KORESPONDENSI PAPER SYARAT KHUSUS GURU BESAR

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The Impact of Various Periods of Mercury Exposure on The Osmoregulatory and Blood Gas Parameters of Tilapia (*Oreochromis niloticus*)

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KORESPONDENSI PAPER SYARAT KHUSUS GURU BESAR

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No	Tanggal	Keterangan	Keterangan
1	4 Februari 2023	**Submission** Mendaftar (submit) pertama manuscript ke Jurnal Emerging Contaminants	Halaman 3
2	4 Februari 2023	**PDF for submission to Emerging Contaminants requires approval -** Manuscript dibuat format PDF oleh Jurnal PDF untuk naskah artikel telah dibuat oleh Jurnal dan memerlukan persetujuan Penulis.	Halaman 31
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5	5 Maret 2023	**Decision on submission to Emerging Contaminants – Review of Manuscript** Naskah dievaluasi oleh Reviewer. Reviewer merekomendasikan pertimbangan ulang naskah setelah revisi dan modifikasi substansial. Naskah dapat dikirimkan ulang setelah menanggapi komentar Reviewer dan Naskah harap dikirimkan ulang setelah direvisi paling lambat 04 Mei 2023. Saat merevisi naskah diharap mempertimbangkan semua masalah yang disebutkan dalam komentar reviewer dengan saksama: harap menguraikan setiap perubahan yang dibuat sebagai tanggapan atas komentar Reviewer dan memberikan sanggahan yang sesuai untuk komentar tersebut.	Halaman 37

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BUKTI KORESPONDENSI NO 1 -
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Emerging Contaminants

The impacts of sublethal mercury concentrations on the osmoregulatory and acid-base parameters of tilapia (Oreochromis niloticus) at various times

--Manuscript Draft--

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Article Type:	Original Research Article
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	Carolyn Melissa Payus, PhD
Abstract:	Mercury (Hg) can contaminate aquatic environments as a result of anthropogenic activity. Hg accumulates quickly in the tissues of fish and has the potential to affect their blood gas and electrolyte contents. Therefore, this study aimed to examine the impacts of sublethal mercury concentrations on blood gas and electrolyte levels of tilapia (Oreochromis niloticus) at various times. Fish were administered to sublethal concentrations of Hg (0.06 and 0.6 mg/L) for 4 and 15 days. At the end of the experiment, fish were collected from each treatment to examine the levels of Hg and carbonic anhydrase (CA) in the gills, plasma osmolality, ions, blood pH, pCO2, pO2, and hematological parameters. Only fish exposed to 0.6 mg/L Hg for 15 days had the greater Hg concentration than in the control gills. Hg inhibited respiration by generating metabolic acidosis, decreasing gill CA, reducing pO2, plasma osmolality, Cl-, Na+, and K+ only at 0.6 mg/L for for 15 days. Similarly, red blood cell, hemoglobin, and hematocrit levels decreased only at 0.6 mg/L for for 15 days. All of these impairments can limit a fish's ability to provide appropriate oxygen to its cells, hence diminishing its physical activity and productivity.
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February 4, 2023

Dear Editor-in-Chief
Emerging Contaminants

We wish to submit our original revised research article entitled "**The impacts of sublethal mercury concentrations on the osmoregulatory and acid-base parameters of tilapia (*Oreochromis niloticus*) at various times**" for consideration by **Emerging Contaminants**.

We confirm that this work is authentic and is not currently submitted or reviewed or published in any other journal.

Our study revealed that the accumulation of Hg in the gills of fish exposed to sublethal Hg concentrations rises with the Hg content of the medium and exposure duration. This accumulation has a considerable effect on acid-base parameters, osmotic and ionic regulation, and blood parameters, especially in fish exposed to greater Hg concentrations and for a longer duration.

This journal publishes works on all areas of toxicology and contaminants, such as the impacts of heavy metals on aquatic organisms, thus we believe this submission is suitable for publishing.

We have no conflicts of interest to disclose. Please address all correspondence concerning this manuscript to me at agoes_soegianto@fst.unair.ac.id and/or agoes_soegianto@unair.ac.id

Thank you for your consideration of this manuscript.
Sincerely,

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The impacts of sublethal mercury concentrations on the osmoregulatory and acid-base parameters of tilapia (*Oreochromis niloticus*) at various times

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Abstract

Mercury (Hg) can contaminate aquatic environments as a result of anthropogenic activity. Hg accumulates quickly in the tissues of fish and has the potential to affect their blood gas and electrolyte contents. Therefore, this study aimed to examine the impacts of sublethal mercury concentrations on blood gas and electrolyte levels of tilapia (*Oreochromis niloticus*) at various times. Fish were administered to sublethal concentrations of Hg (0.06 and 0.6 mg/L) for 4 and 15 days. At the end of the experiment, fish were collected from each treatment to examine the levels of Hg and carbonic anhydrase (CA) in the gills, plasma osmolality, ions, blood pH, pCO_2, pO_2, and hematological parameters. Only fish exposed to 0.6 mg/L Hg for 15 days had the greater Hg concentration than in the control gills. Hg inhibited respiration by generating metabolic acidosis, decreasing gill CA, reducing pO_2, plasma osmolality, Cl^-, Na^+, and K^+ only at 0.6 mg/L for for 15 days. Similarly, red blood cell, hemoglobin, and hematocrit levels decreased only at 0.6 mg/L for for 15 days. All of these impairments can limit a fish's ability to provide appropriate oxygen to its cells, hence diminishing its physical activity and productivity.

Keywords: water pollution; mercury, osmoregulation; acid-base balance; blood, fish

1. Introduction

Mercury is among the most hazardous pollutants that threaten aquatic ecosystems, as it is a strong neurotoxin for fish, wildlife, and humans [1]. Mercury (Hg) is released into the environment as a consequence of the natural weathering of rock or the activity of volcanoes. However, human activity is the principal source of mercury in the environment. This occurs through the combustion of coal to produce electricity and the discharge of waste from industrial processes [2]. Mercury is mostly deposited from the atmosphere in the majority of aquatic environments. The United States Environmental Protection Agency (USEPA) has determined that emissions from coal-fired power plants are the primary contributor to the presence of mercury in the atmosphere [3].

Mercury occurs naturally at very low concentrations in water. Commonly, uncontaminated freshwaters have 5 to 20 ng/L of total Hg, while contaminated waters can contain up to 0.5 µg/L [4]. Hg concentrations in river water near unregulated gold mining are between 12.7 and 13.6 µg/L [5]. In the vast majority of surface waters, the levels of Hg are typically much too low to have any direct adverse effects on either adult fish or the more sensitive early life stages of fish [6]. Acute toxicity of Hg to fish species varied, with 30 µg/L for a guppy *Lebistes reticulatus* [7], 580 µg/L for Mozambique tilapia *Orechromis mossambicus* [8], 327 µg/L for perch *Percocypris pingi* [9], 220 - 1220 µg/L for Nile tilapia *Oreochromis niloticus* [10, 11], 606 µg/L for fresh water climbing perch *Anabas testudineus* [12], and 900 µg/L for common carp *Cyprinus carpio* [13].

The bioavailability of Hg in teleost fish is dependent on the overall chemical concentration of the surrounding environment, which includes pH, salinity, hardness, hydrophobicity, and metals' interactions with biotic and abiotic ligands, as well as the fish's ability to absorb the various Hg forms at the gill, skin, and digestive system [14]. Hg accumulation may impair fish's immune

system [15, 16], respiratory and cardiovascular systems [17, 18], reproductive organs [19, 20], digestive system and excretory system [21], blood system [22], and enzymatic activity [23].

To our knowledge, relatively few researches have examined how Hg affects the acid-base parameters of fish. Prior research has shown that various metals such as zinc, copper, and lead alter the acid-base parameters of several fish species [24, 25, 26]. Metals and other contaminants may destroy gills, causing hypoxia and acid-base imbalance [27, 28].

Acid-base regulation in fish is linked to ionic regulation since it is primarily dependent on the continual exchange of H^+ and HCO_3^- for Na^+ and Cl^- through the gills. Carbonic anhydrase regulates CO_2 release, ion regulation, and acid-base balance [29]. Metals inhibit fish carbonic anhydrase in vitro [30, 31]. Nevertheless, in vivo studies on metals' impact on carbonic anhydrase in fish is lacking. Therefore, the effects of Hg on carbonic anhydrase in fish will be investigated in this study.

Oreochromis niloticus is a commercially significant species of fish, notably in Indonesia. Typically, this species reacts quickly to environmental changes [11]. Due to the fact that tilapia is often reared in freshwater that is continually polluted by metals from human activities, the effects of Hg on tilapia are of great concern. In this study, we evaluated the effects of sub-lethal Hg exposure on acid-base, osmoregulatory, blood parametrs, and gill CA in *O. niloticus* over four and fifteen days.

2. Materials and methods

2.1. Protocol for sample collection and laboratory acclimation

This research used tilapia *O. niloticus* from a fish farm in Pasuruan, East Java that measured 15.5 ± 0.6 cm in length and weighed 68.6 ± 1.2 g. A plastic bag containing oxygenated fresh water

was used to bring them to the laboratory. The animals were then acclimated at least for two weeks in laboratory acclimation tanks (250 L) using dechlorinated tap water at 28-29°C and 12-hour light/12-hour dark photoperiods. A biofilter made up of gravel, sand, and sponge filters, maintained water quality by recirculating the water continuously. Pellet fish meal equal to 1% of the fishes' daily estimated body weight was given to them [11]. To preserve the water quality at an adequate level for fish, excrement, uneaten food scraps, and other undesired things were removed daily. During acclimation and testing, daily measurements showed that the optimal ranges for temperature (28.6 ± 0.5 °C), pH (7.8 ± 0.3), and dissolved oxygen (7.3 ± 0.5 mg/L).

2.2. Experiment solution preparation

By dissolving 1.3539 grams of $HgCl_2$ (Merck, Darmstadt, Germany) in one liter of deionized water, a 1000 mg/L Hg stock solution was made. Our previous study demonstrated that the lethal concentration (96 h LC_{50}) of Hg to *O. niloticus* was 1.22 mg/L [11]. Based on this LC_{50} value the nominal concentrations of Hg used in this experiment were: 0.06 mg/L (5 % of LC_{50}, corresponding to 0.044 mg/L measured level), 0.6 mg/L (50% of LC_{50}, equivalent to 0.49 mg/L measured level), and control (equivalent to 0.001 mg/L measured level, does not contain Hg). Our experiments were conducted across two distinct time periods: 4 days (short term) and 15 days (long term).

2.3. Investigation of the effects of Hg on fish

Following the acclimation period 50 physically active fish were randomly picked from the acclimatized holding tank and put into 10 separate tanks, each containing 5 fish. Each tank had a capacity of 40 liters and was filled with testing medium: 0.06 mg/L of Hg for 4 days and 15 days,

0.6 mg/L of Hg for 4 days and 15 days, and the control. For each concentration, two tanks were used. Half of the test medium was replaced every 48 hours to keep the Hg content constant. At the end of experiment, 5 randomly selected fish were taken from each treatment to collect blood and gill tissue samples. Hg-containing experimental water was collected and kept in a metal waste water storage tank when the experiment was completed. Any operations that necessitated the use of animals were conducted in compliance with the Animal Care and Use Policy of Airlangga University.

2.4. Hg measurement in gills

Candra et al. [32] method was utilized to quantify Hg in the gills of tilapia. To achieve a consistent weight, tilapia gills were removed and oven-dried at 60°C for 48 hours. The dried gills were then ground into a powder. In a Mars 6 microwave digester, 0.5 g of powdered gills were subjected to acid solutions containing 2 mL of HNO_3 - $HClO_4$ (1:1) and 5 mL H_2SO_4 for 3 hours at 80 °C. After cooling, 0.1 mL of $KMnO_4$ and 0.5 mL of $SnCl_2$ were added to stabilize the purple hue of the solution. As a preservative, sufficient NH_2 OH-HCl solution was introduced to neutralize the $KMnO_4$ excess. A determination of mercury utilizing a flameless atomic absorption spectrophotometer (Mercury-Hydride System Analitik Jena, HS 60) was performed on a sample aliquot. Sample Hg concentrations were given as mg/g wet weight. The mercury detection limit was 0.001 mg/kg. Measuring mercury in standard reference material (DORM-4) from the National Research Council of Canada confirmed the analytical procedure. The validation of the analytical method revealed a Hg recovery of 93% of the DORM-4 certificate.

2.5. Evaluation of blood chemistry and physiological variables

Before blood was drawn from the fish, a 200 mg/L clove solution was used to sedate them [33]. With a 1 mm plastic syringe, blood was swiftly collected for every fish through the caudal aorta [26]. The blood was then deposited in vacutainer tubes with tripotassium-ethylene diaminetetraacetic acid (EDTA), an anticoagulant. A SFRI Blood Cell Counter 33 (Jean d'Illac, France) was used to measure red blood cell (RBC) numbers, hematocrit (Ht), and hemoglobin (Hb) concentrations [11]. Blood pH, pCO_2, and pO_2 were determined using a GASTAT-Navi analyzer (Japan). pCO_2 and pO_2 were both given in mmHg. Blood plasma was obtained from blood sample after 10 minutes of centrifugation at 5000 rpm and 4 ^{o}C. An osmometer (Fiske® 210, Norwood, MA, USA) was used to test the osmolality of 20 µL of blood plasma. The result was given in mOsm/kg. To measure the concentrations of plasma electrolytes (Cl^-, Na^+ and K^+) were carried out using a SpotChem EL SE-1520 (Kyoto, Japan). The results were given in mmol/L [11].

CA plays a crucial role in the control of the acid-base balance in fish, which primarily happens through the gills [34]. Hence, CA level in gills exposed to Hg were measured in this study. Using an enzyme-linked immunosorbent assay (ELISA) kit (Catalog Number E0123Fi) and in accordance with the manufacturer's instructions (Bioassay Technology Laboratory Biotech Co. Ltd.), CA levels were determined.

2.6. Analyses of statistical data

All data was provided as mean and standard deviation and tested for normality. The results were then analyzed using two-way ANOVA and Tukey's HSD. When $p < 0.05$, the statistically significant difference existed. All statistical analyses used IBM® SPSS® Statistics version 25.

3. Results

None animals perished during testing. Fish exposed to 0.06 mg/L Hg for 4 days and 15 days, as well as 0.6 mg/L Hg for 4 days, did not differ significantly from the control group. Only fish exposed to 0.6 mg/L Hg for 15 days had the greatest Hg concentration in their gills (Fig. 1).

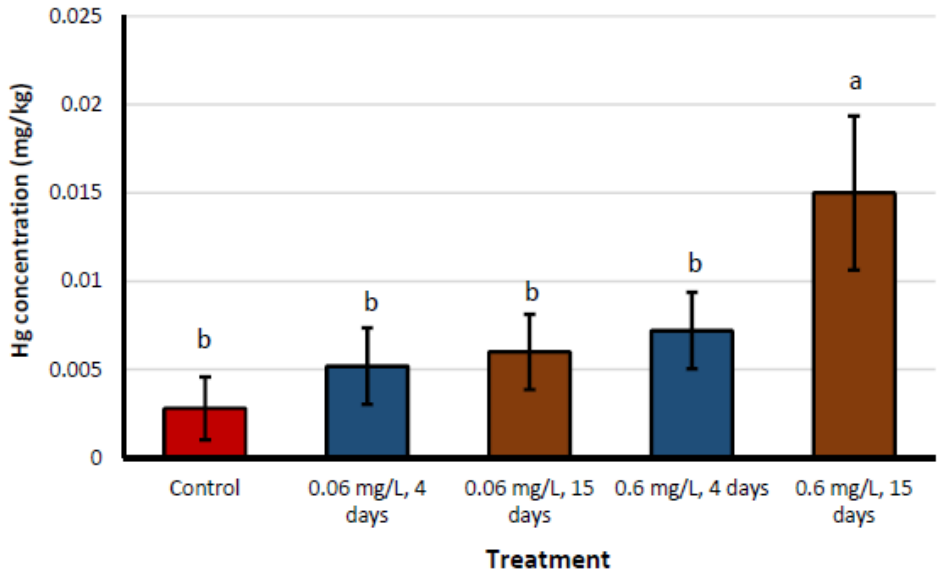

Fig. 1. Hg concentration in fish gills subjected to varying Hg medium concentrations. Lower case letters represent significant differences ($p < 0.05$, a > b).

At 0.6 mg/L for 15 days, Hg had a significant impact on pH, pCO_2, pO_2, and CA, but other treatments did not differ from the control. Fish subjected to 0.6 mg/L Hg for 15 days had the lowest blood pH, pO_2 and CA levels, but the highest blood pCO_2 levels. (Fig. 2).

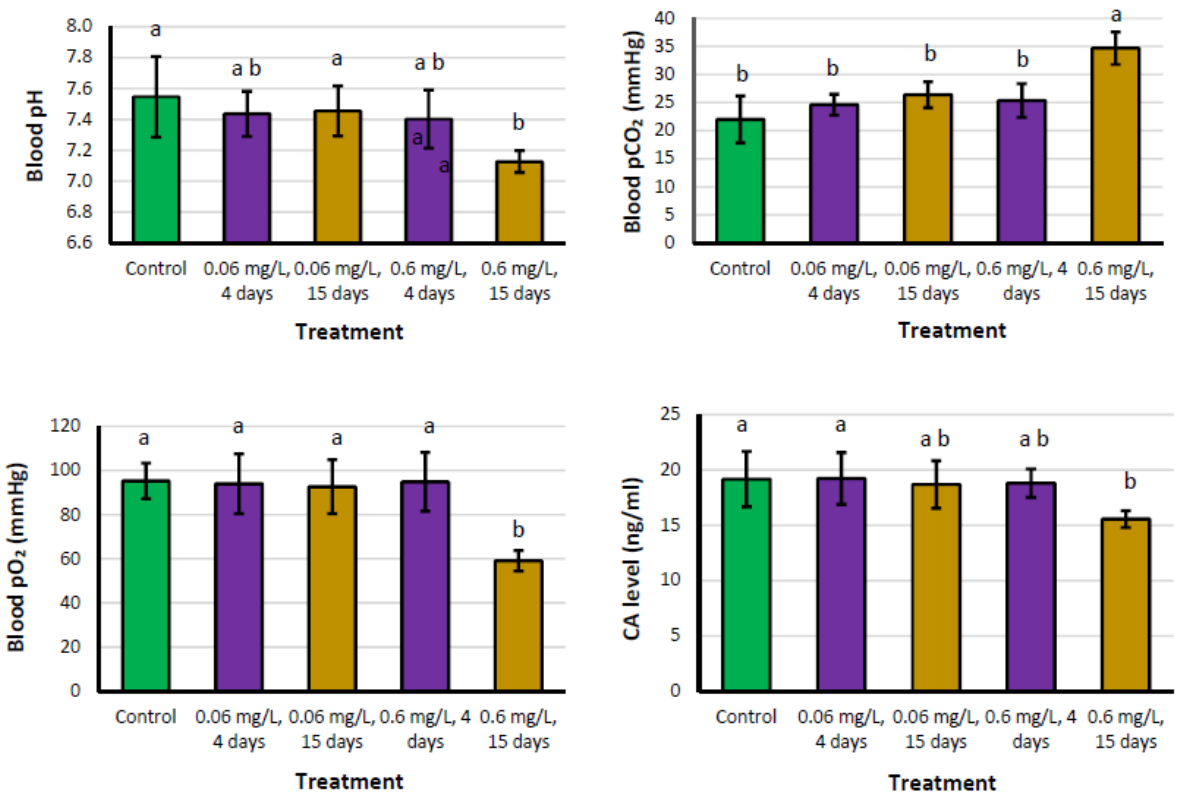

Fig. 2. Blood pH, pCO_2, pO_2, and CA levels in fish subjected to varying Hg medium concentrations. Significant differences are denoted with lowercase letters ($p < 0.05$, a > b).

Fish exposed to 0.6 mg/L Hg for 4 days and 15 days had reduced plasma osmolality, with 15 days exhibiting the lowest level of osmolality. Fish treated with 0.6 mg/L Hg for 15 days had the lowest concentrations of Cl^-, Na^+, and K^+ while other treatments did not significantly differ from the control (Fig. 3).

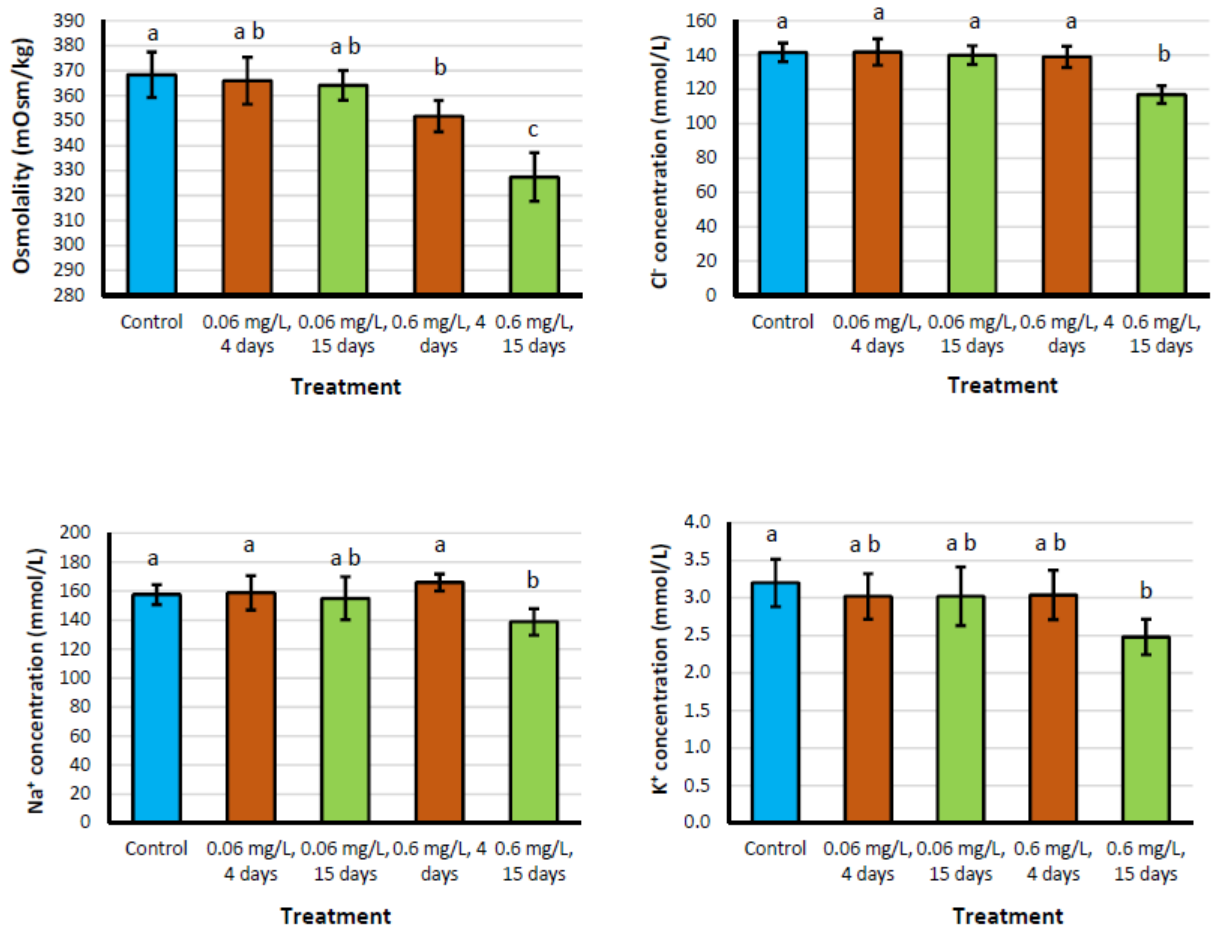

Fig. 3. Osmolality and ion levels in fish subjected to Hg exposure. Lower case letters show significant differences ($p < 0.05$, a > b > c).

Only fish exposed with 0.6 mg/L Hg for 15 days had reduced RBC, Hb, and Ht levels compared to the control, but the other treatments did not significantly differ from the control (Fig. 4).

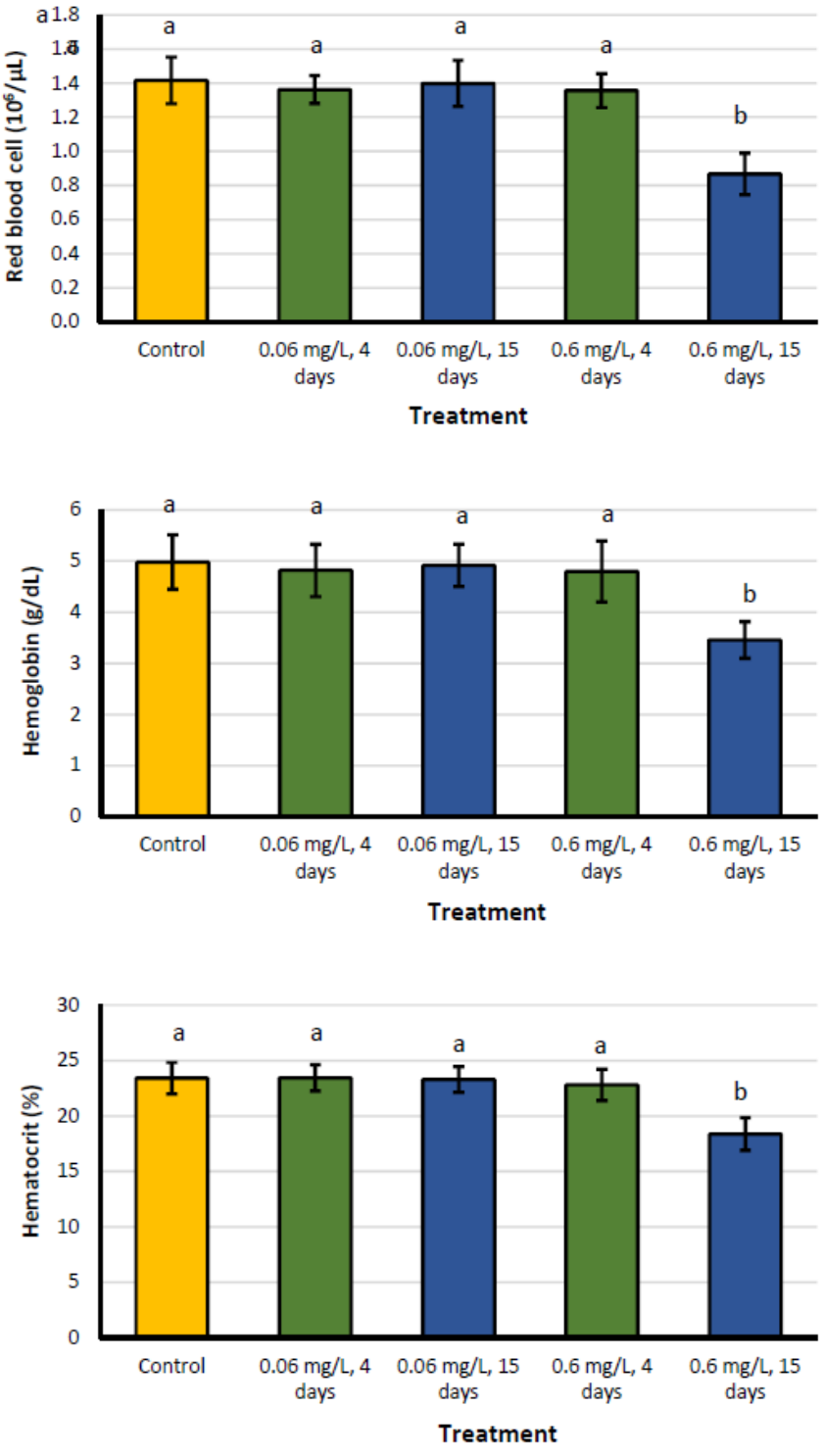

Fig. 4. Red blood cells, hemoglobin and hematocrit in Hg-treated fish. Lower case letters show significant differences ($p < 0.05$, a > b).

4. Discussion

This research utilized a relatively high concentration of Hg since *O. niloticus* has a significant tolerance to Hg [6]. In this investigation, sub-lethal concentrations of 0.06 and 0.6 mg/L of Hg were used. These concentrations may be higher than those present in the natural environment [4], but it is expected that examinations into the effects of Hg on the parameters of the fish blood will be able to be carried out at this concentration, and that the effects will be able to be clearly detected. Variable exposure times and Hg concentrations resulted in varying gill Hg levels in the tilapia used in this investigation, with 15 days of exposure to 0.6 mg/L Hg causing the highest level. Acidosis was noted at fish exposed to 0.6 mg/L Hg for 15 days. This ultimately results in an increase in pCO_2 followed by a decrease in pO_2, most likely due to gill disruption by Hg. The gill plays a vital role in fish respiratory gas transfer as the major site of CO_2 sensing and the known site of O_2 chemoreception [34, 35, 36], therefore this function may be disturbed in gill exposed to pollutants including Hg. In the hemolymph of crabs *Chasmagnathus granulatus* subjected to ammonia, increases in pCO_2 and significant decreases in pO_2 were also identified. These changes suggest that the documented histological impairment to the gills impeded gas exchange [37]. According to the results of our study, at 0.6 mg/L Hg for 15 days the decrease concentration of CA in the gills of tilapia was occurred. This decline is believed due to the result of gill cells losing their ability to convert CO_2 into HCO_3^-, which was also found by Larsen et al. [25] and Shandro and Casey [38].

Maintaining ionic and osmotic equilibrium in fish is dependent on the regulation of NaCl transport across the gills [29]. The uptake of the necessary ion (Na^+ or Cl^-) from the surrounding environment is associated with the transfer of acid–base relevant ions to the water. Clearly, these acid–base exchanges have a direct influence on the ion-regulatory needs of the animal [39].

Freshwater fish can maintain ion and osmoregulatory homeostasis by taking in Na^+ or Cl^- in exchange for their internal H^+ or $HCO3^-$. This allows the fish to make changes to its acid-base balance and keep its ions in balance [29, 39]. Heavy metals alter monovalent ion (Na^+ or Cl^-) regulation, leading ion outflow in freshwater fish [40, 41, 42]. After 15 days of exposure to 0.6 mg/L Hg, plasma Na^+ and Cl^- concentrations in tilapia decreased. At this treatment concentration, Cl^-/HCO_3^- exchange and Na^+/H^+ exchange may occur in the gills during hypercarbia. After being exposed to hypercapnia in freshwater for 96 hours, Atlantic salmon (*Salmo salar*) develop a respiratory acidosis. To combat this, the fish decrease their plasma concentrations of Cl^- and Na^+, likely by branchial Cl^-/HCO_3^- and Na^+/H^+ exchange, and increase their ion difference [43]. These adjustments occur during acidosis adjustment and may be indicative of a more general gill function impairment [43]. In our study, 0.6 mg/L Hg for 15 days resulted in a decrease in plasma Cl^- and Na^+ which is followed by a decrease in osmolality, most likely due to injury to gill cells. Larsen et al. [27] suggested that another possible explanation for the decrease in Cl^- and Na^+ is that heavy metal exposure may modify the Na^+/K^+-ATPase activity in chloride cells, thereby disrupting the branchial ion exchanger. Copper exposure decreased the osmolality and ion content of hemolymph of shore crab (*Carcinus maenas*). This may be due to increased osmoregulating Na^+/K^+-ATPase activity [44]. This study demonstrated that after 15 days of 0.6 mg/L Hg exposure, ionic and osmotic regulations substantially diminishes. Without a doubt, when prolonged exposure is carried out, it will result in mortality; nevertheless, this will require further investigation.

A decrease in K^+ levels was noted in fish exposed to 0.6 mg/L Hg for 15 days. This lower level of K^+ may be due to the damaged gill epithelium and Na^+/K^+-ATPase activity, causing changes passive fluxes. Since fish gills can let K^+ through, efflux is greater than influx. A decrease in K^+ uptake, as proposed by Patridge and Lymbery [45], is more significant than an increase in

K^+ loss. Meanwhile, Nussey et al. [46] suggested that the adaptation of fish to decreased osmolality is responsible for the decrease in serum K^+. The considerable alterations in Na^+/K^+-ATPase activity may explain why the blue mussel (*Mytilus edulis*) is unable to compete against the enhanced passive K^+ efflux that occurs when it is exposed to the antifouling chemical chlorothalonil [47].

Some hematological changes were also associated to acidosis. Our results demonstrated that fish exposed to 0.6 mg/L Hg for 15 days experienced acidosis and ionic imbalance. Under this extreme condition, when the energy requirement surpasses the capacity of aerobic energy production, anaerobic glycolysis is activated to produce extra ATP. As the final product of anaerobic glycolysis, lactic acid causes fatigue and decreases blood plasma pH [48]. Kurbel [49] suggested that low plasma pH may have caused Cl^- to move into red blood cells causing decreased levels of Cl^- in plasma, or Cl^- may have moved into the intracellular layer to restrict lactate efflux. Furthermore, Turner et al. [50] reported a rise in blood lactate and a reduction in plasma Cl^- in highly trained trout, and they hypothesized that these alterations were the result of this exchange. The decline in Ht correlated with erythrocyte shrinkage. In addition to this, there was a correlation between the drop in Ht and the shrinking of the erythrocytes. As shown by tinier carp (*Cyprinus carpio*) red cells, a considerable reduction in blood O_2-affinity may have resulted from a reduction in the size of large red cells in fish exposed to high Hg level and for longer durations. This was observed because larger red cells are more oxygen-binding [51]. There is a potential that the O_2 transport in the blood would become impeded.

Exposure to 0.6 mg/L Hg for 15 days caused in decreases in all blood parameters tested in this study. A disruption in the erythrocytes or erythropoietic function is indicated [52]. Diverse

fish species that were exposed to varying concentrations of heavy metals exhibited lowered red blood cells, hemoglobin, and hematocrit levels [11, 53, 54, 55].

A significant decrease in RBC amount showed that Hg might destroy RBC during erythrocyte circulation. Heath [56] identified a similar phenomenon in fish exposed to heavy metals. Al-Rudainy [57] found that heavy metals impede the enzymatic pathway that produces Hb in fish. The decreases in RBC, Hb, and Ht indicate that tilapia exposed to 0.6 mg/L Hg for 15 days developed anemia or hemodilution. This is consistent with our study's pO_2 testing, that shows that Hg-treated fish exhibit a considerable drop in pO_2. Wepener et al. [58] and Nussey et al. [46] proves that when fish are in this state, they can't get enough oxygen to their tissues, which makes them less active and productive.

5. Conclusion and implication

Hg can enter and contaminate aquatic ecosystems as a result of human activities, and once there, it can be stored and accumulated in the aquatic ecosystem or directly absorbed by aquatic organisms. Tilapia *O. niloticus* is a commercially valuable species that provides protein. Due to the fact that tilapia is frequently farmed in freshwater that is continuously contaminated by metals resulting from human activities, the effects of Hg on tilapia require considerable attention. As our study demonstrated that Hg would have an effect on the reduction of ionic and osmotic regulation, acid-base balance, blood O_2-affinity, blood O_2 delivery, and the fish's ability to provide adequate oxygen to cells, it is recommended that during fish production, source streams that may be contaminated with Hg and other metals be treated before to entry into fish ponds or tanks.

Competing interests

Authors have no conflicting interests.

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Availability of data and materials

Upon request, the corresponding author will provide access to the data used to support the results of this study.

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Abstract:	Mercury (Hg) poses a significant risk to aquatic ecosystems and living organisms as a result of human activities. Hg found in fish can be detrimental to human health when consumed, causing harmful effects. The potential impact of Hg on fish physiology is a significant concern, given its presence in fish. The rapid accumulation of Hg in fish tissues has the potential to impact their blood gas and electrolyte compositions. The objective of this investigation was to analyze the effects of sublethal concentrations of Hg on the blood gas and electrolyte levels of tilapia (Oreochromis niloticus) over different periods of exposure. This research was conducted through laboratory experiments. The study involved subjecting fish to sublethal concentrations of Hg at levels of 0.06 and 0.6 mg/L for a duration of 4 and 15 days. Upon completion of the toxicity test, fish specimens were taken from each testing group for the purpose of analyzing the Hg and carbonic anhydrase (CA) concentrations present in the gills, as well as osmoregulatory, acid-base, and hematological parameters. The results indicate that solely the fish that were subjected to a concentration of 0.6 mg/L Hg for a duration of 15 days exhibited a higher concentration of Hg in their gills compared to the control group. The inhibition of respiration by Hg was observed to be a result of the generation of metabolic acidosis, reduction in gill CA, decrease in pO2, as well as a decrease in plasma osmolality, Cl-, Na+, and K+. The levels of red blood cells, hemoglobin, and hematocrit exhibited a decrease solely at the concentration of 0.6 mg/L over a period of 15 days. The previously mentioned impairments have the potential to restrict the capacity of fish to adequately supply oxygen to their cells, thereby reducing overall performance.
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Response to Reviewers:	

Answer the Reviewer Questions

Manuscript Number: EMCON-D-23-00010

The impacts of sublethal mercury concentrations on the osmoregulatory and acid-base parameters of tilapia (*Oreochromis niloticus*) at various times

Reviewer #1:

1) The first thing to point out is that the title of this article does not attract readers' interest.

I found that the author has studied many indicators in this research, but not all indicators must appear in the title: **Initial title: "The impacts of sublethal mercury concentrations on the osmoregulatory and acid-base parameters of tilapia (Oreochromis niloticus) at various times**"

Answer:

The title was modified to become: "The impact of various periods of mercury exposure on the osmoregulatory and blood gas parameters of tilapia (*Oreochromis niloticus*)"

2) The abstract section should briefly introduce the research background and research significance and clarify the research methods, then introduce the main research results, and finally, give the corresponding conclusions. The abstract of this article looks ok, but some descriptions are redundant, and I hope to make a comprehensive modification.

Answer:

We have added the research background and research significance in Abstract so that it is more meaningful to carry out this research

"Mercury (Hg) poses a significant risk to aquatic ecosystems and living organisms as a result of human activities. Hg found in fish can be detrimental to human health when consumed, causing harmful effects. The potential impact of Hg on fish physiology is a significant concern, given its presence in fish. The rapid accumulation of Hg in fish tissues has the potential to impact their blood gas and electrolyte compositions. The objective of this investigation was to analyze the effects of sublethal concentrations of Hg on the blood gas and electrolyte levels of tilapia (Oreochromis niloticus) over different periods of exposure. This research was conducted through laboratory experiments. The study involved subjecting fish to sublethal concentrations of Hg at levels of 0.06 and 0.6 mg/L for a duration of 4 and 15 days. Upon completion of the toxicity test, fish specimens were taken from each testing group for the purpose of analyzing the Hg and carbonic anhydrase (CA) concentrations present in the gills, as well as osmoregulatory, acid-base, and hematological parameters. The results indicate that solely the fish that were subjected to a concentration of 0.6 mg/L Hg for a duration of 15 days exhibited a higher concentration of Hg in their gills compared to the control group.

The inhibition of respiration by Hg was observed to be a result of the generation of metabolic acidosis, reduction in gill CA, decrease in pO2, as well as a decrease in plasma osmolality, Cl-, Na+, and K+. The levels of red blood cells, hemoglobin, and hematocrit exhibited a decrease solely at the concentration of 0.6 mg/L over a period of 15 days. The previously mentioned impairments have the potential to restrict the capacity of fish to adequately supply oxygen to their cells, thereby reducing overall performance."

3) The material and method section need to be re-integrated and checked to avoid repetition and confusion. All experimental protocols should be better explained.

Answer:

We have refined materials and methods by integrating and reviewing them to avoid repetition and confusion. We have described the experimental protocol better in the revised manuscript.

4) Did you adapt the fish to the lab conditions before the trial?
No information about the feeding regime and type of fish during adaptation.
How did you feed the fish during the trial? What type of food?

Answer:

Yes. The fish underwent a period of no less than 14 days for acclimating to the dechlorinated tap water, maintained at a temperature range of 28-29 oC, and subjected to a photoperiod of 12-hour light and 12-hour dark in the 250 L acclimated tanks located within the testing facility (line 121 - 124).

Throughout the trial, the fish were provided with pellet fish meal at a concentration that corresponded to one percent of their daily estimated body weight (line 167 – 169)
The pellet fish meal consisted of 30% proteins, 3% fat, and 4% fibers (Takari, Indonesia) (Line 126 – 127).

5) How did the authors verify the mercury levels in the water?

Answer:

The process for determining the concentration of Hg in the experimental media was performed according to the methodology outlined in reference [36]. The experimental media were subjected to filtration using a 0.45 µm membrane filter and was subsequently acidified to a pH of less than 2 through the addition of HNO3. A volume of 100 mL of the specimen was transferred into a sample container that has a capacity of 300 mL. Subsequently, a volume of 5 mL H2SO4 and 2.5 mL HNO3 were introduced and homogenized. Subsequently, 15 mL of potassium permanganate solution were introduced into each sample container, agitated, and supplemented with potassium permanganate as required until the persistence of the purple hue for a minimum of 15 minutes.

Subsequently, 8 mL of potassium persulfate were introduced into each vessel and subjected to heating in a water bath maintained at 90 ± 5 °C for 2 hours. Following the cooling process, a 6 mL solution of sodium chloride-hydroxylamine sulfate was introduced into each container and subjected to analysis using a flameless atomic absorption spectrophotometer, specifically the Mercury-Hydride System Analitik Jena HS 60. (Line 144 - 157).

- Did you change the water or keep it as it was throughout the trial?
  Answer:

  To maintain a constant concentration of Hg, 50% of the experimental medium was renewed at intervals of 48 hours (Line 166 – 167)

6) The number of samples used for biochemical testing is not clear. The number of samples given in all figures are not listed.
   Answer:
   Each treatment test contained 5 fish and was duplicated. For each biochemistry test was used 5 randomly selected fish were taken from each treatment to collect and analyze the blood and gill tissue samples.
   "Blood and gill tissue samples were obtained from five fish selected at random from each treatment group following 4 and 15 days of exposure" (Line 169 – 170)

   We have added the number of samples in all figures (Please check the revised figures)

7) On the first mention of a species in the text, give both the common (trivial) and formal name, and make sure that the presentation is correct and consistent.
   Answer:
   The present manuscript includes the common and scientific names of all species mentioned.

8) Make sure that symbols, sub-, and super-scripts, upper- and lower-case are presented correctly and that there is correct and consistent use of italics, brackets and punctuation etc.
   Answer:
   All symbols, subscripts, and superscripts, as well as upper- and lower-case letters, italics, brackets, and punctuation, have been improved and accurately displayed throughout the manuscript.

Reviewer #2:

1) I tried to make an assessment for the manuscript but I found that it is short communication with very limited data. Please make in deep research even though you have limited data, you can write good results section and make a very good interpretation. I can't accept in the current form.

Answer:

We appreciate the reviewer's insightful comments on our manuscript. Our entire manuscript, including the title, abstract, research methods, results, discussion, conclusions, and suggestions, as well as the references, has been refined and expanded. We are expecting that the revisions we have made to our manuscript will meet the Emerging Contaminants journal's requirements for publication.

The impact of various periods of mercury exposure on the osmoregulatory and blood gas parameters of tilapia (*Oreochromis niloticus*)

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Abstract

Mercury (Hg) poses a significant risk to aquatic ecosystems and living organisms as a result of human activities. Hg found in fish can be detrimental to human health when consumed, causing harmful effects. The potential impact of Hg on fish physiology is a significant concern, given its presence in fish. The rapid accumulation of Hg in fish tissues has the potential to impact their blood gas and electrolyte compositions. The objective of this investigation was to analyze the effects of sublethal concentrations of Hg on the blood gas and electrolyte levels of tilapia (*Oreochromis niloticus*) over different periods of exposure. This research was conducted through laboratory experiments. The study involved subjecting fish to sublethal concentrations of Hg at levels of 0.06 and 0.6 mg/L for a duration of 4 and 15 days. Upon completion of the toxicity test, fish specimens were taken from each testing group for the purpose of analyzing the Hg and carbonic anhydrase (CA) concentrations present in the gills, as well as osmoregulatory, acid-base, and hematological parameters. The results indicate that solely the fish that were subjected to a concentration of 0.6 mg/L Hg for a duration of 15 days exhibited a higher concentration of Hg in their gills compared to the control group. The inhibition of respiration by Hg was observed to be a result of the generation of metabolic acidosis, reduction in gill CA, decrease in pO_2, as well as a decrease in plasma osmolality, Cl^-, Na^+, and K^+. The levels of red blood cells, hemoglobin, and hematocrit exhibited a decrease solely at the concentration of 0.6 mg/L over a period of 15 days. The previously mentioned impairments have the potential to restrict the capacity of fish to adequately supply oxygen to their cells, thereby reducing overall performance.

Keywords: water pollution; mercury, osmoregulation; acid-base balance; blood, fish

1. Introduction

Mercury (Hg) is considered to be one of the most perilous pollutants that pose a threat to aquatic ecosystems. This is due to its potent neurotoxicity towards fish, wildlife, and humans [1]. Hg is emitted into the environment as a result of natural rock weathering or volcanic activity. Nevertheless, it is human activity that serves as the primary contributor of mercury in the environment. The aforementioned phenomenon is a result of the process of coal combustion for the generation of electricity and the subsequent release of industrial waste [2]. In the majority of aquatic environments, Hg is primarily deposited from the atmosphere. According to the United States Environmental Protection Agency (USEPA), the biggest source of mercury in the atmosphere is emissions from coal-fired power plants [3]. Hg occurs naturally at very low concentrations in water. Commonly, uncontaminated freshwaters have 5 to 20 ng/L of total Hg, while contaminated waters can contain up to 0.5 µg/L [4]. Hg concentrations in river water near unregulated gold mining are between 12.7 and 13.6 µg/L [5]. In the vast majority of surface waters, the levels of Hg are typically much too low to have any direct adverse effects on either adult fish or the more sensitive early life stages of fish [6]. Acute toxicity of Hg to fish species varied, with 30 µg/L for a guppy *Lebistes reticulatus* [7], 580 µg/L for Mozambique tilapia *Orechromis mossambicus* [8], 327 µg/L for perch *Percocypris pingi* [9], 220 - 1220 µg/L for Nile tilapia *Oreochromis niloticus* [10, 11], 606 µg/L for fresh water climbing perch *Anabas testudineus* [12], and 900 µg/L for common carp *Cyprinus carpio* [13].

The bioavailability of Hg in teleost fish is dependent on the overall chemical concentration of the surrounding environment, which includes pH, salinity, hardness, hydrophobicity, and metals' interactions with biotic and abiotic ligands, as well as the fish's ability to absorb the various Hg forms at the gill, skin, and digestive system [14]. Hg accumulation may impair fish's immune

system [15, 16], respiratory and cardiovascular systems [17, 18], reproductive organs [19, 20], digestive system and excretory system [21], blood system [22], and enzymatic activity [23].

As far as current knowledge is concerned, there exists a paucity of scientific study on the impact of Hg on the state of acid-base equilibrium in fish. Prior research has indicated that certain heavy metals, including Zn, Cu, Pb, and Cd, have the potential to impact the acid-base parameters of various species of fish, for instance rainbow trout (*Salmo gairdneri*) [24], cod (*Gadus morhua*) [25], groovy mullet (*Liza dumerili*) [26], and tilapia *O. niloticus* [27]. Huang and Chen [28] observed that various types of inorganic pollution, including nitrite, led to an increase of nitrite levels in the bloodstream, methemoglobin, as well as partial pressure of oxygen (pO_2) in *Anguilla Anguilla*, commonly known as European eels. Additionally, a negative correlation was observed between nitrite concentrations in media and blood pH, pCO_2, and HCO_3. Chen and Lee [29] observed that the giant prawn *Macrobrachium rosenbergii* exhibited elevated haemolymph pO_2 and ammonia excretion, along with a decrease in haemolymph pH, subsequent to exposure to nitrite. The exposure to heavy metals and other pollutants has been observed to cause alterations in acid-base balances and various hematological parameters. This phenomenon is characterized by a rapid sequence of events, wherein gill damage is expected to be the cause of hypoxemia, leading to tissue hypoxia and a resultant combined acidosis, ultimately proving to be fatal [30].

It can be observed that animals produce a comparable amount of carbon dioxide to the oxygen they consume during metabolic processes. Blood serves the function of conveying oxygen from the external milieu to the body's tissues, whilst the tissues discharge carbon dioxide, which is subsequently transported by the blood back into the external environment. Hemoglobin (Hb) is an essential component of the red blood cell (RBC) and facilitates the transportation of O_2 and CO_2 in the blood of all vertebrates [30]. The process of acid-base compensating in fish is primarily

dependent on the direct transfer of H^+ and HCO_3^- via the gill as a substitute for Na^+ and Cl^-. As a result, the acid-base regulation in fish is closely associated with the regulation of ions. Consequently, the maintenance of ionic and osmotic equilibrium in fish necessitates regulating the flow of NaCl transportation across the gills. Carbonic anhydrase (CA) is responsible for regulating CO_2 excretion, ionic regulation, and acid-base balance [31]. Numerous in vitro investigations have suggested that fish CA activity is inhibited by heavy metals [32, 33]. Nonetheless, there exists a scarcity of in vivo investigations concerning the impact of Hg on CA of fish. As a result, the impacts of Hg on the enzyme carbonic anhydrase found in fish will be the focus of this study.

Oreochromis niloticus is a economic value species of fish, particularly in Indonesia. Tilapia is extensively cultivated in ponds which obtain their water from nearby rivers. In the meantime, there are numerous agricultural, industrial, and residential activities along the river. [34]. Thus, the aquaculture species that depend on river water as their primary resource supply are impacted by the waste, which includes heavy metals, that flows into the rivers from these activities. Typically, this species reacts quickly to environmental changes [11], and it can accumulate Hg from the environment [35]. The potential impact of Hg on tilapia is a significant concern, as the species is commonly cultivated in freshwater environments that are persistently polluted by metals originating from anthropogenic sources. The present investigation aimed to analyze the impact of sub-lethal Hg exposition on the osmoregulation, blood gas contents, hematological characteristics, as well as CA of gills of *O. niloticus* for a duration of four and fifteen days.

2. Materials and methods

2.1. Laboratory acclimatization of experimental animals

The study used *O. niloticus* tilapia specimens that measured 15.5 ± 0.6 cm in length and weighed 68.6 ± 1.2 g. The piscine specimens were procured from a pisciculture establishment located in Pasuruan, East Java. They were transported to the laboratory in a plastic bag containing fresh water with oxygenation. Subsequently, the fish underwent a period of no less than 14 days for acclimating to the dechlorinated tap water, maintained at a temperature range of 28-29 ^{o}C, and subjected to a photoperiod of 12-hour light and 12-hour dark in the 250 L acclimated tanks located within the testing facility. A biofiltration system comprising of gravel, sand, and sponge filters was employed to facilitate the continuous recirculation of water during the acclimating process, thereby ensuring the preservation of water quality. The fish were administered pellet fish meal that consisted of 30% proteins, 3% fat, and 4% fibers (Takari, Indonesia) at a dosage equivalent to one percent of their daily estimated body weight [11]. On a daily basis, the removal of excrement, discarded food scraps, and other undesired materials was carried out to maintain an adequate standard of water quality for the fish. The optimal values for temperature, pH, and dissolved oxygen were established through regular measurements taken during the acclimation and experimentation phases. These values were determined to be 28.6 ± 0.5 °C, 7.8 ± 0.3, and 7.3 ± 0.5 mg/L, respectively.

Answer Question no.4

2.2. Preparing a Hg Solution

A 1000 mg/L Hg stock solution was prepared by dissolving 1.3539 grams of $HgCl_2$ (Merck, Darmstadt, Germany) in one liter of deionized water. As per the findings of our prior research, the lethal concentration (96-h LC_{50}) of Hg for *O. niloticus* was determined to be 1.22 mg/L [11]. The

nominal concentrations of Hg utilized in this experiment were determined based on the LC_{50} value, and were as follows: 0.06 mg/L (equivalent to 5% of LC_{50}), 0.6 mg/L (equivalent to 50% of LC_{50}), and a control group that did not contain Hg. The experiment media were subjected to concentration measurements, resulting values of 0.044 ± 0.07 mg/L, 0.49 ± 0.04 mg/L, and <0.001 – 0.001 mg/L (control), respectively. The study involved conducting experiments across two distinct time frames, namely a short-term period of four days and a long-term period of 15 days. The process for determining the concentration of Hg in the experimental media was performed according to the methodology outlined in reference [36]. The experimental media were subjected to filtration using a 0.45 μm membrane filter and was subsequently acidified to a pH of less than 2 through the addition of HNO_3. A volume of 100 mL of the specimen was transferred into a sample container that has a capacity of 300 mL. Subsequently, a volume of 5 mL H_2SO_4 and 2.5 mL HNO_3 were introduced and homogenized. Subsequently, 15 mL of potassium permanganate solution were introduced into each sample container, agitated, and supplemented with potassium permanganate as required until the persistence of the purple hue for a minimum of 15 minutes. Subsequently, 8 mL of potassium persulfate were introduced into each vessel and subjected to heating in a water bath maintained at 90 ± 5 °C for 2 hours. Following the cooling process, a 6 mL solution of sodium chloride-hydroxylamine sulfate was introduced into each container and subjected to analysis using a flameless atomic absorption spectrophotometer, specifically the Mercury-Hydride System Analitik Jena HS 60.

Answer Question no.5

2.3. Sublethal toxicity test of Hg on O. niloticus

Following a period of acclimation, a sample of 50 fish in good health was randomly chosen from a holding tank and subsequently allocated to 10 separate tanks, each containing five fish. The

experimental setup involved tanks with a volume of 40 L each, which were filled with a testing medium comprising of varying concentrations of Hg for different durations. Specifically, the concentrations of Hg were 0.06 mg/L and 0.6 mg/L, and the exposure periods were 4 and 15 days. Additionally, a control group was included in the study, which did not contain any Hg. Each concentration was tested using two sets of tanks. To maintain a constant concentration of Hg, 50% of the experimental medium was renewed at intervals of 48 hours. Throughout the trial, the fish were provided with pellet fish meal at a concentration that corresponded to one percent of their daily estimated body weight. Blood and gill tissue samples were obtained from five fish selected at random from each treatment group following 4 and 15 days of exposure. Upon completion of the experiment, the experimental water containing Hg was collected and subsequently stored in a metallic tank designed for the storage of wastewater. The experimental procedures that utilized animals were conducted in compliance to the Animal Care and Use Regulations of Airlangga University.

Answer Question no.5

Answer Question no.6

2.4. The measurement of Hg levels in the gills.

The concentration of Hg in tilapia gills was determined using a procedure proposed by Candra et al. [37]. The gills of tilapia were extracted and subjected to a drying process in an oven maintained at 60°C for 48 hours until a consistent weight was achieved. Following desiccation, the gills were pulverized into a fine particulate form. A quantity of 0.5 grams of pulverized gills underwent a heating process in acid solutions consisting of 2 mL of HNO_3 - $HClO_4$ (1:1) and 5 mL of H_2SO_4. The heating process was carried out in a Mars 6 microwave digester for 3 hours at 80 ^oC. Upon cooling of the solution, 0.1 mL of $KMnO_4$ and 0.5 mL of $SnCl_2$ were introduced to preserve the violet coloration. Sufficient NH_2OH-HCl solution was added to neutralize the excess

185 $KMnO_4$, which functioned as a preservative. A singular portion of the specimen was subjected to
186 analysis for the presence of Hg utilizing a flameless atomic absorption spectrophotometer,
187 specifically the Mercury-Hydride System Analitik Jena, HS 60. The concentrations of Hg in the
188 analyzed samples were reported in units of mg/kg of wet weight. The limit of detection for Hg was
189 0.001 mg/kg. The validation of the analytical approach was carried out through the measurement
190 of Hg in the DORM-4 standard reference material of the National Research Council of Canada. In
191 the course of validating the analytical procedure, it was ascertained that the recovery of Hg for the
192 DORM-4 certificate was 93%.
193
194 ***2.5. The measurement of blood chemistry and physiological parameters***
195 The fish were subjected to anesthesia using a clove solution of 200 mg/L prior to the
196 collection of their blood samples. This particular anesthesia was selected due to its minimal impact
197 on physiological parameters [38]. Approximately 1 mL of blood from each fish was extracted
198 through the caudal aorta using a plastic syringe that was not heparinized [27]. The blood sample
199 was collected and preserved using vacutainer plastic tubes containing EDTA as a means of
200 preventing coagulation. Subsequently, the blood samples were promptly collected into an
201 automatic blood analyzer (SFRI Blood Cell Counter 33, Jean d'Illac, France) for the evaluation of
202 hematological parameters, including red blood cell (RBC) counts, hematocrit (Ht) levels, and
203 hemoglobin (Hb) concentrations [11, 39]. In order to measure the blood pH, pCO_2, and pO_2 levels,
204 a blood sample was immediately administered onto a sensor card (Techno Medica 091, Japan),
205 and subsequently inserted into an automatic blood gas analyzer (GASTAT-Navi, Japan). The pCO_2
206 and pO_2 were denoted in mmHg [27]. Blood plasma was isolated from blood cells through
207 centrifugation of a small amount of the blood specimen at 5000 rpm and 4 ^oC for 10 minutes,

before measuring the plasma's osmolality, as well as the concentrations of Na^+, Cl^-, and K^+. The plasma's osmolality was assessed through the introduction of a 20 µL plasma sample into a tube, followed by measurement using a micro-sample osmometer (Fiske® 210, Norwood, MA, USA) and reported in mOsm/kg units. The plasma's Na^+, Cl^-, and K^+ concentrations were assessed by transferring a 22 µL plasma sample into a specialized tube and subsequently analyzing it with an electrolyte analyzer (SpotChem EL SE-1520, Kyoto, Japan), and reported in mmol/L [11, 39]. The manufacturer of each instrument utilized for measurement supplied the requisite chemicals and components for the determination of hematological, acid-base parameters, osmolality, and ion levels.

CA is a crucial factor in regulating the acid-base equilibrium in fish, predominantly taking place in the gills [40]. The present study employed an ELISA kit (Catalog Number E0123Fi) to determine the concentration of CA in gills that were subjected to Hg exposure. The experimental protocol was carried out in accordance with the guidelines provided by the manufacturer (Bioassay Technology Laboratory Biotech Co. Ltd.). The extracted gills were thoroughly rinsed with phosphate-buffered saline (PBS, pH 7.4) to eliminate any remaining blood and weighed. The microtiter plate was pre-coated with Anti-CA antibody before its utilization. In order to ascertain the concentration of CA, standardized samples of 50 µL, blank samples, and 40 µL samples were introduced into every well. Ten microliters of anti-CA antibody and 50 µL of streptavidin-horseradish peroxidase were promptly added to every well, with the exception of the control blank. The contents were homogenized, sealed with a plate cover, and subjected to incubation at 37°C for a duration of 60 minutes. Following the removal of the sealer, the plate underwent automated aspiration and was subsequently rinsed five times with wash buffer. The tissue paper was utilized for the purpose of cleaning the plate. The plates were securely closed with a sealant and

subsequently subjected to incubation at a temperature of 37°C in the absence of light for approximately 10 minutes. During this incubation period, 50 µL of solution A and 50 µL of solution B were added to every well. Stop solution of 50 µL was added to each well to terminate the enzymatic activity. The sample was assessed at a wavelength of 450 nm using an automatic microplate reader (Model iMark by Bio-Rad, Japan) within a time frame of 10 minutes of the administration of the stop solution. The concentration of CA was expressed in units of ng/ml.

2.6. Statistical analysis

The data was presented in terms of mean and standard deviation, and was subjected to normality testing. The data was subjected to statistical analysis through the application of two-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test. A significant statistical difference was observed when the p-value was less than 0.05. The statistical analyses were conducted utilizing IBM® SPSS® Statistics version 25.

3. Results

The findings of the mercury toxicity assessment conducted on tilapia indicate that no mortalities were observed throughout the testing period. The accumulation of Hg in the gills of fish was observed following exposure to Hg-contaminated water. Specifically, Hg levels of 0.0052 and 0.006 mg/kg were recorded after 4 and 15 days of exposure to 0.06 mg/L Hg, respectively. Additionally, a Hg level of 0.0072 mg/kg was observed in fish gills after 4 days of exposure to 0.6 mg/L Hg. However, the concentrations of Hg in the gills did not exhibit a statistically significant difference when compared to the control group (0.0028 mg/kg). The results indicate that the highest concentration of Hg in the gills (0.015 mg/kg Hg) was observed solely in fish that were

subjected to a 0.6 mg/L Hg exposure for 15 days (Fig. 1). The concentration treatment of 0.06 mg/L seems to be statistically insignificant in terms of Hg accumulation in fish gills when compared to the control group after 4 and 15 days of exposure. Similarly, when subjected to treatment involving elevated concentrations of Hg (0.6 mg/L), the accumulation rate in the gills of fish was observed to be greater than that of the control group. However, the difference was considered statistically insignificant when the fish were exposed for a duration of only 4 days. The findings indicate that, in addition to concentration, the duration of exposure is a significant factor in the increase of Hg accumulation in the gills of fish. Upon increasing the duration of exposure to a concentration of 0.6 mg/L of Hg by nearly four-fold (over a period of 15 days), a significant ($p<0.05$) six-fold increase in the accumulation of Hg in fish gills was observed when compared to the control group of fish (Fig. 1).

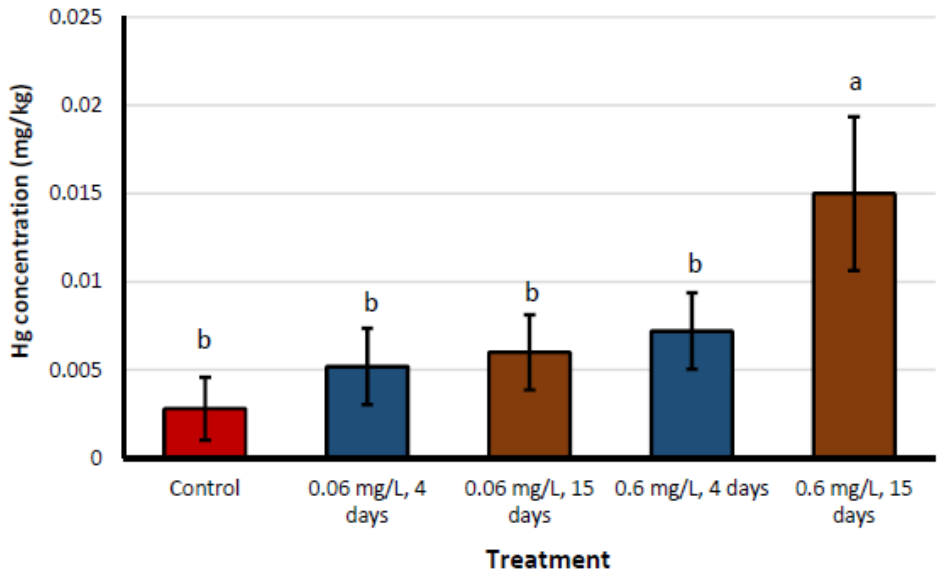


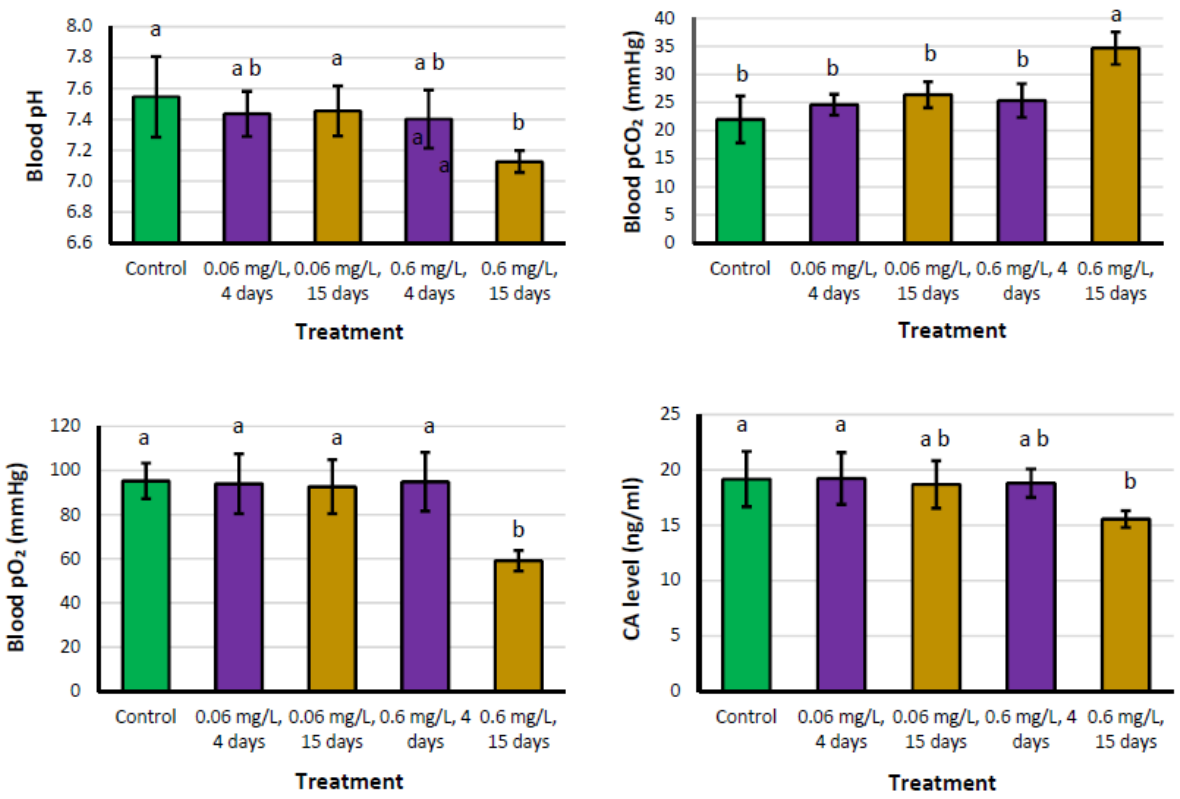
Fig. 1. Hg concentration in fish gills subjected to varying Hg medium concentrations. Lower case characters indicate significant differences ($p < 0.05$, a > b), Number of samples (N) = 5 individual.

269

270 Under a high treatment concentration of 0.6 mg/L for a duration of 15 days, the presence
271 of Hg had a significant impact on pH, pCO_2, pO_2, and CA. However, no significant differences
272 were observed in other treatments as compared to the control. The results of the study indicate that
273 fish exposed to a concentration of 0.6 mg/L Hg for a period of 15 days exhibited a decrease in
274 blood pH (7.1), pO_2 (59.1 mmHg) and CA (15.6 ng/ml) levels, while displaying an increase in
275 blood pCO_2 (34.7 mmHg) levels (Fig. 2). The observed values displayed a statistically significant
276 difference ($p<0.05$) when compared to the control group. The results indicate that the sublethal
277 concentration of 0.06 mg Hg/L had a negligible impact on the blood composition of tilapia.
278 Specifically, the exposure duration ranging from 4 to 15 days did not significantly alter the pH,
279 pCO_2, pO_2, and CA levels in the fish blood.

280

281



282

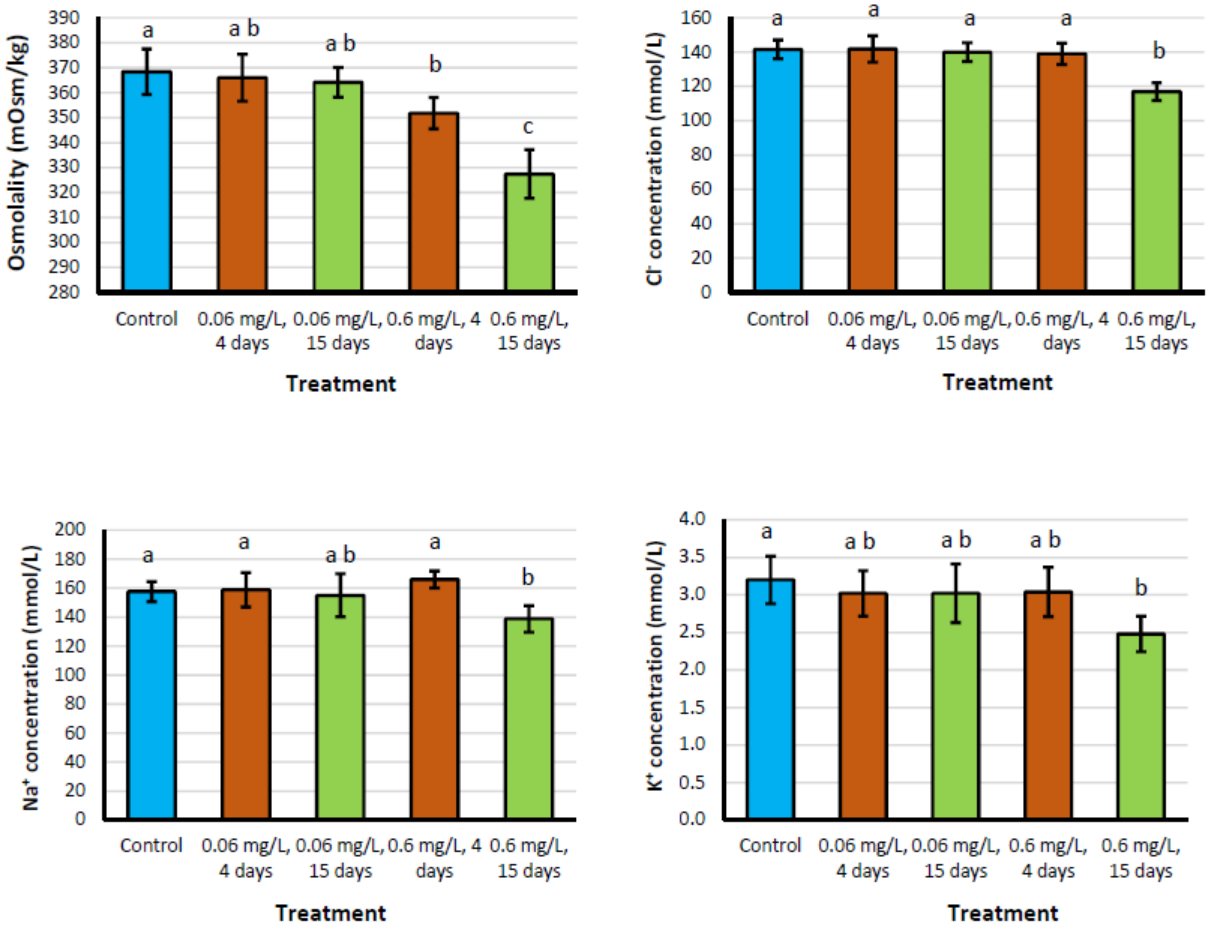
283 **Fig. 2.** Blood pH, pCO_2, pO_2, and CA levels in fish exposed to varying Hg levels. Significant
284 differences are denoted with lowercase letters ($p < 0.05$, a > b), N = 5 individual.

285

286 The blood parameters (osmolality, Cl^-, Na^+ and K^+) of fish subjected to 0.6 mg/L Hg for 4
287 and 15 days exhibited a significant decline ($p<0.05$) in comparison to the control value. A
288 statistically significant reduction ($p<0.05$) in plasma osmolality was observed, with values of 351.8
289 mOsm/kg and 327.4 mOsm/kg in comparison to the control group (368.4 mOsm/kg), respectively.
290 The lowest level was observed after 15 days of exposure. The results of the study indicate that fish
291 exposed to a concentration of 0.6 mg/L of Hg for a period of 15 days experienced a notable
292 reduction ($p<0.05$) in their blood mineral compositions, particularly in the levels of Cl^-, Na^+, and

293 K^+. These concentrations were found to be the lowest among all treatments, while other treatments

294 did not exhibit any significant differences when compared to the control group (Fig. 3).



295

296 **Fig. 3.** Osmolality and ion concentrations in mercury-exposed fish. Lowercase characters show

297 significant statistical differences ($p < 0.05$, a > b > c), N = 5 individual.

298

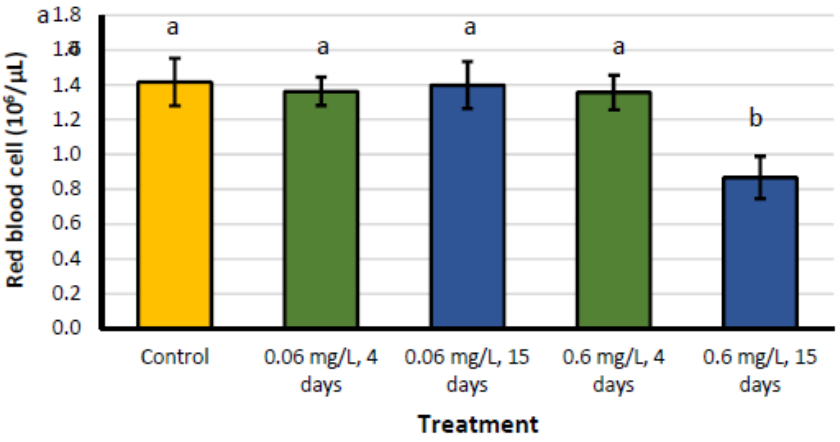
299 The results of the study indicate that only fish that were subjected to a concentration of 0.6

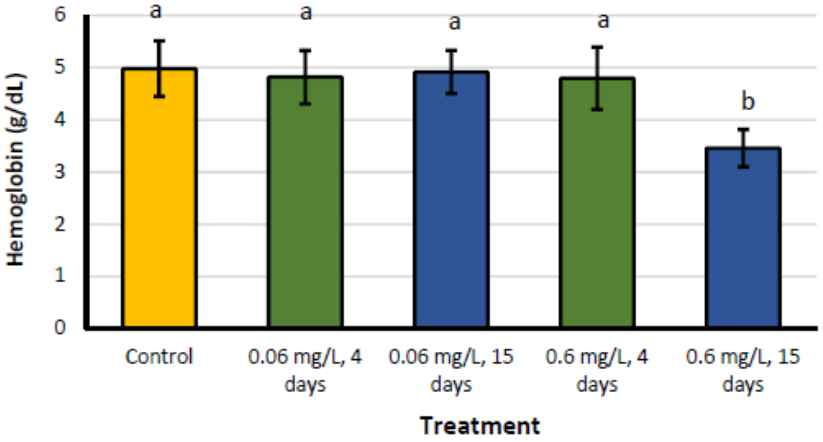
300 mg/L Hg for a period of 15 days exhibited a significant reduction ($p<0.05$) in their RBC (0.87

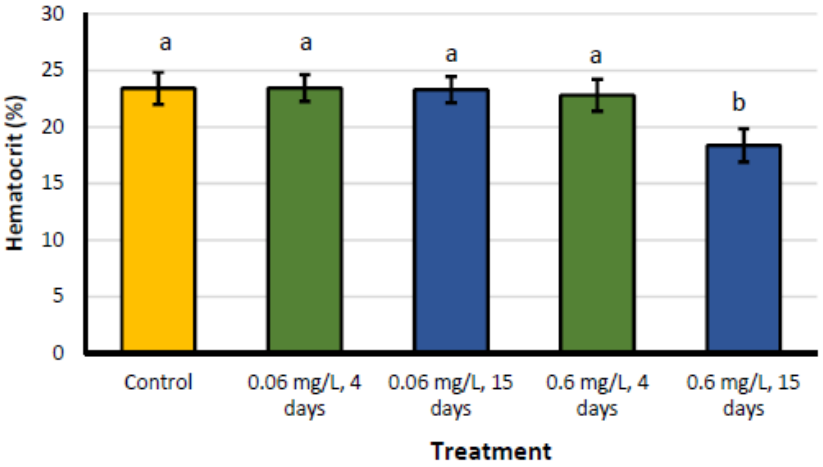
301 106/µL), Hb (3.46 g/dL), and Ht (18.36 %) levels in comparison to the control group. However,

302 the other treatments did not demonstrate any significant differences from the control group (Fig.

303 4).







304

305 **Fig. 4.** The levels of red blood cells, hemoglobin, and hematocrit in fish exposed with Hg. Lower

306 case characters indicate significant statistical differences ($p < 0.05$, a > b), N = 5 individual.

4. Discussion

The study employed a comparatively elevated concentration of Hg due to the notable tolerance of *O. niloticus* towards Hg, as previously reported [6]. The present study employed sub-lethal concentrations of Hg at 0.06 and 0.6 mg/L. Although the concentrations of Hg in concern may exceed those typically found in the natural environment [4], it is anticipated that investigations into the impact of Hg on various parameters of fish blood can be conducted at this level of concentration, and that any resulting effects can be readily observed. The gill Hg levels in the tilapia under investigation were found to vary depending on the exposure time and Hg concentrations. The highest level of Hg was observed in the group exposed to 0.6 mg/L Hg for a duration of 15 days. The study observed the occurrence of acidosis in fish that were subjected to 0.6 mg/L Hg for 15 days. The ultimate outcome of this phenomenon is an elevation in pCO_2 accompanied by a reduction in pO_2, plausibly attributable to the disruption of gill function by Hg. Exposure to Hg resulted in respiratory failure, leading to a reduction in oxygen uptake, accumulation of carbon dioxide in the bloodstream, and an elevation in pCO_2. The respiratory gas transfer process in fish is significantly dependent on the gill, which serves as the primary site for CO_2 sensing and O_2 chemoreception [40, 41, 42]. Consequently, exposure of the gill to pollutants such as Hg may potentially disrupt this crucial function.

The respiration of shrimp larvae was found to be impacted by varying levels of Hg and exposure durations, resulting in a reduction in their oxygen consumption rate (RO_2). Following a 10-h exposure to 160 ppb of Hg, there was a reduction of 43% and 49% in the RO_2 levels in zoeae III and zoeae V stages, respectively. A duration of 27 hours of exposure to 80 ppb of Hg or more resulted in paralytic weakness in almost all of the larvae [43]. The impediment of RO_2 may be elucidated by the diffusion of heavy metal across the permeable surfaces, namely gills and

teguments [44]. According to Hassaninezhad et al. [45], the presence of $HgCl_2$ at concentrations of 0.01 and 0.02 mg/L may result in a reduction of gas exchange ability in yellowfin seabream (*Acanthopagrus latus*) due to fish gill respiration deficiency caused by Hg contamination.

Elevated levels of pCO_2 and notable reductions in pO_2 were observed in the hemolymph of *Chasmagnathus granulatus* crabs exposed to ammonia. The alterations proposed in the study indicate that the histological damage observed in the gills hindered the process of gas exchange [46]. The study findings indicate that a reduction in the CA level in the tilapia gills was observed after 15 days of exposure to 0.6 mg/L Hg. The decrease is thought to be attributable to the gill cells' incapacity to transform CO_2 into HCO_3^-. This finding is consistent with the findings showed by Larsen et al. [25] and Shandro and Casey [47].

The regulation of NaCl transport across the gills is crucial for maintaining ionic and osmotic equilibrium in fish [31]. The process of acquiring the essential ion (either Na^+ or Cl^-) from the adjacent milieu is linked to the transference of ions that are pertinent to acid-base equilibrium into the water. The acid-base interactions mentioned possess a direct impact on the ion-regulatory necessities of the organism [48]. Freshwater fish are capable of achieving ion and osmoregulatory homeostasis through the process of exchanging Na^+ or Cl^- for their internal H^+ or HCO_3^-. The aforementioned mechanism enables the fish to regulate its acid-base equilibrium and maintain homeostasis of its ions [31, 48]. The regulation of monovalent ions (Na^+ or Cl^-) in freshwater fish is altered by heavy metals, resulting in ion outflow [49, 50, 51]. The study found that tilapia exposed to 0.6 mg/L Hg for a period of 15 days experienced a decrease in plasma Na^+ and Cl^- concentrations. At the given concentration of treatment, it is possible for Cl^-/HCO_3^- exchange and Na^+/H^+ exchange to take place in the gills while experiencing hypercarbia. Following a 96-hour exposure to hypercapnia in freshwater, Atlantic salmon (*Salmo salar*) exhibit the development of

353 respiratory acidosis. In response to this situation, the fish employ a mechanism involving the
354 exchange of Cl^-/HCO_3^- and Na^+/H^+ at the branchial level to reduce their plasma levels of Cl^- and
355 Na^+. This leads to an increase in their ion difference [52]. The aforementioned modifications take
356 place during the process of acidosis regulation and could potentially suggest a broader dysfunction
357 in gill functionality [52]. The findings of our investigation indicate that exposure to 0.6 mg/L Hg
358 over a period of 15 days elicited a reduction in plasma Cl^- and Na^+ levels, subsequently leading to
359 a decrease in osmolality. This outcome is likely attributable to damage incurred by gill cells.
360 According to Larsen et al. [25], a plausible reason for the reduction in Cl^- and Na^+ levels could be
361 the alteration of Na^+/K^+-ATPase activity in chloride cells due to exposure to heavy metals, which
362 could potentially disturb the branchial ion exchanger. The hemolymph of shore crab (*Carcinus*
363 *maenas*) experienced a reduction in osmolality and ion content as a result of exposure to copper.
364 The observed phenomenon could potentially be attributed to an elevation in osmoregulatory
365 Na^+/K^+-ATPase activity [53]. The gills of fish seem to experience a disturbance in Na regulation
366 as a result of exposure to inorganic Hg, which is attributed to a reduction in the activity of Na^+/K^+-
367 ATPases in the gills [54, 55]. Previous studies have reported a robust inverse relationship between
368 the concentration of pollutants and the activity of Na^+/K^+-ATPase in the gills of the European
369 flounder (*Platichthys flesus*) inhabiting a region contaminated with mercury [56]. Additionally, a
370 comparable inhibition of Na^+/K^+-ATPase activity has been observed in mrigal carp (*Cirrhinus*
371 *mrigala*) following acute exposure to $HgCl_2$ at concentrations of 0.068 and 0.034 mg/L [57].
372 According to Macirella and Brunelli [58], short exposure to two low concentrations of mercury
373 chloride led to changes in gill morphology and alterations in Na^+/K^+-ATPase. The enhancement
374 was elucidated as a period of adaptation that relies on the sustained metallic impact or maintaining
375 of the ion flow [59]. The findings of this research indicate that the exposure to 0.6 mg/L Hg for a

period of 15 days resulted in a significant reduction in both ionic and osmotic regulations. Undoubtedly, extended exposure may lead to death; however, additional research is necessary to confirm this assertion.

The findings indicate that the exposure of fish to 0.6 mg/L Hg for a duration of 15 days resulted in a reduction in K^+ levels. The potential cause of the reduced level of K^+ ions could be attributed to the impaired gill epithelium and the subsequent impact on the activity of the Na^+/K^+-ATPase, leading to alterations in the passive fluxes. Due to the permeability of fish gills to K^+, the efflux of K^+ is greater than its influx. According to Partridge and Lymbery [60], a reduction in the uptake of K^+ is comparatively more important than an elevation in K^+ loss. Alternately, the reduction in serum K^+ can be attributed to the adjustment of fish to lower osmolality [61]. According to Oliveira-Ribeiro et al. [62], the gill epithelium can be disrupted by exposure to dissolved Hg, which may have an impact on gas exchange and the ability of cell membranes to allow cations to pass through. The observed changes in Na^+/K^+-ATPase activity could potentially account for the competitive disadvantage experienced by the blue mussel species *Mytilus edulis* in the presence of the antifouling agent chlorothalonil, which induces an increased passive K^+ efflux [63]. The study conducted by Prasad et al. [64] revealed that alterations in the levels of Na^+ and K^+ ions could potentially signify a stress-induced response that arises due to extended exposure of fish to harmful substances. The aforementioned exposure possibly initiated distinct physiological and metabolic mechanisms that have the potential to enhance the efflux of ions.

The findings of the study indicate that a reduction in RBC, Hb and Ht levels were observed in tilapia that were subjected to 0.6 mg/L Hg for a duration of 15 days. The values of RBC, Hb, and Ht of Tench (*Tinca tinca*) decreased significantly in response to acute lethal (1.0 mg/L Hg for 48 h) and sublethal (0.25 mg/L Hg for 3 weeks) treatments, whereas at lower sublethal (0.1 mg/L

Hg for 24 h) treatments, RBC and Ht values increased significantly while Hb levels remained stable [65]. Walking catfish (*Clarias batrachus*) exposed for 14 days to varying concentrations of mercuric chloride ($HgCl_2$) exhibited a decline in RBC and Hb levels [66]. A decrease in RBC, Hb and Ht in Silver carp (*Hypophthalmichthys molitrix*) were recorded after exposure to both low (10% LC_{50}) and high (50% LC_{50}) concentrations of $HgCl_2$ for 4 days [67]. *Tinca tinca* exhibited a significant decrease in their RBC, Hb, and Ht levels following exposure to three distinct mercury treatments [68]. Ishikawa et al. [69] reported that there was no significant difference in the levels of RBC, Hb, and Hct in *Oreochromis niloticus* when exposed to varying concentrations of Hg (0.02, 0.002, 0.0002 mg/L).

Hematological alterations have been confirmed in association with acidosis. Our findings indicate that fish subjected to 0.6 mg/L Hg over a period of 15 days exhibit a reduction in RBC, Hb, and Ht concentrations, along with acidosis and ion imbalance. Under these critical circumstances, when the heightened energy demand exceeded the capacity of aerobic energy production, the organism initiates anaerobic glycolysis as a means of generating additional ATP. Lactic acid, which is the end result of anaerobic glycolysis, is known to induce fatigue and reduce the pH of blood plasma [70]. The reduction in Cl^- levels in plasma could be attributed to the movement of Cl^- into red blood cells, potentially due to low plasma pH. Otherwise, Cl^- could have been transported into the intracellular membrane, thereby limiting lactic efflux [71]. Moreover, Turner et al. [72] found that highly trained trout experienced an increase in lactate levels in their blood and a decline in blood plasma Cl^-. They suggested that this exchange could be the reason for these changes. The reduction in Ht level may be associated with the diminution of erythrocyte size. The findings suggest that prolonged exposure to high levels of Hg in tilapia may lead to a significant decrease in blood O_2-affinity, possibly due to a reduction in the size of red blood cells.

This is evidenced by the observation of smaller red cells in carp (*Cyprinus carpio*) that were exposed to high levels of toxicant [73]. The increased size of red blood cells may potentially increase their ability to bind with oxygen [74]. As a result, it is likely that the presence of Hg could impede the transportation of oxygen through the bloodstream of fish.

The study findings indicate that a 15-day exposure to 0.6 mg/L Hg resulted in a reduction of all blood parameters that were examined. Hence, an indication of a disturbance in the erythrocytes or erythropoietic function is present [75]. Several studies have reported that different fish species subjected to different levels of heavy metals experienced a reduction in their red blood cell count, hemoglobin levels, and hematocrit levels [11, 27, 76, 77, 78].

The observed reduction in red blood cell count suggests that Hg may have a detrimental effect on erythrocytes during circulation. A comparable occurrence was observed by Heath [79] in fish that were subjected to high levels of metallic substances. According to Al-Rudainy's research [80], the enzymatic pathway responsible for the production of Hb in fish is inhibited by heavy metals. The findings suggest that the tilapia that were subjected to 0.6 mg/L Hg for a duration of 15 days exhibited a state of anemia or hemodilution, as evidenced by the observed reductions in RBC, Hb, and Ht levels. The findings align with the results of our study's pO_2 examination, which indicates a significant decrease in pO_2 levels among fish treated with Hg. Fish with such a condition experience insufficient oxygen supply to their tissues, leading to reduced levels of activity and productivity [61, 81].

5. Conclusions and recommendations

Mercury (Hg) has the potential to infiltrate and pollute aquatic ecosystems due to anthropogenic actions. Once present, it has the capacity to be sequestered and concentrated within

445 the aquatic ecosystem or assimilated by aquatic organisms. Ultimately, the substance will enter the
446 food chain through both water and food sources, resulting in adverse health effects for both animals
447 and humans. *Oreochromis niloticus*, is a species of significant commercial importance due to its
448 high protein content. The potential impact of Hg on *O. niloticus* is an issue of significant concern,
449 as the species is often cultivated in freshwater environments that are vulnerable to contamination
450 by metallic substances originating from anthropogenic sources. The study findings indicate that
451 the presence of Hg can lead to a decrease in the levels of electrolyte, osmoregulation, acid-base
452 equilibrium, hematological parameters, and the capacity of tilapia to supply sufficient oxygen to
453 the cells. Hence, the prevention of pollution by heavy metals including Hg in aquatic environments
454 is considered one of the greatest crucial strategies to tackle this issue. The unregulated discharge
455 of sewage and industrial effluents has significantly impaired the overall aquatic environment and
456 water quality, thereby impacting the biochemical and physiological well-being of aquatic
457 organisms, including fish. To preserve ecological equilibrium, it is recommended that industrialists
458 refrain from disposing of their waste without prior treatment. In the long run, the reduction of
459 pollution emanating from these specific sources would result in a corresponding decrease in the
460 concentration of toxic metals in fish, as the level of pollutants in their natural environment
461 diminishes. To address this issue, it is imperative to devise strategies or measures that can offer a
462 viable solution. Consequently, it is imperative to take action at present to guarantee that
463 forthcoming pollution and discharges of heavy metals in aquatic ecosystems are reduced to the
464 greatest possible degree to avert contamination of aquatic ecosystems on a global scale and to
465 avoid harmful effects on fish and humans.
466
467

Competing interests

Authors have no conflicting interests.

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Availability of data and materials

Upon request, the corresponding author will provide access to the data utilized to substantiate the findings of this research.

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The impact of various periods of mercury exposure on the osmoregulatory and blood gas parameters of tilapia (*Oreochromis niloticus*)

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ABSTRACT

Mercury (Hg) poses a significant risk to aquatic ecosystems and living organisms as a result of human activities. Hg found in fish can be detrimental to human health when consumed, causing harmful effects. The potential impact of Hg on fish physiology is a significant concern, given its presence in fish. The rapid accumulation of Hg in fish tissues has the potential to impact their blood gas and electrolyte compositions. The objective of this investigation was to analyze the effects of sublethal concentrations of Hg on the blood gas and electrolyte levels of tilapia (*Oreochromis niloticus*) over different periods of exposure. This research was conducted through laboratory experiments. The study involved subjecting fish to sublethal concentrations of Hg at levels of 0.06 and 0.6 mg/L for a duration of 4 and 15 days. Upon completion of the toxicity test, fish specimens were taken from each testing group for the purpose of analyzing the Hg and carbonic anhydrase (CA) concentrations present in the gills, as well as osmoregulatory, acid-base, and hematological parameters. The results indicate that solely the fish that were subjected to a concentration of 0.6 mg/L Hg for a duration of 15 days exhibited a higher concentration of Hg in their gills compared to the control group. The inhibition of respiration by Hg was observed to be a result of the generation of metabolic acidosis, reduction in gill CA, decrease in pO_2, as well as a decrease in plasma osmolality, Cl^-, Na^+, and K^+. The levels of red blood cells, hemoglobin, and hematocrit exhibited a decrease solely at the concentration of 0.6 mg/L over a period of 15 days. The previously mentioned impairments have the potential to restrict the capacity of fish to adequately supply oxygen to their cells, thereby reducing overall performance.

1. Introduction

Mercury (Hg) is considered to be one of the most perilous pollutants that pose a threat to aquatic ecosystems. This is due to its potent neurotoxicity towards fish, wildlife, and humans [1]. Hg is emitted into the environment as a result of natural rock weathering or volcanic activity. Nevertheless, it is human activity that serves as the primary contributor of mercury in the environment. The aforementioned phenomenon is a result of the process of coal combustion for the generation of electricity and the subsequent release of industrial waste [2]. In the majority of aquatic environments, Hg is primarily deposited from the atmosphere. According to the United States Environmental Protection Agency (USEPA), the biggest source of mercury in the atmosphere is emissions from coal-fired power plants [3]. Hg occurs naturally at very low concentrations in water. Commonly, uncontaminated freshwaters have 5–20 ng/L of total Hg, while contaminated waters can contain up to 0.5 μg/L [4]. Hg concentrations in river water near unregulated gold mining are between 12.7 and 13.6 μg/L [5]. In the vast majority of surface waters, the levels of Hg are typically much too low to have any direct adverse effects on either adult fish or the more sensitive early life stages of fish [6]. Acute toxicity of Hg to fish species varied, with 30 μg/L for a guppy *Lebistes reticulatus* [7], 580 μg/L for Mozambique tilapia *Orechromis mossambicus* [8], 327 μg/L for perch *Percocypris pingi* [9], 220–1220 μg/L for Nile tilapia *Oreochromis niloticus* [10,11], 606 μg/L for fresh water climbing perch *Anabas testudineus* [12], and 900 μg/L for common carp *Cyprinus carpio* [13].

The bioavailability of Hg in teleost fish is dependent on the overall chemical concentration of the surrounding environment, which includes pH, salinity, hardness, hydrophobicity, and metals' interactions with biotic and abiotic ligands, as well as the fish's ability to absorb the various Hg forms at the gill, skin, and digestive system [14]. Hg accumulation may impair fish's immune system [15,16], respiratory and cardiovascular systems [17,18], reproductive organs [19,20], digestive system and excretory system [21], blood system [22], and enzymatic activity [23].

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As far as current knowledge is concerned, there exists a paucity of scientific study on the impact of Hg on the state of acid-base equilibrium in fish. Prior research has indicated that certain heavy metals, including Zn, Cu, Pb, and Cd, have the potential to impact the acid-base parameters of various species of fish, for instance rainbow trout (*Salmo gairdneri*) [24], cod (*Gadus morhua*) [25], groovy mullet (*Liza dumerili*) [26], and tilapia *O. niloticus* [27]. Huang and Chen [28] observed that various types of inorganic pollution, including nitrite, led to an increase of nitrite levels in the bloodstream, methemoglobin, as well as partial pressure of oxygen (pO_2) in *Anguilla Anguilla*, commonly known as European eels. Additionally, a negative correlation was observed between nitrite concentrations in media and blood pH, pCO_2, and HCO_3. Chen and Lee [29] observed that the giant prawn *Macrobrachium rosenbergii* exhibited elevated hemolymph pO_2 and ammonia excretion, along with a decrease in hemolymph pH, subsequent to exposure to nitrite. The exposure to heavy metals and other pollutants has been observed to cause alterations in acid-base balances and various hematological parameters. This phenomenon is characterized by a rapid sequence of events, wherein gill damage is expected to be the cause of hypoxemia, leading to tissue hypoxia and a resultant combined acidosis, ultimately proving to be fatal [30].

It can be observed that animals produce a comparable amount of carbon dioxide to the oxygen they consume during metabolic processes. Blood serves the function of conveying oxygen from the external milieu to the body's tissues, whilst the tissues discharge carbon dioxide, which is subsequently transported by the blood back into the external environment. Hemoglobin (Hb) is an essential component of the red blood cell (RBC) and facilitates the transportation of O_2 and CO_2 in the blood of all vertebrates [30]. The process of acid-base compensating in fish is primarily dependent on the direct transfer of H^+ and HCO_3^- via the gill as a substitute for Na^+ and Cl^-. As a result, the acid-base regulation in fish is closely associated with the regulation of ions. Consequently, the maintenance of ionic and osmotic equilibrium in fish necessitates regulating the flow of NaCl transportation across the gills. Carbonic anhydrase (CA) is responsible for regulating CO_2 excretion, ionic regulation, and acid-base balance [31]. Numerous in vitro investigations have suggested that fish CA activity is inhibited by heavy metals [32,33]. Nonetheless, there exists a scarcity of in vivo investigations concerning the impact of Hg on CA of fish. As a result, the impacts of Hg on the enzyme carbonic anhydrase found in fish will be the focus of this study.

Oreochromis niloticus is a economic value species of fish, particularly in Indonesia. Tilapia is extensively cultivated in ponds which obtain their water from nearby rivers. In the meantime, there are numerous agricultural, industrial, and residential activities along the river [34]. Thus, the aquaculture species that depend on river water as their primary resource supply are impacted by the waste, which includes heavy metals, that flows into the rivers from these activities. Typically, this species reacts quickly to environmental changes [11], and it can accumulate Hg from the environment [35]. The potential impact of Hg on tilapia is a significant concern, as the species is commonly cultivated in freshwater environments that are persistently polluted by metals originating from anthropogenic sources. The present investigation aimed to analyze the impact of sub-lethal Hg exposition on the osmoregulation, blood gas contents, hematological characteristics, as well as CA of gills of *O. niloticus* for a duration of four and fifteen days.

2. Materials and methods

2.1. Laboratory acclimation of experimental animals

The study used *O. niloticus* tilapia specimens that measured 15.5 ± 0.6 cm in length and weighed 68.6 ± 1.2 g. The piscine specimens were procured from a pisciculture establishment located in Pasuruan, East Java. They were transported to the laboratory in a plastic bag containing fresh water with oxygenation. Subsequently, the fish underwent a period of no less than 14 days for acclimating to the dechlorinated tap water, maintained at a temperature range of 28–29 °C, and subjected to a photoperiod of 12-h light and 12-h dark in the 250 L acclimated tanks located within the testing facility. A biofiltration system comprising of gravel, sand, and sponge filters was employed to facilitate the continuous recirculation of water during the acclimating process, thereby ensuring the preservation of water quality. The fish were administered pellet fish meal that consisted of 30% proteins, 3% fat, and 4% fibers (Takari, Indonesia) at a dosage equivalent to one percent of their daily estimated body weight [11]. On a daily basis, the removal of excrement, discarded food scraps, and other undesired materials was carried out to maintain an adequate standard of water quality for the fish. The optimal values for temperature, pH, and dissolved oxygen were established through regular measurements taken during the acclimation and experimentation phases. These values were determined to be 28.6 ± 0.5 °C, 7.8 ± 0.3, and 7.3 ± 0.5 mg/L, respectively.

2.2. Preparing a Hg solution

A 1000 mg/L Hg stock solution was prepared by dissolving 1.3539 g of $HgCl_2$ (Merck, Darmstadt, Germany) in 1 L of deionized water. As per the findings of our prior research, the lethal concentration (96h LC_{50}) of Hg for *O. niloticus* was determined to be 1.22 mg/L [11]. The nominal concentrations of Hg utilized in this experiment were determined based on the LC_{50} value, and were as follows: 0.06 mg/L (equivalent to 5% of LC_{50}), 0.6 mg/L (equivalent to 50 % of LC_{50}), and a control group that did not contain Hg. The experiment media were subjected to concentration measurements, resulting values of 0.044 ± 0.07 mg/L, 0.49 ± 0.04 mg/L, and <0.001–0.001 mg/L (control), respectively. The study involved conducting experiments across two distinct time frames, namely a short-term period of four days and a long-term period of 15 days. The process for determining the concentration of Hg in the experimental media was performed according to the methodology outlined in Ref. [36]. The experimental media were subjected to filtration using a 0.45 μm membrane filter and was subsequently acidified to a pH of less than 2 through the addition of HNO_3. A volume of 100 mL of the specimen was transferred into a sample container that has a capacity of 300 mL. Subsequently, a volume of 5 mL H_2SO_4 and 2.5 mL HNO_3 were introduced and homogenized. Subsequently, 15 mL of potassium permanganate solution were introduced into each sample container, agitated, and supplemented with potassium permanganate as required until the persistence of the purple hue for a minimum of 15 min. Subsequently, 8 mL of potassium persulfate were introduced into each vessel and subjected to heating in a water bath maintained at 90 ± 5 °C for 2 h. Following the cooling process, a 6 mL solution of sodium chloride-hydroxylamine sulfate was introduced into each container and subjected to analysis using a flameless atomic absorption spectrophotometer, specifically the Mercury -Hydride System Analitik Jena HS 60.

2.3. Sublethal toxicity test of Hg on O. niloticus

Following a period of acclimation, a sample of 50 fish in good health was randomly chosen from a holding tank and subsequently allocated to 10 separate tanks, each containing five fish. The experimental setup involved tanks with a volume of 40 L each, which were filled with a testing medium comprising of varying concentrations of Hg for different durations. Specifically, the concentrations of Hg were 0.06 mg/L and 0.6 mg/L, and the exposure periods were 4 and 15 days. Additionally, a control group was included in the study, which did not contain any Hg. Each concentration was tested using two sets of tanks. To maintain a constant concentration of Hg, 50% of the experimental medium

was renewed at intervals of 48 h. Throughout the trial, the fish were provided with pellet fish meal at a concentration that corresponded to one percent of their daily estimated body weight. Blood and gill tissue samples were obtained from five fish selected at random from each treatment group following 4 and 15 days of exposure. Upon completion of the experiment, the experimental water containing Hg was collected and subsequently stored in a metallic tank designed for the storage of wastewater. The experimental procedures that utilized animals were conducted in compliance to the Animal Care and Use Regulations of Airlangga University.

2.4. The measurement of Hg levels in the gills

The concentration of Hg in tilapia gills was determined using a procedure proposed by Candra et al. [37]. The gills of tilapia were extracted and subjected to a drying process in an oven maintained at 60 ˚C for 48 h until a consistent weight was achieved. Following desiccation, the gills were pulverized into a fine particulate form. A quantity of 0.5 g of pulverized gills underwent a heating process in acid solutions consisting of 2 mL of HNO_3 - $HClO_4$ (1:1) and 5 mL of H_2SO_4. The heating process was carried out in a Mars 6 microwave digester for 3 h at 80 ˚C. Upon cooling of the solution, 0.1 mL of $KMnO_4$ and 0.5 mL of $SnCl_2$ were introduced to preserve the violet coloration. Sufficient NH_2OH–HCl solution was added to neutralize the excess $KMnO_4$, which functioned as a preservative. A singular portion of the specimen was subjected to analysis for the presence of Hg utilizing a flameless atomic absorption spectrophotometer, specifically the Mercury-Hydride System Analitik Jena, HS 60. The concentrations of Hg in the analyzed samples were reported in units of mg/kg of wet weight. The limit of detection for Hg was 0.001 mg/kg. The validation of the analytical approach was carried out through the measurement of Hg in the DORM-4 standard reference material of the National Research Council of Canada. In the course of validating the analytical procedure, it was ascertained that the recovery of Hg for the DORM-4 certificate was 93%.

2.5. The measurement of blood chemistry and physiological parameters

The fish were subjected to anesthesia using a clove solution of 200 mg/L prior to the collection of their blood samples. This particular anesthesia was selected due to its minimal impact on physiological parameters [38]. Approximately 1 mL of blood from each fish was extracted through the caudal aorta using a plastic syringe that was not heparinized [27]. The blood sample was collected and preserved using vacutainer plastic tubes containing EDTA as a means of preventing coagulation. Subsequently, the blood samples were promptly collected into an automatic blood analyzer (SFRI Blood Cell Counter 33, Jean d'Illac, France) for the evaluation of hematological parameters, including red blood cell (RBC) counts, hematocrit (Ht) levels, and hemoglobin (Hb) concentrations [11,39]. In order to measure the blood pH, pCO_2, and pO_2 levels, a blood sample was immediately administered onto a sensor card (Techno Medica 091, Japan), and subsequently inserted into an automatic blood gas analyzer (GASTAT-Navi, Japan). The pCO_2 and pO_2 were denoted in mmHg [27]. Blood plasma was isolated from blood cells through centrifugation of a small amount of the blood specimen at 5000 rpm and 4 ˚C for 10 min, before measuring the plasma's osmolality, as well as the concentrations of Na^+, Cl^-, and K^+. The plasma's osmolality was assessed through the introduction of a 20 μL plasma sample into a tube, followed by measurement using a microsample osmometer (Fiske® 210, Norwood, MA, USA) and reported in mOsm/kg units. The plasma's Na^+, Cl^-, and K^+ concentrations were assessed by transferring a 22 μL plasma sample into a specialized tube and subsequently analyzing it with an electrolyte analyzer (SpotChem EL SE-1520, Kyoto, Japan), and reported in mmol/L [11,39]. The manufacturer of each instrument utilized for measurement supplied the requisite chemicals and components for the determination of hematological, acid-base parameters, osmolality, and ion levels.

CA is a crucial factor in regulating the acid-base equilibrium in fish, predominantly taking place in the gills [40]. The present study employed an ELISA kit (Catalog Number E0123Fi) to determine the concentration of CA in gills that were subjected to Hg exposure. The experimental protocol was carried out in accordance with the guidelines provided by the manufacturer (Bioassay Technology Laboratory Biotech Co. Ltd.). The extracted gills were thoroughly rinsed with phosphate-buffered saline (PBS, pH 7.4) to eliminate any remaining blood and weighed. The microtiter plate was pre-coated with Anti-CA antibody before its utilization. In order to ascertain the concentration of CA, standardized samples of 50 μL, blank samples, and 40 μL samples were introduced into every well. Ten microliters of anti-CA antibody and 50 μL of streptavidin-horseradish peroxidase were promptly added to every well, with the exception of the control blank. The contents were homogenized, sealed with a plate cover, and subjected to incubation at 37 ˚C for a duration of 60 min. Following the removal of the sealer, the plate underwent automated aspiration and was subsequently rinsed five times with wash buffer. The tissue paper was utilized for the purpose of cleaning the plate. The plates were securely closed with a sealant and subsequently subjected to incubation at a temperature of 37 ˚C in the absence of light for approximately 10 min. During this incubation period, 50 μL of solution A and 50 μL of solution B were added to every well. Stop solution of 50 μL was added to each well to terminate the enzymatic activity. The sample was assessed at a wavelength of 450 nm using an automatic microplate reader (Model iMark by Bio-Rad, Japan) within a time frame of 10 min of the administration of the stop solution. The concentration of CA was expressed in units of ng/ml.

2.6. Statistical analysis

The data was presented in terms of mean and standard deviation, and was subjected to normality testing. The data was subjected to statistical analysis through the application of two-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test. A significant statistical difference was observed when the p-value was less than 0.05. The statistical analyses were conducted utilizing IBM® SPSS® Statistics version 25.

3. Results

The findings of the mercury toxicity assessment conducted on tilapia indicate that no mortalities were observed throughout the testing period. The accumulation of Hg in the gills of fish was observed following exposure to Hg-contaminated water. Specifically, Hg levels of 0.0052 and 0.006 mg/kg were recorded after 4 and 15 days of exposure to 0.06 mg/L Hg, respectively. Additionally, a Hg level of 0.0072 mg/kg was observed in fish gills after 4 days of exposure to 0.6 mg/L Hg. However, the concentrations of Hg in the gills did not exhibit a statistically significant difference when compared to the control group (0.0028 mg/kg). The results indicate that the highest concentration of Hg in the gills (0.015 mg/kg Hg) was observed solely in fish that were subjected to a 0.6 mg/L Hg exposure for 15 days (Fig. 1). The concentration treatment of 0.06 mg/L seems to be statistically insignificant in terms of Hg accumulation in fish gills when compared to the control group after 4 and 15 days of exposure. Similarly, when subjected to treatment involving elevated concentrations of Hg (0.6 mg/L), the accumulation rate in the gills of fish was observed to be greater than that of the control group. However, the difference was considered statistically insignificant when the fish were exposed for a duration of only 4 days. The findings indicate that, in addition to concentration, the duration of exposure is a significant factor in the increase of Hg accumulation in the gills of fish. Upon increasing the duration of exposure to a concentration of 0.6 mg/L of Hg by nearly four-fold (over a period of 15

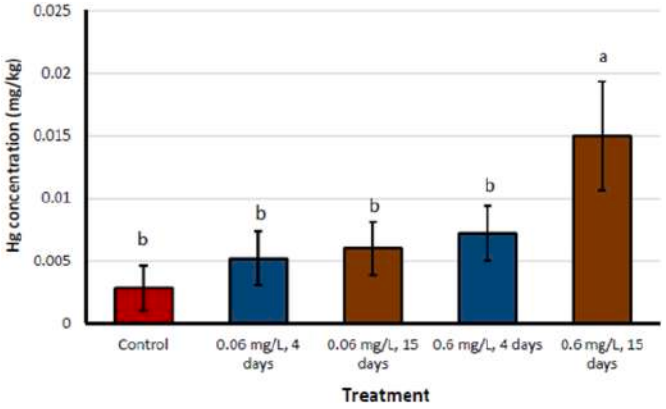

Fig. 1. Hg concentration in fish gills subjected to varying Hg medium concentrations. Lower case characters indicate significant differences ($p < 0.05$, a > b), Number of samples (N) = 5 individual.

days), a significant ($p < 0.05$) six-fold increase in the accumulation of Hg in fish gills was observed when compared to the control group of fish (Fig. 1).

Under a high treatment concentration of 0.6 mg/L for a duration of 15 days, the presence of Hg had a significant impact on pH, pCO_2, pO_2, and CA. However, no significant differences were observed in other treatments as compared to the control. The results of the study indicate that fish exposed to a concentration of 0.6 mg/L Hg for a period of 15 days exhibited a decrease in blood pH (7.1), pO_2 (59.1 mmHg) and CA (15.6 ng/ml) levels, while displaying an increase in blood pCO_2 (34.7 mmHg) levels (Fig. 2). The observed values displayed a statistically significant difference ($p < 0.05$) when compared to the control group. The results indicate that the sublethal concentration of 0.06 mg Hg/L had a negligible impact on the blood composition of tilapia. Specifically, the exposure duration ranging from 4 to 15 days did not significantly alter the pH, pCO_2, pO_2, and CA levels in the fish blood.

The blood parameters (osmolality, Cl^-, Na^+ and K^+) of fish subjected to 0.6 mg/L Hg for 4 and 15 days exhibited a significant decline ($p < 0.05$) in comparison to the control value. A statistically significant reduction ($p < 0.05$) in plasma osmolality was observed, with values of 351.8 mOsm/kg and 327.4 mOsm/kg in comparison to the control group (368.4 mOsm/kg), respectively. The lowest level was observed after 15 days of exposure. The results of the study indicate that fish exposed to a concentration of 0.6 mg/L of Hg for a period of 15 days experienced a notable reduction ($p < 0.05$) in their blood mineral compositions, particularly in the levels of Cl^-, Na^+, and K^+. These concentrations were found to be the lowest among all treatments, while other treatments did not exhibit any significant differences when compared to the control group (Fig. 3).

The results of the study indicate that only fish that were subjected to a concentration of 0.6 mg/L Hg for a period of 15 days exhibited a significant reduction ($p < 0.05$) in their RBC ($0.87\ 10^6/\mu L$), Hb (3.46 g/dL), and Ht (18.36%) levels in comparison to the control group. However, the other treatments did not demonstrate any significant differences from the control group (Fig. 4).

4. Discussion

The study employed a comparatively elevated concentration of Hg due to the notable tolerance of *O. niloticus* towards Hg, as previously reported [6]. The present study employed sub-lethal concentrations of Hg at 0.06 and 0.6 mg/L. Although the concentrations of Hg in concern may exceed those typically found in the natural environment [4], it is anticipated that investigations into the impact of Hg on various parameters of fish blood can be conducted at this level of concentration, and that any resulting effects can be readily observed. The gill Hg levels in the tilapia under investigation were found to vary depending on the exposure time and Hg concentrations. The highest level of Hg was observed in the group exposed to 0.6 mg/L Hg for a duration of 15 days. The study observed the occurrence of acidosis in fish that were subjected to 0.6 mg/L Hg for 15 days. The ultimate outcome of this phenomenon is an elevation in pCO_2 accompanied by a reduction in pO_2, plausibly attributable to the disruption of gill function by Hg. Exposure to Hg resulted in respiratory failure, leading to a reduction in oxygen uptake, accumulation of carbon dioxide in the bloodstream, and an elevation in pCO_2. The respiratory gas transfer process in fish is significantly dependent on the gill, which serves as the primary site for CO_2 sensing and O_2 chemoreception [40–42]. Consequently, exposure of the

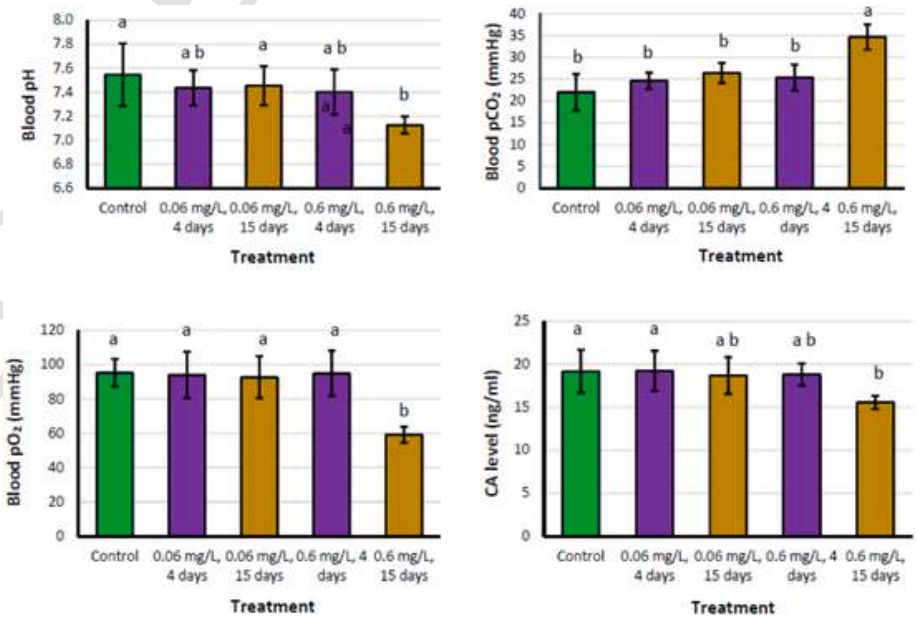

Fig. 2. Blood pH, pCO_2, pO_2, and CA levels in fish exposed to varying Hg levels. Significant differences are denoted with lowercase letters ($p < 0.05$, a > b), N = 5 individual.

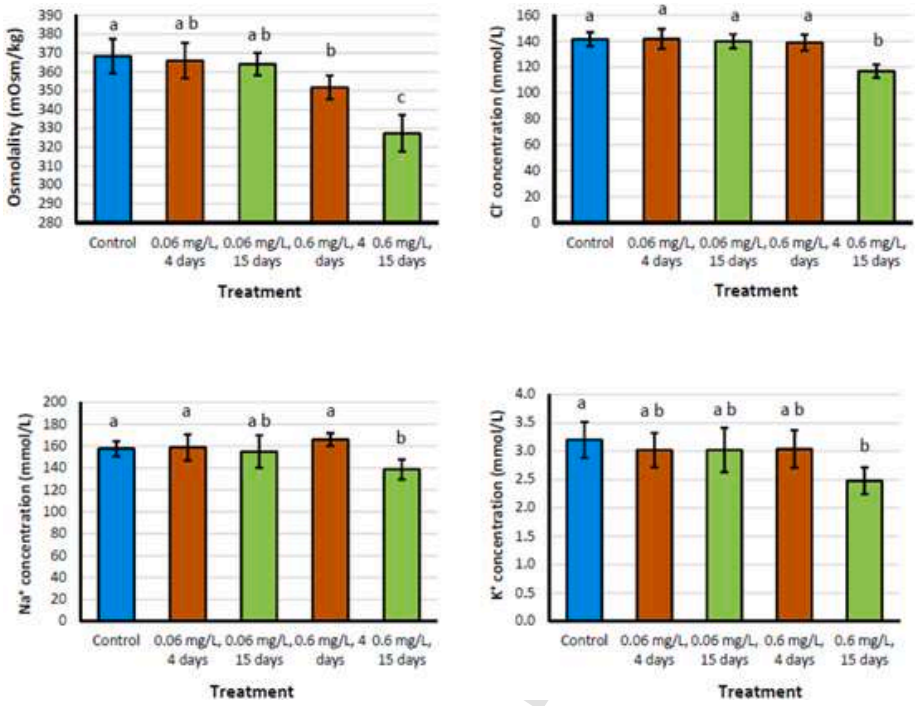

Fig. 3. Osmolality and ion concentrations in mercury-exposed fish. Lowercase characters show significant statistical differences ($p < 0.05$, a > b > c), N = 5 individual.

gill to pollutants such as Hg may potentially disrupt this crucial function.

The respiration of shrimp larvae was found to be impacted by varying levels of Hg and exposure durations, resulting in a reduction in their oxygen consumption rate (RO_2). Following a 10h exposure to 160 ppb of Hg, there was a reduction of 43% and 49% in the RO_2 levels in zoeae III and zoeae V stages, respectively. A duration of 27 h of exposure to 80 ppb of Hg or more resulted in paralytic weakness in almost all of the larvae [43]. The impediment of RO_2 may be elucidated by the diffusion of heavy metal across the permeable surfaces, namely gills and teguments [44]. According to Hassaninezhad et al. [45], the presence of $HgCl_2$ at concentrations of 0.01 and 0.02 mg/L may result in a reduction of gas exchange ability in yellowfin seabream (*Acanthopagrus latus*) due to fish gill respiration deficiency caused by Hg contamination.

Elevated levels of pCO_2 and notable reductions in pO_2 were observed in the hemolymph of *Chasmagnathus granulatus* crabs exposed to ammonia. The alterations proposed in the study indicate that the histological damage observed in the gills hindered the process of gas exchange [46]. The study findings indicate that a reduction in the CA level in the tilapia gills was observed after 15 days of exposure to 0.6 mg/L Hg. The decrease is thought to be attributable to the gill cells' incapacity to transform CO_2 into HCO_3^-. This finding is consistent with the findings showed by Larsen et al. [25] and Shandro and Casey [47].

The regulation of NaCl transport across the gills is crucial for maintaining ionic and osmotic equilibrium in fish [31]. The process of acquiring the essential ion (either Na^+ or Cl^-) from the adjacent milieu is linked to the transference of ions that are pertinent to acid-base equilibrium into the water. The acid-base interactions mentioned possess a direct impact on the ion-regulatory necessities of the organism [48]. Freshwater fish are capable of achieving ion and osmoregulatory homeostasis through the process of exchanging Na^+ or Cl^- for their internal H^+ or HCO_3^-. The aforementioned mechanism enables the fish to regulate its acid-base equilibrium and maintain homeostasis of its ions [31,48]. The regulation of monovalent ions (Na^+ or Cl^-) in freshwater fish is altered by heavy metals, resulting in ion outflow [49–51]. The study found that tilapia exposed to 0.6 mg/L Hg for a period of 15 days experienced a decrease in plasma Na^+ and Cl^- concentrations. At the given concentration of treatment, it is possible for Cl^-/HCO_3^- exchange and Na^+/H^+ exchange to take place in the gills while experiencing hypercarbia. Following a 96-h exposure to hypercapnia in freshwater, Atlantic salmon (*Salmo salar*) exhibit the development of respiratory acidosis. In response to this situation, the fish employ a mechanism involving the exchange of Cl^-/HCO_3^- and Na^+/H^+ at the branchial level to reduce their plasma levels of Cl^- and Na^+. This leads to an increase in their ion difference [52]. The aforementioned modifications take place during the process of acidosis regulation and could potentially suggest a broader dysfunction in gill functionality [52]. The findings of our investigation indicate that exposure to 0.6 mg/L Hg over a period of 15 days elicited a reduction in plasma Cl^- and Na^+ levels, subsequently leading to a decrease in osmolality. This outcome is likely attributable to damage incurred by gill cells. According to Larsen et al. [25], a plausible reason for the reduction in Cl^- and Na^+ levels could be the alteration of Na^+/K^+-ATPase activity in chloride cells due to exposure to heavy metals, which could potentially disturb the branchial ion exchanger. The hemolymph of shore crab (*Carcinus maenas*) experienced a reduction in osmolality and ion content as a result of exposure to copper. The observed phenomenon could potentially be attributed to an elevation in osmoregulatory Na^+/K^+-ATPase activity [53]. The gills of fish seem to experience a disturbance in Na regulation as a result of exposure to inorganic Hg, which is attributed to a reduction in the activity of Na^+/K^+-ATPases in the gills [54,55]. Previous studies have reported a robust inverse relationship between the concentration of pollutants and the activity of Na^+/K^+-ATPase in the gills of the European flounder (*Platichthys flesus*) inhabiting a region contaminated with mercury [56]. Additionally, a comparable inhibition of Na^+/K^+-ATPase activity has been observed in mrigal carp (*Cirrhinus mrigala*) following acute exposure to $HgCl_2$ at concentrations of 0.068 and 0.034 mg/L [57]. According to Macirella and Brunelli [58], short exposure to two low concentrations of mercury chloride led to changes in gill morphology and alterations in Na^+/K^+-ATPase. The enhancement was elucidated as a period of adaptation that relies on the sustained metallic impact or maintaining of the ion flow [59]. The findings of this research indicate that the exposure to 0.6 mg/L Hg for a period of 15 days resulted in a significant

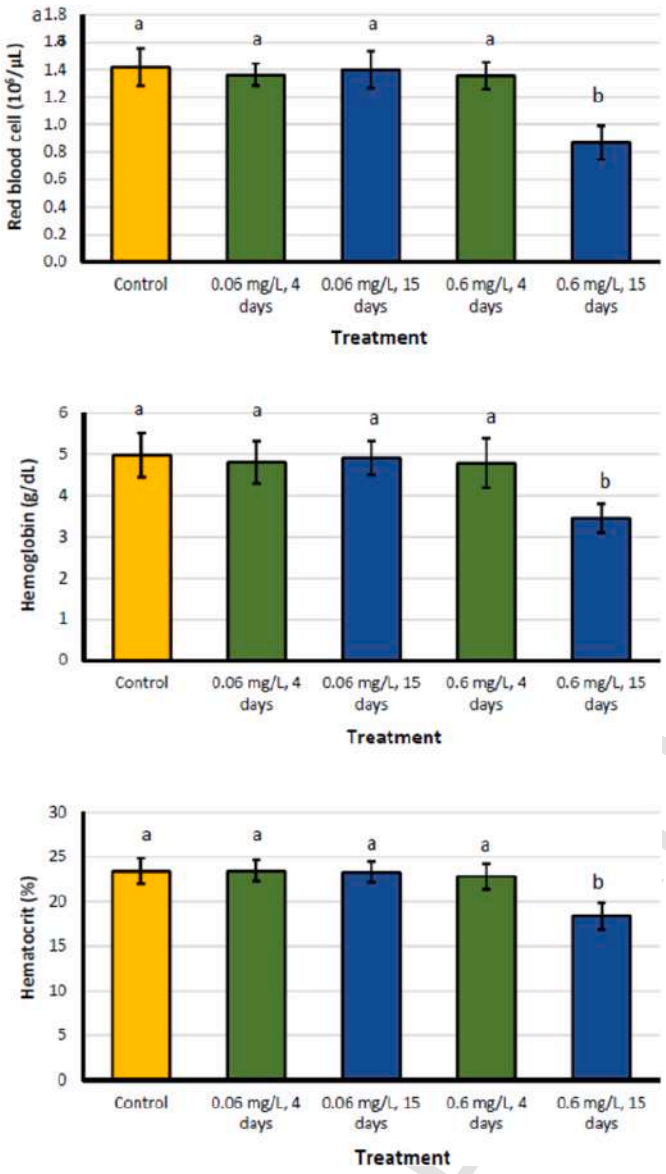

Fig. 4. The levels of red blood cells, hemoglobin, and hematocrit in fish exposed with Hg. Lower case characters indicate significant statistical differences ($p < 0.05$, a > b), N = 5 individual.

reduction in both ionic and osmotic regulations. Undoubtedly, extended exposure may lead to death; however, additional research is necessary to confirm this assertion.

The findings indicate that the exposure of fish to 0.6 mg/L Hg for a duration of 15 days resulted in a reduction in K^+ levels. The potential cause of the reduced level of K^+ ions could be attributed to the impaired gill epithelium and the subsequent impact on the activity of the Na^+/K^+-ATPase, leading to alterations in the passive fluxes. Due to the permeability of fish gills to K^+, the efflux of K^+ is greater than its influx. According to Partridge and Lymbery [60], a reduction in the uptake of K^+ is comparatively more important than an elevation in K^+ loss. Alternately, the reduction in serum K^+ can be attributed to the adjustment of fish to lower osmolality [61]. According to Oliveira-Ribeiro et al. [62], the gill epithelium can be disrupted by exposure to dissolved Hg, which may have an impact on gas exchange and the ability of cell membranes to allow cations to pass through. The observed changes in Na^+/K^+-ATPase activity could potentially account for the competitive disadvantage experienced by the blue mussel species *Mytilus edulis* in the presence of the antifouling agent chlorothalonil, which induces an increased passive K^+ efflux [63]. The study conducted by Prasad et al. [64] revealed that alterations in the levels of Na^+ and K^+ ions could potentially signify a stress-induced response that arises due to extended exposure of fish to harmful substances. The aforementioned exposure possibly initiated distinct physiological and metabolic mechanisms that have the potential to enhance the efflux of ions.

The findings of the study indicate that a reduction in RBC, Hb and Ht levels were observed in tilapia that were subjected to 0.6 mg/L Hg for a duration of 15 days. The values of RBC, Hb, and Ht of Tench (*Tinca tinca*) decreased significantly in response to acute lethal (1.0 mg/L Hg for 48 h) and sublethal (0.25 mg/L Hg for 3 weeks) treatments, whereas at lower sublethal (0.1 mg/L Hg for 24 h) treatments, RBC and Ht values increased significantly while Hb levels remained stable [65]. Walking catfish (*Clarias batrachus*) exposed for 14 days to varying concentrations of mercuric chloride ($HgCl_2$) exhibited a decline in RBC and Hb levels [66]. A decrease in RBC, Hb and Ht in Silver carp (*Hypophthalmichthys molitrix*) were recorded after exposure to both low (10% LC_{50}) and high (50% LC_{50}) concentrations of $HgCl_2$ for 4 days [67]. *Tinca tinca* exhibited a significant decrease in their RBC, Hb, and Ht levels following exposure to three distinct mercury treatments [68]. Ishikawa et al. [69] reported that there was no significant difference in the levels of RBC, Hb, and Ht in *Oreochromis niloticus* when exposed to varying concentrations of Hg (0.02, 0.002, 0.0002 mg/L).

Hematological alterations have been confirmed in association with acidosis. Our findings indicate that fish subjected to 0.6 mg/L Hg over a period of 15 days exhibit a reduction in RBC, Hb, and Ht concentrations, along with acidosis and ion imbalance. Under these critical circumstances, when the heightened energy demand exceeded the capacity of aerobic energy production, the organism initiates anaerobic glycolysis as a means of generating additional ATP. Lactic acid, which is the end result of anaerobic glycolysis, is known to induce fatigue and reduce the pH of blood plasma [70]. The reduction in Cl^- levels in plasma could be attributed to the movement of Cl^- into red blood cells, potentially due to low plasma pH. Otherwise, Cl^- could have been transported into the intracellular membrane, thereby limiting lactic efflux [71]. Moreover, Turner et al. [72] found that highly trained trout experienced an increase in lactate levels in their blood and a decline in blood plasma Cl^-. They suggested that this exchange could be the reason for these changes. The reduction in Ht level may be associated with the diminution of erythrocyte size. The findings suggest that prolonged exposure to high levels of Hg in tilapia may lead to a significant decrease in blood O_2-affinity, possibly due to a reduction in the size of red blood cells. This is evidenced by the observation of smaller red cells in carp (*Cyprinus carpio*) that were exposed to high levels of toxicant [73]. The increased size of red blood cells may potentially increase their ability to bind with oxygen [74]. As a result, it is likely that the presence of Hg could impede the transportation of oxygen through the bloodstream of fish.

The study findings indicate that a 15day exposure to 0.6 mg/L Hg resulted in a reduction of all blood parameters that were examined. Hence, an indication of a disturbance in the erythrocytes or erythropoietic function is present [75]. Several studies have reported that different fish species subjected to different levels of heavy metals experienced a reduction in their red blood cell count, hemoglobin levels, and hematocrit levels [11,27,76–78].

The observed reduction in red blood cell count suggests that Hg may have a detrimental effect on erythrocytes during circulation. A comparable occurrence was observed by Heath [79] in fish that were subjected to high levels of metallic substances. According to Al-Rudainy's research [80], the enzymatic pathway responsible for the production of Hb in fish is inhibited by heavy metals. The findings suggest that the tilapia that were subjected to 0.6 mg/L Hg for a duration of 15 days exhibited a state of anemia or hemodilution, as evidenced by the observed reductions in RBC, Hb, and Ht levels. The findings align with the results

of our study's pO_2 examination, which indicates a significant decrease in pO_2 levels among fish treated with Hg. Fish with such a condition experience insufficient oxygen supply to their tissues, leading to reduced levels of activity and productivity [61,81].

5. Conclusions and recommendations

Mercury (Hg) has the potential to infiltrate and pollute aquatic ecosystems due to anthropogenic actions. Once present, it has the capacity to be sequestered and concentrated within the aquatic ecosystem or assimilated by aquatic organisms. Ultimately, the substance will enter the food chain through both water and food sources, resulting in adverse health effects for both animals and humans. *Oreochromis niloticus*, is a species of significant commercial importance due to its high protein content. The potential impact of Hg on *O. niloticus* is an issue of significant concern, as the species is often cultivated in freshwater environments that are vulnerable to contamination by metallic substances originating from anthropogenic sources. The study findings indicate that the presence of Hg can lead to a decrease in the levels of electrolyte, osmoregulation, acid-base equilibrium, hematological parameters, and the capacity of tilapia to supply sufficient oxygen to the cells. Hence, the prevention of pollution by heavy metals including Hg in aquatic environments is considered one of the greatest crucial strategies to tackle this issue. The unregulated discharge of sewage and industrial effluents has significantly impaired the overall aquatic environment and water quality, thereby impacting the biochemical and physiological well-being of aquatic organisms, including fish. To preserve ecological equilibrium, it is recommended that industrialists refrain from disposing of their waste without prior treatment. In the long run, the reduction of pollution emanating from these specific sources would result in a corresponding decrease in the concentration of toxic metals in fish, as the level of pollutants in their natural environment diminishes. To address this issue, it is imperative to devise strategies or measures that can offer a viable solution. Consequently, it is imperative to take action at present to guarantee that forthcoming pollution and discharges of heavy metals in aquatic ecosystems are reduced to the greatest possible degree to avert contamination of aquatic ecosystems on a global scale and to avoid harmful effects on fish and humans.

Availability of data and materials

Upon request, the corresponding author will provide access to the data utilized to substantiate the findings of this research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The impact of various periods of mercury exposure on the osmoregulatory and blood gas parameters of tilapia (*Oreochromis niloticus*)

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ABSTRACT

Mercury (Hg) poses a significant risk to aquatic ecosystems and living organisms as a result of human activities. Hg found in fish can be detrimental to human health when consumed, causing harmful effects. The potential impact of Hg on fish physiology is a significant concern, given its presence in fish. The rapid accumulation of Hg in fish tissues has the potential to impact their blood gas and electrolyte compositions. The objective of this investigation was to analyze the effects of sublethal concentrations of Hg on the blood gas and electrolyte levels of tilapia (*Oreochromis niloticus*) over different periods of exposure. This research was conducted through laboratory experiments. The study involved subjecting fish to sublethal concentrations of Hg at levels of 0.06 and 0.6 mg/L for a duration of 4 and 15 days. Upon completion of the toxicity test, fish specimens were taken from each testing group for the purpose of analyzing the Hg and carbonic anhydrase (CA) concentrations present in the gills, as well as osmoregulatory, acid-base, and hematological parameters. The results indicate that solely the fish that were subjected to a concentration of 0.6 mg/L Hg for a duration of 15 days exhibited a higher concentration of Hg in their gills compared to the control group. The inhibition of respiration by Hg was observed to be a result of the generation of metabolic acidosis, reduction in gill CA, decrease in pO_2, as well as a decrease in plasma osmolality, Cl^-, Na^+, and K^+. The levels of red blood cells, hemoglobin, and hematocrit exhibited a decrease solely at the concentration of 0.6 mg/L over a period of 15 days. The previously mentioned impairments have the potential to restrict the capacity of fish to adequately supply oxygen to their cells, thereby reducing overall performance.

1. Introduction

Mercury (Hg) is considered to be one of the most perilous pollutants that pose a threat to aquatic ecosystems. This is due to its potent neurotoxicity towards fish, wildlife, and humans [1]. Hg is emitted into the environment as a result of natural rock weathering or volcanic activity. Nevertheless, it is human activity that serves as the primary contributor of mercury in the environment. The aforementioned phenomenon is a result of the process of coal combustion for the generation of electricity and the subsequent release of industrial waste [2]. In the majority of aquatic environments, Hg is primarily deposited from the atmosphere. According to the United States Environmental Protection Agency (USEPA), the biggest source of mercury in the atmosphere is emissions from coal-fired power plants [3]. Hg occurs naturally at very low concentrations in water. Commonly, uncontaminated freshwaters have 5–20 ng/L of total Hg, while contaminated waters can contain up to 0.5 μg/L [4]. Hg concentrations in river water near unregulated gold mining are between 12.7 and 13.6 μg/L [5]. In the vast majority of surface waters, the levels of Hg are typically much too low to have any direct adverse effects on either adult fish or the more sensitive

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early life stages of fish [6]. Acute toxicity of Hg to fish species varied, with 30 μg/L for a guppy *Lebistes reticulatus* [7], 580 μg/L for Mozambique tilapia *Orechromis mossambicus* [8], 327 μg/L for perch *Percocypris pingi* [9], 220–1220 μg/L for Nile tilapia *Oreochromis niloticus* [10,11], 606 μg/L for fresh water climbing perch *Anabas testudineus* [12], and 900 μg/L for common carp *Cyprinus carpio* [13].

The bioavailability of Hg in teleost fish is dependent on the overall chemical concentration of the surrounding environment, which includes pH, salinity, hardness, hydrophobicity, and metals' interactions with biotic and abiotic ligands, as well as the fish's ability to absorb the various Hg forms at the gill, skin, and digestive system [14]. Hg accumulation may impair fish's immune system [15,16], respiratory and cardiovascular systems [17,18], reproductive organs [19,20], digestive system and excretory system [21], blood system [22], and enzymatic activity [23].

As far as current knowledge is concerned, there exists a paucity of scientific study on the impact of Hg on the state of acid-base equilibrium in fish. Prior research has indicated that certain heavy metals, including Zn, Cu, Pb, and Cd, have the potential to impact the acid-base parameters of various species of fish, for instance rainbow trout (*Salmo gairdneri*) [24], cod (*Gadus morhua*) [25], groovy mullet (*Liza dumerili*) [26], and tilapia *O. niloticus* [27]. Huang and Chen [28] observed that various types of inorganic pollution, including nitrite, led to an increase of nitrite levels in the bloodstream, methemoglobin, as well as partial pressure of oxygen (pO_2) in *Anguilla Anguilla*, commonly known as European eels. Additionally, a negative correlation was observed between nitrite concentrations in media and blood pH, pCO_2, and HCO_3. Chen and Lee [29] observed that the giant prawn *Macrobrachium rosenbergii* exhibited elevated hemolymph pO_2 and ammonia excretion, along with a decrease in hemolymph pH, subsequent to exposure to nitrite. The exposure to heavy metals and other pollutants has been observed to cause alterations in acid-base balances and various hematological parameters. This phenomenon is characterized by a rapid sequence of events, wherein gill damage is expected to be the cause of hypoxemia, leading to tissue hypoxia and a resultant combined acidosis, ultimately proving to be fatal [30].

It can be observed that animals produce a comparable amount of carbon dioxide to the oxygen they consume during metabolic processes. Blood serves the function of conveying oxygen from the external milieu to the body's tissues, whilst the tissues discharge carbon dioxide, which is subsequently transported by the blood back into the external environment. Hemoglobin (Hb) is an essential component of the red blood cell (RBC) and facilitates the transportation of O_2 and CO_2 in the blood of all vertebrates [30]. The process of acid-base compensating in fish is primarily dependent on the direct transfer of H^+ and HCO_3^- via the gill as a substitute for Na^+ and Cl^-. As a result, the acid-base regulation in fish is closely associated with the regulation of ions. Consequently, the maintenance of ionic and osmotic equilibrium in fish necessitates regulating the flow of NaCl transportation across the gills. Carbonic anhydrase (CA) is responsible for regulating CO_2 excretion, ionic regulation, and acid-base balance [31]. Numerous in vitro investigations have suggested that fish CA activity is inhibited by heavy metals [32,33]. Nonetheless, there exists a scarcity of in vivo investigations concerning the impact of Hg on CA of fish. As a result, the impacts of Hg on the enzyme carbonic anhydrase found in fish will be the focus of this study.

Oreochromis niloticus is a economic value species of fish, particularly in Indonesia. Tilapia is extensively cultivated in ponds which obtain their water from nearby rivers. In the meantime, there are numerous agricultural, industrial, and residential activities along the river [34]. Thus, the aquaculture species that depend on river water as their primary resource supply are impacted by the waste, which includes heavy metals, that flows into the rivers from these activities. Typically, this species reacts quickly to environmental changes [11], and it can accumulate Hg from the environment [35]. The potential impact of Hg on tilapia is a significant concern, as the species is commonly cultivated in freshwater environments that are persistently polluted by metals originating from anthropogenic sources. The present investigation aimed to analyze the impact of sub-lethal Hg exposition on the osmoregulation, blood gas contents, hematological characteristics, as well as CA of gills of *O. niloticus* for a duration of four and fifteen days.

2. Materials and methods

2.1. Laboratory acclimation of experimental animals

The study used *O. niloticus* tilapia specimens that measured 15.5 ± 0.6 cm in length and weighed 68.6 ± 1.2 g. The piscine specimens were procured from a pisciculture establishment located in Pasuruan, East Java. They were transported to the laboratory in a plastic bag containing fresh water with oxygenation. Subsequently, the fish underwent a period of no less than 14 days for acclimating to the dechlorinated tap water, maintained at a temperature range of 28–29 °C, and subjected to a photoperiod of 12-h light and 12-h dark in the 250 L acclimated tanks located within the testing facility. A biofiltration system comprising of gravel, sand, and sponge filters was employed to facilitate the continuous recirculation of water during the acclimating process, thereby ensuring the preservation of water quality. The fish were administered pellet fish meal that consisted of 30% proteins, 3% fat, and 4% fibers (Takari, Indonesia) at a dosage equivalent to one percent of their daily estimated body weight [11]. On a daily basis, the removal of excrement, discarded food scraps, and other undesired materials was carried out to maintain an adequate standard of water quality for the fish. The optimal values for temperature, pH, and dissolved oxygen were established through regular measurements taken during the acclimation and experimentation phases. These values were determined to be 28.6 ± 0.5 °C, 7.8 ± 0.3, and 7.3 ± 0.5 mg/L, respectively.

2.2. Preparing a Hg solution

A 1000 mg/L Hg stock solution was prepared by dissolving 1.3539 g of $HgCl_2$ (Merck, Darmstadt, Germany) in 1 L of deionized water. As per the findings of our prior research, the lethal concentration (96h LC_{50}) of Hg for *O. niloticus* was determined to be 1.22 mg/L [11]. The nominal concentrations of Hg utilized in this experiment were determined based on the LC_{50} value, and were as follows: 0.06 mg/L (equivalent to 5% of LC_{50}), 0.6 mg/L (equivalent to 50% of LC_{50}), and a control group that did not contain Hg. The experiment media were subjected to concentration measurements, resulting values of 0.044 ± 0.07 mg/L, 0.49 ± 0.04 mg/L, and <0.001–0.001 mg/L (control), respectively. The study involved conducting experiments across two distinct time frames, namely a short-term period of four days and a long-term period of 15 days. The process for determining the concentration of Hg in the experimental media was performed according to the methodology outlined in Ref. [36]. The experimental media were subjected to filtration using a 0.45 μm membrane filter and was subsequently acidified to a pH of less than 2 through the addition of HNO_3. A volume of 100 mL of the specimen was transferred into a sample container that has a capacity of 300 mL. Subsequently, a volume of 5 mL H_2SO_4 and 2.5 mL HNO_3 were introduced and homogenized. Subsequently, 15 mL of potassium permanganate solution were introduced into each sample container, agitated, and supplemented with potassium permanganate as required until the persistence of the purple hue for a

minimum of 15 min. Subsequently, 8 mL of potassium persulfate were introduced into each vessel and subjected to heating in a water bath maintained at 90 ± 5 °C for 2 h. Following the cooling process, a 6 mL solution of sodium chloride-hydroxylamine sulfate was introduced into each container and subjected to analysis using a flameless atomic absorption spectrophotometer, specifically the Mercury-Hydride System Analitik Jena HS 60.

2.3. Sublethal toxicity test of Hg on O. niloticus

Following a period of acclimation, a sample of 50 fish in good health was randomly chosen from a holding tank and subsequently allocated to 10 separate tanks, each containing five fish. The experimental setup involved tanks with a volume of 40 L each, which were filled with a testing medium comprising of varying concentrations of Hg for different durations. Specifically, the concentrations of Hg were 0.06 mg/L and 0.6 mg/L, and the exposure periods were 4 and 15 days. Additionally, a control group was included in the study, which did not contain any Hg. Each concentration was tested using two sets of tanks. To maintain a constant concentration of Hg, 50% of the experimental medium was renewed at intervals of 48 h. Throughout the trial, the fish were provided with pellet fish meal at a concentration that corresponded to one percent of their daily estimated body weight. Blood and gill tissue samples were obtained from five fish selected at random from each treatment group following 4 and 15 days of exposure. Upon completion of the experiment, the experimental water containing Hg was collected and subsequently stored in a metallic tank designed for the storage of wastewater. The experimental procedures that utilized animals were conducted in compliance to the Animal Care and Use Regulations of Airlangga University.

2.4. The measurement of Hg levels in the gills

The concentration of Hg in tilapia gills was determined using a procedure proposed by Candra et al. [37]. The gills of tilapia were extracted and subjected to a drying process in an oven maintained at 60 °C for 48 h until a consistent weight was achieved. Following desiccation, the gills were pulverized into a fine particulate form. A quantity of 0.5 g of pulverized gills underwent a heating process in acid solutions consisting of 2 mL of HNO_3 - $HClO_4$ (1:1) and 5 mL of H_2SO_4. The heating process was carried out in a Mars 6 microwave digester for 3 h at 80 °C. Upon cooling of the solution, 0.1 mL of $KMnO_4$ and 0.5 mL of $SnCl_2$ were introduced to preserve the violet coloration. Sufficient NH_2OH—HCl solution was added to neutralize the excess $KMnO_4$, which functioned as a preservative. A singular portion of the specimen was subjected to analysis for the presence of Hg utilizing a flameless atomic absorption spectrophotometer, specifically the Mercury-Hydride System Analitik Jena, HS 60. The concentrations of Hg in the analyzed samples were reported in units of mg/kg of wet weight. The limit of detection for Hg was 0.001 mg/kg. The validation of the analytical approach was carried out through the measurement of Hg in the DORM-4 standard reference material of the National Research Council of Canada. In the course of validating the analytical procedure, it was ascertained that the recovery of Hg for the DORM-4 certificate was 93%.

2.5. The measurement of blood chemistry and physiological parameters

The fish were subjected to anesthesia using a clove solution of 200 mg/L prior to the collection of their blood samples. This particular anesthesia was selected due to its minimal impact on physiological parameters [38]. Approximately 1 mL of blood from each fish was extracted through the caudal aorta using a plastic syringe that was not heparinized [27]. The blood sample was collected and preserved using vacutainer plastic tubes containing EDTA as a means of preventing coagulation. Subsequently, the blood samples were promptly collected into an automatic blood analyzer (SFRI Blood Cell Counter 33, Jean d'Illac, France) for the evaluation of hematological parameters, including red blood cell (RBC) counts, hematocrit (Ht) levels, and hemoglobin (Hb) concentrations [11,39]. In order to measure the blood pH, pCO_2, and pO_2 levels, a blood sample was immediately administered onto a sensor card (Techno Medica 091, Japan), and subsequently inserted into an automatic blood gas analyzer (GASTAT-Navi, Japan). The pCO_2 and pO_2 were denoted in mmHg [27]. Blood plasma was isolated from blood cells through centrifugation of a small amount of the blood specimen at 5000 rpm and 4 °C for 10 min, before measuring the plasma's osmolality, as well as the concentrations of Na^+, Cl^-, and K^+. The plasma's osmolality was assessed through the introduction of a 20 μL plasma sample into a tube, followed by measurement using a micro-sample osmometer (Fiske® 210, Norwood, MA, USA) and reported in mOsm/kg units. The plasma's Na^+, Cl^-, and K^+ concentrations were assessed by transferring a 22 μL plasma sample into a specialized tube and subsequently analyzing it with an electrolyte analyzer (SpotChem EL SE-1520, Kyoto, Japan), and reported in mmol/L [11,39]. The manufacturer of each instrument utilized for measurement supplied the requisite chemicals and components for the determination of hematological, acid-base parameters, osmolality, and ion levels.

CA is a crucial factor in regulating the acid-base equilibrium in fish, predominantly taking place in the gills [40]. The present study employed an ELISA kit (Catalog Number E0123Fi) to determine the concentration of CA in gills that were subjected to Hg exposure. The experimental protocol was carried out in accordance with the guidelines provided by the manufacturer (Bioassay Technology Laboratory Biotech Co. Ltd.). The extracted gills were thoroughly rinsed with phosphate-buffered saline (PBS, pH 7.4) to eliminate any remaining blood and weighed. The microtiter plate was precoated with Anti-CA antibody before its utilization. In order to ascertain the concentration of CA, standardized samples of 50 μL, blank samples, and 40 μL samples were introduced into every well. Ten microliters of anti-CA antibody and 50 μL of streptavidin-horseradish peroxidase were promptly added to every well, with the exception of the control blank. The contents were homogenized, sealed with a plate cover, and subjected to incubation at 37 °C for a duration of 60 min. Following the removal of the sealer, the plate underwent automated aspiration and was subsequently rinsed five times with wash buffer. The tissue paper was utilized for the purpose of cleaning the plate. The plates were securely closed with a sealant and subsequently subjected to incubation at a temperature of 37 °C in the absence of light for approximately 10 min. During this incubation period, 50 μL of solution A and 50 μL of solution B were added to every well. Stop solution of 50 μL was added to each well to terminate the enzymatic activity. The sample was assessed at a wavelength of 450 nm using an automatic microplate reader (Model iMark by Bio-Rad, Japan) within a time frame of 10 min of the administration of the stop solution. The concentration of CA was expressed in units of ng/ml.

2.6. Statistical analysis

The data was presented in terms of mean and standard deviation, and was subjected to normality testing. The data was subjected to statistical analysis through the application of two-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test. A significant statistical difference was observed when the p-value was less than 0.05. The statistical analyses were conducted utilizing IBM® SPSS® Statistics version 25.

3. Results

The findings of the mercury toxicity assessment conducted on tilapia indicate that no mortalities were observed throughout the testing period. The accumulation of Hg in the gills of fish was observed following exposure to Hg-contaminated water. Specifically, Hg levels of 0.0052 and 0.006 mg/kg were recorded after 4 and 15 days of exposure to 0.06 mg/L Hg, respectively. Additionally, a Hg level of 0.0072 mg/kg was observed in fish gills after 4 days of exposure to 0.6 mg/L Hg. However, the concentrations of Hg in the gills did not exhibit a statistically significant difference when compared to the control group (0.0028 mg/kg). The results indicate that the highest concentration of Hg in the gills (0.015 mg/kg Hg) was observed solely in fish that were subjected to a 0.6 mg/L Hg exposure for 15 days (Fig. 1). The concentration treatment of 0.06 mg/L seems to be statistically insignificant in terms of Hg accumulation in fish gills when compared to the control group after 4 and 15 days of exposure. Similarly, when subjected to treatment involving elevated concentrations of Hg (0.6 mg/L), the accumulation rate in the gills of fish was observed to be greater than that of the control group. However, the difference was considered statistically insignificant when the fish were exposed for a duration of only 4 days. The findings indicate that, in addition to concentration, the duration of exposure is a significant factor in the increase of Hg accumulation in the gills of fish. Upon increasing the duration of exposure to a concentration of 0.6 mg/L of Hg by nearly four-fold (over a period of 15 days), a significant ($p < 0.05$) six-fold increase in the accumulation of Hg in fish gills was observed when compared to the control group of fish (Fig. 1).

Under a high treatment concentration of 0.6 mg/L for a duration of 15 days, the presence of Hg had a significant impact on pH, pCO_2, pO_2, and CA. However, no significant differences were observed in other treatments as compared to the control. The results of the study indicate that fish exposed to a concentration of 0.6 mg/L Hg for a period of 15 days exhibited a decrease in blood pH (7.1), pO_2 (59.1 mmHg) and CA (15.6 ng/ml) levels, while displaying an increase in blood pCO_2 (34.7 mmHg) levels (Fig. 2). The observed values displayed a statistically significant difference ($p < 0.05$) when compared to the control group. The results indicate that the sublethal concentration of 0.06 mg Hg/L had a negligible impact on the blood composition of tilapia. Specifically, the exposure duration ranging from 4 to 15 days did not significantly alter the pH, pCO_2, pO_2, and CA levels in the fish blood.

The blood parameters (osmolality, Cl^-, Na^+ and K^+) of fish subjected to 0.6 mg/L Hg for 4 and 15 days exhibited a significant decline ($p < 0.05$) in comparison to the control value. A statistically significant reduction ($p < 0.05$) in plasma osmolality was observed, with values of 351.8 mOsm/kg and 327.4 mOsm/kg in comparison to the control group (368.4 mOsm/kg), respectively. The lowest level was observed after 15 days of exposure. The results of the study indicate that fish exposed to a concentration of 0.6 mg/L of Hg for a period of 15 days experienced a notable reduction ($p < 0.05$) in their blood mineral compositions, particularly in the levels of Cl^-, Na^+, and K^+. These concentrations were found to be the lowest among all treatments, while other treatments did not exhibit any significant differences when compared to the control group (Fig. 3).

The results of the study indicate that only fish that were subjected to a concentration of 0.6 mg/L Hg for a period of 15 days exhibited a significant reduction ($p < 0.05$) in their RBC ($0.87\ 10^6$/μL), Hb (3.46 g/dL), and Ht (18.36%) levels in comparison to the control group. However, the other treatments did not demonstrate any significant differences from the control group (Fig. 4).

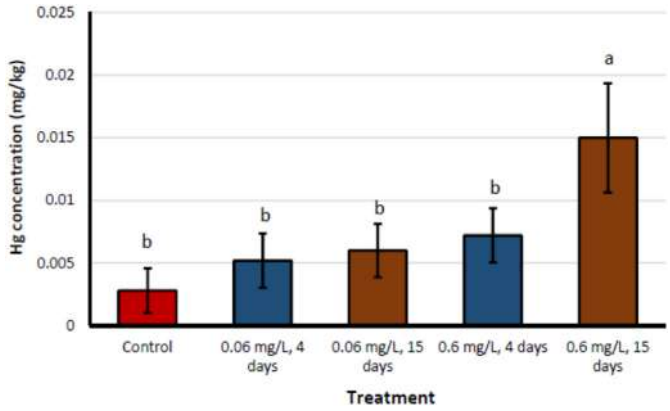

Fig. 1. Hg concentration in fish gills subjected to varying Hg medium concentrations. Lower case characters indicate significant differences ($p < 0.05$, a > b), Number of samples (N) = 5 individual.

4. Discussion

The study employed a comparatively elevated concentration of Hg due to the notable tolerance of *O. niloticus* towards Hg, as previously reported [6]. The present study employed sub-lethal concentrations of Hg at 0.06 and 0.6 mg/L. Although the concentrations of Hg in concern may exceed those typically found in the natural environment [4], it is anticipated that investigations into the impact of Hg on various parameters of fish blood can be conducted at this level of concentration, and that any resulting effects can be readily observed. The gill Hg levels in the tilapia under investigation were found to vary depending on the exposure time and Hg concentrations. The highest level of Hg was observed in the group exposed to 0.6 mg/L Hg for a duration of 15 days. The study observed the occurrence of acidosis in fish that were subjected to 0.6 mg/L Hg for 15 days. The ultimate outcome of this phenomenon is an elevation in pCO_2 accompanied by a reduction in pO_2, plausibly attributable to the disruption of gill function by Hg. Exposure to Hg resulted in respiratory failure, leading to a reduction in oxygen uptake, accumulation of carbon dioxide in the bloodstream, and an elevation in pCO_2. The respiratory gas transfer process in fish is significantly dependent on the gill, which serves as the primary site for CO_2 sensing and O_2 chemoreception [40–42]. Consequently, exposure of the gill to pollutants such as Hg may potentially disrupt this crucial function.

The respiration of shrimp larvae was found to be impacted by varying levels of Hg and exposure durations, resulting in a reduction in their oxygen consumption rate (RO_2). Following a 10h exposure to 160 ppb of Hg, there was a reduction of 43% and 49% in the RO_2 levels in zoeae III and zoeae V stages, respectively. A duration of 27 h of exposure to 80 ppb of Hg or more resulted in paralytic weakness in almost all of the larvae [43]. The impediment of RO_2 may be elucidated by the diffusion of heavy metal across the permeable surfaces, namely gills and teguments [44]. According to Hassaninezhad et al. [45], the presence of $HgCl_2$ at concentrations of 0.01 and 0.02 mg/L may result in a reduction of gas exchange ability in yellowfin seabream (*Acanthopagrus latus*) due to fish gill respiration deficiency caused by Hg contamination.

Elevated levels of pCO_2 and notable reductions in pO_2 were observed in the hemolymph of *Chasmagnathus granulatus* crabs exposed to ammonia. The alterations proposed in the study indicate that the histological damage observed in the gills hindered the process of gas exchange [46]. The study findings indicate that a reduction in the CA level in the tilapia gills was observed after 15 days of exposure to 0.6 mg/L Hg. The decrease is thought to be attributable to the gill cells' incapacity to transform CO_2 into HCO_3^-. This finding is consistent with the findings showed by Larsen et al. [25] and Shandro and Casey [47].

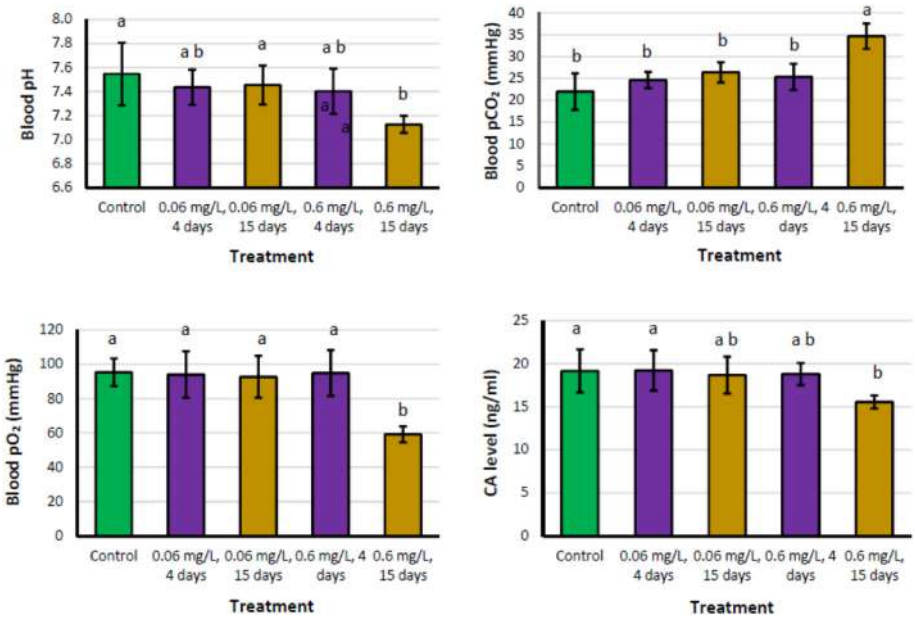

Fig. 2. Blood pH, pCO_2, pO_2, and CA levels in fish exposed to varying Hg levels. Significant differences are denoted with lowercase letters ($p < 0.05$, a > b), N = 5 individual.

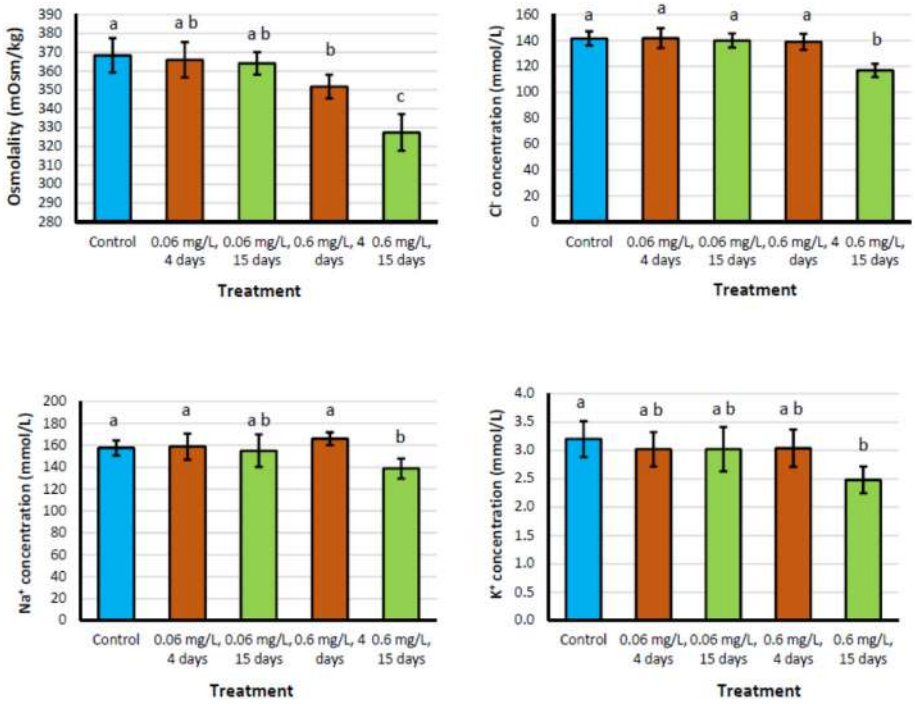

Fig. 3. Osmolality and ion concentrations in mercury-exposed fish. Lowercase characters show significant statistical differences ($p < 0.05$, a > b > c), N = 5 individual.

The regulation of NaCl transport across the gills is crucial for maintaining ionic and osmotic equilibrium in fish [31]. The process of acquiring the essential ion (either Na^+ or Cl^-) from the adjacent milieu is linked to the transference of ions that are pertinent to acid-base equilibrium into the water. The acid-base interactions mentioned possess a direct impact on the ion-regulatory necessities of the organism [48]. Freshwater fish are capable of achieving ion and osmoregulatory homeostasis through the process of exchanging Na^+ or Cl^- for their internal H^+ or HCO_3^-. The aforementioned mechanism enables the fish to regulate its acid-base equilibrium and maintain homeostasis of its ions [31,48]. The regulation of monovalent ions (Na^+ or Cl^-) in freshwater fish is altered by heavy metals, resulting in ion outflow [49–51]. The study found that tilapia exposed to 0.6 mg/L Hg for a period of 15 days experienced a decrease in plasma Na^+ and Cl^- concentrations. At the given concentration of treatment, it is possible for Cl^-/HCO_3^- exchange and Na^+/H^+ exchange to take place in the gills while experiencing hypercarbia. Following a 96-h exposure to hypercapnia in freshwater, Atlantic salmon (*Salmo salar*) exhibit the development of respiratory acidosis. In response to this situation,

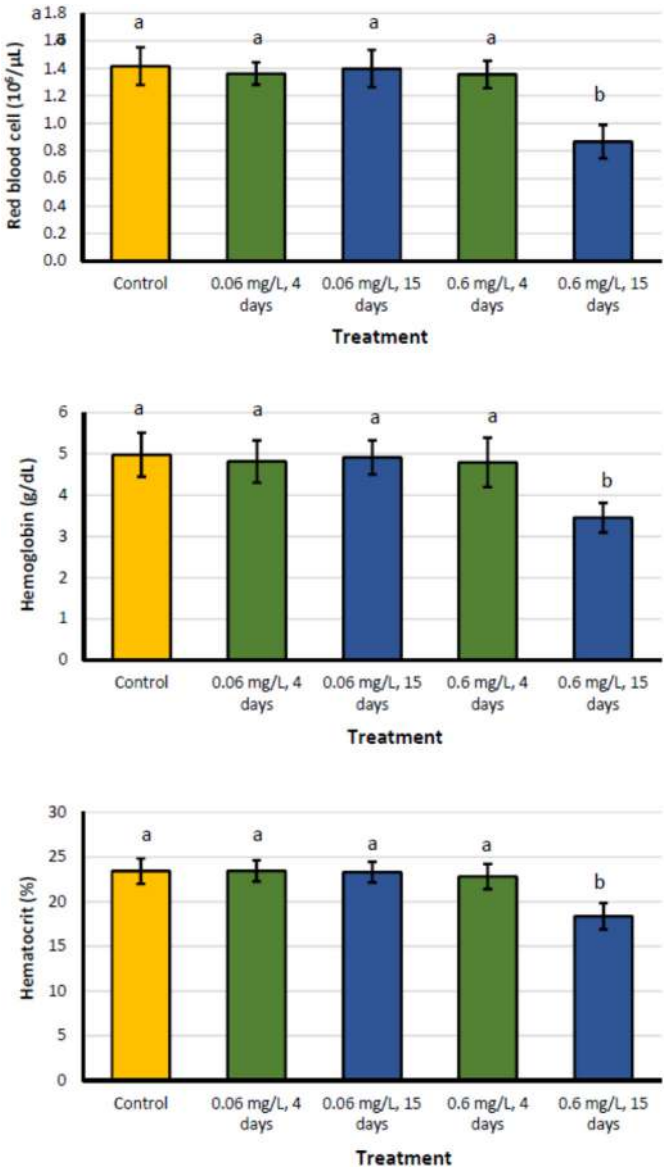

Fig. 4. The levels of red blood cells, hemoglobin, and hematocrit in fish exposed with Hg. Lower case characters indicate significant statistical differences ($p < 0.05$, a > b), N = 5 individual.

the fish employ a mechanism involving the exchange of Cl^-/HCO_3^- and Na^+/H^+ at the branchial level to reduce their plasma levels of Cl^- and Na^+. This leads to an increase in their ion difference [52]. The aforementioned modifications take place during the process of acidosis regulation and could potentially suggest a broader dysfunction in gill functionality [52]. The findings of our investigation indicate that exposure to 0.6 mg/L Hg over a period of 15 days elicited a reduction in plasma Cl^- and Na^+ levels, subsequently leading to a decrease in osmolality. This outcome is likely attributable to damage incurred by gill cells. According to Larsen et al. [25], a plausible reason for the reduction in Cl^- and Na^+ levels could be the alteration of Na^+/K^+-ATPase activity in chloride cells due to exposure to heavy metals, which could potentially disturb the branchial ion exchanger. The hemolymph of shore crab (*Carcinus maenas*) experienced a reduction in osmolality and ion content as a result of exposure to copper. The observed phenomenon could potentially be attributed to an elevation in osmoregulatory Na^+/K^+-ATPase activity [53]. The gills of fish seem to experience a disturbance in Na regulation as a result of exposure to inorganic Hg, which is attributed to a reduction in the activity of Na^+/K^+-ATPases in the gills [54,55]. Previous studies have reported a robust inverse relationship between the concentration of pollutants and the activity of Na^+/K^+-ATPase in the gills of the European flounder (*Platichthys flesus*) inhabiting a region contaminated with mercury [56]. Additionally, a comparable inhibition of Na^+/K^+-ATPase activity has been observed in mrigal carp (*Cirrhinus mrigala*) following acute exposure to $HgCl_2$ at concentrations of 0.068 and 0.034 mg/L [57]. According to Macirella and Brunelli [58], short exposure to two low concentrations of mercury chloride led to changes in gill morphology and alterations in Na^+/K^+-ATPase. The enhancement was elucidated as a period of adaptation that relies on the sustained metallic impact or maintaining of the ion flow [59]. The findings of this research indicate that the exposure to 0.6 mg/L Hg for a period of 15 days resulted in a significant reduction in both ionic and osmotic regulations. Undoubtedly, extended exposure may lead to death; however, additional research is necessary to confirm this assertion.

The findings indicate that the exposure of fish to 0.6 mg/L Hg for a duration of 15 days resulted in a reduction in K^+ levels. The potential cause of the reduced level of K^+ ions could be attributed to the impaired gill epithelium and the subsequent impact on the activity of the Na^+/K^+-ATPase, leading to alterations in the passive fluxes. Due to the permeability of fish gills to K^+, the efflux of K^+ is greater than its influx. According to Partridge and Lymbery [60], a reduction in the uptake of K^+ is comparatively more important than an elevation in K^+ loss. Alternately, the reduction in serum K^+ can be attributed to the adjustment of fish to lower osmolality [61]. According to Oliveira-Ribeiro et al. [62], the gill epithelium can be disrupted by exposure to dissolved Hg, which may have an impact on gas exchange and the ability of cell membranes to allow cations to pass through. The observed changes in Na^+/K^+-ATPase activity could potentially account for the competitive disadvantage experienced by the blue mussel species *Mytilus edulis* in the presence of the antifouling agent chlorothalonil, which induces an increased passive K^+ efflux [63]. The study conducted by Prasad et al. [64] revealed that alterations in the levels of Na^+ and K^+ ions could potentially signify a stress-induced response that arises due to extended exposure of fish to harmful substances. The aforementioned exposure possibly initiated distinct physiological and metabolic mechanisms that have the potential to enhance the efflux of ions.

The findings of the study indicate that a reduction in RBC, Hb and Ht levels were observed in tilapia that were subjected to 0.6 mg/L Hg for a duration of 15 days. The values of RBC, Hb, and Ht of Tench (*Tinca tinca*) decreased significantly in response to acute lethal (1.0 mg/L Hg for 48 h) and sublethal (0.25 mg/L Hg for 3 weeks) treatments, whereas at lower sublethal (0.1 mg/L Hg for 24 h) treatments, RBC and Ht values increased significantly while Hb levels remained stable [65]. Walking catfish (*Clarias batrachus*) exposed for 14 days to varying concentrations of mercuric chloride ($HgCl_2$) exhibited a decline in RBC and Hb levels [66]. A decrease in RBC, Hb and Ht in Silver carp (*Hypophthalmichthys molitrix*) were recorded after exposure to both low (10% LC_{50}) and high (50% LC_{50}) concentrations of $HgCl_2$ for 4 days [67]. *Tinca tinca* exhibited a significant decrease in their RBC, Hb, and Ht levels following exposure to three distinct mercury treatments [68]. Ishikawa et al. [69] reported that there was no significant difference in the levels of RBC, Hb, and Ht in *Oreochromis niloticus* when exposed to varying concentrations of Hg (0.02, 0.002, 0.0002 mg/L).

Hematological alterations have been confirmed in association with acidosis. Our findings indicate that fish subjected to 0.6 mg/L Hg over a period of 15 days exhibit a reduction in RBC, Hb, and Ht concentrations, along with acidosis and ion imbalance. Under these

critical circumstances, when the heightened energy demand exceeded the capacity of aerobic energy production, the organism initiates anaerobic glycolysis as a means of generating additional ATP. Lactic acid, which is the end result of anaerobic glycolysis, is known to induce fatigue and reduce the pH of blood plasma [70]. The reduction in Cl^- levels in plasma could be attributed to the movement of Cl^- into red blood cells, potentially due to low plasma pH. Otherwise, Cl^- could have been transported into the intracellular membrane, thereby limiting lactic efflux [71]. Moreover, Turner et al. [72] found that highly trained trout experienced an increase in lactate levels in their blood and a decline in blood plasma Cl^-. They suggested that this exchange could be the reason for these changes. The reduction in Ht level may be associated with the diminution of erythrocyte size. The findings suggest that prolonged exposure to high levels of Hg in tilapia may lead to a significant decrease in blood O_2-affinity, possibly due to a reduction in the size of red blood cells. This is evidenced by the observation of smaller red cells in carp (*Cyprinus carpio*) that were exposed to high levels of toxicant [73]. The increased size of red blood cells may potentially increase their ability to bind with oxygen [74]. As a result, it is likely that the presence of Hg could impede the transportation of oxygen through the bloodstream of fish.

The study findings indicate that a 15day exposure to 0.6 mg/L Hg resulted in a reduction of all blood parameters that were examined. Hence, an indication of a disturbance in the erythrocytes or erythropoietic function is present [75]. Several studies have reported that different fish species subjected to different levels of heavy metals experienced a reduction in their red blood cell count, hemoglobin levels, and hematocrit levels [11,27,76–78].

The observed reduction in red blood cell count suggests that Hg may have a detrimental effect on erythrocytes during circulation. A comparable occurrence was observed by Heath [79] in fish that were subjected to high levels of metallic substances. According to Al-Rudainy's research [80], the enzymatic pathway responsible for the production of Hb in fish is inhibited by heavy metals. The findings suggest that the tilapia that were subjected to 0.6 mg/L Hg for a duration of 15 days exhibited a state of anemia or hemodilution, as evidenced by the observed reductions in RBC, Hb, and Ht levels. The findings align with the results of our study's pO_2 examination, which indicates a significant decrease in pO_2 levels among fish treated with Hg. Fish with such a condition experience insufficient oxygen supply to their tissues, leading to reduced levels of activity and productivity [61,81].

5. Conclusions and recommendations

Mercury (Hg) has the potential to infiltrate and pollute aquatic ecosystems due to anthropogenic actions. Once present, it has the capacity to be sequestered and concentrated within the aquatic ecosystem or assimilated by aquatic organisms. Ultimately, the substance will enter the food chain through both water and food sources, resulting in adverse health effects for both animals and humans. *Oreochromis niloticus*, is a species of significant commercial importance due to its high protein content. The potential impact of Hg on *O. niloticus* is an issue of significant concern, as the species is often cultivated in freshwater environments that are vulnerable to contamination by metallic substances originating from anthropogenic sources. The study findings indicate that the presence of Hg can lead to a decrease in the levels of electrolyte, osmoregulation, acid-base equilibrium, hematological parameters, and the capacity of tilapia to supply sufficient oxygen to the cells. Hence, the prevention of pollution by heavy metals including Hg in aquatic environments is considered one of the greatest crucial strategies to tackle this issue. The unregulated discharge of sewage and industrial effluents has significantly impaired the overall aquatic environment and water quality, thereby impacting the biochemical and physiological well-being of aquatic organisms, including fish. To preserve ecological equilibrium, it is recommended that industrialists refrain from disposing of their waste without prior treatment. In the long run, the reduction of pollution emanating from these specific sources would result in a corresponding decrease in the concentration of toxic metals in fish, as the level of pollutants in their natural environment diminishes. To address this issue, it is imperative to devise strategies or measures that can offer a viable solution. Consequently, it is imperative to take action at present to guarantee that forthcoming pollution and discharges of heavy metals in aquatic ecosystems are reduced to the greatest possible degree to avert contamination of aquatic ecosystems on a global scale and to avoid harmful effects on fish and humans.

Availability of data and materials

Upon request, the corresponding author will provide access to the data utilized to substantiate the findings of this research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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