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# Insights into the CuO nanoparticle ecotoxicity with suitable marine model species

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ABSTRACT

Metal oxide nanoparticles, among them copper oxide nanoparticles (CuO NPs), are widely used in different applications (e.g. batteries, gas sensors, superconductors, plastics and metallic coatings), increasing their potential release in the environment. In aquatic matrix, the behavior of CuO NPs may strongly change, depending on their surface charge and some physical-chemical characteristics of the medium (e.g. ionic strength, salinity, pH and natural organic matter content). Ecotoxicity of CuO NPs to aquatic organisms was mainly studied on freshwater species, few tests being performed on marine biota.

The aim of this study was to assess the toxicity of CuO NPs on suitable indicator species, belonging to the ecologically relevant level of consumers. The selected bioassays use reference protocols to identify Effect/Lethal Concentrations (E(L)C), by assessing lethal and sub-lethal endpoints. Mortality tests were performed on rotifer (*Brachionus plicatilis*), shrimp (*Artemia franciscana*) and copepod (*Tigriopus fulvus*). While moult release failure and fertilization rate were studied, as sub-lethal endpoints, on *T. fulvus* and sea urchin (*Paracentrotus lividus*), respectively. The size distribution and sedimentation rates of CuO NPs, together with the copper dissolution, were also analyzed in the exposure media.

The CuO NP ecotoxicity assessment showed a concentration-dependent response for all species, indicating similar mortality for *B. plicatilis* ($48hLC_{50} = 16.94 \pm 2.68$ mg/l) and *T. fulvus* ($96hLC_{50} = 12.35 \pm 0.48$ mg/l), followed by *A. franciscana* ($48hLC_{50} = 64.55 \pm 3.54$ mg/l). Comparable $EC_{50}$ values were also obtained for the sub-lethal endpoints in *P. lividus* ($EC_{50} = 2.28 \pm 0.06$ mg/l) and *T. fulvus* ($EC_{50} = 2.38 \pm 0.20$ mg/l). Copper salts showed higher toxicity than CuO NPs for all species, with common sensitivity trend as follows: *P. lividus* ≥ *T. fulvus (sublethal endpoint)* ≥ *B. plicatilis* > *T. fulvus (lethal endpoint)* > *A. franciscana*.

CuO NP micrometric aggregates and high sedimentation rates were observed in the exposure media, with different particle size distributions depending on the medium. The copper dissolution was about 0.16% of the initial concentration, comparable to literature values.

The integrated ecotoxicological-physicochemical approach was used to better describe CuO NP toxicity and behavior. In particular, the successful application of ecotoxicological reference protocols allowed to produce reliable L(E)C data useful to identify thresholds and assess potential environmental hazard due to NPs.

## 1. Introduction

In the last few decades, the rapid growth of nanotechnology applications, including electronics, optics, textiles, medical devices, drug delivery systems, chemical sensors, biosensors, and environmental remediation, has increased the release of nanoparticles (NPs) into the environment (Bondarenko et al., 2013). This rapid increase in NP release has not been accompanied by accurate investigations of their environmental safety (Corsi et al., 2014). Concerns have been raised on the toxic impact of NPs on the environmental compartments (Khosravi-Katuli et al., 2017). Aquatic ecosystems are the major sink of NPs, ending into the marine environment, through several direct and

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indirect routes. An extensive research has been performed on freshwater species, while still few studies and a limited amount of data is available for gauging the ecotoxicological effects of NPs on marine wildlife (Baker et al., 2014; Gallo et al., 2016; Minetto et al., 2014, 2016a).

Copper oxide (CuO) NPs are among the most employed NPs, they found effective application in metallic coating production, wood preservation, air and liquid filtration, inks production, skin products and textiles (Maisano et al., 2015). Moreover, CuO NPs are promising antimicrobial and antifouling agents and their introduction in aquaculture has been supposed to ensure advantages in the near future (Hanna et al., 2013).

Although copper is an essential element playing an important role in metabolism, it is extremely toxic (Buffet et al., 2011). It has been largely demonstrated that Cu affects negatively physiological processes in many marine organisms, such as mussels, fish, and crustaceans (e.g. Tornero and Hanke, 2016). This suggests a potential risk of CuO NPs if released into the environment and the issue should be thoroughly investigated. Nevertheless, the ecotoxicity of CuO NPs, particularly to marine organisms, is still little explored (Rossetto et al., 2014; Park et al., 2014; Wu et al., 2015). Only few studies have been conducted to identify Effect/Lethal Concentrations (E(L)C) (Abdel-Khalek et al., 2015; Rotini et al., 2016; Torres-Duarte et al., 2016), although the E(L)$C_{50}$ values are crucial to calculate thresholds and assess potential environmental hazard due to NPs (Ray et al., 2009).

Crustaceans and particularly the brine shrimp, *Artemia* spp., are among the most common model organisms in acute bioassays for toxicity assessments, mortality and growth being the main endpoints (Libralato et al., 2016). Nowadays, *Artemia* species are increasingly used as test organisms in nanoecotoxicology (Ates et al., 2013; Libralato, 2014; Rajabi et al., 2015) with successful results. The exposition to silver NPs caused mortality, ingestion and accumulation in the gut region, DNA damage in nauplii, and reduction of the hatching rate (Arulvasu et al., 2014). The exposition to different metal oxide NPs caused changes in nauplii behavioural and biochemical responses (Gambardella et al., 2014) and induced oxidative stress (Ates et al., 2013). Rotifers, major components of marine zooplankton, are extensively used in ecotoxicological research, being useful indicator species. They can be properly utilized for nanotoxicological evaluation, providing valuable insight into the effects of NPs on microinvertebrate grazers. In particular, a standardized acute toxicity test for marine *Brachionus plicatilis* has been described (ASTM, 2004; ISO, 2016). The genus *Brachionus* can be proposed as indicator species for assessing toxicity of NPs because of their rapid reproduction, short generation times, sensitivity to environmental changes, and the commercial availability of dormant eggs (Snell and Janssen, 1995). Few NP toxicity studies are available on rotifers and indicated lethal effect in *Brachionus plicatilis* (Clément et al., 2013) and alteration on reproductive rate, feeding behavior, and offspring fitness in *Brachionus manjavacas* (Snell and Hicks, 2011). Ecotoxicological tests aimed to evaluate sub-lethal endpoints are commonly used for routine environmental monitoring and were applied to study the biological impact of NPs on marine invertebrates, demonstrating that gametes and early life stages of marine organisms are extremely sensitive to NPs (Maisano et al., 2015). Copepods, and particularly the harpacticoid *Tigriopus fulvus*, is widely distributed in the Mediterranean and has been successfully used in ecotoxicological studies and ecological risk assessments (Faraponova et al., 2007, 2016; Manfra et al., 2010; Mariani et al., 2006; Prato et al., 2012, 2013, 2015; Tornambè et al., 2012), due to the easy to use and inexpensiveness, the good sensitivity to different toxicants and the reproducibility of the test (Faraponova et al., 2005). Test on *T. japonicus* clearly showed the high acute toxicity of ZnO NPs (Wong et al., 2010). Echinoderms have been so far the most studied group of marine invertebrates for assessing NP developmental toxicity (Canesi and Corsi, 2016). In particular, some studies reported the use of sea urchin gametes, embryos and/or larvae of sea urchins to evaluate the effects of engineered NPs (Falugi et al., 2012; Gambardella et al., 2015; Manzo et al., 2013; Matranga and Corsi, 2012). To our best knowledge, no literature data are available on CuO NP effects on *Artemia* spp., rotifers and copepods, while two studies demonstrated the sub-lethal toxic effects of CuO NPs on sea urchin development (Maisano et al., 2015; Torres-Duarte et al., 2016).

This study aims to evaluate CuO NP ecotoxicity, using intercalibrated procedures, on suitable indicator species, belonging to the ecologically relevant level of consumers. In particular, we performed static tests as follows:

- acute mortality test with shrimp (*Artemia franciscana*), rotifer (*B. plicatilis*) and copepod (*T. fulvus*);
- acute moult release test with *T. fulvus;*
- short-chronic fertilization test with sea urchin (*Paracentrotus lividus*).

The ecotoxicity assessment was also extended to the ionic form of Cu, as positive/solubility control. Aggregation and stability of CuO NPs in the exposure media, including dissolved copper, were also measured to better understand their behavior and toxicity.

## 2. Material and methods

### 2.1. CuO NPs and testing suspensions

The copper (II) oxide NPs were purchased from *US Research Nanomaterials, Inc.* as a water dispersion (20 wt%, purity of 99.95%) with a nominal particle size in the range of 25–55 nm. CuO NP stock suspension (1 g/L) was prepared in 0.22 µm filtered milli-Q water (mQW, reference medium) from the 20% dispersion and sonicated for 15′ in a bath sonicator (60 W, 47 kHz, 25 °C; Branson Ultrasonic Baths), then stored in the dark at 4 °C. Cellulose acetate membrane filters were used in all the preparations (Schleicher & Schuell, Germany). CuO NP stock suspension was used for preparation of all the final testing suspensions; this stock suspension was sonicated for 15′ immediately prior to each preparationM. Final suspensions were prepared in seawater vortexed prior to use, without sonication. According to reference protocols, natural seawater (NSW) was used for sea urchins and synthetic

**Table 1**
Main aspects of ecotoxicological procedures used in this study.

| Species | Life-stage | Test medium | Nominal concentrations (mg/L) | End-points | Exposure time (h) | Reference Protocols |
|---|---|---|---|---|---|---|
| *Artemia franciscana* | Nauplii (48 h) | SSW | **CuO NPs:** 10–20–40–80<br>**$CuSO_4·5H_2O$:** 5.00–7.10–10.10–14.40 | Mortality | 48 | Artoxkit (2014) Manfra et al. (2015) |
| *Brachionus plicatilis* | Nauplii (28 h) | SSW | **CuO NPs:** 6–12–25–50<br>**$CuSO_4·5H_2O$:** 0.08–0.16–0.32–0.64- | Mortality | 48 | ASTM (2004)<br>ISO (2016) |
| *Tigriopus fulvus* | Nauplii (24 h) | SSW | **CuO NPs:** 3–6–12–25<br>**$CuCl_2$:** 0.06–0.12–0.25–0.50 | Mortality, Moult release | 96 | ISO 14669 (1999), Faraponova et al., (2005, 2016) |
| *Paracentrotus lividus* | Gametes | NSW | **CuO NPs:** 1–5–10–20<br>**$Cu(NO_3)_2·5H_2O$:** 0.03–0.05–0.06–0.09 | Fertilization | 2 | Lera et al. (2006)<br>Manfra et al. (2011) |