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LC_{50} Determination and Copper and Cadmium Accumulation in the Gills of Kutum (*Rutilus frisii kutum*) Fingerlings

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ABSTRACT

Water pollution by metals and subsequent fish contamination are considered to be severe problems with detrimental ecological consequences. The purpose of the present study was to determine the median acute toxicity (LC_{50}) of copper (Cu) and cadmium (Cd) in kutum, *Rutilus frisii kutum* fingerlings. Also, using sub-lethal tests, metal accumulation and gill ion changes were evaluated. Fingerlings (1.1 ± 0.25 g) were exposed to different concentrations of $CuSO_4 \cdot 5H_2O$ and $CdCl_2.2 \cdot 5H_2O$ in the static bioassay OECD test. In definitive tests, fish were exposed to nominal concentrations of 0.127, 0.229, 0.330, 0.432, 0.534, and 0.636 mg/l of Cu and 3.938, 6.893, 9.847, 12.801, 15.755, and 18.709 mg/l of Cd. Results from a Probit analysis showed that 96-h LC_{50} values were 0.45 and 12.22 mg/l for Cu and Cd, respectively. Semi-static sub-lethal tests were conducted with nominal concentrations of 0, 12.7, 25.4, 50.9, and 101.8 µg/l of Cu and 0, 0.1, 0.25, 0.5, and 1 mg/l of Cd for 4 days. Significant accumulations were observed in gill Cu/Cd levels in all treatments in comparison to the controls ($P < 0.05$) with a maximum average of 22.32 ± 1.25 µg Cu/g gill wet weight at 12.7 µg Cu/l treatment and 6.18 ± 0.44 µg Cd/g gill wet weight at 1.0 mg Cd/l treatment. Finally, no significant ($P > 0.05$) changes were observed in gill sodium and calcium contents during Cu/Cd exposure. In conclusion, Cu is more toxic than Cd for kutum, and it has higher uptake in the gills.

Keywords: Copper, cadmium, sub-lethal, kutum

INTRODUCTION

Environmental pollutants such as heavy metals, pesticides, and other organic materials have detrimental effects on aquatic organisms. Metals as non-degradable pollutants are considered serious threat for aquatic environments by entering through different anthropogenic and natural sources (Moore 1991). Copper (Cu) and cadmium (Cd) are two existent metals in aquatic ecosystems, and numerous studies focus on different aspects of their toxicity for aquatic biota. They are cumulative pollutants, which exert a wide range of biochemical, physiological and genetic alterations in fish and other aquatic organisms (Heath 1995; Khangarot and Rathore 2003; Di Giulio and Hinton 2008; Eyckmans *et al*. 2012). Nowadays, the extensive use of Cu in aquaculture, agriculture, industry, and mining has resulted in its release and subsequent increase in receiving waters (Khangarot and Rathore 2003; Guardiola *et al*. 2012). Similarly, Cd as a non-essential heavy metal is used widely in industry for producing paints, dyes, cement and phosphate fertilizers causing increased amounts in aquatic ecosystems (Jarrup 2003; Burger 2008).

LC_{50} tests measure the susceptibility and survival potential of organisms to a particular toxic substance. Pollutants with higher LC_{50} values are less toxic because greater concentrations are required to induce mortality in organisms (Eaton *et al*. 2005). In addition, in most natural waters, metals are usually present only at sub-lethal concentrations, and such a contamination in aquatic environments is a widespread problem particularly in rivers and estuaries. Pollution of rivers, lakes, coastal, and marine waters by metals like Cu and Cd is widely observed in aquatic environments which leads to a considerable increase of their concentrations in aquatic organisms (Simpson 1981; Ravera 1984; Ray 1984; Harrison 1986; Lopes *et al*. 2001; Jarrup 2003; Agusa *et al*. 2004; Burger 2008).

Numerous investigations point out the behavioral changes in fish exposed to lethal or sub-lethal concentrations of metals like Cu and Cd (Sloman *et al*. 2003; Vutukuru *et al*. 2005). In addition, metals accumulate in different organs of aquatic animals, and produce deleterious effects on fish (Asagba *et al*. 2008; Isani *et al*. 2009; Javed 2012). However, their accumulation levels in living organisms depends on species, the size of individuals, type of tissue or organ, and the metal itself (Lloyd 1992; Finn 2007). Generally, the uptake of water-borne Cu and Cd in freshwater fish occurs mainly through the gills, and this organ has crucial role in ion uptake and homeostasis (McGeer *et al*. 2000). Water-borne Cu/Cd can accumulate in fish gill cells, and then, affect the function of branchial pumps like Na^+K^+-ATPase and Ca^{+2}-ATPase. These pumps are active in chloride cells, and have vital role in fish osmoregulation (Shephard and Simkiss 1978; Pelgrom *et al*. 1995; Wong and Wong 2000).

The Caspian Sea, the largest continental water body on the earth (Dumont 1998), is the habitat for numerous commercial fishes, but some investigators have documented the accumulation of contaminants like metals in Caspian Sea fish populations (Moore *et al*. 2003; Agusa *et al*. 2004; Anan *et al*. 2005). Moreover, chemical contamination is described as one of the most significant factors influencing the commercial fish populations in Caspian Sea (Karpinsky 1992). Accordingly, some investigations have documented water and sediment pollution in southern parts of the Caspian Sea (De Mora *et al*. 2004; Charkhabi *et al*. 2005; Parizanganeh *et al*. 2006; Saeedi and Karbassi 2006; Parizanganeh *et al*. 2008; Saeedi *et al*. 2010; Bagheri *et al*. 2011) that some commercial fishes spend part of their larval and fingerling stages in such polluted environments.

Kutum, *Rutilus frisii kutum* is a species with great ecological and commercial value, and its stocks in the Caspian Sea are replenished through artificial breeding. Every year more than hundreds of millions fries are produced by Ira-

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nian Fisheries Organization, and then, fingerlings are released into the southern rivers and estuaries of the Sea (Farabi *et al*. 2007; Abdolhay *et al*. 2010) in which they probably experience different sub-lethal concentrations of pollutants like metals. The first objective of the present study was to determine the LC_{50} values of Cu and Cd and the second objective was to study their accumulations and gill sodium (Na^+) and calcium (Ca^+) changes during sub-lethal exposure in kutum fingerlings.

MATERIALS AND METHODS

Fish

The kutum fingerlings with average body weight of 1.1 ± 0.25 g were obtained from the Shahid Rajaee Fish Hatchery Center, Sari, Iran. Fish were transferred to aquaculture laboratory of the same center on July, 2008. Fish acclimated to the laboratory conditions with ambient photoperiod in the 1000 l stock tanks for 2 weeks before the experimental use. The fish were fed 3% of body weight by commercial food once daily in the morning (at 9:00 a.m.).

Acute exposure

Cu and Cd stock solutions were prepared by using $CuSO_4 \cdot 5H_2O$ and $CdCl_2.2 \cdot 5H_2O$, and were stored at 4°C. Before commencing the experiments, stock solutions were diluted to the desired concentrations. Following a two week acclimation period and for pilot experiments, fish were transferred from the stock tanks to the 20 l experimental ones based on a static bioassay test following the OECD No. 203 protocol (OECD 1992). Each tank contains 10 fish/10 l of oxygenated well water with maintaining constant dissolved oxygen at 7.6 ± 0.2 mg/l, temperature at 23.5 ± 0.9°C, pH at 8.1 ± 0.2 and water hardness at 275 ± 8 mg $CaCO_3$/l. The fish were starved for 24 h prior to and during the experiment. Healthy kutum fingerlings were exposed to various concentrations of metals for range-finding tests to choose concentrations that resulted in mortality of fish within the range of 5 to 95%.

Thereafter, in definitive tests, fish were treated in the same conditions with 0.127, 0.229, 0.330, 0.432, 0.534, and 0.636 mg/l of Cu (equivalent to 0.5, 0.9, 1.3, 1.7, 2.1, and 2.5 mg/l of $CuSO_4 \cdot 5H_2O$) and 3.938, 6.893, 9.847, 12.801, 15.755, and 18.709 mg/l of Cd (equivalent to 8, 14, 20, 26, 32, and 38 mg/l of $CdCl_2.2 \cdot 5H_2O$) concentrations. Acute toxicity of Cu and Cd to kutum was estimated as the median lethal concentration, LC_{50}, after exposing of fish for 96 h to the mentioned concentrations. Fish mortality was recorded after 0, 24, 48, 72, and 96 h of exposure to Cu/Cd solutions. Fishes were considered dead when gill opercula and body movement ceased, and when these characteristics occurred, fishes were immediately collected. LC_{50} values were calculated by the Probit Analysis test (Finney 1971).

Sub-lethal exposure

For sub-lethal tests, fish were randomly distributed in thirty 20 l tanks (three replicates per treatment) to perform the 4 day period sub-lethal tests. Every tank containing 10 fishes was exposed to test solutions with the following concentrations of 12.7, 25.4, 50.9, and 101.8 µg/l of Cu (equivalent to 50, 100, 200 and 400 µg/l of $CuSO_4 \cdot 5H_2O$) and 0.1, 0.25, 0.5, and 1 mg/l of Cd (equivalent to 0.2, 0.5, 1 and 2 mg/l of $CdCl_2.2 \cdot 5H_2O$) and 0.0 mg/l (control), respectively. Sub-lethal concentrations derived as about 3, 6, 12 and 22%, and also 0.8, 2, 4 and 8% of 96-h LC_{50} value of Cu and Cd, respectively. About 90% of water was changed from stock tanks daily to keep concentrations of Cd and Cu solution near the nominal level. All fishes of both experimental and control groups were captured and sacrificed after 4 days of exposure. Then, the gill tissues were quickly removed and washed with 0.9 % NaCl solution. Samples were maintained at -20°C until metal analysis.

Analysis

For metal ion determination, gill samples were dry-ashed in silica vessels according to Cinier *et al*. (1999). Then, 1 ml of 65% super pure nitric acid (Merck, Darmstadt, Germany) was added to each ashed sample in test tube. After complete digestion, ultra pure water was added to each sample to reach volume of 15 ml. Then, sample filtered (0.22 µ Cellulose acetate, Sandic, S&S, Germany), and the metal concentrations determined by ICP-OES (GBC, Integra XL). The concentration of metal in gill was reported as µg/g wet weight (WW). Also, accumulation factor (AF) was calculated based on the following definition (Kim *et al*. 2004):

Accumulation Factor (AF): $C_t - C_c/C_w$

where C_t, C_c, C_w are the metal concentration in the experimental groups, controls, and water, respectively.

Statistical analysis

Statistical analyses were performed using SPSS software (ver. 17.0, SPSS Co., Chicago, IL, USA). Data are presented as mean ± SE. All the data were tested for normality (Kolmogorov–Smirnov test) and homogeneity (Levene's test). Data were analyzed by One-way analysis of variance (ANOVA). Means were compared by Duncan's multiple comparison test ($P < 0.05$) (Sokal and Rohlf 1995).

RESULTS

Results of the LC_{50} experiments have presented in **Table 1**. Under our experimental conditions, the 96-h LC_{50} values of Cu and Cd for *R. frisii kutum* was found to be approx. 0.45 ± 0.024 and 12.22 ± 0.64 mg/l, respectively.

As soon as the fish were exposed to acute and sub-lethal concentrations of Cd/Cu, they have become slightly excited and swam erratically, becoming normal only a few hours later. In sub-lethal test, after a 4 day exposure, there was a consistent increase in the rate of opercular movement, and also, excess mucus secretion was partly evident. It should be noted that behavioral and swimming patterns in control groups were normal, and there was not any mortality in this group during the experimental period.

According to the detailed results shown in **Figs. 1** and **2**, it is clear that Cu and Cd could accumulate in the gills of

Table 1 Median acute toxicity (LC_{50}) and 95% confidence limits of Cu and Cd to fingerlings kutum, *R. frisii kutum*.

LC_{50}	Time (h)			
	24	**48**	**72**	**96**
Cu	0.7403 ± 0.0779 (0.6310-1.0183)	0.5596 ± 0.0329 (0.5043-0.6442)	0.4537 ± 0.0237 (0.4090-0.5065)	0.4537 ± 0.0237 (0.4090-0.5065)
Cd	23.7233 ± 3.6554 (18.9961-40.4886)	16.4807 ± 1.2847 (14.4055-20.1075)	13.5012 ± 0.7964 (12.0165-15.324)	12.2206 ± 0.6415 (10.9566-13.5721)

Table 2 Gill ion concentrations (µmol/g gill W.W) on day 4 of exposure to single sub-lethal concentration of Cu and Cd.

	$CdCl_2.2 \cdot 5H_2O$ (mg/l)					$CuSO_4 \cdot 5H_2O$ (µg/l)				
	0	**0.2**	**0.5**	**1**	**2**	**0**	**50**	**100**	**200**	**400**
Na	84.63 ± 7.5 (8)	79.8 ± 4.2 (9)	80.9 ± 5.7 (8)	80.23 ± 8.8 (6)	85.44 ± 5.7 (9)	72.55 ± 6.76 (7)	76.03 ± 7.89 (8)	75.20 ± 5.91 (6)	77.80 ± 3.48 (7)	80.74 ± 4.37 (9)
Ca	35.06 ± 2.8 (8)	28.77 ± 1.7 (9)	33.56 ± 1.79 (10)	29.70 ± 1.93 (7)	36.05 ± 3.6 (7)	40.12 ± 6.83 (8)	38.12 ± 5.98 (9)	36.05 ± 5.57 (8)	36.90 ± 2.57 (10)	37.22 ± 3.08 (10)

Data were analyzed by one-way ANOVA with Duncan comparisons for different metal concentrations. Data are presented as mean ± SE, n = 6-9.

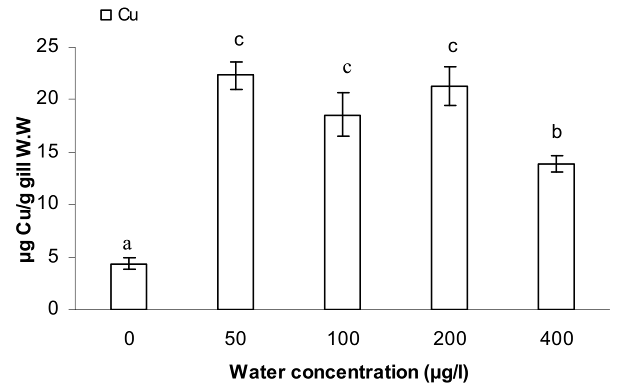

Fig. 1 Cu concentrations in gills of *R. frisii kutum* fingerlings exposed to different water-borne sub-lethal copper sulphate concentrations for 4 days (mean ± SE, n= 6-9). The significant difference between groups was analyzed by one-way analysis of variance with Duncan comparisons ($P < 0.05$).

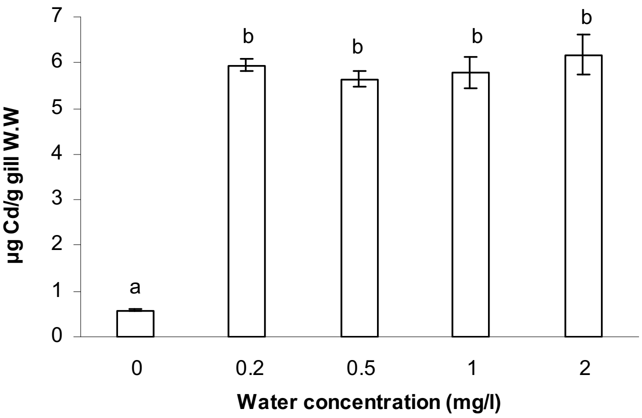

Fig. 2 Cd concentrations in gills of *R. frisii kutum* fingerlings exposed to different water-borne sub-lethal cadmium chloride concentrations for 4 days (mean ± SE, n=6-9). The significant difference between groups was analyzed by one-way analysis of variance with Duncan comparisons ($P < 0.05$).

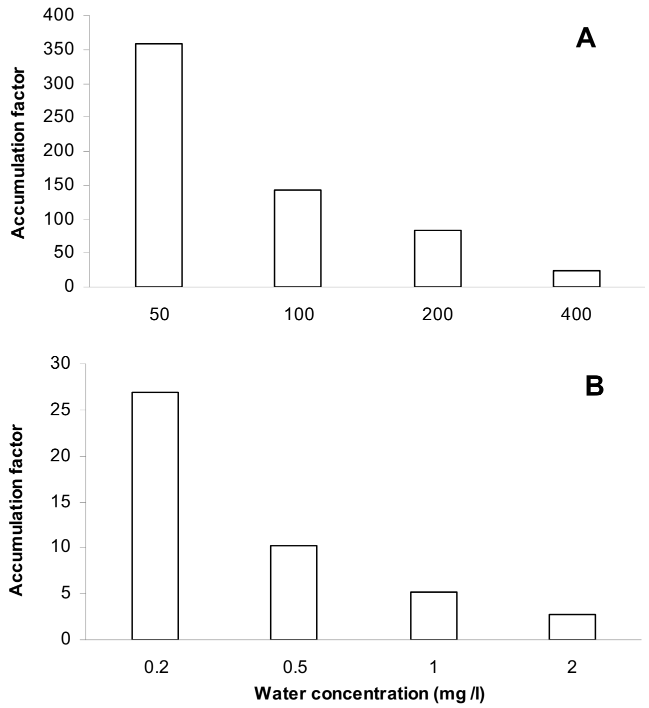

Fig. 3 Accumulation factor (AF) in gills of *R. frisii kutum* fingerlings exposed to different water-borne sub-lethal copper sulphate (µg/l, A) and cadmium chloride (mg/l, B) concentrations for 4 days (mean ± SE, n= 6-9).

exposed fish at all treatments compared to control group after 96-h sub-lethal exposure significantly ($P > 0.05$). Also, significant difference was observed between the result of 101.8 µg Cu/l (400 µg $CuSO_4{\cdot}5H_2O$/l) dose compared to other Cu doses ($P < 0.05$), but significant differences was not observed in gill Cd concentrations among experimental ones ($P > 0.05$). The highest levels of Cu and Cd compounds were 22.32 ± 1.25 µg/g wet weight (12.7 µg Cu/l treatment equivalent to 50 µg $CuSO_4{\cdot}5H_2O$/l) and 6.18 ± 0.44 µ/g wet weight (1 mg Cd/l treatment equivalent to 2 mg $CdCl_2.2{\cdot}5H_2O$/l), respectively. Accumulation factors (AF) of Cu and Cd in fish gills are shown in **Fig. 3**. The AFs were decreased with increasing in exposure concentration, and showing an inverse relationship between AFs and exposure concentrations. A comparison between groups revealed neither difference in gill sodium nor calcium levels during Cd/Cu exposures for 4 days ($P > 0.05$, **Table 2**).

DISCUSSION

Acute toxicity

The susceptibility of fish to a particular metal is a very important factor for LC_{50} determination and subsequent values. In this study, the toxicity of Cu and Cd for *R. frisii kutum* increased with increasing concentration and exposure time. When fish were exposed to 0.127 mg/l of Cu and 3.938 mg/l of Cd, only 6.7% and 10% of fish died after 96 h, respectively, whereas 83.3% and 86.7% of fish died after 96 h when fish were exposed to concentrations of 0.636 mg/l of Cu and 18.709 of mg/l of Cd. Results obtained from the acute toxicity tests obviously demonstrated that Cu is extremely more toxic than Cd for kutum, and the 96-h LC_{50} value of Cd is about 27.2-fold higher than Cu one. Acute toxicity tests can detect the toxic damages of special pollutants in a short period of time. They ascribe the degree of toxicity among various pollutants, and the relative sensitivities of species to a particular one (Buikema *et al*. 1982).

The obtained LC_{50} values showed that kutum fingerlings are more sensitive to Cu and Cd than some other fishes. Values of 1.402 mg/l of Cu for *Esomus danricus* and 17.1 mg/l of Cd for *Cyprinus carpio* have already been reported (Suresh *et al*. 1993; Vutukuru *et al*. 2005). Also, Chen *et al*. (2012) reported a 96-h LC_{50} of 2.55 ± 0.03 mg/l for 1-month old Juvenile tilapia *Oreochromis mossambicus* during pulsed Cu exposure. Moreover, Tsay and Yu (1981) showed a 96-h LC_{50} of 23.20 mg/l in *Anguilla japonica* (0.65 g) during copper sulphate exposure. The 96-h LC_{50} values of Cu and Cd for fish differ from species to species and according to the type of metal. However, metal toxicities depend upon various factors such as water hardness and pH (Lauren and McDonald 1986; Perschbacher and Wurts 1999). It is worth noting that in the present study, high water hardness (275 ± 8 mg $CaCO_3$/l) and to some extent pH (8.1 ± 0.2) have affected LC_{50} values because high water hardness minimizes the bioavailability of Cu and Cd to fish and waterborne calcium and magnesium have a protective effect against Cu and Cd toxicity (Pagenkopf 1983; Perschbacher and Wurts 1999).

Sub-lethal exposure

According to obtained LC_{50} values of Cu, and its higher toxicity to kutum, its concentrations in the present study were lower compared to Cd in sub-lethal tests. Besides, some of the tested Cu concentrations in sub-lethal tests occur in Iranian surface water occasionally (Varedi *et al*. 2010). No mortality was observed in *R. frisii kutum* fingerlings exposed to all sub-lethal Cd concentrations over a 4 day period, but one fish died in one of 3 replications at the both 25.4 and 50.9 µg Cu/l treatments during the 96 h sub-lethal experiments.

During this study, behavioral changes in exposed fish were slight at sub-lethal tests when comparing to acute ones. Behavioral changes are of the most sensitive indicators of potential effects of intoxication. Increasing of mucus that was observed on the gill surface during metal exposure may contribute to lower uptake of metal through gills. Thus, mucus is thought to act as buffer and to prevent the metal from interacting with site of the toxicity (Reid and McDonald 1991; Hmoud 1995; Lee *et al*. 2010). Vutukuru *et al*. (2005) also reported behavioral responses such as copious mucus secretion, loss of scales, grouping and loss of equilibrium in *Esomus danricus* when they exposed fishes to water-borne sub-lethal level of 0.1402 mg/l of Cu. These results also agree with the finding by other authors around Cd exposure (Thophon *et al*. 2003; Pandey *et al*. 2008).

In the present study, the results show that Cu/Cd exposures produce significant accumulations in gills of experimental groups in comparison to control, but there was no obvious trend between increasing of water-borne metal doses and subsequent gill metal accumulations in experimental groups. For example, in Cd exposures, unlike the 10-fold incresease in the water nominal concentrations in the 2 mg/l treatment compared to 0.2 mg/l one, no significant changes were observed in the gill Cd accumulations (**Fig. 2**). The results of present study are contrary to some studies that have shown such a relationship for branchial metal accumulations (Cinier *et al*. 1999; Kim *et al*. 2004; Atli and Canli 2008). Their findings corroborate those of Asagba *et al*. (2008) who showed an increasing of gill cadmium accumulations with a 4-fold increasing (0.4 ppm compared to 0.10 ppm) in water Cd dose after 7 and 21 days. Water-borne metals enter the fish mainly through the gills because gills are in direct contact with aquatic environment (Lemaire-Gony and Mayer-Gostan 1994; Jayakumar and Paul 2006). Fish gills, as physiologically complex and vulnerable structures, are the first target tissue to accumulate metals. These properties make them as target organs for water-borne toxicants (Wu *et al*. 2007). Moreover, gills are one of the main sites for metallothionein (MT) production in fishes (Klavercamp *et al*. 1984). MT influences the uptake, distribution, and toxicity of metal by binding it, and can describe the cause of subsequent tolerance (De la Torre *et al*. 2000; Wimmer *et al*. 2005). Numerous studies show that the accumulation of a metal in fish tissues is related to numerous factors such as exposure dose and time, temperature, water chemistry, metal type, metal interactions, fish age, and metabolism as well as food habits (Heath 1995; Chen and Folt 2000; Kim *et al*. 2004; Erickson *et al*. 2008; Burridge *et al*. 2010).

AF is a useful factor to compare the body burden of an organism with the degree of exposure dose (Kim *et al*. 2004). Obtained AFs were about 358.6, 142, 84.4, and 23.7 for 50, 100, 200, and 400 μg $CuSO_4{\cdot}5H_2O$/l and about 26.9, 10.1, 5.2, and 2.8 for 0.2, 0.5, 1, and 2 mg $CdCl_2.2{\cdot}5H_2O$/l, respectively. Obtained data demonstrated that water metal concentration influences the branchial AF in kutum. These results are in accordance with calculated bioconcentration factor in *Cyprinus carpio* where at both concentrations (53 and 443 μg/l Cd) lower gill bioconcentration factor was observed in higher one (Cinier *et al*. 1999). Also, gill AF of olive flounder, *Paralichthys olivaceus* was inversely related to the exposure concentration as higher AF was reported at 10 μg/l of Cd after sub-chronic exposure to 10, 50, and 100 μg/l Cd for 10, 20, and 30 days (Kim *et al*. 2004). Similar patterns of AF were indicated in Japanese eel, *Anguilla japonica* during Cd exposure (Yang and Chen 1999). McGeer *et al*. (2000) showed the ability of gills of rainbow trout to absorb and store large amounts of Cd in a short time. However, it is supposed that the short period of exposure during this study and the capacity of gill ion channels for metal uptake at this limited time resulted in a relatively high AF at the lowest metal concentration compared to other concentrations.

Both Cu and Cd disturb either branchial Na^+ or Ca^{2+} transport by affecting branchial ionic pumps like Na^+/K^+-ATPase, Ca^{2+}-ATPase, and carbonic anhydrase (Christensen and Tucker 1976; Shephard and Simkiss 1978; Pelgrom *et al*. 1995; Pratap and Wendlaar Bonga 1993; Li *et al*. 1998; Wong and Wong 2000), and therefore, metals change the ion balances of fish osmoregulatory organs (Ay *et al*. 1999; Grosell *et al*. 2003). In oppose to our supposition for gill Na^+/Ca^{2+} changes during Cu/Cd concentration exposure, significant differences comparing to correspondence control value was not observed. In this study, because plasmatic ionic changes were not studied in fingerlings, comparison with others plasmatic ionic data during metal exposure was impossible. It was proposed that short duration of exposure period was certainly effectual on obtained results and thus, it is likely that the sufficient time was not provided for manifestations of ionic changes in fingerling gills.

In conclusion, LC_{50} results clearly show that Cu/Cd toxicity for *R. frisii kutum* increased with increasing concentration and exposure time, and more importantly, Cu is more toxic than Cd for fingerlings. Also, water concentration influences the branchial metal concentrations and AF in kutum but not in a dose-dependent manner. Moreover, Cu has a greater potential for entering through the fish gills. According to the results, we need more concern around environmental metal concentrations especially in rivers and estuaries for protecting the fish larva and juveniles from metal contamination.

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