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Chapter 1 General introduction

1.1 The era of nanotechnology and nanomaterials

The term “nanotechnology” was firstly used by Drexler in his book “Engines of Creation: The coming Era of Nanotechnology” in 1986 (Drexler, 1986). Nanotechnology is specified to manipulate matter on an atomic and molecular as well as supramolecular scale through integrating science and technology to create new and unique materials and products. After twenty years of theoretical and technical development, the nanotechnology field is gaining a substantially increasing attention, not only from scientific and technological fields, but also from political, commercial and environmental fields. As the products of nanotechnology, nanomaterials (NMs) are generally defined as materials containing 50% or more particles with a size in at least one dimension between 1 and 100 nm (European Comission, 2012). NMs often own unique electronic, optical and mechanical properties stemming from their nanoscale dimensions (Narayan et al., 2004). Due to these novel properties relative to their bulk counterparts, NMs currently are widely manufactured and applied in over 1600 commercial products already introduced to the global market (Vance et al., 2015). For example, nanoscale silver materials are incorporated in food packaging (Arora and Padua, 2010), paints (Kumar et al., 2008), wound dressings (Rai et al., 2009), cosmetics (Kokura et al., 2010) and clothing (Emam et al., 2013), due to their antibacterial properties. Titanium dioxide NMs are widely used in sunscreens (Galloway et al., 2010) and pigments (Allen et al., 2005) as well as groundwater remediation (Savage and Diallo, 2005). Carbon nanotubes are widely used to increase the life cycle of batteries as an additive (Shapira et al., 2013) and to improve the performance of solar cells (Lu et al., 2013). A more extensive schematic overview of where and to what purpose NMs are used is given in Figure 1.1.

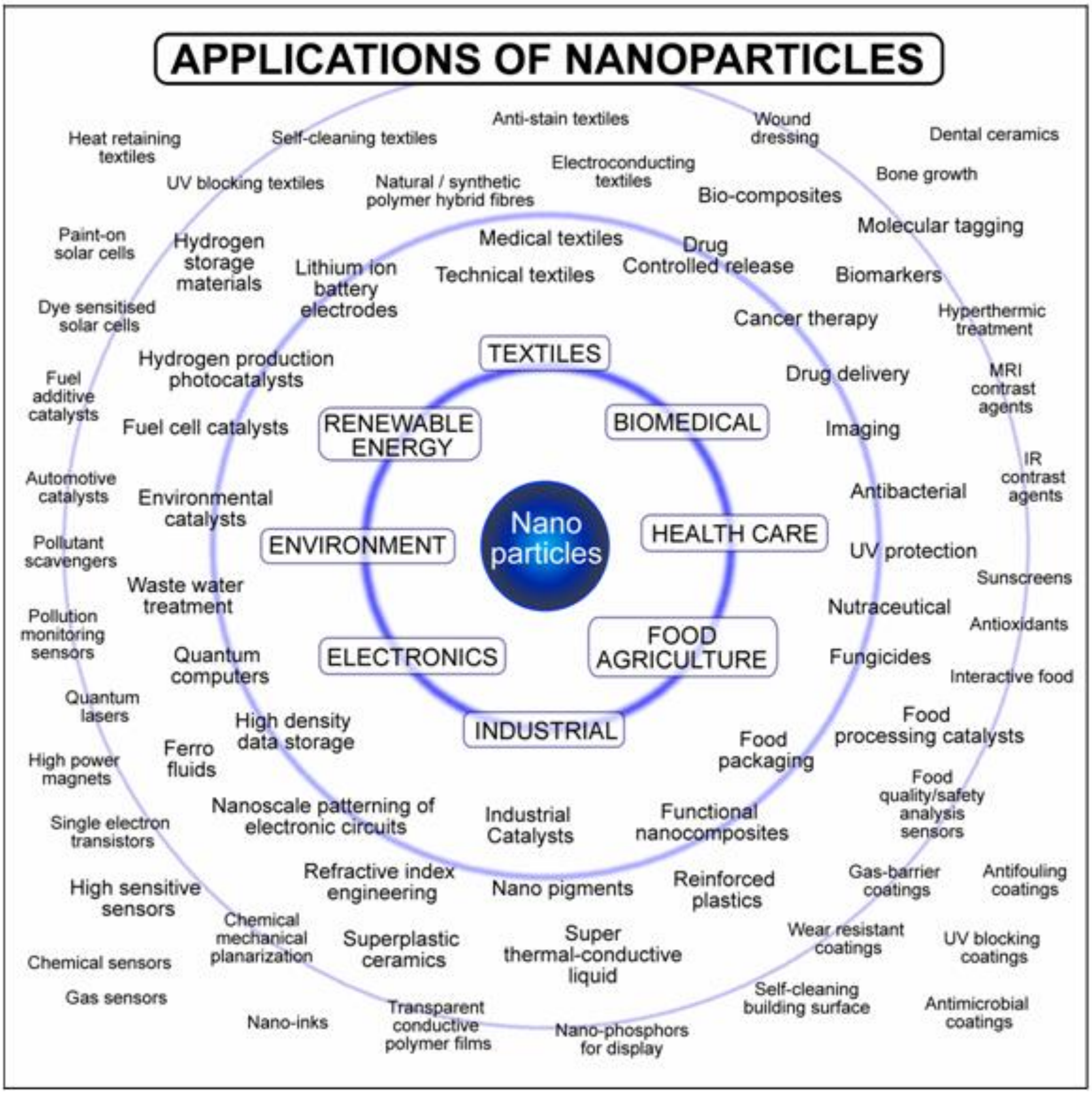


Figure 1.1 The major applications of nanoparticles (cited from Tsuzuki, 2009).

In the European Union (EU), studies on the sustainable development of nanotechnology have already become an essential part of many European policy initiatives and the Framework Program 7 (FP7). Its successor Horizon 2020 has even addressed that nanotechnology and the resulting NMs may provide a key influence in solving the most important challenges that humans are facing today, like energy, climate, efficient use of resources, information and communication as well as health and food supplies. Similar to the EU which has been putting many efforts in the development of nanotechnology, China has also regarded nanotechnology as a very promising area that could bring China a great chance of development since 2001. In July 2001, the National Programme on the Development of Nanoscience and Nanotechnology (MOST 2001) was released to provide specific planning for the overall development of nanotechnologies. In 2006, the outline of the National Medium- and Long-Term Science and Technology Development Plan" (MOST 2006) was released. In 2012, a special five-year nano research plan (MOST 2012) was formulated in order to deepen the implementation of the outline plan (MOST 2006). To date, 1.0 billion Renminbi (RMB) has been investigated by the Nanoscience Research program to support 28 nanotechnology projects, which brings the rapid development of nanotechnology in China.

1.2 Nanoparticles

Nanoparticles (NPs) are particles of any shape with one or more dimensions between 1 and 100 nm (European Commission, 2012). Based on the source of NPs, they can be classified into two major types: natural NPs and synthetic NPs. NPs have naturally existed since the beginning of the Earth's history (Handy et al., 2008). The naturally occurring NPs can be found in volcanic dust (Rietmeijer and Mackinnon, 1997), the ocean surface microlayer (Obernosterer et al., 2005), soils (Reid et al., 2000), ice cores (Murr et al., 2004) and sediments (Verma et al., 2002). Synthetic NPs can normally be categorized into incidental and engineered NPs (Figure 1.2). Incidental NPs are produced as a side product of anthropogenic activities, such as diesel exhaust and welding fumes and often possess irregular shapes (O'Brien and Cummins, 2008). Nowadays, incidental NPs are regarded as a major source of air pollution (Kumar et al., 2013). Engineered NPs have been specifically designed and deliberately manufactured, which provide tremendous benefits and advances in human life and technology. The vast majority of engineered NPs have regular shapes, such as spheres and tubes. According to the chemical composition, NPs can also be categorized as metal-based NPs and non-metal-based NPs. Currently, the most commonly used NP types are metal-based NPs, such as silver NPs, zinc oxide NPs, titanium dioxide NPs and copper NPs.

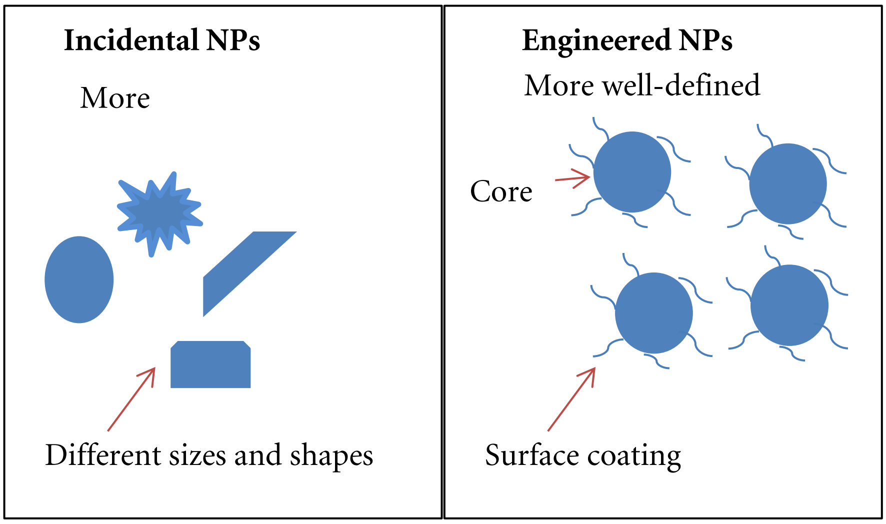


Figure 1.2 Overview of the incidental and engineered nanoparticles.

Due to their small sizes, NPs commonly possess unique properties relative to their bulk particles (larger than one micrometer), including unique physical (Faure et al., 2013), optical (Huang and E1-Sayed, 2010), magnetic (Park et al., 2007) and electronic properties (Pardo-Yissar et al., 2001). These unique properties result in the wide manufacture and applications of NPs in many industrial and commercial products (see Figure 1.1). The focus of this thesis is on copper NPs (designated as CuNPs, hereafter). CuNPs are currently widely used in antimicrobials, semiconductors, catalysis and skin products (Grifftt et al., 2007; Lee et al., 2008; Radi et al., 2010) and they are the major constituent of conductive inks and pastes (Griffitt et al., 2007).

1.3 Fate of nanoparticles in aqueous environment

Due to the wide manufacture and applications of nano-enabled products, it appears to be inevitable for humans and the environment to be exposed to NPs. For instance, workers can be exposed to NPs during production processes (laboratory and/or workshop) as well as during waste treatment processes, whereas consumers can be exposed to NPs directly through the use of NPs-containing products. Environmental compartments, like waters and soils, can become a sink of NPs via the degradation of the nano-enabled products at the end of their life, discharging factories effluents, as well as via the use of NPs for remediation.

Surface waters, as one of the important sinks for NPs, can receive NPs from atmospheric deposition, leaching from soils and through direct inputs, such as wastewater discharges (Scown et al., 2010). Given the many unknowns in risks and the predicted unprecedented growth of the nanotech market and hence the nanoparticle emissions in future, it is critical to thoroughly understand the behavior and fate of NPs in aquatic environments. Once NPs have been released into the water systems, they are subject to undergo a series of environmental processes. Due to their high surface energy, NPs tend to adsorb to particulate matter and to aggregate in aquatic media. Aggregation is one of the most important environmental processes of NPs, which to a large extent determines their transport ability and ultimate fate. According to the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Verwey, 1947; Derjaguin and Landau, 1993), the stability and aggregation of NPs in media depend on the attractive van der Waals forces and repulsive electrostatic forces. Repulsive electrostatic forces are dependent on the zeta potential and the thickness of the electrical double layer (Minocha and Mumper, 2012). Aggregation of NPs is expected to occur if the attractive forces override the repulsion forces. NPs can aggregate to other particles with similar composition (homo-aggregation), whereas they also can hetero-aggregate to particles present in waters like particulate natural organic matter (Furtado et al., 2015). In the course of aggregation, NP aggregates will grow in size, and they may deposit to the bottom of the exposure environment under gravity. After depositing on the sediment, the transport and bioavailability of NPs will be largely restricted (Lin et al., 2010).

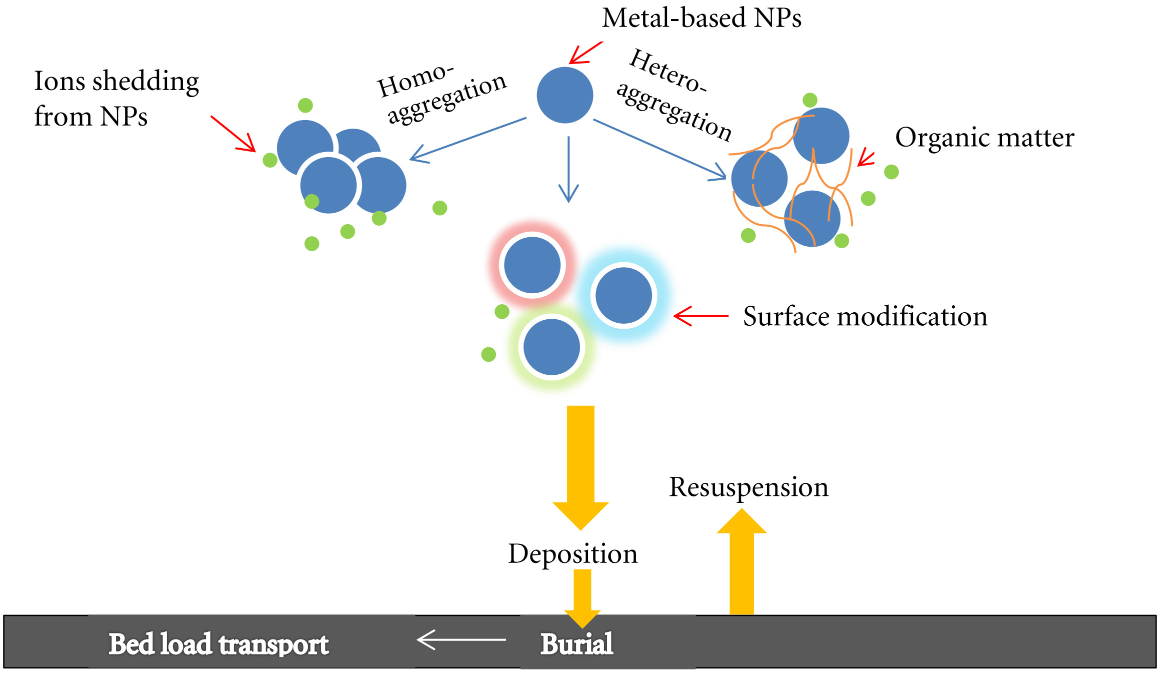


Figure 1.3 The possible environmental processes that metallic NPs may undergo after being released in aqueous environments.

Figure 1.3 shows the possible environmental processes that metallic NPs may undergo after being released in aquatic environment. Dissolution may occur in oxic environments after being submerged into waters, by which a particle merges into the solution phase to form a homogeneous mixture (Borm et al., 2006)0. The potential of dissolution of NPs can affect their persistence in the water column. It has been generally recognized that stability and dissolution of metallic NPs in aquatic environments depend not only on particle-specific characteristics (e.g., size, surface coating and shape), but also on environmental parameters, like pH, electrolyte content, dissolved organic carbon (DOC) as well as shear forces.

pH is an important parameter among water chemistry of aquatic environments. Previous studies have shown that pH can affect the stability and dissolution of NPs by impacting their surface charge directly (Zhang et al., 2010; Mohd et al., 2014). Moreover, pH can also influence the fate of NPs indirectly by changing the interactions between NPs and natural organic materials in the exposure environment. For example, Mohd et al. (2014) found that at a low concentration of Suwannee River Humic Acid (SRHA) (< 0.05 mg/L), anionic SRHA was rapidly adsorbed on the positively charged ZnO NPs consequently promoting aggregation when pH was below 9.3. The point of zero charge of the ZnO NPs was occurred at pH 9.3. When pH ≥ 9.3, the ZnO NPs was however stabilized by the addition of SRHA. It is worth to note that pH not only can influence the stability and solubility, but also can change the speciation of ions released from NPs (Peeters et al., 2016). Different species may hold different mobility and bioavailability to aquatic biota. For example, Adeleye et al. (2014) found that at pH 7, free Cu^{2+} released from Cu NPs accounted for up to 55% of total dissolved Cu. Free Cu^{2+} is the most bioavailable species, compared to other dissolved Cu species (Sunda et al., 1976). At pH 4, the concentration of Cu^{2+} increased to ≥ 72%, whereas it was non-detectable at pH 11. Hence, pH deserves to be considered when evaluating the fate of NPs in specific environmental compartments.

Electrolytes can also influence the physicochemical characteristics of NPs in water systems and consequently affect the exposure and hazards of NPs to organisms. Generally, electrolyte presence in waters can compress the electro-double layer of NPs, resulting in the aggregation of NPs (Mukherjee and Weaver, 2010). In particular, divalent electrolytes are more pronounced to promote the contraction of the double-layer of NPs, compared to monovalent electrolytes, which is known as the Schulze-Hardy rule. For example, Huynh and Chen (2011) found that $CaCl_2$ at a concentration of 2.1 mM could induce the aggregation of citrate coated Ag NPs, while the concentration for a monovalent electrolyte (NaCl) being capable of destabilizing the citrate coated Ag NPs, was 47.6 mM. Electrolytes have also been reported to affect the dissolution of NPs. Adeleye et al. (2014) found that CuO NPs dissolved more when the electrolyte content (NaCl) of the exposure media increased; however, Park et al. (2014) found that the dissolved Cu and Zn released from CuO NPs and ZnO NPs decreased with increasing electrolyte contents. Therefore, more systematical research needs to be conducted in order to better understand the specific impact of electrolytes on the fate of NPs.

Natural organic matter (NOM) composed of organic compounds originating from the residues of organisms such as plants and animals and their waste products, is ubiquitous in terrestrial and aquatic environments. To date, the effect of NOM on the fate of NPs has been widely studied. However, contradictory results on this topic still continue to emerge. On one hand, it was widely reported that NOM can bring stabilization effect on NPs via electrostatic repulsion and/or steric hindrance (Grillo et al., 2015). The stabilization effect arising from NOM facilitates the dispersion and transport of NPs in water systems. On the other hand, NOM was observed to accelerate the aggregation of NPs at high divalent cation concentrations (e.g., Ca^{2+}). The possible mechanism underlying the enhancement of aggregation of NPs is the occurrence of cation-NOM bridging effects (Figure 1.4).

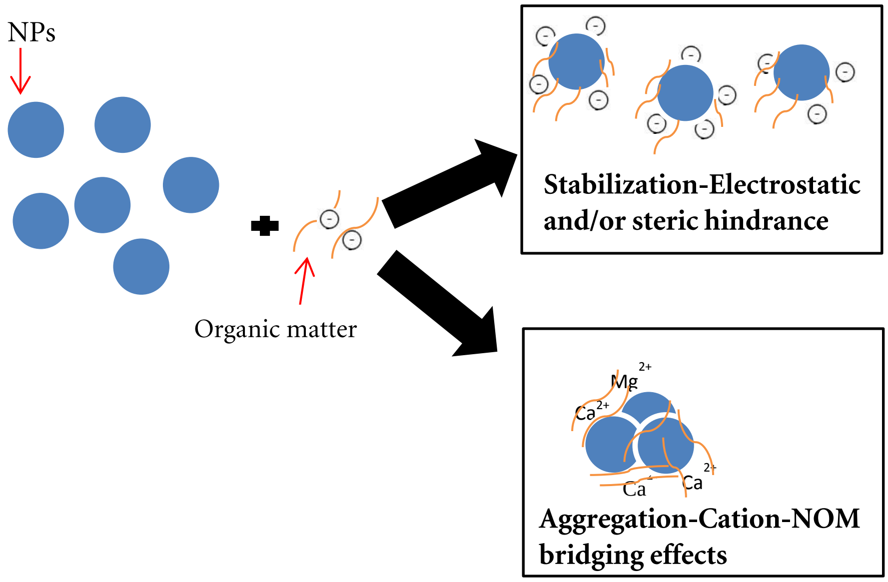


Figure 1.4 The possible interactions between NPs and natural organic matter (NOM).

Besides affecting the aggregation behavior of NPs, the interactions between NOM and NPs are also likely to influence the dissolution process of NPs. In some cases, the presence of NOM was reported to promote the dissolution rate of soluble NPs, because of the chelation of ionic species by NOM (Wang et al., 2011; Adeleye et al., 2014; Majedi et al., 2014; Miao et al., 2015). However, inhibiting effects of NOM on dissolution were also reported by some other researchers (Conway et al., 2015; Collin et al., 2016; Liu et al., 2016). The inhibiting effects of NOM were attributed to the surface adsorption of NOM, consequently blocking the oxidation sites of NPs (Dubas and Pimpan, 2008), to the steric exclusion of water from the surface of the particles by the coating of NOM, and/or to the reduction of the availability of H^+, which was consumed by NOM molecules (Yoon et al., 2005; Adeleye et al., 2014). The inconsistent findings suggest that the specific effect of NOM on the fate of NPs is currently far from clear and hence deserves to be continuously and systematically studied.

1.4 Accumulation of metallic nanoparticles

As mentioned above, after being submerged in aqueous environments, unstable metallic NPs are subject to aggregate and dissolve concurrently. As a consequence, both particles and ions released from NPs are likely to be taken up by organisms. For the dissolved ions, it has been reported that they can be taken up through membrane protein pumps and channels (Oparka et al., 1991; Conner and Schmid, 2003; Bianchini and Wood, 2008). As for the particles, ingestion was reported to be the dominant pathway for them to be taken up by organisms, like crustaceans (Rosenkranz et al., 2009; Zhao and Wang, 2012). Particles have been found to be able to cross cellular barriers through active endocytic pathways, passive diffusion, ion channels and carrier mediated transport (Hwang and Kim 2014; Skjolding et al., 2014; Li et al., 2016). In fact, for both NPs and their released ions, the essential process to ignite adverse effects on an organism is to be taken up by the organism or to approach its immediate vicinity (von Moos et al., 2014). It is therefore critical to investigate the profile of NP accumulation in organisms in order to better understand the nature of toxicity of NP suspensions. Although it has been identified that the chemical composition, size, shape, surface coating, and concentration of NPs have the potential to influence their accumulation in organisms (Zhao and Wang, 2012; Li and Wang, 2013; Pavagadhi et al., 2014; Carnovale et al., 2016), there is still very limited information in terms of the accumulation of NPs in organisms, especially that very few research has differentiated the relative contributions of NPs and their released ions to the accumulation.

1.5 Ecotoxicity of metallic nanoparticles

Considering the broad application of NPs, there is inevitably an increasing chance for humans and environmental organisms to be exposed to NPs through different routes as mentioned above. Hence, it is of paramount importance to thoroughly understand their potential risk to the human health and environment. It is recognized that properties of NPs and characteristics of the receiving environment can greatly affect the toxicity of NPs. For example, it has been reported that the toxicity of CuNPs was significantly affected by the size (Hua et al., 2014), shape (Carnovale et al., 2016), surface coating (Zhao and Wang, 2012) and also by natural organic matter (Yang et al., 2014), ionic strength (Chambers et al., 2014) and pH of the exposure environment (Van Hoecke et al., 2011). Due to the propensity of unstable-metallic NPs to dissolve after being emitted into aqueous environment, the NP suspension is generally a combination of NPs themselves (designated as $NP_{(particle)}$ hereafter) and their released ions (designated as $NP_{(ion)}$ hereafter). Although for the same metallic NPs, the toxicity of the $NP_{(ion)}$ is commonly reported to be higher than the same amount of $NP_{(particle)}$ (Kittler et al., 2010; Rainville et al., 2014; Song et al., 2015a), it remains unclear whether the observed toxicity of unstable-metallic NP suspensions mainly originates from $NP_{(ion)}$ or from $NP_{(particle)}$. It is clear that determining the main source of toxicity of NPs upon varying exposure environments is meaningful for environmental risk assessment and management of NPs.

1.5.1 Ecotoxicity of nanoparticles originating from the $NP_{(ion)}$

For the soluble NPs, such as CuNPs, Ag NPs and ZnO NPs, partial or even complete dissolution is expected to occur after being submerged in aqueous environments. Many studies have shown that $NP_{(ion)}$ would at least partially contribute to the toxicity induced by NP suspensions. For instance, toxicity of ZnO NPs to crustaceans and algae was reported to be largely or even solely attributed to the ions released from ZnO NPs (Franklin et al., 2007; Aruoja et al., 2009; Adam et al., 2015). For CuO NPs and Ag NPs, $NP_{(ion)}$ have also been revealed by many researchers to be an important source for driving the adverse effects of the NP suspensions (Heinlaan et al., 2008; Aruoja et al., 2009; Gunawan et al., 2009; Mortimer et al., 2010; Bondarenko et al., 2012; Xiu et al., 2012). However, some studies have found that the toxicity of NPs was not related to $NP_{(ion)}$. Santo et al. (2014), for example, found that the dissolved Zn released from ZnO NPs did not make any contribution to the 48-h toxicity of ZnO NPs to *D. magna*. Hao et al. (2013) found that the contribution of $NP_{(ion)}$ to the toxicity of ZnO NP suspensions to juvenile carp was negligible. Piret et al. (2014) disclosed that $NP_{(ion)}$ contributed only to a small extent to the toxicity induced by CuO NPs on HepG2 cells.

1.5.2 Ecotoxicity of nanoparticles originating from $NP_{(particle)}$

According to the existing research, the mechanisms of actions of $NP_{(particle)}$ include inducing a higher level of reactive oxygen species, inflammatory responses, membrane leakage and damage, DNA damage, lipid peroxidation, and mitochondrial damage (Pulskamp et al., 2007; Lin et al., 2009; Fruijtier-Pölloth, 2012; Passagne et al., 2012; Poynton et al., 2012). Some recent published literatures even found that the toxicity of $NP_{(particle)}$ differed from that of $NP_{(ion)}$ at the mechanistic level (Poynton et al., 2011; Rainville et al., 2014). Nevertheless, the issue whether $NP_{(particle)}$ contributes to the toxicity of NP suspensions remains questionable. Indeed, studies that found the adverse effects of NP suspensions mainly or even solely resulting from $NP_{(ion)}$ are not few, as mentioned above. These contradictory conclusions hinder our understanding and predictive potential on NP hazards to environments.

1.5.3 Approach to differentiate the toxic contribution of $NP_{(ion)}$ and $NP_{(particle)}$

As mentioned above, for the unstable- metallic NPs, the NP suspension is generally viewed as a mixture of $NP_{(particle)}$ and $NP_{(ion)}$. It is still difficult to clearly differentiate the contribution of $NP_{(ion)}$ and $NP_{(particle)}$ to the observed toxicity by lab work (Ivask et al., 2013). Although, the specific modes of action of the $NP_{(ion)}$ and $NP_{(particle)}$ remain unclear, some recently published papers found that the modes of toxicity of $NP_{(particle)}$ differed from that of $NP_{(ion)}$ (Poynton et al., 2011; Poynton et al., 2012; Rainville et al., 2014; Zhao et al., 2016). Hence, the modes of action of $NP_{(ion)}$ and $NP_{(particle)}$ were assumed to be dissimilar in this thesis. For toxic chemicals, if the modes of action or mechanisms of action of the chemicals were dissimilar, the response addition model could be used to estimate the combined effects, which has been mechanistically supported by conceptual findings in the area of pharmacology (Altenburger et al., 2003). Liu et al. (2016) verified that the response addition model performed well in estimating the toxicity of mixtures of $Cu(NO_3)_2$-CuNPs, $Zn(NO_3)_2$-ZnONPs, and $Zn(NO_3)_2$-CuNPs to lettuce roots. This indicates that the use of the response addition model to quantify the relative contribution of $NP_{(ion)}$ and $NP_{(particle)}$ to the toxicity of NP suspensions is reasonable.

$$E_{(\mathrm{total})} = 1 - \left[\left(1 - E_{(\mathrm{ion})}\right)\left(1 - E_{(\mathrm{particle})}\right)\right] \tag{1.1}$$

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

1.6 Experimental observations in aquatic systems

1.6.1 The test species, waterflea *Daphnia magna*

Daphnia magna is a small planktonic crustacean, which belongs to the subclass Phyllopoda. *D. magna* is also called water flea. This might originate from its jump-like swimming behavior. The length of daphnia adults is in the range from 1.5 to 5 mm. *D. magna* is widespread in a variety of freshwater systems from swamps to lakes and streams. A water current within the thoracic opening of the carapace could be generated due to the formation of a specialized filtering apparatus. This allows the organisms to collect and ingest suspended particles (mainly algae) as large as 70 µm (Geller and Müller, 1981). Furthermore, due to the high efficiency of filtering water, *D. magna* has a significant interaction with the aquatic environment, resulting in a high risk of exposure to pollutants.

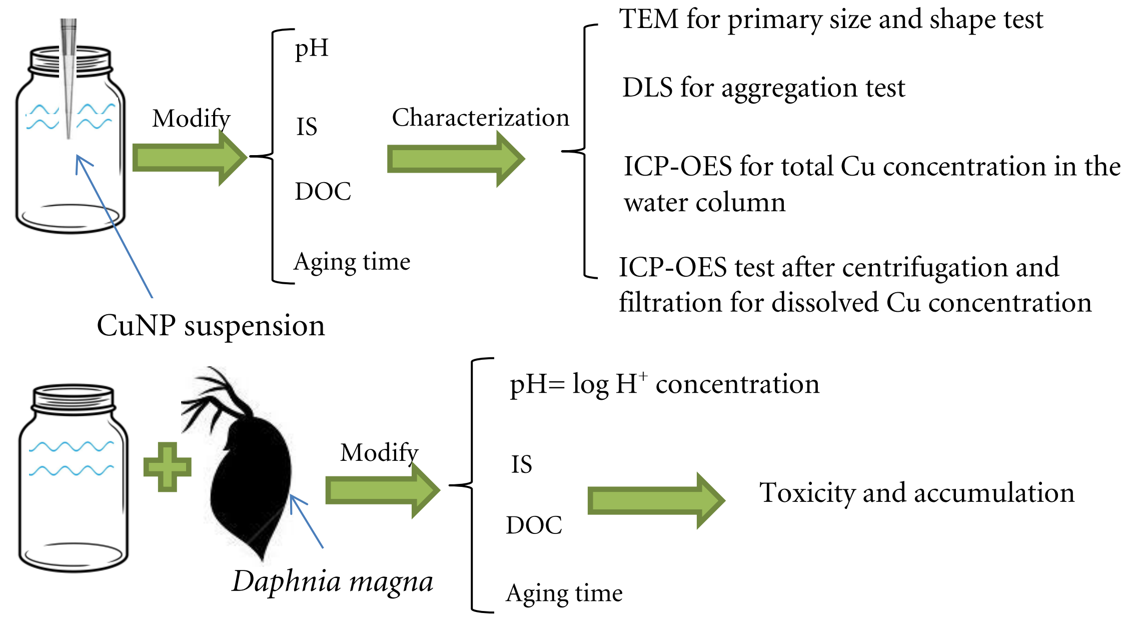
D. magna has been widely used as a test organism in ecotoxicology. This is mainly because of its ecological importance. The daphnid families are generally the food and energy link between primary producers (e.g., algae) and secondary consumers (e.g., fish and fish larvae) (Baun et al., 2008). In addition, *D. magna* also displays other advantages when used as experimental organism. Its transparency allows for the observations of its inner anatomical structures at the microscope; *D. magna* is relatively easy to keep in the laboratory; it has a fast generation time, which allows a large scale experiment relatively easy to perform. The Organization for Economic Cooperation and Development (OECD) has even developed two guidelines for testing chemical toxicity on *D. magna*. Test No. 202 "Daphnia sp., Acute Immobilization Test" is applied to assess the acute toxicity of a chemical; test No. 211 "*Daphnia magna* Reproduction Test" is used to determine the 21-day chronic toxicity of a chemical. Therefore, *D. magna* is an obvious first choice as an experimental organism to reflect the ecotoxicology of NPs (Baun et al., 2008).

1.6.2 Test nanoparticles

Considering their widespread applications as mentioned above, CuNPs are selected as the test NPs. Although CuNPs and Cu-based NPs have been found to be highly toxic to a wide range of aquatic organisms, such as algae (Aruoja et al., 2009), crustacean (Song et al., 2015a) and fish species (Song et al., 2015b), information on the toxic actions of CuNPs is still limited, compared to other metallic NPs, like Ag NPs, TiO_2 NPs and ZnO NPs. Hence, fate, accumulation and toxicity of CuNPs upon various water chemistry were systematically studied in this thesis.

1.6.3 Exposure conditions tested in this thesis

For a comprehensive assessment of the fate, accumulation and toxicity of CuNPs, suspensions of CuNPs with a range of water chemistry were prepared by diluting the stock CuNP suspensions into various exposure conditions (Figure 1.5). The prepared CuNP suspensions for fate, toxicity and accumulation assessment were kept in a climate chamber under a 16:8-h light-dark cycle (20 ± 1 °C) statically and dynamically. At each sampling time point, CuNP suspensions were used for fate testing and assessment of mortality of *D. magna*. As for the fate investigation, the morphology and primary size of CuNPs were measured by using transmission electron microspectroscopy (TEM, JEOL 1010, JEOL Ltd., Japan). The hydrodynamic diameters of the NPs core were determined using dynamic light scattering (DLS) on a Zetasizer Nano-ZS instruments (Malvern, Instruments Ltd., UK). The zeta potential of each copper suspension at the same time point was evaluated by ZetaPALS software based on the Smoluchowski equation. The total amount of Cu remaining in the water column within incubation was determined by collecting 5 mL samples from the position around 2 cm below the surface of each suspension and then digested by 65% nitric acid for at least 1 d before analysis by inductively coupled plasma optical emission spectrometry (ICP-OES). The concentration of dissolved Cu shedding from CuNPs was determined by pipetting 10 mL of the CuNP suspensions from the water column and subsequently centrifugation of the samples at 30392 g for 30 min at 4 °C (Sorvall RC5Bplus centrifuge, Fiberlite F21-8 × 50y rotor). The samples were then filtered through a syringe filter with 0.02 µm pore diameter (Anotop 25, Whatman). The filtrate was digested by nitric acid and the dissolved Cu concentration was determined using ICP-OES. All the acute toxicity tests in this study were carried out according to OECD Guideline 202.



Stored in climate chamber: 16:8-h light-dark cycle (20 ± 1 °C) statically and dynamically.

Figure 1.5 An overall scheme of the experimental set-up of the thesis. IS means the ionic strength of the suspensions. DOC is the abbreviation of dissolved organic carbon. DLS is the abbreviation of dynamic light scattering. TEM means transmission electron microscopy. ICP-OES is inductively coupled plasma optical emission spectrometry.

1.7 Objectives of this thesis

As described above, the fate, accumulation and toxicity of CuNPs across a range of water chemistry are still poorly known. Hence, this PhD study aimed to disclose the behavior, fate, accumulation and toxicity of CuNPs as well as the relative contribution of $NP_{(ion)}$ and $NP_{(particle)}$ to the observed accumulation and toxicity of CuNP suspensions to *D. magna* under environmentally relevant water chemistry conditions.

To achieve the research objectives, the following sub-questions were addressed:

(1) Are particles themselves or their released ions the main source of the overall observed toxicity and accumulation of CuNPs in *D. magna*? (Chapter 2)

(2) How does water chemistry affect the toxicity of CuNP suspensions to *D. magna*? (Chapter 3)

(3) How does water chemistry affect the relative contribution of $NP_{(ion)}$ and $NP_{(particle)}$ to the observed toxicity of suspensions of CuNPs to *D. magna*? (Chapter 3 and 5)

(4) How does water chemistry affect the behavior and fate of CuNPs? (Chapter 4)

(5) How does water chemistry affect the particle-specific toxicity of CuNPs to *D. magna*? (Chapter 5)

1.8 Outline of this thesis

Chapter 1 A general introduction regarding NPs and their fate, accumulation and toxicity. The objectives of the thesis are mentioned.

Chapter 2 The toxicity and accumulation of Cu and ZnO NPs in *D. magna* were investigated and compared. Furthermore, the specific role of $NP_{(ion)}$ and $NP_{(particle)}$ in the observed toxicity and accumulation was differentiated by using the response addition model.

Chapter 3 The toxicity of suspensions of CuNPs to *D. magna* upon different exposure conditions was assessed. Meanwhile, the variations of the relative contribution of $NP_{(ion)}$ and $NP_{(particle)}$ to the overall toxicity of CuNP suspensions upon modification of the exposure conditions were analyzed.

Chapter 4 The fate of CuNPs in aqueous environment at the field relevant conditions was determined and the key factors among the water chemistry parameters in determining the fate of CuNPs were identified.

Chapter 5 The impact of water chemistry on the particle-specific toxicity of CuNPs to *D. magna* was investigated.

Chapter 6 This chapter summarizes the main findings of this thesis. Based on the conclusions, the key factors in determining the fate and toxicity among environmental parameters, the main driver of the toxicity of CuNP suspensions upon various water chemistry, and how the water chemistry affect the particle-specific toxicity of CuNPs were understood. In addition, recommendations for future research in terms of NP risk assessment are briefly discussed in this chapter.

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Chaper 2 Toxicity and accumulation of Cu and ZnO nanoparticles in *Daphnia magna*

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Abstract

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

Key words: copper nanoparticles, zinc oxide nanoparticles, acute toxicity, accumulation, *Daphnia magna*

2.1 Introduction

Nanoparticles (NPs) are defined as particles with at least one dimension between 1 and 100 nm. Due to the small size of NPs, they usually show unique physicochemical properties, such as high surface area and high mechanical strength. NPs are increasingly used in many applications. Cu nanoparticles (CuNPs) and ZnO nanoparticles (ZnONPs) have been manufactured on a large scale in different areas. For instance, CuNPs have been widely employed in catalysis and batteries (Zhang et al., 2005; Radi et al., 2010), and ZnONPs have been extensively used in cosmetics and UV-absorbers (Becheri et al., 2008). Like other types of NPs, the recent increasing use of CuNPs and ZnONPs has also started to induce concern on their toxicity to some specific aquatic organisms, such as mussel (Hu et al., 2014), and juvenile carp (Hao et al., 2013). Hence, there is a necessity to investigate the underlying processes resulting in the toxicity of CuNPs and ZnONPs to aquatic organisms.

Toxicity of NPs may be exerted by particles (designated as $NP_{(particle)}$ hereafter) (Cronholm et al., 2013; Santo et al., 2014), by dissolved ions released from NPs (designated as $NP_{(ion)}$ hereafter) (Jo et al., 2012; Adam et al., 2014a), or by both $NP_{(particle)}$ and $NP_{(ion)}$ (Navarro et al., 2008). Undoubtedly, determining the contribution of $NP_{(particle)}$ and $NP_{(ion)}$ to the overall toxicity of nanoparticle suspensions (designated as $NP_{(total)}$ hereafter), is a crucial step in assessing and managing the possible adverse effects of NPs. Based on the existing literature, it is still hard to get a uniform conclusion about what is the major source of toxicity of NPs. This may be due to the fact that many factors can affect the toxicity performance of NPs, not only the characteristics of the NPs, but also the exposure conditions (Heinlaan et al., 2008). Thus the underlying physicochemical mechanisms leading to the toxicity of different NPs to aquatic organisms should be investigated on a case by case basis, which suggests that more research should be performed to make a comprehensive consideration about this research question.

In the present study, *Daphnia magna* was selected as a model organism. The 48 h toxicity of CuNPs and ZnONPs to *D. magna* was determined. To analyze the relative contribution of $NP_{(ion)}$ to the overall toxicity of CuNPs and ZnONPs (designated as $CuNP_{(total)}$ and $ZnONP_{(total)}$ hereafter, respectively), centrifugation and filtering methods were combined to separate the dissolved ions released from the NPs. Subsequently, the 48 h toxicity of the supernatants only containing $NP_{(ion)}$ to daphnids was investigated. In order to test whether using metal salts as substitutes for the dissolved ions released from NPs is effective, the toxicity of dissolved Cu released from CuNPs (designated as $CuNP_{(ion)}$ hereafter) and dissolved Zn released from ZnONPs (designated as $ZnONP_{(ion)}$, hereafter) to daphnia neonates was compared to the toxicity of $Cu(NO_3)_2$ and $Zn(NO_3)_2$. Additionally, the issue which species, $NP_{(particle)}$ or $NP_{(ion)}$, plays a major role in the accumulation process was investigated in this study.

2.2 Materials and methods

2.2.1 Test materials, test medium and test species

CuNPs with a nominal size of 50 nm (advertised specific surface area, 6-8 m^2/g; purity, 99.8%) and ZnONPs with a nominal size of 43 nm (advertised specific surface area, 27 m^2/g; purity, 99.5%) were purchased from IoLiTec (Heibronn, Germany). Both CuNPs and ZnONPs were spherically shaped. $Cu(NO_3)_2$ and $Zn(NO_3)_2$ were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands).

Stock nanoparticle suspensions and salts were freshly prepared in ISO standard test medium (STM) after 20 min sonication in a water bath sonicator. The STM used in this study (pH 7.8 ± 0.2) contained (mg/L MilliQ water): $CaCl_2 \cdot 2H_2O$: 294; $MgSO_4 \cdot 7H_2O$: 123.25; $NaHCO_3$: 64.75; KCl: 5.75 (Griffitt et al., 2007).

D. magna, originally obtained from the Dutch National Institute for Public Health and the Environment (RIVM), was selected as the test species. Artificial ElendtM4 medium was used to culture *D. magna* (OECD, 2004), which was refreshed three times a week. The test organisms were cultured in plastic containers at a density of 1 individual/10 ml of ElendtM4 medium under a 16:8 light-dark cycle (20 ± 1 °C) and fed with *Pseudokirchneriella subcapitata* every two days.

2.2.2 Physicochemical analysis

The morphology and size of CuNPs and ZnONPs in STM were characterized by using transmission electron microspectroscopy (TEM, JEOL 1010, JEOL Ltd., Japan). The samples were analyzed directly (which was around 1 h after submerging NPs into the STM, to which we will refer to as 1 h) and after 24 h and 48 h. The size distribution of suspensions of CuNPs and ZnONPs at 1 mg/L were analyzed at 1 h, 24 h and 48 h after incubation in the test medium by dynamic light scattering (DLS) on a zetasizer Nano-ZS instrument (Malvern, Instruments Ltd., UK). At the same point, the zeta potential of each suspensions was determined by the ZetaPALS software based on the Smoluchowski equation. The actual exposure concentrations of CuNPs and ZnONPs, as well as $Cu(NO_3)_2$ and $Zn(NO_3)_2$ in the STM were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) after digestion in 65% HNO_3 for at least 1 day. In order to obtain the $CuNP_{(ion)}$ and $ZnONP_{(ion)}$, the two nanoparticle suspensions firstly were centrifuged at 30392 g for 30 min at 4 ℃ (Sorvall 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). We also applied ultracentrifugation and then performed DLS to confirm that the concentration of $NP_{(particle)}$ in the supernatants obtained by centrifugation at 30392 g for 30 min at 4 ℃ and subsequent filtration through a syringe filter with 0.02 μm pore diameter was less than the detection limit, and the detailed information on the confirmation is given in the Supplementary Information 2.1.

2.2.3 Assessment of the 48 h release profiles of dissolved Cu and Zn

The release profiles of dissolved Cu released from the CuNPs at 0.1 mg/L and 10 mg/L for a maximum of 48 h of exposure, and the release of dissolved Zn released from the ZnONPs at 1 mg/L and 10 mg/L for a maximum of 48 h of exposure in the STM were investigated. At different times, namely after being exposed to the medium for 1, 12, 24, 36 and 48 h, CuNPs and ZnONPs samples were centrifuged at 30392 g for 30 min at 4 ℃ (Sorvall RC5Bplus centrifuge, Fiberlite F21-8 × 50y rotor) and then the supernatants were filtered through a syringe filter with 0.02 μm pore diameter (Antop 25, Whatman). Subsequently, the Cu concentrations and Zn concentrations in the supernatants after filtration were measured by ICP-OES. To analyze the losses of NPs and $NP_{(ion)}$ after centrifugation and filtration, experiments were conducted and the detailed information on the analysis is given in Supplementary Information 2.2.

2.2.4 The 48-h acute toxicity test

OECD Guideline 202 with slight modifications was used to test the acute toxicity of the two nanoparticle suspensions. Before the start of the acute toxicity test, daphnids were kept in the STM for 1-2 h to evacuate their guts. The toxicity tests were performed using neonates (< 24 h). Five individuals were transferred into a test vial, containing 20 ml of CuNPs or ZnONPs, or metal salt solutions, or control. Based on the results of range finding experiments, exposure concentrations from 0.02 mg/L to 0.16 mg/L for CuNPs and from 0.4 mg/L to 2.6 mg/L for ZnONPs, as well as from 0.01 mg/L to 0.06 mg/L for $Cu(NO_3)_2$ and from 0.5 mg/L to 1.5 mg/L for $Zn(NO_3)_2$ were selected. Each test was composed of 8 to 10 different exposure concentrations and each exposure concentration was tested with 4 replicates. Daphnids were incubated under a 16:8 h light/dark photoperiod (20 ± 1 ℃) without feeding during the 48 h exposure period. To avoid significant changes in the concentrations of the nanoparticle suspensions, the exposure media were refreshed every 24 h.

Besides investigating the toxicity of the suspensions of CuNPs and ZnONPs, the relative contribution of $NP_{(particle)}$ and $NP_{(ion)}$ to the toxicity induced by the suspensions of CuNPs and ZnONPs was also examined. In order to better simulate the contribution of $CuNP_{(ion)}$ and $ZnONP_{(ion)}$ to the overall toxicity, the supernatants after filtration of the two NPs in the STM were prepared as described above. The neonate daphnids were then exposed to the supernatants according to the same procedures as described above. To compare with the toxicity of the freshly prepared solutions of $CuNP_{(ion)}$ and $ZnONP_{(ion)}$, the toxicity of $Cu(NO_3)_2$ and of $Zn(NO_3)_2$ to *D. magna* was tested with the same procedures as described above, as well.

2.2.5 Accumulation experiments

Experiments were conducted to identify the accumulation profiles of *D. magna* exposed to CuNPs, ZnONPs and their corresponding dissolved ions. In brief, *D. magna* (8 d old) were transferred to the suspensions of CuNPs and ZnONPs at a density of 1 individual/10 ml with 3 replicates. The concentrations of CuNPs applied in the accumulation experiments were 0.1 and 0.05 mg/L and the concentrations of ZnONPs were 1 and 0.5 mg/L, respectively. These concentrations were selected based on the results of the acute toxicity tests described above, which concerned the LC50. Adult daphnids (8 d old) were utilized. We first of all selected adult daphnids, because they are more easy to handle, compared to neonates. Moreover, due to physiological (e.g., surface-volume ratio) and behavioral aspects adults are often less susceptible to NPs compared to juvenile and neonate life stages in ecotoxicity studies (Ates et al., 2013; Wang et al., 2013), which allowed us to analyze accumulation characteristics at high concentration of nanoparticle suspensions. Finally, it is advantageous to use adults as growth dilution may be reduced (He et al., 2007; Karimi et al., 2007). In order to distinguish between the contribution of $NP_{(particle)}$ and $NP_{(ion)}$ to accumulation, *D. magna* were also exposed to the supernatants of the CuNPs at the initial CuNPs concentrations of 0.1 mg/L and 0.05 mg/L and to the supernatants of ZnONPs at the initial ZnONPs concentrations of 1 mg/L and 0.5 mg/L. These supernatants were prepared as described above. Many recent studies have shown that the accumulation of NPs in *D. magna* is a rapid process (Tab et al., 2012; Zhao and Wang, 2012; Li et al., 2013). Hence, a 48 h exposure period was selected in our test. Similar to the acute toxicity tests, the accumulation experiments were conducted under a 16:8 h light/dark photoperiod (20 ± 1 °C) without feeding during the 48 h exposure period. The exposure media were refreshed every 24 h. After the 48 h exposure period, 10 mobile daphnids were sampled from each exposure medium and they then were transferred to MilliQ water for 1-3 min. Subsequently, they were rinsed three times with fresh MilliQ water. After rinsing, they were dried at 80 °C overnight in pre-weighed glass containers before weighing on a microbalance and then digested in 69% HNO_3 at 80 °C overnight. The Cu and Zn concentrations in the digested samples were subsequently determined by ICP-OES.

To clarify in this study, accumulation is defined as the absorbed and adsorbed metals after rinsing the daphnids. As after rinsing the daphnids with MilliQ water, NPs may not be completely removed, and may still be adsorbed to the outside of organisms. Surface adsorption of NPs onto the exterior of *D. magna* limits their biological activity and also may pose risks to their health development. For example, Dabrunz et al. (2011) found that adsorbed NPs caused mortality through reducing the molting rate of *D. magna*. Hence, both absorbed and adsorbed fraction are considered in this study.

2.2.6 Relative contribution to toxicity of $NP_{(particle)}$ and $NP_{(ion)}$

The neonates of *D. magna* were exposed to the nanoparticle suspensions containing a mixture of $NP_{(particle)}$ and $NP_{(ion)}$. The behavior and toxicity of chemicals in a mixture may not conform to that predicted from data on pure compounds (Altenburger et al., 2003). Complicated and remarkable changes in the apparent properties of its constituents can be induced by interactions of components in a mixture, leading to increased or decreased effects compared with the ideal reference case of additive behavior. For evaluation of the joint toxicity of mixtures, the concentration addition (CA) model and the independent action (IA) model are two prominent reference models, both of which have been mechanistically supported by pharmacology (Altenburger et al., 2003). Which model is preferably employed to analyze the combined effects of chemicals in a mixture is based on the mode of action (Altenburger et al., 2003). Specifically, the CA model can be utilized to estimate the combined effects of chemicals in a mixture with a similar mode of action, whereas the IA model is employed to analyze the joint effects of chemicals with dissimilar mode of actions (Altenburger et al., 2003). The response addition model is often used as a synonym for the IA model (Faust et al., 2003). Based on the previous literature, it is widely believed that the modes of actions of $NP_{(particle)}$ and $NP_{(ion)}$ are likely to be dissimilar (Hua et al., 2014a and 2014b). Thus the response addition model was selected in the present study to calculate the relative contribution to toxicity of $NP_{(particle)}$ and $NP_{(ion)}$. The response addition model is defined as follows:

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

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

2.2.7 Statistical analysis

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

2.3 Results

2.3.1 Physicochemical characterization of CuNPs and ZnONPs

TEM images of CuNPs and ZnONPs are presented in Figure 2.1. The images demonstrate that the CuNPs and ZnONPs used in this study were spherical particles. However, the CuNPs and ZnONPs aggregated intensely into irregular shapes in the STM. DLS was performed for determining the hydrodynamic diameter of the CuNPs and ZnONPs suspended in STM. Data on size distributions after 1, 24 and 48 h are given in Table 2.1. Both NPs aggregated as soon as being submerged into the STM. The particle size of CuNPs in the STM was found to increase from 568 ± 72 nm after 1 h of incubation to 953 ± 525 nm after 48 h of incubation and the particle size of ZnONPs shifted from 1154 ± 252 nm after 1 h of incubation to 1871 ± 509 nm after 48 h of incubation. The zeta potential of the two nanoparticle suspensions increased after NPs being submerged into the medium. The zeta potential of the CuNPs increased from -14 ± 4 after 1 h of incubation to -4 ± 2 after 48 h of incubation. Similarly, the zeta potential of the ZnONPs increased from -10 ± 2 after 1 h of incubation to -3 ± 2 after 48 h of incubation.

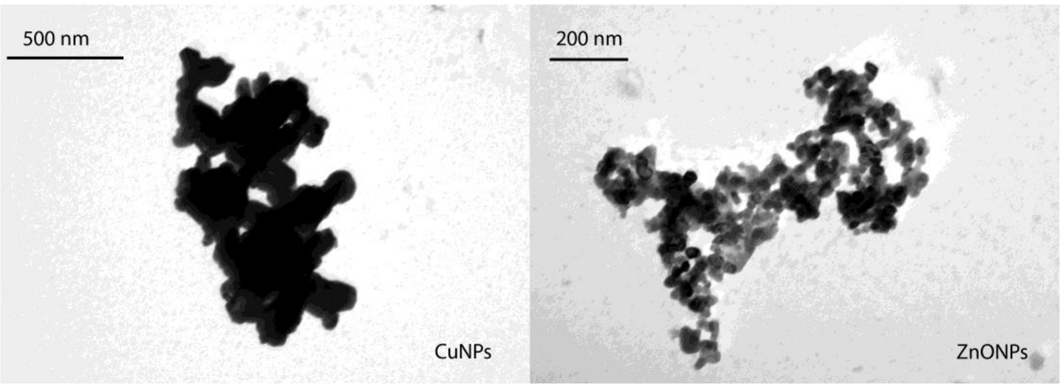

Figure 2.1 TEM images of CuNPs and ZnONPs after 1 h of incubation in the standard test medium (STM).

Table 2.1 Hydrodynamic diameter and zeta-potential of 1 mg/L suspensions of CuNPs and ZnONPs in the STM.

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

Hydrodynamic size and zeta potential were expressed as mean ± SD (*n* = 3).

2.3.2 The 48 h ion release profiles of CuNPs and ZnONPs

The 48 h release profiles of CuNPs of ZnONPs are shown in Figure 2.2. The percentage of $CuNP_{(ion)}$ in the CuNPs suspension at 0.1 mg/L shifted from 18% after 1 h of incubation to 20% after 48 h of incubation. The ZnONPs at 1 mg/L showed a relatively high degree of dissolution. The percentage of the $ZnONP_{(ion)}$ increased from 59% after 1 h of incubation to 65% after 48 h of incubation in the STM. As for the percentages of the $CuNP_{(ion)}$ and $ZnONP_{(ion)}$ in the nanoparticle suspensions at 10 mg/L, both of them remained around 10% during the 48 h of incubation.

2.3.3 The 48 h acute toxicity of CuNPs and ZnONPs

All the exposures of neonate daphnids to the nanoparticle suspensions and their dissolved ions induced significant toxicity (Figure 2.3). CuNP$_{(total)}$ showed a much lower 48 h LC50 value of 0.093 mg/L (with a 95% CI of 0.86-0.101 mg/L), compared to ZnONP$_{(total)}$ (0.99 mg/L, with a 95% CI of 0.92-1.07 mg/L). The LC50 value of CuNP$_{(ion)}$ was 0.030 mg/L (with a 95% CI of 0.027-0.034 mg/L) and the LC50 value of the ZnONP$_{(ion)}$ was 1.15 mg/L (with a 95% CI of 1.02-1.30 mg/L), which indicated that the CuNP$_{(ion)}$ was also much more toxic (over 10 times) to *D. magna*, compared to the ZnONP$_{(ion)}$. The dose-response curves for $Cu(NO_3)_2$ and $Zn(NO_3)_2$ are also given in Figure 2.3. The LC50 of $Cu(NO_3)_2$ to the neonates was 0.028 mg/L (with a 95% CI of 0.026-0.029 mg/L), which was similar to the LC50 value of the CuNP$_{(ion)}$. Likewise, the LC50 of $Zn(NO_3)_2$ (1.01 mg/L, with a 95% CI of 0.94-1.09 mg/L) was similar to the LC50 level of the ZnONP$_{(ion)}$.

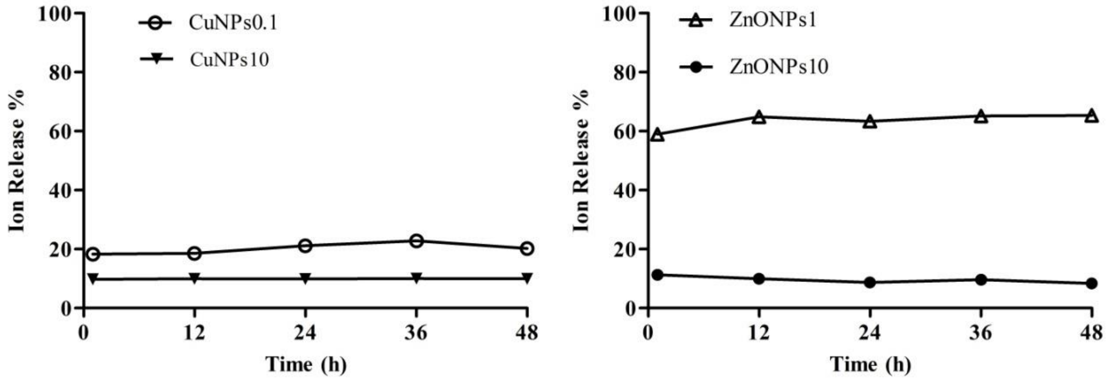

Figure 2.2 The relative percentages of dissolved Cu released from CuNPs at the concentrations of 0.1 mg/L and 10 mg/L, and of dissolved Zn released from ZnONPs at the concentrations of 1 mg/L and 10 mg/L, during 48 h of incubation in the STM. Data are mean ± SD (*n* = 3).

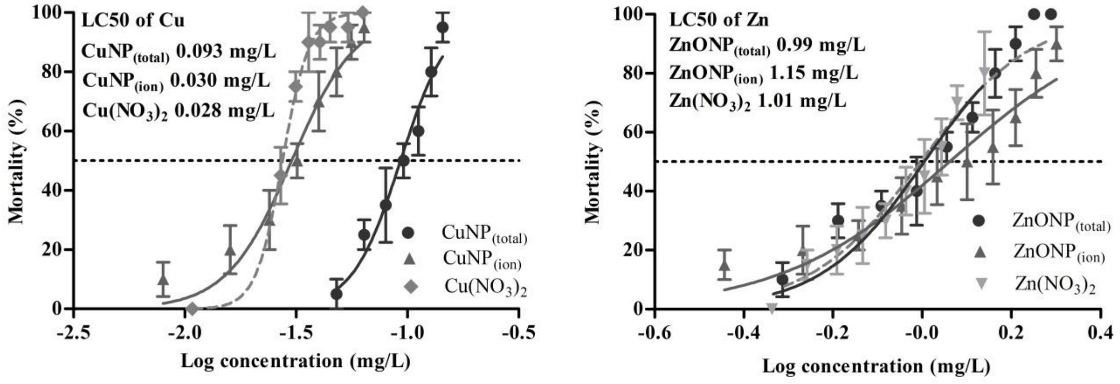

Figure 2.3 Dose-response curves of mortality (%) of *D. magna* exposed to different concentrations of CuNP$_{(total)}$, ZnONP$_{(total)}$, CuNP$_{(ion)}$, ZnONP$_{(ion)}$, $Cu(NO_3)_2$ and $Zn(NO_3)_2$ for 48 h. Actual log-transformed Cu or Zn concentrations are plotted on the x-axis. Data are mean ± SD (*n* = 4).

2.3.4 The 48 h accumulation characteristics of CuNPs and ZnONPs

The accumulation profiles of CuNPs and ZnONPs are shown in Figure 2.4. At the low CuNPs concentration (0.05 mg/L), the overall accumulation of Cu (105 ± 39 μg/g dry weight) in daphnids was higher than the accumulation of the $\text{CuNP}_{(\text{ion})}$ (49 ± 14 μg/g dry weight). Moreover, at the high CuNPs concentration (0.1 mg/L), the overall accumulation of Cu was 264 ± 60 μg/g dry weight, which was significantly higher than the accumulation of the $\text{CuNP}_{(\text{ion})}$ (68 ± 15 μg/g dry weight, $p < 0.05$). As for the Zn accumulation, the overall accumulation of Zn at 0.5 mg/L of ZnONPs (558 ± 106 μg/g dry weight) was significantly higher than the accumulation of $\text{ZnONP}_{(\text{ion})}$ (301 ± 82 μg/g dry weight, $p < 0.05$). Likewise, the overall accumulation of Zn at 1 mg/L of ZnONPs (1345 ± 331 μg/g dry weight) was significantly higher than the accumulation of $\text{ZnONP}_{(\text{ion})}$ (484 ± 85 μg/g dry weight, $p < 0.05$).

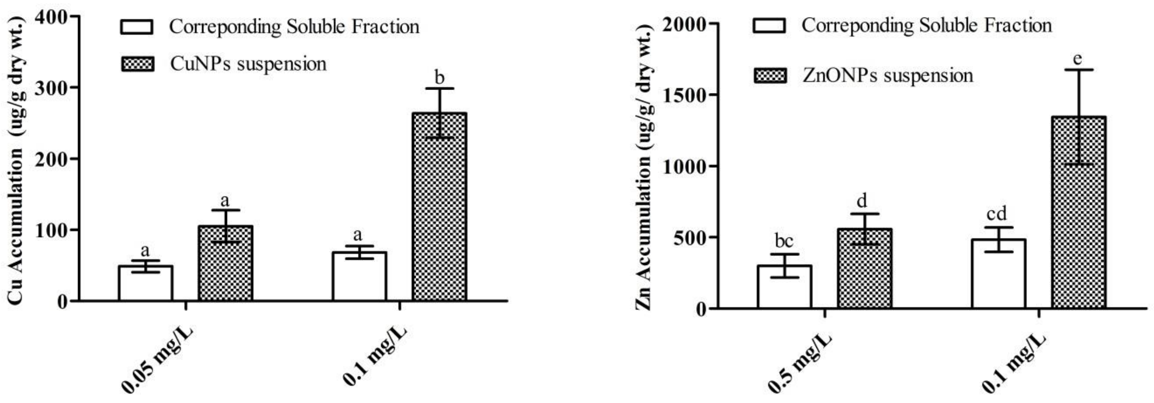

Figure 2.4 The accumulation (μg/g dry wt.) of Cu and Zn in *D. magna* after exposure for 48 h to 0.05 mg/L and 0.1 mg/L of CuNP suspensions and to 0.5 mg/L and 1.0 mg/L of ZnONP suspensions, the corresponding dissolved Cu released from the above concentrations of CuNPs, and the corresponding dissolved Zn released from the above concentrations of ZnONPs. The different letters indicate the significant differences of accumulation levels, $p < 0.05$. The *p*-value between different accumulation groups was determined by means of the *t*-test.

2.3.5 Relative contribution of $\text{NP}_{(\text{particle})}$ and $\text{NP}_{(\text{ion})}$ to toxicity and accumulation

The relative contribution of $\text{NP}_{(\text{particle})}$ and $\text{NP}_{(\text{ion})}$ to the overall toxicity at the LC50 levels of CuNPs and ZnONPs to neonates is given in Table 2.2. The relative contribution of $\text{CuNP}_{(\text{ion})}$ to toxicity was only 26%, whereas $\text{CuNP}_{(\text{particle})}$ accounted for about 74% of the relative contribution to mortality at the LC50 level of $\text{CuNP}_{(\text{total})}$. $\text{ZnONP}_{(\text{ion})}$ only contributed a fraction of 31% to the overall toxicity, compared to the contribution of $\text{ZnONP}_{(\text{particle})}$. In addition, the relative contribution to accumulation of $\text{NP}_{(\text{particle})}$ and $\text{NP}_{(\text{ion})}$ is calculated and shown in Table 2.3. At the low concentration of CuNPs (0.05 mg/L), $\text{CuNP}_{(\text{ion})}$ contributed around 52% to the overall accumulation, whereas at the concentration of 0.1 mg/L of CuNPs, $\text{CuNP}_{(\text{ion})}$ merely accounted for about 28%. Likewise, at the low concentration of ZnONPs (0.5 mg/L), $\text{ZnONP}_{(\text{ion})}$ could explain about 53% of the overall Zn accumulation, while at the high concentration (1 mg/L of ZnONPs), $ZnONP_{(ion)}$ only contributed around 36% to the overall Zn accumulation.

Table 2.2 The relative contribution of $NP_{(particle)}$ and $NP_{(ion)}$ to mortality at the LC50 level of the CuNPs and ZnONPs exposed to neonate daphnids.

Type	Relative contribution to mortality (%)	
	$NP_{(particle)}$	$NP_{(ion)}$
Cu	74 ± 5	26 ± 5
Zn	69 ± 4	31 ± 4

The relative contribution of $NP_{(particle)}$ and $NP_{(ion)}$ to mortality was expressed as mean ± SD (*n* = 4).

Table 2.3 The relative contribution of $NP_{(particle)}$ and $NP_{(ion)}$ to accumulation at different concentrations of nanoparticle suspensions.

Suspensions	Relative contribution to accumulation (%)	
	$NP_{(particle)}$	$NP_{(ion)}$
CuNPs at 0.05 mg/L	48 ± 25	52 ± 25
CuNPs at 0.1 mg/L	72 ± 12	28 ± 12
ZnONPs at 0.5 mg/L	47 ± 5	53 ± 5
ZnONPs at 1 mg/L	64 ± 3	36 ± 3

The relative contribution of $NP_{(particle)}$ and $NP_{(ion)}$ to accumulation was expressed as mean ± SD (*n* = 3).

2.4 Discussion

2.4.1 Ion release profiles of CuNPs and ZnONPs

In this study, ZnONPs were demonstrated to be more soluble than CuNPs. ZnONPs showed an especially rapid dissolution process at a low concentration in the STM. We found that 59% of the ZnONPs at 1 mg/L were already dissolved after 1 h of incubation (Figure 2.2). This result was similar with the recent result obtained by Adam et al. (2014b), who detected that ZnONPs (with a nominal size of 30 nm) showed 60% of dissolution within 1-2 h in the ISO medium. However, it is worth to note that the percentage of $ZnONP_{(ion)}$ is reported to vary considerably in the existing literature. For example, Merdzan et al. (2014) found that the percentage of dissolved Zn released from bare ZnONPs with a nominal size of 20 nm was to a large extent (> 85%) present in solution (10^{-2} M HEPES, 10^{-5} M Ca, pH 7.0) after 24 h of exposure. This is higher than the extent of dissolution we found in our study. However, another study reported that the percentages of ZnONP$_{(ion)}$ were only 30% at 0.5 mg/L of ZnONPs and 20% at 2 mg/L of ZnONPs after 24 h of exposure in simplified M7 medium (Li et al., 2013). This discrepancy is a reflection of the combined effects exerted by the characteristics of NPs and exposure medium, such as, ionic strength and pH of the test medium (Li et al., 2013), presence of coatings (Merdzan et al., 2014), and particle size (Meulenkamp et al., 1998). The ion-release profiles of NPs should thus be analyzed on a case-by-case basis. At the high concentration applied in this study (10 mg/L), the percentages of CuNP$_{(ion)}$ and ZnONP$_{(ion)}$ were almost constant around 10%, much lower than the percentages of ZnONP$_{(ion)}$ and CuNP$_{(ion)}$ at the low concentrations (namely 1 mg/L ZnONPs and 0.1 mg/L CuNPs). Similarly, Zhao and Wang (2012) found that at a low concentration of AgNPs (10 μg/L) in SM7 medium, the relative percentage of the dissolved Ag released from AgNPs could be beyond 50%, while at a high concentration of AgNPs (1000 μg/L) the percentage of dissolved Ag remained constant at less than 10%. These results indicate that the concentration of NPs is also an important factor to consider regarding the dissolution characteristics of NPs, since the equilibrium between dissolved and adsorbed ions might potentially play a role. Furthermore, it needs to be noted that, especially at high nanoparticle concentrations, the percentage of NP$_{(ion)}$ remains constant during the whole exposure time.

2.4.2 Acute toxicity of CuNPs and ZnONPs

In the present study, CuNPs were found to have a LC50 level (0.093 mg/L), which was over 10 times lower than that of ZnONPs (0.99 mg/L). It is thus obvious that *D. magna* is much more vulnerable to dispersions of CuNPs than to dispersions of ZnONPs. Previous toxicity assessments of NPs have primarily focused on probing into the effects of different exposure routes, such as the respiratory or gastrointestinal tracts (Chang et al., 2015). Most previous studies did not distinguish between the relative contributions of NP$_{(particle)}$ and NP$_{(ion)}$ to the overall toxicity induced by NPs, or have simply compared the overall toxicity of metal-based NPs with that of corresponding metal salts. However, metal salts import other types of ionic species into solutions, compared to the NP$_{(ion)}$ directly, which may exert effects on acute toxicity through combined effects on physiological characteristics and metal speciation (Lopes et al., 2014). In this study, to investigate whether using metal salts to replace NP$_{(ion)}$ is effective, comparison of acute toxicity between freshly prepared CuNP$_{(ion)}$ and ZnONP$_{(ion)}$, and the corresponding metal salts was conducted, respectively. Similar LC50 values of the CuNP$_{(ion)}$ and $Cu(NO_3)_2$ and of the ZnONP$_{(ion)}$ and $Zn(NO_3)_2$ were obtained. Our results indicate that using $Cu(NO_3)_2$ and $Zn(NO_3)_2$ to substitute for CuNP$_{(ion)}$ and ZnONP$_{(ion)}$ is effective. Furthermore, the results of assessment of the relative contribution to mortality revealed that the CuNP$_{(ion)}$ and ZnONP$_{(ion)}$, were not the major source of acute toxicity of CuNPs and ZnONPs. Similarly, Li and Wang (2013) also concluded that the toxicity of ZnONPs to *D. magna* cannot only be attributed to the ZnONP$_{(ion)}$ and Santo et al. (2014) even found that the ZnONP$_{(ion)}$ did not make any contribution to the toxicity of ZnONPs to *D. magna*. However, there are also studies which attributed the toxicity of metal-based or metaloxide-based NPs to the NP$_{(ion)}$. For instance, Adam et al. (2014a) concluded that the toxicity of ZnONPs to *D. magna* can be largely attributed to the NP$_{(ion)}$ rather than the NP$_{(particle)}$; Jo et al. (2012) reported that the dissolved Cu released from CuONPs largely contributed to the observed acute toxicity to *D. magna*. The different characteristics of NPs and exposure conditions, such as the size of NPs (Lopes et al., 2014) or the pH value of the exposure medium (Bian et al., 2011), may lead to the apparent discrepancy. Thus, more toxicity tests involving a wider range of NPs, exposure conditions, and model organisms, should be conducted in the future. Thereupon, a more explicit description of the test condition is needed.

2.4.3 Accumulation characteristics of CuNPs and ZnONPs

While the mechanisms underlying the CuNPs and ZnONPs mediated toxicity in *D. magna* are poorly known, a significant accumulation of CuNPs and ZnONPs in daphnids was observed in this study. The concentrations of Cu and Zn detected in daphnids were quite high (up to 0.1%). These findings are similar to but lower than the maximum body burdens observed for uptake studies with *D. magna* for carbon nanotubes (6.8%, Petersen et al., 2009), fullerenes (0.7%, Pakarinen et al., 2013) and graphene (0.7%, Guo et al., 2013). Furthermore, the accumulation results display that the internal concentrations of Cu and Zn were proportional to the concentrations of CuNPs and ZnONPs administered in this study (Figure 2.4). Actually *D. magna* are filter feeders, enabling them to ingest particles smaller than the size of 70 μm (Geller and Müller, 1981). In our study, both CuNPs and ZnONPs aggregates were far smaller than 70 μm (median particle size about 1 μm), thus they could be readily taken up by daphnids. On the other hand, the aggregation of CuNPs and ZnONPs to approximately 1 μm makes sedimentation likely. There is evidence that sediment particles are ingested by daphnids (Petersen et al., 2009; Lee et al., 2012), but the accumulating rate might be different to suspended particles (Tervonen et al., 2010), and furthermore the adsorption to daphnids' carapace and appendages might also be different to dispersions if the particles are applied as large aggregates and agglomerates (Lee et al., 2012). Therefore also the size of NPs' aggregates might be of importance in the accumulation study. At the high concentration of nanoparticle suspensions applied, both $\mathrm{CuNP_{(ion)}}$ and $\mathrm{ZnONP_{(ion)}}$ contributed only to a limited extent to the accumulation of Cu and Zn, respectively (Figure 2.4). These results indicate that $\mathrm{NP_{(particle)}}$ play a dominant role in the accumulation process at the high concentration of CuNPs and ZnONPs, in line with the conclusion obtained from the toxicity assessment of the $\mathrm{NP_{(particle)}}$ exposed to neonates. This dominant role of $\mathrm{NP_{(particle)}}$ in accumulation process might be reflected in the following manifestations. *D. magna* ingested metals in the particle form more than in the ion form in this study; $\mathrm{NP_{(particle)}}$ existing in nanoparticle suspensions may facilitate the intake of $\mathrm{NP_{(ion)}}$ by adsorbing metal ions on their surface areas (Tan et al., 2012); it also might be that $\mathrm{NP_{(particle)}}$ became predominately packed in organism gut tract; there was adsorption of $\mathrm{NP_{(particle)}}$ to the outer shell of daphnids partly; finally, all the above manifestations could coexist. However, which manifestation is the major reason that results in the leading role of $\mathrm{NP_{(particle)}}$ requires further research. At the low concentration of the nanoparticle suspensions applied in this study (namely 0.05 mg/L CuNPs and 0.5 mg/L of ZnONPs), both the relative contributions to accumulation of $\mathrm{CuNP_{(ion)}}$ and $\mathrm{ZnONP_{(ion)}}$ increased to around 50% (Table 2.3). The different contribution to accumulation of $\mathrm{NP_{(ion)}}$ might be caused by the different dissolubility of NPs. At a low particle concentration, the proportion of dissolved NPs tends to be higher, whereas it tends to be lower at a high particle concentration (Hua et al., 2014a and 2014b; Mwaanga et al., 2014). Thus, a higher proportion of $NP_{(ion)}$ might be ingested by organisms at a lower concentration of NPs than at a higher concentration of NPs. Consequently, when the concentrations of CuNPs and ZnONPs were below 0.05 mg/L and 0.5 mg/L, respectively, $NP_{(ion)}$ were predominantly accumulated. The results indicate that at a low concentration of nanoparticle suspensions, ions released from metallic nanoparticles like Cu and ZnO are overshadowing the effects of NPs.

2.5 Environmental implications

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

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Supplementary information

Supplementary information 2.1

To analyze whether there were still $NP_{(particle)}$ remaining in the supernatants after centrifugation, we firstly conducted an ultracentrifugation experiment. Specifically, ultracentrifugation of nanoparticle suspensions was performed at 192700 g for 30 min at 4 °C (Kontron Centrikon T-2070, TFT 50.38 rotor). Subsequently, the dissolved ion concentration in the supernatants prepared by the two methods of centrifugation was compared. If there was no significant difference of dissolved ion concentration in the supernatants obtained by regular centrifugation and ultracentrifugation, it was to be concluded that regular centrifugation can be applied in this study and $NP_{(particle)}$ may be completely removed in the supernatant after centrifugation, on the other hand if there was a significant difference, indeed $NP_{(particle)}$ would be remaining in the supernatants after regular centrifugation and ultracentrifugation should replace regular centrifugation to be employed in this study. After comparing, it was found that there was no significant difference between the ion concentration in the supernatants disposed by the two different centrifugation methods (Figure S2.1), which means that the supernatants obtained by centrifugation at around 30000 g and subsequent filtration through a syringe filter with 0.02 μm pore diameter, may only contain $NP_{(ion)}$. Furthermore, besides comparing the ion concentration by means of ICP-OES in the supernatants after regular centrifugation and ultracentrifugation, DLS was used to confirm $NP_{(particle)}$ were removed in the supernatants after filtration. From Figure S2.2, it is clear that the particle profile of the cultural media (STM) was similar to the profiles of the supernatants of CuNPs and ZnONPs. Moreover, the polydispersity indexes (PDI) of the supernatants of the STM, CuNPs and ZnONPs were very high (higher than the recommended PDI values for the DLS measurement: 0-0.7) and the count rates of them were very low (lower than the recommended count rates for the DLS measurement: 100-500 kcps), which did not meet the quality criteria of the DLS test. These results indicate that the particle concentration in the supernatants obtained by centrifugation at around 30000 g and subsequent filtration through a syringe filter with 0.02 μm pore diameter was less than the combined detection limit of the ICP-OES and DLS of 0.01 mg/L.

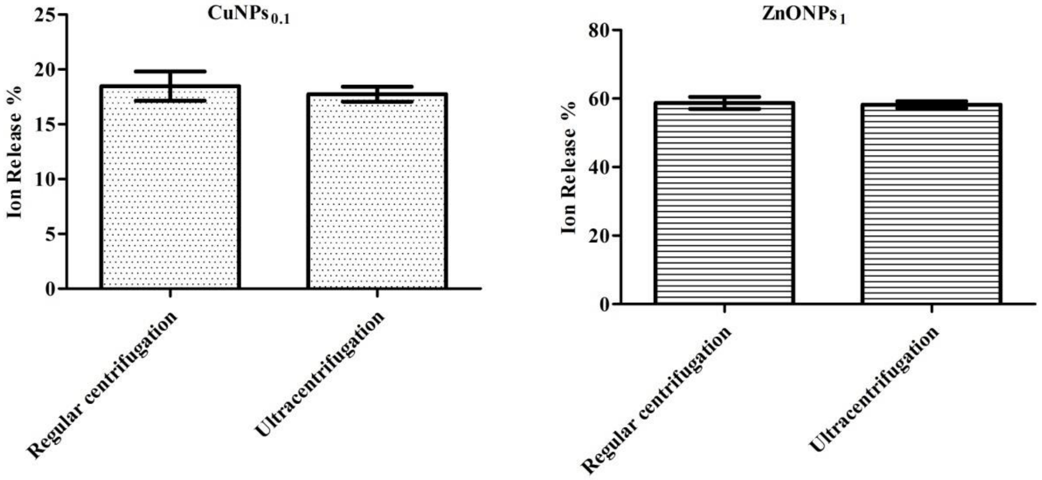

Figure S2.1 The comparison of the ion release profiles in the supernatants obtained by regular centrifugation for 30 min at 4 °C with 30392 g and ultracentrifugation for 30 min at 4 °C with 192700 g, respectively. The left graph exhibits the relative percentage of dissolved Cu released from the CuNPs at the concentration of 0.1 mg/L and the right graph displays the relative percentage of dissolved Zn released from the ZnONPs at the concentration of 1 mg/L. Results are expressed as mean ± SD (*n* = 3).

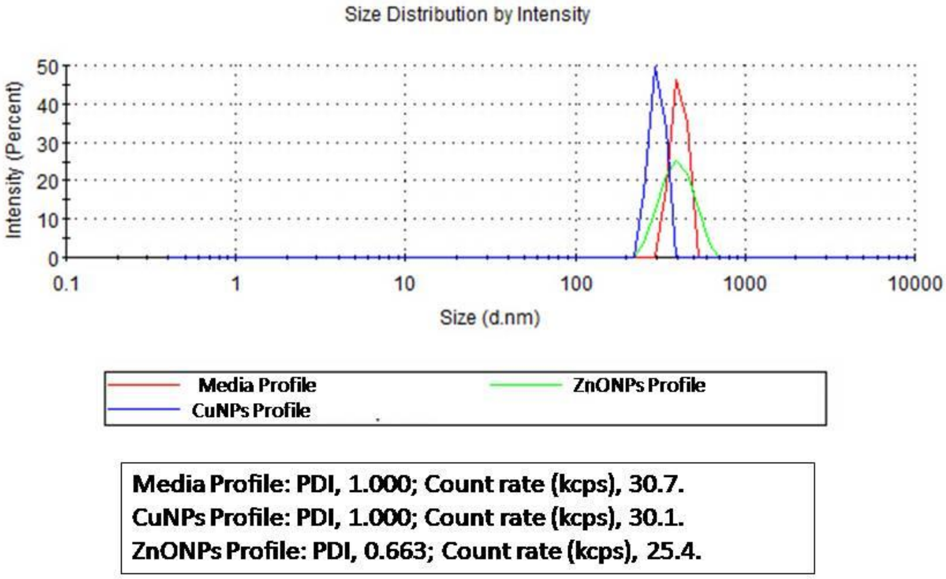

Figure S2.2 Dynamic light scattering data for particle profiles of the supernatants of STM, CuNPs and ZnONPs. As control, the particle profile of the supernatant of the cultural media (STM) obtained by centrifugation at 30392 g and subsequent filtration through a syringe filter with 0.02 μm pore diameter has been measured first, which was shown in the figure with red line. Then the particle profiles of the supernatants of CuNPs and ZnONPs also obtained by centrifugation at 30392 g and subsequent filtration through a syringe filter with 0.02 μm pore diameter were represented by blue and green lines, respectively.

Supplementary information 2.2

During the procedures of centrifugation and filtration, NPs and $NP_{(ion)}$ may be adsorbed to the sidewalls of centrifuge tubes or on the filter membrane. To analyze the losses of NPs and $NP_{(ion)}$ after centrifugation and filtration, experiments were conducted. Specifically, freshly prepared nanoparticle suspensions were sampled and the nanoparticle concentration in the samples was detected by ICP-OES after digestion in 65% HNO_3 for at least 1 day. Moreover, to compare the difference of nanoparticle concentration before and after centrifugation, the nanoparticle concentration after centrifugation at 30392 g for 30 min at 4 °C was also tested by ICP-OES after digestion in 65% HNO_3. The difference of $NP_{(ion)}$ concentration before and after filtration was also detected. After centrifugation at 30392 g for 30 min at 4 °C, Concentration of $NP_{(ion)}$ in the supernatant was detected by ICP-OES. Furthermore, supernatants obtained by centrifugation at 30392 g for 30 min at 4 °C were filtered through a syringe filter with 0.02 μm pore diameter (Antop 25, Whatman). Subsequently, the concentration of $NP_{(ion)}$ in the supernatants obtained by centrifugation and subsequent filtration through a syringe filter with 0.02 μm pore diameter was tested. The results were shown in Figure S2.3. It reported that there indeed exist decrease of nanoparticle concentration after centrifugation and decrease of dissolved ion concentration after filtration. However, the difference of the ZnONPs concentration before and after centrifugation and of the dissolved Zn concentration before and after filtration, as well as of the CuNPs concentration before and after centrifugation and of the dissolved Cu concentration before and after filtration was not significantly different ($p > 0.05$, the *p*-value between different groups was tested by mean of the *t*- test).

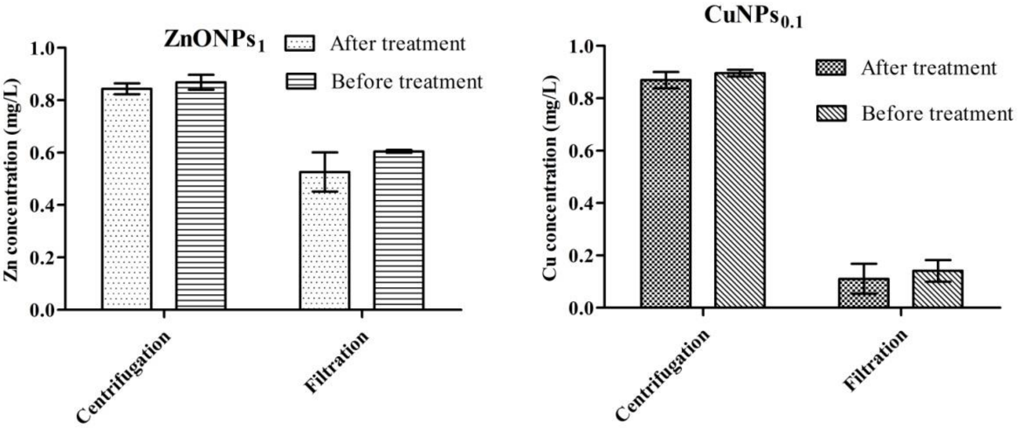

Figure S2.3 The losses of NPs and $NP_{(ion)}$ after centrifugation and filtration. The left graph shows the concentration of ZnONPs at 1 mg/L before and after centrifugation and the concentration of dissolved Zn released from ZnONPs at 1 mg/L before and after filtration. The right graph exhibits the concentration of CuNPs at 0.1 mg/L before and after centrifugation and the concentration of dissolved Cu released from CuNPs at 0.1 mg/L before and after filtration. Results are expressed as mean ± SD ($n = 3$).

Chapter 3 Toxicity of copper nanoparticles to *Daphnia magna* under different exposure conditions

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Abstract

Although the risks of metallic nanoparticles (NPs) to aquatic organisms have already been studied for more than 10 years, our understanding of the link between the fate of particles in exposure medium and their toxicity is still in its infancy. Moreover, most of the earlier studies did not distinguish the contribution of particles and soluble ions to the toxic effects caused by suspensions of metallic NPs. In this study, the toxicity of CuNPs to *Daphnia magna* upon modification of the exposure conditions, achieved by aging the suspensions of CuNPs and by altering water chemistry parameters like the pH and levels of dissolved organic carbon (DOC), was investigated. The LC50 values for CuNPs exposure decreased by about 30 % after 7 days of aging. The LC50 values increased by more than 12-fold upon addition of DOC at concentrations ranging from 0 to 10 mg/L to the exposure medium. Changing the pH from 6.5 to 8.5 resulted in a 3-fold higher LC50 value. Furthermore, it was found that during 7 days of aging of the exposure medium (without addition of DOC and at pH 7.8), the toxicity could be mostly ascribed to the particles present in the suspension (around 70%). However, adding DOC or decreasing the pH of the exposure medium reduced the contribution of the particles to the observed toxicity. Thus, we found that the effective concentration regarding the toxicity was mainly driven by the contribution of the soluble ions in the presence of DOC or at pH 6.5. Our results suggest that the toxicity results of CuNPs obtained from laboratory tests may overestimate the risk of the particles in polluted waters due to the common absence of DOC in laboratory test solutions. Moreover, the role of the ions shedding from CuNPs is very important in explaining the toxicity in natural waters.

Key words: copper nanoparticles; water chemistry; acute toxicity; fate; *Daphnia magna*

3.1 Introduction

The toxicity of metallic nanoparticles (NPs) has been studied quite intensively during the last few years, as evidenced by a large number of publications on this topic (Chen et al., 2015). Most of the earlier studies focused on the influence of the physicochemical properties of metallic NPs, such as size, shape and surface coating, on their toxicity. Yet, conflicting and inconsistent results on the factors determining toxicity are commonly reported. For example, Hoheisel et al. (2012) observed that the toxicity of Ag NPs to *Daphnia magna* increased with decreasing particle size in Lake Superior (St, Louis County, MN, USA) water with low to moderate ionic strength and dissolved organic carbon (DOC) no greater than 2 mg C/L, whereas Li et al. (2010) found that there was no reletion between size of Ag NPs and toxicity to *D. magna* in standard synthetic freshwater prepared according to the EPA protocol. Once being released into the aquatic environment, NPs in fact are subject to environmentally driven modifications (e.g., dissolution, aggregation and sedimentation) over time (referred to as aging hereafter), which may affect the bioavailability of ions shedding from the metallic NPs (designed as $NP_{(ion)}$ hereafter) and particles themselves (indicated as $NP_{(particle)}$ hereafter) and then their toxicity to specific organisms (Lin et al., 2012; Sørensen and Baun, 2015). It has been reported that exposure conditions including the aging time (e.g., freshly prepared or aged suspensions) and water chemistry parameters like the pH and DOC levels of the aqueous environment can affect the process of modifications of NPs (Gao et al., 2012; Li et al., 2013; Cupi et al., 2015). Thus, an emphasis on the effects of the exposure conditions on the fate and toxicity of NPs is needed to gain a better understanding of the risk of metallic NP in natural water systems.

To date, it is generally recognized that both $NP_{(ion)}$ as well as $NP_{(particle)}$ potentially contribute to the overall toxicity observed for organisms following exposure to suspensions of metallic NPs (Adam et al., 2014a; Hua et al., 2014a; Song et al., 2015). However, most of the existing studies have not separated the contributions of $NP_{(ion)}$ and $NP_{(particle)}$ to the toxic effects caused by suspensions of metallic NPs, let alone that they have investigated the influence of the exposure conditions on the relative contribution of $NP_{(ion)}$ and $NP_{(particle)}$ to the observed toxicity caused by metallic NP suspensions. Thus, the issue which form, $NP_{(ion)}$ or $NP_{(particle)}$, is the major source of the effective concentration that is actually causing the toxicity of metallic NPs is still under debate.

CuNPs were reported to be highly toxic to a wide range of aquatic organisms (Smith et al., 2014; Xiao et al., 2015), whereas the information concerning the toxic action of CuNPs in water systems under different exposure conditions (e.g., under different aging time as well as at different pH and DOC levels) is far less available compared to other types of metal-based NPs, such as Ag, TiO_2 and ZnO NPs. The aim of this study was to determine the fate and toxicity of CuNPs to *D. magna* upon modification of the exposure conditions. Furthermore, the relative contribution of ions released from CuNPs and of particles themselves in inducing toxic effects to *D. magna* upon modification of the exposure conditions was investigated. The modification of the exposure conditions in this study was achieved by storing the NP suspensions for different time periods, by changing the levels of DOC in the exposure solutions, and by changing the pH of the exposure medium in the range found in natural freshwaters.

3.2 Materials and methods

3.2.1. Test organisms

Daphnia magna, originally obtained from the Dutch National Institute for Public Health and the Environment (RIVM), was selected as the test species. According to the OECD guideline 202, *D. magna* were cultured in plastic containers with Artificial ElendtM4 medium at a density of 1 individual/10 ml of ElendtM4 medium (OECD, 2004). The culture medium was refreshed three times a week. The test organisms were fed with *Pseudokirchneriella subcapitata* every two days and maintained inside a controlled-temperature chamber under a 16:8 light-dark cycle (20 ± 1 °C).

3.2.2. Preparation and characterization of the exposure CuNP suspensions

Spherical CuNPs (purity, 99.8%) were purchased from Iolitec GmbH with an advertised primary size of 50 nm and a specific surface area of 6-8 m^2/g. ISO standard testing medium (STM) recommended by OECD was used to prepare CuNP suspensions. The STM applied in this study (pH 7.8 ± 0.2) contained (mg/L MilliQ water): $CaCl_2 \cdot 2H_2O$: 294; $MgSO_4 \cdot 7H_2O$: 123.25; $NaHCO_3$: 64.75; KCl: 5.75. The morphology and size of CuNPs in STM were assessed by transmission electron microscopy (TEM, JEOL 1010, JEOL Ltd., Japan) and are shown in Figure S3.1. To monitor the change of toxicity of CuNP suspensions towards *D. magna* within 7 days after preparation of suspensions of CuNPs in STM, a stock suspension (nominal 100 mg/L) was prepared after 30 min of sonication in a water bath sonicator. Thereafter, several identical sets of particle suspensions were prepared immediately by a series of dilution from the stock CuNP solution with STM and subsequently stored undisturbed in a climate chamber at 20 ± 1 °C under a 16 h light:8 h dark photoperiod cycle. Each set of exposure suspensions consisted of a series of actual exposure concentrations that ranged from 36 to 216 μg/L and a control. The application of the exposure concentration range was based on a preliminary range finding test. Meanwhile, 7 groups of suspension of 1 mg/L of CuNPs with three replicates were prepared, which were employed for determining the change of physicochemical properties of CuNP suspensions during storage process. Specifically, the size distribution of CuNPs at different sampling time points (i.e., immediately after preparation, which we will refer to as 0 d, and after 1, 2, 3, 4, 7 and 9 d of preparation) was determined by dynamic light scattering (DLS) on a Zetasizer Nano-ZS instrument (Malvern, Instruments Ltd., UK). At the same point, the zeta potential of each suspension was determined by ZetaPALS software based on the Smoluchowski equation. Meanwhile, to quantify the amount of Cu ions remaining in the water column released from CuNPs at 0.1 mg/L during 9 d of aging, a sample with three replicates was obtained by pipetting 15 ml of solution from the position around 3 cm below the surface (the volume of the solution in the beaker was 100 ml). The sample was subsequently centrifuged at 30392 g for 30 min at 4 °C (Sorvall RC5Bplus centrifuge, Fiberlite F21-8 × 50y rotor) to obtain the supernatant. After centrifugation, the supernatant was filtered through a syringe filter with 0.02 μm pore diameter (Anotop 25, Whatman). The supernantant was digested by 69% HNO_3 at room temperature for at least 1 d and the concentration of Cu in the supernatant was then determined by inductively coupled plasma optical emission spectrometry (ICP-OES).

To investigate the influence of pH on the fate and toxicity of CuNPs in media, a series of exposure media were prepared by diluting the stock CuNP suspension (nominal 100 mg/L), after which the pH levels of the exposure medium were adjusted to 6.5 and 8.5, respectively, with 0.1 M NaOH or 0.1 M HCl. To maintain the pH value of the exposure medium constant during 48 h exposure, the pH of the exposure medium was adjusted every 24 h with 0.1 M NaOH or 0.1 M HCl. The application of the pH range from 6.5 to 8.5 (namely, 6.5, 7.8 and 8.5) was because they are commonly found in freshwaters (Vijver et al., 2008), which is suited for healthy, diverse, and productive fish and macroinvertebrates communities (Hirst et al., 2002). The physicochemical analysis and characterization of CuNPs in STM at pH 6.5 and 8.5, including the size distribution and zeta potential and dissolution results during 48 h, were also conducted by the above mentioned methods.

In addition, the effect of DOC on the fate and toxicity of CuNPs in the exposure solution was also explored. Suwannee River humic acid II (SRHA) was used as a model DOC in this study. A SRHA stock solution was prepared by dissolving SRHA in 0.002 N NaOH in deionized water. The SRHA solution was then stirred overnight and filtered through a 0.2 µm cellulose acetate membrane and stored at 4 °C. The total organic carbon (TOC) content in the prepared stock solution was measured by a TOC analyzer (TOC-VCPH, Shimadzu Corporation). In order to determine the influence of DOC on the physicochemical properties of CuNPs, CuNPs were suspended in STM in the presence of DOC by diluting the SRHA stock solution to obtain the four desired DOC concentrations (i.e., 0.5, 2, 5 and 10 mg DOC/L). All of the suspensions were adjusted to pH 7.8 ± 0.2 with 0.1 M HCl or 0.1 M NaOH. Then, the suspensions were sonicated for 30 min. After 24 and 48 h of equilibration, the size distribution and zeta potential of the CuNP suspensions with addition of DOC were determined by the above mentioned methods. Meanwhile, the influence of DOC on dissolution of CuNPs was also tested. CuNPs at 0.1, 1 and 10 mg/L were suspended into STM with addition of DOC at the above mentioned concentrations (i.e., 0.5, 2, 5 and 10 mg DOC/L). There were three replicates in each treatment. The suspensions were subsequently sonicated for 30 min. After 48 h of equilibration, the amount of Cu ions in the water column was analyzed by the above mentioned methods.

3.2.3. Acute toxicity test

The acute toxicity test was carried out according to OECD Guideline 202. In the aging effect assay, at each sampling time point (i.e., 0, 1, 2 and 7 d), neonates (< 24 h) were selected and then exposed to the prepared exposure medium for 48 h. The exposure suspensions were prepared in the way as described above. Similarly, in the pH and DOC effect assay, neonates of age less than 24 h were selected and then exposed to the exposure medium with different pH and DOC levels. In addition, to investigate the toxic effects of Cu ions released from the CuNPs, $Cu(NO_3)_2$ was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) and the acute toxicity of $Cu(NO_3)_2$ to daphnids under varying DOC and pH levels (i.e., 0, 0.5, 2, 5 and 10 mg/L of DOC and at pH 6.5, 7.8 and 8.5) was also determined as a positive control. Before starting all the toxicity tests, neonates were kept in STM for 1-2 h to evacuate their guts. Each exposure medium contained 5 daphnids, which included 4 replicates. During the 48 h acute toxicity test, daphnids were incubated under a 16:8 h light/dark photoperiod (20 ± 1 °C) without feeding. After the 48 h toxicity testing, the mortality of the daphnids in each suspension was recorded.

3.2.4. Data analysis and statistics

Data are expressed as the mean with the corresponding standard deviation (SD). The LC50 values and 95% confidence intervals (95% CI) were calculated using GraphPad Prism 5. In order to analyze the speciation of dissolved Cu in STM at pH 6.5, 7.8 and 8.5, the software of Visual MINTEQ 3.1 was used, which was downloaded from http://vminteq.lwr.kth.se/ and used without modification. Statistical analyses were conducted using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons (SPSS 16.0, SPSS Inc., Chicago, IL, USA) to compare the influence of DOC and pH levels and aging time on the dissolution of CuNPs. The significantly different level in all calculations was set at $p < 0.05$.

Moreover, in order to determine the major source of effective concentration upon modification of the exposure conditions, the toxic contribution of Cu ions shedding from CuNPs and the particulate Cu under different exposure conditions was calculated. Generally, the nanoparticle suspension is a mixture of soluble ions released from NPs and particles. The precise mechanisms of toxicity of metal ions versus metal-based NPs are not fully understood, but some recent studies have reported that likely the mode of action of metal ions differed from that of the metal-based NPs (Poynton et al., 2012; Ivask et al., 2014; Boudreau et al., 2016). Hence, we assumed that the modes of action of CuNP$_{(particle)}$ and CuNP$_{(ion)}$ were likely dissimilar. In this circumstance, the response addition model was employed in this study. The response addition model (Backhaus et al., 2000), is as follows:

$$E_{(total)} = 1 - \left[\left(1 - E_{(ion)}\right)\left(1 - E_{(particle)}\right)\right] \tag{3.1}$$

Where $E_{(total)}$, $E_{(ion)}$ and $E_{(particle)}$ represent the toxic effects caused by the nanoparticle suspensions, their corresponding released ions and the corresponding particulate Cu (scaled from 0 to 1), respectively. In the present study, $E_{(total)}$ was measured experimentally; $E_{(ion)}$ was calculated according to the concentration-response curve of $Cu(NO_3)_2$ towards *D. magna*. This makes $E_{(particle)}$ as the only unknown, allowing for direct calculation of the effects caused by the NP$_{(particle)}$.

3.3 Results and discussion

3.3.1 Effects of exposure conditions on the fate of CuNPs

The physicochemical properties of CuNPs were investigated in order to better understand the fate of CuNPs in the exposure medium under different exposure conditions. The DLS results showed a decrease in absolute zeta-potential value of the CuNP suspensions from around -11 mV after being freshly prepared to about -3 mV after being stored for 9 d in STM (Figure 3.1A). The decrease of the absolute zeta-potential value may be due to the adsorption of divalent cations present in the STM onto the surface of the CuNPs (Son et al., 2015). The zeta-potential value indicates that the CuNPs in STM upon storage was unstable and tended to aggregate to a larger extent. This indication was supported by the observed increase of hydrodynamic diameter of CuNPs in STM during storage (Figure 3.1B). The CuNPs in STM aggregated rapidly to around 600 nm once being submerged into the exposure solution (Figure 3.1B). Due to the rapid aggregation of CuNPs in the exposure medium, the CuNPs in the medium presented a rapid sedimentation trend, which was found that the total Cu concentration in the water column decreased as a function of time (Figure 3.2A). The actual Cu concentration in the water column showed a decrease of 43% after 1 day of storage. Thus, the rapid aggregation of CuNPs in STM reduced the bioavailability of CuNPs to daphnids, which may lead to a lower toxicity of CuNPs to daphnids compared to the isolated CuNPs. However, the comparison of the toxic effects to organisms between the aggregate states and the isolated particles was not addressed in this study and needs to be further investigated. In case of the dissolution profiles of CuNPs upon storage, the concentration of Cu-ions increased gradually during storage (expressed as the percentage of the total actual Cu added into the STM, Figure 3.2B). Specifically, the amount of Cu ions increased significantly from 15% after preparation of the suspension (as measured around 1-2 h after submerging CuNPs into the exposure medium, to which we will refer to as 0 d) to 36% after 9 days of storage of the suspensions (one-way ANOVA, $p < 0.05$).

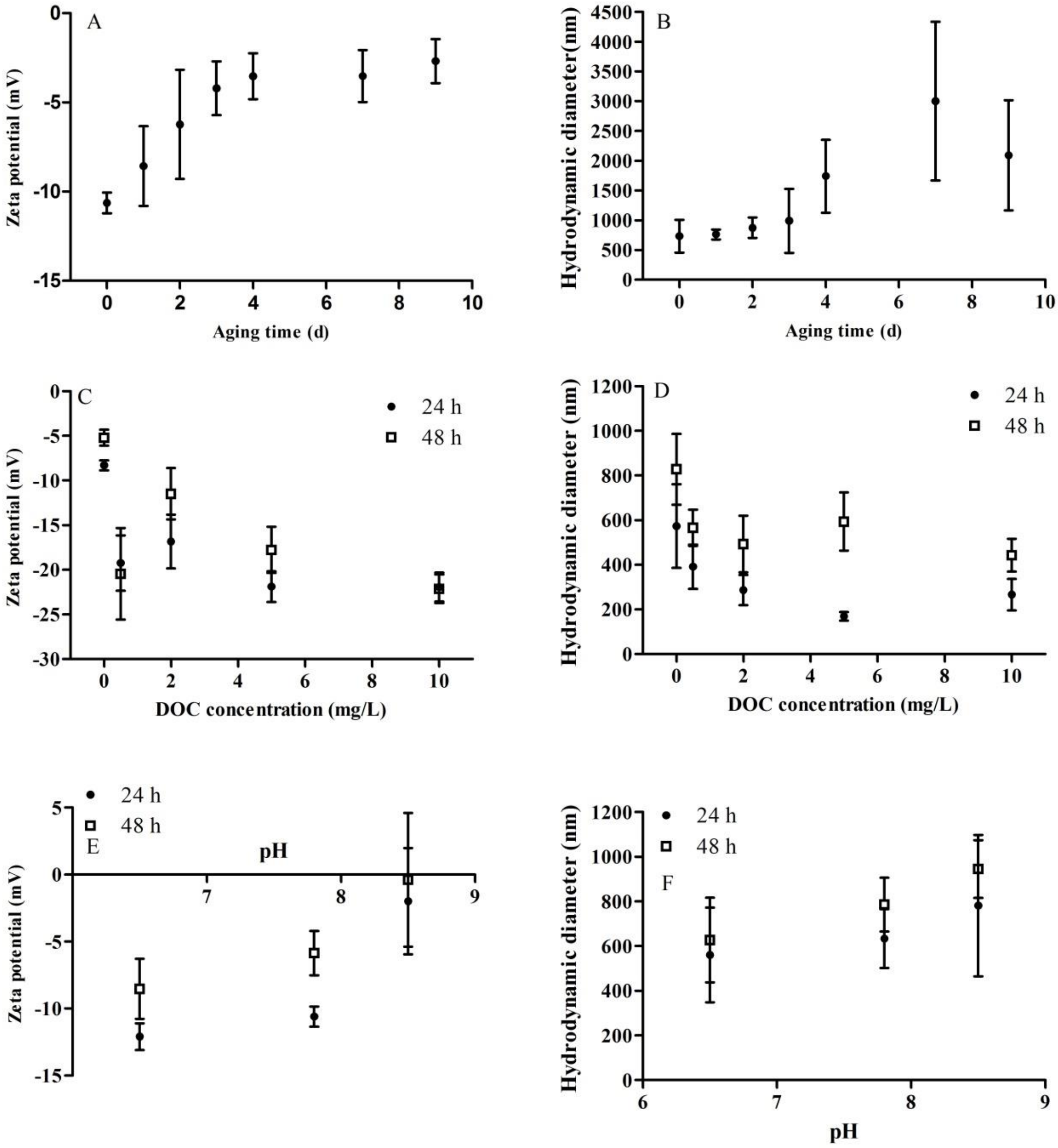

Figure 3.1 Zeta-potential and hydrodynamic diameter of CuNPs incubation in the standard test medium (STM) with aging for up to 9 d without stirring (A and B); zeta-potential and hydrodynamic diameter of CuNPs after 24 and 48 h of incubation in STM with varying DOC and pH levels, respectively (C, D, E and F). The data are expressed as mean ± SD ($n = 3$).

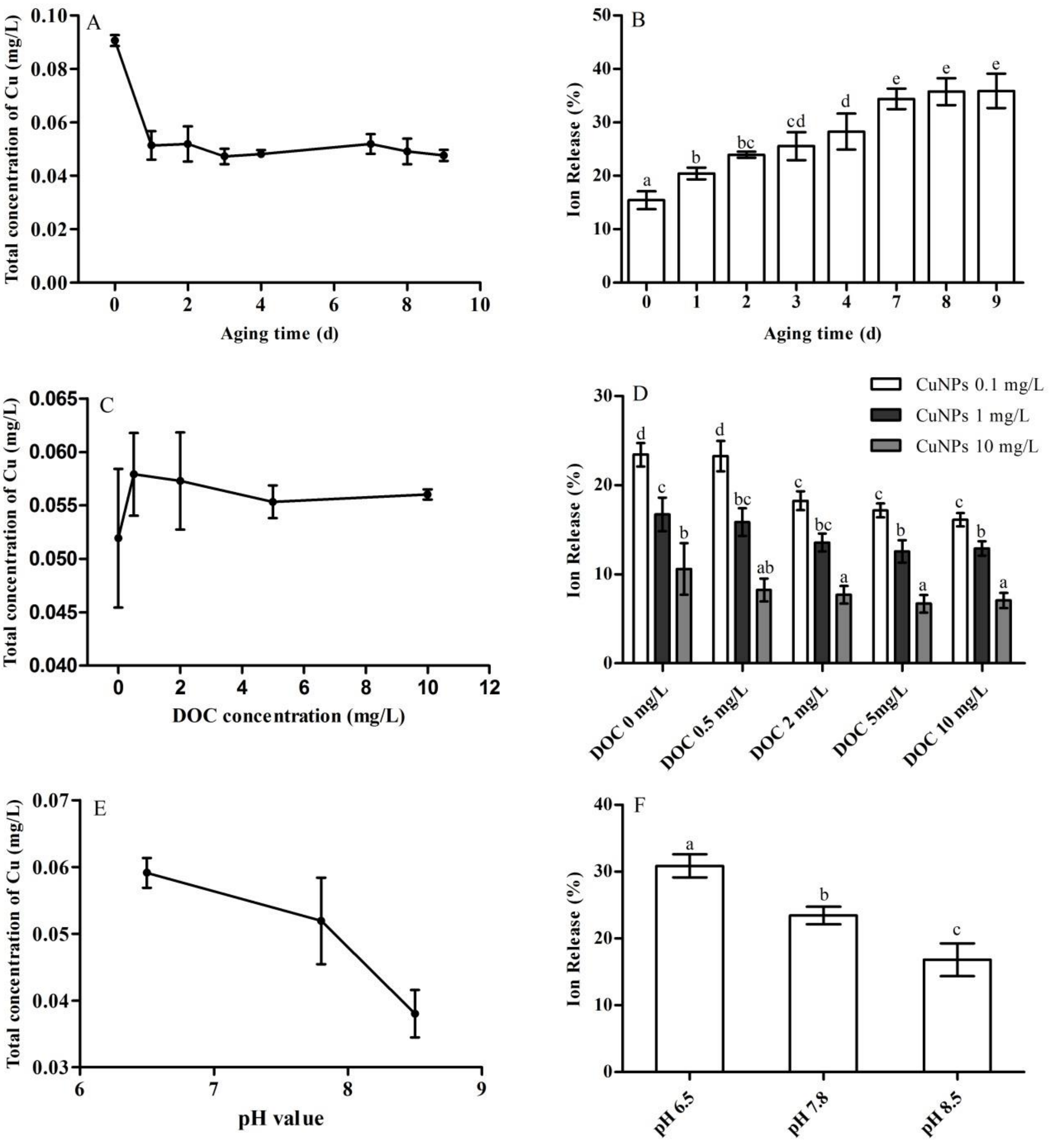

Figure 3.2 Total Cu concentration remaining in the water column during 9 d of aging (A); (B) shows the dissolution profiles of CuNPs (0.1 mg/L) during 9 d of aging in STM without stirring; the total Cu concentration after 48 h of incubation in STM in the presence of DOC (C); (D) presents the amount of ions released from CuNPs at 0.1, 1 and 10 mg/L after 48 h of incubation in STM in the absence and presence of DOC (i.e., 0.5, 2, 5 and 10 mg DOC/L); (E) shows the total Cu concentration after 48 h of incubation in STM at pH 6.5, 7.8 and 8.5; the amount of ions released from CuNPs (0.1 mg/L) after 48 h of incubation in STM at pH 6.5, 7.8 and 8.5 (F). The data are expressed as mean ± SD ($n = 3$). The different letters in graphs of B, D and F indicate the significant difference of the amount of ions expressed as the percentage of the total Cu added into the exposure medium (i.e., STM).

Besides investigating the fate of CuNPs in the exposure medium as a function of time, the effect of water chemistry (e.g., DOC and pH values in the exposure solution) on the fate of CuNPs was also studied. In line with the results of other studies (Fabrega et al., 2009; Rosenfeldt et al., 2015), we found that the absolute zeta-potential value of CuNPs increased, whereas the hydrodynamic diameter of the particles in the exposure solution decreased in the presence of DOC (Figure 3.1C and D). These results indicate that the stability of CuNPs in the exposure medium increased upon addition of DOC. The increased stability of CuNPs in the exposure medium may increase the amount of CuNPs dispersed in the exposure medium. The assessment of the concentration of Cu after 48 h of incubation remaining in STM supports this conclusion (Figure 3.2C). In order to understand the influence of DOC on the dissolution profiles of CuNPs, the amount of Cu ions released from CuNPs at 0.1, 1 and 10 mg/L after incubation in STM for 48 h with addition of an increasing concentration of DOC was investigated. The results are presented in Figure 3.2D. It is clear that the percentage of soluble Cu (expressed as % of the total Cu) increased with decreasing concentrations of CuNPs in STM, which was in agreement with the results reported by Hua et al. (2014b), indicating that the concentration of CuNPs is an important factor in determining the dissolution extent. Furthermore, it is worth to note that the presence of DOC (for up to 10 mg DOC/L) did not strongly change the amount of Cu ions released from CuNPs at all the three CuNPs concentrations tested (i.e., 0.1, 1 and 10 mg/L), even though the percentage of soluble Cu in STM without addition of DOC was significantly higher than that with addition of DOC of concentrations higher than 2 mg DOC/L (one-way ANOVA, $p < 0.05$). Similarly, Gao et al. (2012) found that SRHA did not strongly impact the amount of ionic Ag released from Ag NPs (with a nominal size of 20-30 nm) after being exposed in STM for 2 days. On the one hand, DOC increased the stability of CuNPs in STM leading to a higher chance to release more soluble Cu-ions from the surface of CuNPs. On the other hand, DOC may inhibit the dissolution by blocking the oxidation sites of NPs (Dubas et al., 2008), and by eliminating the superoxide for its further oxidation of metal-based NPs (Goldstone and Voelker, 2000). The net effect of the opposite processes suggested above may result in the findings that the dissolution of CuNPs in the presence of DOC did not change strongly in this study.

It was found that the absolute zeta-potential value decreased in this study and the hydrodynamic diameter increased with an increase in pH value in STM (Figure 3.1E and F), indicating a lower stability of CuNPs in exposure medium with a higher pH value. The surface charge of CuNPs at pH 8.5 was very close to zero (Figure 3.1E), implying that the point of zero charge (PZC) of CuNPs in STM is located around pH 8.5. pH is considered as a main factor influencing aggregation behavior of NPs through posing effects on the surface charge of NPs. Particles tend to aggregate most when the pH approaches the PZC (Adam et al., 2014b). Previous studies reported that the PZC values of Cu-based NPs were situated at pH from 7.9 to 10 (Li and Chang, 2004; Guedes et al., 2009; Sousa et al., 2013). The zeta-potential of CuNPs at pH 8.5 found in our study confirmed this conclusion. On the other hand, the percentage of dissolution of CuNPs at lower pH was significantly higher (one-way ANOVA, $p < 0.05$), and was 31%, 23% and 17% at pH 6.5, 7.8 and 8.5 after incubation in STM for 48 h, respectively (Figure 3.2F). The higher dissolution and stability of CuNPs in the exposure medium at a lower pH resulted in the observed result of a higher amount of Cu remained in the water column after 48 h of incubation (Figure 3.2E).

3.3.2 Effects of exposure conditions on the toxicity of suspensions of CuNPs

To date, most published literatures concerning the risks of NPs have focused on the effects of freshly prepared NPs (i.e., pristine NPs). In fact, the pristine NPs generally are structurally and chemically different from their aged counterparts (Valsami-Jones et al., 2015), which therefore may cause different toxicity to organisms. In our study, we found that the 48 h LC50 values of CuNPs decreased with increasing aging days, indicating that the toxicity of suspensions of CuNPs increased gradually during 7 days of aging (Figure 3.3). Specifically, the LC50 value of the freshly prepared CuNPs was 0.093 mg/L (with a 95% CI: 0.086-0.101 mg/L), while after 1, 2, and 7 days of aging, the LC50 values were 0.089 mg/L (with a 95% CI: 0.078-0.102 mg/L), 0.070 mg/L (with a 95% CI: 0.059-0.084 mg/L) and 0.060 mg/L (with a 95% CI: 0.049-0.074 mg/L), respectively (Table S3.1). The increased toxicity of suspensions of metal-based NPs with aging found in our study was in line with some other researches. For example, Kittler et al. (2010) found that the toxicity of citrate-stabilized and poly (vinylpyrrolidone)-stabilized Ag NPs to human mesenchymal stem cells in ultrapure water increased during 6 months of aging and the authors ascribed the increased toxicity with time to the increased amount of ions released from Ag NPs. In our study, an increased amount of Cu ions in the exposure medium over time was also found (Figure 3.2B). On the other hand, the CuNPs aggregated more as the hydrodynamic diameter increased with time (Figure 3.1B). It has been reported that the aggregates of NPs could inhibit the availability of NPs for *D. magna* (Oleszczuk et al., 2015). Thus, the increased toxicity of CuNP suspensions with aging in our study may be attributed to the increased amount of Cu ions in the suspension.

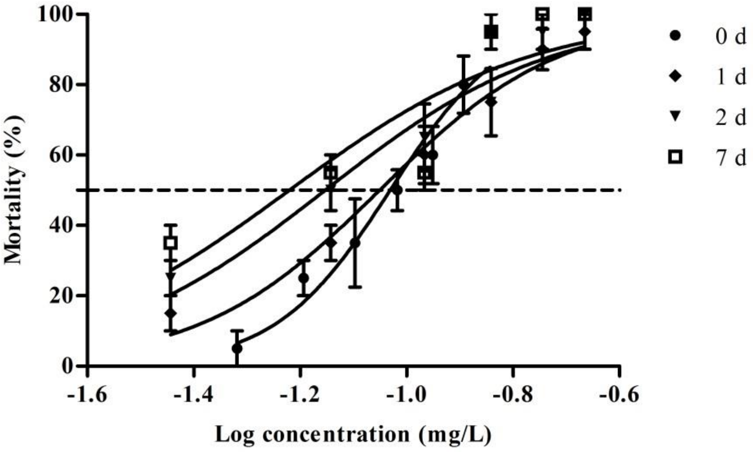

Figure 3.3 Mortality of *D. magna* neonates in response to 48 h of exposure to different aged CuNP suspensions (i.e., freshly prepared, 1, 2 and 7 day aged). The data are expressed as mean ± SD (*n* = 4).

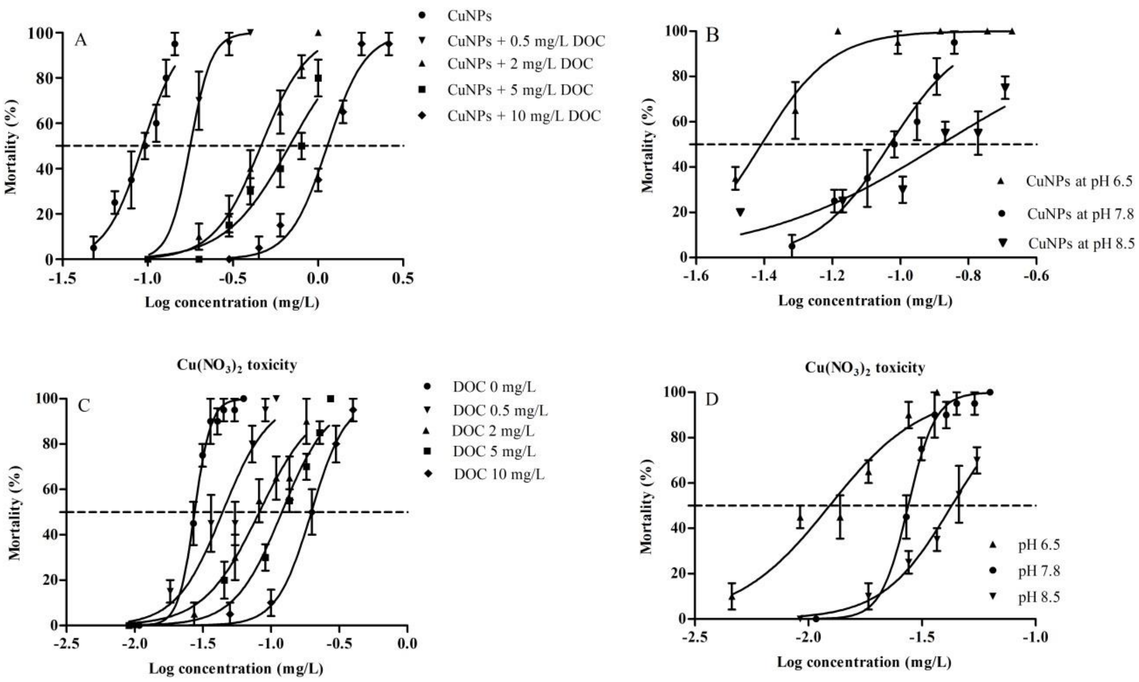

Figure 3.4 Mortality of *D. magna* neonates in response to 48 h of exposure to CuNP suspensions in the presence of DOC at 0, 0.5, 2, 5 and 10 mg/L (A) and at pH 6.5, 7.8 and 8.5 without addition of DOC (B); mortality of *D. magna* neonates in response to 48 h of exposure to $Cu(NO_3)_2$ with addition of DOC at 0.5, 2, 5 and 10 mg/L and without addition of DOC (C); mortality of *D. magna* neonates in response to 48 h exposure to $Cu(NO_3)_2$ at pH 6.5, 7.8 and 8.5 (D). The toxicity data of suspensions of CuNPs and $Cu(NO_3)_2$ to daphnids are expressed as mean ± SD (*n* = 4).

Figure 3.4A presents the influence of DOC on the toxicity of suspensions of CuNPs towards daphnids. It is obvious that the LC50 value of CuNPs strongly increased with increasing DOC concentration in STM. Specifically, the 48 h LC50 value of CuNPs to daphnids was 0.09 mg/L, whereas it increased to 0.18, 0.46, 0.68 and 1.13 mg/L in the presence of DOC at 0.5, 2, 5, and 10 mg DOC/L, respectively (Figure 3.4A and Table S3.2), indicating that the acute toxicity of CuNPs towards daphnids decreased with increasing DOC concentration in the exposure medium. Combined with the finding that the addition of DOC in STM did not strongly impact the dissolution profiles of CuNPs (Figure 3.2D), this suggests that the reduction of toxic potential of CuNPs in the presence of DOC is not caused mainly by the decrease of the amount of Cu ions released from CuNPs.

pH is one of the most important environmental parameters affecting the fate and toxicity of NPs in the environment (Long et al., 2004; Waalewijn-Kool et al., 2013). In the present study, the results about the influence of pH on the toxicity of CuNPs are presented in Figure 3.4B. It is obvious that the LC50 value of suspensions of CuNPs to *D. magna* increased with increasing pH values of the exposure medium (i.e., STM), with 0.039 mg/L (95% CI: 0.036-0.043 mg/L), 0.093 mg/L (95% CI: 0.085-0.101 mg/L) and 0.132 mg/L (95% CI: 0.112-0.154 mg/L) at pH 6.5, 7.8 and 8.5, respectively (Figure 3.4B and Table S3.2). Our results imply that the toxicity of suspensions of CuNPs decreased with increasing pH levels in the exposure medium. This is in line with other studies involved in investigating the effects of pH on toxicity of metal-based NPs (Li et al., 2013; Waalewijn-Kool et al., 2013). Besides influencing the physicochemical characteristics of CuNPs (e.g., dissolution, aggregation and sedimentation of CuNPs, as mentioned above), pH also can influence the speciation of dissolved Cu in the exposure medium (Adeleye et al., 2014). The speciation of dissolved Cu in the exposure medium was calculated using Visual MINTEQ 3.1. The calculation shows that the percentage of free Cu^{2+}-ions among all the species of the total dissolved Cu decreased from 74% at pH 6.5 to merely 2% at pH 8.5, whereas the percentage of $CuOH^+$ was the highest at pH 8.5 (higher than 13%), compared to pH 6.5 (4%) and pH 7.8 (13%) (Figure S3.2). This may also result in the lower toxicity to daphnids observed at pH 8.5, compared to the toxicity at pH 6.5 and 7.8, as the toxicity of Cu^{2+} is higher than that of $CuOH^+$ (de Schamphelaere and Janssen, 2002).

3.3.3 Relative contribution of $\text{CuNP}_{(\text{ion})}$ and $\text{CuNP}_{(\text{particle})}$ to toxicity

As soon as metal-based NPs are within an exposure medium, they tend to release metal ions, whereas the particles aggregate spontaneously and then they may sediment (Zhao et al., 2012; Hua et al., 2014a). This may change the bioavailability of $\text{NP}_{(\text{particle})}$ and $\text{NP}_{(\text{ion})}$ to organisms and subsequently affect the relative contribution of $\text{NP}_{(\text{particle})}$ and $\text{NP}_{(\text{ion})}$ to the overall toxicity caused by metal-based NP suspensions. In the present study, the relative contribution of $\text{CuNP}_{(\text{particle})}$ and $\text{CuNP}_{(\text{ion})}$ upon modification of the exposure conditions to toxicity was analyzed by the response addition model (Equation 3.1). To determine the contribution of $\text{CuNP}_{(\text{ion})}$ to toxicity, the dose-response relationships for $Cu(NO_3)_2$ to *D. magna* under different exposure conditions were analyzed. The LC50 value of $Cu(NO_3)_2$ without addition of DOC at pH 7.8 was 28 μg/L, whereas it decreased to 12 μg/L at pH 6.5 and increased to 43 μg/L at pH 8.5 (Figure 3.4D and Table S3.3). In the presence of DOC, the LC50 values of $Cu(NO_3)_2$ at pH 7.8 showed an approximately 2, 3, 4, and 7 fold increase with addition of

DOC at 0.5, 2, 5, and 10 mg/L, respectively (i.e., 44, 82, 120 and 198 μg/L, Figure 3.4C and Table S3.3), relative to the LC50 value without addition of DOC.

Table 3.1 The relative contribution of $CuNP_{(particle)}$ and $CuNP_{(ion)}$ to toxicity caused by freshly prepared and aged CuNP suspensions at the LC50 values to *D. magna*.

Aging time (d)	Relative contribution to toxicity %	
	$CuNP_{(particle)}$	$CuNP_{(ion)}$
0	75	25
1	64	36
2	85	15
7	74	26

Table 3.2 The relative contribution of $CuNP_{(particle)}$ and $CuNP_{(ion)}$ to toxicity caused by the CuNP suspension under different compositions of the exposure medium at the LC50 values towards *D. magna*.

DOC concentration (mg/L)	pH value	Relative contribution to toxicity %	
		$CuNP_{(particle)}$	$CuNP_{(ion)}$
0	8.5	76	24
0	6.5	5	95
0	7.8	75	25
0.5	7.8	17	83
2	7.8	27	73
5	7.8	38	62
10	7.8	54	46

For the response addition model, at the LC50 values of the CuNP suspensions to *D. magna*, the lethality of the freshly prepared and aged CuNP suspensions could be ascribed over 64% to the $CuNP_{(particle)}$ (Table 3.1). This predominant contribution by $NP_{(particle)}$ was also found by Santo et al. (2014) for ZnO NPs (with a primary size < 50 nm), who reported that ZnO $NP_{(particle)}$ dominated the toxicity of ZnO NPs exposed in STM to *D. magna*. Our results thus conclude that the effective concentration regarding the toxicity of CuNPs to *D. magna* in the exposure medium (pH: 7.8 ± 0.2; without addition of DOC) was dominated by the contribution of $CuNP_{(particle)}$. However, the water chemistry parameters (i.e., DOC and pH levels of the exposure medium) exerted a strong effects on the role of $CuNP_{(ion)}$ and $CuNP_{(particle)}$ in the observed toxicity caused by CuNP suspension (Table 3.2). The relative contribution of $CuNP_{(particle)}$ to toxicity decreased from 76% at pH 8.5 to 5% at pH 6.5; meanwhile, the relative contribution to toxicity of $CuNP_{(particle)}$ decreased from 75% without addition of DOC to 17%, 27%, 38% and 54% with addition of DOC at 0.5, 2, 5 and 10 mg DOC/L, respectively. Because the percentage of Cu-ions in the exposure medium upon addition of 0.5 mg DOC/L did not change compared to the percentage of soluble Cu without addition of DOC in STM (Figure 3.2D), the significant change of the contribution to toxicity of $CuNP_{(particle)}$ at 0.5 mg DOC/L in the exposure medium may mechanistically be attributed to a decrease of attachment of particles to the surface of organisms through both steric and electronic repulsion (Rosenfeldt et al., 2015). The increase of the absolute zeta-potential value of CuNPs in the exposure medium at 0.5 mg DOC/L, as mentioned above, supported this suggestion. On the other hand, it is interesting to note that the contribution to toxicity of $CuNP_{(particle)}$ increased with addition of DOC higher than 0.5 mg/L in the exposure medium, relative to the contribution to toxicity of $CuNP_{(particle)}$ at 0.5 mg DOC/L. One of the explanations for this result may be directly due to the gradual reduction of the percentage of Cu-ions released from CuNPs after 48 h of incubation in the exposure medium from 23% with addition of DOC at 0.5 mg/L to 16% with the addition of DOC at 10 mg/L (Figure 3.2D). Consequently, the contribution of $CuNP_{(ion)}$ to toxicity in the exposure medium with addition of DOC higher than 0.5 mg/L decreased, relative to that at 0.5 mg DOC/L. In addition, free Cu^{2+} in exposure medium was reported to a large extent determined by complexation on DOC (de Schamphelaere and Janssen, 2002), which may also lead to the reduction of the relative contribution of $CuNP_{(ion)}$ to toxicity with an increase in DOC concentration in STM. The concentration of DOC in the majority of water systems, like rivers, canals and lakes, is in general in the range of 3 to 8 mg/L (Vijver et al., 2008). The findings in our study suggest that in natural waters the effective concentration regarding the toxicity of suspensions of CuNPs is likely to be mainly ascribed to the Cu ions released from CuNPs rather than to the particulate Cu.

3.4 Conclusions

This study investigated how the toxicity of CuNPs and the relative contribution of $CuNP_{(ion)}$ and $CuNP_{(particle)}$ to the overall toxicity of CuNPs towards *D. magna* changed upon modification of the exposure conditions. Our results demonstrate that the exposure conditions (i.e., the aging time of NP suspensions and water chemistry parameters like the pH and DOC values) influenced the toxicity of CuNPs to organisms. Compared to the factors of pH and storage time in this study, DOC exerted the highest extent of effect on toxicity, and significantly alleviated the toxicity of CuNPs to *D. magna*. Therefore, the toxicity of metal-based NPs may be overestimated in laboratory tests due to the common absence of DOC in laboratory test solutions. Our results also emphasize that by evaluating the effect of the composition of the exposure medium on the physicochemical properties of CuNPs, connections between the fate and toxicity of CuNPs can be drawn.

Furthermore, in the case that CuNPs were added to the standard test medium (pH: 7.8 ± 0.2; without addition of DOC), the particulate Cu played a dominant role in the toxicity caused by the CuNP suspension. Nevertheless, adding DOC or decreasing the pH of the exposure medium reduced the contribution of particles to toxicity. We thus found that the effective concentration regarding the toxicity of CuNP suspension to *D. magna* was dominantly driven by the contribution of ions rather than by the contribution of particulate Cu in the presence of DOC with a concentration less than 10 mg DOC/L or at pH 6.5. The findings in our study suggest that the effect of water chemistry parameters like the DOC and pH values need to be considered when evaluating the contribution of $NP_{(ion)}$ and $NP_{(particle)}$ to toxicity.

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Supplementary information

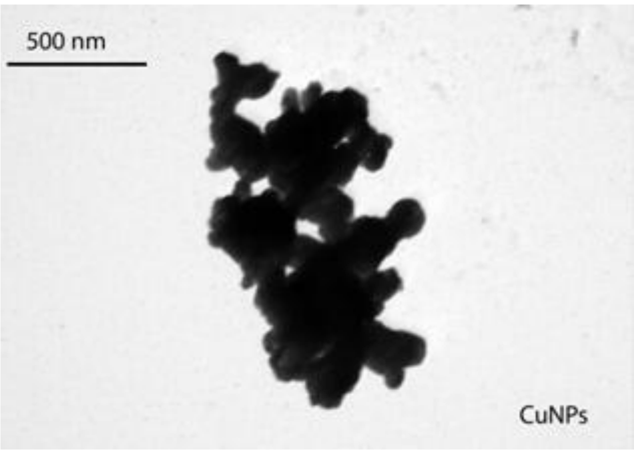

Figure S3.1 TEM image of CuNPs after 1 h of incubation in the standard test medium (STM) which was cited from Xiao *et al.* 2015.

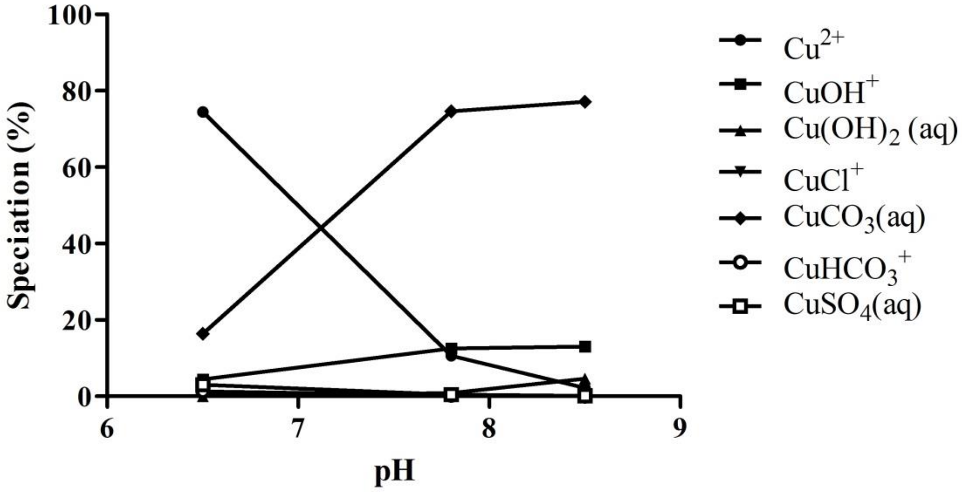

Figure S3.2 pH-dependent speciation of Cu-ions in STM estimated by Visual MINTEQ.

Table S3.1 The median lethal concentration (LC50) values and corresponding 95% confidence intervals of CuNP suspensions with aging for up to 7 days to *D. magna*

Aging time (d)	LC50 of CuNPs (mg/L)	
	LC50	95% CI
0	0.093	0.086-0.101
1	0.089	0.078-0.102
2	0.070	0.059-0.084
7	0.060	0.049-0.074

Table S3.2 The median lethal concentration (LC50) values and corresponding 95% of confidence intervals of CuNPs with varying DOC and pH levels in STM towards *D. magna.*

DOC Conc. (mg/L)	pH value	LC50 of CuNPs (mg/L)	
		LC50	95% CI
0	6.5	0.04	0.04-0.04
0	7.8	0.09	0.09-0.10
0	8.5	0.13	0.11-0.15
0.5	7.8	0.18	0.15-0.21
2	7.8	0.46	0.42-0.51
5	7.8	0.68	0.60-0.76
10	7.8	1.13	1.05-1.22

Table S3.3 The median lethal concentration (LC50) values and corresponding 95% of confidence intervals of suspensions of $Cu(NO_3)_2$ towards *D. magna*

DOC Conc. (mg/L)	pH value	LC50 of $Cu(NO_3)_2$ (µg/L)	
		LC50	95% CI
0	6.5	12	11-14
0	7.8	28	26-29
0	8.5	43	39-47
0.5	7.8	44	37-52
2	7.8	82	69-97
5	7.8	120	107-135
10	7.8	198	175-223

Reference

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Chapter 4 Impact of water chemistry on the behavior and fate of copper nanoparticles

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Abstract

A full-factorial test design was applied to systematically investigate the contribution and significance of water chemistry parameters (pH, divalent cations and dissolved organic carbon (DOC) concentration) and their interactions on the behavior and fate of copper nanoparticles (CuNPs). The total amount of Cu remaining in the water column after 48 h of incubation was mostly influenced by divalent cation content, DOC concentration and the interaction of divalent cations and DOC. DOC concentration was the predominant factor influencing the dissolution of CuNPs, which was far more important than the effect of pH in the range from 6 to 9 on the dissolution of the CuNPs. The addition of DOC at concentrations ranging from 5 to 50 mg C/L resulted in a 3-5 fold reduction of dissolution of CuNPs after 48 h of incubation, compared to the case without addition of DOC. Divalent cation content was found to be the most influential factor regarding aggregation behavior of the particles, followed by DOC concentration and the interaction of divalent cations and DOC. In addition, the aggregation behavior of CuNPs was more influential than their dissolution behavior over the variation of sedimentation profiles of CuNPs. The results provided in this study are meaningful for improved understanding and prediction of the behavior and fate of metallic NPs in aqueous environments.

Key words: copper nanoparticles; water chemistry; aggregation; dissolution; sedimentation

4.1. Introduction

Metallic nanoparticles (NPs) are increasingly applied in industrial and consumer products. The release of NPs into the aquatic environment can occur via different routes like industrial and municipal wastewater discharges (Boxall et al., 2007), runoff (Osmond et al., 2010) and leaching from consumer products (Hendren et al., 2011). Fueled by the inevitable release of NPs into the aqueous environment, concern about the adverse effects of these materials on aquatic biota has been growing. Among other particles, copper NPs (CuNPs) have been demonstrated to be highly toxic to a wide range of aquatic organisms (Hua et al., 2014; van Moos and Slaveykova, 2014; Song et al., 2015). It is therefore essential to comprehensively understand the behavior and fate of CuNPs in aquatic environment across a range of water chemistry, in order to better interpret and predict their hazards to organisms.

After being emitted into aquatic environments, NPs are subject to undergo a series of environmental processes. These processes include dissolution and aggregation and subsequent sedimentation, which can consequently affect their ultimate fate, bioavailability and hazards to organisms. Currently, it is widely known that the behavior and fate of NPs are highly dependent on the water chemistry. In particular, environmental parameters like pH, electrolytes (especially divalent cations) and natural organic matter (NOM) can strongly influence the ultimate fate of NPs in the environment. For example, pH can affect the aggregation and dissolution of metallic NPs by influencing the surface potential of the NPs (von der Kammer et al., 2010; Peng et al., 2017). The divalent cations of Ca^{2+} and Mg^{2+} are able to efficiently compress the electrical double-layer of NPs and consequently enhance aggregation of NPs (Zhang et al., 2009; Van Hoecke et al., 2011). NOM has been widely reported to enhance the stability of metallic NPs via electrostatic and/or steric repulsion (Gao et al., 2012; Romanello et al., 2013), and to alter their dissolution profiles through chelation and/or complexation of the metallic NPs (Majedi et al., 2014; Wang et al., 2015). Destabilizing effects of NOM on metallic NPs have also been reported, especially in the presence of divalent cations. This is due to the formation of bridges between NOM and cations (Stankus et al., 2011). Nevertheless, the issue of how and to what extent environmental parameters affect the behavior and fate of NPs is still insufficiently resolved. This is amongst others due to a lack of full-factorial experiments to quantitatively investigate the individual and interactive effects of each environmental parameter on the behavior and fate of NPs in the environment.

In this study, a factorial test design was applied to systematically investigate the behavior and fate of CuNPs in various aqueous matrices. The aim of this study is to evaluate the contribution and significance of each environmental parameter (i.e., pH, divalent cation content, and NOM concentration) and their interactions to the variations of behavior and fate of CuNPs across a range of water chemistry. This study is meaningful for improving our understanding and predictive potential of behavior and fate of NPs in freshwater environments.

4.2 Materials and methods

4.2.1 Preparation of CuNP suspensions

CuNPs (spherical surface area 30-50 m^2/g; purity 99.9%) were purchased from IoLiTec, with a nominal size of 25 nm. Stock suspensions of CuNPs (250 mg/L) were freshly prepared in MilliQ water after 30 min of bath-sonication to disperse the particles, prior to each experiment.

For the study design, CuNP suspensions under a range of water chemistry conditions were prepared. The pH values of the exposure media were adjusted by 0.1 M NaOH or 0.1 M HCl to reach values in between 6 and 9. The divalent cation composition of the exposure media was modified by adding $CaCl_2 \cdot 2H_2O$ and $MgSO_4 \cdot 7H_2O$ in a fixed molar ratio of 4:1. Aldrich humic acid (sodium salt) (HA) was used as a surrogate to natural dissolved organic carbon (DOC). A stock solution was prepared by dissolving HA in 0.002 N NaOH solution, followed by stirring for 24 h, filtration through a 0.2 μm cellulose acetate membrane, and storage at 4 ℃ prior to use. The total organic carbon (TOC) content of the prepared stock HA solutions was determined by a TOC analyzer (TOC-VCPH, Shimadzu Corporation). CuNP suspensions were prepared freshly for all aqueous matrices tested. A factorial experiment design was adopted with 3 pH (6, 7.8, 9) × 3 divalent cation concentrations (0, 2.5, 10 mM) × 4 DOC concentrations (0, 5, 25, 50 mg C/L, Table 4.1). The range of each parameter employed in this study encompasses the range commonly observed in natural environments (Vijver et al., 2008; Ottofuelling et al., 2011; Hammes et al., 2013).

4.2.2 CuNP characterization

The kinetics of hydrodynamic diameters of CuNP suspensions within 48 h of incubation, were determined in triplicate by dynamic light scattering (DLS) on a Zetasizer Nano-ZS instruments (Malvern, Instruments Ltd., UK). At the same time point, the zeta-potential of each NP suspension was measured by ZetaPALS software based on the Smoluchowski equation. During the 48 h of incubation, all the prepared NP suspensions were stored in a climate chamber under a 16:8-h light-dark cycle (20 ± 1 °C). To maintain the desired pH value during the 48 h incubation, the pH of the exposure media was adjusted carefully with 0.1 M NaOH or 0.1 M HCl every 24 h. In addition, the actual divalent cation concentration (i.e., Ca^{2+} and Mg^{2+}) and actual DOC concentration in exposure suspensions were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) and listed (Table S4.1).

The variations of the concentration of total Cu and dissolved Cu shedding from CuNPs in the water column, were monitored within 48 h of incubation. The actual concentration for each CuNP exposure suspension was around 800 μg/L, which is in the range (10-920 μg/L) of the predicted CuNP concentration in aquatic environments (Chio et al., 2012). The prepared CuNP suspensions across a range of water chemistry, as prepared according to Table 4.1, were stored in the climate chamber (16:8-h light-dark cycle; 20 ± 1 °C). At each sampling point (1, 12, 24, 36 and 48 h after preparation), 2 independent CuNP suspensions with the same water chemistry as duplicates were used to analyze the concentration of each Cu fraction. For each exposure suspension, a 5 mL sample collected carefully from the position around 2 cm below the surface of each NP suspension, was digested by 65% nitric acid at room temperature for at least 1 d prior to ICP-OES analysis. In this way, the total amount of Cu remaining in the water column, including ions released from CuNPs and suspended particulate Cu could be determined. Kinetics of the sedimentation of CuNPs within 48 h of incubation could be analyzed by determining the change of concentration of total Cu remaining in the water column. At each sampling point, after drawing the 5 mL sample for total Cu concentration analysis, 10 mL sample was pipetted from the water column of each CuNP exposure suspension and centrifuged at 30392 g for 30 min at 4 °C (Sorvall RC5Bplus centrifuge, Fiberlite F21-8 × 50y rotor). The supernatant was then filtered through a syringe filter with 0.02 um pore diameter (Anotop 25, Whatman). The filtrate was digested by nitric acid and analyzed for the concentration of dissolved Cu shedding from CuNPs in the water column via ICP-OES.

4.2.3 Data analysis

A univariate general linear model with a Tukey's post Hoc test was developed using SPSS 16.0 to analyze the significance and the contribution (%) of each treatment and their interaction effects on aggregation, zeta-potential and dissolution of CuNPs and on the total amount of Cu remaining in the water column after 48 h of incubation. Data were checked for normality and the homogeneity before analysis of variance (ANOVA). The significant level in all calculations was set at $p < 0.05$.

Table 4.1 Overview of the systematic modification of the matrices used for testing the fate of CuNPs.

Trial no.	pH value	Cation conc. (mM)	DOC conc. (mg/L)
1	6	0	0
2	6	0	5
3	6	0	25
4	6	0	50
5	6	2.5	0
6	6	2.5	5
7	6	2.5	25
8	6	2.5	50
9	6	10	0
10	6	10	5
11	6	10	25
12	6	10	50
13	7.8	0	0
14	7.8	0	5
15	7.8	0	25
16	7.8	0	50
17	7.8	2.5	0
18	7.8	2.5	5
19	7.8	2.5	25
20	7.8	2.5	50
21	7.8	10	0
22	7.8	10	5
23	7.8	10	25
24	7.8	10	50
25	9	0	0
26	9	0	5
27	9	0	25
28	9	0	50
29	9	2.5	0
30	9	2.5	5
31	9	2.5	25
32	9	2.5	50
33	9	10	0
34	9	10	5
35	9	10	25
36	9	10	50

Conc. = concentration

4.3 Results and discussion

4.3.1 Total Cu suspending in the water column upon modification of water chemistry

The total amount of Cu suspending in the water column represents the level of Cu to which pelagic organisms are likely exposed (Adeleye et al., 2014). It includes the dissolved Cu shedding from CuNPs which is freely suspending in the water column, and suspending particulate Cu and may also include the dissolved Cu complexed by DOC. The dynamics of the total amount of Cu remaining in the water column within 48 h of incubation upon various environmental conditions are presented in Figure 4.1. Due to sedimentation, the total amount of Cu in the water column dropped after suspending the particles in the various media tested in this study. Especially in the DOC containing-suspensions at 10 mM of divalent cations, the majority of the total added Cu was removed from the water column by sedimentation within the first 12 h of incubation (Figure 4.1). The average Cu concentration remaining in the water column after 48 h of incubation corresponding to the level of each factor, is given in Figure 4.2. The average value for each factor enables comparison among the levels of that factor (Majedi et al., 2014). It is clear that the average total Cu concentration decreased with the addition of DOC and with increasing divalent cation content, especially at 10 mM of divalent cations. The lowest amount of Cu determined in the water column was observed for the cases of 10 mM of divalent cations and 50 mg C/L at pH 7.8 and pH 9 (Figure 4.1), at which only 14% of the initially added CuNPs remained in the water column after 48 h of incubation.

Our results demonstrated that the divalent cation content was the most influential factor regarding the total concentration of Cu suspending in the water column, which explained around 60% of the variation of total concentration of Cu remaining in the water column after 48 h of incubation (Table 4.2). The next most important factor was DOC concentration, as this parameter explained approximately 15% of the change of concentration of Cu suspending in the water column after 48 h of incubation. Our results are consistent with the findings of Peeters et al. (2016), who found that in landfill leachate the settling effect of ionic strength prevailed the dispersion effect of NOM on Fe-based NPs. The pH was shown to insignificantly affect the concentration of Cu remaining in the water column after 48 h ($p > 0.05$, Table 4.3). pH has been reported to significantly affect the sedimentation of NPs only when it approaches the point of zero charge (PZC) of NPs, at which NPs are expected to aggregate most and aggregation-accompanied sedimentation occurs (Hsiung et al., 2016; Peng et al., 2017). In this study, pH in the range of 6 to 9 did not significantly affect the zeta-potential of CuNPs and the PZC of CuNPs was not included in the pH range investigated (Table 4.3). Probably that is why pH had an insignificant effect on the total Cu concentration in the water column. The interaction between divalent cations and DOC could explain around 13% of the variation of the total Cu concentration in the water column after 48 h of incubation (Table 4.2). The interactive effect of divalent cations and DOC may arise from their effect on aggregation, as discussed in more detail below.

Table 4.2 Results of ANOVA analyses showing the treatments and their interaction effects on the total amount of Cu[a] remaining in the water column and dissolution[b] of CuNPs and hydrodynamic diameter[c] and zeta-potential[d] of CuNP suspensions after 48 h of incubation.

Source	Sum of square	Mean square	*F*-value	*p*-value	PC(%)
Total amount of Cu					
pH (A)	357	178	1.05	0.361	0.01
Divalent cation conc. (B)	1469155	734577	4311.00	< 0.001	59.93
DOC conc. (C)	377406	125802	738.32	< 0.001	15.39
A × B	77219	19305	113.30	< 0.001	3.15
A × C	177427	29571	173.55	< 0.001	7.24
B × C	306866	51144	300.16	< 0.001	12.52
A × B × C	36946	3079	18.07	< 0.001	1.51
Error	6134	170	-	-	0.25
Total	2451511	-	-	-	-
Dissolution					
pH (A)	1351	676	220	< 0.001	4.56
Divalent cation conc. (B)	195	94	32	< 0.001	0.66
DOC conc. (C)	26688	8896	2902	< 0.001	90.05
A × B	415	104	34	< 0.001	1.40
A × C	774	129	42	< 0.001	2.61
B × C	55	9	3	< 0.001	0.19
A × B × C	48	4	1	0.262	0.16
Error	110	3	-	-	0.37
Total	29636	-	-	-	-
HDD					
pH (A)	3483836	1741918	49.3	< 0.001	7.47
Divalent cation conc. (B)	21420000	10710000	302.98	< 0.001	45.95
DOC conc. (C)	10860000	3620919	102.42	< 0.001	23.30
A × B	1046549	261637	7.40	< 0.001	2.25
A × C	1267398	211233	5.98	< 0.001	2.72
B × C	4908854	818142	23.14	< 0.001	10.53
A × B × C	1080975	90081	2.55	< 0.001	2.32
Error	2545557	35355	-	-	5.46
Total	46620000	-	-	-	-
Zeta-potential					
pH (A)	15	8	1.58	0.213	0.09
Divalent cation conc. (B)	14031	7015	1474.00	< 0.001	86.20
DOC conc. (C)	1042	347	72.96	< 0.001	6.40
A × B	173	43	9.08	< 0.001	1.06
A × C	56	9	1.96	0.08	0.34
B × C	505	84	17.69	< 0.001	3.10
A × B × C	113	9	1.99	0.038	0.69
Error	343	5	-	-	2.10
Total	16277	-	-	-	-

PC = Percentage contribution. Conc. = concentration. HDD = Hydrodynamic diameter.

[a] The coefficient of determination (R^2) of the model for total amount of Cu was 0.997, suggestive of good model fit with sufficient predictive power.

[b] The coefficient of determination (R^2) of the model for dissolution was 0.996, suggestive of good model fit with sufficient predictive power.

[c] The coefficient of determination (R^2) of the model for aggregation was 0.945, suggestive of good model fit with sufficient predictive power.

[d] The coefficient of determination (R^2) of the model for zeta-potential was 0.979, suggestive of good model fit with sufficient predictive power.

Table 4.3 Hydrodynamic diameter and zeta-potential of CuNPs during 48 h of incubation in waters across a range of pH, divalent cations and DOC concentrations.

Trial no.	Hydrodynamic diameter (nm)[a]			Zeta-potential (mV)[a]		
	1 h	24 h	48 h	1 h	24 h	48 h
1	413 ± 29	556 ± 49	600 ± 72	-31 ± 1	-26 ± 5	-29 ± 4
2	134 ± 47	122 ± 37	127 ± 18	-39 ± 2	-35 ± 1	-35 ± 1
3	82 ± 16	93 ± 9	87 ± 8	-41 ± 5	-39 ± 2	-40 ± 3
4	80 ± 12	72 ± 21	77 ± 8	-45 ± 5	-41 ± 1	-42 ± 4
5	754 ± 217	903 ± 194	1383 ± 360	-13 ± 2	-14 ± 2	-9 ± 1
6	141 ± 34	127 ± 16	159 ± 25	-14 ± 1	-11 ± 1	-12 ± 1
7	110 ± 24	115 ± 18	149 ± 16	-12 ± 1	-12 ± 2	-12 ± 1
8	89 ± 19	105 ± 17	117 ± 13	-12 ± 1	-13 ± 2	-12 ± 2
9	780 ± 106	947 ± 61	854 ± 47	-7 ± 1	-8 ± 1	-9 ± 1
10	815 ± 80	988 ± 58	1047 ± 56	-11 ± 1	-9 ± 1	-10 ± 2
11	1099 ± 145	1047 ± 88	1135 ± 199	-11 ± 1	-10 ± 2	-11 ± 1
12	1156 ± 183	1138 ± 64	993 ± 162	-13 ± 1	-11 ± 1	-11 ± 2
13	465± 84	641 ± 173	518 ± 80	-34 ± 4	-27 ± 1	-26 ± 1
14	323 ± 65	191 ± 5	156 ± 14	-36 ± 12	-26 ± 2	-27 ± 4
15	191 ± 30	159 ± 14	236 ± 27	-42 ± 1	-42 ± 2	-39 ± 4
16	157 ± 14	168 ± 10	148 ± 8	-40 ± 4	-41 ± 3	-43 ± 6
17	637± 105	1008 ± 116	1650 ± 335	-17 ± 1	-12 ± 3	-10 ± 1
18	369 ± 41	486 ± 21	512 ± 23	-18 ± 1	-16 ± 1	-16 ± 2
19	373 ± 60	457 ± 23	468 ± 16	-19 ± 1	-18 ± 1	-17 ± 1
20	359 ± 19	445 ± 27	495 ± 17	-19 ± 1	-16 ± 3	-15 ± 1
21	795 ± 138	1301 ± 356	1773 ± 221	-10 ± 1	-8 ± 1	-7 ± 1
22	1004 ± 198	1273 ± 83	1287 ± 442	-15 ± 1	-14 ± 1	-13 ± 0
23	1217 ± 189	1441 ± 300	1398 ± 191	-16 ± 1	-15 ± 0	-14 ± 1
24	1540 ± 107	1208 ± 214	1605 ± 446	-16 ± 1	-14 ± 1	-14 ± 1
25	633 ± 58	824 ± 78	669 ± 119	-29 ± 1	-28 ± 1	-32 ± 1
26	385 ± 27	323 ± 38	324 ± 53	-32 ± 3	-31 ± 1	-31 ± 2
27	274 ± 43	236 ± 39	223 ± 4	-40 ± 3	-39 ± 4	-42 ± 3
28	263 ± 18	203 ± 13	212 ± 21	-42 ± 4	-42 ± 3	-42 ± 2
29	745 ± 93	1307 ± 172	2436 ± 490	-9 ± 2	-6 ± 3	-5 ± 4
30	456 ± 38	567 ± 45	553 ± 47	-14 ± 1	-14 ± 0	-13 ± 1
31	411 ± 13	559 ± 48	579 ± 22	-14 ± 0	-10 ± 6	-13 ± 0
32	403 ± 14	534 ± 50	559 ± 40	-14 ± 0	-14 ± 0	-13 ± 0
33	646 ± 141	1319 ± 163	2278 ± 407	-11 ± 1	-8 ± 1	-7 ± 1
34	1200 ± 113	1396 ± 74	1377 ± 35	-12 ± 1	-12 ± 1	-11 ± 0
35	1369 ± 45	1313 ± 101	1293 ± 158	-12 ± 0	-12 ± 0	-11 ± 0
36	1528 ± 52	1173 ± 106	1393 ± 99	-12 ± 0	-12 ± 0	-11 ± 0

[a] Hydrodynamic diameters and zeta-potentials are expressed as means ± SD (*n* = 3).

4.3.2 Dissolution of CuNPs upon modification of water chemistry

Dissolution of NPs is a key process that determines to a considerable extent the bioavailability and toxicity of NPs to organisms. The results of the quantification of ions released from CuNPs after 48 h of incubation in media across a range of water chemistry are given in Figure 4.3. In the absence of DOC, around 75%, 65% and 55% of the total added Cu were dissolved after 48 h of incubation at pH 6, 7.8 and 9, respectively (Figure 4.3). The CuNPs used in this study owned a relatively high solubility. In our previous study, less than 25% of ions released from CuNPs (with a pristine size of 50 nm) after 48 h of incubation in the suspension with 2.5 mM of divalent cations and pH of 7.8, were determined (Xiao et al., 2016). This difference in terms of solubility may arise from the different pristine size. On the other hand, the percentage of ions released from CuNPs in the water column was decreased 3-5 fold by the addition of DOC ranging from 5 to 50 mg C/L after 48 h of incubation, compared to the case without addition of DOC (Figure 4.3). At pH 6, for example, the percentage of dissolution of CuNPs decreased from 75% without addition of DOC to 27% upon addition of 5 mg DOC/L after 48 h of incubation (Figure 4.3). The reduction of dissolution of metallic NPs by the addition of DOC has also been reported by some other studies (Liu and Hurt, 2010; Conway et al., 2015). The NOM-dependent reduction of the concentration of dissolved Cu shedding from CuNPs in the water column was possibly due to the combination of complexation of dissolved Cu by DOC and of coating of DOC on the surface of Cu particles (Conway et al., 2015).

The average percentages of ions released from CuNPs ranked according to the importance of the water properties considered, are given in Figure 4.2. The dominant factor was DOC at 0 mg C/L, at which CuNPs exhibited the highest percentage of dissolved Cu shedding from CuNPs. The addition of DOC at a concentration of 5 mg C/L largely reduced the percentage of dissolved Cu from 65% without addition of DOC to around 29%. The highest solubility of CuNPs occurred at the situation with 2.5 mM of divalent cations, pH of 6 and without addition of DOC (Figure 4.3). Furthermore, our results showed that more than 90% of the variation of the dissolution of CuNPs could be explained by the DOC concentration (Table 4.2). It is worth to note that pH, which is often considered as the most influential factor on NP dissolution (Majedi et al., 2014; Odzak et al., 2014), only accounted for 5% of the variation of the dissolution of CuNPs in this study (Table 4.2). According to the dissolution mechanisms of metallic NPs (Bian et al., 2011; Wang et al., 2016), reactions of metal-NPs with protons are expected under acidic conditions on the basis of the reactivity of nanomaterials. The CuNPs used in this study displayed a relatively high reactivity due to their small pristine size (as mentioned above), which hence may overshadow the influence of pH on the dissolution process of CuNPs. Bian et al. (2011) found that pH was more likely to affect dissolution of metallic NPs under highly acidic and alkaline conditions (lower than pH 6 and higher than pH 9) than in the pH range from 6 to 9. Hence, consideration of the role of pH in the dissolution of metallic NPs needs to be interpreted with care. It is interesting to point out that even though the parameter of divalent cation content was the most influential regarding the aggregation behavior of CuNPs (discussed in more detail below, Table 4.2), it played a negligible role in the dissolution of CuNPs (Table 4.2). Similarly, despite the interaction between divalent cations and DOC accounting for around 11% of the aggregation of CuNPs (discussed in more detail below, Table 4.2), less than 1% of the dissolution of CuNPs could be explained by their interaction. These results corroborate that aggregation of NPs does not necessarily result in a change of dissolution of NPs (Son et al., 2015; Wang et al., 2015).

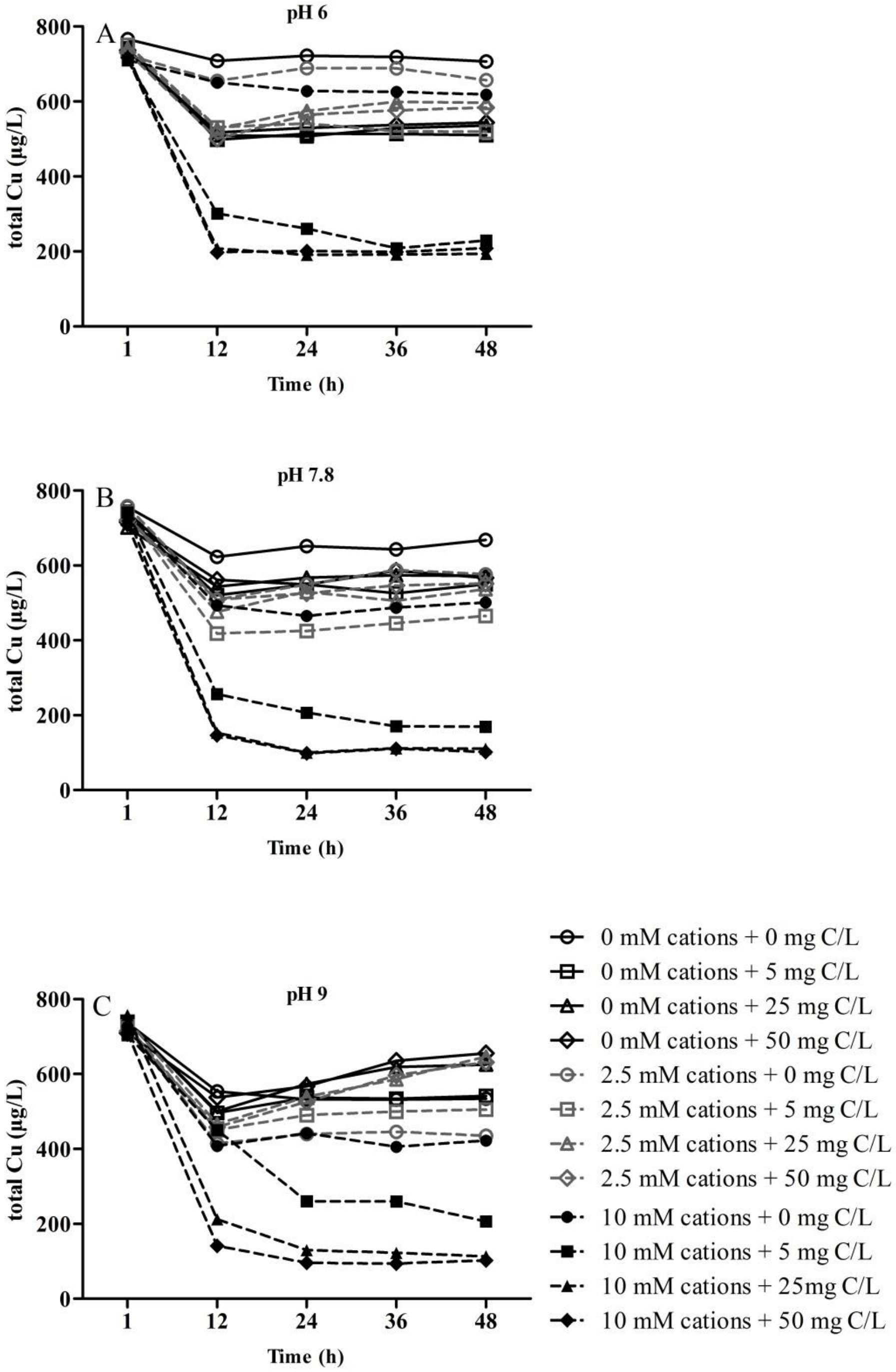

Figure 4.1 The total amount of Cu remaining in the water column during 48 h of incubation of CuNPs across a range of pH, divalent cations and DOC concentrations. Each data point represents the mean of duplicate measurements at the same experimental condition.

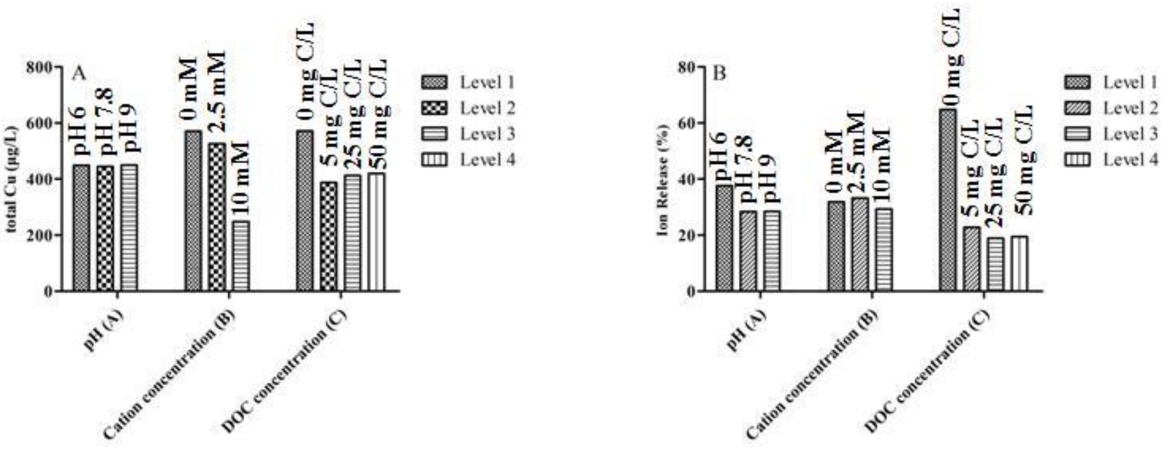

Figure 4.2 Effect of each level of pH, divalent cations and dissolved organic carbon on the average of total amount of Cu remaining in the water column and on the percentages of ions released from CuNPs after 48 h of incubation. For pH treatments, level 1, 2 and 3 represent pH 6, 7.8 and 9, respectively. For divalent cation treatments, level 1, 2 and 3 mean 0, 2.5 and 10 mM of divalent cations, respectively. For DOC treatments, level 1, 2 ,3, and 4 indicate 0, 5, 25 and 50 mg C/L, respectively.

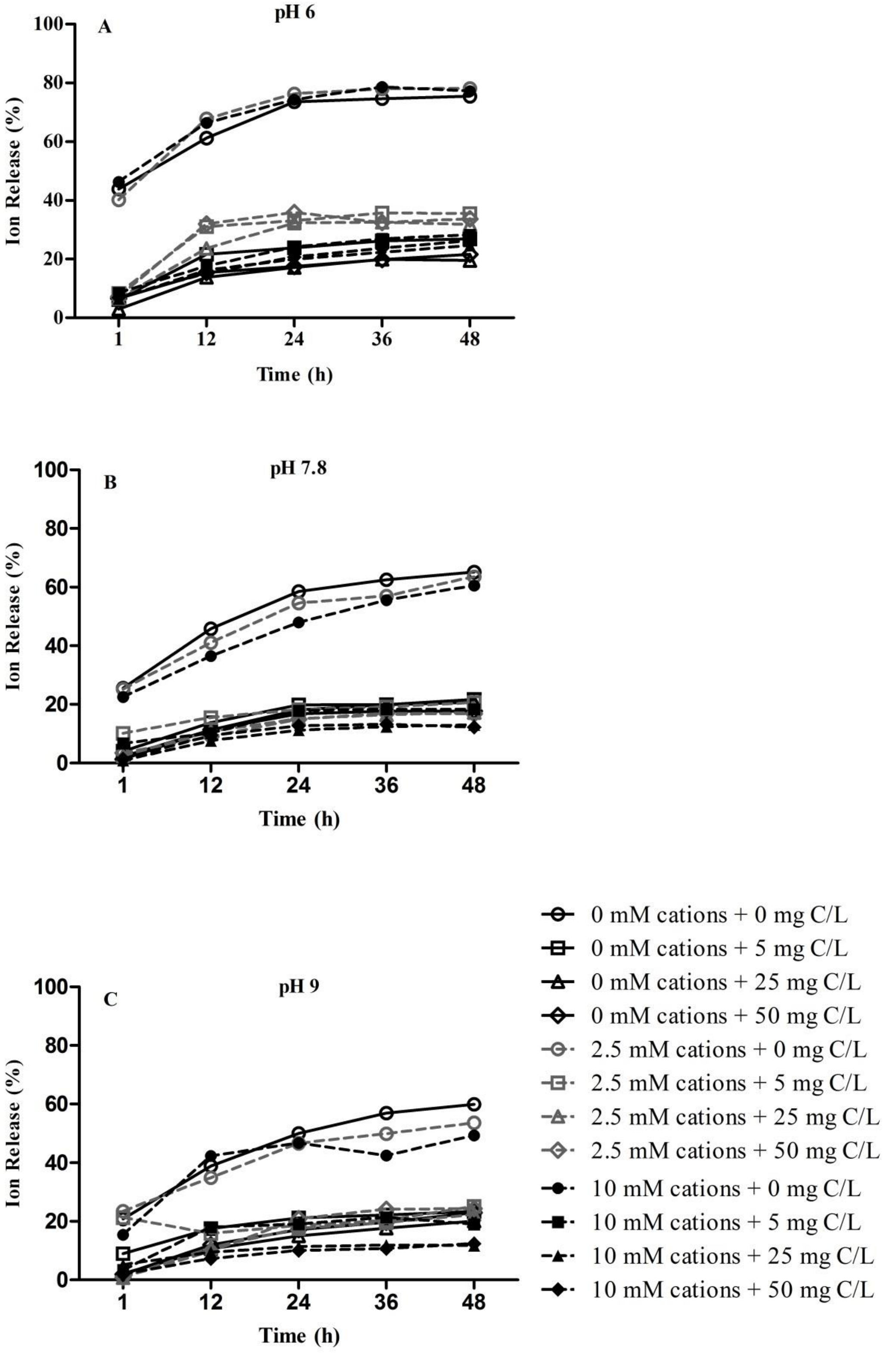

Figure 4.3 Dissolution profiles of CuNPs across a range of pH, divalent cations and DOC concentrations. Each data point represents the mean of duplicate measurements at the same experimental condition.

4.3.3 Aggregation of CuNPs upon modification of water chemistry

In this study, the aggregation behavior of the CuNPs was strongly modified by the composition of the media (Table 4.3). The average hydrodynamic diameter and zeta-potential of the CuNP suspensions as a function of the environmental parameters tested in this study, are presented in Figure 4.4. It is evident that the hydrodynamic diameters of CuNPs increased with a rise in pH and with increasing divalent cation content, while the hydrodynamic diameter of CuNPs dropped with the addition of DOC. The smallest hydrodynamic diameter was around 80 nm after 48 h of incubation in the suspension with 50 mg DOC/L and pH of 6 when no divalent cations were added (trial 4 in Table 4.3). The addition of divalent cations largely reduced the absolute value of the zeta-potential of CuNP suspensions (Figure 4.4).

The aggregation behavior of the CuNPs was significantly modified by factors of the divalent cation content, the DOC concentration and pH as well as by their interactions ($p < 0.05$, Table 4.2). Among these three properties, the divalent cation content was the most significant factor responsible for inducing variation of the aggregate size of suspensions of CuNPs. The factor of divalent cation content explained around 46% of the change of hydrodynamic diameter of CuNPs in exposure media across a range of water chemistry (Table 4.2). Meanwhile, the divalent cation content was also the predominant factor in influencing the zeta-potential of CuNP suspensions ($p < 0.05$, Table 4.2), which was far more important than DOC in determining the zeta-potential of the suspensions. This is probably due to a rapid adsorption of divalent cations on the surface of DOC and NPs, neutralizing the negative charges and reducing the absolute value of the zeta-potential of the suspensions. According to the Derjaguin- Landau-Verwey-Overbeek theory, the stability of colloidal particles in aqueous environments is determined by the sum of Van der Waals forces and electrical double layer repulsions. In this study, the addition of divalent cations significantly decreased the absolute values of the zeta-potential of CuNP suspensions ($p < 0.05$, Table 4.2), leading to the reduction of electrical double layer repulsion between particles. Enhanced aggregation of CuNPs with the addition of divalent cations was hence likely due to net attractive force between particles as a result of prevailing Van der Waals attraction.

The second most important factor was the DOC concentration, which explained around 23% of the variation in aggregate size of CuNPs (Table 4.2). The interaction between the divalent cations and DOC was also important in influencing the aggregation of CuNPs, accounting for 11% of the variation of aggregate sizes (Table 4.2). The interaction between divalent cations and DOC can influence the aggregation of NPs via affecting the surface charge of NPs and via their bridging effect (Delay et al., 2011). In this study, the stabilizing effect of DOC on CuNPs decreased with a rise in divalent cation content (Table 4.3 and Figure 4.4). At 2.5 mM of the cations, the average hydrodynamic diameter of the CuNP aggregates was approximately 500 nm upon addition of DOC with a concentration in between 5 and 50 mg C/L (trials 18, 19 and 20 in Table 4.3), which was larger than the case in which no cations were added (around 200 nm, trials 14, 15 and 16 in Table 4.3) and much smaller than the case in which no DOC was added (around 1650 nm, trail 17 in Table 4.3). These results suggest that DOC could still stabilize the NPs at 2.5 mM of divalent cations. In fact, when the cations were added the zeta-potential of CuNP suspensions was higher than -20 mV even in the presence of DOC for up to 50 mg C/L (Table 4.3). Colloidal suspensions are generally inherently unstable and tend to aggregate if the absolute value of the zeta-potential is smaller than 30 mV (Schramm, 2005). This suggests that the stabilization effect of DOC on CuNPs in the divalent cations-containing suspensions is likely to result from steric hindrance imparted by DOC. Similarly, Delay et al. (2011) also found that even though addition of 1 mM of Ca^{2+} into aqueous Ag NP systems containing NOM resulted in electrostatic destabilization of the particles, the steric repulsion imparted by NOM still stabilized the particle aggregates and allowed them to disperse uniformly in suspension. However, in our study, the addition of DOC into the CuNP suspensions containing 10 mM of divalent cations led to the formation of HA-flocs, which then settled quickly. The formation of flocs is attributed to the bridging effects between divalent cations and DOC, which can result in an enhanced aggregation and sedimentation of NPs (Stankus et al., 2011; Romanello et al., 2013). The massive sedimentation of CuNPs observed in DOC-containing suspensions at 10 mM of divalent cations (Figure 4.1), as mentioned above, hence could arise from the interparticle bridging by the formed HA-flocs. In order to quantify the interaction between DOC and divalent cations, we first determined the concentrations of DOC in the water column across a range of divalent cation contents within 48 h. The results showed that the DOC concentrations in the water column at 0 and 2.5 mM of divalent cations maintained similar within 48 h of incubation, while at 10 mM of cations, the concentration of DOC within the range between 5 and 50 mg C/L dropped significantly over time (Figure S4.1). Furthermore, the concentration of the divalent cations in the presence of 25 mg DOC/L was also monitored within 48 h of incubation. It was found that in the CuNP suspension containing 25 mg/L of DOC and 10 mM of divalent cations, both the concentrations of Ca^{2+} and Mg^{2+} in the water column decreased with time (Figure S4.2). Our results support the findings reported by Stankus et al. (2011) that both Ca^{2+} and Mg^{2+} could bridge with DOC, resulting in an enhanced aggregation of Au NPs. In addition, it is worth to point out that the average aggregate sizes measured at 1 h of the DOC-containing suspensions of CuNPs at 10 mM of divalent cations may mainly arise from the HA-flocs rather than from CuNPs, as the hydrodynamic diameters of suspensions without CuNPs (combination of DOC and 10 mM of divalent cations) were similar to the suspensions with CuNPs (Table 4.3 and Figure S4.3). However, the HA-flocs sediment rapidly and intensively, which resulted in the average hydrodynamic diameters of the suspensions without CuNPs measured at 24 h and 48 h being similar (around 600 nm, Figure S4.3), smaller than the average hydrodynamic sizes of suspensions with CuNPs (Table 4.3). Hence, employing the average hydrodynamic diameters measured at 48 h to analyze the impact of environmental parameters on the aggregation of CuNPs, could reduce the influence of HA-flocs on the average sizes of CuNP aggregates.

Although pH in the range from 6 to 9 was found to have an insignificant effect on the zeta-potential of the CuNP suspensions ($p > 0.05$, Table 4.2), it still significantly influenced the hydrodynamic diameter of CuNPs, accounting for 7% of the variation of the average aggregate size of the CuNPs ($p < 0.05$, Table 4.2). This may result from the effect of pH on the dissolution of CuNPs, which could further affect the hydrodynamic diameter of particles (Domingos et al. 2009; Song et al., 2014). It is interesting to point out that the interactions between pH and other factors could also significantly affect the aggregation of CuNPs ($p <$

0.05, Table 4.2). For instance, DOC was more pronouncedly inhibiting aggregation at pH 6 than at pH 7.8 and 9, as the average aggregate sizes at pH 6 were much smaller than at pH 7.8 and 9 after 48 h of incubation (Table 4.3). This may arise from the effect of pH on the deprotonation/protonation and adsorption capacity of DOC (Yang et al., 2009; Van Hoecke et al., 2011), leading to a change of stabilization effect of DOC on CuNPs. It is important to point out that the order in terms of the significance of each factor in influencing the aggregation behavior of CuNPs was similar to the order of significance of each factor in influencing the total amount of Cu in the water column (Table 4.2). These results indicate that the aggregation behavior of CuNPs was more influential than their dissolution behavior over the variation of sedimentation profiles of CuNPs.

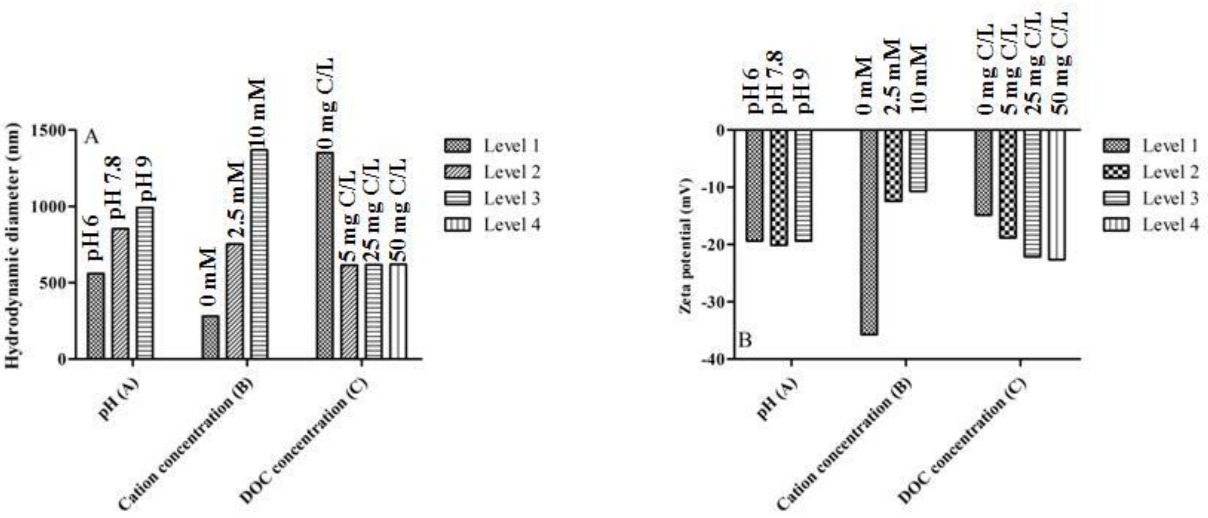

Figure 4.4 Effect of each level of pH, divalent cations and dissolved organic carbon (DOC) on the average of hydrodynamic diameter and zeta-potential of CuNP suspensions. For pH treatments, level 1, 2 and 3 represent pH 6, 7.8 and 9, respectively. For divalent cation treatments, level 1, 2 and 3 mean 0, 2.5 and 10 mM of divalent cations, respectively. For DOC treatments, level 1, 2 ,3, and 4 indicate 0, 5, 25 and 50 mg C/L, respectively.

4.4 Conclusions

This study systematically investigated the main and interactive effects of pH and concentrations of divalent cations and DOC of the exposure medium on the behavior and fate of CuNPs. The results demonstrated that the total amount of Cu remaining in the water column after 48 h of incubation was mostly influenced by divalent cation content, DOC concentration and the interaction of divalent cations and DOC. DOC concentration was the predominant factor in explaining the variation of dissolution of CuNPs, which was far more important than the effect of pH in the range from pH 6 to pH 9 on the dissolution of CuNPs. The divalent cation content was the most influential factor regarding the aggregation behavior, followed by the DOC concentration. The interaction between the parameter of divalent cations and DOC also played an important role in influencing the aggregation of CuNPs. In addition, the sedimentation profile of Cu was more influenced by the aggregation behavior of CuNPs than by their dissolution.

The results provided in this study are helpful for improving our understanding and the prediction potential on how and to what extent environmental factors affect the behavior and fate of metallic NPs upon a range of environmental conditions. In addition, our results can help to optimize the testing guidelines for assessing the toxicity of NPs for the aim of improving the reproducibility of NP toxicity tests and making sure that the test organisms are exposed mainly to NPs rather than to larger aggregates and to the ions released from the NPs.

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Supplementary information

Table S4.1 Nominal and measured DOC and divalent cation concentrations in CuNP suspensions at pH 7.8.

[a]DOC conc. (mg C/L)	[b]DOC conc. (mg C/L)	[a]Ca^{2+} conc. (mM)	[b]Ca^{2+} conc. (mM)	[a]Mg^{2+} conc. (mM)	[b]Mg^{2+} conc. (mM)
0	0	0	0.16	0	0.05
5	4.7	0	0.21	0	0.07
25	26.4	0	0.58	0	0.15
50	49.7	0	1.49	0	0.33
0	0	2	2.24	0.5	0.51
5	5.0	2	2.37	0.5	0.54
25	24.7	2	2.68	0.5	0.58
50	46.8	2	3.29	0.5	0.78
0	0	8	7.91	2	2.19
5	4.4	8	8.02	2	2.23
25	23.8	8	8.44	2	2.32
50	48.3	8	9.37	2	2.48

Conc. = concentration.

[a] Nominal concentration.

[b] Measured concentration.

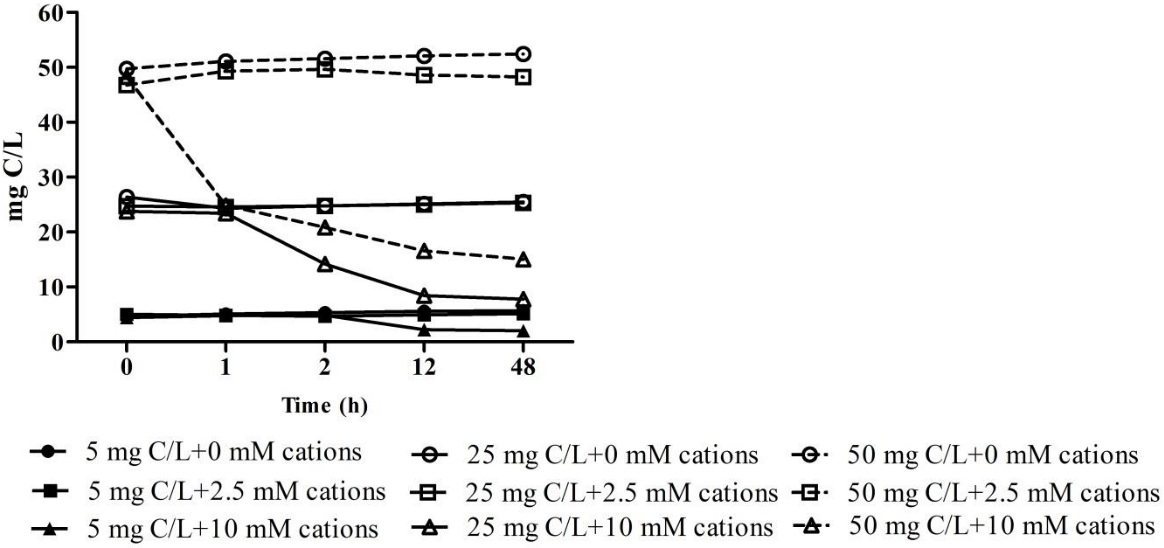

Figure S4.1 The dynamics of DOC concentrations within a range from 5 to 50 mg C/L in the water column during 48 h of incubation at pH 7.8 across a range of divalent cation concentrations. Each data point represents the mean of duplicate measurements at the same experimental condition.

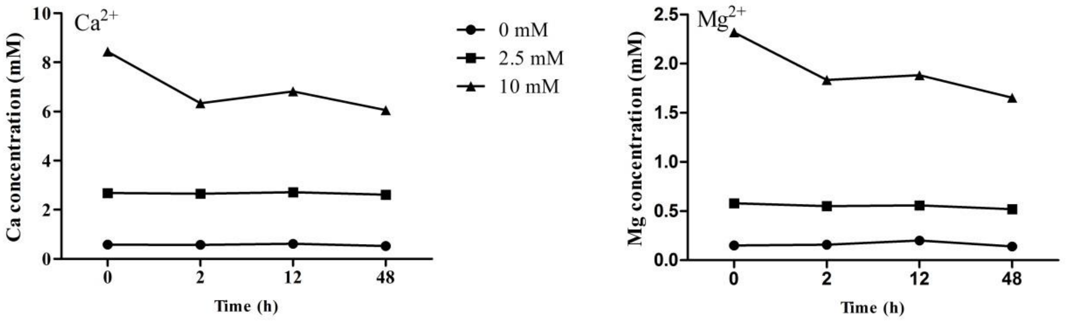

Figure S4.2 The dynamics of Ca^{2+} and Mg^{2+} concentrations in the water column within 48 h of incubation at pH 7.8 and 25 mg DOC/L. Each data point represents the mean of duplicate measurements at the same experimental condition.

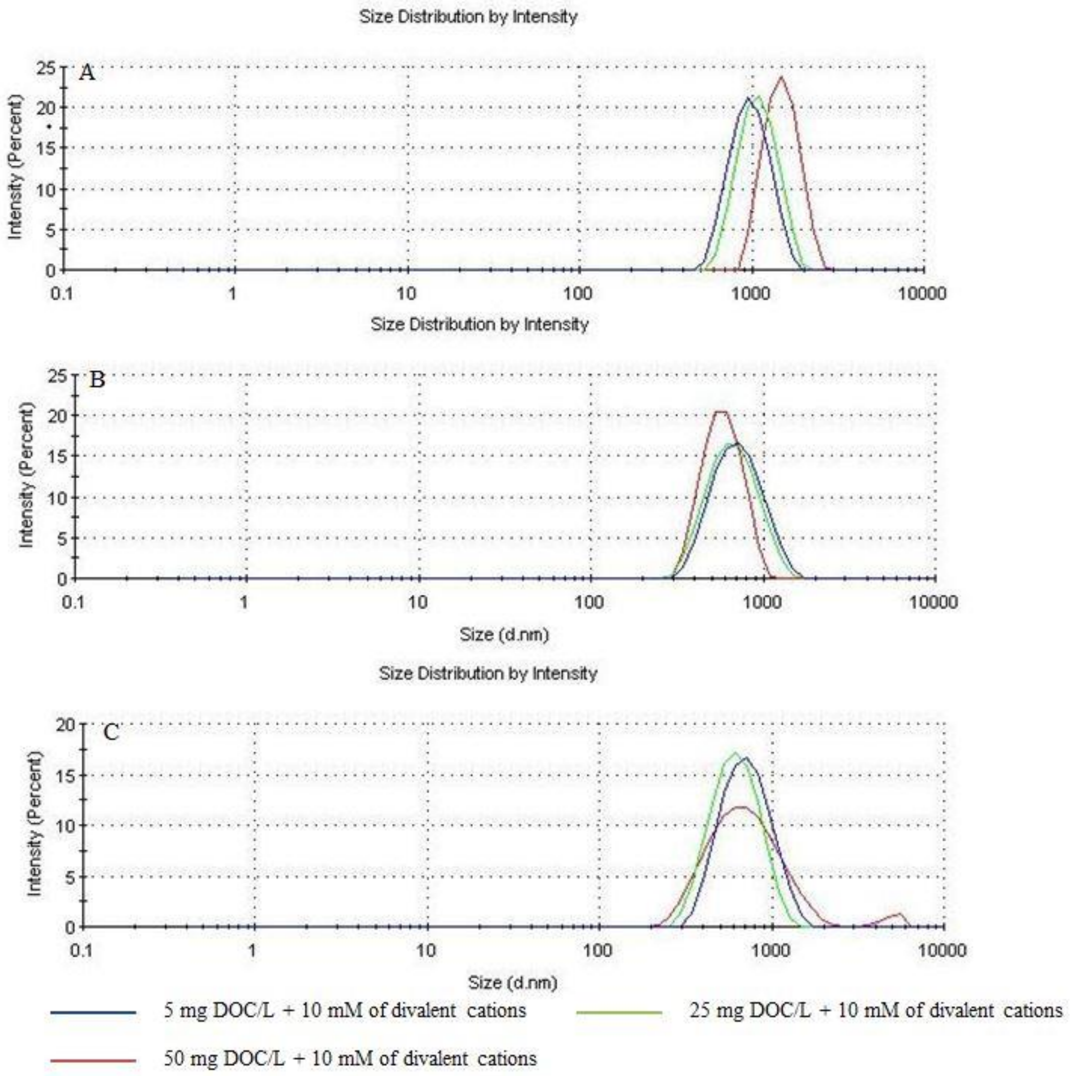

Figure S4.3 Hydrodynamic diameters of suspensions of combination of DOC and 10 mM of divalent cations and pH of 7.8 measured at 1 h (A), 24 h (B) and 48 h (C).

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

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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 ≥ 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; *Daphnia 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 $NP_{(particle)}$ and $NP_{(ion)}$ 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 m^2/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 μm 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). *Daphnia magna* 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 $K_2Cr_2O_7$ 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 $K_2Cr_2O_7$) (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 ± 0.2) contained (mM MilliQ water): $CaCl_2·2H_2O$: 2; $MgSO_4·7H_2O$: 0.5; $NaHCO_3$: 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 $CaCl_2·2H_2O$ and $MgSO_4·7H_2O$ 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 ± 1 ℃) 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 ℃). 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 μg/L, which is in the range (10-920 μg/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 µm 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 $\text{CuNP}_{\text{(total)}}$ 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 $\text{CuNP}_{\text{(total)}}$ to daphnids, a series of exposure concentrations for $\text{CuNP}_{\text{(total)}}$ 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 $\text{CuNP}_{\text{(ion)}}$ 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 $\text{NP}_{\text{(ion)}}$ and $\text{NP}_{\text{(particle)}}$ remain unclear. Nevertheless, some recent published papers found that the mode of action of $\text{NP}_{\text{(particle)}}$ differed from that of $\text{NP}_{\text{(ion)}}$ (Poynton et al., 2011; Poynton et al., 2012; Rainville et al., 2014). Hence, it was assumed that the modes of action of $\text{CuNP}_{\text{(ion)}}$ and $\text{CuNP}_{\text{(particle)}}$ would be dissimilar. In this circumstance, the toxicity of $\text{CuNP}_{\text{(particle)}}$ can be deduced by using the response addition model (Backhaus et al., 2000):

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

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 $Cu(NO_3)_2$ 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 $\text{NP}_{\text{(particle)}}$.

$$C_{\text{T}} = \frac{C_1 T_1 + C_2 T_2 + C_3 T_3 + \cdots C_{\text{n}} T_{\text{n}}}{T_1 + T_2 + T_3 + \cdots T_{\text{n}}} \qquad 5.2$$

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

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

Trial no.	Condition	pH	Cation conc. (mM)	DOC conc. (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

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 $\text{CuNP}_{(\text{ion})}$ 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 $\text{CuNP}_{(\text{ion})}$ in the water column. In both the static and the dynamic treatments, the amount of $\text{CuNP}_{(\text{ion})}$ 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).

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

Trial no.	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

[a] Hydrodynamic diameter and zeta-potential are expressed as the mean ± standard deviation (*n* = 3).

ND means not determined.

5.3.2 Acute toxicity of CuNPs to *Daphnia magna*

The LC50 of CuNP$_{(total)}$ across a range of water chemistry is provided in Table 5.3. In the static treatments, the LC50 values of CuNP$_{(total)}$ were 0.024, 0.050 and 0.094 mg/L at pH 6, 7.8 and 9, respectively. The LC50 values of CuNP$_{(total)}$ 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 CuNP$_{(total)}$ increased from 0.026 mg/L without addition of cations to 0.076 mg/L at 5 mM of cations. The LC50 of CuNP$_{(total)}$ 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 CuNP$_{(total)}$ 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 CuNP$_{(total)}$ significantly increased upon addition of DOC. In the static treatments, the LC50 of CuNP$_{(total)}$ 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 CuNP$_{(total)}$ 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 CuNP$_{(particle)}$ 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 CuNP$_{(particle)}$ 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 CuNP$_{(particle)}$ 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 CuNP$_{(particle)}$ 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 CuNP$_{(particle)}$ decreased with increasing pH and with increasing concentrations of cations and DOC in both the static and dynamic exposure treatments.

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

Trial no.	**LC50 (95% CI, mg/L)**		**Relative contribution at LC50 (%)**	
	$CuNP_{(total)}$	**$CuNP_{(particle)}$**	**$CuNP_{(ion)}$**	**$CuNP_{(particle)}$**
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)	~ 0.015[a]	62	38
13	0.152 (0.132-0.176)	~ 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

CI: Confidence intervals

Conc. = concentration

[a] means the data is not accurate.

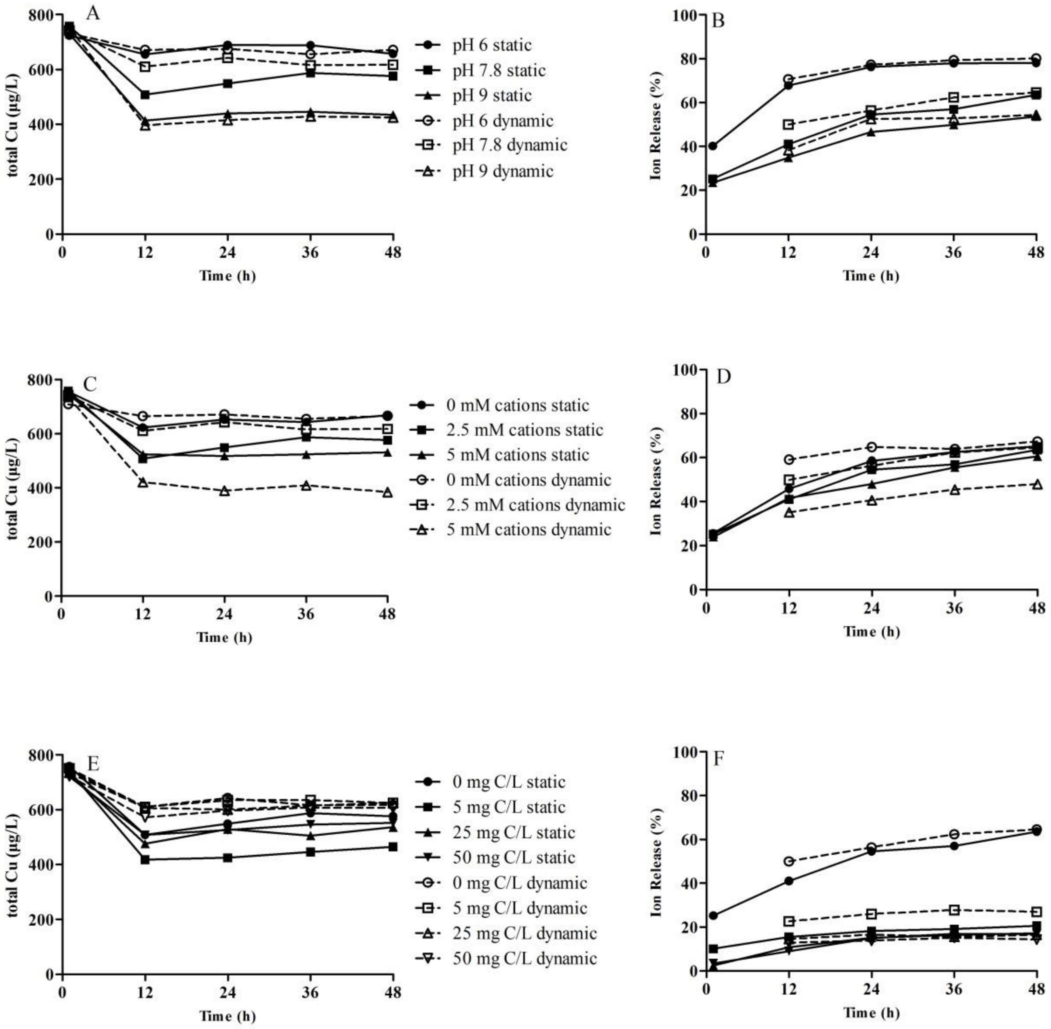

Figure 5.1 Time profiles of the total amount of Cu and dissolved Cu in suspensions of CuNPs within 48 h of incubation in the static and dynamic exposure treatment as a function of pH (A-B), of concentrations of divalent cations (C-D) and of DOC (E-F). All data are presented as the mean of duplicate.

5.3.3 Relative contribution of CuNP$_{\text{(particle)}}$ and CuNP$_{\text{(ion)}}$ to toxicity

The relative contribution of CuNP$_{\text{(particle)}}$ and CuNP$_{\text{(ion)}}$ to the toxicity of 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 CuNP$_{\text{(total)}}$ to *D. magna* at the LC50 level in the absence of DOC was mainly caused by CuNP$_{\text{(ion)}}$. In both the static and dynamic treatments, more than 53% of the toxicity of CuNP$_{\text{(total)}}$ could be explained by 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 CuNP$_{\text{(ion)}}$ upon the addition of 0, 2.5 and 5 mM of cations, respectively. Similarly, in the dynamic treatments toxicity of CuNP$_{\text{(total)}}$ was predominantly contributed by 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 CuNP$_{\text{(particle)}}$ to the overall toxicity was higher than that of CuNP$_{\text{(ion)}}$. In the static exposure treatments, the relative contribution of 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 $CuNP_{(ion)}$ and $CuNP_{(particle)}$ were more toxic at lower pH. The higher toxicity of $CuNP_{(ion)}$ 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 $CuNP_{(particle)}$ under reduced pH may be explained by the sensitivity changes of freshwater zooplankton due to low pH and consequent reduction of their tolerance to $NP_{(particle)}$ (Locke, 1991; Haven et al., 1993; Hu et al., 2017). The toxicity of $CuNP_{(total)}$ increased with a reduction of the divalent cation concentrations. This finding is the net effect of the reduction of the toxicity of both $CuNP_{(ion)}$ and $CuNP_{(particle)}$ upon increasing concentrations of the cations. The reduced toxicity of $CuNP_{(particle)}$ 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 Ca^{2+} and Mg^{2+} 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 $CuNP_{(particle)}$ 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 $CuNP_{(ion)}$ (≥ 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 $NP_{(ion)}$. 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 $CuNP_{(particle)}$ (Table 5.3). The alteration of the roles of $CuNP_{(particle)}$ 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 $CuNP_{(ion)}$ and $CuNP_{(particle)}$ was decreased upon increasing pH and increasing concentrations of divalent cations. Toxicity of $CuNP_{(total)}$ was mainly driven by $CuNP_{(ion)}$ when no DOC. In addition, toxicity of CuNP suspensions with addition of DOC at concentrations from 5 to 50 mg C/L under the dynamic exposure modality was approximately 25%-40% higher than that under the static exposure modality. The toxicity of $CuNP_{(ion)}$ and $CuNP_{(particle)}$ with addition of DOC was largely mitigated. As a result of the large reduction of $CuNP_{(ion)}$ and the enhancement of the stability of $CuNP_{(particle)}$ when DOC was added, the toxicity of $CuNP_{(total)}$ was mainly attributable to the $CuNP_{(particle)}$ 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

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

DOC Conc. (mg/L)	pH value	Cation Conc. (mM)	LC50 of $Cu(NO_3)_2$ (mg/L)	
			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

Conc. = concentration.

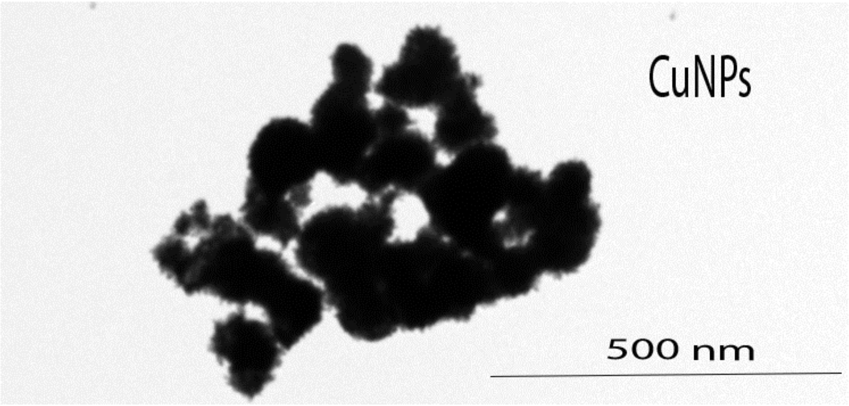
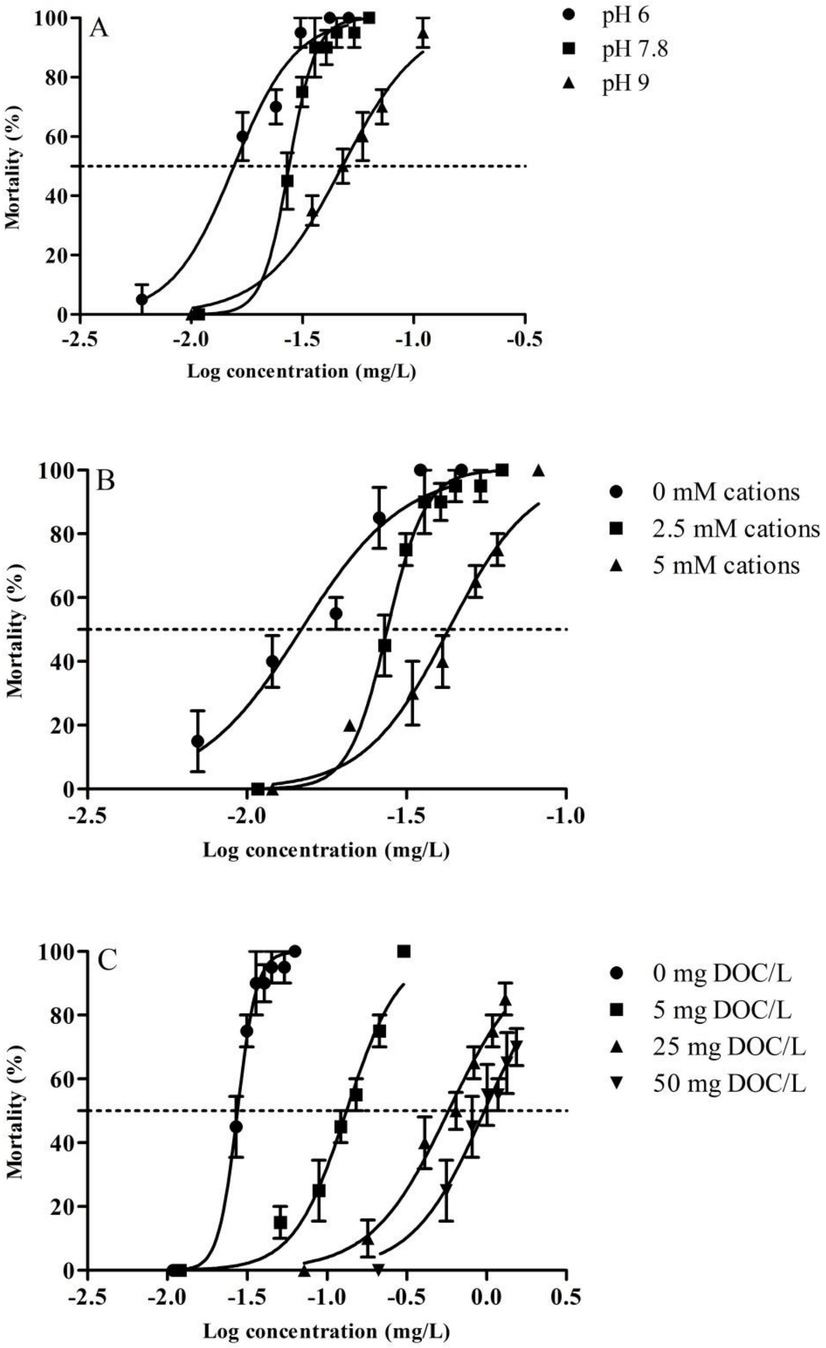


Figure S5.1 TEM images of CuNPs after 1 h of incubation in the exposure medium at pH 7.8 and 2.5 mM of cations.



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 ± standard deviation (*n* =4).

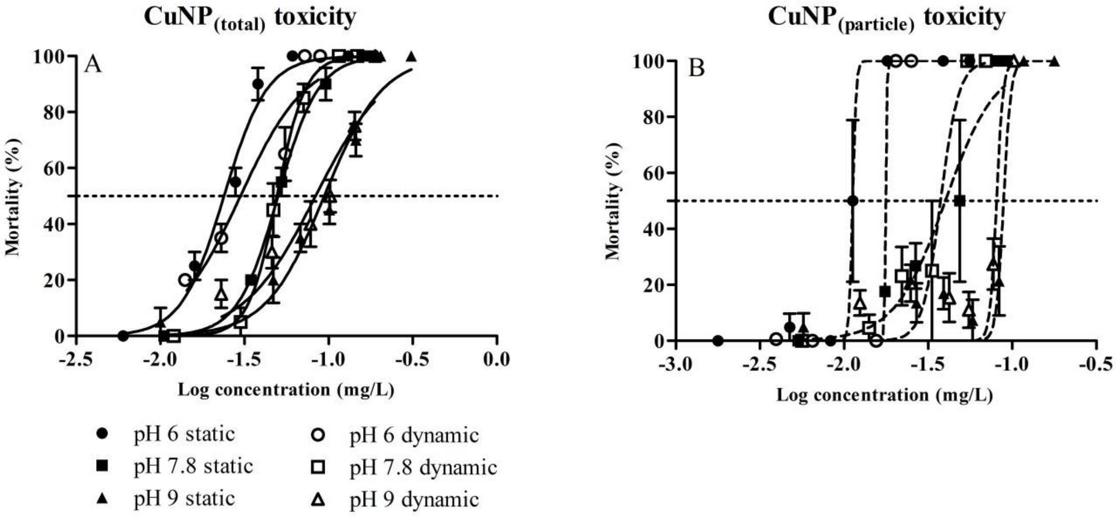


Figure S5.3 Dose-response curves of *D. magna* in response to 48 h of exposure to CuNP suspensions (A) and CuNP$_{(particle)}$ (B) as a function of pH in both the dynamic and static exposure treatments. Each data point is expressed as mean ± standard deviation ($n = 4$).

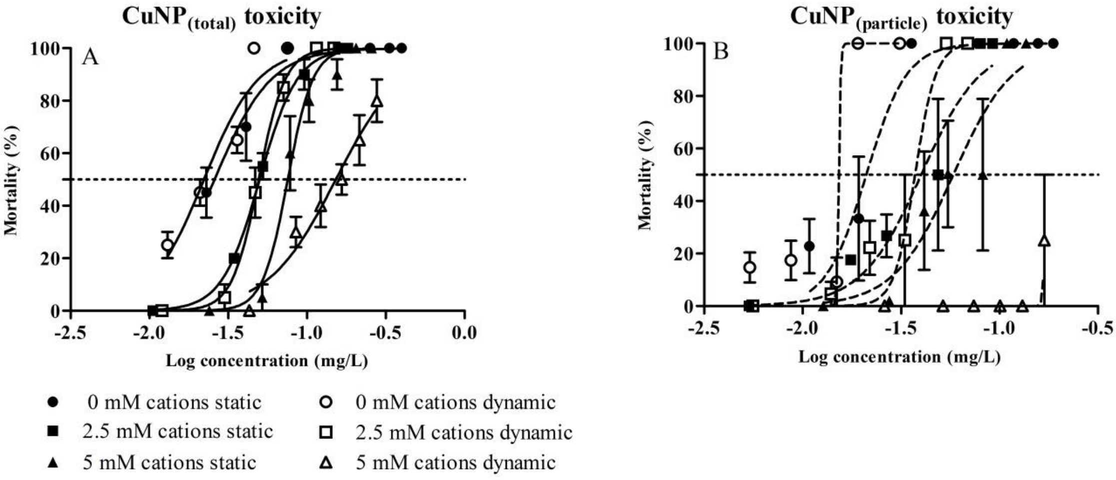


Figure S5.4 Dose-response curves of *D. magna* in response to 48 h of exposure to CuNP suspensions (A) and CuNP$_{(particle)}$ (B) as a function of concentrations of the divalent cations in both the dynamic and static exposure treatments. Each data point is expressed as mean ± standard deviation ($n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

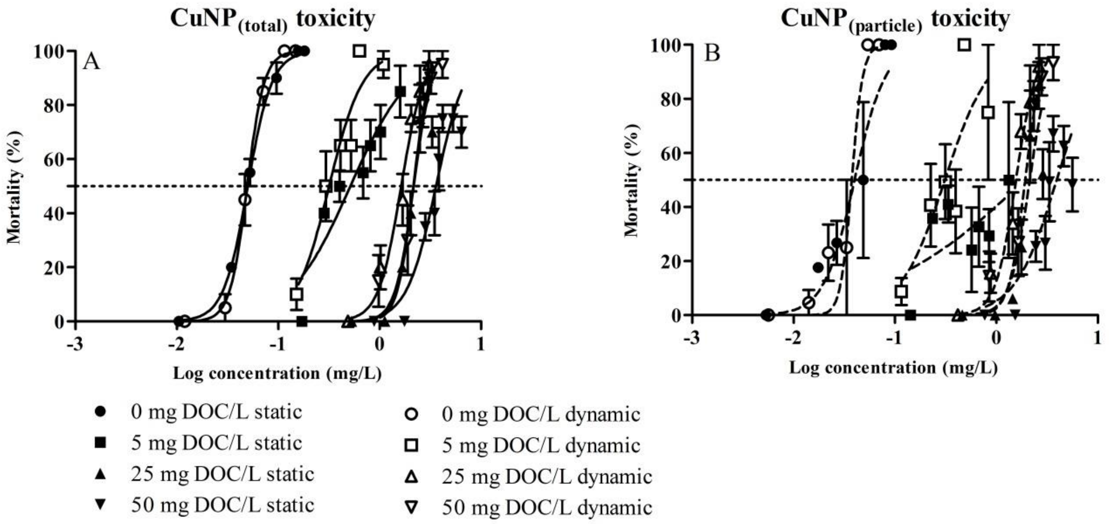


Figure S5.5 Dose-response curves of *D. magna* in response to 48 h of exposure to CuNP suspensions (A) and CuNP$_{(particle)}$ (B) as a function of concentrations of DOC in both the dynamic and static exposure treatments. Each data point is expressed as mean ± standard deviation (*n* = 4).

Chapter 6 General discussion

It is widely recognized that metallic NPs have the potential to induce adverse effects on humans and the environment. Metallic NPs can be toxic due to their small size (Rivera et al., 2010; Wang et al., 2011; Sajid et al., 2015). In addition to their small size, some unstable metallic NPs, such as Ag NPs, ZnO NPs and Cu-based NPs, can release metal ions as a result of oxidation by oxygen and protons present in exposure environments (Sotiriou et al., 2012; Zhao and Wang, 2012). The metal ions shedding from NPs also have the potential to cause toxicity to aquatic biota (Ivask et al., 2014; Valsami-Jones and Lynch, 2015). Assessment of the main source of toxicity caused by NP suspensions (particles themselves or their released ions) is meaningful for environmental risk assessment and management. However, most of the existing studies on the toxicity of metallic NPs to aquatic biota have not attempted to separate the contributions of $NP_{(particle)}$ and $NP_{(ion)}$ to the overall observed toxicity of suspensions of unstable metallic NPs. One of the aims of this thesis was therefore to determine the relative contribution of $NP_{(particle)}$ and $NP_{(ion)}$ to suspension toxicity across different exposure conditions.

For the purpose of development of quick and cost-effective risk assessment, it is required that NPs are grouped and ranked on the basis of their physicochemical and hazardous properties, and of information on the exposure conditions. NPs are subject to undergo complex transformation and redistribution processes (e.g., aggregation, sedimentation, and dissolution), resulting in the change of physicochemical characteristics of NPs over time. These transformation and redistribution processes are reported to be highly dependent on the characteristics of the exposure medium (Valsami-Jones and Lynch 2015; Holden et al., 2016; Selck et al., 2016). Although studies regarding the nano-toxicity have been increasing, the vast majority of these studies only determined their toxicity without providing thorough information regarding behavior, fate and accumulation of NPs, let alone that efforts were undertaken to integrate this information with the observed toxic effects of NPs. This hinders the process of identification of the key factors among physicochemical properties of NPs and water chemistry parameters that determine the toxic effects of NPs, and consequently blocks the development of grouping and ranking schemes for NPs. To address this issue, integrated studies of fate, accumulation and toxicity of CuNPs upon various exposure conditions were systematically performed in this thesis.

The research of this PhD thesis was conducted based on the following research questions, which were answered in the preceding four chapters:

(1) Are particles themselves or their released ions the main source of the observed toxicity and accumulation of CuNPs in *D. magna*?

(2) How does water chemistry affect the toxicity of CuNP suspensions to *D. magna*?

(3) How does water chemistry affect the role of $NP_{(particle)}$ and $NP_{(ion)}$ in the observed toxicity of suspensions of CuNPs to *D. magna*?

(4) How does water chemistry affect the behavior and fate of CuNPs?

(5) How does water chemistry affect the particle-specific toxicity of CuNPs to *D. magna*?

6.1 Answers to research questions

(1) Are particles themselves or their released ions the main source of the overall observed toxicity and accumulation of CuNPs in *D. magna*?

The relative contribution of $NP_{(particle)}$ and $NP_{(ion)}$ to the observed toxicity and accumulation was quantified based on the response addition model. Results provided in chapter 2 demonstrated that $NP_{(particle)}$ was mostly responsible for the observed toxic effects of CuNPs (with a pristine size of 50 nm) and ZnONPs (with a pristine size of 43 nm) in ISO standard test medium, accounting for around 70% of the toxic effects at the LC50 values of the suspensions of CuNPs and ZnONPs. Consistent with the roles of $NP_{(particle)}$ in toxicity, it was also found that $NP_{(particle)}$ was the predominant contributor to the accumulation in *D. magna* rather than $NP_{(ion)}$, at the LC50 of the suspensions of CuNPs and ZnONPs. However, at low exposure concentrations of CuNPs and ZnONPs (0.05 and 0.5 mg/L, respectively), $NP_{(ion)}$ was the major source accumulated by daphnids, rather than $NP_{(particle)}$. The different contribution to accumulation of $NP_{(ion)}$ and $NP_{(particle)}$ might arise from the variations of solubility of NPs under different exposure concentrations of NPs. The percentage of ions released from NPs tends to be higher at lower particle concentration (Zhao and Wang 2012; Hue et al., 2014). Thus, a higher likelihood of $NP_{(ion)}$ might be ingested by organisms at a lower exposure concentration of NPs. Our results indicate that the relative contribution of $NP_{(particle)}$ and $NP_{(ion)}$ to accumulation and toxicity could be impacted by the concentration of NPs.

(2) How does water chemistry affect the toxicity of CuNP suspensions to *D. magna*?

Toxicity of CuNP suspensions decreased with increasing pH and with increasing concentrations of divalent cations and DOC (Chapters 3 and 5). The toxicity of CuNP suspensions was also dependent on the exposure modality, as the exposure modality could exert impact on the environmental behavior and fate of NPs. In the presence of DOC at a concentration ranging from 5 to 50 mg C/L, the toxicity of CuNP suspensions to *D. magna* in the dynamic exposure treatment (i.e., suspensions were kept on a shaker with a vibration speed of 140 rpm along the exposure duration) was 25%-40% higher than the toxicity of CuNP suspensions with the same chemical compositions kept under static exposure condition (i.e., the suspensions were left undisturbed along the exposure duration, Chapter 5). The dissolution profiles of CuNPs upon the addition of DOC in the static and dynamic exposure treatments were found to be similar within 48 h of incubation, 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. This is probably due to the fact that the dynamic flow increased the shear forces and consequently resulted in the disaggregation of NPs (Metreveli et al., 2015; Lv et al., 2016). Hence, the higher toxicity of CuNP suspensions as found in the dynamic exposure treatments, compared to in the static exposure treatments when DOC was added, could result from the enhanced stability of CuNPs. The toxicity of CuNP suspensions to daphnids decreased with dispersion time, resulting in a 30% reduction of the toxicity of CuNPs, expressed on the basis of the initially added CuNP concentration, after 7 days of dispersion (Chapter 3). The reduction of adverse effect of CuNP suspensions to *D. magna* over time could be attributed to the aggregation-accompanied sedimentation behavior of CuNPs, which reduces the bioavailability of CuNPs to *D. magna.* In addition, the pristine size of NPs also had influence on their toxicity. The toxicity of suspensions of CuNPs with a pristine size of 25 nm was higher than the toxicity of suspensions of CuNPs with a pristine size of 50 nm at the same exposure conditions (Chapters 3 and 5). The CuNPs with a pristine size of 25 nm had much higher solubility than CuNPs with a pristine size of 50 nm, due to the larger surface area of the CuNPs with smaller pristine size. These results emphasize that connections between behavior and fate of NPs and toxicity of NPs can be drawn.

(3) How does water chemistry affect the relative contribution of $NP_{(ion)}$ and $NP_{(particle)}$ to the observed toxicity of suspensions of CuNPs to *D. magna*?

The relative contribution of $NP_{(particle)}$ and $NP_{(ion)}$ to the observed toxicity was highly dependent on the water chemistry for exposure (Chapters 3 and 5). At the LC50 of the suspensions of CuNPs (with a pristine size of 50 nm), the main driver of the toxicity of CuNP suspensions was $NP_{(particle)}$ rather than $NP_{(ion)}$ in the absence of DOC and at pH of 7.8. However, the $NP_{(ion)}$ was the main contributor to the overall toxicity at pH 6.5, as a result of higher solubility of CuNPs at a lower pH when no DOC was added. The relative contribution of $NP_{(particle)}$ to the overall toxicity decreased initially and increased afterwards with addition of DOC ranging from 0.5 to 10 mg C/L (Chapter 3). Compared to the case without addition of DOC, the significant reduction of the relative contribution of $NP_{(particle)}$ to the observed toxicity at 0.5 mg DOC/L, was likely attributed to a decrease of attachment of particles on the surface of organisms because of electrostatic repulsion and steric hindrance imparted by DOC (Rosenfeldt et al., 2015). When the DOC was higher than 0.5 mg/L, the increasing contribution of $NP_{(particle)}$ to the observed toxicity was due to the stronger inhibition effect of DOC on dissolution of CuNPs, compared to the case at 0.5 mg DOC/L. The initial size of CuNPs also had an influence on the role of $NP_{(ion)}$ and $NP_{(particle)}$ in the overall toxicity. As a follow-up study, it was found that the toxicity of suspensions of CuNPs (with a pristine size of 25 nm) was mainly driven by $NP_{(ion)}$ rather than by $NP_{(particle)}$ in the same exposure conditions of without addition DOC and pH of 7.8 (Chapter 5). This is probably due to the higher solubility of smaller initial size of CuNPs. With the addition of DOC at a concentration ranging from 5 to 50 mg C/L, the dissolution of CuNPs (with a pristine size of 25 nm) was significantly inhibited and meanwhile the stability of CuNPs was enhanced, which led to the $NP_{(particle)}$ being the main driver of the overall toxicity of suspensions of CuNPs.

(4) How does water chemistry affect the behavior and fate of CuNPs?

Among the water chemistry parameters of pH in the range from 6 to 9, divalent cations at a concentration from 0 to 10 mM and DOC at a concentration from 0 to 50 mg C/L, the divalent cation content was the most significant factor influencing the aggregation behavior of CuNPs, which explained around 46% of the variations of hydrodynamic diameter of CuNPs in exposure media across a range of water chemistry (Chapter 4). This was due to the efficient compression of the double-layer of CuNPs by the addition of divalent cations, as the zeta-potential of CuNP suspensions was found to be significantly decreased by the addition of divalent cations (Chapter 4). DOC was the next most influential factor for the variation of aggregation, which could explain 23% of the variations of aggregation of CuNPs (Chapter 4). Meanwhile, DOC also significantly affected the zeta-potential of CuNP suspensions (Chapter 4). This corroborates that DOC can stabilize NPs via electrostatic repulsion and/or steric hindrance (Gao et al., 2012; Romanello and Fidalgo de Cortalezzi, 2013). The interaction between DOC and divalent cations was also of importance in influencing the aggregation of CuNPs, accounting for 11% of the change of the aggregation of CuNPs (Chapter 4). This is because their interactions can affect aggregation via affecting the surface charge of NPs and via their bridging action (Delay et al., 2011). DOC played a predominant role in influencing the dissolution of CuNPs (Chapter 4). The percentage of ions released from CuNPs in the water column was decreased by 3-5-fold by the addition of DOC ranging from 5 to 50 mg C/L after 48 h of incubation, compared to the case without addition of DOC (Chapter 4). The NOM-dependent reduction of the concentration of ions released from CuNPs in the water column was possibly due to the combination of complexation of dissolved Cu by DOC and of coating of DOC on the surface of Cu particles (Liu and Hurt 2010; Conway et al., 2015). pH, which is often considered as the most influential factor on NP dissolution (Majedi et al., 2014; Odzak et al., 2014), only accounted for 5% of the variations of the dissolution of CuNPs (Chapter 4). Probably because pH can affect the dissolution on the basis of reactivity of nanomaterials (Bian et al., 2011). Even at pH 9 when no DOC was added, more than 50% of the CuNPs (with a pristine size of 25 nm) was dissolved after 48 h of incubation (Chapter 4). This indicates that the CuNPs with a pristine size of 25 nm owned a relatively high reactivity due to their small pristine size, which thus overshadowed the influence of pH in dissolution process of CuNPs. The aggregation behavior of CuNPs was more influential than the dissolution behavior for the variations of sedimentation profiles of CuNPs, as the order of significance of environmental parameters for influencing the total amount of Cu remaining in the water column after 48 h of incubation, was similar to the order of significance of environmental parameters for influencing the aggregation behavior of CuNPs (Chapter 4).

(5) How does water chemistry affect the particle-specific toxicity of CuNPs to *D. magna*?

It was found that the particle-specific toxicity of CuNPs strongly depends on the water chemistry for exposure (Chapter 5). The particle-specific toxicity of CuNPs in line with the trends in toxicity for $\text{CuNP}_{\text{(ion)}}$, decreased with increasing pH and with increasing concentrations of divalent cations and DOC. The increased toxicity of $\text{CuNP}_{\text{(particle)}}$ under reduced pH may be explained by the sensitivity changes of freshwater zooplankton (Locke et al., 1991; Havens et al., 1993; Hu et al., 2017), and consequently reduced the tolerance of *D. magna* to $\text{CuNP}_{\text{(particle)}}$. The reduced toxicity of $\text{CuNP}_{\text{(particle)}}$ with addition of cations may result from the enhanced aggregation imparted by the cations, which decreased the effective surface area of $\text{CuNP}_{\text{(particle)}}$ to *D. magna* and reduced the toxicity of $\text{CuNP}_{\text{(particle)}}$. The decrease in toxicity for $\text{CuNP}_{\text{(particle)}}$ with the addition of DOC may be a function of passivation of the particle surface by DOC adsorption (Fabrega et al., 2009).

6.2 Implications for environmental risk assessment

In "Nanosafety in Europe 2015-2025: Towards Safe and Sustainable Nanomaterials and Nanotechnology Innovations" (Strategic Research Agenda; SRA), the key issues in terms of nanosafety requiring rapid solutions in the near future have been described. In general, the research needs and priorities for the coming 10 years constitute four thematic areas: 1) nanomaterial identification and classification; 2) nanomaterial exposure and transformation; 3) mechanisms underlying effects of nanomaterials on humans and environment; 4) development of tools for risk assessment and management (Savolainen et al., 2013). In this thesis, the environmental behavior, fate, accumulation and toxicity of CuNPs to *D. magna* across different exposure conditions were systematically investigated. This is clearly meaningful to improve our understanding of transformation and hazards of NPs upon various exposure environments. Hence, two of the key issues described in SRA were addressed in this thesis.

Nanosafety has been increasingly studied, while thousands of the publications on this issue have directly focused on the toxicity of NPs with limited focus on transformation and fate of NPs in related environmental compartments (Baalousha et al., 2016). Figure 6.1 shows a summary of the relationships between characteristics of the receiving environment and the behavior, fate and hazards of NPs, as described in this thesis. It demonstrated that dissolution, aggregation and sedimentation are key environmental processes determining the fate of NPs in environmental compartments. Furthermore, it was found that links between the fate of NPs and the toxicity of the particles can be laid. Hence, this thesis highlights that integrated research regarding fate, accumulation and hazards of NPs is needed in order to comprehensively understand the hazards of NPs to environment and to decrease the uncertainties of data regarding the ecotoxicology of NPs.

It has been recognized that hazard assessment of NPs through current standardized testing guidelines for evaluating the risk of conventional chemicals is applicable with some adaptation (Diez-Ortiz et al., 2015). The use of a standard test medium can make the toxicity results comparable, while concerns regarding the environmental relevance of the standardized protocols have been raised. This thesis demonstrates that characteristics of the receiving environment, especially NOM, can largely affect the fate and toxic effects of NPs. NOM is ubiquitous in the natural environment. However, NOM is not taken into consideration in current standard protocols for evaluating adverse effects of NPs. Hence, the parameter NOM is recommended to be included in the hazard testing guidelines for NPs in order to make toxicity data of NPs more environmentally relevant. In addition, it was found that the toxicity of CuNP suspensions decreased over time, while the toxicity of CuNP suspensions was higher under dynamic exposure modality than under statically exposure modality. In the above mentioned cases, the change of the toxicity of CuNP suspensions was corresponding to the variations of concentrations of particles stabilized in suspensions. These results indicate that the stabilized particles are more bioavailable to *D. magna* than non-stabilized particles. As NPs would partially or even fully deposit from the media under gravity or simply agglomerate or aggregate with environmental solids, the dose-response curves of NPs to *D. magna* plotted with the initially added concentration of NPs are likely to underestimate the toxic effects of NPs to the organisms. Therefore, dose-response curves of NPs to aquatic organisms are recommended to be evaluated according to the concentrations of NPs in the most relevant environment that the testing organisms distribute in.

This thesis demonstrated that the relative contribution of NPs themselves and their released ions to the observed toxicity caused by NP suspensions was highly dependent on the pristine size of NPs and on the characteristics of the receiving environment. These findings imply that the roles of NPs and their released ions in the observed toxicity should be interpreted with care. In addition, the toxicity data of CuNPs with two different pristine sizes and of ZnONPs corroborates that modifying the pristine size and chemical composition of NPs is useful for the purpose of the safe by design of new NPs.

6.3 Future research outlook and recommendations

The accurate quantification of environmental release and concentrations of NPs in environment compartments is a critical pre-requirement for evaluating their potential risks. However, it is currently quite challenging to accurately quantify and monitor the concentrations of NPs in natural environments due to the analytical limitations. Hence, research aiming to improve the accuracy of detection and quantification of the actual environmental concentrations of NPs, is certainly needed in the future. Quantification of accumulation of NPs in organisms is meaningful for elucidating the real dose (effective dose) responsible for a certain toxic effect. In this thesis, the accumulation of CuNPs and ZnONPs in *D. magna* was measured. However, the mechanisms underlying the accumulation process are still unclear, let alone the mechanisms governing the distribution and elimination of NPs from organisms. It is therefore critical to conduct further studies in this field, as such studies can advance our understanding of the toxicokinetics of NPs.

Understanding the mode(s) of actions of NPs leading to toxic effects is beneficial for identifying the most appropriate dose-metrics for NPs and for grouping and ranking of different NPs. In my thesis, the particle-specific contribution to the observed toxicity of CuNP suspensions upon varying exposure environments was quantified through the response addition model. However, the specific mode(s) of actions of NPs underlying the toxicity of NPs upon various environmental conditions were not investigated in this thesis and the mode(s) of actions of NPs are far from clearly known. Hence, further efforts should be devoted to determine the mode(s) of actions of NPs related to the adverse effects of NPs.

All toxicity studies reported in this thesis focused only on one single type of NPs (either spherical CuNPs or ZnONPs). In fact, different types of NPs generally coexist in the environment due to the widespread applications of various NPs. It has been found that the coexistence of different types of NPs has the potential to induce different toxicity on exposed organisms, compared to their individual effects (Li et al., 2015; Hua et al., 2016). Nonetheless, the combined toxic effects of NP mixtures are still only partly understood. Hence, further assessment of the interactions of more binary and even ternary mixtures of metallic NPs should be conducted in order to better interpret and predict the toxicity of NPs in natural environments.

The toxicity of CuNPs on the model organisms (*Daphnia magna*) were systematically investigated in this thesis, while knowledge gaps regarding the toxic effects of CuNPs on the food chain and biomagnification are still existing. As a result of food web interactions, uptake of NPs by organisms which would not be directly exposed is expected to occur. Hence, the trophic transfer and biomagnification of CuNPs require further investigation.

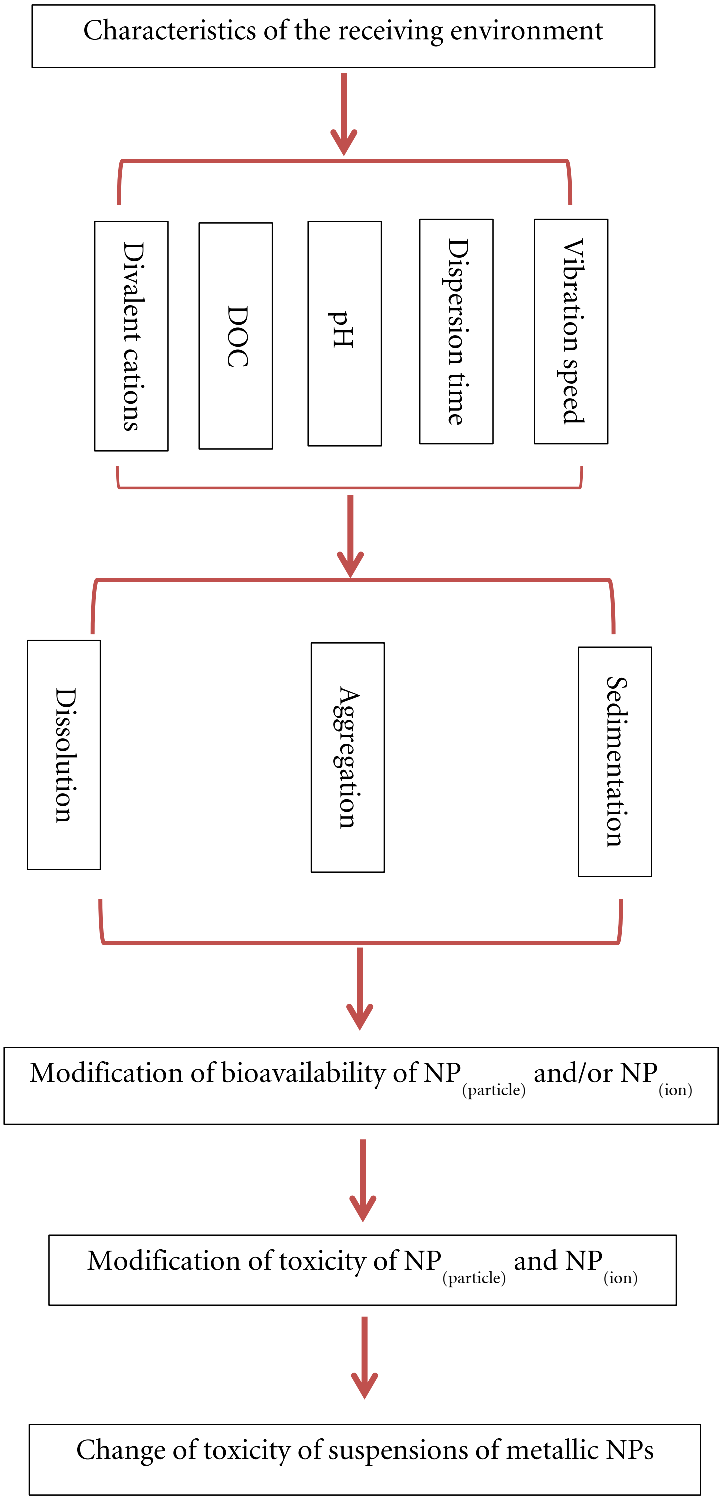


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Figure 6.1 Scheme of how the characteristics of the receiving environment affect the toxicity of NP suspensions. The characteristics of the receiving environment can affect the behavior and fate of NPs and further influence the bioavailability of NPs and their released ions to *D. magna*, which could result in the variations of adverse effects of NPs themselves and their released ions and consequently change the toxicity of suspensions of NPs to organisms. DOC is the abbreviation of dissolved organic carbon.

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Summary

Considering the widespread applications of nanoparticles (NPs) in consumer and industrial products, concern regarding the toxic effects of NPs to humans and the environment has been sharply growing in recent years. Although the growing concern has led to increasing efforts to assess nanotoxicology, the underlying mechanisms of toxicity of metallic NPs are still largely unclear. To date, it has been widely recognized that the toxicity of NPs is highly dependent on the physicochemical characteristics of the environment in which the NPs are introduced (the exposure medium) and the physicochemical properties of the metallic NPs. To improve our understanding of the toxicity of NPs, research is required that integrates the impact of the physicochemical characteristics of NPs and the impact of differences in water composition with the toxicity profiles of NPs. However, the majority of hazard assessment studies of metallic NPs only determined their toxicity without providing thorough information regarding behavior, fate and accumulation of NPs, let alone that efforts were undertaken to integrate this information with the observed toxicity of NPs. Suspensions of metallic NPs typically are a mixture of particles and ions. Evaluation of the issue whether the main sources of toxicity of NP suspensions are either the NPs or their released ions, is critical for environmental risk assessment and management of released NPs. Nevertheless, most of the studies regarding nanotoxicology have not separated the relative contribution of the particles themselves and their released ions to the observed toxicity. In this thesis, studies integrating fate, accumulation and the related toxicity of CuNPs to *Daphnia magna* across different exposure regimes were systematically conducted. Furthermore, the impact of water chemistry on the relative contribution of particles and their released ions to the observed toxicity of suspensions of CuNPs, and on the particle-specific toxicity of CuNPs was evaluated.

In Chapter 2, the relative contribution of particles and their released ions to the toxicity of suspensions and accumulation of CuNPs in *D. magna* was quantified by using the response addition model. For this study, CuNPs (with a pristine size of 50 nm; spherical shape; advertised surface area, 6-8 m^2/g) and ZnONPs (with a pristine size of 43 nm; spherical shape; advertised surface area, 27 m^2/g) were used to prepare exposure suspensions. It was found that at the LC50 (lethal concentration at which 50% of the organisms dead) of the CuNP and ZnONP suspensions, the relative contributions of ions released from CuNPs and ZnONPs to the observed toxicity were around 26% and 31%, respectively. This indicates that particles rather than their released ions were the dominant descriptor for the observed toxicity. Taking accumulation as an endpoint, similar patterns of relative contributions of particles and their released ions were determined at exposure concentrations close to the LC50 (approximately 0.1 mg/L and 1 mg/L of CuNPs and ZnONPs, respectively). This implies that there is likely a direct causal relationship between accumulation and toxic effects of NPs. On the other hand, at low exposure concentrations of CuNPs and ZnONPs (around 0.05 and 0.5 mg/L, respectively), the dissolved ions shedding from the NPs were predominantly accumulated by the daphnids. The relative contributions of particles and their released ions to the overall

observed accumulation in organisms are hence dependent on the concentration of NPs to which the organisms are exposed.

In Chapter 3, the toxicity of suspensions of CuNPs (with a pristine size of 50 nm) to *D. magna* upon modification of the exposure conditions was investigated. This was done by aging the suspensions of CuNPs for up to 7 days and by varying the water chemistry parameters including pH and dissolved organic carbon (DOC) concentration. The LC50 value for suspensions of CuNPs to *D. magna* decreased by about 30% after 7 days of aging, compared to the LC50 of freshly prepared suspensions of CuNPs. The LC50 value of suspensions of CuNPs increased by more than 12-fold upon addition of DOC at concentrations ranging from 0 to 10 mg C/L. There was a 3-fold increase in the LC50 value of suspensions of CuNPs upon changing the pH of the exposure medium from 6.5 to 8.5. Furthermore, it was found that within 7 days of aging of the exposure medium (at pH 7.8 and without addition of DOC), the overall observed toxicity could be mostly ascribed to the particles present in suspension (around 70%). However, the presence of DOC at concentrations ranging from 0.5 to 10 mg C/L or the reduction of pH of the exposure medium from 8.5 to 6.5 reduced the relative contribution of particles to the observed toxicity. We thus conclude that the toxicity of CuNP suspensions and the roles of particles and their released ions regarding the observed toxicity are highly dependent on the exposure conditions.

In Chapter 4, the effects of water chemistry parameters (pH, divalent cations and DOC) on the behavior and ultimate fate of CuNPs (with a pristine size of 25 nm; specific surface area, 30-50 m^2/g; shape spherical), were systematically investigated. The results demonstrated that divalent cation content was the most influential factor for the aggregation behavior of CuNPs, which explained around 46% of the variations of the aggregation of CuNPs in exposure media across a range of water chemistry. DOC concentration was the next most influential factor, which could explain 23% of the variation of the aggregation of CuNPs. The interaction between divalent cations and DOC also played an important role in influencing the aggregation of CuNPs, accounting for 11% of the change of aggregation of CuNPs. DOC concentration was the predominant factor for the variation in dissolution profiles of CuNPs. The percentage of ions released from CuNPs in the water column was decreased by 3-5-fold by the addition of DOC at concentrations ranging from 5 to 50 mg C/L after 48 h of incubation, compared to the case without addition of DOC. pH in the range from 6 to 9 only accounted for 5% of the variation of dissolution of CuNPs. In addition, the sedimentation profile of CuNPs was mostly influenced by divalent cation content and DOC concentration and the interaction of divalent cations and DOC. These results are helpful for improving our understanding and prediction potential on how and to what extent environmental factors affect the behavior and fate of metallic NPs upon a range of environmental conditions.

In Chapter 5, the impact of water chemistry on the particle-specific toxicity of CuNPs to *D. magna* was determined. CuNPs with a pristine size of 25 nm were exposed to *D. magna* upon modification of the water chemistry parameters (i.e., pH, divalent cations and DOC). It corroborated that the toxicity of the suspensions of CuNPs decreased with increasing pH and with increasing concentrations of divalent cations and DOC. This is the net effect of water chemistry on the toxicity of particles and ions released from CuNPs. Based on the response addition model, it was found that the toxicity of the particles was in most cases lower than the toxicity of ions released from CuNPs. The particle-specific toxicity of CuNPs decreased with increasing pH and with increasing concentrations of divalent cations and DOC. The variations of the particle-specific toxicity were related to their behavior and fate upon varying exposure conditions. The toxicity of CuNP suspensions was also dependent on the exposure modality: in the presence of DOC at a concentration ranging from 5 to 50 mg C/L, the toxicity of CuNP suspensions to *D. magna* in the dynamic exposure treatment was 25%-40% higher than the toxicity of CuNP suspensions with the same chemical composition under static exposure conditions. The dissolution profiles of CuNPs upon the addition of DOC in the static and dynamic exposure treatments were found to be similar within 48 h of incubation, whereas the extent of aggregation of CuNPs was smaller within the 48 h of incubation in the dynamic treatments than in the static treatments when DOC was added. Hence, the higher toxicity of CuNP suspensions as found in the dynamic exposure treatments, could be attributed to the enhanced stability of CuNPs in the aquatic medium. These results indicate that stabilized particles are more bioavailable to *D. magna* than non-stabilized particles.

To conclude, this thesis provides further insight in understanding the behavior, fate, accumulation and toxicity of CuNPs across different exposure conditions. Furthermore, our study emphasizes that studies integrating the dynamic physico-chemical characteristics of NPs into their toxicity profiles are needed in order to comprehensively interpret the hazards of NPs to the environment. Also, the importance of water chemistry in determining the relative contributions of particles and their released ions to the observed toxicity of NP suspensions and in determining the particle-specific toxicity of NPs, is clearly highlighted.

Samenvatting

Vanwege de vele toepassingen van nanodeeltjes (nano's) in consumenten- en industriële producten, is de vraag of er effecten van nano's te verwachten zijn op mens en milieu in de afgelopen jaren sterk actueel geworden. Hoewel de groeiende bezorgdheid heeft geleid tot meer studies gericht op nanotoxicologie, zijn de onderliggende mechanismen van toxiciteit van metaalhoudende nano's nog steeds onduidelijk. Tot op heden is algemeen bekend dat de toxiciteit van nano's sterk afhankelijk is van de fysisch-chemische eigenschappen van het blootstellingsmedium en de kenmerken van de metaalhoudende deeltjes. Om ons begrip van de effecten van nano's te verbeteren, is het nodig om onderzoek te doen naar het gedrag van nano's in relatie tot de fysisch-chemische eigenschappen van milieucompartimenten. Ondanks dit alles is in het merendeel van de risicobeoordelingsstudies vaak het effect gerapporteerd zonder grondige informatie te geven over gedrag, lot en accumulatie van nano's, laat staan dat er inspanningen worden gedaan om deze informatie te integreren met de waargenomen toxische effecten van nano's. Metallische nanodeeltjes zijn veelal een mix van deeltjes en ionen. Toxische effecten van suspensies van nanodeeltjes kunnen worden beschreven op basis van de toxiciteit van de deeltjes in suspensie en de toxiciteit van de ionen die van de nanodeeltjes vrijkomen, waarbij het verschil tussen beiden van cruciaal belang is voor het milieurisicobeoordeling. Niettemin hebben de meeste studies over nanotoxicologie de relatieve bijdrage van de deeltjes en hun vrijgegeven ionen niet gescheiden. In dit proefschrift werden studies over het integreren van het lot, de accumulatie en de daarmee verband houdende toxiciteit van koper-nano's (CuNPs) voor Daphnia magna over verschillende blootstellingsregimes systematisch uitgevoerd. Bovendien werd de impact van waterchemie op de relatieve bijdrage van deeltjes en hun vrijgegeven ionen aan de waargenomen toxiciteit van suspensies van CuNPs en op de deeltjesspecifieke toxiciteit van CuNPs geëvalueerd.

In hoofdstuk 2 werden de relatieve bijdragen van deeltjes en hun vrijgegeven ionen aan toxiciteit en accumulatie van NP's in D. magna gekwantificeerd door gebruik te maken van het response-additie model. Voor deze studie werden CuNPs (50 nm, bolvormig, oppervlakte 6-8 m2/g) en ZnONPs (43 nm, bolvormig, oppervlakte 27 m2/g) gebruikt. Gevonden werd dat de relatieve bijdrage van de ionen op de lethale concentratie (LC50 waarde) van CuNP en ZnONP respectievelijk 26% en 31% was. Dit wijst erop dat deeltjes in plaats van hun vrijgegeven ionen de dominante parameter zijn voor het beschrijven van de waargenomen toxiciteit. In geval van accumulatie als eindpunt werd een vergelijkbare relatieve bijdrage gevonden (concentraties op LC50 niveau van respectievelijk 0,1 mg/L en 1 mg/L CuNPs en ZnONPs). Dit betekent dat er waarschijnlijk een direct causaal verband bestaat tussen accumulatie en effecten van nano's. Bij lage blootstellingsconcentraties CuNPs en ZnONPs (respectievelijk 0,05 en 0,5 mg/L) zijn de opgeloste ionen vanuit de nano's de meest dominante parameter die de accumulatie in daphnia beschrijft. De relatieve bijdragen van deeltjes en hun vrijgegeven ionen aan de waargenomen accumulatie in organismen zijn derhalve afhankelijk van de concentratie van nano's waaraan de organismen blootgesteld zijn.

In hoofdstuk 3 wordt de invloed van blootstellingsomstandigheden op de toxiciteit van suspensies van CuNPs (50 nm) voor D. magna onderzocht bij wijziging van de verhouding tussen nanodeeltjes en metaalionen. Dit werd mogelijk door de suspensies van CuNPs gedurende 7 dagen te verouderen en door de waterchemie te variëren, waaronder verandering van pH en de DOC concentratie. De LC50 waarde van suspensies van CuNPs voor D. magna daalde ongeveer 30% na 7 dagen veroudering, vergeleken met de LC50 van vers bereide suspensies van CuNPs. De LC50 waarde van suspensies van CuNPs steeg meer dan twaalf keer na toevoeging van DOC in concentraties variërend van 0 tot 10 mg C/L. Er was een 3-voudige toename in de LC50-waarde van suspensies van CuNPs bij het veranderen van de pH van het blootstellingsmedium van 6,5 tot 8,5. Verder bleek dat de totale waargenomen toxiciteit binnen 7 dagen na het verouderen van het blootstellingsmedium (bij pH 7,8 en zonder toevoeging van DOC) meestal kan worden toegeschreven aan de deeltjes die aanwezig zijn in suspensie (ongeveer 70%). De aanwezigheid van DOC in concentraties van 0,5 tot 10 mg C/L of verlaging van de pH van het blootstellingsmedium van 8,5 tot 6,5 verminderde echter de relatieve bijdrage van deeltjes tot de waargenomen toxiciteit. Wij concluderen derhalve dat de toxiciteit van CuNP suspensies en de rol van deeltjes en hun vrijgegeven ionen in de waargenomen toxiciteit sterk afhankelijk waren van de blootstellingsomstandigheden.

In hoofdstuk 4 worden de effecten van waterchemie (pH, kationen en opgeloste organische koolstof (DOC)) op het gedrag en het uiteindelijke lot van CuNPs (25 nm, oppervlak 30-50 m2/g , bolvormig), systematisch onderzocht. De resultaten toonden aan dat kationen bij een concentratie van 0 tot 10 mM de meest invloedrijke factor (46%) waren voor het beschrijven van de variatie op het aggregatiegedrag van CuNPs. DOC was de daaropvolgende invloedrijke factor (23%) voor het beschrijven van de variatie van aggregatie van CuNPs. De interactie tussen kationen en DOC speelde hierbij ook een belangrijke rol (11%). DOC was de overheersende factor voor het beschrijven van de variaties van gehaltes van CuNPs in oplossing. Het percentage vrije ionen afkomstig uit de CuNP's werd 3-5 keer verlaagd door toevoeging van DOC in concentraties die varieerden van 5 tot 50 mg C/L na 48 uur incubatie, vergeleken met de situatie waarin geen DOC was toegevoegd. De zuurgraad (pH 6 tot 9) kon 5% van de variaties beschrijven van de gehaltes aan CuNPs in suspensie. Daarnaast werd het sedimentatieprofiel van CuNPs voornamelijk beïnvloed door de kationen en de DOC concentratie en door de interactie van deze concentraties. Deze resultaten zijn nuttig voor het verbeteren van ons begrips- en voorspellingspotentieel over hoe en in welke mate milieufactoren het gedrag en het lot van metaalhoudende nano's beïnvloeden.

In hoofdstuk 5 werd de invloed van waterchemie op de deeltjes-specifieke toxiciteit van CuNPs voor de watervlooi D. magna bepaald. D. magna werd blootgesteld aan CuNP's (25 nm) en de waterchemie (pH, kationen en DOC) werd gemanipuleerd. De resultaten tonen aan dat de toxiciteit van de CuNP suspensies afneemt met toenemende pH en met toenemende kation en DOC concentraties. Dit alles is het netto effect van veranderingen van waterchemie op de toxiciteit van deeltjes en ionen die uit CuNP's vrijkomen. Op basis van het response-additiemodel bleek dat de toxiciteit van de deeltjes in de meeste gevallen lager was dan de toxiciteit van ionen die vrijkomen van CuNP's. De deeltjes-specifieke toxiciteit van CuNP's daalde met toenemende pH en met toenemende concentraties kationen en DOC. De variaties van de deeltjes-specifieke toxiciteit waren gerelateerd aan hun gedrag bij verschillende blootstellingsomstandigheden. De toxiciteit van CuNP suspensies was ook afhankelijk van de blootstellingsmodaliteit, omdat aangetoond werd dat de blootstellingsmodaliteit invloed uitoefent op het gedrag en het lot van NP's: In de aanwezigheid van DOC bij een concentratie van 5 tot 50 mg C/L was de toxiciteit van CuNP suspensies voor D. magna onder dynamische blootstelling 25-40% hoger dan de toxiciteit van CuNP suspensies met dezelfde chemische samenstellingen die onder statische condities (niet geroerd) werden gehouden. De oplos-profielen van CuNP's bij de toevoeging van DOC bij de statische en dynamische blootstellingsbehandelingen bleken gelijk te zijn binnen 48 uur incubatie, terwijl de mate van aggregatie van CuNP's kleiner was bij de dynamische behandelingen dan in de statische behandelingen onder toevoeging van DOC. Hieruit blijkt dat de hogere toxiciteit van CuNP suspensies zoals gevonden in de dynamische blootstellingsbehandelingen, kan worden toegeschreven aan de verhoogde stabiliteit van CuNPs in suspensie. Deze resultaten geven aan dat gestabiliseerde deeltjes meer biobeschikbaar zijn voor D. magna dan niet-gestabiliseerde deeltjes.

Dit proefschrift geeft inzicht in het gedrag, het lot, de accumulatie en toxiciteit van CuNP's onder verschillende blootstellingscondities. Daarnaast benadrukt ons onderzoek dat studies die de dynamische fysisch chemische karakterisering van NP's integreren in hun effecten nodig zijn om de risico's van nano's in het milieu grondig te interpreteren. Tevens wordt het belang van waterchemie onderstreept bij het bepalen van de relatieve bijdragen van deeltjes en hun vrijgegeve ionen om de toxiciteit van NP suspensies te beschrijven.

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Yinlong Xiao

Sep 2017, Leiden

Curriculum Vitae

Yinlong Xiao was born on 25th Feb. 1988, in Ziyang, Sichuan Province, China. After 3-year high school study in Anyue Middle School, he started his 4-year university education in College of Forestry, at Sichuan Agricultural University, from where he graduated with a bachelor degree in Forestry Science in 2011. After that, he continued his master study in the College of Forestry, at Sichuan Agricultural University. During this period, he was supervised by Prof. Tingxing Hu and Dr. Lihua Tu and his master research mainly focused on the effects of nitrogen deposition on forestry soil ecosystems. After 2-years of study, he obtained his master degree in 2013. Following completion of his master thesis, he was awarded a government scholarship by China Scholarship Council (CSC) to continue his PhD research at the Institute of Environmental Sciences (CML), Leiden University. From 2013 to 2017, he undertook a PhD research on determining the fate, accumulation and ecotoxicity of nanoparticles in aquatic environments under the supervision of Prof. Willie Peijnenburg and Dr. Martina Vijver. This thesis is the outcome of his PhD research. After his PhD journey, Yinlong will continue his research career.

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