

Fate, accumulation and ecotoxicity of copper nanoparticles under environmentally relevant conditions

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Citation

Xiao, Y. (2017, September 13). *Fate, accumulation and ecotoxicity of copper nanoparticles under environmentally relevant conditions*. Retrieved from https://hdl.handle.net/1887/56155

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Author: Xiao, Y. Title: Fate, accumulation and ecotoxicity of copper nanoparticles under environmentally relevant conditions Issue Date: 2017-09-13

Chapter 5 Impact of water chemistry on the particle-specific toxicity of copper nanoparticles to *Daphnia magna*

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Submitted to Science of the Total Environment

Abstract

Toxicity of metallic nanoparticle suspensions (NP_(total)) is generally assumed to result from the combined effect of the particles present in suspensions (NP_(particle)) and their released ions (NP_(ion)). Evaluation and consideration of how water chemistry affect the particle-specific toxicity of NP_(total) are critical for environmental risk assessment of nanoparticles. In this study, it was found that the toxicity of Cu NP_(particle) to *Daphnia magna*, in line with the trends in toxicity for Cu NP_(ion), decreased with increasing pH and with increasing concentrations of divalent cations and dissolved organic carbon (DOC). Without addition of DOC, the toxicity of Cu NP_(total) to *D. magna* at the LC50 was mainly driven by Cu NP_(ion) (accounting for $\geq 53\%$ of the observed toxicity). However, toxicity of Cu NP_(total) in the presence of DOC at a concentration range from 5 to 50 mg C/L largely resulted from the NP_(particle) (57%-85%), which could attribute to the large reduction of the concentration of Cu NP_(ion) and the enhancement of the stability of Cu NP_(particle) when DOC was added. Our results indicate that water chemistry needs to be explicitly taken into consideration when evaluating the role of NP_(particle) and NP_(ion) in the observed toxicity of NP_(total).

Key words: Copper nanoparticles; water chemistry; fate; toxicity; Daplinia magna

5.1 Introduction

The fast development of nanotechnology over the past decade has boosted the manufacture and application of engineered nanomaterials in industrial and consumer products. For example, Cu nanoparticles (CuNPs) currently are widely utilized in antimicrobials, semiconductors, catalysis and skin products. The rapid increase in their manufacture, use and disposal inevitably results in an increasing likelihood for CuNPs to be released into aquatic environments. Many studies have found that CuNPs are highly toxic to a wide range of organisms, such as algae (Adam et al., 2015a; Zhao et al., 2016), mussels (Hu et al., 2014), crustaceans (Song et al., 2015a; Xiao et al., 2016), and fishes (Hua et al., 2014; Song et al., 2015b). Hence, concerns regarding the environmental safety of CuNPs deserve to be emphasized.

Although a growing number of studies involved in nanotoxicology have been conducted over the past decade, issues regarding the mechanisms of toxicity of NPs are still under debate, especially the topic whether particles themselves or their released ions are the main driver for the toxicity of suspensions of slowly dissolving metallic NPs. Some recent studies found that the toxicity of metallic NPs was mainly due to their released ions (referred to as $NP_{(ion)}$ hereafter) (Jo et al., 2012; Adam et al., 2015b), while others revealed that the cause underlying the NPs toxicity was largely attributable to the NPs themselves (referred to as $NP_{(particle)}$) hereafter) (Hua et al., 2014; Santo et al., 2014; Wang et al., 2016). These inconsistent conclusions may result from ignoring the effects of the physicochemical properties of test medium on the fate and toxicity of $NP_{(particle)}$ and $NP_{(ion)}$. In fact, once emitted to aquatic environments, metallic NPs are commonly subject to undergo a series of environmental processes, such as dissolution and aggregation followed by sedimentation. As a consequence of these processes, a metallic NP suspension is generally a mixture of $NP_{(particle)}$ and $NP_{(ion)}$. Factors capable of influencing these environmental processes have the potential to affect the fate and toxicity of $NP_{(particle)}$ and $NP_{(ion)}$ in water systems, which may further result in the change of the contribution of $NP_{(particle)}$ and $NP_{(ion)}$ to the toxicity of NP suspensions. Currently, it is widely known that water chemistry parameters, such as pH (Mohd et al., 2014), electrolytes (especially divalent cations) and natural organic matter (NOM) (Mukherjee and Weaver, 2010; Grillo et al., 2015) can impact the environmental behavior and fate of NPs and the toxicity of NP suspensions to biota. However, very few studies have systematically investigated the impact of water chemistry on the particle-specific toxicity and on the relative contribution of $NP_{(particle)}$ to the observed toxicity of NP suspensions.

In this study, the behavior, fate and toxicity of CuNPs and copper ions to *Daphnia magna* across a range of water chemistry parameters were assessed. Furthermore, the relative contribution of $NP_{(particle)}$ and $NP_{(ion)}$ to the toxicity of CuNP suspensions upon varying water chemistry was determined.

5.2 Materials and methods

5.2.1 Testing materials and organisms

CuNPs (nominal size, 25 nm; specific surface area, 30-50 m²/g; purity, 99.9%; shape, spherical) were obtained from IoLiTec. Aldrich humic acid (sodium salt) (HA) was used as a standardized natural dissolved organic carbon (DOC). A stock solution was prepared by dissolving HA in 0.002 N NaOH in deionized water. The HA solution was then stirred overnight and filtered through a 0.2 μ m cellulose acetate membrane and subsequently stored at 4 °C prior to experiments. The total organic carbon (TOC) content of the prepared stock solution was measured by a TOC analyzer (TOC-VCPH, Shimadzu Corporation). *Dapluia magua* was selected as the model organism for toxicity testing. The test organisms were fed with freshly cultured *Pseudokirchneriella subcapitata* every three days and maintained inside a controlled-temperature chamber under a 16:8 light-dark cycle (20 ± 1 °C). At intervals of about 4 months, the sensitivity of the daphnid culture was checked with the reference toxicant K₂Cr₂O₇ to ensure the sensitivity of the daphnid culture remained within the limits as set by the OECD guideline (24 h 50% effective concentration = 0.6 – 2.1 mg/L K₂Cr₂O₇) (OECD, 2004).

5.2.2 Preparation of suspensions of CuNPs

ISO standard testing medium (STM), recommended by OECD, was used to prepare CuNP suspensions. The STM (pH 7.8 \pm 0.2) contained (mM MilliQ water): CaCl₂·2H₂O: 2; MgSO₄·7H₂O: 0.5; NaHCO₃: 0.77; KCl: 0.08. In order to understand the influence of water chemistry on the fate and toxicity of CuNPs, CuNP suspensions in which the water chemistry was modified, were also prepared. The modification of water chemistry of the exposure media

was achieved by altering the most critical environmental factors assumed to affect NP toxicity, which are pH, and divalent cation and DOC concentrations. The overview of the testing scheme with the details on the different trials is presented in Table 5.1. For the effects of pH, besides at pH 7.8, suspensions of CuNPs at pH 6 and 9 (adjusted by addition of 0.1 M NaOH or 0.1 M HCl) were also prepared; for the divalent cation treatments, suspensions of CuNPs with 0, 2.5 and 5 mM of cations were prepared by adding CaCl₂·2H₂O and MgSO₄·7H₂O in a fixed molar ratio of 4:1; for assessing the effects of DOC on toxicity, CuNP suspensions with 0, 5, 25 and 50 mg C/L were prepared by diluting the stock HA solutions. The ranges of the water chemistry parameters were selected to accommodate the optimal conditions for growth of D. magna and they encompass the range commonly observed in natural environments (Vijver et al., 2008; Ottofuelling et al., 2011; Hammes et al., 2013). Moreover, most previous studies regarding the fate and toxicity of NPs were performed under static conditions (i.e., stored without disturbance along the exposure duration). However, by definition the 'real' environment is dynamic (Godinez and Darnault, 2011; Lv et al., 2016), and accordingly fate and toxicity of NPs under dynamic exposure conditions deserve to be studied. To compare the fate and toxicity of CuNPs to D. magna under static and dynamic conditions, one set of the prepared CuNP suspensions was maintained statically under a 16:8-h light-dark cycle (20 \pm 1 °C) during 48 h of incubation and the other set of CuNP suspensions was stored on a laboratory shaker with a vibration speed of 140 rpm under identical conditions (i.e., 16:8-h light-dark cycle and 20 ± 1 °C). It was verified (visual observation) that the vibration speed applied (140 rpm) had no adverse effects on the well-being of *D. magna* throughout the 48 h of exposure.

5.2.3 Physicochemical characterization of CuNPs

The morphology and primary size of the CuNPs in the STM were characterized using transmission electron microscopy (TEM, JEOL 1010, JEOL Ltd., Japan). The hydrodynamic diameters of CuNPs upon various exposure scenarios prepared above were measured in triplicate immediately after preparation (which was around 1 h for the preparation of CuNP suspensions, to which we will refer to as 1 h in this study) and after 24 and 48 h of preparation by dynamic light scattering (DLS) on a Zetasizer Nano-ZS instrument (Malvern, Instruments Ltd., UK). The zeta potential of each copper suspension at the same time point was measured by ZetaPALS software based on the Smoluchowski equation.

The changes of the total Cu concentration and dissolution profile in the exposure suspensions upon modification of pH, cation and DOC concentrations within 48 h were monitored separately. This was done at an actual CuNP concentration of about 800 μ g/L, which is in the range (10-920 μ g/L) of the predicted CuNP concentration in aquatic environments (Chio et al., 2012). The prepared CuNP suspensions across a range of water chemistry, as presented in Table 5.1, were kept for increasing time periods (1, 12, 24, 36 and 48 h). At each sampling time point, 2 independent CuNP suspensions with the same water chemistry as duplicates were used to measure the concentration of each Cu fraction. For each suspension, a 5 mL sample was collected carefully from the position around 2 cm below the surface of each suspension and then digested by 65% nitric acid at room temperature for at least 1 d before being analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES). In this way, the total Cu concentration in the water column (i.e., the sum of the dissolved Cu

and particulate Cu) could be measured. After sampling for the total Cu concentration measurement, 10 mL of each suspension was pipetted from the water column and subsequently centrifuged at 30392 g for 30 min at 4 °C (Sorvall RC5B plus centrifuge, Fiberlite F21-8 × 50y rotor). The supernatants were then filtered through a syringe filter with 0.02 μ m pore diameter (Anotop 25, Whatman). The filtrates were digested by nitric acid and ICP-OES was used to determine the dissolved Cu concentration.

5.2.4 Acute toxicity testing

All acute toxicity tests in this study were carried out according to OECD Guideline 202. Five neonates (< 24 h) were exposed for 48 h to each suspension of CuNPs (referred to as $CuNP_{(total)}$ hereafter) prepared according to Table 5.1. During the 48 h acute toxicity test, daphnids were not fed. In order to obtain the dose-response curves of $CuNP_{(total)}$ to daphnids, a series of exposure concentrations for $CuNP_{(total)}$ with the same water composition were employed to expose the daphnids. Each concentration tested, consisted of 4 replicates. To calculate the toxicity of the dissolved ions released from CuNPs (referred to as $CuNP_{(ion)}$ hereafter), the dose-response curves of $Cu(NO_3)_2$ solutions to daphnia neonates for 48 h across a range of water chemistry were also determined.

5.2.5 Data analysis

The specific modes of action of $NP_{(ion)}$ and $NP_{(particle)}$ remain unclear. Nevertheless, some recent published papers found that the mode of action of $NP_{(particle)}$ differed from that of $NP_{(ion)}$ (Poynton et al., 2011; Poynton et al., 2012; Rainville et al., 2014). Hence, it was assumed that the modes of action of $CuNP_{(ion)}$ and $CuNP_{(particle)}$ would be dissimilar. In this circumstance, the toxicity of $CuNP_{(particle)}$ can be deduced by using the response addition model (Backhaus et al., 2000):

$$E_{(\text{total})} = 1 - \left[(1 - E_{(\text{ion})}) (1 - E_{(\text{particle})}) \right]$$
 5.1

Where $E_{\text{(total)}}$, $E_{\text{(ion)}}$ and $E_{\text{(particle)}}$ represent the toxicity caused by the nanoparticle suspensions, and the ions and the NPs present in the suspensions (scaled from 0 to 1), respectively. In the present study, $E_{\text{(total)}}$ was measured experimentally. The time weighted average (TWA) ion concentration at each exposure concentration of CuNPs, calculated from Eq. 5.2, was used to analyze the toxicity caused by copper ions (i.e., $E_{\text{(ion)}}$) in the suspensions of CuNPs, according to the concentration-response curves of Cu(NO₃)₂ towards *D. magna*. This makes $E_{\text{(particle)}}$ as the only unknown, allowing for direct calculation of the effects caused by a specific concentration of NP_(particle).

$$C_{\rm T} = \frac{C_1 T_1 + C_2 T_2 + C_3 T_3 + \dots + C_n T_n}{T_1 + T_2 + T_3 + \dots + T_n}$$
 5.2

Where $C_{\rm T}$ is the TWA concentration and $C_{\rm i}$ is the analyte concentration observed for time $T_{\rm i}$, and so on, until time $T_{\rm n}$.

The median lethal concentration (LC50) and the related 95% confidence intervals (CI) were calculated using Graphpad Prism 5.

5.3 Results

5.3.1 Physicochemical characterization of CuNPs

The images captured by the transmission electron microscopy demonstrated that the pristine shape of the CuNPs was spherical and CuNPs aggregated rapidly after submersion into the exposure medium (Figure S5.1). The hydrodynamic diameters and zeta-potentials of CuNP suspensions across a range of water chemistry are presented in Table 5.2. At a cation concentration of 2.5 mM, in both the static and the dynamic exposure treatments the NPs aggregated to micro-size aggregates after 48 h of incubation in the testing media with pH ranging from 6-9 and without addition of DOC (trials 1-3 and 9-11). The hydrodynamic diameters of CuNPs remained around 518 nm after 48 h of incubation in the static treatment without addition of cations (trial 4). However, the addition of divalent cations enhanced the extent of aggregation of the NPs (trials 2, 4 and 5). The zeta-potential of the NP suspension without addition of divalent cations in the static treatment was around -30 mV within 48 h of incubation, while it increased to around -10 mV at 5 mM of cations. In the static treatments, the aggregate size of CuNPs after 48 h of incubation was around 500 nm with addition of DOC at a concentration ranging from 5 to 50 mg C/L (trails 6-8). In the dynamic treatments, the aggregate sizes of CuNPs were around 200 nm at 5 mg C/L and 100 nm at 25 and 50 mg C/L within 48 h of incubation (trials 14-16).

T. 1		рН	Cation conc.	DOC conc.
Trial no.	Condition		(mM)	(mg/L)
1	Static	6	2.5	0
2	Static	7.8	2.5	0
3	Static	9	2.5	0
4	Static	7.8	0	0
5	Static	7.8	5	0
6	Static	7.8	2.5	5
7	Static	7.8	2.5	25
8	Static	7.8	2.5	50
9	Dynamic	6	2.5	0
10	Dynamic	7.8	2.5	0
11	Dynamic	9	2.5	0
12	Dynamic	7.8	0	0
13	Dynamic	7.8	5	0
14	Dynamic	7.8	2.5	5
15	Dynamic	7.8	2.5	25
16	Dynamic	7.8	2.5	50

Table 5.1 Overview of the experimental setup for testing the fate and toxicity of CuNPs across a range of water chemistry.

Conc. = concentration

After 48 h of incubation, 91%, 76% and 60% of the total added CuNPs still remained in the water column in the static exposure treatments at pH 6, 7.8 and 9, respectively (Figure 5.1A). In the static treatments, 78%, 64% and 54% of the total added CuNPs were dissolved after 48 h of incubation at pH 6, 7.8 and 9, respectively (Figure 5.1B). The profiles of the total amount of Cu and CuNP_(ion) remaining in the water column during 48 h of incubation in the static and dynamic exposure settings across the range of pH from 6 to 9 were similar (Figure 5.1A-B). At the cation concentrations of 0, 2.5 and 5 mM, the total amount of Cu remaining in the water column after 48 h was 88%, 76% and 71% in the static treatments and 94%, 83% and 52% in the dynamic treatments, respectively (Figure 5.1C). Around 65% of the total added Cu was dissolved at the cation concentrations ranging from 0 to 5 mM in both the static and the dynamic treatments, except at the concentration of 5 mM in the dynamic treatment, as 48% of the CuNPs was dissolved after 48 h of incubation (Figure 5.1D). Around 63%, 73% and 76% of the total added CuNPs remained in the water column after 48 h of incubation in the static treatments at 5, 25 and 50 mg DOC/L, respectively (Figure 5.1E). In the dynamic treatments, approximately 85% of the initially added CuNPs remained in the water column after 48 h of incubation across the DOC concentration range from 0 to 50 mg/L. The addition of DOC significantly reduced the amount of CuNP_(ion) in the water column. In both the static and the dynamic treatments, the amount of CuNP_(ion) decreased to around 20% after 48 h of incubation in all the cases with the addition of DOC from 5 to 50 mg/L (Figure 5.1F).

Trial no. –	Hydrod	Hydrodynamic diameter (nm) ^a			Zeta-potential (mV) ^a		
	1 h	24 h	48 h	1 h	24 h	48 h	
1	754 ± 217	903 ± 194	1383 ± 360	-13 ± 2	-14 ± 2	-9 ± 1	
2	637 ± 105	1008 ± 116	1650 ± 335	-17 ± 1	-12 ± 3	-10 ± 1	
3	745 ± 93	1307 ± 172	2436 ± 490	-9 ± 2	-6 ± 3	-5 ± 4	
4	465 ± 84	641 ± 173	518 ± 80	-34 ± 4	-27 ± 1	-26 ± 1	
5	715 ± 134	1474 ± 144	1865 ± 132	-10 ± 2	-10 ± 2	-8 ± 1	
6	369 ± 41	486 ± 21	512 ± 23	-18 ± 1	-16 ± 1	-16 ± 2	
7	373 ± 60	457 ± 23	468 ± 16	-19 ± 1	-18 ± 1	-17 ± 1	
8	359 ± 19	445 ± 27	495 ± 17	-19 ± 1	-16 ± 3	-15 ± 1	
9	ND	1078 ± 219	1617 ± 293	ND	-9 ± 3	-7 ± 3	
10	ND	1029 ± 239	1761 ± 985	ND	-8 ± 4	-6 ± 3	
11	ND	2050 ± 319	1203 ± 562	ND	-7 ± 1	-3 ± 2	
12	ND	414 ± 82	891 ± 390	ND	-23±4	-19 ± 1	
13	ND	879 ± 169	1237 ± 219	ND	-5 ± 3	-4 ± 3	
14	ND	282 ± 10	221 ± 23	ND	-12 ± 2	-12 ± 1	
15	ND	142 ± 25	118 ± 14	ND	-12 ± 1	-11 ± 1	
16	ND	127 ± 10	127 ± 9	ND	-11 + 1	-11 + 2	

Table 5.2 Hydrodynamic diameter and zeta-potential of CuNPs during 48 h of incubation in system	S
with various water chemistry.	

^a Hydrodynamic diameter and zeta-potential are expressed as the mean \pm standard deviation (n = 3).

ND means not determined.

5.3.2 Acute toxicity of CuNPs to Daphnia magna

The LC50 of $\text{CuNP}_{(\text{total})}$ across a range of water chemistry is provided in Table 5.3. In the static treatments, the LC50 values of $\text{CuNP}_{(\text{total})}$ were 0.024, 0.050 and 0.094 mg/L at pH 6, 7.8 and 9, respectively. The LC50 values of $\text{CuNP}_{(\text{total})}$ in the dynamic treatments were similar to those in the static treatments at the same pH, which were 0.030, 0.049 and 0.084 mg/L at pH 6, 7.8 and 9, respectively. In the static treatments, the LC50 of $\text{CuNP}_{(\text{total})}$ increased from 0.026 mg/L without addition of cations to 0.076 mg/L at 5 mM of cations. The LC50 of $\text{CuNP}_{(\text{total})}$ upon the dynamic exposure trial was similar to that upon the static trial at the same cation concentration, except at the cation concentration of 5 mM, at which the LC50 of $\text{CuNP}_{(\text{total})}$ was 0.152 mg/L in the dynamic treatment, about a factor of 2 higher than the LC50 obtained in the static treatment. The LC50 of $\text{CuNP}_{(\text{total})}$ significantly increased upon addition of DOC. In the static treatments, the LC50 of $\text{CuNP}_{(\text{total})}$ increased from 0.050 mg/L without addition of DOC to 0.515, 2.166 and 3.591 mg/L at 5, 25 and 50 mg DOC/L, respectively; in the dynamic treatments, the LC50 values of $\text{CuNP}_{(\text{total})}$ were 0.318, 1.634, and 2.153 mg/L at 5, 25, and 50 mg DOC/L, respectively.

The dose-response curves of $Cu(NO_3)_2$ across the ranges of pH, divalent cation and DOC concentrations used in this study for CuNPs are presented in the supplementary information (Figure S5.2). In the absence of DOC, the 48-h LC50 of $Cu(NO_3)_2$ increased with increasing pH, which were 0.016, 0.028 and 0.048 mg/L at pH 6, 7.8 and 9, respectively (Table S5.1). At pH 7.8 and in the absence of DOC, the LC50 values of $Cu(NO_3)_2$ were 0.015, 0.028 and 0.043 mg/L with addition of 0, 2.5 and 5 mM of the divalent cations, respectively. Toxicity of $Cu(NO_3)_2$ to *D. magna* was greatly mitigated by the addition of DOC, with the LC50 increasing from 0.028 mg/L without addition of DOC to 0.133, 0.577 and 0.970 mg/L with addition of 5, 25 and 50 mg DOC/L, respectively (Table S5.1).

The dose-response curves with the endpoint mortality of *D. magna* were calculated based on the response addition model (see supplementary information Figure S5.3-5.5). In the static treatments, the LC50 of $\text{CuNP}_{(\text{particle})}$ increased from 0.011 mg/L at pH 6 to 0.040 mg/L at pH 7.8 and 0.089 mg/L at pH 9; the LC50 of $\text{CuNP}_{(\text{particle})}$ increased from 0.021 mg/L without addition of divalent cations to 0.058 mg/L upon addition of 5 mM of cations; the LC50 of $\text{CuNP}_{(\text{particle})}$ increased from 0.040 mg/L in the absence of DOC to 3.939 mg/L upon addition of 50 mg DOC/L (Table 5.3). Similar to the LC50 in the static exposure treatments, the LC50 of $\text{CuNP}_{(\text{particle})}$ in the dynamic exposure treatments also showed increasing trends with increasing pH and with increasing concentrations of cations and DOC (Table 5.3), indicating that the toxicity of $\text{CuNP}_{(\text{particle})}$ decreased with increasing pH and with increasing concentrations of cations and DOC in both the static and dynamic exposure treatments.

Trial	LC50 (95%	₀ CI, mg/L)	Relative contribution at LC50 (%)		
no.	CuNP _(total)	CuNP _(particle)	CuNP _(ion)	CuNP _(particle)	
1	0.024 (0.022-0.026)	0.011 (0.011-0.012)	100	0	
2	0.050 (0.048-0.053)	0.040 (0.031-0.052)	53	47	
3	0.094 (0.084-0.106)	0.089 (0.061-0.130)	68	32	
4	0.026 (0.022-0.031)	0.021 (0.018-0.025)	72	28	
5	0.076 (0.069-0.082)	0.058 (0.044-0.076)	60	40	
6	0.515 (0.414-0.640)	1.913 (0.309-11.850)	43	57	
7	2.166 (2.009-2.335)	2.142 (1.916-2.393)	38	62	
8	3.591 (3.273-3.939)	3.939 (3.324-4.669)	33	67	
9	0.030 (0.025-0.036)	$0.018\ (0.018 - 0.018)$	100	0	
10	0.049 (0.046-0.053)	0.038 (0.031-0.045)	70	30	
11	$0.084\ (0.071 - 0.098)$	0.081 (0.063-0.104)	64	36	
12	0.022 (0.019-0.025)	$\sim 0.015^{a}$	62	38	
13	0.152 (0.132-0.176)	$\sim 0.171^{a}$	100	0	
14	0.318 (0.266-0.380)	0.311 (0.219-0.441)	33	67	
15	1.634 (1.470-1.817)	1.568 (1.404-1.750)	28	72	
16	2.153 (1.923-2.411)	1.930 (1.717-2.169)	15	85	

Table 5.3 The median lethal concentration (LC50) of $\text{CuNP}_{(\text{total})}$ and $\text{CuNP}_{(\text{particle})}$ after 48 h of exposure to *D. magna* upon various exposure conditions and the relative contribution of $\text{CuNP}_{(\text{particle})}$ and $\text{CuNP}_{(\text{ion})}$ to toxicity of $\text{CuNP}_{(\text{total})}$ at the LC50.

CI: Confidence intervals

Conc. = concentration

^a means the data is not accurate.



Figure 5.1 Time profiles of the total amount of Cu and dissolved Cu in suspensions of CuNPs within 48 h of incubation in the static and dynamic exposure treatment as a function of pH (A-B), of concentrations of divalent cations (C-D) and of DOC (E-F). All data are presented as the mean of duplicate.

5.3.3 Relative contribution of CuNP_(particle) and CuNP_(ion) to toxicity

The relative contribution of $\text{CuNP}_{(\text{particle})}$ and $\text{CuNP}_{(\text{ion})}$ to the toxicity of $\text{CuNP}_{(\text{total})}$ to *D. magna* at the LC50 levels is given in Table 5.3. According to the calculation results based on the response addition model, the toxicity of $\text{CuNP}_{(\text{total})}$ to *D. magna* at the LC50 level in the absence of DOC was mainly caused by $\text{CuNP}_{(\text{ion})}$. In both the static and dynamic treatments, more than 53% of the toxicity of $\text{CuNP}_{(\text{total})}$ could be explained by $\text{CuNP}_{(\text{ion})}$ at pH ranging from 6 to 9. At pH 7.8 and in the static exposure treatments, 72%, 53% and 60% of the observed toxicity could be attributed to $\text{CuNP}_{(\text{ion})}$ upon the addition of 0, 2.5 and 5 mM of cations, respectively. Similarly, in the dynamic treatments toxicity of $\text{CuNP}_{(\text{total})}$ was predominantly contributed by $\text{CuNP}_{(\text{ion})}$ ($\geq 62\%$) at the divalent cation concentrations from 0 to 5 mM. However, upon addition of DOC at concentrations from 5 to 50 mg/L, the relative contribution of $\text{CuNP}_{(\text{particle})}$ to the overall toxicity was higher than that of $\text{CuNP}_{(\text{ion})}$. In the static exposure treatments, the relative contribution of $\text{CuNP}_{(\text{ion})}$ to the overall toxicity decreased from 53% without addition of DOC to 43%, 38% and 33% upon addition of 5, 25 and 50 mg DOC/L, respectively; in the dynamic exposure treatments, the relative contribution of $CuNP_{(ion)}$ to the overall toxicity shifted from 70% without addition of DOC to 33%, 28% and 15% with addition of 5, 25 and 50 mg DOC/L, respectively.

5.4 Discussion

5.4.1 Behavior and fate of CuNPs upon modification of water chemistry

In this study, CuNPs aggregated to a higher extent in the exposure matrices with a higher concentration of divalent cations (Table 5.2). This enhancement of aggregation was due to the compression of the double-layer of NPs imposed by the cations, as the absolute value of the zeta-potential of CuNP suspensions decreased with addition of the cations (Table 5.2). In natural waters, DOC is ubiquitous and has been identified in many studies being key for determining the fate of metallic NPs in environments (Conway et al., 2015; Zou et al., 2015; Lawrence et al., 2016; Joo and Zhao, 2017). Consistent with the findings of other studies (Adeleye et al., 2014; Conway et al., 2015), we also found that the addition of DOC inhibited the further aggregation of CuNPs. Furthermore, the inhibiting effect of DOC on aggregation of the CuNPs was stronger in dynamic exposure treatments than in static exposure treatments, as reflected by the smaller average sizes of CuNPs in the dynamic exposure treatments (Table 5.2). This is probably by the fact that the dynamic flow increased the shear forces, which consequently resulted in the disaggregation of NPs (Metreveli et al., 2015; Lv et al., 2016). In agreement with other studies (Dubas and Pimpan, 2008; Adeleye et al., 2014), dissolution of the CuNPs was enhanced with increasing pH. The addition of DOC significantly reduced the concentration of CuNP_(ion) in both the static and dynamic exposure treatments. The reduction of the concentration of NP_(ion) upon addition of DOC in the water column was also reported by some other studies (Conway et al., 2015; Zhou et al., 2016). The possible mechanisms underlying the reduction effects of DOC include complexation and surface adsorption to block the oxidation sites of NPs by DOC (Dubas and Pimpan, 2008; Rosenfeldt et al., 2015), steric exclusion of water from the surface of the particles by DOC coating, and/or reduction of the availability of H⁺ which may bind to DOC molecules (Yoon et al., 2005; Adeleye et al., 2014).

5.4.2 Toxicity of CuNPs upon modification of water chemistry

In this study, the toxicity of CuNPs to *D. magna* was strongly dependent on the water chemistry of the exposure medium. Both $\text{CuNP}_{(\text{ion})}$ and $\text{CuNP}_{(\text{particle})}$ were more toxic at lower pH. The higher toxicity of $\text{CuNP}_{(\text{ion})}$ at lower pH is due to the higher percentage of free Cu^{2+} ions at lower pH (Odzak et al., 2014; Xiao et al., 2016), which is generally considered to be the most toxic species among all dissolved Cu species (de Schamphelaere and Janssen, 2002). The increasing toxicity of $\text{CuNP}_{(\text{particle})}$ under reduced pH may be explained by the sensitivity changes of freshwater zooplankton due to low pH and consequent reduction of their tolerance to $\text{NP}_{(\text{particle})}$ (Locke, 1991; Haven et al., 1993; Hu et al., 2017). The toxicity of $\text{CuNP}_{(\text{total})}$ increased with a reduction of the divalent cation concentrations. This finding is the net effect of the reduction of the toxicity of $\text{CuNP}_{(\text{ion})}$ and $\text{CuNP}_{(\text{particle})}$ upon increasing concentrations of the cations. The reduced toxicity of $\text{CuNP}_{(\text{particle})}$ upon increasing concentrations of the cations. The reduced toxicity of $\text{CuNP}_{(\text{particle})}$ with addition of cations may result from the enhanced aggregation imparted by the cations as mentioned above,

which decreases the effective surface area of CuNP_(particle) to D. magna and consequently reduces the toxicity of CuNP_(particle). According to the biotic ligand model (BLM) (Di Toro et al., 2001), the enhanced competition between Ca²⁺ and Mg²⁺ and the CuNP_(ion) for binding sites on the biotic ligands of daphnids upon increasing cation concentrations results in the mitigation of the toxicity of CuNP_(ion). In the presence of DOC, consistent with many other studies (Blinova et al., 2010; Gunsolus et al., 2015), the toxicity of CuNP_(total) was mitigated to a high extent. In the static exposure modality, the toxicity of CuNP_(total) decreased around 10, 43, and 72 times with addition of 5, 25 and 50 mg DOC/L, respectively, compared to the situation in which no DOC was added. The mitigation effects of DOC on the observed toxicity were derived from the findings that both the toxicity of CuNP_(ion) and CuNP_(varticle) to D. magna was decreased with addition of DOC. The decrease in toxicity for CuNP_(ion) and CuNP_(particle) with the addition of DOC may be a function of complexation of CuNP_(ion) and passivation of the particle surface by DOC adsorption (Fabrega et al., 2009). Under the dynamic exposure modality, the mitigating effects of DOC on toxicity were weakened, compared to those under the static modality. The toxicity of CuNP_(total) in the dynamic exposure treatments was around 38%, 25% and 40% higher than the toxicity of CuNP_(total) in the static treatments upon addition of 5, 25 and 50 mg DOC/L, respectively. The dissolution profiles upon the addition of DOC in the static and dynamic exposure treatments were similar within 48 h of incubation (Figure 5.1F), whereas the aggregation extents of CuNPs were smaller within the 48 h of incubation in the dynamic treatments than in the static treatments when DOC was added (Table 5.2). Hence, the higher toxicity of CuNP_(total) as found in the dynamic exposure treatments, compared to in the static exposure treatments when DOC was added, could result from the increased stability of CuNPs.

5.4.3 Relative contribution of CuNP_(particle) and CuNP_(ion) to toxicity

Evaluation of the relative contribution of NP_(particle) and NP_(ion) to the suspension toxicity upon varying water chemistry is critical for environmental risk assessment. This would allow us to understand the mechanisms of toxicity of NPs and to make process-based predictions of fate and ecological responses. Our results clearly evidenced that even for the same type of CuNPs, the relative contribution of CuNP_(particle) and CuNP_(ion) to the observed toxicity was greatly altered by the physicochemical characteristics of the exposure medium. In the absence of DOC, the toxicity of CuNP_(total) to D. magna at the LC50 level was mainly caused by CuNP_(ion) $(\geq 53\%$ of the observed toxicity). Similarly, Jo et al. (2012) found that the toxicity of CuO NPs (with a nominal size < 50 nm) to *D. magna* in exposure media without DOC and at pH 7.6 was largely attributable to the NP_(iop). Also, Heinlaan et al. (2008) found that in exposure medium without addition of DOC and at pH within the range 7.3-7.8, the main driver for the toxicity of CuO NPs (with a nominal size around 30 nm) to D. magna was the NP_(ion). In those cases that ions are dominating the toxicity, BLMs can give large insights in the NP suspensions-induced response assessment. On the other hand, in the presence of DOC at concentrations ranging from 5 to 50 mg/L, the toxicity of CuNP_(total) was largely explained by the contribution of $\text{CuNP}_{(\text{particle})}$ (Table 5.3). The alteration of the roles of $\text{CuNP}_{(\text{particle})}$ and CuNP_(ion) in the toxicity of CuNP suspension by DOC could result from the large reduction of dissolution of the particles on top of the observed enhancement of the stability of CuNP_(particle) in the water column. It is worth to note that the relative contribution of CuNP_(particle) to

toxicity with the addition of DOC at concentrations from 5 to 50 mg/L in the dynamic exposure treatments was 10-18% higher than that in the static exposure treatments. This may be derived from the additional stabilization effects of DOC on CuNPs in the dynamic treatments. These observations imply that the particle dynamics in aqueous environment are of importance as well. Our results highlight the importance of water chemistry on the roles of NP_(particle) and NP_(ion) in the observed toxicity.

5.5 Conclusions

This study demonstrates that the particle-specific toxicity of CuNPs strongly depends on water chemistry of the exposure medium. In the absence of DOC, the toxicity of $\text{CuNP}_{(\text{ion})}$ and $\text{CuNP}_{(\text{particle})}$ was decreased upon increasing pH and increasing concentrations of divalent cations. Toxicity of $\text{CuNP}_{(\text{total})}$ was mainly driven by $\text{CuNP}_{(\text{ion})}$ when no DOC. In addition, toxicity of $\text{CuNP}_{(\text{total})}$ was mainly driven by $\text{CuNP}_{(\text{ion})}$ when no DOC. In addition, toxicity of $\text{CuNP}_{(\text{total})}$ was approximately 25%-40% higher than that under the dynamic exposure modality was approximately 25%-40% higher than that under the static exposure modality. The toxicity of $\text{CuNP}_{(\text{ion})}$ and $\text{CuNP}_{(\text{particle})}$ with addition of DOC was largely mitigated. As a result of the large reduction of $\text{CuNP}_{(\text{ion})}$ and the enhancement of the stability of $\text{CuNP}_{(\text{particle})}$ when DOC was added, the toxicity of $\text{CuNP}_{(\text{total})}$ was mainly attributable to the $\text{CuNP}_{(\text{particle})}$ in case of addition of DOC, especially under the dynamic exposure modality. Our results highlight the need of dynamic fate characterization of metallic NPs in aquatic environments along the exposure duration in order to interpret their ecotoxicity.

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Supplementary information

DOC Conc. (mg/L)	pH value	Cation Conc.	LC50 of $Cu(NO_3)_2$ (mg/L)		
	privade	(mM)	LC50	95% CI	
0	6	2.5	0.016	0.014-0.018	
0	7.8	2.5	0.028	0.026-0.029	
0	9	2.5	0.048	0.043-0.053	
0	7.8	0	0.015	0.013-0.017	
0	7.8	5	0.043	0.039-0.047	
5	7.8	2.5	0.133	0.121-0.146	
25	7.8	2.5	0.577	0.507-0.657	
50	7.8	2.5	0.970	0.845-1.114	

Table S5.1 The median lethal concentration (LC50) and corresponding 95% confidence intervals (CI) of $Cu(NO_3)_2$ to *D. magna* after 48 h of exposure upon modification of pH, and divalent cation and DOC concentrations

Conc. = concentration.



Figure S5.1 TEM images of CuNPs after 1 h of incubation in the exposure medium at pH 7.8 and 2.5 mM of cations.



Figuer S5.2 Dose-response curves of *D. magna* neonates in response to 48 h of exposure to $Cu(NO_3)_2$ across a range of pH (A), divalent cation concentrations (B) and dissolved organic carbon (DOC) concentrations (C). Each data is expressed as mean \pm standard deviation (n = 4).



Figure S5.3 Dose-response curves of *D. magna* in response to 48 h of exposure to CuNP suspensions (A) and $\text{CuNP}_{(\text{particle})}$ (B) as a function of pH in both the dynamic and static exposure treatments. Each data point is expressed as mean ± standard deviation (n = 4).



Figure S5.4 Dose-response curves of *D. magna* in response to 48 h of exposure to CuNP suspensions (A) and $\text{CuNP}_{(\text{particle})}$ (B) as a function of concentrations of the divalent cations in both the dynamic and static exposure treatments. Each data point is expressed as mean ± standard deviation (n = 4).



Figure S5.5 Dose-response curves of *D. magna* in response to 48 h of exposure to CuNP suspensions (A) and CuNP_(particle) (B) as a function of concentrations of DOC in both the dynamic and static exposure treatments. Each data point is expressed as mean \pm standard deviation (n = 4).