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**Title:** Towards understanding the toxicity of copper nanoparticles in aquatic ecosystems  
**Issue Date:** 2015-07-02
Chapter 4 Assessing toxicity of copper nanoparticles across five cladoceran species

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*Environmental Chemistry and Toxicology. 2015*

*(doi: 10.1002/etc.3000)*

**Abstract**
Due to ever increasing applications, nanoparticles will eventually end up in the environment. However, currently no common principle has been established to help understand the toxicity of NPs across species. Therefore, it is difficult to estimate the potential risks of nanoparticles to untested species in the environment. We exposed four different sizes of copper nanoparticles and one submicron sized copper particle to five cladoceran species (*Daphnia magna*, *Daphnia pulex*, *Daphnia galeata*, *Ceriodaphnia dubia* and *Chydorus sphaericus*) to investigate if morphological attributes of species can help to assess the acute toxicity of copper nanoparticles across species. Results showed that rod shaped copper nanoparticles caused much lower toxicity to all species than spherical copper nanoparticles. Both the particles and ions contributed to the total toxicity of the CuNPs suspensions. Moreover, the toxicity caused by particles in five different copper suspensions increases with decreasing body length, surface area and body volume of neonates of five cladoceran species. Especially the correlations between body volume of the five cladoceran species tested and the corresponding toxicity caused by five different CuNPs were statistically significant and in all cases $R_{adj}^2$ was higher than 0.51 ($p < 0.001$). The highest correlation was found between the body volume and the toxicity of the 78 nm CuNPs ($R_{adj}^2 = 0.95$, $p < 0.001$). To conclude, the correlations between attributes of cladoceran species and the toxicity of CuNPs reported in this study evoke the possibility to assess and extrapolate the toxicity of NPs across species with similar attributes.

**Key words:** Copper nanoparticles, Cladoceran species, Acute toxicity, Attributes of species
4.1 Introduction

The fast development in nanotechnology provides many possibilities for industries and nanoparticles (NPs) with special properties have been manufactured and applied in many products. For instance, zinc-based NPs have been extensively applied in sunscreens because of their anti-ultraviolet properties (Osmond and Mccall 2010). Copper-based NPs can be used to improve the electron-transfer efficiency of electrochemical sensors because of their catalytic properties (Wang et al. 2004). With the increasing application of NPs, concerns regarding the fate and potential adverse effects of NPs in the environment raise sharply (Scheufele et al. 2007).

Plenty of studies elucidated that adverse effects of NPs are largely associated with NP properties like size, shape and surface coating (Yu et al. 2012), and with environmental characteristics like pH, water chemistry and temperature (Yu et al. 2012, Auffan et al. 2009). However, toxicity data generated in different studies are hardly comparable as often different NPs, different testing species and different testing strategies were adopted. Little is known regarding the toxicity of NPs across species. A recent study supported that morphological attributes may play an important role in affecting the toxicity of NPs to invertebrates (Hund-Rinke and Simon 2006). Another study also reported that physiological attributes of cells, like the thickness of the cellular barrier, can significantly affect the DNA damage caused by cobalt-chromium NPs (29 nm ± 6.3 nm) and titanium dioxides NPs (50 nm ± 20 nm) (Sood et al. 2011). Despite those studies suggesting that attributes of testing organisms/cells can affect the toxicity of NPs, no common principle has been established to help understand the toxicity of NPs across species (Nel et al. 2006).

Previous studies have proven that there are quantitative links between attributes of species and the toxicokinetic endpoints measured in organisms following exposure to metals/pesticides (Rubach 2010). For instance, it was found that the values of the 96-h LC50 caused by copper increases with the increase of body mass of diverse fish species, with an r² of 0.54 (Grosell et al. 2002). A positive correlation was also found between the acute toxicity of zinc ions (LC50) in four types of cladoceran species and the body size of those species (Vesela and Vijverberg 2007). It would be even more meaningful to explore the relation between the attributes of species and the toxicity of NPs. Because currently the toxicity data on NPs are far scarcer than the data for ‘regular’ chemicals, and understanding the relationship between the attributes of species and the toxicity of NPs can assist in extrapolating the toxicity of NPs across species with similar attributes.

We exposed five cladoceran species to four sizes of copper nanoparticles (CuNPs) and one submicron sized copper particle (SMP) to investigate if morphological attributes of cladoceran species play an important role in affecting the toxicity of CuNPs across species. Cladoceran species are selected to illustrate our approach because cladoceran species are filter feeders and they have simple feeding mechanisms. NPs in water can interact directly with the carapace of cladoceran species or be filtered and transported directly to the gut.
by thoracic appendages (Hund-Rinke and Simon 2006, Gophen and Geller 1984). Our hypothesis is that the acute toxicity of different sizes of CuNPs increases with decreasing body length, surface area and body volume of five cladoceran species.

4.2 Materials and Methods

4.2.1 CuNPs suspensions
CuNPs with a nominal size of 25, 50 and 100 nm stored in inert gas, respectively, were purchased from IoLiTec, Inc., Germany. CuNPs of 78 nm (nominal) and one type of submicron-sized copper particles (SMPs with nominal size of 500 nm) stored in inert gas were purchased from NanoAmor®, USA. All the particles were uncoated.

Stock suspensions of all CuNPs, the SMPs and of Cu(NO$_3$)$_2$ were freshly prepared in ISO standard test medium (STM) after 10 min of sonication in a water bath sonicator (ISO 1996). The STM used in this study (pH 7.8 ± 0.2) contained (mg/L MilliQ water): CaCl$_2$.2H$_2$O: 294; MgSO$_4$.7H$_2$O:123.25; NaHCO$_3$: 64.75; KCl: 5.75 (ISO 1996).

4.2.2 Physico-chemical Characterization
Initial sizes of all five particles were analyzed at 100 mg/L in MilliQ water using CPS disk centrifugation (CPS Instruments Europe., The Netherlands). The morphology of all CuNPs in STM was characterized by TEM in STM (JEM1010, JEOL Ltd., Japan) at 0 h. Size distributions of all copper suspensions at 1mg/L in STM were measured at 0, 24 and 48 h under testing conditions by DLS on a Zetasizer Nano-ZS instrument (Malvern, Instruments Ltd., UK). Three independent measurements were taken with each measurement consisting of three measurements. The zeta potential of all copper suspensions at the same time point was measured by this instrument as well.

4.2.3 Actual exposure concentrations and copper ion release
The actual Cu concentration in CuNPs suspensions and in solutions of Cu(NO$_3$)$_2$ with nominal concentrations of 0.1 mg/L and 0.01 mg/L were measured by Graphite furnace atomic absorption spectrometry (GFAAS, Perkin Elmer 1100 B) and listed (SI 4.1). The normalized percentage of measured concentrations versus nominal concentrations of each CuNPs suspension was applied to calculate the actual exposure concentrations of all suspensions and solutions used in this study.

Ion release of each copper suspension was measured at a concentration close to the estimated LC$_{50}$ (concentration causing a 50 % of immobilization of crustaceans with respect to the controls) value of each suspension. Therefore, 2 ml of 0.1 mg/L 78 nm and 0.01 mg/L all other copper suspensions were sampled at time 0 and 48 h after incubation and centrifuged at 16 100 g for 20 min at 4°C (5415 R series centrifuge, Eppendorf, Germany) to remove aggregates from suspensions (Song et al. 2014, Fernandez et al. 2013). Research carried out by a colleague in the same group confirmed that there were
no particle was presented in the supernatants using DLS measurement (Xiao et al. 2015). Each measurement consisted of two replicates. The total concentration of prepared CuNPs suspensions and the supernatants were then acidified using 10% HNO$_3$ and then analyzed using AAS. Copper ion release (%) was calculated as percentage of the total copper concentration.

4.2.4 Cladoceran species

*Daphnia magna, Daphnia pulex, Daphnia galeata, Ceriodaphnia dubia* from the family of Daphniidae and *Chydorus sphaericus* from the family of Chydoridae were selected as test organisms in this experiment in view of the variation of their body length, surface area and body volume. All cladoceran species were cultured in STM medium in plastic containers with a volume of 1 liter under 16:8 light-dark cycle (20 °C ± 1 °C) and fed with 3:1 mixture of *Pseudokirchniella subcapitata* and *Chlamydomonas reinhardtii* three times a week.

Ten neonates replicates (< 24h), corresponding in size to those in the toxicity tests of each species were randomly selected and morphological modalities of these neonates were observed by a microscope equipped with a digital camera in the beginning of the experiment. Details about how the morphology was measured and calculated are shown in SI 4.2.

4.2.5 Exposure

We exposed all five cladoceran species to suspensions of the five different sizes of copper particles for 48 h to test the acute toxicity of copper particles according to the OECD 202 guidelines (OECD 2004). Cu(NO$_3$)$_2$ was used as a positive control. Eight groups containing five individuals per group were exposed to a series of test concentrations of each CuNPs suspension and SMPs suspension as well as of the positive and negative controls with a volume of 20 mL in glass vials. Subsequently, these vials were incubated for 48 h under 16:8 light-dark cycle (20 °C ± 1 °C). Test organisms were not fed during the experiments. Immobilization (%) of each species was recorded after 48 h. Less than 10% immobilization of organisms in the control was considered to be valid as a qualified control.

4.2.6 Data analysis and statistics

All exposures were performed with eight replicates. Normality and homoscedasticity of all data was checked prior to carrying out statistical analysis. The lethal concentration causing 50 % of mortality during 48h of exposure (48 h-LC$_{50}$sus) to each copper suspension and to Cu(NO$_3$)$_2$ was calculated based on dose response curves using SPSS 16.0 using the function of the Probit regression (IBM SPSS, Armonk, NY, USA). Multiple comparisons among LC$_{50}$sus of different CuNPs suspensions to each species were carried out using a one-way ANOVA followed by a Holm-Sidak method (p < 0.05) using Sigmaplot 12.0 (Systat Software
The toxic contribution of the particle and of the released ion of CuNPs and SMPs suspensions was calculated and plotted separately as described in the SI. Based on this calculation, the dose response curve caused by the particle in CuNPs and SMPs suspensions were obtained. Subsequently, LC50 values of the particle in CuNPs and SMPs suspensions were calculated as the method mentioned above.

Linear regression analysis was carried out to analyze the relations the 48-h LC50 of each copper suspension and Cu(NO3)2 versus body length, surface area and body volume of five cladoceran species using SPSS (IBM SPSS, Armonk, NY, USA). The same regression technique is also used to analyze the relation between the 48-h LC50 caused by NPs only in each copper suspension and morphological attributes of the five cladoceran species as well as the relation between initial particle size and the 48-h LC50 caused by NPs in each copper suspension. The regression significance p < 0.01 was considered as statistical significant.

4.3 Results

4.3.1 Physico-chemical characterization of CuNPs

Transmission electron microscopy (TEM) pictures revealed that the 25, 50 and 100 nm CuNPs and the 500 nm particles were spherical, whereas the 78 nm CuNPs had a rod shaped nanostructure that was visible within aggregates in the Standard Test Medium (STM) (Figure 4.1). The size distributions of all particles were determined by CPS Disc Centrifugation (Figure 4.1). The size of the 25 nm CuNPs was predominantly distributed between 25 nm and 95 nm (70 %), with a mean size of 48 nm. The size distribution of the 50 nm CuNPs was in between 50 nm and 531 nm (98 %) with a mean value of 144 nm. 70 % of the 78 nm CuNPs had a size between 50 nm and 150 nm (mean size: 134 nm). Around 90 % of the 100 nm CuNPs had a size between 40 nm and 190 nm and the mean size detected by CPS was 113 nm for the 100nm CuNPs. All particles were monodispersed, except the 500 nm CuNPs. The suspension of the 500 nm CuNPs in STM had approximately 30 % of particles smaller than 136 nm and about 40 % of the particles were distributed between 260 nm and 730 nm.

All particles underwent aggregation and dissolution processes simultaneously in STM during 48 h (Figure 4.2). Dynamic Light Scattering (DLS) results showed that all copper particles were present as large aggregates in the STM at 0 h and retained their aggregation status after 48 h of incubation under culture conditions (Figure 4.2(a)). Ion release of different sizes of CuNPs and SMPs in STM at time 0, 24 and 48 h was time-dependent (Fig 4.2(c)).
Figure 4.1 Morphology and size distribution of the 25 nm, 50 nm, 78 nm, 100 nm and 500 nm CuNPs in MilliQ water, measured by TEM and CPS. Notice that the X axes have different data ranges for each CuNPs. The relative particle number is shown on the Y axes in the graph, as generated by the default software of CPS. The number of particles at the peak was defined as 100%. The number of particles with other sizes was normalized as the percentage of the particle number at the peak. Therefore, peak area indicates the percentage of particle size distribution.
Chapter 4 Toxicity of copper nano in cladocerans

Copper ions were already present in the STM after sonication (0 h) with the concentrations ranging from 1.9 % ± 0.96 % to 13.4 % ± 6.3 % depending on the type of copper particle. The majority of ions were released during the first 24 h in the STM. The dissolution processes slowed down after 24 h and around 20 – 30 % of the total amount was dissolved after 48 h for all CuNPs and the SMPs except the 78 nm CuNPs. The 78 nm CuNPs released the lowest amount of ions (6 % on a mass basis) in STM and the aggregation size of 78 nm NPs was most stable during 48 h incubation as compared with the other particles tested.

There was no obvious trend for the variation of zeta potential over 48 h (Figure 4.2(b)). The zeta potential of all copper suspensions over 48 h was between -10 to -25 mV. Therefore, aggregation and dissolution processes were expected to continue with increase in timespan.

4.3.2 Acute toxicity of CuNPs

Less than 10% of mortality was observed in negative control, indicating the validation of our experiment according OECD 202 guidelines (OECD 2004). It was observed that all particle suspensions showed high toxicity to all tested species (Figure 4.3, the underlying data are listed in SI 4.4). The 25 nm CuNPs suspension expressed the highest toxicity to D. pulex (LC₅₀sus of 0.009 ± 0.001 mg/L), D. galeata (LC₅₀sus of 0.0012 ± 0.001 mg/L) and C. dubia (LC₅₀sus of 0.003 ± 0.001 mg/L), compared to Cu(NO₃)₂ and all other particle suspensions. Cu(NO₃)₂ was the most toxic to D. magna and C. sphaericus, with an 48-h LC₅₀sus of 0.020 ± 0.001 mg/L and 0.013 ± 0.002 mg/L, respectively. Compared to spherical CuNPs suspensions and the SMPs suspension, the rod shaped particle (78 nm) suspension expressed the lowest toxicity to all cladoceran species. The 48-h LC₅₀sus values of the 78 nm suspension were 0.791 ± 0.035 mg/L to D. magna, 0.166 ± 0.014 mg/L to D. pulex, 0.148 ± 0.017 mg/L to D. galeata, 0.015 ± 0.002 mg/L to C. dubia and 0.077 ± 0.005 mg/L to C. sphaericus, respectively.

C. dubia showed the highest vulnerability among all tested species, when exposed to copper suspensions and Cu(NO₃)₂. C. sphaericus, D. galeata and D. pulex showed intermediate values and D. magna was the most invulnerable species.
Figure 4.2. (a) Hydrodynamic diameter and (b) Zeta potential of CuNPs and SMPs suspensions before (0 h) and after 24 and 48 h incubation in the STM. Results are expressed as the mean of three replicates with standard deviation. (c) Ion release (%) of CuNPs and SMPs before (0 h) and after 24 h and 48 h incubation in STM. Results are expressed as the mean of two replicates in STM with their standard deviation (SD).

Figure 4.3 48-h LC$_{50}$ of the five copper particle suspensions and Cu(NO$_3$)$_2$ to five cladoceran species, plotted as the mean of eight replicates ± standard deviation. Significant differences (P < 0.05) between the LC$_{50}$ of different particles to the same cladoceran species are indicated using different characters.
 Attributes of species and toxicity of CuNPs

To investigate the correlation between morphological attributes of species and the toxicity of copper suspensions, we measured the body length, calculated the surface area and body volume of the neonates of five cladoceran species tested (SI 4.5), and plotted these attributes parameters against the eight replicates of 48-h LC$_{50}$ of each particle suspension (48-h LC$_{50\text{sus}}$) as well as the eight replicates of 48-h LC$_{50}$ caused by Cu(NO$_3$)$_2$ for all five cladoceran species in Figure 4.4. The statistical parameters and correlation coefficients are listed in Table 4.1.

The 48-h LC$_{50\text{sus}}$ of each copper suspension and of Cu(NO$_3$)$_2$ is statistically significantly correlated with the body length, surface area and body volume of the five tested species regardless the size and shape of copper particles as well as the different percentage of ions released from each particle, except for the correlation between the toxicity of 25 nm CuNPs suspension and body length. Body length ($R^2_{\text{adj}} = 0.63, p < 0.001$) and surface area ($R^2_{\text{adj}} = 0.62, p < 0.001$) are better parameter to demonstrate the 48-h LC$_{50}$ of Cu(NO$_3$)$_2$ across five species than body volume ($R^2_{\text{adj}} = 0.49, p < 0.001$), whereas body volume is the best parameter to illustrate the toxicity of CuNPs suspensions across five species indicating that both particles and ions in these CuNPs suspensions were responsible for the toxicity to cladoceran species.

Nevertheless, since the same type of correlation also exists between the toxicity caused by Cu(NO$_3$)$_2$ and the body size of the five tested species (Figure 4.4), it is still unclear whether the ion or the particles in CuNPs suspensions are predominantly driving the relationship between body size of species and the acute toxicity of CuNPs suspensions. In order to gain insight into the toxicity contributed by the particles only in CuNPs suspensions, and to better understand the relationship between the toxicity of particles only in each CuNPs suspension and the body size of the five tested species, the relative toxic contribution of particles and ions in all copper suspensions was calculated separately based on the average ion release at 0 h and 48 h using the dose response curve of Cu(NO$_3$)$_2$ (SI 4.3 , SI 4.6). Our results showed that particles contributed significantly to the total toxicity of CuNPs suspensions. Subsequently, we calculated the LC$_{50}$ caused by particles only in each suspension (LC$_{\text{50particle}}$) to five cladoceran species and the relationship between LC$_{\text{50particle}}$ and the body length, body surface area and body volume of the five tested species (Figure 4.5, Table 4.1 and SI 4.7). It was found that all LC$_{\text{50particle}}$ values were significantly correlated to the body size of the five tested species ($P < 0.01$). Especially, LC$_{\text{50particle}}$ values shows the best correlation with body volume of the five cladoceran species and in all cases $R^2_{\text{adj}}$ was higher than 0.51 ($p < 0.001$). The highest correlation was found between the body volume and the toxicity of the 78 nm CuNPs ($R^2_{\text{adj}} = 0.95, p < 0.001$).
Figure 4.4 The 48-h LC$_{50_{sus}}$ of each CuNPs suspension and the SMPs suspension as well as Cu(NO$_3$)$_2$ to five cladoceran species is plotted against the body length, surface area and body volume of five cladoceran species. The regression line is plotted.
### Table 4.1 The Statistical parameters of the correlations between length, surface area and volume of five cladoceran species and the corresponding LC$_{50\text{us}}$ caused by each particle suspension and Cu(NO$_3$)$_2$ (upper part), as well as the corresponding LC$_{50\text{particle}}$ caused by NPs only in each suspension (lower part) (n = 40)

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Note: C represents intercept, while B represents slope in linear regression. f represents significance f from ANOVA analysis. p < 0.01 is statistically significant.
Figure 4.5 The 48-h LC_{50_{particle}} of NPs in CuNPs suspension to five cladoceran species is plotted against the body length, surface area and body volume of the five cladoceran species. The regression line is plotted.

4.4 Discussion

4.4.1 Particle properties and toxicity of CuNPs

NPs with different sizes and different shapes of the particles were used in this study to make sure that the relationship between the attributes of species and the toxicity of NPs is not a matter of coincidence. Our results showed that different shapes of the NPs have very distinct fate in the testing medium. The amount of ions released by the rod shaped 78 nm CuNPs was much lower than the amount released by the other spherical CuNPs. These differences indicated that the rod-shaped CuNPs are more stable among all particles tested. These findings with regard to the stability and toxicity of rod shape NPs are consistent with previous findings. A previous study from our laboratory showed that the ion release from rod-shaped CuNPs was three times less than from spherically shaped CuNPs after 48 h incubation in cell culture medium (Song et al. 2014). Another recent study also revealed similar information, showing that dissolution of rod-shaped CuNPs was significantly lower than of spherical CuNPs (Misra et al. 2012).

The physiochemical properties of CuNPs also affected their toxicity on the testing organisms. Compare to other spherical CuNPs, the rod shaped 78 nm CuNPs was the least toxic particle used in this study according to our calculation (SI 4.7). Previous literature unfolded similar finding, showing that dendritic nickel-based NPs expressed dramatically higher toxicity to zebrafish embryos than spherical nickel-based NPs (Ispas et al. 2009).
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This finding indicates NPs do bring additional nano-specific effects and shape of NPs is one of the important factors need to be considered when investigating the toxicity profile of CuNPs.

We also investigated the relation between physicochemical properties of CuNPs and the corresponding toxicity caused by particles in each suspension. Due to the wide distribution of particle sizes, the difference of particle shape and different sensitivity of species, the relationship between particle size and the toxicity caused by particles in each suspension vary from species to species (SI 4.8). No clear trend was found between the actual mean size of CuNPs and their corresponding toxicities to the five tested species in this study. Similar result was also revealed by another study, showing no relationship can be established between the acute toxicity of zinc-based NPs suspensions and their initial particle sizes (Lopes et al. 2014).

4.4.2 Attributes of species and toxicity of CuNPs

All CuNPs and SMPs suspensions showed high toxicity to all tested species. Even though the size, shape of CuNPs and the amount of ions released from each CuNPs suspension can affect the link between morphological attributes of the family of daphniidae and toxicity of copper suspensions, our results illustrated that the attributes of testing organisms also play an important role in the interaction with NPs. The smaller organisms were more vulnerable to CuNPs. The main reasons which can explain the inverse relationship between toxicity of CuNPs and body size of cladoceran species can be summarized in two aspects. Firstly, proportionally, smaller cladocerans were exposed to more particles than bigger ones because of the high surface area/volume ratio of smaller organisms. Secondly, particles and ions can be collected faster through water filtration and transferred faster into the gut and potentially be taken up faster by smaller cladocerans due to their higher respiration rate and circulation rate than in case of larger organisms (Peters 1986, Scanlan et al. 2013). The smaller organisms are thus more sensitive to chemical stressors, which is in line with previous studies (Grosell et al. 2002).

In this study, we found that body length and body surface area are better correlated to the toxicity caused by Cu(NO$_3$)$_2$ than the particles in CuNPs suspensions. Body volume has a better correlation with the toxicity of the particles in CuNPs suspensions. Possible reasons explaining the differences observed include the fact that copper ions and particles have different physiochemical properties. Previous studies illustrated that the physiochemical properties of chemical stressors would significantly influence their uptake route (Shakweh et al. 2005) and toxicokinetics (López-Serrano et al. 2014). For instance, the uptake of metal ions through the carapace of cladoceran species contributes significantly to the initial uptake of metal in aquatic invertebrates, rather than from a dietary source (Robinson et al. 2003). However, currently no evidence is available showing the uptake of NPs through carapace. NPs are more likely to be taken up through the epithelial cells of the gut (Lovern et al. 2008, Heinlaan et al. 2011). A recently
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toxicokinetical analysis reported that silver NPs have a very distinct uptake and depuration rate in zebrafish eleutheroembryos, compared with silver ions (López-Serrano et al. 2014). The difference in uptake routes and toxicokinetics may intrigue the different relations between the stressors and the attributes of species. Our results revealed that the attributes that can be used to assessing the toxicity of chemical stressors across species may vary between the stressors with different physiochemical properties, which are in line with a previous study (Rubach 2010).

To conclude, the present study suggests that physiochemical properties of NPs can dramatically affect the toxicity of NPs suspensions. The correlation between attributes of cladoceran species and the toxicity of CuNPs reported in the present studies further emphasis the importance of considering attributes of testing organisms in future studies to better understand the toxicity of NPs across species, and evokes the possibility to assess and extrapolate the toxicity of NPs across species with similar attributes.

Acknowledgements

Lan Song is sponsored by the Environmental ChemOinformatics Marie Curie Initial Training Network (ECO-ITN) within the seventh research framework programme of the European Union (238701). Part of the work was performed within the frameworks of the RIVM sponsored project “IRAN” and the NATO sponsored project “Ecotoxicity of metal and metal oxide nanoparticles: Experimental study and modelling”, project number SFPP•984401. We thank Eric van Genderen for his help with the TEM characterization.
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Bibliography


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Xiao Y, Vijver MG, Chen G and Peijnenburg WJGM. 2015. Toxicity and Accumulation of Cu and ZnO nanoparticles in Daphnia magna. Environmental Science & Technology. In press. DOI: 10.1021/acs.est.5b00538

**Supplementary Information**

**SI 4.1** Measured concentrations of the percentage of nominal concentration of all CuNPs particles, expressed as (%).

<table>
<thead>
<tr>
<th>Percentage (%)</th>
<th>25 nm</th>
<th>50 nm</th>
<th>78 nm</th>
<th>100 nm</th>
<th>500 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>132.7 ± 0.2</td>
<td>109.7 ± 18.9</td>
<td>70.8 ± 10.4</td>
<td>62.1 ± 2.6</td>
<td>158.2 ± 2.7</td>
</tr>
</tbody>
</table>

**SI 4.2** Body length was defined as the length from the top of the eyespot to the base of the tail spine (a) was measured for each organism (Oda et al. 2011). Width of carapace (b) and the thickness of the body (c) were measured as well.

![Diagram of Daphnia morphology](image)

**Figure SI 4.2.1** The Simplified morphology of daphnids

Surface area of the species from family of daphniidae was treated as ellipsoid and calculated by Knud Thomsen approximation (Klamkin 1976a, Klamkin 1976b) (Eq. 1). Body volumes were calculated according Eq. 2

\[
S = 4\pi \left( \frac{a^p b^p c^p}{3} \right)^{1/p} \quad \text{Eq. 1}
\]

\[
V = \frac{4}{3} \pi \left( \frac{a}{2} \times \frac{b}{2} \times \frac{c}{2} \right) \quad \text{Eq. 2}
\]

Where S is the surface area of organism. a, b and c are shown in Figure SI 4.2.1 1. p ≈ 1.61.

The body of C. sphaericus is close to spherical. The surface area and body volume of C. sphaericus were calculated as Eq. 3 and Eq. 4

\[
S = 4\pi \left( \frac{a}{2} \right)^2 \quad \text{Eq. 3}
\]

\[
V = \frac{4}{3} \pi \left( \frac{a}{2} \right)^3 \quad \text{Eq. 4}
\]

where S and V are the surface area and body volume of C. sphaericus, respectively.

a is one-half of body length of C. sphaericus.
SI 4.3 The relative toxic contribution of particulates in the CuNPs suspensions and the relative toxic contribution of ions, expressed as mean ± standard deviation. LC$_{50}$ caused by particulates in each suspension is listed in table below.

The calculation detail has been reported in previous study (Song et al. 2014). Briefly, it was assumed that the release of copper ions is independent of the concentration of CuNPs and there are no interactions between Cu$^{2+}$ and CuNPs. The toxicity of Cu$^{2+}$ ($E_{Cu^{2+}}$) in the copper suspensions could be determined according to the concentration–response curve of Cu(NO$_3$)$_2$. The total toxicity of copper suspensions was assessed experimentally. Therefore, the toxic effect of the particle form of the CuNPs ($E_{CuNP}$) can be estimated using the response addition model:

$$E_{CuNP} = 1 - \frac{1 - E_{total}}{1 - E_{Cu^{2+}}}$$

Where $E_{total}$ represents the total toxicity caused by the copper suspensions. $E_{CuNP}$ and $E_{Cu^{2+}}$ represent the toxicity caused by the particle form of CuNPs and Cu$^{2+}$, respectively.

Reference


### S14.4 48-h LC50sus of CuNPs suspensions, SMP suspension and Cu(NO3)2 to five cladoceran species, plotted as the mean of eight replicates ± standard deviation.

<table>
<thead>
<tr>
<th>LC50sus (mg/L)</th>
<th>25 nm</th>
<th>50 nm</th>
<th>78 nm</th>
<th>100 nm</th>
<th>500 nm</th>
<th>Cu²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. magna</td>
<td>0.100 ± 0.02</td>
<td>0.136 ± 0.017</td>
<td>0.791 ± 0.035</td>
<td>0.096 ± 0.012</td>
<td>0.099 ± 0.01</td>
<td>0.020 ± 0.001</td>
</tr>
<tr>
<td>D. pulex</td>
<td>0.009 ± 0.001</td>
<td>0.044 ± 0.004</td>
<td>0.166 ± 0.014</td>
<td>0.062 ± 0.004</td>
<td>0.034 ± 0.004</td>
<td>0.017 ± 0.003</td>
</tr>
<tr>
<td>D. galeata</td>
<td>0.012 ± 0.001</td>
<td>0.061 ± 0.006</td>
<td>0.148 ± 0.017</td>
<td>0.040 ± 0.005</td>
<td>0.020 ± 0.003</td>
<td>0.015 ± 0.002</td>
</tr>
<tr>
<td>C. sphaericus</td>
<td>0.052 ± 0.011</td>
<td>0.045 ± 0.01</td>
<td>0.077 ± 0.005</td>
<td>0.032 ± 0.005</td>
<td>0.017 ± 0.002</td>
<td>0.013 ± 0.002</td>
</tr>
<tr>
<td>C. dubia</td>
<td>0.003 ± 0.001</td>
<td>0.002 ± 0.0003</td>
<td>0.015 ± 0.002</td>
<td>0.003 ± 0.001</td>
<td>0.006 ± 0.001</td>
<td>0.006 ± 0.001</td>
</tr>
</tbody>
</table>

### S14.5 Body length, surface area and body volume of neonates of each tested species, expressed as mean of ten replicates ± standard deviation.

<table>
<thead>
<tr>
<th>Morphological data</th>
<th>D. magna</th>
<th>D. pulex</th>
<th>D. galeata</th>
<th>C. sphaericus</th>
<th>C. dubia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body length (mm)</td>
<td>1.10 ± 0.04</td>
<td>0.86 ± 0.03</td>
<td>0.73 ± 0.01</td>
<td>0.23 ± 0.04</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>Surface area (mm²)</td>
<td>1.73 ± 0.11</td>
<td>0.93 ± 0.07</td>
<td>0.72 ± 0.10</td>
<td>0.14 ± 0.03</td>
<td>0.06 ± 0.005</td>
</tr>
<tr>
<td>Body volume (mm³)</td>
<td>0.19 ± 0.02</td>
<td>0.07 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.005 ± 0.002</td>
<td>0.001 ± 0.001</td>
</tr>
</tbody>
</table>
SI 4.6 The total toxicity (%) caused by copper suspensions (green), and the toxicity (%) caused by particle forms of each copper suspension (CuNPs) (red) and Cu$^{2+}$ (blue) plotted against the total copper concentrations, respectively. Results are expressed as means ± standard deviation.
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**SI 4.7** The LC$_{50}$ particle caused by NPs only in each suspension (mg/L), the calculation is based on the results in SI 4.2.

<table>
<thead>
<tr>
<th>LC$_{50}$ particle (mg/L)</th>
<th>25 nm</th>
<th>50 nm</th>
<th>78 nm</th>
<th>100 nm</th>
<th>500 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. magna</em></td>
<td>0.103 ± 0.012</td>
<td>0.152 ± 0.032</td>
<td>1.054 ± 0.103</td>
<td>0.099 ± 0.026</td>
<td>0.106 ± 0.032</td>
</tr>
<tr>
<td><em>D. pulex</em></td>
<td>0.007 ± 0.001</td>
<td>0.040 ± 0.005</td>
<td>0.164 ± 0.016</td>
<td>0.052 ± 0.003</td>
<td>0.030 ± 0.002</td>
</tr>
<tr>
<td><em>D. galeata</em></td>
<td>0.010 ± 0.001</td>
<td>0.055 ± 0.008</td>
<td>0.149 ± 0.022</td>
<td>0.033 ± 0.005</td>
<td>0.017 ± 0.002</td>
</tr>
<tr>
<td><em>C. sphaericus</em></td>
<td>0.052 ± 0.024</td>
<td>0.045 ± 0.021</td>
<td>0.075 ± 0.005</td>
<td>0.027 ± 0.004</td>
<td>0.015 ± 0.002</td>
</tr>
<tr>
<td><em>C. dubia</em></td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.015 ± 0.002</td>
<td>0.003 ± 0.001</td>
<td>0.006 ± 0.001</td>
</tr>
</tbody>
</table>

**SI 4.8** The actual sizes of CuNPs are plotted against the corresponding toxicities caused by particles in each CuNPs suspension to five cladoceran species.