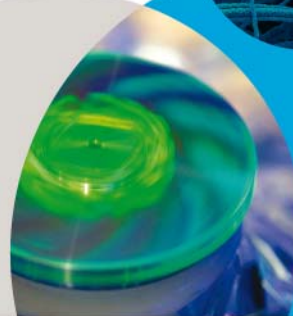
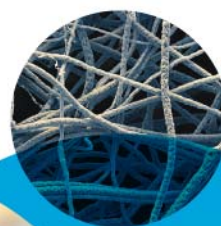
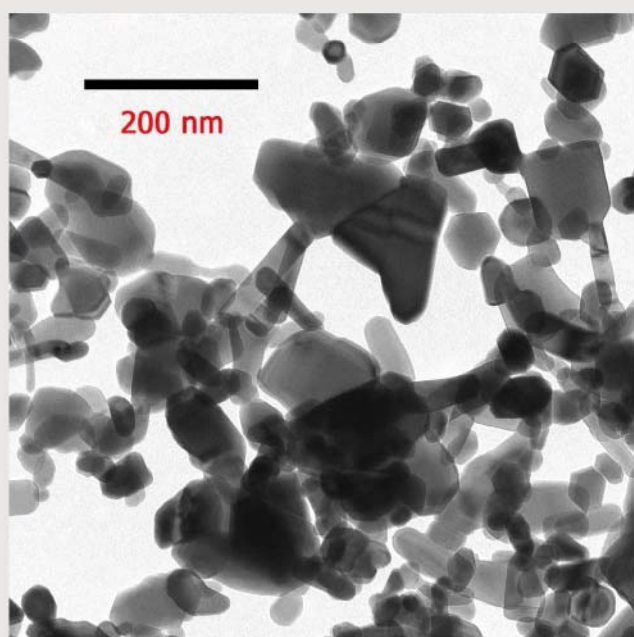


## Interim Report for Ecotoxicology Testing of Manufactured ZnO and CeO<sub>2</sub> Nanoparticles



September 2010

IN SUPPORT OF PROSPECT:

Ecotoxicology Test Protocols for Representative  
Nanomaterials in Support of the OECD Sponsorship Programme

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Cover photo: BASF SE Z-COTE® zinc oxide nano particles (Image courtesy of BASF SE)

## Executive summary

### Background

The PROSPECT Link project provides a direct contribution to the global safety assessment of nanomaterials described by the OECD Working Party on Manufactured Nanomaterials (WPMN). The project aims to develop fit for purpose ecotoxicology test methods and data on two selected nanomaterials: zinc oxide (ZnO) and cerium oxide (CeO<sub>2</sub>) manufactured nanoparticles (MNPs), which have been identified as commercially relevant to the global economic impact of nanotechnology. Within PROSPECT, we are investigating the ecotoxicology of the test MNPs to fish, invertebrates and algae inhabiting both aquatic and terrestrial environments.

This review summarises the work undertaken to date (month 19), which has focused on the ecotoxicology of ZnO and CeO<sub>2</sub> MNPs to two invertebrates: the model organisms *Daphnia magna* (waterflea) and *Corophium volutator* (mudshrimp).

The MNPs selected for study were those of relevant size and/or most comprehensive physical-chemical characterisation. These were:

1. Cerium oxide (supplied by Antaria, particle size ~ 10 nm),
2. Zinc oxide-Nanosun (supplied by Micronisers Ltd, particle size ~ 30 nm)
3. Zinc oxide-Zcote (supplied by BASF, particle size ~ 170 nm)

### Results

#### Nanoparticle dissolution

- Rapid dissolution of ZnO MNPs (Nanosun) occurred in seawater and in organism test water. At a concentration of 20 mg l<sup>-1</sup> about 25% of ZnO MNP was dissolved in seawater, whereas 10-12% of the ZnO MNP was dissolved in the organism test water. This is important since the dissolution rate and formation of Zn ions (Zn<sup>+</sup>) is likely to influence the bioavailability and hence the toxicity of different forms of zinc. Future work will replicate these experiments with CeO<sub>2</sub> MNP (Antaria).

#### Water column toxicity-D.magna

- Acute exposure (48h) of *D. magna* to CeO<sub>2</sub> MNPs (modified OECD test guideline 202) had no effect on mortality at concentrations up to 100 mg l<sup>-1</sup>.
- Acute exposure (48h) of *D. magna* to ZnO MNPs (modified OECD test guideline 202) caused a concentration-dependent increase on mortality (LC50 1.55 mg l<sup>-1</sup>).
- *D. magna* exposed to sublethal concentrations of ZnO MNPs had a lower feeding rate compared to bulk ZnO and soluble zinc. This did not appear to be related to physical impairment or obstruction of the organism's feeding apparatus.
- Bioimaging using Coherent Anti-stokes Raman Scattering (CARS) and optical light microscopy confirmed that *D. magna* ingested large quantities of both MNPs, but in the case of CeO<sub>2</sub> MNPs the high uptake did not affect survival.

#### Sediment toxicity –C. volutator

- Acute exposure (10 days) of *C. Volutator* to ZnO MNPs (modified ASTM test guideline 2000) caused a concentration-dependent increase in mortality which was similar for bulk (micron sized), soluble and nanoscale ZnO irrespective of particle size (average particle sizes tested: 30 -170 nm).
- Acute exposure through the overlying water was 10-fold more toxic than acute exposure through the sediment. This would suggest an increase in bioavailability of ZnO MNPs when delivered through the water column, although this has not yet been confirmed.

- Chronic exposure (100 days) of *C. volutator* to ZnO in micron, nano or soluble form had no effect on survival, but inhibited reproduction at all concentrations ( $\geq 1 \text{ mg l}^{-1}$ ). Specific growth rate and age of sexual differentiation were significantly delayed by increasing concentrations of zinc, but after 100 days all populations were sexually differentiated. Sublethal toxicity (DNA damage in hemolymph cells) was significantly lower for nanosun ZnO ( $\geq 0.5 \text{ mg l}^{-1}$ ) than for all other forms of zinc. This suggests that ZnO MNPs may have lower bioavailability than micron or soluble Zn to sediment dwelling organisms.
- Bioimaging was used to identify zinc granule accumulation in the hepatopancreas, suggesting this is a major site of metal detoxification in this species.

### **Future work**

- Dissolution rates of bulk ZnO and all forms of CeO<sub>2</sub> MNPs in test media are being calculated. This is important to help differentiate the effects of free metal and size related nano effect of the nanoparticles.
- More detailed investigation of bioaccumulation and biological effects (growth, survival reproduction, sublethal toxicity including free radical damage) are being performed for both invertebrate test species. This is important to help in the interpretation of the results.
- Future work will extend ecotoxicology testing to algae, fish and terrestrial invertebrate species and may consider interspecies differences and food web effects.

## 1. Background

Nanotechnology industries aim to generate significant benefits for society, ranging from new materials for the creation and storage of alternative energy technologies, to effective remedial soil and water treatments, to products and equipment for personalised healthcare. Industries and consumers are already benefiting from the performance-enhancing properties of manufactured nanomaterials (MNPs) in a number of products; and many more processes and products enhanced by properties that occur exclusively on the nanoscale are anticipated to enter the market within the next 10 to 20 years, providing a significant contribution to the economic growth of a country and to its high-technology status within the global market.

The OECD (Organisation for Economic Co-Operation and Development) Working Party on Manufactured Nanomaterials (WPMN) was established in 2006, to help member countries efficiently and effectively address the safety challenges of nanomaterials. This Working Party, which brings together more than 100 experts from governments and other stakeholders, launched the *OECD Sponsorship Programme of Manufactured Nanomaterials*, a global programme that pools expertise and funding to test the human health and environmental safety effects from 14 types of nanomaterial, which have been identified as commercially relevant to the global economic impact of nanotechnology.

The *PROSPECT LINK* project helps to fulfil the UK's contribution to the OECD programme to examine the environmental safety of manufactured nanomaterials important to UK industry, zinc oxide (ZnO) and cerium oxide (CeO<sub>2</sub>) nanoparticles. It addresses gaps in the current level of knowledge on the physico-chemical and ecotoxicological properties of these materials, and supports fundamental scientific research to establish robust scientific test methodologies for hazard identification. Available standard tests for bulk chemicals (*i.e.* not in nano form) may not adequately protect for the effects of nanomaterials, although it is not yet known whether indeed this is the case. A review of the current understanding of the physico-chemical and ecotoxicological properties of these materials has been performed to identify knowledge gaps (Prospect deliverables 1 and 2). In the current work and in the accompanying report (Imperial College London Prospect report-Mark Rehkamper), we are working to identify new nanomaterial-specific test methodologies for cerium and zinc nanoparticles.

## 2. Role of University of Exeter in PROSPECT

The University of Exeter is responsible for investigating the ecotoxicology of ZnO and CeO<sub>2</sub> MNPs to OECD-listed fish, invertebrate and algae species representing aquatic and terrestrial environments. The testing regime takes into account different routes of exposure and measures OECD relevant endpoints (such as growth, survival and reproduction). This review summarises the work undertaken to date (month 19), which has focused on the testing of ZnO and CeO<sub>2</sub> MNPs in two invertebrate test organisms: the planktonic freshwater *Daphnia magna* and the sediment dweller amphipod *Corophium volutator*.

## 3. Organisms

### 3.1 *Daphnia magna*

*D. magna* (Figure 1) is a small (0.2-5 mm) freshwater planktonic crustacean of the order cladocera that inhabits the water column. Under ideal environmental (laboratory) conditions *D. magna* reproduce parthenogenetically to produce clonal offspring. This parthenogenetic mode of reproduction (isolating genetic variability) and short life cycle (egg to adult in ~10 days) make *D. magna* an ideal organism for studying the physiological impacts of nanomaterials in the water column.



Figure 1. Light micrograph of a 3-day old *Daphnia magna*. At 20°C *D. magna* reach sexual maturity in 6-8 days releasing their eggs into a brood chamber. Eggs are nurtured in the brood chamber inside the carapace. The embryos complete their development inside the brood chamber and hatch as free-swimming neonates at day 8-10. In the following 2-4 days the mature females release a 2<sup>nd</sup> brood of neonates with reproduction peaking around the 3<sup>rd</sup> brood (day 12-14) or 4<sup>th</sup> brood (day 14-17).

### 3.2 *Corophium volutator*

*C. volutator* (Figure 2) is a small amphipod (6-9 mm) that lives the sediment of estuaries and coasts. *C. volutator* lives burrowed in U-shaped tubes dug in upper sediment layers, and feeds by ingesting sediment particles as well as on plankton that flows though its tube during high tides. The behaviour and ecological niche of *C. volutator* makes it an excellent model for NMP, since the ultimate environmental fate of most nanomaterials, including ZnO and CeO<sub>2</sub> MNPs, is likely to be the sediments.



Figure 2. *Corophium volutator* (female) in sediment. Notice the U-shaped borrow used for protection (from <http://www.speciesatrisk.ca/fundyshorebirds/region/corophium.html>)

## 4. Methods

### 4.1 CeO<sub>2</sub> and ZnO nanoparticles

Based on the information available prior to starting this study (Table 1) the MNPs selected for study were: CeO<sub>2</sub> (Antaria, ~ 10 nm), ZnO-Nanosun (Micronisers, ~ 30 nm), and ZnO Z-COTE (BASF, ~100 nm). Further physico-chemical characterisation of both particles in selected organisms' test media is being investigated by the National Physics Laboratory (see separate report).

	Primary particle size (nm)	Surface area	Porosity	Crystal structure	Impurities (% purity)
ZnO-Nanosun	30-50	x	x	x	X
ZnO-Zcote	100	x	x	x	X
ZnO Zcote HP1	130	x	x	x	X
ZnO Sigma Aldrich	>1000	x	x	x	x
CeO <sub>2</sub> - Umicore	50-70	x	x	x	X
CeO <sub>2</sub> -Antaria	10	x	x	x	x
CeO <sub>2</sub> -Sigma Aldrich	>1000	x	x	x	x

Table 1. Reported size and required physicochemical properties for ecotoxicology testing of MNPs (x: values not yet determined).

### 4.2 Preparation of nanoparticle dispersion

Experimental test concentrations were prepared following the protocol suggested by the National Physics Laboratory and available online at <http://www.nanotechia-prospect.org/>. In brief, a known concentration of MNP was measured out and made into a paste by addition of a drop or two of deionised water. Subsequently, 9 more drops of water were added to the paste and mixed prior to increasing the volume up to 15 ml with DDI water. The suspension was sonicated twice for 10 seconds (Cole-Parmer® 130-Watt Ultrasonic Processors (50/60 Hz, VAC 220)) and used as the stock suspension. Known volumes of stock suspension were aliquoted out, added to the experimental beakers, at the required dosing regimes, and mixed with a glass rod.

### 4.3 Nanoparticle dissolution

Equilibrium dialysis was used as described in Frankin et al. (2007) in order to investigate the dissolution rate of ZnO MNP in *C. volutator* test media (25 ppt seawater) and in *D. magna* test medium. In brief, Cole Parmer (Vernon Hills, IL) Spectra/Por 7 dialysis membranes of 1000 Da molecular weight cut-off (nominal pore size) and 20 mm diameter were cut into 10 cm lengths and rinsed thoroughly in ultrapure water prior to use. The dialysis cells were formed by filling the membrane tubes with Milli-Q water and sealing them with plastic dialysis clips which had been cleaned by soaking in 10% (v/v) nitric acid for 24 h and thoroughly rinsed in Milli-Q water. The volume of the cells was approximately 10-15 mL. Test solutions were prepared by dosing ZnO MNP (20 mg l<sup>-1</sup>) both test media. Dialysis cells were added to the test suspension which was stirred continuously for 5-7 days, and maintained at the same temperature and pH values as required for the ecotoxicology tests performed. At each sampling time, two dialysis cells were removed from the suspensions, and the content acidified with ultrapure HNO<sub>3</sub> and the total zinc was measured by ICP-OES.

## 4.4 Experimental Setup of Ecotoxicity Tests for *D. magna*- Water column toxicity

### 4.4.1 Husbandry

In accordance with OECD guideline 202 (OECD, 2004), *D. magna* (obtained from the University of Birmingham, UK) were cultured at a density of 20 animals per 1200 ml media (OECD-recommended ISO test media for exposures: 195.87 mg l<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O 82.20 mg l<sup>-1</sup>, mgSO<sub>4</sub>·7H<sub>2</sub>O mg l<sup>-1</sup>, 64.80 mg l<sup>-1</sup> NaHCO<sub>3</sub>, 5.80 mg l<sup>-1</sup> KCl, including 0.002 mg l<sup>-1</sup> sodium selenite, pH 7.5 and 20°C) in a 16:8h light: dark photoperiod and temperature of 20 °C. Media was renewed 3 times a week and supplemented with an organic seaweed extract: Marinure (Wilfrid-Smith Limited, Oakley Hay, UK). Cultures were fed on suspensions of the green alga *Chlorella vulgaris*, and this was further supplemented daily with 8µg l<sup>-1</sup> of dried baker's yeast.

### 4.4.2 Acute exposure effects of ZnO MNPs on *D. magna*

*D. magna* was used in 48h acute immobility test in accordance with the OECD guideline 202 with modifications. To determine the 48h acute LC50 and LC100 concentrations of ZnO MNP to *D. magna*, a range-finding experiment was performed. Groups of 10 \* 3-day old daphnids were transferred to 250 ml glass beakers containing 100 ml of test media with 0-10 mg l<sup>-1</sup> of ZnO, or 1-100 mg l<sup>-1</sup> of CeO<sub>2</sub>. The same mass of soluble zinc ions (from ZnCl<sub>2</sub>) and bulk ZnO or bulk CeO<sub>2</sub> were used to compare and discriminate amongst potential size and soluble fraction effects. The organisms were not fed during the experiment and after 48h the number of immobile organisms was counted.

### 4.4.3 Effects of sublethal concentrations of ZnO MNPs on the feeding rate of *D. magna*

Ten 3-day old daphnids were placed in 250 ml glass beaker containing 100 ml test media (pH 7.5, 20°C). A 24 h exposure was performed to sublethal concentrations of ZnO MNP, bulk ZnO and the same mass of Zn ions (from ZnCl<sub>2</sub>). Thereafter, organisms were transferred to fresh and aerated media and their feeding rate assessed by quantifying the uptake of *C. vulgaris* by UV spectroscopy (OD<sub>440</sub>). A known concentration of algae was added to each beaker to a desired final algae density (OD<sub>440</sub> 0.04±0.002). After 24h the optical density of the test media (OD<sub>440</sub>) and the algae density of the suspension were measured and the total algae uptake quantified.

### 4.4.4 Bioimaging of ZnO and CeO<sub>2</sub> uptake by *D. magna* by Coherent Anti Stokes Raman Scattering and light microscopy

After a 48h exposure to ZnO and CeO<sub>2</sub> MNP, organisms were harvested, rinsed twice with fresh *D. magna* test medium and visualised using an optical light microscope Nikon SMZ and imaged with a 20x objective. To further observe a pattern of uptake and a potential translocation of the MNP from the gut to nearby tissues coherent anti-stokes Raman scattering (CARS) microscopy was used. The imaging microscope is a multi-photon 3D imaging technique with subcellular resolution based on contrasting structures on a distinctive molecular vibration within the sample. It is a non-invasive technique and does not require labelling or staining of the samples. This technique has proven useful for localising metal oxide nanoparticle aggregates within tissues (Moger, 2008; Galloway et al., 2010; Johnston et al., 2010). Sample preparation involved harvesting exposed and non exposed daphnids, rinsing them three times with fresh test medium, anaesthetising them with a 10-15 drops of tricaine (0.4 g l<sup>-1</sup>) and embedding in a 1% soft agar gel and placing in glass bottomed Petri dishes. The forward CARS signal was collected by an air condenser (NA) 0.55 and directed onto a red-sensitive photomultiplier tube (R3896, Hamamatsu). The epi-CARS signal was collected by use of the objective lens and separated from the pump and Stokes beams by a long-wave pass dichroic mirror (z850rdc-xr, Chroma Technologies) and directed onto a second R3896 photomultiplier tube.

## 4.5 Experimental Setup of Ecotoxicity Tests for *C. volutator*- Sediment toxicity

### 4.5.1 Husbandry

Sediment and *C. volutator* were collected from the Otter estuary, Devon (ordinance survey grid reference: SY065820). Organisms were transported back to the laboratory and acclimated at 12°C in trays containing sediment and overlaying 25 ppt seawater in a 12:12h light:dark cycle for 7–10 days to acclimate them prior to any experimental work.

### 4.5.2 Acute exposure to ZnO nanoparticles

Acute sediment tests were based on standard 10-day sediment toxicity tests (ASTM, 2000; Roddie and Thain, 2001; USEPA, 2001) with a light regime modifications, as suggested by Scarlett et al. (2007). Adult *C. volutator* (size range 4–7 mm, n = 20) were exposed to increasing concentrations of ZnO MNP, zinc ions and bulk zinc. Dosing and exposures were done either via water or via sediment. The animals were not fed during the test. At the end of the test the sediment was gently sieved (300 µm) and the number of alive, dead and missing amphipods in each vessel recorded.

### 4.5.3 Chronic exposure to ZnO nanoparticles

Chronic tests were performed based on the USEPA (2001) amphipod chronic test, and modified as in Conradi and Depledge (1999) and Scarlett et al. (2007) and *C. volutator* neonates were harvested by sieving the sediment through a 500 µm nominal pore size sieve to remove larger animals. The sieved fraction was then re-sieved using a 300 µm sieve where neonates were then collected. 20 organisms were transferred to 2L glass beakers containing 7 mm of sieved natural sediment and 500 mL of continuously aerated fresh seawater (pH 8, 25 ppt, 12 °C). Dosing of ZnO MNP, bulk ZnO and the same mass of Zn ions (from ZnCl<sub>2</sub>) was done via the water. Organisms were fed weekly, and water changes and dosing via the water were undertaken 24h after feeding. Every 28-35 days three replicate beakers were sampled from each treatment and control for a period of 100 days.

### 4.5.4 Survival, growth and reproductive output

Survival, growth and reproduction (quantified based on the release of neonates) was measured at every sampling point (28, 63 and 100 days). Three beaker replicates per treatment were sampled by sieving the sediment with a sediment sieve of nominal pore sizes of 500 and 300 µm. Adults and juveniles were collected at the 500 µm fraction whereas neonates were collected at the 300 µm fraction. The total number of live, dead and missing individuals was recorded and their size measured. Reproduction was assessed by counting the number of neonates in the 300 µm sediment fraction. Specific growth rate was assessed, considering it as the rate of increase in length related to the length of the animal, and expressed as a percentage of that of the control (E:C ratio) (Conradi and Depledge, 1998), where

$$E:C = \ln L_{\text{exp}} * 100 / \ln L_{\text{control}} \quad (L: \text{length of the organisms})$$

### 4.5.5 Sublethal effects: DNA damage

Heavy metals can cause free radical damage to cellular macromolecules including proteins, lipids and DNA. Damage to DNA has been reported following exposure to numerous MNPs (Hoshino et al., 2004; Karlsson et al., 2008), and hence was selected as a method to determine sublethal ZnO MNP toxicity to *C. volutator*. Damage to DNA (single strand breaks) was measured in individual hemolymph cells using a modification of the method of Singh (1988), as described in Lewis and Galloway (2009). Briefly, 2 organisms from the *in vivo* assays were homogenised in a 1 ml buffer solution. 200 µl of the suspension was centrifuged (3 min, 1000 rpm) and the supernatant removed. The pellet (cell concentrate) was gently mixed with 1% low melting point agarose (heated to 37 °C) and placed onto 1% agarose-coated slides. The comet assay (that measures single stranded breakage in DNA) was performed using alkaline conditions at 5 °C. Slides were stained with 20 mg l<sup>-1</sup> ethidium

bromide and examined with a fluorescent microscope (420-490 nm excitation-520 nm emission). The percentage of DNA in the comet tail (quantity of DNA with strand breaks) was quantified (n=100) using Kinetic V COMET Software.

#### **4.5.6 Bioimaging of ZnO and CeO<sub>2</sub> uptake by *D. magna* by Coherent Anti Stokes Raman Scattering and light microscopy**

*C. volutator* stores Zn in the hepatopancreas, excreting metal rich detoxified granules as the ventral hepatopancreas cells pass through the cell cycle (Gallay and Rainbow, 1998; Rainbow, 2002). To explore the detoxification and accumulation of different forms of Zn, light microscopy images of the hepatopancreas were obtained using an optical light microscope Nikon SMZ and imaged with a 20x objective. Samples were prepared by dissecting the hepatopancreas of exposed and unexposed organisms to 1 mg l<sup>-1</sup> ZnO MNP, bulk ZnO and Zn ions. To further assess potential accumulation of Zn MNP or metal granules in the hepatopancreas, coherent anti-stokes Raman scattering (CARS) microscopy was used. Hepatopancreas from exposed and non exposed organisms were fixed in 4% formalin and placed in glass bottom Petri dishes for imaging. CARS imaging was performed as previously described for *D. magna*. Histological analyses of the hepatopancreas were performed to assess any sign of tissues level changes. Samples were prepared by fixing the whole organisms with Bouin's solution for 24h, dehydrated with IMS series and embedded into paraffin wax. Organisms embedded in paraffin blocks were then sectioned (6 µm sections) using a rotary microtome and stained with haematoxylin and eosin. A Zeiss Axioskop 40 light microscope coupled to an Olympus DP70 Digital Microscope Camera and AnalySIS Image Processing Software were used for image processing.

## 5. Results

### 5.1 Nanoparticle dissolution

ZnO MNP and micron size (bulk) ZnO dissolved more rapidly in seawater than in *D. magna* test water over a period of 120-170 hours. At a concentration of 20 mg l<sup>-1</sup> approximately 25 % and 12 % of the ZnO MNP were dissolved in seawater and in *D. magna* test water, respectively. Dissolution of bulk ZnO has been investigated in seawater, and the dissolution rate was considerably lower than for ZnO MNP (Figure 3 and Figure 4).

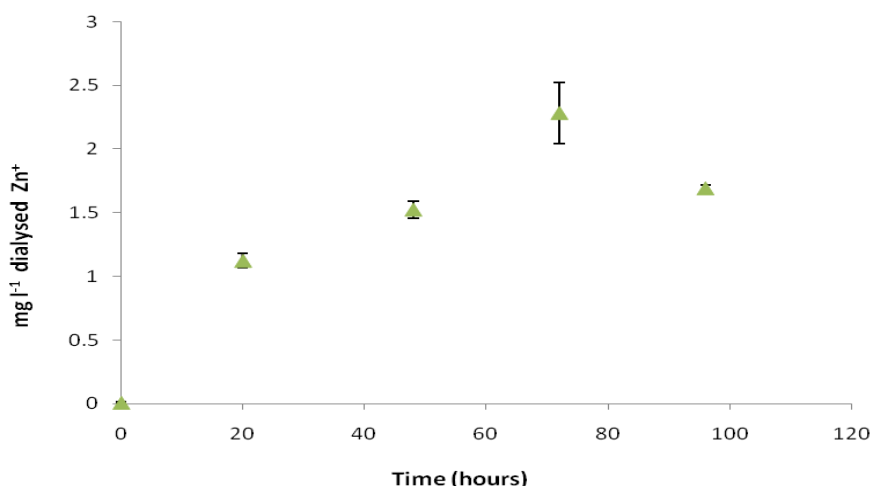


Figure 3. Determination of ZnO MNP dissolution rates by equilibrium dialysis in *D. magna* test media (pH 7.55-7.60, 20 °C). Values represent the mean  $\pm$  SD of three samples. Dialysed Zn<sup>2+</sup> represents the total ionised zinc from the metal MNP.

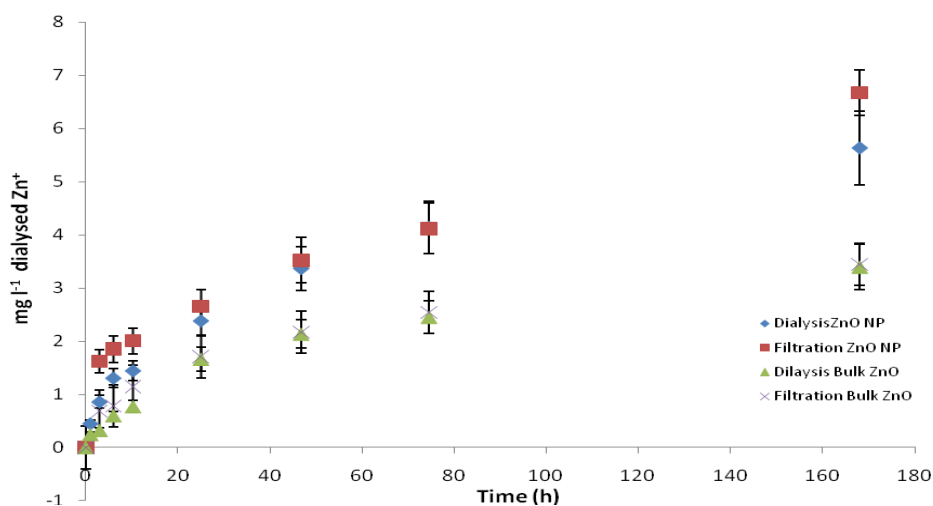


Figure 4. Determination of ZnO MNP dissolution rates in 25 ppt seawater (12 °C, pH 8) by equilibrium dialysis. Values represent the mean  $\pm$  SD of three samples. Dialysed Zn<sup>2+</sup> represents the total ionised zinc from the metal MNP.

### 5.2 *D. magna*- Water column toxicity

CeO<sub>2</sub> MNP (Antaria APS 10 nm) and bulk (micron sized) CeO<sub>2</sub> were not toxic after an acute exposure (48h) up to and including the highest concentration tested of 100 mg l<sup>-1</sup> (Figure 5). LC50 values could not be determined. However, organisms ingested large quantities of both materials as detected with light and CARS imaging (Figure 6).

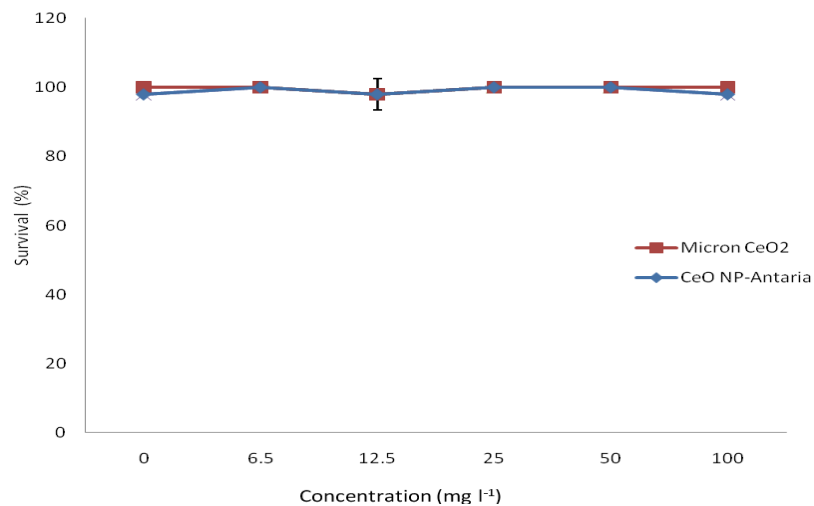


Figure 5. Effects on survival (%) of an acute (48h) exposure of different concentrations (mg l<sup>-1</sup>) of CeO<sub>2</sub> MNP and micron sized (bulk) CeO<sub>2</sub> on 3-d old *D. magna*.

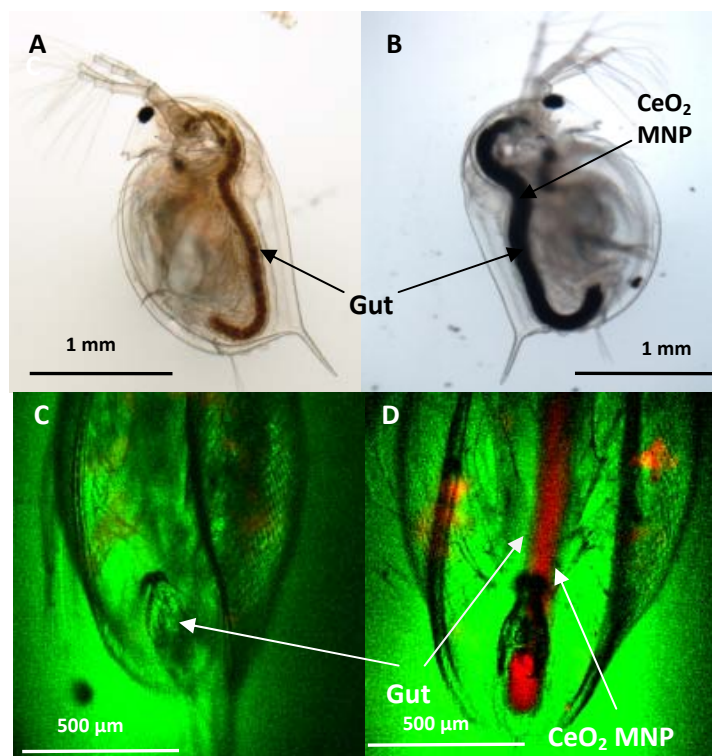


Figure 6. Light microscopy and CARS images of *D. magna* following a waterborne exposure to CeO<sub>2</sub> MNP. (A) and (C) unexposed and (B) and (D) exposed to 10 mg l<sup>-1</sup> CeO<sub>2</sub> MNP for 48h.

The toxicity of ZnO MNP (Micronisers, APS 30 nm) was determined in 3-day old *D. magna* after an acute exposure (48h) (Figure 7). The effect of ZnO MNP on survival of *D. magna* differed across different types of Zn (Table 2), with LC50 values for Zn ions and ZnO MNP were 3.3 and 1.5 mg l<sup>-1</sup>, respectively. Organisms ingested ZnO MNP as detected with Coherent Anti-Stokes Raman Scattering imaging (Figure 8).

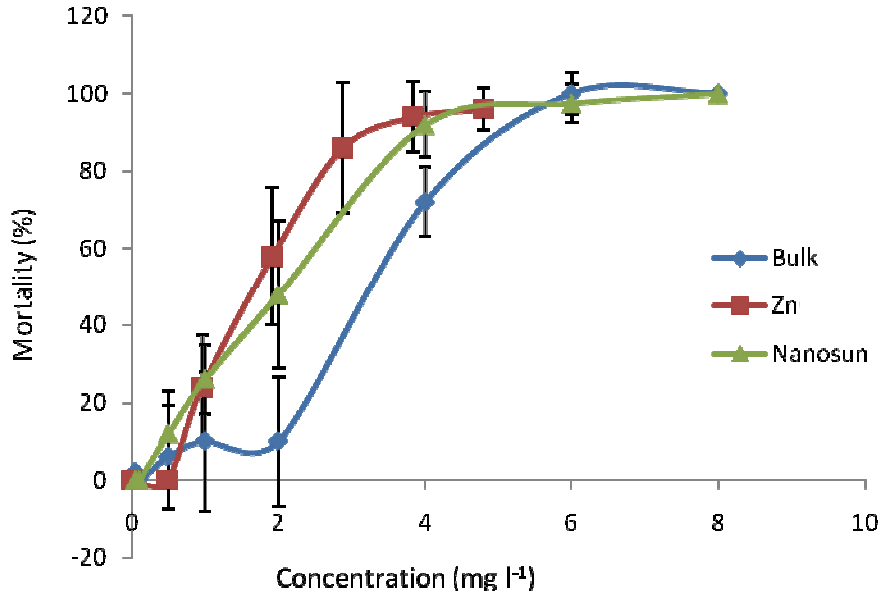


Figure 7. Effects on mortality (%) of an acute (48h) exposure of different concentrations ( $\text{mg l}^{-1}$ ) of ZnO MNP on 3-d old *D. magna*.

<b>ZnCl<sub>2</sub></b>		95% confidence interval.		<b>ZnO MNP</b>		95% confidence interval.	
	Exposure conc ( $\text{mg l}^{-1}$ )	lower	Upper		Exposure conc ( $\text{mg l}^{-1}$ )	lower	Upper
LC1	0.887	0.62	1.143	LC1	0.282	0.183	0.386
LC5	1.305	0.989	1.596	LC5	0.465	0.333	0.595
LC10	1.604	1.265	1.91	LC10	0.608	0.457	0.752
LC15	1.844	1.493	2.159	LC15	0.728	0.565	0.882
<b>LC50</b>	<b>3.321</b>	<b>2.932</b>	<b>3.713</b>	<b>LC50</b>	<b>1.557</b>	<b>1.335</b>	<b>1.796</b>
LC85	5.98	5.294	6.944	LC85	3.33	2.827	4.086
LC90	6.873	6.015	8.149	LC90	3.987	3.33	5.031
LC95	8.447	7.238	10.377	LC95	5.205	4.224	6.88
LC99	12.436	10.157	16.457	LC99	8.582	6.543	12.485

Table 2. LC50s for an acute (48h) exposure of ZnO MNP and bulk ZnO to 3-d old *D. magna* (EPA Probit analysis).

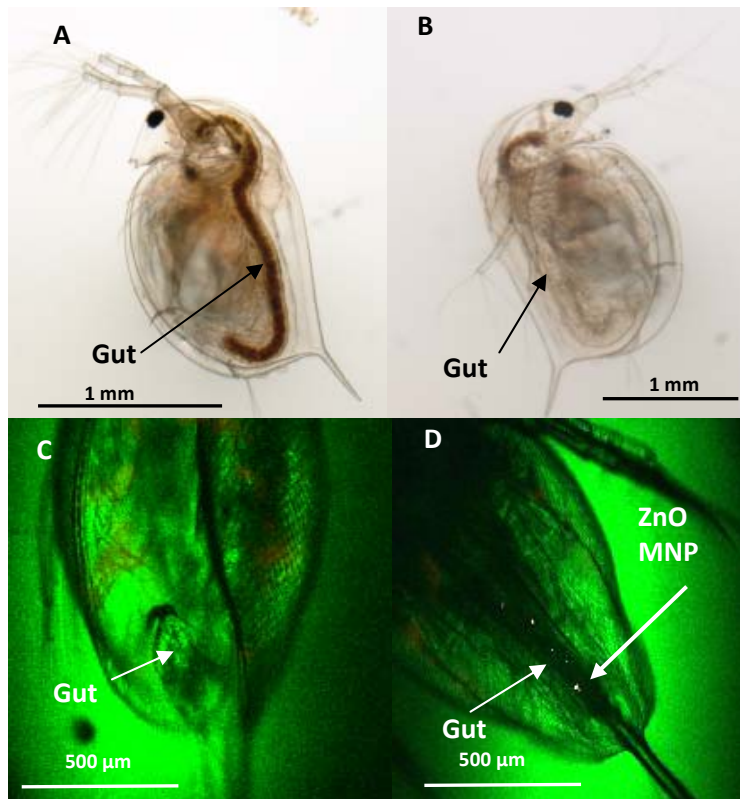


Figure 8. Light microscopy and CARS images of *D. magna* following a waterborne exposure to ZnO MNP. (A) and (C) unexposed and (B) and (D) exposed to  $10 \text{ mg l}^{-1}$  ZnO MNP for 48h.

### 5.3 Sublethal effects of ZnO MNP to *D. magna*

The feeding rate (uptake of the algae *Chlorella vulgaris*) was measured as a sublethal effect of exposure of *D. Magna* to ZnO MNP (Micronisers, APS 30 nm). Organisms exposed to ZnO MNP had a lower feeding rate (reduction of up to 30% at  $1 \text{ mg l}^{-1}$ ) compared to bulk ZnO and soluble zinc (Figure 9). Scanning electron micrographs suggested that the decrease in feeding rate was not related to a physical impairment or obstruction as a result of binding of ZnO MNP or ZnO MNP aggregates to the antenna nor thoracic appendages of the organisms (Figure 10).

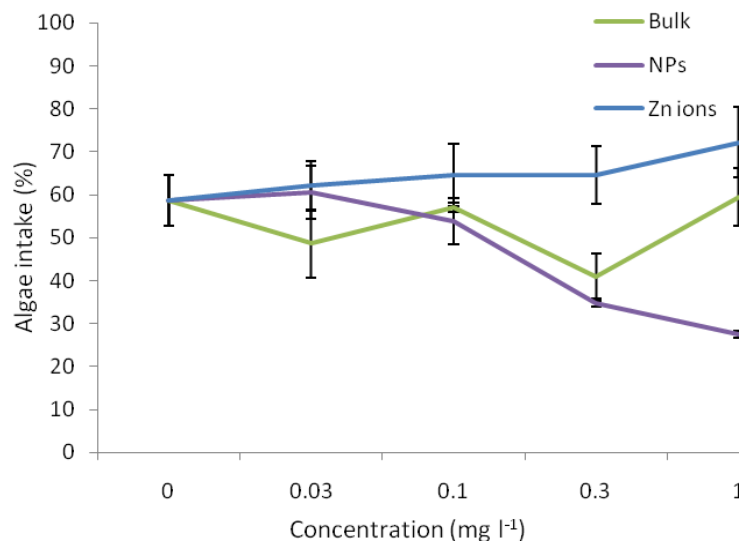


Figure 9. Effect of a 24 h exposure to the same mass of ZnO MNP, bulk ZnO and Zn ions on feeding rate (algae uptake) in 3-d old *D. magna*.

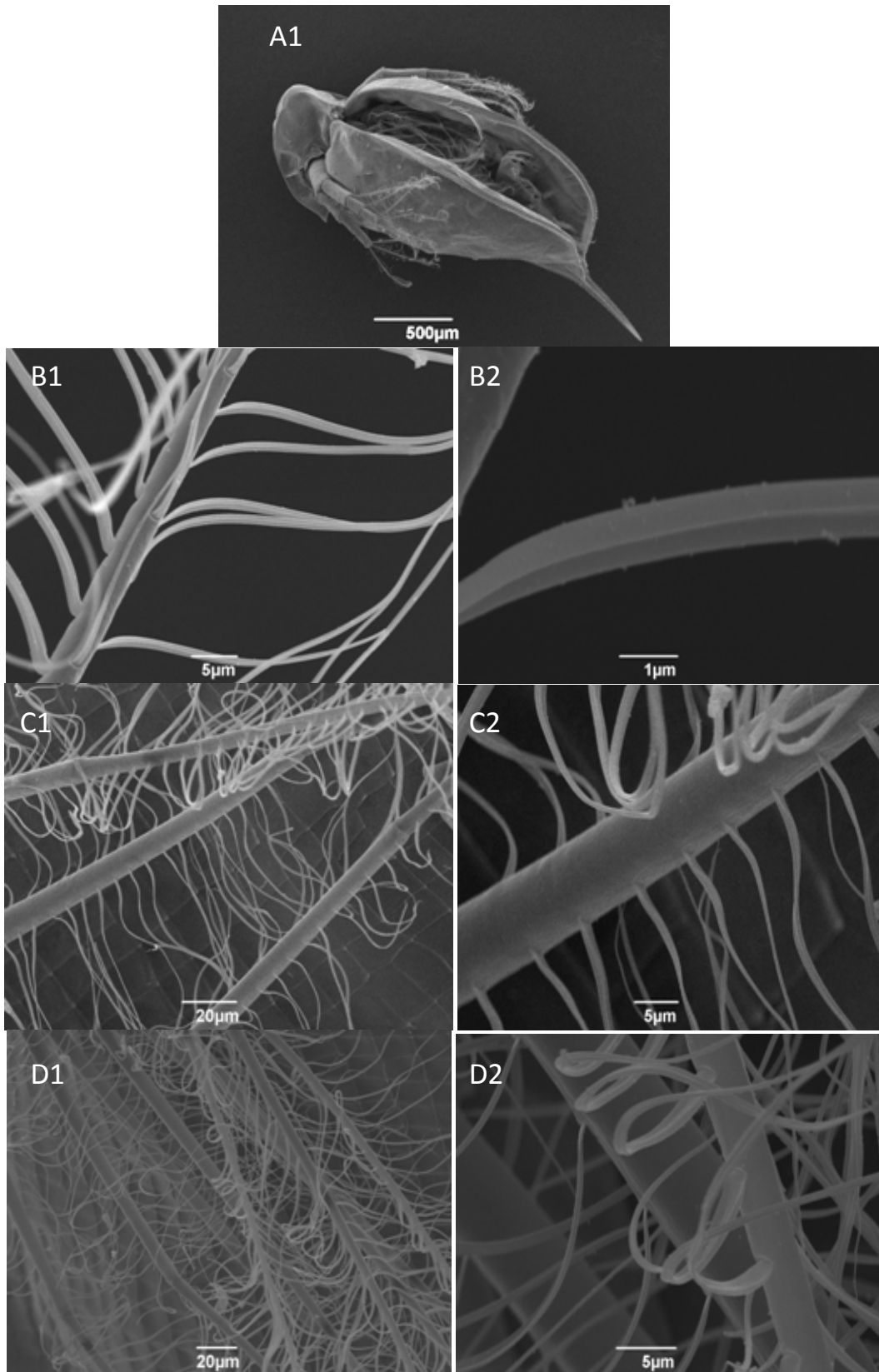


Figure 10. Scanning electron micrographs of 4-d old *D. magna* exposed for 24h to ZnO MNP. A) Whole organisms. B and C) second antenna, and D) thoracic appendages.

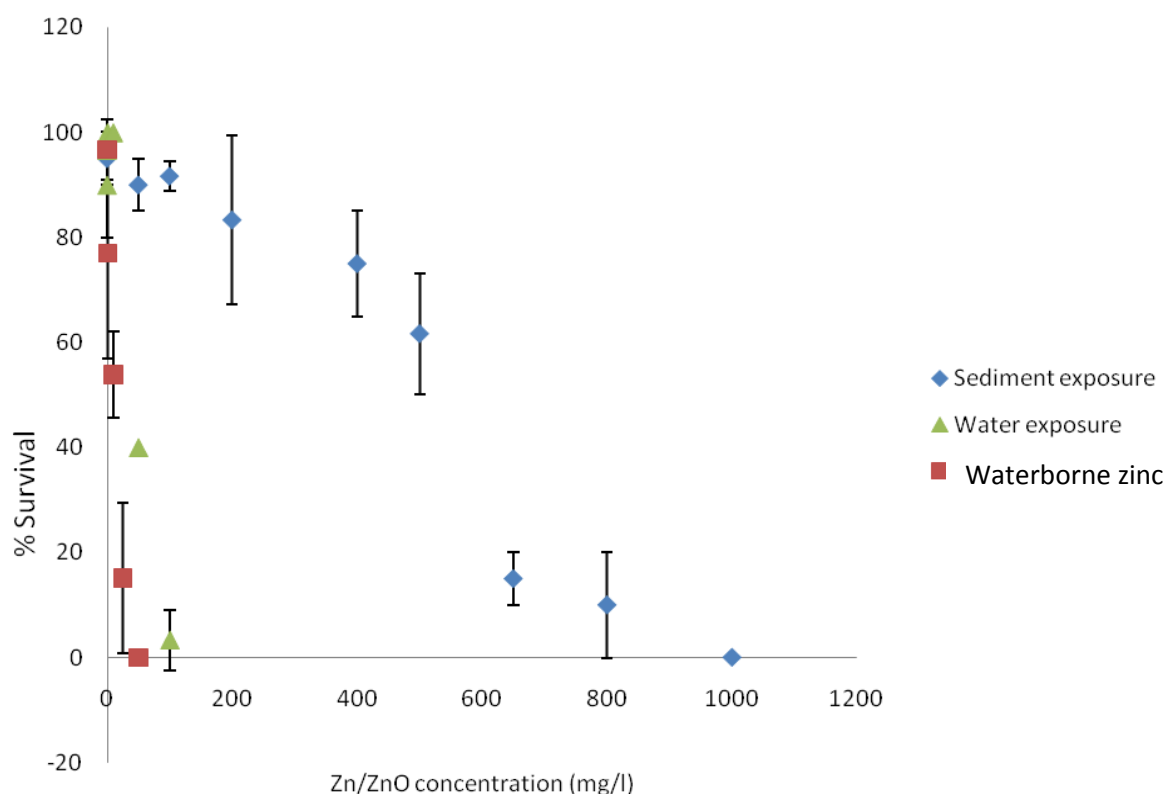


Figure 11. A comparison of the toxicity (represented as survival (%)) to adult *C. volutator* of 10 day waterborne and sediment exposures to ZnO (Micrometrics) and soluble zinc.

## 5.4 Chronic exposures- Survival and growth

Chronic exposures across the full lifecycle (100 days) were performed using sublethal concentrations of ZnO MNP, soluble Zn and bulk ZnO. Effects on survival, growth, reproduction and sublethal toxicity (DNA damage) were recorded. Population mortality was not significantly affected under any treatment and concentration after 100 days (< 40 % under all conditions), yet those populations exposed to soluble zinc always had the highest mortality rates after 28, 63 and 100, days when compared to the same mass of bulk and MNP ZnO (Table 3).

Days	Treatment ( $\text{mg l}^{-1}$ )									
	Control	MNP 0.2	MNP 0.5	MNP 1	Bulk 0.2	Bulk 0.5	Bulk 1	Zn <sup>+</sup> -0.2	Zn <sup>+</sup> -0.5	Zn <sup>+</sup> -1
28	88.75±2.5	80±14.1	83.3±12.6	93.3±5.8	82.5±3.5	92.5±3.5	92.5±3.5	96.7±5.8	88.4±5.8	85±8.7
63	85±4.1	87.5±10.6	86.7±11.5	86.7±15.3	81.6±27.5	87.5±3.5	80±22.9	80±21.8	80±5.0	86.7±7.6
100	91.7±5.8	72.5±3.5	78.3±5.8	66.7±10.4	71.6±10.4	78.3±7.6	63.3±16.1	56.7±10.4	70±5.0	63.3±10.4

Table 3. Survival (%) of *C. volutator* after 28, 63 and 100 days to a waterborne exposure to different forms of Zn (nanoparticle (MNP), bulk and soluble (Zn<sup>+</sup>) and concentrations (0.2, 0.5 and 1  $\text{mg l}^{-1}$ ).

Figure 12 shows a box-and-whisker plot for the size (body length) of *C. volutator* in the populations of organisms exposed to all three forms of zinc. The specific growth rate (SGR) decreased with increasing concentrations of any form of zinc, and was most significant 28 and 63 days after the onset of the exposure (Table 4), whereas after 100 days most organisms had reached the adult size corresponding to the unexposed populations ( $5.11 \pm 0.84$  mm). The exception to this was for the population exposed to the highest concentration of bulk ZnO ( $1 \text{ mg l}^{-1}$ ), for which the body length of the organisms was significantly smaller ( $p < 0.05$ ). In other words, zinc leads to a reduction in the SGR, which directly affected the age of sexual differentiation as quantified and described in Figure 13.

Treatment	28 days	63 days	100 days
MNP 0.2	13.34	0.44	2.46
MNP 0.5	16.09	5.03	2.97
MNP 1	10.82	7.94	5.45
Bulk 0.2	-0.30	11.68	0.37
Bulk 0.5	8.11	1.48	3.00
Bulk 1	13.00	12.56	9.25
ZnCl <sub>2</sub> 0.2	3.92	10.64	3.19
ZnCl <sub>2</sub> 0.5	15.30	13.27	3.92
ZnCl <sub>2</sub> 1	20.36	17.51	4.39

Table 4. Specific growth rates of *C. volutator* exposed to different concentrations and forms of zinc over 100 days.

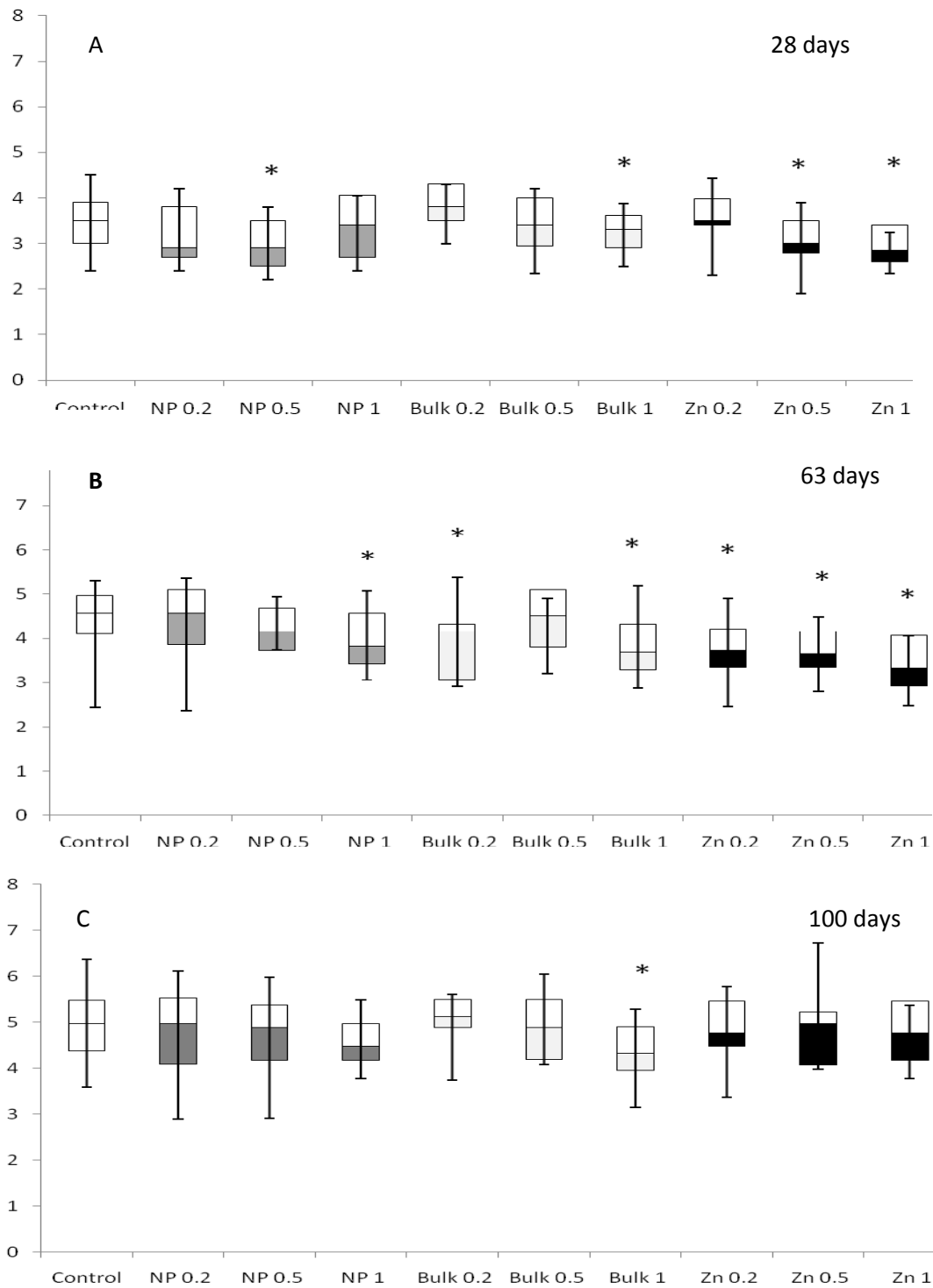


Figure 12. Multiple box-and-whisker plots showing the size (body length, mm) of *C. volutator* in populations exposed to 0, 0.2, 0.5 and 1 mg l<sup>-1</sup> ZnO MNP, bulk ZnO and soluble zinc after A) 28 days; B) 63 days and D) 100 days of exposure. Asterisks (\*) represent treatments significantly different from controls. (R) Demarks treatments where adult *C. volutator* reproduced after 100 days.

## 5.5 Reproduction

28 days after exposure, 50% of the unexposed population of *C. volutator* was sexually differentiated, and the time for sexual differentiation was delayed with increasing concentration of zinc. At the end of the exposure all populations reached sexual maturity, however at the time of sampling (100 days) only unexposed populations had reproduced ( $8.87 \pm 3.3$  neonates per female).

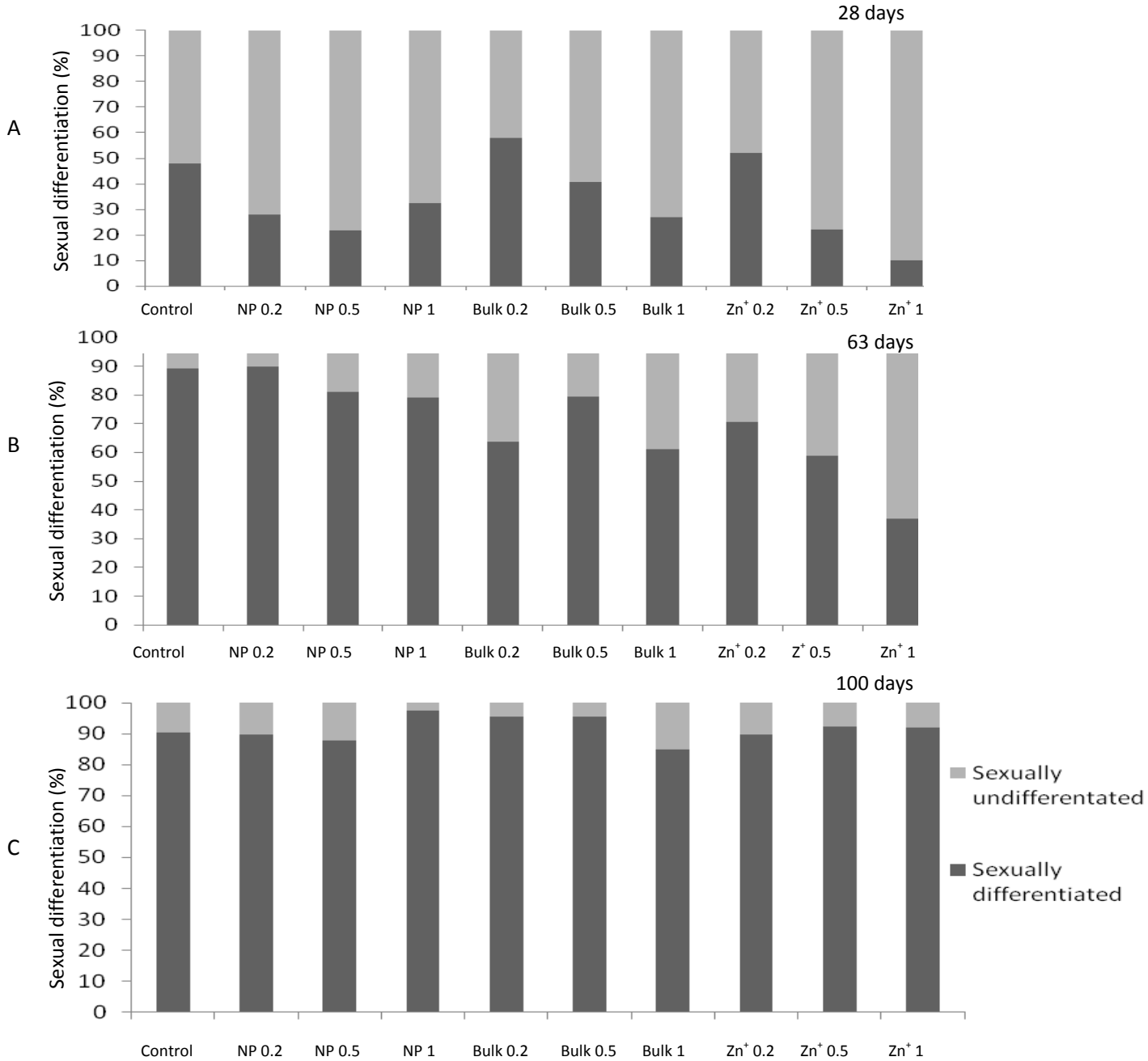


Figure 13. Temporal variations in sexual differentiation of *C. volutator* exposed to different concentration ( $\text{mg l}^{-1}$ ) and forms of zinc. A) 28 days; B) 63 days and C) 100 days

## 5.6 Sublethal effects- Damage to DNA

Results show that exposure to increasing concentrations of any form of Zn significantly increased DNA damage, and this was evident at both 63 and 100 days of exposure (ANOVA,  $p < 0.05$ ) (Figure 14). After 100 days, the effect of zinc on DNA damage were significantly different among MNP exposed populations and the bulk and soluble zinc exposed ones (ANOVA  $p < 0.01$ ), indicating a potential different mode(s) of toxicity for this effect between soluble and bulk zinc, and nanoparticulated zinc.

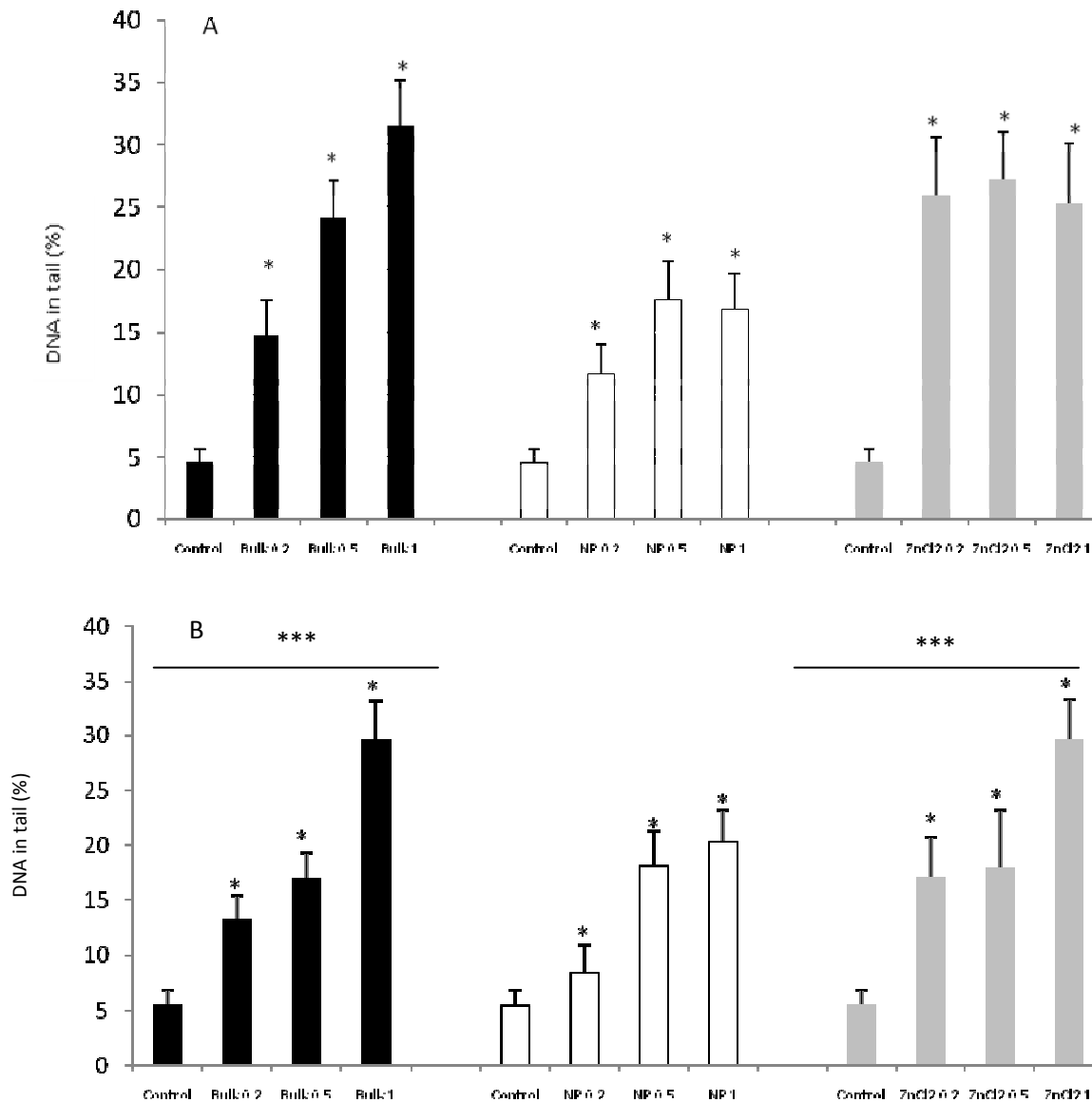


Figure 14. DNA damage quantified as the percentage (%) of DNA in the tail of hemolymph cells of *C. volutator* organisms exposed for 63 (A) and 100 (B) days to different forms and concentrations of Zn.

## 5.7 Zinc uptake and detoxification

Light microscopy images show the presence of granules in the hepatopancreas of *C. volutator* exposed to bulk Zn and ZnO MNP. Notice also the darker yellowish colour of the hepatopancreas of exposed organisms as a result to Zn exposure (Figure 15), indicating the role of this organ in bioaccumulation and detoxification of zinc. The metal composition of the granules formed in the hepatopancreas is currently being investigated with TEM-EDX<sup>1</sup> analysis and will aid determining the sublethal implications of ZnO MNP exposures and potential toxicological differences to soluble and bulk counterparts (see also EM image in Figure 16B, of TEM of hepatopancreas of *C. volutator* exposed to ZnO MNPs). Histological sections of the hepatopancreas showed a higher number of vacuolated cells. Histological samples are still being processed and analysed (Figure 16).

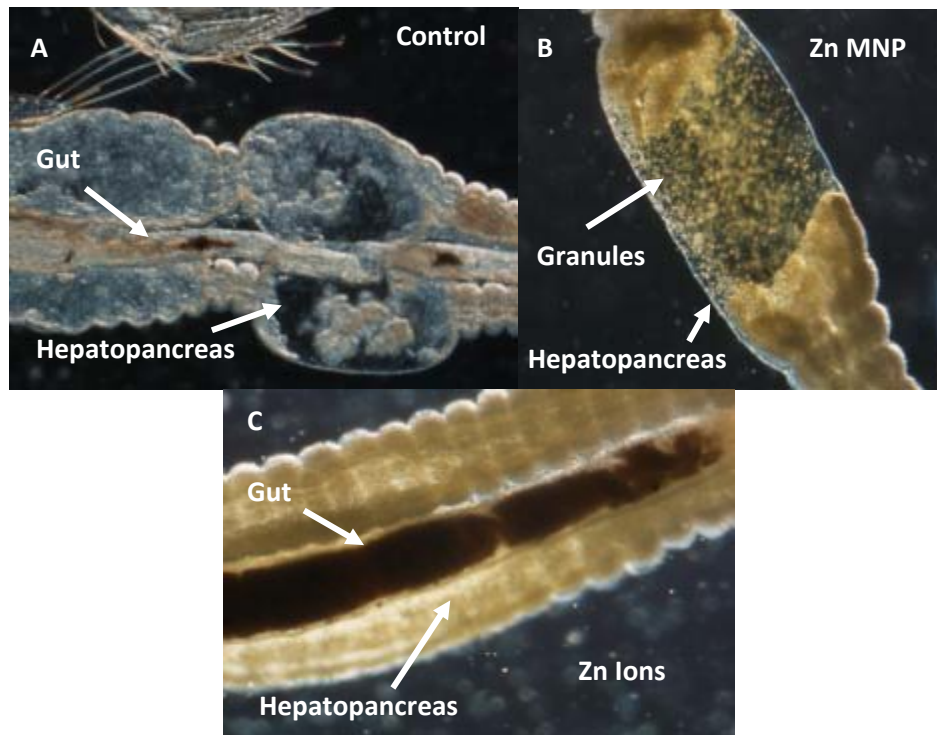


Figure 15. Light microscopy images of the hepatopancreas of exposed (B-D) and unexposed (A) *C. volutator* to different forms of zinc.

<sup>1</sup> TEM-EDX: Transmission Electron Scanning Microscopy coupled to energy dispersive X-ray spectroscopy

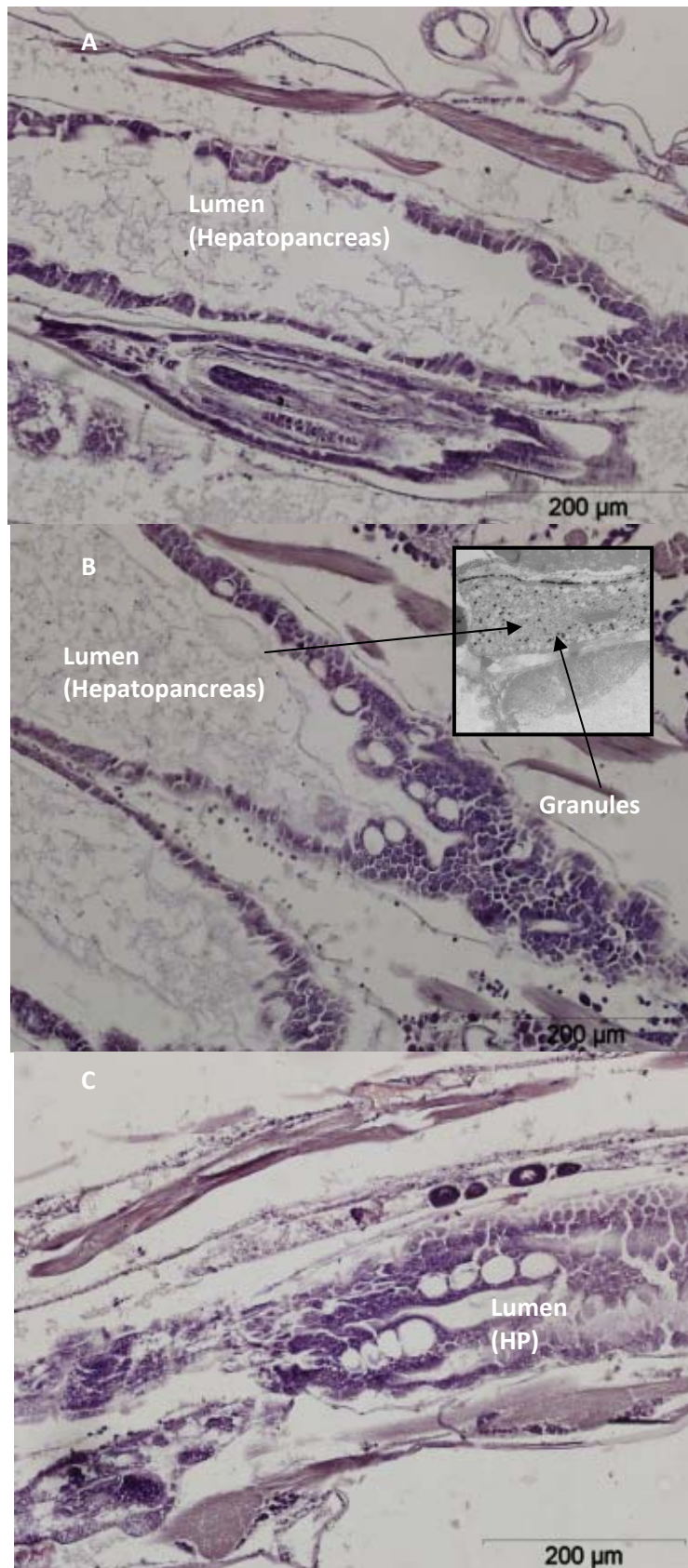


Figure 16. Hepatopancreas morphology as visualised by light microscopy of A) control; B) 1mg l<sup>-1</sup> ZnO MNP and C) 1 mg l<sup>-1</sup> bulk ZnO after a 63 day exposure. Insert in B shows TEM of granules in hepatopancreas.

## 6. Discussion

This work has investigated the effects of exposure to ZnO and CeO<sub>2</sub> MNPs to the planktonic and freshwater *D. magna* and to the sediment dwelling amphipod *C. volutator*.

The results confirm that the toxicity of CeO<sub>2</sub> MNP (Antaria, APS 10 nm) did not cause any acute (48 h) toxicity to *D. magna* at concentrations as high as 100 mg l<sup>-1</sup>, as other studies have already shown (Van Hoecke et al., 2009). CeO<sub>2</sub> MNP were present at seemingly high density in the gut of *D. magna*, as assessed by light and CARS imaging. Ingestion of MNP by this organism has also been reported for many other MNP, such as carbon nanotubes (Roberts et al., 2007), fullerene (Zhu et al., 2009), titanium dioxide (Baun et al., 2008; Zhu et al., 2009), and quantum dots (Bouldin et al., 2008), to name a few. Yet, their ability to translocate to other tissues once in the gut is still unknown.

In order to determine ZnO MNP toxicity in aquatic systems it is necessary to know the rate of ZnO MNP/particle dissolution (Franklin et al., 2007), given that under certain environmental conditions high rates of ZnO MNP dissolution can lead to a pool of free Zn ions which are highly toxic to aquatic organisms (Santore et al., 2002; van Straalen, 2002). In our study, the rate of ZnO MNP dissolution was taken into consideration during the experimental design, and control exposures of soluble Zn ions were performed to compare their toxicological effects with those seen with bulk and MNP. Indeed, in *D. magna* test medium (pH 7.5, 20°C), and after 72 h, only 10 % of the ZnO MNP was dissolved. The overall results show that acute exposures (48h) of different forms of zinc to *D. magna* were within the range reported by other authors (Heinlaan et al., 2008; Zhu et al., 2009). However, ZnO MNP (Micronisers, APS 30 nm) were more toxic than the same mass of soluble zinc ions, with LC50 of 1.55 mg l<sup>-1</sup> and 3.32 mg l<sup>-1</sup>, respectively. Based on the results obtained from ZnO MNP dissolution rates this indicates that there was a lower concentration of soluble Zn<sup>+</sup> in the system to account for the level of biological effects seen, indicating a potential MNP-mediated effect. A MNP-mediated effect also appeared to be involved with the significant decrease in feeding rate of *D. magna*; a 30% decrease on feeding rate was observed in comparison to bulk ZnO and soluble Zn<sup>+</sup>, and scanning electron microscopy images did not show any association or binding of ZnO aggregates on the antenna or thoracic appendages of *D. magna* that could physically compromise swimming and thus feeding ability.

The acute and chronic effects of zinc to *C. volutator* have previously been reported. Low zinc concentrations (<0.8 mg l<sup>-1</sup>) delayed population growth and sexual maturation (Conradi and Depledge, 1999). Our work is the first one that investigates and compares the effects of zinc ions with ZnO MNP in this species. Waterborne exposure of *C. volutator* to ZnO MNP resulted in a far higher toxicity than when dosed via the sediment (by an order of magnitude) indicating that bioavailability of ZnO MNP or Zn complexes is greater via the water column. Acute toxicity of waterborne ZnO MNP did not differ significantly from bulk ZnO toxicity. Yet, the rate of dissolution of bulk and ZnO MNP varied significantly, with the latter having a higher rate of dissolution than bulk ZnO. Indeed, after a week and under the same experimental conditions 25% of the MNP dissolved, whereas for bulk ZnO only about 12 % of the particle had dissolved. This has important implications when discriminating nano-effects from soluble metal effects, and setting up experimental control exposures with Zn<sup>+</sup>. Based on preliminary information on solubility rates and toxicity, further research is being done to determine specific mode of uptake, bioaccumulation and detoxification of ZnO MNP.

The effects of ZnO MNP on the life cycle of *C. volutator* (100 days) show that survival was not significantly affected at the end of the exposure, yet specific growth rate (SGR) and age of sexual differentiation were directly affected. SGR was reduced with increasing the concentration of any form of zinc, and sexual differentiation was delayed with increasing zinc concentrations, and was

delayed most in those populations exposed to soluble zinc and bulk ZnO. These sublethal effects were more evident at the 28 and 63 days of exposure, whereas after 100 days in all exposed and unexposed zinc more than 90% of the populations had reached sexual maturity. Reproduction however (presence of neonates), only occurred unexposed populations at the end of the experiment (100 days;  $8.87 \pm 3.3$  neonates per female). DNA damage was also induced by exposure to zinc, and increased with increasing the concentration of zinc. Overall, DNA damage was significantly higher in populations exposed to bulk ZnO and soluble zinc ions ( $p < 0.01$ ). The toxicological differences for the sublethal concentrations of the different Zn particles (adjusted for mass of zinc ions) indicated that soluble zinc and bulk ZnO have a higher toxicological impact. Yet, considering that the dissolution rate of bulk ZnO is lower than the dissolution rate of ZnO MNP, that might suggest that the mode of action of these compounds differ, yet further experiments on uptake and detoxification processes are necessary to confirm this hypothesis.

Currently, further research is being done in order to investigate the detoxification processes undertaken by *C. volutator* under the three forms of zinc organisms were exposed to. Based on light microscopy imaging the effects of metal bioaccumulation in *C. volutator* hepatopancreas were evident, and further research investigated the metal composition of the granules formed in the hepatopancreas is being conducted using X-ray diffraction analysis.

## 7. Conclusion

From the obtained results we can conclude that:

- Acute exposure (48h) to CeO<sub>2</sub> MNP did not affect survival of *D. magna*.
- Acute exposure (48h) to ZnO MNP did significantly affect survival of *D. magna* with LC50s lower than those of Zn ions. This indicates that although MNP dissolution rate is high (up to 10% in *D. magna* medium, after 72h), the pool of free Zn ions formed is not sufficient to cause the observed mortality.
- Both, CeO<sub>2</sub> and ZnO nanoparticles can be ingested by *D. magna*, indicating the potential risks of bioaccumulation associated with a waterborne MNP exposure.
- Type of exposure (sediment vs. waterborne) has a strong implications on toxicity to the amphipod *C. volutator*, with waterborne exposures being significantly more toxic (possibly due to the enhanced bioavailability of the MNP or dissolved Zn from the MNP).
- Chronic exposure of sublethal Zn MNP concentrations (< 1mg l<sup>-1</sup>) to *C. volutator* results in delayed growth rate and delayed sexual maturation, with implications for the time of reproduction.
- Accumulation of sublethal concentrations of ZnO MNP and other forms of zinc indicate the hepatopancreas as a site for zinc detoxification and storage. Further analysis is being performed to determine if ZnO MNP uptake and detoxification processes for different forms of zinc.

## 8. Future work

Future work will concentrate on defining the bioaccumulation and effects of the test MPS in more detail. This will include further studies with the test invertebrates described here and will also extend to fish, algae and terrestrial invertebrates. Results from these studies will be delivered in 2011.

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