

Toxicity and Accumulation of Cu and ZnO Nanoparticles in *Daphnia magna*

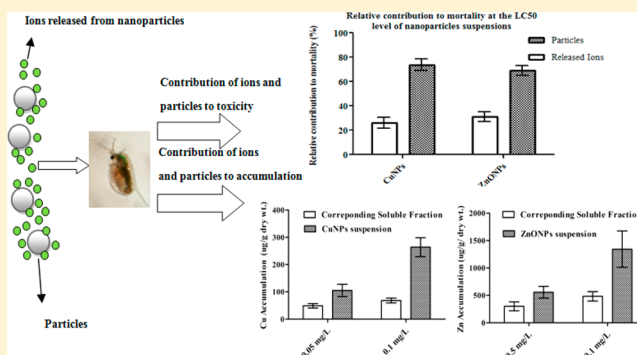
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Supporting Information

ABSTRACT: There is increasing recognition that the wide use of nanoparticles, such as Cu (CuNPs) and ZnO nanoparticles (ZnONPs), may pose risks to the environment. Currently there is insufficient insight in the contribution of metal-based nanoparticles and their dissolved ions to the overall toxicity and accumulation. To fill in this gap, we combined the fate assessment of CuNPs and ZnONPs in aquatic test media with the assessment of toxicity and accumulation of ions and particles present in the suspensions. It was found that at the LC50 level of *Daphnia magna* exposed to the nanoparticle suspensions, the relative contributions of ions released from CuNPs and ZnONPs to toxicity were around 26% and 31%, respectively, indicating that particles rather than the dissolved ions were the major source of toxicity. It was additionally found that at the low exposure concentrations of CuNPs and ZnONPs (below 0.05 and 0.5 mg/L, respectively) the dissolved ions were predominantly accumulated, whereas at the high exposure concentrations (above 0.1 mg/L and 1 mg/L, respectively), particles rather than the released ions played a dominant role in the accumulation process. Our results thus suggest that consideration on the contribution of dissolved ions to nanoparticle toxicity needs to be interpreted with care.



INTRODUCTION

Nanoparticles (NPs) are defined as particles with at least one dimension between 1 and 100 nm. Due to NPs' small size, they usually show unique physicochemical properties, such as high surface area and high mechanical strength. NPs are increasingly used in many applications. Cu nanoparticles (CuNPs) and ZnO nanoparticles (ZnONPs) have been manufactured on a large scale in different areas. For instance, CuNPs have been widely employed in catalysis and batteries,^{1,2} and ZnONPs have been extensively used in cosmetics and UV-absorbers.³ Like other types of NPs, the recent increasing use of CuNPs and ZnONPs has also started to induce concern on their toxicity to some specific aquatic organisms, such as mussel⁴ and juvenile carp.⁵ Hence, there is a necessity to investigate the underlying processes resulting in the toxicity of CuNPs and ZnONPs to aquatic organisms.

Toxicity of NPs may be exerted by particles (designated as NP_(particle) hereafter),^{6,7} by dissolved ions released from NPs (designated as NP_(ion) hereafter),^{8,9} or by both NP_(particle) and NP_(ion).¹⁰ Undoubtedly, determining the contribution of NP_(particle) and NP_(ion) to the overall toxicity of nanoparticle suspensions (designated as NP_(total) hereafter), is a crucial step in assessing and managing the possible adverse effects of NPs. Based on the existing literature, it is still hard to get a uniform conclusion about what is the major source of toxicity of NPs.

This may be due to the fact that many factors can affect the toxicity performance of NPs, not only the characteristics of the NPs, but also the exposure conditions.¹¹ Thus, the underlying physicochemical mechanisms leading to the toxicity of different NPs to aquatic organisms should be investigated on a case by case basis, which suggests that more research should be performed to make a comprehensive consideration about this research question.

In the present study, *Daphnia magna* was selected as a model organism. The 48 h toxicity of CuNPs and ZnONPs to *D. magna* was determined. To analyze the relative contribution of NP_(ion) to the overall toxicity of CuNPs and ZnONPs (designated as CuNP_(total) and ZnONP_(total) hereafter, respectively), centrifugation and filtering methods were combined to separate the dissolved ions released from the NPs. Subsequently, the 48 h toxicity of the supernatants only containing NP_(ion) to daphnids was investigated. In order to test whether using metal salts as substitutes for the dissolved ions released from NPs is effective, the toxicity of dissolved Cu released from CuNPs (designated as CuNP_(ion) hereafter) and

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dissolved Zn released from ZnONPs (designated as ZnONP_(ion) hereafter) to daphnia neonates was compared to the toxicity of Cu(NO₃)₂ and Zn(NO₃)₂. Additionally, the issue which species, NP_(particle) or NP_(ion), plays a major role in the accumulation process was investigated in this study.

MATERIALS AND METHODS

Test Materials, Test Medium, and Test Species. CuNPs with a nominal size of 50 nm (advertised specific surface area, 6–8 m²/g; purity, 99.8%) and ZnONPs with a nominal size of 43 nm (advertised specific surface area, 27 m²/g; purity, 99.5%) were purchased from IoLiTecGmbH (Heibronn, Germany). Both CuNPs and ZnONPs were spherically shaped. Cu(NO₃)₂ and Zn(NO₃)₂ were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Stock nanoparticle suspensions and salts were freshly prepared in ISO standard test medium (STM) after 20 min sonication in a water bath sonicator. The STM used in this study (pH 7.8 ± 0.2) contained (mg/L Milli-Q water): CaCl₂·2H₂O: 294; MgSO₄·7H₂O: 123.25; NaHCO₃: 64.75; KCl: 5.75.¹²

Daphnia magna, originally obtained from the Dutch National Institute for Public Health and the Environment (RIVM), was selected as the test species. Artificial ElendtM4 medium was used to culture *D. magna*,¹³ which was refreshed three times a week. The test organisms were cultured in plastic containers at a density of 1 individual/10 mL of ElendtM4 medium under a 16:8 light-dark cycle (20 ± 1 °C) and fed with *Pseudokirchneriella subcapitata* every 2 days.

Physicochemical Analysis. The morphology and size of CuNPs and ZnONPs in STM were characterized by using transmission electron microscopy (TEM, JEOL 1010, JEOL Ltd., Japan). The samples were analyzed directly (which was around 1 h after submerging NPs into the STM, to which we will refer to as 1 h) and after 24 and 48 h. The size distribution and zeta potential of suspensions of CuNPs and ZnONPs at 1 mg/L were analyzed at 1, 24, and 48 h after incubation in the test medium by dynamic light scattering (DLS) on a zetasizer Nano-ZS instrument (Malvern, Instruments Ltd., UK). The actual exposure concentrations of CuNPs and ZnONPs, as well as Cu(NO₃)₂ and Zn(NO₃)₂ in the STM, were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) after digestion in 65% HNO₃ for at least 1 day. In order to obtain the CuNP_(ion) and ZnONP_(ion), the two nanoparticle suspensions first were centrifuged at 30392 g for 30 min at 4 °C (Sorvall RCSBplus centrifuge, Fiberlite F21-8 × 50y rotor) to obtain the supernatants, which were then filtered through a syringe filter with 0.02 μm pore diameter (Antop 25, Whatman). We also applied ultracentrifugation and then performed DLS to confirm that the concentration of NP_(particle) in the supernatants obtained by centrifugation at 30392 g for 30 min at 4 °C and subsequent filtration through a syringe filter with 0.02 μm pore diameter was less than the detection limit, and the detailed information on the confirmation is given in the Supporting Information 1.

Assessment of the 48 h Release Profiles of Dissolved Cu and Zn. The release profiles of dissolved Cu released from the CuNPs at 0.1 mg/L and 10 mg/L for a maximum of 48 h of exposure and the release of dissolved Zn released from the ZnONPs at 1 mg/L and 10 mg/L for a maximum of 48 h of exposure in the STM were investigated. At different times, namely after being exposed to the medium for 1, 12, 24, 36, and

48 h, CuNPs and ZnONPs samples were centrifuged at 30392 g for 30 min at 4 °C (Sorvall RCSBplus centrifuge, Fiberlite F21-8 × 50y rotor), and then the supernatants were filtered through a syringe filter with 0.02 μm pore diameter (Antop 25, Whatman). Subsequently, the Cu concentrations and Zn concentrations in the supernatants after filtration were measured by ICP-OES. To analyze the losses of NPs and NP_(ion) after centrifugation and filtration, experiments were conducted, and the detailed information on the analysis was given in the Supporting Information 2.

The 48 h Acute Toxicity Test. OECD Guideline 202 with slight modifications was used to test the acute toxicity of the two nanoparticle suspensions. Before the start of the acute toxicity test, daphnids were kept in the STM for 1–2 h to evacuate their guts. The toxicity tests were performed using neonates (<24 h). Five individuals were transferred into a test vial, containing 20 mL of CuNPs or ZnONPs, or metal salt solutions, or control. Based on the results of range finding experiments, exposure concentrations from 0.02 mg/L to 0.16 mg/L for CuNPs and from 0.4 mg/L to 2.6 mg/L for ZnONPs, as well as from 0.01 mg/L to 0.06 mg/L for Cu(NO₃)₂ and from 0.5 mg/L to 1.5 mg/L for Zn(NO₃)₂ were selected. Each test was composed of 8 to 10 different exposure concentrations, and each exposure concentration was tested with 4 replicates. Daphnids were incubated under a 16:8 h light/dark photoperiod (20 ± 1 °C) without feeding during the 48 h exposure period. To avoid significant changes in the concentrations of the nanoparticle suspensions, the exposure media were refreshed every 24 h.

Besides investigating the toxicity of the suspensions of CuNPs and ZnONPs, the relative contribution of NP_(particle) and NP_(ion) to the toxicity induced by the suspensions of CuNPs and ZnONPs was also examined. In order to better simulate the contribution of CuNP_(ion) and ZnONP_(ion) to the overall toxicity, the supernatants after filtration of the two NPs in the STM were prepared as described above. The neonate daphnids were then exposed to the supernatants according to the same procedures as described above. To compare with the toxicity of the freshly prepared solutions of CuNP_(ion) and ZnONP_(ion), the toxicity of Cu(NO₃)₂ and of Zn(NO₃)₂ to *D. magna* was tested with the same procedures as described above, as well.

Accumulation Experiments. Experiments were conducted to identify the accumulation profiles of *D. magna* exposed to CuNPs, ZnONPs, and their corresponding dissolved ions. In brief, *D. magna* (8 d old) were transferred to the suspensions of CuNPs and ZnONPs at a density of 1 individual/10 mL with 3 replicates. The concentrations of CuNPs applied in the accumulation experiments were 0.1 and 0.05 mg/L, and the concentrations of ZnONPs were 1 and 0.5 mg/L, respectively. These concentrations were selected based on the results of the acute toxicity tests described above, which concerned the LC50. Adult daphnids (8 d old) were utilized. We first of all selected adult daphnids because they are more easy to handle, compared to neonates. Moreover, due to physiological (e.g., surface-volume ratio) and behavioral aspects adults are often less susceptible to NPs compared to juvenile and neonate life stages in ecotoxicity studies,^{14,15} which allowed us to analyze accumulation characteristics at high concentration of nanoparticle suspensions. Finally, it is advantageous to use adults as growth dilution may be reduced.^{16,17} In order to distinguish between the contribution of NP_(particle) and NP_(ion) to accumulation, *D. magna* were also exposed to the supernatants

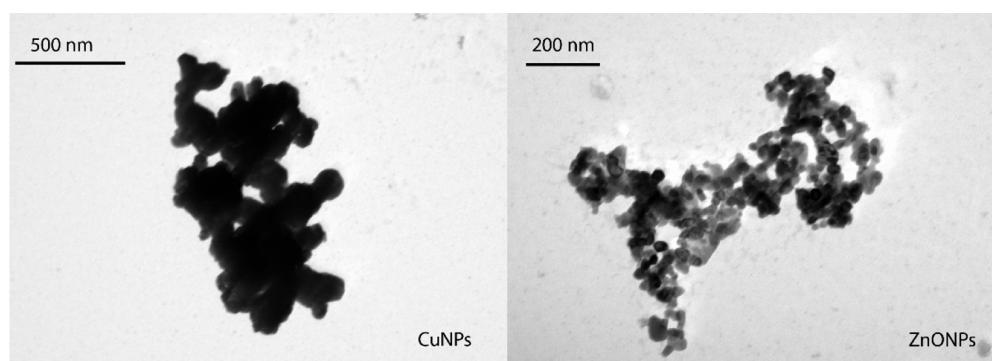


Figure 1. TEM images of CuNPs and ZnONPs after 1 h of incubation in the standard test medium (STM).

Table 1. Hydrodynamic Diameter and Zeta-Potential of 1 mg/L Suspensions of CuNPs and ZnONPs in the STM^a

type	hydrodynamic diameter (nm)			zeta potential (mV)		
	1 h	24 h	48 h	1 h	24 h	48 h
CuNPs	568 ± 72	879 ± 228	953 ± 525	−14 ± 4	−9 ± 3	−4 ± 2
ZnONPs	1154 ± 252	1647 ± 129	1871 ± 509	−10 ± 2	−6 ± 1	−3 ± 2

^aHydrodynamic size and zeta potential were expressed as mean ± SD ($n = 3$).

of the CuNPs at the initial CuNPs concentrations of 0.1 mg/L and 0.05 mg/L and to the supernatants of ZnONPs at the initial ZnONPs concentrations of 1 mg/L and 0.5 mg/L. These supernatants were prepared as described above. Many recent studies have shown that the accumulation of NPs in *D. magna* is a rapid process.^{18–20} Hence, a 48 h exposure period was selected in our test. Similar to the acute toxicity tests, the accumulation experiments were conducted under a 16:8 h light/dark photoperiod (20 ± 1 °C) without feeding during the 48 h exposure period. The exposure media were refreshed every 24 h. After the 48 h exposure period, 10 mobile daphnids were sampled from each exposure medium, and they then were transferred to Milli-Q water for 1–3 min. Subsequently, they were rinsed three times with fresh Milli-Q water. After rinsing, they were dried at 80 °C overnight in preweighed glass containers before weighing on a microbalance and then digested in 69% HNO₃ at 80 °C overnight. The Cu and Zn concentrations in the digested samples were subsequently determined by ICP-OES.

To clarify in this study, accumulation is defined as the absorbed and adsorbed metals after rinsing the daphnids. As after rinsing the daphnids with Milli-Q water, NPs may not be completely removed and may still be adsorbed to the outside of organisms. Surface adsorption of NPs onto the exterior of *D. magna* limits their biological activity and also may pose risks to their health development. For example, Dabrunz et al.²¹ found that adsorbed NPs caused mortality through reducing the molting rate of *D. magna*. Hence, both absorbed and adsorbed fractions are considered in this study.

Relative Contribution to Toxicity of NP_(particle) and NP_(ion). The neonates of *D. magna* were exposed to the nanoparticle suspensions containing a mixture of NP_(particle) and NP_(ion). The behavior and toxicity of chemicals in a mixture may not conform to that predicted from data on pure compounds.²² Complicated and remarkable changes in the apparent properties of its constituents can be induced by interactions of components in a mixture, leading to increased or decreased effects compared with the ideal reference case of additive behavior. For evaluation of the joint toxicity of mixtures, the concentration addition (CA) model and the

independent action (IA) model are two prominent reference models, both of which have been mechanistically supported by pharmacology.²² Which model is preferably employed to analyze the combined effects of chemicals in a mixture is based on the mode of action.²² Specifically, the CA model can be utilized to estimate the combined effects of chemicals in a mixture with a similar mode of action, whereas the IA model is employed to analyze the joint effects of chemicals with dissimilar mode of actions.²² The response addition model is often used as a synonym for the IA model.²³ Based on the previous literature, it is widely believed that the modes of actions of NP_(particle) and NP_(ion) are likely to be dissimilar.^{24,25} Thus, the response addition model was selected in the present study to calculate the relative contribution to toxicity of NP_(particle) and NP_(ion). The response addition model is defined as follows

$$E_{(\text{total})} = 1 - [(1 - E_{(\text{ion})})(1 - E_{(\text{particle})})]$$

where $E_{(\text{total})}$ and $E_{(\text{ion})}$ represent the toxicity caused by the nanoparticle suspensions and their corresponding released ions (scaled from 0 to 1). In the present study, $E_{(\text{total})}$ and $E_{(\text{ion})}$ were quantified experimentally. This makes $E_{(\text{particle})}$ as the only unknown, allowing for direct calculation of the effects caused by the NP_(particle).

Statistical Analysis. All data are expressed as the mean with the corresponding standard deviation (SD). The LC50 values and 95% confidence intervals (95% CI) were calculated by GraphPad Prism 5 using nonlinear regression. Based on normality and homogeneity of variance, statistically significant differences between accumulation groups were determined by *t*-test. The significance level in all calculations was set at $p < 0.05$.

RESULTS

Physicochemical Characterization of CuNPs and ZnONPs. TEM images of CuNPs and ZnONPs are presented in Figure 1. The images demonstrate that the CuNPs and ZnONPs used in this study were spherical particles. However, the CuNPs and ZnONPs aggregated intensely into irregular shapes in the STM. DLS was performed for determining the

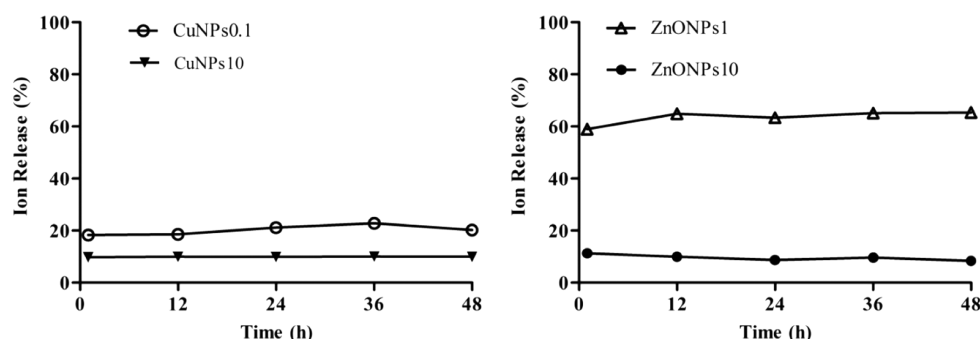


Figure 2. Relative percentages of dissolved Cu released from CuNPs at the concentrations of 0.1 mg/L and 10 mg/L and of dissolved Zn released from ZnONPs at the concentrations of 1 mg/L and 10 mg/L, during 48 h of incubation in the STM. Data are mean $\pm$ SD ($n = 3$).

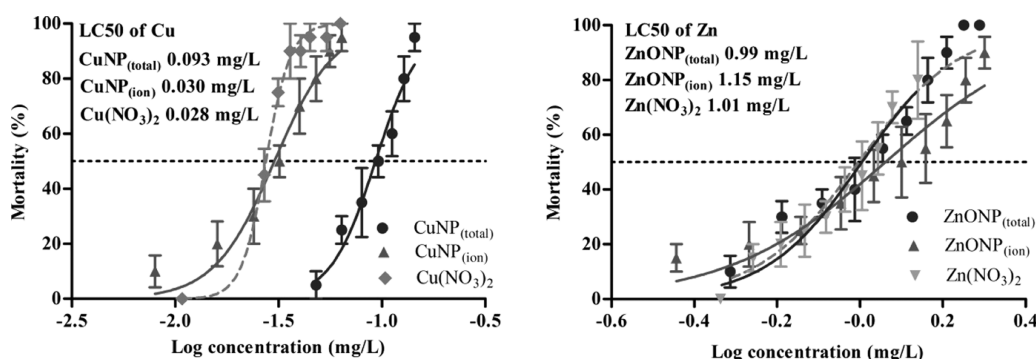


Figure 3. Dose–response curves of mortality (%) of *D. magna* exposed to different concentrations of CuNP_(total), ZnONP_(total), CuNP_(ion), ZnONP_(ion), Cu(NO₃)₂, and Zn(NO₃)₂ for 48 h. Actual log-transformed Cu or Zn concentrations are plotted on the x-axis. Data are mean $\pm$ SD ($n = 4$).

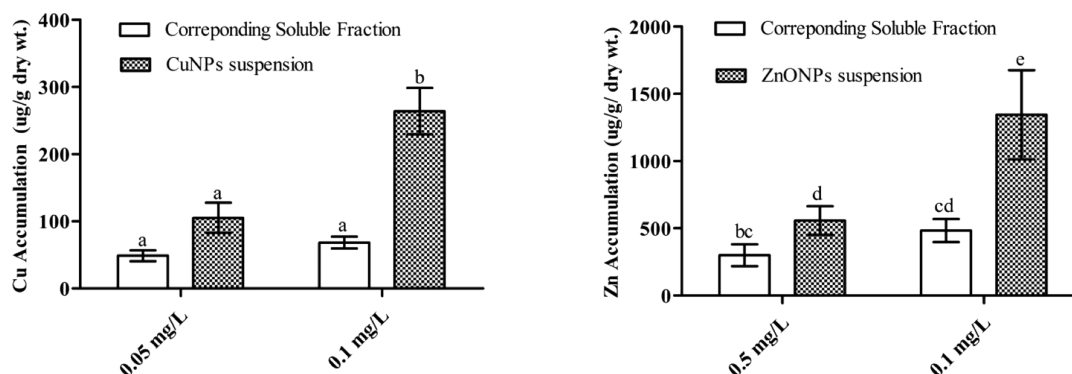


Figure 4. Accumulation ($\mu\text{g/g}$ dry wt.) of Cu and Zn in *D. magna* after exposure for 48 h to 0.05 mg/L and 0.1 mg/L of CuNPs suspensions and to 0.5 mg/L and 1.0 mg/L of ZnONPs suspensions, the corresponding dissolved Cu released from the above concentrations of CuNPs, and the corresponding dissolved Zn released from the above concentrations of ZnONPs. The different letters indicate the significant differences of accumulation levels, $p < 0.05$; The p -value between different accumulation groups was determined by means of the t -test.

hydrodynamic diameter of the CuNPs and ZnONPs suspended in STM. Data on size distributions after 1, 24, and 48 h are given in Table 1. Both NPs aggregated as soon as being submerged into the STM. The particle size of CuNPs in the STM was found to increase from 568 ± 72 nm after 1 h of incubation to 953 ± 525 nm after 48 h of incubation, and the particle size of ZnONPs shifted from 1154 ± 252 nm after 1 h of incubation to 1871 ± 509 nm after 48 h of incubation. The zeta potential of the two nanoparticle suspensions increased after NPs being submerged into the medium. The zeta potential of the CuNPs increased from -14 ± 4 after 1 h of incubation to -4 ± 2 after 48 h of incubation. Similarly, the zeta potential of the ZnONPs increased from -10 ± 2 after 1 h of incubation to -3 ± 2 after 48 h of incubation.

The 48 h Ion Release Profiles of CuNPs and ZnONPs.

The 48 h release profiles of CuNPs of ZnONPs are shown in Figure 2. The percentage of CuNP_(ion) in the CuNPs suspension at 0.1 mg/L shifted from 18% after 1 h of incubation to 20% after 48 h of incubation. The ZnONPs at 1 mg/L showed a relatively high degree of dissolution. The percentage of the ZnONP_(ion) increased from 59% after 1 h of incubation to 65% after 48 h of incubation in the STM. As for the percentages of the CuNP_(ion) and ZnONP_(ion) in the nanoparticle suspensions at 10 mg/L, both of them remained around 10% during the 48 h of incubation.

The 48 h Acute Toxicity of CuNPs and ZnONPs. All the exposures of neonate daphnids to the nanoparticle suspensions and their dissolved ions induced significant toxicity

(Figure 3). CuNP_(total) showed a much lower 48 h LC50 value of 0.093 mg/L (with a 95% CI of 0.86–0.101 mg/L), compared to ZnONP_(total) (0.99 mg/L, with a 95% CI of 0.92–1.07 mg/L). The LC50 value of CuNP_(ion) was 0.030 mg/L (with a 95% CI of 0.027–0.034 mg/L), and the LC50 value of the ZnONP_(ion) was 1.15 mg/L (with a 95% CI of 1.02–1.30 mg/L), which indicated that the CuNP_(ion) was also much more toxic (over 10 times) to *D. magna*, compared to the ZnONP_(ion). The dose–response curves for Cu(NO₃)₂ and Zn(NO₃)₂ are also given in Figure 3. The LC50 of Cu(NO₃)₂ to the neonates was 0.028 mg/L (with a 95% CI of 0.026–0.029 mg/L), which was similar to the LC50 value of the CuNP_(ion). Likewise, the LC50 of Zn(NO₃)₂ (1.01 mg/L, with a 95% CI of 0.94–1.09 mg/L) was similar to the LC50 level of the ZnONP_(ion).

The 48 h Accumulation Characteristics of CuNPs and ZnONPs. The accumulation profiles of CuNPs and ZnONPs are shown in Figure 4. At the low CuNPs concentration (0.05 mg/L), the overall accumulation of Cu (105 ± 39 μg/g dry weight) in daphnids was higher than the accumulation of the CuNP_(ion) (49 ± 14 μg/g dry weight). Moreover, at the high CuNPs concentration (0.1 mg/L), the overall accumulation of Cu was 264 ± 60 μg/g dry weight, which was significantly higher than the accumulation of the CuNP_(ion) (68 ± 15 μg/g dry weight, *p* < 0.05). As for the Zn accumulation, the overall accumulation of Zn at 0.5 mg/L of ZnONPs (558 ± 106 μg/g dry weight) was significantly higher than the accumulation of ZnONP_(ion) (301 ± 82 μg/g dry weight, *p* < 0.05). Likewise, the overall accumulation of Zn at 1 mg/L of ZnONPs (1345 ± 331 μg/g dry weight) was significantly higher than the accumulation of ZnONP_(ion) (484 ± 85 μg/g dry weight, *p* < 0.05).

Relative Contribution of NP_(particle) and NP_(ion) to Toxicity and Accumulation. The relative contribution of NP_(particle) and NP_(ion) to the overall toxicity at the LC50 levels of CuNPs and ZnONPs to neonates is given in Table 2. The

Table 2. Relative Contribution of NP_(particle) and NP_(ion) to Mortality at the LC50 Level of the CuNPs and ZnONPs Exposed to Neonate Daphnids^a

type	relative contribution to mortality (%)	
	NP _(particle)	NP _(ion)
Cu	74 ± 5	26 ± 5
Zn	69 ± 4	31 ± 4

^aThe relative contribution of NP_(particle) and NP_(ion) to mortality was expressed as mean ± SD (*n* = 4).

relative contribution of CuNP_(ion) to toxicity was only 26%, whereas CuNP_(particle) accounted for about 74% of the relative contribution to mortality at the LC50 level of CuNP_(total). ZnONP_(ion) only contributed a fraction of 31% to the overall toxicity, compared to the contribution of ZnONP_(particle). In addition, the relative contribution to accumulation of NP_(particle) and NP_(ion) is calculated and shown in Table 3. At the low concentration of CuNPs (0.05 mg/L), CuNP_(ion) contributed around 52% to the overall accumulation, whereas at the concentration of 0.1 mg/L of CuNPs, CuNP_(ion) merely accounted for about 28%. Likewise, at the low concentration of ZnONPs (0.5 mg/L), ZnONP_(ion) could explain about 53% of the overall Zn accumulation, while at the high concentration (1 mg/L of ZnONPs), ZnONP_(ion) only contributed around 36% to the overall Zn accumulation.

Table 3. Relative Contribution of NP_(particle) and NP_(ion) to Accumulation at Different Concentrations of Nanoparticle Suspensions^a

suspensions	relative contribution to accumulation (%)	
	NP _(particle)	NP _(ion)
CuNPs at 0.05 mg/L	48 ± 25	52 ± 25
CuNPs at 0.1 mg/L	72 ± 12	28 ± 12
ZnONPs at 0.5 mg/L	47 ± 5	53 ± 5
ZnONPs at 1 mg/L	64 ± 3	36 ± 3

^aThe relative contribution of NP_(particle) and NP_(ion) to accumulation was expressed as mean ± SD (*n* = 3).

DISCUSSION

Ion Release Profiles of CuNPs and ZnONPs. In this study, ZnONPs were demonstrated to be more soluble than CuNPs. ZnONPs showed an especially rapid dissolution process at a low concentration in the STM. We found that 59% of the ZnONPs at 1 mg/L were already dissolved after 1 h of incubation (Figure 2). This result was similar to the recent result obtained by Adam et al.²⁶ who detected that ZnONPs (with a nominal size of 30 nm) showed 60% of dissolution within 1–2 h in the ISO medium. However, it is worth noting that the percentage of ZnONP_(ion) is reported to vary considerably in the existing literature. For example, Merdzan et al.²⁷ found that the percentage of dissolved Zn released from bare ZnONPs with a nominal size of 20 nm was to a large extent (>85%) present in solution (10^{−2} M HEPES, 10^{−5} M Ca, pH 7.0) after 24 h of exposure. This is higher than the extent of dissolution we found in our study. However, another study reported that the percentages of ZnONP_(ion) were only 30% at 0.5 mg/L of ZnONPs and 20% at 2 mg/L of ZnONPs after 24 h of exposure in simplified M7 medium.¹⁸ This discrepancy is a reflection of the combined effects exerted by the characteristics of NPs and exposure medium, such as, ionic strength and pH of the test medium,¹⁸ presence of coatings,²⁷ and particle size.²⁸ The ion-release profiles of NPs should thus be analyzed on a case-by-case basis. At the high concentration applied in this study (10 mg/L), the percentages of CuNP_(ion) and ZnONP_(ion) were almost constant around 10%, much lower than the percentages of ZnONP_(ion) and CuNP_(ion) at the low concentrations (namely 1 mg/L ZnONPs and 0.1 mg/L CuNPs). Similarly, Zhao and Wang²⁰ found that at a low concentration of Ag NPs (10 μg/L) in SM7 medium, the relative percentage of the dissolved Ag released from Ag NPs could be beyond 50%, while at a high concentration of Ag NPs (1000 μg/L) the percentage of dissolved Ag remained constant at less than 10%. These results indicate that the concentration of NPs is also an important factor to consider regarding the dissolution characteristics of NPs, since the equilibrium between dissolved and adsorbed ions might potentially play a role. Furthermore, it needs to be noted that, especially at high nanoparticle concentrations, the percentage of NP_(ion) remains constant during the whole exposure time.

Acute Toxicity of CuNPs and ZnONPs. In the present study, CuNPs were found to have a LC50 level (0.093 mg/L), which was over 10 times lower than that of ZnONPs (0.99 mg/L). It is thus obvious that *D. magna* is much more vulnerable to dispersions of CuNPs than to dispersions of ZnONPs. Previous toxicity assessments of NPs have primarily focused on probing into the effects of different exposure routes, such as the respiratory or gastrointestinal tracts.²⁹ Most previous research

did not distinguish between the relative contributions of $\text{NP}_{(\text{particle})}$ and $\text{NP}_{(\text{ion})}$ to the overall toxicity induced by NPs or have simply compared the overall toxicity of metal-based NPs with that of corresponding metal salts. However, metal salts import other types of ionic species into solutions, compared to the $\text{NP}_{(\text{ion})}$ directly, which may exert effects on acute toxicity through combined effects on physiological characteristics and metal speciation.³⁰ In this study, to investigate whether using metal salts to replace $\text{NP}_{(\text{ion})}$ is effective, comparison of acute toxicity between freshly prepared $\text{CuNP}_{(\text{ion})}$ and $\text{ZnONP}_{(\text{ion})}$ and the corresponding metal salts was conducted, respectively. Similar LC50 values of the $\text{CuNP}_{(\text{ion})}$ and $\text{Cu}(\text{NO}_3)_2$ and of the $\text{ZnONP}_{(\text{ion})}$ and $\text{Zn}(\text{NO}_3)_2$ were obtained. Our results indicate that using $\text{Cu}(\text{NO}_3)_2$ and $\text{Zn}(\text{NO}_3)_2$ to substitute for $\text{CuNP}_{(\text{ion})}$ and $\text{ZnONP}_{(\text{ion})}$ is effective. Furthermore, the results of assessment of the relative contribution to mortality revealed that the $\text{CuNP}_{(\text{ion})}$ and $\text{ZnONP}_{(\text{ion})}$ were not the major source of acute toxicity of CuNPs and ZnONPs. Similarly, Li and Wang¹⁸ also concluded that the toxicity of ZnONPs to *D. magna* can not only be attributed to the $\text{ZnONP}_{(\text{ion})}$, and Santo et al.⁶ even found that the $\text{ZnONP}_{(\text{ion})}$ did not make any contribution to the toxicity of ZnONPs to *D. magna*. However, there are also studies which attributed the toxicity of metal-based or metaloxide-based NPs to the $\text{NP}_{(\text{ion})}$. For instance, Adam et al.⁹ concluded that the toxicity of ZnONPs to *D. magna* can be largely attributed to the $\text{NP}_{(\text{ion})}$ rather than the $\text{NP}_{(\text{particle})}$; Jo et al.⁸ reported that the dissolved Cu released from CuO NPs largely contributed to the observed acute toxicity to *D. magna*. The different characteristics of NPs and exposure conditions, such as the size of NPs³¹ or the pH value of the exposure medium,³² may lead to the apparent discrepancy. Thus, more toxicity tests involving a wider range of NPs, exposure conditions, and model organisms should be conducted in the future. Thereupon, a more explicit description of the test condition is needed.

Accumulation Characteristics of CuNPs and ZnONPs.

Although the mechanisms underlying the CuNPs and ZnONPs mediated toxicity in *D. magna* are poorly known, a significant accumulation of CuNPs and ZnONPs in daphnids was observed in this study. The concentrations of Cu and Zn detected in daphnids were quite high (up to 0.1%). These findings are similar to but lower than the maximum body burdens observed for uptake studies with *D. magna* for carbon nanotubes (6.8%),³³ fullerenes (0.7%),³⁴ and graphene (0.7%).³⁵ Furthermore, the accumulation results display that the internal concentrations of Cu and Zn were proportional to the concentrations of CuNPs and ZnONPs administered in this study (Figure 4). Actually *D. magna* are filter feeders, enabling them to ingest particles smaller than the size of 70 μm .³⁶ In our study, both CuNPs and ZnONPs aggregates were far smaller than 70 μm (median particle size about 1 μm), thus they could be readily taken up by daphnids. On the other hand, the aggregation of CuNPs and ZnONPs to approximately 1 μm makes sedimentation likely to have occurred. There is evidence that sediment particles are ingested by daphnids,^{33,37} but the accumulating rate might be different to suspended particles³⁸ and furthermore the adsorption to daphnids' carapace and appendages might also be different to dispersions if the particles are applied as large aggregates and agglomerates.³⁷ Therefore, also the size of NPs' aggregates might be of importance in the accumulation study. At the high concentration of nanoparticle suspensions applied, both $\text{CuNP}_{(\text{ion})}$ and $\text{ZnONP}_{(\text{ion})}$ contributed only to a limited extent to the accumulation of Cu and

Zn, respectively (Figure 4). These results indicate that $\text{NP}_{(\text{particle})}$ play a dominant role in the accumulation process at the high concentration of CuNPs and ZnONPs, in line with the conclusion obtained from the toxicity assessment of the $\text{NP}_{(\text{particle})}$ exposed to neonates. This dominant role of $\text{NP}_{(\text{particle})}$ in the accumulation process might be reflected in the following manifestations. *D. magna* ingested metals in the particle form more than in the ion form in this study. $\text{NP}_{(\text{particle})}$ existing in nanoparticle suspensions may facilitate the intake of $\text{NP}_{(\text{ion})}$ by adsorbing metal ions on their surface areas.³⁹ It also might be that $\text{NP}_{(\text{particle})}$ became predominately packed in an organism gut tract. There was adsorption of $\text{NP}_{(\text{particle})}$ to the outer shell of daphnids partly. Finally, all the above manifestations could coexist. However, which manifestation is the major reason that results in the leading role of $\text{NP}_{(\text{particle})}$ requires further research. At the low concentration of the nanoparticle suspensions applied in this study (namely 0.05 mg/L CuNPs and 0.5 mg/L of ZnONPs), both the relative contributions to accumulation of $\text{CuNP}_{(\text{ion})}$ and $\text{ZnONP}_{(\text{ion})}$ increased to around 50% (Table 3). The different contribution to accumulation of $\text{NP}_{(\text{ion})}$ might be caused by the different dissolubility of NPs. At a low particle concentration, the proportion of dissolved NPs tends to be higher, whereas it tends to be lower at a high particle concentration.^{24,25,40} Thus, a higher proportion of $\text{NP}_{(\text{ion})}$ might be ingested by organisms at a lower concentration of NPs than at a higher concentration of NPs. Consequently, when the concentrations of CuNPs and ZnONPs were below 0.05 mg/L and 0.5 mg/L, respectively, $\text{NP}_{(\text{ion})}$ were predominantly accumulated. The results indicate that at a low concentration of nanoparticle suspensions, ions released from metallic nanoparticles like Cu and ZnO are overshadowing the effects of NPs.

ENVIRONMENTAL IMPLICATIONS

This study investigated the potential environmental effects of CuNPs and ZnONPs and their corresponding dissolved ions. Our results demonstrate that at the LC50 levels of CuNPs and ZnONPs suspensions, the $\text{NP}_{(\text{particle})}$ dominated the toxicity rather than the $\text{NP}_{(\text{ion})}$. Additionally, at the low exposure concentrations of CuNPs and ZnONPs (below 0.05 mg/L of CuNPs and 0.5 mg/L of ZnONPs, respectively) the $\text{NP}_{(\text{ion})}$ was predominantly accumulated, whereas at the high exposure concentration (above 0.1 mg/L of CuNPs and 1 mg/L of ZnONPs, respectively), $\text{NP}_{(\text{particle})}$ not only played a dominant role in the accumulation process but also was the species primarily responsible for toxicity.

ASSOCIATED CONTENT

Supporting Information

Comparison of the ion concentration in the supernatants prepared by regular centrifugation employed in the present study and ultracentrifugation and comparison the particle profiles of the STM and of the supernatants of CuNPs and ZnONPs (Supporting Information 1). The losses of NPs and $\text{NP}_{(\text{ion})}$ after centrifugation and filtration (Supporting Information 2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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