Aquatic Toxicology 144–145 (2013) 105–115

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Interactive effects of copper and dissolved organic matter on sodium uptake, copper bioaccumulation, and oxidative stress in juvenile freshwater mussels (*Lampsilis siliquoidea*)

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ARTICLE INFO

Article history:
Received 1 June 2013
Received in revised form
16 September 2013
Accepted 26 September 2013

Keywords:
Copper
Acute toxicity
Dissolved organic matter
Freshwater mussel
Ionoregulation
Oxidative stress

ABSTRACT

Freshwater mussels are exceptionally sensitive to many contaminants including metals, but the mechanisms of toxicity are not fully understood. Similarly, our understanding of the protective effects of dissolved organic matter (DOM) is also undergoing revision, since recent studies have found that DOM may also directly affect organism physiology, in addition to its well known capability in complexing and reducing bioavailability of metals. In the present study, these issues were investigated in juvenile (6–12 months old) freshwater mussels (*Lampsilis siliquoidea*) in moderately-hard reconstituted water (Ca^{2+} = 0.406 mmol/L; Mg^{2+} = 0.537 mmol/L; Na^+ = 1.261 mmol/L; K^+ = 0.077 mmol/L; hardness = 80–100 mg/L $CaCO_3$; pH = 8.02 and DOM = 0.3 mg C/L). Mussels were acutely exposed (24 and 96 h) to Cu (0, 2 or 12 μg Cu/L) combined with three concentrations (0, 3 or 6 mg C/L) of DOM of terrigenous origin (Luther Marsh). We analyzed unidirectional Na^+ influx, whole-body ion content (Na^+, K^+, Ca^{2+} and Mg^{2+}), enzyme (Na^+/K^+-ATPase, H^+-ATPase and carbonic anhydrase) activities, copper bioaccumulation and oxidative stress-related parameters. Exposure to DOM alone caused a marked increase in the unidirectional Na^+ influx rate and a decrease in v-type H^+-ATPase activity, suggesting that DOM alone can cause alterations in membrane transport functions and therefore, whole-body Na^+ metabolism. Unidirectional Na^+ influx rate and Na^+K^+-ATPase activity were inhibited when mussels were exposed to the higher Cu concentration tested (12 μg Cu/L). The influx inhibition was ameliorated by the simultaneous presence of DOM. At this same Cu concentration, DOM also significantly protected mussels against whole-body Na^+ and K^+ losses associated with Cu exposure, as well as against Cu bioaccumulation. Oxidative stress parameters did not show clear trends across treatments. Overall, our results indicate that Cu is a potent ionoregulatory toxicant to freshwater mussels. They also demonstrate that natural DOM protects against both Cu bioaccumulation and ionoregulatory toxicity, and that at least part of this protection results from direct positive effects of DOM on Na^+ metabolism.

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1. Introduction

Copper (Cu) is a transition metal and an essential element for all aerobic organisms due to its role as cofactor of mitochondrial enzymes (Solomon and Lowery, 1993). Also, in aquatic arthropods and molluscs, Cu is a component of haemocyanin, the oxygen carrier protein (Taylor and Anstiss, 1999). Although it is essential, Cu at elevated concentrations in the water acts as a potent toxicant for aquatic animals. In many freshwater organisms, Cu appears to be an ionoregulatory toxicant (e.g. Grosell et al., 2002; Grosell, 2012), reducing branchial Na^+ uptake and inhibiting key osmoregulatory enzymes such as Na^+/K^+-ATPase (Laurén and McDonald, 1987) and carbonic anhydrase (Zimmer et al., 2012). Cu is also a strong oxidative toxicant in many organisms (reviewed by Lushchak, 2011; Grosell, 2012).

Although the toxic effects of Cu have been reported for a several species belonging to different phyla, freshwater mussels have gained particular attention, due to their very high sensitivity to this metal. Freshwater mussels are considered to be one of the most threatened groups in the world, with approximately 70% of the North American species listed as endangered, threatened or of special concern (Williams et al., 1993; Neves et al., 1997). This situation is due to several factors including the complex life cycle of most

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http://dx.doi.org/10.1016/j.aquatox.2013.09.028

species (involving a parasitic larval stage called glochidia) (Kat, 1984; Barnhart et al., 2008), introduction of exotic invasive species (Gillis and Mackie, 1994), loss of habitats by the construction of dams (Hovingh, 2004) and, subtle, widespread, chronic environmental contamination (Bogan, 1993; Strayer et al., 2004). With respect to Cu, freshwater mussels are more sensitive than all other previously tested organisms (Wang et al., 2011). Recently, Jorge et al. (2013) reported that the mechanism underlying chronic Cu toxicity (evaluated at environmentally relevant concentrations) to juvenile *Lampsilis siliquoidea* appeared to be ionoregulatory disruption, through a reduction in whole-body Na^+ concentrations and a decrease in Na^+/K^+-ATPase activity, rather than oxidative stress and damage.

It is well established that Cu bioavailability and, therefore, Cu toxicity, are directly related to water chemistry (Playle, 1998; Di Toro et al., 2001; Niyogi and Wood, 2004). Cations such as Na^+, Ca^{2+}, and Mg^{2+} can compete with other metal ions such as Cu for binding sites on aquatic organisms, and can provide protection by decreasing metal uptake (Pagenkopf, 1983; Playle, 1998). Similarly, waterborne ligands such as dissolved organic matter (DOM) can bind to positively charged metal cations, reducing their bioavailability, and thus preventing them from binding at the site of toxic action on the organism (Playle et al., 1993). According to several authors, the protection offered by DOM against metal toxicity appears to be source-dependent, with terrigenous DOM (i.e. allochthonous, originating from land-based sources outside the water body) being more protective than autochthonous DOM (produced within the water body) (De Schamphelaere et al., 2004; Ryan et al., 2004; Al-Reasi et al., 2012). In fact, it has been shown that terrigenous DOM, as well as a commercial surrogate (humic acid), can linearly increase the EC50 of Cu to juvenile freshwater mussels (Wang et al., 2009) and glochidia larvae (Gillis et al., 2008, 2010).

The protective effect of DOM against metal toxicity is usually attributed to the ability of these large anionic molecules to complex metals, reducing their bioavailability (e.g. Playle et al., 1993; Al-Reasi et al., 2011, 2012). However, the direct biological actions of these compounds have been largely overlooked. The investigations of Campbell et al. (1997), Vigneault et al. (2000), and Al-Reasi et al. (2013a,b) have provided evidence that DOM molecules may directly interact with biological surfaces. They also suggested that this interaction appears to be associated with altered membrane permeability, due to both the hydrophobic and hydrophilic moieties of these compounds. This idea was supported by Galvez et al. (2008) who demonstrated, using both in vivo and in vitro approaches, that exposure to natural organic matter (NOM) decreased the transepithelial potential in rainbow trout gills. More recently, Wood et al. (2011) have suggested that some of the so-called indirect protective effects of DOM on metal toxicity may actually be due to the ability of DOM to promote alterations in gill physiology, such as changes in the fluidity of the lipoprotein bilayer in the transcellular pathway, altering accessibility of the Na^+ transport sites in short-term exposures, and the number of transport sites in longer term exposures. Indeed, studies on both freshwater fish (McGeer et al., 2002; Wood et al., 2003; Matsuo et al., 2004) and crustaceans (Glover and Wood, 2005; Glover et al., 2005a,b,c; Al-Reasi et al., 2013b) have demonstrated that DOM alone results in biologically significant effects on both the ionoregulatory physiology and nitrogenous waste excretion of the organisms in a time-dependent manner.

With this background in mind, the present investigation examined the physiological responses of juvenile freshwater mussels (*L. siliquoidea*) acutely exposed to environmentally relevant Cu concentrations in combination with DOM isolated from a terrigenous source. Mussels were exposed for two different experimental periods (24 and 96 h), at 3 distinct environmentally relevant Cu concentrations (nominally 0, 2 and 12 μg Cu/L), in combination with 3 different DOM concentrations (nominally 0, 3 and 6 mg C/L as dissolved organic carbon). We hypothesized that acute Cu exposure at these concentrations would negatively affect animal physiology, mainly through a disruption in Na^+ homeostasis, rather than through the induction of oxidative stress, in accord with the recent chronic Cu exposure study on *L. siliquoidea* (Jorge et al., 2013). Cu-induced disturbances in Na^+ regulation have been previously reported for many fish and crustaceans (Laurén and McDonald, 1985; Bianchini and Wood, 2003; Grosell et al., 2002, 2004; Pinho et al., 2007; Bianchini et al., 2008), as well as for the marine clam *Mesodesma mactroides* (Lopes et al., 2011), but with variable mechanisms. One of our goals was to understand the mechanism(s) of this effect in freshwater mussels. Endpoints evaluated were unidirectional Na^+ influx rate, whole-body ion content (Na^+, K^+, Ca^{2+} and Mg^{2+}), the activities of ionoregulatory enzymes (Na^+/K^+-ATPase, v-type H^+-ATPase, and carbonic anhydrase), Cu bioaccumulation, and oxidative stress parameters (total reactive oxygen species concentration and antioxidant capacity against peroxyl radicals). We further hypothesized that DOM, at environmentally realistic levels, would reduce or prevent the physiological disturbances in Na^+ homeostasis caused by Cu exposure, as well as reduce the bioaccumulation of this metal. Finally, we postulated that DOM itself, in the absence of Cu, would have direct positive effects on Na^+ metabolism, thereby contributing to its protective effects against Cu toxicity to these sensitive freshwater animals.

2. Materials and methods

2.1. Mussels

Juvenile freshwater mussels (*Lampsilis siliquoidea*; 6–12 months old; 87–186 mg) were purchased from Missouri State University (Springfield, MO, USA). Mussels were held in 1-L beakers, each containing 50 individuals and 1 L of aerated moderately-hard reconstituted water (Ca^{2+} = 0.406 mmol/L; Mg^{2+} = 0.537 mmol/L; Na^+ = 1.261 mmol/L; K^+ = 0.077 mmol/L; hardness = 80–100 mg/L $CaCO_3$; pH = 8.02 and DOM = 0.3 mg C/L) (USEPA, 1994), and acclimated for at least 15 days prior to experiments. During this period, mussels were fed once daily with 1 mL of an algal mixture consisting of 0.5 mL non-viable algae – shellfish diet (Reed Mariculture, Campbell CA, USA) and 1 mL of *Nannochloropsis oculata* (Reef Crew, Aurora, ON, CA) diluted in 900 mL of acclimation water (Ingersoll et al., 2006; Wang et al., 2007). Each batch of algal food was used for one week and kept in refrigerator at 4 °C (Wang et al., 2007). Partial (80%) water changes were completed every 2 days. Mussels were held at room temperature (21 °C) with a photoperiod of 16 h light:8 h dark.

2.2. Experimental design

The present study consisted of three sets of experiments as described below. An initial 6 h experiment was performed in order to determine appropriate exposure concentrations for subsequent experiments. The following nominal concentrations were tested: 0, 2, 12 and 30 μg/L of Cu combined with 0, 3, 6 or 12 mg/L DOM (as dissolved organic carbon (DOC)). Based on the results obtained, subsequent 24 and 96 h exposures were performed using 0, 2 and 12 μg Cu/L combined with 0, 3, or 6 mg DOC/L. All DOM used was derived from a concentrated solution extracted by reverse osmosis from water collected at Luther Marsh (Ontario, Canada). Al-Reasi et al. (2012) described the extraction procedure and the physicochemical characterization of this DOM source. In all cases, DOM and Cu (as $CuCl_2$) were added 24 h prior to experimentation to allow them to stabilize with the experimental media, at room temperature. For the 96 h exposure, one water change took

place at 48 h, following the ASTM recommendations (ASTM, 2006). All experiments were conducted under the same temperature and photoperiod conditions as the acclimation (as described above). Mussels were fasted for 24 h prior to experimentation.

Immediately before the mussels were introduced into the experimental media and also at 24 and 48 h after the start of the experiments, samples from the experimental media were collected for Cu and DOM analyses. pH measurements were taken using a handheld pH meter (Accumet Basic®, AB15 pH meter, Fisher Scientific, Ottawa, ON, Canada) (Table S1, supplementary data). All samples were filtered using a 0.45 μm syringe-tip filter (Acrodisc Supor membrane; Pall Life Sciences, Ville St. Laurent, QC, Canada) prior to acidification to reveal dissolved metal concentration. All samples for Cu analysis were acidified (HNO_3 trace metal grade; Fisher Scientific, Ottawa, ON, Canada) to a final concentration of 1% HNO_3. Samples taken for DOM measurements were not acidified. Dissolved Cu concentrations in the water were measured by graphite furnace atomic absorption spectroscopy (SpectrAA220 with a SpectrAA GTA110, Varian, Mulgrave, Victoria, Australia). A Cu reference solution (Fisher Scientific, Ottawa, ON, Canada) was used to create internal standard curves. In addition, certified reference materials TM24 and TM25 (National Water Research Institute, Environment Canada, Burlington, ON, Canada) were used to demonstrate that Cu recovery was within 20% of certified values (average for TM24 ($n = 19$) = 83.30%; TM25 ($n = 16$) = 90.74%). DOM was measured using a total organic carbon (TOC – $V_{CPH/CPN}$) analyzer (Shimadzu Corporation, Kyoto, Japan; detection limit: 50 μg C/L) and standards were prepared and run according to manufacturer instructions.

2.3. Sodium influx experiments

Sodium influx experiments were performed during the final 6 h of the 24-h and 96-h period of exposures. The radioactive ^{22}Na isotope (Eckert & Ziegler Isotope Products, Valencia, CA, USA) was employed to determine the unidirectional sodium influx rate (J_{in}^{Na}). Juvenile mussels were transferred to individual 50-mL experimental flasks containing the appropriate test media for 3 h prior to the beginning of the experiment. Afterwards, a ^{22}Na solution was added to each flask, leading to a final concentration of 0.05 μCi ^{22}Na/mL. This concentration was chosen based on the mussel weight and results obtained by Dietz (1979) for measuring basal levels of Na^+ influx in another freshwater mussel (*Ligumia subrostrata*). Mussels were held in the experimental media for 6 h after the addition of radioactive ^{22}Na. Following the 6 h exposure, they were removed from the exposure medium and placed in a rinsing solution containing 20 mM "cold" NaCl for 2 min. This "cold displacement rinse" was performed to remove any loosely bound radioisotope from the mussel's shell. Then, the entire live mussel (shell and soft tissue) was blotted dry, placed in a plastic vial and assayed for gamma radioactivity. For all isotope flux experiments, a water sample (1 mL) was obtained 5 min after the addition of ^{22}Na to the experimental media (initial), and again following 6 h of exposure (final). Radioactivity from ^{22}Na (measured as counts per minute, cpm) in the water and entire live mussel was determined using a Canberra Packard Minaxi Auto gamma 5000 series gamma-counter (Meridien, CT, USA).

The Na^+ influx rate (nmol/g/h) was calculated as:

$$J_{in}^{Na} = \mathrm{cpm}(A)/SA/W/T, \quad (1)$$

where cpm (A), W, and T indicate the radioactivity measured in the entire animal, mussel weight (g) and experimental time (h), respectively. SA represents the average specific activity calculated as:

$$SA = \left[\frac{(\mathrm{cpm/total\ Na^+})_i + (\mathrm{cpm/total\ Na^+})_f}{2}\right] \quad (2)$$

where cpm value represents the ^{22}Na radioactivity/L measured by gamma counting and total Na^+ value corresponds to the total Na^+ concentration (μmol/L) measured by atomic absorption spectroscopy (Varian Spectra AA220) in the initial and final water samples obtained from the experimental media.

At the end of each exposure and subsequent radioactive measurements, the whole soft tissue mass (i.e. no internal tissue differentiation) was removed from the shell of each animal with forceps and flash frozen in liquid nitrogen for subsequent enzymatic activity and ion content analyses. The remaining shells were discarded. Samples used to measure reactive oxygen species (ROS) were held on ice for a maximum of 3 h until assay, as described below. As the mass of tissue in each mussel was small, different animals were employed for the different analyses (Sections 2.4, 2.5 and 2.6).

2.4. Whole-body ion content and Cu bioaccumulation

Whole soft tissues were thawed, blotted dry, weighed (wet weight), dried to a constant weight in a 68 °C oven and weighed again (dry weight). Tissues were digested with 1 mL of 77% HNO_3 (HNO_3 trace metal grade; Fisher Scientific, Ottawa, ON, Canada) in sealed vials for 48 h at room temperature. From this main solution, aliquots were taken for measurements of whole-body ion content (Na^+, K^+, Mg^{2+}, Ca^{2+}) by atomic absorption spectroscopy (Varian Spectra AA220), and Cu accumulated in tissues by graphite furnace atomic absorption spectroscopy (Varian, SpectrAA220 with a SpectrAA GTA110 furnace). Certified standards and reference materials were employed, as previously described (Section 2.2).

2.5. Enzymatic analyses

Prior to enzymatic analyses, whole-body mussel samples were thawed and homogenized (1:10 w/v) in ice-cold buffer solution containing 50 mM imidazole, 125 mM sucrose and 5 mM EGTA with pH adjusted to 7.3. Samples were then centrifuged (5000 × *g*, 4 °C, for 3 min) and the respective supernatants collected and kept on ice throughout the analysis. Na^+/K^+-ATPase and v-type H^+-ATPase activity in homogenates were determined concomitantly, following the protocols described in Bianchini and Wood (2003) and Lin and Randall (1993), respectively. Briefly, both methods calculate the difference in the amount of adenosine diphosphate (ADP) produced by the samples when assayed in a control reaction (no inhibitor added) and in an inhibition reaction. Ouabain (Sigma–Aldrich, St. Louis, MO, USA) and sodium azide with N-ethylmaleimide (NEM) (Sigma–Aldrich, St. Louis, MO, USA) were used as inhibitors of Na^+/K^+-ATPase and v-type H^+-ATPase, respectively. Absorbance (340 nm) from the reaction mixture was evaluated at 20 s intervals over 45 min at 20 °C using a microplate reader (SpectraMAX Plus; Molecular Devices, Menlo Park, CA, USA). Enzyme activity was expressed as μmol ADP/mg protein/h.

Carbonic anhydrase (CA) activity was measured according to Henry (1991). The same homogenate described above was added to a reaction buffer (225 mM mannitol; 75 mM sucrose; 10 mM Tris buffer; 10 mM NaH_2PO_4; pH 7.4) and kept at 2–4 °C throughout the analysis. The reaction was initiated by adding 1 mL of CO_2-saturated distilled water. Change in pH of the reaction mixture was measured every 5 s during the following 30 s with an electrode connected to a handheld pH meter (Accumet Basic®, AB15 pH meter, Fisher Scientific, Ottawa, ON, Canada). Enzyme activity

was calculated by dividing the pH slope of the reaction in the presence of the sample homogenate by the slope of a blank reaction (i.e., no sample homogenate addition). The protein concentration in the homogenates was determined using the Bradford Reagent (Sigma–Aldrich, St. Louis, MO, USA), and calculated based on a standard curve built with bovine serum albumin (Sigma–Aldrich, St. Louis, MO, USA).

2.6. Reactive oxygen species and total antioxidant capacity against peroxyl radicals

Immediately after dissection, whole-body soft tissues were weighed and homogenized (1:10 w/v) in ice-cold buffer solution (Tris–HCl 100 mM, EDTA 2 mM, $MgCl_2 \cdot 6H_2O$ 5 mM, pH adjusted to 7.75). Samples were centrifuged (10,000 × g, 20 min, 4 °C) and the supernatant collected and employed in analyses. Total reactive oxygen species (ROS) were determined as previously described by Amado et al. (2009). Briefly, the supernatant (10 μL) was transferred to a black 96-well microplate, followed by a reaction buffer containing 30 mM HEPES, 200 mM KCl and 1 mM $MgCl_2 \cdot 6H_2O$ (pH 7.2). Immediately before reading, 40 mM H_2DCF-DA (2′,7′-dichlorofluorescein diacetate) solution was added to each well. Fluorescence (excitation: 488 nm; emission: 525 nm) produced by the cleavage of H_2DCF-DA into the fluorescent probe DCF (2′,7′-dichlorofluorescein) was monitored over 45 min, with readings every 5 min, using a microplate reader with controlled temperature (35 °C) (Biotek FLx800, Biotek Instrument Inc., Winooski, VT, USA). ROS concentration was calculated after adjusting total fluorescence production over the 45 min into a second order polynomial function, and integrating fluorescence data over time. ROS concentration was normalized considering the total protein content in the supernatant.

Total antioxidant capacity against peroxyl radicals (ACAP) was also assayed following procedures described by Amado et al. (2009). The ACAP assay employed the same homogenate, reaction buffer and fluorescent probe described above for measurement of ROS concentration. Briefly, six replicates of each soft tissue homogenate were transferred to a black 96-well microplate. In half of the replicates, 4 mM ABAP solution (2,2′-azobis 2 methylpropionamidine dihydrochloride, Sigma–Aldrich) was added. ABAP causes ROS generation at temperatures higher than 35 °C. Therefore, this method measures the sample capacity to scavenge the ROS generated by comparing the total fluorescence production (calculated as described above) in untreated samples against that in ROS-induced samples by ABAP addition. A higher difference indicates a lower antioxidant capacity. Therefore, data were expressed as 1/total fluorescence production, and normalized considering the total protein content in homogenates, as previously described by Jorge et al. (2013).

2.7. Data analysis

In general, data have been expressed as means ± 1 SEM. Mean values were compared through one-way analysis of variance (ANOVA), firstly among different Cu treatments within a given DOM concentration, and secondly, among different DOM treatments within a given Cu concentration. For these analyses, data were grouped according to nominal values of Cu and DOM, but actual measured values of these water chemistry parameters have been reported in Tables 1 and 2. ANOVA was followed by the Tukey test. In all cases, mean values were considered significantly different when $p < 0.05$. ANOVA assumptions (data normality and homogeneity of variances) were previously checked, and if not achieved, data were transformed using a square root transformation (Zar, 1984).

Table 1
Dissolved Cu and dissolved organic carbon (DOC) concentrations measured in the experimental media for the 6-h duration experiment. Data are expressed as mean ± standard error. Mean values sharing the same upper case letter are not significantly different among Cu treatments within a given DOC concentration. Mean values sharing the same lower case letter are not significantly different among DOC treatments within a given Cu concentration.

Experimental treatments	Dissolved Cu (μg/L)	DOC (mg/L)
	Mean (0–6 h) ± SEM (n = 10–20)	Mean (0–6 h) ± SEM (n = 10–20)
Control	0.16 ± 0.06 ^{Aa}	0.59 ± 0.07 ^{Aa}
3 mg/L DOC	0.23 ± 0.20 ^{Aa}	2.30 ± 0.01 ^{Ab}
6 mg/L DOC	1.30 ± 0.1 ^{Cb}	4.65 ± 0.05 ^{ABc}
12 mg/L DOC	0.61 ± 0.07 ^{Ac}	8.70 ± 0.07 ^{Ad}
2 μg/L Cu	1.65 ± 0.19 ^{Ba}	1.06 ± 0.16 ^{Ba}
2 μg/L Cu + 3 mg/L DOC	2.16 ± 0.11 ^{Ba}	2.99 ± 0.18 ^{Bb}
2 μg/L Cu + 6 mg/L DOC	2.31 ± 0.20 ^{Ba}	4.94 ± 0.12 ^{Ac}
2 μg/L Cu + 12 mg/L DOC	3.04 ± 0.29 ^{Bb}	8.63 ± 0.07 ^{Ad}
12 μg/L Cu	7.43 ± 0.4 ^{Ca}	0.51 ± 0.07 ^{Aa}
12 μg/L Cu + 3 mg/L DOC	10.34 ± 0.1 ^{Cb}	2.47 ± 0.04 ^{Bb}
12 μg/L Cu + 6 mg/L DOC	11.95 ± 0.2 ^{Cc}	4.25 ± 0.25 ^{Ac}
12 μg/L Cu + 12 mg/L DOC	12.03 ± 0.0 ^{Ccd}	8.60 ± 0.09 ^{Ad}
30 μg/L Cu	21.18 ± 0.94 ^{Da}	0.44 ± 0.04 ^{Aa}
30 μg/L Cu + 3 mg/L DOC	25.46 ± 0.49 ^{Dbc}	2.37 ± 0.02 ^{Ab}
30 μg/L Cu + 6 mg/L DOC	24.69 ± 0.48 ^{Db}	4.92 ± 0.04 ^{Ac}
30 μg/L Cu + 12 mg/L DOC	28.18 ± 0.69 ^{Dc}	8.70 ± 0.14 ^{Ad}

3. Results

3.1. Experimental conditions and mussel survival

Dissolved Cu and DOM concentrations measured in each exposure solution during the experiments are summarized in Tables 1 and 2. Two consistent trends are noteworthy. Firstly, within any nominal Cu concentration, measured Cu concentrations in the exposure water were lower in the absence of added DOM than when DOM was raised to nominal values of 3, 6, or 12 mg C/L. Secondly, measured DOM concentrations (as DOC) were consistently lower than nominal values by 10–30%.

The mortality rate of exposed mussels across all experiments was <1%, demonstrating the good overall health of test organisms. All observed effects were therefore considered sublethal.

3.2. Unidirectional Na^+ influx rate during 6 h acute exposure

In the initial experiment, a non-significant trend of increasing unidirectional Na^+ influx was observed when mussels were exposed to the three increasingly higher levels of DOM for 6 h in the absence of added Cu (Fig. 1). This trend became significant in the presence of all three concentrations of added Cu, and was most pronounced at 30 μg/L Cu. Na^+ influx was strongly inhibited when mussels were exposed to both 12 and 30 μg/L Cu (Fig. 1). Regardless of the Cu concentration used, when Cu exposure was combined with any amount of DOM, Na^+ influx recovered partially or completely, indicating a protective effect of DOM (Fig. 1).

3.3. Unirectional Na^+ influx rate after 24 h and 96 h Cu exposures

In order to determine effects of Cu and DOM on Na^+ uptake, Na^+ influx rate was measured during the final 6 h of the 24-h and 96-h exposures. After 24 h of exposure, Na^+ uptake rate in Cu-exposed mussels only was not significantly different from that of control mussels (0 μg/L Cu). Nevertheless, Na^+ uptake rate in mussels exposed to 12 μg/L Cu was significantly lower than in those exposed to 2 μg/L Cu (Fig. 2A) in the absence or in the presence of 3 mg C/L of added DOM. Mussels exposed to the highest Cu concentration tested combined with DOM at 6 mg C/L were able to recover

Table 2
Dissolved Cu and dissolved organic carbon (DOC) concentrations measured in the experimental media used for the 24- and 96-h duration experiments. Data are expressed as mean ± standard error. Mean values sharing the same upper case letter are not significantly different among Cu treatments within a given DOC concentration. Mean values sharing the same lower case letter are not significantly different among DOC treatments within a given Cu concentration.

Experimental treatments	24-h experiment		96-h experiment	
	Dissolved Cu (μg/L) Mean (0–24 h) ± SEM (n = 9 – 10)	DOC (mg/L) Mean (0–24 h) ± SEM (n = 100–20)	Dissolved Cu (μg/L) Mean (0–96 h) ± SEM (n = 5)	DOC (mg/L) Mean (0–96 h) ± SEM (n = 5)
Control	0.11 ± 0.03 ^{Aa}	0.51 ± 0.10 ^{ABa}	0.43 ± 0.13 ^{Aa}	0.40 ± 0.07 ^{Aa}
3 mg/L DOC	0.84 ± 0.08 ^{Ab}	2.97 ± 0.39 ^{ABb}	0.20 ± 0.09 ^{Aa}	2.85 ± 0.04 ^{Ab}
6 mg/L DOC	0.73 ± 0.30 ^{Ab}	4.82 ± 0.04 ^{Ac}	1.39 ± 0.31 ^{Ab}	5.70 ± 0.52 ^{Ac}
2 μg/L Cu	0.27 ± 0.29 ^{Aa}	0.99 ± 0.32 ^{Aa}	0.63 ± 0.11 ^{Aa}	0.36 ± 0.08 ^{Aa}
2 μg/L Cu + 3 mg/L DOC	2.00 ± 0.12 ^{Bb}	3.32 ± 0.33 ^{Ab}	1.69 ± 0.13 ^{Ab}	2.80 ± 0.06 ^{Ab}
2 μg/L Cu + 6 mg/L DOC	2.36 ± 0.14 ^{Ab}	5.52 ± 0.26 ^{Bc}	2.50 ± 0.10 ^{Ac}	5.12 ± 0.49 ^{Ac}
12 μg/L Cu	9.30 ± 0.13 ^{Ba}	0.21 ± 0.10 ^{Ba}	8.61 ± 0.92 ^{Ba}	1.22 ± 0.54 ^{Ba}
12 μg/L Cu + 3 mg/L DOC	11.09 ± 0.10 ^{Cab}	2.47 ± 0.02 ^{Bb}	12.53 ± 0.62 ^{Bb}	3.27 ± 0.05 ^{Bb}
12 μg/L Cu + 6 mg/L DOC	13.00 ± 1.29 ^{Bb}	4.78 ± 0.04 ^{Ac}	12.79 ± 0.64 ^{Bb}	5.54 ± 0.45 ^{Ac}

the Na^+ influx rate to the control level (0 μg/L Cu + 0 mg/L DOM added), indicating a protective effect of DOM (Fig. 2A).

At 96 h of exposure, a very clear positive effect of DOM on Na^+ metabolism was seen, where mussels exposed to 6 mg C/L as DOM had their mean Na^+ uptake rate enhanced by 2.7-fold relative to that of control mussels (0 μg/L Cu + 0 mg/L DOM) in the absence of added Cu (Fig. 2B). This pattern was also consistent across both Cu concentrations tested (2 and 12 μg/L Cu) combined with DOM (Fig. 2B).

3.4. *Activity of ionoregulatory enzymes after 24 and 96 h exposure*

No significant change in Na^+/K^+-ATPase activity was observed in mussels exposed to Cu alone for 24 h, regardless of the added DOM concentration (Fig. 3A). Furthermore, the Na^+/K^+-ATPase activity pattern at 96 h (Fig. 3B) was similar to that seen for Na^+ uptake at 24 h (cf. Fig. 2A). In this case, enzyme activity at 12 μg/L Cu in the absence of added DOM was lower than that observed at 2 μg/L Cu under the similar condition, but neither of the mean enzyme activity values were significantly different from that observed for

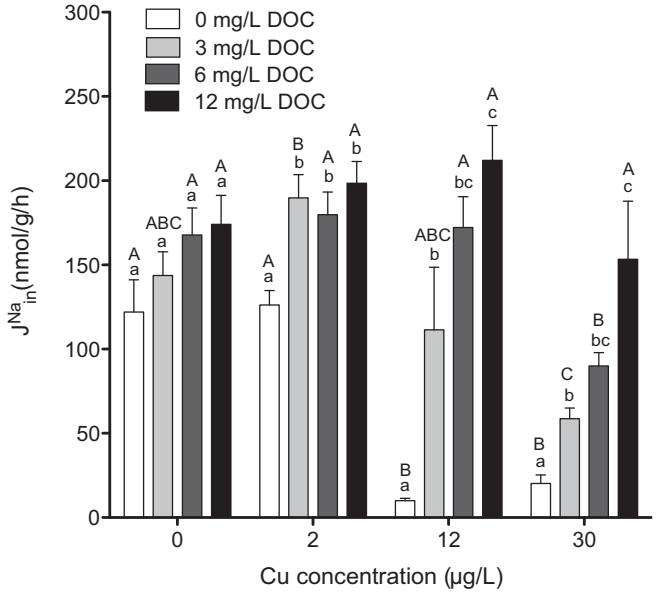

Fig. 1. Na^+ influx rate in mussels exposed to Cu and dissolved organic matter for 6 h. Data are expressed as mean ± SEM (n = 5–13). Mean values sharing the same upper case letter are not significantly different among Cu treatments within a given DOC concentration. Mean values sharing the same lower case letter are not significantly different among DOC treatments within a given Cu concentration.

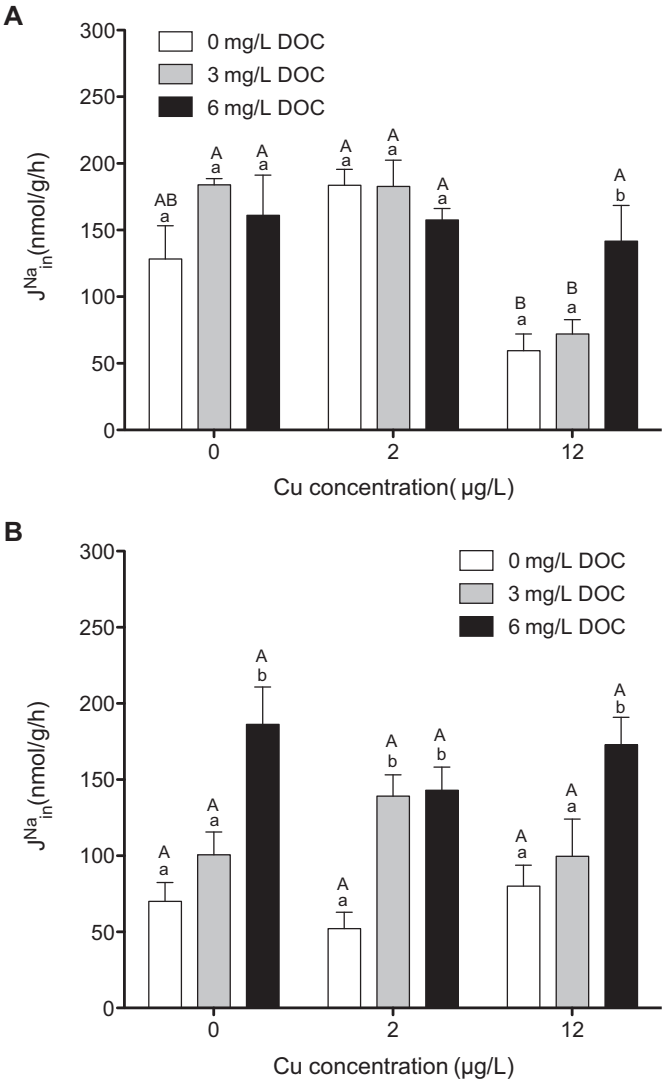

Fig. 2. Na^+ influx rate in mussels exposed to Cu and dissolved organic matter for 24 h: (A) (n = 5–10) and 96 h and (B) (n = 5–10). Data are expressed as mean ± SEM. Mean values sharing the same upper case letter are not significantly different among Cu treatments within a given DOC concentration. Mean values sharing the same lower case letter are not significantly different among DOC treatments within a given Cu concentration.

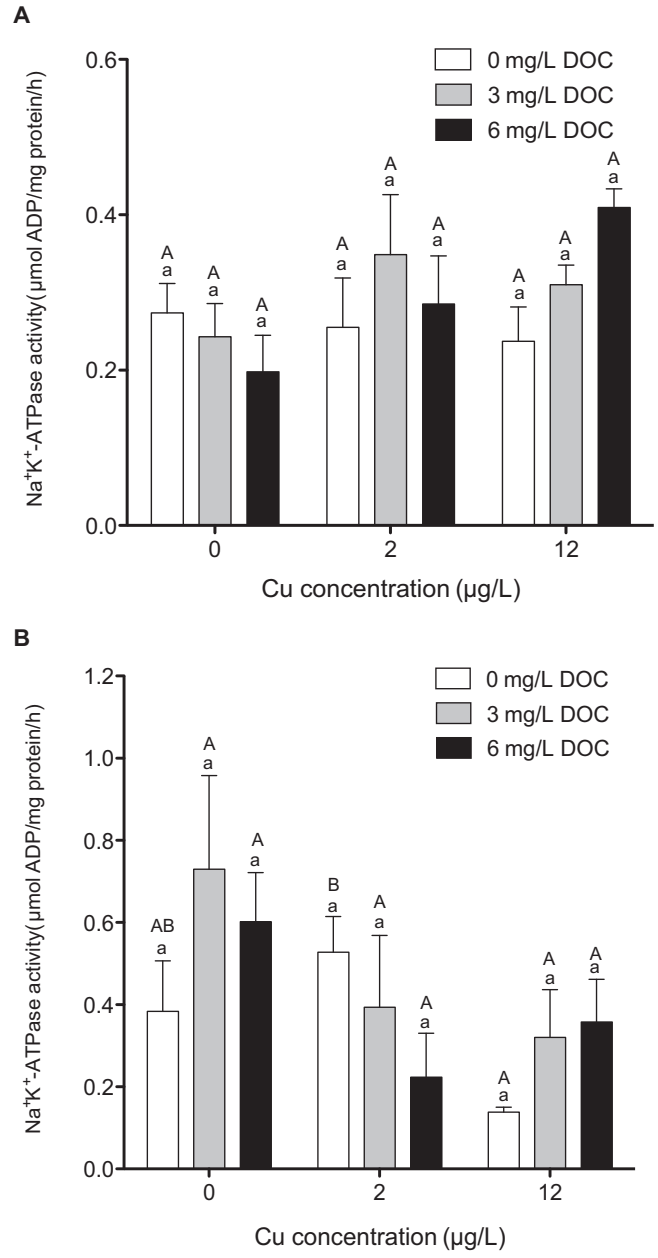

Fig. 3. Whole-body Na^+K^+-ATPase activity in mussels exposed to Cu and dissolved organic matter for 24 h: (A) (n = 5–15) and 96 h (B) (n = 3–9). Data are expressed as mean ± SEM. Mean values sharing the same upper case letter are not significantly different among Cu treatments within a given DOC concentration. Mean values sharing the same lower case letters are not significantly different among DOC treatments within a given Cu concentration.

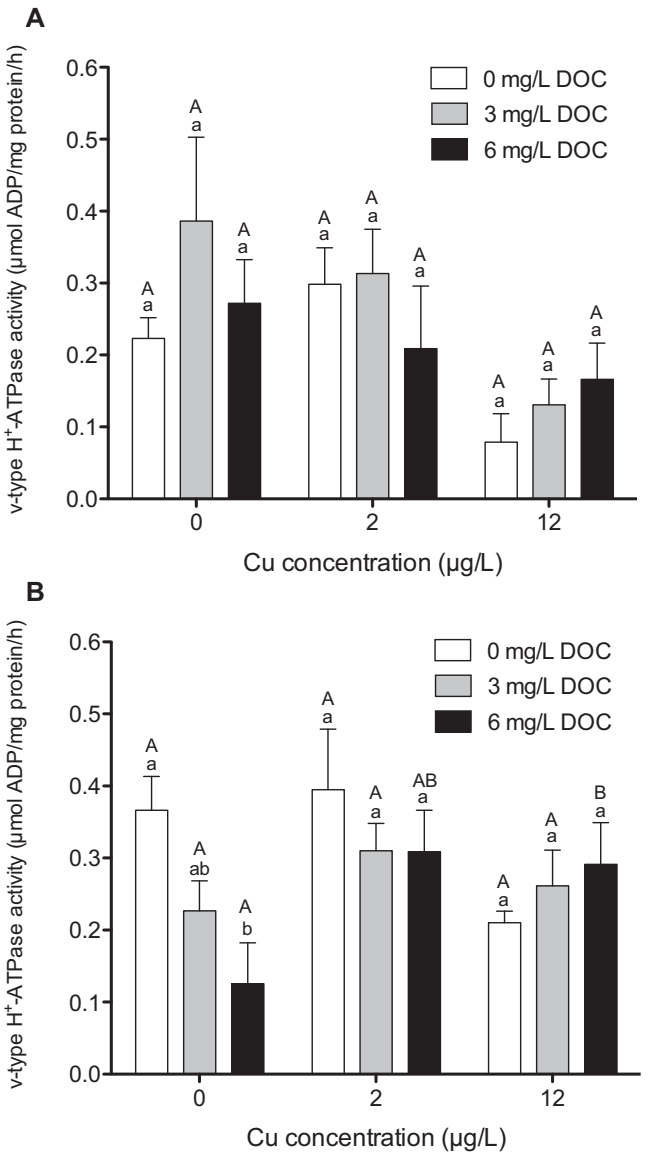

Fig. 4. Whole-body v-type H^+-ATPase activity in mussels exposed to Cu and dissolved organic matter for 24 h (A) (n = 5–15) and 96 h (B) (n = 3–9). Data are expressed as mean ± SEM. Mean values sharing the same upper case letter are not significantly different among Cu treatments within a given DOC concentration. Mean values sharing the same lower case letters are not significantly different among DOC treatments within a given Cu concentration.

control mussels (no Cu added). In mussels exposed to DOM alone, no significant change in Na^+/K^+-ATPase activity was observed, although there was a trend toward increasing enzyme activity. Na^+/K^+-ATPase activity was significantly lower in mussels exposed to 12 µg/L Cu than in those exposed to 2 µg/L. Therefore exposure to Cu in the presence of DOM does not seem to alter activity in a clear way (Fig. 3B).

For both experimental durations (24 and 96 h), there was no significant change in carbonic anhydrase activity in mussels exposed to any treatment when compared to control levels (6.67 ± 1.07 enzyme activity/mg protein) (Figs. S1A and S1B, supplementary data). Similarly, there was no significant difference in v-type H^+-ATPase activity in mussels exposed for 24 h, although there was a non-significant trend for a lowered enzyme activity in mussels exposed to 12 µg/L Cu at each DOM concentration tested (Fig. 4A). However, mussels exposed for 96 h to DOM alone (no Cu addition) showed a significant concentration-dependent decrease in H^+-ATPase activity. Indeed, H^+-ATPase activity in mussels exposed to 6 mg C/L DOM was only ~30% of that measured in control mussels (0 µg/L Cu + 0 mg/L DOM) (Fig. 4B). This DOM effect was not seen in the presence of either 2 or 12 µg/L Cu.

3.5. *Whole-body ion concentrations after 24 and 96 h exposures*

At 24 h, there was no significant change in whole-body Na^+ content in Cu-exposed mussels in any treatment. However, the presence of 3 or 6 mg C/L DOM significantly increased (~15%) the whole-body Na^+ content in the presence of 2 µg/L Cu. This effect was not seen at 12 µg/L Cu (Fig. 5A). However, there was a significant decrease in whole-body Na^+ content by about 15% at 2 µg/L Cu, and 35% at 12 µg/L Cu in the absence of added DOM after 96 h of

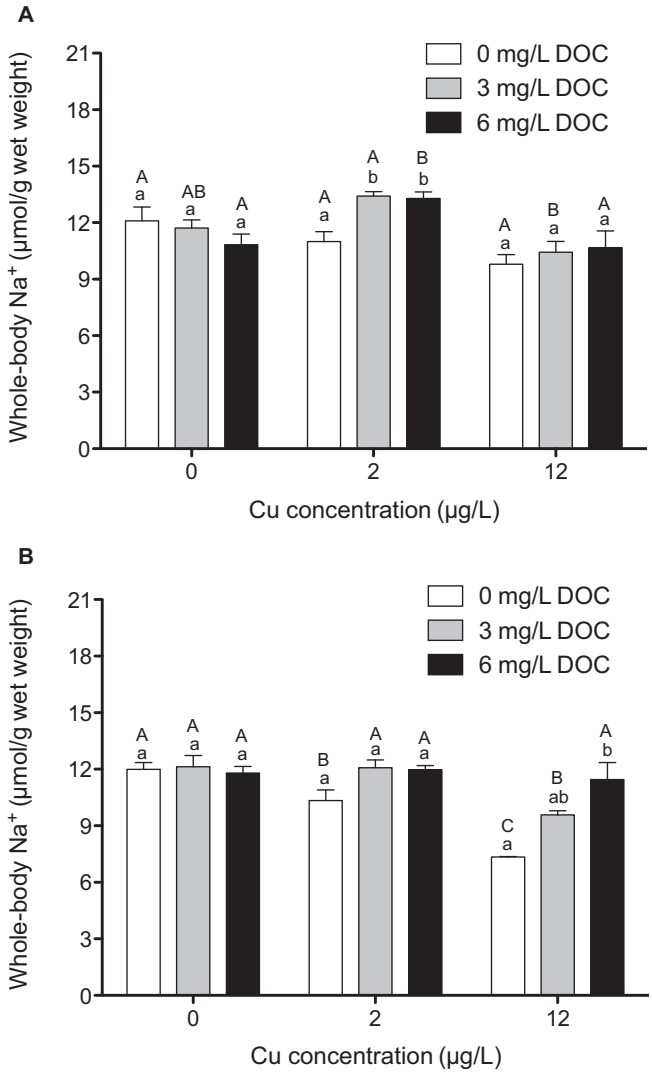

Fig. 5. Whole-body Na^+ content in mussels exposed to Cu and dissolved organic matter for 24 h: (A) (n = 3–10) and 96 h (B) (n = 3–10). Data are expressed as mean ± SEM. Mean values sharing the same upper case letter are not significantly different among Cu treatments within a given DOC concentration. Mean values sharing the same lower case letters are not significantly different among DOC treatments within a given Cu concentration.

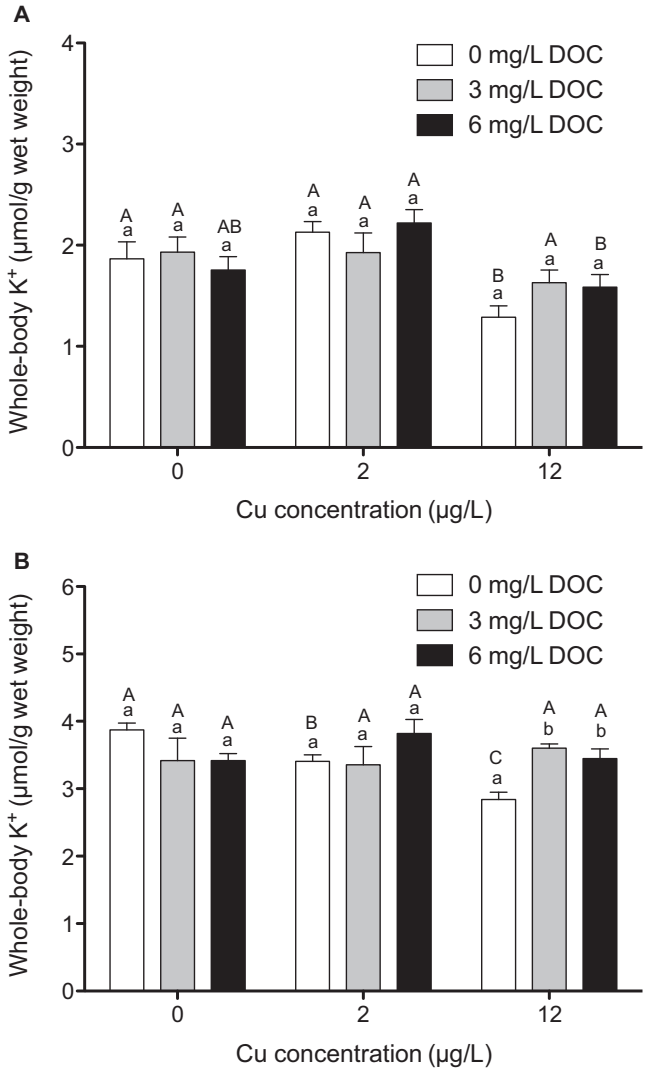

Fig. 6. Whole-body K^+ content in mussels exposed to Cu and dissolved organic matter for 24 h: (A) (n = 4–9) and 96 h (B) (n = 3–10). Data are expressed as mean ± SEM. Mean values sharing the same upper case letter are not significantly different among Cu treatments within a given DOC concentration. Mean values sharing the same lower case letters are not significantly different among DOC treatments within a given Cu concentration.

exposure (Fig. 5B). When either 3 or 6 mg C/L DOM was combined with the highest Cu concentration tested (12 µg/L), whole-body Na^+ content was similar to that observed in control mussels (0 µg/L Cu + 0 mg/L DOM), evidence again of the protective effect of DOM. Whole-body Na^+ content did not change in mussels exposed to DOM alone for 96 h in the absence of added Cu (Fig. 5B).

In the absence of added DOM, whole-body K^+ content in mussels exposed to 12 µg/L Cu for 24 h was significantly reduced (Fig. 6A). At 96 h of exposure, reduction was significant at both 2 and 12 µg/L Cu (Fig. 6B). These effects were prevented by the presence of both concentrations of DOM tested (Fig. 6A and B).

No significant change in whole-body Ca^{2+} content was observed in mussels exposed to Cu for 24 h (Table 3). Mussels exposed for 96 h to 12 µg Cu/L had significantly lower whole-body Ca^{2+} content when compared to control mussels (0 µg/L Cu), an effect that was prevented by the two DOM concentrations tested (Table 3). Similarly, no change in whole-body Mg^{2+} content was observed, at either exposure time (24 and 96 h), regardless of the Cu concentration tested (Table 3). The reason for the consistently lower whole body Mg^{2+} values in the 96-h exposures relative to the 24-h exposures is unknown, but checks showed that this was not due to analytical error. The experiments were performed at different times, suggesting temporal variation in mussel physiology.

3.6. *Oxidative stress-related parameters after 24 and 96 h of exposure*

There was negligible evidence for oxidative stress in mussels subjected to the various treatments tested. No significant difference in ROS concentration was found among treatments, when compared to the general control condition (34,024 ± 4469 fluorescence units/mg protein) for both experimental durations (Figs. S2A and S2B, supplementary data). Although no major difference was found for the antioxidant capacity against peroxyl radicals (ACAP, control mean = 0.89 ± 0.11 fluorescence units/mg protein), a significant decrease was observed in mussels exposed to 12 µg Cu/L combined with 6 mg C/L DOM when compared to those exposed to DOM at 6 mg C/L and no Cu added for 24 h (Fig. S3A, supplementary data). In mussels exposed for 96 h, no significant change in ACAP was observed (Fig. S3B, supplementary data).

Table 3
Whole-body Ca^{2+} and Mg^{2+} content in mussels exposed to Cu and dissolved organic matter for 24 and 96 h. Data are expressed as mean ± standard error. Mean values sharing the same upper case letter are not significantly different among Cu treatments within a given DOC concentration. Mean values sharing the same lower case letter are not significantly different among DOC treatments within a given Cu concentration. Mean values were compared only within the same experimental time.

Experimental treatments	Whole-body Ca^{2+} content (μmol/g/wet weight)		Whole-body Mg^{2+} content (μmol/g/wet weight)	
	24 h Mean ± SEM (n = 5)	96 h Mean ± SEM (n = 5)	24 h Mean ± SEM (n = 5)	96 h Mean ± SEM (n = 5)
Control	76.37 ± 3.05 ^{Aa}	89.70 ± 2.26 ^{Aa}	23.85 ± 2.61 ^{Aa}	15.60 ± 0.70 ^{Aa}
3 mg/L DOC	82.68 ± 1.46 ^{Aa}	77.61 ± 3.04 ^{Ab}	24.71 ± 5.03 ^{Aa}	14.86 ± 0.78 ^{Aa}
6 mg/L DOC	70.15 ± 4.24 ^{Aa}	80.51 ± 2.40 ^{Aab}	24.23 ± 3.94 ^{Aa}	14.49 ± 0.82 ^{Aa}
2 μg/L Cu	82.69 ± 12.27 ^{Aa}	97.52 ± 1.78 ^{Aa}	21.00 ± 3.76 ^{Aa}	12.00 ± 0.72 ^{Ba}
2 μg/L Cu + 3 mg/L DOC	67.74 ± 4.26 ^{Aa}	86.71 ± 4.12 ^{Aa}	19.43 ± 3.38 ^{Aa}	12.88 ± 0.97 ^{Aa}
2 μg/L Cu + 6 mg/L DOC	70.39 ± 4.56 ^{Aa}	87.37 ± 9.18 ^{Aa}	26.96 ± 6.83 ^{Aa}	13.29 ± 0.83 ^{Aa}
12 μg/L Cu	83.31 ± 5.88 ^{Aa}	70.14 ± 2.88 ^{Ba}	25.79 ± 5.72 ^{Aa}	12.19 ± 0.82 ^{ABa}
12 μg/L Cu + 3 mg/L DOC	94.26 ± 14.79 ^{Aa}	89.76 ± 6.27 ^{Aa}	24.44 ± 5.81 ^{Aa}	13.45 ± 0.42 ^{Aa}
12 μg/L Cu + 6 mg/L DOC	85.14 ± 6.87 ^{Aa}	75.49 ± 6.76 ^{Aa}	27.87 ± 5.13 ^{Aa}	13.89 ± 0.99 ^{Aa}

3.7. Cu bioaccumulation after 24 and 96 h exposure

At 24 h, whole-body Cu content was significantly higher in mussels exposed to 12 μg Cu/L than in those exposed to 2 μg Cu/L (Fig. 7A). This effect was not prevented by the presence of either 3 or 6 mg C/L DOM. At 96 h, whole-body Cu content was similar to that observed at 24 h. However, DOM appeared to exhibit a protective role against Cu accumulation at 96 h. Indeed, whole-body Cu content was significantly decreased when 6 mg C/L DOM was combined with 12 μg/L Cu (Fig. 7B).

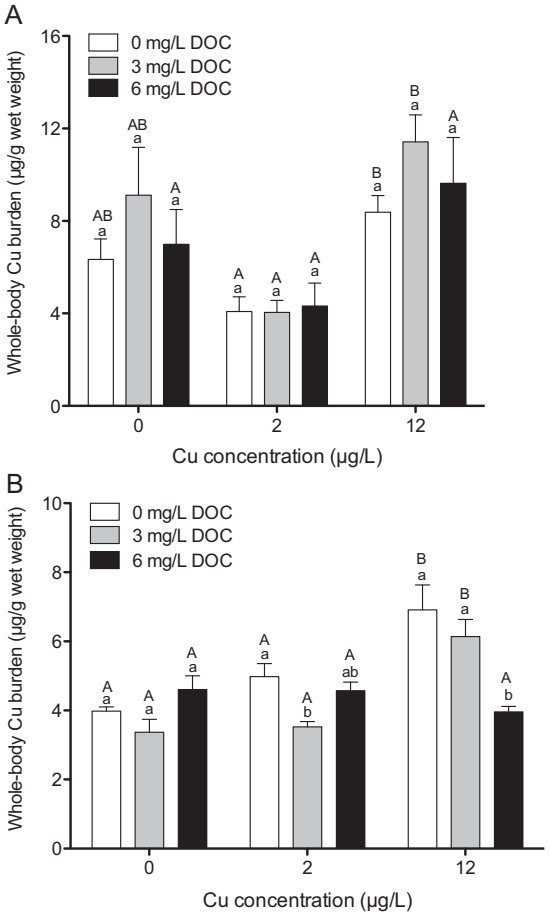

Fig. 7. Whole-body Cu content in mussels exposed to Cu and dissolved organic matter for 24 h: (A) (n = 5–10) and 96 h (B) (n = 3–10). Data are expressed as mean ± SEM. Mean values sharing the same upper case letter are not significantly different among Cu treatments within a given DOC concentration. Mean values sharing the same lower case letters are not significantly different among DOC treatments within a given Cu concentration.

4. Discussion

4.1. Overview and implications for mussel survival

Results obtained support our original hypothesis that acute exposure to environmentally realistic concentrations of Cu (2–30 μg/L; Grosell, 2012; Jorge et al., 2013) would negatively affect the physiology of freshwater mussels, through a disruption of Na^+ homeostasis, rather than through an induction of oxidative stress. Also, these results help clarify the mechanism of this Cu-induced ionoregulatory effect, which can be explained considering the inhibited active unidirectional Na^+ influx from the water (Figs. 1 and 2), reduced whole-body Na^+/K^+-ATPase activity (Fig. 3) (with no change in v-type H^+-ATPase and carbonic anhydrase activity), and decreased whole-body Na^+ (Fig. 5) and K^+ (Fig. 6) content. Our results also support our hypothesis that DOM, at environmentally realistic levels, would reduce or prevent the physiological disturbance in Na^+ homeostasis caused by exposure to low level of Cu (2–12 μg/L) (Figs. 1, 2, 3 and 5), as well as reduce whole-body Cu accumulation (Fig. 7). Finally, they strongly support our idea that DOM itself, in the absence of Cu, would have direct positive effects on Na^+ metabolism (Figs. 1 and 2B), thereby contributing to its protective role against the acute Cu toxicity in sensitive freshwater mussels.

There was negligible mussel mortality during the 6, 24 and 96 h experiments. Therefore, all effects reported in the present study were considered as being sub-lethal within this short time frame. However, in comparable 28-day experiments performed by Jorge et al. (2013) with the same life stage of *Lampsilis siliquoidea*, but in the absence of added DOM (a background DOM concentration of ~0.4 mg C/L), cumulative mortality was significantly higher at both 2 μg Cu/L (21%) and 12 μg Cu/L (70%) when compared to the control condition (0 μg/L Cu) (12.5%), with clear evidence of Cu-induced ionoregulatory disturbance (marked reduction in whole-body Na^+ content and decreased Na^+/K^+-ATPase activity). Therefore, inhibitions in Na^+ influx and Na^+/K^+-ATPase activity observed in the present study could have led to significant mortality had the experiment been extended. While these inhibitions were largely prevented by addition of DOM up to 96 h of exposure, it is unknown whether the protective role of DOM would remain effective during chronic exposures.

4.2. Experimental conditions

Direct measurements revealed that dissolved Cu concentrations in water samples taken from the exposure containers were lower when DOM was not added into the experimental medium, and were usually well below the nominal values (Tables 1 and 2). While not

ideal, this is the practical reality of closed system exposures. Free Cu is innately "sticky", adsorbing readily to the external surface of the organism and the walls of the exposure chamber, whereas added DOM complexes Cu and helps keep it in solution. The hypothesis that the DOM source could be Cu-loaded was refuted, since in every experiment, Cu concentration in the DOM stock solution was measured, and only background levels of Cu were detected (0.20–1.39 μg/L). Regardless, the discrepancy is a conservative error, because the greatest negative effects on the animal physiology (i.e. ionoregulatory disturbances) generally occurred in those treatments lacking added DOM, where the dissolved Cu concentration was the lowest (Figs. 1–3, 5 and 6). Therefore, added DOM offered protection against the acute Cu toxicity, despite keeping more Cu in solution. The fact that measured DOM concentrations (as DOC) were consistently lower than nominal values by 10–30% (Tables 1 and 2) is another experimental reality. In closed system exposures, a small amount of DOM may come out of solution onto surfaces or be taken up by the organism (Roditi et al., 2000; Baines et al., 2005). Nevertheless, the percentage loss appeared to be uniform across treatments, so no systematic bias was induced.

4.3. Effects of Cu exposure alone

As sedentary filter feeding animals, freshwater mussels are exposed via multiple routes to dissolved metals in the water, and therefore, can accumulate these metals to concentrations that exceed those dissolved in water (Ray, 1984). After 96 h of exposure to Cu at 12 μg/L, mussels accumulated up to twice the amount of metal found in those kept under no Cu conditions (Fig. 7B). Although mortality has been highly associated with whole-body metal accumulation in other studies with invertebrates (e.g. Borgmann et al., 1991; Adams et al., 2011; Ng et al., 2012), no significant mortality was observed in the present experiments. Recently, Jorge et al. (2013) similarly reported no correlation between Cu accumulation and mortality in juvenile *L. siliquoidea* after chronic exposure to identical Cu concentrations.

In order to determine appropriate exposure concentrations, we performed the initial 6 h duration experiments using a series of Cu and DOM levels, with unidirectional Na^+ influx measurements (Fig. 1). Exposure to 12 and 30 μg/L of Cu for only 6 h resulted in strong inhibition of Na^+ uptake rate in juvenile *L. siliquoidea*, indicating the high sensitivity and rapid response of this freshwater mussel species to metal exposure. However, the same marked effects were attenuated or disappeared when the exposure time was raised to 24 and 96 h (Fig. 2). Nevertheless, whole-body Na^+ concentration had declined significantly in mussels exposed for 96 h to either 2 μg/L Cu (by about 15%) or to 12 μg/L Cu (by about 35%, Fig. 5B). We know from the chronic exposure studies performed by Jorge et al. (2013) that this slow decline continues for up to 28 d, eventually reaching 50–70% losses of whole-body Na^+, which are associated with mortality. Thus, one could infer that the mechanism by which Cu exerts toxicity to these freshwater mussels is mainly through a disruption in Na^+ metabolism. The initial cause may be the competition by Cu for the epithelial Na^+ uptake sites in the mussel, thereby explaining the reduction in unidirectional Na^+ influx. However, the later recovery of Na^+ influx observed at 24–96 h (Fig. 2) suggests that some recovery or acclimation occurs. Notably however, there was some indication that an inhibition of Na^+/K^+-ATPase activity had started after 96 h of exposure. In fact, Jorge et al. (2013) demonstrated that after exposure for 7–14 days to the same two Cu concentrations tested in the present study, inhibition of Na^+/K^+-ATPase activity became highly significant. Na^+/K^+-ATPase is the key enzyme creating the electrochemical gradients needed for the performance of active Na^+ uptake. Potentially this could result in a second phase of inhibition of active Na^+ uptake. Alternatively or additionally, it is possible that Cu exposure causes an increase in unidirectional Na^+ efflux. This has been often described in Cu-exposed fish (e.g. Laurén and McDonald, 1985), and could be an important factor contributing to net Na^+ loss in freshwater mussels. Interestingly, Cu had no apparent detrimental effects on two other enzymes thought to be intimately involved in active Na^+ uptake, the v-type H^+-ATPase (Fig. 4) and carbonic anhydrase (Supplementary Fig. S1). Grosell (2012) noted that as yet there is no evidence that Cu interferes with H^+-ATPase, but recently Zimmer et al. (2012) have shown that exposure to low level of Cu can inhibit the branchial carbonic anhydrase activity in teleost fish.

Oxyradicals, or reactive oxygen species (ROS), are thought to be continuously produced in biological systems by a number of processes (Diguiseppi and Fridovich, 1984), including redox reactions with transition metals (Aust et al., 1985). Cu is a powerful inducer of oxidative stress in many organisms (reviewed by Lushchak, 2011; Grosell, 2012). However, the present study revealed no alteration in oxidative stress parameters (ROS concentration, ACAP; Supplementary Figs. S2 and S3) in all treatments employed for the two Cu exposures performed. These findings complement those of Jorge et al. (2013) who reported that a variety of oxidative stress parameters, such as antioxidant enzymes, ACAP, and total ROS concentration, did not respond in a clear way in the freshwater mussel *L. siliquoidea* during chronic exposure to low level of Cu. Freshwater mussels are able to cope with ROS formation promptly, by upregulating superoxide dismutase, catalase, and selenium-dependent glutathione peroxidase mRNA levels, increasing the activity of the lysosomal system, and the concentration of neutral lipids (Bigot et al., 2011), as well as through the utilization of GSH and induction of metallothionein synthesis (Nugroho and Frank, 2012). Overall, data from the present study suggest that oxidative stress may not be an important contributor to the mechanism of Cu toxicity in the freshwater mussel *L. siliquoidea*, at the environmentally relevant concentrations of Cu tested.

4.4. Effects of exposure to Cu combined with DOM

Because freshwater mussels are found in a wide range of habitats from small urbanized streams to large oligotrophic lakes, it is important to understand how diverse water quality conditions may affect their vulnerability to contaminants (Gillis et al., 2010). DOM is now recognized as a water quality factor that has a major influence on Cu toxicity to many organisms, including invertebrates (Playle et al., 1993; Di Toro et al., 2001; De Schamphelaere et al., 2004; Niyogi and Wood, 2004; Ryan et al., 2004; Al-Reasi et al., 2011, 2012, Monteiro et al., 2013). Several studies have evaluated the protective effects of DOM against Cu-induced toxicity to the early life stages of freshwater mussels (Hanstén et al., 1996; Gillis et al., 2008, 2010; Wang et al., 2009). While those studies aimed to determine threshold values for effect concentrations, the present study had the aim of uncovering the physiological effects of Cu and DOM exposure and the mechanisms underlying them. With respect to the relevance of these findings to wild mussels, given the inherent level of DOM in many of the significant mussel habitats in Ontario (mean values range: 2–13 mg C/L), Gillis et al. (2010) concluded that DOM would protect the sensitive glochidia (mussel larvae) against the potential toxicity associated with the typical levels of Cu found in those habitats (mean values range: 0.6–2.4 μg/L). The present results suggest that this protection by DOM would also be extended to the sensitive juvenile stages, although the potential toxicity of a Cu spill would of course depend upon the concentration of both parameters.

It is not surprising that the mussels exposed to Cu combined with DOM had lower levels of bioaccumulated Cu at 96 h (Fig. 7B). This might be attributed to the fact that less free Cu was available in solution due to binding with DOM molecules, and therefore, less Cu would be incorporated by the animals (Pagenkopf, 1983;

Playle et al., 1992; Richards et al., 1999). The results of the initial 6 h exposure experiment (Fig. 1) showed that every DOM concentration, when tested in combination with Cu, resulted in a partial or complete recovery of the whole-body Na^+ influx rate. Similar trends were seen at 24 h (Fig. 2A) and 96 h (Fig. 2B) of exposure where added DOM sometimes increased the Na^+ influx rate even above that recorded when Cu was absent. Indeed, there was a partial or complete recovery in whole-body Na^+ (Fig. 5B) and K^+ contents (Fig. 6B) in mussels exposed for 96 h to Cu combined with DOM, when compared to those exposed to only Cu (as discussed above). Our results are in complete agreement with the findings of Gheorghiu et al. (2010) on fish, who reported that DOM exposure in the presence of Cu could result not only in a reduction in the bioavailability of Cu, but also in an alteration in fish gill physiology in order to maintain Na^+ balance. Taken altogether, these findings indicate that DOM not only affects Cu bioavailability, but also acts directly on the organism producing enhancements in Na^+ balance, the main target for Cu toxicity.

4.5. Effects of DOM exposure alone

Interest in the direct effects of DOM on the ionoregulatory physiology of freshwater animals has been increasing recently (reviewed by Wood et al., 2011). Several studies have indicated that terrigenous DOM, relative to autochthonous DOM, has both better ameliorative effects against metal toxicity, and more potent direct effects on organism physiology (e.g. Matsuo et al., 1994; Richards et al., 1999, 2001; Wood et al., 2003; De Schamphelaere et al., 2004; Ryan et al., 2004; Glover and Wood, 2005; Glover et al., 2005a,b,c; Galvez et al., 2008; Al-Reasi et al., 2011, 2012). At a physicochemical level, these two aspects may well be related mechanistically (Al-Reasi et al., 2013a). Terrigenous DOM appears to be composed of larger molecules with a higher aromatic content and a greater chemical reactivity, while autochthonous DOM has a lower aromatic content, composed of smaller molecules and appears to be optically lighter (Richards et al., 2001; De Schamphelaere et al., 2004; Ryan et al., 2004; Galvez et al., 2008; Al-Reasi et al., 2011, 2013a). The optical and physicochemical properties of the DOM source used in the present study, i.e. Luther Marsh, were recently characterized (Al-Reasi et al., 2011, 2013a). It is known that Luther Marsh DOM has mainly a terrigenous origin, and it is optically dark and high in chemical reactivity. In an experiment conducted by Al-Reasi et al. (2012), this same DOM solution tested at 6 mg C/L, increased the 48-h LC_{50} value for Cu to *Daphnia magna* by 31-fold.

In the present study, DOM exposure alone had very interesting effects on Na^+ metabolism in *L. siliquoidea*. Exposure to 6 mg C/L for 96 h clearly increased Na^+ influx rate in the absence of Cu (Fig. 2B), and a similar though non-significant trend was seen in the initial 6 h exposure experiment (Fig. 1). The 96-h exposure to 6 mg C/L also decreased the v-type H^+-ATPase activity (Fig. 4B). Galvez et al. (2008) found that exposure to DOM induced a hyperpolarization of the transepithelial potential in rainbow trout gills tested both in vivo and in vitro, which could have led to an increase of active Na^+ uptake to counterbalance the effects of increased diffusive permeability (Vigneault et al., 2000). Luther Marsh DOM was highly effective in this regard. It has been proposed that active Na^+ uptake in fish is driven not only by the basolateral Na^+/K^+ pump, but also by the electrogenic, apically localized H^+ pump, which energizes Na^+ channels (Lin and Randall, 1993; Marshall and Grosell, 2005). It is difficult to reconcile the current observations of reduced H^+-ATPase activity but increased Na^+ influx caused by DOM exposure. However, to our knowledge, it is not yet known whether the v-type H^+-ATPase mechanism contributes to Na^+ uptake in freshwater mussels. Regardless, the novel inhibition of the H^+ pump activity revealed by this study is yet another indicator of direct actions of DOM on animal physiology, illustrating "the two faces of DOC" (Wood et al., 2011).

Acknowledgements

This research was supported by an award from the International Development Research Centre (IDRC, Ottawa, Canada) and the Canada Research Chair Program to AB and CM Wood. CMW is supported by the Canada Research Chair Program. A. Bianchini is a Research Fellow from the Brazilian 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq, Proc. #304430/2009-9, Brasília, DF, Brazil), and is supported by the International Research Chair Program from IDRC. PL Gillis is supported by Environment Canada.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2013.09.028.

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