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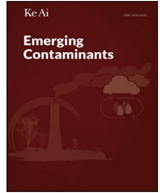

Change of osmoregulatory and hematological parameters in tilapia (*Oreochromis niloticus*) after exposure to sublethal mercury concentrations

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The effects of Hg exposure on blood parameters and gill physiology of tilapia (*Oreochromis niloticus*) were analyzed. Fish maintained in freshwater were exposed for 7 days (d) to sublethal mercury concentrations (0.1 and 1 mg/L). Blood serum osmolality (SO), sodium (Na^+), potassium (K^+) and chloride (Cl^-) ionic concentrations, and hematological parameters were assessed after 1 up to 7 d of exposure. Serum osmolality and ionic concentrations of exposed fish appeared differently affected throughout the experimental period compared to the controls. Osmolality was reduced at the 2 tested concentrations but Na^+ and Cl^- contents were only altered at 1 mg/L of Hg after 1 d of exposure and values rapidly returned to the control values thereafter. K^+ content was also modified and significantly increased at both concentrations after 1 d of exposure but returned to the control values after 3 d of exposure. Red blood cell (RBC), white blood cell (WBC) and hemoglobin (Hb) levels were significantly increased throughout the experiment but returned to control values after 7 d of exposure only for the 0.1 mg/L concentration. The hematocrit (Ht) levels remained unaffected due to Hg exposure. Therefore, tilapias exposed to sublethal concentrations of Hg present a marked osmotic imbalance with ionic and hematological disorders that are rapidly compensated.

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1. Introduction

Mercury is ubiquitously distributed in the environment, present in natural products, and exists extensively in items encountered in daily life. There are three forms of mercury, i.e., elemental (or metallic) mercury, inorganic mercury compounds, and organic mercury compounds. Common inorganic Hg sources include water and air pollution, skin lightning products containing mercury and mercury release from dental amalgam filling, two issues that happen in daily life, bear significant public health importance, and yet undergo extensive debate on their safety. Moreover, Hg emissions have been increasing each year due to rapid industrialization [1]. Specifically, 25 studies on fish were reviewed, and assimilation efficiencies ranged from 10% to 100% for MeHg and from 2% to 51% for Hg(II) [2]. The biggest incident caused by Hg contamination (Minamata disaster) that occurred in Japan in the 1950s, raised enormous concerns about Hg contamination. For example various concentrations of mercury in fish muscle have been recorded from canned fish marketed in the USA (Georgia and Alabama) [3]. More recently, it was found that mercury concentrations in panga fillets can range from 0.1 to 0.69 mg/kg [4]. Some of the analyzed samples were close to or above the limit of mercury set by the European legislation at 0.5 mg/kg. A widespread Hg contamination was also observed in different fish communities throughout the Western US and Canada, reflecting a broad gradient in Hg availability and cycling in the environment [5]. In Thailand, a Provisional Tolerable Weekly Intake (PTWI) established by the Joint FAO/WHO Expert Committee on Food Additives is 0.3 mg of total mercury per capita of which no more than 0.2 mg should be present as methylmercury [6].

Compared to terrestrial animals, fish have to cope with more challenging osmotic and ionic gradients from aquatic environment due to ionic composition fluctuations. Teleosts need to have highly

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efficient osmoregulatory mechanisms in order to maintain their body fluid homeostasis, which is necessary for optimal physiological processes. Several studies selected fish as an experimental model to assess mercury effects in aquatic organism [5,7–9]. It appears that carnivorous fish have higher levels of mercury than non-carnivorous (omnivorous and benthophagic) fish [7]. Cardoso et al. [8] also reported that inorganic mercury [Hg(II)] and MeHg follow an identical accumulation pattern in the brain overtime, showing a significant increase after 3 d of exposure. However, differences in mean Hg concentrations and accumulation rates have been reported between four different fish species (*Mycteroperca microlepis*, *Lutjanus campechanus*, *Caulolatilus microps*, and *Serioli dumerili*) [9]. Some results on plasma osmolality of tilapias exposed to sublethal concentration of mercury are also available but different responses were observed after 1 d of exposure and after 7 d of exposure [10]. One of several studies used 1 mg/L concentration of Hg and they found it was accumulated in gills, muscle, liver, testes, and ovaries [11]. Knowledge on hematological characteristics is also a potential key biomarker tool that can be used as an effective and sensitive index to monitor physiological changes in fish but no data are yet available on tilapias exposed to mercury [12,13].

In this study, the East Java strain of the tilapia *Oreochromis niloticus* was chosen as a model fish species, not only because the existence of Hg from gold mining activities in East Java, but, also, because it is one of the most consumed freshwater fish in Indonesia and Food Agriculture Organization of United Nations stated tilapia is the second most important fish in the world after carps. It can face a wide range of environmental conditions and is abundant in many rivers of the area [14,15]. Nevertheless, since freshwater ponds used in tilapia farms are often polluted with significant amounts of Hg from both atmospheric and anthropogenic sources, there is a considerable concern regarding the daily effects of Hg in tilapias. As reported by Soegianto et al. [16] the concentrations of Hg in cockles in certain location of East Java Coast were found to be higher than the permissible limit for human consumption, according to Indonesia and international standards. Therefore, we experimentally explored the physiological and hematological effects of short-term accumulation of mercury on the East Java strain of *O. niloticus* kept in freshwater, by measuring blood serum osmolality, ion concentration levels and hematological parameters over a 7 d exposure time. Gill Na^+-K^+-ATPase (NKA) concentration was also analyzed after a 4 d exposure. This study further enhances the comprehensive understanding of tilapia physiology when exposed to sublethal concentrations of mercury. It may also be useful for health risk assessment in the fish industry because Hg exposure can be a critical factor in areas where tilapias are cultured.

2. Materials and methods

2.1. Fish acclimation

Nile tilapia (*Oreochromis niloticus*) (East Java strain, local name: Jatimbulan), were obtained from the Freshwater Aquaculture Development Unit of East Java (UPT-PBAT) in Pasuruan, East Java, Indonesia. After transporting the fish to the laboratory rearing facility of Universitas Airlangga, animals were maintained in fiber tanks filled 250 L of continuously aerated freshwater (FW) and natural light system. After fish were fasted 2 d, fish were fed with commercial dried food (fish pellets containing 30% of proteins, 3% of fat and 4% of fibers) (Takari, Sidoarjo, Indonesia) at a daily ratio of 1% of the fish estimated body weight [17]. As suggested by Nursanti et al. [18], fish were acclimated for 14 days under laboratory conditions before the start of the experiment. Feces and other waste materials were siphoned off daily to reduce ammonia content in the water. Temperature ranged from 28 to 30 °C. The experiment in this study were conducted with the principles and procedures that were approved by the Institutional Animal Care of Universitas Airlangga.

2.2. Acute toxicity concentration estimation

A stock solution of Hg (1000 mg/L) was prepared by dissolving 1.3539 g $HgCl_2$ (Merck, Germany) in 1 L deionized water and stored in borosilicate glass containers. Range-finding test in order to obtain information about the range of concentrations to be used in the main test were done. The 4 d LC_{50} for Hg in *O. niloticus*, an acute toxicity bioassay was conducted in a static system. The chosen Hg concentrations were 0.1, 0.5, 1, 1.5 and 2 mg/L along with a control condition (dechlorinated tap water). For each condition, ten fish were used for each designed concentration and mortality was thoroughly monitored, with the test being repeated two times (twenty fish for each concentration). During the toxicity test, observations of the fish test sample were performed based on fish mortality. The 4 d LC_{50} value was determined using the Spearman-Karber method.

2.3. In vivo exposure experiment

Based on the LC_{50} values obtained with the acute toxicity test, a control, a lower (0.1 mg/L) and a higher (1 mg/L) sublethal concentrations of Hg were chosen for the *in vivo* exposure experiment. The Hg concentrations used in this study were potentially encountered by fish in the contaminated aquatic environment (0.27–0.39 mg/L) [6,19]. A total of 150 fish (total length 15.8 ± 0.7 cm; body weight 69.8 ± 1.1 g) were randomly selected and transferred to different aquariums containing 40 L of water (2 aquariums per concentration and time period) continuously aerated in natural light system. Fifty percent of the water in the tank was changed every 2 d. The experiment lasted 7 d. During that period, water parameters (temperature, pH, and dissolved oxygen concentration) were obtained. Temperature ranged from 28 to 30 °C, dissolved oxygen concentrations and pH values were comprised between 7.1 and 7.7 mg/L (Lutron DO 5510, Taiwan) and 7.85 and 8.20 (Hanna HI 98150, Beijing, China), respectively. Blood and gill samples from 60 fish in total were collected at different times (after 1 d up to 7 d exposure) and from different individuals (5 fish that were randomly collected for each time period from the 2 replicate experiments) while blood and gills sampling from the control group was conducted after 1 d (Table 1). There were five fish in each tank and there were 30 tank used in total, five fish were randomly collected from two tanks of each treatment (0.1 and 1 mg Hg/L) at the intervals of 1 d (D1 group), 2 d (D2 group), 3 d (D3 group), 4 d (D4 group), 5 d (D5 group), 6 d (D6 group), and 7 d (D7). The sampling from the control group was conducted at once (after 1 d). The control group was collected once because according to the previous researcher there was no different parameter of the fish in the control group (without treatment), the fish without treatment had stable condition. In addition, gill and kidney samples were collected from fish after a 4 d Hg exposure for NKA determination.

2.4. Serum osmolality, ion concentration, hematology and NKA concentration assay

Prior to blood sampling, fish were anesthetized with clove solution (200 mg/L) according to the method described by Soegianto et al. [20]. Blood from each fish was obtained by puncturing the heart using a non-heparinized syringe. For each individual, one blood sample was introduced to Vacutainer tubes containing 10.5 mg of anti-coagulant K3-EDTA (tripotassium-ethylene

Table 1
The experimental design and number of fish analyzed from each experiment group.

Hg (mg/L)	Number of Tanks Used	Treatment Day (Fish Sampled/Total Fish)						
		D1	D2	D3	D4	D5	D6	D7
0	2	5/10						
0.1	14	5/10	5/10	5/10	5/10	5/10	5/10	5/10
1	14	5/10	5/10	5/10	5/10	5/10	5/10	5/10

Note: Five fish were randomly collected from two tanks of each treatment (0.1 and 1 mg Hg/L) at the intervals of 1 d (D1 group), 2 d (D2 group), 3 d (D3 group), 4 d (D4 group), and 5 d (D5 group), 6 d (D6 group), and 7 d (D7). The sampling from the control group was conducted at once (after 1 d).

diaminetetraacetic acid) in order to determine different hematological parameters. Another blood sample was inserted into microtubes for serum osmolality and ion concentration determinations. Tubes containing blood samples were centrifuged at 5000 rpm for 10 min at 4 °C to separate blood serum from blood cells. Fish sera were measured for osmolality, Na^+, Cl^- and K^+ concentrations. Medium and serum osmolalities were measured using an automated freezing point depression osmometer (Fiske® 210 Micro-Sample Osmometer, Norwood, Massachusetts, USA) and expressed as mOsm/kg. In a freezing point depression osmometer, a sample of serum is introduced to a cooling chamber where it is cooled to a temperature below its expected freezing point. The sample is stirred with a wire, which causes the sample to become partially crystallized. The heat generated from this process brings the sample to a temperature that is equal to its freezing point. Serum Na^+, Cl^- and K^+ concentrations were measured by electrolyte analyzer (SpotChem EL SE-1520, Kyoto, Japan). Blood samples from Vacutainer tubes containing EDTA were aspirated directly into the automated hematology analyzer (AHA) [21,22]. AHA (SFRI Blood Cell Counter 33, Jean d'Illac, France) was used to assess the hematological parameters: RBC counts, Ht levels, Hb concentrations. The SFRI blood cell counter 33 uses the electric resistance detecting method (impedance technology) with hydro dynamic focusing to measure RBC and Ht. Hb is measured photocolorimetrically using sodium lauryl sulfate-Hb, a cyanide-free method. The reagents required for the operation of the Blood Cell Counter 33 are supplied by SFRI Corporation. Because fish has nucleated RBC, which potentially interfere with the WBC count on many types of automated hematology analysers [23]. This makes it necessary to count the WBC manually. The total leukocyte count was determined by the haemocytometer method of Campbell [24]. Blood sample (20 μL of blood) was added to 0.38 ml of white blood cell diluting fluid in a clean test tube, to make a 1:19 dilution. A drop of the diluted blood was charged onto a Neubaeur chamber and allowed to settle for 2 min. The ×40 objective lens of the light microscope was used in making a total count of leukocytes on the four corner squares. The number of cells counted for each blood sample was multiplied by 50 to obtain the total leukocyte count per microlitre of blood [24].

Dissected gills and kidneys were rinsed in PBS (pH 7.4) to remove excess blood thoroughly and weigh before homogenization. They were homogenized individually in PBS (pH 7.4) with a glass homogenizer on ice and centrifuged at 2000–3000 rpm for approximately 20 min. We assessed NKA concentration after D4 to provide acute toxicity assessment. A sandwich-ELISA was used to measure NKA according to the instructions from Bioassay Technology Laboratory biotech Co. Ltd, Shangai, China. All microtiter plates provided in the kit were pre-coated with an antibody specific to NKA. To measure NKA concentration, 50 μL of a standard sample, a blank and 40 μlL of tissue sample were added to each well. Immediately after, 10 μL of biotinylated detection Ab working solution (the detection antibody in the NKA kit is a fish monoclonal Ab) and 50 μL of horseradish peroxidase (HRP) were added to each well but not to the blank control, mixed well, and covered with the plate sealer supplied by kit manufacture. The microplates were incubated for 60 min at 37 °C. The sealer was removed and each plate was aspired and washed 5 times with wash buffer, automatically. The plates were blotted onto paper towels. Then, 50 μL of substrate solution A and 50 μL of substrate solution B were added to each well, and the plates were resealed and incubated for about 10 min at 37 °C in the dark. To terminate the enzyme reaction, 50 μL of stop solution was added to each well, the blue color changed into yellow immediately and optical density was determined using an automatic microplate reader (Bio-Rad, model iMark, Japan) at 450 nm within 10 min after adding the stop solution. The NKA concentrations in the gills and kidneys were determined using the appropriate standard curves and data were expressed as ng/mL.

2.5. Hg concentration in gills

Five fish form each treatment group were dissected and the gills of each animal were removed, dried at 60 °C for 48 h to constant weight. The measurement of Hg in gills followed the Candra et al. [25]. Approximately 2 g of homogenized tissue sample from each fish was digested in acid solutions 2 mL of $HNO_3 - HClO_4$ (1:1) and 5 mL H_2SO_4 at 80 °C for 3 h in Mars 6 microwave digester. After cooling, 0.1 mL of $KMnO_4$ and 0.5 mL of $SnCl_2$ solutions were added until the purple color of the solutions stabilized. Sufficient hydroxylamine hydrochloride solution was added to neutralize the excess potassium permanganate as a preservative. The solution was adjusted to 50 mL with deionized water. An aliquot was taken for mercury determination using flameless atomic absorption spectrophotometer (Mercury-Hydride System Analitik Jena, HS 60). Hg concentrations of samples were expressed as μg/g wet weight. The detection limit of mercury was 0.003 μg/g.

Analytical blanks were run in the same way as the samples and the concentrations were determined using standard solutions prepared in the same acid matrix. The accuracy and precision of the analytical performance were validated by measuring the dogfish muscle reference material (DORM-4) provided by the National Research Council of Canada. The recoveries for Hg in the tissue standard reference material DORM-4 were 97 ± 5%. All reagents used for this analysis were of analytical grade.

2.6. Statistical analysis

Data are expressed as mean ± standard error and were verified their normality and homogeneity before used for statistical analysis. All statistical analyses were performed using IBM SPSS version 21 (IBM Corp. Armonk, New York, USA). If data did not meet the assumption of normality and homogeneity of variance, data were log transformed. Statistical analysis of the data was performed using one-way ANOVA followed by a Tukey's HSD post hoc comparison test to evaluate daily effect of Hg on serum osmolality, serum ions, hematological parameters and Hg level in gills. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Accute toxicity concentration estimation

Fish were exposed to different Hg concentrations (0.1, 0.5, 1, 1.5 and 2 mg/L of Hg) and the percentage of mortality was: 10, 15, 15, 95 and 100%, respectively. We observed that fish maintained at the concentration of 2 mg/L can only survive for a few hours. Based on these data, the 4 d LC_{50} value for Hg was calculated to be 1.22 ± 0.743 mg/L.

3.2. Serum osmolality

No fish death during experiment. Serum osmolalities in fish exposed to Hg at 0.1 mg/L were significantly lower than the osmolality value of the controls at d 2, d 5, and d 6. Serum osmolalities in fish exposed to Hg at 1 mg/L were significantly lower than the osmolality value of the controls from d 1 up to d 4 (Fig. 1a). The decrease appeared constant (between 16 and 23%) when fish were exposed to the lower Hg concentration. For fish exposed to 1 mg/L the decrease was up to 31% at the beginning of the exposure period and was then similar to the decrease observed for the lower Hg concentration.

3.3. Serum ions

Sodium content in blood serum of control fish and those exposed up to 7 d with 0.1 mg/L Hg remained constant (Fig. 1b). However, Na^{+} concentration in the serum of fish exposed to 1 mg/L of Hg was significantly lower than the controls after 1 d of exposure ($p < 0.05$) (Fig. 1b). Sodium concentration then increased and reached the sodium content observed in the control group and in fish exposed to the lower Hg concentration.

Similarly, chloride concentration in the serum of control and exposed fish to 0.1 mg/L of Hg remained constant (Fig. 1c) but at the higher tested concentration, a significant decrease in Cl^{-} content was observed after 1 d of exposure (almost 0.25 fold decrease). Values then reached the chloride concentration observed in the control group with no significant difference for the fish exposed for d 2, d 3, d 4, d 5, d 6, and d 7 with 0.1 and 1 mg/L of Hg and with the controls.

However, K^{+} concentrations in blood serum significantly increased in fish exposed to 0.1 and 1 mg/L of Hg compared to the controls. The maximum potassium content was observed after d 1 and d 7 of exposure with the higher and the lower Hg concentration, respectively (Fig. 1d).

3.4. Gill NKA concentration

After 4 d of exposure, gill and kidney NKA concentrations were not significantly different among the different treatments. Tilapia fish exposed with the lower Hg concentration showed a slight increase in gill NKA concentration compared to the controls (up to 23%) but fish exposed to the higher Hg concentration had similar values compared to the controls (Fig. 2).

3.5. Hematological parameters

The hematocrit value of fish exposed up to 7 d to the sublethal concentrations of 0.1 and 1 mg/L of Hg remained constant up to d 5

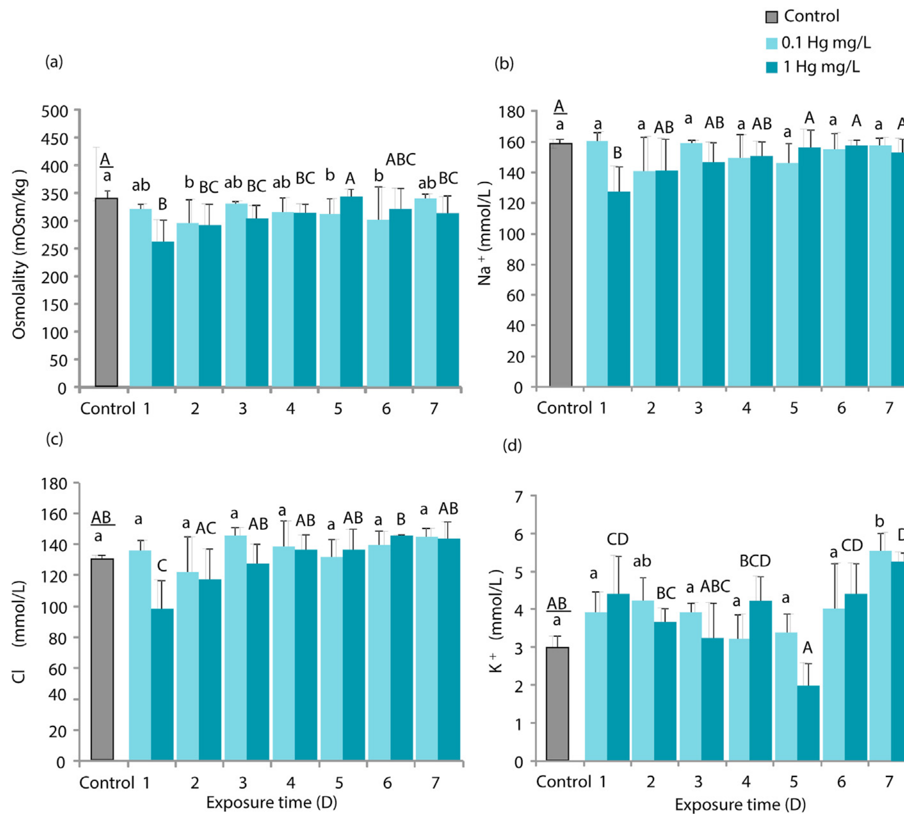

Fig. 1. Blood serum osmolality and ion concentration of *O. niloticus* exposed to lower and higher concentration of Hg: (a) Daily blood serum osmolality; (b) Daily Na^{+} concentration; (c) Daily Cl^{-} concentration; and (d) Daily K^{+} concentration. Lowercase letters indicate significant differences at different exposure times for tilapias kept at the lower concentration. Uppercase letters indicate significant differences at different exposure times for tilapias kept at the higher concentration. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's HSD post hoc comparison test, $p < 0.05$).

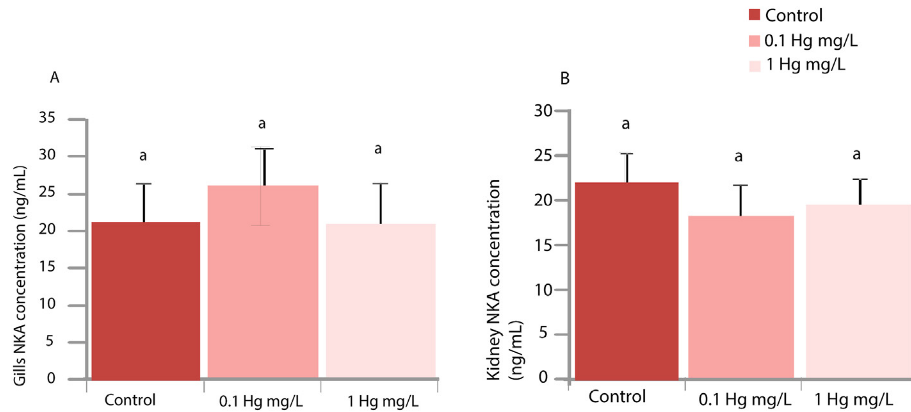

Fig. 2. NKA concentration in gills and kidneys of *O. niloticus* exposed to lower and higher concentration of Hg for 4 d. Lowercase letters indicate significant differences.

and decreased compared to the control group at d 6 and d 7 groups (Fig. 3a). However, RBC, Hb, and WBC content of exposed fish were not significantly different at d 6 and d 7 groups. (Fig. 3a).

For RBC content of fish exposed to the lowest Hg concentration, the RBC values increased after 1 d, 2 d, 3 d and 4 d of exposure compared to the controls (Fig. 3b). The highest values were recorded after 2 d of exposure. For the higher Hg concentration, values were also increased compared to the controls. Red blood cell content remained elevated up to 7 d of exposure, with a peak after 3 d of exposure and a slight decrease observed after 4 d of exposure.

Fish hemoglobin present a similar pattern (Fig. 3c). For fish exposed to 1 mg/L of Hg, an immediate increase occurs after 1 d of exposure reaching a plateau with elevated values up to 7 d of exposure. At the lower Hg concentration, hemoglobin content is also increased but recorded values decrease after 7 d of exposure to reach values that are similar to the controls. White blood cell content is also increased due to Hg exposure (Fig. 3d).

3.6. Hg accumulation in gills

The Hg accumulation in gills of fish exposed up to 7 d to the sublethal concentration (0.1 and 1 mg/L) of Hg decreased starting at D2 to reach values that are similar to the control group at D5-D7 (Fig. 4). The highest values were recorded after 1 d of exposure.

4. Discussion

As seen in this study, short-term exposure to low concentrations of mercury down to 0.1 mg/L affects blood serum osmolality of freshwater *O. niloticus* fish. Disruption of the osmotic balance of fish

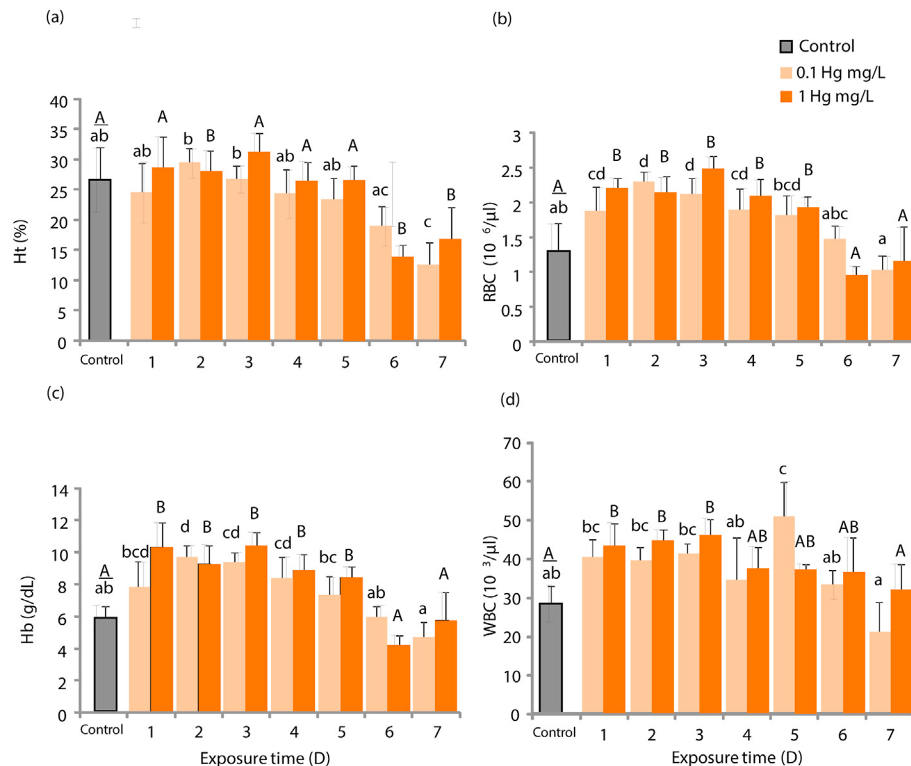

Fig. 3. Hematology of *O. niloticus* exposed to lower and higher concentration of Hg: (a) Daily hematocrit value (Ht); (b) red blood cell (RBC) content; (c) hemoglobin (Hb) content, and (d) white blood cell (WBC) content. Similar statistical labelling as in Fig. 1.

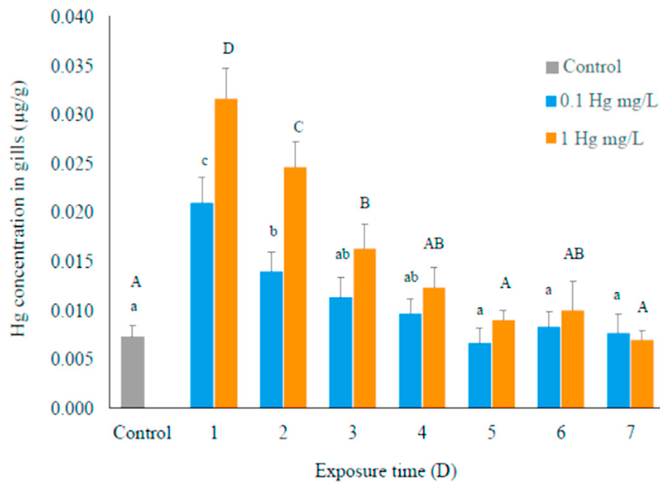

Fig. 4. Daily Hg accumulation in gills of *O. niloticus* exposed to lower and higher concentration of Hg. Similar statistical labelling as in Fig. 1.

exposed to lethal and sublethal concentrations of mercury is typically observed with a decrease in plasma concentrations of Na^+ and Cl^- [10,26]. For example, the first day of exposure with 0.5 mg/L of mercury causes osmoregulatory failure in the freshwater cichlid *Oreochromis aureus* with also a significant decrease in blood and plasma osmolality [10]. In freshwater-adapted fish, maintenance of a proper ionic balance of the blood is typically performed at the gill level with the active uptake of Na^+ and Cl^- to compensate for the urinary salt excretion and passive loss through permeable body surfaces. Since Na^+ and Cl^- are the major ions in the blood accounting for 80–90% of the blood osmolality, their tight regulation is critical for osmoregulation [27]. As expected, Na^+ and Cl^- content for *O. niloticus* exposed to lower and higher Hg concentrations was significantly decreased after 1 d of exposure while K^+ content in the blood was inversely increased. Within 7 d of exposure, ionic concentrations for these major osmolytes returned to values that were observed in control fish. Therefore, after an initial osmotic imbalance, exposed tilapias can quickly restore their ionic concentration although fish are still exposed to mercury. Furthermore, blood osmotic pressure remains affected despite an ionic adjustment.

This can be indicate MeHg is the greatest contributor to the overall mercury bio-accumulation and that dietary exposure is the predominant pathway compare to waterborne Hg treatment, waterborne Hg plays a relatively small role in overall bio-accumulation by fish in most conditions [28,29]. Previous study reported that the highest Hg accumulation was at the first 24 h exposure and after 4 d exposure Hg accumulation in gills decreased [30]. Flienko et al [31] also did not find significant morphological changes in carp in sublethal Hg concentration. In the lethal concentration, as long as the fish can increase the oxygen consumption, fish would not severe damage to gill's structure permanently. In this study, we observed the large quantity of mucous secretion, this also acts as a defense mechanism against several toxic substances [30].

In a hyper-osmotic environment, fish gills are permeable to K^+ and that K^+ efflux is greater than influx [32]. This would indicate that in unpolluted freshwater, reduced K^+ uptake, rather than increased loss of K^+, is the most important factor [33]. Thus, the initial increase and subsequent decrease in blood serum K^+ concentration due to sublethal Hg exposure could be ascribed as an osmotic adaptation in order to counterbalance the loss of Na^+ [34]. Changes in osmoregulation in fish exposed to some stressor have been generally elucidated by measuring the blood plasma (or serum) electrolytes and/or total osmolality [35]. Our previous study found that salinity can reduce the lethal effects of mercury [36] therefore, to maximize the prevention of osmolality and ion imbalances due to mercury exposure it is recommended that salinity be given to live fish media as soon as possible because osmolality and ion imbalances occur from the first day mercury exposure.

Disruption of the osmotic balance and ionic imbalance in fish blood exposed to mercury has been attributed to a decreased activity of the NKA in the gill epithelial ionocytes that are specialized in ion transfer between the surrounding environment and the blood [26,37,38]. A significant decrease in gene expression for NKA has also been described in the gills of zebrafish *Danio rerio* after 4 d of exposure to a low concentration (7.7 μg/L) of mercury although, gene and protein expressions are increased at a high concentration (38.5 μg/L) [39]. Furthermore, the number of ionocytes is greatly increased when fish are exposed to the high concentration of mercury, these cells being located along the primary gill filament and along the respiratory lamellae [39]. Concentration of Hg 1 mg/L was exposed to *Tinca tinca* and accumulated in gills after d 4 exposure [11]. However, in our study, for freshwater tilapia *O. niloticus* exposed to sublethal concentrations of mercury (0.1 and 1 mg/L), no change in NKA content was observed after d 4 exposure. Therefore, more studies are needed to better understand the reasons that underpin the variable responses observed in the gene and protein expressions of this enzyme due to mercury exposure.

Gills have been established as the main route of mercury uptake [40]. It induces osmoregulatory dysfunctions that precede the appearance of gross morphological changes such as hyperplasia and ectopia of ionocytes, lamellar fusion, increased mucous secretion, alteration of pavement cells (PVCs), detachment of the secondary epithelium, pillar cell degeneration, degeneration, and apoptosis. For example, the fourth day mercury exposure at a concentration as low as 7.7 μg l^{-1} induces severe morphological and ultrastructural changes in zebrafish gills [39]. In some areas of the primary epithelium, the typical microridge arrangement on the apical side of the PVCs is missing. The gill secondary lamellae can also be folded and fused in the distal portion, thus occluding the interlamellar space. Other histological damages have also been observed in the gills of *Tilapia mossambica* and *Lates calcarifer*, such as tissue necrosis and ionocyte hypertrophy [41]. Modifications in gill water permeability have also been observed with an increased rate of water uptake by the gills [39]. The expression pattern of metallothionein (MTs) is also modified [18]. These alterations may play a defense role against contamination and may not represent the consequence of an irreversible toxic effect [41]. Furthermore, all these morphological and physiological changes at the gill level might cause respiratory disorders.

The binding action of inorganic mercury appears to occur in two phases. The rapid binding phase has a half-life of only a few minutes and is completed in less than 1.5 h. During this phase, mercury is easily removed with various complexing agents, affecting cellular structures rather than enzyme properties. The slow binding phase occurs with higher concentrations of mercury and binding is essentially [42]. Also, biokinetic modeling results indicate that MeHg is the greatest contributor to the overall mercury bio-accumulation and that dietary exposure is the predominant pathway. After 8 h of waterborne exposure, most of the accumulated mercury is in the gills, suggesting that the uptake of dissolved Hg is primarily through the gills as well as surface adsorption but, in tilapia, inorganic mercury is rapidly egested during the first day [43]. Such a rapid intake of Hg by the fish tissues that are also quickly egested by the digestive system may initially disrupt gene and protein expression of NKA. However, this appears to be rapidly compensated even if it leads to a profound and durable osmotic imbalance.

A significant change in plasma osmolality is the first measurable indicator of mercury toxicity, leading later to other hematological stress responses [10]. In our study, the Ht value, i.e., the volumetric fraction of RBC in the blood [44], remained constant during the short-term Hg exposure despite an increased number of RBC and WBC observed after d 1 exposure at the 2 tested concentrations but at last day of experiment Ht decreased while RBC and WBC were adapting to normal condition. It is assumed that during this exposure period, fish express genes that act on the immune system in red blood cells with the result an increased of RBC while Ht decreasing at the last day of experiment. This is in accordance with published data indicating that in fish, RBC not only play a major role in the transport of gas to cells and tissues but are also associated with the immune response [45]. Furthermore, the presence of heavy metals in fish is known to interfere with the synthesis of Hb [46]. Therefore, stress due to an inappropriate environment can affect hemoglobin level in fish and can impair physiological integrity. According to Clarkson et al. [47], about half of the mercury in the blood is associated with RBC and the remaining half forms a complex with serum albumin by combination with sulfhydryl groups. Thus, the accumulated mercury could produce some hematological and biochemical changes in the blood [48] leading for example to osmotic disorders. If Hb synthesis is inhibited, then, the ability to bind oxygen is reduced. Therefore, an increase in the number of RBCs and Hb content, as observed in our study, could compensate for such a reduced binding capacity. Hussan et al. [49] obtained a significant decrease in the O2 consumption rate in the Indian carp *Catla catla* with increasing mercury concentrations. The frequency of coughs and yawns can also be increased to compensate the increasing demand of oxygen and reduced supply of oxygen; this behavior increasing the gaseous exchange through the gills [50]. However, oxygen consumption measured in the shrimp *Penaeus setiferus* with postlarvae that had been exposed to sublethal concentrations of mercury for 60 days was not significantly different compared to the control group. This suggests that these very low concentrations of mercury (0.5 and 1.0 μg/L) are not physiologically stressful to the shrimp, or, that these animals have the ability to adapt to these low concentrations [51]. The increased number of leukocytes as seen in our study for tilapias exposed to mercury has also been observed for other fish species and with other pollutants. For example, an increase in the number of leukocytes was observed in *Channa punctatus* exposed to lead [52] and in *Clarias batrachus* exposed to Hg [53]. This increase has been attributed to a non-specific immune response to stress because stress hormones such as prolactin and cortisol are also involved to restore ionic balance [54].

5. Conclusion

In this study, data on osmotic and hematological parameters collected from tilapias exposed to sublethal concentrations of mercury revealed that the fish general physiology and immune system balance are rapidly affected (within the first day of exposure). However, exposed fish manage to rapidly restore their blood ionic concentration and defense mechanism, although blood osmolality is still impaired up to 7 d. This indicates that the overall energy expenditure of the fish must be increased even if they can withstand a short-term sublethal exposure with mercury. Future studies should identify the metabolic cascade affecting the fish within the first hours of exposure, and better determine the effects of blood cell proliferation and increased blood volume on the fish hydromineral balance and energy use. Also, the temporal control mechanism of NKA as a key enzyme in osmoregulation, should be further investigated in the gills but also in several other tissues (kidney, digestive tract). For the immune system, the ratio between the heterophils and the lymphocytes (H/L ratio) should be further investigated since it may indicate a stimulation of the innate immune system. Finally, longer exposure times should be considered to better understand the consequence of these defense mechanisms against mercury contamination on life-history traits.

Author contributions

K.S.H., A.S., conceived and designed the study; K.S.H. performed the laboratory work and laboratory analyses, A.S. supervised the laboratory work, and K.S.H, J.H.L and A.S. wrote this paper equally.

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Declaration of competing interest

The authors declare no conflict of interest.

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