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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

Lisa Magdalena Rossbach

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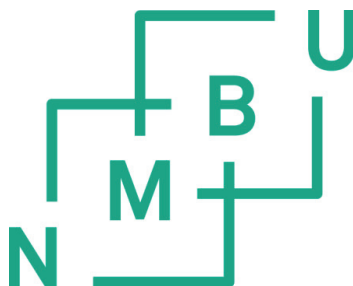
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Ås (2019)



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

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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 AgNO₃. 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 AgNO₃.**
- 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 AgNO₃ was used as a positive control.

For AgNO₃ 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 AgNO₃ 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 AgNO₃. 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 AgNO₃. However, the concentrations necessary to achieve a comparable dose-response were 7 – 10 times higher for the NPs compared to AgNO₃. 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 AgNO₃. Ag uptake by the nematodes was comparable between the two forms of Ag. However, following depuration approximately 2 fold higher concentrations of AgNO₃ were retained by nematodes.

In the multigenerational, chronic exposure, nematodes were continuously exposed to three concentrations of either AgNO₃ (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 AgNO₃ 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 AgNO₃ exposed nematodes only produced offspring at > 1 mm. Furthermore, the continuous exposure towards Ag NPs led to an increased sensitivity towards AgNO₃. Comparatively, the multigenerational AgNO₃ exposure resulted in no change in toxic response towards AgNO₃, 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, AgNO₃ 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ and Ce³⁺, 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 produkttegenskaper 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 adaptasjon 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 eksponeringslutt (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 toleranserrespons, vist ved økt reproduksjon i nærvær av Ag NP sammenlignet med kontroll/andre behandlinger. Den økte reproduksjonskapasiteten medførte en kostnad i form av redusert vekst. Disse nematodene produserte avkom ved en total

kroppslengde på 0.8-1 mm, mens kontroll og AgNO₃-eksponerte nematoder reproduserte avkom kun når kroppslengden var >1 mm. I tillegg viste det seg at Ag NP eksponerte nematoder ble mer sensitive for AgNO₃. Til sammenligning førte multigenerasjon AgNO₃-eksponering ikke til noen endring i respons mot AgNO₃, 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 inducere ROS. Dette antyder en adaptasjon 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* genen, 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 AgNO₃ celler og vev i hele kroppen. Multigenerasjonstudien viste økt *sod-1* genuttrykk i F3 generasjonen antyder en potensiell rolle for antioksidant forsvarsmekanismer i adaptasjon 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 AgNO₃ 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 inducerer ROS som fører til observerte toksiske effekter inkludert redusert reproduksjon, fertilitet og vekst, mens AgNO₃ inkorporeres lettere i cellene og dermed kan påvirke enzymer og proteiner direkte, med påfølgende ROS produksjon. Samlet sett viser resultatene at AgNO₃ og Ag NP inducerer 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

reduisert 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 AgNO₃. (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 AgNO₃ 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
EC _x	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 $0.001 \mu\text{g Ag L}^{-1}$ (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).

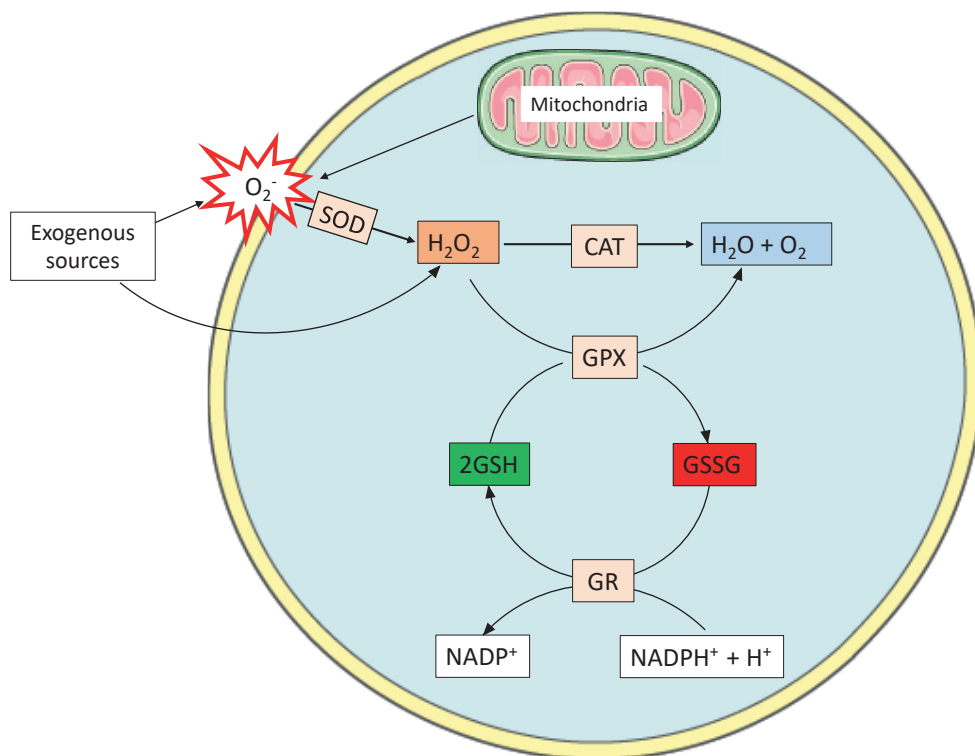


Figure 1: Schematic representation of the production and removal of ROS by antioxidant defense systems in the nematode *C. elegans*. Abbreviations are explained in the text.

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

H₂O₂ leading to superoxide (O₂^{•-}) 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 (O₂^{•-}) and peroxide radicals (O₂^{2•-}), as well as hydroxyl radicals (•OH), hydrogen peroxide (H₂O₂) and singlet oxygen (¹O₂) (Hwang *et al.*, 2008, He *et al.*, 2012a, He *et al.*, 2012b, Choi *et al.*, 2018). The formation of •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 AgNO₃ (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 adaptation 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 (Muysen 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 O₂ 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 H₂O₂ 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 constraints. 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 (Muysen 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 <i>C. elegans</i> studies	Multigenerational nanoparticle studies	Multigenerational Ag NP studies	Multigenerational Ag NP <i>C. elegans</i> 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 AgNO₃. 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 AgNO₃.
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 AgNO₃ in the nematode *C. elegans* (Paper I and II).
- II. To investigate the reproducibility of Ag NP toxicity tests with *C. elegans*, using AgNO₃ 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 AgNO₃), 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 AgNO₃ 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 AgNO₃ 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.

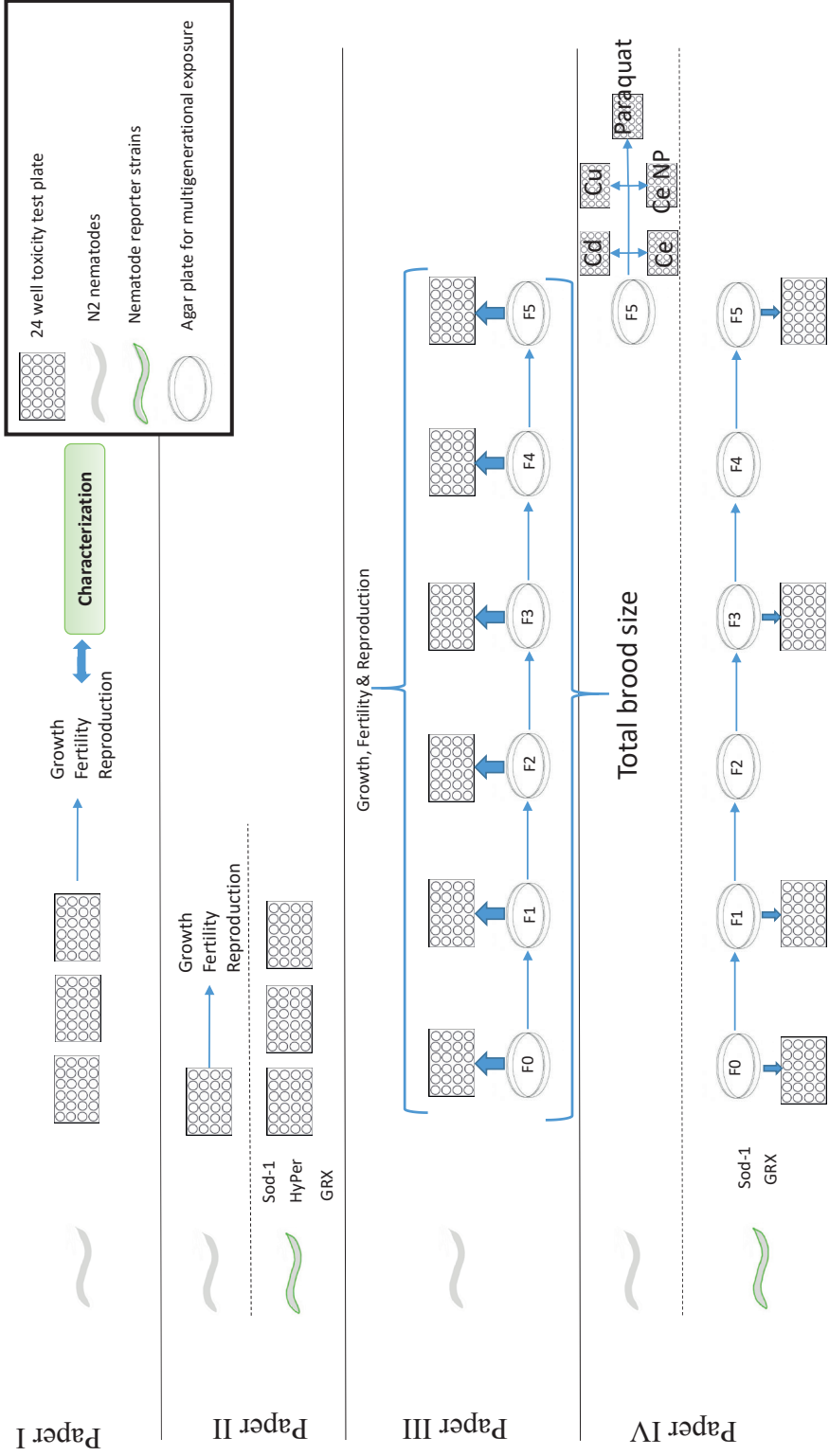


Figure 2: Overview of the different exposure set ups performed.

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 ddH₂O (15 M Ω 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 ddH₂O.

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.

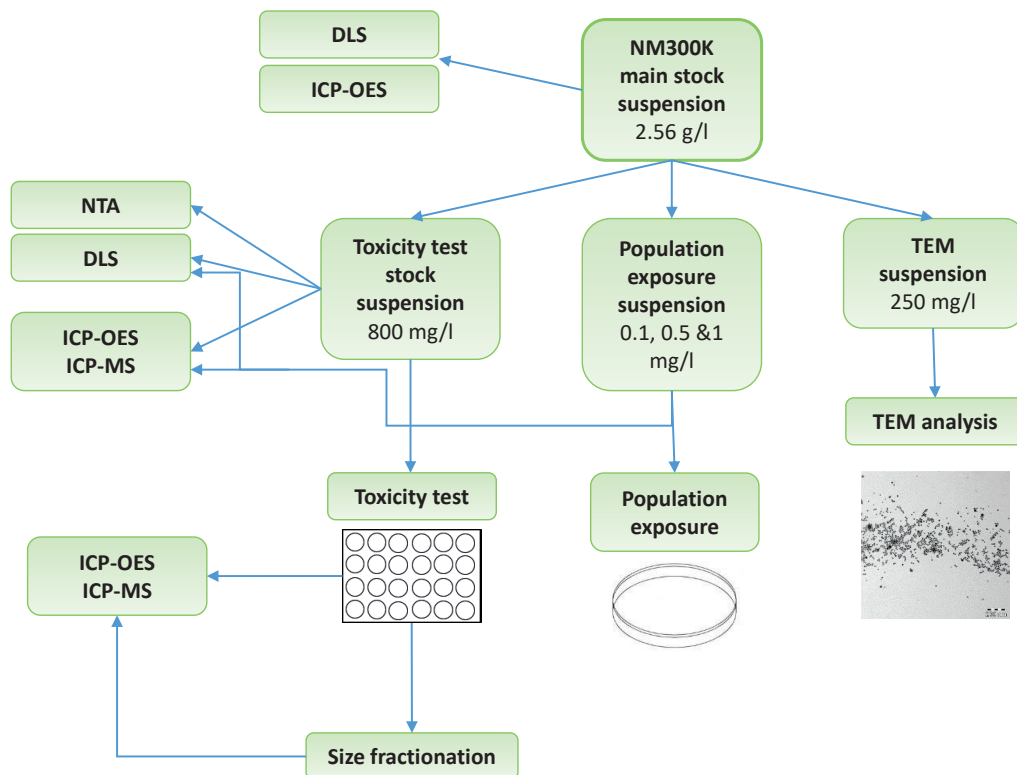


Figure 3: Outline of sampling and techniques for analysis of particle characterization and behavior during the exposures. Abbreviations are explained in the text.

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 μ l 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 \sim 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 \sim 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 $^{\circ}$ 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 AgNO₃ 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 AgNO₃ populations, however, due to delayed development, had to be adjusted accordingly for all Ag NP populations (table 2).

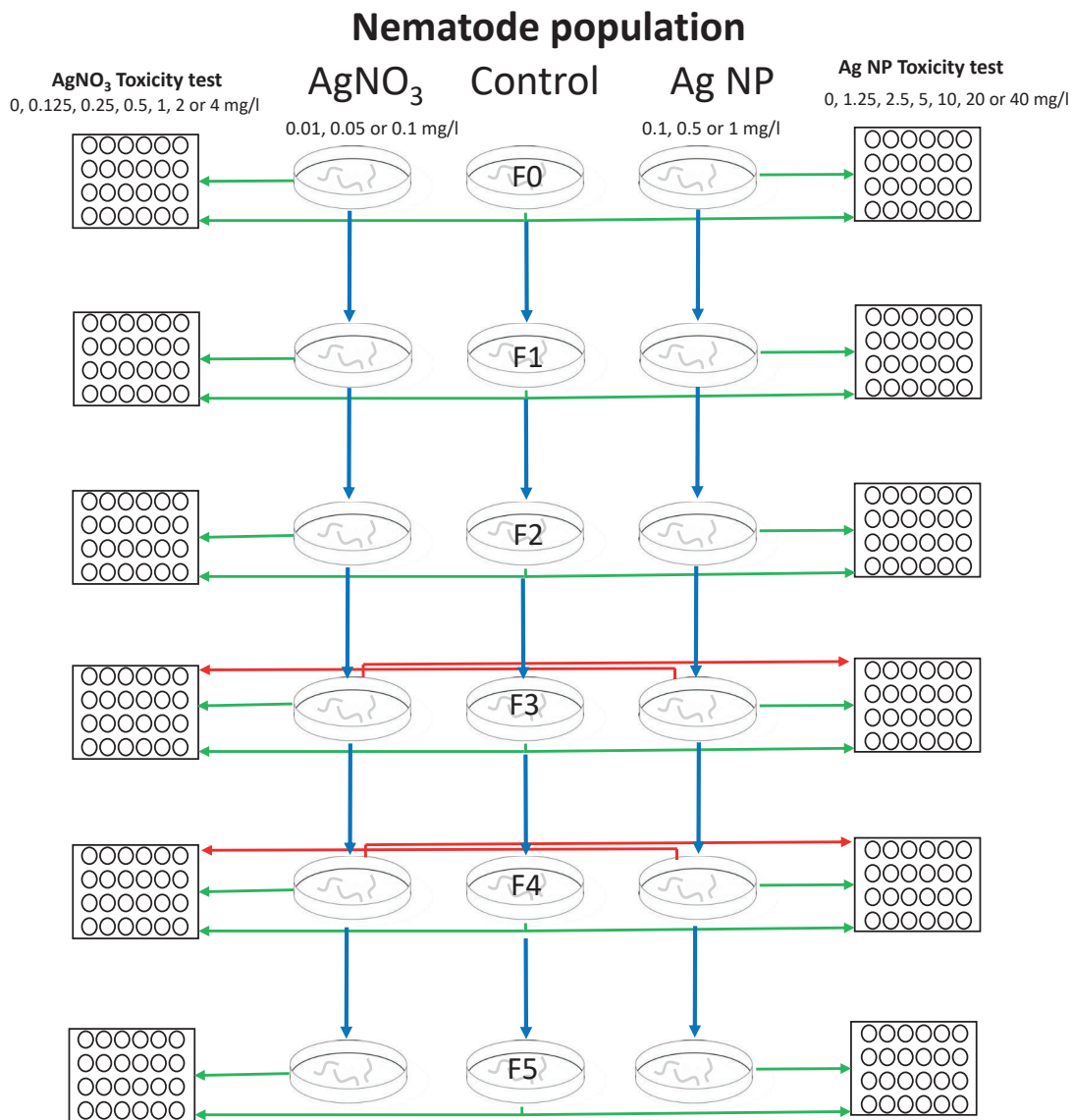


Figure 4: Experimental design of the continuous exposure to either AgNO₃ or Ag NPs, or control populations on agar plates. Offspring from each generation were subjected to standard toxicity tests to a range of concentrations of either AgNO₃ or Ag NPs.

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

Population	Number of days																											
	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																
AgNO ₃ 0.01 mg/l	72h					72h				72h		72h																
AgNO ₃ 0.05 mg/l	72h					72h				72h		72h																
AgNO ₃ 0.1 mg/l	72h					72h				72h		72h																
Ag NP 0.1 mg/l	72h					72h				120h		96h																
Ag NP 0.5 mg/l	72h					72h				120h		96h																
Ag NP 1 mg/l	72h					72h				144h																		
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 AgNO₃ 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 (Cu²⁺ - 0, 0.06, 0.13, 0.25, 0.5, 1 and 2 mg L⁻¹), cadmium (Cd²⁺ - 0, 0.09, 0.19, 0.38, 0.75, 1.5 and 3 mg L⁻¹), cerium (Ce³⁺ - 0, 3.13, 6.25, 12.5, 25, 50 and 100 mg L⁻¹), cerium dioxide nanoparticles (CeO₂ NPs - 0, 0.156, 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, lose 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).

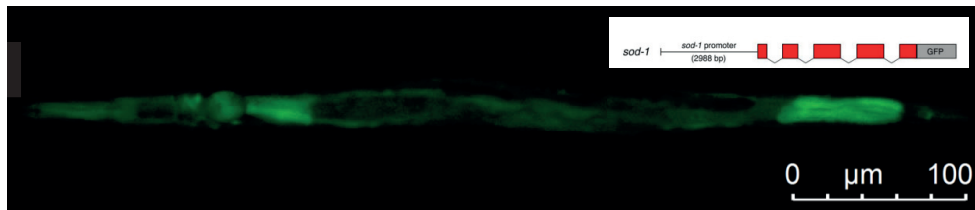


Figure 5: GFP labelled SOD-1 reporter strain (Doonan *et al.*, 2008) (photo L. Rossbach).

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).

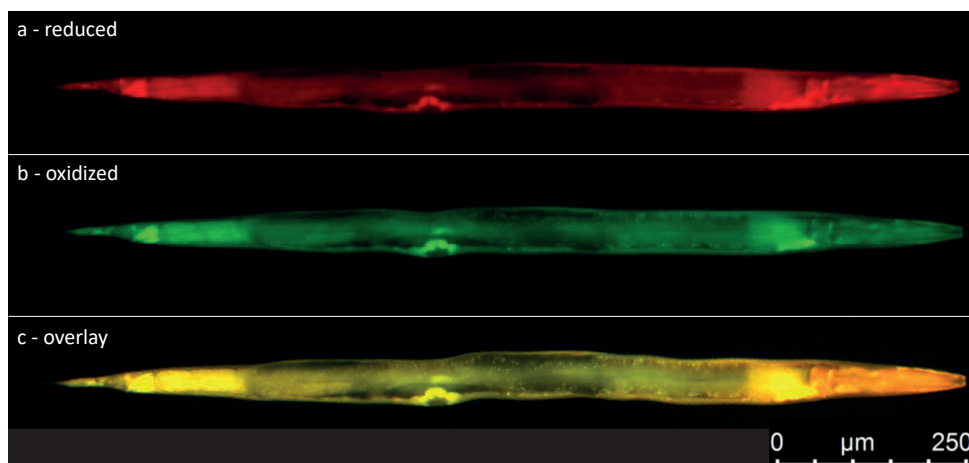


Figure 6: YFP labelled peroxide biosensor strain HyPer, showing here high levels of cellular hydrogen peroxide, taken with a 405 nm (reduced - a) and 490 nm (oxidized - b) excitation filter, and a 535 emission filter (Back *et al.*, 2012) (photo L. Rossbach).

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).

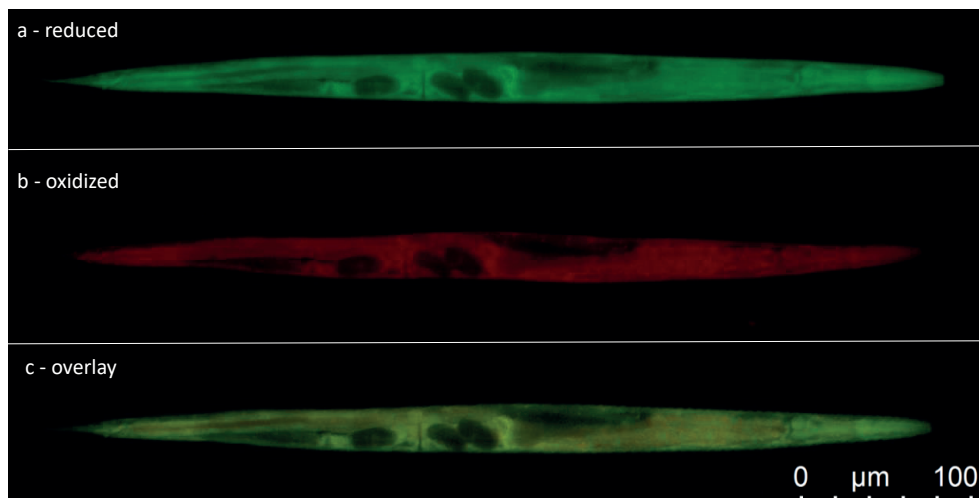


Figure 7: The GFP labelled GRX (Grx1-roGFP2) biosensor strain, showing here a heightened GSSG/GSH ratio (overlay – c), taken with a 490 nm (reduced – a) and 405 nm (oxidized – b) excitation filter, and a 535 emission filter (Back *et al.*, 2012) (photo L. Rossbach).

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 AgNO₃ or Ag NPs. However, for this exposure a single concentration of 0.1 and 0.5 mg Ag L⁻¹, for AgNO₃ 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.

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

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

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 \pm SD) for experiments 2 and 3, respectively. Dynamic light scattering analysis of the stock suspension (in ddH₂O) 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ 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 (AgNO₃ 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 AgNO₃ 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 AgNO₃ could potentially mean higher Ag associated with *E. coli*, increasing dietary uptake in this exposure.

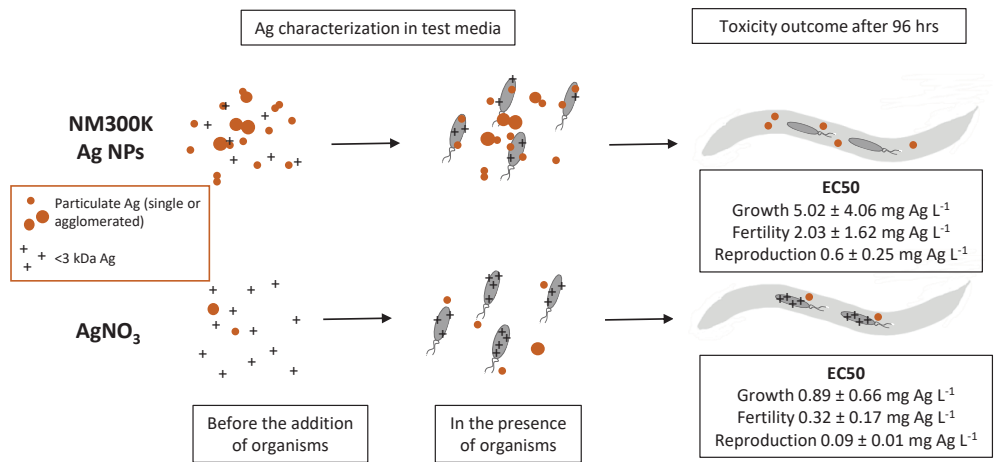


Figure 8: Graphical summary of exposure and main studied endpoints and results in Paper I.

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 AgNO₃ 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 \pm 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ (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 AgNO₃ (1 mg Ag L⁻¹).

A significant increase in *sod-1* expression was found at 0.5 and 1 mg Ag L⁻¹ AgNO₃, 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ exposure.

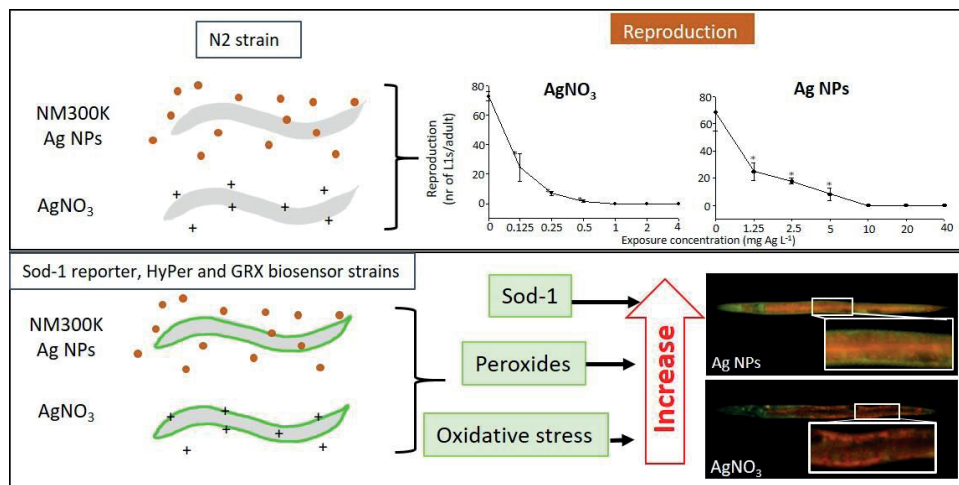


Figure 9: Graphical summary of exposure and main results in Paper II.

3.3. Paper III.

Adaptive tolerance to a multigenerational silver nanoparticle (NM300K) exposure by the nematode *Caenorhabditis elegans* is associated with increased sensitivity to AgNO₃

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), AgNO₃ (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 AgNO₃ 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 AgNO₃ from the AgNO₃ 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 AgNO₃ 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 AgNO₃, compared to the AgNO₃ population. For the AgNO₃ 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.

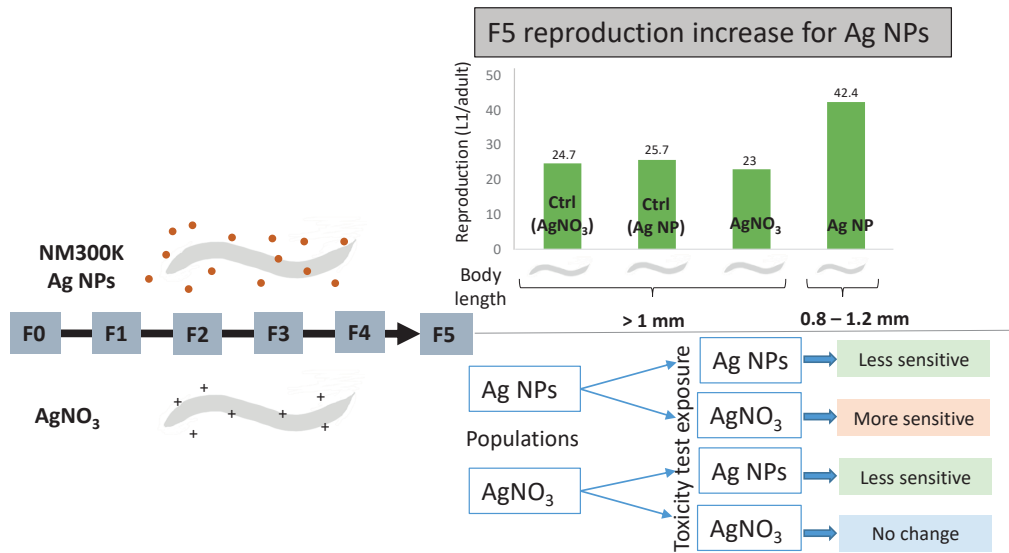


Figure 10: Graphical summary of exposure and main results in Paper III.

3.4. Paper IV.

Effects on the nematode *Caenorhabditis elegans* antioxidant defense and reactive oxygen species (ROS) metabolism following multigenerational exposure to AgNO₃ 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 AgNO₃ or Ag NPs of the N2 strain (as presented in Paper III), nematodes were exposed to cerium (Ce³⁺), cerium dioxide nanoparticles (CeO₂ NPs), cadmium (Cd²⁺), copper (Cu²⁺) and paraquat, in standard toxicity tests. Following the multigenerational exposure towards either AgNO₃ or Ag NPs, nematodes showed no changes in sensitivity when exposed to cadmium, copper, and CeO₂ NPs. Both Ag NP and AgNO₃ 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ or Ag NPs in the toxicity test, *sod-1* gene expression showed a significant increase in the F3 generation from all three populations (control, AgNO₃ 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ 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.

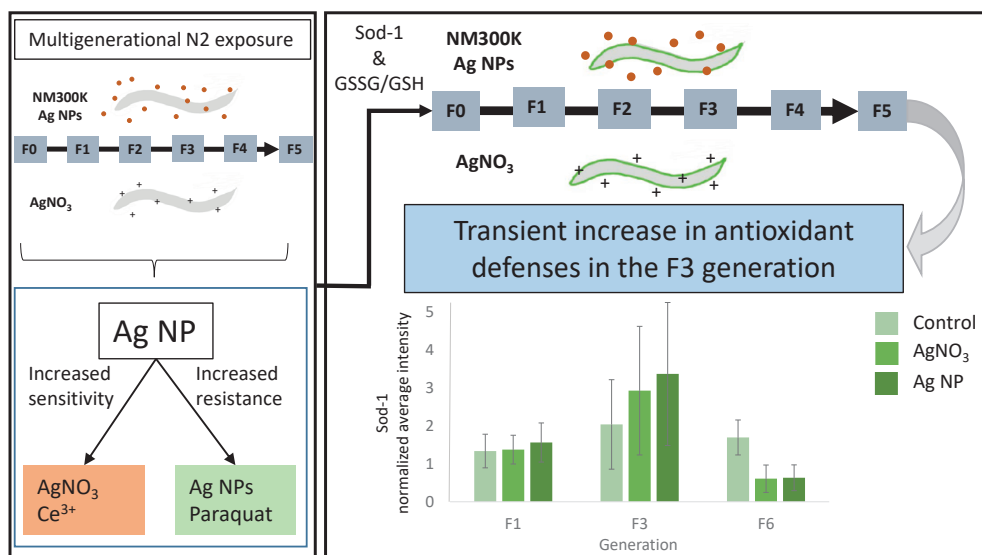


Figure 11: Graphical summary of exposure and main results in Paper IV.

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 AgNO₃. Thus the exposures were set up to a) characterize the toxic effect of the Ag NPs compared to AgNO₃ 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 AgNO₃, 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					
I	1	0-4	0-4	0-4 and 0-40	NP characterization, growth, fertility, reproduction, uptake, EC-estimations	<ul style="list-style-type: none"> TEM: 12.5 – 16.7 nm DLS: 33.8 – 82 nm Zeta pot.: -1.02 – -7.32 mV 	<ul style="list-style-type: none"> Presence of larger (<200nm) particles in AgNO₃ exposure Presence of <3 kDa Ag fraction at T=0 in both exposures 	<ul style="list-style-type: none"> Up take from both forms of Ag, higher retention of AgNO₃ after depuration Higher relative toxicity from AgNO₃
II	1	0-4 and 0-10	0-4 and 0-10	NP characterization, growth, fertility, reproduction, ROS and oxidative stress (<i>sod-1</i> expression, peroxide levels, GSSG/GSH ratios), localization, EC-estimations	<ul style="list-style-type: none"> TEM: 16.7 ± 6.5 nm DLS: 93.3 ± 1.3 nm Zeta pot.: -8.77 mV 	<ul style="list-style-type: none"> High aggregated Ag fraction from both Ag exposures at 72 hrs 	<ul style="list-style-type: none"> Both forms of Ag induce <i>sod-1</i> Increase in peroxides Low Ag NPs produce oxidative stress Ag NPs primarily produce ROS within the lumen 	
III	6	0.01, 0.05 and 0.1	0-4 and 1	NP characterization, growth, fertility, reproduction, cross-toxicity test exposure, EC-estimations	<ul style="list-style-type: none"> TEM: 16.7 ± 6.5 nm DLS: 73 ± 1.1 nm Zeta pot.: -5.5 ± 2.7 mV 	<ul style="list-style-type: none"> At 96 hrs low <3 kDa Ag fraction from both Ag exposures High degree of aggregation, or association with <i>E. coli</i> from the Ag NPs 	<ul style="list-style-type: none"> Adaptation towards Ag NPs; but at the cost of reduced growth as well as increased sensitivity towards AgNO₃ No adaptation towards AgNO₃ AgNO₃ leads to reduced sensitivity towards Ag NP exposure 	
IV	6	0.1 and 0.5	0.1, 0.5 and 1	NP characterization, external damages, total brood size, N2 sensitivity towards other toxicants, ROS and oxidative stress (<i>sod-1</i> and GSSG/GSH ratios)	<ul style="list-style-type: none"> TEM: 23.9 ± 21.8 nm DLS: 79 ± 4.42 nm 	<ul style="list-style-type: none"> At 96 hrs low <3 kDa Ag fraction from both Ag exposures High degree of aggregation, or association with <i>E. coli</i> from the Ag NPs 	<ul style="list-style-type: none"> Reduced sensitivity towards paraquat following six generational Ag NP exposure Temporary increase in <i>sod-1</i> expression in the F3 generation from both Ag populations compared to the control population 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 AgNO₃, 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ and Ag NP exposures, where approximately 30 – 60 % and 40 – 70 % of the Ag was aggregated in the AgNO₃ 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 Ag₂S (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 (SO₄²⁻) 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 AgNO₃ 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, AgNO₃ 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ (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 AgNO₃. 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 AgNO₃ 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 AgNO₃. 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 AgNO₃ than Ag NPs in liquid media.

The reduced toxicity of the Ag NPs compared to the AgNO₃ 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 AgNO₃ 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 AgNO₃. 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 AgNO₃, while the multigenerational exposure to AgNO₃ 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ population compared to control and Ag NP populations. These results indicate transferable changes in the antioxidant defenses in response to AgNO₃ 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 AgNO₃ 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 AgNO₃ 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 peroxy 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ 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 AgNO₃. 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 AgNO₃ 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 AgNO₃.

Although, both AgNO₃ 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 AgNO₃ 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 AgNO₃ 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 AgNO₃. 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

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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 10872
- 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

Environmental Chemistry

Characterizing the Behavior, Uptake, and Toxicity of NM300K Silver Nanoparticles in *Caenorhabditis elegans*

Merethe Kleiven,^{a,*} Lisa M. Rossbach,^a Julian A. Gallego-Urrea,^{a,b} Dag A. Brede,^a Deborah H. Oughton,^{a,*} and Claire Coutris^c^aCenter for Environmental Radioactivity (CERAD CoE), Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, Ås, Norway^bDepartment of Marine Sciences, University of Gothenburg, Kristineberg, Fiskebäckskil, Sweden^cDivision of Environment and Natural Resources, Norwegian Institute of Bioeconomy Research, Høgskoleveien, Ås, Norway

Abstract: Using *Caenorhabditis elegans* as a model organism, we addressed the potential linkage among toxicity of NM300K Ag nanoparticles (AgNPs), their particle size distribution, and the presence of dissolved Ag in the test media. Of the 3 endpoints assessed (growth, fertility, and reproduction), reproduction was the most sensitive, with the 50% effect concentration (EC50) ranging from 0.26 to 0.84 mg Ag L⁻¹ and 0.08 to 0.11 mg Ag L⁻¹ for NM300K and AgNO₃, respectively. Silver uptake by *C. elegans* was similar for both forms of Ag, whereas bioaccumulation was higher in AgNO₃ exposure. The observed differences in toxicity between NM300K and AgNO₃ did not correlate with bioaccumulated Ag, which suggests that toxicity is a function of the type of exposing agent (AgNPs vs AgNO₃) and its mode of action. Before addition of the food source (*Escherichia coli*), size fractionation revealed that dissolved Ag comprised 13 to 90% and 4 to 8% of total Ag in the AgNO₃ and NM300K treatments, respectively. No dissolved Ag was detectable in the actual test media due to immediate Ag adsorption to bacteria. The results of the present study indicate that information on behavior and characterization of exposure conditions is essential for nanotoxicity studies. *Environ Toxicol Chem* 2018;37:1799–1810. © 2018 SETAC

Keywords: Toxic effects; Nanoparticles; Bioaccumulation; Characterization; Reproducibility

INTRODUCTION

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

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

Both the initial particle characteristics and the associated transformations of the particles have the potential to significantly impact the interaction of the particles with biological systems (Montes-Burgos et al. 2010). A range of studies have suggested that the observed toxic effects of AgNPs can be largely, or solely, attributed to Ag ion release following particle dissolution, whereas others provide evidence for particle-specific effects, for example, from reactive oxygen species generated on the surface

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

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of the particle (Borm et al. 2006; Carlson et al. 2008). Thus, monitoring of particle dissolution, for instance by means of ultrafiltration, needs to be taken into consideration, and to be followed as a function of time to identify the source of the toxic response measured (Sørensen and Baun 2015).

The AgNP NM300K used in the present study is a representative Ag nanomaterial provided by the European Commission Joint Research Centre and is thus one of the best characterized sources of AgNPs available. However, despite well-developed synthesis methods and thorough characterization by the supplier, physicochemical changes such as agglomeration, aggregation, and surface charge variations arise during the preparation of stock suspensions and additions to the exposure media (Lundqvist et al. 2008). Thus, further characterization during these stages is essential.

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

Furthermore, the toxicity of NM300K AgNPs has been studied in a range of species, including *Daphnia magna* (Poynton et al. 2012), the gram-negative bacterium *Pseudomonas putida* (Malleve et al. 2016), the marine diatom *Chaetoceros curvisetus*, the Enchytraeid *Enchytraeus crypticus* (Gomes et al. 2017), and an earthworm *Lumbricus rubellus* (van der Ploeg et al. 2014; Gomes et al. 2017). To our knowledge, this is the first study to have explored the toxic effects of NM300K on the nematode *Caenorhabditis elegans* with the aim of investigating the linkage between characterization and toxicity.

Living in the soil porewater, the nematode *C. elegans* is a relevant model organism for a range of environmental contaminants. Detailed knowledge about its physiology and biology allows for extensive measurements of a wide range of toxicological endpoints, including fecundity, reproduction, and development (O'Reilly et al. 2014; Hunt 2017). As a result of its short life cycle (96 h at 20 °C), *C. elegans* represents a perfect *in vivo* model for nanoparticle toxicology because exposure time is minimized and thus aging effects of the particles are reduced (Handy et al. 2012). Furthermore, the impact of different test media and impacts on the particle toxicity to *C. elegans* have been recognized, and different media have been proposed, such as the low-ionic-strength US Environmental Protection Agency moderately hard reconstituted water (Cressman and Williams 1997; Tyne et al. 2013). However, despite increased use of these low-ionic-strength media in toxicity tests, particles are still rarely characterized in media in the actual test. More importantly, information is lacking on speciation and fractions of silver (irrespective of the original source) as well as the associated dynamical behavior.

Therefore, the present study aims to ascertain the potential linkage between toxicity of the AgNPs NM300K to the nematode *C. elegans* and the behavior of the particles before

and after deposit into the test medium. The approach consisted of measuring standardized endpoints (survival, growth, fecundity, and reproduction), in combination with monitoring of particle behavior in stocks and exposure media over time. The experiment consists of a series of 3 experiments set up over 3 consecutive years using the same standard toxicity test, but 2 different stock preparation methods. This allowed us to investigate the reproducibility of these toxicity tests, as well as the influence of stock preparation methods on particle size.

MATERIALS AND METHODS

Experimental design

Caenorhabditis elegans were exposed to NM300K AgNPs and silver nitrate (AgNO₃) in 3 separate experiments (experiments 1–3 in Table 1, hereafter termed E1, E2, and E3) following the International Organization for Standardization (ISO, 2010) 10872 guideline with some modifications, including changes in exposure media. Stocks of wild-type nematodes N2 Bristol (*Caenorhabditis* Genetic Centre, Minneapolis, MN, USA) were kept in liquid cultures before a synchronized culture was obtained by treating gravid hermaphrodites with hypochlorite to extract eggs. Eggs were hatched on agar plates overnight to obtain synchronized L1 stage larvae before the start of the exposure.

All 3 experiments were carried out as standard 96-h toxicity tests at 20 °C in the dark, in 24-well culture plates, gently shaken to ensure sufficient oxygenation. Each well contained 495 µL of the bacteria *Escherichia coli* OP50 resuspended in moderately hard reconstituted water (US Environmental Protection Agency 2002), 5 µL of *C. elegans* at the L1 larval stage in liquid medium M9 (density 11 ± 5.5 L1/µL), and 500 µL AgNO₃ solution or AgNP suspension in moderately hard reconstituted water (at twice the nominal concentration). The nematodes were exposed in triplicate to AgNPs and AgNO₃. In experiments 1 and 2, a concentration range of 0.1 to 4 mg L⁻¹ was chosen for both AgNO₃ and AgNP to match concentrations for both exposures (Table 1). In an attempt to reach similar toxic effects for AgNP and AgNO₃ exposures, the concentration range of AgNP in experiment 3 was increased (Table 1). In addition, separate exposure plates were set up for the characterization of exposure suspensions, with sampling performed at 0 and 96 h for E1, at 0, 20, and 96 h for E2, and at 96 h for E3. The experiments were carried out over a time span of 3 y, allowing us to investigate reproducibility over time.

The potential effects of the stabilizing agents present in the NM300K AgNP material (NM300K DIS) were tested separately. No effects were observed at concentrations equivalent

TABLE 1: Nominal exposure concentrations of AgNO₃ and NM300K silver nanoparticles (AgNPs) in 3 separate experiments

Experiment	AgNO ₃ (mg Ag L ⁻¹)	AgNPs (mg Ag L ⁻¹)
1	0.1, 0.5, 1, 2, and 4	0.1, 0.5, 1, 2, and 4
2	0.1, 0.25, 0.5, 1, 2, and 4	0.1, 0.25, 0.5, 1, 2, and 4
3	0.125, 0.25, 0.5, 1, 2, and 4	0.9, 1.8, 3.6, 7.3, 14.5, and 29

to those found in the highest NM300K AgNP exposure concentrations.

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

Preparation and characterization of Ag suspensions

The nanomaterial used in all 3 experiments was the Organisation for Economic Co-operation and Development (OECD) representative AgNP NM300K (Fraunhofer IME). These are spherical Ag nanoparticles dispersed in a mix of 2 stabilizing agents, 4% each of polyoxyethylene glycerol trioleate and Tween 20. The average particle size is reported to be 15 nm, with 90% of the particles $<20 \text{ nm}$. Silver nitrate (pro analysis Merck) was used to compare the toxicity and behavior of AgNPs with those of a Ag salt. In E1, the AgNP stock suspension with a concentration of 400 mg Ag L^{-1} was prepared under anaerobic conditions (in a glove box) by adding the original NM300K suspension to Milli-Q water ($15 \text{ M}\Omega/\text{cm}$) and mixing by gentle resuspensions with the pipette. The AgNP stock suspension in E2 and E3 was prepared according to Jensen et al. (2016). Briefly, a 2.56 g Ag L^{-1} stock suspension was prepared by dispersing the original NM300K suspension in Milli-Q water and sonicating for 13 min at 15% amplitude (depositing $7.35 \pm 0.05 \text{ W}$) using a 400-W Branson Sonifier S-450D (Branson Ultrasonics) equipped with a standard 13-mm disruptor horn (model 101-147-037). In all experiments, the subsequent suspensions were all prepared from the stock suspension.

Transmission electron microscope. Transmission electron microscope (TEM; Morgagni 268, FEI) was used to assess the core diameter of AgNPs in the stock suspension, in E2 and E3. Ten μL of AgNP stock suspension (E2) or of a diluted stock suspension to obtain an optimized particle concentration on the grid (250 mg L^{-1} ; E3) were added to a 400-mesh Cu-coated Piloform film (Agar Scientific), and the specimens were allowed to air dry. The TEM images were acquired with the instrument operating at 80 keV. Analysis of the TEM images was performed using the iTEM software (Olympus), according to the protocol by Mast and de Temmeman (2016). The particle size provided is the Ferret minimum defined as the minimum distance of parallel tangents at opposing particle borders.

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

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

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

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

Nanoparticle tracking analysis. Nanoparticle tracking analysis was used to assess the hydrodynamic diameter of individual particles. The nanoparticle tracking analysis measurements of the hydrodynamic diameter were carried out on a NanoSight LM10 device. The light source was a solid-state, single-mode laser diode (radiation output maximum power $<50 \mu\text{W}$, 635-nm continuous wave, maximum power $<35 \text{ mW}$). The standard camera Marlin F-033B (Allied Vision Technologies) was used. All data were analyzed using the instrument software (NanoSight™ Ver 2.2). The nanoparticle tracking analysis was done on 5 videos each 1 min long. The measurements with nanoparticle tracking analysis were performed on the samples from the final day of E1 after mild centrifugation ($\sim 1000 \text{ g}$).

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

All samples were digested with acid and appropriately diluted before ICP-MS measurements. In E1, all samples were digested in a solution of aqua regia ($40\% \text{ HNO}_3$ and $11\% \text{ HCl v/v}$) at high temperature (260°C) and pressure (120 bar; UltraCLAVE 3, Milestone) before diluting to suitable concentrations for ICP-MS measurements. Because this was subsequently found to be

unnecessary to achieve complete dissolution, in E2 and E3, samples were digested in ultrapure HNO₃ (sample:HNO₃ volume ratio of 1:5 in E2 and 1:7.5 in E3) at 80 °C for 4 h, before dilution to a final acid concentration of 10 vol %.

Uptake of Ag

For determination of the potential uptake of AgNPs, Ag⁺, and/or transformation products by the nematodes, an uptake study was conducted during E2. Nematodes were exposed in triplicates to NM300K and AgNO₃ for 65 h before ICP–MS analysis. To measure total uptake (including gut content), half of the nematodes were washed twice in moderately hard reconstituted water without further depuration. To assess the Ag fraction tightly/stably bound to organisms, the other half of the nematodes were subjected to 2 h of depuration on agar plates seeded with *E. coli*. Subsequent to depuration, nematodes were recovered from the agar plates by carefully flushing them from the dish into an Eppendorf tube using moderately hard reconstituted water. All samples (undepurated and depurated organisms) were washed thoroughly with moderately hard reconstituted water followed by a gentle centrifugation (250 g) and supernatant removal. This step was repeated twice. Samples were then evaporated to dryness, 1.5 mL ultrapure HNO₃ (65%) was added to them, and they were heated at 90 °C for 2 h. Following the digestion, samples were diluted to reach 10 vol % HNO₃ and analyzed by ICP–MS. To produce concentrations as ng Ag μg⁻¹ wet weight nematode, the ICP–MS results in ng Ag were divided by the exact number of nematodes in each of the 3 replicate dishes (average 12, range 7–20) and then multiplied by the wet weight of a nematode using the formula $W = (L \times D^2) / (1.6 \times 10^6)$, where W is the mass (wet wt in μg) per individual, L is the nematode length (μm), and D is the greatest body diameter (μm; Andrassy 1956).

Estimation of effect concentrations

The estimation of effect concentrations at 10 and 50% (EC10 and EC50) on growth, fertility, and reproduction was performed using the free software RegTox developed by Eric Vindimian (2016). The values used for estimating EC10 and EC50 were the average values from all replicate wells ($n = 3–8$) expressed as % of controls. The Hill model was used for the estimation, the model parameters were calculated by nonlinear regression, and their confidence intervals (CIs) were estimated using a Monte Carlo simulation. The values reported are the optimal values for EC10 and EC50 with their 95% CIs.

RESULTS AND DISCUSSION

Ag characterization in exposure media

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

As measured by TEM, the mean particle size of the stock suspension was 12.5 ± 4.1 nm (mean \pm standard deviation [SD], $n = 70$) in E2 and 16.7 ± 6.5 nm (mean \pm SD, $n = 32241$; Table 2 and Supplemental Data, Figure S1). However, aggregates/agglomerates, varying in size, were also present in the samples. The presence of aggregates/agglomerates was also indicated by the difference in zeta-average particle size and number mean, as well as the polydispersity index in the size measurements conducted with dynamic light scattering (Table 2). Interestingly, dynamic light scattering measurements suggested a higher aggregation of the NM300K in the sonicated stock suspensions (E2 and E3) than the stock suspension that was homogenized by repeated pipetting. For suspensions that are produced from powders, sonication might help to break down larger aggregates, but dispersions that have been synthesized in liquid media can be induced to aggregation by the addition of sonicating power (Handy et al. 2012; Petersen et al. 2014). However, the E1 stock was less concentrated than the E2 and E3 stocks, potentially also influencing the zeta-average hydrodynamic diameter.

Ag characterization in exposure media without organisms.

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

To gain information on the influence of moderately hard reconstituted water on the AgNP size in NM300K exposures, and on the formation of AgNPs or other Ag(I) complexes in AgNO₃ exposures, a range of concentrations of either form of Ag were analyzed by dynamic light scattering. The results showed that the mean zeta-average particle size in the higher exposure concentrations was close to that seen in the stock suspension (Table 3 and Figure 1). In the low exposure concentration

TABLE 2: Size characterization of stock suspensions measured by DLS and TEM^a

Experiment	Stock concentration (g L ⁻¹)	Z-average diameter (nm)	Number mean diameter (nm)	Polydispersity index	Zeta potential (mV)	TEM diameter Ferret min (nm)
1	0.02	33.8 \pm 1.7	ND	0.461 \pm 0.005	-1.02	ND
2	2.56	82.0 \pm 6.0	28 \pm 5.0	0.293 \pm 0.010	ND	12.5 \pm 4.1
3	2.56	71.7 \pm 0.6	28 \pm 8.6	0.272 \pm 0.003	-7.32	16.7 \pm 6.5

^aResults are provided as mean \pm 1 standard deviation.

DLS = dynamic light scattering; TEM = transmission electron microscopy; ND = not determined.

TABLE 3: Particle size (mean \pm 1 standard deviation) measured by DLS in exposure suspensions prior to addition of *Escherichia coli* or *Caenorhabditis elegans*

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

^aCalculated using general purpose algorithm (normal resolution) in Malvern Zetasizer software.

^bData from experiment 2.

^cData from experiment 1.

DLS = dynamic light scattering; NA = not available.

(0.5 mg L⁻¹), the zeta-average particle size was significantly larger than in the stock suspension. In samples containing more than one size population of particles or aggregates, dynamic light scattering tends to overestimate the mean particle size, due to the higher intensity signals reflected by larger particles. This artifact is even more evident at lower particle concentrations, and hence increases uncertainties in the measurements (Handy et al. 2008). Measurements of change in particle size over time showed that the intermediate concentrations (2 and 4 mg L⁻¹) were stable over time, whereas in both the lowest (0.5 mg L⁻¹) and highest (10 mg L⁻¹) concentrations, the particles were less stable, and a time-dependent increase in zeta-average particle size was observed (Figure 1). The presence of larger particles or Ag complexes (e.g., AgCl_(s)) in AgNO₃ exposures was evident

from the dynamic light scattering results, which also showed higher polydispersity than in the NM300K exposures (Table 3).

Time-resolved dynamic light scattering measurements of the zeta-average diameter of NM300K performed over a period of a few minutes showed an initial high degree of instability before stabilizing at approximately 30 nm, and a polydispersity index of approximately 0.450 in all suspensions tested (Figure 2 and Supplemental Data, Figure S2). These polydispersity indices indicate the presence of larger aggregates, which was also the case for the starting material. However, no increase in the hydrodynamic diameter was observed even at 10 mg L⁻¹, which indicates no contribution of collision-induced aggregation (Gallego-Urrea et al. 2016). The aggregation experiment in moderately hard reconstituted water did not show any increase in diameter during a lapse of 10 min when 10 mg L⁻¹ NM300K was added. Interestingly, the AgNO₃ solution containing 10 and 1 mg Ag L⁻¹ in moderately hard reconstituted water showed an increase in the particle size and reached a steady-state value after a few minutes; the value of the steady-state hydrodynamic diameter varied with the initial AgNO₃ concentration (Figure 2A and B) and mixing ratios with the medium. This behavior can be explained by the formation of AgCl particles, which is consistent with the speciation calculations performed with visual Minteq (see the next section, *Ag inorganic speciation modeling using Minteq*) and was also observed by other authors in media containing chloride ions (Gonçalves et al. 2017).

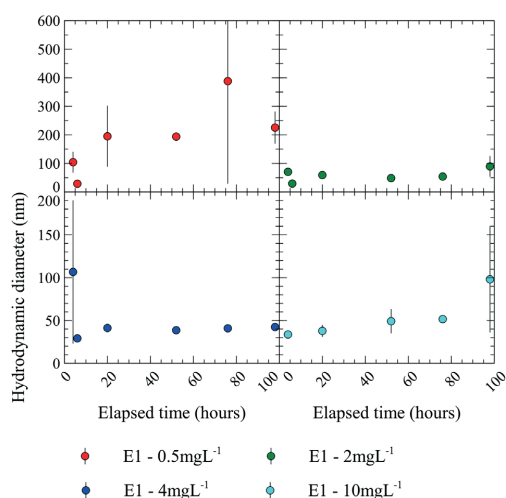


FIGURE 1: Zeta-average diameters obtained from dynamic light scattering measurements in experiment 1 (E1) without organisms for the NM300K silver nanoparticle exposures. Averages of 3 replicated measurements are presented, and error bars represent 1 standard deviation.

Ag inorganic speciation modeling using Minteq. The results also showed the importance of characterizing not only the AgNP exposure suspensions, but also the Ag salt solutions. Although the assumption is often that these represent an ionic exposure, Ag speciation is also affected by the chemical conditions of the media, and particles and colloids can also be formed, as shown by dynamic light scattering measurements. To control for possible formation of inorganic solid-phase species (e.g., AgCl_(s)), chemical speciation of Ag(I) with Minteq in moderately hard reconstituted water without organisms revealed the possible formation of AgCl⁰ after approximately 100 μ g L⁻¹ of free Ag ion was added to the medium. The dissolved Ag, which in this case means all forms of silver other

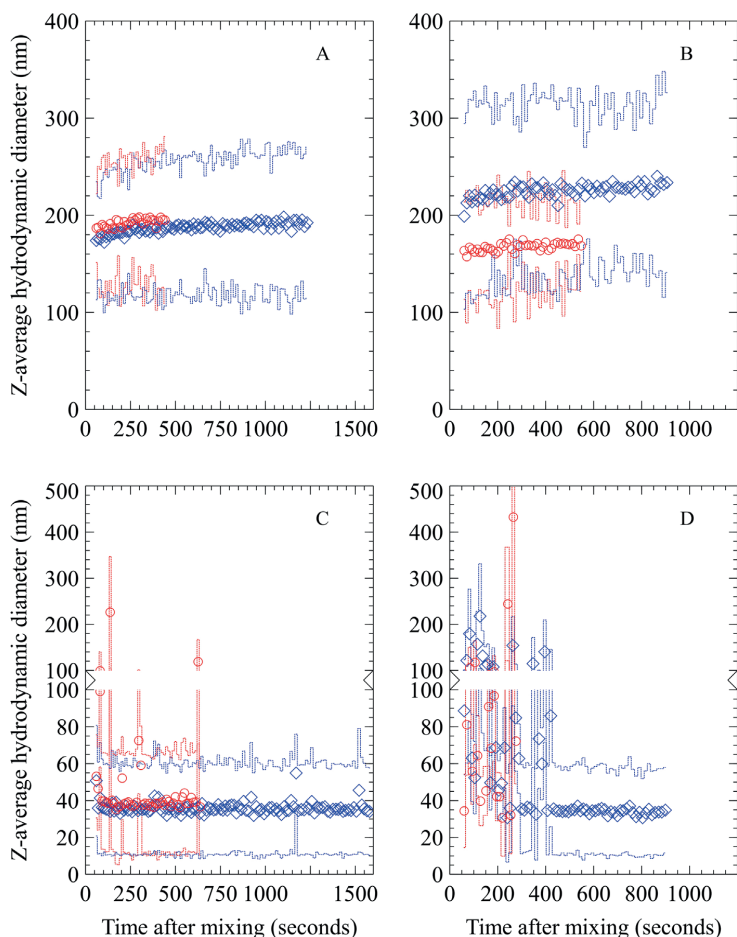


FIGURE 2: Time-resolved dynamic light scattering measurements performed on AgNO_3 solutions in moderately hard reconstituted water (A and B) and NM300K in moderately hard reconstituted water (C and D). (A) and (C) correspond to measurements done at 10 mg Ag L^{-1} , and (B) and (D) at 1 mg Ag L^{-1} . Zeta-average hydrodynamic diameters (d_H) were obtained as explained in the section *Preparation and characterization of Ag suspensions*, and duplicate values are presented with the markers. The dotted lines correspond to the corresponding color-matched standard deviation (SD) obtained from polydispersity index (PDI) values assuming a Gaussian profile distribution ($\text{SD} = (d_H^2 \times \text{PDI})^{0.5}$).

than $\text{AgCl}_{(s)}$, remained relatively constant (concentrations between 100 and $500 \mu\text{g L}^{-1}$) when the total Ag was below $2000 \mu\text{g L}^{-1}$. In moderately hard reconstituted water in the absence of organisms, the results indicate that most of the nondissolved Ag corresponds to $\text{AgCl}_{(s)}$.

Ag characterization in exposure media in the presence of organisms. The presence of the test organisms in a toxicity test system (in the present study, the nematodes *C. elegans* and their food source the bacteria *E. coli*) influenced the test system, including Ag speciation and particle behavior. In the present study, an additional characterization of the actual exposure system was thus performed to address possible changes in Ag speciation and provide insight into time-dependent changes in particle aggregation and dissolution. This included size

measurements using dynamic light scattering and nanoparticle tracking analysis, as well as total and $<3 \text{ kDa}$ Ag concentrations using ICP-MS.

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

The nanoparticle tracking analysis measurements of the exposure suspensions after 96 h of exposure were all in accordance with the dynamic light scattering measurements with regard to the presence of large material, but also showed

that the presence of small particles (<200 nm) in the exposure media with NM300K was significantly greater than in the control (Figure 3). The presence of particulate material in the control was probably due to organic particles coming from the organisms. The 2 mg L⁻¹ AgNO₃ exposure suspension contained a large amount of particles in the 200-nm range (Figure 3) compared with the control, probably originating from AgCl_(s) formed in the medium.

In AgNO₃ treatments, the measured concentration of total Ag at the beginning of the exposure was within a 10% deviation (0–8%) from nominal concentrations (Supplemental Data, Table S2 and S3). In NM300K treatments, the measured concentration of total Ag at the beginning of the exposure was within a 5 to 33% deviation from nominal concentrations. In both AgNO₃ and NM300K treatments, there was a reduction in measured total Ag after 96 h at low concentrations (<1 mg Ag L⁻¹). In general, the recovery was decreasing with decreasing concentrations (Supplemental Data, Table S2 and S3).

The size fractionation showed that the initial concentration of dissolved Ag (<3 kDa) varied among treatments. In AgNO₃ treatments, the dissolved Ag content varied between 13 and 90% of the total Ag measured in exposure media without *E. coli* (Supplemental Data, Table S2). In contrast, the <3 kDa Ag fraction was reduced to less than the limit of detection within 2 h after addition of *E. coli* (Supplemental Data, Table S3). In NM300K treatments, the initial <3 kDa Ag fraction was 4–8% of the total Ag concentration in the absence of bacteria in the medium (Supplemental Data, Table S2). These results are consistent with previous investigations of the behavior of NM300K in various test media, showing an initial input of dissolved Ag from NM300K of <8% in all tested media (Köser et al. 2017). In that study, the highest dissolved Ag fraction was found in the medium with the lowest Cl⁻ concentration (Steinberg medium), a medium with a composition and Cl⁻ content similar to the moderately hard reconstituted water used in the present study. Köser et al. (2017) reported that the dissolved Ag was present in the original stock suspension provided by the manufacturer, bound to the dispersant agents, and was therefore not a result of further particle dissolution.

Furthermore, the same authors suggested that after this initial release of dissolved Ag, the dispersants would help to prevent any further release of Ag(I) from the particles by limiting the access of O₂ to the surface of AgNPs. They thus concluded that NM300K toxicity attributed to ionic Ag was related to the ionic fraction found in the dispersion before the start of the toxicity test, and could not be related to the further oxidation of the particles and subsequent ionic releases (Köser et al. 2017). As a consequence of the work of Köser et al. (2017), oxidation and subsequent release of Ag(I) from NM300K would not be expected during short-term exposure periods. In the present study, exposure suspensions were continuously shaken to maintain sufficient O₂ levels, which are necessary for the metabolism of *C. elegans*. The constant oxygenation together with the presence of *E. coli* and *C. elegans* might influence the protective effects of the dispersants, and potentially even enhance the dissolution of AgNPs. However, the initial dissolved Ag fraction (<3 kDa) in the exposure suspensions containing *E. coli* and *C. elegans* was very low (<0.65 µg L⁻¹) in all AgNO₃ and NM300K treatments (Supplemental Data, Table S3), and remained so during the whole duration of the experiment (Supplemental Data, Tables S3 and S4). This finding strongly suggests an interaction between dissolved Ag and *E. coli* present in the exposure suspensions. Silver ions are well known for their antibacterial properties, which are closely connected to their ability to interact with the negatively charged bacterial surface and translocate to the interior cell where they interfere with enzymatic functions and metabolic processes (Yamanaka et al. 2005). Mullen et al. (1989) reported that 89% of a 108 mg L⁻¹ Ag(I) solution was removed from solution by binding to bacteria. This interaction is highly efficient and is a likely explanation for the low dissolved Ag content found in the present study.

Uptake and toxicity

Ag uptake by nematodes. Toxicity of NPs to *C. elegans* is highly dependent on the uptake and residence time, both of which are related to surface chemistry and particle size (Meyer

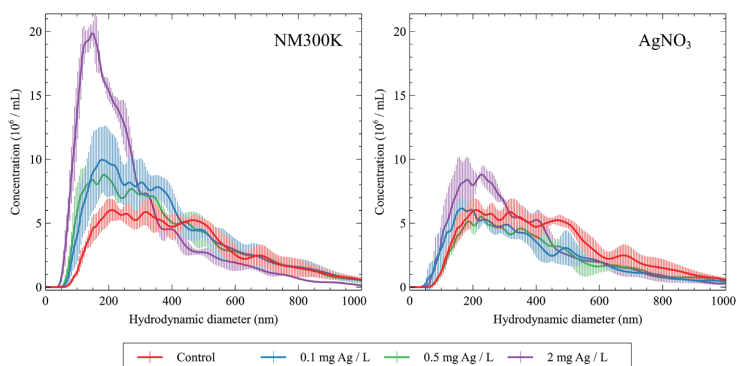


FIGURE 3: Particle size distribution in exposure media in experiment 1 containing 0, 0.1, 0.5, and 2 mg Ag L⁻¹, 96 h after addition of NM300K (left) or AgNO₃ (right). Complete lines correspond to average values of 5 videos of 60 s each. Error bars represent the 95% confidence interval among the videos.

et al. 2010; Ellegaard-Jensen et al. 2012). Image analysis has shown that NP uptake occurs predominantly via ingestion, and that coated monodispersed NPs are taken up by intestinal cells (Meyer et al. 2010). However, there are few or no quantitative data on the uptake of AgNPs. To further characterize the bioaccumulation of Ag, we thus devised an experiment to quantify the uptake of Ag in *C. elegans* from NM300K and AgNO₃ exposures. The uptake was measured after 65 h of exposure to avoid the interference of offspring. The digestion process in *C. elegans* is very rapid, and it has been shown that the residency time for *E. coli* is on average 2 min, with defecation on average every 50 s (Ghafouri and McGhee 2007). Thus, to determine the Ag fraction retained, the exposed nematodes were depurated by feeding on uncontaminated food for 2 h. This would facilitate the removal of any unbound Ag from the intestinal lumen and enable depuration of Ag that might be removed by other defense mechanisms. The remaining Ag should thus be incorporated in the nematode.

In undepurated nematodes, ICP–MS measurements showed that the concentration in nematodes was correlated with the exposure concentrations for both NM300K and AgNO₃ (Table 4). The same dose dependency was observed for the depurated nematodes. However, the overall Ag content (given as ng mg⁻¹ wet wt) in the nematodes after depuration was reduced by >98% in both NM300K and AgNO₃ exposures (Table 4). The remaining 0.6 to 2% fraction was retained in the organisms, indicating a strong binding into tissues and possibly translocation into cells from the intestinal lumen. Yang et al. (2014) exposed *C. elegans* to AgNO₃ at 0.3 mg Ag L⁻¹ and to citrate-stabilized AgNPs at 10 mg Ag L⁻¹ for 24 h in moderately hard reconstituted water and reported internal concentrations 3 to 10 times higher than reported in the present study, but suggested that their ICP–MS measurements were dominated by AgNPs retained in the gut. Through TEM analysis, the authors also identified damage to intestinal epithelial cells and effects to cell organelles like mitochondria and lysosomes. However, translocation of Ag into the cells is not prerequisite for toxicity of nanomaterial. Formation of reactive oxygen species as a consequence of oxidation of the AgNP surface has been associated with oxidative stress in the intestine leading to toxicity (Yang et al. 2012). It should be mentioned that such oxidation of the AgNP surface also involves release of Ag(I), which again could partly be the cause of the toxicity seen in the NM300K exposures. Despite observed uptake of the citrate-stabilized AgNPs into cells, most

of the AgNPs remained in the intestinal lumen in the study by Yang et al. (2014).

Toxicity. The toxicity of NM300K AgNPs and AgNO₃ was assessed by measuring the effects on the standardized endpoints growth, fertility, and reproduction after 96 h of exposure in moderately hard reconstituted water. Dose-dependent effects were observed for all endpoints in both NM300K and AgNO₃ exposures (Figure 4), with reproduction as the most sensitive endpoint. The toxic effects of AgNO₃ were consistent for all the experiments (E1–E3), indicating reproducibility of the experimental setup. The nematode development was assessed in a stereomicroscope after 96 h, and it was evident that exposure to both AgNO₃ and NM300K affected the growth of *C. elegans*. It appeared that development was delayed and that all nematodes reaching the adult stage were fertile and able to reproduce, although the number of offspring per adult was reduced. However, the fertility results were characterized by a very abrupt EC10 to EC50 dose response over a narrow concentration range in both AgNO₃ and NM300K exposures. This finding indicates that fertility might be highly vulnerable to interexperimental variation and not as robust an endpoint as growth or reproduction.

We observed a stimulation of reproduction by NM300K at the lowest exposure concentration, and a stimulation of growth by both AgNO₃ and NM300K lowest exposure concentrations. Such compensatory effects are frequently reported when organisms like *C. elegans* are challenged by low-level environmental stressors, including Ag (Cypser and Johnson 2002). Overall, AgNO₃ induced toxicity at lower exposure concentrations compared with NM300K, with the EC50 for growth, fertility, and reproduction 2 to 9 times lower for AgNO₃ compared with NM300K (Table 5). The wide range is due to the higher variation in NM300K-induced toxic effects among experiments. This is particularly visible on EC10 values for reproduction, where very little variation was seen across the years for AgNO₃ treatments (0.06–0.08 mg Ag L⁻¹) compared with NM300 treatments (0.09–0.52 mg Ag L⁻¹). Previous studies on the toxicity of AgNPs to *C. elegans* in aqueous exposures (K⁺ medium or moderately hard reconstituted water) have reported EC50 values for growth, reproduction, and mobility ranging from 0.09 to 50 mg Ag L⁻¹ (Ellegaard-Jensen et al. 2012; Yang et al. 2012; Starnes et al. 2015). Not surprisingly, the toxicity of AgNPs as well as AgNO₃ was higher in moderately

TABLE 4: Measured Ag concentrations in undepurated and depurated nematodes for NM300K and AgNO₃ exposures during experiment 2^a

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

^aConcentrations in nematodes are given as mean ± standard deviation.

NA = not applicable, no surviving nematodes; LOD = limit of detection = 2.7 ng Ag mg⁻¹ wet weight nematode.

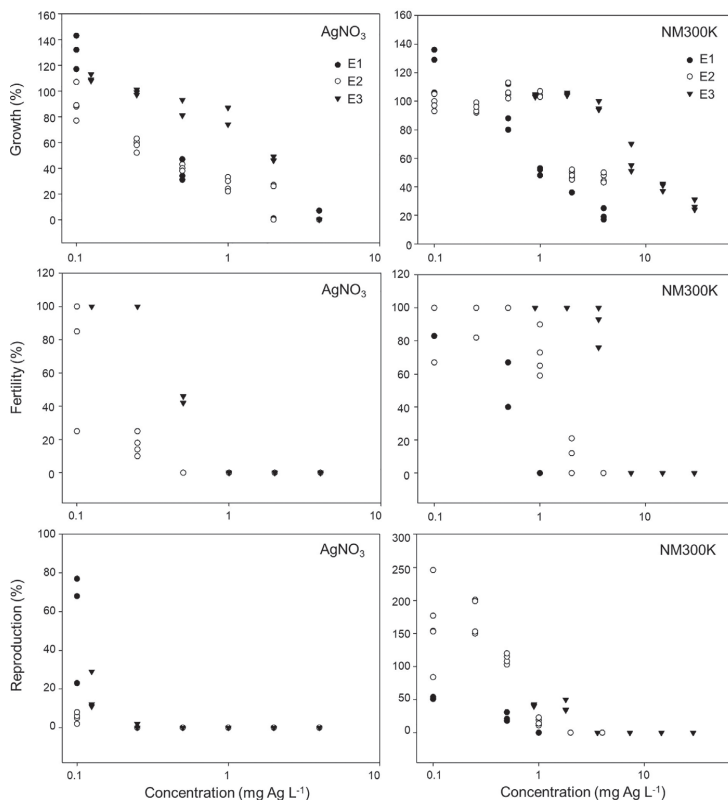


FIGURE 4: Growth, fertility, and reproduction of *Caenorhabditis elegans* after 96 h exposure to AgNO₃ and NM300K, expressed as percentage of controls. E1, E2, E3 = experiments 1, 2, and 3, respectively.

hard reconstituted water, which has a lower chloride content and a lower conductivity than K⁺ medium.

To our knowledge, the present study is the first to report EC values for *C. elegans* exposed to NM300K. Köser et al. (2017) reported EC50 values for NM300K in other organisms: growth inhibition in *Pseudokirchneriella subcapitata* at 0.62 ± 0.37 mg Ag L⁻¹ and in *Lemna minor* at 0.50 mg Ag L⁻¹ (95% CI

0.19–1.11 mg Ag L⁻¹); and immobilization of *Daphnia magna* at 0.04 ± 0.01 mg Ag L⁻¹.

The toxicity induced by NM300K tended to be higher in E1 compared with E2 and E3, particularly when compared with AgNO₃ toxicity (with E1 showing at most a factor of 3 between EC50 values for NM300K and AgNO₃, compared with a factor of 6–9 in E2 and E3; Table 5). It is possible that the observed

TABLE 5: Effect concentration 10 and 50% (EC10 and EC50) on growth, fertility, and reproduction for NM300K and AgNO₃ exposures in experiments 1 to 3 (E1–E3)^a

Endpoint		NM300K		AgNO ₃	
		EC10 (mg Ag L ⁻¹)	EC50 (mg Ag L ⁻¹)	EC10 (mg Ag L ⁻¹)	EC50 (mg Ag L ⁻¹)
Growth	E1	0.37 (0.28–0.73) A	1.45 (1.33–1.87) A	0.36 (0.16–0.41) B	0.47 (0.43–0.59) A
	E2	0.85 (0.51–1.38) A	2.91 (2.37–3.90) B	0.07 (0.05–0.11) A	0.38 (0.32–0.47) A
	E3	2.43 (1.54–3.63) A	10.70 (8.91–12.71) C	0.87 (0.79–1.28) C	1.82 (1.64–1.96) B
Fertility	E1	0.45 (0.38–0.75) A	0.56 (0.48–0.77) A	NA	NA
	E2	0.80 (0.65–0.94) A	1.23 (1.05–1.36) B	0.08 (0.05–0.13) A	0.15 (0.11–0.20) A
	E3	3.64 (3.18–3.79) B	4.29 (3.90–4.39) C	0.38 (0.35–0.41) B	0.49 (0.47–0.49) B
Reproduction	E1	0.09 (0.01–0.35) A	0.26 (0.09–0.44) A	0.07 (0.042–0.094) A	0.11 (0.078–0.121) A,B
	E2	0.52 (0.22–0.82) A	0.74 (0.48–0.92) B	0.06 (0.057–0.066) A	0.08 (0.072–0.080) A
	E3	0.18 (0.15–0.49) A	0.84 (0.69–1.18) B	0.08 (0.074–0.076) A	0.09 (0.086–0.091) B

^aResults are provided with their 95% confidence interval (CI) in parentheses. Parameter estimations were based on nominal concentrations and calculated using the Hill model. For each endpoint, EC values with overlapping CIs are indicated by similar capital letters. NA = not available.

difference could in part be ascribed to the protocol used for the preparation of NM300K stock suspension, which had a lower particle size in E1, but because this size difference did not carry over to the exposure suspensions, it could also reflect differences in nematode batch sensitivity. There was no obvious correlation between toxicity and measured concentrations or size distribution in the exposure solutions.

Linking exposure, uptake, and toxicity

Speciation of molecular form and size fractionation in exposure characterization for organisms like *C. elegans* is of utmost importance because their normal feeding behavior entails ingesting bacteria and particles in the size range 100 nm to 3 μm from the water column (Yang et al. 2012). Although the initial particle size in the present exposure was between 8 and 23 nm as measured by TEM, and thus <100 nm, the presence of larger particle aggregates (~200 nm) was well within the feeding range of *C. elegans*. There were clear differences between the AgNO₃ and NM300K exposures with respect to particle size and the presence of <3 kDa Ag in suspension (in the absence of *E. coli* and *C. elegans*; Figure 1 and Supplemental Data, Table S2). As expected, nanosized particles could be identified in NM300K exposures (Figures 1–3 and Table 3). However, AgNO₃ exposures also contained larger particulate matter (Figures 2 and 3 and Table 3), which indicates formation of Ag complexes.

Prior to the addition of *E. coli* to the exposure suspensions, the <3 kDa Ag fraction was significantly lower in NM300K treatments (4–8% of total Ag) than in AgNO₃ treatments (13–90% of total Ag; Supplemental Data, Table S2). However, at the actual beginning of the toxicity test (i.e., after addition of *E. coli*) no <3 kDa Ag was found, suggesting rapid affiliation of ionic Ag with the bacteria (Supplemental Data, Table S3). The fact that a significant proportion of Ag was associated with *E. coli* indicates that bacteria act as a vehicle to promote the uptake of ions, and possibly also AgNPs, via ingestion. The observed differences in particle size, the presence of aggregates, and the <3 kDa Ag difference between NM300K and AgNO₃ exposures were not reflected in the Ag concentrations accumulated in *C. elegans* (Table 4). Although incorporated Ag (still present in nematodes after depuration) was very similar in AgNO₃ and NM300K treatments, toxicity correlated strictly with exposure concentration. The fact that similar levels of Ag uptake in the nematodes caused significantly different effect levels showed that toxicity was most likely determined by the type of exposing agent (NM300K vs AgNO₃). The toxicity associated with Ag uptake from AgNO₃ exposures (both total and incorporated Ag) was higher than that associated with NM300K exposures (Table 4). This observation is consistent with previous reports indicating that AgNPs act mostly in the intestine (Yang et al. 2014), whereas Ag(I) is probably more effectively taken up into the intestinal cells where it interferes with enzymes and organelle functions.

In line with this model, reproduction, the most sensitive endpoint measured in the present study, was already strongly affected at the lowest AgNO₃ concentration (44–95% reduction at 0.1 mg Ag L⁻¹). In contrast, similar effects on reproduction

were only observed from 0.5 to 1 mg Ag L⁻¹ in NM300K exposures (Figure 4). This could potentially be linked to the initial differences seen in the <3 kDa Ag fraction. The higher concentration of <3 kDa Ag in AgNO₃ exposures presumably led to a higher concentration of Ag associated with the bacteria *E. coli*. As the food source for nematodes, *E. coli* act as vehicles, enhancing the bioavailability of Ag(I) and consequently also the toxicity. Likewise, the toxicity observed in NM300K exposures could potentially also be directly related to the concentration of dissolved Ag, either through dissolution of NM300K (Yang et al. 2012), or release of Ag(I) bound to the surfactants in the original NM300K nanomaterial (Köser et al. 2017). Although NM300K AgNPs have been reported to be relatively stable over short exposure periods, the physicochemical environment and processes in the digestive tract of *C. elegans* (e.g., an acidic environment with pH values of 3.6–6.0; Chauhan et al. 2013) could potentially speed up the release of Ag(I). However, as discussed previously and supported by size fractionation measurements, dissolution of Ag(I) from the nanoparticles is not necessarily or likely the sole explanation for the observed differences in toxicity. A nanospecific toxicity (e.g., oxidative stress generated from the formation of reactive oxygen species on the surface of the nanoparticles) is another mechanism of toxicity (Yang et al. 2012). In the present study we unfortunately cannot offer firm conclusions on the mechanistic pathways leading to toxicity.

CONCLUSIONS

The last decade of nanotoxicity research has generated a large number of published, available toxicity data. However, due to the lack of harmonized testing, the data are often not considered as reliable for risk assessment (Kos et al. 2016). Standardized operating protocols (ISO or OECD) used for traditional soluble chemicals with high reproducibility do not work as well for nanotoxicity studies. These questions have been addressed in several European Union projects (CO-NANOMET, NANOREG, Nanofate, NanoTest), but the lack of harmonized protocols is still a problem. Although the 3 separate experiments (E1–E3) reported in the present study did not follow a fully harmonized testing protocol, they were conducted in the same way with respect to key points such as experimental media, temperature, type of Ag nanoparticles, experimental setup, strain of *C. elegans*, and so on. Despite some variation in EC values, the present study showed a relatively good agreement among the different experiments, with EC values in the same order of magnitude. Especially in terms of the most sensitive endpoint, reproduction, the EC values were similar over a period of 2 or 3 yr for both AgNO₃ and NM300K exposures. Characterization of the exposure solutions for both AgNPs and AgNO₃ treatments suggested that, in this case, variations in toxicity did not appear to be correlated with differences in particle size, aggregation, or dissolution among the 3 experiments. In addition, differences in AgNP and AgNO₃ exposure could not be explained solely by differences in Ag speciation.

Although 3 studies performed by the same laboratory provides far from enough data to form conclusions about

reproducibility, it is clear that the differences in reproducibility of tests between laboratories, both in the same and across different species, cannot be analyzed without information on the behavior and characterization of the exposure media. Likewise, understanding differences between NP and ion mechanisms requires that characterization also be carried out on ion exposure solutions, which is often overlooked in nanotoxicity tests.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4144.

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Data availability—Research data pertaining to this article are accessible by request to the authors (merethe.kleiven@nmbu.no or deborah.oughton@nmbu.no).

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1 Supporting information

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

4

5 Merethe Kleiven^{a*}, Lisa M. Rossbach^a, Julian A. Gallego-Urrea^{ab}, Dag Anders Brede^a, Deborah H.
6 Oughton^a and Claire Coutris^c

7 ^a *Norwegian University of Life Sciences, Faculty of Environmental Sciences and Natural Resource*
8 *Management, Center for Environmental Radioactivity (CERAD CoE), P.O. Box 5003 NMBU, 1432 Ås,*
9 *Norway*

10 ^b *Department of Marine Sciences, University of Gothenburg, Kristineberg 566, SE-45178 Fiskebäckskil*
11 *Sweden*

12 ^c *Division of Environment and Natural Resources, Norwegian Institute of Bioeconomy Research,*
13 *Høgskoleveien 7, 1431 Ås, Norway*

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

15

16 Contains 2 tables and 3 figures

17 **TABLES**

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

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

20 **Table S2.** Silver concentrations (total and <3 kDa) in AgNO₃ and NM300K treatments, in E1, at the start
 21 (without bacteria), and end of the experiment. ND: not determined.
 22

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

23

24 **Table S3.** Silver concentrations (total and <3 kDa) in AgNO₃ and NM300K treatments, in E2, at the start
 25 (with bacteria), after 20 h (E2) and end of the experiment. ND: not determined.
 26

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

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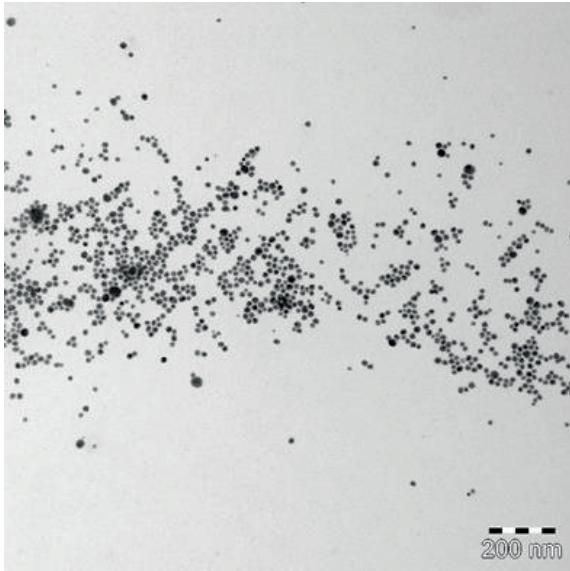
29 **Table S4.** Silver concentrations (total and <3 kDa) in AgNO₃ and NM300K treatments at end of the
 30 experiments (E1-3). ND: not determined.
 31

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

32 *2000xg, 15 min

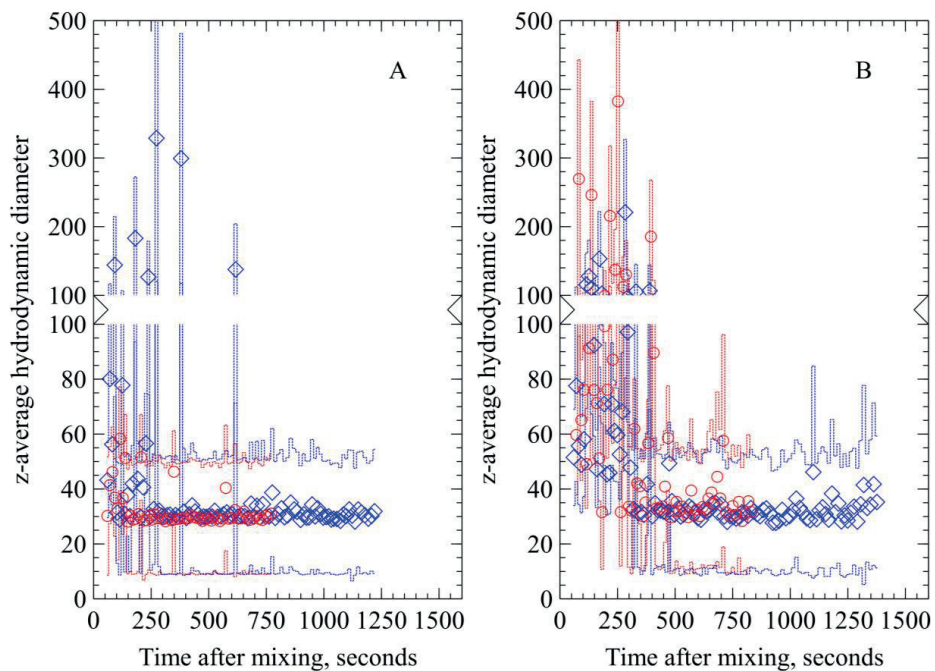
33

34 **FIGURES**

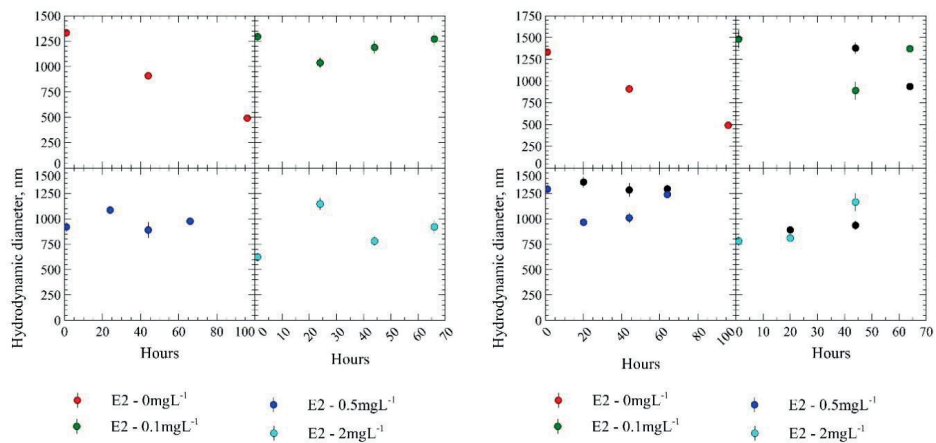


35

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



37
 38 **Figure S2.** Time resolved DLS measurements performed on NM300K suspensions in MilliQ water. A
 39 corresponds to measurements done at 10 mg Ag L⁻¹, and B at 1 mg Ag L⁻¹. Zeta-average hydrodynamic
 40 diameters, d_H , were obtained as explained in the text and duplicate values are presented with the
 41 markers. The dotted lines correspond to the corresponding color-matched standard deviation obtained
 42 from polydispersity-index, PDI, values assuming a Gaussian profile distribution ($SD = (d_H^2 \cdot PDI)^{0.5}$).



43

44 **Figure S3.** Zeta-average diameters obtained from DLS measurements (in E2) of NM300K AgNPs (left) and
 45 AgNO₃ (right) exposure suspensions, in presence of bacteria *E. coli*. Averages of three replicated
 46 measurements are presented and error bars represent one standard deviation. Black symbols
 47 correspond to the same sample measured after resuspension.

Paper II

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

4

5 Lisa M. Rossbach^{1,*}, Deborah H. Oughton¹, Claire Coutris² and Dag A. Brede¹

6 Norwegian University of Life Sciences, Faculty of Environmental Sciences and Natural
7 Resource Management, P.O. BOX 5003 NMBU, No-1432 Ås, Norway

8 ²Norwegian Institute of Bioeconomy Research, Division of Environment and Natural
9 Resources, Høgskoleveien 7, Ås, Norway

10 *Corresponding author: Lisa.rossbach@nmbu.no

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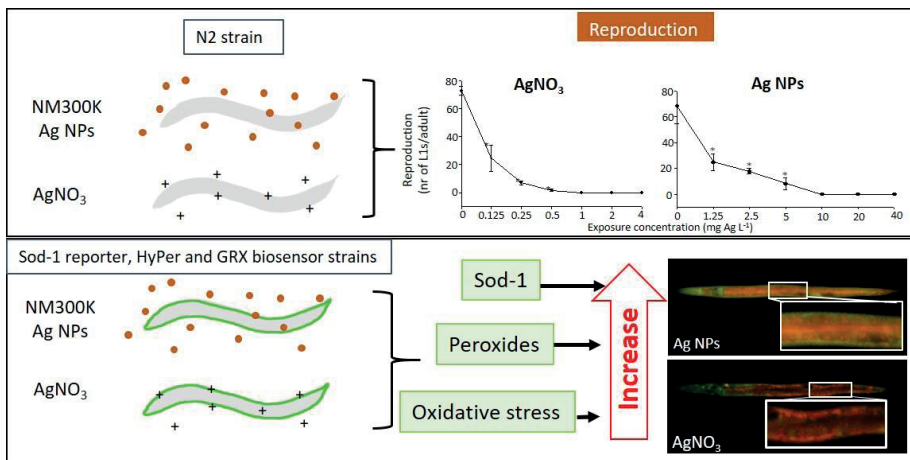
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23

24 **Abstract**

25 The current study provides an *in vivo* analysis of the production of reactive oxygen
26 species (ROS) and oxidative stress in the nematode *Caenorhabditis elegans* following
27 exposure to EU reference silver nanoparticles NM300K and AgNO₃. Induction of
28 antioxidant defenses was measured through the application of a SOD-1 reporter, and the
29 HyPer and GRX biosensor strains to monitor changes in the cellular redox state. Both
30 forms of Ag resulted in an increase in *sod-1* expression, elevated H₂O₂ levels and an
31 imbalance in the cellular GSSG/GSH redox status. Microscopy analysis of the strains
32 revealed that AgNO₃ induced ROS related effects in multiple tissues, including the
33 pharynx, intestinal cells and muscle tissues. In contrast, NM300K Ag NPs resulted in
34 localized ROS production and oxidative stress, specifically in tissues surrounding the
35 intestinal lumen. This indicates that Ag from AgNO₃ exposure was readily transported
36 across the whole body, while Ag or ROS from NM300K exposure was predominantly
37 confined within the luminal tissues. The observed increase in ROS production and the
38 changes in GSSG/GSH ratio coincided with increased physiological toxic effects. However,
39 *sod-1* was not induced at the lowest Ag concentrations, although reprotoxicity was seen
40 at these levels. While both forms of Ag caused oxidative stress, impaired development,
41 and reprotoxicity, the results indicate different roles of ROS production in the toxic modes
42 of action of AgNO₃ versus NM300K.



43

44 **Keywords:** reporter strains, biosensor strain, oxidative stress, NM300K

45 **1. Introduction**

46 Due to their wide applications, silver nanoparticles (Ag NPs) are one of the most
47 intensively studied nanomaterials available (Syafiuddin et al., 2017). Characteristics,
48 such as size, surface charge, chemistry, and dissolution state of the NPs can influence
49 bioavailability, reactivity and toxicity of the material (Foldbjerg et al., 2009, Völker et al.,
50 2015, Zhao and Wang, 2011, Ivask et al., 2014, Batista et al., 2017). The EU reference
51 material NM300K has been provided by the European Commission Joint Research Centre,
52 and is one of the best characterized Ag NPs available (Köser et al., 2017, Wasmuth et al.,
53 2016). Furthermore, the toxicity of NM300K has been assessed in a wide range of species,
54 such as bacteria, daphnia, annelids (Poynton et al., 2012, van der Ploeg et al., 2014, Gomes
55 et al., 2017, Ribeiro et al., 2015), and the nematode *Caenorhabditis elegans* (Kleiven et al.,
56 2018). Results from Kleiven et al. (2018) show distinct differences in toxicity between
57 AgNO₃ and the Ag NP NM300K, with higher retention of Ag from AgNO₃ following the
58 depuration of the nematodes. These differences have been supported by a follow-up
59 study on multigenerational effects Ag NPs versus AgNO₃, which also suggests a clear
60 difference in the toxic mode of action between the two forms of Ag (Rossbach et al., 2019).

61 Silver nanoparticles have been shown to induce physical damages to the cuticle of *C.*
62 *elegans* (Kim et al., 2012), and negatively affect physiological processes, including
63 reproduction and development. At the molecular level, Ag NPs interfere with metabolism
64 by inhibition of enzyme activities, which may damage cellular constituents including
65 mitochondria and DNA (Kleiven et al., 2018, Nel et al., 2006, Kim et al., 2009, Piao et al.,
66 2011, Roh et al., 2009). Moreover, one of the most important toxic mechanisms of Ag NPs
67 is the production of free radicals on the surface of the particle, which may impact
68 biological molecules and cellular structures, potentially leading to oxidative stress (Kim
69 et al., 2009, Roh et al., 2009, Ribeiro et al., 2015, Jiang et al., 2009, Lüersen et al., 2013).

70 A range of studies highlight differences in toxic mechanism between ionic Ag and Ag NPs
71 (Choi et al., 2008, Choi et al., 2018, Hunt et al., 2013), possibly explained by differences in
72 reactive oxygen species (ROS) formation and oxidative stress development between the
73 two forms of Ag (McShan et al., 2014). Negative impacts on chloroplast structure and
74 function of *Spirodela polyrhiza* have been related to oxidative stress caused by Ag NP
75 exposure (Jiang et al., 2009). Lim et al. (2012) highlight the importance of ROS formation
76 by Ag NPs in *C. elegans*, and suggest that oxidative stress plays an important role in Ag NP

77 reproductive toxicity, whereas AgNO₃ exposure did not result in a significant increase in
78 ROS. A study in the soil organism *Enchytraeus crypticus* showed distinct differences in
79 the oxidative stress defense mechanisms and response patterns between the Ag NPs
80 NM300K and AgNO₃ (Ribeiro et al., 2015). Comparatively, it has been shown that any
81 oxidative stress measured following the exposure of *Daphnia magna* to either Ag NPs or
82 AgNO₃, could almost entirely be attributed to ionic releases from the NPs, due to similar
83 response patterns from the two forms of Ag (Li et al., 2015).

84 Due to extensive knowledge about its biology and physiology, the nematode *C. elegans* is
85 an excellent model organism for studying ROS production, and oxidative stress
86 mechanisms. Studies suggest that *C. elegans* possesses a highly complex, but nevertheless
87 specialized, ROS and redox control system (Braeckman et al., 2016). For instance, their
88 genomes encode for five superoxide dismutase (SOD) forms, used for the detoxification
89 of superoxides, with SOD-1, SOD-4 and SOD-5 being Cu/ZnSOD isoforms, while SOD-2 and
90 SOD-3 are mitochondrial MnSOD isoforms (Braeckman et al., 2016, Hoogewijs et al., 2008,
91 McCord and Fridovich, 1969). SOD-1, found in the cytosol and mitochondrial
92 intermembrane space, is evenly expressed and is responsible for ~75% of total SOD
93 activity in *C. elegans* (Doonan et al., 2008). Also involved in *C. elegans* oxidative stress
94 defense are catalase (CTL) and glutathione peroxidase (GPX) enzymes. While the well
95 studied isoforms CTL-1 and CTL-2 are responsible for 80% of the cellular CTL activity
96 (Petriv and Rachubinski, 2004, Togo et al., 2000), information on the cytosolic CTL-3 is
97 still lacking (Braeckman et al., 2017). Similarly, although several glutathione S-
98 transferases (GSTs) seem to be involved in oxidative stress defenses as ROS scavengers,
99 in most cases, less is known about specific functions, substrate and localization
100 (Braeckman et al., 2016, Dröge, 2003). Under normal conditions, glutathione (GSH)
101 molecules are mostly reduced, with only a low proportion of the oxidized glutathione
102 disulphide (GSSG) present (Lüersen et al., 2013). The NADPH-dependent GSSG reductase
103 (GSR) contributes to recycling of excess GSSG (Lüersen et al., 2013). Furthermore, γ -
104 glutamylcysteine synthetase (GCS) as well as GSH synthetase (GSS) act as catalysts to
105 maintain intracellular GSH homeostasis (Lüersen et al., 2013, Townsend et al., 2003).
106 Hence, due to the high intracellular abundance of this redox couple, the quantification of
107 changes in the GSSG/GSH ratios allows for a reliable indication of the total redox state of
108 the cell (Schafer and Buettner, 2001, Braeckman et al., 2017, Braeckman et al., 2016).

109 Through the use of a reporter strain SOD-1 (GA508 wuls54[pPD95.77 sod-1::GFP, rol-
110 6(su1006)]) and two biosensors HyPer and Grx1-roGFP2 (GRX), the current study aimed
111 to elucidate changes in the intracellular redox state in response to either AgNO₃ or
112 NM300K Ag NP exposure, by the nematode *C. elegans*, and relate these findings to
113 standard toxicity test endpoints. It was hypothesized that differences in the localization
114 of oxidation signals by the strains would be governed by differences in retention and
115 distribution of the Ag from the AgNO₃ and Ag NPs by *C. elegans*. Furthermore, building on
116 the results of previous studies with *C. elegans* (Lim et al., 2012), it was hypothesized that
117 antioxidant defenses would come at the expense of other biological processes such as
118 growth, fertility and/or reproduction.

119 **2. Methods**

120 2.1. Nanoparticle stock preparation and characterization

121 The OECD representative Ag nanomaterial NM300K (Fraunhofer IME, Munich, Germany)
122 stocks were prepared according to a Standard Operating Procedure developed by EU
123 NanoReg (Jensen, 2016). Briefly, a 2.56 g Ag L⁻¹ stock was prepared in ddH₂O (15 MΩ·cm)
124 using a probe sonicator (Branson S-450 D sonicator, disruptor horn 13 mm) for 13
125 minutes at 15 % amplitude. Fresh stocks were prepared for each exposure.

126 For the characterization of the nanoparticles, a range of tools was employed. Dynamic
127 light scattering (DLS, Malvern PN3702 Zetasizer Nanoseries) for hydrodynamic diameter
128 and zeta potential, and transmission electron microscopy (TEM, Morgagni 268, FEI) for
129 core diameter were carried out on stock solutions. For the TEM analysis, a 250 mg Ag L⁻¹
130 stock was prepared in ddH₂O (15 MΩ·cm), before addition to the grid and subsequent
131 TEM imaging.

132 Assessment of the size distribution and particle dissolution at T-0 and 72 h of exposure
133 was carried out by ultrafiltration of the exposure media using 3 kDa Millipore Centrifugal
134 filters (Amicon, Millipore). Triplicate samples of the exposure media were first
135 centrifuged (2000 g for 5 min) to remove *E. coli* and larger particle aggregates, with
136 subsequent ultrafiltration of the supernatants following pre-conditioning of the filters.
137 For the dissolved (LMM) Ag fraction, 400 µl of sample was added, and centrifuged at
138 14 000 g for 30 min, with a subsequent collection of 200 µl of the filtrate for analysis.

139 All samples were analyzed by Inductively Coupled Plasma Optical Emission Spectrometry
140 (Agilent Technologies 5110 ICP-OES) or Mass Spectrometry (ICP MS Agilent 8800,
141 Mississauga, ON, Canada), using oxygen as a collision gas, and tuned using the
142 manufacturer's tuning solution (#5188-6564, Agilent Technologies, Mississauga, ON,
143 Canada). Measurements were carried out on two Ag isotopes (107 and 109), with
144 detection limits at 0.007 and 0.005 $\mu\text{g L}^{-1}$, respectively, and limits of quantification at 0.02
145 $\mu\text{g L}^{-1}$.

146 2.2. Nematode culture and exposure

147 All nematodes were exposed to either AgNO_3 or the silver nanoparticle NM300K in either
148 96 h (wild type N2), or 72 h (SOD-1, HyPer and GRX) standard toxicity tests, according to
149 the International Organization for Standardization guideline 10872 (ISO, 2010), with
150 some modification. Prior to the start of the exposure, nematodes, previously kept in liquid
151 cultures, were treated with a hypochlorite solution, for egg extraction and
152 synchronization of the culture. Eggs were allowed to hatch on agar plates overnight to
153 obtain synchronous L1-stage larvae.

154 2.3. Toxicity test exposure and sampling

155 All nematodes were exposed in 24-well culture plates, from L1 stage, at 20 °C in the dark,
156 with continuous gentle shaking for sufficient oxygenation. Each well contained 1 mL of
157 the bacteria *Escherichia coli* OP50 re-suspended in moderately hard reconstituted water
158 (MHRW) (United States Environmental Protection Agency, 2002), and 11 ± 6 L1 stage
159 nematodes. Toxicity tests were carried out using the N2 Bristol strain, while ROS
160 production and oxidative stress effects were assessed using one reporter and two
161 biosensor strains.

162 The N2 Bristol strain *C. elegans* (*Caenorhabditis Genetic Centre*, Minneapolis, USA) was
163 exposed in triplicate to nominal concentrations of 0.125, 0.25, 0.5, 1, 2 and 4 mg Ag L^{-1} for
164 AgNO_3 , or 1.25, 2.5, 5, 10, 20 and 40 mg Ag L^{-1} for NM300K Ag NPs. To terminate toxicity
165 tests, 0.5 mL of Rose Bengal was added and toxicity test plates were kept for 10 minutes
166 at 80 °C. A stereo microscope (Leica M205C) was used for subsequent assessment of
167 growth, fertility and reproduction, using a hand held tally counter. The raw data obtained

168 from this toxicity test have also been used as part of a multigenerational study studying
169 the adaptive tolerance of *C. elegans* to NM300K Ag NPs (Rossbach et al., 2019).

170 Through the measurement of the emitted green fluorescent protein (GFP) signal, the
171 SOD-1 strain (GA508 wuls54[pPD95.77 sod-1::GFP, rol-6(su1006)]) (Institute of Healthy
172 Ageing Genetics, University College London) allows for a direct *in vivo* measurement of
173 the induction of *sod-1* gene expression. The HyPer strain expresses a peroxide-specific
174 sensor protein, consisting of a redox sensitive yellow fluorescent protein fused to the
175 H₂O₂ sensing domain of *E. coli* OxyR (Back et al., 2012). The HyPer biosensor strain thus
176 facilitates the *in vivo* quantitative assessment of H₂O₂ levels with cell specific resolution.
177 When exposed to peroxide, an intramolecular disulfide bridge is formed which leads to a
178 conformational change of the protein that results in the shift of the 420/500 nm
179 excitation to 516 nm emission ratio. Similarly, the Grx1-roGFP2 (GRX) transgenic strain
180 enables assessment of the ratio between the oxidized (GSSG) and reduced (GSH) forms of
181 glutathione (Back et al., 2012). In this manner, changes in the GRX emission ratio are
182 directly determined by the GSSG/GSH ratio, and thus reflect the intracellular redox
183 potential in the nematode. Quantification of the changes in GRX emission ratio thus
184 provides information about the manifestation of oxidative stress (Back et al., 2012).

185 Due to slight differences in sensitivity of the reporter and biosensor strains to Ag
186 exposure, lower concentration ranges were chosen of 0.1, 0.5 and 1 mg Ag L⁻¹ for AgNO₃
187 or 1, 5 and 10 mg Ag L⁻¹ for Ag NPs, in triplicate. Immobilized nematodes were imaged
188 using a fluorescent light microscope (LEICA DM6 B), equipped with a 405 nm excitation
189 and 535 nm emission filter. For oxidized to reduced ratios of the biosensor strains, a
190 second image (Ex 490 nm and Em 535 nm) was taken. Ratios were calculated as described
191 in Back et al (2012). The SOD-1 average intensity was normalized to nematode size, to
192 account for possible variation in signal strength due to developmental stages. All images
193 were quantified using the LAS X Leica application suit X imaging software for pixel based
194 average intensity measurements.

195 2.4. Statistical analysis

196 For the statistical analysis of all the data, Minitab® 18 software was used. A significance
197 level of 0.05 was used in all tests. Differences between treatments were analyzed using
198 ANOVA, followed by a Tukey's range test. Alternatively, a Kruskal-Wallis test was done

199 when the residuals of the ANOVA were not normally distributed. For the comparison of
200 the various nematode strains, a Spearman rank or Pearson product moment correlation
201 test was used.

202 Effect concentrations (EC10 and EC50) were calculated with the open source software
203 RegTox, using the Hill model (Vindimian, 2016) and are reported as the optimal value for
204 EC10 and EC50 with corresponding 95 % confidence intervals.

205 **3. Results**

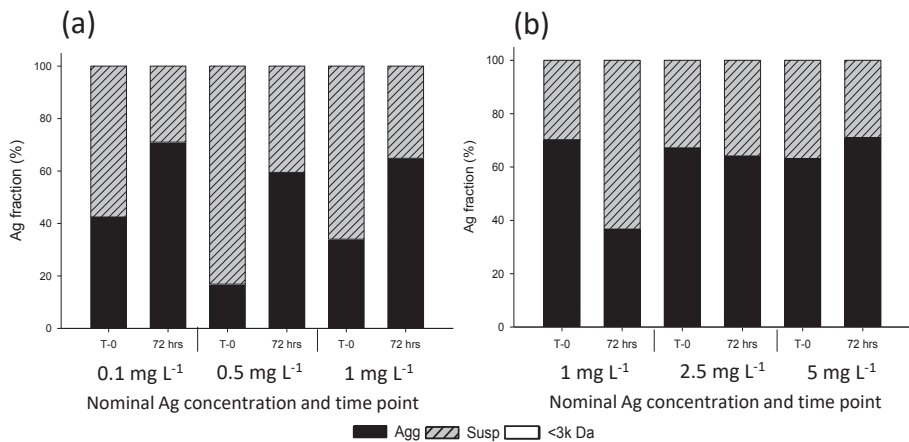
206 3.1. Nanoparticle characterization

207 Since the importance of physicochemical characteristics of Ag NPs in explaining toxic
208 responses has been shown in previous studies (Kleiven et al., 2018), we conducted
209 detailed characterization of the NM300K Ag NPs, prior and post application to the
210 exposure media, as well as monitored changes over time. TEM analysis of stock
211 suspensions showed a median size of 23.2 ± 17.2 nm (mean \pm SD, throughout the text)
212 (Figure S1), which is in good agreement with supplier characteristics. DLS analysis
213 showed an average diameter of 93.3 ± 1.3 nm and 87.6 ± 1 nm and a PDI of 0.3 and 0.25,
214 for the initial 256 mg Ag L^{-1} (Figure S2) and the diluted 100 mg Ag L^{-1} stocks in ddH₂O
215 ($15 \text{ M}\Omega\cdot\text{cm}$), respectively. The measurement of the electrophoretic mobility of the Ag NPs
216 in the stocks showed a zeta potential of -8.77 and -14.3 mV for the 256 mg Ag L^{-1}
217 stocks, respectively.

218 ICP-OES or ICP-MS analysis of stocks and exposure media showed reasonable agreement
219 with nominal concentrations, with some differences between AgNO₃ and Ag NPs. While
220 AgNO₃ exposure stocks showed recoveries of 100 ± 0.0 % and 107 ± 0.9 %, for the 80 mg
221 Ag L^{-1} and 10 mg Ag L^{-1} , respectively, Ag NP stocks were comparatively lower, with
222 recoveries of 81.2 ± 0.0 % and 78.7 ± 22.2 %, for the 800 mg Ag L^{-1} and 100 mg Ag L^{-1} ,
223 respectively. Exposure media showed recoveries of 84.9 ± 29.8 % and 64.4 ± 22.6 % for
224 AgNO₃, at T-0 and after 72 h. The variation in the measurements stems from lower
225 recoveries at higher exposure concentrations (46 – 95 % of nominal concentrations),
226 while lower exposure concentrations were much more consistent with nominal
227 concentrations (98 – 136 %). Similarly, a 10 % time dependent decrease in Ag
228 concentration was seen in the Ag NP exposure, with a reduction from 84 ± 21 % at T-0 to
229 74 ± 23 % after 72 h, in recovery of nominal Ag concentrations. The observed decreases

230 in Ag concentration over the duration of the exposure are probably due to aggregation,
231 precipitation and/or sorption to the well surfaces.

232 To monitor changes in the behavior of silver in the exposure, a fractionation experiment
233 was conducted at the beginning (T-0) and end (72 h) of the exposure (Figure 1). This was
234 conducted on both the N2 and biosensor strains exposures. Findings from the N2
235 exposure are presented in Rossbach et al. (2019), and lay in accordance with results
236 presented in the current article (Figure 1). No dissolved (<3 kDa) Ag fraction was found
237 at either time point. It is highly probable that the positively charged Ag ions are associated
238 with the negatively charged surface of the *E. coli*, and hence removed in the first
239 centrifugation step, removing the aggregated fraction, before ultrafiltration. Overall, both
240 forms of Ag show a propensity for aggregation and/or association with *E. coli* over the
241 course of the exposure, as seen in the aggregated fraction (Figure 1). While larger,
242 aggregated Ag particles might reduce the direct exposure of nematodes, Ag associated
243 with *E. coli* might facilitate an increased dietary exposure. This coincides with the
244 conclusions from Kleiven et al. (2018). Assuming that Ag associated with *E. coli* is still
245 capable of exerting a toxicological response, all toxicity results in the following section
246 are based on nominal concentrations in exposure suspensions.



247

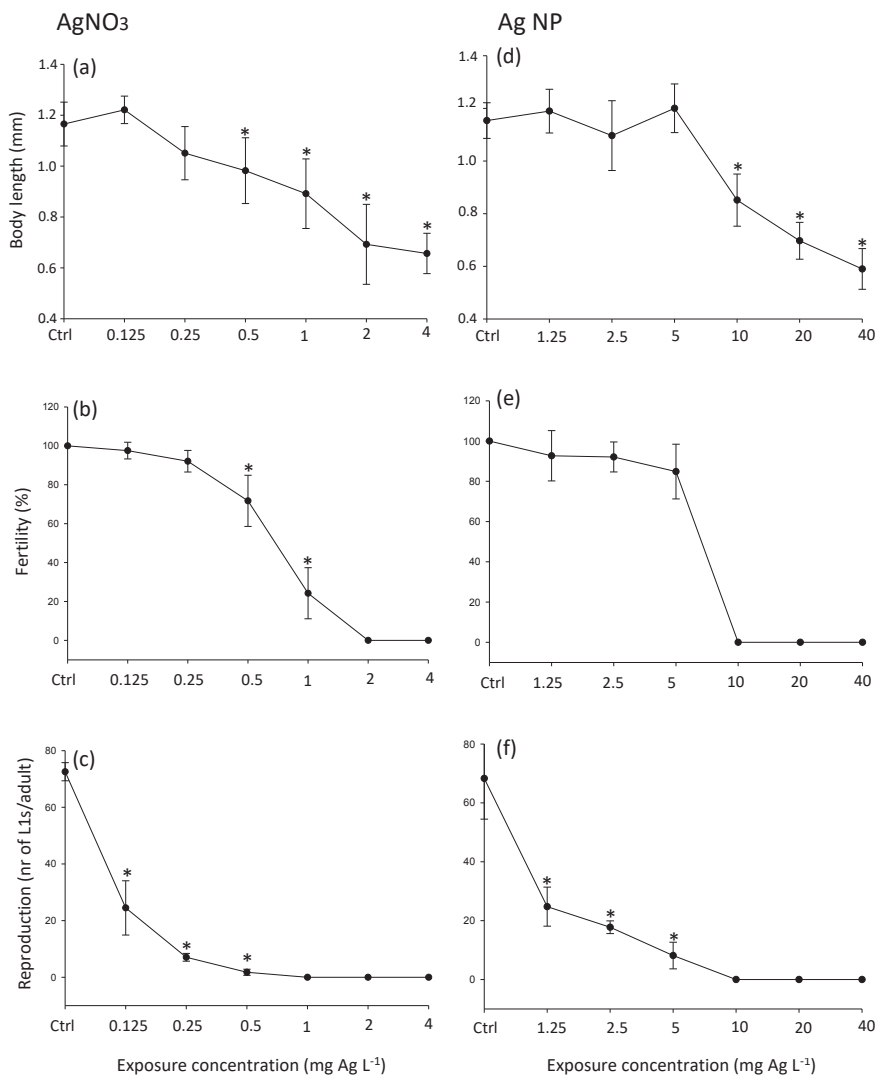
248 **Figure 1.** Characterization of the exposure media from the biosensor strain exposure, at
249 T-0 and 72 h, showing the aggregated (Agg), suspended (Susp) and dissolved (< 3 kDa)
250 Ag fraction of either AgNO₃ (a) or Ag NPs (b), in moderately hard reconstituted water
251 containing *E. coli* and nematodes.

252 3.2. Dose-response toxicity test

253 The toxicity of AgNO₃ or Ag NPs was assessed in a standard 96 h toxicity test, in MHRW,
254 by measuring growth, fertility and reproduction. AgNO₃ exposed nematodes displayed a
255 dose dependent decrease in growth with increasing concentrations (Figure 2a). All
256 AgNO₃ concentrations up to 4 mg Ag L⁻¹ showed statistically significant decrease in
257 growth compared to controls, except for the lowest two concentrations. At 4 mg Ag L⁻¹ a
258 56 % reduction in growth was found. These findings correspond to growth EC10 and
259 EC50 estimations for AgNO₃ exposed nematodes of 0.34 mg Ag L⁻¹ (0.10 – 0.89) and 2.58
260 mg Ag L⁻¹ (1.82 – 3.77), respectively (Table 1). The Ag NP exposure on the other hand,
261 resulted in no decrease in growth up to 5 mg Ag L⁻¹, but rather a fluctuation at lower
262 concentrations. This was followed by a subsequent dose dependent reduction in
263 development up to 40 mg Ag L⁻¹ (ANOVA, $p < 0.05$), with a 51.2 % reduction in total body
264 length compared to controls ($n = 10$). The EC10 and EC50 estimations are given in Table
265 1.

266 Similar to growth, fertility of nematodes exposed to AgNO₃ remained unaffected at 0.125
267 and 0.25 mg Ag L⁻¹ (Figure 2b). A statistically significant reduction in fertility to 73 % of
268 the control was observed at 0.5 mg Ag L⁻¹ (ANOVA, $p = 0.008$), followed by a reduction to
269 28.1 % in nematodes exposed to concentrations of 1 mg Ag L⁻¹ and 0 % fertility at 2 and
270 4 mg Ag L⁻¹. The impacts of Ag NP on fertility showed a reduction down to 83 % fertility
271 at 5 mg Ag L⁻¹ (Figure 2e), although this was not statistically significant from controls.
272 Fertility was 0 % at concentrations ≥ 10 mg Ag L⁻¹.

273 Reproduction data for both AgNO₃ and Ag NP represents the most sensitive endpoint
274 measured in the toxicity test. This was further confirmed by the EC10 and EC50 estimates
275 (Table 1). At the lowest (0.125 mg Ag L⁻¹) AgNO₃ concentration a statistically significant
276 (ANOVA, $p = 0.005$) decrease in reproduction, to below 35 % of that in controls, was
277 measured (Figure 2c). At 0.5 mg Ag L⁻¹ reproduction was less than 4% of that in controls
278 (ANOVA, $p = 0.001$), followed by no reproduction at 1 mg Ag L⁻¹ or higher. An impairment
279 in reproduction was observed in the Ag NP exposure, with a dose dependent reduction
280 down to 12.6 % compared to controls at 5 mg Ag L⁻¹ and a total absence of reproduction
281 at concentrations ≥ 10 mg Ag L⁻¹ (Figure 2f).



282

283

284 **Figure 2.** Growth (a and d), fertility (b and e) and reproduction (c and f) of *C. elegans*,
 285 exposed to either AgNO₃ or the Ag NPs NM300K, in MHRW containing *E. coli* in a standard
 286 96 h toxicity test. Data is presented as mean ± SD (n = 10). Asterisks indicate significant
 287 differences (Tukey's range test) between exposed nematodes and controls.
 288 Concentrations are given as nominal values.

289 **Table 1.** Reproduction, fertility and growth EC10 and EC50 value for *C. elegans* exposed
 290 to either AgNO₃ or Ag NPs in a standard 96 h toxicity test, in MHRW. 95 % confidence
 291 intervals are provided in parentheses. All values were calculated using the Hill model.

		AgNO ₃ (mg Ag L ⁻¹)	Ag NP (mg Ag L ⁻¹)
Reproduction	EC10	0.04 (0.02 - 0.06)	0.11 (0.04 - 0.44)
	EC50	0.10 (0.07 - 0.12)	0.71 (0.41 - 1.21)
Fertility	EC10	0.33 (0.25 - 0.42)	3.50 (2.78 – 3.67)
	EC50	0.70 (0.59 - 0.79)	4.30 (3.90 – 4.45)
Growth	EC10	0.34 (0.10 – 0.89)	4.09 (1.51 – 8.30)
	EC50	2.58 (1.81 – 3.77)	18.6 (13.9 – 24.6)

292

293 3.3. ROS production and oxidative stress effects by AgNO₃ or Ag NPs

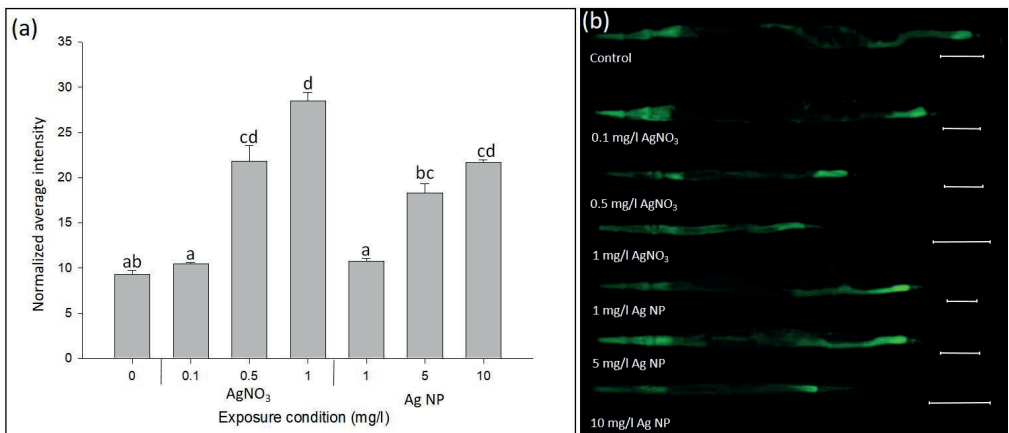
294 To investigate the potential role of free radicals in the toxic response observed in the N2
 295 Bristol strain, three parallel tests were set up to obtain *in vivo* measurements of AgNO₃
 296 and Ag NPs exposure on *sod-1* gene expression and redox balance within the nematode,
 297 using the SOD-1 reporter strain, and the two biosensors HyPer and GRX. While all three
 298 strains showed a dose response from both the AgNO₃ and Ag NP exposure, no correlation
 299 between different strains was found, neither for AgNO₃ (p = 0.03) nor Ag NPs (p = 0.046)
 300 (Pearson's correlation coefficient).

301 3.3.1. Concentration dependent increase in *sod-1* expression

302 For the investigation of *sod-1* gene expression in *C. elegans* in response to AgNO₃ or Ag
 303 NP exposure, the GFP labelled reporter strain GA508 provides an accurate tool for the *in*
 304 *vivo* measurement of *sod-1* gene expression (Doonan et al., 2008). Both AgNO₃ and Ag NP
 305 exposure resulted in development delays of the SOD-1 strain (data not shown).
 306 Therefore, average intensity was normalized to the size of the nematodes in each
 307 exposure condition, to account for the variation in signal strength related to differences
 308 in developmental stages.

309 The AgNO₃ exposed nematodes showed no statistically significant differences from
 310 controls at the lowest (0.1 mg Ag L⁻¹) exposure concentrations (ANOVA, p = 0.7),
 311 indicating no increase in Ag induced *sod-1* expression (Figure 3). However, a significant

312 increase in *sod-1* expression for 0.5 and 1 mg Ag L⁻¹ (ANOVA, p = 0.01 and p < 0.001)
 313 exposed nematodes, compared to controls was found. Similarly for the Ag NP exposed
 314 nematodes, no differences were found between controls and 1 mg Ag L⁻¹ exposure
 315 (ANOVA, p = 0.4), however a statistically significant increase was seen for the 5 and 10
 316 mg Ag L⁻¹ Ag NP exposure (ANOVA, p = 0.003 and p < 0.001) (Figure 3a). Visual image
 317 analysis showed minor differences in the expression pattern of the intestine, pharynx and
 318 muscle tissue, between nematodes exposed to AgNO₃ or Ag NPs.



319

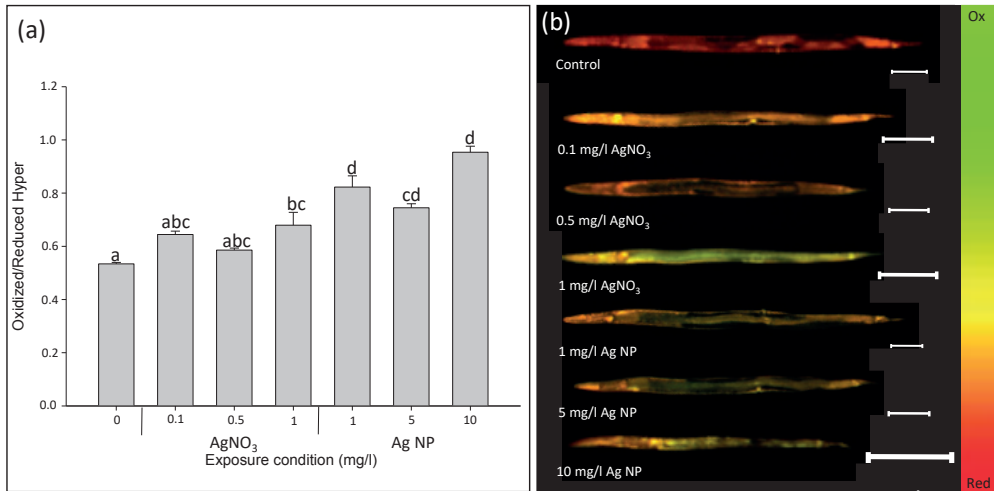
320 **Figure 3.** *In vivo* measurement of *sod-1* expression pattern in the SOD-1 reporter strain
 321 GA508 (a) in response to either AgNO₃ or Ag NP exposure with corresponding images (b)
 322 (n = 10/concentration). Values are normalized to controls for direct comparison. Letters
 323 indicate statistically significant differences among exposure groups. Bars represent 100
 324 μm.

325

326 3.3.2. Exposure to Ag results in tissue specific cellular H₂O₂ production

327 The dismutation of O₂⁻ and 2H⁺ by superoxide dismutase antioxidant enzymes is the main
 328 source of H₂O₂ in the nematode (Back et al., 2012) Therefore, to quantify potential effects
 329 of this increase, the biosensor HyPer was employed in the current experiment. The dose
 330 response of HyPer was non-monotonous, unlike that observed from SOD-1. However, an
 331 overall significant increase compared to controls was seen from all Ag NP exposure
 332 concentrations (p < 0.001), while only the 1 mg Ag L⁻¹ AgNO₃ exposure resulted in a
 333 significant increase (p < 0.001) (Figure 4a).

334 Controls expressed evenly low levels of H₂O₂ across the whole body, including the
 335 pharynx, intestine and tail, but no signal was seen in the gonads (Figure 4b). Notably, all
 336 Ag NP exposures appeared to cause pronounced increased H₂O₂ levels in the intestine,
 337 while AgNO₃ exposures increased H₂O₂ levels across the whole body, including the
 338 intestine, particularly at 1 mg Ag L⁻¹ (Figure 4b). At 10 mg Ag L⁻¹ Ag NP exposure, elevated
 339 H₂O₂ levels were also visible outside the intestine.

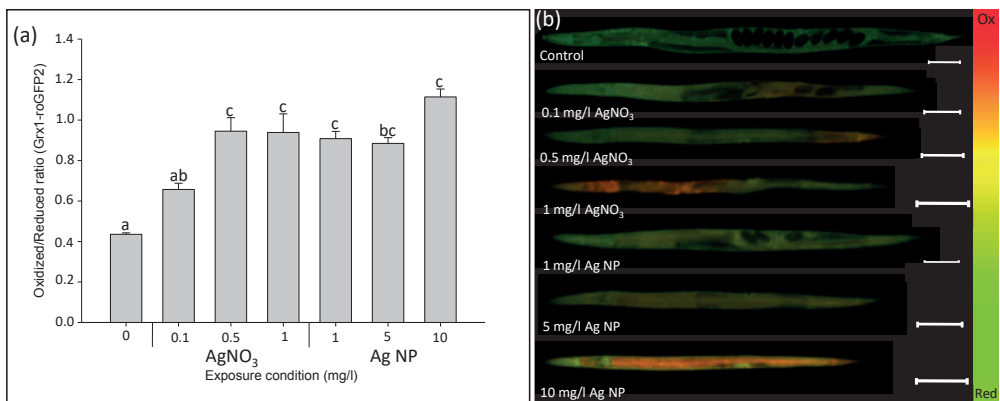


340
 341 **Figure 4.** *In vivo* measurement (a) of the hydrogen peroxide biosensor HyPer in response
 342 to either AgNO₃ or Ag NP exposure with corresponding images (b) (n = 10 per
 343 concentration). Values are normalized to controls for direct comparison. Letters indicate
 344 statistically significant differences among exposure groups. Bars represent 100 μm.

345
 346 3.3.3. Nanoparticle exposure influences to the GSSG/GSH redox status and
 347 oxidative stress in the intestine of *C. elegans*

348 One of the reducing agents of H₂O₂ in nematodes is glutathione (GSH) (Back et al., 2012).
 349 The GRX strain enables the measurement of the cellular GSSG/GSH redox balance in the
 350 nematode, and may thus serve as a proxy for *in vivo* measurement of oxidative stress
 351 (Back et al., 2012, Braeckman et al., 2016). In the current experiment, both AgNO₃ and Ag
 352 NPs resulted in statistically significant increases in oxidized to reduced GSSG/GSH ratios,
 353 compared to controls (Figure 5). Increases were significantly different from controls at

354 0.5 and 1 mg Ag L⁻¹ AgNO₃ exposure (p < 0.05). Similarly, all three Ag NP exposure
 355 concentrations resulted in higher GSSG/GSH ratios compared to controls (p < 0.001). A
 356 visibly increased oxidation was seen in nematodes exposed to the higher AgNO₃ (0.5 and
 357 1 mg Ag L⁻¹) and Ag NP (5 and 10 mg Ag L⁻¹) concentrations, compared to controls (Figure
 358 5b). In all Ag concentrations, Ag NP showed a consistent pattern with increased oxidation
 359 primarily within the intestinal lining of the nematodes, similar to the pattern observed
 360 from the HyPer strain (Figure 4b).

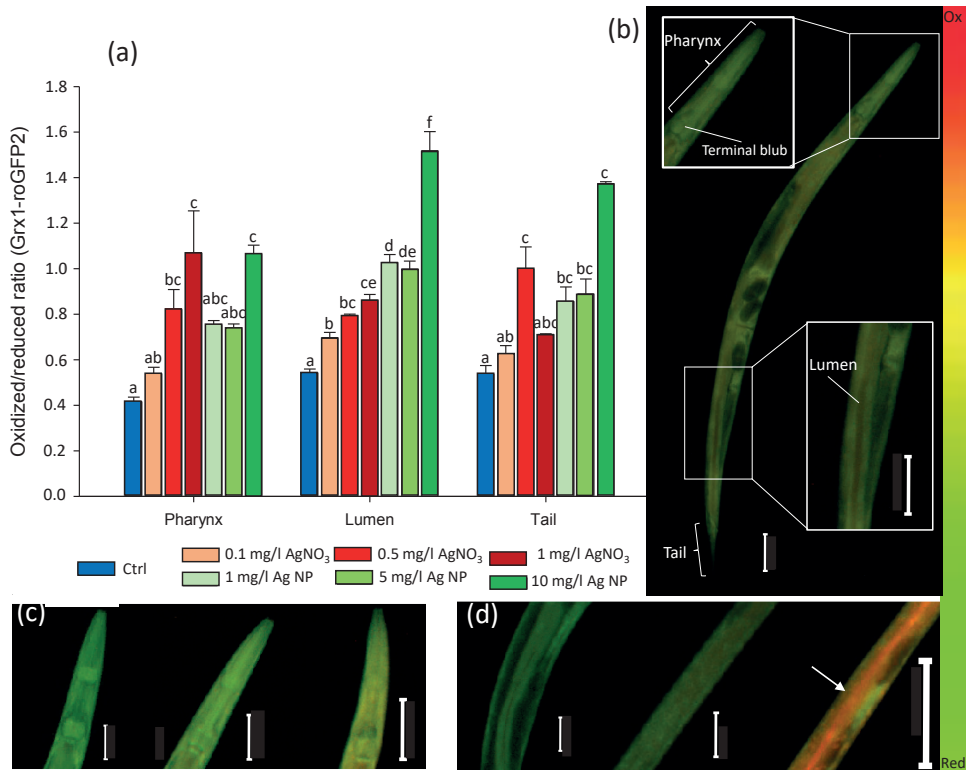


361
 362 **Figure 5.** Oxidized to reduced ratios of the biosensor Grx1-roGFP2 (a) with
 363 corresponding images (b), measured *in vivo*, in response to the exposure to either AgNO₃
 364 or Ag NPs (n = 10 per concentration). Values are normalized to controls for direct
 365 comparison. Letters indicate statistically significant differences among exposure groups.
 366 Bars represent 100 μm.

367 Due to observable increases in oxidation in tissues connected to the lumen in both the
 368 GRX and HyPer strains, samples of the lumen, pharynx and tail were analyzed in detail
 369 for GSSG/GSH ratios, to identify variations in organ and tissue specific establishment of
 370 Ag induced oxidative stress (Figure 6a and b). Due to higher quality of the images, the
 371 quantification of tissue specific patterns was focused on the GRX strain. Compared to
 372 controls, all exposure concentrations resulted in increased GSSG/GSH ratios in the
 373 pharynx, intestinal lining, and the tail (p < 0.05) (Figure 6a). Both 1 mg Ag L⁻¹ AgNO₃
 374 (Figure 6c middle) and 10 mg Ag L⁻¹ Ag NP (Figure 6c right) resulted in a comparable
 375 increase in oxidized to reduced ratios in the pharynx, compared to controls (figure 6c
 376 left). A significantly higher effect on redox status was measured in the luminal tissues

377 from nematodes exposed to either 1 and 10 mg Ag L⁻¹ Ag NP (Figure 6d right), compared
 378 to controls (Figure 6d left) and 1 mg Ag L⁻¹ AgNO₃ (Figure 6d middle). In other parts of
 379 the body, there were no significant differences between the AgNO₃ and Ag NP exposures.
 380 However, during image analysis, it was noted that several nematodes exposed to either
 381 0.5 or 1 mg Ag L⁻¹ AgNO₃ had regions of elevated oxidized ratios, specifically in the head
 382 or the tail (Figure S3). This was not observed after Ag NP exposure.

383



384

385 **Figure 6.** Spatial patterns of GSSG/GSH ratios within the nematode *C. elegans* taken from
 386 either control, AgNO₃ or Ag NP treatments (mean ± SEM, n = 5) (a). Measurements were
 387 made of the pharynx, a representative sample of the lumen, and the tail (b, 0.1 mg Ag L⁻¹
 388 Ag NP). Increased GSSG/GSH ratios were observed in all nematodes exposed to either
 389 AgNO₃ or Ag NPs, compared to controls, most significantly in the pharynx (c, from left to
 390 right: control, 1 mg Ag L⁻¹ AgNO₃ and 10 mg Ag L⁻¹ Ag NPs), and the lumen (d, from left to

391 right: control, 1 mg Ag L⁻¹ AgNO₃ and 10 mg Ag L⁻¹ Ag NPs). Arrows indicate areas of
392 interest with high levels of oxidation. Letters indicate significant differences (ANOVA, p <
393 0.05). All scale bars are 50 μm in length.

394

395 **4. Discussion**

396 There is now a consensus that ROS production and oxidative stress are important modes
397 of action of NP induced toxicity. It appears that the mechanism for ROS generation
398 depends on the nature of each NP (Manke et al., 2013). The mechanisms underlying
399 cellular ROS generation are insufficiently understood, but there are two main ways by
400 which NPs generate free radicals, acting either as catalysts or indirectly via interference
401 with metabolic processes (enzymes), which in turn leads to ROS formation (Huang et al.,
402 2010). In addition, ion release contributes to the ROS mediated toxicity of certain NPs
403 (Fabrega et al., 2011).

404 A range of studies have shown that both silver ions and Ag NPs may produce superoxide
405 anions (O₂⁻), hydroxyl radicals (-OH), hydrogen peroxide (H₂O₂), hydroperoxyl radicals
406 (HO₂[·]) and singlet oxygen (¹O₂) (Hwang et al. 2008, He, Garg and Waite 2012a, He et al.
407 2012b, Choi et al. 2018), which implies a significant potential to cause oxidative stress.
408 Moreover, Ag NPs may result in oxidant-mediated response to ROS, by binding to thiol
409 groups found in thiol proteins/peptides such as GSH, and consequently limiting ROS
410 neutralization (Navarro et al., 2008, Piao et al., 2011). However, there is little information
411 on how NPs affect *in vivo* ROS production in the exposed organisms. In the current study,
412 we capitalized on recent advances in Fluorescent Protein Sensors tagged *C. elegans*
413 (Braeckman et al., 2016, Back et al., 2012) to investigate the effects of Ag NPs on redox
414 biology in relation to measured toxic effects on development and reproduction.

415 4.1. NM300K Ag NPs and AgNO₃ affect cytosolic superoxide dismutase 416 expression

417 A number of studies have shown that NPs may affect cellular superoxide levels,
418 particularly if mitochondrial function is compromised (Grzelak et al., 2018, Abdal Dayem
419 et al., 2017, Masoud et al., 2015). By employing the GA508 SOD-1 reporter strain we were
420 able to firmly demonstrate that NM300K Ag NPs induced *sod-1* superoxide antioxidant
421 defense in a dose dependent manner (Figure 4), which unequivocally is a response to

422 elevated cytosolic superoxide (Doonan et al., 2008). This implies that either NM300K
423 particles, released ions, or superoxide have been translocated from the lumen to the
424 intracellular compartment. In previous studies, Ag ions on the particle surface, or their
425 releases following dissolution, have been related to the increased ROS in response to Ag
426 NP exposure (Foldbjerg et al. 2009, Carlson et al. 2008, Kim et al. 2009). Superoxide is an
427 intrinsic product of metabolism, and physiological *sod-1* expression is highly dependent
428 on developmental stage. Young adult stage larvae are typified by high expression in the
429 pharynx and the distal part of the intestine (Doonan et al., 2008). In this study, the
430 NM300K Ag NPs caused a dose dependent inhibition of development also in the SOD-1
431 strain (Figure 3b), which potentially could have a major impact on *sod-1* response. It was
432 evident that the lowest NM300K Ag NP exposure had no effect on *sod-1*, both with respect
433 to the expression levels and cells or tissue patterns (Figure 3b). The medium level
434 exposure (5 mg Ag L⁻¹) caused a more pronounced effect, especially in the midgut and
435 intestinal cells. The expression pattern in the intestinal cell was heterogenous. The
436 highest NM300K Ag NP exposure caused a marked effect on growth, accompanied by a
437 more uniformly enhanced *sod-1* expression in intestinal cells. In comparison, AgNO₃
438 exposure elicited a significantly higher *sod-1* response (Figure 3). The observable
439 differences in expression patterns in both intestine, pharynx and muscle tissues, suggest
440 that AgNO₃ might be taken up more efficiently from the gastrointestinal tract and thereby
441 cause higher superoxide production in tissues outside the intestine. The observation that
442 both NM300K Ag NPs and AgNO₃ exposure generated excess superoxide demonstrates a
443 significant potential to cause disturbance to physiological processes regulated by ROS
444 signaling, and to cellular redox homeostasis. In line with these observations, we
445 hypothesized that Ag generated superoxide would cause a downstream effect on ROS
446 metabolism in *C. elegans*.

447 4.2. NM300K Ag NP-induced hydrogen peroxide production patterns do not 448 correlate to *sod-1* expression

449 In normal physiological situation, sequestering of superoxide is the main source of H₂O₂
450 (Braeckman et al., 2017, Back et al., 2012). We therefore hypothesized that Ag induced
451 *sod-1* expression would result in corresponding elevated cytosolic H₂O₂ as measured by
452 the HyPer ratiometric biosensor. In this respect, the two exposure agents elicited
453 different responses (Figure 4). In agreement with the *sod-1* expression, the lowest AgNO₃

454 exposure concentrations showed a slight, but not statistically significant, increase in
455 H₂O₂. The medium AgNO₃ exposure, resulted in a decreased response, although not
456 statistically significant, which was unexpected based on the corresponding *sod-1*
457 expression. However, compared to the control, significantly increased H₂O₂ levels were
458 seen in the highest exposure concentration. Assessment of the H₂O₂ patterns indicate that
459 peroxide production occurred in most tissues, with a predominance in the intestine from
460 the highest AgNO₃ exposure concentration. In comparison, the NM300K particles caused
461 a much stronger production of H₂O₂. Overall, the results revealed significantly higher
462 peroxide levels than AgNO₃, even at the 1 mg L⁻¹ NM300K concentration (Figure 4a). In
463 contrast to *sod-1* expression (Figure 3), no dose response was measured. The observation
464 that H₂O₂ levels were, although not statistically significantly, lower at 5 mg L⁻¹ could hint
465 at the mobilization of antioxidant defenses, which successfully sequestered ROS to
466 partially restore the redox homeostasis. The fact that 10 mg L⁻¹ NM300K concentration
467 showed a further increase in H₂O₂ is a strong indication that antioxidant defenses were
468 unable to counteract the total ROS burden. Interestingly, the images suggested a gradient
469 of H₂O₂ within the intestinal cells, with highest levels in tissues closest to the lumen
470 (Figure 5b). This indicated a potential of a tissue specific oxidative stress.

471 4.3. Ag influences *in vivo* cellular redox balance

472 To test the hypothesis that increases in ROS would influence the redox balance and
473 produce oxidative stress in the nematodes, changes in the GSSG/GSH ratios were
474 measured using the GRX biosensor strain. GSH is central for maintaining cellular redox
475 homeostasis as a substrate for oxidative stress defense enzymes. Glutathione S-
476 transferase and peroxidases play a key role in the removal of H₂O₂, through the reducing
477 agent GSH (Back et al., 2012, Wu et al., 2004). Changes in GSSG/GSH ratio are directly
478 related to oxidative stress (Back et al., 2012, Braeckman et al., 2016). Ag NPs have been
479 shown to increase ROS levels and deplete glutathione in rat liver cells and the fruit fly
480 *Drosophila melanogaster* (Hussain et al., 2005, Ahamed et al., 2010). Moreover, Piao et al.
481 (2011) showed that Ag NP may increase cellular ROS levels by directly inhibiting GSH
482 synthesizing enzymes. In line with this, assessment of the whole organism conclusively
483 showed that the cellular redox potential was significantly impacted by the exposure to
484 either form of Ag (Figure 5). The data showed that the lowest (1 mg Ag L⁻¹) Ag NP
485 concentration resulted in an imbalance of the cellular redox potential, similar to the

486 highest (1 mg Ag L⁻¹) AgNO₃ concentration. The remaining exposures showed an overall
487 comparable significant increase in GSSG/GSH ratios.

488 The compiled HyPer and GRX analysis indicates significant expenditure of glutathione to
489 maintain cellular redox homeostasis, which enables the elimination of ROS, including
490 H₂O₂. At the highest exposure concentrations, GRX shows significant oxidation,
491 demonstrating a depletion of the glutathione pool. The loss of redox homeostasis strongly
492 suggests that the antioxidant defense systems are overloaded, that oxidative stress is
493 manifested and that cellular damage is imminent. Notably, a significant concentration
494 dependent growth effect and considerable variation between individual nematode
495 GSSG/GSH ratios within exposure groups was observed. The images revealed clear
496 differences related to the types of Ag, and distinct spatial GSSG/GSH ratio patterns
497 between exposure groups. These patterns were similar to the visually observable H₂O₂
498 levels in the HyPer strain, which suggested tissue specific oxidative stress effects from
499 the NM300K Ag NPs.

500 4.4. Tissue specific oxidative stress effects in response to NM300K Ag NP 501 exposure

502 Detailed analysis of specific tissues demonstrated distinct anatomical patterns of
503 GSSG/GSH ratios, in a concentration dependent manner. This signifies a concentration
504 dependent increased potential for oxidative stress damage. To identify tissue related
505 effects, the analysis focused on three regions of the nematode. Controls showed
506 uniformity with respect to GSSG/GSH ratios, irrespective of tissues. The AgNO₃ exposed
507 nematodes showed significantly higher oxidative stress in the pharynx compared to the
508 intestine and tail regions. In the Ag NP exposure, the pharynx was the least affected part
509 of the nematode. The most profound effects were related to the intestine and tissues
510 surrounding the lumen, particularly evident for the 10 mg Ag L⁻¹ Ag NP treatment (Figure
511 6). This confirms that the NM300K Ag NP exposure resulted in increased ROS generation,
512 with high levels of oxidative stress, predominantly localized to the intestinal lumen
513 (Figure 6). These observations are in accordance with findings by Yang et al. (2014), who
514 found Ag NP toxicity to act primarily in the intestine of the nematodes, whilst dissolved
515 Ag was taken up by the intestinal cells, allowing for interferences with enzymes or
516 organelles. Furthermore, Hunt et al. (2013) found Ag NPs (10 nm) to be primarily

517 associated with the pharynx, lumen tissues and the intestine, where increases in
518 exposure concentration did not significantly alter Ag NP localization.

519 The toxicity of nanoparticles is highly dependent on uptake and retention of the particles
520 by the organism (Meyer et al., 2010). These parameters, however, are influenced by
521 surface chemistry and hence reactivity, as well as the size of the Ag NPs (Meyer et al.,
522 2010). Citrate coated Ag NPs (7 ± 11 nm) have been shown to be taken up and transferred
523 to offspring in *C. elegans* (Meyer et al. 2010). Kleiven et al (2018) showed high intake, but
524 low retention of ~0.6 to 2% of the NM300K Ag NPs by *C. elegans* after depuration
525 indicating negligible levels of cellular internalization of the NM300K Ag NPs. Differences
526 in the uptake and internal distribution of the AgNO₃ compared to Ag NPs by the
527 nematodes were also observed in the current study.

528 Potentially, ionic releases from the particles could result in intercellular uptake of Ag, and
529 cause toxic effects. Ribeiro et al. (2015) explained differences in oxidative stress response
530 mechanisms between AgNO₃ and NM300K in the soil invertebrate *Enchytraeus crypticus*
531 by the slow dissolution of NM300K, leading to delayed exposure to ionic Ag, with a
532 subsequent delay in toxic effect compared to AgNO₃. A range of studies have investigated
533 the dissolution state of the uncoated NM300K Ag NPs, and showed time and media
534 composition dependent ionic releases, varying between 0.07 – 29 % (Wasmuth et al.,
535 2016, Lodeiro et al., 2017, Köser et al., 2017). Nevertheless, the fractionation in the
536 current study showed only low or no ionic fraction in either the beginning or following
537 72 hours of exposure, independent of the added form of Ag (Figure 1). It should be noted,
538 however, that any LMM Ag fraction released during the exposure in the media, may be
539 subject to rapid sorption to the bacterial cells, and hence may therefore contribute to
540 toxicity. Moreover, despite results indicating low dissolution of the NM300K Ag NPs in
541 the current exposure, this does not exclude further dissolution of the particles, with
542 subsequent ionic releases inside the gut of the nematode. The fact that the NM300K Ag
543 NPs induced oxidative stress associated with cells directly surrounding the
544 gastrointestinal tract, however, suggests that neither translocated NPs, nor the dissolved
545 Ag fraction in the Ag NP exposure, produced any significant oxidative stress outside the
546 lumen. On the other hand, at the highest exposure concentrations (10 mg Ag L⁻¹), slight
547 oxidation is visible in the anterior part of the nematode, surrounding the pharynx, as well
548 as along the body outside the intestinal lumen (Figure 6d). The observed increased

549 oxidation may be attributed to either the cellular uptake of ROS, like $O_2^{\cdot-}$ and H_2O_2
550 produced on the surface of the particles, or translocation of the particles themselves (Choi
551 et al., 2018, Goldstein et al., 2005). Alternatively, the increase in H_2O_2 may aid the
552 oxidation of the particles themselves, increasing ionic releases (Navarro et al., 2008),
553 which could explain the observation of oxidation outside the pharynx and intestinal
554 lumen at the highest Ag NP concentrations in the current study (Figure 6c). Ingested Ag
555 NPs have been shown to result in physical damages to the microvilli in mice (Shahare et
556 al., 2013). Such direct damages, although not measured in the current study, may also
557 significantly add to the observed impacts of the Ag NPs on the luminal tissues of the
558 nematodes.

559 On the other hand, results show that $AgNO_3$ exposure produced measureable levels of
560 oxidation more evenly throughout the whole body of the nematodes (Figures 5 and 6).
561 Although low LMM Ag fraction and an overall increase in aggregated Ag fraction is
562 observed from the $AgNO_3$ exposure in the test media, internal dissolution of such
563 aggregates may still be possible. Further, the lack of ionic fraction in the $AgNO_3$ exposure
564 illustrates that any dissolved ions are likely to be removed by either sorption or
565 precipitation. The fact that $AgNO_3$ is capable of inflicting oxidative damage (GRX strain)
566 to most tissues in the entire nematode, however, could be indicative of Ag uptake into
567 these cells (Ratte, 1999, Connell et al., 1991). It could also be a consequence of
568 interference with metabolic processes that results in an accumulation of radicals or
569 provokes a systemic production of ROS (Cortese-Krott et al., 2009, Yoshimaru et al.,
570 2006). Moreover, increased ROS production has been shown to cause lipid peroxidation,
571 DNA damage or enzyme and protein modification or inactivation (Cortese-Krott et al.,
572 2009). It is thus highly likely that the observed oxidative stress produced by Ag in the
573 current study has the potential to cause similar damages. The distinct difference in
574 biodistribution of the two forms of Ag, linked with comparable toxic response outcomes,
575 makes the further study of toxic mechanism of $AgNO_3$ compared to the NM300K Ag NPs
576 of interest. Moreover, antioxidant defense and damage repair is energy demanding and
577 thus may negatively impact growth and reproduction.

578 4.5. Ag induced redox effects in relation to toxic development and reprotoxic
579 effects

580 The toxicity of NM300K has been studied in a range of terrestrial and aquatic organisms
581 (Poynton et al., 2012, Kleiven et al., 2018, Gomes et al., 2017, van der Ploeg et al., 2014,
582 Rossbach et al., 2019). Moreover, studies show that Ag NPs, including the NM300K
583 applied in the current study, induce oxidative stress in various organisms, including *C.*
584 *elegans* (Ahamed et al., 2010, Foldbjerg et al., 2009, Roh et al., 2009, He et al., 2012,
585 Carlson et al., 2008, Kim et al., 2009, Ribeiro et al., 2015, Nguyen et al., 2016, Gomes et al.,
586 2017). Furthermore, an increased ROS production has been related to DNA breakages
587 and increased levels of apoptosis and necrosis, as well as reprotoxicity (Foldbjerg et al.,
588 2009, Lim et al., 2012). In the current study, both AgNO₃ and NM300K produced a dose-
589 dependent increase in ROS and oxidative stress, as well as a nano-specific ROS induction,
590 as measured by the reporter strain GA508 (SOD-1), and the two biosensors HyPer and
591 Grx1-roGFP2 (GRX) (Figures 3 – 5). Moreover, both forms of Ag show a similar dose
592 dependent response pattern in terms of growth, fertility and reproduction, however, Ag
593 NP concentrations 5 - 10 fold higher than AgNO₃ were needed to produce similar levels
594 of effect (Table 1, Figure 2).

595 Organisms are well equipped at producing and eliminating ROS within cells (Lushchak,
596 2014). If exposed to external ROS producers, it has been suggested that organisms are
597 able to reduce other biological processes, such as reproduction or biosynthesis, and
598 instead increase antioxidant defense mechanisms (Lushchak, 2014). In the current study,
599 reproductive toxicity was observed at concentrations equal to or lower than those that
600 induced the *sod-1* antioxidant defense (Figures 2 and 3). This is in line with results found
601 by Roh et al. (2009), who reported increased expression of the *sod-3* gene (mitochondrial
602 MnSOD), but not *sod-1* (cytosolic CuZnSOD), in response to exposure of *C. elegans* to 0.1
603 and 0.5 mg Ag L⁻¹ Ag NPs (PVP coated, 14 - 20 nm) for 24 hours. Notably, the exposure
604 time was significantly shorter than in the current experiment (24 hours compared to 72
605 hours). Other studies suggest that reproductive Ag NP toxicity is related to *sod-3* and *daf-*
606 *12* gene expression, and that oxidative stress plays a key role in Ag NP toxicity (Roh et al.,
607 2009, Lim et al., 2012). Additionally, Völker et al. (2015) found that *sod* activity was
608 unaffected by low concentrations (µg L⁻¹) of NM 300 Ag NP (15 nm, PVP coated) exposure
609 in the freshwater clam *Sphaerium corneum*, and only a small increase was seen in
610 response to AgNO₃ exposure.

611 HyPer data show that the lowest (1 mg Ag L⁻¹) Ag NP concentration results in an
612 imbalance of the cellular redox potential, while AgNO₃ (1 mg Ag L⁻¹) shows no such trend.
613 This suggests that the nematode is much more able to maintain a balanced redox status
614 within the cell, and more effectively fight the ROS production by the AgNO₃ compared to
615 Ag NPs. In accordance with this, Mendes et al. (2015) found that AgNO₃ and NM300K Ag
616 NPs induce different oxidative stress defense mechanisms in the springtail *Folsomia*
617 *candida*, where both CAT and GST show a clear particle specific effect. However, overall,
618 results suggested a delayed reaction to the Ag NP exposure compared to AgNO₃.
619 Nevertheless, both forms of Ag resulted in significantly reduced reproductive capacity at
620 these concentrations (Mendes et al., 2015). Similarly, results from the current study
621 imply a relationship between oxidative stress development and reproductive toxicity
622 from the Ag NP exposure, where increases in peroxide levels, as well as oxidative stress
623 manifestation occur at similar Ag NP concentrations as reduced reproduction. Fertility
624 and growth are unaffected at such low concentrations. This was not seen from the AgNO₃
625 exposure, where reproductive toxicity occurs at lower concentrations than oxidative
626 stress manifestation. Thus, results from the current study indicate differences in the toxic
627 mode of action related to the reproductive toxicity of *C. elegans*, from the two forms of Ag,
628 and a nano-specific tissue effect.

629 **5. Conclusion**

630 In the current study we addressed the toxicity of the NM300K Ag NPs compared to that
631 of AgNO₃ and, through the application of the reporter strain SOD-1 and two biosensors
632 HyPer and GRX, investigated changes in intracellular redox state in the nematode *C.*
633 *elegans*. To the best of our knowledge, this is the first study to demonstrate nanoparticle-
634 induced ROS production, induction of antioxidant defense and evidence of oxidative
635 stress *in vivo* in an intact organism. Results showed that low concentrations of NM300K
636 Ag NPs resulted in an imbalance in cellular redox potential, suggesting the manifestation
637 of oxidative stress. It was also evident that NM300K Ag NPs induced superoxide
638 production, and triggered the expression of cytosolic *sod-1*. Furthermore, the Ag NP
639 exposure caused cellular peroxide levels, and significant changes to cellular redox
640 balance. The stress patterns showed different distributions of oxidative stress within the
641 organism, suggesting that in contrast to AgNO₃, the Ag NPs predominantly act from within
642 the lumen, presumably by interacting with the epithelium of intestinal cells. The results

643 also suggested that ROS production and oxidative stress from Ag NP exposure is an
644 important mode of action that could be related to the observed reproductive toxicity. The
645 compiled results on uptake, retention, toxic effects and oxidative stress patterns,
646 consistently point to differences in modes of action between the two forms of Ag. Silver
647 from AgNO₃ exposures may enter cells, inhibit enzymes, and perturb metabolic functions,
648 which in turn leads to ROS production as a secondary effect, contributing to toxicity. For
649 Ag NPs, and their transformation products, majorly confined to the lumen, ROS
650 production and associated damage to the intestine and surrounding tissues appear to
651 constitute the primary toxic mechanism.

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670 7. References

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899 **Supplementary Material**

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

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906 Lisa M. Rossbach^{1,*}, Deborah H. Oughton¹, Claire Coutris² and Dag A. Brede¹

907 Norwegian University of Life Sciences, Faculty of Environmental Sciences and Natural
908 Resource Management, P.O. BOX 5003 NMBU, No-1432 Ås, Norway

909 ²Norwegian Institute of Bioeconomy Research, Division of Environment and Natural
910 Resources, Høgskoleveien 7, Ås, Norway

911 *Corresponding author: Lisa.rossbach@nmbu.no

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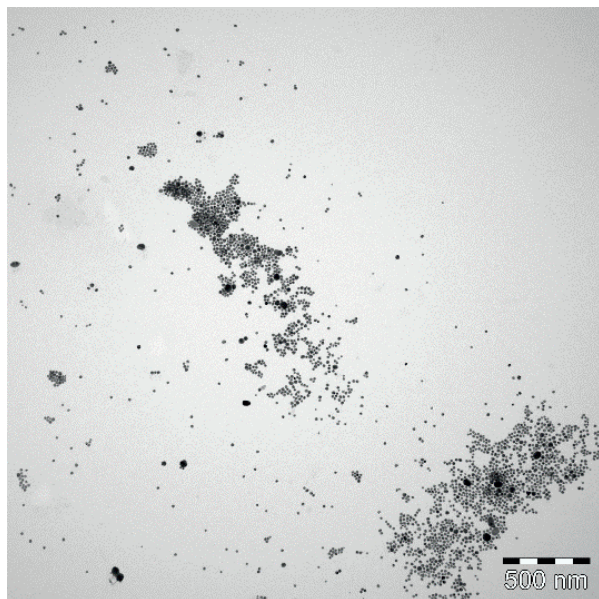
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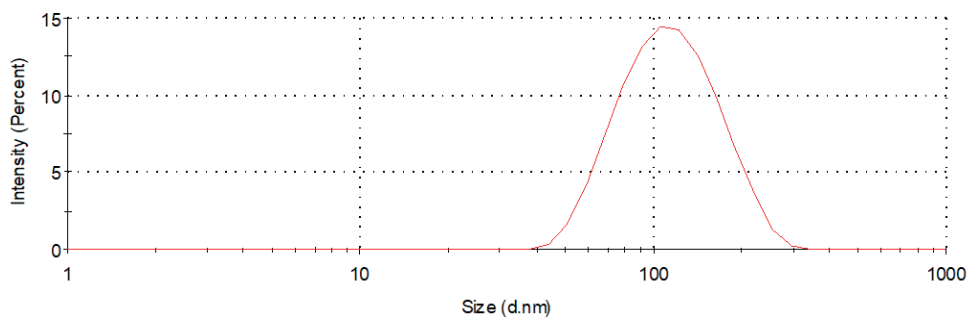
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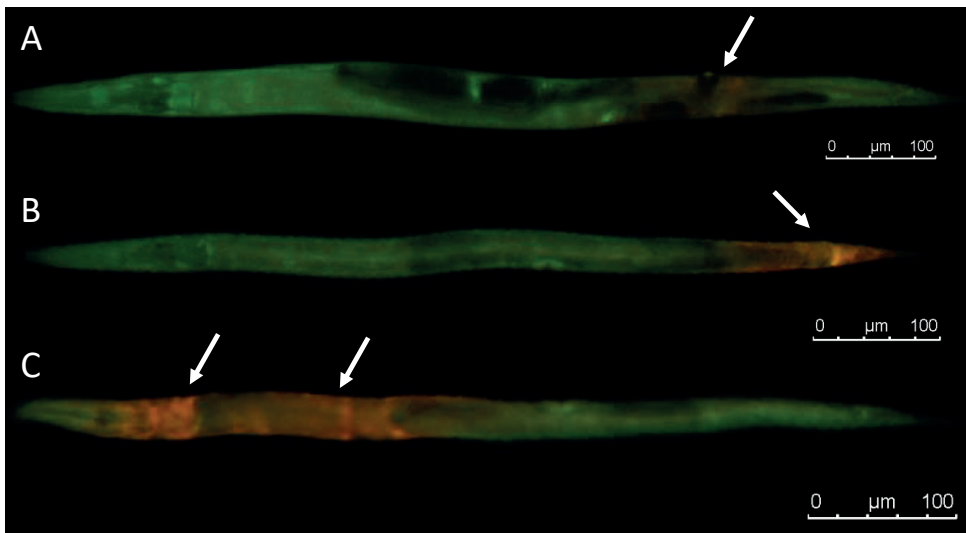
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925 **Figure S1:** Transmission Electron microscopy image of the NM300K silver nanoparticles
926 in the stock suspension (in ddH₂O) with a median particle size of 23.2 ± 17.2 nm (n =
927 361).



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929 **Figure S2:** Size distribution of the NM300K silver nanoparticles in the stock suspension
930 (in ddH₂O), as measured by Dynamic Light Scattering.



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932 **Figure S3.** Increase in GSSG/GSH ratios within the nematode *C. elegans* exposed to
933 AgNO₃ at 0.1 mg Ag L⁻¹ (A), 0.5 mg Ag L⁻¹ (B), and 1 mg Ag L⁻¹ (C). Arrows indicate areas
934 of interest with high levels of oxidation.

935

Paper III

ARTICLE



Adaptive tolerance to multigenerational silver nanoparticle (NM300K) exposure by the nematode *Caenorhabditis elegans* is associated with increased sensitivity to AgNO₃

Lisa M. Rossbach^a, Erica Maremonti^a, Dag M. Eide^b, Deborah H. Oughton^a and Dag A. Brede^a

^aFaculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, Aas, Norway;

^bNorwegian Institute of Public Health, Oslo, Norway

ABSTRACT

Toxic effects of silver nanoparticles (Ag NPs) are, in most cases, measured within a single generation, while information regarding multigenerational exposure remains scarce. The current study assessed changes in toxic response (reproduction, fertility, and development) towards Ag NPs (NM300K; uncoated, 16.7 ± 6.5 nm) compared to AgNO₃ over six generations, following chronic exposure of the model organism *Caenorhabditis elegans*. This revealed that AgNO₃ exposure was associated with no changes in susceptibility to Ag. In contrast, multigenerational exposure to sub-lethal concentrations of Ag NPs resulted in persistent delayed development, but rendered increased tolerance to Ag NP with respect to fertility and fecundity. The results thus permit inference of a difference in toxic mode of action of the two forms of Ag, which instigate different response patterns. Results reveal a novel mechanism for the adaptation toward Ag NPs, where increased reproductive fitness occurs at the expense of somatic growth. This adaptive mechanism was, however associated with increased susceptibility to AgNO₃ with respect to growth, fertility and reproduction. The current study thus demonstrates that a nano-specific resistance can be developed by *C. elegans*. Importantly, this adaptation renders increased vulnerability to another environmental stressor, and thus exposure to a second contaminant could be detrimental to such populations.

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KEYWORDS

Ag nanoparticles; *C. elegans*;
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Introduction

Due to their wide range of applications, silver nanoparticles (or Ag engineered nanomaterials) have become one of the most widely used nanomaterial in industry and consumer products. A range of studies relate silver nanoparticle (Ag NP) toxicity to their relatively high solubility and consequent ionic releases (Meyer et al. 2010; Yang et al. 2012; Starnes et al. 2015; Recordati et al. 2016). However, clear differences in toxicity between ionic Ag and Ag NPs have been observed (Navarro et al. 2008; Kleiven et al. 2018). Such differences are related to particle-specific effects linked to oxidative stress mechanisms that produce adversity ranging from DNA damage and genetic changes to inhibition of physiological properties, such as growth and reproduction (Kim et al. 2009; Roh et al. 2009; Hackenberg et al. 2011; Völker et al. 2015). Furthermore, lesions and bursts to the

outer cuticle of nematodes exposed to Ag NPs have been reported (Kim, Nam, and An 2012). Nanoparticle toxicity has been reported in a wide range of aquatic and terrestrial species; however, most studies only cover a limited timescale of an organisms' life stage, rather than the whole lifespan, and few have looked at impacts across generations (Goussen et al. 2013). Therefore, effects resulting from multigenerational, chronic exposures are relatively unknown.

Ecotoxicology aims to translate effects, such as embryonic death or decreased fertility, observed at an organism level, to alterations in population size and structure (Anderson and Wild 1994). However, some exposures, particularly long term, may result in heritable impacts, with effects only materializing in successive generations (Anderson and Wild 1994; Yu et al. 2013). This is of particular importance for nanoparticle toxicity, due to their intracellular uptake and intergenerational

CONTACT Lisa M. Rossbach  Lisa.rossbach@nmbu.no  Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, P.O. BOX 5003 NMBU, No-1432 Aas, Norway

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transfer (Meyer et al. 2010; Choi et al. 2014), as well as hereditary adverse effects in response to nanoparticle exposure (Kim, Kwak, and An 2013).

The nematode *Caenorhabditis elegans* is, due to its short life span, high fecundity and short generation time, a suitable model organism for ecotoxicological studies, in particular for multigenerational exposures (Handy et al. 2012; Goussen et al. 2013). Additionally, the short generation time of *C. elegans* also prevents aging effects of the nanomaterials (Handy et al. 2012). Generally, *C. elegans* will reproduce hermaphroditically; however, in response to stressors, genetic diversity may be facilitated by cross-fertilization with males (occurrence increased through starvation of populations), increasing adaptation (Morran, Parmenter, and Phillips 2009).

Adaptive responses have been observed in a variety of organisms including *C. elegans*, where the adaptation is defined as the ability to cope with higher concentrations or doses, when previously exposed to lower concentrations or doses of the same agent (Yanase et al. 1999; Dutilleul et al. 2014). Decreases in fertility, lifespan and mortality have been observed following a four generational exposure to three different sizes of Ag NPs, where nematodes showed acclimation to lower concentrations and cumulative damage at higher concentrations (Contreras et al. 2014). Schultz et al. (2016) found an increased sensitivity in the second generation in response to both Ag NP and AgNO₃ exposure over a range of concentrations with no recovery or resistance development for 10 generations, implying significant transgenerational epigenetic effects. Furthermore, following transgenerational exposure of the bacteria *Escherichia coli* to PVP coated Ag NPs, Luo et al. (2016) measured significant decreases in life span and reproduction, however, a recovery was observable after four generations. Additionally, Dutilleul et al. (2014) concluded that *C. elegans* are able to develop increased resistance to pollutants following only a few generations, however, with strong dependency on exposure and pollutant type. Likewise, it has been shown that cumulative effects from continuous exposures may have major impacts on an organisms' ability to cope with the toxicity of Ag NPs (Contreras et al. 2014).

Thus, the main hypothesis of the present study is that the adaptive ability of *C. elegans* to continuous chronic exposure to either AgNO₃ or Ag NPs will

depend on the concentration as well as the type of silver. The continuous chronic exposure to low concentrations will result in rapid adaptation of successive generations. Exposure to higher concentrations will cause a deteriorating effect over generations. Therefore, a multigenerational exposure to sublethal concentrations of either AgNO₃ or the Ag NP NM300K was conducted to determine whether this would alter the sensitivity to silver by assessing toxic effects in reproduction, fertility and development.

Materials and methods

Silver nanoparticle preparation and characterization

Nanoparticles used in the current study were the OECD representative Ag nanomaterial NM300K (Fraunhofer IME, Munich, Germany) and stocks were prepared according to a Standard Operating Procedure developed by EU NanoReg (Jensen et al. 2016). Briefly, a 2.56 g L⁻¹ stock was prepared in ddH₂O (15 MΩ·cm) using a probe sonicator (Branson S-450 D sonicator, disruptor horn 13 mm) for 13 min at 15% amplitude. Fresh stocks were prepared, characterized and appropriately diluted immediately prior to exposure of each population or for toxicity tests.

For the characterization of the nanoparticles, a range of tools was employed. Dynamic light scattering (DLS, Malvern PN3702 Zetasizer Nanoseries, Malvern Inc., Malvern, UK) for hydrodynamic diameter and zeta potential, and transmission electron microscopy (TEM, Morgagni 268) for core diameter was carried out on stock solutions. Assessment of the size fractionation and particle dissolution after 96 h of exposure was carried out by ultrafiltration (14,000 g for 30 min) of the exposure media using <3 kDa Millipore Centrifugal filters (Amicon, Millipore, Billerica, MA) at F0, F2 and F5. Triplicate samples of the exposure media were first centrifuged (2000 g for 5 min) to remove *Escherichia coli* and larger particle aggregates, with subsequent ultrafiltration of the supernatants following pre-conditioning of the filters.

Concentrations for the multigenerational exposure of the populations were selected to be sublethal, ranging from 0.1, 0.5, and 1 mg Ag L⁻¹ for nanoparticles, and 0.01, 0.05, and 0.1 mg Ag L⁻¹ for the AgNO₃ exposures. Concentrations for toxicity

testing were 1.25, 2.5, 5, 10, 20, and 40 mg Ag L⁻¹ and 0.125, 0.25, 0.5, 1, 2, and 4 mg Ag L⁻¹ for Ag NPs and AgNO₃, respectively. To confirm the exposure, triplicate samples for total Ag determination were taken at each generation. All Ag concentrations were determined by Inductively Couple Plasma Mass Spectrometry (ICP-MS, Agilent 8800, Mississauga, ON, Canada), using oxygen as a collisional gas, tuned using manufacturer tuning solution (#5188-6564, Agilent Technologies, Mississauga, ON, Canada), measuring two Ag isotopes (107 and 109), at a detection limit of 0.003 ppm.

***Caenorhabditis elegans* culture and maintenance**

N2-stock of nematode was obtained from *Caenorhabditis* Genetic Centre, Minneapolis, MN. Liquid populations were kept for two months prior to the experiment to maintain a healthy stock. To obtain synchronized populations, gravid hermaphrodites were treated with alkaline hypochlorite to extract eggs (Stiernagle 2006). Eggs were immediately transferred to previously Ag spiked NGM agar plates, seeded with an *E. coli* OP50 lawn. Plates were continuously monitored for development of nematodes into L4 stage and the onset of egg laying. A stereo microscope (Leica M205C) was used for all monitoring, imaging and assessment of *C. elegans*.

Multigenerational exposure

Prior to experiment, triplicate founder populations were generated, which were used to establish three biological replicate populations for each exposure condition. Multigenerational exposures (Figure 1) were carried out with 250 nematodes per culture plate in triplicate, for each of the population exposure concentrations (controls, 0.01, 0.05, and 0.1 mg L⁻¹ AgNO₃ or 0.1, 0.5, and 1 mg L⁻¹ Ag NPs), on 3 ml NGM agar plates, seeded with 300 µl of fresh 10 × concentrated *E. coli* OP50 lawn. On the day of transfer to new generations, 300 µl Ag stock solutions were added on top of *E. coli* lawn on the agar plates, and allowed to air dry for 2 h inside a laminar flow cabinet, before the addition of the new population. Concentrations were chosen according to previously conducted pilot experiments, in order to avoid lethality, on a wide range of concentrations of either Ag NPs (0.1–100 mg Ag L⁻¹) or AgNO₃ (0.01–50 mg

Ag L⁻¹). All generations were continuously exposed from egg stage.

Following 72 h of exposure, nematode size was recorded, before washing of each plate using 3 ml of moderately hard reconstituted water (MHRW; 1.14 mM NaHCO₃, 0.44 mM CaSO₄·2H₂O, 0.5 mM MgSO₄ and 0.05 mM KCl; pH 7.6) (United States Environmental Protection Agency 2002) allowing only eggs to remain on the plate. Half of the eggs were transferred onto new, previously prepared culture plates, for the continuation of the population. The remaining eggs were allowed to hatch on the plate for 12 h at 20 °C for subsequent toxicity testing.

Toxicity testing and cross toxicity test exposure

Standard 96 h toxicity tests were carried out at 20 °C in the dark in 24 well cell culture plates at each generation for all populations. Each well contained 1 ml of MHRW with ca. 5*10⁹ *E. coli* and 11 ± 5.7 L1 stage nematodes. Nematodes were exposed in triplicate to 1.25, 2.5, 5, 10, 20, and 40 mg Ag L⁻¹ and 0.125, 0.25, 0.5, 1, 2, and 4 mg Ag L⁻¹ of Ag NPs or AgNO₃, respectively. Additionally, at F3 and F4, nematodes previously exposed to AgNO₃ were also subject to an Ag NP toxicity test, and *vice versa*, to establish whether any changes in toxicity response would apply to both forms of Ag (from here on referred to as the cross-toxicity test). To terminate the test, wells were stained using 0.5 ml of Rose Bengal, and placed at 80 °C for 10 min, before the assessment of the endpoints. Further, samples were collected for total Ag concentrations, and, at F0, F2, and F5 for <3 kDa fractionation of the exposure medium. At the end of toxicity tests, nematodes on all plates were measured for size, and counted for total number of offspring per recovered adult and number of pregnant nematodes using a hand-held tally counter (International Organization of Standardization 2010).

Statistical analysis

One-way ANOVA was applied to assess for simple group comparison when error terms were normally distributed, using Minitab® 18 (Minitab Inc. 2010). The remaining analyses were done in JMP Pro v14 (SAS institute, Cary, NC). Within each generation, reproduction toxicity test measurements were normalized to measured Ag concentrations, and data presented as

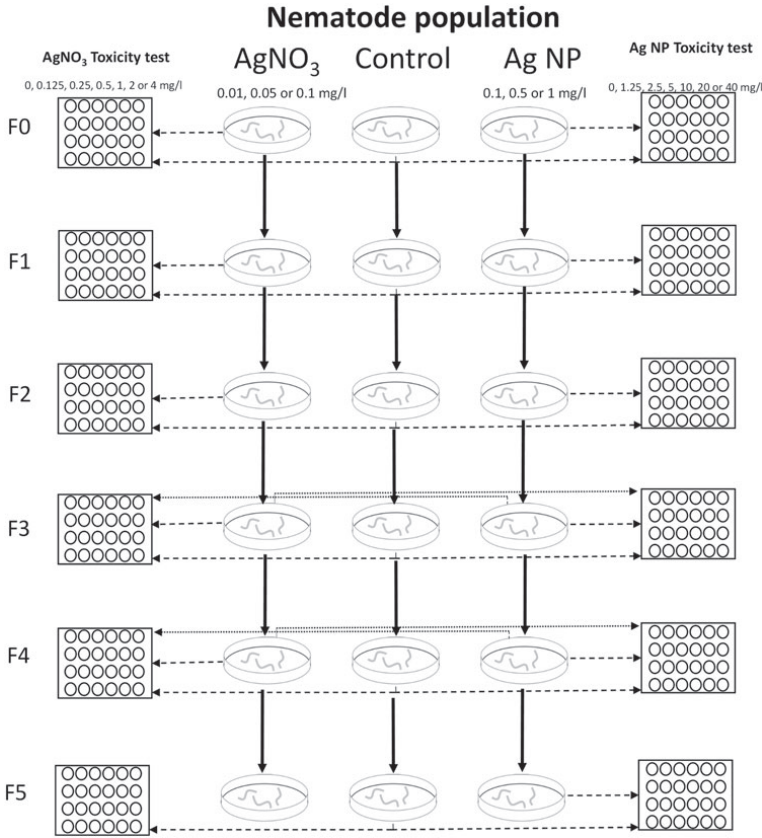


Figure 1. Experimental design of the continuous exposure to three concentrations (mg Ag L^{-1}) of either AgNO_3 or NM300K Ag NPs, or controls (no added Ag), was carried out on NGM agar plates, seeded with *E. coli*. Offspring from each generation was either transferred onto the next generation or employed in toxicity testing (dashed lines) to a range of concentrations of either AgNO_3 or Ag NPs with subsequent measurements of growth, fertility and reproduction. Cross-toxicity tests (dotted lines) were conducted at F3 and F4.

relative to respective toxicity test controls (Yu et al. 2012; Moon et al. 2017). Toxic effects on the three responses (percent fertility, offspring counts (reproduction), and body length) were checked for optimum curve fitness. Different logistic curves, including the Hill equation, showed best fit to data (lowest AIC/BIC values and highest *R*-square). The body size data could in some instances be analyzed in a linear model, but not always. Poisson regression (generalized linear model with over dispersion) was eventually chosen for the analysis of all responses to simplify comparisons between different outcomes, with either concentration or generation as continuous regressors, and either population or cross-toxicity group as class variable. Values were considered significantly different at *p*-values lower than 0.05. Curves in figures are the mean

response of each population within one entire toxicity test (\pm SEM), with a lambda of 0.05, to emphasize the group differences and are not the regression curves from the Poisson model.

Effect concentration (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.

Results

Nanoparticle characterization and exposure concentrations

Stocks and exposure solutions were characterized for particle size distribution and total Ag concentration

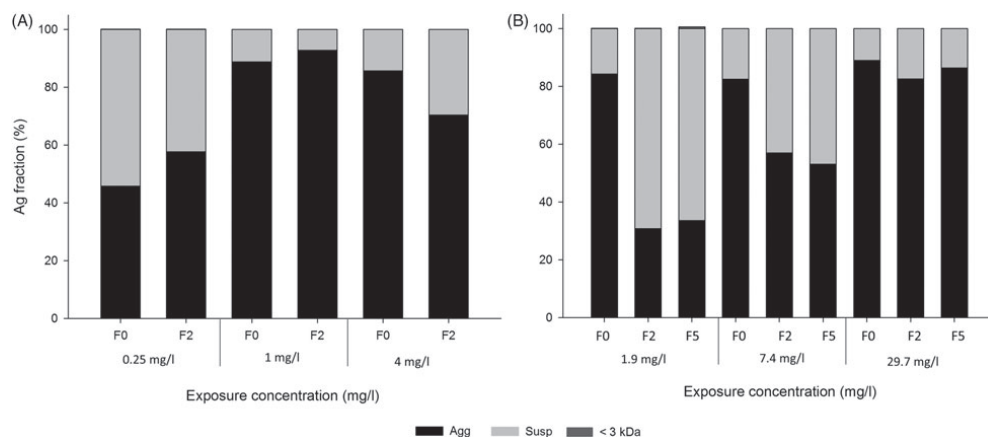


Figure 2. Aggregated (Agg), suspended (Susp), and dissolved (<3 kDa) Ag fraction of exposure media containing either AgNO₃ (A) or NM300K Ag NPs (B), after 96 h of exposure in MHRW containing *E. coli* and nematodes. For suspended Ag fraction *E. coli* was removed. Note, dissolved (<3 kDa) Ag fraction was in most cases too low to be visible in the graph.

to both confirm the concentration of Ag in exposure solutions and to follow any changes in speciation during exposure. Transmission Electron microscopy (TEM) of the NM300K SOP ddH₂O stock suspensions showed that the nanoparticles were spherically shaped with a median particle size of 16.7 ± 6.5 nm (median \pm SD), with a small fraction of $> 1 \mu\text{m}$ sized agglomerates or aggregates (Figure S1).

Dynamic light scattering (DLS) of the same stock solutions showed an average size of 73 ± 1.1 nm, and a zeta potential of -5.5 ± 2.7 mV for the 800 mg L⁻¹ toxicity test stocks and 74.5 ± 2.1 nm (mean \pm SD) and a zeta potential of -4.27 ± 0.4 mV (mean \pm SD) for the population exposure stocks (Figure S2). This indicates a relatively low electrostatic stability of the particles in ddH₂O, and a consequent agglomeration and settlement of aggregates was observed within few hours. Therefore, stocks were diluted immediately after sonication.

To monitor behavior of silver during the exposure, all Ag-stocks and toxicity test exposure media were assessed using ICP-MS or -OES. AgNO₃ solutions showed 61 ± 1.6 and 100% (\pm SD) recovery compared to nominal concentrations, for population exposure stocks (0.01, 0.05, and 0.1 mg Ag L⁻¹) and toxicity stocks (80 mg Ag L⁻¹), respectively, while Ag NP solutions showed 79.2 ± 8.1 and $72 \pm 5.5\%$ (\pm SD) recoveries for population exposure stocks (0.1, 0.5, and 1 mg Ag L⁻¹) and toxicity test stocks, respectively. Consequently, Ag NP toxicity

exposure concentrations are reported as measured concentrations hereafter. Total concentrations of Ag in toxicity test exposure solutions decreased significantly over 96 h with recoveries of 68.7 ± 18.7 and $65.2 \pm 14.2\%$ (\pm SD) for AgNO₃ and Ag NPs, respectively (ANOVA, $p > 0.05$) (Table S1), indicating significant aggregation, precipitation, and sorption to well surfaces during the 96 h exposure period.

Changes in total Ag concentrations were consistent with fractionation analysis of toxicity test media samples. At 96 h of exposure, low (<1.5%) levels of dissolved Ag were recovered in the <3 kDa fraction, for both AgNO₃ and Ag NPs (Figure 2). Since most of the Ag was removed with the *E. coli* centrifugation (either due to sorption or aggregation of colloids) the suspended fraction of Ag in most samples was low at $30.6 \pm 20.5\%$ (\pm SD) total Ag. With respect to Ag NP, approximately $67 \pm 22\%$ (\pm SD) of the Ag was aggregated or associated with *E. coli*. Furthermore, dissolved Ag (<3 kDa) comprised $0.09 \pm 0.2\%$ (\pm SD) of the total and $0.3 \pm 0.3\%$ (\pm SD) of the suspended Ag fractionations for AgNO₃ and Ag NPs, respectively. Due to higher Ag concentrations within the pre-filtrate, results show much more reliable recoveries in terms of total Ag levels. However, results indicate a lower relative fraction of low molecular mass (LMM) Ag in the form of dissolved Ag (<3 kDa), and a decrease in dissolved Ag with increased pre-filtrate Ag concentrations. In conclusion, the formation of larger particles and sorption

to *E. coli* effectively removed LMM Ag from solution.

Multigenerational exposure

In order to assess the ability of an organism to develop an adaptive response toward AgNO₃ or Ag NPs, the nematode *C. elegans* was exposed for six consecutive generations to either form of Ag. Further, to identify potential differences between toxic mechanisms from the two forms of Ag, a cross toxicity test exposure was set up. Across the generations a slight increase in size and reproduction was observed from control populations (data not shown); however, Poisson regression showed that the set up and the data were consistent for the entire experiment, with no significant differences in development of control populations ($p = 0.9$).

Multigenerational Ag exposure caused no impact on subsequent generations' performance in unexposed control conditions

Since Ag NP exposed nematodes showed delayed development compared to the control and AgNO₃ populations, we anticipated that this could potentially influence the performance of their offspring. However, all organisms in toxicity test control conditions, across all generations and independent of previous exposure conditions (control, AgNO₃, or Ag NPs), showed a uniform average size of 1.2 ± 0.05 mm, as well as no significant differences (Poisson regression model. $p = 0.26$) in fertility and reproduction at 96 h development (Figure S3).

Developmental delays due to multigenerational Ag NP exposure

In the current exposure regime, the onset of egg laying was unaffected in control or AgNO₃ exposed populations. Egg laying in the N2 hermaphrodite cultured on NGM-agar with OP50 as food source has been shown to start at ~65 h at 20 °C (Byerly, Cassada, and Russell 1976). Therefore, at 72 h, there were sufficient amount of eggs, to allow the culture transfer to subsequent generations, as well as provide synchronized L1 offspring for toxicity testing. Relative to the control, AgNO₃ exposed nematodes showed normal growth and reproduction patterns.

In contrast, chronic population exposure to sub-lethal Ag NPs resulted in a substantial delayed onset of egg laying (Table S2), and therefore, the 0.1 and 0.5 mg L⁻¹ Ag NP populations could only be transferred to the next generation at 120 h at F2 to F3. For all subsequent generations, populations were transferred at 96 h. A similar delay in reproduction was observed for the 1 mg L⁻¹ Ag NP population with F2 transfer at 144 h, followed by a slight recovery to 120 h at F3 and 96 h for F4 and F5 generations. Consistently, the NP exposed nematodes were visibly less mobile on the population plates (data not shown).

Minor impact on toxic responses following multigenerational chronic AgNO₃ exposure

The current study was aimed at developmental and reproductive effects. We observed an increase in total body length of all AgNO₃ populations across the generations (linear regression model, $p > 0.001$), irrespective of population exposure (Figure 3(a)). However, rather than an adaptation toward the AgNO₃ exposure, this might signify a recovery from the initial decrease in growth seen in the F1 generation.

Developmental changes were monitored using the total body length of nematodes (Figure S4). The growth of all AgNO₃ populations showed a consistent dose–response relationship in the AgNO₃ toxicity test, with $45.3 \pm 18.7\%$ (mean \pm SD, $n = 50$) decreased body size for all nematodes exposed to 4 mg L⁻¹ compared to controls, independent of generation and the concentration of the population exposure (Figure 3(a)). The fact that nematodes previously exposed to 0.01 and 0.1 mg L⁻¹ AgNO₃ were significantly smaller than controls across generations (linear model, $p = 0.02$ and $p = 0.005$, respectively) (Figure 3(a)), can most likely be attributed to the increased sensitization at the F1 generation, followed by a subsequent recovery in generations F2–F5.

Toxicity tests indicated a general increased ability to maintain reproduction in the AgNO₃ exposure from F0 to F5 for all populations, however, not statistically significant (linear model, $p = 0.07$), but all exposure populations remained below or equal to the control populations (Figure 3(b)). Nematodes derived from control populations (no previous Ag

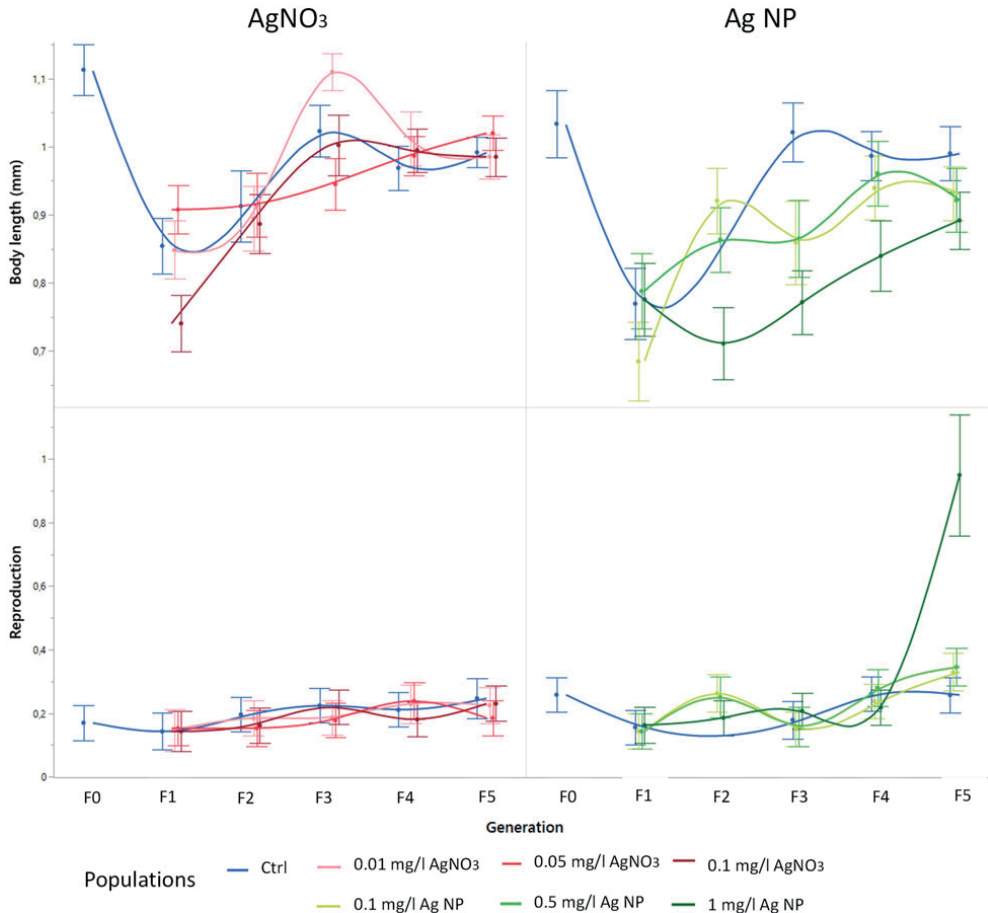


Figure 3. Mean response of individual populations (either Control, AgNO_3 , or Ag NPs), in standard toxicity test. Each curve represents the average response of exposed and unexposed nematodes within one entire standard toxicity test, in terms of body length or number of offspring per adult nematode, as measured by growth (mm, \pm SEM, $n = 21$) and reproduction (normalized to toxicity test controls, \pm SEM, $n = 21$), of *C. elegans* across six successive generations. Individual toxicity tests were normalized to their toxicity test controls. Toxicity test concentrations were either AgNO_3 or Ag NPs. Note the 1 mg L^{-1} Ag NP group produces more offspring, despite smaller size in F5.

exposure) produced offspring up to 0.25 mg L^{-1} AgNO_3 in F0 to F2, while in the F3 and F4 generation produced offspring at concentrations as high as 0.5 mg L^{-1} AgNO_3 . Notably, all populations showed a transient increased sensitivity towards AgNO_3 in the second generation (F1). At this generation, all populations only produced offspring at the lowest AgNO_3 concentrations (Figure S5).

As measured by reproduction, no statistically significant change in sensitivity towards AgNO_3 of control populations was found (linear regression, $p = 0.16$) in the F0 to F5 generations (Figure 3).

Furthermore, AgNO_3 populations showed comparable reproductive capacity in all generations in relation to control populations, with only minor changes in susceptibility relative to controls observable (Figure 3). Additionally, mean effect concentration (EC50) values showed that for all generations, AgNO_3 exposed nematodes were equally sensitive as controls (Table 1). The 0.01 mg L^{-1} nematode populations showed a decrease in sensitivity toward AgNO_3 at F3 and F4, with nematodes exposed to concentrations as high as 1 mg L^{-1} still producing offspring (Figure S5). In contrast, the 0.1 mg L^{-1}



Table 1. Effective concentrations (10% and 50%) for reproduction, for AgNO₃ and Ag NPs exposed *C. elegans* across six generations, following the continuous chronic exposure to three concentrations of AgNO₃ or three concentrations of Ag NPs, and subsequent exposure to six concentrations of either.

	AgNO ₃					Ag NP				
	Ctrl	0.01 mg/L	0.05 mg/L	0.1 mg/L	Ctrl	0.1 mg/L	0.5 mg/L	1 mg/L		
F0										
EC10	0.07 (0–0.10)				0.17 (0.17–0.48)					
EC50	0.10 (0.01–0.12)				0.83 (0.70–1.22)					
F1										
EC10	–	0.07 (0–0.11)	0.07 (0–0.07)	–	0.50 (0–0.57)	–	–	0.59 (0–0.68)		
EC50	–	0.08 (0–0.12)	0.09 (0–0.10)	–	0.65 (0–0.71)	–	–	0.74 (0–0.86)		
F2										
EC10	0.06 (0.03–0.10)	0.04 (0–0.10)	0.07 (0–0.09)	0.07 (0–0.09)	–	0.28 (0.06–0.74)	0.44 (0.07–1.40)	0.79 (0–0.82)		
EC50	0.11 (0.08–0.12)	0.08 (0.01–0.12)	0.09 (0–0.11)	0.09 (0–0.12)	–	0.96 (0.45–1.54)	1.02 (0.54–1.71)	0.85 (0–0.89)		
F3										
EC10	0.04 (0.02–0.07)	0.07 (0.01–0.09)	0.07 (0.02–0.09)	0.05 (0.02–0.07)	–	0.50 (0–0.54)	0.50 (0–0.69)	0.42 (0.13–0.79)		
EC50	0.11 (0.09–0.13)	0.11 (0.06–0.12)	0.10 (0.05–0.11)	0.11 (0.09–0.13)	–	0.64 (0–0.69)	0.67 (0–0.88)	0.80 (0.53–0.97)		
F4										
EC10	0.04 (0.02–0.06)	0.03 (0–0.11)	0.06 (0.03–0.09)	0.09 (0.08–0.10)*	0.11 (0.04–0.44)	0.11 (0.04–0.34)	0.48 (0.36–0.64)	0.15 (0.03–0.67)		
EC50	0.10 (0.07–0.12)	0.10 (0.01–0.16)	0.13 (0.10–0.16)	0.11 (0.10–0.12)	0.71 (0.41–1.21)	0.60 (0.36–0.86)	1.20 (1.06–1.36)	0.60 (0.24–0.96)		
F5										
EC10	0.08 (0.03–0.16)	0.03 (0.01–0.10)	0.10 (0–0.11)	0.03 (0.01–0.08)	0.08 (0.2–0.41)	0.44 (0.24–1.05)	0.58 (0.34–1.48)	3.38 (0.20–5.85)		
EC50	0.14 (0.09–0.22)	0.10 (0.05–0.15)	0.11 (0.02–0.12)	0.10 (0.05–0.14)	0.63 (0.29–1.12)	1.53 (1.08–2.27)	1.89 (1.33–2.68)*	3.87 (1.26–6.01)*		

Bold values indicate EC values with 96% confidence intervals that do not overlap with control EC 95% confidence intervals. The * indicates significant differences from controls.

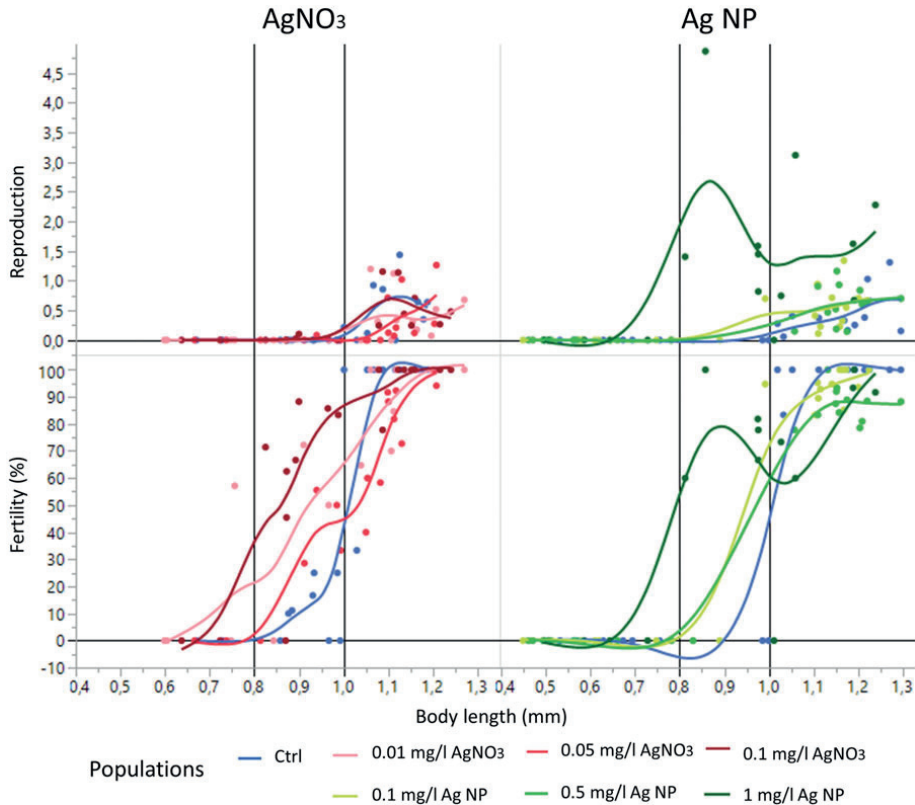


Figure 4. Normalized reproduction and fertility relative to body length (mm) for F5 nematode populations exposed to either AgNO_3 or Ag NPs in a standard toxicity test. Vertical lines indicate minimum size (1 mm) of control nematodes needed for reproduction and the lower limit (0.8 mm) where reproduction was measured in the current exposure regime.

AgNO_3 populations exhibited an increased sensitivity where the levels of offspring remained similar or marginally lower than control populations (Figure S5). At F5, a decrease in offspring production was observed for all populations.

The number of pregnant nematodes was used as a measure of fertility (Figure S6). The number of pregnant nematodes challenged with AgNO_3 showed a similar trend to reproduction, where pregnant nematodes were only found in concentrations up to 0.5 mg L^{-1} at F0 and up to 1 mg L^{-1} at F3 and F5 (Figure S6). To investigate whether nematodes were able to modulate their fertility in response to AgNO_3 exposure over time, fertility was compared across generations with a Poisson regression analysis. In generations F0–F5, no increased fertility was measured ($p = 0.98$). Nevertheless, a clear increase in fertile nematodes is seen in the F5

generation from smaller (0.8–1 mm) organisms from the 0.01 and 0.1 mg L^{-1} AgNO_3 populations, compared to controls within the same size range (Figure 4). However, despite fertile nematodes at this size range, no reproduction was observed for these populations.

Chronic multigenerational Ag NP exposure results in adaptation toward Ag NPs

A substantial decreased development was observed for all nematodes in the 14.9 and 28.8 mg L^{-1} exposure concentrations, and specifically for the populations exposed to 1 mg L^{-1} Ag NPs (Figure S7). Across all generations, the 0.1 and 1 mg L^{-1} Ag NP population nematodes were significantly shorter than the control population (linear model, Tukey's HSD, $p = 0.01$ and $p = 0.02$, respectively) (Figure 3).

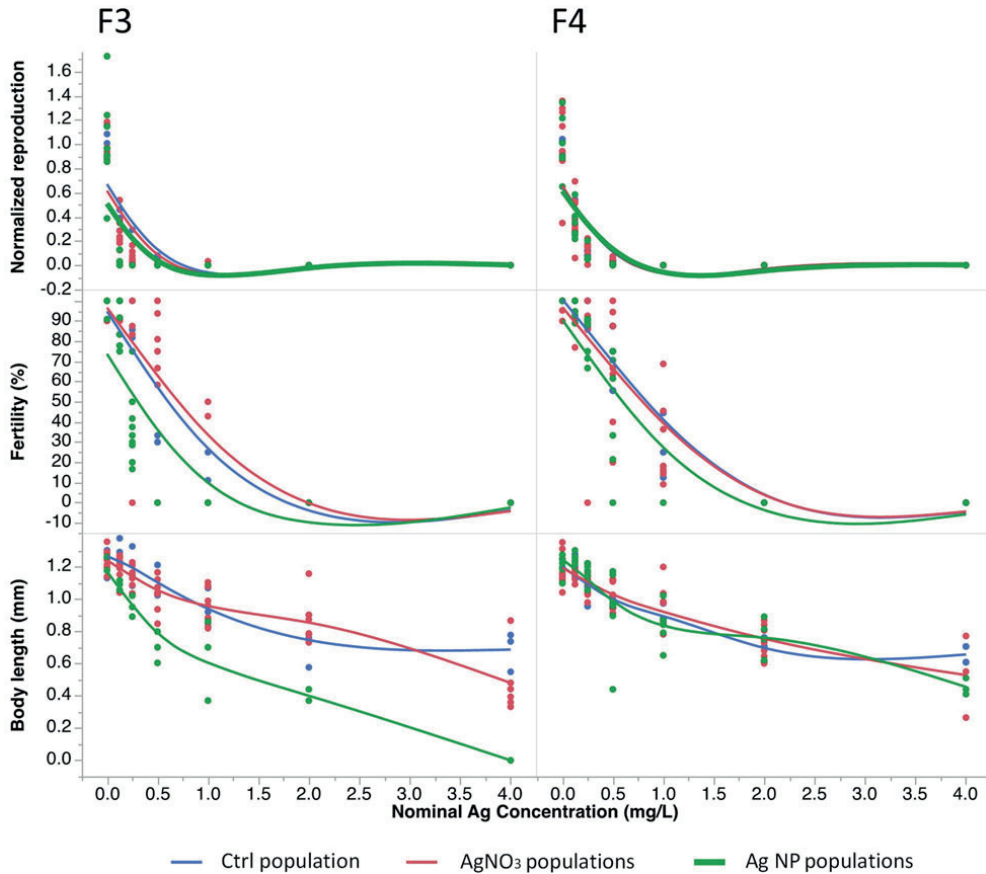


Figure 5. Cross-toxicity test with AgNO_3 . Growth, fertility, and reproduction of *C. elegans* from the cross-toxicity test exposure at F3 and F4, taken from either the AgNO_3 , Ag NPs, or control populations, exposed to six concentrations of AgNO_3 in a standard toxicity test. Curves are cubic spline fits. The concentrations of Ag in either population did not influence nematodes performance in the toxicity test, and hence AgNO_3 or Ag NP exposed populations were pooled ($p > 0.9$).

With respect to reproduction, the Ag NP exposed populations during generations F0 to F3 appeared to develop an initial increased sensitivity, compared to control nematodes, but this effect was not statistically significant (Poisson regression, $p = 0.8$) (Figure S5). Both the 0.1 and 0.5 mg L^{-1} populations behaved similar to the control population (Figure S8). In contrast, F5 showed a significant increase in offspring produced at higher Ag NP toxicity test concentrations with numbers exceeding control populations reproduction (Poisson regression model, $p = 0.01$) (Figure 3(d)). As anticipated, the observed adaptive responses were dependent on the populations' exposure concentrations.

Following six generations (F5) of exposure, nematodes from the 1 mg L^{-1} Ag NP population showed a 425% and 283% increase in reproduction, in toxicity test concentration 1.9 and 3.7 mg L^{-1} , respectively. At lower concentrations, the reproduction was equal to the control populations (Figure S8). This was further corroborated by the calculated reproduction EC_{50} values, which demonstrate an overall adaptation, signifying a decrease in sensitivity of the Ag NP exposed population nematodes to reproductive toxicity of Ag NP (Table 1).

For all generation, independent of population concentration, pregnant nematodes were present at concentrations up to 3.7 mg L^{-1} (Figure S9).

Fertility showed no change in generations F0–F3, for neither AgNO₃ nor Ag NP exposed populations. At the later generations F4 and F5, the numbers of pregnant nematodes were consistent and comparatively higher than in previous generations. Interestingly, at the F5 generation, the 1 mg L⁻¹ Ag NP population exhibited higher fertility and reproduction than controls at a substantially smaller body size (0.8–1 mm) (Poisson regression model, $p = 0.006$) (Figure 4). Moreover, fertility and reproduction at this size interval were significantly higher in the Ag NP than in the AgNO₃ exposed population (Poisson model, $p = 0.006$).

Multigenerational AgNO₃ exposure results in increased resistance towards Ag NP while Ag NP exposure exacerbates sensitivity to AgNO₃

In order to investigate whether multigenerational AgNO₃ or Ag NP exposure instigate different adaptive changes to the *C. elegans* populations, a cross-toxicity test was performed. There was no significant effect of the population exposure concentration, for neither Ag NP nor the AgNO₃ on the response of the nematodes in the cross-toxicity test (Poisson model, $p > 0.9$), hence all AgNO₃ or Ag NPs populations were pooled (Figures 5 and 6). For growth, the AgNO₃ toxicity tests revealed that Ag NP exposed populations were significantly more sensitive than the controls or AgNO₃ populations ($p < 0.0001$) in the F3 generation (Figure 5). Likewise, a similar significant decrease in fertility was observed ($p = 0.0002$). In the F4 generation, neither growth ($p = 0.8$) nor fertility ($p = 0.4$) was significantly impacted by population exposure. On the other hand, reproduction measurements showed a statistically significant decrease between the Ag NP population and the AgNO₃ population in both F3 ($p = 0.0002$) and F4 ($p = 0.02$) (Figure 5). Nematodes previously exposed to Ag NPs showed increased sensitization, where offspring was only produced at the lowest (0.125 mg L⁻¹) AgNO₃ concentration, while AgNO₃ and control populations produced offspring at concentrations as high as 3.6 mg L⁻¹.

The Ag NP cross toxicity test data revealed a statistically significant increase in growth by the AgNO₃ populations compared to the Ag NP populations for F3 ($p = 0.03$), but not F4 ($p = 0.6$) generations (Figure 6). Furthermore, a significant ($p = 0.02$)

increase in fertility from the F3 AgNO₃ populations, compared to the Ag NP populations was found. No such difference was found for the F4 generation (0.2) (Figure 6). A statistically significant increase in reproduction was found for the AgNO₃ population compared to the Ag NP population, in the F3 ($p < 0.0001$) and F4 ($p = 0.04$) generations (Figure 6). Control populations as well as the AgNO₃ populations were able to produce offspring at concentrations as high as 3.6 mg L⁻¹ Ag NP in the toxicity test, while the Ag NP populations were incapable of producing offspring at 1.8 mg L⁻¹ Ag NP exposure. Thus, confirming that pre-exposure to AgNO₃ rendered nematodes less sensitive to Ag NP exposure, while Ag NP pre-exposure exacerbated the effects of AgNO₃.

Discussion

Due to their longevity and continuous release into the environment, the study of multigenerational effects of Ag NPs presents a realistic exposure scenario. Further, understanding the behavior and toxic modes of action of such contaminants is vital for safe use and management of nanoparticles. To this end, we devised a scenario where the effects of sub-lethal exposures to either Ag NP or AgNO₃ were investigated over the course of six generations. We thus hypothesized that the effects caused by Ag NP would be significantly different from AgNO₃.

The results showed that the overall effect patterns on AgNO₃ and Ag NP exposed populations were characterized by several important differences between the two forms of Ag. A commonality, for all exposed populations across all generations, was the fact that they performed equal to the control populations in clean environments, represented by the toxicity test control conditions. This was true for all measured parameters including growth, fertility, and reproduction (Figure S3). This indicates a high capacity to recover from environmental stress by *C. elegans* populations.

Interaction of Ag with *E. coli* an important facilitator of Ag uptake by *C. elegans*

To investigate whether the differences in effects could be related to uptake, size characterization of the Ag NPs was conducted (16.7 nm ± 6.5 nm;

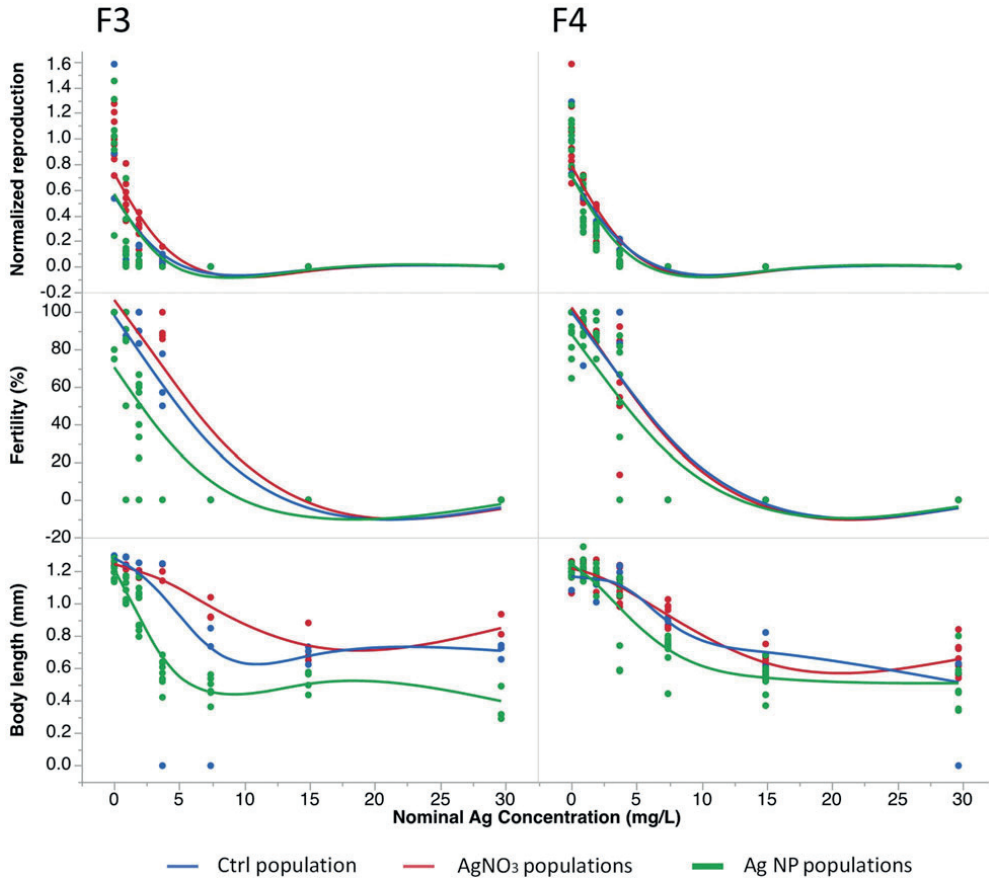


Figure 6. Cross-toxicity test with Ag NP. Growth, fertility, and reproduction of *C. elegans* from the cross toxicity test exposure at F3 and F4, taken from either the AgNO₃, Ag NPs, or control populations, exposed to six concentrations of Ag NPs, in a standard toxicity test. Ag NPs or control populations, exposed to six concentrations of AgNO₃ in a standard toxicity test. Curves are cubic spline fits. The concentrations of Ag in either population did not influence nematodes performance in the toxicity test, and hence AgNO₃ or Ag NP exposed populations were pooled ($p > 0.9$).

mean \pm SD). Speciation by size fractionation analysis showed changes in Ag speciation during the 96 h of exposure for both AgNO₃ and Ag NP (Figure 2). It has been suggested that ionic silver released from the Ag NPs could account for a high degree of any toxic effect observed (Meyer et al. 2010; Beer et al. 2012; Li et al. 2015). However, in the current study, both forms of Ag showed transformations to larger particles (<30 nm) and only low proportions of dissolved Ag complexes (<3 kDa) remained after 96 h, in the toxicity test exposure media. These observations are consistent with observations from a previous study that showed that both AgNO₃ and Ag NP were found to be

predominantly associated with *E. coli* during exposure (Kleiven et al. 2018). Kleiven et al. (2018) also investigated the uptake and depuration of Ag NP compared to AgNO₃. This indicated that AgNO₃ and Ag NP uptake was equal, but AgNO₃ retention by the nematodes was much higher. Additionally, results from Köser et al. (2017) only detected low levels of dissolved Ag when testing NM300K in a range of different test media. However, the authors suggested ions present in the exposure media, to be associated to the stock dispersant, rather than dissolution from the NPs, where the dispersant hinders the further oxidation of the particles when introduced in the test media (Köser et al. 2017).

Thus, this could explain low ionic fractions observed in the current experiment. Alternatively, rapid adsorption of free ions to the *E. coli* (Kleiven *et al.* 2018) might account for the low detection of dissolved Ag (<3 kDa) in the current exposure.

These results, together with the toxic response developments from F0 to F5 generations and results from the cross-toxicity exposure (Figures 5 and 6), consistently point towards different mode of action (MoA) from AgNO₃ versus Ag NPs. To further investigate the differences in toxic MoA and nature of the potential adaptive responses we investigated whether exposure to Ag NP would affect tolerance to AgNO₃ and vice versa.

Pre-exposure to Ag NPs induced increased resistance toward Ag NP toxicity

We hypothesized that exposure to high levels of Ag NP that cause significant effects in F0 would cause progressively increased severity of effects during the successive generations. Conversely, we hypothesized that low level Ag NP would facilitate an increased tolerance to Ag NP. The effects of multigenerational Ag NPs, in general, reveal increased sensitivity during F1 to F3 generations regardless of exposure regime. This trend changed profoundly from F4. The low and medium level pre-exposures resulted in slight adaptation with respect to growth, accompanied with moderately improved tolerance to Ag NP with respect to reproduction in F4, and pronounced higher reproduction compared to control in F5 (Figures S7 and S8). The highest level of pre-exposure caused severe persistent impairment of growth throughout all generations (Figure 3). Nevertheless, despite the reduced growth, nematodes were able to maintain their reproductive status, possibly due to energy reallocation.

Other multigenerational exposure scenarios (Goussen *et al.* 2013; Schultz *et al.* 2016), report an increasing resistance from control groups, which may be explained by an adaptation toward the experimental conditions. We did not observe any significant change in control population behavior and sensitivity to the exposure scenario (Figure 3). Thus, the increasing adaptation of the Ag NP populations to the Ag NP exposure was not due to acclimation towards exposure conditions but was actual adaptive response toward the NPs.

Similar to findings by Schultz *et al.* (2016), the increased sensitization of the F1 generation in the current study is also in accordance with the trans-generational study conducted by Yu *et al.* (2013) (Figures 3). Exposure to Cd, Cu, Pb, and Zn induced significant inhibitory effects in the parent generation, with increased severity of effects and sensitization in the subsequent daughter generation (Yu *et al.* 2013). However, and contrary to the current study, Schultz *et al.* (2016) did not observe recovery of neither the PVP-coated Ag NP nor the AgNO₃ exposed nematodes in successive generations.

Despite the reduced growth the fertility and reproduction of both Ag NP and AgNO₃ populations recovered by F5 (Figures 3, S5, S6, S8, and S9). Notably, the 1 mg L⁻¹ Ag NP population showed the highest fecundity of all populations in the F5 generation (Figure 3). Moreover, at F5, smaller nematode (0.8–1 mm), from either AgNO₃ or Ag NP populations, expressed fertility ($p = 0.02$), but only the NP population nematodes produced offspring, compared to control with no fertility or reproduction observed at this larval size interval (Figure 4). This implies that *C. elegans* is capable of adapting their reproductive strategy to the presence of Ag NP and AgNO₃, at the expense of growth. This further supports the need to study multigenerational exposure scenarios, where significant effects of the toxicant remain unaccounted for, rather than single generation exposures.

The results of toxicity tests and calculated EC50 values consistently showed that the continuous chronic exposure to AgNO₃ resulted in a slightly increased sensitization of *C. elegans* towards AgNO₃, with respect to growth, fertility and reproduction (Tables 1 and S2). It thus appears that *C. elegans* was less capable of adapting to AgNO₃, at least within the number of generations and the exposure set up applied in the current study. It is thus conceivable that an adaptive response might occur at lower AgNO₃ exposure concentrations, than applied in the current study.

Luo *et al.* (2016) showed that ingested PVP-coated Ag NPs (25 nm) were effectively transferred to offspring and further generations, where a recovery from toxic effects was only observed after four generations. Contrasting this, the current results suggest no heritable effects, since nematodes were able to maintain normal reproduction despite

parental exposure to any of the three Ag NP concentrations (Figure S8). This may be explained by differences in surface chemistry, or uptake and retention of the Ag NPs by the nematodes (Kleiven et al. 2018). The study by Kleiven et al. (2018) showed that less than $\sim 0.6\%$ of the NM300K particles were retained by *C. elegans*. This could potentially explain why NM300K renders toxicity primarily to the directly exposed generation and allows for adaptive development of tolerance (Figure 3).

The aforementioned observations imply a distinct difference in toxic response of the nematodes to the two forms of Ag. The distinction in response seen in the cross-toxicity exposure tests support the notion that there are important differences in toxic MoA and effects of the Ag NPs compared to AgNO_3 . Such differences indicate that AgNO_3 and Ag NP affect growth, development and reproduction by either difference in uptake, as shown by Kleiven et al. (2018), and/or toxic MoA mechanisms.

The developmental delays observed in all nanoparticle exposed populations throughout the six generations (Figure 6) could potentially be related to transgenerational toxicity. The fact that the Ag NP exposed nematode populations suffered significant developmental delays necessitated adjustment of the experimental protocol for generational transfers. Such delays may inadvertently result in the selection of less sensitive offspring, thus inducing tolerance in a yet unexplained manner. Therefore, increased adaptive responses may be due to selection induced by the Ag NP, allowing individuals with more suitable detoxification mechanisms to remain and successively dominate the population.

While resistance within a population to a pollutant may be seen as a positive development, it often comes at an associated physiological cost, affecting the performance of a population (Medina, Correa, and Barata 2007). The accumulation and possible ROS production by the particles inside the cells is likely to result in an energy reallocation by the organism and in turn increase developmental time or reduced reproduction (Álvarez et al. 2005). Furthermore, as shown in the current study, the resistance development can come at the expense of increased vulnerability toward another environmental stressor, with potentially negative impacts on the population. It is important when discussing adaptation of a species to a toxicant, to distinguish

between acclimation (physiological adaptation) and genetic adaptation through genetically inherited tolerance selection (Medina et al. 2007). Genetic changes, such as micro-evolution, where the exposure to a toxicant causes alterations to the genetic code, may occur after just a few generations (Medina et al. 2007). While Ellegaard-Jensen, Jensen, and Johansen (2012) concluded that *C. elegans* are not able of adaptive response to a pollutant, Dutilleul et al. (2014) showed rapid resistance development after just a few generations to depleted Uranium and NaCl. Schultz et al. (2016) suggest epigenetic changes as a possible explanation for their findings of increased sensitization with no recovery. The resistance pattern observed in the current study hints at acclimation rather than genetic adaptation due to rapid changes of initial increased sensitivity in earlier generations to an increased resistance at later generations. However, due to the possibility of the selection of more resilient individuals in generational transfers, a definite conclusion cannot be reached. Nevertheless, this pattern is consistent with previous observations from uranyl nitrate and gold nanoparticles (Goussen et al. 2013; Moon et al. 2017). Additionally, when removed from the exposure, as in the toxicity test control, all populations performed equally with respect to growth, fertility, and reproduction.

Conclusion

In the current study, we address toxic effects of Ag NPs NM300K compared to AgNO_3 during six consecutive generations to investigate whether the nematode *C. elegans* would become sensitized or develop a resistance. Despite the fact that low chronic exposure to Ag NP resulted in developmental delays, we provide evidence strongly suggesting that exposure to either form of Ag resulted in adaptive response towards Ag NP, after a few generations. In contrast, the nematodes showed no such adaptive response towards AgNO_3 . The current study highlights novel mechanisms for the adaptation toward Ag NPs, where reproductive capacity occurs at the expense of somatic growth, which is characterized by the fertility and higher reproduction in smaller nematodes. Furthermore, the current study confirms the difference in toxic mechanisms between the two forms of Ag (AgNO_3 or

nanoparticulate), possibly resulting from differences in exposure, due to a high degree of interaction of the dissolved Ag with the *E. coli*, facilitating the Ag uptake by the nematode. Importantly, we show that the adaptation to Ag NPs, leaves nematodes more vulnerable towards other environmental stressors. Findings of this study will aid to further improve the understanding of the toxicity of nanoparticles, as well as contribute to our knowledge about the physiological consequences of *C. elegans* in response to toxicants.

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Disclosure statement

The authors report no conflict of interest.

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Supplementary Material

Multigenerational Adaptive tolerance of the nematode *C. elegans* to Silver nanoparticles is associated with increased sensitivity to SilverNO₃ (Ag^(I))

Lisa M. Rossbach¹, Erica Maremonti¹, Dag M. Eide², Deborah H. Oughton¹ and Dag A. Brede¹

¹Norwegian University of Life Sciences, Faculty of Environmental Sciences and Natural Resource Management, P.O. BOX 5003 NMBU, No-1432 Aas, Norway

²Folkehelseinstituttet, Lovisenberggata 8, 0456 Oslo, Norway.

*Corresponding author: Lisa.rossbach@nmbu.no

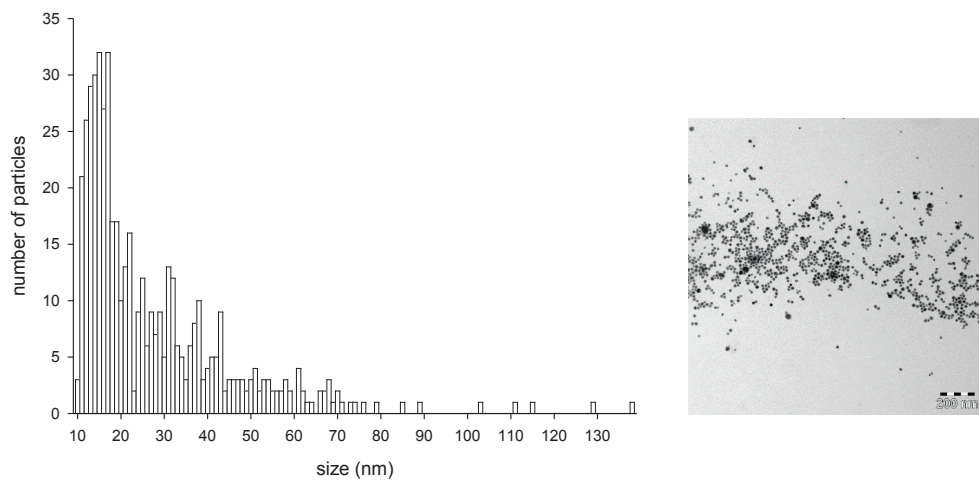


Figure S1: Transmission Electron microscopy image and size distribution of the NM300K silver nanoparticles in ddH₂O with a median particles size of 16.7 ± 6.5 nm.

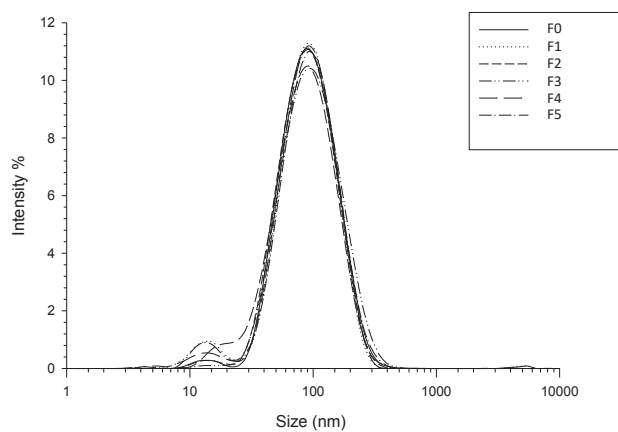


Figure S2: Size distribution of the NM300K Silver nanoparticles applied in the current study for generations F0 – F5, in ddH₂O, as measured by Dynamic Light Scattering (DLS).

Table S1: Total Ag (either AgNO₃ or Ag NPs) concentrations (\pm SD) following 96 hrs of exposure of the nematode *C. elegans* in MHRW containing *E. coli*, measured in generation F0, F2 and F5. Concentrations are given as a mean of triplicate samples.

Generation	AgNO ₃			Ag NP		
	Nominal Ag (mg/l)	Ag recoveries at 96 hrs	SD	Nominal Ag (mg/l)	Ag recoveries at 96 hrs	SD
F0	0.25	0,18	0,01	2.5	1,40	0,08
	1	0,76	0,02	10	4,20	0,14
	4	3,97	0,09	40	14,67	2,05
F2	0.25	0,13	0,01	2.5	1,09	0,15
	1	0,61	0,02	10	5,60	1,13
	4	3,53	0,09	40	16,33	1,70
F5	0.25	0,01	0,00	2.5	1,40	0,16
	1	0,03	0,00	10	5,20	0,64
	4	0,14	0,00	40	14,33	0,94

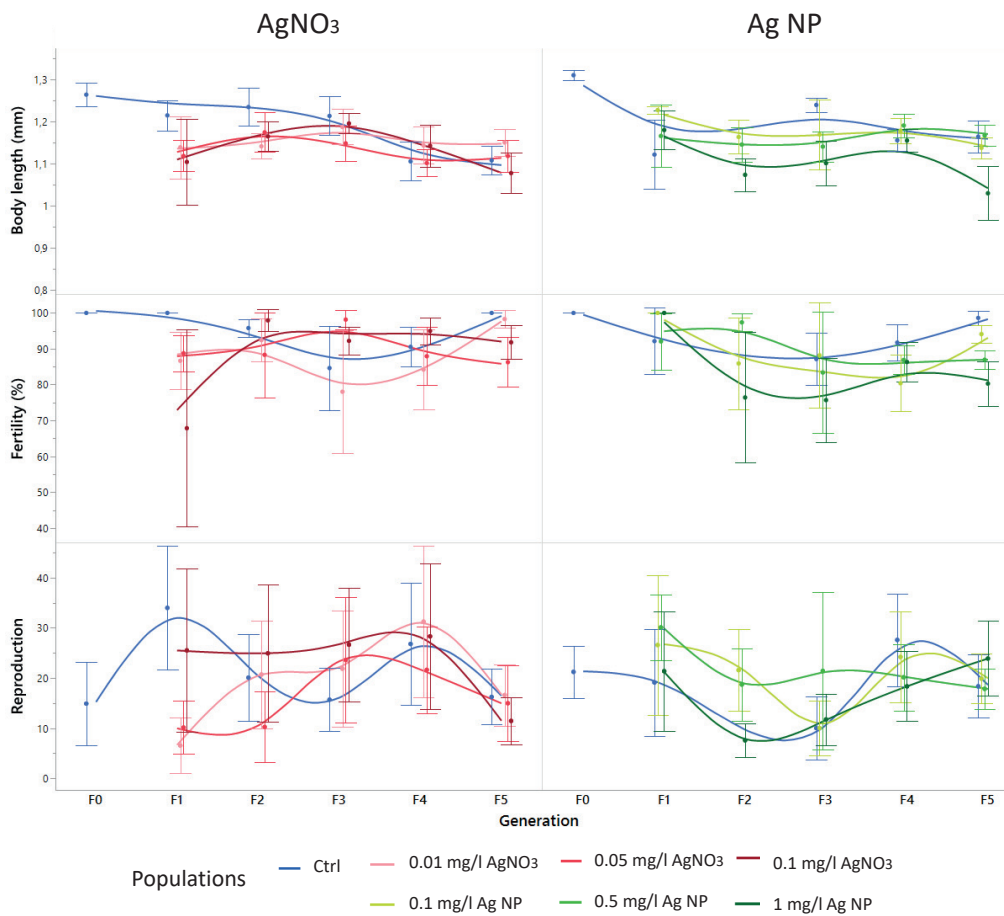


Figure S3: Total body length, fertility and reproduction of *C. elegans* offspring from chronic exposure populations (either 0.01, 0.05 or 0.1 mg L⁻¹ AgNO₃ or 0.1, 0.5 or 1 mg L⁻¹ Ag NPs) in toxicity test control conditions (no Ag added). Results present means ± SEM.

Table S2: Transfer times of nematode populations between each generation.

Population	Number of days																											
	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			72h							
AgNO ₃ 0.01 mg/l	72h					72h			72h			72h			72h			72h			72h							
AgNO ₃ 0.05 mg/l	72h					72h			72h			72h			72h			72h			72h							
AgNO ₃ 0.1 mg/l	72h					72h			72h			72h			72h			72h			72h							
Ag NP 0.1 mg/l	72h					72h			120h			120h			96h			96h			96h			96h				
Ag NP 0.5 mg/l	72h					72h			120h			120h			96h			96h			96h			96h				
Ag NP 1 mg/l	72h					72h			144h			144h			120h			120h			96h			96h				

AgNO₃

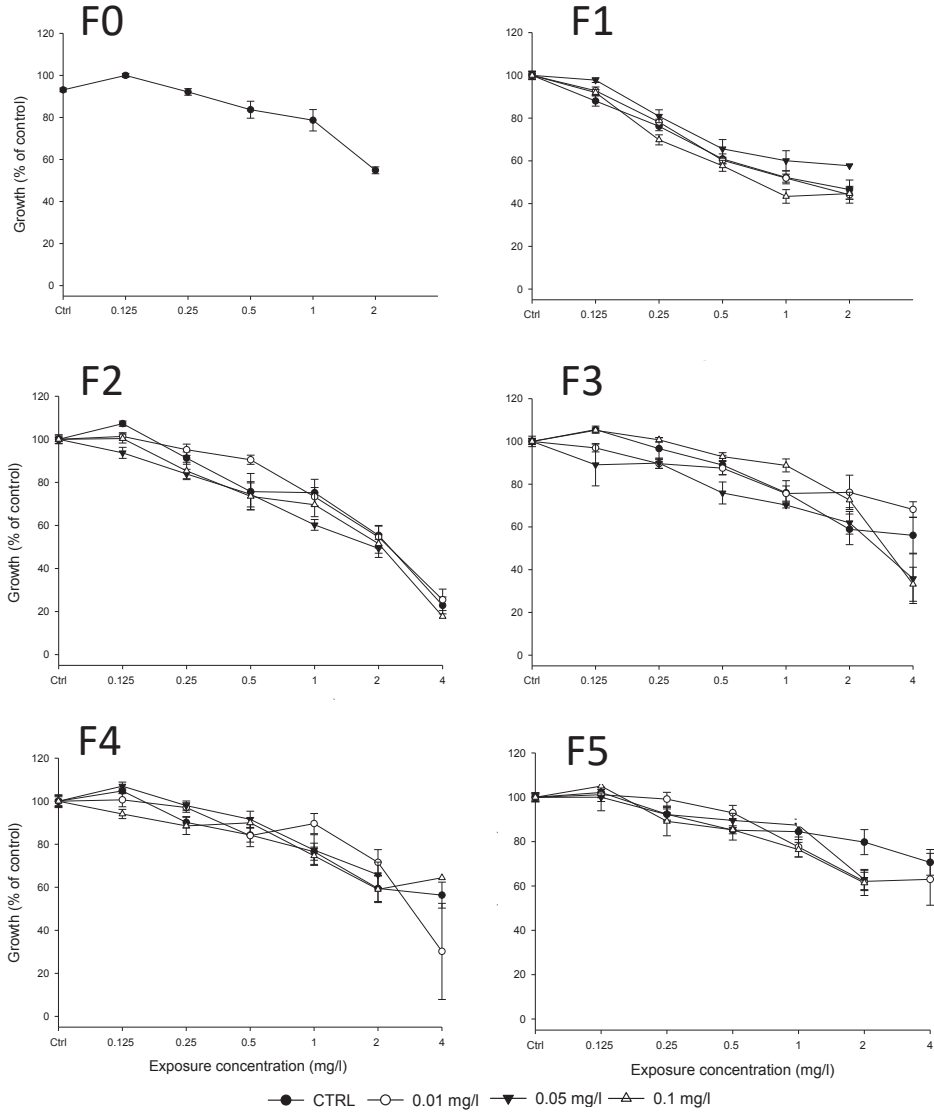


Figure S4: Effect on growth of *C. elegans* offspring from chronic exposure populations to AgNO₃ during six generations. Results represent mean of triplicates ± SD, normalized to controls, using a standardized 96 h toxicity test exposure to size concentrations of AgNO₃ in MHRW containing 1 ml *E. coli* re-suspended, with 10 nematodes/exposure well.

AgNO₃

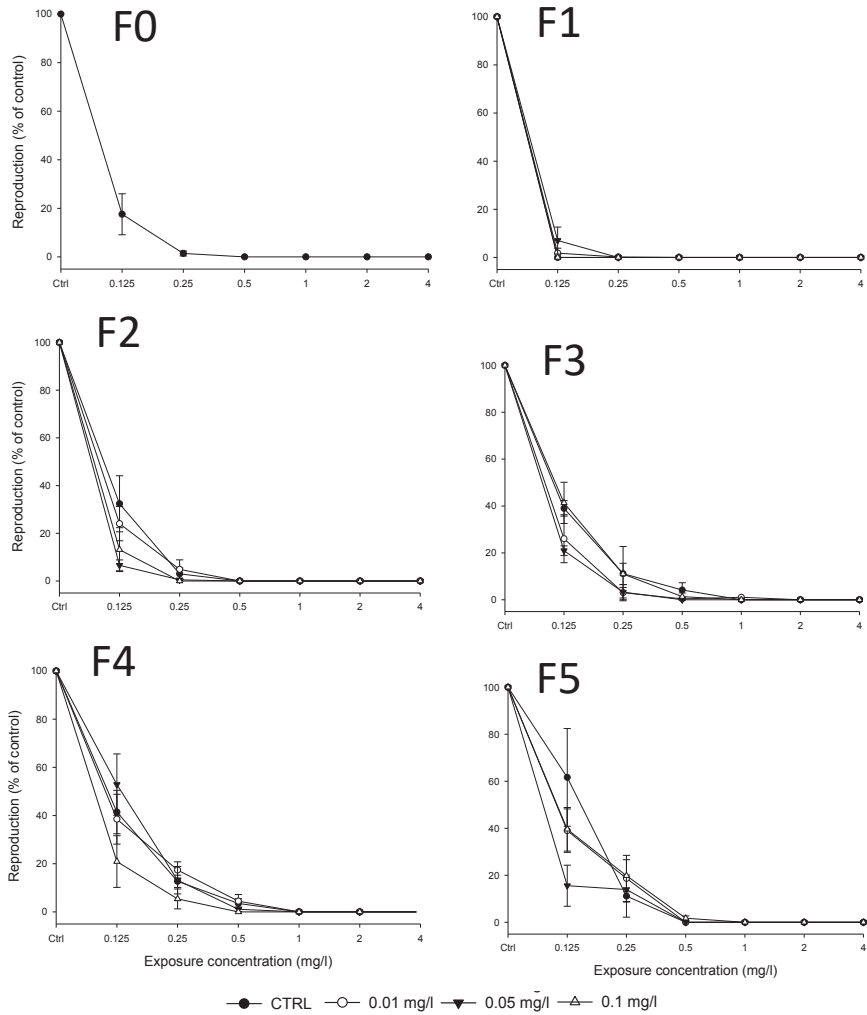


Figure S5: Effect on reproduction of *C. elegans* offspring from chronic exposure populations to AgNO₃ during six generations. Results represent mean of triplicates ± SD, normalized to controls, using a standardized 96 h toxicity test exposure to size concentrations of AgNO₃ in MHRW containing 1 ml *E. coli* re-suspended, with 10 nematodes/exposure well.

AgNO₃

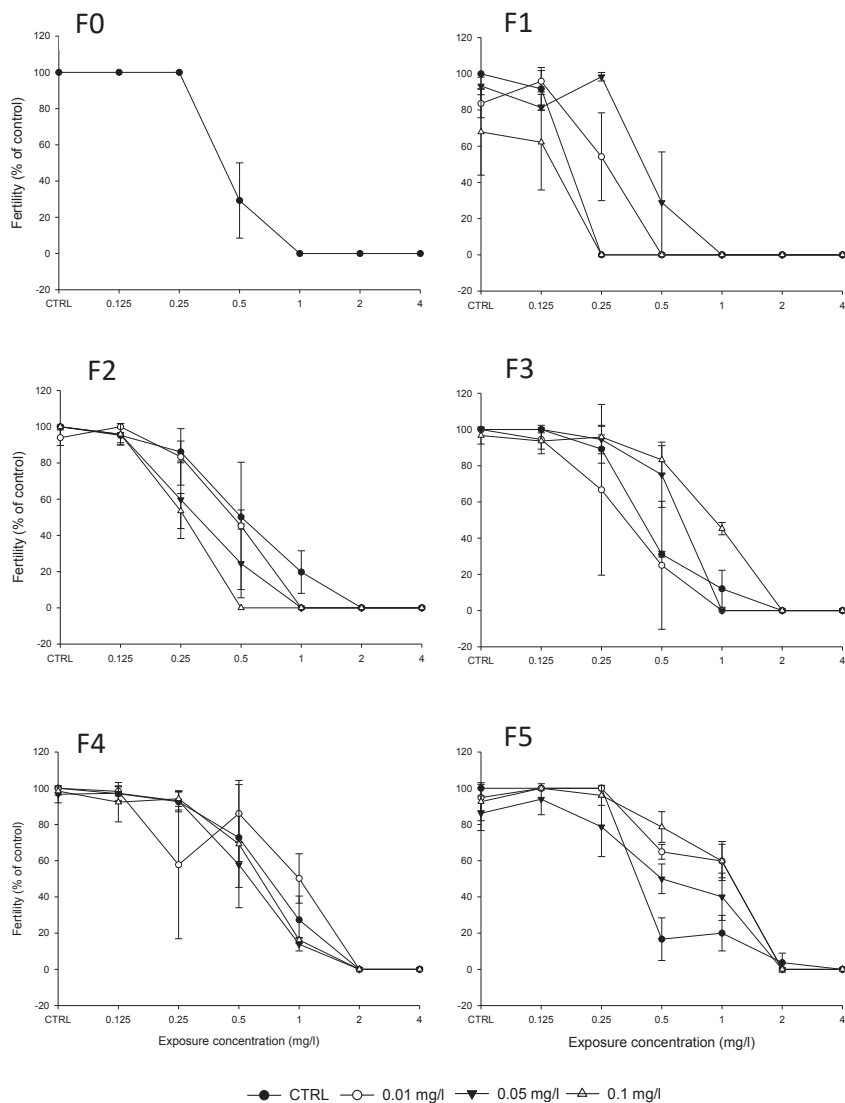


Figure S6: Effect on fertility of *C. elegans* offspring from chronic exposure populations to AgNO₃ during six generations. Results represent mean of triplicates ± SD, normalized to controls, using a standardized 96 h toxicity test exposure to size concentrations of AgNO₃ in MHRW containing 1 ml *E. coli* re-suspended, with 10 nematodes/exposure well.

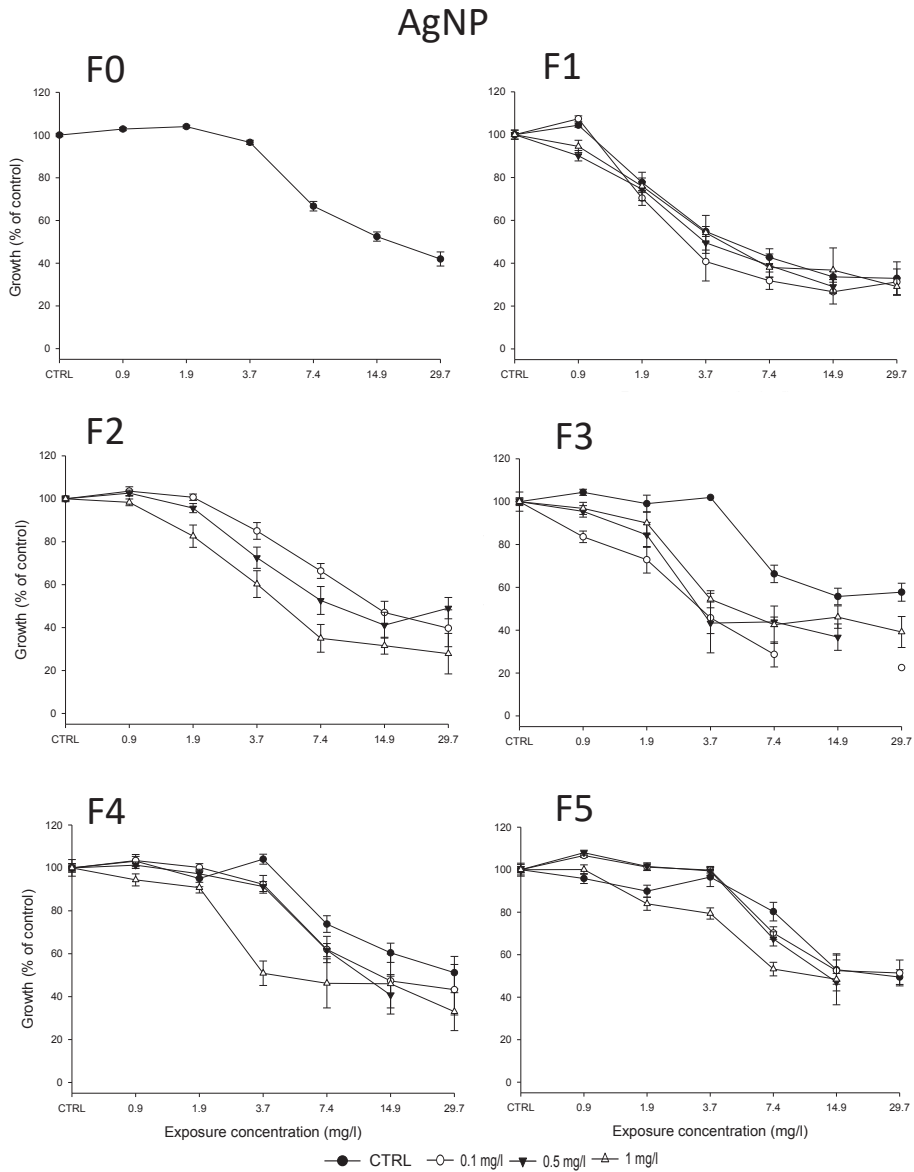


Figure S7: Effect on growth of *C. elegans* offspring from chronic exposure populations to Ag NP during six generations. Results represent mean of triplicates \pm SD, normalized to controls, using a standardized 96 h toxicity test exposure to size concentrations of Ag NP in MHRW containing 1 ml *E. coli* re-suspended, with 10 nematodes/exposure well.

AgNP

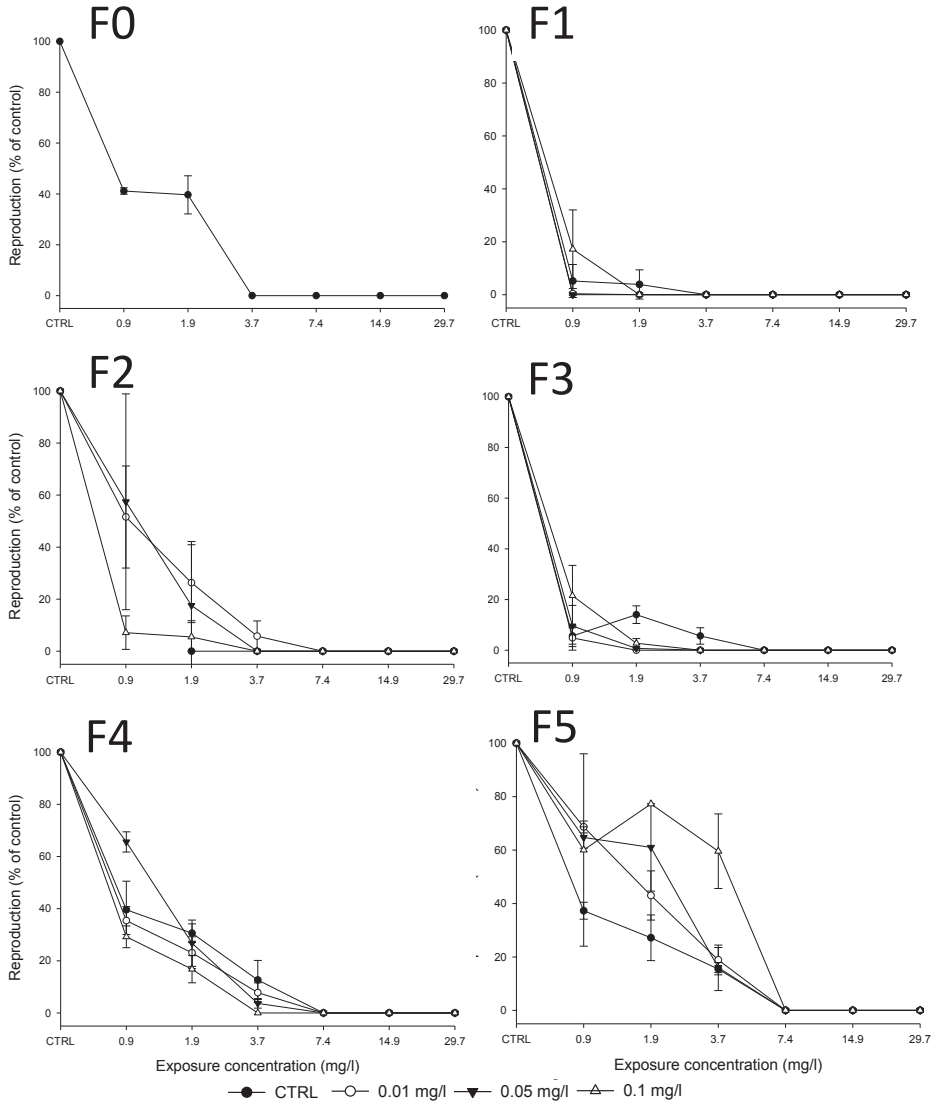


Figure S8: Effect on reproduction of *C. elegans* offspring from chronic exposure populations to Ag NP during six generations. Results represent mean of triplicates \pm SD, normalized to controls, using a standardized 96 h toxicity test exposure to size concentrations of Ag NP in MHRW containing 1 ml *E. coli* re-suspended, with 10 nematodes/exposure well.

Ag NP

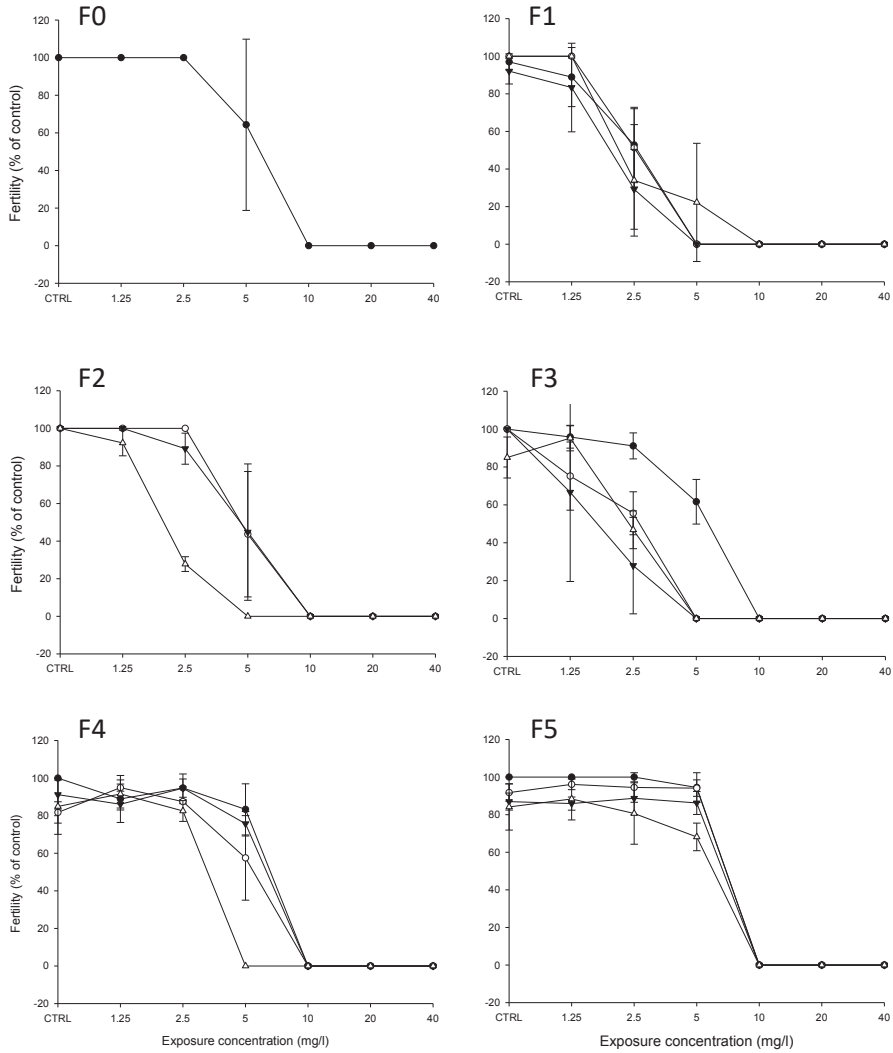


Figure S9: Effect on fertility of *C. elegans* offspring from chronic exposure populations to Ag NP during six generations. Results represent mean of triplicates \pm SD, normalized to controls, using a standardized 96 h toxicity test exposure to size concentrations of Ag NP in MHRW containing 1 ml *E. coli* re-suspended, with 10 nematodes/exposure well.

Paper IV

Impact of multigenerational exposure to AgNO₃ or NM300K silver nanoparticles on *Caenorhabditis elegans* antioxidant defense and oxidative stress

Lisa M. Rossbach, Deborah H. Oughton and Dag A. Brede

Norwegian University of Life Sciences, Faculty of Environmental Sciences and Natural Resource Management, P.O. BOX 5003 NMBU, No-1432 Ås, Norway

*Corresponding author: Lisa.rossbach@nmbu.no

Abstract

Adaptation of the nematode *Caenorhabditis elegans* towards NM300K Ag NPs has previously been demonstrated. In the current study, the consequence of multigenerational exposure to silver nanoparticles (Ag NPs NM300K) on the sensitivity to a range of secondary stressors (CeO₂ NP, Ce³⁺, Cu²⁺, Cd²⁺ and paraquat) was investigated using the nematode *C. elegans* as a model organism. This revealed an increased sensitivity towards Ce³⁺ ions with reduced reproduction from both AgNO₃ and NM300K. A Ag NP specific improved tolerance to paraquat with higher fecundity, indicated an involvement of reactive oxygen species (ROS) metabolism in the adaptive response to NM300K. The potential involvement of the antioxidant defenses related to adaptive responses was investigated across six generations of exposure using the *sod-1::GFP* reporter (GA508), and the Grx1-roGFP2 (GRX) biosensor strains. Results showed an increase in *sod-1* expression by the F3 generation, accompanied by a reduction of GSSG/GSH ratios, from both AgNO₃ and Ag NP exposures. The continuous exposure until the F6 generation resulted in a decreased *sod-1* expression from AgNO₃ and Ag NP compared to controls, associated with an increase in GSSG/GSH ratios. This demonstrated that the continuous exposure to Ag results in a temporary enhancement of the antioxidant defense in *C. elegans*. The results thus suggest that reproductive capacity is a more advantageous selective trait compared to oxidative stress response as an adaptive response towards NM300K Ag NPs.

Keywords: Reactive oxygen species, *sod-1*, glutathione, reporter strain, biosensor strain

1. Introduction

Silver nanoparticles (Ag NPs) have been given a great deal of attention in ecotoxicological studies due to their wide use, acting as antibacterial agents in consumer products (Fung and Bowen 1996, Park et al. 2009). Due to the well known toxic properties of ionic Ag, environmental releases and consequential exposure of organisms to Ag NPs is still of great concern (Cleveland et al. 2012, McGillicuddy et al. 2017, Klaine et al. 2008). The EU reference Ag NPs NM300K have been subject to a wide range of toxicological studies, and hence is amongst the best characterized nanomaterials available (Bicho et al. 2016, van der Ploeg et al. 2014, Völker et al. 2015, Köser et al. 2017, Kleiven et al. 2018). In the nematode *Caenorhabditis elegans*, Ag NPs have been shown to negatively impact physiological processes, such as reproduction, development and locomotion (Hunt et al. 2014, Ellegaard-Jensen, Jensen and Johansen 2012, Kleiven et al. 2018). Moreover, the production of free radicals by the particles, resulting in oxidative stress, NP have the potential to damage lipids, proteins and DNA (Hwang et al. 2008, He, Garg and Waite 2012a, He et al. 2012b, Choi et al. 2018, Foldbjerg et al. 2009). Furthermore, the ability of *C. elegans* to develop an adaptation towards NM300K Ag NP exposure over the course of six generations has been demonstrated in our previous work (Roszbach et al. 2019).

Considered one of the most important toxic mechanisms of Ag NPs, oxidative stress has been the focus of a range of Ag NPs toxicity studies focusing on various species (Ribeiro et al. 2015, Jiang et al. 2014, Kim et al. 2009), also including the nematode *C. elegans* (Lim et al. 2012b, Roh et al. 2009, Ahn et al. 2014). Oxidative stress is a substantial mechanistic contributor of Ag NP induced reproductive toxicity (Lim et al. 2012b). Roh et al. (2009) showed increased expression of the *sod-3* gene by *C. elegans*, in response to uncoated Ag NP (< 100 nm) exposure. A comparative toxicity study showed highest toxic response in *C. elegans* from uncoated Ag NPs (< 100 nm) and AgNO₃, associated with oxidative mitochondrial and DNA damages, by distinct toxic mechanisms for the NPs, compared to the AgNO₃ (Ahn et al. 2014).

The nematode *C. elegans* presents the perfect model for the study of the antioxidant defense systems, both enzymatic and non-enzymatic, including glutathione peroxidase (GPX), superoxide dismutase (SOD) and peroxiredoxin (Finkel and Holbrook 2000, Hernández-García et al. 2010, Miranda-Vizuete and Veal 2017). Understanding the role of reactive oxygen species (ROS) in toxicity testing is of vital importance, as ROS have

been associated with immune response, cell proliferation, differentiation, and apoptosis (Schieber and Chandel 2014). Furthermore, the change in redox status may consequently negatively affect development, and impact senescence and death (Veal, Day and Morgan 2007). *C. elegans* possess a highly specialized and complex ROS and redox control system, where their genome encodes for five SOD enzymes (Braeckman et al. 2016, McCord and Fridovich 1969, Doonan et al. 2008). Further, the catalase enzymes (CTL) and glutathione-S-transferases (GST) facilitate ROS scavenging (Braeckman et al. 2017). Moreover, the ratio of the reduced to oxidized glutathione (GSSG/GSH) has been shown to be a good indicator of the intercellular redox status (Braeckman et al. 2016, Braeckman et al. 2017, Storey 1996).

The adaptation towards oxidative stress has been well documented in single organisms or across generations (Demple and Halbrook 1983, Wojcik et al. 1996, Yanase et al. 1999). In a biochemical context, adaptation to changes in ROS levels, is focused primarily on preventative measures, rather than repair mechanisms (Storey 1996). Furthermore, it has been proposed that such exposure scenarios will evoke a physiological response in *C. elegans* which in turn allows for a cross-adaptation towards other stressors (Cypser and Johnson 2002b). Lastly, “memory effects”, when said stressor is removed, should play a key role in understanding mechanisms of NP toxicity (Schultz et al. 2016).

In our previous experiments, we demonstrated *in vivo* production of ROS accompanied by oxidative stress manifestation despite the induction of the *sod-1* gene as an antioxidant defense mechanisms in *C. elegans* in response to NM300K and AgNO₃ exposure (Rossbach et al. submitted). Further we provided evidence for the development of an adaptation, as shown by increased reproduction despite a significant reduction in size, towards the Ag NP NM300K by *C. elegans*, following six generations of continuous exposure (Rossbach et al. 2019). Therefore, this study aims to elucidate the underlying adaptive mechanistic processes in terms of ROS production and oxidative stress manifestation, through the measurement of the *sod-1* expression and changes in cellular redox status, in response to the multigenerational exposure to either the Ag NPs NM300K or AgNO₃.

2. Method

2.1. Nanoparticle preparation and characterization

Ag NP stock solutions were prepared, with adaptations, according to the Standard Operating Procedure, developed by EU NanoReg project (Jensen, 2016) using the OECD representative Ag Nanomaterials NM300K (Fraunhofer, IME, Munich, Germany). Briefly, 256 mgL⁻¹ aliquots were weighed into individual containers prior to the start of the exposure in anoxic conditions inside a nitrogen tent. Each day of exposure, individual stocks were prepared freshly in ddH₂O (15 MΩ·cm) and sonicated for 13 minutes at 15 % amplitude using a probe sonicator (Branson S-450 D sonicator, disruptor horn 13 mm). All stocks, AgNO₃ and Ag NPs, applied in both the cultures and toxicity tests were diluted from the initial stock and applied immediately after preparation.

Size distribution of all stocks was measured for hydrodynamic diameter using Dynamic light scattering (DLS, Malvern PN3702 Zetasizer Nanoseries). Transmission electron microscopy (TEM, Morgagni 268) was conducted on 100 mg L⁻¹ stocks in ddH₂O (15 MΩ·cm), for particle size, shape and aggregation state of the particles. Particle dissolution and size fractionation of either form of Ag (Ag NPs and AgNO₃) in the exposure media, was carried out by ultrafiltration using <3 kDa Millipore Centrifugal filters (Amicon, Millipore), at T-0 and 72 h of the toxicity test exposure. Samples of the exposure media were first centrifuged at 2000 g for 5 mins to remove *E. coli* and larger aggregates. The resulting supernatant was subjected to ultrafiltration to measure the <3 kDa fraction. The <3 kDa filters were pre-conditioned with supernatant prior to ultrafiltration.

All samples were analyzed by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-OES) or Mass Spectrometry (ICP MS Agilent 8800, Mississauga, ON, Canada), using oxygen as a collision gas, tuned using manufacturer tuning solution (#5188-6564, Agilent Technologies, Mississauga, ON, Canada), measuring two Ag isotopes (107 and 109), at 0.0001 ppm detection limit and limit of quantification at 0.0004 µg L⁻¹.

2.2. Multigenerational exposure

All three *C. elegans* strains, N2 Bristol strain *Caenorhabditis elegans* (*Caenorhabditis Genetic Centre*, Minneapolis, USA), SOD-1 (GA508 wuls54[pPD95.77 sod::1GFP, rol-6(su1006)]) (Institute of Healthy Ageing Genetics, University College London) and the Grx1-roGFP2 (GRX) strain (Back et al. 2012), were continuously exposed for six

generations on NMG agar plates seeded with 10 x concentrated *Escherichia coli*, 250 nematodes per exposure plate, in triplicate. N2 nematodes were either unexposed or exposed to 0.01, 0.05 or 0.1 mg L⁻¹ AgNO₃, or 0.1, 0.5 or 1 mg L⁻¹ Ag NPs. Concentrations had to be adjusted for the SOD-1 and GRX strains, due to differences in sensitivity towards Ag, compared to the N2 Bristol strain. Both the strains were either unexposed or exposed to either 0.1 or 0.5 mg L⁻¹ AgNO₃ and Ag NPs, respectively. All concentrations were based on previously conducted pilot experiments.

The Ag was applied onto the exposure plates on the day of culture transfers. Plates were kept at 20 °C in the dark for 96 hours before collection of pregnant nematodes. Pregnant nematodes were treated with alkaline hypochlorite for egg extraction. Eggs were washed and immediately transferred onto new NGM agar plates. At F1, F3 and F6, a proportion of the eggs were hatched over night for subsequent use of synchronized L1 in toxicity tests.

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.

2.3. Toxicity test exposure and sampling

All standard toxicity tests were conducted in 24 well cell culture plates, each well containing 25 nematodes in 1 ml of *E. coli* re-suspended in MHRW water (US Environmental Protection Agency, 2002). Plates were kept at 20 °C in the dark on a shaking table, before sampling.

Following the six generational chronic exposure of the N2 *C. elegans*, as presented in Rossbach *et al.* (2019), changes in sensitivity towards other stressors were assessed. In the F6 generation, nematodes previously exposed to either AgNO₃ or Ag NPs, were exposed to either copper (0, 0.06, 0.13, 0.25, 0.5, 1 or 2 mg L⁻¹), cadmium (0, 0.09, 0.19, 0.38, 0.75, 1.5 or 3 mg L⁻¹) or cerium (0, 3.13, 6.25, 12.5, 25, 50, 100 mg L⁻¹), as well as cerium nanoparticles (0, 0.156, 3.13, 6.25, 12.5, 25 or 50 mg L⁻¹). Furthermore, to assess the involvement of ROS and oxidative stress production in the adaptive response observed in our previous study (Rossbach *et al.* 2019), nematodes were exposed to 0, 0.08, 0.16, 0.31, 0.63, 1.25 or 2.5 mM of the known ROS inducer paraquat for 96 h before sampling. To terminate the test, wells were stained using 0.5 ml of Rose Bengal, and

placed at 80 °C for 10 minutes, before the assessment of growth, fertility and reproduction (ISO 2010).

For both the SOD-1 and GRX strain, toxicity tests were set up at F0, F1, F3 and F6. Nematodes, independent of multigenerational culture exposure, were exposed to either, 0.1, 0.5 and 1 mg L⁻¹ AgNO₃, 1, 5 and 10 mg L⁻¹ of Ag NPs or an unexposed control, for 72 h, before sampling.

For sampling, nematodes were immobilized with NaAzide and analyzed using a fluorescent light microscope (LEICA DM6 B), equipped with a 405 nm excitation and 535 nm emission filter. For assessment of oxidized to reduced ratios of the GRX biosensor strain, a second image (Ex 490 and Em535 nm) was taken. Ratios were calculated as described in Back et al (2012). Tissue specific analysis of changes in the redox status was conducted as described by Back et al. (2012). The SOD-1 expression, the average intensity was normalized to nematode size, to account for possible variance in signal strength related to developmental stages. All images were quantified by pixel based average intensity measurements using the LAS X Leica application suit X imaging software (LEICA DM6 B).

2.4. Statistical analysis

All statistical analysis was carried out using MiniTab® 18 (Minitab Inc. 2010). For group comparison One-way ANOVA (Tukey's range test) was applied, when error terms were normally distributed. Where appropriate, to stabilize the variance, a Box-Cox transformation of the data was conducted. For non parametric analysis of the data, a Kruskal-Wallis one-way analysis of variance was conducted. General Linear Model analysis was done in JMP Pro v14 (SAAS institute Cary, NC, USA). Values were considered significantly different at p-values lower than 0.05. To account for inherent variations between generations, data was normalized to mean toxicity test control values (Yu et al. 2012, Moon et al. 2017).

3. Results

3.1. Nanoparticle Characterization

Nanoparticle toxicity has been shown to be highly dependent on NP behavior (Jiang, Oberdörster and Biswas 2009, Kleiven et al. 2018). To ensure consistency between studies, particle characteristics and behavior during exposures were characterized in

ddH₂O (15 MΩ·cm) as well as monitor changes over time in the test media. Transmission electron microscopy (TEM) analysis of the NM300K Ag NPs in ddH₂O (15 MΩ·cm) showed particles to be spherical with a median size of 23.9 ± 21.8 nm (Figure S1). Dynamic light scattering in ddH₂O (15 MΩ·cm) showed an average hydrodynamic diameter of 79 ± 4.42 nm (mean ± SD) and a Pdi of 0.3, for the initial 256 mg L⁻¹ Ag NP stock (Table S1), followed by an increase in hydrodynamic diameter with decreasing Ag NP concentrations in diluted toxicity test working solutions (Table S1). Recoveries of Ag concentration in the toxicity test exposure media showed a slight decrease over time (Supplementary materials, section 1.3). Size fractionation data is consistent with our previous findings presented in Kleiven et al. (2018) (Supplementary materials, section 1.4, Figure S2).

3.2. Multigenerational exposure of N2 nematodes to 1 mg L⁻¹ Ag NPs decreases susceptibility towards paraquat

Analysis of Ag NP exposed nematodes revealed no external damages, cuts or lesion of the cuticle of the nematodes (Figure S4). The development of a cross-tolerance stemming from the exposure of a primary stressor, followed by the exposure towards a secondary stressor has been shown (Rossbach 2019). To test the cross tolerance development in the current study, N2 nematodes were exposed to Ce NPs, Cd²⁺, Cu²⁺ and Ce³⁺, as well as the herbicide and well known ROS inducer, paraquat, following the six generational exposure to either AgNO₃ or Ag NPs. The six generational exposure towards AgNO₃ or Ag NPs did not alter the nematodes response towards Ce NPs, Cd²⁺ or Cu²⁺ (data not shown), compared to control population. However, increased sensitivity towards Ce³⁺ (supplementary materials, section 2.1, Figure S3) from either Ag exposure, compared to the control population, was found, further supporting a change in toxic response resulting from the long term chronic exposure towards either form of Ag. *C. elegans* have been shown to develop a cross resistance towards two different oxidative stress inducers (Cypser and Johnson 2002a). A similar phenomenon was also observed in the results from the current study (Figure 3). The six generational exposure to Ag NPs of the N2 strain, led to changes in response towards paraquat, compared to control population nematodes (Figure 1). The pre-exposure towards AgNO₃ did not result in such effects. Despite lower fertility at 0.078 mM Paraquat exposure, from the Ag NP populations, compared to the control and AgNO₃ population nematodes, overall, a statistically significant ($p < 0.001$) increase in fertility is measured for the Ag NP population

nematodes. The Ag NP population nematodes were the only population showing fertility at 0.156 mM paraquat concentration. Moreover, the Ag NP population was the only population able to produce offspring at 0.156 mM of Paraquat, albeit, the observed effect was below the statistical significance level ($p = 0.07$).

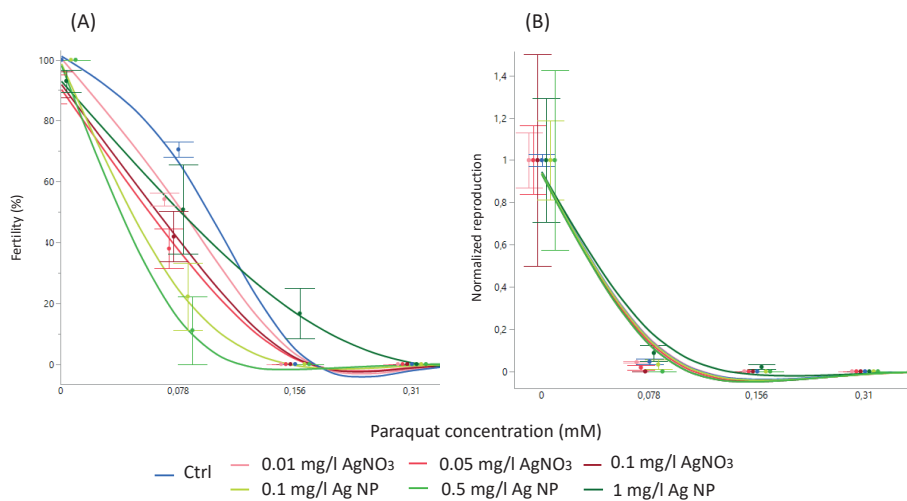


Figure 1. Paraquat tolerance of *C. elegans* after six generations continuous exposure to either of three concentrations of AgNO₃ or Ag NPs. (A) Fertility (%; mean \pm SEM). (B) Reproduction (normalized to individual toxicity test controls, mean \pm SEM). Nematodes were exposed in a standard 96 h toxicity test to six concentrations of paraquat. Note: graphs only show four concentrations reproduction was zero at higher concentrations.

3.3. Antioxidant defenses and oxidative stress manifestation

The *sod-1::GFP* reporter strain and biosensor Grx1-roGFP2 were continuously exposed to either AgNO₃ (0.1 mg L⁻¹) or Ag NPs (0.5 mg L⁻¹) for six generations to investigate changes in antioxidant defenses and oxidative stress tolerance. The increased *sod-1* expression is directly connected to production of H₂O₂, which is sequestered by either catalase or gpx enzymes, and is as such directly related to glutathione homeostasis. Thus, it was expected that changes in *sod-1* expression would be related to changes in the cellular redox status measured as GSSG/GSH ratio. In line with results from a previous study (Roszbach et al. submitted), both the *sod-1::GFP* and GRX strains showed an Ag concentration dependent increase in *sod-1* expression and GSSG/GSH ratios in all generations. Both Ag populations

show comparable *sod-1* expression patterns to the control population in the F1 generation. A significantly increased expression was measured in the F3 generation, followed by a significant decrease in the F6 generations from both Ag populations compared to controls.

3.4. Alterations to *sod-1* expression in response to the continuous chronic exposure towards AgNO₃ or Ag NP

The GA508 reporter strain that measures *sod-1* gene expression can be used to assess changes in ROS formation in the form of superoxide (Doonan et al. 2008). Overall, the *sod-1* induction in the current exposure is consistent with previous observations, with a clear dose response for all populations (Roszbach et al. submitted). The induction patterns were quite uniform and did not indicate any tissue or cell specific responses.

Assessment of the *sod-1* expression of nematodes in toxicity test control conditions confirmed consistency of the exposures across generations (Figure 2). In toxicity test control conditions nematodes taken from control populations (i.e. nematodes that had never been previously exposed to Ag), showed no change in *sod-1* expression across generations (Figure 2). In comparison, both Ag NP and AgNO₃ populations show a statistically significant ($p < 0.005$) decrease in *sod-1* expression compared to previous generations, and the control population expression levels, in the F6 generation (Figure 2). This suggests a substantial impact of the Ag on the SOD-1 antioxidant defense system.

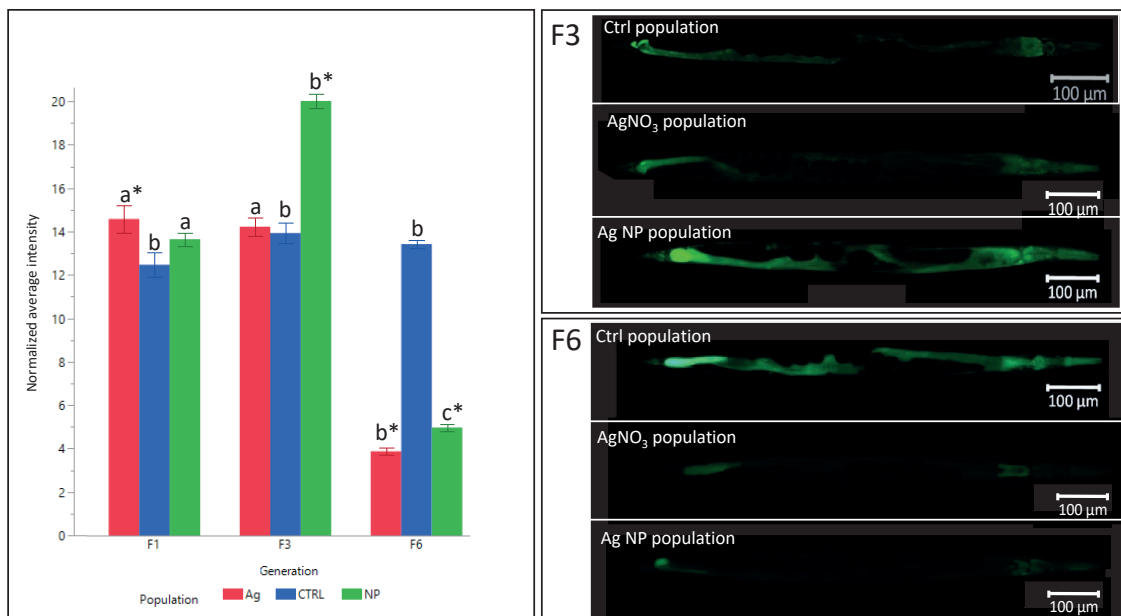


Figure 2. *In vivo* measurement of *sod-1* expression pattern in the *sod-1::gfp* reporter strain GA508 wuls54[pPD95.77 *sod-1::GFP*, *rol-6(su1006)*], in toxicity test control conditions (no added Ag), following the multigenerational exposure towards either no Ag, 0.1 mg L⁻¹ AgNO₃ or 0.5 mg L⁻¹ Ag NPs. Across the generations for each population, different letters (a, b, c) indicate statistically significant differences within that population. Asterisks indicates significant differences of either Ag population compared to control population within a generations.

The toxicity test results measured *sod-1* expression after exposure to both Ag NP and AgNO₃ in the chronically exposed populations. All the chronically exposed populations showed a dose dependent increase in expression in all generations (Figure 3). An overall increase in expression in the F3 generation, compared to F1 and F6 expression levels, was measured for all populations. For both toxicity test exposures, AgNO₃ and Ag NPs, the Ag NP population showed the overall highest *sod-1* expression levels, compared to the other two populations. The AgNO₃ population expression levels did not show a linear dose response dose response, observed in all other exposures. Moreover, AgNO₃ population expression levels were comparable to those of the control population in the AgNO₃ toxicity test, while comparatively increased levels were seen in the Ag NPs toxicity test.

In the F3 generation, at 5 mg L⁻¹ Ag NPs both Ag populations show increased expression compared to the control population, only the Ag NP expression levels were significantly higher than controls ($p = 0.036$). In the F6 generation on the other hand, expression levels of the control population are comparable to levels measured in the F1 generation (Figure 3). However, both Ag populations show a significantly lower expression, compared to the control population in the F6 generation, in all toxicity test exposures (Figure 3). This may indicate an adjustment of antioxidant defenses by the nematodes in response to the six generational exposure. Furthermore, expression levels are comparable between the two Ag exposed populations. It should be noted, however, that Ag exposure lead to a slight concentration dependent decrease in size of the nematodes, which suggests an impact on the organisms development which, in turn could interfere with antioxidant defenses.

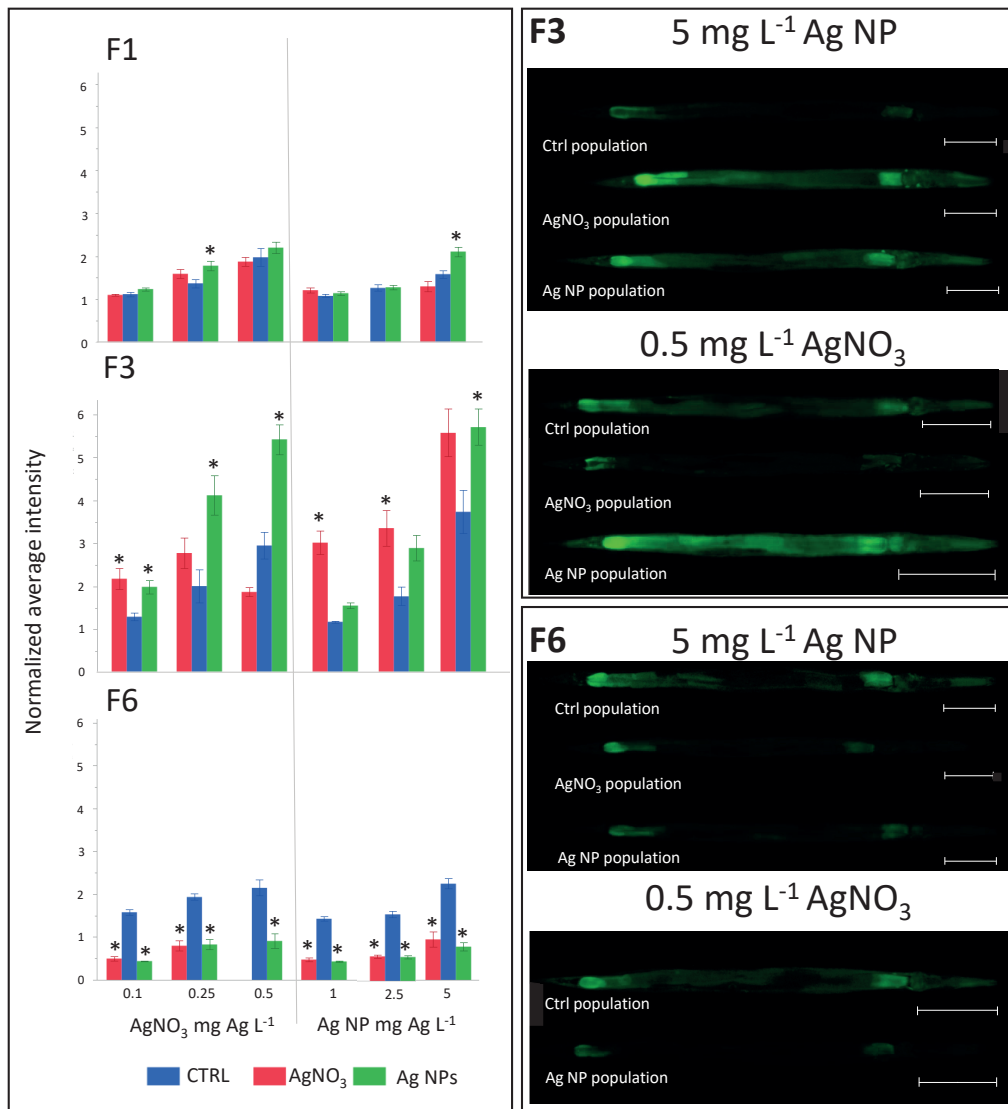


Figure 3. *In vivo* measurement of *sod-1* expression pattern, quantitative measurements on the left, and corresponding representative images on the right, in the *sod-1::gfp* reporter strain GA508 wuls54[pPD95.77 *sod::1GFP*, *rol-6(su1006)*] in response to the exposure of either three concentrations of AgNO₃ or Ag NP, following the multigenerational exposure to either controls (no added Ag), 0.1 mg L⁻¹ AgNO₃ or 0.5 mg L⁻¹ Ag NPs. Values are normalized to toxicity test controls for direct comparison. Asterisks indicate significant differences to control population within individual toxicity test exposure concentrations. All scale bars represent 100 μm.

3.5. Changes in cellular redox status following the multigenerational exposure towards Ag

The efficiency of the glutathione cycle combined with high intracellular GSH concentrations (1 – 11 Mm) are essential to maintain cellular redox homeostasis. Measurements of the GSSG/GSH ratios may thus act as a suitable proxy for the total cellular redox state (Back et al. 2012). Therefore, for the *in vivo* measurement of the redox biology of the nematodes, the biosensor Grx1-roGFP2 (Back et al. 2012) was used in the current experiment.

Across generations, in toxicity test control conditions, the control population showed no statistically significant changes in redox state levels ($p = 0.9$), indicating consistency of the multigenerational set up (Figure 4). Similarly, the AgNO_3 population showed a tendency towards decreased GSSG/GSH ratios, compared to controls. However, in the F6 generations oxidation levels of the AgNO_3 population were statistically significant lower ($p = 0.039$) than control populations. The Ag NP population nematodes show an increase in GSSG/GSH ratios in the F3 and F6 generation compared to the F1 generation levels. However, levels are not statistically significantly different to control population levels ($p > 0.05$). Due to low number of replicates from the control population in the F3 generation, data for this group were not included in figure 4.

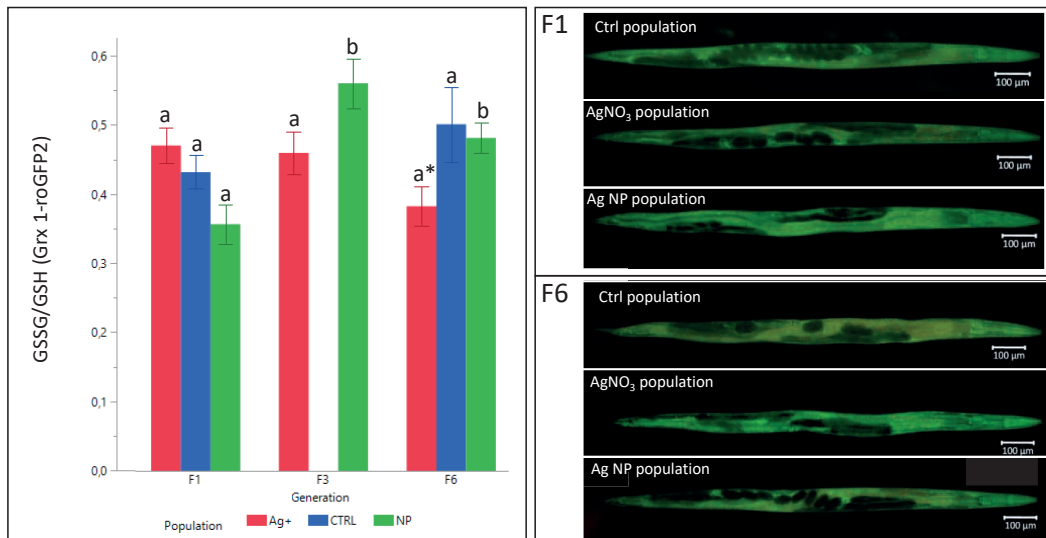


Figure 4. Oxidized to reduced GSSG/GSH ratios, quantitative measurements on the left, and corresponding representative images on the right, of the biosensor Grx1-roGFP2 in toxicity test control conditions, following the multigenerational exposure to either controls (no added Ag), 0.1 mg L⁻¹ AgNO₃ or 0.5 mg L⁻¹ Ag NPs. Across the generations for one population, different letters (a, b, c) indicate statistically significant differences within that population. Asterisks indicates significant differences of either Ag population compared to control population within a generations.

Assessing the toxicity test responses (Figure 5), GSSG/GSH levels overall, although a dose response was measured, it is not as evident as in the *sod-1* exposure. Furthermore, the control population showed a similar increase in the F3 generation compared to the F1 and F6 generations, as observed in the *sod-1* exposure. Both Ag populations followed a similar trend in terms of GSSG/GSH levels in all generations. In the F3 generation, both Ag populations showed reduced GSSG/GSH ratios compared to control population, in both toxicity tests. In the F6 generation on the other hand, the AgNO₃ population showed consistently higher GSSG/GSH ratios compared to the control populations. However, GSSG/GSH ratios measured from the Ag NP population nematodes were lower compared to the controls when exposed to AgNO₃ in the toxicity test, and higher when exposed to Ag NPs.

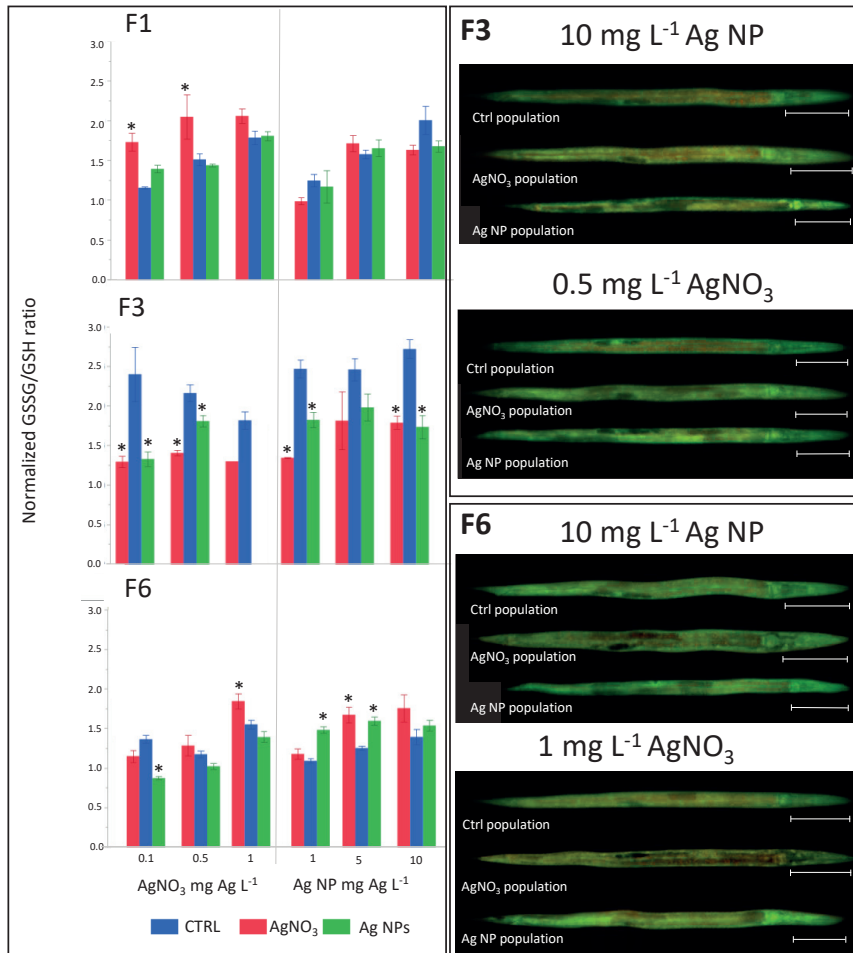


Figure 5. Oxidized to reduced ratios, quantitative measurements on the left, and corresponding representative images on the right, of the biosensor Grx1-roGFP2 in response to the exposure of either three concentrations of AgNO₃ or Ag NP, following the multigenerational exposure to either controls (no added Ag), 0.1 mg L⁻¹ AgNO₃ or 0.5 mg L⁻¹ Ag NPs. Values are normalized to individual toxicity test controls for direct comparison. Asterisks indicate significant differences to control population within one toxicity test exposure concentration. All scale bars represent 100 μm. Note, due to low replicates from the control population in the F3 generation, values were normalized to control population average from the F1 and F6 generation.

4. Discussion

Silver NPs are the one of the most commonly studied nanomaterial to date. Multiple differences between ionic Ag and Ag NPs have been found in terms of biodistribution and toxic mode of action (Kleiven et al. 2018, Navarro et al. 2008b, Choi et al. 2018, Hunt et al. 2013, Rossbach et al. submitted). Some of the observed differences have been attributed to ROS formation and oxidative stress development (Cortese-Krott et al. 2009, Roh, Eom and Choi 2012, Lim et al. 2012b, McShan, Ray and Yu 2014). Understanding underlying toxic mechanisms of Ag nanomaterials compared to ionic Ag, is therefore of vital importance for the further management and safe use of nanomaterials. In a recent study, we showed that *C. elegans* exposed continuously for six generations to NM300K Ag NPs resulted in increased reproductive capacity in presence of Ag NP. However, the adaptive tolerance was associated with higher susceptibility to AgNO₃, as well as reduced growth (Rossbach et al. 2019). In the current study, we therefore investigated how the multigenerational Ag exposure affected tolerance to a range of other stressors including an engineered nanomaterial (Ce NP) and corresponding ion (Ce³⁺), divalent cationic metal (Cd²⁺ and Cu²⁺), and oxidative stress inducer (paraquat). Further, changes in antioxidant defense mechanism by the nematode *C. elegans* in response to the continuous chronic exposure to either AgNO₃ or NM300K Ag NPs was investigated. In line with finding from other studies, results from the current study overall show dose dependent *sod-1* induction and oxidative stress development from all populations exposed to either AgNO₃ or Ag NPs in the toxicity test in all generations (Roh et al. 2009, Lim et al. 2012a, Limbach et al. 2007).

4.1. Transformation of the Ag in the exposure media over time

To allow for a comprehensive assessment of toxic effects of nanomaterials, the characterization prior and post application in the toxicity test is of vital importance (Navarro et al. 2008a). Possible dissolution of particles, and interactions with the *E. coli* are of particular importance in the current study, in order to ascertain the exposure and uptake of the Ag by *C. elegans* (Navarro et al. 2008a, Köser et al. 2017, Kleiven et al. 2018). It has been suggested that, if toxicity could primarily be attributed to the dissolution, meaning ionic releases, of the particles, it would make the study of nanoparticles a less pressing matter (Ratte 1999). However, a range of studies, with contradicting results on the Ag NP NM300K dissolution state in the exposure media, highlights the importance of

monitoring of the behavior in the exposure media of these NPs (Wasmuth et al. 2016, Köser et al. 2017, Lodeiro et al. 2017). In the exposure media, the fractionation experiment shows a large transformation from suspended to aggregated fraction over time, with only low free low molecular mass (LMM) (< 3kDa) Ag fractions measured at T-0, for both forms of Ag. This may be the result of the higher affinity of the ionic Ag to bind to the *E. coli* in the exposure media. It is hypothesized that the aggregated fraction in the current exposure consists of both, transformation of the Ag to larger particles, as well as interactions of the positively charged Ag with the negatively charged surface of *E. coli*. While larger particles are assumed to reduce the direct exposure and uptake of the Ag by the nematodes, Ag fractions associated with the *E. coli* are thought to be facilitating uptake and exposure (Kleiven et al. 2018, Li, Nikaido and Williams 1997, Dakal et al. 2016). Köser et al. (2017) revealed, in an analysis of the NM300K stock dispersant, the presence of a low (~8 %) ionic Ag fraction, possibly explaining the T-0 <3 kDa Ag fraction in the Ag NP exposure, in the current study. Moreover, the fractionation of the Ag NP exposure revealed a comparatively lower transformation of the Ag NP to the aggregated fractions, compared to the AgNO₃ exposure. Overall, results suggest that any dissolved ions present in the exposure media, from either form of Ag, are rapidly removed by either sorption or precipitation. Nevertheless, further dissolution within the lumen of the nematodes is possible in either exposure.

4.2. No cuticle damages from Ag NP exposure

To exclude changes in response to the exposure, by the two strains, resulting from possible external damages to the cuticle of the nematodes, scanning electron microscope images were taken of the two highest nanoparticle nematode populations in order to check for possible phenotypic changes or damages to the cuticle, as described by Kim, Nam and An (2012) for nematodes exposed to 10 and 100 mg L⁻¹. SEM analysis did not show any exterior damages resulting from the exposure conditions (Figure S4) and therefore, all effects measured, originated from the internalization of the Ag.

4.3. Nano-specific increased paraquat tolerance following the six generational Ag NPs exposure

The six generational exposure towards the NM300K Ag NPs, resulted in an increased susceptibility towards the exposure of AgNO₃ (Rossbach et al. 2019). Conversely, the

multigenerational exposure towards AgNO₃ resulted in a decrease in sensitivity towards Ag NPs (Rossbach et al. 2019). Therefore, the current study investigated whether such changes in toxic response, or the development of a cross resistance, as described by Cypser and Johnson (2002b), is stressor specific. We thus measured the response of nematodes towards three metal ions, Cu²⁺, Cd²⁺, or Ce³⁺, an oxide nanoparticle, CeO₂ NPs, and the herbicide and ROS inducer, paraquat, following the six generational exposure towards either AgNO₃ or Ag NPs. Results showed that changes in toxic response stemming from the continuous Ag exposure were stressor specific. Neither Cu, Cd or CeO₂ NP exposure resulted in any changes in sensitivity of the Ag exposed nematode populations, compared to control population nematodes. However, both Ag (AgNO₃ and Ag NP) exposures resulted in an increase in sensitivity towards Ce ions (Figure S3). Alternatively, the six generational exposure towards Ag NPs resulted in an increase in reproduction compared to control and AgNO₃ population nematodes in the paraquat exposure.

Studies show that certain organisms are more adapted at dealing with oxidative stress and its harmful consequences than others (Koch and Hill 2017, Monaghan, Metcalfe and Torres 2009). Cypser and Johnson (2002b) showed the development of a cross-resistance towards two different oxidative stress inducing agents in their exposure study on *C. elegans*. Additionally, a study by Yanase et al. (1999) showed a resistance development towards oxidative stress inducers, by means of pre-treatment to 90 % oxygen leading to decrease sensitivity towards x-ray irradiation. In line with this, results from the current study show that the continuous exposure of *C. elegans* to Ag NPs resulted in an increased ability to withstand the exposure of the known ROS inducer paraquat (Figure 3).

The exposure to the herbicide paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) is amongst the most commonly used methods of inducing oxidative stress through increased ROS production in organisms (Koch and Hill 2017, Suntres 2002). Once ingested, paraquat is known to rapidly distribute amongst tissues across the whole body, and interfere with redox cycling through the generation of superoxide anions, leading to consequential increased levels of hydrogen peroxide and hydroxyl radicals (Suntres 2002, Gram 1997). Further paraquat will oxidize cellular NADPH, and lead to lipid peroxidation (Suntres 2002, Gram 1997). The increase in superoxide anions would indicate that systems with heightened superoxide dismutase levels would be more

resistant to paraquat or other superoxide anion producing agents (Fridovich and Hassan 1979).

Both AgNO₃ and Ag NPs are known to produce superoxide anions, as well as peroxide radicals (O₂^{·-}), hydroxyl radicals (·OH), hydrogen peroxide and hydrogen peroxide (H₂O₂) and singlet oxygen (O₂), on the surface of the particles (Hwang et al. 2008, He et al. 2012a, He et al. 2012b, Choi et al. 2018). The continuous chronic exposure towards Ag NPs, however, did not result in a significant increase in *sod-1* gene expression, compared to the AgNO₃ toxicity test exposure in the F6 generation. Rather a significant decrease in *sod-1* expression was measured for either Ag population compared to the control populations, in the F6 generation. Nevertheless, a temporal increase in *sod-1* expression was measured in the F3 generation. This was observable in an earlier generation, compared to the adaptive effects observed on reproduction in our previous work (Rossbach et al. 2019).

Glutathione has also been shown to be highly efficient at reducing the toxicity of paraquat exposure (Nakagawa et al. 1995, Djukic et al. 2012). Acting as an endogenous antioxidant scavenger, GSH protects cells from oxidative stress, making GSH of critical importance to cell survival (Habib, Shi and Lieberman 2007, Pena-Llopis, Ferrando and Pena 2003, Piao et al. 2011a, Sies 1999). Piao et al. (2011b) showed in their Ag NP (5 – 10 nm) study on human liver cells, that exposure to the particles produced oxidative stress through the inhibition of GSH synthesizing enzymes (γ -glutamate cysteine ligase and GSH synthetase). This, however, was not observable in the current study. In toxicity test control conditions, the AgNO₃ population exposed nematodes showed a significant decrease in GSSG/GSH ratios in the F6 generation, compared to the control population, indicating an improved antioxidant defense system.

4.4. Changes in antioxidant defenses following the six generational exposure towards Ag NPs

Through biochemical and metabolic adaptations, organisms are known to be able to deal with periods of low oxygen (Storey 1996). Similarly, organisms are adapted to deal with the overproduction of ROS by maintaining high antioxidant enzyme activities, as well as large glutathione pools (Storey 1996). A maternal *C. elegans* study on Au NPs (10 nm) showed that exposure of the parent generation, resulted in decreased reproduction in

unexposed offspring in the F2 generation, while no effects were observed in the F1 generation, and a slow recovery up to the F4 generation (Kim, Kwak and An 2013). Similar heritable effects of parental exposure (uncoated Ag NPs; 29 ± 4 nm), have been observed in the fruit fly *Drosophila melanogaster* in the F2 generation, but no effects were measured in the F1 generation (Panacek et al. 2011). The decrease in the F2 generation was followed by a recovery at F5 - F8, and therefore it was concluded that Ag NP exposure does not result in any long term heritable changes. In the current exposure, evidence for the transfer of effects from exposed adults to unexposed offspring is provided by measurements in toxicity test control conditions. These show only minor changes in *sod-1* gene expression, and cellular redox status, up until the F3 generation. This is in conjunction with no changes in GSSG/GSH ratios, where there is no effect in earlier generations. In the F6 generation however, a decrease in *sod-1* gene expression is measured for populations exposure to both forms of Ag.

It has been shown that Ag ions might reach developing embryos and thus be transferred to the next generation (Furr et al. 1994, Park et al. 2009). It is possible that Ag ion transfer to offspring might explain the small but significant decrease in GSSG/GSH ratios from the AgNO₃ population, in the F6 generation compared to control. Notably, no such effect was observed for the Ag NP exposure. While *sod-1* expression of Ag NP populations is lower than control population levels, GSSG/GSH ratios are comparable to those of control population. Nevertheless, as indicated by no change in total brood size (Figure S5), the observed changes do not affect phenotypic traits (i.e. fertility and reproduction).

When challenged with higher concentrations of either AgNO₃ or Ag NP, the continuous exposure towards AgNO₃ nor Ag NPs resulted in significantly lower GSSG/GSH ratios, compared to the control population, in the F1 and F3 generation (Figure 5). This, in conjunction with increases in *sod-1* expression, identifies *sod-1* as an important antioxidant, protecting the cells from oxidative stress, when these had been exposed to either AgNO₃ or Ag NPs in earlier generations (Figure 4). Nevertheless, only the Ag NP populations showed significantly increased *sod-1* expression in the toxicity test control conditions in the F3 generation. This could therefore suggest that at this stage the Ag NPs have had a “priming effect” on antioxidant defenses. However, by the F6 generation, responses had changed.

Contrary to expectations an overall increase in GSSG/GSH ratios was measured in the F6 Ag NP toxicity test exposure compared to control populations. Results from our previous work (Rossbach et al. 2019) showed that the continuous chronic exposure towards 1 mg L⁻¹ Ag NPs resulted in increased reproduction when exposed to Ag NPs in the toxicity test, compared to control populations. This however came at the cost of reduced growth. Therefore, in the current work, it was hypothesized that the continuous chronic exposure towards Ag NPs would lead to an increase in antioxidant defenses, in order to avoid oxidative stress manifestation, allowing for increased in reproductive capacity. Results from the current study show an increase in the sod-1 antioxidant defenses in the F3 generation, but and not in the F6 generation. This could reflect that the antioxidant defense response of the reporter strain would have preceded any adaptive response at a phenotypic level, as observed by the significant increase in reproduction seen in the F5 generation in the N2 strain (Rossbach et al. 2019). It is thus conceivable that an enhancement of reproductive traits is most beneficial, and therefore the dominating selective force.

5. Conclusion

The current study was set up in order to monitor changes of the antioxidant enzyme sod-1 and the manifestation of oxidative stress in response to the multigenerational exposure towards either AgNO₃ or Ag NP. Findings from the current study show that the continuous chronic exposure to Ag NPs increased the ability of the exposed nematodes to withstand the known ROS inducer paraquat, indicating a change in the sod antioxidant defenses. Further, results show an effect of both forms of Ag on the nematodes sod-1 antioxidant defense system. It was hypothesized that the maintenance of reproduction in response to the continuous chronic exposure towards Ag NPs by *C. elegans*, would necessitate a change in ROS defense mechanisms. While an increase in *sod-1* gene expression was seen measured in the F3 generation, by the F6 generation, *sod-1* expression levels were significantly below those of the control population. This could indicate that sod-1 is involved in the adaptive responses of nematodes towards Ag. Nevertheless, the change in gene expression occurs earlier than measurable phenotypic adaption seen in other experiments. This therefore suggests that, although ROS production and oxidative stress are important modes of action of Ag NP toxicity, the

maintenance of reproductive capacity is the dominating effect, possibly at the expense of antioxidant stress defense.

6. Acknowledgements

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Supplementary Material

Impact of multigenerational exposure to AgNO₃ or NM300K Ag NPs on *Caenorhabditis elegans* antioxidant defense and oxidative stress

Lisa M. Rossbach, Deborah H. Oughton and Dag A. Brede

Norwegian University of Life Sciences, Faculty of Environmental Sciences and Natural Resource Management, P.O. BOX 5003 NMBU, No-1432 Ås, Norway

*Corresponding author: Lisa.rossbach@nmbu.no

1. Characterization of the Ag NPs

1.1. Transmission electron microscopy

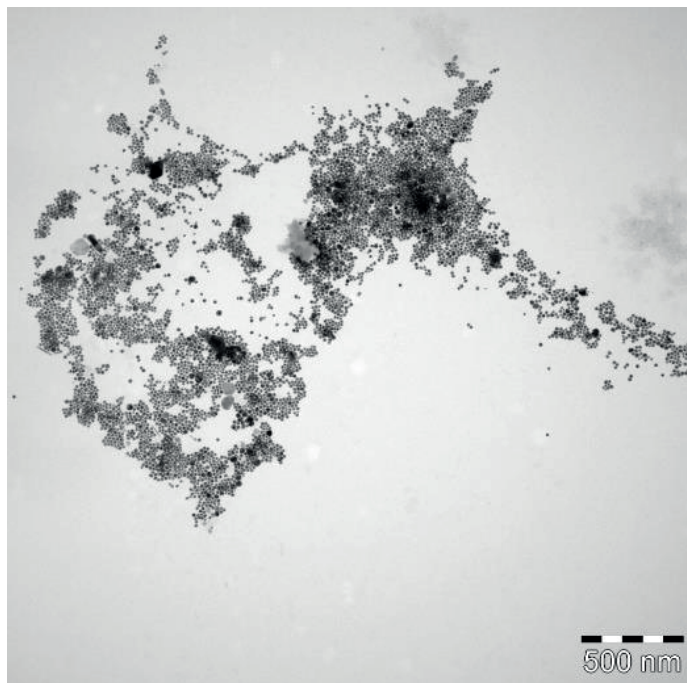


Figure S1: Transmission electron microscopy image of the NM300K Ag NPs in ddH₂O.

1.2. Dynamic light scattering

Table S1: Hydrodynamic diameter measurements (Z-average diameter (nm)) and polydispersity index (\pm SD) for NM300K Ag NP initial and toxicity test stocks prepared for the current experiment.

Ag NP stocks (mg/l)	Z-average diameter (nm)	Polydispersity index
256	78.98 (\pm 4.42)	0,30 (\pm 0,05)
10	81,01 (\pm 2.91)	0,29 (\pm 0.03)
0.5	155,83 (\pm 42.95)	0,22 (\pm 0.05)
0.1	235,12 (\pm 33.8)	0,35 (\pm 0.01)

1.3. Recoveries

The Ag exposure of the nematodes in the toxicity test was confirmed by ICP-MS analysis of total Ag concentrations of the Ag exposures. The AgNO₃ exposure showed a recovery of 102.3 ± 7.8 % of nominal Ag concentrations, while the Ag NP exposure showed recoveries of 90.7 ± 5.5 % of nominal concentrations, measured at the start of the experiment. Recoveries for the AgNO₃ exposure showed a 16% decrease following 72 hrs of exposure down to 85.4 % of nominal concentrations. Interestingly, recoveries for Ag NPs show a 14 % increase with 95.1 % of nominal concentrations, following 72 hrs of exposure.

1.4. Size fractionation

For the assessment of changes in the behavior of the Ag in the exposures media over time, a fractionation experiment was conducted at T-0 and 72 h of exposure. A small dissolved (< 3 kDa) Ag fraction was found at T-0 in the AgNO₃ exposure for all three concentrations (Figure 1). Nevertheless, no such Ag fraction was detected at 72 h for any of the exposure concentrations, where the majority of the Ag was in the suspended fraction. Furthermore and increase in the aggregated fraction is observed at all three exposure concentrations over time. For the Ag NP exposure, low dissolved (< 3kDa) Ag fraction was measured at 5 and 10 mg L⁻¹ exposure concentration at T-0, but not at 72 hrs. Further, at the lowest (1 mg L⁻¹) exposure concentration a decrease in aggregated fraction is observed, while the opposite is true for the highest exposure concentration (10 mg L⁻¹).

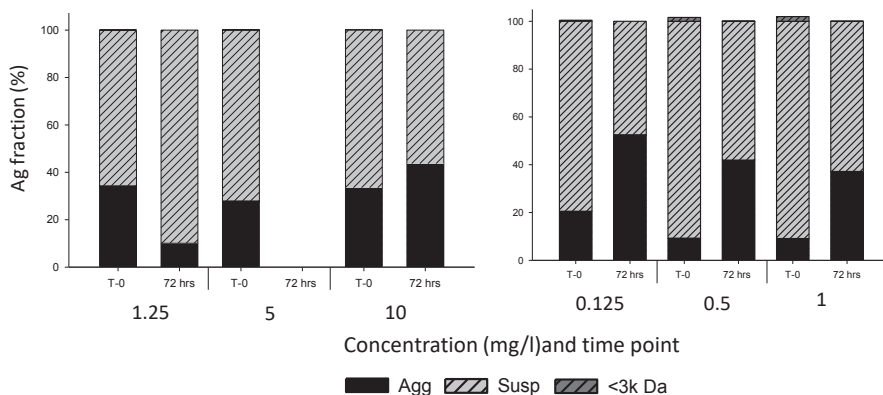


Figure S2: Aggregated (Agg), suspended (Susp) and dissolved (<3 kDa) Ag fraction of toxicity test exposure media containing either AgNO₃ (A) or NM300K Ag NPs (B), at T-0 and 72 hrs of exposure in MHRW containing *E. coli* and nematodes. For suspended Ag fraction *E. coli* was removed.

2. Toxicity test

2.1. Cerium exposure

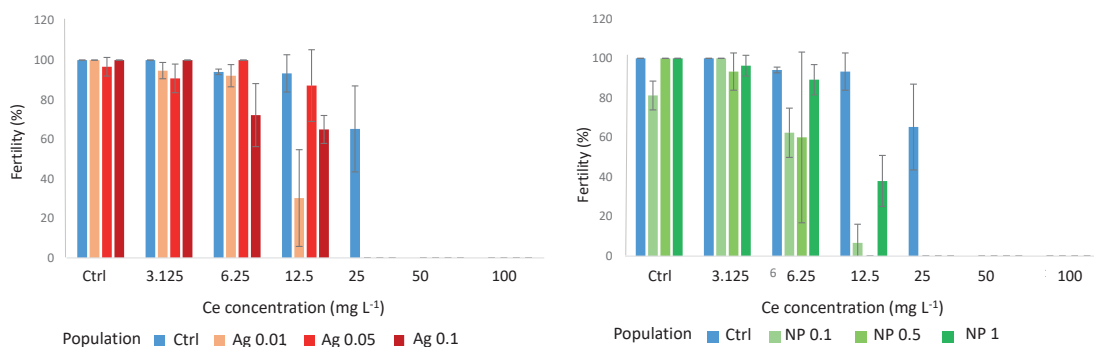


Figure S3: Cerium tolerance of *C. elegans* after six generations continuous exposure to either of three concentrations of AgNO₃ or Ag NPs, as measured by fertility (%), mean ± SD). Nematodes were exposed in a standard 96 h toxicity test to six concentrations of cerium.

3. SEM imaging

Sample preparation

For the assessment of potential external damages, cuts or lesions to the cuticle of the nematodes, caused by the Ag NPs, a sample of roughly 50 nematodes was taken from each population and washed three times in 5 mM PIPES buffer and fixed until further handling. For Scanning Electron Microscope (SEM, Zeiss, EVO50), washed nematodes were kept in 0.05 M PIPES buffer (pH 7), before overnight fixing in freshly prepared fix solution (2 % PF + 1.25 % GA in 0.05 M PIPES buffer, pH 7). Samples were washed three times in 0.05 M PIPES buffer, before osmium stabilization, followed by stepwise ethanol dehydration at 30, 50, 70, 90, 95 %, and finally 4 x 100 % at 15 minutes each. Using the critical point dryer (BAL-TEC CPD030) samples were dried and mounted for SEM analysis.

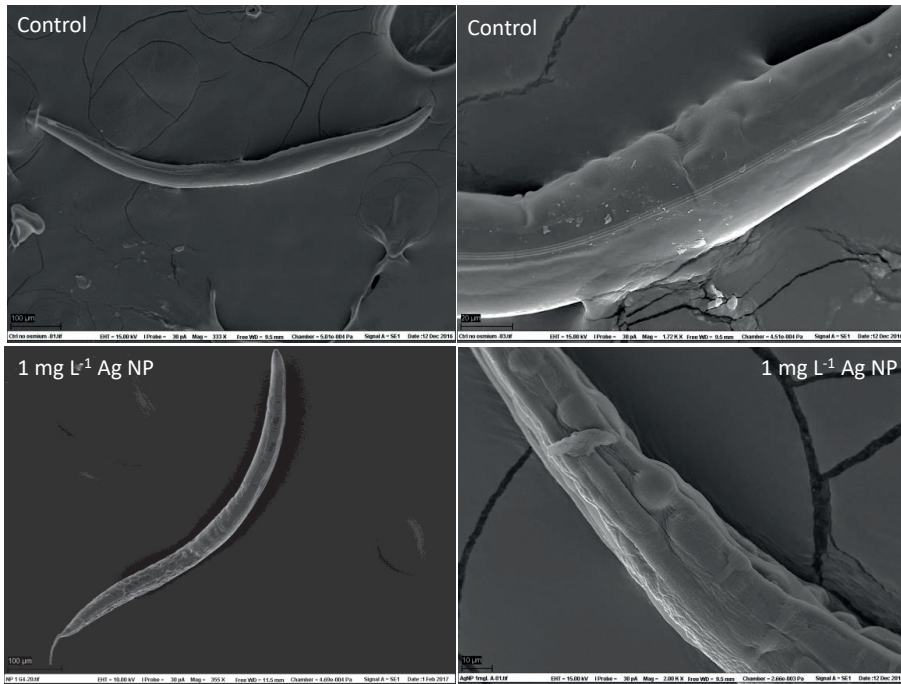


Figure S4: Scanning electron microscope images of either control or 1 mg L⁻¹ Ag NP exposed nematodes. Analysis of nematodes from all exposure concentrations showed no exterior damages, including cuts or lesions, from the NP exposure.

4. Total brood size

Method

To determine changes in total brood size, at each generation five N2 nematodes were randomly hand picked at 48 h from the multigenerational culture exposure and placed onto clean (no added Ag) NGM agar plates seeded with 100µl of *E. coli*. Nematodes were allowed to lay eggs for 72 h before moving onto new, unexposed plates. This was repeated three times. Once adults had been removed, plates were stained using Bengal Rose and left at 80 °C for 10 minutes to halt further hatching or development of the eggs and embryos. All plates were counted for eggs and offspring using a hand held tally counter. Total brood size was determined by the sum of total offspring produced by individual hermaphrodites.

Results

While, under normal conditions, *C. elegans* will produce approximately 280 offspring during their reproductive period, under unfavorable conditions, nematodes may reduce their reproduction and increase their lifespan until the conditions improve (Byerly *et al.*, 1976, Hodgkin, 1991, Mukhopadhyay and Tissenbaum, 2007). To assess long lasting toxic effects of the multigenerational Ag exposure on reproduction of the N2 nematode populations, a measure of changes in the total brood size (number of offspring per hermaphrodite) of individuals was conducted (Figure 2). Although the Ag NP exposed populations had a tendency to produce less offspring compared to the AgNO₃ populations, the total brood size measurements did not show any statistically significant difference between total number of offspring produced and generations ($p = 0.429$), or population exposure ($p = 0.827$) (figure 1).

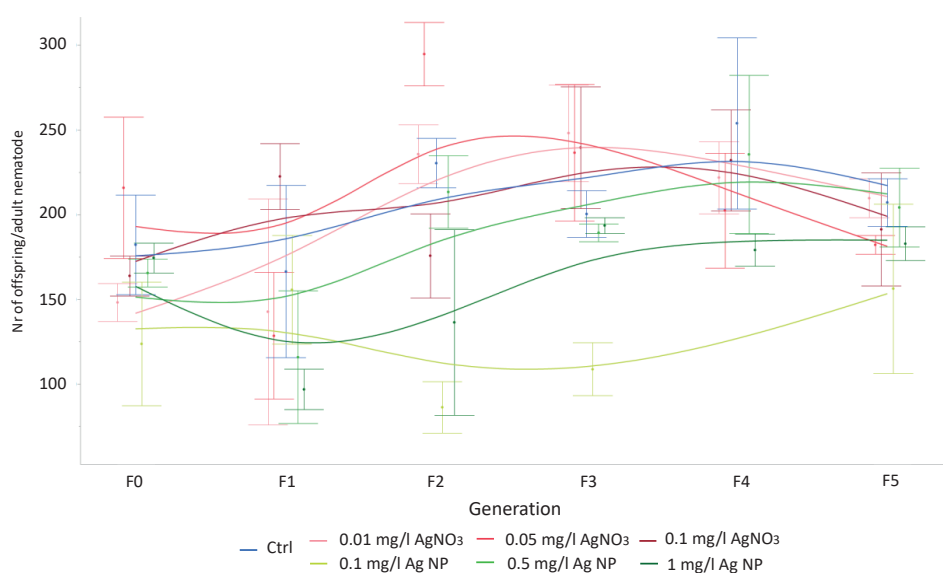


Figure S5: Total Brood size measurement (mean \pm SEM) for five adult N2 *C. elegans* previously exposed in the multigenerational exposure regime, to either three concentrations of AgNO₃ or Ag NP, or controls. Total brood size was assessed by the total number of offspring produced by the selected adult nematodes over nine consecutive days.

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Norwegian University
of Life Sciences

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