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Effects of salinity on short-term waterborne zinc uptake, accumulation and sub-lethal toxicity in the green shore crab (*Carcinus maenas*)

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ABSTRACT

Waterborne zinc (Zn) is known to cause toxicity to freshwater animals primarily by disrupting calcium (Ca) homeostasis during acute exposure, but its effects in marine and estuarine animals are not well characterized. The present study investigated the effects of salinity on short-term Zn accumulation and sub-lethal toxicity in the euryhaline green shore crab, *Carcinus maenas*. The kinetic and pharmacological properties of short-term branchial Zn uptake were also examined. Green crabs (n = 10) were exposed to control (no added Zn) and 50 μM (3.25 mg L^{-1}) of waterborne Zn (~25% of 96 h LC_{50} in 100 seawater) for 96 h at 3 different salinity regimes (100%, 60% and 20% seawater). Exposure to waterborne Zn increased tissue-specific Zn accumulation across different salinities. However, the maximum accumulation occurred in 20% seawater and no difference was recorded between 60% and 100% seawater. Gills appeared to be the primary site of Zn accumulation, since the accumulation was significantly higher in the gills relative to the hepatopancreas, haemolymph and muscle. Waterborne Zn exposure induced a slight increase in haemolymph osmolality and chloride levels irrespective of salinity. In contrast, Zn exposure elicited marked increases in both haemolymph and gill Ca levels, and these changes were more pronounced in 20% seawater relative to that in 60% or 100% seawater. An *in vitro* gill perfusion technique was used to examine the characteristics of short-term (1–4 h) branchial Zn uptake over an exposure concentration range of 3–12 μM (200–800 μg L^{-1}). The rate of short-term branchial Zn uptake did not change significantly after 2 h, and no difference was recorded in the rate of uptake between the anterior (respiratory) and posterior (ion transporting) gills. The *in vitro* branchial Zn uptake occurred in a concentration-dependent manner across different salinities. However, the rate of uptake was consistently higher in 20% seawater relative to 60% or 100% seawater – similar to the trend observed with tissue Zn accumulation during *in vivo* exposure. The short-term branchial Zn uptake was found to be inhibited by lanthanum (a blocker of voltage-independent Ca channels), suggesting that branchial Zn uptake occurs *via* the Ca transporting pathways, at least in part. Overall, our findings indicate that acute exposure to waterborne Zn leads to the disruption of Zn and Ca homeostasis in green crab, and these effects are exacerbated at the lower salinity.

1. Introduction

Zinc (Zn) is an essential micronutrient to all known living organisms, but becomes toxic at elevated exposure concentrations. The toxicity of Zn to aquatic biota is well known, but much of our current understanding is based on studies conducted with freshwater organisms, primarily fish (Hogstrand, 2012 for review). Zn is

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known to be particularly toxic to freshwater fish during waterborne exposure, and can act as an inonoregulatory toxicant, specifically during acute exposure (Spry and Wood, 1985; Hogstrand et al., 1995, 1996). Waterborne Zn is bioavailable as a free divalent ion, Zn^{++}, which shares, at least in part, a common uptake pathway with Ca^{++} in the gills of freshwater fish reflecting "ionic mimicry" (Spry and Wood, 1989; Hogstrand et al., 1994, 1995, 1998). As a consequence of this, exposure to waterborne Zn at elevated concentrations causes disruption of branchial Ca^{2+} uptake *via* competitive interaction, leading to hypocalcemia, which may eventually result in death depending on the exposure concentration (Spry and Wood, 1985; Hogstrand et al., 1995, 1996). In contrast to freshwater organisms, the mechanisms of Zn uptake and toxicity in marine or estuarine organisms have been studied sporadically. Although a shared transport system for Zn^{++} and Ca^{++} has been suggested in marine crustacean gill (Sá et al., 2009), it is unclear whether the acute exposure to waterborne Zn elicits similar inonoregulatory disruptions in marine animals as observed in freshwater animals. Recently, it has been reported that waterborne Zn exposure causes toxicity in estuarine killifish (*Fundulus heteroclitus*) by disrupting ionic homeostasis (Ca^{++} and Na^{+}), however the effect was salinity-dependent and decreased with increasing salinity (Loro et al., 2014).

Unlike marine fish, marine and estuarine crustaceans generally do not drink sea water (Henry et al., 2012). Consequently, during waterborne exposure to elevated trace metals, crustacean gills become the major site of uptake and accumulation of metals (Burke et al., 2003; Lee et al., 2010; Martins et al., 2011). In crustacean animals, gills carry out multiple vital physiological functions including gas and ion exchange with the environment, and thus are likely the key site of toxic action for metals. Since crustacean gills play a major role in regulating internal homeostasis, their properties change with alterations in environmental conditions such as pCO_2 or salinity (Lucu, 1990; Fehsenfeld et al., 2011). Many marine euryhaline crustaceans, such as the green shore crab, *Carcinus maenas*, are moderate osmoregulators and maintain their internal osmolality slightly above the ambient seawater. However, they can also withstand wide salinity fluctuations in estuarine habitats, and attempt to maintain ionic homeostasis by upregulating the absorption of ions *via* gills in order to compensate for the diffusive ion loss of ions from the body during environmental dilution (Towle et al., 1997, 2001). Crustacean gill epithelia have two main cell types: respiratory cells and ion-transporting cells. These two cell types are heterogeneously distributed among the gills, with the ion transporting gills more concentrated in the posterior 3–4 pairs of gills (6–9) (Freire et al., 2008). Thus, the posterior gills might play a more prominent role than the anterior gills in promoting metal uptake and toxicity of metals in crustaceans during waterborne exposures. Moreover, the uptake of ions by the posterior gills is likely to increase during low salinity regimes, thus potentially leading to greater uptake and toxicity of metals as a consequence.

The availability of Zn^{++} in sea water is mainly regulated by the complexation of Zn^{++} with inorganic anions, particularly chloride (Cl^{-}) and hydroxide (OH^{-}) (Zirino and Yamamoto, 1972; Rainbow et al., 1993) (cf. Table 3). A decrease in salinity reduces the amount of inorganic ions available for complexation of Zn^{++}, and also decreases the concentrations of potentially competing cations (*e.g.* Ca^{++}, Mg^{++}, Na^{+}), thereby increasing its availability for biological uptake. Thus, a decrease in salinity can increase the uptake and accumulation of Zn in marine organisms simply due to the changes in water chemistry. However, previous studies indicated that the relationship between Zn uptake and salinity does not always follow a linear pattern in marine crustaceans. For example, it has been reported that lower salinities caused an increase in Zn uptake in the extremely euryhaline Chinese mitten crab (*Eriocheir sinensis*), but resulted in reduced Zn uptake in the euryhaline green shore crab (*C. maenas*) and stenohaline velvet swimming crab (*Necora puber*) (Rainbow and Black, 2002). Moreover, an increase in Zn uptake with decreasing salinity was observed in the crustacean amphipod *Orchestia gammarellus*, but only in the salinity range of 36–25 ppt, with no further increase below 25 ppt (Rainbow et al., 1993; Rainbow and Kwan, 1995). These observations indicate that the uptake and toxicity of Zn in marine crustaceans can be influenced by their physiological response to change in ambient salinity, offsetting the physicochemical effect of Zn speciation in seawater.

The overall goal of this study was to elucidate how salinity influences the uptake, accumulation and toxicity of Zn during acute waterborne exposure in a model euryhaline decapod crustacean species, the green shore crab (*C. maenas*). The biology and ecology of this species are well known, and it has been extensively used in aquatic ecotoxicology research (Leignel et al., 2014; Rodrigues and Pardal, 2014). The specific objectives of this study were: (i) to examine how the changes in salinity affect the tissue-specific accumulation of Zn, (ii) to understand how salinity influences the kinetics of short-term waterborne Zn uptake in the crustacean gill and also to characterize the pharmacological properties of branchial Zn uptake pathway, and (iii) to determine the ionoregulatory and osmoregulatory effects of acute waterborne Zn exposure across different salinity regimes. We hypothesized that the uptake, accumulation and toxicity of Zn in the green crab would increase with decreasing salinity, and the toxicity of Zn would occur due to the disruption of ionic (*e.g.*, Ca^{++}) homeostasis.

2. Methods

2.1. Animal care

Male green crabs (*Carcinus maenas*; 50–70 g) were collected from two uncontaminated sites just outside of Pipestem Inlet (N 49°02. 274 – W 125°20. 710 and N 49°01.749 – W 125°21.515) in Barkley Sound (BC, Canada) using baited crab pots. Animals were transferred and held at the Bamfield Marine Sciences Centre (Bamfield, BC, Canada) in outdoor tanks (~200-L) maintained with flow-through seawater (SW; ~35 ppt) under constant aeration on a 12 h D:12 h L photoperiod. All crabs were allowed to acclimate to the holding conditions in 100% seawater (35 ppt) (SW) for 7 days prior to their use in this study. Crabs were then randomly distributed into one of three salinity exposure groups [20% SW (7 ppt), 60% SW (21 ppt), 100% SW (35nppt)] where they were held for a 7-day acclimation period. Each group (N = 20) was maintained in a plastic container (68 L) with aeration and filtration. Water changes were made every 3 days to avoid the accumulation of deleterious nitrogenous waste (Regnault, 1987). Crabs were fed twice a week on salmon fish heads, but were starved 48 h prior to any experimentation. All procedures were approved by Bamfield Animal Research Ethics Board and were in accordance with the Guidelines of the Canadian Council on Animal Care.

2.2. In vivo *acute waterborne Zn exposure*

Crabs were exposed to a nominal waterborne Zn concentration of 55 μM (3.60 mg L^{-1}) for 96 h in 3 different salinity regimes (20% SW, 60% SW, and 100% SW). The Zn exposure concentration used here represented approximately the 25% of the 96 h LC_{50} of waterborne Zn in *C. maenas* in 100% SW (Elumalai et al., 2007). The experimental exposures were performed in large polyethylene containers filled with 40 L of SW under continuous aeration. The targeted Zn concentration was achieved by spiking the exposure water with the appropriate amount of a concentrated $ZnCl_2$ stock solution, and the water was then allowed to equilibrate for