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Impact of multigenerational exposure to $AgNO_3$ or NM300K Ag NPs on antioxidant defense and oxidative stress in *Caenorhabditis elegans*

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ABSTRACT

Adaptation of the nematode *Caenorhabditis elegans* towards NM300K silver nanoparticles (Ag NPs) has previously been demonstrated. In the current study, the sensitivity to a range of secondary stressors (CeO_2 NP, Ce^{3+}, Cu^{2+}, Cd^{2+}, and Paraquat) following the multigenerational exposure to silver nanoparticles (Ag NPs NM300K) or $AgNO_3$ was investigated. This revealed improved tolerance to the ROS inducer Paraquat with higher fecundity after pre-exposure to Ag NP, indicating an involvement of reactive oxygen species (ROS) metabolism in the adaptive response to NM300K. The potential contribution 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 $AgNO_3$ and Ag NP exposures. Continuous exposure to $AgNO_3$ and Ag NP until the F6 generation resulted in a decreased *sod-1* expression, with a concomitant increase in GSSG/GSH ratios. The results thus show that despite an initial enhancement, the continuous exposure to Ag caused a severe impairment of the antioxidant defense capacity in *C. elegans*.

1. Introduction

Silver nanoparticles (Ag NPs) have received 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; Klaine et al., 2008; Mcgillicuddy et al., 2017). 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; Kleiven et al., 2018; Köser et al., 2017; van der Ploeg et al., 2014; Völker et al., 2015). In the nematode *Caenorhabditis elegans*, Ag NPs have been shown to negatively impact physiological processes, such as reproduction, development, and locomotion (Ellegaard-Jensen et al., 2012; Hunt et al., 2014; Kleiven et al., 2018). Moreover, the production of free radicals induced by nanoparticles exposure has the potential to induce oxidative stress response and produce damage to lipids, proteins and DNA (Choi et al., 2018; Foldbjerg et al., 2009; He et al., 2012a; He et al., 2012b; Hwang et al., 2008; Rossbach et al., 2020). In our previous work we showed that the chronic six generational exposure towards the NM300K Ag NPs, resulted in an increased susceptibility towards $AgNO_3$ exposure, while the multigenerational exposures towards $AgNO_3$ resulted in a decrease in sensitivity towards Ag NPs (Rossbach et al., 2019).

Oxidative stress is considered an important toxic mechanisms of Ag NPs and has been the focus in a range of toxicity studies in various species (Jiang et al., 2014; Kim et al., 2009; Ribeiro et al., 2015), including the nematode *C. elegans* (Ahn et al., 2014; Lim et al., 2012a; Roh et al., 2009). Oxidative stress is a substantial mechanistic contributor of Ag NP induced reproductive toxicity (Lim et al., 2012a). 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 study where *C. elegans* exposed to coated or uncoated Ag NPs (< 100 nm), or $AgNO_3$, showed a significant toxic response from $AgNO_3$ and uncoated Ag NPs, which was associated with increased mitochondrial membrane permeability and oxidative DNA damage, underlying the oxidative stress response from both forms of silver (Ahn et al., 2014).

The nematode *C. elegans* presents the perfect model for the study of both enzymatic and non-enzymatic antioxidant defense systems, 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

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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, changes of the redox status may have adverse consequences for the physiological development, with negative impact on metabolism, cell senescence, and apoptosis (Finkel and Holbrook, 2000; Jones, 2008; Veal et al., 2007). *C. elegans* possess a highly specialized and complex ROS and redox control system, where their genome encodes for five isoforms of the SOD enzyme (Braeckman et al., 2016; Doonan et al., 2008; Mccord and Fridovich, 1969). Further, this nematode possesses three catalase encoding genes (CTL) and over 50 putative glutathione-S-transferases (GST) genes, which facilitate ROS scavenging (Braeckman et al., 2017). The glutathione-S-transferases (GSTs) together with GSH are important cellular detoxification enzymes. 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).

An adaptation towards oxidative stress has been demonstrated in single organisms or across generations in bacterial and human cells (Demple and Halbrook, 1983; Wojcik et al., 1996), as well as in *C. elegans* (Contreras et al., 2014; Dutilleul et al., 2014; Helmcke and Aschner, 2010; 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, 2002). 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 mechanism in *C. elegans* in response to NM300K and $AgNO_3$ exposure (Rossbach et al., 2020). Further we provided evidence that *C. elegans* could develop an adaptation, as shown by increased reproduction despite a significant reduction in size, towards the Ag NP NM300K, following six generations of continuous exposure (Rossbach et al., 2019). Therefore, this study aimed to elucidate the underlying adaptive processes in terms of ROS production and oxidative stress manifestation. We investigated changes in tolerance towards secondary stressors, as well as measured the *sod-1* expression and changes in cellular redox status, following multigenerational exposure to either the Ag NPs NM300K or $AgNO_3$.

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 (< 20 nm, dispersed in 4% each of Polyoxyethylene Glycerol Trioleate and Polyoxyethylene (20) Sorbitan mono-Laurat (Tween 20)) (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 ddH_2O (15 MΩ cm) and sonicated for 13 min at 15% amplitude using a probe sonicator (Branson S-450 D sonicator, disruptor horn 13 mm). All stocks, $AgNO_3$ 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 ddH_20 (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 $AgNO_3$) 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 (Ø 6 cm) seeded with 10 x concentrated *Escherichia coli,* 250 nematodes per exposure plate, in triplicate (Fig. S1). N2 nematodes were either unexposed or exposed to 0.01, 0.05, or 0.1 mg L^{-1} $AgNO_3$, or 0.1, 0.5, or 1 mg L^{-1} Ag NPs. Equitoxic Ag concentrations were chosen for the SOD-1 and GRX strains, due to slight differences in sensitivity towards Ag, compared to the N2 Bristol strain. Both the strains were either unexposed (controls), or exposed to either 0.1 or 0.5 mg L^{-1} $AgNO_3$ and Ag NPs, respectively (Fig. S1). Selection of all concentrations was based on pilot experiments.

On the day of culture transfers, 300 μl of the appropriate Ag stock was applied onto the exposure plates (containing 3 ml of NGM agar and an *E. coli* lawn), prior to transferring the nematodes. Plates were kept at 20 °C in the dark for 96 h before collection of pregnant nematodes. Pregnant nematodes were treated with alkaline hypochlorite solution for egg extraction (Stiernagle, 2006). 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.

2.3. Toxicity test exposure and sampling

All standard toxicity tests were conducted in triplicate on 24-well cell culture plates, each well containing 25 nematodes in 1 ml of *E. coli* resuspended in moderately hard reconstituted water (MHRW) (Kleiven et al., 2018; 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 $AgNO_3$ or Ag NPs, were exposed to either concentration of three ions, 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, or 100 mg L^{-1}), as well as an alternative nanoparticle, cerium nanoparticles (0, 01.56, 3.13, 6.25, 12.5, 25, or 50 mg L^{-1}), in standard toxicity tests (Rossbach et al., 2019). Stocks were prepared in ddH_2O 24 h prior the application in the toxicity tests. 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 in a standard toxicity test, 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 min, before the assessment of growth (three biological replicates and 10 individuals per replicate, total body length), fertility (three biological replicates and 10

individuals per replicate, number of pregnant nematodes/exposure total number of L4 stage nematodes in each well), and reproduction (three biological replicates and 10 individuals per replicate, number of life offspring/total number of L4 stage nematodes/exposure well) using a stereomicroscope (Leica M205C) (ISO, 2010).

For both the SOD-1 and GRX strain, toxicity tests were set up at F1, F3, and F6. Both nematode strains, independent of multigenerational culture exposure, were exposed to either, 0.1, 0.5, and 1 mg L^{-1} $AgNO_3$, 1, 5, and 10 mg L^{-1} of Ag NPs, or to unexposed control (three biological replicates and 10 individuals per replicate) for 72 h, before sampling.

For sampling, 10 nematodes were randomly chosen from each exposure concentrations (pooled from triplicate exposure wells) and immobilized with sodium azide (NaN_3). Analyses of the expression signals were performed 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, at excitation 490 and emission 535 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). For the SOD-1 expression, the average intensity was normalized to nematode total body length, to account for possible variance in signal strength related to developmental stages (Doonan et al., 2008; Rossbach et al., 2020). All images were quantified using the LAS X Leica application suit X imaging software (LEICA DM6 B) for pixel based average intensity measurements.

2.4. Statistical analysis

All statistical analyses were carried out using either MiniTab® 18 (Minitab Inc. 2010) or Jmp Pro v14 (SAS institute, Cary, NC). For group comparison when error terms were normally distributed, a one-way ANOVA (Tukey's HSD) was applied. For non-parametric analysis of the data, a Kruskal-Wallis one-way analysis of variance was conducted. For fold-change analysis, multigenerational data was log2 transformed. For regression analysis of the multigenerational data, a standard least square model was applied. To account for inherent variations between generations, data was normalized to toxicity test control values (Moon et al., 2017; Yu et al., 2012).

3. Results

3.1. Nanoparticle characterization

Nanoparticle toxicity has been shown to be highly dependent on NP behavior (Jiang et al., 2009; Kleiven et al., 2018). To ensure consistency between studies, particle characteristics and behavior during exposures were characterized in ddH_2O (15 MΩ·cm) and changes over time were monitored in the test media. Transmission electron microscopy (TEM) analysis of the NM300K Ag NPs in ddH_2O (15 MΩ cm) showed particles to be spherical with a median size of 23.9 ± 21.8 nm (Fig. S2). Dynamic light scattering in ddH_2O (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 the exposure period (72 hrs) (Supplementary materials, Section 2.3). Size fractionation data is consistent with our previous findings presented in Kleiven et al. (2018) (Supplementary materials, Section 2.4, Fig. S3).

3.2. Multigenerational exposure of N2 nematodes to 1 mg L^{-1} Ag NPs decreases susceptibility towards Paraquat

To exclude changes in response to the exposure resulting from possible external damages to the cuticle of the nematodes, scanning electron microscope images were taken of the nematode populations exposed to the two highest concentrations of nanoparticles (5 and 10 mg L^{-1} Ag NPs in N2 strain nematodes). Results revealed no external damages, cuts, or lesions of the cuticle of the nematodes (Fig. S4). To test the cross-tolerance development in the current study, N2 nematodes were exposed to Ce NPs, Cd^{2+}, Cu^{2+} and Ce^{3+}, as well as the herbicide and well-known ROS inducer, Paraquat, following the six generational exposure to either $AgNO_3$ or Ag NPs. The six generational exposure towards $AgNO_3$ or Ag NPs did not alter the nematodes response (growth, fertility, and reproduction) towards Ce NPs, Cd^{2+}, or Cu^{2+} (data not shown), compared to control population. However, increased sensitivity ($p < 0.05$, Kruskal-Wallis post hoc) towards Ce^{3+} (supplementary materials, Section 3.1, Fig. S5) 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.

The six generational exposure to Ag NPs of the N2 strain, led to changes in response towards Paraquat, compared to control population nematodes (Fig. 1). The pre-exposure towards $AgNO_3$ did not result in such effects. A significant increase compared to controls ($p < 0.001$, Kruskal-Wallis post hoc) in fertility was measured for the highest Ag NP (1 mg L^{-1}, dark green line Fig. 1A) population exposed to 0.156 mM Paraquat. In contrast, the two lower Ag NP populations showed lower fertility at similar concentrations of the herbicide, compared to the control and $AgNO_3$ population nematodes (Fig. 1A). Moreover, the Ag NP populations nematodes were the only population showing fertility and production of offspring at 0.156 mM of Paraquat, albeit, the observed effect was below the statistical significance level ($p = 0.07$, Kruskal-Wallis post hoc Fig. 1B).

3.3. Alterations to sod-1 expression in response to the continuous chronic exposure towards $AgNO_3$ or Ag NP

The GA508 reporter strain that measures *sod-1* gene expression has been validated as a reporter for ROS formation in the form of superoxide (Doonan et al., 2008). Sod-1 is an important constituent of the nematodes' antioxidant defense system, responsible for approximately 80% of the total SOD activity (Doonan et al., 2008). Overall, the *sod-1* induction in the current study is consistent with previous observations (Rossbach et al., 2020), with a clear dose response for all populations (Figs. 3 and 5). The induction patterns from both, $AgNO_3$ and Ag NPs, 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 (Fig. 2). In toxicity test control conditions, nematodes taken from the unexposed populations (i.e. nematodes not previously exposed to Ag), showed no change in *sod-1* expression across generations (Fig. 2). In comparison, nematodes previously exposed to Ag NPs showed a temporal statistically significant increase in *sod-1* expression in the F3 generation (Fig. 2). On the other hand, both Ag NP and $AgNO_3$ populations show a significant decrease in *sod-1* expression compared to both previous generations, and to the control population expression levels, in the F6 generation (Fig. 2).

All pre-exposed populations, except the $AgNO_3$ population in the F3 generation, showed a dose dependent increase in expression within all generations (Fig. 3). For both $AgNO_3$ and Ag NPs toxicity test exposures, the Ag NP population showed a slight but significant increase of *sod-1* expression compared to the control populations in the F1, and a ~2 fold increase in expression in the F3 generation compared to controls (Fig. 3). The $AgNO_3$ population on the other hand, only showed increased *sod-1* expression when exposed to Ag NPs in the toxicity test from the F3 generation, while it remained equal to control population levels in the F1, and the F3 $AgNO_3$ toxicity test (Fig. 3). In the F6 generation, both Ag populations show a significantly lower expression compared to the control population in all toxicity test exposures concentrations (Fig. 3). Data from the $AgNO_3$ pre-exposed nematodes in the highest Ag NP toxicity test exposure concentration is not shown, due to a lack of

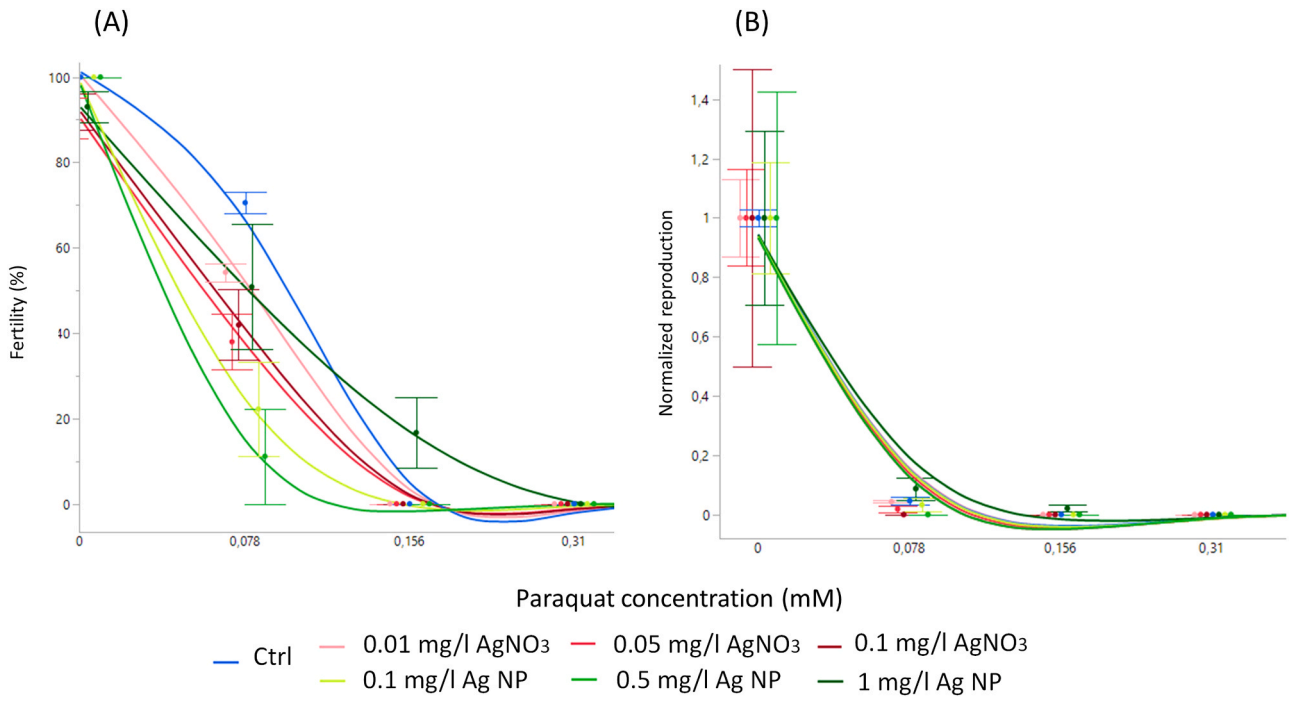

Fig. 1. Paraquat tolerance of *C. elegans* after six generations continuous exposure to either of three concentrations of $AgNO_3$ or Ag NPs. (A) Fertility (% mean ± SEM). (B) Reproduction (normalized to individual reproductive values of toxicity test controls, mean ± SEM). Nematodes were exposed in a standard 96 h toxicity test to six concentrations of Paraquat. Note: graphs only show four concentrations; reproduction was zero at higher concentrations.

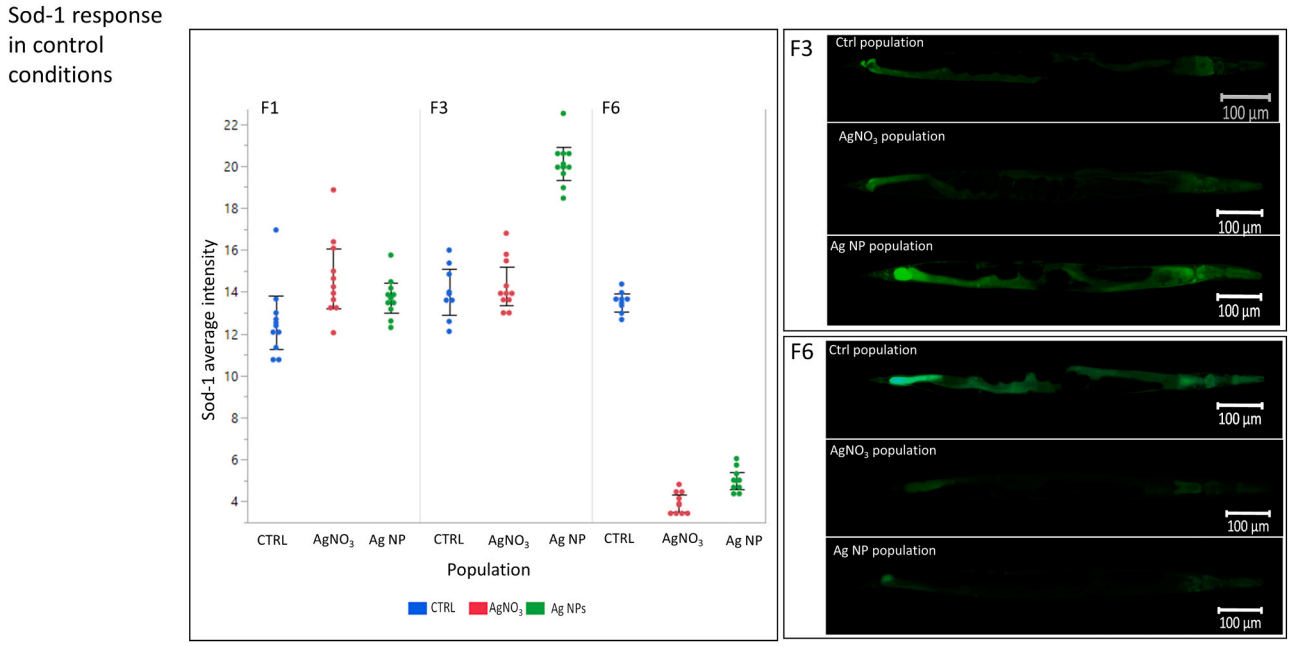

Fig. 2. *In vivo* measurement of *sod-1* expression pattern in the *sod-1*::gfp reporter strain GA508 in toxicity test control conditions (no added Ag), following the multigenerational exposure towards either no Ag (control), 0.1 mg L^{-1} $AgNO_3$ or 0.5 mg L^{-1} Ag NPs, in generations F1, F3 and F6 (n = 10 nematodes/treatment). Results present individual measurements, as well as means ± 95% confidence interval. Nematode images on the right present examples of expression patterns.

replicates.

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

The biosensor Grx1-roGFP2 (Back et al., 2012) was used in the current experiment for in vivo measurements of the cellular redox potential in different tissues of the nematodes. Across generations, in toxicity test control conditions, the unexposed population (blue dots) showed no significant changes of the redox state levels ($p = 0.9$, Kruskal-Wallis post hoc), indicating consistency of the multigenerational set up (Fig. 4). In the F6 generation, the $AgNO_3$ population, showed significantly lower ($p = 0.039$, Kruskal-Wallis post hoc) oxidation levels, than the control population. The Ag NP population nematodes showed an increase in GSSG/GSH ratios in the F3 and F6 generation compared to the F1 generation levels. However, levels were not significantly different to control

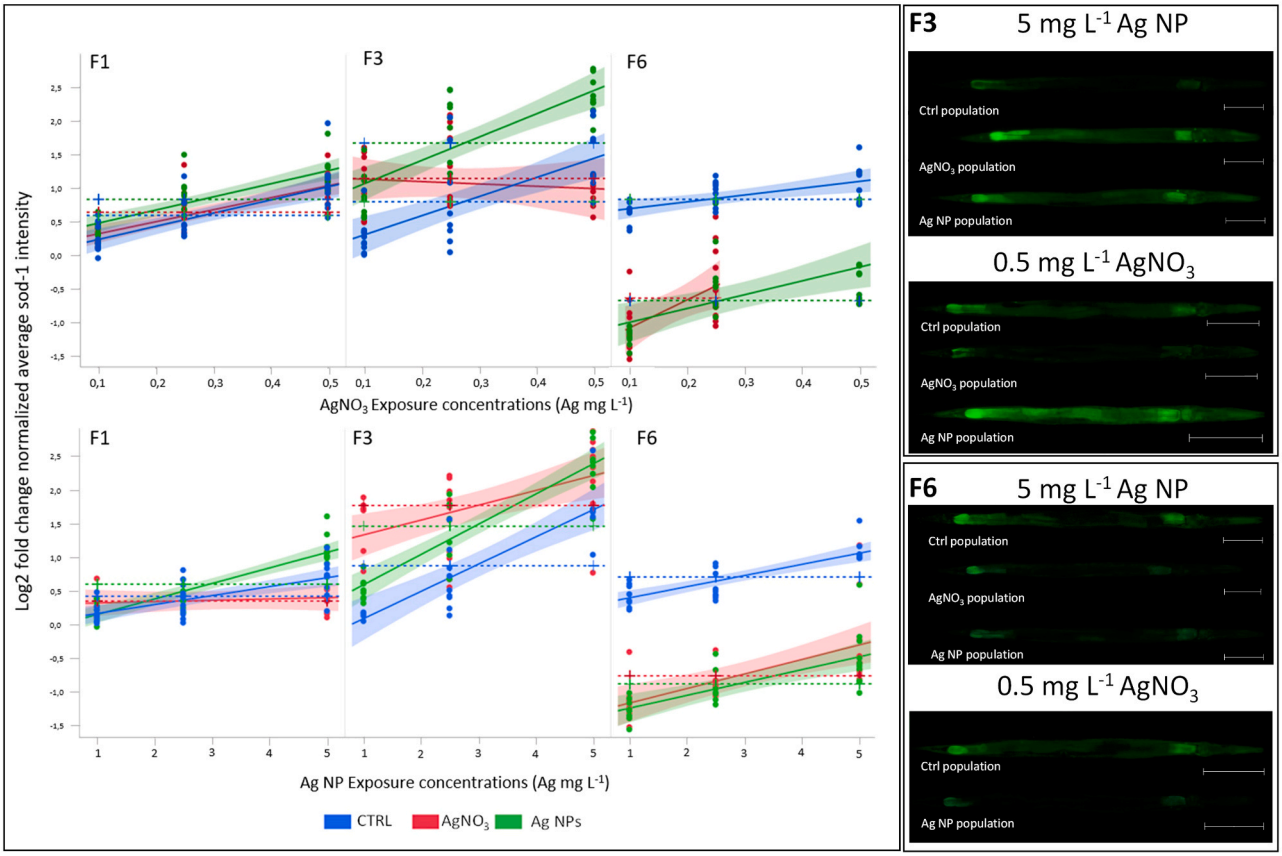

Fig. 3. *In vivo* measurement of *sod*-1 expression patterns in the *sod-1*::gfp reporter strain GA508 populations following the multigenerational exposure. Control (no added Ag), 0.1 mg L^{-1} $AgNO_3$, or 0.5 mg L^{-1} Ag NPs. Dose response effect measured to three concentrations of $AgNO_3$ or Ag NP. Quantitative measurements on the left (solid lines) with least square means (dotted lines), and corresponding representative images on the right. Values are normalized to toxicity test controls for direct comparison. Scale bars represent 100 μm.

population levels. Unfortunately, for technical reasons, there were insufficient number of observations for the controls.

All populations showed a dose dependent increase of GSSG/GSH ratios in all generations' toxicity tests (Fig. 5). In the F1 generation both Ag populations show statistically significant increased oxidation levels compared to control population, when exposed to $AgNO_3$ in the toxicity test (Fig. 5). When exposed to Ag NPs in the toxicity test however, only the Ag NP population showed significantly increased oxidations levels compared to the controls in the F1 generation, while the $AgNO_3$ exposed nematodes were significantly reduced (Fig. 5). In the F6 generation, the $AgNO_3$ population showed a statistically significantly increase compared to the control population. On the other hand, the Ag NP populations' oxidation levels were significantly reduced, when exposed to $AgNO_3$ (Fig. 5). In the F6 generation on the other hand, the $AgNO_3$ population showed consistently higher GSSG/GSH ratios compared to the control population, when exposed to either form of Ag. However, GSSG/GSH ratios measured from the Ag NP population nematodes were lower compared to the controls when exposed to $AgNO_3$ in the toxicity test, and higher when exposed to Ag NPs (Fig. 5). Unfortunately, the F3 toxicity test results for the unexposed population had to be excluded due to a lack of proper toxicity test control organisms for the normalization.

4. Discussion

Silver NPs are one of the most extensively studied nanomaterial to date. Multiple differences between ionic Ag and Ag NPs have been found in terms of biodistribution and toxic mode of action (Choi et al., 2018; Hunt et al., 2013; Kleiven et al., 2018; Navarro et al., 2008b; Rossbach et al., 2020). Certain differences have been attributed to ROS formation and oxidative stress response (Cortese-Krott et al., 2009; Lim et al., 2012a; Mcshan et al., 2014; Roh et al., 2012; Rossbach et al., 2020).

Our previous work has shown the ability of the nematode to develop an adaptation, in terms of increased reproduction, towards the exposure of the NM300K Ag NPs, however underlying mechanisms, leading to the increased resistance were not investigated (Rossbach et al., 2019). Understanding the underlying toxic mechanisms of Ag nanomaterials across multiple generations compared to ionic Ag, can therefore provide vital information for the further management and safe use of nanomaterials. 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 (Ce^{3+}), divalent cationic metal (Cd^{2+} and Cu^{2+}), and oxidative stress inducer (Paraquat). Subsequently, changes to the antioxidant defense system in response to the multigenerational exposure to either $AgNO_3$ or NM300K Ag NPs was investigated. In line with findings from other studies, our results show an overall dose-dependent *sod-1* induction and oxidative stress response in all populations exposed to either forms of silver ($AgNO_3$ or Ag NPs) in the toxicity tests for all generations (Lim et al., 2012a; Limbach et al., 2007; Roh et al., 2009; Rossbach et al., 2020).

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

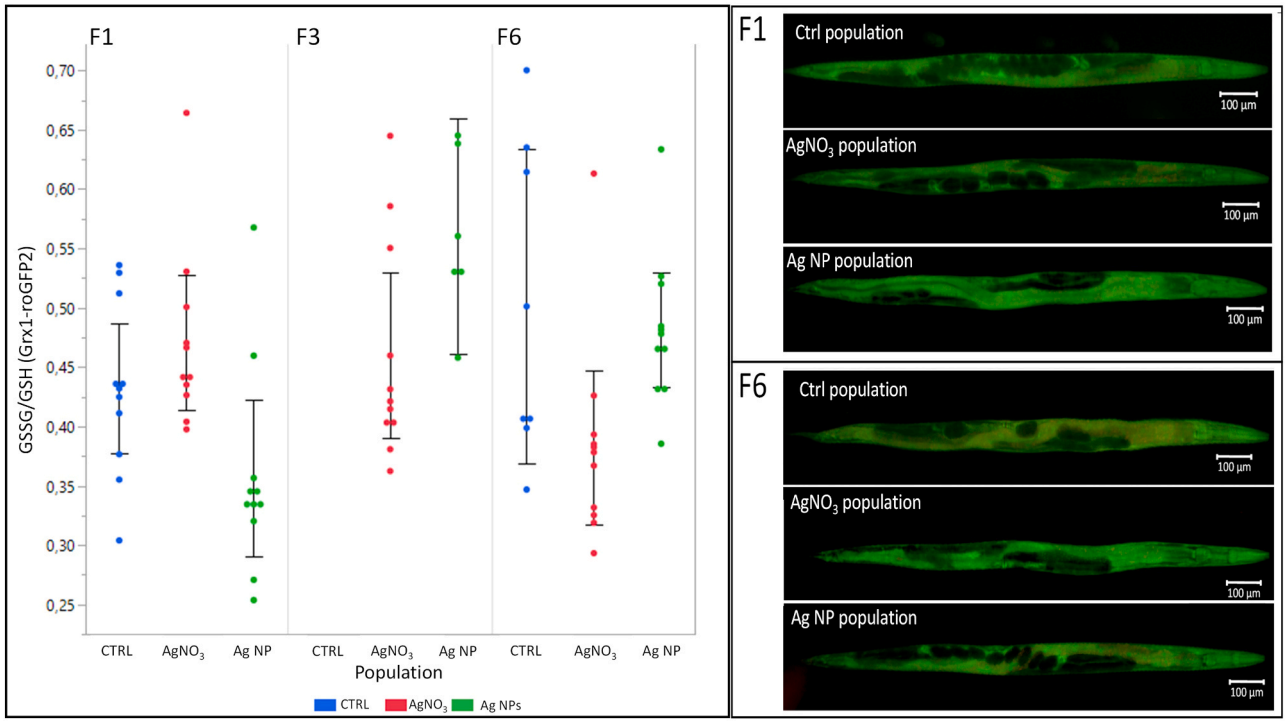

Fig. 4. Oxidized to reduced GSSG/GSH ratios, quantitative measurements on the left (error bars = 95% CI), and corresponding representative images on the right, of the biosensor Grx1-roGFP2 in toxicity test control conditions, following the multigenerational exposure to either controls (no added Ag), 0.1 mg L^{-1} $AgNO_3$, or 0.5 mg L^{-1} Ag NPs (n = 10 nematodes/treatment).

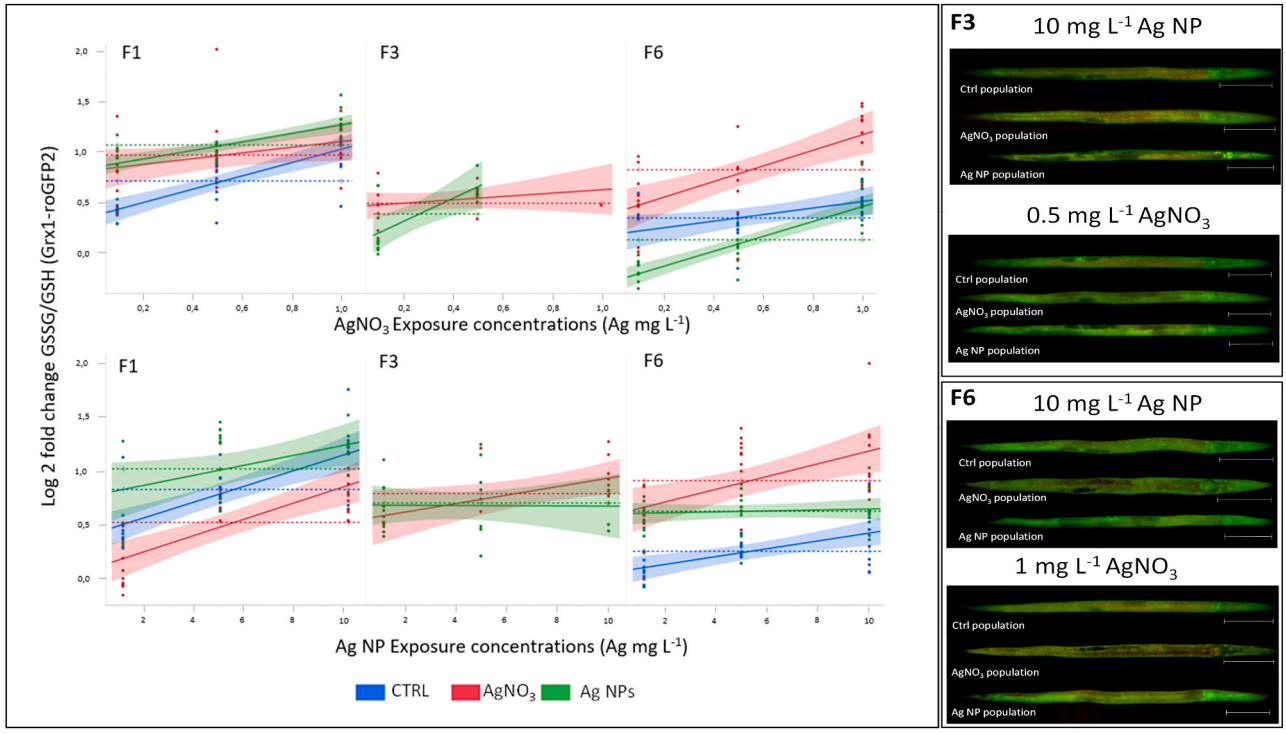

Fig. 5. Oxidized to reduced ratios, quantitative measurements on the left (solid lines) with least square means (dotted lines), and corresponding representative images on the right, of the biosensor Grx1-roGFP2 populations following the multigenerational exposure. Control (no added Ag), 0.1 mg L^{-1} $AgNO_3$, or 0.5 mg L^{-1} Ag NPs. Dose response effect measured to either three concentrations of $AgNO_3$ or Ag NP. Values are normalized to individual toxicity test controls for direct comparison. All scale bars represent 100 µm. Note, due to insufficient number of replicates data is not presented for the control population in the F3 generation.

by *C. elegans* (Kleiven et al., 2018; Köser et al., 2017; Navarro et al., 2008a). Furthermore, it should be noted that the antibacterial properties of Ag and Ag NPs, presumably affects the viability of *E. coli* (Contreras et al., 2014), and that the measured effects onto the *C. elegans* comprises both the direct Ag toxicity as well as the impact onto the food source. 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). A range of studies, with contradicting results on the Ag NP NM300K dissolution state in the exposure media, highlights the importance of monitoring the behavior in the exposure media of these NPs (Köser et al., 2017; Lodeiro et al., 2017; Wasmuth et al., 2016). In the present study fractionation of exposure media shows a large transformation from suspended to aggregated fraction over time, with only low free low molecular mass (LMM) (< 3 kDa) Ag fractions measured at T-0, for both forms of Ag. Köser et al. (2017) revealed, in an analysis of the NM300K stock dispersant, the presence of a low (~8%) ionic Ag fraction. This could explain the < 3 kDa Ag fraction measured at T-0 in the Ag NP exposure, while the decrease in time as well as low LMM fractions in $AgNO_3$ exposures, may reflect the high affinity of the ionic Ag with the *E. coli* cells 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 facilitate uptake and exposure (Dakal et al., 2016; Kleiven et al., 2018; Li et al., 1997). Moreover, the fractionation of the Ag NP exposure revealed a comparatively lower transformation of the Ag NP to the aggregated fractions, compared to the $AgNO_3$ exposure. The overall low measured LMM fractions in the exposures, 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. Nano-specific increased Paraquat tolerance following the six generational Ag NPs exposure

Studies show that certain organisms have adapted specific mechanisms to deal with higher levels of oxidative stress to counteract potential adverse biological consequences (Koch and Hill, 2017; Monaghan et al., 2009). Cypser and Johnson (2002) 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 acquired resistance towards oxidative stress inducers, by means of pre-treatment to 90% oxygen leading to decreased sensitivity towards x-ray irradiation. In our previous work, the six generational exposure towards the NM300K Ag NPs resulted in an increased susceptibility towards $AgNO_3$ exposure, as measured by a decrease in growth, fertility, and reproduction (Rossbach et al., 2019). Conversely, the multigenerational exposure towards $AgNO_3$ 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 described by Cypser and Johnson (2002), is stressor specific.

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 to Paraquat (Fig. 3). In toxicology, the exposure to the herbicide Paraquat (1,1 ′-dimethyl-4,4 ′-bipyridinium dichloride) is amongst the most commonly used methods for 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 Paraquat redox cycles through the generation of superoxide anions, leading to consequential increased levels of hydrogen peroxide and hydroxyl radicals (Gram, 1997; Suntres, 2002). Internalized Paraquat will oxidize cellular NADPH, and lead to lipid peroxidation (Gram, 1997; Suntres, 2002). 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 $AgNO_3$ and Ag NPs are known to produce superoxide anions ($O2^{\cdot\cdot}$), as well as peroxide radicals, hydroxyl radicals ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2) on the surface of the particles (Choi et al., 2018; He et al., 2012a; He et al., 2012b; Hwang et al., 2008; Rossbach et al., 2020). The continuous chronic exposure towards Ag NPs, however, did not result in a significant increase in *sod-1* gene expression compared to the $AgNO_3$ toxicity test exposure in the F6 generation. In contrast, a significant decrease in *sod-1* expression was measured for both Ag pre-exposed populations compared to the control populations in the F6 generation.

Glutathione has also been shown to be highly efficient at reducing the toxicity of Paraquat exposure (Djukic et al., 2012; Nakagawa et al., 1995). Acting as an endogenous antioxidant scavenger, GSH protects cells form oxidative stress, making this antioxidant of critical importance to cell survival (Habib et al., 2007; Pena-Llopis et al., 2003; Piao et al., 2011a; Sies, 1999). Piao et al. (2011a) 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 $AgNO_3$ population pre-exposed nematodes showed a significant decrease in GSSG/GSH ratios in the F6 generation, compared to the control population, possibly indicating an improved antioxidant defense capacity.

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

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 was shown up to the F4 generation (Kim et al., 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 change (Panacek et al., 2011). In the current study, evidence for the transfer of effects from exposed adults to unexposed offspring is provided by measurements in toxicity test control conditions. Unexposed offspring, however, only show 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. On the other hand, in the F6 generation, a decrease in *sod-1* gene expression was observed for populations exposure to both forms of Ag. This may indicate an adjustment of antioxidant defenses by the nematodes in response to the six generational exposure. It should be noted that Ag exposure (both $AgNO_3$ and Ag NP) 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 the antioxidant defenses activation.

Increased *sod-1* expression is directly connected to production of H_2O_2, which is sequestered by either catalase (CAT) or glutathione peroxidase (GPx) enzymes regenerating water and molecular oxygen, and is as such directly related to glutathione homeostasis (Finkel and Holbrook, 2000). 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). Contrary to expectations, an overall increase in GSSG/GSH ratios was measured in the F6 Ag NP toxicity test exposure compared to control populations (Fig. 5). Results from our previous work (Rossbach et al., 2019) indicated 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 with the added cost of reduced growth. We therefore hypothesized that the continuous chronic exposure towards Ag NPs would lead to an increase in antioxidant defenses, in order to avoid oxidative stress manifestation, which in turn is beneficial for the nematode reproductive capacity. However, results from the current study showed an increase in the *sod-1* antioxidant defenses in the F3 generation, but not in the F6 generation. This could reflect that the antioxidant defense response preceded the onset of adaptive response at a phenotypic level, as observed by the significant increased reproduction seen in the F5 generation of wild type (Rossbach et al., 2019). It appears that, although ROS production and oxidative stress are important modes of action of Ag NP toxicity, the fortification of reproductive capacity is the dominating adaptive effect, conceivably at the expense of antioxidant defenses.

5. Conclusion

The current study was undertaken to investigate the activation of the antioxidant defenses and the effects on oxidative stress response following a multigenerational exposure towards either $AgNO_3$ or NM300K 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 that implicate changes in the SOD-1 antioxidant defense system. It was therefore 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. The multigenerational exposure of GA508 and GRX to Ag NPs verified a strong modulation effect on *sod-1* expression and on the glutathione redox balance at F3, respectively. This effect was temporal, and by F6 the antioxidant response towards Ag was reduced significantly below that of the unexposed population. The fact that nematodes exposed to Ag for six consecutive generations showed a higher reproductive capacity (fertility) when challenged with Paraquat, despite a reduced overall antioxidant defense capacity, suggests a profound adaptive change in the nematode physiology.

CRediT authorship contribution statement

Dr Lisa M. Rossbach: Planning and conducting of the experiment, sampling, data analysis, writing of the manuscript. **Prof. Deborah H. Oughton:** Funding of the project, data interpretation, and writing of the manuscript. **Dr. Erica Maremonti**: Sampling and writing of the manuscript. **Dr. Dag M. Eide**: Statistical modeling for data analysis, data presentation, and manuscript revisions. **Dr. Dag A. Brede**: Planning of the experiment, data interpretation, and writing of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112178.

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