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RESEARCH PAPER

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Investigations on the Osmoregulation System of Freshwater Fish (*Oreochromis niloticus*) Exposed to Mercury in Differing Salinities

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Abstract

Increases in salinity affect fish physiology and metal uptake. Thus, freshwater fish Oreochromis niloticus were exposed to Hg2+ in different salinities (0, 1, 10 ppt) for acute (0,3 µM Hg2+, 3 days) and chronic (0,03 µM Hg2+, 30 days) exposure protocols. Following the exposures, activities of Na+/K+-ATPase, Mg2+-ATPase and Ca2+-ATPase in the gill and Ca2+-ATPase in muscle of fish were measured and significant ($P<0.05$) results were remarked. Salinity increase alone did not cause alterations in ATPase activities in the gill and muscle in both acute and chronic exposures. However, there were increases in ATPase activities in gill and muscle tissues of fish exposed to Hg2+ in different salinities both in acute and chronic exposures. In acute exposures, only Mg2+-ATPase activities in the gill increased at 1 and 10 ppt salinities ($P<0.05$). In chronic exposures, activities of gill Na+/K+-ATPase at 10 ppt, Mg2+-ATPase at 1 and 10 ppt and Ca2+-ATPase at all salinities increased. Similarly, activity of muscle Ca2+-ATPase also increased at 1 and 10 ppt. The most alterations occurred in Mg2+-ATPase activity, followed by Ca2+-ATPase activity and Na+/K+-ATPase activity in tissues of fish exposed to salinity+Hg2+. Overall, chronic exposures affected fish physiology much more that acute ones.

Introduction

The aquatic environments are the sinks for most discharges produced by anthropogenic and natural activities. It is well known that heavy metals are accumulated from water by fish and are toxic at high levels. There are environmental quality standard levels set to estimate the maximum concentrations in the aquatic environment. This is <0.5 µg/L for mercury in the estuaries and coastal waters (McLusky, 1989). Mercury is perhaps the most toxic metal for aquatic organisms, organic mercury being 10 times more toxic than inorganic mercury (Boening, 2000). These toxicities become unpredictable in estuarine organisms where salinity changes, as the bioaccumulation of mercury changes in different salinities (Dutton and Fisher, 2014). Together with heavy metal stress, freshwaters are also loaded with salt from anthropogenic activities such as industry, domestic, agriculture and road salting (approximately 15 million tons in USA and 200 thousand tons in Turkey). Most of those salts eventually find their ways in nearby water bodies like lakes and rivers, making salinity increase one of the most important problems in the freshwater ecosystems (Lin *et al.,* 2005, Choi, An & An, 2008). Rainbow and Luoma (2011) indicated that metal toxicity is not generally related to the metal burdens in tissues, but always related to metals that are metabolically available. Bioavailability of metals is also affected by salinity presence and consequently metal

uptake is altered depending on salinity levels of waters (Kulac, Atli & Canli, 2012; Baysoy, Atli & Canli, 2013; Dutton and Fisher, 2014). Therefore, the toxic effects of metals should be tested realistically in different salinity, hardness, or pH levels to understand better the consequences that fish face in the freshwater environments.

Osmoregulation means actively maintaining osmotic concentrations in the extracellular fluids against the osmolarity of water in the surrounding (Baysoy *et al.,* 2013; Saglam, Atli & Canli, 2013). In this context, it is a physiological adaptation of which animals must cope with properly in the estuarine environments and also in salinity increased freshwaters (Heath, 1987). The respiration, excretion, osmotic and ionic regulation in fish are carried out by the gills, because the branchial epithelium is a tissue where both active and passive exchange occurs between the animal and the environment (Schmidt-Nielsen, 1990). Thus branchial Na^+/K^+-ATPase (EC 3.6.3.9) plays a key role in whole body ion regulation in fish (Neufeld, Holliday & Prichard, 1980; Towle, 1981). To maintain cytoplasmic concentrations of Na+ below and K+ above those in the cellular fluid in fish, active transport of these cations against their electrochemical gradients is dependent on metabolic energy which is generally in the form of ATP. Mg^{2+}-ATPase (EC 3.6.3.2) has an important role in oxidative phosphorylation, involving in the transepithelial regulation of Mg^{2+} ions (Parvez, Sayeed & Raisuddin, 2006). Mg-ATPase is found in two forms in the cell, meaning an oligomycin sensitive fraction in the mitochondria, which is thought to play key role in the oxidative phosphorylation and oligomycin insensitive Mg-ATPase, which is known to play key roles in ion transport in the endoplasmic reticulum (Boyer *et al.*, 1977). The process of oxidative phosphorylation occurs in the inner membranes of mitochondria in animals and other eukaryotes. Ca^{2+}-ATPase (EC 3.6.3.8) has important roles in functioning to remove the Ca^{2+} ions from cytoplasm to protect the low Ca^{2+} levels (Watson and Beamish, 1981).

Several biomarkers have gained importance in metal toxicity studies as they may supply beneficial data in evaluation of fish physiology before toxic effects occur. Therefore, as a sensitive biomarker, the response of ATPases in freshwater fish may be used as an early warning signal in salinity increased waters (Dogan, Atli & Canli, 2015; Atli, Canli, Eroglu, Dogan, & Canli, 2016). The Nile tilapia (*O. niloticus*) is widely cultured fish in freshwater systems, though it is one of the least salt tolerant tilapia (Kamal and Mair, 2005). This species is suitable biological models for studying the osmoregulatory mechanisms in teleost fish due to their adaptive capacity to different salinities depending upon the integrated osmoregulatory function of several organs, the adaptation to saline water involving some functional changes in gill epithelium chloride cells and ATPases (Cioni *et al* (1991). Thus, the present study aimed to estimate the effects of mercury on ATPases in the gill and muscle of freshwater fish (*O. niloticus*) in different salinities, using 2 experimental protocols (acute: 0.3 µM Hg^{2+} for 3 days and chronic: 0.03 µM Hg^{2+} for 30 days).

Materials and Methods

Experimental Protocol

Freshwater fish *O. niloticus* were supplied from the culture pools in Cukurova University (Turkey) that they were reproduced for 30 years. Fish were moved to the experimental laboratory for acclimatization processes. Fish were kept for one month before the experiments in experimental conditions given in Table 1, using glass aquariums (40×40×100 cm) that contained 120 L test medium.

After the adaptation period, fish were exposed to different salinities (NaCl) alone (nominal values of 0, 1 and 10 ppt) and together with salinity and mercury ($HgCl_2$) for acute (0,3 µM Hg for 3 d.) and chronic durations (0,03 µM Hg for 30 d.). The measured salinities for 0, 1 and 10 ppt nominal salt levels were 0,24±0,05, 1,35±0,22 and 10,3±0,04 ppt for acute exposures, respectively, while these measurements for 0, 1 and 10 ppt nominal salt levels were 0,25±0,03, 1,34±0,15 and 10,4±0,03 ppt for chronic exposures, respectively. Measurements of medium salinities were done with a multimeter probe (Orion 5-Star). A total of 6 fish was used for each exposure case and fish were fed (2% of their weight) with commercial fish food (Pinar Sazan, Izmir, Turkey) just 1 h before the cleaning of aquaria every 3 days to renew the exposure medium. Mean length (19,8±0,40 cm) and weight (125,7±5,14 g) of fish did not differ significantly (P>0.05) among different exposures and controls. After acute and chronic durations, all fish were killed by

Table 1. Physicochemical characteristics of water used during the experiments. Data are the means and associated standard errors.

Exposure Duration	pH	Oxygen mg O_2/L	Hardness mg Ca_2CO_3/mL	Alkalinity mg Ca_2CO_3/mL	Temperature °C	Salinity ppt
Acute	7,36±0,18	6,05±0,11	334,7±3.1	403,3±18,47	20.0±1.0	0,24±0,05
Chronic	7,49±0,90	6,16±0,21	335,6±2.0	402,2±12,45	20.1±1.1	0,25±0,03

transaction of spinal cord, according to the decision of the Ethic Committee of Çukurova University. The gill and muscle of fish were taken out and stored at -80 ^{0}C until the analyses.

ATPase Activity Assay

Frozen tissues were thawed and homogenized in ice-cold buffer containing 20 mM Tris-HCl, 0.25 M sucrose, and 1 mM EDTA (pH 7.7) with a ratio of 1/10 at 9500 rpm, then homogenates were centrifuged at 13,000 g (+4 ^{0}C) for 20 minutes to obtain the supernatants. The measurements of ATPase activity and protein were done in the supernatants. The final assay concentrations and conditions were optimized in our previous research (Atli and Canli, 2008). The activities of ATPases in the gill and muscle of fish were measured by the method of Atkinson, Gatenby & Lowe (1973). ATPase activity was calculated from the inorganic phosphate liberated from ATP. Na^+/K^+-ATPase activity was calculated from the differences between the presence (Mg^{2+}-ATPase activity) and absence (Total-ATPase activity) of the ouabain. Ca^{2+}-ATPase activity was measured as the absorbance differences between the presence and absence of $CaCl_2$. All assays were carried out in triplicate. The total protein levels were determined by the method of Lowry, Rosebrough, Farr, & Randall, (1951) using bovine serum albumin as a standard. A detailed description of the method used was given in our previous papers (Atli and Canli, 2011; Kulac *et al.,* 2012).

Statistical Analysis

A statistical package program (SPSS 13) was used to analyze the data using one-way Anova test. Each parameter was analyzed separately using its own control to estimate the changes in each condition. If there is a significant difference ($P<0.05$) in comparing group, then those data were analyzed by Duncan tests to determine which individual group was significantly different. All data were presented as figures (mean±se).

Results

Fish exposed to salinity alone did not cause any significant ($P>0.05$) alteration in gill and muscle ATPase activities in both acute and chronic exposures (Figures. 1-3). Likewise, there was no fish mortality during the experiments, including salinity alone and salinity+mercury exposure experiments.

In acute exposures, there were increases in Mg^{2+}-

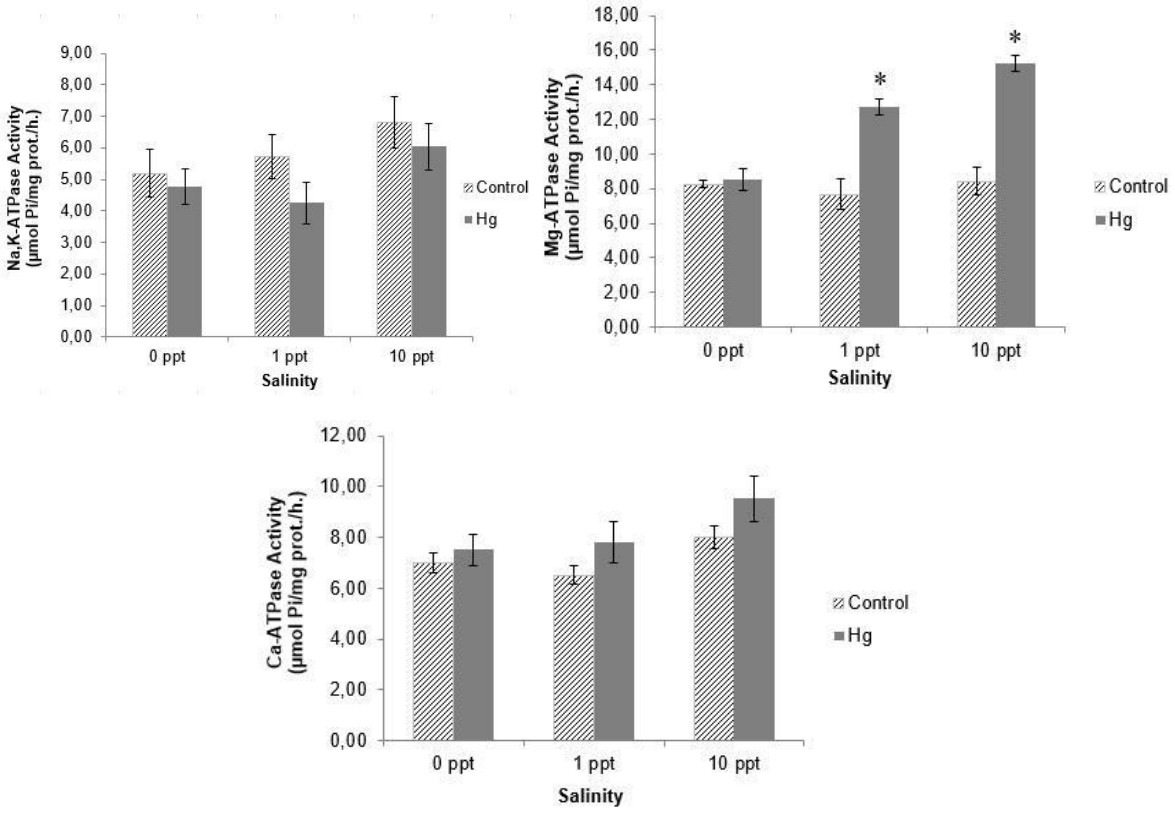

Figure 1. Effects of salinity and salinity+mercury exposures on (a) Na^+/K^+-ATPase activity, (b) Mg^{2+}-ATPase activity, and (c) Ca^{2+}-ATPase activity in the gill of *O. niloticus*exposed to acute experimental protocol (0.3 µM Hg^{2+} for 3 days). Data are expressed as mean (n=6)±standard errors. * indicate significant ($P<0.05$) differences resulted from the Duncan tests between salinity controls and salinity+mercury exposures.

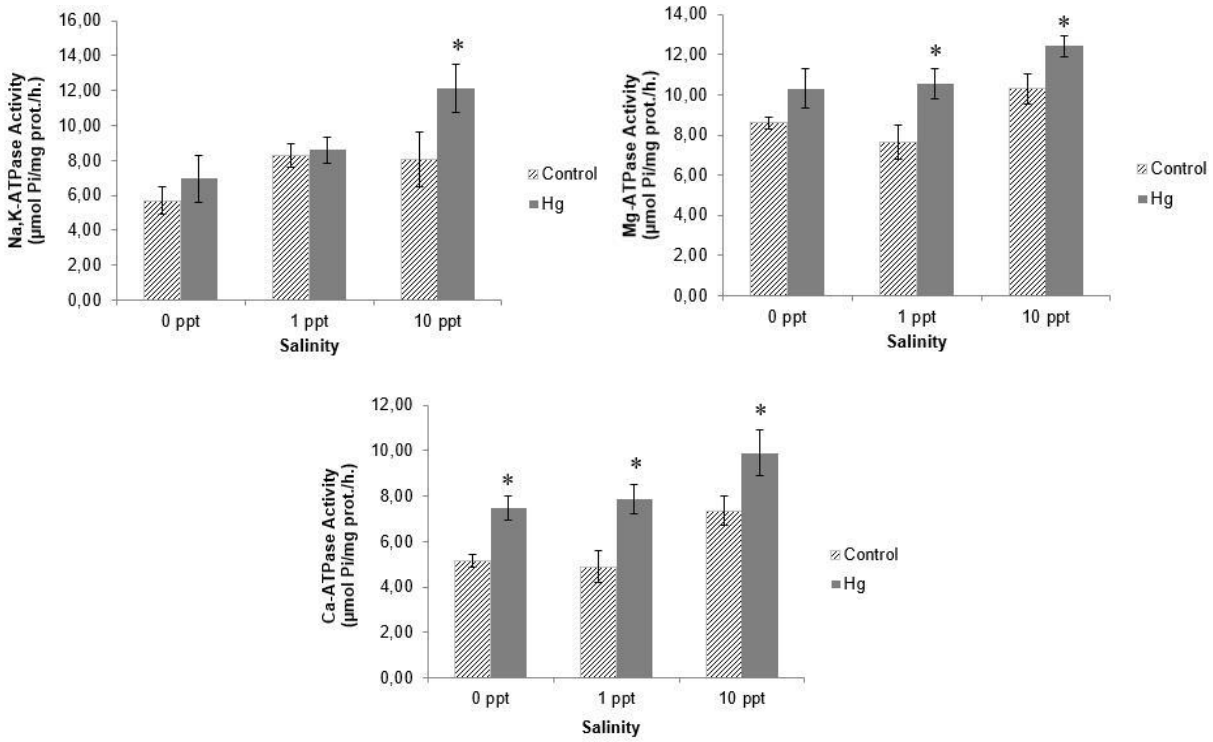

Figure2. Effects of salinity and salinity+mercury exposures on (a) Na^+/K^+-ATPase activity, (b) Mg^{2+}-ATPase activity, and (c) Ca^{2+}-ATPase activity in the gill of *O. niloticus*exposed to chronic experimental protocol (0.03 µM Hg^{2+} for 30 days). See details in Figure 1.

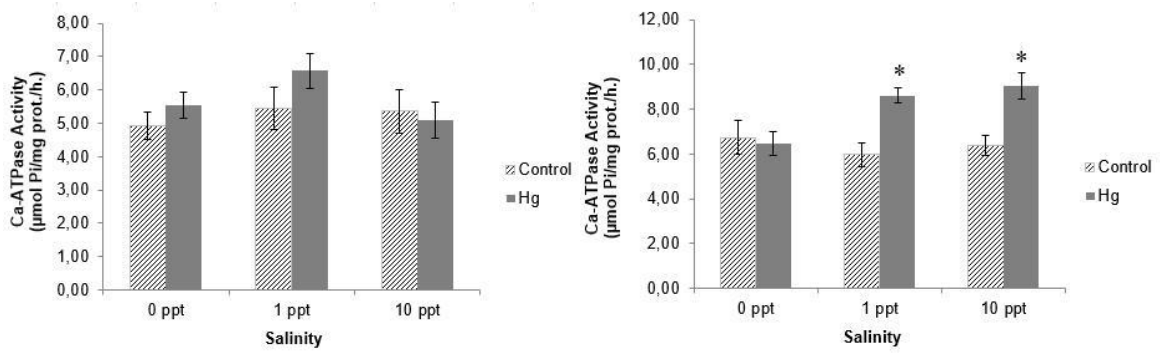

Figure 3. Effects of salinity and salinity+mercury exposures on (a) Ca^{2+}-ATPase activity in the muscle of *O. niloticus*exposed to (a) acute and (b) chronic experimental protocols. See details in Figure 1 and Figure 2.

ATPase activities in fish exposed to mercury in different salinities compared to controls (Figure. 1). Highest increases occurred in Mg^{2+}-ATPase activity in the gill of fish exposed to acute mercury concentration at 1 ppt (66%) and 10 ppt (81%) salinities. Na^+,K^+-ATPase activity in the gill did not change significantly in acute exposures. In chronic exposures, there were increases in Na^+,K^+-ATPase activity (at 10 ppt) and Mg^{2+}-ATPase activity (at 1 and 10 ppt) in the gill of fish exposed to mercury (Figure. 2). Ca^{2+}-ATPase activity in the gill increased at all salinities in chronic exposures, highest increase being (60%) at 1 ppt. However, there was no significant change in gill Ca^{2+}-ATPase activity in acute exposures. Similarly, there was no significant alteration in muscle ATPase activities in acute exposures, though there were increase in Ca^{2+}-ATPase activity at 1 ppt (42%) and 10 ppt (41%) salinities in chronic exposures (Figure 3).

Discussion

No fish mortality occurred in the present study at all exposure durations, despite increased salinities of experimental waters. Likewise, fish also did not die during 30 days of exposure period when they were exposed to mercury or mercury plus elevated salinities.

Literature data showed that salinity adaptation of freshwater fish may be deadly if fish cannot cope with the increased osmolality (Heath, 1987; Blanchard and Grosell, 2006). However, tilapia species are known by their resistances against certain levels of changes in the environmental factors (Kamal and Mair, 2005). This was also supported by the response of ATPases in the present study to salinity increase, as ATPase activities in salinity increased conditions (without mercury) did not cause significant alterations in enzyme activities. This may support the fact that tilapias are resistant against salinity increase or in another word, they can adapt easily to increased salinities (Almeida *et al.,* 2002).

Metal toxicity is largely determined by metal bioavailability, which is affected by several factors of fresh waters such as salinity, pH, hardness, organic matter and temperature (Dutton and Fisher, 2011). All these factors also determine the conductivity values of waters. It seems that the bioavailability of metals decreases when the water conductivity increases, suggesting a negative relationship between the conductivity and metal toxicity (Canli and Canli, 2015). Mercury is different than most heavy metals, in terms of bioavailability, toxicity, tissue accumulation and physico-chemical characteristics (Dutton and Fisher, 2011; 2014). Mercury mostly accumulates in muscle tissue as a methylated form which makes it highly toxic to aquatic organisms and consumers (Canli and Furness, 1993; Canli and Erdem 1994; Smylie, McDonough, Reed & Shervette, 2016).

This study demonstrated that there were significant increases in the activities of ATPase following exposure to mercury in elevated salinities. Percent alterations in ATPase activities were summarized in Table 2. According to this table, elevated salinities alone did not cause any significant alteration in ATPase activities in both acute and chronic exposures. However, there were significant increases in fish exposed to Hg^{2+}+elevated salinities in both acute and chronic exposures. Data showed that most significant alterations occurred in Mg^{2+}-ATPase activity, followed by Ca^{2+}-ATPase activity and Na^{+}/K^{+}-ATPase activity in the gill and muscle of fish exposed to salinity+Hg^{2+} exposures. Overall, chronic exposures affected fish physiology much more that acute ones. Salinity is one of the most important environmental factors in freshwaters, as it causes physiological alterations in fish metabolism. It seems that fish required more energy to cope with mercury toxicity at increased salinities so that Mg^{2+}-ATPase activity increased in salinity+Hg^{2+} combination exposures.

In the literature, there were conflicting data when ATPase activities compared from different studies. This may be mostly due to the differences in exposure conditions such as metal type, concentrations, durations and fish biology. In our previous studies, we demonstrated different results for other metals such as, copper, lead, chromium and cadmium, comparable to mercury (Kulac *et al.,* 2012; 2013; Baysoy *et al.,* 2013). This is possible due to different binding site of metals and also a specific stress addition of each metal to fish metabolism. Shivkamat and Roy (2005) indicated that alteration in Na^{+}/K^{+}-ATPase activity in the gill of tilapia may be a result of the changes in membrane structure and fluidity which may also cause alterations in the activity of Na^{+}/K^{+}-ATPase. Changes in Na^{+}/K^{+}-ATPase activities were also evident in euryhaline fish species, suggesting the influence of salinity changes in

fish metabolism (Nolan, Op't Veld, Balm & Bonga, 1999). Kulac *et al.,* (2013) indicated that metals and salinity alone and in combination may alter ATPases activities, as both factors are important in metal uptake. Differences in ATPase activities from different studies may also be the results of the differences in the turnover rates of enzymes which directly influence enzyme activity.

Studies carried out with different heavy metals showed different trends in enzyme activities, possibly due to exposure conditions such as metal type, duration, salinity and exposure concentration and fish species in concern. Kulac *et al.,* (2012) showed that Cd accumulation in the tissues of *O. niloticus* decreased as the salinity of the medium increased and activities of

Table 2. Percent variations in the activities of ATPases in tissues of*Oreochromis niloticus* following acute and chronic exposures to mercury in differing salinities. Asterisks indicate significant (P<0.05) changes over control values. (↑: Increase; ↓: Decrease; —: Not significant).

Conditions		Gill			Muscle
Exposure Duration	Salinity	Na^{+}/K^{+}-ATPase	Mg^{+2}-ATPase	Ca^{+2}-ATPase	Ca^{+2}-ATPase
Acute	0 ppt	-	-	-	-
	1 ppt	-	*66 ↑	-	-
	10 ppt	-	*81 ↑	-	-
Chronic	0 ppt	-	-	*46 ↑	-
	1 ppt	-	*38 ↑	*60 ↑	*42 ↑
	10 ppt	*50 ↑	*21 ↑	*34 ↑	*41 ↑

ATPases were affected from the exposure conditions.They demonstrated that salinity+Cd combine exposures decreased Na^+/K^+-ATPase activity in 2 ppt medium in the gill while the activity increased at 8 ppt medium and the activity in the intestine decreased in relation to salinity increase. However, the activities of Mg^{2+}-ATPase and Ca^{2+}-ATPase showed a declining trend with the increase in salinity. Baysoy *et al.,* (2013) showed that ATPase activities were affected both from salinity and metals (Pb, Cr), pointing the ATPases in the gills were more affected from the exposures compared to the ATPases in the intestine. They also demonstrated that there were increasing trends in ATPase activities in salinity alone group, whereas there were decreasing trends in the metal+salinity groups. Similar trends were also shown in the tissues of *O. niloticus* exposed to copper in elevated salinities (Kulac *et al.,* 2013). Poopal, Ramesh & Dinesh (2013) found opposite data in Indian major carp (*Cirrhinus mrigala*) exposed to inorganic mercury (0.068 and 0.034 mg Hg/L) in an acute (96 h) experiment, as Na^+/K^+-ATPase activity in gill and brain of fish decreased significantly. They emphasized that the binding capacity of mercury on the sulfhydryl groups of enzymes which make it an effective enzyme inhibitor. In the present study, Ca^{2+}-ATPase activity in the gill and muscle increased when fish were exposed to mercury in increased salinities, suggesting the breakdown of the active transport mechanism and also due to the disturbed Ca^{2+} homeostasis (Verbost, Flik, Pang, Lock & Bonga, 1988). Ca^{2+}-ATPase activity in the gill of *Periophthalmus dipes* following exposure to chromium was a result of the blocked active transport system by chromium (Thaker *et al.*, 1996). Similar data were also demonstrated by Wong and Wong (2000). They showed that the activity of Ca^{2+}-ATPase in the gill of *O. mossambicus* decreased following cadmium exposures, suggesting an inhibition of calcium transport.

Conclusion

In conclusion, the present study demonstrated that the response of ATPases in the gill and muscle of *O. niloticus* varied in relation to the type of stress factors, tissues and exposure durations and provided valuable information concerning mercury toxicity in fish exposed to mercury in elevated salinities. The response of ATPases from both acute and chronic exposures were the same, as all significant alterations in ATPase activities in the gill and muscle were increasing manners. The most significant alterations occurred in Mg^{2+}-ATPase activity, followed by Ca^{2+}-ATPase activity and Na^+/K^+-ATPase activity in tissues of fish exposed to salinity+Hg^{2+} exposures. Overall, chronic exposures affected ATPase activities more that acute ones. The current results also emphasized that during ecotoxicological investigations, salinity of waters should be taken into account before having a conclusion in the field. However, the present data also emphasized that more data are needed to estimate better the effects of salinity and other physico-chemical factors on the osmoregulation systems of freshwater fish.

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