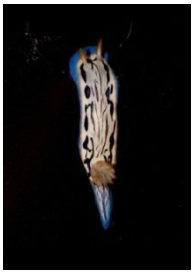

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Comparative Toxicity of Copper Oxide Nanoparticles and Dissolved Copper to Freshwater Mussels

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Abstract: The commercial use of copper oxide nanoparticles (nCuO) has raised concerns about the toxic risk to filter feeders such as bivalves. The purpose of this study was to compare the toxicity of nCuO and dissolved Cu^{2+} to the freshwater mussel *Dreissena bugensis*. Mussels were exposed to a range of concentrations of both forms of Cu (2, 10 and 50 µg/L) for 96 h at 15 C. After the exposure period, some mussels were kept aside to determine the air survival time as a measure of resistance to stress. The remaining mussels were processed for total and labile Cu determination in their tissues and the following effects: lipid peroxidation (LPO), DNA damage, arachidonate cyclooxygenase (COX), protein-ubiquitin levels (UB), acetylcholinesterase (AChE) and glutathione S-transferase (GST) activity. The data revealed that only exposure to Cu^{2+} led to the accumulation of total Cu in tissues with a decrease in labile Cu^{2+} levels suggesting that Cu was strongly bound to tissues. Exposure to nCuO led to specific effects on COX activity (inflammation) and UB levels (damaged protein turnover). It is concluded that although no significant changes in Cu levels in mussels were detected, exposure to nCuO produced different effects than Cu^{2+} in freshwater mussels.

Keywords: Nanoparticle, Copper Oxide, Bioaccumulation, Oxidative stress, Inflammation, Protein damage, Toxicity, Freshwater mussel

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Introduction

Nanotechnology is a rapid growing field with many applications in many key areas of our economy. They have application in diverse area such as medical diagnostics, therapeutics, electronics and personal care products. Nanotechnology produce materials that display at least one dimension in the nanoscale at size range between 1-100 nm.

The increasing commercial activities of these products could lead to the inadvertent release in the aquatic environment and threaten aquatic life (Rather *et al.*, 2018). The unique properties of nanoparticles such as size, surface area, and surface coating and charge could influence the fate, bioavailability across different tissues and toxicity in aquatic organisms. Copper oxide nanoparticles (nCuO) are particularly of concern given their multiple uses in our economy (Raiput *et al.*, 2020). At the nanoscale, nCuO is increasingly used as bioactive paints, integrated circuits, batteries, and for liquid/air filtration. They also have interesting bactericidal properties and are added in skin care products and clothes. More research is needed to better understand the fate of nCuO in the environment, bioavailability and toxicity mechanisms especially if nCuO will degrade and release Cu^{2+} ions and contribute to toxicity. The identification of key physiological targets that respond to nCuO and Cu^{2+} is of interest. Indeed, the better understanding of the mechanisms of nanoparticle toxicity is crucial in risk assessment since conventional toxicity tests (i.e, using ionic Cu) may not answer the above questions.

Ionic Cu^{2+} and Cu^{1+} are biological essential metals and are found in reaction centres of many enzymes involved in redox reactions such as superoxide dismutase and cytochrome c oxidase. Cu could become toxic when present at concentrations that overwhelms storage capacity and regulatory mechanisms in cells. Indeed, Cu is not usually found in its free ionic form because it is strongly bound to thiol-containing proteins such as metallothioneins and glutathione which are considered as storage pools of Cu (Amiard *et al.*, 2006). Bivalves are especially at risk to nanoparticles given their filtering activity where they can trap important quantities of suspended materials including nanoparticles and their aggregates. They are also sessile and live for relatively long periods, which makes them targets to nanotechnology pollution. This is in keeping with bivalves are considered relevant organisms towards potentially toxic compounds associated to dissolved solids and colloids (Gagné *et al.*, 2007; Canesi *et al.*, 2012). Exposure of mussels to Cu^{2+} and nCuO revealed uptake in tissues when exposed at relatively high concentrations in mg/L range (Hanna *et al.*, 2014). The toxicity of Cu involves protein and DNA damage from oxidative stress and altered immune responses in mussels (Hu *et al.*, 2014). Nanoparticles also produce reactive oxygen species and oxidative stress, which is considered as one of the major toxic effects although these are not always explained by dissolved Cu^{2+}. This suggests that toxicity also results from other properties such as size, surface charge, reactivity and corona properties (Gagné *et al.*, 2007; Duran *et al.*, 2015). Recent investigations revealed that nanoparticles could induce inflammation from ingested particles and protein denaturation and turnover (Gagné *et al.* 2012; Hu *et al.*, 2014). However, it is not known whether inflammation and protein turnover were related or were the results from independent pathways. The origin of oxidative stress (i.e., from release of Cu^{2+} or inflammation) also needs further investigations. In this respect, the identification of specific toxic effects that could discriminate between nCuO and dissolved Cu^{2+} would be of value for the risk assessment and management of Cu-based products in order to track their effects in the environment.

The purpose of this study was therefore to examine and compare the toxicity of nCuO and Cu^{2+} to quagga mussel *Dreissena bugensis*. Toxicity was examined by following changes in oxidative stress (glutathione S-transferase and lipid peroxidation), inflammation (arachidonate cyclooxygenase), genotoxicity (DNA stand breaks), protein turnover (ubiquitin tagging to proteins) and neuromuscular activity by following acetylcholinesterase (AChE) activity. The null hypothesis consists in the statement that the toxicity of nCuO and Cu^{2+} is the same. An attempt was made to identify biomarkers that could discriminate between the effects of each Cu forms.

Materials and Methods

Copper oxide nanoparticle:

A stock solution of Copper oxide nanoparticle (nCuO) was obtained from US Research Materials (USA). This commercial nCuO suspension composed nanoparticles in the 25-55 nm diameter size range according to the supplier information that was confirmed Dynamic Light Scattering analysis as described below. For the exposure regime, quagga mussels (*Dreissena bugensis*) were exposed to increasing concentrations of either nCuO or Cu^{2+} as $CuSO_4$ in dechlorinated tap water (controls): 0, 2, 10 and 50 µg/L as total Cu. Controls consisted of aquarium water which was obtained from tap water from the city of Montreal after additional UV-treatment and charcoal filtration. The nanoparticle's size and Zeta potential of nCuO were determined in aquarium water using a dynamic light scattering instrument (Mobius Instrument, Wyatt Technologies, Santa Barbara, CA, USA) operating with a laser at a wavelength of 532 nm. The instrument was previously calibrated with standard suspensions of latex nanoparticles (Polyscience, USA).

The total levels of Cuin the exposure media were determined after 1 hr of dissolution of nCuO using ICP-MS spectrometry following acidification with 1% v/v nitric acid (Seastar grade BC, Canada). Quagga mussels (n = 20) were exposed to either nCuO or Cu^{2+} as $CuSO_4$for 96 h at 15 C under constant aeration. The exposure media were not renewed and the mussels were not fed during that time. After the exposure period, mussel mortality was checked and a subgroup of mussels (N=10) were kept aside for air-time survival assessment. For total Cu levels in mussels, a subgroup of 20 individuals were placed in clean aquarium water overnight as a depuration step and the mussels were removed for total Cu determination using ICP-mass spectrometry as described above. The tissues were first acid-digested with concentrated HNO_3, HCl and 30% H_2O_2 , then heat-digested in microwave vessels for 2 h and diluted with MilliQ water to a volume of 12 mL. For the biomarker analyses, the remaining group of 10 mussels were analyzed for total weight shell length, total and soft tissues weights. The soft tissues were quickly stored at -85 C with 5 volumes of homogenization buffer. The homogenization buffer consisted of 50 mM NaCl containing 2 mM KH_2PO_4, 1 mM $NaHCO_3$, 25 mM Hepes-NaOH, pH 7.4, 1 µg/mL apoprotininand 1 mM dithiothreitol.

Air survival test:

After the exposure period, 10 mussels were kept aside to determine the air-time survival as previously described (Gagné *et al.*, 2015). Briefly, mussels were randomly chosen and

placed individually into plastic plates under 80% humidity at 18 C. They were maintained as such and weighed each day until mortality was determined by shell opening. The time of death in days was determined for each individual over the 7 treatment groups: 1 control, 3 nCuO concentrations and 3 Cu^{2+} concentrations. The weight loss during air emersion at day 3 (the last day before manifestation of mortality) was also measured and the data were expressed as the ratio of weight loss (weight at given day/initial weight)

Biomarker assessments:

The soft tissues were allowed to thaw on ice for 15-20 min and homogenized still in melting ice using a Teflon pestle tissue grinder at 4 C. A portion of the homogenate was set aside for lipid peroxidation (LPO), DNA damage and total proteins. The remainder of the homogenate was centrifuged at 15000 x g for 20 min at 4 C and the supernatant (S15) was removed for arachidonate cyclooxygenase (COX), glutathione S-transferase (GST), acetylcholinesterase (AChE), labile Cu^{2+} and protein-ubiquitin level evaluations. Total proteins were determined in the homogenate and S15 fraction using the protein-dye binding principle using standard solutions of serum bovine albumin for calibration (Bradford, 1976).

Lipid peroxidation (LPO) was determined in soft tissue homogenates using the thiobarbituric acid method (Wills, 1987). A volume of 10 µL of the homogenate was mixed with 175 µL of 10% trichloroacetic acid containing 1 mM $FeSO_4$ and 50 µL of 0.7% thiobarbituric acid. The mixture was heated at 75 C for 10 min. The mixture was cooled to room temperature and centrifuged at 10 000 x g for 5 min to remove any precipitates. A 150 µL volume was transferred to a 96-well dark microplate, and fluorescence readings were taken at 540 nm excitation and 590 nm emission. Standard solutions of malonaldehyde (tetramethoxypropane, Sigma Chemical Company, ON, Canada) were prepared for calibration. Results were expressed as µg thiobarbituric acid reactants (TBARS)/mg total proteins in the homogenate. The levels of DNA strand breaks were also determined in the homogenate using the fluorescence DNA precipitation assay (Olive, 1988; Gagné *et al.*, 2008). Briefly, 20 µL of the homogenate from each tissues were mixed with 100 µL of 50 mM NaOH, 10 mM Tris base, 10 mM ethylenediamine tetraacetate and 2% sodium dodecyl sulphate (SDS) for 5 min and 120 µL of 0.12 M KCl was added. The mixture was heated at 60 C for 10 min, cooled on ice for 5 min and centrifuged at 8 000 x g for 10 min to precipitate SDS-associated protein and genomic DNA. The supernatant (DNA strands) was mixed with SYTO Green dye in 3 mM sodium cholate, 0.4 M NaCl and 100 mM Tris-acetate pH 8 to control for the traces of SDS in the supernatant which could interference with fluorescence readings (Bester *et al.*, 1994). Fluorescence was measured at 485 nm excitation and 530 nm emission (Microplate, Synergy-4, Bioteck, USA) using standard solutions of salmon sperm DNA for calibration. The data were expressed as µg supernatant DNA/mg proteins.

Levels of labile Cu^{2+} were determined using a fluorescent probe methodology (Udhayakumaria *et al.*, 2014). A 20 µL sample of the S15 fraction was mixed with 180 µL of 50 µM of 1,2-diaminoanthraquinone in 100

mM NaCl, 5 mM KH_2PO_4 and 10 mM Hepes-NaOH, pH 7.4. Fluorescence was measured at 400 nm excitation and 475 nm emission (Synergy-4, Biotek Instuments, USA) using standard solutions of $CuSO_4$ for instrument calibration. Data were expressed as relative fluorescence units (RFU)/mg proteins. The activity of arachidonate-dependent cyclooxygenase (COX) activity was determined in the S15 fraction of soft tissues using a fluorescence microplate reader (Gagné, 2014). The S15 fraction was mixed with 50 µM arachidonate, 2 µM of dichlrofluorescein and 0.1 µg/mL of horseradish peroxidase in 50 mM Tris-HCl, pH 8.0 and 0.05% Tween-20 for 20 min at 20 C in dark microplates. Fluorescence measurements at each 5-min were taken at 485 nm excitation and 528 nm emission (Synergy 4, Biotek Instruments, USA). The data were expressed as the increase in relative fluorescence units/min/mg proteins in the S15 fraction. Acetylcholinesterase (AChE) activity was determined in the S15 fraction using acetylthiocholine as the substrate analogue for acetylcholine. The formation of thiocholine were determined using the Ellman's reagent according to Gélinas *et al.* (2013). Standard solutions of reduced glutathione were used for calibration. The data were expressed as absorbance increase at 412nm/min/mg proteins. The activity of glutathione S-transferase (GST) activity were determined in the S15 fraction using a microplate spectrometric assay (Boryslawskyj *et al.*, 1988). The activity was determined using reduced glutathione and 2,4-dichlorodinitro-benzene as the chromophore substrate at 340 nm. The data were expressed as the increase in absorbance at 340 nm/min/mg total proteins in the S15 fraction. The levels of polyubiquitinylated proteins were determined by enzyme-linked immuno-sorbent assay (ELISA) in the S15 fraction as described in a previous study (Auclair *et al.*, 2019). Standards of polyubiquitin (Ub2-7, K48-linked, Enzo Life Sciences, Farmingdale, NY) or the S15 fraction were used to coat the microplate wells (Immulon-4). The antibody ubiquitin lys48-specific rabbit monoclonal antibody (clone Apu2; EMD Millipore, Billerica, USA) was diluted 1/2000 in phosphate buffered saline (140 mM NaCl, 5 mM KH_2PO_4 and 1 mM $NaHCO_3$, pH 7.4) containing 0.5 % albumin and was added to each wells. After incubation for 60 min, the wells were washed with 0.5% albumin and the secondary antibody (anti-rabbit Igg-linked with to peroxidase; ADI-SAB-300, Enzo, USA) diluted 1/5000 and incubated for another hour. After well washing, the activity of peroxidase was detected using a highly sensitive Chemiluminescence assay kit (Roche Diagnostics, QC, Canada). Data were expressed as ng of polyubiquitin/mg proteins.

Data analysis:

The study design examines the bioavailability and toxicity of nCuO and Cu^{2+} in quagga mussels where the null hypothesis is that the bioavailability and toxicity of both forms of Cu are identical. In this study, there were in total 7 treatments: mussels exposed to aquarium water only (controls); mussels exposed to 3 concentrations Cu as nCuO in aquarium water; and mussels exposed to 3 concentrations of Cu as $CuSO_4$ in aquarium water. Data normality and homogeneity of variance were determined using the Shapiro-Wilk and Bartlett tests, respectively. The influence of exposure concentration and Cu forms (control, nCuO and Cu^{2+}) were examined using

2-way factorial analysis of variance. Critical differences between treatments were determined using the Least Square Difference (LSD) test. The trends between the data were also analyzed using the Pearson moment correlation test. The biomarker data were also analyzed by discriminant function analysis to determine which biomarkers best discriminate between the forms of Cu. Significance was set at $p < 0.05$. All statistical analyses were performed with the SyStat software package (version 13.2, USA).

Results

The levels of Cu in solution were determined in the aquarium water after 1 h of dissolution of nCuO (Table 1). For nCuO, 90% of Cu was detected in the MilliQ water with a mean diameter of 79 nm with a Zeta potential of -15 mvolts. In the aquarium water, 70% of the Cu was detected with no changes in the mean diameter and Zeta potential for nCuO. The mussel weigth/shell length and soft tissues ratios were not significantly affected by either $Cu^{2+}$and nCuO (data not shown). The same was observed for air-time survival and weight loss after 4 days suggesting that no important systemic effects and survival was caused by exposure to 2-50 µg/L total Cu as either Cu^{2+} and nCuO. Air-time survival was significantly correlated with weight loss at day 4 (r=-0.59) indicating a stronger initial weight loss at the third day was associated with lower air survival time in the mussel samples. The levels of total Cu and labile Cu^{2+} were determined in soft tissues in mussels exposed to each Cu forms (Fig. 1). For total Cu in soft tissues, the levels changed over the exposure concentrations used (ANOVA $p<0.01$). The total Cu levels were significantly increased in mussels exposed to dissolved Cu^{2+} at 10 and 50 µg/L Cu compared to controls. Cu levels were also significantly higher in the 10 µg/L Cu^{2+} group compared to the 10 µg/L nCuO group. For labile Cu levels, the only significant effect was a decrease in labile Cu in gills in mussels exposed to 50 ug/L of Cu^{2+}.

Oxidative stress and inflammation were examined in mussels exposed to each forms of Cu by following changes in GST and COX activities, respectively (Fig. 2). GST activity was not significantly changed by exposure concentrations and Cu forms (2-way factorial ANOVA $p>0.05$ for concentration and Cu forms). COX activity was significantly influenced by concentrations and forms of Cu as determined by 2-way ANOVA. COX activity was higher in mussels exposed to 10 µg/L nCuO compared to controls or to 10 ug/L Cu^{2+} group. GST activity was significantly correlated with soft tissues ratio (r=-0.30) and LPO levels with COX activity (r=-0.32).

Table 1: Physico-chemical properties of nCuO in the exposure media

Parameter	MilliQ water	Aquarium water
Cu concentration	200 ug/L (nCuO)	200 ug/L (nCuO)
% remaining	90%	70%
Mean diameter (nm)	79 ± 10	74 ± 5
Zeta potential (mvolt)	-15±5	-13 ± 3

Tissue damage was examined at the protein (polyubiquitinylation), lipids (LPO) and DNA levels (Fig. 3). Protein-ubiquitin level was significantly increased in mussels

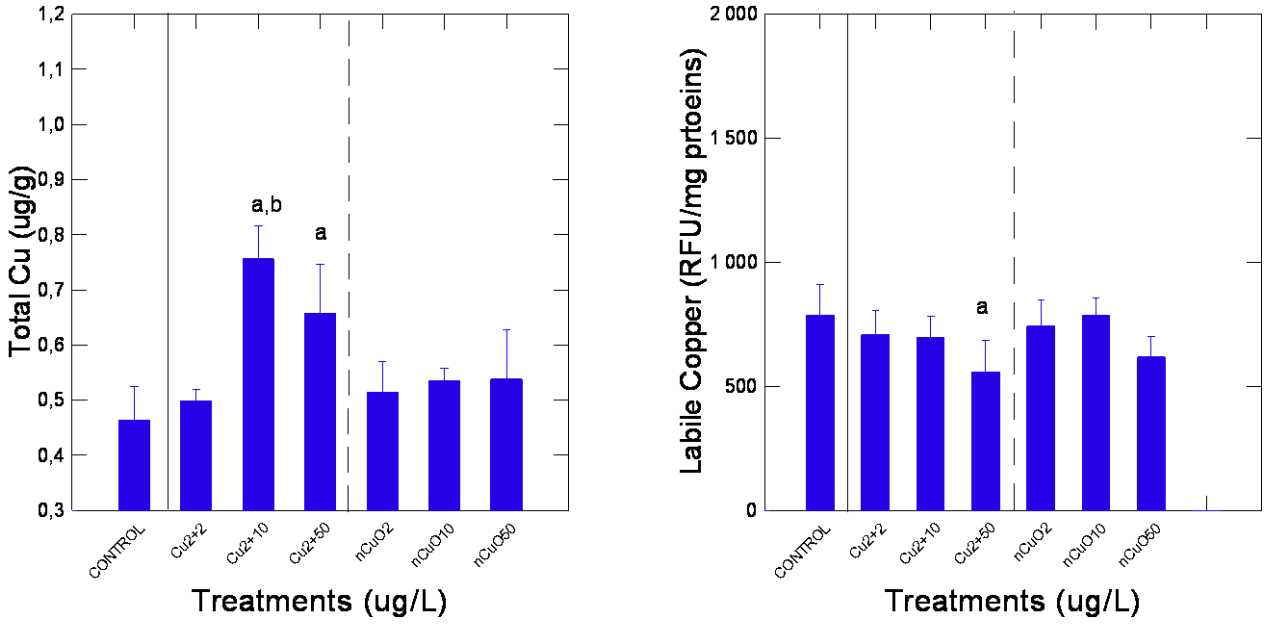


Fig. 1: Tissue levels of labile Cu^{2+} in mussels exposed to nCuO and dissolved Cu.
a and b indicate significance from control and between the forms of Cu (at the same concentrations).

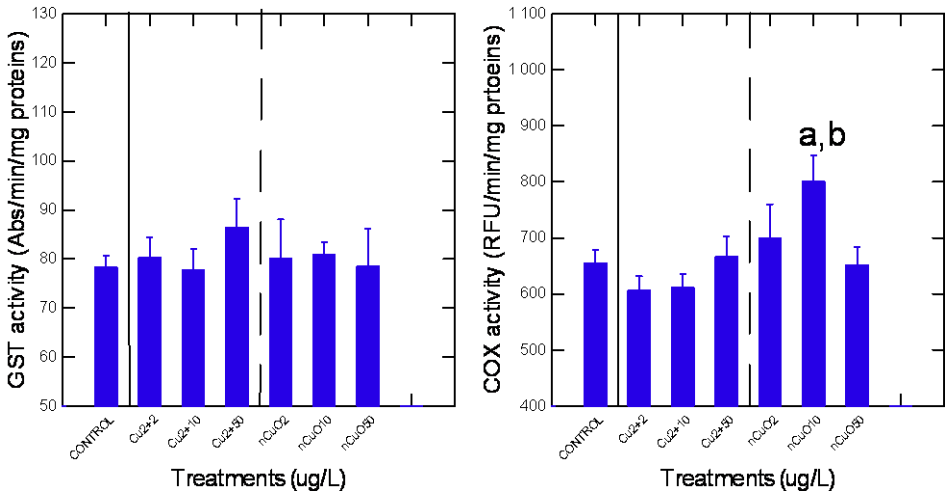


Fig. 2: Oxidative stress in mussels exposed to Cu forms.
a and b indicates significance from control and between the forms of Cu (at the same concentrations).

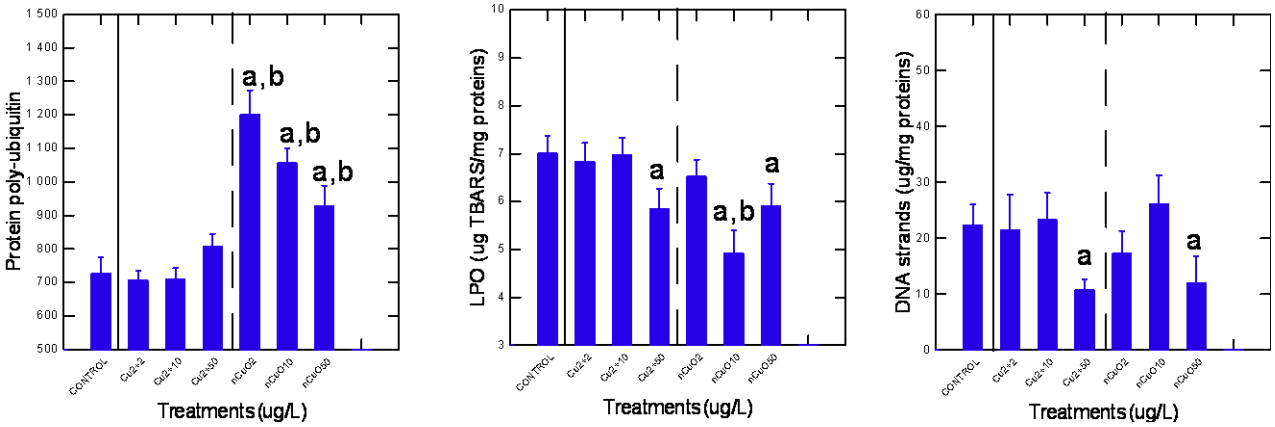
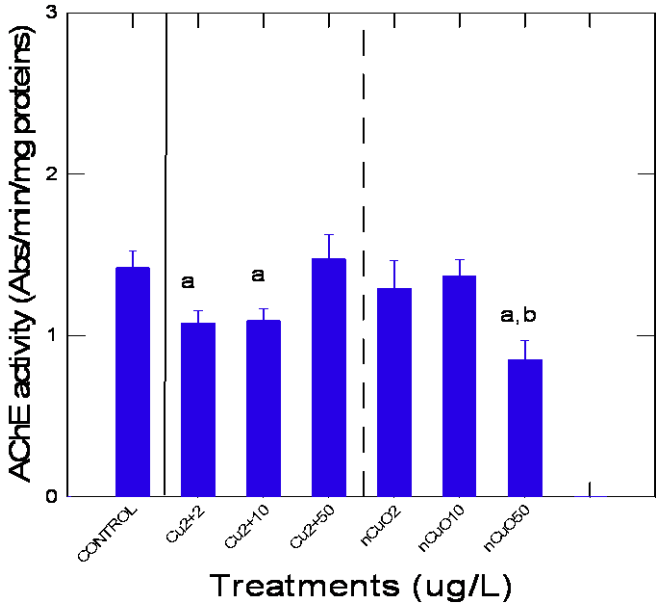


Fig. 3: Biomarker of tissue damage in mussels exposed to Cu forms.
Tissue damage was determined by following changes in poly-ubiquitinylated proteins, LPO and DNA damage. a and b indicates significance from control and between the forms of Cu (at the same concentrations).

exposed to nCuO only in respect to control or to the equivalent Cu^{2+} concentration. Protein ubiquitin levels were significantly correlated with Cox activitiy (r=0.61). LPO levels were generally decreased by either dissolved or nanoparticulate Cu with nCuO decreasing LPO levels at lower concentrations than Cu^{2+} (10 instead of 50 µg/L). Cu levels were significantly correlated with LPO levels (r=0.48). The levels of DNA strand breaks were also determined in mussels treated to Cu forms and concentrations. DNA strand breaks were significantly decreased in mussels exposed to 50 µg/L Cu^{2+} and nCuO. DNA strand breaks were also significantly correlated with total Cu levels (r=-0.34). The activity in AChE was also determined in mussels exposed to either forms of Cu concentrations (Fig. 4). AChE activity was significantly decreased in mussels exposed to 10 and 50 ug/L nCu). AChE activity was also somewhat decreased in the first 2 concentrations of dissolved Cu^{2+} compared to the controls but did not differed from nCuO at the corresponding concentrations. Correlation analysis revealed that AChE was significantly correlated with COX (r=-34) and GST (0.59) activities.

Fig. 4: Change in acetylcholinesterase activity in mussels exposed to both forms of Cu.
a and b indicates significance from control and between the forms of Cu (at the same concentrations).



In the attempt to gain a global view on the mussels' response to each forms of Cu, a discriminant function analysis was performed (Fig. 5). The analysis revealed that all the variance was explained with a mean classification performance of 92 % indicating

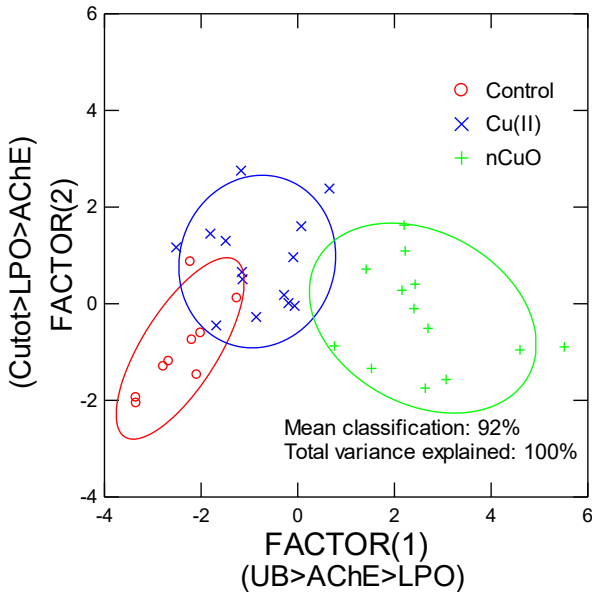


Fig. 5: Multivariate analysis of biomarker data. Discriminant function analysis was performed on the biomarker data. The effects from nCuO were discriminated (92 % classification; 100 % of the variance explained) from the observed effects of Cu^{2+} and controls.

that the effects between controls, Cu^{2+} and nCuO could be discriminated with each other at the sublethal level. The biomarkers more closely associated were protein ubiquitin, AChE, LPO and total Cu levels in mussels. The effects between nCuO and Cu^{2+} were best separated by protein ubiquitin, AChE and LPO levels. Given that protein-ubiquitin levels were strongly correlated with COX activity (r=0.61), this activity was also able to respond more closely to nCuO.

Discussion

Based on the Cu bioaccumulation data, the Cu loadings were significantly increased in mussels exposed to dissolved Cu^{2+} only. Mussels exposed to low concentrations of nCuO did not display significant Cu bioaccumulation in tissues. However, nCuO was reported as bioavailable on Swan mussels *Anodonta cygnea* exposed to 0.25, 2.5 and 25 ug/L as total Cu for 12 days (Moezzi *et al.*, 2019). The foot accumulated more Cu than the mantle with bioaccumulation factors of 5.4 and 3.25, respectively. Hence, the accumulation of nCuO in mussels could be related to the size, exposure time and species which prevent us to generalize bioavailabitly of nCuO with only one test species. Another factor is that tissue analyses were performed in the whole soft tissues (i.e., not in specific tissues) because of the small size of quagga mussels compared to Swan mussels. In another study with marine mussels exposed to nCuO, mussels were able to ingest and effectively excrete the nanoparticles which could explain the lack of total Cu bioaccumulation in tissues when exposed to low concentrations of nCuO (Hanna *et al.*, 2014). Notwithstanding this, exposure to a higher concentration of 3 mg/L nCuO led to bioaccumulation factor of 26. There were no changes in labile Cu^{2+} in mussels exposed to either forms of Cu suggesting that Cu was readily (strongly) bound in mussel tissues. Cu^{2+} binds strongly to thiol-containing proteins such as metallothioneins (MT) which is induced by divalent heavy metals (Farrell *et al.*, 1993). However, if the induction of MT is sufficient to prevent the spill-over of Cu ions to the intracellular protein pool, the increase in labile Cu would be limited. Based on the present data, no increase of labile Cu^{2+} was observed which suggests no spill-over of Cu occurred. This was corroborated by the lack of correlations between labile Cu^{2+} and LPO or GST. LPO was actually reduced by either Cu^{2+} or nCuO which suggests a general reduction of ROS production in tissues in mussels exposed to low concentrations of Cu. In a previous study, exposure of mussels to nCuO could led to induction of MT suggesting that Cu^{2+} was released or that ROS production increased

(Gomes *et al.*, 2011). Indeed, both forms of Cu was able to induce MT in the Mediterranean mussels which suggests that nCuO is instable in cells and releases ionic Cu in the process. However, the toxicity of nCuO differed from Cu^{2+} in mussels in this study.

Table 2: Correlation analysis of biomarker data

	CF	SFT	Air survival	Weight loss	LPO	DNA	COX	UB	AChE	GST	Cu^{2+}
SFT	-0.13	1									
Air Survival	-0.25	0.08	1								
Weight loss	0.01	-0.27	**-0.59**	1							
LPO	0.07	**0.32**	0.15	-0.08	1						
DNA	-0.06	0.07	0.15	0.09	0.03	1					
COX	**-0.32**	-0.22	0.1	0.18	**-0.33**	0.16	1				
UB	-0.23	-0.04	-0.05	0.12	-0.1	0.01	**0.61**	1			
AChE	-0.01	-0.12	-0.04	0.03	-0.02	0.17	0.34	0.03	1		
GST	0.1	**-0.3**	-0.03	-0.11	-0.25	-0.23	0.20	0.01	**0.59**	1	
Cu2+	0.03	-0.13	-0.09	-0.02	0.07	0.03	0.09	0.06	-0.1	0.1	1
Total Cu	-0.18	0.17	-0.11	-0.11	**0.48**	**-0.34**	0.08	0.08	-0.1	-0.2	0.01

Significant correlations are indicated in bold

According to the discriminant function analysis data, the toxicity of nCuO differed from those of Cu^{2+} and controls. This is especially of interest since the observed effects occurred without any changes in total Cu loadings in mussel tissues. Indeed, protein-ubiquitin levels and COX activity were specifically influenced by nCuO but not by Cu^{2+}, which suggest that nanoparticles were more specifically linked to the inflammatory and protein turnover (proteasome-ubiquitin protein removal) pathways. Interestingly protein-ubiquitin levels were significantly correlated with COX activity (r=0.61) which suggests that these endpoints were not only specific to nCuO but related with each other. A transcriptomic analysis was performed in blue mussels exposed to nCuO and revealed similar effects with Cu^{2+} although some specific changes with nCuO were observed (Châtel *et al.*, 2018). Indeed, nCuO produced stronger changes in genes involved in protein assembly-disassembly, reaction oxygen species removal (potentially from COX activity), metallothionein (Cu^{2+} sequestration) and energy production (ATP synthase production). The gene expression data revealed that the observed effects of nCuO exposure could not be explained by the release of Cu^{2+}. A comparative study of the effects of nCuO and Cu^{2+} on the Mediterranean mussel *Mytulis galloprovincialis* revealed similar and other changes at the proteomic level (Gomes *et al.*, 2014). Nanoparticulate Cu produced marked effects on GST (oxidative stress), protein degradation (cathepsin L) and apoptosis while Cu^{2+} affected hemocyte proteins involved in motility and adhesion and precollagen D which could be involved in inflammation. This study also concluded that the toxicity of nCuO was not solely due to Cu^{2+}. The effects of nCuO and Cu^{2+} to the immune system were also reported in mussel hemocytes (Katsumiti *et al.*, 2018). Increased phagocytosis and reactive oxygen species production were induced only by nCuO which suggest a “particle effect” and inflammation. In marine mussels exposed to nCuO and Cu^{2+}, the

latter caused significant Cu accumulation in gills and the hemolymph (Torres-Duarte *et al.*, 2019). Although nCuO did not cause accumulation of Cu in tissues, damage to gill tissues and ingestion by hemocyte were observed. Decreased phagocytosis of pathogenic bacteria *Vibrio tubiashii* was also observed in hemocytes which could render them more susceptible to bacterial infection. Inflammation and decreased AChE activity in muscle in fish chronically exposed to (1-3 mg/L) nCuO was observed (Mani *et al.*, 2019). Decreased AChE was also observed in the mussel *Mytilus galloprovincialis* to 10 µg/L for 15 days (Gomes *et al.*, 2011). AChE activity was also reduced in mussels exposed to 10 µg/L Cu^{2+} which suggests that the effects were mediated, at least in part, by dissolved Cu. Decreased AChE activity was also observed at the highest concentration (500 µg/L) of nCuO in the present study. This suggests that neuro-muscular activity could be affected by the nanoparticles perhaps through interference at the protein (F-actin) assembly-disassembly level.

Another specific effect of nCuO that was not observed with dissolved Cu^{2+}, in addition with COX activity, was ubiquitin tagging of proteins which suggest increased turnover of damaged proteins. Moreover, protein-ubiquitin levels were significantly correlated with COX activity indicating that the damage also involved inflammation. The specific effect (nanoparticle but not the dissolved component) was also observed in freshwater mussels *Elliptio complanata* exposed to nanosilver (Gagné *et al.*, 2013). Increased protein ubiquitinylation was also observed in marine mussels *Mytilus galloprovincialis* exposed to iron oxide nanoparticles (Taze *et al.*, 2016). In addition to this specific effect, increased LPO and DNA damage were also observed. This suggests that metallic and oxide nanoparticles could alter the dynamic space of proteins where denatured proteins are tagged by ubiquitin for elimination via autophagosomes. Altered proteins aggregates, which are composed of ubiquitinylated proteins (synuclein), lead to the formation of Lewy bodies in the brain of zebrafish larvae exposed to titanium dioxide nanoparticles (Hu *et al.*, 2017). The formation and accumulation of Lewy bodies are often observed in neurodegenerative disease such as Parkinson's disease. Increased protein aggregation was recently observed in mussel exposed to "inert" polystyrene nanoparticles which was associated with changes in the fractal organization of cytoplasmic proteins (Auclair *et al.*, 2020). This is consistent with the notion that nanoparticles contaminate the space domain of macromolecules, which can change the cytoplasmic organization in cells. Ubiquitin could also form a corona on various inorganic nanoparticles and contribute to the alteration of this signaling pathway in protein removal and turnover (Duran *et al.*, 2015). A distinct protein-corona structure at the surface of the nanoparticles was shown to influence resulting effects such as oxidative stress, inflammation, endocytosis and cytotoxicity. This is keeping with studies showing specific toxicity of nCuO that were not explained by Cu^{2+} (Sun *et al.*, 2017). Nanoparticles could exacerbate the production of reactive oxygen species leading to inflammation, altered protein conformation and cytotoxicity compared to the equivalent dissolved ions counterpart.

Conclusion

The bioaccumulation and toxicity of nCuO and Cu^{2+} were investigated in freshwater mussel.

Mussels exposed to dissolved Cu^{2+} accumulated Cu in tissues but not with nCuO exposed mussels. On the one hand, both forms of Cu cause similar changes in DNA strand breaks, LPO and AChE activity. On the other hand, even though accumulation of Cu was not observed in mussels exposed to low concentrations of nCuO, specific effects were observed at the inflammation and protein-ubiquitin levels. The null hypothesis that Cu toxicity is the same for nanoparticulate and dissolved Cd is therefore rejected.

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