Marine and Freshwater Behaviour and Physiology, 2014
Vol. 47, No. 4, 253–264, http://dx.doi.org/10.1080/10236244.2014.926077

Taylor & Francis
Taylor & Francis Group

The effect of sub-lethal exposure to copper and the time course of recovery in clean water on biochemical changes in juvenile fish (*Acipenser persicus*)

Saeed Zahedi[a]*, Alireza Mirvaghefi[b], Maryam Rafati[c], Gholamreza Rafiee[b], Bagher Mojazi Amiri[b], Mehdi Hedayati[d], Changiz Makhdoomi[e] and Musa Zarei Dangesaraki[e]

[a]*Faculty of Marine and Atmospheric Sciences and Technologies, Department of Fisheries Sciences, University of Hormozgan, Bandar Abbas, Iran;* [b]*Faculty of Natural Resources, Department of Fisheries and Environmental Sciences, University of Tehran, Karaj, Iran;* [c]*Department of Natural Resources, Savadkooh Branch, Islamc Azad University, Savadkooh, Iran;* [d]*Obesity Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran;* [e]*Shahid Rajaee Sturgeon Hatchery Center, Sari, Iran*

(*Received 31 March 2012; accepted 17 May 2014*)

This study reports the 96-h LC_{50} value and tissue copper (Cu) levels and biochemical changes in juvenile fish (*Acipenser persicus*) exposed to 0.026 mg/l ambient Cu for 1, 7 and 14 days. It then examined the recovery of the same parameters after placing the juvenile fish in clean water for a further period of 28 days. The intestine, kidney and gill Cu levels, plasma glucose, total protein, triglyceride, cortisol, triiodothyronine and thyroxine concentrations, liver protein contents, liver catalase, superoxide dismutase (SOD) and glutathione S-transferase activities were studied. The 96-h LC_{50} value of Cu was 0.502 mg/l for juvenile *A. persicus*. The results indicate that Cu exposure produced significant accumulations of Cu in gills and kidney over the treatment time. Sublethal dose of Cu resulted in a short-term increase in plasma glucose, total protein and cortisol levels that decreased with time. After the 28-day recovery phase, there were significant differences in kidney Cu levels and triglyceride concentrations as well as SOD activities between recovery fish treatments and their control groups on day 42. The 28-day recovery phase caused significant decreases in total protein levels and SOD activities of Cu-exposed fish on day 42 compared to day 14. The results suggest that 28 days are insufficient for complete recovery to Cu exposure by juveniles and a longer period would be required for full recovery. Moreover, the study showed that the recovery phase following Cu exposure could change biochemical parameters to levels that are not close to those seen during exposure or control levels.

Keywords: copper; toxicity; recovery; biochemistry; juvenile; sturgeon; *Acipenser persicus*

Introduction

Copper (Cu) is an essential micronutrient for living systems but it is toxic in excess concentrations (Wood 2012). Cu is broadly dispersed at the sublethal concentrations in aquatic bodies (Harrison 1986; De Mora et al. 2004). Exposure of fish to aqueous Cu leads

*Corresponding author. Email: szahedi.phd@hormozgan.ac.ir

to its bioaccumulation (Shaw et al. 2012), disruption of ion homeostasis (McGeer et al. 2000a), histopathologic changes (Al-Bairuty et al. 2013) and an alteration of stress-related parameters (Gagnon et al. 2006). In addition, Cu exposure causes oxidative stress (Pretto et al. 2014) and then, antioxidant enzymes could be used as sensitive biomarkers to test water for the presence of Cu toxicity (Atli & Canli 2007). Catalase (CAT) and superoxide dismutase (SOD) enzymes protect fish against oxidative stress by converting the reactive oxygen species to less reactive ones (Winston & Di Giulio 1991). Moreover, glutathione S-transferase (GST) that catalyzes the conjugation of glutathione with a variety of electrophilic metabolites involves in the detoxification process (Varo et al. 2007).

Anadromous fishes spend short period of their life especially larval and juvenile stages in rivers and estuaries before entering to brackish/marine waters. However, numerous investigations have documented the existence of metal pollution in rivers and estuaries (Axtmann & Luoma 1991; He et al. 1998; Koukal et al. 2004; Ridgway & Shimmield 2002). As long as juveniles of anadromous fish pass through these polluted riverine and coastal waters during their downstream migration, they presumably experience transient doses of a metal(s) or their mixtures will consequently increase the risk of metal toxicity in such stressful environments. Juveniles are characteristically more sensitive than adults to contamination; they have higher weight specific metabolic rate and thus, can accumulate larger quantities of metals per unit of volume (Farkas et al. 2003). It might probably occur in southern Caspian Sea basin because low concentrations of metals have been reported in water and sediments of rivers, estuaries and coastal zones (De Mora et al. 2004; Charkhabi et al. 2005; Saeedi & Karbassi 2006; Parizanganeh et al. 2008).

Persian sturgeon, *Acipenser persicus,* is an anadromous species with great conservational, ecological and commercial value in the Caspian Sea basin (Bahmani et al. 2001) and its juveniles migrate from freshwaters of the rivers to brackish water of the Caspian sea during downstream migration. Juvenile *A. persicus* is therefore an appropriate native species for short-term Cu toxicity assays. Since the contamination logically decreases with distance from the coast, juvenile would presumably clear this contamination in the deep sea. Little is presently known about the effect of a short-term Cu exposure on anadromous fish juveniles at the level of biochemical changes and, more importantly, subsequent recovery in clean water or indeed, the time required for the complete recovery process. Knowledge of the time required for complete recovery of fish to pre-exposure levels would be helpful to understanding their health (Sampath et al. 1998).

The purpose of the present investigation was to study the effects of a single, sublethal Cu exposure (14 days) on juvenile sturgeon at an environmentally relevant concentration (Varedi et al. 2010) and the subsequent recovery in Cu-free water (28 days). We firstly investigated median acute toxicity of Cu for finding a sublethal concentration. Second, we investigated the effects of sublethal Cu exposure on its Cu bioaccumulation and biochemical responses under laboratory conditions. Third, we studied the subsequent recovery phase to find out when and to what extent juveniles would recover from short-term exposure. To this purpose, we studied tissue metal bioaccumulations, blood/tissue stress-related biochemical responses and liver antioxidant enzyme activities as well as gill ions on days 1, 7, 14 and subsequent 28-day recovery on day 42.

Materials and methods

Fish

Persian sturgeon juveniles (body weight: 130 ± 19 g; body length: 34.2 ± 2.1 cm (mean ± SD); age: 14 months) were obtained from the Ecology Faculty of the Caspian Sea

(Sari, Mazandaran, Iran) and transferred to the laboratory of Shahid Rajaee Sturgeon Hatchery Center (Sari, Mazandaran, Iran) in mid-June, 2008. Fish stocked at 2000 l fish tanks under laboratory conditions with natural photoperiod (15 L, 9 D) for at least four weeks before exposure. The tanks were aerated with air stones attached to an air compressor to saturate the water with oxygen. Fish were then transferred from the stock tanks to experimental ones for acute and sublethal tests as well as subsequent recovery in Cu-free water. The differences in weights of the fish used in the experiments were not significant. The fish were fed 3% of body weight once daily in the morning (at 9:00–9:30 h).

Exposures and recovery

Copper stock solution was prepared by dissolving copper sulphate ($CuSO_4 \cdot 5H_2O$, Merck, Germany) in 1 l of double-deionized water. The stock solution was held at 4 °C until needed. Stock solution was diluted to the desired concentration with tank water prior to each experiment. The measured physicochemical characteristics of experimental water were: temperature 22.3 ± 0.73 °C, dissolved oxygen 7.7 ± 0.33 mg/l, pH 7.8 ± 0.2 and hardness 278 ± 6.4 mg $CaCO_3$/l. No alterations were observed in water quality parameters with metal contamination. Water Cu concentrations were monitored daily by inductively coupled plasma optical emission spectrometer (ICP-OES, GBC, Integra XL) and showed that actual Cu concentrations were stable between two water changes and throughout the sublethal exposure and they were 0.002 ± 0.0 and 0.0239 ± 0.003 mg/l ($n = 56$) for control and experimental mediums, respectively.

A static bioassay test was performed to determine the 96-h LC_{50} of Cu to *A. persicus*, following the Organization for Economic Cooperation and Development protocol No. 203. Seven healthy fish were randomly placed in the solution prepared for each concentration in three replicates. The fish were starved for 24 h prior to and during the course of the experiment. Fish were exposed to nominal concentrations of 0 (control), 0.05, 0.25, 0.45, 0.65, 0.85 and 1.05 mg/l of Cu for definitive tests. The mortality of fish was recorded for 0, 24, 48 and 96 h after exposure to Cu. The data were used to find the median lethal concentration (LC_{50}) adopting Prohibit analysis with the Statistical Analysis System, SAS.

In sublethal tests, juveniles (eight fish/tank, four replicates) were exposed to a sublethal concentration of 0.026 mg Cu/l (5% of 96-h LC_{50}) for 1, 7 and 14 days as semi-static conditions in 1000 l of contaminated test solutions or non-contaminated water as a control. Water (90%) was replaced with fresh medium just after feeding to minimize metal loss and to reduce the water contamination by food remains. For the recovery experiment, both remaining Cu-exposed and control fish were immediately held in Cu-free water for further 28 days as recovery and control treatments, respectively (seven fish/tank, two replicates) and sampled on day 42 of the experiment. During sublethal exposure and subsequent recovery, fish were fed daily at 3% of body weight and then, they were starved for 24 h prior to sampling to avoid prandial effects (Basha & Rani 2003).

Sampling

On sampling days (1, 7, 14 and 42), six fish were removed randomly from each treatment and immediately anaesthetized in clove-essence solution (at 9:00–9:30 h). After equilibrium was lost, fish were removed, measured and weighed. Blood

samples were taken from the caudal vein by means of heparinized capillaries and held on ice until centrifugation. Immediately after blood collection, liver, intestine, kidney and gill tissues were dissected, rinsed by physiological serum, weighed, frozen in liquid nitrogen and stored at −80 °C until further analysis. Blood samples were centrifuged at 10,000 rpm for 3 min (+4 °C) to obtain plasma, aliquoted and stored in −20 °C. The livers were homogenized by homogenizer (TRI-I instrument, England) in 100 mM phosphate buffer (pH 7.4, 1:10, *w*/*v*) containing 2 mM EDTA and 150 KIU/ml aprotinin as protease inhibitors. Homogenates were centrifuged at 10,000 rpm (Beckman, Avanti^{TM} 30, USA) for 45 min (+4 °C) and supernatant was used as an enzyme source.

Analysis

For metal ion determination, samples of intestine, kidney and gills were dry-ashed in silica vessels according to Cinier et al. (1999). The ashed samples were dissolved in 2 ml 1:1 mixture of 65% super pure nitric acid (Merck, Germany) and hydrochloric acid 37% (Fluka chemika, Switzerland). The resulting solutions were subsequently diluted to 10 ml with ultra-pure water, filtered (0.22 µ Cellulose acetate, Sandic, S&S, Germany) and kept in a refrigerator until analyzed for metals using ICP-OES.

The glucose and triglycerides levels in plasma samples were measured with enzymatic colorimetric assay kits and total protein levels with a chemical colorimetric assay kit (Pars Azmoon, Tehran, Iran). Plasma cortisol, T_3 and T_4 were assayed with commercial ELISA kits (Diagnostics Biochem Canada Inc, Ontario, Canada). Liver CAT (EC.1.11.1.6) and SOD (SOD, EC.1.15.1.1) activities were measured using colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing City, P.R. China) in microtiter plate format and using ELISA Reader (Sunrise, Tecan, Austria) for optical density recording. All the assays were performed according to manufacturer guidelines. One unit of enzyme activity is the amount of enzyme that catalyzes the oxidation of 1 µmol substrate per minute. The enzyme activities are expressed as U/mg protein. Liver GST (EC.2.5.1.18) activities were measured by monitoring the formation of the product of the reaction between GSH and 1-chloro-2, 4-dinitrobenzene (CDNB) at 340 nm (Habig et al. 1974). One unit of GST activity was calculated as µmol CDNB conjugate formed/min/mg protein at 25 °C. The solutions of glutathione (GSH) 50 mM in phosphate buffer 0.1 M, pH 6.5, and 1-chloro-2,4-dinitrobenzene (CDNB) 50 mM in ethanol were prepared just before the assay. The reaction mixture consisting of 100 µl of GSH solution and 100 µl of CDNB solution was added to 70 µl of the sample, and the GST activity was measured at 340 nm after 1 min incubation. The results were given as µmol/min/mg protein.

Statistical analysis

Statistical analyses of data were carried out using the SPSS statistical package program (ver. 17.0, SPSS Company, Chicago, IL, USA). The values are reported as mean ± SE. Student's *t* test was used to test the difference between control and treatment groups at each sampling time as well as between days 14 and 42. Data from different days of sublethal exposure were also compared by one-way analysis of variance and differences in the means were tested by Duncan's multiple range tests.

Results

Cu exposure effects

According to the acute tests, the Cu 96-h LC_{50} value for juvenile *A. persicus* was 0.502 mg/l. At the sublethal exposure (0.026 mg/l of Cu) and during the recovery phase, no mortality occurred. Behavioral and swimming patterns in control groups were normal but juveniles displayed some behavioral changes when subjected to the sublethal Cu concentration. The most important behavioral changes in sublethal Cu exposure were a slight elevation of excitement levels, sudden jerks and secretion of extra gill mucus. When approaching the fish tanks, some juveniles frequently showed the escape behavior and sometimes, one or two of them collided with a tank wall and then, they return to normal swimming. Some sort of restlessness was also apparent in the experimental groups.

The result of Cu concentrations in intestine, kidney and gills as a function of single sublethal Cu concentration and exposure time is shown in Figure 1. There were no significant changes in intestine Cu levels between treatments and after 1, 7 and 14 days of Cu exposure, and a classic time–response relationship was not apparent ($p > 0.05$). There were significant differences between the control and Cu-exposed groups in kidney and gills Cu concentrations on days 7 and 14 ($p < 0.05$). In addition, in Cu-exposed groups and on day 14, kidney Cu burdens were significantly higher compared to days 1 and 7 ($p < 0.05$) with the maximum average during experimentation (8.13 µg Cu/g wet tissue).

The effects of Cu on plasma glucose, total protein and triglyceride levels as well as liver protein contents are shown in Table 1. Plasma glucose levels appeared markedly elevated in Cu treatment fish only at first day of sampling compared to the

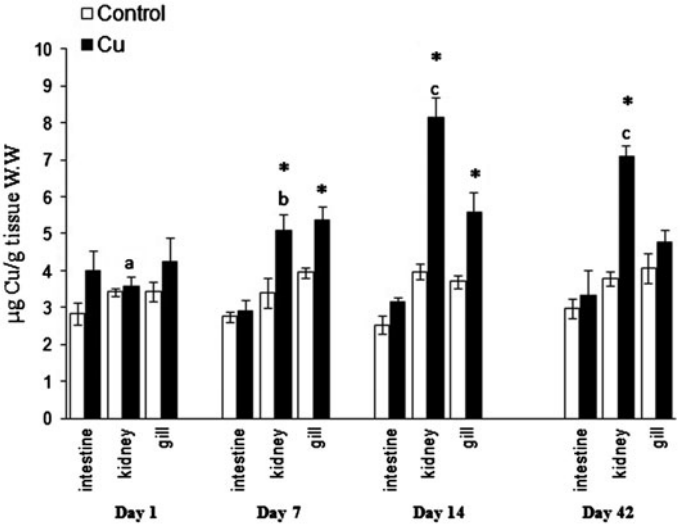

Figure 1. Changes in Cu concentrations (µg/g tissue wet weight (*w*/*w*)) of juvenile *A. persicus* exposed to 0.026 mg/l of Cu on days 1, 7 and 14 and subsequent recovery on day 42 (mean ± SE, $n = 5–6$). Significant differences among treatments in each sampling day were donated by asterisks ($p < 0.05$). Different letters indicate a significant difference among different days of sub-lethal exposure ($p < 0.05$).

Table 1. Changes in blood biochemical parameters in juvenile *A. persicus* exposed to a sub-lethal concentration of Cu (0.026 mg/l) on days 1, 7 and 14 and subsequent recovery on day 42.

	Control				Cu			
Parameters	Day 1	Day 7	Day 14	Day 42	Day 1	Day 7	Day 14	Day 42
Plasma glucose (mg/dl)	53 ± 2.01	41.33 ± 5.93	54 ± 3.21	51 ± 4.16	80.75 ± 6.54^{*b}	52.67 ± 5.99^{a}	50 ± 2.93^{a}	54.5 ± 3.62
Plasma protein (g/dl)	2.9 ± .058	2.2 ± 0.41	2.47 ± .54	2.26 ± 0.44	4.08 ± .14^{*b}	2.8 ± .26^{a}	2.75 ± .21^{a}	2.05±.17$^{\#}$
Plasma triglyceride (mg/dl)	329.3 ± 44.7	296 ± 25.15	319.67 ± 40.89	302.66 ± 14.88	552.75 ± 73.24^{b}	231.1 ± 34.05^{a}	266.88 ± 40.77^{a}	219 ± 20.96*
Liver protein (mg/mg)	0.126 ± 0.001	0.130 ± 0.003	0.137 ± 0.009	0.129 ± 0.011	0.135 ± 0.004	0.126 ± 0.005	0.126 ± 0.004	0.142 ± 0.006

Note: Data were analyzed by Student's *t*-test to compare the control and treatment groups at the same treatment time as well as between days 14 and 42, and by one-way ANOVA with Duncan comparisons for different days of sub-lethal exposure. Significant differences among treatments in each sampling day were donated by asterisks ($p < 0.05$). Different letters indicate a significant difference among different days of sub-lethal exposure and # symbol indicates differences between Cu-exposed versus recovery fish ($p < 0.05$). Data are presented as mean ± SE, $n = 5–6$.

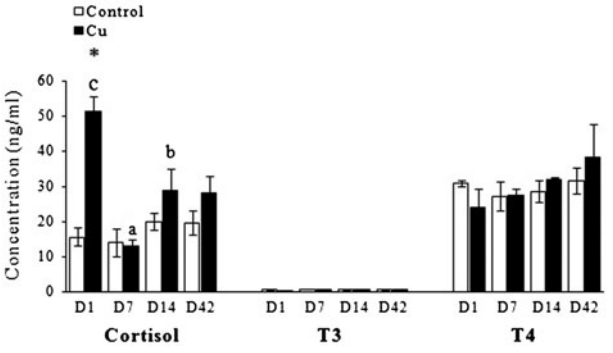

Figure 2. Plasma hormonal concentrations of juvenile *A. persicus* exposed to 0.026 mg/l of Cu on days 1, 7, 14 and subsequent recovery on day 42 (mean ± SE, $n = 5–6$). Significant differences among treatments in each sampling day were donated by asterisks ($p < 0.05$). Different letters indicate a significant difference among different days of sub-lethal exposure ($p < 0.05$).

control groups ($p < 0.05$). Also, a significant decrease was observed on days 7 and 14 in comparison to day 1 ($p < 0.05$). The highest mean plasma glucose level on day 1 was 80.75 mg/dl followed by declines at both the second and third sampling and reached 50 mg/dl on day 14. There was also a significant increase ($p < 0.05$) in plasma total protein concentration at Cu treatment compared to the control group on day 1 ($p < 0.05$). In addition, in Cu-exposed fish, plasma total protein concentration decreased significantly at the second and the third sampling compared to the first one ($p < 0.05$). During the experiment and with the Cu-exposed groups, plasma triglyceride levels decreased significantly compared with day 1, and reached their lowest level at the second stage of experimentation ($p < 0.05$). There were no significant differences in liver protein contents with sublethal dose or with the time course ($p > 0.05$).

On the first sampling, Cu caused significant increase in plasma cortisol concentrations in Cu-exposed fish compared to control groups (Figure 2, $p < 0.05$) and as time elapsed, significant decreases were observed at other sampling stages ($p < 0.05$). Neither T_3 nor T_4 levels and liver CAT or SOD activities changed significantly with sublethal dose or with the time course (Figures 2 and 3(A) and (B)). GST activity increased in experimental groups in comparison to the control groups but these differences were not statistically significant ($p > 0.05$, Figure 3(C)). Neither did a comparison between groups reveal significant differences in gill ion levels for calcium, potassium or sodium for two weeks ($p > 0.05$, Table 2).

Recovery phase effects

After the 28-day recovery phase, significant differences were shown in renal Cu concentrations between recovery groups and their control groups on day 42 ($p < 0.05$, Figure 1). Likewise, plasma triglyceride concentrations and hepatic SOD activities showed significant decreases between recovery treatments and their controls on day 42 ($p < 0.05$, Table 1 and Figure 3(B)). In addition, there were no significant differences in all measured biochemical factors in control groups between days 14 and 42 ($p > 0.05$).

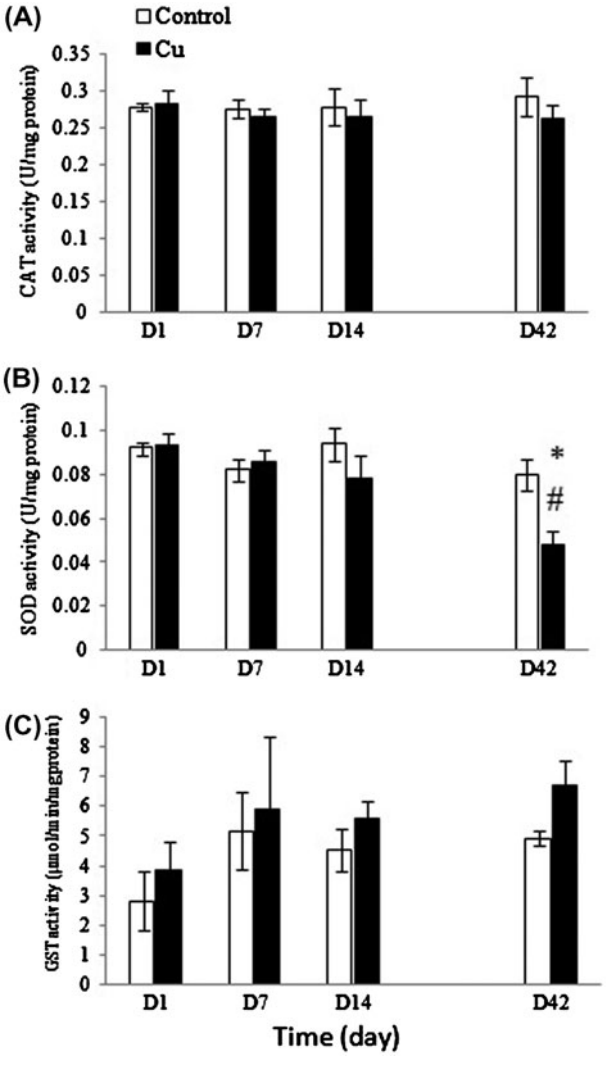

Figure 3. Liver CAT (A), SOD (B) (U/mg protein) as well as GST (C) activities (μmol/min/mg protein) of juvenile *A. persicus* exposed to 0.026 mg/l of Cu on days 1, 7, 14 and subsequent recovery on day 42 (mean ± SE, $n = 5–6$). Significant differences among treatments in each sampling day were donated by asterisks ($p < 0.05$). Different letters indicate a significant difference among different days of sub-lethal exposure and a # symbol indicates differences between Cu-exposed vs. recovery fish treatment ($p < 0.05$).

In contrast, the 28-day recovery period revealed significant decreases in two biochemical parameters in Cu-exposed fish. First, a significant decrease was observed in plasma total protein concentration of Cu-exposed fish on day 42 compared to day 14 ($p < 0.05$) and it reached its lowest level (2.05 ± 0.17 g/dl) during the course of the study (Table 1). Second, recovery phase caused a reduction in liver SOD activities. This became a statistically significant difference by day 42 compared to day 14 ($p < 0.05$, Figure 3(B)).

Table 2. Gill ion concentrations (μmol/g gill *w/w*) in juvenile *A. persicus* exposed to a sub-lethal concentration of Cu (0.026 mg/l) on days 1, 7 and 14 and subsequent recovery on day 42.

	Calcium		Potassium		Sodium	
Day	Control	Cu	Control	Cu	Control	Cu
1	32.07±1.91	35.12±3.48	31.82±1.57	27.31±1.08	82.98±0.79	63.299±4.4
7	48.11±6.83	63.97±17.32	29.86±.89	29.62±3.98	67.63±17.02	84.99±13.2
14	30.69±5.2	48.46±4.55	30.56±8.53	25.74±2.43	78.07±4.43	83.15±6.95
42	45.81±6.85	49.05±6.28	37.28±9.38	25.16±3.24	75.23±12.49	82.14±8.92

Note: Data were analyzed by Student's *t*-test to compare the control and treatment groups at the same treatment time as well as between days 14 and 42, and by one-way ANOVA with Duncan comparisons for different days of sub-lethal exposure. No significant differences were detected ($p < 0.05$). Data are presented as mean ± SE, $n = 5–6$.

Discussion

Cu exposure effects

A previous study showed that Cu had the lowest 96-h LC_{50} value among tested metals and it led to the highest toxicity for *A. persicus* (Mirzaei et al. 2004). Our LC_{50} values were higher because the water hardness, pH and fish age were different between the two studies, which might affect the calculated LC_{50} (Chen et al. 2012; Esbaugh et al. 2013).

Results obtained from previous experiments documented behavioral changes in fish exposed to sublethal metal concentrations (Scott & Sloman 2004; Vutukuru et al. 2005). Some of the reported changes were not observed here. The low concentration of Cu quite probably accounts for at least some of this, particularly since we observed no severe effects. The copious secretion of gill mucus and the restlessness of the juveniles during Cu exposure were, however, consistent with the previous report (Vutukuru et al. 2005).

On days 1 and 7, the highest Cu concentrations were detected in the gills, but on day 14, it was highest in the kidney. During waterborne metal exposure in the freshwater, the metals enter the fish body mainly through the gills as a temporary target organ for metal accumulation (Grosell et al. 1997; McGeer et al. 2000b). Thereafter, the metal is distributed to other internal organs (McGeer et al. 2000b; Wu et al. 2007). Accordingly, we observed redistribution of the Cu to the kidney so that the concentrations of Cu increased in the kidney over time and reached their highest levels on day 14. Thus, exposure of *A. persicus* juveniles to the 0.026 mg/l of Cu induced significant but differently distributed metal accumulations depending on the length of exposure period and the tissue.

Plasma glucose and total protein but not triglyceride and liver protein concentrations increased significantly on day 1 which indicates that severe stress response has produced during initial phase of Cu exposure. These responses return to normal values by day 7 suggest that juveniles are able to adapt themselves to new condition during time. Short-term increase in plasma cortisol levels was observed in Cu-exposed group on day 1, but it showed a significant decrease on day 7. Metal exposure can induce an osmo-ionic disturbance that elevates plasma cortisol levels. Cu exposure had no effect on T_3 or T_4 levels probably because the used concentration may be below the limit, which can influence thyroid function.

In this study, there were no significant differences in antioxidant enzyme activities. Fish can adapt physiologic conditions during pollution exposure by varying the

expression of the antioxidant enzymes. It is likely that the observed responses were related to binding of Cu to these proteins or converting of active enzyme compounds to inactive ones as described by others (Basha & Rani 2003; Wong & Whitaker 2003).

No significant differences were observed in gill ion contents of *A. persicus*. Generally, fish ionic regulation impairs during Cu exposure because of structural and functional damage to gills and activities of its chloride cell pumps (Pelgrom et al. 1995). The observed changes here may be related to physicochemical characteristics of the used water like its high hardness (278 ± 6.4 mg $CaCO_3$/l) that has probably affected Cu bioavailability in the medium and has mitigated gill metal uptake.

The selected concentration (0.026 mg/l of Cu) was physiologically stressful for juvenile *A. persicus*. It was verified by metabolic changes as part of a nonspecific stress response. However, the observed changes were rapid and transient following exposure and returned to control levels. This matter indicates that the juvenile fish might partly adapt themselves to the new environment in the laboratory. But, in natural environments like rivers and especially estuaries and coastal areas, other factors such as water chemistry can be vastly variable. This make them stressful environments for fishes that could lead to more sensitivity to toxicity and exacerbation of the stress of juvenile fish that exposed to elevated Cu levels especially in long-term exposures.

Recovery phase effects

Cu levels were significantly higher only in kidney but not in intestine and gills compared to their controls on day 42. Both gills and kidney participate in Cu excretion (Mazon & Fernandes 1999). The gill Cu level of experimental fish was a little higher than the controls compared to their kidney levels where it was near twofold greater than their controls on day 14. Thus, after a 28-day recovery period, gill Cu levels reached to the control levels because of clearance but kidney Cu levels could not be eliminated and might require further time. The decrease of plasma triglyceride can clarify the pivotal role of lipid sources in energetic expense during and after metal exposure that did not recover even after 28 days. Moreover, the observed decreases in SOD activity can be justified by mobilization of stored Cu from different tissues particularly osmoregulatory ones to liver and its effect on hepatic SOD activities.

Recovery period in Cu-exposed fish affected their plasma total protein levels and liver SOD activities on day 42 compared to day 14 demonstrating that the fish did not fully recover. Increasing in protein breakdown is a functional response to deal with the extra energy requirements during stress response (Reddy & Bhagyalakshmi 1994). We interpreted this response as compensatory and adaptive response to metal stress that activated restoration of some special functions by the cells and tissues leading to protein consumption.

In conclusion, based on a set of biomarkers measured in this study, a 28-day time course of recovery was not long enough for complete recovery in the laboratory and more time is probably required, and more importantly, recovery phase could change some biomarkers to the new levels, which are not necessarily near exposure or control ones. Thus, recovery phase responses are not predictable from the previous exposure period similar to what observed for SOD changes that it continues to decrease during recovery. On the other hand, the less contamination of deep sea compared to coastal area would possibly provide an opportunity for recovery of juveniles if the previous stresses were not severe and the required time has passed. Therefore, even low sublethal Cu concentration in high hardness and pH is effective on *A. persicus* that cannot recover even

over 28 days in Cu-free water and in the light of the results gained, we need to be more cautious about the protection of the natural resources against sublethal effects of metals.

Acknowledgments

We thank Dr Ehsan Shahriary and Dr Mohsen Navari for their insightful comments. We also express our deep sense of gratitude to Saeed Mahdavi Sahebi, Ahmad Eslamifar, Hossein Vaezzade, Maryam Mardane Khatooni and A. Ghasemi for their help during the course of this work.

References

Al-Bairuty GA, Shaw BJ, Handy RD, Henry TB. 2013. Histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trout (*Oncorhyncus mykiss*). Aquat Toxicol. 126:104–115.

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

Axtmann EV, Luoma SN. 1991. Large-scale distribution of metal contamination in the fine-grained sediments of the Clark Fork River, Montana, USA. Appl Geochem. 6:75–88.

Bahmani M, Kazemi R, Donskaya P. 2001. A comparative study of some hematological features in young reared sturgeons (*Acipenser persicus* and *Huso huso*). Fish Physiol Biochem. 24:135–140.

Basha PS, Rani AU. 2003. Cadmium-induced antioxidant defense mechanism in freshwater teleost *Oreochromis mossambicus* (Tilapia). Ecotoxicol Environ Saf. 56:218–221.

Charkhabi AH, Sakizadeh M, Rafiee G. 2005. Seasonal fluctuation of heavy metal pollution in Iran's Siahrood River. Environ Sci Pollut Res. 12:264–270.

Chen WY, Lin CJ, Ju YR, Tsai JW, Liao CM. 2012. Assessing the effects of pulsed waterborne copper toxicity on life-stage tilapia populations. Sci Total Environ. 417–418:129–137.

Cinier CC, Petit-Ramel M, Faure R, Garin D, Bouvet Y. 1999. Kinetics of cadmium accumulation and elimination in carp, *Cyprinus carpio* tissues. Comp Biochem Physiol C. 122:345–352.

De Mora S, Sheikholeslami MR, Wyse E, Azemard S, Cassi R. 2004. An assessment of metal contamination in coastal sediments of the Caspian Sea. Mar. Pollut. Bull. 48(1–2):61–77.

Esbaugh AJ, Mager EM, Brix KV, Santore R, Grosell M. 2013. Implications of pH manipulation methods for metal toxicity: not all acidic environments are created equal. Aquat Toxicol. 130–131:27–30.

Farkas A, Salanki J, Specziar A. 2003. Age- and size-specific patterns of heavy metals in the organs of freshwater fish *Abramis brama* L. populating a low contaminated site. Water Res. 37:959–964.

Gagnon A, Jumarie C, Hontela A. 2006. Effects of Cu on plasma cortisol and cortisol secretion by adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*). Aquat Toxicol. 78:59–65.

Grosell MH, Hogstrand C, Wood CM. 1997. Cu uptake and turnover in both Cu-acclimated and non-acclimated rainbow trout (*Oncorhyncus mykiss*). Aquat Toxicol. 38:257–276.

Habig WH, Pabst MJ, Jakoby WB. 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J Biol Chem. 249:7130–7139.

Harrison FL. 1986. The impact of increased copper concentrations on freshwater ecosystems. In: Hodgson E, editor. Reviews in environmental toxicology 2. Amsterdam: Elsevier; p. 117–250.

He M, Wang Z, Tang H. 1998. The chemical, toxicological and ecological studies in assessing the heavy metal pollution in Le An River. Water Res. 32:510–518.

Koukal B, Dominik J, Vignati D, Arpagaus P, Santiago S, Ouddane B, Benaabidate L. 2004. Assessment of water quality and toxicity of polluted Rivers Fez and Sebou in the region of Fez (Morocco). Environ. Pollut. 131:163–172.

Mazon AF, Fernandes MN. 1999. Toxicity and differential tissue accumulation of copper in the tropical freshwater fish, *Prochilodus scrofa* (Prochilodontidae). Bull Environ Contam Toxicol. 63:797–804.

McGeer JC, Szebedinszkey C, McDonald DG, Wood CM. 2000a. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout I: iono-regulatory disturbance and metabolic costs. Aquat Toxicol. 50:231–243.

McGeer JC, Szebedinszkey C, McDonald DG, Wood CM. 2000b. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout II: tissue specific metal accumulation. Aquat Toxicol. 50:245–256.

Mirzaei J, Nezami S, Mehdinejad K, Pajand ZO, Alinejad R. 2004. Acute toxicity (96-h LC50) of heavy metals (Pb, Zn, Cu and Cd) in two species of sturgeon (*Acipenser persicus* and *Acipenser stellatus*). Paper presented at: 5th Symposium of Sturgeon Fish; Ramsar, Iran.

Parizanganeh A, Lakhan VC, Jalalian H, Ahmad SR. 2008. Contamination of nearshore surficial sediments from the Iranian coast of the Caspian Sea. Soil Sediment Contam. 17:19–28.

Pelgrom SMGJ, Lock RAC, Balm PHM, Wendelaar Bonga SE. 1995. Effects of combined waterborne cadmium and copper exposures on ionic composition and plasma cortisol in tilapia *Oreochromis mossambicus*. Comp Biochem Physiol C. 111:227–235.

Pretto A, Loro VL, Silva VMM, Salbego J, Menezes CC, Souza CF, Gioda CR, Baldisserotto B. 2014. Exposure to sublethal concentrations of copper changes biochemistry parameters in Silver Catfish, *Rhamdia quelen*, Quoy & Gaimard. Bull Environ Contam Toxicol. 92:399–403.

Reddy PS, Bhagyalakshmi A. 1994. Changes in oxidative metabolism in selected tissues of the crab (*Scylla serrata*) in response to cadmium toxicity. Ecotoxicol Environ Saf. 29:255–264.

Ridgway J, Shimmield G. 2002. Estuaries as repositories of historical contamination and their impact on shelf seas. Estuar Coast Shelf Sci. 55:903–928.

Saeedi M, Karbassi AR. 2006. Heavy metals pollution and speciation in sediments of southern part of the Caspian Sea. Pak J Biol Sci. 9:735–740.

Sampath K, James R, Akbar Ali KM. 1998. Effects of copper and zinc on blood parameters and prediction of their recovery in *Oreochromis mossambicus* (Pisces: Cichlidae). Indian J Fish. 45:129–139.

Scott GR, Sloman KA. 2004. The effects of environmental pollutants on complex fish behavior: integrating behavioural and physiological indicators of toxicity. Aquat Toxicol. 68:369–392.

Shaw BJ, Al-Bairuty G, Handy RD. 2012. Effects of waterborne copper nanoparticles and copper sulphate on rainbow trout, (*Oncorhynchus mykiss*): physiology and accumulation. Aquat Toxicol. 116–117:90–101.

Varedi SE, Gholamipoor S, Rezaei M. 2010. The heavy metals concentrations in water column of 5, 10 and 50 m in the southern part of the Caspian Sea. Paper presented at: 1st National–Regional Conference on Ecology of the Caspian Sea (FCECS2010); Sari, Iran. Persian.

Varo I, Nunes B, Amat F, Torreblanca A, Guilhermino L, Navarro JC. 2007. Effect of sublethal concentrations of copper sulphate on seabream *Sparus aurata* fingerlings. Aquat Living Resour. 20:263–270.

Vutukuru SS, Suma C, Madhavi KR, Juveria J, Pauleena JS, Rao JV, Anjaneyulu Y. 2005. Studies on the development of potential biomarkers for rapid assessment of copper toxicity to freshwater fish using *Esomus danricus* as model. Int J Environ Res Publ Health. 2:63–73.

Winston GW, Di Giulio RT. 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat Toxicol. 19:137–161.

Wong DWS, Whitaker JR. 2003. Catalase. In: Whitaker JR, Voragen AGJ, Wong DWS, editors. Handbook of food enzymology. New York, NY: Marcel Dekker; p. 389–401.

Wood CM. 2012. An introduction to metals in fish physiology and toxicology: basic principles. In: Wood CM, Farrell AP, Brauner C, editors. Homeostasis and toxicology of essential metals – fish physiology, vol. 31A. San Diego, CA: Elsevier; p. 1–51.

Wu SM, Shih MJ, Ho YC. 2007. Toxicological stress response and cadmium distribution in hybrid tilapia (*Oreochromis* sp.) upon cadmium exposure. Comp Biochem Physiol C. 145:218–226.