



Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga *Chlamydomonas reinhardtii*

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ABSTRACT

Copper oxide nanoparticles (CuO NP) are frequently employed for their antimicrobial properties in antifouling paints. Their extensive use can contaminate aquatic ecosystems. However, the toxicological effects of this NP in the environment are poorly known. In this study, we evaluated the toxicity and oxidative stress induced by CuO NP on *Chlamydomonas reinhardtii* using several toxicological assays. CuO NP was found to induce growth inhibition and a significant decrease in carotenoids levels. From data on cells density after 72 h of CuO NP exposure in light, the EC50 value was calculated to be $150.45 \pm 1.17 \text{ mg L}^{-1}$ and the NOEC $\leq 100 \text{ mg L}^{-1}$. Evaluation of esterase activity demonstrates a decrease in cell metabolism activity with the increase of CuO NP concentration. The CuO NP induced an increase of reactive species level ($190 \pm 0.45\%$ at 1000 mg L^{-1} after 72 h of exposition, compared to control) and lipid peroxidation of cellular membranes ($73 \pm 2\%$ at 1000 mg L^{-1} of CuO NP in 72 h of exposition, compared to control). Investigation of CuO NP uptake showed the presence of NP into *C. reinhardtii* cells in different sites of the cell and, biomarkers of enzymatic antioxidants showed a change of activity after CuO NP exposition. In conclusion, *C. reinhardtii* was shown to be sensitive to the presence of CuO NP in their environment and CuO NP treatments induced a toxic response from 0.1 mg L^{-1} after 72 h of treatment.

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1. Introduction

The advance of nanotechnology in the last decade impelled the fast growth of the nanotoxicology domain and the need for information on potential hazards of this technology in the environment (Oberdörster et al., 2005). The interest in metallic nanoparticles (NP) has amplified due to its increasing employment in anti-fouling paints, applied on hulls to reduce the growth and colonization of marine macro and microorganisms that are responsible for reducing the speed of boats and for increasing their fuel consumption (Almeida et al., 2007; Sandberg et al., 2007). The NP employed to this function are principally titanium, zinc and copper (Almeida et al., 2007).

The presence of NP in anti-fouling paints can lead to their dispersion in the aquatic ecosystem (Curtis et al., 2006; Handy et al., 2008). Concerns with the toxic effects of copper oxide NP (CuO NP) have led to the investigation of CuO NP toxic mechanisms in various

aquatic organisms. The toxic effects of CuO NP were reported in different microorganisms, higher plants and cell lines (Heinlaan et al., 2008; Aruoja et al., 2009; Kasemets et al., 2009; Mortimer et al., 2010; Saison et al., 2010; Baek and An, 2011; Buffet et al., 2011; Shi et al., 2011). Overall, CuO NP toxicity seems to be related to particles solubilization into toxic Cu^{2+} ions, with evidences of inductions of cytotoxicity, genotoxicity and oxidative stress. Furthermore, surface properties related to NP were also recognized as important factors in the toxicity of nanomaterials (Verma and Stellacci, 2010; Zhu et al., 2010). The possibility of aquatic environment contamination by NP has led to diverse studies that evaluate the toxic effects and bioaccumulation in microalgae (Navarro et al., 2008; Wang et al., 2008; Aruoja et al., 2009; Saison et al., 2010; Perreault et al., 2012).

Microalgae represent important ecotoxicological models since they constitute the main source of biomass production which supports all the other aquatic trophic levels. Unicellular algae present a high superficial area of contact in relation to their volume, which amplify its exposition to the toxicant studied (Torres et al., 2008; Saison et al., 2010). *Chlamydomonas reinhardtii* is a unicellular green microalga that meets the above requirements, and has a rapid time of adaptation and reproduction (Merchant et al., 2007), therefore it

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was chosen as a model microorganism in this study. The response of *C. reinhardtii* to NP was previously reported for different types of metallic NP. Wang et al. (2008) observed the induction cellular damage and lipid peroxidation in *C. reinhardtii* exposed to titanium dioxide NP. Navarro et al. (2008) evaluated the toxic effects of silver NP (AgNP) on *C. reinhardtii* and demonstrated that the toxicity of AgNP is mediated by Ag⁺ release from the NP. Saison et al. (2010) reported that core-shell CuO NP causes the induction of cell aggregation and the inhibition of photosystem II by chlorophyll degradation, probably via the formation of reactive species (RS).

A major process in the inhibition of algal growth and photosynthetic processes by pollutants is the generation of RS (Pinto et al., 2003; Dewez et al., 2005; Elbaz et al., 2010). To cope with RS formation, several mechanisms are used by algae to prevent oxidative stress. Carotenoids are essential for the prevention of chlorophyll oxidation by photochemical and non-photochemical mechanisms of energy dissipation (Niyogi, 2000). Moreover, some enzymes work as a cellular defense mechanisms against RS and others important biomarkers to evaluate the presence of this oxidative stress (Pinto et al., 2003). An investigation of change in their activity brings significant information about the action mechanism of toxic chemicals on cellular system.

The main objective of our report was to characterize the toxic effects of CuO NP in *C. reinhardtii*. To evaluate these effects, we investigated the growth, esterase activity, pigment of *C. reinhardtii* cells exposed to CuO NP. Furthermore, we also investigated several biomarkers related to oxidative stress as well, as RS formation, lipid peroxidation (LPO) and enzymatic antioxidant activities of catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione S-transferase (GST) induced by CuO NP exposition. NP characterization was performed in culture media employing techniques of zeta potential of NP suspension by electrophoretic mobility, transmission electron microscopy (TEM) and dynamic light scattering (DLS).

2. Materials and methods

2.1. Nanoparticles preparation and characterization

CuO NP was obtained from MTI Corporation (Richmond CA; 30–40 nm of diam.; purity: 99.9%; shape: nearly spherical; crystal-lite: monoclinic). Prior to every assay, fresh NP stock suspensions (10,000 mg L⁻¹ of CuO NP in HSM media) were prepared by sonication in an ultrasonic cell disruptor (VibraCell, 400 W) for 2 min with 40% of power. CuO NP was suspended in HSM media in a concentration of 0.1 and 1.0 mg L⁻¹. NP size was also evaluated by TEM imaging (Philips CM200 200 kV) and pictures taken with an AMT 2k × 2k CCD camera. The elemental nature of the metallic aggregates was determined by energy-dispersive X-ray spectroscopy using a Philips CM200 200 kV TEM equipped with an EDAX Genesis EDS system. In parallel, a determination of particle size distribution in solution was performed by DLS analysis with a ZetaPlus particle sizer (Brookhaven Instruments Corporation, USA). Zeta potential of NP in the HSM media was determined by electrophoretic mobility method with Zeta Plus system.

2.2. Algal culture

C. reinhardtii wild type (CC-125) strain was obtained from the Chlamydomonas Genetic Center (Duke University, Durham, NC, USA). Cells were cultivated in batch culture of 1 L of HSM media (Harris, 2009). Cultures were exposed to continuous illumination (100 μmol of photons m⁻² s⁻¹) provided by white fluorescent lamps (Sylvania GroLux F 36 W) at 23 °C ± 1. The culture was permanently aerated to obtain a constant CO₂ concentration in the growing medium.

2.3. Algal exposure to copper oxide nanoparticles

Aliquots of 20 mL of the algal culture (2.5 × 10⁵ cells mL⁻¹) in the exponential growth phase were exposed to 0.1, 1, 10, 100 and 1000 mg L⁻¹ of CuO NP for 24, 48 and 72 h under the same illumination and temperature conditions used for growing cultures. All the NP suspensions were prepared in HSM media. For the control samples, the same media and conditions were used but in the absence of CuO NP. All samples were evaluated in triplicates.

2.4. Microscopy of exposed algae

Morphological changes in algal culture were determined on samples exposed 72 h to CuO NP using a Nikon Eclipse TS100 microscope, and pictures were captured with a PixelINK Camera. TEM analysis of intracellular CuO NP was performed 72 h after treatment. 50 mL of control and NP-treated cultures (CuO NP 10 mg L⁻¹) were centrifuged and the supernatant discarded. Without disturbing the pellet, cells were washed, fixed and stained with 1% OsO₄ and 1.5% KFeCN in water (Perreault et al., 2012). Samples were dehydrated with acetone and infiltrated with epon. Hardened pellets were cut into 0.6 μm thick slices and placed on a gold grid for TEM analysis. No secondary fixation was used to obtain a better visualization of NP inside the cell. Samples were visualized with a FEI Tecnai 12 120 kV microscope and pictures taken with a Gatan 792 Bioscan 1k × 1k Wide Angle Multiscan CCD camera.

2.5. Algal culture cell viability and granulosity

Algal culture cell density and granulosity were measured by flow cytometry (FACScan; Becton Dickinson Instruments) using a suspension of Fluospheres[®] carboxylate-modified microbeads 2 μm (Molecular Probes, cat. #F8827) at a known concentration of microbeads. Size threshold of the cytometer was fixed just below the size of the microbeads. Algae cell count was made using the algae/microbeads ratio: cell density = (% algal cell/%) × microbeads density. The cell viability was determined by a comparison between cell density on CuO NP expositions and the control test on different exposition times. The dose-response curve was plotted at 72 h (chart data not shown) to calculate the half maximal effective concentration (EC50) and to estimate the no observed effect concentration (NOEC) of CuO NP. The scattering of light by the cell, which is dependent on the amount of cellular content, was used as an indicator of cellular granulosity. Granulosity was obtained by plotting side-scattering value in function of size, with a gate on algal cells population.

2.6. Chlorophyll pigment determination

Total chlorophylls and carotenoids were extracted in methanol 100% at 65 °C and quantitative determination was done by measurement of absorbance from extracts on the wavelengths 470, 652.4, 665.2 and 750 nm (Lichtenthaler, 1987).

2.7. Toxicity evaluation by esterase activity

For analysis of esterase activity, fluorescein diacetate (FDA) was used (Regel et al., 2002; Yu et al., 2007). Cell staining was performed by adding 10 μL FDA 10 μM (Sigma, dissolved in acetone) in 500 μL of cells. As esterase activity of cells changed continuously in fluorescence intensity, the data were expressed as the mean fluorescence intensity and the results as a percentage of the control (Yu et al., 2007). Three states were defined (see electronic supplementary material, Fig. 1): a low activity state, M1, corresponded to the activity of heat treated cells (1 min, 90 °C), the normal state, M2, corresponded to the activity distribution of an untreated algal

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