Journal of Applied Veterinary Sciences, 10 (4): (2025) proof
ISSN: Online: 2090-3308, Print: 1687-4072
Journal homepage : https://javs.journals.ekb.eg

Quantifying Organ-Specific Toxicity of Dietary Heavy Metals in Zebrafish Using a Histopathological Scoring Index

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ABSTRACT

This study investigates sublethal effects of eight dietary heavy metals—copper (Cu), zinc (Zn), iron (Fe), cobalt (Co), chromium (Cr), aluminum (Al), manganese (Mn), and molybdenum (Mo)—on juvenile zebrafish (*Danio rerio*). Over a three-month period, fish were fed diets supplemented with each metal at two concentrations (1 mg/kg and 5 mg/kg), resulting in 16 exposure groups plus a control. Histological sections of gills, intestine, axial muscle, liver, kidney, and brain were evaluated across 49 histopathological parameters. A Metal Impact Index (MI) was used to quantify organ-specific toxicity. Both tested concentrations induced significant alterations in multiple organs, with severity varying by metal and tissue. Gill tissues exhibited the highest MI values, with Cu, Al, and Cr causing epithelial hyperplasia, lamellar degeneration, and pillar cell collapse. Cr exposure led to significant intestinal damage, while Fe caused the most prominent muscle pathology. Liver lesions (vacuolation, necrosis) increased with Cu, Cr, and Fe, and kidney damage (tubular degeneration) was evident in Cr- and Fe-treated groups. Brain tissue showed no significant MI variation. Hierarchical clustering based on cumulative MI scores grouped Cu, Cr, and Fe as high-toxicity metals, particularly in gills, liver, and kidney, while Mn, Zn, Al, and Mo formed lower-toxicity clusters. The MI approach provides a standardized framework for detecting early organ-specific toxicity and may support aquaculture health monitoring, environmental risk assessment, and fish welfare management in metal-contaminated ecosystems.

Original Article:

Received
Accepted
Published in

Keywords: Aquaculture; Fish; Gills; Heavy Metals; Toxicity.

***J. Appl. Vet. Sci., 10(4)*: Proof**

INTRODUCTION

Heavy metals are persistent environmental pollutants that pose a significant risk to aquatic life through waterborne and dietary exposure routes. Fish are especially vulnerable, as toxic metals accumulate in their organs and tissues, causing cellular and histopathological damage **(Dane and Sisman, 2020; Durai *et al.*, 2021)**.Among these, sublethal effects are of particular concern in aquaculture, where chronic exposure may impair growth, physiological functions, and internal organ structure, and in some cases elevate the flesh content of particular heavy metals through dietary or water content of heavy metals **(Majid *et al.*, 2019; Emenike *et al.*, 2022).**

In this study, eight heavy metals were selected for evaluation: copper (Cu), zinc (Zn), iron (Fe), cobalt (Co), chromium (Cr), aluminum (Al), manganese (Mn), and molybdenum (Mo). These elements are commonly found in aquafeed ingredients, especially fishmeal, and are subject to regulation in animal diets by agencies such as the European Commission and the FAO; for example, directive 2002/32/EC suggests a dietary benefit threshold of 5 mg/kg for several heavy metals in animal and fish feeds.

Chromium (Cr) exists in essential (trivalent) and highly toxic (hexavalent) forms, with contamination linked to industrial discharge and bioaccumulation in aquatic food chains **(Ali *et al.*, 2019).** Aluminum (Al), though non-essential, is a significant aquatic pollutant from anthropogenic sources (e.g., mining, acid rain) and causes gill damage in fish **(Karlsson-Norrgren *et al.*, 1985).** These metals are considered common contaminants in aquaculture systems, entering via feed; for example, fishmeal containing 0.67–4.67 mg/kg Cr was reported by **Janbakhsh *et al.*, (2018).**

Furthermore, Cr can be found in water or sediment, posing risks even at sublethal levels **(Yin *et al.*, 2018).** Their selected heavy metals used in the present work are based on their reported sublethal toxicity, which is the reason that these are included in regulatory frameworks (e.g., Directive 2002/32/EC) and

mirrors real-world exposure scenarios where metals coexist, enabling comparative assessment of organ-specific toxicity profiles **(Kamunde *et al.,* 2002; Durai *et al.,* 2021).T**oxic elements, such as cadmium (Cd), lead (Pb), and mercury (Hg), were deliberately excluded due to ethical concerns over their lethality and the impracticality of using them in long-term sublethal studies **(Gülden *et al.,* 2005).**

Heavy metal sublethal toxicity can be assessed using histopathological analysis, which is a widely accepted tool for evaluating the toxic effects of heavy metals in fish tissues **(Ali *et al.,* 2019; Ahmed *et al.,* 2020).** Histopathological evaluation is a valuable method for detecting such early effects, but in many studies, it is limited to qualitative descriptions or focused on single organs, frequently the gills. A potential alternative tool is the Combination Index **(Sönmez *et al.,* 2025)** which advanced our understanding of chemical interactions in mixture toxicity. However, this is typically designed for systemic or dose-response modeling rather than for assessing localized, organ-specific responses. The present study builds on this framework by applying a structured histopathological scoring system. The Metal Impact Index (MI) to quantify damage across multiple organs following dietary exposure. This approach enables early detection of tissue-specific toxicity and facilitates direct comparisons among metals and organs, offering a practical tool for environmental monitoring and aquaculture risk assessment.

This is particularly important for assessing early, sublethal effects in target organs such as gills, liver, kidney, and brain, which are known to respond differently to various metal exposures **(Dane and Şişman, 2020).** We hypothesize that MI values reflect the relative sensitivity of each organ and may offer a standardized metric for environmental risk assessment, aquaculture health monitoring, and welfare management.

MATERIALS AND METHODS

1. Ethical Approval

Ethical approval for this study was obtained from the Scientific Ethics Committee of the Faculty of Veterinary Medicine at the University of Thessaly (Protocol No. 548/05-04–2016). Juvenile zebrafish (*Danio rerio*) were housed in 12 L aquaria at 28 °C. Each group was maintained in duplicate tanks, with 50% of the water replaced daily.

2. Experimental Groups

The zebrafish used in this study originated from a breeding stock held at the Laboratory of Aquaculture in the Department of Agriculture, University of Ioannina, NW Greece. Water quality parameters, such as temperature (28 ± 1°C), NOX (NO_3 < 100 mg/L; NO_2 < 0.01 mg/L), NHX (NH_3 < 0.09 mg/L; NH_4 < 0.05 mg/L), GH (general hardness: 7–140), carbonate hardness (60), pH (7.2–7.3), and dissolved oxygen (>6.5 mg/L), were monitored daily.

The fish were housed in 12 L aquarium tanks, with each tank containing 20 zebrafish. Two tanks served as the control group, where fish were fed a standard diet free of metals. The remaining sixteen tanks (eight experimental groups) were set up in duplicate, with each group exposed to a different heavy metal. In each tank, 50% of the water was replaced daily. This design enabled a comprehensive evaluation of the toxicological effects of each metal at the specified dietary concentrations over the course of the three-month exposure period.

At the start of the trial, fish had an average body weight of approximately 0.3 ± 0.05 g. All experimental procedures were performed at the Aquatic Toxicology Laboratory, Department of Agriculture, University of Ioannina, Greece, under controlled conditions (temperature: 28 ± 1°C; photoperiod: 14 h light:10 h dark; pH: 7.2 ± 0.2). These methods are consistent with those previously described by the same research group in an earlier study on embryonic metal toxicity in zebrafish **(Gouva *et al.,* 2020).**

The experimental groups received feed supplemented with one of eight heavy metals: copper (Cu), zinc (Zn), iron (Fe), cobalt (Co), chromium (Cr), aluminum (Al), manganese (Mn), or molybdenum (Mo). The metals were obtained from VWR Chemicals BDH® and applied as follows: Cu (copper sulfate pentahydrate), Zn (zinc sulfate heptahydrate), Fe (iron chloride hexahydrate), Co (cobalt chloride hexahydrate), Cr (chromium chloride hexahydrate), Al (aluminum sulfate tetradecahydrate), Mn (manganese sulfate monohydrate), and Mo (ammonium molybdate tetrahydrate). Each metal was dissolved in distilled water, sprayed onto the feed, and dried as described by **Dang *et al.,* (2012)** and **Tsai *et al.,* (2013).** Two dietary concentrations were tested per metal: 1 mg/kg and 5 mg/kg, resulting in 16 exposure groups plus the control. Fish were fed twice daily at 4% of their body weight, with feeding amounts adjusted every ten days based on biomass.

3. Sample Collection

Histological samples were collected after three months of dietary exposure. Fish were euthanized, and tissues were sampled after opening the abdominal cavity. After dissection, tissues were immediately fixed in 10% neutral buffered formalin (NBF) at 4°C for 48 hours, using a minimum 10:1 fixative-to-tissue volume ratio. Samples were then processed through a standard dehydration series (graded ethanol), cleared in xylene,

and embedded in paraffin. Sections were cut using a rotary microtome at thicknesses appropriate to each tissue type: 4 µm for gills, muscle, liver, and kidney; 5 µm for intestine and brain. All sections were stained with hematoxylin and eosin (Hand E) and examined under a light microscope using x40 magnification. Randomly selected three fields of view form each organ from eahc sample (n=4 fish per metal per month).

4. Investigated Parameters

The histopathological findings are described for six organs—gills, intestine, muscle, liver, kidney, and brain-based on the observed tissue alterations following dietary exposure to the tested heavy metals. Histopathological evaluation followed established criteria **(Hardisty, 1976; Griffitt *et al.*, 2007; Haque *et al.*, 2012; Ahmed *et al.*, 2020; Durai *et al.*, 2021).** The Metal Impact Index (MI) was calculated per organ as the proportion of observed pathological findings relative to the total criteria defined for each organ: gills (11), intestine (12), muscle (6), liver (9), kidney (5), and brain (6). Four fish per metal per month were analyzed with a minimum of three histological sections per fish.

More specifically, the analysis focused on the following parameters:Gills:Primary lamellae Degeneration of Supporting Structures (PL); Hyperplasia of epithelial cells (HCE); Telangiectasis at the tips of secondary lamellae (TSL); Curling of secondary lamellae (CSL); Degeneration of pillar cells (BPC); Lifting of the secondary lamellae epithelium (LSGE); Extensive degeneration of pillar cells (CDPCS); Rupture of secondary lamellae tips (RSLT); oedema of epithelial cells (ECC); disruption of pillar cell-supported capillary structure (RBPC); oedema and rupture of epithelial cells (EREC).

Intestine: Fusion of villi (FV); Dilation of the lumen (LL); Flattened villi (FLV); Damaged enterocytes (DEC); Distended lumen; Damaged longitudinal muscle layer (DLML); Vacuole formation (VF); Swelling of the lamina propria (SLP); disintegration of villi and epithelial cracking (TFC); swelling of the longitudinal muscle layer (SLML); Damaged goblet cells (DGC); Disarrangement of the muscularis mucosa (DMM), Axial Muscle Tissue:Myofibril gap formation (MGF); Inter-myofibrillar space (IMFS); Myofibrillar disintegration (MD); Oedema between muscle fibers (OMF); Muscle necrosis (MN); Muscle oedema (MO). Liver: Cytoplasmic degeneration (CD); Epithelial cell necrosis (ECN); Hydropic swelling of hepatocytes (HSHC); Blood congestion (BC); Nuclear pyknosis (NP); Cytoplasmic vacuolation (CV); Nuclear degeneration (ND); Accumulation of dark granules (ADG); Cellular necrosis (CN). Kidneys: Enlarged glomeruli (EG); Reduced glomeruli (RG); Hyaline droplets (HD); Interstitial degeneration (IND); Tubular degeneration and necrosis (TDG).

Brain (Cerebellum): Purkinje cell degeneration (PCD); Neutrophil loss (NL); Gliosis (GL); Spongiosis (necrosis) (N); Cerebral hemorrhage (CHAEM). Organ-specific sensitivity to each heavy metal was assessed using the Metal Impact Index (MI), which was calculated as the proportion of observed histopathological alterations out of the total number of predefined criteria for each organ. MI scores provided a standardized, numerical representation of tissue damage severity. No metal accumulation analysis was performed, as the focus of the study was on the comparative histological response to sublethal dietary exposure levels.

To confirm that pathologies across different body parts were statistically independent, a chi-square test of independence was conducted. Subsequently, for each organ under examination, the Metal Impact Index (MI) was calculated for every sample unit. This index represents the proportion of histopathological alterations observed in each organ, calculated as the number of observed pathological changes divided by the total number of predefined histopathological criteria assessed for that organ (e.g., 11 criteria for gills, 12 for intestines, 6 for muscles, 9 for liver, 5 for kidney, and 6 for brain.

The Metal Impact Index (MI) is a metric used to evaluate the effects of heavy metal exposure on zebrafish by quantifying the extent of pathological changes observed in various organs and tissues. The histopathological parameters assessed using across three months for fish exposed to concentrations of the metals administered *per os* (oral administration). The organs and tissues assessed in this study include Gills; Intestines; Muscle; Liver; Kidneys and Brain

Each organ was evaluated for a set of different histopathological symptoms, with 4 fish per metal per month used for the samples. The Metal Impact Index (MI) is a summary metric which can quantify and compare the effects of different metals across the organs. MI is calculated as the proportion of pathological findings observed in each organ relative to the total number of parameters assessed for that organ. In the present study, the Metal Impact (MI) index was calculated as follows: MI_{Gills} = (number of observed pathological changes on Gills)/11; $MI_{Intestines}$ = (number of observed pathological changes on Intestines)/12; $MI_{Muscles}$ = (number of observed pathological changes on Muscles)/6; MI_{Liver} = (number of observed pathological changes on Liver)/7; MI_{Kidney} = (number of observed pathological changes on Kidney)/5; MI_{Brain} = (number of observed pathological changes on Brain /6.

The specific pathologies included in the Metal Impact Index for each organ are detailed in **Table 1**

Table 1. Pathologies associated with each organ's Metal Impact Index.

Gills	Intestines	Muscles	Liver	Kidney	Brain
G-PL	I-FV	M-GFMF	L-CD	K-EG	BL-GBC
G-HCE	I-LL	M-IMFS	L-EN	K-RG	BR-PCD
G-TSL	I-FLV	M-DMF	L-HSHC	K-HD	BR-NL
G-CSL	I-DEC	M-EMF	L-BC	K-IND	BR-GL
G-BPC	I-DL	M-MD	L-NP	K-TDG	BR-N
G-LSGE	I-DLML	M-ME	L-CV		BR-CHAEM
G-CDPCS	I-VF		L-ND		
G-RSLT	I-SLP		L-ADG		
G-ECC	I-CCA		L-CN		
G-RBPC	I-SLML				
G-EREC	I-DGC I-DMM				

Gills: PL—Primary lamellae—Degeneration of Supporting Structures; HCE—Hyperplasia of epithelial cells; TSL—Telangiectasis at the tips of secondary lamellae; CSL—Curling of secondary lamellae; BPC—Degeneration of pillar cells; LSGE—Lifting of the secondary lamellae epithelium; CDPCS—Complete damage to the pillar cell system; RSLT—Rupture of secondary lamellae tips; ECC—Oedema of epithelial cells; RBPC—Rupture and degeneration of the pillar cell system; EREC—Oedema and rupture of epithelial cells. Intestine: FV—Fusion of villi; LL—Large lumen; FLV—Flattened villi; DEC—Damaged enterocytes; DLML—Damaged longitudinal muscle layer; VF—Vacuole formation; SLP—Swelling of the lamina propria; TFC—Tissue fragmentation and cracking; SLML—Swelling of the longitudinal muscle layer; DGC—Damaged goblet cells; DMM—Disarrangement of the muscularis mucosa. Axial Muscle Tissue: MGF—Myofibril gap formation; IMFS—Inter-myofibrillar space; MD—Myofibrillar disintegration; OMF—Oedema between muscle fibers; MN—Muscle necrosis; MO—Muscle oedema. Liver: CD—Cytoplasmic degeneration; ECN—Epithelial cell necrosis; HSHC—Hydropic swelling of hepatocytes; BC—Blood congestion; NP—Nuclear pyknosis; CV—Cytoplasmic vacuolation; ND—Nuclear degeneration; ADG—Accumulation of dark granules; CN—Cellular necrosis. Kidneys: EG—Enlarged glomeruli; RG—Reduced glomeruli; HD—Hyaline droplets; IND—Interstitial degeneration; TDG—Tubular degeneration and necrosis. Brain (Cerebellum): PCD—Purkinje cell degeneration; NL—Neutrophil loss; GL—Gliosis; N—Spongiosis (necrosis); CHAEM—Cerebral hemorrhage. GBC; Spindle-like red blood cells.

5. Statistical analysis

Hierarchical cluster analysis with Ward's linkage method **(Ward, 1963)** was performed on the aggregated time data to group metals based on minimized within-group variation across the 49 observed histopathological changes. The clustering was based on the cumulative morbidity rates of 49 histopathological symptoms across all examined organs. For each metal group, cumulative morbidity (%) was calculated using 12 observations derived from triplicate fish sampled at monthly intervals over the 3-month exposure period. The results were visualized in a heatmap where the intensity of red shading reflects the degree of cumulative pathological findings, with darker red indicating higher morbidity.

To refine and interpret the cluster analysis results, a beta regression model (**Ferrari and Cribari-Neto, 2004)** was applied separately to each body part. This model tested significant differences in the effects of eight metals on each specific organ. Prior to applying the beta regression, the MI index values of 0 and 1 were transformed following the method proposed by **Smithson and Verkuilen (2006)** to accommodate the model's requirements. Tukey's post hoc test was then used to identify groups of metals with similar toxicity profiles, while the procedure outlined by **Piepho (2004)** provided an indicative grouping of metals based on their effects on each organ. Binomial logistic regression model was applied in order to assess whether metal type and exposure time significantly influenced each of the 49 histopathological parameters. For factors demonstrating a statistically significant effect on a given pathology, the Marascuilo procedure **(Marascuilo, 1966)** was applied on all pairwise combinations of proportion differences to find homogeneous groups among the factor's levels. Statistical analyses were carried out using SPSS version 21 and the R statistical programming language.

RESULTS

Zebrafish exposed to heavy metals at both dietary concentrations (1 mg/kg and 5 mg/kg) exhibited statistically significant histopathological alterations in several organs. Only organ-specific lesions that were statistically significant ($p < 0.05$) were considered indicative of metal toxicity in this study. Although other minor alterations were occasionally observed, these did not meet the significance threshold and were therefore excluded from interpretation.

Gills, the primary site of metal uptake, showed severe changes epithelial hyperplasia, telangiectasis, and curling of secondary lamellae—particularly after exposure to Cu, Al, and Cr. Liver damage, observed diffusely across the hepatic parenchyma, included cytoplasmic degeneration, epithelial necrosis, and blood congestion, most notably from Cu and Cr exposure. Kidneys displayed tubular degeneration and necrosis, primarily with Cr and Fe. Intestinal tissue showed villus blunting, epithelial erosion, and ulceration, with signs of vascular injury and inflammatory cell infiltration. Severe ulcerative enteropathy and necrosis at the villus tips were also observed. Although less pronounced, brain pathology included gliosis; however, no

statistically significant differences were observed in brain MI indices across metal treatments.

Representative histological images of these significant organ-specific lesions are presented in Figures 1–5. These include telangiectasis and lamellar distortion in gills **(Fig. 1),** necrotizing and fragmented myofibers in axial muscles **(Fig. 2)**, and intestinal epithelial damage with villus blunting and ulceration **(Fig. 3).**

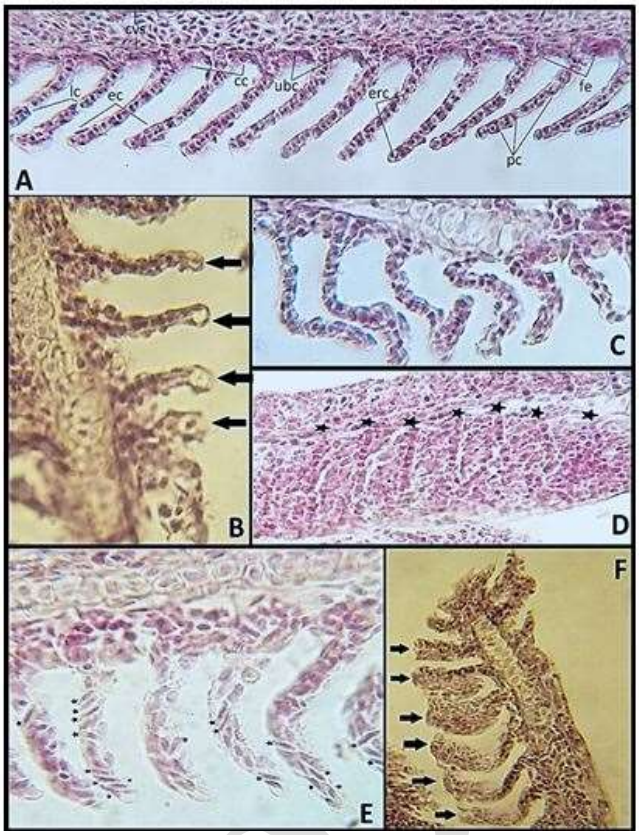

Fig.1: (a) Normal gill filament, sagittal section, Central venous sinus (cvs), chloride cells (cc), epithelial cells (ec), lacunae (lc), erythrocytes (erc), pillar cells (pc), filamental epithelium (fe), undifferentiated basal cells (ubc) (Hand E stain, 100×). (B) Oedema (OED)at the tip of the secondary lamellae (arrows) of fish treated with Al (Hand E stain, 100x) (C) Curling of secondary lamellae (CSL) of Co treatment (Hand E stain, 100×). (D) Gill epithelial hyperplasia of secondary lamellae (HCE), blood channels (afferent and efferent arterioles) filled with erythrocytes are unaffected (asterisks), between them, increased lamellar internal cellularity is obvious. Fish were treated with Mn (Hand E stain, 100×). (E) Degeneration of pillar cells (BPC) on early telangiectatic processes of secondary lamellae. Note the spindle cell morphology of erythrocytes (asterisks). Here, the blood channels (lacunae) have lost their ability to regulate blood flow, due to the completely damaged pillar cells system (CDPCS). Fish were treated with Zn (Hand E stain, 100×). (F) Telangiectasia (TSL) at the distal 2/3 of secondary lamellae (arrows) on fish treated with Co (Hand E stain).

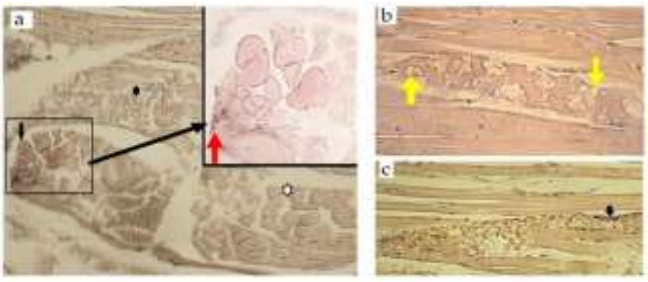

Fig.2: Representative axial muscle histopathological issues of zebrafish due to dietary exposure to dietary Co (3mg/kg). (a): Severe necrotizing myositis in zebrafish following dietary cobalt (Co) exposure. Affected muscle fibers appear enlarged and hyper-eosinophilic (Hya; black arrow), with marked loss of cross-striations and fragmentation (asterisk). Fully hyalinized fibers and localized infiltration of macrophages are also evident (red arrow). (b) Muscle fiber exhibits severe fragmentation (black arrows) (sparing areas along the fiber). (c) Early infiltration of macrophages (yellow arrows) on degenerative muscle fiber exhibiting severe fragmentation.

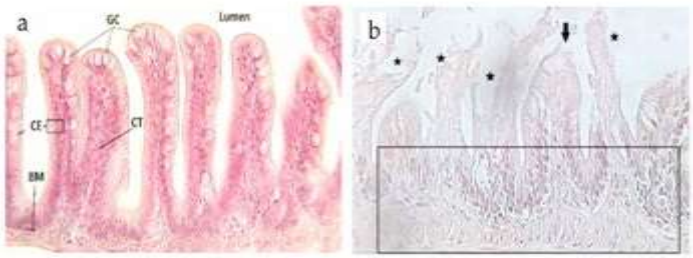

Fig. 3: (a): Normal intestine, goblet cells (GC), columnar epithelium (CE), connective tissue (CT) basement membrane (BM). (b **(b)** Intestinal section from zebrafish exposed to dietary aluminum (Al), exhibiting epithelial erosions (ERS; arrows) and ulceration (ULC; asterisk) at the peripheral tips of the villi.

Additionally, kidney sections revealed vascular congestion and leukocytic infiltration, indicative of inflammatory responses and impaired renal function, particularly in fish exposed to dietary iron **(Fig.4).** Hepatic tissue exhibited a centrilobular zone of pallor with cytoplasmic vacuolation and loss of hepatic cord organization, consistent with zonal degeneration and early steatosis, especially in chromium-exposed groups **(Fig.5).**

These histopathological alterations corroborate the quantitative findings of the Metal Impact Index (MI), which highlighted gills, liver, kidney, and intestine as primary target organs for sublethal metal toxicity. Notably, the distribution and severity of lesions were metal-specific and support the organ-level trends described in the regression analysesThe specific pathologies included in the MI for each organ are presented in detail in **Table 1.**

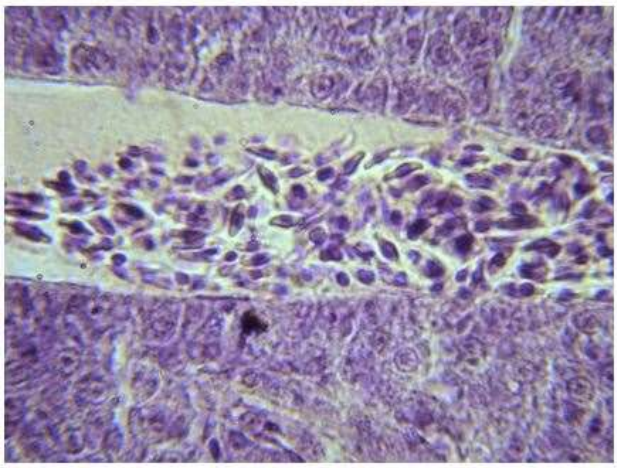

Fig. 4: (a) Histological section of zebrafish kidney exposed to dietary iron (3months with 5 mg/kg). The vessel lumen is filled with densely packed erythrocytes, and spindle-shaped leukocytes with oval nuclei and moderate cytoplasm infiltrate the surrounding tissue, indicating vascular congestion and an active inflammatory response.

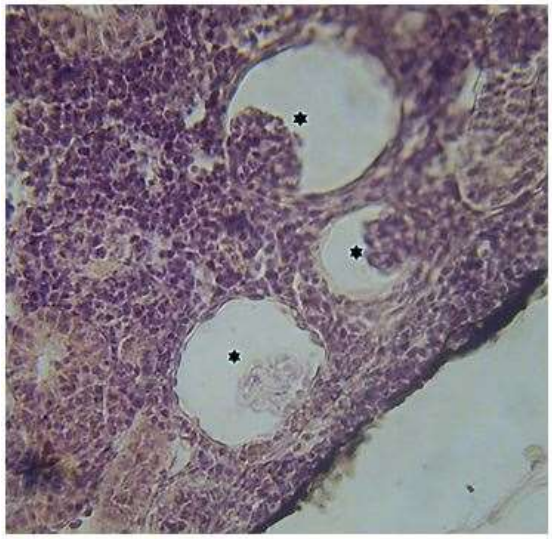

Fig. 4: (b) Histological section of kidney of fish exposed tovChromium ((3 months with 5 mg/kg)., showing altered glomerular structures. The tissue section reveals renal parenchyma containing multiple glomeruli, among which several exhibit pathological alterations. Glomeruli marked with black stars (*) demonstrate evident signs of degeneration, including loss of cellular detail, shrinkage or collapse of the glomerular tuft, and increased Bowman's space. Surrounding hematopoietic tissue appears densely cellular, as is typical in teleost renal tissue

Figure 4 presents the statistical dependence among pathological symptoms (chi-square test). **Figure 5** shows hierarchical cluster analysis (Ward's method) of the eight metals based on cumulative morbidity rates. Cu, Al, and Cr exhibited the highest MI values in gills. Beta regression confirmed significantly higher MI scores in gills for Cu ($p < 0.001$), Al ($p = 0.002$), Cr ($p = 0.013$), Mn ($p = 0.045$), and Mo ($p = 0.037$). Cr had a significantly greater effect on intestines ($p = 0.034$), and Fe had the largest effect on muscles ($p = 0.039$). In the liver, Cu ($p = 0.019$), Cr ($p = 0.011$), and Fe ($p = 0.048$) caused significantly elevated MI scores. For kidneys, Cr ($p = 0.037$) and Fe ($p = 0.041$) produced significant histopathological changes. No metal significantly affected brain MI scores

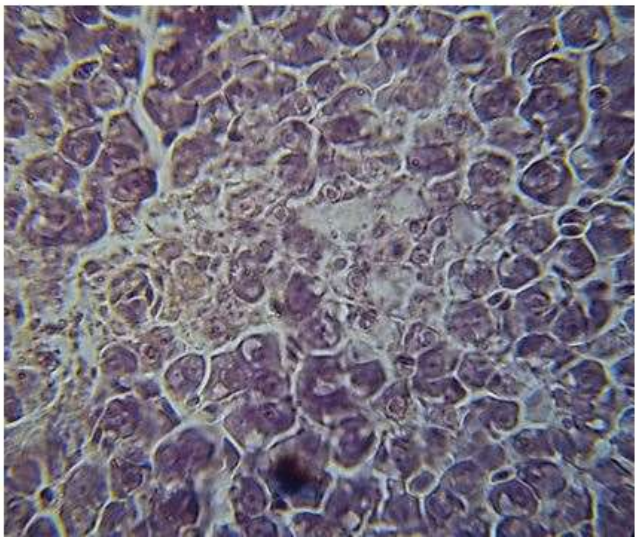

Fig. 5: Lliver section from zebrafish exposed to dietary Chromium (3 months with 5 mg/kg). A centrilobular zone of pallor is evident, marked by cytoplasmic vacuolation of hepatocytes and disorganization of hepatic cords. These alterations indicate zonal hepatic degeneration and steatosis.

Logistic regression **(Fig. 6)** assessed each of the 49 histopathological characteristics using metal type and time as explanatory variables. Significant effects were observed for gills CSL (Wald χ^2 (8) = 16.270, $p = 0.039$), gills EREC ($p = 0.009$), kidney IND ($p = 0.028$), and kidney TDG ($p = 0.041$). Time was significant for gills TSL ($p < 0.001$), gills CDPCS ($p = 0.041$), kidney IND ($p = 0.011$), kidney TDG ($p = 0.006$), and brain GL ($p = 0.017$). No significant time-dependent trends were found for liver pathologies.

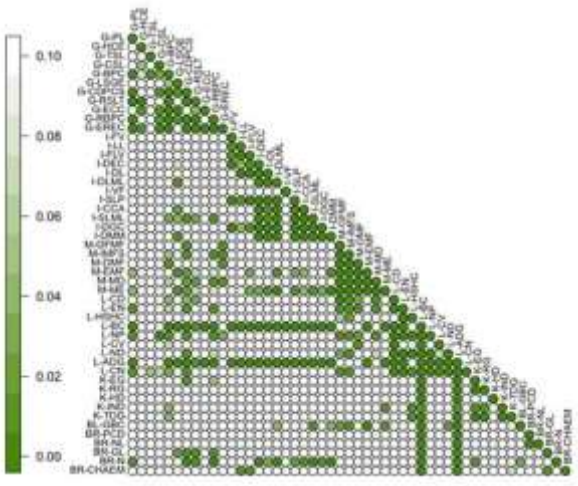

Fig.6: Statistical dependence among pathological changes (shades of green represent statistical dependence among pathologies, with the significance assessed by the chi-square test).

Further analysis indicated that Mn and Zn, Al and Mo, and Fe and Co formed three groups with similar toxicity profiles, while Cr, Cu, and the control group remained distinct clusters. These groupings, derived from hierarchical cluster analysis, are illustrated in **Fig. 7.**

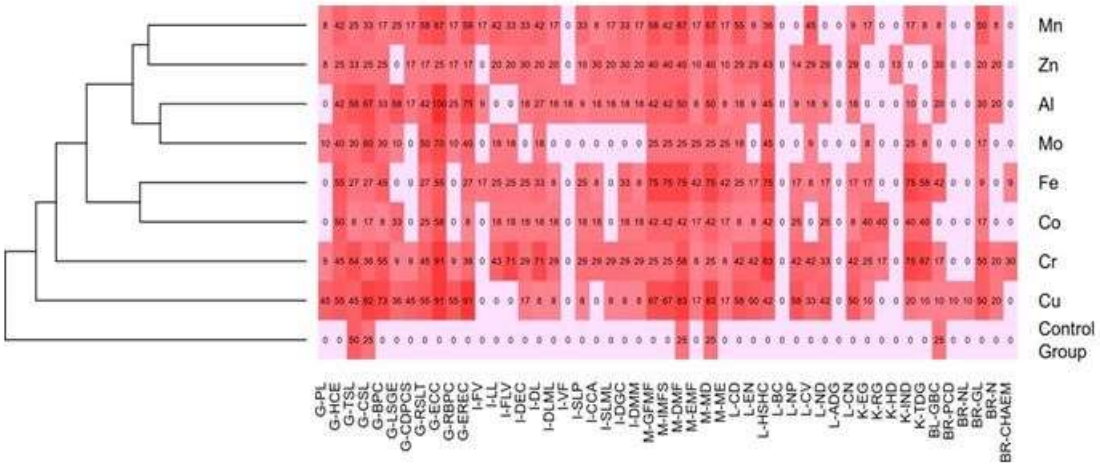

Fig. 7: Hierarchical cluster analysis (Ward's method) of eight heavy metals based on the morbidity rates of 49 pathological symptoms in zebrafish. The heatmap illustrates the cumulative morbidity rates (%) across all symptoms for each metal, calculated from 12 observations (n=4 each week for each group) over three time (monthly samples) points. Shades of red are used, with darker red indicating higher morbidity.

Regarding effects on gills, Cu ($p < 0.001$), Al ($p = 0.002$), Cr ($p = 0.013$), Mn ($p = 0.045$), and Mo ($p = 0.037$) exhibited significantly higher MI indices than the control group. Tukey's HSD post hoc tests further identified Cu, Al, and Cr as a group with elevated toxicity in gills among the eight metals. Cr showed a significantly greater effect on intestines compared to the control group ($p = 0.034$), while Fe had a significantly larger impact on muscles ($p = 0.039$). For the liver, Cu ($p = 0.019$), Cr ($p = 0.011$), and Fe ($p = 0.048$) demonstrated significantly higher effects, while Cr ($p = 0.037$) and Fe ($p = 0.041$) similarly affected the kidneys. No metal showed a statistically significant difference from the control group in its effect on brain and blood tissues. A summary of the beta regression model results is provided in **Table 2.**

Table 2. Metal effects on MI index.ns: non significant effect.

	Gills	Intestines	Muscles	Liver	Kidney	Brain
Cu	<0.001	0.034	Ns	0.019	ns	Ns
Mo	0.037	ns	Ns	Ns	ns	Ns
Al	0.002	ns	Ns	Ns	ns	Ns
Mn	0.045	ns	Ns	Ns	ns	Ns
Zn	ns (1)	ns	Ns	Ns	ns	Ns
Fe	ns	ns	0.039	0.048	0.041	Ns
Cr	0.013	ns	Ns	0.011	0.037	Ns
Co	ns	ns	Ns	Ns	ns	Ns

(1) Not significant at 0.05 level.

The occurrences of pathological observations for each metal and time measurement are depicted in **Fig. 8.** A logistic regression model was applied to each of the 49 pathological characteristics, with metal type and measurement time designated as explanatory variables. For both factors—metal type and time—the control group served as the reference level. Metal type emerged as a significant factor for several pathologies: Gills CSL (Curling of Secondary Lamellae) (Wald $\chi^2(8) = 16.270$, $p = 0.039$), Gills EREC (Oedema and Rupture of Epithelial Cells) (Wald $\chi^2(8) = 20.247$, $p = 0.009$), Kidney IND (Interstitial Degeneration) (Wald $\chi^2(8) = 17.240$, $p = 0.028$), and Kidney TDG (Tubular Degeneration-Necrosis) (Wald $\chi^2(8) = 16.106$, $p = 0.041$). Conversely, time was identified as a significant explanatory variable for Gills TSL (Telangiectasis at the Tip of Secondary Lamellae) (Wald $\chi^2(2) =$

19.527, $p < 0.001$), Gills CDPCS (Complete Damage to Pillar Cell System) (Wald $\chi^2(2) = 6.408$, $p = 0.041$), Kidney IND (Interstitial Degeneration) (Wald $\chi^2(2) = 9.050$, $p = 0.011$), Kidney TDG (Tubular Degeneration-Necrosis) (Wald $\chi^2(2) = 10.195$, $p = 0.006$), and Brain GL (Gliosis) (Wald $\chi^2(2) = 8.141$, $p = 0.017$).

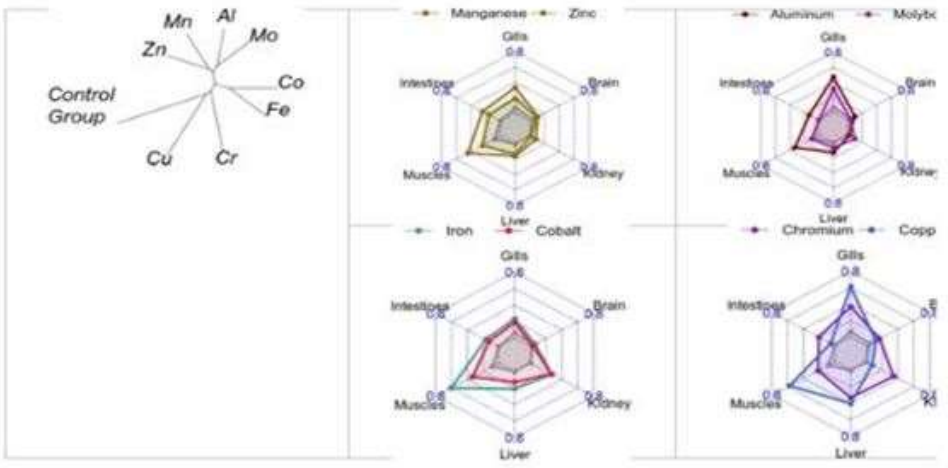

Fig.8: Hierarchical cluster analysis (Ward's method) of cumulative Metal Impact Index (MI) values across organs. The dendrogram groups the eight heavy metals into clusters based on their similarity in organ-specific toxicity profiles. Three metal pairs; Mn and Zn, Al and Mo, Fe and Co—formed distinct toxicity clusters, while Cr, Cu, and the control group remained separate, reflecting their unique impact on zebrafish tissues.

Marascuilo post hoc comparisons revealed that fish exposed to Cu solutions exhibited a significantly higher likelihood of developing Curling of Secondary Lamellae (CSL) and Oedema and Rupture of Epithelial Cells (EREC) in gills. Similarly, exposure to Fe and Cr was associated with a significantly greater probability of Interstitial Degeneration (IND) and Tubular Degeneration-Necrosis (TDG) in kidneys. This time-effect of exposure are illustrated in **Figs. 9-10.** The prevalence of Interstitial Degeneration and Tubular Degeneration-Necrosis in kidneys increased over time. In contrast, Telangiectasis at the Tip of Secondary Lamellae (TSL), Complete Damage to Pillar Cell System (CDPCS), and Gliosis (GL) did not exhibit a clear temporal trend **(Figs. 11 and 12).** Although Fe, Cr, and Cu induced histopathological alterations in the liver, logistic regression analysis did not reveal a statistically significant time-dependent pattern in liver pathology over the three-month exposure period.

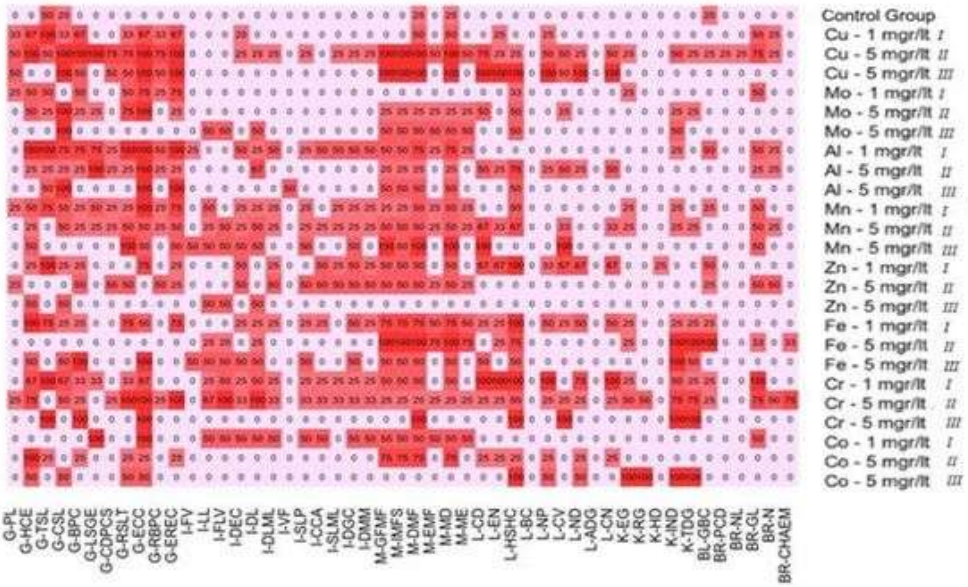

Fig. 9: Percent of pathological occurrences for each metal and measurement. Different shades of red color indicate the intensity of the morbidity after one month (I), two months (II) and three months (III) exposure.

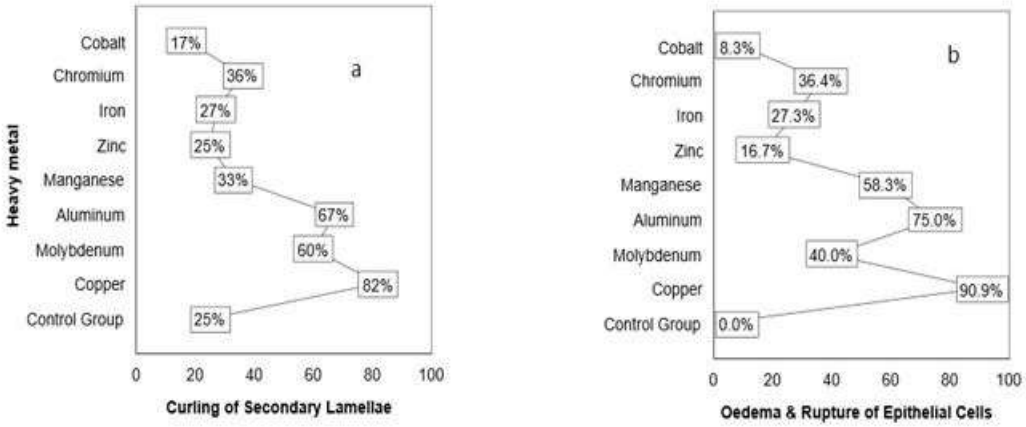

Fig. 10: Metal effect on curling of secondary lamellae and oedema and rupture of epithelial cells.

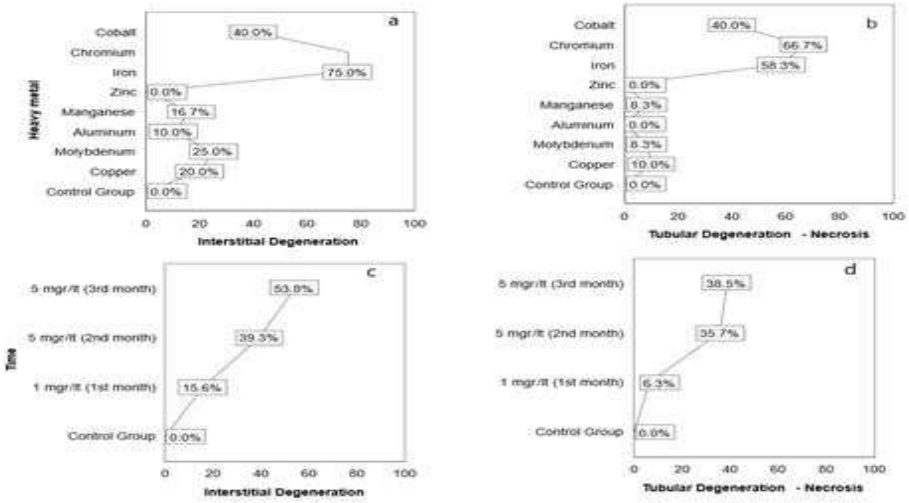

Fig. 11: Metal and time effects on interstitial and tubular degenerations.

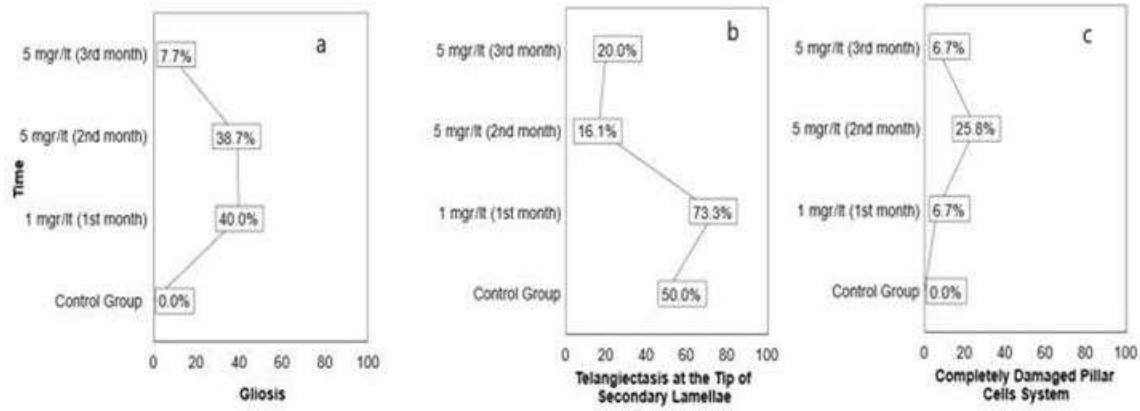

Fig.12: Time effect on Gliosis, TSL and CDPCS.

The results of the binomial logistic regression models, assessing the influence of metal type and exposure time on each of the 49 histopathological changes, are summarized in **Table 3.**

Table 3. Logistic regression models findings.

Nagelkerke R^2	Significance		Post hoc test	
	Metal	Time	Metal	Time
Gills				
Telangiectasis at the Tip of Secondary Lamellae				
0.555	Ns	<0.001	Co < Cu	1st < 2nd & 3rd
Curling of Secondary Lamellae				
0.278	0.039	Ns	Co < Cu	
Completely Damaged Pillar Cells System				
0.436	Ns	0.041	Co < Cu	Ctr < 2nd
Oedema & Rupture of Epithelial Cells				
(Ctr, Co, Fe, Zn) < Cu				
0.449	0.009	Ns	Ctr < Mn	
Ctr, Co < Al				
Kidney				
Interstitial Degeneration				
0.546	0.028	0.011	(Ctr, Zn, Al) < (Fe, Cr)	Ctr < 2nd & 3rd 1st < 3rd
Tubular Degeneration necrosis				
0.633	0.041	0.006	(Ctr, Al, Zn) < (Fe, Cr)	Ctr < 2nd &3rd 1st < 3rd
Brain *Gliosis*				Ctr < 1st & 2nd
0.356	Ns	0.017		

ns, not significant at 0.05. 'Ctr' refers to the control group, '1st' to the first month of exposure, '2nd' to the second month, and '3rd' to the third month of exposure. The symbol '<'represent the relative change in severity of the observed changes at each time point.

DISCUSSION

The observed pathological changes reflect the differential sensitivity of zebrafish organs to dietary heavy metal exposure. The severity of gill lesions—including hyperplasia, telangiectasis, and pillar cell damage—supports their role as the primary interface for metal uptake, consistent with prior findings by **Naz *et al.,* (2021).** Cr and Cu emerged as the most aggressive toxicants, followed by Al, with the highest MI scores across organs. These results align with mammalian studies: **Ahmad Baker *et al.,* (2025)** reported similar degenerative and necrotic alterations in rat liver and kidneys following chronic dimethyl mercury exposure, suggesting conserved vertebrate responses to heavy metals.

The MI-based clustering reinforced these toxicity patterns. Cu, Mn, Fe, and Cr grouped into high-toxicity clusters, while Mo and Zn exhibited moderate effects. These patterns were consistent with the observed organ-specific damage. For example, Cu, Al, and Cr caused significant gill lesions such as telangiectasis and epithelial hyperplasia, indicating disrupted gas exchange and osmoregulatory function. Cr and Fe showed pronounced renal toxicity, evidenced by tubular degeneration and interstitial infiltration, while Cu and Fe were linked to hepatocellular vacuolation and disorganized liver architecture.

The dietary exposure model applied in this study simulates realistic intake through fishmeal-based feeds, a known source of metals like chromium **(Janbakhsh *et al.,* 2018).** Although the study's concentrations exceed natural background levels, they remain within regulatory feed limits (Directive 2002/32/EC; FAO Technical Paper No. 384) and permit investigation of cumulative sublethal effects. This model also reflects the heightened susceptibility of juvenile fish, which are especially vulnerable due to their high metabolic rates and immature detoxification systems **(Bambino and Chu, 2017).** Dietary exposure routes differ substantially from waterborne ones in terms of uptake dynamics and organ targeting. **Kamunde *et al.,* (2002)** found that dietary copper uptake can exceed waterborne uptake, and **Liu *et al.,***

(2025) demonstrated liver pathology from dietary iron in carp. Although **Merrifield *et al.,* (2013)** studied metal nanoparticles rather than bulk forms, their use of the same dietary exposure route and their observation of gut epithelial damage justify comparison with our findings. **Craig *et al.,* (2009)** observed that varying dietary iron levels modulated gene expression responses to waterborne copper exposure, highlighting the interactive role of dietary metals in shaping overall metal toxicity profiles.

The cumulative effects reported here support prior evidence that heavy metals interfere with oxidative balance, induce inflammatory responses, and trigger structural damage in metabolically active tissues. Cu is known to generate reactive oxygen species (ROS), promoting lipid peroxidation and cellular apoptosis **(Shaw *et al.,* 2019).** Cr, while essential at trace levels, exhibits genotoxic and pro-oxidant properties at higher concentrations. Similarly, Fe catalyzes the formation of hydroxyl radicals via the Fenton reaction, aggravating tissue degeneration **(Fernando *et al.,* 2015).** These biochemical mechanisms underlie the histopathological findings reported in the present study. The absence of statistical effects in brain may reflect lower sensitivity or reduced accumulation in these organs, possibly due to barriers like the blood–brain barrier. Further studies employing tissue metal quantification (e.g., ICP-MS) and molecular biomarkers are needed to clarify the toxicokinetics and accumulation profiles of dietary metals.

The pathological patterns observed particularly in gills, liver, and kidneys also reinforce the value of the Metal Impact Index (MI) as a diagnostic tool for organ-specific sublethal toxicity. The MI results align with logistic regression findings, which further validated the significance of individual pathologies, such as Curling of Secondary Lamellae (CSL), Telangiectasis (TSL), and Tubular Degeneration (TDG). This dual analytical framework strengthens confidence in histological endpoints as reliable markers for environmental and dietary metal toxicity.

Notably, this study focused on eight metals commonly present in fishmeal and aquaculture feed ingredients. While cadmium (Cd), lead (Pb), and mercury (Hg) are well-known for their high toxicity, they were deliberately excluded from this dietary trial. These metals can induce acute toxic effects at relatively low concentrations, making them unsuitable for sublethal, chronic exposure models. In addition, their presence in regulated aquafeeds is typically minimal compared to essential trace metals like Fe, Zn, and Cu. Ethical considerations regarding their lethality in long-term feeding trials also influenced their exclusion.

The observed gill lesions including epithelial hyperplasia, pillar cell damage, and telangiectasis parallel those described in field studies involving Nile tilapia from polluted aquatic environments (Ahmed et al., 2020) and corroborate reports from Cu exposure studies in zebrafish and other fish species **(Karlsson-Norrgren *et al.,* (1985); Chen *et al.,* 2023; Wu *et al.,* 2023). Karlsson (1983)** and further documented gill histopathology as a primary consequence of heavy metal stress in salmonids. Likewise, manganese-induced gill pathology observed in this study aligns with the work of **Rodrigues *et al.,* (2018).** Such structural damage to the gills can compromise respiratory efficiency and gas exchange, ultimately affecting systemic oxygen transport. This functional impairment is consistent with alterations in hematocrit and hemoglobin reported in other fish species exposed to heavy metal intoxication, which reflect anemia-like conditions and reduced oxygen-carrying capacity **(Ahmed *et al.,* 2022).**

The liver and kidney damage exhibited by the experimental groups, marked by vacuolation, congestion, and tubular necrosis, is also consistent with heavy metal bioaccumulation effects described by **El-Sherif *et al.,* (2025**) and **Fernando *et al.,* (2015).** These findings reinforce earlier claims that the liver, kidney, and gills are target organs for sublethal heavy metal toxicity. The results support the use of histopathology as a sensitive early biomarker for dietary metal exposure, as also highlighted by **Gouva *et al.,* (2020a; 2020b).**

Taken together, these findings support the use of zebrafish as a relevant vertebrate model for dietary metal toxicity studies and highlight the diagnostic utility of histopathological assessment and MI scoring in environmental monitoring and aquaculture risk assessment. Field studies **(e.g., Yacoub *et al.,* 2021; Eneji *et al.,* 2011; Perez-Ruzafa *et al.,* 2018; Lu *et al.,* 2018)** further affirm histopathology as a practical, non-lethal endpoint to monitor metal stress and health risks in aquatic organisms. The histopathological alterations observed in the gills, liver, and kidneys of zebrafish exposed to dietary Cu, Cr, and Al in this study are consistent with previous reports linking heavy metal toxicity to immune system impairment. Structural lesions such as lamellar fusion, hepatocellular degeneration, and reduced glomerular integrity can compromise essential physiological functions, indirectly weakening immune defenses. Similar to the mechanisms described by Morcillo ***et al.,* (2015), Rajeshkumar *et al.,* (2017)** and **Raeeszadeh *et al.,* (2023),** these metals may disrupt leukocyte activity, reduce phagocytic capacity, and alter cytokine expression, thereby increasing susceptibility to infections. The organ damage documented here likely

exacerbates such immunotoxic effects by impairing the functional capacity of tissues critical for maintaining homeostasis and pathogen defense.

One limitation of the present study is that it focused on lesions that were statistically significant, thereby avoiding speculative interpretation. Nonetheless, the occurrence of non-significant but observable alterations in other organs suggests the possibility of subclinical or early-stage damage that may not have progressed to a level detectable by our scoring and statistical criteria within the 3-month period. Such changes, although not meeting our significance threshold, may represent cumulative or delayed effects that could become more pronounced with longer exposure or higher doses, as has been noted in other chronic toxicity studies. Future investigations incorporating more sensitive biomarkers, longitudinal sampling, or higher-resolution histopathological scoring could help to clarify the progression and broader systemic impact of chronic metal exposure.

CONCLUSION

This study highlights the serious risks heavy metals like Cu, Cr, and Fe pose to fish health in aquaculture. These metals caused marked histopathological changes. The MI can be used as a tool for metal toxicity detection and disease management strategies in aquaculture.

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