ISJ 18: 33-45, 2021 ISSN 1824-307X

RESEARCH REPORT

The influence of surface waters on the bioavailability and toxicity of copper oxide nanoparticles to freshwater mussels

J Auclair, P Turcotte, C Gagnon, F Gagné*

Aquatic Contaminants Research Division, Environment and Climate Change Canada, 105 McGill, Montréal, Québec, Canada H2Y 2E7

 Accepted January 22, 2021

Abstract

The increased commercial use of copper oxide nanoparticles (nCuO) led to the release of nanoparticles in wastewaters potentially harming the aquatic biota. The purpose of this study was to determine the toxic action of nCuO and dissolved Cu (II) to *Dreissena bugensis* freshwater mussels placed in 4 types of surface waters: aquarium, green (high conductivity), brown (high organic carbon) and 10 % municipal effluent (high conductivity and anthropogenic source of organic carbon). Mussels were exposed to 50 µg/L of nCuO or Cu (II) for 96 h at 15 °C in the above waters. The results revealed that the total Cu loadings were higher in mussels placed in organic-rich waters (brown and effluent) and exposed to either forms of Cu. Tissue Cu contents were correlated with air-time survival, lipid peroxidation, protein-ubiquitin levels and DNA strand breaks. Both surface water types and Cu forms influenced Zn (II) mobilization, glutathione S-transferase activity and protein turnover (ubiquitin binding). Based on the surface water properties, Cu (II) was more influenced by the levels and origin of the organic carbon content while nCuO was more influenced by the total suspended solids. In conclusion the toxicity of nCuO could be influenced by surface waters properties especially when similar physiological targets are impacts by these treatments.

Key Words: Quagga mussels; surface waters; copper oxide nanoparticles; oxidative stress; ubiquitin

Introduction

The exponential commercial application of nanoparticles have lead to the mobilization of nanoparticles in the environment. Nanoparticles are currently used in various consumer products because of their new properties (Lee *et al*., 2010). The global production of nanoparticles is estimated in the order 320 metric tons (Keller and Lazareva, 2013). The continuous release of metallic nanoparticles could reach levels in the environment that exceed the toxic threshold in aquatic organisms (Garner *et al.,* 2017). Given their intrinsic properties, metal oxide nanoparticles that are produced at large scales are cerium, titanium, zinc and copper oxide nanoparticles (nCuO) where zinc oxide nanoparticles and nCuO are the most commonly used metal-based nanoparticles. These metal oxide nanoparticles are poorly soluble and subject to aggregation when the surface charges are neutralised by salt or other conterions (Liu *et al.*, 2018). Conversely, in the presence of natural organic matter (humic and fulvic acids), an increased proportion of monomeric nanoparticles could be observed (Keller *et al.*, 2010). The predicted environmental concentration of nCuO in municipal wastewaters was estimated between the µg/L to mg/L range based on modeling approaches (Musee *et al*., 2011). The levels of metal oxides nanoparticles could reach values well above the mg/L range (Walden and Zhang, 2016).

The toxicity of nCuO to aquatic residents such as bacteria, algae, invertebrates and fish has been examined in numerous studies (Gomes *et al.*, 2011; Isani *et al.*, 2013; Adam *et al.*, 2015). While the acute and chronic lethality of nCuO are in the mg/L range, which are not expected to occur often in the environment. Effects at molecular level were shown to occur at concentrations in the µg/L range which is of concern in the context of long-term exposure to these nanomaterials (Buffet *et al.*, 2011; Cuillel *et al.*, 2014; Giannetto *et al.*, 2019). Dissolved Cu (II) are more acutely toxic to rainbow trout (96 h LC50=15 µg/L) indicating that the nanoforms of Cu oxides are much less toxic (Marr *et al*., 1999). The

Corresponding author:
François Gagné
Aquatic Contaminants Research Division
Environment and Climate Change Canada
105 McGill, Montréal, Québec, Canada H2Y 2E7
E-mail: francois.gagne@canada.ca

96 h toxicity of nCuO for the copepod *Tigriopus fulvus* was at 12 mg/L with sublethal effects (copepod moult and sea urchin egg fertilization) at 2 mg/L (Rotini *et al.*, 2018). The study also found that in the presence of sea water (salt-rich), only 0.16 % of nCuO were in the dissolved phase. The toxicity also depends on the environmental conditions, which could influence the dissolution, aggregation, surface charge states of the nanoparticles. Bivalves are species particularly at risk to suspended particle in the water column since they are filter-feeders and sessile organisms. The gills filter water to trap suspended material (nanoparticles and their aggregates) which are then directed to the digestive system for assimilation. The toxicity of nanoparticles in bivalves species has been extensively examined over the years (Gagné *et al.*, 2007; Canesi *et al.*, 2012; Rocha *et al.*, 2015). These studies revealed the release of elements from the nanoparticle is an important driver of toxicity but also by other characteristic properties of the nanoparticles such as size, form, surface charges and coatings. Nanoparticles and their aggregates were shown to accumulate in the digestive gland and not only producing oxidative stress but inflammatory responses as well in addition to cellular injury to membrane, proteins and DNA. However, it was not determined whether the nature of freshwater could also influence exposure and effects of nanomaterials in mussels. Indeed, in high salt conditions, the surface charge (Zeta potential) could be canceled out and favor aggregation mechanisms which could be trapped in the digestive gland and initiate inflammatory/immune responses. Conversely, the presence of natural organic matter could act as surfactants and favor dissolution of nanoparticles which favor transfer in cells (Dominigo *et al.*, 2009). These co-occurring mechanisms complicate the prediction of nanoparticle state in surface waters, which could in turn, influence bioavailability and toxicity in organism. Indeed, the influence of water conductivity, the nature (natural, municipal, agricultural) and levels of total organic carbon is less understood at the present time. A recent review was recently published solely on the influence of organic matter on the state of engineered nanoparticles (Grillo *et al.*, 2015). Natural organic matter are mainly composed of humic and fulvic acids while organic matter from urban areas (effluents) is mainly composed of protein compounds from human/animal wastes.

The purpose of this study was therefore to determine the influence of surface water properties and form of Cu (nCuO or Cu (II)) on the bioavailability and toxicity in freshwater mussels *Dreissena bugensis*. In this study, 4 types of freshwater were examined in mussels: aquarium (dechlorinated tap water), high conductivity/low total organic carbon (TOC) water (green waters), low conductivity/high TOC water (brown water) and a diluted municipal effluent in aquarium water as another source of organic matter. Toxicity was examined by following changes in oxidative stress, inflammation, genotoxicity and turnover of damaged proteins in the soft tissues of mussels. An attention was given on the combined influence of Cu forms (dissolved vs nanoparticles) and surface water properties towards bioavailability and toxic effects in freshwater mussels.

Materials and Methods

Copper oxide nanoparticle

A stock solution of copper oxide nanoparticle (nCuO) from US Research Materials (USA) was used in this study. According to the manufacturer's specifications, the size distribution of nCuO suspension was between 25-55 nm according to the supplier information. For the exposure regime, quagga mussels (*Dreissena bugensis*) were exposed to a nominal concentration of 50 µg/L total Cu as either nCuO or Cu (II) as CuSO4 in dechlorinated tap water (controls), and three other types of surface water as described below. This concentration range was selected based on previously reported total copper concentrations in municipal effluents (10-50 µg/L) (Gagnon *et al*., 2014; Dietler *et al*., 2019). The hydrodynamic diameter and zeta potential of nCuO were measured at least three times 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 latex beads at 50 nm diameter (Polyscience, USA).

Two types of natural water samples were sampled in the St. Lawrence River: green and brown waters (20 L each). The aquarium water consisted of UV and charcoal-treated dechlorinated tap water and was considered as the reference water. A 10 % dilution of a physico-chemically (primary) treated municipal effluent from a city of 2 million inhabitants was also prepared in aquarium water to simulate a water sample with same total organic carbon content (TOC) than green and aquarium waters but of different (anthropogenic) origin. In each of water samples, TOC, total suspended solids (TSS), pH and conductivity were determined according to standard methods (APHA, 2010). Green water is characterized by a relatively high conductivity (200-250 µS/cm) and TSS, low TOC (< 4 mg/L), and a slightly alkaline pH (pH > 7.2) (Table 1). Brown water is characterized by low

Table 1 Surface water characteristics data

Water type	TOC (mg/L)	Conductivity µScm-1	Total Suspended Solid (mg/L)	pH
Aquarium	3.1 ± 1	297 ± 30	< 1	8.1 ± 0.5
Green	3.4 ± 1	245 ± 20	19 ± 2*	8.3 ± 0.5
Brown	7.3 ± 2*	108 ±10*	4 ± 0.5*	7.5 ± 0.5
Effluent	3.2 ± 0.75	319 ± 30	2 ± 0.2*	8.2 ± 0.5

* significant from aquarium water

conductivity (100-160 µS/cm) and TSS, higher TOC levels (> 5 mg/L) and a slightly acidic pH (pH < 8). The surface waters (20 L) were collected 2-3 weeks before the onset of exposure; they were stored in the dark at 4 °C until the exposure experiments at 15 °C. The total levels of Cu in the 4 surface waters (green, brown, 10 % municipal effluent and aquarium) were determined before and after 1 h of dissolution of nCuO using ICP-MS spectrometry following acidification with 1 % v/v nitric acid (Seastar grade BC, Canada). Quagga mussels (n = 30) were exposed to each type of water with and without 50 µg/L total Cu as either nCuO or Cu (II) as $CuSO_4$ for 96 h at 15 °C under constant aeration. Control mussels were exposed to each type of surface water only with no addition of either forms of Cu. The exposure experiment was repeated 2 times. The surface waters were not renewed and the mussels were not fed during the exposure period. A subgroup of 10 mussels was kept aside for air-stress survival while the remaining 20 mussels were processed as follows. For Cu assessments in mussels, a subgroup of 10 individuals were placed in clean aquarium water overnight as a depuration step to remove loosely bound Cu at the surfaces of the mussels. The soft tissues were removed for total Cu determination using ICP-mass spectrometry as described above. The tissues were acid-digested in 10 % HNO_3 at 70-80 °C for 12 h and diluted to 1 % with MilliQ water. For the biomarker analyses, the remaining group of 10 mussels were analyzed for shell length, total weight and soft tissues weight. The soft tissues were then stored at -85 °C with the homogenization buffer (at 20 % weight/volume). The homogenization buffer consisted of 100 mM NaCl, 25 mM Hepes-NaOH, pH 7.4, 1 µg/mL apoprotin and 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). In brief, the mussels were weighed and the shell length determined, and they were placed individually into plastic containers under 80 % humidity at 20 °C. They were maintained as such and weighed each day until mortality (opened shells). The time of death in days was determined for each individual over the 12 treatment groups: 4 types of surface water alone, 4 types of surface water with nCuO and 4 types of surface water with nCu (II). The weight loss during air emersion were also measured and the data were expressed as the ratio of weight loss: (initial weight-weight at given day)/initial weight.

Biomarker analyses

The soft tissues were allowed to thaw on ice for 30 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) evaluation, glutathione S-transferase (GST), acetylcholinesterase (AChE), labile zinc (Zn) and protein-ubiquitin levels. 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 25 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 10000 x g for 5 min to remove any precipitates. A 200 µL volume was transferred to a 96-well dark microplate, and fluorescence readings were taken at 540 nm excitation and 600 nm emission. Standard solutions of malonaldehyde (tetramethoxypropane, Sigma Chemical Company, ON, Canada) were made for calibration in the homogenization buffer. 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, 25 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). After 5 min, one volume of 0.12 M KCl was added and heated at 60 °C for 10 min. The mixture was cooled on ice for 10 min and centrifuged at 8000 x g for 10 min to precipitate SDS-associated proteins 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 the 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 Zn were determined using a fluorescent probe methodology (Gagné and Blaise, 1996). Briefly, a 20 µL sample of the S15 fraction was mixed with 180 µL of 50 µM of TSQ probe in 20 % DMSO, 140 mM NaCl, 5 mM KH_2PO_4 and 10 mM Hepes-NaOH, pH 7.4. Fluorescence was measured at 370 nm excitation and 490 nm emission (Synergy-4, Biotek Instuments, USA) using standard solutions of $ZnSO_4$ 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). Briefly, 25 µL of the S15 fraction was added to 175 µL of the assay mixture composed of 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. The reaction was allowed to proceed for 20 min at 20 °C and fluorescence readings were made at each 5-min interval at 485 nm excitation and 528 nm emission

in dark microplates (Synergy 4, Biotek Instruments, USA). The data were expressed as the increase in relative fluorescence units/min/mg proteins in the S15 fraction. The activity of acetylcholinesterase (AChE) activity was determined in the S15 fraction using acetylthiocholine as the substrate. The formation of thiocholine were determined using the Ellman's reagent according a previously published methodology (Gélinas *et al.*, 2013). Standard solutions of reduced glutathione were used for calibration. The data were expressed as RFU/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-dichlorodinitrobenzene 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 S_{15} fraction. The levels of polyubiquitinylated proteins were determined by enzyme-linked immunosorbent 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 influence of surface waters on two chemical forms of Cu -(nCuO and Cu (II)- in terms of toxicity in quagga mussels. There were 12 treatments: mussels exposed to each type of water: aquarium, brown, green, effluent 10 % alone (treatments 1-4); mussels exposed to 50 µg/L Cu as nCuO in each type of water (treatments 5-8); and mussels exposed to 50 µg/L of Cu as $CuSO_4$ in each type of water (treatments 9-12). In each treatment, 10 mussels were used for air-time surivival, biomarkers and chemical analysis for a total of N=30 mussels per treatment and the experiment was repeated twice. Data normality and homogeneity of variance were verified using the Shapiro-Wilk and Bartlett tests respectively. The influence of surface water types (aquarium, green, brown and 10 % effluent) and Cu forms (nCuO and Cu (II)) were examined using 2-way factorial analysis of variance. Critical differences between treatments were determined using the Least Square Difference (LSD) test. Relationships between the biomarker data were also analyzed using the Pearson moment correlation test. The data were also analyzed by discriminant function analysis to determine which biomarkers could discriminate between the influence of surface waters and forms of Cu in mussels. Significance was set at $p<0.05$. All statistical analyses were performed with the SysStat software package (version 13.2, USA).

Results

The surface water physico-chemical properties were determined (Table 1). In respect to total organic carbon (TOC), brown waters had the highest level at 7.3 mg/L compared to green (3.4 mg/L), diluted municipal effluent (3.2 mg/L) and aquarium waters (3.1 mg/L). Although the levels of TOC in green waters were similar to the diluted municipal effluents sample, the nature differed in the following manner: the TOC in green water is of natural origin (coined natural organic matter) while of municipal wastewater origin in the diluted municipal effluent. In respect to conductivity, the municipal effluent and aquarium waters had the highest conductivity at 319 and 295 $\mu S*cm^{-1}$ respectively compared to green (245 $\mu S*cm^{-1}$) and brown waters (108 $\mu S*cm^{-1}$). In respect to total suspended solids (TSS), green waters had the highest value (19 mg/L) compared to brown (4 mg/L) and municipal effluent (2 mg/L). Aquarium waters had no measurable levels of TSS. The pH was statistically the same for all water types. Based on these properties, the influence of the origin of TOC could be resolved by comparing effects between green and diluted municipal effluent. Effects caused by an increase in TOC of natural origin or by a decrease in conductivity (effect in the levels of TOC and conductivity) could be estimated between responses of green and brown waters. The influence of TSS could be appraised by comparing the responses between aquarium and green waters. The behavior of nCuO in the various water types was also analyzed using DLS (Table 2). Dilution in

Table 2 Behavior of nCuO in the water samples

Water sample	nCuO concentration	Mean diameter 1h (nm)	Zeta potential 1h (mvolts)	Mean diameter 48h (nm)	Zeta potential 48h (mvolts)
MilliQ water	200 ug/L	79 ± 10	Not analyzed	80 ± 10	Not analyzed
Aquarium	200 ug/L	74 ± 5	-13 ± 3	132 ± 46[2]	-19 ± 4
Green water	200 ug/L	100 ± 36	-20 ± 3	133 ± 18	-14 ± 2
Brown water	200 ug/L	69 ± 8	-14 ± 4	140 ± 66	-19 ± 4
Effluent 10 %	200 ug/L	92 ± 26	-14 ± 4	220 ± 80[1,2]	-14 ± 3

1. Significant from MilliQ water; 2. Significant compared to 1h

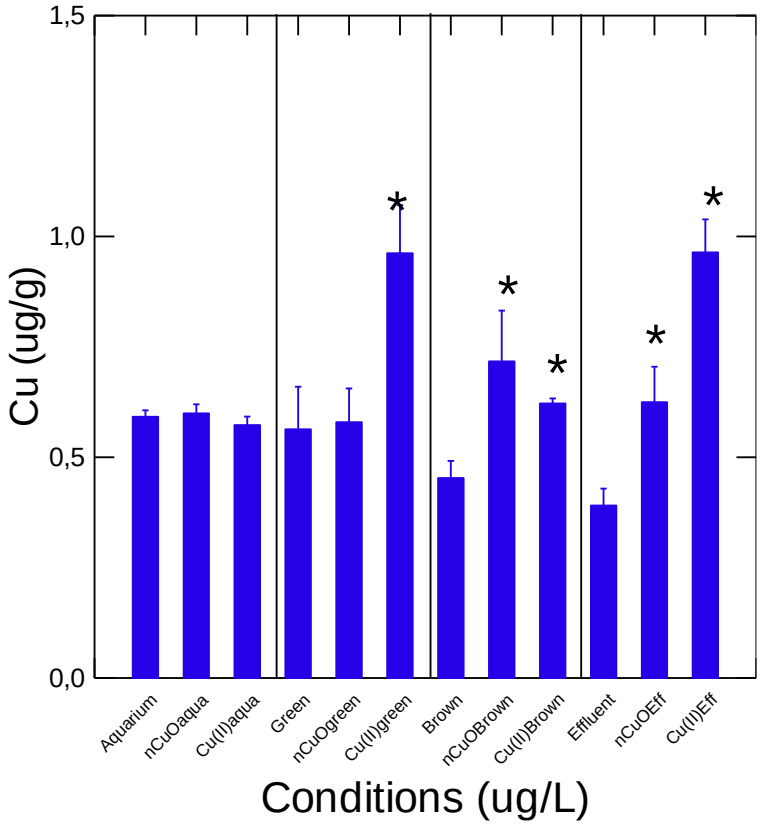

Fig. 1 Cu levels in mussels exposed to Cu forms and surface water types. Total Cu levels were measured in soft tissues of mussels following a 12 h depuration period in clean aquarium water. The data represent the mean and standard deviation expressed as ug metal/g dry weight. The letters a, b, c and d represents significance in the following: a difference between water control types with no added Cu (water type effect); b difference between the forms of Cu (Cu (II) and nCuO) from aquarium water (reference water); c difference between the forms of Cu (nCuO or Cu (II)) from corresponding water type control (e.g., nCuo-green vs green); d difference between forms of Cu within each water type (e.g., nCuO brown vs Cu (II) brown)

MilliQ water produced no change in the mean diameter in respect to the supplier's specifications. After dissolution for 1 h in the 4 types of water, the mean diameter (70 nm) did not significantly change between the water types although significantly more variation in the size distribution was observed especially for green and diluted municipal effluent waters. After 48 h, the mean diameter of nCuO in MilliQ water did not significantly changed. In the diluted municipal effluent, the mean diameter of nCuO was significantly higher than aquarium water and with the mean diameter after 1 h of dissolution in the same water. The mean diameter in aquarium water was also significantly increased at 48h (132 nm) compared to 1 h (74 nm) in aquarium water. No significant changes were observed for the Zeta potential between the water types and between 1 and 48 h in solution.

The total Cu levels were determined in tissues of mussels following a 12 h depuration period (Figure 1). In respect to water controls, Cu-levels in tissues were significantly lower in mussels placed in brown and the diluted municipal effluent compared to aquarium water controls. The data revealed that total Cu tissue levels were significantly increased in mussels exposed to Cu (II) and nCuO in brown water and effluent compared to the respective controls. The increase in Cu in tissues were also higher than those in mussels exposed to nCuO or Cu (II) in aquarium water suggesting that bioavailability is increased in organic-rich waters (brown water and the diluted effluent). The levels of Cu in tissues were significantly higher in mussels exposed green water and Cu (II) compared to mussels exposed to green water and nCuO. In green water, Cu-tissue levels was significantly increased in mussels exposed to Cu (II) compared to green water controls. The estimated bioavaibility for nCuO/Cu (II) in green, brown and diluted effluent waters were not determined/20, 14/12 and 13/19 L/kg respectively. This suggests that the presence of organic matter readily increased nCuO bioavaibility and compares to Cu (II).

The condition factor (mussel weight/shell length ratio) and the soft tissues weight ratio (soft tissue wet weight/mussel weight) were not significantly influenced by either surface water type or Cu forms (2-way ANOVA $p>0.1$ for Cu forms and water types;

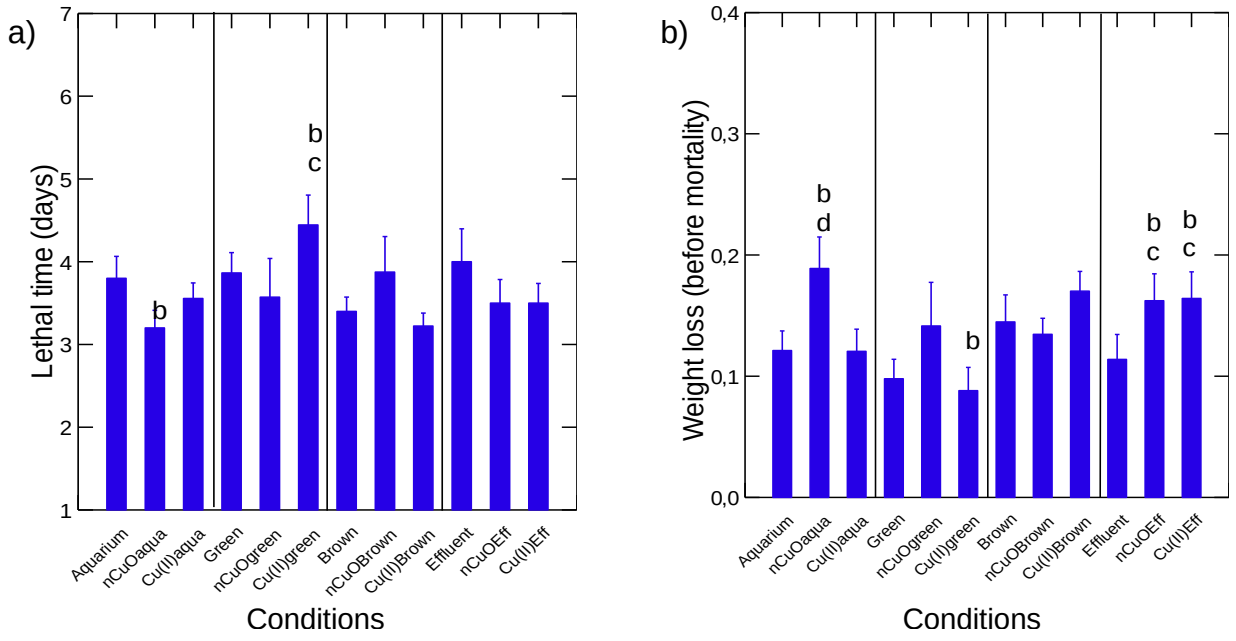

Fig. 2 Air-time survival in mussels exposed to Cu forms and surface waters. The air-time survival a) and weight loss at day 3 b) are schown. The letters a, b, c and d represents significance effect in the following: a difference between water control types with no added Cu (water type effect); b difference between the forms of Cu (Cu (II) and nCuO) relative to aquarium water (reference water); c difference between the forms of Cu (nCuO or Cu (II)) from corresponding water type control (e.g., nCuo-green vs green); d difference between forms of Cu within each water type (e.g., nCuO brown vs Cu (II) brown

data not shown). The lethal time was mostly influenced by water type and significant interaction was observed between Cu forms and the type of surface waters (Figure 2A). The lethal time was significantly reduced in mussels exposed to nCuO in aquarium water compared to aquarium water (controls). The lethal time was significantly increased in mussels exposed to Cu (II) in green water compared to controls and mussels exposed to nCuO in green water. The weight loss during the air survival time test was also determined as this endpoint occurs before mortality response. (Figure 2). The data revealed that the Cu forms had greater effects than surface water types but no significant interaction between the 2 treatments. Weight loss at the 3rd day (i.e., the last day before appearance of mortality) was more important in mussels exposed to nCuO in aquarium water compared to controls (aquarium water) and mussels exposed to Cu (II) in aquarium water. The weight loss was lower in mussels exposed to Cu (II) in green water compared to aquarium water but marginally so in green water control. The weight loss was more important in mussels exposed to either forms of Cu in the diluted effluent compared to mussels in aquarium water and the diluted effluent water controls. Weight loss was significantly correlated with the lethal time (r = -0.69) where mussels losing weight more quickly (after 3 days) survive less to air emersion.

Oxidative stress was examined in mussels exposed to Cu forms and surface waters by following changes in labile Zn levels, COX activity and GST activity (Figures 3A, 3B and 3C). Factorial 2-way ANOVA revealed a significant interaction between surface water types and forms of Cu. In respect to water types, the levels of labile Zn were significantly reduced in mussels placed in green and brown waters compared to aquarium water control. In the presence of Cu forms, labile Zn was significantly decreased in mussels exposed to nCuO and Cu (II) compared to aquarium water. Labile Zn were lower in mussels exposed to Cu (II) in aquarium water compared to nCuO in aquarium water. In mussels placed in green and brown waters, labile Zn levels were decreased compared to aquarium water suggesting that brown water masked the effects of Cu forms on labile Zn levels. Inflammation in mussels was measured by following COX activity in soft tissues (Figure 3B). Two-way ANOVA revealed that COX activity was significantly influenced by the type of surface water (p = 0.01) and marginally so with the Cu forms (p = 0.07). COX activity in mussels exposed to brown water control was significantly lower relative to mussels in aquarium water control. COX activity was significantly lower in mussels exposed to nCuO and Cu (II) in green water compared to mussels placed in green water alone. COX activity was significantly lower in mussels exposed to both forms of Cu in brown water in respect to aquarium water but not so when compared to brown water control suggesting that the water properties was the main driver for this effect. COX activity in mussels exposed to nCuO placed in the diluted municipal effluent was

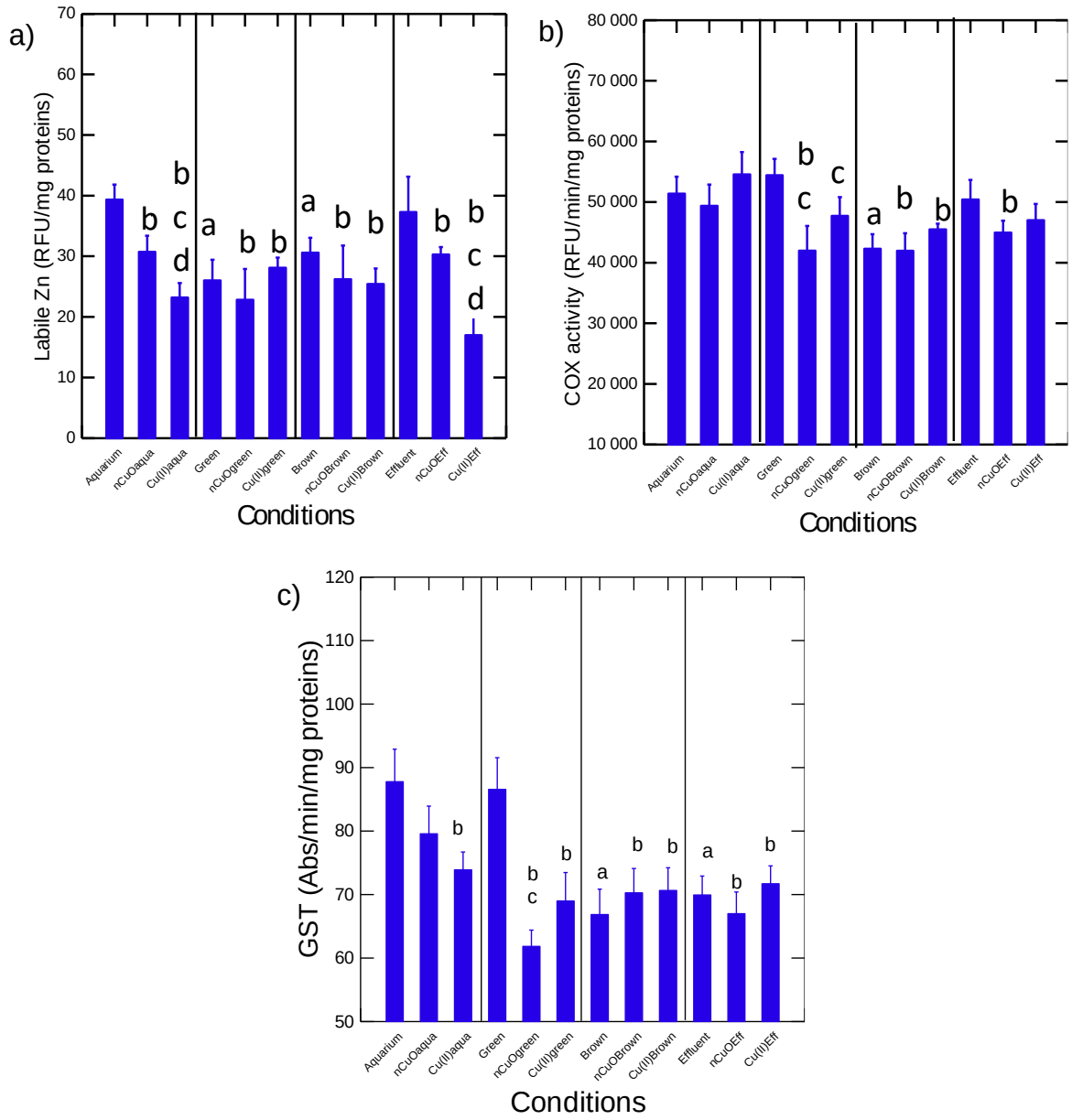

Fig. 3 Oxidative stress in fish exposed to dissolved Cu and nCuO. After the exposure period, the levels of labile Zn and LPO were determined in the soft tissues. The letters a, b, c and d represents significance effect in the following: a difference between water control types with no added Cu (water type effect); b difference between the forms of Cu (Cu (II) and nCuO) from aquarium water (reference water); c difference between the forms of Cu (nCuO or Cu (II)) from corresponding water type control (e.g., nCuo-green vs green); d difference between forms of Cu within each water type (e.g., nCuO brown vs Cu (II) brown)

marginally (p = 0.1) lower that aquarium water control and the diluted effluent control. Correlation analysis revealed that COX activity was significantly correlated with weight loss (r = -0.24) and GST activity (r = 0.38) (Table 3). A significant interaction between surface water and Cu forms was obtained for GST activity (Figure 4C). GST activity was reduced in mussels placed in brown and the diluted municipal effluent controls compared to aquarium control. In aquarium and green waters, the activity was significantly reduced by both nCuO and Cu (II). In brown and diluted municipal waters, neither forms of Cu produced any significant changes.

Toxic effects of mussels exposed to forms of Cu in different surface waters were examined by following damage at the level of proteins (ubiquitin tagging of damaged proteins), lipids (LPO) and the genetic material (DNA strand breaks) (Figures 4A, B and C). The levels of protein-ubiquitin were significantly influenced both surface water types and Cu forms according to 2-way ANOVA (Figure 4A). Protein-ubiquitin levels were significantly lower in

Table 3 Correlation analysis of biomarker data

	Time (air)	Weight loss	CF	Tissue weight	LPO	DNA	COX	UB	ACHE	GST	Labile Zn	
Time (air)	1											
Weight loss	**-0.69**	1										
CF	0.042	-0.07	1									
Tissue	0.13	-0.11	**0.58**	1								
LPO	0.01	0.01	0.17	0.19	1							
DNA	-0.03	0.02	0.11	**0.24**	**0.57**	1						
COX	0.22	**-0.28**	-0.10	0.01	0.01	-0.06	1					
UB	0.08	-0.10	-0.18	-0.14	0.08	-0.15	**0.25**	1				
ACHE	0.11	-0.06	-0.02	-0.08	**-0.23**	-0.11	0.13	**0.33**	1			
GST	0.10	-0.16	-0.14	-0.10	-0.17	**-0.25**	**0.44**	**0.53**	**0.43**	1		
Labile Zn	0.08	**-0.24**	0.01	0.11	-0.11	0.03	0.16	**0.22**	0.07	**0.37**	1	
Cu (total)	0.05	-0.02	0.04	-0.1	**0.34**	**-0.58**	0.07	**0.25**	-0.1	0.10	-0.15	

Significant correlations are indicated in bold

mussels exposed to brown and diluted effluent controls compared to aquarium water control. Their levels were significantly reduced in mussels exposed to Cu (II) in aquarium water compared to aquarium water (control). In green waters, protein damage was significantly decreased in mussels exposed to nCuO and Cu (II). In brown waters, the levels of protein-ubiquitin in mussels exposed to nCuO were significantly lower compared to aquarium water controls but not with the brown water controls suggesting again that the driver of effects was from surface water type. In mussels exposed to the diluted effluent, the levels were decreased in mussels exposed to Cu (II) compared to the diluted effluent and aquarium water controls. Correlation analysis revealed that protein-ubiquitin levels were significantly correlated with many endpoints: COX activity (r = 0.25), AChE (0.33), GST activity (r = 0.53) labile Zn levels (r = 0.22) and total Cu levels in tissues (r = 0.25). Factorial ANOVA revealed that LPO levels were mainly influenced by the Cu forms but not by the surface water types (Figure 4B). The levels of LPO was significantly elevated only in mussels exposed to green water and Cu (II) compared to green water and aquarium water controls. Correlation analysis revealed that LPO was significantly correlated with tissue Cu levels (r = 0.34), AChE (r = -0.23) and DNA strand breaks (r = -0.58). The levels of DNA damage (genotoxicity) was also examined in mussels (Figure 4C). The data revealed that a marginal interaction between water types and Cu forms was found. In green water, the levels of DNA strand breaks were increased in mussels exposed to nCuO compared to green water controls. The levels of DNA strand breaks were lower in mussels exposed to Cu (II) in brown water compared to brown water controls. Correlation analysis revealed that DNA damage was correlated with LPO (r = 0.57), tissues weight (r = 0.24) and GST activity (r = -0.25).

The levels of AChE activity was also determined in mussels to detect changes in neural activity (Figure 5). Analysis of the data revealed that the water types significantly influenced the activity while the Cu forms had no effect. AChE activity was significantly decreased in green, brown and diluted effluent waters compared to aquarium water control. The decreased did not differ from the corresponding water controls which indicates absence of Cu effects regardless of the form. AChE activitiy was significantly correlated with GST activity (r = 0.43).

In the attempt to gain a more global understanding, a discriminant function analysis was performed to seek out relationships between the influence of Cu forms in the different surface water types (Figure 6). Based on this analysis, 99 % of the variance was explained and the classification performance was at 25 % which indicates that some conditions were not always discriminated (i.e., similar effects were obtained). The biomarkers with the highest factorial weight, explaining the total variance were protein-ubiquitin, labile Zn and GST activity. In respect on the water types only, the surface waters were all well separated from each other suggesting influence of water properties such as pH, conductivity, organic matter content and suspended solids on the biomarkers used in this study. In aquarium water, the addition of nCuO did not produced important changes compared to Cu (II), which were mostly in the direction of the x axis with labile Zn and protein-ubiquitin levels and GST activity. In green water, the effects of Cu forms markedly differ from controls but were rather similar with each other based on labile Zn, protein-ubiquitin levels and GST activity. In brown water, the effects between brown water controls, nCuO and Cu (II) were the least discriminated showing the dampening influence of this organic matter-rich water on the toxicity of Cu. In respect to the diluted municipal effluent, the effects of Cu (II) were markedly different than those produced by nCuO and controls which were more closely related with each other. In summary, mussels placed in green and the aquarium waters led to more important effects when mussels were exposed to Cu (II) and to nCuO to a lesser extent compared to mussels in brown water and the diluted municipal effluent. The influence of organic carbon origin could be evaluated by comparing the responses between green waters and diluted municipal effluent which

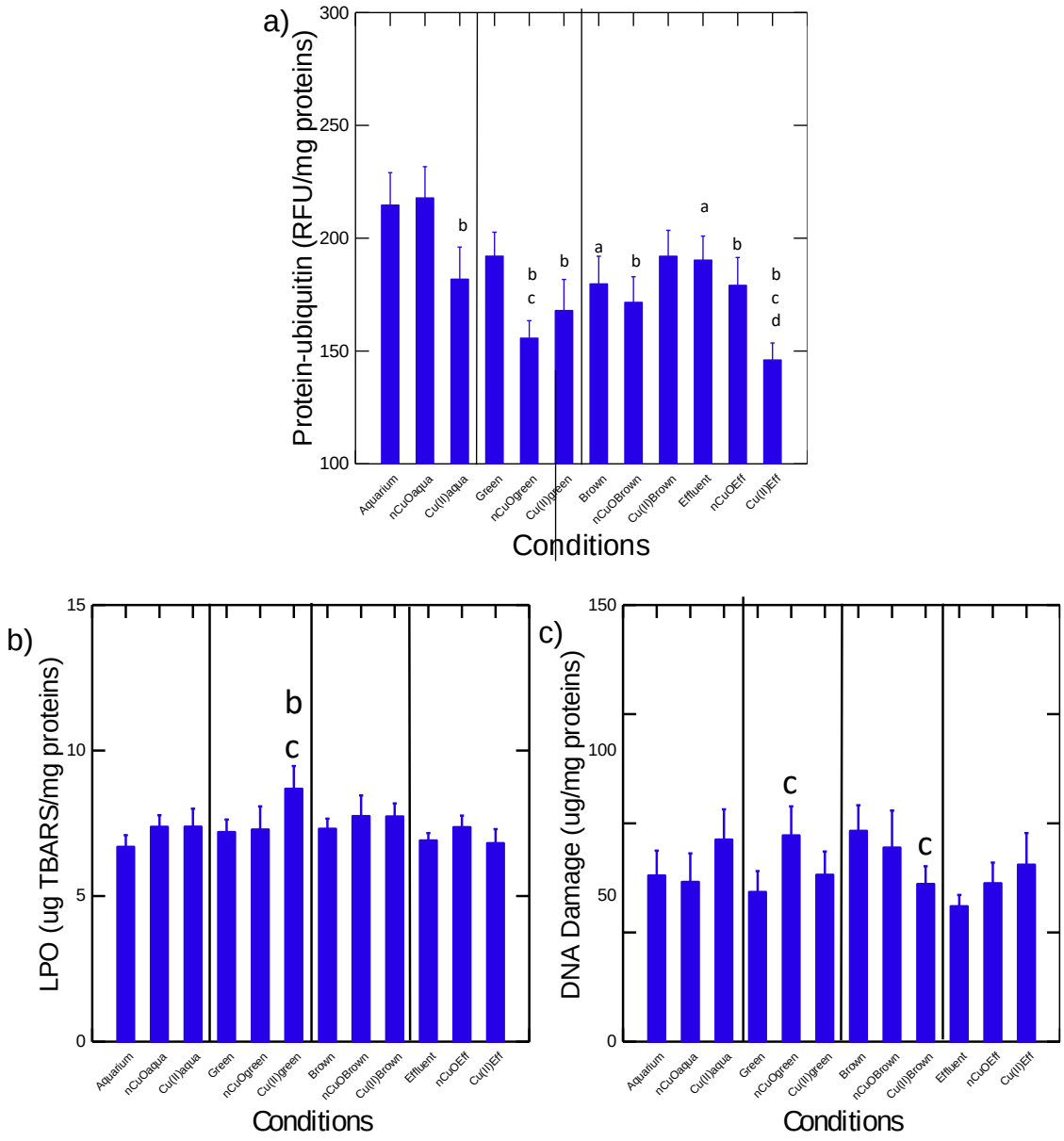

Fig. 4 Tissue damage in mussels exposed to Cu forms and surface waters. After the exposure period, tissue damage were studied at the protein (ubiquitinylation), LPO, DNA damage levels in soft tissues. The letters a, b, c and d represents significance effect in the following: a difference between water control types with no added Cu (water type effect); b difference between the forms of Cu (Cu (II) and nCuO) from aquarium water (reference water); c difference between the forms of Cu (nCuO or Cu (II)) from corresponding water type control (e.g., nCuo-green vs green); d difference between forms of Cu within each water type (e.g., nCuO brown vs Cu (II) brown)

contain relatively the same amount of organic carbon content but from different origin (natural vs municipal). The data revealed the influence of the organic carbon origin influence more the responses for dissolved Cu (II) than nCuO. The influence of total organic carbon (TOC) levels could be evaluated by comparing the responses between green and brown waters which as different levels of natural TOC content (brown waters have 2.1 times more TOC than green waters) but the conductivity was also inversely influenced (green water have 2.2 times more conductivity than brown water). The analysis also revealed that the Cu (II) responses were more strongly influenced than with nCuO but this effect could be related to both TOC and conductivity. The influence of total suspended solids (TSS) could be evaluated by comparing the response of aquarium and green waters which contains the highest levels of TSS for the latter. The analysis revealed that TSS influenced more the responses of nCuO than Cu (II). Hence, the effects of nCuO are more influenced by TSS than TOC and conductivity while the effects of Cu (II) are more influenced by the levels/origin of the TOC and conductivity.

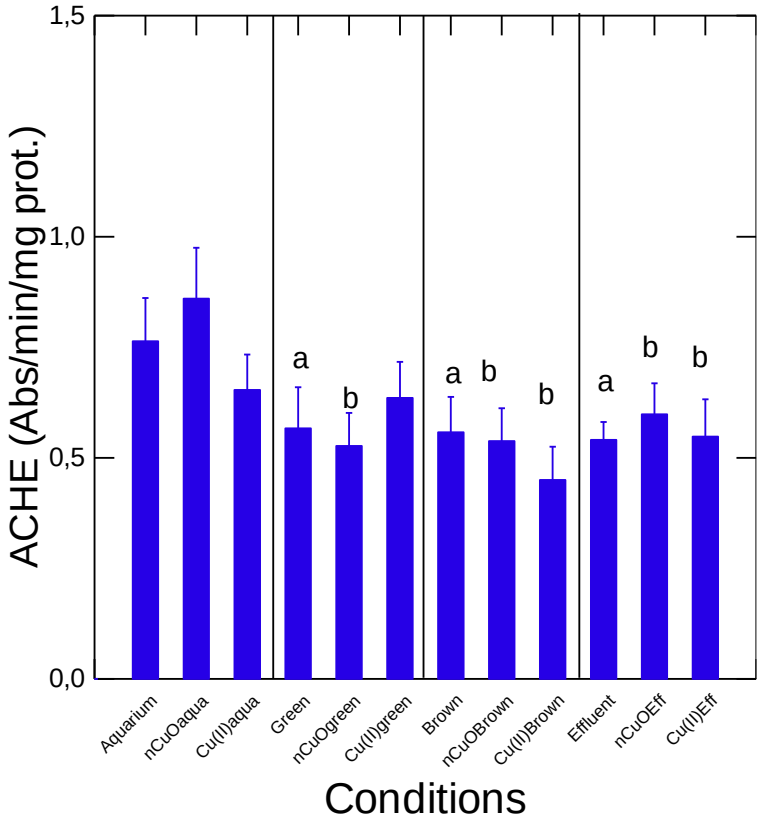

Fig. 5 AChE in mussels exposed to Cu forms in different surface waters. After the exposure period, AChE and GAT activities were determined in soft tissues. The letters a, b, c and d represents significance effect in the following: a difference between water control types with no added Cu (water type effect); b difference between the forms of Cu (Cu (II) and nCuO) from aquarium water (reference water); c difference between the forms of Cu (nCuO or Cu (II)) from corresponding water type control (e.g., nCuo-green vs green); d difference between forms of Cu within each water type (e.g., nCuO brown vs Cu (II) brown)

Discussion

The organic carbon content was the highest in brown water and is composed of natural organic matter such as humic and fulvic acids. The presence of organic matter was shown to favor nanoparticle suspension and increase bioavailability towards Cu (II) which was associated to LPO, decreased DNA strand breaks (reduced DNA repair activity), protein-ubiquitin levels. Indeed, the bioavailability of nCuO was always lower than Cu (II) in green and diluted effluent suggesting the influence of the type and concentration of organic matter. Interestingly in brown waters, rich in natural organic matter, nCuO and Cu (II) were equally bioavailable for mussels. This is in keeping with the DLS analysis showing no significant increase in mean particle diameter after 48 h suspension in brown water. However, the dispersity of nCuO was higher (coefficient of variation of 46 % of the mean diameter) at 48 h compared to 12 % at 1 h in brown water (Table 2). This suggests that the nCuO intereacted with the organic matter matrix of the brown water samples but not in a coherent manner (increased disorder in the distribution of size). In a former study, dissolution experiments in 5 different types of surface waters revealed that nCuO could form large aggregates over time (24 h) in lake water reaching 400 nm diameter (Liu *et al*., 2018). Although the organic matter content was 11 mg/L, which was higher that the brown water sample at 7 mg/L in the present study, aggregation was less apparent. Exposure to low concentrations of Cu (II) at 50 µg/L as total Cu only increased tissue Cu loadings in musses placed in brown water and which suggests that nCuO did not accumulate in mussels although Cu tissue levels were correlated with biomarkers cited above. It was shown that the presence of natural organic matter could influence the dissolution state of nCuO which could, in turn, influence bioavailability in the Gulf killifish (Jiang *et al.*, 2017). Indeed, increased solubilisation of nCuO by natural organic matter was shown to decrease its bioavailability. In another study with the invertebrate shredder *Allogamus ligonifer* exposed to nCuO and humic acids mixtures, exposure to nCuO increased the feeding rate and this response was inversely proportional to the nanoparticle's size (Pradhan *et al*., 2015). The addition of humic acids alone decreased feeding activity but this was dampened

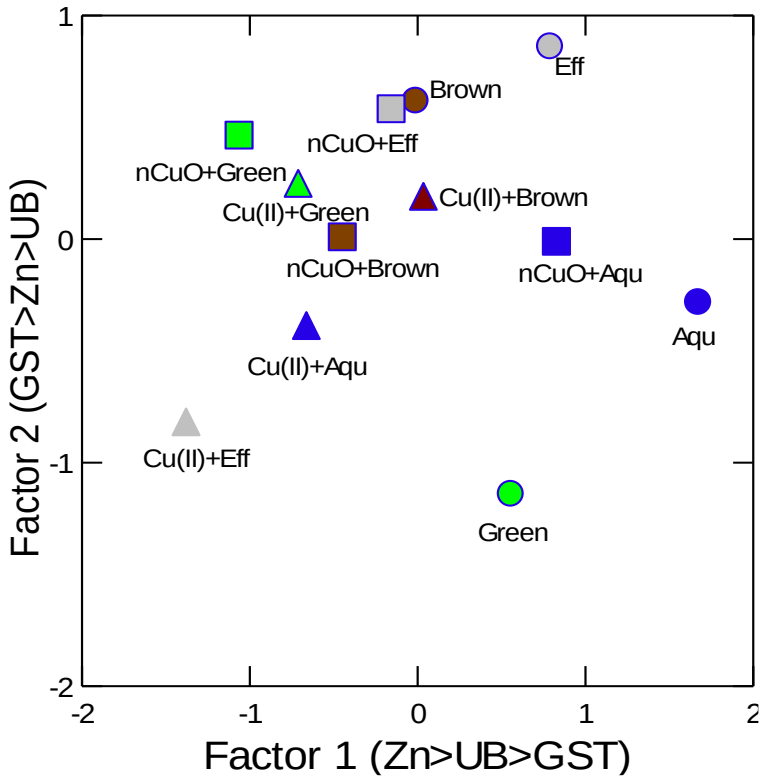

Fig. 6 Discriminant function analysis of mussels responses to dissolved and nanoparticulate Cu in different surface waters. Discriminant function analysis was performed with the biomarker responses. The canonical scores for the surface waters types O, Cu (II) Δ and nCuO□ are shown for clarity

by the addition of nCuO again by smaller size nanoparticles (≤ 50 nm). In the water flea *Daphnia magna* exposed to nCuO and nZnO for 72h, decreased GST activity, increased metallothioneins and LPO was observed (Mwaanga *et al*., 2014). These effects were also dampened when natural organic matter was added at a concentration of 0.5 mg/L which was below the organic matter content in the present study. This is consistent with the observation that the biomarker responses obtained in mussels in brown water (organic matter-rich) where close to those observed with brown water controls. The decrease in the toxicity of nCuO in the presence of organic matter to rice was also observed (Peng *et al*., 2015). It was postulated that organic matter forms a coating on the nanoparticles which enhance electrostatic repulsion between nanoparticles and interaction at the surface of the contact tissues. In this respect, the addition of anionic charges at the surface of the nanoparticle could limit interaction at the surface of membranes, which normally contains negative charges. Conversely, positively-charged coatings of nanoparticles are usually more toxic in organisms. This was seemingly the same situation with metallic silver nanoparticles (Fuman *et al*., 2013; Auclair *et al*., 2019). Humic and fulvic acids limited nanoparticle aggregation where silver nanoparticles with positively-coated surface were more toxic to juvenile trout than negatively-coated nanoparticles. In the attempt to determine if the toxic properties of Cu could be changed by organic matter i.e., not only by reduced uptake, a metabolomic study was undertaken in *Daphnia pulex* (Taylor *et al*., 2016). The study revealed that the natural organic matter readily decreased Cu toxicity but although Cu increased oxidative stress in these organisms (mode of action of Cu), the presence of organic matter increased metabolic energy status which could have mitigated Cu toxicity.

It is noteworthy that Cu tissue levels were significantly related to oxidative damage (LPO), decreased DNA strand breaks and the removal of damaged protein by ubiquitin tagging. In addition to the binding of organic matter on the nanoparticles, ubiquitin and other proteins could form a stable corona on metallic nanoparticles which could disrupt the turnover of damaged/denatured proteins in cells (Duran *et al*, 2015). Ubiquitin tagging of proteins was one of the major responses based from discriminant function analysis in the present study. Protein-ubiquitin levels were significantly decreased in green waters for nCuO while Cu (II) decreased the levels in mussels placed in aquarium and the diluted municipal effluent. In respect to organic carbon-rich brown water, protein-ubiquitin levels were not influenced by either nCuO or Cu (II). The levels were also lower in brown water controls compared

to aquarium water controls which, suggests a surface water-mediated effect. Because protein ubiquitin levels were not correlated with LPO, protein turnover was not the results of oxidative stress caused by Cu. However, the correlation with labile Zn in the post-mitochondrial fraction suggests the displacement of Zn(II) by other cations or by oxidative stress, perhaps Cu (II) or reactive oxygen species. Protein-ubiquitin levels were also decreased in the digestive gland of freshwater mussels *Elliptio complanata* exposed to 20 nm nano-silver but were induced by 80 nm nano-silver (Gagné *et al*., 2013). This suggests that the size of the nanoparticles could influence protein ubiquitin levels in mussels. If this holds true for nCuO, the size distribution of the nanoparticles should encompass the 20 nm diameter range. Protein-ubiquitin levels were also significantly related to AChE indicating decreased neuro-muscular activity, however the effects were rather associated to a surface water effect than exposure to Cu forms. Indeed, exposure of mussels to brown and diluted municipal effluent had decreased AChE activity and Cu produced no significant effect in respect to each water controls. Hence, protein turnover changes observed by Cu forms is also influenced with surface water types which suggests interaction or combined effects of surface water properties and exposure to Cu. This forms the basis of cumulative effects when the various environmental conditions (surface water types and Cu contamination) acts at the same biochemical targets in cells. Nevertheless, it was shown in a previous study with the marine mussel *Mytilus galloprovincialis*, that nCuO and Cu (II) decreased AChE in mussels (Gomes *et al*., 2011).The mussels were exposed to 10 µg/L of Cu as nCuO and Cu (II) for 15 days which was longer than the exposure time used in the present study (96 h). Metallothioneins and LPO was induced which suggests the release of Cu (II) in mussels exposed to nCuO after 15 days. In conclusion, the toxicity of nCuO and Cu (II) could be influenced by the surface waters properties and the form of Cu in freshwater mussels. Protein-ubiquitin, labile Zn and GST activity were the major effects of mussels exposed to nCuO and Cu (II) in 4 types of freshwater. Both GST activity and labile Zn levels were decreased by surface waters and forms of Cu as with protein-ubiquitin levels as these were common targets from which cumulative effects could be obtained. The biomarkers of damage LPO and DNA strand breaks, representing an irreversible damage compared tot the other sublethal effects, were only affected by Cu forms and not by the surface water properties. The same was also observed at the individual level with the air survival time and weight loss. Based on the responses obtained for nCuO and Cu (II) in the 4 types of surface waters, it appears that the TOC origin (natural vs anthopogenic) influence more the response of mussels exposed to Cu (II) while the TSS influence more the response of mussels exposed to nCuO. More research will be needed to better understand the interplay between biomarkers at different levels of biological organisation in the context of multiple environmental stressor.

Acknowledgements

This work was funded by the Chemical Management Plan of Environment and Climate Change Canada. The technical assistance of Joanne Kowalczyk for tissue preparation and biomarker analyses is dully recognized.

References

Adam N, Leroux F, Knapen D, Bals S, Blust R. The uptake and elimination of ZnO and CuO nanoparticles in *Daphnia magna* under chronic exposure scenarios. Water Res. 68: 249-261, 2015.

APHA (American Public Health Association). Standard methods for the examination of water and wastewater. 17th Edition. Washington DC, United States, http://hdl.handle.net/1969.3/24401, 2010.

Auclair J, Turcotte P, Gagnon C, Peyrot C, Wilkinson KJ, Gagné F. The influence of surface coatings on the toxicity of silver nanoparticle in rainbow trout. Comp Biochem Physiol C Toxicol Pharmacol. 226: 108623, 2019.

Bester MJ, Potgieter HC, Vermaak WJH. Cholate and pH reduce interference by SDS in the determination of DNA with Hoescht. Anal Biochem. 223: 299-305, 1994.

Boryslawskyj M, Garrood AC, Pearson JT, Woodhead D. Elevation of glutathione-S-transferase activity as a stress response to organochlorine compounds, in the freshwater mussel, *Sphaerium corneum*. Marine Environ Res. 24: 101-104, 1988.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72: 248-254, 1976.

Buffet PE, Richard M, Caupos F, Vergnoux A, Perrein-Ettajani H, Luna-Acosta A, *et al*. A mesocosm study of fate and effects of CuO nanoparticles on endobenthic species (*Scrobicularia plana*, *Hediste diversicolor*). Environ Sci Technol. 47: 1620-1628, 2013.

Canesi L, Ciacci C, Fabbri R, Marcomini A, Pojana G, Gallo G. Bivalve molluscs as a unique target group for nanoparticle toxicity. Mar Environ Res. 76: 16-21, 2012.

Cuillel M, Chevallet M, Charbonnier P, Fauquant C, Pignot-Paintrand I, Arnaud J, *et al*. Interference of CuO nanoparticles with metal homeostasis in hepatocytes under sub-toxic conditions. Nanoscale 6: 1707-1715, 2014.

Dietler D, Babu M, Cissé G, Halage AA, Malambala E, Fuhrimann S. Daily variation of heavy metal contamination and its potential sources along the major urban wastewater channel in Kampala, Uganda. Environ Monit Assess 191: 52, 2019.

Domingos RF, Tufenkji N, Wilkinson KI. Aggregation of titanium dioxide nanoparticles: role of a fulvic acid. Environ Sci Technol. 43: 1282-1286, 2009.

Durán N, Silveira CP, Durán M, Martinez DS. Silver nanoparticle protein corona and toxicity: a mini-review. J Nanobiotechnology. 13: 55, 2015.

Furman O, Usenko S, Lau BL. Relative importance of the humic and fulvic fractions of natural organic matter in the aggregation and deposition of silver nanoparticles. Environ Sci Technol. 47: 1349-1356, 2013.

Gagné F, Blaise C. Available intracellular Zn as a potential indicator of heavy metal exposure in rainbow trout hepatocytes. Environ Toxicol Wat Qual. 11: 319-325, 1996.

Gagné F, Gagnon C, Blaise C. Aquatic Nanotoxicology: A review. Current Top Toxicol. 4; 51-64, 2007.

Gagné F, Auclair J, Turcotte P, Gagnon C. Sublethal effects of silver nanoparticles and dissolved silver in freshwater mussels. J Toxicol Environ Health A. 76: 479-490, 2013.

Gagné F. Biomarkers of infection and diseases. Biochemical Ecotoxicology-Principles and Methods, First Edition Elsevier Inc., USA (Chapter 11), 2014.

Gagné F, Auclair J, Peyrot C, Wilkinson KJ. The influence of zinc chloride and zinc oxide nanoparticles on air-time survival in freshwater mussels. Comp Biochem Physiol. 172-173C: 36-44, 2015.

Gagnon C, Turcotte P, Trépanier S, Gagné F, Cejka PJ. Impacts of municipal wastewater oxidative treatments: changes in metal physical speciation and bioavailability. Chemosphere 97: 86-91, 2014.

Garner KL, Suh S, Keller AA. Assessing the Risk of Engineered Nanomaterials in the Environment: Development and Application of the nanoFate Model. Environ Sci Technol. 51: 5541-5551, 2017.

Gélinas M, Lajeunesse A, Gagnon C, Gagné F. Temporal and seasonal variation in acetylcholinesterase activity and glutathione-S-transferase in amphipods collected in mats of *Lyngbya wollei* in the St-Lawrence River (Canada). Ecotoxicol Environ Saf. 94: 54-59, 2013.

Giannetto A, Cappello T, Oliva S, Parrino V, De Marco G, Fasulo S, *et al*. Copper oxide nanoparticles induce the transcriptional modulation of oxidative stress-related genes in *Arbacia lixula* embryos. Aquat Toxicol. 201: 187-197, 2018.

Gomes T, Pinheiro JP, Cancio I, Pereira CG, Cardoso C, Bebianno MJ. Effects of copper nanoparticles exposure in the mussel *Mytilus galloprovincialis*. Environ Sci Technol. 45: 9356-9362, 2011.

Grillo R, Rosa AH, Fraceto LF. Engineered nanoparticles and organic matter: a review of the state-of-the-art. Chemosphere 119: 608-619, 2015.

Isani G, Falcioni ML, Barucca G, Sekar D, Andreani G, Carpenè E, *et al*. Comparative toxicity of CuO nanoparticles and CuSO4 in rainbow trout. Ecotoxicol Environ Saf. 97: 40-46, 2013.

Jiang C, Castellon BT, Matson CW, Aiken GR, Hsu-Kim H. Relative Contributions of Copper Oxide Nanoparticles and Dissolved Copper to Cu Uptake Kinetics of Gulf Killifish (*Fundulus grandis*) Embryos. Environ Sci Technol. 51: 1395-1404, 2017.

Keller AA, Wang H, Zhou D, Lenihan HS, Cherr G, Cardinale BJ, *et al*. Stability and aggregation of metal oxide nanoparticles in natural aqueous matrices. Environ Sci Technol. 44: 1962-1967, 2010.

Keller AA, Lazareva A. Predicted releases of engineered nanomaterials: from global to regional to local. Environ Sci Technol Lett. 1: 65-70, 2013.

Lee J, Mahendra S, Alvarez PJ. Nanomaterials in the construction industry: a review of their applications and environmental health and safety considerations. ACS Nano 4: 3580-3590, 2010.

Liu Z, Wang C, Hou J, Wang P, Miao L, Lv B, *et al*. Aggregation, sedimentation, and dissolution of CuO and ZnO nanoparticles in five waters. Environ Sci Pollut Res Int. 25: 31240-31249, 2018.

Marr JC, Lipton J, Cacela D. Hansen JA, Meyer JS, Bergman HL. Bioavailability and acute toxicity of copper to rainbow trout (*Oncorhynchus mykiss*) in the presence of organic acids simulating natural dissolved organic carbon. Can J Fish Aquat Sci. 56: 1471-1483, 1999.

Musee N, Thwala M, Nota N. The antibacterial effects of engineered nanomaterials: implications for wastewater treatment plants. J. Environ. Monitor. 13: 1164-1183, 2011.

Mwaanga P, Carraway ER, van den Hurk P. The induction of biochemical changes in *Daphnia magna* by CuO and ZnO nanoparticles. Aquat Toxicol. 150: 201-209, 2014.

Olive PL. DNA precipitation assay: a rapid and simple method for detecting DNA damage in mammalian cells. Environ Mol Mutagen. 11: 487-495, 1988.

Peng C, Zhang H, Fang H, Xu C, Huang H, Wang Y, *et al*. Natural organic matter-induced alleviation of the phytotoxicity to rice (*Oryza sativa* L.) caused by copper oxide nanoparticles. Environ Toxicol Chem. 34: 1996-2003, 2015.

Pradhan A, Geraldes P, Seena S, Pascoal C, Cássio F. Natural organic matter alters size-dependent effects of nanoCuO on the feeding behaviour of freshwater invertebrate shredders. Sci Total Environ. 535: 94-101, 2015.

Rocha TL, Gomes T, Sousa VS, Mestre NC, Bebianno MJ. Ecotoxicological impact of engineered nanomaterials in bivalve molluscs: An overview. Mar Environ Res. 111: 74-88, 2015.

Rotini A, Gallo A, Parlapiano I, Berducci MT, Boni R, Tosti E, *et al*. Insights into the CuO nanoparticle ecotoxicity with suitable marine model species. Ecotoxicol Environ Saf. 147: 852-860, 2018.

Taylor NS, Kirwan JA, Yan ND, Viant MR, Gunn JM, McGeer JC. Metabolomics confirms that dissolved organic carbon mitigates copper toxicity. Environ Toxicol Chem. 35: 635-644, 2016.

Walden C, Zhang W. Biofilms Versus Activated Sludge: Considerations in Metal and Metal Oxide Nanoparticle Removal from Wastewater. Environ Sci Technol 50: 8417-8431, 2016.

Wills ED. Evaluation of lipid peroxidation in lipids and biological membranes. In: Snell K, Mullock B, editors. Biochemical toxicology: a practical approach. Washington D.C: IRL Press; p. 127-150, 1987