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Effects of natural organic matter on the joint toxicity and accumulation of Cu nanoparticles and ZnO nanoparticles in *Daphnia magna*[☆]

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ABSTRACT

Various modern products have metallic nanoparticles (MNPs) embedded to enhance products performance. Technological advances enable nowadays even multiple hybrid nanoparticles. Consequently, the future co-release of multiple MNPs will inevitably result in the presence of MNP mixtures in the environment. An important question is if the responses of mixtures of MNPs can be dealt with in a similar way as with the responses of biota to mixtures of metal salts. Moreover, natural organic matter (NOM) is an important parameter affecting the behavior and effect of MNPs. Herein, we determined the joint toxicity and accumulation of copper nanoparticles (CuNPs) and zinc oxide nanoparticles (ZnONPs) in *Daphnia magna* in the absence and presence of Suwannee River natural organic matter (SR-NOM), compared to the joint toxicity and accumulation of corresponding metal salts. The results of toxicity testing showed that the joint toxicity of CuNPs + ZnONPs was greater than the single toxicity of CuNPs or ZnONPs. The joint toxic action of CuNPs + ZnONPs was additive or more-than-additive for *D. magna*. A similar pattern was found in the toxicity of the mixtures of Cu- and Zn-salts from the literature data. The presence of SR-NOM had no significant impact on the joint toxicity of CuNPs + ZnONPs. The calculated component-specific contribution to overall toxicity indicated that SR-NOM increased the relative contribution of dissolved ions released from the MNPs to the toxicity of the binary mixtures at high-effect concentrations of individual MNPs. Moreover, dissolved Zn-ions released from the ZnONPs were found to dominate the joint toxicity of CuNPs + ZnONPs in the presence of SR-NOM. Furthermore, the results of the accumulation experiment displayed that the presence of SR-NOM significantly enhanced the accumulation of either CuNPs or ZnONPs in *D. magna* exposed to the MNP mixtures.

1. Introduction

With the rapid progress in nanotechnology, various metallic nanoparticles (MNPs) are embedded in industrial and domestic products (Guinée et al., 2017; Mitrano et al., 2015). Nowadays, hybrid nanoparticles are constantly emerging to achieve multiple functionalities for single-component nanoparticles (Ma, 2019). Consequently, the potential co-release of multiple-component MNPs will inevitably bring out the presence of MNP mixtures in the environment. Nanotoxicological studies on multiple MNPs are gradually becoming a topic of interest. Current studies indicated that the toxic potential of multiple MNP mixtures is likely to be distinct from the summed toxicity of the individual MNPs. For instance, synergistic effects were found after exposure of *Escherichia coli* to a mixture of AgNPs + TiO_2NPs (Wilke et al., 2018), and for *Nitrosomonas europaea* after exposure to a mixture of CeO_2NPs + ZnONPs (Yu et al., 2016). On the other hand, some binary mixtures such as TiO_2NPs + CeO_2NPs (Yu et al., 2016), and AgNPs + CuONPs (Ogunsuyi et al., 2019) induced antagonistic toxicity to *N. europaea* and *Clarias gariepinus*, respectively.

Many MNPs undergo dissolution, namely the shedding of metal ions from MNPs, in an aqueous medium. Thus, particles ($MNP_{particle}$), dissolved ions released from MNPs (MNP_{ion}), or both $MNP_{particle}$ and MNP_{ion}, may contribute to the overall toxicity of MNPs ($MNP_{overall}$) (Wang et al., 2012; Cronholm et al., 2013; Adam et al., 2014; Xiao et al.,

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2015; Ye et al., 2018). It is also realized that the assessment of the ecotoxicological effects of mixtures of MNPs is more complicated because of the coexistence of different $MNP_{particle}$ and different MNP_{ion} in suspensions. Given that total MNP_{ion} in the mixtures of MNPs contributes to the joint toxicity of multiple MNPs mainly, it is necessary to unravel if the responses of mixtures of MNPs can be dealt with in a similar way as with the responses of biota to mixtures of metal salts.

Natural organic matter (NOM) plays an important role in modulating the ecotoxicological effects of metals (Nogueira et al., 2017) and nanoparticles (Wang et al., 2011). Previous studies have shown that the addition of NOM reduced the toxicity of metals such as Cu and Zn to aquatic organisms, due to the complexation of metal ions with NOM and thereby decreasing the bioavailability of the metals (Hyne et al., 2005; Nadella et al., 2009; Clifford and McGeer, 2009). Moreover, NOM is known to have a significant impact on the dissolution and toxicity of individual MNPs in the aquatic environment (Zhang et al., 2009; Wang et al., 2016; Sani-Kast et al., 2017; Sharma et al., 2019). The impacts of NOM on the toxicity of nanoparticles depend on several mechanisms, including altered electrostatic repulsion between nanoparticles and/or between nanoparticles and cells, scavenging of nanoparticles-induced reactive oxidative species, and the formation of complexes with MNPs-released ions (Deng et al., 2017). In addition, NOM can show different influences on the aquatic toxicity of MNPs with different types. For example, in the presence of NOM, the mitigation of the mortality of CuNPs on *Daphnia magna* occurred by reducing the contribution of dissolved Cu^{2+} to the toxicity of the suspension (Xiao et al., 2018). However, the addition of NOM stabilized ZnONP suspensions and did not decrease toxicity (Cupi et al., 2015). It is interesting to reveal whether there is an alteration of mixture toxicity due to the changes in bioavailability of MNPs when the amount of the ions versus particle fraction changed in the presence of NOM.

CuNPs and ZnONPs are not only widely applied in numerous products, but they are also reported to be toxic to a wide range of aquatic organisms (Bondarenko et al., 2013; Xiao et al., 2015; Ho et al., 2018). The objectives of the present study are to: (1) determine the joint toxicity of binary mixtures of CuNPs and ZnONPs to the zooplankton species *Daphnia magna*; (2) evaluate the contribution of each mixture constituent to the overall toxicity of the binary mixtures; (3) elucidate the differences in toxicity and accumulation between mixed metal-salt exposures and mixed MNP exposures; (4) use a surrogate for NOM which provides binding sites to modulate the fate, toxicity, contribution to toxicity of the individual constituents present in the mixture, and metal accumulation following exposure to binary MNP mixtures. Our starting hypothesis is that the toxicity and accumulation are associated with the dissolved fraction of the MNPs, as dependent on the type of MNPs, the number of nanoparticle constituents (individual or multiple MNPs), and the properties of the exposure medium such as in the presence of NOM.

2. Materials and method

2.1. Test materials

CuNPs (reported specific surface area of 30–50 m^2/g; purity >99.5%) and ZnONPs (reported specific surface area of 19 ± 5 m^2/g; purity >99.5%) purchased from IoLiTecGmbh (Heibronn, Germany) and Plasmachem GmbH (Berlin, Germany) were selected in this study. The two MNPs have the same primary size of 25 nm and they are both spherically shaped. $Cu(NO_3)_2$ and $Zn(NO_3)_2$ obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) were used as reference materials, which have previously been reported to be an effective approach of assessing the impacts of dissolved ions released from MNPs (Xiao et al., 2015). Suwannee River NOM (SR-NOM, 2R101N) as the surrogate for NOM was supplied by the International Humic Substances Society (IHSS). The selected SR-NOM was acquired from filtered river water, which was concentrated using two portable reverse osmosis systems (Green et al., 2015). The preparation procedures of the MNP and SR-NOM stocks are described in the Supplementary data.

2.2. Physicochemical characterizations

The morphology, size distribution (Z-average hydrodynamic diameter), and zeta-potential of MNPs in suspensions were determined to characterize their physicochemical properties in the test medium. Details for all of these characterizations are given in the Supplementary data. The actual concentrations of Zn- and Cu-ions released from ZnONPs, CuNPs, $Cu(NO_3)_2$, and $Zn(NO_3)_2$ were measured using Atomic Absorption Spectroscopy (AAS; PerkinElmer 1100B).

For modeling dissolved Cu and Zn speciation, a geochemical code, Visual MINTEQ (version 3.1) (Gustafsson, 2013) was used. The NICA-Donnan model was applied for describing the binding of metals to humic substances (Minteq and Agency, 2000). The NICA-Donnan model uses a bimodal, continuous distribution of affinities for protons and metal ions (Unsworth et al., 2006). This model has been successfully used for evaluating Cu and Zn complexation to dissolved organic matter in a previous study (Baker, 2012). The modeling of metal ions, metal-inorganic complexes, and NOM-bound complexes (Metal-NOM complexes) could be performed at the same time. Hence, a direct comparison of each species of dissolved ions in the suspensions was possible.

2.3. Experimental outline

Fig. 1 depicts the outline of toxicity and accumulation testing. The cultures (Supplementary data) and 48 h acute toxicity assays with *D. magna* were performed following the OECD Guideline 202 (OECD, 2004).

2.3.1. Single and joint toxicity tests

Neonates (<24 h) were used in the test after cleaning their guts for around 2 h in ElendtM7 medium. Ten individuals were randomly placed in each beaker, containing either 100 mL of a suspension of the MNPs or a control. Daphnids were exposed to increasing initial concentrations of CuNPs (ranging from 0.01 to 7 mg L^{-1}), ZnONPs (from 0.5 to 100 mg L^{-1}), $Cu(NO_3)_2$ (from 0.1 to 0.88 mg L^{-1}), and $Zn(NO_3)_2$ (from 1 to 8 mg L^{-1}). Three replicates were included for each treatment. Each acute toxicity test was repeated with different batches of *D. magna* at three different times and the data presented are the mean of the runs ($n = 3$). The tests were maintained under a 16:8 h light/dark photoperiod (22 ± 1 °C) without feeding during the 48 h exposure period. The actual exposure concentrations of particles and ions in each treatment were measured by AAS after incubation in the test medium without daphnids. The detailed sampling procedures for the determination of the actual exposure concentrations of particles and ions were described in the Supplementary data.

The concentration–response curves (CRCs) were constructed via GraphPad Prism 8.0 for each of the single toxicants (CuNPs, ZnONPs, Cu^{2+}, and Zn^{2+}). From the CRCs, the estimated LC_{10} and LC_{50} (concentrations at which 10% and 50% of mortality of test species are observed) of CuNPs and ZnONPs were selected as exposure concentrations used in tests for single and mixture toxicity in the presence of SR-NOM (1, 10 and 20 mg L^{-1}). The LC_{10} and LC_{50} values of each MNP were employed to give the fixed concentration ratios that were used in the toxicity tests performed on the binary mixtures of CuNPs and ZnONPs.

2.3.2. Relative contributions to toxicity of $MNP_{particle}$ and MNP_{ion}

It is widely believed that the modes of actions of $MNP_{particle}$ and MNP_{ion} are likely to be independent (Xiao et al., 2015; Liu et al., 2016), which is in line with the assumption of the response addition (RA) model (Bliss, 1939). Thus, the RA model is selected as a simplified way of estimating the toxicity of $MNP_{particle}$ from the experimentally determined overall toxicity of MNP suspensions ($MNP_{overall}$) to evaluate the relative contribution of $MNP_{particle}$ and MNP_{ion} to toxicity (Xiao et al.,

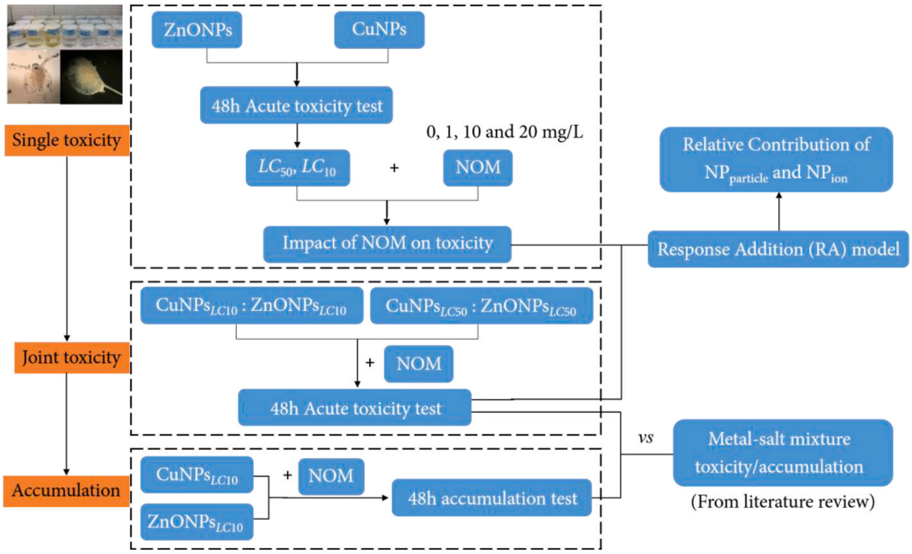

Fig. 1. Scheme of the experimental outline.

2015; Wu et al., 2020). The RA model is defined as follows:

$$E_{overall} = 1 - (1 - E_{ion})(1 - E_{particle}) \tag{1}$$

where $E_{overall}$ and E_{ion} represent the toxic effects caused by the MNP suspensions and their corresponding released ions (scaled from 0 to 1), respectively. $E_{overall}$ was quantified by the mixture toxicity testing. The combined effect (E_{ion}) of the two dissolved ions released from the MNPs present in the binary MNP mixtures was calculated as follows:

$$E_{ion(Cu^{2+}+Zn^{2+})} = 1 - (1 - E_{Cu^{2+}})(1 - E_{Zn^{2+}}) \tag{2}$$

Then, the effects caused by the particles ($E_{particle}$) were directly calculated by the RA model.

2.3.3. *Accumulation tests*

The accumulation profile of the MNPs by *D. magna* was measured through a 48 h accumulation test. Following the acute toxicity test, the concentrations of CuNPs and ZnONPs used in the accumulation experiments were the actual particle concentrations of MNPs at the LC_{10} level for the exposure to both single and binary MNPs. For the accumulation test, the exposure time and all other conditions were the same as in the toxicity test. The pre-treatment of daphnid accumulation was based on the procedure described by Bossuyt and Janssen (2005) with slight modification. Briefly, after the 48 h exposure period, healthy *D. magna* (150–200 daphnids for each replicate) were selected and transferred to 5% EDTA solution for around 15 min. They were then washed two times with 5% EDTA and then two times with fresh Milli-Q water to remove the absorbed particles and ions upon the surface of the daphnids. After absorbing the water left behind on the organisms using tissue paper, the animals were dried at 80 °C overnight in pre-weighed glass containers before weighing on a microbalance and then digested in 65% HNO_3 at 80 °C overnight. The Cu and Zn concentrations in the digested samples were subsequently determined by AAS. The statistical analyses were listed in the Supplementary data.

3. Results and discussion

3.1. *Characterization of CuNPs and ZnONPs in the absence and presence of SR-NOM*

The morphology of the CuNPs and ZnONPs in the single and binary mixtures and the impact of SR-NOM in the test medium are presented in Fig. S1 (Supplementary data). Analysis of the TEM images indicates that the CuNPs (Fig. S1A) or ZnONPs (Fig. S1B) agglomerated intensely and formed irregular shapes in the test medium. However, in the presence of SR-NOM, the CuNPs (Fig. S1D) or ZnONPs (Fig. S1E) showed a greater tendency to disagglomerate. In addition, the CuNPs or ZnONPs co-agglomerated regardless of the absence (Fig. S1C) or presence (Fig. S1F) of SR-NOM.

The hydrodynamic diameter (D_H in nm) and zeta potential (ZP in mV) of CuNPs and ZnONPs and their binary mixtures in the absence and presence of 1, 10, and 20 mg L^{-1} SR-NOM in the test medium are depicted in Fig. 2 and Fig. S2 (Supplementary data). After 48 h of exposure, the degree of agglomeration of individual and binary mixtures of CuNPs and ZnONPs in the absence of SR-NOM decreased in the order of CuNPs $>$ ZnONPs $\approx$ mixture of CuNPs and ZnONPs (Fig. 2A). For the individual CuNPs and ZnONPs, the addition of SR-NOM reduced the extent of agglomeration remarkably, corresponding to the results shown in the TEM images (Figs. S1D and S1E). The inhibition of agglomeration implied that SR-NOM stabilized the individual MNPs, which facilitated their dispersion. The stabilization effect arising from SR-NOM depended upon the exposure concentration of SR-NOM. The co-agglomeration behavior of the binary mixtures was significantly modified only after adding 20 mg L^{-1} SR-NOM. Meanwhile, no significant change in the ZP values of MNPs was observed in the absence and presence of SR-NOM. The only exception was the case of 20 mg L^{-1} SR-NOM as this concentration of SR-NOM reduced the absolute ZP value of CuNPs over 48 h of incubation (Fig. 2B), which might be due to the reduction of electrical double layer repulsion between particles.

The sedimentation of the particles was evaluated by relating the actual concentrations (C) of the MNPs (based on total metal) after 48h of exposure in the suspensions to the initial concentrations (C_0) of the MNP suspensions. According to Fig. 2C, the actual concentrations of CuNPs and ZnONPs decreased by around 20–25% after 48 h of settling in the absence of SR-NOM. Upon the addition of 1 and 10 mg L^{-1} SR-NOM, the actual concentration of CuNPs dropped by 28–35% in the test medium due to the sedimentation of particles. This is in good agreement with the change of D_H values of CuNPs as aforementioned, suggesting that the reduction of aggregation of CuNPs by SR-NOM (1 and 10 mg L^{-1}) was mainly caused by the increased sedimentation of the particles. The actual concentration of ZnONPs after 48 h of incubation in the absence and presence of SR-NOM did not differ significantly, irrespective of the concentration of SR-NOM. In the binary system, the actual concentration of CuNPs after 48 h of exposure in the mixture suspensions dropped dramatically by 54% compared to the concentration of the individual CuNPs suspension, while the actual concentration of ZnONPs after 48 h

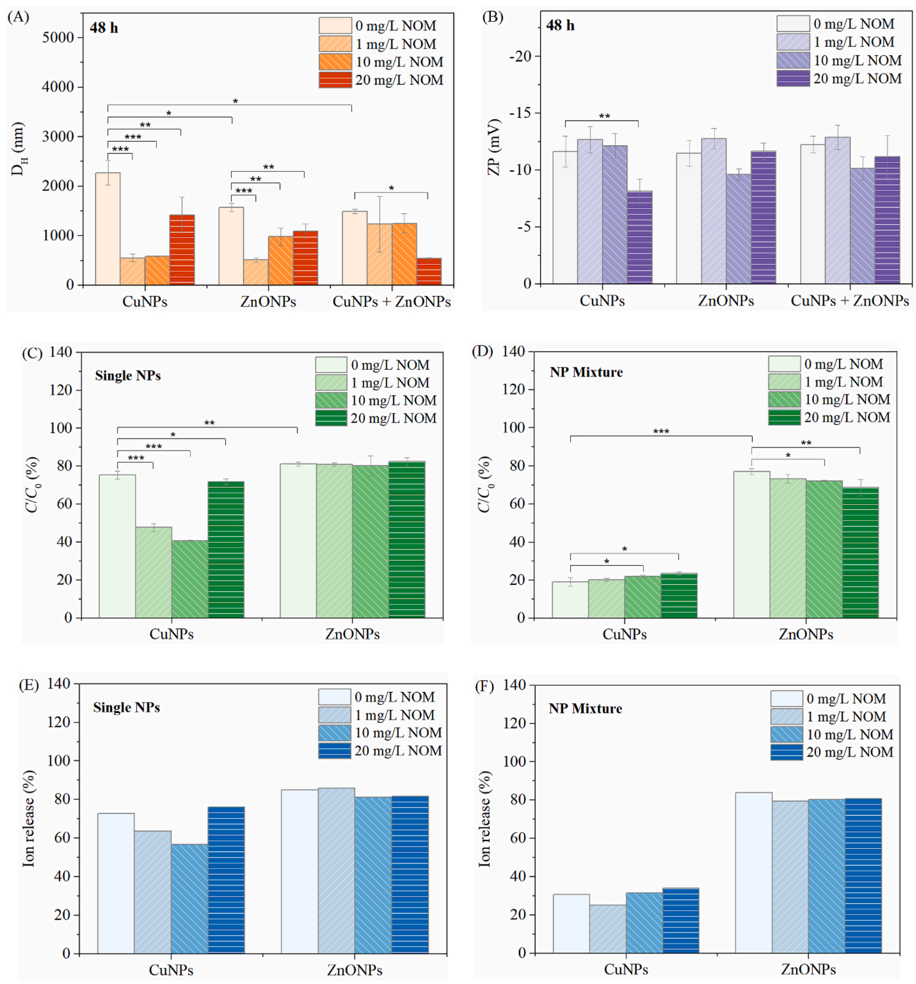

Fig. 2. Variation in D_H (A) and ZP (B) of CuNPs and ZnONPs and their binary mixtures in the absence and presence of SR-NOM after 48 h of exposure; ratios (%) of actual concentrations (C) of CuNPs and ZnONPs (based on total metal) at 48 h (C) and of their mixture (D) to initial exposure concentrations (C_0) of the nanoparticle suspensions in the test medium; the ion release (%, based on C_{TWA}, the average value expressed as a single value calculated based on concentrations at 0, 24 and 48h) released from CuNPs and ZnONPs (E) and from the binary mixture (F). Values shown in A-D are expressed as mean ± standard deviation ($n = 3$). $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ indicating significant differences between the treatments.

settling did not change significantly when comparing the concentrations of single MNPs and mixtures.

The profiles of ion release shedded from the individual and binary MNPs in the absence and presence of SR-NOM are shown in Fig. 2E and F, where it is expressed based on the concentration of the time-weighted average C_{TWA}. The high percentage (>70%) of dissolved ions in the individual MNPs suspensions demonstrated that ions from both CuNPs and ZnONPs to a high degree dissolved (Fig. 2E). The concentration of Zn-ions was not influenced by the addition of SR-NOM, and the concentration of Cu-ions only decreased by about 9 and 16% when 1 and 10 mg L^{-1} SR-NOM were added into the suspensions, respectively. The dissolution of ZnONPs in the mixture suspensions was similar to the dissolution in the individual exposure system, as shown in Fig. 2F. However, the ion release of CuNPs shifted from 73% to 31% when they were incubated in the mixture suspensions. The presence of SR-NOM only slightly influenced the degree of dissolution of both CuNPs and ZnONPs in the mixtures. In addition, the prediction of the SR-NOM-dependent speciation as obtained using Visual MINTEQ 3.1 is shown in Fig. S5. It is found that the ratio of ions (Cu^{2+} and Zn^{2+}) to the total dissolved metal (Cu and Zn) hugely decreased in the presence of SR-NOM in both individual MNP suspensions and mixture suspensions. Note that the Cu-NOM complex accounted for almost all of the dissolved Cu-ions with the addition of any concentration of SR-NOM, confirming that Cu^{2+} was easily bound to SR-NOM through weak electrostatically binding and chemical complexation, as previously suggested (Field and Sea, 2014). The ratio of the concentration of Zn^{2+} to the concentration of total dissolved Zn-ions shifted from 84% to 67% after adding 1 mg L^{-1} SR-NOM and to 0% after adding 10 and 20 mg L^{-1} SR-NOM, respectively. Similar interactions between SR-NOM and dissolved ions were observed in the mixture suspensions.

3.2. Single and joint acute toxicity of CuNPs and ZnONPs to D. magna in the absence and presence of SR-NOM

3.2.1. Single and joint toxic effects

CRCs of CuNPs, ZnONPs, $Cu(NO_3)_2$, and $Zn(NO_3)_2$ are presented in Fig. S3. The LC_{50} and LC_{10} values of single compounds calculated from the CRCs are provided in Table 1. Based on the LC_{50} and LC_{10} values, the toxicity decreased in the order of $Cu(NO_3)_2$ > CuNPs > $Zn(NO_3)_2$ > ZnONPs. Xiao et al. (2015) also found that the acute toxicity of CuNPs was greater than that of ZnONPs. Furthermore, *D. magna* was more sensitive to metal ions than to the corresponding MNPs, with Cu^{2+} being the most toxic to daphnids. The difference in the sensitivity of *D. magna* to Cu/Zn-ions and Cu/ZnNPs was also observed by Xiao et al. (2015).

In the presence of SR-NOM, a significant reduction in the mortality of *D. magna* exposed to CuNPs was observed (Fig. 3A). It is obvious that

Table 1
Lethal concentrations (mgCu L^{-1} or mgZn L^{-1}) of suspensions of Cu and Zn NPs, and of solutions of $Cu(NO_3)_2$ and $Zn(NO_3)_2$ towards *D. magna*, expressed based on time-weighted average concentrations of Cu and Zn after 48 h of exposure.

Test materials	LC_{50} (95% confidence limits)	LC_{10} (95% confidence limits)
CuNPs	0.40 (0.26–0.57)	0.11 (0.02–0.22)
$Cu(NO_3)_2$	0.04 (0.03–0.06)	0.02 (0.01–0.03)
ZnONPs	4.01 (2.90–54.35)	1.29 (0.12–2.61)
$Zn(NO_3)_2$	0.75 (0.58–0.96)	0.27 (0.17–0.41)

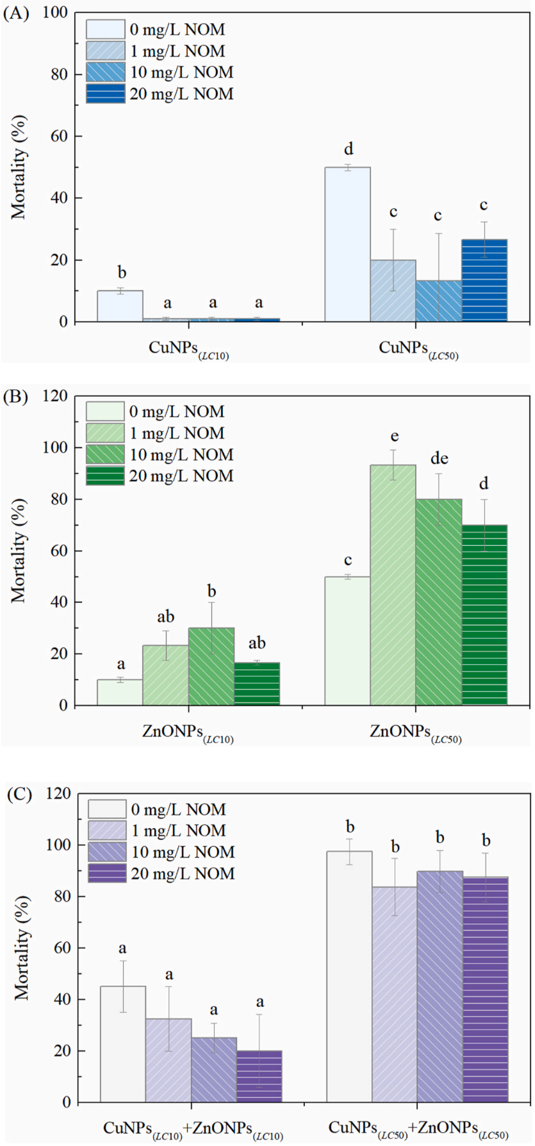

Fig. 3. Mortality (%) of *D. magna* exposed to individual CuNPs (A) and ZnONPs (B) in the presence of 0, 1, 10, and 20 mg L^{-1} SR-NOM; and the impact of SR-NOM (0, 1, 10 and 20 mg L^{-1}) on mixture toxicity of CuNPs and ZnONPs, based on the LC_{10} and LC_{50} ratios (C) at their individual LC_{10} and LC_{50} concentration. Values are expressed as mean ± standard deviation ($n = 3$). Letters indicate statistically significant ($p < 0.05$) differences between the treatments.

different concentrations of SR-NOM significantly reduced the mortality of CuNPs to a similar degree. Xiao et al. (2018) also found that the toxicity of CuNPs to *D. magna* decreased with increasing the concentrations of humic acid. The decrease in toxicity for CuNPs with the addition of SR-NOM may be due to the complexation of released ions with NOM and the passivation of the particle surface by NOM adsorption (Fabrega et al., 2009). Noteworthy, the opposite, hence, an increase in the mortality of daphnids exposed to ZnONPs (Fig. 3B) was observed. These results are in line with Cupi et al. (2015) who found a similar effect of SR-NOM on the toxicity towards daphnids, namely that the presence of SR-NOM significantly enhanced the toxicity (in terms of 48h-EC_{50} values) of ZnONPs test suspensions prepared from nanoparticle Stock I to *D. magna*. The enhancement of the toxicity might be explained by the fact that the stabilization of the ZnONP suspensions in the presence of SR-NOM is significant. Organisms can take in stabilized nanoparticles in the aqueous medium, and the bio-accumulation/biological effects are likely enhanced (Deng et al., 2017).

As can be seen in Fig. S4, the mortality of the binary mixtures of CuNPs and ZnONPs at the LC_{10} and LC_{50} ratios was 45% and 98%, respectively. This means that the co-exposure of CuNPs and ZnONPs exerted more-than-additive or additive toxic effects on *D. magna*. To investigate the differences in the modes of joint toxic action between the co-exposure of CuNPs and ZnONPs and their corresponding metal-ion counterparts, we summarized literature data on the joint toxicity of Cu- and Zn-salts to freshwater organisms in Table S1. As shown in Table S1, additive or more-than-additive effects on *D. magna* were found in most studies on the joint toxicity of Cu- and Zn-salts (Komjarova and Blust, 2008; Cooper et al., 2009; Meyer et al., 2015; Lari et al., 2017). This indicated that the two studied MNPs in the mixtures interacted in a manner similar to their corresponding metal salts.

The impact of SR-NOM on the mortality of the binary mixtures of CuNPs and ZnONPs at the LC_{10} and LC_{50} ratios is presented in Fig. 3C. Generally, no significant impact ($p < 0.05$) on the joint toxicity of CuNPs and ZnONPs was found in the presence of SR-NOM, regardless of the mixture ratios and the SR-NOM concentration.

3.2.2. *Relative contribution of MNP_{ion} and $MNP_{particle}$ to overall toxicity*

Fig. 4 depicts the relative contribution of MNP_{ion} and $MNP_{particle}$ to the overall toxicity of the individual and binary mixtures of CuNPs and ZnONPs. As shown in Fig. 4A, $CuNPs_{particle}$ and $CuNPs_{ion}$ accounted for 82% and 18% of the toxicity of $CuNPs_{overall}$ at the LC_{50} level, respectively, while $CuNPs_{particle}$ and $CuNPs_{ion}$ accounted for 0% and 100% of the toxicity of $CuNPs_{overall}$ at the LC_{10} level. The high contribution of dissolved Cu-ions to the toxicity of $CuNPs_{overall}$ at the LC_{10} level is due to the relative high ion release as mentioned above. Note that in the presence of SR-NOM no data were obtained for the relative contribution of $CuNPs_{particle}$ and $CuNPs_{ion}$ to the toxicity of $CuNPs_{overall}$ at the LC_{10} level, which is due to the fact that no mortality was observed for the $CuNPs_{overall}$ in the presence of SR-NOM (Fig. 4A). Upon the addition of SR-NOM, the relative contribution of $CuNPs_{ion}$ to the toxicity of $CuNPs_{overall}$ at the LC_{50} level decreased owing to the reduction of dissolved Cu^{2+}-concentrations by SR-NOM. As shown in Fig. 4B, $ZnONPs_{ion}$ accounted for 100% of the relative contribution to the toxicity of $ZnONPs_{overall}$ in the absence of SR-NOM, implying that the single toxicity of ZnONPs was to be fully ascribed to the dissolved Zn-ions. In contrast, some previous studies found that the particles of ZnONPs were the main source of toxicity (Xiao et al., 2015; Ye et al., 2018). This difference might be explained by a difference in dissolution rates due to the studied MNPs' characteristics and the exposure conditions (Lopes et al., 2014). It was found that SR-NOM had no impact on the relative contribution of $ZnONPs_{ion}$ to the toxicity of $ZnONPs_{overall}$ at the LC_{10} level, whereas SR-NOM had different impacts on the relative contribution of $ZnONPs_{ion}$ to the toxicity of $ZnONPs_{overall}$ at the LC_{50} level. Generally, decreasing the concentration of SR-NOM increased the relative contribution of $ZnONPs_{particle}$ to the toxicity of $ZnONPs_{overall}$ at the LC_{50} level.

Fig. 4C presents the relative contribution of MNP_{ion} and $MNP_{particle}$ to the toxicity of the binary mixtures of CuNPs and ZnONPs at the LC_{10} and LC_{50} ratios to *D. magna* in the absence and presence of SR-NOM. The contribution of MNP_{ion} to the joint toxicity of the binary mixtures at the LC_{10} ratio was 100%, irrespective of whether SR-NOM was present. This implies that dissolved ions play an absolute role in the joint toxicity of CuNPs + ZnONPs, which can be regarded as a mixture of Cu^{2+} and Zn^{2+}. For the toxicity of the binary mixtures at the LC_{50} ratio, the relative contribution of MNP_{ion} (47%) to the joint toxicity of the binary mixtures was lower than that of $MNP_{particle}$ (53%). However, a contribution of MNP_{ion} of more than 50% was observed in the presence of SR-NOM, implying that the contribution of dissolved ions to the joint toxicity of the binary mixtures was enhanced by SR-NOM.

The specific contribution of Cu-ions and Zn-ions to the toxicity induced by total dissolved ions was further separated, as shown in Fig. 4D. Generally, the contribution of Zn-ions to the overall toxicity induced by MNP_{ion} in the binary mixtures in the absence and presence of

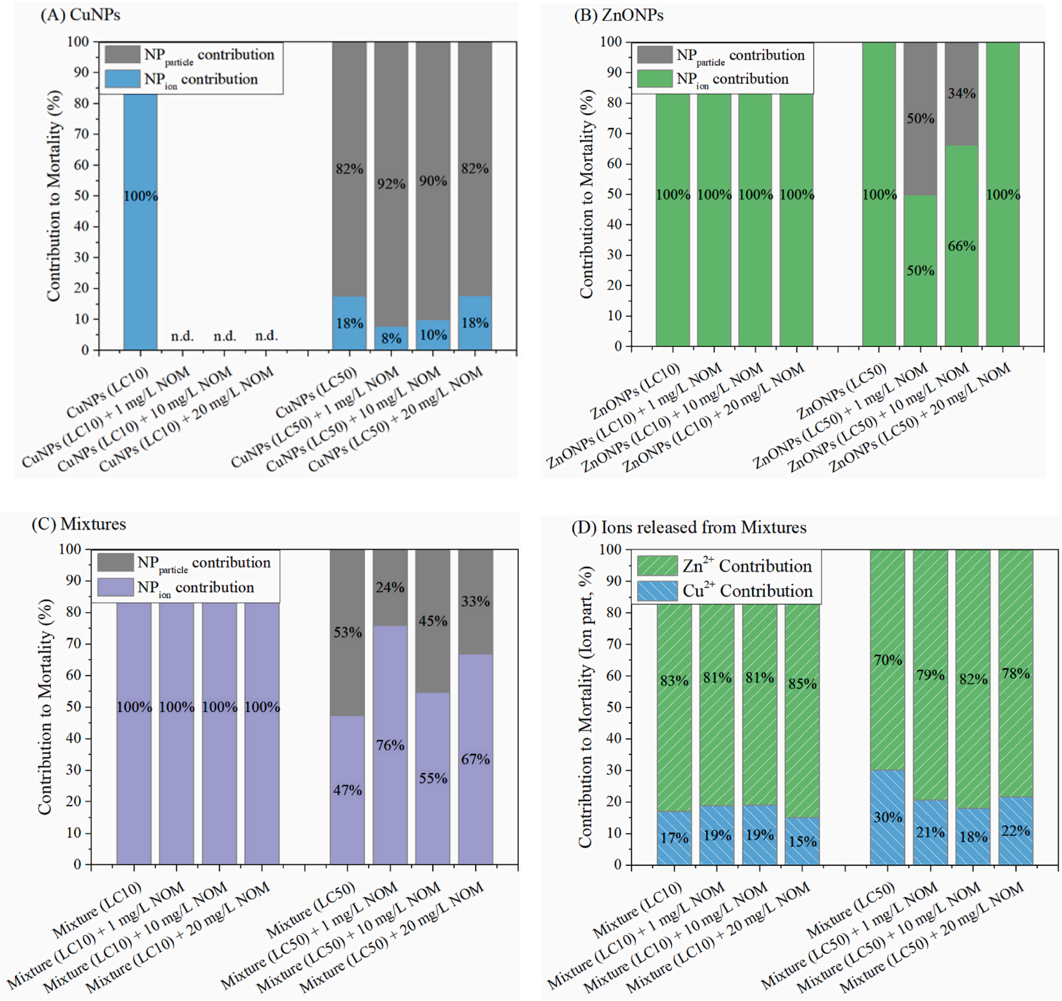

Fig. 4. Relative contribution to toxicity of MNP$_{particle}$ and MNP$_{ion}$ of the individual CuNPs (A) and ZnONPs (B), and the binary mixtures (C) at the LC_{10} and LC_{50} ratios in the absence and presence of 1, 10 and 20 mg L^{-1} SR-NOM. (D) depicts the relative contribution of Cu-ions and Zn-ions released from the MNPs to the toxicity caused by the total dissolved ions. n. d. = not determined.

SR-NOM was markedly greater than Cu-ions. This means that Zn-ions dictated the toxicity of MNP$_{ion}$ in the binary mixtures no matter whether SR-NOM was present or absent. It can be concluded that the concentration of SR-NOM used was inadequate to counteract the impacts of ions on the overall toxicity of the binary mixtures of CuNPs and ZnONPs.

3.3. Single and joint accumulation of CuNPs and ZnONPs in D. magna in the absence and presence of SR-NOM

The accumulation of MNPs (based on ingested and internalized metal) in *D. magna* exposed to the individual CuNPs and ZnONPs and the binary mixtures at the LC_{10} ratio in the absence and presence of 20 mg L^{-1} SR-NOM is shown in Fig. 5. After 48 h of exposure, the amounts of Cu and Zn accumulated in the daphnids were 0.79 ± 0.04 and 1.09 ± 0.01 mg g^{-1} dry weight for CuNPs and ZnONPs, respectively. It is obvious that the total Zn accumulation was higher than the total Cu accumulation. This is consistent with the experimental results of Xiao et al. (2015).

The accumulated amount of Zn in the binary mixtures of CuNPs and ZnONPs increased by a factor of 2.5 compared to the accumulated amount of Zn in the individual ZnONPs, as shown in Fig. 5. A similar phenomenon in which the accumulated amount of Zn increased in the presence of Cu was also found for the case of exposure of daphnids to Cu- and Zn-salts (Komjarova and Blust, 2008). This also means that CuNPs

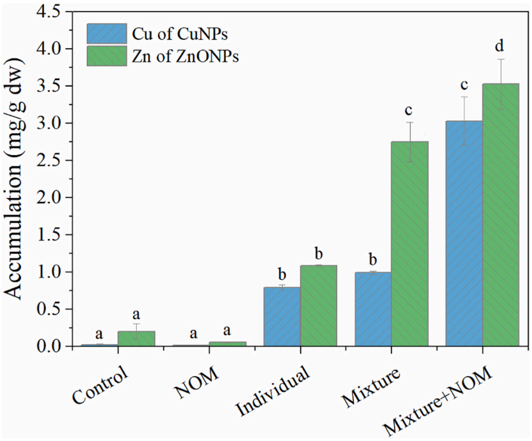

Fig. 5. Accumulation (mg g^{-1} dry weight) of total Cu and Zn in *D. magna* after 48 h exposed to the single and binary mixtures of CuNPs and ZnONPs in the presence of 20 mg L^{-1} SR-NOM. Values are expressed as mean ± standard deviation ($n = 3$). Different letters indicate significant differences between the treatments for the accumulation of the same elementary ($p < 0.05$).

and ZnONPs acted in an interaction mode similar to Cu-salts and Zn-salts. However, there is no significant change in the amounts of CuNPs (in terms of total Cu) accumulated in daphnids after the addition of ZnONPs.

The amounts of Cu and Zn in the binary mixtures of CuNPs and ZnONPs accumulated in *D. magna* were 3.1 and 1.3 times higher in the presence of SR-NOM than when SR-NOM was not present. This means that the addition of SR-NOM increased the accumulation of the MNPs. In particular, in the presence of SR-NOM the accumulation of Cu increased more obviously. NOM was previously shown to reduce metal bioavailability via chelating and sequestering metal cations (Gheorghiu et al., 2010; Al-Reasi et al., 2011; DePalma et al., 2011; Nogueira et al., 2017; Qiao et al., 2019). Nevertheless, it cannot be excluded that metal ions bound with NOM are available accumulation by aquatic organisms (Wang et al., 2016), especially for *D. magna* which can ingest suspended and sediment particles smaller than the reported size limit of 70 μm (Geller and Müller, 1981; Tervonen et al., 2010; Lee and Ranville, 2012). The maximum size of detected metal-NOM complexes and agglomerates of MNP mixtures was smaller than 2 μm in this work, hence the complexes, agglomerates, or their sediments, could be taken up by *D. magna*. Moreover, similar results suggested that some organisms could provide indirect routes for the uptake of Cu-NOM complexes (Lorenzo et al., 2005), or some active biological processes in organisms could counteract the chelation of Cu by NOM (Lores et al., 1999), thus increasing the accumulation of Cu in presence of NOM. Consequently, an analogous mechanism might be responsible for the enhancement of accumulation of either Cu or Zn in the binary mixtures of CuNPs and ZnONPs when NOM was present. In addition, the increase in bioavailability and accumulation of mixed MNPs in the presence of SR-NOM are in good agreement with a deduction that the competition of Cu–Zn binding with NOM will enhance the accumulation of Cu or Zn ions (Meyer et al., 2015; Crémazy et al., 2019).

4. Conclusions

The joint toxicity effects of CuNPs and ZnONPs were determined to be additive or more-than-additive in *D. magna*, which is similar to the joint toxicity effects of Cu- and Zn-ions reported in the literature. SR-NOM had no significant impact on the apparent toxicity of the binary mixtures of CuNPs and ZnONPs. However, the addition of SR-NOM increased the relative contribution of total dissolved ions released from the MNPs compared to the particles to the toxicity of the binary mixtures at high-effect concentrations of individual MNPs. Moreover, the Zn-ions released from the ZnONPs had a dominant role in the binary mixtures of CuNPs and ZnONPs as a result of the agglomeration and sedimentation of CuNPs and the complexation of the CuNPs released Cu-ions with SR-NOM. Furthermore, SR-NOM remarkably enhanced the bioaccumulation of both Cu and Zn in the binary mixtures of CuNPs and ZnONPs. Overall the results corroborate the importance of natural environmental factors in determining the ecotoxicity of multiple MNPs in the aquatic environment.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Author statement

Qi Yu: Investigation, Data curation, Writing – original draft, Visualization, Funding acquisition. Zhuang Wang: Conceptualization, Investigation, Methodology, Formal analysis, Validation, Writing – review & editing, Project administration. Guiyin Wang: Investigation, Visualization, Formal analysis. Willie J.G.M. Peijnenburg: Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition, Project administration. Martina G. Vijver: Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition, Project administration.

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