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

Philosophiae Doctor (PhD)
Thesis 2019:37

# Single and multigenerational studies of silver nanoparticle toxicity and adaptive mechanisms in the nematode Caenorhabditis elegans

## Enkelt og multigenerasjonsstudies av toksistet og adaptive mekanismer I nematoden Caenorhabditis elegans

Lisa Magdalena Rossbach

# Single and multigenerational studies of silver nanoparticle toxicity and adaptive mechanisms in the nematode *Caenorhabditis elegans*

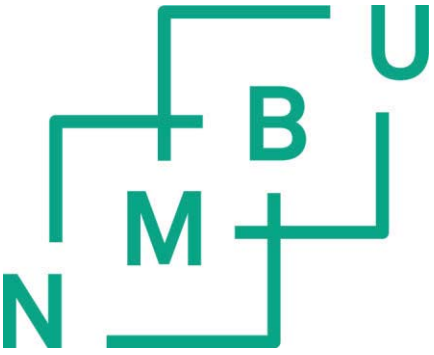
Enkelt og multigenerasjonsstudier av toksisitet og adaptive mekanismer i nematoden *Caenorhabditis elegans*

Philosophiae Doctor (PhD) Thesis

Lisa Magdalena Rossbach

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

Ås (2019)




Thesis number 2019:37
ISSN 1894-6402
ISBN 978-82-575-1596-6

# PhD supervisors

Professor Deborah H. Oughton

Department of Environmental Sciences,
Faculty of Environmental Sciences and Natural Resource Management (MINA),
Norwegian University of Life Sciences (NMBU)
E-mail: deborah.oughton@nmbu.no

Research Professor Dag A. Brede,

Department of Environmental Sciences,
Faculty of Environmental Sciences and Natural Resource Management (MINA),
Norwegian University of Life Sciences (NMBU)
E-mail: dag.anders.brede@nmbu.no

Researcher Claire Coutris

Division of Environmental and Natural Resources
Department of Soil Quality and Climate Change
Norwegian Institute of Bioeconomy Research (NIBIO)
E-mail: Claire.coutris@nibio.no

# Evaluation Committee

Professor Teresa Fernandes

Institute of Life and Earth Sciences
Heriot-Watt University
Edinburgh, EH14 4AS,
United Kingdom
Phone: +44 131 451 4599
E-mail: T.Fernandes@hw.ac.uk

Dr. Espen Mariussen

Norwegian Institute of Air Research (NILU)
Instituttveien 18
2007 Kjeller
Norway
Phone: +47 63 89 82 29
E-mail: Espen.Mariussen@nilu.no

Dr. Hans-Christian Teien

Department of Environmental Sciences,
Faculty of Environmental Sciences and Natural Resource Management (MINA),
Norwegian University of Life Sciences (NMBU)
E-mail: hans-christian.teien@nmbu.no

# Contents

# Acknowledgements

The current PhD work was primarily funded by the NANoREG (grant number 29199) and NANOCHARM (grant number 221391) projects, as well as the Centre of Environmental Radioactivity (CERAD), funded by the Norwegian Research Council (2013-2023) through its Centres of Excellence (CoE) funding scheme (223268/F50). All of the experimental work and sample analysis was conducted at the Isotope laboratory at the Norwegian University of Life Sciences.

I would like to give special thanks to my supervisors, Prof. Deborah Oughton (NMBU, main supervisor), Dr. Dag Anders Brede (NMBU, co-supervisor), and Dr. Claire Coutris (NIBIO, co-supervisor), for all the scientific advisement, support, and encouragement during the last 4 years. Dag, without you, none of this would have been possible. I truly appreciate that you always took the time to discuss (and re-discuss) my research. To Claire, thank you for the friendly assistance all these years, and seeing my work through to the end. And, lastly, a PhD cannot be done without a main supervisor. Deborah, all the kind words throughout the PhD were always highly appreciated (and often needed)! I could not have asked for a more caring, patient, and supportive main supervisor.

Throughout this PhD, I had the pleasure to work with all of the lovely staff from the Isotope laboratory; Thank you to you all! Additionally, all my gratitude to Ian, Yevgeniya, Erica, Estela, Frøydis, Shane, Seb and Quentin, aka "The Gang", for all the laughter, support and unforgettable times. Special thanks to Merethe, Ulva and Erica, for helping me through some of the hardest bits. Special thanks to Ian for the never-ending patience, help with English, and always finding a way to make me smile.

Also, many thanks to my co-authors, and old and new colleagues, in particular Pablo, Keke, Yet, Anike, Lene, Julian, Mirian, Marit, Karl Andreas, Signe, Lene and Hilde. Special thanks to Dag Markus, for all the patience and the long, sometimes seemingly never-ending, days, working on my data.

Thank you to my family for believing in me and always providing me with all the opportunities to follow my dreams.

Lisa Rossbach, Ås, 2019

# SUMMARY

A substantial increase in nanomaterial production and a rise in novel applications are predicted in the coming years. There are clearly a number of positive aspects of a range of different "nano-applications". However, the inevitable release into the environment of nanomaterials from such applications also carries a potential risk. Silver nanoparticles (Ag NPs) are the most commonly used nanoparticles to date, where their antibacterial properties are used to enhance a number of commercial products, such as in wound dressings, cosmetics, textiles, and food packaging. The high degree of leaching of the Ag NPs from consumer products results in accumulations in landfills and the terrestrial environment. Despite being amongst the most extensively studied nanomaterial to date, there is still a certain amount of controversy about their toxicity. A multitude of studies ascribed toxic effects to ionic releases, while other studies have identified particle specific effects. Furthermore, results from Ag NP toxicity tests are hard to compare, due to differences in exposure media, organisms, particle characteristics and the endpoints studied. Lastly, toxicity studies primarily focus on the exposure of a specific life stage or a single generation of an organism. The lack of multigenerational studies could mean a large uncertainty about long-term effects.

This PhD research project was aimed at understanding different aspects of the toxicity of the reference Ag NP NM300K towards the nematode *Caenorhabditis elegans*, in comparison to $AgNO_3$. To address this aim, a range of single and multigenerational studies were set up using *C. elegans* as a model organism. Studies were designed to test three hypotheses:

1. **NM300K Ag NPs would be toxic to *C. elegans*, but the relatively low dissolution of these NPs would make these less toxic than $AgNO_3$.**
2. **Multigenerational exposure will lead to an adaptation towards Ag NPs at lower concentrations, but an increase in sensitization at higher concentrations, across generations.**
3. **The production of reactive oxygen species (ROS) involved in the toxic mechanisms of Ag NPs, would trigger antioxidant defenses following exposure, and changes in toxic responses over generations could be related to these defense mechanisms.**

Standard toxicity tests were carried out to test for toxic response, while the chronic multigenerational exposures were carried out on agar plates. For all exposures $AgNO_3$ was used as a positive control.

For $AgNO_3$ and Ag NPs exposures, size fractionation measurements showed substantial changes in Ag speciation over time, characterized by an increase in larger aggregates, coupled with a decrease in the low molecular mass (LMM) Ag fraction (<3 kDa). In the $AgNO_3$ exposures the LMM Ag fraction was 0 - 54 % of total Ag concentration at time zero (T-0), but only 0 - 0.06 % at 96 h. Comparatively, in the Ag NP exposure, a reduction from ~16 % at T-0, to 0.05 % at 96 h was observed. This confirmed a low dissolution of Ag NPs and a higher initial LMM concentration in exposure media for $AgNO_3$. Furthermore, results suggest a high interaction of Ag with *Escherichia coli*, the nematodes' food source, which, in turn, facilitate the dietary Ag uptake by *C. elegans*.

In the single generation exposures, a concentration-dependent decrease in nematode reproduction, fertility, and growth was measured for both Ag NP and $AgNO_3$. However, the concentrations necessary to achieve a comparable dose-response were 7 – 10 times higher for the NPs compared to $AgNO_3$. This is further supported by EC50 estimations, for which Ag NP showed 3 - 7 times higher levels for growth, 8 fold higher for fertility, and 2 – 9 fold higher for reproduction, as compared to $AgNO_3$. Ag uptake by the nematodes was comparable between the two forms of Ag. However, following depuration approximately 2 fold higher concentrations of $AgNO_3$ were retained by nematodes.

In the multigenerational, chronic exposure, nematodes were continuously exposed to three concentrations of either $AgNO_3$ (0.01, 0.05 or 0.1 mg Ag $L^{-1}$) or Ag NPs (0.1, 0.5 or 1 mg Ag $L^{-1}$), prior to exposing different generations to higher concentrations (of $AgNO_3$ or Ag NPs) in standard toxicity tests. The continuous chronic exposure to 1 mg Ag $L^{-1}$ Ag NPs resulted in an adaptive response, as measured by an increase in reproduction compared to controls, to Ag NPs. However, the adaptation came at a cost of reduced growth. These nematodes were producing offspring at a total body length of 0.8 – 1 mm, while control and $AgNO_3$ exposed nematodes only produced offspring at > 1 mm. Furthermore, the continuous exposure towards Ag NPs led to an increased sensitivity towards $AgNO_3$. Comparatively, the multigenerational $AgNO_3$ exposure resulted in no change in toxic response towards $AgNO_3$, but a decreased sensitivity towards Ag NPs. Lastly, a decreased sensitivity towards the known ROS inducer paraquat was measured following the six

generational exposure to Ag NPs, hinting at changes in the involvement of the superoxide (SOD) antioxidant defense system in the observed adaptive response.

Studies using fluorescently labelled *C. elegans* reporter and biosensor strains showed that the exposure to both forms of Ag resulted in an Ag concentration-dependent increase in antioxidant defenses, as well as an increase in peroxide levels and changes in the cellular redox status in the nematodes. Furthermore, findings showed distinct differences in the biodistribution of Ag. Changes in cellular redox status in the luminal cells, suggest Ag NPs were primarily contained within the intestine. In comparison, $AgNO_3$ acted more evenly across the whole body. Across generations, however, an increase in *sod-1* expression in the F3 generations suggests the involvement of antioxidant defenses in the adaptive response. Nevertheless, a decrease in *sod-1*, combined with a simultaneous increase in oxidative stress development in the F6 generations, suggest that the maintenance of reproductive capacity is the more beneficial adaptive response of nematodes, compared to oxidative stress responses.

To conclude, both forms of Ag showed transformations over time in the exposure media with a decrease in the LMM Ag fraction, in conjunction with an increase in the aggregated fraction. This highlights the importance of monitoring the Ag speciation throughout the exposure period. Differences in toxicity between $AgNO_3$ and Ag NPs could be related to differences in the initial LMM fractions as well as clear differences in biodistribution. Moreover, low dissolution of the Ag NPs may prevent the incorporation of Ag from the Ag NPs into cellular components. Comparing the two forms of Ag suggests that Ag NPs lead to increases in ROS production that, in turn, could lead to the observed toxic effects, as measured by decreases in reproduction, fertility and growth. Additionally, it was concluded that Ag from $AgNO_3$ exposure is more readily incorporated into cells, leading to the intracellular production of ROS across the whole body of the nematodes. Overall, the results suggested that $AgNO_3$ had a different toxic mode of action to that of Ag NPs. Furthermore, data suggested that *C. elegans* was able to develop an adaptive response towards the exposure of Ag NPs. This response came with the associated cost of reduced growth, increased sensitivity towards $AgNO_3$ and $Ce^{3+}$, and reduced oxidative stress defense.

# SAMMENDRAG

De nærmeste årene forventes en betydelig økning i produksjon av nanopartikler, og en rekke nye bruksområder for disse. Det er mange positive effekter knyttet til bruk av ulike nanomaterialer. Det er imidlertid uunngåelig at bruken vil medføre utslipp av nanomaterialer og dermed utgjøre en risiko for miljøet. Sølvnanopartikler (Ag NP) er per nå blant de mest kommersielt anvendte nanopartiklene. Deres antibakterielle egenskaper brukes til å forbedre produktegenskaper i sårbandasjer, kosmetikk, tekstiler og matemballasje. Utlekking av Ag NP fra slike produkter har ført til akkumulering av sølv i avfallsdeponier og terrestriske miljøer. Selv om sølvnanopartikler er blant de best vitenskapelig studerte nanomaterialer som finnes, er det likevel knyttet stor usikkerhet og mangel på konsensus vedrørende toksiske effekter av Ag NP. En rekke studier knytter toksisiteten til lekkasje av ioner, mens andre forsøk har vist partikkelspesifikke effekter. Det har også vist seg vanskelig å sammenligne slike studier, på grunn av ulikheter i forsøksoppsettene relatert til eksponeringsmedium, testorganismer, partikkelegenskaper og hvilke effektparametere som er målt. Majoriteten av eksponeringsstudiene har vært fokusert på en enkelt generasjon eller et spesifikt livsstadium hos testorganismen. Det er derfor stor mangel på data fra multigenerasjonsstudier, og dermed liten kunnskap om potensielle effekter av langtidseksponering.

Målsettingen for dette doktorgradsarbeidet har vært å undersøke og forstå sentrale aspekter ved toksisitet av referansenanopartikkelmateriale Ag NM300K sammenlignet med sølvnitrat ($AgNO_3$) ved bruk av *Caenorhabditis elegans* som testorganisme. Studien har bestått av en rekke enkeltgenerasjons- og multigenerasjonseksponeringer med *C. elegans*.

Eksperimentene ble designet til å teste tre hypoteser:

1. **NM300K Ag NP er toksiske for *C. elegans*, men toksisiteten er lavere enn for $AgNO_3$ fordi ioner det i relativt liten grad frigjøres ioner fra partiklene**.
2. **Eksponering over flere generasjoner vil føre til en adapsjon til lave konsentrasjoner av Ag NP, mens høye konsentrasjoner vil medføre økt sensitivitet.**

3. **Reaktive oksygenforbindelser (ROS) generert som resultat av Ag NP eksponering, vil aktivere antioksidantsystemer, og vil over generasjoner medføre endringer i toksiske responser relatert til forsvarsmekanismer mot oksidativt stress.**

*C. elegans* ble eksponert ved bruk av standard toksisitetstester eller på agarplater for kronisk multigenerasjonseksponering. Sølvnitrat ($AgNO_3$) ble brukt som positiv kontroll ved alle eksponeringsforsøkene. Størrelsesfraksjoneringen endret seg over tid for både $AgNO_3$ og Ag NP. Resultatene viste betydelig dannelse av større aggregater, koblet med en reduksjon i lavmolekylært (LMM) Ag (<3 kDa). Ved start (T-0) av $AgNO_3$ eksponeringene utgjorde LMM 0-54 % av total mengde Ag, mens ved eksponeringsslutt (T-96) var andelen LMM redusert til 0 – 0.06 %. Til sammenligning viste Ag NP eksponeringen en reduksjon fra 16 % ved T-0, til 0.05 % (T-96). Dette bekreftet lav frigjøring av sølvioner fra NM300K partiklene og som forventet en betydelig høyere initial LMM fraksjon i eksponeringsmediet fra $AgNO_3$ eksponering. Resultatene viser en høy interaksjon mellom sølv og nematodenes næringskilde (*Escherichia coli*). Dette kan forklare hvorfor Ag i $AgNO_3$ eksponeringene i såpass begrenset grad foreligger som frie ioner, og bidrar til økt opptak via diett.

Eksponering over en enkelt generasjon viste doseavhengige reduksjoner i nematodenes reproduksjon, fertilitet og vekst i respons til både Ag NP og $AgNO_3$. Det var en signifikant forskjell i toksisitet, ettersom det krevdes 7- 10 ganger høyere dose av Ag NP for å få sammenlignbar effekt med $AgNO_3$. Dette understøttes av estimerte $EC_{50}$ konsentrasjoner som viser 3-7 ganger forskjell for vekst, 8 ganger forskjell for fertilitet, og 2-9 ganger forskjell i reproduksjon mellom Ag NP og $AgNO_3$. Analyser av eksponerte nematoder viste relativt likt opptak fra begge typer sølv, mens analyser etter depurering viste ca. 2 ganger høyere retensjon av sølv hos $AgNO_3$ eksponerte nematoder.

For multigenerasjonstudien ble nematodene kontinuerlig (kronisk) eksponert for tre ulike konsentrasjoner av enten $AgNO_3$ (0.01, 0.05 eller 0.1 mg $L^{-1}$) eller Ag NP (0.1, 0.5 eller 1.0 mg $L^{-1}$). Effekter av eksponeringen ble målt for hver generasjon ved bruk av en standard toksisitetstest. Kontinuerlig (kronisk) eksponering ved 1.0 mg $L^{-1}$ resulterte i en adaptiv toleranserespons, vist ved økt reproduksjon i nærvær av Ag NP sammenlignet med kontroll/andre behandlinger. Den økte reproduksjskapasiteten medførte en kostnad i form av redusert vekst. Disse nematodene produserte avkom ved en total

kroppslengde på 0.8-1 mm, mens kontroll og $AgNO_3$-eksponerte nematoder reproduserte avkom kun når kroppslengden var >1 mm. I tillegg viste det seg at Ag NP eksponerte nematoder ble mer sensitive for $AgNO_3$. Til sammenligning førte multigenerasjon $AgNO_3$-eksponering ikke til noen endring i respons mot $AgNO_3$, men en redusert sensitivitet ovenfor Ag NP. Nematoder eksponert for Ag NP over seks generasjoner viste også redusert sensitivitet for Paraquat som ofte brukes for å indusere ROS. Dette antyder en adapsjon relatert til antioksideringsforsvarsmekanismer inkludert superoksid dismutase (SOD).

Ved bruk av fluorescensmerkede *C. elegans* reporter- og biosensorstammer, ble det vist at begge typer sølv induserte konsentrasjonsavhengig økning i uttrykk av *sod-1* genet, økt intracellulær peroksidkonsentrasjon og endringer i cellenes redoksstatus. Resultatene viser distinkte forskjeller relatert til biodistribusjon av Ag. Endringene i redoks-status i intestinale celler tyder på at Ag NP primært interagerer med tarmepitelet. I sammenligning påvirket $AgNO_3$ celler og vev i hele kroppen. Multigenerasjonstudien viste økt *sod-1* genuttrykk i F3 generasjonen antyder en potensiell rolle for antioksidant forsvarsmekanismer i adapsjon på dette stadiet i eksponeringen. Denne endringen var ikke varig, og i F6 generasjonen var *sod-1* genuttrykket nedregulert under kontroll nivå, samtidig som nematodene viste økt oksidativt stress. Dette tyder på at opprettholdelse av reproduksjonssuksess er en viktigere adaptiv respons for nematodene enn økt forsvar mot oksidativt stress.

Det konkluderes med at begge typer Ag transformeres i løpet av eksponeringstiden, ved at den lavmolekylære fraksjonen reduseres i takt med dannelse av aggregater, hvilket understreker viktigheten av Ag spesiering gjennom hele eksponeringen. Forskjellen i toksisitet mellom $AgNO_3$ og Ag NP kan være knyttet til den initiale LMM fraksjonen, samt forskjeller i biodistribusjonen. Den lave frigjøringen av ioner fra Ag NP forhindrer translokasjon av sølv intracellulært. Sammenligning av de to typene sølv tyder på at Ag NP induserer ROS som fører til observerte toksiske effekter inkludert redusert reproduksjon, fertilitet og vekst, mens $AgNO_3$ inkorporeres lettere i cellene og dermed kan påvirke enzymer og proteiner direkte, med påfølgende ROS produksjon. Samlet sett viser resultatene at $AgNO_3$ og Ag NP induserer toksisitet ved ulike virkningsmekanismer. Studien viser også at *C. elegans* er i stand til å utvikle økt toleranse mot Ag NP eksponering via adaptive prosesser. Denne adaptive responsen medfører en kostnad i form av

redusert vekst, økt sensitivitet mot $AgNO_3$ og $Ce^{3+}$ og redusert kapasitet mot oksidativt stress.

## List of Papers

**Paper I. Characterizing the behavior, uptake and toxicity of NM300K silver nanoparticles in *Caenorhabditis elegans***. (Kleiven, M., Rossbach, L.M., Gallego-Urrea, J.A., Brede, D.A., Oughton, D.H., & Coutris, C., 2018. *Environmental Toxicology and Chemistry*. 37(7): 1799 – 1810, DOI: 10.1002/etc.4144)

**Paper II. *In vivo* assessment of silver nanoparticle induced reactive oxygen species reveals tissue specific effects on cellular redox status in the nematode *Caenorhabditis elegans*.** (Submitted to *Environmental Science: Nano*. Rossbach, L.M., Oughton, D.H., Coutris, C., & Brede, D.A.)

**Paper III. Adaptive tolerance to a multigenerational silver nanoparticle (NM300K) exposure by the nematode *Caenorhabditis elegans* is associated with increased sensitivity to $AgNO_3$.** (Rossbach, L.M., Maremonti, E., Eide, D.M., Oughton, D.H. & Brede, D.A., 2019. *Nanotoxicology* 18:1 – 16, DOI: 10.1080/17435390.2018.1557272)

**Paper IV. Effects on *Caenorhabditis elegans* antioxidant defense and reactive oxygen species (ROS) metabolism following multigenerational exposure to $AgNO_3$ or NM300K Ag NPs** (Manuscript - Rossbach, L.M., Oughton, D.H., & Brede, D.A.)

# Abbreviations

| | |
|---|---|
| Ag | Silver |
| Ag NP | Silver nanoparticles |
| Da | Dalton, atomic mass unit |
| DLS | Dynamic light scattering |
| ECx | Effective concentration x |
| GFP | Green fluorescent protein |
| GSH | Glutathione |
| GR | Glutathione reductase |
| GSSG | Glutathione disulfide |
| GST | Glutathione s-transferase |
| GPX | Glutathione peroxidase |
| ICP-MS | Inductively coupled plasma mass spectrometry |
| ICP-OES | Inductively coupled plasma optical emission spectroscopy |
| LMM | Low molecular mass |
| LOEC | Lowest observed effect concentration |
| MHRW | Moderately hard reconstituted water |
| NMBU | Norwegian University of Life Sciences |
| NOEC | No observed effect concentration |
| NOM | Natural organic matter |
| PVP | Polyvinylpyrrolidone |
| ROS | Reactive oxygen species |
| SOD | Superoxide dismutase |
| spICP-MS | Single particle ICP-MS |

| | |
|---|---|
| TEM | Transmission electron microscopy |
| YFP | Yellow fluorescent protein |

# 1. Introduction

## 1.1. Background

Toxicology deals with negative impacts of chemicals and toxicants on humans, organisms and the environment, and is often traced back to Paracelsus. Paracelsus coined the phrase "*Solely the dose determines that a thing is not a poison*" (Sola dosis facit venenum) introducing the concept of dose and dose response. Concentration and exposure time are two major aspects of toxicology, both of which determine the effects of single chemicals, and thereby criteria for assessing the risk of specific chemicals (Walker, 2006). In recent years, nanotoxicology has emerged as a sub-field of toxicology, which aims to identify the link between the nanoparticle physicochemical characteristics, such as size, surface properties and charge, and their toxicity (Donaldson *et al.*, 2004, Jiang *et al.*, 2009).

The term "nano" originates from the Greek word for "dwarf". Richard Feynman is by many acclaimed for predicting the rise of the innovative field of nanotechnology in a talk given back in 1959 "*There is plenty of room at the bottom: An invitation to enter a new field of Physics*" (Khan *et al.*, 2017), recognizing the unique properties of materials manipulated at the atomic scale. Today, modern nanotechnology is defined as "*creating products and applications based primarily upon the synthesis of molecules in the nanoscale ($10^{-9}$ m) size range*" (Warheit, 2018). The changing properties of a material when its size range falls below 100 nm, and hence the manipulation of material properties, makes nanotechnology an industrially important field of research, with an interesting range of applications and uses.

Although nanomaterials occur naturally, incidental and manufactured nanomaterials attract the main focus in terms of health and safety of humans, for instance occupational workers and consumers, as well as adverse effects on the environment (Hunt *et al.*, 2013, Lazareva and Keller, 2014, Warheit, 2018). Naturally occurring nanomaterials, such as from volcanic eruptions, and incidentally produced, such as by-products of combustion processes, are often termed ultrafine particles, and have a tendency to be heterogeneous in terms of their physical and chemical properties. On the other hand, engineered nanomaterials are designed with physicochemical properties for a specific function.

In the early 2000's, more than 35 countries initiated research into nanoscale production (Roco, 2003). This was followed by a steady increase in nanomaterial production, with

around 4000 registered nanomaterials listed in the Nanowerk database to date (Nanowerk, 2019). In 2005 the total global investment in nanotechnologies was approximately $10 billion globally, and expected to increase to 1 trillion by 2011 - 2015. In 2004, around $10^3$ tons was produced annually, with a further predicted increase to $10^4$ – $10^5$ tons annually by 2010 (Science Policy Section, 2004, Harrison, 2007, Navarro *et al.*, 2008a). Production of nanomaterials is estimated to triple by 2020, where metal oxide nanoparticle production alone is estimated to increase to up to 1.6 million tons annually by 2020 (Forster *et al.*, 2011, Piccinno *et al.*, 2012, Future Markets, 2017, Sun *et al.*, 2017, Lead *et al.*, 2018). Due to vast differences in the way market research quantifies nanomaterials applied by producers, uncertainty by producers on the exact amounts used, as well as a lack of comparative historical data, the estimation of exact quantities of nanomaterials currently used in production is difficult (Piccinno *et al.*, 2012, Giese *et al.*, 2018).

The increasing use and application makes it necessary to define and categorize nanomaterials. Governmental organizations as well as industries have made efforts to define nanomaterials, resulting in a wide range of available definitions to date (Boverhof *et al.*, 2015). The International Organization for Standardization (ISO) defines nanomaterials as a "*Material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale"*, with an additional definition of "nanoscale" lying in the size range of 1 – 100 nm (ISO, 2015). The European Commission on the other hand, has included additional factors to the size limit, in their definition: *"A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm – 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 % and 50 %" (European Commission, 2011).* Common to all definitions is that, for a particle to be considered "nano", one of its dimensions must be below 100 nm (Boverhof *et al.*, 2015). However, critics point to inconsistencies between different definitions, as well as the strong focus on the size limit of 100 nm, which has little theoretical or environmental relevance (Boverhof *et al.*, 2015). It has been proposed that, for definitions to be relevant for risk assessment, factors like distribution

threshold, size-dependent properties, the state of agglomeration/de-agglomeration and aggregation/disaggregation should be included (Boverhof et al., 2015).

Due to their small size, meaning a higher surface to volume ratio, nanomaterials have increased reactivity and different surface chemistry compared to their larger particles of the same material, possibly leading to adverse effects on humans and organisms (Brown *et al.*, 2000). Therefore, regulatory agencies are confronted with the question about whether the nanoparticulate form of a compound needs to be managed differently than in its bulk or dissolved phases (Hund-Rinke *et al.*, 2016).

## 1.2. Silver nanoparticles

Because of their antibacterial properties, silver nanoparticles (Ag NPs) are a versatile material, used in a wide range of products, for instance as wound dressing, in sports equipment, as well as baby products (Fung and Bowen, 1996, Park *et al.*, 2009, Nowack, 2010). Most of the nano silver released into the environment originates primarily from textiles, with additional sources from cleaning agents, cosmetics and medical products (Giese *et al.*, 2018). Nevertheless, although environmental releases of Ag NPs, from for instance textiles (Völker *et al.*, 2015), has been shown experimentally, data on actual environmental release scenarios is scarce (Gottschalk *et al.*, 2013). Difficulties arise from a lack of appropriate analytical techniques able to distinguish Ag NPs from naturally occurring particles and colloids, as well as the low concentrations in environmental media, hindering accurate quantification (Sun *et al.*, 2016).

Attempts have been made to estimate fluxes using modelling tools. Giese *et al.* (2018) calculated the annual production of Ag NPs to be < 1000 tons. Modelled release data predicted environmental concentrations in freshwater systems in the pg to ng $L^{-1}$ range, with a roughly 2 - 6 fold estimated increase by 2050 (Giese *et al.*, 2018). For agricultural soils, however, modeled concentrations range between 30 pg/kg (minimum in 2017) up to 10 µg/kg soil (maximum in 2015) (Giese *et al.*, 2018). However, a large uncertainty within these modeled estimations is acknowledged. Sun *et al.* (2016) calculated highest releases of Ag NPs from electronics and appliances with ~38 % of the total nano Ag application and ~25 % from textiles, collecting in landfills and sediment. However, estimates presented should be considered with care, as models used do not consider chemical transformations and dissolution of the NPs, which are of particular importance in the case of Ag NPs (Sun *et al.*, 2016).

## 1.3. Silver nanoparticle toxicity to biota

Despite high uncertainties about actual environmental concentrations, previous knowledge about the toxic properties of the ionic forms of different materials, led to increasing concern about the potential adverse effects of nanomaterials (Oberdörster *et al.*, 2005, Piccinno *et al.*, 2012). In response, the number of published studies on nanoparticle toxicity has increased by 600 % over the last decade (Vazquez-Muñoz *et al.*, 2017). Therefore, obtaining detailed knowledge about the potential toxic effects and toxic mechanisms of such exposures is of clear interest. Despite being one of the most intensively studied nanomaterials to date, controversy still exists about the toxic properties of Ag NPs, with results differing or even contradicting each other (Vazquez-Muñoz *et al.*, 2017). However, the potential environmental hazard of Ag NPs is attributed to the well-known antibacterial properties of Ag, together with their ability to produce reactive oxygen species (ROS) on the surface of the particles (Vazquez-Muñoz *et al.*, 2017). The antibacterial action of Ag NPs has been attributed, almost entirely, to $Ag^+$ releases by the NPs, as well as the production of reactive oxygen species by the NPs, making factors like shape, coating or size of the NPs secondary in terms of toxicity towards bacteria (Kim *et al.*, 2007, Xiu *et al.*, 2011, Durán *et al.*, 2016). Nevertheless, with increasing complexity of organisms, the mechanisms of the toxicity is not as clear cut, where ion toxicity does not solely explain toxic response (Lead *et al.*, 2018). Therefore, while many studies conclude that toxicity can be solely attributed to ionic releases a range of studies have found particle specific effects, or a combination of the two (Navarro *et al.*, 2008b, Kim *et al.*, 2009, Fabrega *et al.*, 2011, Beer *et al.*, 2012, Choi *et al.*, 2018). This further highlights the high influence of the particle characteristics on toxicity.

Available studies are hard to compare due to differences in exposure media, choice of organisms, test conditions, and different Ag NPs with varying shape, size, charge or coating, all characteristics that impact NP behavior (Vazquez-Muñoz *et al.*, 2017). Nevertheless, Ag NPs appear to be toxic to most organisms, with a LOEC of 5 µg Ag $L^{-1}$ in the freshwater clam *Sphaerium corneum*, an acute EC50 of 121 µg Ag $L^{-1}$ for *Daphnia magna*, and 8.95 and 13.9 µg Ag $L^{-1}$ for *D. pulex and D. galeata*, respectively (Li *et al.*, 2010a, Völker *et al.*, 2013, Ahn *et al.*, 2014, Völker *et al.*, 2015). However, most studies report higher toxicity from Ag ions, with an EC50 for *D. pulex* of 0.68 µg Ag $L^{-1}$, and 2.13 µg Ag $L^{-1}$ for *D. galeata* (Völker et al., 2013*)*, and the lowest NOEC values for Daphnia

spp of 0.001 µg Ag $L^{-1}$ (Bielmyer *et al.*, 2002). Völker *et al.* (2013) relate differences in toxicity back to the coatings of the particles, for instance PVP, preventing particle dissolution, and hence a lower ionic fraction in the exposure. However, following the ingestion, Ag NPs may either exert their toxicity directly in the intestine, or be taken up into cells and transferred across different tissues. Furthermore, Ag NPs often show high dissolution, leading to localized ionic releases (Völker *et al.*, 2015).

Additional toxic mechanisms of Ag NPs can be related to their high affinity to sulfur. Ag (either ionic or particulate) can have direct interaction with macromolecules, possibly leading to conformational changes, including protein unfolding or the adsorption of proteins onto the surface of the particles, also referred to as the formation of a protein corona (Choi *et al.*, 2009, Liu *et al.*, 2011, Saptarshi *et al.*, 2013). Changes in the protein structure have the potential to impact downstream protein-protein interactions, cellular signaling or DNA transcription (Saptarshi *et al.*, 2013). Further, direct interaction of the nanoparticles with membrane proteins and active signaling pathways may lead to inhibition of cell proliferation (Asharani *et al.*, 2009, Gopinath *et al.*, 2010, Roh *et al.*, 2012). Ag may also bind to iron sulfur clusters and inhibit enzyme activities, with silver sulfide precipitation, resulting in succinate dehydrogenase inhibition, consequently leading to further metabolic interferences (Ghandour *et al.*, 1988). Moreover, the binding of Ag to sulfhydryl (thiol) groups has been shown to promote iron releases, leading to the formation of hydroxyl radicals (Gordon *et al.*, 2010).

## 1.4. Reactive oxygen species and oxidative stress

Most organisms require oxygen ($O_2$) to live, but the partial reduction of the $O_2$ may lead to the formation of ROS. In eukaryotic cells, ROS are continuously produced by the mitochondria (Murphy, 2009, Lushchak, 2011). This production of ROS is continuously counterbalanced by a finely tuned antioxidant defense system, in order to avoid oxidative stress (Livingstone, 2003, Murphy, 2009). Exposure to a wide range of environmental stressors, including Ag NPs (Hwang *et al.*, 2008), UV radiation or herbicides (such as paraquat)(Suntres, 2002), may potentially induce an additional production of ROS. An imbalance between ROS production and ROS neutralization results in an imbalance in the redox status of a cell. Hence, a disturbance in the redox homeostasis may lead to the formation of oxidative damage to biomolecules, including protein, lipids and nucleic acid, as well as interferences in cellular signaling mechanisms (Ray *et al.*, 2012).

Although ROS are natural byproducts of cellular oxidative metabolism, in general, ROS are considered to be "*unwanted and toxic by-products of living in an aerobic environment*" (Finkel, 1998), and to have destructive properties (Lushchak, 2014, Abdal Dayem *et al.*, 2017). However, studies show that ROS may also play a significant part in maintaining cellular homeostasis (Finkel and Holbrook, 2000). Furthermore, ROS have been involved in regulating proliferative responses, fighting certain infections, or functioning as a signaling molecule (Finkel, 1998, Finkel and Holbrook, 2000, Dröge, 2003).

Cellular ROS levels are strongly regulated by the antioxidant defense system, including enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT), as well as antioxidants such as flavonoids, ascorbic acids, vitamin E and glutathione (GSH) (Wu *et al.*, 2014). Oxygen radicals like superoxide ($O_2^-$) are highly reactive, have a short biological lifespan (nano to micro seconds), and need to be rapidly reduced by SOD into $H_2O_2$ , which may then be sequestered into water ($H_2O$) and oxygen ($O_2$) by the enzymes CAT and GPX (Figure 1) (Abdal Dayem *et al.*, 2017, Braeckman *et al.*, 2017).

The superoxide dismutase enzymes are important components of the antioxidant defenses, found in nearly all oxygen exposed cells (Abdal Dayem *et al.*, 2017). The SOD enzymes act as a catalyst for the dismutation of superoxide anions ($O_2^-$), a process where one electron of the $O_2^-$ is transferred to another $O_2^-$, producing a molecule of hydrogen peroxide ($H_2O_2$) and oxygen ($O_2$) (Lumb, 2017). SODs are classified by their metals, iron (Fe) or manganese (Mn), copper (Cu) and zinc (Zn), or nickel (Ni), used for stability, catalysis and structure (Case, 2017). In *C. elegans,* there are five forms of SOD, with SOD-1, SOD-4 and SOD-5 being Cu/ZnSOD isoforms, and SOD-2 and SOD-3 being mitochondrial MnSOD isoforms (McCord and Fridovich, 1969, Hoogewijs *et al.*, 2008, Braeckman *et al.*, 2017).

Furthermore, increases in ROS are mitigated by expenditure of GSH as an electron donor for antioxidant enzymes. This leads to an oxidation of the reduced GSH into its oxidized form, glutathione disulfide (GSSG) (Figure 1) (Braeckman *et al.*, 2017). Organic hydroperoxides and hydrogen peroxides are broken down by glutathione s-transferase (GST) and GPX, using GSH as a reducing agent (Braeckman *et al.*, 2017).

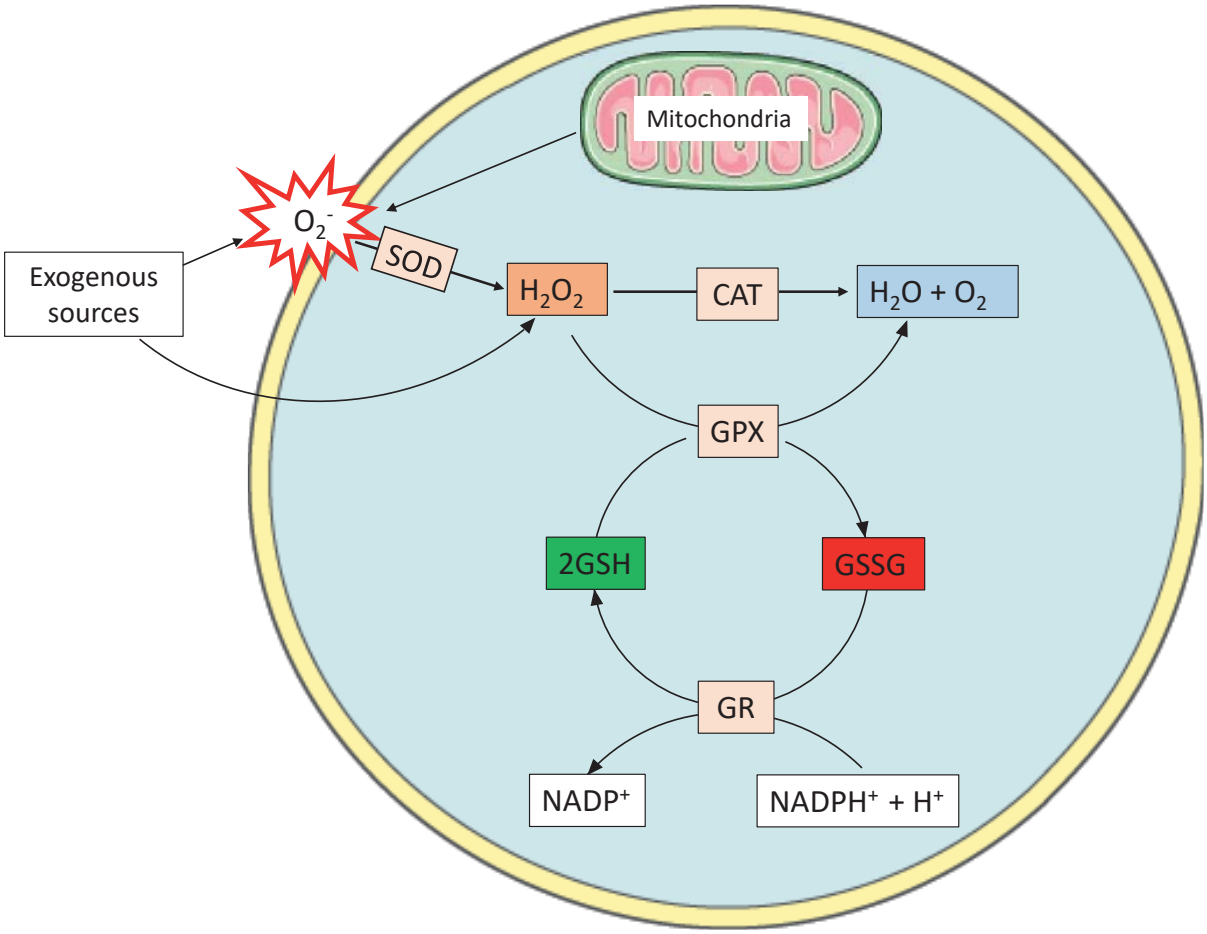



**Figure 1:** Schematic representation of the production and removal of ROS by antioxidant defense systems in the nematode *C. elegans.* Abbreviations are explained in the text.

## 1.5. Silver nanoparticles and reactive oxygen species

Chemically speaking, any compound can potentially be an oxidizing agent by accepting electrons (Kermanizadeh *et al.*, 2015). The compound donating the electrons becomes oxidized, while the oxidizing agent is reduced. Ionic Ag is known to produce ROS, potentially leading to oxidative stress (Cortese-Krott *et al.*, 2009, Park *et al.*, 2009). It has been suggested that Ag ions impair enzymes in the respiratory chain, and therefore increase cellular superoxide radicals (Park *et al.*, 2009). Further, it has been shown that Ag NPs may produce free radicals on the surface of the particles (Hwang *et al.*, 2008, He *et al.*, 2012a, He *et al.*, 2012b, Ribeiro *et al.*, 2015, Choi *et al.*, 2018). Structural modifications, as well as alterations in the electronic properties on the surface of the particles result in the formation of reactive groups (Donaldson and Tran, 2002, Oberdörster *et al.*, 2005). The reaction of Ag NPs with oxygen will produce $H_2O_2$ (Liu and Hurt, 2010, He *et al.*, 2011). On the other hand, Ag NP may act as a catalyst to break down

$H_2O_2$ leading to superoxide ($O_2^{\cdot-}$) production (Endo *et al.*, 2008, Guo *et al.*, 2008, He *et al.*, 2011). Therefore, both Ag NPs and silver ions may lead to the production of superoxide anions ($O_2^-$) and peroxide radicals ($O_2^{-2\cdot}$), as well as hydroxyl radicals ($^{\cdot}OH$), hydrogen peroxide ($H_2O_2$) and singlet oxygen ($^1O_2$) (Hwang *et al.*, 2008, He *et al.*, 2012a, He *et al.*, 2012b, Choi *et al.*, 2018). The formation of $^{\cdot}OH$ by Ag, either particulate or ionic, has a high potential for DNA damages, through the interaction of the free radical with the DNA forming 8-hydroxyl-2'-deoxyguanosine (8-OHdG) (Valavanidis *et al.*, 2009, Song *et al.*, 2012). ROS production by nanoparticles is a factor of characteristics including size, charge, surface area and chemical structure, and may lead to a sequence of pathological events such as inflammation, fibrosis, genotoxicity and carcinogenesis (Shvedova *et al.*, 2012, Abdal Dayem *et al.*, 2017).

Ag NPs have been shown to induce oxidative stress in a wide range of species, including zebrafish (Choi *et al.*, 2010, Massarsky *et al.*, 2013), *C. elegans* (Roh et al., 2009, Lim et al., 2012a, Roh et al., 2012), nitrifying bacteria (Choi and Hu, 2008), mice (Song *et al.*, 2012) and duckweed (Jiang *et al.*, 2014). Jiang *et al.* (2014) measured an increase in SOD activity in duckweed, when exposed to 6 and 20 nm Ag NPs. Similar increases in superoxide dismutase activity were found in *Escherichia coli*, however these were attributed to ionic releases (Hwang *et al.*, 2008). Nevertheless, in the freshwater clam *Sphaerium corneum,* SOD activity was unaffected by the exposure to PVP coated Ag NPs (15 nm), and $AgNO_3$ (Völker *et al.*, 2015). Moreover, results showed moderate changes in GST and GPX activities (Völker *et al.*, 2015). In *C. elegans,* reproductive failure resulting from Ag NP exposure (20 – 30 nm), has been related to ROS formation and oxidative stress manifestation (Roh *et al.*, 2009, Lim *et al.*, 2012b). Furthermore, Roh *et al.* (2009) showed that the production of ROS and Ag NPs (20 nm) induced the expression of the *sod-3* gene in *C. elegans*. Citrate coated Ag NPs (26.2 ± 7.6 nm) caused a rapid depletion of reduced cellular GSH, and apoptosis in mice (Lee *et al.*, 2014b).

## 1.6. Adaptation towards stressors

More and more research is focused on the stress, and acclimation and adaptive responses of organisms towards stressors (Bijlsma and Loeschcke, 2005). Environmental stress results from changes of abiotic factors, such as temperature, climate factors or chemical components (Lindgren and Laurila, 2005, Sorensen *et al.*, 2005). A wide range of possible adaptive processes, such as morphological, behavioral, physiological, neuro-endocrine,

blood biochemistry, metabolic, molecular and cellular responses promote the survival of an organism in an environment under stressful conditions (Bijlsma and Loeschcke, 2005, Sejian *et al.*, 2018). Therefore adaptation may be defined as "*the process of change in an organism to conform better with (new) environmental conditions, whereby the organism (or group of organisms) acquires characteristics, involving changes in morphology, physiology or behaviour, that improve their survival and reproductive success in the particular environment*" (Bijlsma and Loeschcke, 2005). This adaptive process, however, includes both the acclimation of an individual organism within its life span towards a stressor, as well as adaption across generations by a population (Sun *et al.*, 2014). Therefore, studies to date use the term "adaptation" interchangeably between single organisms and changes across multiple generations of a population. In the current work, a distinction was made, where the term adaptation refers to changes in the toxic response towards a stressor, developed by a population across multiple generations. Changes in sensitivity within the lifespan of the same organism are referred to as acclimation, i.e. the adaptation of an organism towards a secondary stressor, following the exposure to a primary stressor.

Acclimation processes in *C. elegans* have been shown by Zhao and Wang (2012). The ability to increase the resistance towards a stressor is highly governed by the life stage of the exposed organism. Further, the duration of the primary exposure, and the concentration and duration of the secondary exposure will govern changes in sensitivity towards the stressor (Zhao and Wang, 2012). On the other hand, several laboratory based studies show the adaptive abilities of organisms to man-made stressors, such as temperature (Hoffmann *et al.*, 2003), uranium (Dutilleul *et al.*, 2014), cadmium (Muyssen and Janssen, 2004), quantum dots and cadmium salts (Contreras *et al.*, 2014), and methylmercury (Helmcke and Aschner, 2010), over few generations. However, there are still questions about the dynamics of the underlying processes at play (Bijlsma and Loeschcke, 2005).

It has been suggested that a dose of roughly 25 % of the minimum lethal dose of an agent is necessary to induce increased resistance towards that same agent (Calabrese and Baldwin, 1997a, Calabrese and Baldwin, 1997b). Cypser and Johnson (2002a) demonstrated the ability of *C. elegans* to rapidly develop an acclimation response, stemming from the exposure to multiple stressors (heat, oxygen and juglone), towards

the exposure to more severe challenges by the same agent. Furthermore, a cross tolerance development was shown where the exposure to the xenobiotic juglone, increased the resistance towards $O_2$ exposure (Cypser and Johnson, 2002a). A range of other studies have reported a similar cross tolerance development within the lifespan of the same organism. The pre-treatment to UV irradiation resulted in an increased ability of the nematodes to withstand exposure to mercury, lead and chromium, with a significant reduction in oxidative damages and the prevention of locomotive defects (Wang *et al.*, 2010). Similarly, UV irradiation pre-treatment reduced the toxic effects of cadmium exposure (Wang and Xing, 2010). Heat pretreatment of maize seedlings resulted in decreased sensitivity towards chilling, heat, drought and salt stress of the same seedlings (Gong *et al.*, 2001, Gibson *et al.*, 2017). It has been suggested that the mechanism underlying the cross tolerance response, is related to changes in the $H_2O_2$ signaling (Gong *et al.*, 2001). Overall, findings suggest that the acclimation process is not governed by the physical nature of the stressor, but by the physiological and mechanistic responses of the organism, including antioxidant defenses, stress protein induction, signaling pathway modulation and metabolic regulation, responses that are shared by several stressors (Zhao and Wang, 2012, Gibson *et al.*, 2017).

It has been suggested that oxidative stress induction by low dose exposure of a toxicant is underlying the mechanism of a decrease in sensitivity to higher doses of the same or other oxidative stress inducing agents (Zhao and Wang, 2012). Increase in the antioxidant defense system may be a result of an increase in ROS formation by the mitochondria, leading to an acclimation response, which may in turn accumulate across generations leading to long-term reductions in oxidative stress (Zhao and Wang, 2012). Young nematodes have been shown to have an increased ability to acclimate to oxidative stress (induced by either the quinone plumbagin or hypoxia) by increasing their superoxide dismutase (SOD) activity, while more mature/older nematodes were not able to adjust their antioxidant defenses in such a manner (Darr and Fridovich, 1995). However, a study by Yanase *et al.* (1999) showed that pre-treatment by hyperoxia of *C. elegans* did not affect the gene expression of *sod-1* and *sod-3,* demonstrating the specificity of such acclimation responses.

## 1.7. Ag NP multigenerational studies

Conventional toxicity testing will often be limited in terms of duration of the exposure. Most of the exposure scenarios only cover a limited number of life stages of an organism, making predictions on chronic long-term exposures difficult (Goussen *et al.*, 2013). Multigenerational exposure studies, particularly studies focusing on nanoparticles, although available, remain scarce, due to time constrains. Many model organisms used in toxicological studies, have a long maturation time, making the exposure of multiple generations tedious. However, to assess the potential for adaptation on an ecologically relevant time scale, it would be necessary to cover at least four generations of exposure, to avoid acclimation effects (Muyssen and Janssen, 2004).

The multigenerational exposure studies that are available to date, cover a variety of stressors and organism, ranging from experiments on ocean acidification, diet, antibiotics and metals, on copepods, rats, daphnia and earthworms (Flynn *et al.*, 2000, Andre *et al.*, 2009, Kim *et al.*, 2012, Völker *et al.*, 2013, Pedersen *et al.*, 2014) (table 1). Despite Ag NPs being amongst the most widely studied nanomaterials, only four studies have been carried out to date, where three focus on the multigenerational exposure of *C. elegans* (Contreras *et al.*, 2014, Luo *et al.*, 2016, Schultz *et al.*, 2016), and one on daphnia species (Völker *et al.*, 2013). In a recovery study, Luo *et al.* (2016) primarily focused on the trophic transfer of the PVP coated Ag NPs (25 nm, -12.6 ± 0.99 mV, and 75 nm, -25.7 ± 2.02 mV), from *E. coli* to *C. elegans*, and the transfer of effects up to the unexposed F4 generation. A higher toxicity, in terms of apoptosis, total brood size, life span and population size, from the 25 nm Ag NPs towards *C. elegans* was found. The parental nematodes, fed with *E. coli* containing Ag NPs showed significant germ cell death. A transfer of effects was seen in subsequent unexposed generations, with a recovery only observed at the F3 generation for the 75 nm Ag NPs, and the F4 generation for the 25 nm Ag NPs (Luo *et al.*, 2016). In contrast, the multigenerational Ag NP exposure study by Schultz *et al.* (2016) concluded that nematodes did not recover from the increased sensitization resulting from the PVP coated Ag NP (58.3 ± 12.9 nm, -11.6 mV) exposure across generations, even when exposure was removed. Nematodes were continuously exposed for 10 generations, and changes in total brood size, growth and lifespan in toxicity tests (Simulated Soil Pore Water) were measured (Schultz *et al.*, 2016). The authors suggest that possible epigenetic inheritance may play a role in the lack of

recovery across generations (Schultz *et al.*, 2016). Similarly, Contreras *et al.* (2014) found a size dependent increase in sensitivity when measuring life span and fertility across four generations of exposure of *C. elegans* to three different sized Ag NPs (2, 5 and 10 nm). Only growth and neurodegenerative endpoints showed evidence of adaptation at lower (1 and 10 mg $L^{-1}$) concentrations (Contreras *et al.*, 2014). Overall, these studies showed limited adaptive abilities of *C. elegans* towards Ag NP exposure across generations, with increased sensitization and heritable effects dominating the findings. Nevertheless, results from Contreras *et al.* (2014) support the notion that nematodes are in fact able to develop an adaptation, however, this is highly dependent on concentrations.

**Table 1:** Number of multigenerational exposure studies available to date.

| Search engine | Multigenerational studies | Multigenerational *C. elegans* studies | Multigenerational nanoparticle studies | Multigenerational Ag NP studies | Multigenerational Ag NP *C. elegans* studies |
|---|---|---|---|---|---|
| **Google scholar** | 1,280 | 66 | 38 | 4 | 3 |
| **Web of science** | 76 | 11 | 2 | 1 | 1 |
| **Oria** | 336 | 20 | 4 | 1 | 1 |

## 1.8. Aims and Objectives

Based on previous findings on Ag NP toxicity, the overall aim of the current work was to increase the understanding of the different aspects of the toxicity of the reference Ag NP NM300K towards the nematode *C. elegans*, in comparison to $AgNO_3$. This included single and multigenerational exposure studies, as well as investigations into the role of ROS production, changes in the antioxidant defenses and oxidative stress development in the toxicity mechanisms. The work was centered around three linked hypotheses:

1. NM300K Ag NPs would be toxic to *C. elegans*, but the relatively low dissolution of these NPs would make these less toxic than $AgNO_3$.
2. Multigenerational exposure will lead to an adaptation towards Ag NPs at lower concentrations, but an increase in sensitization at higher concentrations.
3. ROS production involved in the toxic mechanisms of Ag NPs, would trigger antioxidant defenses following exposure to the Ag NPs, and changes in toxic responses across generations could be related to these defense mechanisms.

To address these, the following tests and experiments were set up:

I. To characterize the toxic effect of the reference Ag NPs NM300K compared to $AgNO_3$ in the nematode *C. elegans* (Paper I and II).

II. To investigate the reproducibility of Ag NP toxicity tests with *C. elegans*, using $AgNO_3$ as a positive control, and link the characterization of Ag in test media to the toxicity (Paper I).

III. To examine the changes in toxic response towards Ag (either NPs or $AgNO_3$), and a range of other stressors (Ce, Cd, Cu, Ce-NPs and paraquat), following the chronic multigenerational exposure to either form of Ag, in *C. elegans* (Paper III and IV).

IV. To monitor changes in antioxidant defenses and oxidative stress manifestation in the nematode, in response to NM300K or $AgNO_3$ exposure, and attempt to link these to changes in the toxic response following the multigenerational exposure towards either form of Ag (Paper II and IV).

# 2. Methodology

## 2.1. General outline

To address the objectives of the current PhD research, a range of exposures were set up (Figure 2). All exposures were conducted using the model organism *C. elegans,* the EU reference Ag NP NM300K, and $AgNO_3$ as a positive control. The nanoparticles were characterized for size distribution, electrostatic stability, size, and particle shape, as well as, changes in the size distribution of Ag as a function of time in the exposure media and the presence of organisms. A multigenerational exposure scenario was set up to test for changes in the response towards both forms of Ag. The toxic response was assessed in standard 96 h toxicity tests following the ISO guideline, performed within each generation of the exposure. Changes in growth, fertility and reproduction were measured at the end of each toxicity test. At the end of the six generational exposure, nematodes were additionally exposed to a range of other metal cations, another nanoparticle type and a known ROS inducer, in order to test for possible changes in toxic response towards other toxicants. Due to indications of changes in the antioxidant defenses, another multigenerational exposure scenario was set up with a GFP labelled superoxide dismutase-*1* gene expression reporter, and a glutathione biosensor strain. The methods are outlined below, however, a more technical and detailed description can be found in the corresponding papers.

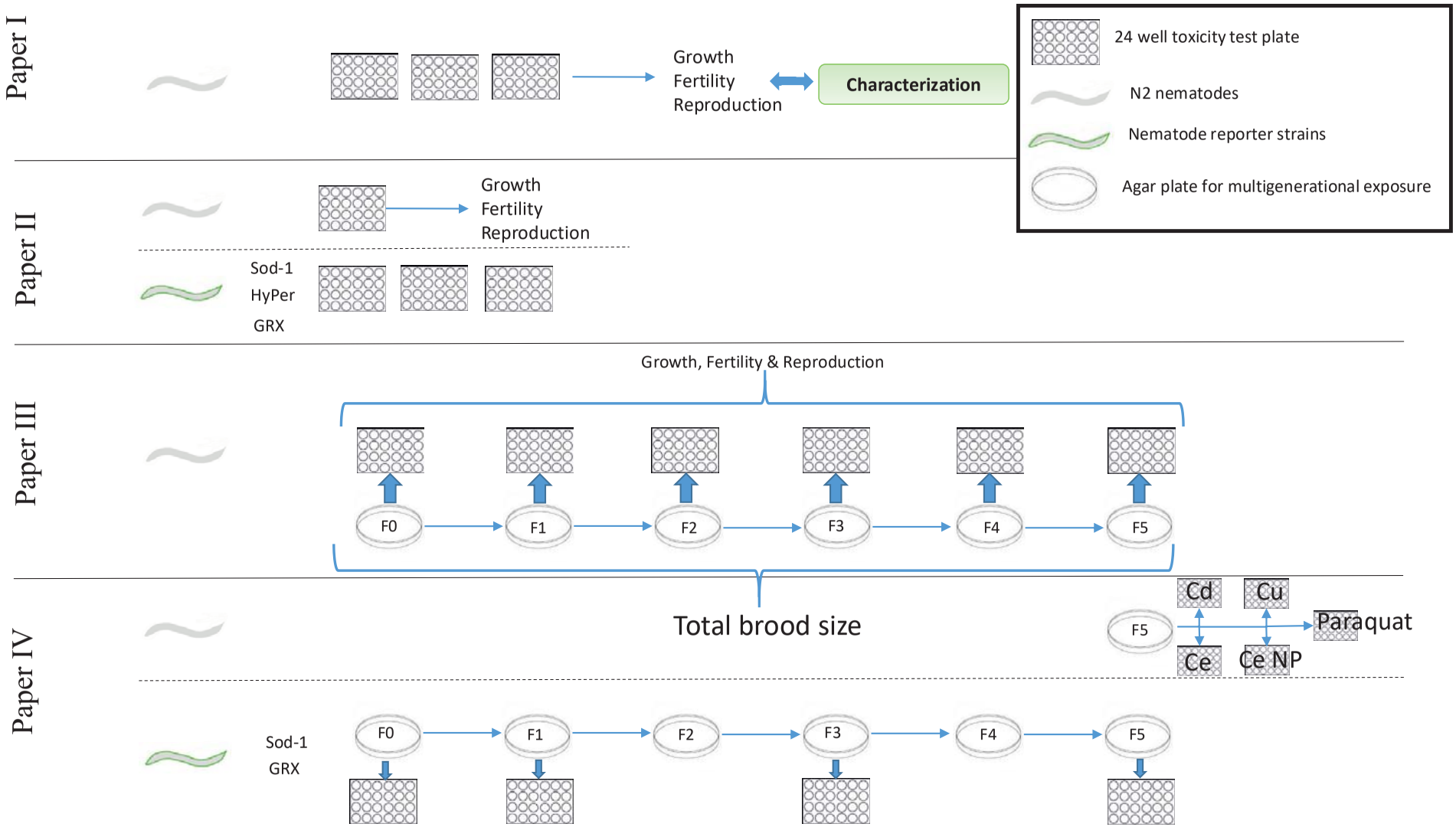



**Figure 2:** Overview of the different exposure set ups performed.

## 2.2. NM300K stock preparation and characterization

Due to their high antibacterial properties, Ag NPs are of great concern in terms of environmental releases or the exposure to organisms and their toxic effects. For the present work, the EU reference material NM300K Ag NPs were chosen, partly to contribute to the knowledge base of this well-characterized material, but also because of their low dissolution, providing the possibility to measure NP specific effects.

### 2.2.1. Stock preparation of the NM300K stock suspensions

All stocks for all exposures were prepared in dd$H_2O$ (15 MΩ cm) water. For stocks in Papers I, II and III, the NANoREG standard operating procedure for stock suspension of the NM300K Ag NPs was followed (Jensen *et al.*, 2016). In Paper IV, however, the SOP was adjusted to reduce the concentrations of the main stocks by 10 fold. Nevertheless, for all stocks, Ag NPs were sonicated using a sonicator probe (Branson S-450 D sonicator, disruptor horn 13 mm). All subsequent working solutions and exposure stocks, as well as those for TEM analysis, were directly diluted from the initial stock in dd$H_2O$.

### 2.2.2. Nanoparticle characterization

Due to a lack of understanding on the exact relationship between the characteristics of nanoparticles and their toxicity, it is essential to fully characterize the nanoparticles throughout the exposure (i.e. prior to the administration and during the exposure) to obtain meaningful and reproducible results (Navarro *et al.*, 2008a, Jiang *et al.*, 2009, Köser *et al.*, 2017). Nevertheless, it is difficult to cover all possible characteristics, and hence, a list of priority characteristics has been proposed, including measurements on the size, dispersion state, surface charge, shape, chemical composition, surface area, surface chemistry, and dissolution state (Navarro *et al.*, 2008a, Jiang *et al.*, 2009, Köser *et al.*, 2017). Despite manufacturers providing detailed information on nanoparticle physicochemical properties upon purchase, it is necessary to monitor physicochemical changes of the particles, such as agglomeration, dissolution, and surface charge variations, in the actual exposure media, and with test organisms present (Powers *et al.*, 2007, Jiang *et al.*, 2009). Absorption, distribution, metabolism, and excretion of the nanoparticles by the organisms can be affected by the size, surface charge, as well the dissolution state (Jiang *et al.*, 2009, Djurišić *et al.*, 2015, Sørensen and Baun, 2015). However, monitoring the changes of particle characteristics in the exposure media is challenging, since many of the characterization techniques are only applicable to pristine

particles. A general outline of different characterization techniques applied to stocks and working solutions in the present work is given in figure 3.

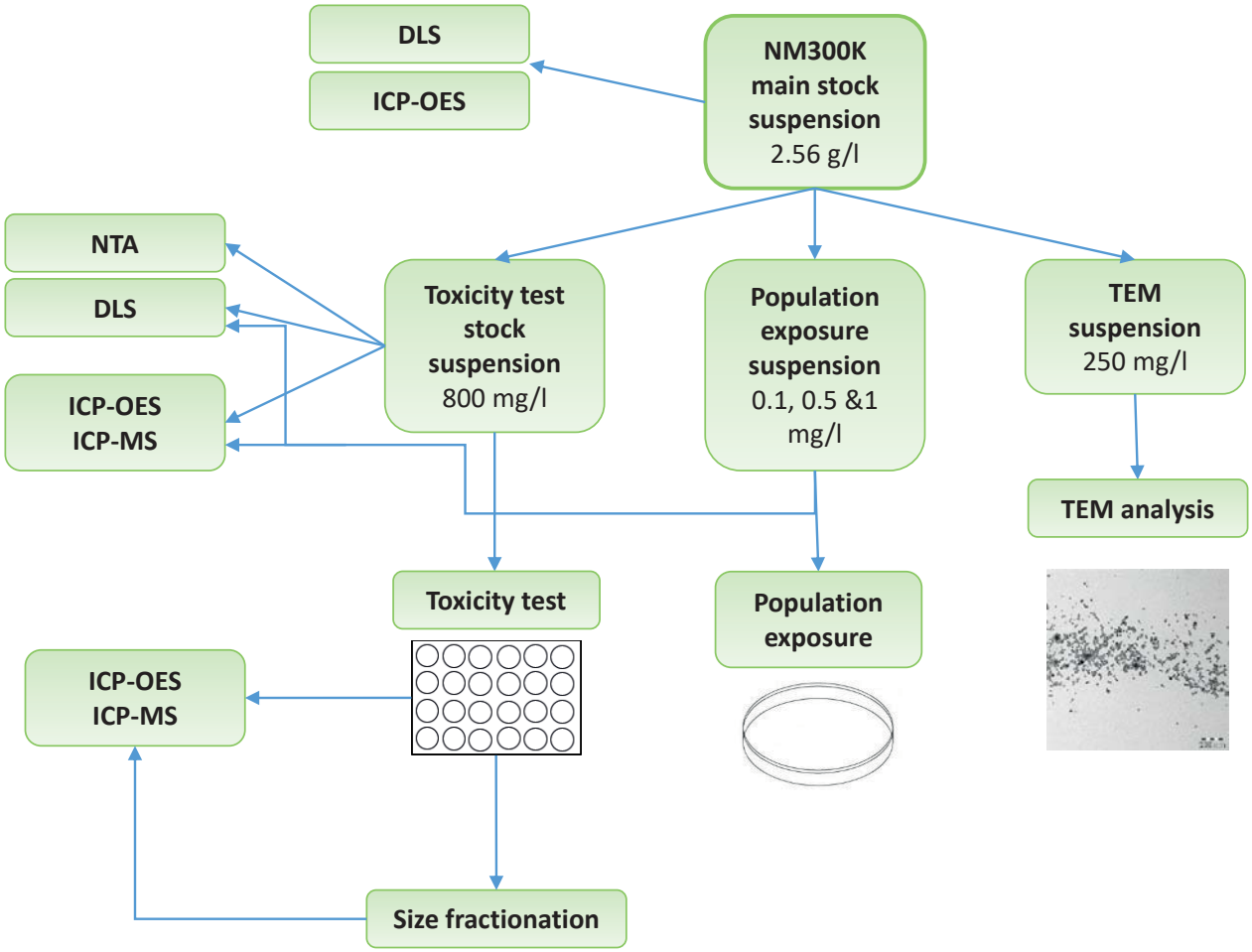



**Figure 3:** Outline of sampling and techniques for analysis of particle characterization and behavior during the exposures. Abbreviations are explained in the text.

### 2.2.3. Transmission Electron Microscopy analysis

To identify the size and shape of the NM300K Ag particles, samples were prepared for transmission electron microscopy (TEM) analysis, using the Morgagni 268 (FEI, Eindhoven, Netherlands) at an acceleration voltage of 80 keV. In TEM an electron beam is directed at a ultrathin sample and the resulting interactions are observed. If the electrons experience a high density area (including Ag NPs) within the sample, the electrons are scattered back. It is important to note that the image is created by the electrons that are transmitted through the sample, rather than the back scattered

electrons. Samples were prepared on a 400 mesh carbon coated copper grid and dried at room temperature. TEM imaging of nanoparticles is a useful characterization technique as it also provides visual evidence of the presence of nanomaterials, and allows the identification of the shape of individual monomers as well as aggregates. Nevertheless, it should be noted that artifacts due to preparation techniques may occur (Fabrega *et al.*, 2011).

### 2.2.4. Hydrodynamic diameter and zeta potential

The size distribution and zeta potential of all Ag NP stock suspensions was measured using a Malvern Zetasizer ZS (DLS – Malvern Instruments Ltd, Worcestershire, UK). The measurement is based on the principle of Brownian motion of particles, using a laser that is passed through the sample and scattered around particles and/or aggregates. The zeta potential, on the other hand, provides an indication of the stability of the particles. There are several limitations to the DLS measurements, such a low sensitivity at lower concentrations, non-selective material detection, and the inability to distinguish mixtures. Furthermore, the presence of larger aggregates or particles will be more effective at scattering the light, and hence, skewing the size distribution (Domingos *et al.*, 2009). Nevertheless it may be used as a relatively quick method, compared to, for instance, TEM analysis, to validate the stock preparation method and allow for the simple comparison of different stock suspensions (Hagendorfer *et al.*, 2012).

### 2.2.5. Centrifugation, ultrafiltration and size fractionation

Agglomeration and aggregation, as well as oxidation of Ag NPs leading to the dissolution of the Ag NPs to dissolved $Ag^{+}$ ions, are amongst the most important mechanisms impacting the interaction of Ag NPs with the organisms in a toxicity test (Lowry *et al.*, 2012, Behra *et al.*, 2013, Starnes *et al.*, 2015). Therefore, an assessment of changes in the Ag NPs colloidal dispersion in the exposure media is vital. The low molecular mass (LMM) Ag fraction in the exposure media was assessed by centrifugal ultrafiltration using Amicon Ultra-15 centrifugal filters with a 3 kDa membrane filter (Millipore). Due to the presence of the *E. coli* and nematodes, as well as larger particles in the samples, and to avoid the clogging of the 3 kDa filter units, samples were centrifuged for 5 min at 2000 g prior to ultrafiltration. A sample of the supernatant was taken and analyzed for the total Ag content; defined as the suspended Ag fraction of the samples.

To control for the possible binding of the Ag to the filter units, the 3 kDa filters were pre-conditioned by centrifugation of a sample aliquot, which was discarded before the ultrafiltration of the samples to be analyzed. Filter units were then filled with 400 µl of the sample and centrifuged for 30 min at 14000 g. The Ag content of the filtrate (<3 kDa) was analyzed by ICP-MS. Since 3 kDa corresponds to approximately ~1 nm (Sasaki *et al.*, 2006), it was assumed that this fraction contained mostly ionic or LMM Ag species.

## 2.3. Choice of organism

The nematode *C. elegans* is a free-living organism found worldwide. Although often considered to be living in the soil pore water, they may be found in rotting fruit and vegetable matter, feeding on bacteria (Corsi *et al.*, 2015). They are approximately ~1 mm in length and, due to its short life span, high fecundity, and well-annotated genome, very suitable for a wide range of exposure studies (Handy *et al.*, 2012, Goussen *et al.*, 2013). Although males may occur (at a frequency of roughly 0.2 %), *C. elegans* exist primarily as a self-fertilizing hermaphrodite (Corsi *et al.*, 2015). In the laboratory, *C.* elegans are primarily grown on agar plates, fed with the bacterium *Escherichia coli.* They will reach their reproductive adult stage (L4) within 3 days at 25 °C and will produce 280 eggs per adult in the absence of males, and up to 1000 with males present (Corsi, 2006). In response to a lack of food, they may enter an arrested larval stage, called the dauer stage, and survive without food for approximately one month. Due to their transparent body, *C. elegans* may be used for studies on individual cells and subcellular details, as well as *in vivo* studies using fluorescent labels (Corsi *et al.*, 2015).

*C. elegans* was chosen for a number of reasons as a model organism for the current work. Keeping in mind that a high amount of Ag NPs will concentrate in the terrestrial environment (Sun *et al.*, 2016, Giese *et al.*, 2018), a soil organism, such as *C. elegans*, is a well suited model for determining toxic effects of prolonged exposure towards Ag NPs. Further, living in water films and water-filled pore spaces in the soil, liquid exposure is the most common way to expose nematodes, which may also give indications of effects of the Ag NPs in the aquatic environment (Leung *et al.*, 2008, ISO, 2010). Resulting from the easy maintenance, short generation time, and self-fertilizing ability to produce a large amount of offspring, *C. elegans* has been used in a wide range of toxicological studies. Further, it has been suggested as the perfect model for toxicity testing due to its short lifespan, avoiding the aging of the particles in prolonged exposures (Handy, 2012).

Additionally, the short generation time is highly preferable for multigenerational studies (Hunt, 2017). Hence, organisms with longer maturation time may not be as suitable for such studies. Due to their small size, it is easy to maintain large cultures, and high numbers of biological replicates, thus achieving robust experimental design. *C. elegans* are a model organism for a wide range of studies, since a number of molecular signals controlling their development are also found in higher, more complex organisms, also including humans (Corsi, 2006, Markaki and Tavernarakis, 2010). Therefore, results shown in the current work may provide evidence for similar changes in other organisms.

## 2.4. Toxicity test

Age synchronized *C. elegans* were exposed in a standard 96 hrs toxicity test, according to the ISO 10872 guideline (ISO, 2010) with some modification. Traditionally, *C. elegans* exposure studies are either conducted on agar plates seeded with *E. coli* or in high ionic strength media, such as M9 or K+. However, the exposure media was changed to the US EPA moderately hard reconstituted water (MRHW) (United States Environmental Protection Agency, 2002), due to its low ionic strength, minimizing the agglomeration and aggregation of the particles, as well as, the complexation and precipitation of $Ag^+$ with, for instance, chlorides (El Badawy *et al.*, 2010). Furthermore, MHRW is considered to be a more environmentally relevant media for toxicity testing on nanomaterials (see Tyne *et al.* (2013)). Exposure stocks were directly applied to the exposure media. All plates were kept at 20 °C in the dark, on shaking plates (for appropriate aeration) throughout the entire exposure period.

## 2.5. Exposure and concentrations

All concentrations, for the toxicity test and the population exposure on agar plates, were chosen on the basis of findings from pilot experiments on a wide range of concentrations. Concentration ranges were then chosen in order to achieve similar toxic effects by both forms of Ag. Chronic Ag concentrations for the multigenerational exposure were chosen in order to elicit a minor toxic response at highest concentrations, however, avoiding major toxicity to reproduction or development of the organism.

## 2.6. Endpoints

Toxicity tests on the N2 (wild type) nematode strain, described in Papers I, II and III, were carried out according to ISO guidelines 10872, with slight modifications (ISO, 2010), and nematodes were sampled for growth, fertility, and reproduction. Following 96 h of exposure, all wells were stained with Rose Bengal, and kept at 80 °C for 10 minutes. Nematodes were then assessed for growth (total body length of the nematodes), fertility (number of gravid nematodes per total number of recovered adults), and reproduction (number of offspring per total number of recovered adults) counted using a stereo microscope (Leica M205C). Additionally, an uptake experiment was conducted, as described in Paper I, comparing the uptake and retention of the $AgNO_3$ by the nematodes to that of Ag NPs.

## 2.7. Chronic multigenerational exposure

The chronic multigenerational exposure, as described in Paper III, was set up in triplicate, for six generations. Exposures of the N2 strain were set up with three concentrations for both forms of Ag (Figure 4). Ten times concentrated *E. coli*, re-suspended in MHRW, was applied and allowed to dry on the agar plates to create an *E. coli* lawn, prior to the start of the experiment. Before each culture transfer, the appropriate Ag exposure stock suspension was applied and allowed to dry on top of the *E. coli* lawn. This was done because a high interaction between the Ag and the *E. coli* is assumed and, hence, the exposure of the nematodes living on top of the agar in the *E. coli* lawn should be assured.

Nematode populations were allowed to hatch, mature, and lay eggs on each exposure plate. Adults and eggs were then separated. Eggs were either directly applied onto exposure plates to establish the next generation (Figure 4, blue arrows), or allowed to hatch and synchronize overnight and used in the toxicity test (Figure 4, green arrows).

Population transfer times between generations were kept to 72 h for controls and $AgNO_3$ populations, however, due to delayed development, had to be adjusted accordingly for all Ag NP populations (table 2).

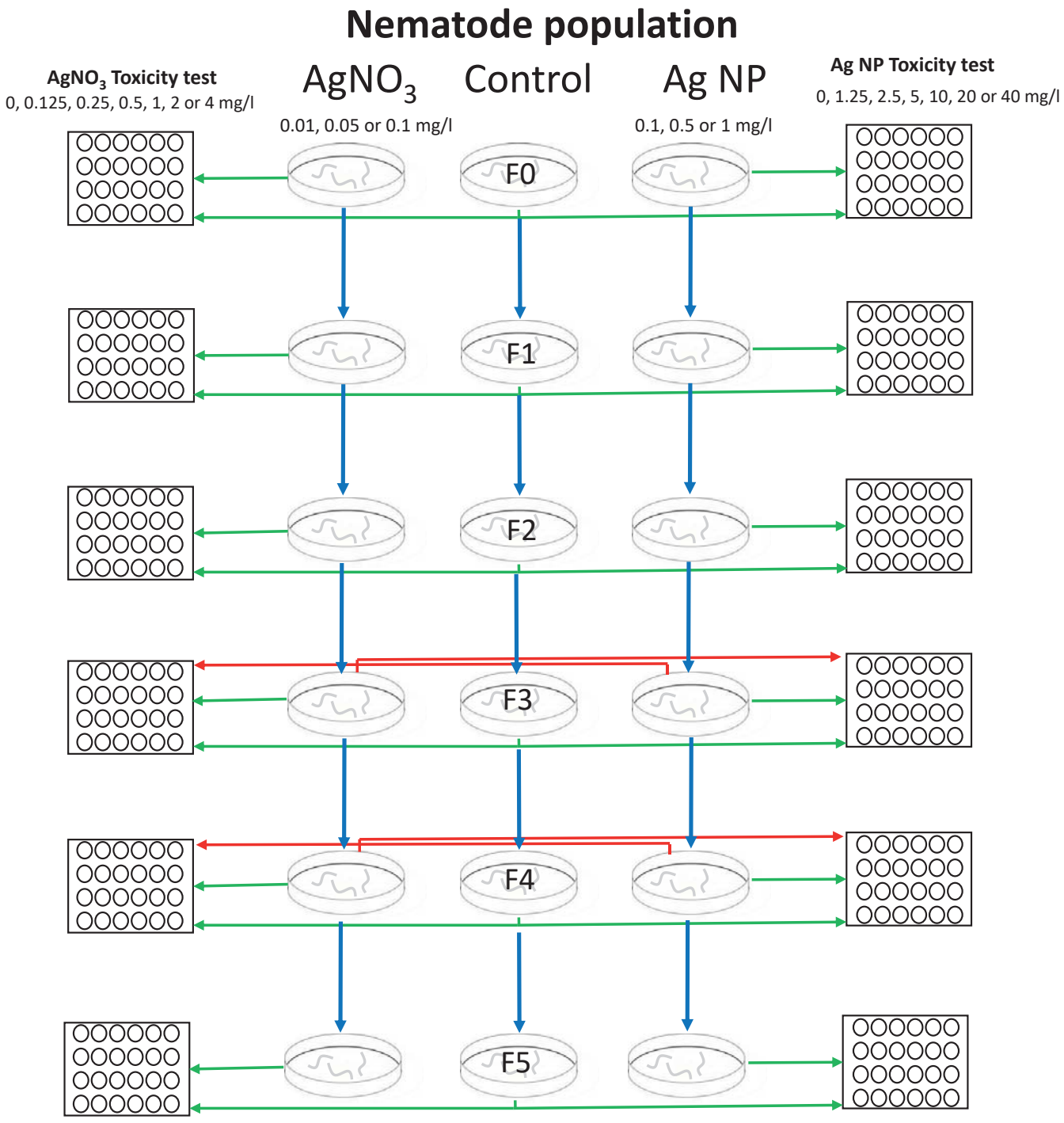



**Figure 4:** Experimental design of the continuous exposure to either $AgNO_3$ or Ag NPs, or control populations on agar plates. Offspring from each generation were subjected to standard toxicity tests to a range of concentrations of either $AgNO_3$ or Ag NPs.

**Table 2:** Transfer times of nematode populations, between different generations.

| | Number of days | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Population | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
| Control | 72h | | | | 72h | | | | 72h | | | 72h | | | 72h | | | 72h | | | | | | | | | |
| $AgNO_3$ 0.01 mg/l | 72h | | | | 72h | | | | 72h | | | 72h | | | 72h | | | 72h | | | | | | | | | |
| $AgNO_3$ 0.05 mg/l | 72h | | | | 72h | | | | 72h | | | 72h | | | 72h | | | 72h | | | | | | | | | |
| $AgNO_3$ 0.1 mg/l | 72h | | | | 72h | | | | 72h | | | 72h | | | 72h | | | 72h | | | | | | | | | |
| Ag NP 0.1 mg/l | 72h | | | | 72h | | | | 120h | | | | | 96h | | | | 96h | | | 96h | | | | | | |
| Ag NP 0.5 mg/l | 72h | | | | 72h | | | | 120h | | | | | 96h | | | | 96h | | | 96h | | | | | | |
| Ag NP 1 mg/l | 72h | | | | 72h | | | | 144h | | | | | | 120h | | | | 96h | | | | | 96h | | | |
| | F0 | | | | F1 | | | | F2 | | | F3 | | | F4 | | | | F5 | | | | | | | | |

### 2.7.1. Cross toxicity test exposure

In addition to the standard toxicity tests set up at each generation, a cross toxicity test exposure was set up at generation F3 and F4 (Figure 5, red arrows), in order to assess whether the exposure to one form of Ag will change the response to the other. Nematodes exposed to $AgNO_3$ were exposed to six concentrations of Ag NPs and vice versa in standard toxicity tests and sampled for growth, fertility, and reproduction.

### 2.7.2. Sampling

For the chronic multigenerational exposure, the N2 nematodes were sampled for total brood size. Additionally, nematodes were sampled from the multigenerational exposure plates at generation F5 for scanning electron microscope (SEM, Zeiss EVO 50 EP) imaging, to exclude external damages to the cuticle of the nematode by the NPs.

## 2.8. F6 generation toxicity test towards other toxicants

Additional, to test for changes in the response of the nematodes towards other toxic compounds, three metal cations, another nanoparticle type, and a known ROS inducer were chosen. In the F6 generation, nematodes were exposed in a standard 96 h toxicity test to six concentrations of either copper ($Cu^{2+}$ - 0, 0.06, 0.13, 0.25, 0.5, 1 and 2 mg $L^{-1}$), cadmium ($Cd^{2+}$ - 0, 0.09, 0.19, 0.38, 0.75, 1.5 and 3 mg $L^{-1}$), cerium ($Ce^{3+}$ - 0, 3.13, 6.25, 12.5, 25, 50 and 100 mg $L^{-1}$), cerium dioxide nanoparticles ($CeO_2$ NPs - 0, 01.56, 3.13, 6.25, 12.5, 25 and 50 mg $L^{-1}$), or the herbicide paraquat (0, 0.08, 0.16, 0.31, 0.63, 1.25 and 2.5 mM). Nematodes were analyzed for growth, fertility, and reproduction effects, as described above.

## 2.9. ROS production and antioxidant defenses mechanisms

### 2.9.1. Nematode strains

*C. elegans* present the perfect model for the *in vivo* measurement of redox sensor based on fluorescent probes, due to their transparent body (Doonan *et al.*, 2008, Back *et al.*, 2012, Miranda-Vizuete and Veal, 2017). The biosensor and reporter strains used in Papers II and IV were specifically chosen to monitor changes in antioxidant defense induction and changes in the cellular redox balance and oxidative stress manifestation. The ability to measure changes in a quick, efficient, and visual way in live organisms, avoids inaccuracy in measurements due to complicated staining processes, time resolved changes, and/or interferences due to sample preparation methods. Nevertheless, as

organisms are alive during sampling and measurement, and recovery and damage repair mechanisms are still on going, measurements from reporter strains and biosensors may still be subject to confounding factors influencing measurements. Furthermore, fluorescently labelled strains may, if cultures are not maintained properly, loose the GFP transgene across generations, thus compromising measurements of changes.

### 2.9.2. SOD-1

Superoxide dismutase, first described by McCord and Fridovich (1969), presents the first line of defense against free radicals in *C. elegans*. For the determination of changes in the antioxidant defense system, a GFP labelled SOD-1 (GA508 wuls54[pPD95.77 sod-1::GFP, rol-6(su1006)]) reporter strain was chosen (Figure 5) (Doonan *et al.*, 2008). The choice of this specific reporter strain was based on the fact that in *C. elegans*, SOD-1 is the most abundant, with 75 % of total sod transcription, and, therefore, contributes most of the total SOD activity (Doonan *et al.*, 2008). The SOD-1::GFP transgenic line was created to express a GFP-tagged SOD fusion protein for the *sod-1* gene and may act as a reporter for *sod-1* gene expression (Figure 5) (Doonan *et al.*, 2008).

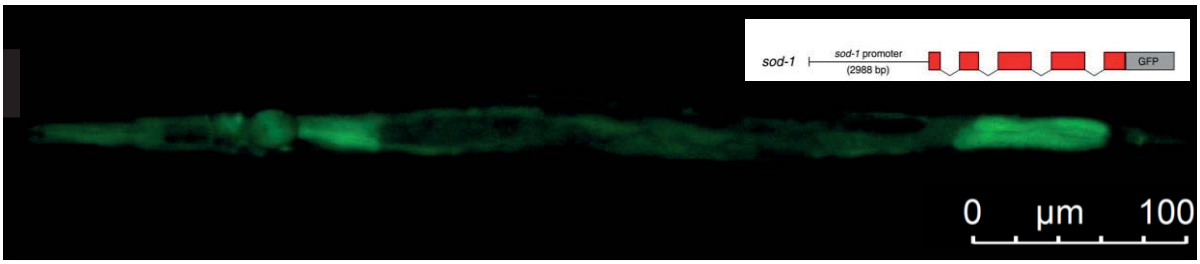



**Figure 5:** GFP labelled SOD-1 reporter strain (Doonan *et al.*, 2008) (photo L. Rossbach).

### 2.9.3. HyPer

As an end product of the dismutation of the $O_2^{\cdot-}$ by the SOD-1 enzyme, hydrogen peroxide ($H_2O_2$) is produced (Braeckman *et al.*, 2017). Therefore, the biosensor strain HyPer was applied to monitor changes in cellular peroxide levels (Figure 6). This strain expresses a peroxide-specific sensor protein, consisting of a yellow fluorescent protein (YFP) fused to the $H_2O_2$ sensing domain of the *E. coli* OxyR (Back *et al.*, 2012). Therefore, this strain acts as a proxy for changes in peroxide levels. Increases in peroxide levels lead to the formation of a disulfide bridge, and, hence, a conformational change of the protein. Therefore, two fluorescent images were taken with a 405 nm (reduced – Figure 6a) and

490 nm (oxidized – Figure 6b) excitation filter, and a 535 emission filter. In the overlay image (combination of reduced and oxidized images), red areas in the nematode contain low levels of the $H_2O_2$, while increase in $H_2O_2$ levels are shown by a shift towards green (Figure 6c).

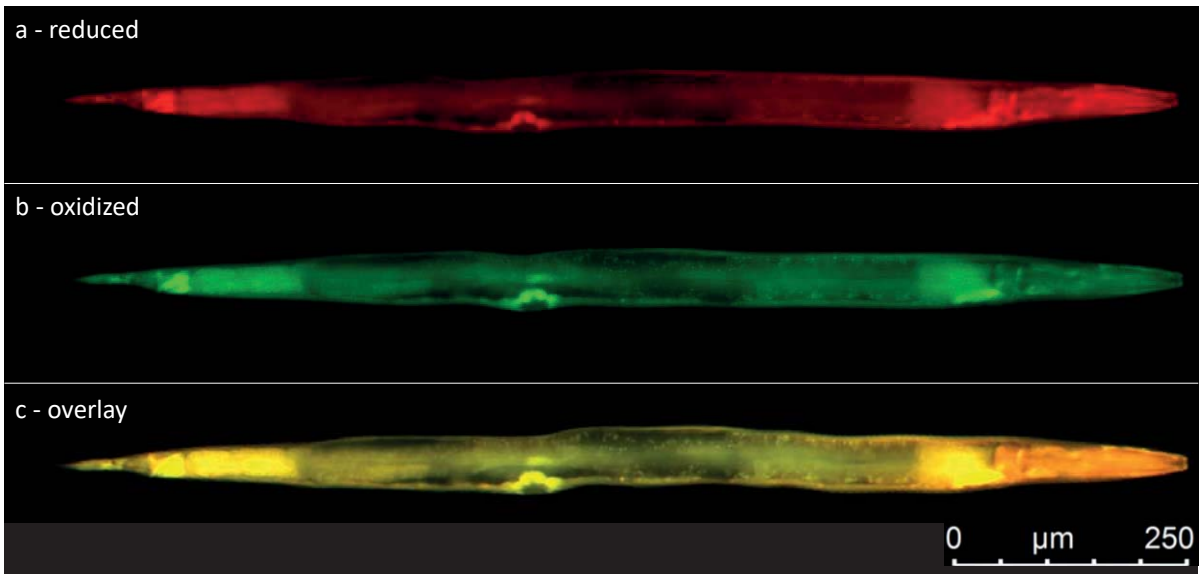



**Figure 6:** YFP labelled peroxide biosensor strain HyPer, showing here high levels of cellular hydrogen peroxide, taken with a 405 nm (reduced - a) and 490 nm (oxidized – b) excitation filter, and a 535 emission filter (Back *et al.*, 2012) (photo L. Rossbach).

### 2.9.4. GRX

Glutathione (GSH) plays a fundamental role in the breakdown of peroxides into $H_2O$ and $O_2$. The Grx1-roGFP2 strain (referred to as GRX) allows for the measurement of the ratio between the oxidized (GSSG) and reduced (GSH) form of the peptide, due to its redox sensitive GFP-reporter enzyme (Figure 8). If the cellular homeostasis is disturbed by increased amounts of ROS, it creates a shift in the signal from roGFP and, thereby, serves as a real time, *in vivo* indicator of an imbalance in the GSSG-GSH redox cycle. Due to the high intracellular GSH concentrations (1 – 11 mM) and its pivotal role in antioxidant defense, the GRX strain may act as a reliable proxy for the total cellular redox state (Back *et al.*, 2012). Therefore, two fluorescent images were taken with a 490 nm (reduced – Figure 7a) and 405 nm (oxidized – Figure 7b) excitation filter, and a 535 emission filter. In the overlay image (combination of reduced and oxidized images), visually green areas in the nematode represent low ratios of GSSG/GSH (balanced redox state), while increase

in GSSG/GSH ratios are shown by an increase in red areas (imbalanced redox state) (Figure 7c).

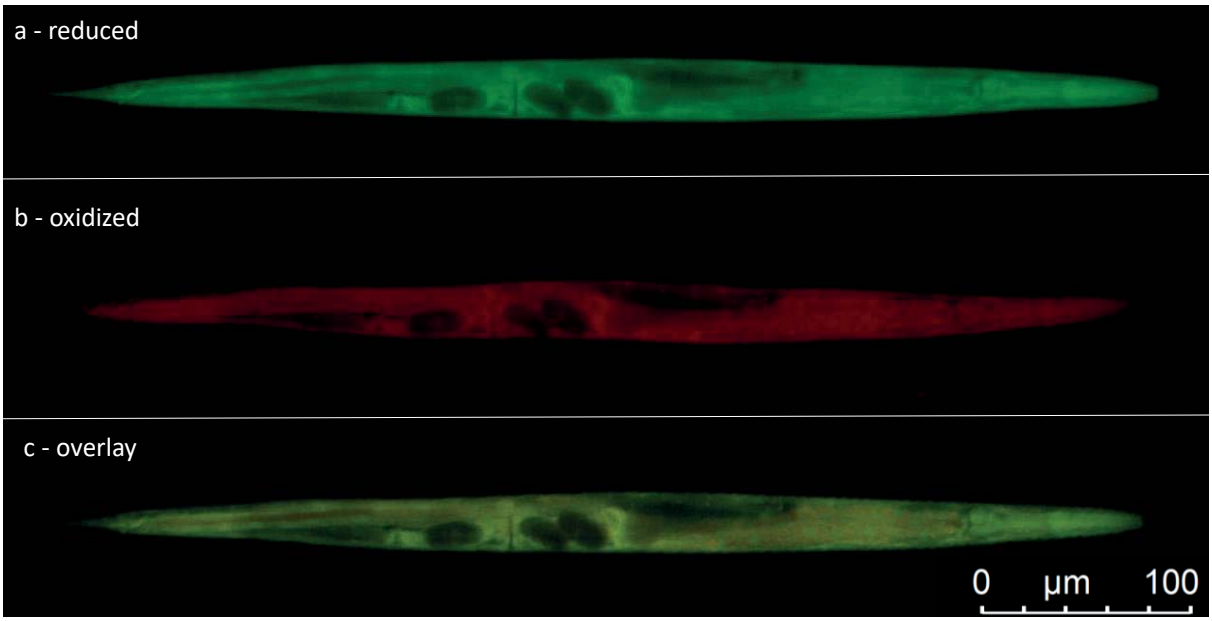



**Figure 7:** The GFP labelled GRX (Grx1-roGFP2) biosensor strain, showing here a heightened GSSG/GSH ratio (overlay – c), taken with a 490 nm (reduced – a) and 405 nm (oxidized – b) excitation filter, and a 535 emission filter (Back *et al.*, 2012) (photo L. Rossbach).

### 2.9.5. Multigenerational exposure

The SOD-1 and GRX strains were both subjected to a multigenerational exposure set up, as described in Paper IV. Similar to the exposure regime described above and in Paper III, they were exposed for six generations to a chronic concentration of either $AgNO_3$ or Ag NPs. However, for this exposure a single concentration of 0.1 and 0.5 mg Ag $L^{-1}$, for $AgNO_3$ and Ag NPs, respectively, was chosen.

The generational transfer times for the strains had to be adjusted, with decreased development observed, for both Ag populations, compared to controls. The experimental set up in Paper IV was slightly amended to the nature of the strains, and gravid nematodes were washed off the plates and bleached, for synchronization, between each generation. At generations F1, F3, and F6, nematodes were exposed in standard toxicity tests and analyzed by fluorescence microscopy.

### 2.9.6. Toxicity test and sampling

The toxicity tests for the strains described in Papers II and IV were carried out on nematodes sampled at 72 h. 72 h proved to provide the most accurate measurements in terms of development and signal strength for all three strains. Nematodes were directly imaged on a light microscope (LEICA DM6 B), equipped with a 405 nm (reduced HyPer and oxidized GRX) and 490 nm (oxidized HyPer and reduced GRX) excitation and 535 nm emission filter. Nematodes were immobilized using sodium azide. Images were then individually analyzed for signal strength using the LAS X Leica application suit X imaging software for pixel based average intensity measurements. Ratios for GRX and HyPer were calculated as shown below.

$$Oxidized{:}\,reduced\ ratio\ HyPer = \frac{\text{HyPer Ex 490/Em535}}{\text{HyPer Ex405/EM535}}$$

$$Oxidized{:}\,reduced\ ratio\ GRX = \frac{\text{GRX Ex 405/Em535}}{\text{GRX Ex490/Em535}}$$

## 2.10. Data analysis

All data was analyzed using either MiniTab® 18 (Minitab Inc. 2010) or JMP Pro v14 (SAS Institute, Cary, NC). For normally distributed data, the parametric ANOVA was done, as well as, Tukey test for multiple comparison of the data. Where appropriate, to stabilize the variance, a Box-Cox transformation of the data was conducted. Kruskal-Wallis was applied for non-normally distributed data. For the comparison of data across generations, results were normalized to individual toxicity test controls (Yu *et al.*, 2012, Moon *et al.*, 2017). The analysis of the multigenerational changes presented in Paper III were carried out in collaboration with a statistician, and analyzed either with a linear model or with a Poisson regression (generalized linear model with over dispersion). Effect concentrations (EC10 and EC50) were calculated with the open source software RegTox, using the Hill model (Vindimian, 2016) and are reported as the optimal value for EC10 and EC50 with corresponding 95 % confidence intervals.

# 3. Results

This section provides a summary of main results presented in the individual papers. Results for the characterization with the TEM and DLS show only small variation between different studies, and, hence, are not described for each individual manuscript in detail. For a more detailed description of all results, as well as figures and tables, the reader is referred to the corresponding papers.

## 3.1. Paper I.

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

This paper investigated the potential linkage between toxicity towards the nematode *C. elegans* and the characterization of the NM300K Ag NPs. Furthermore, the reproducibility of the standard 96 h toxicity test with the NM300K Ag NPs was assessed. Three experiments were set up over the course of three consecutive years, using two different nanoparticle stock preparation methods. Further, a range of characterization techniques were employed and compared between different experiments. Endpoints measured were growth, fertility, and reproduction, as well as the uptake and retention of the nanoparticles by the nematodes.

Transmission electron microscope analysis showed a mean particle size of 12.5 ± 4.1 nm and 16.7 ± 6.5 nm (mean ± SD) for experiments 2 and 3, respectively. Dynamic light scattering analysis of the stock suspension (in dd$H_2O$) revealed a high mean particle size of 82.0 ± 6.0 nm and 71.7 ± 0.6 nm for experiments 2 and 3, while experiment 1 had a mean particle size of 33.8 ± 1.7 nm (Z-average diameter). When comparing the number based mean, experiments 2 and 3 had similar particle size (table 2 in Paper I).

The characterization in the exposure media (MHRW) showed that in the higher concentrations NM300K had a similar mean hydrodynamic diameter to that measured for the stock suspensions, while lower concentrations were significantly larger (table 3 in Paper I). Further, at 0.5 and 10 mg Ag $L^{-1}$ a time dependent (across four days) increase (roughly 2 fold) in zeta-average particle size was observed, while concentrations of 2 and 4 mg Ag $L^{-1}$ remained stable over time. DLS measurements of the $AgNO_3$ exposure media showed a zeta average diameter of 893 ± 108, 425 ± 36 and 404 ± 8 nm at 0.2, 1 and 4 mg Ag $L^{-1}$, respectively. Time resolved DLS measurements of the NM300K over the

course of a few minutes showed an initial fluctuation of the zeta average diameter, which stabilized at around 30 nm and a polydispersity index (PdI) of 0.450 for all suspensions.

In absence of bacteria, the LMM Ag fraction in the Ag NP treatments was 4 – 8 % of the total Ag concentration, in the exposure media. In the $AgNO_3$ exposure, the dissolved fraction at T-0 showed a wide variation ranging between 13 and 54 % of the total Ag concentrations, without *E. coli* present. The size fractionation of the $AgNO_3$ and Ag NPs exposure media in presence of organisms (*E. coli* and *C. elegans*) showed that within 2 hours of adding the Ag, the <3 kDa fraction fell below the limit of detection of the ICP-MS, indicating a high interaction of the ionic Ag with the organisms (Figure 8).

Toxicity test endpoints showed a consistent and comparable dose response relationship for all measured parameters including growth, fertility, and reproduction. Despite some variation in EC values, there was a relatively good agreement among the three separate experiments. ECs were in the same order of magnitude, especially in terms of reproduction, the most sensitive endpoint, for both $AgNO_3$ and NM300K Ag NP exposure.

Uptake of both forms of Ag by the nematode was comparable, with concentrations reaching 1 µg Ag $mg^{-1}$ wet weight in nematodes exposed to 0.5 mg Ag $L^{-1}$ ($AgNO_3$ and Ag NPs). However, following depuration, a roughly 98 % decrease in Ag concentration was found, and a ~2 fold higher Ag retention was measured for the $AgNO_3$ exposure, compared to the Ag NPs (see table 4 in Paper I). This, combined with different levels of toxicity indicates that the toxic response might be governed by the exposing agent (Figure 8), which may be traced back to differences in the LMM (<3 kDa) fraction, where higher LMM fraction in the $AgNO_3$ could potentially mean higher Ag associated with *E. coli*, increasing dietary uptake in this exposure.

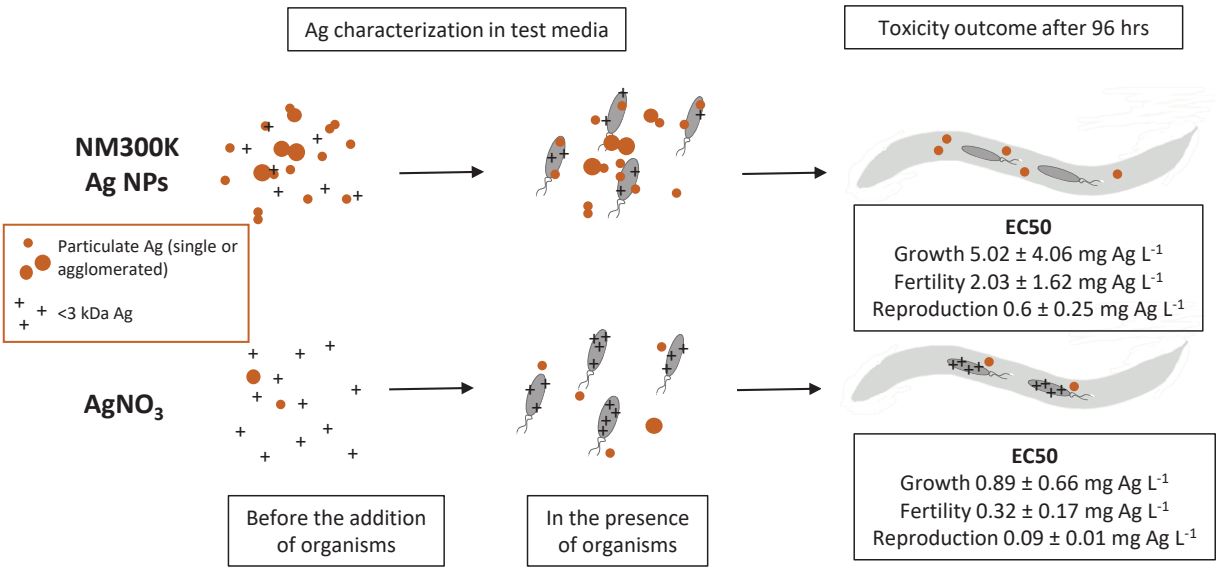



**Figure 8:** Graphical summary of exposure and main studied endpoints and results in Paper I.

## 3.2. Paper II.

*In vivo* assessment of silver nanoparticle induced reactive oxygen species reveals tissue specific effects on cellular redox status in the nematode *Caenorhabditis elegans*

The aim of this study was to assess changes in the intracellular redox state as well as links to toxicity test endpoints in the nematode *C. elegans*, following the exposure to either $AgNO_3$ or Ag NPs. A *sod-1::gfp* reporter strain, and a peroxide and a glutathione related biosensor strain, allowed for the *in vivo* measurement of *sod-1* gene expression, ROS production and changes in the intracellular redox status. Further, differences in internal localization of the two forms of Ag were analyzed. To our knowledge, this is the first study to demonstrate nanoparticle-induced ROS production, induction of antioxidant defense, and evidence of oxidative stress *in vivo* in intact live organisms.

In stocks, the characterization showed a mean particle size of 16.7 ± 6.5 nm (mean ± SD) as measured by TEM, and 93.3 ± 1.3 nm zeta average diameter for the NM300K Ag NPs, as measured by DLS. Size fractionation of the exposure media showed that no (or below detection) LMM Ag (<3 kDa) was present in any of the $AgNO_3$ and Ag NP exposures, even at the beginning of the experiment, once bacteria *E. coli* were present. In addition, the aggregated Ag fraction was comparable ~60 – 70 % of total Ag) in all exposures after 72 h.

Toxicity test effects showed a dose response comparable to those presented in Paper I. However, distinct differences in tissue distribution between the two forms of Ag were apparent (Figure 9). Localized analysis of the GSSG/GSH ratios showed significantly higher oxidation levels in the tissues lining the intestinal lumen from the Ag NP exposure, compared to controls and $AgNO_3$ exposed nematodes. A ~60 % increase in oxidation levels located in the epithelial cells surrounding the lumen were observed at 10 mg Ag $L^{-1}$ Ag NPs compared to 1 mg Ag $L^{-1}$ $AgNO_3$ (Figure 9). A ~15 - 20 % increase in oxidation levels located in the epithelial cells surrounding the lumen were observed at the lowest Ag NPs (1 mg Ag $L^{-1}$) compared to the highest $AgNO_3$ (1 mg Ag $L^{-1}$).

A significant increase in *sod-1* expression was found at 0.5 and 1 mg Ag $L^{-1}$ $AgNO_3$, while no increase was found at 0.1 mg Ag $L^{-1}$. For the Ag NPs exposure, *sod-1* expression was significantly increased at 5 and 10 mg Ag $L^{-1}$, but not at 1 mg Ag $L^{-1}$. Peroxide levels were significantly increased, compared to controls, following the exposure to all

concentrations of Ag NPs, while the exposure to $AgNO_3$ only resulted in a significant increase at 1 mg Ag $L^{-1}$. In addition, peroxide levels in the Ag NP exposure were significantly higher at 1 and 10 mg $L^{-1}$ compared to all $AgNO_3$ levels. Compared to controls, all concentrations, irrespective of type of Ag, showed a significant increase in GSSG/GSH ratios, except for the 0.1 mg Ag $L^{-1}$ $AgNO_3$ exposure.

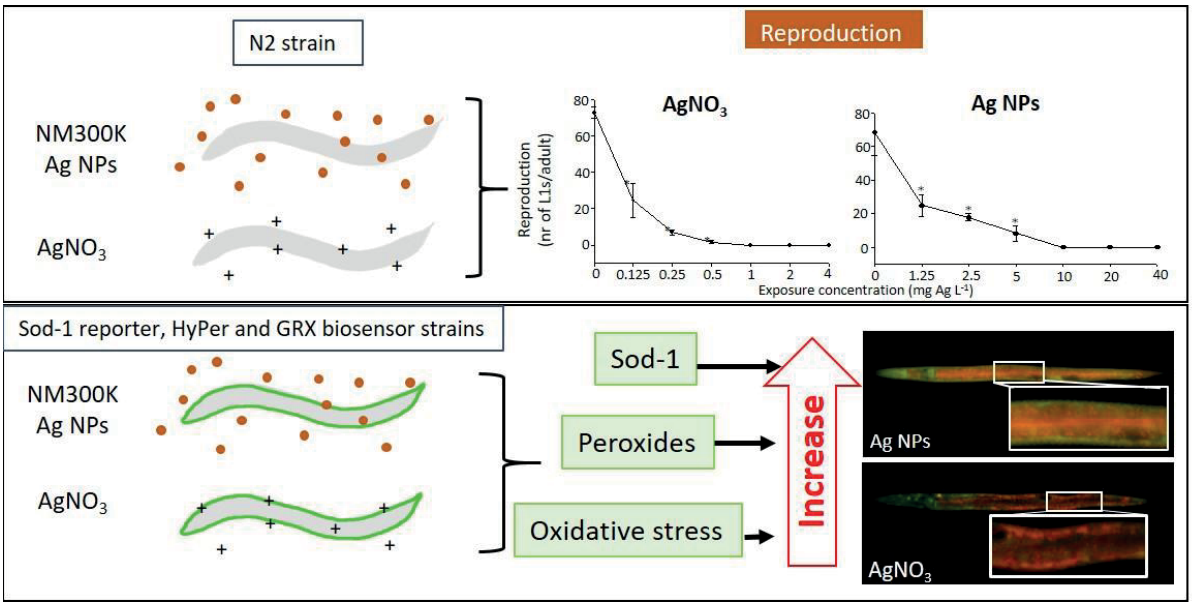



**Figure 9:** Graphical summary of exposure and main results in Paper II.

## 3.3. Paper III.

Adaptive tolerance to a multigenerational silver nanoparticle (NM300K) exposure by the nematode *Caenorhabditis elegans* is associated with increased sensitivity to $AgNO_3$

To investigate the long term effects of Ag NP exposure by the nematode, a chronic multigenerational exposure was set up. Nematodes were kept for six generations in either control (no added Ag), $AgNO_3$ (0.01, 0.05 or 0.1 mg Ag $L^{-1}$) or Ag NP (0.1, 0.5 or 1 mg Ag $L^{-1}$) populations. Toxicity tests at the end of each generation were used to monitor changes in growth, fertility, and reproduction. Further, at generations F3 and F4 nematodes were subjected to a cross-toxicity test: nematodes from $AgNO_3$ populations were exposed to Ag NPs, and *vice versa*, to identify whether changes in toxic response would apply to either form of Ag. Characterization of the Ag NPs showed comparable results as presented in Papers I and II, thus confirming consistent exposure.

The chronic multigenerational exposure showed no change for unexposed controls across generations. Only the Ag NP populations expressed an increase in development, and, hence, delaying transfer to subsequent generations. All populations experienced a sudden increase in sensitivity towards the Ag exposures in the F1 generation, followed by a steady recovery across the generations. Nevertheless, no change in toxic response towards $AgNO_3$ from the $AgNO_3$ pre-exposed population was measured. The 1 mg $L^{-1}$ Ag NP population, however, was significantly less sensitive to Ag NP exposure in the toxicity test, as demonstrated by significantly increased reproduction in the F5 generation, compared to control populations. This was further supported by EC50 estimations (see Paper III, Table 1 for more details). The observed adaptive response of Ag NP populations came at the cost of reduced growth, however, where nematodes from the 0.1 and 1 mg $L^{-1}$ Ag NP populations were significantly shorter than control population nematodes (Figure 10).

The toxicity test revealed that the Ag NP population was significantly more sensitive, in terms of growth and fertility, towards $AgNO_3$ exposure, compared to the control population, in the F3 generation, but not the F4 generation. Reproduction was significantly decreased in both generations, for Ag NP population exposed to $AgNO_3$, compared to the $AgNO_3$ population. For the $AgNO_3$ population exposed to Ag NPs in the toxicity test, a significant increase in fertility and growth was found in the F3 generation,

but not the F4. Reproduction, on the other hand, showed a significant increase in both generations.

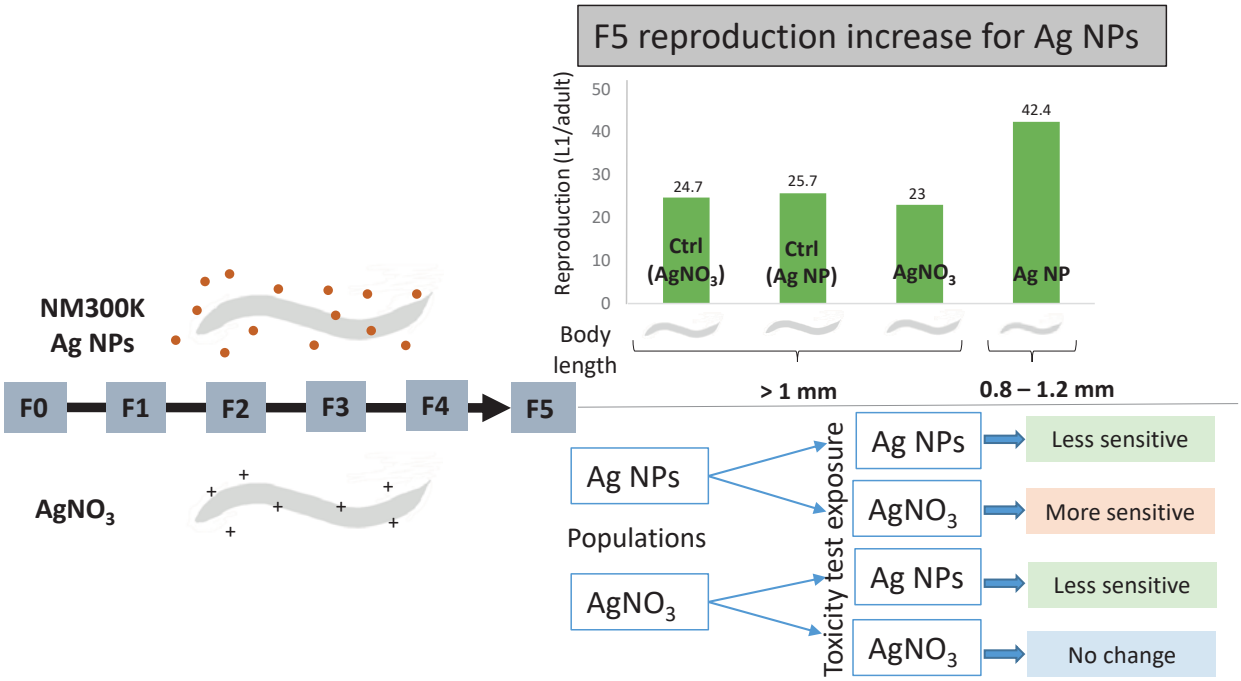



**Figure 10:** Graphical summary of exposure and main results in Paper III.

## 3.4. Paper IV.

Effects on the nematode *Caenorhabditis elegans* antioxidant defense and reactive oxygen species (ROS) metabolism following multigenerational exposure to $AgNO_3$ or NM300K Ag NPs

Exposure to one stressor has been shown to have the potential to change the toxic response to a second stressor (Calabrese and Baldwin, 1997b, Calabrese and Baldwin, 1997a). Following the multigenerational exposure to either $AgNO_3$ or Ag NPs of the N2 strain (as presented in Paper III), nematodes were exposed to cerium ($Ce^{3+}$), cerium dioxide nanoparticles ($CeO_2$ NPs), cadmium ($Cd^{2+}$), copper ($Cu^{2+}$) and paraquat, in standard toxicity tests. Following the multigenerational exposure towards either $AgNO_3$ or Ag NPs, nematodes showed no changes in sensitivity when exposed to cadmium, copper, and $CeO_2$ NPs. Both Ag NP and $AgNO_3$ population nematodes exhibited increased sensitivity towards Ce ions, compared to controls. The six generational exposure towards 1 mg $L^{-1}$ Ag NPs however, rendered nematodes less sensitive towards paraquat in terms of fertility (Figure 11). Nematodes previously exposed to 1 mg $L^{-1}$ Ag NP for six generations, showed statistically significant increased fertility, compared to control and $AgNO_3$ populations. Paraquat is known to produce superoxide anion, leading to the assumption, that systems with increased superoxide defenses will be more resistant towards the exposure to paraquat. Hence, to identify changes in ROS metabolism and the involvement of oxidative stress mechanisms, a multigenerational exposure scenario was set up, exposing either the SOD-1 reporter strain or the GRX biosensor strain to chronic concentrations of either $AgNO_3$ or Ag NPs for six generations. At generation F1, F3, and F6 toxicity tests were set to investigate changes in the response to increasing concentrations of either form of Ag.

In toxicity tests, the expression of *sod-1* in the control population showed no significant changes across generations in toxicity test control conditions, confirming the reproducibility and consistency of the exposure set up. Overall, when exposed to $AgNO_3$ or Ag NPs in the toxicity test, s*od-1* gene expression showed a significant increase in the F3 generation from all three populations (control, $AgNO_3$ and Ag NP), compared to the expression measured in the F1 or F6 generation. Moreover, in the F3 generation both Ag populations revealed increased *sod-1* expression compared to the control population. However, a significant decrease in *sod-1* expression levels in the F6 generation from both

Ag populations was measured, when exposed to either $AgNO_3$ or Ag NPs in the toxicity test.

In terms of GSSG/GSH ratios, the control population showed no changes in ratios across generations in toxicity tests control conditions, while the Ag NP population showed an increase in ratios in the F3 and F6 generation compared to the F1 generation. The $AgNO_3$ population, on the other hand, showed a statistically significant decrease in the F6 generation, compared to F1 and F3, in the toxicity test control conditions. In the $AgNO_3$ and Ag NP toxicity tests, both Ag populations showed a statistically significant decrease in GSSG/GSH ratios in the F3 generations, compared to the control population, indicating an increase in antioxidant defenses resulting from the Ag exposure. In the F6 generation, however, an overall increase in ratios was measured for the Ag populations compared to controls.

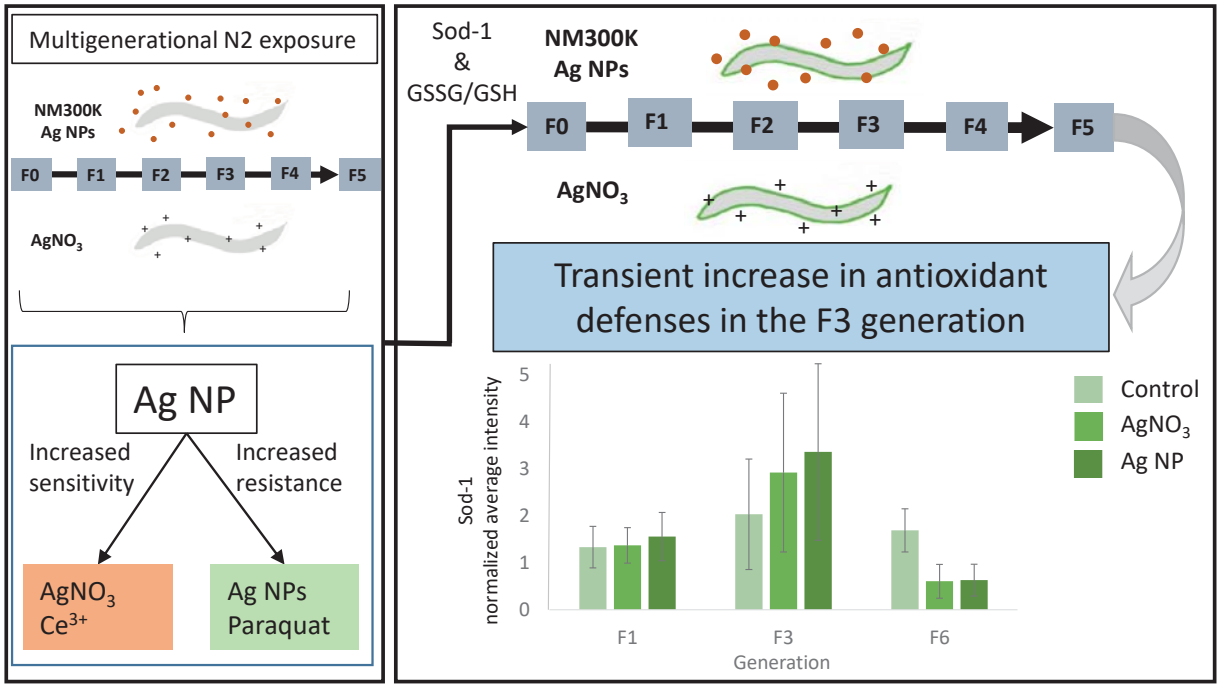



**Figure 11:** Graphical summary of exposure and main results in Paper IV.

# 4. Discussion

The rapidly increasing quantities of Ag NPs produced, applied, and released into the environment, make it almost impossible to test and make risk assessment for every type of particle (Gomes *et al.*, 2017). Further, as research to date has shown, there is a lack of comparative data, where changes in exposure media, as well as particle characteristics may highly impact toxicity (Vazquez-Muñoz *et al.*, 2017). Hence, the use of a reference material, as used in the current study (NM300K), to add to the collection of information of these particle is important. Further, identifying commonalities on a biochemical level between different Ag NPs and their toxic mode of action may help the comparison to ionic Ag, and evaluate whether current legislation regulating environmental release of Ag, are sufficient to protect the environment and organisms.

The concentrations of Ag NPs in agricultural soils concentrations are estimated to range between 30 pg/kg (minimum in 2017) up to 10 µg/kg soil (maximum in 2050)(Giese *et al.*, 2018). Overall, by 2050 a 2 – 6 fold increase of environmental Ag NP concentrations is predicted by Giese et al (2018). Concentrations used in the current exposure studies are above the current predictions. For the purpose of the current exposures, concentrations high enough to induce an effect, without causing acute toxicity had to be chosen for the multigenerational exposures presented in Paper III and IV. Despite higher concentrations, the exposure of organisms for multiple generations holds great importance. Most exposure studies will only cover a specific life stage or one generation of an organism, where long term and heritable effects of multigenerational exposures may be missed (Goussen *et al.*, 2013). Further, as described in table 1 in section 1.7, only a limited number of Ag NP related multigenerational exposure studies are available, limiting the comparison between results. This highlights the need for further Ag NP multigenerational studies for the validation of results.

This project aimed to increase the understanding of the different aspects of the toxicity of the reference Ag NP NM300K towards the nematode *C. elegans,* in comparison to $AgNO_3$. Thus the exposures were set up to a) characterize the toxic effect of the Ag NPs compared to $AgNO_3$ in *C. elegans*, b) investigate the reproducibility of such toxicity tests, and try to link the characterization of the Ag to the toxicity, c) examine changes in toxic response towards Ag NPs or $AgNO_3$, as well as a range of other stressors (Ce, Cd, Cu, Ce-NPs and paraquat), following the chronic multigenerational exposure towards either

form of Ag, and d) examine changes in the antioxidant defenses and oxidative stress manifestation across generations, aiming to link such changes to changes in toxic response observed in the multigenerational exposure. The exposure set up and main findings are summarized in table 3.

**Table 3:** Exposure conditions, endpoints and studied effects of this project.

| Paper | Number of generations | Population exposure concentration (mg $L^{-1}$) | | Toxicity test exposure concentration (mg $L^{-1}$) | | Studied endpoints | Ag NP characteristics (Size, size distribution, charge) | Behavior of Ag in the exposure (MHRW) | Observed effects |
|---|---|---|---|---|---|---|---|---|---|
| | | $AgNO_3$ | Ag NPs | $AgNO_3$ | Ag NPs | | | | |
| I | 1 | | | 0 – 4 | 0 – 4 and 0 – 40 | NP characterization, growth, fertility, reproduction, uptake, EC-estimations | TEM: 12.5 – 16.7 nm<br>DLS: 33.8 – 82 nm<br>Zeta pot.: -1.02 - -7.32 mV | - Presence of larger (<200nm) particles in $AgNO_3$ exposure<br>- Presence of <3 kDa Ag fraction at T-0 in both exposures | - Up take from both forms of Ag, higher retention of $AgNO_3$ after depuration<br>- Higher relative toxicity from $AgNO_3$ |
| II | 1 | | | 0 – 4 and 0 - 10 | 0 – 40 and 0 - 10 | NP characterization, growth, fertility, reproduction, ROS and oxidative stress (*sod-1* expression, peroxide levels, GSSG/GSH ratios), localization, EC-estimations | TEM: 16.7 ± 6.5 nm<br>DLS: 93.3 ± 1.3 nm<br>Zeta pot.: -8.77 mV | - High aggregated Ag fraction from both Ag exposures at 72 hrs | - Both forms of Ag induce *sod-1*<br>- Increase in peroxides<br>- Low Ag NPs produce oxidative stress<br>- Ag NPs primarily produce ROS within the lumen |
| III | 6 | 0.01, 0.05 and 0.1 | 0.1, 0.5 and 1 | 0 – 4 | 0 - 40 | NP characterization, growth, fertility, reproduction, cross-toxicity test exposure, EC-estimations | TEM: 16.7 ± 6.5 nm<br>DLS: 73 ± 1.1 nm<br>Zeta pot.: -5.5 ± 2.7 mV | - At 96 hrs low <3 kDa Ag fraction from both Ag exposures<br>- High degree of aggregation, or association with *E. coli* from the Ag NPs | - Adaptation towards Ag NPs, but at the cost of reduced growth as well as increased sensitivity towards $AgNO_3$<br>- No adaptation towards $AgNO_3$<br>- $AgNO_3$ leads to reduced sensitivity towards Ag NP exposure |
| IV | 6 | 0.1 | 0.5 | 0.1, 0.5 and 1 | 1, 5 and 10 | NP characterization, external damages, total brood size, N2 sensitivity towards other toxicants, ROS and oxidative stress (*sod-1* and GSSG/GSH ratios) | TEM: 23.9 ± 21.8 nm<br>DLS: 79 ± 4.42 nm | - At 96 hrs low <3 kDa Ag fraction from both Ag exposures<br>- High degree of aggregation, or association with *E. coli* from the Ag NPs | - Reduced sensitivity towards paraquat following six generational Ag NP exposure<br>- Temporary increase in *sod-1* expression in the F3 generation from both Ag populations compared to the control population<br>- Increased oxidative stress in the F6 generation from both Ag populations, compared to the control population |

## 4.1. Changes in size distribution in the stock and media

Particle aggregation is quite common for Ag NPs in exposure media, and may result in the sedimentation of larger particles, hence reducing the total Ag concentration in solution, as well as, changing the size distribution. Data from TEM and DLS measurements showed high consistency between studies. A reduction of total Ag concentrations in the media was observed, possibly stemming from the aggregation and/or the adsorption of the Ag to the exposure well surface. In all studies, and for both Ag NP and $AgNO_3$, a change in the Ag size distribution was observed in the exposure media over time. Size fractionation showed an overall transformation from ionic form to colloids and/or larger particles. A wide range of factors may influence the behavior of both Ag NP and $AgNO_3$ in the exposure media, such as the presence of organisms, Ag concentrations, particle properties or the test media composition (Römer *et al.*, 2011).

A slight increase in the zeta average diameter, from 71.7 ± 0.6 nm to 79 ± 4.42 nm was measured in Papers II and IV, compared to Papers I and III. This was related back to a decrease in the Ag NP concentration used in the initial stock preparation, as presented in Papers I and III, compared to Papers II and IV. The analysis of the particles by TEM revealed particles to be spherically shaped with a mean size of 12.5 – 23.9 nm (Papers I – IV), where stock concentrations and preparation method appears to be influencing the mean size measurements. On average, for all measurements, DLS size distributions were 3 - 4 times larger than the TEM size measurements, probably because TEM measurements are number based (Fabrega et al., 2011). Further, DLS measurements may be more influenced by the presence of larger aggregates in the suspension, while it is possible to exclude these manually in the TEM analysis (Fabrega et al., 2011).

### 4.1.1. Recovery of Ag concentrations in stocks and exposure media

Overall the recoveries for $AgNO_3$ were consistently high as were the lower Ag NP stocks. The lower recoveries of 72 ± 5.5 % from the 2.56 g Ag $L^{-1}$ stock suspensions of Ag NPs suggest an increased removal of the particles at higher concentrations, due to higher aggregation caused by an increased rate of collision between the particles (Hassellöv *et al.*, 2008). Silver concentrations in the exposure media, in the presence of the *E. coli* and *C. elegans*, measured at T-0 showed high recoveries ranging between 80 – 90.7 % of nominal concentrations in Paper I, II and IV. However, as recoveries of below 80 % Ag were measured in Paper III, concentrations presented are measured rather than nominal

concentrations. ICP-MS analysis of exposure media for both forms of Ag showed a similar percentage loss over the course of the exposure, ranging between 0 – 34.8 % of the total Ag concentrations measured at T-0 (Paper II, III and IV). The decrease in the total Ag concentrations as compared to nominal concentrations was anticipated, and is in accordance with a range of studies reporting 60.2 ± 4.2 % decrease in total concentrations over time in MHRW (Schultz *et al.*, 2016). The decrease in Ag concentration, however, does not appear to be dependent on exposure concentrations, and might therefore be attributed to the binding of the Ag to the well surface, as well as increased aggregation, as observed in the fractionation experiments, with subsequent sedimentation of the Ag.

### 4.1.2. Size distribution of the Ag in the exposure media

Size fractionation of exposure media showed a high removal of Ag from the exposure media, with the removal of *E. coli*, for both $AgNO_3$ and Ag NP exposures, where approximately 30 – 60 % and 40 – 70 % of the Ag was aggregated in the $AgNO_3$ and Ag NP exposures, respectively, at 72 hours of exposure (Paper II and IV). The removal of a large Ag fraction, by centrifugation, suggested a high interaction of both forms of Ag in the exposure media with the *E. coli*, hence facilitating the uptake of the Ag. *C. elegans* have been shown to feed on particles in the size range of 100 nm to 3 µm, leading to the assumption that even larger aggregates, not directly associated with *E. coli* might be ingested and contributing to the Ag toxicity. Although at all time points, in all studies, a suspended Ag fraction was measured, generally, only low or no LMM Ag fraction (<3 kDa) was observed at T-0, and non at subsequent time points.

A factor influencing the size distribution of the Ag in the media is likely to be the media composition, with high concentration of chlorides, sulfides and organic matter being highly influential factors in the speciation of the Ag. Therefore, it is possible that any dissolution, or free ion fraction in the exposure could not be detected as an effect of the rapid sorption of any released Ag ions to the *E. coli*, as well as complexation with other compounds, such as chlorides. At low concentrations of chloride ions ($Cl^-$), as present in the MHRW used in the current experiments, as well as low Cl:Ag ratio, Ag NP have been shown to have a low dissolution rate, due to the formation of a AgCl layer on the particle surface, increasing their stability (Ho et al., 2010, Li et al., 2010b, Levard et al., 2013). The complexation of Ag ions with chlorides, forming AgCl(s) could explain the increase in

aggregation observed in both exposures, where bridging structures between the Ag NPs have been reported to facilitate aggregation (Levard et al., 2012).

Sulfide is considered to be one of the main Ag complexing agents in natural waters (Reinsch *et al.*, 2012), where reduced sulfur is primarily present under anaerobic conditions. Ag is a "soft metal", and will preferentially bind to inorganic sulfides and sulfur containing organic molecules, and therefore be transformed into $Ag_2S$ (Reinsch *et al.*, 2012). Due to their low solubility, such complexes may have great influence on the toxicity of the Ag (Choi and Hu, 2009, Zhang *et al.*, 2011). Although MHRW contains relatively high sulfur concentrations, 27 mg $L^{-1}$, sulfidation of the Ag is rather unlikely, as the sulfur is most likely in the form of sulfate ($SO_4^{2-}$) and levels of reduced sulfur are expected to remain low, as the media is well oxygenated (for details on MHRW recipe see United States Environmental Protection Agency (2002)). The Ag in the current exposures is more likely to precipitate as chlorides due to the lower solubility product.

Moreover, natural organic matter (NOM) is known to increase the stability of the particles in exposure (Cumberland and Lead, 2009, Delay *et al.*, 2011). Although the organic matter content in the MHRW is considered to be low, it is probably increased in the presence of the *E. coli* and *C. elegans*.

Even if the interaction of the Ag NP with the sulfur or chloride remains low, changes in the surface charge due to the ionic strength of the media may still explain the increased aggregation over time. The surface charge of a particle can have major influence on the way an organism interacts with the NPs (El Badawy *et al.*, 2011). Furthermore, in order to stabilize the particles and avoid aggregation, many particles have an organic coating, such as Tween 20 for the NM300K Ag NPs used in the current work, providing an electrostatic, or electrosteric repulsion force between the particles (Phenrat *et al.*, 2008, Hotze *et al.*, 2010, Sharma *et al.*, 2014). An increase in ionic strength of a media (above 0.1 mM), has been related to a decrease in the repulsive properties of the electrostatic barrier between and particles, and hence leading to an increase in aggregation (Jiang *et al.*, 2009, El Badawy *et al.*, 2010). Although sterically stabilized Ag NPs are in general less influenced by changes in ionic strength, the ionic strength of the MHRW may lead to an increase in formation of larger particles over time in the NP exposures (El Badawy *et al.*, 2010).

The anticipated higher levels of LMM Ag fraction (<3 kDa) in the $AgNO_3$ exposure, compared to the Ag NP exposure, was only observed in media in the absence of organisms (Paper I). The NM300K Ag NPs used in the current experiment, have low ionic releases, possibly due to the stabilizing agent (Tween-20) in the dispersant, acting as a type of coating around the NPs, preventing the oxidation of the particles in the exposure media over time (Köser *et al.*, 2017). The NM300K Ag NPs stock suspension has been shown, by Köser *et al.* (2017), to contain roughly 8 % of dissolved Ag, which can account for the small <3 kDa Ag fraction measured in the Ag NP exposures, as presented in Papers I and IV.

Overall, a similar change from smaller to larger particles was observed from both, $AgNO_3$ and Ag NP, exposures, in all studies. Although the NM300K Ag NPs have a steric stability, and despite the dispersing agent, a high degree of aggregation was measured. Further, rapid binding to *E. coli*, as well as complexation, may have prevented the measurement of any LMM fraction present in the media.

## 4.2. Toxicity

Toxicity tests were set up to compare $AgNO_3$ and Ag NP toxicity in the F0 generation, as well as across generations, combined with the investigation into Ag mediated ROS production, and antioxidant defenses by the nematode for single and multiple generations. Overall, 96 h exposure to both forms of Ag resulted in a concentration dependent decrease in development, fertility and reproduction. Over the course of multiple generations, an adaptation towards the Ag NPs was measured in terms of increased reproduction, however, at the cost of reduced growth. Furthermore, changes in the response towards other environmental stressors were recorded. Investigation into the antioxidant defenses showed a possible increased ability to counteract ROS, by the nematodes following the multigenerational exposure to both forms of Ag in the F3 generation, preventing oxidative stress manifestation when challenged with Ag NP and $AgNO_3$ in toxicity tests. This was, however, followed by an overall decrease in antioxidant defenses in the F6 generation.

### 4.2.1. F0 toxicity

One of the aims of this study was to compare the toxic response by *C. elegans*, in terms of growth, fertility and reproduction, of the NM300K Ag NPs to that of $AgNO_3$ (Papers I and II). More specifically, it was hypothesized that NM300K Ag NPs would be toxic to *C.*

*elegans*, but that the relatively low dissolution of these NPs would make these less toxic than $AgNO_3$. Nematodes feed via pharyngeal pumping, ingesting liquid and food from their surrounding into their digestive tract, making them the perfect model for dietary exposure studies (Hunt *et al.*, 2014). Developmental endpoints were chosen for this specific toxicity test, as lethal dose estimations (e.g. LD50) are hard to obtain from nematode adults, in this kind of exposure study. Higher toxicity of the $AgNO_3$ exposure was measured, where EC50 estimations for growth were 3 – 10 times, fertility 8 times and reproduction 2 – 9 times higher for the Ag NPs, than for $AgNO_3$. This is in accordance with findings by Völker *et al.* (2013) (PVP coated, NM300, <15 nm) and Connolly *et al.* (2015) (NM300K), who found a comparatively higher toxicity from $AgNO_3$ than Ag NPs in liquid media.

The reduced toxicity of the Ag NPs compared to the $AgNO_3$ in the current experiments, may also be explained by differences in the bioaccumulation. Although results showed that both forms of Ag were taken up in a dose dependent manner, following depuration twice as much Ag was retained by the nematodes from the $AgNO_3$ exposure, compared to the Ag NPs (Paper I, table 4). Although translocation of Ag NPs into cells has been reported, the internalization is highly dependent on size, surface charge, shape, functionalization and protein corona (Stoehr *et al.*, 2011, Butler *et al.*, 2015, Milić *et al.*, 2015, Panzarini *et al.*, 2018). Positively or neutrally charged NPs are generally more easily taken up by cells than negatively charged particles (Durán *et al.*, 2016, Panzarini *et al.*, 2018). Furthermore, cellular uptake is highly shape dependent, where different cells show preference towards different shapes, e.g. rods, spheres or tubes (Stoehr *et al.*, 2011, Panzarini *et al.*, 2018). Cellular uptake of spherical Ag NPs (12 - 30 nm) has been demonstrated (Farkas *et al.*, 2011, Milić *et al.*, 2015). However, as shown by results presented in Paper II, the Ag NP exposure resulted in a clear increase in oxidative stress primarily confined within the intestinal lumen, indicating low cellular internalization as well as distribution of the NPs, or ionic Ag released from the NPs, by the nematodes. This is similar to findings by Luo *et al.* (2016), who, in their dietary Ag NP (25 nm, -12 mV) exposure study on *C. elegans*, showed that the majority of the ingested Ag NPs remained within the lumen, with only a limited number of particles transferred to cells.

However, cellular uptake is not essential for the toxic effects of Ag NPs. Due to the high antibacterial properties, a wealth of studies have focused on the interaction of Ag NPs and

bacterial cells, including effects on microbial gut communities (Williams *et al.*, 2015, Pietroiusti *et al.*, 2016, van den Brule *et al.*, 2016). Antibacterial action of Ag NPs have been shown in a wide range of *in vitro* studies. The oxidation of the Ag NPs in the low intestinal pH, as well as the generation of reactive oxygen species on the surface of the particles have been linked to Ag NP toxicity (Yang *et al.*, 2012). It can be assumed, that, if the Ag NPs exert their toxicity primarily within the intestinal lumen, as shown from findings in Paper II, it may potentially have negative impacts on the gut bacteria. The reaction with thiol-groups (-S-H) of either ionic or particulate form results in the disturbance of electron transport, leading to growth inhibition and death of bacterial cells (Davies and Etris, 1997). Further, structural change and dissipation of the proton motive force are related to the interactions of the Ag NPs with compounds of the bacterial membrane (Sondi and Salopek-Sondi, 2004). Further, the introduction of oxygen species, found in the silver crystalline lattice by the Ag NPs could have consequences on anaerobic microorganisms (Sawosz *et al.*, 2007).

Results from *in vivo* animal studies are less consistent. Although no reduction in microbial content in quails following dietary exposure to Ag NPs was found in a study by Sawosz *et al.* (2007), it was observed that the Ag NPs caused destruction of cell membranes of both gram-positive and gram-negative bacteria. This indicates an overall toxicity towards cellular membranes, rather than specific interactions with cell membrane components of either group of bacteria (Sawosz *et al.*, 2007). Moreover, changes in the overall microbial environment within the gut of quail, was observed by a change in ratio of aerobic to anaerobic bacteria (Sawosz *et al.*, 2007). However, it appears that toxicity towards bacteria is size dependent with highest toxicity from 1 – 10 nm (Morones *et al.*, 2005, Williams *et al.*, 2016, Choi *et al.*, 2018). Therefore, in the current study, although increased oxidative stress was measured in the intestine of the nematodes from the NM300K Ag NPs in paper II, particles showed a median size ranging from 16.7 – 23.9 nm, and therefore may limit the antibacterial interactions. Nevertheless, the NM300K increased ROS production and oxidative stress manifestation may in turn induce programmed cell death of bacterial cells (Lee *et al.*, 2014a).

In accordance with the hypothesis stated above, the NM300K showed lower toxicity compared to $AgNO_3$. Differences in toxicity may be related back to the surface charge as well as the coating and low dissolution of the NM300K Ag NPs, which may in turn, limit

the biodistribution of the particles, confining the NPs to the lumen of the nematode. Furthermore, results support previous studies that suggest ROS production by the NPs to be a significant contributor to the toxic response observed.

## 4.3. Multigenerational exposure

Changes in the response of an organism following the multigenerational exposure towards a compound may manifest themselves in different ways: general changes in the performance of the organism during the multigenerational exposure, changes to challenges of the same stressor, changes to challenges of a different stressor, or through the transfer of effects from exposed adults to unexposed offspring. Results from the current exposures show clear differences in response of the nematode towards the two forms of Ag, as observed in Paper I and II. Therefore, as presented in Paper III, a multigenerational exposure was set up in order to further investigate changes in the toxic response towards either form of Ag, across multiple generations. It was hypothesized that the multigenerational exposure will lead to an adaptation towards Ag NPs at lower concentrations, but an increase in sensitization at higher concentrations, across generations. Only few studies have investigated the multigenerational effects of Ag NPs on *C. elegans* (Völker *et al.*, 2013, Contreras *et al.*, 2014, Luo *et al.*, 2016, Schultz *et al.*, 2016). However, results from Schultz *et al.* (2016) and Contreras *et al.* (2014) are in contradiction to results from the current study (Paper III), where evidence for an adaptation towards the Ag NP exposure was found for nematodes exposed to 1 mg $L^{-1}$ Ag NP following six generational exposure. Multigenerational exposure to one toxicant has been shown, through changes in physiological responses, to evoke altered sensitivity towards other stressors (Cypser and Johnson, 2002b). This has also been shown in the current studies, where the multigenerational exposure to Ag NPs resulted in increased sensitivity towards $AgNO_3$, while the multigenerational exposure to $AgNO_3$ decreased sensitivity towards Ag NPs (Paper III). Furthermore, increased sensitization was found towards the Cerium exposure for both Ag populations, and a decrease in the sensitivity towards the known ROS inducer, paraquat, from Ag NP exposed nematodes (Paper IV).

### 4.3.1. Transfer of effects of parental exposure to unexposed offspring

Multigenerational exposure studies are of great importance, as in many cases effects of an exposure are only observable in offspring generations. Alternatively, however, effects can be detected in both adult and offspring. This may be the result of the transfer of

effects, or, alternatively, the transfer of the stressor from exposed adults to offspring. The transfer of reproductive effects induced by gold nanoparticles in *C. elegans* from an exposed parental generation to unexposed offspring, was seen across four generations (Kim *et al.*, 2013). This transfer was explained by interactions of the developing gonad and embryo germ cells within the parent nematodes, resulting in abnormalities of the reproductive system in the offspring generations (Kim *et al.*, 2013). Luo *et al.* (2016) showed the transfer of effects, as characterized by germ cell apoptosis, from an exposed parental *C. elegans* population to offspring generations, where a recovery from effects was only observed in the F3 or F4 generation, depending on size of the Ag NPs. Furthermore, Schultz *et al.* (2016) showed no recovery from effects of the nematodes, even following 10 generations in unexposed conditions. These stand in stark contrast to findings from the current study, which showed a remarkable capacity of recovery. In the current work, the best evidence for potential transfer of effects to unexposed populations comes from measurement of toxicity test control condition nematodes, as presented in Paper III. The offspring from exposed (either $AgNO_3$ or Ag NPs) adults performed comparable to control population nematodes, in terms of both development and reproduction. Furthermore, as evidenced by the total brood size measurements (Paper III), once exposure was removed, nematode development and reproduction was comparable to that of control population nematodes. Differences between findings from the current exposure and other nanoparticle studies may indicate the importance of the nanoparticle characteristics, and how small changes in size, surface charge, and/or dissolution state, may have major influences on the toxicity and the biological effects, as well as the transfer of the particles from parent to offspring generations. Nevertheless, results presented in Paper IV provide evidence for the generational transfer of effects. Six generational exposure to both $AgNO_3$ and Ag NP lead to a significant decrease in *sod-1* expression compared to controls, in toxicity test control conditions. Moreover, in the F6 generation a decrease in cellular redox status (GSSG/GSH ratio) was measured from the $AgNO_3$ population compared to control and Ag NP populations. These results indicate transferable changes in the antioxidant defenses in response to $AgNO_3$ exposure, leading to decreases in oxidative damages in offspring nematodes. However, such changes do not interfere with development or reproduction, as shown in Paper III.

Alternatively, physical transfer of particles from parents to offspring could contribute to the observation of effects in successive generations. Luo *et al.* (2016) observed a transfer

from parent to offspring of 25 nm Ag NPs, similar to the Ag NP size used in the current study (16.7 ± 6.5 nm). However, these Ag NPs had a PVP coating and a zeta potential (-12.6 ± 0.99 mV) approximately 3 times lower than that in the current exposure (-1.02 to -8.77 mV; Papers I, II and III) (Luo *et al.*, 2016). This coupled with the relatively low retention of the NM300K Ag NPs in the current study, inside the nematode following depuration (Paper I), indicates that the coating and charge may be the governing physicochemical processes, influencing the internalization and generational transfer of the particles, and hence limiting the transfer of NM300K Ag NPs from exposed adults to unexposed offspring.

### 4.3.2. Toxic response across generations

Interestingly, findings from the current study, as presented in Paper III, stand in contradiction to findings by Contreras *et al.* (2014). Contreras *et al.* (2014) exposed nematodes to a wide range of Ag NP (mPEG-SH coated) concentrations (1 – 100 mg $L^{-1}$) in a similar manner as done in the current exposure. It was concluded that nematodes in the lower (1 – 10 mg $L^{-1}$) concentration ranges showed slightly reduced sensitivity (in terms of growth and neurological endpoints) following a mere four generations of exposure. This was assessed, not in standard toxicity tests as done in the current experiment (Paper III), but directly from the exposed populations on NGM-agar plates. In the current exposure (Paper III) however, adverse effects in terms of development, leading to an increase in the generational transfer times, were shown for all Ag NP populations (0.1, 0.5 and 1 mg Ag $L^{-1}$), and an adaptive tolerance was only measurable in the subsequent toxicity test set ups in the F5 generation. A more comparable exposure set up, to that used in the current exposure, was used by Schultz *et al.* (2016). Nevertheless, findings remain contradictory. Schultz *et al.* (2016) assessed changes in sensitivity to PVP coated Ag NPs, in toxicity tests in Simulated Soil Pore Water (SSPW). Although SSPW is considered to be more environmentally relevant than MHRW, both medias are considered low ionic strength (~4 mM and 10 mM for MHRW and SSPW respectively). Meyer *et al.* (2010) showed cellular uptake in *C. elegans* of PVP coated Ag NP (25 ± 7 nm). Therefore, difference between the two exposures may arise from differences in the NP coatings, hence meaning differences in uptake and distribution. This, coupled with their larger size (58.3 ± 12.9 nm), could potentially lead to decreased toxicity compared to the NM300K, meaning that higher concentrations would be needed to induce an adaptive response.

### 4.3.3. Antioxidant defenses and ROS production by the Ag NPs

In accordance with the hypothesis that ROS production is involved in the toxic mechanisms of Ag NPs, and would trigger antioxidant defenses, results from the current studies confirmed that the Ag NP NM300K are ROS producers (Paper II and IV). They induced the sod-1 antioxidant defense system, increased peroxide levels, and led to the manifestation of oxidative stress (Paper II and IV). At the highest concentrations the exposure to $AgNO_3$ and Ag NPs resulted in an overwhelmed glutathione antioxidant defense system and hence led to changes in cellular redox status (Paper II). It has been suggested that ROS formation and/or oxidative stress are two factors underlying acclimation responses, with increases in the SOD antioxidant defense system observed in young *C. elegans* exposed to the quinone 'plumbagin', or hypoxia, increasing their resistance towards these two ROS inducers at a later age (Darr and Fridovich, 1995, Zhao and Wang, 2012). This is consistent with findings from the current study, where the six generational exposure to Ag NPs resulted in an increased ability to withstand the exposure to the known ROS inducer paraquat, compared to control and $AgNO_3$ populations (Paper IV). Paraquat is a quaternary nitrogen herbicide, applied for weed control (Suntres, 2002). To date, it is amongst the most commonly used compounds for oxidative stress related studies in organisms (Suntres, 2002, Koch and Hill, 2017). Through the inhibition reaction of NADP to NADPH, it will lead to the formation of superoxide anion and singlet oxygen, and hence increase levels of hydroxyl and peroxyl radicals (Gram, 1997, Suntres, 2002). This implies that organisms with a heightened sod antioxidant defense system are more able to withstand the exposure to paraquat, and hence led to the hypothesis that changes in toxic responses across generations could be related to changes in the antioxidant defense system, specifically the sod related defenses.

However, based on findings presented in paper II, it appears that the single exposure to either form of Ag induces an alternative antioxidant defense, different to sod-1, and sod-1 would only be induced as an additional defense at higher Ag concentrations. This is in accordance with findings by Roh *et al.* (2009), who found an induction of *sod-3* but not *sod-1* gene in *C. elegans*, exposed to Ag NP. Findings from the multigenerational exposure on the other hand, presented in paper IV, suggest the involvement of *sod-1* as an antioxidant defense, in response to the long term exposure to Ag. Overall, both Ag populations followed a similar trend in terms of *sod-1* expression levels, and changes of

GSSG/GSH ratios across generations. The overall increase in *sod-1* expression in the F3 generation from both Ag exposures hints at Ag induced changes to the SOD-1 antioxidants. This is in accordance with findings by Darr and Fridovich (1995), who showed that nematodes are able to acclimate to oxidative stress, through the increase in SOD activity. Moreover, it has been suggested that organisms are able to reduce other biological processes, such as reproduction or biosynthesis, and instead increase antioxidant defense mechanisms (Lushchak, 2014). This may explain reduced development and reproduction in earlier generations from Ag exposed nematodes compared to the control population, as shown in Paper III. Nevertheless, the low *sod-1* expression levels from both Ag populations in the F6 generation measured in the current study, suggests that this effect is temporary.

Therefore, results from the current study suggest that the *sod-1* antioxidant is not the only mechanism involved in short-term defense against Ag induced ROS. In the short term exposure, results hint at differences in distribution and hence may mean differences in toxic mode of action. However, the adaptation of the antioxidant defense is observable earlier than the phenotypic adaptation. Contrary to expectations however, the multigenerational exposure towards either form of Ag resulted in a decreased ability to protect against oxidative stress, and statistically significantly lower *sod-1* expression levels, in the F6 generation (paper IV). This, in combination with findings of an adaptation towards Ag NPs by the nematodes in the F5 generation (Paper III), suggests that the maintenance of reproductive capacity is the dominating adaptive effect, and occurs at the expense of growth, and potentially antioxidant defenses.

# 5. Limitations of the work

Several limitations and uncertainties to the work should be taken into consideration. Considering that the media composition and exposure characteristics are the driving force behind the bioavailability and toxic response of the Ag, a more detailed analysis of the behavior of the Ag in the exposure media would have been beneficial. Rather than limiting the size fractionation to the start and the end of the exposure, additional size fractionation, for instance at > 200 nm fraction, as well as additional time points, throughout the exposure period may have been useful at interpreting changes in the behavior of the Ag over time. Additional more advanced characterization techniques, such as the analysis with spICP-MS and/or field flow fractionation (FFF), would have provided more detail and descriptive results for the changes of the Ag in the exposure media.

In addition, it would have been beneficial to have had analysis of the Ag distribution on the agar population plates, in order to identify exact exposure concentrations of the nematodes on the agar plates. It is assumed, due to observable and replicable effects on the exposure plates, that nematodes were sufficiently exposed, however, the Ag content of the *E. coli* lawn and agar should have been analyzed by ICP-MS, to eliminate possible variations between the different forms and exposure conditions.

Furthermore, the $AgNO_3$ did not result in any changed toxic response by the nematodes, across the six generations. However, although no significant differences were found, data hints at changes in the response of the $AgNO_3$ exposed population nematodes in the later generations. Therefore, an increase in generations, possibly up to 10, may have resulted in observable changes in the toxic response of the $AgNO_3$ nematodes. Further, as the adaptive response of the Ag NP exposed nematodes was only measured in the last (F5) generation, an increase in generations would have provided a confirmation of these findings.

Considering results found in Papers II and IV, indicating the involvement of alternative antioxidant defenses, it would have been beneficial to apply other reporter strains, such as a sod-3 reporter. Alternatively, gene expression of exposed nematodes could have, not only validated findings from the current studies, but also provided insights into alternative antioxidant defenses.

Further, more imaging analysis, such as TEM or SEM, identifying and confirming Ag NP uptake by the nematode, as shown in Paper I. Although the imaging of Ag NPs in the lumen of the nematodes was attempted using dark field imaging, a more detailed analysis of the Ag NPs within the lumen, as presented in Paper II, would have been valuable additions at confirming findings from the present studies. Additionally, more details on interaction of the Ag NPs with the *E. coli* could have been provided by TEM-analysis. The interaction of the Ag with the *E. coli* is of great importance in order to confirm the dietary exposure of the *C. elegans* in the toxicity test, but also in the population exposures, on the agar plates. Although the interaction of Ag NPs with *E. coli* bacterial cells has been shown by e.g. Sondi and Salopek-Sondi (2004), changes in particles characteristics may result in differences in cell-particle interactions. More detailed analysis of this interaction would have provided valuable information on the exposure.

Lastly, throughout the multigenerational exposure, additional uptake studies were conducted, but failed. This information could have allowed more concrete discussion on multigenerational effects, identifying differences in the uptake between Ag NPs and $AgNO_3$. Further, uptake analysis in offspring generations of exposed adults would have provided additional interesting insights in interpreting total brood size data.

# 6. Conclusion

The current PhD work investigated the toxic effects of the Ag NP NM300K on the nematode *C. elegans,* in single and multiple generations of exposure. All studies used $AgNO_3$ as a positive control. Therefore, the overall aim of the PhD was to increase the understanding of the different aspects of the toxicity of the reference Ag NP NM300K towards the nematode *C. elegans*, in comparison to $AgNO_3$.

Although, both $AgNO_3$ and the NM300K Ag NPs were toxic to *C. elegans*, a distinctly different effect patterns between the two forms of Ag was shown. Both Ag NPs and $AgNO_3$ experienced a comparable transformation over time in the exposure media, with increasing aggregated fractions, and decreasing LMM Ag species. The lower toxicity of the Ag NPs may be a result of the high initial LMM fraction in the $AgNO_3$ exposure. The low dissolution of the Ag NPs resulted in limited transfer of Ag, from Ag NPs, into cells, leading to more localized toxicity.

Across generations, the Ag NP exposure lead to an adaptive response by the nematodes at the highest population exposure concentration. The adaptive response, however, came at the cost of reduced growth, as well as increased sensitivity towards $AgNO_3$. Moreover, results suggest that SOD-1 is involved in the adaptation development in earlier generations. However, due to a significant reduction in *sod-1* expression by the F6 generation, results imply that the maintenance of the reproductive capacity is the dominating adaptive response.

The current PhD work adds novel information to the field of Ag NP toxicity with respect to mechanisms leading to long term effects. Furthermore, the importance of multigenerational exposure studies, as well as the involvement of ROS in Ag NP toxicity are highlighted. The results from this study reveal that adaption to primary stressor may have profound and detrimental effects with respect to secondary challenges, and thus emphasizes the need for further evaluation of long term effects of NPs. The complex nature of the antioxidant defense responses demonstrates a need for further investigation of the molecular mechanisms affected by NP exposure.

To further increase the environmental relevance of such studies, aging and transformation products of Ag NPs should be employed. Additionally, more exposure studies on environmentally relevant concentrations will be required for the

improvement of predictions on Ag NP toxicity towards organisms in the environment. This, in combination with standard operating procedures, could potentially reduce the uncertainties between different Ag NP studies, and thus help risk assessments and environmental regulation of Ag NPs.

# 7. References


Abdal Dayem, A., Hossain, M.K., Lee, S.B., Kim, K., Saha, S.K., Yang, G.-M., Choi, H.Y. & Cho, S.-G., 2017. The Role of Reactive Oxygen Species (ROS) in the Biological Activities of Metallic Nanoparticles. *International Journal of Molecular Sciences,* 18**,** 120.

Ahn, J.-M., Eom, H.-J., Yang, X., Meyer, J.N. & Choi, J., 2014. Comparative Toxicity of Silver Nanoparticles on Oxidative Stress and DNA Damage in the Nematode, Caenorhabditis elegans. *Chemosphere,* 108**,** 343-352.

Andre, J., Charnock, J., Stürzenbaum, S.R., Kille, P., Morgan, A.J. & Hodson, M.E., 2009. Accumulated Metal Speciation in Earthworm Populations with Multigenerational Exposure to Metalliferous Soils: Cell Fractionation and High-Energy Synchrotron Analyses. *Environmental Science & Technology,* 43**,** 6822-6829.

Asharani, P.V., Hande, M.P. & Valiyaveettil, S., 2009. Anti-proliferative Activity of Silver Nanoparticles. *BMC Cell Biology,* 10**,** 65.

Back, P., De Vos, W.H., Depuydt, G.G., Matthijssens, F., Vanfleteren, J.R. & Braeckman, B.P., 2012. Exploring Real-Time in vivo Redox Biology of Developing and Aging Caenorhabditis elegans. *Free Radical Biology and Medicine,* 52**,** 850-859.

Beer, C., Foldbjerg, R., Hayashi, Y., Sutherland, D.S. & Autrup, H., 2012. Toxicity of Silver Nanoparticles—Nanoparticle or silver ion? *Toxicology Letters,* 208**,** 286-292.

Behra, R., Sigg, L., Clift, M.J.D., Herzog, F., Minghetti, M., Johnston, B., Petri-Fink, A. & Rothen-Rutishauser, B., 2013. Bioavailability of Silver Nanoparticles and Ions: From a Chemical and Biochemical Perspective. *Journal of the Royal Society, Interface,* 10**,** 20130396-20130396.

Bielmyer, G.K., Bell, R.A. & Klaine, S.J., 2002. Effects of Ligand-bound Silver on Ceriodaphnia dubia. *Environmental Toxicology & Chemistry,* 21**,** 2204-8.

Bijlsma, R. & Loeschcke, V., 2005. Environmental Stress, Adaptation and Evolution: an Overview. *Journal of Evolutionary Biology,* 18**,** 744-749.

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.

Braeckman, B., Back, P.U., Matthijssens, F.G.E., Olsen, A.E. & Gill, M.S.E., 2017. Oxidative Stress. *In* S.I.S. Rattan (ed.) *Healthy Ageing and Longevity.* Springer, 219 - 244.

Brown, L.M., Collings, N., Harrison, R.M., Maynard, A.D., Maynard, R.L. & Jefferson, D.A., 2000. The Surface Activity of Ultrafine Particles. *Philosophical Transactions of the Royal Society of London. Series A: Mathematical, Physical and Engineering Sciences,* 358**,** 2683-2692.

Butler, K.S., Peeler, D.J., Casey, B.J., Dair, B.J. & Elespuru, R.K., 2015. Silver Nanoparticles: Correlating Nanoparticle Size and Cellular Uptake with Genotoxicity. *Mutagenesis,* 30**,** 577-591.

Calabrese, E.J. & Baldwin, L.A., 1997a. The Dose Determines the Stimulation (and Poison): Development of A Chemical Hormesis Database. *International Journal of Toxicology,* 16**,** 545-559.

Calabrese, E.J. & Baldwin, L.A., 1997b. A Quantitatively-based Methodology for the Evaluation of Chemical Hormesis *Human and Ecological Risk Assessment: An International Journal,* 3**,** 545-554.

Case, A.J., 2017. On the Origin of Superoxide Dismutase: An Evolutionary Perspective of Superoxide-Mediated Redox Signaling. *Antioxidants (Basel, Switzerland),* 6**,** 82.

Choi, J.E., Kim, S., Ahn, J.H., Youn, P., Kang, J.S., Park, K., Yi, J. & Ryu, D.Y., 2010. Induction of Oxidative Stress and Apoptosis by Silver Nanoparticles in the Liver of Adult Zebrafish. *Aquatic Toxicology,* 100**,** 151-9.

Choi, O., Clevenger, T.E., Deng, B., Surampalli, R.Y., Ross, L., Jr. & Hu, Z., 2009. Role of Sulfide and Ligand Strength in Controlling Nanosilver Toxicity. *Water Research,* 43**,** 1879-86.

Choi, O. & Hu, Z., 2008. Size Dependent and Reactive Oxygen Species Related Nanosilver Toxicity to Nitrifying Bacteria. *Environmental Science & Technology,* 42**,** 4583-4588.

Choi, O.K. & Hu, Z.Q., 2009. Nitrification Inhibition by Silver Nanoparticles. *Water Science & Technology,* 59**,** 1699-702.

Choi, Y., Kim, H.-A., Kim, K.-W. & Lee, B.-T., 2018. Comparative Toxicity of Silver Nanoparticles and Silver Ions to Escherichia coli. *Journal of Environmental Sciences,* 66**,** 50-60.

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.

Contreras, E.Q., Puppala, H.L., Escalera, G., Zhong, W.W. & Colvin, V.L., 2014. Size-Dependent Impacts of Silver Nanoparticles on the Lifespan, Fertility, Growth and Locomotion of Caenorhabditis elegans. *Environmental Toxicology and Chemistry,* 33**,** 2716-2723.

Corsi, A.K., 2006. A Biochemist's Guide to Caenorhabditis elegans. *Analytical Biochemistry,* 359**,** 1-17.

Corsi, A.K., Wightman, B. & Chalfie, M., 2015. *A Transparent Window into Biology: A Primer on Caenorhabditis elegans* [online]. Worm Book. Available from: http://www.wormbook.org/chapters/www_celegansintro/celegansintro.html [Accessed Access Date

Cortese-Krott, M.M., Münchow, M., Pirev, E., Heßner, F., Bozkurt, A., Uciechowski, P., Pallua, N., Kröncke, K.-D. & Suschek, C.V., 2009. Silver Ions Induce Oxidative Stress and Intracellular Zinc Release in Human Skin Fibroblasts. *Free Radical Biology and Medicine,* 47**,** 1570-1577.

Cumberland, S.A. & Lead, J.R., 2009. Particle Size Distributions of Silver Nanoparticles at Environmentally Relevant Conditions. *Journal of Chromatography A,* 1216**,** 9099-9105.

Cypser, J.R. & Johnson, T.E., 2002a. Multiple Stressors in Caenorhabditis elegans Induce Stress Hormesis and Extended Longevity. *The Journals of Gerontology: Series A,* 57**,** B109-B114.

Cypser, J.R. & Johnson, T.E., 2002b. Multiple Stressors in Caenorhabditis elegans Induce Stress Hormesis and Extended Longevity. *The Journal of Gerontology. Series A, Biological Sciences and Medical Sciences,* 57**,** B109-14.

Darr, D. & Fridovich, I., 1995. Adaptation to Oxidative Stress in Young, but not in Mature or Old, Caenorhabditis elegans. *Free Radical Biology and Medicine,* 18**,** 195-201.

Davies, R.L. & Etris, S.F., 1997. The Development and Functions of Silver in Water Purification and Disease Control. *Catalysis Today,* 36**,** 107-114.

Delay, M., Dolt, T., Woellhaf, A., Sembritzki, R. & Frimmel, F.H., 2011. Interactions and Stability of Silver Nanoparticles in the Aqueous Phase: Influence of Natural Organic Matter (NOM) and Ionic Strength. *Journal of Chromatography A,* 1218**,** 4206-4212.

Djurišić, A.B., Leung, Y.H., Ng, A.M.C., Xu, X.Y., Lee, P.K.H., Degger, N. & Wu, R.S.S., 2015. Toxicity of Metal Oxide Nanoparticles: Mechanisms, Characterization, and Avoiding Experimental Artefacts. *Small,* 11**,** 26-44.

Domingos, R.F., Baalousha, M.A., Ju-Nam, Y., Reid, M.M., Tufenkji, N., Lead, J.R., Leppard, G.G. & Wilkinson, K.J., 2009. Characterizing Manufactured Nanoparticles in the Environment: Multimethod Determination of Particle Sizes. *Environmental Science & Technology,* 43**,** 7277-7284.

Donaldson, K., Stone, V., Tran, C.L., Kreyling, W. & Borm, P.J.A., 2004. Nanotoxicology. *Occupational and Environmental Medicine,* 61**,** 727.

Donaldson, K. & Tran, C.L., 2002. Inflammation Caused by Particles and Fibers. *Inhalation Toxicology,* 14**,** 5-27.

Doonan, R., Mcelwee, J.J., Matthijssens, F., Walker, G.A., Houthoofd, K., Back, P., Matscheski, A., Vanfleteren, J.R. & Gems, D., 2008. Against the Oxidative Damage Theory of Aging: Superoxide Dismutases Protect Against Oxidative Stress but have Little or no Effect on Life Span in Caenorhabditis elegans. *Genes & Development,* 22**,** 3236-3241.

Dröge, W., Year. Oxidative Stress and Aging. *In*: R.C. Roach, P.D. Wagner & P.H. Hackett, ed.^eds. *Hypoxia*, Boston, MA: Springer US, 191-200.

Durán, N., Durán, M., De Jesus, M.B., Seabra, A.B., Fávaro, W.J. & Nakazato, G., 2016. Silver Nanoparticles: A New View on Mechanistic Aspects on Antimicrobial Activity. *Nanomedicine: Nanotechnology, Biology and Medicine,* 12**,** 789-799.

Dutilleul, M., Bonzom, J.M., Lecomte, C., Goussen, B., Daian, F., Galas, S. & Reale, D., 2014. Rapid Evolutionary Responses of Life History Traits to Different Experimentally-Induced Pollutions in Caenorhabditis elegans. *Bmc Evolutionary Biology,* 14**,** 252.

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**,** 1260-6.

El Badawy, A.M., Silva, R.G., Morris, B., Scheckel, K.G., Suidan, M.T. & Tolaymat, T.M., 2011. Surface Charge-Dependent Toxicity of Silver Nanoparticles. *Environmental Science & Technology,* 45**,** 283-287.

Endo, T., Yanagida, Y. & Hatsuzawa, T., 2008. Quantitative Determination of Hydrogen Peroxide using Polymer Coated Ag Nanoparticles. *Measurement,* 41**,** 1045-1053.

European commission, 2011. Definition of a Nanomaterial. *Available at: http://ec.europa.eu/environment/chemicals/nanotech/faq/definition_en.htm [accessed on: 17.02.2019]*.

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.

Farkas, J., Christian, P., Gallego-Urrea, J.A., Roos, N., Hassellov, M., Tollefsen, K.E. & Thomas, K.V., 2011. Uptake and Effects of Manufactured Silver Nanoparticles in Rainbow Trout (Oncorhynchus mykiss) Gill Cells. *Aquatic Toxicology,* 101**,** 117-125.

Finkel, T., 1998. Oxygen Radicals and Signaling. *Current Opinions in Cell Biology,* 10**,** 248-53.

Finkel, T. & Holbrook, N.J., 2000. Oxidants, Oxidative Stress and the Biology of Ageing. *Nature,* 408**,** 239.

Flynn, K.M., Ferguson, S.A., Delclos, K.B. & Newbold, R.R., 2000. Multigenerational Exposure to Dietary Genistein has no Severe Effects on Nursing Behavior in Rats. *Neurotoxicology,* 21**,** 997-1001.

Forster, S., Olveira, S. & Seeger, S., 2011. *Nanotechnology in the Market: Promises and Realities*.

Fung, M.C. & Bowen, D.L., 1996. Silver Products for Medical Indications: Risk-Benefit Assessment. *Journal of Toxicology: Clinical Toxicology,* 34**,** 119-26.

Future markets, 2017. The Global Market for Metal and Metal Oxide Nanoparticles 2010 - 2027. *Future Markets, Inc.*

Ghandour, W., Hubbard, J.A., Deistung, J., Hughes, M.N. & Poole, R.K., 1988. The Uptake of Silver Ions by Escherichia coli K12: Toxic Effects and Interaction with Copper Ions. *Applied Microbiology and Biotechnology,* 28**,** 559-565.

Gibson, O.R., Taylor, L., Watt, P.W. & Maxwell, N.S., 2017. Cross-Adaptation: Heat and Cold Adaptation to Improve Physiological and Cellular Responses to Hypoxia. *Sports Medicine,* 47**,** 1751-1768.

Giese, B., Klaessig, F., Park, B., Kaegi, R., Steinfeldt, M., Wigger, H., Von Gleich, A. & Gottschalk, F., 2018. Risks, Release and Concentrations of Engineered Nanomaterial in the Environment. *Scientific Reports,* 8**,** 1565.

Gomes, S.I.L., Roca, C.P., Scott-Fordsmand, J.J. & Amorim, M.J.B., 2017. High-Throughput Transcriptomics Reveals Uniquely Affected Pathways: AgNPs, PVP-Coated AgNPs and Ag NM300K Case Studies. *Environmental Science: Nano,* 4**,** 929-937.

Gong, M., Chen, B.O., Li, Z.-G. & Guo, L.-H., 2001. Heat-Shock-Induced Cross Adaptation to Heat, Chilling, Drought and Salt Stress in Maize Seedlings and Involvement of H2O2. *Journal of Plant Physiology,* 158**,** 1125-1130.

Gopinath, P., Gogoi, S.K., Sanpui, P., Paul, A., Chattopadhyay, A. & Ghosh, S.S., 2010. Signaling Gene Cascade in Silver Nanoparticle Induced Apoptosis. *Colloids and Surfaces B: Biointerfaces,* 77**,** 240-5.

Gordon, O., Vig Slenters, T., Brunetto, P.S., Villaruz, A.E., Sturdevant, D.E., Otto, M., Landmann, R. & Fromm, K.M., 2010. Silver Coordination Polymers for Prevention of Implant Infection: Thiol Interaction, Impact on Respiratory Chain Enzymes, and Hydroxyl Radical Induction. *Antimicrobial Agents and Chemotherapy,* 54**,** 4208-4218.

Gottschalk, F., Sun, T. & Nowack, B., 2013. Environmental Concentrations of Engineered Nanomaterials: Review of Modeling and Analytical Studies. *Environmental Pollution,* 181**,** 287-300.

Goussen, B., Parisot, F., Beaudouin, R., Dutilleul, M., Buisset-Goussen, A., Pery, A.R. & Bonzom, J.M., 2013. Consequences of a Multi-Generation Exposure to Uranium on Caenorhabditis elegans Life Parameters and Sensitivity. *Ecotoxicology,* 22**,** 869-78.

Gram, T.E., 1997. Chemically Reactive Intermediates and Pulmonary Xenobiotic Toxicity. *Pharmacological Reviews,* 49**,** 297-341.

Guo, J.-Z., Cui, H., Zhou, W. & Wang, W., 2008. Ag Nanoparticle-Catalyzed Chemiluminescent Reaction between Luminol and Hydrogen Peroxide. *Journal of Photochemistry and Photobiology A: Chemistry,* 193**,** 89-96.

Hagendorfer, H., Kaegi, R., Parlinska, M., Sinnet, B., Ludwig, C. & Ulrich, A., 2012. Characterization of Silver Nanoparticle Products Using Asymmetric Flow Field Flow Fractionation with a Multidetector Approach - a Comparison to Transmission Electron Microscopy and Batch Dynamic Light Scattering. *Analytical Chemistry,* 84**,** 2678-2685.

Handy, R., 2012. Environmental Toxicity of Engineered Nanomaterials: Focus on Aquatic Species. *Toxicology Letters,* 211**,** S12-S13.

Handy, R.D., Van Den Brink, N., Chappell, M., Muehling, M., Behra, R., Dusinska, M., Simpson, P., Ahtiainen, J., Jha, A.N., Seiter, J., Bednar, A., Kennedy, A., Fernandes, T.F. & Riediker, M., 2012. Practical considerations for conducting ecotoxicity test methods with manufactured nanomaterials: what have we learnt so far? *Ecotoxicology,* 21**,** 933-972.

Harrison, P., 2007. Emerging Challenges: Nanotechnology and the Environment. *GEO Year Book***,** 61-68.

Hassellöv, M., Readman, J.W., Ranville, J.F. & Tiede, K., 2008. Nanoparticle Analysis and Characterization Methodologies in Environmental Risk assessment of Engineered Nanoparticles. *Ecotoxicology,* 17**,** 344-361.

He, D., Garg, S. & Waite, T.D., 2012a. H2O2-Mediated Oxidation of Zero-Valent Silver and Resultant Interactions among Silver Nanoparticles, Silver Ions, and Reactive Oxygen Species. *Langmuir,* 28**,** 10266-10275.

He, D., Jones, A.M., Garg, S., Pham, A.N. & Waite, T.D., 2011. Silver Nanoparticle−Reactive Oxygen Species Interactions: Application of a Charging−Discharging Model. *The Journal of Physical Chemistry C,* 115**,** 5461-5468.

He, W., Zhou, Y.-T., Wamer, W.G., Boudreau, M.D. & Yin, J.-J., 2012b. Mechanisms of the pH Dependent Generation of Hydroxyl Radicals and Oxygen Induced by Ag Nanoparticles. *Biomaterials,* 33**,** 7547-7555.

Helmcke, K.J. & Aschner, M., 2010. Hormetic Effect of Methylmercury on Caenorhabditis elegans. *Toxicology and Applied Pharmacology,* 248**,** 156-64.

Ho, C.M., Yau, S.K., Lok, C.N., So, M.H. & Che, C.M., 2010. Oxidative Dissolution of Silver Nanoparticles by Biologically Relevant Oxidants: a Kinetic and Mechanistic Study. *Chemistry - An Asian Journal,* 5**,** 285-93.

Hoffmann, A., Sørensen, J. & Loeschcke, V., 2003. Adaptation of Drosophila to Temperature Extremes: Bringing Together Quantitative and Molecular Approaches. *Journal of Thermal Biology,* 28**,** 175-216.

Hoogewijs, D., Houthoofd, K., Matthijssens, F., Vandesompele, J. & Vanfleteren, J.R., 2008. Selection and Validation of a Set of Reliable Reference Genes for Quantitative sod Gene Expression Analysis in C. elegans. *BMC Molecular Biology,* 9**,** 9-9.

Hotze, E.M., Phenrat, T. & Lowry, G.V., 2010. Nanoparticle Aggregation: Challenges to Understanding Transport and Reactivity in the Environment. *Journal of Environmental Quality,* 39**,** 1909-24.

Hund-Rinke, K., Baun, A., Cupi, D., Fernandes, T.F., Handy, R., Kinross, J.H., Navas, J.M., Peijnenburg, W., Schlich, K., Shaw, B.J. & Scott-Fordsmand, J.J., 2016. Regulatory Ecotoxicity Testing of Nanomaterials – Proposed Modifications of OECD Test Guidelines Based on Laboratory Experience with Silver and Titanium Dioxide Nanoparticles. *Nanotoxicology,* 10**,** 1442-1447.

Hunt, G., Lynch, I., Cassee, F., Handy, R.D., Fernandes, T.F., Berges, M., Kuhlbusch, T.a.J., Dusinska, M. & Riediker, M., 2013. Towards a Consensus View on Understanding Nanomaterials Hazards and Managing Exposure: Knowledge Gaps and Recommendations. *Materials (Basel),* 6**,** 1090-1117.

Hunt, P.R., 2017. The C. elegans Model in Toxicity Testing. *Journal of Applied Toxicology,* 37**,** 50-59.

Hunt, P.R., Keltner, Z., Gao, X., Oldenburg, S.J., Bushana, P., Olejnik, N. & Sprando, R.L., 2014. Bioactivity of Nanosilver in Caenorhabditis elegans: Effects of Size, Coat, and Shape. *Toxicology Reports,* 1**,** 923-944.

Hwang, E.T., Lee, J.H., Chae, Y.J., Kim, Y.S., Kim, B.C., Sang, B.I. & Gu, M.B., 2008. Analysis of the Toxic Mode of Action of Silver Nanoparticles using Stress-Specific Bioluminescent Bacteria. *Small,* 4**,** 746-50.

Iso, 2010. Water quality -- Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of Caenorhabditis elegans (Nematoda). *ISO 10872:2010. Geneva, Switzerland.*

Iso, 2015. International Organization for Standardization. Nanotechnologies – Vocabularly-Part 1: Core terms. . *ISO/TS 80004-1:2015*.

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, H.-S., Qiu, X.-N., Li, G.-B., Li, W. & Yin, L.-Y., 2014. Silver Nanoparticles Induced Accumulation of Reactive Oxygen Species and Alteration of Antioxidant Systems in the Aquatic Plant Spirodela polyrhiza. *Environmental Toxicology and Chemistry,* 33**,** 1398-1405.

Jiang, J., Oberdörster, 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.

Kermanizadeh, A., Chauche, C., Brown, D.M., Loft, S. & Moller, P., 2015. The Role of Intracellular Redox Imbalance in Nanomaterial Induced Cellular Damage and Genotoxicity: a Review. *Environmental and Molecular Mutagenesis,* 56**,** 111-24.

Khan, I., Saeed, K. & Khan, I., 2017. Nanoparticles: Properties, Applications and Toxicities. *Arabian Journal of Chemistry*.

Kim, H.Y., Lee, M.J., Yu, S.H. & Kim, S.D., 2012. The Individual and Population Effects of Tetracycline on Daphnia magna in Multigenerational Exposure. *Ecotoxicology,* 21**,** 993-1002.

Kim, J.S., Kuk, E., Yu, K.N., Kim, J.-H., Park, S.J., Lee, H.J., Kim, S.H., Park, Y.K., Park, Y.H., Hwang, C.-Y., Kim, Y.-K., Lee, Y.-S., Jeong, D.H. & Cho, M.-H., 2007. Antimicrobial Effects of Silver Nanoparticles. *Nanomedicine: Nanotechnology, Biology and Medicine,* 3**,** 95-101.

Kim, S., Choi, J.E., Choi, J., Chung, K.H., Park, K., Yi, J. & Ryu, D.Y., 2009. Oxidative Stress-Dependent Toxicity of Silver Nanoparticles in Human Hepatoma Cells. *Toxicology In Vitro,* 23**,** 1076-84.

Kim, S.W., Kwak, J.I. & An, Y.J., 2013. Multigenerational Study of Gold Nanoparticles in Caenorhabditis elegans: Transgenerational Effect of Maternal Exposure. *Environmental Science & Technology,* 47**,** 5393-9.

Koch, R.E. & Hill, G.E., 2017. An Assessment of Techniques to Manipulate Oxidative Stress in Animals. *Functional Ecology,* 31**,** 9-21.

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.

Lazareva, A. & Keller, A.A., 2014. Estimating Potential Life Cycle Releases of Engineered Nanomaterials from Wastewater Treatment Plants. *ACS Sustainable Chemistry & Engineering,* 2**,** 1656-1665.

Lead, J.R., Batley, G.E., Alvarez, P.J.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,* 37**,** 2029-2063.

Lee, W., Kim, K.J. & Lee, D.G., 2014a. A Novel Mechanism for the Antibacterial Effect of Silver Nanoparticles on Escherichia coli. *Biometals,* 27**,** 1191-201.

Lee, Y.H., Cheng, F.Y., Chiu, H.W., Tsai, J.C., Fang, C.Y., Chen, C.W. & Wang, Y.J., 2014b. Cytotoxicity, Oxidative Stress, Apoptosis and the Autophagic Effects of Silver Nanoparticles in Mouse Embryonic Fibroblasts. *Biomaterials,* 35**,** 4706-15.

Leung, M.C.K., Williams, P.L., Benedetto, A., Au, C., Helmcke, K.J., Aschner, M. & Meyer, J.N., 2008. Caenorhabditis elegans: An Emerging Model in Biomedical and Environmental Toxicology. *Toxicological Sciences,* 106**,** 5-28.

Levard, C., Hotze, E.M., Lowry, G.V. & Brown, G.E., 2012. Environmental Transformations of Silver Nanoparticles: Impact on Stability and Toxicity. *Environmental Science & Technology,* 46**,** 6900-6914.

Levard, C., Mitra, S., Yang, T., Jew, A.D., Badireddy, A.R., Lowry, G.V. & Brown, G.E., 2013. Effect of Chloride on the Dissolution Rate of Silver Nanoparticles and Toxicity to E. coli. *Environmental Science & Technology,* 47**,** 5738-5745.

Li, T., Albee, B., Alemayehu, M., Diaz, R., Ingham, L., Kamal, S., Rodriguez, M. & Bishnoi, S.W., 2010a. Comparative toxicity study of Ag, Au, and Ag-Au bimetallic nanoparticles on Daphnia magna. *Analytical and Bioanalytical Chemistry,* 398**,** 689-700.

Li, X., Lenhart, J.J. & Walker, H.W., 2010b. Dissolution-Accompanied Aggregation Kinetics of Silver Nanoparticles. *Langmuir,* 26**,** 16690-16698.

Lim, D., Roh, J.-Y., Eom, H.-J., Choi, J.-Y., Hyun, J. & Choi, J., 2012a. 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.

Lim, D., Roh, J.Y., Eom, H.J., Choi, J.Y., Hyun, J. & Choi, J., 2012b. 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**,** 585-92.

Lindgren, B. & Laurila, A., 2005. Proximate Causes of Adaptive Growth Rates: Growth Efficiency Variation among Latitudinal Populations of Rana temporaria. *Journal of Evolutionary Biology,* 18**,** 820-8.

Liu, J. & Hurt, R.H., 2010. Ion Release Kinetics and Particle Persistence in Aqueous Nano-Silver Colloids. *Environmental Science & Technology,* 44**,** 2169-2175.

Liu, J., Pennell, K.G. & Hurt, R.H., 2011. Kinetics and Mechanisms of Nanosilver Oxysulfidation. *Environmental Science & Technology,* 45**,** 7345-53.

Livingstone, D.R., 2003. *Oxidative Stress in Aquatic Organisms in Relation to Pollution and Aquaculture*.

Lowry, G.V., Gregory, K.B., Apte, S.C. & Lead, J.R., 2012. Transformations of Nanomaterials in the Environment. *Environmental Science & Technology,* 46**,** 6893-6899.

Lumb, A.B., 2017. Chapter 24 - Oxygen Toxicity and Hyperoxia. *In* A.B. Lumb (ed.) *Nunn's Applied Respiratory Physiology (Eighth Edition).* Elsevier, 341-356.e2.

Luo, X., Xu, S.M., Yang, Y.N., Li, L.Z., Chen, S.P., Xu, A. & Wu, L.J., 2016. Insights into the Ecotoxicity of Silver Nanoparticles Transferred from Escherichia coli to Caenorhabditis elegans. *Scientific Reports,* 6**,** 36465.

Lushchak, V.I., 2011. Environmentally Induced Oxidative Stress in Aquatic Animals. *Aquatic Toxicology,* 101**,** 13-30.

Lushchak, V.I., 2014. Free Radicals, Reactive Oxygen Species, Oxidative Stress and its Classification. *Chemico-Biological Interactions,* 224**,** 164-75.

Markaki, M. & Tavernarakis, N., 2010. Modeling Human Diseases in Caenorhabditis elegans. *Biotechnology Journal,* 5**,** 1261-76.

Massarsky, A., Dupuis, L., Taylor, J., Eisa-Beygi, S., Strek, L., Trudeau, V.L. & Moon, T.W., 2013. Assessment of Nanosilver Toxicity during Zebrafish (Danio rerio) Development. *Chemosphere,* 92**,** 59-66.

Mccord, J.M. & Fridovich, I., 1969. Superoxide Dismutase. An Enzymic Function for Erythrocuorein (Homecuprein) *Journal of Biological Chemistry,* 244**,** 6049-6055.

Meyer, J.N., Lord, C.A., Yang, X.Y.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.

Milić, M., Leitinger, G., Pavičić, I., Zebić Avdičević, M., Dobrović, S., Goessler, W. & Vinković Vrček, I., 2015. Cellular Uptake and Toxicity Effects of Silver Nanoparticles in Mammalian Kidney Cells. *Journal of Applied Toxicology,* 35**,** 581-592.

Miranda-Vizuete, A. & Veal, E.A., 2017. Caenorhabditis elegans as a Model for Understanding ROS Function in Physiology and Disease. *Redox Biology,* 11**,** 708-714.

Moon, J., Kwak, J.I., Kim, S.W. & An, Y.-J., 2017. Multigenerational Effects of Gold Nanoparticles in Caenorhabditis elegans: Continuous versus Intermittent Exposures. *Environmental Pollution,* 220**,** 46-52.

Morones, J.R., Elechiguerra, J.L., Camacho, A., Holt, K., Kouri, J.B., Ramirez, J.T. & Yacaman, M.J., 2005. The Bactericidal Effect of Silver Nanoparticles. *Nanotechnology,* 16**,** 2346-53.

Murphy, Michael p., 2009. How Mitochondria Produce Reactive Oxygen Species. *Biochemical Journal,* 417**,** 1 - 13.

Muyssen, B.T.A. & Janssen, C.R., 2004. Multi-Generation Cadmium Acclimation and Tolerance in Daphnia magna Straus. *Environmental Pollution,* 130**,** 309-316.

Nanowerk, 2019. Nanomaterials Database. *Available at: https://www.nanowerk.com/nanomaterial-database.php [accessed 18.02.2019]*.

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.

Nowack, B., 2010. Nanosilver Revisited Downstream. *Science,* 330**,** 1054-1055.

Oberdörster, G., Maynard, A., Donaldson, K., Castranova, V., Fitzpatrick, J., Ausman, K., Carter, J., Karn, B., Kreyling, W., Lai, D., Olin, S., Monteiro-Riviere, N., Warheit, D., Yang, H. & Group, A.R.F.T.I.R.F.R.S.I.N.T.S.W., 2005. Principles for Characterizing the Potential Human Health Effects from Exposure to Nanomaterials: Elements of a Screening Strategy. *Particle and Fibre Toxicology,* 2**,** 8.

Panzarini, E., Mariano, S., Carata, E., Mura, F., Rossi, M. & Dini, L., 2018. Intracellular Transport of Silver and Gold Nanoparticles and Biological Responses: An Update. *International Journal of Molecular Science,* 19**,** 1305.

Park, H.-J., Kim, J.Y., Kim, J., Lee, J.-H., Hahn, J.-S., Gu, M.B. & Yoon, J., 2009. Silver-Ion-Mediated Reactive Oxygen Species Generation Affecting Bactericidal Activity. *Water Research,* 43**,** 1027-1032.

Pedersen, S.A., Håkedal, O.J., Salaberria, I., Tagliati, A., Gustavson, L.M., Jenssen, B.M., Olsen, A.J. & Altin, D., 2014. Multigenerational Exposure to Ocean Acidification

during Food Limitation Reveals Consequences for Copepod Scope for Growth and Vital Rates. *Environmental Science & Technology,* 48**,** 12275-12284.

Phenrat, T., Saleh, N., Sirk, K., Kim, H.-J., Tilton, R.D. & Lowry, G.V., 2008. Stabilization of Aqueous Nanoscale Zerovalent Iron Dispersions by Anionic Polyelectrolytes: Adsorbed Anionic Polyelectrolyte Layer Properties and their Effect on Aggregation and Sedimentation. *Journal of Nanoparticle Research,* 10**,** 795-814.

Piccinno, F., Gottschalk, F., Seeger, S. & Nowack, B., 2012. Industrial Production Quantities and Uses of Ten Engineered Nanomaterials in Europe and the World. *Journal of Nanoparticle Research,* 14**,** 1109.

Pietroiusti, A., Magrini, A. & Campagnolo, L., 2016. New Frontiers in Nanotoxicology: Gut Microbiota/Microbiome-Mediated Effects of Engineered Nanomaterials. *Toxicology and Applied Pharmacology,* 299**,** 90-95.

Powers, K.W., Palazuelos, M., Moudgil, B.M. & Roberts, S.M., 2007. Characterization of the Size, Shape, and State of Dispersion of Nanoparticles for Toxicological Studies. *Nanotoxicology,* 1**,** 42-51.

Ray, P.D., Huang, B.W. & Tsuji, Y., 2012. Reactive Oxygen Species (ROS) Homeostasis and Redox Regulation in Cellular Signaling. *Cell Signal,* 24**,** 981-90.

Reinsch, B.C., Levard, C., Li, Z., Ma, R., Wise, A., Gregory, K.B., Brown, G.E. & Lowry, G.V., 2012. Sulfidation of Silver Nanoparticles Decreases Escherichia coli Growth Inhibition. *Environmental Science & Technology,* 46**,** 6992-7000.

Ribeiro, M.J., Maria, V.L., Scott-Fordsmand, J.J. & Amorim, M.J.B., 2015. 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.

Roco, M.C., 2003. Nanotechnology: Convergence with Modern Biology and Medicine. *Current Opinion in Biotechnology,* 14**,** 337-346.

Roh, J.-Y., Eom, H.-J. & Choi, J., 2012. Involvement of Caenohabditis elegans MAPK Signaling Pathways in Oxidative Stress Response Induced by Silver Nanoparticles Exposure. *Toxicological Research,* 28**,** 19-24.

Roh, J.Y., Sim, S.J., Yi, J., Park, K., Chung, K.H., Ryu, D.Y. & Choi, J., 2009. Ecotoxicity of Silver Nanoparticles on the Soil Nematode Caenorhabditis elegans Using Functional Ecotoxicogenomics. *Environmental Science & Technology,* 43**,** 3933-3940.

Römer, I., White, T.A., Baalousha, M., Chipman, K., Viant, M.R. & Lead, J.R., 2011. Aggregation and Dispersion of Silver Nanoparticles in Exposure Media for Aquatic Toxicity Tests. *Journal of Chromatography A,* 1218**,** 4226-4233.

Saptarshi, S.R., Duschl, A. & Lopata, A.L., 2013. Interaction of Nanoparticles with Proteins: Relation to Bio-Reactivity of the Nanoparticle. *Journal of Nanobiotechnology,* 11**,** 26.

Sasaki, T., Kobayashi, T., Takagi, I. & Moriyama, H., 2006. Solubility Measurement of Zirconium(IV) Hydrous Oxide. *International Journal for Chemical Aspects of Nuclear Science and Technology,* 94**,** 489 - 494.

Sawosz, E., Binek, M., Grodzik, M., Zielinska, M., Sysa, P., Szmidt, M., Niemiec, T. & Chwalibog, A., 2007. Influence of Hydrocolloidal Silver Nanoparticles on Gastrointestinal Microflora and Morphology of Enterocytes of Quails. *Archives of Animal Nutrition,* 61**,** 444-51.

Schultz, C.L., Wamucho, A., Tsyusko, O.V., Unrine, J.M., Crossley, A., Svendsen, C. & Spurgeon, D.J., 2016. Multigenerational Exposure to Silver Ions and Silver Nanoparticles Reveals Heightened Sensitivity and Epigenetic Memory in

Caenorhabditis elegans. *Proceedings of the Royal Society B-Biological Sciences,* 283**,** 20152911.

Science policy section, 2004. *Nanoscience and nanotechnologies: opportunities and uncertainties*.

Sejian, V., Bhatta, R., Gaughan, J.B., Dunshea, F.R. & Lacetera, N., 2018. Review: Adaptation of Animals to Heat Stress. *Animal,* 12**,** s431-s444.

Sharma, V.K., Siskova, K.M., Zboril, R. & Gardea-Torresdey, J.L., 2014. Organic-Coated Silver Nanoparticles in Biological and Environmental Conditions: Fate, Stability and Toxicity. *Advances in Colloid and Interface Science,* 204**,** 15-34.

Shvedova, A.A., Pietroiusti, A., Fadeel, B. & Kagan, V.E., 2012. Mechanisms of Carbon Nanotube-Induced Toxicity: Focus on Oxidative Stress. *Toxicology and Applied Pharmacology,* 261**,** 121-133.

Sondi, I. & Salopek-Sondi, B., 2004. Silver Nanoparticles as Antimicrobial Agent: a Case Study on E. coli as a Model for Gram-Negative Bacteria. *Journal of Colloid and Interface Science,* 275**,** 177-182.

Song, M.F., Li, Y.S., Kasai, H. & Kawai, K., 2012. Metal Nanoparticle-Induced Micronuclei and Oxidative DNA Damage in Mice. *Journal of Clinical Biochemistry and Nutrition,* 50**,** 211-6.

Sorensen, J.G., Norry, F.M., Scannapieco, A.C. & Loeschcke, V., 2005. Altitudinal Variation for Stress Resistance Traits and Thermal Adaptation in Adult Drosophila buzzatii from the New World. *Journal of Evolutionary Biology,* 18**,** 829-37.

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.

Stoehr, L.C., Gonzalez, E., Stampfl, A., Casals, E., Duschl, A., Puntes, V. & Oostingh, G.J., 2011. Shape Matters: Effects of Silver Nanospheres and Wires on Human Alveolar Epithelial Cells. *Part Fibre Toxicol,* 8**,** 36.

Sun, P.Y., Foley, H.B., Handschumacher, L., Suzuki, A., Karamanukyan, T. & Edmands, S., 2014. Acclimation and Adaptation to Common Marine Pollutants in the Copepod Tigriopus californicus. *Chemosphere,* 112**,** 465-471.

Sun, T.Y., Bornhöft, N.A., Hungerbühler, K. & Nowack, B., 2016. Dynamic Probabilistic Modeling of Environmental Emissions of Engineered Nanomaterials. *Environmental Science & Technology,* 50**,** 4701-4711.

Sun, T.Y., Mitrano, D.M., Bornhöft, N.A., Scheringer, M., Hungerbühler, K. & Nowack, B., 2017. Envisioning Nano Release Dynamics in a Changing World: Using Dynamic Probabilistic Modeling to Assess Future Environmental Emissions of Engineered Nanomaterials. *Environmental Science & Technology,* 51**,** 2854-2863.

Suntres, Z.E., 2002. Role of Antioxidants in Paraquat Toxicity. *Toxicology,* 180**,** 65-77.

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.

Valavanidis, A., Vlachogianni, T. & Fiotakis, C., 2009. 8-hydroxy-2' -deoxyguanosine (8-OHdG): A Critical Biomarker of Oxidative Stress and Carcinogenesis. *Journal of Environmental Science and Health Part C Enviornmental Carcinogenesis & Ecotoxicology Reviews,* 27**,** 120-39.

Van Den Brule, S., Ambroise, J., Lecloux, H., Levard, C., Soulas, R., De Temmerman, P.-J., Palmai-Pallag, M., Marbaix, E. & Lison, D., 2016. Dietary Silver Nanoparticles can Disturb the Gut Microbiota in Mice. *Particle and Fibre Toxicology,* 13**,** 38.

Vazquez-Muñoz, R., Borrego, B., Juárez-Moreno, K., García-García, M., Mota Morales, J.D., Bogdanchikova, N. & Huerta-Saquero, A., 2017. Toxicity of Silver Nanoparticles in Biological Systems: Does the Complexity of Biological Systems Matter? *Toxicology Letters,* 276**,** 11-20.

Völker, C., Boedicker, C., Daubenthaler, J., Oetken, M. & Oehlmann, J., 2013. Comparative Toxicity Assessment of Nanosilver on Three Daphnia Species in Acute, Chronic and Multi-Generation Experiments. *PLOS ONE,* 8**,** e75026.

Völker, C., Kämpken, I., Boedicker, C., Oehlmann, J. & Oetken, M., 2015. Toxicity of Silver Nanoparticles and Ionic Silver: Comparison of Adverse Effects and Potential Toxicity Mechanisms in the Freshwater Clam Sphaerium corneum. *Nanotoxicology,* 9**,** 677-685.

Walker, C.H., Hopkins, S.P., Sibly, R.M., Peakall, D.B., 2006. Principles of Ecotoxicology, 3rd ed. *Taylor & Francis Group, CRC Press, Boca Raton, FL*.

Wang, D., Liu, P. & Xing, X., 2010. Pre-treatment with Mild UV Irradiation Increases the Resistance of Nematode Caenorhabditis elegans to Toxicity on Locomotion Behaviors from Metal Exposure. *Environmental Toxicology and Pharmacology,* 29**,** 213-222.

Wang, D. & Xing, X., 2010. Pre-treatment with Mild UV Irradiation Suppresses Reproductive Toxicity Induced by Subsequent Cadmium Exposure in Nematodes. *Ecotoxicology and Environmental Safety,* 73**,** 423-9.

Warheit, D.B., 2018. Hazard and Risk Assessment Strategies for Nanoparticle Exposures: How Far have we come in the past 10 years? *F1000Res,* 7**,** 376.

Williams, K., Milner, J., Boudreau, M.D., Gokulan, K., Cerniglia, C.E. & Khare, S., 2015. Effects of Subchronic Exposure of Silver Nanoparticles on Intestinal Microbiota and Gut-Associated Immune Responses in the Ileum of Sprague-Dawley Rats. *Nanotoxicology,* 9**,** 279-289.

Williams, K.M., Gokulan, K., Cerniglia, C.E. & Khare, S., 2016. Size and Dose Dependent Effects of Silver Nanoparticle Exposure on Intestinal Permeability in an in vitro Model of the Human Gut Epithelium. *Journal of Nanobiotechnology,* 14**,** 62.

Wu, H., Yin, J.J., Wamer, W.G., Zeng, M. & Lo, Y.M., 2014. Reactive Oxygen Species-Related Activities of Nano-Iron Metal and Nano-Iron Oxides. *Journal of Food and Drug Analysis,* 22**,** 86-94.

Xiu, Z.-M., Ma, J. & Alvarez, P.J.J., 2011. Differential Effect of Common Ligands and Molecular Oxygen on Antimicrobial Activity of Silver Nanoparticles versus Silver Ions. *Environmental Science & Technology,* 45**,** 9003-9008.

Yanase, S., Hartman, P.S., Ito, A. & Ishii, N., 1999. Oxidative Stress Pretreatment Increases the X-radiation Resistance of the Nematode Caenorhabditis elegans. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 426**,** 31-39.

Yang, X., 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**,** 1119-27.

Yu, Z., Chen, X., Zhang, J., Wang, R. & Yin, D., 2012. *Transgenerational Effects of Heavy Metals on L3 Larva of Caenorhabditis elegans with Greater Behavior and Growth Inhibitions in the Progeny*.

Zhang, W., Yao, Y., Sullivan, N. & Chen, Y., 2011. Modeling the Primary Size Effects of Citrate-Coated Silver Nanoparticles on their Ion Release Kinetics. *Environmental Science & Technology,* 45**,** 4422-8.

Zhao, Y.L. & Wang, D.Y., 2012. Formation and Regulation of Adaptive Response in Nematode Caenorhabditis elegans. *Oxidative Medicine and Cellular Longevity,* 2012**,** 564093.

# *Errata*

1) The section numbering has been updated in the table of contents as it was incorrect
2) Page 6: “in liquid media” was added for clarification
3) Page 35, section 2.6: The reference to the ISO guidelines 10972 was corrected to 10**8**72
4) Page 38, section 2.6.1: “*Figure 5*” has been changed to “*Figure 4*”
5) Following suggestions by opponent #2 two sentences were added to Paper IV to refer to the supplementary materials, section 3:
    i. **Page 6, Section 2.2:** “*For the assessment of potential external damages, cuts or lesions to the cuticle of the nematodes, caused by the Ag NPs, nematodes were analyzed using the scanning electron microscope. For more detail see supplementary materials, section 3.*”
    ii. **Page 8, Section 3.2:** “*Analysis of Ag NP exposed nematodes revealed no external damages, cuts or lesion of the cuticle of the nematodes (Figure S4).*”

**Paper I**
Kleiven, M., Rossbach, L.M., Gallego-Urrea, J.A., Brede, D.A., Oughton, D.H. & Coutris, C. 2018. Characterizing the Behavior, Uptake, and Toxicity of NM300K Silver Nanoparticles in *Caenorhabditis elegans*. - Environmental Toxicology and Chemistry 37: 1799-1810.
DOI: 10.1002/etc.4144

**Paper II**
Rossbach, L.M., Oughton, D.H., Coutris, C. & Brede, D.A. *In vivo* assessment of silver nanoparticle induced reactive oxygen species reveals tissue specific effects on cellular redox status in the nematode *Caenorhabditis elegans*. - Environmental Science: Nano.
(Submitted)

**Paper III**
Rossbach, L.A., Maremonti, E., Eide, D.M., Oughton, D.H. & Brede, D.A. 2019. Adaptive tolerance to multigenerational silver nanoparticle (NM300K) exposure by the nematode *Caenorhabditis elegans* is associated with increased sensitivity to $AgNO_3$. - Nanotoxicology 13: 527-542.
DOI: 10.1080/17435390.2018.1557272

**Paper IV**
Rossbach, L.A., Oughton, D.H. & Brede, D.A. Impact of multigenerational exposure to $AgNO_3$ or NM300K silver nanoparticles on *Caenorhabditis elegans* antioxidant defense and oxidative stress.
(Manuscript)

ISBN: 978-82-575-1596-6
ISSN: 1894-6402

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

103277 / ANDVORDGRAFISK.NO