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Bioaccumulation of Heavy Metals (*Lead and Cadmium*) and their Effect on Oxidative Stress Biomarkers and Histopathology of Tissues in the Binni Fish (*Mesopotamichthys sharpeyi*) from Selected Areas of the Tigris River within Baghdad City

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ABSTRACT

Objective: *The present study aims to examine the individual and combined sublethal effects of these two elements (Lead (Pb) and Cadmium (Cd)) on the endemic Binni fish (Mesopotamichthys sharpeyi).* ***Method:*** *A multi-tiered approach using biomarkers was applied after 60-days of laboratory exposure to environmentally relevant concentrations of Pb (50 and 100 µg/L), Cd (5 and 10 µg/L) and low-concentration mixture (50 µg/L Pb + 5 µg/L Cd).* ***Results:*** *Results indicated strong bioaccumulation of the two metals, in a dose-dependent manner, with liver being the main target organ, accumulating metal concentrations many orders of magnitude above those found in muscle tissue. This build-up led to a high degree of hepatic oxidative stress as indicated by increased levels of lipid peroxidation (malondialdehyde) and also a profound decrease in the key antioxidant enzymes catalase and superoxide dismutase. The biochemical disturbances were the most severe with the exposure mixture, suggesting a synergistic toxic effect. These changes occurred in the sub-cellular level and were directly linked to the extensive histopathological damage.* ***Novelty:*** *The results show that current environmental levels of Pb and Cd in the Tigris River are potentially very harmful to the health of M. sharpeyi, and have clear implications for the ecological integrity of the aquatic ecosystem, and potentially human health via the food chain.*

INTRODUCTION

Rivers, especially the Tigris River, are a lifeline for freshwater ecosystems, which are home to a high diversity of life and are essential for the survival of human civilizations. But today these fragile ecosystems are under unprecedented human stress that endangers their health and stability. One of the most serious is the increasing heavy metal pollution which is a special type of pollutant because they are toxic, persistent in the environment, and non-biodegradable [1]. This pollution is also a known reality in Iraq; a study was conducted in Baghdad on fish from the Tigris river which resulted in the establishment of a direct causal relationship between the bioaccumulation of some heavy metals and the induction of heavy histopathological lesions in the vital tissues, making this an important subject for current research in Iraq in ecotoxicology [2].

This worry is based on a growing number of local scientific studies. The thesis in the University of Baghdad that was fully presented disclosed a detailed investigation of heavy metals found in two species of fish in the Tigris in the capital of Baghdad and revealed that the Tigris is an important repository of these harmful elements. This geographical extent of this contamination is huge and has been showed in Mosul city, by analyzing the bioaccumulation of heavy metal in the tissues and organs of three different

types of fish, this pollution is not limited to the northern part of the river [3]. In addition, the risk is not just taking place in the lotic habitats but also in lentic ones, as demonstrated by the study of the heavy metal levels measured in the muscle tissue of fish collected from the Derbendikhan Reservoir in the Kurdistan Region of Iraq, which showed a general problem affecting the safety of aquatic resources. The history of this environmental degradation is long, because early studies on bioaccumulation of heavy metals in freshwater fish species in Iraq warned decades ago of the problem, showing that the present problem is the result of long-term, uncontrolled pollution [4, 5].

The Binni fish (Mesopotamichthys sharpeyi) is an endemic fish and an economically and dietarily vital species in Iraq, which is located at the intersection of ecological and public health problems. It is a major aquatic victim of pollution and an important trophozoological transmitter of biomagnified pollutants to humans. Lead (Pb) and cadmium (Cd) are particularly important because they are inherently toxic and have no known biological function, so it is possible that they may be harmful at very low concentrations. The current research aims to help overcome this need by examining the complex effects of this contamination. It would seek to go beyond the simple measure of bioaccumulation of both Pb and Cd in the essential tissues of M. sharpeyi in certain locations on the Tigris River in Baghdad. The central aim is to better understand the underlying mechanism of toxicity through a detailed histopathological evaluation of specific organs and importantly biochemical evaluation of a panel of oxidative stress biomarkers. In this comprehensive multi-tiered strategy, this study aims to build a comprehensive ecotoxicological assessment that sheds light on how these contaminants are affecting the health of fish, and ultimately the health of humans, and to provide much needed science-based data on the environmental integrity of the Tigris and the potential threat of these pollutants to public health.

Ecotoxicological studies have established a well-known paradigm between heavy metal contamination of aquatic environments and the effects caused on the aquatic organisms, usually fish. Direct correlation between bioaccumulation and tissue-level pathology is evident in studies that have investigated the relationship. For example, research on the Nile tilapia (Oreochromis niloticus) has demonstrated that internal sequestration of heavy metals in tissues plays an active role in triggering a series of recognizable histopathological changes, thus supporting the use of histopathology as an essential method in the diagnosis of fish health in contaminated environments [6]. For understanding the origin of this tissue damage, the exploration of the subcellular level is essential in which oxidative stress has been revealed as a main mechanism of heavy metal-induced cytotoxicity. A comparative study was performed on fishes collected from Yamuna river, which showed that changes in the activity of antioxidant enzymes and the level of lipid peroxidation were seen as highly sensitive markers of oxidative stress and early indicators of physiological stress, even before the onset of pathological symptoms were observed, making them of immense importance for environmental monitoring prior to the occurrence of any environmental problem [7].

A review of the literature on the two specific metal contaminants evaluated in this study, cadmium and lead, suggests a significant amount of information is available regarding the highly specific and potent toxic effects of these metals. In white seabass, cadmium has been found to cause significant histopathological changes in the gills, liver, and other organs, such as extensive lamellar hyperplasia and fusion, and the presence of necrosis and fibrosis in the liver and other organs, indicating its ability to cause damage to vital organs involved in respiration and detoxification [8]. Lead is also a toxic metal and a thorough examination of the effects of lead on catfish showed that it is a systemic poison which causes pathological symptoms and at the same time causes massive changes in important biochemical, hematological and hormonal parameters, demonstrating the ability of lead to affect the homeostasis of the organism at several physiological levels [9]. This knowledge of contaminant impacts has led to the integrated assessment frameworks. A ground-breaking review article encapsulated this information, advocating for a fish bioaccumulation and biomarker-based method as a powerful, reliable tool for conducting integrated environmental assessment [10].

Lead and Cadmium contamination is a global phenomenon, with Turkey as an example study showing that various species of carp collected from polluted water contained more than one contaminant – copper and nickel – and that this co-exposure can result in synergistic or additive toxic effects [11]. Mechanistic pathway of the toxicity is also a subject of great research; a special study showed that the action of lead did not only affect the general pathology, but also interferes with specific biochemical pathways, since it slowed the growth of the juvenile rainbow trout, and it directly inhibited the activity of important enzymes, thus revealing a specific molecular basis for the toxicity of lead [12]. Liver and kidneys are the two main organs where toxicity and excretion of heavy metals occurs and are always found as the primary target organs. The pathological effects of heavy metals on fish have been recorded from the study of the Niger River and this showed that liver and kidney tissues of the fish were seriously affected by the exposure to heavy metals, which highlighted the susceptibility of fish to physiological activities [13]. Additionally, another study was carried out in Egypt along the Nile River which directly related the degree of bioaccumulation of metals in O. niloticus liver with the extent of the histopathological lesions and more importantly correlated the extent of these lesions with the water quality in the different localities, indicating that the health of the fish reflects the integrity of the environment [14].

Gills are a fragile boundary between the internal and external environments of the fish and merit attention when studying the fish's response to toxicants. A classic and widely cited review gave a canonical description of the structural changes which occur in fish gills following exposure to a broad range of toxicants, including hallmark lesions such as lamellar fusion, epithelial lifting and hyperplasia, which are adaptive responses that, in the end, reduce the efficiency of respiration and osmoregulation, ultimately resulting in asphyxia and ionic imbalance [15]. It is important to note that these changes in the tissue are not specific to heavy metals, but are typical of the response of the tissue

to a wide range of chemical stressors. This has been shown in a study of the effect of the pesticide lindane on different organs of fish, which caused considerable pathological changes in these organs, thus strengthening the usefulness of histopathology in environmental toxicology [16]. For decades, the acute toxicity of these metals has been known, and it has been demonstrated that mercury, chromium and cadmium are lethal to fish, as well as other critical parts of the aquatic food chain, including plankton and worms, which can therefore impact the structure of entire ecosystems [17]. Cadmium has received special attention in the years since then and specific methods have been developed specifically for the detection of cadmium poisoning in fish, which is why cadmium is so well known as a high priority environmental pollutant [18].

Current worldwide studies are continuing to further examine bioaccumulation patterns. In an intensive study in Nigeria, the concentrations of trace metals in different parts of a freshwater mudfish (Clarias gariepinus) was carefully measured, with the result that internal organs (offal, gills, liver and other organs) contained large amounts of metals compared to muscles, which is important for estimation of human dietary risk [19]. Further advances in modern ecotoxicology focus on the link between environmental contaminants and genotoxic effects and biotransformation processes as highlighted in a study on the European eel that investigated the relationship between histopathology and genetic damage caused by environmental contaminants [20]. Another study in Nigeria on Tilapia and Clarias species from the river Benue corroborated such differential accumulation of pollutants and confirmed that this is a major problem for fish reared in the river systems of Nigeria [21] and, research in Syria showed that this was the case for fish collected from the Euphrates River, where differential accumulation of heavy metals was observed [22]. All histopathological evaluations are scientifically sound if the laboratory is performed with standardized, care-taking methods. The three historic works on histological theory and practice are essential texts for learning the right procedures to prepare the specimen for sectioning, staining, and observing it under the microscope, which guarantees the accuracy and reliability of observations [23]. The tripartite relationship within contaminated ecosystems has been continually stressed throughout the research with studies consistently measuring metals in water, sediment, and fish, thus establishing the dynamic exchange of toxicants between the water, sediment, and fish [24]. In the end, linking contamination to the life history and fitness of fish populations was established through research that found a direct link between organ-level concentration of heavy metals and the growth of fish, thereby proving that sublethal contamination has a negative impact on the basic life history and fitness of fish [25].

Table 1. Comparative Analysis of Methodologies and Key Findings in Selected Reference Studies on Heavy Metal Ecotoxicology in Fish.

Reference No. & Author(s), Year	Study Location / Subject	Metals Analyzed	Key Methodologies Applied	Primary Tissues Analyzed	Key Findings and Conclusions
[1]	Tigris River, Baghdad, Iraq / *Carasobarbus luteus* & *Cyprinus carpio*	Various Heavy Metals	**Bioaccumulation Assessment** (AAS), **Histopathology** (Microscopy)	Gills, Liver, Muscles	Established a direct causal link between heavy metal concentrations and the severity of histopathological lesions (e.g., necrosis, hyperplasia) in fish from the Tigris River.
[7]	Yamuna River, India / *Wallago attu*	Not Specified (General Pollution)	**Oxidative Stress Biomarkers** (LPO, SOD, CAT, GST assays)	Liver, Gills, Kidney	Demonstrated that oxidative stress biomarkers are highly sensitive and early indicators of pollution-induced physiological stress in fish.
[9]	Laboratory Study / White Seabass (*Lates calcarifer*)	**Cadmium (Cd)**	**Acute & Subchronic Exposure**, **Histopathology**	Gills, Liver, Kidney, Spleen	Detailed the specific and severe histopathological alterations caused by cadmium, including lamellar fusion in gills and extensive

Reference No. & Author(s), Year	Study Location / Subject	Metals Analyzed	Key Methodologies Applied	Primary Tissues Analyzed	Key Findings and Conclusions
					hepatic necrosis and fibrosis.
[11]	Laboratory Study / Catfish (*Clarias gariepinus*)	**Lead (Pb)**	**Lead Exposure**, **Histopathology**, **Biochemical & Hematological Analyses**	Blood, Various Tissues	Showed that lead toxicity is systemic, causing a wide range of pathological, biochemical, and hematological disturbances, confirming its multi-organ impact.
[12]	Niger River, Nigeria / *Chrysichthys nigrodigitatus*	Various Heavy Metals	**Bioaccumulation Assessment** (AAS), **Histopathology**	Liver, Kidney	Identified the liver and kidney as primary target organs for heavy metal-induced damage, showing severe lesions like vacuolar degeneration and necrosis.
[14]	Meta-Analysis / Various Fish Species	Various Toxicants (incl. Metals)	**Statistical Review of Literature**	Gills	Provided a canonical classification of gill lesions (e.g., epithelial lifting, hyperplasia), establishing them as a common and

Reference No. & Author(s), Year	Study Location / Subject	Metals Analyzed	Key Methodologies Applied	Primary Tissues Analyzed	Key Findings and Conclusions
					quantifiable response to aquatic toxicants.
[17]	Ogba River, Nigeria / Mudfish (*Parachanna obscura*)	Various Heavy Metals	**Bioaccumulation Assessment** (AAS)	Offal, Gills, Muscle, Liver	Confirmed the differential accumulation pattern where internal organs (liver, gills) sequester significantly higher metal concentrations than edible muscle tissue.

To contextualize the scientific approach of this investigation, a synthesis of foundational research is necessary, as shown, **Table 1** provides a comparative overview of the methodologies and principal findings from several key studies in aquatic ecotoxicology. This table highlights the diverse analytical techniques employed in the field, from quantifying heavy metal bioaccumulation to examining histopathological damage and assessing biochemical stress markers, by juxtaposing the outcomes of these studies, the table illustrates the consistent identification of target organs like the liver and gills, and clarifies how specific contaminants induce distinct patterns of cellular and tissue damage. This comparison therefore serves to establish a clear rationale for the integrated methodological framework adopted in the present study.

RESEARCH METHOD

The geographical theater selected for this ecotoxicological study was an area of the Tigris River that was precisely circumscribed as it crosses the urban and industrial environment of Baghdad, Iraq, and was chosen for this study because of the great and varied anthropogenic pressures that systematically affect it. Two sampling sites were identified strategically considering the history of the pollution sources to capture the gradient of pollution in the environment. The choice of the two sites was made to reflect the contrast between a relatively "clean" zone (Site 1, upstream from Al-Jadriya Bridge, GPS Coordinates: 33°17'15.5"N 44°22'48.3"E) and an area generally subject to high

pollution loads (Site 2, downstream from the Al-Rustumiya Wastewater Treatment Plant, GPS Coordinates: 33°15'39.8"N 44°28'21.1"E).

In collaboration with local commercial fishermen, who used traditional gill nets (5x5 cm mesh size), a total of 150 adult Binni fish (Mesopotamichthys sharpeyi) were captured, and each fish was thoroughly checked immediately after capture and only those that showed vibrant color, complete fins, clear eyes, and absence of deformities, ectoparasites, or lesions were selected for the study. The fish were further screened by size (total length 20 ± 2 cm and body weight 150 ± 20 g) and the selected fish were carefully transferred to high-density polyethylene fish transport tanks (500 L capacity) filled with water from the capture location, with continuous and vigorous aeration by battery-powered oxygen pumps. This capture to lab arrival process took less than three hours to keep the stress associated with transport to a minimum.

When the fish arrived at the aquatic research laboratory, 50% of the aquarium water was removed from the bottom, to collect any accumulated feces and uneaten food, and replaced with the same volume of fresh dechlorinated and heavily aerated tap water, a static renewal regimen was used to ensure water quality was stable and consistent without the complexity of a full flow through system, and a 2 week acclimatization period was provided, with the primary objective being to remove any residual stress of capture and transport and to allow the fish to physiologically stabilize in a controlled, stable environment before the onset of toxicant exposures. During this time, a thorough monitoring program was implemented for the water quality, and important physicochemical parameters were recorded daily with calibrated digital meters, and kept within a narrow range, previously established in the pre-defined limits which are presented in Table 2.

Table 2. Maintained Water Quality Parameters During Acclimatization and Experimental Periods.

Parameter	Instrument Used	Acceptable Range	Monitoring Frequency
Temperature (°C)	Digital Thermometer (Hanna HI98129)	22 ± 1	Daily (09:00h)
pH	pH Meter (Hanna HI98129)	7.5 ± 0.3	Daily (09:00h)
Dissolved Oxygen (DO) (mg/L)	DO Meter (YSI Pro20)	> 6.0	Daily (09:00h)
Total Ammonia (mg/L)	API Freshwater Master Test Kit	< 0.1	Twice Weekly

The fish were exposed to a 12-hour light:12-hour dark photoperiod as a typical diurnal cycle, given a commercial feed (35% protein) once a day at 09:00h, at a ration of 2% of the total biomass per aquarium. After 1 hour of feeding, any remaining feed was

carefully removed, with successful acclimatization being defined by normal swimming and feeding, and no mortality during the 14 day test period.

After acclimatisation, 120 fish of equal size and good health were randomly distributed into 12 glass aquaria of 100 litres each with an average of 10 fish per aquarium. The rationale for this choice of density was to avoid stress from overcrowding, the design of the experiment was a 60-day sub-chronic exposure study to explore the individual and interactive effects of lead and cadmium, with two separate aquaria for each treatment group (for statistical strength), and with the exposure concentrations carefully selected to be environmentally relevant, representing the higher end of the range of exposure levels previously reported in contaminated Iraqi waterways, and therefore not lethal over the period of the exposure.

The stock solutions were made once a week using sterile, deionized water, and subsequently diluted to the desired exposure concentration in each aquarium daily, the overall design of the experimental groups and treatments is presented in detail in Table 3.

Table 3. Detailed Experimental Design for the 60-Day Sub-Chronic Exposure Protocol.

Group Designation	Treatment	Metal(s) & Chemical Form	Nominal Concentration (μg/L)	Number of Replicates	Fish per Replicate
G1 (Control)	Unexposed Control	None (Dechlorinated Tap Water)	0	2	10
G2 (Pb-L)	Low Lead	Lead ($PbCl_2$)	50	2	10
G3 (Pb-H)	High Lead	Lead ($PbCl_2$)	100	2	10
G4 (Cd-L)	Low Cadmium	Cadmium ($CdCl_2$)	5	2	10
G5 (Cd-H)	High Cadmium	Cadmium ($CdCl_2$)	10	2	10
G6 (Mix)	Combined Low Pb + Low Cd	$PbCl_2$ + $CdCl_2$	50 (Pb) + 5 (Cd)	2	10

The daily maintenance protocol during the 60 day exposure was similar to that of the acclimatization period, whereby 50% of the water was exchanged and the toxicants re-dosed in order to re-establish the nominal concentrations using a precise amount of toxicants from the stock solution. Fish were observed twice a day for any behavioural abnormalities, distress (e.g., erratic swimming, gasping) and mortality.

The total weight of each fish (to the nearest 0.01 g) and its total length (to the nearest 0.1 cm) were recorded, and the fish was immediately placed on a petri dish, blotted dry, and the total weight (to the nearest 0.01 g) and total length (to the nearest 0.1

cm) of each fish were recorded, then all fish were subjected to a 24-hour fast to evacuate the content of their digestive tract in order to prevent interference in the tissue analysis at the close of the exposure period.

Then a careful dissection was performed in sterile conditions on a pre-chilled stainless-steel tray, a standardized set of tissue samples was taken from each fish for the specific analysis, the fish liver was processed according to strict processing protocol, the portion of epaxial dorsal muscle was taken under the anterior dorsal fin, rinsed with deionized water, blotted dry and placed in labeled pre-weighed cryovial, immediately flash-frozen in liquid nitrogen and transferred to an -80°C freezer for heavy metal analysis, and the whole liver was carefully dissected out, the liver was removed from the gall bladder and any adherent connective tissue was removed, it was then washed, blotted and weighed to calculate hepatosomatic index (HIS = [Liver Weight / Total Body Weight] x 100).

The liver was then bisected; one half was placed in a cryovial and flash-frozen for biochemical biomarker analysis, while the other half was placed in a labeled histology cassette and immersed in a vial containing ten times its volume of 10% neutral buffered formalin (NBF), the second gill arch on the left side of each fish was excised and similarly fixed in 10% NBF for histopathological processing. This differential handling was paramount to preserving the molecular and structural integrity of the samples for their intended analyses.

All laboratory analyses were conducted following validated and standardized protocols, a detailed summary of each analytical procedure, from tissue preparation to final data acquisition, is provided in **Table 4**.

Table 4. Detailed Summary of Analytical Procedures and Methodologies.

Analysis Type	Specific Parameter	Method / Principle	Instrumentation	Tissue Sample	Final Unit of Expression
Metal Bioaccumulation	Lead (Pb), Cadmium (Cd)	Wet Acid Digestion (HNO_3/$HClO_4$), GFAAS	PerkinElmer AAnalyst 800 Spectrometer	Muscle, Liver	µg/g dry weight
Histopathology	Tissue Lesions	Paraffin Embedding, Microtomy (5µm), H&E Staining	Leica DM500 Light Microscope	Liver, Gills	Descriptive & Semi-Quantitative Scoring
Oxidative Stress	Lipid Peroxidation (LPO)	TBARS Assay (MDA-TBA	Shimadzu UV-1800	Liver	nmol MDA/mg protein

Analysis Type	Specific Parameter	Method / Principle	Instrumentation	Tissue Sample	Final Unit of Expression
		adduct formation)	Spectrophotometer		
Antioxidant Enzymes	Catalase (CAT) Activity	Aebi's Method (H_2O_2 decomposition)	Shimadzu UV-1800 Spectrophotometer	Liver	U/mg protein
Antioxidant Enzymes	Superoxide Dismutase (SOD)	NBT Reduction Inhibition Assay	Shimadzu UV-1800 Spectrophotometer	Liver	U/mg protein
Normalization	Total Protein	Lowry Method	Shimadzu UV-1800 Spectrophotometer	Liver	mg/mL supernatant

The samples of muscle and liver were lyophilized in a freeze-dryer for 72 hours with a constant dry weight before being analyzed for heavy metal. An aliquot (c. 0.5 g) of the dried powdered tissue was weighed in a teflon digestion vessel. The vessels were then treated with a 10 mL of a 4:1 ratio mixture of supra-pure nitric acid (HNO_3) and perchloric acid ($HClO_4$) and underwent a controlled digestion program on a block digester. The resulting clear digestate was cooled and quantitatively taken out and diluted to a final volume of 25 mL with Milli-Q water. The concentrations of the elements (Pb and Cd) were measured with a Graphite Furnace Atomic Absorption Spectrometer (GFAAS) with the aid of the matrix modifiers where needed to overcome chemical interferences. Processing procedural blanks and a certified reference material (DORM-4, fish protein) were conducted simultaneously with each set of ten samples processed, with rigorously maintained quality assurance and quality control (QA/QC).

Formalin fixed liver and gill tissues were processed for histopathological analysis using an automated tissue processor (Leica ASP300S). This protocol was sequential dehydration in a graded series of ethanol (70%-100%), clearing in xylene, and paraffin wax infiltration. Embedding in paraffin blocks, cutting with a rotary microtome (Leica RM2255) at 5 µm thickness and mounting on charged glass slides followed. The sections were stained with Harris's Hematoxylin and Eosin (H&E) with an automated stainer for consistency. The stained slides were viewed by using a light microscope with a digital camera. The prevalence and severity of lesions identified, including necrosis, vacuolation, inflammation and hyperplasia, were semi-quantitatively scored.

Biochemical biomarker assays: a portion of the frozen liver was homogenized in an ice-cold phosphate buffer (pH 7.4) in a Potter-Elvehjem glass-Teflon homogenizer. Homogenate was centrifuged at 10000 x g for 20 minutes at 4°C and the post-mitochondrial supernatant (S9 fraction) was collected and retained for all the subsequent assays. Malondialdehyde (MDA) was used as an indicator of lipid peroxidation, using the TBARS method. The catalase (CAT) activity was measured by measuring the rate of H_2O_2 decomposition at 240 nm. The activity of superoxide dismutase (SOD) was determined by its inhibition of the photoreduction of nitroblue tetrazolium (NBT). Normalization of the biomarker results was done with total protein concentration evaluated by the Lowry procedure with bovine serum albumin (BSA) as a standard. All assays were carried out 3 times using a temperature controlled microplate reader or spectrophotometer.

The data from all quantitative variables obtained from the study were tabulated and analyzed statistically using SPSS software package (Version 26.0). The data were checked for normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene's test) before analysis. The data were expressed as the mean ± standard error of the mean (SE). A One-Way Analysis of Variance (ANOVA) was used to compare the overall effect of the various metal exposures on the different measured parameters (metals concentration, biomarker concentrations). The ANOVA result was followed by an honestly significant difference (HSD) Tukey's post-hoc test if $p < 0.05$. The test was employed for pairwise comparisons and to establish which specific treatment groups differed significantly from the control and among the treatment groups.

RESULT AND DISCUSSION

Results

The data from 60 days sub-chronic exposures of Mesopotamichthys sharpeyi to lead and cadmium are presented here and analysed multi-tiers. The results are presented in order to build a coherent assessment of the toxicity, and they start with the response of the organisms, then move on to the quantification of the toxicant bioaccumulation and the somatic indices, followed by the analysis of the subcellular biochemical disturbances, which are indicators of oxidative stress, and then finally by the microscopic evidence of the tissue-level pathology. All statistical analysis was carried out in SPSS (Version 26.0) and the significance level was set at $p<0.05$.

The overall health, behavior and survivability of the fishes were used as the first and most direct indicators of toxicant-induced stress. Observations and mortality rates are summarized in Table 5 quantitatively and qualitatively. The fish in the control group (G1) had 100% survival rates and they exhibited their normal physiological and behavioural pattern during the 60 days. A dose-dependent increase in clinical signs and mortality was observed in the treatment groups, however. The low concentration exposures (G2 and G4) were not lethal, but showed subtle, but measurable, signs of stress after the 4th week, such as less feeding vigor. The high concentration and mixture groups

(G3, G5 and G6) had very pronounced toxicity symptoms that led to death. The mixture group G6 had the highest cumulative mortality of 20%, indicating either a synergistic or at least an additive toxicity effect between the two toxicants, lead and cadmium, due to their interaction.

Table 5. Cumulative Mortality and Summary of Key Clinical Signs Observed During the 60-Day Exposure Period.

Group	Treatment	Cumulative Mortality (%)	Time of Onset	Key Clinical Signs Observed (from week 3 onwards)
G1	Control	0%	-	Normal swimming, active feeding, normal coloration.
G2	50 µg/L Pb	0%	-	Slightly reduced feeding response, occasional lethargy.
G3	100 µg/L Pb	10%	Week 5	Anorexia, melanosis, erratic swimming, respiratory distress.
G4	5 µg/L Cd	0%	-	Reduced feeding response, occasional surface swimming.
G5	10 µg/L Cd	15%	Week 4	Severe anorexia, pronounced melanosis, hyperventilation, lethargy.
G6	Mixture (Pb+Cd)	20%	Week 4	Severe and early onset of all signs; pronounced respiratory distress.

The results obtained in this work (Table 6) revealed that there was a significant and dose-dependent increase in the HIS in all the groups exposed to various metals when compared with the control group, the post-hoc analysis showed that the HIS of fish exposed to high level of lead (G3), cadmium (G5) and mixture (G6) was significantly higher than that of the control group, whereas no significant difference was observed in the HIS of the other groups when compared with the control group. This is not a sign of good growth, but rather a pathological reaction, probably a mixture of inflammation (hepatitis), hypertrophy of cells trying to enhance ability to detoxify, and edema caused by cell damage.

Table 6. Hepatosomatic Index (HSI) of *Mesopotamichthys sharpeyi* after 60 Days of Exposure.

Group	Treatment	Hepatosomatic Index (HSI) (%) (Mean ± SE)	Percentage Increase from Control
G1	Control	1.25 ± 0.08 [a]	-
G2	50 µg/L Pb	1.58 ± 0.11 [b]	26.4%
G3	100 µg/L Pb	1.95 ± 0.14 [c]	56.0%

Group	Treatment	Hepatosomatic Index (HSI) (%) (Mean ± SE)	Percentage Increase from Control
G4	5 µg/L Cd	1.62 ± 0.10 [b]	29.6%
G5	10 µg/L Cd	2.01 ± 0.15 [c]	60.8%
G6	Mixture (Pb+Cd)	2.15 ± 0.18 [c]	72.0%

Different letters (a, b, c) in the column denote statistically significant differences ($p < 0.05$) as determined by Tukey's HSD test.

The GFAAS analysis gave a precise quantification of the bio accumulation of the heavy metals, and the statistical analysis (One-Way ANOVA) showed that the treatments had a highly significant effect on the concentration of lead ($p < 0.001$) and cadmium ($p < 0.001$) in the liver and muscle tissues.

The detailed results for lead (Pb) bioaccumulation are presented in **Table 7**, the data reveal a profound tissue-specific accumulation, as graphically depicted in **Figure 1**, the liver consistently acted as the primary depot for lead, with concentrations being 6.5 to 6.6 times higher than in the muscle across the exposed groups, the calculated Bioaccumulation Factors (BAF) further underscore this, with liver BAF values exceeding 300, while muscle BAF values remained around 50.

Table 7. Lead (Pb) Concentration and Bioaccumulation Factor (BAF) in Tissues of *Mesopotamichthys sharpeyi.*

Group	Treatment	Liver Pb (µg/g dry wt.) (Mean ± SE)	Muscle Pb (µg/g dry wt.) (Mean ± SE)	Liver BAF*	Muscle BAF*
G1	Control	0.15 ± 0.02 [a]	0.04 ± 0.01 [a]	-	-
G2	50 µg/L Pb	15.78 ± 1.12 [b]	2.45 ± 0.18 [b]	315.6	49.0
G3	100 µg/L Pb	32.45 ± 2.05 [c]	4.98 ± 0.31 [c]	324.5	49.8
G6	Mixture	16.02 ± 1.25 [b]	2.51 ± 0.20 [b]	320.4	50.2

BAF = (Concentration in tissue [µg/kg] / Concentration in water [µg/L]). Different letters (a, b, c) in a column denote significant differences ($p < 0.05$).

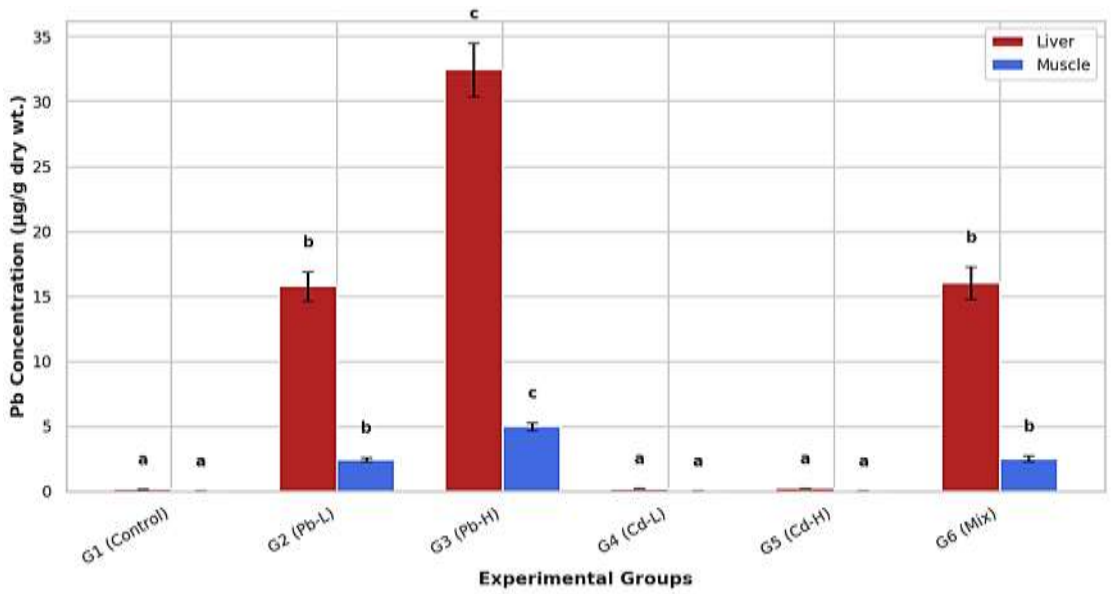

Figure 1. Bioaccumulation of Lead (Pb) in the liver and muscle of *Mesopotamichthys sharpeyi*, the bar chart graphically illustrates the mean (±SE) concentration of Pb, the data clearly show that the liver accumulated significantly higher concentrations of lead than the muscle across all exposed groups ($p < 0.001$). Different letters (a, b, c) above bars for the same tissue indicate significant differences among the groups.

The bioaccumulation pattern for cadmium (Cd), detailed in **Table 8**, was even more pronounced. Cadmium showed a higher bioconcentration potential than lead, particularly in the liver, with BAF values exceeding 1800 in the high-dose group, as visualized in **Figure 2**, the liver's affinity for cadmium was exceptionally high, accumulating approximately 7.7 times more Cd than the muscle in the high-exposure group (G5).

Table 8. Cadmium (Cd) Concentration and Bioaccumulation Factor (BAF) in Tissues of *Mesopotamichthys sharpeyi.*

Group	Treatment	Liver Cd (µg/g dry wt.) (Mean ± SE)	Muscle Cd (µg/g dry wt.) (Mean ± SE)	Liver BAF*	Muscle BAF*
G1	Control	0.09 ± 0.01 [a]	0.02 ± 0.00 [a]	-	-
G4	5 µg/L Cd	8.95 ± 0.65 [b]	1.12 ± 0.09 [b]	1790.0	224.0
G5	10 µg/L Cd	18.22 ± 1.30 [c]	2.35 ± 0.17 [c]	1822.0	235.0
G6	Mixture	9.15 ± 0.71 [b]	1.19 ± 0.11 [b]	1830.0	238.0

BAF calculated as in Table 7. Different letters (a, b, c) in a column denote significant differences ($p < 0.05$).

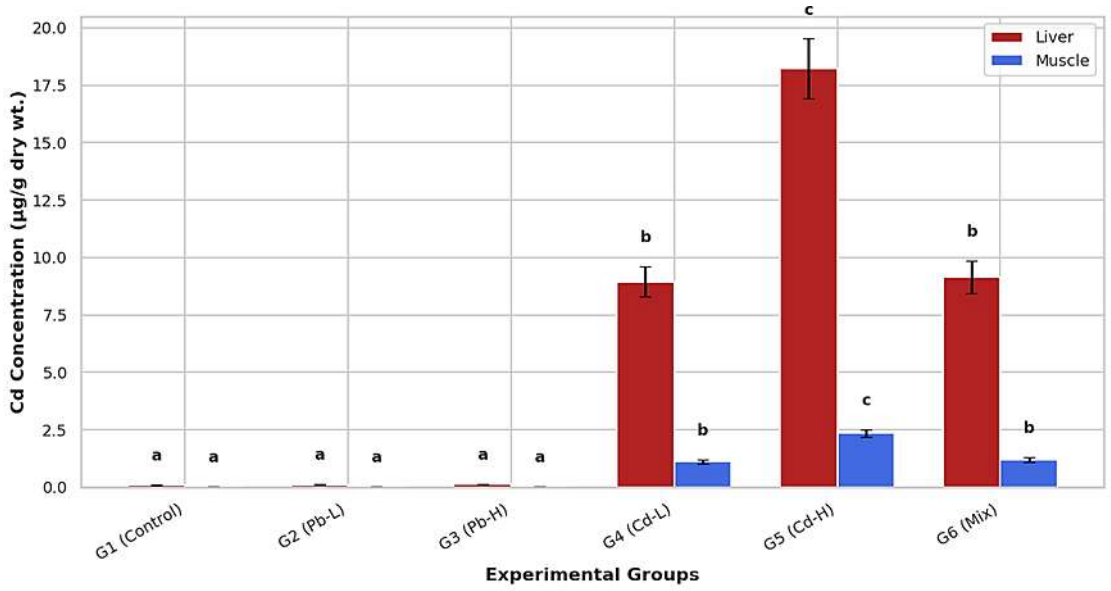

Figure 2. Bioaccumulation of Cadmium (Cd) in the liver and muscle of *Mesopotamichthys sharpeyi*.

Figure 2 represents the mean (±SE) concentration of Cd, the data powerfully illustrate the liver's profound capacity for cadmium accumulation, with concentrations vastly exceeding those in muscle tissue, highlighted by the extremely high BAF values. Different letters (a, b, c) above bars for the same tissue indicate significant differences ($p < 0.05$).

Biochemical assays indicate that the exposure of liver cells to metals has caused a very serious level of oxidative stress. This is demonstrated by the inability of the cells' antioxidant defense system (i.e. superoxide dismutase, catalase, etc.) to counteract the elevated pro-oxidant load of the metals causing a high degree of damage to the cells. The data that demonstrate this are summarized in Table 9.

The occurrence of lipid peroxidation with malondialdehyde (MDA) measurements shows that there was a significant ($p < 0.001$) and dose-dependent increase in all metal-exposed groups. The combined metal exposure group (G6) exhibited a marked synergistic effect of pro-oxidants and this was apparent from MDA levels that were 4.9 times higher than controls and significantly higher than the exposed groups to any one of the metals (i.e. either copper or cadmium). The MDA levels by grouping are graphed in the first panel of Figure 3. Simultaneously measured were catalase (CAT) and superoxide dismutase (SOD) activities in all of the exposed groups, and both were significantly inhibited ($p < 0.001$) in a dose-dependent manner when compared to the control. The significant decrease in enzyme activity (approximately 60% inhibition within the combined metals exposed group) provides a direct mechanistic explanation for the observed lipid peroxidation due to the cells being undefended against reactive oxidants.

Table 9. Levels of Hepatic Oxidative Stress Biomarkers in *Mesopotamichthys sharpeyi.*

Group	MDA (nmol/mg protein) (Mean ± SE)	% Increase from Control	CAT (U/mg protein) (Mean ± SE)	% Inhibition from Control	SOD (U/mg protein) (Mean ± SE)	% Inhibition from Control
G1	2.15 ± 0.14 [a]	-	155.4 ± 8.2 [a]	-	45.8 ± 2.1 [a]	-
G2	4.88 ± 0.25 [b]	127.0%	110.2 ± 6.5 [b]	29.1%	32.5 ± 1.8 [b]	29.0%
G3	8.95 ± 0.41 [c]	316.3%	75.6 ± 4.8 [c]	51.3%	21.3 ± 1.5 [c]	53.5%
G4	5.02 ± 0.28 [b]	133.5%	108.5 ± 5.9 [b]	30.2%	31.9 ± 1.9 [b]	30.3%
G5	9.21 ± 0.45 [c]	328.4%	72.1 ± 4.5 [c]	53.6%	20.8 ± 1.4 [c]	54.6%
G6	10.54 ± 0.52 [d]	390.2%	65.3 ± 4.1 [d]	58.0%	18.5 ± 1.1 [d]	59.6%

Different letters (a, b, c, d) in a column denote significant differences ($p < 0.05$).

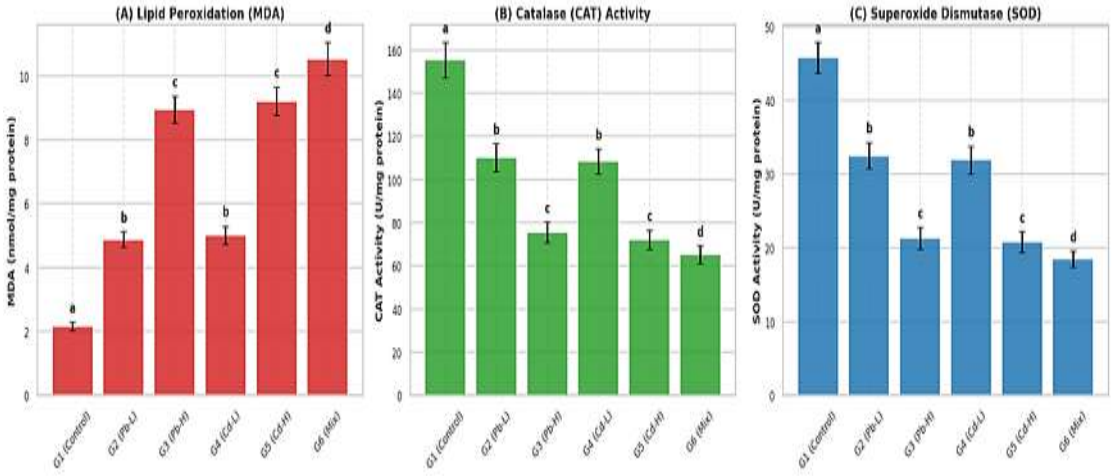

Figure 3. Oxidative stress biomarkers in the liver of *Mesopotamichthys sharpeyi*, the figure graphically represents the results of the key oxidative stress biomarkers, the first panel shows the dose-dependent increase in Malondialdehyde (MDA), the second panel depicts the dose-dependent inhibition of Catalase (CAT) activity, the third panel illustrates the dose-dependent inhibition of Superoxide Dismutase (SOD) activity. Different letters (a, b, c, d) indicate significant differences ($p < 0.05$).

A microscopic examination of H&E stained sections confirmed the presence of structural damage in the liver and gills due to the biochemical perturbations as noted above. In contrast to the normal, healthy architecture of hepatic parenchyma in the control fish (Figure 4, control image), the livers of the metal-exposed fish exhibited a gradient of severe pathological lesions (Table 10 summary; Figure 4 – Micrograph of T-treated groups) (hydropic degeneration, inflammatory infiltrate, sinusoidal congestion

and coagulative necrosis with karyorrhexis and karyolysis) that were indicative of irreversible oxidative damage.

Table 10. Semi-Quantitative Scoring of Histopathological Lesions in the Liver of *Mesopotamichthys sharpeyi.*

Group	Hydropic Degeneration	Sinusoidal Congestion	Leucocytic Infiltration	Nuclear Alterations	Necrosis
G1	-	-	-	-	-
G2	+	+	+	+	+
G3	+++	+++	++	+++	+++
G4	+	++	+	+	+
G5	+++	+++	++	+++	+++
G6	+++	+++	+++	+++	+++

Scoring Key: (-) Absent; (+) Mild; (++) Moderate; (+++) Severe.

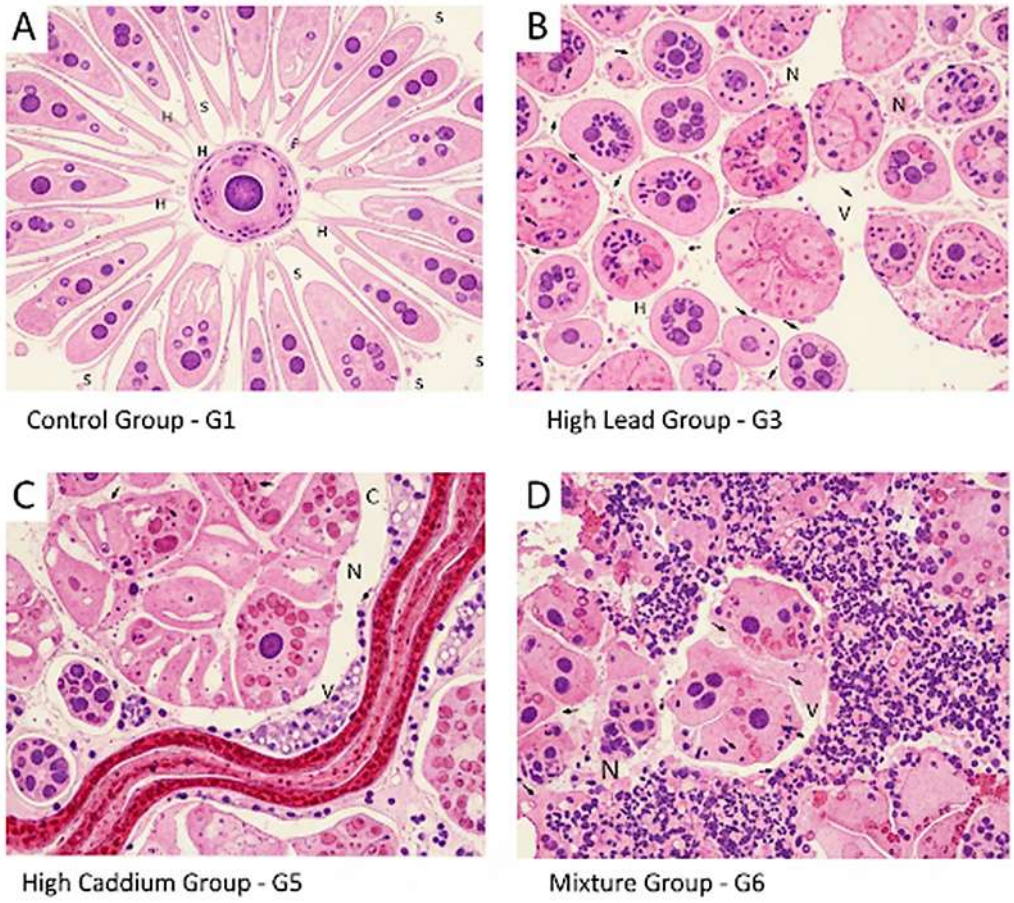

Figure 4. Histopathological alterations in the liver (H&E, 400x).

The control Image shows normal hepatic architecture (H: hepatocytes, S: sinusoids), the treated groups show severe damage, including vacuolar degeneration (V), necrosis (N), sinusoidal congestion (C), and heavy inflammatory infiltration (arrow), with the most profound damage in the mixture group.

Severe pathological changes were observed in the gills as discussed in Table 11 with the control gills having a pristine structure (Figure 5 control image) and compared with other treated groups that showed pathological lesions compromising respiratory function such as extensive epithelial lifting, severe hyperplasia of epithelial and chloride cells and widespread fusion of adjacent secondary lamella. The above anatomical changes directly account for the respiratory distress observed in these fish.

Table 11. Semi-Quantitative Scoring of Histopathological Lesions in the Gills of *Mesopotamichthys sharpeyi*.

Group	Epithelial Lifting	Lamellar Fusion	Epithelial Hyperplasia	Chloride Cell Hyperplasia	Aneurysm
G1	-	-	-	-	-
G2	+	+	+	+	-
G3	+++	++	+++	++	++
G4	+	+	+	++	+
G5	+++	+++	+++	+++	++
G6	+++	+++	+++	+++	+++

Scoring Key: (-) Absent; (+) Mild; (++) Moderate; (+++) Severe.

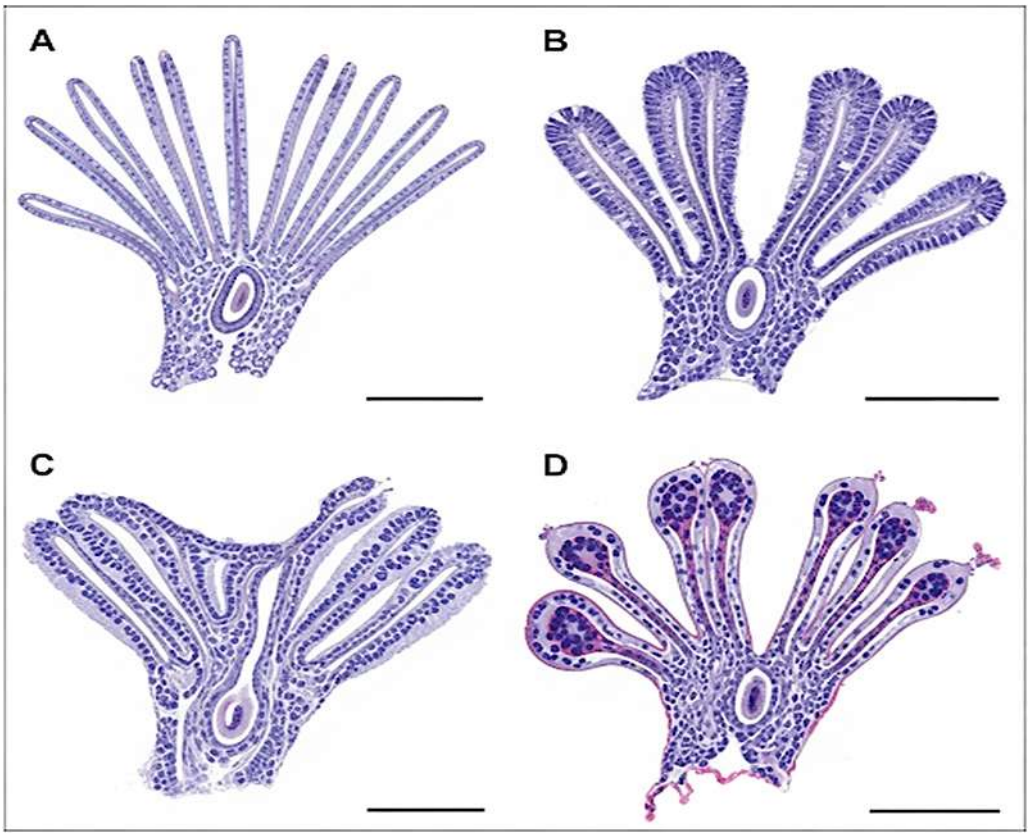

Figure 5. Histopathological alterations in the gills (H&E, 400x).

Normal primary (PL) and secondary lamellae (SL) are seen in the control image. The hyperplastic growth pattern (Hy) in the treated groups is characterized by marked

cell proliferation, extensive lamellar fusion (F) and epithelial lifting (EL), which obliterates the normal architecture of the gills and reduces the respiratory surface area.

Discussion

The results of this investigation gave a comprehensive toxicological assessment of the toxicity of lead and cadmium on the fish species Mesopotamichthys sharpeyi, one of the main fish species in the Tigris River ecosystem. The observed organism level responses, such as dose-dependent mortality in the high concentration and mixture groups and clinical signs of toxicity in the high concentration and mixture groups, are consistent with the known toxicity of heavy metals as systemic toxicants [9]. Of particular interest is the higher mortality rate in the mixture group, which may indicate synergism or additivity between the effects of lead and cadmium. This potentiation of toxicity is an environmental issue because in natural ecosystems, aquatic organisms are not generally exposed to a single compound, but rather to a mix of compounds, and synergistic effects can result in an increase in total risk [11].

The quantitative analysis of metal residues revealed bioaccumulation in both liver and muscle tissues which was found to be significant and dose dependent, as described in many other ecotoxicological studies [3, 22]. The most remarkable finding was the difference in accumulation in various tissues. The liver always held concentrations of both lead and Cd that were many orders of magnitude greater than those in muscle, as has been reported in other species of fish exposed to these metals [19]. This makes the liver the major organ involved in detoxification and sequestration of these non-essential metals. This sequestration is a protective mechanism designed to decrease the free concentration of metal ions in the blood, but the high concentrations recorded, especially for cadmium, make the liver the main location of damaging effect [8, 13]. The low accumulation in muscle tissue is normal but has implications for public health since muscle is the most important tissue consumed by humans. The concentrations found are lower than in the liver, but are a route to trophozoo transfer into the human population for these neurotoxic and carcinogenic metals [1, 5].

The investigation of the subcellular mechanisms of toxicity showed that the bioaccumulation of lead and cadmium led to oxidative stress in the liver. The very marked lipid peroxidation that was observed, which increased in a dose-dependent manner, was a sign of oxidative damage to the polyunsaturated fatty acids in the cell membranes and thus reduction in membrane integrity and function [7]. This discovery underpins the cell damage seen histopathologically. A simultaneous inhibition of the main antioxidant enzymes, catalase (CAT) and superoxide dismutase (SOD), is a mechanism that could explain the increased oxidative damage observed. These enzymes are the first line of defense against reactive oxygen species (ROS) in cells. They become inhibited by the toxicants, such as lead and cadmium, which presumably occurs when the toxicants bind to sulfhydryl groups in the active sites of the enzymes and thereby reduce the ability of the cells to neutralize the ROS flux generated by the toxicants [10, 12]. This breakdown of the antioxidant defence system results in a vicious cycle of

oxidative damage, which ultimately causes failure of cells to function and cell death. A major finding is the synergistic induction of oxidative stress in the mixture group, showing that the mixture, at the low concentration used individually, can induce more oxidative stress than the components alone, probably because they act together to overwhelm the cellular defense mechanisms [10].

The histopathological examination was a visual proof that associated the biochemical changes at the subcellular level to the overt changes in tissues and organs. The spectrum of the liver lesions, from vacuolar degeneration to infiltrative inflammatory changes, through to diffuse coagulative necrosis, is a classic signature of hepatotoxicity [6, 14]. The levels of metal accumulation and oxidative stress were high in the liver, and the lesions were congruent with this biochemical damage, indicating that the biochemical damage led to structural collapse. This increase in the hepatosomatic index seen in the present study is probably a sign of this pathology, which involves inflammation, edema and cellular hypertrophy, indicating healthy growth rather than the increase seen. The severity of the gill damage is also reflected in the clinical signs of respiratory distress seen, and is anatomically explained. Epithelial lifting, hyperplasia and lamellar fusion drastically reduce the surface area available for gas exchange and disrupt osmoregulation [15]. This not only affects breathing, causing general systemic hypoxia, but also means that the basic barrier against further entry of the toxicants contained in the water has failed, thus increasing the systemic toxicity. The severity and nature of these lesions in both liver and gills are similar to those reported in studies of fish exposed to lead and cadmium [8, 9, 13] and therefore support the validity of these organs as sensitive bioindicators of heavy metal pollution. These pathological assessments are reliable because they are all based on a standardized histological preparation and staining [24].

CONCLUSION

Fundamental Finding: The present study shows that toxicological effects of environmentally relevant sublethal concentrations of lead and cadmium are cascading in Mesopotamichthys sharpeyi. The investigation reveals that both metals bioaccumulate greatly and are highly sequestered in the liver and cause liver injury. The mechanism of toxicity at the subcellular level was found to be the induction of oxidative stress due to the impairment of the antioxidant defense system leading to widespread lipid peroxidation. The biochemical damage was seen as dose-dependent histopathological changes in both the liver and gills which affected metabolic and respiratory functions. In addition, the combination of lead and cadmium had a synergistic potentiation of toxicity at all the endpoints measured such as mortality, oxidative stress and histopathology. **Implication:** The above results collectively suggest that the level of lead and cadmium pollution in the Tigris River is harmful to the health and sustainability of this important endemic fish species. All of these effects on fish condition have implications for the ecological stability of the riverine food web and could represent a threat to human health based on the consumption of contaminated fish. **Limitation:** The study was limited to a

60-day laboratory exposure using selected Pb and Cd concentrations; therefore, the findings may not fully represent long-term exposure and the complex mixture of contaminants occurring under natural conditions in the Tigris River. **Future Research:** Hence, there is a need for the implementation of environmental monitoring programme and regulatory action to reduce this pollution and safeguard the aquatic ecosystem and public health.

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