Environmental Pollution 243 (2018) 723–733

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Toxicity of copper oxide nanoparticles to Neotropical species *Ceriodaphnia silvestrii* and *Hyphessobrycon eques*☆

Adrislaine S. Mansano*, Jaqueline P. Souza, Juliana Cancino-Bernardi, Francine P. Venturini, Valéria S. Marangoni, Valtencir Zucolotto

Nanomedicine and Nanotoxicology Group, Physics Institute of São Carlos (IFSC), University of São Paulo (USP), Brazil

ARTICLE INFO

Article history:
Received 25 May 2018
Received in revised form
12 August 2018
Accepted 3 September 2018
Available online 8 September 2018

Keywords:
Copper oxide nanoparticles
Chronic toxicity
Oxidative stress
Cytotoxicity
Environmental risk

ABSTRACT

The increase of production and consumption of copper oxide nanostructures in several areas contributes to their release into aquatic ecosystems. Toxic effects of copper oxide nanoparticles (CuO NPs), in particular, on tropical aquatic organisms are still unknown, representing a risk for biota. In this study, the effects of rod-shaped CuO NPs on the Neotropical species *Ceriodaphnia silvestrii* and *Hyphessobrycon eques* were investigated. We also compared the toxicity of CuO NPs and $CuCl_2$ on these species to investigate the contribution of particles and cupper ions to the CuO NPs toxicity. Considering the low copper ions release from CuO NPs (<1%), our results revealed that the toxicity of CuO NPs to *C. silvestrii* and *H. eques* was mainly induced by the NPs. The 48 h EC_{50} for *C. silvestrii* was 12.6 ± 0.7 μg Cu L^{-1} and for *H. eques* the 96 h LC_{50} was 211.4 ± 57.5 μg Cu L^{-1} of CuO NPs. There was significant decrease in reproduction, feeding inhibition and increase in reactive oxidative species (ROS) generation in *C. silvestrii* exposed to CuO NPs. In fish *H. eques*, sublethal exposure to CuO NPs caused an increase in ROS generation in gill cells and an increase in cells number that were in early apoptotic and necrotic stages. Our results showed that CuO NPs caused toxic effects to *C. silvestrii* and *H. eques* and ROS play an important role in the toxicity pathway observed. Data also indicated that *C. silvestrii* was among the most sensitive species for CuO NPs. Based on predicted environmental concentration in water bodies, CuO NPs pose potential ecological risks for *C. silvestrii* and *H. eques* and other tropical freshwater organisms.

1. Introduction

Copper oxide nanoparticles (CuO NPs) have been applied in several areas due to their excellent thermophysical, catalytic and antibacterial properties (Ebrahimnia-Bajestan et al., 2011; Majumder and Neogi, 2016; Prasad et al., 2016). CuO NPs are widely used in electronics, catalysis, sensors, solar cells, drug delivery, agriculture, food preservation, textiles, paints, coatings and water treatment (Bondarenko et al., 2013; Keller et al., 2017). More specifically, rod-shaped CuO have been demonstrated enhanced properties due to their greater surface to volume ratio compared to the spherical ones (Jia et al., 2015). Inevitably, the increasing production and widespread utilization of CuO NPs lead to their release into the aquatic environment, posing a potential hazard to non-target organisms (Lu et al., 2017; Wu et al., 2017).

Concerns regarding the potential impacts of NPs in the aquatic environments have risen sharply. Although CuO NPs present a low dissolution rate in water, they may cause toxic effects on aquatic organisms (Keller et al., 2017). It is still controversial about what is the major source of toxicity of CuO NPs. Based on the literature, toxicity of CuO NPs may be exerted by particles specific effects (Cronholm et al., 2013; Manusadzianas et al., 2012), by copper ions released from NPs (Jo et al., 2012) or by both particle and ions (Xiao et al., 2015, 1016). The dissolved Cu^{2+} from CuO NPs can enter the cells through ion channels and active transport. In addition, CuO NPs may interact with cells due to their small size, and cross the cell membranes through ion channels and transporter proteins, endocytosis and/or phagocytosis (Chang et al., 2012). Thus, CuO NPs can cause toxicity by one or more pathways, inducing cell membrane damage, mitochondrial injury, protein denaturation, modification of nucleic acids, cellular damage through reactive oxygen species (ROS) generation,

☆ This paper has been recommended for acceptance by Baoshan Xing.

* Corresponding author. Nanomedicine and Nanotoxicology Group, Physics Institute of São Carlos, University of São Paulo, Av. Trabalhador Sãocarlense, 400, São Carlos, SP, 13560-970, Brazil.
E-mail address: laine_mansano@yahoo.com.br (A.S. Mansano).

https://doi.org/10.1016/j.envpol.2018.09.020
0269-7491/

oxidative stress, DNA damage and apoptosis (Hou et al., 2017; Peng et al., 2017; Thit et al., 2017).

Exposure of aquatic organisms to CuO NPs has evidenced the NPs accumulation and trophic transfer through the food chain (Ates et al., 2014, 2015). Aquatic organisms may be in contact with CuO NPs either by direct uptake from water or by dietary intake, which can result in adverse consequences to organisms and ecosystem. Previous studies have reported the effects of CuO NPs on various species, such as bacteria (Bondarenko et al., 2012), cyanobacteria (Lone et al., 2013), algae (Melegari et al., 2013; Zhao et al., 2016), protozoans (Mortimer et al., 2010), cladocerans (Xiao et al., 2015; Wu et al., 2017), fish (Sun et al., 2016; Xu et al., 2017) and aquatic plants (Shi et al., 2011; Zhao et al., 2017). Most data regarding the toxicity of CuO NPs has focused on acute exposure of organisms at relatively high concentrations, which implies little ecological realism. Some studies have investigated the long-term CuO NPs toxicity on cladoceran and fish. Adam et al. (2015) and Rossetto et al. (2014) observed that CuO NPs chronic exposure affected the growth and reproduction of *Daphnia magna* at different concentrations (>1 mg L^{-1}). Zhao et al. (2011) reported *Cyprinus carpio* fish growth was inhibited after 30 day sublethal exposure to CuO NPs (100 mg L^{-1}), while Ates et al. (2015) found that CuO NPs induced oxidative stress in *Carassius auratus* liver and gills after 21 day exposure (1 and 10 mg L^{-1}). However, toxicity data on long-term and chronic effects of CuO NPs at low exposure levels are still insufficient (Hou et al., 2017).

In addition, most toxicity studies on aquatic invertebrates and vertebrates were conducted on standard test species (OECD, 1992; USEPA, 2011), such as the cladocerans *D. magna* and *Ceriodaphia dubia*, and fish *Danio rerio* (zebrafish) and *Oncorhynchus mykiss* (rainbow trout). Only few studies have investigated the effects of NPs on tropical native species (e.g. Yoo-iam et al., 2014). Toxicological effects of CuO NPs on tropical aquatic organisms are still unknown, representing a risk for biota. Therefore, there is a growing need in obtaining ecotoxicological data under tropical conditions, using native test organisms for the environmental risk assessment of NPs.

The aim of this study was to evaluate the toxicity of rod-shaped CuO NPs to Neotropical species *Ceriodaphnia silvestrii* and *Hyphessobrycon eques*. Rod-shaped CuO NPs were selected due to their wide application and different way of interacting with a biological system since the particle shape seems to have effects on biological responses (Misra et al., 2014; Venkataraman et al., 2011). In addition, we also compared the toxicity of CuO NPs and $CuCl_2.2H_2O$ on these species to investigate the contribution of particles and cupper ions to the CuO NPs toxicity. *C. silvestrii* (Cladocera) has a wide geographic distribution in South America; a short life cycle and is easy to maintain in laboratory conditions; and belongs to one of the most sensitive group of species for a variety of contaminants (Fonseca and Rocha, 2004; Mansano et al., 2016). *Hyphessobrycon eques* (Characidae) is a Neotropical fish widely distributed in South America, especially in regions with pH levels between 6.5 and 7.0, temperature between 26 °C and 28 °C; and due to its peculiar body coloration, it is highly appreciated as an aquarium fish (Aguinaga et al., 2014; Fujimoto et al., 2013). The toxicity of CuO NPs and $CuCl_2$ to *C. silvestrii* and *H. eques* was evaluated through lethal and sublethal exposure: for cladoceran, the endpoints immobility, reproduction, feeding rates and reactive oxygen species (ROS) generation were investigated, while for fish the mortality, ROS generation, apoptosis and necrosis induction and uptake in the gill cells were assessed. We also evaluated the sensitivity of these species compared to other species through species sensitivity distribution curves and discussed the importance of using native species in risk assessments.

2. Material and methods

2.1. Synthesis and characterization of CuO NPs

CuO nanoparticles were synthesized by modification of a previous method (Misra et al., 2014). For this, 335 μL of glacial acetic acid were added to 100 mL of a 0.02 M $CuSO_4.5H_2O$ aqueous solution. The system was heated to 100 °C followed by a rapid addition of 0.45 g NaOH under vigorous stirring. After 15 min, the system was cooled to room temperature and the black precipitated was washed in ultrapure water by centrifugation. The particles were then resuspended in solution containing 1% m/v of sodium citrate. After 10 min of sonication, the systems were washed by centrifugation twice and resuspended in ultrapure water.

The CuO NPs functionalized with sodium citrate were structurally characterized by powder X-ray diffraction (XRD). The hydrodynamic diameter and polydispersity index value (PdI) of the NPs were analyzed by dynamic light scattering (DLS) in Malvern Zetasizer Nano ZS90, which was also used for zeta potential measurements. Transmission electron microscopy (TEM) images were acquired using a JEOL JEM-2100 operating at 200 kV.

2.2. Copper exposure concentrations and ion release

The actual copper concentrations in CuO NPs suspensions and in $CuCl_2.2H_2O$ solutions used in the toxicity tests were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 7000 Series, Thermo Scientific). Samples were taken from the exposure medium, acidified to 1% HNO_3 and frozen at −20 °C until analysis.

To evaluate the ion release from CuO NPs, dissolution assays were performed at a concentration of 100 μg Cu L^{-1} in the cladoceran and fish exposure media, under the same toxicity test conditions. Samples were collected at three time points (0, 24 and 48 h) throughout the experiment and centrifuged at 7500 × *g* for 30 min through a 3 kDa Amicon centrifugal filter (Merck Millipore, Darmstadt, Germany) to remove any CuO NPs or agglomerates. $CuCl_2$ solution was similarly centrifuged to determine the ionic Cu loss during centrifugation. The samples were acidified to 1% HNO_3 and the copper concentrations were analyzed by ICP-OES.

2.3. Test organisms

The cladoceran *Ceriodaphnia silvestrii* was maintained in reconstituted water (pH 7.0–7.6, conductivity 160 μS cm^{-1} and hardness 40–48 mg $CaCO_3$ L^{-1}) at 25 ± 1 °C and 12:12 h light/dark photoperiod, as recommended by the Brazilian Association of Technical Standards (ABNT, 2005). The organisms were fed daily with the algae *Raphidocelis subcapitata* (10^5 cells mL^{-1}) and a food supplement containing yeast and fish food was added (ABNT, 2005).

The adult fish *Hyphessobrycon eques* (4–6 months old, body weight 0.39 ± 0.11 g, body size 3.08 ± 0.23 cm) was obtained commercially and kept in stock 50-L aquaria with dechlorinated tap water and aeration for 15 days prior to the tests. The animals were maintained under controlled temperature (25.0 ± 1 °C), pH (7.0 ± 1.0), dissolved oxygen (80% air saturation), and photoperiod (12:12 h light/dark). The organisms were fed once a day with commercially available artificial fish diet (Tetra Color - Tropical Flakes), which was suspended 24 h before the beginning of the toxicity tests (OECD, 1992). This study was approved by the Ethics Committee for Animal Use of the Physics Institute of São Carlos of the University of São Paulo under protocol number 17/2016.

2.4. Acute and chronic toxicity tests with C. silvestrii

The acute and chronic toxicity tests followed the ABNT guidelines (2004, 2005). In the acute assays, the concentrations of 0.0, 7.0, 10.0, 13.0, 16.0 and 19.0 μg Cu L^{-1} for both CuO NPs and $CuCl_2.2H_2O$ were tested. The acute exposure test was performed in four replicates per concentration and five neonates (6–24 h old) per replicate (n = 20). The assays were maintained at 25 ± 1 °C, without addition of food. After 48 h of exposure, the number of immobile individuals was counted and used to calculate the median effective concentration (EC_{50}).

Based on the acute toxicity tests, the chronic assays were performed at the following concentrations: 0.0, 0.5, 1.0, 2.0, 4.0, 8.0 and 10.0 μg Cu L^{-1} for CuO NPs and 0.0, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 μg Cu L^{-1} for $CuCl_2.2H_2O$. Chronic toxicity tests were conducted using ten replicates, each containing one neonate (6–24 h old) in 15 mL of test solution (n = 10). The cladocerans were fed and kept under the same conditions as described above for culture maintenance. The duration of the chronic test was 8 days (control organisms produced three broods of offspring). The test solutions were renewed every other day after the number of surviving adults and neonates had been registered. The variables pH, temperature and dissolved oxygen were measured at the start and at the end of the toxicity tests.

2.5. Feeding inhibition and post-exposure tests with C. silvestrii

The feeding inhibition and post-exposure assays were based on the method described by McWilliam and Baird (2002). Adult organisms (48 h old) were exposed to different sublethal concentrations of CuO NPs and $CuCl_2.2H_2O$ and a fixed concentration of 5×10^5 cells mL^{-1} of algae *R. subcapitata*, provided as food. The cladocerans were fed during 24 h in the dark (exposure period). The experimental design had four replicates per treatment, and five individuals per replicate (n = 20). An additional replicate with media and algae but without cladoceran was performed for each treatment, to determine any changes from the initial algal concentration. After 24 h, organisms were transferred to flasks containing clean medium (not contaminated) with *R. subcapitata* suspension at a concentration of 5×10^5 cells mL^{-1} and allowed to feed for 4 h in the dark (post-exposure period). After the 24 h exposure and the 4 h post-exposure times, algae concentration was measured by fluorometric determination of the chlorophyll-a concentration using a microplate reader (SpectraMax M3). The fluorescence values were converted to cells mL^{-1} using calibration curve. Feeding rates were calculated by the equation given by Allen et al. (1995): $F = V(C_0 - C_{24\ or\ 4})/t$, where F is the feeding rate (cells $individual^{-1}\ h^{-1}$); V is volume of medium in the test vessel (mL); C_0 is initial algal cell concentration (cells mL^{-1}); $C_{24\ or\ 4}$ is final algal cell concentration (cells mL^{-1}) and t is duration of the experiment (24 or 4 h).

2.6. ROS generation in C. silvestrii

ROS production was determined using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). The ROS bioassays performed were adapted from the method described by Souza et al. (2018) and Xie et al. (2006). Adult organisms (48 h old) were exposed to different sublethal concentrations of NPs CuO and $CuCl_2.2H_2O$ during 24 h. Tests were carried out using four replicates per treatment and 40 organisms per replicate (n = 160). After exposure, organisms were transferred to containers in 1 mL of 0.1 M phosphate buffer (pH 7.4) and subsequently homogenized (Ultra-Turrax T8 IKA) under ice bath. Homogenates were centrifuged at 10,000 × *g* at 4 °C for 10 min. The supernatant was collected (20 μL) and incubated in phosphate buffer with 5 μM H_2DCF-DA for 30 min at 25 °C in the dark. As a positive control, a subset was exposed to 0.5% H_2O_2 (v/v). Fluorescence intensity was measured using a microplate reader (SpectraMax M3) with excitation at 485 nm and emission at 530 nm. ROS levels were expressed as a percentage of fluorescence intensity relative to control group.

2.7. Acute toxicity tests with H. eques

The acute toxicity tests were performed according to the Organization for Economic Cooperation and Development (OECD, 1992). Adult organisms were exposed to concentrations of 0, 10, 30, 60, 120, 240 and 480 μg Cu L^{-1} for CuO NPs and 0, 12.5, 25, 50, 100, 200 and 300 μg Cu L^{-1} for $CuCl_2.2H_2O$. Experiment was carried out in glass aquaria filled with 3 L static exposure medium with constant aeration and without feeding. Three replicates were used for each treatment, each containing five individuals (n = 15). The duration of the test was 96 h and the fish were maintained at 25 ± 2 °C and a 12:12 h light/dark photoperiod. Fish mortalities were recorded every 24 h and any dead fish was removed from the exposure aquaria. The number of dead individuals was used to determine the median lethal concentration (LC_{50}). Dissolved oxygen, pH, and temperature were measured at the start and at the end of the toxicity tests.

2.8. ROS generation in H. eques gill cells

For ROS production evaluation in gill cells, fish were exposed to three sublethal concentrations of CuO NPs and $CuCl_2.2H_2O$. The exposure conditions were the same as described previously for acute toxicity tests (item 2.7). Experiments were performed in three replicates per treatment with six fish per replicate (n = 18). After 24 h of exposure, fish were collected and anesthetized in cold water (4 °C), and gills from each group were harvested. Following, gills were homogenized in ice-cold 0.01 M phosphate buffered saline (PBS, pH 7.4) in proportion 1:10 (tissue mass:volume). Homogenates were centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatant containing gill cells was collected (20 μL) and incubated in PBS buffer with 5 μM H_2DCF-DA at 25 °C for 30 min in the dark. As a positive control, a subset was exposed to 0.5% H_2O_2 (v/v). Fluorescence intensity was measured at 485 nm excitation and 530 nm emission using a microplate reader (SpectraMax M3). ROS levels were expressed as a percentage of fluorescence intensity relative to control group.

2.9. Apoptosis, necrosis and uptake assays in H. eques gill cells

After 24 h of exposure to CuO NPs and $CuCl_2.2H_2O$, gill cell suspensions were prepared as described previously (item 2.8) and used for the apoptosis, necrosis and uptake assays. Apoptosis and necrosis were determined by flow cytometry (BD FACSCalibur® Flow Cytometer, USA) after staining with FITC Annexin V/Propidium Iodide (PI) (FITC Annexin V Apoptosis Kit, BD Bioscience®, USA) following the manufacturer's instructions. All samples were analyzed by acquisition of 10,000 events.

The cellular uptake was investigated by side scattering (SSC) intensity value, a cell granularity measurement tool performed by flow cytometry (Park et al., 2017; Suzuki et al., 2007). Gill cell suspensions (control and treatments) were diluted in PBS buffer (pH 7.4) at a concentration of 1×10^6 cells ml^{-1} and analyzed by flow cytometry with acquisition of 10,000 events.

2.10. Species sensitivity distribution

Species sensitivity distribution (SSD) curve was constructed to compare acute toxicity values obtained from the *C. silvestrii* and

H. eques tests evaluating cupper nanoparticles with values for other aquatic species. Toxicity data were compiled from the USEPA ECOTOX database, supplemented with available literature data. Only laboratory toxicity data for freshwater organisms that could be confirmed from original publications were included in the SSD. Log-normal distributions of toxicity values were constructed using the ETX 2.0 software (Van Vlaardingen et al., 2004). The fraction of species potentially affected (potentially affected fraction - PAF) and the 5th percentile (hazardous concentration for the 5% of species - HC_5) and 50th percentile (hazardous concentration for the 50% of species - HC_{50}) with their confidence limits were determined with this software according to the method described by Aldenberg and Jaworska (2000). Log-normality at 5% significance level was evaluated with the Anderson-Darling test included in the ETX software package.

2.11. Data analysis

The EC_{50} and LC_{50} values for the acute toxicity tests were calculated by nonlinear regression using logistic curve in the Statistica 7.0 software (Statsoft, 2004). Data from sublethal assays were assessed for normality (Chi-squared test) and homogeneity of variances (Bartlett's test), and then analyzed using one-way ANOVA. A post hoc multiple comparisons Dunnett's test was performed to verify significant differences between treatments and control at 95% confidence. All statistical tests were carried out using Statistica 7.0 software (Statsoft, 2004).

3. Results

3.1. Characterization of CuO NPs

The characterization of CuO NPs by X-ray diffraction (XRD) is shown in Fig. 1A. The characteristic peaks corresponding to (−111), (111), (−202), (202), (−311), and (220) planes are located at $2\theta = 35.57°$, 38.74°, 48.76°, 58.35°, 66.28° and 68.13°, respectively. They correspond to CuO monoclinic phase by comparison with the Joint Committee on Powder Diffraction Standards (JCPDS) card files no. 48−1548. The absence of peaks related to Cu_2O_3, Cu_2O, and Cu confirmed the formation of single-phase CuO. TEM images showed that CuO NPs were rod-shaped nanoparticles (Fig. 1B); the average transversal and longitudinal sizes were about 8 and 28 nm respectively. Zeta potential measures revealed a high negative potential (−23.5 ± 0.7 mV) due to the coating with sodium citrate. The hydrodynamic diameter was around 30 nm (Fig. 1C).

The characterization of CuO NPs in the cladocerans and fish exposure media are presented in Supplementary Material Tables S1 and S2, respectively. In this study, CuO NPs underwent aggregation in test medium of the organisms, and the particle aggregation was higher in the fish medium (average hydrodynamic size: 264.2 ± 52.5 nm) than in the cladoceran exposure medium (average hydrodynamic size: 247.8 ± 18.8 nm). The zeta potential of all CuO NPs suspensions was between −19 mV and −37 mV.

3.2. Abiotic variables of the toxicity tests and chemical analysis

During the acute and chronic toxicity tests, the pH of the test solutions ranged from 7.06 to 7.52, water temperature varied between 24.7 and 25.4 °C and dissolved oxygen from 6.02 to 6.89 mg L^{-1} for cladoceran. For fish, the pH in all toxicity tests ranged from 7.01 to 7.29, water temperature varied between 24.6 and 25.1 °C and dissolved oxygen from 6.17 to 7.71 mg L^{-1}. No mortality was observed in the control group during exposures. Thus, all tests met the validity criteria set by ABNT (2005) and OECD (1992).

After analyzing the test solutions in ICP-OES, our results showed that the actual copper exposure concentrations in toxicity tests with CuO NPs and $CuCl_2$ differed by less than 10% from the nominal concentrations (Supplementary Material Figs. S1 and S2). The average percentage recovery of Cu in the all the toxicological media samples was 98.2 ± 6.7%. Therefore, the results were calculated based on nominal concentrations, as suggested by International Organization for Standardization (ISO, 2000).

Dissolved Cu^{2+} concentration was below the 1 μg Cu L^{-1} detection limit of the ICP-OES in both the cladoceran and fish exposure medium, indicating that the freely dissolved Cu fraction was less than 1% of the total CuO NPs added (100 μg Cu L^{-1}).

3.3. Acute and chronic effects to C. silvestrii

Acute exposure to CuO NPs and $CuCl_2$ caused high immobility for *C. silvestrii* at low concentrations (Table 1). The results also indicated that the Cu^{2+} ions added as $CuCl_2$ caused greater toxicity than the CuO NPs to *C. silvestrii*. In chronic exposure, survival of *C. silvestrii* females after 8 days of exposure to CuO NPs (Fig. 2A) and $CuCl_2$ (Fig. 2B) was not affected at the concentrations tested (survival ≥ 90%). Regarding the fertility of *C. silvestrii*, CuO NPs (Fig. 2A) caused a significant decrease in the number of neonates per female at the concentration from 10 μg Cu L^{-1}. The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values for CuO NPs were 8 μg Cu L^{-1} and 10 μg Cu L^{-1}, respectively. For $CuCl_2$ (Fig. 2B), there was a significant decrease in *C. silvestrii* fertility at concentrations of 4 and 8 μg Cu L^{-1}. The NOEC and LOEC values for $CuCl_2$ were 2 μg Cu L^{-1} and 4 μg Cu L^{-1}, respectively.

The feeding rates of *C. silvestrii* after 24 h of exposure to increasing concentrations of CuO NPs and $CuCl_2$ were affected in a

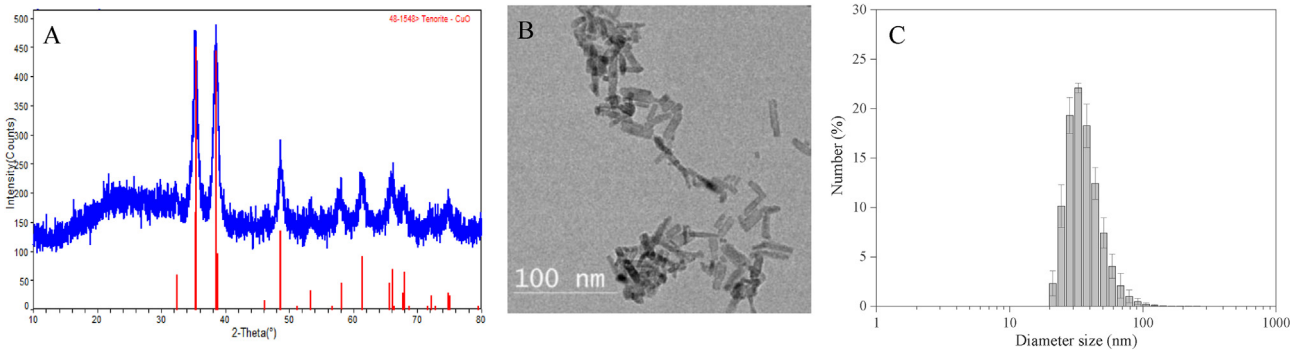

Fig. 1. X-ray diffraction patterns (A), TEM image (B) and DLS of the copper oxide nanoparticles (CuO NPs). The vertical red lines in (A) correspond to patterns of CuO (Tenorite, JCPDS 48−1548). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Acute toxicity values (μg Cu L^{-1}) for the cladoceran *Ceriodaphnia silvestrii* and the fish *Hyphessobrycon eques* exposed to copper oxide nanoparticles (CuO NPs) and copper chloride dihydrate ($CuCl_2.2H_2O$).

Compounds tested	*Ceriodaphnia silvestrii*			*Hyphessobrycon eques*		
	48 h EC_{10}	48 h EC_{20}	48 h EC_{50}	96 h LC_{10}	96 h LC_{20}	96 h LC_{50}
CuO NPs (μg Cu L^{-1})	9.9 ± 0.6	10.8 ± 0.5	12.6 ± 0.7	90.1 ± 58.0	123.5 ± 58.2	211.4 ± 57.5
$CuCl_2.2H_2O$ (μg Cu L^{-1})	7.6 ± 0.6	8.4 ± 0.5	9.8 ± 0.6	25.0 ± 14.6	32.2 ± 14.8	49.8 ± 14.2

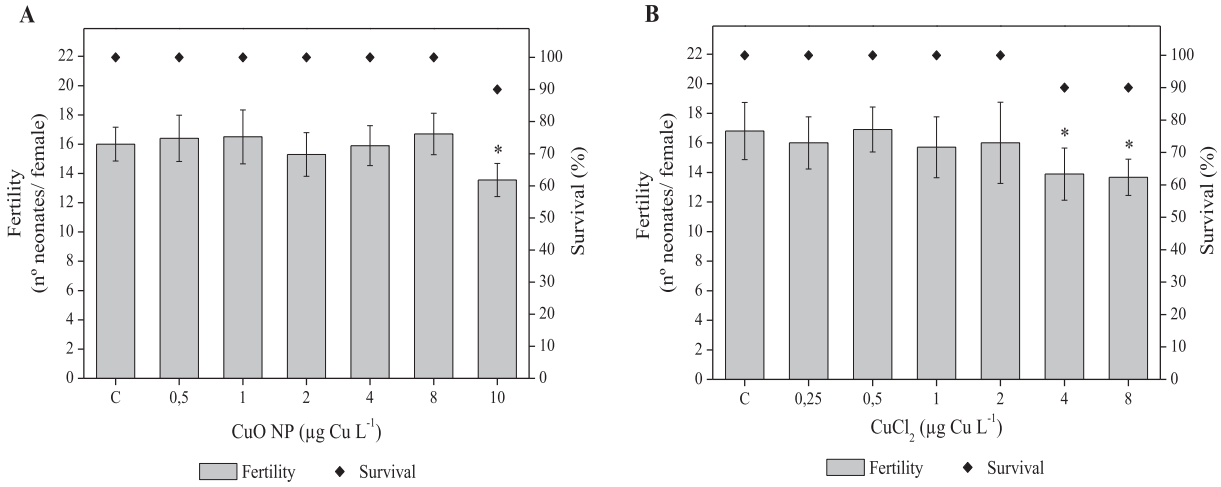

Fig. 2. Survival percentage and fertility of *Ceriodaphnia silvestrii* (mean ± SD number of neonates per female) after exposure 8 days to copper oxide nanoparticles (CuO NPs) (A) and copper chloride ($CuCl_2$) (B). Asterisk (*) indicates value significantly different from control ($p < 0.05$, Dunnett's test).

concentration dependent way for both exposures. For cladocerans exposed to CuO NPs, the feeding rate decreased significantly at concentrations of 6, 8 and 10 μg Cu L^{-1} (Fig. 3A) and the 24 h EC_{50} was 6.2 ± 1.9 μg Cu L^{-1}. For $CuCl_2$, there was a significant decrease in feeding rate at concentrations of 4, 6 and 8 μg Cu L^{-1} (Fig. 3B) and the 24 h EC_{50} was 7.8 ± 3.7 μg Cu L^{-1}. After 4 h of post-exposure (Fig. 3C–D), cladocerans significantly affected by CuO NPs and $CuCl_2$ did not recover, presenting feeding rates still lower than control.

Sublethal concentration exposures of CuO NPs or $CuCl_2$ induced significant ROS generation in *C. silvestrii* after 24 h (Fig. 4). The results showed that Cu added as $CuCl_2$ caused greater oxidative stress on cladoceran than NPs CuO suspension. While the 8 μg Cu L^{-1} concentration of CuO NPs induced an increase of 57% in ROS level (Fig. 4A), this same $CuCl_2$ concentration increased 185% ROS generation, when compared to control group (Fig. 4B). However, after 24 h exposure to 10 μg Cu L^{-1} of NPs CuO, ROS production was greatly increased (208%) in *C. silvestrii*.

3.4. Lethal and sublethal effects to H. eques

The acute toxicity results of CuO NPs and $CuCl_2$ to *H. eques* are shown in Table 1. The 96 h LC_{50} values for CuO NPs were about 4.2 times higher than for $CuCl_2$, demonstrating that the NPs suspension was less toxic than Cu salt solution on the basis of mass of Cu added.

The ROS assays showed a concentration dependent increase in

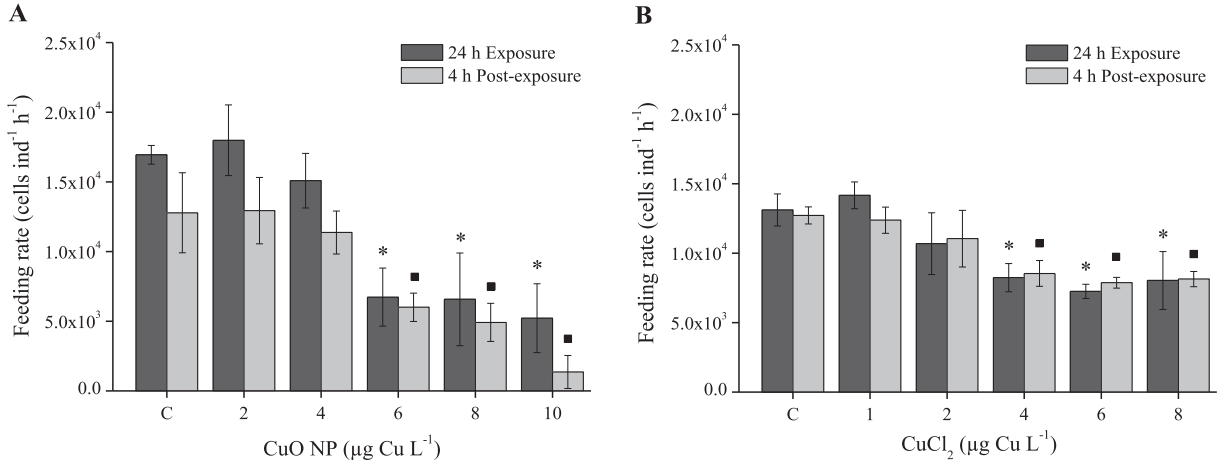

Fig. 3. Feeding rate of *Ceriodaphnia silvestrii* on microalgae cells after 24 h of exposure and 4 h of post-exposure to copper oxide nanoparticles (CuO NPs) (A) and copper chloride ($CuCl_2$) (B). * and ■ indicates value significantly different from control ($p < 0.05$, Dunnett's test).

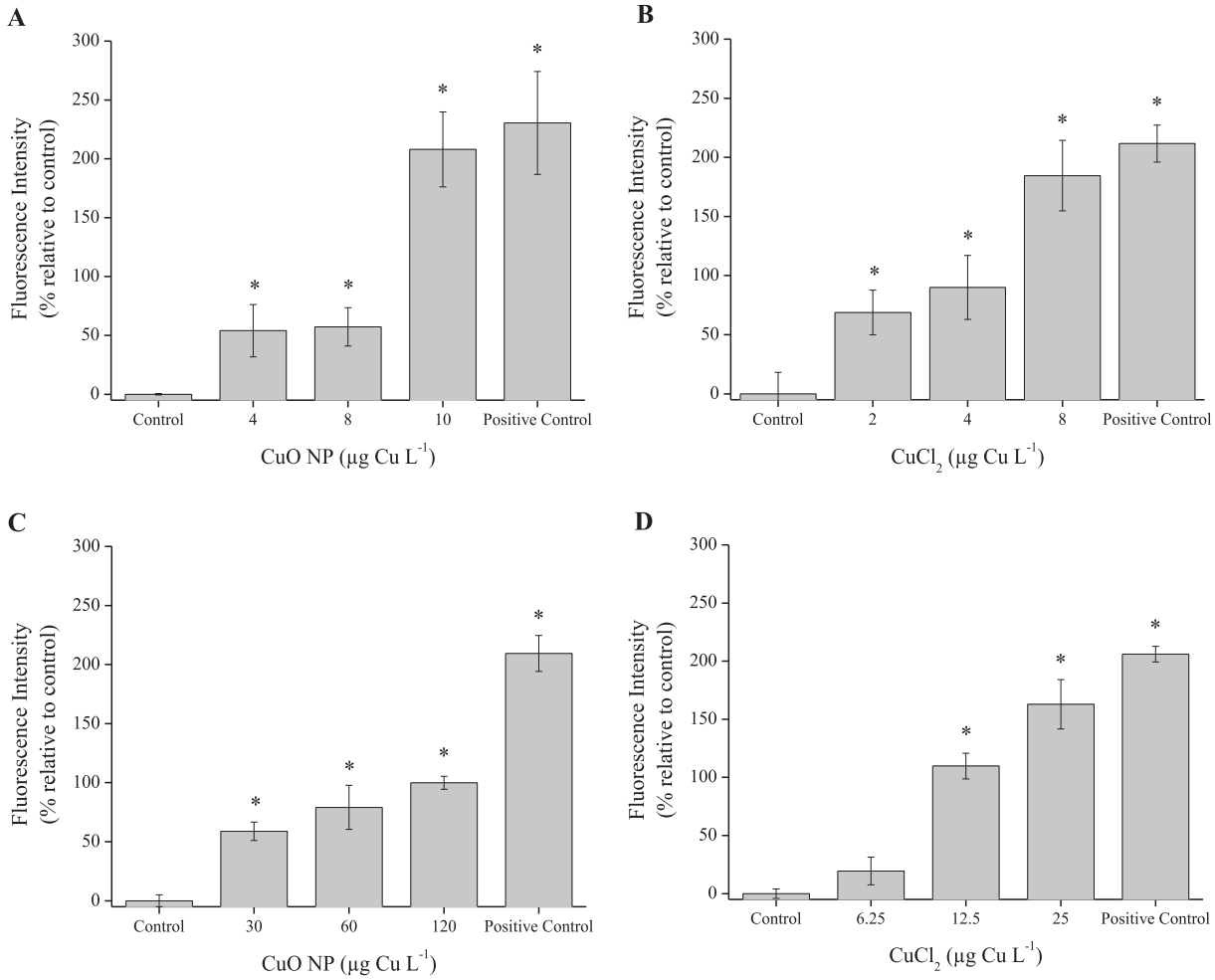

Fig. 4. ROS generation expressed as the percentage of fluorescence intensity relative to the control in *Ceriodaphnia silvestrii* after exposure to copper oxide nanoparticles (CuO NPs) (A) and copper chloride ($CuCl_2$) (B) and in *Hyphessobrycon eques* after exposure to CuO NPs (C) and $CuCl_2$ (D). Asterisk (*) indicates value significantly different from control ($p < 0.05$, Dunnett's test).

oxidative stress on the fish after exposure to CuO NPs (Fig. 4C) and $CuCl_2$ (Fig. 4D). The results also indicated that Cu added as $CuCl_2$ caused greater toxicity than CuO NPs to *H. eques*. While 24 h exposure to 120 µg Cu L^{-1} of CuO NPs induced an increase of 100% in ROS levels, 12.5 µg Cu L^{-1} of $CuCl_2$ caused an increase of 110% in ROS generation.

The induction of apoptosis and necrosis in the *H. eques* gill cells by CuO NPs and $CuCl_2$ are brought by Fig. 5. Low concentrations of Cu as CuO NPs or $CuCl_2$ caused apoptosis and necrosis in gill cells compared to control group. For CuO NPs, 30 µg Cu L^{-1} induced an increase of 9.4% and 13.1% in early apoptotic and necrotic cells, respectively, compared to control (Fig. 5A). With the increasing CuO NPs dose, more serious apoptosis and necrosis events occurred in the gills. For $CuCl_2$, 25 µg Cu L^{-1} caused an increase of 6.7 and 12.2% in apoptotic and necrotic cells, respectively, compared to control (Fig. 5B).

Flow cytometry was used to quantify the uptake in the gill cells of *H. eques* exposed to CuO NPs and $CuCl_2$ (Fig. 6). By measuring the SSC intensity, it can be verified that the gill cells internalized CuO NPs after 24 h of exposure and significantly changed the cell granularity in all sublethal concentrations tested (30, 60 and 120 µg Cu L^{-1}) (Fig. 6A). Interestingly, gill cells of fish exposed to different concentrations of $CuCl_2$ did not present significant differences in SSC intensity in relation to control (Fig. 6B).

3.5. Species sensitivity distribution

From the SSD curve constructed for CuO NPs (Fig. 7), the mean values and their 95% confidence intervals of HC_5 and HC_{50} for aquatic organisms were 15.1 µg Cu L^{-1} (2.7–48.1 µg Cu L^{-1}) and 621.9 µg Cu L^{-1} (250.3–1545.1 µg Cu L^{-1}), respectively. According to HC_5 value, *H. eques* would be protected against the toxic effects of CuO NPs, but the NPs could still cause acute and chronic toxicity to *C. silvestrii*. Furthermore, the large HC confidence intervals showed great differential sensitivity of the species involved. By analyzing the SSD curve (Fig. 7), the cladoceran *C. silvestrii* was more sensitive to short-term CuO NPs exposure than species commonly used in ecotoxicological studies, such as the cladocerans *Daphnia similis*, *Daphnia pulex*, *Daphnia magna* and *Ceriodaphnia dubia*. Regarding the fish, *H. eques* was also more sensitive than the standard test species, such as *Danio rerio*, *Pimephales promelas* and *Oncorhynchus mykiss*.

4. Discussion

The results regarding the dynamics of the NPs showed a very small dissolved copper fraction (<1%) from CuO NPs in exposure suspensions. Different authors confirmed this low dissolution value from Cu NPs. Griffitt et al. (2007, 2008) reported that ion release

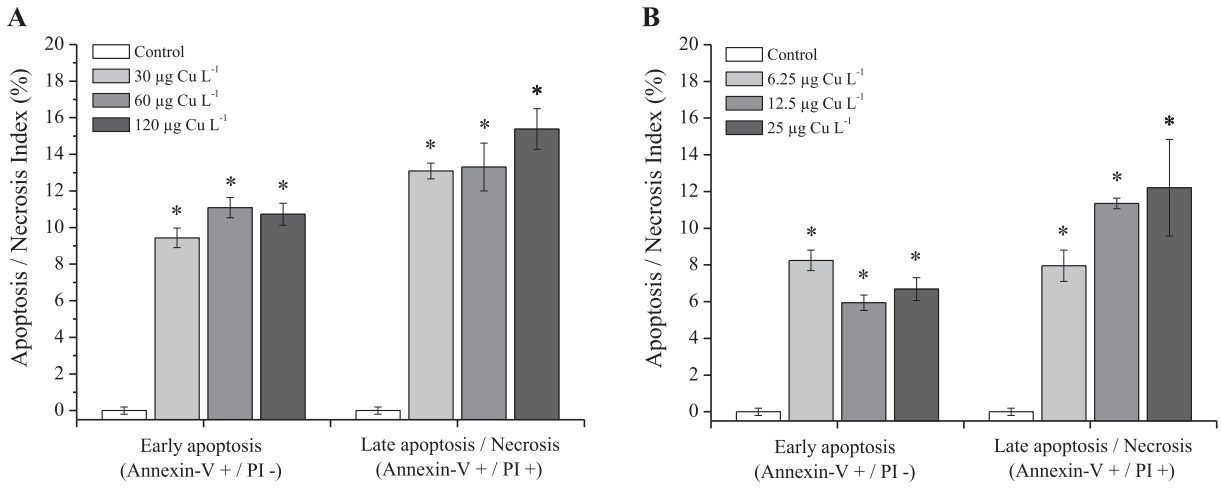

Fig. 5. Number of gill cells (percentage) in adult fish *Hyphessobrycon eques* in early apoptosis and late apoptosis/necrosis relative to the total population after 24 h of exposure to copper oxide nanoparticles (CuO NPs) (A) and copper chloride ($CuCl_2$) (B). Asterisk (*) indicates value significantly different from control ($p < 0.05$, Dunnett's test). The symbols "+" and "-" mean positive and negative, respectively.

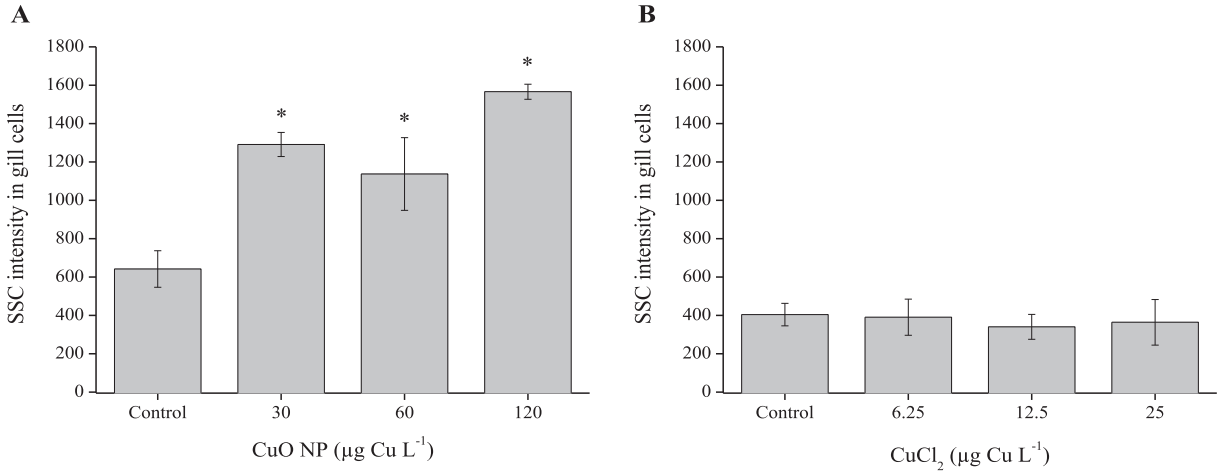

Fig. 6. Flow cytometry side scattering (SSC) intensity in gill cells of fish *Hyphessobrycon eques* after 24 h of exposure to copper oxide nanoparticles (CuO NPs) (A) and copper chloride ($CuCl_2$) (B). Asterisk (*) indicates value significantly different from control ($p < 0.05$, Dunnett's test).

from Cu NPs was <0.1% and Misra et al. (2014) verified that copper fraction released from rod-shaped CuO NPs was 1.1%. Previous findings also revealed that different shaped NPs release distinct amounts of ions in the testing medium, where the dissolution of rod-shaped Cu NPs can be significantly lower than that of spherical Cu NPs (Misra et al., 2014; Song et al., 2015a). Our results also showed that most of the CuO NPs in exposure media was present as larger aggregates (between 200 and 340 nm). Aggregation of CuO NPs has been reported in several studies (e.g. Adam et al., 2015; Song et al., 2015a). The formation of aggregates depends on aqueous media characteristics, specifically pH, ionic strength and humic acids (Conway et al., 2015; Sousa and Teixeira, 2013). Therefore, aggregation behaviour of NPs is common in aqueous media where other ionic components are present, such as in standardized test media. Bondarenko et al. (2013) found that the agglomeration was especially high in media used for ecotoxicological assays (algae, crustaceans and fish), as verified in our study.

In this study we assessed the toxicity of CuO NPs and $CuCl_2$ to two Neotropical species and compared their effects at relevant endpoints for each species, focusing on different induced effects between ionic Cu^{2+} and CuO NPs. One of the concerns about the environmental risk assessment of NPs is whether toxicity is specifically related to the intrinsic properties of particles, or if toxicity is mainly related to the copper ions released from NPs, which would indicate that the risk assessment of NPs could be carried out within the framework of existing regulations. Considering the low copper ions release from CuO NPs (<1%), our results indicated that the toxicity of CuO NPs to *C. silvestrii* and *H. eques* was mainly induced by the NPs.

In general, both CuO NPs and $CuCl_2$ caused lethal and sublethal effects to cladoceran and fish. However, for all endpoints evaluated in this study, $CuCl_2$ caused greater toxicity to *C. silvestrii* and *H. eques* than CuO NPs (based on the mass of copper added). Although Cu is an essential element for many biological processes, Cu doses above the required levels can be toxic to organisms, as it is already well known. Thus, Cu^{2+} ions released by CuO NPs can induce ROS by various chemical processes, but intracellular CuO NPs may also interact directly with oxidative organelles such as mitochondria, and stimulate ROS production (Hou et al., 2017). As expected, the mechanism of toxicity attributed to CuO NPs in this study was the ROS generation (Fig. 4).

Acute exposure of cladoceran *C. silvestrii* to CuO NPs resulted in

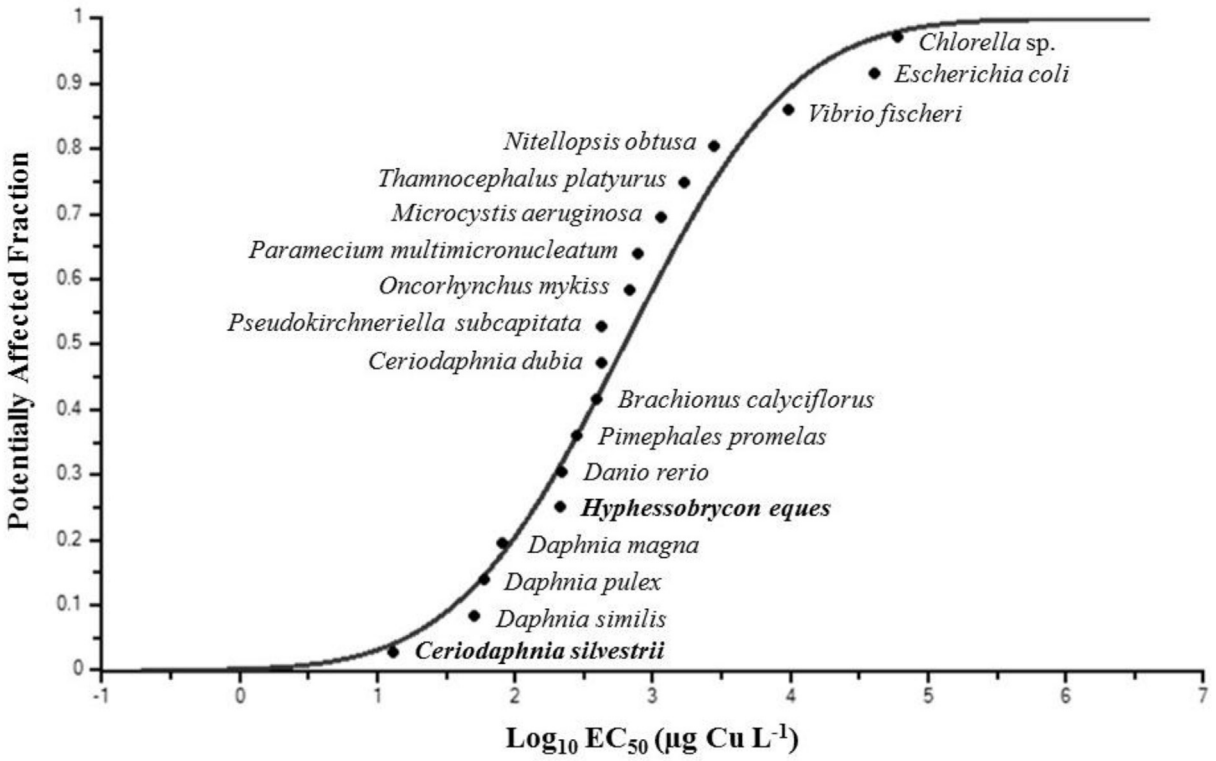

Fig. 7. Species sensitivity distribution (SSD) constructed based on EC_{50} values for CuO NPs in this study for *Ceriodaphnia silvestrii* and *Hyphessobrycon eques* and from literature for other aquatic organisms. Data and their respective references used in the SSD curve are presented in Supplementary Material Table S3.

high immobility at low concentrations (48 h $EC_{50} = 12.6 \pm 0.7$ μg Cu L^{-1}). To the best of our knowledge, this species was the most sensitive to CuO NPs recorded in the literature up to now (Fig. 7). *C. silvestrii* was approximately 5 and 33 times more sensitive than *Daphnia magna* (60 μg Cu L^{-1}; Xiao et al., 2016) and *Ceriodaphnia dubia* (419 μg Cu L^{-1}, Griffitt et al., 2008), respectively. Experimental factors related with species (e.g. size and age), experimental conditions (e.g. temperature, photoperiod, hardness, pH, exposure time) and characteristics of CuO NPs (e.g. size, shape, surface functionalization) may explain the differences in species sensitivity (Fig. 7).

Regarding chronic exposure, the number of neonates per *C. silvestrii* female was significantly reduced at 10 μg Cu L^{-1}. Few studies that investigated chronic effects of CuO NPs in cladoceran (Adam et al., 2015; Rossetto et al., 2014; Zhao et al., 2012) demonstrated a significant inhibition of *D. magna* reproduction at different exposure concentrations (0.002–7.8 mg L^{-1}). Effects on fertility of *C. silvestrii* (reduction in number of offspring) can be coupled with oxidative stress (measured by the production of ROS) in this study. Exposure to NPs probably disrupted the physiological integrity of cladoceran and induced defense and repair mechanisms (detoxification and antioxidant system activity), resulting in energy costs during these processes (Saebelfeld et al., 2017; Smolders et al., 2005). The metabolic costs incurred by oxidative stress probably led to a reduction of energy available for reproduction. This disproportionate energy allocation caused by the CuO NPs exposure could have severe consequences for species maintenance at the population level. Therefore, effects on reproduction of *C. silvestrii* could represent a threat to the population maintenance and potentially alter zooplankton community structure to long-term.

Low concentrations of CuO NPs (6–10 μg Cu L^{-1}) significantly decreased the feeding rates of *C. silvestrii* after 24 h exposure. In that case, the influence of CuO NPs on feeding inhibition of *C. silvestrii* can be related to NP. The presence of adsorbed NPs in the filtering appendages and the accumulation of CuO NPs in cladocerans might have impaired their feeding capacity. Previous studies reported that NPs may negatively affect cladoceran feeding behaviour due to high accumulation and low depuration of NPs in the body of organisms (Zhao and Wang, 2011; Zhu et al., 2010), and the agglomerates of NPs adhered to the filtering apparatus (Asghari et al., 2012; Ribeiro et al., 2014). Long-term feeding inhibition could eventually lead to reduced growth and reproduction in cladocerans due to poor food intake and malnutrition (Lu et al., 2017; Ribeiro et al., 2014; Souza et al., 2018). Food intake reduction verified in the present study may be one of the causes of the observed chronic toxicity (Fig. 2) after exposure to CuO NPs. Therefore, CuO NPs presence in freshwater environments may cause damages on cladocerans at both individual and population levels, including oxidative stress, feeding inhibition and reproduction reduction, posing risks to aquatic ecosystems.

Acute exposure of fish *H. eques* to CuO NPs showed that the NPs were toxic at low concentrations (96 h $LC_{50} = 211.4 \pm 57.5$ μg Cu L^{-1}). The 96 h LC_{50} value for *H. eques* in this study was similar to the 96 h LC_{50} reported by Song et al. (2015b) for *D. rerio* (zebrafish) (96 h $LC_{50} = 220 \pm 80$ μg Cu L^{-1}), but compared to species *Pimephales promelas* and *Oncorhynchus mykiss*, our value was lower than those reported by those authors. The similarity between the toxicity of *H. eques* and zebrafish may be due to the comparable length between these species and the similarities of the test conditions (temperature and photoperiod) for tropical organisms. Relatively small differences can be explained by differences in exposure medium and NPs used in the tests. Our findings suggest that the exposure conditions, species biological parameters and characteristics of NPs need to be detailed and considered in any comparative study and in the risk assessment process for NPs.

Our data revealed that the toxicity of CuO NPs to fish *H. eques* was attributed mainly to nanoparticles. These results are in accordance with some research of copper NPs toxicity for fish (e.g. Griffitt et al., 2007; Hua et al., 2014; Wang et al., 2015). High reactivity, as well as specific surface characteristics, confer NPs the capacity to generate ROS by interacting with subcellular structures (Gomes et al., 2011; Manke et al., 2013). This was observed in our study, since exposure of fish *H. eques* to the CuO NPs increased the gill ROS production. CuO NPs can cross the cell membrane and, once inside the cell, induce the ROS production and lead to the development of oxidative stress (Fahmy and Cormier, 2009; Gupta et al., 2016). It is known that copper has the capacity to generate ROS through Fenton-type reactions, leading to the production of oxyradicals that activate the antioxidant defense system (Heinlaan et al., 2008; Wang et al., 2014). Furthermore, nanoparticle-cell interactions may induce pro-oxidant effects via intracellular ROS generation involving mitochondrial respiration and activation of NADPH-like enzyme systems (Driscoll et al., 2001; Manke et al., 2013).

The evidence of CuO NPs internalization in the gill cells and may be interacting with the subcellular structures was verified by changes in the SSC intensity, measured by flow cytometry. Generally, it is considered that the SSC provides information on granularity/internal complexity (Shapiro, 2001). In a similar manner to granules in granulocytes, nanoparticles located inside cells can increase the scatter of light in the 90-degree direction in a dose-dependent manner (Zucker et al., 2010). Previous studies reported that the SSC intensity is proportional to the concentration of NPs inside the cells (Park et al., 2017; Yoo and Yoon, 2014; Zucker et al., 2010). However, this measure is limited to semiquantitative analysis of cell-associated NPs (Park et al., 2017). In this study, the SSC intensity was increased in the fish gill cells at all concentrations tested (Fig. 6A), demonstrating that the NPs were internalized. Regarding the copper salt, cell granularity was not altered probably because the Cu^{2+} ions present strong affinity toward intracellular metal-binding proteins (Theillet et al., 2014).

The increase in the gills cells number undergoing apoptosis and necrosis was likely due to oxidative stress caused by exposure to CuO NPs. Induction of oxidative stress has been considered as one of the important regulatory factors of cytotoxicity by NPs (Kumari et al., 2017). According to these authors, the antioxidant mechanism may fail to cope with the increased oxidative stress due to abnormal functionality of responsible proteins (sod1, p53), leading to apoptosis and necrosis of cells. Furthermore, excessive damages to lysosomal membranes by NPs can result in leakage of hydrolytic enzymes, which promotes cell apoptosis and necrosis (Kurz et al., 2008; Ostaszewska et al., 2018). As in our study, Wang et al. (2014) also reported that Cu NPs induced apoptosis in the gills at low concentrations (20 and 100 μg Cu L^{-1}). Considering that environmentally relevant concentrations the CuO NPs may cause oxidative stress and increase apoptosis and necrosis in the fish gill cells, the environmental concern increases considerably, since these damages can cause a cascade of events, impairing physiological functions that rely heavily in proper gill functioning, like respiration and ionic balance, beyond survival and reproduction.

5. Conclusions on the potential risks of CuO NPs

Currently, few studies have reported on the concentrations of Cu NPs present in aquatic environment (e.g. Chio et al., 2012). The predicted environmental concentration (PEC) for Cu NPs in some receiving waters was 60 μg L^{-1} with a 95% confidence interval of 10–920 μg L^{-1} (Chio et al., 2012). Given these predicted concentrations in some aquatic systems, Cu NPs may represent potential risks for some species of cladocerans and fish in natural systems (Fig. 7). According to the PEC values, all the concentrations tested in this study are environmentally relevant. Furthermore, our results revealed that the average PEC reported by Chio et al. (2012) exceeds the 48 h EC_{50}, NOEC and LOEC values of cladoceran *C. silvestrii*, which could cause mortality, oxidative stress, feeding inhibition and reproduction decrease. Moreover, our results show that in these predicted Cu NPs concentrations, sublethal effects on fish *H. eques*, including oxidative stress and increased apoptosis and necrosis in the gill cells could occur in natural aquatic environments. It should be noted that the laboratory tests were conducted under controlled conditions, whereas organisms in the field may be subjected to several additional stressors (e.g. additional pollutants, competition for resources and predation) that may increase their sensitivity. Our findings evidence the ecological relevance of tropical species in ecotoxicity testing and support the use of native species to better tune ecological risk assessment of CuO NPs in tropical ecosystems. Several environmental factors, such as pH, temperature, organic substances (Quik, 2013), can strongly influence the fate and toxicity of NPs in aquatic systems, which may reduce or increase their toxic capacity. Therefore, extrapolations of CuO NPs toxicity through different species, even under standardized test conditions, requires caution and emphasizes the need to report details of the exposure conditions and characteristics of NPs, to allow an adequate evaluation of the potential risks of NPs. This study may contribute to environmental risk assessment and support regulatory actions, improving the protection of tropical aquatic biota.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (Proc. No.150395/2016-7) and the Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (Proc. No. 2017/03165-1). We thank Brazilian Nanotechnology National Laboratory - LNNano/CNPEM/MCTIC (Campinas, SP, Brazil) for the use of TEM. We thank the Analytical Chemistry Laboratory - CENA/USP (Piracicaba, SP, Brazil) for the use of ICP-OES.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.09.020.

References

ABNT - Associação Brasileira de Normas Técnicas, 2004. NBR 12713. Aquatic Ecotoxicology – Acute Toxicity – Test with *Daphnia* spp. (Crustacea, Cladocera), Rio de Janeiro, Brazil.

ABNT - Associação Brasileira de Normas Técnicas, 2005. NBR 13373. Aquatic Ecotoxicology – Chronic Toxicity – Test with *Ceriodaphnia* spp. (Crustacea, Cladocera), Rio de Janeiro, Brazil.

Adam, N., Vakurov, A., Knapen, D., Blust, R., 2015. The chronic toxicity of CuO nanoparticles and copper salt to *Daphnia magna*. J. Hazard Mater. 283, 416–422.

Aguinaga, J.Y., Claudiano, G.S., Marcusso, P.F., Ikefuti, C., Ortega, G.G., Eto, S.F., Fernandes, J.B., 2014. Acute toxicity and determination of the active constituents of aqueous extract of *Uncaria tomentosa* Bark in *Hyphessobrycon eques*. J. Toxicol. 4, 1–5.

Aldenberg, T., Jaworska, J.S., 2000. Uncertainty of hazardous concentrations and fraction affected for normal species sensitivity distributions. Ecotoxicol. Environ. Saf. 46, 1–18.

Allen, Y., Calow, P., Baird, D.J., 1995. A mechanistic model of contaminant-induced feeding inhibition in *Daphnia magna*. Environ. Toxicol. Chem. 14 (9), 1625–1630.

Asghari, S., Johari, S.A., Lee, J.H., Kim, Y.S., Jeon, Y.B., Choi, H.J., Yu, I.J., 2012. Toxicity of various silver nanoparticles compared to silver ions in *Daphnia magna*.

J. Nanobiotechnol. 10 (14), 1–11.
Ates, M., Arslan, Z., Demir, V., Daniels, J., Farah, I.O., 2015. Accumulation and toxicity of CuO and ZnO nanoparticles through waterborne and dietary exposure of goldfish (*Carassius auratus*). Environ. Toxicol. 30, 119–128.
Ates, M., Dugo, M.A., Demir, V., 2014. Effect of copper oxide nanoparticles to Sheepshead Minnow (*Cyprinodon variegatus*) at different salinities. Digest J. Nanomat. Biostruct. 9, 369–377.
Bondarenko, O., Ivask, A., Käkinen, A., 2012. Sub-toxic effects of CuO nanoparticles on bacteria: kinetics, role of Cu ions and possible mechanisms of action. Environ. Pollut. 169, 81–89.
Bondarenko, O., Juganson, K., Ivask, A., Kasemets, K., Mortimer, M., Kahru, A., 2013. Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: a critical review. Arch. Toxicol. 87 (7), 1181–1200.
Chang, Y.N., Zhang, M., Xia, L., Zhang, J.E., Xing, G., 2012. The toxic effects and mechanisms of CuO and ZnO nanoparticles. Materials 5, 2850–2871.
Chio, C.P., Chen, W.Y., Chou, W.C., Hsieh, N.H., Ling, M.P., Liao, C.M., 2012. Assessing the potential risks to zebrafish posed by environmentally relevant copper and silver nanoparticles. Sci. Total Environ. 420, 111–118.
Conway, J.R., Adeleye, A.S., Gardea-Torresdey, J.L., Keller, A.A., 2015. Aggregation, dissolution and transformation of copper nanoparticles in natural waters. Environ. Sci. Technol. 49 (5), 2749–2756.
Cronholm, P., Karisson, H.L., Hedberg, J., Lowe, T.A., Winnberg, L., Elihn, K., Wallinder, I.O., Moller, L., 2013. Intracellular uptake and toxicity of Ag and CuO nanoparticles: a comparison between nanoparticles and their corresponding metal ions. Small 9 (7), 970–982.
Driscoll, K.E., Howard, B.W., Carter, J.M., Janssen, Y.M., Mossman, B.T., Isfort, R.J., 2001. Mitochondrial-derived oxidants and quartz activation of chemokine gene expression. In: Dansette, P.M., et al. (Eds.), Biological Reactive Intermediates VI. Advances in Experimental Medicine and Biology. Springer, Boston, MA, pp. 489–496.
Ebrahimnia-Bajestan, E., Niazmand, H., Duangthongsuk, W., Wongwises, S., 2011. Numerical investigation of effective parameters in convective heat transfer of nanofluids flowing under a laminar flow regime. Int. J. Heat Mass Tran. 54 (19), 4376–4388.
Fahmy, B., Cormier, S.A., 2009. Copper oxide nanoparticles induce oxidative stress and cytotoxicity in airway epithelial cells. Toxicol. Vitro 23 (7), 1365–1371.
Fonseca, A.L., Rocha, O., 2004. The life-cycle of *Ceriodaphnia silvestrii* Daday, 1902, a neotropical endemic species (Crustacea, Cladocera, Daphnidae). Acta Limnol. Bras. 16 (4), 319–328.
Fujimoto, R.Y., Barros, Z.N., Diniz, D.G., Marinho-Filho, A., Eiras, J.C., 2013. First occurrence of *Quadrigyrus nickoli* (Acanthocephala) in the ornamental fish *Hyphessobrycon eques*. Rev. Bras. Parasitol. Vet. 22, 110–113.
Gomes, T., Pinheiro, J.P., Cancio, I., Pereira, C.G., Cardoso, C., Bebianno, M.J., 2011. Effects of copper nanoparticles exposure in the mussel *Mytilus galloprovincialis*. Environ. Sci. Technol. 45, 9356–9362.
Griffitt, R.J., Luo, J., Gao, J., Bonzongo, J.C., Barber, D.S., 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. Environ. Toxicol. Chem. 27 (9), 1972–1978.
Griffitt, R.J., Weil, R., Hyndman, K.A., Denslow, N.D., Powers, K., Taylor, D., Barber, D.S., 2007. Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*). Environ. Sci. Technol. 41 (23), 8178–8186.
Gupta, Y.R., Sellegounder, D., Kannan, M., Deepa, S., Senthilkumaran, B., Basavaraju, Y., 2016. Effect of copper nanoparticles exposure in the physiology of the common carp (*Cyprinus carpio*): biochemical, histological and proteomic approaches. Aquac. Fish. 1, 15–23.
Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.C., Kahru, A., 2008. Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Chemosphere 71 (7), 1308–1316.
Hua, J., Vijver, M.G., Ahmad, F., Richardson, M.K., Peijnenburg, W.J.G.M., 2014. Toxicity of different-sized copper nano-and submicron particles and their shed copper ions to zebrafish embryos. Environ. Toxicol. Chem. 33 (8), 1774–1782.
Hou, J., Wang, X., Hayat, T., Wang, X., 2017. Ecotoxicological effects and mechanism of CuO nanoparticles to individual organisms. Environ. Pollut. 221, 209–217.
ISO - International Organization for Standardization, 2000. Water Quality e Determination of Long Term Toxicity of Substances to *Daphnia magna* Straus (Cladocera, Crustacea). ISO 10706. International Organization for Standardization, Geneva, Switzerland.
Jia, W., Liu, Y., Hu, P., Yu, R., Wang, Y., Ma, L., Wang, D., Li, Y., 2015. Ultrathin CuO nanorods: controllable synthesis and superior catalytic properties in styrene epoxidation. Chem. Commun. (J. Chem. Soc. Sect. D) 51, 8817–8820.
Jo, H.J., Choi, J.W., Lee, S.H., Hong, S.W., 2012. Acute toxicity of Ag and CuO nanoparticle suspensions against *Daphnia magna*: the importance of their dissolved fraction varying with preparation methods. J. Hazard Mater. 227, 301–308.
Keller, A.A., Adeleye, A.S., Conway, J.R., Garner, K.L., Zhao, L., Cherr, G.N., Hong, J., Gardea-Torresdey, J.L., Godwin, H.A., Hanna, S., Ji, Z., Kaweeteerawat, C., Lin, S., Lenihan, H., Miller, R., Nel, A., Peralta-Videa, J., Walker, S.L., Taylor, A.A., Torres-Duarte, C., Zink, J., Zuverza-Mena, N., 2017. Comparative environmental fate and toxicity of copper nanomaterials. NanoImpact 7, 28–40.
Kumari, P., Panda, P.K., Jha, E., Kumari, K., Nisha, K., Mallick, M.A., Verma, S.K., 2017. Mechanistic insight to ROS and Apoptosis regulated cytotoxicity inferred by Green synthesized CuO nanoparticles from *Calotropis gigantea* to Embryonic Zebrafish. Sci. Rep. 7 (16284), 1–17.
Kurz, T., Terman, A., Gustafsson, B., Brunk, U.T., 2008. Lysosomes in iron metabolism, ageing and apoptosis. Histochem. Cell Biol. 129, 389–406.
Lone, J.A., Kumar, A., Kundu, S., 2013. Characterization of tolerance limit in *Spirulina platensis* in relation to nanoparticles. Water Air Soil Pollut. 224, 1670–1676.
Lu, G., Yang, H., Xia, J., Zong, Y., Liu, J., 2017. Toxicity of Cu and Cr nanoparticles to *Daphnia magna*. Water Air Soil Pollut. 228, 1–13.
Majumder, S., Neogi, S., 2016. Antimicrobial activity of copper oxide nanoparticles coated on cotton fabric and synthesized by one-pot method. Adv. Sci. Eng. Med. 8, 102–111.
Manke, A., Wang, L., Rojanasakul, Y., 2013. Mechanisms of nanoparticle-induced oxidative stress and toxicity. BioMed Res. Int. 2013, 942916.
Mansano, A.S., Moreira, R.A., Dornfeld, H.C., Diniz, L.G.R., Vieira, E.M., Daam, M.A., Rocha, O., Seleghim, M.H.R., 2016. Acute and chronic toxicity of diuron and carbofuran to the neotropical cladoceran *Ceriodaphnia silvestrii*. Environ. Sci. Pollut. Res. 1–12.
Manusadzianas, L., Caillet, C., Fachetti, L., Gylytė, B., Grigutytė, R., Jurkonienė, S., Férard, J.F., 2012. Toxicity of copper oxide nanoparticle suspensions to aquatic biota. Environ. Toxicol. Chem. 31, 108–114.
McWilliam, R.A., Baird, D.J., 2002. Postexposure feeding depression: a new toxicity endpoint for use in laboratory studies with *Daphnia magna*. Environ. Toxicol. Chem. 21 (6), 1198–1205.
Melegari, S.P., Perreault, F., Costa, R.H., Popovic, R., Matias, W.G., 2013. Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga *Chlamydomonas reinhardtii*. Aquat. Toxicol. 142, 431–440.
Misra, S.K., Nuseibeh, S., Dybowska, A., Berhanu, D., Tetley, T.D., Valsami-Jones, E., 2014. Comparative study using spheres, rods and spindle-shaped nanoplatelets on dispersion stability, dissolution and toxicity of CuO nanomaterials. Nanotoxicology 8 (4), 422–432.
Mortimer, M., Kasemets, K., Kahru, A., 2010. Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*. Toxicology 269, 182–189.
OECD - Organization for Economic Cooperation and Development, 1992. Guideline for Testing of Chemicals Section 2: Effects on Biotic Systems. Test No. 203: Fish, Acute Toxicity Test. OECD Publishing.
Ostaszewska, T., Śliwiński, J., Kamaszewski, M., Sysa, P., Chojnacki, M., 2018. Cytotoxicity of silver and copper nanoparticles on rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Environ. Sci. Pollut. Res. 25, 908–915.
Park, J., Ha, M.K., Yang, N., Yoon, T.H., 2017. Flow cytometry-based quantification of cellular Au nanoparticles. Anal. Chem. 89 (4), 2449–2456.
Peng, C., Shen, C., Zheng, S., Yang, W., Hu, H., Liu, J., Shi, J., 2017. Transformation of CuO nanoparticles in the aquatic environment: influence of pH, electrolytes and natural organic matter. Nanomaterials 7 (10), 326.
Prasad, P.R., Kanchi, S., Naidoo, E.B., 2016. In-vitro evaluation of copper nanoparticles cytotoxicity on prostate cancer cell lines and their antioxidant, sensing and catalytic activity: one-pot green approach. J. Photochem. Photobiol. B Biol. 161, 375–382.
Quik, J.T., 2013. Fate of Nanoparticles in the Aquatic Environment. Removal of Engineered Nanomaterials from the Water Phase under Environmental Conditions. PhD Thesis. Radboud University Nijmegen, The Netherlands.
Ribeiro, F., Gallego-Urrea, J.A., Jurkschat, K., Crossley, A., Hassellöv, M., Taylor, C., Soares, A.M.V.M., Loureiro, S., 2014. Silver nanoparticles and silver nitrate induce high toxicity to *Pseudokirchneriella subcapitata, Daphnia magna* and *Danio rerio*. Sci. Total Environ. 466, 232–241.
Rossetto, A.L., Melegari, S.P., Ouriques, L.C., Matias, W.G., 2014. Comparative evaluation of acute and chronic toxicities of CuO nanoparticles and bulk using *Daphnia magna* and *Vibrio fischeri*. Sci. Total Environ. 490, 807–814.
Saebelfeld, M., Minguez, L., Griebel, J., Gessner, M.O., Wolinska, J., 2017. Humic dissolved organic carbon drives oxidative stress and severe fitness impairments in *Daphnia*. Aquat. Toxicol. 182, 31–38.
Shapiro, H.M., 2001. Optical measurements in cytometry: light scattering, extinction, absorption, and fluorescence. Meth. Cell Biol. 63, 107–129.
Shi, J., Abid, A.D., Kennedy, I.M., Hristova, K.R., Silk, W.K., 2011. To duckweeds (*Landoltia punctata*), nanoparticulate copper oxide is more inhibitory than the soluble copper in the bulk solution. Environ. Pollut. 159 (5), 1277–1282.
Smolders, R., Baillieul, M., Blust, R., 2005. Relationship between the energy status of *Daphnia magna* and its sensitivity to environmental stress. Aquat. Toxicol. 73, 155–170.
Song, L., Vijver, M.G., Snoo, G.R., Peijnenburg, W.J., 2015a. Assessing toxicity of copper nanoparticles across five cladoceran species. Environ. Toxicol. Chem. 34 (8), 1863–1869.
Song, L., Vijver, M.G., Peijnenburg, W.J., Galloway, T.S., Tyler, C.R., 2015b. A comparative analysis on the in vivo toxicity of copper nanoparticles in three species of freshwater fish. Chemosphere 139, 181–189.
Sousa, V.S., Teixeira, M.R., 2013. Aggregation kinetics and surface charge of CuO nanoparticles: the influence of pH, ionic strength and humic acids. Environ. Chem. 10, 313–322.
Souza, J.P., Venturini, F.P., Santos, F., Zucolotto, V., 2018. Chronic toxicity in *Ceriodaphnia dubia* induced by graphene oxide. Chemosphere 190, 218–224.
Sun, Y., Zhang, G., He, Z., Wang, Y., Cui, J., Li, Y., 2016. Effects of copper oxide nanoparticles on developing zebrafish embryos and larvae. Int. J. Nanomed. 11, 905–918.
Suzuki, H., Toyooka, T., Ibuki, Y., 2007. Simple and easy method to evaluate uptake potential of nanoparticles in mammalian cells using a flow cytometric light scatter analysis. Environ. Sci. Technol. 41 (8), 3018–3024.
Statsoft, 2004. Statistica, v. 07 (accessed 16.04.18.). www.statsoft.com.
Theillet, F.X., Binolfi, A., Frembgen-Kesner, T., Hingorani, K., Sarkar, M., Kyne, C., Elcock, A.H., 2014. Physicochemical properties of cells and their effects on

intrinsically disordered proteins (IDPs). Chem. Rev. 114 (13), 6661–6714.
Thit, A., Skjolding, L.M., Selck, H., Sturve, J., 2017. Effects of copper oxide nanoparticles and copper ions to zebrafish (*Danio rerio*) cells, embryos and fry. Toxicol. Vitro 45, 89–100.
USEPA - US Environmental Protection Agency, 2011. Region 1: Freshwater Acute Toxicity Test Procedure and Protocol. Washington, DC.
Van Vlaardingen, P., Traas, T.P., Wintersen, A.M., Aldenberg, T., 2004. ETX 2.0. A Program to Calculate Hazardous Concentrations and Fraction Affected, Based on Normally Distributed Toxicity Data. RIVM Report No. 601501028/2004. National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands.
Venkataraman, S., Hedrick, J.L., Ong, Z.Y., Yang, C., Ee, P.L.R., Hammond, P.T., Yang, Y.Y., 2011. The effects of polymeric nanostructure shape on drug delivery. Adv. Drug Deliv. Rev. 63, 1228–1246.
Wang, T., Long, X., Cheng, Y., Liu, Z., Yan, S., 2014. The potential toxicity of copper nanoparticles and copper sulphate on juvenile *Epinephelus coioides*. Aquat. Toxicol. 152, 96–104.
Wang, T., Long, X., Liu, Z., Cheng, Y., Yan, S., 2015. Effect of copper nanoparticles and copper sulphate on oxidation stress, cell apoptosis and immune responses in the intestines of juvenile *Epinephelus coioides*. Fish Shellfish Immunol. 44 (2), 674–682.
Wu, F., Bortvedt, A., Harper, B.J., Crandon, L.E., Harper, S.L., 2017. Uptake and toxicity of CuO nanoparticles to *Daphnia magna* varies between indirect dietary and direct waterborne exposures. Aquat. Toxicol. 190, 78–86.
Xiao, Y., Peijnenburg, W.J., Chen, G., Vijver, M.G., 2016. Toxicity of copper nanoparticles to *Daphnia magna* under different exposure conditions. Sci. Total Environ. 563, 81–88.
Xiao, Y., Vijver, M.G., Chen, G., Peijnenburg, W.J., 2015. Toxicity and accumulation of Cu and ZnO nanoparticles in *Daphnia magna*. Environ. Sci. Technol. 49 (7), 4657–4664.
Xie, F., Koziar, S.A., Lampi, M.A., Dixon, D.G., Norwood, W.P., Borgmann, U., Huang, X.D., Greenberg, B.M., 2006. Assessment of the toxicity of mixtures of copper, 9,10-phenanthrenequinone, and phenanthrene to *Daphnia magna*: evidence for a reactive oxygen mechanism. Environ. Toxicol. Chem. 25, 613–622.
Xu, J., Zhang, Q., Li, X., Zhan, S., Wang, L., Chen, D., 2017. The effects of copper oxide nanoparticles on dorsoventral patterning, convergent extension, and neural and cardiac development of zebrafish. Aquat. Toxicol. 188, 130–137.
Yoo, H.J., Yoon, T.H., 2014. Flow cytometric assessment of reactive oxygen species generations that are directly related to cellular ZnO nanoparticle uptake. J. Nanosci. Nanotechnol. 14 (7), 5395–5401.
Yoo-iam, M., Chaichana, R., Satapanajaru, T., 2014. Toxicity, bioaccumulation and biomagnification of silver nanoparticles in green algae (*Chlorella sp.*), water flea (*Moina macrocopa*), blood worm (*Chironomus spp.*) and silver barb (*Barbonymus gonionotus*). Chem. Speciat. Bioavailab. 26 (4), 257–265.
Zhao, C.M., Wang, W.X., 2011. Comparison of acute and chronic toxicity of silver nanoparticles and silver nitrate to *Daphnia magna*. Environ. Toxicol. Chem. 30 (4), 885–892.
Zhao, H., Lu, G., Xia, J., Jin, S., 2012. Toxicity of nanoscale CuO and ZnO to *Daphnia magna*. Chem. Res. Chinese 28 (2), 209–213.
Zhao, J., Cao, X., Liu, X., Wang, Z., Zhang, C., White, J.C., Xing, B., 2016. Interactions of CuO nanoparticles with the algae *Chlorella pyrenoidosa*: adhesion, uptake, and toxicity. Nanotoxicology 10 (9), 1297–1305.
Zhao, J., Ren, W., Dai, Y., Liu, L., Wang, Z., Yu, X., Zhang, J., Wang, X., Xing, B., 2017. Uptake, distribution, and transformation of CuO NPs in a floating plant *Eichhornia crassipes* and related stomatal responses. Environ. Sci. Technol. 51 (13), 7686–7695.
Zhao, J., Wang, Z., Liu, X., Xie, X., Zhang, K., Xing, B., 2011. Distribution of CuO nanoparticles in juvenile carp (*Cyprinus carpio*) and their potential toxicity. J. Hazard Mater. 197, 304–310.
Zhu, X., Chang, Y., Chen, Y., 2010. Toxicity and bioaccumulation of TiO_2 nanoparticle aggregates in *Daphnia magna*. Chemosphere 78 (3), 209–215.
Zucker, R.M., Massaro, E.J., Sanders, K.M., Degn, L.L., Boyes, W.K., 2010. Detection of TiO_2 nanoparticles in cells by flow cytometry. Cytometry 77 (7), 677–685.