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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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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.

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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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