



Trace Metal Contamination and Biomarker Responses in Fish from a Mining-Impacted River Basin in Cebu, Philippines

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Abstract: Mining activities in Toledo City, Cebu, have created extensive mined-out lands and left a legacy of metal contamination in surrounding aquatic systems. This study assessed trace metal concentrations and molecular biomarker responses in fish from the downstream section of the Sapangdaku River and adjacent Tañon Strait coastline. Pore water and sediments were analyzed for Cd, Cr, Cu, Pb, and Zn using FAAS and ICP-MS, while biomarker assays measured metallothionein (MT) induction in liver tissues and genotoxicity through the assessment of micronuclei (MN) and nuclear abnormalities (NA) in erythrocytes. Results revealed that Cu was the dominant pollutant in pore water (mean 173 µg/L; peak 831 µg/L at Station 3), exceeding the U.S. EPA and Philippine DENR criteria by more than tenfold. Sediment indices (Igeo, CF, PLI) confirmed very high contamination from Cu, with Pb and Zn contributing moderately, while Cd and Cr remained near background levels. Fish analyses showed substantial bioaccumulation of Cu and Zn, particularly in benthic feeders (*Mugil cephalus*, *Eubleekeria jonesi*), which also exhibited the strongest biomarker responses. MT levels reached 299 µg/g in *M. cephalus*, while MN and NA frequencies were strongly correlated with Cu concentrations ($R > 0.98$, $p < 0.05$). NA were consistently more frequent than MN, suggesting their greater sensitivity as early markers of genotoxic stress. These results demonstrate that legacy mining continues to exert significant ecological stress on the Sapangdaku River system. The combined use of chemical analyses and biomarkers provides valuable baseline data, with MT, MN, and NA emerging as sensitive indicators of metal contamination and ecological risk.

Keywords: Porewater toxicity; metallothionein induction; risk assessment; mining; biomarker responses

1. Introduction

Mining is among the most significant anthropogenic activities contributing to environmental contamination worldwide. While mining operations provide economic benefits, they often result in long-term ecological disturbances, including acid mine drainage, soil enrichment with trace metals, and contamination of freshwater systems. These impacts are particularly severe in river basins where heavy metals accumulate in sediments and aquatic organisms, threatening ecosystem integrity and human health through the food

chain. Toledo City in Cebu, Philippines, is a major mining hub, encompassing approximately 35,000 hectares of mined-out land primarily from extensive copper extraction [1]. The legacy of mining has left a persistent ecological footprint, with copper and associated trace metals contaminating soils and river systems. Elevated concentrations of heavy metals in stream ecosystems near mining sites pose considerable ecological risks, particularly for aquatic organisms and higher trophic levels. Despite this, rehabilitation and monitoring initiatives in the Toledo River basin remain limited.

Several studies have already documented the extent of contamination in the region. Lo and Sakamoto [2] reported high levels of copper (>700 ppm) and zinc (>100 ppm) in sediments from the Sapangdaku River and the tailings outfall in Barangay Ibo. Aggangan et al. [1] described extremely acidic soils (pH ~2.5) with copper concentrations (154–638 mg/kg) far exceeding the permissible limit of 36 mg/kg. More recently, Sanchez et al. [3] confirmed that copper levels in the midstream and upstream sections of the Sapangdaku River remain above acceptable limits, primarily due to copper mining and the erosion of copper-rich landmasses. In addition, metal accumulation has been reported in plants, mollusks, and bivalves inhabiting the Toledo coastal area, but relatively few studies have examined fish populations that integrate contamination across habitats. Fish are considered effective sentinels of aquatic ecosystem health because they integrate exposure to contaminants over time and space, allowing for a comprehensive assessment of environmental health. Downstream fish, in particular, provide an important measure of cumulative pollution, as contaminants transported by river currents tend to accumulate in these areas with minimal dilution [4–6]. Studies from Alberta, Japan, and Vietnam have demonstrated that downstream sites reflect the highest contamination risks, making them reliable locations for biomonitoring.

Traditional monitoring has focused on quantifying metal concentrations in water, sediments, and biota. However, such measurements provide only part of the picture, as they cannot always reveal early biological stress. Molecular and cellular biomarkers—including metallothionein induction and genotoxicity assays (micronucleus and nuclear abnormalities)—offer sensitive indicators of sub-lethal toxic effects. They detect physiological stress responses in organisms before population-level declines occur, thereby serving as valuable tools for ecotoxicological monitoring and environmental risk assessment. Despite the ecological and human health significance of the Toledo River basin, recent work investigating biomarker responses in fish remains scarce. The absence of updated biomonitoring data poses challenges for environmental management, particularly in assessing rehabilitation needs and guiding local policy. This study, therefore, aimed to assess trace metal contamination and associated biomarker responses in fish from the downstream section of the Toledo River basin. Specifically, we quantified the levels of copper, zinc, lead, cadmium, and chromium in fish tissues and evaluated biomarker responses, including metallothionein induction and genotoxicity. By establishing baseline data, this study aims to contribute to the development of local environmental policies and provide a scientific foundation for future rehabilitation and monitoring programs.

2. Materials and Methods

2.1 Sampling sites.

The Sapangdaku River, also known as the Hinulawan River, is situated at an estimated terrain elevation of 5 meters above sea level, spanning a long stretch of 9 kilometers across 10 barangays in Toledo City. The three sampling sites, including the mouth of the Sapangdaku River and one from each side of the river mouth in the Tañon Strait, were used in the study, as shown in Figure 1. Sampling Site 1 was in the coastline of Barangay Dumlog near the Balamban Coast, Station 2 was located at the mouth of Sapangdaku River, and Station 3 was in the coastline of Daanglungsod near Atlas Fertilizer Plant (AFC) and Toledo City Port. Both coastlines are part of the Tañon Strait.

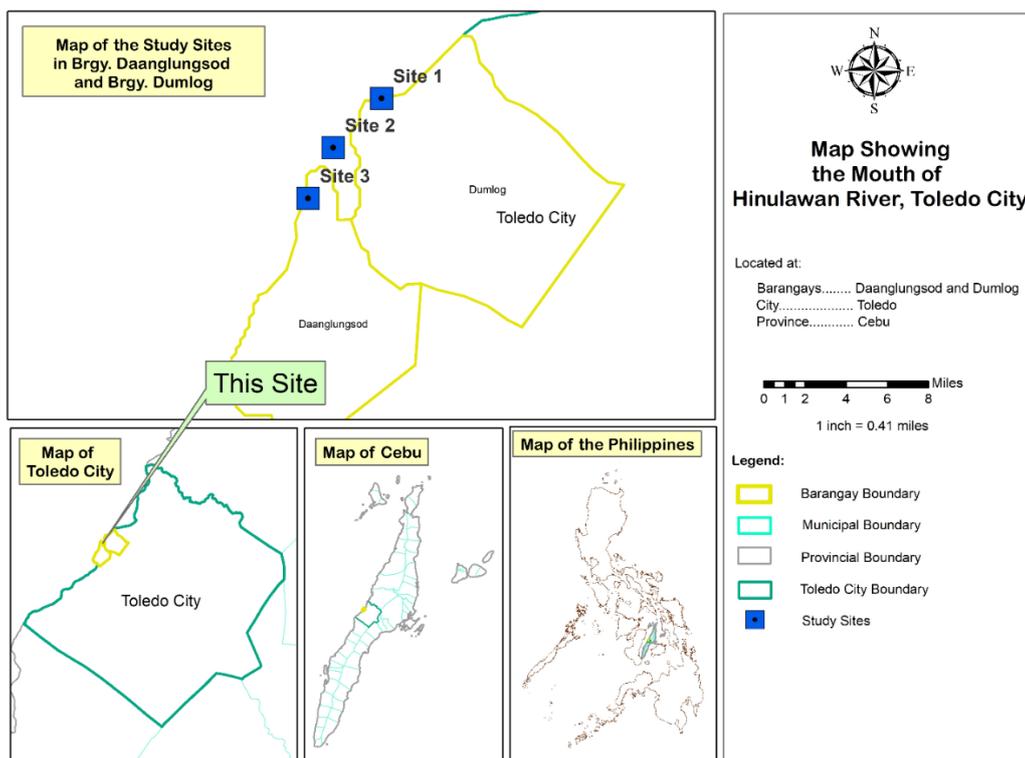


Figure 1. Location map of the sampling stations in the Hinulawan river.

The GPS coordinates of the sampling sites, along with a technical description of each site, are tabulated in Table 1. Station 1 in Barangay Dumlog is located near a ship-building facility, while Station 3 is situated near the Atlas Fertilizer Corporation (AFC). The color of the water in the sampling sites ranged from dark brown to black. All three sampling sites are abundant with Mangrove trees.

Table 1. Technical and Qualitative Description of Stations 1, 2, and 3 at Sapangdaku River, Toledo City, Cebu, Philippines.

Stations	GPS Coordinates	Description of Site
Station 1 Barangay Dumlog	N 10° 24.191' E 123° 38.745'	Observable suspended solids Abundant Mangrove trees Coastline is black and brown Ship-building facility a few kilometers north
Station 2 Sapangdaku Estuary	N 10° 24.093' E 123° 38.665'	The mouth of the river is covered with suspended solids Abundant Mangrove trees Coastline is dark brown The bed of the mouth of the river is soft
Station 3 Barangay DaangLunsod	N 10° 23.952' E 123° 38.552'	Observable suspended solids Abundant Mangrove trees Coastline is dark brown AFC is a few kilometers south

2.2 Sampling Protocol and Design.

Sampling was conducted every other month from May to September 2017 at the mouth of the Sapangdaku River, as well as on both the left and right sides of the river's mouth along the coastline of the Tañon Strait. A 20-meter stretch of the site was designated as the study area for each sampling station. Within the study area, random samples of pore water and sediments were collected. Fifteen core samplers, each containing pore water and sediments, were collected per river to achieve a highly reliable composite sample.

2.3 Pore Water and Sediment Sample Collection and Analyses.

Pore water was collected using the method of Azcue et al. [8] and Kalnejais et al. [9]. Fifteen cores of undisturbed sediment were collected in each river using a previously acid-washed hand-held polyvinyl chloride corer with a diameter of 5 cm and a length of 20 cm, as shown in Figure 2. The cores were collected with at least 20 cm of sediment columns. The cores were securely covered with plastic and tightened with a rubber band, then placed in labeled ziplock plastic bags. These were then stored in a standing position in ice boxes and brought back to the laboratory.



Figure 2. Core samplers used in pore water collection.

In the laboratory, the sediments in core samplers were slowly removed and centrifuged at 4000 rpm for 30 min to separate the pore water. The pore water samples from each river were combined and filtered through a cellulose membrane with 0.45- μm pores, then preserved by adding 10% (v/v) reagent-grade nitric acid. The samples were placed in an ice bath and transported to TechnoLab, Labangon, Cebu, for analysis of heavy metals and total hardness. The samples were digested using the USEPA Method 3050B for sediments and the APHA-AWWA method for porewater. The metal concentrations were determined using a Flame Atomic Absorption Spectrophotometer (FAAS) via an external calibration method. Recovery tests were performed in triplicate per station per sampling month.

2.4 Pore Water Toxicity Analysis.

2.4.1. *Interstitial water criteria toxicity unit (IWCTU)*. The pore water quality can be evaluated for each metal using IWCTU as a water quality index, defined as:

$$IWCTU_{Me} = \frac{[Me]_{i.w.}}{FCV_{Me}} \quad (1)$$

Where $[Me]_{i.w.}$ is the dissolved metal (Me) concentration and FCV_{Me} is the hardness-dependent final chronic value for the metal [10, 11]. While the $IWCTU_{Me}$ exceeds 1, it implies a risk of toxicity to aquatic organisms. The method for calculating FCV_{Me} is presented in Table 1.

2.4.2 *Nemeraw Index (NI)*. The Nemerow index was also used to estimate the pore water quality, and it was calculated as shown in Equation 2:

$$(NI) = \sqrt{\frac{(IWCTU)_{max}^2 + (IWCTU)_{mean}^2}{2}} \quad (2)$$

Five grades of toxic impact were used: no impact, slight impact, moderate impact, strong impact, and serious impact, with NI values of 5, respectively (Liu et al. [12]). These two indices, the total IWCTU and the NI, which combine the results for multiple metals, can be used to estimate pore water quality (Zhu et al. [13]).

2.5 Sediment Toxicity Risk Assessment.

The degree of contamination of heavy metals in the sediments was assessed by determining the geoaccumulation index (Igeo), contamination factor (CF), and pollution load index (PLI) (Islam et al. [14]).

2.5.1 *Geo-accumulation index (Igeo)*. Geoaccumulation index (Igeo) values were calculated using equation 3:

$$I_{geo} = \log_2 \frac{C_n}{1.5 B_n} \quad (3)$$

Where C_n is the measured concentration of metal n in the sediment, and B_n is the geochemical background value of element n in the background sample.

2.5.2 *Contamination Factor (CF)*. CF was calculated using equation 4 or by dividing the content of each metal by the background values in the sediment.

$$CF_{metal} = \frac{C_{metal}}{C_{background}} \quad (4)$$

2.5.3 *Pollution load index (PLI)*. The PLI is defined as the n th root of the multiplication of the contamination factor of metals (CF), as shown in Equation 5. The greater its value, the more polluted is the river system (Islam et al. [16]).

$$PLI = (CF_1 \times CF_2 \dots CF_n)^{1/n} \quad (5)$$

2.6 Fish Sample collection and blood extraction.

The sampling method and sample handling procedures prior to analysis in this study were approved by the University of San Carlos Institutional Animal Care and Use Committee. A total of four or more different fish species, available in all stations, were randomly collected from each sampling station using nets or fishhooks. Blood of each fish was extracted and smeared on a slide before the extraction of the liver. The fish samples were washed with distilled water and were then packed in plastic bags, labeled, and transported in ice boxes to the laboratory. The fish samples were rewashed with distilled water, placed in aluminum foil, and then in a ziplock bag, and will be frozen at -10°C until further treatment.

2.6.1 Sample Preparation and Heavy Metal Analysis in Fish.

Fish samples were oven-dried at 110°C until a constant weight was reached to remove water. Fish samples were pooled to obtain approximately 0.25-0.35 g of dry weight for microwave digestion, based on the AOAC Method 999.10, using 1 mL of 30% H_2O_2 and 7 mL of concentrated HNO_3 . The microwave-digested samples were filtered using Whatman 42 filter paper and diluted to the mark in 25-mL volumetric flasks. The samples were analyzed with FAAS coupled with an external calibration method. A recovery test was also conducted.

2.6.2 Genotoxicity.

A blood smear was prepared according to the procedures described by Bacolod et al. [15]. A blood smear was air-dried for 24 hours and then fixed in absolute methanol for 30 seconds. It was washed with distilled water and air-dried for an additional 24 hours before staining. The dried smear was added with Gemsa stain in phosphate buffer solution and counterstained with May-Grunwald solution. Micronuclei (MN) and nuclear abnormalities were identified based on Al-Sabti and Metcalf [16] and Fenech et al. [17] using a microscope at 1000x magnification, with a total of 1000 erythrocytes observed at each slide. The final frequency was obtained after getting the average MN and NA of the three replicates per fish.

2.6.3 Metallothionein Assay.

To quantify metallothionein (MT) concentration in cells, the method was adapted from Linde and Garcia-Vazquez [18]. Briefly, the fish liver was homogenized in a 3-mL buffer (0.5 M sucrose, 20 mM Tris-HCl, pH 8.6, 0.01% β-mercaptoethanol), then centrifuged at 30,000 rpm for 10 minutes to obtain the supernatant. Cold ethanol (1.05 mL) and chloroform (80 μL) were added per mL of supernatant, followed by centrifugation at 6,000 rpm for 10 minutes at 0-4°C. The pellets were washed with a mixture of ethanol, chloroform, and buffer, then centrifuged again. The dried pellet was resuspended in 150 μL of 0.25 M NaCl, and 150 μL of 1N HCl with 4 mM EDTA was added. Next, 4.2 mL of 2 M NaCl containing 0.43 mM DTNB buffered with 0.2 M Na-phosphate (pH 8) was added, followed by centrifugation at 3,000 × g for 5 minutes. The supernatant absorbance at 412 nm was measured using a UV-Vis spectrophotometer. MT concentration was calculated using a standard curve of reduced glutathione (GSH) and assuming 1 mole of MT contained 20 moles of cysteine. The final MT content was determined by dividing the concentration by 20.

2.7 Statistical Treatment of Data.

Differences in the mean values of metal concentrations in pore water, sediments, fish tissues, genotoxicity, and metallothionein (MT) levels between stations and fish species were analyzed using two-way ANOVA with GraphPad Prism 7.04. The Pearson correlation coefficient was employed to assess the relationship between metal concentrations in fish, genotoxicity, and MT. Spearman's rank correlation coefficient (Spearman's rho) was used to evaluate the monotonic relationships between genotoxicity and metal accumulation, as well as between MT levels and metal accumulation.

3. Results and Discussion

3.1 Metal in Pore Water of Sapangdaku River and Tañon Strait Coastline

The pore water analysis revealed copper as the dominant contaminant across all stations and sampling months, with mean concentrations of $173 \pm 9.23 \mu\text{g/L}$ and peaks reaching $831 \pm 50.55 \mu\text{g/L}$ at Station 3. Zinc followed, while chromium, lead, and arsenic occurred at comparatively lower levels. A two-way ANOVA revealed significant differences in metal concentrations among stations ($p < 0.001$) and among metals ($p < 0.001$), confirming both spatial variability and metal-specific patterns. Post-hoc tests indicated that Cu concentrations at Station 3 were significantly higher ($p < 0.05$) than at Stations 1 and 2. These differences reflect site-specific conditions: Station 2 (estuary) receives riverine discharges and tailings, Station 1 (Dumlog) experiences seawater dilution, while localized industrial discharges influence Station 3 (near the Atlas Fertilizer Corporation and port).

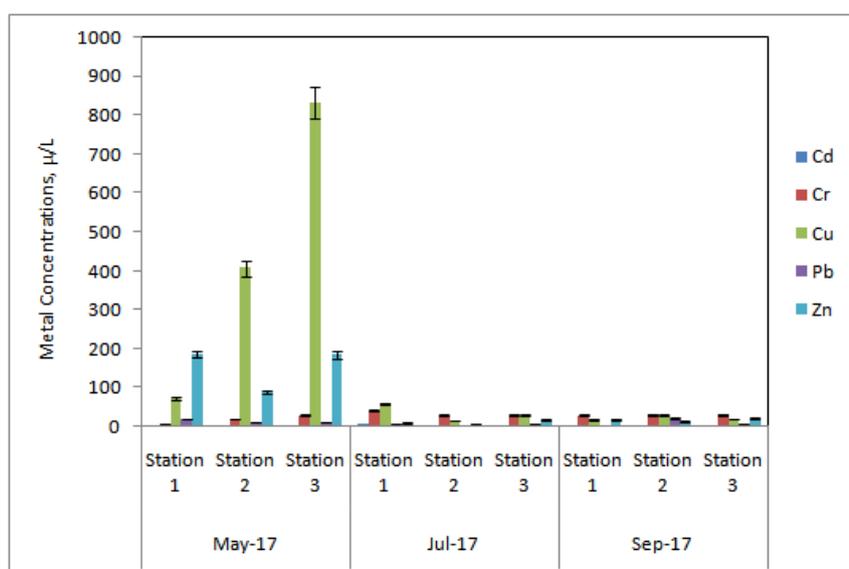


Figure 3. Trace metal concentrations in pore waters of Sapangdaku River and Tañon Strait Coastline

The dominance of copper is consistent with the geological and mining legacy of Toledo, which has resulted in extensive mined-out lands and soils enriched in copper with acidic properties. The relatively low pH and reducing conditions of sediments in this basin enhance copper solubility, allowing greater partitioning into pore water compared to other metals. Previous reports of highly acidic soils (pH ~2.5) with Cu concentrations ranging from 154 to 638 mg/kg [1] and sediments with Cu levels exceeding 700 ppm [2] support this enrichment. Compared with other regions, the severity in Toledo is striking: Deriner Dam Lake, Turkey, recorded only 9.0 µg/L Cu [22], while Shaocun River, China (123 µg/L) [13], and Cipeles River, Indonesia (322 µg/L) [23], still fall well below the values observed here. From a toxicological standpoint, the observed Cu levels are ecologically alarming. The U.S. EPA chronic water quality criterion for Cu is 16 µg/L, which was exceeded by more than an order of magnitude at all sites. IWCTU values also confirmed Cu toxicity risks across stations, while Cd exceeded thresholds at Station 1. Such exceedances suggest adverse impacts on aquatic organisms, including gill damage, reduced growth, and impaired reproduction in fish and invertebrates. Because pore water represents the most bioavailable fraction of metals, these concentrations indicate direct exposure risks to benthic fauna. Collectively, the statistical evidence highlights the strong influence of mining-derived Cu contamination in the Sapangdaku River and highlights its potential ecological consequences for the adjacent Tañon Strait.

3.2 Metal Concentrations in Sediments of Sapangdaku River and Tañon Strait Coastline

The sediment analysis revealed that Cu was the most abundant metal at all stations, except in July 2017, when the concentrations of Cu and Zn were nearly equal. Across sampling periods, the general trend was Cu > Zn > Pb > Cr > Cd, which is consistent with previous findings from the Balamban coastline [20]. Statistical testing indicated that metal concentrations were marginally significantly different between sampling sites ($p = 0.0837$). However, differences in metal concentrations were highly significant ($p < 0.001$), confirming that variation in overall sediment contamination is primarily driven by the type of metal rather than the location of collection.

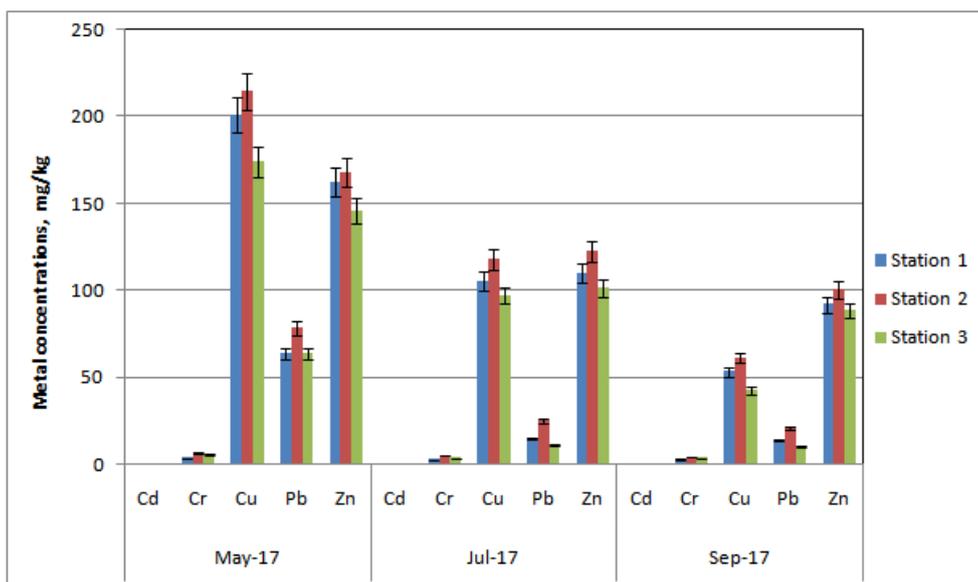


Figure 4. Heavy metal concentrations in sediments.

Copper and zinc were the dominant contributors to this variation, with Cu showing consistently higher concentrations across most stations and months, while Zn occasionally approached similar levels. Lead, chromium, and cadmium were present at much lower concentrations, contributing minimally to the observed differences. The predominance of Cu in sediments reflects the legacy of mining inputs, while elevated Zn may be linked to both natural sources and agricultural/industrial activities in the coastal zone.

Generally, higher concentrations of each metal were recorded in May 2017, which is attributed to the low water flow in May, resulting in the precipitation of the metals in the sediment and thereby increasing their

concentration [14]. This is supported by Duman and Kar [24], who reported that the combined effects of increased evaporation and decreased rainfall may indeed lead to higher metal concentrations in sediments. Considering the toxicity reference values (TRVs) proposed by the USEPA [25], Cu, Pb, and Zn in all stations exceeded the limits (16, 31, and 110 µg/L, respectively), while both Cd and Cr are below the limits. This means that the levels of the former metals in the rivers can harm the aquatic ecosystems associated with these rivers, especially after they receive urban water and wastewater, as seen in the downstream of the Tsurumi River in Japan [6].

3.3 Pore Water Toxicity Analysis

The Final chronic values (FCV) of the selected metals (Cd, Cu, Pb, and Zn) were determined in Table 2, and the IWCTU index was calculated from Eq. (1) for each metal at each river. The FCV for all metals in this study was higher compared to the values reported by Mehdizadeh et al. [26] in the pore water of Anzali wetland located in the southwestern part of the Caspian Sea.

Table 2. Hardness-dependent final chronic value (FCVMe) determined for Cd, Cu, Pb, and Zn in pore water.

Sites	CaCO ₃ , mg/L			FCVCd			FCVCu			FCVPb			FCVZn		
	May	Jul	Sep	Ma	Jul	Sep	Ma	Jul	Sep	Ma	Jul	Sep	Ma	Jul	Sep
1	4256	12775	11011	16.2	36.1	32.4	290	716	630	298	1208	1000	2508	6366	5612
2	7310	5676	15512	24.1	20.0	45.6	444	358	845	298	430	1547	3966	3205	7504
3	12987	14540	11559	36.5	39.7	33.6	726	799	657	1234	1424	1063	6455	7103	5848

Table 3 shows that the IWCTU values for Cd, Cu, Pb, and Zn in the porewater of the Sapangdaku River and Tañon Strait coastline exceeded the recommended water quality criteria set by the US Environmental Protection Agency. The IWCTU for Cu at all stations was >1, as was the IWCTU value of Cd at Station 1.

Table 3. Toxic unit analysis results for the pore water metals in Sapangdaku River and Tañon Strait Coastline

Site	Mean Interstitial Water Criteria Toxicity Unit (IWCTU)				ΣIWCTU	NI	Impact grade
	Cd	Cu	Pb	Zn			
1	2.24	1.13	0.049	0.32	3.53	1.58	Slight
2	0.39	2.43	0.77	0.17	2.42	1.72	Slight
3	0.27	1.51	0.32	0.41	1.58	1.08	slight

Data in bold exceeded the corresponding water quality criteria recommended by EPA (2002)

The IWCTU values indicated that copper posed potential toxicity risks across all stations, while cadmium exceeded thresholds at Station 1. Notably, the mean Cu concentrations in pore water (173 µg/L, peaking at 831 µg/L) were more than ten times higher than the U.S. EPA chronic water quality criterion of 16 µg/L for aquatic life [25]. Even the lowest Cu values observed in this study exceeded the EPA guideline by several orders of magnitude, highlighting the severity of Cu enrichment in the system. By contrast, Zn and Pb generally remained below their respective thresholds, and Cd exceedances were limited to Station 1. When compared to other international benchmarks, the ecological risk remains evident. The Canadian Council of Ministers of the Environment (CCME) sets a chronic guideline for Cu at 2–4 µg/L (depending on hardness), meaning that the observed values in the Sapangdaku River were up to two orders of magnitude higher. Similarly, the Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZG, 2018) recommend Cu concentrations not exceeding 1.4 µg/L in freshwater, far below the levels measured in this study. Philippine national standards (DENR DAO 2016-08) set a Class C freshwater guideline for Cu at 50

$\mu\text{g/L}$, which was also consistently exceeded at all stations. Mehdizadeh et al. [26] reported Nemeraw Index values below 1 for the Anzali wetland, indicating no adverse impacts. While the release of heavy metals from sediment into the water column appears to be limited, transformation and migration processes require further investigation. The potential toxicity near surface sediments is likely due to the release of metals from biogenic material during aerobic degradation of organic matter [27]. These comparisons highlight the toxicological significance of the findings: pore water Cu concentrations not only surpass U.S. EPA thresholds but also significantly exceed those of Canadian, Australian/New Zealand, and Philippine water quality criteria. Given that pore water represents the most bioavailable fraction of metals, these exceedances suggest substantial risks to benthic organisms and higher trophic levels, with likely consequences for ecological health and fisheries in the Sapangdaku River and adjacent Tañon Strait.

3.4 Sediment Toxicity Risk Assessment of Metal Contamination

3.4.1 Geo-accumulation Index, *I*_{geo}.

The *I*_{geo} values revealed clear differences among metals and sampling months. Copper showed the highest contamination, with *I*_{geo} values ranging from 2.1 to 3.4 in May and 1.5 to 2.8 in July, indicating *moderate to high contamination* according to Müller's [28] classification. Lead also exhibited contamination during these months, with *I*_{geo} values ranging from 1.0 to 1.6 in May and from 0.7 to 1.2 in July, corresponding to *moderate contamination*. By September, *I*_{geo} values for both Cu and Pb had declined (<1.0), indicating dilution effects from increased rainfall and seasonal sediment flushing. In contrast, cadmium and chromium recorded *I*_{geo} values consistently below zero throughout all sampling periods, indicating that they are *uncontaminated*. Both source and geochemical behavior can explain this pattern. Cd inputs are likely minimal in the Toledo basin compared to Cu and Zn, as there are no major anthropogenic Cd sources reported in the area. For Cr, its strong affinity for sediment particles and tendency to form stable mineral phases reduce its mobility and bioavailability, keeping concentrations near natural background levels. These findings suggest that Cu and Pb are the primary contaminants of concern, driven by mining-derived inputs and industrial activities, while Cd and Cr remain at or near geochemical background levels. The dominance of Cu is consistent with the area's mining legacy and acidic sediment conditions, which enhance its solubility and accumulation. This emphasizes the need to prioritize ecological risk assessments for Cu and Pb, while acknowledging that not all trace metals contribute equally to sediment contamination in the Sapangdaku River and Tañon Strait.

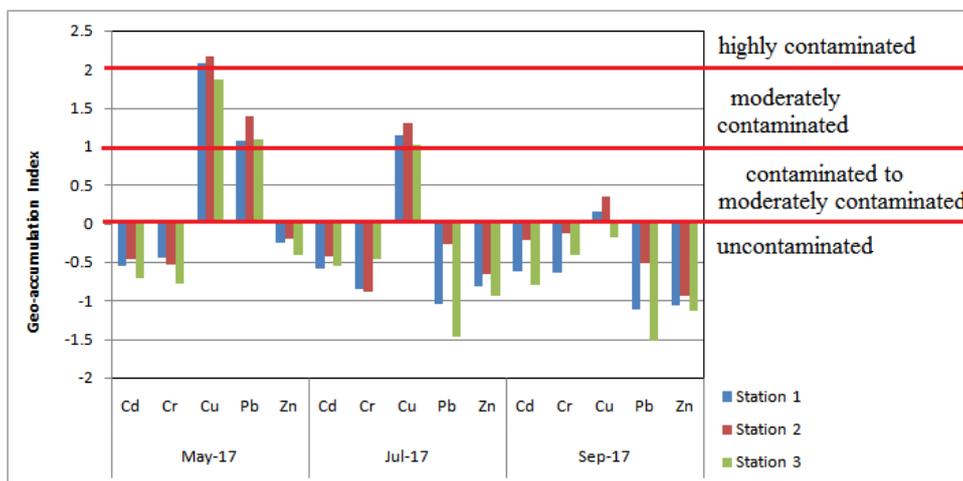


Figure 5. Geoaccumulation index (*I*_{geo}) values of heavy metals in the Tañon Strait.

3.4.2 Contamination factor, *CF*.

The *CF* values for all metals followed the order $\text{Cu} > \text{Pb} > \text{Zn} > \text{Cd} > \text{Cr}$, as shown in Figure 6. According to Hakanson's classification, $\text{CF} < 1$ indicates *low contamination*, 1–3 *moderate*, 3–6 *considerable*, and >6 *very high contamination*. In this study, copper consistently showed the highest *CF* values, reaching 6.2 at Station 2 in May, which falls into the *very high contamination* category. Pb also exhibited *considerable contamination* in May, with *CF* values between 3.1 and 4.8 across stations. By contrast, Zn recorded *CF* values of 1.8–2.4 in May,

corresponding to *moderate contamination*, while Cd and Cr remained below 1 throughout, indicating *low contamination*. The CF results confirm Cu and Pb as the dominant pollutants of concern, with Zn contributing moderately, particularly in May. The temporal variation reflects both local mining and industrial activities, as well as seasonal hydrological conditions. Meanwhile, Cd and Cr levels remain near background values, indicating that their ecological risks are minimal compared to those of the other metals in the Sapangdaku River and Tañon Strait.

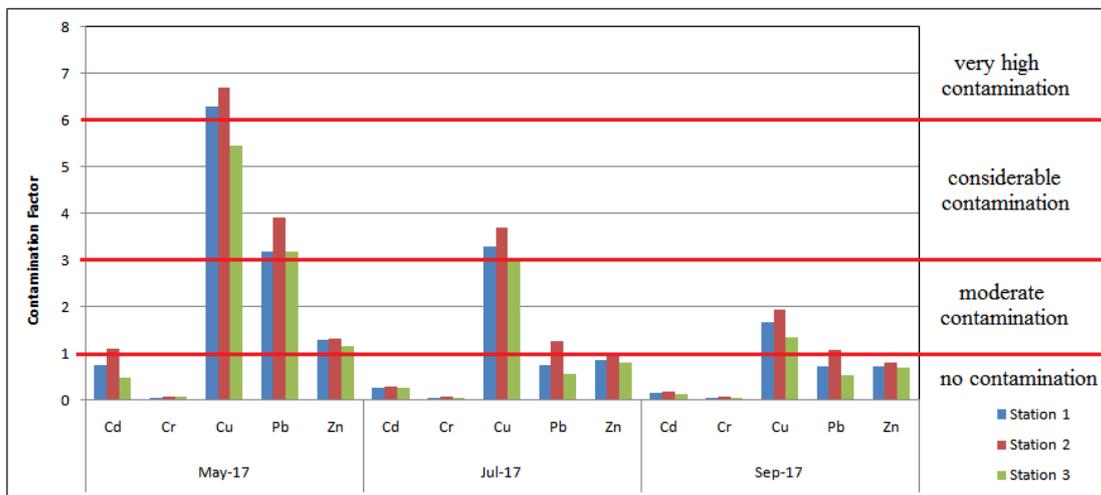


Figure 6. Contamination factor (CF) values of heavy metals in Tañon strait sediments.

3.4.3 Pollution Load Index.

Figure 7 shows the PLI values, which provide a composite measure of sediment quality by integrating CF values across metals; a PLI value greater than 1 indicates progressive site deterioration. In this study, only Station 2 in May recorded a PLI above 1 (1.23), signifying overall pollution pressure. This elevated value was primarily driven by copper, which showed very high contamination (CF > 6), and was further reinforced by *considerable contamination* from Pb (CF > 3). Zinc, while only *moderate* (CF ~2), also contributed to the cumulative index, whereas Cd and Cr remained negligible. Thus, the exceedance of PLI > 1 at Station 2 reflects the combined effects of multiple metals, with Cu as the dominant contributor. When compared to other regional and global studies, the severity at Station 2 is noteworthy but not unprecedented. For example, Mohiuddin et al. [5] reported PLI values ranging from 1.1 to 2.8 in downstream sections of urban rivers in Japan, while Islam et al. [14] documented values between 1.2 and 3.5 in contaminated river sediments in Bangladesh. In contrast, relatively unpolluted river systems typically exhibit PLI < 1, as observed in French riverine sediments by Lourião-Cabana et al. [11]. Locally, in Alburo and Villegas [29], a PLI of 41 was reported for Station 3 of the Lahug River in Cebu, attributed to intense urban activities, underscoring the potential for extreme contamination in urbanized catchments. By comparison, Villacarlos et al. [30] reported PLI values of ~2.3 in April and ~2.45 in November 2018 for the Balamban Coast, Cebu, Philippines, indicating moderate but persistent metal pollution in a coastal environment adjacent to industrial activities. Taken together, the PLI analysis highlights Station 2 in May as the site of the most significant ecological concern. The combined effect of elevated Cu, Pb, and Zn emphasizes the need for targeted monitoring at this estuarine location, which serves as both a sink for upstream contamination and a conduit for pollutant transport into the Tañon Strait.

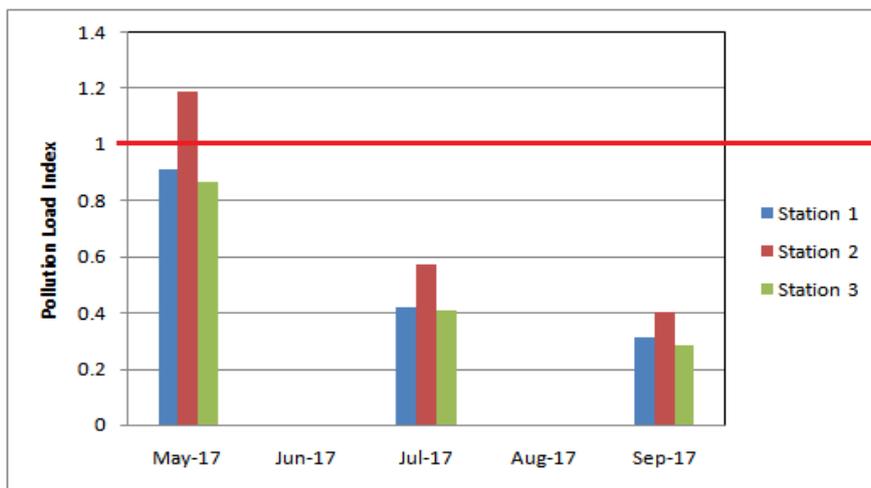


Figure 7. Pollution load index value of heavy metals in Tañon strait sediments.

3.5 Fish Analysis

Several different fish species were collected, with a maximum of four species available in situ in all three sampling sites in July 2017. The four fish species were identified by the curator of the Marine Biological Collection, Department of Biology, University of San Carlos, based on their visible external characteristics, as *Mugil cephalus* Linnaeus, *Trichiurus lepturus* Linnaeus, *Strongylura leiura*, and *Eubleekeria jonesi*.

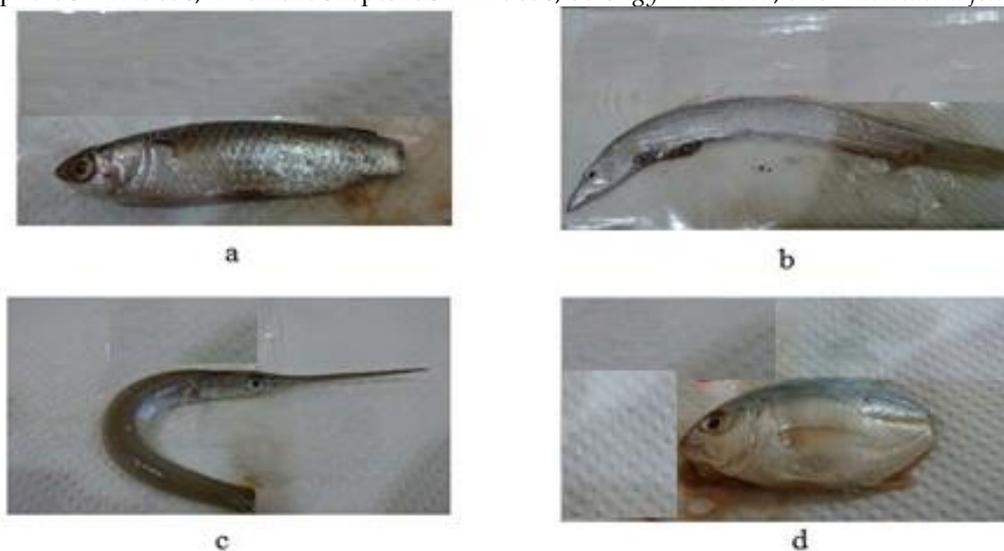


Figure 8. Identification of fish species: (a) *Mugil cephalus* Linnaeus, (b) *Trichiurus lepturus* Linnaeus, (c) *Strongylura leiura*, and (d) *Eubleekeria jonesi*.

3.5.1 Heavy Metal Concentration in Fishes.

The metal content analysis of fish samples, presented in Figure 9, revealed significantly high accumulation levels of Cu and Zn across all species. The mean metal concentrations followed a descending order: Zn > Cu > Cd > Cr = Pb, with Pb and Cr below the detection limit of 0.01 mg/kg. Minor variations in metal concentrations were observed between sites, likely due to the mobility of fish between locations. As highlighted by Kalay and Canli [31], non-essential metals such as Pb, Cr, and Cd generally occur at very low levels.

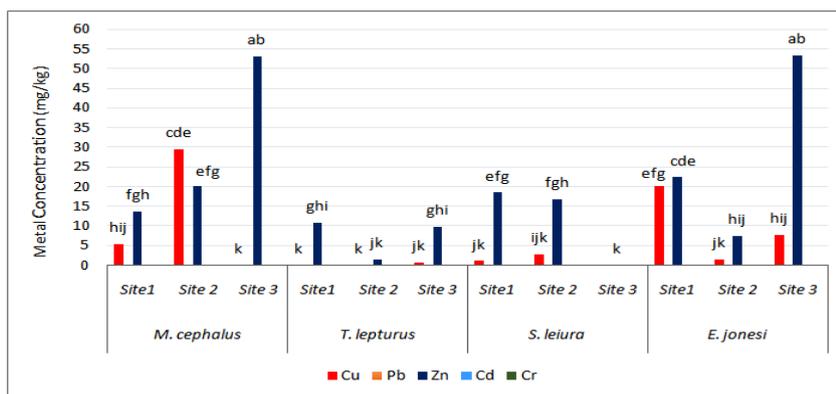


Figure 9. Heavy metal accumulation of the four fish species. Data are expressed in mg/kg. Different letters show significant differences in the concentration of Cu and Zn among species and sites.

3.5.1.1 Zinc

Zn levels in fish species were highest in *M. cephalus* (13.6–53.1 mg/kg) and *E. jonesi* (7.5–53.4 mg/kg), with lower levels detected in *T. lepturus* and *S. leiura* (below detection to 18.5 mg/kg). The elevated Zn levels in *M. cephalus* and *E. jonesi* can be explained by their feeding modes and habitat use. *M. cephalus* is a benthic detritivore, ingesting sediments and organic detritus where Zn accumulates, while *E. jonesi*, with its relatively larger muscle mass and benthic feeding habits, tends to store more trace metals. Both species are frequently found in sediment-rich estuarine zones, increasing their exposure to Zn-laden particulates compared to more pelagic feeders such as *T. lepturus*. Statistical analysis revealed significant differences in Zn concentrations between sites and species ($P < 0.05$), except for the comparison between *T. lepturus* and *S. leiura*. Although Zn levels were below the permissible limits set by Canadian (100 mg/kg), Hungarian (80 mg/kg), and Australian (150 mg/kg) standards, they exceeded the FAO limit of 40 mg/kg for potable fish, which could restrict their export potential and raise concerns about long-term consumption risks for local communities. Chronic dietary exposure to elevated zinc, while less acutely toxic than copper or lead, may still contribute to gastrointestinal distress, immune dysfunction, and metabolic imbalances in humans. Compared with other studies, Zn accumulation in the Sapangdaku basin was lower than that reported for *E. suratensis* in Indian reservoirs (98–303 mg/kg) but higher than values observed in some Philippine freshwater systems (e.g., *P. reticulata* from Butuanon River, 16.4–140 mg/kg) [32]. Thus, while not the highest globally, the Zn levels detected here are ecologically and commercially significant, particularly for species closely linked to benthic habitats.

3.5.1.2 Copper

River basin. As shown in earlier sections, pore water Cu concentrations reached up to 831 $\mu\text{g/L}$ at Station 3 and sediments exceeded 700 ppm near the tailings outfall [2], both far above background levels and indicative of long-term mining discharges. The high Cu accumulation in fish, therefore, aligns with the extreme enrichment of surrounding sediments and pore water, confirming that benthic and detritivorous feeders, such as *M. cephalus*, are particularly vulnerable to exposure. The mean Cu concentrations in fish exceeded international food safety limits by approximately 12 times (mean, not maximum) relative to the WHO (3 mg/kg) [33], FAO (4 mg/kg) [34], EU (1 mg/kg) [35], and FEPA (1.3 mg/kg) [36] permissible thresholds. This consistent exceedance underscores a clear risk to food safety, with potential implications for both local consumption and compliance with export standards. While copper (Cu) is an essential trace element, excessive intake can disrupt gastrointestinal and hepatic functions in humans, and the concentrations measured in this study exceed tolerable dietary levels. From an ecological perspective, the Cu levels detected are high enough to elicit sub-lethal physiological stress in fish. Previous studies have demonstrated that chronic Cu exposure causes gill damage, impaired ion regulation, oxidative stress, and reduced growth rates in freshwater and estuarine fish [46, 44]. Similar Cu accumulation patterns were reported in *C. anguillari* and *O. niloticus* from the Nile River (22.87 and 18.37 mg/kg, respectively) and in *Esox lucius* and *Abramis brama* in Polish lakes (0.14–7.76 mg/kg) [38]. However, the concentrations in the present study were markedly higher. These effects

are consistent with the elevated metallothionein induction and genotoxic responses observed here, supporting the conclusion that Cu contamination in the Sapangdaku River is both bioaccumulative and biologically active at the molecular level.

Table 4. Permissible limit of metals set by different health organizations and toxicology values.

Standards	Cu (mg/kg)	Cd (mg/kg)	Cr (mg/kg)	Pb (mg/kg)	Zn (mg/kg)
FAO (2000)	4.00	0.50	1.00	6.00	40.0
WHO (2006)	3.00	0.50	0.15	2.00	-
EU (2001)	1.00	-	1.00	-	-
FEPA (2003)	1.30	-	0.15	2.00	-

3.5.1.3 Cadmium

Cadmium (Cd) concentrations in the sampled fish ranged from below the detection limit (<0.01 mg/kg) to 0.111 mg/kg in *M. cephalus*, 0.099 mg/kg in *E. jonesi*, 0.056 mg/kg in *T. lepturus*, and 0.052 mg/kg in *S. leiura*. Statistical analysis indicated no significant differences in Cd levels between fish species or sampling sites. Importantly, all values remained within the acceptable limits established by WHO and FAO (0.5 mg/kg) [32,33], suggesting no immediate food safety risks from Cd exposure in fish harvested from the Sapangdaku River basin. Both source and geochemical factors can explain the consistently low Cd levels. Unlike Cu and Zn, which are heavily influenced by mining discharges in Toledo, there are no major anthropogenic Cd inputs documented in the catchment. Cd also exhibits a strong affinity for sulfide phases under reducing conditions, which immobilizes it in sediments and reduces its bioavailability to aquatic organisms. This explains why Cd remained below toxic thresholds despite its well-recognized status as a highly toxic, non-essential metal. When compared globally, the Cd levels in this study are relatively low. Fish from contaminated freshwater systems often show much higher concentrations, such as *Oreochromis niloticus* from Egyptian rivers (0.2–1.2 mg/kg) or commercial species from Indian reservoirs (0.3–0.9 mg/kg) [37]. In contrast, the values reported here (<0.12 mg/kg) are closer to background levels typical of less polluted systems. Thus, while Cu and Zn clearly drive contamination risk in the Sapangdaku basin, Cd appears to pose minimal ecological or human health risk at present.

3.6 Metallothionein Assay.

Metallothionein (MT), a key metal-detoxifying protein in fish, was quantified to assess species-specific responses to heavy metal exposure. The highest MT levels were observed in *M. cephalus* (299.16 µg/g at Site 2), followed by *E. jonesi* (200.02 µg/g at Site 1), while *T. lepturus* and *S. leiura* exhibited comparatively lower responses, as shown in Figure 10. Statistical analysis confirmed significant variation in MT levels among species and sites ($p < 0.0001$), consistent with differences in both exposure pathways and physiological stress responses. These results align with findings in *O. niloticus* from the metal-contaminated Barra Mansa River, Brazil, where Linde and Garcia-Vazquez [18] reported MT values of 212.63 µg/g, suggesting that benthic-associated species generally show higher induction under polluted conditions.

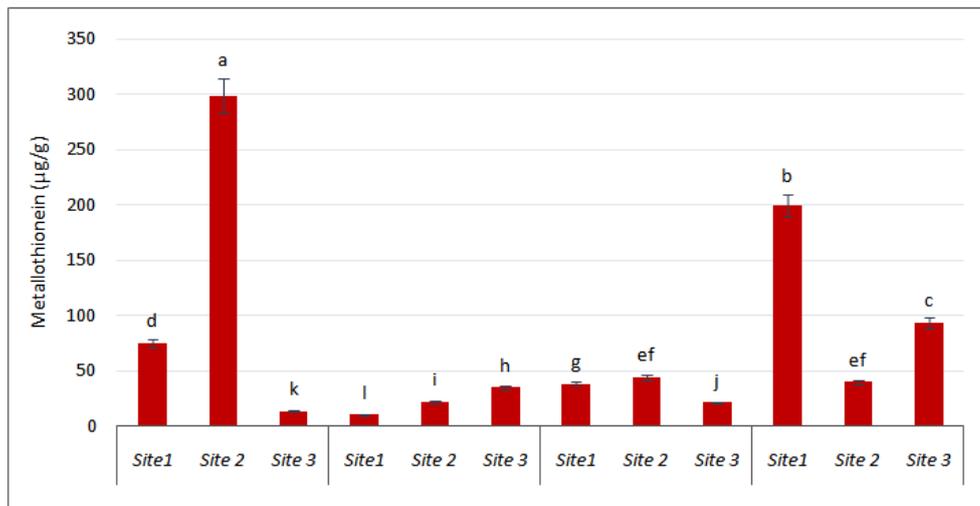


Figure 10. MT levels in the four fish species. Data are expressed in $\mu\text{g/g}$. Different letters show significant differences in the concentration of Cu and Zn among species and sites.

In addition to intrinsic metabolic differences, feeding ecology and habitat use strongly influence MT induction. Benthic detritivores such as *M. cephalus* ingest sediments and organic detritus that contain bioavailable metals, directly increasing their internal metal burden and stimulating MT production. Similarly, *E. jonesi*, which forages in benthic habitats and has larger muscle mass for storage, also exhibited elevated MT levels. In contrast, more pelagic feeders, such as *T. lepturus*, are less exposed to contaminated sediments, which explains their comparatively lower MT responses. These species-specific differences are consistent with earlier reports that benthic and detritivorous fish accumulate metals more efficiently due to direct interaction with sediment [30]. The pronounced MT induction in *M. cephalus*, therefore, reflects both its ecological niche and dietary habits. By resuspending and ingesting fine sediments at the estuary, this species is exposed to the elevated Cu and Zn concentrations documented in pore water and sediments (Sections 3.1 and 3.2). This sediment-associated exposure pathway likely explains why *M. cephalus* consistently showed the strongest biomarker response, reinforcing its role as a reliable sentinel species for monitoring heavy metal contamination in estuarine environments. Similar conclusions have been drawn in previous biomonitoring studies where high MT induction corresponded to Cu enrichment and sediment contact [20, 18].

3.7 Genotoxicity

Figure 11 illustrates genotoxicity parameters affected by excessive heavy metal uptake in fish. The micronucleus assay, widely used to measure genotoxicity in both laboratory and field conditions (Carrasco et al. [40]), reveals the formation of nuclear abnormalities such as lobed, blebbed, and notched nuclei in fish erythrocytes. These abnormalities result from exposure to environmental and chemical contaminants with cytotoxic, genotoxic, mutagenic, or carcinogenic activity. Micronuclei, shown in Figure 11a, form during cellular division and reflect cytogenetic effects, including the loss of chromosomal fragments or whole chromosomes that are excluded from the main nucleus during anaphase. The MN test in fish can detect clastogenic and aneugenic effects of environmental agents in aquatic media. Since teleost erythrocytes are nucleated, MN are counted in fish erythrocytes to assess clastogenic activity (Al-Sabti and Metcalfe [41]). Additionally, NA, including blebbed, lobed, and notched nuclei, as well as bi-nucleated cells, have been identified as potential indicators of genotoxicity [42]. While the mechanisms behind NA formation (shown in Figure 11b) are not fully understood, these abnormalities are regarded as indicators of genotoxic damage, complementing MN scoring in routine genotoxicity assessments.

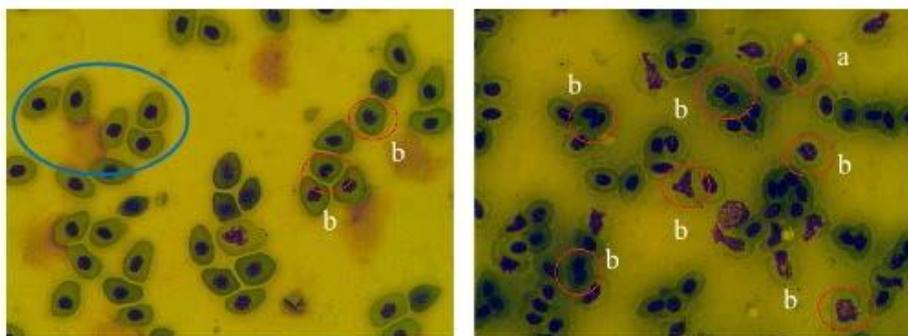


Figure 11. Erythrocytes were studied for the Environmental Genotoxicity test. Cells inside the blue circle are normal cells. Cells inside the red circles are (a) micronuclei and (b) nuclear abnormalities.

3.7.1 Micronuclei Test.

The average number of micronuclei (MN) varied across species and sampling sites, ranging from 0.111 to 3.667 per 1000 erythrocytes for *M. cephalus*, 0.000 to 0.0667 per 1000 erythrocytes for *T. lepturus*, 0.222 to 0.0889 per 1000 erythrocytes for *S. leiura*, and 0.778 to 2.556 per 1000 erythrocytes for *E. jonesi*. Elevated Cu concentrations were significantly correlated with increased MN formation ($R > 0.98, p < 0.05$), consistent with earlier reports linking Cu exposure to clastogenic and aneugenic effects in fish erythrocytes [43, 15]. The highest MN frequencies were observed in *M. cephalus* at Site 2 and *E. jonesi*, which aligns with their benthic feeding habits and higher accumulation of Cu and Zn.

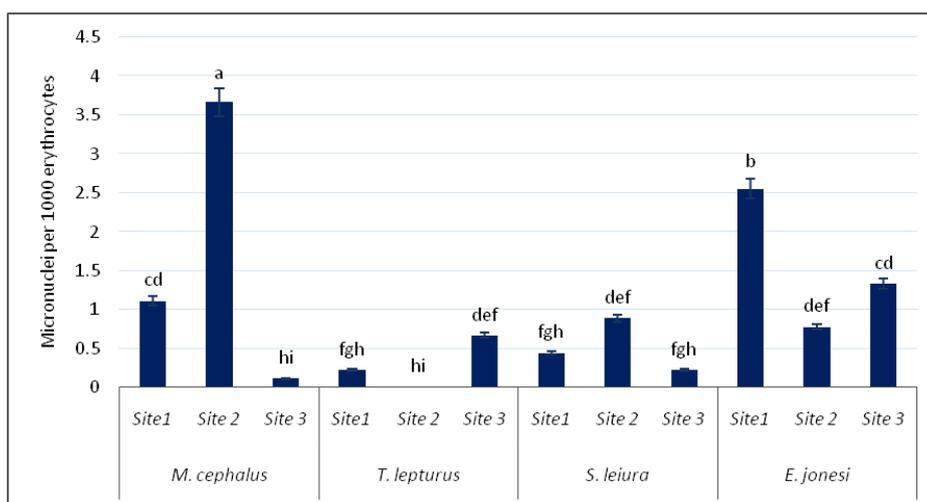


Figure 12. Frequency of MN in the erythrocytes of fish between the three sites. Data are expressed in the number of micronuclei formed per 100 erythrocytes. Different letters indicate significant differences in the number of micronuclei among species and sites.

Figure 12 illustrates the frequency of MN per 1000 erythrocytes, with the highest MN frequencies observed in *M. cephalus* at site 2 and *E. jonesi*, which aligns with the MT response and metal accumulation. The results show that as metal uptake increases, the MN frequency per 1000 erythrocytes also increases. These findings are consistent with those of Stankevičiūtė et al. [44], who reported a similar trend in *Oncorhynchus mykiss* exposed to Cu and Zn, where the highest MN frequency observed was 0.67 per 1000 erythrocytes, further confirming that higher metal accumulation correlates with increased MN formation.

3.7.2 Nuclear Abnormalities.

Figure 13 illustrates the impact of Cu concentration on nuclear abnormalities in fish, revealing significant differences between species and sampling sites. The study reveals that higher Cu concentrations are associated with a greater number of nuclear abnormalities, exhibiting a strong correlation ($R > 0.98, P < 0.05$). The frequency of nuclear abnormalities per 1000 erythrocytes ranged from 1.556 to 19.333 in *M. cephalus*,

1.222 to 1.556 in *T. lepturus*, 1.778 to 5.778 in *S. leiura*, and 4.778 to 11.889 in *E. jonesi*. NA frequencies were strongly correlated with Cu levels ($R > 0.98$, $p < 0.05$), reflecting higher sensitivity to metal stress. These findings are in agreement with previous studies that reported elevated NA frequencies under heavy metal exposure, often exceeding MN responses [44, 45].

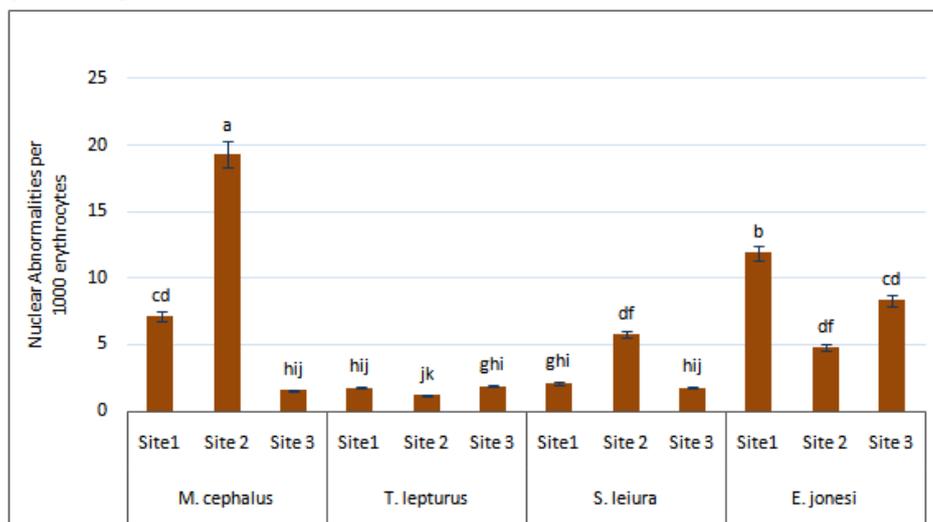


Figure 13. Frequency of NA in the erythrocytes of fish between the three sites. Data are expressed in the number of nuclear abnormalities per 100 erythrocytes. Different letters indicate significant differences in the number of nuclear abnormalities among species and sites.

The results in this study show that both MN and NA frequencies were significantly higher at higher Cu concentrations, which aligns with similar findings in other studies. For instance, Stankevičiūtė et al. [44] reported the highest NA frequency of 14.33 per 1000 erythrocytes in *Oncorhynchus mykiss* exposed to Cu and Zn, while [45] found the highest NA frequency of 6.22 per 1000 erythrocytes in *Parablennius sanguinolentus* exposed to heavy metals. These studies also observed that as metal uptake increased, so did the frequency of NA.

The present study also showed that the increase in MN and NA was more pronounced at higher Cu concentrations, with NA being more frequently observed than MN. This observation is consistent with previous studies, such as [45], where *Prochilodus scrofa* exposed to high Cu concentrations displayed elevated MN and NA frequencies. Both MN and NA frequencies showed a significant correlation with MT levels ($R > 0.98$, $P < 0.05$), indicating that the higher the MT levels, the greater the number of MN formed in the erythrocytes of the fish. This relationship likely stems from the correlation between MT and Cu levels; as Cu levels increase, so do MT levels, leading to a corresponding rise in MN formation.

Taken together, NA were consistently more frequent than MN across all species, suggesting that NA formation may be a more sensitive or earlier genotoxic indicator of heavy metal stress. While MN reflect permanent chromosomal damage caused by clastogenic or aneugenic events, NA are thought to result from disturbances in the mitotic spindle or nuclear envelope stability, which may occur more rapidly after exposure [42]. The predominance of NA in *M. cephalus* and *E. jonesi*, therefore, supports the idea that benthic feeders not only accumulate more metals but also manifest earlier cytogenetic stress responses. This comparative evidence highlights the utility of using both MN and NA assays together, with NA potentially serving as a more sensitive biomarker for detecting early genotoxic effects in metal-polluted environments.

4. Conclusions

This study demonstrated that the Sapangdaku River and its adjacent Tañon Strait coastline remain heavily influenced by mining-derived copper contamination, with pore water and sediment analyses confirming that copper is the dominant pollutant, followed by zinc and lead. Pore water Cu concentrations exceeded U.S. EPA, CCME, ANZG, and DENR criteria by more than an order of magnitude. At the same time,

sediment indices (Igeo, CF, PLI) consistently identified Cu as the primary driver of ecological risk, with localized contributions from Pb and Zn. In contrast, Cd and Cr remained close to background levels, indicating limited anthropogenic input and low bioavailability. Fish analyses revealed substantial bioaccumulation of Cu and Zn, particularly in benthic feeders such as *M. cephalus* and *E. jonesi*, which also exhibited the strongest biomarker responses. The induction of metallothionein in liver tissues and elevated frequencies of micronuclei and nuclear abnormalities in erythrocytes confirmed that metal contamination was biologically active, inducing detoxification mechanisms and genotoxic stress. Notably, nuclear abnormalities occurred more frequently than micronuclei, suggesting their utility as an earlier or more sensitive biomarker of heavy metal exposure. Overall, the integration of chemical and biological endpoints provides compelling evidence of ongoing ecological stress in the Sapangdaku River basin. The findings underscore the importance of ongoing biomonitoring and enhanced regulatory oversight to safeguard aquatic ecosystems and protect communities that rely on these resources. As *M. cephalus* emerged as a reliable sentinel species due to its sediment-feeding habits and consistent biomarker responses, its use in long-term monitoring could support environmental management and policy decisions aimed at mitigating the impacts of legacy mining and associated industrial activities in Toledo City. These findings also revealed the utility of MT, MN, and NA as sensitive indicators for assessing metal contamination and its potential ecological risks in aquatic environments.

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