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Mortality, bioaccumulation and physiological responses in juvenile freshwater mussels (*Lampsilis siliquoidea*) chronically exposed to copper

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ABSTRACT

Several studies have indicated that the early life stages of freshwater mussels are among the most sensitive aquatic organisms to inorganic chemicals, including copper. However, little is known about the toxic mode of action and sub-lethal effects of copper exposure in this group of imperiled animals. In this study, the physiological effects of long-term copper exposure (survival, growth, copper bioaccumulation, whole-body ion content, oxygen consumption, filtration rate, ATPase activities, and biomarkers of oxidative stress) were evaluated in juvenile (6 month old) mussels (*Lampsilis siliquoidea*). The mussels' recovery capacity and their ability to withstand further acute copper challenge were also evaluated in secondary experiments following the 28 day exposure by assessing survival, copper bioaccumulation and whole-body ion content. Mussels chronically exposed to 2 and 12 μg Cu/L showed significantly higher mortality than those held under control conditions (mortality 20.9, 69.9 and 12.5%, respectively), indicating that juvenile *L. siliquoidea* is underprotected by the U.S. Environmental Protection Agency (USEPA) biotic ligand model (BLM)-derived chronic water quality criteria (WQC) (2.18 μg Cu/L) and the hardness-derived USEPA WQC (12.16 μg Cu/L). Soft tissue copper burden increased equally for both copper exposures, suggesting that chronic toxicity is not associated with copper bioaccumulation. Several physiological disturbances were also observed during chronic copper exposure. Most relevant was a decrease in whole-body sodium content paralleled by an inhibition of Na^+ K^+-ATPase activity, indicating a metal-induced ionoregulatory disturbance. Filtration and oxygen consumption rates were also affected. Redox parameters (reactive oxygen production, antioxidant capacity against peroxyl radicals, glutathione-S-transferase (GST) activity, and glutathione (GSH) concentration) did not show clear responses, but membrane damage as lipid peroxidation (LPO) was observed in both copper exposures. Mussels previously held in control conditions or pre-exposed to 2 μg dissolved Cu/L were able to maintain their ionic homeostasis and did not experience mortality after the 4-d recovery period. In contrast, those previously exposed to 12 μg dissolved Cu/L exhibited 50% mortality indicating that they had already reached a 'point of no return'. Pre-exposure to copper did not influence mussel response to the copper challenge test. As observed for the chronic exposure, mortality of mussels held in the absence of copper and submitted to the challenge test was also associated with an ionoregulatory disturbance. These results indicate that ionoregulatory disruption in freshwater mussels chronically exposed to copper is the main mechanism of toxicity and that redox parameters do not appear to be useful as indicators of sub-lethal copper toxicity in these animals.

1. Introduction

During the past 30 years, species diversity and population density of native freshwater mussels (Unionidae) have declined substantively throughout the United States and Canada (Williams et al., 1993; Neves et al., 1997). This alarming decline has been attributed to a range of factors, including habitat destruction (Miller et al., 1989), changes in fish-host distribution (Isom and Yokley, 1968), invasive species (Gillis and Mackie, 1994), and environmental degradation associated with anthropogenic activities (Williams

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et al., 1993; Fuller, 1974; Havlik and Marking, 1987; Bogan, 1993). Surveys have revealed that toxic substances were one of the top five stressors cited as limiting freshwater mussels (Richter et al., 1997). It is known that chemical spills and other point sources of contaminants can cause localized mortality; however, the widespread decrease in freshwater mussel populations may result in part from the subtle, pervasive effects of chronic, low-level contamination (Strayer et al., 2004).

Several studies have indicated that the early life stages of freshwater mussels are among the most sensitive aquatic organisms to inorganic chemicals, including copper (Jacobson et al., 1993, 1997; Milam et al., 2005; Gillis et al., 2008). Notably, recent investigations (Wang et al., 2007a,b,c) have confirmed that juvenile freshwater mussels are more sensitive to acute and chronic copper exposure than most of the commonly tested freshwater organisms including cladocerans (*Daphnia magna* and *Ceriodaphnia dubia*), an amphipod (*Hyalella azteca*), fathead minnow (*Pimephales promelas*), and rainbow trout (*Oncorhynchus mykiss*). According to Wang et al. (2007a), the 50% effect concentration (EC_{50}) of copper for the early life stages of mussels often were at or below the final acute value (FAV) used to derive the United States Environmental Protection Agency (USEPA, 1996) 1996 acute Water Quality Criteria (WQC) for copper. March et al. (2007), using consensus-based methods, determined that multiple species of freshwater mussels are sensitive to particular waterborne contaminants (e.g. copper) at concentrations lower than the current federal U.S. Water Quality Criteria and the state of Oklahoma's water quality standards.

As benthic filter-feeding organisms, freshwater mussels are exposed to metals that are dissolved in water, associated with suspended particles and deposited in sediments. Because of these multiple routes of exposure, freshwater mussels can bioaccumulate certain metals to concentrations that substantially exceed those dissolved in water (Ravera, 1984; Ray, 1984). The acute toxicity of metals to freshwater mussels has been examined in a number of studies, but the sub-lethal effects of long-term exposure to low level, environmentally relevant concentrations are little understood. Sub-lethal effects of metal exposure include impairment in growth, filtration efficiency, enzyme activity and behaviour (Naimo, 1995; Vijayavel et al., 2007; March et al., 2007), with subsequent ecological impacts. Therefore, there is a need to investigate the physiological effects of long-term copper exposure, particularly biomarker responses capable of predicting ecological impacts from metal exposure in freshwater mussels. In contrast to the simple measurement of toxicant bioaccumulation, biomarkers can offer biologically relevant information on the potential impact of toxic pollutants on the health of organisms (Van der Oost et al., 1996). Copper toxicity, for example, can be attributed to a range of biological dysfunction like the generation of reactive oxygen species (Harris and Gitlin, 1996), ionoregulatory disturbance (Laurén and McDonald, 1985, 1987a,b; Vitale et al., 1999; Grosell and Wood, 2002; Bianchini et al., 2004; Grosell et al., 2004a), acid–base balance disturbances (Vitale et al., 1999; Skaggs and Henry, 2002; Grosell et al., 2003, 2004b) and impairment of ammonia excretion (Blanchard and Grosell, 2006; Grosell et al., 2003, 2004b; Wilson and Taylor, 1993).

In light of the above, the objectives of the present study were to evaluate the physiological effects of chronic copper exposure (28 days) to juvenile freshwater mussels (*Lampsilis siliquoidea*) by assessing survival, biometric parameters, copper bioaccumulation, whole-body ion content (Na^+, K^+, Mg^{2+}, Ca^{2+}), oxygen consumption, filtration rate, ATPase activities, and biomarkers of oxidative stress. To evaluate the recovery capacity of freshwater mussels and their ability to withstand further acute copper challenge (20 μg/L), 4 day recovery tests and acute copper exposures were performed following the 28 day chronic copper exposure by assessing survival, copper bioaccumulation and whole-body ion content (Na^+, K^+, Mg^{2+}, Ca^{2+}). Chronic exposures were conducted with three groups of mussels, control (0 μg Cu/L), low concentration of copper (2 μg Cu/L) and high concentration (12 μg Cu/L). The recovery test and acute copper challenge were performed with mussels that had survived the 28 d chronic exposure.

Lampsilis siliquoidea was chosen for this study because it is widespread throughout North America (Watters et al., 2009) and because it is closely related, and thus a potential surrogate for *Lampsilis fasciola*, Canadian Species at Risk. Because of its broad distribution and success in laboratory culture, *L. siliquoidea* has been frequently used in toxicity studies (Wang et al., 2007a,b,c, 2010; Gillis et al., 2008, 2010; Gillis, 2011; Bringolf et al., 2007). Although *L. siliquoidea* has been shown to be more sensitive to some contaminants than standard aquatic test organisms (Wang et al., 2007b) very little is known about the actual mechanisms of toxicity and thus the physiological reasons for their heightened sensitivity.

2. Materials and methods

2.1. Experimental animals

Juvenile freshwater mussels (*Lampsilis siliquoidea*, Barnes, 1823) (Fatmucket), were obtained from laboratory culture at Missouri State University (Springfield, MO, USA), where glochidia isolated from at least three females were pooled for production of juvenile mussels with host fish (Wang et al., 2007a). Juvenile mussels were reared in the laboratory for six months with live algae (*Neochloris oleoabundans*) in a compact system (Barnhart, 2006) before shipping to Environment Canada (Burlington, ON) for testing. Once in the laboratory, mussels were kept in an aerated 3-L polyethylene aquarium with 2 L of reconstituted moderately hard water (80–100 mg/L $CaCO_3$) (USEPA, 1994). About 70% of the culture water was replaced every 2 days over a 20-day acclimation period. Water temperature and photoperiod were maintained at 20 ± 1 °C and 16:8 h light:dark, respectively. Mussels were fed twice daily during the acclimation period with commercially nonviable algae (Shellfish Diet, Reed Mariculture, Campbell, CA, USA), according to Ingersoll et al. (2006) and Wang et al. (2007c). They were fed at a rate of 1 μL concentrated food per 1 L of moderately hard reconstituted water.

2.2. Chronic toxicity tests

An early-life stage chronic copper exposure (28 d) was performed using juvenile mussels (~7 month-old). Copper exposures were performed in reconstituted moderately hard water (80–100 mg/L $CaCO_3$) (USEPA, 1994) under semi-static conditions with full renewal of test solutions every day. The test was conducted with three groups of mussels: control (n = 75), exposed to a low copper concentration (nominal 5 μg Cu/L) (n = 105), and exposed to a high copper concentration (nominal 15 μg Cu/L) (n = 135). Fifteen juvenile mussels were randomly distributed in 1-L glass beakers for each treatment. Exposure vessels were kept at 20 ± 1 °C and under a 16:8 h (light:dark) photoperiod regime. Exposure media were gently aerated using 10 μL pipette tips attached to airlines to maintain an acceptable concentration of dissolved oxygen (>6.8 mg/L).

At the beginning of the experiment, juveniles with foot movement were randomly weighed, measured (length and width) and identified with numbered tags attached to their shells. Exposure solutions were prepared by adding a copper stock solution (see below) to reconstituted moderately hard water which had been previously aerated for 24 h to achieve CO_2 equilibrium and stabilization (USEPA, 1994). Exposure solutions were kept at 20 °C to equilibrate for 24 h prior to adding mussels. Copper chloride (Baker

Analyzed, J.T. Baker Inc, Phillipsburg, NJ, USA) was added from a stock solution (0.85 g Cu/L) prepared in acidified Milli-Q water (0.1% HNO_3; Suprapur®, Merck, Haar, Germany).

Mussels were fed once a day by adding 10 mL of algal mixture into 1 L of exposure solution (Wang et al., 2007c). The algal mixture was a combination of *Nannochloropsis* (Instant Algae®) and Shellfish Diet (Reed Mariculture, Campbell, CA, USA) prepared by adding 1 mL of *Nannochloropsis* concentrate and 2 mL of Shellfish Diet concentrate to 1.8 L of exposure solution (Wang et al., 2007c). Each batch of algal food was allowed to equilibrate with copper for 24 h prior to its first use to feed mussels. The same batch of algal food (kept at 4 °C) was used to feed mussels for one week (Wang et al., 2007c). A new batch of algal food was prepared weekly as previously described.

Mussel survival was monitored daily. Every 7 days, the total body mass (wet weight) was measured using an analytical scale (Mettler AJ100, Switzerland), and size (length and width) was determined using digital calipers. Whole body copper concentration, whole body ion content, filtration rate and oxygen consumption were also determined on days 7, 14, 21 and 28 (see below for details). At these experimental times, five mussels of very similar size from each test concentration (nominal 0, 5, 15 μg Cu/L) were also collected and dissected for further analyses as described below. For biochemical analyses, soft tissues were homogenized and centrifuged according to Gallagher et al. (1992). Sample was placed in 1.5 mL microcentrifuge tubes containing a buffer solution (Tris–HCl 100 mM; EDTA 2 mM; $MgCl_2{\cdot}6H_2O$ 5 mM; pH 7.75; 1:10 weight:volume; 4 °C), homogenized (~1 min) using a teflon pestle, and centrifuged (10,000 × *g*, 20 min, 4 °C). It is important to note that homogenate supernatant contained both mitochondrial fractions and cytosol, thus corresponding to the post-mitochondrial fraction (S9). Also, it is worth to note that test performed using the cationic dye JC1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolyl carbocyanine iodide) indicated that no viable mitochondria were observed in crude homogenates of mussel soft tissue. All procedures were performed on ice. Several aliquots of the supernatant were collected for biomarkers measurements, as described below. One aliquot was immediately used to quantify the reactive oxygen species (ROS). The other aliquots were frozen (−80 °C) for measurements of antioxidant capacity against peroxyl radicals, lipid peroxidation (LPO), glutathione-S-transferase (GST) activity, glutathione (GSH) concentration, Na^+ K^+ ATPase activity, Ca^{2+} ATPase activity and Mg^{2+} ATPase activity. Total protein content in the homogenate supernatant was also determined (Bradford, 1976) for data normalization.

Samples of the experimental media were collected every 3 days (i.e. every third water change). Collections were performed before (i.e., 24 h) and after (i.e., 0 h) the water was changed. Samples were used to determine the effective copper concentrations in the experimental media and to assess the water quality of the exposure (see below for details).

2.3. Recovery test and copper challenge experiment

Following the 28-day chronic exposure, surviving mussels were collected and used to conduct two additional experiments. A recovery test was performed in clean reconstituted moderately hard water (USEPA, 1994) under semi-static conditions with full renewal of test solutions every day. Six juvenile mussels from each treatment (control, low and high copper concentration) were distributed across three glass beakers (250 mL; 2 mussels per beaker) and kept in reconstituted moderately hard water without copper addition for 4 days. Also, an acute copper challenge was performed using eleven mussels from each treatment (control, low and high copper concentration). Mussels were transferred to separate 250-mL glass beakers (3 beakers with 3 mussels and 1 beaker with 2 mussels) and exposed (4 days) to copper (25 μg Cu/L—nominal concentration) in reconstituted moderately hard water. This copper concentration was selected based on the acute copper LC_{50} value determined in an earlier study with juvenile mussels under similar experimental conditions (Wang et al., 2007b). Because of the expected (50%) mortality at the 25 μg/L exposure, a higher number of the chronically exposed mussels ($n = 11$) were used in this acute copper challenge experiment when compared to the number of mussels used in the recovery experiment. At the end of the exposure (96 h), surviving mussels from both experiments (recovery and acute copper challenge tests) were counted, and their soft tissues were dissected for copper accumulation and whole-body ion content measurements. It is important to note that some endpoints were not measured after the secondary experiments because of the limited number of surviving mussels. All procedures for processing the mussels employed in the 4-d exposures followed those described above for the chronic exposure experiment.

2.4. Copper bioaccumulation and whole body ion content

On days 7, 14, 21 and 28, juvenile mussels ($n = 5$) from each treatment (nominal 0, 5, and 15 μg Cu/L) were collected and dissected. The soft tissue was dried (60 °C for 48 h), weighed, digested in 500 μL of 65% HNO_3 (Suprapur®, Merck, Haar, Germany) for 48 h, and diluted up to 2 mL with Milli-Q water. Copper content in digested samples was measured by graphite furnace atomic absorption spectroscopy (GF AAS; Varian Spectra AA-220 with a Spectra AA GTA-110 furnace, Mulgrave, Australia). Whole-body copper concentration is expressed as μg Cu/g dry weight of mussel soft tissue. Whole-body ion contents (Na^+, K^+, Mg^{2+}, Ca^{2+}) were measured on the same tissue digests by flame atomic absorption spectrophotometry (Varian AA 220FS, Mulgrave, Australia) and are expressed as μmol/g dry weight of soft tissue, except for Ca^{2+} which is expressed as mmol/g dry weight of soft tissue.

2.5. Filtration rate

Filtration rate was measured while holding mussels in 200 mL reconstituted moderately hard water with 6 μL of Shellfish Diet. Diet concentration (algae) was monitored by means of the chlorophyll concentration in the water. Chlorophyll was determined in water samples collected from each glass beaker ($n = 5$ per treatment: control, 5 and 15 μg Cu/L) at the beginning and the end of the 2 hour experiment. Chlorophyll concentration was determined following the method described by Jeffrey and Humphrey (1975), which quantifies chlorophyll pigments in 90% acetone. Filtration rate was calculated based on the decrease in chlorophyll concentration in the water according to Coughlan (1969): $m = \frac{M}{nt} \times \ln \frac{C_0}{C_t}$ where, m is the filtration rate (mL/h), M is the volume of the experimental medium (mL), n is the number of animals in each test vessel, t is the duration of the experiment (h), and C_0 and C_t are the algal concentrations at the beginning and the end of the test, respectively.

2.6. Oxygen consumption

Oxygen consumption of each mussel was individually measured using a respirometer (20-mL syringes) filled with moderately hard reconstituted water. Five mussels from each treatment (control, 5 and 15 μg Cu/L) were used for measurements. A recovery period of 2 hour in the respirometer chamber was adopted to reduce the effects of handling stress. Water samples (5 mL) were drawn from the closed respirometer at the beginning (0 h) and the end (2 h) of the experiment for measurement of the oxygen partial pressure (PO_2). Oxygen concentrations in chambers during

the experiment were not allowed to fall below 75% saturation, to avoid oxygen stress effects. In each sample, PO_2 was measured using a water-jacketed O_2 electrode (Cameron E101, Port Aransas, TX, USA) thermostatized to the acclimation temperature (20 °C), and connected to a digital dissolved oxygen meter (AM Systems Polarographic Amplifier—model 1900; Sequim, WA, USA). To ensure thermal equilibrium, respirometers were submerged in a water bath at the desired temperature. Absolute oxygen consumption rates were calculated using the following formula: $MO_2 = \frac{\Delta PO_2 \times \alpha O_2 \times V}{M \times t}$ where MO_2 is the molar rate of oxygen consumption (μmol O_2/g/h), ΔPO_2 is the measured change in PO_2 values between the start and the end of each oxygen consumption test period (mmHg; 1 mmHg = 0.133 kPa), αO_2 is the solubility constant for O_2 in water (μmol/L/mmHg) (Boutilier et al., 1984), V is the volume of water in the respirometer, M is the mass of the mussel (g wet weight), and t is time (h).

2.7. Reactive oxygen species (ROS)

Among different techniques (for review: Lushchak, 2011), ROS measurement was performed using the fluorescent dye 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) in the homogenate supernatant (S9) prepared as described above. All procedures were done following the protocol described by Amado et al. (2009). Total reactive oxygen species (ROS) were determined based on the cleavage of H_2DCF-DA (40 mM) by esterases present in the S9 fraction (post-mitochondrial fraction) in a reactive buffer containing 30 mM HEPES (pH 7.2), 200 mM KCl and 1 mM $MgCl_2$. The resulting fluorescent compound, i.e., 2′,7′-dichlorofluorescein (DCF), was quantified using a fluorometer (Biotek FLx800, Biotek Instrument Inc., Winooski, VT, USA) at wavelengths of 488 nm and 525 nm for excitation and emission, respectively. Total fluorescence production (45 min, with readings every 5 min) was calculated after adjusting data to a second-order polynomial function by integrating the fluorescence units over time. ROS was expressed as fluorescence area normalized by the total protein content in the homogenate supernatant (area/mg protein).

2.8. Antioxidant capacity against peroxyl radicals

Total antioxidant competence against peroxyl radicals was evaluated according to Amado et al. (2009). The reaction occurred by thermal decomposition of 2,2′-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich) in a buffer solution containing 30 mM HEPES (pH 7.2), 200 mM KCl and 1 mM $MgCl_2$. The non-fluorescent compound H_2DCF-DA (40 mM) was oxidized by ROS generating the fluorescent compound DCF, which was quantified as described above for ROS measurement. The thermal decomposition of ABAP and ROS formation was monitored for 30 min, with readings every 5 min. Total fluorescence production was calculated as described above for ROS measurement. The relative difference between ROS area in the presence and absence of ABAP was considered as a measure of the tissue antioxidant capacity, with a larger difference in area indicating a lower antioxidant capacity (Amado et al., 2009). Therefore, data are expressed as 1/relative fluorescence area, and normalized considering the total protein content in the tissue homogenate supernatant (1/relative area/mg protein).

2.9. Lipid peroxidation (LPO)

Lipid peroxidation was analyzed using the Thiobarbituric Acid Reactive Substances (TBARS) assay modified by Oakes and Van der Kraak (2003). This test quantifies the peroxidative damage in lipids generated by the action of ROS through malondialdehyde (MDA) production. MDA reacts with thiobarbituric acid (TBA) under conditions of high temperature and acidity, producing a chromogen that can be measured by fluorescence (excitation: 515 nm; emission 553 nm). TMP (1,1,3,3-tetramethoxypropane) was used as an external standard. LPO was expressed as nmol TMP/mg protein.

2.10. Glutathione-S-transferase (GST) activity and glutathione (GSH) concentration

The glutathione-S-transferase (GST) activity was measured spectrophotometrically (340 nm) using a microplate reader (Biotek ELx800, Biotek Instrument Inc., Winooski, VT, USA) following procedures described by Habig et al. (1974) and using 1-chloro-2,4-dinitrobenzene (CDNB, Sigma Aldrich) as a substrate. Values were expressed as μmol GS-DNB using an extinction coefficient of 9.6 mM/cm. Enzyme activity was then expressed as enzyme units/mg protein.

Glutathione (GSH) concentration was determined according to Ellman (1959) using 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB). This water soluble aromatic disulfide reacts with aliphatic thiol compounds at pH 8.0 to produce a colored anion that can be read at 412 nm. Results are expressed as μmol GSH/mg protein.

2.11. ATPases activities

Na^+ K^+ ATPase activity was measured according to the Bianchini and Castilho (1999) method which itself was a modification of the Morris and Edwards (1995) method. Measurements were determined as the difference between the inorganic phosphate liberated from ATP in the presence of K^+ (medium A, 77 mM NaC1; 19 mM KCl; 6 mM $MgCl_2$; 3 mM ATP and Tris–HCl 0.1 M at pH 7.6) and in the absence of K^+ with ouabain (medium B, 96 mM NaCl; 6 mM $MgCl_2$; 3 mM ATP; 1 mM ouabain and Tris–HCl 0.1 M at pH 7.6). Concentration of inorganic phosphate (Pi) was determined through absorbance readings (620 nm) in the reaction mixture after 30 min of incubation at 90 °C, following procedures described by Fiske and SubbaRow (1925). Mg^{2+} ATPase activity was calculated from the difference between the amounts of inorganic phosphate released in the absence of K^+ before and after the incubation period. Ca^{2+} ATPase activity was assayed using the method described by Vajreswari et al. (1983). The reaction medium contained 80 mM NaCl, 5 mM $MgCl_2$, 3 mM ATP, 20 mM Tris–HCl (pH 7.4), 0.5 mM $CaCl_2$ and 1 mM ouabain in a final volume of 0.5 mL. In all cases, enzyme activity was expressed as μmoles Pi/mg protein/h.

2.12. Water chemistry analyses

Every 3 days, pH (Accumet Basic AB15, Fisher Scientific, Toronto, ON, Canada) and dissolved oxygen concentration (YSI Meter, Model 8510, Yellow Springs, OH, USA) were measured in the exposure media. Measurements were performed prior the introduction of mussels in the exposure media, and after they had been kept in that solution for 24 h (i.e. exposed to copper).

Filtered (0.45-μm syringe-tip filters, Acrodisc Supor membrane, Pall Life Sciences, USA) samples (10 mL) of the test media were also collected and acidified (0.5% HNO_3; Suprapur®, Merck, Haar, Germany) for copper and water chemistry analyses, as described below. The concentrations of total (non-filtered samples) and dissolved copper (filtered samples) in the exposure solutions were determined using graphite furnace atomic absorption (GF AAS; Varian Spectra AA-220 with a Spectra AA GTA-110, Mulgrave, Australia). Internal copper standards were made using Copper Reference Solution (Fisher Scientific, Nepean, ON). Copper recovery was within ±10% based on certified reference materials TM24 and TM25 (Environmental Canada, National Water Research Institute, Burlington, ON). The concentrations of Na^+, K^+, Mg^{2+} and

Ca^{2+} in the exposure solutions were determined by flame atomic absorption spectrophotometry (Varian AA 220FS, Mulgrave, Australia). Dissolved organic carbon (DOC) concentration was measured in filtered water samples using a Total Organic Carbon (TOC) analyzer (Shimadzu 5050A, Mandel Scientific, Canada) and the standards were prepared according to manufacturer specifications.

2.13. Data analysis

The LC_{50} value after 28 days of copper exposure was calculated based on cumulative mortality data over the test period using the Trimmed Spearman Karber Method. Mortality data was calculated accumulating the percentage of mussels that died over each 7-day interval of exposure. Differences in mortality rates among treatments (control, 2 and 12 μg Cu/L) after 28 days exposure to copper were detected through the Chi square test (two-tailed). Whole-body Na^+ concentration and mortality (%) data were subjected to a piecewise linear regression to determine the whole-body Na^+ concentration below which mussel mortality starts to occur (breakpoint regression), Correlation analysis was then performed between these two parameters considering only the whole-body Na^+ concentration values lower than the corresponding breakpoint value. Also, correlation analysis was performed using all mortality (%) and mean tissue copper accumulation data. These analyses were performed using the software Statistica v. 7 (StatSoft Inc., USA). A 'site specific' WQC (i.e. Criterion Maximum Concentration) was produced for the chronic exposure using the specific measured water chemistry of the 28 d exposure with the BLM model (version 2.2.3). The hardness-derived WQC, was calculated as USEPA (2007), considering the measured hardness of the experimental medium used for the 28 d chronic exposure.

Data for biometric parameters and biomarkers analyses are expressed as mean ± SE ($n = 5$). Differences among treatments were assessed by two-way analysis of variance (ANOVA) followed by the Tukey's test. When necessary, data were mathematically transformed to meet ANOVA assumptions of normality and homogeneity of variances. Transformations used are specified in the respective figure legends. In all cases, the significance level adopted was 5% ($\alpha = 0.05$).

3. Results

Total and dissolved copper concentrations in the experimental medium were similar and did not show significant variation over the duration of the juvenile mussel chronic experiment (28 d). The mean measured total and dissolved values for the nominal concentrations of 5 and 15 μg Cu/L were 2.12 ± 0.34, 2.0 ± 0.60, 12.58 ± 0.99 and 12.0 ± 1.58 μg Cu/L, respectively. Approximately 95% of the total copper was dissolved in both treatments. No copper was detected in the control medium (detection limit = 0.2 μg/L). Because dissolved forms of copper usually include most of the toxic forms of this metal, all results are expressed in terms of the dissolved copper concentration.

Water chemistry parameters (Ca^{2+} = 13.58 ± 0.62 mg/L, Na^+ = 25.48 ± 3.87 mg/L, Mg^{2+} = 11.41 ± 0.75 mg/L, K^+ = 2.16 ± 0.15 mg/L, Cl^- = 2.17 ± 0.28 mg/L, SO_4^- = 81.19 ± 0.50 mg/L concentration, hardness = 86.15 ± 0.64 mg $CaCO_3$/L and alkalinity = 58.85 ± 0.49 mg/L) were within the range expected for reconstituted moderately hard water (USEPA, 1994). Over the experimental time, mean values of water pH, dissolved oxygen content and DOC were 7.60 ± 0.09, 7.36 ± 0.10 mg/L, and 0.409 ± 0.04 mg C/L, respectively.

In the chronic toxicity test, 12.5% mortality was observed in the control treatment (Fig. 1), thus meeting the acceptability criterion for a 28-day mussel test of ≤20% control mortality at the end of the exposure (ASTM, 2009). For juvenile mussels exposed to copper, 20.9% and 69.9% mortality was observed after 28 days of exposure to 2 and 12 μg Cu/L, respectively (Fig. 1). These mortality values were significantly different from that observed in control mussels (12.5%). The chronic dissolved LC_{50} for *Lampsilis siliquoidea* juveniles was calculated as 7.27 μg Cu/L (5.96–8.86 μg Cu/L, 95% confidence limits). It is important to note that no difference in biometric parameters was observed for any control or copper exposed mussels over the course of the experiment (Table 1). Water parameters in both the recovery test and the acute challenge experiment were similar to those measured in the chronic toxicity test (temperature: 20 °C, pH: 7.5–7.7, dissolved oxygen: 7.36 mg/L, and DOC: 0.468 ± 0.06 mg C/L). Hardness, alkalinity and ionic composition were the same as the chronic toxicity test (28 d) described above.

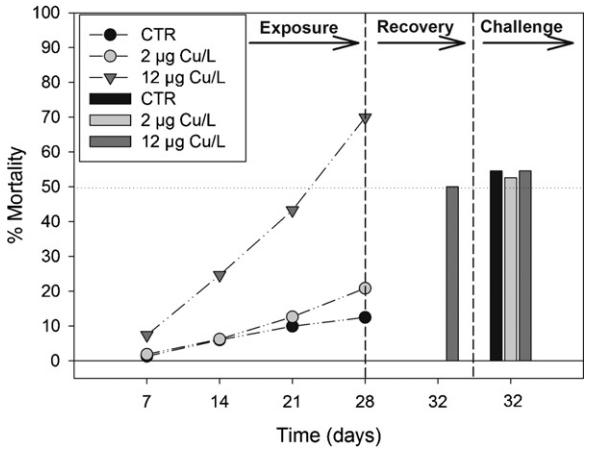

Fig. 1. Lines and symbols represent mean chronic mortality (%) of juvenile *Lampsilis siliquoidea* either held under control condition (no copper addition; CTR) or exposed to different dissolved copper concentrations (2 and 12 μg Cu/L) for a maximum of 28 days. Bars represent the mean mortality of mussels after either a recovery test in clean (no copper addition) reconstituted moderately hard water or after an acute (4 d) copper challenge experiment (exposure to 20 μg Cu/L). Both secondary experiments employed mussels previously used in the 28 d chronic exposure. Treatments without bars represent 0% mortality.

No mortality was observed throughout the 4-day recovery test in either the control or the low copper pre-exposure treatment (2 μg Cu/L). However, 50% mortality was observed in the high copper pre-exposure concentration (12 μg Cu/L) (Fig. 1). High mortality was also observed at the end of the acute copper challenge (20 μg Cu/L dissolved concentration) for the three different pre-treatments (control, 2 and 12 μg Cu/L) (Fig. 1). Mussels kept under control condition (no copper addition) and those exposed to different dissolved copper concentrations (2 and 12 μg Cu/L) in the initial 28 day exposure showed 54.5, 52.6 and 54.5% mortality, respectively, at the end of the 4-day period of copper challenge.

Whole body copper concentration (27.9 ± 9.4 μg/g dry weight) in control mussels was constant throughout the experiment (Fig. 2).

Table 1
Mean (± standard deviation) total length (mm), width (mm) and whole animal weight (g) of juvenile *Lampsilis siliquoidea* held under control condition (no copper addition; CTR) or chronically exposed to different dissolved copper concentrations (2 and 12 μg Cu/L) for 28 days. Same small case letters indicates no significant difference among treatments ($P < 0.05$).

Treatment	Time (days)	Length (mm)	Width (mm)	Weight (g)
CTR	0	8.96 ± 0.68[a]	5.52 ± 0.04[a]	0.11 ± 1.16[a]
CTR	28	9.22 ± 0.68[a]	5.79 ± 0.04[a]	0.11 ± 1.84[a]
2 μg Cu/L	0	8.99 ± 0.92[a]	5.61 ± 0.62[a]	0.10 ± 0.02[a]
2 μg Cu/L	28	9.17 ± 0.85[a]	5.74 ± 0.60[a]	0.10 ± 0.02[a]
12 μg Cu/L	0	8.92 ± 0.87[a]	5.62 ± 0.53[a]	0.10 ± 0.02[a]
12 μg Cu/L	28	9.02 ± 0.85[a]	5.65 ± 0.53[a]	0.10 ± 0.02[a]

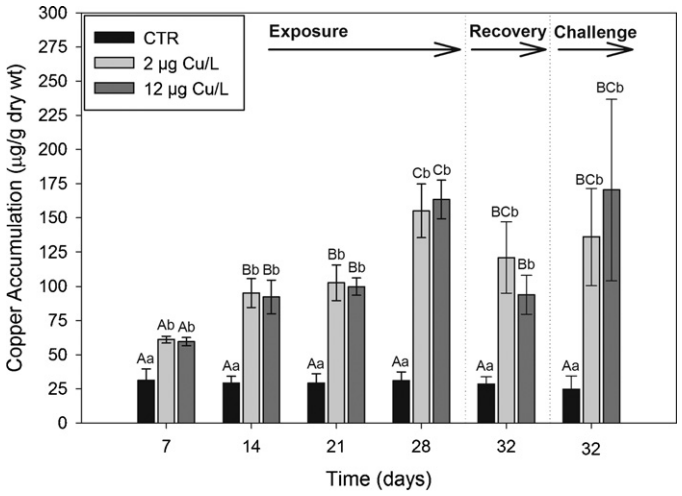

Fig. 2. Whole-body copper bioaccumulation in juvenile *Lampsilis siliquoidea* held under control condition (no copper addition; CTR) or exposed to different dissolved copper concentrations (2 and 12 µg Cu/L) for 28 days followed by 4 days of either a recovery period in reconstituted moderately hard water (without copper addition) or a copper challenge exposure (20 µg Cu/L). Data are expressed as mean ± standard deviation ($n = 5$). Different capital letters indicate significant differences over time for the same treatment while different small case letters indicate significant difference among treatments for the same experimental time. Data were previously transformed (square root transformation) to meet the ANOVA assumptions of normality and homogeneity of variances.

However, it increased over time in copper-exposed mussels, rising from 63.8 ± 5.2 (day 7) to 164.9 ± 35.8 (day 28) µg/g dry weight in those exposed to 2 µg Cu/L and from 56.2 ± 31.0 (day 7) to 158.5 ± 25.4 (day 28) µg/g dry weight in those exposed to 12 µg Cu/L (Fig. 2). Although copper bioaccumulation was time-dependent, there was no significant difference in the whole-body copper concentrations between the two experimental treatments (2 and 12 µg Cu/L). In addition, no correlation ($R^2 = 0.56$, $p = 0.6$) between copper toxicity and copper accumulation, was found. The same pattern was observed for the pre-treated mussels (from 28-d chronic exposure) after the recovery test and the acute copper challenge (Fig. 2).

Whole-body Na^+ concentration decreased significantly as a function of the copper exposure concentration and over the exposure time (Fig. 3A). The piecewise linear regression analysis indicated that mussel mortality starts to occur when whole-body Na^+ concentration is below 43.8 µmol/g dry weight of soft tissue. Correlation analyses using the whole-body Na^+ concentration values lower than the breakpoint value (43.8 µmol/g dry weight of soft tissue) indicated a strong and significant negative correlation ($R^2 = 0.77$, $p < 0.05$) between whole-body Na^+ concentration and mussel mortality. Notably, mussels pre-exposed to copper for 28 days and further submitted to the recovery test showed a significant increase in the whole-body Na^+ concentration, suggesting that these mussels were once again able to regulate their whole-body Na^+ concentration. In turn, mussels kept under control condition or pre-exposed to copper (2 and 12 µg Cu/L) for 28 days and further submitted to the acute copper challenge (20 µg Cu/L) showed a decreased whole body Na^+ concentration (Fig. 3A). As observed for Na^+, whole-body K^+ concentration decreased significantly as a function of the copper exposure concentration and over the 28-days exposure period (Fig. 3B). Also, a recovery of the whole-body K^+ concentration was observed after mussels pre-exposed to copper were submitted to the recovery test in clean water (no copper addition). However, only mussels pre-exposed to 2 µg Cu/L for 28 days showed a significant decrease in whole-body K^+ concentration when submitted to the acute copper challenge (20 µg Cu/L) (Fig. 3B). In contrast to Na^+ and K^+, whole-body Ca^{2+} and Mg^{2+} concentrations did not vary across the copper concentrations tested and over time (28 d), keeping a median concentration of 2.41 ± 0.47 mmol/g dry weight for Ca^{2+} and 221.66 ± 30.30 µmol/g dry weight for Mg^{2+}. Also, they did not vary after the acute copper challenge.

Relatively constant profiles of Ca^{2+} ATPase (Fig. 4A) and Mg^{2+} ATPase (Fig. 4B) activity in both copper treatments (2 and 12 µg Cu/L) support the conclusion that mussels were capable of regulating Ca and Mg homeostasis during the 28-days period of exposure to copper. However, Na^+,K^+ATPase activity (Fig. 4C) decreased in both copper concentrations (2 and 12 µg Cu/L). This inhibition of Na^+ K^+ ATPase activity is in agreement with the observed decrease in whole-body Na^+ (Fig. 3A) and K^+ (Fig. 3B) concentrations, and strongly indicates that copper toxicity may be related to ionoregulatory disturbances.

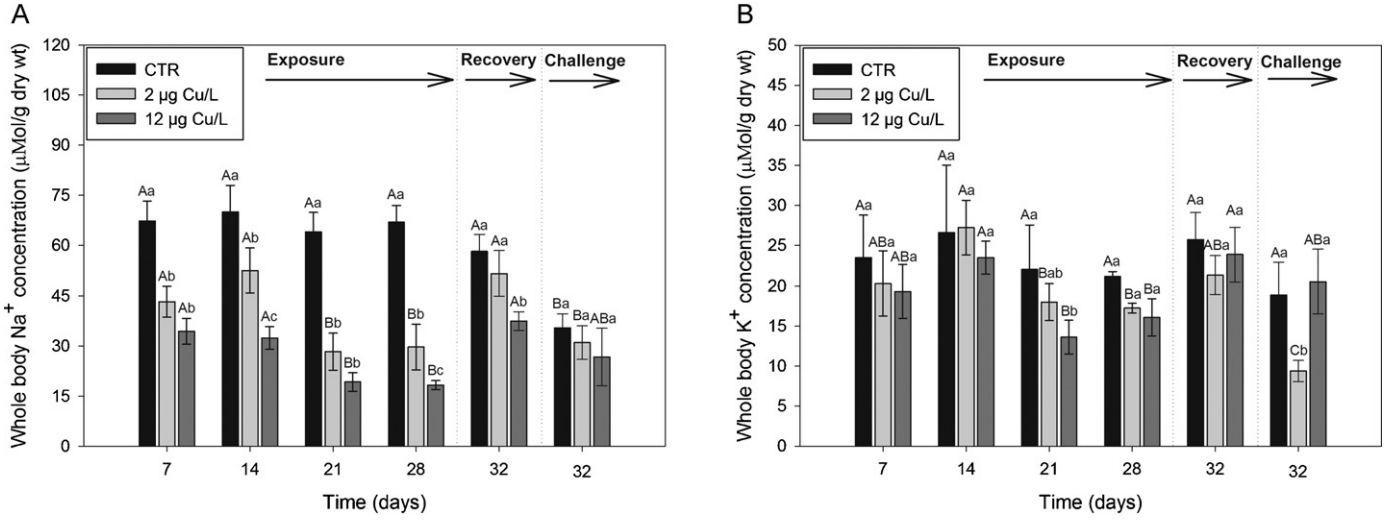

Fig. 3. Whole-body ion concentrations in juvenile *Lampsilis siliquoidea* held under control condition (no copper addition; CTR) or exposed to different dissolved copper concentrations (2 and 12 µg Cu/L) for 28 days followed by 4 days of either a recovery period in reconstituted moderately hard water (without copper addition) or a copper challenge exposure (20 µg Cu/L). (A) Sodium (Na^+) concentration expressed in µMol/g dry weight. (B) Potassium (K^+) concentration expressed in µMol/g dry weight. Data are expressed as mean ± standard deviation ($n = 5$). Different capital letters indicate significant differences over time for the same treatment while different small case letters indicate significant difference among treatments for the same experimental time. Data were previously transformed (square root transformation) to meet the ANOVA assumptions of normality and homogeneity of variances.

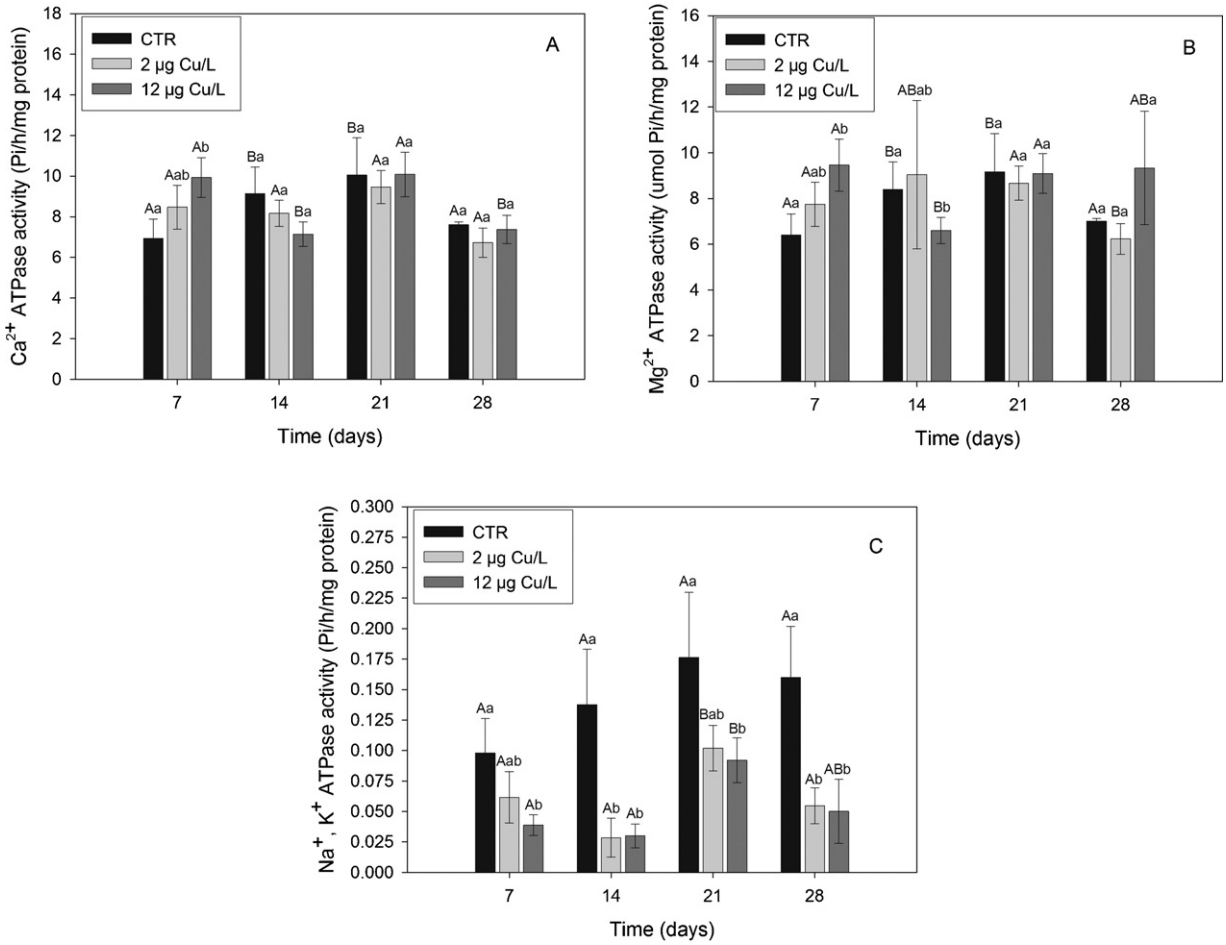

Fig. 4. Activity of ATPases in juvenile *Lampsilis siliquoidea* held under control condition (no copper addition; CTR) or exposed to different dissolved copper concentrations (2 and 12 µg Cu/L) for 28 days. (A) Ca^{2+} ATPase activity, (B) Mg^{2+} ATPase activity, and (C) Na^{+} K^{+} ATPase activity. Data are expressed as mean ± standard deviation ($n = 5$). Different capital letters indicate significant differences over time for the same treatment while different small case letters indicate significant difference among treatments for the same experimental time. Data met the ANOVA assumptions (data normality and variances homogeneity) without any mathematical transformation.

Filtration and oxygen consumption rates were impacted by chronic copper exposure, the responses were variable depending on copper concentration and time of exposure. There was a significant increase in filtration rate of mussels exposed to 12 µg Cu/L for 7 days. However, mussels exposed to copper (2 and 12 µg Cu/L) for 14 and 28 days showed a significantly lower filtration rate than those kept under control condition (no copper addition) (Fig. 5A). Oxygen consumption rate was also lower on day 7, but only in mussels exposed to 2 µg Cu/L, and on day 28 in mussels exposed to 2 and 12 µg Cu/L (Fig. 5B).

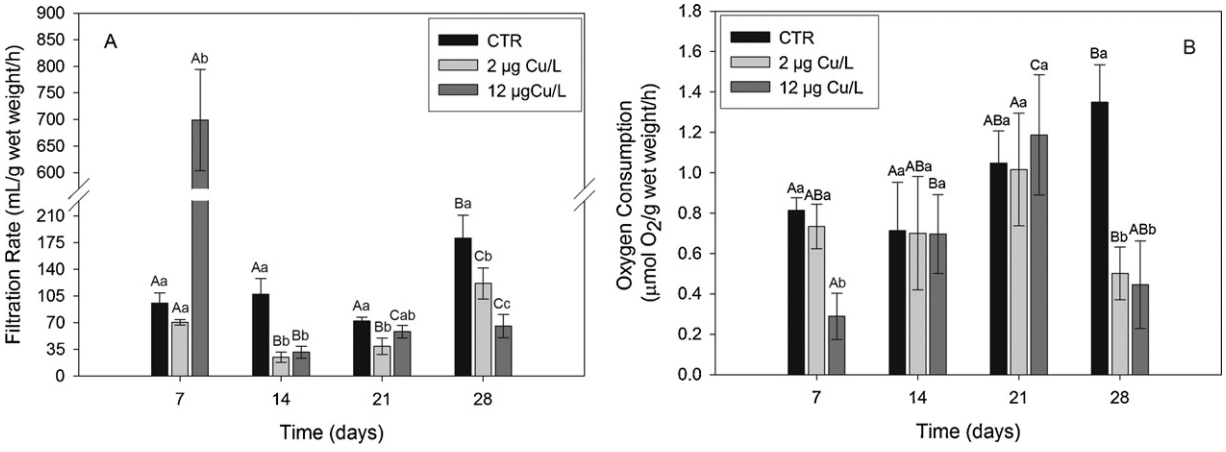

Fig. 5. (A) Filtration rate and (B) oxygen consumption rate in juvenile *Lampsilis siliquoidea* held under control condition (no copper addition; CTR) or exposed to different dissolved copper concentrations (2 and 12 µg Cu/L) for 28 days. Data are expressed as mean ± standard deviation ($n = 5$). Different capital letters indicate significant differences over time for the same treatment while different small case letters indicate significant difference among treatments for the same experimental time. Filtration rate data were previously transformed (square root transformation) to meet the ANOVA assumption of normality and homogeneity of variances. No mathematical transformation was needed for the oxygen consumption data.

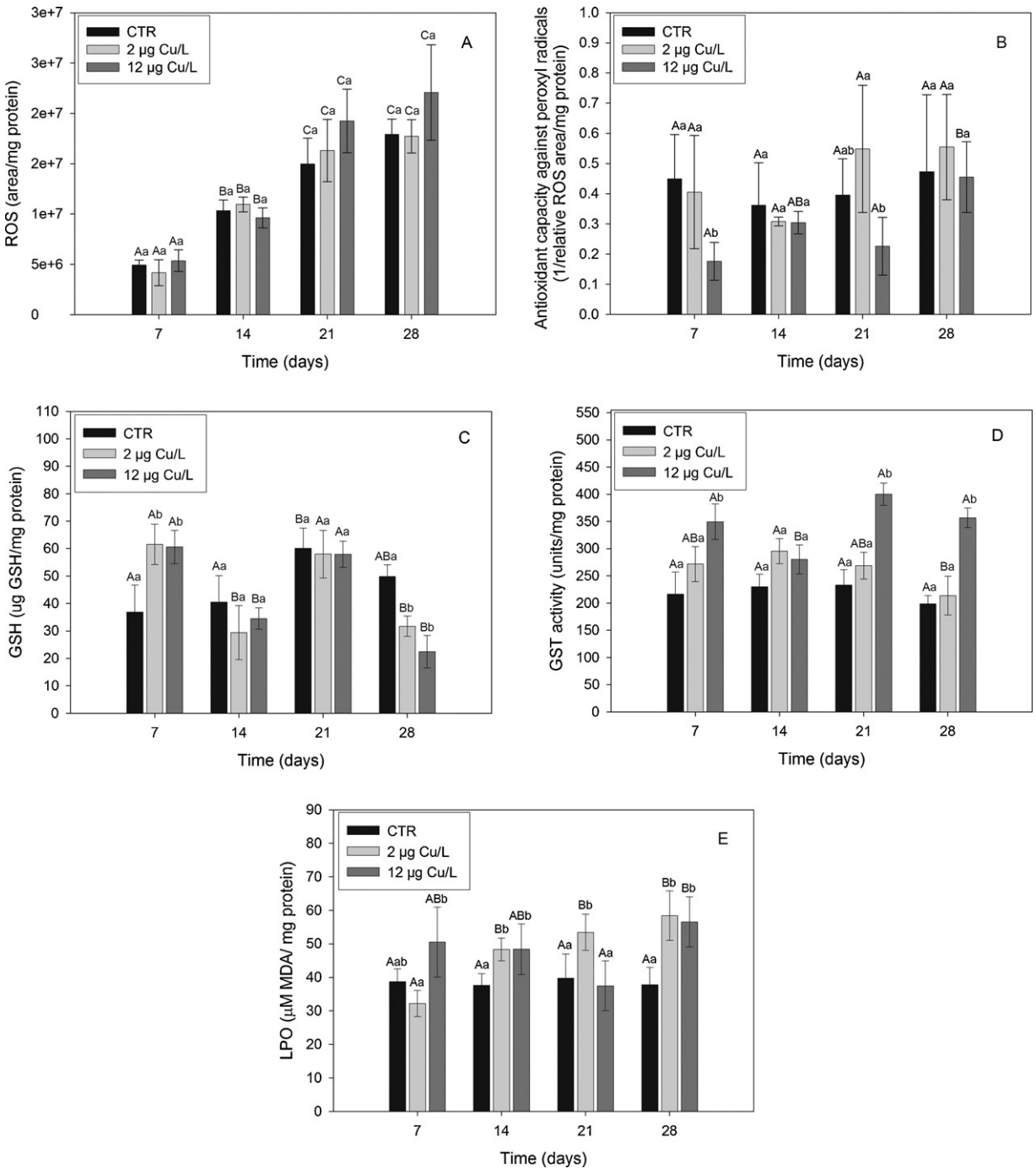

Fig. 6. (A) Reactive oxygen species (ROS), (B) antioxidant capacity against peroxyl radicals, (C) glutathione (GSH) concentration, (D) glutathione-S-transferase (GST) activity, and (E) lipid peroxidation (LPO) levels in juvenile *Lampsilis siliquoidea* kept under control conditions (no copper addition; CTR) or exposed to different dissolved copper concentrations (2 and 12 µg Cu/L) for 28 days. Data are expressed as mean ± standard deviation ($n = 5$). Different capital letters indicate significant differences over time for the same treatment while different small case letters indicate significant difference among treatments for the same experimental time. Data were previously transformed (square root transformation) to meet the ANOVA assumptions of normality and homogeneity of variances.

Because of the redox properties of copper, we examined whether this metal would cause oxidative stress in mussels. There were no significant differences in the amount of ROS produced by control and copper-exposed mussels (Fig. 6A); however there was a marked trend for an increase in this parameter over the exposure time in mussels from all treatments, including those kept under control condition (no copper addition). The antioxidant capacity against peroxyl radicals value, an indicator of the tissue capacity to scavenge ROS, was slightly lower in mussels exposed to 12 µg Cu/L for 7 and 21 days, but no significant difference was observed over time (Fig. 6B).

With respect to the detoxifying mechanisms for ROS, there were some significant differences, but not very marked responses. There was a significant increase of the GST activity in mussels exposed to 12 µg Cu/L for 7, 21 and 28 days when compared to mussels kept under control condition (no copper addition). The control animals showed a constant GST activity value throughout the experiment (Fig. 6D). In contrast, GSH concentration increased significantly

in mussels exposed for 7 days to 2 and 12 μg Cu/L (Fig. 6C) and decreased at after 28 days of copper treatment. LPO, an indicator of oxidative damage, was higher in mussels from the two copper treatments on day 14 and 28 (Fig. 6E).

4. Discussion

The resulting 28-d LC_{50} from the current study showed that juveniles of the fatmucket mussel *L. siliquoidea* are one of the most sensitive freshwater species to chronic copper exposure compared to those already reported for juvenile freshwater mussels (Wang et al., 2007c) and other freshwater organisms (Birge, 1978; Birge et al., 1978; Belanger et al., 1990; Cowgill and Milazzo, 1991; West et al., 1993). It is important to note that mussels exposed to the 2 μg Cu/L treatment experienced significantly higher mortality ($P = 0.01$; Chi square test) than those kept under control conditions for 28 days (mortality 20.9 and 12.5%, respectively). This finding indicates that (under the tested water chemistry conditions) *L. siliquoidea* would be under protected by the USEPA BLM-derived WQC of 2.18 μg Cu/L or the USEPA hardness-derived WQC of 12.16 μg Cu/L (USEPA, 2007) and marginally protected by the Canadian Water Quality Guideline (WQG) of 2 μg Cu/L for waters with hardness of less than 120 mg $CaCO_3$/L (CCME, 2005). Although the site specific USEPA BLM-derived criterion offers more protection than the USEPA hardness-derived criteria, the reality is that no U.S. state has actually adopted the BLM-derived WQC as a standard, and Cu is currently being regulated throughout the United States using the hardness-derived WQC (Brix et al., 2011).

According to the USEPA (2007), the toxicological endpoint used to derive the chronic value from data generated in early life stage tests can be survival, reproduction, growth, emergence, or intrinsic growth rate. In the present study, mussel survival was one of the most sensitive indicators of chronic copper toxicity to *L. siliquoidea* (Fig. 1). In contrast with other chronic metal toxicity studies performed with freshwater organisms that found growth rate as the most sensitive endpoint (Grosell, 2006; De Schamphelaere et al., 2008; Schlekat et al., 2010; Brix et al., 2011), no significant effect on growth rate (biometric parameters) was observed in juveniles of the fatmucket *L. siliquoidea* exposed to 2 or 12 μg Cu/L. However, Wang et al. (2007c) concluded that growth was a more sensitive endpoint than survival after they observed significant growth in control *L. siliquoidea* and a lack of growth in copper exposed mussels over the course of a chronic (28 days) copper experiment that employed two month old mussels.

An increase in whole-body copper concentration was observed, over the duration of the experiment in mussels exposed to 2 and 12 μg Cu/L, however, no significant difference in bioaccumulation was observed between these two exposure concentrations. Moreover, no correlation was found between copper accumulation and mortality, suggesting that chronic copper toxicity in juvenile mussels was not directly associated with copper accumulation in the body. The scarce accumulation at the highest dose tested could be due to the activation of detoxification mechanisms through metallothioneins and lysosomes. Both mechanisms play a role in copper detoxification and its elimination from the digestive gland of mussels (Viarengo et al., 1981, 1985; George and Viarengo, 1985). Other hypothesis for the differential mortality observed is that mussels with high Cu burdens had died and metal bioaccumulation was only analyzed in the surviving organisms. Also, it is important to note that the steady-state accumulation was not necessarily achieved, making interpretation of accumulation data difficult.

As mentioned above, no correlation between mortality and copper bioacumulation was observed. According to Borgmann et al. (1993), at lower metal concentrations and long-term exposures, such as those reported here, gradual metal uptake could result in metal deposition in non-critical tissues leading to a higher overall metal concentration, but the majority of the metal accumulates in the fractions with lower metabolic activity. In contrast, at elevated water metal concentrations, most of the copper accumulates in sensitive tissues, causing damage and death (e.g. respiratory epithelia) before extensive metal accumulation occurs. Several disturbances that potentially identify the toxic mechanism of action were observed. Most straightforward were the reduction of whole-body Na^+ concentration, corresponding with exposure time (Fig. 2), copper concentration (Fig. 2), mortality rate (positive and significant correlation) and the inhibition of the Na^+ K^+ ATPase activity. These findings are in complete agreement with the known etiology of copper toxicity in freshwater fish, where copper has been shown to inhibit Na^+ K^+ ATPase activity thereby reducing or eliminating the motive force which is necessary for Na^+ uptake (Wood, 2001; Grosell et al., 2002; Grosell and Wood, 2002). With a reduced ability to actively take up Na^+ from the environment, freshwater aquatic organisms are unable to counteract the diffusive Na^+ loss, and in consequence, the extracellular Na^+ concentration declines accordingly. Many researchers have shown that the loss of sodium from adult fish during copper exposure is dependent upon the concentration of the cupric ion (i.e., as Cu^{2+} concentration increases, whole-body sodium decreases) and the duration of the exposure (i.e., as exposure time increases, the whole-body sodium decreases) (Laurén and McDonald, 1985, 1986, 1987a; Grippo and Dunson, 1991; McGeer et al., 2000; Croke and McDonald, 2002; Taylor et al., 2003). Some investigators have suggested that a 30% loss of plasma or whole-body sodium is associated with mortality in adult freshwater fish (Laurén and McDonald, 1985; Paquin et al., 2002, Wood, 2001). The present data (Fig. 3A) suggest that freshwater mussels may be able to tolerate sodium losses as high as 50–70% before mortality occurs.

In the present study, filtration and oxygen consumption rates were also measured as possible biomarkers of copper exposure. However, results obtained suggest that the response of these parameters do not appear to be related to the exposure duration. This finding may indicate that the copper concentrations tested (2 and 12 μg Cu/L) affect the metabolic state of mussels during metal exposure, more specifically on day 28 when a significant reduction in these parameters was observed. Also, the oxidative stress-related parameters (ROS, antioxidant capacity against peroxyl radicals, GST activity, GSH concentration and LPO) measured in whole (i.e. soft tissue) mussels showed no clear relationship with the long-term copper effects in mussels exposed to the copper concentrations tested (2 and 12 μg Cu/L). In fact, copper-induced changes observed in the physiological/biochemical biomarkers analyzed in the present study could be attributed to some adaptive or compensatory mechanisms expressed in chronically exposed mussels. Furthermore, the expression of oxidative stress-related biomarkers can be tissue-dependent or likely be influenced by environmental factors other than metal exposure (Monserrat et al., 2007). For example, in the present study we observed that ROS formation had a general tendency to increase over time in both control and copper-exposed mussels, suggesting the response was associated more with the laboratory and test holding conditions than with the metal exposure. Taken altogether, these findings suggest that the biomarkers evaluated (on the whole-body soft tissue) would not be suitable tools to monitor the biological impact of the chronic copper exposure on juvenile freshwater mussels under the conditions tested in the present study.

Pre-exposure to copper did not influence the acute toxicity of this metal when mussels were acutely challenged with 20 μg Cu/L (Fig. 1). Therefore, mussels previously exposed to 2 and 12 μg Cu/L for 28 days, and thereby potentially pre-selected (the 'survivor effect') or acclimated to copper, suffered the same mortality (52.6–54.5%) as mussels held in a control condition (not pre-exposed to copper). Clearly, there was no evidence of acclimation

or selection for copper tolerance. The mortality observed could be explained by a significant decrease in the whole-body Na^+ levels, just as in the mussels previously held in the absence of copper (control). It is important to note that whole-body copper burden in control mussels remained constant during the 4-day period of acute exposure suggesting that toxicity is not associated with copper accumulation but rather an ionoregulatory disturbance. These results are in agreement with those observed in the juvenile mussels from the chronic toxicity experiment (28 d).

Data from the recovery test showed that mussels previously held in control conditions or pre-exposed to a lower copper concentration (2 μg Cu/L) were able to maintain ionic homeostasis and did not experience mortality after the 4-day recovery period. In contrast, those previously exposed to the higher copper concentration (12 μg Cu/L), still exhibited 50% mortality, despite showing improvement in some parameters after the 4-day recovery period, i.e. decreased copper accumulation and increased whole body Na^+ levels when compared to values observed after the 28-day period of pre-exposure to copper. This finding indicates that these mussels had already achieved a 'point of no return'.

5. Conclusions

The present study aimed to characterize the chronic sensitivity of juvenile freshwater mussels (*L. siliquoidea*) to waterborne copper and to understand the toxic mechanism(s) of action. We demonstrated that *L. siliquoidea* is one of the most sensitive freshwater species tested to date and is under protected by current (USEPA) BLM-derived chronic WQC or the USEPA hardness-derived WQC and marginally protected by the Canadian Water Quality Guideline (WQG). Our data also suggest that copper concentrations in *L. siliquoidea* under chronic conditions in the laboratory, cannot be used to accurately infer the presence or absence of copper toxicity. Regarding the underlying mechanisms of this sensitivity to copper, we made several important observations. The most important was the significant reduction of whole-body Na^+ concentration and inhibition of Na^+ K^+ ATPase activity in mussels exposed to 2 or 12 μg Cu/L for 28 days. These findings strengthen the use of these parameters (whole-body Na^+ concentration and Na^+ K^+ ATPase) as sensitive indicators of copper-induced impairment. We suggest that ionoregulatory disruption in freshwater mussels chronically exposed to copper is the main mechanism of toxicity. In contrast, redox parameters do not seem to be useful biomarkers of sub-lethal copper stress in freshwater mussels.

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