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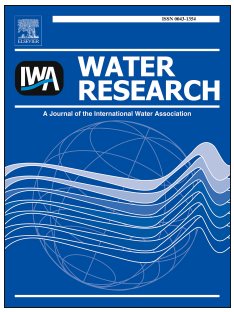

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The uptake and elimination of ZnO and CuO nanoparticles in *Daphnia magna* under chronic exposure scenarios

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Abstract

In this study, the uptake and elimination of ZnO and CuO nanoparticles in *Daphnia magna* was tested. Daphnids were exposed during 10 days to sublethal concentrations of ZnO and CuO nanoparticles and corresponding metal salts ($ZnCl_2$ and $CuCl_2.2H_2O$), after which they were transferred to unexposed medium for another 10 days. At different times during the exposure and none-exposure, the total and internal zinc or copper concentration of the daphnids was determined and the nanoparticles were localized in the organism using electron microscopy. The exposure concentrations were characterized by measuring the dissolved, nanoparticle and aggregated fraction in the medium. The results showed that the ZnO nanoparticles quickly dissolved after addition to the medium. Contrarily, only a small fraction (corresponding to the dissolved metal salt) of the CuO nanoparticles dissolved, while most of these nanoparticles formed large aggregates. Despite an initial increase in zinc and copper concentration during the first 48 hour to 5 day exposure, the body concentration reached a plateau level that was comparable for the ZnO nanoparticles and $ZnCl_2$, but much higher for the CuO nanoparticles (with visible aggregates accumulating in the gut) than $CuCl_2.2H_2O$. During the remaining exposure and subsequent none-exposure phase, the zinc and copper concentration decreased fast to concentrations comparable with the unexposed daphnids. The results indicate that *D. magna* can regulate its internal zinc and copper concentration after exposure to ZnO and CuO nanoparticles, similar as after exposure to metal salts. The combined dissolution, accumulation and toxicity results confirm that the toxicity of ZnO and CuO nanoparticles is caused by the dissolved fraction.

Keywords: nano, zinc, copper, dissolution, aggregation; electron microscopy

1. Introduction

Metal oxide nanoparticles exhibit specific physical and chemical properties as a result of their small sizes (1 – 100 nm). These specific properties make metal oxide nanoparticles useful for application in many household and industrial products. The last decade, a drastic increase in the production and use of CuO and ZnO nanoparticles has occurred. As such, ZnO nanoparticles are being widely used in sunscreens, cosmetics, paints, plastics (Ma et al., 2013), while some applications of CuO nanoparticles include gas sensors (Chowdhuri et al., 2004), batteries (Zhang et al., 2005), plastics and metallic coatings (Hernández Battez et al., 2010). This increased application of nanoparticles has caused environmental concerns since their specific physical and chemical properties may cause them to behave reactive in aquatic and other environmental compartments.

It is known that metal oxide nanoparticles entering the aquatic environment behave in a highly dynamic manner. As such, ZnO and CuO nanoparticles have been shown to dissolve (Kasemets et al., 2009; Mortimer et al., 2010) and aggregate (Jo et al., 2012; Keller et al., 2010; Zhao et al., 2011) rapidly. The dissolution of nanoparticles depends on factors such as the exposure concentration (Li and Wang, 2013), chemical composition, nanoparticle size (e.g. smaller particles have been shown to dissolve faster (Bian et al., 2011; David et al., 2012)) and water chemistry (Li and Wang, 2013). The formation of aggregates depends largely on the surface charge of the nanoparticles, which can be influenced by the water chemistry as well. If all

nanoparticles have a high negative or positive charge, they will repel each other. Contrarily, nanoparticles tend to aggregate when the surface charge is low (Bagwe et al., 2006). As a result of these dynamics, aquatic organisms are not only exposed to nanoparticles but also to their dissolution and/or aggregation products.

ZnO and CuO have been shown to be toxic to different aquatic species (Adam et al., 2014b; Aruoja et al., 2009; Baek and An, 2011; Chen et al., 2011). However, up till now there is still some controversy on which mechanisms cause the observed toxicity in aquatic species. Due to the nanoparticle dynamics (dissolution and aggregation), it is possible that multiple mechanisms may be responsible for the observed toxicity. However, these mechanisms also largely depend on the organism that is exposed.

When exposing the filter feeder *D. magna* to ZnO and CuO nanoparticles, these nanoparticles or their derivates may adsorb on the carapace or may be taken in by the organisms. The nanoparticles or their aggregates, attaching to the carapace may cause hinder to the daphnids. The intake may include the uptake of toxic ions, dissolved from the nanoparticles. Toxic ions can be taken up by ion channels (passive) or by ion pumps (active) located in the membranes of gill epithelial cells (Bianchini and Wood, 2008; Simkiss and Taylor, 1989). Several authors have suggested that the toxicity of ZnO (Adam et al., 2014b; Franklin et al., 2007; Heinlaan et al., 2008) and CuO (Aruoja et al., 2009; Heinlaan et al., 2008) nanoparticles to this species is caused by the released free metal ions. It is also possible that nanoparticles or nanoparticle aggregates are ingested by the daphnids. This ingestion of nanoparticle aggregates is possible through the

filter feeding mechanism of *D. magna*, with average filter mesh sizes of 0.4 – 0.7 µm (Gophen and Geller, 1984). Nanoparticle aggregates that are taken in may occur as dispersed nanoparticles or aggregates in the gut or dissolve in the gut or in the cells (e.g. after uptake by endocytosis) due to lower pH values. Subsequently, the nanoparticles (or their derivates) can either become incorporated or eliminated from the body. Under acute exposure scenarios, ZnO (Li and Wang, 2013) and CuO (Adam et al., 2014a; Heinlaan et al., 2011) nanoparticles have been shown to be ingested by *D. magna*. Under these exposure conditions, CuO nanoparticles occurred in the gut as dispersed particles but were not able to penetrate the epithelial cells (Adam et al., 2014a; Heinlaan et al., 2011) and ZnO nanoparticles, which were expected to dissolve in the gut, were eliminated fast from the daphnids, after a 30 min exposure to the nanoparticles (Li and Wang, 2013).

Despite the current knowledge on the acute uptake and elimination of ZnO and CuO nanoparticles, it remains unclear whether under long-term exposure scenarios, nanoparticles are ingested or attach to the outside of *D. magna* and can become incorporated in the body or are eliminated by this species. Therefore, in the current study, the chronic uptake and elimination of ZnO and CuO nanoparticles was studied in *D. magna*. The uptake and elimination of nanoparticles (or its aggregated or dissolved form) was characterized by measuring total and internal metal concentrations. Electron microscopic techniques were used to localize the nanoparticles in the daphnids. To characterize the nanoparticle specific effect, parallel exposures were run with corresponding metal salts. It can be hypothesized that, similar as

under acute exposure conditions, ZnO and CuO nanoparticles can be ingested and eliminated by *D. magna* when exposed under long-term exposure conditions.

2. Methods

2.1. Tested nanoparticles and metal salts

Different types of ZnO and CuO nanoparticles and their corresponding metal salts were tested. A ZnO nanodispersion (NanoTek 40 weight % in water colloidal dispersion, Alfa Aesar Germany, 40 nm) and nanopowder (NanoSun, Micronisers PTY Australia, 30 nm) were compared with $ZnCl_2$ (Sigma-Aldrich Belgium, ≥98 %). The tested CuO nanopowder (Sigma-Aldrich Belgium, <50 nm) was compared with $CuCl_2.2H_2O$ (ICN Biomedicals Belgium). The size and shape of the nanoparticles were characterized by transmission electron microscopy (FEI Philips CM30 equipped with a Gatan imaging filter).

2.2. Test species

The freshwater crustacea *Daphnia magna* was used as a test species. Daphnids were reared in bio-filter treated tap water (pH 8.4 – 8.5, conductivity 513 µS/cm) at 20 °C under a constant light-dark cycle (14 h light – 10 h dark). The water was refreshed three times a week and the daphnids were fed with 4 x 10^5 algae cells/ml (*Raphidocelis subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio; the added volumes were calculated based on measured (Multisizer 3 Coulter Counter; Beckman Coulter) algae concentrations in algae stock solutions).

2.3. Exposure of *Daphnia magna* to nanoparticles and metal salts

125 A 20 day chronic experiment, including an exposure and none-exposure phase, was performed.
126 Juvenile *Daphnia magna* (<24 h) were exposed to the nanoparticles and metal salts during 10
127 days in OECD recommended ISO test medium ($CaCl_2.2H_2O$: 0.294 g/l, $MgSO_4.7H_2O$: 0.123 g/l,
128 $NaHCO_3$: 0.065 g/l, KCl: 0.006 g/l, water hardness 250 mg $CaCO_3$/l, pH 7.8 – 8.2, conductivity
129 617 µS/cm (OECD, 2004)), after which they were transferred to clean test medium for another
130 10 days. During the first 10 days, daphnids were exposed to the earlier determined chronic
131 nominal EC_{50} concentrations for reproduction of the ZnO nanodispersion (0.064 mg Zn/l), ZnO
132 nanopowder (0.137 mg Zn/l), $ZnCl_2$ (0.096 mg Zn/l), CuO nanopowder (1.04 mg Cu/l) and
133 $CuCl_2.2H_2O$ (0.02 mg Cu/l) (unpublished data). For this, stock solutions of 50 mg/l nanoparticles
134 (100 ml for the ZnO nanodispersion; 200 ml for the ZnO and CuO nanopowder) or metal salt
135 (200 ml) were freshly prepared in ISO test medium from the dispersion or dry powder. The
136 nanoparticle stock solutions were sonicated for 30 min in a sonication bath (Branson 2510) to
137 obtain optimal particle dispersion (Chowdhury et al., 2010), while the metal salt stocks were
138 not sonicated. Small volumes of these stocks were added to ISO medium to obtain the above
139 mentioned concentrations in a starting volume of 1900 ml in plastic (polypropylene) beakers in
140 triplicate. The blanks (unexposed) were also run in triplicate. Per beaker, 190 daphnids were
141 added (10 ml per daphnid). The daphnids were fed on the algae species *Raphidocelis*
142 *subcapitata* (4×10^5 cells/ml). Every 48 hours, the daphnids were transferred to freshly spiked
143 (during exposure) or clean (during none-exposure, including the blanks) medium (10
144 ml/daphnid) and fed.
145

146 The exposure concentrations were measured directly after addition of the daphnids (to which
147 we will refer to as 0 hours; 1 to 2 hours after spiking of the stock solutions) and 48 hours later in
148 the ISO medium. Unfiltered, 450 nm syringe filtered (Acrodisc PP, Pall life sciences), 100 nm
149 syringe filtered (Puradisc PTFE, Whatman) and 3 kDa ultrafiltered (Microsep centrifuge filters
150 Pall Life Sciences) using a 1 h centrifugation at 7500 g (Beckman Avanti J25; time and maximal
151 centrifugal force as indicated by the manufacturer) samples were taken from three replicates
152 for the different exposures. All samples were taken from the water column. As a result,
153 nanoparticle aggregates precipitated to the bottom of the vessel were not included. After
154 acidification to 1 % HNO_3, the Zn or Cu concentration of the different unfiltered and filtered
155 samples was measured by ICP-MS (Thermo Scientific Element 2 XR) or ICP-OES (Thermo
156 Scientific 6000 series). Physicochemical parameters such as pH, temperature, O_2 were
157 measured regularly during the experiment (Hach HQ30d-flexi). At different times during the
158 exposure and none-exposure phase, daphnids were sampled from the medium to determine
159 the metal body concentrations and for the electron microscopic localization of the
160 nanoparticles in the daphnids.
161
162 2.4. Uptake and elimination of nanoparticles and metal salts in *Daphnia magna*
163 After 0 h, 24 h, 48 h, 5 days and 10 days during the exposure and none-exposure phase, 20
164 surviving daphnids were sampled from the different replicates of each treatment. These were
165 washed for a few seconds in pure water to wash of the surrounding exposure medium. Ten of
166 these daphnids were used for length determination by measuring the distance from the head to
167 the apical spine (microprojector, Projectina), after which they were put in 1.5 ml bullet vials.

168 The other ten daphnids were washed in 5 mM Na_2EDTA for 20 minutes to remove externally
169 bound nanoparticles, aggregates or inorganic metal species. After washing quickly in pure water
170 to remove the EDTA, the daphnids were put in bullet vials. All vials were placed in a dry oven at
171 60 °C for at least 48 h until a constant dry weight. To each vial, containing dried daphnids, 50 µL
172 HNO_3 (69 %) and (after 12 hours) 50 µL H_2O_2 (30 %) was added. The daphnids were dissolved
173 four hours later by microwave digestion (4 min 100 W, 3 min 180 W, 2 min 180 W, 2 min 300
174 W, 2 min 300 W, 2 min 450 W; Samsung combi CST1660ST) (Blust et al., 1988), after which the
175 samples were diluted to 1 – 2 % HNO_3. The internal zinc or copper concentration (washed with
176 Na_2EDTA) and total zinc or copper concentration (not washed with Na_2EDTA) of the daphnids
177 was measured by ICP-MS (Thermo Scientific Element 2 XR). In this study, the internal metal
178 concentration includes all zinc or copper inside *D. magna*. The total metal concentration
179 includes the internal metal concentration and the metal attached to the outside of the
180 daphnids. The metal body concentration is expressed in terms of dry weight. The dry weight of
181 the daphnids was obtained by extrapolation from the measured length (using the formula:
182 weight = 0.0028 x length$^{3.6819}$, as experimentally determined for the daphnids in our culture see
183 Appendix A).
184
185 2.5. Electron microscopic localization of nanoparticles in *Daphnia magna*
186 After 10 days of exposure to the ZnO and CuO nanoparticles, 4 surviving daphnids were
187 sampled from the different replicates of each treatment. The daphnids were rinsed for a few
188 seconds in pure water, after which they were directly placed overnight in fixation buffer (21 g/l
189 sodium cacodylate, 1/10 dilution of glutaraldehyde (25 %), 1/10 dilution of paraformaldehyde

(20 %), 500 mg/l $CaCl_2.2H_2O$, pH 7.4). Subsequently they were washed three times for 15 min with rinsing buffer (21 g/l sodium cacodylate, 500 mg/l $CaCl_2.2H_2O$, 75 g/l sucrose, pH 7.4). The *D. magna* samples were maintained at 4 °C until further analysis. Dehydration of the daphnids was done with ethanol (15 min 50 %, 15 min 70 % x2, 20 min 90 %, 15 min 100 % x3), after which they were washed in propylene oxide (100 %, 3x 1 h). Subsequently, the daphnids were impregnated in epoxy resin (24 h, Spurr's low viscosity resin) which was polymerized in an oven at 60 °C. Ultrathin slices (100 nm) were cut with an ultramicrotome (Leica UC7; with a histo diamond knife (Diatome)) through the main organs and were studied by Scanning Transmission Electron Microscopy (STEM: FEI Tecnai F20, Fischione annular detector type 3000; equipped with an energy-dispersive (EDX) X-ray detector) to visualize the nanoparticles in *D. magna*. Images were acquired using a Fischione annular detector in STEM mode.

2.6. Data analysis and statistics

GraphPad Prism (version 6) was used for data visualization and statistics. One-way ANOVA tests were performed to test for significant differences in length between the exposed and blank daphnids. The differences in zinc or copper concentrations obtained by the different filtrations were compared in a one-way ANOVA, with Tukey´s post test. Two-way ANOVA tests (with Tukey's post test) were done to test simultaneously for significant differences in the internal (samples washed with EDTA) and total (samples not washed with EDTA) zinc (or copper) concentration between the exposed and blank daphnids and for the effect of exposure time and for the interaction between the exposure and exposure time. The Tukey´s post tests were used to test for significant differences in the internal and total zinc (or copper) concentration

between the exposed and blank daphnids at each time point. The uptake and elimination data were modelled using first-order kinetic models. The uptake (increase in total and internal zinc or copper concentration) was fitted using equation 1 (with C_t the concentration in *Daphnia* at time t; C_0 the concentration in *Daphnia* at time 0; C_m the concentration in the medium; k_u the uptake rate constant and k_e the elimination rate constant during the uptake-phase; t the time of uptake). The elimination (decrease in total and internal zinc or copper concentration) was fitted using equation 2 (with C_t the concentration in *Daphnia* at time t; C_i the initial zinc or copper concentration of *Daphnia*; k the elimination rate constant during the elimination-phase; t the time of elimination) (Ardestani et al., 2014; Newman and Unger, 2003).

$$\text{Equation 1: } C_t = C_0 + C_m\left(\frac{k_u}{k_e}\right)(1 - e^{-k_e t})$$

$$\text{Equation 2: } C_t = C_i\, e^{-kt}$$

3. Results

3.1. Nanoparticle characteristics

The size and shape of the metal oxide nanoparticles were characterized by electron microscopy (Fig. 1). The measured average sizes with standard deviations were 19.1 ± 4.5 nm for the ZnO nanopowder (Fig. 1a), 39.2 ± 22.3 nm for the ZnO nanodispersion (Fig. 1b) and 21.3 ± 10.2 nm for the CuO nanopowder (Fig. 1c). The ZnO nanodispersion showed large differences in size and shape of the nanoparticles. The other nanoparticle types consisted of more homogenous, mostly round, particles.

232

233 3.2. Exposure of *Daphnia magna* to nanoparticles and metal salts

234 The zinc concentrations after filtration over a 3 kDa, 100 nm and 450 nm filter and in the
235 unfiltered samples are presented for the ZnO nanopowder (Fig. 2a), ZnO nanodispersion (Fig.
236 2b) and $ZnCl_2$ (Fig. 2c) after 0 and 48 hours of exposure. Higher zinc concentrations were
237 measured in the 3 kDa and 100 nm filtered samples than in the 100 nm and 450 nm filtered
238 samples. These concentration differences are consistent in the different nanoparticle and metal
239 salt exposures. Since zinc salt is known to completely dissolve under these conditions, the zinc
240 salt exposure can serve as a reference for the nanoparticle exposures. Most of the ZnO
241 nanoparticles from the nanodispersion dissolved instantly in the medium, with full dissolution
242 measured after 48 hours of exposure. For the ZnO nanopowder, somewhat different results
243 were obtained. Directly after exposure (0 hours) only 66.8 % (with min: 63.1 – max: 69.9 %) of
244 the nanoparticles was dissolved. At this time, nanoparticle aggregates larger than 450 nm were
245 still present in the medium. However, within 48 hours of exposure, these aggregates had
246 completely dissolved.

247

248 The copper concentrations were measured in the filtered (3 kDa, 100 nm, 450 nm) and
249 unfiltered samples after 0 hours and 48 hours of exposure to the CuO nanopowder (Fig. 3a) and
250 Cu salt (Fig. 3b). The copper salt dissolved instantly in the medium. Directly after spiking (0
251 hours of exposure), only a small fraction of the nanoparticles was dissolved (on average 0.63 %
252 with min: 0.43 % – max: 0.83 %; 0.0069 ± 0.0022 mg Cu/l). This dissolved fraction stayed
253 constant throughout the exposure (0.99 % with min: 0.79 – max: 1.13 %; 0.0079 ± 0.0001 mg

254 Cu/l after 48 hours of exposure) and corresponded very well with the dissolved fraction of the
255 copper salt (0.0075 ± 0.0001 mg Cu/l at 0 hours, 0.0079 ± 0.0001 mg Cu/l at 48 hours, fraction
256 passing through a 3 kDa filter). Upon entering the medium (0 hours), most of the CuO
257 nanoparticles formed aggregates with sizes larger than 450 nm. During the exposure, the
258 nanoparticles remained aggregated with visual precipitation of aggregates on the bottom of the
259 exposure vessels after 48 hours.
260
261 In the zinc experiment, the average pH was 7.91 ± 0.18, while the oxygen and temperature
262 were 8.42 ± 0.05 mg/l (97.7 ± 0.38 %) and 19.3 ± 0.5 °C. Similar values were found in the copper
263 experiment. Here pH, oxygen and temperature were 7.95 ± 0.15, 8.29 ± 0.29 mg/l (91.8 ± 3.69
264 %), 19.6 ± 0.6 °C.
265
266 3.3. Uptake and elimination of nanoparticles and metal salts in *Daphnia magna*
267 The length of the daphnids (mm) is given for the (unexposed) blank daphnids and the ones
268 exposed to the ZnO nanopowder, nanodispersion and $ZnCl_2$ (Fig. 4a) during the 20 day
269 experiment. A clear increase in length could be seen during the first 10 days of exposure, while
270 after 10 days, the daphnids had reached their adult size and did no longer grow during the next
271 10 days. No significant differences were observed in length between the blank and exposed
272 daphnids (one-way ANOVA indicated no significant differences at most time points). For the
273 copper exposures the *D. magna* lengths are presented in Fig. 4b. Similar to the zinc exposure,
274 an increase in length could be seen during the first 10 days of exposure while afterwards the

lengths stayed constant. Here as well, no effect of the exposure (nanoparticle or metal salt) could be seen on the *Daphnia* length.

An initial increase in *D. magna* zinc concentration was observed during the 10 day exposure to the ZnO nanopowder, nanodispersion and $ZnCl_2$ (Fig. 5). During this period, both the total and internal (which were not significantly different) concentrations of zinc increased. Afterwards, a fast decrease in the *Daphnia* zinc concentration was observed. First-order kinetics (curves for uptake and elimination) are indicated on the different graphs. The uptake and elimination rate constants of these curves are indicated in Tab. 1.

For the daphnids exposed to the ZnO nanopowder (Fig. 5b), a high increase in zinc concentration was observed during the first 48 hours of exposure with the internal and total zinc concentrations reaching plateau levels up to 0.42 ± 0.06 and 0.50 ± 0.27 µg Zn/mg dry weight (two-way ANOVA with Tukey's post test indicating significant differences between the nanopowder exposures and blanks for internal and total zinc after 48 hours of exposure). Afterwards, the *Daphnia* zinc concentration decreased but significant differences from the blank were still observed after 5 days and 10 days (two-way ANOVA with Tukey's post tests indicating significant differences between the blank and exposure for internal and total zinc at both time points) of exposure. During the none-exposure phase, the zinc concentrations decreased to similar concentrations as in the unexposed daphnids. After 24 hours of none-exposure, Tukey's post tests indicated no more significant differences in zinc concentration between the daphnids previously exposed to the nanopowder and the unexposed blanks. The kinetic models indicate total zinc uptake rate constants and elimination rate constants of

297 0.1061 ml.(mg dry weight.h)$^{-1}$ and 0.0379 h^{-1} during the uptake phase and elimination rate
298 constants of 0.0044 h^{-1} during the elimination phase.
299
300 For the daphnids exposed to the ZnO nanodispersion (Fig. 5c), a steady increase in zinc
301 concentration occurred during the first 5 days of exposure (two-way ANOVA with Tukey's post
302 tests indicated significant differences between the exposed and blank daphnids in internal zinc
303 concentration after 48 hours of exposure and in internal and total zinc concentration after 5
304 days of exposure), reaching maximal internal and total zinc concentrations of 0.22 ± 0.06 and
305 0.27 ± 0.08 µg Zn/mg dry weight. After 10 days of exposure the total and internal zinc
306 concentrations decreased, but were still significantly higher than the zinc concentration in the
307 blank daphnids (two-way ANOVA with Tukey's post tests indicating significant differences
308 between the blanks and exposures for internal and total zinc). During the none-exposure phase,
309 the concentration of the previously exposed daphnids decreased to concentrations similar as in
310 the blank daphnids (with no significant differences after 24 hours of none-exposure indicated
311 by two-way ANOVA with Tukey's post tests). Based on the total zinc concentrations, uptake rate
312 constants and elimination rate constants of 0.0201 ml.(mg dry weight.h)$^{-1}$ and 0.0107 h^{-1} during
313 the uptake phase and elimination rate constants of 0.0028 h^{-1} during the elimination phase
314 were obtained.
315
316 For the zinc salts (Fig. 5d) a comparable pattern of uptake and elimination was observed. During
317 the first 5 days of exposure, the zinc concentration increased and reached maximal internal and
318 total concentrations of 0.28 ± 0.03 and 0.31 ± 0.01 µg Zn/mg dry weight. Significant differences

319 between the zinc concentration in the exposure and the blank were observed after 24 hours
320 (two-way ANOVA with Tukey's post tests indicating significant differences between the blank
321 and exposed daphnids for total zinc concentration), 48 hours and 5 days (two-way ANOVA with
322 Tukey's post tests indicating significant differences between the blank and exposed daphnids
323 for internal and total zinc concentration at both time points) of exposure to the metal salt. After
324 10 days, the zinc concentration decreased but was still significantly different from the zinc
325 concentration in the blank daphnids (two-way ANOVA with Tukey's post tests indicating
326 significant differences between the blank and exposed daphnids for internal and total zinc
327 concentration). Afterwards, the zinc concentration decreased within 24 hours of none-exposure
328 and reached concentrations as low as in the blank daphnids (two-way ANOVA with Tukey's post
329 tests indicating no significant differences between the exposures and blanks). Uptake rate
330 constants and elimination rate constants of 0.1009 ml.(mg dry weight.h)$^{-1}$ and 0.0741 h^{-1} during
331 the uptake phase and elimination rate constants of 0.0036 h^{-1} during the elimination phase
332 were obtained for the modelled total zinc data.
333
334 An initial increase in the internal and total copper concentration (with no significant difference
335 between internal and total concentration) of the daphnids was observed during the exposure to
336 the CuO nanopowder and Cu salt (Fig. 6). During the subsequent part of the exposure and
337 none-exposure period, a decrease in the copper concentration was observed. The modelled
338 first-order kinetics are indicated on the graphs, while the corresponding rate constants are
339 indicated in Tab. 1.
340

341 During the 10 day exposure to the CuO nanopowder (Fig. 6b), maximum internal (6.03 ± 1.63 µg
342 Cu/mg dry weight) and total (6.20 ± 1.03 µg Cu/mg dry weight) copper concentrations were
343 reached after 5 days. Significant differences from the blank (Fig. 6a) were observed after 24
344 hours (two-way ANOVA with Tukey's post tests indicating significant differences in total copper
345 concentration between the blank and exposure), 48 hours and 5 days (two-way ANOVA with
346 Tukey's post tests indicating significant differences in internal and total copper concentration
347 between the blank and exposure). After 10 days of exposure, these concentrations were lower
348 (two-way ANOVA with Tukey's post tests still indicating significant differences in total copper
349 concentration between the blank and exposure). During the none-exposure phase, the copper
350 concentration decreased fast to concentrations comparable with the blank daphnids (two-way
351 ANOVA with Tukey's post tests indicating no significant differences with the blank after 24
352 hours of none-exposure). Based on the kinetic models, total copper uptake rate constants and
353 elimination rate constants of 0.0602 ml.(mg dry weight.h)$^{-1}$ and 0.0037 h^{-1} during the uptake
354 phase and elimination rate constants of 0.0095 h^{-1} during the elimination phase were obtained.
355
356 During the 10 day exposure of the daphnids to the copper salt (Fig. 6c), an increase in internal
357 (maximum at 0.73 ± 0.07 µg Cu/mg dry weight) and total (maximum at 0.95 ± 0.08 µg Cu/mg
358 dry weight) copper concentration was observed during the first 48 hours of exposure (two-way
359 ANOVA with Tukey's post tests indicating significant differences in internal and total copper
360 concentration between the blank and exposure after 24 and 48 hours of exposure). After 5 days
361 and 10 days (two-way ANOVA with Tukey's post tests indicating significant differences in
362 internal and total copper concentration between the unexposed and exposed daphnids) of

363 exposure, the copper concentration dropped and kept decreasing during the none-exposure
364 phase, reaching similar concentrations as in the blank daphnids (Tukey's post tests indicating no
365 significant differences between the exposed daphnids and blanks after 24 hours of none-
366 exposure). Uptake rate constants and elimination rate constants of 0.8284 ml.(mg dry
367 weight.h)$^{-1}$ and 0 h^{-1} during the uptake phase and elimination rate constants of 0.0080 h^{-1}
368 during the elimination phase were obtained for the modelled total copper data.
369
370 3.4. Electron microscopic localization of nanoparticles in *Daphnia magna*
371 In the different ZnO nanoparticle exposures, no nanoparticles could be localized. However, CuO
372 nanoparticles could be seen in the gut of *D. magna* (Fig. 7) after 10 days of exposure to 1.10 ±
373 0.02 mg Cu/l. In the electron microscopic image, the epithelium with microvilli and the gut
374 lumen can be seen on the left (Fig. 7a) while gut lumen with debris can be seen on the right
375 (Fig. 7b). It is clearly visible that nanoparticle aggregates (visible as white aggregates) are
376 situated in the gut lumen. However, these nanoparticles were not visible in the epithelial cells.
377 The EDX spectrum (Fig. 7c) confirms that it are indeed CuO nanoparticles in the gut and not
378 artefacts occurring as a result of the *Daphnia* treatment.
379

380 4. Discussion

381 4.1. Exposure conditions, uptake, elimination and internalization of ZnO nanoparticles in *D.*
382 *magna*
383 As expected, $ZnCl_2$ was completely dissolved when added to the exposure solution. After 0
384 hours of exposure, a large fraction of the ZnO nanoparticles was already dissolved in the

exposure medium. As such, most of the nanodispersion dissolved instantly. At this time (1 to 2 hours after spiking of the stock solution) lower dissolution was observed for the nanopowder (on average 66.8 % with min: 63.1 – max: 69.9 %). Initially, some of the nanoparticles from the nanopowder aggregated to large sizes (> 450 nm). In our previous study (Adam et al., 2014b) similar aggregation processes were observed when exposed under the same conditions as in the current study. As a result, the dynamic light scattering results (measured average aggregate sizes of 244 - 280 nm) from Adam et al. (2014b) are expected to give a good indication of the initial aggregation sizes of the ZnO nanopowder in the current study. Factors that can explain this initial aggregation in our exposure include pH (Dunphy Guzman et al., 2006) and ionic strength (Zhou and Keller, 2010). When the pH reaches the point of zero charge, the overall surface charge of the nanoparticles is zero and as a result the particles will no longer repel each other and aggregate. The ionic strength was 0.0119 M in our exposure. Zhou and Keller (2010) have shown that at values above 0.01 M, aggregation of nearly spherical ZnO nanoparticles (of 20 nm) was induced, with aggregates of more than 300 nm directly after spiking to about 1350 nm after 170 min of exposure. During the exposure, the nanoparticle aggregates started to dissolve and after 48 hours of exposure, no aggregates occurred in the exposure medium. The high initial dissolution and complete dissolution after 48 hours of exposure was as expected due to the low chronic exposure concentrations used, similar to Adam et al. (2014b). As a result, the daphnids exposed to the ZnO nanopowder were exposed for a large part to dissolved zinc and to a small fraction of aggregated particles, while the daphnids exposed to the ZnO nanodispersion were mostly exposed to dissolved zinc and the daphnids exposed to the zinc salt were solely exposed to dissolved zinc.

407

408 During the 20 day uptake and elimination experiment, an initial increase and subsequent
409 decrease of the total and internal zinc was observed in the daphnids exposed to the ZnO
410 nanoparticles and the metal salts. The zinc from the ZnO nanoparticles and $ZnCl_2$ attaching to
411 the outside carapace was negligible, compared to the amount of internal zinc. This high
412 ingestion of ZnO nanoparticles in *D. magna,* compared to a negligible adsorption onto the
413 carapace was also described by Li and Wang (2013). The zinc concentration of the daphnids
414 increased similarly during the first 5 days of exposure to comparable concentrations for the ZnO
415 nanodispersion (0.064 mg Zn/l) and the $ZnCl_2$ (0.096 mg Zn/l). During the subsequent exposure,
416 the zinc body burden decreased. Since the nanoparticles of the ZnO dispersion dissolved fast
417 and the uptake and elimination of zinc was very similar with $ZnCl_2$, no nanoparticle specific
418 uptake appears to occur. The daphnids exposed to higher ZnO nanopowder concentrations
419 (0.137 mg Zn/l) reached higher maximum zinc concentrations after 48 hours of exposure,
420 followed by a decrease of the zinc concentration during the subsequent exposure. Since the
421 dissolution rate of the aggregated ZnO nanoparticles from the nanopowder is lower than that
422 of the nanodispersion and the metal salt and its initial zinc concentration is higher, it is possible
423 that the ZnO aggregates were ingested. However, even if these aggregates were ingested, they
424 did not cause any additional toxicity. In addition, the very similar uptake rates for zinc from the
425 ZnO nanopowder (0.1061 ml.(mg dry weight.h)$^{-1}$) and zinc salt (0.1009 ml.(mg dry weight.h)$^{-1}$)
426 also suggests no nanoparticle specific uptake. Slightly lower average uptake rate constants
427 (0.045 and 0.051 ml.(mg dry weight.h)$^{-1}$) for zinc in *D. magna* were observed by Yu and Wang
428 (2002) and Komjarova and Blust (2009a). A recent study (Adam et al., 2014a) indicated that

429 when *D. magna* was exposed during 48 hours to acute concentrations (immobilization EC_{50}) of
430 the ZnO nanodispersion (1.70 ± 0.05 mg Zn/l), ZnO nanopowder (1.78 ± 0.02 mg Zn/l) and zinc
431 salt (1.88 ± 0.09 mg Zn/l), a similar increase in total zinc was also observed for the nano (3.87 ±
432 1.31 µg Zn/mg dry weight for nanodispersion; 4.23 ± 2.51 µg Zn/mg dry weight for
433 nanopowder) and zinc salt exposure (2.62 ± 1.39 µg Zn/mg dry weight). The results of this study
434 (Adam et al., 2014a) indicate that, similar as in the current study, no nanoparticle specific
435 uptake was observed. The initial increase in zinc concentration in the current study can be
436 explained by the active accumulation of essential metals for the metabolic requirements of the
437 daphnids (Muyssen and Janssen, 2002). During the initial exposure (first 48 hours or 5 days of
438 exposure), the daphnids grew (Fig. 4a) and took in zinc as an essential element. However, after
439 this period, the internal zinc concentrations reached maximum values (0.499 ± 0.265 mg Zn/mg
440 dry weight when exposed to nanopowder; 0.266 ± 0.080 mg Zn/mg dry weight when exposed
441 to nanodispersion; 0.312 ± 0.014 mg Zn/mg dry weight when exposed to $ZnCl_2$). It has been
442 shown that zinc becomes toxic to daphnids at a zinc body content of ≥ 0.468 ± 0.080 mg Zn/mg
443 dry weight (Muyssen and Janssen, 2002). To limit excessive accumulation of zinc, the daphnids
444 try to keep their body content below this concentration by regulating their internal zinc
445 concentration, either by elimination or by lowering the uptake of zinc. This can be seen after 5
446 days (nanopowder) and 10 days (nanodispersion, $ZnCl_2$) of exposure. *D. magna* has been shown
447 capable to actively regulate its zinc concentration up to 0.6 mg Zn/l (Muyssen and Janssen,
448 2002). Also in the case of acute exposures to ZnO nanoparticles (0.5 and 2 mg/l), an active
449 regulation of zinc has been shown (Li and Wang, 2013). The elimination of zinc by the daphnids
450 has been shown to be regulated by molting, in which the exoskeleton occurs as a metal sink

451 which is removed after molting (Muyssen and Janssen, 2002). This can explain for a large part
452 the decrease in zinc concentration of the daphnids. During the none-exposure phase, the
453 *Daphnia* zinc concentration decreased fast (with similar elimination rate constants for the ZnO
454 nanopowder (0.0044 h^{-1}), ZnO nanodispersion (0.0028 h^{-1}) and zinc salt (0.0036 h^{-1})) and the
455 zinc concentration of the previously exposed daphnids did not significantly differ from the
456 unexposed ones after 24 hours of none-exposure. At the acute level, the elimination of ZnO
457 nanoparticles from *D. magna* has been shown to be fast as well (Li and Wang, 2013). In general
458 it appears that *D. magna* can regulate its zinc concentration after exposure to ZnO
459 nanoparticles, similar as after exposure to metal salts. Due to the observed similar dissolution
460 and uptake of the ZnO nanoparticles and the metal salt, the observed effects on reproduction
461 (daphnids were exposed to reproduction EC_{50}) are expected to be caused by the dissolved
462 fraction.
463
464 4.2. Exposure conditions, uptake, elimination and internalization of CuO nanoparticles in *D.*
465 *magna*
466 Directly after exposing *D. magna* to sublethal chronic CuO nanoparticle concentrations (1.10 ±
467 0.02 mg Cu/l), only a very small fraction had dissolved (on average 0.63 % with min: 0.43 – max:
468 0.83 %). This dissolved fraction (<1 %) only slightly increased throughout the exposure. Heinlaan
469 et al. (2011) found similar dissolution values (0.16 to 0.63 %), when exposed to comparable
470 concentrations of CuO nanoparticles (3.2 mg Cu/l). In our study, the dissolved nanoparticle
471 fraction (0.0069 ± 0.0022 mg Cu/l at 0 hours, 0.011 ± 0.0019 mg Cu/l at 48 hours) corresponded
472 very well with the dissolved fraction (0.0075 ± 0.0001 mg Cu/l at 0 hours, 0.0079 ± 0.0001 mg

473 Cu/l at 48 hours) of the copper salt (fraction passing through a 3 kDa filter). During the initial
474 exposure (0 hours), most of the CuO nanoparticles had formed large aggregates (with sizes
475 larger than 450 nm). In a recent study (Adam et al. submitted to Journal of Hazardous
476 Materials) we observed a similar aggregation as in this current study. Dynamic light scattering
477 results from this recent study indicate that under these exposure conditions (which were
478 similar for both studies), directly after addition of the nanoparticles to the medium, CuO
479 nanopowder formed aggregates with average size of 312 – 364 nm. After 48 hours of exposure,
480 high aggregation still occurred but the measured concentration in the unfiltered samples was
481 lower than the concentration measured directly after exposure (0 hours). This can be explained
482 by the high aggregation of CuO nanoparticles. Due to this aggregation, the nanoparticles
483 precipitated to the bottom of the vessel and as a result were not included in the sampling of
484 the medium. The increasing aggregation of CuO nanoparticles over time has also been observed
485 by Gomes et al. (2012). Factors that influence this CuO aggregation include pH, ionic strength
486 and humic acids (Sousa and Teixeira, 2013). The aggregation is highest when the pH approaches
487 the point of zero charge. A recent study has shown that the aggregation of CuO nanoparticles
488 increases with increasing ionic strength (with maximum values up to 0.15M) (Sousa and
489 Teixeira, 2013). However, the ionic strength of the test medium was only 0.0119 M. Humic acid
490 has been shown to adsorb to nanoparticles and thus stabilize them and reduce aggregation
491 (Sousa and Teixeira, 2013). As a result, in the nanoparticle exposure, the daphnids were mostly
492 exposed to CuO aggregates and only to very low concentrations of dissolved copper, whereas in
493 the metal salt exposure, the daphnids were solely exposed to dissolved copper.
494

495 During the 10 day exposure to the CuO nanoparticles and the copper salt and 10 day none-
496 exposure, a clear increase and subsequent decrease in the total and internal copper
497 concentration of the daphnids was found (Fig. 6), similar to the zinc exposures (Fig. 5).
498 Throughout the exposure to the nanoparticles and metal salt, the amount of copper attaching
499 to the carapace was negligible compared to what was ingested.
500 For the copper salt (exposure of 0.026 mg Cu/l), an increase in the copper concentration was
501 observed during the first 48 hours of exposure (Fig. 6c), with a high uptake rate constant of
502 0.8284 ml.(mg dry weight.h)$^{-1}$. High uptake rate constants (0.625 ml.(mg dry weight.h)$^{-1}$) for
503 copper in *D. magna* were also observed by Komjarova and Blust (2009b). During the
504 subsequent exposure and none-exposure (Fig. 6c), the copper concentration decreased in the
505 current study. The observed initial increase and subsequent decrease of copper in the daphnids
506 is due to the active regulation of copper. Initially, daphnids take up copper as an essential
507 element to meet their metabolic requirements. However, when the internal body burden
508 becomes too high, the daphnids are able to lower their copper concentrations by reduced
509 intake or elimination. This active regulation of copper by *Daphnia* was also shown by Bossuyt
510 and Janssen (2005). As such, daphnids have been shown to regulate copper at concentrations
511 of up to 0.035 mg Cu/l (Bossuyt and Janssen, 2005). However, toxicity (e.g. effects on
512 reproduction, growth, survival) was observed when the internal copper concentrations reached
513 0.175 ± 0.017 mg Cu/mg dry weight (Bossuyt and Janssen, 2003; Bossuyt and Janssen, 2005).
514 Throughout our exposure experiments the internal copper concentrations were higher so that
515 toxic effects were to be expected. Since no effects were observed on the growth (Fig. 4b) and
516 survival of the daphnids in the experiment, it is possible that the active regulation of the

daphnids involves elimination of the copper excess through their carapace (or eggs) after molting, causing the observed effects on reproduction (at these exposure concentrations the reproduction was inhibited by 50 %). It has been shown that when exposed to a toxic stressor, the basal metabolism (including survival and growth) of *D. magna* can maintained by reduced reproduction (Arzate-Cárdenas and Martínez-Jerónimo, 2012; Villarroel et al., 2009).

When exposed to the CuO nanopowder, an increase in the body copper concentration was observed during the first 5 days of exposure (Fig. 6b). However, after 10 days of exposure to the nanoparticles, the *Daphnia* copper concentration decreased. During the none-exposure, the copper was eliminated fast from the daphnids. The copper levels in the nanoparticle exposed daphnids reached much higher concentrations than in the daphnids exposed to the copper salt. Under acute exposure scenarios, much higher *Daphnia* copper concentrations were also observed when exposed to the CuO nanoparticles than when exposed to the copper salt (Adam et al., 2014a). In this study, after 48 hours of exposure to immobilization EC_{50} values of the CuO nanoparticles (13.35 ± 0.10 mg Cu/l) and copper salt (0.031 ± 0.001 mg Cu/l), copper concentrations of 6.73 ± 0.92 µg Cu/mg dry weight (nano) and 0.35 ± 0.03 µg Cu/mg dry weight (metal salt) were measured. In the current study, differences in uptake rate between the nanoparticle and metal salt exposure may suggest nanoparticle specific effects. Taking into account the much higher exposure concentrations in the nanoparticle exposure, lower uptake rate constants were observed in these exposures (0.0602 ml.(mg dry weight.h)$^{-1}$) compared with the salt exposure (0.8284 ml.(mg dry weight.h)$^{-1}$). These results indicate that the copper from the nanoparticles is taken in less efficient than the copper from the copper salt. Since CuO nanoparticles are only taken in by ingestion (as indicated below; copper salt may be taken in by

539 additional mechanisms), a trade-off may occur between ingestion of algae and ingestion of
540 nanoparticles. Similarly, Skjolding et al. (2014) indicates that when fed on algae, the uptake of
541 gold nanoparticles by *D. magna* is less efficient than when not fed. The high copper
542 concentrations in the nanoparticle exposure and the electron microscopic study indicate that
543 the nanoparticle aggregates are ingested by the daphnids and can be localized in the gut lumen
544 but do not appear to penetrate the cells. Based on the measured *Daphnia* copper
545 concentration after 10 days of exposure and the total theoretical copper concentration in the
546 gut if it were to be completely filled with CuO nanoparticles (taking into account a *Daphnia* gut
547 volume of 0.018 mm^3 and a CuO nanoparticle density of 6.4 g CuO/cm^3 (EPRUI Nanoparticles &
548 Microspheres)), 0.6 % of the gut is expected to be filled with CuO nanoparticles after 10 days of
549 exposure. The ingestion of CuO nanoparticles in *D. magna* has been shown by Heinlaan et al.
550 (2011) and Fan et al. (2012) under acute exposure scenarios (4 mg CuO/l during 48 hours and
551 0.006 – 0.111 mg CuO/l for 72 hours). In addition, Heinlaan et al. (2011) showed that the
552 ingested particles occurred as dispersed unaggregated CuO nanoparticles in the midgut but no
553 internalization in the midgut epithelial cells was observed. In our study, CuO nanoparticles were
554 exposed in much higher concentrations than the copper salt to cause the same effects on *D.*
555 *magna* reproduction. However, the dissolved nanoparticle fraction in the medium
556 corresponded with the dissolved copper salt fraction. As a results, when expressed on a
557 dissolved scale, similar effect concentrations (reproduction EC_{50}) are observed for the CuO
558 nanoparticles and Cu salts. Therefore, the only fraction responsible for the toxicity was this
559 dissolved fraction, indicating that the ingested CuO nanoparticles did not dissolve in the

560 *Daphnia* gut nor caused any additional toxicity. The fast decrease in copper concentration
561 indicates that the ingested nanoparticles were excreted by the daphnids quickly.
562

563 5. Conclusions

564 Our results showed a comparable uptake and elimination of the ZnO nanoparticles and the zinc
565 salt, with no evidence of ZnO nanoparticles accumulating in the gut or internalization in the
566 cells, due to the fast dissolution of these nanoparticles. The combined fast dissolution in the
567 medium, uptake and toxicity results indicate that the toxicity of the ZnO nanoparticles to *D.*
568 *magna* was caused by the dissolved fraction. Under the tested conditions, the CuO
569 nanoparticles were ingested by *D. magna* and could be localized in the gut but were not
570 internalized in the cells and were easily eliminated. Despite this high ingestion of CuO
571 nanoparticles, the similar dissolution in the medium and toxicity of the nanoparticles and the
572 copper salt indicate that the caused toxicity is due to the dissolved fraction as well. Future work
573 should focus on long-term accumulation studies of different types of nanoparticles in different
574 species.
575

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582

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Tables

Tab. 1 - Uptake and elimination rate constants obtained by first-order kinetic modelling. For both internal and total zinc and copper concentrations the uptake rate constant (K_u) and elimination rate constant (K_e) during the uptake phase and elimination rate constant (K) during the elimination phase with corresponding 95 % confidence intervals and R^2 values are indicated. If negative values were obtained, 0 is indicated in the table.

Figures

Fig. 1 - Transmission electron microscopic image of the ZnO nanopowder (a), ZnO nanodispersion (b) and CuO nanopowder (c).

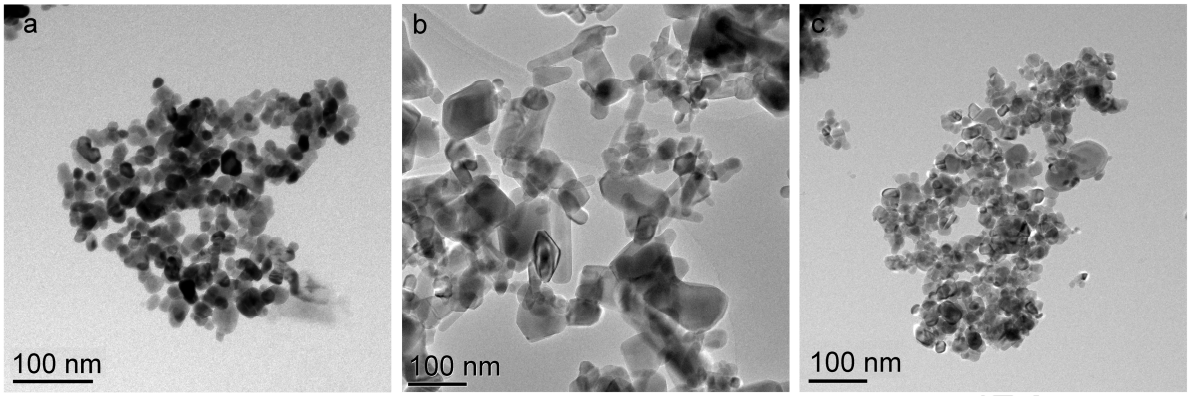
Fig. 2 - Measured zinc concentration (with standard deviations of three replicates) in the 3 kDa, 100 nm, 450 nm and unfiltered samples after 0 hours (1 to 2 hours after spiking of the stock solutions) and 48 hours of exposure to the ZnO nanopowder (a), ZnO nanodispersion (b) and $ZnCl_2$ (c). One-way ANOVA tests (indicating differences between the zinc concentrations of different (un)filtered samples) are indicated for 0 and 48 hours of exposure. The blanks (not indicated on graph) in the unfiltered samples had average concentrations of 0.005 ± 0.002 mg Zn/l.

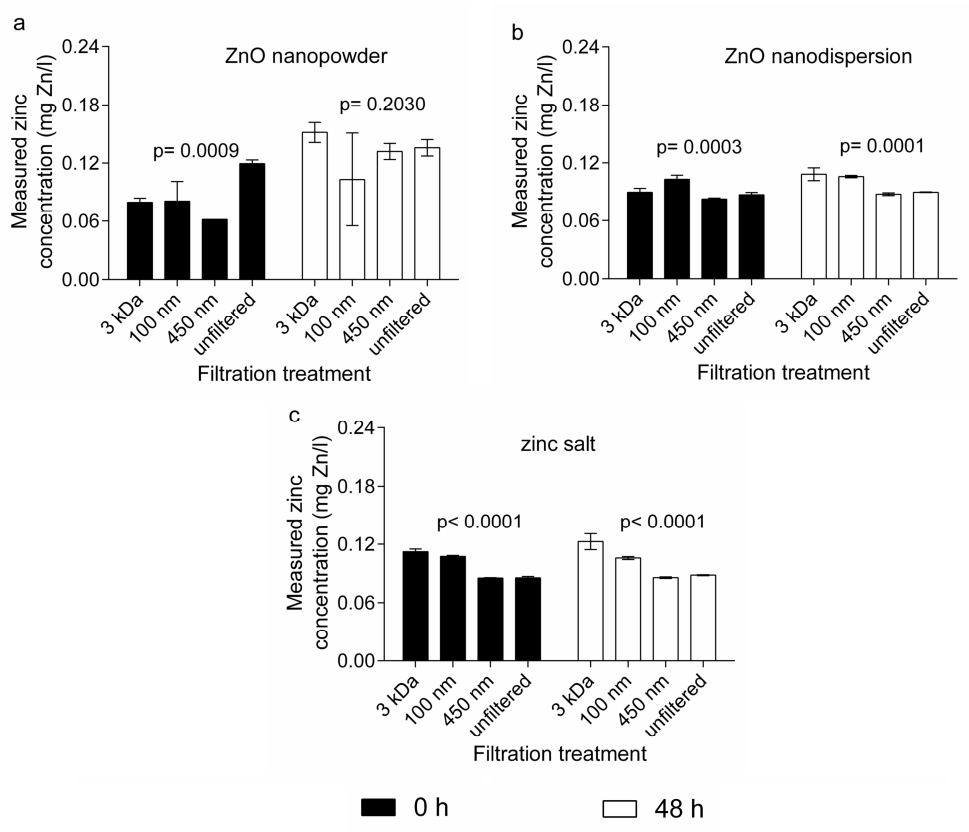
Fig. 3 - Measured copper concentration (with standard deviations of three replicates) in the 3 kDa, 100 nm, 450 nm and unfiltered samples after 0 and 48 hours of exposure to the CuO nanopowder (a) and $CuCl_2.2H_2O$ (b). One-way ANOVA showed significant differences between the different (un)filtered samples ($p< 0.0001$ at 0 and 48 hours for nano and salt). The blanks (not indicated on graph) in the unfiltered samples had average concentrations of 0.0075 ± 0.003 mg Cu/l.

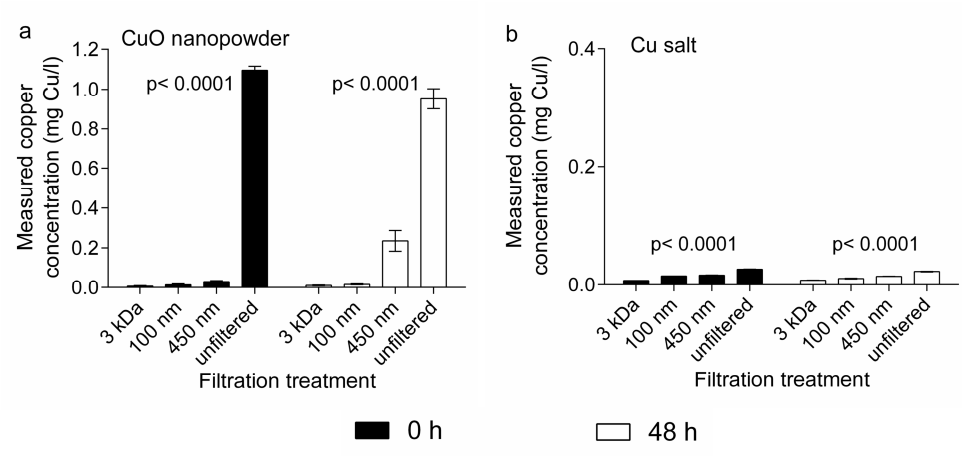
Fig. 4 - The length (with standard deviations of thirty replicates) of *D. magna* when a: not exposed (blank) and exposed to the ZnO nanopowder, nanodispersion and $ZnCl_2$ and b: not exposed (blank) and exposed to the CuO nanopowder and $CuCl_2.2H_2O$, during 10 days followed by 10 days of none-exposure. For the zinc exposure, one-way ANOVA indicated no significant differences in length between the unexposed and exposed daphnids at most time points ($p> 0.0601$; with an exception after 5 days of exposure $p= 0.0008$ and 10 days of none-exposure p=

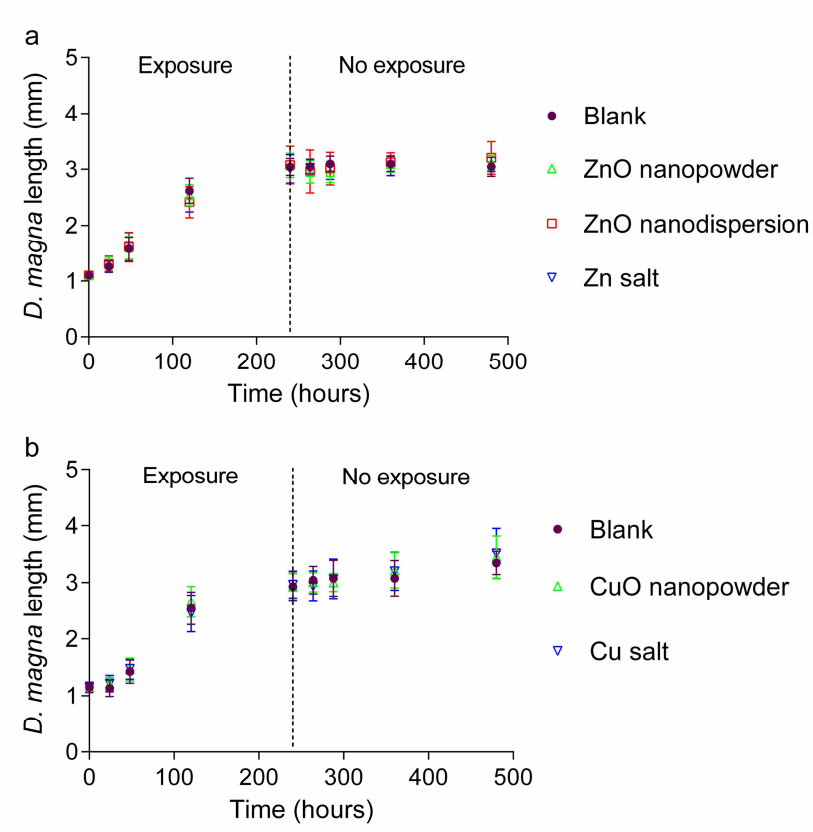
734 0.0320). For the copper exposure, one-way ANOVA indicated no significant differences in length between the
735 unexposed and exposed daphnids at most time points (p> 0.2020; with an exception after 24 hours p= 0.0342 and
736 5 days p= 0.0328 of exposure).
737
738 Fig. 5 - Total and internal zinc concentration (with standard deviations of three replicates containing 10 daphnids
739 each) of the unexposed *D. magna* and of *D. magna* exposed to the ZnO nanopowder (b), ZnO nanodispersion (c),
740 $ZnCl_2$ (d) during 10 days, followed by 10 days of none-exposure. Two-way ANOVA tests (studying effects of
741 exposure i.e. zinc concentration in the exposed and blank daphnids during the exposure and none-exposure phase,
742 exposure time and the interaction between exposure and time; with Tukey's post test) showed significant effects
743 of the exposure (p< 0.0001 for nanopowder, p< 0.0001 for nanodispersion, p< 0.0001 for zinc salt), exposure time
744 (p< 0.0001 for nanopowder, p< 0.0001 for nanodispersion, p< 0.0001 for zinc salt) and interaction between
745 exposure and exposure time (p< 0.0001 for nanopowder, p= 0.0394 for nanodispersion, p< 0.0001 for zinc salt).
746 The modelled curves (solid lines for total and dashed lines for internal zinc) are indicated.
747
748 Fig. 6 - Total and internal copper concentration (with standard deviations of three replicates containing 10
749 daphnids each) of the unexposed *D. magna* and of *D. magna* exposed to the CuO nanopowder (b), $CuCl_2.2H_2O$ (c)
750 during 10 days, followed by 10 days of none-exposure. Two-way ANOVA tests (studying effects of exposure i.e.
751 copper concentration in the exposed and blank daphnids during the exposure and none-exposure phase, exposure
752 time and the interaction between exposure and time; with Tukey's post test) showed significant effects of the
753 exposure (p< 0.0001 for nanopowder, p< 0.0001 for copper salt), exposure time (p< 0.0001 for nanopowder, p<
754 0.0001 for copper salt) and interaction between exposure and exposure time (p< 0.0001 for nanopowder, p<
755 0.0001 for copper salt). The modelled curves (solid lines for total and dashed lines for internal copper) are
756 indicated.
757
758 Fig. 7 - STEM image of aggregated CuO nanoparticles in the gut lumen (a and b) and corresponding EDX-spectrum
759 (c, of image b) after 10 days of exposure.

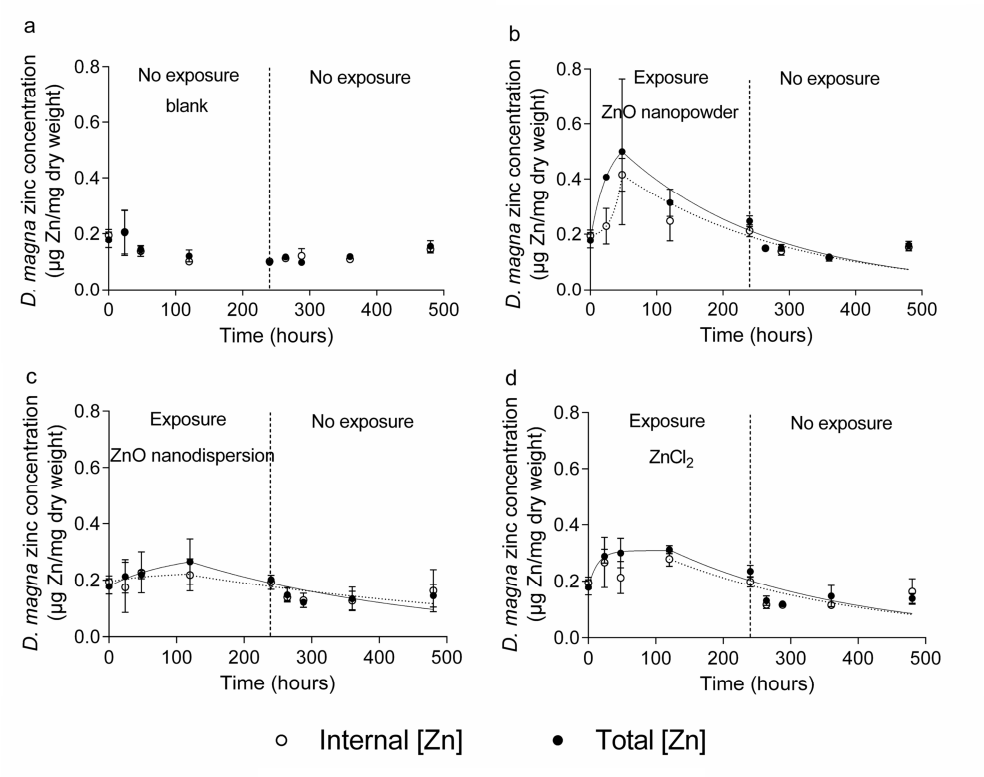
Chemical		Uptake phase					Elimination phase		
		K_u ml.(mg dry weight.h)$^{-1}$	95% CI K_u	K_e (h^{-1})	95% CI K_e	R^2	K (h^{-1})	95% CI K	R^2
ZnO nanopowder	Internal	0.0041	0 to 0.0191	0	0 to 0.0342	0.8294	0.0040	0.0033 to 0.0047	0.7401
	Total	0.1061	0 to 0.6941	0.0379	0 to 0.3857	0.6005	0.0044	0.0034 to 0.0055	0.6469
ZnO nanodispersion	Internal	0.0077	0 to 0.0671	0.0144	0 to 0.2193	0.0526	0.0018	0.0009 to 0.0026	0.1526
	Total	0.0201	0 to 0.0719	0.0107	0 to 0.0705	0.3498	0.0028	0.0020 to 0.0037	0.4384
Zn salt	Internal	ND	ND	ND	ND	ND	0.0034	0.0024 to 0.0044	0.4165
	Total	0.1009	0 to 0.2377	0.0741	0 to 0.1859	0.7771	0.0036	0.0027 to 0.0045	0.6282
CuO nanopowder	Internal	0.0317	0.0090 to 0.0545	0	0 to 0.0048	0.9161	0.0101	0.0053 to 0.0149	0.6761
	Total	0.0602	0.0346 to 0.0859	0.0037	0 to 0.0121	0.9347	0.0095	0.0053 to 0.0136	0.6950
Cu salt	Internal	0.4977	0.1860 to 0.8093	0	0 to 0.0128	0.9558	0.0066	0.0059 to 0.0072	0.9679
	Total	0.8284	0.4156 to 1.2410	0	0 to 0.0196	0.9660	0.0080	0.0070 to 0.0090	0.9544

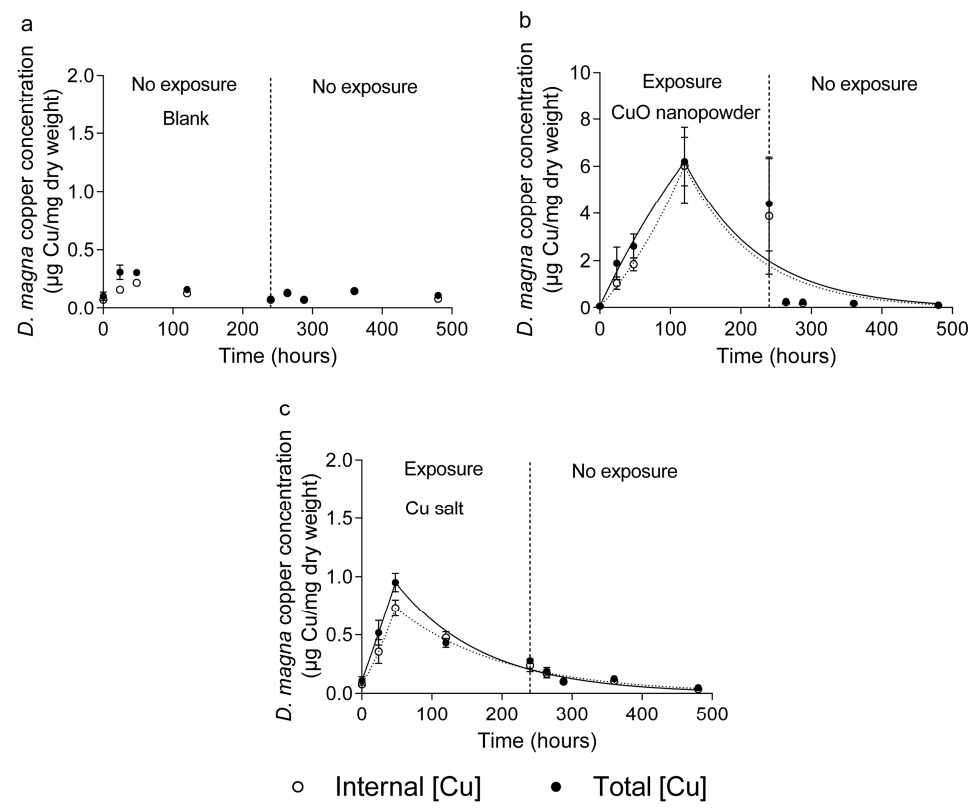


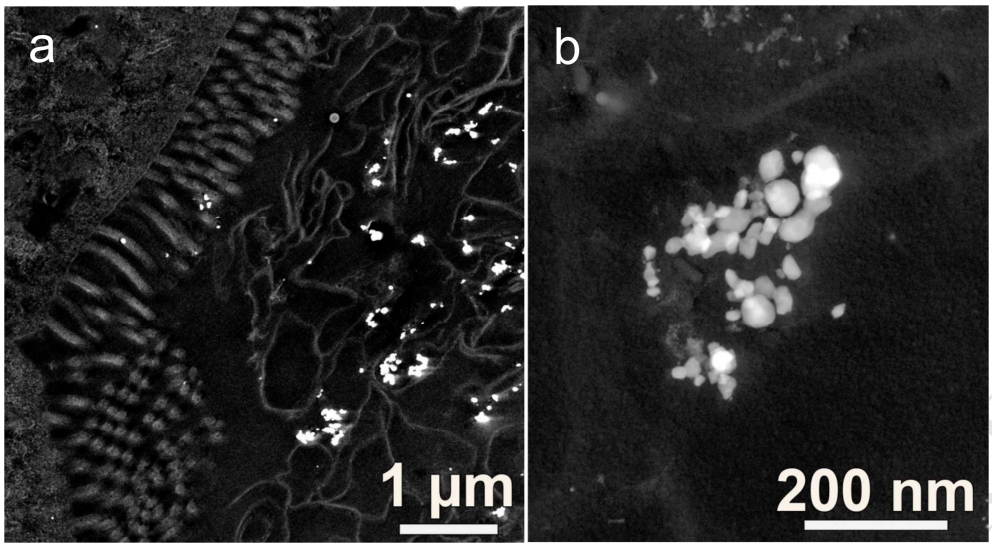


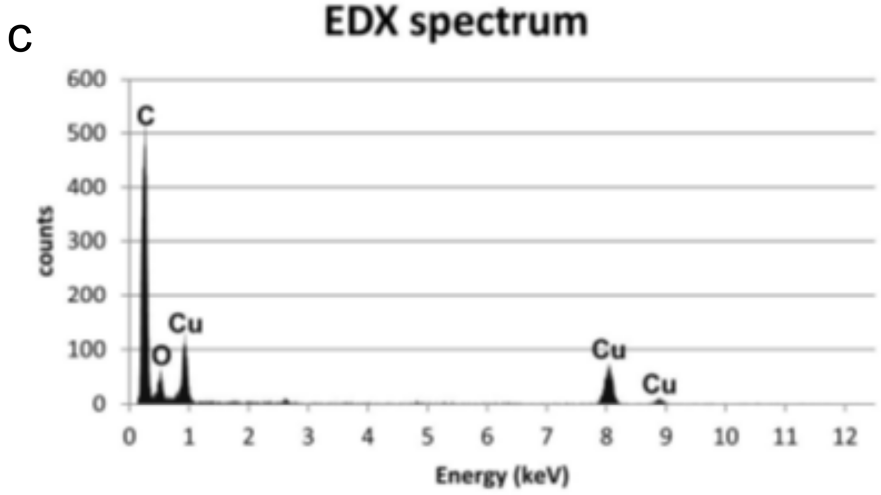












Highlights

ZnO nanoparticles (NPs) quickly dissolve after addition to the medium

Most CuO NPs aggregate in the medium, while only a small fraction dissolves

Daphnids exposed to the NPs can regulate their internal zinc and copper concentration

CuO NPs accumulate in the gut but do not penetrate cells or tissues

The toxicity of ZnO and CuO NPs to daphnids is caused by the dissolved fraction

Appendix A

To determine the zinc or copper concentration (µg metal/mg dry weight) of the daphnids, the mg dry weight of the daphnids needed to be known. However, to avoid contamination of the samples and since these organisms are very small we chose to extrapolate the dry weights from the length data.

For this a standard curve was made. From our Daphnia culture, *D. magna* of different lengths were sampled. Their lengths (measured from head to apical spine) were determined and daphnids of exactly the same length were pooled together (they were put together on a small filter paper). The filter papers containing the daphnids were put in a dry oven for 48 hours. Afterwards, the total weight of the pooled daphnids was determined (by transferring the daphnids to a pre-calibrated balance containing a different small filter paper). To determine the individual daphnia weight, the total weight was divided by the number of daphnids present in each pool (containing about 6 daphnids). Afterwards a standard curve was made (see Fig. A.1). According to Bird and Prairie (1985), the curve between weight and length can be described as $W=a*L^b$. The formula weight = 0.0028 x length$^{3.6819}$, obtained from our curve, was used to determine the dry weight of the daphnids in the different tests, based on the measured lengths.

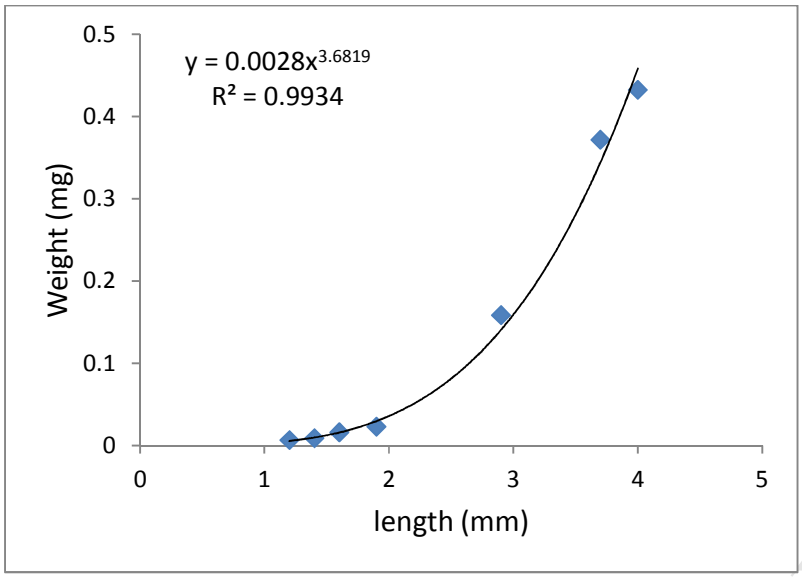


Fig. A.1: Standard curve describing the relation between dry weight and length of *D. magna*.

Reference

Bird, D.F. and Prairie, Y.T., 1985. Practical guidelines for the use of zooplankton length-weight regression equations. Journal of Plankton Research 7(6), 955-960.