The chronic toxicity of ZnO nanoparticles and ZnCl₂ to Daphnia magna and the use of different methods to assess nanoparticle aggregation and dissolution

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Abstract
In this study, the effect of ZnO nanoparticles and ZnCl₂ on growth, reproduction and accumulation of zinc in Daphnia magna was determined in a 21-day chronic toxicity test. A variety of techniques were used to distinguish the free zinc ion, dissolved, nanoparticle and aggregated zinc fraction in the Daphnia test medium. The results showed similar chronic effects on growth, reproduction and accumulation for the ZnO nanoparticles (EC₁₀, 20, 50 reproduction: 0.030, 0.049, 0.112 mg Zn/l) and the ZnCl₂ (EC₁₀, 20, 50 reproduction: 0.014, 0.027, 0.082 mg Zn/l). A large fraction of the nanoparticles rapidly dissolved after introduction in the exposure medium. Aggregation of nanoparticles was also observed but within 48 h of exposure most of these ZnO aggregates were dissolved. Based on the combined dissolution kinetics and toxicity results, it can be concluded that the toxicological effects of ZnO nanoparticles at the chronic level can be largely attributed to the dissolved fraction rather than the nanoparticles or initially formed aggregates.

Keywords: nanotoxicity, zinc, reproduction, Daphnia

Introduction
Metal oxide nanoparticles are defined as particles of metal oxides with at least one dimension between 1 and 100 nm. As a result of their small size, they demonstrate unique properties such as a high strength, transparency, surface reactivity and UV absorption characteristics. These specific properties make nanoparticles of interest for use in many applications such as sunscreens, cosmetics and solar cells. However, due to the increasing production of nanoparticles for commercial applications, nanoparticles will somehow end up in the aquatic environment, where their small sizes and specific properties may have adverse effects. One of the most widely used and toxic metal oxide nanoparticles is ZnO (Kahru & Dubourguier 2010). At the acute level, the toxicity of these nanoparticles has already been characterised by several studies. ZnO nanoparticles have been proven to be toxic to bacteria (0.29 mg Zn/l < EC₅₀ < 800 mg Zn/l), 30 min < exposure time < 24 h (Adams et al. 2006; Heinlaan et al. 2006; Li et al. 2011), multiple generations of algae (0.042 mg Zn/l < EC₅₀ < 0.068 mg Zn/l, with exposures lasting 72 h) (Aruoja et al. 2009; Franklin et al. 2007), protozoa (4.3 mg Zn/l < EC₅₀ < 8.3 mg Zn/l, 4 h < exposure time < 24 h) (Mortimer et al. 2010), nematoda (1.8 mg Zn/l < EC₅₀ < 789 mg Zn/l, 2 h < exposure time < 72 h) (Ma et al. 2009; Wang et al. 2009), crustacea (0.15 mg Zn/l < EC₅₀ < 18 mg Zn/l, 24 h < exposure time < 48 h) (Wiench et al. 2009; Heinlaan et al. 2008; Poynton et al. 2011) and fish (1.4 mg Zn/l < EC₅₀ < 18.5 mg Zn/l, 84 h < exposure time < 96 h) (Bai et al. 2010; Zhu et al. 2008, 2009a). The chronic toxicity of ZnO nanoparticles is less well documented and include some ZnO nanoparticle toxicity studies on soil species (Hooper et al. 2011; Kool et al. 2011) and a limited number of studies on aquatic species (Hao & Chen 2012; Zhao et al. 2012). The effect of ZnO nanoparticles has been tested on Daphnia magna mortality and immobilization at the acute level with EC₅₀ concentrations ranging from 0.5 to 18 mg Zn/l (Heinlaan et al. 2008; Wiench et al. 2009; Zhu et al. 2009b, 2012; Poynton et al. 2011). So far, at the chronic level, only one study has investigated the effect of ZnO nanoparticles (with a very low NOEC (no observed effect concentration) value of 0.00064 mg Zn/l and a LOEC (lowest observed effect concentration) value of 0.0032 mg Zn/l) on D. magna reproduction (Zhao et al. 2012).
Several authors have indicated that the acute toxicity of ZnO nanoparticles to bacteria (Heinlaan et al. 2008; Blinova et al. 2010), algae (Franklin et al. 2007), protozoa (Mortimer et al. 2010), nematoda (Ma et al. 2009) and crustacea (Heinlaan et al. 2008) is caused by the Zn\(^{2+}\) ions, formed after dissolution of the particles. These findings were mainly based on comparisons of the acute toxicity of zinc oxide nanoparticles and the corresponding inorganic salts. The toxicity caused by zinc salts has already been thoroughly studied in D. magna. Acute EC\(_{50}\) concentration values (obtained from the USEPA Ecotox database) were mostly between 0.1 and 14 mg Zn/l (Bringmann & Kühn 1977; Biesinger & Christensen 1972). At the chronic level, 21-day exposure EC\(_{10}\) reproduction values were between 0.09 and 0.99 mg Zn/l (Heijerick et al. 2003), while EC\(_{20}\) values were between 0.091 and 1 mg Zn/l (Heijerick et al. 2003; Muyssen & Janssen 2005) and NOEC values ranged from 0.08 to 1 mg Zn/l (Heijerick et al. 2003; Muyssen & Janssen 2007).

It is known that nanoparticles show a highly dynamic and complex behaviour when they enter the aquatic environment and do not just remain as single particles. Zinc oxide nanoparticles have been shown to rapidly dissolve (e.g. Kasemets et al. 2009) but at the same time aggregate (e.g. Keller et al. 2010). It was recently demonstrated that, while the dissolved concentration in equilibrium with ZnO nanoparticles depends on the size of the primary particles, the rate of the dissolution is controlled by the size of the aggregate (David et al. 2012). The formation of aggregates depends largely on the surface charge of the particles. If all the nanoparticles have a high negative or positive charge, they will repel each other, resulting in a higher stability. If the surface charge is low, nanoparticles tend to aggregate and form larger conglomerates (Bagwe et al. 2006). Additionally, different environmental factors such as the ion composition and ionic strength of the medium, the pH and natural organic matter (NOM) influence this balance. At pH values near the point of zero charge, some nanoparticles tend to aggregate (Domingos et al. 2009; Dunphy Guzman et al. 2006). NOM absorbs onto the surface of the particles and reduces aggregation (Keller et al. 2010; Zhang et al. 2009). By contrast, higher ionic strengths (e.g. 10 mM NaCl in ultrapure water) enhance the aggregation of some nanoparticles (e.g. Zn; Zhou & Keller 2010).

Several techniques are being used to measure the dissolution and aggregation of nanoparticles. These techniques include biological detections and concentration measurements after (or without) physical separation. For biological detection methods, metal ion sensing bacteria or yeasts are commonly used to measure the dissolved fraction (Kasemets et al. 2009; Baek & An 2011; Mortimer et al. 2010; Heinlaan et al. 2008). These metal ion detection tests are mostly done at pH values ranging from 5.5 to 6.5. However, most toxicity tests on model organisms (e.g. algae, fish, crustacea) are done at higher pH values, more common to natural aquatic ecosystems, under which the metal oxide nanoparticle dissolution is different. Furthermore, it cannot be excluded that these bacteria and yeasts are affected by individual nanoparticles or aggregates in addition to metal ions. For the physical separation, filters of different pore size are often used. When using a small pore ultrafilter of 1–2 kDa (Poynton et al. 2011) or dialysis (Franklin et al. 2007), it is possible to differentiate between the nanoparticles and the dissolved fraction. Filters of 100 nm can be used to differentiate between the aggregated and nanoparticle/dissolved fraction (Laurent et al. 2004).

Other techniques have been used to directly measure or visualise nanoparticles and their dissolved or aggregated fraction without physical separation. As such, dynamic light scattering (DLS) (Kato et al. 2010), flow-field-flow fractionation (Calzolai et al. 2011), ultraviolet-visible analysis (Seo et al. 2011) and scanning- (SEM) and/or transmission electron microscopy (TEM) (Soto et al. 2005; Hondow et al. 2011) have been used to measure the size of nanoparticles and their aggregates. David et al. (2012) recently studied the kinetics and thermodynamics of ZnO nanoparticles dissolution with the AGNES (Absence of Gradients and Nernstian Equilibrium Stripping) technique (Galceran et al. 2004) which allows the measurement of the free Zn concentration without any previous physical separation. A combination of several of the above-mentioned techniques can be used to distinguish between the different fractions that are formed.

Given the highly dynamic nature of ZnO nanoparticles, the central aim of this study was to investigate the chronic toxicity of the nanoparticles to D. magna, a filter feeding freshwater crustacean used as a model organism in ecotoxicological testing. It has previously been reported that the acute toxicity of ZnO nanoparticles is largely attributable to the dissolution of the zinc ions from the nanoparticles. However, whether this is also the case in chronic scenarios remained an open question. As per the authors’ knowledge, this is one of the first papers studying the chronic toxicity of ZnO nanoparticles in D. magna in combination with a dynamic nanoparticle characterisation in the exposure medium.

**Methods**

**Test materials and characterisation**

ZnO nanopowder NanoSun, with an advertised particle size of 30 nm, was obtained from Micronisers PTY (Australia). A corresponding zinc salt ZnCl\(_2\) (≥98%) was obtained from Sigma-Aldrich. The commercial nanoparticle size was checked by different techniques. The ZnO nanopowder was first added to pure water (Millipore) to obtain a stock solution of 10 g/l, which was shaken and sonicated for 5–6 h in a sonication bath. The size and shape of the particles were visualised by TEM (Philips CM200 FEGTEM) after drying of the suspension (required for this microscopic technique). The size distribution of the particles in suspension was characterised by DLS (Malvern Z-sizer NS) directly after sonication.

**Test species**

The freshwater crustacean D. magna was used as a test species. Daphnids were reared in biofilter-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 afterwards the daphnids were fed with $4 \times 10^5$ algae cells/ml (Pseudokirchneriella subcapitata and Chlamydomonas reinhardtii in a 3:1 ratio).

**Chronic toxicity of ZnO nanoparticles and ZnCl₂ to D. magna**

The effect of the ZnO nanoparticles and ZnCl₂ was tested on the survival, growth and reproduction of D. magna in a chronic test scenario according to OECD guidelines 211 (OECD 2008). Immediately before the exposure, a freshly prepared stock solution (from dry powder) was made (50 mg/l in OECD recommended ISO test medium: CaCl₂·2H₂O: 0.294 g/l, MgSO₄·7H₂O: 0.123 g/l, NaHCO₃: 0.065 g/l, KCl: 0.006 g/l, water hardness 250 mg CaCO₃/l, pH 7.8–8.2, conductivity 617 µS/cm (OECD 2004)). The suspension of the ZnO nanoparticles was sonicated for 30 min in a sonication bath to maximise the particle dispersion and obtain a homogeneous distribution without visually aggregated nanoparticles. The ZnCl₂ stock solution (50 mg/l in ISO test medium) was not sonicated. Subsequently, small volumes of these stocks were added to OECD recommended ISO test medium to obtain concentrations of 0.0024, 0.008, 0.024, 0.064, 0.16 mg Zn/l (0.037, 0.123, 0.369, 0.983, 2.457 µM) for the ZnO nanoparticles. The size of the nanoparticles in the three highest exposure concentrations (0.024, 0.064, 0.16 mg Zn/l) was visualised by DLS directly (measured 5 min after a 30-min sonication) after spiking. For the zinc salts, exposure experiment solutions of 0.01, 0.024, 0.048, 0.14, 0.38 mg Zn/l (0.147, 0.367, 0.734, 2.20, 5.87 µM) were prepared. One daphnid (< 24 h) was transferred to 100 ml of test medium, in 10 replicate test vessels, for the different exposure concentrations and blanks (OECD 2008). D. magna were fed with $4 \times 10^5$ cells/ml of the algae species P. subcapitata. The daphnids were transferred to freshly spiked medium (from newly made stock solutions) and fed every 48 h with a fresh spike of algae. During the exposure period of 21 days, the number of live offspring was counted daily and removed from the medium. At the end of the experiment, the length of the adult daphnids was also determined.

**Accumulation of ZnO nanoparticles and ZnCl₂ in and on D. magna**

The accumulation of ZnO nanoparticles and ZnCl₂ in and on D. magna was analysed by measuring the zinc concentration of the adults. After 21 days of exposure, the adult daphnids were pooled per concentration, rinsed with pure water and transferred to polypropylene bullet tubes. The samples were dried in an oven at 60°C for at least 48 h until a constant dry weight. To each tube containing dried daphnids, 50 µL HNO₃ (69%) and (after 12 h) 50 µL H₂O₂ (30%) was added. Four hours later, the daphnids were dissolved by microwave digestion (4 min 100 W, 3 min 180 W, 2 min 180 W, 2 min 300 W, 2 min 300 W, 2 min 450 W) (Blust et al. 1988). After cooling down and dilution of the samples to 1% HNO₃, the zinc concentration of the daphnids was measured by inductively coupled plasma optical emission spectrometry (ICP-OES; Thermo scientific 6000 series).

**Zinc concentrations and exposure conditions**

Samples of the exposure medium were taken to determine zinc concentrations and speciation of the ZnO nanoparticles and ZnCl₂. A series of filtration methods were used to distinguish between the total, nanoparticle and dissolved zinc concentration, that is, the aggregated fraction (retained on a 100 or 450 nm filter), the nanoparticle fraction (retained on a 1 kDa filter but passing through a 100 nm filter) and the dissolved fraction (passing through a 1 kDa filter). As the D. magna medium was renewed every 48 h, samples were taken 1–2 h after spiking of the stock solution (0–1 h after addition of the daphnids, to the different solutions) to which the authors refer to as 0 h (directly after renewal) and 48 h later, to which the authors will refer to as 48 h (directly before renewal), in three replicate test vessels. The 100 nm (Pall Puradisc PTFE) and 450 nm (Whatman Acrodisc PP) filtrations were performed with syringe filters and the 1 kDa ultrafiltrations with Microsep centrifuge filters (Pall Life Sciences), using a 1 h centrifugation at 7500 g (Beckman Avanti J25). All these samples were acidified to 1% HNO₃ and the zinc concentration was analysed by Inductively coupled plasma mass spectrometry (ICP-MS; Agilent technologies 7700 series). Additionally, voltammetric measurements were carried out to measure the ionic zinc (Zn²⁺) concentration in the medium. AGNES (Galceran et al. 2004) was used to measure the Zn²⁺ concentrations. During the measurement, the pH was buffered with Tris (0.02 M) and brought to the same value as measured in the exposure. The effect of zinc complexation by the buffer was taken into consideration using the appropriate thermodynamic stability constants. The measurements were carried out with a voltammetric cell (Metrohm 633 VA Stand) coupled to a potentiostat (µAutolab III), attached to a computer with GPES software (version 4.9.007). For exposure concentrations equal to or lower than 0.024 mg Zn/l, the two-pulses strategy (Companys et al. 2005) was used, for higher exposures only one pulse was used. The shifted blank was distracted from the faradaic current measurements (David et al. 2012) to eliminate background measurements. The pH (7.8–8.2), temperature (19–21°C) and oxygen concentrations (>80%) of the exposure medium was checked regularly.

**Data analysis and statistics**

Graphpad Prism (version 5.04) was used for data visualisation and statistics. Based on the chronic toxicity data, brood size, time to first brood, time between broods and number of broods per female were calculated for the surviving daphnids after 21 days of exposure. Concentration–response curves were constructed and EC₁₀, ₂₀, ₅₀ values for reproduction (exposure concentration at which 10, 20 or 50% of the reproduction was inhibited) were calculated. One-way ANOVA tests with Tukey’s multiple comparison post test were performed on reproduction data to compare the significant differences in total number of produced neonates between the different exposure concentrations. Similar statistical analyses were performed on the adult size and the zinc accumulation data. The differences in zinc concentrations obtained by the different filtration procedures were compared in a one-way ANOVA, with Tukey’s multiple
A TEM analysis (Figure 1, left) showed that the ZnO nanoparticles were spherical particles with monophasic hexagonal wurtzite crystal structure and with primary diameters between 20 and 40 nm. DLS results (Figure 1, right) indicated that the nanoparticles were aggregated with most sizes between 60 and 70 nm in pure water.

Zinc concentrations and exposure conditions
As expected, the zinc salt (no data shown) had completely dissolved (100%) directly after addition to the OECD medium. In most cases, no significant difference was detected when comparing the different unfiltered and filtered treatments in a one-way ANOVA. The measured (AGNES) and modelled (Visual Minteq) free zinc ion concentrations were in good agreement (Figure 2A).

Immediately after introducing the ZnO nanoparticles in the Daphnia exposure medium, the particles started to dissolve. Measurements of the dissolved zinc concentration (fraction passing through a 1 kDa filter) show that on average 85.5 (average minimum: 69–average maximum: 100) % of the zinc was found in solution within hours after spiking (0 h (samples taken 1–2 h after spiking), Figure 3A). However, at this time, some particles were still present in the medium and had not dissolved in the time elapsed between preparation of the dispersion and the sampling for the different measurements (one-way ANOVA p < 0.001; Tukey’s Multiple Comparison Tests indicated significant differences between unfiltered and filtered sample concentrations). Moreover, no individual nanoparticles but rather aggregates larger than 100 nm appear to be present in the medium, since zinc concentrations measured after filtration over the 100 and 450 nm filter (or unfiltered samples) were significantly different. DLS analysis performed directly after spiking of the exposure solutions confirms this initial aggregation. At the nominal exposure concentration of 0.024 mg Zn/l, 0.064 mg Zn/l and 0.16 mg Zn/l, the average sizes of these aggregates were 373 (min 255–max 712) nm, 280 (min 190–max 459) nm and 244 (min 190–max 396) nm.

Figure 1. Characterisation of the ZnO nanopowder with a TEM image (left) and DLS analysis (right) in pure water. The TEM image shows that the nanoparticles are spherical particles with sizes between 20 and 40 nm. In pure water they form aggregates with average values of 66.5 and 153.1 nm in size.

Figure 2. The measured free zinc concentration (Zn²⁺) for ZnCl₂ (A) and the ZnO nanoparticles (B) after 0 and 48 h of exposure in function of the measured total Zn exposure concentration (unfiltered samples after 0 h of exposure). The continuous line represents Visual Minteq theoretical free zinc calculations (taking into account the complexation with Tris and the OECD medium composition) based on the total Zn concentration corresponding to the unfiltered sample after 0 h of exposure.
After 48 h of exposure (Figure 3B), most of these aggregates had dissolved (on average 90.9% (average minimum: 88%–average maximum: 100) %), which is also indicated by the decrease in concentration difference measured after filtration over a 100 and 450 nm filter (or in unfiltered samples). For the highest exposure concentration these differences were not even significant. The higher dissolution (similar to the zinc salt) is also translated in the slight increase in the free Zn²⁺ concentration after 48 h (Figure 2B).

The other exposure medium characteristics showed little variation during the 48 h of exposure and were not dependent on the zinc exposure concentration. In the ZnO nanoparticle exposure, average pH values of 8.03 ± 0.09 were measured, while in the ZnCl₂ exposure these values were 8.01 ± 0.09. The temperature was 19.5 ± 0.6 °C (for the nanoparticle exposure) and 19.3 ± 0.6 °C (for the salt exposure). The O₂ concentrations were 99.5 ± 2.4% for ZnO nanoparticles and 97.1 ± 3.0% for ZnCl₂. The ionic strength of the blank and the exposed OECD medium was 0.0119 M as calculated with Visual Minteq.

Chronic toxicity of ZnO nanoparticles and ZnCl₂ to D. magna

During the 21-day exposure of the daphnids, the average number of neonates decreased as the exposure concentration of the ZnO nanoparticles and the zinc salts increased. The concentration-response curves show the reproduction (% of blank) as function of the exposure concentration (Figure 4). For both nanoparticles and salt, the number of neonates in the two highest concentrations was significantly different from the blank (one-way ANOVA \( p < 0.01 \)). Based on measured total zinc concentrations, EC₁₀, EC₂₀ and EC₅₀ were calculated.

| Concentration (mg Zn/l) | EC₁₀ | EC₂₀ | EC₅₀ |
|-------------------------|------|------|------|
| ZnO nanoparticles       | 0.030| 0.049| 0.112|
| ZnCl₂                   | 0.014| 0.027| 0.082|

Figure 3. The measured Zn concentration (with standard deviations) after 0 (A) and 48 (B) h in the unfiltered and filtered (1 kDa, 100 nm, 450 nm) samples when exposed to different concentrations of ZnO nanoparticles (measured unfiltered samples after 0 h of exposure are indicated on the x-axis). Significant differences are indicated with different letters per exposure concentration (Tukey’s post tests, one-way ANOVA \( p < 0.01 \)). The blank is indicated with C.

Figure 4. The chronic toxicity of ZnO nanoparticles (A) and ZnCl₂ (B) presented as a concentration-response curve (reproduction (% of blank)) with standard deviation in function of measured total zinc exposure concentration (unfiltered samples taken after 0 h of exposure, on a log-scale, mg Zn/l). A one-way ANOVA showed significant differences. The calculated effect concentration values for ZnO nanoparticles were 0.030 (95% confidence interval: 0.011–0.084) mg Zn/l (EC₁₀), 0.049 (0.025–0.096) mg Zn/l (EC₂₀) and 0.112 (0.074–0.170) mg Zn/l (EC₅₀). For ZnCl₂, these values were 0.014 (0.006–0.037) mg Zn/l (EC₁₀), 0.027 (0.014–0.053) mg Zn/l (EC₂₀), 0.082 (0.050–0.133) mg Zn/l (EC₅₀).
values of 0.030 (95% confidence interval (CI): 0.011–0.084) mg Zn/l, 0.049 (0.025–0.096) mg Zn/l and 0.112 (0.074–0.170) mg Zn/l for ZnO nanoparticles were calculated (Figure 4A). A NOEC of 0.058 mg Zn/l was found, while the LOEC was 0.131 mg Zn/l. In the case of ZnCl2 (Figure 4B), an EC10 value of 0.084 (0.006–0.037) mg Zn/l, an EC20 value of 0.27 (0.014–0.053) mg Zn/l and an EC50 value of 0.082 (0.050–0.133) mg Zn/l was obtained, similar to the nanoparticle values. Here, a NOEC value of 0.040 mg Zn/l was found while the LOEC was 0.126 mg Zn/l. The unexposed daphnids started reproducing faster and had more broods per female than the ones exposed to the highest concentrations of nanoparticles and the salt (Table I). The average brood size in the two highest exposure concentrations of ZnO was lower than in the blank. For the salts, a steady decrease in brood size was observed with increasing exposure to zinc (Table I). When looking at the survival of the adult daphnids, an effect of exposure could only be detected for the highest zinc salt concentration (Table I).

The adult daphnids had average lengths of 4.6 ± 0.3 mm (blank), 4.7 ± 0.2 mm (0.0024 mg Zn/l), 4.3 ± 0.4 mm (0.008 mg Zn/l), 4.6 ± 0.2 mm (0.024 mg Zn/l), 4.5 ± 0.4 mm (0.064 mg Zn/l), 4.1 ± 0.8 mm (0.16 mg Zn/l) when exposed to the different ZnO nanoparticle concentrations. When exposed to ZnCl2, these lengths were 4.5 ± 0.5 mm (blank), 4.4 ± 0.4 mm (0.01 mg Zn/l), 4.2 ± 0.4 mm (0.024 mg Zn/l), 4.4 ± 0.4 mm (0.048 mg Zn/l), 4.4 ± 0.4 mm (0.14 mg Zn/l) and 3.6 ± 0.4 mm (0.38 mg Zn/l). A one-way ANOVA showed no significant differences in length between the exposure and the blank. However, for the highest zinc salt exposure a significant difference with the blank was seen (Tukey’s post test).

Table I. Chronic effects (average brood size, time to first brood, time between broods, broods per female with standard deviations) of ZnO nanoparticles and ZnCl2 exposure at different measured exposure concentrations (unfiltered samples after 0 h of exposure which is 0-1 h after addition of the daphnids) and the survival of the daphnids after 21 days of exposure (in the initial exposure 10 daphnids were present per concentration).

| Measured exposure (mg Zn/l) | Average brood size (number) | Time to first brood (days) | Time between broods | Broods per female (number) | Survival (number) |
|-----------------------------|-----------------------------|---------------------------|---------------------|---------------------------|------------------|
| ZnO nanoparticle            |                             |                           |                     |                           |                  |
| 0.008 (blank)               | 24 ± 10                     | 9.3 ± 0.8                 | 2.3 ± 0.9           | 3.5 ± 0.7                 | 8                |
| 0.009                       | 26 ± 13                     | 9.5 ± 0.7                 | 2.2 ± 0.4           | 3.9 ± 0.3                 | 9                |
| 0.014                       | 22 ± 10                     | 9.3 ± 0.5                 | 2.4 ± 0.5           | 3.5 ± 1.3                 | 7                |
| 0.027                       | 27 ± 13                     | 9.6 ± 0.7                 | 2.3 ± 0.5           | 3.9 ± 0.3                 | 9                |
| 0.058                       | 20 ± 8                      | 9.5 ± 0.7                 | 2.3 ± 0.5           | 3.3 ± 0.5                 | 10               |
| 0.131                       | 16 ± 6                      | 10.2 ± 1.0                | 2.3 ± 0.5           | 2.4 ± 1.0                 | 9                |
| ZnCl2                       |                             |                           |                     |                           |                  |
| 0.006 (blank)               | 23 ± 13                     | 9.0 ± 0.9                 | 2.3 ± 0.4           | 4.2 ± 0.4                 | 10               |
| 0.013                       | 21 ± 12                     | 9.0 ± 1.0                 | 2.2 ± 0.5           | 4.1 ± 0.4                 | 7                |
| 0.023                       | 21 ± 11                     | 9.2 ± 0.9                 | 2.3 ± 0.5           | 3.9 ± 0.3                 | 9                |
| 0.04                        | 19 ± 12                     | 9.0 ± 1.3                 | 2.3 ± 0.5           | 3.8 ± 1.2                 | 8                |
| 0.126                       | 18 ± 8                      | 10.2 ± 1.3                | 2.4 ± 0.5           | 2.1 ± 0.8                 | 8                |
| 0.313                       | 0.3                         | 11.7 ± 3.8                | 2.5 ± 0.7           | 1.0 ± 1.0                 | 3                |

Accumulation of ZnO nanoparticles and ZnCl2 in and on D. magna

After 21 days, there was a significant (one-way ANOVA) influence of the exposure on the accumulation of zinc (Figure 5) and this was the same for the ZnO nanoparticles (p < 0.001, Figure 5A) and ZnCl2 (p < 0.001, Figure 5B). The zinc concentration of the adults increased with increasing exposure concentration. The concentration in the blanks was

Figure 5. The accumulation of Zn in daphnia when exposed to ZnO nanoparticles (A) and ZnCl2 (B). The measured Zn concentration per mg dry weight in function of the total Zn exposure concentration (measured unfiltered samples after 0 h of exposure) is indicated. One-way ANOVA tests indicated significant differences in Zn accumulation for the different nanoparticle (p < 0.001) and salt (p < 0.001) exposures. The significant differences with the blank (C) are indicated (Tukey’s post test).
0.069 ± 0.009 μg Zn/mg dry weight and increased to 0.223 ± 0.003 μg Zn/mg dry weight for the highest nanoparticle exposure (0.131 mg Zn/l). When comparing across the exposure ranges of the nanoparticles and the salt, accumulation of zinc was similar. For the highest salt exposure, no accurate accumulation values could be calculated due to the high daphnia mortality.

Discussion

The dissolution study showed that the zinc salt dissolved immediately in the stock solution (50 mg/l) and therefore only dissolved zinc was added to the exposure medium. In addition, the dissolution kinetics of ZnO nanoparticles were fast, resulting in rapid dissolution of the nanoparticles so that D. magna were already from the onset exposed to a large part of dissolved zinc. However, not all the particles had dissolved in the OECD medium, since some were present as aggregates larger than 100 nm. For ZnO nanoparticles, with diameters of 20–40 nm, one would expect a small enhancement of the solubility compared with bulk ZnO (David et al. 2012). Even if complete dissolution is expected, one must still consider the time needed for such a process. David et al. (2012) indicated that the dissolution of ZnO is strongly dependent on the aggregate size. The aggregate formation has its own kinetics and is strongly dependent on pH (Dunphy Guzman et al. 2006) and ionic strength (Zhou & Keller 2010). Tso et al. (2010) indicated that the point of zero charge for ZnO nanoparticles is close to pH 8 (Tso et al. 2010), at which pH the overall surface charge is zero and thus nanoparticles are no longer repelling each other and can aggregate. The initial aggregation of the particles in the authors’ experiments directly after spiking (0 h of exposure) can be explained by the proximity of the pH (around 8) to the point of zero charge. Another factor facilitating this initial aggregation is the ionic strength, which was 0.0119 M. Zhou & Keller (2010) have shown that at a pH of 8.1 (comparable with the authors’ setup), aggregation of nearly spherical ZnO nanoparticles (of 20 nm) was induced at values above 0.01 M (aggregates of more than 300 nm directly after exposure to about 1350 nm at 0.01 M after 170 min of exposure).

Within the first 48 h of exposure, the initially formed aggregates had mostly dissolved. This high dissolution can be explained by the low chronic exposure concentrations that were used (below the maximal solubility of ZnO at this pH). At these low concentrations, the nanoparticles tend to dissolve faster since the dissolution rate is proportional to the difference between the current free Zn ions and the free Zn2+ concentration in equilibrium after dissolution of ZnO. This can be presented by a first order reaction (i.e. Noyes–Whitney equation with $C = \text{the concentration in solution at time } t, C_0 = \text{the solubility of ZnO in equilibrium, } k = \text{first order release rate constant (Peng et al. 2011 from Costa & Sousa Lobo (2001)).}$). As such, if the concentration in solution (C) is lower, the time (t) to reach equilibrium (C0) will be faster, than when the concentration in solution (C) is higher.

\[
C = C_0 \left(1 - e^{-kt}\right)
\]

After 48 h, on average 90.9 ± 2.5% of the nanoparticles had dissolved. Similar solubility values were found by Heinlaan et al. (2008) who indicated that at concentrations below 1 mg/l 69–97% of the ZnO nanoparticles had dissolved using Escherichia coli as a metal sensing bacteria at pH 6.5 (Heinlaan et al. 2008). Other solubility studies (Poynton et al. 2011; Wang et al. 2009) that measured the dissolved fraction after physical separations (centrifugation or ultrafiltration) at pH values of 7–8.1 showed lower solubility (4-18%) values. However, the exposure concentrations in these studies were more than 16 times higher (minimum 2.2 mg/l) than the concentrations used in the current chronic experiment representing lower and more environmentally realistic exposure ranges.

For both the nanoparticles and the salts, Zn2+ concentrations measured by AGNES were considerably lower than the dissolved concentrations measured after filtration over a 1 kDa filter. In a system without nanoparticles, the 1 kDa filter provides the soluble fraction (i.e. the free Zn2+ and dissolved Zn complexes formed with components of the OECD medium, e.g. ZnCl+, ZnHCO3−), while AGNES provides just the free Zn2+ ion concentration. For ZnCl2, taking into account the non-negligible complexation of Zn by the Tris buffer (added during the measurements) and the inorganic species present in OECD medium the measured concentrations agreed with chemical speciation modelling (using Visual Minteq). The proximity of the ZnO nanoparticles and ZnCl2 measured free zinc concentration and the theoretical dissolution supports the statement that all ZnO nanoparticles were dissolved after 48 h.

The chronic toxicity of the ZnO nanopowder (NanoSun) to D. magna was compared with ZnCl2. The results showed that, at sublethal exposure concentrations, ZnO nanoparticles affected the reproduction of D. magna, in association with an increasing zinc accumulation. A clear decrease in the number of neonates (Figure 4A) and increase in the accumulation of zinc (Figure 5A) was seen with an increasing exposure concentration. These chronic nanoparticle effects are of high importance to the aquatic environment since they represent more realistic exposure scenarios than acute ones. When these results are compared with the inorganic salt ZnCl2, a similar toxicity (Figure 4B) and zinc accumulation (Figure 5B) is observed. The comparable toxicity of ZnO nanoparticles and ZnCl2 has already been shown before in several acute toxicity studies (Mortimer et al. 2010; Franklin et al. 2007). Therefore, different authors (Franklin et al. 2007; Mortimer et al. 2010; Kool et al. 2011) indicate that the toxicity of ZnO nanoparticles is due to the formation of toxic zinc ions. In soil species, the toxicity of these particles was lower than the zinc salt at the chronic level (Hooper et al. 2011; Kool et al. 2011). According to Muyssten & Janssen (2002), D. magna adults are able to regulate zinc at accumulated concentrations of up to 254 ± 79 μg Zn/g dry weight, while higher concentrations lead to mortality. In agreement with these results, an accumulation of 223 ± 3 μg Zn/g dry weight was measured in the highest ZnO nanoparticle exposure for which no significant mortality could be seen.
Overall, the accumulation of the nanoparticles in and on the daphnids was similar to the accumulation of the zinc salts within similar exposure ranges. This confirms that the toxicity of the nanoparticles is caused by toxic ions.

Based on theoretical predicted environmental concentrations (PEC) of ZnO nanoparticles (0.01 μg/l) in European surface water (Gottschalk et al. 2009), there is currently no risk for adverse environmental effects on the basis of the chronic toxicity results obtained with D. magna. The lowest calculated effect of ZnO nanoparticles on Daphnia reproduction (EC10: 0.030 mg Zn/l) was 3000 times higher than this PEC value.

Although, the NanoSun type of ZnO nanoparticle showed a similar toxicity as the inorganic salt and was mostly dissolved within 48 h of exposure, different ZnO nanoparticles under different environmental exposures may show other dissolution patterns and toxicities. As such, not only factors such as size and shape (Zhou & Keller 2010), but the nanoparticle coating needs to be taken into consideration as well. The ZnO NanoSun particles are not coated. However, different coatings may increase or decrease the solubility of the particles, which will influence the dissolution and aggregation and possibly also the toxicity. It has been shown that the type of coating can influence the toxicity of ZnO nanoparticles to cancer cells (Nair et al. 2009). Additionally, the exposure conditions and concentration may influence dissolution and aggregation and thus also the uptake and toxicity (i.e. dissolution rates also depend on pH and dissolution may be slower and formation of aggregates stronger in more alkaline conditions). As such, nanoparticles have been shown to be taken up by the soil organism Eisenia veneta when exposed to high acute ZnO concentrations (196 mg/l) (Hooper et al. 2011). The difference in toxic effect was also shown in an article by Poynton et al. (2011), in which the gene expression of Daphnias exposed to sublethal ZnO nanoparticles and zinc salts was followed. These results showed that distinct sets of genes were activated by the nanoparticles and zinc salts.

Conclusions

This study shows that under the tested circumstances, ZnO nanoparticles and zinc salts cause a similar chronic toxicity and accumulation in D. magna. In addition, the fast dissolution of ZnO nanoparticles indicates that the toxicity is mostly linked to free Zn ions. As per the authors’ knowledge, this is one of the first papers to study the chronic toxicity of ZnO nanoparticles in an aquatic species and to characterise the aggregation, individual nanoparticles and dissolution.

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Declaration of interest

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