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Giti Kashi

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Article

Effects of cadmium on metallothionein and histology in gills of tilapia (*Oreochromis niloticus*) at different salinities ›

Lisda Nursanti, Eka Nofitasari, Alfiah Hayati, Sucipto Hariyanto, Bambang Irawan & Agoes Soegianto

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Blood clinical-chemical parameters and feeding history in growing Japanese quail (*Coturnix japonica*) chicks exposed to Tris(1,3-dichloro-2-propyl) phosphate and Dechlorane Plus *in ovo* ›

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Activity of nucleoside triphosphate diphosphohydrolase, 5′-nucleotidase, and adenosine deaminase in cattle fed on pastures treated with different nitrogen fertilizers ›

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Article

Arsenic speciation in rice consumed in south-western Nigeria, and estimation of dietary intake of arsenic species through rice consumption ›

Joseph A. Adeyemi, Chris O. Adedire, Airton da Cunha Martins-Junior, Ana Carolina Paulelli, Akinsola F. Awopetu, Fabiana Roberta Segura, Vanessa C. de Oliveira-Souza, Bruno L. Batista & Fernando Barbosa Jr

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Changes over time in persistent organic pollutants (POP) concentrations in human milk in the Republic of Moldova ›

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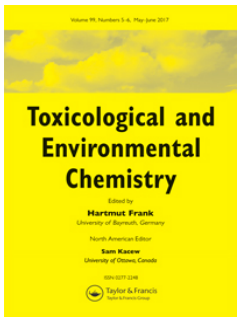

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Effects of cadmium on metallothionein and histology in gills of tilapia (Oreochromis niloticus) at different salinities

Lisda Nursanti, Eka Nofitasari, Alfiah Hayati, Sucipto Hariyanto, Bambang Irawan & Agoes Soegianto

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Effects of cadmium on metallothionein and histology in gills of tilapia (*Oreochromis niloticus*) at different salinities

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ABSTRACT

The objectives of this study were to evaluate the effects of sublethal cadmium concentrations on the levels of cadmium, metallothionein (MT) and histological changes in gills of East Java strain tilapia (*Oreochromis niloticus*) at different salinity levels. The levels of cadmium in control gills were not significantly different at 0, 5 and 10 practical salinity unit (PSU). The cadmium concentrations in gills of cadmium-exposed fish were significantly higher at 0 PSU than at 5, 10 and 15 PSU. The MT concentrations in control gills were not significantly different at 0, 5, and 10 PSU. The MT concentrations of cadmium-exposed fish were significantly higher than those in respective control groups at 0, 5 and 10 PSU. Significant gill damage occurred in fish exposed to cadmium at lower salinity. The epithelial lifting was noted at gills of fish exposed to 2.5 mg/L of cadmium at 0 PSU, and telangiectasis was observed at gills exposed to 5 mg/L of cadmium at 0 PSU. The level of gill damage decreased with increasing salinity of media. The increased MT and histological changes in gills of our findings could be a protective response of animals to toxic effect of cadmium.

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Cadmium; accumulation; metallothionein; gills; histology; *Oreochromis niloticus*

1. Introduction

Cadmium is a pollutant that enters the environment from anthropogenic and natural sources. In natural waters, Cd can occur at concentrations <0.1 μg/L, but in heavily polluted estuaries, it may reach 2–16 mg/L (Soegianto et al. 1999; Cao et al. 2012). Fish are highly sensitive to Cd contamination (EPA 2001). Exposure to sub-lethal concentrations of Cd may cause biochemical, osmotic and ionic disturbances, and adaptive responses in blood and tissues (Pelgrom et al. 1995).

Tilapia (*Oreochromis niloticus*) tolerates a wide range of salinity, and can be cultured in freshwater, brackish water and seawater. Different tilapia strains vary considerably to salinity tolerance from 0 to 32 practical salinity unit (PSU) (Chervinski 1982; Suresh and Lin 1992; Avella, Berhaut, and Bornancin 1993). Baroiller et al. (2000) reported that *O. niloticus* did not tolerate salinity above 20 PSU and might not be suitable for culture in full-strength seawater (37–40 PSU).

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In nature, particularly in regions which are impacted by industrial, agricultural and domestic activities, tilapia are potentially exposed to a variety of toxic substances including cadmium. In this region, fish often encounter both water salinity changes and elevated cadmium level; therefore, the interaction between salinity acclimation and toxicant becomes important (Erk et al. 2008; Adeyemi et al. 2012). It has been generally accepted that the toxicity of cadmium to aquatic animals changes as a function of ambient salinity, with the metal generally being more toxic at low salinities (Erk et al. 2008; Bielmyer et al. 2012), primarily due to greater complexation of Cd^{2+} by Cl^{-} (De Lisle and Roberts 1988; Santore et al. 2002). Anions in higher salinity waters may bind to and change the speciation of the metal, thus altering its availability for the gills (Santore et al. 2002). Tilapia (*O. niloticus*) is a hyper- and hypo-osmoregulator; at low salinities it actively maintains its blood hyper-osmotic to the external environment and at high salinities the blood is maintained in a hypo-osmotic state (Fontainhas-Fernandes et al. 2003). Consequently, the trace metal uptake can be facilitated due to increased activity of ionic pumps when the organism is hyper-osmoregulating. Uptake can also occur via the gut when the organism is hypo-osmoregulating (Erk et al. 2008).

Accumulation of metals does not necessarily result in deleterious effects since organisms have possibilities to protect themselves from metal toxicity by increased excretion, differential allocation among organs and by binding metals intercellularly (Bervoets et al. 2013). Metallothioneins (MTs) are involved in the binding and regulation of essential metals such as copper and zinc, and the detoxification of non-essential metals such as cadmium and mercury (Coyle et al. 2002). The induction of MTs as a response to elevated levels of metal exposure has been frequently used as a biomarker for metal exposure (Dallinger et al. 1997; Chowdhury, Baldisserotto, and Wood 2005). Given the different way of exposure, both metal accumulation and MT induction in fish might differ depend on the levels of salinities and metals in the media.

Gills of fish are known as primary effector site for both active and passive exchanges occur between the organism and its environment (Gilles and Delpire 1997); it is likely, therefore, to be a site of action of metals. This study is aimed to evaluate the MT concentration in gills of *O. niloticus* resulting from water–borne Cd exposure at different salinities. Further, the Cd concentration in the gills was examined to assess whether its level was proportional to the level of MT protein. The structural change of gills was also evaluated in order to know the impact of Cd in this organ.

2. Materials and methods

2.1. Fish collection and acclimation

Fish *O. niloticus* (East Java strain, local name: Jatimbulan), approximately 10.7 ± 0.8 cm, were collected from a local commercial farm. The fish were transported to the laboratory in a plastic bag containing aerated ambient water. The fish were fed with commercial pellets (30% protein, 3% fat and 4% fiber) (Takari, Sidoarjo, Indonesia), acclimated during 2 weeks to different salinities from 0 to 5, 10, and15 PSU with a 5 PSU daily increase in order to avoid osmotic shock, and maintained for 14 additional days at photoperiods of 12 h light and 12 h dark. High mortality was noted at 20 PSU. Seawater from the Surabaya

coast adjacent to the university and municipal tap water aerated for dechlorination (Putranto et al. 2014) and filtered through gravel, sand, and sponge filter was used. Acclimation and experimentation were performed at a temperature of 28–30 °C, pH 7.6–8.1, and dissolved oxygen of 7.2–7.4 mg/L.

2.2. Assessment of lethal Cd concentration

A stock solution of Cd (1000 mg /L) was prepared by dissolving 2.744 g $Cd(NO_3)_2 \bullet 4H_2O$ (Merck, Darmstadt, Germany) in 1000 mL deionized water. The median lethal concentration (LC_{50}) was determined with Cd^{2+} at concentrations of 0, 1.25, 2.5, 5, 10, and 20 mg/L and 10 fish in triplicate in 63 L plastic tanks containing 50 L de-ionized waters which was aerated continuously by an air stone without water renewal. Each day, dead fish (immobile, lack of opercular movement, no response when touched with glass rod) were counted and removed. The fish were not fed during the experiment. The 96 h LC_{50} was estimated using the trimmed Spearman-Karber method (Putranto et al. 2014). The 96 h LC_{50} and 95% confidence intervals of Cd to *O. niloticus* were 7.53 (6.11–9.28) mg/L. From an ecotoxicological point of view, the Cd concentrations used in this study were 2.5 and 5 mg/L. These concentrations can potentially be found by fish in their natural environment (Cao et al. 2012).

2.3. Sub-lethal toxicity

Sub-lethal tests were conducted using a static renewal method with 80% test solutions being renewed every 48 h.

Duplicates of 12 groups of 8 fish each were exposed for 7 days to Cd at concentrations of 2.5 and 5 mg/L at 0, 15, 10, and 15 PSU under continuous aeration. The fish were fed ad libitum twice daily with commercial fish food, with uneaten food and debris being removed daily.

At the end of exposure, six fish were randomly removed from each tank to determine MT, and six fish for measuring Cd concentrations. The remaining 4 fish were used for histology.

2.3.1. Determination of Cd

Fish were sacrificed by rapid spinal transection and the gills were dissected and stored at −20°C until Cd determination as described by Asmysari, Irawan, and Soegianto (2013).

Cd concentrations were expressed as mg/kg dry weight (dw). Analytical blanks were run in the same way as the samples, and concentrations were determined using standard solutions prepared in the same acid matrix. The accuracy of the Cd determination was verified using dogfish muscle reference material (DORM-2) provided by the National Research Council of Canada (Ottawa, Canada), with Cd recovery of 106% and detection limit of 0.002 mg/kg dw.

2.3.2. Determination of MT

After thawing, 2 g of gill tissues were homogenized with 500 μL buffer containing radioimmunoprecipitation assay total protein extraction lysis buffer (Bioworld Technology,

Nanjing, P.R. China) using a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 5000 g for 10 min at 4 °C. The supernatants were transferred into 1.5 mL tubes, heat denatured at 100 °C for 10 minutes in a boiling water bath, and the denatured proteins were collected by centrifugation at 10,000*g* for 10 min at 4 °C. The pellets were dissolved in 100 μL phosphate-buffered saline (PBS) (Nacalai Tesque, Kyoto, Japan), pH 7.4, and subjected to MT assay as antigen using indirect enzyme-linked immunosorbent assay.

Flat-bottom, 96-well microtiter plates (SPL Life Sciences, Gyeonggi-do, Korea) were coated with antigen diluted in 1:40 with carbonate buffer (0.1 mol/L, pH 9.6), incubated overnight at 4 °C, decanted, and washed 6 times with 100 μL PBS containing 0.2% Tween-20. Plates were incubated with 2% bovine serum albumin (Sigma-Aldrich Chimie, Deisenhofen, Germany) in PBS, 100 μL/well, for 1 h at room temperature, then washed 3 times with 100 μL PBS-Tween-20. A dilution (1:800) of the rabbit polyclonal MT antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with assay buffer (BSA-PBS-Tween-20) was added at 100 μL/well as a primary antibody. After 1 h at room temperature, the plates were washed 6 times with PBS-Tween-20. A dilution (1:800) of biotinylated anti-rabbit IgG (H+L) antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) with assay buffer was added at 100 μL/well, and incubated for 1 hour at room temperature as secondary antibody. The plates were washed 6 times with PBS-Tween-20. Aliquots of 100 μL Streptavidin-horseradish peroxidase (Dako Agilent Technologies, Glostrup, Denmark) diluted with assay buffer (1:800) were added to each well, incubated for 1 h at room temperature, and washed 6 times with 200 μL PBS-Tween-20. Then, 100 μL substrate sure blue tetramethylbenzidine Microwell Substrate (Kirkegaard and Perry Laboratories) was added to each well, and incubated for 30 min at room temperature in a dark room. Enzyme action was stopped by adding 100 μL 1 mol/L HCl to each well, and after 10 min, absorbance was measured at 450 nm in an automatic microtiter plate reader (Stat Fax 3200, Awareness Technology, Palm City, FL, USA).

2.3.3. Histology

The gills were dissected and immediately fixed in 10% neutral buffered formalin for 24 h, dehydrated in a graded ethanol series, cleared in xylene and embedded in paraffin wax (Bancroft and Gamble 2002). The samples were sectioned to a thickness of 6 μm with a microtome (Microm HM 315, Walldorf, Germany) and mounted on glass slides. Sections were de-paraffinized in xylene, hydrated in ethanol and stained with hematoxylin and eosin. Histological slides were examined with a light microscope (Olympus CX41, Tokyo, Japan) and pictures were taken with the digital camera installed on the microscope.

2.4. Statistical analysis

All data were expressed as mean ± standard deviation and their normality and homogeneity were verified before statistical analysis. Statistical analysis of the data was performed using two-way ANOVA followed by a Duncan's post hoc analysis for multiple comparisons to evaluate effects of cadmium on Cd and MT concentrations. $p < 0.05$ was considered as the level of significance.

3. Results and discussion

3.1. Effects of salinity on Cd and MTs in gills

Cd concentrations in the control groups at all tested salinities ranged insignificantly from 28 to 35 μg/L (Table 1). The Cd concentrations in gills of Cd-exposed fish were significantly higher than those in the control groups at salinity 0 and 5 PSU ($p< 0$ .05). There were no significant differences in the Cd concentrations in gills of fish exposed to the salinity 10 PSU ($p > 0.05$). At salinity 15 PSU the highest level of Cd was noted in gills of fish exposed to 5 mg/L ($p < 0.05$), while there were no significant differences in Cd levels in gills of fish exposed to 0 and 2.5 mg/L ($p > 0.05$).

The accumulation of Cd in gills of *O. niloticus* exposed to the same concentration of Cd is dependent on the salinity (Table 1). The Cd concentrations in gills were significantly higher at 0 PSU than at 5, 10, and 15 PSU. The possible mechanism that can explain this result is the complexation of Cd^{2+} with chloride ions (De Lisle and Roberts 1988; Blust, Kockelbergh, and Baillieul 1992; Wright 1995; Santore et al. 2002). At low salinity, the level of free Cd ion was increase because of reduced formation of chloro-complexes (Verslycke et al. 2003). They observed that free Cd ion percentages were 19.7 and 3.4 at salinities 5 and 25 PSU, respectively. Blust, Kockelbergh, and Baillieul (1992) investigated that the uptake of Cd by brine shrimp *Artemia franciscana* decreased with increasing salinity. Cd accumulation in carapace, gills, hepatopancreas, and muscle rose pronouncedly in the crabs (*Uca rapax*) treated in dilute sea water (≈8.65 PSU) than in concentrated sea water (≈43.35 PSU) (Zanders and Rojas 1996). Roast, Widows, and Jones (2001) demonstrated the uptake different of Cd in the benthic mysid at the two salinities were caused by the differences in the free Cd ion.

In addition to the above physico-chemical factors, physiological factors also regulate the response of organisms to metal toxicity (Erk et al. 2008). For example, the killifish *Fundulus heteroclitus* is more sensitive to metal ions in freshwater than at a salinity of 10 PSU, which is close to its isosmotic point (Grosell et al. 2007). Euryhaline species appear to be more resistant to metal at or near their isosmotic points (De Lisle and Roberts

Table 1. Cadmium and MT concentrations in gills of control and Cd-exposed fish (μg/kg) in different salinity levels. Different letters indicate significant differences ($p < 0.05$; a < b < c < d < e < f). Data are means of six measurements.

Media		Gills	
Salinity (PSU)	Cd (mg/L)	Cd (μg/kg)	MT (μg/kg)
0	0	$31 \pm 4^{a,b}$	520 ± 172^{a}
0	2.5	69 ± 4^{e}	1590 ± 82^{d}
0	5	77 ± 6^{f}	2030 ± 289^{e}
5	0	$35 \pm 1^{b,c}$	$713 \pm 224^{a,b}$
5	2.5	53 ± 7^{d}	1496 ± 263^{d}
5	5	58 ± 6^{d}	1625 ± 435^{d}
10	0	$35 \pm 2^{b,c}$	$631 \pm 93^{a,b}$
10	2.5	39 ± 3^{c}	$1266 \pm 173^{c,d}$
10	5	40 ± 3^{c}	1485 ± 268^{d}
15	0	28 ± 4^{a}	$948 \pm 164^{b,c}$
15	2.5	$33 \pm 5^{a,b}$	$1323 \pm 527^{c,d}$
15	5	$35 \pm 3^{b,c}$	$1271 \pm 495^{c,d}$

1988; Hall and Anderson 1995) because the osmolality between blood and external medium are in equilibrium. This physiological response indicates that osmoregulatory mechanisms play an important role in metal bioavailability (Hall and Anderson 1995; Ardiansyah, Irawan, and Soegianto 2012). Our result was consistent with this physiological phenomenon, at salinities 10 and 15 PSU (near the isosmotic point of this species (Febry and Lutz 1987; Hassan et al. 2013)), the accumulations of Cd in gills were lower than at 0 and 5 PSU.

MT levels were determined on the gills of control and Cd-exposed fish at different salinities (Table 1). In the control organisms (without Cd), there were no significant differences in the MT levels in gills of fish exposed to salinities 0, 5, and 10 PSU ($p > 0.05$). Although the level of MT in gills of fish exposed to salinity 15 presented the highest level; however, statistically was not different with those of fish exposed to salinities 5 and 10 PSU ($p > 0.05$). The MT concentrations in gills of Cd-exposed fish were significantly higher than those in respective control groups at salinities 0, 5, and 10 PSU ($p < 0.05$), while the cadmium exposure did not influence the MT levels in gills of fish exposed to salinity 15 PSU ($p > 0.05$). The highest level of MT was observed at gills of fish exposed to 5 mg/L at salinity 0 PSU. The MT concentrations of gills of fish exposed to 2.5 and 5 mg/L at salinity 0 PSU increased significantly by 110% and 128%, respectively, when compared to that of the controls ($p < 0.05$). At salinities 5 and 10 PSU, the MT concentrations of gills of control and Cd-exposed fish have similar tendency. The MT concentrations in gills of fish exposed to 2.5 and 5 mg/L were higher than those of the control fish ($p < 0.05$); however, the levels of MT in gills of fish exposed to 2.5 mg/L were not different than those exposed to 5 mg/L. There were no significant differences in the MT levels in gills of fish exposed to the different Cd concentrations at salinity 15 PSU ($p > 0.05$).

The changes in Cd accumulation due to salinity were reflected in the MT levels (Table 1). The MT concentrations substantially increased with decreasing salinity, with fish exposed to 5 mg/L at 0 PSU presented the highest MT concentration. The hybrid tilapia demonstrated the higher level of MT in hepatic tissue after 5 and 15 days exposure to Cd compared to the control, but there were no significant difference between the 5 and 15 days treatment groups (Wu, Shih, and Ho 2007). Atli and Canli (2008) reported that MT levels in liver of *O. niloticus* increased significantly with increasing Cd level in media. While Erk et al. (2008) reported the different results, i.e. no significant differences in MT levels between the control and Cd-exposed groups of mysid *Neomysis integer* were observed. They suggested that Cd concentration used in their study was too low to induce significant increases in MT levels. They also demonstrated that high molar ratios of essential metals versus Cd indicated an excess of metals other than Cd (especially Cu and Zn) that can bind to MTs. Hogstrand and Haux (1991) suggested that if ions of Cd enter the cell, the synthesis of MT will increase and homeostasis will be restored by incorporation of Cd into MT. In fish MT has a prominent role in reducing the toxicity of heavy metals (Hamilton and Mehrle 1986). The sequestration of metals by MT is not a static system, and the rate of MT synthesis increased with increasing time and level of exposure to water-borne metals (McCarter and Roch 1984; Hogstrand and Haux 1991). The increased MT in gills of Cd-exposed fish in our findings is suggested that MT played a prominent protection against Cd toxicity. From mammalian studies, there is some evidence of a

decreased toxicity of Cd when it is bound to MT (Roberts and Schnell 1982; Beattie, Marion, and Denizeau 1987).

It has been suggested that the toxic effects of Cd will occur when the binding capacity of MT is exceeded (Brown and Parsons 1978). At low doses, metal ions bind to MT, whereas at higher doses the metal ions spill-over to bind to high-molecular-weight proteins and cause tissue damage (Winge, Premakumar, and Rajagopaean 1975). Our results showed that the binding capacity of MT to Cd is not yet exceeded the threshold level, because the level of Cd in gills still increased with increasing the concentration of Cd in medium at all salinity groups. The increased rate of MT synthesis seems to be similar to the rate of Cd influx, thus it offered protection against Cd toxicity (Hogstrand and Haux 1991).

3.2. Histology

Lifting of the outer layer of the lamellar epithelium was observed in the gills of fish exposed to 2.5 mg/L for 7 days at salinity 0 PSU (Figure 1(c)), meanwhile a pronounced alteration of secondary lamellae resulting telangiectasis was noted in the gills of fish exposed to 5 mg/L for 7 days at salinity 0 PSU (Figure 1(d)). Hyperplasia of the epithelial cells of secondary lamellae was observed in fish exposed to 2.5 mg/L for 7 days at salinity 5 PSU (Figure 1(e)), whereas epithelial lifting of secondary lamellae was observed in fish exposed to 5 mg/L for 7 days at salinity 5 PSU (Figure 1(f)). The gills of fish exposed to 2.5 and 5 mg/L at salinities 10 and 15 PSU presented similar structural alteration, with lower level of damage when compared to those exposed to the same Cd levels at salinities 0 and 5 PSU. Epithelial lifting and hyperplasia of the secondary lamellae were observed only at certain gills (Figure 1(g,h)).

Our results showed that the environmental Cd concentrations were adequate to cause histological changes in the gills. Significant gill damage occurred in fish exposed to Cd at lower salinity. Epithelial lifting was noted at gills exposed to 2.5 mg/L at 0 PSU, and telangiectasis was observed at gills of fish exposed to 5 mg/L at 0 PSU. The level of gill damage decreased with increasing salinity of media. Histological alterations of gills such as lamellar epithelium lifting, epithelial hypertrophy and hyperplasia, fusion of lamellae, and lamellar telangiectasis have been also observed by several authors in fish exposed to metals (De Boeck et al. 2001; Cerqueira and Fernandes 2002; Heerden, Vosloo, and Nikinmaa 2004; Martinez et al. 2004; Figueiredo-Fernandes, Ferreira-Cardoso, and Garcia-Santos 2007; Aldoghachi et al. 2016). The lifting and hyperplasia of lamellar epithelium could serve as a mechanism of defense, because separation epithelial of the lamellae increases the distance across which waterborne metals must diffuse to reach the bloodstream (Mallat 1985; Arellano, Storch, and Saraquete 1999). Heerden, Vosloo, and Nikinmaa (2004) more specifically reported that gill alteration such as thickening in gill epithelium and lamellar telangiectasis occurred in rainbow trout *Oncorhynchus mykiss* after being exposed to copper for 4 h; however, after 48 h in copper-free water, recovery took place to a certain extent. The changes in gill morphology due to exposure to Cd in our findings could be a compensatory response to keep metal from entering through gill cells.

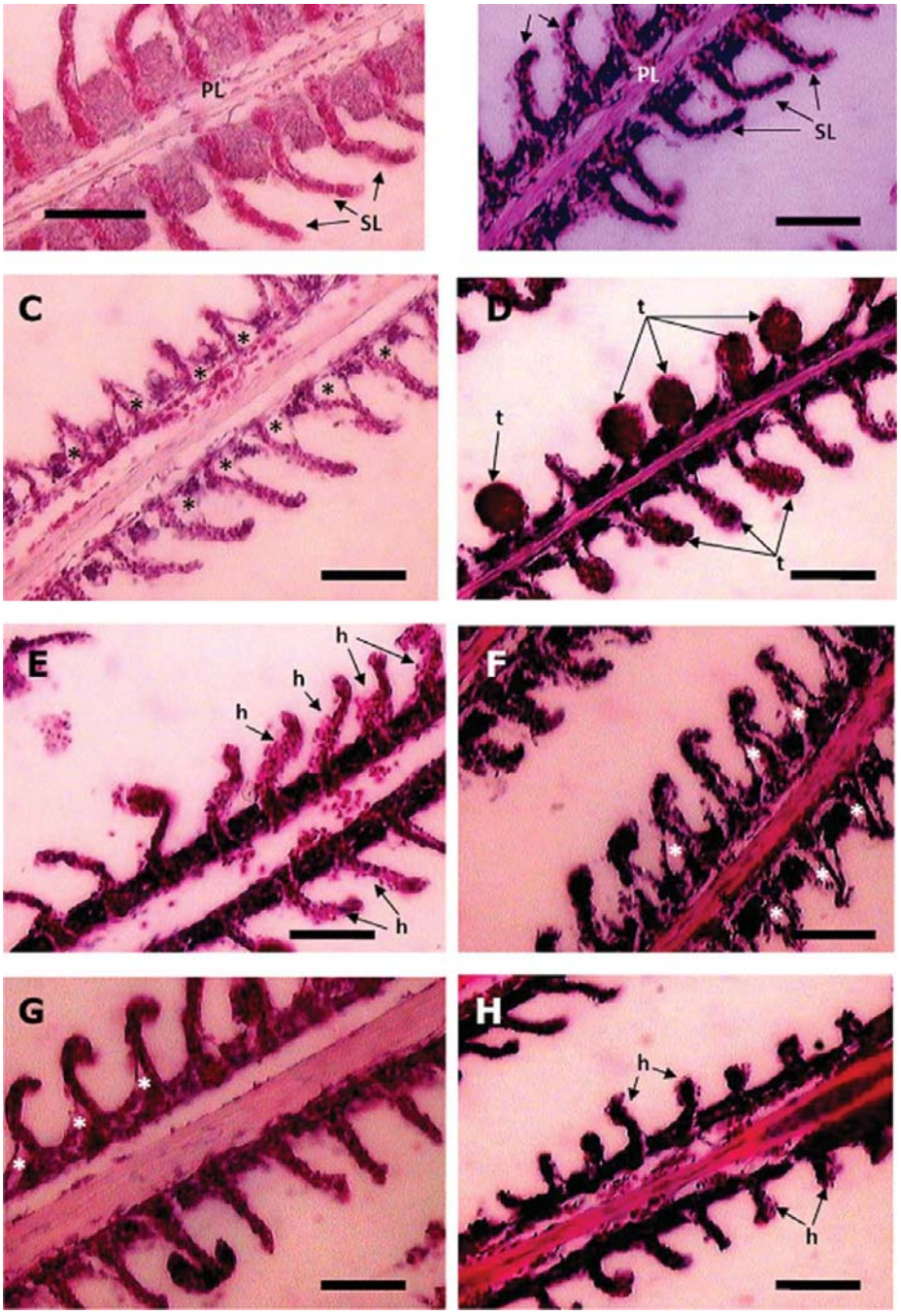

Figure 1. Histological sections of gills of controls and Cd-exposed *Oreochromis niloticus* at different salinities. (a) Gills of control fish (without Cd) exposed to salinity 0 PSU; (b) Gills of control fish exposed to salinity 15 PSU; (c) Gills of fish exposed to 2.5 mg Cd/L at salinity 0 PSU; (d) Gills of fish exposed to 5 mg Cd/L at salinity 0 PSU; (e) Gills of fish exposed to 2.5 mg Cd/L at salinity 5 PSU; (f) Gills of fish exposed to 5 mg Cd/L at salinity 5 PSU; (g) Gills of fish exposed to 5 mg Cd/L at salinity 10 PSU; (h) Gills of fish exposed to 5 mg Cd/L at salinity 15 PSU. PL, primary lamellae; SL, secondary lamellae; h, hyperplasia; t, telangiectasis; *epithelial lifting. Bar = 50 μm.

4. Conclusion

Our results demonstrate that the physico-chemical and physiological factors affect the accumulation of Cd in gills of tilapia at different salinities. The Cd concentrations in gills of Cd-exposed fish decreased with increasing salinity of media. These results confirm that Cd accumulation occurs primarily due to complexation of the free Cd ion by chloride ion. At the physiological point of view, the Cd uptake by this euryhaline fish most probably occurs via osmoregulatory mechanisms. Our results also show that MT levels in gills of *O. niloticus* increased significantly in Cd-exposed fish which support the hypothesis that MT is a significant factor for the accumulation of heavy metals in fish and the level of MT reflects the degree of exposure at least at the concentration used in our experiments. In the histological level, we noted that significant gill damage occurred in fish exposed to Cd at lower salinity. The level of gill damage decreased with increasing salinity of media. Like MT, the histological changes in our findings could be a protective response of animals to toxic effect of Cd.

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Disclosure statement

The authors declare that there are no conflicts of interest.

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