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Distribution of CuO nanoparticles in juvenile carp (*Cyprinus carpio*) and their potential toxicity

Jian Zhao[a,b], Zhenyu Wang[a,*], Xiaoyun Liu[c], Xiaoyan Xie[a], Kai Zhang[a], Baoshan Xing[b,*]

[a] College of Environmental Science and Engineering, Ocean University of China, Qingdao 266100, China
[b] Department of Plant, Soil and Insect Sciences, University of Massachusetts, Amherst, MA 01003 USA
[c] Institute of Materials Science and Engineering, Ocean University of China, Qingdao 266100, China

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ABSTRACT

Adverse effect of engineered nanoparticles (NPs) on the aquatic environment and organisms has recently drawn much attention. This paper reports on the toxicity of CuO NPs to juvenile carp (*Cyprinus carpio*) and their distribution in the fish. CuO NPs and its counterpart bulk particles (BPs) (10, 50, 100, 200, 300, 500 and 1000 mg L^{-1}) exhibited no acute toxicity (96 h), while during the 30 day sub-acute toxicity test, carp growth was significantly inhibited by CuO NPs (100 mg L^{-1}) in comparison to control, CuO BPs and Cu^{2+} groups. CuO NPs (or released Cu^{2+} ions inside the fish body) could distribute in various tissues/organs and followed an order: intestine > gill > muscle > skin and scale > liver > brain. For time-related distribution, Cu content (expressed on a dry mass basis) in intestine, gill and liver increased faster (within 1 day) and they had obviously higher Cu content than other tissues/organs at all exposure times. CuO NPs could be excreted by carp to lower their toxicity. Cholinesterase activity was inhibited during CuO NPs exposure, suggesting NPs exposure could have potential neurotoxicity, and free Cu^{2+} ions dissolved inside the carp body was responsible for the cholinesterase inhibition. Finally, actual suspended NPs concentrations should be used instead of initially added concentrations whenever possible in nanotoxicity studies.

1. Introduction

Nanotechnology consumer products inventory reports 1317 products or product lines by March 2011, 5 times more than that in 2006 [1]. These nanoproducts have various applications such as electronics, biomedicines, sensors and environmental remediation [2,3]. Production and use of engineered nanomaterials likely result in their release into aquatic environments and lead to unexpected hazards on aquatic organisms [4].

For metal oxide nanoparticles (NPs), toxicity to fish has been investigated. Zhu et al. found that ZnO had higher acute toxicity to zebrafish embryos than TiO_2 and Al_2O_3 NPs [5]. Similarly, the toxicity of ZnO NPs (96 h LC_{50}, 4.9 mg L^{-1}) to zebrafish was much higher than that of TiO_2 NPs (96 h LC_{50}, 124.5 mg L^{-1}) [6]. The high toxicity of ZnO NPs was partly attributed to the dissolved Zn^{2+} ions [7]. For other fish, TiO_2 at high concentrations (1000 mg L^{-1} and 200 mg L^{-1}, respectively) did not cause significant mortality in the fathead minnow embryos [8] and juvenile carp [9]. As another common metal oxide NPs, CuO NPs could also release dissolved ions and their toxicity to fish has not been investigated, yet. Karlsson et al. observed that CuO NPs were highly toxic to human lung epithelial cells compared to carbon nanotubes and other oxide NPs [10]. In addition, most of the emerging literature on the toxicity of NPs has focused on acute exposure to fish. It is likely that long-time exposure may be of more ecological importance. NPs through long-time exposure could be distributed and dissolved in fish body, thus having chronic toxicity. To date, the contribution of NPs in each organ/tissue to whole fish body NPs burden is still unclear and to our knowledge, there is no data available for time-dependent effect on NPs distribution in fish body.

It was reported that surface functionalized Au NPs and latex NPs could distribute in the brain of medaka (*Oryzias latipes*) due to their hydrophobic surface [11,12]. Metal oxide NPs (e.g., TiO_2) were hard to pass through the blood–brain barrier to enter the brain of zebrafish [13]. However, metal ions could easily enter the circulatory system, both CuO NPs and released ions may reach brain and have toxicity to central nervous system [14,15]. In the vertebrate brain, cholinesterase (ChE) plays a key role in the central nervous system by hydrolyzing the neurotransmitter acetylcholine (ACh) in cholinergic synapses [16,17]. When ChE is inhibited, ACh accumulates and interferes with the function of the nervous system. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are two types of ChE in the brain. Commonly, AChE is predominant in the brain while BChE plays a backup role in supporting and regulating cholinergic transmission when AChE is absent or inhibited

* Corresponding authors. Tel.: +1 413 545 5212; fax: +1 413 545 3958.
E-mail addresses: bx@pssci.umass.edu (B. Xing), wang0628@ouc.edu.cn (Z. Wang).

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[17]. Both enzymes could hydrolyze ACh. Therefore, the change of total ChE activity after NPs exposure could reflect the degree of neurotoxicity and could be measured using ACh as the substrate.

Therefore, juvenile carp (*Cyprinus carpio*) was used as a test organism to investigate acute and sub-acute toxicity of CuO NPs and their counterpart bulk particles (BPs). Time-dependent distribution of NPs in tissue/organ of carp was determined. ChE activity of brain tissues was examined as a biomarker to evaluate neurotoxicity of CuO NPs. Results of this study will provide insight into the toxicity mechanisms of CuO NPs and their distribution in aquatic organisms.

2. Materials and methods

2.1. Particles and suspension preparation

CuO NPs and BPs were purchased from Beijing Nachen S&T Ltd., China. Surface area of both particles was measured by the Brunauer–Emmer–Teller (BET) method (Autosorb 1, Quantachrome, USA), based on the adsorption–desorption isotherm of N_2 at 77 K [18]. Particle morphology was visualized using transmission electron microscopy (TEM, JEM-2100, Japan) operated at 200 kV. Samples were prepared by suspending particles in ethanol and evaporating one drop on a nickel grid with carbon support film [19]. Particle suspensions at the nominal concentrations of 10, 50, 100, 200, 300, 500 and 1000 mg L^{-1} were prepared by weighing dry CuO NPs or BPs into the tap water (pH 7.1), then ultrasonicated (100 W, 40 kHz) for 1 h to increase their dispersion. Hydrodynamic diameter and zeta potential of the particles in the suspensions were estimated using a Nano Zetasizer (Malvern Instrument). Dissolution test was conducted to determine Cu ion concentration in the CuO NPs and BPs suspensions at all tested concentrations. Particles were suspended in the tap water for 2 days and then separated according to our previous study [16]. Concentration of Cu^{2+} in the solution was measured by graphite furnace atomic absorption spectrometry (AAS) (Solaar M6, Thermo Electron). Individual Cu solutions were then prepared by dissolving $CuSO_4{\cdot}5H_2O$ in the tap water according to the Cu ion dissolution result determined with graphite furnace AAS.

2.2. Fish and toxicity assessment

Juvenile carp (*C. carpio*) were obtained from an aquaculture farm in Qingdao, China. The initial body length and weight of the carp were 5.95 ± 0.50 cm and 2.50 ± 0.45 g, respectively. Carp were kept in several 40 L tanks supplied with continuously aerated tap water. Water temperature was maintained at 25 ± 1 °C. Dissolved oxygen, hardness and pH were 6.5–7.8 mg L^{-1}, 76.4–79.1 mg L^{-1} as CaO, and 7.1–7.3, respectively. Acute toxicity tests were carried out to obtain the dose-response relationship for carp exposed to CuO NPs and BPs. Fish were fasted for 24 h prior to the test. For 96 h acute exposure, 48 tanks (3 L) with three replicates for the CuO NPs and BPs treatments were used. Every 10 fish were put into each tank. In each treatment, the nominal particle concentrations were 0, 10, 50, 100, 200, 300, 500 and 1000 mg L^{-1}. Fish behavior such as swinging frequency was observed every 24 h and mortality was carefully recorded.

Sub-acute toxicity tests (30 days) were conducted at the nominal concentration of 100 mg L^{-1} based on the acute toxicity results in which CuO NPs at 100 mg L^{-1} had no acute toxicity but the fish behaviors such as swinging frequency were obviously changed (Fig. S4b). Water or treated solutions were changed every 2 days, and fish were fed 30 min before each water change. Four exposure groups were defined: CuO NPs, CuO BPs, soluble Cu^{2+} and control (tap water only). The Cu^{2+} concentration in the Cu^{2+} group was obtained from the dissolution test described above. Twelve tanks (40 L) with three replicates for these 4 treatments were used. For each treatment, 150 carp were put into each tank. Body lengths and weights were measured before and during the exposure time. Three fish were randomly removed from each tank at 0, 1, 3, 5, 10, 15, 20, 25 and 30th day, and were stored in freezer at −20 °C. After 30 days exposure, the fish from each time point were dried at 105 °C to constant weight, ground, quantified and stored at −20 °C for whole body Cu content analysis.

2.3. Tissue distribution and excretion of NPs

During the sub-acute toxicity tests, 10 more fish each time were collected from each tank, washed and dissected. Part of brain tissue was separated immediately for ChE analysis. The remaining brain, intestine, gill, liver, muscle, skin and scale and others (all the rest parts of the fish) were dried and ground for Cu content analysis.

For excrement collection, 30 fish were put into a tank and three replicates were performed for each group (CuO NPs, CuO BPs, soluble Cu^{2+} or control group). Five fish were removed from each tank after exposing for 0, 6, 12, 24, 36 and 48 h, washed 3 times, and then placed into other tanks with clean tap water. These fish were cultivated continually for 48 h and excrements were collected, dried and ground for Cu content analysis.

Wet digestions were carried out for all samples. Four mL of concentrated HNO_3 were used to digest each sample for 8 h at 50 °C, and then diluted to 16 mL [20]. All acids and water used were of suprapure quality. Graphite furnace AAS was used for Cu concentration determination.

2.4. TEM observation

Gill, liver, intestine and brain tissues of fish were sampled after CuO NPs exposure for 30 days, and fish excrements were collected after exposure for 2 days. All samples were then placed in 5% glutaraldehyde overnight, postfixed in 1% osmic acid for 2 h, dehydrated in a graded series of ethanol for 20 min, and embedded in EPON812 resin overnight. Samples were then sectioned to 60–80 nm using an ultra-microtome (LKB Nova), stained with uranyl acetate, and placed on Ni grids instead of Cu grids to avoid the interference of Cu when using energy dispersive spectroscopy (EDS, INCA100, Oxfordshire, UK) equipped with TEM.

2.5. Cholinesterase activity assay

Fresh brain tissue samples removed from the sub-acute exposure were homogenized in 1:10 (w/v) ice-cold physiological saline. The homogenate was then centrifuged at 3000 rpm at 4 °C for 10 min [21]. Clear supernatant was stored at −80 °C until ChE assay. ChE activity was determined by an Ellman method [22] and modified in our previous study [16]. Briefly, in the enzyme reaction, substrate (acetylthiocholine iodide, Sigma) was hydrolyzed by ChE in the supernatant and produced yellowish product in the presence of color reagent (5,5′-dithiobis (2-nitro-benzoic acid), Sigma). The yellowish product was measured using an UV/vis spectrophotometer (Hitachi 2001). The ChE activity of carp brain in the CuO NPs, CuO BPs or soluble Cu^{2+} group was expressed as percent of that in the control group, and was calculated as follows:

$$\text{ChE activity (\% control)} = \frac{A}{A_0} \times 100$$

where A is the OD (optical density) difference between 0 and 3 min in the CuO NPs, CuO BPs or soluble Cu^{2+} group, A_0 is the OD difference between 0 and 3 min in the control group.

2.6. Statistical analysis

All data were presented with mean ± SD deviation. A one-way analysis of variance (ANOVA) was used to determine statistical significance ($p < 0.05$) of the differences between measured values using Excel 2003 and SPSS for Windows 12.0.

3. Results and discussion

3.1. Particle characterization

Characterization of CuO NPs and BPs is showed in Table 1. TEM imaging indicates the average particle size of individual CuO NPs as 20–40 nm, larger than that provided by producer (Table 1, Fig. S1). Hydrodynamic diameter of CuO NPs in the suspension was concentration-dependent (Fig. S2). CuO NPs at nominal concentration of 100 mg L^{-1} had an average hydrodynamic diameter of 394 ± 35 nm, much larger than the size measured by TEM, due to CuO aggregation and hydration. For oxide NPs, electrostatic repulsion was an important stabilization mechanism, and the NPs tend to aggregate as the surface charge approaches neutral [23]. Therefore, the zeta potential close to zero (only −1.65 ± 0.35 mV) of CuO NPs in tap water could be responsible for its high aggregation. Aeration used in the toxicity tests might decrease aggregation and sedimentation of NPs to a certain extent. CuO NPs and BPs were dissolved in the tap water (Fig. S3), with 14.65 μg L^{-1} and 12.80 μg L^{-1} of released Cu^{2+} ions in 100 mg L^{-1} CuO NPs and BPs suspensions (nominal concentration), respectively after 2 day exposure. Thus, the soluble Cu^{2+} group (0.015 mg L^{-1} Cu^{2+}) was used to examine the toxicity of dissolved ions to the carp.

3.2. Toxicity of CuO NPs to carp

As shown in Fig. S4, during the 96 h acute toxicity, tail beat frequency of carp was lowered after exposure to CuO NPs (>100 mg L^{-1}) compared to BPs, consistent with the mortality rate data (Fig. S5). Tail beat frequency of carp after 4 days of CuO NPs exposure was lower than that after 1 day exposure, indicating that toxicity of CuO NPs was time-dependent. However, the mortality rates were less than 30% at all CuO exposure concentrations (nominal concentrations, 0–1000 mg L^{-1}) (Fig. S5), suggesting that CuO NPs had no obvious acute toxicity to carp up to 1000 mg L^{-1}. Our previous study showed that TiO_2 NPs (200 mg L^{-1}) did not present acute toxicity to carp [9]. The 48 h median lethal concentration (LC_{50}) of TiO_2 NPs to zebrafish was larger than 10 mg L^{-1} (the largest test concentration) [24]. For CeO_2 NPs, no obvious toxicity was found for zebrafish embryos at 200 mg L^{-1} [25]. All these results indicated that these metal oxide NPs including CuO had low acute toxicity to fish. However, NPs could enter the fish body and accumulate in organs and tissues. In addition, metal ions could be dissolved from these NPs after NPs uptake and have sub-acute and chronic toxic effects [26].

A 30 day sub-acute toxicity to carp was further conducted in 100 mg L^{-1} of CuO NPs suspension (with 51 mg L^{-1} of suspended CuO NPs, Fig. S6). Carp growth was inhibited after 30 day exposure (Table 2). Increased length and weight of carp body followed an order: tap water > soluble Cu^{2+} > CuO BPs > CuO NPs, showing that CuO NPs had higher growth inhibition, which was in good agreement with the acute toxicity results. Soluble Cu^{2+} group did not show significant growth change compared to control ($p < 0.05$), suggesting that Cu^{2+} dissolved from CuO NPs in the tap water (i.e., 0.015 mg L^{-1}) had minimal toxicity. Total Cu content in the carp body was up to 2480 μg g^{-1} dry weight after 30 day CuO NPs exposure, 170 times higher than that of fish in the control groups (Fig. 1). Cu content could reach a relatively high value (930 μg g^{-1} dry weight) only after 1 day exposure. The following slow increase of Cu content mirrors Cu excretion by fish, which is discussed below. Uptake of CuO BPs also occurred although the Cu content in carp bodies was lower than that of CuO NPs, suggesting that particles uptake was at least partly dependent on the particle size.

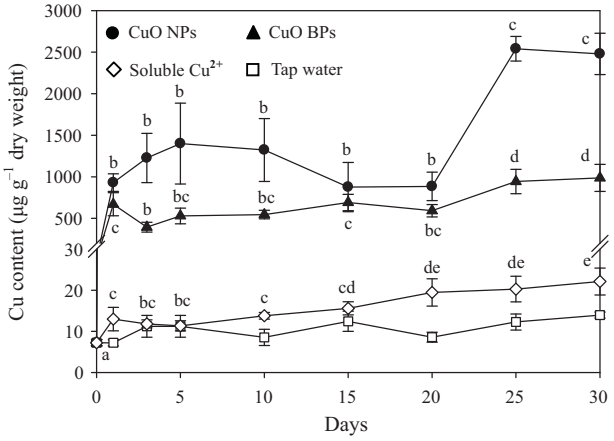

Fig. 1. Cu concentrations in the whole body of carp after exposure to CuO NPs (at a nominal concentration of 100 mg L^{-1}), CuO BPs (at a nominal concentration of 100 mg L^{-1}) and soluble Cu^{2+} (0.015 mg L^{-1}). Error bars indicate the standard deviation ($n = 3$). In the figure, for a given treatment (CuO NPs, CuO BPs or soluble Cu^{2+}), points followed by different letters (a–e) indicate significant differences at different concentrations by the Student–Newman–Keuls test ($p < 0.05$).

Because of aggregation and sedimentation, the actual fish exposure concentration of CuO NPs could be lower than the added nominal concentration. The relationship between the nominal CuO NPs concentrations (0–1000 mg L^{-1}) and the suspended CuO NPs concentrations was linear, and around 70% of CuO NPs was settled down after 48 h aeration (Fig. S6). Thus, fish were likely exposed to 268 mg L^{-1} of suspended CuO NPs in the CuO NPs suspension with a nominal concentration of 1000 mg L^{-1}. However, the actual fish exposure concentration would be a bit higher than the suspended concentration because the settled CuO NPs could be re-suspended as a result of fish swimming to the bottom of the tanks. In the nanotoxicology studies, the actual concentrations of suspended NPs would be more useful than the added nominal concentrations. Therefore, the toxicity of tested NPs could be erroneously concluded to be low if only nominal concentrations are used to evaluate nanotoxicity.

3.3. Distribution and excretion of NPs

To understand the distribution of CuO NPs in carp body, Cu contents in gill, intestine, brain, liver, skin and scale, and muscle were analyzed and Cu level was detected in all test tissues. Over 30 days, the order of Cu contribution after CuO NPs exposure was intestine > others > gill > muscle > skin and scale > liver > brain (Fig. 2). The intestine that only represented 5.7% of the body mass (Table S1) contributed more than 50% of the whole body Cu amount after CuO NPs exposure, much higher than Cu contribution in the tap water (11.1%). After CuO NPs exposure, the gill kept almost the same proportion of Cu despite the increased contribution of intestine, suggesting the gill also played an important role in CuO NPs accumulation. All the other organs/tissues had lower Cu contributions compared to the control.

Time-dependent CuO NPs distribution is expressed on a dry mass basis (Fig. 3). There was a sharp increase of Cu concentration for all test tissues by day 1, and intestine and gill had relative constant accumulations over time. This tendency is consistent with the whole body Cu content results (Fig. 1), suggesting that CuO NPs were internalized across the mouth and gill. Actually, intestine had

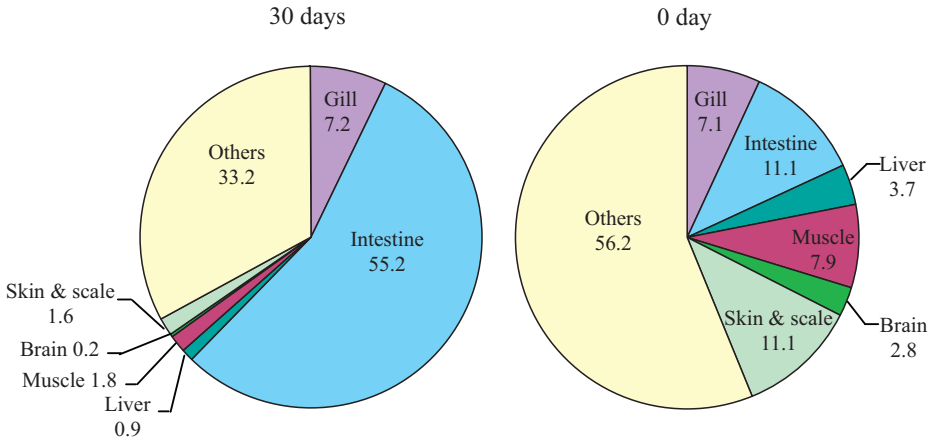

Fig. 2. The Cu percentage contribution of different tissues/organs to the total body Cu burden after CuO NPs exposure (30 days) and before exposure (0 day). "Others" include all the rest parts of the fish such as bones. The percentage contribution after 30 days had significant difference from that before exposure for all tissues/organs except the gill.

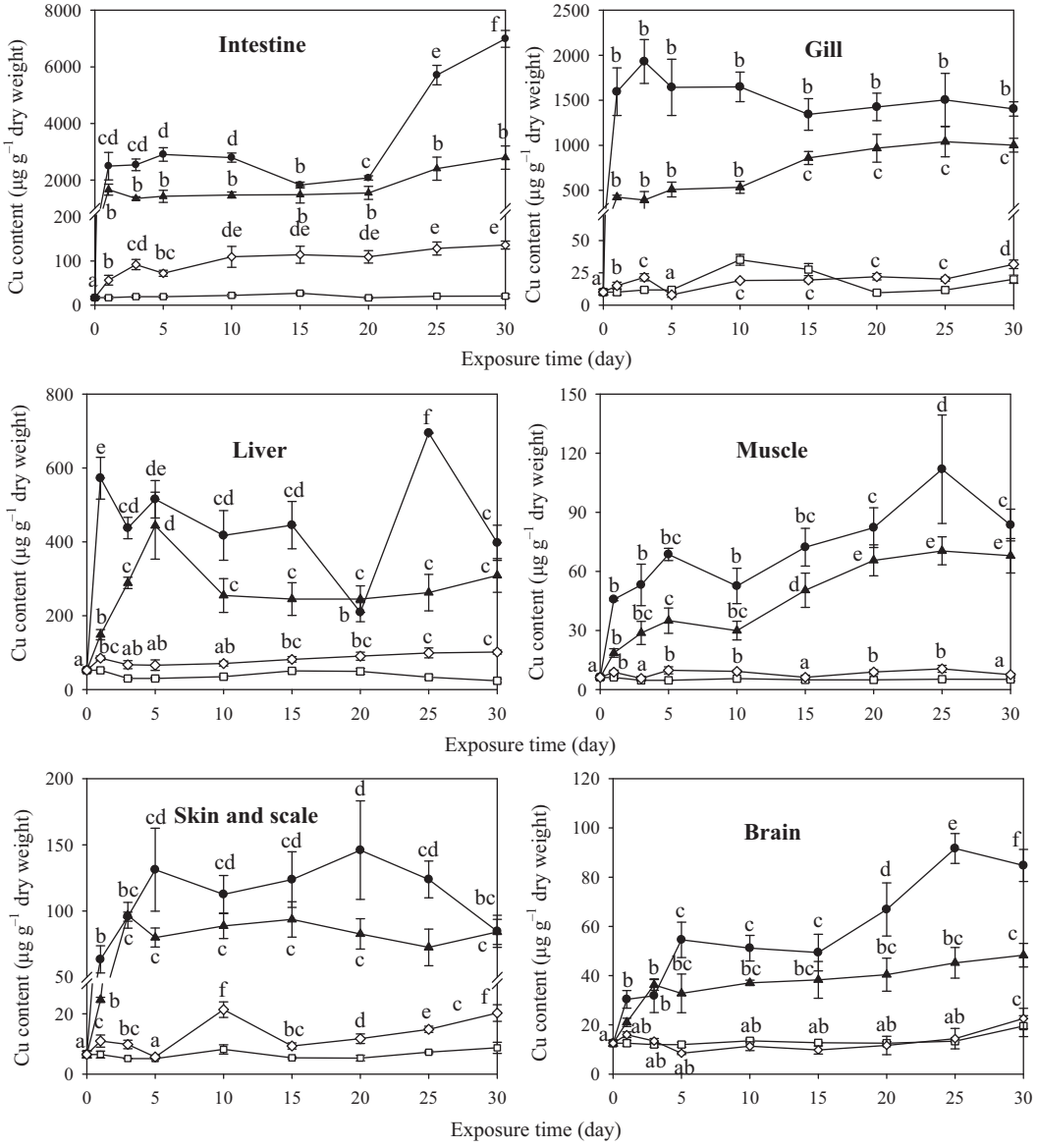

Fig. 3. Cu contents in intestine, gill, liver, muscle, skin and scale, and brain after exposure to CuO NPs for 30 days. Fish were exposed to 100 mg L^{-1} CuO NPs (●), 100 mg L^{-1} CuO BPs (▲), 0.015 mg L^{-1} Cu^{2+} (◊), and the tap water without particles or Cu^{2+} as the control (□). "100 mg L^{-1}" is the nominal concentration. Error bars indicate the standard deviation ($n = 3$). In the figure, for a given treatment (CuO NPs, CuO BPs or soluble Cu^{2+}), points followed by different letters (a–f) indicate significant differences at exposure times by the Student–Newman–Keuls test ($p < 0.05$). (Note: the scale of y-axis on each panel is different.)

Table 1
Characteristics of CuO NPs and BPs used in this study.

Particle	Size[a] (nm)	Size from TEM[b] (nm)	Purity (%)	Surface area ($m^2\ g^{-1}$)	Zeta potential[c] (mV)	Hydrodynamic diameter[c] (nm)	Shape
CuO NPs	<10	20–40	>99.9	13.31	−1.65 ± 0.35	394 ± 35	Granular
CuO BPs	1500	1000–2000	>99.9	0.52	−8.51 ± 1.17	–	Granular

[a] The size was provided by producer.
[b] The size was measured in our lab, TEM images were showed in Fig. S1.
[c] The nominal concentration of particles suspension was 100 mg L^{-1} for the measurements of zeta potential and hydrodynamic diameter; the hydrodynamic diameter of CuO BPs was unavailable because of poor suspension; the hydrodynamic diameters of CuO NPs at other concentrations were showed in Fig. S2.

Table 2
Effects of CuO NPs on the body weight and length of carp after 30 day exposure.

Growth parameter	Tap water	CuO NPs	CuO BPs	Soluble Cu^{2+}
Length (cm)[a]	1.01 ± 0.13	0.29 ± 0.25*	0.70 ± 0.19	0.90 ± 0.26
Weight (g)[b]	0.99 ± 0.01	0.77 ± 0.09*	1.07 ± 0.10	1.00 ± 0.10

*Significant diffidence from the increased length or weight of carp in tap water ($p < 0.05$).
[a] Increased length of carp by day 30.
[b] Increased weigh of carp by day 30.

the highest Cu level, 2490 and 6990 μg g^{-1} dry weight after 1 and 30 day CuO NPs exposure, with the highest bioaccumulation factors (Fig. S7). Fish were fed 30 min before water change, CuO NPs could be adsorbed on fish food and enter intestine together (Fig. S8). This may be an important reason for the highest Cu level in intestine. The highest level of TiO_2 in the intestine of rainbow trout was also found after dietary exposure [13]. Unlike rainbow trout, carp is a stomachless fish and cannot provide an extreme acidic environment before intestine [27,28]. Thus, the Cu presented in the intestines was probably in the form of CuO NPs. This is supported by the TEM image, in which CuO NPs were found in the intestinal cells (Fig. 4c).

High level of Cu was also found in the gill and liver which are known to be target organs for Cu [29]. After 30 day Cu^{2+} solution exposure, accumulated Cu in the liver (101.4 μg g^{-1} dry weight) was higher than that in the gill (31.7 μg g^{-1} dry weight) while the gill showed higher accumulation than liver for CuO NPs. NPs Uptake in the gill is a combination of adsorption on the gill surface and the subsequent penetration across the gill [30]. Gills have a large

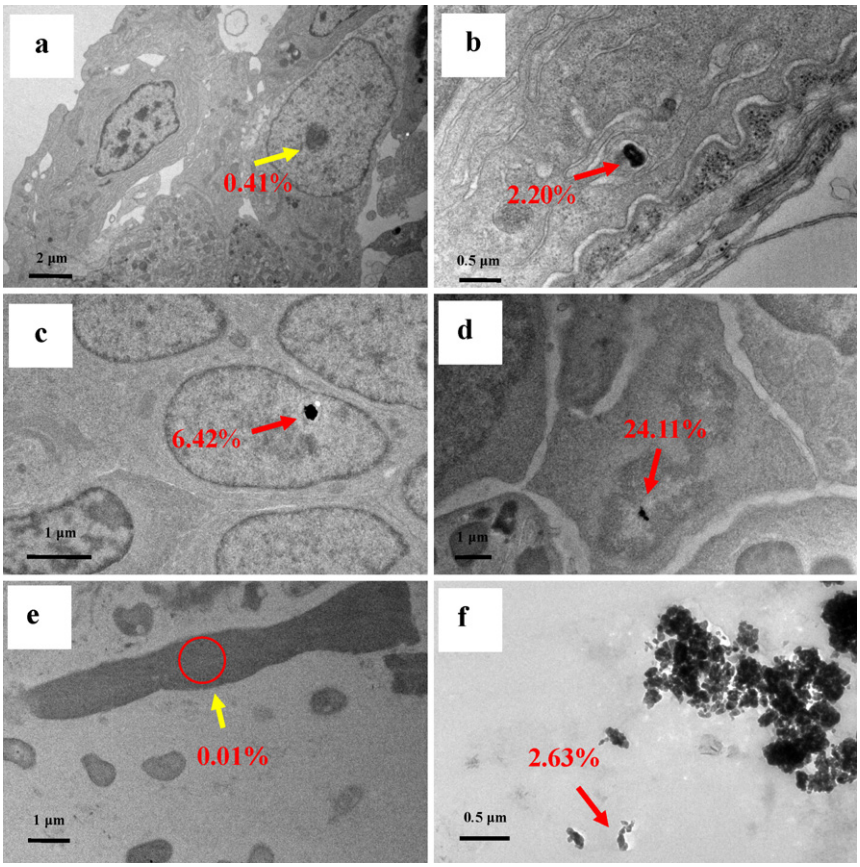

Fig. 4. TEM observations of NPs distribution in gill, liver, intestine and excrement of carp after 30 days exposure in CuO NPs suspension at a nominal concentration of 100 mg L^{-1}. a: gill control; b: gill after exposure; c: intestine after exposure; d: liver after exposure; e: excrement control; f: excrement after exposure. The point showed by arrow or circle was analyzed by EDS, and the NPs-accumulated point was showed by red arrow. Cu atomic percent of different points was also showed in red color.

surface area and could greatly accumulate CuO NPs. Moreover, CuO NPs in the tap water were aggregated (Table 1) and could also limit the further transfer from gill to bloodstream, which may be anther reason for higher Cu accumulation in gill. However, CuO NPs were observed in the macrophage-like cells of gill (Fig. 4b), intracellular NPs were mainly present in the lysosomes which could provide low pH environment and enhance the NPs dissolution [26]. The released free Cu^{2+} ions could enter the circulatory system of the fish and lead to widespread distribution. CuO NPs were also detected in liver from TEM observation and EDS analysis (Fig. 4d). CuO NPs in the intestine and gill would reach the liver and lead to a high Cu level in liver via blood stream. C_{60} [12] and TiO_2 [13] NPs were reported to be in the liver of medaka and rainbow trout, respectively. It has been demonstrated that the role of the liver is central in Cu storage and metabolism [31], therefore it is possible that Cu^{2+} ions could also be present in the liver. Cu ions released from CuO NPs could bind to proteins in the liver such as metallothionein [32,33]. This reaction would increase the dissolution of CuO NPs and bring more toxicity to liver and other organs.

In contrast to the intestine and gill, Cu levels in the muscle kept increasing until the 25th day. It is probably because Cu was taken up and stored by other organs such as gill primarily before transporting to the muscle, and low Cu levels were thus present at all time points. Lack of binding proteins may be another reason for low level of Cu in the muscle. While the muscle is not the main target of CuO NPs or Cu ions, long period of exposure may result in accumulation of significant Cu levels, which is important from the point of human food safety.

Cu was detected in the skin and scale, probably because of the adsorption of CuO NPs and binding of Cu ions. CuO NPs can hardly go through the skin because of the effective barrier provided by the external epithelium. Mucus produced on the fish skin would also prevent the penetration and injury of CuO NPs. Therefore, unlike gill, direct contact with water did not make high accumulation of Cu on the skin. This may be another reason for low Cu level in the muscle.

Interestingly, Cu was also detected in the brain, and the Cu content was dependent on the exposure time. The contribution of Cu ions dissolved in the tap water to total Cu content in the brain appears insignificant because Cu content was not increased in the soluble Cu^{2+} group in comparison to the control group (Fig. 3). Further, CuO NPs was not detected in the brain from TEM observation and EDS analysis (Fig. S9). The solubility of CuO NPs could be highly enhanced by biological solutions after entering the fish body. The release of Cu from Cu-based particles in synthetic biological fluids (i.e., blood) is above 5 mg L^{-1} after 48 h of exposure [34], much higher than that from CuO NPs in the tap water (0.015 mg L^{-1}). CuO NPs in the gill could be taken up by lysosomes and dissolved inside [26]. Therefore, CuO NPs firstly entered the fish body via gill and mouth, and then may be partially dissolved in the body and finally the solubilized ions entered the brain. For all tissues/organs, the Cu levels and bioaccumulation factors in the CuO NPs group were higher than those in the CuO BPs group (Fig. 3, Fig. S7), clearly showing that CuO NPs were transported more readily and had higher toxicity to carp.

Excrements were collected and analyzed after carp were exposed to CuO NPs at different times (Fig. 5). Clearly, the Cu content in the excrements increased with increasing exposure time (0–96 h), while the Cu contents of main cumulative organs (intestine and gill) and whole body only sharply increased by day 1, indicating that Cu (ions or NPs) could be eliminated together with excrements during the exposure time. Furthermore, CuO NPs were observed in the excrements by TEM imaging (Fig. 4e and f), these NPs may not participate in physiological metabolism, thus having low toxic impact.

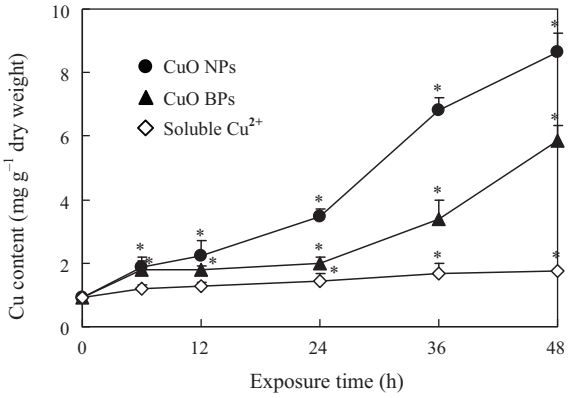

Fig. 5. Cu concentrations in the carp excrements after exposure to CuO NPs (nominal concentration = 100 mg L^{-1}), CuO BPs (nominal concentration = 100 mg L^{-1}) and Cu^{2+} (0.015 mg L^{-1}) at different times. After exposure at different times, carp were washed with clean water and cultivated continually for 48 h before excrement collection. Error bars indicate the standard deviation ($n = 3$). For each treatment, asterisk (*) indicates statistically significant difference ($p < 0.05$) from the Cu content before exposure.

3.4. ChE activity and potential neurotoxicity

For the control group, ChE activity of the carp brain remained constant during 30 days of cultivation (Fig. 6). However, for the CuO NPs treated group, ChE activity initially decreased significantly ($p < 0.05$) and remained constant till the fifth day. Also in the CuO NPs treated group, the Cu contents in the carp brain rapidly increased over all 5 days (Fig. 3), indicating that ChE inhibition was highly related to the uptake of Cu by brain. After that, ChE activities of CuO NPs and BPs groups recovered gradually but were still significantly lower than the control at the last time point (30th day) ($p < 0.05$). The recovery was probably attributed to the fish adaptability and relatively steady Cu contents in the brain after day 5 (Fig. 3) due to possible removal of Cu by metabolism and excretion. Higher rapid ChE inhibition and faster recovery in rats has also been reported following the exposure of a neurotoxin, chlorpyrifos [35].

During the whole exposure time, ChE inhibition followed the order: CuO NPs > CuO BPs > soluble Cu^{2+}, showing that CuO NPs may have toxicity potential to nervous system because ChE (mainly AChE) present in brain are crucial enzymes for neural transmission and are biomarkers for neurotoxicity. For CuO NPs, as discussed

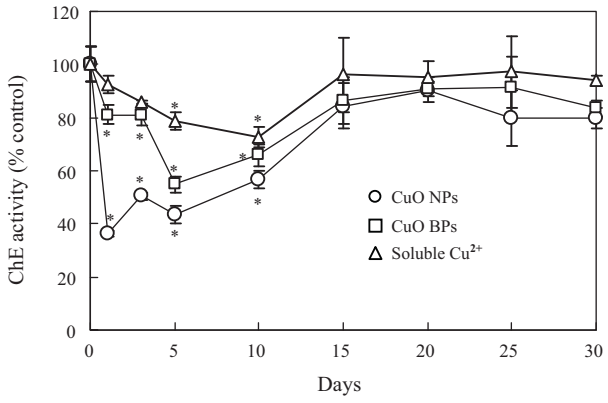

Fig. 6. ChE activity in the brain of carp after exposure to CuO NPs (nominal concentration = 100 mg L^{-1}), CuO BPs (nominal concentration = 100 mg L^{-1}) and soluble Cu^{2+} (0.015 mg L^{-1}) ($n = 3$). The ChE activity of carp brain in the CuO NPs, CuO BPs or soluble Cu^{2+} group was expressed as percent of that in the control group (tap water only). Error bars indicate the standard deviation ($n = 3$). For each treatment, asterisk (*) indicates statistically significant difference ($p < 0.05$) from the Cu content before exposure.

above, Cu in the brain was mainly in the form of Cu ions because CuO NPs could be dissolved during their transport in the carp body. Our previous study showed that Cu^{2+} ions dissolved from Cu NPs highly inhibited in vitro AChE activity (about the half of AChE inhibition) [16]. Therefore, it is probable that ChE inhibition in the brain was mainly caused by free Cu^{2+} ions dissolved from CuO NPs inside the carp body.

4. Conclusions

CuO NPs had no obvious acute toxicity to juvenile carp. Growth of carp was significantly inhibited by CuO NPs during the 30 day sub-acute toxicity tests. Intestine and gill of the carp contributed to most of whole body Cu amounts after CuO NPs exposure, suggesting intestine and gill were the cumulative organs to CuO NPs. CuO NPs could be distributed in intestine, gill and livers and partially eliminated from the body via the excrement as shown from TEM observations. ChE activity in the carp brain was highly inhibited after CuO NPs exposure, probably because of the free Cu^{2+} ions released from CuO NPs inside the carp body. The ChE inhibition suggested that CuO NPs in water have neurotoxicity potential to carp. For all the tests, CuO NPs had higher toxicity than CuO BPs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.09.094.

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