Aquatic Toxicology 110–111 (2012) 187–193

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

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

Oxidative stress parameters and antioxidant response to sublethal waterborne zinc in a euryhaline teleost *Fundulus heteroclitus*: Protective effects of salinity

Vania Lucia Loro[a,b,*], Mariana Basso Jorge[a,c], Kassio Rios da Silva[a,d], Chris M. Wood[a]

[a] Dept. of Biology, McMaster University, 1280 Main Street West, Hamilton, ON, L8S 4K1, Canada
[b] Departamento de Química, Universidade Federal de Santa Maria (UFSM), Av. Roraima 1000, Santa Maria, RS, 97105-900, Brazil
[c] Universidade Federal do Rio Grande (FURG), Instituto de Ciências Biológicas, Rio Grande, RS, 96201-900, Brazil
[d] Universidade Federal do Rio Grande (FURG), Instituto Oceanografico, Rio Grande, RS, 96201-900, Brazil

ARTICLE INFO

Article history:
Received 31 October 2011
Received in revised form 11 January 2012
Accepted 16 January 2012

Keywords:
Metals
Zinc
Oxidative parameters
Antioxidants

ABSTRACT

Zinc is an essential trace metal, but many aspects of its toxicity remain unclear. In this study, we investigated zinc effects on oxidative stress parameters and antioxidant profile in four tissues (gill, liver, kidney, and white muscle) of *Fundulus heteroclitus*. Possible interactive effects of salinity were also studied. Killifish were exposed to sublethal level (500 $\mu g\,L^{-1}$) of waterborne zinc for 96 h in 0% (fresh water), 10% (3.5 ppt), 30% (10.5 ppt) and 100% sea water (35 ppt). Salinity per se had no effect on any parameter in the control groups. Zinc exposure clearly induced oxidative stress, and responses were qualitatively similar amongst different tissues. Salinity acted as a strong protective factor, with the highest levels of reactive oxygen species (ROS) and greatest damage (protein carbonyls, lipid peroxidation as indicated by thiobarbituric acid reactive substances (TBARS) in 0 ppt, the least in 100% sea water (35 ppt), and gradations in between in many of the observed responses. Increases in total oxidative scavenging capacity (TOSC) occurred at higher salinities, correlated with increases in the activities of superoxide dismutase (SOD) and glutathione-S-tranferase (GST), as well as in tissue glutathione (GSH) concentrations. However, TOSC was depleted in zinc-exposed fish at 0 ppt, accompanied by decreases in SOD, GST, GSH, and also catalase (CAT) activity. Our results confirm that sublethal waterborne zinc is an oxidative stressor in fish, and highlight the important protective role of higher salinities in ameliorating the oxidative stress associated with zinc toxicity in this model estuarine teleost.

1. Introduction

Many industrial and agricultural processes have contributed to increased metal levels in the environment over the years. Aquatic ecosystems are frequently contaminated with metals such as zinc and copper which are essential in low concentrations but toxic in higher concentrations (Lushchak, 2011; Wood, 2012). Metals are able to disrupt the integrity of the physiological and biochemical mechanisms in fish. The disturbances include loss of appetite, reduced growth, decreased aerobic scope, and mortality (McGeer et al., 2000; Gioda et al., 2007). Zinc is an essential trace element of biological and health importance. It plays an important role in cellular metabolism acting as a co-factor in a number of enzymatic reactions. However, prolonged and excessive zinc uptake may lead to toxic effects (Atli and Canli, 2007).

Exposure of fish to metals may result in increases in reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radicals, and hydroxyl radicals leading to impairment of normal oxidative metabolism and finally to oxidative stress (Lushchak, 2011). While this has been well-studied for copper (Grosell, 2012), there is almost no information available for zinc on this topic in fish (Hogstrand, 2012; Lushchak, 2011). A number of response parameters can be used to evaluate the oxidative stress state generated by metal exposure. These include lipid peroxidation (LPO), formation of protein carbonyls, as well as enzymes and other moieties that act as defensive mechanisms in fish tissues (Campana et al., 2003; Farombi et al., 2007). The latter antioxidant system include various enzymes such as superoxide dismutases (SOD) which catalyze the dismutation of superoxide radical to hydrogen peroxide, as well as catalase (CAT) and glutathione peroxidase (GPx) which act to degrade hydrogen peroxide. The glutathione S-transferase (GST) family exhibits important detoxifying activities against lipid hydroperoxides generated by inorganic pollutants such as metals (Meyer et al., 2003; Dautremepuits et al., 2004; Farombi et al., 2007). Glutathione (GSH) is an important non-enzymatic antioxidant which protects fish tissues against oxidative damage by neutralizing free radicals and other types of ROS.

* Corresponding author at: Departamento de Química, Universidade Federal de Santa Maria (UFSM), Av. Roraima 1000, Santa Maria, RS, 97105-900, Brazil. Tel.: +55 55 3220 9456/+1 905 525 9140x23537; fax: +55 55 3220 8240.
E-mail address: Vania.Loro@gmail.com (V.L. Loro).

0166-445X/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2012.01.012

Fish are widely used for assessment of the quality of aquatic environments, and some fish species serve as good bioindicators of environmental pollution. *Fundulus heteroclitus*, the Atlantic killifish, lives in estuaries and salt marshes of the eastern coast of North America, moving diurnally in and out on the tidal front to feed (Marshall, 2003). This species exhibits a variety of physiological adaptations which allow them to easily withstand large fluctuations of salinity in their natural environment (Griffith, 1974; Prodocimo et al., 2007; Scott et al., 2008; Wood and Grosell, 2009). Recently *F. heteroclitus* has become a model organism in physiology and toxicology, especially for studying euryhalinity in estuarine teleosts (Burnett et al., 2007). This reflects a large body of research on its ionoregulatory, reproductive and developmental physiology, its responses to a wide range of toxicants, together with rapid progress on its genome.

In the context of the present study, we have selected the killifish as a bioindicator to study the potential interactions between waterborne zinc exposure and environmental salinity. Salinity as well as calcium, pH and DOC (dissolved organic carbon) will alter both the physiology of the organism, and the speciation and bioavailability of zinc (Hogstrand, 2012). The present paper focuses on oxidative stress responses, and a parallel study focuses on zinc bioaccumulation and ionoregulatory impacts (Loro et al., 2012). The first hypothesis of the present study was that sublethal waterborne zinc exposure (500 $\mu g\,L^{-1}$) for 96 h would induce oxidative stress. This was evaluated by measuring ROS levels, lipid peroxidation, protein carbonyl formation, and detoxification mechanisms in various tissues of the killifish. In line with this, antioxidant parameters such as oxyradical scavenging capacity (TOSC) and antioxidant enzyme activities were also evaluated. The second hypothesis was that salinity would act as protective factor against zinc toxicity if indeed killifish tissues do suffer oxidative injury from zinc. This was conducted by acclimating the fish to fresh water (0), 3.5 ppt, 10.5 ppt or 35 ppt (100% sea water), and then exposing them to this same zinc level at the four different salinities.

2. Material and methods

2.1. Animal collection and acclimation

Atlantic killifish of the northern subspecies *Fundulus heteroclitus macrolepidotus* of both sexes (weight: 3.5 ± 0.6 g; length: 4.2 ± 0.8 cm) were obtained from Aquatic Research Organisms (ARO) Ltd. (Hampton, NH, U.S.A.), who collected them by beach-seining of local tidal flats. At McMaster University they were kept in aerated tanks (250 L) operated as static systems with carbon filtration under a natural photoperiod (12 h light–12 h dark) at 18.5 °C. Initially they were held at a salinity of 10% (3.5 ppt) for several weeks. Saline waters were made by the addition of Instant Ocean sea salt (Big Al's Aquarium Supercenter, Woodbridge, ON, Canada) to fresh water, considering 35 $g\,L^{-1}$ as 35 ppt of salinity. The fresh water was dechlorinated Hamilton, Ontario tap water (moderately hard: $[Na^+] = 0.6$ mequiv L^{-1}, $[Cl^-] = 0.8$ mequiv L^{-1}, $[Ca^{2+}] = 1.8$ mequiv L^{-1}, $[Mg^{2+}] = 0.3$ mequiv L^{-1}, $[K^+] = 0.05$ mequiv L^{-1}; titration alkalinity 2.1 mequiv L^{-1}; pH ~8.0; hardness ~140 $mg\,L^{-1}$ as $CaCO_3$ equivalents). The fish were then acclimated to different salinities (0, 10, 30 and 100%) under these conditions for seven days in the 250 L tanks prior to experiments. During acclimation, fish were fed once a day to satiation with a mix of 50% commercial flakes (Wardley Total Tropical Gourmet Flake Blend, Hartz Mountain Corp., Secausus, NJ, U.S.A.) and 50% frozen brine shrimp (San Francisco Bay Brand, Newark, CA, U.S.A.). Food was withheld for 48 h prior to the start of experiments and throughout the 96 h tests.

2.2. Zinc exposures at different salinities

After acclimation to the different salinities, fish were divided into eight groups and transferred to separate 8 L aquaria (6 fish in each) pre-cleaned with 1% HNO_3. Four control groups of salinity (0 = Hamilton fresh water, 3.5 ppt, 10.5 ppt and 35 ppt = 100% sea water) were used, and four experimental groups at these same salinities were exposed to 500 $\mu g\,L^{-1}$ zinc for 96 h. To maintain good water quality, 80% of the water was renewed every day. In order to prevent zinc loss, no filters were used during the experimental period.

Zinc concentrations were monitored daily to ensure relatively constant metal exposures. Water samples for ions and dissolved zinc measurements were obtained by passage through 0.45 μM syringe filters (Acrodisc syringe filter; Pall Life Sciences, Houston, TX, U.S.A.). Unfiltered water samples were also taken for total zinc measurements. Zinc concentrations were analyzed using a Varian FS220 flame atomic absorption spectrophotometer (Varian, Mulgrave, Victoria, Australia) using certified zinc standards (Fisher Scientific, Toronto, Canada). Other chemical characteristics and zinc concentrations of the test waters are shown in Table 1. Dissolved oxygen was measured using a portable Accumet® polarographic electrode and AB15 meter (Fisher Scientific, Missisauga, Ontario), total DOC concentration using a Shimadzu TOC-$V_{CPH/CPN}$ total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan), and alkalinity by titration to pH 4.0 using standardized HCl. The ionic compositions of the various test waters have been reported in Loro et al. (2012), and were close to nominal values. Based on these ionic analyses and the additional measurements shown in Table 1, Zn speciation analysis for each salinity (Table 2) was performed using Visual MINTEQ software (ver. 3.0, beta, KTH, Department of Land and Water, Resources Engineering, Stockholm, Sweden; courtesy of J.P. Gustafsson, Royal Institute of Technology, Stockholm, Sweden).

After 96 h exposure, all fish were euthanized with a lethal dose of NaOH-neutralized MS-222 (Syndel Laboratories Ltd., Vancouver, B.C., Canada). Gill, liver, intestine and muscle were quickly removed by dissection, frozen in liquid nitrogen, and stored at −80 °C. For measurements of ROS and oxyradical scavenging capacity (TOSC), a slice of each tissue was removed before freezing.

2.3. Oxidative parameters and enzyme measurements

Reactive oxygen species (ROS) were determined in the fresh supernatant fraction of gill, liver, intestine, and muscle by the method of Viarengo et al. (1999). Tissue samples were prepared through homogenization (1:5, w/v) in a buffer containing Tris-HCl (100 mM, pH 7.75), EDTA (2 mM) and $MgCl_2$ (5 mM). The homogenates were centrifuged at 10,000 × *g* for 20 min at 4 °C. ROS was expressed as area of ROS per mg of protein. In the same supernatant, oxyradical scavenging capacity (TOSC) was determined using the method described by Amado et al. (2009). TOSC was expressed as the relative area of remaining ROS per milligram of protein, an index which is inversely proportional to total oxyradical scavenging capacity. Supernatants resulting from centrifugation were also used for all measurements described above. Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) as described previously by Buege and Aust (1978). TBARS levels are expressed as nmol MDA per mg of protein. Protein carbonyl content was assayed by the method described by Yan et al. (1995) with some modifications. Briefly the changes were to increase initial dilution and solubilize the protein carbonyl without using guanidine. The total amount of protein carbonyls formed was calculated using a molar extinction coefficient of 22,000 $M\,cm^{-1}$ and expressed as nmol carbonyl per mg protein.

Superoxide dismutase activity was determined by measuring the inhibition of the auto-oxidation of epinephrine at pH 10.2

Table 1
Water chemistry and zinc concentrations of the experimental media used in the study. Data were expressed as mean ± SEM (n = 6). DOC = dissolved organic carbon. Means sharing the same letter are not significantly different.

Parameter	Salinity 0 ppt	Salinity 3.5 ppt	Salinity10.5 ppt	Salinity 35 ppt
Total zinc ($\mu g\,L^{-1}$)	486.6 ± 8.5^{A}	490 ± 8.0^{A}	495 ± 9.0^{A}	487.5 ± 8.5^{A}
Dissolved zinc ($\mu g\,L^{-1}$)	476.5 ± 9.0^{A}	480 ± 8.5^{A}	485 ± 8.5^{A}	477.8 ± 7.5^{A}
Temperature (°C)	18.3 ± 0.3^{A}	18.2 ± 0.2^{A}	18.6 ± 0.3^{A}	18.5 ± 0.2^{A}
pH	7.61 ± 0.11^{A}	7.85 ± 0.15^{A}	8.1 ± 0.11^{B}	8.35 ± 0.2^{B}
Dissolved oxygen ($mg\,L^{-1}$)	8.0 ± 0.5^{A}	7.8 ± 0.4^{A}	7.5 ± 0.4^{A}	8.0 ± 0.6^{A}
Alkalinity ($mg\,CaCO_3\,L^{-1}$)	68 ± 0.8^{A}	78 ± 0.6^{B}	108 ± 1.5^{B}	130 ± 3.8^{C}
DOC ($mg\,L^{-1}$)	2.48 ± 0.45^{A}	2.55 ± 0.35^{A}	2.65 ± 0.4^{A}	2.75 ± 0.45^{A}

Table 2
Chemical speciation of zinc (chemical species (%) in experimental media of study).

Species of zinc (%)	Salinity 0%	Salinity 10%	Salinity 30%	Salinity 100%
Zn^{2+}	79.4	73.6	50.5	25.9
$ZnOH^{+}$	2.4	1.7	4.1	4.5
$Zn(OH)_2$	3.1	2.2	18.9	43.5
$ZnCl^{+}$	0.2	3.8	6.2	9.8
$ZnCl_4^{2-}$	0	0	0	0.4
$ZnCl_3^{-}$	0	0	0.1	0.9
$ZnCl_2$	0	0.1	0.4	1.6
$ZnSO_4$	2.6	10.8	7.4	4.6
$Zn(SO_4)_2^{-2}$	0	0.5	0.6	0,5
$ZnCO_3$	10.0	6.0	11.2	7.7
$ZnHCO_3^{+}$	2.0	1.2	0.6	0.2
$Zn(CO_3)_2^{-2}$	0	0.	0.2	0.2

and 25 °C as described by Magwere et al. (1997). One unit of SOD activity is the amount of SOD necessary to cause 50% inhibition of epinephrine auto-oxidation. The activity of catalase (CAT) was determined according to the procedure of Clairborne (1995) by following the absorbance of hydrogen peroxide at 240 nm pH 7.0 at 25 °C and expressed as $\mu mol\,min^{-1}$(U) per mg protein. The activity of glutathione S-transferase (GST) was measured according to Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. GST activity of was expressed as μmol GS-DNB min^{-1} per mg protein using an extinction coefficient of 9.6 $mM\,cm^{-1}$. Glutathione (GSH) formation was determined in the same supernatant of enzymes according to Ellman (1959) at 412 nm using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and expressed as μmol GSH per mg of protein. Protein content was assayed according to Bradford (1976) using bovine serum albumin as standard.

2.4. Statistical analyses

Data have been expressed as mean ± standard error of mean (n = 6). All data were tested and exhibited normal distribution. Comparisons among mean values were performed using one-way analysis of variance (ANOVA) followed by the Fisher's LSD test to verify differences among control groups at different salinities, and among zinc exposure groups at different salinities. Differences between zinc-exposed and control animals at the same salinity were assessed using Student's two-tailed unpaired t-test. In all cases, the significance level adopted was 95% (α = 0.05).

3. Results

3.1. Waterborne zinc and water parameters

Total zinc concentrations were close to the nominal value of 500 $\mu g\,L^{-1}$ at all salinities, and dissolved zinc concentrations were very close to total values, (Table 1). Dissolved O_2 and DOC concentrations did not vary amongst salinities, but water pH and alkalinity both increased with increasing salinity. These differences in water chemistry caused differences in Zn speciation. Most notably, free Zn^{2+} decreased from 79.4% to 25.9% as salinity increased from fresh water to 35 ppt (100% sea water). These changes reflected increases in hydroxide, carbonate, and chloride complexes, especially $Zn(OH)_2$ as pH, alkalinity, and chloride concentration all increased.

3.2. ROS and TOSC levels

Salinity itself had no effect on tissue ROS levels under control conditions, though ROS levels tended to be slightly higher in liver than in gill, intestine and muscle (Fig. 1). Significantly increased ROS concentrations were observed in gill, liver, intestine and muscle tissues of killifish exposed to 500 $\mu g\,L^{-1}$ of zinc for 96 h at some salinities. For all four tissues, the highest ROS production in relation to control occurred in the group exposed to zinc at 0 ppt. Fish exposed to zinc at salinity 3.5 ppt also exhibited increased ROS

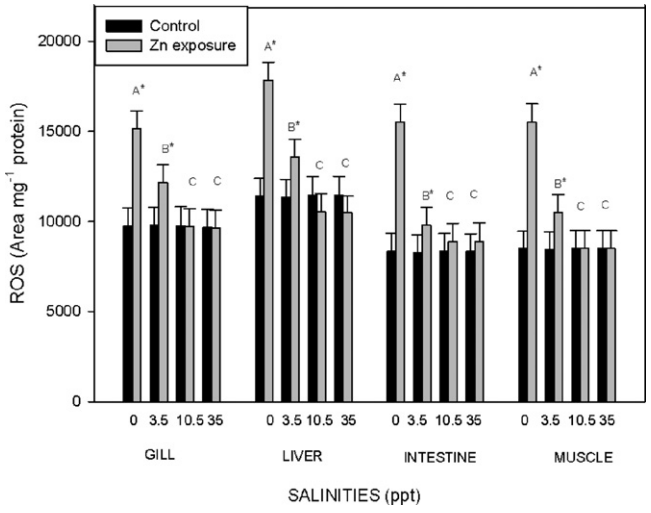

Fig. 1. Reactive oxygen species (area of ROS mg^{-1} protein) in tissues of *F. heteroclitus* exposed to 500 $\mu g\,L^{-1}$ of zinc at different salinities for 96 h. (*) indicates significant differences between Zn-exposed groups and respective control. Capital letters indicate significant differences among the Zn-exposed groups at different salinities. ($P \leq 0.05$) (n = 6).

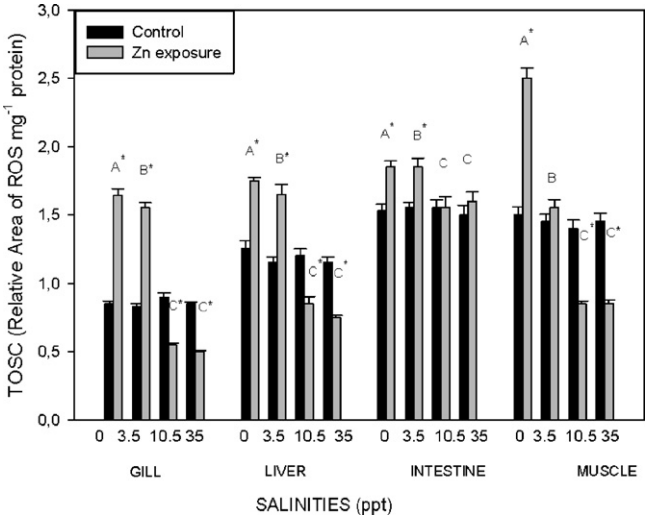

Fig. 2. TOSC (relative area of ROS mg^{-1} protein) in tissues of *F. heteroclitus* exposed to 500 $\mu g\,L^{-1}$ of zinc at different salinities for 96 h. (*) indicates significant differences between Zn-exposed groups and respective control. Capital letters indicate significant differences among the Zn-exposed groups at different salinities. ($P \leq 0.05$) ($n = 6$).

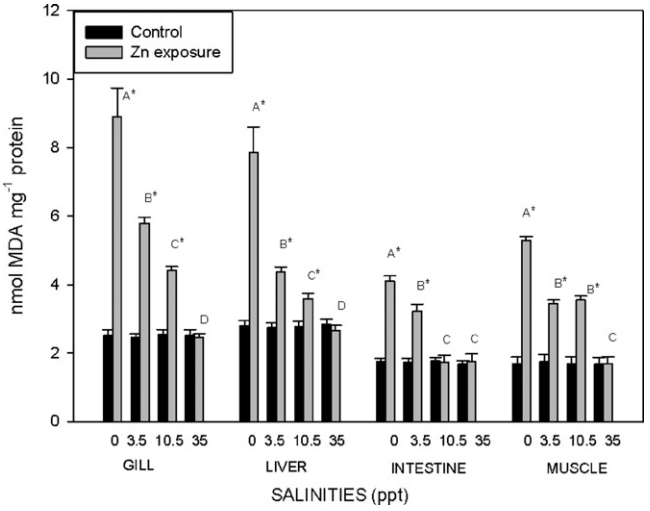

Fig. 3. TBARS (nmol MDA mg^{-1} protein in tissues of *F. heteroclitus* exposed to 500 $\mu g\,L^{-1}$ of zinc at different salinities for 96 h. (*) indicates significant differences between Zn-exposed groups and respective control. Capital letters indicate significant differences among the Zn-exposed groups at different salinities. ($P \leq 0.05$) ($n = 6$).

formation. However, at salinities of 10.5 and 35 ppt, ROS levels in zinc-exposed fish were similar to control values (Fig. 1).

With respect to TOSC levels, there were again no significant differences with respect to salinity in the control groups, though relative area tended to be lower in gills than in the other three tissues, indicating a greater total ROS scavenging capacity in the gills (Fig. 2). Gill, liver and intestine of animals exposed to zinc at 0 and 3.5 ppt showed significantly increased relative area, signifying a loss of total ROS scavenging capacity in response to zinc exposure at low salinities. Muscle tissue showed an increased area (i.e. decreased ROS scavenging capacity) in fish exposed to zinc only at 0 ppt. In contrast, killifish exposed to zinc at salinities of 10.5 or 35 ppt showed significantly decreased relative area in liver, gills, and muscle, but not in intestine (where there was return to control values at these salinities), indicating an augmentation of total ROS scavenging capacity (Fig. 2).

3.3. Lipid peroxidation (LPO) and protein carbonyls

TBARS are an index of LPO. Salinity per se had no effects on TBARS levels in control groups for all tissues studied, though values tended to be higher in gill and liver, and lower in intestine and muscle. In accord with the results for ROS and TOSC, *F. heteroclitus* exhibited somewhat parallel changes in TBARS in all tissues, indicative of lipid peroxidation, in response to zinc exposure (Fig. 3). In the intestine, TBARS were significantly increased relative to the controls only at 0 and 3.5 ppt, whereas in gill, liver, and muscle, a significant response persisted up to 10.5 ppt. The largest increases in all four tissues occurred in fresh water (0 ppt), with progressively smaller responses at higher salinities, such that there was no evidence of elevated LPO at salinity of 35 ppt.

Within each tissue, salinity had no effect on protein carbonyl levels under control conditions, though levels were highest in gill and liver, intermediate in intestine, and lowest in muscle (Fig. 4). In response to zinc exposure, the gill exhibited an increase in protein carbonyls only at 0 ppt, and not at higher salinities, whereas the liver and intestine exhibited responses at both 0 and 3.5 ppt. The tissue most affected was muscle, with significant increases in protein carbonyl concentrations at all four salinities (Fig. 4).

3.4. Antioxidant enzymes

All enzyme activities have been normalized to protein content; note that there were no significant changes in protein concentration as a result of the experimental treatments. Under control conditions, catalase (CAT) activities were very consistent across all tissues and salinities (Fig. 5). The only response to zinc exposure was a substantial reduction (50–60%) in CAT activity in all four tissues in fresh water (0 ppt). There were no other changes.

Another important anti-oxidant defense mechanism, superoxide dismutase (SOD), exhibited rather different patterns (Fig. 6). Under control conditions, SOD activity did not vary with salinity, but activity levels were higher in gill and liver, and lower in intestine and muscle. At 0 ppt, zinc exposure reduced SOD activity by 25–50% in all four tissues, and in this respect the responses were somewhat similar to those with CAT. However at all higher

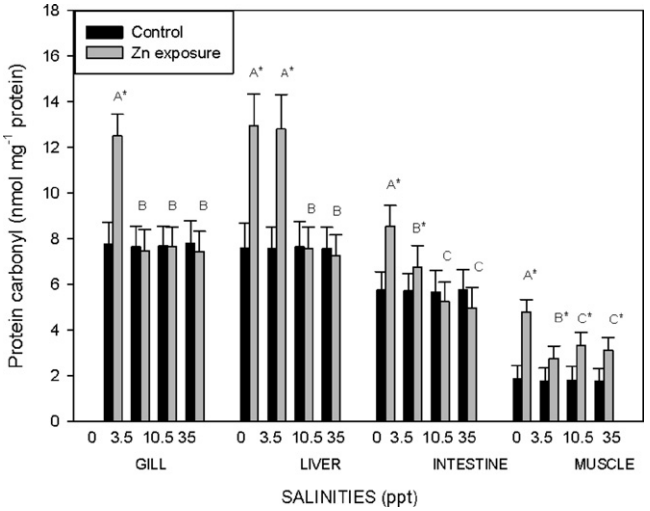

Fig. 4. Protein carbonyls (nmol mg^{-1} protein) in tissues of *F. heteroclitus* exposed to 500 $\mu g\,L^{-1}$ of zinc at different salinities for 96 h. (*) indicates significant differences between Zn-exposed groups and respective control. Capital letters indicate significant differences among the Zn-exposed groups at different salinities. ($P \leq 0.05$) ($n = 6$).

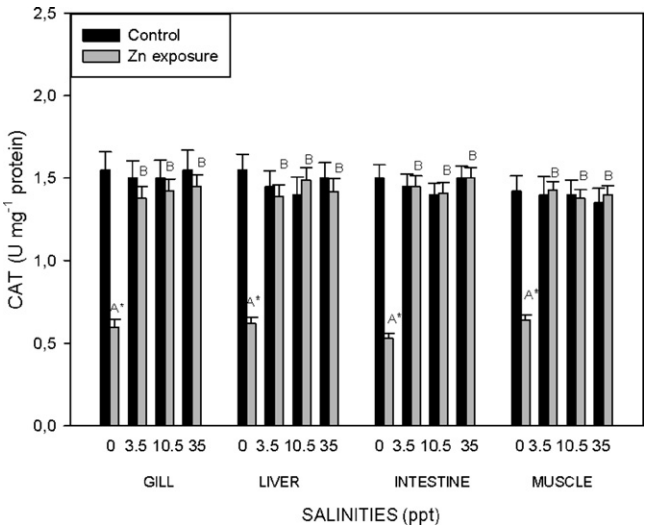

Fig. 5. Catalase activity (CAT; U mg^{-1} protein) in tissues of *F. heteroclitus* exposed to 500 μg L^{-1} of zinc at different salinities for 96 h. (*) indicates significant differences between Zn-exposed groups and respective control. Capital letters indicate significant differences among the Zn-exposed groups at different salinities. ($P \leq 0.05$) ($n = 6$).

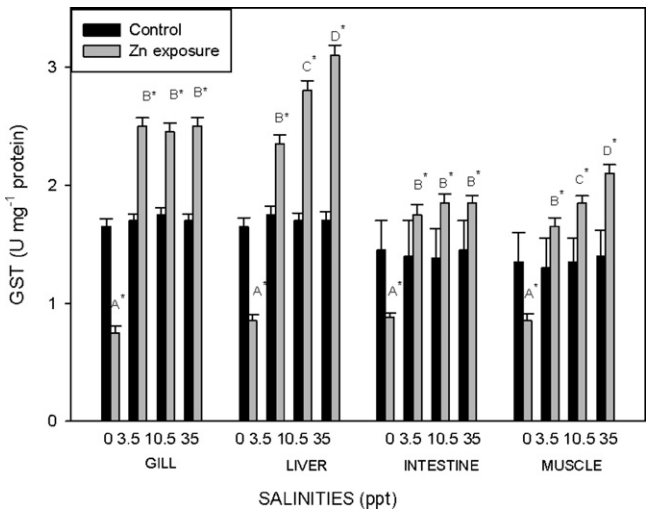

Fig. 7. Glutathione S-transferase activity (GST; U mg^{-1} protein) in tissues of *F. heteroclitus* exposed to 500 μg L^{-1} of zinc at different salinities for 96 h. (*) indicates significant differences between tested groups and respective control. Capital letters indicate significant differences among the Zn-exposed groups at different salinities. ($P \leq 0.05$) ($n = 6$).

salinities, SOD activity increased significantly in all tissues, with the greatest relative responses occurring in muscle.

Under control conditions, glutathione S-transferase (GST) activities were similar in all tissues and unaffected by salinity (Fig. 7). In zinc-exposed fish, the pattern of response was very similar to that seen with SOD (Fig. 6). Thus GST activity was reduced by 30–50% in response to zinc exposure in all four tissues in fresh water (0 ppt), but significantly increased relative to control values at all higher salinities, again in all four tissues. However, GST activities increased at all higher salinities. The responses tended to be progressive in liver and muscle, with the largest increases at 35 ppt (Fig. 7).

3.5. Glutathione

Baseline glutathione (GSH) levels were not affected by salinity in any tissue, but tended to be higher in gill and liver, and lower in intestine and muscle under control conditions (Table 3). As with CAT and GST, there was depletion of GSH reserves in all four tissues in fish exposed to zinc in water (0 ppt), but significant increases at all higher salinities. Again the response to zinc exposure appeared to be progressive with salinity, with the greatest increases seen in 35 ppt (100% sea water).

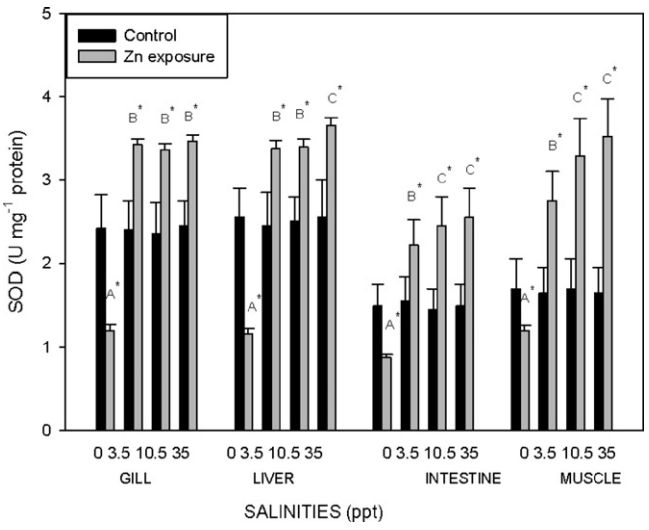

Fig. 6. Superoxide dismutase activity (SOD; U mg^{-1} protein) in tissues of *F. heteroclitus* exposed to 500 μg L^{-1} of zinc at different salinities for 96 h. (*) indicates significant differences between Zn-exposed groups and respective control. Capital letters indicate significant differences among the Zn-exposed groups at different salinities. ($P \leq 0.05$) ($n = 6$).

4. Discussion

Both of the original hypotheses of this study were confirmed. Firstly, exposure of killifish to a sublethal level of waterborne zinc (~500 μg L^{-1}) for 96 h clearly induced oxidative stress. This was demonstrated by an increase in ROS levels in all four tissues, and associated with this, increases in ROS-associated damage (LPO, protein carbonyls), alterations in ROS scavenging capacity (TOSC), and responses in antioxidant enzymes and glutathione. Secondly, salinity clearly acted as a protective factor against zinc toxicity, with the highest levels of ROS and greatest indications of damage in fresh water (0 ppt), the least in 100% sea water (35 ppt), and indications of gradations in between in many of the observed responses. Salinity in itself appeared to have no effect on oxidative stress parameters in *F. heteroclitus*, in contrast to several reports on other species (summarized by Lushchak, 2011), but the fish in our study had been acclimated to salinity, rather than challenged by salinity.

There were some subtle differences amongst the four tissues studied and amongst the various oxidative stress responses, the latter particularly in the muscle tissue. However, the overall the response patterns were remarkably similar. This agrees with a recent study on oxidative stress in freshwater zebrafish exposed to another nutritive metal, Cu, in various water chemistries, where many of the same endpoints were similar in gill and liver tissue (Craig et al., 2007). This suggests that the effects of oxidative stress occur generally throughout the fish, and in future toxicological studies where the goal is to assess the occurrence (or non-occurrence) of oxidative stress, it may be sufficient to sample just one tissue or a whole body extract.

It is important to note that the oxidative stress responses observed in the present study occurred at a zinc exposure concentration (500 μg L^{-1}) that was very sublethal – 40–200-fold lower than acutely toxic concentrations. The 96 h LC_{50} values for zinc

Table 3
GSH levels (μmol mg^{-1} of protein) in tissues of *F. heteroclitus* exposed to 500 μg L^{-1} of zinc at different salinities (96 h). Means ± SEM (n = 6). (*) in column indicates significant differences between tested groups and respective control. Capital letters in a row indicate significant differences between zinc exposed groups at different salinities ($P \leq 0.05$).

Gill				
Salinity	0%	10%	30%	100%
Control	0.48 ± 0.03	0.55 ± 0.05	0.5 ± 0.04	0.55 ± 0.06^{A}
Zinc exposure	0.30 ± 0.03^{A*}	0.7 ± 0.06^{B*}	0.78 ± 0.06^{B*}	0.85 ± 0.07^{C*}
Liver				
Salinity	0%	10%	30%	100%
Control	0.512 ± 0.06	0.5 ± 0.04	0.45 ± 0.02	0.50 ± 0.05
Zinc exposure	0.35 ± 0.05^{A*}	0.65 ± 0.05^{B*}	0.65 ± 0.06^{B*}	0.9 ± 0.07^{C*}
Intestine				
Salinity	0%	10%	30%	100%
Control	0.32 ± 0.03	0.3 ± 0.02	0.35 ± 0.02	0.34 ± 0.02
Zinc exposure	0.10 ± 0.015^{A*}	0.55 ± 0.025^{B*}	0.55 ± 0.03^{B*}	0.6 ± 0.04^{B*}
Muscle				
Salinity	0%	10%	30%	100%
Control	0.33 ± 0.02	0.32 ± 0.03	0.32 ± 0.02	0.3 ± 0.025
Zinc exposure	0.15 ± 0.015^{A*}	0.5 ± 0.05^{B*}	0.52 ± 0.04^{B*}	0.7 ± 0.06^{C*}

measured on the same batch of killifish, under the same exposure conditions (Loro et al., 2012), ranged from 20,000 μg L^{-1} in fresh water (0 ppt) to 106,000 μg L^{-1} in 100% sea water (35 ppt). Thus oxidative stress is a very sensitive endpoint.

Oxidative stress responses to zinc in fish have received very little study (Hogstrand, 2012). The exact mechanism involved in zinc-induced oxidative stress is still unknown, but it may relate to DNA fragmentation, and zinc's interaction with endogenous low molecular weight thiols and proteins (Gioda et al., 2007). Another possible explanation is that zinc induces changes in the activity of enzymes, resulting in ROS generation (Pedrajas et al., 1995; Gioda et al., 2007). However, an alternate explanation for the observed responses involves the Zn^{2+} ion in itself. It is now clear that free Zn^{2+} plays an important role in the cell signaling pathways accompanying oxidative stress. ROS releases Zn^{2+} from metallothionein, and the increase in cytosolic Zn^{2+} is detected by the intracellular zinc sensor (Mtf-1) which sets in a motion a chain of events resulting in increased gene expression of a suite of target genes coding for antioxidant enzymes, either through direct induction or downstream events (reviewed by Hogstrand, 2012). These include CAT, SOD, and GST. Thus it is possible that the observed increases in SOD (Fig. 6) and GST activities (Fig. 7), and increase in ROS scavenging capacity (decreased TOSC area; Fig. 2) accompanying zinc exposure at higher salinities reflected this direct action of Zn^{2+} as a cell signaling molecule, especially as there was no evidence of ROS accumulation at the higher salinities (Fig. 1). Increased levels of TOSC have been correlated with adaptation to life in pro-oxidant environments (Regoli, 2000), so this response could prime these zinc-exposed fish to respond effectively if ROS levels were to increase at a future point.

In our companion study (Loro et al., 2012) we found there was no increase in either plasma or tissue concentrations of total zinc in these killifish exposed to zinc at salinities of 3.5, 10.5, or 35 ppt. Ostensibly this would argue against the direct action explanation, but it must be remembered that free Zn^{2+} levels in the cytoplasm would be in the sub-micromolar range (Maret, 2011), and therefore undetectable in whole tissue zinc measurements. However, these total zinc concentration measurements do indicate that all of the responses seen at 3.5, 10.5, and 35 ppt occurred without any appreciable increase in tissue or plasma zinc levels, and with little (at 3.5 ppt) or no buildup (at 10.5 or 35 ppt) of ROS in the tissues (Fig. 1).

However, in killifish exposed to zinc in fresh water (0 ppt), there were measurable increases (by approximately 50%) in total zinc concentrations in the blood plasma, and in all tissues except the liver. This was accompanied by ROS accumulation (Fig. 1), oxidative damage to lipids (TBARS; Fig. 3) and proteins (protein carbonyls; Fig. 4), reductions in the activities of detoxifying enzymes (CAT, Fig. 5; SOD, Fig. 6: GST, Fig. 7), and decreases in GSH in all tissues (Table 3). As a result, TOSC relative area increased (Fig. 2), so total ROS scavenging capacity decreased in all tissues. It would appear that in fresh water, the level of zinc exposure and internal accumulation caused oxidative stress that was so severe as to exhaust the defensive mechanisms, such that substantial damage occurred. However, again it is not clear whether zinc was acting to inhibit or damage the enzymes, or whether this was a response to high levels of ROS (Fig. 1).

It is well known that increased salinity protects against zinc uptake in euryhaline fish (Hogstrand, 2012), but to our knowledge this is the first demonstration that it also protects against zinc-induced oxidative stress responses. There may be several factors involved. The first is that increased salinity alters the speciation of Zn, such that for any given total zinc concentration, there is much less free Zn^{2+} ion, the form generally considered the most bioavailable and reactive, due to complexation with various anions (Table 2). The greater ionic strength should theoretically reduce its activity even more. In this regard it is noteworthy that despite an increase in SOD levels and almost doubling of GSH levels (Table 3) at 3.5 and 10.5 ppt in Zn-treated animals, lipid peroxidation was still observed (Fig. 3). Perhaps Zn in the form of $Zn(OH)_2$, $ZnCl^+$, or $ZnSO_4$, the levels of which are increased at intermediate salinities (Table 2), are particularly potent oxidants for the lipid bilayer, but not for the cytosolic compartment? In this regard, nothing appears to be known as yet about the cytosolic distribution of various Zn forms between the lipid and the aqueous phase. Other possible explanations for these differential responses of redox state markers with salinity should also be sought in future work. A second important factor is that higher salinity increases the concentration of potentially competitive cations (Na^+, K^+, Ca^{2+}, Mg^{2+}; Table 1). Of these, Ca^{2+} appears to be the most effective (Zhang and Wang, 2007), as it directly competes with the Zn^{2+} uptake mechanism at the gills (Spry and Wood, 1989). In future studies, it will be of interest to assess whether the Ca^{2+} component alone of salinity protects against oxidative stress. In studies on Cu-exposed zebrafish, Craig et al. (2007) reported that both waterborne Ca^{2+} and waterborne Na^+ protected against oxidative stress caused by Cu exposure, but that the latter was in fact more effective. Overall, the current results add to mounting evidence that salinity is an important factor affecting metal toxicity in estuarine environments (see Grosell et al., 2007), and should be taken into account in future revisions of ambient water quality criteria.

Acknowledgments

Procedures were approved by the McMaster University Animal Care Committee and complied with the regulations of the Canada

Council for Animal Care. This work was supported by an NSERC CRD grant (Scott Smith, P.I.) with co-funding from the International Zinc Association (IZA), the International Lead Zinc Research Organization (ILZRO), the Nickel Producers Environmental Research Association (NiPERA), the International Copper Association (ICA), the Copper Development Association (CDA), Teck Resources, and Vale Inco. We thank Eric Van Genderen (IZA) and Joe Gorsuch (CDA) for constructive comments on the manuscript. The work was also supported by an award from the International Development Research Centre (IDRC) and the Canada Research Chair Program to Adalto Bianchini (University of Rio Grande, Brazil) and CMW. CMW is supported by the Canada Research Chair Program. Vania Lucia Loro received a post-doctoral scholarship from the Brazilian agency CAPES (Proc. 1331-10-8).

References

Amado, L.L., Garcia, M.L., Ramos, P.B., Freitas, R.F., Freitas, R.F., Zafalon, B., Ferreira, J.L.R., Yunes, J.S., Monserrat, J.M., 2009. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: application to evaluate microcystins toxicity. Sci. Total Environ. 407, 2115–2123.

Atli, G., Canli, M., 2007. Enzymatic responses to metal exposures in a freshwater fish *Oreochromis niloticus*. Comp. Biochem. Physiol. C 145, 282–287.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302–309.

Burnett, K.G., Bain, L.J., Baldwin, W.S., Callard, G.V., Cohen, S., Di Giulio, R.T., Evans, D.H., Crawford, D.L., 2007. *Fundulus* as the premier teleost model in environmental biology: opportunities for new insights using genomics. Comp. Biochem. Physiol. D 2, 257–286.

Campana, O., Sarasquete, C., Blasco, J., 2003. Effect on ALA-D activity, metallothionein levels, and lipid peroxidation in blood, kidney, and liver of the toadfish *Halobatrachus didactylus*. Ecotoxicol. Environ. Saf. 55, 116–125.

Clairborne, A., 1995. Catalase activity. In: Greewald, A.R. (Ed.), Handbook of Methods for Oxygen Radical Research. CRC Press, Florida, pp. 237–242.

Craig, P.M., Wood, C.M., McClelland, G.B., 2007. Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*). Am. J. Physiol. 293, R1882–R1892.

Dautremepuits, C., Paris-Palacios, S., Betoulle, S., Vernet, G., 2004. Modulation in hepatic and head kidney parameters of carp (*Cyprinus carpio* L.) induced by copper and chitosan. Comp. Biochem. Physiol. C 137, 325–333.

Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 70–77.

Farombi, E.O., Adelowo, O.A., Ajimoko, Y.R., 2007. Biomarkers of oxidative stress and heavy metal levels as indicators of environment pollution in African cat fish (*Clarias gariepinus*) from Nigeria Ogun River. Environ. Res. Public Health 4, 158–165.

Gioda, C.R., Lissner, L.A., Pretto, A., da Rocha, J.B.T., Schetinger, M.R.C., Neto, J.R., Morsch, V.M., Loro, V.L., 2007. Exposure to sublethal concentrations of Zn(II) and Cu(II) changes biochemical parameters in *Leporinus obtusidens*. Chemosphere 69, 170–175.

Griffith, R.W., 1974. Environment and salinity tolerance in the genus *Fundulus*. Copeia 1974, 319–331.

Grosell, M., 2012. Copper. In: Wood, C.M., Farrell, A.P., Brauner, C.A. (Eds.), Homeostasis and Toxicology of Essential Metals – Fish Physiology, vol. 31A. Elsevier, San Diego, pp. 53–133.

Grosell, M., Blanchard, J., Brix, K.V., Gerdes, R., 2007. Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. Aquat. Toxicol. 84, 162–172.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Hogstrand, C., 2012. Zinc. In: Wood, C.M., Farrell, A.P., Brauner, C.A. (Eds.), Homeostasis and Toxicology of Essential Metals – Fish Physiology, vol. 31A. Elsevier, San Diego, pp. 135–200.

Loro, V.L., Nadella, S.R., Wood, C.M., 2012. Zinc bioaccumulation and ionoregulatory impacts in Fundulus heteroclitus exposed to sublethal waterborne zinc at different salinities. Comp. Biochem. Physiol. C Toxicol.

Lushchak, V., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30.

Magwere, T., Naik, Y.S., Hasler, J.A., 1997. Effect of chloroquine treatment on antioxidant enzymes in rat liver and kidney. Free Radic. Biol. Med. 22, 321–327.

Maret, W., 2011. Redox biochemistry of mammalian metallothioneins. J. Biol. Inorg. Chem., doi:10.1007/s00775-011-0800-0.

Marshall, W.S., 2003. Rapid regulation of NaCl secretion by estuarine teleost fish: coping strategies for short-duration freshwater exposures. Biochim. Biophys. Acta 1618, 95–105.

McGeer, J.C., Szebedinszky, C., McDonald, D.G., Wood, C.M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1: iono-regulatory disturbance and metabolic costs. Aquat. Toxicol. 50, 231–243.

Meyer, J.N., Smith, J.D., Winston, G.W., Di Giulio, R.T., 2003. Antioxidant defenses in killifish (*Fundulus heteroclitus*) exposed to contaminated sediments and model pro-oxidants: short-term and heritable responses. Aquat. Toxicol. 65, 377–395.

Pedrajas, J.R., Peinaldo, J., López-Barea, J., 1995. Oxidative stress in fish exposed to model xenobiotics. Oxidatively modified forms of Cu, Zn-superoxide dismutase as potential biomarkers. Chem. Biol. Interact. 98, 267–282.

Prodocimo, V., Galvez, F., Freire, C.A., Wood, C.M., 2007. Unidirectional Na^+ and Ca^{2+} fluxes in two euryhaline teleost fishes, *Fundulus heteroclitus* and *Oreochromis mykiss*, acutely submitted to a progressive salinity increase. J. Comp. Physiol. B 177, 519–528.

Regoli, F., 2000. Total oxyradical scavenging capacity (TOSC) in polluted and translocated mussels: a predictive biomarker of oxidative stress. Aquat. Toxicol. 50, 351–361.

Scott, G.R., Baker, D.W., Schulte, P.M., Wood, C.M., 2008. Physiological and molecular mechanisms of osmoregulatory plasticity in killifish after seawater transfer. J. Exp. Biol. 211, 2450–2459.

Spry, D.J., Wood, C.M., 1989. A kinetic method for the measurement of zinc influx in the rainbow trout and the effects of waterborne calcium. J. Exp. Biol. 142, 425–446.

Viarengo, A., Burlando, B., Cavaletto, M., Marchi, B., Ponzano, E., Blasco, J., 1999. Role of metallothionein against oxidative stress in the mussel *Mytillus galloprovincialis*. Am. J. Physiol. 277, R1612–R1619.

Wood, C.M., 2012. An introduction to metals in fish physiology and toxicology: basic principles. In: Wood, C.M., Farrell, A.P., Brauner, C.A. (Eds.), Homeostasis and Toxicology of Essential Metals – Fish Physiology, vol. 31A. Elsevier, San Diego, pp. 1–51.

Wood, C.M., Grosell, M., 2009. TEP on the tide in killifish (*Fundulus heteroclitus*): effects of progressively changing salinity and prior acclimation to intermediate or cycling salinity. J. Comp. Physiol. B 179, 459–467.

Yan, L.J., Traber, M.G., Packer, L., 1995. Spectrophotometric method for determination of carbonyls in oxidatively modified apolipoprotein B of human low-density lipoproteins. Anal. Biochem. 228, 349–351.

Zhang, L., Wang, W.-X., 2007. Waterborne cadmium and zinc uptake in a euryhaline teleost *Acanthopagrus schlegeli* acclimated to different salinities. Aquat. Toxicol. 84, 173–181.