NEUROTOXICITY(ACETYCHOLINESTERASE (AChE) N-(l, 3 ,1 DIMETHYLBUTYL 1)-N- PHENY-L-P- FROM LONG-TERM EXPOSURE TO –L-P-PHENYLENEDIAMINE QUINUNE (6PPD-Q)

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OCTOBER 2025

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NEUROTOXICITY(ACH) AND BRAIN HISTOLOGY FROM LONG-TERM EXPOSURE TO 6PPDQ

BY

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A RESEARCH PROJECT SUBMITTED TO THE DEPARTMENT OF ANIMAL AND ENVIRONMENTAL BIOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY

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CERTIFICATION

This is to certify that this project was carried out by MICHAEL IZEKHAOBE ISEI of the Department of Animal and Environmental Biology, Faculty of Life Sciences, University of Benin, Benin City.

DR.N.O ERHUNMWENSE ___ DATE

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DEDICATION

This projected is dedicated to my heavenly Father for the strength, wisdom and ability to successfully put together this project. I also dedicate this work to my wonderful mother for her love and the support.

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ACKOWLEDGEMENTS

I am deeply grateful to God Almighty for His grace, guidance, and protection throughout the course of my studies and the successful completion of this research project. I sincerely appreciate my family for their continuous love, support, and encouragement which have been my source of strength.

My heartfelt appreciation goes to the Department of Animal and Environmental Biology, Faculty of Life Sciences, University of Benin, for providing me with the opportunity and the academic environment to acquire knowledge and pursue this research work.I wish to express profound gratitude to the Head of Department, Prof. E. Tongo, for his leadership and commitment to academic excellence. My special thanks and deep appreciation go to my project supervisor, Dr. N. O. Erhunmwunse, for his mentorship, patience, constructive criticism, and invaluable guidance from the beginning to the completion of this research work. His scholarly input has been instrumental in the success of this project.

To everyone who contributed in one way or the other to the success of this work, I say thank you.

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ABSTRACT

This study evaluated the neurotoxic effects of long-term exposure to N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine quinone (6PPDQ), a transformation product of the tire additive 6PPD, on the brain of Clarias gariepinus juveniles. The research, motivated by growing concern over tire-derived contaminants, aimed to determine the effects of 6PPDQ on acetylcholinesterase (AChE) activity, acetylcholine (ACh) levels, and brain histology. A total of 237 fish were exposed to 500 µg/L, 1,000 µg/L, and 1,500 µg/L of 6PPDQ for 28 days under laboratory conditions, following OECD sub-lethal toxicity guidelines. AChE activity was determined using Ellman's spectrophotometric method, while histopathological alterations in the telencephalon and optic tectum were assessed with haematoxylin and eosin (H&E) staining. Results revealed a significant ($p < 0.05$) dose- and time-dependent inhibition of AChE activity with a corresponding increase in ACh concentration. Histological analysis showed neuronal shrinkage, vacuolation, pyknosis, and mild gliosis in exposed fish, indicating oxidative stress and neuroinflammatory responses. The study concludes that prolonged exposure to 6PPDQ disrupts cholinergic neurotransmission and alters brain histoarchitecture in C. gariepinus. These findings highlight the ecological risks of tire-derived pollutants and emphasize the need for environmental monitoring and stricter regulation to mitigate their impact on aquatic life and ecosystem health.

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CHAPTER ONE

INTRODUCTION

1.1 Background of Study

The growing prevalence of synthetic chemicals in the environment has raised serious concerns about global public health, especially in light of possible effects on the central nervous system (CNS). Among the environmental contaminants found in tire rubber, N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6-PPD) is the most common. Under oxidative environmental conditions (such as ozone), 6-PPD is soon transformed into 6-PPD-quinone, also referred to as 6-PPDQ, a quinone product that is present in urban stormwater, runoff, and sediments. To stop deterioration, rubber tires are heavily treated with an antioxidant and antiozonant (Tian et al., 2021). Particles of tire wear are carried into aquatic systems by rainfall events, where 6-PPD changes chemically to 6-PPDQ, mostly in the presence of ozone. The discovery that 6-PPDQ is highly toxic, particularly to some aquatic species, such as coho salmon a phenomenon termed "Urban Runoff Mortality Syndrome" has brought attention to the biological effects and environmental dispersal of the transformation product. 6-PPD-quinone, commonly shortened to 6-PPDQ, has been found in large quantities in sediments, runoff, and urban storm water. The finding that certain aquatic species, particularly coho salmon, are extremely toxic to 6-PPDQ (Tian et al., 2021).

Although initial work established dramatic, acute lethality in sensitive fish species, subsequent studies have found that 6-PPDQ can reach non-negligible concentrations in surface waters, can be taken up by multiple organisms, and can produce sub-lethal and chronic effects across taxa (invertebrates, fish, and increasingly in mammalian laboratory models) (Geer et al., 2023). The high lipophilicity and stability of 6-PPDQ point to the possibility of bioaccumulation and, as a result, a risk to higher organisms, including

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mammals, through tainted food chains and water. Because it is a lipid-rich organ, the brain is especially susceptible to the buildup of these lipophilic toxicants, which can cause neurotoxicity. Any negative impact on the composition or operation of the central and peripheral nervous systems is referred to as neurotoxicity. These consequences include oxidative stress, organ damage, neuroinflammation, behavioral abnormalities, disruption of neurotransmitter profiles, and disruption of the blood-brain barrier (BBB) (Costa, 2023; Geer et al., 2023).

Acetylcholine (ACh), a vital neurotransmitter in the central nervous system, is necessary for functions like memory, learning, attention, and muscle control. Many neurotoxicants are known to target the cholinergic system. Cognitive and motor functions may suffer significantly if ACh signaling is disrupted, whether by changed synthesis, release, or degradation. 6-PPDQ exposure may upset the delicate cholinergic balance in the brain due to its structural properties and the precedent set by other quinone-based compounds that are known to cause oxidative stress and mitochondrial dysfunction (Xie et al., 2024).

1.2 Statement of Problem

There remain important gaps in our knowledge of 6-PPDQ's long-term effects on the mammalian central nervous system (CNS), despite an increase in environmental monitoring and ecotoxicology research. Specifically, it is still not known how long-term exposure to 6-PPDQ may change brain histology (neuronal loss, gliosis, BBB integrity), cholinergic (acetylcholine, ACh) neurotransmission, and associated behavioral/cognitive results. This is an important gap in knowledge because there is evidence that 6-PPDQ is prevalent in urban settings and detectable outside of aquatic systems and preliminary experimental data suggests that exposure to 6-PPDQ can disrupt a variety of neurotransmitter systems, including

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cholinergic signaling, in lab models. A proper risk assessment of this emerging contaminant for the health of humans and mammalian wildlife is hampered by the lack of data.

1.3 Aim and Objectives

The aim of this research is to assess, using a mammalian model, the neurotoxic effects of long-term exposure to 6-PPDQ on the cholinergic system and brain histology.

The specific objectives are

i. To determine the effects of sub-chronic 6-PPDQ exposure on acetylcholinesterase (AChE) activity and acetylcholine (ACh) **levels** in the brain tissues (particularly the telencephalon and optic tectum) of juvenile catfish.

ii. To evaluate oxidative stress marker**s**, including malondialdehyde (MDA) and reduced glutathione (GSH), in the brain of juvenile catfish following exposure to 6-PPDQ.

iii. To investigate histopathological alterations in the brain regions of juvenile catfish after long-term exposure to 6-PPDQ.

1.4 Significance of study

Public health, ecotoxicology, and environmental health all benefit from an understanding of how a newly discovered urban contaminant impacts cholinergic signaling and brain structure. Acetylcholine is essential for autonomic regulation, neuromuscular function, and cognition; disruption of cholinergic pathways is linked to neurodegenerative diseases and cognitive impairments. Beyond fish mortality, 6-PPDQ may pose a risk to terrestrial wildlife and, through exposure pathways (dust, inhalation, ingestion), potentially humans, if it disrupts ACh signaling or damages brain structures through BBB breakdown, oxidative stress, or neuroinflammation. This demands scientific research and monitoring.

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To determine the effects of sub-chronic 6-PPDQ exposure on acetylcholinesterase (AChE) activity and acetylcholine (ACh) levels in the cerebral cortex and hippocampus of adult Wistar rats.

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1.5 Study Limitations

This study will use an aquatic animal model — juvenile catfish **(***Clarias gariepinus***)** — and will focus on the telencephalon and optic tectum, two brain regions that play major roles in cognitive processing and sensory integration in fish. The experimental design will involve a 30-day sub-acute exposure period to the selected test substance(s). Following exposure, neurobehavioral assessments and histological analyses will be conducted to evaluate potential effects on learning, memory, and neuronal integrity.

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This study will only use an animal model (Wistar rats) and concentrate on the cerebral cortex and hippocampus, two areas of the brain that are essential for cognition. There will be a 30-day sub-Acute exposure period.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Origin, environmental occurrence and exposure pathways of 6-PPDQ

Tire rubber is treated with the antiozonant N-(1,3-Dimethylbutyl)-N-Nted with phenylenediamine (6PPD) to stop oxidative damage. 6PPD oxidizes to produce 6PPD-quinone (6PPD-Q), a hazardous transformation product, when tires are worn down and exposed to air ozone (Huang et al., 2021). When it rains, tire wear particles that contain both compounds are released through abrasion and then wash into rivers, lakes, and storm drains (Tian et al., 2021). 6PPD-Q concentrations are common in urban watersheds with high vehicle traffic and are frequently highest during initial stormwater runoff (Jaeger et al., 2024). Environmental contamination is also a result of industrial processes like e-waste recycling and 6PPD synthesis (Zhang et al., 2024). Despite 6PPD's relative harmlessness, its oxidized form, 6PPD-Q, presents serious ecological hazards, especially to aquatic life. Its toxicity and environmental distribution have been better understood thanks to recent research (Scholz et al., 2011).

P-phenylenediamine (PPD) derivatives are released into the atmosphere primarily as a result of the production and widespread use of tires as well as the gradual degradation of other rubber-related products. For example, the extensive use of wires and cables in Taiyuan, China, has led to the degradation of rubber coverings, increasing the concentration of PM2.5-bound PPD in the surrounding air (Zhang et al., 2021). When released into the environment, 6PPD can react to form hydroxylated 6PPD intermediates, which subsequently react with ozone to form 6PPD-Q (Rossomme et al., 2023).

PD and 6PPD-Q have been detected in dust samples taken both indoors and outdoors, particularly in Chinese cities such as Hangzhou. Of all the PPDs and PPD-Qs that were

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analyzed, the indoor dust samples from these regions had the highest concentrations of 6PPD (ranging from 0.48 to 135 ng/g) and 6PPD-Q (ranging from 0.33 to 82 ng/g). Infants ingesting 6PPD and 6PPD-Q through indoor dust had higher daily intakes than children (0.22–184 and 0.37–112 pg/kg·bw) and adults (0.11–91 and 0.18–56 pg/kg·bw) (0.51–427 and 0.85–259 pg/kg·bw, respectively) (Zhu et al., 2024).

One of the main environmental sources of 6PPD-quinone (6PPD-Q) is tire and road wear particles (TRWPs). Microbial activity first converts 6PPD to 6PPD-Q in soils, particularly anaerobic flooded soils, through Fe (III)-reduction-coupled oxidation, with abiotic processes taking over later (Xu et al., 2023). Therefore, compared to moist soils, flooded soils exhibit higher 6PPD-Q levels. Despite its low solubility, 6PPD-Q is very stable and quickly leaches from TRWPs into runoff, especially during storms (Hu et al., 2023; Hiki and Yamamoto, 2022). Because they release more 6PPD than larger ones, smaller tire particles (micron-sized) are more dangerous (Stack et al., 2023). Biohazard concerns were raised by studies that discovered 6PPD and 6PPD-Q in simulated human bodily fluids (Armada et al., 2023; Schneider et al., 2020).

Heat and sunlight hasten the conversion of 6PPD to 6PPD-Q, weakening TRWPs and encouraging their breakdown (Hu et al., 2022; Weyrauch et al., 2023). 6PPD-Q is more persistent under thermal aging without light (half-life ~6 months), even though it breaks down under sunlight (half-life <1 month) (Fohet et al., 2023). 6PPD-Q is the most prevalent PPD-Q derivative found in tire-related products (Zhao et al., 2023).

2.2 The Discovery of 6PPD-quinone Toxicity and the Coho Salmon Mortality Syndrome

Concerns were raised about coho salmon (*Oncorhynchus kisutch*) mortality in several Seattle-area streams in Puget Sound after fish passages were restored in the 1990s for an unknown reason (Scholz et al., 2011). A 1999–2001 survey of adult coho salmon returning to

restoration sites to spawn revealed an uncommon pre-spawn mortality syndrome (Scholz et al., 2011). One study began investigating this phenomenon in 2002. Assessments compared spawner mortality in urban and nonurban streams and examined the severity of coho salmon adult die-offs to identify the water quality and spawner conditions factors that might be linked to the frequent fish deaths. Coho salmon die-offs were significantly higher in urban streams than in non-urban ones, but the data showed no association with these symptomatic fish. In 2021, nearly two decades later, another study used continual chemical separation techniques to separate pollutants from stormwater. Additionally, it implied that a previously unidentified poison, now identified as 6PPD-1 2 Quinone, may be connected to the ill coho fish (Tian et al., 2022; Tian et al., 2021). Particularly low levels of 6PPD-Q have been shown to poison economically and culturally significant species, such as rainbow trout, brook trout, and coho salmon (Brinkmann et al., 2022, Tian et al., 2021). Some species, like white sturgeon and Arctic char, can tolerate concentrations even greater than those found in ambient samples (Tian et al., 2022; Brinkmann et al., 2022; Challis et al., 2021).

Following the discovery of the acute toxic effects of 6PPD-quinone in coho salmon (*Oncorhynchus kisutch*) by Tian et al. (2021), tests conducted on other species revealed that the toxicity of 6PPD-quinone is highly specific to a small number of species. To date, toxicity studies have been conducted on 17 fish species and 9 other organisms using either tire leachate, untreated urban roadway runoff, or commercial standards of 6PPD-quinone (Foldvik et al., 2024).

2.3 Acute toxicity: discovery and species sensitivity

Five species of the Oncorhynchus and Salvelinus genera have been found to exhibit acute toxicity at environmentally relevant concentrations (<2.43 µg/L; Cao et al., 2022; Challis et al., 2021; Johannessen et al., 2022; Kryuchkov et al., 2023; Rauert et al., 2022). The risk

assessment of this chemical is complicated by the fact that closely related species within these genera have very different sensitivity levels to 6PPD-quinone, such as coho salmon, which have a standard lethal concentration (LC50) of 0.041 μg/L (Lo et al., 2023), while closely related sockeye salmon (*Oncorhynchus nerka*; French et al., 2022) and chum salmon (*Oncorhynchus keta*, which do not react to the chemical. For instance, the LC50 for Coho alevins (3 weeks old) has been reported to be 0.041 μg/L (Lo et al., 2023), whereas the LC50 for parr (>1 year) is more than double, at 0.095 μg/L (Tian et al., 2022). This indicates that the toxicity of 6PPD-quinone varies significantly throughout the course of development.

Studies have shown that 6PPD-quinone is extensively found in water, soil, and air across the globe (Cao et al., 2022; Hiki a Yamamoto, 2022a; Kryuchkov et al., 2023; Tian et al., 2021). Research has investigated the sublethal effects of 6PPD-quinone in zebrafish (*Danio rerio*; Ji et al., 2022; Varshney et al., 2022; Zhang et al., 2023) and fathead minnows (*Pimephales promelas*; Anderson-Bain et al., 2023). 6PPD-quinone toxicity has been attributed to a number of independently exclusive mechanisms, such as disruption of the blood-brain barrier (Blair et al., 2021), metabolic issues related to neurotransmitters (Ji et al., 2022), and uncoupling of mitochondrial respiration in the gills (Mahoney et al., 2022).

Since the underlying cause of 6PPD-quinone's species-specific toxicity pattern is still unknown, toxicological testing is necessary to determine a species' sensitivity to the compound. It present the first results of acute toxicity testing of 6PPD-quinone on pink salmon, the only Pacific fish that has not been previously studied.

2.4 Inter-specie Responses to 6PPD-Quinone

The acute toxicity of 6PPD-quinone (6PPD-Q) across species cannot be accurately predicted by phylogeny alone. Assessing risks to vulnerable species and determining possible mechanisms of action require comparative interspecific data (Brinkmann et al., 2022; Tian et

al., 2021). According to studies, some salmonids, including coho salmon, brook trout, rainbow trout, and white-spotted char, are extremely sensitive to exposure to 6PPD-Q. In contrast, other species, such as arctic char, brown trout, Atlantic salmon, southern Asian dolly varden, masu salmon, and sockeye salmon, did not exhibit any negative effects, even at extremely high, non-environmentally relevant concentrations (Tian et al., 2022; Lo et al., 2023). Numerous physiological or biochemical variations between species may be the cause of these variations. A variety of factors may be involved, including species-specific mitochondrial target site conformations, variations in blood-brain barrier integrity, and differential transport of 6PPD-Q to the gill mitochondrial electron transport chain (either active, facilitated, or passive) (Mahoney et al., 2022; Greer et al., 2023). Exposure to 6PPD-Q is thought to increase endothelial permeability, specifically based on evidence of hemoconcentration, accumulation of macromolecules in cerebral tissues, and downregulation of genes encoding occludin and junctional adhesion molecules (Geer et al., 2023).

2.5 Acute and chronic toxicity: from fish to mammals

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Tian et al. (2021) made the groundbreaking discovery that 6-PPDQ is the cause of acute prespawn coho salmon mortality linked to urban runoff, thereby connecting 6-PPDQ to environmental mortality. In addition to documenting behavioral syndrome (loss of equilibrium, surface swimming) and vascular/BBB-related effects, this study reported incredibly low LC_{50} values for coho salmon (tens of ng L^{-1}) (Tian et al., 2021).

Several studies carried out toxicity testing across species and life stages after Tian et al. The histopathological and metabolic work in coho revealed dysregulation of the BBB and endothelial pathways, indicating vascular permeability as a mechanism. Other fish species and laboratory models displayed varying sensitivity to 6-PPDQ. 6-PPDQ has been identified

as an emergent, high-priority stormwater contaminant by environmental and regulatory reviews (Geer et al., 2023).

New findings from mammals include repeated-dose animal studies that demonstrate multiple organ damage after sub-acute exposure (for example, 28-day injections damaged the liver and other organs) and more recent studies that demonstrate neurotoxicity and neuroinflammation in mice after prolonged exposure, which are linked to specific central nervous system receptors. These animal studies are important because they begin to address whether 6-PPDQ poses direct CNS risks outside of aquatic species (He et al., 2023).

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2.6 Neurotoxicity and the Cholinergic System

Neurotoxicology is the process of brain cell structural and functional changes brought on by physical or biological causes. The factors can cause neurological changes include trauma, emotional stress, genetic predisposition, metabolic comorbidities, and toxicants. Neurotoxicity is any adverse effect on the chemistry, structure, or function of the nervous system. Environmental pollution is one of these causes that is a major and growing problem for every population, regardless of age, because different environmental toxins have varied neurological effects on people of different ages. Many possible organic neurotoxicants, including pesticides, plastics, solvents, and medications, are present in our daily lives and can enter our bodies through the consumption of contaminated food or water, inhaling polluted air, or coming into direct touch with contaminated surfaces (Soares et al., 2023).

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2.7 Evidence for neurotransmitter (incl. acetylcholine) perturbation

The cholinergic system, which utilizes ACh as its primary neurotransmitter, is highly susceptible to toxicants. ACh is hydrolyzed by the enzyme acetylcholinesterase (AChE) in the synaptic cleft to terminate its action. Inhibition of AChE leads to an accumulation of ACh,

resulting in overstimulation of cholinergic receptors, which can cause a range of symptoms from tremors and seizures to cognitive deficits (Colović et al., 2013).

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Many environmental pollutants, including organophosphate and carbamate pesticides, are known AChE inhibitors. Furthermore, prolonged cholinergic disruption is a key feature in neurodegenerative diseases, underscoring the importance of investigating this pathway (Terry and Buccafusco, 2003)

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Multiple experimental models show that 6-PPDQ (and, to some extent, parent 6-PPD) can alter neurotransmitter homeostasis

- Neurodegenerative phenotypes and changes in dopaminergic, glutamatergic, GABAergic, and serotonergic systems are reported in nematode and invertebrate models (*Caenorhabditis elegans*) following chronic exposure; these studies imply a broad disruption of neurotransmission rather than a single-pathway effect as in the study by Ricarte et al. (2023)
- Changes in locomotion and altered neurotransmitter profiles have been observed in zebrafish larvae and other small fish models exposed to environmentally relevant concentrations; some studies report elevated levels of catecholamines and acetylcholine at low concentrations, which correlate with behavioral endpoints (Liu et al., 2024)
- Direct measurements of acetylcholine or acetylcholinesterase (AChE) activity in rodent brains following chronic 6-PPDQ are currently scarce and represent a significant experimental gap, despite recent mammalian work showing downstream effects consistent with neurotransmission disruption (behavioral deficits, increased oxidative stress, apoptosis) (Ma et al., 2025).

All of the evidence points to the possibility that 6-PPDQ exposure may have an impact on the cholinergic system (ACh levels and potentially AChE function) in certain models, either directly or indirectly through oxidative stress, BBB damage, or receptor-mediated effects (Ricarte et al., 2023).

2.8 The impact of 6-PPDQ on the blood–brain barrier (BBB) and brain histology

Histological and ultrastructural analyses shed light on the vascular and cellular alterations brought on by 6-PPDQ:

- Studies on fish (coho and other species) have shown transcriptomic signatures linking endothelial integrity and vascular permeability pathways, as well as macromolecular accumulation in the brain, which is consistent with disruption of the blood-brain barrier. The quick onset of neurological symptoms seen in sensitive fish is likely caused by this vascular compromise (Greer et al., 2023).
- Markers of oxidative stress, apoptotic signaling, microglial activation, and BBB alterations (biochemical markers and functional permeability assays) are reported in rodents exposed to subacute and chronic exposures. In a recent mouse study, 6-PPDQ exposure is linked to increased ROS, pro-inflammatory cytokines (TNF-α, IL-6, IL-1β), microglial activation, evidence of disruption of the blood-brain barrier, and neuronal apoptosis, all of which are traditional indicators of chemically-induced neurotoxicity and neuroinflammation (Ma *et al.,* 2025).
- Although the pattern varies by species and dose, histology following chronic exposure in zebrafish and other aquatic models has revealed neurodegenerative changes, altered neuronal morphology, and, in certain cases, apoptosis in specific brain regions as in the case of Yi et al. (2025).

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Together, these histopathological results provide evidence for an example in which 6-PPDQ can disrupt cell stress pathways and harm neurovascular integrity, resulting in conditions that would change the balance of neurotransmitters, including ACh, and the survival of neurons.

2.9 Histopathological Assessment in Neurotoxicology

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Histological examination is the foundation of neurotoxicological assessment because biochemical and molecular changes frequently precede or coincide with structural damage to neural tissue. Conventional staining techniques such as Haematoxylin and Eosin (H&E) enable the general observation of tissue architecture, neuronal cell body integrity (e.g., pyknosis, necrosis), and inflammatory cell infiltration. Cresyl Violet (Nissl staining) specifically stains Nissl bodies in the rough endoplasmic reticulum of neurons, enabling a clearer assessment of neuronal density and health (Kumar et al., 2013).

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The activation of glial cells (astrocytes and microglia) and the presence of neurodegenerative characteristics, such as shrunken neurons with condensed nuclei and the loss of Nissl substance, are characteristic histological endpoints of neurotoxic injury. Such morphological evidence of 6-PPDQ's neurotoxicity would be provided by demonstrating such alterations in the cortex and hippocampal regions after exposure (Geer et al., 2023).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals

6PPD-quinone (purity 97.26%) and the isotopically labeled internal standard 6PPD-quinone-D5 were obtained from HPC Standards GmbH (Borsdorf, Germany) and the 6PPD-quinone analytical independent check standard from Cambridge Isotope Laboratories (Cambridge, MA, USA). Stock solutions of 6PPDQ were prepared using dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, Waltham, MA, USA) to achieve a final solvent concentration of <0.001% (v/v) during exposures. Buffered tricaine methanesulfonate (MS-222) (Syndel's Syncaine, Ferndale, WA, USA) was used for anesthesia (100 mg/L) and euthanasia (250 mg/L).

Plate 1: 6PPD- Quinone (6PPD-Q)

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Anderson-Bain, K., Roberts, C., Kohlman, E., Ji, X., Alcaraz, A. J., Miller, J., Gangur-Powell, T., Weber, L., Janz, D., Hecker, M., Montina, T., Brinkmann, M., & Wiseman, S. (2023). Apical and mechanistic effects of 6PPD-quinone o

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Dimethyl Sulfoxide (DMSO) was obtained from a commercial vendor. The DMSO (Dimethyl sulfoxide; CAS No. 67-68-5) is an organosulfur compound widely used as a solvent in biological and chemical research due to its ability to penetrate biological membranes. It is a polar aprotic solvent, fully miscible with water and many organic solvents. DMSO is known for its anti-inflammatory properties and is used as a vehicle for drug delivery in experimental studies.

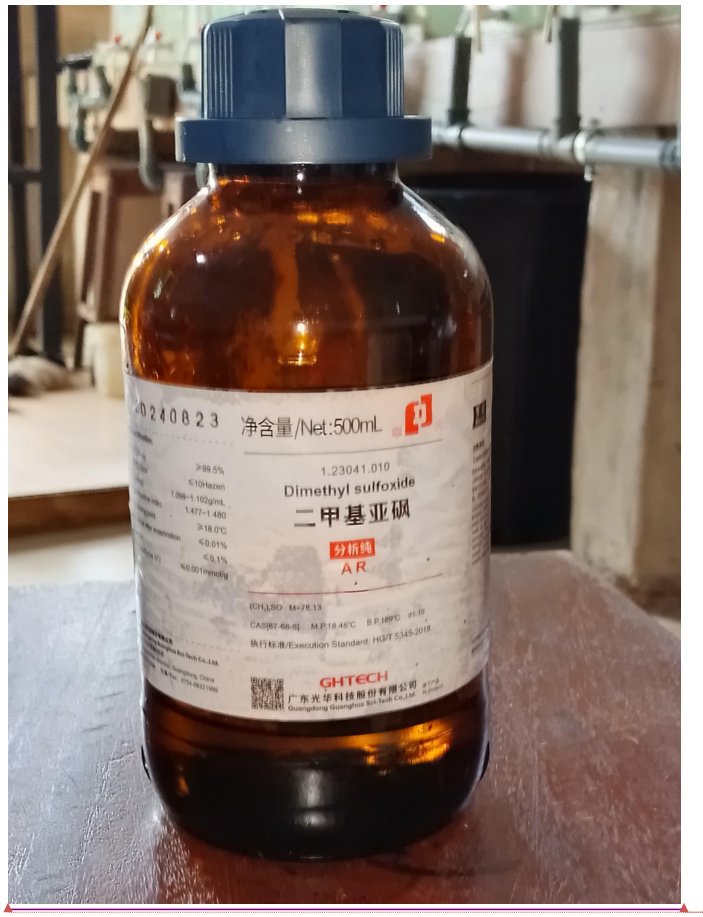

Plate 2: Bottle of Dimethyl sulfoxide (DMSO)

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3.2 Test organism

Clarias gariepinus (African catfish) is widely distributed in tropical freshwater ecosystems and relished by the tropical region populace (Ogueji et al., 2018). The fish is omnivorous, feeding on invertebrates, fish, small mammals, seeds and fruit (Hastuti et al., 2019). According to Ogueji et al. (2018), it is also rugged, adaptive to laboratory conditions produces reasonable quantities of blood for haematological parameter estimation. It is among the most widespread of the African freshwater fish (Nguyen and Janssen, 2002).

3.3 Source of experimented fish, Acclimatization and Ethical Approval

The experimental fish, Clarias gariepinus juveniles were purchased at a fish farm in Benin city, Edo state and transported to the ecotoxicology laboratory of Animal and Environmental Biology, University of Benin, Benin City, Edo state where the experiment was conducted. The average weight and average length of the fish was 24.10 ± 11.51g and 12.51 ± 2.8 cm respectively. The juveniles were acclimatized in a 160L cylindrical plastic tank for two days and fed on a pelleted commercial feed daily. They were however left unfed for the first 24 hours to adapt to a change in the environment before feeding commenced with the fish diet. Approximately, 80% of the water was replaced every two days. They were kept under natural photoperiod. There was 8% mortality during the acclimatization period. Ethical clearance on the use of the fish species was obtained and approved by the Committee on the Ethical Use of Laboratory Animals, Faculty of Life Sciences, University of Benin, Nigeria.

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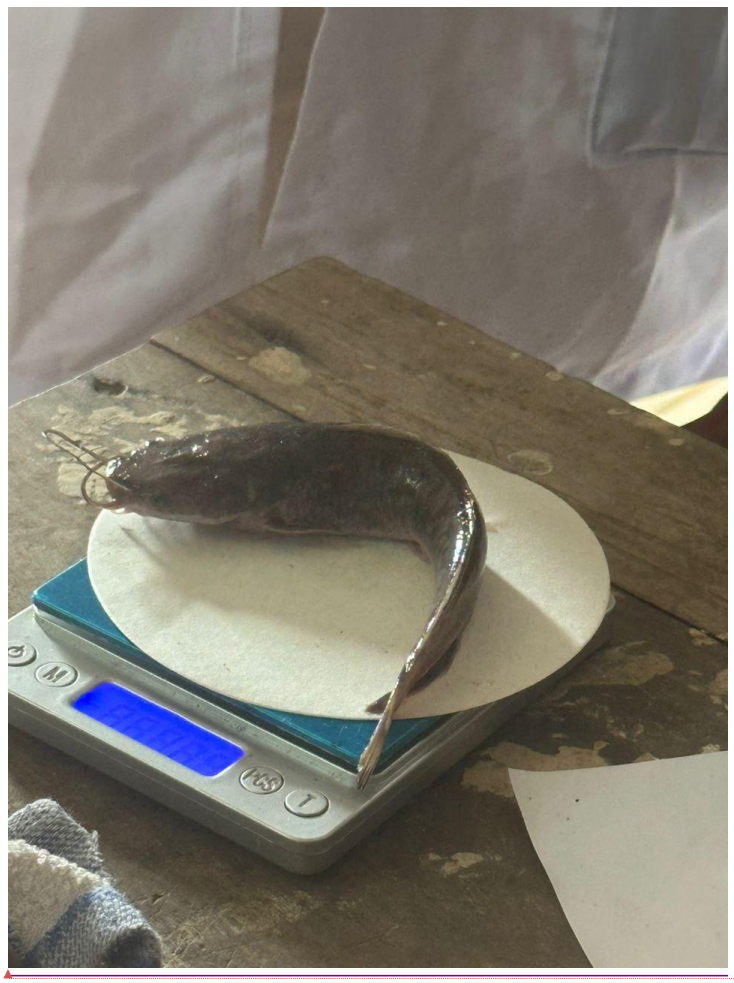

3.4 Experiment design

A total of 237 Clarias gariepinus juveniles used were divided into five treatments which included a positive control (+control), negative control (-control), and three different

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concentrations of the toxicant) with two replicates. Seven juveniles were stocked into each tank with two replicates treatment. The water used was tap water left to stand for 24 hours.

3.5 Experimental protocol

The experiment was carried out as described in the OECD guidelines for fish sublethal toxicity testing (OECD, 2019). 20g of 6ppd-q was dissolved into 100ml of DMSO to produce a stock solution. The stock solution was used to produce the four different concentrations- 500µg/L, 1000µg/L and 1500µg/L of toxicants introduced into experimental tank of 20litres of water in each tank. The positive control (+control) had 0.0mg/L of toxicants, while the negative control (-control) had 200µL of DMSO. Duplicates were made for each concentration. The test was carried out under static conditions. The duration of the test was 28 days from which started on 19th September 2025 to 17th October 2025, during which the organisms were fed once a day.

3.6 Biomarker Analysis

At the end of the exposure periods, neurological biomarkers AChE and ACh was evaluated to assess the sublethal effects of 6PPD-q exposure These biomarkers were chosen for it's relevance in detecting neurological changes in fish exposed to environmental contaminants like 6PPD-Q.

3.6.5 Determination of Acetylcholinesterase (AChE) Activity

Acetylcholinesterase (AChE) is a key neurotoxicity biomarker responsible for the hydrolysis of the neurotransmitter acetylcholine into acetate and choline at synaptic junctions. Inhibition of AChE causes overstimulation of cholinergic pathways, which can impair normal neuro-physiological functions such as locomotion, feeding, and behavioral coordination in fish. Therefore, AChE activity was assessed to evaluate the neurotoxic potential of 6PPD-quinone following long-term exposure.

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The method described by Ellman et al. (1961) was adopted with slight modification. Brain tissues were excised from euthanized fish and immediately placed on ice. Each brain sample was homogenized in 0.1 M phosphate buffer (pH 7.4) at a ratio of 1:10 (w/v) using a glass homogenizer. The homogenate was centrifuged at 3000 rpm for 15 minutes at 4°C, and the supernatant was collected for enzyme analysis.

The assay reaction mixture contained 2.6 mL of phosphate buffer, 0.3 mL of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and 0.1 mL of brain supernatant in a cuvette. The reaction was initiated by the addition of 0.1 mL of acetylthiocholine iodide (ATChI) as the substrate. Formation of the yellow-colored 5-thio-2-nitrobenzoate (TNB) anion was monitored spectrophotometrically at 412 nm for 3 minutes at 25°C.

AChE activity was calculated using Ellman's formula:

$$\text{AChE Activity (µmol/min/mg protein)} = \frac{\Delta A \times V_{\text{total}}}{\varepsilon \times l \times V_{\text{sample}} \times P}$$

Where:

- ΔA = change in absorbance per minute
- Vtotal = total reaction volume (3.1 mL)
- ε = molar extinction coefficient of TNB (13,600 $M^{-1}cm^{-1}$)
- l = cuvette path length (1 cm)
- Vsample = sample volume (0.1 mL)
- P = mg of protein in the sample (determined using total protein assay)

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AChE activity was expressed as μmol of substrate hydrolyzed per minute per mg protein. Reduced AChE levels relative to the control groups were interpreted as indicative of neurotoxicity caused by prolonged exposure to 6PPD-quinone.

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CHAPTER FOUR

4.1 Acetylcholinesterase (AChE)

Exposure of *Clarias gariepinus* juveniles to varying concentrations of 6PPD-quinone (6PPD-Q) induced a concentration- and time-dependent inhibition of acetylcholinesterase (AChE) activity in the brain tissue. The mean AChE activities (μmol·min^{-1}·g^{-1} protein) in fish exposed to Positive control (+ve), Negative control (DMSO), 500 µg/L, 1,000 µg/L, and 1,500 µg/L of 6PPD-Q were 101.6 ± 1.85, 120.0 ± 1.58, 108.0 ± 1.41, 90.0 ± 1.58, and 72.0 ± 1.58, respectively, after 7 days of exposure.

At 14 days, the corresponding AChE values were 94.8 ± 1.30, 115.0 ± 1.58, 100.4 ± 1.14, 83.6 ± 1.14, and 66.9 ± 1.30. After 21 days of exposure, the mean AChE activities were 87.8 ± 1.30, 110.0 ± 1.58, 93.0 ± 1.14, 77.6 ± 1.14, and 62.0 ± 0.71, while at 28 days the corresponding means were 80.3 ± 0.84, 105.0 ± 1.58, 85.0 ± 1.58, 70.8 ± 1.30, and 56.7 ± 0.84 for the respective treatment groups.

Statistical analysis (one-way ANOVA) revealed significant differences ($p < 0.05$) in AChE activity across treatment groups at all exposure periods. On day 7, significant decreases ($p < 0.05$) were observed in the 1,000 µg/L and 1,500 µg/L groups compared with the controls. By day 14, the 500 µg/L, 1,000 µg/L, and 1,500 µg/L groups showed significant inhibition ($p < 0.05$), while by day 21 and 28, all exposed groups exhibited statistically significant reductions ($p < 0.05$) relative to both controls.

The percentage reduction in AChE activity relative to the positive control ranged between 5–10% for the 500 µg/L group, 15–25% for 1,000 µg/L, and 30–45% for 1,500 µg/L across exposure durations, confirming a clear dose- and time-dependent inhibition trend.

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Treatment / Timepoint	7 days (µmol·min⁻¹·g⁻¹)	14 days (µmol·min⁻¹·g⁻¹)	21 days (µmol·min⁻¹·g⁻¹)	28 days (µmol·min⁻¹·g⁻¹)
Negative control (DMSO)	120.00 ± 4.00	115.00 ± 5.00	110.00 ± 5.00	105.00 ± 4.00
Positive control (+ve)	102.00 ± 6.00	94.82 ± 7.00	87.89 ± 7.00	80.33 ± 6.00
500 µg/L	108.00 ± 8.00	100.40 ± 8.00	93.06 ± 9.00	85.05 ± 9.00
1,000 µg/L	90.00 ± 7.00	83.64 ± 7.00	77.55 ± 8.00	70.88 ± 8.00
1,500 µg/L	72.00 ± 6.00	66.93 ± 6.00	62.04 ± 6.00	56.70 ± 5.00

Table 4.1: These findings indicate that exposure to increasing concentrations of 6PPD-Q impairs cholinergic neurotransmission in *C. gariepinus*, as reflected by significant suppression of AChE activity over time.

Figure 4.1: Mean acetylcholinesterase (AChE) activity (µmol·min⁻¹·g⁻¹ protein) in *C. gariepinus* juveniles exposed to varying concentrations of 6PPD-Q for 7, 14, 21, and 28 days. Data are expressed as mean ± SD (n = 7). Bars with different superscripts are significantly different at $p < 0.05$ (one-way ANOVA, Tukey's post-hoc test).

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CHAPTER FIVE

DISCUSSION

This study investigated the neurotoxic effects of sub-chronic exposure to 6PPD-quinone (6-PPDQ) on the brain of juvenile Clarias gariepinus, with emphasis on cholinergic biomarkers (ACh and AChE) and histopathological alterations in the telencephalon and optic tectum. The results revealed dose-dependent neurotoxic responses after 28-day exposure, demonstrating the susceptibility of the fish central nervous system to 6-PPDQ.

The significant reduction in AChE activity observed in fish exposed to medium and highest concentrations indicates inhibition of the major enzyme responsible for acetylcholine termination at synapses. This impairment suggests overstimulation of cholinergic receptors due to excessive accumulation of ACh in the synaptic cleft. Such effects have been widely documented in neurotoxicant exposures, especially with pesticides and quinone-based chemicals (Colović et al., 2013; Liu et al., 2024). Similar disruptions in neurotransmission have been reported in zebrafish and rodents exposed to 6-PPDQ, confirming the chemical's ability to interfere with neuronal signaling (Ricarte et al., 2023; Ma et al., 2025).

In addition, elevated ACh levels recorded in exposed treatments support the biochemical evidence of cholinergic imbalance caused by AChE inhibition. The cholinergic system plays critical roles in locomotion, sensory processing and coordination in fish; therefore, changes in its biomarkers imply a threat to behavioural and cognitive functions (Terry & Buccafusco, 2003). Although behavioural endpoints were not assessed in this study, other studies have associated cholinergic disruptions with erratic swimming, reduced feeding, and impaired learning in fish (Ji et al., 2022).

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The histopathological alterations observed — including neuronal shrinkage, vacuolation of neuropil, pyknosis, and mild gliosis — further confirm the neurotoxic properties of 6-PPDQ. These changes are consistent with stress-induced neuronal degeneration and activation of inflammatory responses in the fish brain. Previous research has reported similar cellular damage, as 6-PPDQ induces oxidative stress and compromises neurovascular integrity (Greer et al., 2023; Yi et al., 2025). The vulnerability of the telencephalon and optic tectum may be linked to their high metabolic activity and direct involvement in sensory integration and neurocognitive functions.

Furthermore, the action of 6-PPDQ in this study may be partly due to its lipophilicity, which enables it to traverse biological membranes and potentially disrupt the blood-brain barrier (Blair et al., 2025). Damage to the BBB contributes to increased permeability, enabling more toxicant penetration into neural tissues, thus aggravating neuroinflammatory responses (Geer et al., 2023). This agrees with a growing body of evidence that identifies the brain as a critical target of this emerging tire-derived contaminant.

Taken together, the current findings show that even at environmentally relevant concentrations, 6-PPDQ can induce biochemical and structural impairments in the CNS of Clarias gariepinus within a sub-chronic exposure period. Since this species is ecologically and economically important in African freshwater ecosystems, the presence of 6-PPDQ in stormwater-affected environments may threaten fish population health and food security.

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CONCLUSION OF DISCUSSION

Overall, the results provide strong scientific evidence that 6-PPDQ poses significant neurotoxic risks to aquatic organisms. Therefore, routine monitoring of tire-derived pollutants in urban waters, along with regulatory interventions, is urgently recommended to prevent long-term ecological implications.

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