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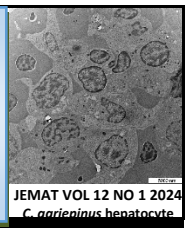

Physical and Biochemical Toxicity Assessment of Acute Lead Nitrate ($Pb(NO_3)_2$) Exposure on Liver Tissues of *Clarias gariepinus*

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ABSTRACT

Lead pollution is a major global concern due to its harmful effects on the environment and human health, particularly within the aquaculture industry. This study examines the impact of lead exposure on *Clarias gariepinus*, a commercially significant catfish species used as a biomarker for environmental pollution. Lead exposure can disrupt enzyme activity, causing hematological, gastrointestinal, and neurological issues in both humans and animals. The research involved exposing *C. gariepinus* to varying concentrations of lead nitrate ($Pb(NO_3)_2$) and assessing behavioral, morphological, and physiological changes. This study revealed dose-dependent impairments in swimming, breathing, and feeding behaviors, highlighting the effect of lead exposure. Histological analysis of liver tissues showed cellular stress and damage, including vacuolization and mitochondrial irregularities. Additionally, the study measured cholinesterase (ChE) activity to assess the biochemical impact on liver function, noting a significant decrease of up to 40% in butyrylcholinesterase (BChE) activity with increased lead exposure. This supports the use of ChE activity as a potential biomarker for environmental pollution. Employing sentinel species like *C. gariepinus* in environmental risk assessments is crucial.

INTRODUCTION

Lead pollution is an increasingly pressing global concern due to its detrimental effects on both environmental and human health. As environmental pollution continues to increase, the adverse consequences of lead exposure have cast a shadow over essential activities like aquaculture, particularly in regions like Sabah, Malaysia [1]. Lead exposure can lead to hematological dysfunction, gastrointestinal issues, and neurological disturbances in both humans and animals. Furthermore, lead can interfere with enzyme activity, disrupt cellular calcium metabolism, and hinder nerve conduction. The hepatotoxicity of lead is particularly concerning, as it can cause damage to critical liver tissues, resulting in cellular stress, oxidative damage, and mitochondrial irregularities. This damage can lead to various liver disorders, including impaired detoxification, altered enzyme activity, liver fibrosis, and potentially contribute to the development of conditions such as fatty liver disease, cirrhosis, and liver cancer. In addition, lead exposure has been found to have genotoxic effects on the brain, bone marrow, and lung cells. These findings highlight the need for monitoring and addressing lead pollution to protect the health of both human populations and aquatic ecosystems. Fish represent a vital source of dietary protein and are recognized as efficient bio-accumulators of heavy metals [2]. Understanding the effects of these pollutants on fish populations is crucial, particularly considering increasing concerns about lead contamination in human liver and hair tissues.

Particularly, lead nitrate limits in the environment are regulated under various international and national guidelines to protect human health and environmental integrity. In the United States, the Environmental Protection Agency (EPA) enforces the Safe Drinking Water Act, setting an action level of 15 ppb for lead at customer taps, with a goal of reducing this concentration to zero, as lead is known to be harmful with no safe level of exposure [3]. Similarly, the European Union regulates lead concentrations in drinking water to a stricter standard of 10 µg/L, in accordance with the Drinking Water Directive (Council Directive 98/83/EC), which is under review for further reduction. The World Health Organization (WHO) also recommends a provisional guideline value of 10 µg/L for lead in drinking water, highlighting the global consensus on the need for stringent control of lead levels [4]. These regulations

are particularly significant for compounds like lead nitrate, a soluble form of lead that can contribute significantly to total lead exposure when present in water bodies. Compliance with these standards is critical to prevent the toxicological impacts associated with lead. Fish is a good biomarker research subject for heavy metals pollution monitoring because it can easily accumulate pollutants and also contains organs (gills, liver, blood, muscle and brain) with high sensitivity towards the presence of xenobiotics [5].

It also has a high sensitivity towards temperature changes, natural surroundings, water quality deterioration and aquatic contamination antagonistically that influences the fish health as well as mortalities and ecosystem degradation. Fish biomarkers including the assessment of biochemical, cellular and physiological alteration were utilized for monitoring the biological effect of toxicants especially metal exposure [6]. In this present study, *Clarias gariepinus*, a catfish known to have high commercial value, will be used as a biomarker to study the effect of heavy metals through its behaviour, morphology and physical changes which are very useful for environmental contamination monitoring. *C. gariepinus* is one of the most widely consumed freshwater fish in Malaysia and often known due to their hardiness.

However, contamination of freshwater may cause the detrimental effect on this species as it affects their physical, behavioural and morphology conditions. A few studies have been conducted to observe the effect of heavy metals (lead, copper, cadmium, zinc) on *C. gariepinus* [7–9]. In this study, *C. gariepinus*, commonly known as catfish and respected for its commercial value, will serve as a biomarker to analyze the consequences of heavy metal exposure on fish through assessments of behavioral changes, morphological alterations, and physical changes. This investigation holds immense promise for the field of environmental contamination monitoring.

Cholinesterase (ChE) is an important enzyme belongs to the serine hydrolases group, which catalyses the hydrolytic cleavage of acyl group in various esters of choline. It is a family of enzyme that comprises acetylcholinesterase (AChE; EC 3.1.1.7), butyrylcholinesterase (BChE; EC 3.1.1.8) and propionylcholinesterase (PChE; EC 3.1.1.8) classified based on their preference for specific substrates. Acetylcholinesterase (AChE) has a high preference for acetylcholine; Butyrylcholinesterase (BChE) is better in degrading butyrylcholine, while Propionylcholinesterase prefers propionylcholine [10,11]. Cholinesterase present in both vertebrates and invertebrates. Vertebrates contain acetylcholine neurotransmitter in their neuromuscular junctions and synapses [12].

There are two types of gene exist in vertebrates, which are AChE and BChE [13,14]. The use of extracted cholinesterase from aquatic organisms as biomarker for the detection of anti-cholinesterase effect has been developed based on the alteration in a biological response (molecular, cellular and physiological alterations to behavioural changes) due to the exposure of contaminants in aquatic system [15]. This is because ChE is the crucial enzyme that abundantly presents in brain, skeletal muscles, and erythrocyte membrane and catalyze the hydrolysis of acetylcholine to choline and acetic acid.

Cholinesterase is present throughout the body, particularly at the neural junction where the nerve fibres are terminated. It is important in signal termination at cholinergic synapses by rapid hydrolysis of neurons acetylcholine in the brain [16]. ChE may also serve as a detoxifier in the liver as it performs an important role in biosynthesis [17]. BChE belongs to the esterase family of enzymes, which is slightly different than AChE. BChE is basically used to hydrolyze butyrylcholine (BCh) substrate. However, the significant function of BChE was not well characterized compared to that of AChE [18].

BChE contains peripheral anionic site, omega loop, choline binding site, oxyanion hole, catalytic triad of esteratic site, and acyl binding site [19]. BChE is also found in plasma, and it's involved in the regulation of cells proliferation. It also exists in cholinergic synapses with the ability to hydrolyze AChE. Furthermore, Propionylcholinesterase (PrChE) is a nonspecific cholinesterase, which is known as pseudocholinesterase like BChE. Its function could be similar as BChE where it can substitute AChE being depleted in their ability to hydrolyze AChE [20]. The important role of metal ions includes about one-third of the enzymes which benefits the enzyme-substrate complexes by modifying the electron flow in substrate or enzyme and effectively controlling the enzyme-catalyzed reaction.

The VOS Viewer visualization generated from the Scopus document search using keywords such as "Lead toxicity," "fish," "acetylcholinesterase," and "biomarker" spanning from 2003 to 2024 offers a rich visual representation of the research area in this interdisciplinary field (**Fig. 1**). Through bibliometric analysis, the VOS Viewer unveils clusters of interconnected keywords, elucidating prevalent studies within the literature. "Lead toxicity" emerges as a central keyword, underscoring its significance as a primary research focus. Surrounding keywords such as "fish," "acetylcholinesterase," and "biomarker" indicate crucial components of the studies.

Moreover, the visualization identifies influential authors and collaborative networks, indicating prominent figures and research groups focusing the understanding of lead toxicity in fish. By analyzing co-authorship patterns and keyword clusters, researchers gain valuable insights into collaborative trends and the dissemination of knowledge within the field. Furthermore, the temporal analysis provided by the VOS Viewer enables researchers to track the evolution of research trends over time, identifying emerging topics in focus.

This comprehensive overview facilitates a deeper understanding of the complex interactions between lead toxicity, fish physiology, and biomarker responses, ultimately contributing to the advancement of environmental science and toxicology. As research in this area continues to evolve, the VOS Viewer remains an indispensable resource for navigating the complex landscape of lead toxicity research in fish, driving innovation and fostering sustainable solutions for mitigating environmental pollutants' adverse effects on aquatic ecosystems [21]. The Scopus analysis as illustrated in **Fig. 2** conducted on the keywords "Lead AND toxicity AND fish AND acetylcholinesterase AND biomarker" reveals a comprehensive landscape of scholarly literature focused on understanding the impact of lead toxicity on fish physiology, particularly emphasizing acetylcholinesterase (AChE) as a biomarker.

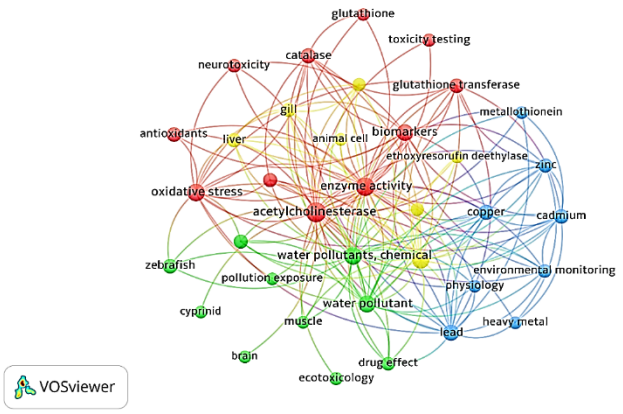


Fig. 1. Network visualization of research keywords related to the effects of lead toxicity on fish, focusing on acetylcholinesterase as a biomarker. This bibliometric analysis includes studies from Scopus using the search keywords: "Lead AND toxicity AND fish AND acetylcholinesterase AND biomarker".

Among the retrieved documents, 35 are from the field of environmental science, highlighting the significance of this research area within the broader scientific community. The exploration extends across interdisciplinary boundaries, drawing insights from environmental science, toxicology, fisheries biology, and biomonitoring deepen our understanding of the mechanisms underlying lead toxicity in fish and to create innovative strategies for environmental monitoring and conservation.

The abundance of research findings underscores the urgency and importance of continued investigation into the effects of lead pollution on aquatic ecosystems. By synthesizing scholarly literature, researchers can identify gaps in knowledge, propose hypotheses for further exploration, and contribute to the development of effective mitigation strategies aimed at safeguarding aquatic environments from the detrimental effects of lead contamination. This synthesis of research findings not only informs academic discourse but also serves as a crucial foundation for evidence-based policymaking and environmental management practices, ultimately fostering the preservation and sustainability of aquatic ecosystems for future generations.

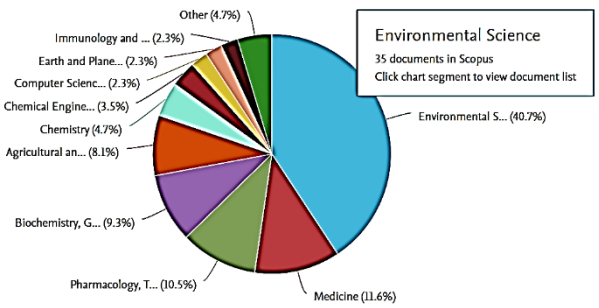


Fig. 2. Distribution of research documents across various academic disciplines related to the study of lead toxicity in fish, highlighting the interdisciplinary approach to this environmental issue. The pie chart, derived from a Scopus search analysis. This visualization underscores the diverse scientific perspectives contributing to the understanding of lead toxicity impacts.

MATERIALS AND METHODS

Ethical Statement

Before the commencement of this study, approval was granted by the Universiti Putra Malaysia Institutional Animal Care and Use Committee with the approval number UPM/IACUC/AUP-R033/2017

Fish Acquisition and Acclimatisation

Healthy *Clarias gariepinus (C. gariepinus)* specimens, weighing 100 ± 20 g and measuring 20 ± 2 cm in length, were procured from a commercial supplier in Semenyih, Selangor. They were carefully transported to the laboratory in a well-aerated container to prevent physical injury and minimize stress. Upon arrival, the fish were placed in a glass aquarium containing 40 L of dechlorinated water and allowed to acclimatize for 10 days. During this acclimation period, the fish were fed commercial fish pellets to maintain their health and vitality.

Chemical Exposure

The treatment for acute, non-renewal toxicity testing was conducted using *C. gariepinus,* weighing between 200–300 g, obtained from the Department of Fisheries, Semenyih. The fish were transported to the Bioremediation, Biomonitoring and Ecotoxicology Laboratory (BBE) at Universiti Putra Malaysia and acclimatized for approximately 10 days before treatment. The water supply used was 60 L of chlorine-free tap water, filtered with a top filter pump (Ki–Ki, China). Anti-chlorine was added to minimize chlorine exposure to the fish.

The temperature and pH of the water were maintained between 27 and 30 °C and pH 6–7, respectively, to replicate natural conditions. The fish were kept in the dark to mimic their natural habitat, and the water was renewed twice a week to ensure cleanliness and hygiene. The treatment was conducted under controlled conditions for 96 hours to generate LC_{50} data. The fish were grouped into six animals per aquarium. The treatment involved exposing the fish to lead nitrate ($Pb(NO_3)_2$) in the aquarium at various concentrations: 0, 5, 10, 20, and 30 mg/L , with triplicates for each concentration. Behavioural changes were monitored throughout the treatment period. A semi-quantitative analysis was performed by observing several changes in the fish over the prolonged exposure period.

Sample preparation for Transmission Electron Microscope (TEM) microscopy

The liver sample was fixed with 4% glutaraldehyde for 24 h. It was then centrifuged at 3000 ×g for a 5 min. The liver was subsequently cut into 1 mm³ pieces prior to washing with 0.1 M sodium cacodylate buffer three times for 10 min each. The liver was then post-fixed with 1% osmium tetroxide for 2 h at 4°C. Following this, the sample was washed again. Dehydration was achieved using a series of acetone concentrations of 35, 50, 75, and 90%, each for 10 min. The liver was then immersed in absolute acetone concentration three times for 10 min each. The dehydrated liver was infiltrated with a mixture of acetone and resin with ratios of 1:1 for 1 h and 1:3 for 2 h. 100% resin was used to infiltrate the liver overnight. The liver was then embedded onto the beam capsule along with the resin. Polymerization took place for 24–48 hours at 60°C.

The liver sample was sectioned by following the ultrathin sectioning process. The selected area of the sectioned liver was cut and dried. The liver was placed on a glass slide and stained with uranyl acetate for 15 min. The slide was washed with distilled water two times prior to staining with lead stain for 10 min. The prepared sample of treated and untreated liver was viewed under TEM, and the morphology was observed.

Preparation of Crude Homogenate

C. gariepinus specimens were freeze-killed and their liver were extracted and weighed. The liver tissues were homogenized in 0.1 M sodium phosphate buffer (pH 7) containing 2 mM phenylmethylsulfonyl fluoride (PMSF). The homogenization process was carried out using a homogenizer (Ultra-Turrax T25, Germany). The resulting homogenate was then centrifuged at 10,000 xg in a high-speed centrifuge (Beckman Coulter, Germany) for 20 min at 4°C. The supernatant was collected and stored at -25°C for further analysis.

Cholinesterase (ChE) Activity Determination

ChE activity in *C. gariepinus* was assessed using a modified method derived from Ellman et al. [22]. In this assay, 10 µL of the crude sample was added to a 96-well microplate containing 200 µL of 0.1 M sodium phosphate buffer (pH 7.0) and 20 µL of 5,5'-dithiobis(2-nitrobenzoic acid) (0.1 M).

The mixture was incubated for 15 minutes before adding 20 µL of substrates (5.0 mM acetylthiocholine iodide, butyrylthiocholine iodide, and propionylthiocholine iodide). After an additional 10-minute incubation, the ChE activity was determined based on the substrate (mM) hydrolyzed per minute (U), with the specific activity expressed as µmol min^{-1} mg^{-1} of protein or U mg^{-1} of protein. Protein content was determined using the Bradford method, with bovine serum albumin as the standard [23].

RESULTS AND DSICUSSION

Behavioral response of *C. gariepinus* affected by $Pb(NO_3)_2$

Fish behaviour reflects the physiological response of fish to environmental factors [24]. Behavioural alterations are considered sensitive biomarkers for evaluating the toxic exposure and effects on fish health [25]. Parameters such as swimming performance, avoidance behaviour, and feed intake were studied during a 96-h exposure to lead nitrate ($Pb(NO_3)_2$). **Table 1** presents an observation of the behavioral responses of *C. gariepinus* to varying concentrations of lead (Pb) over a 96-h exposure period.

The physical behaviors showed by *C. gariepinus* indicate the effect of increasing in exposure dosage on physiological and cognitive functions (**Table 1**). Lower concentrations of ($Pb(NO_3)_2$) subtly affect swimming and feeding behaviours, while higher concentration leads to significant impairments in breathing, movement, and overall vitality. These changes, reflecting neurotoxic impacts, highlight the critical risk that leads to aquatic life, affecting their ability to forage, escape predators, and survive in contaminated environments. Such findings underscore the need for stringent environmental controls to mitigate lead pollution [26]. The detailed explanation of the selected observed criteria are as follows:

- **Swimming Performance:** The analysis indicates a dose-dependent decline in swimming performance as lead concentration increases. At the lowest concentration (5 mg/L), fish exhibit only slight deviations from normal behavior, which progressively worsen at higher concentrations (20 mg/L and 30 mg/L), culminating in significant lethargy and reduced navigational efficacy at the highest tested concentration. Similarly, contaminants such as aluminium are found to significantly impair cognitive functions in fish, such as spatial memory and learning ability [27]. This impairment affects fish's capability to navigate their surroundings, which is crucial for escaping predators and foraging.

- **Breathing Difficulty: Table** 1 highlights a direct correlation between lead concentration and breathing difficulty, marked by increased opercula and mouth movements. These symptoms suggest that the fish are struggling to obtain enough oxygen, a condition that intensifies with increasing lead levels. At 30 mg/L, fish experience severe respiratory distress, often surfacing more frequently to access air. The finding can be compared with the study conducted by Mahi et al. [28], where significant behavioral changes related to breathing style was prominently observed in lead-treated groups of *Oreochromis niloticus*.

- **Feeding Behavior:** Lead exposure adversely affects feeding behavior, with a noticeable decrease in feed intake as lead levels rise. This is due to the neurotoxic effects, which impair the neurological functions governing hunger and foraging behavior. Exposure to pollutants has related with the increase of boldness and altered foraging patterns in fish species like perch. Such changes lead to increased consumption of contaminated prey, raising their pollutant load and potentially impacting their health and survival rates [26,29].

- **General Behavior:** Lower concentrations of lead (5 mg/L) result in slight avoidance behaviors, whereas higher concentrations lead to more pronounced changes. At 10 mg/L, fish display increased avoidance behavior, including swimming to the surface, possibly to escape higher concentrations of lead accumulated in lower water strata. By 30 mg/L, behavioral responses show significant deterioration, with fish becoming mostly motionless or sinking to the bottom, indicating severe neurotoxic effects and muscular impairment [30].

Table 1. An observation of the behavioral responses of *Clarias gariepinus* to varying concentrations of lead (Pb) over a 96-h exposure period.

[Pb] (mg/L)	Swimming Performance	Breathing Difficulty	Feeding Behavior	General Behavior
Control	Normal	No difficulty	Normal intake	Normal activity
5	Slight decrease	Increased opercula and mouth movement	Slight decrease	Slight avoidance behavior
10	Moderate decrease	Increased opercula and mouth movement	Slight decrease	Slight avoidance behavior
20	Moderate decrease	More pronounced difficulty in breathing	Reduced intake	Increased avoidance, some may swim to surface
30	Significantly decrease and swim upwards	Severe difficulty, frequent surface	Significantly reduced	Mostly motionless, some sink to bottom

swimming

This observation coincides with that of Meng et al [31] who mentioned that the nutritional status of fish is influenced by the accumulation of copper in the primary organs including liver and kidney, which are the sites for the excretory system. ($Pb(NO_3)_2$) suppresses fish appetite by elevating the glucose level in the blood, which will trick its body system to abnormally receive more calorie intake and eventually utilise it into liver glycogen. In the present study, the fish excretion was also increased when subjected to prolonged exposure. Mortality was recorded at 20 mg/L of Pb exposure was recorded as the highest concentration tested. Comparably, in certain study, the initial response of *Pangasius hypophthalmus* was observed at 40 mg/L after 48 h to the Pb exposure [32]. According to Jagadeshwarlu et al [33], the rate of fish mortality is related to the ($Pb(NO_3)_2$) retention time in water. Thus, the higher the retention time, the lower survival rate of the fish.

Histological alteration of *C. gariepinus* liver tissue affected by $Pb(NO_3)_2$

Histological alteration is aimed at understanding the sub-lethal and lethal effects of environmental pollutants, with a focus on how these stressors affect fish health, behavior, and physiology. The transmission electron microscopy (TEM) micrograph displays a detailed view of healthy liver cells from *C. gariepinus* (**Fig.** 3A). The image shows the endoplasmic reticulum and possibly Golgi apparatuses, indicating active protein synthesis. Notably, the nuclei are prominent, with clearly defined nuclear envelopes and heterochromatin, suggesting healthy cellular function.

The overall morphology and lack of disrupted organelles signify a well-preserved, functioning liver cell environment. TEM image in **Fig**. **3B** shows liver cells from *C. gariepinus* with noticeable differences compared to the first image, likely indicating the effects of lead exposure at a concentration of 30 mg/L. This micrograph exhibits irregularities such as unclear mitochondrial structures, potential vacuolations, and altered cellular morphology which are not as distinct and organized as in the first image. These signs suggest cellular stress or damage, which is typical of lead toxicity effects in liver cells. In contrast, the first image displayed a healthier state with well-preserved cellular structures, clear organelles, and overall normal hepatocyte appearance.

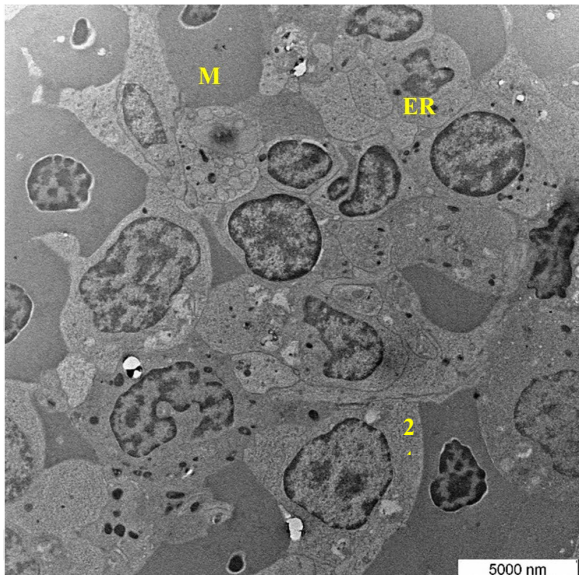
Nuclear destruction (4) as depicted in **Fig. 5B** was normally due to the elevation of relative oxygen species (ROS) production due to the declining defensive mechanism required to scavenge ROS [19]. The ROS induced by recalcitrants such as transitional metal ions, pesticides and petroleum sludge and in this case Pb have caused an imbalance between the production of ROS and antioxidant defences in the living organism, which results in oxidative stress. The induction of ROS by redox-active metals including Pb occurs through the redox cycling process, thus generating the third mechanism of free radicals production known as the Fenton reaction, a reaction that involves the reduction of Pb into their complexes [34,35].

In this case, the induction of ROS by Pb would have more toxic effects on the fish as it has been supported by the study of [35], which also proposes that ($Pb(NO_3)_2$) can increase the cellular ROS generation. Moreover, ROS production and reaction are considered responsible for the decrease in permeability of the nuclear envelope and the loss of the main barrier function, which leads to the destruction of the membrane bilayer. This is the main reason contributing to the occurrences cell necrosis.

The elevation of vacuolation upon. ($Pb(NO_3)_2$) exposure acts as a defence mechanism as it is an isolated noxious agent that enters the hepatic system, exporting them to be scavenged through the autophagy process. These alterations suggest significant cellular stress and potential toxicity effects, highlighting the impact of heavy metal exposure on liver tissue. These effects are consistent with findings from [6] who reported similar histopathological changes in liver tissues of fish exposed to heavy metals, such as vacuolar degeneration and necrosis of hepatocyte nuclei. Padrilah et al. [36] also observed significant cellular alterations in *C. gariepinus* liver cells exposed to lead, supporting these observations.

Table 2 summarised various studies that use of different organs of *C. gariepinus* as biomarkers for detecting heavy metal contamination in aquatic environments. These studies collectively underscore the critical role of fish organs in environmental monitoring and pollution assessment. For instance, the research by Ogamba et al. [37] highlights the bioaccumulation of heavy metals in the gills and liver of *C. gariepinus*, demonstrating these organs' sensitivity to environmental contaminants. Similarly, Abowei and Ogamba [38] examined the histological changes in the liver and gills of fish due to water pollution, emphasizing the detrimental effects of contaminants on these vital organs. Additionally, the study by Izah et al. [39] investigated the bioaccumulation of chromium, lead, and cadmium in the bones and tissues of *C. camerunensis* and *O. niloticus*, further demonstrating the widespread impact of pollutants on different anatomical parts. These studies collectively validate the use of various fish organs as reliable biomarkers for detecting heavy metal contamination, highlighting the consistency of findings across different regions and reinforcing the importance of these indicators in environmental and public health assessments [40,41].

Recent research continues to highlight the critical impacts of heavy metals on the liver of fish, a vital organ often used to assess environmental toxicity [42]. Studies show that chronic exposure to metals like arsenic can significantly impair liver function, leading to disruptions in metabolic activities and structural damage within the liver tissues of fish [43]. The liver's role in detoxification makes it particularly sensitive to contaminants like arsenic, cadmium, and mercury [44]. These metals can induce oxidative stress in liver cells, disrupting normal cellular processes and causing inflammation and necrosis. Lead accumulation in the bodies of animals, including fish like *C. gariepinus*, can have detrimental physical and biochemical effects on their liver. These effects can be observed both in vivo and in vitro, providing valuable insights into the toxicity of lead.



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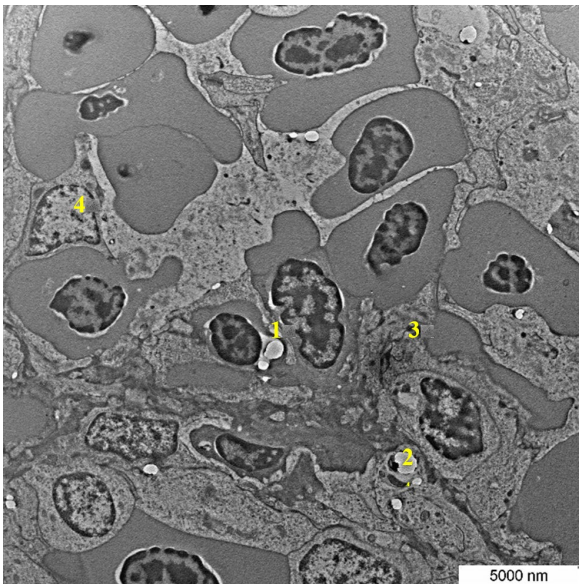

B

Fig. 3. Comparative analysis of *C. gariepinus* hepatocyte under heavy metal stress. **A)** TEM micrograph of a healthy *C. gariepinus* liver cell, displaying well-preserved cellular structures with distinct mitochondria (M), clear nuclear membranes (N), and intact endoplasmic reticulum (ER). This image serves as a control to demonstrate the normal ultrastructure of liver cells. **B)** TEM micrograph of *C. gariepinus* liver cells after exposure to lead. Formation of lipidosis during the exposure 30 mg/L (1), vesicles formation (2), necrotic area (3), and nuclear karryohexis (4) were observed on affected liver section under TEM.

Table 1. Summary of various studies the effect different organs of *C. gariepinus* as biomarkers for detecting heavy metal contamination in aquatic environments.

Heavy metals	Targeted Organ	Effect	Reference
Lead Copper	Gill, Liver	Liver necrosis Lamella degeneration	[37]
Chromium	Liver, Gill	Liver necrosis Lamella degeneration	[38]
Chromium Lead	Muscle	Muscle degeneration	[39]
Arsenic	Kidney Brain Liver	Intracellular oedema Necrosis Hepatic degeneration	[45–47]
Copper	Liver	Liver necrosis Discoloration, pyknosis	[36]
Copper	Gills	Lamella fusion Aneurysm on primary lamella	[48]

Enzyme inhibition study

Enzyme-based biomarker was chosen as a preferable estimation tool for toxicant existence as it provides rapid determination by which it is detectable even in low toxicant concentration [49]. **Fig. 4** presents the activity of cholinesterase enzymes extracted from untreated fish liver, quantified against three distinct substrates: acetylthiocholine (ATC), butyrylthiocholine (BTC), and propionylthiocholine (PTC). Enzyme activity is measured in units (U), where one unit is defined as the amount of enzyme that catalyses the conversion of one micromole of substrate per minute under specified conditions. In this analysis, BTC shows the highest enzyme activity with 98 U, indicating a strong affinity or effectiveness of cholinesterase towards this substrate, followed by PTC with 29 U and ATC with 21 U where the BChE activity was expressed as the quantity of substrate 200 µM hydrolysed by ChE per minute (U) with the extinction coefficient of 13.6 mM^{-1} cm^{-1} (**Fig. 4**). The significant variance in enzymatic activity among the substrates. This indicates a strong affinity of cholinesterase for BTC, suggesting higher efficacy or affinity towards this substrate compared to the others [49].

This selectivity in enzymatic activity highlights the substrate specificity of cholinesterase, which is crucial for further biochemical investigations and potential therapeutic developments. For instance, Sabullah et al. [51] observed decreased cholinesterase activity in fish liver when exposed to metal ions, with a more significant impact on acetylcholine than butyrylcholine. This suggests that environmental stressors can cause substrate-specific enzyme inhibition, altering enzyme dynamics. [50]. This differential activity profile is essential for elucidating the substrate specificity of cholinesterase, facilitating further biochemical investigations and the development of potential therapeutic interventions that target enzyme modulation.

Comparatively, similar studies have analyzed cholinesterase activity from fish exposed to environmental stressors. These findings underscore the sensitivity of cholinesterase activity to external chemical influences and provide a crucial comparison for understanding how environmental factors might alter enzyme dynamics. Such comparative analyses are vital for biochemical investigations and the development of potential therapeutic interventions that target enzyme modulation. Based on crude profile in **Fig. 4**, ChE extracted from *C. gariepinus* liver was seen dominant with BChE. Hence, the activity determination was proceeded using BTC as substrate.

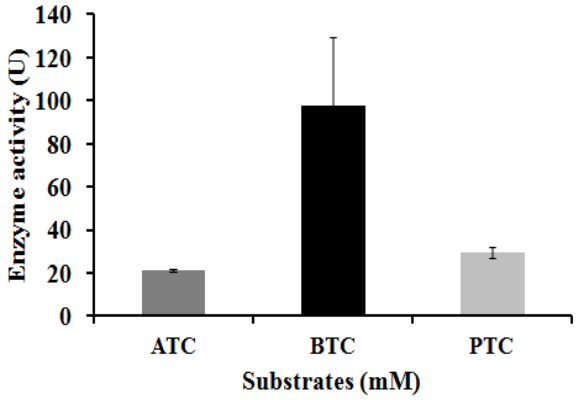

Fig. 4. The enzyme activity of cholinesterase (ChE) extracted from *C. gariepinus* liver in units (U) against three different substrates: acetylthiocholine (ATC), butyrylthiocholine (BTC), and propionylthiocholine (PTC**).**

Fig. 5 illustrates the *in vitro* effects of lead nitrate ($Pb(NO_3)_2$) on butyrylcholinesterase (BChE) activity extracted from the liver of *C. gariepinus*, show a significant decrement in enzyme activity with increasing concentrations of the metal. Activity levels are expressed as a percentage of the control condition, where 0 mg/L of lead represents 100% enzymatic activity, serving as the baseline. As depicted, exposure to 5 mg/L of lead nitrate results in a slight reduction in BChE activity and as the concentration increases to 10 and 20 mg/L, there is a noticeable progressive decrease in activity, indicating a dose-dependent inhibitory effect. The most substantial reduction is observed at 30 mg/L, where the enzyme activity drops significantly until it reaches less than 50% of activity compared to baseline, shows the significant impact of higher concentrations of lead on BChE efficiency.

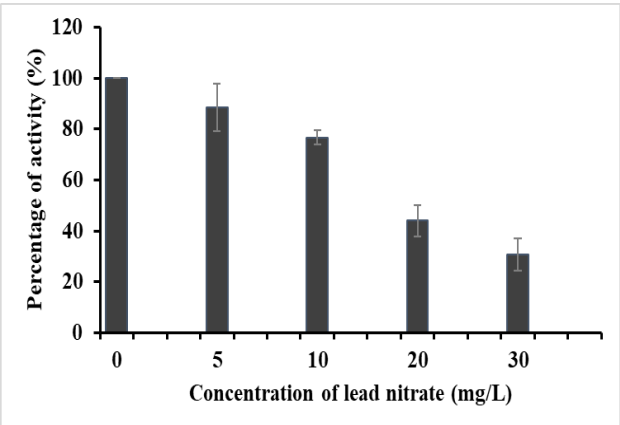

Fig. 5. Percentage inhibition of butyrylcholinesterase (BChE) activity in *Clarias gariepinus* liver exposed to lead nitrate ($Pb(NO_3)_2$).

Previous research has shown that pesticides like carbamate and organophosphate that are also known as nerve agents, inhibit the AChE activity through the catalytic steps involved in carbamylation and phosphorylation at the active site of AChE [19]. In contrast, metal ions bind to amino acid side chains, which include the tryptophan (ring nitrogen), carbonyl groups, cysteine (thiol), serine, methionine (thioether), asparagine threonine, tyrosine (hydroxyl groups) and glutamine, and if these amino acids are involved in the catalytic activity, the enzyme is rendered inactive [6]. This is because the protein groups containing histidine residues are most susceptible to the metal binding as the imidazole group of histidine allows the strongest cation-$\pi\pi$ attraction which can interact with nitrogenous cations of substrates or free metal ions [50]. The inhibition of AChE by free metal ions might be the result of the attraction of the negative charge of amino acid side chains comprised carboxyl groups such as glutamate and aspartate that exist at the catalytic triad of AChE which causes the structural change of the active site.

Similar scientific evidence supports these findings. For instance, Pariza et al. [52] that cadmium exposure in *C. gariepinus* showed significant inhibition of cholinesterase activity in both the brain and blood. This inhibition was dose-dependent, with higher concentrations of cadmium leading to greater reductions in enzyme activity, mirroring the pattern observed with lead nitrate in this study. Similarly, research by [53] on chronic cadmium toxicity in *C. gariepinus* liver tissue showed that prolonged exposure to the metal significantly decreased cholinesterase activity, reinforcing the enzyme's sensitivity to heavy metal contamination.. These studies collectively highlight the vulnerability of cholinesterase to heavy metal exposure and underscore the enzyme's potential as a biomarker for environmental pollution monitoring.

CONCLUSION

This study highlights the significant impact of lead pollution on environmental and human health, using *Clarias gariepinus* as a biomarker to assess the effects of heavy metal exposure. Behavioral observations revealed dose-dependent impairments in swimming, breathing, and feeding behaviors, demonstrating the neurotoxic effects of lead nitrate ($Pb(NO_3)_2$) on aquatic life. Histological analysis confirmed severe cellular stress and damage in liver tissues, including vacuolization and mitochondrial irregularities, which indicate oxidative stress and disrupted cellular processes. Biochemical assessments showed a marked decrease in butyrylcholinesterase (BChE) activity with increasing lead concentrations, supporting the use of enzyme as a sensitive biomarker for environmental pollution monitoring. The study enhances the use of butyrylcholinesterase (BChE) activity as a sensitive biomarker for environmental pollution, reinforcing its applicability in real-world monitoring scenarios. By proposing the integration of bioremediation techniques and innovative technologies like nanotechnology and biosensors for pollution management, this research not only contributes to understanding the mechanisms of lead toxicity but also paves the way for developing more effective strategies for mitigating heavy metal contamination in aquatic environments. These novel insights underscore the findings' contribution to environmental risk assessment and the protection of aquatic ecosystems and public health.

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