 1.2 ± 0.4 μg g^{-1} dry weight for $AgNO_3$ and NM300K Ag NPs, respectively) (unpublished material).

Transfer factors (concentration of Ag in tissue or organism divided on the Ag concentration in the exposure media (water or food)) are presented in Table 5. Within each waterborne experiment, there was a higher transfer of Ag in the $AgNO_3$ exposure compared to the Ag NP exposures. The transfer was also higher in waterborne exposure compared to dietary.

4.3 Toxicity endpoints

In all tests (Paper I, II and IV), $AgNO_3$ exposures induced higher toxicity than any of the Ag NPs tested (Table 6 and 7). The algae *R. subcapitata* was the most sensitive organism towards $AgNO_3$ induced toxicity with EC_{10} of 3.36 (95 % CI: 1.58-4.70) and EC_{50} of 7.09 (95 % CI: 3.83-10.52) μg Ag L^{-1} for growth inhibition after 72 h of exposures. The nematode *C. elegans* was the least sensitive organism, with EC_{10} for growth being one order of magnitude higher than in the algae (20 fold increase), with a similar difference seen for the most sensitive endpoint, reproduction (Table 6). In the waterborne exposure to brown trout, toxicity was assessed by blood plasma parameters (blood glucose and plasma ions), as well as biomarkers for oxidative stress, apoptosis, and metallothionein. The most sensitive endpoint in the $AgNO_3$ exposure was blood glucose, with increased concentration seen following exposures to 5 μg Ag L^{-1} (Table 7). Also plasma sodium and chloride were affected, but not significantly reduced until 10 μg Ag L^{-1}. The genetic biomarkers for oxidative stress (Glutathione reductase, GR; Glutathione peroxidase x3, GPx3; Glutathione S transferase, GST) and apoptosis (Caspase 6A, *Casp6a*; Bcl2 associated x protein, *Bax*) were significantly upregulated in liver of fish exposed to the highest concentration of $AgNO_3$ after waterborne exposure. However, after dietary exposure there were no significant effects on the expression of these genetic biomarkers (unpublished material).

Table 5. Transfer factors of Ag after exposure of *C. elegans*, Atlantic salmon and brown trout to $AgNO_3$ and different Ag NPs. In *C. elegans* the transfer factors are calculated based on the Ag accumulation in the whole organism. Depurated refer to nematodes kept on agar plates seeded with *E. coli* to allow feeding of uncontaminated food for 2 h, while "undepurated" refer to nematodes collected prior to depuration. The transfer factors in the salmonids is based on Ag accumulation in gill, liver and kidney. For gill and liver in brown trout, only dry weight was obtained in this study. To enable direct comparison of the transfer factors, the wet weight was estimated assuming dry weight to be approximately 20 % of wet weight (Rosseland et al., 2007).

C. elegans (65 h)[a]					
			Undepurated	Depurated	
$AgNO_3$	Combined dietary and waterborne exposure	0.10 mg L^{-1}	2.2 (±0.1) $\times10^{-3}$		
		0.52 mg L^{-1}	1.8 (±0.1) $\times10^{-3}$	2.9 (±0.1) $\times10^{-5}$	
NM300K Ag NPs		0.08 mg L^{-1}	1.6 (±0.2) $\times10^{-3}$		
		0.40 mg L^{-1}	2.6 (±0.1) $\times10^{-3}$	1.8 (±0.5) $\times10^{-5}$	
		1.56 mg L^{-1}	2.1 (±0.1) $\times10^{-3}$	1.3 (±0.7) $\times10^{-5}$	
Salmo salar (48 h)[b]					
			Gill	Liver	Kidney
$^{110m}AgNO_3$	Waterborne	3 µg Ag L^{-1}	5.5 (±1.3) $\times10^{2}$	4.8 (±0.9) $\times10^{2}$	6 (±2.9) $\times10^{1}$
	Dietary	0.6 mg Ag kg^{-1} fish		2.3 (±1.5) $\times10^{-2}$	4 (±2.6) $\times10^{-4}$
Citrates ^{110m}Ag NPs	Waterborne	3 µg Ag L^{-1}	1.4 (±0.6) $\times10^{2}$	2.3 (±0.8) $\times10^{2}$	2.2 (±1.6) $\times10^{1}$
	Dietary	0.6 mg Ag kg^{-1} fish		2.7 (±0.96) $\times10^{-2}$	9 (±7) $\times10^{-4}$
Uncoated ^{110m}Ag NPs	Dietary	0.6 mg Ag kg^{-1} fish		3 (±1.9) $\times10^{-3}$	1 (±2) $\times10^{-4}$
Salmo trutta (96 h)[c]					
			Gill	Liver	Kidney
$AgNO_3$	Waterborne	2 µg Ag L^{-1}	8.2 (±4.1) $\times10^{1}$	5.5 (±2.6) $\times10^{2}$	2,0 (±1.1) $\times10^{2}$
		5 µg Ag L^{-1}	1.2 (±0.4) $\times10^{2}$	5.3 (±1.9) $\times10^{2}$	2,7 (±1.3) $\times10^{2}$
		10 µg Ag L^{-1}	1.3 (±0.2) $\times10^{2}$	4.1 (±0.6) $\times10^{2}$	1.9 (±1.2) $\times10^{2}$
	Dietary[#]	15±2 µg Ag kg^{-1} fish	0.3 ± 4.9	2.5 (±0.9) $\times 10^{1}$	3.2±1.7
NM300K Ag NPs	Waterborne	2 µg Ag L^{-1}	4 ± 3	NA	NA
		5 µg Ag L^{-1}	2 ± 1	NA	NA
		10µg Ag L^{-1}	2 ± 1	NA	NA
	Dietary[#]	11±1 µg Ag kg^{-1}g fish	0.2 ± 2.4	2.3 (±0.6) $\times 10^{1}$	4.4 ± 3.0

[a] Transfer factor: (mg Ag g^{-1} wet weight nematode)/(mg Ag g^{-1} water)
[b] Transfer factor: (ccpm ^{110m}Ag g^{-1} organ)/(ccpm ^{110m}Ag g^{-1} water or food)
[c] Transfer factor: (µg Ag g^{-1} organ)/(µg Ag g^{-1} water or food)
NA Not applicable due to no significant accumulation of Ag compared to control fish
[#] unpublished material

The NM300K Ag NP was tested in all three organisms (*C. elegans, R. subcapitata*, and *Salmo trutta*), and induced toxicity in both *C. elegans* (Figure 4 in Paper I) and *R. subcapitata* (Figure 3 in Paper II), the algae being the most sensitive (Table 6). In *C. elegans*, the EC values were between 7 and 12 times higher for growth and 5 to 7 times higher for reproduction after 96 h than in the $AgNO_3$ exposures (Table 6). In the exposure to the algae *R. subcapitata*, the EC values for growth inhibition obtained after 72 h for NM300K (Table 6) were around 3 times higher than for $AgNO_3$. In brown trout, neither the waterborne nor the dietary exposure to NM300K Ag NPs induced a toxic response (Table 7). There were no changes in blood plasma parameters, and due to lack of systemic accumulation, the genetic biomarkers were not analysed for the waterborne exposed fish. After dietary exposure to NM300K, there were no differentiated regulation of the genetic biomarkers compared to the controls (unpublished material).

Also the Mesosilver induced toxicity in *R. subcapitata*, similar to the toxicity observed in $AgNO_3$ exposures (Table 6, and Figure 3 in Paper II), and was by far the Ag nanomaterial that induced the highest toxicity of the ones tested in this research. It should however be noted that the toxicity of the citrate stabilized and the uncoated Ag nanoparticles included in Paper III was not assessed for toxicity, only for their accumulation and distribution in brown trout.

Table 6. Effect concentrations 10 % and 50 % (EC_{10} and EC_{50}) (*C. elegans* and *R. subcapitata*) and no-observed-effect concentrations (NOEC) and lowest-observed-effect concentrations (LOEC) (*Salmo trutta*), after exposure to $AgNO_3$ and different Ag NPs.

	***C. elegans* (96 h)**			***R. subcapitata* (72 h)**
	Growth mg Ag L^{-1}	Fertility mg Ag L^{-1}	Reproduction mg Ag L^{-1}	Growth inhibition µg Ag L^{-1}
$AgNO_3$	EC10 0.07 (0.05-0.11) EC50 0.38 (0.32-0.47)	EC10 0.08 (0.05-0.13) EC50 0.15 (0.11-0.20)	EC10 0.08 (0.05-0.13) EC50 0.15 (0.11-0.20)	EC10 3.4 (1.6-4.7) EC50 7.1 (3.8-10.5)
NM300K	EC10 0.85 (0.51-1.38) EC50 2.91 (2.37-3.90)	EC10 0.80 (0.65-0.94) EC50 1.23 (1.05-1.36)	EC10 0.52 (0.22-0.82) EC50 0.74 (0.48-0.92)	EC10 9.2 (0.7-21.9) EC50 24.2 (15.7-98.2)
Mesosilver	-	-	-	EC10 8.5 (NC) EC50 9.7 (NC)
NM302	No observed toxicity			

* The exposure of *C. elegans* was a combined waterborne and dietary exposure, however, dietary was concluded to be the main route of exposure.

Table 7. No effect concentration (NOEC) and Lowest observed effect concentration (LOEC) for *Salmo trutta* after 96 h exposure to waterborne or dietary exposure to $AgNO_3$ or NM300K Ag NPs. The NOEC is the lowest concentration where no significant difference between exposed and control fish were observed. ND = not determined.

		Blood glucose		**Blood plasma Na and Cl**		**Gene expression**	
		NOEC	LOEC	NOEC	LOEC	NOEC	LOEC
$AgNO_3$	Waterborne	2 µg Ag L^{-1}	5 µg Ag L^{-1}	5 µg Ag L^{-1}	10 µg Ag L^{-1}	5 µg Ag L^{-1}	10 µ Ag L^{-1}
	Dietary	ND	> 11±1 µg Ag kg^{-1} fish	ND	> 11±1 µg Ag kg^{-1} fish	ND	> 11±1 µg Ag kg^{-1} fish
NM300K	Waterborne	ND	> 10 µg Ag L^{-1}	ND	> 10 µg Ag L^{-1}	ND	ND
	Dietary	ND	> 15±2 µg Ag kg^{-1} fish	ND	> 15±2 µg Ag kg^{-1} fish	ND	> 15±2 µg Ag kg^{-1} fish

5. GENERAL DISCUSSION

5.1 Exposure

It was hypothesized that there would be time dependent changes in the nanoparticle fraction, defined as > 3/10 kDa and < 220 nm, in the exposure suspensions over time. In all waterborne exposures, changes in all three Ag size fractions were observed over time, for both Ag NP and $AgNO_3$ exposures, and in all test media. As described in section 4.1, the general findings were a decrease in total Ag concentration and shift in size fractions towards larger particulate matter (> 220 nm still remaining in suspension) with time, as well as higher concentration of dissolved Ag species (< 3 and 10 kDa) in $AgNO_3$ than in Ag NP exposures. The observed changes in the NP fraction in the exposures can influenced by various factors, including both changes in total concentrations as well as the relative size distribution between fractions. In turn, the results suggest that these processes depend on particle properties, concentration of NPs, test media composition and the presence of test organisms.

5.1.1. Factors influencing changes in total concentration

Observations of a reduction in total Ag concentrations in exposure media during NP toxicity test are quite common, and usually attributed to the tendency of NPs to aggregate leading to sedimentation of the larger particles. Previous studies have reported total concentrations of Ag NPs to be 35-55 % if the nominal concentration (Scown et al. 2010, Farmen et al. 2012, Baccehetta et al. 2017), which is in line with results obtained in the different experiments included in this PhD research: a reduction of 20-50 % over time, dependent on Ag NP and concentration. The reduction in total Ag concentration was generally higher for Ag NP than $AgNO_3$ exposures. In the *C. elegans* $AgNO_3$ exposure, the maximum reduction was 20 %, in comparison to a maximum of 10 % in the other test systems. In both $AgNO_3$ and Ag NP exposures, with exception of the NM300K Ag NPs, the reduction in total Ag was inversely related to the initial Ag concentration (i.e., there was a larger % reduction in low than in high initial Ag concentrations. This could reflect loss by sorption for low concentrations of $AgNO_3$ and Ag NPs, and loss due to both sorption and aggregation for Ag NPs.

5.1.2. Factors influencing changes in size distribution

In addition to reducing the total Ag concentrations in solution, particle aggregation also changed the size distribution in all test media. Since chemical properties are known to influence NP aggregation, the chemical composition of media would also be expected to influence the change in size distribution. Key factors that influence the aggregation of ENMs in natural waters, especially electrostatically stabilized ENMS, include pH, ionic strength, presence of divalent cations, presence of organic matter (type and concentration), and the concentration of the ENM (Lead et al., 2018). For Ag speciation in natural waters, pH, as well as water hardness (Ca^{2+} and Mg^{2+}), have been reported to be less important than for other trace metals. While chloride, sulfide, and organic materials strongly influence the speciation (Wood et al., 2012).

The media used in the experiments conducted during this research are all considered relatively soft waters in an international context. However, the low ionic strength waters are more relevant for the Nordic areas. The media cover a range of cationic concentrations, chloride and sulfur concentrations as well as ionic strength (Table 2). However, as they all are either synthetically made waters (MHRW, VSRW, OECD TG 201 without EDTA) or local drinking water with a relative low concentration of total organic material (3 mg L^{-1}), it was assumed that NOM (generally reported to enhance nanoparticle stability) would not be expected to play a major role in the transformation/stabilization of the Ag exposures ($AgNO_3$ or Ag NPs) under these experimental conditions. Although, as discussed below, the presence of organisms, might have released organic material. Silver is known to have high affinity towards sulfur, and complexation to sulfide (S^{2-}) is expected to occur mainly under anaerobic conditions such as, for example, in sediments and not in surface waters. Although sulfides were previously not thought to play a role in Ag behavior in surface waters, more recent studies suggest that the presence of low nanomolar levels of reduced sulfur in oxygenated freshwaters make Ag sulfide complexes the most likely speciation of Ag also in natural waters (Wood et al., 2012). The sulfur concentrations in the media used in present research were in the range of 1.3 – 3.4 mg L^{-1}, with the exception of the MHRW used in the *C. elegans* experiments which had a sulfur concentration of 27 mg L^{-1} (Table 2). However, in these synthetic, oxygenated waters, sulfur was most likely to have been present as sulfate (SO_4^{2-}) rather than reduced sulfur. Thus, formation of silver sulfide is unlikely in any of the exposures ($AgNO_3$ or Ag NPs), and sedimentation of silver sulfide would not be expected to contribute to the reduction of total Ag concentration observed in all exposures.

The algae media, OECD TG 201, had by far the highest concentrations of chloride (17.8 mg L^{-1}) compared to the media used in the experiments with Atlantic salmon (0.2 mg L^{-1}) and brown trout (2.3 mg L^{-1}). Dissolution of Ag NPs have been reported to increase with increasing chloride concentrations for both citrate stabilized and PVP coated Ag NPs (Angel et al., 2013). Thus, the dissolution of the Ag NPs in the algae experiment (Mesosilver, NM300K, and NM3002) could be expected to be higher than in the fish experiments. In the pilot study conducted for Paper III (unpublished material), an increase in dissolved Ag species (<3 kDa) from 20 to 50 % (approximately from 0.8 to 2 µg L^{-1}) of total Ag concentration was observed for the citrate stabilized ^{110m}Ag NPs. Also in the algae experiment (Paper II), an increase in dissolved Ag species (< 10 kDa) from < 1 % in all concentrations to 1.2 to 15 % was observed after the first 24 h of the NM300K Ag NP exposure. Köser et al. (2017) reported a maximum initial concentration of dissolved Ag species (< 3kDa) of 8 % of total Ag concentration, which they ascribed to dissolved Ag present in the original stock suspension bound to the dispersant agent, rather than dissolution of the NM300K Ag NPs. They further conclude that the dispersant agent would limit the access of O_2 to the surface of the NM300K Ag NPs, and hence protect it against any further release of Ag ions, at least over a short time period as a toxicity test (96 h). This would appear to contradict the results seen in the algae paper (Paper II) where both dissolution and aggregation were observed, with aggregation being the net dominant process. But, as discussed in paper II, Köser et al (2017) used orders of magnitude higher concentrations and different test media conditions. It is, however, important that free Ag ions released from the Ag NP surface would not remain as free Ag ions in the exposures for long, but rather bind to the surface of an organism or complex with, for example, chloride, thus potentially masking the dissolution of the Ag NPs. An increased dissolution of Ag NPs with higher chloride concentrations could at the same time facilitate the aggregation of the NPs by complexation of free Ag ions with chloride forming $AgCl_{(s)}$, which has been reported to form bridging structures between Ag nanoparticles and thereby facilitate aggregations (Levard et al., 2012, Li and Lenhart, 2012).

The ionic strength was also considerably higher than in the two media used in the fish experiments (1.7 mM vs approx. 0.7 mM). Increased ionic strength is known to cause aggregation of nanoparticles, as is the increased Cl and cation concentrations (El Badawy et al., 2010). These factors, and especially the higher Cl concentrations and ionic strength, could partly explain the higher degree of aggregation of the NM300K Ag NPs seen in the algae (Paper II) exposure compared to that of the brown trout (Paper III), despite the fact that sterically stabilized nanoparticles generally are less influenced by factors like ionic strength (El Badawy et al., 2010). Comparing the

citrate stabilized Ag NP with Mesosilver, they were similar in both size, shape and surface charge. However, despite the higher ionic strength and much higher Cl and Na concentrations in the algae media, the Mesosilver were more stable over time compared to the citrate stabilized Ag NPs in the Atlantic salmon exposure. This indicates that the coating of the particles, and possibly also the higher exposure concentrations used in the Mesosilver NP exposure, to be the most important factors explaining the differences in stability of these two particles. Although they both had negative relative surface charge (-40 mV), differences could reflect a possible electrosteric stabilization mechanism of the Mesosilver NPs, i.e., the particle coating has a steric stabilizing mechanism in addition to the electrostatic forces identified by the Zeta potential. Nanomaterials with electrosteric stabilization have previously been reported to show higher degree of stability (Levard et al., 2012). For an electrostatically stabilized nanomaterials, increasing concentrations of the counter ions would reduce this stability leading to aggregation. Thus additional stabilization by steric mechanisms could counteract some of this instability. It is also important to keep in mind that a lack of detected dissolved Ag (< 3/10 kDa) in the exposure media does not necessarily mean a lack of dissolution of the NPs, but rather a transformation of the Ag ions to colloidal and particulate species as result of complexation. Also association to organisms would remove the Ag ions from solution.

In addition to influencing the reduction in total Ag concentration over time, the initial total Ag concentration also was an important factor in the transformation from smaller to larger size fractions. This was well illustrated in the algae study, which demonstrated both the effect of Ag concentration on changes in size distribution as well as secondary effects caused by changes in organism effects due to toxicity effects (Paper II). For the concentration ranges used in this study: $AgNO_3$ (0.3-25 µg Ag L^{-1}) and Ag NP (4-53, and 2- 24 µg Ag L^{-1} for Mesosilver and NM300K, respectively), the general trend was the higher the total Ag concentration, the lower the generation of larger particulate matter (Paper II Figure 1, Table S2). At the end of exposure, groups with the lowest total Ag concentration (0.25 and 2.4 µ Ag L^{-1} for $AgNO_3$ and 5.4 and 10.7 µg Ag L^{-1} for Mesosilver) had >80 % and >50 % of total Ag concentration in the larger particulate fraction (>220 nm) for $AgNO_3$ and Mesosilver, respectively. In the highest exposure concentrations, only 30 % and 20 % of total Ag concentrations was found in the particulate fraction for $AgNO_3$ and Mesosilver exposures, respectively. However, the presence of the algae themselves could also facilitate the transformation of the NPs and $AgNO_3$ exposures, through algae facilitation/initiation of particle aggregation through interactions with either the algae surfaces or their secretions. It was therefore hypothesized that, in addition to the total Ag concentration itself, concentration-dependent changes

in Ag size fraction could also be influenced by toxicity driven differences in algae density. The higher total Ag concentrations induced high initial toxicity, and algae death thus highly reduced algae densities after the initial 24 h of exposure (> 80 % growth inhibition in both $AgNO_3$ and Mesosilver exposures). In the lower exposure concentrations, however, there was no or very low toxicity observed, therefore increasing algae densities with time, which could facilitate an increased removal of Ag (for further discussion see Paper II). Also in the *C. elegans* exposure the added organisms influenced the Ag size fractions present in the media. The concentrations of dissolved Ag (< 3/10 kDa) in the $AgNO_3$ exposures prior to addition of *E. coli* were between 800 and 2000 µg Ag L^{-1} depending on the total Ag concentration. After addition of *E. coli*, measured concentrations of the dissolved Ag fractions were < 0.65 µg Ag L^{-1}. Although these measurements were conducted in two different replicate experiments, they strongly indicate that *E. coli* influenced the waterborne exposure by adsorption/absorption of dissolved Ag (< 3kDa) from the exposure solution. This could have changed the route of exposure from what was initially a combined waterborne and dietary exposure to a mainly dietary exposure. Similar findings were reported by Conine and Frost (2017), where exposure of *Daphnia magna* with and without the presence of algae changed the toxicity induced by the nanoparticles. The NPs interacted with the algae, transforming the exposure from waterborne to dietary, resulting in lower toxicity to the Daphnia. These results show the importance of considering the whole exposure regime, including the test organisms themselves, as well as added organisms like *E. coli*, which is a prerequisite for the toxicity testing with *C. elegans*.

To conclude, although the dominating transformation process in the Ag NP and $AgNO_3$ exposures was aggregation, differences were seen between different Ag NPs, and in comparison to $AgNO_3$. Of all the Ag NPs tested, the Meosilver was most unaffected by the experimental conditions (e.g., chemical composition of media and the presence of organisms), which probably was an effect of the coating agent. The NM300K was the largest spherical Ag NP tested in this study as well as the only one with dispersant agents providing an initial steric stability. Despite the dispersant agent, there was a higher degree of aggregation in the NM300K Ag NP exposures over time compared to the other tested Ag NP, regardless of media. Finally, the presence of organisms influenced the Ag size distribution, initiating and facilitating aggregation, as well as adsorbing/absorbing Ag ions, thus in turn influencing the level of exposure.

5.2 Accumulation and effects

All Ag NPs tested in waterborne exposures (Mesosilver, NM300K, citrate stabilized Ag NPs), except NM302, were accumulated and/or induced toxicity in one or more of the tested organisms. However, differences were seen between different Ag NPs, between Ag NPs and $AgNO_3$, as well as for the same Ag NP in different organisms and routes of exposure. All organisms showed accumulation of Ag in tissue/whole organism after dietary exposure for all the tested Ag NPs (Citrate stabilized and Uncoated (Paper III), NM300K (Paper I and IV)), as well as $AgNO_3$, (Paper I, III, IV).

5.2.1 Bioavailability and accumulation

It was hypothesized that variation in the size fractions of Ag ions and Ag NPs in the test media would result in different bioaccumulation in the organisms. This was confirmed in the waterborne exposure of Atlantic salmon (Paper III) and brown trout (IV). The higher concentrations of Ag in gill and liver of fish exposed to $AgNO_3$ compared to Ag NPs (in both Paper III and IV) correlates with the higher concentration of dissolved Ag species (< 3/10 kDa) present in the $AgNO_3$ exposures during the exposure period. The two Ag NPs tested in the fish experiments in this PhD research, citrate stabilized Ag NPs and the NM300K Ag NPS, expressed different bioavailability, which to some degree could be linked to the Ag size fractions present in the exposure media. In the exposure to NM300K there were no dissolved Ag (< 3/10 kDa) measured in the exposure and no systemic uptake of Ag, despite Ag associated to gills. This indicates that the NM300K Ag NPs were not accumulated under the current experimental conditions, possibly as a consequence of their size and surface coating providing protection against dissolution. The citrate stabilized Ag NPs on the other hand were bioavailable, which could be linked to the initial concentration of dissolved Ag (< 3/10 kDa).

In the waterborne exposure of brown trout, the measured concentration of Ag in gill and liver after 96 h increased with increasing exposure concentration of $AgNO_3$ (Figure 2 in Paper IV). Although the gill is the primary site for interaction (and also the site of toxicity) in waterborne exposures, the accumulated Ag in liver was fivefold higher than in gill (0.8 ± 0.4 and 2.9 ± 0.9 μg g^{-1} dry weight gill versus 13 ± 5 and 20 ± 3 μg g^{-1} dry weight liver, in the 5 and 10 μg Ag L^{-1} $AgNO_3$ exposures, respectively). Also in the exposure to Atlantic salmon, the $AgNO_3$ exposure lead to higher

concentrations in the liver than in gills, thus confirming liver as the organ in freshwater fish to accumulate the highest concentration of Ag. This is in accordance with previously published studies (Galvez and Wood, 1999, Galvez et al., 2001).

Generally speaking, the accumulation of Ag NPs was lower than for $AgNO_3$ exposures, but differences were seen between different NPs. The waterborne exposure of brown trout to the NM300K Ag NP (Paper IV) showed no significant systemic uptake over the 96 h period of exposure (Figure 2 in Paper IV). However, low levels of Ag were detected in gills. Thus, the Ag associated with gills after NM300K Ag NP exposure was probably mainly a result of Ag NPs adsorption to the gill epithelia structures (e.g., mucus layer) rather than actual accumulation of Ag NPs in gill cells. Accumulation of Ag in fish after exposure to Ag NPs has been shown in several studies (Scown et al., 2010, Farmen et al., 2012, Ribeiro et al., 2014, Bruneau et al., 2016), and was also observed in Atlantic salmon (Paper III) after 48 h exposure to waterborne citrate stabilized Ag NP, resulting in Ag absorption to gill, and transfer to liver and kidney in the same order of magnitude as $AgNO_3$ (Table 5). However, considering the relative low concentration of dissolved Ag compared to the NP fraction and the small size of these Ag NPs, an uptake of the citrate Ag NPs themselves could be possible and were not ruled out in this experiment. However, free Ag ions could also dissolve from the nanoparticles attached to the gills due to the possible reduction in pH of the water when passing over the gill (Playle and Wood 1989).

We also hypothesized that diet could be a significant route of silver uptake from Ag NPs in fish. All Ag compounds ($AgNO_3$ and Ag NPs) tested in dietary exposure to Atlantic salmon, brown trout and *C. elegans* were taken up into the organisms. In the dietary exposure of Atlantic salmon (Paper III), the accumulation of Ag in liver was in the same order of magnitude for the citrate stabilized Ag NPs and $AgNO_3$, while the uncoated Ag NPs were lower, with transfer factor one order of magnitude lower than in the two other exposures (Table 5, Figure 3 in paper III). Also in brown trout (Paper IV), the accumulation of Ag in liver after dietary exposure to NM300K Ag NPs and $AgNO_3$ was similar (mean ± s.d; 1.2 ± 0.4 µg Ag/g dry weight and 1.9 ± 0.7 µg Ag/g dry weight, for NM300K and $AgNO_3$, respectively), and in the same range as reported for Zebrafish (*Danio rerio*) after exposure to biologically incorporated Ag NPs in Brine shrimp used as food (Lacave et al., 2017). Galvez et al. (1999, 2001), reported the accumulation of Ag in liver after dietary exposure of Rainbow trout (*Oncorhynchus mykiss*) to be dependent on whether the Ag was biologically incorporated into the food source, or not. When fed with food spiked with Ag_2S, the hepatic Ag concentration was 4 fold compared to the control fish. However, with biologically incorporated Ag, at much lower concentrations, the hepatic Ag concentration was 12 fold higher than in control. In this PhD

research the fold changes observed in the exposure of brown trout were 4 fold and 2 fold increase compared to the control fish for $AgNO_3$ and NM300K Ag NPs, respectively, agreeing well with the numbers reported by Galvez and coauthors.

The calculated transfer factors based on the dietary exposures of salmonids, were significantly different in the two experiments (Table 5). Differences in transfer of Ag after exposure to different Ag NPs would not necessarily be unexpected, however, one would expect the transfer of Ag after exposure to $AgNO_3$ to be more similar than the results reported here. The reasons for these differences could be related to the differences in experimental design: specifically, the differences in exposure concentrations of Ag (Table 1), preparation and distribution of the food (directly added to slurry vs processed pellets, force-feeding vs normal feeding), and exposure duration (48 vs 96 h) (detailed information in the respective papers (Paper III, and IV)). Since the waterborne transfers of $AgNO_3$ showed similar uptake between the two experiments, this is not a factor of possible changes in Ag speciation caused by the radiolabeling, and more likely due to the short exposure and single dose used in the Atlantic salmon study. A study by Clearwater et al. (2000) reported dietary uptake and distribution of Cu in Rainbow trout to occur in two phases. First, a rapid uptake by gut tissue, followed by a slower uptake by internal organs. By 72 h after the infusion only 12 % had been absorbed and distributed to internal organs. Although the experiment by Clearwater and coauthors addressed Cu, the same pattern of slower distribution to internal organs, could also be valid for Ag. Together with the differences in exposure duration in the two dietary experiments with salmonids in the current study, 48 and 96 h, this could reflect the large difference seen in dietary transfer factors between the two studies. However, comparisons between the dietary exposures for the two experiments should be done with care. The first, radiolabeled experiment was designed primarily to compare the bioavailability of Ag NP and $AgNO_3$, wherein radiolabeling offered the sensitivity needed to study gut uptake of Ag directly from the different Ag species to a variety of organs. While in the second experiment, continuous feeding allowed for a longer exposure time, but required more processing to incorporate the Ag NP and $AgNO_3$ into the food, which could also change the speciation of the Ag.

In *C. elegans* both NM300K Ag NPs and $AgNO_3$ were ingested, and the Ag concentration in the organisms before depuration (as well as after) was correlated with exposure concentrations, and reached similar levels after 65 h of exposure (Paper I, Table 4). Depuration (feeding on uncontaminated food for two hours) reduced the overall Ag content in the nematodes with > 98 % in both exposures, hence low transfer factors (Table 5) of Ag, and the remaining 0.6-2 % fraction

retained in the organisms was possibly translocated into cells from the intestinal lumen. Uptake of Ag NPs into intestinal cells has been reported by e.g., Meyer et al. (2010).

Results from the this PhD research support previous claims that, although aggregation often reduces bioavailability from waterborne NM exposures, there is no evidence that it affects bioavailability from ingested NMs (Croteau et al., 2011, 2014). In some cases aggregation can even enhance accumulation by making particles accessible or by increasing ingestion rates (Croteau et al., 2014). The accumulation of NM300K after dietary exposure to brown trout, is higher than observed in *C. elegans*. Although the nematode exposure was a combined waterborne and dietary exposure where the relative contribution to the accumulation and toxicity could not be distinguished with the current experimental design, there was strong indication of a dietary dominance. To conclude, route of exposure did influence the bioavailability and accumulation of Ag NPs and both of the dietary exposure experiments showed a similar organ accumulation for Ag NP and $AgNO_3$, thus confirming that dietary exposure is a relevant exposure route for Ag NP, but one where the exposure risks from Ag NP are likely to be similar to that of $AgNO_3$.

The factors influencing the Ag size fractions in the waterborne exposure media also influence the bioavailability and the degree of Ag transfer from media to organism. For example, the main difference between citrate stabilized Ag NPs, Mesosilver and NM300K are their size (1 vs 1 vs 31 nm), stabilizing mechanism (electrostatic for the citrate stabilized and NM300K Ag NPs vs sterically in NM300K), surface charge (-40 vs -40 vs -4.5 mV), and dissolution. However, these factors seem to be less important, or at least influenced in a different way, for the bioavailability of the Ag NPs in dietary exposures.

5.2.2 Toxicity

The overall aim of the research was to address the toxicity of Ag to different organisms after exposure to $AgNO_3$, included as a positive control due to its well-known toxicity to aquatic organisms, and different Ag NPs. More specifically, it was hypothesized that a nanospecific component of toxicity could be linked with exposure to Ag NPs. But before addressing this hypothesis, a discussion of the general toxicity results is presented first

In all tests, $AgNO_3$ exposures induced higher toxicity than any of the Ag NPs tested (Table 6), which is also commonly reported in the literature (Fabrega et al., 2011). The algae *R. subcapitata* was the

most sensitive organism towards $AgNO_3$ with induced growth inhibition after 72 h of exposure, and EC_{10} of 3.36 (95 % CI 1.58-4.70) and EC_{50} of 7.09 (95 % CI 3.83-10.52) µg Ag L^{-1}. This is in the lower range of previously reported EC_{50} values, 4.9-34 µg Ag L^{-1}, for $AgNO_3$ *in R. subcapitata* (Ribeiro et al., 2014, Sørensen and Baun, 2015, Hund-Rinke et al., 2018). The nematode *C. elegans* was the least sensitive organism with EC_{10} for growth one order of magnitude higher than in the algae (a 20 fold increase) after exposure to AgNO3, with a similar difference seen for the most sensitive endpoint, which was reproduction (Table 6).

In the waterborne exposure of brown trout, toxicity was assessed by blood plasma parameters (blood glucose and plasma ions), as well as biomarkers for oxidative stress, including metallothionein, and apoptosis. The most sensitive endpoint in the $AgNO_3$ exposure was blood glucose, with increased concentration following exposures to 5 and 10 µg Ag L^{-1}. Also plasma sodium and chloride were affected, but only reduced at the highest exposure concentration, 10 µg Ag L^{-1}. The combined results of Ag accumulation in gill, liver and kidney (i.e., elevated blood glucose and reduced plasma sodium and chloride concentrations) fit well with expectations about established Ag toxicity mechanisms. Waterborne Ag ions are related to a possible change in permeability of the gill membrane and inhibition of Na^+/K^+ ATPase, and known to result in disruption of osmoregulation (Hogstrand and Wood, 1998, Morgan et al., 2004a, Bury and Wood, 1999). In addition to impairment of osmoregulation, Ag is known to induce oxidative stress and apoptosis, which was also seen in hepatic gene expression in brown trout at the highest exposure concentrations (10 µg Ag L^{-1}). The biomarkers for oxidative stress (glutathione reductase, glutathione peroxidase x3, and glutathione S transferase) as well as apoptosis (Caspase 6A and Bcl2 associated x protein) were significantly upregulated in liver of fish exposed to the highest concentration of $AgNO_3$ (10 µg Ag L^{-1}). This shows that exposure to dissolved Ag species (after addition of $AgNO_3$) activate protection mechanisms against ROS. And although, under the current experimental conditions, the upregulation of these protective mechanisms were sufficient to protect the organisms against severe damage, they could be rendered insufficient for protection towards ROS under prolonged chronic exposure.

With respect to toxicity of Ag NPs, the general findings were a reduced toxicity compared to $AgNO_3$, regardless of organism and type of Ag NP. It should, however, be kept in mind, that in the algae study accumulation was not assessed, thus the question of whether the observed differences in response were a result of higher accumulation of Ag in the $AgNO_3$ exposures, or if the Ag NPs are less toxic despite similar accumulated concentrations of Ag could not be assessed. The NM300K Ag NPs were tested in all three organisms, and were found to induce toxicity in both *C. elegans* and *R.*

subcapitata, but not in brown trout. Levels of Ag associated with the gills of brown trout exposed to waterborne NM300K Ag NP were low, and did not result in any effects on physiological blood parameters like blood glucose, Na and Cl blood plasma levels. This further supports that the Ag was only adsorbed to the gill epithelia structures (e.g., mainly the mucus layer) rather than actually taken up in gill tissue, or transported through the gill membrane. In the exposure to the algae *R. subcapitata*, the EC values obtained after 72 h for NM300K (Table 6) were in agreement with concentrations previously reported in literature (15-140 µg Ag L^{-1}) (Ribeiro et al., 2014, Sørensen and Baun, 2015, Hund-Rinke et al., 2018), and were around 3 times higher than for $AgNO_3$. Also the Mesosilver induced toxicity in *R. subcapitata* was similar to the toxicity observed in $AgNO_3$ exposures (Table 6, and Figure 3 in Paper II). The results provided two lines of evidence that the toxicity observed in the Mesosilver and NM300K Ag NPs exposures could not be explained by the presence of Ag(I) (<10 kDa) alone, but rather a nanospecific toxicity or a combination of the two, which has also been reported by for example Sendra et al. (2017). The toxicity observed in the $AgNO_3$ and Mesosilver exposures in our study were correlated with the concentration of dissolved Ag species (<10 kDa). However, in the Mesosilver a much larger growth inhibition was seen compared to $AgNO_3$ groups with similar concentrations of dissolved Ag, thus strongly indicating an additional nanospecific toxicity. After 24 h of exposure, NM300K exposure groups displayed a significant growth inhibition in the highest exposure concentrations (14 and 24 µg Ag L^{-1}), however, the algae populations recovered over time. There was also evidence of a nanospecific induced toxicity from NM300K due to the negligible concentrations of dissolved Ag measured during the first 24 h. Nanospecific toxicity, or in a combination with Ag ion induced toxicity, have been reported for several species, including algae (Navarro et al., 2008b, Sendra et al., 2017). The general trend seen in algae growth inhibition over time (i.e., reduced effect on growth over time) was in line with the size fractionation results showing reduced concentrations of in the dissolved Ag and colloidal/NP Ag over time and an increased particulate matter >220 nm (Table 4).

Neither waterborne nor dietary exposure to NM300K Ag NPs in brown trout induced a toxic response within the experimental period, despite being bioavailable in the dietary exposure with Ag detected in liver (Figure 2 and 3 in Paper IV). Despite very low transfer factors (Table 5) and accumulated Ag concentrations (Table 4 in Paper I), the NM300K Ag NPs induced toxicity to *C. elegans* with. The EC values were between 7 and 12 times higher for growth and 5 to 7 times higher for reproduction compared with $AgNO_3$ exposures (Table 6). Accumulation and translocation of Ag from the intestinal lumen into the cells is not a prerequisite for induction of toxicity by the Ag NPs. Oxidation of the Ag NP surface, generation of reactive oxygen species and

consequently also Ag ions, have been associated with oxidative stress in the intestine leading to toxicity (Yang et al., 2012). In the *C. elegans* tests all dissolved Ag (< 3 kDa) was adsorbed to/absorbed by the *E. coli*, which could potentially increase the intake and bioavailability of Ag since the nematode feed on the bacteria. Since *C. elegans* normally feed on bacteria and particles in the size range of 100 nm to 3 µm, both dissolution of Ag ions before adsorption to *E. coli* and aggregation of NM300K Ag NPs during the exposure period, could increase the exposure and the potential for toxicity through higher uptake of Ag particles. This is in contrast to the more typical reduction in toxicity that would follow aggregation in other waterborne exposures.

The final nanomaterial tested in *C. elegans* and *R. subcapitata* studies was the NM302 Ag rods. These Ag nanorods did not induce any toxicity in either of the tested organisms. Their rather large size and instability in suspension caused aggregation and sedimentation, resulting in a lack of or low bioavailability. The results also indicate the dissolution of the NM302 Ag rods to be low considering no toxicity was observed in any of the organisms. Toxicity is often reported to increase with decreasing particle sizes, thus the lack of toxicity could be explained by the NM302 Ag rods large size. However Chae and coauthors (2016) reported a nanospecific toxicity in *D. magna* after exposure to Ag nanowires of similar size and shape to the NM302 Ag rods tested in this PhD research.

To conclude, $AgNO_3$ induced toxicity at lower exposure concentrations than any of the Ag NPs, with Mesosilver as an exception, showing similar EC values as for $AgNO_3$ exposure of algae. In the waterborne exposures of $AgNO_3$ both bioavailability, accumulation and toxicity were linked to the presence of dissolved Ag species (< 3 or 10 kDa) in the exposures. However, for both the Mesosilver and NM300K Ag NPs, there was evidence of an additional nanospecific toxicity in the algae exposures.

5.3 Environmental relevance

There are several aspects to address regarding the environmental relevance of this PhD research, specifically in light of the choice of organisms, exposure concentrations and route of exposure, and these will be briefly discussed in the following paragraphs.

With respect to the choice of organisms, these were partly dictated by the research programs funding the research, but the different organisms also reflect different aspects of environmental relevance. The algae *R. subcapitata* has an important role in freshwater ecosystems due to its role

as a primary producer and its widespread distribution. Thus, severe toxic responses such as, for example, growth inhibition of the algae population could lead to detrimental effects on parts of the ecosystem due to food web collapses (Ribeiro et al., 2015a). In addition, algae in general are known for their ability to adsorb and absorb metals, including Ag (Ratte, 1999). Thus, as well as being relatively sensitive organisms in waterborne exposures, they can also act as a dietary source of exposure. The Atlantic salmon and brown trout are both salmonids and known to be among the most valuable freshwater species of fish (Poléo et al., 1997), and relatively sensitive to environmental stressors compared to other organisms. For example, all life stages of Atlantic salmon are very sensitive to acidic waters, but the smolt stage (preparing for a life in seawater) is recognized as the most sensitive life stage towards aluminum so far tested (Rosseland and Skogheim 1984, Rosseland and Staurnes, 1994, Nilsen et al. 2010). *Chironomide* larvae which feed in the sediments, is an important food source for brown trout (Brown et al. 1980, Pechlaner and Zaderer 1985, Jenssen et al. 2010), which can be a direct link between sediment concentrated pollutants and dietary exposure to fish. Aggregation and subsequent sedimentation and removal from the water column to the sediments is a likely fate of Ag NPs in aquatic systems.

Exposure concentrations of several magnitudes above predicted environmental concentrations are often used in studies addressing toxicity of ENMs, including Ag NPs. Predicted environmental concentrations are associated with large uncertainties, but reported to be in the low μg L^{-1} range. The behavior of trace metals like Ag, at very low concentrations can be very different from at high concentrations, hence it is important to also conduct studies at environmentally relevant concentrations. In all exposures, except the *C. elegans* study (Paper I), exposure concentrations in the waterborne exposures were in the low μg Ag L^{-1} range (Table 1). The obtained EC values were, however, higher than predicted environmental concentrations. In more complex natural environments, the exposure, and thus also the bioavailability, accumulation and toxicity could also change. Most likely these changes would not lead to increased waterborne toxicity. None of the tested Ag NPs were more bioavailable, accumulated to higher levels in organisms or induced toxicity at lower concentrations than $AgNO_3$, regardless of exposure route (waterborne or dietary). However, considering the relatively acute nature of the exposures in this study, chronic exposures could result in accumulated Ag concentrations high enough to induce chronic effects.

Finally, regarding the question of whether current risk assessment criteria for Ag would be likely to protect the environment, since the results of the present research suggest that acute exposures to Ag-NPs are not more toxic than $AgNO_3$, the existing risk assessment regime is unlikely to underestimate the environmental hazards of Ag NP. However, the evidence of an Ag NP specific

component for algae toxicity, combined with the affinity of algae for absorption of Ag NP, means that care should be taken in extrapolating this conclusion to chronic exposures.

5.4 Limitations of the work

There was a number of limitations and uncertainties in the current research that should be considered, and are addressed in the following paragraphs. In general, the research would have benefitted by a more systematic testing of several Ag ENMs in the test media, to generate a more complete data basis and thus make it easier to evaluate potential correlations between factors.

It needs to be recognized that both the characteristics of the pristine nanoparticles as well as the media composition would be the driving forces behind the time dependent changes in nanoparticle fraction. With the current experimental design, identification of the individual factors' contribution to this change could not be assessed. To be able to identify the most important characteristic of the pristine nanoparticle, and how this characteristic could influence time dependent changes, one characteristic (e.g., particle size or particle coating) would have to be changed at a time, and not multiple which is the case in this PhD research. However, changing one nanoparticle characteristic at the time is rather difficult to achieve experimentally, simply because the different parameters are often interdependent on each other, so changing one leads to the change of another.

As the main research question in this research was not only to characterize the pristine Ag NPs and stock suspensions, but also to characterize them in the exposure media, a more thorough analysis of the Ag speciation would improve the ability to decipher the contribution of the individual media components relative to the observed changes in exposure, and their influence on toxicity.

In addition to size fractionation, a more qualitative and quantitative analysis of the different Ag species present could be obtained by using more advanced characterization techniques like, for example, field flow fractionation and spICP-MS. Also chemical identification (e.g., how Ag complexes were formed) of the transformation products in the exposures, characterization of the Ag NPs after incorporation into the commercial fish food, as well as their interaction with algae and the *E. coli* in the *C elegans* study would have helped to understand the mechanisms and processes better.

Accumulation of Ag after Ag NP exposure was assessed in *C. elegans*, Atlantic salmon and brown trout, by measuring total Ag concentration in organism/organs after acid digestion with ICP-MS. Technically, the accumulated Ag could be a result of Ag ion accumulation after release from the

surface of the Ag NP and not accumulation of the Ag NPs themselves. Thus, identification of Ag NPs in organism/organs by the means of imaging techniques would have been an advantage. However, identification of a nanoscale particle in an image is not necessarily evidence of biological uptake. Nanoscale particles identified inside tissue could be an actual uptake of nanoparticles, but it could also be a result of formation of a nanoscale particle after uptake of free Ag ions released from the Ag NP surface.

In the experimental design for exposure of the salmonids, the level of replication was limited. There were six to eight fish in each waterborne exposure group, and individual fish were considered as biological replicates. However, they were exposed in the same exposure tank, and should be considered as pseudo-replicates, which could potentially influence the statistical analysis. The limited number of replicates would also limit the ability of gene expression analysis (qPCR) to identify significant results when the variability between replicates is large (Paper IV).

The short exposure times 48 and 96 h will always be a challenge when we want to predict the consequences in nature. This is illustrated in the experiment on dietary exposure of brown trout, where the liver concentration of Ag NPs continued to increase until the end of the exposure period, without any systemic effects on physiological parameters (Figure 2, Table 4 in Paper IV). Chronic exposure, as could be expected in nature, might have led to a "saturation" of the metallothionein binding resulting in cell death, release of Ag(I) from the liver tissue, and then led to systemic effects and maybe mortality.

Algae in general are known for their ability to adsorb and absorb metals, including Ag (Ratte, 1999), which is a challenge in toxicity tests with static set ups. Because the algae so effectively interact with silver (as well as other trace metals) they remove Ag from suspension. At lower exposure concentrations this removal could lead to a recovery of the algae population, which could mask an initial toxicity of the compound tested. In nature, chronic exposure could eventually lead to a toxic accumulation. Thus, the optimal setup would be a flow-through system. However, this is not without its practical limitations, and challenging when testing nanoparticles due to the instability of many nanoparticle suspensions.

Other sources of uncertainties in this PhD research include those related to the chemical analysis (uncertainty of ICP-MS), size characterization of Ag NPs with DLS as well as TEM. In TEM, the particle size obtained for Mesosilver was most likely an overestimation due to the very small size which made it difficult to include in the image analysis. In addition TEM is known for underestimating the size of NPs with organic coatings due to the lack of visibility of the coating with

this technique. However, despite the contribution of uncertainty from the analytical methods, these contribute a rather minor part of the overall uncertainty associated with NP ecotoxicological studies, and more likely eclipsed by instability of the exposure conditions.

6. CONCLUSIONS AND PERSPECTIVES

In all exposures, across organisms, there was a change in Ag size fractions over time. Aggregation being the net dominant process, resulting in a decrease in NP (> 3 kDa and < 220 nm) and dissolved Ag fractions (< 3 or 10 kDa) and an increase in larger particulate matter (> 220 nm) with time. In the waterborne exposures accumulation, bioavailability and toxicity were linked to the presence of dissolved Ag species in the exposure. However, for the Mesosilver NPs and NM300K Ag NPs, there were also indications of additional nanospecific toxicities in the algae exposures. Silver nanoparticles show a potential for dietary uptake, with hepatic Ag concentrations reaching similar levels as those seen after exposure to $AgNO_3$. However, no negative effects were detected in fish after dietary exposure.

Since the toxicity was always highest after exposure to $AgNO_3$, the hazard assessment of Ag ENMs should be sufficiently covered by the already existing risk assessment and regulations for Ag, however, knowledge gaps remain concerning chronic exposure and different exposure routes.

Identifying the main transformation processes, their products and linking these to the pristine NP characteristics on one side and the bioavailability and toxicity on the other side is one of the major topics for future research. Studies of bioavailability, accumulation and toxicity could be made more applicable by using environmentally relevant concentrations as well as relevant NPs (e.g., NPs leaching from consumer products). Conducting toxicity studies using aged/transformed NPs, in addition to being environmentally relevant, might also reduce the variability and uncertainties seen in toxicity testing with NPs today. In combination with the use of standardized operating procedures and defined reference NP materials this could improve the basis of risk assessment and regulation.

REFERENCES

ADAMS, N. W. & KRAMER, J. R. 1999. Determination of silver speciation in wastewater and receiving waters by competitive ligand equilibration/solvent extraction. *Environmental toxicology and chemistry,* 18**,** 2674-2680.

ANGEL, B. M., BATLEY, G. E., JAROLIMEK, C. V. & ROGERS, N. J. 2013. The impact of size on the fate and toxicity of nanoparticulate silver in aquatic systems. *Chemosphere,* 93**,** 359-365.

AQION 2018. *http://www.aqion.de/site/130*.

ASHARANI, P., WU, Y. L., GONG, Z. & VALIYAVEETTIL, S. 2008. Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology,* 19**,** 255102.

BAALOUSHA, M., CORNELIS, G., KUHLBUSCH, T., LYNCH, I., NICKEL, C., PEIJNENBURG, W. & VAN DEN BRINK, N. 2016. Modeling nanomaterial fate and uptake in the environment: current knowledge and future trends. *Environmental science: nano,* 3**,** 323-345.

BENN, T., CAVANAGH, B., HRISTOVSKI, K., POSNER, J. D. & WESTERHOFF, P. 2010. The release of nanosilver from consumer products used in the home. *Journal of environmental quality,* 39**,** 1875-1882.

BENN, T. M. & WESTERHOFF, P. 2008. Nanoparticle silver released into water from commercially available sock fabrics. *Environmental science & technology,* 42**,** 4133-4139.

BIELMYER, G. K., BELL, R. A. & KLAINE, S. J. 2002. Effects of ligand-bound silver on *Ceriodaphnia dubia*. *Environmental toxicology and chemistry,* 21**,** 2204-2208.

BLEEKER, E. A. J., DE JONG, W. H., GEERTSMA, R. E., GROENEWOLD, M., HEUGENS, E. H. W., KOERS-JACQUEMIJNS, M., VAN DE MEENT, D., POPMA, J. R., RIETVELD, A. G., WIJNHOVEN, S. W. P., CASSEE, F. R. & OOMEN, A. G. 2013. Considerations on the EU definition of a nanomaterial: Science to support policy making. *Regulatory toxicology and pharmacology,* 65**,** 119-125.

BOVERHOF, D. R., BRAMANTE, C. M., BUTALA, J. H., CLANCY, S. F., LAFRANCONI, M., WEST, J. & GORDON, S. C. 2015. Comparative assessment of nanomaterial definitions and safety evaluation considerations. *Regulatory toxicology and pharmacology,* 73**,** 137-150.

BRUNEAU, A., TURCOTTE, P., PILOTE, M., GAGNE, F. & GAGNON, C. 2016. Fate of silver nanoparticles in wastewater and immunotoxic effects on rainbow trout. *Aquatic toxicology (Amsterdam, Netherlands),* 174**,** 70-81.

BROWN, A.E., OLDHAM, R.S.AND WARLOW, A. 1980. *Chironomidae*. In: Murray, A. (Ed.) Ecology, Systematics Cytology and Physiology. Page 323-329. (https://doi.org/10.1016/B978-0-08-025889-8.50050-7)

BURY, N. R. & WOOD, C. M. 1999. Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na+ channel. *American journal of physiology-regulatory integrative and comparative physiology,* 277**,** R1385-R1391.

CARLSON, C., HUSSAIN, S. M., SCHRAND, A. M., BRAYDICH-STOLLE, L. K., HESS, K. L., JONES, R. L. & SCHLAGER, J. J. 2008. Unique Cellular Interaction of Silver Nanoparticles: Size-Dependent Generation of Reactive Oxygen Species. *Journal of physical chemistry B,* 112**,** 13608-13619.

CHAE, Y. & AN, Y.-J. 2016. Toxicity and transfer of polyvinylpyrrolidone-coated silver nanowires in an aquatic food chain consisting of algae, water fleas, and zebrafish. *Aquatic Toxicology,* 173**,** 94-104.

CHAE, Y. J., PHAM, C. H., LEE, J., BAE, E., YI, J. & GU, M. B. 2009. Evaluation of the toxic impact of silver nanoparticles on Japanese medaka (*Oryzias latipes*). *Aquatic toxicology,* 94**,** 320-327.

CHRISTIAN, P., VON DER KAMMER, F., BAALOUSHA, M. & HOFMANN, T. 2008. Nanoparticles: structure, properties, preparation and behaviour in environmental media. *Ecotoxicology,* 17**,** 326-343.

CLEARWATER, S. J., BASKIN, S. J., WOOD, C. M. & MCDONALD, D. G. 2000. Gastrointestinal uptake and distribution of copper in rainbow trout. *Journal of experimental biology,* 203**,** 2455-2466.

COMMISSION., E. 2011. Commission recommendation of 18 October 2011 on the definition of nanomaterial (2011/696/EU). *https://ec.europa.eu/research/industrial_technologies/pdf/policy/commission-recommendation-on-the-definition-of-nanomater-18102011_en.pdf*.
CONINE, A. L. & FROST, P. C. 2017. Variable toxicity of silver nanoparticles to *Daphnia magna*: effects of algal particles and animal nutrition. *Ecotoxicology,* 26**,** 118-126.
COUTRIS, C., JONER, E. J. & OUGHTON, D. H. 2012. Aging and soil organic matter content affect the fate of silver nanoparticles in soil. *Science of the total environment,* 420**,** 327-333.
CRANE, M., HANDY, R. D., GARROD, J. & OWEN, R. 2008. Ecotoxicity test methods and environmental hazard assessment for engineered nanoparticles. *Ecotoxicology,* 17**,** 421.
CROTEAU, M.-N., MISRA, S. K., LUOMA, S. N. & VALSAMI-JONES, E. 2011. Silver bioaccumulation dynamics in a freshwater invertebrate after aqueous and dietary exposures to nanosized and ionic Ag. *Environmental science & technology,* 45**,** 6600-6607.
CROTEAU, M.-N. L., MISRA, S. K., LUOMA, S. N. & VALSAMI-JONES, E. 2014. Bioaccumulation and toxicity of CuO nanoparticles by a freshwater invertebrate after waterborne and dietborne exposures. *Environmental science & technology,* 48**,** 10929-10937.
DOTY, R. C., TSHIKHUDO, T. R., BRUST, M. & FERNIG, D. G. 2005. Extremely stable water-soluble Ag nanoparticles. *Chemistry of materials,* 17**,** 4630-4635.
ECHAVARRI-BRAVO, V., PATERSON, L., ASPRAY, T. J., PORTER, J. S., WINSON, M. K. & HARTL, M. G. 2017. Natural marine bacteria as model organisms for the hazard-assessment of consumer products containing silver nanoparticles. *Marine environmental research,* 130**,** 293-302.
EL BADAWY, A. M., LUXTON, T. P., SILVA, R. G., SCHECKEL, K. G., SUIDAN, M. T. & TOLAYMAT, T. M. 2010. Impact of Environmental Conditions (pH, Ionic Strength, and Electrolyte Type) on the Surface Charge and Aggregation of Silver Nanoparticles Suspensions. *Environmental science & technology,* 44**,** 1260-1266.
ELLEGAARD-JENSEN, L., JENSEN, K. A. & JOHANSEN, A. 2012. Nano-silver induces dose-response effects on the nematode *Caenorhabditis elegans*. *Ecotoxicology and environmental safety,* 80**,** 216-223.
EVANS, D. H., PIERMARINI, P. M. & CHOE, K. P. 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological reviews,* 85**,** 97-177.
FABREGA, J., FAWCETT, S. R., RENSHAW, J. C. & LEAD, J. R. 2009. Silver nanoparticle impact on bacterial growth: effect of pH, concentration, and organic matter. *Environmental science & technology,* 43**,** 7285-7290.
FABREGA, J., LUOMA, S. N., TYLER, C. R., GALLOWAY, T. S. & LEAD, J. R. 2011. Silver nanoparticles: Behaviour and effects in the aquatic environment. *Environment international,* 37**,** 517-531.
FARMEN, E., MIKKELSEN, H. N., EVENSEN, O., EINSET, J., HEIER, L. S., ROSSELAND, B. O., SALBU, B., TOLLEFSEN, K. E. & OUGHTON, D. H. 2012. Acute and sub-lethal effects in juvenile Atlantic salmon exposed to low mu g/L concentrations of Ag nanoparticles. *Aquatic toxicology,* 108**,** 78-84.
FEYNMAN, R. 1960. There's plenty of room at the bottom. *Engineering and Science* 23**,** 22-36.
GALVEZ, F., HOGSTRAND, C., MCGEER, J., BUREAU, D. & WOOD, C. M. The physiological effects of dietary silver exposure in rainbow trout (*Oncorhynchus mykiss*). Proceedings, 1996. 25-28.
GALVEZ, F., HOGSTRAND, C., MCGEER, J. C. & WOOD, C. M. 2001. The physiological effects of a biologically incorporated silver diet on rainbow trout (*Oncorhynchus mykiss*). *Aquatic toxicology,* 55**,** 95-112.
GALVEZ, F. & WOOD, C. M. 1999. Physiological effects of dietary silver sulfide exposure in rainbow trout. *Environmental toxicology and chemistry,* 18**,** 84-88.

GIESE, B., KLAESSIG, F., PARK, B., KAEGI, R., STEINFELDT, M., WIGGER, H., GLEICH, A. & GOTTSCHALK, F. 2018. Risks, Release and Concentrations of Engineered Nanomaterial in the Environment. *Scientific reports,* 8**,** 1565.

GOTTSCHALK, F., LASSEN, C., KJOELHOLT, J., CHRISTENSEN, F. & NOWACK, B. 2015. Modeling flows and concentrations of nine engineered nanomaterials in the Danish environment. *International journal of environmental research and public health,* 12**,** 5581-5602.

HANDY, R. 2012. Environmental toxicity of engineered nanomaterials: Focus on aquatic species. *Toxicology letters,* 211**,** S12-S13.

HANDY, R. D., AL-BAIRUTY, G., AL-JUBORY, A., RAMSDEN, C. S., BOYLE, D., SHAW, B. J. & HENRY, T. B. 2011. Effects of manufactured nanomaterials on fishes: a target organ and body systems physiology approach. *Journal of fish biology,* 79**,** 821-853.

HANDY, R. D., HENRY, T. B., SCOWN, T. M., JOHNSTON, B. D. & TYLER, C. R. 2008b. Manufactured nanoparticles: their uptake and effects on fish-a mechanistic analysis. *Ecotoxicology,* 17**,** 396-409.

HANDY, R. D. & SHAW, B. J. 2007. Toxic effects of nanoparticles and nanomaterials: implications for public health, risk assessment and the public perception of nanotechnology. *Health, risk & society,* 9**,** 125-144.

HASSELLÖV, M. & KAEGI, R. 2009. *Analysis and characterization of manufactured nanoparticles in aquatic environments*, John Wiley & Sons, Inc.: United Kingdom.

HIRIART-BAER, V. P., FORTIN, C., LEE, D.-Y. & CAMPBELL, P. G. 2006. Toxicity of silver to two freshwater algae, *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata*, grown under continuous culture conditions: influence of thiosulphate. *Aquatic toxicology,* 78**,** 136-148.

HOEKSTRA, J. & VAN EWIJK, P. 1993. Alternatives for the no-observed-effect level. *Environmental toxicology and chemistry,* 12**,** 187-194.

HOGSTRAND, C., GROSELL, M., WOOD, C. M. & HANSEN, H. 2003. Internal redistribution of radiolabelled silver among tissues of rainbow trout (*Oncorhynchus mykiss*) and European eel (*Anguilla anguilla*): the influence of silver speciation. *Aquatic toxicology,* 63**,** 139-157.

HOGSTRAND, C. & WOOD, C. M. 1998. Toward a better understanding of the bioavailability, physiology and toxicity of silver in fish: Implications for water quality criteria. *Environmental toxicology and chemistry,* 17**,** 547-561.

HUND-RINKE, K., SCHLICH, K., KÜHNEL, D., HELLACK, B., KAMINSKI, H. & NICKEL, C. 2018. Grouping concept for metal and metal oxide nanomaterials with regard to their ecotoxicological effects on algae, daphnids and fish embryos. *NanoImpact,* 9**,** 52-60.

HUNT, P. R. 2017. The *C. elegans* model in toxicity testing. *Journal of applied toxicology,* 37**,** 50-59.

ISO 2010. Water quality: Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans* (Nematoda).

ISO 2015. International Organization for Standardization. Nanotechnologies -Vocabulary-Part 1: Core terms. ISO/TS 80004-1:2015.

KAEGI, R., SINNET, B., ZULEEG, S., HAGENDORFER, H., MUELLER, E., VONBANK, R., BOLLER, M. & BURKHARDT, M. 2010. Release of silver nanoparticles from outdoor facades. *Environmental pollution,* 158**,** 2900-2905.

KLAINE, S. J., ALVAREZ, P. J., BATLEY, G. E., FERNANDES, T. F., HANDY, R. D., LYON, D. Y., MAHENDRA, S., MCLAUGHLIN, M. J. & LEAD, J. R. 2008. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. *Environmental toxicology and chemistry,* 27**,** 1825-1851.

KLAINE, S. J., KOELMANS, A. A., HORNE, N., CARLEY, S., HANDY, R. D., KAPUSTKA, L., NOWACK, B. & VON DER KAMMER, F. 2012. Paradigms to assess the environmental impact of manufactured nanomaterials. *Environmental toxicology and chemistry,* 31**,** 3-14.

KROGLUND, F., FINSTAD, B., STEFANSSON, S. O., NILSEN, T. O., KRISTENSEN, T., ROSSELAND, B. O., TEIEN, H. C. & SALBU, B. 2007. Exposure to moderate acid water and aluminum reduces Atlantic salmon post-smolt survival. *Aquaculture,* 273**,** 360-373.

KÖSER, J., ENGELKE, M., HOPPE, M., NOGOWSKI, A., FILSER, J. & THOMING, J. 2017. Predictability of silver nanoparticle speciation and toxicity in ecotoxicological media. *Environmental science-nano,* 4**,** 1470-1483.

JENSSEN, M.T.S, BORGSTRØM, R., SALBU, B. AND ROSSELAND, B.O. 2010. The European minnow *Phoxinus phoxinus* (L.) in the the subalpine lake, Øvre Heimdalsvatn, has higher mercury concentrations than the top predator, brown trout *Salmo trutta* L. *Hydrobiologia,* 642, 115 – 126.

LACAVE, J. M., FANJUL, A., BILBAO, E., GUTIERREZ, N., BARRIO, I., AROSTEGUI, I., CAJARAVILLE, M. P. & ORBEA, A. 2017. Acute toxicity, bioaccumulation and effects of dietary transfer of silver from brine shrimp exposed to PVP/PEI-coated silver nanoparticles to zebrafish. *Comparative biochemistry and physiology Part C-Toxicology & pharmacology,* 199**,** 69-80.

LEAD, J. R., BATLEY, G. E., ALVAREZ, P. J., CROTEAU, M. N., HANDY, R. D., MCLAUGHLIN, M. J., JUDY, J. D. & SCHIRMER, K. 2018. Nanomaterials in the Environment: Behavior, Fate, Bioavailability, and Effects—An Updated Review. *Environmental toxicology and chemistry*.

LEE, D.-Y., FORTIN, C. & CAMPBELL, P. G. 2005. Contrasting effects of chloride on the toxicity of silver to two green algae, *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*. *Aquatic toxicology,* 75**,** 127-135.

LEVARD, C., HOTZE, E. M., LOWRY, G. V. & BROWN JR, G. E. 2012. Environmental transformations of silver nanoparticles: impact on stability and toxicity. *Environmental science & technology,* 46**,** 6900-6914.

LI, X. & LENHART, J. J. 2012. Aggregation and dissolution of silver nanoparticles in natural surface water. *Environmental science & technology,* 46**,** 5378-5386.

LIM, D., ROH, J. Y., EOM, H. J., CHOI, J. Y., HYUN, J. & CHOI, J. 2012. Oxidative stress-related PMK-1 P38 MAPK activation as a mechanism for toxicity of silver nanoparticles to reproduction in the nematode *Caenorhabditis elegans*. *Environmental toxicology and chemistry,* 31**,** 585-592.

LIU, J., WANG, Z., LIU, F. D., KANE, A. B. & HURT, R. H. 2012. Chemical transformations of nanosilver in biological environments. *ACS nano,* 6**,** 9887.

LOWRY, G. V., GREGORY, K. B., APTE, S. C. & LEAD, J. R. 2012. Transformations of nanomaterials in the environment. ACS Publications.

MAIER, M. GIERIG, M. & WEGENKE, M. 2016. Mobilität und Verhalten von Nanopartikeln in der Umwelt. Bayerisches Landesamt für Umwelt (LfU). Augsburg.

MAIER, M. & WEGENKE, M. 2017. Untersuchungen zum Vorkommen von Nanopartikeln in Wasser und Luft – 1. Zwischenbericht zum Forschungsvorhaben. Bayerisches Landesamt für Umwelt,
Augsburg (unpublished).

MAYNARD, A. D. 2011. Don't define nanomaterials. *Nature,* 475**,** 31-31.

MEYER, J. N., LORD, C. A., YANG, X. Y., TURNER, E. A., BADIREDDY, A. R., MARINAKOS, S. M., CHILKOTI, A., WIESNER, M. R. & AUFFAN, M. 2010. Intracellular uptake and associated toxicity of silver nanoparticles in *Caenorhabditis elegans*. *Aquatic toxicology,* 100**,** 140-150.

MOORE, M. N. 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environment International,* 32**,** 967-976.

MORGAN, T. P., GROSELL, M., PLAYLE, R. C. & WOOD, C. M. 2004a. The time course of silver accumulation in rainbow trout during static exposure to silver nitrate: physiological regulation or an artifact of the exposure conditions? *Aquatic toxicology,* 66**,** 55-72.

NAVARRO, E., BAUN, A., BEHRA, R., HARTMANN, N. B., FILSER, J., MIAO, A. J., QUIGG, A., SANTSCHI, P. H. & SIGG, L. 2008a. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. *Ecotoxicology,* 17**,** 372-386.

NAVARRO, E., PICCAPIETRA, F., WAGNER, B., MARCONI, F., KAEGI, R., ODZAK, N., SIGG, L. & BEHRA, R. 2008b. Toxicity of Silver Nanoparticles to *Chlamydomonas reinhardtii*. *Environmental science & technology,* 42**,** 8959-8964.

NILSEN, T.M., EBBESSON, L.O., KVERNELAND, O.G., KROGLUND, F. FINSTAD, B. AND STEFANSSON, S. 2010. Effects of acid water and aluminium exposure on gill Na^+, K^+-ATPase α-subunit isoforms, enzyme activity, physiology and return rates in Atlantic salmon (*Salmo salar* L.). *Aquatic toxicology,* 97, 250-259.

NOTTER, D. A., MITRANO, D. M. & NOWACK, B. 2014. ARE NANOSIZED OR DISSOLVED METALS MORE TOXIC IN THE ENVIRONMENT? A META-ANALYSIS. *Environmental toxicology and chemistry,* 33**,** 2733-2739.

OECD 2011. OECD 201 Freshwater Alga and Cyanobaceria, Growth Inhibition Test. OECD Guidelines for the testing of chemicals. Adopted:23 March 2006. Annex 5 corected: 28 July 2011.

OUGHTON, D. H., HERTEL-AAS, T., PELLICER, E., MENDOZA, E. & JONER, E. J. 2008. Neutron activation of engineered nanoparticles as a tool for tracing their environmental fate and uptake in organisms. *Environmental toxicology and chemistry,* 27**,** 1883-1887.

PAQUIN, P. R., GORSUCH, J. W., APTE, S., BATLEY, G. E., BOWLES, K. C., CAMPBELL, P. G., DELOS, C. G., DI TORO, D. M., DWYER, R. L. & GALVEZ, F. 2002. The biotic ligand model: a historical overview. *Comparative biochemistry and physiology Part C: Toxicology & pharmacology,* 133**,** 3-35.

PECHLANER, R. AND ZADERER, P. 1985. Interrelations between brown trout and chironomids in the alpine lake Gossenköllesee. *Verhandlungen des internationalen verein limnologie,*. 22, 2620-2627.

POLÉO, A. B., ØSTBYE, K., ØXNEVAD, S. A., ANDERSEN, R. A., HEIBO, E. & VØLLESTAD, L. A. 1997. Toxicity of acid aluminium-rich water to seven freshwater fish species: a comparative laboratory study. *Environmental pollution,* 96**,** 129-139.

RATTE, H. T. 1999. Bioaccumulation and toxicity of silver compounds: A review. *Environmental toxicology and chemistry,* 18**,** 89-108.

RIBEIRO, F., GALLEGO-URREA, J. A., GOODHEAD, R. M., VAN GESTEL, C. A., MOGER, J., SOARES, A. M. & LOUREIRO, S. 2015a. Uptake and elimination kinetics of silver nanoparticles and silver nitrate by *Raphidocelis subcapitata*: The influence of silver behaviour in solution. *Nanotoxicology,* 9**,** 686-695.

RIBEIRO, F., GALLEGO-URREA, J. A., JURKSCHAT, K., CROSSLEY, A., HASSELLOV, M., TAYLOR, C., SOARES, A. & LOUREIRO, S. 2014. Silver nanoparticles and silver nitrate induce high toxicity to *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Danio rerio*. *Science of the total environment,* 466**,** 232-241.

RIBEIRO, M. J., MARIA, V. L., SCOTT-FORDSMAND, J. J. & AMORIM, M. J. 2015b. Oxidative stress mechanisms caused by Ag nanoparticles (NM300K) are different from those of $AgNO_3$: Effects in the soil invertebrate *Enchytraeus Crypticus*. *International journal of environmental research and public health,* 12**,** 9589-9602.

ROSSELAND, B.O. & SKOGHEIM, O.K. 1984. A comparative study on salmonid fish species in acid aluminium-rich water. II. Physiological stress and mortality of one and two year old fish. *Report: Institute of fresh-water research,* Drottningholm 61: 186-194.

ROSSELAND, B.O. & STAURNES, M. 1994. Physiological mechanisms for toxic effects and resistance to acidic water: An ecophysiological and ecotoxicological approach. Pp 227-246 in: Steinberg, C.E.W. & Wright, R.W. (eds.) *Acidification of Freshwater Ecosystems: Implications for the Future*. John Wiley & Sons, Ltd. ISBN 0-471-94206-5.

ROSSELAND, B. O., MASSABUAU, J.-C., GRIMALT, J. O., HOFER, R., LACKNER, R., RADDUM, G. G., ROGNERUD, S. & VIVES, I. 2001. Fish Exotoxicology: The EMERGE Fish Sampling Manual for Live Fish. The EMERGE Project (European Mountain lake Ecosystems: Regionalisation, diaGnostic and socio economic valuation).

ROSSELAND, B. O., ROGNERUD, S., COLLEN, P., GRIMALT, J. O., VIVES, I., MASSABUAU, J.-C., LACKNER, R., HOFER, R., RADDUM, G. G. & FJELLHEIM, A. 2007. Brown trout in Lochnagar: population and contamination by metals and organic micropollutants. *Lochnagar: The natural history of a mountain lake*, 253-285.

SALBU, B., BJØRNSTAD, H., LINDSTRØM, N., BREVIK, E., RAMBAEK, J., ENGLUND, J., MEYER, K., HOVIND, H., PAUS, P. & ENGER, B. 1985. Particle discrimination effects in the determination of trace metals in natural fresh waters. *Analytica chimica acta,* 167, 161-170.

SCOWN, T. M., SANTOS, E. M., JOHNSTON, B. D., GAISER, B., BAALOUSHA, M., MITOV, S., LEAD, J. R., STONE, V., FERNANDES, T. F., JEPSON, M., VAN AERLE, R. & TYLER, C. R. 2010. Effects of Aqueous Exposure to Silver Nanoparticles of Different Sizes in Rainbow Trout. *Toxicological sciences,* 115, 521-534.

SELCK, H., HANDY, R. D., FERNANDES, T. F., KLAINE, S. J. & PETERSEN, E. J. 2016. Nanomaterials in the Aquatic Environment: A European Union-United States Perspective on the Status of Ecotoxicity Testing, Research Priorities, and Challenges Ahead. *Environmental toxicology and chemistry,* 35, 1055-1067.

SENDRA, M., YESTE, M., GATICA, J., MORENO-GARRIDO, I. & BLASCO, J. 2017. Direct and indirect effects of silver nanoparticles on freshwater and marine microalgae (*Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum*). *Chemosphere,* 179, 279-289.

SØRENSEN, S. N. & BAUN, A. 2015. Controlling silver nanoparticle exposure in algal toxicity testing - A matter of timing. *Nanotoxicology,* 9, 201-209.

TYNE, W., LOFTS, S., SPURGEON, D. J., JURKSCHAT, K. & SVENDSEN, C. 2013. A new medium for *Caenorhabditis elegans* toxicology and nanotoxicology studies designed to better reflect natural soil solution conditions. *Environmental toxicology and chemistry,* 32, 1711-1717.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY, U. E. 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 5th edition. EPA-821-R-02-012. Office of water, Washington DC, USA. .

VANCE, M. E., KUIKEN, T., VEJERANO, E. P., MCGINNIS, S. P., HOCHELLA, M. F., JR., REJESKI, D. & HULL, M. S. 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. *Beilstein journal of nanotechnology,* 6, 1769-1780.

VERWEY, E. & OVERBEEK, J. T. G. 1948. Theory of Stability of Lyophobic Colloids' Elsevier Publishing" Co. Amsterdam Holland.

VOELKER, D., SCHLICH, K., HOHNDORF, L., KOCH, W., KUEHNEN, U., POLLEICHTNER, C., KUSSATZ, C. & HUND-RINKE, K. 2015. Approach on environmental risk assessment of nanosilver released from textiles. *Environmental research,* 140, 661-672.

VON DER KAMMER, F., FERGUSON, P. L., HOLDEN, P. A., MASION, A., ROGERS, K. R., KLAINE, S. J., KOELMANS, A. A., HORNE, N. & UNRINE, J. M. 2012. Analysis of engineered nanomaterials in complex matrices (environment and biota): general considerations and conceptual case studies. *Environmental toxicology and chemistry,* 31, 32-49.

WANG, S., LV, J., MA, J. & ZHANG, S. 2016. Cellular internalization and intracellular biotransformation of silver nanoparticles in *Chlamydomonas reinhardtii*. *Nanotoxicology,* 10, 1129-1135.

WARHEIT, D. B. 2018. Hazard and risk assessment strategies for nanoparticle exposures: how far have we come in the past 10 years? *F1000Research,* 7.

WOOD, C. M., FARRELL, A. & BRAUNER, C. 2012. Fish Physiology: Homeostasis and Toxicology of Non-Essential Metals. , 1st Edition. *Elsevier Inc.,* 31B.

WOOD, C. M., HOGSTRAND, C., GALVEZ, F. & MUNGER, R. S. 1996. The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) .1. The effects of ionic Ag+. *Aquatic toxicology,* 35, 93-109.

WOOD, C. M., PLAYLE, R. C. & HOGSTRAND, C. 1999. Physiology and modeling of mechanisms of silver uptake and toxicity in fish. *Environmental toxicology and chemistry,* 18, 71-83.

YANG, X. Y., GONDIKAS, A. P., MARINAKOS, S. M., AUFFAN, M., LIU, J., HSU-KIM, H. & MEYER, J. N. 2012. Mechanism of Silver Nanoparticle Toxicity Is Dependent on Dissolved Silver and Surface Coating in *Caenorhabditis elegans*. *Environmental science & technology,* 46**,** 1119-1127.

Paper I

KLEIVEN, M., ROSSBACH, L. M., GALLEGO-URREA, J. A., BREDE, D. A., OUGHTON, D. H. & COUTRIS, C. 2018. Characterizing NM300K Silver Nanoparticles Behavior, Uptake and Toxicity in Caenorhabditis elegans. *Environmental toxicology and chemistry. Which has been* published in final form at http://dx.doi.org/10.1002/etc.4144

Characterizing NM300K silver nanoparticles behavior, uptake and toxicity in *Caenorhabditis elegans*

Merethe Kleiven[a*], Lisa M. Rossbach[a], Julian A. Gallego-Urrea[ab], Dag Anders Brede[a], Deborah H. Oughton[a] and Claire Coutris[c]

[a] *Norwegian University of Life Sciences, Center for Environmental Radioactivity (CERAD CoE), Postboks 5003 NMBU, 1432 Ås, Norway*

[b] *Department of Marine Sciences, University of Gothenburg, Kristineberg 566, SE-45178 Fiskebäckskil, Sweden*

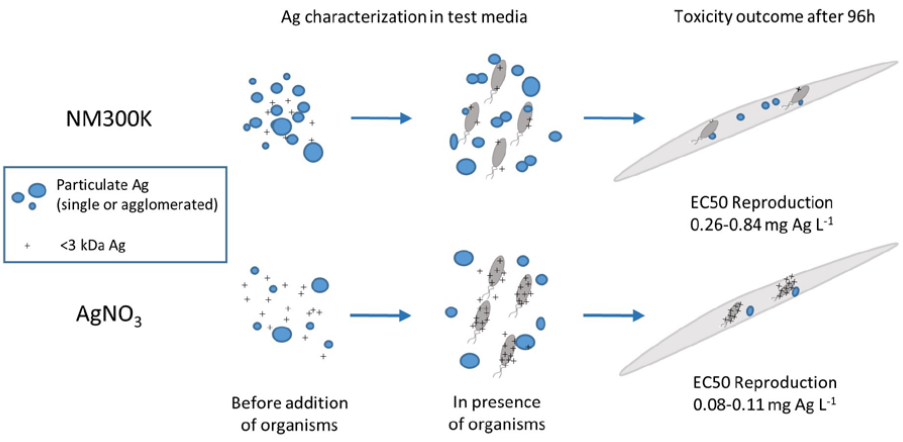
[c] *Division of Environment and Natural Resources, Norwegian Institute of Bioeconomy Research, Høgskoleveien 7, 1431 Ås, Norway*

[*] Address correspondence to *merethe.kleiven@nmbu.no or deborah.oughton@nmbu.no*

Abstract

Using *C. elegans* as a model organism, this study addresses the potential linkage between toxicity of NM300K AgNPs and particle size distribution and presence of dissolved Ag in the test media. Of the three endpoints assessed (growth, fertility and reproduction), reproduction was the most sensitive, with EC50s ranging from 0.26-0.84 mg Ag L^{-1} and 0.08-0.11 mg Ag L^{-1} for NM300K and $AgNO_3$, respectively. Silver uptake by *C. elegans* was similar for both forms of Ag, while bioaccumulation was higher in $AgNO_3$ exposure. The observed differences in toxicity between NM300K and $AgNO_3$ did not correlate to bioaccumulated Ag, which suggests the toxicity to be a function of the type of exposing agent (AgNPs vs $AgNO_3$) and their mode of action. Before addition of the food source, *E. coli*, size fractionation revealed that dissolved Ag comprised 13-90 % and 4-8 % of total Ag in the $AgNO_3$ and NM300K treatments, respectively. No dissolved Ag was detectable in the actual test media, due to immediate Ag adsorption to bacteria. Results from the current study highlight that information on behavior and characterization of exposure conditions is essential for nanotoxicity studies.

Graphical abstract



Keywords Toxic effects, nanoparticles, bioaccumulation, characterization, reproducibility

1. Introduction

Due to their antibacterial properties, silver nanoparticles (AgNPs) are amongst the most commonly used nanomaterials in consumer products, with increased application in medical devices, as well as general consumer products, such as clothing or sports equipment. In part because of the well-established toxicity of ionic silver, the environmental release of AgNPs and its potential toxicity to organisms has attracted a great deal of attention in both terrestrial and aquatic toxicity testing in recent years (Ratte, 1999, Sørensen and Baun, 2015). Demarcation of particles and ion effects highlights the importance of detailed characterization of both the particles and the exposure media, prior and under the test. Furthermore, since the relationship between toxic effect and particle characteristics remains unclear, it is vital to measure a range of potentially significant aspects, such as surface chemistry, charge, size, shape and chemical composition (Jiang et al., 2009).

Knowledge on the dispersion state and its controlling parameters is of great importance when preparing nanoparticle suspensions for toxicological studies. Nanoparticles are known to have a high propensity to form agglomerates or aggregates, both of which have the potential to severely impact the interaction of the particles with the organisms in question (Jiang et al., 2009). The degree of aggregation and/or dissolution and subsequent ionic releases will depend on the exposure media used in toxicity testing. Factors like pH, salinity or the presence of humic substances play a significant role in toxicokinetics (Wasmuth et al., 2016). These well-known influences of exposure media on particle chemistry call for more harmonized nano-specific approaches to toxicity testing, such as the EU NanoReg Standard Operating Procedure for nanomaterials (Jensen et al., 2016).

Both the initial particle characteristics and the associated transformations of the particles have the potential to significantly impact the interaction of the particles with biological systems (Montes-Burgos et al., 2010). A range of studies have suggested that the observed toxic effects of AgNPs can be largely, or solely, attributed to Ag ion release following particle dissolution, while others provide evidence for particle specific effects, for example from reactive oxygen species generated on the surface of the particle (Borm et al., 2006, Carlson et al., 2008). Thus monitoring of particle dissolution, for instance by means of ultrafiltration, needs to be taken into consideration, and to be followed as a function of time in order to identify the source of the toxic response measured (Sørensen and Baun, 2015).

The AgNP NM300K used in the current study is a representative Ag nanomaterial provided by the European Commission Joint Research Centre, and thus one of the best characterized sources of AgNPs

66 available. However, despite well-developed synthesis methods and thorough characterization by the
67 supplier, physicochemical changes, such as agglomeration, aggregation and surface charge variations
68 arise during the preparation of stock suspensions and addition to the exposure media (Lundqvist et al.,
69 2008). Thus further characterization during these stages is essential.

70 To date NM300K AgNPs have been used in a wide variety of studies ranging from investigations into
71 speciation, characterization as well as textile retention time of the NPs (Voelker et al., 2015). Köser et al.
72 (2017) suggested that the high dispersion and redox stability of NM300K AgNPs in a series of different
73 ecotoxicity media could partly be attributed to the coating of the particles. They also showed that the
74 initial Ag ion concentrations measured in the media originated from Ag ions present in the dispersant,
75 and found no evidence for further particle dissolution.

76 Furthermore, their toxicity has been studied in a range of species, including *Daphnia magna* (Poynton et
77 al., 2012), the gram-negative bacterium *Pseudomonas putida* (Mallevre et al., 2016), marine diatoms
78 *Chaetoceros curvisetus*, enchytraeids *Enchytraeus crypticus* (Gomes et al., 2017) and earthworms
79 *Lumbricus rubellus* (van der Ploeg et al., 2014, Gomes et al., 2017). To our knowledge, this is the first
80 paper looking at toxic effects of NM300K towards the nematode *Caenorhabditis elegans* with the aim of
81 investigating the linkage between characterization and toxicity.

82 Living in the soil pore water, the nematode *C. elegans* is a relevant model organism for a range of
83 environmental contaminants. Detailed knowledge about their physiology and biology allows for
84 extensive measurements of a wide range of toxicological endpoints, including fecundity, reproduction
85 and development (Hunt, 2017, O'Reilly et al., 2014). Resulting from its short lifecycle (96 h at 20 °C),
86 *C. elegans* represents a perfect *in vivo* model for nanoparticle toxicology by minimizing exposure time,
87 and hence reducing aging effects of the particles (Handy et al., 2012). Furthermore, the impact of
88 different test media and impacts on the particle toxicity towards *C. elegans* has been recognized and
89 different media have been proposed, such as the low ionic strength U.S. EPA moderately hard,
90 reconstituted water (Cressman and Williams, 1997, Tyne et al., 2013). However, despite increased use of
91 these low ionic strength media in toxicity tests, particles are still rarely characterized in media in the
92 actual test. More importantly, information on speciation and fractions of silver (irrespectively of the
93 original source) as well as the associated dynamical behavior is lacking.

94 Therefore, the current study aims to ascertain the potential linkage between toxicity of the AgNPs
95 NM300K towards the nematode *C. elegans* and behavior of the particles prior and post application into
96 the test medium. The approach consisted in measuring standardized endpoints (survival, growth,

fecundity and reproduction), in combination with monitoring of particle behavior in stocks and exposure media over time. Furthermore, the reproducibility of these toxicity tests was investigated, and two stock preparation methods were compared.

2. Materials and methods

2.1 Experimental design

Caenorhabditis elegans were exposed to NM300K AgNPs and silver nitrate ($AgNO_3$) in three separate experiments (Experiments 1-3 in Table 1, hereafter E1, E2 and E3) following the ISO 10872 guideline with some modifications, including changes in exposure media (ISO, 2010).

Stocks of wild-type nematodes N2 Bristol (*Caenorhabditis Genetic Centre*, Minneapolis, USA) were kept in liquid cultures before obtaining a synchronized culture by treating gravid hermaphrodites with hypochlorite to extract eggs. Eggs were hatched on agar plates overnight to obtain synchronized L1 stage larvae before the start of the exposure.

All three experiments were carried out as standard 96 h toxicity tests at 20 °C, in the dark, in 24-well culture plates, gently shaken to ensure sufficient oxygenation. Each well contained 495 µL bacteria *E. coli* OP50 re-suspended in moderately hard reconstituted water (MHRW) (United States Environmental Protection Agency, 2002), 5 µL *C. elegans* at L1 larval stage in liquid medium M9 (density 11 ± 5.5 L1 per 5 µL), and 500 µL $AgNO_3$ solution or AgNP suspension in MHRW (at twice the nominal concentration). The nematodes were exposed, in triplicate, to AgNPs and $AgNO_3$ in the concentration range of 0.1-29 mg L^{-1} and 0.1-4 mg L^{-1}, respectively (Table 1). Additionally, separate exposure plates were set up for the characterization of exposure suspensions, with sampling performed at 0 and 96 h for E1, at 0, 20 and 96 h for E2, and at 96 h for E3. The experiments were carried out over a time span of 3 years.

The potential effects of NM300K DIS, the stabilizing agents found in the NM300K material were controlled for by testing the concentration present when exposed to the highest exposure concentration of NM300K. No effects were observed.

Table 1. Nominal exposure concentrations of $AgNO_3$ and NM300K AgNPs in three separate experiments.

	$AgNO_3$ (mg Ag L^{-1})	**AgNPs (mg Ag L^{-1})**
Experiment 1	0.1, 0.5, 1, 2 and 4	0.1, 0.5, 1, 2 and 4
Experiment 2	0.1, 0.25, 0.5, 1, 2 and 4	0.1, 0.25, 0.5, 1, 2 and 4
Experiment 3	0.125, 0.25, 0.5, 1, 2 and 4	0.9, 1.8, 3.6, 7.3, 14.5 and 29

The toxicity tests were terminated by addition of 0.5 mL of Rose Bengal (300 mg L^{-1}) to all wells and heating at 80 °C for 10 min. Survival, growth, fertility and reproduction were assessed using a stereomicroscope (Leica M205C) equipped with a camera, and pictures were analyzed using the open source image processing program ImageJ (https://imagej.net) or the Leica software (LAS vs 4.4.0). Nematodes were considered fertile when they contained at least one embryo.

2.2 Preparation and characterization of silver suspensions

The nanomaterial used in all three experiments was the OECD representative AgNPs NM300K (Fraunhofer IME, Munich, Germany). These are spherical Ag nanoparticles dispersed in a mix of two stabilizing agents, 4 % each of Polyoxyethylene Glycerol Trioleate and Tween 20. The average particle size is reported to be 15 nm, with 90 % of the particles <20 nm. Silver nitrate (pro analysis Merck, Darmstad, Germany) was used to compare the toxicity and behavior of AgNPs to those of a silver salt. In E1, the AgNP stock suspension with a concentration of 400 mg Ag L^{-1} was prepared under anaerobic conditions (in a glove box) by adding the original NM300K suspension to MilliQ water (15 MΩ·cm) and mixing by gentle resuspensions with the pipette. The AgNP stock suspension in E2 and E3 was prepared according to Jensen et al. (2016). Briefly, a 2.56 g Ag L^{-1} stock suspension was prepared by dispersing the original NM300K suspension in MilliQ water and sonicating for 13 min at 15 % amplitude (depositing 7.35 ± 0.05 W) using a 400 Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, USA) equipped with a standard 13 mm disruptor horn (Model number: 101-147-037). In all experiments, the subsequent suspensions were all prepared from the stock suspension.

2.2.1 Transmission electron microscope

Transmission electron microscope (TEM, Morgagni 268, FEI, Eindhoven, Netherlands) was used to assess the core diameter of AgNPs in the stock suspension, in E2 and E3. Ten µL of AgNP stock suspension (E2) or of a diluted stock suspension to obtain an optimized particle concentration on the grid (250 mg L^{-1}, E3) were added to a 400-mesh Cu coated Piloform film (Agar Scientific, Essex, UK) and the specimens were allowed to air dry. TEM images were acquired with the instrument operating at 80 keV.

The analysis of the TEM images was performed using the iTEM software (Olympus), according to the protocol by Mast and de Temmeman (2016). The particle size provided is the Ferret minimum defined as the minimum distance of parallel tangents at opposing particle borders.

2.2.2 Dynamic light scattering

Dynamic light scattering (DLS) measurements were performed on a Malvern Zetasizer ZS (Malvern instruments Ltd, Worcestershire, UK) equipped with a laser source with wavelength 633 nm. Zeta-average hydrodynamic diameters and size distributions were determined using the "multiple narrow modes (high resolution)" algorithm supplied by Malvern. Measurements were done in triplicates of 3 to 5 runs with autocorrelation functions of 10 seconds.

Measurements of the hydrodynamic diameter were performed on stock suspensions, as well as exposure suspensions both with and without *E. coli* present, throughout the duration of the experiments.

The same instrument was used for the measurements of electrophoretic mobility and the Smoluchowski approximation was used for determining zeta-potentials (in E1 and E3). Three measurements with 5 runs per measurement were obtained.

An aggregation experiment was conducted to explore the aggregation rates of Ag particles in MHRW in both NM300K and $AgNO_3$ exposures. Aggregation rates were measured using time-resolved DLS. Stock suspensions were directly mixed with MHRW in a proportion 1:20, followed by mixing 1:1 or 1:10 in MHRW (so that the final concentrations were 1 and 10 mg Ag L^{-1}), immediately mixed on a vortex shaker for 10 s and measured with fixed attenuator and measurement position. The time until the first measurement was completed was recorded. A variable number of time points of 10 s autocorrelations were taken for the study.

2.2.3 Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was used to assess the hydrodynamic diameter of individual particles. The NTA measurements of the hydrodynamic diameter were carried out on a Nanosight LM10 (NanoSight Ltd, Amesbury, UK). The light source was a solid-state, single-mode laser diode (radiation output max power <50 μW, 635 nm continuous wave, max power <35 mW). The standard camera Marlin F-033B (Allied Vision Technologies GmbH, Stadtroda, Germany) was used. All data were analyzed using the instrument software (NanoSight™ version 2.2). The analysis with NTA was done on 5 videos

with 1 min length each. The measurements with NTA were performed on the samples from the final day of E1 after mild centrifugation (ca. 1000×g).

2.2.4 Total and dissolved Ag

Total and dissolved (defined as <3 kDa) Ag were determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 8800, Mississauga, Canada). For determination of total Ag concentrations, 200 µL of the samples were collected before being digested and measured by ICP-MS according to the specifications in Table S1. Dissolved Ag was determined by filtration through a preconditioned 3 kDa Amicon cellulose membrane filter (Amicon Millipore), centrifugation at 14000×g for 30 min and subsequent collection of 200 µL of the filtrate for digestion and ICP-MS measurements. To avoid clogging of the filter in the presence of organisms, the nematodes and *E. coli* were removed by centrifugation (2000×g, 15 min) prior to 3 kDa filtration. The supernatant of the centrifugation prior to 3 kDa filtration was also sampled and measured by ICP-MS in E3.

All samples were digested with acid and appropriately diluted before ICP-MS measurements. In E1, all samples were digested in a solution of Aqua Regia (40 % HNO_3 and 11 % HCl v/v) at high temperature (260 °C) and pressure (120 bar) (UltraCLAVE 3, Milestone Ltd.) before diluting to suitable concentrations for ICP-MS measurements. Since this was subsequently found to be unnecessary to achieve complete dissolution, in E2 and E3, samples were digested in ultrapure HNO_3 (sample:HNO_3 volume ratio of 1:5 in E2 and 1:7.5 in E3) at 80 °C for 4 h, before dilution to a final acid concentration of 10 vol %.

2.3 Uptake of Ag

For the determination of the potential uptake of AgNPs, Ag^+ and/or transformation products by the nematodes, an uptake study was conducted during E2. Nematodes were exposed in triplicates to NM300K and $AgNO_3$ for 65 h before ICP-MS analysis. To measure total uptake (including gut content), half of the nematodes were washed twice in MHRW without further depuration. To assess the Ag fraction tightly/stably bound to organisms, the other half of the nematodes were subjected to 2 h of depuration on agar plates seeded with *E. coli*. Subsequent to depuration, nematodes were recovered from the agar plates by carefully flushing them from the dish into an Eppendorf tube using MHRW. All samples (undepurated and depurated organisms) were washed thoroughly with MHRW followed by a gentle centrifugation (250×g) and supernatant removal. This step was repeated twice. Samples were then evaporated to dryness, added 1.5 mL ultrapure HNO_3 (65 %), and heated at 90 °C for 2 h. Following the digestion, samples were diluted to reach 10 vol % HNO_3 and analyzed by ICP-MS. To produce

concentrations as ng Ag μg^{-1} wet weight nematode, the ICP-MS results in ng Ag were divided by the exact number of nematodes in each of the three replicate dishes (average 12, ranging from 7 to 20) and then multiplied by the wet weight of a nematode, using the formula $W = (L \times D^2)/(1.6 \times 10^6)$, where *W* is the mass (wet weight in µg) per individual, *L* is the nematode length (µm) and *D* is the greatest body diameter (µm) (Andrassy, 1956).

2.4 Estimation of effect concentrations

The estimation of effect concentrations (EC10 and EC50) on growth, fertility and reproduction was performed using the free software RegTox, developed by Eric Vindimian (http://www.normalesup.org/~vindimian/en_download.html). The values used for estimating EC10 and EC50 were the average values from all replicate wells (n=3 to 8), expressed as % of controls. The Hill model was used for the estimation, and the values reported are the optimal value for EC10 and EC50 with their 95% confidence intervals.

3 Results and discussion

3.1 Silver characterization in exposure media

3.1.1 TEM and DLS analysis of NM300K in stock suspensions

To assess the particle size, surface charge and hydrodynamic diameter distributions, the NM300K stock suspensions (in MilliQ water) of each experiment were analyzed by TEM and/or DLS. The TEM analysis showed good agreement with manufacturers specifications (15 nm), while the DLS measurements indicated a higher mean particle size.

As measured by TEM, the mean particle size of the stock suspension was 12.5 ± 4.1 nm (mean ± SD, n = 70) in E2 and 16.7 ± 6.5 nm (mean ± SD, n = 32241) (Table 2, Figure S1). However, aggregates/agglomerates, varying in size, were also present in the samples. The presence of aggregates/agglomerates was also indicated by the difference in Z-average particle size and number mean, as well as the polydispersity index (PDI) in the size measurements conducted with DLS (Table 2). Interestingly, DLS measurements suggested a higher aggregation of the NM300K in the sonicated stock suspensions than the stock suspension that was homogenized by repeated pipetting. For suspensions that are produced from powders, sonication might be of aid to breakdown larger aggregates; however, dispersions that have been synthesized in liquid media can be induced to aggregation by the addition of sonicating power (Petersen et al., 2014, Handy et al., 2012). However, the E1 stock was less

concentrated than E2 and E3 stocks, potentially also influencing the zeta-average hydrodynamic diameter.

Table 2. Size characterization of stock suspensions measured by DLS and TEM. Results are provided as mean ± one standard deviation. ND: not determined

	Stock concentration (g L^{-1})	Z-Average diameter (nm)	Number mean diameter (nm)	Polydispersity index	Zeta potential (mV)	TEM diameter Ferret min (nm)
E1	0.02	33.8 ± 1.7	ND	0.461 ± 0.005	-1.02	ND
E2	2.56	82.0 ± 6.0	28 ± 5.0	0.293 ± 0.010	ND	12.5 ± 4.1
E3	2.56	71.7 ± 0.6	28 ± 8.6	0.272 ± 0.003	-7.32	16.7 ± 6.5

3.1.2 Ag characterization in exposure media without organisms

Although characterization of stock suspensions can give information about the initial particle size, shape, charge etc., several of these parameters change when the particles are added to the exposure medium used in a toxicity test. Thus, efforts were made to gain information about the NM300K AgNP behavior in the exposure medium.

To gain information on the influence of MHRW on the AgNP size in NM300K exposures, and on the formation of AgNPs or other Ag(I) complexes in $AgNO_3$ exposures, a range of concentrations of either form of Ag were analyzed by DLS. Results showed that the mean Z-average particle size in the higher exposure concentrations was close to that seen in the stock suspension (Table 3 and Figure 1). In the low exposure concentration (0.5 mg L^{-1}), the Z-average particle size was significantly larger than in the stock suspension. In samples containing more than one size population of particles or aggregates, DLS tends to overestimate the mean particle size, due to the higher intensity signals reflected by larger particles. This artefact is even more evident at lower particle concentrations, and hence increases uncertainties in the measurements at lower particle concentrations (Handy et al., 2008). Measurements of change in particle size over time showed that the intermediate concentrations (2 and 4 mg L^{-1}) were stable over time, while in both the lowest (0.5 mg L^{-1}) and highest (10 mg L^{-1}) concentrations the particles were less stable and a time dependent increase in Z-average particle size was observed (Figure 1). The presence of larger particles or Ag complexes (e.g. $AgCl_{(s)}$) in $AgNO_3$ exposures was evident from the DLS results, with higher polydispersity than seen in the NM300K exposures (Table 3).

Table 3. Particle size (mean ± one standard deviation) measured by DLS in exposure suspensions prior to addition of *E. coli* or *C. elegans*. NA: not available.

	Nominal Ag concentrations (mg L^{-1})	Z-Average diameter (nm)	Number mean diameter [c] (nm)	Polydispersity index
NM300K	0.2 [b]	121 ± 22	51 ± 4	0.219 ± 0.034
	0.5 [a]	104 ± 37	NA	0.166 ± 0.033
	1 [b]	79 ± 3	43 ± 8	0.253 ± 0.036
	2 [a]	71 ± 14	NA	0.126 ± 0.011
	4 [a]	107 ± 84	NA	0.186 ± 0.058
	4 [b]	74 ± 1	43 ± 5	0.304 ± 0.014
	10 [a]	34 ± 0.4	NA	0.461 ± 0.007
$AgNO_3$	0.2 [b]	893 ± 108	154 ± 11	0.811 ± 0.086
	1 [b]	425 ± 36	205 ± 8	0.444 ± 0.023
	4 [b]	404 ± 8	276 ± 12	0.375 ± 0.020

[a] Data from E1, [b] Data from E2, [c] Calculated using general purpose algorithm (normal resolution) in Malvern zetasizer software.

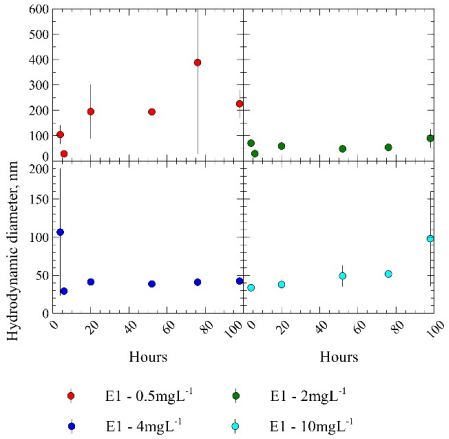


Figure 1. Zeta-average diameters obtained from DLS measurements in E1 without organisms for the NM300K AgNPs exposures. Averages of three replicated measurements are presented and error bars represent one standard deviation.

Time-resolved DLS measurements of the z-average diameter of NM300K performed over a period of a few minutes showed an initial high degree of instability, before stabilizing at approximately 30 nm and a

PDI of *ca*. 0.450 in all suspensions tested (Figure 2, Figure S2). These PDIs indicate the presence of larger aggregates, which was also the case for the starting material. However, no increase in the hydrodynamic diameter was observed even at 10 mg L^{-1}, which indicates no contribution of collision induced aggregation (Gallego-Urrea et al., 2016). The aggregation experiment in MHRW did not show any increase in diameter during a lapse of 10 min when 10 mg L^{-1} NM300K was added. Interestingly, the $AgNO_3$ solution containing 10 and 1 mg Ag L^{-1} in MHRW showed an increase in the particle size and reached a steady-state value after a few minutes; the value of the steady state hydrodynamic diameter varied with initial particle size distribution and mixing ratios with the medium. This behavior can be explained by the formation of AgCl particles, which is consistent with the speciation calculations performed with visual Minteq (see paragraph 3.1.3) and was also observed by other authors in media containing chloride ions (Gonçalves et al., 2017).

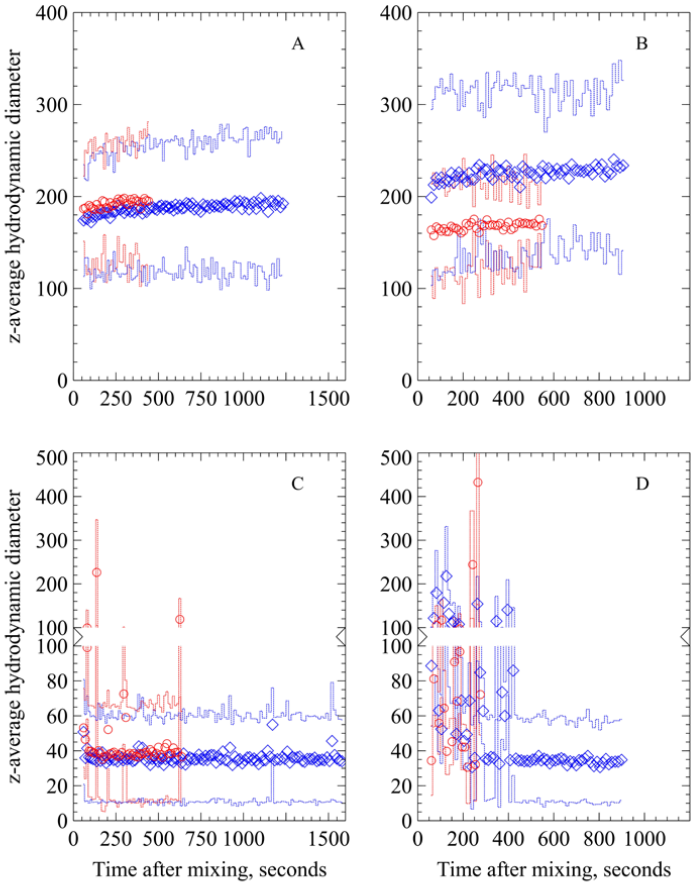


Figure 2. Time resolved DLS measurements performed on $AgNO_3$ solutions in MHRW (A and B) and NM300K in MHRW (C and D). A and C correspond to measurements done at 10 mg Ag L^{-1}, and B and D at 1 mg Ag L^{-1}. Zeta-average hydrodynamic diameters, d_H, were obtained as explained in the text and duplicate values are presented with the markers. The dotted lines correspond to the corresponding color-matched standard deviation obtained from polydispersity-index, PDI, values assuming a Gaussian profile distribution ($SD = (d_H^2.PDI)^{0.5}$).

3.1.3 Ag inorganic speciation modelling using Minteq

The results also showed the importance of characterizing not only the AgNP exposure suspensions, but also the Ag salt solutions. While the assumption is often that these represent an ionic exposure, Ag speciation is also affected by the chemical conditions of the media, and particles and colloids can also be formed, as shown by DLS measurements. To control for possible formation of inorganic solid phase species (e.g. $AgCl_{(s)}$), chemical speciation of Ag(I) with Minteq in MHRW without organisms revealed the possible formation of $AgCl^0$ after ~100 µg L^{-1} of free Ag ion was added to the medium. The dissolved Ag, which in this case means all forms of silver other than $AgCl_{(s)}$, remains relatively constant (concentrations between 100 and 500 µg L^{-1}) when the total Ag is below 2000 µg L^{-1}. In MHRW in absence of organisms, results indicate that most of the non-dissolved Ag corresponds to $AgCl_{(s)}$.

3.1.4 Ag characterization in exposure media in presence of organisms

The presence of the test organisms in a toxicity test system, here the nematodes *C. elegans* and their food source the bacteria *E. coli*, influenced the test system, including Ag speciation and particle behavior. In this study, an additional characterization of the actual exposure system was thus performed to address possible changes in Ag speciation, and provide insight into time dependent changes in particle aggregation and dissolution. This included size measurements using DLS and NTA, as well as total and <3 kDa Ag concentrations using ICP-MS.

3.1.4.1 DLS

In an attempt to gain information on NM300K behavior as well as formation of AgNPs and other Ag complexes in the $AgNO_3$ exposures under toxicity test conditions, DLS measurements were performed on exposure suspensions. As expected, *E. coli* strongly affected the analysis, even at the highest exposure concentrations, and did not produce intelligible data about the actual particle size (Figure S3).

3.1.4.2 NTA

The NTA measurements of the exposure suspensions after 96 h exposure were all in accordance with DLS measurements with regards to the presence of large material, but also showed that the presence of small particles (<200 nm) in the exposure media with NM300K was significantly greater than in the control (Figure 3). The presence of particulate material in the control was probably due to organic particles coming from the organisms. The 2 mg L^{-1} $AgNO_3$ exposure suspension contained a large amount of particles in the 200 nm range (Figure 3) compared to the control, probably originating from $AgCl_{(s)}$ formed in the medium.

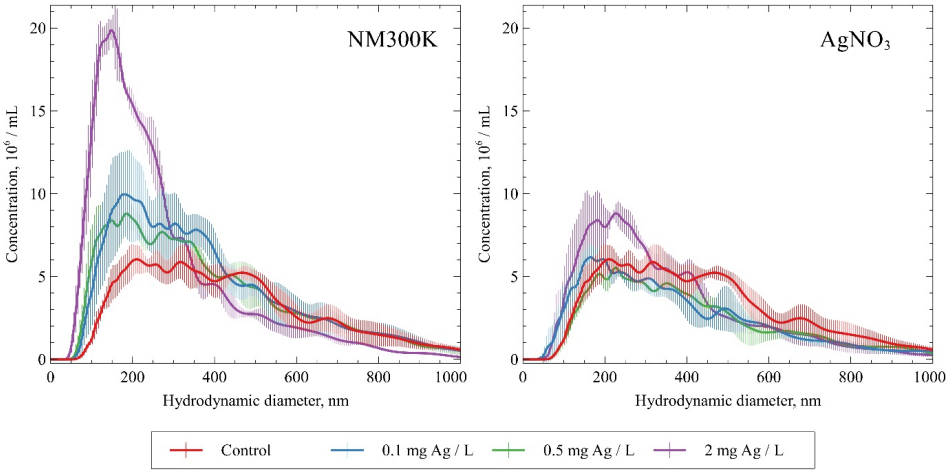


Figure 3. Particle size distribution in exposure media in E1 containing 0, 0.1, 0.5 and 2 mg Ag L^{-1}, 96 h after addition of NM300K (left) or $AgNO_3$ (right). Complete lines correspond to average values of five videos of 60 s each. Error bars represent the 95% confidence interval among the videos

3.1.4.3 Exposure concentrations and size fractionation

In $AgNO_3$ treatments, the measured concentration of total Ag at the beginning of the exposure was within 10 % deviation (0-8 %) from nominal concentrations (E1 and E2, Table S2). In NM300K treatments, the measured concentration of total Ag at the beginning of the exposure was within 5 to 33 % deviation from nominal concentrations. In both $AgNO_3$ and NM300K treatments, there was a reduction in measured total Ag after 96 h at low concentrations (<1 mg Ag L^{-1}). In general, the recovery was decreasing with decreasing concentrations (Table S2).

The size fractionation showed that the initial concentration of dissolved Ag (<3 kDa) varied among treatments. In $AgNO_3$ treatments, the dissolved Ag content varied between 13 and 90 % of the total Ag measured in exposure media without *E. coli* (Table S2, E1). In contrast, the <3 kDa Ag fraction was reduced to <LOD within 2 h upon addition of *E. coli* (Table S2, E2). In NM300K treatments, the initial <3 kDa Ag fraction was 4-8 % of the total Ag concentration, in absence of bacteria in the medium (Table S2, E1). These results are consistent with previous investigations on the behavior of NM300K in various test media, showing an initial input of dissolved Ag from NM300K <8 % in all tested media (Köser et al. (2017). In the study by Köser at al., the highest dissolved Ag fraction was found in the medium with the

lowest Cl^- concentration (Steinberg medium), a medium with a composition and Cl^- content similar to the MHRW used in the present study. Köser at al. reported that the dissolved Ag was present in the original stock suspension provided by the manufacturer, bound to the dispersant agents, and therefore not a result of further particle dissolution. Furthermore, the authors suggested that after this initial release of dissolved Ag, the dispersants would help to prevent any further release of Ag(I) from the particles, by limiting the access of O_2 to the surface of AgNPs. They thus concluded that NM300K toxicity attributed to ionic Ag was related to the ionic fraction found in the dispersion before the start of the toxicity test, and could not be related to the further oxidation of the particles and subsequent ionic releases (Köser et al., 2017). As a consequence of the work of Köser et al. (2017), oxidation and subsequent release of Ag(I) from NM300K would not be expected during short-term exposure periods. In the present study, exposure suspensions were continuously shaken to maintain sufficient O_2 levels, which are necessary for the metabolism of *C. elegans*. The constant oxygenation together with the presence of *E. coli* and *C. elegans* might influence the protective effects of the dispersants, and potentially even enhance the dissolution of AgNPs. However, the initial dissolved Ag fraction (<3 kDa) in the exposure suspensions containing *E. coli* and *C. elegans* was very low (<0.65 µg L^{-1}) in all $AgNO_3$ and NM300K treatments (Table S2, E2), and remained so during the whole duration of the experiment (Table S2, E2 and E3). This strongly suggests an interaction between dissolved Ag and *E. coli* present in the exposure suspensions. Silver ions are well-known for their antibacterial properties, which are closely connected to their ability to interact with the negatively charged bacterial surface and translocate to the interior cell where they interfere with enzymatic functions and metabolic processes (Yamanaka et al., 2005). Mullen et al. (1989) reported that 89 % of a 108 mg L^{-1} Ag(I) solution was removed from solution by binding to bacteria. This interaction is highly efficient and a likely explanation for the low dissolved Ag content in our study.

3.2 Uptake and toxicity

3.2.1 Silver uptake by nematodes

Toxicity of NPs to *C. elegans* is highly dependent on the uptake and residence time, both of which are related to surface chemistry and particle size (Meyer et al., 2010, Ellegaard-Jensen et al., 2012). Image analysis has shown that NP uptake occurs predominantly via ingestion, and that coated monodispersed NPs are taken up by intestinal cells (Meyer et al., 2010). However, there is little or no quantitative data on the uptake of AgNPs. To further characterize the bioaccumulation of Ag, we thus devised an experiment to quantify the uptake of Ag in *C. elegans* from NM300K and $AgNO_3$ exposures The uptake

was measured after 65 h exposure to avoid the interference of offspring. The digestion process in *C. elegans* is very rapid and it has been shown that the residency time for *E. coli* is on average two minutes, with defecation on average every 50 s (Ghafouri and McGhee, 2007). Thus, to determine the Ag fraction retained, the exposed nematodes were depurated by feeding on uncontaminated food for two hours. This would facilitate the removal of any unbound Ag from the intestinal lumen, and enable depuration of Ag that might be removed by other defense mechanisms. Remaining Ag should thus be incorporated in the nematode.

In undepurated nematodes, ICP-MS measurements showed that the concentration in nematodes was correlated with the exposure concentrations for both NM300K and $AgNO_3$ (Table 4). The same dose dependency was observed for the depurated nematodes. However, the overall Ag content (given as ng mg^{-1} wet weight) in the nematodes after depuration was reduced by >98 % in both NM300K and $AgNO_3$ exposures (Table 4). The remaining 0.6-2 % fraction was retained in the organisms, indicating a strong binding into tissues and possibly translocation into cells from the intestinal lumen. Yang et al. (2014) exposed *C. elegans* to $AgNO_3$ at 0.3 mg Ag L^{-1} and to citrate stabilized AgNPs at 10 mg Ag L^{-1} for 24 h in MHRW and reported internal concentrations 3-10 times higher than reported in the current study, but suggested that their ICP-MS measurements were dominated by AgNPs retained in the gut. Through TEM analysis, the authors also identified damage to intestinal epithelial cells and effects to cell organelles like mitochondria and lysosomes. However, translocation of Ag into the cells is not prerequisite for toxicity of nanomaterial. Formation of reactive oxygen species as a consequence of oxidation of the AgNP surface has been associated to oxidative stress in the intestine and consequently leading to toxicity (Yang et al., 2012). It should be mentioned that such oxidation of the AgNP surface also involves release of Ag(I), which again could partly be the cause of toxicity seen in the NM300K exposures. Despite observed uptake of the citrate stabilized AgNPs into cells, most of the AgNPs remained in the intestinal lumen in the study by Yang et al. (2014).

Table 4. Measured Ag concentrations in undepurated and depurated nematodes for NM300K and $AgNO_3$ exposures during E2. Concentrations in nematodes are given as mean ± SD. NA: not applicable, no surviving nematodes. LOD = 2.7 ng Ag mg^{-1} wet wt nematode.

	Ag concentration in exposure media		**Ag concentration in nematodes**	
	Nominal ($mg\ L^{-1}$)	Measured ($mg\ L^{-1}$)	Undepurated ($ng\ mg^{-1}$ wet wt)	Depurated ($ng\ mg^{-1}$ wet wt)
NM300K	0.1	0.08	134 ± 13.8	<LOD
	0.5	0.40	1053 ± 31	7 ± 2
	2.0	1.56	3250 ± 292	21 ± 12
$AgNO_3$	0.1	0.10	223 ± 11	<LOD
	0.5	0.52	945 ± 65	15 ± 3
	2.0	2.03	NA	NA

3.2.2 Toxicity

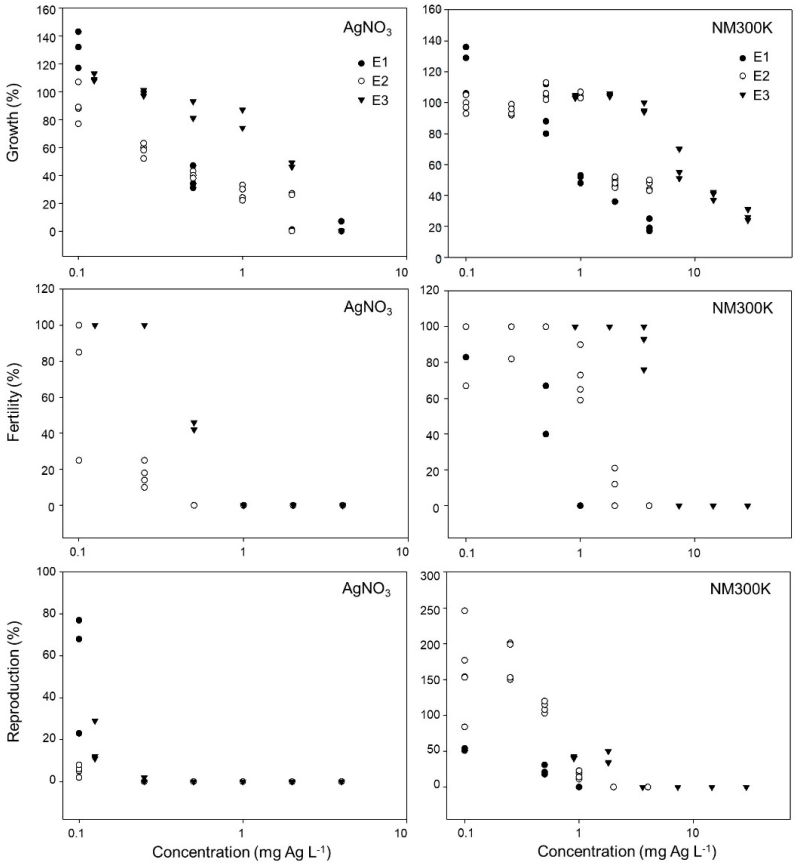
The toxicity of NM300K AgNPs and $AgNO_3$ was assessed by measuring the effects on the standardized endpoints growth, fertility and reproduction after 96 h exposure in MHRW. Dose dependent effects were observed for all endpoints in both NM300K and $AgNO_3$ exposures (Figure 4), with reproduction as the most sensitive endpoint. The toxic effects of $AgNO_3$ were consistent for all the experiments (E1-3), indicating reproducibility of the experimental set up. The nematode development was assessed in a stereomicroscope after 96 h and it was evident that exposure to both $AgNO_3$ and NM300K affected the growth of *C. elegans*. It appeared that development was delayed and that all nematodes reaching the adult stage were fertile and able to reproduce, although the number of offspring per adult was reduced. However, the fertility results were characterized by a very abrupt EC10 to EC50 dose response over a narrow concentration range in both $AgNO_3$ and NM300K exposures. This indicates that fertility might be highly vulnerable to inter experimental variation and not as robust an endpoint as growth or reproduction.

We observed a stimulation of reproduction by NM300K at the lowest exposure concentration, and a stimulation of growth by both $AgNO_3$ and NM300K lowest exposure concentrations. Such compensatory effects are frequently reported when organisms like *C. elegans* are challenged by low level environmental stressors, including Ag (Cypser and Johnson, 2002). Overall, $AgNO_3$ induced toxicity at lower exposure concentrations compared to NM300K, with EC50 for growth, fertility and reproduction

2-9 times lower for $AgNO_3$ compared to NM300K (Table 5). The wide range is due to the higher variation in NM300K induced toxic effects among experiments. This is particularly visible on EC10 values for reproduction, where very little variation was seen across the years for $AgNO_3$ treatments (0.06-0.08 mg Ag L^{-1}) compared to NM300 treatments (0.09-0.52 mg Ag L^{-1}). Previous studies on toxicity of AgNPs to *C. elegans* in aqueous exposures (K+ medium or MHRW) have reported EC50 values for growth, reproduction, and mobility ranging from 0.09-50 mg Ag L^{-1} (Yang et al., 2012, Ellegaard-Jensen et al., 2012, Starnes et al., 2015). Not surprisingly, the toxicity of AgNPs as well as $AgNO_3$ was higher in MHRW, which has a low chloride content and a low conductivity, than in K+ medium.

To our knowledge, the current study is the first to report EC values for *C. elegans* exposed to NM300K. Köser et al. (2017) reported EC50 values for NM300K in other organisms: growth inhibition of *Pseudokirchneriella subcapitata* at 0.62 ± 0.37 mg Ag L^{-1}, and *Lemna minor* at 0.50 mg Ag L^{-1} (95 % CI 0.19-1.11 mg Ag L^{-1}); immobilization of *Daphnia magna* at 0.04 ± 0.01 mg Ag L^{-1}.

NM300K induced toxicity tended to be higher in E1 as compared to E2 and E3, particularly when compared to $AgNO_3$ toxicity (E1 showing at most a factor of 3 between EC50 values for NM300K and $AgNO_3$, compared to a factor of 6-9 in E2 and E3, Table 5). It is possible that the observed difference could in part be ascribed to the protocol used for the preparation of NM300K stock suspension, which had a lower particle size in E1, but as this size difference did not carry over to the exposure suspensions, it could also reflect differences in nematode batch sensitivity. There was no obvious correlation between toxicity and measured concentrations or size distribution in the exposure solutions.



440

441 **Figure 4**. Growth, fertility and reproduction of *C. elegans* after 96 h exposure to $AgNO_3$ and NM300K,

442 expressed as % of controls.

Table 5. Effect concentration 10 % and 50 % (EC10 and EC50) on growth, fertility and reproduction for NM300K and $AgNO_3$ exposures in experiments 1-3 (E1-E3). Results are provided with their 95 % confidence interval (CI) in parentheses. Parameter estimations were based on nominal concentrations. For each endpoint, EC with overlapping CI are indicated by similar letters. NA: not available.

		NM300K		**$AgNO_3$**	
		EC10 (mg Ag L^{-1})	EC50 (mg Ag L^{-1})	EC10 (mg Ag L^{-1})	EC50 (mg Ag L^{-1})
Growth	E1	0.37 (0.28-0.73) a	1.45 (1.33-1.87) a	0.36 (0.16-0.41) b	0.47 (0.43-0.59) a
	E2	0.85 (0.51-1.38) a	2.91 (2.37-3.90) b	0.07 (0.05-0.11) a	0.38 (0.32-0.47) a
	E3	2.43 (1.54-3.63) b	10.70 (8.91-12.71) c	0.87 (0.79-1.28) c	1.82 (1.64-1.96) b
Fertility	E1	0.45 (0.38-0.75) a	0.56 (0.48-0.77) a	NA	NA
	E2	0.80 (0.65-0.94) a	1.23 (1.05-1.36) b	0.08 (0.05-0.13) a	0.15 (0.11-0.20) a
	E3	3.64 (3.18-3.79) b	4.29 (3.90-4.39) c	0.38 (0.35-0.41) b	0.49 (0.47-0.49) b
Reproduction	E1	0.09 (0.01-0.35) a	0.26 (0.09-0.44) a	0.07 (0.042-0.094) a	0.11 (0.078-0.121) ab
	E2	0.52 (0.22-0.82) a	0.74 (0.48-0.92) b	0.06 (0.057-0.066) a	0.08 (0.072-0.080) a
	E3	0.18 (0.15-0.49) a	0.84 (0.69-1.18) b	0.08 (0.074-0.076) a	0.09 (0.086-0.091) b

3.3 Linking exposure, uptake and toxicity

Speciation of molecular form and size fractionation in exposure characterization for organisms like *C. elegans* is of upmost importance, in which normal feeding behavior entails ingesting bacteria and particles in the size range 100 nm to 3 µm from the water column (Yang et al., 2012). Although the initial particle size in the current exposure was between 8-23 nm as measured by TEM, and thus <100 nm, the presence of larger particle aggregates (*ca*. 200 nm) were well within the feeding range of *C. elegans*. There were clear differences between the $AgNO_3$ and NM300K exposures with respect to particle size and the presence of <3 kDa Ag in suspension (in the absence of *E. coli* and *C. elegans*) (Figure 1 and Table S2, E1). As expected, nanosized particles could be identified in NM300K exposures (Figures 1-3, Table 3). However, $AgNO_3$ exposures also contained larger particulate matter (Figures 2 and 3, Table 3), which indicates formation of Ag-complexes.

Prior to the addition of *E. coli* to the exposure suspensions, the <3 kDa Ag fraction was significantly lower in NM300K treatments (4-8 % of total Ag) than in $AgNO_3$ treatments (13-90 % of total Ag) (Table S2, E1). But at the actual beginning of the toxicity test, *i.e.* upon addition of *E. coli*, no <3 kDa Ag was

found, suggesting rapid affiliation of ionic Ag with the bacteria (Table S2, E2). The circumstance that a significant proportion of Ag was associated to *E. coli* indicates that bacteria act as a vehicle to promote the uptake of ions, and possibly also AgNPs, via ingestion. The observed differences in particle size, presence of aggregates and <3 kDa Ag between NM300K and $AgNO_3$ exposures were not reflected in the Ag concentrations in accumulated in *C. elegans* (Table 4). Although incorporated Ag (still present in nematodes after depuration) was very similar in $AgNO_3$ and NM300K treatments, toxicity correlated strictly to the exposure concentrations. The fact that similar levels of Ag uptake in the nematodes caused highly different effect levels, showed that toxicity was here highly determined by the type of exposing agent (NM300K vs $AgNO_3$). The toxicity associated to Ag uptake from $AgNO_3$ exposures (both total and incorporated Ag) was higher than from NM300K exposures (Table 4). This observation is consistent with previous reports indicating that AgNPs act mostly in the intestine (Yang et al., 2014), while Ag(I) is probably more effectively taken up into the intestinal cells where it interferes with enzymes and organelle functions.

In line with this model, reproduction, the most sensitive endpoint measured in the current study, was already strongly affected at the lowest $AgNO_3$ concentration (44-95 % reduction at 0.1 mg Ag L^{-1}). In contrast, similar effects in reproduction were only observed from 0.5-1 mg Ag L^{-1} in NM300K exposures (Figure 4). This could potentially be linked to the initial differences seen in the <3 kDa Ag fraction. The higher concentration of <3 kDa Ag in $AgNO_3$ exposures presumably led to a higher concentration of Ag associated with the bacteria *E. coli*. As the food source for nematodes, *E. coli* act as vehicles enhancing bioavailability of Ag(I), and consequently also the toxicity. Likewise, the toxicity observed in NM300K exposures could potentially also be directly related to the concentration of dissolved Ag, either through dissolution of NM300K (Yang et al., 2012), or release of Ag(I) bound to the surfactants in the original NM300K nanomaterial (Köser et al., 2017). Although, NM300K AgNPs have been reported to be relatively stable over short-time exposure periods, the physicochemical environment and processes in the digestive tract of *C. elegans* (e.g. acidic environment with pH between 3.6 and 6.0 (Chauhan et al., 2013)) could potentially speed up the release of Ag(I). However, as discussed above and supported by size fractionation measurements, dissolution of Ag(I) from the nanoparticles is not necessarily or likely the sole explanation to the observed differences in toxicity. A nanospecific toxicity, e.g. oxidative stress generated from ROS formation on the surface of the nanoparticles, is another mechanism of toxicity (Yang et al., 2012). In the current study we unfortunately cannot conclude on the mechanistic pathways leading to toxicity.

4. Conclusions

The last decade of nanotoxicity research has generated a large number of published, available toxicity data. However, due to the lack of harmonized testing, the data are often not considered as reliable for risk assessment (Kos et al., 2016). Standardized operating protocols (ISO or OECD) used for traditional soluble chemicals with high reproducibility do not work as well for nanotoxicity studies. These questions have been addressed in several EU projects (CO-NANOMET, NANoREG, Nanofate, NanoTest) however the lack of harmonized protocols is still a problem. Although the three separate experiments (E1-E3) reported in this paper did not follow a fully harmonized testing protocol, they were conducted in the same way with respect to key points such as experimental media, temperature, type of Ag nanoparticles, experimental set up, strain of *C. elegans* etc. Despite some variation in EC values, the current study showed a relatively good agreement among the different experiments, with EC values in the same order of magnitude. Especially when it comes to the most sensitive endpoint, reproduction, the EC values were similar over a period of three/two years for both $AgNO_3$ and NM300K exposures. Characterization of the exposure solutions for both AgNPs and $AgNO_3$ treatments, suggested that, in this case, variations in toxicity did not appear to be correlated to differences in particle size, aggregation or dissolution between the three experiments. In addition, differences in AgNPs and $AgNO_3$ exposure could not be explained solely by differences in Ag speciation.

Although three studies performed by the same laboratory is far from enough data to conclude about reproducibility, it is clear that understanding differences in reproducibility of tests between laboratories, both in the same and across different species, cannot be analyzed without information of the behavior and characterization of the exposure media. Likewise understanding differences between NP and ion mechanisms requires that characterization is also carried out on ion exposure solutions, which is often overlooked in nanotoxicity tests.

5. Acknowledgments

This work was funded by the Research Council of Norway through the NANoREG (grant number 310584) and NANOCHARM (grant number 221391) projects. The authors would also like to thank K.A. Jensen and S. Lohne for assistance with the ICP-MS measurements and E. Maremonti for technical support in the lab.

6. Data Accessibility

Research data pertaining to this article is located at figshare.com: DOIs for all figures will be uploaded accordingly.

References

ANDRASSY, I. 1956. Die rauminhalts-und gewichtsbestimmung der fadenwürmer (Nematoden). *Acta Zoologica Hungarica,* 2**,** 1-5.

BORM, P., KLAESSIG, F. C., LANDRY, T. D., MOUDGIL, B., PAULUHN, J., THOMAS, K., TROTTIER, R. & WOOD, S. 2006. Research strategies for safety evaluation of nanomaterials, Part V: Role of dissolution in biological fate and effects of nanoscale particles. *Toxicological Sciences,* 90**,** 23-32.

CARLSON, C., HUSSAIN, S. M., SCHRAND, A. M., BRAYDICH-STOLLE, L. K., HESS, K. L., JONES, R. L. & SCHLAGER, J. J. 2008. Unique Cellular Interaction of Silver Nanoparticles: Size-Dependent Generation of Reactive Oxygen Species. *Journal of Physical Chemistry B,* 112**,** 13608-13619.

CHAUHAN, V. M., ORSI, G., BROWN, A., PRITCHARD, D. I. & AYLOTT, J. W. 2013. Mapping the pharyngeal and intestinal pH of Caenorhabditis elegans and real-time luminal pH oscillations using extended dynamic range pH-sensitive nanosensors. *ACS nano,* 7**,** 5577-5587.

CRESSMAN, C. P. & WILLIAMS, P. L. 1997. Reference toxicants for toxicity testing using Caenorhabditis elegans in aquatic media. *Environmental Toxicology and Risk Assessment: Modeling and Risk Assessment Sixth Volume.* ASTM International.

CYPSER, J. R. & JOHNSON, T. E. 2002. Multiple stressors in Caenorhabditis elegans induce stress hormesis and extended longevity. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 57**,** B109-B114.

ELLEGAARD-JENSEN, L., JENSEN, K. A. & JOHANSEN, A. 2012. Nano-silver induces dose-response effects on the nematode Caenorhabditis elegans. *Ecotoxicology and environmental safety,* 80**,** 216-223.

GALLEGO-URREA, J. A., HAMMES, J., CORNELIS, G. & HASSELLÖV, M. 2016. Coagulation and sedimentation of gold nanoparticles and illite in model natural waters: Influence of initial particle concentration. *NanoImpact,* 3**,** 67-74.

GHAFOURI, S. & MCGHEE, J. D. 2007. Bacterial residence time in the intestine of Caenorhabditis elegans. *Nematology,* 9**,** 87-91.

GOMES, S. I., ROCA, C. P., SCOTT-FORDSMAND, J. J. & AMORIM, M. J. 2017. High-throughput transcriptomics reveals uniquely affected pathways: AgNPs, PVP-coated AgNPs and Ag NM300K case studies. *Environmental Science: Nano,* 4**,** 929-937.

GONÇALVES, S. F., D PAVLAKI, M., LOPES, R., HAMMES, J., GALLEGO-URREA, J. A., HASSELLÖV, M., JURKSCHAT, K., CROSSLEY, A. & LOUREIRO, S. 2017. Effects of silver nanoparticles on the freshwater snail Physa acuta: The role of test media and snails' life cycle stage. *Environmental toxicology and chemistry,* 36**,** 243-253.

HANDY, R. D., CORNELIS, G., FERNANDES, T., TSYUSKO, O., DECHO, A., SABO-ATTWOOD, T., METCALFE, C., STEEVENS, J. A., KLAINE, S. J., KOELMANS, A. A. & HORNE, N. 2012. Ecotoxicity test methods for engineered nanomaterials: Practical experiences and recommendations from the bench. *Environmental Toxicology and Chemistry,* 31**,** 15-31.

HANDY, R. D., VON DER KAMMER, F., LEAD, J. R., HASSELLOV, M., OWEN, R. & CRANE, M. 2008. The ecotoxicology and chemistry of manufactured nanoparticles. *Ecotoxicology,* 17**,** 287-314.

HUNT, P. R. 2017. The C-elegans model in toxicity testing. *Journal of Applied Toxicology,* 37**,** 50-59.

ISO 2010. Water quality: Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of Caenorhabditis elegans (Nematoda).

JENSEN, K. A., BOOTH, A., KEMBOUCHE, Y. & BORASCHI, D. 2016. Validated protocols for test item preparation for key in vitro and ecotoxicity studies, NANoREG Deliverable D2.06.

JIANG, J. K., OBERDORSTER, G. & BISWAS, P. 2009. Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. *Journal of Nanoparticle Research,* 11**,** 77-89.

KOS, M., KAHRU, A., DROBNE, D., SINGH, S., KALČÍKOVÁ, G., KÜHNEL, D., ROHIT, R., GOTVAJN, A. Ž. & JEMEC, A. 2016. A case study to optimise and validate the brine shrimp Artemia franciscana immobilisation assay with silver nanoparticles: The role of harmonisation. *Environmental Pollution,* 213**,** 173-183.

KÖSER, J., ENGELKE, M., HOPPE, M., NOGOWSKI, A., FILSER, J. & THOMING, J. 2017. Predictability of silver nanoparticle speciation and toxicity in ecotoxicological media. *Environmental Science-Nano,* 4**,** 1470-1483.

LUNDQVIST, M., STIGLER, J., ELIA, G., LYNCH, I., CEDERVALL, T. & DAWSON, K. A. 2008. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 14265-14270.

MALLEVRE, F., ALBA, C., MILNE, C., GILLESPIE, S., FERNANDES, T. F. & ASPRAY, T. J. 2016. Toxicity Testing of Pristine and Aged Silver Nanoparticles in Real Wastewaters Using Bioluminescent Pseudomonas putida. *Nanomaterials,* 6.

MAST, J. & DE TEMMEMAN, P.-J. 2016. Protocol(s) for size-distribution analysis of primary NM particles in air, powders, and liquids, NANoREG Deliverable D2.10.

MEYER, J. N., LORD, C. A., YANG, X. Y., TURNER, E. A., BADIREDDY, A. R., MARINAKOS, S. M., CHILKOTI, A., WIESNER, M. R. & AUFFAN, M. 2010. Intracellular uptake and associated toxicity of silver nanoparticles in Caenorhabditis elegans. *Aquatic toxicology,* 100**,** 140-150.

MONTES-BURGOS, I., WALCZYK, D., HOLE, P., SMITH, J., LYNCH, I. & DAWSON, K. 2010. Characterisation of nanoparticle size and state prior to nanotoxicological studies. *Journal of Nanoparticle Research,* 12**,** 47-53.

MULLEN, M., WOLF, D., FERRIS, F., BEVERIDGE, T., FLEMMING, C. & BAILEY, G. 1989. Bacterial sorption of heavy metals. *Applied and Environmental Microbiology,* 55**,** 3143-3149.

O'REILLY, L. P., LUKE, C. J., PERLMUTTER, D. H., SILVERMAN, G. A. & PAK, S. C. 2014. C. elegans in high-throughput drug discovery. *Advanced Drug Delivery Reviews,* 69**,** 247-253.

PETERSEN, E. J., HENRY, T. B., ZHAO, J., MACCUSPIE, R. I., KIRSCHLING, T. L., DOBROVOLSKAIA, M. A., HACKLEY, V., XING, B. & WHITE, J. C. 2014. Identification and avoidance of potential artifacts and misinterpretations in nanomaterial ecotoxicity measurements. *Environ. Sci. Technol,* 48**,** 4226-4246.

POYNTON, H. C., LAZORCHAK, J. M., IMPELLITTERI, C. A., BLALOCK, B. J., ROGERS, K., ALLEN, H. J., LOGUINOV, A., HECKMAN, J. L. & GOVINDASMAWY, S. 2012. Toxicogenomic Responses of Nanotoxicity in Daphnia magna Exposed to Silver Nitrate and Coated Silver Nanoparticles. *Environmental Science & Technology,* 46**,** 6288-6296.

RATTE, H. T. 1999. Bioaccumulation and toxicity of silver compounds: A review. *Environmental Toxicology and Chemistry,* 18**,** 89-108.

STARNES, D. L., UNRINE, J. M., STARNES, C. P., COLLIN, B. E., OOSTVEEN, E. K., MA, R., LOWRY, G. V., BERTSCH, P. M. & TSYUSKO, O. V. 2015. Impact of sulfidation on the bioavailability and toxicity of silver nanoparticles to Caenorhabditis elegans. *Environmental Pollution,* 196**,** 239-246.

SØRENSEN, S. N. & BAUN, A. 2015. Controlling silver nanoparticle exposure in algal toxicity testing A matter of timing. *Nanotoxicology,* 9**,** 201-209.

TYNE, W., LOFTS, S., SPURGEON, D. J., JURKSCHAT, K. & SVENDSEN, C. 2013. A new medium for Caenorhabditis elegans toxicology and nanotoxicology studies designed to better reflect natural soil solution conditions. *Environmental Toxicology and Chemistry,* 32**,** 1711-1717.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY, U. E. 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 5th edition. EPA-821-R-02-012. Office of water, Washington DC, USA. .

VAN DER PLOEG, M. J., HANDY, R. D., WAALEWIJN-KOOL, P. L., VAN DEN BERG, J. H., HERRERA RIVERA, Z. E., BOVENSCHEN, J., MOLLEMAN, B., BAVECO, J. M., TROMP, P. & PETERS, R. J. 2014. Effects of silver nanoparticles (NM-300K) on Lumbricus rubellus earthworms and particle characterization in relevant test matrices including soil. *Environmental toxicology and chemistry,* 33**,** 743-752.

VOELKER, D., SCHLICH, K., HOHNDORF, L., KOCH, W., KUEHNEN, U., POLLEICHTNER, C., KUSSATZ, C. & HUND-RINKE, K. 2015. Approach on environmental risk assessment of nanosilver released from textiles. *Environmental Research,* 140**,** 661-672.

WASMUTH, C., RUDEL, H., DURING, R. A. & KLAWONN, T. 2016. Assessing the suitability of the OECD 29 guidance document to investigate the transformation and dissolution of silver nanoparticles in aqueous media. *Chemosphere,* 144**,** 2018-2023.

YAMANAKA, M., HARA, K. & KUDO, J. 2005. Bactericidal actions of a silver ion solution on Escherichia coli, studied by energy-filtering transmission electron microscopy and proteomic analysis. *Applied and environmental microbiology,* 71**,** 7589-7593.

YANG, X., JIANG, C., HSU-KIM, H., BADIREDDY, A. R., DYKSTRA, M., WIESNER, M., HINTON, D. E. & MEYER, J. N. 2014. Silver nanoparticle behavior, uptake, and toxicity in Caenorhabditis elegans: effects of natural organic matter. *Environmental science & technology,* 48**,** 3486-3495.

YANG, X. Y., GONDIKAS, A. P., MARINAKOS, S. M., AUFFAN, M., LIU, J., HSU-KIM, H. & MEYER, J. N. 2012. Mechanism of Silver Nanoparticle Toxicity Is Dependent on Dissolved Silver and Surface Coating in Caenorhabditis elegans. *Environmental Science & Technology,* 46**,** 1119-1127.

Supporting information

Characterizing NM300K silver nanoparticles behavior, uptake and toxicity in *Caenorhabditis elegans*

Merethe Kleiven[a*], Lisa M. Rossbach[a], Julian A. Gallego-Urrea[ab], Dag Anders Brede[a], Deborah H. Oughton[a] and Claire Coutris[c]

[a] *Norwegian University of Life Sciences, Postboks 5003 NMBU, 1432 Ås, Norway*

[b] *Department of Marine Sciences, University of Gothenburg, Kristineberg 566, SE-45178 Fiskebäckskil Sweden*

[c] *Division of Environment and Natural Resources, Norwegian Institute of Bioeconomy Research, Høgskoleveien 7, 1431 Ås, Norway*

[*] *Corresponding author email address: merethe.kleiven@nmbu.no or deborah.oughton@nmbu.no*

Contains 2 tables and 3 figures

Tables

Table S1. Sample preparation and ICP-MS measurement parameters for E1, E2 and E3.

	E1	**E2**	**E3**
Sample volume	400 µL	200 µL	200 µL
Volume ratio sample:acid for digestion	5:4(HNO_3):1(HCl)	1:5	1:7.5
Digestion conditions	260 °C, 120 bar	80 °C, 4 h	80 °C, 4 h
Digestion method	Microwave (UltraClave 3, Milestone Ltd.)	Heating cabinet	Heating cabinet
Final acid concentration	2-4 vol % HCl 6.5 % HNO_3	10 vol % HNO_3	10 vol % HNO_3
Ag Isotopes	107, 109	107, 109	107, 109
Limit of detection	0.04 µg L^{-1}	0.65 µg L^{-1}	0.007 µg L^{-1}
Limit of quantification	0.135 µg L^{-1}	1.94 µg L^{-1}	0.02 µg L^{-1}
Internal Standard	Indium	Indium	none
Online standard	Rhodium	Rhodium	Rhodium
Gas mode	Oxygen	Oxygen	Oxygen

Table S2. Silver concentrations (total and <3 kDa) in $AgNO_3$ and NM300K treatments at the start (without bacteria in E1 and with bacteria in E2), after 20 h (E2)and end of the experiments (E1-3). ND: not determined.

		Start (0 h) without *E.coli*				End (96 h)	
E1	Nominal (µg L^{-1})	Total (µg L^{-1})	*% deviation from nominal concentration*	<3 kDa (µg L^{-1})	*% of total Ag*	Total (µg L^{-1})	<3 kDa (µg L^{-1})
$AgNO_3$	100	93	*-8*	79	*86*	75	<0.135
	500	475	*-5*	110	*23*	435	<0.135
	1000	975	*-3*	123	*13*	ND	ND
	2000	2000	*0*	1794	*90*	1950	1
	4000	3750	*-6*	2178	*58*	ND	ND
NM300K	100	105	*5*	4	*4*	75	<0.04
	500	525	*5*	33	*6*	415	<0.135
	1000	1325	*33*	72	*5*	ND	ND
	2000	2375	*19*	104	*4*	2500	1
	4000	4875	*22*	397	*8*	ND	ND
Control	0	<0.04	-	<0.04	-	<0.04	<0.04

		Start (2 h) with *E.coli*				After 20 h		End (96 h)	
E2	Nominal (µg L^{-1})	Total (µg L^{-1})	*% deviation from nominal concentration*	<3 kDa (µg L^{-1})	*% of total Ag*	Total (µg L^{-1})	<3 kDa (µg L^{-1})	Total (µg L^{-1})	<3 kDa (µg L^{-1})
$AgNO_3$	100	95	*-5*	<0.65	*ND*	67	<0.65	57	<0.65
	500	518	*4*	<0.65	*ND*	423	<0.65	427	<0.65
	2000	2027	*1*	<0.65	*ND*	1960	<0.65	2045	<0.65
NM300K	100	81	*-19*	<0.65	*ND*	79	<0.65	72	<0.65
	500	397	*-21*	<0.65	*ND*	387	<0.65	317	<0.65
	2000	1555	*-22*	<0.65	*ND*	1614	<0.65	1584	<0.65

		End (96 h)				
E3	Nominal (µg L^{-1})	Total (µg L^{-1})	After centrifugation*	*% of total Ag*	<3 kDa (µg L^{-1})	*% of total Ag*
$AgNO_3$	250	185	100	*54*	0.11	*0.06*
	1000	760	85	*11*	0.14	*0.02*
	4000	3967	566	*14*	0.93	*0.02*
NM300K	1800	1400	221	*16*	0.68	*0.05*
	7300	4200	723	*17*	0.59	*0.01*
	29000	14667	1586	*16*	0.42	*<0.01*

*2000×g, 15 min

Figures

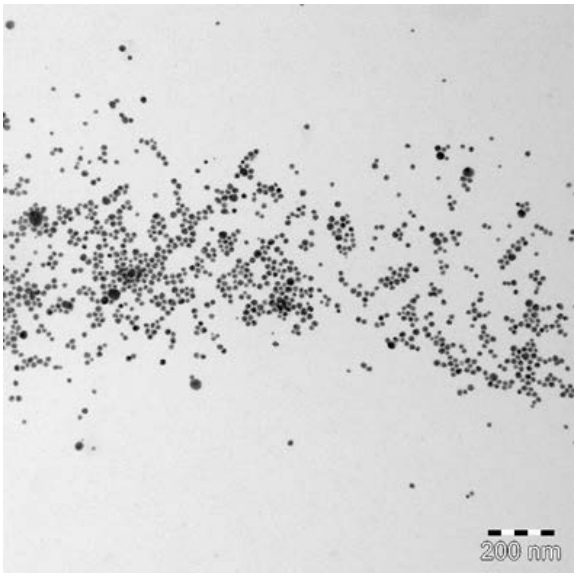
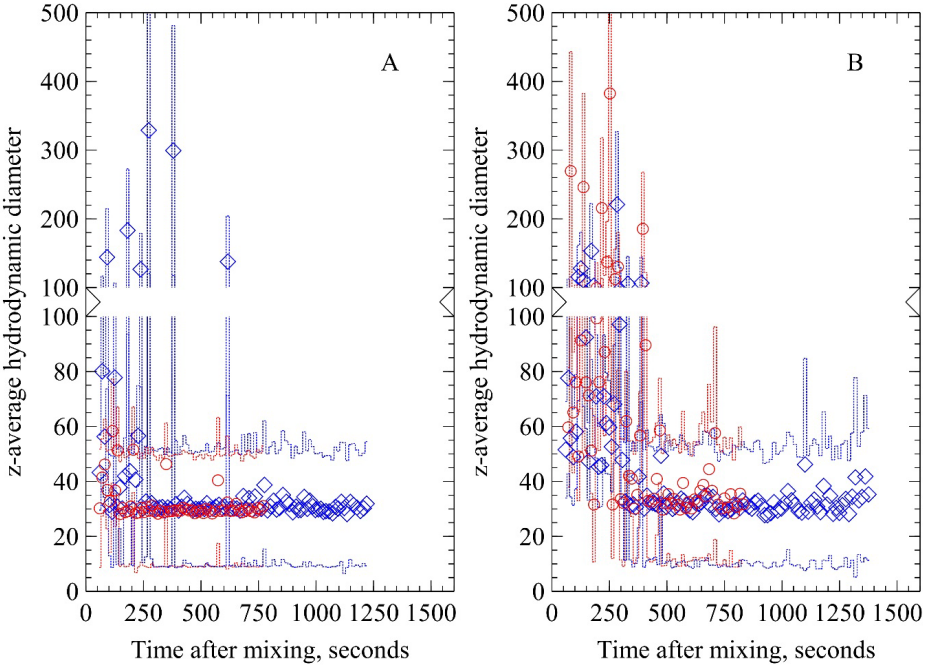
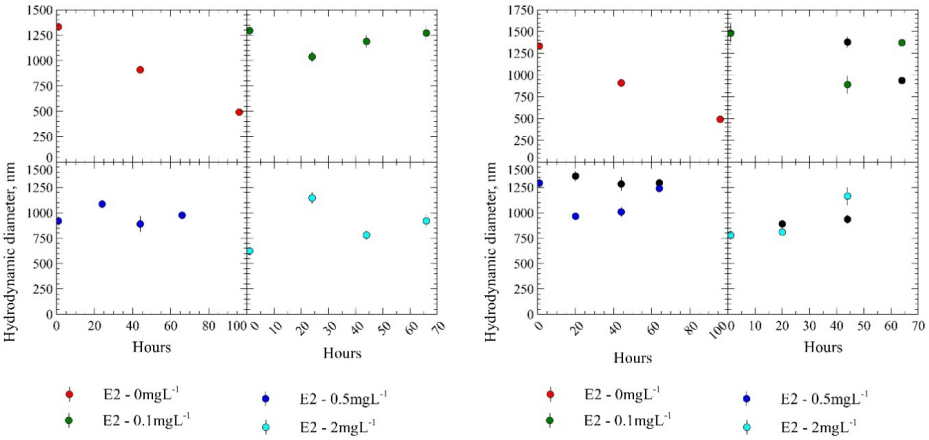


Figure S1. TEM image of NM300K AgNPs suspended in MilliQ water.



665

666 **Figure S2**. Time resolved DLS measurements performed on NM300K suspensions in MilliQ water. A
667 corresponds to measurements done at 10 mg Ag L^{-1}, and B at 1 mg Ag L^{-1}. Zeta-average hydrodynamic
668 diameters, d_H, were obtained as explained in the text and duplicate values are presented with the
669 markers. The dotted lines correspond to the corresponding color-matched standard deviation obtained
670 from polydispersity-index, PDI, values assuming a Gaussian profile distribution ($SD = (d_H^2.PDI)^{0.5}$).



671

672 **Figure S3**. Zeta-average diameters obtained from DLS measurements (in E2) of NM300K AgNPs (left) and
673 $AgNO_3$ (right) exposure suspensions, in presence of bacteria *E. coli*. Averages of three replicated
674 measurements are presented and error bars represent one standard deviation. Black symbols
675 correspond to the same sample measured after resuspension.

676

Paper II

Growth inhibition in Raphidocelis subcapita – evidence of nanospecific toxicity of silver nanoparticles

Merethe Kleiven[a*], Ailbhe Macken[b], Deborah H. Oughton[a]

[a] *Norwegian University of Life Sciences, Center for Environmental Radioactivity, P.O.Box 5003 NMBU, 1432 Ås, Norway*

[b] *Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway*

[*] Address correspondence to *merethe.kleiven@nmbu.no* or *deborah.oughton@nmbu.no*

ABSTRACT

Silver, known for its antibacterial properties and for its toxicity to aquatic organisms, is one of the most frequently used nanomaterials and silver nanoparticles can be found in a range of consumer products as well as medical applications. The present study investigated the toxicity of three different silver nanomaterials (Mesosilver (M-Ag), NM300K and NM302) and $AgNO_3$, in the algae *Raphidocelis subcapitata.* Exposures in the low µg L^{-1} range were combined with characterization of exposure media to determine whether differences in toxicity could be linked to changes in Ag speciation and/or any nanospecific mechanisms. All tested Ag compounds, except the NM302, reduced growth in the following order $AgNO_3$ ≥ M-Ag > NM300K > NM302 with 50% effect concentrations of 7.09 (3.83-10.52), 9.7 (range not calculated) and 24.18 (15.66-98.16) µg L^{-1}, for $AgNO_3$, Mesosilver and NM300K, respectively. Characterization of exposure media showed that both concentration and time influenced the speciation and stability of Ag in algal test media, regardless of Ag source, and also affected the toxicity to *R. subcapitata*. In both $AgNO_3$ and Mesosilver exposure the toxicity was correlated with the presence of Ag(I) (< 10 kDa), however levels of Ag(I) were too low to account for the observed Mesosilver effects, indicating a nanospecific contribution. Nanospecific toxicity was also observed for NM300K after 24 h of exposure, however the algae population recovered over time probably due to changes in exposure caused by aggregation of the nanoparticles.

Keywords: Silver nanoparticles, *Raphidocelis subcapitata,* Characterisation, aquatic toxicity.

1. INTRODUCTION

The antimicrobial properties of silver have been known for centuries, and this is the reason why silver nanoparticles (AgNPs) are amongst the most frequently used nanomaterials on the market (Vance et al., 2015). In addition to medical applications (e.g. wound dressings, surface coatings of medical devices), AgNPs are increasingly being used in consumer products, for example in cosmetics, cloths, cleaning agents, and as food additives (Echavarri-Bravo et al., 2017). With the increase in AgNP applications follows an increased risk of environmental release of AgNPs and their transformation products (e.g. Ag_2S and AgCl) potentially posing a risk to biota (Ribeiro et al., 2015).

The toxicity of Ag in aquatic environments is well-documented (Ratte, 1999), and AgNPs have been reported to induce toxicity to a range of different organisms: bacteria (Fabrega et al., 2009, Echavarri-Bravo et al., 2017), algae and invertebrates (Ribeiro et al., 2015, Sørensen and Baun, 2015), and fish (Chae et al., 2009, Bruneau et al., 2016). However, despite the attention nanoparticle toxicity has received in the last decades there still are uncertainties regarding toxicity mechanisms, particularly whether observed effects can be attributed to nanospecific mechanisms, be explained completely by the release of ions, or by a combination of the two (Fabrega et al., 2011, Sendra et al., 2017). Understanding this will depend on the type of particle and its physicochemical properties (e.g. size, surface charge, coating) as well as exposure conditions (e.g. media composition, pH, temperature, conductivity). Highlighting the importance of exposure characterization throughout the experimental test period in order to try and elucidate and understand the possible mechanisms of toxicity

As primary producers, algae play an essential role in aquatic ecosystems (Ribeiro et al., 2015, Wang et al., 2016), and alterations to these communities are likely to influence species at higher tropic levels, and therefore potentially impact the whole ecosystem (Ribeiro et al., 2015). In the present study the freshwater green algae *Raphidocelis subcapitata*, a commonly used species in regulatory testing and a key constituent in aquatic systems, were exposed to three different AgNPs (NM300K,

Mesosilver, NM302), as well as $AgNO_3$. The nanomaterials selected for testing represented different sizes, shapes and stabilizing agents. The selected materials included two OECD reference nanomaterials (NM300K, NM302) and a commercial cosmetic product (Mesosilver skin conditioner). The objective of the current study was to try to link differences in toxicity between the tested Ag compounds to the exposure characteristics obtained through thorough exposure characterization over the experimental exposure period. We hypothesized that aggregation and changes in concentrations of dissolved Ag(I), which was expected to be the active component, would be the main factors influencing toxicity.

2. MATERIALS AND METHODS

2.1. Preparation and characterization of silver suspensions

The nanomaterials used in this study were OECD reference Ag nanomaterials, specifically NM300K and NM302 (provided by the Joint Research Centre Reference Nanomaterial Repository, Ispra, Italy). Both nanomaterials were supplied as aqueous dispersants, the NM300K with 4% Polyoxyetylene Glycerol Trioleate and Tween 20, with a total Ag content of 10.16% (w/w) and the NM302 in a dispersion with the additives Rheology modifiers (≤ 2 wt%), polymers and surfactants (≤ 1 wt%) and a total Ag content of 7.4 wt%. In addition, a commercial silver colloidal suspension, Mesosilver (M-Ag NP) (Purest Colloids, Inc, Westampton, NJ, USA) was used, and finally silver nitrate (p.a. quality, Sigma-Alrich) was included as a positive control, a reference for dissolved silver toxicity.

The NM300K and NM302 stock suspensions were prepared according to Jensen et al. (2016). Briefly, a 2.56 g Ag L^{-1} stock suspension was prepared by dispersing the original suspensions in MilliQ water (18 MΩ·cm) and sonicating for 10 min at 10% amplitude using a 400 Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, USA) equipped with a standard 13 mm disruptor horn (Model number: 101-147-037). The M-Ag NP, provided as a colloidal suspension from the manufacturer with a concentration of 20 mg L^{-1}, was used without any sonication. A 1 g L^{-1} stock solution of $AgNO_3$ was prepared in MilliQ water. Intermediate stock suspensions of 50 µg Ag L^{-1} were prepared from the

main stock suspensions of NM300K and NM302, while for $AgNO_3$ an intermediate stock solution of 10 Ag mg L^{-1} was prepared. These intermediate stock solutions were used to prepare the exposure suspensions by direct addition to the algal test medium.

Particle size of the AgNPs in stock suspensions were obtained by transmission electron microscopy (TEM) and dynamic light scattering (DLS). Characterization of exposure suspensions was conducted with DLS and size fractionation coupled with inductively coupled plasma mass spectrometry (ICP-MS, Agilent 8800) for measurements of Ag concentrations.

*2.1.1.*Transmission electron microscope

The three AgNP stock suspensions were added (10 µl) to a 400-mesh Cu coated Piloform film (Agar Scientific, Essex, UK) and the samples were left to evaporate overnight in the dark. The images were acquired, on a FEI Morgani 268 transmission electron microscope (FEI, Eindhoven, Netherlands) operating at 80 keV. The images were analyzed with the software Adobe Photoshop CS5 to obtain the average particle size for the three tested nanoparticles.

2.1.2. Dynamic light scattering

Particle size measurements were performed on a Malvern Zetasizer ZS (Malvern instruments Ltd, Worcestershire, UK) equipped with a laser source with a wavelength of 633 nm. The Zeta-averaged hydrodynamic diameters and size distributions were obtained for the main stock suspension of AgNPs (2.56 g L^{-1} for NM300K and NM302, 20 mg L^{-1} for M-Ag), as well as intermediate stocks and exposure suspensions at 0, 24, 48, and 72 h. The measurements were performed on samples pre and post centrifugation (4000 rpm, 10 min) conducted to remove the algae from suspension.

2.1.3. Total Ag concentrations and size fractionation

To obtain information on Ag concentrations and size fractions present in exposure suspensions over time, membrane filtration followed by ICP-MS were performed. For determination of total Ag concentrations samples were collected from the exposure suspensions prior to addition of algae at time zero, and then at 24, 48, and 72 h of exposure. For the samples collected during the exposure (24-72 h) an additional sample was collected for determination of total Ag concentration after algae was removed by centrifugation at 4000 rpm for 10 min, prior to fractionation and analysis. After removal of the algae, a size fractionation was conducted by membrane filtration with a cut-off of 0.22 µm (membrane syringe filter, Millipore) and an ultracentrifugation filter (centrifuged at 5000xg for 15 min) with a 10 kDa cut-off (Amicon Ultra-15 centrifugal filters). The size fraction > 0.22 µm was defined as particulate, fraction < 0.22 µm and > 10 kDa was defined as nanoparticles/colloidal, while the fraction < 10 kDa was defined as low molecular mass (LMM) and assume to be dissolved Ag. All fractionated water samples for Ag analysis were acidified with 10 vol% ultrapure HNO_3. Samples were then stored in the dark at 4 °C prior to ICP-MS measurements (ICP-MS, Agilent 8800).

2.2. Test organisms, culture preparations and growth inhibition test

The freshwater algae *Raphidocelis subcapitata* (NIVA strain CHL 1 from The Norwegian Culture Collection of Algae, NORCCA, owned by NIVA, Oslo, Norway) was exposed to three different Ag NPs (NM300K, NM302, and M-Ag NPs) and $AgNO_3$. The tests were conducted according to the OECD 201 (OECD, 2011), with slight modifications according to Cerrillo and Mendoza (2015).

2.2.1. Algal growth inhibition test

The algae were cultivated in 250 ml Erlenmeyer flasks (100 ml culture volume), capped with air permeable cellulose stoppers to allow gas exchange. Both pre-culturing and the experiments were conducted under test conditions: at 23 °C and at an illumination 60 µE m^{-2} s^{-1} (cool fluorescent light). The flasks were continuously shaken at 90 rpm. The duration of the tests were 72 h. The pH was measured at the beginning and end of the tests. The algae were pre-cultured in OCED 201 media for

3 days prior to test initiation, to ensure algae were growing exponentially. Exposure media was a modified OCED medium without Fe-EDTA. The initial algal density of all test vessels was $5x10^6$ cells L^{-1} (density demined by a cell counter, Coulter counter) in a final volume of 1 ml.

Preliminary range finding tests were conducted with all compounds to determine the range of concentrations to be used in the definitive tests. Based on these results five concentrations, arranged in a geometrical series were selected for each compound. Six replicates for the control, (containing only medium and algae), were included in each test together with three replicates of each exposure concentration. An additional two replicates for each concentration were added for exposure characterization after 24 and 48 h. These additional replicates were not used for the calculation of the toxicological endpoints and were used as sacrificial vessels for characterization only. The nominal exposure concentrations were in the range of 0.32 to 32 µg Ag L^{-1} for $AgNO_3$, 5 to 50 µg Ag L^{-1} for M-Ag NPs, 2.56 to 25.6 µg Ag L^{-1} for NM300K, and 0.26 to 25.6 mg Ag L^{-1} for NM302. In addition to the control group, a dispersant control was included for the NM300K and NM302 toxicity tests to assess the potential negative/positive effects of the dispersant used to stabilize these two nanomaterials. The concentration of dispersants was equal to the concentration present in the highest exposure concentrations of NM300K and NM302, and was found not to induce any toxicity.

The toxicity endpoint investigated was growth inhibition compared to the control as a response to exposure to different silver nanomaterials and silver nitrate. The growth/growth inhibition was quantified by measuring the algal biomass as a function of time. This was conducted by chlorophyll-a-extraction in accordance to the method specified in Mayer et al. (1997). In short, 1 ml of all exposure suspensions were sampled and placed into individual foil-wrapped plastic tubes, 0.1 ml Locust Bean Gum suspension (30 mg in 20 ml H_20), and 4.4 ml acetone (100% with magnesium carbonate) were added to each tube. Samples were mixed well and stored in the dark at room temperature until the next day. Following storage, chlorophyll concentration was determined with a

fluorescence spectrophotometer (Agilent Technologies. Cary Eclipse Fluorescence Spectrophotometer), excitation wavelength 430 nm and emission wavelength of 670 nm.

The growth inhibition was calculated by converting the obtained fluorescence values into algae biomass by the means of a calibration curve. The calibration curve was obtained by measuring three replicate algae inoculums with four different algae densities ranging from $3x10^3$ to $5x10^5$ cells ml^{-1} both with a cell counter (Coulter counter) and with the fluorescence spectrophotometer. The obtained calibration curve had a R^2 of 0.99.

The reference substance $K_2Cr_2O_7$ was employed as a positive control and followed the same procedure as described above. The EC50 value for the reference substance $K_2Cr_2O_7$ was 0.6 mg/L (95% CI 0.187-1.972). In the controls for all tests, the change in pH was < 0.5 units during the test, well within the 1.5 units given as the maximum allowed change in the guideline (Table S3).

2.3. Statistical analysis

The statistical testing of growth inhibition was performed with GraphPad Prism 6 (GraphPad Software, La Jolla, CA 92037, USA). Statistical analysis was carried out using a one-way analysis of variance followed by a Tukey-Kramer means comparison test to identify significant differences compared to the controls. Statistical significance was accepted at *p* 0.05.

The EC10 and EC50 (concentration that elicits an estimated 10 and 50% toxic effect) values for all compounds were calculated using REGTOX-EV7.0.6.xls (Eric Vindimian http://eric.vindimian.9online.fr), a curve fitting macro for Microsoft Excel. Toxicity data for all compounds were fitted to a sigmoidal curve and either the Weibull or Hill models were used to calculate the effective concentration (EC) values.

3. RESULTS AND DISCUSSION

3.1. Particle characterization

3.1.1. TEM

The M-Ag and NM300 Ag NPs were both spherical (Table 1, Figure S1 a and b) and had a primary particle size of 11 ± 3 (n=425, n being the particle number) and 16 ± 5 (n=383, n being the particle number) nm, respectively. The NM302 Ag nanomaterial mainly contained long rod-shaped Ag NPs (µm range), measuring 176 ± 41 nm (n=30) in their smallest dimension (Table 1, Figure S1 c). Aggregates/agglomerates exceeding the size range of the primary particles were observed for all three nanomaterials. The particle sizes obtained for NM300K and NM302 are both according to the sizes given by the manufacturer (15 nm and 100-200 nm thick for NM300K and NM302, respectively). For M-Ag NPs the obtained particle size was larger than expected compared to the manufacturers information (0.6 nm). The most probable cause for this discrepancy is an overestimation in the size measurements, due to the difficulties of measuring the size of the smallest particles present in the TEM images.

3.1.2. DLS

The hydrodynamic particle sizes obtained for the stock suspensions are reported in Table 1. The Z-averaged particle sizes are generally larger than particle sizes obtained by TEM, according to expectations. The 'Number mean' particle (calculated from the intensity-based particle size) sizes were in accordance with the manufactures specifications of 0.6 and 15 nm for M-Ag and NM300K, respectively. (Table 1). Dynamic light scattering was not a suitable method for size characterization of the NM302 Ag NPs due to their rod shape, as well as their rather large size and instability in suspension (sedimentation of the particles).

As expected, the presence of algae in the exposure suspensions heavily influenced the particle size measurements obtained by DLS, and DLS was not able to give a reliable Z-averaged particle

diameter. Centrifugation of the samples to remove the algae did not improve the obtained results (s.d., and PDI did not decrease) (Table S1). As was seen in stock solutions, the Number mean particle size was considerably smaller than the Z-averaged particle size. However, low particle concentrations result in increasing measurement uncertainties, thus making DLS for particle characterization in exposure suspensions with low exposure concentrations (µg L^{-1}) challenging.

The Zeta potential for the M-Ag and NM300K stock suspensions (Table 1) indicate different stabilizing mechanisms for the two particles. The M-Ag seem to be electrostatically stabilized, which is in accordance with the findings of Echavarri-Bravo et al. (2017). The NM300K Ag NPs are known to be sterically stabilized through the adsorbance of non-ionic surfactants. The Zeta potential obtained for the NM300K AgNPs in the current study were close to zero and thus confirming the lack of electrostatic stabilization. These results agree with Lodeiro et al. (2017) and Kleiven et al. (2018) which both reported Zeta potentials for NM300K in MQ water to be slightly negative, but close to zero. However, other studies have reported zeta potentials of -22 ± 3 (Echavarri-Bravo et al., 2017) and -15 mV (Hund-Rinke et al., 2017), albeit at different concentrations and preparation methods. These discrepancies between reported studies highlight the importance of following standardized preparation methods for test solutions for use in ecotoxicity testing. This standardization is needed to improve the reproducibility and comparability of test results obtained by different researchers and organizations testing the same nanoparticle.

Table 1. Characterization of stock suspensions of the three Ag nanomaterials tested (Mesosilver (M-Ag), NM300K, and NM302) measured by TEM and DLS. Results provided as mean ± one standard deviation. NA: Not applicable.

	Stock concentration	TEM diameter (nm)	Z-Average diameter (nm)	Number mean diameter (nm)	Polydispersity index	Zeta potential (mv)
M-Ag	0.020 g L^{-1}	11 ± 3 (N= 425)	38.3 ± 0.3	1.0 ± 0.4	0.6 ± 0.04	-39 ± 2
NM300K	2.56 g L^{-1}	16 ± 5 (N=383)	73.6 ± 0.5	22 ± 16	0.284 ± 0.006	-0.2
NM302	2.56 g L^{-1}	176 ± 41 (N=30)	NA	NA	NA	NA

3.2. Exposure concentrations and size fractionation

Concentration and time were important factors influencing the speciation and stability of Ag in the test media, regardless of Ag source, thus potentially affecting the toxicity to *R. subcapitata*. Measured total Ag concentrations (Table S2) at the beginning of the exposure were between 70 and 90% of nominal concentrations in the $AgNO_3$ (0.3 ± 0.05, 0.7 ± 0.06, 2.4 ± 0.6, 8 ± 1, and 25 ± 2 µg Ag L^{-1}) and NM300K (2.0 ± 0.43, 3.7 ± 0.7, 7 ± 1, 14 ± 3, 24 ± 6 µg Ag L^{-1}) exposures. In the M-Ag exposures, measured concentrations were close to nominal (5.4 ± 0.1, 10.7 ± 0.6, 19 ± 0.0, 35 ± 1, and 53 ± 2 µg Ag L^{-1}). In the NM302 Ag NP exposures the measured concentrations were far from nominal, ranging from 79-250 µg Ag L^{-1} (the closest was 30% of nominal measured at the highest concentration), and sedimentation of the NPs could be observed in the exposure vessels. Therefore, all exposure concentrations are hereafter reported as measured concentrations.

A decrease in total measured Ag in the test media (i.e., the total sample including algae) was observed for all exposures over the 72 h duration of the test (Table S2), which has also been reported in other studies with AgNPs (Echavarri-Bravo et al., 2015, Ribeiro et al., 2015). For $AgNO_3$ and M-Ag the decrease was concentration dependent, with only a 10% decrease in the highest

concentrations. While NM300K showed no concentration dependence, with an average concentration decrease of 50±10%.

There is general agreement that the speciation of Ag is more important for the toxicity than the total concentration of Ag (Ratte, 1999, Köser et al., 2017). Thus, size fractionation was performed for all exposures to assess the change in Ag size fractions over time. The trend was the same for all exposure groups and all exposure concentrations, with aggregation and complexation resulting in a shift towards larger particulate matter over time (Figure 1). This pattern of aggregation over time has also been reported in studies on other organisms (Farmen et al., 2012, Kleiven et al., 2018).

In the highest $AgNO_3$ exposure concentration (25 µg Ag L^{-1}) 98% (24 µg Ag L^{-1}) of the Ag was present as LMM Ag at the beginning of the exposures (Figure 1). However, the LMM fraction decreased in a concentration dependent manner with a decrease in the percentage found as LMM as the initial concentration decreased, and also a relatively lower reduction over time as the initial exposure concentration increased. After 72 h, LMM Ag was only detectable in significant quantities in the two highest exposures concentrations (12 and 1 µg L^{-1} in the 25 and 8 µg Ag L^{-1} exposures, respectively).

In the M-Ag exposures NPs/colloids was the main Ag fraction present at time zero, however LMM Ag also constituted on average 33±2% of the total measured Ag (Figure 1, Table S2), although given the range seen in the Number Mean diameter, this fraction could have included small nanoparticles. The M-Ag exposures were relatively stable over time, with the exception of the two lowest concentrations (5.4 and 10.7 µg L^{-1}) where larger particles (> 220 nm) increased with time. Farmen et al. (2012) exposed Atlantic salmon (*Salmo salar*) to this Ag NP in a low ionic strength lake water, and also observed a relatively stable NP/colloidal Ag fraction over time (48 h) with a reduction in the LMM Ag as the most significant change in exposure.

Close to 100% of the Ag in the NM300K exposures was present as NPs at time zero, independent of concentration. With time, aggregation occurred and the particulate Ag (>220 nm) increased from zero to 80-90% after 72 h (Figure 1). Interestingly, the LMM Ag (< 10 kDa) also increased slightly over

time in a concentration dependent manner (ranging from 0.005 to 0.788 µg L^{-1} in the lowest to the highest exposure concentration) (Table S2). Köser et al. (2017) investigated the stability of the NM300K Ag NPs in different ecotoxicological media, including the OECD media used in this study. They found that the NM300K had a high dispersion and redox stability with little aggregation or dissolution over time (72 h). The dissolved Ag was reported to be 1-2% (total Ag concentration of 7.9 mg L^{-1}) which is in accordance with the findings reported in the current study. However, Köser et al. (2017) did not observe any further increase in dissolved Ag over time (3 days), nor any evidence of aggregation. This was contradictory to the current study where an increase in dissolved Ag (LMM Ag) (from 1% to 8%) was detected in the two highest exposure concentrations, plus evidence of aggregation. However, there were some differences between the two studies, specifically, the start concentrations differed (orders of magnitude higher in Köser et al. (2017) than in the current study), Fe-EDTA was removed from the media that was used in the current study and the presence of algae in the characterized exposure media. These differences could very well explain the contradictory results described above.

To assess the association of the Ag to the algae, either by adsorption or accumulation, the algae were removed by gentle centrifugation. For $AgNO_3$ and M-Ag the relative fraction of Ag associated with the algae increased with decreasing Ag concentration (Figure 2). For NM300K the association to algae seemed to be independent of exposure concentration (Figure 2). However, after 72 h larger particulate matter > 220 nm was the dominant fraction in the lower concentrations of $AgNO_3$ and M-Ag, as well as in all concentrations of NM300K, thus the results could reflect a co-removal of larger particles with the algae during centrifugation rather than association to the algae, or a combination of the two. In the higher exposure concentrations of $AgNO_3$ and M-Ag, where the colloidal/NP and LMM Ag remained the dominant fractions after 72 h, the reduction of Ag after removal of the algae most likely reflects the Ag bound to the algae. However, these results might be confounded by the fact that the amount of algae present in the highest exposure concentrations are considerably lower

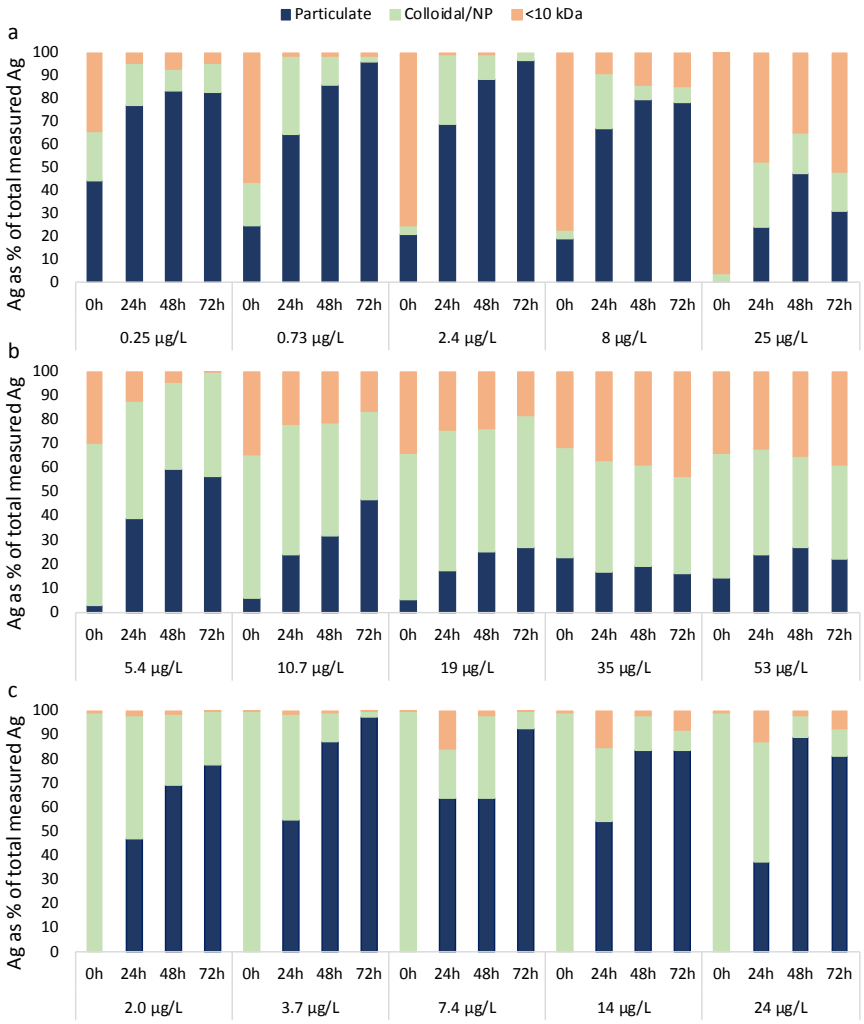
than in the lower concentrations due to the toxicity induced at these high exposure concentrations. Thus, less Ag would be bound to algae merely due to the lower number of algal cells available.

3.3 Growth inhibition

All tested Ag compounds, except the NM302 Ag rods, reduced growth in *R. subcapitata* in the following order $AgNO_3$ ≥ M-Ag > NM300K > NM302.

Increasing concentrations of $AgNO_3$, M-Ag and NM300K caused a decrease in algae growth. For $AgNO_3$ and NM300K exposures there was a concentration-dependent increase in growth inhibition after 24 h (Figure 3), with the exception of the lowest concentration (0.25 µg L^{-1}) in the $AgNO_3$ exposure where an increased growth was observed. A similar increased growth in the lowest exposure were observed in all treatments after 72 h of exposure, although only significant in the M-Ag NPs treatment ($p = 0.0005$). This is a commonly reported phenomenon, hormesis, also reported for nanomaterials (Iavicoli et al., 2014).

In the $AgNO_3$ exposure the growth inhibition observed after 24 h was still significant after 72 h ($p = 0.0146$ and <0.0001 for 8 and 25 µg Ag L^{-1}, respectively). While in the NM300K exposures, all significant growth inhibition at 24 h ($p = 0.0326$ and 0.0071 for 14 and 24 µg Ag L^{-1}, respectively) was no longer present after 48 and 72 h of exposure (Figure 3). In the M-Ag exposure significant growth inhibition ($p < 0.05$) was observed at all exposure concentrations, except the lowest (5.4 µg L^{-1}), after 24 h and remained relatively stable throughout the exposure with significant growth inhibition in the three highest exposure concentrations remaining after 72 h ($p < 0.05$).



307

308 Figure 1. Changes in size fractions of Ag (as % of total measured Ag) present in the a) $AgNO_3$, b)

309 Mesosilver (M-Ag NP), and c) NM300K Ag NP test media over time. The 'Particulate' fraction is

310 defined as > 220 nm, the 'Colloidal/NP' as > 10 kDa and < 220 nm , and last the '<10 kDa is the low

311 molecular mass fractions assumed to be dissolve Ag species.

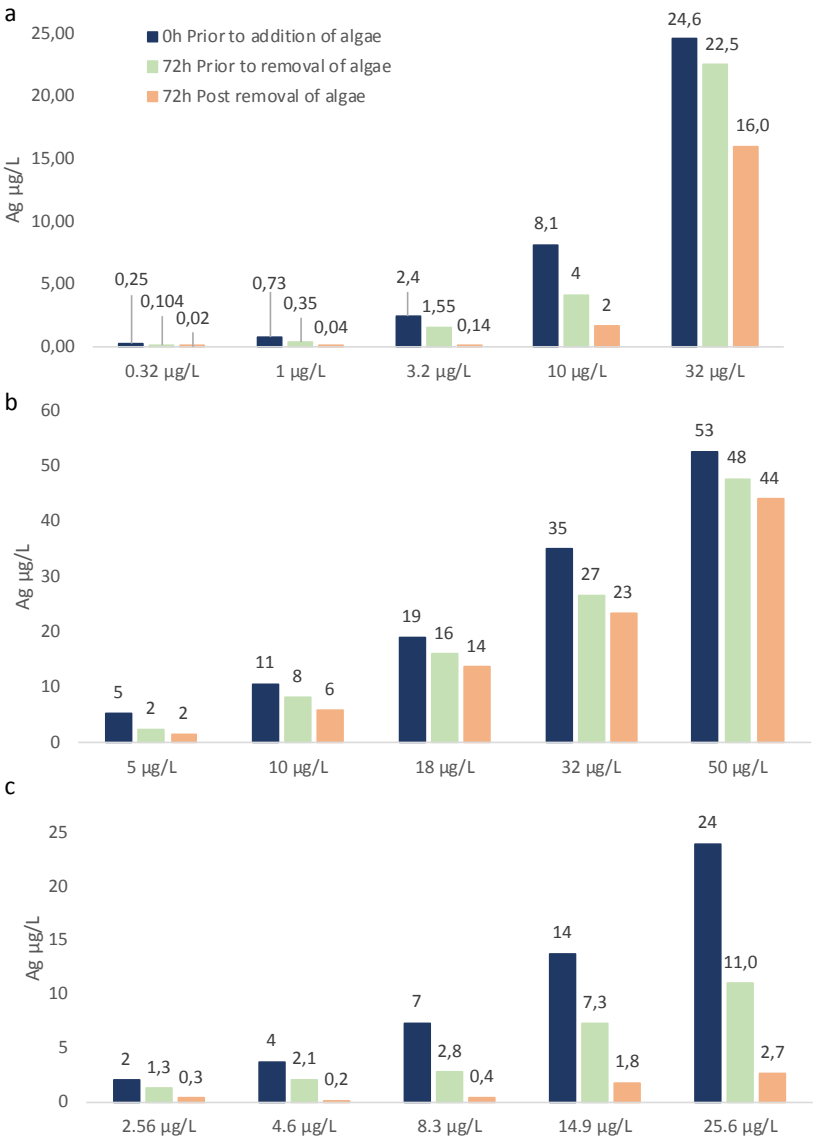
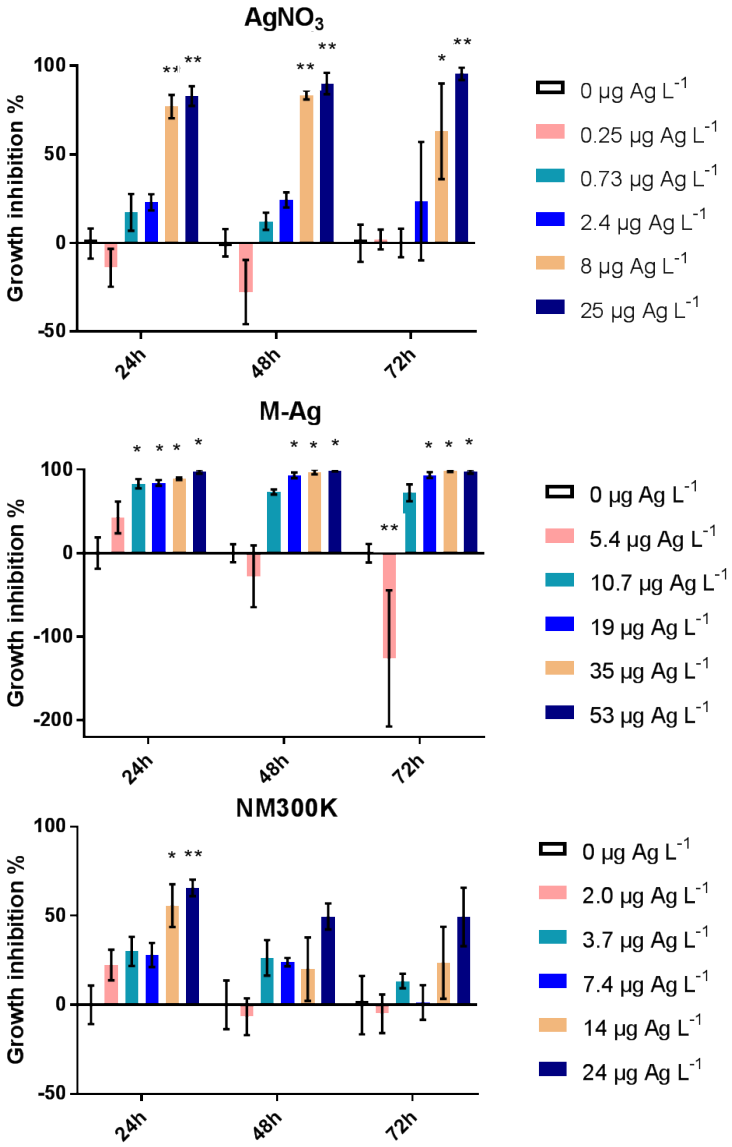


Figure 2. Measured total Ag concentration at t=0 h prior to addition of *R. subcapitata,* and after 72 h before and after removal of the algae by low speed centrifugation in a) $AgNO_3$, b) Mesosilver (M-Ag), and c) NM300K. The numbers over the data points represent the measured total Ag concentrations of each nominal concentration. Be aware the different scales on the Y-axis.

The EC50 for 72 h growth inhibition values obtained in the current study (Table 2) are among the lowest reported for $AgNO_3$ and Ag NPs in general and NM300K specifically. The EC50 values for *R. subcapitata* have previously been reported in the range 4.9-34 and 15-140 µg L^{-1} for $AgNO_3$ and NM300K, respectively (Ribeiro et al., 2014, Sørensen and Baun, 2015, Hund-Rinke et al., 2018). The EC50 reported for NM300K in the current study (13-24 µg L^{-1}) agrees well with the EC50 of 15-81 µg L^{-1} reported by Hund-Rinke et al. (2018). When it comes to the M-Ag NPs there are not much data available in the literature to compare with, especially in relation to algae. Ellegaard-Jensen et al. (2012) reported an LC50 value of 4.4 mg L^{-1} for *Caenorhabditis elegans*, however this is a species known to be highly tolerant to a range of contaminants. Farmen et al. (2012) reported a NOEC of 20 µg L^{-1} for Atlantic salmon exposed for 48 h, a species known for its sensitivity. Echavarri-Bravo et al. (2017) exposed marine algae species to $AgNO_3$, Mesosilver (Mesosilver Hot tub™ cleaner) and NM300K, and reported EC50 (growth inhibition) in the range of <10 to 50, 145 to above 1000 and as low as 72 µg L^{-1} for $AgNO_3$, NM300K and Mesosilver, respectively. Compared to these studies the EC values obtained in the present study are low, and of the four Ag compounds tested in the present study, $AgNO_3$ was the most potent growth inhibitor (Table 2).

Differences in toxicity between the different AgNPs could be linked to differences in size and stability seen in stock solutions, particularly the lack of toxicity observed for NM302K. Although Mesosilver and NM300K showed a similar size range for both Z-averaged diameter and TEM (11 and 16 nm, respectively), the 'Number mean' particle size was lower for Mesosilver (1.0 ± 0.4 nm) and in agreement with particle size given by the manufacturer (0.6 nm). Toxicity is known to be influenced by the size of the nanoparticles, usually increasing with decreasing particle size, which might be a contributing factor to the higher toxicity observed for these particles. However, these measurements do not explain all the observed toxicity, and characterization of the exposure solutions can provide additional information to better understand the changes in time and impact of Ag speciation on toxicity.



342

343 Figure 3. Growth inhibition (%) of *R. subcapitata* during 72 h of exposure to $AgNO_3$, M-Ag NPs and
344 NM300K Ag NPs. One way analysis of variance followed by Tukey-Kramer means comparison test
345 was used to identify significant differences compared to the control ,* $p < 0.05$, ** $p < 0.01$. Growth
346 inhibition (%) is the positive bar, thus negative bars are stimulation of growth.

Table 2. Effect concentration (µg Ag L^{-1}) 10% and 50% (EC10 and EC50) on growth *R. subcapitata* exposed to $AgNO_3$, M-Ag NPs and NM300K Ag NPs. Results are provided with their 95 % confidence interval (CI) in parentheses. Parameter estimations calculated using the Hill model.

Test substance	Exposure time (h)	EC10 (95% CI) (µg Ag L^{-1})	EC50 (95% CI) (µg Ag L^{-1})	Model (Regtox)[¤]
$AgNO_3$	24	0.8 (0.21-2.04)	4.19 (2.55-6.68)	Hill
	48	1.03 (0.24-2.78	3.36 (2.24-6.81)	Hill
	72	3.36 (1.58-4.70)	7.09 (3.83-10.52)	Weibull
M-Ag NP	24	1.65 (6.4E-07 – 7.67)	5.77 (0.91-11.90)	Hill
	48	7.99 (NC)	9.67 (NC)	Hill
	72	8.48 (NC)	9.74 (NC)	Hill
NM300K	24	0.93 (0.06-4.45)	12.83 (7.07-30.19)	Hill
	48	2.77 (0.01-18.42)	29.51 (13.40-298.53)	Hill
	72	9.23 (0.68-21.9)	24.18 (15.66-98.16)	Hill

NC: Not Calculated
[¤]REGTOX-EV7.0.6.xls (Eric Vindimian http://eric.vindimian.9online.fr), a curve fitting macro for Microsoft Excel.

3.4 Linking toxicity to exposure characterization

The results presented in the current study provide two lines of evidence that the toxicity observed in the M-Ag and NM300K AgNP exposures cannot be explained by the presence of LMM Ag, but rather a nanospecific toxicity or a combination of the two. Similar findings have also been reported by, for example, Sendra et al. (2017) and Huang et al. (2017).

359 The toxicity of $AgNO_3$ was linked to the presence of LMM Ag. Toxicity was only observed in the two
360 highest exposure concentrations, which were the only groups that still contained LMM Ag after 72 h.
361 A concentration dependent trend in toxicity was observed at 24 and 48 hours. However, by 72h no
362 toxicity was seen in the lower concentration exposures, by which time the LMM Ag had disappeared.
363 The LMM decreased from 6.3 and 24 μg L^{-1} in the two highest exposures at time 0 to 0.6 and
364 11.8 μg L^{-1} at 72 h, and the growth inhibition changed from 72 and 83% to 63 and 96% after 72 h.

365 The similarites in the observed toxicity over time at all concentrations of M-Ag reflects the stability
366 seen in the size fractionation results. Also here there are indications that the toxicity was linked to
367 the presence of LMM Ag, since the significant growth inhibition disappears with the reduction of
368 LMM Ag in the lowest concentrations. However, despite similar concentrations of LMM Ag (10 and
369 11 μg Ag L^{-1}) in the highest exposure (25 μg L^{-1}) of $AgNO_3$ and the 35 μg L^{-1} M-Ag exposure,
370 respectively, the growth inhibition was much higher in the M-Ag (89%) than in the $AgNO_3$ (53%) after
371 24h. This could indicate an additional NP induced toxicity in the M-Ag exposure.

372 In the NM300K exposure, the toxicity present after 24 h of exposure could not be linked to the
373 presence of LMM Ag since close to 100% of the Ag at time zero were present as nanoparticles in the
374 two highest exposure concentrations. There was a slight increase in LMM Ag over time (a maximum
375 LMM Ag concentration of 1.4 μg L^{-1} at 24 h), however this concentration of LMM Ag was not high
376 enough to be responsible for the level of toxicity on its own (growth inhibition of 66%) at 24 h,
377 strongly indicating a nanospecific effect of these particles.

378 It should however be kept in mind that in natural waters Ag will not exist as Ag^+ for long, but form
379 complexes with inorganic (e.g. chloride and thiosulphate) and organic (e.g. natural organic matter)
380 ligands (Hiriart-Baer et al., 2006). These ligands could influence the fate of different AgNPs in
381 different ways, and thus also their toxicity. For example, the presence of chloride has been reported
382 to reduce the toxicity of $AgNO_3$ towards *R. subcapitata* (Lee et al., 2005). Hiriart-Baer et al. (2006)
383 found that Ag-thiosulphate complexation increased the uptake of Ag into the algae, but that the

toxicity of these complexes was lower than for Ag^{+}. Components believed to influence AgNP behavior and fate in the environment (e.g. ionic strength, Total Organic Carbon, Dissolved Organic Carbon, chloride etc.) have been frequently studied. However, they are usually studied separately in controlled laboratory experiments and cannot account for the potential interactions between all of these different factors and the complexity found in natural waters (Conine et al., 2017). It is also important to consider seasonal changes in natural systems since they influence the condition of the organisms and thus potentially their sensitivity towards contaminants (Conine et al., 2017). Despite the low EC values reported in the current study (low µg L^{-1}), they are however still higher after 72 h than expected environmental concentrations of 0.5-2 µg L^{-1}(Gottschalk et al., 2011). The acute 24 h EC10 values are however < 2 µg L^{-1} for all Ag exposures, except the NM302 Ag rods. Whether or not these AgNPs in natural environments would pose a risk to the algae communities would depend on the environmental conditions (abiotic and biotic), however the higher complexity of the exposure media in natural systems would most likely reduce toxicity rather than enhance it.

4. CONCLUSIONS

The objective of this study was to investigate the effects of several types of silver nanomaterials to the freshwater algae *R. supcapitata* in combination with characterization to investigate changes in speciation and/or aggregation state over time and if relevant identify any possible nanospecific effects or mechanism. The tested particles were different in shape, size and stabilizing material. Using a robust set of fractionation and characterization techniques to monitor changes in nanoparticle behavior over the exposure period, insight into the aggregation processes in the test media that effected algal growth was possible. Our results successfully identified a combination of factors that appeared to be responsible for the observed toxicity. The toxicity of M-Ag and NM300K AgNPs could not be explained by the presence of LMM Ag alone, but rather a nanospecific effect or a combination of the two is more likely. The results also showed the importance, and dynamic nature of exposure duration and the need for characterization in toxicity testing of nanomaterials such as

409 silver. This study directly showed a change in the growth inhibition of the test algae with changes in
410 the behavior and speciation of the silver in solution. For example, in the case of AgNO2 and M-Ag at
411 low concentrations, significant effects that were observed early in the exposure period (after 24 and
412 48 hours) were seen to diminish over time. The importance of exposure duration is therefore
413 highlighted, especially when trying to extrapolate to possible realistic environmentally relevant
414 exposure scenarios. In the case of nanoparticles at low concentrations (as would be the case in the
415 aquatic environment) there may be the possibility that affects that are elicited after a short
416 exposure period may be lost and the algae may recover which would obviously lessen the
417 environmental risk. Overall the characterization of the test media suggests that changes in
418 speciation can be influenced by both time and concentration, as well as the algae concentration,
419 which can act as confounding factors for toxicity tests, but the information gained through this study
420 may also be important in understanding the behavior and risks of such metallic particles entering
421 into freshwater environments.

422 5. ACKNOWLEDMENT

423 The Research Council of Norway through the NANoREG (grant number 310584) and NANOCHARM
424 (grant number 221391) projects funded this work. The authors would also like to thank Ø. Enger for
425 assistance with the ICP-MS measurements.

426 6. DATA ACCESSIBILITY

427 Research data pertaining to this article is accessible upon request to the authors.

REFERENCES

BRUNEAU, A., TURCOTTE, P., PILOTE, M., GAGNE, F. & GAGNON, C. 2016. Fate of silver nanoparticles in wastewater and immunotoxic effects on rainbow trout. *Aquatic toxicology (Amsterdam, Netherlands),* 174**,** 70-81.

CERRILLO, C. & MENDOZA, G. 2015. Standard Operating Procedure. Toxicity Test with microalgae Pseudokirchneriella subcapitata for NANoREG core nanomaterials, NANoREG Deliverable D4.12. http://www.nanoreg.eu/media-and-downloads/deliverable-reports/312-nanoreg-deliverables-sops.

CHAE, Y. J., PHAM, C. H., LEE, J., BAE, E., YI, J. & GU, M. B. 2009. Evaluation of the toxic impact of silver nanoparticles on Japanese medaka (Oryzias latipes). *Aquatic toxicology,* 94**,** 320-327.

CONINE, A. L., REARICK, D. C., XENOPOULOS, M. A. & FROST, P. C. 2017. Variable silver nanoparticle toxicity to Daphnia in boreal lakes. *Aquatic Toxicology,* 192**,** 1-6.

ECHAVARRI-BRAVO, V., PATERSON, L., ASPRAY, T. J., PORTER, J. S., WINSON, M. K. & HARTL, M. G. 2017. Natural marine bacteria as model organisms for the hazard-assessment of consumer products containing silver nanoparticles. *Marine environmental research,* 130**,** 293-302.

ECHAVARRI-BRAVO, V., PATERSON, L., ASPRAY, T. J., PORTER, J. S., WINSON, M. K., THORNTON, B. & HARTL, M. G. 2015. Shifts in the metabolic function of a benthic estuarine microbial community following a single pulse exposure to silver nanoparticles. *Environmental pollution,* 201**,** 91-99.

ELLEGAARD-JENSEN, L., JENSEN, K. A. & JOHANSEN, A. 2012. Nano-silver induces dose-response effects on the nematode Caenorhabditis elegans. *Ecotoxicology and environmental safety,* 80**,** 216-223.

FABREGA, J., FAWCETT, S. R., RENSHAW, J. C. & LEAD, J. R. 2009. Silver nanoparticle impact on bacterial growth: effect of pH, concentration, and organic matter. *Environmental science & technology,* 43**,** 7285-7290.

FABREGA, J., LUOMA, S. N., TYLER, C. R., GALLOWAY, T. S. & LEAD, J. R. 2011. Silver nanoparticles: Behaviour and effects in the aquatic environment. *Environment international,* 37**,** 517-531.

FARMEN, E., MIKKELSEN, H. N., EVENSEN, O., EINSET, J., HEIER, L. S., ROSSELAND, B. O., SALBU, B., TOLLEFSEN, K. E. & OUGHTON, D. H. 2012. Acute and sub-lethal effects in juvenile Atlantic salmon exposed to low mu g/L concentrations of Ag nanoparticles. *Aquatic toxicology,* 108**,** 78-84.

GOTTSCHALK, F., ORT, C., SCHOLZ, R. W. & NOWACK, B. 2011. Engineered nanomaterials in rivers - Exposure scenarios for Switzerland at high spatial and temporal resolution. *Environmental pollution,* 159**,** 3439-3445.

HIRIART-BAER, V. P., FORTIN, C., LEE, D.-Y. & CAMPBELL, P. G. 2006. Toxicity of silver to two freshwater algae, Chlamydomonas reinhardtii and Pseudokirchneriella subcapitata, grown under continuous culture conditions: influence of thiosulphate. *Aquatic toxicology,* 78**,** 136-148.

HUND-RINKE, K., SCHLICH, K., KÜHNEL, D., HELLACK, B., KAMINSKI, H. & NICKEL, C. 2017. Grouping concept for metal and metal oxide nanomaterials with regard to their ecotoxicological effects on algae, daphnids and fish embryos. *NanoImpact*.

HUND-RINKE, K., SCHLICH, K., KÜHNEL, D., HELLACK, B., KAMINSKI, H. & NICKEL, C. 2018. Grouping concept for metal and metal oxide nanomaterials with regard to their ecotoxicological effects on algae, daphnids and fish embryos. *NanoImpact,* 9**,** 52-60.

IAVICOLI, I., FONTANA, L., LESO, V. & CALABRESE, E. J. 2014. Hormetic dose–responses in nanotechnology studies. *Science of the total environment,* 487**,** 361-374.

JENSEN, K. A., BOOTH, A., KEMBOUCHE, Y. & BORASCHI, D. 2016. Validated protocols for test item preparation for key in vitro and ecotoxicity studies, NANoREG Deliverable D2.06.

KLEIVEN, M., ROSSBACH, L. M., GALLEGO-URREA, J. A., BREDE, D. A., OUGHTON, D. H. & COUTRIS, C. 2018. Characterizing NM300K Silver Nanoparticles Behavior, Uptake and Toxicity in Caenorhabditis elegans. *Environmental toxicology and chemistry*.

KÖSER, J., ENGELKE, M., HOPPE, M., NOGOWSKI, A., FILSER, J. & THOMING, J. 2017. Predictability of silver nanoparticle speciation and toxicity in ecotoxicological media. *Environmental science-nano,* 4**,** 1470-1483.

LEE, D.-Y., FORTIN, C. & CAMPBELL, P. G. 2005. Contrasting effects of chloride on the toxicity of silver to two green algae, Pseudokirchneriella subcapitata and Chlamydomonas reinhardtii. *Aquatic toxicology,* 75**,** 127-135.

LODEIRO, P., BROWNING, T. J., ACHTERBERG, E. P., GUILLOU, A. & EL-SHAHAWI, M. S. 2017. Mechanisms of silver nanoparticle toxicity to the coastal marine diatom Chaetoceros curvisetus. *Scientific reports,* 7**,** 10.

MAYER, P., CUHEL, R. & NYHOLM, N. 1997. A simple in vitro fluorescence method for biomass measurements in algal growth inhibition tests. *Water Research,* 31**,** 2525-2531.

OECD 2011. OECD 201 Freshwater Alga and Cyanobaceria, Growth Inhibition Test. OECD Guidelines for the testing of chemicals. Adopted:23 March 2006. Annex 5 corected: 28 July 2011. .

RATTE, H. T. 1999. Bioaccumulation and toxicity of silver compounds: A review. *Environmental toxicology and chemistry,* 18**,** 89-108.

RIBEIRO, F., GALLEGO-URREA, J. A., GOODHEAD, R. M., VAN GESTEL, C. A., MOGER, J., SOARES, A. M. & LOUREIRO, S. 2015. Uptake and elimination kinetics of silver nanoparticles and silver nitrate by Raphidocelis subcapitata: The influence of silver behaviour in solution. *Nanotoxicology,* 9**,** 686-695.

RIBEIRO, F., GALLEGO-URREA, J. A., JURKSCHAT, K., CROSSLEY, A., HASSELLOV, M., TAYLOR, C., SOARES, A. & LOUREIRO, S. 2014. Silver nanoparticles and silver nitrate induce high toxicity to Pseudokirchneriella subcapitata, Daphnia magna and Danio rerio. *Science of the total environment,* 466**,** 232-241.

SENDRA, M., YESTE, M., GATICA, J., MORENO-GARRIDO, I. & BLASCO, J. 2017. Direct and indirect effects of silver nanoparticles on freshwater and marine microalgae (Chlamydomonas reinhardtii and Phaeodactylum tricornutum). *Chemosphere,* 179**,** 279-289.

SØRENSEN, S. N. & BAUN, A. 2015. Controlling silver nanoparticle exposure in algal toxicity testing A matter of timing. *Nanotoxicology,* 9**,** 201-209.

VANCE, M. E., KUIKEN, T., VEJERANO, E. P., MCGINNIS, S. P., HOCHELLA, M. F., JR., REJESKI, D. & HULL, M. S. 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. *Beilstein journal of nanotechnology,* 6**,** 1769-1780.

WANG, S., LV, J., MA, J. & ZHANG, S. 2016. Cellular internalization and intracellular biotransformation of silver nanoparticles in Chlamydomonas reinhardtii. *Nanotoxicology,* 10**,** 1129-1135.

SUPPLEMENTARY INFORMATION

Growth inhibition in Raphidocelis subcapita – evidence of nanospecific toxicity of silver nanoparticles

Merethe Kleiven[a*], Ailbhe Macken[b], Deborah H. Oughton[a]

[a] *Norwegian University of Life Sciences, Center for Environmental Radioactivity, P.O.Box 5003 NMBU, 1432 Ås, Norway*

[b] *Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway*

[*] Address correspondence to merethe.kleiven@nmbu.no

Figures

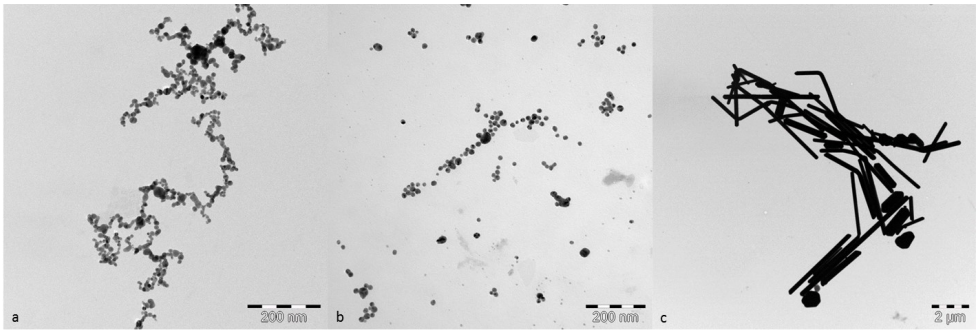


Figure S1. Size and shape of Ag NPs tested in the current paper, images obtained by transmission electron microscopy. Mesosilver (M-Ag) (a), NM300K (b), and NM302 (c) AgNPs in MQ water. The scale bare on a) and b) is 200 nm, on c) it is 2 µm. The sizes after imaging analysis were

Tables

Table S1. Hydrodynamic diameter (mean ± one standard deviation) of particles in the exposure suspensions prior and post removal of algae by centrifugation (4000 rpm, 10 min), measured by Dynammic light scattering. NA: not available

		Prior to removal of algae			Post removal of algae		
		Z-averaged diameter (nm)	Number mean diameter (nm)	PDI	Z-averaged diameter (nm)	Number mean diameter (nm)	PDI
$AgNO_3$	0h	NA	NA	NA	207±112	66±24	0.4±0.1
	24h	504±551	60±12	0.7±0.3	77±335	26±9	0.8±0.1
	48h	110±28	62±7	0.3±0.2	551±351	37±10	0.6±0.2
	72h	1094±1550	73±22	0.8±0.2	150±73	61±16	0.7±0.3
M-Ag	0h	NA	NA	NA	102±92	1.8±0.3	0.3±0.1
	24h	75±55	3±1	0.3±0.2	250±252	3±1	0.4±0.2
	48h	71±16	20±24	0.25±0.09	300±136	43±7	0.33±0.1
	72h	157±101	67±9	0.2±0.1	296±304	23±20	0.4±0.2
NM300K	0h	NA	NA	NA	NA	NA	NA
	24h	NA	NA	NA	119±13	88±9	0.19±0.06
	48h	1171±1171	45±12	0.9±0.2	1676±1727	48±19	0.8±0.2
	72h	250±136	55±9	0.5±0.2	347±351	38±8	0.8±0.2

Table S2. Measured Ag concentrations in $AgNO_3$, Mesosilver (M-Ag), NM300K, and NM302 exposures throughout the duration of the experiment, and for the different size fraction. LOD 0.0049 µg L^{-1}.

Measured Ag concentrations (µg Ag L^{-1})																				
		0h				24h					48h					72h				
Ag exposure	Nominal concentration	Total (prior to algae)	Particulate	Colloidal/ NP	<10 kDa	Total (prior to algae removal)	Total (post algae removal)	Particulate	Colloidal/ NP	<10 kDa	Total (prior to algae removal)	Total (post algae removal)	Particulate	Colloidal/ NP	<10 kDa	Total (prior to algae removal)	Total (post algae removal)	Particulate	Colloidal/ NP	<10 kDa
AgNO3	0,32 µg/L	0,25±0.05	0,11	0,05	0,09	0,11	0,04	0,09	0,02	<0,0049	0,065	0,021	0,05	0,01	<0,0049	0,104±0.009	0,024±0.005	0,09	0,01	< 0,0049
	1 µg/L	0,73±0.06	0,18	0,14	0,41	0,31	0,15	0,20	0,11	<0,0049	0,32	0,07	0,28	0,04	<0,0049	0,35±0.03	0,04±0.01	0,34	0,01	<0,0049
	3,2 µg/L	2,4±0.6	0,50	0,10	1,80	0,71	0,37	0,49	0,22	<0,0049	0,82	0,18	0,73	0,09	<0,0049	1,55±0.07	0,14±0.06	1,49	0,06	<LOD
	10 µg/L	8±1	1,54	0,30	6,30	4,2	1,7	2,80	1,01	0,39	6,3	2,2	5,00	0,41	0,89	4±2	2±2	3,25	0,30	0,60
	32 µg/L	25±2	0,00	1,00	24,00	21	17	5,00	6,00	10,00	21	13	10,00	3,70	7,30	22,5±0.7	16	7,00	3,75	11,75
M-Ag	5 µg/L	5,4±0.1	0,17	3,60	1,60	5,1	3,7	2,00	2,47	0,63	4,7	3	2,80	1,69	0,21	2,5±0.3	1.6±0.06	1,39	1,07	0,01
	10 µg/L	10,7±0.6	0,67	6,30	3,70	9,6	8,3	2,30	5,20	2,10	8,8	7	2,80	4,10	1,90	8±1	6±1	3,83	3,05	1,35
	18 µg/L	19±0	1,00	11,50	6,50	17	16	3,00	9,90	4,10	16	13	4,00	8,20	3,80	16±2	14±2	4,33	8,70	2,97
	32 µg/L	35±1	8,00	16,00	11,00	30	27	5,00	14,00	11,00	31	27	6,00	13,00	12,00	27±2	23±2	4,33	10,73	11,60
	50 µg/L	53±2	7,67	27,00	18,00	50	47	12,00	22,00	16,00	48	43	13,00	18,00	17,00	47±4	44±3	10,67	18,67	18,33
NM300K	2,56 µg/L	2,0±0.4	<0.0049	2,284	0,016	0,7	0,5	0,340	0,368	0,012	0,6	0,1	0,430	0,183	0,007	1,25±0.07	0,4±0.4	0,969	0,276	<0,0049
	4,6 µg/L	3,7±0.7	<0.0049	4,283	0,017	1,7	1,1	0,930	0,749	0,021	1,1	0,3	0,960	0,132	0,008	2±2	0,17±0.08	2,065	0,050	0,006
	8,3 µg/L	7±1	<0.0049	7,770	0,030	3,9	1,8	2,500	0,790	0,610	1,1	0,7	0,700	0,379	0,021	3±1	0,4±0.1	2,545	0,196	0,009
	14,9 µg/L	14±3	<0.0049	13,940	0,060	6,6	3,8	3,600	2,000	1,000	5,1	1,6	4,270	0,739	0,091	7.3±0.6	2±1	6,130	0,606	0,565
	25,6 µg/L	24±6	<0.0049	23,830	0,170	11,0	7,9	4,100	5,500	1,400	25,0	4,6	22,300	2,250	0,450	11±7	3±2	8,955	1,257	0,788
NM302	256 µg/L	82				20	0,8				36	0,7				24±6	0,8±0.3			
	800 µg/L	79				61	2,4				47	3,6				65±7	3±1			
	2500 µg/L	86				72	5,8				73	16				74±2	7±5			
	8000 µg/L	110				75	54				80	64				78±2	45±8			
	25600 µg/L	250				85	73				86	77				89±3	72,3±0.6			

545 Table S3. Growth rates and pH in control groups in toxicity testing with *R. subcapitata*, all replicates
546 merged.

Exposure	pH in controls		Growth rates in controls
	0h	72h	72 h
$K_2Cr_2O_7$	8	8.3	1.8
$AgNO_3$	7.9	7.8	0.8
M-Ag	8.1*	7.8	1.0
NM300K	7.9	7.8	1.0
NM302	7.9	7.8	1.3

547 *pH measured at 24 h.

548

549

Paper III

Route of exposure has a major impact on uptake of silver nanoparticles in Atlantic salmon (*Salmo salar*)

Merethe Kleiven[a*], Bjørn Olav Rosseland[a], Hans-Christian Teien[a], Erik J. Joner[b] and Deborah Oughton[a]

[a] *Norwegian University of Life Sciences, Faculty of Environmental Sciences and Natural Resource Management, P.O. Box5003 NMBU, 1432 Ås, Norway*

[b] *Norwegian Institute of Bioeconomy Research, P.O. Box 115, 1431 Ås, Norway*

[*] *Address correspondence to merethe.kleiven@nmbu.no or* deborah.oughton@nmbu.no

ABSTRACT

The potential impact of Ag nanoparticles on aquatic organisms is to a large extent determined by their bioavailability through different routes of exposure. In the present study juvenile Atlantic salmon (Salmo salar) were exposed to different sources of radiolabeled Ag (radiolabeled ^{110m}Ag nanoparticles and $^{110m}AgNO_3$). After 48 h waterborne exposure to 3 µg/L citrate stabilized ^{110m}Ag nanoparticles or $^{110m}AgNO_3$, or a dietary exposure to 0.6 mg Ag/kg fish (given as citrate stabilized or uncoated ^{110m}Ag nanoparticles, or $^{110m}AgNO_3$), Ag had been taken up in fish regardless of route of exposures and source of Ag (Ag nanoparticles or $AgNO_3$). Waterborne exposure led to high Ag concentrations on the gills, and dietary exposure to high concentrations in the gastrointestinal tract. Silver distribution to the target organs was similar for both dietary and waterborne exposure, with liver as the main target organ. The accumulation level of Ag was 2-3 times higher for $AgNO_3$ than for Ag nanoparticles when exposed through water, whereas dietary exposure led to no significant differences. The transfer (Bq/g liver/g food or water) from exposure through water was four orders of magnitude higher than from feed using the smallest, citrate stabilized Agnanoparticles (4 nm). The smallest nanoparticles had a five times higher bioavailability in food compared to the larger and uncoated Ag nanoparticles (20 nm). Despite the relatively low transfer of Ag from diet to fish, the short lifetime of Ag nanoparticles in water and transfer to sediment, feed or sediment dwelling food sources such as larvae and worms, could make diet a significant long-term exposure route.

Keywords Nanoparticles, bioavailability, silver, waterborne and dietary exposure, *Salmo salar*

INTRODUCTION

The use of nanomaterials (NMs) in industry and consumer products has been increasing over the last decades and continues to rise (Vance et al. 2015). Most research on the possible negative environmental effects of NMs in aquatic organisms has mainly focused on waterborne exposures and relatively high exposure concentrations in order to assess the potential for toxicity. Studies utilizing lower exposure concentrations, as well as the introduction of the dietary route of exposure, improve understanding and assessment of the environmental risks of NMs.

Silver (Ag) nanomaterials are one of the most common nanomaterials in use, and 24 % of all products listed in the Nanotechnology Consumer Products Inventory (http://www.nanotechproject.org/cpi) contain Ag nanoparticles (Vance et al. 2015). Silver nanoparticles are also among the most frequently studied nanomaterials. Silver is well known to induce toxic responses in aquatic organisms, including fish (Hogstrand and Wood 1998). However, the speciation of Ag have a major influence on toxicity. There is a general consensus that the toxicity of Ag in freshwater fish is caused by the presence of free Ag^+ in the water, and that the mechanism of the toxicity involves an inhibition of the Na^+/K^+ ATPase leading to osmoregulatory problems (Hogstrand and Wood 1998). Nano-specific toxicity of waterborne Ag nanoparticles has been frequently studied in aquatic organisms, and a range of results have been reported featuring differences in Ag species (ranging from ions through nano-forms to larger particles), water chemistry, etc. (Bruneau et al. 2016; Chae and An 2016; Ribeiro et al. 2014; Scown et al. 2010). The overall trend seems to be that the toxicity observed for Ag nanoparticle exposed organisms is lower than the toxicity observed in groups exposed to dissolved/free Ag^+ only. As a general conclusion, it has been proposed that the toxicity of metal nanomaterials such as Ag nanoparticles might mainly be caused by the release of ions and subsequent free ion induced toxicity (Notter et al. 2014). This is however likely to be strongly affected by uptake routes and the bioavailability of different Ag species, and in some cases it will be a combination of nano-specific and Ag^+ induced toxicity as reported by Bruneau et al. (2016).

Uptake, organ distribution, and underlying mechanisms of Ag toxicity in fish is generally well known when it comes to waterborne exposure to Ag^+ (usually added as $AgNO_3$) (Farmen et al. 2012; Hogstrand et al. 2003; Webb and Wood 1998; Wood et al. 1999). Ingestion of nanoparticles has been linked to impacts on gut microbiota (Merrifield et al. 2013), and uptake of Ag has been reported in zebrafish following dietary exposure to Ag nanoparticle contaminated daphnia and shrimp larvae (Chae and An 2016; Lacave et al. 2017; Merrifield et al. 2013). However, the significance of dietary Ag is not as well studied or understood as that of waterborne exposures

(DeForest and Meyer 2015). The dietary exposure of Ag nanoparticles could be significant compared to waterborne exposures due to the relatively short lifetime of Ag nanoparticles in waters, their tendency of sorption to aquatic food organisms such as phytoplankton/zooplankton, as well as removal from the water to sediments and subsequent uptake in benthic organisms. A large proportion of the diet for freshwater fish (such as brown trout) can come from sediment dwelling organisms such as larvae, which is accompanied with direct ingestion of sediments (Pechlaner and Zaderer 1985; Rosseland et al. 2007). Silver has been shown to accumulate in benthic invertebrates (Garnier-Laplace et al. 1992; Ramskov et al. 2015), and could have the potential of bioaccumulation through food (trophic transfer). There are large uncertainties associated with the concentrations of engineered nanomaterials in the environment due to the technical challenges of identifying and quantify nanomaterials in environmental samples (Lead et al. 2018). Thus the environmental concentrations on nanomaterials are mostly based on models and are reported to be in the pg/L to low µg/L range (Chio et al. 2012; Dumont et al. 2015; Gottschalk et al. 2009). To assess the hazard of nanoparticles, exposure concentrations orders of magnitude above environmental concentrations are needed to quantify internal transfer to target organs. This can result in a number of artefacts, such as agglomeration and precipitation out of solution or stimulation of biological exclusion responses to reduce uptake. Exposure concentrations closer to those of environmental relevance would give more realistic data for environmental risk assessment. The radiolabeled Ag sources used in the current study allow the use of a relatively low exposure concentrations and detection of accumulated Ag in a range of organs in a time-and cost-efficient way.

Our aim was to compare waterborne exposure of juvenile Atlantic salmon (*Salmo salar*) to different sources of Ag (two types of radiolabeled ^{110m}Ag nanoparticles and $^{110m}AgNO_3$) at low µg Ag/L concentrations, with the other environmentally relevant route of exposure for fish, which is through diet. The study looked at direct ingestion of nanoparticles, rather than via tropic transfer in feed organisms (which would avoid changes in speciation with Ag during trophic transfer), and covered a larger range of organs than previously studied. Our hypothesis was that 1) there would be differences in uptake and transport to target organs for Ag nanoparticles and $AgNO_3$ exposures, and 2) that the uptake efficiency and pattern of organ distribution would be influenced by the route of exposure.

METHOD AND MATERIALS

Experimental setup

Juvenile Atlantic salmon (1 year old) of both genders (n=168) were obtained from the hatchery at the Norwegian University of Life Sciences (NMBU), from the same age-synchronized stock of

different sizes. The average size in dietary exposure was 21±5.1 g (n=96), and in the waterborne exposure 9.0±1.9 g (n=72). The weight difference reflects the fact that bigger fish were selected for the dietary exposure to ease the force-feeding. There is no indication in the literature, that the uptake and distribution of metals in fish in the same life stage and age is size dependent. The fish were acclimatized in US EPA very soft reconstituted water (United States Environmental Protection Agency 2002) at 10 °C for 7 days before start of exposure to Ag in similar water and temperature conditions. Fish were fed once within the acclimation period.

The fish were exposed, through diet and water, to different sources of Ag as radiolabeled $AgNO_3$ ($^{110m}AgNO_3$), laboratory synthesized citrate-stabilized ^{110m}Ag nanoparticles (C-Ag NPs), and uncoated ^{110m}Ag nanoparticles (U-Ag NPs). The C-Ag NPs was labelled during synthesis, and the U-Ag NPs was labelled by neutron activation. The U-Ag NPs was only included in the dietary exposure due to low dispersibility in water. By including both coated and uncoated nanoparticles into the current study, we were able to compare the effect of coating and to some degree also particle size (C-Ag 4 nm, U-Ag 20 nm).

The fish were kept in dark exposure containers (180 L) through the 48 h static exposure using aerated water (CO_2 < 2 mg/L). The dissolved oxygen (O_2) and pH were monitored to make sure the conditions were satisfactory (Binde 2004). Throughout the 48 h exposure period, six fish were sampled from each exposure group after 3, 7, 24 and 48 h.

Fish were given a one-time dose of radiolabeled feed (1 % of body weight) directly into the gut via syringe for the dietary exposure, whilst the waterborne exposure was a 48 h static exposure. Force feeding rather than conventional feeding was chosen to reduce the risk of waterborne transfer of dissolved Ag from food added to water, and thus also ensure optimal water quality (Total ammonia nitrogen < 1 mg/L). The feed was prepared as a slurry made of commercial food (Skretting startfor, Skretting Norway) spiked with the radiolabeled Ag solutions/suspensions ($AgNO_3$, C-Ag NPs, and U-Ag NPs) in a ratio 50:50, this to enable the feeding with a syringe. The diet-exposed groups were anesthetized with metomidate hydrochloride (Aquacalm, Western Chemical, Ferndale, USA) before being force fed by the means of a tube connected to a 1 mL syringe. After feeding, they were placed in aerated recovery tanks (volume 10 L, 0.5 L/g fish for 15 min), before being moved to the exposure containers. During the experiment, whole body measurements with a Ge-detector (Canberra Industries Inc., USA) was performed on all sacrificed fish before sampling of organs were done.

In the waterborne exposure, the nominal Ag concentration in the $AgNO_3$ and C-Ag NP exposure was 6 µg/L. In the dietary exposure, we aimed for the same activity in Ag contaminated feed, 3 kBq/g food, which is approximately 60 µg Ag/g food, which gave a dietary exposure of 0.6 mg Ag/kg fish for

the Ag sources ($AgNO_3$, C-Ag NPs, and U-Ag NPs) tested in the present study. The exposure concentrations in the current study were selected to be as low as possible, but still high enough to detect Ag in organs other than the primary contact organs (gill, gut), and the expected main target organ (liver), after only 48 h of exposure. The intention was to conduct a sub-lethal exposure for both routes, thus the Ag concentrations in the waterborne exposures were considerably lower than in the dietary exposure.

Silver materials and characterization

The $^{110m}AgNO_3$ solution, as well as the synthesized citrate stabilized Ag nanoparticles (C-Ag NPs; primary particle size of 4 nm avg. diameter) were made from neutron activated $AgNO_3$ (Merck, *pro analysi*). The, uncoated Ag nanoparticles (U-Ag NPs, primary particle size of 20 nm avg. diameter) (QSI-nano silver, Quantum Sphere), delivered as a powder, were also radiolabeled by neutron activation (see supplementary information). The C-Ag NPs were synthesized from $^{110m}AgNO_3$ according to Doty et al. (Doty et al. 2005) (see supplementary material).

Ag nanoparticles in stock solutions and dispersions were characterized for hydrodynamic diameter, polydispersity index and zeta potential with a Malvern Zetasizer ZS (Malvern Instruments, UK) while particle size and shape was determined with transmission electron microscopy (FEI Morgani 268 TEM) with image analysis according to the protocol developed by Mast and de Temmeman (Mast and de Temmeman 2016). Full details of methods can be found in supplementary materials.

Water Sampling procedures and analytical techniques

Water temperature, pH, conductivity, and O_2 were monitored daily before and during the experiment (WTW, Hanna Instruments). Water samples were collected at times of fish sampling to determine the concentration of Ag, quantify general water chemistry parameters and total organic carbon (TOC) (Shimadzu TOC cpn, Kyoto, Japan). No significant differences were detected within or between the waterborne and dietary exposure (temperature, pH, conductivity, O_2 and TOC) (supplementary information).

The Ag concentration of the exposure water was measured along with particle size fractionation to obtain information of Ag speciation. A pilot study was carried out to ensure the stability of the C-Ag NPs (synthesized in the same way as in the current study) in US EPA very soft water without the presence of fish, and waters were also characterized throughout the fish exposure experiments. In both studies (pilot and main experiment) the in situ size fractionation was performed at 0, 3, 7, 24 and 48 h, coinciding with the time points for fish sampling. The filtration was performed partly as described in Farmen et al. (Farmen et al. 2012). The water was filtered through a 0.22 µm membrane syringe filter (Millipore) to exclude bigger particles, and ultra-filtration to exclude NP using a hollow

fiber (Pall Microza Hollow Fiber Module, Pall Corporation, New York, USA) with nominal molecular mass cutoff at 3 kDa (equivalent to approx. 1 nm). The size fraction >0.22 µm was defined as particulate, fraction <0.22 µm and >3 kDa was defined as nanoparticles/colloidal, while the fraction <3 kDa was defined as low molecular mass (LMM)/ions. In the dietary exposure, only the unfiltered water was analyzed to ensure no contamination of the water from feces or regurgitation from Ag fed fish.

All collected water samples (20 mL) were measured using a NaI detector (Wallac Wizard 3'' 1480 Automatic Gamma Counter, PerkinElmer) for detection of ^{110m}Ag during the experiment, and later the total Ag concentration was measured after acidification (2 vol % ultra-pure HNO_3) by ICP-MS (ELAN 6000; Perkin Elmer, Waltham, MA, USA) in order to control the specific activity.

Fish sampling

After 3, 7, 24, and 48 h of exposure, six fish were collected for sampling. The fish were killed by a blow to the head, weight and length was measured, before a range of organs were sampled following the EMERGE protocol (Rosseland et al. 2001). Organs sampled were gills, spleen, gallbladder, liver, heart, kidney, gastrointestinal tract, gut content and brain. All organs were weighted to determine wet weight before determination of ^{110m}Ag content using the NaI detector. In addition, autoradiography was used to visualize the presence of Ag in organs and surface of exposed fish.

Statistics

The statistical testing for organ accumulation was performed on GraphPad Prism 6. A two-way ANOVA test was conducted, and in the case of significant result, a Tukey-Kramer means comparison test was performed to find out how many and which groups differed significantly from each other. For the water parameter (pH, conductivity and temperature) an ANOVA test was conducted followed by the Tukey-Kramer means comparison test. Significance levels was set to 0.05 in all statistical test. All results are presented as mean ± standard deviation, and ^{110m}Ag activity in tissue samples are given as corrected counts per minute (ccpm, corrected for background activity)/g wet weight tissue.

RESULTS AND DISCUSSION

Particle characterization

The primary particle size of the C-Ag NPs were 4 nm (main size population), and for the uncoated nanoparticles U-Ag NPs it has previously been measured to be 20 nm (Coutris et al. 2012; Oughton et al. 2008). However, it was clear that the uncoated particles (U-Ag NPs) was mainly found in larger aggregates (see supplementary information).

Characterization of the Ag exposures

For the dietary exposure, no Ag was detected in the water by NaI measurements over the 48 h exposure period, nor for stable Ag using ICP-MS (Ag < 0.6 µg/L). Thus, we conclude that there was no confounding exposure of waterborne Ag for dietary exposed fish. For waterborne exposure, the average Ag concentration was similar for C-Ag NPs and $AgNO_3$ exposures, namely 3.0±1.2 and 3.0±0.4 µg/L, respectively. The average concentrations over time were not significantly different, but the total Ag concentration varied more over time for C-Ag NPs exposure compared to $AgNO_3$ (Figure 1). The average reduction in effective Ag concentration in the C-Ag NPs exposure over the 48 h time period was from 77 to 42 % of the nominal concentrations, while in the $AgNO_3$ exposure this reduction was only from 52 to 47 %. The deviation between effective and nominal Ag concentration in the water at the beginning of the exposure was most likely caused by sorption of the Ag to the walls of the exposure containers. The sorption seems to be higher in the $AgNO_3$ exposure, probably reflecting the higher affinity of Ag^+ to sorb to the plastic used. The observed reduction in total Ag concentration over time, especially observed in the NP exposure, was probably a result of agglomeration/aggregation of nanoparticles with subsequent sedimentation.

It is well known that the speciation of a metal is often more important than the total concentration, in determining the bioavailability, uptake and toxicity to organisms (Paquin et al. 2002). Thus, size fractionation with respect to Ag in the exposure waters was performed to provide information on the initial size distribution and changes during exposure. The results showed that there was a clear reduction over time (from 3 h to 48 h) in the size fraction <0.22 µm in both C-Ag NPs (from 68 % to 23 %) and $AgNO_3$ groups (from 82 % to 40 %), indicating a decrease in the nanoparticle fraction (Figure 2, Table S1). Only 8 % of the Ag in the nanoparticle group (C-Ag NPs) was below 3 kDa (LMM), and this was reduced to 4 % after 7 h. In contrast, as much as 59 and 23 % of the Ag in the $AgNO_3$ group was present as LMM (< 3 kDa) at 3 and 7 h, respectively (Figure 2). Due to technical problems, it was not possible to carry out <3 kDa fractionation at 24 and 48h. Previous studies carried out by our group (Farmen et al. 2012), showed that the LMM fraction decreased from 10 to 25 % at the beginning of the exposure to 0.2 to 1 % after 24 h. Nanoparticles are known to

agglomerate/aggregate (Schaumann et al. 2015), and thus a shift in particle size towards larger particles over time, with a subsequent sedimentation and reduction in exposure concentrations, could be expected. However, Ag nanoparticles have also been shown to have the potential to dissolve over time (Angel et al. 2013). How easily an Ag NP would dissolve depends on the type of NP (e.g. coating, size) and the exposure conditions (e.g. pH, temperature, conductivity, organic matter etc.). Angel and coauthors (Angel et al. 2013) observed a dissolution of Ag^+ from citrate stabilized Ag nanoparticles in synthetic freshwater of 20-27 % of total NP concentration in the range 1 to 100 µg/L. They also showed that the dissolution rate plateaued out in the presence of humic acids, and that PVP coated Ag nanoparticles had an initially higher dissolution rate compared to the citrate stabilized nanoparticles, but that the dissolution of ions after 72 h was similar for the two particles. Also our pilot study with citrate stabilized Ag nanoparticles (C-Ag NPs), looking at their behavior in US EPA very soft water without the presence of salmon, confirmed that these Ag nanoparticles might dissolve over time. An increase in the LMM fraction over the 48 h time period from 20 % to 50 % of the total Ag concentration at the start of exposure was observed for the C-Ag NPs, demonstrating dissolution of NP. For the $AgNO_3$ exposure, the LMM fraction (approx. 70 %) remained stable over the same time period. It is important to keep in mind that these results were obtained without test organisms. Especially in static exposures the presence of fish can lead to a slight increase in organic matter (production of mucus, feces etc.) which can lead to changes in speciation of Ag (Morgan et al. 2004a). This was observed by Mikkelsen (2009), where the introduction of test organism (Atlantic salmon) initiated and accelerated the agglomeration and aggregation of two different Ag nanoparticles, one of which were the same citrate stabilized particles as used in the experiment reported here. The concentration of TOC was however not increased during the 48 h of exposure in the current study.

Uptake and distribution of Ag in Atlantic salmon organs

Silver was taken up in the fish, regardless of route of exposure and source of Ag. Waterborne exposure led to high Ag concentrations on the gills (1300±400 and 500±150 ccpm/g for $AgNO_3$ and C-Ag NP, respectively), and dietary exposure to high concentrations in the gastrointestinal tract (700±400, 900±300, and 700±650 ccpm/g for $AgNO_3$, C-Ag NP and U-Ag NPs, respectively) (Figure 3, Table S2). However, the elevated levels of Ag in the gill and gastrointestinal tract does not necessarily mean it is absorbed into the epithelium, as it could merely be adsorbed to the apical membrane of the epithelium or interact with the mucus layer (Galvez and Wood 1999). Whether the Ag in organs of fish exposed to Ag nanoparticles are a result of an uptake of the nanoparticles themselves, or Ag ions released from the nanoparticles adsorbed to gill/gut epithelium could not be revealed in the present study. The high Ag levels could also be due to food particles still left in the

gastrointestinal tract. The pattern of Ag distribution to the organs was similar for both dietary and waterborne exposure, with liver as the organ with the highest Ag concentration (Table S2 and S3), which was according to expectations and previously published studies (Galvez et al. 2001; Galvez and Wood 1999). Silver was also detected in kidney and bile (whole gallbladder with content sampled) for both waterborne and dietary exposed fish, but was not detected in the spleen, heart, or brain (Table S2 and S3). For the dietary exposure, ^{110m}Ag was not detected in gills either. Low, but significant, levels of ^{110m}Ag were detected in the gut content of fish from the waterborne exposure (50±80 and 60±100 ccpm/g for $AgNO_3$ and C-Ag NP, respectively) (Table S3), probably caused by excretion of bile (Wood et al. 2012) or occasional swallowing of exposure water. The main target organs in the waterborne exposure was visualized by autoradiography (Figure 4), and the highest activity was shown to be in gills and liver.

Comparison of $AgNO_3$ and Ag nanoparticles. In the waterborne exposure, the levels of Ag in gills was higher in fish exposed to $AgNO_3$ compared to C-Ag NP exposure (Table S3). The transfer ratio for gills after 48 h was 5.5 (±1.03) × 10^2 and 1.4 (±0.55) × 10^2 (Bq/g liver)/(Bq/g water) for $AgNO_3$ and C-Ag NPs, respectively. Also in the liver the Ag accumulated to higher concentrations in the $AgNO_3$ exposure compared to C-Ag NP exposure with transfer ratios of 4.8 (±0.93) × 10^2 and 2.3 (±0.75) × 10^2 (Bq/g liver)/(Bq/g water), respectively.

In the dietary exposures, the accumulation of Ag in the liver after 24 h was higher in fish exposed to C-Ag NPs than in those exposed to $AgNO_3$. However, after 48 h exposure the Ag levels in these two groups had reached the same level, with transfer ratios of 2.3 (±1.5) × 10^{-2} and 2.7 (±0.96) × 10^{-2} (Bq/g liver)/(Bq/g feed) for $AgNO_3$ and C-Ag NPs, respectively. For the uncoated U-Ag NPs the accumulation in liver was minimal, with a transfer ratio one order of magnitude lower than the two other treatments, 3 (±1.9) × 10^{-3} (Bq/g liver)/(Bq/g feed).

The differences in Ag accumulation in waterborne $AgNO_3$ and Ag NP exposures could indicate that uptake of Ag^+ across gills occurred, rather than uptake of nanoparticles, as the differences in uptake closely reflected the amounts of Ag in the LMM fraction during the first part of the exposure period. A certain uptake of nanoparticles may also have taken place, as the LMM fraction was approximately five times higher in the water of the $AgNO_3$ treatment while the uptake was only four times higher than for the C-Ag NP treatment. The difference possibly being due to uptake of nanoparticles. Also the differences seen between the C-Ag NPs and the $AgNO_3$ in the dietary exposure could indicate different uptake mechanisms, or just different accessibility caused by a higher binding affinity of the Ag^+ to compounds in the feed and/or tissue, or both.

Extensive research has been conducted on the underlying routes of uptake of Ag over the gill (Bury et al. 1999; Bury and Wood 1999; Farkas et al. 2011; Osborne et al. 2015) and Ag ions (when added as $AgNO_3$) have been found to be an effective inhibitor of Na^+ gill uptake (Morgan et al. 2004a). If free Ag^+ is the active inhibitor, one might expect the Ag concentration and accumulation in the gills and liver, as well as signs of osmoregulatory problems, to be more rapid and reach higher levels in $AgNO_3$ exposed fish compared to NP exposed fish. However, this assumption only holds if the main route of Ag NP exposure is through dissolution and subsequent uptake of Ag^+ from the nanoparticles, and not by a different uptake mechanism of the nanoparticles themselves. Dissolution of Ag^+ could not be ruled out in this experiment as in many others. Even if the presence of Ag^+ in the water decreased rapidly during the first part of the exposure period, free Ag^+ could dissolve from the nanoparticles attached to the gills due to the possible reduction in pH of the water when passing over the gill (Playle and Wood 1989). However, this might not be the only, or even the main uptake mechanism. Endocytosis and vesicular transport as well as paracellular diffusion through tight junctions between the cells into the blood are other suggested mechanisms of uptake of nanoparticles (Handy et al. 2008b; Moore 2006). Schultz et al. (Schultz et al. 2012) demonstrated a nano-specific inhibition of Na^+ uptake in juvenile Rainbow trout exposed to citrate coated Ag nanoparticles. However, the exact mechanisms of uptake of Ag nanoparticles in fish need further investigations.

Comparison of waterborne and dietary exposure routes. After water exposure, the main activity of ^{110m}Ag was found on gills, followed by liver and bile, with trace levels also found in kidney. After 48 h, the transfer ratios were in the same order of magnitude for liver from $AgNO_3$ and C-Ag NP exposed fish (Figure 3). In diet exposure, the main activity of ^{110m}Ag was found in the gastrointestinal tract, and the transfer to liver was in the same order of magnitude for $AgNO_3$ and C-Ag NPs. The dietary transfer of ^{110m}Ag to liver was however lower from U-Ag NPs. The transfer ratios at 48 h for $AgNO_3$ and C-Ag NPs were in the same order of magnitude for the dietary exposure, whilst the U-Ag NPs was one order of magnitude lower (Figure 3).

Hepatic and renal excretion are two main pathways for excretion of contaminants, excreted through the bile and urine, respectively (Handy et al. 2008b). Since levels of ^{110m}Ag were higher in the liver than in the kidney for all exposures, it would appear hepatic excretion was the main pathway, irrespective of whether the Ag source was waterborne of diet, $AgNO_3$ or Ag NP. The hepatic excretion depends on exocytosis and vesicular transport to form the bile. These vesicles are around 200 nm in size so primary particles as well as small aggregates <200 nm could be transported via these vesicles into the bile, which then enters into the intestine. The renal excretion on the other hand involves filtration of the blood in the vertebrate kidney which has a molecular weight cut-off

around 60 kDa (approx. 2 nm) (Handy et al. 2008b), which would not allow for excretion of most nanoparticles.

Although the target organs and the activity level in the accumulatory organs were similar for dietary and waterborne exposure, as well as between different sources of Ag, the transfer ratios were four orders of magnitude higher for fish exposed through water: 1.2×10^{2} g/g from water and 2.6×10^{-2} g/g from diet for C-Ag NPs, and 4.9×10^{2} g/g from water and 2.2×10^{-2} g/g from diet for $AgNO_3$. These are slightly lower than previously reported concentration ratios for Ag from sediments to freshwater biota, which have been reported to be 7.3×10^{-1} (N=40) for transfer of Ag from sediments to invertebrates. However, these studies confirm that Ag in sediments is bioavailable and can be taken up by a range of organisms.

There could be several explanations for the far lower bioavailability of Ag in dietary exposure compared to the waterborne exposure. Ag is known to form complexes with both inorganic (e.g. chloride, thiosulphate, sulphide) and organic (e.g. natural organic matter) ligands present in natural waters (Hiriart-Baer et al. 2006). Similar complexation processes could potentially also occur during the preparation of the Ag contaminated fish fodder. Silver are known to have a strong binding affinity to for example sulfur (Hiriart-Baer et al. 2006). Hence, instead of uptake over the intestinal epithelia layer and subsequent distribution to the blood, Ag would rather follow the feces due to potential high binding affinity to components in the food. On the other hand, Ag might actually be free to be taken up (e.g. after acid digestion of the fodder in the gut), but ion competition due to higher ionic concentrations in the gut or less effective uptake mechanism in the intestine compared to the gill could lead to lower uptake. Contrary to uptake of gills, studies on intestinal uptake mechanisms of $AgNO_3$ and Ag nanoparticles are rather rare. However, there are some data comparing effectiveness of different dietary sources of Ag. For example, it has been reported that Ag biologically incorporated in food was more bioavailable to rainbow trout than food spiked with Ag_2S (Galvez et al. 2001; Galvez and Wood 1999). Similarly, Ag(I) associated to the internal compartments of algal cells was found to be more accessible for the herbivorous zooplankton than when Ag(I) was adsorbed to the cell surface (Bielmeyer and Klaine (1999) in Andren and Bober, 1999). It has been suggested that Ag biologically incorporated in food is most likely bound to amino acids, which could mean uptake of Ag via amino acid transporters, thus leading to increased accumulation compared to Ag spiked fish fodder (Galvez and Wood 1999). Since the contaminated food in the current study was commercial food spiked with Ag, rather than biologically incorporated Ag in food, this could have reduced transfer, albeit not to the two orders of magnitude difference as compared to water.

Nevertheless, our results clearly demonstrate that Ag can be taken up through the intestine, both from a source of $AgNO_3$ and Ag nanoparticles even though the specific uptake mechanisms involved remain to be described.

Although the transfer ratios for dietary exposure were several orders of magnitude lower than for waterborne exposure, on a weight-to-weight basis, it should be stressed that in contaminated environments, Ag concentrations in sediments, organic materials and diet would be expected to be much higher than in water. An extensive review of the behavior of Ag in freshwater systems cites a range in K_D distribution coefficients from $9.5(\pm2.3) \times 10^4$ to $4.4(\pm1.7) \times 10^5$ (g/kg)/(g/L) (International Atomic Energy Agency 2010). Although these represent sources of Ag^+ rather than Ag NP, sediments are considered to be the primary sink for Ag nanoparticles in the environment, meaning that dietary uptake through ingestion of organic material in contaminated sediments has the potential to be a significant contribution. There is a further possibility for dietary exposure through food-chain transfer from sediment dwelling organisms. Previous studies have reported concentrations ratios of close to one for sediment invertebrates. As many fish species feed on sediments and sediment living invertebrates, exposure to fish from this route could be similar to that from ingestion of sediments or contaminated organic material directly.

CONCLUSION

The use of ^{110m}Ag labelling enabled the determination of transfer ratios for a number of organs following 48 h exposure to Ag nanoparticles and $AgNO_3$, both from waterborne exposure at low Ag concentrations (3.9±1.9 µg/L), and through diet (a more environmentally relevant route of exposure to Ag nanoparticles, 0.6 mg Ag/kg fish). Results indicated differences in transfer for the two Ag nanoparticles tested. Considering the number of different types of Ag nanomaterials on the market, differentiated by one or more characteristics (size, type of coating, surface charge etc.), a variation in their potential for uptake and induction of toxicity would also be expected. This could include higher transfer ratios than reported in the current study for other Ag nanomaterials.

Although the experimental design of the current study has some limitations (only two different types of Ag nanoparticles, one exposure concentration, force feeding, 48 h exposure time), the results show the potential for transfer of Ag from Ag nanoparticle sources to the liver after dietary exposure of Atlantic salmon after only 48 h of dietary exposure. Further work should focus on more environmentally relevant exposure conditions, such as effects of biological incorporation of Ag nanoparticles and chronic exposures, to further increase the knowledge regarding factors influencing the environmental risk of Ag nanoparticles. Proper exposure characterization identifying the differences in Ag-complexation in the food between the Ag salts and Ag nanoparticle contamination would also be valuable.

ACKNOWLEDGMENT

This work was financed by the projects NANOREG (NFR project 310584) and NANOCHARM (NFR project 221391). The authors would also like to thank everybody assisting with sampling; M.N. Pettersen, L.S. Heier and T.H. Aas.

DATA ACCESSIBILITY

Research data pertaining to this article is accessible upon request to the authors.

399 **REFERENCES**

400 Andren AW, Bober TW. 1999. Proceedings. The 6th international conference proceedings: Transport,
401 fate and effects of Silver in the environment. University of Wisconsin System, Sea Grant
402 Institute, USA. August 1999. pp 69-100. Available from:
403 http://digital.library.wisc.edu/1711.dl/EcoNatRes.Argentumv06
404 Angel BM, Batley GE, Jarolimek CV, Rogers NJ. 2013. The impact of size on the fate and toxicity of
405 nanoparticulate silver in aquatic systems. Chemosphere 93(2):359-365.
406 Binde M. 2004. Akvakulturdriftsforeskriften. Vannmiljø, fiskehelse og fiskeveldferd (Aquaculture act.
407 Water quality, fish health and welfare).
408 Bruneau A, Turcotte P, Pilote M, Gagne F, Gagnon C. 2016. Fate of silver nanoparticles in wastewater
409 and immunotoxic effects on rainbow trout. Aquatic toxicology (Amsterdam, Netherlands)
410 174:70-81.
411 Bury NR, Grosell M, Grover AK, Wood CM. 1999. ATP-dependent silver transport across the
412 basolateral membrane of rainbow trout gills. Toxicology and Applied Pharmacology 159(1):1-
413 8.
414 Bury NR, Wood CM. 1999. Mechanism of branchial apical silver uptake by rainbow trout is via the
415 proton-coupled Na+ channel. American Journal of Physiology-Regulatory Integrative and
416 Comparative Physiology 277(5):R1385-R1391.
417 Chae Y, An Y-J. 2016. Toxicity and transfer of polyvinylpyrrolidone-coated silver nanowires in an
418 aquatic food chain consisting of algae, water fleas, and zebrafish. Aquatic Toxicology 173:94-
419 104.
420 Chio C-P, Chen W-Y, Chou W-C, Hsieh N-H, Ling M-P, Liao C-M. 2012. Assessing the potential risks to
421 zebrafish posed by environmentally relevant copper and silver nanoparticles. Science of The
422 Total Environment 420:111-118.
423 Coutris C, Joner EJ, Oughton DH. 2012. Aging and soil organic matter content affect the fate of silver
424 nanoparticles in soil. Science of the Total Environment 420:327-333.
425 DeForest DK, Meyer JS. 2015. Critical Review: Toxicity of Dietborne Metals to Aquatic Organisms.
426 Critical Reviews in Environmental Science and Technology 45(11):1176-1241.
427 Doty RC, Tshikhudo TR, Brust M, Fernig DG. 2005. Extremely stable water-soluble Ag nanoparticles.
428 Chemistry of Materials 17(18):4630-4635.
429 Dumont E, Johnson AC, Keller VD, Williams RJ. 2015. Nano silver and nano zinc-oxide in surface
430 waters–Exposure estimation for Europe at high spatial and temporal resolution.
431 Environmental pollution 196:341-349.
432 Farkas J, Christian P, Gallego-Urrea JA, Roos N, Hassellov M, Tollefsen KE, Thomas KV. 2011. Uptake
433 and effects of manufactured silver nanoparticles in rainbow trout (Oncorhynchus mykiss) gill
434 cells. Aquatic Toxicology 101(1):117-125.
435 Farmen E, Mikkelsen HN, Evensen O, Einset J, Heier LS, Rosseland BO, Salbu B, Tollefsen KE, Oughton
436 DH. 2012. Acute and sub-lethal effects in juvenile Atlantic salmon exposed to low mu g/L
437 concentrations of Ag nanoparticles. Aquatic Toxicology 108:78-84.
438 Galvez F, Hogstrand C, McGeer JC, Wood CM. 2001. The physiological effects of a biologically
439 incorporated silver diet on rainbow trout (Oncorhynchus mykiss). Aquatic Toxicology 55(1-
440 2):95-112.
441 Galvez F, Wood CM. 1999. Physiological effects of dietary silver sulfide exposure in rainbow trout.
442 Environmental Toxicology and Chemistry 18(1):84-88.
443 Garnier-Laplace J, Baudin J, Foulquier L. 1992. Experimental study of 110m Ag transfer from
444 sediment to biota in a simplified freshwater ecosystem. Sediment/Water Interactions.
445 Springer. p. 393-406.
446 Gottschalk F, Sonderer T, Scholz RW, Nowack B. 2009. Modeled environmental concentrations of
447 engineered nanomaterials (TiO2, ZnO, Ag, CNT, fullerenes) for different regions.
448 Environmental science & technology 43(24):9216-9222.

Handy RD, Henry TB, Scown TM, Johnston BD, Tyler CR. 2008b. Manufactured nanoparticles: their uptake and effects on fish-a mechanistic analysis. Ecotoxicology 17(5):396-409.

Hiriart-Baer VP, Fortin C, Lee D-Y, Campbell PG. 2006. Toxicity of silver to two freshwater algae, Chlamydomonas reinhardtii and Pseudokirchneriella subcapitata, grown under continuous culture conditions: influence of thiosulphate. Aquatic toxicology 78(2):136-148.

Hogstrand C, Grosell M, Wood CM, Hansen H. 2003. Internal redistribution of radiolabelled silver among tissues of rainbow trout (Oncorhynchus mykiss) and European eel (Anguilla anguilla): the influence of silver speciation. Aquatic Toxicology 63(2):139-157.

Hogstrand C, Wood CM. 1998. Toward a better understanding of the bioavailability, physiology and toxicity of silver in fish: Implications for water quality criteria. Environmental Toxicology and Chemistry 17(4):547-561.

International Atomic Energy Agency I. 2010. Handbook of Parameter Values for the Prediction of Radionuclide Transfer in Terrestrial and Freshwater Environments. Technical report series no 472.

Jensen KA, Booth A, Kembouche Y, Boraschi D. 2016. Validated protocols for test item preparation for key in vitro and ecotoxicity studies, NANoREG Deliverable D2.06.

Lacave JM, Fanjul A, Bilbao E, Gutierrez N, Barrio I, Arostegui I, Cajaraville MP, Orbea A. 2017. Acute toxicity, bioaccumulation and effects of dietary transfer of silver from brine shrimp exposed to PVP/PEI-coated silver nanoparticles to zebrafish. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 199:69-80.

Lead JR, Batley GE, Alvarez PJ, Croteau MN, Handy RD, McLaughlin MJ, Judy JD, Schirmer K. 2018. Nanomaterials in the Environment: Behavior, Fate, Bioavailability, and Effects—An Updated Review. Environmental toxicology and chemistry.

Mast J, de Temmeman P-J. 2016. Protocol(s) for size-distribution analysis of primary NM particles in air, powders, and liquids, NANoREG Deliverable D2.10.

Merrifield DL, Shaw BJ, Harper GM, Saoud IP, Davies SJ, Handy RD, Henry TB. 2013. Ingestion of metal-nanoparticle contaminated food disrupts endogenous microbiota in zebrafish (Danio rerio). Environmental pollution 174:157-163.

Mikkelsen H. 2009. Toxicity of silver nanoparticles to Atlantic salmon (Salmo salar): Linking chemical speciation to biological effects. Master thesis. Norwegian University of Life Sciences, Ås, Norway.

Moore MN. 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environment International 32(8):967-976.

Morgan TP, Grosell M, Playle RC, Wood CM. 2004a. The time course of silver accumulation in rainbow trout during static exposure to silver nitrate: physiological regulation or an artifact of the exposure conditions? Aquatic Toxicology 66(1):55-72.

Notter DA, Mitrano DM, Nowack B. 2014. Are nanosized or dissolved metals more toxic in the environment? A meta-analysis. Environmental Toxicology and Chemistry 33(12):2733-2739.

Osborne OJ, Lin SJ, Chang CH, Ji ZX, Yu XC, Wang X, Lin S, Xia T, Nel AE. 2015. Organ-Specific and Size-Dependent Ag Nanoparticle Toxicity in Gills and Intestines of Adult Zebrafish. Acs Nano 9(10):9573-9584.

Oughton DH, Hertel-Aas T, Pellicer E, Mendoza E, Joner EJ. 2008. Neutron activation of engineered nanoparticles as a tool for tracing their environmental fate and uptake in organisms. Environmental Toxicology and Chemistry 27(9):1883-1887.

Paquin PR, Gorsuch JW, Apte S, Batley GE, Bowles KC, Campbell PGC, Delos CG, Di Toro DM, Dwyer RL, Galvez F et al. . 2002. The biotic ligand model: a historical overview. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 133(1-2):3-35.

Pechlaner R, Zaderer P. 1985. Interrelations between brown trout and chironomids in the alpine lake Gossenköllesee (Tyrol) With 5 figures and 1 table in the text. Internationale Vereinigung für theoretische und angewandte Limnologie: Verhandlungen 22(4):2620-2627.

Playle RC, Wood CM. 1989. Water chemistry changes in the gill microenvironment of rainbow-trout - experimental-observations and theory. Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 159(5):527-537.

Ramskov T, Forbes VE, Gilliland D, Selck H. 2015. Accumulation and effects of sediment-associated silver nanoparticles to sediment-dwelling invertebrates. Aquatic Toxicology 166:96-105.

Ribeiro F, Gallego-Urrea JA, Jurkschat K, Crossley A, Hassellov M, Taylor C, Soares A, Loureiro S. 2014. Silver nanoparticles and silver nitrate induce high toxicity to Pseudokirchneriella subcapitata, Daphnia magna and Danio rerio. Science of the Total Environment 466:232-241.

Rosseland BO, Massabuau J-C, Grimalt JO, Hofer R, Lackner R, Raddum GG, Rognerud S, Vives I. 2001. Fish Exotoxicology: The EMERGE Fish Sampling Manual for Live Fish.The EMERGE Project (European Mountain lake Ecosystems: Regionalisation, diaGnostic and socio economic valuation).

Rosseland BO, Rognerud S, Collen P, Grimalt JO, Vives I, Massabuau J-C, Lackner R, Hofer R, Raddum GG, Fjellheim A. 2007. Brown trout in Lochnagar: population and contamination by metals and organic micropollutants. Lochnagar: The Natural History of a Mountain Lake:253-285.

Schaumann GE, Philippe A, Bundschuh M, Metreveli G, Klitzke S, Rakcheev D, Gruen A, Kumahor SK, Kuehn M, Baumann T et al. . 2015. Understanding the fate and biological effects of Ag- and TiO2-nanoparticles in the environment: The quest for advanced analytics and interdisciplinary concepts. Science of the Total Environment 535:3-19.

Schultz AG, Ong KJ, MacCormack T, Ma G, Veinot JGC, Goss GG. 2012. Silver Nanoparticles Inhibit Sodium Uptake in Juvenile Rainbow Trout (Oncorhynchus mykiss). Environmental Science & Technology 46(18):10295-10301.

Scown TM, Santos EM, Johnston BD, Gaiser B, Baalousha M, Mitov S, Lead JR, Stone V, Fernandes TF, Jepson M et al. . 2010. Effects of Aqueous Exposure to Silver Nanoparticles of Different Sizes in Rainbow Trout. Toxicological Sciences 115(2):521-534.

Shaw BJ, Handy RD. 2011. Physiological effects of nanoparticles on fish: A comparison of nanometals versus metal ions. Environment International 37(6):1083-1097.

United States Environmental Protection Agency UE. 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 5th edition. EPA-821-R-02-012. Office of water, Washington DC, USA. .

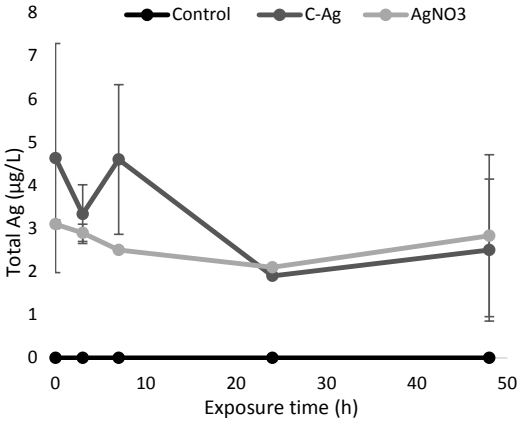
Vance ME, Kuiken T, Vejerano EP, McGinnis SP, Hochella MF, Jr., Rejeski D, Hull MS. 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. Beilstein Journal of Nanotechnology 6:1769-1780.

Webb NA, Wood CM. 1998. Physiological analysis of the stress response associated with acute silver nitrate exposure in freshwater rainbow trout (Oncorhynchus mykiss). Environmental Toxicology and Chemistry 17(4):579-588.

Wood CM, Farrell A, Brauner C. 2012. Fish Physiology: Homeostasis and Toxicology of Non-Essential Metals. , 1st Edition. Elsevier Inc. 31B, pp 1-54.

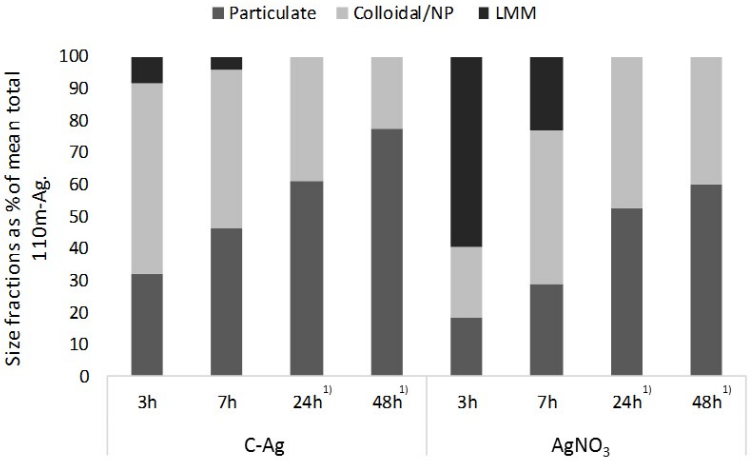
Wood CM, Playle RC, Hogstrand C. 1999. Physiology and modeling of mechanisms of silver uptake and toxicity in fish. Environmental toxicology and chemistry 18(1):71-83.

542 **FIGURES**



543

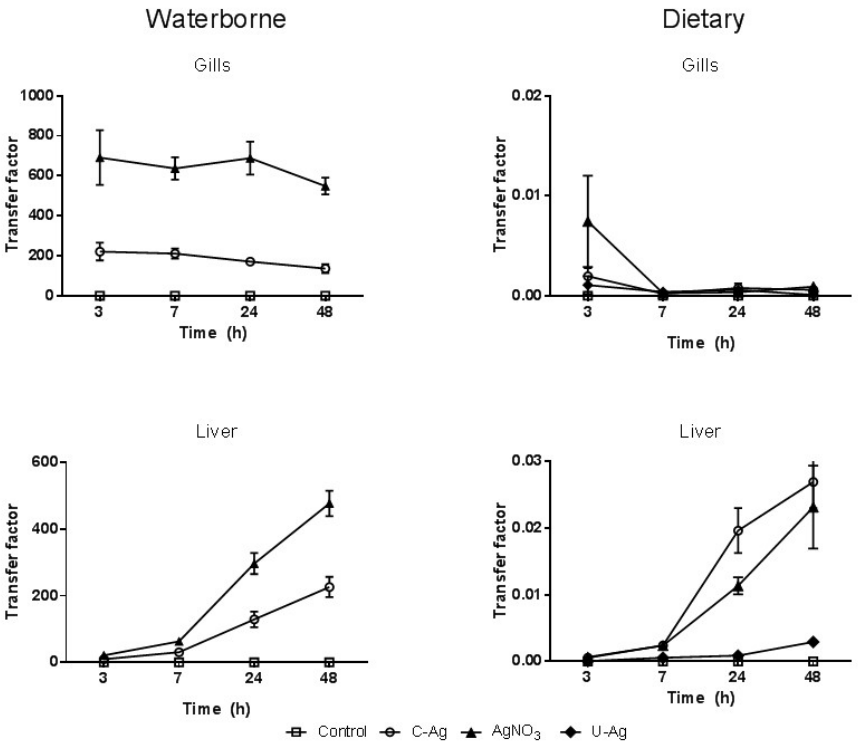
544 Figure 1: Average Ag concentration (µg/L±SD) in unfiltered exposure water over time for the
545 waterborne exposure (N=3), measured at time 0, 3, 7, 24, and 48 h.



546

547 Figure 2: Changes in the size fractions, particulate (> 0.22 µm), colloidal/NP (< 0.22 µm, > 3 kDa) and
548 low molecular mass (LMM, < 3 kDa) of ^{110m}Ag present in the exposure water in the waterborne
549 exposure over time (U-AgNPs, $AgNO_3$). [1)] No LMM, < 3 kDa samples could be collected after 24 and
550 48 h due to technical problems, thus the colloidal/np fractions potentially also contain LMM Ag.

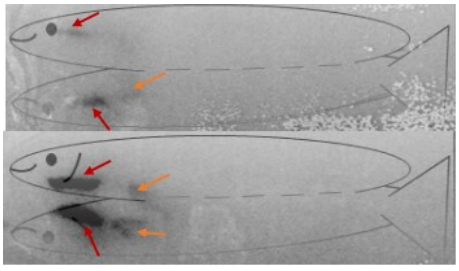
551



552

553 Fig 3. Time dependent transfer ratio((Bq/g tissue)/(Bq/g feed) and (Bq/kg tissue)/(Bq/L water)) of
554 ^{110m}Ag to gills and liver from dietary and waterborne exposure of radiolabeled $AgNO_3$, synthesized
555 citrate stabilized silver nanoparticles (C-Ag NPs), and commercially uncoated silver nanoparticles (U-
556 Ag NPs, only in dietary exposure) and controls. Data presented as mean±SEM, Be aware of the
557 difference in scale on Y-axis between waterborne and dietary graphs.

558



559

560 Figure 4. Autoradiography showing ^{110m}Ag in gills (red) and liver (orange) of Atlantic salmon exposed
561 through water to Ag nanoparticles (C-Ag NPs, top) and $AgNO_3$ (bottom). The fish was frozen before
562 being sectioned along the dorsal side from the anterior to the posterior end.

SUPPLEMENTARY INFORMATION

Route of exposure has a major impact on uptake of silver nanoparticles in Atlantic salmon (*Salmo salar*)

Merethe Kleiven[a*], Bjørn Olav Rosseland[a], Hans-Christian Teien[a], Erik J. Joner[b] and Deborah Oughton[a]

[a] *Norwegian University of Life Sciences, Faculty of Environmental Sciences and Natural Resource Management, P.O. Box5003 NMBU, 1432 Ås, Norway*

[b] *Norwegian Institute of Bioeconomy Research, P.O. Box 115, 1431 Ås, Norway*

[*] *Address correspondence to merethe.kleiven@nmbu.no or deborah.oughton@nmbu.no*

METHOD AND MATERIALS

Silver materials – synthesis and radiolabelling

Silver nitrate (Merck, pro analysis) and the uncoated Ag nanoparticles (QSI-nano silver, Quantum Sphere) were radiolabeled by neutron activation. Before activation, the powders were weighed into 1 mL polyethylene vials, heat sealed, and packed into an Al container. The neutron activation took place at the Institute of Energy Technology (Kjeller, Norway) at a flux of 10^{12} neutrons/cm^2 for 48 h. Particle characterization, both of stock solutions and of exposure solutions, was carried out by DLS, TEM and ultrafiltration (supplementary information).

The $^{110m}AgNO_3$ stock solution for the water exposure (specific activity 39 kBq/mg) was made by dissolving 9.751 mg of radiolabeled $AgNO_3$ in 1mL of 3M NH_4OH before further dilution with Type 2 water (15 MΩ·cm) to a final volume of 20 mL and a Ag concentration of 2.9 mM (0.5 g/L). The C-Ag NPs were synthesized from $^{110m}AgNO_3$ according to Doty et al. (Doty et al. 2005). The final activity added to the exposure containers was 50 kBq giving an activity of approx. 278 Bq/L and nominal concentration of 6 µg/L in the exposure water. For the dietary exposure, the C-Ag NPs were mixed with the fish food to give an activity of 3 kBq/g (nominal concentrations 60 µg Aq/g). U-Ag NPs with a specific activity of 34 kBq/mg was added to the fish food to obtain a similar activity and Ag concentration as for the other Ag exposed groups.

The $^{110m}AgNO_3$ stock solution for the water exposure (specific activity 39 kBq/mg) was made by dissolving 9.751 mg of radiolabeled $AgNO_3$ in 1mL of 3M NH_4OH before further dilution with Type 2 water (15 MΩ·cm) to a final volume of 20 mL and a Ag concentration of 2.9 mM (0.5 g/L).The C-Ag NPs were synthesized from $^{110m}AgNO_3$ according to Doty et al. (Doty et al.2005), with sodium borohydride ($NaBH_4$) reduction (see supplementary material). A solution of $NaBH_4$ (100 µL, 10 mM) was added to a mixture of 2.5 mL of $^{110m}AgNO_3$ (2.9 mM) and 2.5 mL of tri-sodium citrate (2.5 mM) by continually stirring. The final Ag concentration of the C-Ag NP stock solution was 153.2 mg/L before dilution (50 mL with exposure water) and addition to exposure containers.

Particle characterization

The Ag NPs were characterized for hydrodynamic diameter, polydispersity index and zeta potential with a Malvern Zetasizer ZS (Malvern Instruments, UK). The characterization of the C-Ag NPs was conducted on the stock suspension, while the U-Ag NPs prepared according to (Jensen et al.2016). Briefly, U-Ag NPs dispersed in Type 2 water (15 MΩ·cm) was sonicated for 13 min at 15 % amplitude using a 400 Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a standard 13 mm disruptor horn (Model number: 101-147-037). The commercial powders (U-Ag NPs) have been characterized in detail in earlier publications (Coutris et al.2012; Oughton et al.2008). Particle size and shape was also determined with transmission electron microscopy (TEM). The specimens were prepared by adding 10 µL of a nanoparticle suspension on a 400-mesh Cu coated Piloform film (Agar Scientific, Essex, UK) and the specimens were left for the liquid to evaporate. The images was acquired on a FEI Morgani 268 transmission electron microscope, operating at 80keV.

The image analysis of the TEM pictures was conducted with the software iTEM (Olympus), according to the protocol developed by Mast and de Temmemann (Mast and de Temmeman 2016) as a part of the European FP7 project NANoREG. The Equivalent Circle diameter (ECD) defined as the diameter of a circle that has an area equal to the area of the particle, Ferret min defined as the minimum distance of parallel tangents at opposing particle borders, as well as the shape and sphericity factors, are size and shape parameters obtained in this image analysis.

Food preparations

The feed was prepared as a slurry made of grinded commercial food (Skretting startfor, Skretting Norway) spiked with the radiolabeled Ag solutions/suspensions ($AgNO_3$, C-Ag NPs, and U-Ag NPs) in a ratio 50:50, this to enable the feeding with a syringe. The $AgNO_3$ and C-Ag NPs were both added to the fodder as solutions/suspensions, and mixed by magnetic stirring, before three aliquots were

624 taken out and measured in a Ge-detector to ensure equal distribution. The U-Ag NP added to the
625 fodder as a powder to avoid aggregation of the uncoated NPs in the water before addition to the
626 food. Homogeneity tests were carried out after mixing and showed homogeny distribution of the Ag.
627

RESULTS

Particle characterization

The citrate stabilized Ag nanoparticles (C-Ag) was generally round in shape with a shape and sphericity factor of 1±0.2 and 1±0.1, respectively. There were two obvious size populations present in the C-Ag stock suspension, the most numerous having an average ECD and Ferret min of 4±1.6 nm, n=2180 (Fig S1a). The larger size population have an ECD of 205±104 nm and a Ferret min of 196±95.7 nm, n=467 (Fig S1b). The bigger size population were also round with a shape and sphericity factor of 1±0.2. The hydrodynamic diameter (Z-avg) was 12±0.69 nm, PDI 0.58±0.013, and the zeta potential -40±2.1 mV.

The uncoated Ag nanoparticles (U-Ag) had more variation in shape than what was observed for the C-Ag, which was reflected in the sphericity factor of 0.7±0.13 (n=53) (Fig S1a). Both primary particle shape and size were harder to estimate for these particles due to the higher degree of agglomeration/aggregation. In the present study the Ferret min were 34±20.8 nm (n=53). The size analysis were based on a low number of particles because most particles were found in agglomerates. However, the results were in accordance with the producers (Quantum Sphere) report giving a primary particle size in the range of 20 to 40 nm. Previous studies conducted by our group (Coutris et al.2012; Oughton et al.2008) reported the particle size of these particles to be 19.2±6.8 and 20.2±2.5 nm, respectively. The presence of agglomerates/aggregates were further supported by the DLS measurements giving a hydrodynamic diameter (Z-avg) 196±4.1 nm. The PDI of 0.5±0.01 indicates a polydisperse sample when it comes to particle size. The zeta potential was -34.5 mV.

The discrepancy between the hydrodynamic diameter (Z-avg) and the size measures obtained from the TEM pictures were expected, and is a well-known artifact of the two different analytical methods. If the sample contain more than one size population or mainly aggregates, the DLS tend to favor the larger particles/aggregates due to the higher intensity signals these larger particles will reflect. Thus, in most cases the DLS will give a higher size estimate than the TEM. For the U-Ag NPs the DLS clearly gave an estimate of the aggregates present in the sample rather than the primary particles. The Z-Avg of C-Ag NPs were also influenced by the presence of the larger particle population.

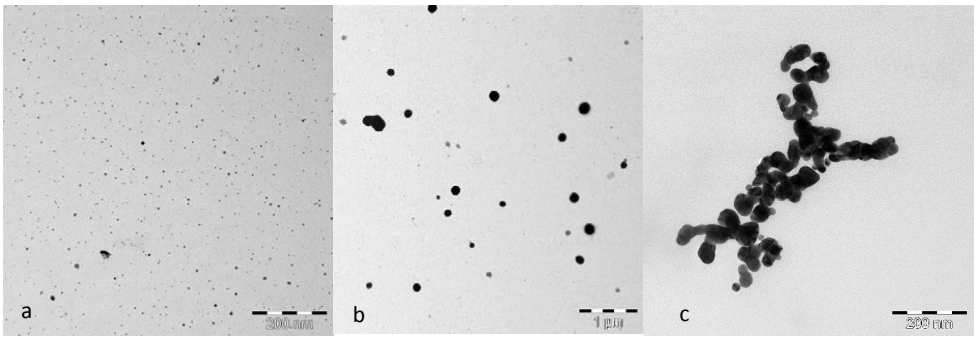


Fig S1. TEM, stock suspension of the citrate stabilized Ag nanoparticles (C-Ag) (a and b), and the uncoated Ag nanoparticles (U-Ag, c). For C-Ag nanoparticles there seem to be two main size classes, ECD of 4±1.6 nm (a) and 205±104 nm (b). For U-Ag the particles mainly seem to be in aggregates/agglomerates (c), however primary particle size seems to be around 23.7±7.14 nm. The scale bare on the left and right picture are 200 nm, on the picture in the middle it is 1 µm.

General water quality parameters

The variation in temperature (10.0±0.3), pH (6.8±0.2) and conductivity (44±1 µS/cm) were minor and non-significant both within and between the waterborne and dietary exposure. The dissolved oxygen was 11±0.1 mg/L (about 95 % saturation). Total organic carbon (TOC) of the control water was <0.5 mg/L at all times. In the exposure water within the waterborne exposure, there were some variation in TOC concentrations at time zero; 0.34 mg/L and 0.52 mg/L for C-Ag and $AgNO_3$, respectively. After 48 h, the variation was no longer present, and all groups had TOC concentration <0.20 mg/L (Limit of quantification). It is well known that organic matter has a high binding affinity to Ag and an increase in the concentration of organic matter usually lead to reduced toxicity (Hogstrand and Wood 1998). The TOC concentrations reported here are very low and stable through the experiment and thus not expected to influence the Ag speciation to any large degree.

Table S1. Changes in size fractions, Particulate (> 0.22 µm), colloidal/NP (< 0.22 µm, > 3 kDa), and low molecular mass (LMM, < 3 kDa), of ^{110m}Ag present in the exposure water in the waterborne exposures (C-Ag NP and $AgNO_3$) over time, given in corrected counts per minute (ccpm) (corrected for background activity), sample volume 20 mL, mean ± s.d.

	C-Ag NP				$AgNO_3$			
	Total	Particulate	Colloidal/NP	LMM	Total	Particulate	Colloidal/NP	LMM
3h	50±14	20±14	28±2	4±2	43±2	11±5	9±5	24±3
7h	80±14	50±14	24±2	2±1	44±2	16±3	19±5	9±4
24h	30±3	13±4	19±3	NA	37±4	17±5	19±2	NA
48h	30±8	20±10	11±5	NA	25±3	9±5	16±4	NA

NA = not applicable, no sample collected due to technical problems

Uptake

No significant accumulation in brain, heart and spleen, although counts were observed occasionally in individual fish. The detected activity of ^{110m}Ag in organs with significant accumulation of Ag over time after exposure to $AgNO_3$ or AgNPs are given in Table S2 and S3 for dietary and waterborne exposures, respectively. Control counts reflects background radioactivity and not contamination of controls.

Table S2. Accumulation of ^{110m}Ag (mean ± s.d., n=6, ccpm/g wet weight tissue, corrected for background activity) in tissue of *Salmo trutta* after dietary exposure to $AgNO_3$ or two different types of Ag NPs (C-Ag NP and U-Ag NP). Also the gut content of the fish were sampled and included in this table.

	Time (h)	Bile ccpm/g	Gill ccpm/g	Gastrointestinal tract ccpm/g	Kidney ccpm/g	Liver ccpm/g	Gut content ccpm/g
$AgNO_3$	3	20±40	200±300	400±300	20±20	17±13	28000±6000
	7	10±20	6±4	600±250	8±14	60±20	26000±5000
	24	40±40	10±10	900±200	10±9	300±70	23000±4000
	48	200±100	20±30	1000±600	10±14	600±400	20000±15000
C-Ag NP	3	10±10	50±50	700±300	5±4	12±7	25000±8000
	7	10±30	5±5	990±90	12±14	60±30	26000±6000
	24	100±50	20±30	1200±400	9±11	500±200	23000±5000
	48	300±200	13±9	800±100	20±20	600±200	20000±5000
U-Ag NP	3	4±5	7±8	800±600	3±5	3±3	40000±20000
	7	20±20	20±20	1400±700	10±12	20±20	40000±20000
	24	20±20	10±10	400±300	10±12	40±30	40000±20000
	48	20±20	4±4	200±100	2±5	120±80	50000±20000
Control	Avg. over time	20±30	3±3	5±3	3±6	6±5	300±400

Table S3. Accumulation of ^{110m}Ag (mean ± s.d., n=6, ccpm/g wet weight tissue, corrected for background activty) in tissue of *Salmo trutta* after waterborne exposure to $AgNO_3$ or Ag nanoparticle (C-Ag NP). Also the gut content of the fish were sampled and included in this table.

	Time (h)	Bile ccpm/g	Gill ccpm/g	Gastrointestinal tract ccpm/g	Kidney ccpm/g	Liver ccpm/g	Gut content ccpm/g
$AgNO_3$	3	5±8	1400±700	20±20	40±70	40±20	50±100
	7	20±30	1300±300	7±8	50±50	130±50	30±70
	24	300±100	1400±400	20±10	50±40	600±160	60±100
	48	800±500	1100±200	20±5	120±60	1000±200	40±50
C-Ag NP	3	20±40	600±100	4±3	6±10	20±20	40±90
	7	90±100	500±150	11±3	20±20	70±40	10±10
	24	120±80	400±60	20±10	30±40	300±140	40±40
	48	400±300	300±100	30±20	60±40	500±200	200±200
Control	Avg. over time	30±30	6±6	2±4	3±7	4±10	30±50

697 **REFRENCES**

698 Coutris C, Joner EJ, Oughton DH. 2012. Aging and soil organic matter content affect the fate of silver
699 nanoparticles in soil. Science of the Total Environment 420:327-333.
700 Doty RC, Tshikhudo TR, Brust M, Fernig DG. 2005. Extremely stable water-soluble Ag nanoparticles.
701 Chemistry of Materials 17(18):4630-4635.
702 Hogstrand C, Wood CM. 1998. Toward a better understanding of the bioavailability, physiology and
703 toxicity of silver in fish: Implications for water quality criteria. Environmental Toxicology and
704 Chemistry 17(4):547-561.
705 Jensen KA, Booth A, Kembouche Y, Boraschi D. 2016. Validated protocols for test item preparation
706 for key in vitro and ecotoxicity studies, NANoREG Deliverable D2.06.
707 Mast J, de Temmeman P-J. 2016. Protocol(s) for size-distribution analysis of primary NM particles in
708 air, powders, and liquids, NANoREG Deliverable D2.10.
709 Oughton DH, Hertel-Aas T, Pellicer E, Mendoza E, Joner EJ. 2008. Neutron activation of engineered
710 nanoparticles as a tool for tracing their environmental fate and uptake in organisms.
711 Environmental Toxicology and Chemistry 27(9):1883-1887.

Paper IV

Characterization of bioconcentration and toxicity of Ag ions and the Ag nanoparticle NM300K in Brown trout (Salmo trutta L.)

Merethe Kleiven[a*], Maria T. Hultman[b], Bjørn Olav Rosseland[a], Pablo Lebed[a], Knut Erik Tollefsen[ab], Hans-Christian Teien[a]

[a] *Norwegian University of Life Sciences(NMBU), Faculty of Environmental Sciences and Nature Resource Management, Center for Environmental Radioactivity (CERAD), P.O.Box 5003 NMBU, 1432 Ås, Norway*

[b] *Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway*

[*] Address correspondence to *merethe.kleiven@nmbu.no or hans-christian.teien@nmbu.no*

Abstract

Silver nanoparticles (Ag NPs) are one of the most frequently used nanomaterials in consumer products due to their antibacterial properties. With the continuously increasing use of nanomaterials follows an increasing risk for environmental release and concern for its potential toxic effects. The aim of the current study was to assess the potential for bioconcentration and effects of Ag ions (added as $AgNO_3$) and the OECD representative Ag nanomaterial NM300K in brown trout (*Salmo trutta,* L.) after 96 h waterborne exposure (2, 5 and 10 µg Ag L^{-1}). The results show bioconcentration of Ag in gills (0.8±0.4, 2.9±0.9 and 6±1 µg Ag g^{-1}), liver (5±3, 13±5 and 20±3 µg Ag g^{-1}), and kidney (0.4±0.2, 1.3±0.6 and 2±1 µg Ag g^{-1}) of brown trout exposed to 2, 5 and 10 µg Ag L^{-1} added as $AgNO_3$. No systemic bioconcentration (i.e., no uptake of Ag in liver and kidney after exposure to NM300K) was detected after exposure to the NM300K Ag NP. However, low levels of Ag was adsorbed to the gills. The differences in bioconcentration of Ag in fish tissue after exposure to $AgNO_3$ and NM300K could be related to the presence of low molecular mass Ag (< 3 kDa) in the $AgNO_3$ exposures. In the NM300K Ag NP exposure suspension no < 3 kDa Ag was detected, while in the $AgNO_3$ exposure as much as 30 % of the total Ag in the highest exposure (10 µg L^{-1}) was present as < 3 kDa Ag, thus presenting a plausible explanation to the differences in bioconcentration seen in the current study. $AgNO_3$ exposure resulted in concentration-dependent change in blood plasma (blood glucose, plasma ions Na^+ and Cl^-) as well as upregulation of genes involved in oxidative stress response (Glutathione reductase, Glutathione peroxidase X3 and Glutathione S transferase) and apoptosis (Caspase 6A, and Bcl2 associated x protein) in liver of fish exposed to 10 µg Ag L^{-1}. We concluded that the differences in accumulation of Ag in fish tissue after exposure to $AgNO_3$ and NM300K was mainly related to concentration of low molecular mass Ag (< 3 kDa) in the exposures. The presence of low molecular mass Ag is therefore proposed to contribute to the reported dose-dependent bioconcentration and toxicity of $AgNO_3$ in present study.

Keywords: bioconcentration, toxicity, qPCR, oxidative stress, apoptosis

1. Introduction

Silver is known to accumulate and induce toxicity to aquatic organisms, including fish (Wood et al., 1996, Farmen et al., 2012, Bruneau et al., 2016). Bioaccumulation and organ distribution of Ag in fish after exposure to Ag ions (Ag(I), usually administered as $AgNO_3$) under different environmental conditions have been reported the last decades (Galvez et al., 1998, Hogstrand et al., 2003). Bioaccumulation of Ag after exposure to Ag nanoparticles (AgNPs) have also been reported, albeit at lower concentrations than their ionic counterparts (Martin et al., 2017). It has been speculated that the accumulated Ag could be a result of dissolution of the nanoparticles followed by accumulation of the released ions rather than accumulation of the nanoparticles (Shaw and Handy, 2011). The main target organs for Ag accumulation in fish after waterborne exposure are gills and liver, and generally reported to be the same after exposure to Ag(I) and AgNPs (Webb and Wood, 2000, Martin et al., 2017). In waterborne exposures, the gill would be the primary organ of Ag interaction, while the liver are expected to be the organs with the highest concentrations of Ag.

For toxicity to fish after waterborne exposure to Ag, the consensus is that it is mainly caused by the presence of free Ag ions leading to inhibition of the Na^+/K^+ ATPase causing severe osmoregulatory problems in freshwater fish (Bury and Wood, 1999, Hogstrand and Wood, 1998), as well as oxidative stress and apoptosis (Farmen et al., 2012, Bruneau et al., 2016). The toxicity of Ag NPs are generally reported to be lower than for Ag(I), and it has been frequently hypothesized that the toxicity of metal nanomaterials is mainly caused by the release of ions followed by a “free ion” or Low Molecular Mass (LMM) metal species induced toxicity (Notter et al., 2014). However, a nanospecific toxicity or a combination of the two, have also been reported (Scown et al., 2010, Bruneau et al., 2016). The mechanism behind observed nano-specific toxicity are often reported to be oxidative stress induced by the formation of reactive oxygen species (ROS) at the surface of the nanoparticle (Carlson et al., 2008). Antioxidant defense systems are activated to protect the cell against ROS induced damage/oxidative stress. These systems involve the glutathione (GSH) detoxification

pathway causing scavenging of ROS and other organic free radicals, the detoxification by metallothionein, as well as the apoptotic pathways resulting in programmed cell death (apoptosis) initiated by cell dysfunction to remove damage cells and thus ensuring normal cellular functioning (Newman, 2009). Molecular biomarkers can be used to identify early responses to contaminants in an organism, and are useful tools for identification of the mechanisms behind observed toxicity.

The lack of consistency in literature regarding the mechanisms behind toxicity of AgNPs can be explained by variations in, for example, water chemistry, Ag speciation, and AgNP characteristics such as coating, size and surface charge. Thus, the importance of thorough exposure characterization that monitor particle transformation (e.g. dissolution, aggregation) cannot be understated. To understand the toxicity of nanomaterials it is important to consider both accumulation and effects, as well as exposure characteristics.

In the last years, an increasing number of studies on the NM300K AgNPs, tested in the current work, have been published, addressing speciation, characterization (Köser et al., 2017) as well as *in vivo* and *in vitro* toxicity to a range of species, including *Pseudokirchneriella subcapitata* (Sørensen and Baun, 2015), soil invertebrates (van der Ploeg et al., 2014, Bicho et al., 2016) and cell lines (Connolly et al., 2015, Bermejo-Nogales et al., 2016). With the basis in these studies, we hypothesize the $AgNO_3$ and NM300K AgNPs exposures to be different. More specifically that the presence of dissolved Ag in the $AgNO_3$ exposures will be higher than in the Ag NP exposure, and that this is reflected in the bioconcnetration and toxicity of Ag. The aim of the current study was to assess the potential of bioconcentration (in gill, liver and kidney) and toxicity (blood plasma parameters and regulation of molecular biomarkers involved in oxidative stress responses and apoptosis) of $AgNO_3$ and NM300K AgNP, an OECD representative Ag nanomaterial, in juvenile brown trout (*Salmo trutta* L.) after waterborne exposure.

2. Material and methods

2.1 Experimental design

Juvenile brown trout (yearlings) were obtained from the hatchery Bjørkelangen Settefisk (1940 Bjørkelangen, Norway), originating from a local wild brown trout strain in Aurskog-Høland (Norway). The fish (10.0±2.3 g, n=10) were acclimated in experimental water (local drinking water, pH 7.2±0.1, conductivity of 43 µS cm^{-1}, calcium 4.5 mg L^{-1}, total organic carbon 3.3 mg L^{-1}) for two weeks in a flow through system at 13.8±0.2 °C prior to the 96 h exposure period. The experiment was conducted at NIVA Marin Research Station Solbergstrand, Norway.

Brown trout were exposed to three concentrations (2, 5, and 10 µg L^{-1}) of either Ag-ions ($AgNO_3$) or Ag nanoparticles (AgNPs: NM300K) in fish tanks (fiberglass) filled with 50 liters of water. The exposure was conducted in a semi-static setup, where 30 of the 50 L were exchanged once a day. An air pump with diffusors ensured oxygen saturation between 80-100 % during the whole experiment. There were six fish per group and they were fed 1 % of the body weight once a day before the renewal of exposure water. The fish were sacrificed after 96 h. Blood were sampled for plasma ion analysis, organs were sampled for determination of Ag bioconcentration (liver, gill and kidney) and gene expression (qPCR analysis of liver tissue). The sampling followed the EMERGE Protocol (Rosseland et al., 2001).

2.2 Exposure water and water quality parameters

Water samples were collected from all exposure containers every second day for characterization of general water chemistry parameters. Concentration of Ag was measured daily. Other potential fish stressor parameters such as temperature, dissolved O_2, pH, conductivity (WTW430i with Sentix 41 pH electrode and TertraCon 325 conductivity probe) and total ammonium nitrogen (Spectrophotometric) were monitored daily throughout the experiment, including the acclimation period.

2.3 Silver materials, exposure and characterization

The Ag ion solution used in the experiment was $AgNO_3$, and a stock solution of 500 mg L^{-1} was prepared. The $AgNO_3$ (Merck, Darmstad, Germany) used was of pro analysis quality, and so were all other chemicals used in this experiment. The silver nanomaterial used was the OECD representative AgNPs NM300K (Joint Research Center Reference Nanomaterial Repository, Ispra, Italy). These nanoparticles are spherical Ag nanoparticles dispersed in stabilizing agents consisting of 4 % (w/w %) of each of Polyoxyethylene Glycerol Trioleate and Tween 20. The average particle size, based on transmission electron microscopy, are reported to be 15 nm, with 90 % of the particles < 20 nm (Klein et al., 2011). The main stock suspensions of AgNPs were prepared according to Jensen et al. (2016). In short, a 2.56 g L^{-1} AgNP stock suspension was prepared by dispersing the original AgNP suspension in Type II water (15 MΩ·cm) and sonicate for 13 min at 15 % amplitude (depositing 7.35±0.05 Watt) using a 400 Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a standard 13 mm disruptor horn (Model number: 101-147-037). An intermediate stock suspension of 50 mg Ag L^{-1} were prepared from the main stock suspensions (for both $AgNO_3$ and Ag NP suspensions), before mixing the Ag contaminants into the water at the beginning of the experiment. Particle size of the AgNPs in stock suspension as well as in exposure media was obtained by transmission electron microscope (TEM), dynamic light scattering (DLS) and single particle inductively coupled plasma mass spectrometry (spICP-MS).

2.3.1 Transmission electron microscope

Two size characteristics were obtain using TEM image analysis; the Equivalent Circle diameter (ECD), defined as the diameter of a circle that has an area equal to the area of the particle, and the minimum distance of parallel tangents at opposing particle borders (Ferret min). The AgNP stock suspensions (2.56 g L^{-1}) were diluted in Type II (15 MΩ·cm) water to obtain the optimal grid particle concentration (250 mg L^{-1}) before 10 µL were added to a 400-mesh Cu coated Piloform film (Agar Scientific, Essex, UK) and the specimens were left for the liquid to evaporate. The images were

acquired on a FEI Morgani 268 transmission electron microscope (FEI, Eindhoven, Netherlands) operating at 80 keV. The image analysis of the TEM pictures was conducted with the software ImageJ (https://imagej.net) according to the protocol developed by Mast and de Temmeman (2016), as a part of the European FP7 project NANoREG.

2.3.2 Dynamic light scattering

Dynamic light scattering measurements were performed on a Malvern Zetasizer ZS (Malvern instruments Ltd, Worcestershire, UK) equipped with a laser source with wavelength 633 nm. Zeta-averaged hydrodynamic diameters and size distributions were determined using the "multiple narrow modes (high resolution)" algorithm supplied by Malvern. The DLS measurements were performed on the freshly made stock suspension of AgNPs (2.56 g L^{-1}), as well as intermediate stocks and exposure suspensions.

2.4 Sampling and analytical techniques

2.4.1 Water

During the exposure period, water samples for total Ag concentration measurements were collected daily from all exposure groups. In addition, *in situ* particle size fractionation were performed to obtain information of the Ag speciation. The size fractionation was performed once at the beginning (0 h) and before renewal of exposures (24 h) in the waterborne exposures. The fractionation was performed following the procedure described by Farmen et al. (2012). Briefly, the water was filtrated through a 0.22 µm membrane syringe filter (Millipore) to exclude larger particulate matter, and hollow-fiber cross-flow ultrafiltration (Pall Microza Hollow Fiber Module, Pall Corporation, New York, USA) with a nominal molecular mass cutoff at 3 and 10 kDa were then performed to exclude the nanoparticles (3 kDa equivalent to approx. 1 nm). The size fraction >0.22 µm was defined as particulate, fraction <0.22 µm and >3 kDa was defined as nanoparticles/colloidal, while the fraction <3 and <10 kDa was defined as low molecular mass (LMM). Ag-ions are included in the LMM fraction. Water samples were acidified with 5 vol % ultrapure HNO_3, except samples for Cl^-

concentration which were added 5 vol % of tetramethylammonium hydroxide (TMAH), prior to storage and ICP-MS measurements (ICP-MS, Agilent 8800, Hachioji, Japan).

2.4.2 Tissue samples of exposed fish

At the end of the 96 h exposure the fish were killed with a blow to the head, weight and length measured before blood were drawn from the caudal vein with heparinized syringe and directly analyzed for blood parameters using I-STAT® portable clinical analyzer (Abbott Point of Care Inc., Princeton, NJ, USA) with EC8+ cassette (Abbott, East Windsor, USA). The fish were dissected and organs were collected for Ag bioconcentration (liver, gills, and kidney) and gene expression (liver) analysis. Tissue samples for Ag bioconcentration analysis were stored at -28 °C before being freeze dried (only gill and liver tissue), added ultrapure HNO_3, internal standard and Type II water (15 MΩ·cm) prior to digestion in an ultraclave (Milestone, Leutkirch, Germany). A certified reference material (Dogfish Liver Certified Reference Material for Trace Material) for Ag in fish tissue was digested with same procedure as the samples. The digested samples were diluted to 10 vol % acidic solution before Ag concentrations were determined by ICP-MS. Tissue sampled for gene expression analysis were stored in RNA later solution (Sigma-Aldrich, St. Louis, MO) at 4 °C for one day before storage at -20°C until further processing.

2.4.3 RNA isolation and quality assessment

Prior to the RNA isolation the liver tissue was weighted, followed by lysis and RNA isolation using Qiagen RNeasy Plus mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer instructions. The isolated total RNA was measured spectrophotometrically (Spec-trophotometer ND 1000, Nanodrop technologies Inc., Wilmington,USA) with quality cut-off criteria as follows: 260/230 ratios of >1.8 and 280/260 ratios of >2.0. The RNA integrity was measured using Agilent BioAnalyzer RNA 6000 Nano series kit (Agilent Technologies, USA) with a RNA Integrity Number (RIN-value) cut-off criteria of >8, indicating high RNA integrity (Fleige and Pfaffl, 2006). The samples were stored at -80^{o}C until further analysis.

2.4.4 Quantitative real-time polymerase chain reaction (qPCR)

Quantitative real-time polymerase chain reaction (qPCR) was performed on a selection of target genes (Table 1) for the three exposure groups of $AgNO_3$ (2, 5 and 10 µg Ag L^{-1}) and the control group in present study. Isolated RNA (1 µg) was reverse transcribed into complementary DNA (cDNA) using Quanta qScript™ cDNA Synthesis Kit (Quanta Biosences Inc., Gaithersburg,USA) according to the manufacturer's instructions, with some modification on the incubation time (1h) at 42 °C. The chosen primers were obtained from previously published studies (Table 1) and were purchased from Eurofins MWG synthesis GmbH (Ebersberg, Germany). All primers were optimized for concentration and annealing temperature using a 5-step cDNA mix pool dilution (2.5-75 ng/well) in a 384 well format on a CFX-384 thermal cycler (Bio-Rad laboratories Inc., USA), yielding a final amplification efficiency of 91.5–104 % (Table 1). All qPCR amplification reactions were performed using the fluorescent dye SYBR®Green Supermix (Quanta Biosences Inc., Gaithersburg, USA) in duplicates in a final reaction of 20 µl/well. The qPCR protocol was performed as follows: Cycle 1: 95°C for 3 min, Cycle 2–40: 95°C for 20 s, followed by the specific primer annealing temperature for 20 s and 72 °C for 20 s (Table 1). The samples were analyzed using 10 ng of cDNA template/well, always including a no-template (NTC) and a no-reverse transcriptase (NRT) control to exclude any contamination in qPCR master mix (NTC) or presence of genomic DNA in RNA samples (NRT). The NTC reported no-detectable amplification (N/A), while NRT reported Cq-values above 33 cycles, ensuring a non-significant amount of quantified genomic DNA in the samples. At the end of each qPCR run a melt curve analysis was performed to identify potential primer-dimer formation or unspecific amplified products formed during the analysis. The expression of target genes were normalized against the total RNA and fold change calculated by comparing the normalized treatment gene expression to the control samples, as reported previously (Song et al., 2012, Tollefsen et al., 2015).

2.5 Data analysis

All statistical testing was performed on GraphPad Prism 6 (GraphPad Software, La Jolla, USA) with a statistical significance level α of 0.05. For bioconcentration, blood plasma parameters, gene expression data and water parameters, one-way ANOVA test was conducted. In the case of significant result, a Tukey-Kramer means comparison test was performed to find out how many and which groups differed significantly from each other. A principal component analysis (PCA) was applied to identify correlations between different parameters measured using XLSTAT2015® using a α of 0.05). Parameters identified by the PCA as relevant were further analyzed by linear regression and correlation analysis using GraphPad prism 6. All results are given as mean ± one standard deviation.

218

Table 1. Genes, primer sequences, accession numbers and analysis protocol used for the qPCR analysis.

Gene	Species		Primer sequence	Amplicone size (bp)	Annealing temperature	Primer conc. (nM)	Amplification efficiency (%)	Genebank accession no.	References
GR	*Salmo salar*	*Forward*	5'-GGCCGAGAGCCCAACAC-3'	NR	60°C	300	103.9	BT045539	Song et al., 2012
		Reverse	5'-TGAAGCCTCTCTCATCAATATCCA-3'			300			
GPx3	*Salmo salar*	*Forward*	5'-GATTCGTTCCAAACTTCCTGCTA-3'	NR	60°C	300	103.5	BT072794	Song et al., 2012
		Reverse	5'-GCTCCCAGAACAGCCTGTTG-3'			300			
GST	*Salmo salar*	*Forward*	5'-ATTTTGGGACGGGCTGACA-3'	81	60°C	300	94.5	BQ036247.1	Olsvik et al., 2007
		Reverse	5'-CCTGGTGCTCTGCTCCAGTT-3'			300			
Bax	*Salmo salar*	*Forward*	5'-ATTGGAAATGAGCTGGATGG-3'	NR	59.4°C	400	94	EG801847	Song et al., 2012
		Reverse	5'-GCCGACAGGCAAAGTAGAAG-3'			400			
MT	*Salmo salar*	*Forward*	5'-CCTTGTGAATGCTCCAAAACTG-3'	101	60°C	300	91.5	x97274	Olsvik et al, 2010
		Reverse	5'-CAGTCGCAGCAACTTGCTTTC-3'			300			
Casp6A	*Salmo salar*	*Forward*	5'-TGAGCCACGGAGAGAACGA-3'	NR	64.5°C	400	100.9	DQ008068	Song et al., 2012
		Reverse	5'-CCCACCAGGCTCTTACACTTG-3'			400			

Abbreviation: NR - Not reported; bp - Base pairs; GR - Glutathione reductase; GPx3 - Glutathione peroxidase x3; GST - Glutathione S transferase; *Casp6a* - Caspase 6A; *Bax* - Bcl2 associatedassosiated x protein; MT - Metallothionein

219

3 Results

3.1 Silver nanoparticle characterization – stock and exposure suspensions

2.4.3 Transmission electron microscope

NM300K AgNPs used in the current study were spherical (Figure S1) and the initial particle size of NM300K AgNPs measured by TEM gave a Ferret min of 16.9±0.1 nm (mean±S.D.) and an equivalent circle diameter (ECD) of 18.7±0.2 nm (n=1833). Aggregates/agglomerates exceeding this size range were also observed in the TEM imaging of the stock suspension (Figure S1).

3.1.2 Dynamic light scattering

The zeta-averaged hydrodynamic diameter (nm) of the main stock were 86±0.7 nm (mean±S.D.) (Table 2). The polydispersity index (PDI) values of the stock suspension, as well as the stock dilution, indicated relatively monodispersed particle populations. It is worth mentioning that the main stock suspension contains two size populations (119 and 19 nm) that represented 95.6 and 4.4 % of the intensity measurements, respectively (Figure S2 A). For the Number mean particle size in the stock suspension the main peak was actually 15 nm (Figure S2 B), in agreement with the particle size obtained from TEM analysis in the current study as well as the size given from the supplier. In both AgNP and $AgNO_3$ exposures larger particulate matter was detected together with high polydispersity indexes (Table 2, Figure S3 and S4).

Table 2. Particle size parameters in stock suspension and stock dilution of NM300K AgNPs as well as all exposure suspensions at t=0 h, obtained by dynamic light scattering. The parameters presented: hydrodynamic diameter by intensity (Z-avg) (nm), by number mean (nm) and polydispersity index (PDI).

	Dynamic light scattering		
	Z-avg nm	Number mean nm	PDI
Stock suspension (2.56 g L^{-1})	86±0.7	33±18	0.244±0.007
Stock dilution (50 mg L^{-1})	88±0.8	50±5	0.231±0.009
AgNP 2.0 µg Ag L^{-1}	753±144	195±60	0.787±0.111
AgNP 5.0 µg Ag L^{-1}	412±164	147±173	0.529±0.122
AgNP 10.0 µg Ag L^{-1}	372±71	39±32	0.458±0.066
$AgNO_3$ 2.0 µg Ag L^{-1}	913±244	2501±61	0.793±0.159
$AgNO_3$ 5.0 µg Ag L^{-1}	643±88	309±23	0.555±0.204
$AgNO_3$ 10.0 µg Ag L^{-1}	6468±9972	152±150	0.781±0.224

3.2 Water chemistry (exposure)

3.2.1 General water parameters and water quality

The main water quality variables for the exposure water used in the current study are listed in Table 3. The water is characterized as low ionic strength, soft freshwater low in total organic carbon. The pH, oxygen, TAN and conductivity did not differ significantly between exposure groups. However, an increase with time was bserved for all parameters (Table S1). The measured values of pH and conductivity after 96 h were significantly different from the other sampling points. The maximum pH increase was <0.2 units. The conductivity increased from 44.9±1.4 µS cm^{-1} to 49.8±0.8 µS cm^{-1} during the

96 h of exposure. The TAN concentrations was in average of 0.3±0.2 mg L^{-1} (n = 20) (Table S2), and well below the advised upper limit of 2 mg L^{-1}.

Table 3. Water chemistry parameters of the exposure water used in the current study (mean±S.D.). Samples collected from all containers during the experiment (n=44), Cl (n=13), pH, conductivity, O_2 and temperature (n=27).

Ca	4.5±0.3 mg L^{-1}
Na	3.9±0.3 mg L^{-1}
Mg	0.40±0.03 mg L^{-1}
S	1.26±0.08 mg L^{-1}
K	0.36±0.07 mg L^{-1}
Cl	2.3±0.2 mg L^{-1}
[#] Ag	< 3.6 ng L^{-1}
[*] Total organic carbon	3.3 mg L^{-1}
[*] NO_3	0.34 mg/L
pH	7.1±0.17
Conductivity	47±2 µS/cm
O_2	10.7±0.26 mg L^{-1}
Temperature	13.4±0.46 °C
Total ammonium nitrogen	0.3±0.2 mg L^{-1}

[*] Parameters obtained from the water suppliers (Frogn kommune), not measured during the experiment.

[#] Ag measured in control groups or during acclimation period prior to addition of Ag.

3.2.2 Ag exposure

The measured Ag concentration in the $AgNO_3$ groups were ≥ 95 % of nominal concentrations, indicating low degree of sorption. In the AgNP exposures the mean measured exposure concentration of Ag were within 80 % of the nominal concentrations at time zero (Table 4). There was an average reduction of 11 ± 4 and 20 ± 2 % in total Ag concentration from 0 h to 24 h in $AgNO_3$ and AgNP treatments, respectively (Table S3). The size fractionation results are presented in Figure 1 and Table S3.

Table 4. Concentrations of Ag (mean±s.d.) at the beginning of the exposure, as % of nominal concentrations at t=0, and % reduction in total silver after 24 h (N=5, LOD 3.6 ng L^{-1}).

	Nominal concentration (µg Ag L^{-1})	Ag concentration t= 0 h, ($\bar{x}$ ± s.d.) (µg L^{-1})	Measured concentration as % of nominal at t= 0 h	% reduction in total Ag after 24 h
Control		<LOD	-	-
$AgNO_3$	2.0	2.0 ± 0.4	98	15
$AgNO_3$	5.0	4.8 ± 0.4	95	12
$AgNO_3$	10.0	9.6 ± 0.4	96	7
AgNP	2.0	1.6 ± 0.2	82	21
AgNP	5.0	4.4 ± 0.3	87	17
AgNP	10.0	7.6 ± 1.9	76	21

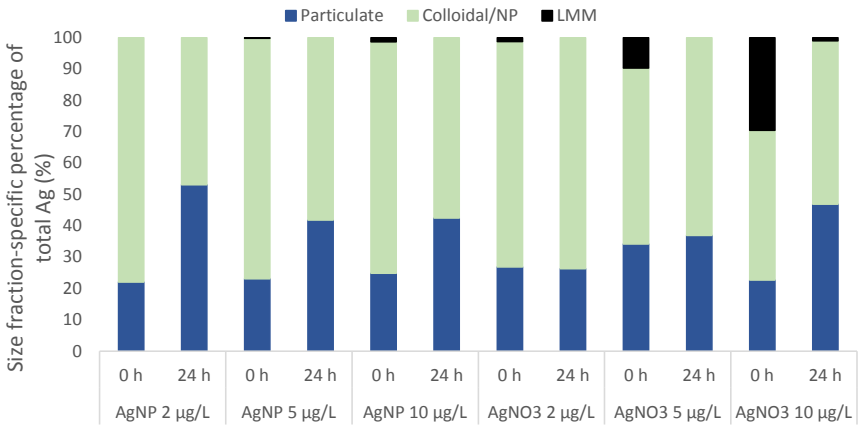


Figure 1. Changes in the size fractions of Ag (as % of total silver) present in the NM300K (AgNP) and $AgNO_3$ exposures at t= 0 h and t= 24 h, total Ag concentrations 2, 5 and 10 µg L^{-1}. The size fractions are defined as followed: Particulate >220 nm, Colloidal/NP <220 nm and >3/10 kDa, LMM < 3 kDa at t=0 and <10 kDa at t=24 h.

3.3 Concentration of Ag in tissue and biological effects

3.3.1 Concentration of Ag in tissue

In the $AgNO_3$ exposed groups, there was a concentration-dependent uptake in gill, liver, and kidney (Figure 2, Table S4), while in the AgNP exposure a significant uptake was only measured in gills for the

two highest exposure concentrations. The concentration of Ag in gills were two orders of magnitude higher in $AgNO_3$ than in AgNP exposures (Figure 2, Table S5).

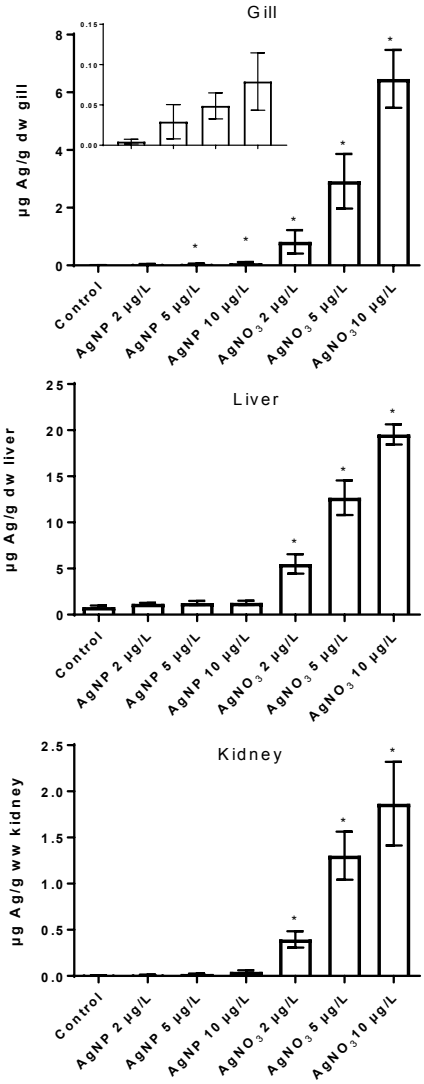
3.3.2 Blood plasma parameters

A clear dose-response in blood glucose was observed after exposure to $AgNO_3$ (Table 5), however the in the AgNP exposure it did not differ significantly from control group. Plasma ions (Na and Cl) were affected at the two highest exposure concentrations of $AgNO_3$ (Table 4). Due to technical problems with the iSTAT at the beginning of the biological sampling, blood plasma parameters were not obtained from the control group and data from unstressed brown trout were used as a proxy (see table 4, for details). Plasma Na and Cl values in the lowest concentrations of $AgNO_3$ (i.e., 2 µg Ag L^{-1}) were found to be similar to that of an "unstressed" brown trout. However, in the highest exposure to $AgNO_3$ (10 µg Ag L^{-1}), the Na and Cl concentrations were lower than all other exposure groups.

Table 5. Total Ag concentration in gill and blood plasma parameters (mean±s.d.) of fish after 96 h of waterborne exposure to either $AgNO_3$ or NM300K AgNPs.

Exposures	Gills µg/g dw	Na mmol/L	Cl mmol/L	Glucose mmol/L[A]	Glucose mmol/L[B]
Control	0.005±0.003	[C]	[C]	-	2.3±0.4
$AgNO_3$ 2 µg Ag L^{-1}	0.8±0.04	131±4	125±5	7±3	5±2
$AgNO_3$ 5 µg Ag L^{-1}	**2.9±1**[*]	121±6	119±10	**18±7**[*]	**14±5**
$AgNO_3$ 10 µg Ag L^{-1}	**6.5±1**[*]	**109±9**[*]	**100±13**[*]	**30±11**[*]	**23±7**[*]
AgNP 2 µg Ag L^{-1}	0.03±0.02	132±6	129±6	5±2	3±1
AgNP 5 µg Ag L^{-1}	0.05±0.02	133±6	128±6	7±3	5±2
AgNP 10 µg Ag L^{-1}	0.08±0.04	132±2	126±7	6±4	4±2

In bold Significant different from the control group.
[*] Significant different from all other exposure groups.
[A] Blood plasma glucose measured by iSTAT (Abbott)
[B] Blood plasma glucose measured by glucometer (FreeStyle Lite, Abbott)
[C] Reference values in brown trout: 144±1 and 132±8 mmol L^{-1} for Na and 131±1 and 119±3 mmol L^{-1} for Cl (Heier et al., 2009, Rosseland et al., 2007)



300

301 Figure 2. Total Ag concentration in gills, liver and kidney in juvenile *Salmo trutta* after 96 h of exposure
302 to $AgNO_3$ and AgNPs (NM300K). For gill and liver the total Ag concentration is given as µg Ag g^{-1} dry
303 weight (dw) and for kidney µg Ag g^{-1} wet weight (ww). The insert figure shows the total Ag concentration
304 in gill after exposure to AgNPs, in more detail with an appropriate scale on the Y axis.

3.3.3 Gene expression

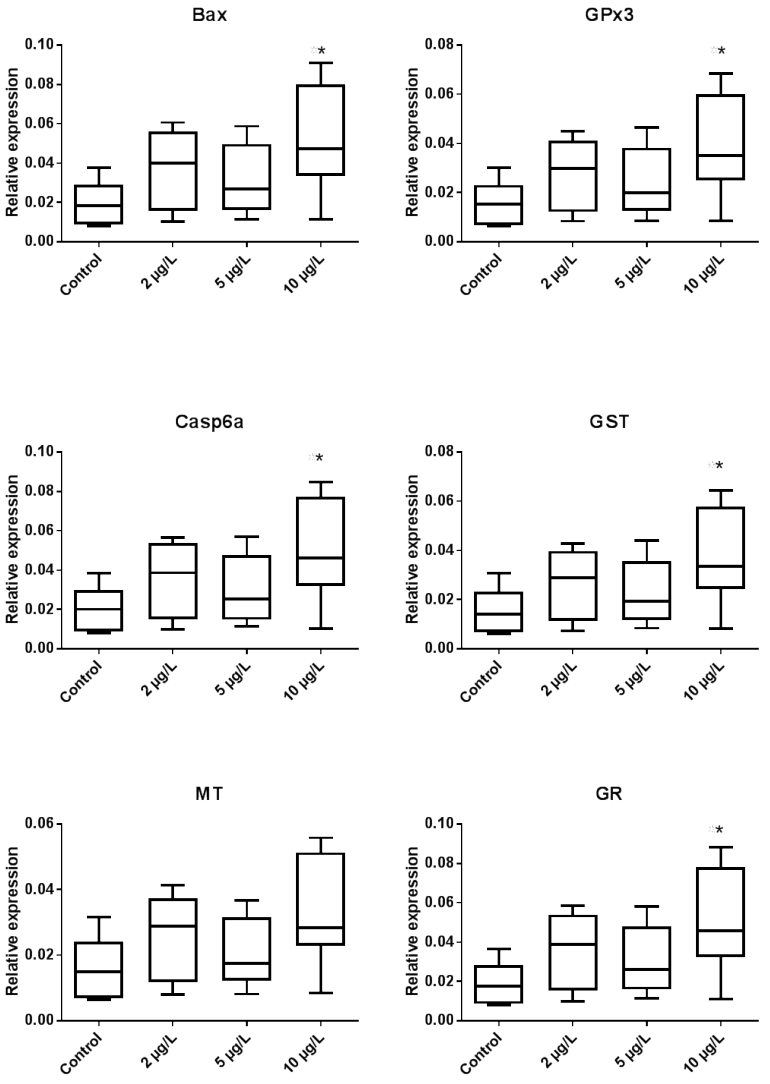
Liver was chosen for the qPCR analysis due to central role in a number of physiological responses. Genes associated with oxidative stress (Glutathione reductase, GR; Glutathione peroxidase x3, GPx3; Glutathione S transferase, GST) and apoptosis (Caspase 6A, *Casp6a*; Bcl2 associated x protein, *Bax*), were all up-regulated in a concentration dependent manner, with the exception of the intermediate concentration (5 µg L^{-1}) (Figure 3). All genes associated with oxidative stress and apoptosis showed an apparent concentration-dependent response, where the highest concentration (10 µg Ag L^{-1}) was also identified to be significantly up-regulated compared to control. The transcriptional expression of Metallothionein (MT) was not significantly up-regulated in any of the exposure concentrations, but had similar concentration-response pattern as the genes associated with oxidative stress and apoptosis (Figure 3). There was no significant accumulation of Ag in liver and kidney of fish exposed to AgNPs (Figure 2), nor any effects on blood plasma glucose levels or other blood plasma parameters (Table 4). Thus, qPCR was only performed on liver samples from $AgNO_3$ exposed fish, as well as the control group.

3.3.4 Principle component analysis (PCA)

A PCA including general toxicity (blood glucose), water chemistry and accumulation of $AgNO_3$ and AgNP (NM300K) is presented in Figure 4, representing 92.4 % (PCA1:72.2 %; PCA2:20.2 %) of the total variance. The first component described most of the data variance, clearly separating high and intermediate concentrations of $AgNO_3$ and the high dose of AgNP from the low dose and control treatment. The high and intermediate doses of $AgNO_3$ were associated in a dose dependent manner with general toxicity, Ag bioconcentration and Ag-fraction. The second component clearly separated general toxicity, bioconcentration and LMM Ag from the colloidal, particulate and total Ag. The overall PCA with a subsequent correlation analysis demonstrated LMM Ag to be the major contributor to the observed increase in blood glucose (r^2= 0.834, $p<0.0001$) and bioconcentration of Ag in gills (r^2= 0.929, $p<0.0001$), liver (r^2= 0.844, $p<0.0001$) and kidney (r^2= 0.723, $p<0.02$) (Table S6). The AgNP exposure was

correlated with the different Ag-fraction's (colloidal, particulate and total Ag) in a concentration-dependent manner, the colloidal/NP Ag were not correlated with the increase in blood glucose or bioconcentration of AgNP, except in gills (r^2: 0.657, $p<0.004$) (Table S7). The PCA demonstrates no correlation between AgNP- and Ag-tissue concertation, nor the toxicity parameter blood glucose.

A second PCA was conducted on specific- (gene expression) and general (blood glucose) toxicity, water chemistry and accumulation of $AgNO_3$ (Figure 5), representing 93.3 % (PCA1:69.4 %; PCA2:23.9 %) of the total variance. The first component clearly separated all the measured parameters from the control and low dose exposure with exception of one sample (Ag 2-3), thus demonstrating that the measured variables to be concentration-dependent. The second component separated the general toxicity, water chemistry and bioconcentration from the gene expression, however the correlation between LMM and the overall gene expression, was weak (r^2: 0.452-0.466, $p<0.034$-0.044) but significant, with exception of MT. The overall PCA demonstrated a concentration-dependent effect of $AgNO_3$ on both specific- and general toxicity, but the specific toxicity could not fully depict which fraction of the $AgNO_3$ that caused the toxicity, due data variability.



344 Figure 3. Hepatic gene expression of *Salmo trutta* after 96 h of waterborne exposure to $AgNO_3$. The data
345 (mean ± s.d.) depicts quantitative real-time PCR (qPCR) of n=6. *Denotes genes being significantly
346 different ($p < 0.05$) from the control. Abbreviation: GR - Glutathione reductase; GPx3 - Glutathione
347 peroxidase x3; GST - Glutathione S transferase; *Casp6a* - Caspase 6A; *Bax* - Bcl2 associated x protein; MT
348 - Metallothionein

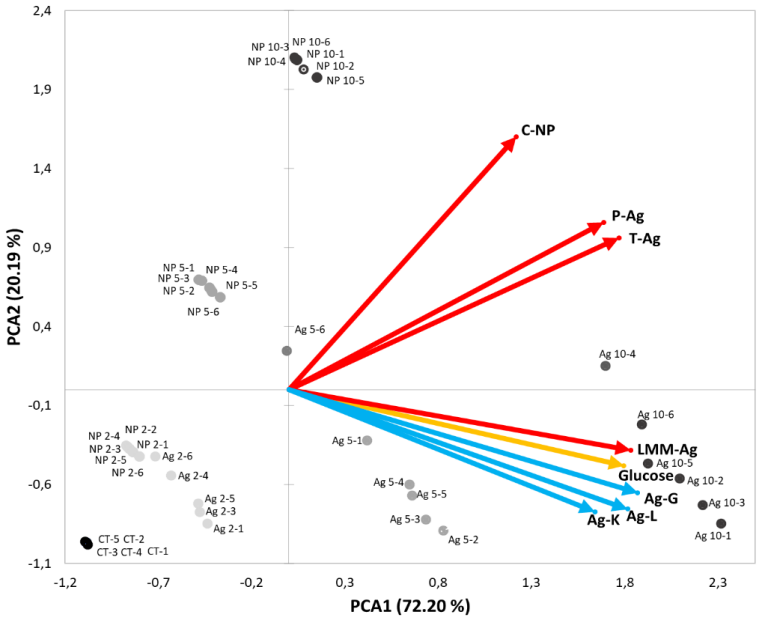


Figure 4. Principal component analysis (PCA) of measured blood glucose, Total (T-Ag), particulate (P-Ag), colloidal/nanoparticles (C-NP) and low molecular mass ions (LMM) Ag and accumulation of Ag in gill (Ag-G), liver (Ag-L) and kidney (Ag-K) in brown trout (*Salmo trutta L.*) exposed to 2, 5 and 10 µg L^{-1} NM300K (NP2, NP5 and NP10) and $AgNO_3$ (Ag2, Ag5 and Ag10), respectively in addition to a control (CT) treatment. The data constitutes of 5-6 independent replicates of fish.

365

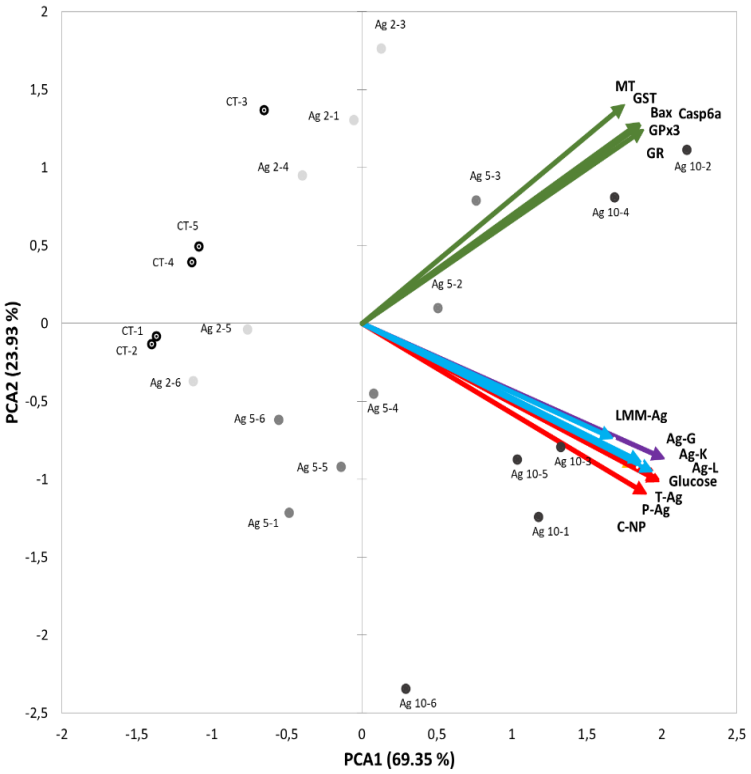


Figure 5. Principal component analysis (PCA) of measured blood glucose, water chemistry parameters (Total (T-Ag), particulate (P-Ag), colloidal (C-NP) and low molecular mass ions (LMM) Ag), tissue accumulation of Ag in gill (Ag-G), liver (Ag-L) and kidney (Ag-K), as well as the genetic markers GR - Glutathione reductase; GPx3 -Glutathione peroxidase x3; GST-Glutathione S transferase; *Casp6a* - Caspase 6A; *Bax* -Bcl2 associated x protein; MT- Metallothionein, in Brown trout exposed to 2-10 µg L^{-1} NM300K and $AgNO_3$, respectively.

DISCUSSION

In the current study, bioconcentration of $AgNO_3$ and AgNPs (NM300K) were demonstrated to be different for gill, liver and kidney and subsequent toxic effects of exposed *brown trout*. With a thorough exposure characterization, observed differences in Ag exposures ($AgNO_3$ and AgNPs) were linked to differences in bioconcentration, and consequently toxicity.

4.1 Exposure

While the initial particle size (16.9±0.1 nm) of the AgNPs obtained with TEM agreed well with the manufactures specifications (mean 15 nm, 90 % of the NPs < 20 nm), the hydrodynamic diameter obtained by DLS measurements gave a higher initial particle size (86±1 nm). However, the number mean particle size (obtained by a calculation from the intensity-based measurements of size by DLS) were in agreement with the TEM results (Table 2). Adding the AgNPs to the exposure water caused aggregation and/or formation of complexes with other chemical substances present in the media (Table 2). The hydrodynamic diameter particle size (Z-average) of the AgNPs in exposure media, increased with decreasing exposure concentrations. Similarly, large particulate matter was present in the $AgNO_3$ exposures, indicating a quick formation of larger Ag complexes when added to the exposure water (this will be further discussed in the next paragraph). It is however important to keep in mind that the nanosized particles present in the different exposures (AgNP and $AgNO_3$) might be similar in size, but most likely very different in surface chemistry. The NM300K AgNPs are coated with stabilizing agents that are not present in the $AgNO_3$ exposure. This difference is likely to influence the particles bioavailability and consequently the toxicity. The exposure concentrations in the current study were low in the context of DLS measurements, which together with aggregation and subsequent sedimentation increased the uncertainties of the particle size reported for the exposure suspensions. The DLS measurements of exposure suspension would be influenced by all particulate matter present in the

389 water, which could include food, faeces, mucus etc. This can possibly be the explanation to the much
390 larger particle size obtained by DLS in the 10 µg Ag L^{-1} $AgNO_3$ exposure compared to the rest of the
391 groups given a possible larger mucus secretion of these fish due to Ag on gill tissue.

392 There was an average reduction of 11 ± 4 and 20 ± 2 % in total Ag concentration from 0 h to 24 h in
393 $AgNO_3$ and AgNP treatments, respectively (Table S3). For the two highest exposure concentrations of
394 $AgNO_3$, 5 and 10 µg Ag L^{-1}, 10 and 30 % of the total Ag concentration was present as LMM Ag at the start
395 of the exposure (0 h), however after 24 h there were no longer any detectable level of LMM Ag present
396 in any of the groups. In the three AgNP groups, there were an approximately twofold decrees (2, 1.5 and
397 1.6 fold for 2, 5 and 10 µg Ag L^{-1}, respectively) in the colloidal/NP fraction over 24 h time period. The
398 LMM fraction was negligible (< 0.0036 µg L^{-1}) in the AgNP exposures. The general trend for both type of
399 exposures, was a shift towards larger particle sizes with an increase in both colloidal/NP and particulate
400 matter over time. In similarity to our results, previous studies have also reported a reduction in
401 concentrations of AgNPs (10-35 nm) between 35-55 % of the nominal exposure concentration (Scown et
402 al., 2010, Farmen et al., 2012, Bacchetta et al., 2017), with an additional 15 % of substrate depletion
403 after 24 h of exposure (Bacchetta et al., 2017). Previous studies suggest aggregation and sedimentation
404 of Ag NPs, resulting in less bioavailable Ag in the water column for the fish to take up and accumulate
405 (Bacchetta et al., 2017).

406 The speciation of a metal is very important for bioavailability, uptake, and toxicity to organisms (Allen
407 and Hansen, 1996, Paquin et al., 2002), this is also true for Ag. Thus, size fractionation with respect to Ag
408 was performed in the current study. The results confirm the main size fraction in both $AgNO_3$ and AgNP
409 exposures at the beginning of the exposure and after 24 h to be the colloidal/NP size fraction (>1 nm,
410 < 220 nm). Even though the dominant size fraction in the different exposures were similar, they might
411 be chemically different. The dissolved Ag(I) in the $AgNO_3$ exposure would most likely react and form
412 complexes to other chemical components in the exposure water (e.g. Cl) more rapidly and in a different

matter than the surface coated AgNPs. In addition to the likely chemical differences in the colloidal/NP fractions, the presence of LMM Ag in the $AgNO_3$ exposures clearly differentiate the $AgNO_3$ and AgNP exposures (Figure 1). Although the LMM Ag are unstable and not detectable after 24 h, the semi-static nature of the current experiment reintroduce LMM Ag every 24 h thus exposing the fish in the $AgNO_3$ groups to much higher concentrations of LMM Ag compared to the fish in the Ag NP exposures. The results reported in the current study are in agreement with Köser et al. (2017) where the initial release of Ag^+ from the AgNPs (NM300K) was less than 8 % over all of the tested exposure media in the study, and no further release of Ag^+ was detected throughout the exposure period. In the current study, as a consequence of the semi-static setup, the renewal of the exposure every 24 h further minimize the effect of time on the surface coating and the potential for oxidation of the AgNP surface. The main difference in exposure between the $AgNO_3$ and Ag NP exposures were the presence of LMM Ag in the $AgNO_3$ exposures.

4.2. Silver accumulation in the internal organs

A concentration-dependent accumulation of Ag in gill, liver and kidney of fish exposed to $AgNO_3$ were demonstrated in the present study (Figure 2, Table S4). Although the gill is the primary site for interaction in waterborne exposures, the accumulated Ag in liver was fivefold higher than in gills (0.8±0.4 and 2.9±0.9 μg g^{-1} dry weight gill versus 13±5 and 20±3 μg g^{-1} dry weight liver, in the 5 and 10 μg Ag L^{-1} $AgNO_3$ exposures, respectively). There was however, no systemic (i.e., liver and kidney) uptake of Ag in fish exposed to AgNPs as the concentrations of Ag in exposed fish was similar to control fish. Exposure to AgNPs caused only a slight increase in concentration of Ag in gills of exposed fish (0.03±0.02, 0.05±0.02, and 0.08±0.04 in 2, 5 and 10 μg Ag L^{-1}, respectively). The results demonstrated that the uptake in gill was between 30 and 80 times higher in $AgNO_3$ exposed fish compared to AgNP exposed fish (Figure 2). Thus, a large difference in bioavailability of Ag from $AgNO_3$ and AgNPs was observed, larger than previously reported results for other AgNPs. Martin et al. (2017) reported less

than 10-fold higher Ag concentration in liver of yellow perch (*Perca flavenscens*) exposed to $AgNO_3$ compared to AgNPs.

Low molecular mass Ag species associated with the $AgNO_3$ treatment was identified by PCA analysis to be the major contributor to the bioconcentration of Ag in gill, liver and kidney (Figure 4). Thus, as expected, the dose-dependent bioconcentration of Ag in tissue samples from $AgNO_3$ exposure groups could be linked to the increase in concentrations of LMM Ag with increasing exposure concentrations. It also agrees well with the low uptake of Ag in fish exposed to AgNPs where LMM Ag was not detectable. The importance of LMM Ag in Ag uptake in waterborne exposures of fish agrees with previous studies (Webb and Wood, 1998, Wood et al., 1999, Morgan et al., 1997).

In nanotoxicology the question of whether the bioconcentration is driven by the release of Ag ions from the NP surface, or by the AgNPs themselves is essential. This most likely depends on the type of AgNP tested with respect to its physical and chemical characteristics, the exposure media used and other experimental conditions, and in many cases it would be a combination of the two, as reported by for example Scown et al. (2010) and Connolly et al. (2015). Both *in vivo* and *in vitro* studies have reported uptake and toxicity in a range of different organisms after exposure to NM300K (Sørensen and Baun, 2015, Connolly et al., 2015, Bermejo-Nogales et al., 2016, Lodeiro et al., 2017). Contrary to these previous studies where toxicity (of which bioavailability is a prerequisite) of NM300K AgNPs were reported, the findings of the current study found these AgNPs not to be bioavailable. Low levels of Ag in gills of AgNP exposed fish were detected, however further systemic transfer of Ag to liver and kidney were not observed (Figure 2). The lack of AgNPs bioconcentrated in the kidney is however not surprising as the primary particle size (16.9±0.1 nm) exceeds the size (> 60 kDa ~ approx. 2 nm) possible to pass through the glomerular filters in vertebrate kidneys (Handy et al., 2008b). The Ag associated with gills of AgNP exposed fish was probably a result of AgNPs adsorption to the gill epithelia structures (e.g. mucus layer) rather than actual tissue accumulation. The low bioavailability could be partly a result of the

surface coating making the particles less reactive to the gill surface, or it could be the initiation of AgNP aggregation after addition to the exposure media thus rendering particle sizes too big for uptake in/transport through the gill with further transfer to liver.

4.3. Toxicity

The current study focus on determining response of biomarkers to similar toxic responses of both $AgNO_3$ and AgNP exposures, and to identify differences in the toxic response between the different exposures. The $AgNO_3$ exposure resulted in bioconcentration of Ag in gill, liver and kidney (Figure 2). Results demonstrated also responses in fish causing increased plasma glucose levels (Figure 3), reduction in plasma Na^+ and plasma Cl^- (Table 4), and significant upregulation of genes involved in oxidative stress response and apoptosis (Figure 3). However, no systemic bioconcentration or effects were detected in the AgNP exposed fish.

Uptake and toxicity of waterborne Ag to freshwater fish have been shown to be linked to the presence of free Ag ions in the water, with inhibition of the Na^+K^+ ATPase as the underlying mechanism leading to disruption of osmoregulation (Hogstrand and Wood, 1998, Morgan et al., 2004a, Bury and Wood, 1999). With such an uptake, one would expect Ag accumulation not only in gills, but also in the systemic organs (e.g., liver and kidney), assuming absorption and transfer to the circulatory system. Liver has previously been reported to be the main target organ for Ag (Handy et al., 2011), thus expected to accumulate the highest concentrations of Ag. The observed accumulation of Ag in gill and liver of $AgNO_3$ exposed fish herein fits well with these expectations. So do the elevated blood levels of glucose, and reduction in plasma chloride and plasma sodium observed in the current study and also seen by for example Farmen et al. (2012) after exposure to two different types of AgNPs as well as $AgNO_3$. Although control values of blood plasma Na^+ and Cl^- are missing in the current study, previously reported reference values in brown trout are 144±1 and 132±8 mmol L^{-1} for Na and 131±1 mmol L^{-1} for Cl) (Heier et al., 2009, Rosseland et

al., 2007). For the closely related Atlantic salmon (*Salmo salar* L.) mean Na and Cl levels of unexposed fish have been reported to be in the range of 130-147 mmol L^{-1} and 124-139 mmol L^{-1}, respectively (Farmen et al., 2012, Olsvik et al., 2010). More importantly, the covariation of Na and Cl levels is a strong indication of osmoregulatory problems in fish exposed to the two highest exposure of $AgNO_3$. The exposure to 2 µg Ag L^{-1} does not seem to cause any ion regulation problems in fish. The reduction in plasma ions (Na and Cl) observed in the two highest exposure concentrations of $AgNO_3$, and the lack of so in the rest of the exposure groups, correlates well with the significant elevation in blood plasma glucose levels seen in the same two $AgNO_3$ exposure groups (Figure 3). Altogether, this strongly indicates osmoregulatory problems at 5 and 10 µg Ag L^{-1} ($AgNO_3$) causing 2.9±0.9 and 6.5±1 µg Ag/g gill, respectively.

The significantly reduction in plasma ions and the high level of glucose indicate sever osmoregulatory problems in the current study. Results indicate good correlation between concentration of Ag in tissue and responses (R^2= 0.698-0.908, $p<0.0001$), indicating that the uptake induce the response. The low Ag concentrations in the gills of AgNP exposed fish did not affect the blood glucose or plasma ions, thus assumed to be associated with gill structures like, for example, mucus, rather than being absorbed/or transported over the gill tissue. This is further supported by the lack of further systemic accumulation of Ag in liver and kidney. The differences in blood glucose response between the $AgNO_3$ and AgNP exposures, as well as the PCA (Figure 4), supports that accumulation of Ag in gills are highly linked to the presence of LMM Ag in waterborne exposure as previously suggested (Luoma, 2008) *in vivo* (Lubick, 2008) and *in vitro* (Connolly et al., 2015). The lack of toxicity observed for the NM300K AgNPs can be explained by the low bioavailability of these AgNPs under the experimental conditions in the current study. Due to the lack of bioconcentration and general toxicity of the AgNPs, gene expression was not conducted on liver tissue of AgNP exposed fish in.

507 The genes selected in the current study were transcriptional biomarkers for three types of early hepatic
508 toxicity responses (Apoptosis, oxidative stress and metal exposure) to contaminants (Ribeiro et al.,
509 2015), previously reported in $AgNO_3$ and AgNP exposed freshwater fish (Scown et al., 2010, Farmen et
510 al., 2012, Bacchetta et al., 2017).

511 **Oxidative stress** is a well-characterized response to several types of metal exposures, including Ag
512 (Bacchetta et al., 2017). The oxidative stress transcriptional biomarkers glutathione reductase (GR),
513 glutathione peroxidase x3 (GPx3) and glutathione S transferase (GST), were all significantly upregulated
514 in fish exposed to the highest $AgNO_3$ concentration (10 µg Ag L^{-1}), indicating dose dependence despite
515 high variation within each treatment group. Transcriptional regulation of the investigated oxidative
516 stress genes where, however, low compared to similar exposure concentrations of $AgNO_3$ in fish (Gagne
517 et al., 2012). Thus indicating the initial transcription occurring mainly during the initial part of exposure
518 as it seem not to be differentially expressed after 96 h of exposure. This is in agreement with previously
519 reported results suggesting optimal transcriptional regulation after 24 to 48 h of exposure to $AgNO_3$ in
520 Japanese medaka (*Oryzias latipes*), being somewhat repressed after 96 h (Chae et al., 2009). Gagne et al.
521 (2012) did however find hepatic transcriptional biomarkers involved in oxidative stress to be significantly
522 affected in rainbow trout after 96 h exposure to $AgNO_3$ (6 µg L^{-1}). However, investigation of downstream
523 molecular events such as enzymatic hepatic activity of e.g. GST is more suitable during a prolonged
524 exposure (>96h) as the enzyme is more robust as the transcriptional regulation is probably
525 compensatory rather than initial.

526 **Apoptosis** is programed cell death initiated by cell dysfunction to remove damage cells, thus ensuring
527 continued normal cellular functioning (Newman, 2009). Both the gene Caspase 6a (*Casp6a*) in the
528 enzyme family for the initiation and execution of the apoptosis, and gene Bcl2 associated x protein
529 (*bax*), involved in the initiation of apoptosis by disrupting the mitochondrial membrane, were
530 significantly up-regulated in fish exposed to the highest concentration (10 µg Ag L^{-1}) of $AgNO_3$ (Figure 4).

Activation and translocation of BAX protein into the mitochondria has previously reported both due to directly (DNA damage) or indirectly (oxidative stress) association with apoptosis (D'Alessio et al., 2005, Song et al., 2012). The results of current study are however indicative of the Ag ions interference with the mitochondrial membrane potential and possible initiation of mitochondrial apoptosis signaling in the liver as *casp6a,* a key protein in the final execution of apoptosis (Fan et al., 2005), also was induced in a similar manner as *bax*. The general toxicity (blood glucose and plasma ion loss) and transcriptional regulation in present study indicates that the highest exposure of $AgNO_3$ may affect and possibly activate the mitochondrial apoptotic signaling in the exposed fish.

Metallothionein (MT) induction is an established indicator of metal induced stress, and has previously been reported for fish gill and liver tissue after exposure to dissolved Ag as well as nano-Ag (Hogstrand et al., 1996, Farmen et al., 2012, Bruneau et al., 2016, Martin et al., 2017). In the current study there was not a significantly different transcriptional regulation of MT in any of the exposure doses after 96 h of exposure to $AgNO_3$ (Figure 4). In similarity to present study, Chae and colleagues (2009) exposed fish to 1 µg L^{-1} $AgNO_3$ and found transcriptionally expressed MT to be at baseline level after 96 h of exposure after initial increase at 24 and 48 h. Similar results were also reported by Ribeiro et al. (2015), where MT protein levels in the soil worm *Enchytraeus Crypticus* peaked (although not significantly different from control) after 3 days of exposure to $AgNO_3$ before decreasing back to control levels, demonstrating its temporal variance. These studies support that the initial activation of hepatic MT in current study may have occurred early in the exposure phase, thus potentially resulting in transcriptional regulation to return to baseline by the time of sampling at 96 h.

Silver could interact with biological targets causing/triggering different mechanisms. However, adverse effects was only studied for osmoregulatory problems and observed at 3 µg Ag/g dw gill or higher. Although osmoregulation failure are assumed to be one of the most sensitive toxicity due to Ag exposure, future work need to identify bioconcentration levels causing adverse effects due to oxidative

555 stress such as lipid peroxidation and DNA damages or apoptosis if more sensitive during chronic
556 exposures.

557 The current study demonstrates differences in exposure, bioavailability, total Ag accumulation, and
558 toxicity of Ag after 96 h of waterborne exposure to 2, 5 and 10 µg Ag L^{-1} of Ag-ions (added as $AgNO_3$) and
559 NM300K AgNPs. The exposure characterization revealed the main difference between the $AgNO_3$ and
560 NM300K Ag NP exposures to be the presence of LMM Ag (< 10 kDa), which was around 30 % of the total
561 Ag concentration in the highest exposures of $AgNO_3$ and negligible in the AgNP exposure.
562 Bioconcentration of Ag after 96 h of exposure to 2, 5 and 10 µg Ag L^{-1}. As a result of the differences in
563 exposure, Ag was only bioconcentrated in gill, liver and kidney of fish exposed to $AgNO_3$. Although low
564 levels of Ag was also associated with gills after exposure to AgNPs, no further internal distribution of Ag
565 indicates the NPs to be adsorbed to the gill structures, rather than absorbed, thus not bioavailable. Fish
566 exposed to $AgNO_3$ displayed a dose-dependent increase of Ag concentration in gill, liver and kidney,
567 causing general toxicity (increased blood plasma glucose, reduced Na and Cl levels) and inducing genes
568 related to oxidative stress and apoptosis in a dose-dependent manner. The second PCA could not
569 describe how the $AgNO_3$ was mediating its specific toxicity (gene expression of the selected biomarkers),
570 as the replicates analyzed reported large variations between the treatment replicate. However
571 considering the strong correlation between the LMM Ag present in the $AgNO_3$ exposures, and the
572 accumulation of Ag in organs as well as the general toxicity parameter, a natural assumption would be
573 that the observed specific toxicity and the mechanisms is also mediated by the LMM Ag. The PCA also
574 confirmed further that the NM300K AgNPs did not accumulate in internal tissue (e.g. liver) or induce
575 toxicity.

576 Although the NM300K AgNPs was not bioavailable under the current experimental conditions (e.g. semi-
577 static set up limiting the effect of time on transformation processes working on the NPs), transformation
578 processes occurring in the environment could result in higher aggregation and sedimentation. Hence

changing the exposure route from waterborne to dietary via sediments and benthic invertebrates. Although they were not bioavailable through gill interactions, they might be through dietary uptake in the intestine.

4 CONCLUSION

The current study demonstrates differences in exposure, total Ag accumulation, and toxicity of Ag after 96 h of waterborne exposure to 2, 5 and 10 µg Ag L^{-1} of Ag-ions (added as $AgNO_3$) and NM300K AgNPs. We concluded that the differences in accumulation of Ag in fish tissue after exposure to $AgNO_3$ and NM300K was mainly related to concentration of low molecular mass Ag (< 3 kDa) in the exposures. The presence of low molecular mass Ag is therefore proposed to contribute to the reported dose-dependent bioconcentration and toxicity of $AgNO_3$ in present study.

5 AKNOWLEDGEMENT

The Research Council of Norway through the NANoREG (grant number 310584) and NANOCHARM (grant number 221391) projects funded this work. The authors would like to thank L.M. Rossbach and E. Maremonti for assisting with the experiment, and all colleagues assisting us with sampling at the end of the experiment.

6 DATA ACCESSIBILITY

Research data pertaining to this article is accessible upon request to the authors.

7 REFERENCES

ALLEN, H. E. & HANSEN, D. J. 1996. The importance of trace metal speciation to water quality criteria. *Water Environment Research,* 68**,** 42-54.

BACCHETTA, C., ALE, A., SIMONIELLO, M. F., GERVASIO, S., DAVICO, C., ROSSI, A. S., DESIMONE, M. F., POLETTA, G., LOPEZ, G., MONSERRAT, J. M. & CAZENAVE, J. 2017. Genotoxicity and oxidative stress in fish after a short-term exposure to silver nanoparticles. *Ecological Indicators,* 76**,** 230-239.

BERMEJO-NOGALES, A., FERNÁNDEZ, M., FERNÁNDEZ-CRUZ, M. & NAVAS, J. 2016. Effects of a silver nanomaterial on cellular organelles and time course of oxidative stress in a fish cell line (PLHC-1). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 190**,** 54-65.

BICHO, R. C., RIBEIRO, T., RODRIGUES, N. P., SCOTT-FORDSMAND, J. J. & AMORIM, M. J. B. 2016. Effects of Ag nanomaterials (NM300K) and Ag salt (AgNO3) can be discriminated in a full life cycle long term test with Enchytraeus crypticus. *Journal of Hazardous Materials,* 318**,** 608-614.

BRUNEAU, A., TURCOTTE, P., PILOTE, M., GAGNE, F. & GAGNON, C. 2016. Fate of silver nanoparticles in wastewater and immunotoxic effects on rainbow trout. *Aquatic Toxicology (Amsterdam, Netherlands),* 174**,** 70-81.

BURY, N. R. & WOOD, C. M. 1999. Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na+ channel. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology,* 277**,** R1385-R1391.

CARLSON, C., HUSSAIN, S. M., SCHRAND, A. M., BRAYDICH-STOLLE, L. K., HESS, K. L., JONES, R. L. & SCHLAGER, J. J. 2008. Unique Cellular Interaction of Silver Nanoparticles: Size-Dependent Generation of Reactive Oxygen Species. *Journal of Physical Chemistry B,* 112**,** 13608-13619.

CHAE, Y. & AN, Y.-J. 2016. Toxicity and transfer of polyvinylpyrrolidone-coated silver nanowires in an aquatic food chain consisting of algae, water fleas, and zebrafish. *Aquatic Toxicology,* 173**,** 94-104.

CHAE, Y. J., PHAM, C. H., LEE, J., BAE, E., YI, J. & GU, M. B. 2009. Evaluation of the toxic impact of silver nanoparticles on Japanese medaka (Oryzias latipes). *Aquatic Toxicology,* 94**,** 320-327.

CONNOLLY, M., FERNANDEZ-CRUZ, M.-L., QUESADA-GARCIA, A., ALTE, L., SEGNER, H. & NAVAS, J. M. 2015. Comparative cytotoxicity study of silver nanoparticles (AgNPs) in a variety of rainbow trout cell lines (RTL-W1, RTH-149, RTG-2) and primary hepatocytes. *International journal of environmental research and public health,* 12**,** 5386-5405.

D'ALESSIO, M., DE NICOLA, M., COPPOLA, S., GUALANDI, G., PUGLIESE, L., CERELLA, C., CRISTOFANON, S., CIVITAREALE, P., CIRIOLO, M. R., BERGAMASCHI, A., MAGRINI, A. & GHIBELLI, L. 2005. Oxidative Bax dimerization promotes its translocation to mitochondria independently of apoptosis. *Faseb Journal,* 19**,** 1504-+.

FAN, T. J., HAN, L. H., CONG, R. S. & LIANG, J. 2005. Caspase family proteases and apoptosis. *Acta Biochimica Et Biophysica Sinica,* 37**,** 719-727.

FARMEN, E., MIKKELSEN, H. N., EVENSEN, O., EINSET, J., HEIER, L. S., ROSSELAND, B. O., SALBU, B., TOLLEFSEN, K. E. & OUGHTON, D. H. 2012. Acute and sub-lethal effects in juvenile Atlantic

salmon exposed to low mu g/L concentrations of Ag nanoparticles. *Aquatic Toxicology,* 108**,** 78-84.

GAGNE, F., ANDRE, C., SKIRROW, R., GELINAS, M., AUCLAIR, J., VAN AGGELEN, G., TURCOTTE, P. & GAGNON, C. 2012. Toxicity of silver nanoparticles to rainbow trout: A toxicogenomic approach. *Chemosphere,* 89**,** 615-622.

GALVEZ, F., HOGSTRAND, C. & WOOD, C. M. 1998. Physiological responses of juvenile rainbow trout to chronic low level exposures of waterborne silver. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology,* 119**,** 131-137.

HANDY, R. D., HENRY, T. B., SCOWN, T. M., JOHNSTON, B. D. & TYLER, C. R. 2008b. Manufactured nanoparticles: their uptake and effects on fish-a mechanistic analysis. *Ecotoxicology,* 17**,** 396-409.

HANDY, R. D., VON DER KAMMER, F., LEAD, J. R., HASSELLOV, M., OWEN, R. & CRANE, M. 2008a. The ecotoxicology and chemistry of manufactured nanoparticles. *Ecotoxicology,* 17**,** 287-314.

HANDY, R. D., AL-BAIRUTY, G., AL-JUBORY, A., RAMSDEN, C. S., BOYLE, D., SHAW, B. J. & HENRY, T. B. 2011. Effects of manufactured nanomaterials on fishes: a target organ and body systems physiology approach. *Journal of Fish Biology,* 79**,** 821-853.

HEIER, L. S., LIEN, I. B., STRØMSENG, A. E., LJØNES, M., ROSSELAND, B. O., TOLLEFSEN, K.-E. & SALBU, B. 2009. Speciation of lead, copper, zinc and antimony in water draining a shooting range—time dependant metal accumulation and biomarker responses in brown trout (Salmo trutta L.). *Science of the Total Environment,* 407**,** 4047-4055.

HOGSTRAND, C., GALVEZ, F. & WOOD, C. M. 1996. Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. *Environmental Toxicology and Chemistry,* 15**,** 1102-1108.

HOGSTRAND, C., GROSELL, M., WOOD, C. M. & HANSEN, H. 2003. Internal redistribution of radiolabelled silver among tissues of rainbow trout (Oncorhynchus mykiss) and European eel (Anguilla anguilla): the influence of silver speciation. *Aquatic Toxicology,* 63**,** 139-157.

HOGSTRAND, C. & WOOD, C. M. 1998. Toward a better understanding of the bioavailability, physiology and toxicity of silver in fish: Implications for water quality criteria. *Environmental Toxicology and Chemistry,* 17**,** 547-561.

HUND-RINKE, K., SCHLICH, K., KÜHNEL, D., HELLACK, B., KAMINSKI, H. & NICKEL, C. 2018. Grouping concept for metal and metal oxide nanomaterials with regard to their ecotoxicological effects on algae, daphnids and fish embryos. *NanoImpact,* 9**,** 52-60.

JENSEN, K. A., BOOTH, A., KEMBOUCHE, Y. & BORASCHI, D. 2016. Validated protocols for test item preparation for key in vitro and ecotoxicity studies, NANoREG Deliverable D2.06.

KLEIN, C. L., COMERO, S., STAHLMECKE, B., ROMAZANOV, J., KUHLBUSCH, T. A. J., VAN DOREN, E., DE TEMMERMAN, P.-J., MAST, J., WICK, P., KRUG, H., LOCORO, G., HUND-RINKE, K., KÖRDEL, W., FRIEDRICHS, S., MAIER, G., WERNER, J., LINSINGER, T. & GAWLIK, B. M. 2011. NM-Series of Representative Manufactured Nanomaterials NM-300 Silver Characterisation, Stability, Homogeneity. *JRC Scientific and Technical Reports*.

KÖSER, J., ENGELKE, M., HOPPE, M., NOGOWSKI, A., FILSER, J. & THOMING, J. 2017. Predictability of silver nanoparticle speciation and toxicity in ecotoxicological media. *Environmental Science-Nano,* 4**,** 1470-1483.

LODEIRO, P., BROWNING, T. J., ACHTERBERG, E. P., GUILLOU, A. & EL-SHAHAWI, M. S. 2017. Mechanisms of silver nanoparticle toxicity to the coastal marine diatom Chaetoceros curvisetus. *Scientific Reports,* 7**,** 10.

LUBICK, N. 2008. Nanosilver toxicity: ions, nanoparticles□or both? : ACS Publications.

LUOMA, S. N. 2008. Silver nanotechnologies and the environment. *The Project on Emerging Nanotechnologies Report,* 15.

MARTIN, J. D., COLSON, T. L. L., LANGLOIS, V. S. & METCALFE, C. D. 2017. BIOMARKERS OF EXPOSURE TO NANOSILVER AND SILVER ACCUMULATION IN YELLOW PERCH (PERCA FLAVESCENS). *Environmental Toxicology and Chemistry,* 36**,** 1211-1220.

MAST, J. & DE TEMMEMAN, P.-J. 2016. Protocol(s) for size-distribution analysis of primary NM particles in air, powders, and liquids, NANoREG Deliverable D2.10.

MORGAN, I. J., HENRY, R. P. & WOOD, C. M. 1997. The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (Oncorhynchus mykiss) is inhibition of gill Na+ and Cl- transport. *Aquatic Toxicology,* 38**,** 145-163.

MORGAN, T. P., GROSELL, M., GILMOUR, K. M., PLAYLE, R. C. & WOOD, C. M. 2004b. Time course analysis of the mechanism by which silver inhibits active Na+ and Cl- uptake in gills of rainbow trout. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology,* 287**,** R234-R242.

MORGAN, T. P., GROSELL, M., PLAYLE, R. C. & WOOD, C. M. 2004a. The time course of silver accumulation in rainbow trout during static exposure to silver nitrate: physiological regulation or an artifact of the exposure conditions? *Aquatic Toxicology,* 66**,** 55-72.

NEWMAN, M. C. 2009. *Fundamentals of ecotoxicology*, CRC press.

NOTTER, D. A., MITRANO, D. M. & NOWACK, B. 2014. ARE NANOSIZED OR DISSOLVED METALS MORE TOXIC IN THE ENVIRONMENT? A META-ANALYSIS. *Environmental Toxicology and Chemistry,* 33**,** 2733-2739.

OLSVIK, P. A., HEIER, L. S., ROSSELAND, B. O., TEIEN, H. C. & SALBU, B. 2010. Effects of combined gamma-irradiation and metal (Al plus Cd) exposures in Atlantic salmon (Salmo salar L.). *Journal of Environmental Radioactivity,* 101**,** 230-236.

OLSVIK, P. A., LIE, K. K., SAELE, O. & SANDEN, M. 2007. Spatial transcription of CYP1A in fish liver. *BMC physiology,* 7**,** 12-12.

PAQUIN, P. R., GORSUCH, J. W., APTE, S., BATLEY, G. E., BOWLES, K. C., CAMPBELL, P. G. C., DELOS, C. G., DI TORO, D. M., DWYER, R. L., GALVEZ, F., GENSEMER, R. W., GOSS, G. G., HOGSTRAND, C., JANSSEN, C. R., MCGEER, J. C., NADDY, R. B., PLAYLE, R. C., SANTORE, R. C., SCHNEIDER, U., STUBBLEFIELD, W. A., WOOD, C. M. & WU, K. B. 2002. The biotic ligand model: a historical overview. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology,* 133**,** 3-35.

RIBEIRO, M. J., MARIA, V. L., SCOTT-FORDSMAND, J. J. & AMORIM, M. J. 2015. Oxidative stress mechanisms caused by Ag nanoparticles (NM300K) are different from those of AgNO3: Effects in the soil invertebrate Enchytraeus Crypticus. *International journal of environmental research and public health,* 12**,** 9589-9602.

ROSSELAND, B. O., MASSABUAU, J.-C., GRIMALT, J. O., HOFER, R., LACKNER, R., RADDUM, G. G., ROGNERUD, S. & VIVES, I. 2001. Fish Exotoxicology: The EMERGE Fish Sampling Manual for Live Fish.The EMERGE Project (European Mountain lake Ecosystems: Regionalisation, diaGnostic and socio economic valuation).

ROSSELAND, B. O., ROGNERUD, S., COLLEN, P., GRIMALT, J. O., VIVES, I., MASSABUAU, J.-C., LACKNER, R., HOFER, R., RADDUM, G. G. & FJELLHEIM, A. 2007. Brown trout in Lochnagar: population and contamination by metals and organic micropollutants. *Lochnagar: The Natural History of a Mountain Lake***,** 253-285.

SCHIRMER, K., FISCHER, B. B., MADUREIRA, D. J. & PILLAI, S. 2010. Transcriptomics in ecotoxicology. *Analytical and bioanalytical chemistry,* 397**,** 917-923.

SCOWN, T. M., SANTOS, E. M., JOHNSTON, B. D., GAISER, B., BAALOUSHA, M., MITOV, S., LEAD, J. R., STONE, V., FERNANDES, T. F., JEPSON, M., VAN AERLE, R. & TYLER, C. R. 2010. Effects of Aqueous Exposure to Silver Nanoparticles of Different Sizes in Rainbow Trout. *Toxicological Sciences,* 115**,** 521-534.

SHAW, B. J. & HANDY, R. D. 2011. Physiological effects of nanoparticles on fish: A comparison of nanometals versus metal ions. *Environment International,* 37**,** 1083-1097.

SHAW, B. J., LIDDLE, C. C., WINDEATT, K. M. & HANDY, R. D. 2016. A critical evaluation of the fish early-life stage toxicity test for engineered nanomaterials: experimental modifications and recommendations. *Archives of toxicology,* 90**,** 2077-2107.

SONG, Y., SALBU, B., HEIER, L. S., TEIEN, H.-C., LIND, O.-C., OUGHTON, D., PETERSEN, K., ROSSELAND, B. O., SKIPPERUD, L. & TOLLEFSEN, K. E. 2012. Early stress responses in Atlantic salmon (Salmo salar) exposed to environmentally relevant concentrations of uranium. *Aquatic Toxicology,* 112**,** 62-71.

SØRENSEN, S. N. & BAUN, A. 2015. Controlling silver nanoparticle exposure in algal toxicity testing A matter of timing. *Nanotoxicology,* 9**,** 201-209.

TOLLEFSEN, K. E., SONG, Y., KLEIVEN, M., MAHROSH, U., MELAND, S., ROSSELAND, B. O. & TEIEN, H.-C. 2015. Transcriptional changes in Atlantic salmon (Salmo salar) after embryonic exposure to road salt. *Aquatic Toxicology,* 169**,** 58-68.

VAN DER PLOEG, M. J., HANDY, R. D., WAALEWIJN-KOOL, P. L., VAN DEN BERG, J. H., HERRERA RIVERA, Z. E., BOVENSCHEN, J., MOLLEMAN, B., BAVECO, J. M., TROMP, P. & PETERS, R. J. 2014. Effects of silver nanoparticles (NM-300K) on Lumbricus rubellus earthworms and particle characterization in relevant test matrices including soil. *Environmental toxicology and chemistry,* 33**,** 743-752.

WARHEIT, D. B. 2018. Hazard and risk assessment strategies for nanoparticle exposures: how far have we come in the past 10 years? *F1000Research,* 7.

WEBB, N. A. & WOOD, C. M. 1998. Physiological analysis of the stress response associated with acute silver nitrate exposure in freshwater rainbow trout (Oncorhynchus mykiss). *Environmental Toxicology and Chemistry,* 17**,** 579-588.

WEBB, N. A. & WOOD, C. M. 2000. Bioaccumulation and distribution of silver in four marine teleosts and two marine elasmobranchs: influence of exposure duration, concentration, and salinity. *Aquatic Toxicology,* 49**,** 111-129.

760 WOOD, C. M., HOGSTRAND, C., GALVEZ, F. & MUNGER, R. S. 1996. The physiology of waterborne silver
761 toxicity in freshwater rainbow trout (Oncorhynchus mykiss) .1. The effects of ionic Ag+. *Aquatic*
762 *Toxicology,* 35**,** 93-109.
763 WOOD, C. M., PLAYLE, R. C. & HOGSTRAND, C. 1999. Physiology and modeling of mechanisms of silver
764 uptake and toxicity in fish. *Environmental toxicology and chemistry,* 18**,** 71-83.

765

766

767 Supplementary Information

768 Characterization of bioconcentration and toxicity of Ag ions and the Ag 769 nanoparticle NM300K in Brown trout (*Salmo trutta* L.)

770

771 Merethe Kleiven[a*], Maria T. Hultman[b], Bjørn Olav Rosseland[a], Pablo Lebed[a], Knut Erik Tollefsen[ab], Hans-
772 Christian Teien[a]

773 [a] *Norwegian University of Life Sciences(NMBU), Faculty of Environmental Sciences and Nature Resource*
774 *Management, Center for Environmental Radioactivity (CERAD), P.O.Box 5003 NMBU, 1432 Ås, Norway*
775 [b] *Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway*
776 [*] Address correspondence to *merethe.kleiven@nmbu.no or hans-christian.teien@nmbu.no*
777

778

Tables

Table S1. Water quality parameters in all exposure groups throughout the 96 h exposure. The values are presented as mean ± S.D. of three replicate samples. For single values, only one individual sample was measured.

Exposure	Time (h)	pH	Conductivity µS/cm	Temperature °C	O_2 mg L^{-1}	O_2 %
Control	24	7.4	-	14.4	10.1	101
Control	48	7.6	48.4	12.5	10.6	101
Control	72	7.3	49.6	13.2	10.3	101
Control	96	7.3	49.7	14.3	10.2	102
		7.4±0.1	49.2±0.7	13.6±0.9	10.2±0.2	101.3±0.5
$AgNO_3$ 2.0 µg L^{-1}	24	7.2	46.0	14.2	9.9	99
$AgNO_3$ 2.0 µg L^{-1}	48	7.1	47.0	13.2	10.5	102
$AgNO_3$ 2.0 µg L^{-1}	72	7.3	46.0	13.7	-	-
$AgNO_3$ 2.0 µg L^{-1}	96	7.3	50.1	14.4	10.2	102
		7.2±0.1	47±2	13.9±0.5	10.2±0.3	100.6±1.8
$AgNO_3$ 5.0 µg L^{-1}	24	7.1	45.0	14.1	10.3	102
$AgNO_3$ 5.0 µg L^{-1}	48	7.2	46.0	13.2	10.6	102
$AgNO_3$ 5.0 µg L^{-1}	72	7.3	46.9	14.0	10.2	102
$AgNO_3$ 5.0 µg L^{-1}	96	7.4	50.5	14.6	10.2	103
		7.2±0.1	47±2	14.0±0.6	10.3±0.2	102.1±0.5
$AgNO_3$ 10.0 µg L^{-1}	24	-	-	-	10.3	102
$AgNO_3$ 10.0 µg L^{-1}	48	7.2	45.0	13.3	10.6	103
$AgNO_3$ 10.0 µg L^{-1}	72	7.3	46.9	13.9	10.2	102
$AgNO_3$ 10.0 µg L^{-1}	96	7.3	49.1	14.4	10.2	103
		7.3±0.1	47±2	13.9±0.6	10.3±0.2	102.2±0.5
AgNP 2.0 µg L^{-1}	24	7.1	44.0	14.2	10.2	-
AgNP 2.0 µg L^{-1}	48	7.1	45.0	13.1	10.6	102
AgNP 2.0 µg L^{-1}	72	7.3	45.0	13.7	10.2	102
AgNP 2.0 µg L^{-1}	96	7.4	48.4	14.6	10.2	102
		7.2±0.1	46±2	13.9±0.6	10.3±0.2	101.9±0.4
AgNP 5.0 µg L^{-1}	24	7.1	46.0	14.1	10.2	101
AgNP 5.0 µg L^{-1}	48	7.2	46.0	13.2	10.6	102
AgNP 5.0 µg L^{-1}	72	7.3	45.0	13.7	10.2	101
AgNP 5.0 µg L^{-1}	96	7.3	49.4	14.4	10.2	102
		7.2±0.1	47±2	13.9±0.5	10.3±0.2	101.7±0.6
AgNP 10.0 µg L^{-1}	24	7.0	46.0	14.1	10.2	102
AgNP 10.0 µg L^{-1}	48	7.1	46.0	13.3	10.5	102
AgNP 10.0 µg L^{-1}	72	7.3	46.4	14.0	10.1	101
AgNP 10.0 µg L^{-1}	96	7.3	50.7	14.5	10.1	101
		7.2±0.2	47.3±2.3	14.0±0.5	10.3±0.2	101.5±0.4

783 Table S2. Concentrations of Total Ammonium Nitrogen (TAN) in the different exposure groups at 24 and
784 96 h of exposure.

TAN (NH_4^+) mg L^{-1}		
	24 h	96 h
Control	0,39	0,4
$AgNO_3$ 2 µg L^{-1}	0,18	0,5
$AgNO_3$ 5 µg L^{-1}	0,28	0,51
$AgNO_3$ 10 µg L^{-1}	0,19	0,55
AgNP 2 µg L^{-1}	0,24	0,35
AgNP 5 µg L^{-1}	0,1	0,44
AgNP 10 µg L^{-1}	0,13	0,54

785

786

Table S3. Concentrations of Ag (µg Ag L^{-1}) at 0h and 24h in the different size fractions in $AgNO_3$ and NM300K AgNP exposures. Particulate > 220 nm, colloidal/NP <220 nm >3 kDa, Low molecular mass (LMM) < 3 kDa at t=0 and <10 kDa at t=24 h.

		Total	Particulate	Colloidal/NP	LMM
Control	0 h	0.0	0.0	0.0	<0.004
	24 h	0.0	0.0	0.0	<0.004
$AgNO_3$ 2 µg Ag L^{-1}	0 h	1.9	0.5	1.3	0.0
	24 h	1.6	0.4	1.2	<0.004
$AgNO_3$ 5 µg Ag L^{-1}	0 h	4.8	1.6	2.7	0.5
	24 h	4.2	1.6	2.7	<0.004
$AgNO_3$ 10 µg Ag L^{-1}	0 h	10.0	2.3	4.8	2.9
	24 h	9.3	4.3	4.8	0.1
AgNP 2 µg Ag L^{-1}	0 h	1.7	0.4	1.4	<0.004
	24 h	1.4	0.7	0.6	<0.004
AgNP 5 µg Ag L^{-1}	0 h	4.7	1.1	3.6	0.0
	24 h	3.9	1.6	2.3	<0.004
AgNP 10 µg Ag L^{-1}	0 h	8.7	2.2	6.5	0.1
	24 h	6.9	2.9	4.0	<0.004

Table S4. Bioconcentration of Ag (µg Ag g^{-1} tissue) in gill (dry weight), liver (dry weight) and kidney (wet weight) of brown trout (*Salmo trutta*) after 96 h of exposure to $AgNO_3$ and NM300K AgNPs.

	Gills	Liver	Kidney
Control	0,005±0.003	0.8±0.4	0.004±0.002
$AgNO_3$ 2 µg Ag L^{-1}	0,8±0.04*	5.5±2.6*	0.4±0.2*
$AgNO_3$ 5 µg Ag L^{-1}	2,9±0.9*	13±5*	1.3±0.6*
$AgNO_3$ 10 µg Ag L^{-1}	6,5±1*	19±3*	2±1*
AgNP 2 µg Ag L^{-1}	0,03±0.02	1.2±0.3	0.012±0.005
AgNP 5 µg Ag L^{-1}	0,05±0.02*	1.3±0.5	0.02±0.01
AgNP 10 µg Ag L^{-1}	0,08±0.04*	1.3±0.5	0.04±0.04

NA = not applicable due to no significant accumulation of Ag compared to control

* Significantly different from control.

Table S5. Transfer factors (mean±s.d.) in *Salmo trutta* after 96 h of waterborne exposure to $AgNO_3$ and AgNPs (NM300K) in gills, liver and kidney.

	Gill ($\mu g\ g^{-1}$ gill /$\mu g\ g^{-1}$ water)	Liver ($\mu g\ g^{-1}$ liver/$\mu g\ g^{-1}$ water)	Kidney* ($\mu g\ g^{-1}$ kidney/$\mu g\ g^{-1}$ water)
$AgNO_3$ 2 µg Ag L^{-1}	4.1 (±2.0) $x10^2$	2.7(±1.3) $x10^3$	2,0 (±1.1) $x10^2$
$AgNO_3$ 5 µg Ag L^{-1}	6.1 (±2.0) $x10^2$	2.6(±1.0) $x10^3$	2,7 (±1.3) $x10^2$
$AgNO_3$ 10 µg Ag L^{-1}	6.7 (±1.0) $x10^2$	2.0(±0.3) $x10^3$	1.9 (±1.2) $x10^2$
AgNP 2 µg Ag L^{-1}	1.8 (±1.3) $x10^1$	NA	NA
AgNP 5 µg Ag L^{-1}	1.1 (±0.4) $x10^1$	NA	NA
AgNP 10 µg Ag L^{-1}	1.0 (±0.5) $x10^1$	NA	NA

NA = not applicable due to no significant accumulation of Ag compared to control
*Based on wet weight, while gill and liver are based on dry weight.

Table S6. Pearson correlation analysis of measured blood glucose, Total (T-Ag), particulate (P-Ag), colloidal/nanoparticles (C-NP) and low molecular mass ions (LMM) Ag and accumulation of Ag in gill (Ag-G), liver (Ag-L) and kidney (Ag-K) in brown trout (*Salmo trutta L.*) exposed to silver nitrate and silver nanoparticles.

Variables	*Glucose*	*T-Ag*	*P-Ag*	*C-NP*	*LMM-Ag*	*Ag-G*	*Ag-L*	*Ag-K*
Glucose	**1**	**0.657**	**0.618**	**0.336**	**0.834**	**0.877**	**0.908**	**0.698**
T-Ag	**0.657**	**1**	**0.973**	**0.906**	**0.727**	**0.663**	**0.604**	**0.522**
P-Ag	**0.618**	**0.973**	**1**	**0.914**	**0.612**	**0.600**	**0.572**	**0.505**
C-NP	**0.336**	**0.906**	**0.914**	**1**	**0.389**	0.299	0.246	0.210
LMM-Ag	**0.834**	**0.727**	**0.612**	**0.389**	**1**	**0.929**	**0.844**	**0.723**
Ag-G	**0.877**	**0.663**	**0.600**	0.299	**0.929**	**1**	**0.924**	**0.871**
Ag-L	**0.908**	**0.604**	**0.572**	0.246	**0.844**	**0.924**	**1**	**0.879**
Ag-K	**0.698**	**0.522**	**0.505**	0.210	**0.723**	**0.871**	**0.879**	**1**

Values in bold are different from 0 with a significance level alpha=0,05

p-values (Pearson):

Variables	*Glucose*	*T-Ag*	*P-Ag*	*C-NP*	*LMM-Ag*	*Ag-G*	*Ag-L*	*Ag-K*
Glucose	**0**	**< 0.0001**	**< 0.0001**	**0.034**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
T-Ag	**< 0.0001**	**0**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.001**
P-Ag	**< 0.0001**	**< 0.0001**	**0**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.000**	**0.001**
C-NP	**0.034**	**< 0.0001**	**< 0.0001**	**0**	**0.013**	0.061	0.126	0.193
LMM-Ag	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.013**	**0**	**< 0.0001**	**< 0.0001**	**< 0.0001**
Ag-G	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.061	**< 0.0001**	**0**	**< 0.0001**	**< 0.0001**
Ag-L	**< 0.0001**	**< 0.0001**	**0.000**	0.126	**< 0.0001**	**< 0.0001**	**0**	**< 0.0001**
Ag-K	**< 0.0001**	**0.001**	**0.001**	0.193	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**

Table S7. Correlation analysis of measured blood glucose, Total (T-Ag), particulate (P-Ag), colloidal/nanoparticles (C-NP) and low molecular mass ions (LMM) Ag and accumulation of Ag in gill (Ag-G), liver (Ag-L) and kidney (Ag-K) in brown trout (*Salmo trutta L.*) exposed to silver nanoparticles.

Variables	*Glucose*	*T-Ag*	*P-Ag*	*C-NP*	*LMM-Ag*	*Ag-G*	*Ag-L*	*Ag-K*
Glucose	**1**	0.310	0.279	0.299	0.120	0.150	0.083	0.145
T-Ag	0.310	**1**	**0.996**	**0.999**	**0.885**	**0.655**	0.155	0.452
P-Ag	0.279	**0.996**	**1**	**0.998**	**0.923**	**0.659**	0.154	0.457
C-NP	0.299	**0.999**	**0.998**	**1**	**0.900**	**0.657**	0.155	0.454
LMM-Ag	0.120	**0.885**	**0.923**	**0.900**	**1**	**0.612**	0.133	0.435
Ag-G	0.150	**0.655**	**0.659**	**0.657**	**0.612**	**1**	0.343	**0.834**
Ag-L	0.083	0.155	0.154	0.155	0.133	0.343	**1**	0.458
Ag-K	0.145	0.452	0.457	0.454	0.435	**0.834**	0.458	**1**

Values in bold are different from 0 with a significance level alpha=0.05

p-values (Pearson):

Variables	*Glucose*	*T-Ag*	*P-Ag*	*C-NP*	*LMM-Ag*	*Ag-G*	*Ag-L*	*Ag-K*
Glucose	**0**	0.226	0.278	0.244	0.648	0.566	0.751	0.579
T-Ag	0.226	**0**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.004**	0.551	0.069
P-Ag	0.278	**< 0.0001**	**0**	**< 0.0001**	**< 0.0001**	**0.004**	0.555	0.065
C-NP	0.244	**< 0.0001**	**< 0.0001**	**0**	**< 0.0001**	**0.004**	0.552	0.067
LMM-Ag	0.648	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**	**0.009**	0.610	0.081
Ag-G	0.566	**0.004**	**0.004**	**0.004**	**0.009**	**0**	0.178	**< 0.0001**
Ag-L	0.751	0.551	0.555	0.552	0.610	0.178	**0**	0.065
Ag-K	0.579	0.069	0.065	0.067	0.081	**< 0.0001**	0.065	**0**

Figures

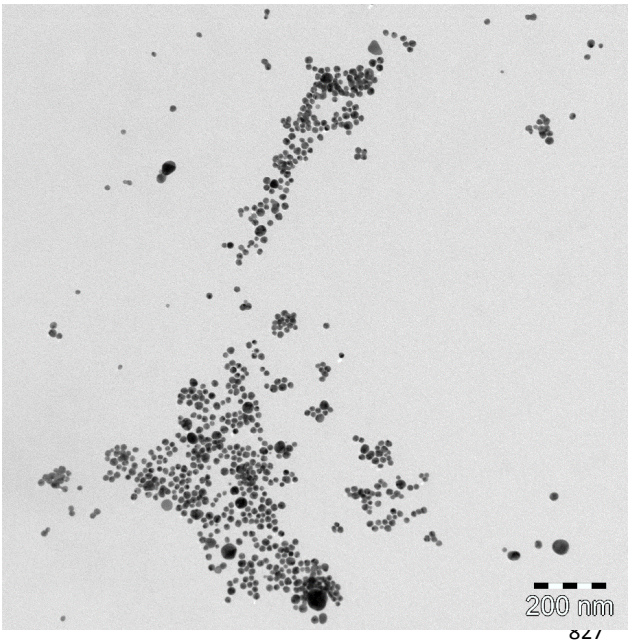
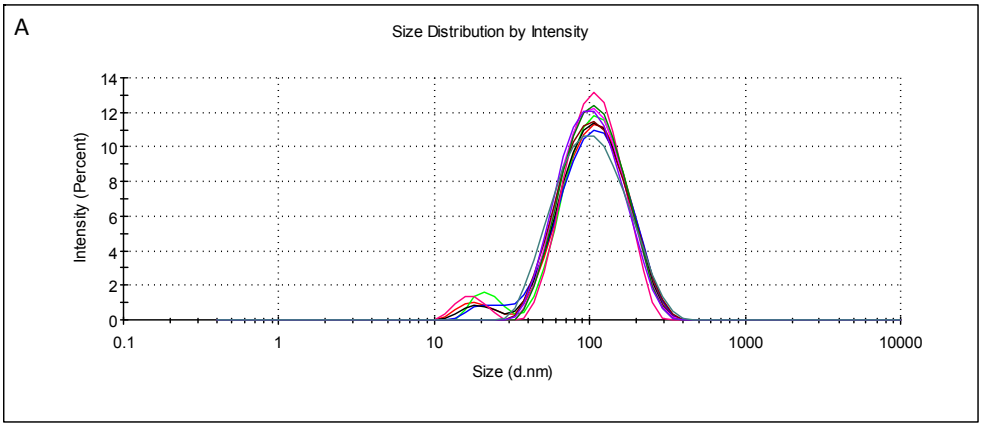
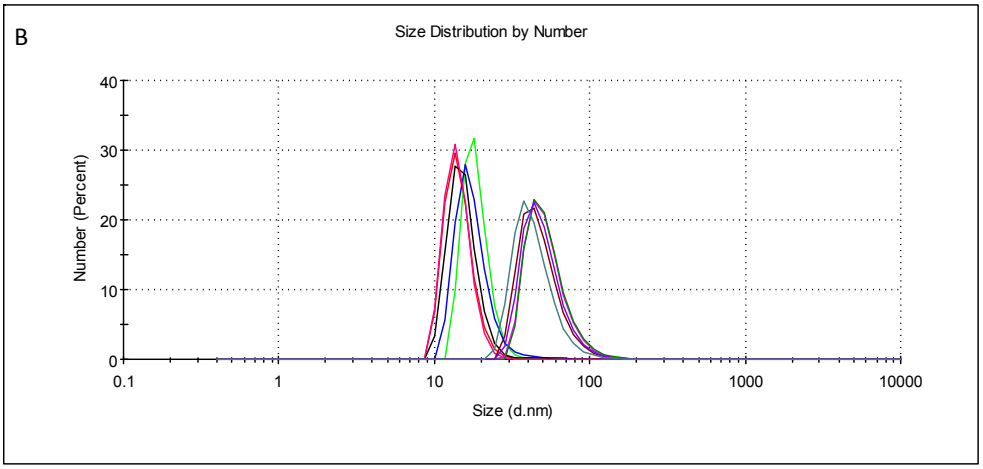


Figure S1. Size and shape of NM300K Ag NP in MilliQ water (250 mg L^{-1}). Image acquired with Transmission Electron Microscopy, and image analysis gave a Ferret min of 16.9±0.1 nm (mean±S.D.) and an equivalent circle diameter (ECD) of 18.7±0.2 nm (n=1833).

831



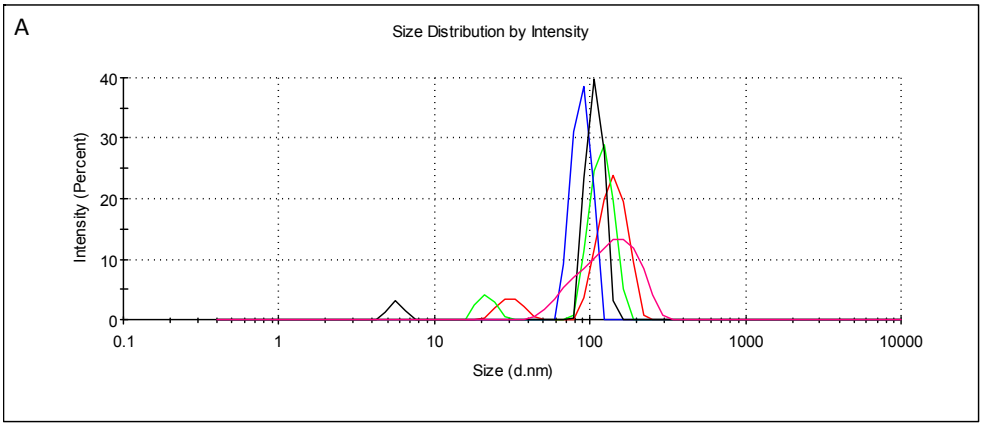
832



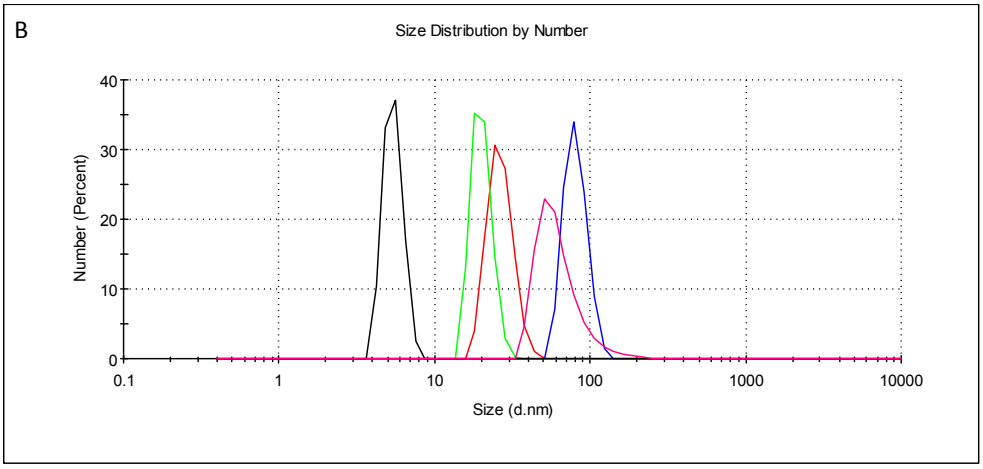
833

834 Figure S2. Size distribution of the NM300K stock suspension (in MilliQ, 2.56 g Ag L^{-1}) given by Intensity

835 (A) and Number (B). Z-average = 86.0±0.7 nm.



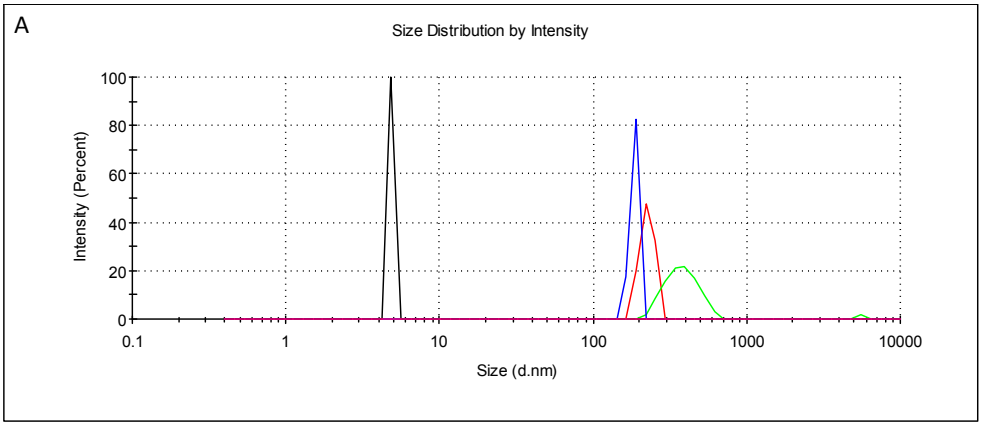
836



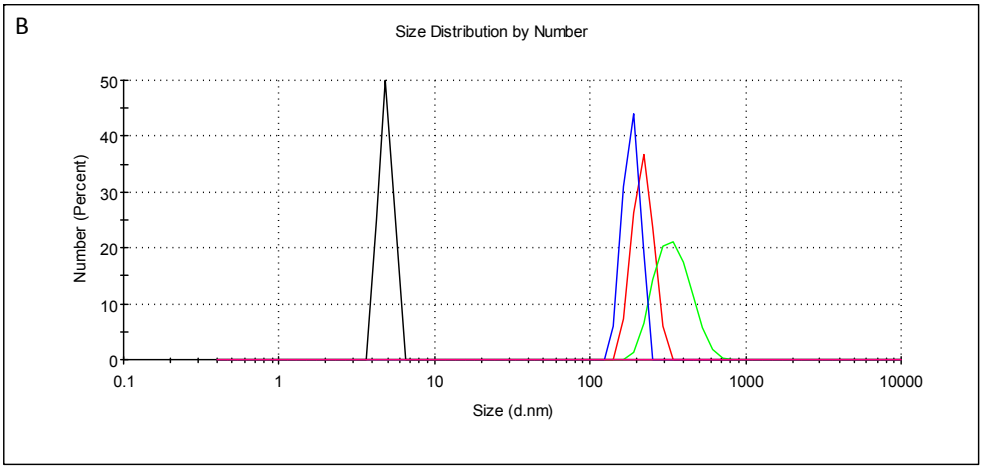
837

838 Figure S3. Size distribution of the 10 µg Ag L^{-1} exposure (local drinking water) of NM300K AgNPs given by
839 Intensity (A) and Number (B). Z-average = 370±70 nm.

840



841



842

843 Figure S4. Size distribution of the 10 µg Ag L^{-1} exposure (local drinking water) of $AgNO_3$ given by Intensity

844 (A) and Number (B). Z-average = 6000±9900 nm.

ISBN: 978-82-575-1527-0
ISSN: 1894-6402

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no