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Comparative evaluation of acute and chronic toxicities of CuO nanoparticles and bulk using *Daphnia magna* and *Vibrio fischeri*

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HIGHLIGHTS

- CuO NPs were more toxic than MPs in acute toxicity with *D. magna* and *V. fischeri*.
- CuO NPs affected reproduction and growth of *D. magna*.
- Morphological changes were observed for *D. magna* after chronic toxicity tests.

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ABSTRACT

Copper oxide (CuO) has various applications, as highlighted by the incorporation of this compound as a biocide of antifouling paints for coating ships and offshore oil platforms. The objective of this study was to evaluate and compare the aquatic toxicity of CuO nanoparticles (NPs) and microparticles (MPs) through acute and chronic toxicity tests with the freshwater microcrustacean *Daphnia magna* and an acute toxicity test with the bioluminescent marine bacteria *Vibrio fischeri*. Acute toxicity results for *D. magna* in tests with CuO NPs ($EC_{50,\ 48\ h}$ = 22 mg L^{-1}) were ten times higher than those for tests with CuO MPs ($EC_{50,\ 48\ h}$ = 223.6 mg L^{-1}). In both periods of exposure of *V. fischeri*, the CuO NPs ($EC_{50,\ 15\ m}$ 248 ± 56.39 — equivalent to 12.40%; $EC_{50,\ 30\ m}$ 257.6 ± 30.8 mg L^{-1} — equivalent to 12.88%) were more toxic than the CuO MPs ($EC_{50,\ 15\ m}$ 2404.6 ± 277.4 — equivalent to 60.10%; $EC_{50,\ 30\ m}$ 1472.9 ± 244.7 mg L^{-1} — equivalent to 36.82%). In chronic toxicity tests, both forms of CuO showed significant effects ($p < 0.05$) on the growth and reproduction parameters of the *D. magna* relative to the control. Additionally, morphological changes, such as lack of apical spine development and malformed carapaces in *D. magna*, were observed for organisms after the chronic test. The toxicity results demonstrate that CuO NPs have a higher level of toxicity than CuO MPs, emphasizing the need for comparative toxicological studies to correctly classify these two forms of CuO with identical CAS registration numbers.

1. Introduction

Although nanotechnology has brought great technological advances, there has been uncertainties regarding the behavior of nanomaterials and their interactions with the environment (Cerqueira et al., 2011; Silva et al., 2011), especially the aquatic ecosystem, which is one of the final destinations where these materials accumulate (Sanchís et al., 2013). The aquatic ecosystem can be contaminated by nanopollutants (Brar et al., 2010), especially metallic nanoparticles, the principle type of nanoparticles (NPs). These NPs are synthesized and utilized in large-scale industrial applications, e.g., copper oxide (CuO) NPs are used as antifouling agents in paints (Perreault et al., 2012) for ships and offshore oil platforms and applications in antimicrobial textiles (Ren et al., 2009; Dastjerdi and Montazer, 2010; Delgado et al., 2011). Thus, these NPs can interact with and affect aquatic organisms.

Comparative studies of nanoscale and microscale materials are important because the intrinsic characteristics of NPs may be directly related to their toxicity, and comprehensive characterization of suspensions of these particles is necessary (Ribeiro et al., 2013). The size, shape, composition, aggregation and solubility of NPs, especially metal-based nanomaterials, may be related to their toxicity (Griffitt et al., 2007). Moreover, several studies have shown that the total dissolved Cu in CuO nanoparticles is the major source of their toxicity (Heinlaan et al., 2008; Aruoja et al., 2009; Kasemets et al., 2009; Mortimer et al., 2010).

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Due to their small size, NPs may cross biological barriers and reach various organs (Perreault et al., 2012). Observations of the effects of size and surface properties on the accumulation of metal NPs in different organisms in vivo have been reported (Li and Chen, 2011). Heinlaan et al. (2008) affirmed that a principle mechanism of the toxicity of ZnO, CuO and TiO_2 NPs is related to oxidative stress, which damages lipids, carbohydrates, proteins and DNA.

Ferreira and Matsubara (1997) demonstrated that reactive oxygen species (ROS) may be formed by radicals of transition metals or other chemical species on particle surfaces or as a consequence of interactions between particles and cellular components. Fenton and Haber–Weiss reactions catalyzed by Cu ions produce ROS in vitro that cause oxidative damage. Knauert and Knauer (2008) recently demonstrated that ROS production plays a key role in the toxicity of Cu and is associated with effects of Cu on photosynthetic activity. Additionally, the formation of ROS can change the cellular integrity of green algae (Saison et al., 2010). Thus, oxidative stress enables the entry of nanomaterials into trophic levels, initially damaging producers, then primary consumers, and finally presenting a risk to humans (Zarbin, 2007).

CuO NPs are highly toxic at different trophic levels: fish (Griffitt et al., 2007), invertebrates (Buffet et al., 2011), protozoa (Mortimer et al., 2010), bacteria (Baek and An, 2011) and yeast (Kasemets et al., 2009). Perreault et al. (2010) used fluorescent imaging of chlorophyll to assess the toxicity of CuO NPs in *Lemna gibba*. Additionally, Perreault et al. (2012) investigated the toxicity of CuO NPs in *Chlamydomonas reinhardtii* green algae by observing the intracellular bioaccumulation of CuO NPs. Both studies reported important toxicological effects of CuO NPs. Furthermore, Wang et al. (2011) evaluated the toxicity of CuO NPs in *Microcystis aeruginosa* algae and observed a greater than 54% growth inhibition after exposure for 4 days to a concentration of 0.5 mg L^{-1}. The above findings demonstrate the need for studies that can confirm the hypothesis that NPs have greater toxicity than MPs.

Although there are several studies in the literature, much is still unknown about the behavior of CuO NPs in direct contact with cells in human beings, animals, bacteria and plants. Furthermore, Karlsson et al. (2009) compared the toxicity of CuO NPs and microparticles (MPs) at the cellular level (human cell line A549) and reported greater toxicity for NPs than MPs.

Microcrustaceans and bacteria are good representatives of different trophic levels, and they are used widely in toxicity tests and as bioindicators in toxicology studies with NPs. The present study evaluated and compared acute and chronic toxicities of CuO in NPs and MPs by exposing freshwater microcrustacean *Daphnia magna* and marine bacteria *Vibrio fischeri* to these materials. Additionally, this study investigated physical interactions of CuO NPs with *D. magna* using light and transmission electronic microscopies to determine the penetration of CuO NPs into this organism.

2. Materials and methods

2.1. Chemicals

CuO nanopowder was obtained from MTI Corporation (Richmond, CA) and, according to the manufacturer, had an average size of 30–40 nm and a minimum purity of 99%. Stock solutions of CuO nanoparticles were prepared in ultrapure water (2000 mg L^{-1}) and sonicated for 30 min using an ultrasonic cell disruptor (Unique — 100 W) at 99% of its maximum power. These suspensions were stored in the dark at 4 °C. The CuO MPs were obtained from Vetec (Rio de Janeiro, Brazil), and their purity was 99%. Stock solutions of CuO MPs were prepared at a concentration of 4000 mg L^{-1}, which was analogous to the stock solutions of NPs, but the stock solutions of MPs were not sonicated. The pH was measured with a potentiometer, a multi-parameter pH analyzer (Consort C535, Belgium), for *D. magna* and *V. fischeri*. The salinity was measured with a salinity refractometer (Impac IPS-10T, Brazil), and *V. fischeri* (NRRL B-11177, recently named *Aliivibrio fischeri*) was purchased from SDI®-Microtox acute reagent. Parameters were monitored during the course of these procedures to minimize any interference in the results of the toxicity tests, which were performed with the particle suspensions.

2.2. Characterization of CuO NPs and MPs

CuO NP and MP suspensions were characterized in ultrapure water at a concentration of 2000 mg L^{-1}. Transmission electronic microscopy (TEM) (JEM-1011 TEM, 100 kV) was used to determine the sizes and shapes of the particles. Drops of the NP suspension were placed on a carbon-Cu grid (300 mesh) and dried in a desiccator under vacuum for 24 h. Additionally, particle sizes and shapes were characterized by scanning electron microscopy (SEM-FEG) (JEOL — JSM-6701F). Droplets of suspensions were placed on stubs, coated with 5 nm of gold and stored in a desiccator under vacuum for 24 h. The zeta potentials (Pz) of the NPs and MPs were determined in ultrapure water using the electrophoretic mobility method with a ZetaPlus system (Brookhaven Instruments Corporation, USA). The hydrodynamic diameter was measured for solutions of 2 g L^{-1} in ultrapure water by dynamic light scattering with a ZetaPlus particle sizer (Brookhaven Instruments Corporation, USA). The concentrations of free fraction Cu ions were evaluated by centrifuging samples (3000 g, 10 min), filtering and acidifying the supernatant (0.20 μ) and performing graphite furnace atomic absorption spectrometry (GFAAS) measurements. The ratios of Cu ions to NPs and MPs were determined using solutions of 2 g L^{-1} in ultrapure water. The surface areas of the original NP and MP powders were determined using a NOVA® surface area analyzer (Quantachrome Instruments) and methodology based on procedures described by Webb and Orr (1997). The crystallite size was determined by X-ray diffraction (XRD); X-ray diffractograms were recorded in the angular range of $2\theta = 20°–80°$ with a step size of 0.05 and a time step of 1 s using a Philips X'Pert diffractometer equipped with a copper tube (CuKα, $\lambda = 1.54056$ Å). The mean crystallite size (D) was calculated for each type of CuO particle using the Scherrer Eq. (1),

$$D = \frac{0.91\lambda}{\beta \cos\theta} \quad (1)$$

where λ is the radiation wavelength, β is the full-width at half-maximum of the principle peak in radians, and θ is half of the diffraction angle.

2.3. Toxicity tests

2.3.1. D. magna culture

D. magna Straus, 1820 (Cladocera, Crustacea), was cultivated according to ISO 6341 (ISO, 2012a) and DIN 38412-30 (DIN, 1989) protocols. These microcrustaceans were cultivated in lots of 25 to 30 adult organisms in M4 culture medium in 2 L beakers with diffuse luminosity, a photoperiod of 16 h of light and a controlled temperature of 20 ± 2 °C. The *D. magna* culture was fed with algae, *Scenedesmus subspicatus* (ISO, 2012b), which were cultivated in CHU growth medium (Chu, 1942) (Supplementary Table S1).

2.3.2. Acute toxicity test with D. magna

Acute toxicity tests with *D. magna* were performed according to ISO 6341 (ISO, 2012a) with neonates (2–26 h) exposed for 48 h. The samples were diluted in reconstituted water called ISO media (235.2 mg L^{-1} $CaCl_2 \cdot 2H_2O$; 98.64 mg L^{-1} $MgSO_4 \cdot 7H_2O$; 4.64 mg L^{-1} KCl; 51.84 mg L^{-1} $NaHCO_3$) at a controlled temperature of 20 ± 2 °C without luminosity. The endpoint used for the toxicological evaluation was the immobility of the organism. Toxicity tests were performed in 50 mL beakers by exposing 20 organisms per dilution. The NP and MP stock solutions were prepared at a concentration of 2000 mg L^{-1}, and dilutions were prepared from 2000 to 3.60 mg L^{-1}. $CuSO_4$ in a concentration range of 125 to 0.48 mg L^{-1} was used as a positive control, and ISO medium was used as a negative control. The EC_{50} was calculated using the trimmed