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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

an hour. No Zn was added to the controls of respective salinity regimes. Each experimental treatment was carried out in 2 replicates, and 10 crabs (5 per replicate) were transferred randomly to the exposure chambers for each treatment. All experimental treatments were conducted using a static renewal system, with a complete (100%) renewal of the exposure water at 24 h. Water samples were collected immediately after the introduction of the fresh exposure water and also at the end of the 24 h cycle, prior to the next exchange. Water samples were filtered through a 0.45 μm syringe filter (Acrodisc; Pall Life Sciences, TX, USA), and later used for the analysis of water chemistry and dissolved Zn concentrations (see below for details). The animals were not fed during the 96-h exposure period, and their survival was monitored every 12 h. At the end of the exposure, animals were anesthetized (~15 min) on ice and then quickly euthanized by a single spike to the ventral ganglion through the ventral wall of the carapace. Gills, muscle, hepatopancreas and haemolymph were collected for the analysis of Zn accumulation, and/or ion (Ca^{++}, Na^{+} and Cl^{-}) and osmolality levels in specific tissues (see below for details).

2.3. In vitro *gill perfusion*

Crabs were removed from salinity acclimation tanks and placed on ice for 15 min to ensure anesthesia. Crabs were then euthanized as described above. The carapace was lifted and removed before the gills 5 (anterior) and 8 (posterior) were excised from the base and placed in a petri dish on ice containing saline solutions which corresponded to the respective salinity (20%, 60% or 100% SW) to which the crabs were acclimated (see below). Gill perfusion experiments were then performed as described by Siebers et al. (1985) with some modifications. Briefly, the excised gill was perfused with an artificial haemolymph based on previous literature (Lignon, 1987; Mantel and Farmer, 1983; Winkler, 1987) for 100% SW crabs: 470 mM NaCl, 12 mM $CaCl_2$, 12 mM $MgCl_2$, 11 mM KCl, 9 mM $NaHCO_3$, 0.1 mM NH_4Cl, 0.3 mM glucose, 0.1 mM glutathione, 0.5 mM glutamine; pH 7.9. For 60% SW and 20% SW crabs the artificial haemolymph was based on recorded values in *C. maenas* from Fehsenfeld and Weihrauch (2013), 60% SW: 332 mM NaCl, 5.3 mM $CaCl_2$, 10.2 mM $MgCl_2$, 8 mM KCl, 4.0 mM $NaHCO_3$, 0.1 mM NH_4Cl, 0.3 mM glucose, 0.1 mM glutathione, 0.5 mM glutamine; pH 7.9, and for 20% SW crabs: 260 mM NaCl, 5 mM $CaCl_2$, 7 mM $MgCl_2$, 7 mM KCl, 7 mM $NaHCO_3$, 0.1 mM NH_4Cl, 0.3 mM glucose, 0.1 mM glutathione, 0.5 mM glutamine; pH 7.9. Perfusion was achieved using a peristaltic pump (Sci 323 Watson-Marlow Bredel Pump, Falmouth Cornwall, England) and elastic tubing that was inserted into afferent and efferent blood vessels of the gill. The rate of perfusion was set at 140 ± 0.1 μL min^{-1} for 1–4 h, as previously employed for similar gill perfusion experiments with *C. maenas* (Siebers et al., 1985; Fehsenfeld and Weihrauch, 2013). To ensure that the rate of *in vitro* branchial Zn uptake was not influenced by changes in the perfusion rate, perfusions with a significantly higher or lower perfusate volume were not included in the analysis. During perfusion, the gills were suspended in a 50-mL ^{65}Zn exposure solution, under continuous aeration. The desired Zn exposure concentrations were achieved by spiking the exposure media (20%, 60% or 100% SW) with appropriate amounts of cold (as $ZnCl_2$) and radioactive Zn (^{65}Zn; 3 μCi L^{-1} as $ZnCl_2$; Perkin Elmer, ON, Canada). At the end of the exposure period, gills were collected, rinsed for 10 s in 1 mM cold $ZnCl_2$ solution to remove loosely bound (not-absorbed) ^{65}Zn, gently blotted dry using Kimwipes, and placed in polyethylene vials for the analysis of ^{65}Zn radioactivity as described below. Perfusate samples were also collected to analyze the movement of Zn from the bathing solution into the perfusate, while samples of the bathing solution were also taken for the determination of specific activity of ^{65}Zn.

We carried out 3 different sets of *in vitro* gill perfusion experiments to characterize the short-term branchial Zn uptake properties in *C. maenas*. In the first experiment, we examined the differences in time-dependent uptake of Zn between the gills 5 and 8. To do this, both gills 5 and 8 from the crabs acclimated to 100% SW were exposed to 3 μM ^{65}Zn in 100% SW for 1–4 h. Since it was observed that Zn uptake in both gills 5 and 8 occurs at the same rate and does not change significantly after 2 h (see Fig. 4), the next two gill perfusion experiments were conducted using only the gills 8 and an exposure period of 2 h. In the second experiment, we examined how salinity influences the concentration-dependent branchial uptake of Zn. For this, gills 8 were exposed to 3, 6 and 12 μM ^{65}Zn in the respective salinity for the exposure (*i.e.* 20%, 60% or 100% SW). In the final experiment, we examined whether epithelial Ca^{++} channels are involved in branchial Zn uptake. Here, gills 8 from the crabs acclimated to 100% SW were exposed to 3 μM ^{65}Zn in 100% SW, in the absence and presence of two different Ca^{++} channel blockers, verapamil and lanthanum chloride ($LaCl_3$), each at 100 μM. Each experimental treatment was replicated 5 times.

2.4. Tissue and water analyses

Osmolalities of SW and haemolymph samples were directly determined using a Wescor VAPRO 5520 vapor pressure osmometer (Logan, UT, USA). Cl^{-} levels in SW and haemolymph were measured using a Labconco digital chloridometer (B5953; Kansas City, MO). Certified standard solutions (Fisher Scientific, ON, Canada) were used to generate standard curves for both osmolality and Cl^{-} analyses. Subsequently, each tissue sample was weighed, and depending on mass, was then placed in either a 50 mL, 15 mL, or a 2 mL centrifuge tube. Tissues were digested with 1 N trace metal grade nitric acid (Sigma-Aldrich, ON, Canada) at volumes of 5 times the weight of the tissue, except for the hepatopancreas and muscle, which were digested in a similar volume of 2 N trace metal grade nitric acid. All tubes were tightly sealed and placed in an incubator at 65 °C for 48 h, with vigorous vortexing at 24 h. The digested samples were then centrifuged for 5 min at 3500 rpm, and the supernatants were collected and used later to measure total Zn and ion concentrations.

Measurements of Zn in water and animal tissue samples were carried out using a flame atomic absorption spectrophotometer (AAnalyst 800; Perkin Elmer, CA, USA) against certified atomic absorption standards (Sigma Aldrich, ON, Canada). The recovery of Zn in water and tissue samples were 102.1% and 96.4%, respectively, as determined by National Research Council of Canada certified reference materials, NASS-6 (SW) and TORT-3 (Lobster hepatopancreas). Final reported Zn concentrations were not corrected for recovery. Ion levels in water (K^{+}, Na^{+}, Mg^{++} and Ca^{++}) and tissue (Na^{+} and Ca^{++} in haemolymph and gill) were also measured using the flame atomic absorption spectrometer against certified standards for each ion, as described previously. Water pH was measured by an Accumet Basic AB15 pH meter (Fisher Scientific, Ottawa, ON). Dissolved organic carbon (DOC) was measured using a Shimadzu TOC-Vcph/CPN total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan).

For the *in vitro* gill perfusion experiments, the radioactivity of ^{65}Zn in gills, perfusates, and bathing solutions was counted on a Wallac 1480 Wizard 3" gamma counter (Perkin Elmer, CA, USA). The actual total Zn concentrations in the bathing solutions were verified by flame absorption spectroscopy as outlined above, and the difference between the measured and the nominal concentrations of Zn never exceeded ±5%.

Table 1
Water chemistry for different experimental salinities. Data are presented as mean ± SEM (N = 8). Different letters indicate the statistically significant differences among treatments ($P \leq 0.05$).

Parameter	20% SW	60% SW	100% SW
pH	7.6 ± 0.04^a	7.9 ± 0.04^a	8.1 ± 0.05^b
Temperature (°C)	18	18	18
DOC (mg L^{-1})	2.6 ± 0.4	2.7 ± 0.4	3.1 ± 0.3
Na^+ (mmol L^{-1})	94.3 ± 1.3^a	327.1 ± 8.3^b	481.4 ± 11.1^c
Mg^{++} (mmolnL^{-1})	7.4 ± 0.3^a	28.3 ± 0.9^b	43.1 ± 0.4^c
K^+ (mmol L^{-1})	1.8 ± 0.03^a	6.5 ± 0.8^b	10.3 ± 0.4^c
Ca^{++} (mmol L^{-1})	1.8 ± 0.2^a	5.4 ± 0.5^b	9.9 ± 0.3^c
Cl^- (mmol L^{-1})	94.8 ± 3.2^a	280.3 ± 6.1^b	516.1 ± 3.8^c
Osmolality (mmol kg^{-1})	355 ± 9.8^a	644 ± 10.1^b	926 ± 11.4^c

Table 2
Measured dissolved Zn concentrations (μM) in 20, 60 and 100% SW used in the 96-h *in vivo* acute waterborne Zn exposure experiment (N = 8). Data are presented as mean ± SEM (N = 8).

Salinity	Control	Zn Exposure
20% SW	0.32 ± 0.03	54.21 ± 2.31
60% SW	0.34 ± 0.02	50.64 ± 3.28
100% SW	0.29 ± 0.03	50.18 ± 5.16

2.5. Calculations and statistical analyses

For gill perfusion experiments, the rate of Zn uptake into the gill tissue (nmol g^{-1} h^{-1}) was calculated as follows:

$$\frac{CPM}{SA} \times \frac{1}{W} \times \frac{1}{T}$$

where CPM is the counts per minute of ^{65}Zn radioactivity in the individual gill tissue, SA is the specific activity of ^{65}Zn (the ratio of ^{65}Zn CPM and total dissolved Zn concentration in the bathing solution (nM)), W is the tissue weight (g), and T is the duration of exposure (h). The rate of Zn uptake into the perfusate (nmol g^{-1} h^{-1}) was also determined by an identical equation except that CPM represented the total accumulated CPM in the perfusate over the exposure period.

All data have been expressed as mean ± SEM (N). Statistical analyses were performed with SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA) and Sigma Stat 3.5 (Systat Software Inc., San Jose, CA, USA). The water chemistry data (Table 1) and the data from the gill perfusion experiment with Ca channel blockers (Fig. 5B) were analyzed using a one-way ANOVA with a Tukey's post-hoc test. The rest of the data were analyzed by two-way ANOVA with Zn concentration and salinity or time as the two independent variables. Where significance ($P < 0.05$) was found, a Tukey's post-hoc test was applied. Significance for all statistical tests was accepted at $\alpha = 0.05$.

For Zn speciation analysis the water chemistry (Table 1) and the measured dissolved Zn concentrations (Table 2) were used to estimate the free ionic Zn (Zn^{++}) concentrations using Visual MINTEQ software (version 3.1, KTH, Department of Land and Water, Resources Engineering, Stockholm, Sweden). The NICA- Donnan model was used to estimate the effect of DOC on Zn speciation (Benedetti et al., 1995) (Table 3).

Table 3
Major chemical species of Zn (% of total Zn) at different salinity regimes as calculated by Visual MINTEQ 3.1 based on measured water chemistry (see Table 1) and dissolved Zn concentrations in the exposure water (see Table 2).

Species of Zn	20% SW	60% SW	100% SW
Zn^{2+}	89.2	78.9	64.9
$ZnCl^+$	8.1	15.6	23.1
$ZnCl_2$ (aq)	0.3	1.4	3.6
$ZnOH^+$	1.0	1.4	1.9
$Zn(OH)_2$ (aq)	0.6	1.7	3.3
Zn-DOC	0.7	0.6	0.6

3. Results

3.1. Effects of salinity on Zn speciation in the exposure water and Zn accumulation in tissues

The measured total Zn concentrations in the exposure water remained consistent both in the control (~0.30 μM) and Zn treatment groups (~50 μM) across 3 different salinity regimes used in this study (20%, 60% and 100% SW) (Table 2). Based on estimation of Zn speciation by Visual MINTEQ (version 3.1), Zn predominantly existed as Zn^{++} in the exposure water of Zn-treatments in all salinities. However, the fraction of Zn^{++} in the exposure water increased with decreasing salinity, with a 25% increase from 100% SW to 20% SW, mainly due to reduced complexation of Zn^{++} by Cl^- and OH^- (Table 3).

The change in salinity did not influence the Zn concentration in any tissues in the control populations of green crab (Fig. 1). Zn accumulation increased significantly relative to the controls in the gills (all gills combined), haemolymph and hepatopancreas following exposure to waterborne Zn for 96 h regardless of the salinity level (Fig. 1A–C). However, for each of these tissues, the maximum Zn accumulation occurred in crabs that were exposed to waterborne Zn at 20% SW, with no difference in Zn accumulation between crabs exposed to Zn at 60% and 100% SW (Fig. 1A–C). In contrast, a significant increase in Zn accumulation in muscle was observed only in crabs exposed to Zn at 20% SW (Fig. 1D). The magnitude of Zn accumulation was found to be the highest in the gill relative to any other tissues. Zinc concentrations in the gill increased by 7–10 fold following exposure to 50 μM waterborne Zn for 96 h, whereas the increase in Zn levels was 2.5–4 fold in the hepatopancreas and haemolymph, and only 1.5 fold in the muscle (Fig. 1).

3.2. Effects of waterborne Zn exposure on ion-osmoregulation and toxicity

Exposure to 50 μM waterborne Zn did not affect the survival of green crabs over 96 h in 60% and 100% SW, but a 20% mortality (2 out of a test population of 10 crabs) was recorded following exposure to Zn at 20% SW.

The concentrations of essential ions (Na^+, Ca^{++} and Cl^-) and osmolality levels in the haemolymph decreased consistently with decreasing salinity in both control and Zn-exposed green crabs (Fig. 2). However, the ion concentrations and osmolality levels in the haemolymph in all of the salinity levels examined were always found to be elevated relative to those in the respective exposure waters, and these differences were more pronounced in 20% SW relative to either 60% or 100% SW (Fig. 2; Table 1).

Exposure to 50 μM waterborne Zn for 96 h resulted in a significant increase in Ca^{++} and Cl^-, but not Na^+, concentrations as well as osmolality levels in the haemolymph regardless of the salinity of the exposure water (Fig. 2). Haemolymph Ca^{++} concentrations in Zn-exposed crabs were elevated by approximately 32%, 37% and 71% in 100%, 60% and 20% SW treatments, respectively, relative to those in the respective control populations (Fig. 2B). In contrast to Ca^{++}, the increases in Cl^- and osmolality levels in the haemolymph following exposure to Zn were modest and varied between 8 and 11% across different salinity levels (Fig. 2C,D).

Similar to the pattern observed in the haemolymph, a salinity-dependent decrease in the gill Na^+ and Ca^{++} concentrations was also recorded (Fig. 3). Exposure to waterborne Zn did not affect gill Na^+ concentration, but significantly increased gill Ca^{++} concentrations in all 3 salinity levels used in this study. The increase in gill Ca^{++}

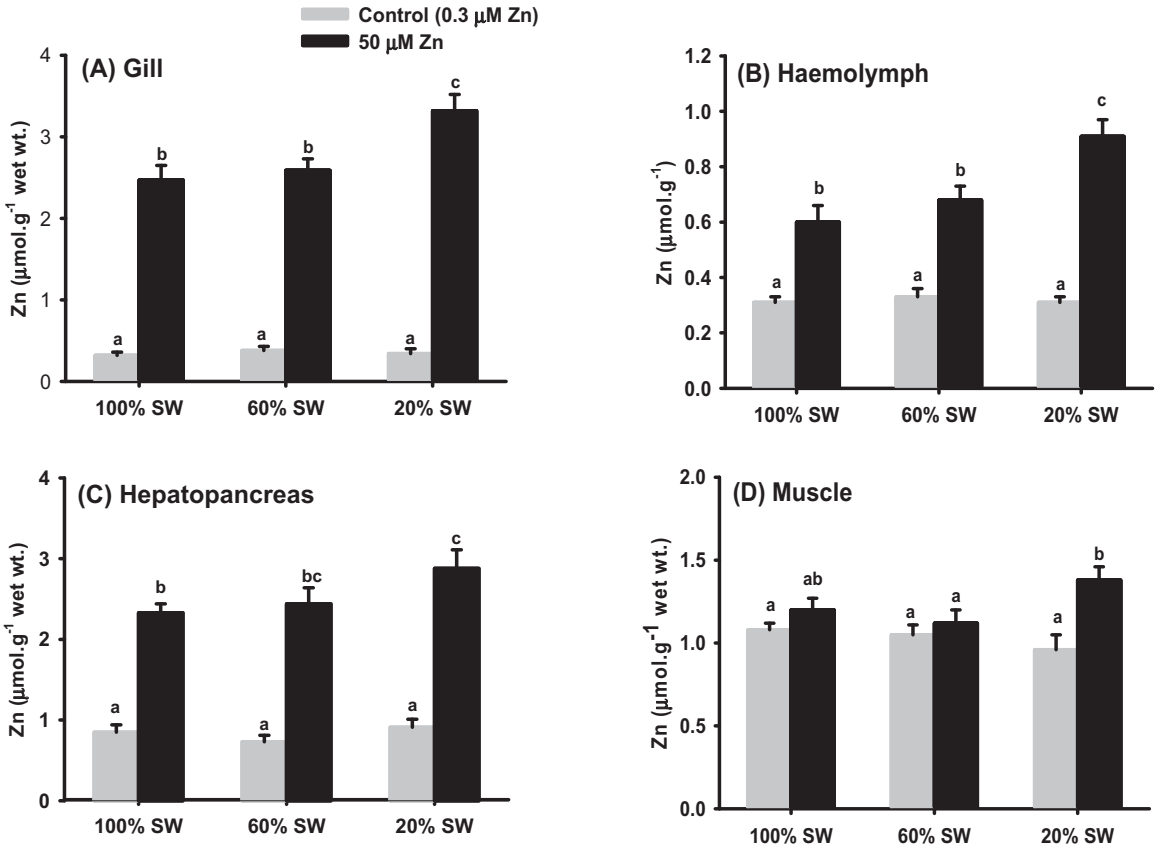

Fig. 1. Tissue-specific Zn accumulation in the green crab (*Carcinus maenas*) exposed to 0.3 μM (control) and 50 μM waterborne Zn for 96 h. Data are presented as mean ± SEM (N = 8–10). Statistically significant differences among the data are indicated by different letters.

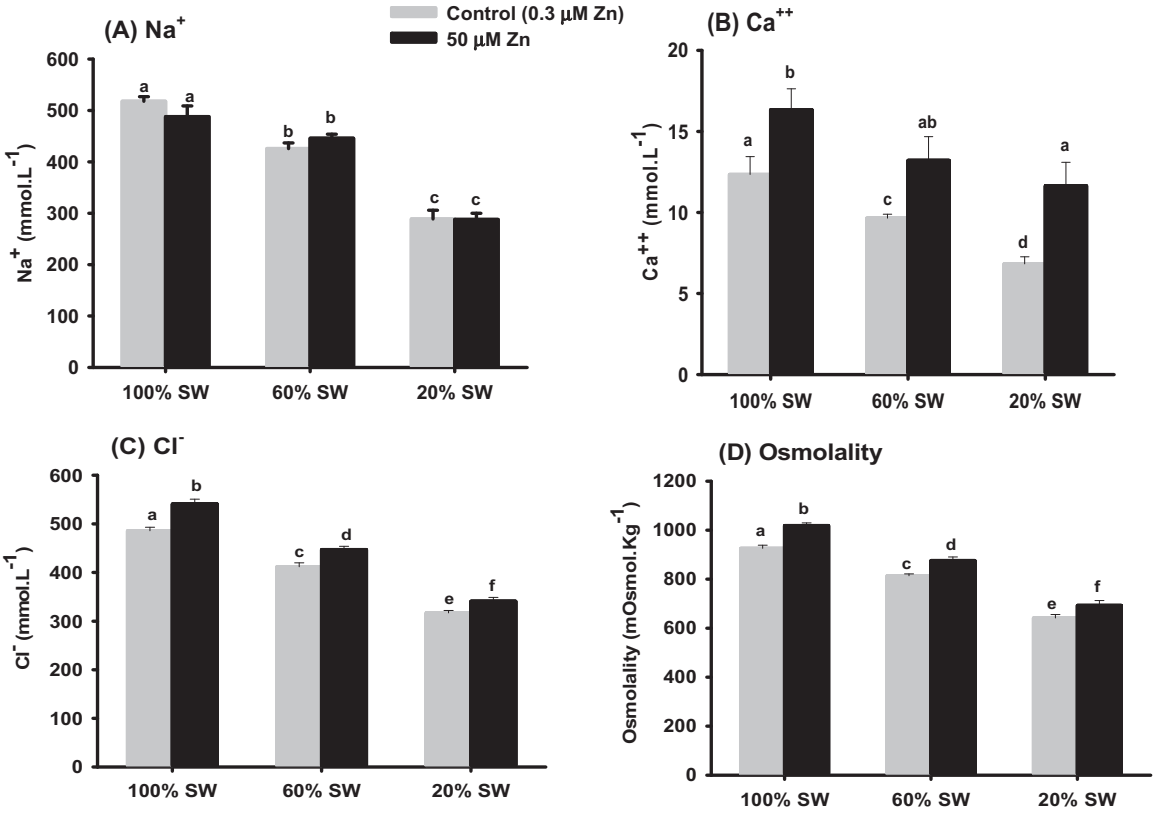

Fig. 2. Haemolymph ion and osmolality levels in the green crab (*Carcinus maenas*) exposed to 0.3 μM (control) and 50 μM waterborne Zn for 96 h. Data are presented as mean ± SEM (N = 8–10). Statistically significant differences among the data are indicated by different letters.

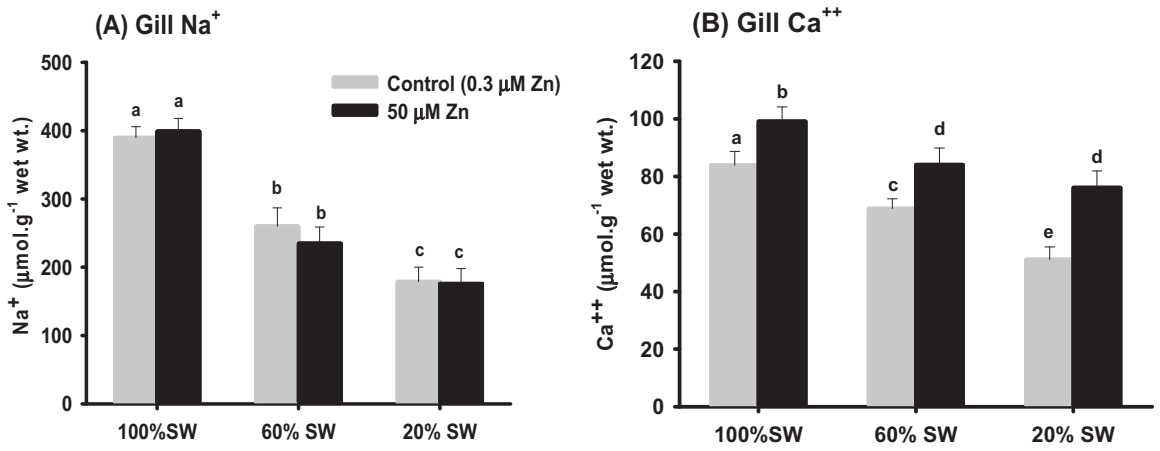

Fig. 3. Gill Ca^{++} and Na^{+} concentrations in the green crab (*Carcinus maenas*) exposed to 0.3 μM (control) and 50 μM waterborne Zn for 96 h. Data are presented as mean ± SEM (N = 8–10). Statistically significant differences among the data are indicated by different letters.

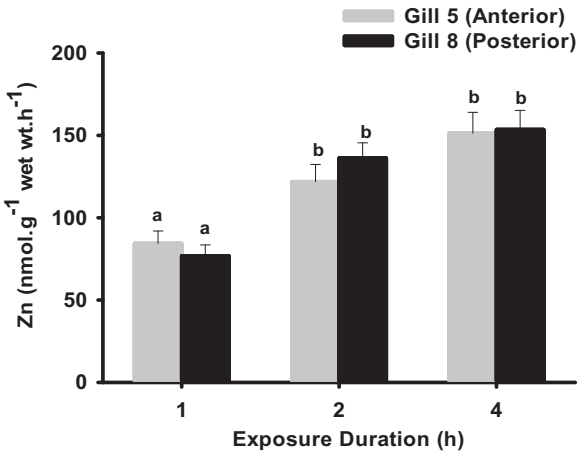

Fig. 4. Time dependent *in vitro* Zn uptake rate into the tissues of two different gill types (anterior and posterior) of the green crab (*Carcinus maenas*) at an exposure concentration of 3 μM ^{65}Zn in 100% SW. Data are presented as mean ± SEM (N = 5). Statistically significant differences among the data are indicated by different letters.

concentration following exposure to Zn was much higher in 20% SW (50%) relative to that in 60% and 100% SW (21 and 18%, respectively).

3.3. *Characteristics of short-term* in vitro *branchial Zn uptake*

The uptake rates of Zn into the perfusate were extremely low (data not shown) and almost negligible (<2%) in comparison to those into the gill tissues, and therefore only the results of uptake in the gill have been described here. The rate of short-term *in vitro* Zn uptake at 3 μM ^{65}Zn exposure increased with time in both gill 5 (anterior) and 8 (posterior), however the magnitude of increase reduced beyond 2 h and the uptake rate approached a steady state by 4 h because rates determined over 2 h were not significantly different from those determined over 4 h (Fig. 4). No difference in the rate of Zn uptake was found between gill 5 and 8 over 4 h of exposure (Fig. 4). In general, the rate of short-term (2 h) *in vitro* Zn uptake in gill 8 increased in a concentration-dependent manner over an exposure range of 3–12 μM ^{65}Zn regardless of salinity, although the uptake seemed to attain saturation at ≥6 μM ^{65}Zn in 20% SW (Fig. 5A). Moreover, the rate of Zn uptake in 20% SW was significantly higher relative to that in 60% or 100% SW at all of the ^{65}Zn exposure concentrations tested, whereas no significant difference in the uptake was observed between 60% and 100% SW at any ^{65}Zn exposure concentration (Fig. 5A). The short-term (2-h) uptake of Zn in gill 8 exposed to 3 μM waterborne ^{65}Zn in 100% SW was significantly decreased (44% reduction) by $LaCl_3$, but not by verapamil, relative to the control (Fig. 5B).

4. Discussion

To the best of our knowledge, this is the first study to characterize the effects of acute waterborne Zn exposure on tissue-specific Zn accumulation and physiology (ion-osmoregulation) in a euryhaline decapod crustacean, the green crab (*C. maenas*). As hypothesized, both Zn accumulation and toxic physiological responses were found to be salinity-dependent, with the major effects being an increase in Zn accumulation in tissues, primarily in the gill, and ion-regulatory (mainly Ca^{++}) disturbances, both of which increased with decreasing salinity.

4.1. *Salinity-dependent tissue-specific patterns of Zn accumulation*

Our study indicated that gill in the green crab becomes one of the primary sites of Zn accumulation during acute exposure to waterborne Zn, as it accumulated Zn in much higher concentration (≥2-fold) than any other tissues examined (hepatopancreas, haemolymph and muscle) (Fig. 1). This is consistent with previous studies which also reported that gill accumulates other metals such as nickel (Ni) and cadmium (Cd) in greater concentrations than hepatopancreas, muscle or haemolymph during acute exposure (Burke et al., 2003; Blewett et al., 2015; Blewett and Wood, 2015). In the present study, the decrease in salinity was found to increase tissue-specific Zn accumulation in green crab acutely exposed to waterborne Zn. In general, our observations are in agreement with several previous studies that reported increased accumulation and toxicity of metals in marine crustaceans with decreasing salinities (Burke et al., 2003; Lee et al., 2010; Leonard et al., 2011; Martins et al., 2011; Blewett et al., 2015; Blewett and Wood, 2015).

Salinity is believed to decrease the bioavailability of Zn due to increased complexation of Zn^{++} with Cl^{-} and OH^{-} in saline water (Zirino and Yamamoto, 1972; Rainbow et al., 1993). In agreement with this, we found that the fraction of Zn^{++} in the Zn exposures declined by 14% from 100% to 60% SW, which was followed by a further 10% decrease in 20% SW (Table 3). Interestingly, however, the relationship between Zn accumulation in tissues and salinity did not follow the same pattern, since we did not observe any significant differences in Zn accumulation in any tissues between crabs exposed to Zn in 60% and 100% SW. In contrast, Zn accumulation in

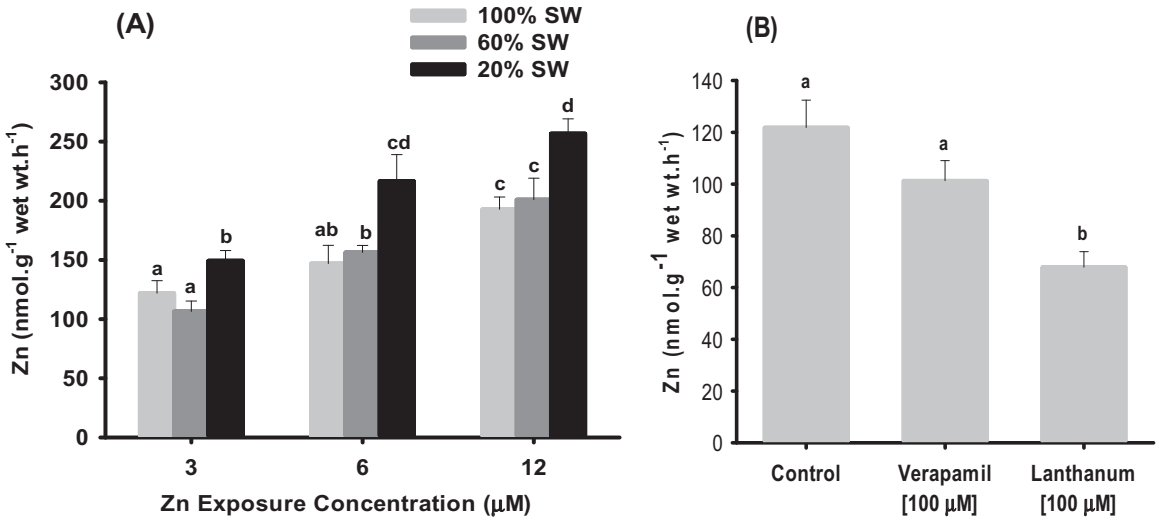

Fig. 5. (A) Concentration dependent *in vitro* Zn uptake rate into the tissue of the posterior gill (gill 8) of the green crab (*Carcinus maenas*) over an exposure period of 2 h to various concentrations of ^{65}Zn; (B) Effects of Ca channel blockers on *in vitro* Zn uptake rate in the posterior gill following exposure to 3 μM ^{65}Zn in 100% SW for 2 h. Data are presented as mean ± SEM (N = 5). Statistically significant differences among the data are indicated by different letters.

all tissues was significantly higher in crabs exposed to Zn in 20% SW relative to that in 60% and 100% SW (Fig. 1). This occurred even in the muscle which did not exhibit any difference in Zn concentration between the control and Zn treatment in either 60% or 100% SW. It is to be noted here that partial mortality (20%) was also observed only in crabs exposed to Zn in 20% SW, which was likely the consequence of higher Zn accumulation in this treatment. Zinc accumulation patterns recorded in the present study were similar to the observations by Blewett et al. (2015); Blewett and Wood (2015) in green crabs exposed to waterborne nickel (Ni), where the maximum Ni accumulation in different tissues were recorded in 20% SW, with no difference in Ni accumulation between 60% and 100% SW. Wright (1977a) also reported a significant increase in tissue-specific Cd accumulation in green crabs exposed to Cd only in low salinities. This likely occurs due to the osmoregulatory strategies employed by the green crab in dilute SW.

Carcinus is a moderate osmoregulator, and always maintains haemolymph osmolality above the osmolality of ambient SW. However, when the external medium gets diluted, the haemolymph osmolality in this species also declines (Siebers et al., 1982), as observed in our study (Fig. 2). Many euryhaline osmoregulators have a critical salinity where *trans*-branchial uptake of NaCl is activated (Henry, 2005). This critical salinity occurs at 26 ppt (~74% SW), for many crustacean species, but higher and lower values have been reported depending on the species. In our study, crabs in both 100% and 60% SW displayed similar Zn accumulation patterns in tissues, possibly because they employ similar osmoregulatory strategies in this salinity range. In contrast, a further increase in Zn accumulation was observed in crabs exposed to Zn 20% SW, likely as a result of the upregulation of active *trans*-branchial uptake of ions in 20% SW. This is supported by the observation that metabolic rates of some euryhaline crabs increase during exposure to low salinities, indicating an increased energetic cost associated with acquiring ions to maintain homeostasis due to environmental dilutions (Normant and Gibowicz, 2008; Wallace, 1972).

Assuming that branchial Zn uptake occurs through ion-specific pathways (described below), an increase in active uptake of ions at the lowest tested salinity would also likely increase the uptake of Zn in the crabs through "ionic mimicry" (see Introduction), and would result in the higher Zn accumulation observed in the present study. Thus, even though the increase in the availability of Zn^{++} from 100% to 60% SW is more or less similar to that from 60% to 20% SW, the change in physiology that is induced at the lowest salinity likely drives the Zn accumulation patterns observed in the green crabs. Contrary to the findings of the present study, Rainbow and Black (2002) reported a decreased Zn accumulation at lower salinities (15–20 ppt vs. 33 ppt) in the haemolymph of green crab exposed to 0.75 μM radiolabeled waterborne Zn for 96 h. The possible reason for the discrepancy between these two studies is that Rainbow and Black (2002) examined Zn accumulation at a much lower Zn exposure concentration along with a more moderate decrease in salinity in the exposure, relative to that employed in the present study (50 μM; salinity range 7–35 ppt), allowing a more efficient regulation of Zn uptake by the experimental animals.

4.2. In vitro *branchial Zn uptake characteristics, and the influence of salinity*

We aimed to study the kinetic and pharmacological characteristics of short-term branchial Zn uptake in *Carcinus* during a phase when the uptake is linear and has not attained saturation. Thus, the *in vitro* branchial Zn uptake was examined at lower Zn exposure concentrations (3–12 μM ^{65}Zn) compared to that used in the *in vivo* exposure (50 μM). Our results revealed that the uptake of Zn in 100% SW occurs at a same rate in both anterior (gill 5) and posterior (gill 8) gills, and that the rate approaches a steady state at 2 h, as it was not significantly different over 4 h (Fig. 4). This suggests that both the anterior (respiratory) and posterior (osmoregulatory) gills in green crab play an equally important role in transporting waterborne Zn into the body. Martins et al. (2011) also reported similar rates of short-term (4 h) *in vitro* branchial Cu uptake between anterior and posterior gills of euryhaline blue crab *Callinectes sapidus* in both high and low salinities. Moreover, Blewett et al. (2015) found that both anterior and posterior gills of green crab absorb Ni at the same rate during short-term (2 h) *in vitro* exposure except at a low salinity (20% SW), where the uptake rate was significantly higher in the posterior gill. We did not examine branchial Zn uptake in low salinities (*e.g.*, 20% SW), nonetheless it appears that our observations on the short-term branchial uptake of Zn are in good agreement with those of Cu or Ni uptake in crustacean decapods.

We observed a concentration-dependent increase in short-term (2 h) branchial Zn uptake in the posterior gill of *Carcinus* in all of the

three salinities tested (20%, 60% and 100% SW). In addition, similar to the pattern of branchial Zn accumulation observed during *in vivo* exposure, the rates of *in vitro* branchial Zn uptake in 20% SW were consistently greater than those in 60% and 100% SW, with no significant difference between 60% and 100% SW at any ^{65}Zn exposure concentration examined (Fig. 5A). This further corroborates the argument that the upregulation of *trans*-branchial ion uptake pathways in 20% SW might have contributed to greater Zn uptake in the green crab during exposure to waterborne Zn across different salinities. Unlike the pattern in 60% and 100% SW, the short-term branchial Zn uptake in 20% SW seemed to approach saturation during exposure to 3–12 μM ^{65}Zn, which likely occurred because of the increased availability of Zn^{++} in 20% SW relative to that in 60% and 100% SW, as described previously. We recorded extremely low rates of Zn uptake in the perfusate relative to that in the gills, possibly because of the absence of Zn-carrier proteins that typically exist in the crustacean haemolymph.

In the present study, the short-term Zn uptake in the posterior gill of green crab was found to be significantly inhibited by $LaCl_3$, but not by verapamil. Lanthanum is a voltage-independent epithelial Ca^{++} channel blocker, whereas verapamil is a L-type voltage-gated Ca^{++} channel blocker. Thus, our results suggest that the branchial uptake of Zn in green crab occurs *via* voltage-independent Ca^{++} channels, at least in part. Sá et al. (2009) also suggested that the short-term Zn uptake in isolated gill epithelial cells of American lobster (*Homarus americanus*) occurs *via* Ca^{++} dependent pathways, although the uptake was found to be sensitive to L-type voltage-gated Ca^{++} channel blockers (verapamil and nifedipine). Nonetheless, it is likely that other epithelial transporters (*e.g.*, Na^+/H^+ exchanger, DMT-1, ZIP) may also be involved in the branchial uptake of Zn in marine crustaceans (Henry et al., 2012), and these should be investigated in future studies.

4.3. Zinc induced disruption of osmoregulation and Ca homeostasis across different salinities

In the present study, a salinity-dependent decrease in haemolymph osmolality as well as in the ion levels in both haemolymph and gill was recorded in the green crab. Similar salinity dependent alterations in osmolality and ion levels in the haemolymph and/or gill of green crab have also been reported in previous studies (Burke et al., 2003; Blewett et al., 2015). Interestingly however, exposure to waterborne Zn resulted in a small but significant increase of haemolymph osmolality and Cl^- levels (not Na^+) regardless of salinity. Quantitatively, Na^+ and Cl^- are the most important ions in crab haemolymph, and therefore the changes in haemolymph osmolality were likely caused by the increases in Cl^- levels, and to a lesser extent by the increases in Ca^{++} levels (see below). Bjerregaard and Vislie (1985) also observed an increase in haemolymph osmolality in the green crab following acute exposure to waterborne Cd, although they implicated a corresponding increase in haemolymph Na^+ level for that change. The mechanism of Cl^- increase stimulated by Zn exposure in the current study is unknown and requires further study.

More importantly, our study revealed that exposure to waterborne Zn resulted in substantial increases in Ca^{++} levels in both gill and haemolymph of green crab, and this effect was particularly exacerbated at the lower salinity (20% SW). It is to be noted that although our findings suggest that Zn^{++} and Ca^{++} may share common uptake mechanisms across the gill, this may not necessarily translate into depletion of Ca^{++} in tissues. Instead, physiological adjustment of Ca^{++} homeostasis may occur in order to maintain tissue Ca^{++} levels in the animal, to the extent that overshoot occurs. Similar to our findings, previous studies also reported marked increases in the haemolymph and gill Ca^{++} in green crab following exposure to waterborne Cd, especially at lower salinities (Wright, 1977b; Bjerregaard and Vislie, 1985; Burke et al., 2003). Cd is a gill Ca^{++} antagonist like Zn^{++} (Niyogi and Wood, 2004). Recently, Loro et al. (2014) similarly observed that acute waterborne exposure to Zn in saline water led to a significant increase in both gill and plasma Ca^{++} levels in killifish. In crabs, Ca^{++} exchange with the exoskeleton could also be involved. Zinc could augment haemolymph Ca^{++} levels in the green crab by inhibiting the transport of Ca^{++} from haemolymph to exoskeleton. This could occur due to the Zn-mediated disruption of Ca^{++}-ATPase and/or Na^+/Ca^{++} exchanger (Hogstrand, 2012; Loro et al., 2014), as both of these membrane transporters are known to be involved in active Ca^{++} transport across the gill and hypodermis in green crab (Roer, 1980). If Zn inhibits transport of Ca^{++} from haemolymph to exoskeleton, haemolymph Ca^{++} level is expected to increase due to passive backflux of Ca^{++} from the exoskeleton. Thus, it can be argued that the higher magnitude of Zn-induced disruption of Ca^{++} homeostasis in 20% SW relative to that in 60% or 100% SW was a consequence of more elevated Zn accumulation – resulting in a greater inhibition of active Ca^{++} transport across the gill and hypodermis of the green crab, and an overshoot in haemolymph Ca^{++} regulation

5. Conclusions

We demonstrated that Zn uptake and accumulation in green crab is only partially salinity-dependent, since a lower salinity (20% SW) resulted in higher uptake and accumulation relative to 60% and 100% SW, whereas no difference was found between the latter two treatments. Our study also revealed that Zn^{++} and Ca^{++} probably share a common branchial uptake pathway in green crab, but this does not translate into the depletion of haemolymph and tissue Ca^{++} levels. Instead, acute exposure to waterborne Zn was found to induce marked increases in the gill and haemolymph Ca^{++}, and the magnitude of these changes were much higher in 20% SW relative to that in 60% or 100% SW. Collectively, these findings suggest that the toxicity of acute exposure to waterborne Zn in green crab is associated with the disruption of Zn and Ca homeostasis. Finally, it is also important to note that the physiology plays a critical role in regulating Zn accumulation and toxicity in osmoregulating marine crustaceans, in addition to the physico-chemistry of Zn in the exposure water.

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References

Benedetti, M.F., Milne, C.J., Kinniburgh, D.G., Van Riemsdijk, W.H., Koopal, L.K., 1995. Metal ion binding to humic substances: application of the non-ideal competitive absorption model. Environ. Sci. Technol. 29, 446–457.

Bjerregaard, P., Vislie, T., 1985. Effects of cadmium on hemolymph composition in the shore crab Carcinus maenas. Mar. Ecol. Prog. Ser. 27, 135–142.

Blewett, T.A., Wood, C.M., 2015. Low salinity enhances Ni-mediated oxidative sress and sub-lethal toxicity to the green shore crab (*Carcinus maenas*). Ecotoxicol. Environ. Saf. 122, 159–170.

Blewett, T.A., Glover, C.N., Fehsenfeld, S., Lawrence, M.J., Niyogi, S., Goss, G.G., Wood, C.M., 2015. Making sense of nickel accumulation and sub-lethal toxic effects in saline waters: fate and effects of nickel in the green crab, *Carcinus maenas*. Aquat. Toxicol. 164, 23–33.

Burke, J., Handy, R.D., Roast, S.D., 2003. Effect of low salinity on cadmium accumulation and calcium homeostasis in the shore crab (*Carcinus maenas*) at fixed free Cd^{2+} concentrations. Environ. Toxicol. Chem. 22, 2761–2767.

Elumalai, M., Antunes, C., Guilhermino, L., 2007. Enzymatic biomarkers in the crab *Carcinus maenas* from the Minho River estuary (NW Portugal) exposed to zinc and mercury. Chemosphere 66, 1249–1255.

Fehsenfeld, S., Weihrauch, D., 2013. Differential acid-base regulation in various gills of the green crab *Carcinus maenas*: Effects of elevated environmental pCO_2. Comp. Biochem. Physiol. 164A, 54–65.
Fehsenfeld, S., Kiko, R., Appelhans, Y., Towle, D.W., Zimmer, M., Melzner, F., 2011. Effects of elevated seawater pCO_2 on gene expression patterns in the gills of the green crab, *Carcinus maenas*. BMC Genomics 12, 488–504.
Freire, C.A., Onken, H., McNamara, J.C., 2008. A structure-function analysis of ion-transport in crustacean gills and excretory organs. Comp. Biochem. Physiol. 151A, 272–304.
Henry, R.P., Lucu, Č., Onken, H., Weihrauch, D., 2012. Multiple functions of the crustacean gill: osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of toxic metals. Front. Physiol. 3, 431.
Henry, R.P., 2005. Critical salinity, sensitivity, and commitment of salinity-mediated carbonic anhydrase induction in the gills of two euryhaline species of decapod crustaceans. J. Exp. Zool. 303A, 45–56.
Hogstrand, C., Wilson, R.W., Polgar, D., Wood, C.M., 1994. Effects of zinc on the kinetics of branchial calcium uptake in freshwater rainbow trout during adaptation to waterborne zinc. J. Exp. Biol. 186, 55–73.
Hogstrand, C., Reid, S., Wood, C., 1995. Ca^{2+} versus Zn^{2+} transport in the gills of freshwater rainbow trout and the cost of adaptation to waterborne Zn^{2+}. J. Exp. Biol. 198, 337–348.
Hogstrand, C., Verbost, P.M., Bonga, S.E., Wood, C.M., 1996. Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. Am. J. Physiol.—Regul. Integr. Comp. Physiol. 270, R1141–R1147.
Hogstrand, C., Webb, N., Wood, C.M., 1998. Covariation in regulation of affinity for branchial zinc and calcium uptake in freshwater rainbow trout. J. Exp. Biol. 201, 1809–1815.
Hogstrand, C., 2012. Zinc. In: Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), Fish Physiology Vol 31A, Homeostasis and Toxicology of Essential Metals. Academic Press, San Diego, CA, pp. 135–200.
Lee, J.A., Marsden, I.D., Glover, C.N., 2010. The influence of salinity on copper accumulation and its toxic effects in estuarine animals with differing osmoregulatory strategies. Aquat. Toxicol. 99, 65–72.
Leignel, V., Stillman, J.H., Baringou, S., Thabet, R., Metais, I., 2014. Overview on the European green crab *Carcinus* spp. (*Portunidae, Decapoda*), one of the most famous marine invaders and ecotoxicological models. Environ. Sci. Pollut. Res. 21, 9129–9144.
Leonard, E.M., Barcarolli, I., Silva, K.R., Wasielesky, W., Wood, C.M., Bianchini, A., 2011. The effects of salinity on acute and chronic nickel toxicity and bioaccumulation in two euryhaline crustaceans: *Litopenaeus vannamei* and *Excirolana armata*. Comp. Biochem. Physiol. 154C, 409–419.
Lignon, J.M., 1987. Ionic permeabilities of the isolated gill cuticle of the shore crab *Carcinus maenas*. J. Exp. Biol. 131, 159–174.
Loro, V.L., Nogueira, L., Nadella, S.R., Wood, C.M., 2014. Zinc bioaccumulation and ionoregulaotory impacts in *Fundulus heteroclitus* exposed to sublethal waterborne zinc at different salinities. Comp. Biochem. Physiol. 166C, 96–104.
Lucu, Č., 1990. Ionic regulatory mechanisms in crustacean gill epithelia. Comp. Biochem. Physiol. 97A, 297–306.
Mantel, L.H., Farmer, L.L., 1983. Osmotic and ionic regulation. In: Mantel, L.H. (Ed.), The Biology of the Crustacea, Vol 5, Internal Anatomy and Physiological Regulation. Academic Press, New York, pp. 53–161.
Martins, C.D.M.G., Barcarolli, I.F., de Menezes, E.J., Giacomin, M.M., Wood, C.M., Bianchini, A., 2011. Acute toxicity, accumulation and tissue distribution of copper in the blue crab *Callinectes sapidus* acclimated to different salinities: *In vivo* and *in vitro* studies. Aquat. Toxicol. 101, 88–99.
Niyogi, S., Wood, C.M., 2004. Kinetic analyses of waterborne Ca and Cd transport and their interactions in the gills of rainbow trout (*Oncorhynchus mykiss*) and yellow perch (*Perca flavescens*), two species differing greatly in acute waterborne Cd sensitivity. J. Comp. Physiol. 174B, 243–253.
Normant, M., Gibowicz, M., 2008. Salinity induced changes in haemolymph osmolality and total metabolic rate of the mud crab *Rhithropanopeus harrisii* Gould, 1841 from Baltic coastal waters. J. Exp. Mar. Biol. Ecol. 355, 145–152.
Rainbow, P.S., Black, W.H., 2002. Effects of changes in salinity and osmolality on the rate of uptake of zinc by three crabs of different ecologies. Mar. Ecol. Prog. Ser. 244, 205–217.
Rainbow, P.S., Kwan, M.K.H., 1995. Physiological responses and the uptake of cadmium and zinc by the amphipod crustacean *Orchestia gammarellus*. Mar. Ecol. Prog. Ser. 127, 87–102.
Rainbow, P.S., Malik, I., O'Brien, P., 1993. Physicochemical and physiological effects on the uptake of dissolved zinc and cadmium by the amphipod crustacean *Orchestia gammarellus*. Aquat. Toxicol. 25, 15–30.
Regnault, M., 1987. Nitrogen excretion in marine and freshwater Crustacea. Biol. Rev. 62, 1–24.
Rodrigues, E.T., Pardal, M.Â., 2014. The crab *Carcinus maenas* as a suitable experimental model in ecotoxicology. Environ. Int. 70, 158–182.
Roer, R.D., 1980. Mechanisms of resorption and deposition of calcium in the carapace of the crab *Carcinus maenas*. J. Exp. Biol. 88, 205–218.
Sá, M.G., Ahearn, G.A., Zanotto, F.P., 2009. $^{65}Zn^{2+}$ transport by isolated gill epithelial cells of the American lobster, *Homarus americanus*. J. Comp. Physiol. 179B, 605–615.
Siebers, D., Leweck, K., Markus, H., Winkler, A., 1982. Sodium regulation in the shore crab *Carcinus maenas* as related to ambient salinity. Mar. Biol. 69 (1), 37–43.
Siebers, D., Winkler, A., Lucu, Č., Thedens, G., Weichart, D., 1985. Na-K-ATPase generates an active transport potential in the gills of the hyperregulating shore crab *Carcinus maenas*. Mar. Biol. 87, 185–192.
Spry, D.J., Wood, C.M., 1985. Ion flux rates, acid-base status, and blood gases in rainbow trout, Salmo gairdneri, exposed to toxic zinc in natural soft water. Can. J. Fish. Aquat. Sci. 42, 1332–1341.
Spry, D.J., Wood, C.M., 1989. A kinetic method for the measurement of zinc influx *in vivo* in the rainbow trout, and the effects of waterborne calcium on flux rates. J. Exp. Biol. 142, 425–446.
Towle, D.W., Rushton, M.E., Heidych, D., Magnani, J.J., Rose, M.J., Amstutz, A., Jordan, M.K., Shearer, D.W., Wu, W.S., 1997. Sodium/proton antiporter in the euryhaline crab *Carcinus maenas*: molecular cloning, expression and tissue distribution. J. Exp. Biol. 200, 1003–1014.
Towle, D.W., Paulsen, R.S., Weihrauch, D., Kordylewski, M., Salvador, C., Lignot, J.H., Spanings-Pierrot, C., 2001. Na^+-K^+-ATPase in the gills of the blue crab, *Collinectes sapidus*: cDNA sequencing and salinity-related expression of α-subunit mRNA and protein. J. Exp. Biol. 204, 4012–5005.
Wallace, J.C., 1972. Activity and metabolic rate in the shore crab *Carcinus maenas*. Comp. Biochem. Physiol. 41A, 523–533.
Winkler, A., 1987. Die Osmoregulation Der Strandkrabbe *Carcinus maenas* (L.) in Konstanten Und Fluktuierenden Salzgehalten. Ph.D Thesis. Universitaet Hamburg.
Wright, D.A., 1977a. The effect of salinity on cadmium uptake by the tissues of the shore crab *Carcinus maenas*. J. Exp. Biol. 67, 137–146.
Wright, D.A., 1977b. The effect of calcium on cadmium uptake by the shore crab *Carcinus maenas*. J. Exp. Biol. 67, 163–173.
Zirino, A., Yamamoto, S., 1972. A pH-dependent model for the chemical speciation of copper, zinc, cadmium and lead in seawater. Limnol. Oceanogr. 17, 661–671.