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Acute toxicity of copper and chromium oxide nanoparticles to *Daphnia similis*

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Abstract

Copper oxide nanoparticles (CuO NPs) are employed in antifouling paints, and chromium oxide nanoparticles (Cr_2O_3 NPs) have been used as a green pigment. Their extensive use can contaminate aquatic ecosystems, and the toxicological effects of these NPs to the biota are poorly known. In this study, we evaluated the acute toxicity induced by CuO and Cr_2O_3 NPs, comparing with $CuSO_4$ and $Cr(NO_3)_3$ as Cu^{2+} and Cr^{3+} ion source, respectively, using the microcrustacean *Daphnia similis*. The mean EC_{50}-48h for CuO NPs was 0.064 mg L^{-1} and for $CuSO_4$ was 0.015 mg L^{-1}. CuO NPs tend to agglomerate, which may have reduced the release of Cu^{2+} in the test medium in relation to $CuSO_4$.The mean EC_{50}-48h for Cr_2O_3 NPs was 6.74 mg L^{-1} and for $Cr(NO_3)_3$ was 11.98 mg L^{-1}. The reduced size of the Cr_2O_3 NPs (15-30 nm) and the higher zeta potential may have contributed to the higher stability in suspension and less potential for agglomeration, partially explaining the higher toxicity of NPs in relation to $Cr(NO_3)_3$. After the tests, we observed morphological damages such as increase in fat droplets, internal organ exposure and partially disintegration in organisms exposed to all tested substances, NPs or the salts.

Keywords: Acute toxicity tests, *Daphnia similis*, Metal oxide nanoparticles, Microcrustaceans, Nanotoxicology.

INTRODUCTION

Metal oxide nanoparticles (NPs) are already manufactured in large scale for both industrial and household use, and are incorporated into a wide range of products such as sunscreens, paints, construction materials, coatings, catalysts and cosmetics (Aruoja *et al*., 2009; Keller *et al*., 2010; Hanna *et al*., 2013). Copper oxide (CuO) NPs have potential to replace noble metal catalysts for carbon monoxide oxidation and CuO NPs suspension (nanofluid) has excellent thermal conductivity for it to be used as a heat transfer fluid in machine tools (Buffet *et al*., 2011). Furthermore, CuO NPs has been used for antimicrobial textiles (Gabbay *et al*., 2006), gas sensors, photovoltaic cells, air and liquid filtration (Sousa & Teixeira, 2013), and as antifouling paints of boats, thus representing an important source of aquatic ecosystems contamination (Melegari *et al*., 2013).Chromium oxide (Cr_2O_3) NPs has also attracted considerable attention in recent years. A wide range of applications such as coating materials for thermal protection, wear resistance, humidity sensing and refractory characteristics have been reported (Makhlouf *et al*., 2013). In addition, this type of NP has been used in green pigment composition (Gibot & Vidal, 2010). As result of the increased use and production of NPs over

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the last years has led to their release in aquatic environments (Kahru *et al*., 2008; Hanna *et al*., 2013).

However, while the novel properties of NPs are increasingly studied, little information is available about their interactions with aquatic organisms (Kahru *et al*., 2008). Additionally, transformations of NPs, such as dissolution, agglomeration, sedimentation, or change of surface moieties, could greatly affect the pathway and extent of NPs environmental fate (Maurer-Jones *et al*., 2013).When added to water, metal NPs can aggregate, sediment out of the water column, adsorb to nutrients, and disassociate to release soluble metal ions (Griffitt *et al*., 2009). For all these reasons, factors such as NPs aggregation, size and surface properties play a crucial role in NPs toxicity because they affect the bioavailability of such materials (Sousa & Teixeira, 2013), and so the characterization of NPs that will be submitted to ecotoxicological evaluation is a fundamental step. Moreover, the knowledge of biological effects, target sites, and especially the modes of action of the engineered particles seems to be unknown yet (Manusadžianas *et al*., 2012).

CuO NPs were identified as being important in ecotoxicological assays due to their relatively low dissolution rate but their potentially high toxicity towards organisms (Buffet *et al*., 2011). Several studies have demonstrated toxicity of CuO NPs to aquatic organisms, including *Allivibrio fischeri*, *Daphnia magna*, *Hediste diversicolor*, *Pseudokirchneriella subcapitata*, *Scrobicularia plana*, *Thamnocephalus platyurus* (Heinlaan *et al*., 2008; Aruoja *et al*., 2009; Kahru & Dubourguier, 2010; Buffet *et al*., 2011; Isani *et al*., 2013), but there is no consensus yet, among authors, regarding the toxic effect observed to be associated with the NPs themselves or just with the release of Cu ions in the test media.

Thus, it is necessary to characterize correctly their effects on aquatic organisms, considering the high toxicity before demonstrated by copper ions to aquatic organisms (Kahru & Dubourguier, 2010). On the order hand, limited data are available in the literature on Cr_2O_3 NPs toxicity. Annarao *et al*. (2008) examined the distribution of Cr_2O_3 NPs in rats and found that its absorption through the skin was efficient and NPs was evenly distributed in the tissues and muscles. Vajpayee *et al.* (2011) evaluated the phytotoxic effects of Cr_2O_3 NPs to wheat (*Triticumae stivum*), and observed that there was inhibition of seed germination and seedling growth in concentration-dependent manner. However, studies published in the international literature on the toxicity of these types of NPs to aquatic organisms are scarce. Lin *et al*. (2012) used zebrafish (*Danio rerio*) embryos and demonstrated that Cr_2O_3 NPs interfered in embryo hatching.

The aim of this work was to evaluate the acute toxicity of CuO and Cr_2O_3 NPs, comparing with $CuSO_4$ and $Cr(NO_3)_3$ as Cu^{2+} and Cr^{3+} ion source, respectively, using *Daphnia similis*. The choice of *D. similis* as test organism is based on your reproduction by parthenogenesis, ease of cultivation in the laboratory, simple handling, sensitivity to wide range of pollutants, life and reproductive short cycle and, for being an internationally standardized testing organism (Knie & Lopes, 2004; Paschoalino *et al.*, 2010). Furthermore, daphnids are considered a keystone species in aquatic toxicology because they are filter-feeders and are able to ingest NPs (Artal *et al*., 2013).Tests employing Daphnia have been used to evaluate the nanomaterials ecotoxicity, due to be important links in the food chain from algae consumed by them, and the fish that are predators (Paschoalino *et al.*, 2010).They have also been proposed as a model organism for the ecotoxicological testing of nanomaterials (Paschoalino *et al.*, 2010; Artal *et al*., 2013).

MATERIAL AND METHODS

Materials

Due the high heterogeneity of commercial NPs, we choose to synthesize the NPs of interest in this study. The following materials were used to synthesized the evaluated NPs: copper sulfate hydrate ($CuSO_4.5H_2O$; 98%), sodium carbonate (Na_2CO_3; 99%), chromium nitrate hydrate ($Cr(NO_3)_3.9H_2O$; 97%) and sodium hydroxide (NaOH; ≥ 97%) purchased from Vetec® (Duque de Caxias, RJ, Brazil), and distilled water. All chemicals were analytical grade and were used as received.

CuO NPs synthesis

CuO NP was prepared via direct thermal decomposition method, adapted from Das *et al*. (2013) to improve the reaction yield and purity of NPs, as well as facilitates the synthesis procedure. An excess of Na_2CO_3 was used, instead of 1 eq., and the final product was separated by centrifugation, instead of filtration. The precursor, $Cu_4(SO_4)(OH)_6$, was synthesized by adding 100 mL of a 60 mmol Na_2CO_3 solution to 100 mL of a 50 mmol $CuSO_4.5H_2O$ solution, and the mixture was ultrasonicated in a ultrasonic cell disruptor (Unique – 100 W, 99% of the maximum power) for 60 min at 60 °C. The precipitate produced was separated by centrifugation and washed several times with warm distilled water to remove any possible remaining ions in the final product. Then, the precipitate was dried in an oven at 70 °C for 12 h. Finally, it was placed in a preheated muffle furnace at 600 °C for decomposition. After 2 h, the CuO NPs were removed from the furnace and allowed to cool to room temperature, and the resulting dark brown powder was ground and sieved.

Cr_2O_3 NPs synthesis

Cr_2O_3 NPs were synthesized using the thermal degradation methodology of $Cr(OH)_3$ adapted from Bañobre-López *et al*. (2003), to facilitate the synthesis procedure and to avoid the oxidation of Cr^{3+} to Cr^{6+}. The temperature of drying $Cr(OH)_3$ was 90 ºC instead of 65 ºC, and the final product was separated by centrifugation, instead of filtration. 21 mmol $Cr(NO_3)_3.9H_2O$ and 50 mmol NaOH were added to 100 mL of distilled water and stirred for 30 min. The product was

separated by centrifugation and washed several times with distilled water. The $Cr(OH)_3$ was dried in an oven for 24 h at 90 °C. Finally the product was calcined at 400 °C for 3 h for obtain Cr_2O_3 NPs.

Characterizations of NPs

All synthesized NPs were characterized by X-ray diffractograms (XRD), which were measured over the angular range of 2θ = 20° - 80° using a Philips X'Pertdiffractometer equipped with a copper tube (CuK α, λ = 1.54056 Å). Images revealing the morphologies and sizes of the NPs were obtained using a transmission electron microscope (TEM; JEM-1011 TEM microscope). The zeta potential (ζ) of the NPs was measured using a Malvern Zetasizer Nano ZS (ZEN 3600 model). The samples for zeta potential and transmission electron microscope were prepared in ultrapure water (UW) (1 g L^{-1}).

Ecotoxicity tests

Suspensions of CuO (100 mg L^{-1}) and Cr_2O_3 (1000 mg L^{-1}) NPs were prepared in mineral water and sonicated for 30 min in an ultrasonic bath (Colle Palmer 8891). Both NPs were initially tested at logarithmic scale of concentration (concentrations ranging 0.001 - 100 mg L^{-1}). Based on the preliminary results of these tests were chosen the final test concentrations. CuO NPs were tested at concentrations 0.02; 0.04; 0.06; 0.08; 0.10; 0.12 and 0.14 mg L^{-1}. Cr_2O_3 NPs were tested at concentrations 10, 15, 20, 25, 30, 35 and 40 mg L^{-1}.

For comparison, solutions of the salts ($CuSO_4$ and $Cr(NO_3)_3$) used in the NPs synthesis were tested as a source of Cu^{2+} and Cr^{3+}, respectively. Both inorganic salts were initially tested at logarithmic scale of concentration (concentrations ranging 0.001 - 500 mg L^{-1}). Based on the preliminary results of these tests were chosen the final test concentrations. $CuSO_4$ was tested using the nominal concentrations of 0.02; 0.04; 0.06; 0.08; 0.10; 0.12 and 0.14 mg L^{-1}, while $Cr(NO_3)_3$ was tested using nominal concentrations of 30, 60, 90, 120, 150 and 180 mg L^{-1}. The tests were performed immediately after the preparation of the suspensions and solutions.

D. similis stock cultures were kept according to procedure NBR 12713 (ABNT, 2009). The sensitivity of the *D. similis* culture was monitored monthly with sodium chloride (NaCl, Sigma Aldrich, ≥99% purity), as a reference substance, and the culture was used if the results were within the range expected toxicity whose EC_{50}-48h should be between 1.6 and 3.6 g L^{-1} (CETESB, 1994). Acute toxicity tests were performed according to the NBR 12713 (ABNT, 2009). For each of four replicates five organisms 6 to 24-h-old were exposed during 48 h in 10 mL of each test concentrations under static conditions at 20 ± 2 °C in the dark. After exposure, immobilized organisms were counted and the EC_{50}-48h estimated by the Trimmed-Spearman Karber method (Hamilton *et al*., 1977). Tests were considered acceptable if *D. similis* immobility in negative controls did not exceed 10% (maximum of two organisms).

RESULTS AND DISCUSSION

Characterizations of NPs

TEM images of the synthesized CuO and Cr_2O_3 NPs are shown in Figure 1 (a and b). The TEM image in Figure 1a shows that the NPs are nearly spherical, with diameters ranging from 50 to 100 nm. Figure 1b shows an irregular morphology, with rare scattered clusters and NPs with sizes ranging between 15 and 30 nm.

The crystalline structures of NPs were examined by XRD (Figure 2 (a) and (b)). For CuO NPs the diffraction peaks from are consistent with the standard structure and can be indexed to the monoclinic phase of CuO (JCPDS No. 89-5898) (Massarotti *et al*., 1998).The results indicated that the products are consisted of pure phase. Different peaks were observed at 2θ = 32.50º (110), 35.42º (002), 38.70º (111), 48.72º (202), 53.49º (020), 58.27º (202), 61.52º (113), 66.22º (311) and 68.12º (220) corresponds to several planes of CuO NP. This confirms the formation of CuO NPs. For Cr_2O_3 NPs the

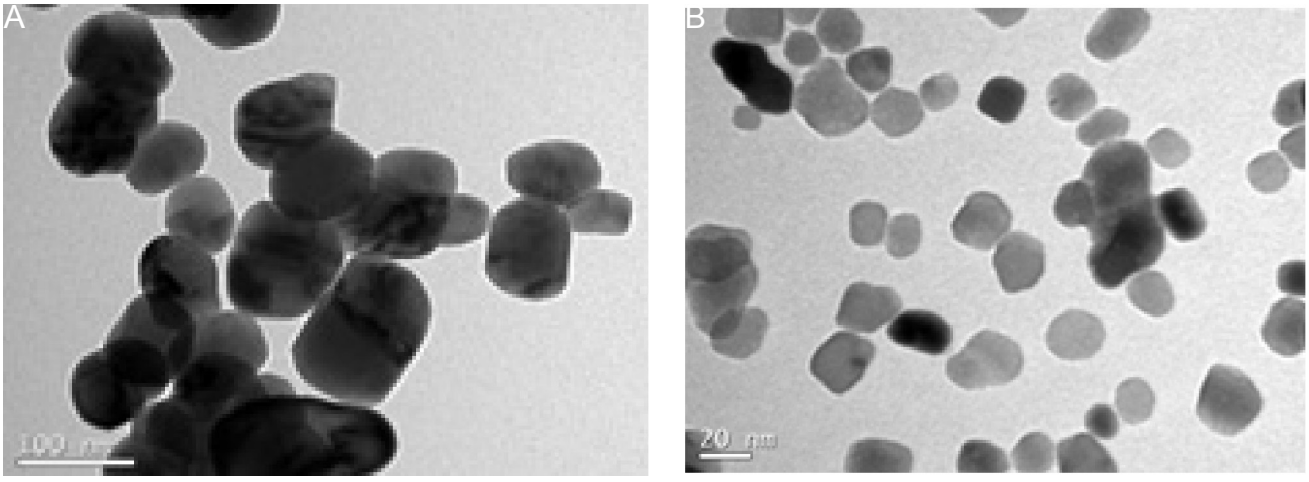

Figure 1 - TEM image of CuO NPs (a) and Cr_2O_3 NPs (b).

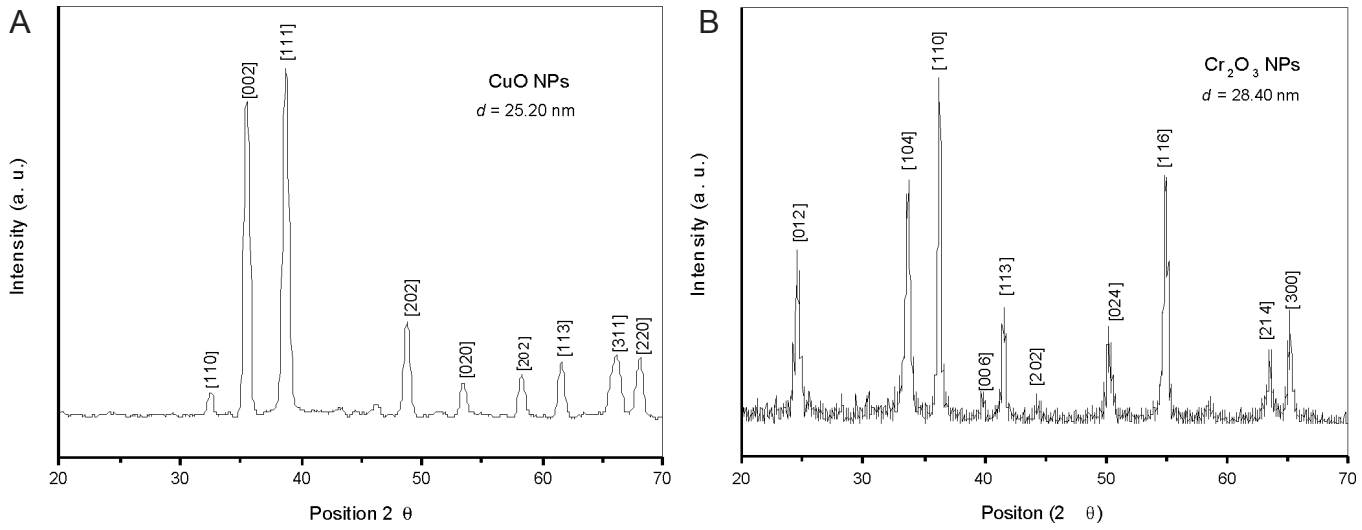

Figure 2 - XRD of CuO NPs (a) and of Cr_2O_3 NPs (b).

diffraction peaks from are consistent with the crystal structure rhombohedral (space group 167, R-3c) and hexagonal lattice parameters (JCPDS No. 082-1484). Different peaks were observed at 2θ = 24.65º (012), 33.70º (104), 36.32º (110), 39.86º (006), 41.46º (113), 44.28º (202), 50.28º (024), 54.86º (116), 63.32º (214) and 65.27º (300) corresponds to several planes of Cr_2O_3 NPs. The crystallite size based on X-ray peak broadening was estimated using Debye-Scherrer's equation (Equation 1) (El-Trass *et al*., 2012):

$$d(\text{Å}) = \frac{k\,\lambda}{\beta\,cos\theta} \quad \text{(Eq. 1)}$$

where *k* is an empirical constant equal to 0.9, λ is the wavelength of the X-ray source (1.5405 Å), β is the full width at half maximum of the diffraction peak, and θ is the angular position of the peak. The average value calculated for the crystallite size for CuO NPs is 25.2 nm and for Cr_2O_3 NPs is 28.40 nm.

Zeta potential value for CuO NPs suspension in ultrapure water was -11.73 mV, and the pH of the suspension was 6.07. The measure of zeta potential of Cr_2O_3 NPs was -19.65 mV and pH = 6.87. Zeta potential is a common indicator of surface charge, which is the electrical potential at the surface of a sphere that includes the particle and adjacent water molecules that travel with the particle during its motion. A common rule of thumb is that the zeta potential must be > 30 mV or < -30 mV for repulsion to be sufficiently strong to avoid agglomeration (Oberdörster *et al*., 2013).

The zeta potential indicates the stability of NPs in solution. The higher the value of the zeta potential greater will be the stability of NPs. These results indicate that Cr_2O_3 is more stable in solution than CuO and both NPs exhibit pH within the range suitable (5-9) to perform toxicological testing.

Ecotoxicity tests

EC_{50}-48h values were determined from the means of results from three independent tests. Since the literature suggests that the toxicity of metal NPs is mainly related to the release of the metallic ions (Heinlaan *et al*., 2008; Aruoja *et al*., 2009; Perreault *et al*., 2014), the EC_{50}-48h were corrected for mass of cooper present in the NPs as well as in the $CuSO_4$ and for mass of chromium present in to Cr_2O_3 NPs and Cr $(NO_3)_3$.

EC_{50}-48h mean for CuO NPs was 0.064 mg L^{-1} and for $CuSO_4$ was 0.015 mg L^{-1} (Table 1). CuO NPs were four times less toxic than the copper sulfate salt (values corrected by mass of copper). EC_{50}-48h of $CuSO_4$ obtained for *D. similis* is in agreement to the results of Bertolleti *et al*. (1992), 0.023 mg L^{-1}. Besides different species of Daphnia present differences in the sensibility to metals, Arauco *et al.* (2005) compare the toxicity of $CuSO_4$ using three different species of Daphnia. The authors obtained similar values of $CuSO_4$ EC_{50}-48h for *D. similis* and *D. magna* (0.0447 and 0.0426 mg L^{-1} respectively - values of EC_{50}-48h without correction for Cu mass). Rodgher *et al.* (2010) evaluated the response of *D. similis* to cadmium and chromium and concluded that this specie is as sensitive to metals as other standardized Daphnia species and behaves as an ideal test organism for ecotoxicological assessments. Based on this data and in the lack of toxicity data for CuO NPs to *D. similis*, we compare our results with data from *D. magna* available in the literature.

Table 1 - EC_{50}-48h values obtained for the three independent tests performed with CuO NPs and $CuSO_4$ using *D. similis.*

	CuO NPs		$CuSO_4$	
Test	**EC_{50}*-48h (mg L^{-1})**	**CI 95%****	**EC_{50}-48h (mg L^{-1})**	**CI 95%**
1	0.051	0.05 – 0.06	0.010	0.01 – 0.01
2	0.065	0.06 – 0.07	0.012	0.01 – 0.01
3	0.077	0.07 – 0.08	0.010	0.01 – 0.01
Mean	0.064		0.010	
Standard deviation	0.013		0.001	
CV (%)***	20.350		11.540	

* EC_{50} – Effective concentration 50%
** CI 95% - Confidence interval of 95%
*** Coefficient of variation

EC_{50}-48h values for CuO NPs and $CuSO_4$ obtained in this work were lower than values available in the literature for *D. magna*. Heinlaan *et al.* (2008) compare the toxicity of CuO NPs with particle size ~ 30 nm and $CuSO_4$ obtaining EC_{50}-48h of 2.6 mg L^{-1} for CuO NPs and 0.07 mg L^{-1} for $CuSO_4$. Blinova *et al.* (2010) also compare the toxicity of CuO NPs with $CuSO_4$ in natural river waters samples collected in six different sampling sites. The objective of the authors was to compare the influence of artificial freshwater and natural waters in the toxicity of the NPs. The authors obtained EC_{50}-48h ranging from 92.7 to > 200 mg L^{-1} for CuO NPs and 0.24 to 0.92 mg L^{-1} to $CuSO_4$. The lower toxicity observed in river water samples in comparison to the test media was attributed to the presence of organic matter that can strongly complex to Cu and reduce the bioavailability of Cu ions (Blinova *et al*., 2010).

Copper sulfate salt was more toxic than the CuO NPs for both *D. similis* and *D. Magna* (Heinlaan *et al.*, 2008; Blinova *et al*., 2010). The same behavior was observed to *Oncorhynchus mykiss* (Isani *et al*., 2013) and *Lemna gibba* (Perreault *et al*., 2014). The higher toxicity of the $CuSO_4$ is associated to the greater bioavailability of Cu ions in the test media in comparison to the release of Cu ions from the CuO NPs. Other important factor is the aggregation of the NPs that can decrease the release of Cu ions (Perreault *et al*., 2014). In this work the lower toxicity of the CuO NPs in relation of $CuSO_4$ may be related also by CuO NPs tendency to agglomerate, which may have reduced the release of Cu^{2+} in the test medium and also a lower intake of the agglomerates by *D. similis*. This tendency to agglomeration and coagulation was observed during the preparation of the suspension and by the low zeta potential (-11.73 mV).

Although the evaluation of morphological parameters is not part of the acute toxicity tests with *D. similis*, during the evaluation of the immobility of tests organisms, it was also observed an increase in the size of fat droplets as a response to exposure to NPs (Figure 3b) compared to control (Figure 3a). Artal *et al*. (2013) also reported small bubbles under the carapace of *D. similis* exposed the silver nanowires that were similar to those observed in this work. The organisms exposed to $CuSO_4$ suffered great damage with exposure of internal organs (Figure 3c).

Although the toxicity of CuO NPs was lower than that of the $CuSO_4$ salt, the values of EC_{50}-48h had become very low, indicating high toxicity. According to Directive 67/548/EEC, the main European Union legislation on chemical safety, substances that exhibit EC_{50} less than 1 mg L^{-1} are classified as very toxic (Kahru & Ivask, 2012), indicating that the CuO NPs and $CuSO_4$ may offer potential risks to aquatic biota.

Until the finishing of this manuscript, few studies investigated the chromium NPs toxicity. Lin *et al.* (2012) investigated the toxicity of chromium NPs to aquatic organisms using *D. rerio*. This study has indicated that Cr_2O_3 NPs could interfere with embryo hatching by a chelator-sensitive mechanism that involves ligation of critical histidine in the ZHE1 (metalloprotease, responsible for degradation of the chorionic membrane) center by the shed metal ions. Moreover, Horie *et al.* (2011) demonstrated that Cr_2O_3 NPs have a high cytotoxic potential to human keratinocyte HaCaT cells.

The mean EC_{50}-48h for Cr_2O_3 NPs was 6.74 mg L^{-1} and for $Cr(NO_3)_3$ was 11.98 mg L^{-1} (Table 2). Cr_2O_3 NPs were approximately two times more toxic that the solution of $Cr(NO_3)_3$. For the Cr_2O_3 NPs the toxicity cannot completely be explained by the Cr^{3+} ions release. The reduced size of the Cr_2O_3 NPs (15-30 nm) and the higher zeta potential may have contributed to the observed toxicity. The tested Cr_2O_3 NPs were more stable in suspension and have less agglomeration potential. Thus, in addition to chromium released in the test medium, the NPs present in the *D. similis* gut may have acted as a direct source of release of Cr ions to the test organism tissues. Hund-Rinke & Simon (2006) found that particles with smaller diameters are more easily ingested by *D. magna*, without any selective mechanism. However, larger particles are more difficult to be processed by the Daphnia (Baudo, 1987), thereby avoiding that the NPs reach the filter chamber.

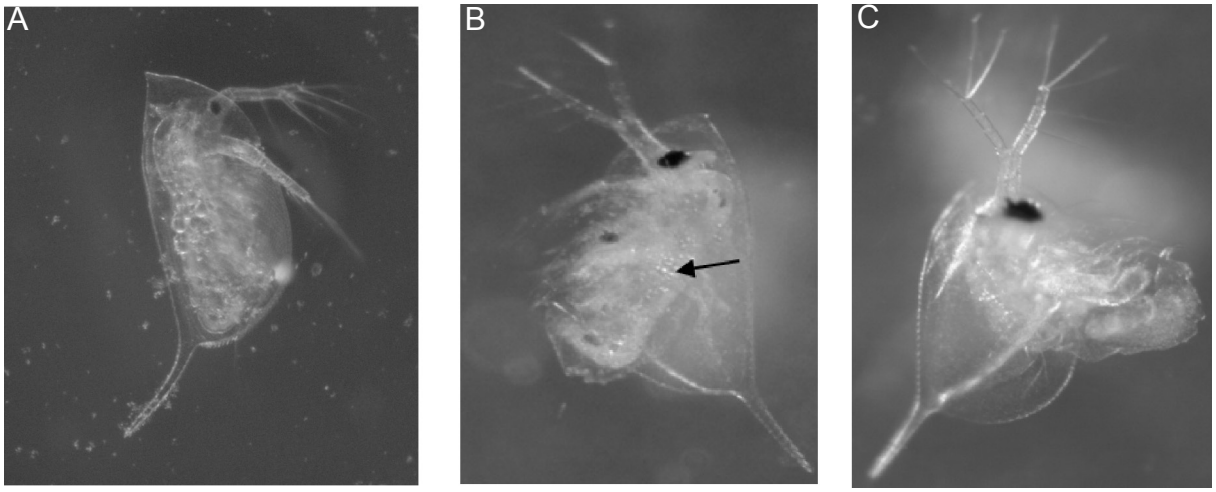

Figure 3 - *D. similis at control (a), D. similis after exposure to CuO NPs (b), and the arrow indicate increases in the size of lipid droplets, and D. similis after exposure to CuSO4 (c)..*

Table 2 - EC_{50}-48h values obtained for the three independent tests performed with Cr_2O_3 NPs and $Cr(NO_3)_3$ using *D. similis.*

Test	Cr_2O_3 NPs		$Cr(NO_3)_3$	
	EC_{50}*-48h (mg L^{-1})	CI 95%**	EC_{50}-48h (mg L^{-1})	CI 95%
1	6.53	5.95 – 7.17	12.26	11.17 – 13.47
2	6.73	5.76 – 7.88	11.03	9.12 – 13.33
3	6.96	6.51 – 7.22	12.67	11.19 – 14.34
Mean	6.74		11.98	
Standard deviation	0.21		0.85	
CV(%)***	3.20		6.96	

* EC_{50} – Effective concentration 50%
** CI 95% - Confidence interval of 95%
*** Coefficient of variation

Organisms exposed to Cr_2O_3 NPs were evaluated after the end of the test, and we could observe that the *D. similis* were partially disintegrated and presented a dark material inside the bodies (Figure 4a), suggesting the intake of the tested NPs. We also observed blue spots in the organisms exposed to $Cr(NO_3)_3$, probably due to deposition of chromium nitrate on the carapace of *D. similis* (Figure 4b).

Some EC_{50}-48h values for trivalent chromium have been reported for invertebrate species. Caloto-Oliveira (2007) evaluated the toxicity of potassium dichromate to *D. similis* and the EC_{50}-48h obtained was 0.081 mg L^{-1} (corrected value by chromium mass). To *D. magna*, the range of EC_{50}-48h obtained to chromic nitrate ranging 2 - 58.7 µg L^{-1}(USEPA, 1980), varying according to the hardness of the water.

Substances with EC_{50} value ranging 1 - 10 mg $L^{-1}$are considered toxic in accordance with Directive 67/548/EEC and dangerous when EC_{50} value ranging 10 - 100 mg L^{-1} (Kahru & Ivask, 2012). Thus, Cr_2O_3 NP and chromium nitrate can be considered as toxic and dangerous, respectively.

CONCLUSIONS

Both NPs tested showed toxicity to *D. similis*, but with distinct behaviors. While the toxicity of CuO NPs appears is primarily associated with the release of Cu ions in the test medium, this mechanism does not appear to be the main cause of the toxicity of Cr_2O_3 NPs. The Cr_2O_3 NPs were approximately two times more toxic than chromium salt indicating that the characteristics of NPs, such as its reduced size, has an important influence on the toxicity observed. In view of the wide use of these NPs and their release into the environment, further ecotoxicological studies of these materials are required, mainly of chromium NPs, due scarce ecotoxicological data.

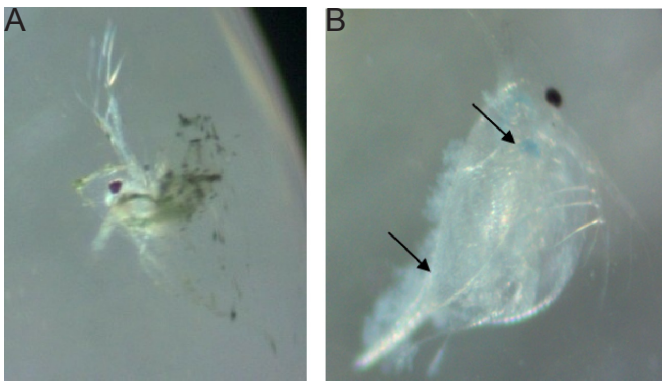

Figure 4 - *D. similis* after exposure to Cr_2O_3 NPs (a) and $Cr(NO_3)_3$(b), and the arrows indicate the blue spots, present probably due to deposition of chromium on the Daphnia carapace.

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