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Toxicity, bioaccumulation and trophic transfer of engineered nanoparticles in the aquatic environment

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Chapter 3

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.

Keywords: Aquatic nanotoxicity; Mixture toxicity; NOM; Metallic nanoparticles; Metal ions

3.1 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 + TiO₂NPs (Wilke et al., 2018), and for *Nitrosomonas europaea* after exposure to a mixture of CeO₂NPs + ZnONPs (Yu et al., 2016). On the other hand, some binary mixtures such as TiO₂NPs + CeO₂NPs (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., 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 et al., 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.

3.2 Materials and Method

3.2.1 Test materials

CuNPs (reported specific surface area of 30-50 m²/g; purity > 99.5%) and ZnONPs (reported specific surface area of 19 ± 5 m²/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(NO₃)₂ and Zn(NO₃)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.

3.2.2 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, $\text{Cu}(\text{NO}_3)_2$, and $\text{Zn}(\text{NO}_3)_2$ were measured using Atomic Absorption Spectroscopy (AAS; Perkin Elmer 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 et al., 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.

3.2.3 Experimental outline

Figure 3.1 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).

3.2.3.1 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(NO₃)₂ (from 0.1 to 0.88 mg L⁻¹), and Zn(NO₃)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, Cu²⁺, and Zn²⁺). 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.

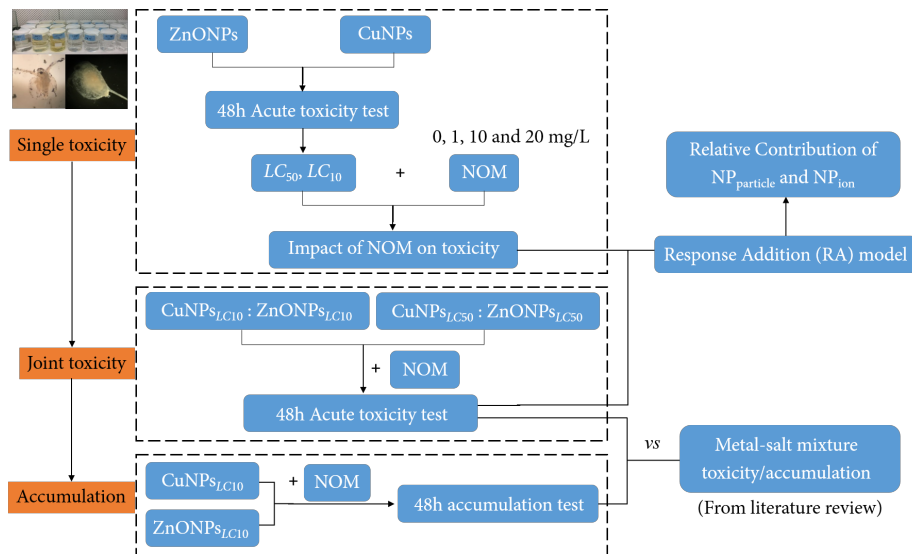


Figure 3.1. Scheme of the experimental outline.

3.2.3.2 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., 2015; Wu et al., 2020). The RA model is defined as follows:

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

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:

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

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

3.2.3.3 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 mins. 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% HNO₃ 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.

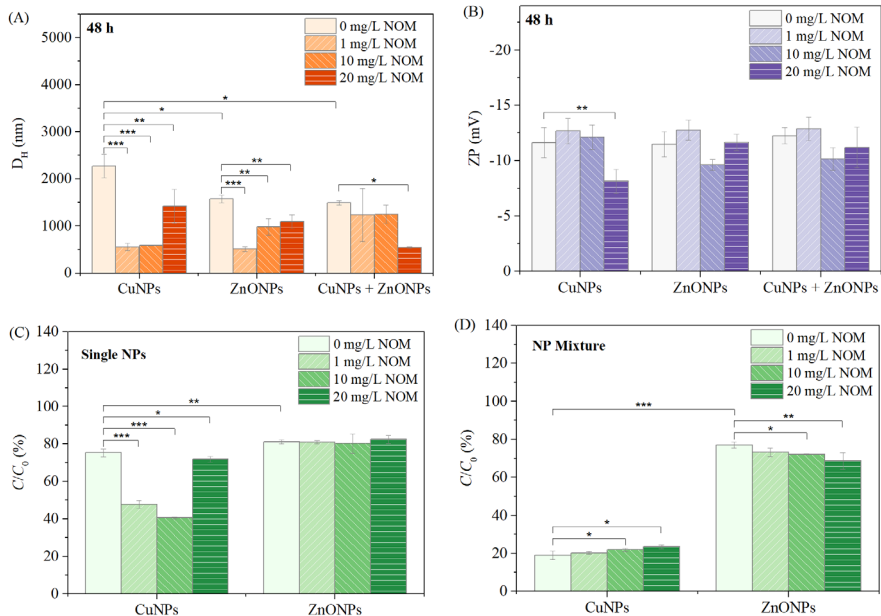
3.3 Results and discussion

3.3.1 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 Figure S3.1 (Supplementary data). Analysis of the TEM images indicates that the CuNPs (Figure S3.1A) or ZnONPs (Figure S3.1B) agglomerated intensely and formed irregular shapes in the test medium. However, in the presence of SR-NOM, the CuNPs (Figure S3.1D) or ZnONPs (Figure S3.1E) showed a greater tendency to disagglomerate. In addition, the CuNPs or ZnONPs co-agglomerated regardless of the absence (Figure S3.1C) or presence (Figure S3.1F) 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 Figure 3.2 and Figure S3.2 (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 increased

in the order of CuNPs > ZnONPs \approx mixture of CuNPs and ZnONPs (Figure 3.2A). 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 (Figure 3.2B), which might be due to the reduction of electrical double layer repulsion between particles.



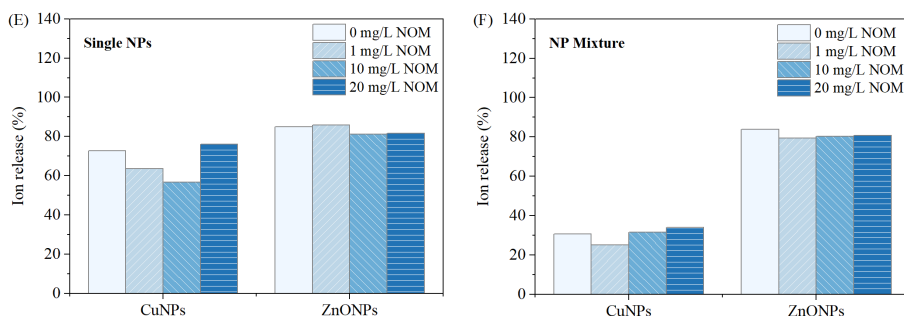


Figure 3.2. Variation in D_H (A) and ZP (B) of CuNPs and ZnONPs and their binary mixtures in the absence and presence of SR-NOM after 48 hours of exposure; ratios (%) of actual concentrations (C) of CuNPs and ZnONPs (based on total metal) at 48 h (C) and of their mixture (D) to initial exposure concentrations (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 \pm standard deviation ($n=3$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicating significant differences between the treatments.

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 Figure 3.2C, 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 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 Figure 3.2E and Figure 3.2F, 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 (Figure 3.2E). 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 Figure 3.2F. 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 Figure S3.5. It is found that the ratio of ions (Cu²⁺ and Zn²⁺) 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 et al., 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.

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

3.3.2.1 Single and joint toxic effects

CRCs of CuNPs, ZnONPs, $\text{Cu}(\text{NO}_3)_2$, and $\text{Zn}(\text{NO}_3)_2$ are presented in Figure S3.3. The LC_{50} and LC_{10} values of single compounds calculated from the CRCs are provided in Table 3.1. Based on the LC_{50} and LC_{10} values, the toxicity decreased in the order of $\text{Cu}(\text{NO}_3)_2 > \text{CuNPs} > \text{Zn}(\text{NO}_3)_2 > \text{ZnONPs}$. 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).

Table 3.1 Lethal concentrations (mgCu L⁻¹ or mgZn L⁻¹) of suspensions of Cu and Zn NPs, and of solutions of Cu(NO₃)₂ and Zn(NO₃)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)

In the presence of SR-NOM, a significant reduction in the mortality of *D. magna* exposed to CuNPs was observed (Figure 3.3A). It is obvious that 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 (Figure 3.3B) 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-EC₅₀ 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 bioaccumulation/biological effects are likely enhanced (Deng et al., 2017).

As can be seen in Figure S3.4, 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 S3.1. As shown in Table S3.1, 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 Figure 3.3C. 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.

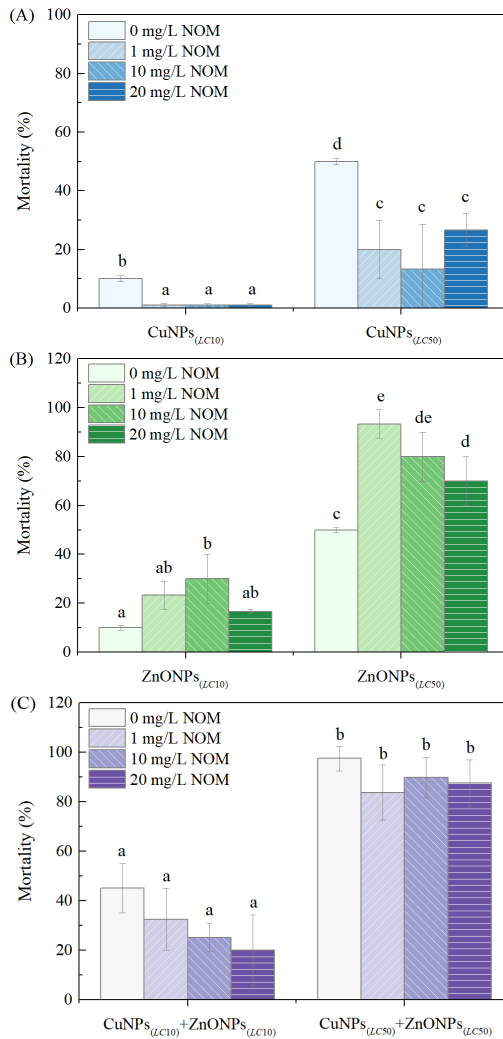


Figure 3.3. Mortality (%) of *D. magna* exposed to individual CuNPs (A) and ZnONPs (B) in the presence of 0, 1, 10, and 20 mg L⁻¹ SR-NOM; and the impact of SR-NOM (0, 1, 10 and 20 mg L⁻¹) on mixture toxicity of CuNPs and ZnONPs, based on the LC_{10} and LC_{50} ratios (C) at their individual LC_{10} and LC_{50} concentration. Values are expressed as mean \pm standard deviation ($n=3$). Letters indicate statistically significant ($p < 0.05$) differences between the treatments.

3.3.2.2 Relative contribution of MNP_{ion} and $\text{MNP}_{\text{particle}}$ to overall toxicity

Figure 3.4 depicts the relative contribution of MNP_{ion} and $\text{MNP}_{\text{particle}}$ to the overall toxicity of the individual and binary mixtures of CuNPs and ZnONPs. As shown in Figure 3.4A, $\text{CuNPs}_{\text{particle}}$ and $\text{CuNPs}_{\text{ion}}$ accounted for 82% and 18% of the toxicity of $\text{CuNPs}_{\text{overall}}$ at the LC_{50} level, respectively, while $\text{CuNPs}_{\text{particle}}$ and $\text{CuNPs}_{\text{ion}}$ accounted for 0% and 100% of the toxicity of $\text{CuNPs}_{\text{overall}}$ at the LC_{10} level. The high contribution of dissolved Cu-ions to the toxicity of $\text{CuNPs}_{\text{overall}}$ 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 $\text{CuNPs}_{\text{particle}}$ and $\text{CuNPs}_{\text{ion}}$ to the toxicity of $\text{CuNPs}_{\text{overall}}$ at the LC_{10} level, which is due to the fact that no mortality was observed for the $\text{CuNPs}_{\text{overall}}$ in the presence of SR-NOM (Figure 3.4A). Upon the addition of SR-NOM, the relative contribution of $\text{CuNPs}_{\text{ion}}$ to the toxicity of $\text{CuNPs}_{\text{overall}}$ at the LC_{50} level decreased owing to the reduction of dissolved Cu^{2+} -concentrations by SR-NOM. As shown in Figure 3.4B, $\text{ZnONPs}_{\text{ion}}$ accounted for 100% of the relative contribution to the toxicity of $\text{ZnONPs}_{\text{overall}}$ 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 $\text{ZnONPs}_{\text{ion}}$ to the toxicity of $\text{ZnONPs}_{\text{overall}}$ at the LC_{10} level, whereas SR-NOM had different impacts on the relative contribution of $\text{ZnONPs}_{\text{ion}}$ to the

toxicity of $\text{ZnONPs}_{\text{overall}}$ at the LC_{50} level. Generally, decreasing the concentration of SR-NOM increased the relative contribution of $\text{ZnONPs}_{\text{particle}}$ to the toxicity of $\text{ZnONPs}_{\text{overall}}$ at the LC_{50} level.

Figure 3.4C presents the relative contribution of MNP_{ion} and $\text{MNP}_{\text{particle}}$ 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 $\text{MNP}_{\text{particle}}$ (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.

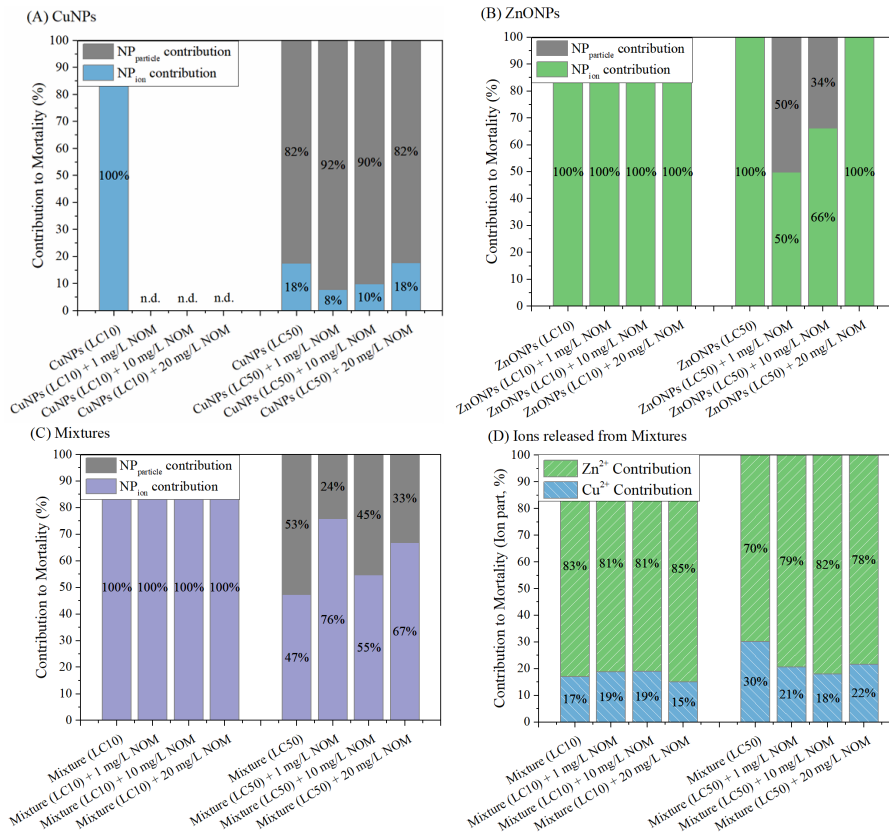


Figure 3.4. Relative contribution to toxicity of $MNP_{particle}$ and MNP_{ion} of the individual CuNPs (A) and ZnONPs (B), and the binary mixtures (C) at the LC_{10} and LC_{50} ratios in the absence and presence of 1, 10 and 20 $mg\ L^{-1}$ SR-NOM. (D) depicts the relative contribution of Cu-ions and Zn-ions released from the MNPs to the toxicity caused by the total dissolved ions. n.d. = not determined.

The specific contribution of Cu-ions and Zn-ions to the toxicity induced by total dissolved ions was further separated, as shown in Figure 3.4D. Generally, the contribution of Zn-ions to the overall toxicity induced by MNP_{ion} in the binary mixtures in the absence and presence of 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.

3.3.3 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 Figure 3.5. 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 Figure 3.5. 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 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.

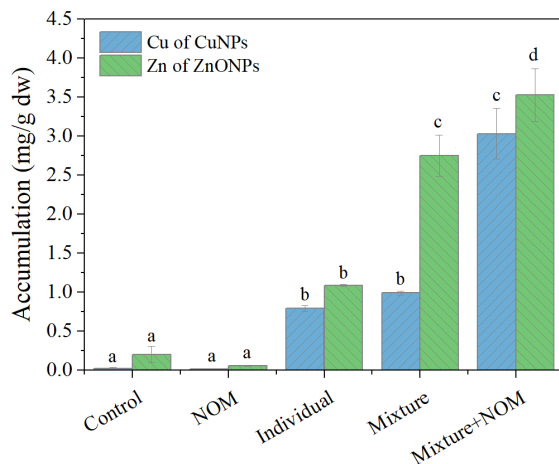


Figure 3.5. Accumulation (mg g^{-1} dry weight) of total Cu and Zn in *D. magna* after 48 h exposed to the single and binary mixtures of CuNPs and ZnONPs in the presence of 20 mg L^{-1} SR-NOM. Values are expressed as mean \pm standard deviation ($n=3$). Different letters indicate significant differences between the treatments for the accumulation of the same elementary ($p < 0.05$).

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).

3.4 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.

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Supplementary Information

Preparation of test suspensions

Stock suspensions containing 100 mg L⁻¹ MNPs or metal salts were freshly prepared in ElendtM7 medium (OECD 202(OECD, 2004)). To disperse the nanoparticles in the medium, each suspension was sonicated for 15 min at 50 Hz in a water bath sonicator (USC200T, VWR, Amsterdam, The Netherlands). A SR-NOM stock solution was prepared by dissolving the SR-NOM in Milli-Q water. After stirring for 24 h at 22 °C in a dark room and filtration through a 0.45 µm cellulose acetate membrane, the stock solution was covered with aluminum-foil and stored at 4 °C prior to use. The SR-NOM contained 43.7% organic carbon².

Physicochemical characterization

The morphology and size of suspensions of 1 mg L⁻¹ of ZnONPs and CuNPs were determined using transmission electron microscopy (TEM, JEOL 1010, JEOL Ltd., Japan). The size distribution (Z-average hydrodynamic diameter), and zeta-potential of suspensions of 1 mg L⁻¹ MNPs with different nominal concentrations of SR-NOM (1, 10, and 20 mg L⁻¹) were analyzed by dynamic light scattering (DLS) using a zetasizer Nano-ZS instrument (Malvern, Instruments Ltd., UK).

The stock solutions were diluted to the appropriate test concentrations in ElendtM7 medium. The pH values of all test suspensions were adjusted by the addition of either 0.1 M NaOH or 0.1 M HCl to maintain a fixed value of around 8.4. During the 48 h of incubation, these suspensions were stored in a climate room under a

16:8 h light-dark cycle (22 ± 1 °C, RH: 80.0%). The samples were collected at the top 0.5 to 1 cm layer of the dispersion after 0, 24, and 48 h. Samples for analysis of the concentration of dissolved ions shedding from the MNPs were firstly centrifuged at 30392 g for 30 min at 4 °C (Sorvall RC5Bplus centrifuge, Fiberlite F21-8 × 50y rotor). Eight mL supernatant (in full) was then decanted into another tube and were digested by the addition of 2 drops of 65% nitric acid for at least 1 d.

Cultures of *Daphnia magna*

Daphnia magna cultures originate from Leiden University. Sensitivity of the organisms is verified every 6 months using potassium dichromate ($K_2Cr_2O_7$), as described within OECD Guideline 202(OECD, 2004). The daphnia culture medium, ElendtM7 medium, was prepared at pH 8.4 ± 0.4 according to OECD Guideline 202(OECD, 2004). *D. magna* was cultured at 22 ± 1 °C with a 16:8 light-dark cycle. Groups of 50-60 adult females were kept in 11 L glass aquariums filled with 5 L of culture medium. Cultures were renewed every week and daphnids were fed with *Pseudokirchneriella subcapitata* every 2 days.

Sampling procedure for the determination of the actual exposure concentrations of particles and ions

The actual exposure concentrations of particles and ions in each treatment were measured by AAS after incubation in the test medium without daphnids. The treatments were digested in 65% HNO_3 for at least 24 h. To obtain the ions released from the nanoparticles, the nanoparticle suspensions firstly were centrifuged at 30392 g for 30 min at 4°C (Sorvall 114 RC5Bplus 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).

Calculation of simplified time weighted average concentrations

To express the actual exposure concentrations over time, a simplified time weighted average (TWA) concentration was calculated based on the measured actual concentrations at 0, 24 and 48 h of exposure. As in our previous study, the TWA concentrations were calculated according to the following formula^{3,4}:

$$C_{TWA} = \frac{\sum_{n=1}^N (\Delta t_n \frac{C_{n-1} + C_n}{2})}{\sum_{n=1}^N \Delta t_n}$$

Where Δt is the time interval, n is the time interval number, N is the total number of intervals, C is the concentration at the end of the time interval. The simulated TWA concentrations were used for all calculations expressing the exposure.

Statistical analyses

A linear model with a Tukey's post Hoc test was developed to analyze the significance level and the contribution (%) of each treatment using SPSS 16.0 (IBM SPSS Statistics for Windows, Ver. 19.0, IBM Corp., Armonk, NY). Data were checked for normality and homogeneity before analysis of variance (ANOVA). The significance level in all calculations was set at $p < 0.05$.

Table S3.1 List of published studies (to date) conducted on joint toxicity of Cu- and Zn-salts to organisms in freshwater

Organism	Species	Cu:Zn ratio	Exposure conditions	Endpoint	Joint effect	References
Fish	<i>Gobiocypris rarus</i>	1:1	pH: 7.8 ± 0.2 25 ± 1°C 16:8 h light:dark photoperiod	75 h Embryo–larval toxicity bioassays (including hatching, lethality,etc.)	synergistic	Zhu et al., 2011 ⁵
Fish	<i>Pimephales promelas</i>	1:2	22.8 – 25.8°C	96 h lethality tests	more-than-additive	Lynch et al., 2016 ⁶
Fish	<i>Danio rerio</i>	series of combination	pH: 7.0 26 ± 0.5 °C 12:12 h light:dark photoperiod	24 h lethality tests Accumulation	antagonistic	Gao et al., 2017 ⁷
Freshwater snail	<i>Lymnaea stagnalis</i>	series of combination	pH: 7.9 ± 0.2 25 ± 1 °C 16:8 h light:dark photoperiod	Short-term accumulation	antagonistic	Crémazy et al., 2019 ⁸
Freshwater Alga	<i>Chlorella sp.</i>	EC ₅₀ ratio	27°C 12:12 h light:dark photoperiod	72 h growth inhibition Accumulation	less-than-additive or antagonistic	Franklin et al., 2002 ⁹
Water flea	<i>Daphnia magna</i>	series of combination	pH: 7.4–7.8 20 ± 1°C 16:8 h light:dark photoperiod 3mg/L DOC	48 h lethality tests	additive or more-than-additive	Meyer et al., 2014 ¹⁰

Water flea	<i>Daphnia magna</i>	<i>EC</i> ₂₀ ratio (1:10)	20 ± 1 °C DOC <1 mg/L	Feeding behaviour 48 h lethality	more-than-additive	Lari et al., 2017(Lari et al., 2017) ¹
Water flea	<i>Daphnia magna</i>	series of combination	pH: 7.0 21 ± 1 °C 16:8 h light:dark photoperiod	96 h accumulation test Bioavailability	additive or less-than-additive	Komjarova and Blust, 2008 ¹²
Water flea	<i>Ceriodaphnia dubia</i> and <i>Daphnia carinata</i>	1:10	pH: 7.5 ± 0.3 25 ± 1 °C 16:8 h light:dark photoperiod	48 h lethality tests 7 d lethality tests	additive or more-than-additive	Cooper et al., 2009 ¹³

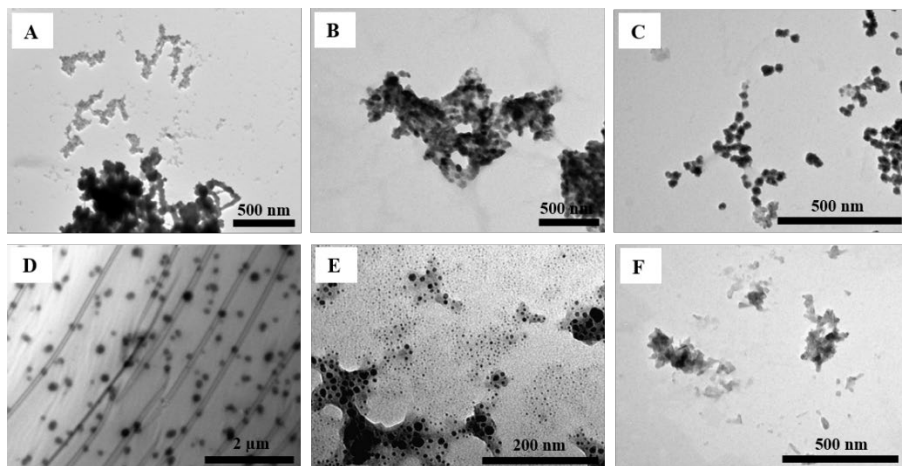


Figure S3.1 TEM images of the MNPs in the absence and presence of SR-NOM (A: CuNPs; B: ZnONPs; C: CuNPs and ZnONPs; D: CuNPs with SR-NOM; E: ZnONPs with SR-NOM; F: CuNPs and ZnONPs with SR-NOM) in the ElendtM7 medium.

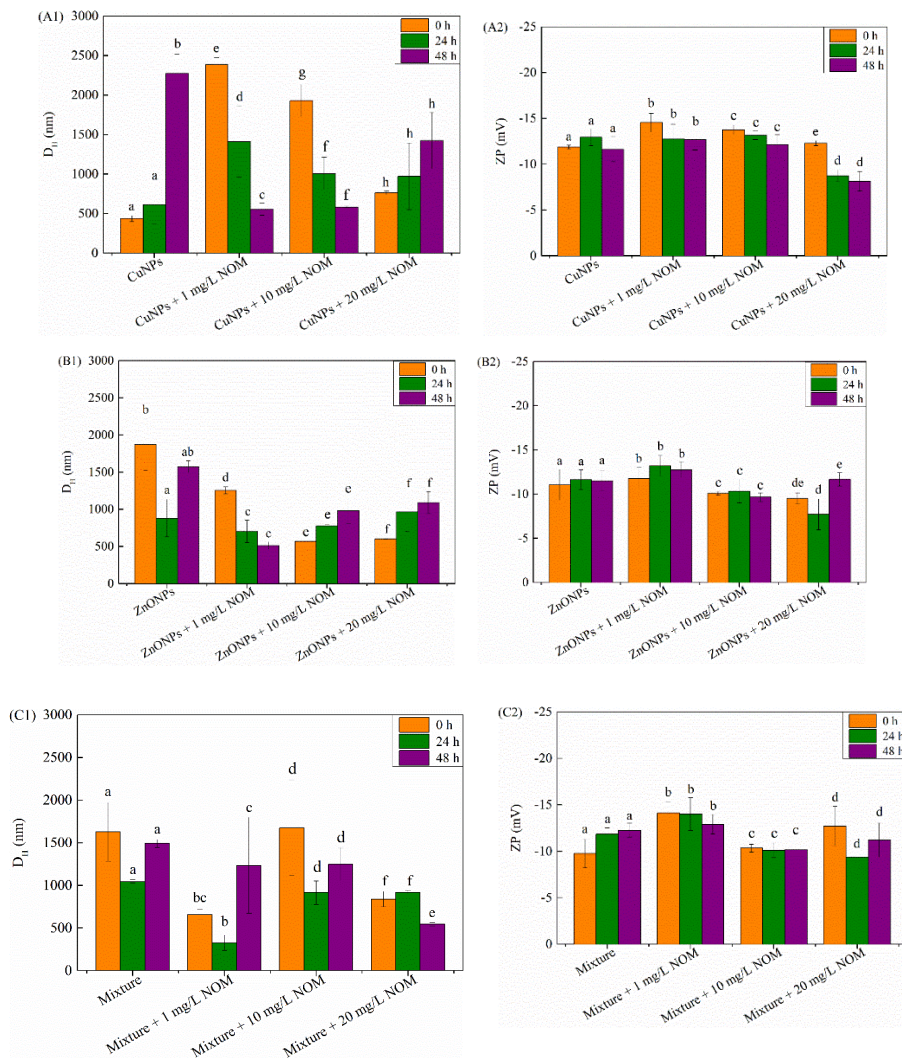
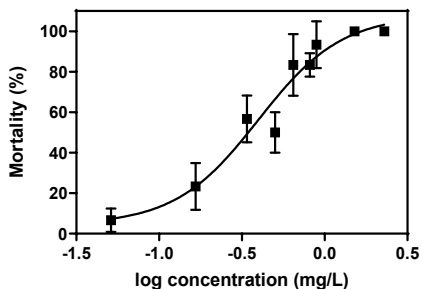
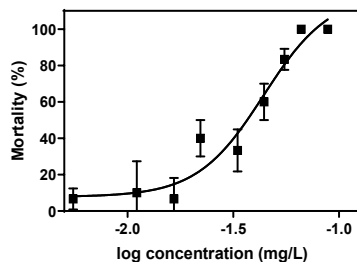


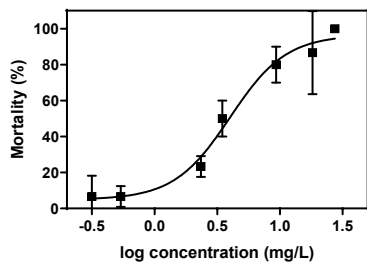
Figure S3.2 Variation in hydrodynamic diameter (D_H in nanometer) and zeta potential (ZP in mVolt) of CuNPs and ZnONPs and their binary mixture in the presence of 0, 1, 10, and 20 mg/L SR-NOM as a function of time (0, 24, and 48 hours). Values are expressed as mean \pm standard deviation ($n = 3$). Letters indicate statistically significant ($p < 0.05$) differences between the treatments.



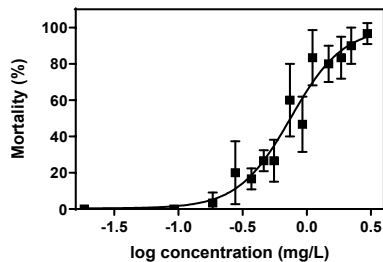
CuNPs



Cu(NO₃)₂



ZnONPs



Zn(NO₃)₂

Figure S3.3 Concentration–response curves of mortality (%) of *D. magna* exposed to individual CuNPs, ZnONPs, Cu(NO₃)₂, and Zn(NO₃)₂. The actual exposure concentration is expressed as the time weighted concentration (C_{TWA}). Actual log-transformed Cu or Zn concentrations are plotted on the x-axis. Data are mean \pm standard deviation (SD) ($n = 3$).

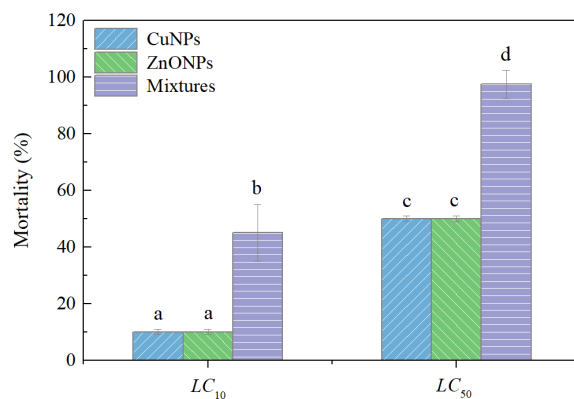


Figure S3.4 Comparison in mortality (%) between the individual and mixtures of CuNPs and ZnONPs. Values are expressed as mean \pm standard deviation ($n = 3$). Letters indicate statistically significant ($p < 0.05$) differences between the treatments.

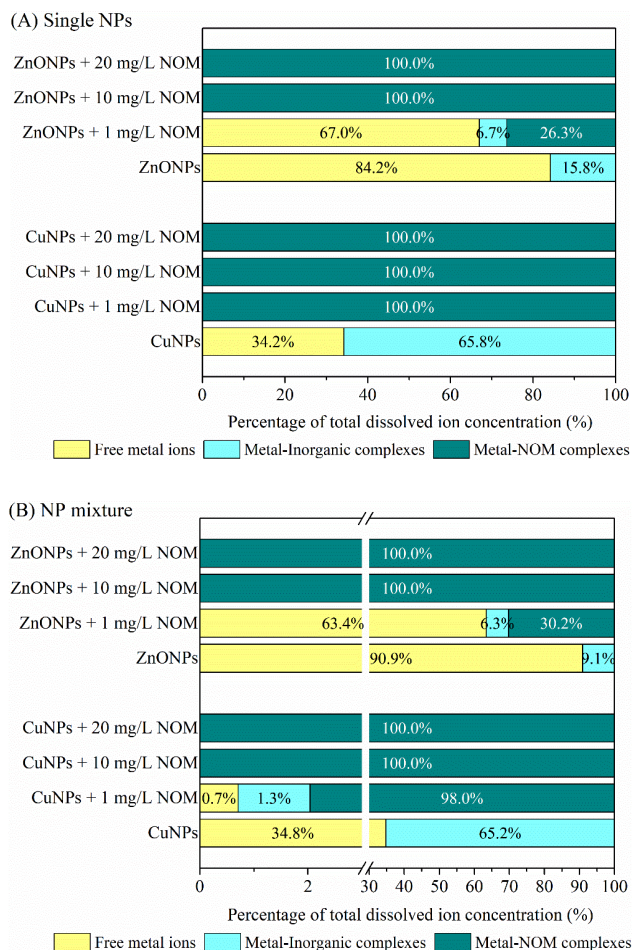


Figure S3.5 Percentage of free metal ions (yellow), metal-inorganic complexes (blue) and metal-NOM complexes (green) calculated with Visual MINTEQ3.1 for dissolved ions in suspensions of CuNPs and ZnONPs(Gustafsson, n.d.)⁴. Complexation to natural organic matter was simulated with state-of-the-art models (NICA-Donnan)(Baker, 2012; Gustafsson, 2001, n.d.; Gustafsson and Berggren Kleja, 2005)⁷.

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