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Comparative toxicity of copper nanoparticles across three Lemnaceae species

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HIGHLIGHTS

- Toxicity of CuNPs to three Lemnaceae species was evaluated at different endpoints.
- The total frond area based relative growth rate was the most sensitive endpoint.
- Both particles and ions contributed to the toxicity effects of CuNP suspensions.
- Intrinsic differences between species can affect the toxicity of CuNP suspensions.

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ABSTRACT

Metallic nanoparticles can end up in aquatic ecosystems due to their widespread application. Even though the toxicological effects of metallic nanoparticles to a diversity of species have been reported extensively, the toxicological data achieved in different studies are not always comparable and little is known regarding the comparative toxicity of nanoparticles across species, as different test strategies and endpoints were applied. To attempt to fill this knowledge gap, *Spirodela polyrhiza*, *Lemna minor* and *Wolffia arrhiza* were exposed to 25 nm spherical copper nanoparticles to investigate the inhibiting effect of copper nanoparticle suspensions across species at three endpoints: total frond area, frond number and dry weight based relative growth rate. The total frond area based relative growth rate was found to be the most sensitive endpoint, with an EC_{50} of 1.15 ± 0.09 mg/L for *S. polyrhiza*, 0.84 ± 0.12 mg/L for *L. minor* and 0.64 ± 0.05 mg/L for *W. arrhiza*. Both the particles and the copper ions contributed to the inhibiting effects of copper nanoparticle suspensions at all endpoints studied. Dose–response related inhibiting effects caused by the copper ions were found at all endpoints studied, whereas the particles only showed dose–response related inhibiting effects on the total frond area based relative growth rate. This suggests that different physiological processes are involved in case of exposure to particles and copper ions. *W. arrhiza* was found to be the most sensitive species tested and *S. polyrhiza* was the least sensitive species tested, when the inhibiting effect was evaluated based on the relative growth rate calculated from total frond area. These findings exemplify the importance of identifying the suitable endpoints of toxicity assessment and considering the intrinsic differences between species when evaluating the toxicological profile of metallic nanoparticles across species.

1. Introduction

The fast development of nanotechnology provides many opportunities for product innovation. Diverse metallic nanoparticles (NPs) are synthesized and applied in many products because of their distinct physico-chemical characteristics. For instance, titanium dioxide NPs are extensively used in sunscreen lotion to protect skin from ultraviolet radiation (Contado and Pagnoni, 2008). Copper nanoparticles (CuNPs) are widely applied in electronics and coating on textiles, because of their optical, electrical, and antifungal properties (Cioffi et al., 2005; Kim et al., 2011).

Metallic NPs will eventually end up into the aquatic environment due to their extensive applications. Because of their unique properties, concerns regarding the fate and potential adverse impacts of metallic NPs in the aquatic environment have increased sharply in recent years (Oberdörster et al., 2005; Schilling et al., 2010). Studies showed that the fate and toxicity of metallic NPs are dependent not only on their composition, size, shape and their capping agent (Handy et al., 2008; Shi et al., 2011), but also on their percentages of dissolution (Schrand et al., 2010). Even though many studies have reported the toxicological effects of metallic NPs to a diversity of species, the results achieved in

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different studies are not always comparable as different endpoints were studied and different testing strategies were used (Blaise et al., 2008; Handy et al., 2008). For instance, many traditional endpoints that are being used to evaluate the toxicity of metal ions to Lemnaceae species, such as photosynthetic activity (Gubbins et al., 2011; Ma et al., 2013; Perreault et al., 2014b), the relative growth rate calculated from dry weight (Perreault et al., 2014a) and from frond number (Shi et al., 2011), have been directly applied to evaluate the toxicological effect of metallic NPs to Lemnaceae species. It is unclear which endpoint is suitable to evaluate the toxicological effects of metallic NPs. In addition, the duration of exposure varied significantly from 48 h to 15 days (Geranio et al., 2009; Perreault et al., 2014a, 2014b; Shi et al., 2011). These inconsistencies increase the difficulty of comparing the toxicity of NPs between studies and little is known concerning the comparative toxicity of NPs across species.

In this study, we investigated the fate and comparative toxicity of CuNPs to three different Lemnaceae species by adopting the OECD 221 guideline (OECD, 2002). Lemnaceae species are a family of macrophytes which are broadly distributed in freshwater lakes. They are widely used for studying the toxicity of chemicals based on total frond area, frond number and dry weight based relative growth rate (OECD, 2002). Three Lemnaceae species from three genera, *Spirodela polyrhiza*, *Lemna minor* and *Wolffia arrhiza* were used in this study in view of the differentiation of their morphology. Our objectives were threefold. Firstly, we wanted to evaluate the acute toxicity of CuNPs based on the endpoints suggested by the OECD 221 guideline to identify the most sensitive endpoint. Secondly, we intended to investigate if both the particles and the ions that were shed from the particles, contribute to the toxicity of CuNP suspensions in water. Thirdly, we wanted to compare the sensitivity of different Lemnaceae species to CuNP suspensions and copper nitrate to assist in understanding and extrapolating the toxicity of the CuNP suspensions across species.

2. Materials and methods

2.1. Stock culture of Lemnaceae species

The three Lemnaceae species, *S. polyrhiza*, *L. minor* and *W. arrhiza* were collected from a freshwater pond (52° 8′33.29″N, 4°23′56.64″E, The Netherlands). The species were then identified according to their morphologies (Landolt, 1998). The composition of the standard ISO growth medium is listed in the supplementary information 1. The identified fronds were cultured separately in ISO growth medium for a week with medium changing every day. Subsequently, the health fronds were picked and rinsed several times using sterile water, and immersed in 0.5% sodium hypochlorite for five minutes (Shi et al., 2011). The fronds were then rinsed three times with 70% ethanol and sterile water (Gordon and Posluszny, 2000). After that, the fronds were placed into fresh sterile ISO growth medium separately for three days. For each species, only one uncontaminated frond was used for further cultivation. The culture of each species was then maintained separately in 5 L containers using the standard ISO growth medium for two months prior to the experiment (ISO, 2006). The growth medium was autoclaved and replaced every 4 days, with a pH maintained at 5.5 ± 0.2. The culture and experiments were conducted at 25 ± 1 °C using a 16 h light:8 h dark photoperiod, with a light intensity of 100 $\mu Em^{-2}\ s^{-1}$ provided by cool-white light fluorescent lamps.

2.2. Experimental set up

CuNPs (chemical formula: Cu, with a specific surface area of 30–50 m^2/g, a purity of 95.5% and a density of 8.92 g/cm^3) with a nominal size of 25 nm stored in inert gas were purchased as dry powder from IoLiTec, Inc., Germany. The CuNPs were dispersed in the culture medium and sonicated in a water bath sonicator for 10 min prior to exposure. Copper nitrate was used as positive control and the culture medium was used as negative control. Eight exposure concentrations were used for investigating the inhibition effects of the CuNP suspensions and copper nitrate in *S. polyrhiza*, *L. minor* (0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 2 and 2.5 mg/L). The exposure concentrations used for investigating the inhibition effects of the CuNP suspensions and copper nitrate in *W. arrhiza* were 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 2 mg/L. For each exposure, three colonies with three fronds each (9 fronds in total) were selected randomly and then exposed to either the CuNP suspensions, copper nitrate or the control in the sterilized glass testing vials with a volume of 100 mL. The testing vials were covered using transparent film to reduce transpiration. Exposures were conducted for 7 days in a semi-static system with the test media renewed every 48 h to help maintain the stability of exposure concentrations. The pH of the test media was maintained at 5.5 ± 0.2 during the exposure using sodium hydroxide or hydrogen chloride solutions. Each exposure was conducted in triplicate.

For each exposure, images were taken with a digital camera at day 0, 3, 5 and 7. Fronds number and total fronds area of each species were measured before and after the experiment by adopting the OECD 221 guideline (OECD, 2002). The images were subsequently analysed using the software Image J (National Institutes of Health, USA). The dry weight of nine colonies with similar total frond area as the colonies used for experiments was measured to represent the dry weight before the experiment. After 7 days of exposure, the plants in each test vial were washed with 2 mL of 10 mmol/L tetrasodium ethylenediaminetetraacetate (Na_4EDTA) to remove the CuNPs and copper ions adsorbed on the surface of the plants (Zhou et al., 2011). Plants were then washed three times with double distilled water (1 mL/time) and dried at 60 °C for 48 h. The dry weight was measured afterwards. The dry weight of *W. arrhiza* was not quantified due to operational difficulties. Colony disintegration and roots abscissions during the experiment were documented every two days.

2.3. Characterization of the CuNP suspensions

The pristine morphology of the CuNPs was measured by using a JEOL 1010 Transmission Electron Microscopy (TEM) (JEOL Ltd., Japan), operating at an accelerating voltage of 70 kV. The initial size distribution of the CuNPs in Milli-Q water (1 mg/L, 0 h) was measured immediately after sonication by using a CPS disk centrifugation (CPS Instruments Europe., The Netherlands), operating at a speed of 14 092 rpm. Number-based hydrodynamic diameters of the CuNPs in the test medium were characterized at 1 mg/L using Dynamic Light Scattering (DLS) at 0, 24 and 48 h on a Zetasizer Nano-ZS instrument (Malvern, Instruments Ltd., UK). The zeta potential of the CuNP suspensions was measured at 1 mg/L using the Zetasizer Nano-ZS instrument as well to indicate the stability of the CuNP suspensions. The measurements of hydrodynamic diameter and zeta potential were conducted in triplicates.

2.4. Chemical exposure concentration and ion release

The actual exposure concentrations of the CuNP suspensions and copper nitrate were quantified by using Graphite Furnace Atomic Absorption Spectrometry (GFAAS, Perkin Elmer 1100 B). As copper ions released from the CuNPs can contribute to the total toxicity of the CuNP suspensions (Song et al., 2014), it is important to quantify the percentage of dissolution of the CuNPs over time. The amount of copper ions released from the CuNPs in the test medium was measured at total copper concentrations of 0.4, 0.8, 2 mg/L at time 0 and 48 h by centrifuging the culture medium samples at 25,000 g for 20 min at 4 °C. Supernatants were subsequently diluted using 10% nitric acid and analysed using GFAAS (Song et al., 2014). The measurements were carried out in triplicate. The percentage of dissolution of the CuNPs was then calculated as the percentage of the actual exposure concentrations.