www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 14: 43-52 (2014) DOI: 10.4194/1303-2712-v14_1_06

Response of the Antioxidant System of Freshwater Fish (*Oreochromis niloticus*) Exposed to Metals (Cd, Cu) in Differing Hardness

Dilek Saglam[1], Gülüzar Atli[1], Zehra Dogan[1], Emine Baysoy[1], Ceren Gurler[1], Ali Eroglu[1], Mustafa Canli[1]

[1] Çukurova University, Faculty of Science and Letters, Department of Biology, 01330, Balcali, Adana, Turkey.

* Corresponding Author: Tel.: +90.322 3386060; Fax: +90.322 3386070;
E-mail: gatli@cu.edu.tr

Received 19 September 2013
Accepted 10 January 2014

Abstract

Freshwater hardness affects metal toxicity and fish physiology. Therefore, the effects of dissolved metals on fish physiology may differ in waters from different geographic regions. In this study, toxic effects of Cd and Cu (1 mg/L) on the antioxidant system of freshwater fish *Oreochromis niloticus* were investigated in two different waters (commercial and tap water). Fish were exposed to metals in hard water (~320 mg $CaCO_3$/ L, conductivity 5.80 mS/cm) and soft water (~80 mg $CaCO_3$/ L, conductivity 1.77 mS/cm) for 1, 7 and 14 days and consequently the activities of antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPX; glutathione reductase, GR and glutathione *S*-transferase, GST) and total glutathione (GSH) levels were measured spectrophotometrically. Cu exposure of fish in soft water caused fish mortality after 8 days, though there was no fish mortality in the other conditions. The antioxidant system of fish responded differently to metal exposures in waters with differing hardness. Metal exposures in soft water showed predominant effects on the antioxidant system of fish comparing to hard water exposures. Similarly, antioxidant enzyme activities also altered in control fish depending on the hardness of waters. In general, CAT was the most sensitive antioxidant enzyme followed by SOD and GPX. CAT and GSH showed an increasing trend while a decreasing trend was observed for SOD and GPX. This study emphasized that the water chemistry affects the fish antioxidant system and metal toxicity that may be useful in environmental monitoring and also evaluating biomarkers in fish from different regions.

Keywords: Antioxidant enzymes, glutathione, *Oreochromis niloticus*, hardness, metal.

Metal (Cd, Cu) Etkisindeki Tatlısu Balığının (*Oreochromis niloticus*) Farklı Sertlikteki Sularda Antioksidan Sistem Cevabı

Özet

Suyun sertliği, metal toksisitesi ve balık fizyolojisini etkilemektedir. Bu nedenle, suda çözünmüş metallerin balık fizyolojisi üzerine olan etkisi farklı coğrafik bölgelerde farklılık gösterebilir. Bu çalışmada, iki farklı su ortamında Cd ve Cu'nun (1 mg/L) tatlısu balığı *Oreochromis niloticus*'un antioksidan sistemi üzerine olan toksik etkileri araştırılmıştır. Balıklar, sert su (~320 mg $CaCO_3$/ L, iletkenlik 5.80 mS/cm) ve yumuşak suda (~80 mg $CaCO_3$/ L, iletkenlik 1.77 mS/cm) 1, 7 ve 14 gün süre ile metal etkisine bırakıldıktan sonra antioksidan enzim (süperoksit dismutaz, SOD; katalaz, CAT; glutatyon peroksidaz, GPX; glutatyon redüktaz, GR ve glutatyon *S*-transferaz, GST) aktiviteleri ve toplam glutatyon (GSH) düzeyleri spektrofotometrik yöntemlerle ölçülmüştür. Yumuşak suda Cu etkisinde kalan balıklarda 8. günden sonra ölümler gözlenirken, diğer koşullarda balık ölümü görülmemiştir. Balık antioksidan sistemi farklı sulardaki metal etkilerine farklı tepkiler vermiştir. Benzer şekilde, kontrol balıkların antioksidan enzim aktiviteleri de suyun sertliğine bağlı olarak değişim göstermiştir. Metaller yumuşak suda, sert su ile karşılaştırıldığında, antioksidan sistem üzerine daha etkili olmuştur. En duyarlı antioksidan enzim CAT olurken, bunu SOD ve GPX izlemiştir. Genel olarak, CAT ve GSH düzeylerinde artış yönünde bir eğilim görülürken, SOD ve GPX düzeylerinde azalış yönünde bir eğilim gözlenmiştir. Bu çalışma, su kimyasının balık antioksidan sistemi ve metal toksisitesi üzerinde etkileri olduğunu vurgulamıştır. Böyle çalışmalar farklı bölgelerden elde edilen balık biyomarkırlarının değerlendirilmesinde ve çevresel gözlemlerde yararlı olabilmektedir.

Anahtar Kelimeler: Antioksidan enzimler, glutatyon, *Oreochromis niloticus*, sertlik, metal.

Introduction

Natural waters have a high potential risk for receiving metals from anthropogenic sources, such as an urban runoff, sewage treatment plants and domestic garbage dumps which eventually cause

adverse effects on biota. Copper occurs naturally in unpolluted freshwaters in a range of 0.2-30 mg/L however, its concentrations ranging from 50 to >560 mg/L have been reported in polluted areas all over the world (USEPA, 2007). In addition, cadmium can occur at concentrations of <0.1 µg/L, but in polluted environments concentrations can be considerably higher (USEPA, 2001). Additionally, aquatic organisms are affected by water hardness and some other characteristics of freshwaters (Heath, 1987; Pinto *et al.*, 2003; Garcia Sampaio *et al.*, 2008; Kulac *et al.*, 2012). Fish communities not only an important ecosystem component, but also used as a food source are shown to be sensitive bioindicators of external factors, such as pollution. Metals are able to disturb the integrity of physiological and biochemical mechanisms such as antioxidant systems (Basha and Rani, 2003; Atli and Canli, 2007, 2008; Ezemonye and Enuneku, 2011).

Water hardness is one of the most important abiotic factors in aquatic systems affecting metal uptake and consequent toxicity. Water hardness can be generalized as the sum of concentration of the divalent cations. The water hardness affects fish physiology and metal bioavailability and consequent metal uptake by fish. Metal bioavailability generally depends on the conductivity of water which is negatively related to free ions in water. Studies show that metals are on an order of magnitude more toxic in soft water than in hard water because they are more soluble in soft water (Heath, 1987; Grosell *et al.*, 2002; Monserrat *et al.*, 2007).

Many studies show that metals can induce oxidative stress by generating free radicals and reactive oxygen species. Fish tissues, particularly liver and kidney have antioxidant defense systems to protect them from oxidative stress caused by metals (Basha and Rani, 2003; Atli *et al.*, 2006; Atli and Canli, 2007). Essential and non-essential metals display different effects on antioxidant enzymes due to their different chemical structures and act as redox active and inactive status (Romeo *et al.*, 2000; Atli *et al.*, 2006; Atli and Canli, 2010). Studies suggest that the antioxidants may play an important role in reducing hazardous effects of metals, and antioxidant responses were suggested to be a useful biomarker for metal exposure of aquatic organisms (Elia *et al.*, 2003; Barata *et al.*, 2005; Atli and Canli, 2010; Srikanth *et al.*, 2013).

Antioxidant enzymes are crucial in the effort to counteract oxidative stress caused by toxicants once the supply of other antioxidant compounds are depleted (Radi and Matkovics, 1988; Martinez-Alvarez *et al.*, 2005). These enzymes, which remove peroxides, and superoxide radicals including SOD (EC 1.15.1.1, converts superoxide anion radical to hydrogen peroxide), CAT (EC 1.11.1.6, reduces hydrogen peroxide to water), GPX (EC 1.11.1.9, detoxifies hydrogen peroxide), GR (EC 1.6.4.2, reduces oxidized glutathione to reduced glutathione GSH) and GST (EC 2.5.1.18, catalysis the conjugation of GSH with xenobiotics) are of the essence in oxidative stress to deal with free radicals causing several disturbances (Pinto *et al.*, 2003; Tripathi *et al.*, 2006).

The aim of this study was to compare effects of Cd and Cu on the liver antioxidant system of fish *Oreochromis niloticus* in differing water hardness.

Materials and Methods

Experimental Protocol

One year old *O. niloticus* (Perciformes: Cichlidae) were obtained from fish culturing pools of Cukurova University and transferred to the laboratory where they were acclimatized to laboratory conditions (20±1°C and illuminated 12 h by fluorescent lamps, daylight 65/80 W) as a stock for one month in dechlorinated tap water (used as hard water). The experiments were carried out in glass aquariums 40×40×100 cm in size and contained 120 L contaminated test solution for metal exposed groups or only test waters for controls after an adaptation of each group in their own water conditions (hard water or soft water) for three days. The aquaria were aerated with air stones attached to an air compressor to saturate with oxygen.

Two different waters as "hard water" (dechlorinated tap water; ~320 mg $CaCO_3$/L) and "soft water" [commercial packed spring water (brand Pinar Gokceagac); ~80 mg $CaCO_3$/L] were used in the experiments. Initially, fish were separated into three groups as control group, Cd and Cu exposed groups for each water conditions. According to this protocol, total of six experimental groups were designed as hard water control group (HW-C) (1), hard water+Cd (HW+Cd) exposed group (2), hard water+Cu (HW+Cu) exposed group (3), soft water control group (SW-C) (4), soft water+Cd (SW+Cd) exposed group (5) and soft water+Cu (SW+Cu) exposed group (6). Based on this, control groups were not exposed to metals. HW+metal and SW+metal groups were individually exposed to 1.0 mg/L of Cd ($CdCl_2.H_2O$) and Cu ($CuCl_2.2H_2O$). Fish were sampled at day 1, 7 and 14 and a total of six fish (replicates) were sampled for each group. During the experiments, measurements in aquaria were done every day with a multimeter (Thermo Orionstar 5) and also measurement of ion levels with a flame atomic absorption spectrophotometer (AAS, Perkin Elmer AS 3100) to determine the quality of hard water and soft water. The values were measured as 5.30±0.83 mg O_2/L, temperature of 20±1°C, pH value of 8.30±0.08, total hardness of 304.2±21.2 mg $CaCO_3$/L, alkalinity of 142.0±11.8 mg $CaCO_3$/L, conductivity of 5.80±0.18 mS/cm, 0.73±0.12 mg Na/L, 28.3±1.20 mg Cl/L and 130.7±2.44 mg Ca/L in hard water. These values were measured as 5.50 ± 0.55 mg O_2/L, temperature of 20±1°C, pH value of

8.58±0.13, total hardness of 82.8±11.2 mg $CaCO_3$/L, alkalinity of 95.5±14.0 mg $CaCO_3$/L, conductivity of 1.77±0.69 mS/cm, 5.5±0.05 mg Na/L, 1.12±0.01 mg Cl/L and 26.1±2.44 mg Ca/L in soft water. Cu and Cd levels in aquaria were also measured (AAS, Perkin Elmer AS 3100) and found that variation between observed and expected levels were less than 10%. Exposure media were renewed every two days to minimize metal loss just after daily feeding (2% of their body weight) with a commercial fish food (Pinar Sazan, Izmir, Turkey) and to reduce contamination with food remains.

After each exposure period, fish were killed by transaction of the spinal cord according to the decision of an Ethic Committee of Cukurova University. Liver tissues were dissected, washed out with physiological saline to remove blood, blotted dry and weighed by using clean equipment before the storage at -80°C until the analysis. The tissues were homogenized (1:10, w/v) in homogenization buffer containing 100 mM potassium phosphate buffer (pH 7.4), 100 mM KCl and 1 mM EDTA at 9500 rpm for 1.5 min on ice. Homogenates were centrifuged at 10000 x g (Sigma 2-16) for 30 min (+4°C). Supernatants were used for the analysis. All chemicals used in this study were obtained from Sigma or Merck (Germany). Total length (12.7±1.40 cm) and weight (28.0±8.70 g) of fish did not differ significantly ($P>0.05$) among different exposure regimes and controls.

Enzyme Activity Assay

CAT activity was measured by using the method of Bessey *et al.* (1946). It was calculated as µmol H_2O_2/mg prot./min. The GPX activity was measured by using the method of Livingstone *et al.* (1992) and calculated as µmol/mg prot./min. The GR activity was analyzed using the method of Carlberg and Mannervik (1975), and calculated as µmol/mg prot./min. SOD activity was measured by the indirect method involving the inhibition of cytochrome *c* reduction at 550 nm for 1 min (McCord and Fridovich, 1969). The SOD activity was calculated as Unit/mg prot. The GST activity was measured using the method of Habig *et al.* (1974) and was calculated as µmol/mg prot./min. The protein contents of the homogenates were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. Methods were described in detail by Atli and Canli (2010).

Total GSH Analysis

Total GSH levels were analyzed according to the method of Beutler *et al.* (1963) and calculated as nmol/mg prot. Methods were described in detail by Atli and Canli (2008).

Statistical Analysis

Statistical analysis of data (Mean±Standard error) was carried out using SPSS statistical package program. Control data were given for each exposure duration in figures. Thus, each parameter from metal exposure experiments was analyzed separately using appropriate control. One-way Anova was used to compare data and significant differences ($P<0.05$) were reanalyzed by Duncan tests to determine which individual group was significantly different from controls. The statistical results were also summarized in Table 1.

Results

There was no fish death in all exposure conditions, except Cu exposure in soft water. Fish death occurred after 8 days in SW+Cu exposure and all fish had died by day 14. Therefore, there is no data regarding 14th day SW+Cu exposure.

The hepatic SOD activity varied in relation to metal species, exposure duration and water hardness. The SOD activity decreased significantly in SW-C group when compared to HW-C group and the lowest activity (45%) was recorded after 1 day exposure. In HW condition, the SOD activity increased (44%) at day 7 in Cd-exposed group comparing to HW-C group. In SW condition, Cu exposure increased the SOD activity (55%) after 1 day exposure compared to SW-C groups. The activity was significantly lower in SW+metal group compared to HW+metal group. The lowest activity was determined at a ratio of 47% after 7 day Cd exposure (Figure 1).

There were outstanding differences between control groups as the CAT activity was many folds higher in SW-C group than in HW-C group, except day 1. In HW condition, the lowest (100%) and the highest CAT activity (103%) were observed following Cd exposures at day 1 and 7, respectively. In SW condition, Cd increased its activity at all durations and the highest increase (970%) was observed after 1 day exposure. However, 1 day Cu exposure caused a total inhibition in CAT activity (Figure 2).

GPX activity varied among the experimental groups. GPX activity in SW-C group was lower comparing to HW-C group. In HW condition, Cd and Cu exposures increased its activity (48% and 94%, respectively) while a decrease (55%) was observed after 7 day Cd exposure. On the other hand, metal exposures in SW condition did not alter GPX activity compared to its control group ($P>0.05$). There were striking differences (307%) between SW+Cu and HW+Cu groups after 1 day of exposures (Figure 3).

GST activity was lower in SW-C group (37%) when compared to HW-C group and the after 7 day. GST activity did not change significantly ($P>0.05$) after HW+metal and SW+metal exposures compared to their control groups. However, GST activity was

Table. 1 summary of the effects of Cd and Cu on the hepatic antioxidant system of *O. niloticus* exposed to metals in different waters. Comparisons were done among hard water (HW), soft water (SW), metal exposed (HW-M and SW-M) and control groups (HW-C and SW-C). ↓: Decrease; ↑:Increase; —: Not significant; ND: Not detected (due to mortality in soft water)

Hardness Comparisons	Metal	Day	SOD	CAT	GPX	GR	GST	GSH
SW-C/HW-C	Control	1	↓	—	↓	↑	—	—
		7	—	↑	↓	—	↓	—
		14	↓	↑	↓	—	—	—
HW+M/HW-C	Cd	1	—	↓	↑	—	—	↑
		7		↑	↓	—	—	↑
		14	—	—	—	—	—	↑
	Cu	1		↓	↑	—	—	↓
		7	—	—	—	—	—	—
		14	—	—	—	—	—	↑
SW+M/SW-C	Cd	1	—	↑	—	—	—	—
		7	—	↑	—	—	—	↑
		14	—	↑	—	—	—	—
	Cu	1		↓	—	—	—	—
		7	—	—	—	—	—	—
		14	ND	ND	ND	ND	ND	ND
SW+M/HW+M	Cd	1	↓	↑	↓	↑	—	↓
		7	↓	↑	—	—	—	↓
		14	↓	↑	↓	—	—	—
	Cu	1	↓	↓	↓	—	—	↑
		7	↓	↑	↓	↓	↓	—
		14	ND	ND	ND	ND	ND	ND

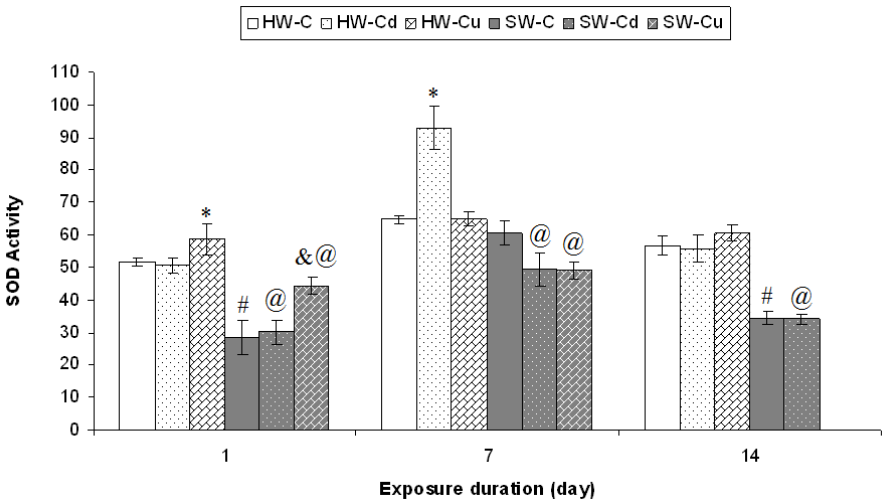

Figure 1. SOD activity (Unit/mg prot.) in the liver of *O. niloticus* exposed to Cd and Cu for 1, 7 and 14 days in waters with different hardness. Data are expressed as mean±standard error (*N*=6). Significant differences (P<0.05) resulted from the Duncan tests between the exposures of HW-C and HW+M with "*", HW-C and SW-C with "#", SW-C and SW+M with "&" and HW+M and SW+M with "@"were symbolized.

significantly lower (45%) following 7 day Cu exposure in SW condition compared to HW condition (Figure 4).

GR activity increased significantly (P<0.05) in SW-C group (54%) at day 1, but there was no significant difference (P>0.05) in the other conditions. GR activity increased (75%) after 1 day Cd exposure in SW condition compared to HW+Cd group. However, it decreased (48%) after 7 day SW+Cu exposure comparing to HW+Cu group (Figure 5).

GSH levels did not differ significantly (P>0.05) over 14 days in both control groups. HW+Cd exposure caused increases in GSH levels and the highest increase was (96%) determined after 7 day Cd exposure compared to HW-C group. However, HW+Cu exposure decreased (100%) GSH level at day 1, but an increase was observed (32%) at the end of the exposure duration compared to their controls. SW+Cd exposure also increased its level (32%) at day 7 compared to the control group in SW condition. When compared to the HW+metal exposures, GSH levels decreased in SW+Cd group at day 1 (30%) and day 7 (22%), whereas there was an increase (100%) in SW+Cu group at day 1 (Figure 6).

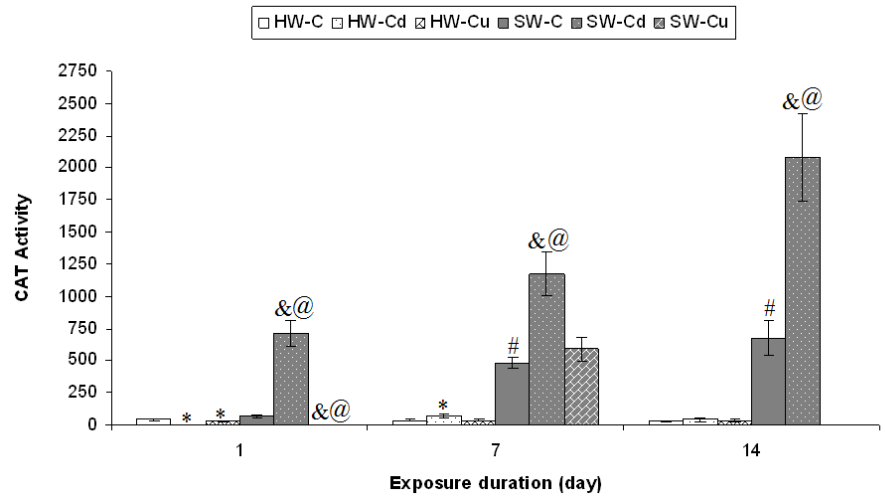

Figure 2. CAT activity (µmol H_2O_2/mg prot./min.) in the liver of *O. niloticus* exposed to Cd and Cu for 1, 7 and 14 days in waters with different hardness. See details in Figure 1.

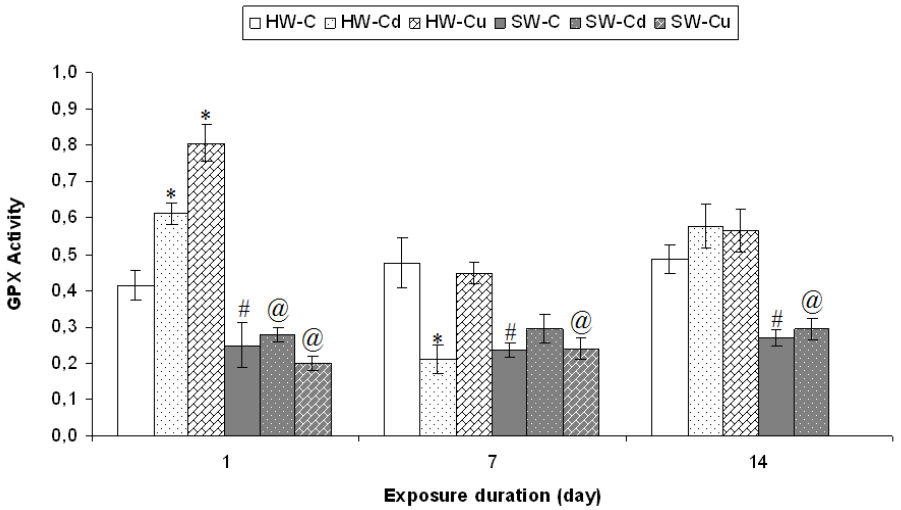

Figure 3. GPX activity (µmol/mg prot./min.) in the liver of *O. niloticus* exposed to Cd and Cu for 1, 7 and 14 days in waters with different hardness. See details in Figure 1.

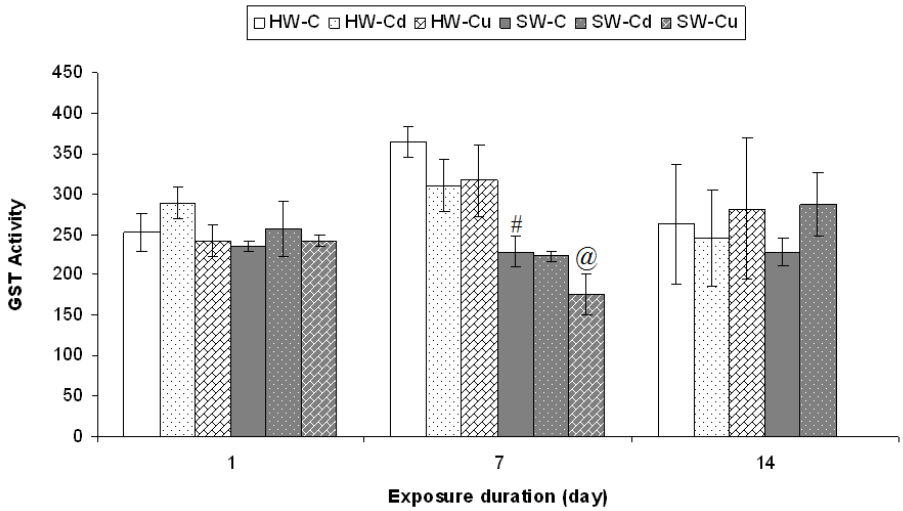

Figure 4. GST activity (µmol/mg prot./min.) in the liver of *O. niloticus* exposed to Cd and Cu for 1, 7 and 14 days in waters with different hardness. See details in Figure 1.

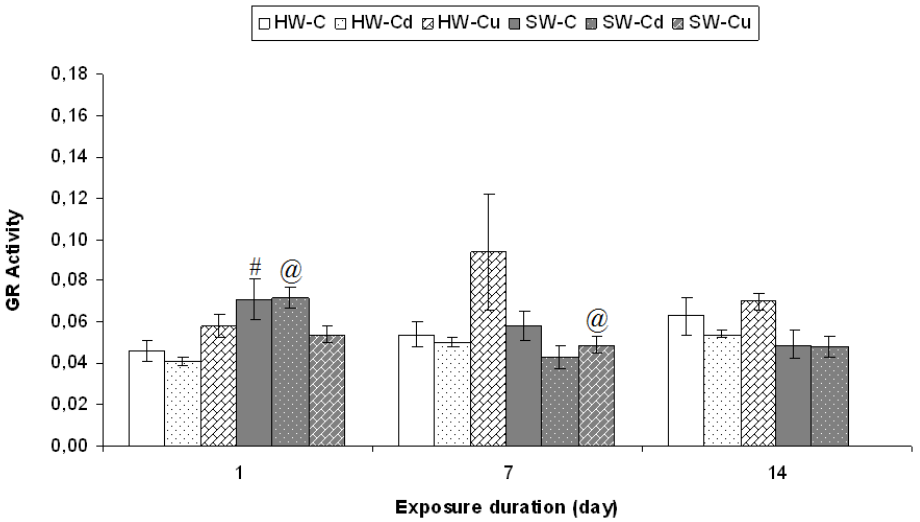

Figure 5. GR activity (µmol/mg prot./min.) in the liver of *O. niloticus* exposed to Cd and Cu for 1, 7 and 14 days in waters with different hardness. See details in Figure 1.

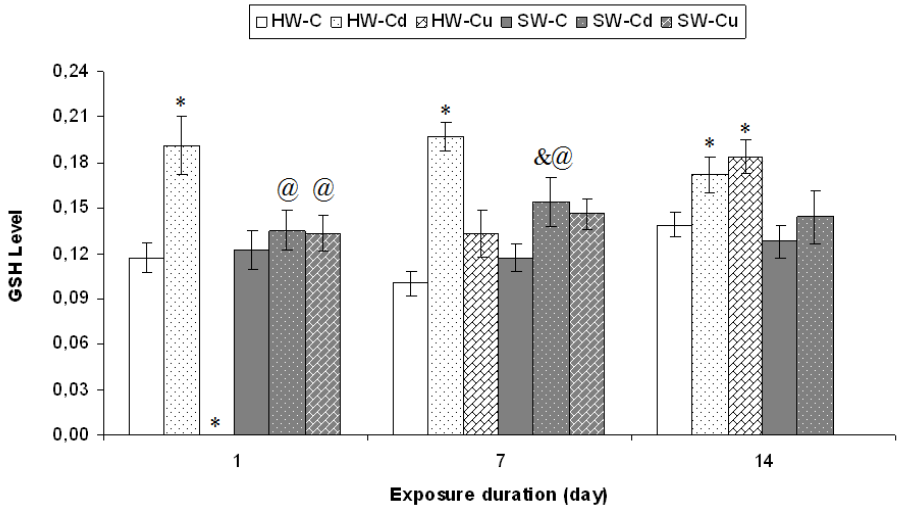

Figure 6. GSH level (nmol/mg prot.) in the liver of *O. niloticus* exposed to Cd and Cu for 1, 7 and 14 days in waters with different hardness. See details in Figure 1.

Discussion

Many metals are known to be powerful oxidants. Redox active metals such as Cr, Cu and Fe, undergo redox cycling, though redox-inactive metals, such as Cd, Hg and Pb deplete major antioxidants in the cell, especially thiol containing antioxidants and enzymes (Pinto *et al.*, 2003). One of the well-known and main mechanisms is a production of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion radical and hydroxyl radical induced by metals through various mechanisms such as Fenton- and Haber-Weiss type reactions. Metals can promote oxidative damage by directly increasing the cellular concentration of ROS and by altering the cellular antioxidant capacity in fish (Radi and Matkovics, 1988; Dautremepuits *et al.*, 2004; Baysoy *et al.*, 2012). Through ROS-mediated reactions, metals cause DNA damage, lipid peroxidation, and protein modification (Nagalakshmi and Prasad, 1998; Dewez *et al.*, 2005). These enzymatic responses are associated with increased ROS production leading to "oxidative stress" occurring when the ROS generation rate exceeds that of their removal (Ercal *et al.*, 2001; Pinto *et al.*, 2003; Martinez-Alvarez *et al.*, 2005). This study has shown that not only metal exposure, but also hardness of water may cause the oxidative stress responses.

Copper, comparing to most metals, is highly toxic for fish even at low exposure concentrations (Heath, 1987; Lauren and McDonald, 1987; Ay *et al.*, 1999; Atli and Canli, 2003). Likewise, in the present study fish mortality occurred after Cu exposure in soft water, beginning at day 8 and all fish had died by day 14. However, there was no fish mortality in hard water exposures, indicating the protective role of ions causing the hardness. Toxicity of metals is influenced by physicochemical properties of aquatic environment

such as salinity, pH and hardness. The previous data demonstrated that uncontrolled loss of Na ions from cells largely determines the toxicity of Cu in fish (Blanchard and Grosell, 2006; De Boeck *et al.*, 2010). Hardness can alter the bioavailability and, by consequence, toxicity of metals as being increased with decreased hardness (Heath, 1987; Bury *et al.*, 2002; Monserrat *et al.*, 2007). Therefore, fish mortality occurred in the present study could be due to the enhanced Cu toxicity, as a result of higher Cu bioavailability in soft water media.

The liver is found to be stronger in view of oxidative stress than the other tissues with the highest SOD and CAT activities (Heath, 1987; Schlenk and Benson, 2001; Atli *et al.*, 2006). This could be related to the fact that the liver is the site of multiple oxidative reactions and maximal free radical generation; therefore liver tissue was thought to be the best to present the response of CAT activity to metal exposure (Hidalgo *et al.*, 2002; Gül *et al.*, 2004; Avci *et al.*, 2005). Antioxidant defense enzymes such as CAT and SOD have a remarkable importance for aquatic organisms because these enzymes protect them from free radicals that cause oxidative stress. The present results showed that SOD activity generally decreased though CAT activity increased. CAT was also found to be the most sensitive antioxidant enzyme when compared to the others**.** The increase in CAT activity may be related to cope with the increased oxidative stress caused by metal exposures, while the decrease may be related to possible direct binding of metal ions to -SH groups on the enzyme molecule. Higher CAT activity was also recorded in different fish species after Cu and Cd exposures (Basha and Rani, 2003; Dautremepuits *et al.*, 2004; Sanchez *et al.*, 2005). Atli *et al.* (2006) demonstrated the significant alterations in CAT activity both *in vivo* and *in vitro* in different tissues of *O. niloticus* after acute and chronic metal exposures. Sensitivity of SOD and CAT activities to metal exposures were also supported with our previous results (Atli and Canli, 2008, 2010). Decreased SOD activity might be an indicator of damage in the antioxidant mechanisms caused by metal exposure and water hardness. Barata *et al.* (2005) also found variations in SOD and CAT activity in *Daphnia magna* after Cd and Cu exposures depending upon metal concentrations. They concluded that toxicants may induce different antioxidant/prooxidant responses depending on their ability to produce ROS. The response of the antioxidant system could differ when organisms exposed to metals and some other factors. For instance, (Garcia Sampaio *et al.*, 2008) showed that single-factor Cu exposure was found to be insufficient to decrease the SOD activity in fish (*Piaractus mesopotamicus*) whereas under hypoxia and combined-factors of hypoxia Cu led a significant decrease in its activity. This situation supports the present data which showed decreases in the activity in SW+metal groups compared to HW+metal groups, indicating enhanced metal toxicity in soft water. Vega-Lopez *et al.* (2008) demonstrated the depressed SOD and increased CAT activity in the liver of fish *Girardinichthys viviparous* obtained from the lakes in Mexico with different habitats. Their results also indicated that the CAT was the main antioxidant defense enzyme. This SOD-CAT activity trend was also recorded in the present study in detoxification of ROS generated by several toxicants including metals. The SOD-CAT system, the first line of defense system against oxidants varied according to the response of fish antioxidant system to counteract with the toxicity of hardness and metal exposures (Basha and Rani, 2003; Vutukuru *et al.*, 2006; Garcia Sampaio *et al.*, 2008; Atli and Canli, 2010).

It was suggested that GSH content showed both increases and decreases in fish tissues exposed to metals due to their organ-specific responses (Berntssen *et al.*, 2000; Sayeed *et al.*, 2003; Atli and Canli, 2008). The increase of hepatic GSH level in fish is probably due to defense system to protect the fish from the oxidative stress. It was also shown that Cd and Cu in different concentrations caused significant GSH level increases in the liver of *O. niloticus* in our previous research (Atli and Canli, 2008). However, in SW+Cd exposure total GSH decreased compared to HW+Cd exposure due to the enhanced Cd toxicity in soft water. Decreased GSH level induced by metals could be depending upon the GSH binding to metals to eliminate the toxic effects (Elia *et al.*, 2003). Lima *et al.* (2006) indicated that increased GSH levels in *O. niloticus* exposed to a contaminated effluent appear to be an antioxidant adaptation to chronic exposure. On the other hand, the decreased GSH levels in the gill and kidney of *Anguilla anguilla* exposed to Cu were related to the increased use of GSH to stabilize Cu in its oxidative stress for preventing the redox cycling and free radicals regeneration (Ahmad *et al.*, 2005). They concluded that GSH first made a rapid protection against oxidative stress via GSH redox cycle or directly detoxifying the ROS generated by oxidative stress (Barata *et al.*, 2005; Ruas *et al.*, 2008). Experimental factors such as time of exposure and dose or environmental factors such as quality of water should be taken into account for possible antioxidant system responses to oxidative stress caused by pollutants (Monserrat *et al.*, 2007; Baysoy *et al.*, 2012; Alak *et al.*, 2013).

The data from the present study showed that activity of GPX showed decreasing trend and it was the most responsive GSH dependent antioxidant enzyme, but it was less affected than CAT. This could be due to the high hydrogen peroxide concentration which associates with CAT activity (Pinto *et al.*, 2003; Trenzado *et al.*, 2006). GPX activity can be also considered complementary to CAT activity that was also supported with the present data. Garcia Sampaio *et al.* (2008) also emphasized the secondary capacity to decompose the peroxides by GPX than

CAT in the liver of *P. mesopotamicus* after the exposure of hypoxia and Cu. Decreasing trend in GPX activity could be attributed to the direct effects of metal ions on the active site of enzyme molecules. Orun *et al.* (2008) also indicated the significant alterations in GPX activity together with SOD and CAT activities in the tissues of fish *Onchorhynchus mykiss* after Cd and Cr exposures. In addition, the effects of soft water were also remarkable for these enzyme activities in this study. One can conclude that differences in hardness of water could also trigger the antioxidant enzyme responses.

In this study, GR activity exhibited little variation in soft water media compared to other active antioxidant enzymes. Reduction of GR activity may result in GSH depletion if extra synthesis of GSH cannot occur to protect its redox status, as a consequence of the prooxidative effects. Additionally, enhancement of GR activity could occur due to re-establishment of the GSH levels that is oxidized (Atli and Canli, 2010). Fei *et al.* (2011) suggested that the response of GR in different tissues of fish *Pampus argenteus* was characterized by tissue specificity and time sequence during the salinity exposures. Nevertheless, GR activity did not alter except for a few cases in the present study. This result could be attributed to the significant changes in CAT, SOD, GPX activities and GSH levels thus we can conclude that sufficient response was provided with these antioxidant parameters that are appeared to be act as an important defense element.

In this study, a decreased in GST activity of *O. niloticus* was recorded only after 7 day Cu exposure in SW condition in contrast to HW+Cu exposure, though there were not so much responses in GST activity to metal exposures in waters with different hardness, similar with GR activity. Decreased GST activity could also be due to compensate the ROS impact on antioxidant system with other antioxidant enzymes. Dautremepuits *et al.* (2004) observed a decrease in antioxidant enzyme activity in the liver of *Cyprinus carpio* exposed to Cu and they indicated that excess Cu causes a rapid GSH oxidation even at low non toxic Cu concentrations in hepatocytes followed by GST depletion. However, it was also interesting to see that total GSH level was not altered in where alterations in GST activity occurred.

Conclusion

The antioxidant enzymes are of great importance against the effects of metals and hardness in fish. Data from the present study showed that water hardness alone and with metal combination affects the response of antioxidant enzymes, possibly due to affecting fish physiology. Cu caused fish mortality after 8th day in soft water, though there was no fish mortality in hard water, suggesting the higher bioavailability of Cu in soft water. Likewise, the antioxidant system of fish was more influenced by metal exposures in soft water, as the hepatic antioxidant enzymes activity decreased. The present study stressed the importance of water quality (hardness) on determination of metal toxicity. This study could be beneficial in ecotoxicological researches in freshwaters as it provides data about antioxidant system response of fish exposed to metal in waters with different hardness. Nevertheless, further researches are necessary to shed more light on characterization of biological responses such as antioxidant enzymes as sensitive biomarkers in fish.

Acknowledgements

This study was supported by a grant of FEF 2010YL6 from Çukurova University.

References

Ahmad, I., Oliveira, M., Pacheco, M., and Santos, M.A. 2005. *Anguilla anguilla* L. oxidative stress biomarkers responses to copper exposure with or without β-naphthoflavone pre-exposure. Chemosphere, 61: 267–275. doi:10.1016/j.chemosphere.2005.01.069.

Alak, G., Atamanalp, M., Topal, A., Arslan, H., Oruç, E., and Altun, S. 2013. Histopathological and biochemical effects of humic acid against cadmium toxicity in brown trout gills and muscles. Turkish Journal of Fisheries and Aquatic Sciences, 13: 315-320. doi: 10.4194/1303-2712-v13_2_13.

Atli, G., and Canli, M. 2003. Natural occurrence of metallothionein-like proteins in the liver of fish *Oreochromis niloticus* and effects of cadmium, lead, copper, zinc, and iron exposures on their profiles. Bulletin of Environmental Contamination and Toxicology, 70: 619–627. doi: 10.1007/s00128-003-0030-4.

Atli, G., Alptekin, Ö., Tükel, S., and Canli, M. 2006. Response of catalase activity to $Ag^{+,}$ Cd^{2+}, Cr^{6+}, Cu^{2+} and Zn^{2+} in five tissues of freshwater fish *Oreochromis niloticus*. Comparative Biochemistry and Physiology, 143C: 218–224. doi:10.1016/j.cbpc.2006.02.003.

Atli, G., and Canli, M. 2007. Enzymatic responses to metal exposures in a freshwater fish *Oreochromis niloticus*. Comparative Biochemistry and Physiology, 145C: 282–287. doi: 10.1016/j.cbpc.2006.12.012.

Atli, G., and Canli, M. 2008. Responses of metallothionein and reduced glutathione in a freshwater fish *Oreochromis niloticus* following metal exposures. Environmental Toxicology and Pharmacology, 25: 33–38. doi:10.1016/j.etap.2007.08.007.

Atli, G., and Canli, M. 2010. Response of antioxidant system of freshwater fish *Oreochromis niloticus* to acute and chronic metal (Cd, Cu, Cr, Zn, Fe) exposures. Ecotoxicology and Environmental Safety, 73: 1884-1889. doi:10.1016/j.ecoenv.2010.09.005.

Avci, A., Kaçmaz, M., and Durak, I. 2005. Peroxidation in muscle and liver tissues from fish in a contaminated river due to a petroleum refinery industry. Ecotoxicology and Environmental Safety, 6: 101–105. doi:10.1016/j.ecoenv.2003.10.003.

Ay, Ö., Kalay, M., Tamer, L., and Canli, M. 1999. Copper and lead accumulation in tissues of a freshwater fish *Tilapia zillii* and its effects on the branchial Na, K-ATPase activity. Bulletin of Environmental

Contamination and Toxicology, 62: 160–168.

Barata, C., Varob, I., Navarro, J.C., Arun, S., Porte, C. 2005. Antioxidant enzyme activities and lipid peroxidation in the freshwater cladoceran *Daphnia magna* exposed to redox cycling compounds. Comparative Biochemistry and Physiology, 140: 175–186. doi:10.1016/j.cca.2005.01.013.

Basha, P.S., and Rani, A.U. 2003. Cadmium-induced antioxidant defense mechanism in freshwater teleost *Oreochromis mossambicus* (Tilapia). Ecotoxicology and Environmental Safety, 56: 218–221. doi:10.1016/S0147-6513(03)00028-9.

Baysoy, E., Atli, G., Gürler, C.Ö., Dogan, Z., Eroglu, A., Kocalar, K., and Canli, M. 2012. The effects of increased freshwater salinity in the biodisponibility of metals (Cr, Pb) and effects on antioxidant systems of *Oreochromis niloticus*. Ecotoxicology and Environmental Safety, 84: 249–253. doi:10.1016/j.ecoenv.2012.07.017.

Berntssen, M.H.G., Lundebye, A.K., and Hamre, K. 2000. Tissue lipid peroxidative responses in Atlantic salmon (*Salmo salar* L.) parr fed high levels of dietary copper and cadmium. Fish Physiology and Biochemistry, 23: 35-48.

Bessey, O.A., Lowry, O.H., and Brock, M.J. 1946. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. The Journal of Biological Chemistry, 164: 321–329.

Beutler, E., Duron, O., and Kelly, B.M. 1963. Improved method for the determination of blood glutathione. The Journal of Laboratory and Clinical Medicine, 61: 882–890.

Blanchard, J., and Grosell, M., 2006. Copper toxicity across salinities from freshwater to seawater in the euryhaline fish *Fundulus heteroclitus*: Is copper an ionoregulatory toxicant in high salinities? Aquatic Toxicology, 80: 131-139. doi:10.1016/j.aquatox.2006.08.001.

Bury, N.R., Shaw, J., Glover, C., and Hogstrand, C. 2002. Derivation of a toxicity-based model to predict how water chemistry influences silver toxicity to invertebrates. Comparative Biochemistry and Physiology, 133: 259–270. doi:10.1016/S1532-0456(02)00096-0.

Carlberg, I., and Mannervik, B. 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. The Journal of Biological Chemistry, 250: 5475–5480.

Dautremepuits, C., Paris-Palacios, S., Betoulle, S., and Vernet, G. 2004. Modulation in hepatic and head kidney parameters of carp (*Cyprinus carpio* L.) induced by copper and chitosan. Comparative Biochemistry and Physiology, 137: 325–333. doi:10.1016/j.cca.2004.03.005.

De Boeck, G., Smolders, R., and Blust, R. 2010. Copper toxicity in gibel carp *Carassius auratus gibelio*: Importance of sodium and glycogen. Comparative Biochemistry and Physiology, 152C: 332–337. doi:10.1016/j.cbpc.2010.05.008.

Dewez, D., Geoffroy, L., Vernet, G., and Popovic, R. 2005. Determination of photosynthetic and enzymatic biomarkers sensitivity used to evaluate toxic effects of copper and fludioxonilin alga *Scenedesmus obliquus*. Aquatic Toxicology, 74: 150–159. doi: 10.1016/j.aquatox.2005.05.007.

Elia, A.C., Galarini, R., Taticchi, M.I., Dörr, A.J.M., and Mantilacci, L. 2003. Antioxidant responses and bioaccumulation in *Ictalurus melas* under mercury exposure. Ecotoxicology and Environmental Safety, 55: 162–167. doi:10.1016/S0147-6513(02)00123-9.

Ercal, N., Gurer-Orhan, H., Aykin-Burns, N. 2001. Toxic metals and oxidative stress part I: mechanisms involved in induced oxidative damage. Current Topics in Medical Chemistry, 1: 529–539. doi: 10.2174/1568026013394831.

Ezemonye, L.I.N. and Enuneku, A.A. 2011. Biochemical alterations in *Hoplobatrachus occipitalis* exposed to sublethal concentrations of cadmium. Turkish Journal of Fisheries and Aquatic Sciences, 11: 485-489. doi: 10.4194/1303-2712-v11_3_21.

Fei, Y., Shiming, P., Peng, S., and Zhaohong, S. 2011. Effect of low salinity on digestive enzyme activities in the intestinal tract of juvenile silver pomfret *Pampus argenteus*. Acta Ecologica Sinica, 31: 55-60. doi: 10.1016/j.chnaes.2010.11.009.

Garcia Sampaio, F.G., Boijink, C.L., Oba, E.T., Santos, L.R.B., Kalinin, A.L., and Rantin, F.T. 2008. Antioxidant defenses and biochemical changes in pacu (*Piaractus mesopotamicus*) in response to single and combined copper and hypoxia exposure. Comparative Biochemistry and Physiology, 147: 43–51. doi:10.1016/j.cbpc.2012.07.002.

Grosell, M., Nielsen, C., and Bianchini, A. 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comparative Biochemistry and Physiology, 133: 287-303. doi: 10.1016/S1532-0456(02)00085-6.

Gul, S., Belge-Kurutas, E., Yıldız, E., Sahan, A., and Doran, F. 2004. Pollution correlated modifications of liver antioxidant systems and histopathology of fish (Cyprinidae) living in Seyhan Dam Lake, Turkey. Environmental International, 30: 605–609. doi: 10.1016/S0160-4120(03)00059-X.

Habig, W.H., Pabst, M.J., and Jakoby, W.B. 1974. Glutathione *S-transferase*s. The first enzymatic step in mercapturic acid formation. Biological Chemistry, 249: 7130–7139.

Heath, A.G. 1987. Water Pollution and Fish Physiology. CRC Press, Florida, USA.

Hidalgo, M.C., Exposito, A., Palma, J.M., Higuera, M. 2002. Oxidative stress generated by dietary Zn-deficiency: Studies in rainbow trout (*Oncorhynchus mykiss*). The International Journal of Biochemistry & Cell Biology, 34: 183–193. doi: 10.1016/S1357-2725(01)00105-4.

Kulac, B., Atli, G., Canli, M. 2012. Investigations on the ATPase activities and cadmium uptake in freshwater fish *Oreochromis niloticus* following exposures to cadmium in ıncreased salinity. Turkish Journal of Fisheries and Aquatic Sciences, 12: 861-869. doi:10.4194/1303-2712-v1241_14.

Lauren, D.J, McDonald, D.G. 1987. Acclimation to copper by rainbow trout, *Salmo gairdneri*: biochemistry. Canadian Journal of Fisheries and Aquatic Science, 44: 105–111.

Lima, P.L., Benassi, J.C., Pedrosa, R.C., Dal Magro, J., Oliveira, T.B., Wilhelm Filho, D. 2006. Time-course variations of DNA damage and biomarkers of oxidative stress in tilapia (*Oreochromis niloticus*) exposed to effluents from a Swine industry. Archives of Environmental Contamination and Toxicology, 50: 23–30. doi: 10.1007/s00244-004-0178-x.

Livingstone, D.R., Lips, F., Martinez, P.G., and Pipe, R.K. 1992. Antioxidant enzymes in the digestive gland of

the common mussel *Mytilus edulis*. Marine Biology, 112: 265–276.

Lowry, O.H., Rosebrough, N.J., Farra, N.J., Randall, R.J. 1951. Protein measurements with the folin phenol reagent. The Journal of Biological Chemistry, 193: 265–275.

Martinez-Alvares, R.M., Morales, A.E., Sanz, A. 2005. Antioxidant defenses in fish: Biotic and abiotic factors. Reviews in Fish Biology and Fisheries, 15: 75-88. doi: 10.1007/s11160-005-7846-4.

McCord, J.M., Fridovich, I. 1969. Superoxide dismutase: An enzymatic function for erythrocuprein (hemocuprein). The Journal of Biological Chemistry, 244: 6049–6055.

Monserrat, J.M., Martinez, P.E., Geracitano, L.A., Amado, L.L., Martins, C.M.G., Pinho, G.L.L., Chaves, I.S.C., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A. 2007. Pollution biomarkers in estuarine animals: critical review and new perspectives. Comparative Biochemistry and Physiology, 146: 221–234. doi:10.1016/j.cbpc.2006.08.012.

Nagalakshmi, N., Prasad, M.N.V. 1998. Copper-induced oxidative stres in *Scenedesmus bijugatus*: protective role of free radical scavengers. Bulletin of Environmental Contamination and Toxicology, 61: 623–628.

Orun, I., Selamoglu, Talas, Z., Ozdemir, I., Alkan, A., Erdogan, K. 2008. Antioxidative role of selenium on some tissues of (Cd^{2+}, Cr^{3+})-induced rainbow trout. Ecotoxicology and Environmental Safety, 71: 71-75. doi:10.1016/j.ecoenv.2007.07.008.

Pinto, E., Sigaud-Kutner, T.C.S., Leitao, M.A.S., Okamoto, O.K., Morse, D., and Colepicolo, P. 2003. Heavy metal-induced oxidative stress in algae. Journal of Phycology, 39: 1008–1018. doi: 10.1111/j.0022-3646.2003.02-193.x.

Radi, A.A.R., and Matkovics, B. 1988. Effects of metal ions on the antioxidant enzyme activities, protein contents and lipid peroxidation of carp tissues. Comparative Biochemistry and Physiology, 90C: 69–72.

Romeo, M., Bennani, N., Gnassia-Barelli, M., Lafaurie, M., and Girard, J.P. 2000. Cadmium and copper display different responses towardsoxidative stress in the kidney of the sea bass *Dicentrarchus labrax*. Aquatic Toxicology, 48: 185–194.

Ruas, C.B.G., Carvalho, C.S., Araujo, H.S.S., Espindola, E.L.G., and Fernandes, M.N. 2008 Oxidative stress biomarkers of exposure in the blood of cichlid species from a metal-contaminated river. Ecotoxicology and Environmental Safety, 71: 86–93. doi:10.1016/j.ecoenv.2007.08.018.

Sanchez, W., Palluel, O., Meunier, L., Coquery, M., Porcher, J.M., and Ait-Aissa, S. 2005 Copper-induced oxidative stress in three-spined stickleback: relationship with hepatic metal levels. Environmental Toxicology and Pharmacology, 19: 177–183. doi:10.1016/j.etap.2004.07.003.

Sayeed, I., Parvez, S., Pandey, S., Bin-Hafeez, B., Haque, R., and Raisuddin, S. 2003. Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, *Channa punctatus*. Bloch. Ecotoxicology and Environmental Safety, 56: 295–301. doi:10.1016/S0147-6513(03)00009-5.

Schlenk, D., and Benson, W.H. 2001. Target organ toxicity in marine and freshwater teleosts. Taylor and Francis, London.

Srikanth, K., Pereira, E., Duarte, A.C., and Ahmad, I. 2013. Glutathione and its dependent enzymes' modulatory responses to toxic metals and metalloids in fish—a review. Environmental Science and Pollution Research International, 20: 2133-2149. doi:10.1007/s11356-012-1459-y.

Trenzado, C., Hidalgo, C.H., Garcia-Gallego, M., Morales, A.E., Furne, M., Domezain, A., Domezain, J., Sanz, A. 2006. Antioxidant enzymes and lipid peroxidation in sturgeon *Acipenser naccarii* and trout *Onchorhynchus mykiss*. A comparative study. Aquaculture, 254: 758-767. doi:10.1016/j.aquaculture.2005.11.020.

Tripathi, B.N., Mehta, S.K., Amar, A., Gaur, J.P. 2006. Oxidative stress in *Scenedesmus* sp. during short-and long-term exposure to Cu^{2+} and Zn^{2+}. Chemosphere, 62: 538–544. doi:10.1016/j.chemosphere.2005.06.031.

USEPA, 2001. Update of ambient water quality criteria for cadmium, EPA-822-R-01–001. Washington, DC, USA.

USEPA, 2007. Aquatic life ambient freshwater quality criteria for copper, EPA-822-R-07-001. Washington, DC, USA.

Vega-Lopez, A., Jimenez-Orozco, F.A., Garcia-Latorre, E., Dominguez-Lopez, M.L. 2008. Oxidative stress response in endangered goodeid fish (*Girardinichthys viviparus*) by exposure to water from its extant localities. Ecotoxicology and Environmental Safety, 71: 94-103. doi:10.1016/j.ecoenv.2007.10.031.

Vutukuru, S.S., Chintada, S., Madhavi, K.R., Rao, J.V., Anjaneyulu, Y. 2006. Acute effects of copper on superoxide dismutase, catalase and lipid peroxidation in the freshwater teleost fish, *Esomus danricus*. Fish Physiology and Biochemistry, 32: 221-229. doi: 10.1007/s10695-006-9004-x.