Global Medical and Health Communication

Online submission: https://ejournal.unisba.ac.id/index.php/gmhc
DOI: https://doi.org/10.29313/gmhc.v13i1.14110

GMHC. 2025;13(1):78–85
pISSN 2301-9123 │ eISSN 2460-5441

RESEARCH ARTICLE

Early Detection of the Heavy Metals Pollution Effect on Citarum River Using Zebrafish Muscle Mitochondria Biomarkers Gene Expression

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Abstract

Citarum is the longest river in West Java and one of the most polluted rivers in Indonesia and the world. Heavy metals from agriculture, industry, and household waste pollute the Citarum river. Heavy metals enter humans and animals by consuming contaminated food and beverages. Several studies have identified mitochondria as a primary target for heavy metal poisoning, resulting in impaired mitochondrial energy production, induction of oxidative stress, apoptosis, and mitophagy. This study aims to investigate the impact of heavy metals from the upstream and downstream areas of the Citarum river on mitochondrial gene expression of *cox4i1*, *sod2*, *baxa*, *mfn1b*, and *ppargc1a* in the skeletal muscle of zebrafish (*Danio rerio*) using in vivo models for biological monitoring of early detection of environmental heavy metal pollution. This experimental study was conducted from December 2019 to March 2020. The study involved four treatment groups and one control group of zebrafish, which were exposed to river water and sediments collected from two sites in the Citarum river area in December 2019. The biomarker levels were analyzed using multivariate analysis. Although all heavy metal levels except mercury were below the WHO threshold in all samples, this study's results showed that the RT-PCR results indicated that the levels of *cox4i1*, *baxa*, and *ppargc1a* in all samples were generally higher than those in the control. There were significant differences ($p<0.05$) using multivariate analysis in *sod2* in Cibeureum water compared to Cibeureum sediment and *ppgarc1a* compared to control and Balekambang sediment. In conclusion, heavy metals from the upstream and downstream areas of the Citarum river had an impact on mitochondrial gene expression of *cox4i1*, *sod2*, *baxa*, *mfn1b*, and *ppargc1a* in the skeletal muscle of zebrafish. Biomarkers *pparc1gc* and *sod2* could be further studied to identify the most valid and reliable parameters for biological monitoring, which may aid in the early detection of environmental damage to humans and animals.

Keywords: Citarum river, heavy metals, mitochondria, muscle, zebrafish

Introduction

Citarum is the longest and widest river in West Java, Indonesia, and was declared one of the National Strategic Rivers by the Indonesian government in 2012. The Citarum river has essential functions for agriculture and fisheries, and is also a groundwater source. Over the years, pollution of the Citarum river has worsened.[1] One of these river pollutants is heavy metal ions (HMI) contamination that exceeds the normal threshold. The concentration of heavy metals (Fe, the highest, Cd, Co, Ni, Pb, Hg, As, Mn) has exceeded the permissible limit in water and sediment of the Citarum river.[2]

Heavy metal ions from contaminated water and aquatic organisms enter the bodies of living organisms. Chronic accumulation of heavy metals in the body can damage nucleic acids, proteins, and cells.[3] Serious effects on animals may include impaired growth and development, cancer, organ damage, and, in extreme cases, leading to death. For example, Hg is commonly responsible for neurological diseases such as Minamata, with clinical signs of ataxia, visual and hearing impairment, sensory impairment, convulsions,

Received: 9 September 2024; Revised: 29 April 2025; Accepted: 29 April 2025; Published: 30 April 2025

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memory impairment, muscle weakness and wasting, and muscle spasms.[4]

Almost all HMI is accumulated and distributed to mitochondria.[5] Heavy metal ions affect mitochondrial oxidative phosphorylation (OXPHOS) by disrupting complex I (NADH dehydrogenase), complex II (succinate dehydrogenase/SDH), complex III (cytochrome oxidase BC/cox), complex IV (cytochrome c oxidase/cox), and complex V (ATP synthase).[6–12] Low ATP levels due to disruption of OXPHOS can cause muscle weakness, and chronic low energy causes wasting and reduces growth and development.[4]

Almost all HMI cause stress oxidative five by increasing reactive oxygen species (ROS)[6–11] and decreasing enzymatic antioxidants such as superoxide dismutase, glutathione peroxidase (gpx), glutathione reductase (gr), catalase, glutaredoxin, thioredoxin, thioredoxin reductase (tax), and peroxiredoxin (pox).[6,7,9,10,12] Superoxide dismutase (sod) converts superoxide to hydrogen peroxide. Two types of sod are Cu/Zn-sod or sod1 and Mn-sod or sod2. Excessively increased reactive oxygen species (ROS) can cause biological damage and trigger oxidative stress. Oxidative stress has been identified as a primary factor in the pathogenesis and pathophysiology of various degenerative diseases such as metabolic syndrome, diabetes, cancer, arthritis, atherosclerosis, coronary heart disease, osteoporosis, obesity, dementia, and stroke.[13]

HMI induces cell death by decreasing mitochondrial membrane potential, releasing cytochrome c (cyt c), reducing Bcl-2, and increasing Bax levels. Bcl-2 is an anti-apoptotic protein, and Bax is a pro-apoptotic protein. Bcl-2 works as an anti-apoptotic protein by binding to Bax. When the bonds of the Bcl-2/Bax complex are separated, Bax undergoes polymerization and forms a transition pore, causing cyt c to exit the cytoplasm and induce apoptosis. High levels of ROS increase peroxisome proliferator-activated receptor gamma coactivator 1α (ppargc1α in humans, ppargc1a in zebrafish), which regulates mitochondrial dynamics through mitochondrial fusion or fission by increasing mfn1.[11,14–16]

This study explores the integration of environmental monitoring with gene-level mitochondrial biomarkers in zebrafish (*Danio rerio*) skeletal muscle mitochondria, focusing on the expression of *cox4i1*, *sod2*, *baxa*, *mfn1b*, and *ppargc1a*, which offers a novel and sensitive approach for the early detection of environmental toxicity before overt pathophysiological changes occur. This study addresses the question: can zebrafish mitochondrial gene expression be an early biomarker for subclinical exposure to heavy metals in river environments?

This study aims to investigate the impact of heavy metals from the upstream and downstream areas of the Citarum river on mitochondrial gene expression of *cox4i1*, *sod2*, *baxa*, *mfn1b*, and *ppargc1a* in the skeletal muscle of zebrafish using in vivo models for biological monitoring of early detection of environmental heavy metal pollution.

Methods

This study utilized zebrafish as experimental animal models. Two areas, Cibeureum and Balekambang, were selected to collect water and sediment samples from the Citarum river (Figure). Cibeureum was chosen for its upstream location near *Situ* Cisanti, the first river source, and is not affected by many residential and industrial activities. Balekambang, on the other hand, was chosen for its downstream location, which has passed through many residential and agricultural areas. The sampling was done in cooperation with the Department of Geophysics of the Faculty of Mathematics and Natural Sciences of Universitas Padjadjaran.

The study also compares mitochondrial gene expression in response to exposure to water and sediments collected from two distinct locations along the Citarum river: Cibeureum, representing the upstream area with minimal industrial influence, and Balekambang, representing a more populated and agriculturally influenced downstream segment. This geographical comparison aims to explore whether environmental context, in addition to pollutant levels, may influence mitochondrial biomarker expression.

Heavy metal examinations were performed using acid destruction, and absorbance was measured using atomic absorption spectroscopy (AAS). These procedures were carried out at *Laboratorium Pusat Survei Geologi* (Geological Survey Central Laboratory), Bandung, West Java, Indonesia.

Seventy zebrafish, 4–6 months old and 380+50

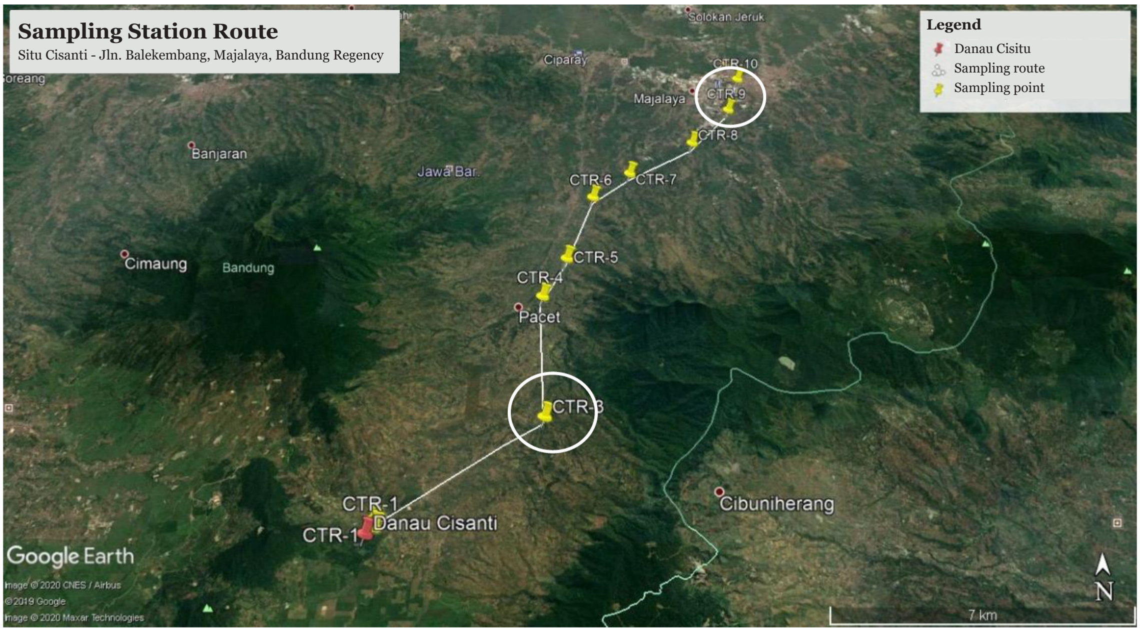

Figure Sampling Site (White Circle)
Note: CTR-3: Cibeureum, Arjasari, West Java; CTR-9: Balekambang, Majalaya, West Java

mg body weight, were selected and maintained in a 22+20°C glass tank compartment system of equal size with a 12-hour light/dark cycle.[17] The animals were fed with the same commercially available fish pellets. The experiment was conducted from December 2019 until March 2020 at the Central Laboratory of Universitas Padjadjaran.

The zebrafish were conditioned for one week for adaptation in a drinking water-filled tank from the Bandung City Drinking Water Company before being randomly divided into five groups for 30 days of experiment exposure as: (1) Group 1: control (drinking water tank), (2) Group 2: tank filled with water from the Cibeureum area of the Citarum river, (3) Group 3: tank filled with water from the Balekambang area of the Citarum River, (4) Group 4: tank filled with drinking water and 800 g sediments from the Cibeureum area of the Citarum river, and (5) Group 5: tank filled with drinking water and 800 gr sediments from area Balekambang of Citarum river.

On day 30 of the experiment, the zebrafish were terminated by the rapid chilling method, which involved submersion the fish in ice water (ice:water=5:1) at a temperature of 1–4°C for 10 minutes after cessation of gill movement.[14,18,19] The muscle tissues were quickly removed, placed in a tube, given an exposure identification label, and stored at −80°C for further use.

The total RNA was extracted from frozen zebrafish muscle using TRIsure reagent (Bioline, United Kingdom) according to the manufacturer's instructions. The concentration and quality of the RNA extraction were analyzed using absorbance spectrophotometry at 260/280 nm (M200 Pro, Tecan, Morrisville, NC). Quantitative PCR was performed using the Ariamx Real-Time PCR (Agilent Technologies) and Meridian Bioscience's MyTaq one-step RT-PCR kit according to the manufacturer's instructions. The GAPDH was used as a housekeeping gene. The primer was designed using a standardized primer design tool. Table 1 shows the sequences of the primers used in this study.

The cycle threshold (Ct) value was calculated from the RT-PCR data, and statistical tests were performed. Data normality was assessed using the Shapiro-Wilk test, and homogeneity of variance was tested using Levene's test. The differences between groups were analyzed using a multivariate one-way ANOVA if the data were homogeneous and normally distributed or a Kruskal-Wallis test if the data were not normally distributed. A post-hoc test was performed using Tukey's and Bonferroni's methods for homogeneous and normally distributed data, as well as pairwise comparisons among groups. Data were analyzed using SPSS V.20.

Table 1 Primer Sequences Used in This Study

Gene	Operational Definition	Product Size (bp)
graph	Forward: CAT CTT TGA CGC TGG TGC TG Reverse: TGG GAG AAT GGT CGC GTA TC	179
cox4i1	Forward: GTT GGT AAA CGG GCC TTG TCC Reverse: AGT CCT CGA CCT TCG CAA CT	146
baxa	Forward: TGC CTG TCG CCT TGT CAT C Reverse: AAT AAC TGC GGA TTC CGT CCC	146
bcl2	Forward: CTG ACT ACC TGA ACG GGC CAC Reverse: AAC GGG TGG AAC ACA GAG TC	106
sod2	Forward: TTC AAC CCC CTG TTA GGT GC Reverse: ATG TTG CAT GGT GCT TGC TG	136
ppargc1a	Forward: CTC GCA ACA TGG ACG AAA GC Reverse: GCT CGT GCT GGT ATT CCT CA	120
mfn1b	Forward: TTC GGT CAG ATT TCC ACC CG Reverse: ACA GCA TCA GAA CAG CGG AA	115

Results

The heavy metal concentrations among the five groups are listed in Table 2. All metals examined had lower levels in samples of river water and water added to Citarum river sediments than the threshold allowed by WHO, except for mercury levels, which were at higher levels. Heavy metal levels in river water tanks are generally higher than in control (drinking water) and water with sediment added.

Heavy metal levels in the Cibeureum river water tank are higher than in the Balekambang tank. The levels of heavy metals in the water added to sediments from Cibeureum were, on average, almost the same as those from Balekambang, except that Cu, Zn, and Hg were higher in water with sediments from Cibeureum.

This study found that *baxa* was higher in almost all samples than the control except for the Balekambang water sample, which was lower (Table 3). The biomarker *mfn1b* was higher in Cibeureum water and 800 g of Cibeureum sediment but lower in Balekambang water and 800 g of Balekambang sediment compared to the control. The level of *prgc1a* was higher than the control in almost all samples. The *sod2* levels were lower than those in the control in

Table 2 Heavy Metal Concentration

HMI	WHO Limit	C	CW	BW	CS	BS
Cu	2	0.007	0.004	0.002	0.011	0.009
Pb	0.01	<0.001	0.004	0.002	<0.001	<0.001
Zn	–	0.1	0.01	0.01	0.15	0.1
Ni	0.07	0.002	0.002	0.001	<0.001	<0.001
Fe	–	0.04	0.03	0.15	0.02	0.02
Cd	0.003	<0.0005	0.0002	0.0003	<0.0005	<0.0005
Cr	0.05	<0.001	0.001	0.001	<0.001	<0.001
Ti	–	<0.01	0.039	0.012	<0.01	<0.01
V	–	–	0.004	<0.001	–	–
As	0.01	0.001	0.002	0.001	<0.001	0.001
Hg	0.006	<0.09	0.09	<0.09	0.09	<0.09

Note: HMI: heavy metal ion, C: control, CW: Cibeureum water, BW: Balekambang water, CS: Cibeureum sediment, BS: Balekambang sediment

Table 3 Gene Expression from RT-PCR

Gene	C	CW	BW	CS	BS
baxa	5.73±1.94	11.39±4.22	2.75±0.999	19.79±5.87	13.93±10.18
mfn1b	26.00±7.099	36.86±7.65	14.54±3.06	35.04±6.48	23.10±3.35
ppargc1a	0.85±0.10[a]	3.55±0.83[b]	2.19±0.81	1.63±0.34	1.17±0.18[a]
sod2	0.86±0.27	0.30±0.16	0.45±0.15	2.11±0.48[a]	0.69±0.22
cox4i1	1.08±0.36	4.12±2.23	0.59±0.35	2.33±0.76	2.99±0.16

Note: [a]compared to sample CW (water of Citarum river from Cibeureum area), [b]compared to control, C: control (drinking water), CW: water of Citarum river from Cibeureum area, BW: water of Citarum river from Balekambang area, CS: drinking water +800 g sediment of Citarum river from Cibeureum area, BS: drinking water +800 g sediment of Citarum river from Balekambang area

nearly all samples, except in the 800 g sample of Cibeureum sediment. The *cox4i1* level was higher than the control in almost all samples except for the Balekambang water samples.

Discussion

Based on the data, all metals examined had lower levels in samples of river water and water added to Citarum river sediments than the threshold allowed by WHO, except for mercury levels, which were at higher levels. Heavy metal levels in river water tanks are generally higher than in control (drinking water) and water with sediment added.

Heavy metal levels in the Cibeureum river water tank are higher than in the Balekambang tank. The levels of heavy metals in the water added to sediments from Cibeureum were, on average, almost the same as those from Balekambang, except that Cu, Zn, and Hg were higher in water with sediments from Cibeureum.

These differences may reflect the contrasting environmental pressures between upstream and downstream areas. Cibeureum, located near the river's source, may contain naturally occurring or agriculture-derived contaminants. In contrast, Balekambang, as a more populated and industrial area, may exert a different pollutant profile or biological stressor environment. Interestingly, despite Cibeureum's status as a less polluted area, biomarker expression levels in zebrafish exposed to Cibeureum water were higher, particularly for *ppargc1a* and *baxa*, suggesting that even upstream environments can carry biologically impactful contaminants.

The results of this study for heavy metals in river water were lower than the WHO threshold, except for the higher Hg level. This variability and the results could be influenced by the rainfall level at the upstream, the hydrological cycle, etc.[20] A previous study found that heavy metal levels exceeded the WHO threshold.[2] An earlier study took samples in November 2016, while this study took samples in December 2019. The sampling time also affects the levels of heavy metals in water or sediment samples.[20,21] The government initiated the Citarum Harum program in 2018 to mitigate pollution and damage to the Citarum river. The concentrations of heavy metals in the control and river water are almost identical, except that Pb is slightly higher.

Generally, the heavy metal levels in the control and tanks with sediment are nearly identical. However, the levels of Ni and Fe were higher in the control water than in the water with sediment. The lower heavy metals in tanks with sediment are due to more than 90% of the heavy metal load in the aquatic system being deposited into the sediment.[22] However, Cu levels were higher in the control water. We have not found a reason why this could happen, as it is the opposite: Cu solubility is low and decreases rapidly when the sediment pH rises above 4.5. The metal solubility for water occurs in the order Ni > Zn > Cd >> Cu >> Cr/Pb.[23]

The levels of heavy metals were almost the same in water with added sediments from the Cibeureum and Balekambang areas, except that Cu and Hg were higher in water with sediments from Cibeureum. Cibeureum is an agricultural and forest area with little housing, while Balekambang is more residential and industrial. Cibeureum is closer to Lake Cisanti as the source of the Citarum river, while Balekambang is farther from Lake Cisanti. The high levels of Hg in the river water in Cibeureum are thought to be due to the use of agricultural products, which carry the Hg into the river through rain.

This study found that *baxa* was higher in almost all samples than the control except for the Balekambang water sample, which was lower. The biomarker *mfn1b* was higher in Cibeureum water and 800 g of Cibeureum sediment but lower in Balekambang water and 800 g of Balekambang sediment compared to the control. The level of *prgc1a* was higher than the control in almost all samples. The *sod2* levels were lower than those in the control in nearly all samples, except in the 800 g sample of Cibeureum sediment. The *cox4i1* level was higher than the control in almost all samples except for the Balekambang water samples.

The difference test showed that all groups had no difference in the *mfn1b* levels ($p>0.05$). At the *ppargc1a* levels, the average values between groups are significantly different ($p<0.05$). Based on Tukey and Bonferroni's post-hoc test, there were significant differences between the variable *ppargc1a* in groups 1 (control) and 2 (Cibeureum water), as well as between groups 2 and 5 (800 g Balekambang sediment added). Meanwhile, the other groups did not show statistically significant differences.

In the variable *sod2* levels, there were significant differences between groups ($p<0.05$), whereas in *baxa* and *cox4i1*, there were no differences ($p>0.05$). Based on the post-hoc test results in the pairwise comparison of group table, it was found that the groups had a difference in the *sod2* variable with a significance value ($p<0.05$), including group 2 (Cibureum water area) and group 6 (800 g Balekambang water with sediments added). There were no differences between the other groups.

There is no difference between control and treatment in the expression of the *baxa*, *mfn1b*, *sod2*, and *cox41* genes. There was a significant difference in *ppargc1a* between Cibeureum water and the control, as well as sediment from Balekambang. There was also a significant difference in *sod2* expression between Cibeureum water and Cibeureum sediments.

The elevated mercury concentration in Cibeureum water, congruent with the result of the genetic examinations, revealed the highest expression levels of *pparcg1a* and the lowest levels of *sod2*. The toxic effects of mercury vary depending on the dose, chemical form, duration, and level of exposure.[24] Mercury induces oxidative stress.[24] Oxidative stress increases *ppargc1a* and mitochondrial antioxidant expression and plays a role in oxidative defense.[25,26] A previous study showed an increase in sod expression as a response to oxidative stress in the liver and skeletal muscle of *Danio rerio*, which gives dietary MeHg. Still, there is no change in the contaminated brain sample, although this organ accumulated the highest Hg concentration.[27]

The previous study demonstrated an increase in sod levels in muscles, an organ identified as a target for mercury accumulation after hair, kidney, and liver in mice.[28] However, this current study reveals inconsistencies, particularly in the case of Cibeureum water samples and Cibeureum sediment-containing water samples, which exhibit an identical mercury concentration of 0.009 ppm. Surprisingly, the results for *sod2* and *ppar1gc* diverge. The sod level in the Cibeureum water sample is the lowest, while in the sediment-containing Cibeureum water sample, it is the highest. Conversely, the *ppar1gc* level is highest in the Cibeureum water sample, whereas it is lower in the water with Cibeureum sediment.

Although some studies indicate an increase in *ppargc1* and sod expression due to mercury exposure, we hypothesize that other factors in the samples may influence these mitochondrial transcription and antioxidant factors, warranting further study. Measurement of the heavy metal concentration in zebrafish could improve the validity of the study in the future by measuring the accumulation effect compared to the water's heavy metal concentration and the mitochondrial gene expression simultaneously.

Although the heavy metal concentrations, except for mercury, remained below WHO standards, this study reveals altered expression in several mitochondrial genes, indicating potential subclinical effects. This suggests that molecular changes precede clinical or ecological consequences, and zebrafish mitochondrial biomarkers may serve as early warning indicators, even in low-concentration exposure. Unlike most previous studies that focused on liver or brain gene expression, this study demonstrates that muscle tissue provides comparable, if not more sensitive, biomarker expression for environmental exposure detection.

Conclusions

The levels of heavy metals in Citarum water and drinking water with added sediment from Citarum were generally below the normal limits of WHO standards for upstream and downstream areas. Still, the mercury levels were above the WHO's normal levels. There are differences in the levels of *pparc1gc* between the water from Cibeureum and the control and water-added Balekambang sediments. There are differences in the *sod2* gene between the control and water samples with sediment from Balekambang. The expression of biomarkers *pparc1gc* and *sod2* could serve as a proposed model for an in vivo biological model to address the early detection of the biological effects of heavy metal-contaminated community water sources in different areas alongside the river stream, even before clinical and pathophysiological changes.

Conflict of Interest

There is no conflict of interest in this study or this manuscript.

Acknowledgment

We are grateful to the staff of the Central Laboratory at Universitas Padjajaran for their assistance with this study.

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