DELIVERABLE REPORT

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Deliverable 6.2: Effects of NM structural and surface features (including coatings) and NM transformation on biological responses

Contents

1. Introduction

In this report we have compiled the results for deliverable 6.2. The task leading to this deliverable report deals with correlating the effects of structure and surface features, including coatings, and transformation of nanomaterials (NMs) in biological media, on the subsequent impacts of the nanomaterials on test organisms. Here we aimed to establish what features of the nanomaterials confer biological effects under the real exposure conditions. The extensive report on D6.1 covers the identification of relative toxicity and the organisms' sensitivity to selected nanomaterials. This report (D6.2) therefore focuses on the effects of coating on the toxicity of the nanoparticles (Table 1) that have been tested in NanoMILE's diverse panel of organisms (Table 2). Later in the project data will be derived on the effects of NM structural and surface features and NM transformation on biological responses and these data can be included in a more extended report at a later date.

1.1 Materials

Across the various labs the manufactured nanomaterials (NMs) were tested for their toxicity towards a range of different study organisms. Tables 1 lists the NMs tested to date, while Table 2 summarises the organisms used in WP6.

Table 1: Nanomaterials with different coatings used in WP6

Nanomaterial	Coating	Primary Size (nm)	Partners
Zinc oxide - JRC NM-110	Uncoated	150	Eawag, KIT, UEXE, IUF
Zinc oxide - JRC NM-111	Triethoxycaprylyl-silane	140	Eawag, KIT
Silver NM – AgNM300K	Dispersant - 4% (w/w) Polyoxyethylene Glycerol Trioleate - 4% (w/w) Polyoxyethylene (20) Sorbitan mono-Laurat (Tween 20)	15	Eawag, KIT, UEXE, IUF, UNI LJ
Silver NM	citrate coated	7	UEXE, Eawag
Silver NM	citrate coated	10	UEXE, Eawag
Silver NM	PVP coated	20	UEXE, Eawag
Cerium oxide (IV) 211	Uncoated	33	KIT, UEXE, IUF
Cerium oxide (IV) 212	Uncoated	20	UEXE, IUF, UNI LJ
Titanium dioxide	Uncoated	10	KIT, UNI LJ
Titanium dioxide	PVP coated	10	KIT, UNI LJ
Titanium dioxide	F127 coated	10	KIT
Titanium dioxide	Dispex AA4040 coated	10	KIT
Titanium dioxide	Uncoated	10	KIT

Titanium dioxide	Uncoated	20	KIT
SiO2-un	Uncoated	<20	KIT
SiO2-NH2	NH2 functional group	<20	KIT
SiO2-COOH	COOH functional group	<20	KIT
SiO2-un RuBPy	RuBPy doped	<20	KIT
SiO2-NH2 RuBPy	NH2 functional, RuBPy doped	<20	KIT
SiO2-COOH RuBPy	COOH functional, RuBPy doped	<20	KIT

Table 2: The organisms on which the differently coated nanoparticles were tested.

Nanomaterial	Organisms
Zinc oxide - JRC NM-110 uncoated	*Chlamydomonas reinhardtii, Caenorhabditis elegans, Danio rerio*
Zinc oxide - JRC NM-111 Triethoxycaprylyl-silane coated	*Chlamydomonas reinhardtii, Caenorhabditis elegans, Danio rerio*
SilverNM – AgNM300K	*Chlamydomonas reinhardtii, Caenorhabditis elegans, Danio rerio, Porcellio scaber*
Silver NM – citrate- 7nm	*Danio rerio*
Silver NM – citrate- 10nm	*Danio rerio*
Silver NM– PVP coated	*Danio rerio*
Cerium oxide (IV) 211	*Eisenia fetida, Folsomia candida, Hypoaspis aculeifer, Caenorhabditis elegans, Danio rerio*
Cerium oxide (IV) 212	*Eisenia fetida, Folsomia candida, Hypoaspis aculeifer, Caenorhabditis elegans, Danio rerio, Porcellio scaber*
Titanium dioxide -Uncoated	*Danio rerio, Porcellio scaber*
Titanium dioxide -PVP coated	*Danio rerio, Porcellio scaber*
Titanium dioxide -F127 coated	*Danio rerio*
Titanium dioxide - Dispex AA4040 coated	*Danio rerio*
Titanium dioxide –Uncoated – 20nm	*Danio rerio*
Titanium dioxide –Uncoated - 10nm	*Danio rerio*
SiO2-un	*Danio rerio*
SiO2-NH2	*Danio rerio*
SiO2-COOH	*Danio rerio*
SiO2-un RuBPy	*Danio rerio*
SiO2-NH2 RuBPy	*Danio rerio*
SiO2-COOH RuBPy	*Danio rerio*

1.2 Exposure Experiments

Toxicity tests of different nanomaterials were conducted via environmental media (water or soil) and effects analysis have focused on apical endpoints, life span, mortality, to development, growth, reproduction, neurological function (e.g. behaviour assays) and photosynthetic yield (algae). In addition, for some organisms a suite of biomarkers of stress responses were measured. The exposure conditions for the NMs for the organisms in the partners' labs are detailed below.

1.2.1 Chlamydomonas reinhardtii

Nanoparticle Suspension Preparation: The ZnO nanoparticles were suspended in nanopure H_2O and ModTalaCa^{2+} F20 medium at nominal concentration of 100 mg L^{-1}. The stock suspension was sonicated for 10 min at 20 kHz and 90% amplitude. The JRC NM-111 particles were first dissolved in 0.5% v/v EtOH (final concentration) and then added to the medium for characterization.

The AgNM-300K nanomaterials stock suspensions with the nominal concentration of 93 µM were freshly prepared in ModTalaCa^{2+} F20. The citrate coated AgNMs' stock suspensions of 100 µM were freshly prepared in ModTalaCa^{2+} F20.

Exposure: The exposure was performed in ModTalaCa^{2+} F20 medium with exponentially growing cells at a final concentration of 2 x 10^5 cells ml^{-1} to different nanoparticle concentrations. After 0.5, 3, 6 and 24 h of exposure samples were taken for the analysis of different physiological and molecular endpoints. After 24 h of incubation, the pH of the control and exposure media was measured.

Endpoints measured: The effects of nanoparticles on *C. reinhardtii* were estimated on different physiological endpoints (cell growth and cell volume, photosynthesis, intracellular ATP content and EPS production).

1.2.2 Danio rerio (KIT)

Nanoparticle Suspension Preparation: Particle dispersions were prepared shortly before exposure. Briefly, dispersed particles were diluted to 5mg/ml in ddH_2O and sonicated for 5min (water bath). Powdered nanomaterials were weighed in to 5mg/ml in ddH_2O and sonnicated for 15min (water bath). Due to the hydrophobicity of ZnO NM111, both ZnO particles were prepared according to the NanoGenotox protocol (15.6mg were pre-wetted with 96% Ethanol and then dispersed in ddH_2O 0.05% BSA before sonification with a tip sonifier for 2x 1min). From there all dispersions were diluted in Holtfreter´s medium to 2x of the exposure concentration. Before each dilution step the dispersions were mixed by pipetting or vortexing.

Exposure: All particles were tested at least twice for toxicity by screening experiments at 1 and 125 µg/ml in embryo medium (5 days of exposure). For those materials showing adverse effects (ZnO, Ag), additional concentrations were tested to obtain dose response curves.

Endpoints measured: Normal development, Hatching, Malformation, Lethality.

1.2.3 *Danio rerio (Exeter)*

Nanoparticle Suspension Preparation: Please see 'WP6 Standard Operation Procedure. Preparation of NP dispersions for exposure to aquatic organisms', for a detailed description of particle handling and preparation. Particle dispersions were prepared freshly shortly before exposure.

Exposure:

Behavioural Assay: Adult wild-type zebrafish (strain: WIK) were allowed to spawn in aquarium water. Batches were collected immediately after spawning and transferred to petri dishes where they were washed and kept in 1:5 OECD zebrafish culture media 5 dpf old larvae from a cleaned Petri dish were transferred into the wells of 24 well multiwell plate, in a total of 600 µl of culture water (one larva per well). The test nanomaterial is then suspended in culture water and serial diluted to the appropriate test concentrations, NMs are dosed in the 4x24 well, via an 80% media change. The positive control is not dosed at this time. The animals are then held under the same culture conditions for 24 hours, food is withheld and the culture water not changed.

48 hour developmental assay: For all exposures, there were >24 embryos (at the 1–2 cell stage, 1–1.5 hpf) per treatment well. The embryos were incubated at 28 +/– 1˚C up to 48 h. After 2 h in culture, the numbers of unfertilised embryos were recorded and these were removed, if the fertilisation success was less than 80% the batch was discarded. At 24 and 48 hpf survival rates and any phenotypic deformities were recorded.

Endpoints measured: 48 hour developmental assay: Body Shape Subtle Variant, Body Shape Malformed, Somites Subtle Variant, Somites Malformed, Notochord Subtle Variant, Notochord Malformed, Tail Subtle Variant, Tail Malformed, Fins Subtle Variant, Fins Malformed, Heart Subtle Variant, Heart Malformed, Face Subtle Variant, Face Malformed, Neural Tube Subtle Variant, Neural Tube Malformed, Arches/Jaws Subtle Variant, Arches/Jaws Malformed, Poor Pigmentation, Excess Pigmentation, Abnormal Pigmentation, Stomach, Not Evident, Liver-Not Evident, Liver–Enlarged, Abdomen distended or yolk sac oedema.

1.2.4 *Caenorhabditis elegans*

Nanoparticle Suspension Preparation: CeO_2 NM212 (powder), CeO_2 NM211 (powder), ZnO NM110 (powder), Ag NM-300K (solution) NPs as well as the dispersant of Ag NM-300K NPs (NM-300KDIS) were obtained from the European Commission Joint Research Centre (JRC). The stock suspensions of uncoated CeO_2 NM212 and NM211 or ZnO NM110 NPs were produced by suspension of 1.6 mg mL-1 powder in ultrapure water and sonication in a cup horn sonicator (Cuphorn Branson) at 200W for 2 minutes to disrupt particles and obtain homogeneous suspensions. The Ag NM-300K NPs (100 mg mL^{-1}) were further diluted to 1 mg mL^{-1} and sonicated as described above. All particle dispersions were serially diluted in ultrapure water.

Exposure: Adult *C. elegans* (wild type, N2) were chronically exposed with increasing concentrations of Ag, ZnO or CeO_2 NMs and subjected to life span analyses and age-resolved observation of

locomotion phenotypes. All NMs were additionally analysed in 24 hour exposures by quantitative observation of the reproductive phenotype internal hatch, e.g. neuromuscular defects of the egg laying system.

***Endpoints measured**:* Life span assays, Quantitative observation of locomotion phenotypes, Quantitative observation of the egg laying defect internal hatch.

1.2.5 Eisenia fétida, Folsomia candida and Hypoaspis aculeifer

Nanoparticle Preparation and exposure:

Eisenia fetida: Test vessels for the tests with Eisenia fetida were Bellaplast containers consisting of inert (non-toxic) plastic (Polystyrene). The earthworms were tested in an artificial soil. The composition of this artificial soil was based on OECD Guideline No. 222 (2006). The Ceria nanoparticles were dissolved in an amount of deionised water sufficient to prepare the respective test solutions. The concentrations to be tested were based on the purity of the Ceria nanoparticles (3.1 % nominal). The test item solutions were incorporated into the soil by thoroughly mixing in the respective test item solution for approx. 5 minutes. The contaminated artificial soil was filled into the test vessels. Per test vessel an amount corresponding to 500 g dw was used. The test item and the reference item were applied at 100 and 1000 mg/kg soil dry weight, respectively. An untreated control group was also tested.

Folsomia candida: Test vessels for the tests with *Folsomia candida* were glass containers (able to be closed tightly) of about 250 mL capacity and with a diameter of about 6.5 cm. The test conditions were maintained as described above for *Eisenia fetida***.** The collembolans were fed with a sufficient amount of granulated dry yeast per test vessel at the beginning of the test and after 14 days *ad libidum.* Ceria nanoparticles and reference material were applied to the test substrate as described for *Eisenia fetida.* The tests were carried out as described in OECD guideline 232. Each test item group included four replicates containing 10 juvenile springtails each. The control group included eight replicates containing 10 juvenile springtails each.

Hypoaspis aculeifer: Test vessels for the tests with Hypoaspis aculeifer were glass containers (able to be closed tightly) of about 100 mL capacity and with a diameter of about 5.5 cm. The mites were tested in an artificial soil as described for Eisenia fetida.

The test conditions were maintained as described for Eisenia fetida. The mites were fed with cheese mites (Tyrophagus putrescentiae) ad libitum 2-3 times a week.Ceria nanoparticles and reference material were applied to the test substrate as described for Eisenia fetida. The tests were carried out as described in OECD guideline 226. Each test item group included four replicates containing 10 adult females each. The control group included eight replicates containing 10 adult females each. The mites were fed with cheese mites (Tyrophagus putrescentiae) ad libitum 2-3 times a week. After 14 days of exposure, the surviving adult females and juveniles were extracted from the soil using a high temperature gradient extractor (McFadyen, 1961).

Endpoints measured: Mortality and Reproduction.

1.2.6 Porcellio scaber

Nanoparticle Preparation: The isopods were collected from uncontaminated locations in Slovenia and 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 ± 2°C and a 16:8-h light:dark photoperiod. Adult animals of both sexes or females with marsupia (weighing more than 30 mg) were used in the experiments. A suspension of particles or distilled water was brushed onto the lower leaf surface to give final nominal concentrations of NPs on the leaves and left until dry. Each individual animal was placed in a Petri dish and one hazelnut leaf 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 are kept in a large glass container under controlled conditions in terms of air humidity (2:80%), temperature (21 ± 1°C), and light regimen (16:8h light:dark photoperiod).

Exposure: Animals in each individual experiment were exposed to different concentrations of NPs (TiO_2, Ag or CeO_2) for 14 days or for different periods in the case of females with marsupia. Animals were fed with leaves dosed with TiO_2, Ag or CeO_2 NPs suspension, providing nominal concentrations of 1000 (for all three NPs) and 5000 (only for CeO_2 NPs) µg NPs/g on a leaf.

Endpoints measured: Feeding parameters (feeding rate, food assimilation efficiency, faecal production rate) and survival were assessed. After the exposure, the animals were anesthetized at a low temperature and then decapitated and their digestive glands and gut isolated for NP analysis.

2. Results and Discussion

Effects of the differently coated nanoparticles are described below and also in detail in the D6.1 report. Table 3 summarises the toxicity of the nanomaterials on the organisms, and the subsequent sections describe the results in more detail. The data are presented per particle type and in terms of their impacts per species type.

Table 3: Summary of the toxicity of the nanomaterials on the test organisms.

	Toxic (?)						
Nanomaterial	C. reinhardtii	C. elegans	*Danio rerio*	*E. fetida*	*F. candida*	*H. aculeifer*	*P. scaber*
Zinc oxide - JRC NM-110 Uncoated	*yes*	*no*	*yes*				
Zinc oxide - JRC NM-111 Triethoxycaprylyl-silane	*no*	*no*	*yes*				
SilverNM – AgNM300K	*yes*	*yes*	*yes*				
SilverNM – citrate- 7nm	*yes*		*yes*				
SilverNM – citrate- 10nm	*yes*		*yes*				
SilverNM– PVP coated			*yes*				
Cerium oxide (IV) 211	*No*	*No*	*No*	*No*	*No*	*No*	
Cerium oxide (IV) 212	*No*	*No*	*No*	*No*	*No*	*No*	
Titanium dioxide -Uncoated			*No*				*No*

Titanium dioxide -PVP coated			*No*				*No*
Titanium dioxide -F127 coated			*No*				
Titanium dioxide -Dispex AA4040 coated			*No*				
Titanium dioxide –Uncoated – 20nm			*No*				
Titanium dioxide –Uncoated - 10nm			*No*				
SiO2-un			*No*				
SiO2-NH2			*No*				
SiO2-COOH			*No*				
SiO2-un RuBPy			*No*				
SiO2-NH2 RuBPy			*No*				
SiO2-COOH RuBPy			*No*				

2.1 Zinc oxide nanomaterials

Two different kinds of zinc oxide nanomaterials obtained from JRC were tested in C. reinhardtii, C. elegans and Danio rerio. One was the uncoated JRC NM-110 and the other the JRC NM-111 coated with Triethoxycaprylyl-silane.

2.1.1 C. reinhardtii:

Characterization and dissolution:

The size and zeta potential of JRC NM 110 and JRC NM 111 in the exposure medium ModTalaCa^{2+} F20 was measured by DLS. The uncoated JRC NM 110 had an initial size of 361.9 ± 4.91 nm. After 6 h, the particles agglomerated to an average size of 646.8 ± 5.26 nm.

The coated JRC NM 111 had a zeta average and zeta potential of 225.01 ± 16.78 nm and -35.6 ± 1.83 µV respectively which stayed stable over a period of 24 h.

The dissolution of ZnO JRC NM-110 (uncoated) and ZnO JRC NM-111 (coated) as a percentage of the added nanoparticle concentration was measured after 0.5, 3, and 6 h. For both types of nanoparticles, the dissolution was not time dependent. With increasing ZnO NP concentrations a higher percentage of nanoparticles dissolved.

Toxicity:

The effects of the coated and uncoated zinc oxide nanomaterials on the photosynthesis of C. reinhardtii was estimated. The inhibition of photosynthetic was observed only when exposed to uncoated to ZnO nanomaterials (Figure 1).

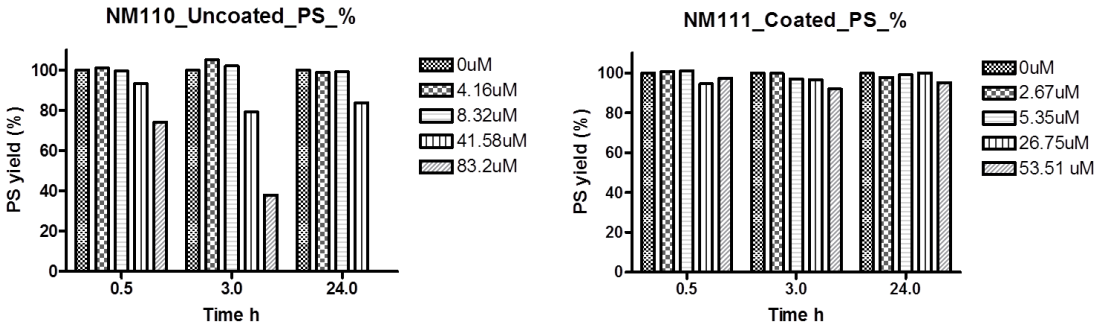

Figure 1: Effects on the photosynthetic yield of C. reinhardtii on exposure to uncoated and coated ZnO nanomaterials

2.1.2 Danio rerio (KIT)

Toxicity

ZnO particles inhibited hatching and reduced larval survival:

Both ZnO particles (NM110 and NM111) showed agglomeration in the high concentration and the agglomerates quickly settled on the bottom of the wells where they got in contact with the chorion (Figure 2). Although the embryonic development appeared normal during the first 4 days of exposure, we frequently observed the formation of a whitish precipitate within the chorion fluid together with an increase in mortality at the highest concentration at 120hpf. Unhatched embryos were also observed at 1 µg/ml but with normal morphology and viability. As the ZnO particles showed inhibition of embryo hatching already at 1 µg/ml, additional concentrations were tested to obtain dose response curves (see Figure 3).

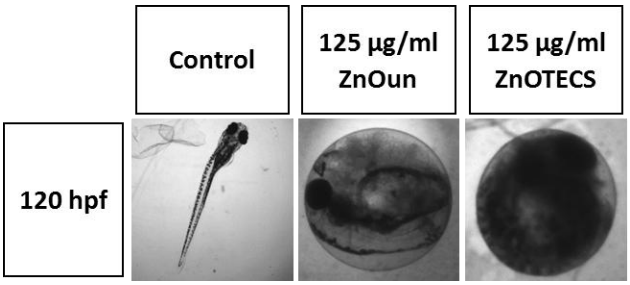

Figure 2: Microscopic images of zebrafish embryos treated with zinc oxide particles (ZnO NM-110, ZnO TECS NM-111) for 5 days. Exposure to both ZnO particles caused complete inhibition of hatching, high mortality and whitish precipitates within the chorion fluid at 125 µg/ml. Note that magnification is lower for the control embryo.

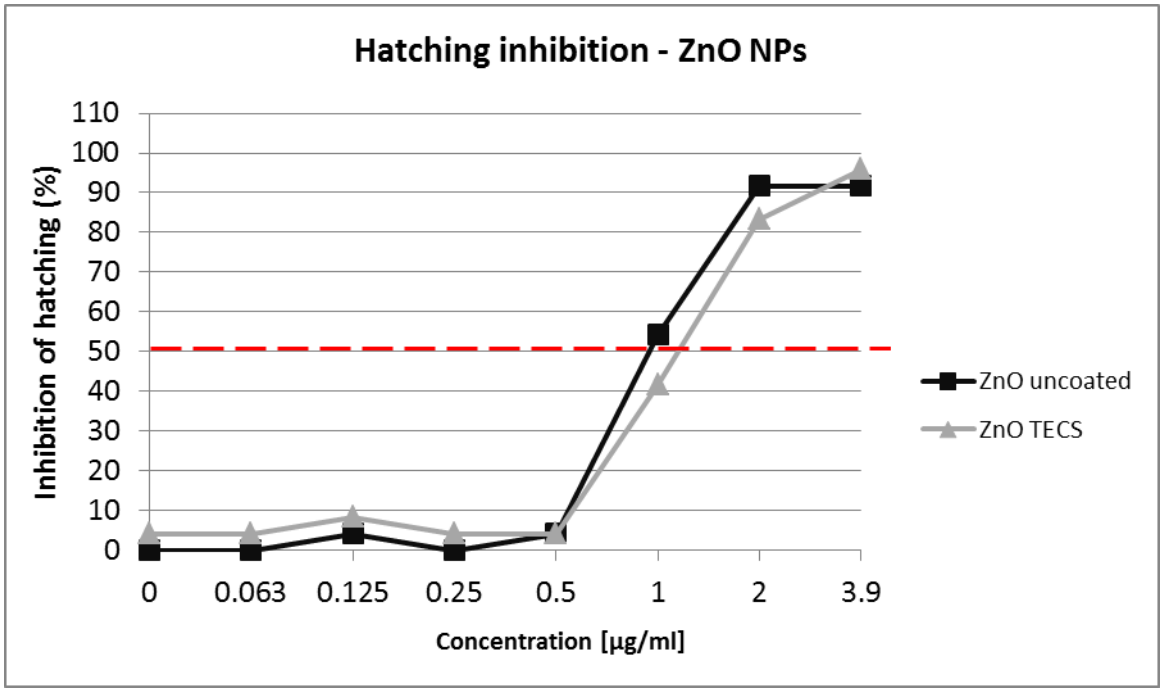

Figure 3: Dose response curves for hatching inhibition at 120hpf of zebrafish embryos treated with zinc oxide particles (ZnO un NM-110, ZnO TECS NM-111). Embryos were exposed to ZnO particles (uncoated NM110 and TECSNM111) in embryo medium in a dilution series ranging from 0.063 to 3.9 µg/ml from gastrulation stage (6hpf) onwards. The percentage of non-hatched embryos is indicated based on analysis of microscopic images taken at 120hpf. The average was calculated from two independent experiments. The EC_{50} value that can be deducted from the curves is approximately 0.75 for NM110 and 1.0 µg/ml and 1 for NM111.

2.1.3 Danio rerio (Exeter)

Behavioural assay

Table 4 shows the behavioural responses of zebrafish exposed to ZnO NMs at 6 dpf. The effects of uncoated ZnO NMs on zebrafish embryos is shown in Figure 4.

Table 4: Behavioural responses of zebrafish exposed to ZnO NMs at 6 dpf

Nanomaterial	Probability of seizure	p=
ZnO NM 110	0.011	0.000279
$ZnCl_2$	0.9652	0.911527

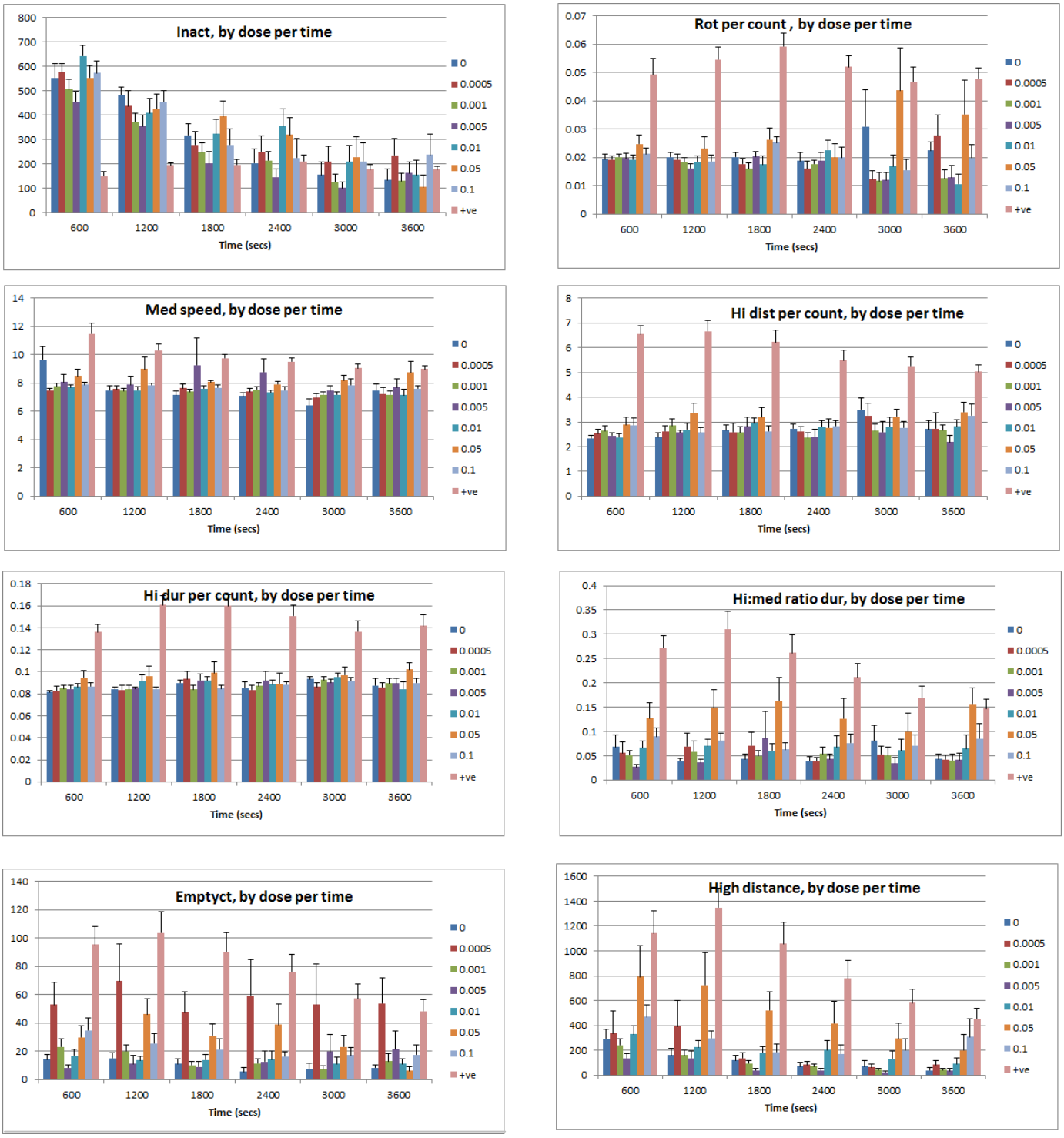

Figure 4. a activity graphs showing concentration response curves during each time interval, across the 1 hour recording period. b) activity graphs showing concentration response curves averaged across all time points by animal. 0 = negative control, +ve = PTZ positive control, 0.0005 represents 5 µg L-1 with the concentration increasing 10 µg L-1 (0.001), 50 µg L-1 (0.005), 100 µg L-1 (0.01), 500 µg L-1 (0.05), up to 1000 µg L-1 (0.1).

Discussion

The differently coated zinc oxide nanomaterials JRC NM 110 and JRC NM111 showed different toxicities in different organisms. The coated nanomaterial was less toxic than the uncoated one to *C. reinhardtii*. However, this is most probably due the differences in the dissolution to ionic zinc. Both the coated and uncoated behaved similarly and showed similar toxicity to *D. rerio.*

2.1.4 C. Elegans

ZnO NM110, NPs were tested in terms of their impact on C. Elegans life span assays. All ZnO NPs applied in concentrations between 20 and 160 μg/ml did not reduce the life span of adult *C. elegans*. As for their untreated counterparts, ZnO NP-exposed *C. elegans* survived for up to 36 days and the respective survival curves show no significant differences (Figure 5). These results are further validated by the observation that survival curves of untreated controls as well as ZnO NP-treated worms run between the ones of short-lived *daf-16* and long-lived *daf-2* mutants, respectively.

As it was shown previously that certain NPs accelerate age-related amyloid protein aggregation and behavioural phenotypes in adult *C. elegans* (*Scharf et al., 2013*) we next analysed if worm survival is correlated with locomotion phenotypes. Typically, wild-type worms show an age-related decline of forward movement (*Huang et al., 2004*). To record all movements, animals were scored according to three modes of locomotion in liquid medium, namely (1) swimming, (2) uncoordinated or (3) head/tail only during their entire life span (Figure 6). Untreated wild-type *C. elegans* show an age-related decline of swimming movements, whereas uncoordinated and head/tail restricted movements increase with age. Worms exposed to 20, 80 and 160 μg/ml ZnO NM110 NPs display similar locomotion phenotypes in comparison to untreated controls (Figure 6).

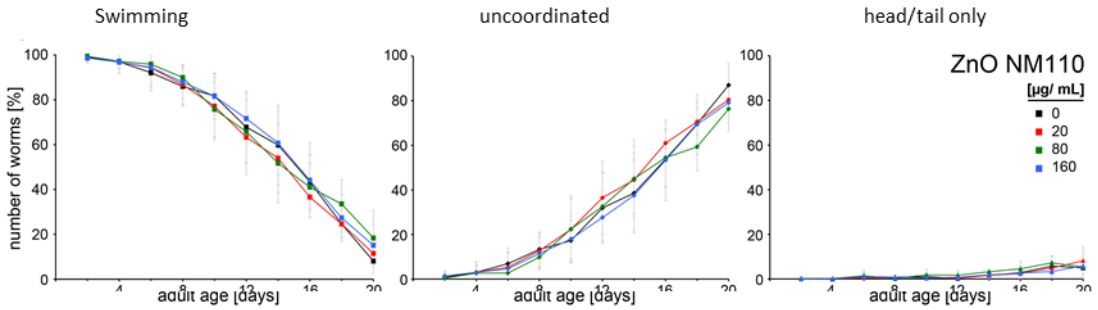

Figure 5. Quantitative observation of locomotion behaviors in NP-exposed *C. elegans*. Locomotion phenotypes of nematodes were classified according to the three categories swimming, uncoordinated or head/ tail only and plotted against adult worm age. All graphs show an age-related decline of swimming nematodes and simultaneous increase of uncoordinated and head/ tail only movements. Phenotypes were scored in nematodes exposed to Ag NPs or the dispersant only control in comparison to untreated controls (black). *, p<0.05; **, p<0.01; Particle addition day 1; dis, dispersant only.

Next we analysed another behavioural phenotype that was previously reported to occur after exposition of adult *C. elegans* to silica nanoparticles (*Pluskota et al., 2009*). The bag of worms (BOW) represents a neural phenotype that is normally age-related and results from neuromuscular defects of the vulva. Hence, egg laying through the vulva is impaired and eggs hatch within the body of the parent worm (internal hatch). Wild type N2 worms were left untreated or exposed to ZnO NM110, NPs and their salts in different concentrations. Neither ZnO NPs nor $ZnCl_2$ induced a significant increase of the BOW phenotype indicating that the neuromuscular function of the vulva is not a target for all nanoparticles or their metal ions (Figure 6). The results of the BOW analyses correspond well with both life span analyses and locomotion scoring in that ZnO NPs show no significant effects.

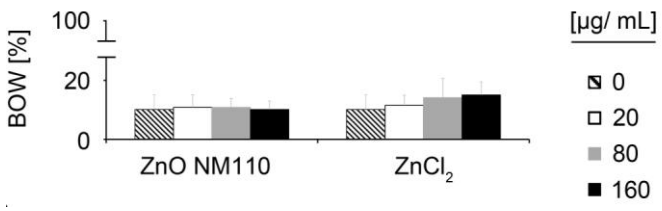

Figure 6. Quantification of the internal hatch (BOW) phenotype in *C. elegans* exposed to different NPs. Nematodes with internally hatched larvae were scored 24 hours after addition of NPs or respective salts. No increase of the BOW phenotype was observed in *C. elegans* treated with ZnO NPs, $ZnCl_2$ (B) or CeO_2 NM212 and NM211 NPs (C). **, p<0.01; BOW, bag of worms.

2.2 Silver nanomaterials

2.2.1 C. reinhardtii

Silver nanomaterial AgNM300k which has as dispersant 4% (w/w) Polyoxyethylene Glycerol Trioleate and 4% (w/w) Polyoxyethylene (20) Sorbitan mono-Laurat (Tween 20) was toxic to the algae. The effects were observed at photosynthesis, **ATP content and growth.**

The effects of AgNP and the dispersant on the growth rate of C. reinhardtii are depicted in Figure 7. The median effective concentration (EC_{50}) was reached at a concentration of 3 µM. The EC_{50} for $AgNO_3$ was 0.16 µM. The growth rate inhibition by AgNP, when plotted as a function of the dissolved Ag^+ content (1.9%) showed that the particles induced a stronger inhibition as compared to $AgNO_3$. The EC_{50} values calculated based on the AgNP exposures were 2.7 times lower than for $AgNO_3$ (0.06 and 0.16 µM respectively, Figure 5B). The AgNP and $AgNO_3$ exposure concentrations that affected the growth rate also had an effect on the cell volume (Figure 7C, D). However, in presence of 0.5 µM of cysteine no effects of AgNP on the growth and cell volume of *C. reinhardtii* could be observed (Figure 7 E, F).

The photosynthetic yield of the cultures exposed to 0.09 and 0.18 µM AgNO3 reduced significantly. Comparable with AgNP, an almost complete recovery was observed after 3 and 24 h. The strongest effect on photosynthetic yield was measured at a concentration of 0.27 µM $AgNO_3$ with no recovery. As already observed in experiments conducted with AgNP, the EC_{50} values increased with time.

The effect of AgNP on photosynthetic yield when plotted as a function of the dissolved Ag^+ content showed that the particles induced a stronger inhibition as compared to that by $AgNO_3$. The EC_{50} values calculated based on the AgNP experiments were more than 2 times lower than for $AgNO_3$. No effects of silver nanoparticles on the photosynthetic yield could be observed in presence of 0.5 µM cysteine (Figure 8).

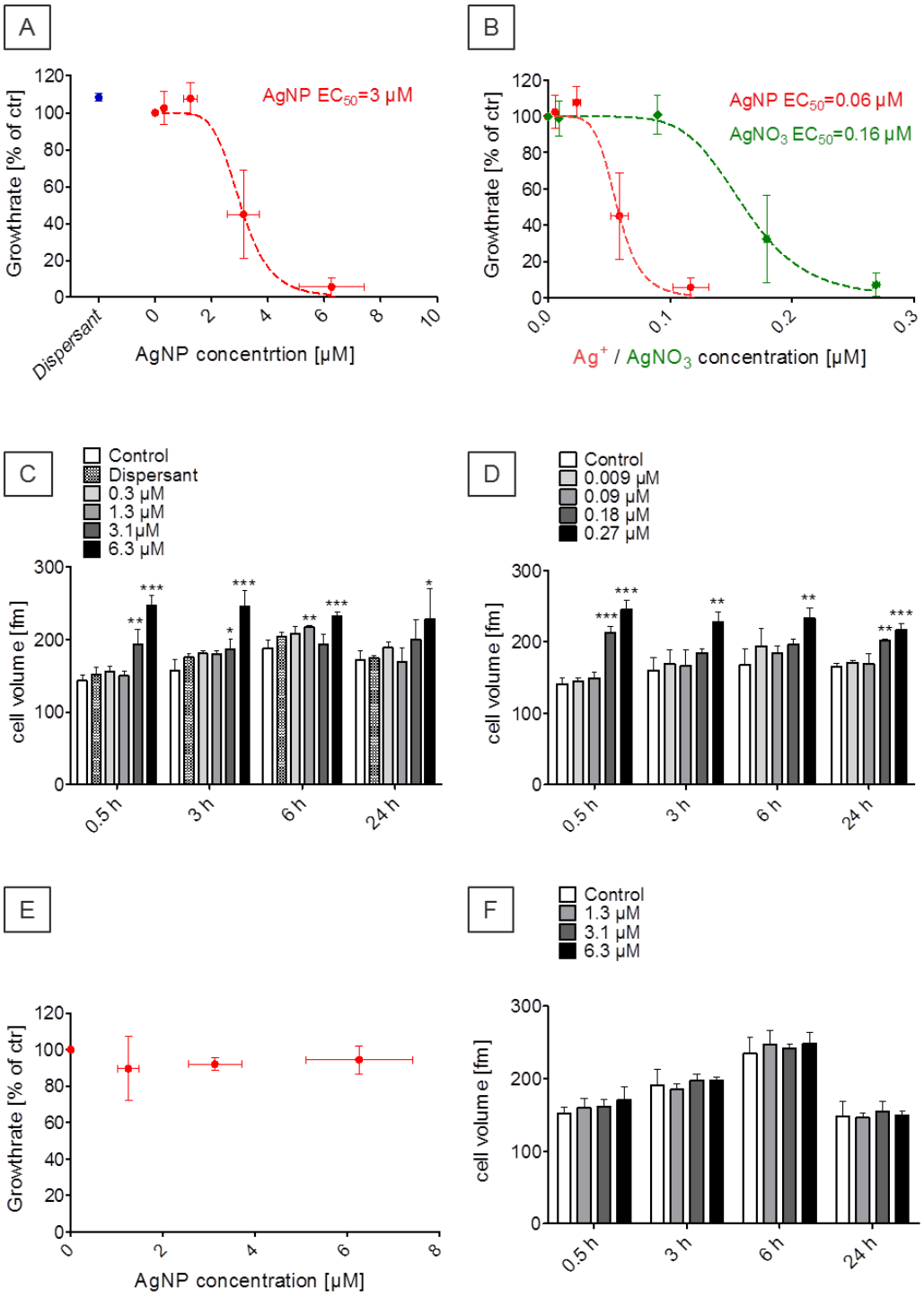

Figure 7: Effects of silver on cell growth and cell volume A: growth rate as a function of concentration of AgNP. The growth rate of cells exposed to the dispersant (blue). B: growth rate as a function of concentration of $AgNO_3$ (green) and the Ag^+ dissolved from the particle surface (red). Volume of cells exposed to AgNP (C) and $AgNO_3$ (D) measured over 24 h. E: growth rate as a function of AgNP concentration in presence of cysteine. F: Volume of cells exposed to AgNP in presence of cysteine.

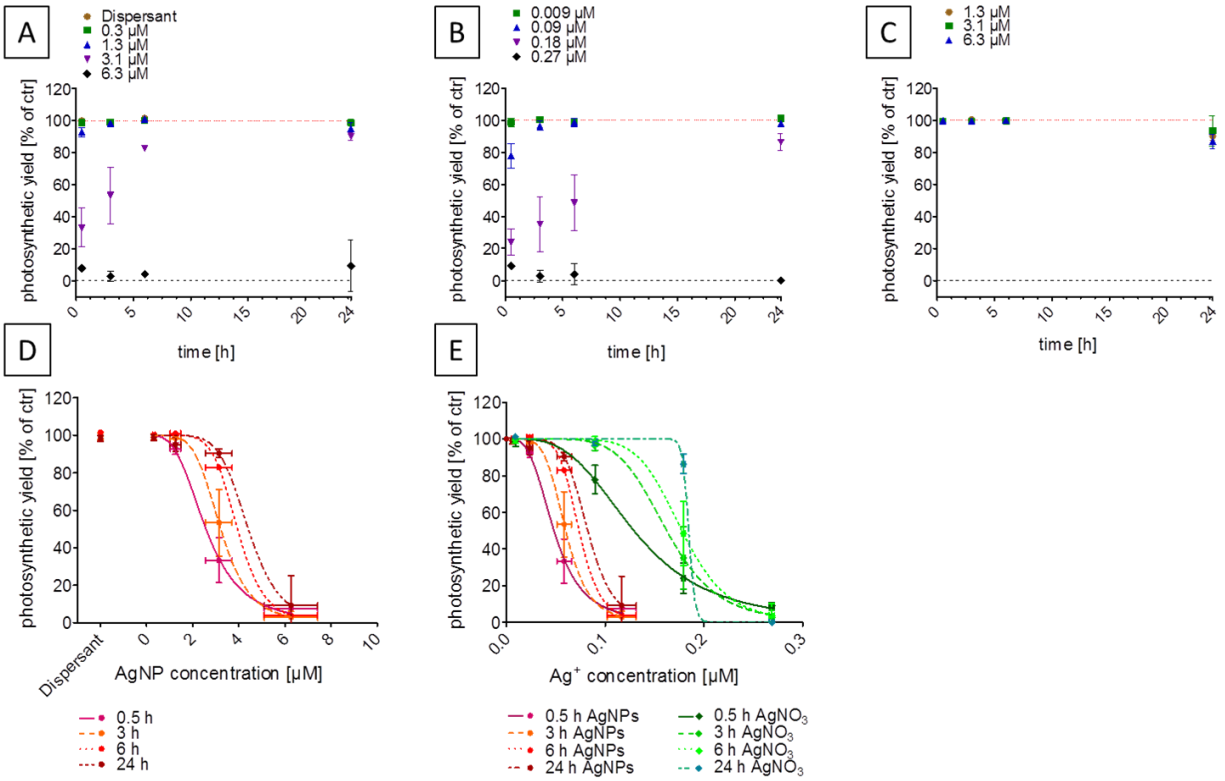

Figure 8: Photosynthetic yield of cultures exposed to silver compounds. Photosynthetic yield upon exposure to AgNP (A), $AgNO_3$ (B) and AgNP in presence of cysteine (C) over the duration of 24 h. Photosynthetic yield as a function of increasing concentration of AgNP (D), $AgNO_3$ (E green) and Ag^+ dissolved from the particle surface by assuming a dissolution of 1.9% (E red) as % of control measured over the duration of 24 h.

2.2.2 Danio rerio (KIT)

Silver particles disturbed development and reduced embryo survival*:* The 20nm Ag 300K NPs showed strong negative effects on hatching rate (Figure 9), morphogenesis (Figure 10) and survival of the zebrafish embryos (Figure 11). The reduced hatching with increasing nanoparticle concentrations could be a consequence of disturbed development or death of the embryos (Figure 12). No adverse effects were observed for the Ag dispersant (JRC NM-300Kdis) when used in the same concentration as in the particle preparation.

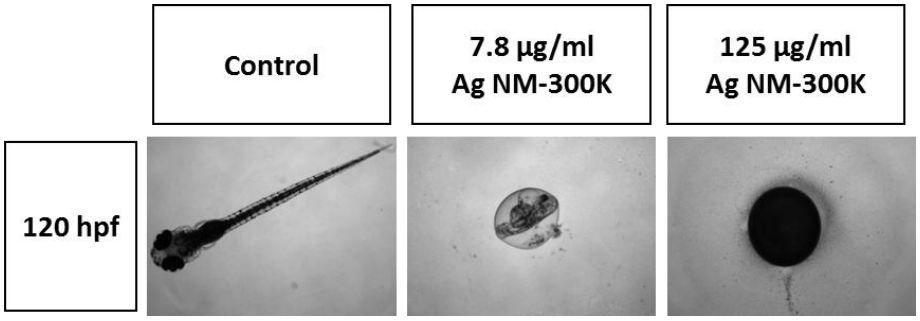

Figure 9: Microscopic images of zebrafish embryos treated with silver particles (Ag NM-300K) for 5 days. Exposure to silver particles caused partial inhibition of hatching, high mortality and malformation rate at 7.8 µg/ml and 100% mortality at 125 µg/ml; note the dark particle agglomerates on the chorion surface at the high concentration. Magnification was the same across the panel.

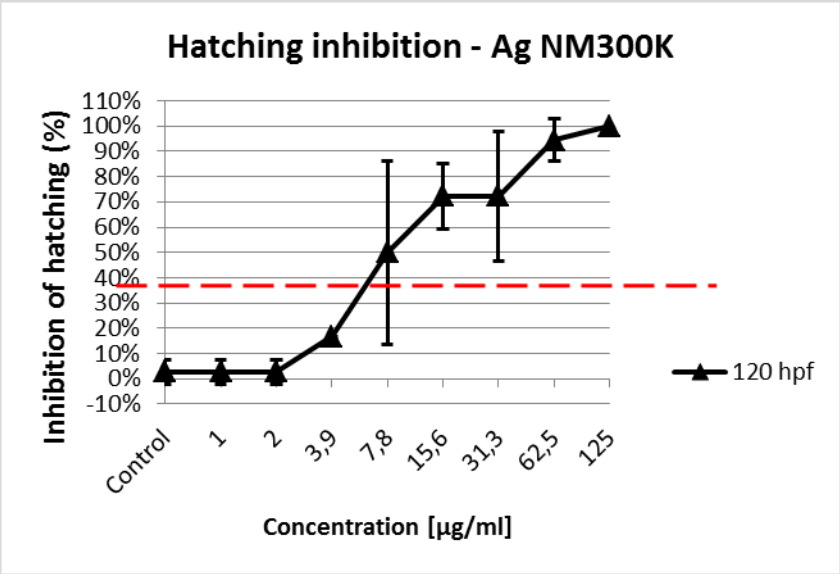

Figure 10: Dose response curves for hatching inhibition at 120hpf of zebrafish embryos treated with silver particles. Embryos were exposed to Ag NM-300K particles in embryo medium in a dilution series ranging from 1 to 125 µg/ml from gastrulation stage (6hpf) onwards. The percentage of non-hatched embryos is indicated based on analysis of microscopic images taken at 120hpf. The average was calculated from three independent experiments except that for the concentration 3.9 µg/ml and 125 µg/ml where only one or two experiments were available. The EC_{50} value (dashed red line) that can be deducted from the curve is approximately 7.8 µg/ml.

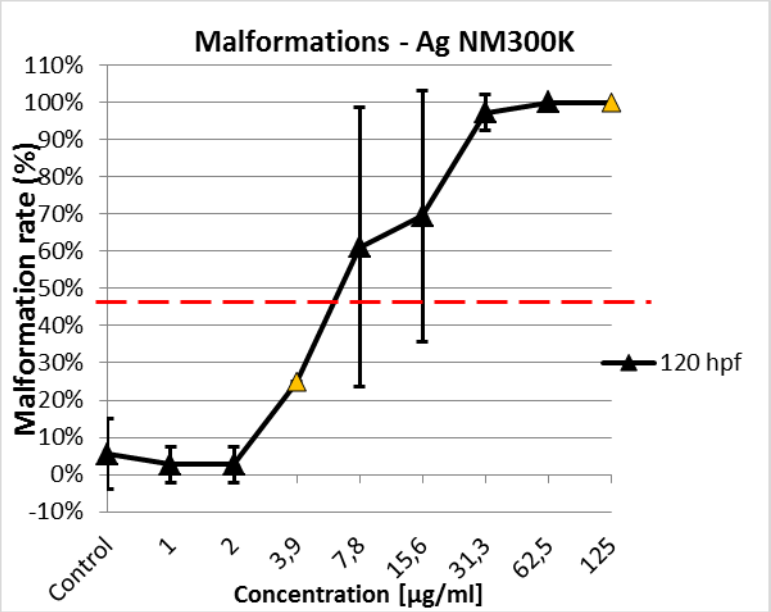

Figure 11: Dose response curve for zebrafish embryo malformation at 120hpf after treatment with silver particles. Embryos were exposed to Ag NM-300K in embryo medium in a dilution series ranging from 1 to 125 µg/ml from gastrulation stage (6hpf) onwards. The percentage of malformed embryos is indicated based on analysis of microscopic images taken at 120hpf. The average was calculated from three independent experiments except that for the concentration 3.9 µg/ml and 125 µg/ml where only one or two experiments were available. The EC_{50} value (dashed red line) that can be deducted from the curve is approximately 7 µg/ml.

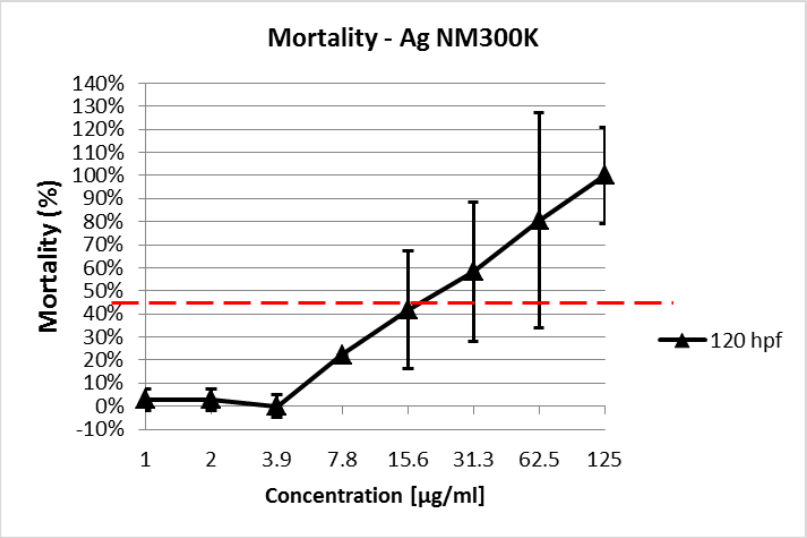

Figure 12: Dose response curve for zebrafish embryo mortality at 120hpf after treatment with silver particles. Embryos were exposed to Ag NM-300K in embryo medium in a dilution series ranging from 1 to 125 µg/ml from gastrulation stage (6hpf) onwards. The percentage of dead embryos is indicated based on analysis of microscopic images taken at 120hpf. The average was calculated from three independent experiments except that for the concentration 3.9 µg/ml and 125 µg/ml where only one or two experiments were available. The EC_{50} value (dashed red line) that can be deducted from the curve is approximately 22 µg/ml.

In summary, for this work of zebrafish, we successfully used nanoparticle exposure and high-content -imaging in a 96 well plate format to obtain quantitative data on several endpoints for zebrafish embryo toxicity. Dose response curves were generated and NOAELs, LOAELs, and EC_{50} values were deducted for the different endpoints. The established exposure conditions and readouts can be applied to other nanomaterials.

2.2.3 D. rerio (Exeter)

Behavioural assay: Table 5 shows the range of AgNPs exposed to zebrafish embryo in order to monitor their impacts on embryo behaviour at 6 dpf. In all cases, behaviour was monitored for 1 hour following exposure at a range of concentrations from 0-1000 µg L^{-1}. Endpoints assessed included: inactivity (Figure 13), rotational events (Figure 14), zebrafish embryo movement measured as the highest distances travelled (Figure 15), medium speed of travel (Figure 16), duration of activity/travel (Figure 17), ratio of high:medium duration activities (Figure 18), number of times where no embryo was observed at all in the field of view (Figure 19) and high activity events (Figure 20) with the AgPVP NPs.

Table 5: Behavioural responses of zebrafish exposed to Ag NMs at 6 dpf

Nanomaterial	Probability of seizure	p=
Ag NM-300K	0.2408	0.988021
Ag PVP	0.011	0.8611
Ag Citrate	0.9652	1
Ag Citrate Bulk	0.4082	1
$AgNO_3$	0.9652	1.0

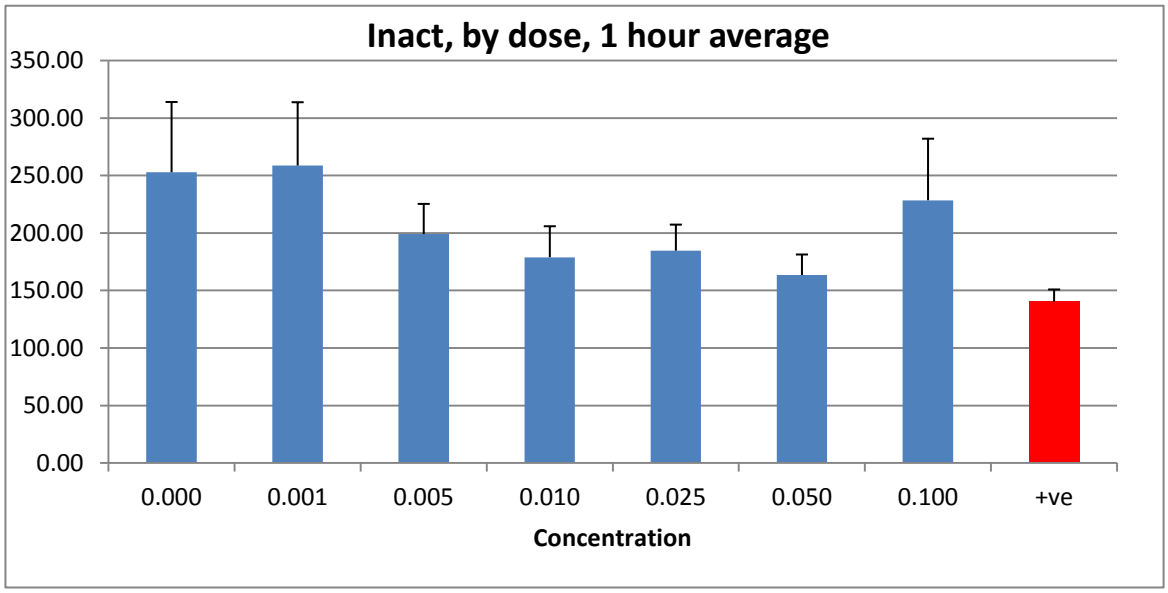

Figure 13. Average inactivity of n=12 zebrafish over 1 hour for increasing concentrations of Ag NM 300k. 0 = negative control, +ve = PTZ positive control, 0.001 represents 10 µg L^{-1} with the concentration increasing 50 µg L^{-1} (0.005), 100 µg L^{-1} (0.01), 250 µg L^{-1} (0.025), 500 µg L^{-1} (0.05) up to 1000 µg L^{-1} (0.1)

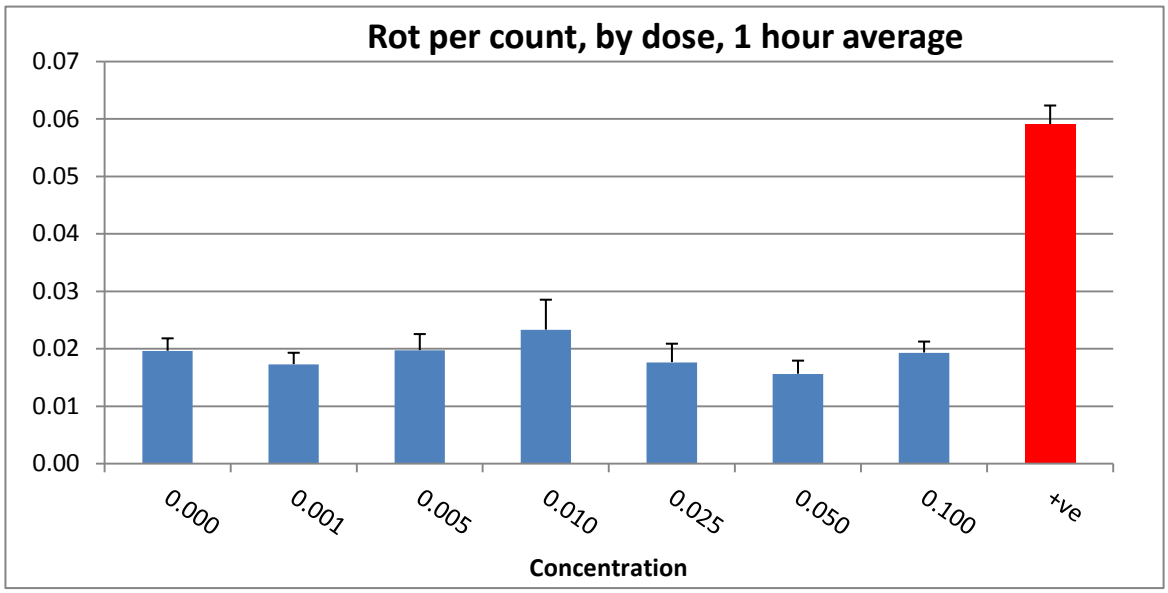

Figure 14. Average counts of 'rotation events' as categorised by the video tracking software of n=12 zebrafish over 1 hour for increasing concentrations of Ag NM 300k. 0 = negative control, +ve = PTZ positive control, 0.001 represents 10 µg L^{-1} with the concentration increasing 50 µg L^{-1} (0.005), 100 µg L^{-1} (0.01), 250 µg L^{-1} (0.025), 500 µg L^{-1} (0.05) up to 1000 µg L^{-1} (0.1)

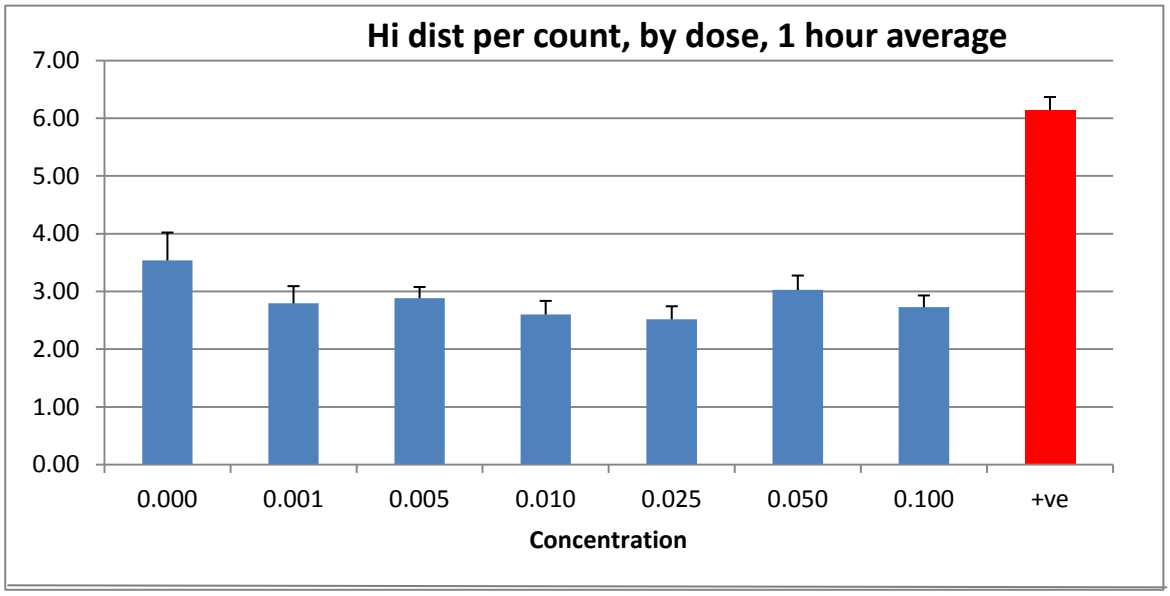

Figure 15. Average counts of zebrafish movement distances categorised as 'high' by the tracking software of n=12 zebrafish over 1 hour for increasing concentrations of Ag NM 300k 0 = negative control, +ve = PTZ positive control, 0.001 represents 10 µg L^{-1} with the concentration increasing 50 µg L^{-1} (0.005), 100 µg L^{-1} (0.01), 250 µg L^{-1} (0.025), 500 µg L^{-1} (0.05) up to 1000 µg L^{-1} (0.1).

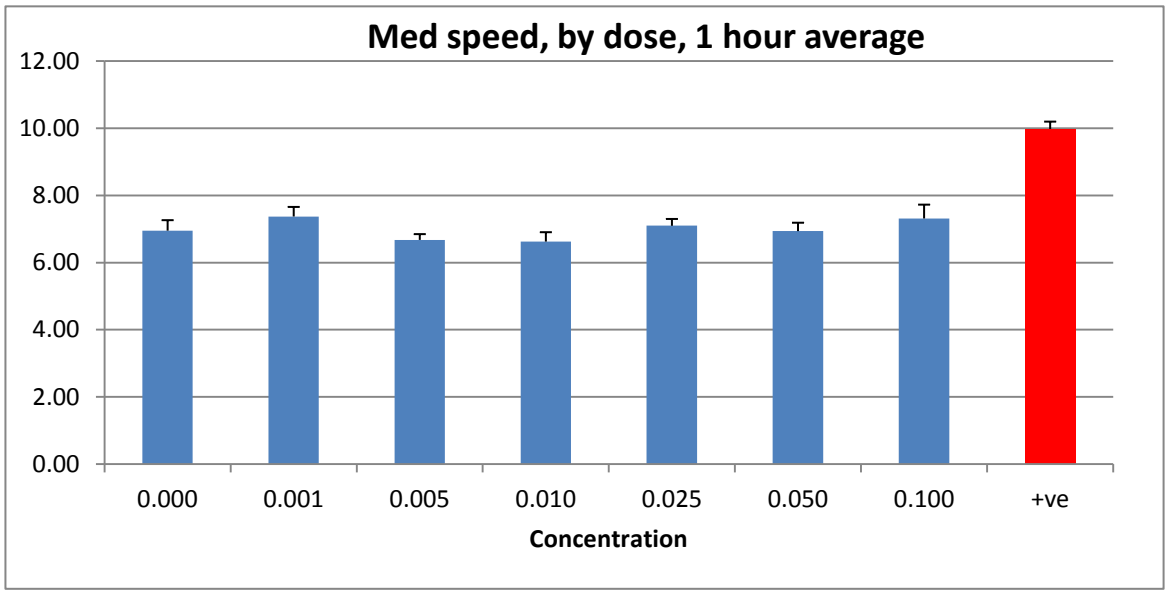

Figure 16. Average counts of zebrafish movement speeds categorised as 'medium' by the tracking software of n=12 zebrafish over 1 hour for increasing concentrations of Ag NM 300k 0 = negative control, +ve = PTZ positive control, 0.001 represents 10 µg L^{-1} with the concentration increasing 50 µg L^{-1} (0.005), 100 µg L^{-1} (0.01), 250 µg L^{-1} (0.025), 500 µg L^{-1} (0.05) up to 1000 µg L^{-1} (0.1)

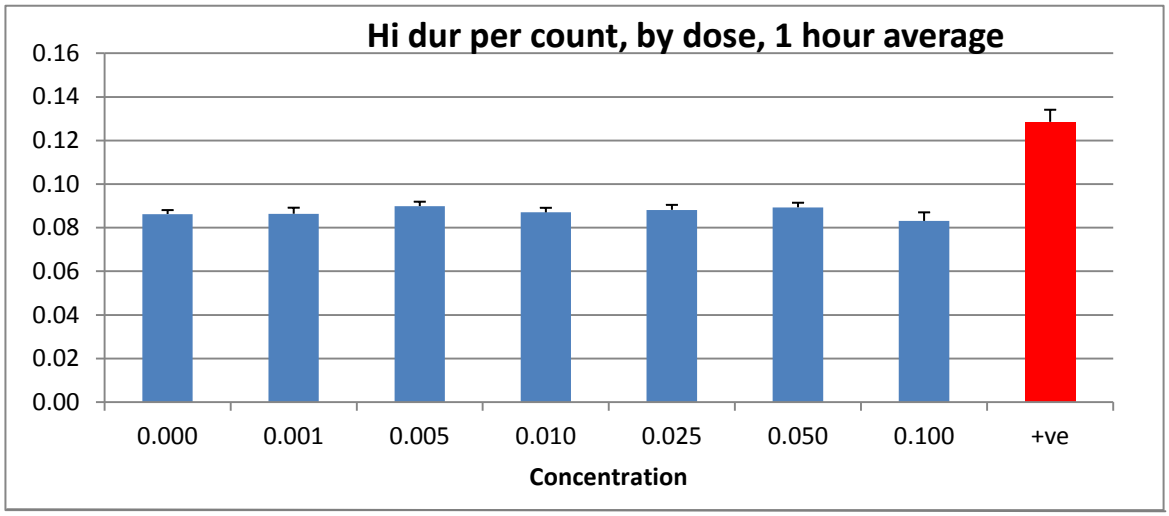

Figure 17. Average counts of 'high duration activities' of zebrafish movement categorised by the tracking software of n=12 zebrafish over 1 hour for increasing concentrations of Ag NM 300k 0 = negative control, +ve = PTZ positive control, 0.001 represents 10 µg L^{-1} with the concentration increasing 50 µg L^{-1} (0.005), 100 µg L^{-1} (0.01), 250 µg L^{-1} (0.025), 500 µg L^{-1} (0.05) up to 1000 µg L^{-1} (0.1).

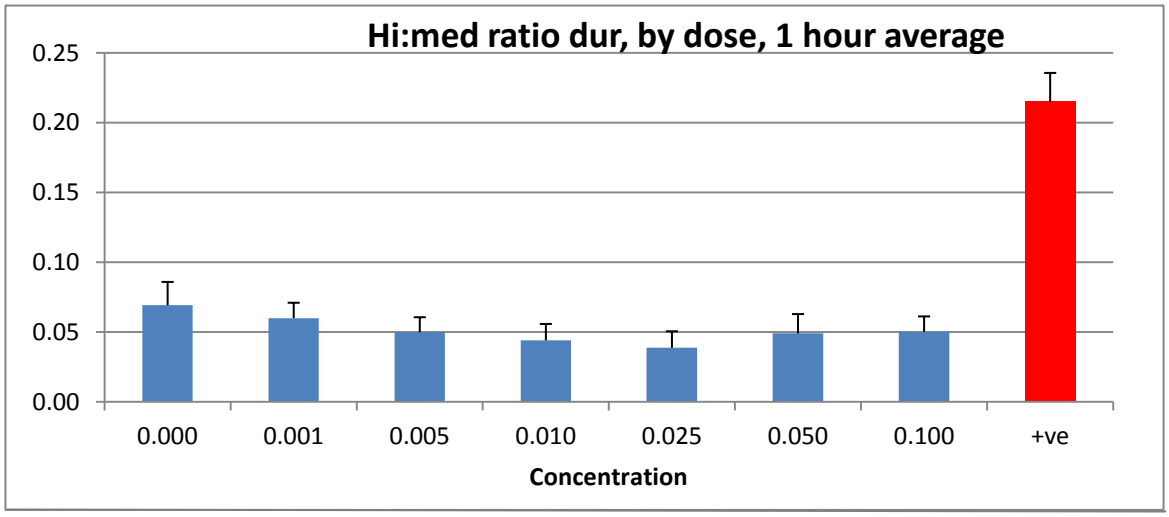

Figure 18. Average ratios of 'high: medium duration activities' of zebrafish movement categorised by the tracking software of n=12 zebrafish over 1 hour for increasing concentrations of Ag NM 300k 0 = negative control, +ve = PTZ positive control, 0.001 represents 10 µg L^{-1} with the concentration increasing 50 µg L^{-1} (0.005), 100 µg L^{-1} (0.01), 250 µg L^{-1} (0.025), 500 µg L^{-1} (0.05) up to 1000 µg L^{-1} (0.1)

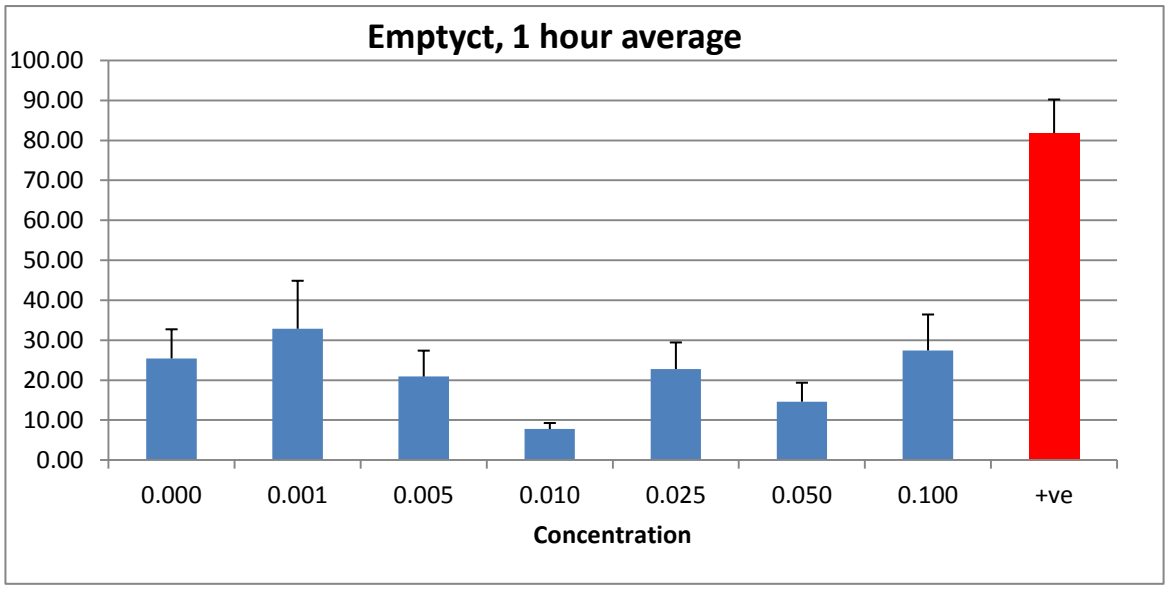

Figure 19. Average number of times during which no object/animal is detected in the screening location by the video capture program

software for n=12 zebrafish over 1 hour for increasing concentrations of Ag NM 300k 0 = negative control, +ve = PTZ positive control, 0.001 represents 10 µg L^{-1} with the concentration increasing 50 µg L^{-1} (0.005), 100 µg L^{-1} (0.01), 250 µg L^{-1} (0.025), 500 µg L^{-1} (0.05) up to 1000 µg L^{-1} (0.1).

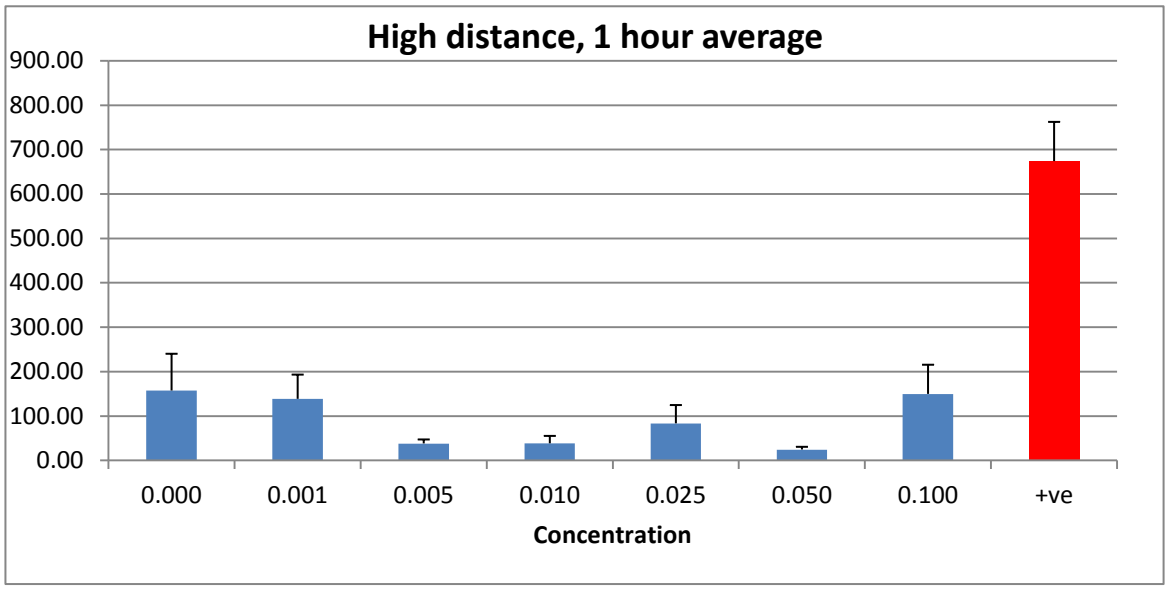

Figure 20. Average 'high distance' events as categorised by the video tracking software for n=12 zebrafish over 1 hour for increasing concentrations of Ag NM. 0 = negative control, +ve = PTZ positive control, 0.001 represents 10 µg L^{-1} with the concentration increasing 50 µg L^{-1} (0.005), 100 µg L^{-1} (0.01), 250 µg L^{-1} (0.025), 500 µg L^{-1} (0.05) up to 1000 µg L^{-1} (0.1). There were 3 treatment specific mortality events across the assay at 1000 µg L^{-1} for $AgNO_3$, AgCit (7nm) and AgPVP. For these 3 treatments there was 100% mortality at the highest concentration after 16 hours of exposure. These studies were then re-run with lower concentrations.

48 hour acute toxicity test

None of the parameters measured were significantly modified by exposure to any of the tested NPs. The toxic concentrations of AgPVP and AgCit (7nm) at 5 dpf were not toxic for 24 and 48 hour embryos. The only toxicity that occurred was for $AgNO_3$ (see Figure 21).

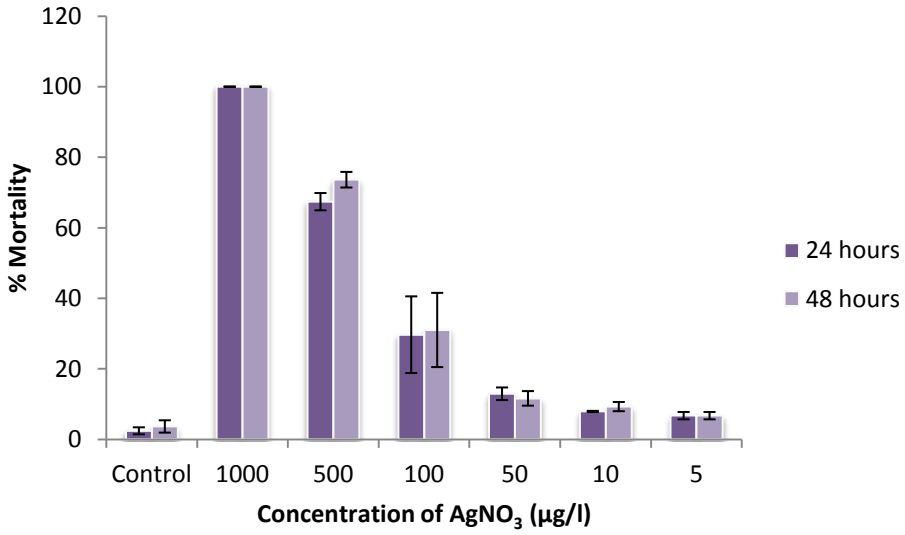

Figure 21. Percentage mortality of zebrafish embryos at 24 and 48 hpf for various concentrations of silver nitrate.

Interestingly, in zebrafish the differently coated silver nanomaterials show varying degrees of toxicity with respect to the effects on behavior. Table 5 shows the probability of seizure in zebrafish adults on exposure to citrate, PVP and dispersant coated silver nanomaterials.

Table 6: Probability of seizure in zebrafish adults on exposure to citrate, PVP and dispersant coated silver NMs

Nanomaterial	Probability of seizure	p=
Ag NM300k	0.2408	0.988021
Ag PVP	0.011	0.8611
Ag Citrate	0.9652	1
Ag Citrate Bulk	0.4082	1
$AgNO_3$	0.9652	1.0

The PVP coated AgNM showed the least probability to cause a seizure and the citrate coated the highest probability. However, whether these differences resulted due to differences in rates of dissolution of the nanomaterials in the medium or due to different uptake rates of the coated nanomaterials has not been determined. The literature overall indicate that the release of Ag^+ and its uptake into the cell is the main cause of AgNP toxicity at least in aquatic organisms.

In further studies if would be interesting to subject AgNM300K to exposures to natural waters containing humic acids that can occur on land to further establish how alterations in surface features affect bioavailability and biological responses in aquatic and terrestial organisms.

2.2.4 Exposures with terrestrial invertebrates

***Feeding parameters and mortality after TiO_2, Ag or CeO_2 NPs ingestion*:** Feeding parameters (feeding rate, food assimilation efficiency, faecal production rate) and survival were not affected below a nominal exposure concentration of 1000 µg TiO_2, Ag or CeO_2 NPs/g dry weight of leaf or 5000 µg CeO_2 NPs/g dry weight of leaf.

***Accumulation of Ag in digestive tissue after NPs ingestion*:** Results of elemental analyses of different body fractions (digestive glands, gut and the rest of the body) with atomic absorption spectroscopy after exposing animals to Ag NPs (JRC) showed that Ag accumulated in the hepatopancreas and not in gut or the rest of body of animals (Figure 22). In contrast Ag accumulated in the hepatopancreas, gut and rest of the body of animals in case of exposure of animals to $AgNO_3$.

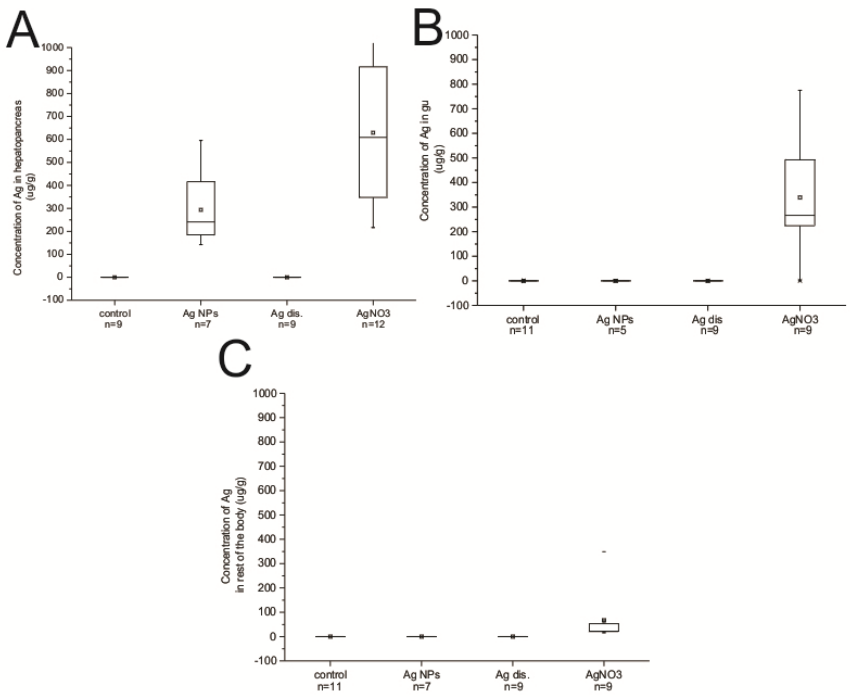

Figure 22: (A) Concentration of Ag in hepatopancreas of animals exposed to Ag NPs, Ag dispersant or $AgNO_3$, (B) Concentration of Ag in gut of animals exposed to Ag NPs, Ag dispersant or $AgNO_3$, (C) Concentration of Ag in rest of the body of animals exposed to Ag NPs, Ag dispersant or $AgNO_3$. Symbols on the box plot represent minimum and maximum data values (whiskers), mean value (□), 75th percentile (upper edge of box), 25th percentile (lower edge of box), median (line in box) and max and min value (-).

2.2.5 C. elegans

Life span experiments were carried out in liquid medium in multi-well plates at 20°C which replicates environmental conditions in the fluid phase of soil. These conditions turned out to be favourable, since untreated wild type N2 worms survived for up to 36 days (Figure 21 A-D). Each experiment included senescence genes mutants *daf-2* or *daf-16* as additional life span controls that define a time window of survival expectancy. An important regulator of *C. elegans* life span is the insulin-like signalling pathway. Life span extensions are caused by mutations in upstream signalling pathway genes such as the *daf-2* insulin-like growth factor (IGF) receptor gene, whereas loss-of function mutations of the downstream *daf-16* forkhead transcription factor gene reduce life span. Accordingly, *daf-2* controls live for up to 44 days in the life span assays, while *daf-16* control worms maximally reach an age of 30 days (Figure 21, A-D).

Addition of a variety of NPs to the life span assays resulted in different worm survival. Addition of 1 μg/ml Ag NM-300K did not alter the life span in comparison to untreated control *C. elegans* (Figure 23). However, incubation with 10 μg/ml Ag NM-300K had already reduced worm survival significantly between days 22 and 32 compared to untreated controls. This outcome was even more dramatic when worms were exposed to 100 μg/ml Ag NM-300K. Here, all *C. elegans* were dead after 8 days. Notably, the concentration of 10 μg/ml Ag NM-300K reduced life span, but NP-exposed worms still survived the short-lived *daf-16* mutants (Figure 21A). The highest concentration of 100 μg/ml Ag NM-300K clearly falls below the survival curve of *daf-16* animals.

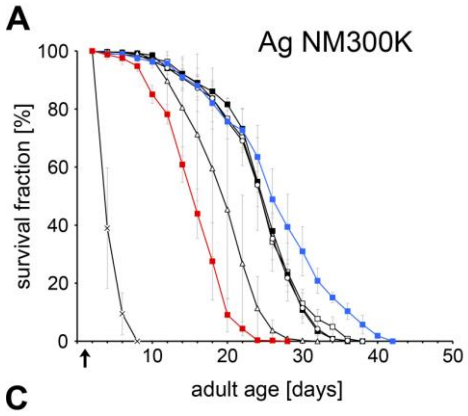

Figure 23. Life span of *C. elegans* exposed to different NPs. Adult *C. elegans* that were exposed to 10 or 100 µg mL^{-1} Ag NPs show a significant decrease ($p<0.01$) of survival in comparison to untreated worms (filled squares). In contrast, the life span of nematodes exposed to 1 µg mL^{-1} Ag NPs or dispersant only is similar compared to untreated controls. Worms exposed to 160, 80 or 20 µg mL^{-1} of

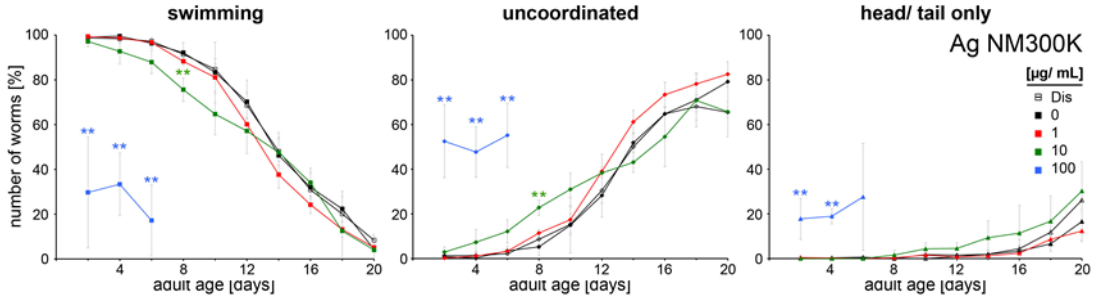

Figure 24. Quantitative observation of locomotion behaviors in NP-exposed *C. elegans*. Locomotion phenotypes of nematodes were classified according to the three categories swimming, uncoordinated or head/ tail only and plotted against adult worm age. All graphs show an age-related decline of swimming nematodes and simultaneous increase of uncoordinated and head/ tail only movements. Phenotypes were scored in nematodes exposed to Ag NPs or the dispersant only control in comparison to untreated controls (black). *, $p<0.05$; **, $p<0.01$; arrows, days of particle addition; dis, dispersant only.

C. elegans treated with 10 µg/ml Ag NM-300K NPs show significantly altered locomotion, *e.g.* significantly reduced swimming on day 8 of the observations (Figure 24; asterisks) compared to the untreated controls. The swimming phenotype is likewise significantly reduced in worms exposed to 100 µg/ml Ag NM-300K NPs between days 2 and 6. Simultaneously these worms show a significant premature acceleration of uncoordinated movements and movements that are restricted to the head and the tail (Figure 24; asterisks). The results of quantitative locomotion scoring suggest that worm survival and locomotion behaviour is closely linked in response to NPs. Ag NM-300K NPs that significantly reduce worm survival also accelerate age-related impairment of forward movement.

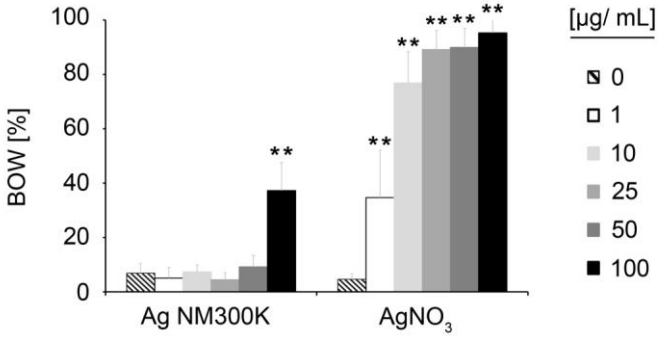

Figure 25. Quantification of the internal hatch (BOW) phenotype in *C. elegans* exposed to different NPs. Nematodes with internally hatched larvae were scored 24 hours after addition of NPs or respective salts. Worms treated with increasing concentrations of $AgNO_3$

showed a significant increase of the BOW phenotype in comparison to untreated controls. The BOW phenotype was likewise significantly increased in nematodes exposed to 100 µg mL^{-1} Ag NPs, but remained unchanged after addition of lower Ag NP-concentrations between 1 - 50 µg mL^{-1}.

2.3 Ceria nanoparticles

2.3.1 Eisenia fetida, Folsomia candida, Hypoaspis aculeifer

Eisenia fetida: Tests need to be repeated due to invalidity of the first tests (Results will be available summer 2015).

Folsomia candida: *Mortality of introduced springtails.* The mortality observed in the control group was 7.5 % and 2.5 % and 10.0 % in the test group with Ceria nanoparticles, and 0.0 % and 5.0 % in the test group with the reference item. No statistically significant difference was seen between the control group and the PROM-CeO_2-20nm and the reference item groups (Fisher's Exact Binomial Test, one-sided greater, $\alpha = 0.05$) was determined for the mortality of the springtails (Table 7).

Table 7**:** Mortality rates for expose to nanomaterials in springtails

Nanomaterial	Concentration [mg/kg sdw]	Total number of adult females introduced	Total number of not recovered adult females	Mean mortality [%]
Control (n=8)	0	80	6	0.988021
PROM-CeO_2-20nm (n=4)	100	40	1	0.8611
	1000	40	4	1
SIGMA-CeO2Bulk-5microns (n=4)	100	40	0	1
	1000	40	2	1.0

n: Number of replicates
sdw: Soil dry weight

Reproduction
The mean number of juveniles in the control group was determined as 778.6 juveniles per replicate. In the treatment groups the mean number of juveniles ranged from 725.6 to 787.7 juveniles per replicate.

No statistically significant difference between the control group and the PROM-CeO_2-20nm and the reference item groups (Student's t-Test, one-sided smaller, $\alpha = 0.05$) was determined for the reproduction of the springtails (Table 8).

Table 8: Impacts on nanomaterial exposure on reproduction in springtails

Treatment group	Concentration [mg/kg sdw]	Mean number of juveniles per replicate	±SD	CV	Reduction in reproduction [1] [%]
Control (n=8)	0	778.6	107.0	13.7	--
PROM-CeO2-20nm (n=4)	100	775.3	67.6	8.7	0.4
	1000	725.6	102.7	14.2	6.8
SIGMA-CeO_2Bulk-5microns (n=4)	100	787.7	114.0	14.5	-1.2
	1000	777.1	146.6	18.9	0.2

[1] Negative values indicate higher reproduction compared to control

CV: Coefficient of Variation, ±SD: Standard Deviation, n: Number of replicates, sdw: Soil dry weight

Hypoaspis aculeifer

Mortality of introduced adult females

The mortality observed in the control group was 10.0 % and 17.5 % and 10.0 % in the test group with Ceria nanoparticles, and 5.0 % and 7.5 % in the test group with the reference item. No statistically significant difference between the control group and the PROM-CeO_2-20nm and the reference item groups (Fisher's Exact Binomial Test, one-sided greater, $\alpha = 0.05$) was determined for the mortality of the mites (Table 9).

Table 9: Mortality rates for exposure to nanomaterials in *Hypoaspis*

Treatment group	Concentration [mg/kg sdw]	Total number of adult females introduced	Total number of not recovered adult females	Mean mortality [%]
Control (n=8)	0	80	8	10.0
PROM-CeO_2-20nm (n=4)	100	40	7	17.5
	1000	40	5	12.5
SIGMA-CeO2Bulk-5microns (n=4)	100	40	2	5.0
	1000	40	3	7.5

n: Number of replicates

sdw: Soil dry weight

Reproduction

The mean number of juveniles in the control group was determined as 234.4 juveniles per replicate. In the treatment groups the mean number of juveniles ranged from 236.8 to 307.5 juveniles per replicate. No statistically significant difference between the control group and the PROM-CeO2-20nm

and the reference item groups (Student's t-Test, one-sided smaller, α = 0.05) was determined for the reproduction of the springtails (Table 10).

Table 10: Impacts of nanomaterial exposure on reproduction in *Hypoaspis*

Treatment group	Concentration [mg/kg sdw]	Mean number of juveniles per replicate	±SD	CV	Reduction in reproduction [1] [%]
Control (n=8)	0	234.4	30.6	13.1	--
PROM-CeO2-20nm (n=4)	100	263.5	44.9	17.0	-12.4
	1000	236.8	31.5	13.3	-1.0
SIGMA-CeO2Bulk-5microns (n=4)	100	307.5	26.5	8.6	-31.2
	1000	301.3	26.5	8.8	-28.5

[1] Negative values indicate higher reproduction compared to control

CV: Coefficient of Variation, ±SD: Standard Deviation, n: Number of replicates, sdw: Soil dry weight

2.3.2 *C. elegans*

CeO_2 NM212 or CeO_2 NM211 NPs were tested in the life span assays. As per ZnO NPs, CeO_2 NPs applied in concentrations between 20 and 160 μg/ml did not reduce the life span of adult *C. elegans*. As compared to their untreated counterparts, CeO_2 NP-exposed *C. elegans* survived for up to 36 days and the respective survival curves show no significant differences (Figure 25). These results are further validated by the observation that survival curves of untreated controls as well as CeO_2 NP-treated worms run between the ones of short-lived *daf-16* and long-lived *daf-2* mutants, respectively. Taken together we show that NM212 or CeO_2 NM211 NPs do not alter worm survival in concentrations between 20 and 160 μg/ml.

To record the impacts of CeO_2 NPs on worm movements, all movements, animals were scored according to three modes of locomotion in liquid medium, namely (1) swimming, (2) uncoordinated or (3) head/tail only during their entire life span (Figure 26). Untreated wild-type *C. elegans* show an age-related decline of swimming movements, whereas uncoordinated and head/tail restricted movements increase with age. Worms exposed to 20, 80 and 160 μg/ml CeO_2 NM212 or CeO_2 NM211 NPs display similar locomotion phenotypes in comparison to untreated controls (Figure 26). Thus, CeO_2 NM212 or CeO_2 NM211 NPs neither reduce *C. elegans* life span nor alter locomotion phenotypes in comparison to untreated control worms.

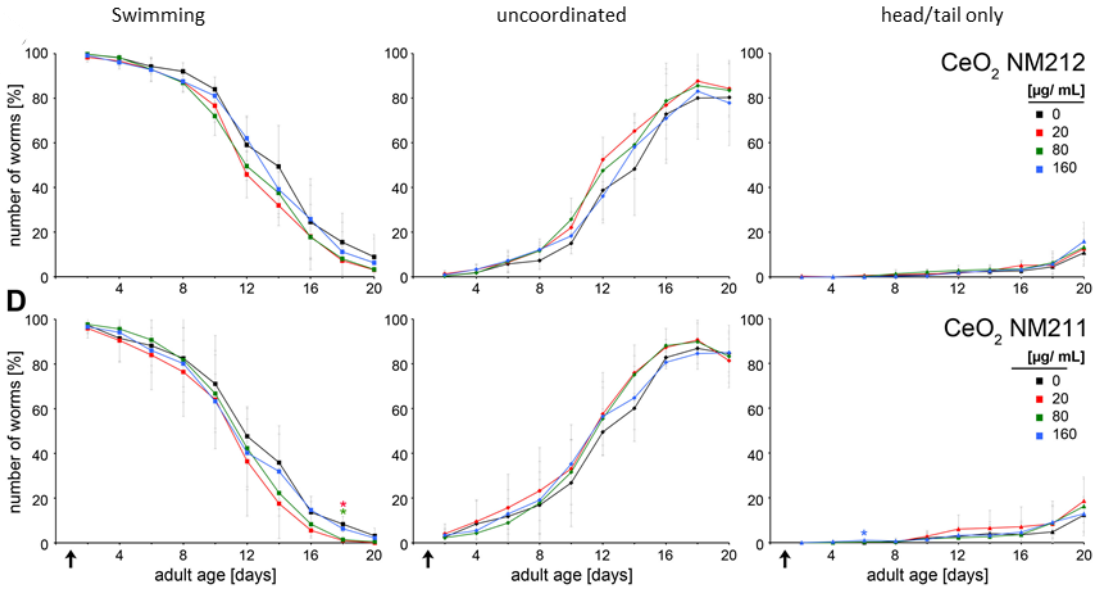

Figure 26. Quantitative observation of locomotion behaviors in NP-exposed *C. elegans*. Locomotion phenotypes of nematodes were classified according to the three categories swimming, uncoordinated or head/ tail only and plotted against adult worm age. All graphs show an age-related decline of swimming nematodes and simultaneous increase of uncoordinated and head/ tail only movements. Phenotypes were scored in nematodes exposed to CeO_2 NM212 NPs and (D) CeO_2 NM211 NPs in comparison to untreated controls (black). *, p<0.05; **, p<0.01; arrows, days of particle addition; dis, dispersant only.

Next we analysed another behavioural phenotype that was previously reported to occur after exposition of adult *C. elegans* to silica nanoparticles (*Pluskota et al., 2009*). The bag of worms (BOW) represents a neural phenotype that is normally age-related and results from neuromuscular defects of the vulva. Hence, egg laying through the vulva is impaired and eggs hatch within the body of the parent worm (internal hatch). Wild type N2 worms were left untreated or exposed to CeO_2 NM212 or CeO_2 NM211 NPs and their salts in different concentrations. Neither CeO_2 NPs induced a significant increase of the BOW phenotype indicating that the neuromuscular function of the vulva is not a target for all nanoparticles (Figure 26). Again, the results of the BOW analyses correspond well with both, life span analyses and locomotion scoring in that only the Ag NM-300K NPs show significant effects. Detailed studies of the role of Ag-ion dissolution are required due to the result that $AgNO_3$ salts likewise significantly induce the BOW phenotype.

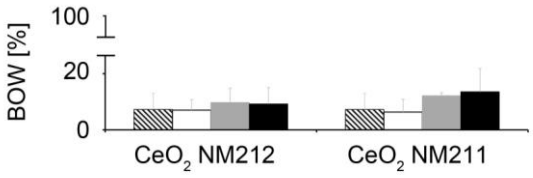

Figure 27. Quantification of the internal hatch (BOW) phenotype in *C. elegans* exposed to different NPs. Nematodes with internally hatched larvae were scored 24 hours after addition of NPs or respective salts. Worms treated with increasing concentrations CeO_2 NM212 and NM211 NPs (C). **, p<0.01; BOW, bag of worms.

Digestive gland cell membrane stability after CeO_2 NPs ingestion: We have previously published that the digestive gland cell membrane stability value was higher than 2 (on a scale from 0 to 9) in only 5% of animals from a stock culture of animals that were in good physiological condition. The cell membranes are considered destabilised when this value is higher than an index of 2. The digestive

gland cell membrane stability of controls or animals exposed to 1000 or 5000 µg CeO_2 NPs/g dry weight of leaf was not affected.

3. Overall conclusions

The work reported here is really just the initial baseline study which will be used in parallel with the subsequent round of studies on the environmentally aged (equivalent) NMs in order to really tease-out the role and effects of structure and surface features, including coatings, and transformation of nanomaterials (NMs) in biological media, on the subsequent impacts of the nanomaterials on test organisms. Thus, the work presented here can be seen as the "before" with the "after" to come as the environmentally aged and transformed NMs are tested. The comparison of the two datasets will provide the key insights into the contributions of the various parameters, including surface coating, to overall toxicity observed, including the link to the role of surface coating and surface composition in determining NM uptake and localisation. Thus, it is still relatively early days in the overall plan of work, although very significant progress has been made, as can be seen here.

In terms of the testing of the aged NMs, as per D3.2 (Experimental protocols for aged MNM) the protocols for ageing the NMs are now fully established, and indeed the aged NMs have been distributed to partners for testing (see also Milestone MS6 which describes the Screening and selection of aged MNM for further analysis in WPs 5-8: GO / NO GO decision"). Thus, rapid progress towards the next phase of testing is expected, with an update on this activity to be provided in the periodic report at M36.

The only nanomaterial found to be toxic at low concentrations across *C. reinhardtii, C. elegans, and Danio rerio* was AgNM300K. It is stabilized in the dispersant which contains 4% (w/w) Polyoxyethylene Glycerol Trioleate and 4% (w/w) Polyoxyethylene (20) Sorbitan mono-Laurat (Tween 20). However, the dispersant alone was not toxic to any of the organisms.

Comparing multiple endpoints such as life span and age-related behaviour phenotypes we show that Ag NPs induce significant toxicity in a concentration-dependent manner. Such toxicity was confirmed by the other endpoints. Nano-Ag likewise reduced life span, accelerated age-related reduction of locomotion and induced the reproductive defect internal hatch. The results are in good agreement with the literature as Ag NPs are generally reported to be toxic in *C. elegans* and across species, *e.g.* in bacteria, algae, crustaceans, and fish. In contrast, we did not observe any adverse effects of CeO_2 or ZnO NPs in our chronic exposure studies, indicating that the particles used do not confer toxicity in adult *C. elegans*. Table 3 above provided a summary of the data.