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The chronic toxicity of ZnO nanoparticles and $ZnCl_2$ to *Daphnia magna* and the use of different methods to assess nanoparticle aggregation and dissolution.

Abstract

In this study, the effect of ZnO nanoparticles and $ZnCl_2$ on growth, reproduction and accumulation of *Daphnia magna* was determined in a 21 day chronic toxicity test. Different techniques were used to distinguish between the Zn^{2+}, dissolved, nanoparticle and aggregated fraction in the daphnia test medium. The results showed similar chronic effects on growth, reproduction and accumulation for the ZnO nanoparticles ($EC_{10,\ 20,\ 50\ reproduction}$: 0.029, 0.048, 0.113 mg Zn/l) and the $ZnCl_2$ ($EC_{10,\ 20,\ 50\ reproduction}$: 0.014, 0.027, 0.082 mg Zn/l). A large fraction of the nanoparticles rapidly dissolved after introduction in the exposure medium. However, also aggregation of nanoparticles was observed but after 48 hours 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 initially formed aggregates.

Introduction

Metal oxide nanoparticles are defined as particles of metal oxides with at least one dimension between 1 and 100 nm. Because of their small sizes, they acquire specific properties such as a high strength, transparency, surface reactivity and UV absorption characteristics. These specific properties make nanoparticles very attractive for their use in many applications such as sunscreens, cosmetics and solar cells. However, due to the increasing production, nanoparticles will somehow end up in the aquatic environment where their small sizes and specific properties may have an adverse effect. One of the most widely used and toxic metal oxide nanoparticles is ZnO (Kahru and Dubourguier 2010). At the acute level, the toxicity of these nanoparticles has already been characterized by several studies. ZnO nanoparticles have been proven to be toxic to bacteria (0.29 mg Zn/l < EC_{50} < 800 mg Zn/l) (Adams et al. 2006; Heinlaan et al. 2008; Li et al. 2010), multiple generations of algae (0.042 mg Zn/l < EC_{50} < 0.068 mg Zn/l) (Aruoja et al. 2009; Franklin et al. 2007), protozoa (4.3 mg Zn/l < EC_{50} < 8.3 mg Zn/l) (Mortimer et al. 2010), nematoda (1.8 mg Zn/l < EC_{50} < 789 mg Zn/l) (Ma et al. 2009; Wang et al. 2009), crustacea (0.15 mg Zn/l < EC_{50} < 18 mg Zn/l) (Wiench et al. 2009; Heinlaan et al. 2008; Poynton et al. 2011) and fish (1.4 mg Zn/l < EC_{50} < 18.5 mg Zn/l) (Bai et al. 2010; Zhu et al. 2008; Zhu et al. 2009a). The chronic toxicity of ZnO nanoparticles is less well documented including some ZnO nanoparticle toxicity studies on soil species (Hooper et al. 2011; Kool et al. 2011) but studies on aquatic species are still very limited (Hao and 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 EC_{50} concentrations ranging from 0.5 to 18 mg Zn/l (Heinlaan et al. 2008; Wiench et al. 2009; Zhu et al. 2009b; Poynton et al. 2011; Zhao et al. 2012). So far, at the chronic level, only one study has studied the effect of

ZnO nanoparticles (with a NOEC value of 0.00064 mg Zn/l and a LOEC value of 0.0032 mg Zn/l) on *Daphnia 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 *Daphnia magna*. Acute EC_{50} concentration values (obtained from the USEPA ecotox database) were mostly between 0.1 mg Zn/l and 14 mg Zn/l (Bringmann and Kühn 1977; Biesinger and 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_{50} values were between 0.091 and 1 mg Zn/l (Heijerick et al. 2003; Muyssen and Janssen 2005) and NOEC values ranged from 0.08 to 1 mg Zn/l (Heijerick et al. 2003; Muyssen and 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, which leads to higher stability. If

these charges are smaller, nanoparticles tend to aggregate (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) are able to 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 particles and stabilizes them by decreasing the aggregation (Keller et al. 2010; Zhang et al. 2009). In contrast, higher ionic strengths (e.g. 10 mM NaCl in ultrapure water) enhance the aggregation of some nanoparticles (eg. Zn; Zhou and Keller (2010)).

Several techniques have been used and developed in order 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 and 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 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. Other techniques have been used to directly measure or visualize nanoparticles and their dissolved or aggregated fraction without physical separation. As such, dynamic light scattering (Kato et al. 2010), flow-field flow fractionation (Calzolai et al. 2011), ultraviolet-visible analysis (Seo et al. 2011) and scanning- and/or transmission electron microscopy (Soto et al. 2005; Hondow et al. 2011) have been used to measure the size of nanoparticles and their aggregates. Recently (David et al. 2012)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 *Daphnia magna*, a filter feeding freshwater crustacean used as model organism in ecotoxicological testing. It was already shown previously that the acute toxicity of ZnO particles was largely attributable to the dissolution of the zinc ions form the nanoparticles. However, whether this is also the case in chronic scenarios remained an open question. To our knowledge this is one of the first papers studying the chronic toxicity of ZnO nanoparticles in *Daphnia magna* in combination with a full dynamic nanoparticle characterisation in the exposure medium.

Methods

Test materials and characterization

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 to obtain a stock solution of 10 g/l, which was shaken and sonicated for 5-6 hours in a sonication bath. The size and shape of the particles were visualized by transmission electron microscopy (TEM, Philips CM200 FEGTEM) after drying of the suspension (required for this microscopic technique). The size of the particles in suspension was characterized by dynamic light scattering (DLS, Malvern Z-sizer NS).

Test species

The freshwater crustacean *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 afterwards the daphnids were fed with $4x10^5$ algae cells/ml (*Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio).

Chronic toxicity of ZnO nanoparticles and $ZnCl_2$ to Daphnia magna

The effect of the ZnO nanoparticles and $ZnCl_2$ salt was tested on the growth and reproduction of *D. magna* in a chronic test scenario according to OECD guidelines 211 (OECD 2008). Immediately before the exposure, a newly (from dry powder) made stock solution (50 mg/l in OECD recommended ISO test medium: $CaCl_2.2H_2O$: 0.294 g/l, $MgSO_4.7H_2O$: 0.123 g/l, $NaHCO_3$: 0.065

g/l, KCl: 0.006 g/l, water hardness 250 mg $CaCO_3$/l, pH 7.8-8.2, conductivity 617 µS/cm (OECD 2004)) of the ZnO nanoparticles was sonicated for 30 min in a sonication bath to maximise the particle dispersion and obtain a homogenous solution without visually aggregated nanoparticles. The $ZnCl_2$ stock solution (50 mg/l in OECD ISO test medium) was not sonicated. Subsequently, small volumes of these stocks were added to OECD 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 (5 minutes after sonication) after spiking. For the zinc salts exposure concentrations 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 made . One daphnid (< 24 h) was transferred to 100 ml of test medium (OECD 2008), in 10 replicate test vessels, for the different exposure concentrations and blanks. *D. magna* were fed with $4x10^5$ 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. During the exposure period of 21 days, the number of living offspring was counted daily and removed from the medium. At the end of the experiment also the length of the adult daphnids was determined.

Accumulation of ZnO nanoparticles and $ZnCl_2$ in and on Daphnia magna

The accumulation of ZnO nanoparticles and $ZnCl_2$ in and on *Daphnia magna* was analysed by measuring the zinc concentration of the adults. After 21 days of exposure the adult daphnids were pooled per concentration, shortly rinsed with pure water and put in polypropylene bullet tubes in a dry oven at 60°C for at least 48 h until a constant dry weight. To each tube containing

dried daphnids, 50 µL HNO_3 (69%) and (after 12 hours) 50 µL H_2O_2 (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). After cooling down and dilution of the samples to 1% HNO_3, the zinc concentration of the daphnids was measured by ICP-OES (Thermo scientific 6000 series).

Zinc concentration and exposure conditions

Samples of the exposure medium were taken to determine zinc concentrations and speciation of the ZnO nanoparticles and $ZnCl_2$. A series of filtration methods were used to distinguish between the total, nanoparticle and dissolved zinc concentration i.e. the aggregated fraction (retained on a 100 nm or 450 nm filter or unfiltered), the nanoparticle fraction (retained on a 1 kDa filter but passing through a 100 nm filter) and the dissolved fraction (passing through a 1kDa filter). As the *D. magna* medium was renewed every 48 h, samples were taken after 0 h (directly after renewal) and after 48 h (directly before renewal) in three replicate test vessels. The 100 nm (Puradisc PTFE) and 450 nm (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 filtered and unfiltered samples were acidified to 1% HNO_3 and the zinc concentration was analysed by ICP-MS (Agilent technologies 7700 series). Additionally, voltammetric measurements were carried out to measure the ionic zinc (Zn^{2+}) concentration in the medium. AGNES (Absence of Gradients and Nernstian Equilibrium Stripping (Galceran et al. 2004)) was used to measure the Zn^{2+} 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 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 discounted from the faradaic current measurements (David et al. 2012). The pH, temperature (°C) and oxygen concentrations (%) of the exposure medium was checked regularly.

Data analysis and statistics

Graphpad Prism (version 5.04) was used for data visualization and statistics. Based on the chronic toxicity data, time to first brood, time between broods and number of broods per female were calculated. Dose-response curves were constructed and $EC_{10, 20, 50}$ reproduction values (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 analysis were performed for the length and the accumulation data. The differences in zinc concentrations obtained by the different filtration procedures were compared in a one-way ANOVA, with Tukey´s multiple comparison post test. The measured free Zn^{2+} concentration was compared with the expected theoretical free zinc concentration (Visual Minteq).

Results

Characterization of test material

A TEM analysis (Figure 1 left) shows that the ZnO nanoparticles are spherical particles with monophasic hexagonal wurtzite crystal structure and with primary diameters between 20 and 40 nm. Based on the DLS analysis (Figure 1 right), it is clear that the nanoparticles formed aggregates 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. Here for most concentrations, no significant difference could be detected when comparing the different unfiltered and filtered treatments in a one-way ANOVA. Taking into account the non-neglible complexation of Zn by the used buffer (TRIS, added to stabilize the pH during the measurement) and other inorganic species present in OECD medium, the measured free Zn concentration (Figure 2a) agreed with theoretical speciation values for the zinc salt. According to speciation computations, the concentration of Zn complexed by TRIS (at a concentration 0.02M and at pH=8.03) in this medium is expected to be 22% higher than the concentration of free Zn.

Directly after exposing the daphnids to ZnO nanoparticles, a large part of the particles appears to have dissolved. An average dissolution (fraction lower than 1 kDa) of 85.5 ± 19.2 % can be seen (0 h, Figure 3a). However, at this time, some particles were still present in the medium (see that the unfiltered fraction is still the highest in Figure 3a at each exposure concentration) and had not dissolved in the time elapsed between dispersion preparation and the different

measurements (samples were taken 1.5 to 2 hours after spiking of the exposure solutions; 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 seem to be present in the OECD medium, since the concentration after filtration over a 1 kDa filter was mostly larger than over a 100 nm filter. DLS-analysis performed directly after spiking of the exposure solutions confirmed these results. At the exposure concentration of 0.024 mg Zn/l, 0.064 mg Zn/l and 0.16 mg Zn the sizes of these aggregates were 372.8 nm, 279.8 nm and 244.4 nm. After 48 hours of exposure (Figure 3b), most of these aggregates had dissolved (on average 90.9 ± 2.5 %), which can be seen by the decline in concentration difference between the unfiltered and the filtered samples. For the highest exposure concentration these differences were not even significant. The higher dissolution (similar to the zinc salt) can be confirmed by the slight increase in the free Zn^{2+} concentration after 48 hours (Figure 2b).

The other exposure medium characteristics showed little variation during the 48 hours of exposure and were not dependent on the exposure concentration. In the ZnO nanoparticle exposure, average pH values of 8.03 ± 0.09 were measured, while in the $ZnCl_2$ 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 salts). The O_2 concentrations were 99.5 ± 2.4 % for ZnO nanoparticles and 97.1 ± 3.0 % for $ZnCl_2$. The ionic strength of the blank and the exposed OECD medium was 0.0119 M (calculated with Visual Minteq).

Chronic toxicity of ZnO nanoparticles and $ZnCl_2$ to Daphnia 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. For both nanoparticles and salts, the number of neonates in the two highest concentrations was significantly different from the blank ($p < 0.01$). In a dose-response curve (Figure 4) the reproduction (% of blank) is indicated in function of the exposure concentration. Based on measured total zinc concentrations, EC_{10}, EC_{20} and EC_{50} values of 0.029 (95% CI: 0.009-0.090) mg Zn/l, 0.048 (0.023-0.099) mg Zn/l and 0.113 (0.072-0.177) mg Zn/l for ZnO nanoparticles were calculated (Figure 4a). A NOEC of 0.058 mg Zn/l was found, while the LOEC was below 0.113 mg Zn/l. In the case of $ZnCl_2$ (Figure 4b) an EC_{10} value of 0.014 (0.004-0.045) mg Zn/l, an EC_{20} value of 0.027 (0.012 – 0.060) mg Zn/l and an EC_{50} value of 0.082 (0.045 – 0.149) 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 below 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 salts (Table 1).

The adult daphnids had average lengths of4.6 ± 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 $ZnCl_2$, 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.

Accumulation of ZnO nanoparticles and $ZnCl_2$ in and on Daphnia 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 $ZnCl_2$ ($p < 0.001$, Figure 5b). The zinc concentration of the adults increased with increasing exposure concentration. As such, the concentration in the blanks was 0.055 ± 0.007 µg Zn/daphnia and increased to 0.116 ± 0.002 µg/daphnia for the highest nanoparticle exposure (0.131 mg Zn/l). Similar Zn accumulation results could be seen for the daphnids exposed to the zinc salt.

Discussion

The results showed that directly after the exposure to the ZnO nanoparticles (0 hours), *D. magna* was for a large part indeed already exposed to dissolved zinc. However, at this time 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 to 40 nm, one would expect a small enhancement of the solubility compared to bulk ZnO (David et al. 2012). Even if the prediction is eventually full dissolution, one must 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 and 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 our 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 and Keller (2010) have shown that at a pH of 8.1 (comparable to our 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). Thus, directly after exposure (samples taken 1.5 to 2 hours after making the stock solution), given the large aggregation, there are still ZnO nanoparticles present in the medium that have not yet dissolved and are retained on the various filters. The zinc salts dissolved immediately in the stock solution (50 mg/l) and therefore only dissolved zinc was added to the exposure medium.

Within the first 48 hours of exposure, these aggregates had mostly dissolved. This high dissolution can be explained by the low chronic exposure concentrations (below the maximal solubility of ZnO at this pH) that were used. At these low concentrations the nanoparticles tend to dissolve faster since the dissolution rate is proportional to the difference between the free Zn^{2+} concentration and the one given by the solubility of ZnO in equilibrium. This can be presented by a first order reaction (i.e. Noyes−Whitney equation with C = the concentration in solution at time t, C_s = the solubility of ZnO in equilibrium, k = first order release rate constant (Peng et al. 2011) from Costa and Sousa Lobo (2001)).

$$C = C_s\left(1 - e^{-kt}\right)$$

After 48 hours, 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 to 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 to 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 exposure experiment.

For both the nanoparticles and the salts, Zn^{2+} concentrations measured by AGNES were much lower than the dissolved concentrations measured after filtration over a1 kDa filter. In a system without nanoparticles the 1 kDa filter provides the soluble fraction (i.e. the free Zn^{2+} and dissolved Zn complexes formed with component of the OECD medium e.g. $ZnCl^{+}$, $ZnHCO^{3+}$, $Zn(SO4)2^{2-}$), while AGNES provides just the free Zn^{2+} ion concentration. For $ZnCl_2$, taking into account the non-neglible 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 modeling (using Visual Minteq). The proximity of the ZnO nanoparticles and $ZnCl_2$ measured free zinc concentration and the theoretical dissolution supports to 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 $ZnCl_2$. Our results showed that at sublethal exposure concentrations ZnO nanoparticles were able to affect the reproduction of *D. magna*, associated 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 $ZnCl_2$, a similar toxicity (Figure 4b) and zinc accumulation (Figure 5b) can be seen. The comparable toxicity of ZnO nanoparticles and $ZnCl_2$ has already been shown before for 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, similar to zinc salts 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 Muyssen and 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. Taking into account that 1 adult 21 day old daphnia equals to 306 µg dry weight (Ghazy and Habashy, 2003), the two highest ZnO nanoparticle exposure concentrations with corresponding Zn accumulations of 379 ± 6 µg Zn/g dry weight and 329 ± 3 µg Zn/g dry weight are toxic to daphnia. For the zinc salt this was the case for the second highest exposure with a corresponding zinc accumulation of 391 ± 3 µg Zn/g dry weight (for the highest exposure not enough daphnids survived to measure accurate accumulation concentrations).Based on

theoretical predicted environmental concentrations (PEC) of ZnO nanoparticles (0.01 µg/l) in European surface water (Gottschalk et al. 2009), we can say that there is no current risk for adverse environmental effects when looking at the chronic toxicity results. The lowest calculated effect of ZnO nanoparticles on daphnia reproduction (EC_{10}: 0.029 mg Zn/l) was more than 3000 times higher than this PEC value.

Taking into account the above-mentioned factors, we would like to highlight that under the tested circumstances, the NanoSun type of ZnO nanoparticle has a similar toxicity as the inorganic salt and is mostly dissolved after 48 hours of exposure. However, 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 and 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 is able to influence the toxicity to cancer cells caused by ZnO nanoparticles (Nair et al. 2009). The exposure concentration may as well influence the uptake and toxicity. 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 daphnids exposed to sublethal ZnO nanoparticles and zinc salts was followed (Poynton et al. 2011). These results showed that distinct sets of genes were activated by the nanoparticles and zinc salts.

In this study, the combination of the similar toxicity and zinc accumulation of ZnO nanoparticles and $ZnCl_2$ and the almost complete dissolution of ZnO NP (after 48 hours of exposure) indicates that the toxicity of the tested ZnO nanoparticles is largely due to soluble zinc, rather than the initially formed aggregates directly after exposure. Future studies should focus on sublethal effects of nanoparticles under different exposure scenarios.

Conclusions

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

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Declaration of Interest

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Tables

Table 1. Chronic effects (time to first brood, time between broods, broods per female with standard deviations) of ZnO nanoparticles and $ZnCl_2$ exposure at different measured exposure concentrations (unfiltered samples after 0 hours of exposure).

	Measured exposure (mg Zn/l)	Time to first brood (days)	Time between broods (days)	Broods per female (number)
ZnO nanoparticle	0.008 (blank)	9.3 ± 0.8	2.3 ± 0.9	3.5 ± 0.7
	0.009	9.5 ± 0.7	2.2 ± 0.4	3.9 ± 0.3
	0.014	9.3 ± 0.5	2.4 ± 0.5	3.5 ± 1.3
	0.027	9.6 ± 0.7	2.3 ± 0.5	3.9 ± 0.3
	0.058	9.5 ± 0.7	2.3 ± 0.5	3.3 ± 0.5
	0.131	10.2 ± 1.0	2.3 ± 0.5	2.4 ± 1.0
$ZnCl_2$	0.006 (blank)	9.0 ± 0.9	2.3 ± 0.4	4.2 ± 0.4
	0.013	9.0 ± 1.0	2.2 ± 0.5	4.1 ± 0.4
	0.023	9.2 ± 0.9	2.3 ± 0.5	3.9 ± 0.3
	0.04	9.0 ± 1.3	2.3 ± 0.5	3.8 ± 1.2
	0.126	10.2 ± 1.3	2.4 ± 0.5	2.1 ± 0.8
	0.313	11.7 ± 3.8	2.5 ± 0.7	1.0 ± 1.0

Figures

Figure 1.

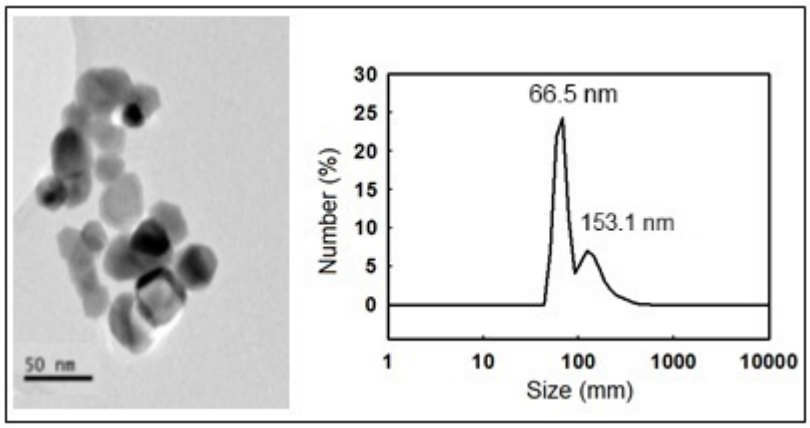
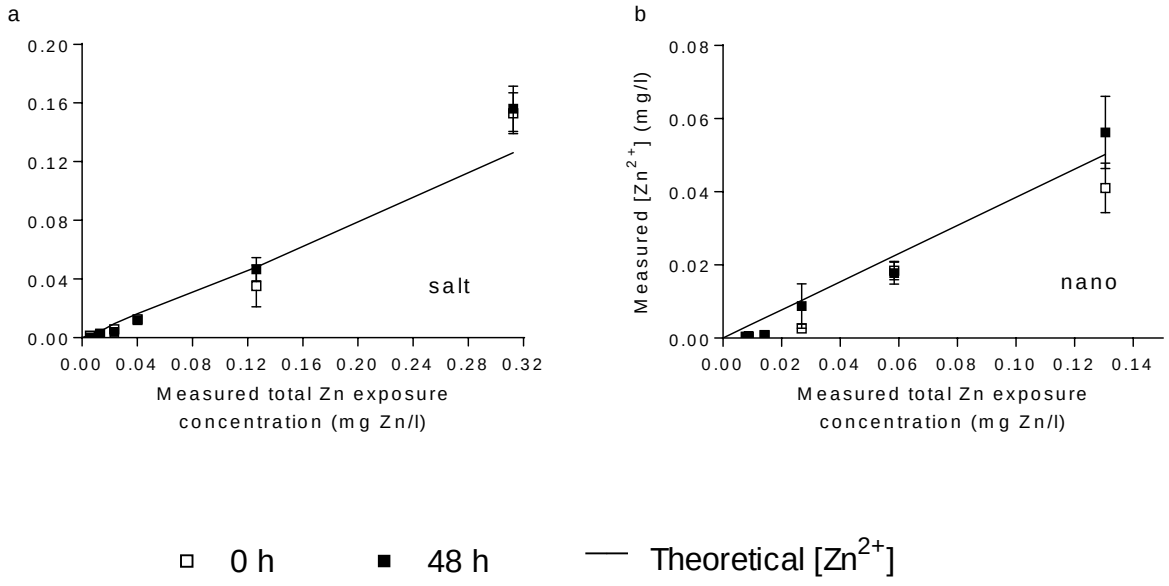


Figure 2.



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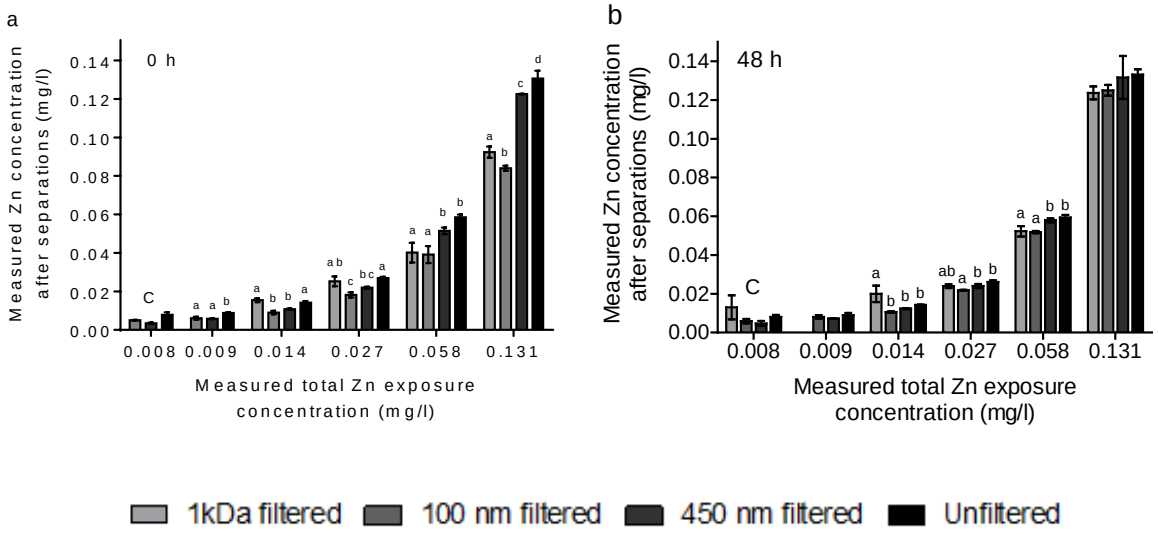

Figure 4.

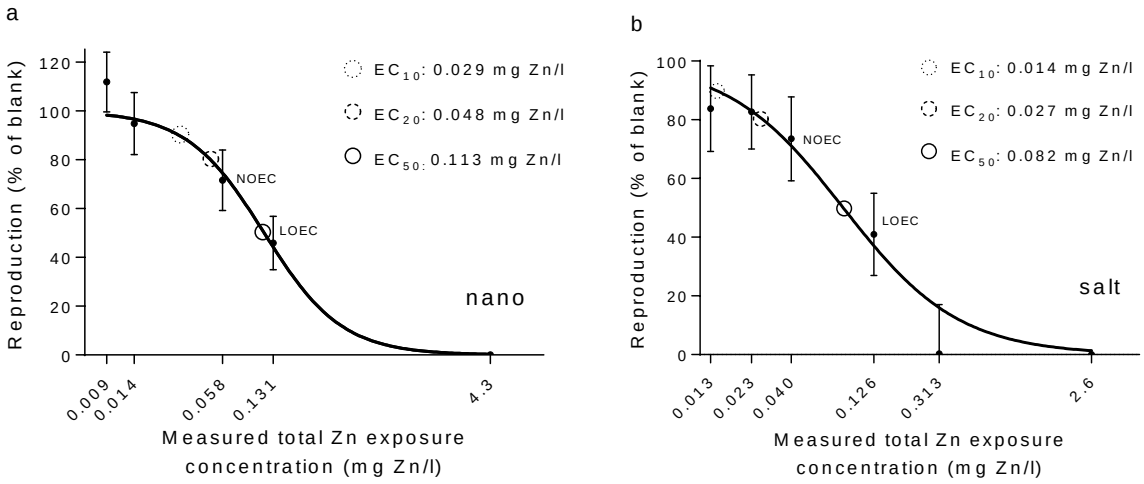

Figure 5.

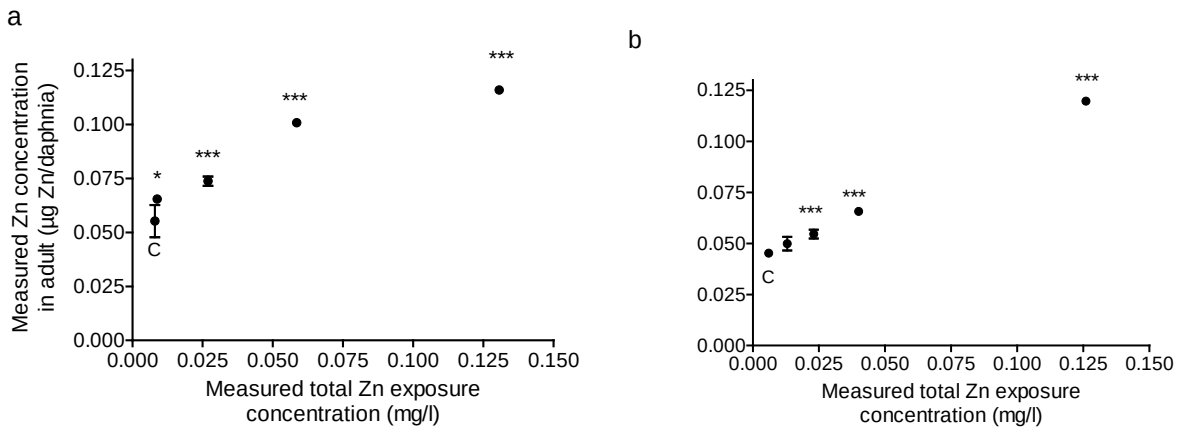

Figure captions

Figure 1. Characterization of the ZnO nanopowder with a TEM image (left) and DLS analysis (right) in pure water.

Figure 2. The measured free zinc concentration (Zn^{2+}) for $ZnCl_2$ (a) and the ZnO nanoparticles (b) after 0 hours and 48 hours of exposure in function of the measured total Zn exposure concentration (unfiltered samples after 0 hours 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 hours of exposure.

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) fractions when exposed to different concentrations of ZnO nanoparticles (measured unfiltered samples after 0 hours 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 $ZnCl_2$ (b) indicated as a dose-response curve (reproduction (% of blank)) with standard deviation in function of measured total zinc exposure concentration (unfiltered samples taken directly after exposure, on a log-scale, mg Zn/l). A one-way ANOVA showed significant differences. The calculated effect concentration values for ZnO nanoparticles were 0.029 (95% confidence interval: 0.009-0.090) mg Zn/l (EC_{10}), 0.048 (0.023-0.099) mg Zn/l (EC_{20}) and 0.113 (0.072-0.177) mg Zn/l (EC_{50}), while NOEC and LOEC values were 0.058 mg Zn/l and <0.113 mg Zn/l For $ZnCl_2$ these values

were 0.014 (0.004-0.045) mg Zn/l (EC_{10}), 0.027 (0.012 – 0.06) mg Zn/l (EC_{20}), 0.082 (0.045 – 0.149) mg Zn/l (EC_{50}), 0.040 mg Zn/l (NOEC) and < 0.126 mg Zn/l.

Figure 5. The accumulation of Zn in daphnia when exposed to ZnO nanoparticles (a) and $ZnCl_2$ (b). The measured Zn concentration per daphnia in function of the total Zn exposure concentration (measured unfiltered samples after 0 hours 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).