Deliverable Report for Grant Agreement Number 760813

Deliverable *5.1*

Advanced long term exposure ecotoxicity bioassays for a variety of species across a food chain.

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Dissemination Level:		
PU	Public	X
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

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1. DESCRIPTION OF TASK

Determine chronic ENM exposure effects focused on the population level; (HWU, UL, DTU); M1-24

Data on effects of ENM for chronic exposures and on animal populations are extremely sparse. In this task, we will measure population relevant level effects for exposures in algae (DTU, HWU), and daphnids (HWU, UL). We will apply Tier 1 ENM (listed in Table 1) for this work. Fitness measures for daphnids will include impacts on growth, mobility and reproductive success (duration: 21 days), and endpoints for algae, will include photosynthesis (duration: over time periods extending between 2h to 72h) and population growth (duration: 72 h, which is equivalent to ~16 generations). Test with daphnids will include those run over multiple generations for a selection of ENMs that cause toxicity (UL). Standardised chronic ecotoxicity tests, as described by OECD guidance documents, will be modified for the nano-specific features explicitly accounting for the fate and behaviour of ENM. Analyses will be conducted on the relevance of waterborne and oral (dietary) routes for a selection of materials, as each can have a major bearing on exposure level at different trophic levels: transfer via the food chain can result in a 10-times greater ENM body burden compared to that via waterborne exposures (Skjolding et al. 2014). Embedded in this work and linking with Task 5.2, we will assess uptake rates and biodistribution in body tissues applying advanced microscopy (to include: confocal microscopy (also fluorescence), electron microscopy, and RAMAN scattering and 2-photon laser (Goodhead et al. 2015). The behaviour of the ENM (including their size distribution and stability) will be evaluated through chemical/particle analyses over the exposure duration in WP1, Task 1.5.*

** Please refer to PATROLS Grant agreement publication*

2. DESCRIPTION OF WORK & MAIN ACHIVEMENTS

2.1. Introduction

Increasing use of engineered nanomaterials (ENMs) in consumer goods have raised concern as to whether they can have detrimental effects on human and/or environmental health. Recent reviews have summarised the effects of ENMs on different organisms (e.g. Do Amaral et al. 2019; Lewis et al. 2019; Hou et al. 2018); however, it remains unclear which organisms are most sensitive and which parameters are most crucial to monitor during the tests.

In order to gain a better understanding of the effects of long term exposures of ENMs on individual animal fitness and population levels a series of Tier 1* ENMs (Centre for Ecotoxicology and Toxicology of Chemicals, Arts et al. 2015) exposure experiments using algae (*Raphidocelis subcapitata*), daphnids (*Daphnia magna*) and zebrafish larvae (*Danio rerio*) were conducted.

Primary producers hold a pivotal role in food chains therefore they are key in the assessment of trophic transfer of ENMs. Indeed, if effects occur at the lower trophic levels the effect can ripple through the food chain. For testing primary producers, the mono-cultured alga *R. subcapitata* was chosen because it is a well-known test organism and because it is ecologically relevant. Additionally, algae likely have a high level of interaction with ENMs in suspension due to their

small size and direct contact with the ENMs.

DTU evaluated the toxicity of Tier 1 materials, namely ZnO (NM-111), TiO_2 (NM-105), CeO_2 (NM-212) and $BaSO_4$ (NM-220) towards mono cultured green alga (*R. subcapitata)* in a series of 72 h growth inhibition tests (using the standardly protocol OECD 201). The population growth of algae was assessed over a period of 72h, which inherently is a chronic test as it covers approximately 16 generations of algae (OECD, 2011). This was done to establish an effect level suitable for bioaccumulation studies (Task 5.1) and also as input for trophic transfer studies from algae to *D. magna* (Task 5.4). The chronic toxicity of the analysed NMs towards algae ranked as follows (from highest toxicity to lowest): ZnO>CeO_2>$BaSO_4$>TiO_2. For compounds with relative low toxicity (EC_{50} > 1 mg/L), namely CeO_2, $BaSO_4$ and TiO_2, a method for determining changes in pigmentation as a more sensitive endpoint is currently under development (Task 5.4).

The freshwater crustacean *D. magna* was used as a standard test species to represent the invertebrates, in determining the population effects of chronic ENM exposure. Both acute and chronic tests (OECD 202 and 211) were conducted to establish baseline information on the concentrations causing effects on mobility, growth and reproduction.

A modified version of the *D. magna* Reproduction Test (OECD 211) was performed at Leiden University (UL). This test allowed for the assessment of the long-term reproductive performance of *D. magna* (21 days) over two-generations exposed to TiO_2 ENMs and CeO_2 ENMs (0, 0.02, 0.2 and 2 mg/L) in the presence of full-spectrum artificial daylight.

At HWU the acute toxicity of ZnO ENMs, CeO_2 ENMs, and $ZnSO_4$ was tested with *D. magna* in acute 48 h tests (OECD test guideline 202) and chronic 21 d tests (OECD guideline 211). For ZnO ENMs tests, the toxicity of triethoxycaprylsilane coated ZnO ENMs (JRCNM01101a/NM-111, 76 nm) and uncoated ZnO NMs (JRCNM62101a/NM-110 new, 70-90 nm) were compared with $ZnSO_4$ as ionic control ($ZnSO_4$*$7H_2O$, Sigma). Unlike ZnO ENMs, CeO_2 (JRCNM02102a/NM-212, <10->100 nm) are known not to dissolve readily and therefore no ionic control was applied.

The rationale for specifically considering long-term exposure when assessing ENM ecotoxicity is based on indications that persistence of some ENMs is inherently high, and that uptake by organisms and resulting effects may be relatively slow. Multigenerational effect assessments (i.e. effect assessments that account for effects manifesting in the offspring of exposed individuals) have frequently been proposed to provide additional value to long-term exposure studies, as they provide additional insight into how exposure may affect the longevity of natural populations that may go unnoticed single-generation studies. Despite the ecological significance of such assessments they are often avoided because of the intensive time and sampling requirements (Castro et al. 2018).

Depending on the crystalline structure of the particles, UVA radiation as present in natural daylight can induce photocatalytic reactions on the particle surface of TiO_2 and CeO_2 ENMs that result in the formation of reactive oxygen species (ROS) in water. ROS induced toxicity is a common mode of action for many toxicants and aquatic organisms (e.g. Lushchak et al. 2011).

Finally, the quantitative uptake of CeO_2 ENMs by *D. magna* was explored in a body burden test (HWU). Scanning electron microscope (SEM) imaging was used to illustrate the distribution of ENMs on the body surface of the daphnids.

In addition to the tasks described in 5.1, HWU did a series of acute 24 h zebrafish larvae (72-96 hpf) exposures to ZnO ENMs, $ZnSO_4$ and CeO_2 ENMs for cross species comparison of sensitivity to ENMs exposure.

The potential trophic transfer of ENMs between organisms is a realistic and important result of chronic exposure. ENMs may transfer through the food chain potentially also resulting in bioaccumulation and biomagnifications to a higher degree than waterborne exposure. Progress on assessment of the toxicity of CeO_2 ENMs via trophic transfer has been made with the aim of establishing bioavailability, dietary uptake, and transfer of CeO_2 ENMs from algae to *Daphnia*, and from *Daphnia* to zebrafish (WP 5.4). Investigation of effects on algae and preliminary investigations of CeO_2 ENM uptake in *D. magna* (aqueous exposure) have been conducted. These experiments assessed effects, uptake and bio distribution in the body tissues by application of advanced ICP-MS measurements (exposures carried out at HWU and body burdens assessed by DTU).

As highlighted in the literature (Lead et al. 2018; Baun et al. 2017; Skjolding et al. 2016), an adequate identification of the exposure requires extensive characterization of the dynamic behaviour of the ENMs in test media to interpret results from exposure studies. This work was carried out using Dynamic Light Scattering (DLS) to describe the dynamic aggregation behaviour in parallel with the studies. In order to quantify the relevance of dietary routes of uptake at environmentally relevant concentrations, a parallel study was setup at DTU to quantify the dynamic fate and behaviour of the tested ENMs in algal medium by analysing content of chosen ENMs on an individual cell level using Single Particle Inductively Coupled Plasma Mass Spectrometry (SP-ICP-MS).

The objectives of the present work were:

1) To establish concentration-response relationships for representative ENMs provided by the JRC repository for ZnO (NM-111), TiO_2 (NM-105), CeO_2 (NM-212) and $BaSO_4$ (NM-220) ENMs in standardized tested with *R. subcapitata*;

2) To characterize the dynamic behaviour of selected ENMs in standard environmental test media;

3) To determine bioaccumulation at environmentally relevant concentrations of selected ENMs in algal cells (*R. subcapitata*) using single-cell-ICP-MS;

4) To explore alternative endpoints for quantifying mode of action of ENM toxicity in algae tests (e.g., change in pigmentation due to shading);

5) To establish the effects of selected ENMs (ZnO and CeO_2) on the fitness parameters growth, mobility and reproduction in daphnids.

6) to develop and test a modified version of the *D. magna* Reproduction Test (OECD 211) to allow for the assessment of the reproductive performance of *D. magna* and their first generation of offspring exposed to TiO_2 ENMs and CeO_2 ENMs in the presence of full-spectrum artificial daylight;

7) To establish the toxicity of selected ENMs in zebrafish larvae (LC50) for cross species comparisons;

8) To establish the effects of medium composition on the toxicity of ENMs in zebrafish larvae.

2.2. Methods

2.2.1. Preparation and Characterization of ENM suspensions

The tier 1 ENMs TiO_2 ENMs (NM-105, JRCNM01005a, primary particle sizes of 15-24 nm), CeO_2 NMs (NM-212, JRCNM02102a, primary particle sizes of 33 nm), ZnO ENMs (coated, NM-111, JRCNM01101a, primary particle sizes of 76 nm) (uncoated, NM-110 new, JRCNM62101a, primary particle size 70-90 nm) and $BaSO_4$ ENMs (NM-220, primary particle size 32 nm), were obtained as a dry powder from the repository for Representative Test Materials (RTMs) of the Joint Research Centre of the European Commission (JRC). These ENMs were derived from a single production batch to enhance the comparability of test results between experiments and laboratories. ROS generation of both materials in the presence of UV radiation was confirmed previously (JRC, 2014a; JRC, 2014b). For SC-ICP-MS experiments 30 nm Au NPs (Jolck et al. 2015) were used.

All tier 1 ENMs tested were dispersed using a modified version of the OECD 318 dispersion guideline, which is largely consistent with the dispersion protocol developed in the FP7 project NANoREG (Jensen et al. 2016). The dispersion protocol is briefly cited verbatim from OECD 318 (2017) below.

"In case the tested nanomaterial is provided in the form of dry powder, it should be pre-wetted in ultrapure water and left in the form of wet-paste for 24 h to insure the proper interaction of nanomaterial surface with ultrapure water. After 24 h of pre-wetting the resulted wet paste is dispersed into known volume of ultrapure water, thus providing a stock dispersion with known nanomaterial concentration. The concentration of the stock dispersion shall be sufficient to allow the further dilution to the required particle number concentration (0.5×10^{12} to 5×10^{12} particles/L) within the analysed samples. The mass concentration of the nanomaterial in the stock dispersion should not be too high to avoid facilitation of nanomaterial agglomeration in the stock before dilution into the test vessels. Thus the recommended concentration of nanomaterial within stock dispersion shall not exceed the concentration of nanomaterial within analysed samples more than 20 times. Usage of a calibrated sonication probe is mandatory. Sonication probes are widely available and allow comparably easy calibration of the energy input to the dispersion. A Microsoft® Excel test preparation tool supporting the calculation of delivered sonication energy via sonication probe is available on the OECD website. Sonication of the dispersion should be performed as follows:

(a) Place a 250 mL glass beaker in an >1 L ice bath and secure against moving.

(b) Add 125 mL of ultrapure water and the required amount of nanomaterial.

(c) Place the tip of the ultrasonic probe (½" or 13 mm diameter), in the centre of the beaker, 2.5 cm below the surface.

(d) Add 40 W output power for 10 min.

(e) Store the prepared dispersion in an amber glass container at 4°C (do not freeze!).

Sonication of high aspect ratio nanomaterials should be performed with care since they tend to break during high energy input. If applicable, high aspect ratio nanomaterials should be sonicated with a method that allows dispersion as far as possible while avoiding destruction of the nanomaterial under investigation. Appropriate dispersion routines, e.g. like for bath sonication, can be found in literature. However, it should be noted that bath sonicators are difficult to calibrate and suffer from a non-uniform distribution of the ultrasonic energy in the bath. As other techniques baths sonicate through the walls of the test vessels which absorb an unknown amount of the sonication energy through damping effects.

In case the investigated nanomaterials are delivered in a form of stable stock dispersion, the stock dispersion is directly diluted to the concentration, convenient for further sample preparation. No additional sonication is required in this case.

The undiluted stock dispersion should not be stored for longer than 14 days and after more than 3 h a re-dispersion by probe sonication has to be performed."

Deviations from this protocol used in this work are presented below in 2.2.3 and 2.2.4.

2.2.2. Characterisation of ENM suspensions prepared for algae experiments

The stability of all ENMs in Milli-Q and ISO 8692 algal medium was analysed by DLS (Malvern, Zetasizer Nano-ZS) at 20°C. A backscattering angle of 173° was used to determine the observed light. Samples of 1 mL of the stock suspensions were measured with three replicates using 10 measurements per run in both Milli-Q, Elendt M7 and ISO 8692 algal media. Stokes-Einstein equation was used to calculate the hydrodynamic diameter of the ENMs using the cumulant method for fitting the autocorrelation function (Kretzschar et al., 1998). For dynamic aggregation behaviour as a function of time the sample was measured at 1 min intervals for 24 h.

Dissolution of ZnO ENMs was determined by single particle ICP-MS using PerkinElmer, NExION 350D set up for SP-ICP-MS with a cyclonic spray chamber and the Syngistix™ software module for single particle application. The transport efficiency was determined using standardized 60 nm Au ENMs (PerkinElmer, Shelton, CT USA). The sample flow rate was 0.02 mL/min, and the dwell time was set to 50 µs, with a total scan time of 100 s.

2.2.3. Preparation and characterisation of ENM suspensions used in chronic and acute D. magna experiments and acute zebrafish experiments

Fish and daphnids were exposed to coated and uncoated ZnO ENMs, $ZnSO_4$ ($ZnSO_4$*$7H_2O$) as ionic control, and CeO_2 ENMs.

The dispersion protocol used for the ZnO ENMs in the *D. magna* studies was modified from NANOGENOTOX (Jensen, 2011), by using a probe sonicator with amplification 10 % and sonication time 16 minutes on 25.6 mg ENMs/10 ml Milli-Q water (3,136 MJ/m^3). This protocol was used as the experiments were started before the OECD 318 was adopted for the project. For experiments using CeO_2 ENM as test compound and all zebrafish experiments, the

OECD 318 test guideline was as described above. A stock solution of 1 mg/mL was prepared by sonication for 10 minutes at amplitude 48%. Size distribution and zeta potential of the ENMs were measured immediately after preparation by DLS (Malvern, Zetasizer Nano-ZS). Moreover, size distribution and zeta potential were measured over time in the different media used in the zebrafish exposure experiments. Dissolution of Zn from the ZnO ENMs were measured by partner laboratory ISTEC-CNR.

For the daphnid exposures, the ENM suspensions were further diluted to obtain the required test concentrations in standard M7 OECD medium (as specified in OECD 202 guideline).

2.2.4. Preparation and characterisation of ENM suspensions used in multi-generational D. magna experiments

Stock suspensions of both ENMs were prepared freshly before every spike in 100 mL glass bottles by dispersing 100mg/ L TiO_2 or CeO_2 ENMs in Milli-Q water (Millipore Milli-Q reference A+ system, Waters-Millipore Corporation, Milford, MA, USA). Prior to use, stocks were ultra-sonicated in a sonication bath for 10 minutes (Sonicor SC-50-22) by applying a calculated delivered acoustic energy of 27±0.2 Watts s^{-1}. Exposure suspensions were prepared in Elendt M7 medium (Elendt, 1990) by pipetting the required volume of stock suspension in the medium followed by three stirs.

Particle size distributions (through DLS) and zeta potential were determined by members of WP1 in Elendt M7 medium. Due to high variability in the data, it was decided to assess particle size distributions and zeta potential on site as well, as this has proved to provide more representative results in the past. Therefore, additional measurements were performed using multi-angle dynamic light scattering (MADLS) which allows for a more accurate assessment of size distributions in poly-disperse samples. Test concentrations at the center of the undisturbed water column were verified at 0 h, 1 h, 24 h and 48 h after preparation by inductively-coupled plasma mass spectrometry (ICP-MS) at ISTEC by WP1.

2.2.5 Hazard studies

2.2.5.1. Toxicity of ENMs test using algae (R. subcapitata)

The test organism used in this study was a laboratory culture of the green alga *R. subcapitata* obtained from the Norwegian Institute for Water Research, Oslo, Norway (NIVA) and grown continuously according to ISO 8692 standard. The algae were cultivated in 20 mL glass vials fitted with a screw cap lid with a hole to allow for CO_2 diffusion from the atmosphere.

The tests were conducted according to the ISO 8692 algal growth inhibition test protocol (ISO, 2012). The exposure duration was 72 hours. Test concentrations were prepared from a stock suspension prepared according to OECD 318 and diluting with ISO 8692 algal medium to obtain the required concentrations. These were then inoculated with appropriate amount of algal culture to obtain an initial density of 10^4 cells mL^{-1}, measured by coulter counter (Beckman Multisizer^{TM} 3, Indianapolis, USA).

Algal medium was prepared according to ISO 8692 (ISO, 2012). The readymade medium was put on a magnetic stirrer for 10-20 minutes to obtain equilibrium with the atmosphere and a pH value of 8±0.2.

Two tests were carried out using the following nominal concentrations of ZnO ENMs: 0.025, 0.25, 1.28, 2.56, 25.6 and 0.1, 0.3, 0.9, 2.36 mg/L for the first and second test, respectively. An initial range-finding test with TiO_2, CeO_2 and $BaSO_4$ ENMs used nominal concentrations of 0.1, 0.5, 1, 5 and 10 mg/L. Due to low growth inhibition of the range-finding test with $BaSO_4$ ENMs a definitive test was carried out using 9.4, 23.5, 58.8, 147.2 and 368 mg/Las nominal concentrations. For each test 6 replicates were used for the control group, and three for the treatments. Each replicate was incubated at conditions identical to the cultivation process and ISO 8692 validity criteria were met for all tests. Samples of 0.4 mL were taken at 0h and 72h and the algal growth rates were calculated based on total algal pigments in each sample quantified by acetone extraction as described by Mayer et al. (1997). The fluorescence of the samples was subsequently measured on a Hitachi Fluorescence Spectrophotometer F-700 (Hitachi High-Technologies, Hitachinaka, Japan). Machine settings were set to 430 and 670 nm for excitation and emission wavelength, respectively. A blank sample containing medium and acetone was used for background correction of the samples.

For data analysis the statistical software R, was used to plot a concentration-response curve using a log logistic fitting and for calculation of 95% confidence intervals. DLS values are reported as mean value ± standard deviation.

2.2.5.2. Bioaccumulation of ENMs in algae (R. subcapitata)

The experiment was set up in 20 mL glass vials using 5 mL inoculum of 10^4 cells/mL (*R. subcapitata*) exposed at a ratio of 1:100 ENMs (Au, CeO2 and BaSO4) which is the theoretical maximum adsorption capacity of 110 nm polystyrene particles (serving as a theoretical threshold for the exposure) based on attraction forces measured with Atomic Force Microscopy (Nolte et al. 2017). The algae were exposed for 72h under similar conditions as described in the ISO 8692 guideline. After 72 h the inoculum was pipetted into 6 mL glass vials which was put into 15 mL Falcon tubes and then centrifuged at 3000g for 30 min to create a pellet of algae. After centrifugation the supernatant was removed and the pellet was resuspended in 5 mL ISO 8962 algae medium and was centrifuged again at the same speed and time. Three rounds of centrifugation and resuspension were carried out in order to minimize the carried over metal from the exposure medium. After the final resuspension of the pellet the samples were ready for measurement on single cell ICP-MS. For SC-ICP-MS measurements was used a PerkinElmer, NExION 350D set up for SC-ICP-MS mode with an Asperon™ spray chamber and the Syngistix™ software module for single cell application. The transport efficiency of the SC-ICP-MS was determined using standardized 60 nm Au ENMs (PerkinElmer, Shelton, CT USA). The sample flow rate was 0.02 mL/min, and the dwell time was set to 50 µs, with a total scan time of 100 s resulting in 2000 replicate windows measured per sample.

2.2.5.3. Acute and chronic toxicity of ENMs to D. magna

The test organism used in this study was a laboratory culture of *D. magna*. In the reproduction

study, two sets of results were obtained since two different *Daphnia* clones, GG4 (Originally from Edinburgh University, Scotland) and the HW1 (originally from Edinburgh Pharmaceutical Processes, Scotland), were used. Daphnids were cultivated in 2 L glass beakers (with plastic covers) containing 1800 mL M7 medium and fed with the microalga *Chlorella vulgaris*, 0.1 - 0.2 mg C/*Daphnia*/day (C: organic carbon).

The OECD 202 *Daphnia* acute immobilisation test is an acute toxicity test aiming at establishing the median effective concentration (EC50) at 48 h. Briefly, 20 neonates (age less than 24 h) per concentration (in 4 replicates), were exposed in 20 mL NMs treatment or control for 48 h. The concentration rage for the ZnO ENMs were 0.16-10 mg/L, 4.67-60 mg/L for $ZnSO_4$ and 10-76 mg/L for CeO_2. The number of immobilized animals were recorded at 24 h and 48 h and the EC50 value was calculated following OECD 202.

The 21 d chronic *Daphnia* reproduction test, with coated and uncoated ZnO (at conc. 180-840 µg/L), was performed following OECD guideline 211 (OECD, 2012). Briefly, *Daphnia* neonates (age less than 24 hours) were placed singularly in 80 ml M7 medium dispersed ENMs (10 replicates/concentration) (control with only medium included). The animals were fed daily and the test suspension replaced twice a week. Mortality and offspring number were recorded daily and the body size of surviving parent *Daphnia* was recorded on day 21.

The median effective concentration (EC50) for *Daphnia* immobilisation was calculated using a nonlinear regression by four-parameter equations using Graphpad Prism 8. The median survival day in the *Daphnia* reproduction test was also calculated by Graphpad Prism 8. All data passed the normality requirements by means of the Anderson-Darling test using Minitab 18. To determine statistical differences between different treatments, data were analysed by Independent-Sample t-tests using SPSS 25. All significant differences were established at $p<0.05$. Homogeneity of variance was checked by means of the Levene's test, using SPSS 25.

2.2.5.4. Multi-generational toxicity of ENMs tests using D. magna

D. magna were obtained from an in-house culture at Leiden University, which is maintained according to the conditions prescribed in OECD guideline 212. In short, cultures are maintained in a climate room at 22±1°C under a 16:8 hours (light:dark) photoperiod and fed with a suspension of *R. subcapitata*. Prior to the experiment the sensitivity of the neonates of the culture was tested through a reference toxicity test using $K_2Cr_2O_7$ and confirmed as being within the recommended boundaries (24 h EC50 immobilization 0.96±0.09mg/L) as stated in the *D. magna* Immobilization Test (OECD 202).

The experiment was initiated by introducing 12 neonates (<24 h) in individual glass beakers containing 50 mL of Elendt M7 medium spiked with TiO_2 or CeO_2 ENMs at concentrations of 0, 0.02, 0.2, and 2 mg/L in the presence or absence of UV radiation (UVR), following a full factorial design. Exposure medium was refreshed every 48 hours and aerated prior to addition of the spike in order to achieve dissolved oxygen (DO) concentrations ≥3 mg/L. DO and pH were measured in three replicates per test concentration before and after every medium renewal. Offspring was counted and removed during every medium renewal. Feeding took place directly after every medium renewal at a rate of 0.1 to 0.2 mg C/Daphnid/day by application of *R. subcapitata*.

UV radiation (UVR) was applied using fluorescent tubes (Terra Exotica UV fluorescent tubes, Terra Exotica, Alfeld, Germany) that emit light at an intensity and spectrum that resembles natural lighting conditions, including 10% UVA and 2% UVB emission. Light intensity (in the UVA, UVB and UVC range) was verified at the surface of the medium of the beakers in the experimental setup using a Flame-S photospectrometer (ser#FLMS02180, Ocean Insight, Duiven, the Netherlands)(Figure 1). In addition, regular fluorescent tubes (Sylvania luxline plus, Osram Sylvania Inc., Massachusetts, USA) were used in the non-UV treatment and their spectral output was analysed as described above. Controls without ENMs exposure but with UVR were included to account for any effects induced by UVR.

Multigenerational effects were assessed by following a modified version of the *D. magna* Reproduction Test (OECD 211) as proposed by Castro et al. (2018). In short, this modification consists of an additional test on top of the 21 day exposure period, in which individuals from the first brood of the F0 generation are reared in clean medium until release of their own first brood. The number of individuals in the first brood of the F1 is considered as a proxy for maternal investment in juvenile fitness, i.e. a multigenerational effect relating to reproductive abilities (Castro et al. 2018).

Mortality, day of 1st reproduction, number of neonates in 1st brood, number of broods and total number of neonates produced over 21 days were recorded for the F0 generation throughout the 21 day exposure period at every medium replacement. In addition, the size of the first brood was recorded for the F1 generation.

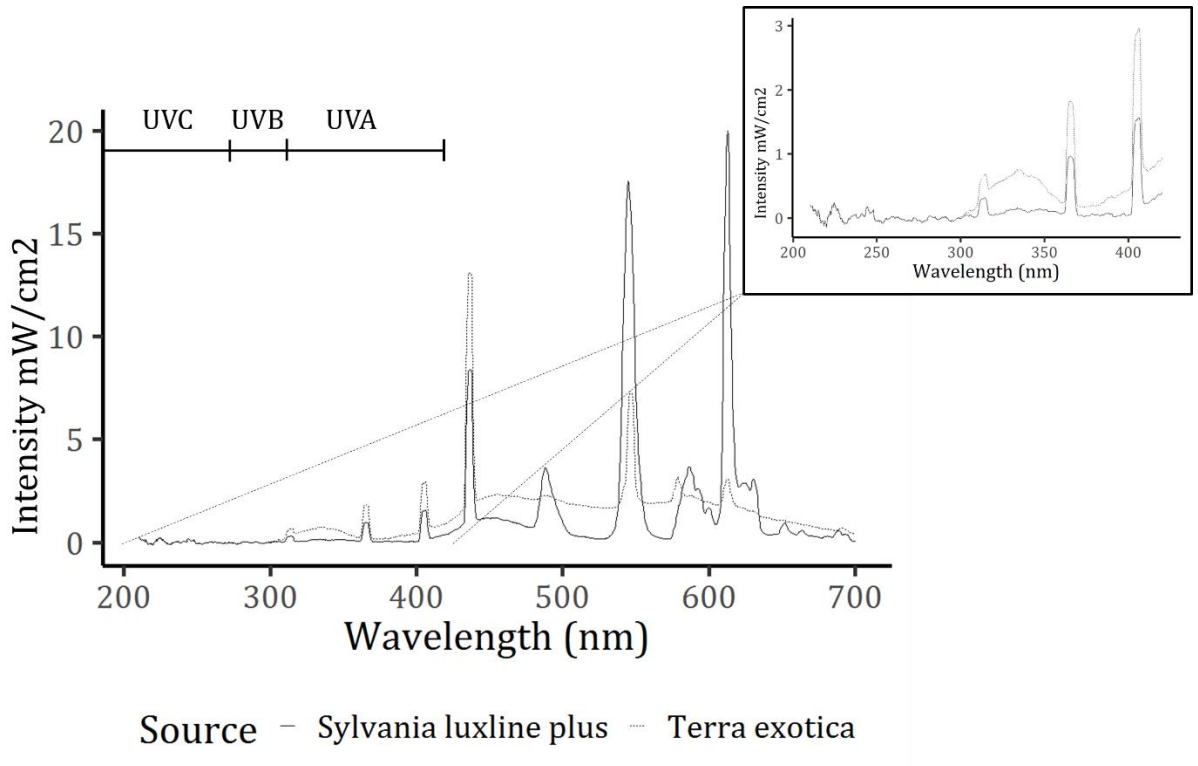

Figure 1. Results from spectrophotometric verification of emission spectra and intensity of Terra Exotica UV fluorescent tubes (Terra Exotica, Alfeld, Germany) and Sylvania luxline plus fluorescent tubes used in the experiment (Osram Sylvania Inc., Massachusetts, USA)

All data were analysed using R version 1.1.419 (R Core Team 2017). Reproduction related endpoints (day of 1st reproduction, number of neonates in 1st brood, number of broods and total number of neonates produced over 21 days) were analyzed by two-way Anova followed by Tukey post-hoc tests to compare between UV and non-UV treatments in addition to the ENM treatment, and to account for possible interactions between UV and the ENM treatment. To do so, a subset of the data was generated prior to the analyses by removing all data points from individuals that died. All data were checked for heteroscedasticity (Bartlett's test) and normal distribution (Shapiro Wilk's test and visual inspection of histograms and QQ-plots) of the residuals prior to analysis. If assumptions for parametric tests were not met, the data were Log10 or Square root transformed and assumption were checked again. In case assumptions were still not met, Kruskal-Wallis tests were performed followed by Bonferroni corrected Dunn's post-hoc tests.

2.2.5.5. Bioaccumulation of ENMs in D. magna

The quantitative uptake of CeO_2 ENMs by *D. magna* was assessed using a modified procedure from Kalman et al. (2015). In summary, juvenile *Daphnia* (8-10 days old) (n = 15/treatment and 3 replicates) were exposed to 60 mL ENM suspension (in M7 medium) at concentrations 0.1-100 mg/L for 24 h and 48 h. From this step, three groups of experiments were conducted:

(a) To determine the amount of ENMs accumulating in the whole body of *Daphnia* the animals were retrieved, washed twice with Milli-Q water on a vacuum filter membrane and weighed;

(b) To determine the amount of internalized ENMs the animals were washed 4 times with 1 mM EDTA, following procedure A;

(c) To determine the amount of ENMs internally accumulated, excluding the gastro-intestinal tract, organisms were transferred to clean medium for 48 hours to depurate before washing with 1 mM EDTA.

The animals were then dried to constant weight. After acid digestion, Ce concentrations were measured by ICP-MS at DTU. The measurements were analysed on an Agilent Technologies 7700 series ICP-MS, equipped with an ASX-500 series ICP-MS autosampler. The aqueous 5 mL samples were digested with 170 µL concentrated HNO_3. For digestion of the *Daphnia* samples, 0.5 mL concentrated HNO_3 were added and heated to 70°C for 2 hours and then left at room temperature until measurement. Right before measuring, the samples were diluted with Milli-Q water to an acid concentration of 2%.

Finally, SEM images were taken of *Daphnia* exposed to one high and one low concentration of ZnO or CeO_2 ENMs to further explore the external body distribution of the ENMs. After 48 hour of exposure the animals were retrieved, washed 3 times in Milli-Q water on filter paper and preserved in 70% ethanol. All samples were examined uncoated, and imaged with a Quanta 650 FEG SEM in low-vacuum mode (0.83 Torr), using a backscattered (BSE) detector Additionally, elemental analysis performed within the SEM using an Oxford Instruments X-Max^N 150mm energy dispersive X-ray (EDX) detector, at 20 kV and working distance of 10 mm, using INCA software.

2.2.5.6. Toxicity of ENMs test using zebrafish larvae *(D. rerio)*

The test organism used in this study was a laboratory culture of *D. rerio* reared at HWU for several generation. Zebrafish males and females was induced to spawning by transferring them to breeding tanks the day before spawning before light was switched off for the night. The light coming on the following morning triggered the fish to begin spawning and the fish were allowed to spawn for 2 hour. The eggs were collected, unfertilized eggs removed and the viable eggs counted into Petri dishes containing approx. 50 mL of OECD water. The density was 50 eggs/dish. The embryos were kept at 28°C. Each day, 50% of the culture water was replaced and the dishes examined for the presence of dead embryos using a compound microscope. If dead embryos were detected they were removed. Embryos hatched approximately 72 hours post fertilisation.

The toxicity of ZnO ENMs, CeO_2 ENMs, and $ZnSO_4$ were tested with the zebrafish larvae in 24 h acute tests. *D. rerio* larvae were exposed to a concentration series of the ENMs and control, in 96-well plates with each well containing an individual fish and exposure occurring from 72-96 hours post fertilization (hpf). The larvae were transferred from the Petri dishes, as much residual liquid from each well as possible was removed and 270 µL of medium was added. Then 30 µL of ENMs suspension dilutions were added.

The following media were tested: Seasalt (Egg water) medium (60 mg/L Instant Ocean), OECD medium ($CaCl_2$ 0.294 g/L, $MgSO_4$ 0.1232 g/L, $NaHCO_3$ 0.0647 g/L, KCl 0.0057 g/L) or E3 medium (60 x stock: NaCl 17.4 g/L, KCl 0.8 g /L, $CaCl_2*2H_2O$ 2.9 g/L, $MgCl_2*6H_20$ 4.89 g/L and 100 µL 1% Methylene blue).

All statistical analyses were conducted with R (R Development Core Team 2015). Zebrafish larvae mortality was modelled by logistic regression with the independent variables ZnO ENMs or $ZnSO_4$ concentration, treatment (presence or absence of ENMs), and concentration and treatment interaction. The logistic regression models were generated by iterative maximization of the likelihood function, and independent variables and their interaction were included if they significantly improved the model (likelihood ratio test, based on Wald x2 distribution). The LC50 was calculated by the logistic regression model, and the 95% confidence interval was calculated using the *logit* model in R. Differences in fish mortality among treatments (i.e., the presence or absence of ENMs) were determined by pairwise contrast statements with $p<0.05$.

2.3. Results

2.3.1. Dynamic fate and behaviour of the tested ENMs in algal medium using DLS and SP-ICP-MS

A series of initial tests were carried out to evaluate the suitability of different dispersion protocols. The simplified OECD 318 protocol described in the materials section was found suitable for dispersion of e.g. TiO_2 ENMs which remained stable for 24 h in Milli-Q without significant changes in size distribution (Figure 2). Furthermore, the determined size corresponded well with the size reported by the distributor (50-150 nm). Consequently, this protocol was used throughout the algal studies.

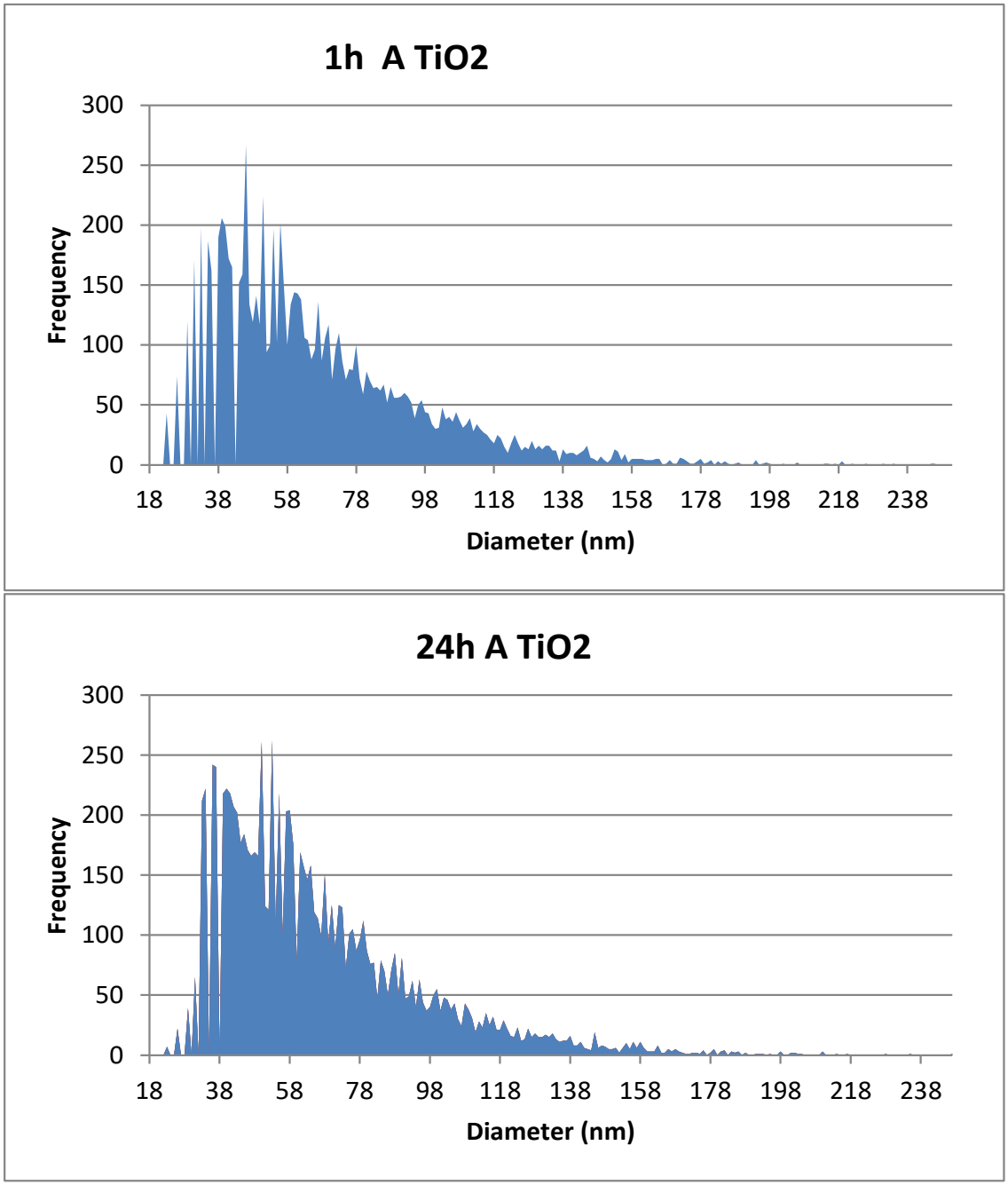

Figure 2: SP-ICP-MS measurements of TiO_2 dispersed in Milli-Q after 1 h (top) and 24 h (bottom) using the simplified OECD 318 protocol.

Table 1 shows the main characteristics of the tier 1 materials 1h after suspension using the OECD 318 protocol. A fraction of the suspended ENMs remains close to the reported primary size. However, all samples had a PdI > 0.2 which means that the suspensions were polydisperse thus containing multiple size distributions of ENMs. It is observed that the ZnO and TiO_2 ENMs had a significantly higher PdI than $BaSO_4$ and CeO_2 ENMs indicating less aggregation of the suspension.

Table 1: Characterization of tier 1 ENMs after 1h suspension in medium and the corresponding Polydispersivity Index (PdI). Primary size was measured using Transmission Electron Microscopy (TEM) (JRC/Frauenhofer) as reported by the distributor. Size in medium was measured using DLS.

Compound	Primary size [nm]	Size in medium [nm]	1st peak size [nm]	PdI
ZnO	76	678±57	182±11	0.7±0.04
TiO_2	15-24	1778±1303	-	0.8±0.2
$BaSO_4$	32	150±20	103±2	0.3±0.03
CeO_2	33	278±12	177±11	0.4±0.01

All the data from the algal toxicity studies were fitted with a log-logistic function to estimate the EC10 and EC50 and the corresponding 95% confidence interval (Table 2). From this Table it is seen that the ranking of toxicity (based on EC50 values) of the tested tier 1 ENMs are ZnO > CeO_2 > $BaSO_4$ > TiO_2.

Table 2: Effect concentrations (10% and 50%) and 95% confidence intervals for Tier 1 ENMs in 72 h algae inhibition experiment according to the ISO 8692 standard.

Compound	Algae	
	EC10 [mg/L]	EC50 [mg/L]
ZnO-NM111	0.02 [0.005-0.08]	0.1 [0.07-0.2]
TiO_2-NM105	0.4 [0.03-4.5]	79 [9-685]
CeO_2-NM212	0.9 [0.5-1.9]	7 [5.9-8.4]
$BaSO_4$-NM220	0.5	26 [15-44]

Measuring the dissolution of ZnO ENMs with SP-ICP-MS yielded 100% dissolved Zn within the first hour of suspension in medium when testing in the µg/L range. The other tier 1 materials tested yielded no or little dissolution. Zn ions are highly toxic to algae (Heijerick et al. 2002), thus the high toxicity observed could be caused by the dissolution of the ENMs. SP-ICP-MS measurements of TiO_2 did not show any dissolution. Similar findings were reported in literature for dissolution measurement of CeO_2 (JRC, 2014) and from WP1 on $BaSO_4$ (BASF, 2019). Consequently, the toxicity of those ENMs is not expected to be due to the release of ions.

2.3.2 Characterization of ENMs stock suspensions used in D. magna and zebrafish experiments

The particle size and zeta potential of the ENM stocks were measured by DLS within 1 hour of preparation (Tables 3, 4 and 5). The mean size of the ENMs suspended in M7 medium is over 10 times larger than the reported nominal size.

Table 3: The particle size and zeta potential of ENMs suspensions used in *D. magna* acute and chronic tests; data are expressed as mean ± standard deviation (3 measurements per sample).

ENMs	Conc. (mg/L)	Medium	Mean particle size (nm)	Polydispersity Index (PdI)	Zeta potential (mV)
ZnO Uncoated	50	M7	733.5 ± 103.2	0.33 ± 0.04	-0.49± 0.82
ZnO Coated	50	M7	969.0 ± 48.5	0.32 ± 0.02	0.01± 0.23
CeO_2	5	M7	1866.4 ± 412.2*	0.72 ± 0.19*	Not provided

* Data were provided by partner Lab, ISTEC-CNR, Italy.

Table 4: The particle size (nm) and polydispersity index (PdI) at different time points, of ENMs suspensions in different media used in the zebrafish larvae exposure. Data are expressed as mean ± standard deviation (3 measurements per sample). It has to be noted that the DLS measurements were done from samples standing in glass scintilation vials and not from the plates used in the experiments.

ENMs	Media	Conc. (mg/L)	Size (nm, ± Stdev) + PdI (± Stdev)						
			0 h	0.5 h	1 h	3 h	6 h	12 h	24 h
ZnO Uncoated	Milli-Q	1000	264.4 ± 4.4 (0.167 ± 0.035)			262.4 ± 5.5 (0.168 ± 0.015)			
	OECD	100	779.3 ± 160.6 (0.221 ± 0.032)	1239 ± 51.5 (0.404 ± 0.045)	2530 ± 217.5 (0.363 ± 0.019)	2077.3 ± 183.3 (0.412 ± 0.113)	2370.3 ± 364.3 (0.351 ± 0.084)	1694.3 ± 164.3 (0.400 ± 0.081)	3154 ± 722.5 (0.350 ± 0.133)
	Seasalt	100	350.6 ± 19.7 (0.220 ± 0.041)	752.6 ± 34.7 (0.274 ± 0.045)	1186 ± 43.6 (0.310 ± 0.010)	1767.7 ± 81.2 (0.471 ± 0.026)	1512.3 ± 45.1 (0.482 ± 0.137)	1243.3 ± 300.6 (0.601 ± 0.056)	2914 ± 1262.1 (0.508 ± 0.142)
	Seasalt	15	422.9 ± 46.2 (0.239 ± 0.008)	674.3 ± 74.7 (0.279 ± 0.026)	912.8 ± 17.0 (0.316 ± 0.023)	1282 ± 45.2 (0.475 ± 0.067)	1215.7 ± 208.8 (0.670 ± 0.087)	864.1 ± 175.8 (0.552 ± 0.050)	791.4 ± 271.0 (0.572 ± 0.049)
	E3	100	741.4 ± 106.8 (0.219 ± 0.029)	1603 ± 65.0 (0.317 ± 0.027)	2135.7 ± 217.8 (0.354 ± 0.098)	2300.7 ± 131.0 (0.361 ± 0.103)	1713 ± 175.8 (0.435 ± 0.062)	2038.7 ± 339.2 (0.401 ± 0.085)	2799.3 ± 427.8 (0.359 ± 0.033)
	E3	15	459.6 ± 20.3 (0.245 ± 0.013)	649.2 ± 37.6 (0.320 ± 0.037)	604.8 ± 44.7 (0.318 ± 0.028)	858.8 ± 84.9 (0.367 ± 0.051)	1041.6 ± 130.4 (0.426 ± 0.065)	851.7 ± 104.6 (0.619 ± 0.022)	735.9 ± 143.4 (0.604 ± 0.072)
ZnO Coated	Milli-Q	1000	255.2 ± 6.85 (0.184 ± 0.012)			256.0 ± 0.265 (0.162 ± 0.014)			
	OECD	100	944.5 ± 153.3 (0.232 ± 0.019)	1530.7 ± 104.9 (0.249 ± 0.020)	1696.7 ± 144.3 (0.293 ± 0.039)	3028 ± 983.5 (0.420 ± 0.081)	1351.7 ± 300.5 (0.439 ± 0.130)	2965 ± 123.5 (0.466 ± 0.312)	2943.3 ± 128.3 (0.372 ± 0.061)
	Seasalt	100	310.8 ± 12.9 (0.213 ± 0.014)	610.0 ± 25.8 (0.361 ± 0.056)	827.9 ± 17.7 (0.329 ± 0.036)	1296.3 ± 102.0 (0.380 ± 0.056)	1061.3 ± 184.4 (0.371 ± 0.009)	1540.7 ± 150.7 (0.437 ± 0.050)	1803.3 ± 134.9 (0.502 ± 0.123)
	Seasalt	14	432.9 ± 14.4 (0.286 ± 0.077)	679.4 ± 30.3 (0.314 ± 0.006)	680.0 ± 98.5 (0.339 ± 0.016)	955.3 ± 60.3 (0.360 ± 0.062)	1228.3 ± 78.9 (0.699 ± 0.177)	1086.0 ± 232.2 (0.477 ± 0.055)	1186.1 ± 264.7 (0.565 ± 0.256)

CeO_2	Milli-Q	1000	200.3 ± 3.027 (0.282 ± 0.024)			180.3 ± 1.2 (0.199 ± 0.190)			
	OECD	100	1143.6 ± 167.1 (0.252 ± 0.021)	2289.3 ± 125.6 (0.357 ± 0.040)	2302.7 ± 170.7 (0.328 ± 0.046)	2544.3 ± 254.1 (0.349 ± 0.010)	2537.7 ± 96.2 (0.274 ± 0.014)	2810.3 ± 148.2 (0.349 ± 0.043)	1864.3 ± 24.26 (0.346 ± 0.031)
	Seasalt	100	886.7 ± 133.5 (0.267 ± 0.021)	2100.7 ± 220.0 (0.368 ± 0.067)	3309 ± 367.2 (0.350 ± 0.105)	2577 ± 125.5 (0.330 ± 0.028)	2654.3 ± 189.0 (0.302 ± 0.040)	2625.3 ± 185.8 (0.377 ± 0.100)	3213.7 ± 460.3 (0.355 ± 0.083)

Table 5: The zeta potential (mV) at different timepoints, of ENMs suspensions in different media used in the zebrafish larvae exposure. Data are expressed as mean ± standard deviation (3 measurements per sample). It has to be noted that the DLS measurements was done from samples standing in glass scintilation vials and not from the plates used in the experiments.

NM	Media	Conc. (mg/L)	Zeta potential (mV)						
			0 h	0.5 h	1 h	3 h	6 h	12 h	24 h
ZnO Uncoated	Milli-Q	1000	28.4 ± 0.56			27.8 ± 0.36			
	OECD	100	6.51 ± 0.08	6.52 ± 0.41	6.43 + 0.21	6.78 ± 0.34	6.69 ± 0.26	5.75 ± 0.55	2.55 ± 0.38
	Seasalt	100	16.20 ± 1.35	17.23 ± 0.59	17.33 ± 0.40	17.8 ± 0.40	14.90 ± 0.78	15.07 ± 0.65	12.57 ± 1.18
	Seasalt	15	8.90 ± 0.98	10.07 ± 0.81	11.97 ± 0.51	11.07 ± 0.21	8.85 ± 1.83	3.06 ± 0.95	-4.50 ± 3.28
	E3	100	-5.83 ± 0.23	-6.36 ± 0.20	-6.20 ± 0.37	-7.49 ± 0.54	-7.98 ± 0.34	-11.37 ± 0.85	-15.23 ± 0.96
	E3	15	-17.13 ± 0.67	-19.87 ± 0.60	-19.27 ± 0.35	-19.87 ± 0.15	-20.6 ± 0.95	-20.07 ± 0.12	-22.33 ± 0.61
ZnO Coated	Milli-Q	1000	30.87 ± 1.34			32.4 ± 0.17			
	OECD	100	6.11 ± 0.11	8.10 ± 0.35	8.60 ± 0.28	9.65 ± 0.34	9.94 ± 0.65	8.50 ± 0.47	6.76 ± 0.32
	Seasalt	100	17.3 ± 0.10	16.9 ± 0.52	16.4 ± 0.2	17.07 ± 0.50	14.77 ± 0.42	15.12 ± 0.23	4.77 ± 1.70
	Seasalt	14	3.49 ± 0.54	6.42 ± 0.14	11.73 ± 0.75	14.40 ± 0.17	12.8 ± 0.00	4.98 ± 1.52	-6.92 ± 1.18
CeO_2	Milli-Q	1000	42.97 ± 0.84			38.53 ± 0.84			
	OECD	100	1.80 ± 0.11	3.39 ± 1.23	3.07 ± 1.12	2.78 ± 0.75	1.92 ± 0.46	-0.33 ± 0.24	-1.84 ± 0.79
	Seasalt	100	-4.36 ± 2.03	4.48 ± 1.36	7.44 ± 0.36	7.37 ± 0.25	5.54 ± 0.60	4.55 ± 0.20	1.48 ± 0.23

For the zebrafish experiments the stocks were diluted with treatment medium within 2 hours of sonication: As can be seen from the DLS results, the stock suspension (1000 mg/L in Milli-Q)

was stable up to at least 3 hours. Repetition of the measurements is planned.

The DLS results indicate that the ENMs suspended in media quickly aggregate. Even within one hour of sonication the size of the particles were about one order of magnitude larger than their nominal size. The high PdI values moreover suggest that the aggregates in the sample have a very broad size distribution.

Dissolution data have been provided by partner Lab, ISTEC-CNR, Italy (Table 6).

Table 6: The dissolution percentage of 5 ppm uncoated and coated ZnO ENMs suspended in M7 medium for 1 h and 72 h.

	Dissolution in M7 1 h (% Zn^{2+}/ Zn_{tot})	SD	Dissolution in M7 72 h (% Zn^{2+}/ Zn_{tot})	SD
Uncoated ZnO ENMs 5 ppm	21.5	3.0	39.7	5.2
Coated ZnO ENMs 5 ppm	25.1	2.3	30.9	3.0

2.3.3. Characterization of ENMs stock suspension for multi-generational Daphnia experiments

The DLS results obtained through WP1 are currently being processed in order to be compared to the MADLS results obtained by LU. ICP-MS measurements of exposure concentrations over time showed a time dependent decrease in exposure concentrations for both TiO_2 and CeO_2 ENMs (Figure 3).

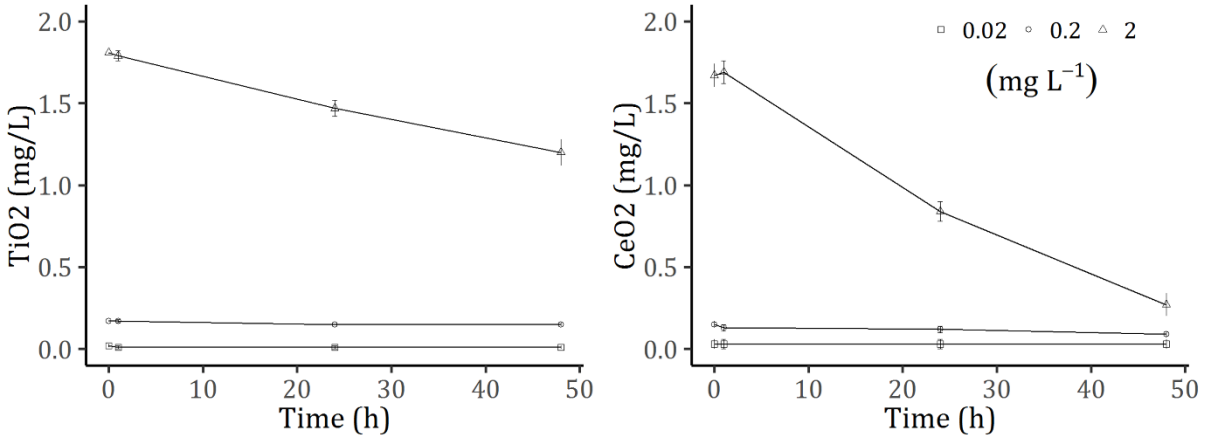

Figure 3. Time-dependent concentrations of TiO_2 ENMs and ENMs in the center of the water column in Elend M7 medium according to the concentrations applied in the experiment. Measurements were performed through ICP-MS at ISTEC by members of WP1. Error bars represent standard deviations.

2.3.4. Bioaccumulation of gold ENMs and tier 1 ENMs in individual algal cells

A series of experiments were carried out to establish the SC-ICP-MS method. The results using 30 nm gold ENMs as exposure are shown in Figure 4. The total mass of the analysed element is shown on the x-axis in attograms, while the frequency of a given mass is shown on the y-axis. It is assumed that the ENMs are spherical and a certain size the mass can be converted to number of particles. In this example the most frequent number of ENMs per cell is within the range of 2-4 ENMs, while cells with >5 ENMs occur less frequently. This means that the potential for bioaccumulation of the tested gold particles is relatively low compared to the theoretical threshold of 100 nanoparticles per algae (Nolte et al. 2017). Further development of the method for the Tier 1 ENMs as well as analysis of aggregated samples is in progress. It can however be concluded that the application of a SP-ICP-MS approach was successful and an important technological advance for quantifying the contribution of algal-associated ENMs to potential trophic transfer of ENMs.

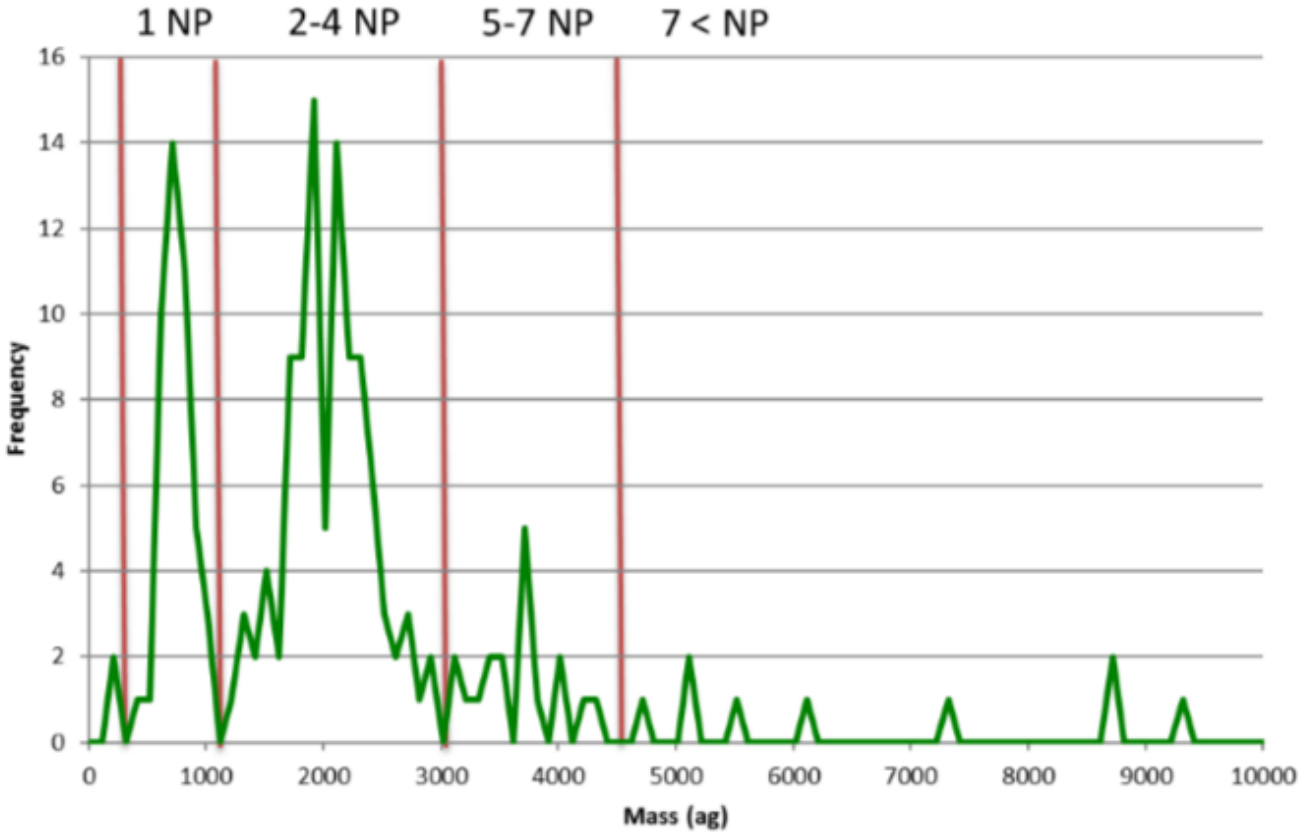

Figure 4: SC ICP-MS measurement of algae (*R. subcapitata*) exposed for 72 h to 30 nm gold ENMs. The red lines mark the intervals for the number of particles associated with each algal cell.

2.3.5. Daphnia acute exposures

The results from the acute toxicity tests showed uncoated and coated ZnO ENMs had similar EC50 values, with the 48 h EC50 value being 2.43 ± 0.24 and 2.35 ± 7.60 mg/L (1.95 ± 0.19 and 1.89 ± 6.10 mg Zn/L) (Mean ± SE), respectively. However, coated ZnO ENMs showed a greater immobilisation effect to *Daphnia* at the three highest concentrations (Figure 5 and 6). The 48 h EC50 value for $ZnSO_4$ was 9.98 ± 4.25 mg/L (2.27 ± 0.97 mg Zn/L) (Mean ± SE) (Figure 7). The toxicity of the ENMs was therefore greater than the Zn ion; this being consistent with results from the literature.

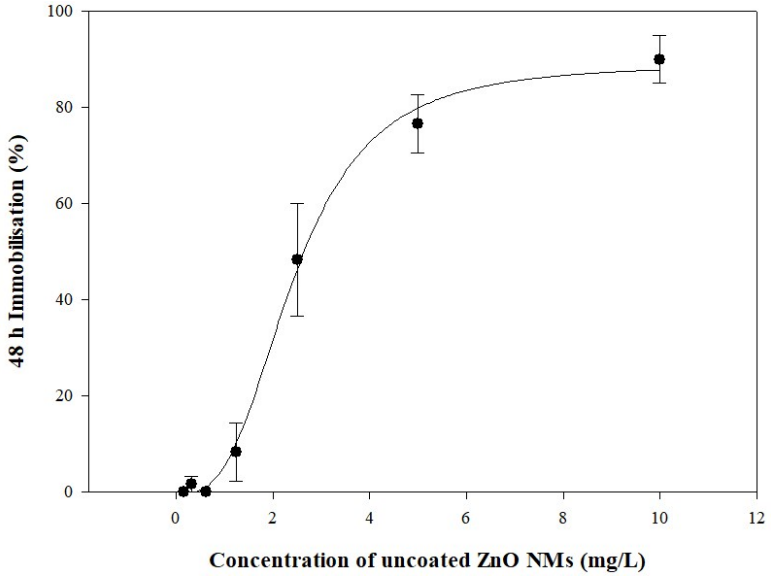

Figure 5: The EC50 of uncoated ZnO ENMs on *D. magna* immobilisation was calculated as 2.43 ± 0.24 mg/L (1.95 ± 0.19 mg Zn/L) after 48 h exposure (n = 3) (Mean ± SE).

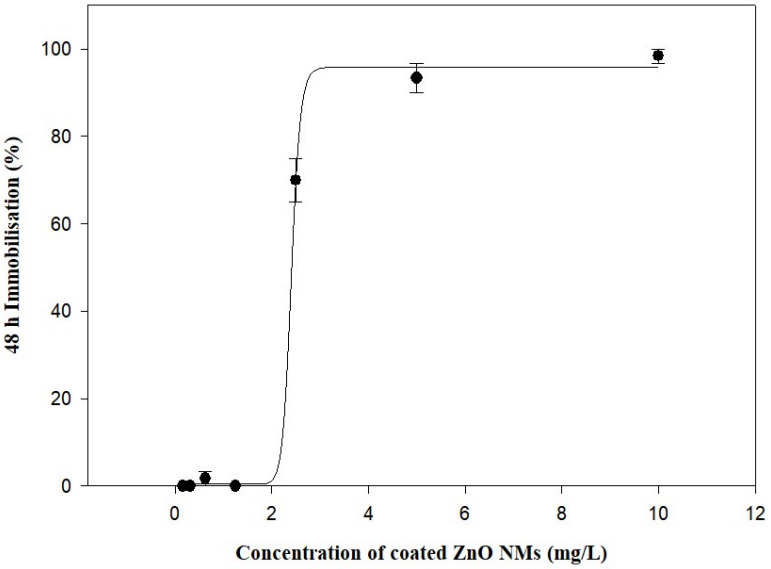

Figure 6: The EC50 of coated ZnO ENMs on *D. magna* immobilisation was calculated as 2.35 ± 7.60 mg/L (1.89± 6.10 mg Zn/L) after 48 h exposure (n = 3) (Mean ± SE).

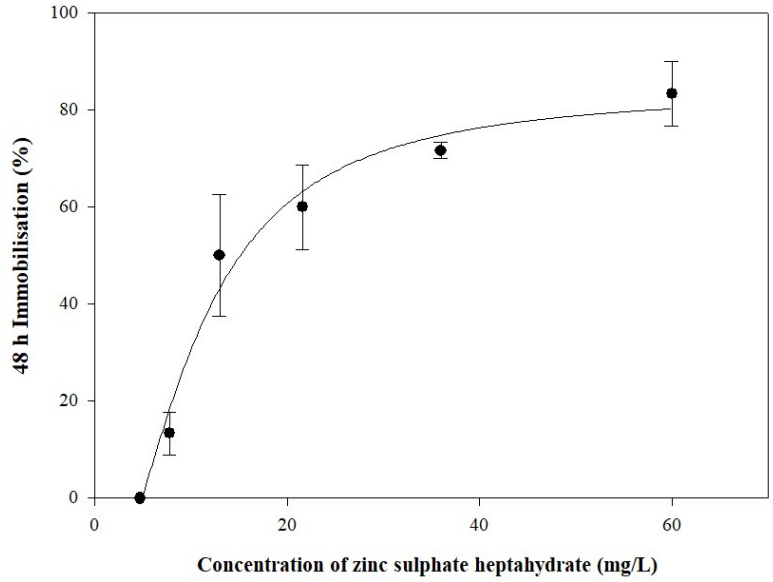

Figure 7: The EC50 value of $ZnSO_4$ on *D. magna* immobilisation was calculated as 9.98 ± 4.25 mg/L (2.27 ± 0.97 mg Zn/L) after 48 h exposure (n = 3) (Mean ± SE).

No mortality of *D. magna* was recorded after acute (48 h) exposure to CeO_2 ENMs (NM 212) at a concentration up to 76 mg/L.

2.3.6. Daphnia chronic exposures

In the reproduction study, two sets of results were obtained since two different *Daphnia* clones, GG4 and the HW1, were used.

For the GG4 clone no significant differences were found between uncoated and coated ZnO ENMs groups on *Daphnia* neonate number and parent body size, except for treatment 300 µg/L which showed a significant difference between groups in terms of parent body size (Figure 8 and 9). Regarding neonate numbers, there was a clear concentration-response relationship within each group. Mortality was higher in the uncoated ZnO ENMs treatments for all concentrations (Table 7). For the same concentration, the median survival day of parent *Daphnia* was 10.0 days for the uncoated ZnO ENMs group, whereas for the coated ZnO ENMs that was 20.0 days (Figure 10).

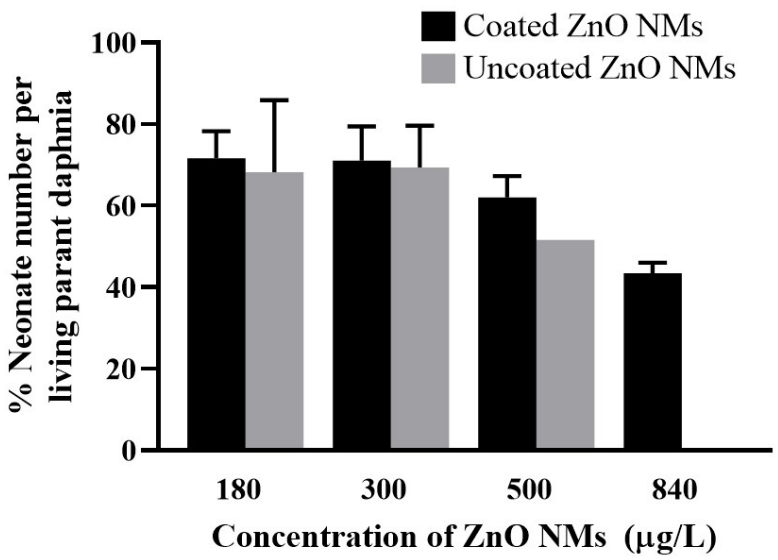

Figure 8: GG4 clone. *D. magna* neonate number after 21 days exposure to uncoated and coated ZnO ENMs. Data are presented as % of the control, and results expressed as the mean ± SE. At 500 µg/L treatment for uncoated ZnO ENMs group only one parent *Daphnia* survived till the end of the test (21 days) (no error bars) and at 840 µg/L 100% mortality was obtained for the uncoated ZnO ENMs treatment (no bars).

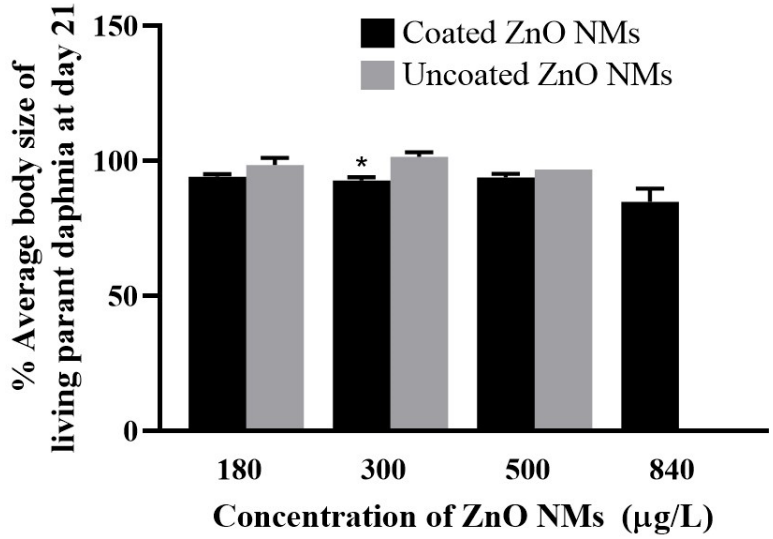

Figure 9: GG4 clone. Parent *D. magna* body size after 21 days of exposure to uncoated and coated ZnO ENMs. Data are presented as % of the control and results expressed as the mean ± SE. * Significant difference compared with treatment uncoated ZnO ENMs 300 µg/L ($p \leq 0.05$). At 500 µg/L treatment for uncoated ZnO ENMs group only one parent *D. magna* survived till the end of the test (21 days) (no error bars) and at 840 µg/L 100% mortality was obtained for the uncoated ZnO ENMs treatment (no error bars).

Table 7: GG4 clone. Mortality of parent *D. magna* after 21 days of exposure to uncoated and coated ZnO ENMs.

ZnO ENMs concentration (µg/L)	Uncoated ZnO ENMs	Coated ZnO ENMs
Control	10%	10%
180	60%	10%
300	50%	10%
500	90%	20%
840	100%	70%

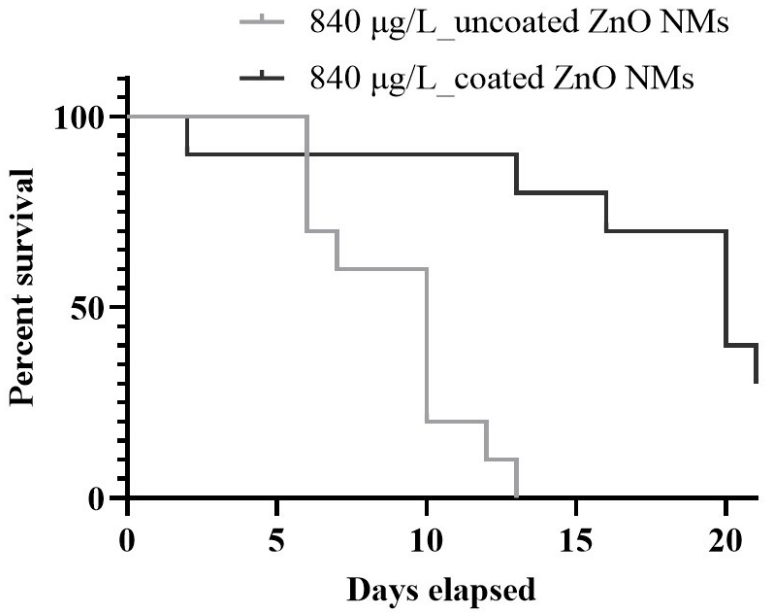

Figure 10: GG4 clone. Survival curve of parent *Daphnia* after 21 days exposure to uncoated and coated ZnO ENMs, at 840 µg/L. Parent *D. magna* median survival days were calculated as 10.0 days for uncoated ZnO ENMs group, and 20.0 days for coated ZnO ENMs group.

For the HW1 clone, no significant difference was found between the uncoated and coated groups in terms of *D. magna* neonate number and parent body size (Figure 11 and 12). As for the GG4 clone, a clear concentration-response relationship in terms of neonate number within each group was observed. For the mortality, no clear trend between exposure to uncoated and coated ZnO ENMs could be detected (Table 8). However, exposure to uncoated ZnO ENMs at the highest concentration of 840 µg/L resulted in shorter median survival of parent *D. magna* at 15.5 days, compared to 19.0 days for animals exposed to coated ZnO ENMs (Figure 13).

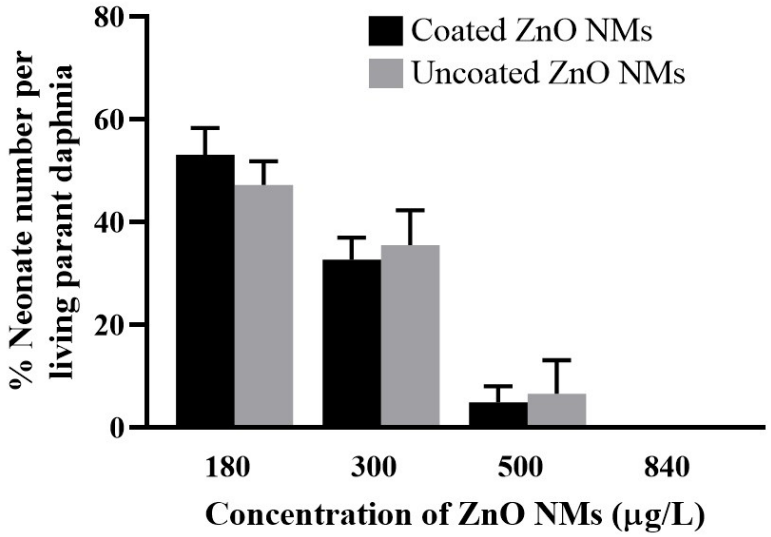

Figure 11: HW1 clone. *D. magna* neonate number after 21 days exposure to uncoated and coated ZnO ENMs. Data are presented as % of the control, and results expressed as the mean ± SE. No error bars are shown at 840 µg/L because no neonates were observed for both uncoated and coated ZnO ENMs treatments.

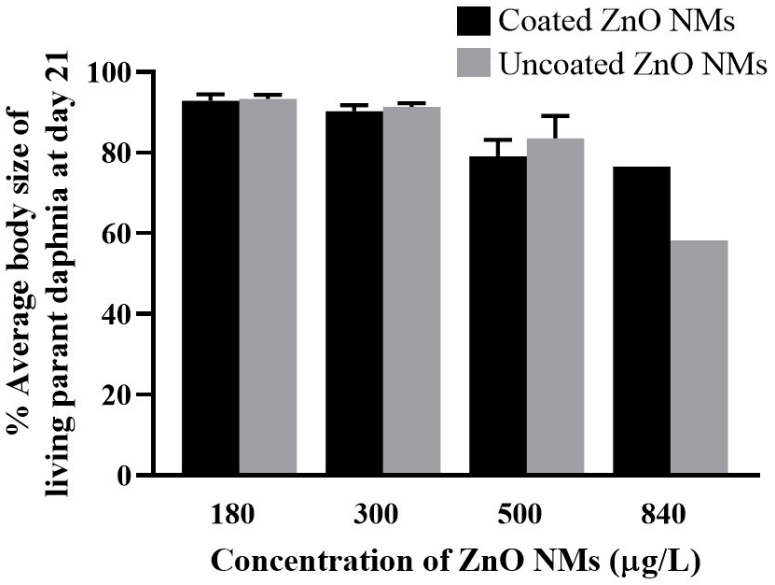

Figure 12: HW1 clone. Parent *D. magna* body size after 21 days of exposure to uncoated and coated ZnO ENMs. Data are presented as % of the control. Results expressed as the mean ± SE. No error bars are shown at the highest concentration (840 µg/L) because only one parent *D. magna* survived till the end of the test (21 days).

Table 8: HW1 clone. Mortality of parent *D. magna* after 21 days exposure to uncoated and coated ZnO ENMs.

ZnO ENMs concentration (μg/L)	Uncoated ZnO ENMs	Coated ZnO ENMs
Control	0%	0%
180	10%	20%
300	30%	30%
500	80%	60%
840	90%	90%

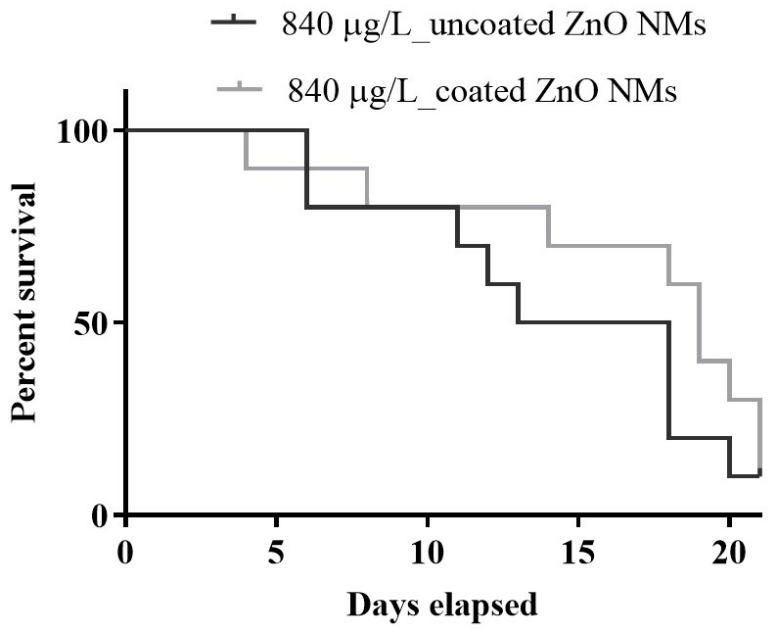

Figure 13: HW1 clone. Survival curve of parent *Daphnia* after 21 days exposure to uncoated and coated ZnO ENMs, 840 μg/L. Parent *Daphnia* median survival day was calculated as 15.5 days for uncoated ZnO ENMs group, 19.0 days for coated ZnO ENMs group.

It can be seen that for the same concentration of coated ZnO ENMs, that the HW1 clone was more sensitive compared to the GG4 clone.

2.3.7. Daphnia multigenerational chronic exposures

Mortality remained >80% for all control groups in the experimental setup and the mean number of neonates produced in the F0 generation was >60 over the 21 day exposure period, thereby meeting the validation criteria as stated for the *D. magna* Reproduction Test (OECD 211). Mortality remained >80% for all treatments and generations, except for the 0.2 mg/ L of TiO_2 ENMs in combination with UVR (Figure 14).

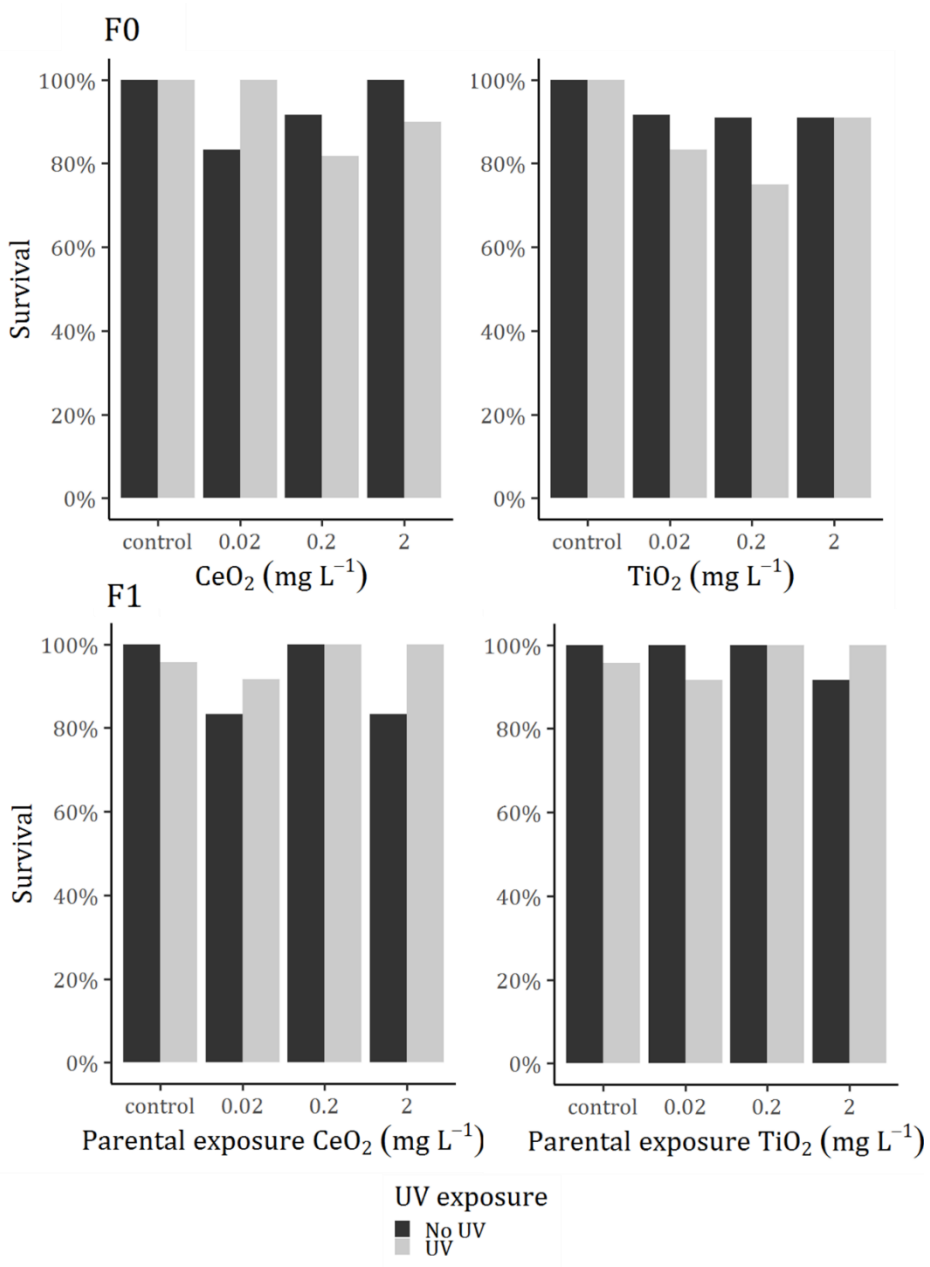

Figure 14. *D. magna* survival rates over the 21 day exposure period (F0) and until release of the first brood (F1)

The output of the performed statistical tests is summarized in Table 9. No statistically significant differences ($P<0.05$) in the mean day of 1st reproduction were observed between any of the treatment groups for either ENM in the first generation. Furthermore, the mean number of broods did not differ between any of the treatment groups for either ENM in the first generation. No statistically significant differences in the mean number of neonates in the 1st brood were observed for any of the treatment groups for either ENM in the first and second generation. A minor statistically significant increase in the mean number of neonates in the 1st brood was observed for the overall UV treatment and the UV * CeO_2 ENM treatment in the F1. The mean number of neonates produced during the 21 day exposure period of the F0 did not differ between treatment groups (Table 9, Figure 15 and 16. A minor statistically significant increase in the mean number of neonates produced was observed for the overall UV treatment in the F1, and for the UV * CeO_2 ENM treatment in the F1 (Table 9).

Table 9: Summary of the output of the parametric analyses (Two-way Anova and Tukey's HSD post hoc tests) comparing ENM and UV treatments including interactions for all endpoints.

	ENM	Dependent variable	Df	Sum Sq	Mean Sq	F value	Pr(>F)
		Day of 1st reproduction					
F0	**TiO_2**	TiO_2	3	0.46	0.15	0.35	0.79
		UV	1	0.83	0.83	1.92	0.17
		UV * TiO_2	3	0.29	0.10	0.23	0.88
	CeO_2	CeO_2	3	0.33	0.10	0.27	0.84
		UV	1	0.26	0.26	0.66	0.42
		UV * CeO_2	3	0.36	0.12	0.30	0.82
		Number of neonates in 1st brood					
F0	**TiO_2**	TiO_2	3	10.88	3.63	1.24	0.30
		UV	1	1.95	1.94	0.67	0.42
		UV * TiO_2	3	15.65	5.23	1.79	0.16
	CeO_2	CeO_2	3	1.64	0.55	0.20	0.90
		UV	1	0.22	0.22	0.08	0.77
		UV * CeO_2	3	17.46	5.82	2.12	0.16
F1	**TiO_2**	TiO_2	3	39.4	13.12	1.23	0.31
		UV	1	0.1	0.09	0.01	0.93
		UV * TiO_2	3	82.6	27.52	2.57	0.06
	CeO_2	CeO_2	3	0.04	0.01	0.34	0.80
		UV	1	0.23	0.23	6.45	0.01*
		UV * CeO_2	3	0.37	0.13	3.51	0.02*
		Number of broods					
F0	**TiO_2**	TiO_2	3	1.13	0.38	0.55	0.65
		UV	1	0.66	0.66	0.97	0.33
		UV * TiO_2	3	3.07	1.02	1.49	0.23
	CeO_2	CeO_2	3	4.24	1.41	1.69	0.18
		UV	1	1.00	0.99	1.19	0.28
		UV * CeO_2	3	1.14	0.38	0.45	0.71
		Total number of neonates produced					
F0	**TiO_2**	TiO_2	3	293	98.8	1.94	0.13
		UV	1	33	33.3	0.66	0.42
		UV * TiO_2	3	112.35	112.35	2.23	0.09
	CeO_2	CeO_2	3	264	87.9	1.57	0.20
		UV	1	293	292.8	5.23	0.03*
		UV * CeO_2	3	247	82.2	1.47	0.23
F1	**TiO_2**	TiO_2	3	39.4	13.12	1.23	0.31
		UV	1	0.1	0.09	0.01	0.93
		UV * TiO_2	3	82.6	27.52	2.57	0.06
	CeO_2	CeO_2	3	0.04	0.01	0.34	0.80
		UV	1	0.23	0.23	6.45	0.01*
		UV * CeO_2	3	0.37	0.13	3.51	0.02*

F0 = 1st generation, *F1* = 2nd generation, Df = Degrees of freedom, Sum Sq = Sum of Squares, Mean Sq = Mean Squared error; * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

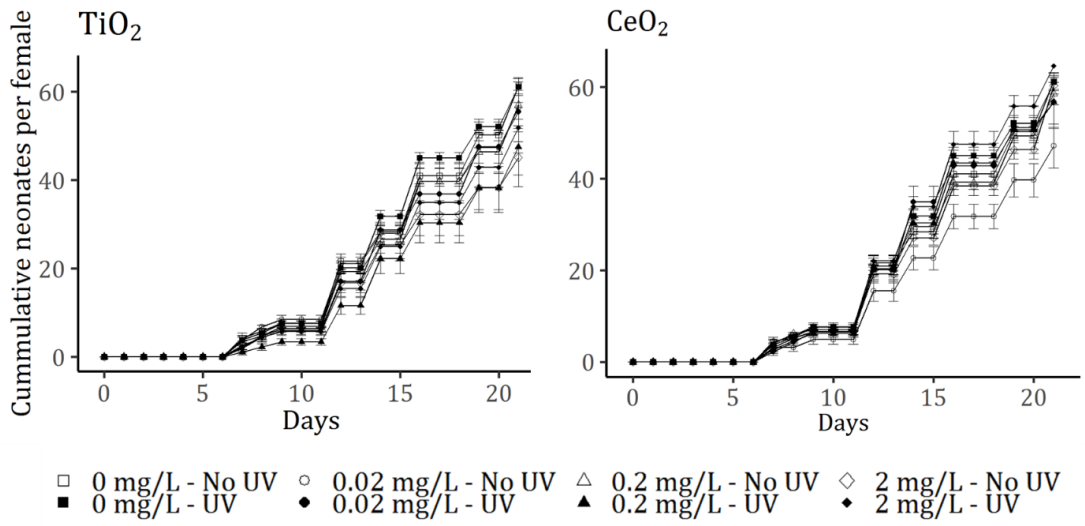

Figure 15. Cumulative number of neonates of *D. magna* produced during the 21 day exposure period of the 1st generation. Error bars represent standard error of the mean (± SE).

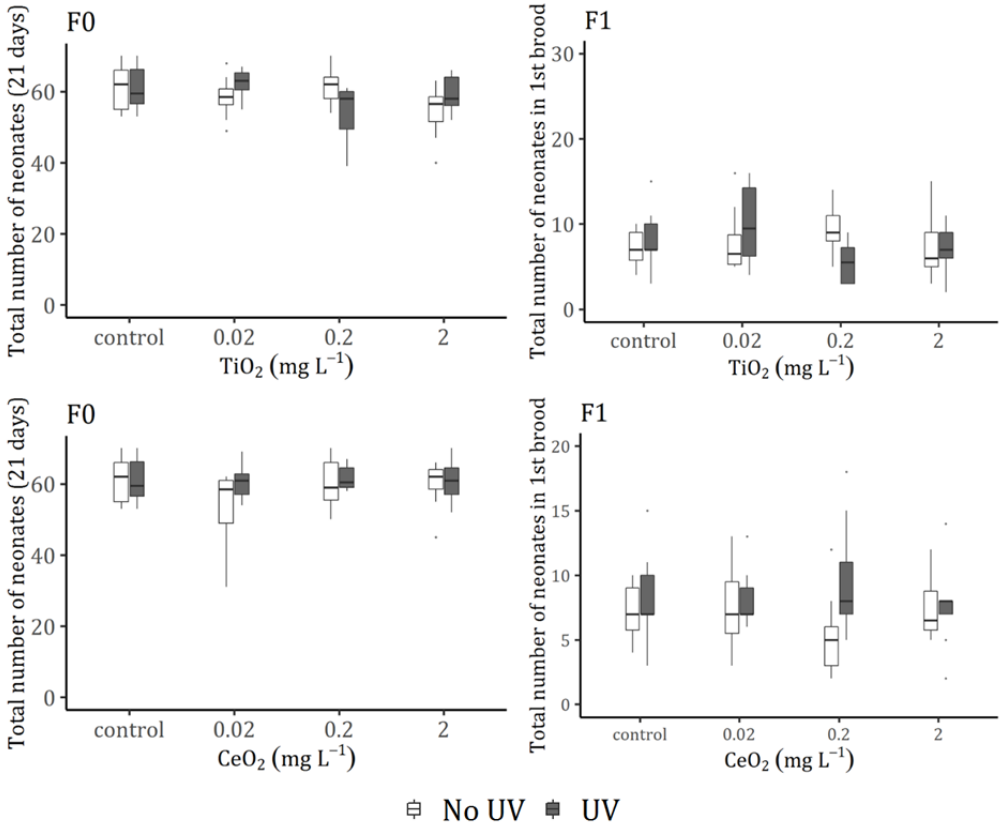

Figure 16. Total number of neonates of *D. magna* produced during the 21 day exposure period of the 1st generation (F0) and in the 1st brood of the second generation (F1). Lines in boxes represent medians for groups, box areas represent upper- and lower quartiles and lines represent the largest/smallest observation that is greater/less than or equal to the upper/lower extremity of the lune + 1.5 * interquartile range.

2.3.8. D. magna body burden

After exposing *D. magna* to CeO_2 ENMs for 24 h following the procedures described in the methods section, the amount of CeO_2 attached to daphnids, or within the organism, was measured by ICP-MS (Table 10).

Table 10: Ce body burden of *D. magna* after exposure to different concentrations of CeO_2 ENMs for 24h and 48 h. Data are expressed as mean ± Standard deviation (n = 3).

CeO_2 ENMs exposure conc. (mg/L)	Exposure time (h)	Ce (µg)/*Daphnia* Washed with Milli-Q water	Ce (µg)/*Daphnia* Washed with EDTA + Milli-Q water	Ce (µg)/*Daphnia* Depurated and Washed with EDTA + Milli-Q water
Control	24	Below detect.	0.000 ± 0.000	0.000 ± 0.000
0.1	24	Not measured	Not measured	0.003 ± 0.003
1	24	0.041 ± 0.003	0.016 ± 0.007	0.045 ± 0.015
10	24	Not measured	Not measured	0.124 ± 0.014
100	24	0.128 ± 0.019	0.199 ± 0.021	0.169 ± 0.009
Control	48	Not measured	Below detect.	0.000 ± 0.000
0.1	48	Not measured	0.004 ± 0.000	0.003 ± 0.000
1	48	0.039 ± 0.019	0.033 ± 0.005	0.010 ± 0.004
10	48	0.099 ± 0.010	0.065 ± 0.022	0.075 ± 0.034
100	48	0.211 ± 0.057	0.155 ± 0.015	0.152 ± 0.030

It is clear that concentrations associated with the daphnids (on the surface or internalized) are directly related to the exposure concentrations. However, washing with EDTA does generally not decrease the measured Ce concentration suggesting this method is not sufficient to remove particles on the carapace of the daphnids. Depuration only decreases the body burden slightly (except for animals exposed to 1 mg/L at 24 h and 10 mg/L for 48 h), again suggesting that the particle sticks to the carapace.

These data will be used to determine the amount of CeO_2 ENMs that can be taken via trophic transfer in future experiments.

2.3.9. D. magna imaging

The distribution of ENMs in externally on the daphnids was evaluated by SEM microscopy (Figure 17). It is clear from the images that the particles are not singularly and evenly dispersed on the *Daphnia* carapace but rather in agglomerated patches, regardless of the exposure concentration. This makes the method unsuitable for external ENMs quantification.

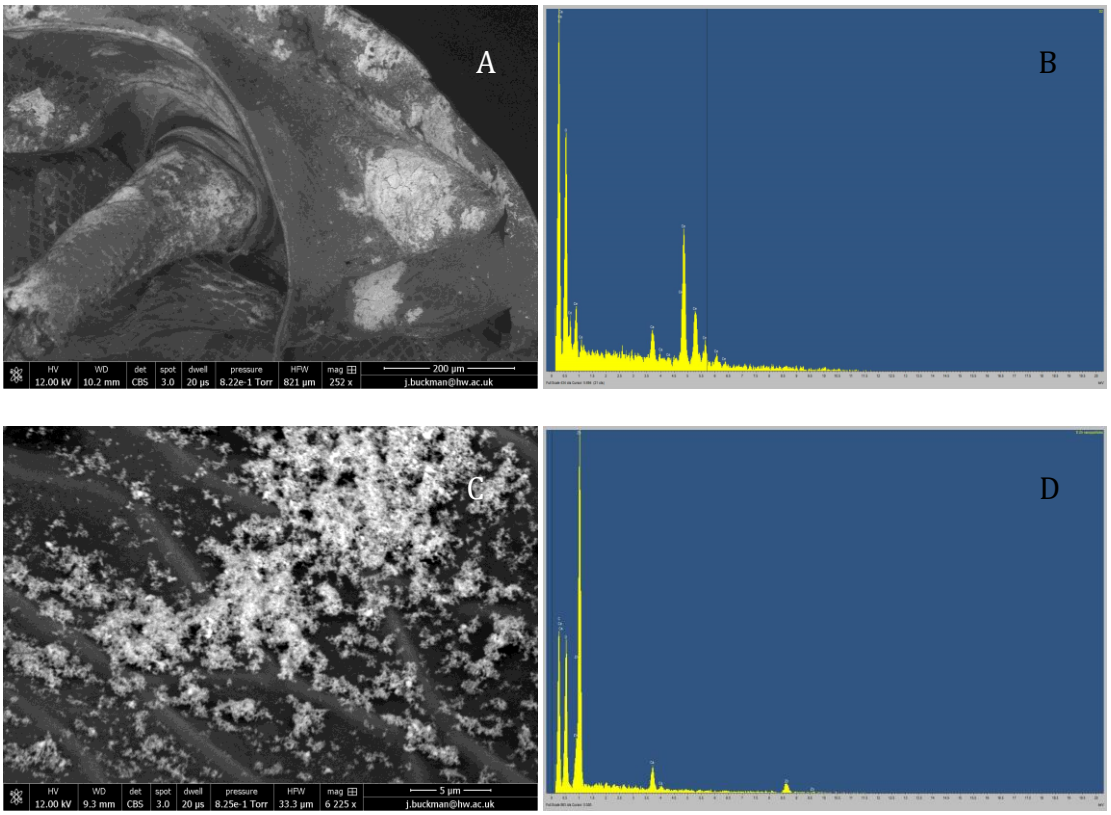

Figure 17: A) SEM image of CeO_2 ENMs on the carapace of *D. magna.* B) Spectrogram of CeO_2 on the carapace of *D. magna.* C) SEM image of ZnO ENMs on the carapace of *D. magna*. B) Spectrogram of ZnO ENMs on the carapace of *D. magna.*

2.3.10. Zebrafish exposures

ZnO ENMs caused mortality in Seasalt (coated ZnO ENMs, LC50 = 13.9 mg ZnO/L (11.1 mg Zn/L); uncoated ZnO ENMs, LC50 = 15.0 mg ZnO/L (12.0 mg Zn/L) and E3 medium (uncoated ZnO ENMs, LC50 = 15.3 mg ZnO/L (12.3 mg Zn/L), but no mortality was observed by either ZnO ENM in OECD medium (Figure 18). It was found that there was no difference in mortality in larvae exposed to either coated or uncoated ZnO ENMs (i.e. similar LC50 values were obtained). $ZnSO_4$ caused mortality in both Seasalt and OECD media, with similar LC50 values obtained (Seasalt LC50: 126.4 mg $ZnSO_4$/L (28.7 mg Zn/L), OECD LC50: 88.5 mg $ZnSO_4$/L (20.1 mg Zn/L)). $ZnSO_4$ was less toxic than ZnO ENMs (when LC50 concentrations are calculated as mg Zn/L). The Zn concentration calculations are based upon molecular percentage and does not take dissolution into account as dissolution values of the ENMs in the different media has not yet been performed (by WP1 partners).

Exposure to CeO_2 ENMs, in Seasalt or OECD media, at concentrations up to 100 mg/L, did not cause any mortality in zebrafish larvae.

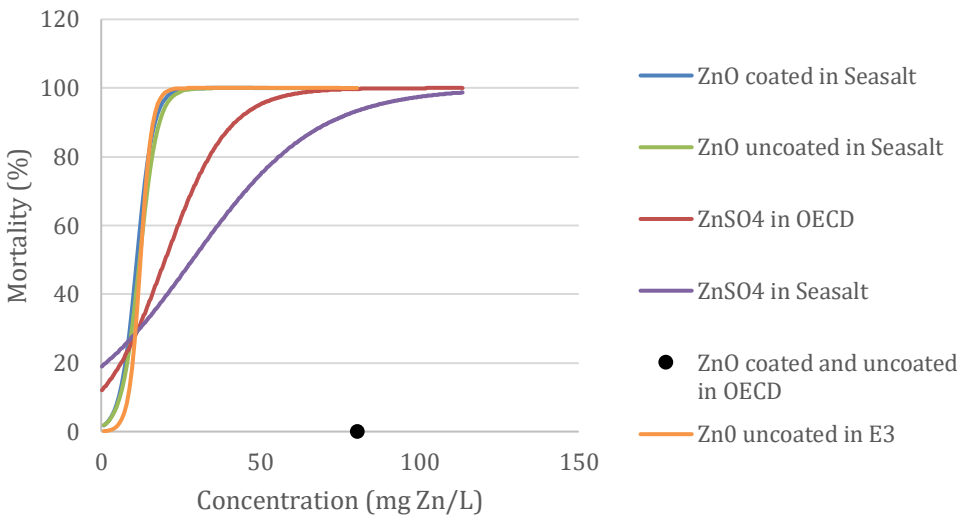

Figure 18: Mortality (%) of zebrafish larvae (72-96 hpf) after exposure to coated and uncoated ZnO ENMs and $ZnSO_4$ in different exposure media. The Zn content is calculated as % of molecular weight and does not take dissolution into account.

2.4. Discussion and Conclusions

Several published studies suggest that the ecotoxicity of ZnO ENMs is mainly due to the dissolved zinc ion. It is therefore necessary to compare the ZnO ENMs to an ionic counterpart like $ZnSO_4$.

Based on these findings it is proposed that the dissolution of Zn ions is the primary driver for ZnO ENM toxicity in algae. Rapid changes in primary size were observed for all the tested ENMs within the first hours of incubation in algal medium. This highlights the need for characterization throughout the experiment to adequately quantify the exposure.

High dissolution was observed for ZnO ENMs at environmentally relevant concentrations (µg/L) while limited dissolution (<10%) was observed for the other ENMs (TiO_2, CeO_2 and $BaSO_4$ ENMs).

It was found that the SP-ICP-MS method was excellent for stable ENMs measuring 2-7 gold ENMs associated with each algal cell. Data interpretation of aggregating ENMs (Tier 1 materials) were more difficult due to assumptions such as spherical shape and constant size. Nonetheless, the method yields direct mass determination on a single cell level which is key for relating subtle effects which is undoubtedly lost if analysing at population level.

ZnO ENMs and $ZnSO_4$ showed comparable acute effects on the basis of Zn content (calculated as % of molecular weight and does not take dissolution into account), in the *D. magna* exposure experiments, and lower toxicity in the zebrafish larvae experiments. In the literature, studies have found similar results (Kim et al 2017, Lin et al 2019). Likewise, Hua et al. (2016), reported that the relative contribution of the ZnO ENMs was higher that the Zn ion in causing toxicity to zebrafish embryos.

The general purpose of surface coating on ENMs is extending their applications or changing their properties such as reducing toxicity, preventing dissolution or aggregation. Given that zinc ions have been proven to play a key role in ZnO ENM toxicity it was hypothesized that the coated ENMs would cause less toxic effects than their uncoated counterparts. Results obtained from both the acute and chronic exposure of *D. magna*, and acute exposure of zebrafish larvae, to coated and uncoated ZnO, however, do not indicate a clear picture regarding the role coatings might have on toxicity. The dissolution data also suggest that there is no difference in dissolution between the two types of ZnO in M7 medium. Based on these results and related literature it is apparent that the relationship between the coating of ENMs and their toxic effects is still unclear.

CeO_2 ENMs showed no toxicity in the *D. magna* and zebrafish acute toxicity tests even at the very high concentration of 100 mg/L. CeO_2 ENMs are not known to release ions.

From the results of acute exposure of zebrafish larvae to different ENMs in different media it is evident that media have a large effect on the toxicity of the particles. One factor that may be contributing to variations in results from different studies may be the test media used. The ionic strength, pH and general chemistry of the test media may influence the dissolution and aggregation of the ENMs. As no Zn dissolution data have been obtained to date from the ZnO ENMs in the different media yet it is not possible to reach clear conclusions from this study on the role of dissolution on the different toxicity of the ZnO ENMs in different media.

The DLS results suggest aggregation of the ENMs in the test medium. This likely results in decreasing bioavailability to the test organisms as discussed in many publications (e.g.Vijver et al. 2018). However, this reflects real exposure scenarios in the environment.

For the multi-generation studies no ENM treatment related effects or interactions with UVR were detected for TiO_2 ENMs and CeO_2 ENMs in either generation on any of the measured endpoints. The majority of previous studies on TiO_2 ENMs and CeO_2 ENMs toxicity in *D. magna* focused on acute exposure scenarios. Comparisons between such studies, but also longer-term exposure studies show that there is large variability between derived effect concentrations, and this is often attributed to differences in the properties (e.g. size, coatings, purity) and preparation (e.g. exposure medium, sonication time) of the ENMs used, or the biological variation between different test populations (see e.g. Handy et al. 2012 for a discussion). Using Representative Test Materials (RTMs) of the Joint Research Centre of the European Commission (JRC) allowed us to obtain results which can be compared with other researchers using the same materials.

Linking exposure concentrations to effects has proven to be challenging in the case of ENMs, and requires analyses of exposure concentrations over time within the test setup. In the current experiment, ICP-MS analysis allowed for the confirmation of a time- and concentration dependent decrease in ENMs concentrations (mass-based) in the water column.

2.5. Continuing work

Results from the uptake experiments with *D. magna* and algae will contribute to the planned study of dietary transfer of CeO_2 ENMs from algae to *D. magna* and from *D. magna* to zebrafish. The low toxicity of these particles and established methods of Ce detection make them ideal for the purpose. The *D. magna* body burden results show that the amount of CeO_2 ENM attached or taken up by *D. magna* is lower but related to exposure concentrations.

Changes in algae pigmentation have previously been proposed as an alternative advanced long term endpoint to consider in algal testing. In WP5 work has been initiated to establish a method for quantifying and analysing for this endpoint. This work is ongoing and may potentially uncover new modes of action for ENMs exhibiting their algal toxicity through other means than release of ions.

Moreover, currently a new SOP for evaluating acute effects of ENMs on daphnids is under development. For the new SOP, an exposure chamber originally designed and proven to work for zebrafish larvae is being modified and tested. The design objective of the exposure chamber is to keep ENMs suspended in the aqueous phase throughout tests (48-h exposure) to enable greater reproducibility of results of ecotoxicity tests. The chamber design has been modified through multiple iterations to ensure that *D. magna,* from age 24 h to 10 days old, are not affected and that ENMs toxicity can be tested.

3. DEVIATION FROM THE WORKPLAN

Characterization of environmental fate and dosimetry linked to WP1 task 1.5 was not provided. This has been discussed within the midterm meeting with the project officer, Carlos-Eduardo Lima da Cunha. It has been shown that ENMs are not stable in the suspensions in regards to their fate, and it is suggested that it is important to measure zeta potential as well hydrodynamic size distribution of particles within exposure samples in parallel with the effect assays. This means that these measurements had to be performed by WP5 partners. Furthermore many chemical measurements (using ICP-MS equipment) were also partly performed by the WP5 partners, which has resulted on some parts of the experiments for sublethal effects, e.g. changes in pigmentation, being delayed. However, in the meantime it was possible to work on activities related to WP5 task 5.5 which was planned to start at M29. So overall no delays have taken place and the end of the project we will have all results as planned in the deliverables.

4. PERFORMANCE OF THE PARTNERS

All partners fulfilled their tasks in a satisfactory time and to the required quality for delivering on the tasks and deliverables outlined above.

5. Conclusions

The Steering Board deems this deliverable to be fulfilled satisfactorily.

6. Annex

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