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

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Chapter 5

Trophic transfer of Cu nanoparticles in a simulated aquatic food chain

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Abstract

The goal of the current study was to quantify the trophic transfer of copper nanoparticles (CuNPs) in a food chain consisting of the microalgae *Pseudokirchneriella subcapitata* as a representative of primary producers, the grazer *Daphnia magna,* and the omnivorous mysid *Limnomysis benedeni*. To quantify the size and number concentration of CuNPs in the biota, tissue extraction with tetramethylammonium hydroxide (TMAH) was performed and quantification was done by single particle inductively coupled plasma mass spectrometry (sp-ICP-MS). The bioconcentration factor (BCF) of the test species for CuNPs varied between 10^2 – 10^3 L/kg dry weight when expressing the internal concentration on a mass basis, which was lower than BCF values reported for Cu^{2+} (10^3 – 10^4 L/kg dry weight). The particle size of CuNPs determined by sp-ICP-MS ranged from 22 to 40 nm in the species. No significant changes in the particle size were measured throughout the food chain. Moreover, the measured number of CuNPs in each trophic level was in the order of 10^{13} particles/kg wet weight. The calculated trophic transfer factor (mass concentration basis) was > 1. This indicates biomagnification of particulate Cu from *P. subcapitata* to *L. benedeni*. It was also found that the uptake of particulate Cu (based on the particle number concentration) was mainly from the dietary route rather than from direct aqueous exposure. Furthermore, dietary exposure to CuNPs had a significant effect on the feeding rate of mysid during their transfer from daphnia to mysid and from algae through daphnia to mysid. This work emphasizes the importance of tracing the particulate fraction of metal-based engineered nanoparticles when studying their uptake and trophic transfer.

Keywords: Cu nanoparticles; uptake route; bioconcentration factor; biomagnification; single particle ICP-MS

5.1 Introduction

Copper nanoparticles (CuNPs) are known for their excellent thermophysical properties and their fairly inexpensive synthesis. As a result, CuNPs have many applications, including in semiconductors, electronic chips, and heat transfer nanofluids (Kim et al., 2016; Li et al., 2021). However, CuNPs can be released into the aquatic environment (Holden et al., 2016; Keller and Lazareva, 2013). This way, CuNPs can be taken up by a variety of organisms and might also be transferred along a food chain (Siddiqui and Bielmyer-Fraser, 2019; Tangaa et al., 2016). Examples reported in literature evidenced that metal-based NPs can affect multiple levels in the food chain, e.g. primary producers and herbivorous consumers following exposure to AgNPs (Kalman et al., 2015; Sharma et al., 2019; Yan and Wang, 2021), quantum dots (Rocha et al., 2017) and TiO_2NPs (Chen et al., 2015; Wang et al., 2017). Several studies reported that dietary uptake of NPs can induce more severe effects compared to direct waterborne exposure (Jackson et al., 2012; Kalman et al., 2015; Wu et al., 2017). However, the data available for the trophic transfer of CuNPs through a multiple-level aquatic food chain are limited and inconclusive.

Distinguishing the differences in uptake pathways and trophic transfer processes between the particulate form of CuNPs and their dissolved-release ions is a critical gap in existing research. Trophic transfer ability can be quantified from the ratio of the mass concentration of NPs in the body of a predator and the mass concentration of these NPs in the prey (Chen et al., 2015; Lee et al., 2015; Yan and Wang, 2021). Body mass concentrations are generally quantified as the total metal content including both the NPs and the dissolved metal ions (Baccaro et al., 2018). However, some studies

have reported that NPs cause as much effect or even more effect than the ionic form of the same metal (Shoults-Wilson et al., 2011; Xiao et al., 2015). For instance, Xiao et al. (2015) found that Cu particles rather than the dissolved Cu ions were the major source of toxicity for water-exposed daphnids. Others found that the dissolved fraction of metal ions causes most of the effects when evaluating NPs toxicity (Adam et al., 2014; Jo et al., 2012). To evaluate the environmental risks of the particulate form of Cu, we expressed the transfer of CuNPs through the food chain based on the particle's number and size distribution in biota of different trophic levels. We also did the same by using mass as the basis for expressing transfer. To date, a limited number of studies (Monikh et al., 2019a; Heringa et al., 2018; Monikh et al., 2021; Taboada-López et al., 2018) have reported on particle number based transfer. This can be explained by the analytical challenges of quantifying particle numbers in complex media such as the whole body and specific tissues of biota.

In the present study, we aim to investigate: (1) whether CuNPs transfer through an aquatic food chain and undergo biomagnification in consumers; (2) to what extent CuNPs can transfer in particulate, and ionic forms and how the particle size and number change in different organisms; and (3) the effect of dietary CuNPs exposure on the survival and feeding rate of the predator (mysids). Accordingly, we used the mass and particle number of the CuNPs as dose metrics. The microalga *Pseudokirchneriella subcapitata* (Kalman et al., 2015; Wang et al., 2019) was selected as a primary producer and the zooplankton *Daphnia magna* (Chen et al., 2015; Dalai et al., 2014; Lee et al., 2015; Ribeiro et al., 2017; Wu et al., 2017) as being representative of consumers grazing on algae. The omnivorous mysid *Lymnomysis benedeni* (Boda and Borza, 2013; Gergs et al., 2008; Hanselmann,

2012) was selected as the predator. The predator *L. benedeni* can provide information on three food transfer cases: case-1 from *P. subcapitata* to *L. benedeni*, case-2 from *D. magna* to *L. benedeni*, and case-3 from *P. subcapitata* to *D. magna* to *L. benedeni*. Moreover, we developed a multistep sample preparation method consisting of the extraction of particles from tissues with tetramethylammonium hydroxide (TMAH). Then, the extracted samples were used to quantify the separated CuNPs by single particle inductively coupled plasma mass spectrometry (sp-ICP-MS). The obtained findings emphasize the roles of particle number and size of particulate Cu in the aquatic organisms in assessing the trophic transfer of CuNPs. Hence, this work will gain a better understanding of the risk of soluble NPs to ecosystems.

5.2 Materials and Method

5.2.1 Test materials

Spherical CuNPs were purchased from IoLiTecGmbh (Heibronn, Germany) with a specific surface area of 30-50 m^2/g, > 99.5% purity and the nominal size of 25 nm. TMAH (25% w/w) and nitric acid (HNO_3, 65%) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

5.2.2 Physicochemical characterization

The morphology and size of the CuNPs were determined using transmission electron microscopy (TEM, JEOL 1010, JEOL Ltd., Japan). The hydrodynamic diameter (Z-average) and zeta potential

(mV) of 1 mg/L NPs in Milli-Q water and in different exposure media were analyzed using a ZetaSizer Nano-ZS instrument (Malvern, Instruments Ltd., UK). Furthermore, sp-ICP-MS (PerkinElmer, NexION 2000 ICP-MS operating in sp mode) was applied to measure the particle number concentration and the size distribution of the particles. The method development and validation have been performed in-house and published lately (Monikh et al., 2021). To measure the number of the particles, CuNPs were mixed with Woods Hole Medium (Janet Stein, 1982) and Elendt M7 medium (OECD, 2004; Samel et al., 1999) (submerging 1 mL of a 100 mg/L stock suspension into 99 mL) for 24 h. During the 24 h of incubation, all suspensions were stored in a climate chamber under a 16:8 h light-dark cycle (temperature: 22 ± 1 °C, RH: 80.0%). The samples were collected at the top 1.5-2 cm layer of the dispersions at 0 and 24 h. Meanwhile, the ion release profiles and the particle aggregation kinetics of the CuNPs were evaluated. The results are shown in Supplementary data.

5.2.3 Trophic transfer experiments

The assembled food chains consisting of either 2 or 3 levels were examined, using *P. subcapitata, D. magna* and *L. benedeni*. The details of the culturing of the organisms are described in the Supplementary data.

Case-1: 2-level food chain from *P. subcapitata* to *L. benedeni.* The algal (*P. subcapitata*) cells at a density of 5.0×10^6 cells/mL were first exposed for 24 h in a suspension of 1 mg/L CuNPs as prepared in the Woods Hole Medium (Janet Stein, 1982). The compositions of Woods Hole Medium are listed in Table S5.1, Supplementary data. The

treatment group contained 10 replicates with 6 controls, containing only test medium and algae. All test glass vials (200 mL) with algae and test solutions (50 mL) were covered using cotton to allow for CO_2 diffusion. The containers were placed on a shaker (110-120 rpm) at 22 ± 2˚C and continuously illuminated at a density of 31-51 μmol m^{-2} s^{-1} as measured via Apogee line quantum sensors (Apogee Instruments, MQ-301). The pH of the test media was measured at the beginning and end of the exposure period. The harvested algal cells pre-contaminated with NPs in the Woods Hole Medium were introduced as food to the mysids for 24 h after washing 3 times. As part of the washing procedure, the algae were centrifuged at 2000 rpm for 5 min with 0.05 M ethylene diamine-tetra acetic acid (EDTA) twice and then once with clean Elendt M7 medium. As a chelating agent, EDTA has been frequently used to complex and remove Cu bound to the surface of organisms in previous studies (Bossuyt and Janssen, 2005; Canuel et al., 2021; Gonzalez-Estrella et al., 2017; Wang et al., 2017; Wu et al., 2017). The targeted algal concentration was around 1.0 × 10^6 cells/mL in each beaker containing one *L. benedeni* individual in 50 mL Elendt M7 medium. The used mysids were allowed to clean their guts one day before exposure. Four replicates were used for each treatment and 20 mysids were included in each replicate. There were also four replicates for the control (without CuNPs or metal nanoparticles). The exposed *P. subcapitata* and *L. benedeni* were washed with 0.05 M EDTA twice and with PBS/Milli-Q water once, then they were directly snap frozen with liquid nitrogen and stored at -80 ˚C before characterization using sp-ICP-MS.

Case-2: 2-level food chain from *D. magna* to *L. benedeni.* *D. magna* (< 24 h) was exposed to 1 mg/L CuNPs for 24 h and washed thrice using clean Elendt M7 medium. Then each *L. benedeni* (without feeding for

24 h) was fed with 5 exposed daphnids once during the 24 h feeding period. Each mysid was placed in a beaker with 50 mL Elendt M7 medium. Four replicates were used for each treatment and 20 mysids were included in each replicate. There were also four replicates for the control (non-exposure). After 24 h, *D. magna* and *L. benedeni* were sampled and washed with 0.05 M EDTA twice and once with Milli-Q water, then directly snap frozen with liquid nitrogen and stored at -80 ˚C for characterization with sp-ICP-MS.

Case-3: 3-level food chain from *P. subcapitata* to *L. benedeni* through *D. magna*. The harvested algal cells pre-contaminated with CuNPs in an aqueous medium were introduced as food to the daphnids for 24 h after washing 3 times with clean Elendt M7 medium. The targeted algal concentration was around 1.0×10^6 cells/mL in each beaker containing 10 *D. magna* individuals (< 24 h). These daphnids were gut cleaned beforehand in 100 mL Elendt M7 medium. Then five of these daphnids were fed to each *L. benedeni* for 24 h as mentioned above for the other food chains.

To quantify the particle or mass concentrations of CuNPs in organisms, three replicates with each treatment containing > 100 daphnids or 15-20 alive mysids were used. To assess the survival of fed mysids, each treatment contained 10 replicates with 15 - 20 alive mysids. For the feeding behavior of mysids on account of the number of ingested daphnia, each treatment contained more than 45 alive mysids.

5.2.4 Extraction of CuNPs from biological tissues

In order to extract CuNPs from the organisms, samples of species of different trophic level were treated with 1 mL of 20% (w/w) TMAH.

This TMAH concentration has already been successfully applied in previous studies (Gray et al., 2013; Jiménez-Lamana et al., 2014; Johnson et al., 2017; Loeschner et al., 2013) and was checked in our test as compared to 5% TMAH. The extraction scheme includes several steps as shown in Figure 5.1. First, biota samples were put into 1 mL of 20% (w/w) TMAH and the suspensions were vortexed for 30 s. Second, the samples were sonicated for 30 minutes in a water bath to speed up the breaking down of the tissue and to prevent particle aggregation. Next, the samples were incubated on a Thermoshaker for 24 h at 70 ˚C and 800 rpm in order to allow the tissue to interact deeply with TMAH instead of allowing the tissue to settle down on the bottom. To extract the particles adsorbed to the carapace, the samples were sonicated for another 30 min after the incubation. After the digestion procedure, all tissues were dissolved whereas the carapaces of the daphnids and the mysids remained in suspension. Therefore, the samples were centrifuged at 4000 rpm for 10 min to separate the solution and the carapaces. The suspension was transferred to a new tube and the remaining carapaces were washed twice with Milli-Q water. The remaining solution was finally diluted to 5 mL and stored at -80 ˚C before measurement. The carapace was digested with HNO_3 (65%) at a temperature above 170 ˚C for around 2 h and then diluted with Milli-Q water to 5 mL. Atomic Absorption Spectroscopy (AAS; Perkin Elmer 1100B) was used to check whether there were still particles or ions left in the digested carapace solutions. The results are shown in Table S5.2.

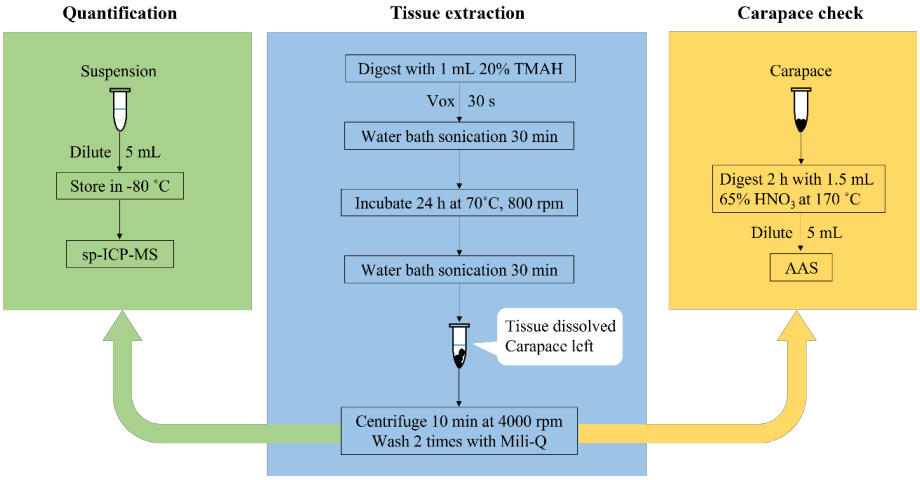

Figure 5.1 Extraction scheme employed in this study.

5.2.5 Particle analysis by sp-ICP-MS

The details of the settings of the instrument and the evaluation of the performance of the extraction method for particles were processed based on previous studies (Monikh et al., 2019a; Cui et al., 2019). The instrumental parameters of the sp-ICP-MS are presented in Table S5.3. The CuNPs suspensions were diluted 1000-fold using ultrapure water to bring the CuNP concentration to a final concentration of within 5000–200000 particles/mL before analysis. The instrument was calibrated using a blank (deionized water) and at least five soluble Cu standards ranging from 0 to 10 ng/g Cu.

5.2.6 Total Cu determination

To measure the total concentration of Cu in the tissues, organism samples were dried to constant weight at 80 ˚C in an oven (MOV-212S,

SANYO Electric Co., Ltd.) and then digested with HNO_3 (65%) at a temperature exceeding 170 ˚C for around 2 h. After samples were fully dissolved and the remaining solutions were transparent. They were then transferred to a clean tube and diluted to 5 mL with Milli-Q water. Cu concentrations in all digested samples were determined by ICP-MS (PerkinElmer, NexION 2000). To evaluate the contamination in the used water, blank samples of Milli-Q water were measured. After running the samples of each treatment, the instrument was cleaned by running 2.5% acid nitric in Milli-Q water followed by samples of Milli-Q water. The biological samples without any treatment were also digested and measured to assure that the samples were free of particulate Cu. There are no standard particles available for CuNPs. Thus, AuNPs of 30, 60, and 100 nm were used for calibration of sp-ICP-MS for measuring the size distribution. Standard Cu solutions of 1 ppb, 10 ppb, 50 ppb, and 100 ppb were used to provide the calibration cure using the ICP-MS. Blanks and standard Cu solutions were determined before analysis and between every 20 samples during the analysis. The limit of detection for Cu was 1 ng/L. The relative standard deviation (RSD) of the measurement was less than 5% for all cases.

5.2.7 Calculation of bioconcentration factor and trophic transfer factor

For aqueous exposure, a bioconcentration factor (BCF) was derived as the ratio between the concentration of NPs in biota and the actual concentration in the medium:

$$\text{BCF (L/kg)} = \frac{C_{\text{biota}}\ (\mu\text{g/kg})}{C_{\text{medium}}(\mu\text{g/L})} \quad (1)$$

where C_{biota} (μg/kg dry weight) is the metal concentration in the organism and C_{medium} (μg/L) is the metal concentration in the exposure medium.

In addition, the trophic transfer factor (TTF) is a measure of the trophic transfer potential of any substance from one trophic level to the next level in a food chain (Ma et al., 2018). The TTF was determined as the ratio of the NP concentration in the higher lever organism to the concentration in the lower level organism:

$$TTF = \frac{C_{predator}}{C_{prey}} \quad (2)$$

A value higher than 1 is indicative of a trend of biomagnification, whereas a value less than or equal to 1 indicates that the extent of transfer is limited. More detail of the data processing and the calculations performed are presented in the Supplementary data.

5.2.8 Statistical analysis

All experiments were performed in four replicates, and the data were expressed as mean ± standard deviation. The differences among various groups were assessed using one-way analysis of variance (ANOVA) by Tukey's range test with the IBM SPSS statistics program, and $p < 0.05$ is defined as the significance level.

5.3 Results and discussion

5.3.1 Physicochemical characterizations of CuNPs

Table 5.1 provides the primary particle size, the mode size, and the hydrodynamic size of CuNPs characterized by TEM, sp-ICP-MS, and dynamic light scattering, respectively. The primary sizes of CuNPs were around 20-30 nm, which were similar to their mode sizes (21-31 nm). The hydrodynamic sizes of CuNPs in the Woods Hole Medium and Elendt M7 medium were greater than their primary particle sizes, implying that CuNPs agglomerated in the test media. The characterization of morphology of pristine CuNPs in the test media also indicated that the spherical CuNPs tended to form irregularly shaped agglomerates (Figure S5.1, Supplementary data).

Table 5.1 Particle sizes and zeta potential of 1 mg/L CuNPs suspended in the Woods Hole Medium and Elendt M7 medium [a]

Test medium	Primary particle size (nm) measured by TEM	Mode size (nm) measured by sp-ICP-MS	Hydrodynamic size (nm) measured by dynamic light scattering	Zeta potential (mV)
Woods Hole Medium	24 ± 6	22 ± 1	682 ± 43	-14 ± 0
Elendt M7	24 ± 4	28 ± 3	1046 ± 81	-8 ± 1

[a] Values are expressed as mean ± standard deviation (n = 3).

The hydrodynamic size of the CuNPs in the Woods Hole Medium was observed to be lower than the hydrodynamic size of CuNPs in the Elendt M7 medium, as shown in Table 5.1. Moreover, the zeta potential of CuNPs in the Woods Hole Medium was more negative than the zeta

potential of CuNPs in the Elendt M7 medium (Table 5.1). The increase in the absolute zeta potential can result in a high rate of particle movement by inducing the growth of the energy barrier, and then preventing the agglomeration of particles (Guo et al., 2018). Furthermore, the hydrodynamic size of CuNPs in the Woods Hole Medium did not change over time (Figure S5.2, Supplementary data). However, the hydrodynamic size of CuNPs in the Elendt M7 medium shifted from 1336 ± 182 nm to 603 ± 82 nm over 30 min of incubation (Figure S5.2). A reasonable explanation for the decrease in the hydrodynamic size may be due to the sedimentation of larger agglomerates (Arenas-Lago et al., 2019). These findings indicated that the stability of CuNPs in the Woods Hole Medium was higher than their stability in the Elendt M7 medium.

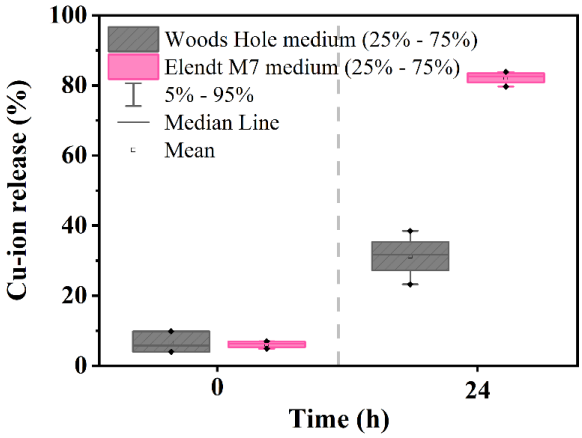

Figure 5.2 Percentage of dissolved Cu released from 1 mg/L CuNPs suspensions after 0 and 24 h of incubation in the Woods Hole Medium and Elendt M7 medium. Values are expressed as mean ± standard deviation (n = 3).

The Cu-ion release profiles from the CuNPs in the test media after 0 and 24 h of incubation are presented in Figure 5.2. The percentages of Cu ions in the CuNPs suspensions were both around 6% at the start of the experiment. However, the percentage of Cu ions in the CuNPs suspensions shifted to 31% in the Woods Hole Medium and even to 82% in the Elendt M7 medium after 24 h of incubation. It can be concluded that particulate Cu might play a more important role in the food chain starting from *P. subcapitata*.

5.3.2 Trophic transfer of CuNPs in the food chain

The actual Cu exposure concentrations in the Woods Hole Medium and Elendt M7 medium were 514 ± 27 µg/L and 355 ± 23 µg/L, respectively, after exposure to CuNPs at a nominal concentration of 1 mg/L. Considering the ion release profiles of CuNPs suspensions, parallel exposure experiments to 10 µg Cu/L $Cu(NO_3)_2$ were conducted to examine the uptake and transfer of dissolved Cu and to compare these properties with the behavior of particulate Cu. Similar to what was done in previous studies (Wang et al., 2015, 2011; Zhang et al., 2018), stable Cu^{2+} ions instead of unstable Cu^{+} ions were selected to represent released ionic Cu. This may be due to the fact that: 1) Cu^{2+} has greater a hydration enthalpy than Cu^{+}, and 2) Cu^{+} can spontaneously form Cu and Cu^{2+} ions ($2Cu^{+}(aq) \rightarrow Cu^{2+} + Cu$). The amount of Cu taken up from the exposure media by each species and the calculated BCF values are presented in Table 5.2. The amount of CuNPs taken up by the organisms directly from the aquatic exposure medium decreased in the order of *L. benedeni* > *D. magna* ≈ *P. subcapitata*. This order was in good agreement with the order of the

degree of uptake of Cu^{2+} in the organisms. Note that the uptake of Cu in *P. subcapitata* and *D. magna* was similar, although the exposure concentration of Cu in the algae medium is higher than the Cu concentration for the daphnids. This provided a similar start for all food chains. As shown in Table 5.2, the BCFs of CuNPs in our test species were all in the range of 10^2 - 10^3 L/kg dry weight. The BCFs of Cu^{2+} were significantly higher than the BCFs of the CuNPs in each trophic level. This suggests a higher bioavailability of Cu in its ionic form than in its particulate form, taking into account the presence of the essential Cu in the organisms. A similar conclusion was reported by Makama et al. (2015) for ionic Ag and particulate Ag in earthworms exposed via (pore) water.

Table 5.2 Cu concentration (μg/g dry weight) in algae (*P. subcapitata*), daphnids (*D. magna*) and mysids (*L. benedeni*), and their bioconcentration factors (BCF, L/kg dry weight) [a]

		Cu. conc. (μg/g dw)	BCF (L/kg dw)
	Control	50 ± 1	
P. subcapitata	CuNPs	344 ± 98	670 ± 191
	Cu^{2+}	84 ± 10	8434 ± 1034
	Control	71 ± 23	
D. magna	CuNPs	380 ± 39	1072 ± 110
	Cu^{2+}	92 ± 4	9199 ± 428
	Control	96 ± 10	
L. benedeni	CuNPs	878 ± 346	2476 ± 975
	Cu^{2+}	527 ± 116	52658 ± 11553

[a] These species were not exposed (control) and exposed to 1 mg/L CuNPs and 0.01 mg/L $Cu(NO_3)_2$ solution, respectively. Values are

expressed as mean ± standard deviation (n = 3). Each replicate contained > 100 daphnids or 15-20 alive mysids.

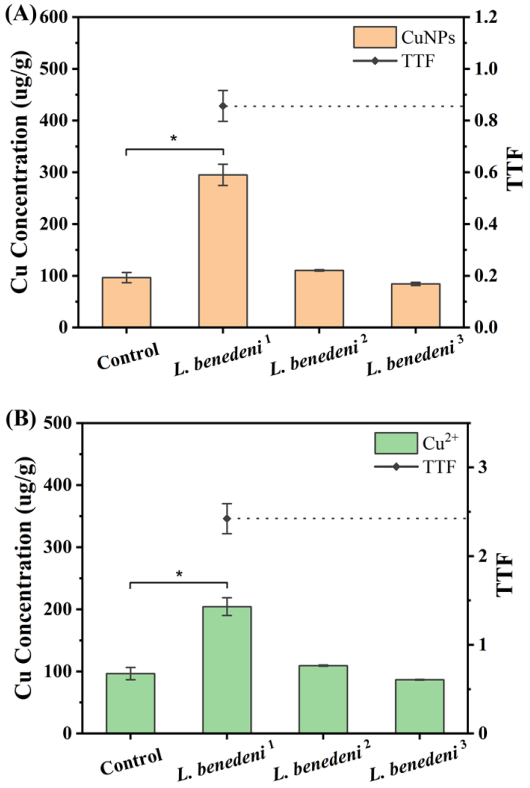

Figure 5.3 Cu concentrations (μg/g dry weight) for CuNPs (A) and Cu^{2+} (B) in mysids (*L. benedeni*) and their mass-based trophic transfer factors (TTF). *L. benedeni* [1], *L. benedeni* [2] and *L. benedeni* [3] stand for the predators in case-1 (from algae to mysid), case-2 (from daphnia to mysid), and case-3 (from algae through daphnia to mysid). Control stands for the mysid fed by unexposed food. Bars represent mean ± standard deviation (n = 3). The asterisk indicates statistical significance versus control group ($p < 0.05$).

The Cu concentrations in the predator from each food chain exposed to CuNPs and Cu^{2+} and the calculated TTFs are presented in Figure 5.3. As shown in Figure 5.3A, the Cu concentration in *L. benedeni*[1] fed with *P. subcapitata* was significantly higher compared to the Cu concentration in the control ($p < 0.05$). Hence, the trophic transfer of CuNPs was observed in case-1, rather than in case-2 and case-3. A similar finding was observed in the trophic transfer of Cu^{2+} (Figure 5.3B). These results indicated that both the food source and the length of the food chain can influence the trophic transfer potential of CuNPs and Cu ions. On the one hand, although the uptake of *L. benedeni*[1] was 3 times higher compared to the control, CuNPs did not biomagnify, as indicated by a TTF < 1. The comparison of TTF values showed that Cu ions were easier transferred across food chains. In addition, aqueous exposure resulted in higher uptake of Cu than in the case of ingestion of Cu-contaminated algae. Croteau et al. (2014) also found that the uptake of CuONPs in a freshwater snail could be accounted for mostly by diet-borne exposures. The accumulation and trophic transfer of NPs are commonly evaluated based on the mass concentration of metal accumulated in the predator. Mass might, however, not be necessarily the best metric to quantify bioaccumulation and trophic transfer of NPs and will for instance not reflect the possibility of preferential uptake of particles of different sizes or different morphology (Monikh et al., 2019b). Therefore, to quantify the size and number concentration of particulate Cu in prey and predator, we determined the particle size and particle number using sp-ICP-MS. Our finding for the performance check of the method showed that the addition of TMAH slightly decreased the sizes of particles and increased the number concentrations of particles (Table S5.4).

Table 5.3 Mode size (nm) and number particle concentrations (# particles/g wet weight) of Cu particles in biota, bioconcentration factors (BCF) in each test species, and trophic transfer factor (TTF) from algae to mysids [a]

	Mode size (nm)	Part. Conc. (10^{13} parts/kg ww)	Part. BCF (L/kg ww)	Part. TTF
P. subcapitata	34 ± 4	2 ± 2[b]	429 ± 364	
D. magna	28 ± 2	2 ± 4	1689 ± 2766	
L. benedeni^0	28 ± 6	2 ± 1[b]	445 ± 102	
L. benedeni^1	31 ± 9	4 ± 2[c]		2 ± 1

[a] *L. benedeni*^{0} and *L. benedeni*^{1} stand for the mysids directly exposed to CuNPs in the aqueous phase and the predators in case-1 (from algae to mysid), respectively. The results are expressed as mean ± standard deviation (n = 3). Each replicate contained > 100 daphnids or 15-20 alive mysids. The different superscript letters indicate statistically significant ($p < 0.05$) differences between the treatments.

The mode size, number concentration, BCF, and TTF of CuNPs in each test species are shown in Table 5.3. The particle size of CuNPs did not change significantly after uptake, with the mode sizes of CuNPs among the test species in the range of 22 - 40 nm. Compared to the uptake order in biota (mysids > daphnia ≈ algae) based on the total mass concentration, the uptake of Cu particles based on number concentration directly from the aqueous phase in the different biota was similar and for all species roughly in the level of 10^{10} particles/g wet weight. The order of the BCF values based on the dose metric of particle number was *D. magna* > *L. benedeni* ≈ *P. subcapitata*. It was

also found that the order of the BCF values based on the dose metric of particle number was different from the order based on mass concentration (*L. benedeni* > *D. magna* > *P. subcapitata*, as shown in Table 5.2). Furthermore, the calculated TTF value of particulate Cu was greater than 1, indicating that the biomagnification of particulate Cu was observed in case-1. Mysids exposed to CuNPs via food (algae) showed higher uptake than mysids exposed via water. This result was in contrast with the total Cu content in the mysids (including both ionic Cu and particulate Cu) which originated mainly via waterborne exposure. Expressed based on the total Cu concentration, biomagnification was not found to occur (TTF < 1). This may be associated with the influence of the biophysiological factors of the predators on the uptake and bioaccumulation of CuNPs. It has been confirmed that several biophysiological factors such as feeding mode, digestive physiology, assimilation, and subcellular fractionation can affect the uptake and bioaccumulation of NPs in organisms (Ohe and Zwart, 2013; Tangaa et al., 2016). For instance, the internal location and subcellar fractionation of soluble metal-based NPs in daphnia distributed in the guts mostly (Yan and Wang, 2021). From this scenario, the extent of Cu uptake by the daphnia and mysid could depend on the conditions in the gut and the gut residence time. In contrast, Brun et al. (2017) found NPs in the lipid droplets around the gut tissue and in the brood pouch rather than in the gut of *D. magna*.

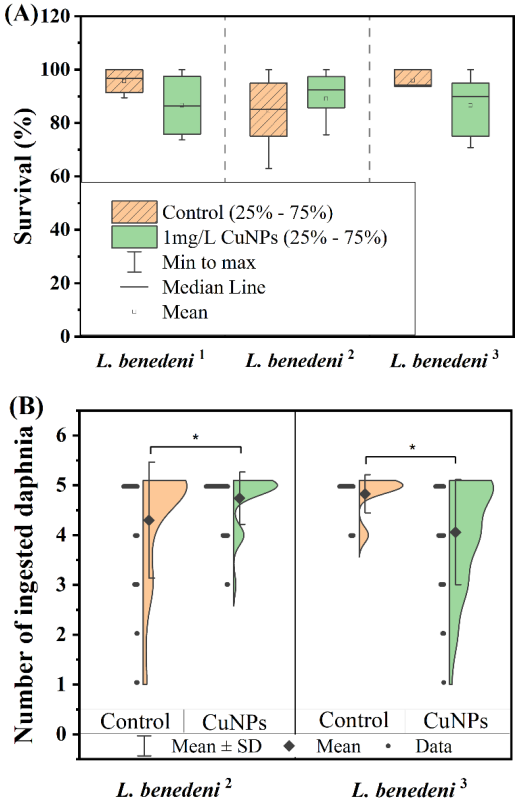

Figure 5.4 Survival (A, %) of mysids (*L. benedeni*) in the three food chains. Each survival test group contained 10 replicates with 15 - 20 alive mysids. The half violin figure (B) describes the distribution of mysids according to the number of ingested daphnia. Dots in the left part of the violin figure stand for the mysids hunting a different number of daphnia. The width of the right part represents the distribution of these data. Each group contained more than 45 alive mysids. *L. benedeni* [1], *L. benedeni* [2] and *L. benedeni* [3] stand for the predators in case-1 (from algae to mysid), case-2 (from daphnia to mysid), and case-3 (from algae through daphnia to mysid), respectively. The asterisk indicates statistical significance versus control group ($p < 0.05$).

Figure 5.4 depicts the effects of dietary CuNPs exposure on the survival (%) and feeding rate (%) of mysids in the food chain. As shown in Figure 5.4A, there was no significant difference in the mysid survival between each treatment and the corresponding control group ($p > 0.05$). In case-1, a high amount of Cu was detected in the mysids and particulate Cu even biomagnified in the predator (Figure 5.3). However, the levels of Cu transferred from algae to mysids did not affect the survival of mysids. In addition, in terms of the feeding, no significant effect of the dietary uptake of CuNPs on the ingestion of algae by the mysids was observed. As shown in Figure 5.4B, a significant effect of the dietary uptake of CuNPs on the feeding rate calculated from the number of hunting the daphnia by the mysids was found in case-2 and case-3 ($p < 0.05$). Compared with the control, the dietary uptake of CuNPs induced an increase in the feeding rate in case-2, while the dietary uptake of CuNPs induced a decrease in the feeding rate in case-3. Since CuNPs were not found to be transferred to the predators (mysids) in the present study, the observed changes in feeding rate may be mainly related to the toxic effects of CuNPs on the prey (algae or daphnia). In our previous studies, the median lethal concentration of CuNPs for *D. magna* was found to be around 0.4 mg Cu/L (Yu et al., 2022). When the studied daphnids were exposed to around 355 μg Cu/L CuNPs, it was observed that some of the alive daphnids tended to swim slowly and became easier to catch during the 24 h exposure period. Therefore, an increase in the feeding ratio was observed in case-2. In addition, the algae (*P. subcapitata*) can compete with *D. magna* for the uptake of CuNPs, which could lead to a decrease in eating the daphnids exposed to CuNPs by the mysids in case-3.

In addition to the hydrosphere, the fate, behavior and effects of CuNPs in other environmental spheres such as the pedosphere and the biosphere are also of equal interest (Al-zharani et al., 2021; Bakshi and Kumar, 2021; Gao et al., 2019; Liu et al., 2018; Rizwan et al., 2017; Wang et al., 2022). A relatively high uptake of Cu and altered nutrient quality in soybean (*Glycine max*) grown in agricultural soil amended with CuNPs (Xiao et al., 2022) were reported. Moreover, natural organic matter increased the dissolution of CuNPs, and mitigated the phytotoxicity of CuNPs more significantly than that of Cu salt (Xiao et al., 2021). The increasing use of Cu-based NP_S in agricultural, industrial and environmental applications will undoubtedly lead to their spread in terrestrial ecosystems (Bakshi and Kumar, 2021). Thus, the dynamic transport of CuNPs may occur from soil to plants and then to food chain. To thoroughly understand the chemical behavior of Cu and potential risk of Cu-based NPs, further research is needed regarding the transport and transformation of CuNPs along terrestrial food chains in plant-soil environments.

5.4 Conclusions

The trophic transfer of CuNPs or Cu^{2+} was found in the direct route from the microalgae *P. subcapitata* to the mysid *L. benedeni*, but the extent of transfer of Cu was limited (TTF < 1). The size of the Cu particles was stable in the range of 22 – 40 nm throughout the food chain. Consequently, particle number and mass were found to be equally suited to express bioaccumulation in the food chains studied. Unique was the quantification of particulate Cu within organisms. The uptake of Cu particles from the exposure medium in the organisms of

different trophic levels based on number concentrations was in the same order of magnitude of 10^{13} particles/kg wet weight. Furthermore, the biomagnification (TTF > 1) of particulate Cu occurred from the algae to the mysids. For the predator mysids, the uptake of total Cu from the aqueous exposure was higher than the uptake via the dietary exposure, while the uptake of particulate Cu was mainly via dietary uptake. Furthermore, the dietary CuNPs exposure showed significant effects on the feeding rates of mysids in the transfer processes from daphnia to mysid and from algae through daphnia to mysid. Taken together, this work exhibited that CuNPs transfer across trophic chains and show a limited extent of biomagnification. It is worthwhile to note that in the real world CuNPs could be excreted by organisms due to their own physiological and biochemical processes, and afterwards CuNPs would re-enter the environment upon excretion and pose an ecological risk to other organisms.

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Supplementary data

Nanoparticle dissolution and aggregation testing

To determine the dissolution profile of CuNPs, we monitored the concentration of ionic Cu in the test media over 24 h. The samples were collected at 0 and 24 h from a suspension of 1 mg/L CuNPs prepared in Woods Hole Medium (Janet Stein, 1982) and in ElendtM7 medium (Samel et al., 1999). The samples were obtained from the top 1.5-2 cm layer of the dispersions., The samples for the measurement of the total Cu concentration were acidified by addition of 2 drops of 65% nitric acid. Samples for the measurement of Cu-ions released from the CuNPs were firstly centrifuged at 15,000 rpm for 30 min at 4 ˚C, and then 5 mL of supernatant was transferred to another tube for analysis. The actual concentration of total Cu and the Cu-ions released from the CuNPs were determined via Atomic Absorption Spectroscopy (AAS; Perkin Elmer 1100B).

Aggregation kinetics during 30 min of changes were monitored using dynamic light scattering (DLS, Malvern, Instruments Ltd., UK). Suspensions of 10 mg/L CuNPs as used for the DLS measurements were prepared in the Wood Hole Medium and in the ElendtM7 medium, respectively. The hydrodynamic diameters of the CuNPs were determined immediately after the dispersions were sonicated for 15 min. During the 30 min observation of aggregation, the DLS measurements were performed with an interval of 2 min.

Culture of test organisms

Pseudokirchneriella subcapitata used as a test organism were maintained in a climate room. OECD Guideline 201 was used as the procedure for culturing of the algae with some slight modification (Andrews and Walsh, 2007). The algae were cultured in autoclaved Woods Hole Medium at 22 ± 1 °C with a 16:8 light-dark cycle. The algae were continuously aerated to provide sufficient CO_2 and the algae suspension was stirred to avoid settling down.

Daphnia magna was originally obtained from Leiden University. According to OECD Guideline 202 (OECD, 2004), the daphnia culture medium ElendtM7 medium (Samel et al., 1999) was prepared at pH 8.4 ± 0.4. *D. magna* were cultured at a temperature of 22 ± 1 °C with a 16:8 light-dark cycle. The density of the culture was around 1 individual/500 mL medium and daphnids were fed with *P. subcapitata* every 2 days.

Lymnomysis benedeni, a widespread species in Western Europe, was collected from a pond (Leiden, The Netherlands) at a size of around 1.1 cm, and the collection included both male and female species. *L. benedeni* was acclimated in the lab for 3 days prior to the test by means of the following 3 steps: 1) culturing in combined natural water and in the ElendtM7 medium with a small amount of sediment originating from the pond for the first day; 2) culturing in the ElendtM7 medium with sediment from the pond for the second day; 3) culturing in the ElendtM7 medium without sediment and without food for the third day to clean the guts. *L. benedeni* was cultured with continuously aeration, using the same condition as for *D. magna*.

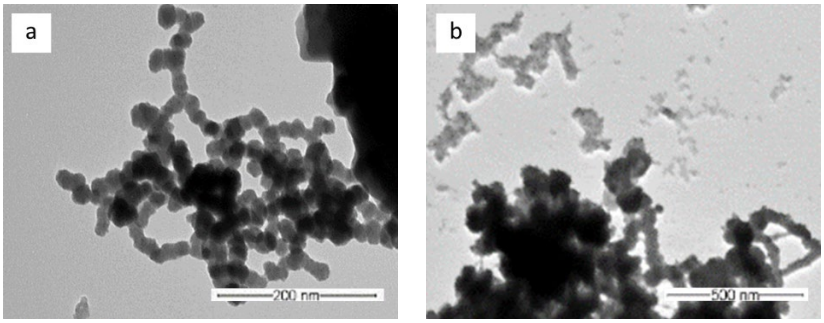

Figure S5.1 TEM images of 1 mg/L Cu NP suspensions in (a) Woods Hole Medium and (b) ElendtM7 medium (Yu et al., 2022)

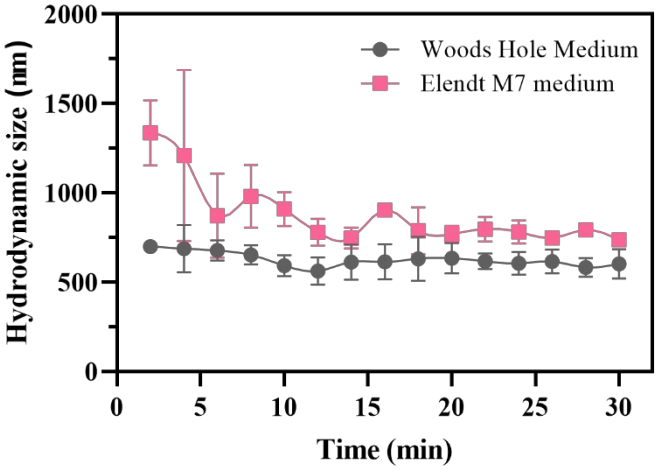

Figure S5.2 Hydrodynamic sizes of the suspension of 10 mg/L CuNPs dispersed in Woods Hole Medium and in ElendtM7 medium over 30 min, as measured by dynamic light scattering. The values reported are expressed as mean ± standard deviation (n = 3)

Table S5.1 Chemical Composition of Woods Hole Medium (Janet Stein, 1982)

Chemicals	Concentration (g/L)
$CaCl{\cdot}2H_2O$	3.676
$MgSO_4{\cdot}4H_2O$	3.698
$NaHCO_3$	1.26
K_2HPO_4	0.872
NaNO3	8.502
$MnCl_2{\cdot}4H_2O$	0.02
$ZnSO_4{\cdot}7H_2O$	0.002
$NaMoO{\cdot}2H_2O$	0.00012
$CoCl_2{\cdot}6H_2O$	0.001
$CuSO_4{\cdot}5H_2O$	0.001
NaEDTA	0.436
$FeCl_3{\cdot}6H_2O$	0.316
Vitamins	0.5 mL of the stock*

*The vitamin stock was prepared according to Lehman (1976).

Table S5.2 Concentration of Cu (μg/g) left on the daphnia and shrimp after extraction

Carapace		Cu concentration (μg/g)
daphnia	Control	8.6 ± 5.6
	Exposure	3.3 ± 1.6
shrimp	Control	0.5 ± 0.2
	Exposure	0.0 ± 0.0

Table S5.3 Instrumental parameters for single particle inductively coupled plasma mass spectrometry (sp-ICP-MS) analysis

Nebulizer Gas Flow [NEB]	1.12 L/min
Auxiliary Gas Flow	1.2 L/min
Plasma Gas Flow	18 L/min
ICP RF Power	1600 W
Flow Rate	0.34 g/min
Transport Efficiency	13.62 %
Dwell Time	50 us

Table S5.4 Particle size and particle number concentration of suspensions of 1 mg/L CuNPs in 20% TMAH and Milli-Q water

Solution	Mean size (nm)	Part. Conc. (10^8 parts/mL)
20% TMAH	25.7 ± 1.0	2.6 ± 0.8
Milli-Q water	39.5 ± 11.1	0.1 ± 0.2

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