DELIVERABLE REPORT

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1. Executive Summary

A series of exposure studies to selected nanomaterials has been conducted on a range of test and sentinel organisms to assess for chronic exposure effects. The test protocols include both standardised OECD test guidelines and newly developed protocols for the specific test organisms. Exposures have been conducted via environmental media (water or soil) and/or the diet and effects analysis have focused on apical endpoints, spanning, mortality, development, growth, reproduction, neurological function (e.g. behaviour assays) and photosynthetic yield (algae). Studies on the aquatic algae *(Clamydomonas reinhardtii,* [EAWAG]) show that dissolution of AgNM300K is much higher in the presence of algae which explains the higher toxicity in silver nanomaterial exposures compared with for larger silver particles. This work has also shown that silver can affect the homeostasis of copper, an essential metal in algae (and other organisms too). In *Caenorhabditis elegans* [IUF] AgNM300K induced a concentration-dependent reduction in the life span and had an inhibitory effect on locomotion. AgNM300K also had a significant inhibitory effect on reproduction and the inhibitory effect on egg laying was associated with accumulation of Ag in the egg-laying organ (vulva). These effects were seen only for high AgNM exposures and it has not been determined if they relate to a specific effect of the Ag nanomaterial or due to dissolution of Ag ions. ZnO and CeO_2 had no effects on longevity, vulval function or age-associated locomotion of the nematodes. It was not possible to draw any firm conclusions on chronic exposure of isopods (*Porcellio scaber* [UNI-LJ]) to nano-TiO_2 as mortality rate in control groups was high, likely linked to stress in the laboratory maintained animals. No evidence was collected however for exposure effects of nano-TiO_2 on the number of juveniles hatched or on feeding behaviour (feeding rate) of females. There was a suggestion of an enhanced destabilisation of the membranes of digestive gland cells in isopods chronically exposed to nano-TiO_2. A pilot study on zebrafish (*Danio rerio*, [UoE]) has shown that adopting the use of isotopically labelled silver we can detect uptake into body tissues for environmentally relevant exposures and this work has provided evidence for maternal transfer to developing eggs. This work has provided the basis for a multigenerational study on the

effects of nano silver in the zebrafish. In this forthcoming study using stabilised silver NMs we propose to investigate for particle- versus silver ion- related effects.

2. Introduction

In this work package we have conducted a series of studies on a range of test and sentinel organisms to assess the chronic exposure effects for exposures to selected nanoparticles (NPs). Studies have been conducted through dose-responses analysis for a selection of NPs that are currently suspected to have biological effects (e.g. nano silver). The tests conducted include those following standardised OECD test guidelines but also newly developed protocols that are better suited for studies on NPs for the specific test organisms. Exposures have been conducted via environmental media (water or soil) and/or the diet and effects analysis have focused on apical endpoints, spanning, mortality, development, growth, reproduction, neurological function (e.g. behaviour assays) and photosynthetic yield (algae). The test organisms include aquatic algae *(Clamydomonas reinhardtii,* [EAWAG]) fish (*Danio rerio*, [UoE and KIT] and terrestrial invertebrates, including *Caenorhabditis elegans* [IUF] and the isopod (*Porcellio scaber,* [UNI-LJ]).

3. Approach

We have structured the report in a manner that is best suited to illustrate the experiments undertaken and effects identified for each of the test species. The report details the experiments conducted and results obtained and conclusions drawn for the different test species separately. In the final conclusions section we illustrate the commonalities and differences in the effects analyses for the different species studied and particle types tested and provide further critical insights to the findings and their implications.

4. Test organisms:

We used a range of organisms, listed above, that include both aquatic and terrestrial species. Details on the biology of these organisms and why they have been selected for study are provided in the deliverables 6.1 report.

5. Selected particles

Various metal and metal oxide based particles were applied in the chronic ecotoxicity exposure studies. They included the core focus particles for work package 6: Ag NM-300K, ZnO NM110, CeO_2 NM212 and CeO_2 NM211 NMs, as well as other particles designated for study under NanoMILE as either produced or sourced and characterised by work package (WP) 2. These particles were obtained from the European Commission Joint Research Centre (JRC) and characterized by various methods (see WP2 for details). Details on the specific particle exposures for the different test organisms are given under the subsequent experiment sections.

6. Exposure Studies

6.1. Effects of chronic exposure of silver nanomaterials (AgNM300K) on the freshwater algae *Chlamydomonas reinhardtii*.

The chronic exposures on *Chlamydomonas reinhardtii* were conducted over 72h, during which time the algae had successfully undergone at least three generations. These experiments are ongoing and have yet to be completed. Here we report only for the effects of AgNM300K at 24h, which corresponds to exposure for one

generation of the algae. We describe the bioavailability of silver in the exposure medium, the amounts of intracellular silver and the transcript profiles of selected metal transporters at 24h.

6.1.1. Materials and Methods

Exposure conditions: The algal cells were grown in ModTalaCa^{2+} F20 medium in Erlenmeyer flasks under controlled axenic (single species culture) conditions (90 x rpm, 23 °C, 276 µE m^{-2} s^{-1}, continuous illumination) in an incubation shaker. Exposures to different nanoparticle concentrations were performed in ModTalaCa^{2+} F20 medium with exponentially growing cells at a final concentration of 2 x 10^5 cells ml^{-}. After 24h, 72h and 96h of exposure, samples were taken for the analysis of different physiological and molecular endpoints.

Bioaccumulation: To ensure no contamination of metals, all experimental materials were soaked in HNO_3 (0.03 M, suprapure) overnight. In addition, the cellulose nitrate filters were boiled in HNO_3 and dried at 50 °C. The cultures exposed to nanoparticles were harvested (3000 rpm, 5 min, RT) and washed in fresh ModTalaCa^{2+} F20 medium three times to remove nanoparticles adsorbed on the surface of the algae. To complex and remove particles as well as free metal ions adsorbed to the cell surface, the samples were washed with 1 mM cysteine for 10 min. Subsequently 10 ml of each culture were collected on cellulose nitrate filters (pore size 0.45 µm, Sartorius, Germany) in triplicates. The filters were dried and digested with 4 ml HNO_3 (65% suprapure) in a High Performance Microwave Reactor (UltraCLAVE, MLS, Germany).

6.1.2. Results and Discussion

Estimation of bioavailable silver in the presence of algae using cysteine, a silver ligand. Our earlier studies, reported in D6.1, indicated that the toxicity of the AgNM300K on *C. reinhardtii* is due to the free silver dissolved from the nanomaterials. This was seen for several endpoints including inhibition of photosynthetic yield, growth and ATP content. However, when the toxicity was estimated as a function of free silver in the exposure medium, in the absence of algae, the nanomaterial seemed more toxic than equi-molar amounts of free silver ions in the medium. Therefore, we concluded that the dissolution of AgNM300K is higher in the presence of algae which in turn explains the higher toxicity in nanomaterial exposures. To estimate the bioavailable silver in the AgNM300K exposures in the presence of algae, cysteine which is a silver ligand was used. *C. reinhardtii* were exposed to 5 µM AgNM300K in the presence of varying concentrations of cysteine and the toxicity was determined by estimating the photosynthetic yield of the algae at 30 min and 24 h (Figure 1). In the absence of cysteine, 5 µM AgNM300K completely inhibited the photosynthetic (PS) yield at 30 min and 24 h. In the presence of 150 nM cysteine, the PS yield at 30 min recovered to about 30% and at 24 h recovered to about 50% of the control untreated algae. In the presence of 500nM cysteine the algae had a PS yield similar to that of the control, both at 30 min and 24h. This indicates that at least 500nM of silver is bioavailable to the algae, assuming each cysteine ligand binds to one silver ion. This suggests that nearly 10% of silver from AgNM300K is dissolved in the presence of *C. reinhardtii,* which is about 5 times higher than that measured in the absence of algae. Thus the presence of the algae fundamentally affects the rate of dissolution.

At 30 min of exposure the algae recover only 10 % of its PS yield when compared to that 24 h. This could be due the presence of other ligands released and also the efflux silver during 24h.

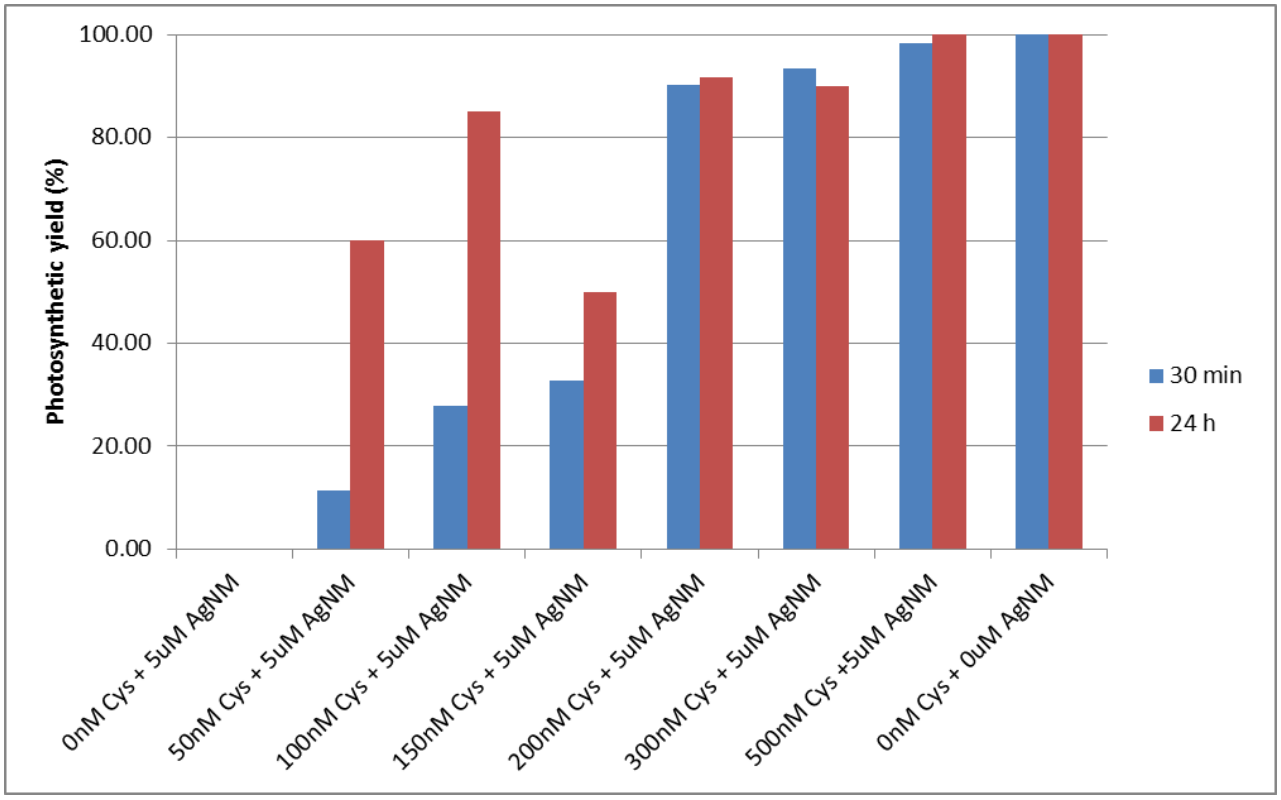


Figure 1: Recovery of photosynthetic yield in algae exposed to 5 µM AgNM300K in the presence of varying amounts of the ligand, cysteine.

Bioaccumulation of silver and its effects on copper homeostasis in algae chronically exposed to AgNM300K: The aim of the experiment was to determine silver bioaccumulation in *C. reinhardtii* during chronic exposure to AgNM300K. Here we present the data for 24 h only as a full analysis of the samples has yet to be conducted. Silver has been suggested to be taken up by *C. reinhardtii* via the copper transporters and this in turn could affect the uptake of the essential metal copper. Estimated intracellular concentrations of silver and copper on exposure to AgNM300K are shown in figure 2 (24h). Increasing amounts of intracellular silver were shown with increasing AgNM300K exposure concentrations. However, the amount of intracellular copper decreased with increasing exposure concentration of AgNM300K (with the exception of the lowest exposure concentration). This suggests that silver can affect the homeostasis of copper with possible implications for silver NP exposure on this essential metal in algae (and possibly other organisms too).

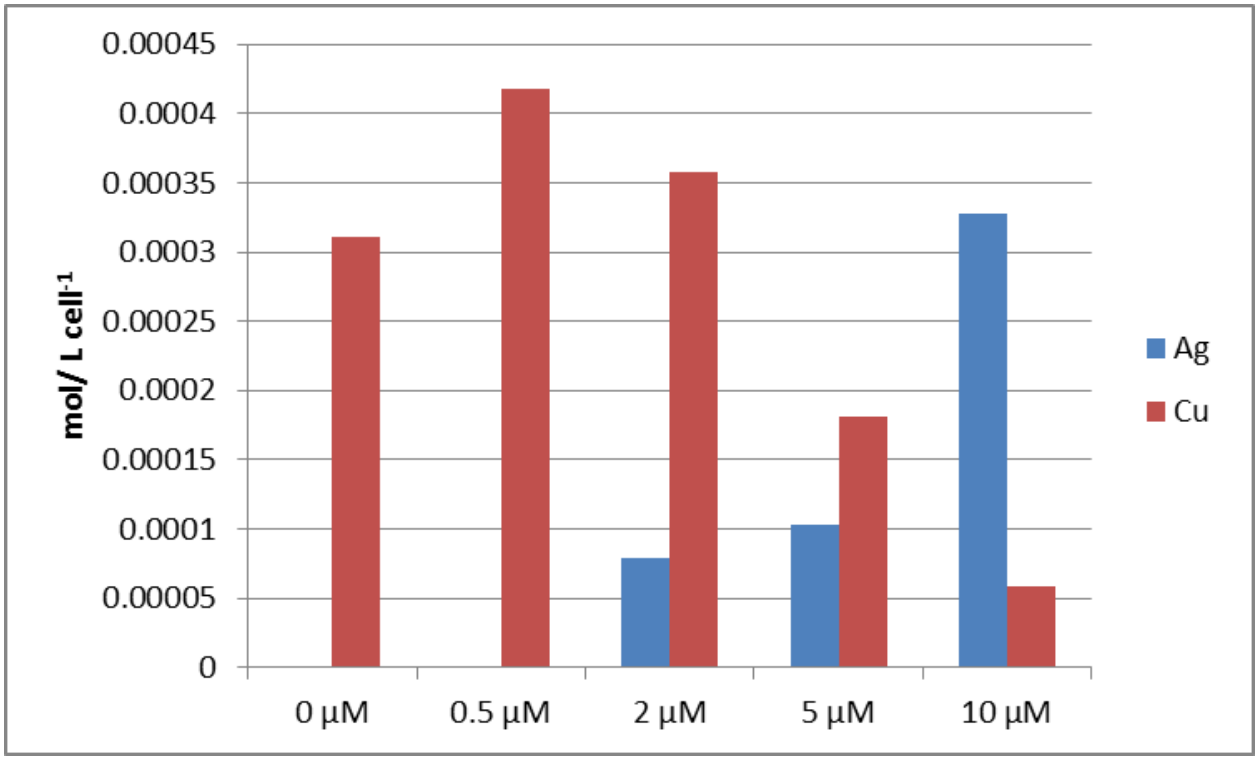


Figure 2: Amounts of intracellular silver and copper in algae exposed to AgNM300K for 24h.

Expression of copper transporters and role in silver transport: The amount of intracellular copper decreased in *C. reinhardtii* on exposure to an increasing amount of AgNM300k indicating that copper homeostasis was affected. We therefore investigated the expression of some copper transporters in the silver nanomaterial exposed algae. Total RNA was isolated from the algae and reversed transcribed to cDNA, which was then used for real-time PCR. RNA extracted from algae exposed to 5 and 10µM was degraded and therefore was not used. The two copper transporters studied were CuATPase, which has been shown in bacteria to efflux silver, and CutA, a copper chaperone involved in the transport of copper from cytoplasm to other organelles. We also studied the expression of the protein Catalase (Cat2) which is involved in oxidizing hydrogen peroxide. 18s was used as reference gene.

Compared with the untreated algae, exposure to 0.5 µM and 2 µM silver NP induced an increase in the expression of Catalase (Cat2), indicating an increase in oxidative stress due to nanomaterial exposure (Figure 3). The CuATPase transcript was also increased in the nanomaterial treatment groups, with a higher transcript level at 0.5 µM compared with that for the 2 µM exposure. The bioaccumulation experiments (Figure 2) indicated no intracellular silver in algae exposed to 0.5 µM of the silver nanomaterial which is probably related to the increased expression of the efflux protein CuATPase. The copper chaperone CutA was overexpressed at 0.5 µM but down-regulated in the algae exposure at 2 µM of silver nanomaterial. A possible explanation for this is that copper homeostasis is affected negatively at the 2 µM exposure, as observed in the bioaccumulation experiments.

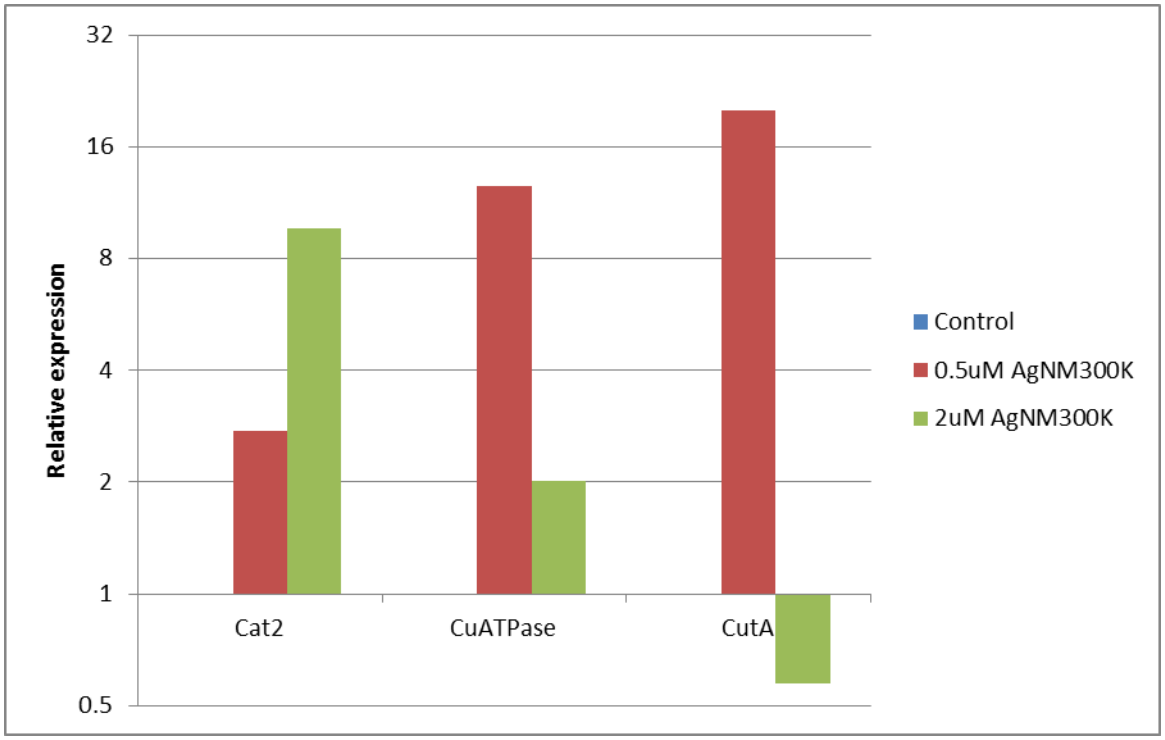


Figure 3: Transcript profile selected genes on exposure to AgNM300k. Cat2- Catalase; CuATPase – Copper ATPase; CutA – copper transporter A

6.1.3. Conclusions

The main conclusions drawn on the studies on *C. reinhardtii* are that the dissolution of AgNM300K is much higher in the presence of algae which explains the higher toxicity in nanomaterial exposures. This work also suggests that silver can affect the homeostasis of copper indicating implications for silver NP exposure on this essential metal in algae (and possibly other organisms too). Ongoing studies will establish the long-term exposure effects of AgNM300K on *C. reinhardtii* over a period of 3 generations (72h). This work will be expanded upon (beyond the requirements for this deliverable in NanoMILE) to investigate the expression of other key transcript and proteins involved in the maintenance of homeostasis in the algae exposed to AgNM300K. A technological challenge in this work has been obtaining good quality RNA from algae exposed to higher concentrations of AgNM300K. We believe this might relate to the silver interfering with the extraction process and this is an issue we are currently looking into to try and resolve.

6.2. Effects of chronic exposure to MNMs, focused on reproduction in *C. elegans*

C. elegans is a 1 mm long nematode that lives in microbe-rich habitats on rotting plant material. Its life cycle consists of embryonic development, four larval stages and a period of 3 to 4 weeks as an adult hermaphrodite worm. This short life span enables chronic exposure studies and age-resolved analyses of nanoparticle-bio-interactions for studies on longevity, fecundity and behavioural phenotypes.

6.2.1. Materials and Methods

For analysis of nanoparticle effects on *C. elegans* longevity wild type worms (N2) and long-lived (daf-2) or short-lived (daf-16) mutants with respective defects in insulin signalling pathways were used. The worms were left untreated or treated with a variety of environmentally relevant MNMs at different concentrations (Figure 1, arrow). The organisms were cultured in 96-well microtiter plates and provided with abundant feed (with bacteria) during

the MNMs exposure studies. Details on the culture conditions adopted for *C.elegans* are published in Scharf et al (in press) and Petrascheck et al (2008).

6.2.2. Results and Discussion

In all experiments short-lived daf-16 worms had a maximal life span of 30 days (red survival curves), whereas long-lived daf-2 mutants lived up to 40 days (blue survival curves). In comparison to untreated worms (N2, light grey) Ag MNMs (NM-300K) induced a concentration-dependent reduction in the life span of *C. elegans* (Figure 1A). Concentrations of 100 μg/mL Ag MNMs were toxic and there was a maximal survival of adult hermaphrodite *C. elegans* to day 8, which fell well below the longevity for short-lived daf-16 worms (Figure 1A, black and red survival curve, respectively).

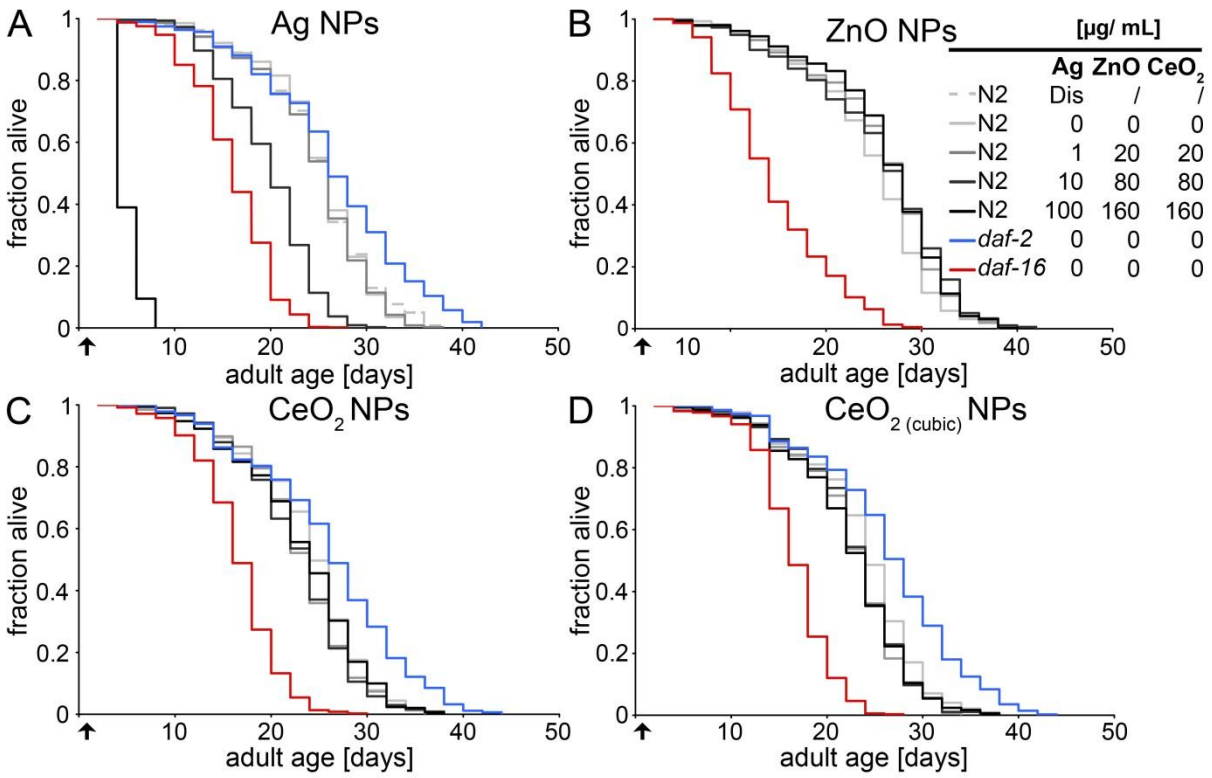


Figure 1. (A) Adult *C. elegans* that were exposed to 10 or 100 μg mL^{-1} Ag MNMs showed a significant decrease ($p<0.01$) in survival in comparison to untreated worms (light grey). In contrast, the life span of nematodes exposed to 1 μg mL^{-1} Ag MNMs or the dispersant control was similar compared with untreated controls. *C. elegans* exposed to 20, 80 or 160 μg mL^{-1} of (B) ZnO MNMs, (C) CeO_2 MNMs or (D) CeO_2 (cubic) MNMs have similar survival curves compared with untreated control worms. Long-lived daf-2 mutants (blue) and short-lived daf-16 mutants (red) show extended or shortened survival curves that define the window of expected survival in *C. elegans*. Arrows, days of particle addition; dis, dispersant only.

In contrast with Ag MNMs neither ZnO (NM-110) nor CeO_2 MNMs (NM-211; NM-212) showed effects on *C. elegans* longevity for concentrations between 20 and 160 μg/mL (Figure 1B-D). All survival curves of MNM-treated worms ran between the short-lived daf-16 and long-lived daf-2 mutants with maximal survival of up to 40 days suggesting that ZnO or CeO_2 MNMs do not reduce the longevity of *C. elegans*.

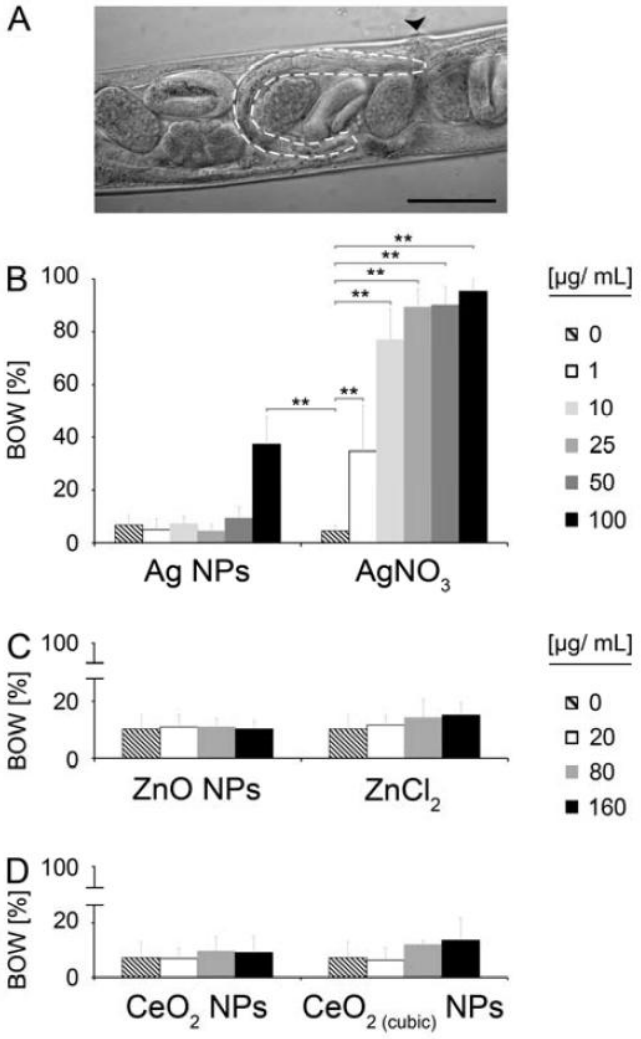


Figure 2. Internal hatch is induced by Ag ions ($AgNO_3$) and toxic concentrations of Ag NPs (NM-300K). (A) Differential interference contrast of internally hatched *C. elegans* larvae. Arrowhead, vulva. Bar, 50 µm. (B-D) Quantification of the BOW phenotype in adult *C. elegans* after 24 hours of treatment with H_2O-, Ag ion- or Ag, ZnO or CeO_2 MNMs. **, significance ($p<0.01$) in comparison to untreated control worms; BOW, bag of worms; NPs, nanoparticles.

Next we investigated effects of MNMs on the reproductive capacity of *C. elegans*. The nematode is a self-fertilizing hermaphrodite that lays eggs through the egg-laying organ vulva (Figure 2A, arrowhead). Malfunction of the vulva is generally correlated with respective mutations, or observed in older *C. elegans,* and manifests in the bag of worms (BOW) phenotype that is characterized by hatching of the progeny within the parent's body (Figure 2A, dashed lines). We have shown previously that certain engineered nanomaterials such as silica NPs significantly induce the internal hatch phenotype and that this effect could be reversed by the anti-aging and anti-convulsant drug ethosuximide suggesting involvement of the neural circuit that controls vulval function (Pluskota et al., 2009). Subsequent analyses have identified a cascade of events that includes accumulation of silica NPs in single vulval cells, induction of axonal protein aggregation in HSN neurons and impairment of serotonergic neurotransmission within the egg-laying circuit (Scharf et al., 2013; Scharf et al., in press).

In the testing of NanoMILE MNMs on induction of internal hatch, worms were cultivated in liquid medium in 96-well plates and left untreated (H_2O) or treated with $AgNO_3$, Ag MNMs (NM-300K), ZnO MNMs (NM-110) or CeO_2 MNMs (NM-211, -212) at increasing concentrations for 24 hours. Ag ions and high concentrations of Ag MNMs (100 μg/mL) induced the BOW phenotype, whereas neither ZnO nor CeO_2 MNMs had any effects on reproduction (Figure 2). Notably, Ag MNMs in concentrations between 1 and 50 μg/mL were ineffective with regard to induction of internal hatch (Figure 2B). These results support the life span analyses findings where concentrations above 10 μg Ag MNM /mL only induced a reduction of *C. elegans* longevity (Figure 1A).

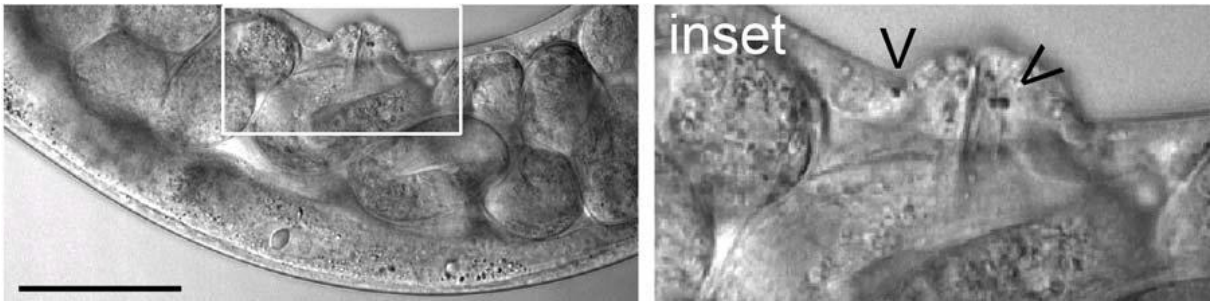


Figure 3. Ag MNMs translocate to the vulva of *C.elegans* (black arrowheads). Representative differential interference contrast micrographs of the *C.elegans* vulva after 24 hours of treatment with Ag MNMs (100 μg mL^{-1}). Bar, 50 μm.

As it was demonstrated that Ag MNMs impairs the function of the egg-laying organ, the vulva we next analysed if nanoparticles or dissolved Ag ions located at the vulva in exposed animals. In this work adult hermaphrodite worms were treated for 24 hours with 100 μg/mL Ag MNMs (NM-300K), immobilized on agarose pads and analysed by differential interference contrast (DIC) microscopy. In these analyses we found localization of Ag MNMs or dissolved Ag ions in vulval cells appearing as focal areas of black staining (Figure 3, inset, arrowheads). Such local distribution suggests that Ag MNMs or dissolved Ag ions penetrate single vulval cells and likewise may penetrate neighbouring HSN neurons. To investigate if Ag MNMs directly interact with HSN neurons as nanoparticles higher resolution imaging, such as transmission electron microscopy (TEM) and coherent anti-stokes Raman scattering (CARS) spectro-microscopy are required and are scheduled for future analyses.

To investigate neural phenotypes in an age-resolved manner, locomotion of adult hermaphrodite worms was monitored and quantified during the entire life span of the worms. The locomotion phenotypes were subdivided into 3 groups, namely swimming, uncoordinated movement and head/tail movement only. Adult *C. elegans* were left untreated or treated with MNMs at a range of concentrations and the locomotion behaviour inspected and quantified by stereo microscopy every second day (Figure 4).

At the start of the experiments all 2-day old worms that were treated with H_2O, ZnO MNMs (NM-110), CeO_2 MNMs (NM-211, -212) or non-toxic concentrations of Ag MNMs (NM-300K) swam. This behaviour declined over time in an age-dependent manner in controls as well as MNM-treated *C. elegans*. In contrast, 2-day old worms that were treated with 100 μg/mL Ag MNMs showed an immediate ($p<0.01$) reduced swimming activity compared with untreated controls (Figure 4A, blue squares). Reduced swimming was correlated with a significant increase of uncoordinated locomotion and movement that is confined to the head and the tail region in *C. elegans* exposed to 100 μg/mL Ag MNMs. Significantly impaired locomotion behaviour was likewise observed in 8-day old worms that were treated with 10 μg Ag MNM/mL suggesting that Ag MNMs may impair neural behaviours in a

concentration- and age-dependent manner. As neither ZnO nor CeO_2 MNMs modified the locomotion of adult *C. elegans* during their life span it can be concluded that these nanomaterials do no impact on longevity, vulval function or age-associated locomotion of the nematodes. However, a concentration-dependent impact on life span and neural phenotypes was seen for exposure to Ag MNMs. We have not established if the observed effects are specific for the Ag nanomaterial or due to Ag ions as a consequence of dissolution.

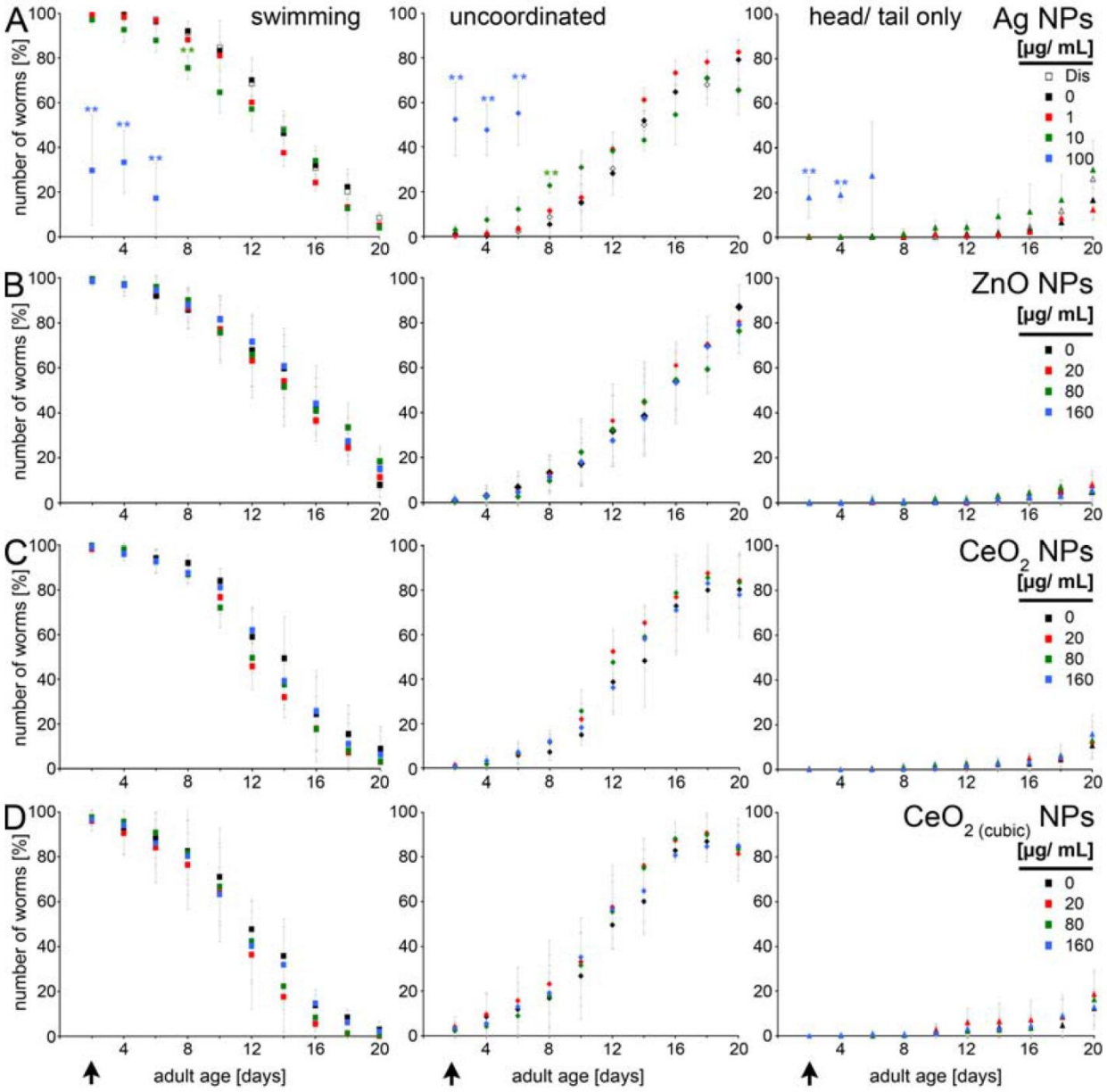


Figure 4. **Quantification of age-related locomotion in adult *C. elegans*.** Locomotion phenotypes were classified according to the three groups 'swimming', 'uncoordinated' or 'head/ tail only' movement and plotted against adult worm age. All graphs show an age-related decline of swimming in nematodes and simultaneous increase of uncoordinated and head/ tail only movements. Phenotypes were scored in nematodes exposed to (A) Ag MNMs (NM-300K) or the dispersant only control, (B) ZnO MNMs, (C) CeO_2 MNMs and (D) CeO_2 (cubic) MNMs in comparison to untreated controls (black). **, $p<0.01$; arrows, addition of MNMs; dis, dispersant only.

6.2.3. Conclusions

The main conclusions drawn on the studies on *C. elegans* are that Ag MNMs show significant effects on longevity, reproduction and neural phenotypes and they do so in a concentration-dependent manner. Such nanoparticle-bio-interactions were not observed however for exposures with ZnO or CeO_2 MNMs. By means of differential interference microscopy Ag was identified in the egg-laying organ, the vulva, and this may help explain the adverse effects of exposure to Ag nanoparticles on reproduction. It has yet to be determined if Ag nanoparticles and / or dissolved Ag ions are responsible for the observed chronic effects. Neither ZnO nor CeO_2 MNMs modified locomotion of adult *C. elegans* during entire their life span and it can be concluded that these nanomaterials do not impact on longevity, vulval function or age-associated locomotion of the nematodes. However, a concentration-dependent impact on life span and neural phenotypes was observed after exposure with Ag MNMs and it will be important to determine in upcoming analyses if the observed effects are specific for the Ag nanomaterial or due to dissolution of Ag ions.

6.3. Effects of chronic exposure to MNMs on isopods

For terrestrial isopods the main route for contaminant intake is via the diet (Drobne 1997). Exposure of terrestrial isopods (prominently *P. scaber*) to contaminant-treated food has been established as a standard toxicity test (Drobne &Hopkin 1994, 1995). Calculation of feeding rate, faecal production rate and food assimilation enables assessment of organism-level physiological response to nanoparticles and their potential toxicity (in case of elevated mortality of test animals). Uptake of nanomaterials (NMs) into isopods however could also occur from the soil.

In this work we conducted a series of chronic exposures of isopods to TiO_2 NMs via the soil and via the diet. A series of studies of shorter duration were conducted exposing adult isopods via the diet to TiO_2 NMs and AgNM to investigate effects on reproduction.

6.3.1. Materials and Methods

Terrestrial isopods *P. scaber* (Isopoda, Crustacea) were collected during May 2014 and September 2014 at an uncontaminated location near Ljubljana, Slovenia. The animals were kept in a terrarium filled with a layer of moistened soil and a thick layer of partly decomposed hazelnut tree leaves (*Corylus avellana*), at a temperature of $20 \pm 2^{o}C$ and on a 16:8-h light:dark photoperiod. Only adult animals (of both sexes) weighing more than 30 mg were used in the experiments. If moulting or the presence of marsupium (brood pouch) were observed, the animals were not included in the experiment in an attempt to use a population as physiologically homogenous as possible.

The materials tested included: (1) nano-TiO_2 (NNM PROm-TiO2un-10nm-121113c), nano-TiO_2 with PVP (NNM PROm-TiO2PVP-10nm-121113c) and $CoCl_2$ as positive control; (2) AgNM300K.

Exposure of isopods to MNMs (in treated soil and via the diet)

Before the experiments, we determined the maximum water holding capacity (WHC) of the Lufa 2.2. soil (based on description given in ISO No. 11268-2 10390). 100% WHC - 42,5 ± 0,3 g H2O/100 g dry soil. We measured pH of the soil (ISO DIS 10390: Soil Quality – Determination of pH) and the soil moisture content was determined.

In the first two soil exposure tests (with TiO_2NM) we used rectangular containers made of chemically inert plastic of about 1-3 L capacity. The design of the container cover permitted gaseous exchange between the substrate

and the atmosphere and access to light (e.g. by means of perforated transparent lid) whilst preventing the animals from escaping. In the third experiment conducted with soil and diet were both dosed with TiO_2 NMs the containers used were smaller, with a 500 mL capacity, and they were set up in two replicates for each exposure group. In the fourth experiment (4a and 4b, assessing for effects of TiO_2 NMs and Ag NMs, respectively, on reproduction for exposures via the diet) we used larger sized plastic terrariums with a capacity of 10 L to allow us to set up a more realistic natural environment with the aim to reduce the stress imposed on the animals during the experiment. In experiments 3 and 4, small stones were added to each container to provide animals with shelter.

We prepared suspensions of nano-TiO_2 and nano TiO_2 PVP in distilled water. The required quantity of nanoparticle dispersion was measured to produce a concentration of nanoparticles in the soil at 1000 mg particles/kg dry soil. After stirring of the nanoparticle dispersion it was added to air-dried soil, and mixed with the soil thoroughly. Air drying of experimental soil took at least 48 h at room temperature, or 24 h in ventilation oven at 30°C. Sufficient water was then added to attain a final soil moisture content of 40% before the experiment.

In the first experiment where the whole test soil was moistened, most of the animals in control group died, and therefore in the remaining experiments only one half of the soil in the test container was moistened to give a desired final moisture content of 40 % and the rest of the soil was left dry. In this scenario, animals could independently select to move between dry or moistened soil.

At the start of the 6 week exposure studies, we collected animals from laboratory culture, weighed them and put 10 animals into each test container. Containers were placed in a cultivation chamber which ensures controlled and stable conditions with a temperature of 20 +/- 2 ºC and natural light cycle of 16 hours of light and 8 hours of darkness. Every week, soil moisture was checked and evaporated water substituted for the soil moisture to keep this at 40 %. Animals were checked every day or at least every second day. Dead animals or females with marsupium were removed from the experiment.

We also exposed females with a marsupium to the MNMs and observed feeding behaviour (feeding rate) and changes in offspring production. We exposed 10 females (in each exposure group) with well-developed marsupium to un-coated and PVP coated nano-TiO_2 and $CoCl_2$ (positive control). The nominal concentrations used were 1000 µg of nano-TiO_2/mg dry leaf. The duration of exposure was between 7 and 21 days, depending of hatching of mancas (post-larval juveniles (having undergone direct development from embryos within the female marsupium)). A similar series of two experiments were conducted with nano-Ag and Ag dispersant (Silver dispersant JRC-NM300KDIS). The nominal concentration of MNMs was calculated on Ag and was 1000 µg Ag/g dry weight of leave. The duration of exposure was between 7 and 21 days, depending of hatching of mancas. Hazelnut leaves were collected from an uncontaminated area and dried at room temperature. Dried leaves were cut into pieces of approximately 100 mg.

Each individual animal was placed in a 9-cm Petri dish. One hazelnut leaf treated with distilled water or nano-TiO_2 suspension with nominal concentration 1000 µg of nano-TiO_2/g was placed in each Petri dish and was the animal's only food source. Humidity in the Petri dish was maintained by spraying tap water on the internal side of the lid every day. All Petri dishes were kept in a large glass container under controlled conditions in terms of air humidity (2:80%), temperature (21 ± 18C), and light regimen (16:8h light:dark photoperiod). The duration of exposure on females with marsupia exposures were run for from 7 to 21 days (depending on when hatching of juveniles occurred).

The feeding rate of isopods was calculated as the mass of consumed leaves per animal's wet weight per day.

The food assimilation efficiency was calculated as the difference between the mass of consumed leaves and the mass of faecal pellets divided by the mass of consumed leaf.

Elemental analyses of animal tissue with ICP-MS and AAS.

After exposure of females via the diet to MNMs, we prepared them and the hatched juveniles for ICP-MS and AAS measurements. AAS relies on an atomic absorption process, while ICP-MS is an atomic/ionic emission spectroscopic technique. The twin method approach is required as the two methods have different sensitivities (Tyler 2000). In the nano-TiO_2 experiment the AAS measurements were performed at the UnLj and samples from the nano-Ag exposure samples were send to University of Birmingham (UoB, NanoMILE partner No 1) for ICP-MS measurements.

In the first experiment on brooding females for exposure to nano-TiO_2, females were exposed from 7 – 21 days (depend on when hatching occurred) and after the hatching juveniles were counted, weighted and prepared for digestion. Females were also weighted and prepared for the AAS analyses. Digestion was conducted as described our previous published work, Romih et al (2015).

In the second experiment on brooding females were left animals exposed to nano-Ag for 7 -14 days and remove the mancas just before hatching in order to avoid contact of offspring with leaves treated with NPs (to exclude measuring the element content of surface of juveniles bodies). Females and offspring were immediately frozen in liquid nitrogen and then send to UoB on dry ice for ICP-MS measurements.

Digestive gland cell membrane stability assay

The membrane integrity of the digestive gland is expected to be directly proportional to the degree of membrane damage caused by the presence of nanomaterials. The acridine orange (AO)/ethidium bromide (EB) assay is based on the assumption that changes in cell membrane integrity result in differences in permeability of cells to AO and EB dyes. Different permeability by the two dyes results in differentially stained nuclei as follows. Acridine orange is taken up by cells with membranes that are intact or destabilized, and in the cell, emits green fluorescence, as a result of its intercalation into double-stranded nucleic acids. Ethidium bromide on the other hand, is taken up only by cells with destabilized cell membranes, and it emits orange fluorescence, after intercalation into DNA. This membrane stability was tested with the modified method previously described by Valant et al (2009). Isolated hepatopancreatic tubes were incubated for 3 min in a mixture of the fluorescent dyes AO and EB and then put on a microscope slide. Fresh samples were examined by an Axioimager.Z1 fluorescent microscope (Zeiss) and photographed with two different sets of filters. The excitation filter 450 to 490 nm and the emission filter 515 nm (filter set 09) were used to visualize AO and EB stained nuclei, while the excitation filter 365 nm and the emission filter 397 nm (filter set 01) were used to visualize nuclei stained with EB only. The cell membrane integrity was assessed by examination of the micrographs. Photographs of intact digestive glands were examined by the same observer twice at intervals over a period of at least 24h. The integrity of cell membrane was assessed visually and classified on the basis of a predefined scale from 1 to 10, where values 1 – 2 represent no effect on digestive glands, values from 3-5 moderate digestive glands membrane destabilization and values from 6 -10 severe membrane destabilization. From preliminary experiments, it was concluded that the non-treated (control) animals show <5% of nuclei stained by EB, values 1 and 2, moderately stressed animals had from 20 – 50 % EB-stained nuclei (value 3 -5), while severely stressed animals have up to 100% of EB-stained nuclei (values 6-10). Our previously published results have demonstrated that in animals in good

physiological condition from a stock culture, the digestive gland cell membrane stability value was higher than 2 in only 5% of animals and this was taken as a benchmark (Valant et al 2009).

Statistical analysis

Differences in the medians of the measured parameters (feeding activity parameters) in the control and the nano-TiO_2-exposed groups were tested using non-parametric Mann-Whitney U tests. All of the calculations were carried out with the Statgraphics Plus 4.0 statistics software.

6.3.2. Results and Discussion

Effects of exposure of isopods to MNMs

In the first and second experiments, where animals were exposed to TiO_2 contaminated soil, the mortality of animals in control group was high with 90 % and 100% respectively. In the first experiment all animals died in the first 14 days of experiment, possibly because of over moistening of the soil. In the second experiment animals died because of a bacterial infection. The high mortality in control groups meant any exposures analyses was invalid.

In the chronic exposure experiment where animals were exposed via the soil and diet to nano- TiO_2 or nano-TiO_2 PVP, mortality of animals in control group after 6 weeks of exposure was 55 %, a level expected in long term exposures, based on our previous studies (Menard et al 2015). The highest level of mortality was observed in the group exposed to nano-TiO_2 (75%). In the surviving animals after 6 weeks exposure we analysed cell membrane stability of isolated digestive glands of survived animals. All of the surviving animals from control group did not have destabilized cell membranes of digestive gland cells (Figure 1). The higher % of destabilized membranes occurred in animals exposed to nano-TiO_2. In the group exposed to nano-TiO_2 PVP 8 out of the 10 remaining animals had destabilized cell membranes of digestive glands (Figure 1).

There was already a high mortality in control groups, because of altered physiological condition of animals. After chronic exposure of isopods to nano-TiO_2 and nano-TiO_2 with PVP via soil and leaves treated with MNMs, a slightly higher percentage of mortality was recorded in group of animals exposed to nano-TiO_2. After chronic exposure of isopods to nano-TiO_2 and nano-TiO_2 with PVP with exposure concentration 1000 ug of Ti/g on soil or on leaves, the membranes of digestive gland cells were moderately destabilised in the case of MNMs exposure and not in the control group. After exposure of isopods to nano-TiO_2 and nano-TiO_2 with PVP via soil and leaves treated with MNMs, accumulation of Ti may have occurred in some females and juveniles, but is not statistically different from that in control group. We cannot exclude the possibility that measured Ti was present on the surface of animals.

During all the experiments with isopods we find the presence of intracellular bacteria in the digestive glands of some tested animals. In their natural environment terrestrial isopods are often invaded by intracellular bacteria with a complex developmental cycle. During the infection hypertrophied cells filled with microorganisms enclosed in vacuoles can be found in the tissue. We cannot exclude that digestive gland cell membrane destabilization observed in the experiments was induced with MNMs exposure. The additional experiments with healthy population of isopods are planned and the set-up protocol for chronic exposures will be upgraded and improved. The infection is considered as a possible state of animals and has to be included into the SOP, but additional data are needed to exclude the interference of infection and NPs.

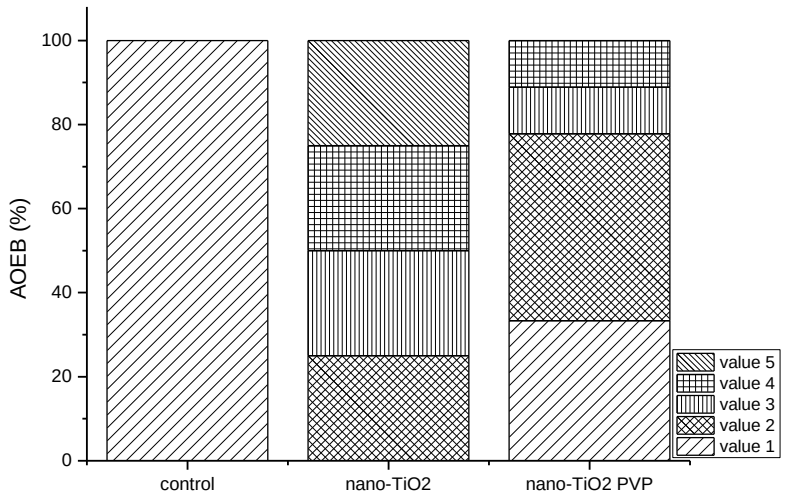


Figure 1. Percentage of animals in each exposure group with different degrees of destabilised cell membrane. Destabilization of the cell membrane was assessed visually and classified from 0 to 10 according to the predefined scale described in Methods. In this experiment the highest scoring value was 5, what represent moderately destabilized membranes.

Effect of MNMs on isopods offspring production

There was no statistically significant difference between controls and animals exposed to nanoparticles. The differences were observed only between control and positive control, $CoCl_2$ (Figure 3).

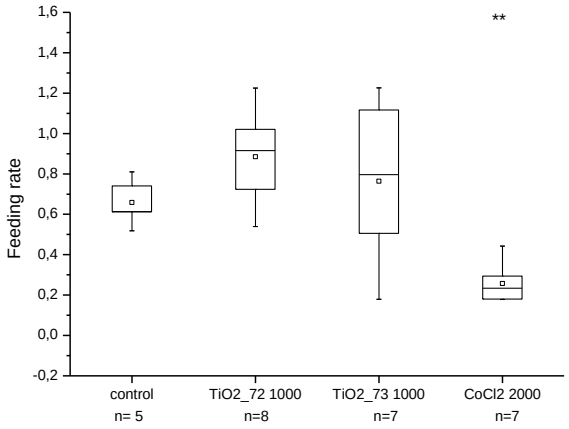


Figure 2: Feeding rate of females exposed to titanium dioxide nanoparticles. Statistically significant differences are marked with (** - $p < 0.005$). n= number of survived animals, TiO2_72 is nano-TiO_2, TiO2_73 is nano-TiO_2 with PVP. 1000 and 2000 are exposure concentrations of TiO_2 nanoparticles and $CoCl_2$ (1000 µg/g dry weight of leave).

There were also no differences in the food assimilation efficiency or faecal production in animals exposed to nanoparticles in comparison to controls. The number of mancas hatched did not change upon female nanoparticle ingestion (Figure 4).

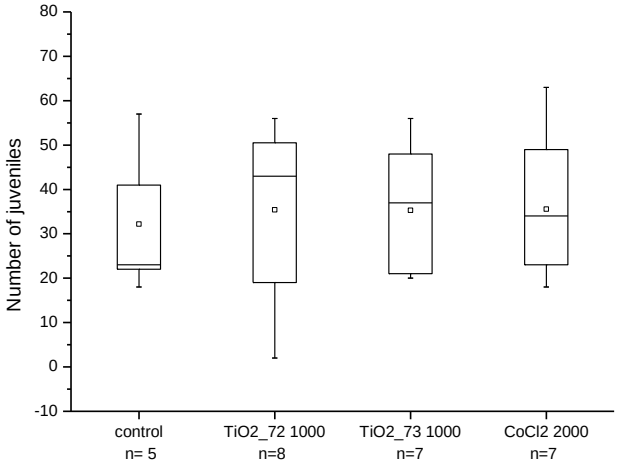


Figure 3: Number of hatched juveniles/female in control and groups exposed to nano-TiO_2 (and positive control – exposed to $CoCl_2$.) n= number of surviving animals, TiO2_72 is nano-TiO_2, TiO2_73 is nano-TiO_2 PVP. 1000 and 2000 are exposure concentrations of TiO_2 nanoparticles and $CoCl_2$.

The accumulation of Ti in juveniles and females was not statistically significantly different from amount of Ti in control animals, which have already consumed Ti from natural environment. We observed, however, that individual animals exposed to nano-TiO_2 PVP accumulated higher concentration of Ti compared with controls in case of juveniles and also for females. This was also the case for nano-TiO_2 PVP. The only statistical significant difference in element concentration was observed in females exposed to positive control, $CoCl_2$ (Figure 4*).*

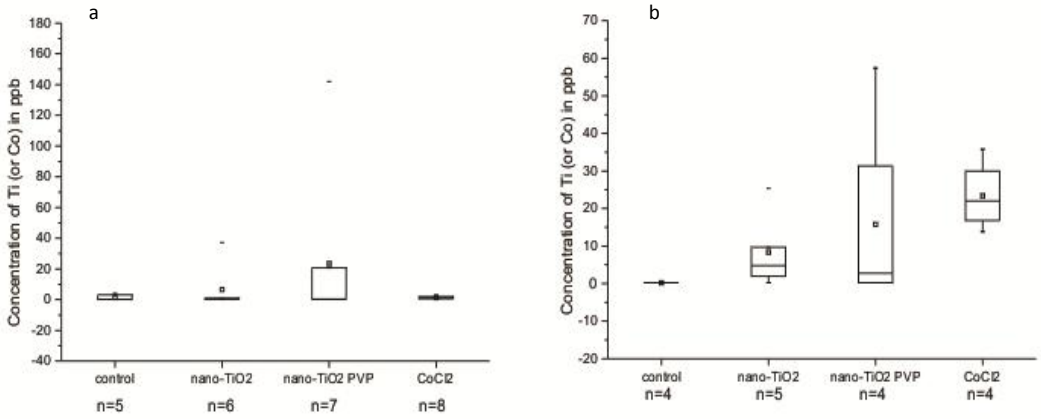


Figure 4: Concentration of Ti and Co in juveniles (a) and females (b) after exposure to nano-TiO_2, nano-TiO_2 PVP and $CoCl_2$. Exposure concentration was 1000 µg TiO_2 and Co/g dry weight of leave, n= number of samples measured. All the juveniles from one female are combined in one sample.

There was no statistically significant differences in feeding rate between control and animals exposed to nano-Ag or Ag dispersant. There was also no effect of Ag MNMs on assimilation efficiency or faecal production rate.

6.3.3. Conclusions

It is not possible to draw any firm conclusions on chronic exposure of isopods to nano-TiO_2 and nano-TiO_2 with PVP via soil and leaves treated with MNMs as mortality rate in control groups was high, likely linked to stress in the maintained animals. Exposure of females to nano-TiO_2 and nano-TiO_2 with PVP however, did not appear to have an effect on the number of juveniles hatched or on feeding behaviour (feeding rate) of females. After exposure of isopods to nano-TiO_2 and nano-TiO_2 with PVP via soil and leaves treated with MNMs, there was some (albeit limited) evidence for accumulation of Ti in some females and in developing juveniles retained in the marsupium. Chronic exposure of isopods to nano-TiO_2 and nano-TiO_2 with PVP also indicated an enhanced destabilisation of the membranes of digestive gland cells.

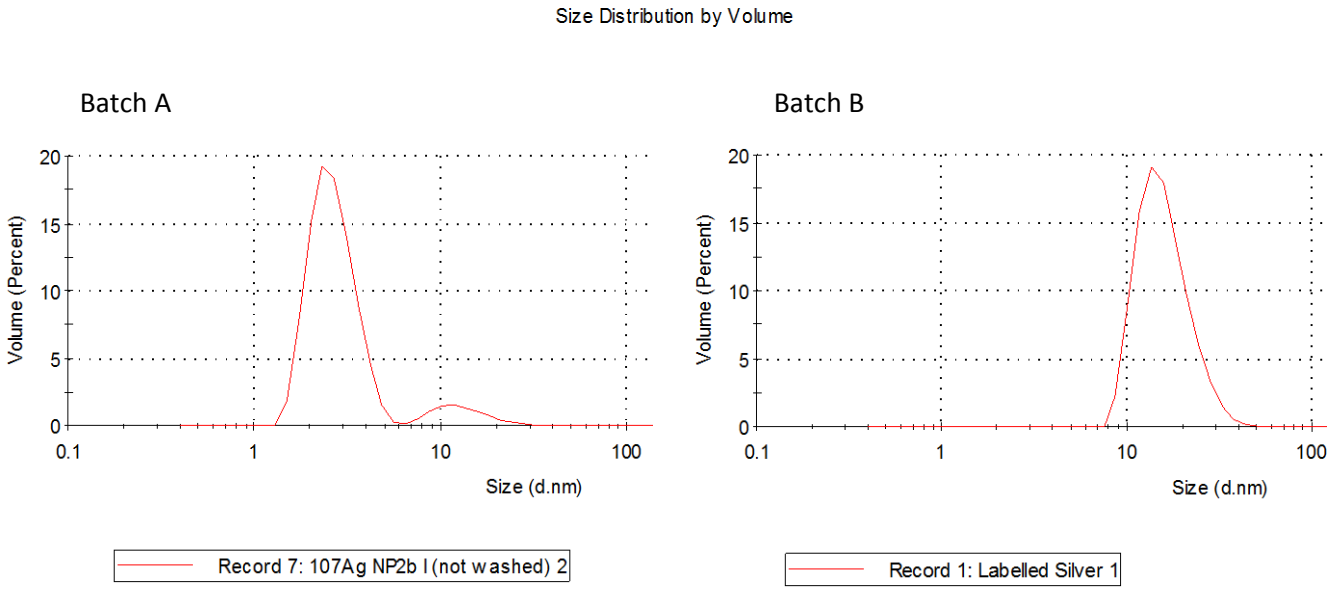
6.4 Effects of chronic exposure to silver nanoparticles in zebrafish

A study has been initiated to assess the chronic exposure effects for silver nanoparticles (NPs) on zebrafish (ZF, *Danio rerio*). Our ambition is to study effects over 3 generations of ZF assessing bioaccumulation of the NPs, reproductive output, effects on development and to conduct targeted analyses on the expression of a series of genes involved in metal handling and oxidative stress. The study materials for this work include 14 nm citrate coated Ag and a second, less-soluble, silver NP. In this manner we should be able to assess the effects (if any) that occur due to dissolution (i.e. silver ions) compared with the intact silver NP. In this work (and reported here) we have undertaken method evaluation and a range finding study for this work.

6.4.1. Materials and Methods

Table 1. Characterisation parameters of 2 trial batches of synthesised Ag particles for isotopic labelling and below (graphs) showing DLS results for particle size.

	Batch A	**Batch B**
Size (DLS)	Particles 4nm in size with some 20nm particles	Highly mono-disperse 14nm particles
Concentration	~23.5 mg l^{-1}	~6.5 mg l^{-1}
Volume	~25ml	~200ml
Yield	~30%	~50%



In Batch A the yield was much lower and the resulting concentration not as high as anticipated. The yield of the suspension in Batch B was much higher (a yield of 50%) and was thus used in this study. Ultrafiltration was applied to reduce the volume of Batch B to 20-25ml (from a volume of 200ml) providing a suspension at a concentration of 69 µg ml^{-1}.

Flake preparation and flake dosing

A major challenge in studies of this nature include the ability to detect silver in the bodies of exposure animals for exposure regimes that bear any environmental relevance. In our pilot study three concentrations of AgNP in artificial flake (fish feed) were made up: 120, 60 and 20 µg g^{-1}. The diet comprised of TetraMin Tropical Flakes-ground to a fine powder mixed with flour and the required amount of Ag NPs. In all treatments 5.4 g of ground flake (based on the requirement to feed 0.5 g zebrafish at a rate of 4% body weight for 25 days) was added to 0.6 g of flour and combined with 12 ml Ag nanoparticle suspension at concentrations of 20, 60 and 120 µg ml^{-1} to provide total Ag^{107} levels of 720, 360, 120 µg in each 6 g food batch. The amalgam for each treatment was mixed together and then spread evenly onto baking paper and dried in an oven at 50^0C. Once dried, flakes were crumbled and placed in a falcon tube for storage at 4^0C. For the pre dosing period, the adult breeding fish were fed TetraMin Tropical Flakes and subsequently during the 21 day exposure period they were fed the artificially synthesised diet containing TetraMin Tropical Flake, flour containing the dosing materials.

Fish source and husbandry

Wild type WIK strain zebrafish embryos were obtained from the Max Planck Institute, Tubingen, Germany and maintained at the University of Exeter. Fry from approximately 2 days post fertilisation (dpf) were fed on a microencapsulated diet (ZM advanced fry feed; ZM Ltd. Hampshire, U.K). This was supplemented from approximately 7 dpf with freshly hatched *Artemia nauplii*. From 21 dpf fish were fed twice daily to satiation with freshly hatched Artemia nauplii and then throughout to adulthood with freshly hatched Artemia nauplii in the morning and with TetraMin Tropical Flake Food in the afternoon. Once the exposure to the Ag materials had been initiated the breeding fish were fed/ dosed TetraMin Tropical Flake in the morning and afternoon.

Fish were maintained in reconstituted water. Mains tap water was filtered by reverse osmosis (Environmental Water Systems (UK) Ltd) and reconstituted with Analar-grade mineral salts to standardized synthetic freshwater (final concentrations to give a conductivity of 300mS: 58mg $^{l-1}$ $CaCl_2.2H_2O$, 24.65mg l^{-1} $MgSO_4.7H_2O$, 12.95mg l^{-1} $NaHCO_3$, 1.15mg l^{-1} KCl, 12.5mg l^{-1} Tropic Marin Sea Salt). This water was aerated, and heated to 28°C in a reservoir before it was supplied to each aquarium using a flow-through system. Aquarium water was routinely monitored for pH, conductivity, ammonia, nitrate, and nitrite, all of which were well within acceptable limits of U.S. EPA guidelines. The photoperiod was set to 13:11h light:dark, with an artificial dawn to dusk transition of 30min. Prior to the experiment, fish of mixed sex were maintained in large holding aquaria, 900x500x300mm in dimension, with a working volume of 112L (approximately 100 fish per aquarium**).**

Sexing and weighing/measuring fish

Fish were sexed using differences in their colouration and differences in the behaviours that exist between males and females. Individual sexually mature fish were placed carefully in a beaker of water to obtain an accurate body weight measurement. They were then placed on a laminated graph paper to measure fork length.

Experimental setup

12 tanks of fish, 6 males and 6 females (tank dimensions: 30cm X 30 cm with a volume of 12 L, with 5 full water tank replacements every 24h) were prepared per treatment (**Figure 1**). Male fish were transferred to the female tank in the evening before a spawning event and removed in the morning to minimise the stress and adverse interactions that can occur when fish are paired together for long periods of time. A pre-exposure week allowed us to ensure that all pair matches were effective (i.e. fish were spawning and behaving normally), with fertilisation and egg output monitored. The 12 tanks of fish showing the most consistent breeding over this time period were then used for the study. The three different treatments varied in their concentration of dosed isotopically labelled Ag nanoparticles per gram of food (120, 60 and 20 µg.g^{-1}).

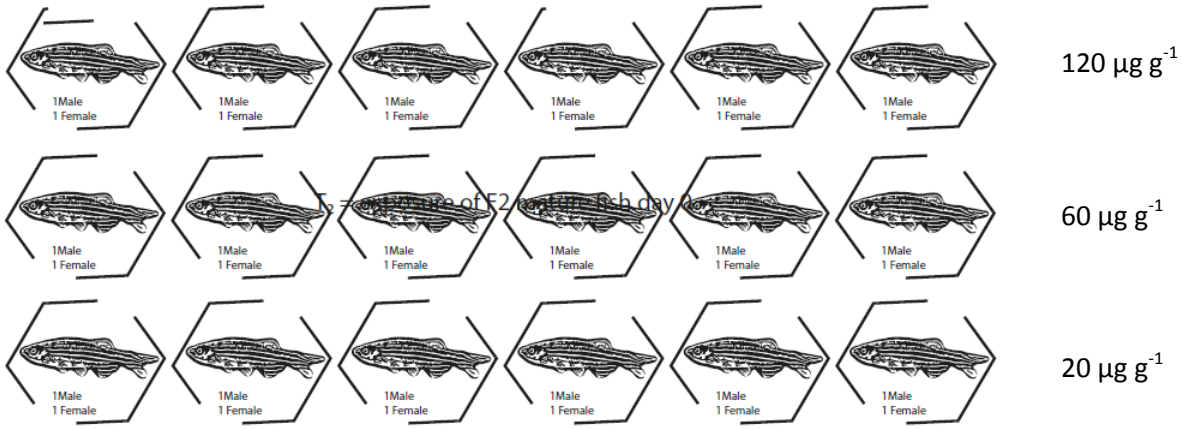


Figure 1. Schematic of experimental set-up.

Measuring egg output and fertilisation

Egg collection was undertaken 3 times a week from the commencement of the experiment, including the pre-exposure week. The glass aquaria were designed with sloping sides to form a 'funnel' to channel any eggs spawned in to an egg-collecting chamber. This facilitated daily embryo collection whilst minimizing disturbance to the fish. Glass marbles of 10mm diameter were placed in the 'funnel' section of the base of the aquaria at a depth of 3–4 marbles to act as a spawning substrate and to minimize oophagy (egg eating). An artificial weed was placed in the tanks rooted in the marbles to act as a refuge and spawning stimulus. Eggs/embryos were collected 1 h after dawn. This was done by opening a valve at the base of the egg collecting chamber, which is fitted with a fine mesh sieve, drawing water through the marbles where the eggs were trapped (marbles were simultaneously agitated). The eggs were captured on the fine mesh sieve and washed to remove any waste food and/or faeces. The eggs/embryos collected were then transferred into a Petri dish with embryo culture water. All embryos from each tank collection were used to calculate fertilisation success. Following collection from all tanks, and at approximately 2 hours post fertilization (hpf), any infertile eggs /dead embryos were easily distinguished and separated from the live embryos. For each of the test aquarium, the numbers of live and dead eggs were counted giving a record of total egg output and overall egg viability for each pair of fish.

Biological Sampling

At the end of the experiment fish were sacrificed by terminal anaesthesia (Benzocaine, Sigma Poole, UK). Measurements of fork length and weight were recorded. Fish were then dissected and the gonads and liver, weighed and stored at -80^{0}C until used for Ag content analysis by ICP-MS. Pools of 1, 2, 5, 10 and 20 embryos from treatment groups were collected at the end of the final week of exposure and snap frozen and stored at -80^{0}C

Ag concentrations in adult fish tissues and embryos determined by MC-ICP-MS

Tissue samples (liver and gonads) were defrosted at room temperature. Samples of livers and gonads were individually processed (12 replicates) giving a total of 6 livers / 6 gonads per treatment for each sex. Samples were then freeze-dried and re-weighed, before undergoing microwave digestion in HNO_3 and H_2O_2. For the embryos, each pooled treatment of 1, 2, 5, 10 and 20 were freeze dried and re-weighed before undergoing microwave digestion. An aliquot (80%) of the whole sample digest solution was processed using chemical separation with ion exchange chromatography before measurement of Ag using multi-collector inductively coupled plasma spectrometry MC-ICP-MS to determine 107Ag/109Ag ratio. Data were analysed to determine contribution of enriched 107Ag and natural Ag concentrations. Due to the expense of the analysis procedure, only selected samples that were processed underwent Ag measurement (see **Table 2** for list of analysed samples). All analysed samples were from female fish. Ag measurements were also conducted on embryos collected from non-exposure zebrafish.

Table 2. Numbers of samples from each treatment group (derived from females only) that were analysed for isotopic Ag content (numbers in the embryo column indicate the numbers of embryos pooled for isotopic Ag analysis).

Treatment	Tissue		
	Liver	*Gonad*	*Embryo*
Control	0	0	5, 10
20	2	2	0
60	2	2	1, 2, 5, 10
120	2	2	1, 5, 10

Statistical analysis

Unless otherwise stated all data are presented as means ±S.E.M. All statistical analysis were performed using Microsoft Excel. Difference between treatment groups were analysed by one-way ANOVA followed by two-sample t-tests. Correlation analysis between with 'time' and 'number of embryos' was also run.

6.4.2 Results and Discussion

There was no mortality in the adult fish for the duration of the experiment. There was no effect of treatment on length or weight of the fish (data not shown).

Measurement of egg output

Average egg output per spawning event was 43.61 ± 4.56, 53.05± 5.76 and 33.81 ± 4.03 for the 20, 60 and 120 µg Ag g^{-1} treatment groups, respectively (**Figure 2**). There was a significant difference in average daily egg production between the exposure treatment groups, p=0.0194, which was accounted for by a significant in egg production between the treatment groups fed 60 µg g^{-1} and 120 µg g^{-1} nano-Ag (p= 0.0153).

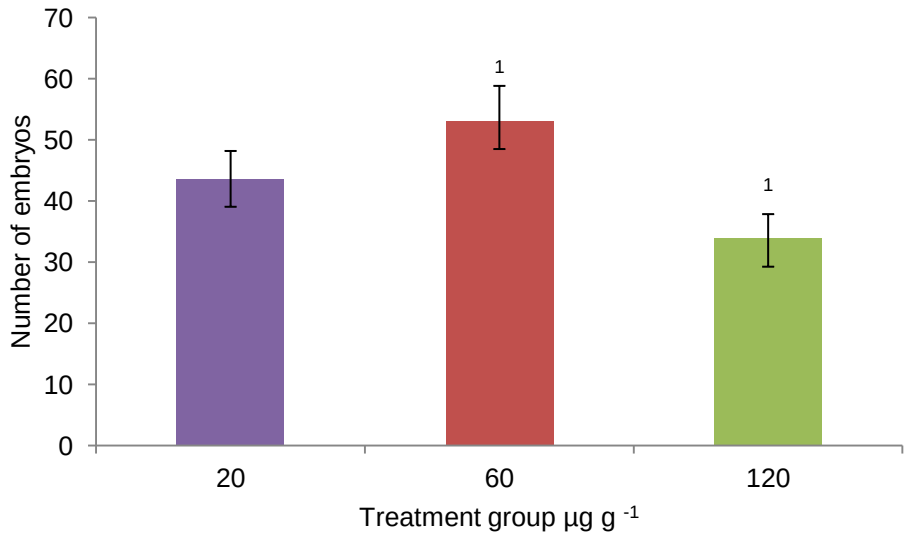
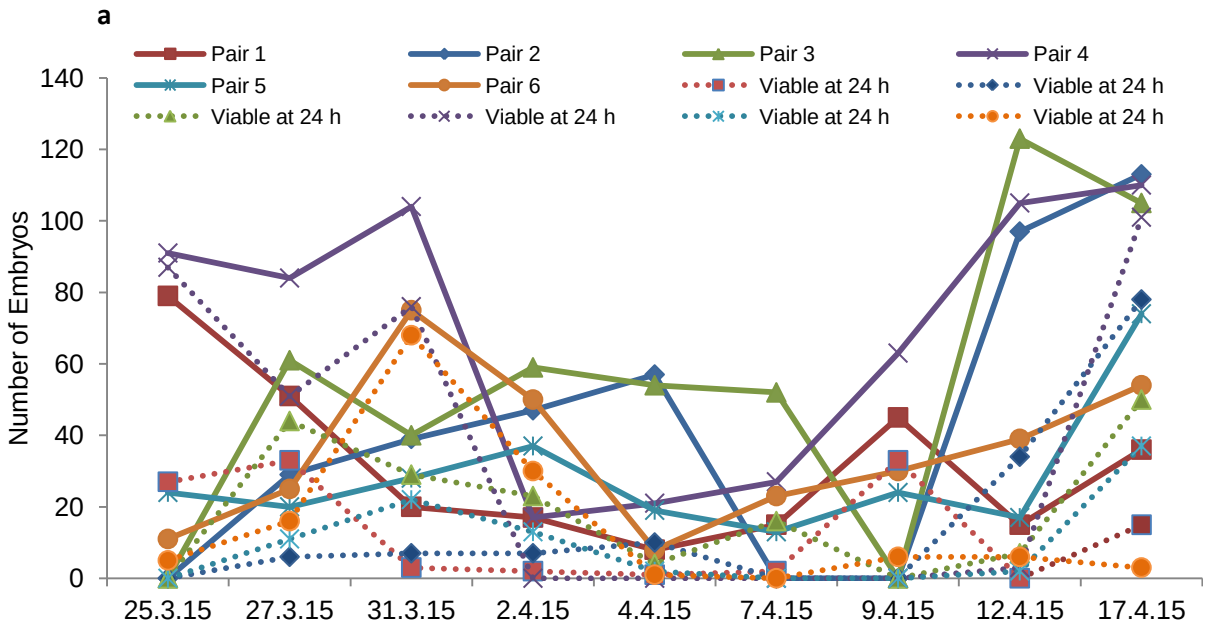
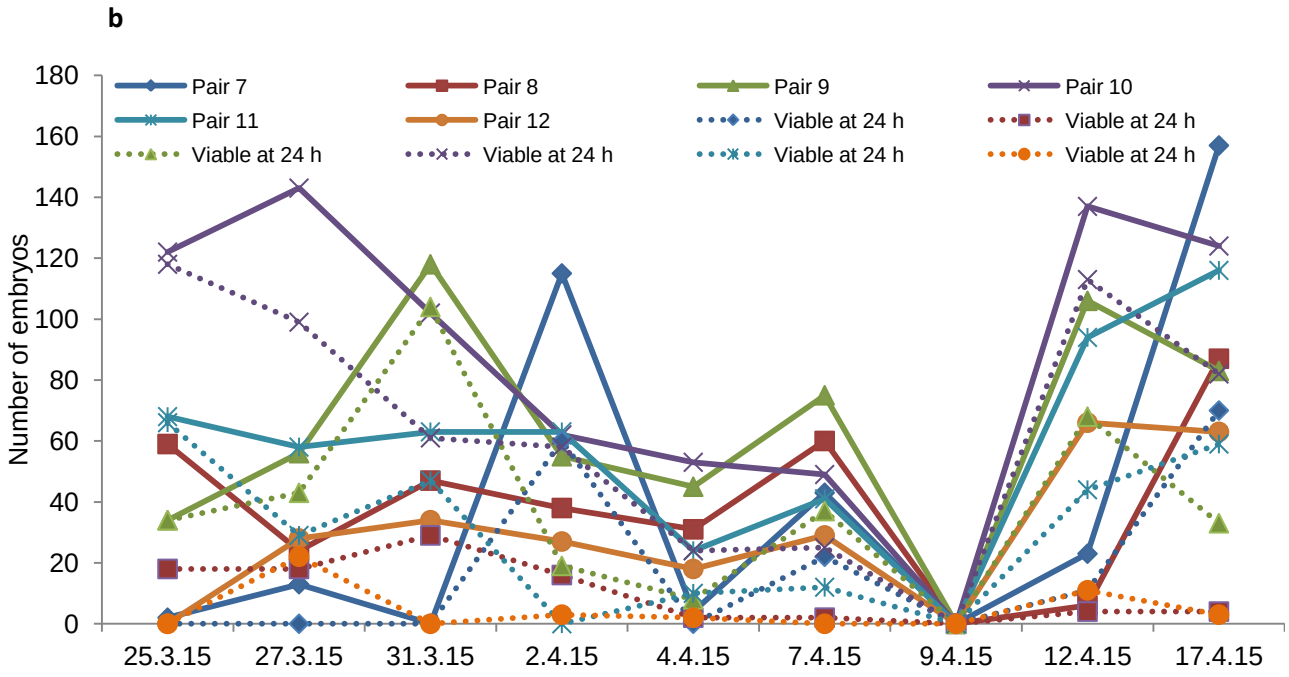


Figure 2. Average egg output, per spawning event, for each treatment (n=6) for the duration (3 weeks) of the experiment. [1] indicates significant difference between groups $p < 0.05$.

Variability in egg production between pairs within the treatment groups was high, with a range of 0 – 157 (**Figure 3a, b, c**). There was no correlation between experimental duration and egg production for any of the pairs in any

of the treatment groups (data not shown). Embryo viability (at 24 hours) also varied greatly between pairs (**Figure 3a, b, c**) although was broadly consistent against the number of embryos produced.





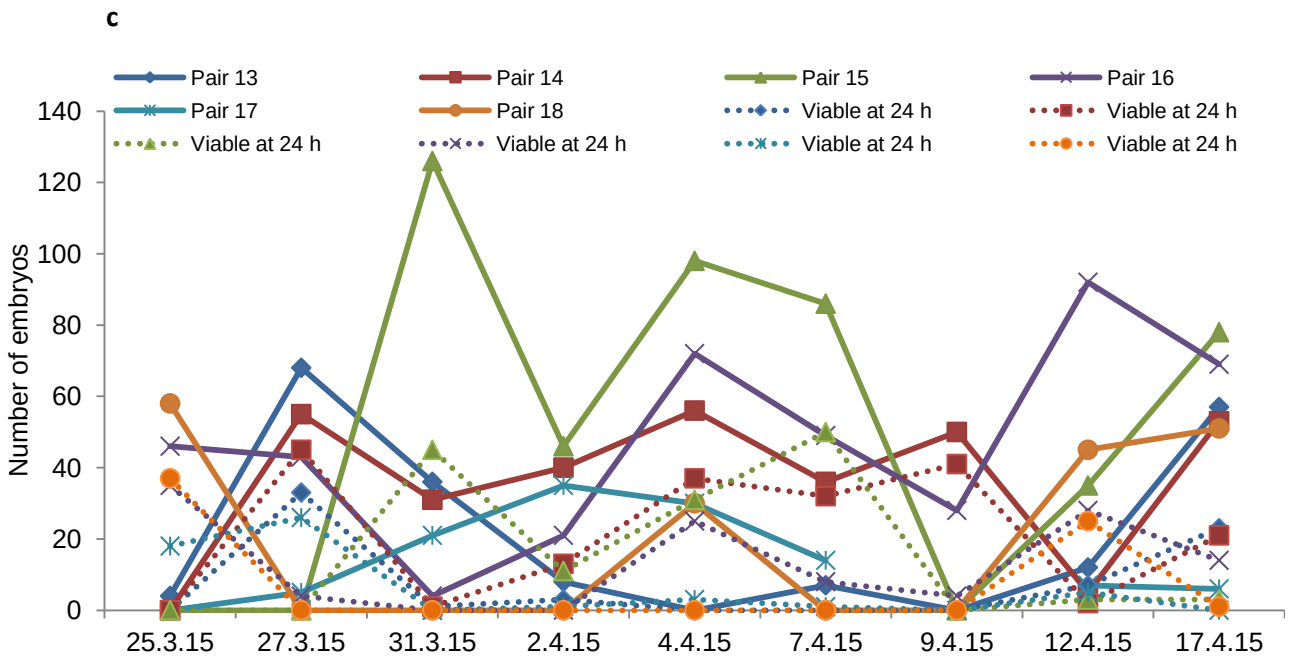
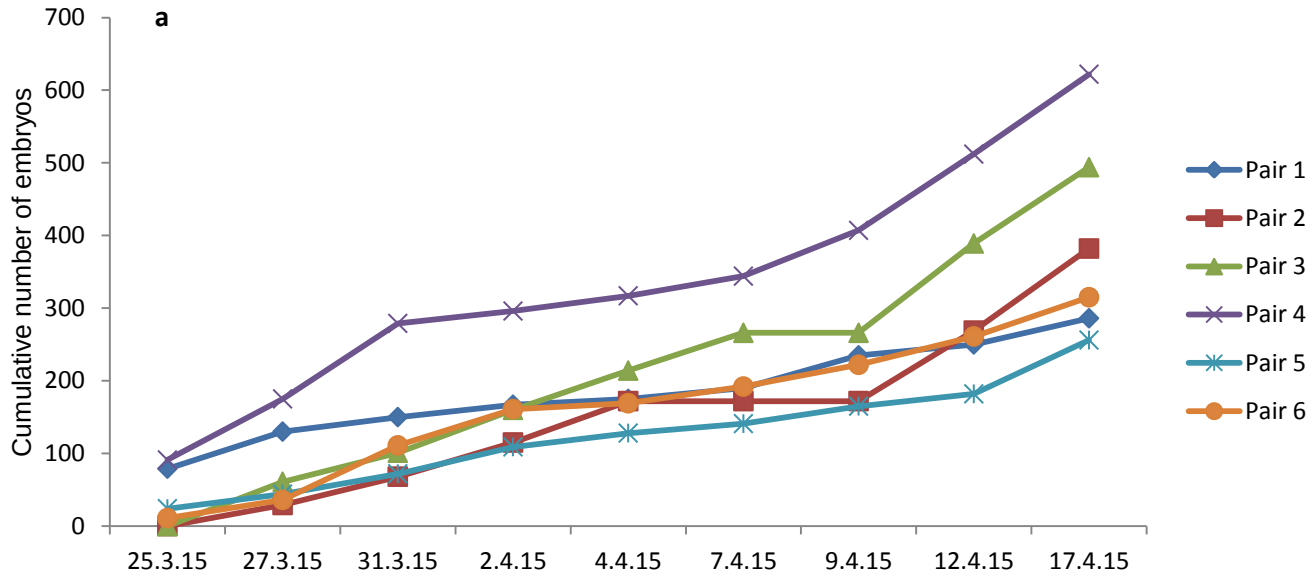


Figure 3. Number of eggs produced (solid line) for each pair over the duration of the experiment for (**a**) 20, (**b**) 60 and (**c**) 120 µg g^{-1} treatments. Coloured dashed lines represent the numbers of viable embryos after 24hours for the respective treatments.

Despite the differences in egg production between pairs both within and between treatments (**Figure 4a, b, c**), for all pairs there was a continual (and cumulative) production of eggs over time, with no evidence for an effect of treatment.



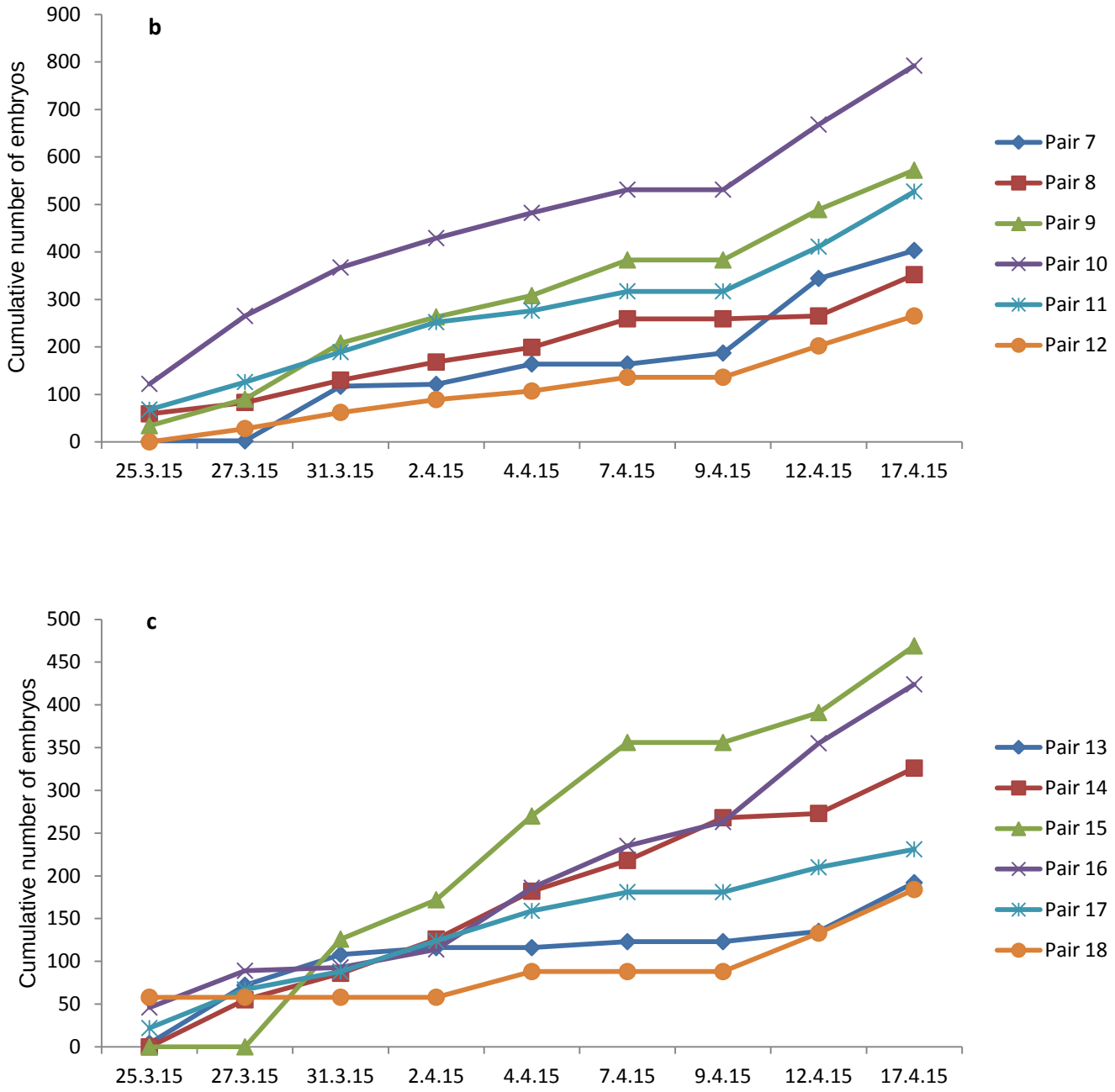
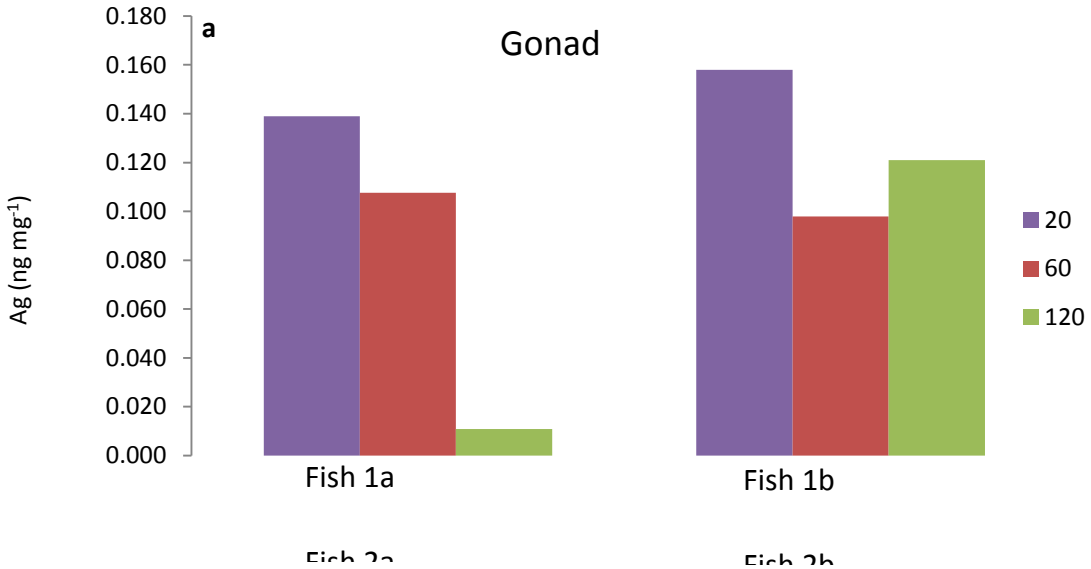


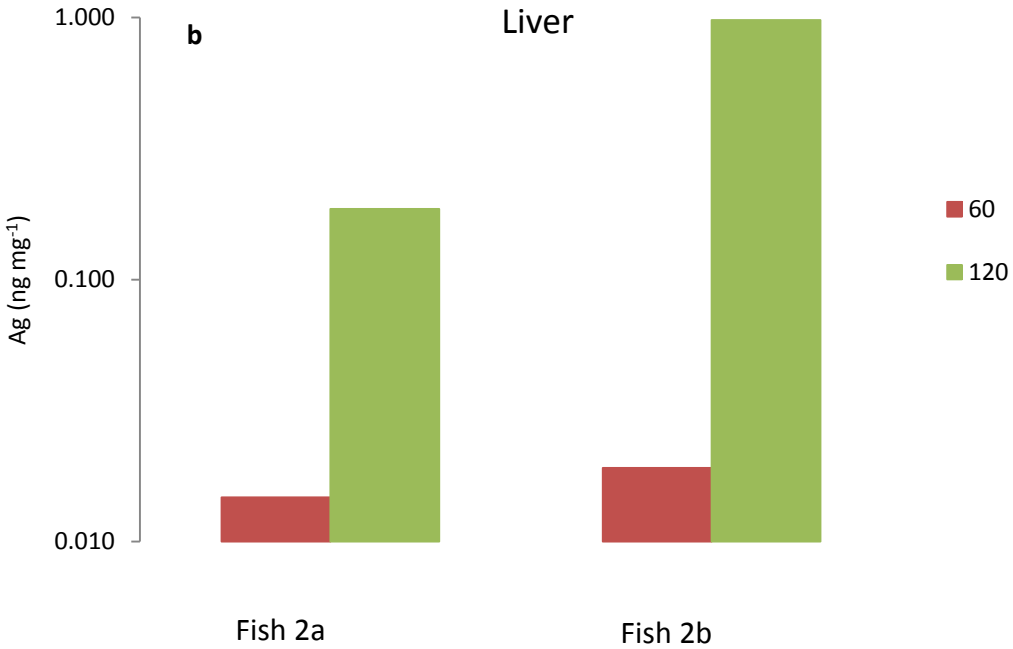
Figure 4. Cumulative number of eggs produced for each pair over the duration of the experiment for (**a**) 20, (**b**) 60 and (**c**) 120 µg g^{-1} treatments.

Background Ag in samples

There was very little background Ag in the samples analysed with a maximum of 0.158, 0.108 and 0.121 ng Ag per mg in the gonadal tissue (**Figure 5a**) in the 20, 60 and 120 µg g^{-1} treatments respectively and 0.978 and 0.019 ng Ag per mg in the liver (**Figure 5b**) for 60 and 120 µg g^{-1} treatments. We were not able to get an accurate weight measurement for the liver from the 20 µg g^{-1} treatment, precluding an accurate calculation of Ag concentration. Total natural Ag in the liver for this treatment however was measured at 0.374 ng, comparative with total natural Ag of 0.268 and 0.138 ng from the two liver samples for the 60 µg g^{-1} treatment and 1.296 and 4.831 ng in the two liver samples for the 120 µg g^{-1} treatment (data not shown). Background levels of Ag were also measured in embryos (**Figure 5c**) and calculated as a concentration (ng mg^{-1} of tissue) and as a value per

embryo (data not shown). The average background Ag level was 0.592, 1.039 and 12.854 ng mg^{-1} and 0.036, 0.070 and 0.590 ng per embryo for the untreated adults, and the 60 and 120 µg g^{-1} AgNP treatments, respectively.





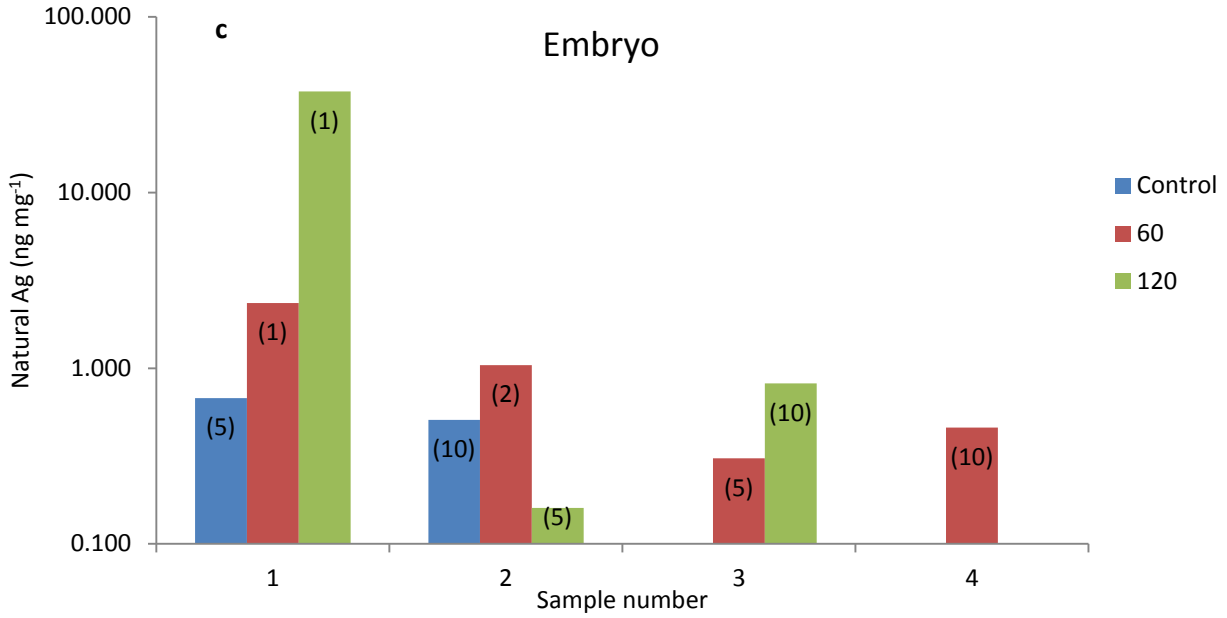
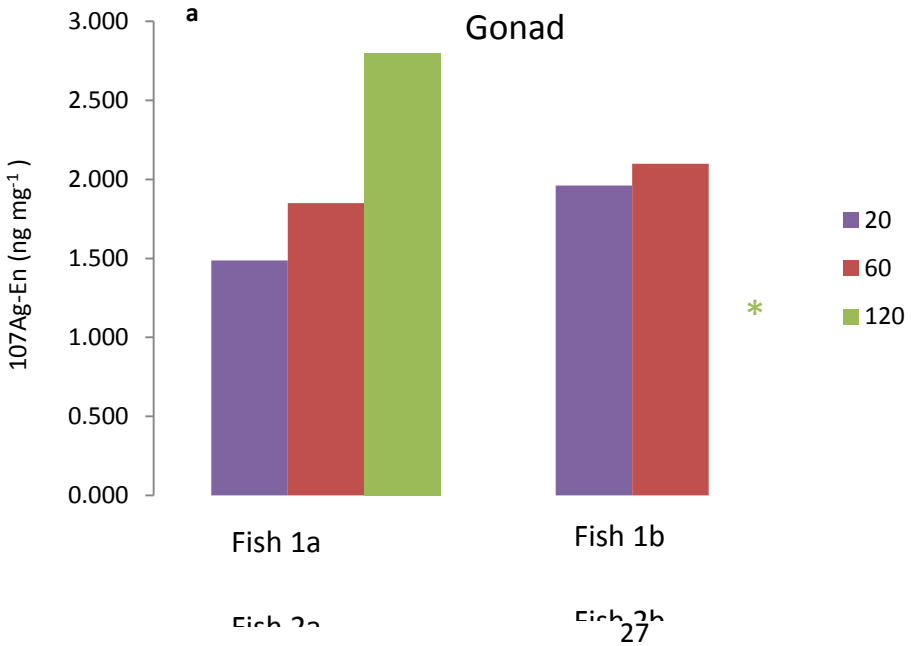


Figure 5. Background Ag levels in experimental samples. Data plotted as single sample measurements, n=2 for gonad and liver tissue and control embryos, n=3 for 60 µg g^{-1} and n=4 for 120 µg g^{-1} treatment embryo samples. (**a**) Gonad tissue plotted as concentration measurement ng mg^{-1} of tissue. (**b**) Liver tissue plotted as a concentration measurement ng mg^{-1} of tissue. (**c**) Embryo concentration measurement ng mg^{-1} of tissue, (number in brackets indicate the number of embryos pooled in that sample).

Ag isotope levels in samples

Levels of Ag isotope measured in the gonad (**Figure 6a**) and liver (**Figure 6b**) tissues suggest higher contents with increasing silver dose. Sample loss and indeterminable weights meant we did not obtain data points for a 60 µg g^{-1} gonad tissue or for both liver samples in the 20 µg g^{-1} treatment. Total Ag^{107} measurements in the liver tissue indicated a dose related uptake with 2.746 ng for the 20 µg g^{-1} treatment , 4.498 and 5.043 for the two samples for the 60 µg g^{-1} treatment and 53.581 and 200.900 ng for the 120 µg g^{-1} treatment (data not shown).



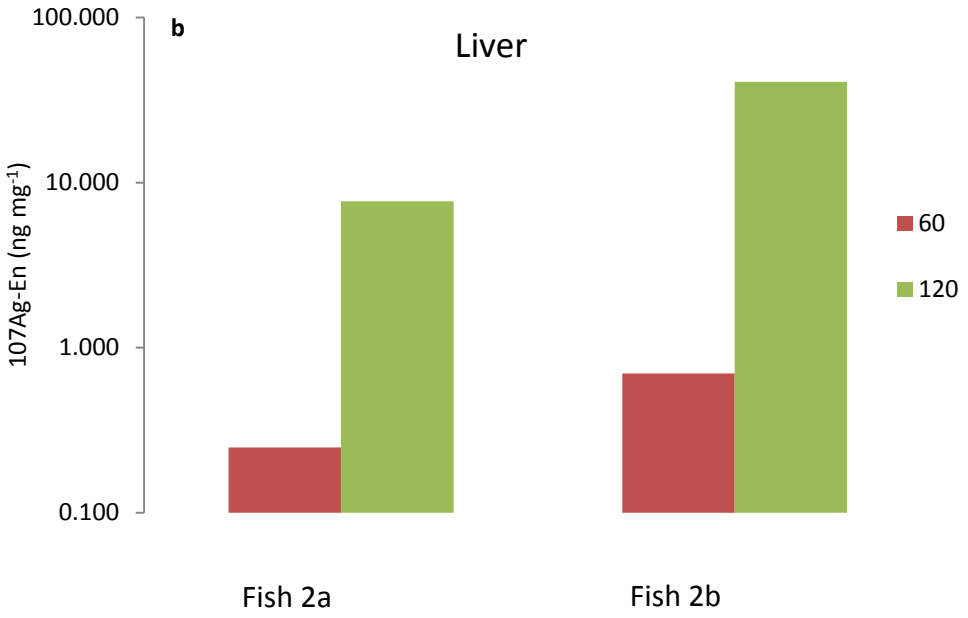


Figure 6. Isotopic Ag^{107} levels in experimental samples. Data plotted as single sample measurements, (**a**) gonad tissue plotted as concentration measurement ng mg^{-1} of tissue. * = sample lost. (**b**) Liver tissue plotted as a concentration measurement ng mg^{-1} of tissue.

Ag^{107} levels were also measured in embryos (**Figure 7**) and calculated as concentration (ng mg^{-1} of tissue) and per embryo (data not shown). The average Ag^{107} level was 0.015, 19.571, 10.099 ng mg^{-1} for the non-exposure 60 and 120 µg g^{-1} treatments respectively. The average Ag^{107} level per embryo was 0.001, 1.385 and 0.519 ng for the non-exposure, 60 and 120 µg g^{-1} treatments, respectively.

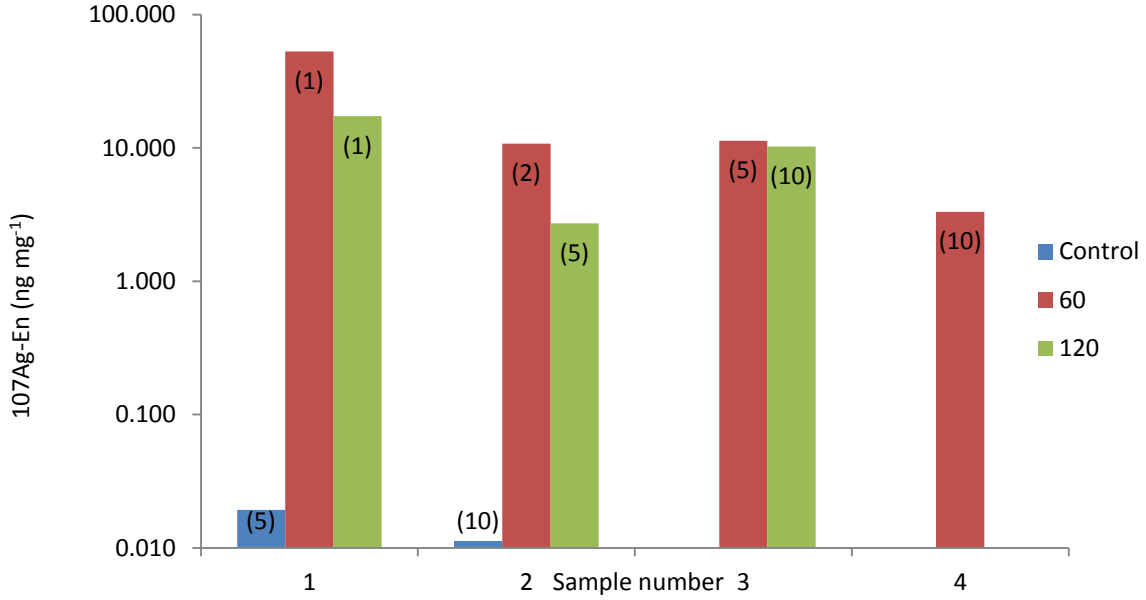


Figure 7. Isotopic Ag^{107} levels in embryo samples. Data plotted as single sample measurements n=2 for control, n=3 for 120 µg g^{-1} and n=4 for 60 µg g^{-1} treatment embryo samples. Number in brackets indicates number of embryos in each sample. Embryo Ag^{107} burdens plotted as a concentration measurement ng mg^{-1} of tissue.

This study has provided the required range finding data and validated the methodology for a long term, multi generations study. There appeared to be a significant effect of the treatment (**Figure 2**) on embryo production – accounted for by a difference between the highest treatment groups (60 and 120 µg g^{-1}). There was no evidence for a concentration related effect on egg production however and we did not have an absolute control in this pilot study. Egg output was as we would expect, but there was a very high variability between pairs and over time within pairs. Egg viability was considerably lower than expected (and was also highly variable, **Figure 4**). Low viability in one collection event (9/14) was due to an unexpected water quality issue - the water system failed overnight. This highlights the requirement for ensuring high water flow rates throughout a study of this nature to avoid such impacts.

In general there was very little non-isotopically labelled Ag in the analysed samples (**Figure 5**). When compared with relative (per mg) levels of ^{107} Ag (**Figure 6, 7**). All the concentrations were low for natural Ag as expected (except in one instance for a single embryo derived from the 120 µg g^{-1} treatment group). Background or natural Ag was measured as part of the study to assess the isotopic ratio which impacts on measurement sensitivity.

Dosed Ag^{107} levels in tissue samples followed a general dose related uptake relationship. Dosed fish produced embryos with a higher Ag^{107} burden than in the control fish. The burden of Ag per mg of tissue was highest in the embryos. This finding is consistent for a concentrating effect for contaminants for various pollutants in fish, examples for this include for polybromodiphenyl ethers (PBDE) in adult female zebrafish (Nyholm *et al.*, 2008) and marine medaka (*Oryzias melastigma*) (van de Merwe *et al.*, 2011). Another example is seen for exposure to perfluorooctane sulfonate (PFOS) where 10% (wt) of the adult PFOS body burden was shown to be transferred to the developing embryos, resulting in a higher total PFOS concentration in eggs (Sharpe *et al.*, 2010). These results, showing positive correlation between dosage and uptake in the adult tissues and also for, maternal transfer to developing eggs in females provides encouraging evidence for the value for undertaking the proposed chronic multigeneration exposure study now proposed. Furthermore, resolution in the isotopic measurement technique has been shown to allow for effective measurement of dosed silver in single embryos. This pilot will allow for a chronic exposure with an environmentally relevant exposure regime.

6.4.3 Conclusions

We have been able to show that adopting the use of isotopically labelled silver we can detect uptake into body tissues for environmentally relevant exposures and have provided evidence for maternal transfer to developing eggs. This pilot study provides us with the information needed to now assess the long term exposure effects for exposure to silver nanoparticles, assessing for maternal transfer and mutligenerational effects. For this work we have revised our pair breeding and husbandry approach to better ensure consistency in egg out put and embryo survival. In collaboration with the University of Birmingham synthesis of 'stable' (low-solubility) Ag NPs is being investigated. We wish to use non-dissolving particles in the long term– multigenerational exposure in addition to the Ag NPs used in the pilot study to investigate for particle- versus silver ion- related effects.

7. Overall conclusions and ongoing work

A main conclusion from the studies on the different test organisms for the chronic exposure studies is that only AgNM300K of the MNMs tested had significant effects on biological function in the different study organisms, and even for this NM, effects were seen only for high concentration exposures. The algal exposures showed that AgNM/silver can affect copper transport mechanisms and this may have significant implications for copper

homeostasis. In aqueous conditions, the studies on algae also indicated that the presence of organisms can affect the rate of dissolution of silver from the MNMs. The chronic exposures to AgNM300K in the nematode, *C. elegans* showed significant effects on life span with inhibitory effects also on reproduction and various neural phenotypes (e..g locomotion). These effects occurred in concentration and time -dependent manner, supporting the hypothesis that exposure effects to this MNM is accumulative. The effects on reproduction in *C elegans* appear to result from an effect on the egg-laying organ vulva, where NMs were seen to be deposited. No firm conclusions can be drawn for the chronic exposure to MNMs in the isopod studies as the mortality rates in control groups were high, likely linked to stress in the maintained animals. Nevertheless, no obvious adverse health effects were apparent on apical endpoints (growth, development, reproduction) for the different MNM exposures. Studies conducted on *Danio rerio* have show that by adopting the use of isotopically labelled silver we can detect uptake into body tissues for environmentally relevant exposures to silver MNMs. This work has furthermore provided evidence for maternal transfer to developing eggs. These data provide the basis for a long term study to establish the effects of exposure to silver nanoparticles, and assessing maternal transfer and mutligenerational effects, that will for the basis of NanoMILE deliverable 6.6 and linking biomarker responses with long term chronic health effects.

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