EXPOSURE TO N-(1,3IDIMETHYLBUTYL1)-N-PHENYL-P-PHENYLENEDIAMINE QUINONE (6PPD-Q) AFFECTS THE SWIMMING KINETICS OF FISH

BY

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CERTIFICATION

This is to certify that this project was carried out by **MARTINS OSAHENRUMWEN AIREMWEN** of the Department of Animal and Environmental Biology, Faculty of Life Sciences, University of Benin, Benin City.

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(HEAD OF DEPARTMENT)

DEDICATION

This research work is dedicated to the loving memory of my late grandfather Airemwen, who in his time nurtured, guided, and sacrificed for me in ways words cannot fully capture. His prayers, encouragement, and unwavering love became the foundation of my journey.

Though he is no longer physically present to witness this academic milestone, his legacy lives on in me. His life continues to inspire resilience, perseverance, and faith reminding me that even in the face of loss, hope endures.

This work is also dedicated to my family, who have remained steadfast in their love and encouragement, and to my friends, colleagues, and communities who have shown me that in times of hardship, one is never truly alone.

ACKNOWLEDGEMENT

First and foremost, I give thanks to God Almighty for the grace, strength, and wisdom that guided me throughout my academic journey.

I wish to sincerely acknowledge myself for my dedication, focus, and perseverance in successfully completing my university education. This achievement is a reflection of the effort, patience, and resilience I invested over the years.

My deepest appreciation goes to my parents, Mr. and Mrs. Augustine Ogbebor, for their unwavering love, prayers, and support from birth till this very moment. Your sacrifices and encouragement have been the backbone of my success.

I am profoundly grateful to my project supervisor, Dr. N. O. Erhunmwunsee, whose guidance, patience, and insightful contributions made this research a success. Without his supervision and mentorship, this work would not have been possible.

My heartfelt thanks also go to my siblings for their encouragement and constant motivation, and to my grandparents, who raised me with love and discipline during my early childhood education. I will forever cherish the words of my late grandfather, Airemwen, who always said, “Anyone who supports you not to learn is not your friend.” His wisdom continues to inspire me to this day.

Finally, I acknowledge myself once more for believing, striving, and never giving up.

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ABSTRACT

The study investigated the effects of 6PPD-quinone (6PPD-Q) on the swimming performance of *Clarias gariepinus* fingerlings over a 28-day exposure period. Fish were exposed to nominal concentrations of 0 µg/L 6PPD-Q (positive control), 200 µg/L DMSO (negative control), and 500, 1000, and 1500 µg/L 6PPD-Q. Swimming speed showed a clear concentration and time dependent reduction across all treatment groups compared to the positive control. The mean swimming speeds (m/s) for the respective treatments were 2.115 ± 1.252, 0.8950 ± 0.2758, 0.8050 ± 0.0778, 0.1100 ± 0.0212, and 0.0250 ± 0.0071 on day 7; 4.110 ± 1.725, 0.4750 ± 0.1061, 0.0600 ± 0.0212, 0.0600 ± 0.0212, and 0.0150 ± 0.0071 on day 14; 0.8950 ± 0.2758, 0.6050 ± 0.0778, 0.1350 ± 0.0212, 0.0550 ± 0.0071, and 0.0350 ± 0.0071 on day 21; and 0.5800 ± 0.1838, 0.2500 ± 0.1556, 0.1650 ± 0.0495, and 0.0400 ± 0.0212 on day 28. Statistically significant differences (p <0.05) were observed between all 6PPD-Q-exposed groups and the positive control, except for the 200 µg/L and negative control groups on days 7, 21, and 28 ($p > 0.05$). Percentage reductions in swimming speed relative to the positive control were 57.7%, 61.9%, 94.8%, and 98.8% on day 7 for the negative control and 500, 1000, and 1500 µg/L groups, respectively. By day 21, reductions reached 32.4%, 84.9%, 93.9%, and 96.1%, and further deepened to 56.9%, 71.6%, 86.2%, and 93.1% by day 28. The observed decline in swimming performance indicates that 6PPD-Q impairs locomotor ability in *C. gariepinus* in a dose and duration dependent manner, potentially compromising survival and ecological fitness in contaminated aquatic environments.

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

Stormwater runoff from urban landscapes has long been a cause for environmental concern due to its chemical complexity, toxicity to aquatic organisms, and temporal and spatial dynamics (Masoner *et al.* 2019). Tire wear particles (TWPs), in addition to road salt, organic pollutants from vehicle emissions and leaks, and hazardous metals from brake pad abrasion, have recently drawn attention from the public and scientific community (McIntyre *et al.* 2021). Tires, which are intricate synthetic matrices made for performance and durability, are widely used as a result of the world's reliance on automobiles. N-(1,3-dimethylbutyl)-N'-phenyl-P-phenylenediamine (6PPD), an antioxidant and antiozonant, is an essential component of contemporary tire rubber that helps stop cracking and degradation brought on by mechanical stress and atmospheric ozone (Halle *et al.* 2021). While 6PPD effectively preserves tire integrity, it is not the compound of ultimate concern. During its protective function or as tires undergo abrasion, 6PPD reacts with ozone, transforming into a transformation product of significant ecological relevance: 6PPD-quinone (6PPD-q) (Tian *et al.* 2021).

Further studies have confirmed that 6PPD-q is a prevalent environmental contaminant by finding it in a variety of environmental matrices, such as air, soil, dust, and waterways worldwide (Baker *et al.* 2025; Johannessen *et al.* 2021). Acute lethality is a dramatic result, but in ecotoxicology, sublethal effects such as those that affect an organism's fitness and health without resulting in immediate death, are frequently more pervasive and subtle. Behaviour, especially swimming performance, is one of the most important sublethal measures for fish and is inextricably linked to foraging, reproduction, survival, and avoiding predators (Tierney, 2011). Since its

discovery, 6PPD-q has been found to be acutely toxic to brook, rainbow/steelhead, lake trout, and coastal cutthroat trout, which are important ecological and recreational species (Nair *et al*. 2023; Brinkmann *et al*. 2022).

One of the main release sources of this chemical pollutant to nearby aquatic ecosystems is modern roadways. Tire-derived chemicals that wash from roads into streams during rain events are one class of contaminants that have recently drawn a lot of attention. This compound which is commonly found in road runoff and urban waters, and it has been linked to acute fish kills in urban streams.

It is estimated that between 26 and 1,900 tons of 6PPD-Q are produced annually worldwide (Hu *et al.* 2022), and the chemical is detected in aquatic environments worldwide up to 2.29 µg/L (Tian *et al.* 2021). It has been demonstrated that the quantity of this contaminant discharged into aquatic environments and the concentrations at which it has been found have a major negative influence on fish populations, especially coho salmon (*Oncorhynchus kisutch*) and other specific salmonid fishes.

Numerous investigations have revealed that in predisposed species exposed to 6PPD-quinone, behavioral abnormalities (such as increased surfacing, loss of equilibrium, and erratic swimming) occur prior to death, indicating neuro-vascular or cardiorespiratory targets (see Mechanisms below). The reductions in swimming ability and endurance after sublethal exposures have been measured explicitly in more recent work. Chronic exposures in model organisms (zebrafish) have shown developmental and neuromotor effects that would be expected to change swimming kinetics, and juvenile salmon exposed to low concentrations showed measurable reductions in critical swimming speed (Ucrit) and endurance even when not immediately lethal. Changes in

vascular integrity and blood parameters have been linked to these sublethal impairments (Jaeger *et al.* 2024).

In 6PPD-quinone toxicity, emerging research findings suggest that vascular injury and disruption of blood–brain and blood–gill barriers are key events. While endothelial dysfunction and vascular permeability pathways may impair oxygen and ion exchange, while blood–brain barrier compromise and direct neuronal impacts may impair motor control, these mechanisms plausibly explain rapid behavioral manifestations like altered swimming and loss of equilibrium that precede mortality (Blair *et al.* 2025).

1.2. Statement of the Problem

The acute lethality of 6PPD-q to coho salmon was discovered, establishing it as a powerful toxicant. In terms of its sublethal neurological behavioural effects, particularly on the swimming kinetics of fish that might not die right away, there is a substantial knowledge gap. A sensitive and comprehensive indicator of neurological and physiological health, swimming kinetics includes metrics like average speed, total distance travelled, acceleration, and angular movement.

Although the exact mechanism of action of 6PPD-q toxicity is still unknown, new research indicates that it may cause oxidative stress and interfere with neurological function. According to Varshney *et al*. (2022), 6PPD-q may affect energy metabolism and mitochondrial function, both of which are essential for the prolonged muscular activity needed for swimming. Additionally, 6PPD-q may directly interfere with the neural circuits controlling movement if it has an impact on neurotransmitter systems or ionoregulation (McIntyre *et al.* 2021).

Understanding these sublethal behavioral effects is essential for several reasons. First, they may influence a wide range of species and developmental stages, often occurring at concentrations well below acute lethal levels. Second, impairments in swimming performance can have significant ecological consequences, reducing individual fitness and potentially altering population dynamics. Finally, although many fish species inhabiting urban waterways are chronically exposed to low concentrations of 6PPD-quinone (6PPD-Q), there is limited knowledge regarding the behavioral impacts of such exposure.

1.3. Aim of Study

This study aims to investigate the sublethal effects of environmentally relevant concentrations of 6PPD-quinone on the swimming kinetics of a model fish species.

1.4 Objectives of Study

- To quantify the acute toxicity (LC_{50}) of 6PPD-quinone to the model species (e.g., Danio rerio or another suitable species) to establish sublethal exposure concentrations.
- To analyse the effects of 24-hour and 96-hour exposures to sublethal concentrations of 6PPD-quinone on key swimming parameters, including:
- Mean swimming velocity
- Total distance moved

1.5. Significance of the Study

Swimming kinetics are fundamental physiological-ecological traits. Changes in routine swimming, sustained swimming capacity and escape performance affect energy budgets, predator–prey interactions, migration success and reproductive outcomes. The findings of this research will provide critical insights into the ecological risks posed by 6PPD-quinone beyond

acute mortality. By demonstrating its capacity to disrupt essential fish behaviour, this study will highlight a more pervasive threat to aquatic ecosystems.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin, use and environmental occurrence of 6PPD and 6PPD-quinone

2.1.1. Use and Function in Rubber Manufacturing

N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD) is an antiozonant widely used in tire rubber formulations to prevent oxidative degradation of polymers during storage and service. This quinone derivative, also known as 6PPD-quinone (6PPD-Q, 6PPDQ, or 6PPD-q), has been found in urban stormwater and receiving surface waters on multiple occasions. Tire wear and road runoff release tire particles containing 6PPD into the environment, where it undergoes oxidative transformation (most notably via ozonation) to produce several transformation products (Tian *et al.* 2021).

2.1.2 Formation and Release of 6PPD-quinone

6PPD itself does not pose a threat to the environment; rather, its transformation product does. 6PPD is transformed into 6PPD-quinone when it combines with atmospheric ozone (Huang *et al.* 2021). On the tire's surface, this reaction never stops. Tire wear particles (TWPs) containing 6PPD and its quinone derivative are released during driving as a result of subsequent mechanical abrasion. These particles are subsequently washed into storm drains and eventually into receiving waters like lakes, rivers, and streams by rainfall events Concentrations can increase during the initial flush of storm events, according to spatial and temporal monitoring, and detectable residues are common in urban watersheds with substantial vehicle traffic and

stormwater inputs (Jaeger *et al.* 2024). Furthermore, industrial activities, including the synthesis of 6PPD and the recycling of E-waste, may also contribute to the dissemination of 6PPD and 6PPD-Q into the environment (Zhang *et al.* 2024). While research on this subject commenced several decades ago (Scholz *et al.* 2011), recent years have witnessed an increased understanding of the widespread distribution of 6PPD-related compounds in the environment and their pronounced toxicity to aquatic organisms.

2.1.2 6PPD in the Atmosphere

The manufacture and extensive use of tires, together with the slow deterioration of other rubber-related products, are major contributors to the atmospheric emission of p-phenylenediamine (PPD) derivatives. In Taiyuan, China, for instance, the widespread use of wires and cables has caused rubber coverings to deteriorate, raising the amount of PM2.5-bound PPD in the surrounding air (Zhang *et al.* 2021). 6PPD can react when released into the environment to make hydroxylated 6PPD intermediates, which then quickly react with ozone to produce 6PPD-Q (Rossomme *et al.* 2023).

2.1.2 Indoor and outdoor dust

In both indoor and outdoor dust samples, PD and 6PPD-Q have been found, especially in Chinese cities like Hangzhou. These regions' indoor dust samples 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) of all the PPDs and PPD-Qs that were examined. The human daily intake of infants consuming 6PPD and 6PPD-Q through indoor dust was found to be higher than that of 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). Consequently, it is essential to understand the possible risks connected to these two substances.

2.1.3 Tire and road wear particles

Tire and road wear particles (TRWPs) are a major source of 6PPD-quinone (6PPD-Q) in the environment. In soils, especially anaerobic flooded soils, microbial activity initially drives the conversion of 6PPD to 6PPD-Q via Fe (III)-reduction-coupled oxidation, while abiotic processes dominate later (Xu *et al.* 2023). Flooded soils therefore show higher 6PPD-Q levels compared to moist soils.

Although 6PPD-Q has low solubility, it is highly stable and leaches rapidly from TRWPs into runoff, particularly during storm events (Hu *et al.* 2023; Hiki and Yamamoto, 2022). Smaller tire particles (micron-sized) pose a greater risk as they release more 6PPD compared to larger ones (Stack *et al.* 2023). Studies also found 6PPD and 6PPD-Q in simulated human body fluids, raising biohazard concerns (Armada *et al.* 2023; Schneider *et al.* 2020).

Sunlight and heat accelerate the transformation of 6PPD to 6PPD-Q, weakening TRWPs and promoting their degradation (Hu *et al.* 2022; Weyrauch *et al.* 2023). Despite its breakdown under sunlight (half-life <1 month), 6PPD-Q is more persistent under thermal aging without light (half-life ~6 months) (Fohet *et al.* 2023). Among PPD-Q derivatives in tire-related products, 6PPD-Q is the most abundant (Zhao *et al.* 2023).

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

Following the restoration of fish passages in the 1990s, coho salmon (*Oncorhynchus kisutch*) mortality in a number of Seattle-area streams in Puget Sound raised concerns due to an unidentified cause (Scholz *et al.* 2011). An uncommon pre-spawn mortality syndrome was found in adult coho salmon returning to restoration locations to spawn, according to a 1999–2001 survey (Scholz *et al.* 2011). In 2002, one study started looking into this phenomenon. In

order to determine the factors related to water quality and spawner conditions that may be connected to the frequent fish deaths, assessments focused on the severity of coho salmon adult die-offs and compared spawner mortality in urban and nonurban streams. Though the data gathered revealed no connection to these symptomatic fish, coho salmon die-offs were significantly higher in urban streams than in non-urban ones. Nearly twenty years later, in 2021, a different study isolated pollutants from stormwater using iterative chemical fractionation techniques. It also suggested that the sick coho fish might be linked to a hitherto unknown toxicant, now known as 6PPD-1 2 Quinone (Tian *et al.* 2022; Tian *et al.* 2021). Research indicates that species that are important to culture and the economy, like coho salmon, brook trout, and rainbow trout, are poisoned by particularly low levels of 6PPD-Q (Brinkmann *et al.* 2022, Tian *et al.* 2021). Other species, such Arctic char and white sturgeon, can withstand amounts even higher than those in ambient samples (Tian *et al.* 2022; Brinkmann *et al.* 2022; Challis *et al.* 2021). .

Tests on additional species following Tian *et al*. (2021) first identified the acute toxic effects of 6PPD-quinone in coho salmon (*Oncorhynchus kisutch*) showed that the toxicity of 6PPD-quinone is extremely specific to only a few species. Thus far, 17 fish species and 9 other organisms have been subjected to toxicity studies using either commercial standards of 6PPD-quinone, tire leachate, or untreated urban roadway runoff (Foldvik *et al.* 2024).

2.3 Acute toxicity: discovery and species sensitivity

Acute toxicity has been reported in five species of the Oncorhynchus and Salvelinus genera 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 fact that closely similar species within these genera have considerably different sensitivity levels to 6PPD-

quinone complicates the risk assessment of this chemical. For instance, coho salmon have a median lethal concentration (LC50) of 0.041 µg/L (Lo *et al.* 2023), while chum salmon (*Oncorhynchus keta*; McIntyre *et al.* 2021) and sockeye salmon (*Oncorhynchus nerka*; French *et al.* 2022), which are closely related, do not react to the chemical. The toxicity of 6PPD-quinone has also been shown to vary significantly during the course of development; for example, the LC50 for *Coho alevins* (3 weeks old) has been reported to be 0.041 µg/L (Lo *et al.* 2023), while the LC50 for parr (>1 year) is more than double, at 0.095 µg/L (Tian *et al.* 2022).

According to studies (Cao *et al.* 2022; Hiki an Yamamoto, 2022a; Kryuchkov *et al.* 2023; Tian *et al.* 2021), 6PPD-quinone is widely present in water, soil, and air worldwide. These effects go beyond death; research has examined the sublethal effects of 6PPD-quinone in fathead minnows (*Pimephales promelas*; Anderson-Bain *et al.* 2023) and zebrafish (*Danio rerio*; Ji *et al.* 2022; Varshney *et al.* 2022; Zhang *et al.* 2023). Various independently exclusive mechanisms of 6PPD-quinone toxicity have been proposed, including disruption of the blood-brain barrier (Blair *et al.* 2021), neurotransmitter-related metabolic problems (Ji *et al.* 2022), and uncoupling of mitochondrial respiration in gills (Mahoney *et al.* 2022).

Toxicological testing is required to ascertain a species' sensitivity to 6PPD-quinone because the underlying reason of its species-specific toxicity pattern is still unknown. As the only Pacific fish that has not been examined before, we report the first findings from acute toxicity testing of 6PPD-quinone on pink salmon.

2.4 Inter-specie Responses to 6PPD-Quinone

Phylogeny alone does not seem to be a reliable indicator of 6PPD-Q's acute toxicity. The identification of the mechanism or mechanisms of action may be aided by key distinctions with interspecific data, which have the potential to be highly valuable and relevant in assessing danger

to species of concern. Certain salmon species, including coho salmon, brook trout, rainbow trout, and white-spotted char, were extremely vulnerable to 6PPD-Q exposure in water. However, at very high non-environmentally relevant concentrations (Brinkmann *et al.* 2022; Tian *et al.* 2021; Tian *et al.* 2022; Lo *et al.* 2023), no discernible negative reaction was seen by other salmonids, including arctic char, brown trout, Atlantic salmon, southern Asian dolly varden, masu salmon, and congeneric sockeye salmon. It seems sense that variations in sensitivity should be further studied and utilized to provide light on the mechanisms underlying 6PPD-Q toxicity.

The interspecific reactions to 6PPD-Q exposure could be caused by a variety of factors, including variations in the delivery of 6PPD-Q to the electron transport chain in gill mitochondria (via active, facilitated, or passive transport), the conformation of mitochondrial targets, which could affect how the toxicant interacts with the target site, or the disruption of the blood-brain barrier, as indicated by hemoconcentration and macromolecule accumulation in cerebral tissues as well as transcriptomic downregulation of occludin and junctional adhesion molecules, which would dramatically increase endothelial permeability (Mahoney *et al.* 2022; Greer *et al.* 2023). One element that could also contribute to interspecies differences is how 6PPD-Q is biologically processed.

2.5 Toxicokinetics (TK) of 6PPD-Q

Toxicokinetics (TK), described by the ADME processes (absorption, distribution, metabolism, excretion), governs how the body interacts with xenobiotics like 6PPD-quinone (6PPD-Q)26. A key TK process is hepatic biotransformation, which can detoxify a xenobiotic or bioactivate it into a more toxic metabolite (Noedberg *et al.* 2004; Hosey *et al.* 2014).

To understand interspecific differences in fish sensitivity to 6PPD-Q, researchers can characterize its metabolite profiles in tissues like gallbladder bile, where metabolites accumulate (Hosey *et al.* 2014). Hypotheses for these differences include variations in absorption, distribution, metabolism, and excretion. Quantifying internal concentrations of 6PPD-Q and its metabolites in vivo can reveal a species' capacity for detoxification or bioactivation, linking biotransformation to sensitivity. This data could be used to create parent-to-metabolite ratios as a tool for predicting a species' sensitivity in the field and to inform regulations on 6PPD use.

Furthermore, ex situ and in vitro methods—such as measuring hepatic clearance, extraction fraction, or intrinsic clearance using S9 fractions, microsomes, or hepatocytes—can provide a quantitative understanding of 6PPD-Q biotransformation (Nordberg *et al.* 2004; Hosey *et al.* 2014; Stadnicka-Michalak *et al.* 2018). These parameters are influenced by factors like hepatic blood flow (QH), plasma protein binding (fu(b)), and enzymatic activity (Nichols *et al.* 2009). Integrating data on plasma concentrations of 6PPD-Q with its biotransformation is crucial for examining its action on the gills and its hepatic processing, as interspecific TK differences are a hypothesized driver of sensitivity (Mahoney *et al.* 2022).

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).

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.

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.

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 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

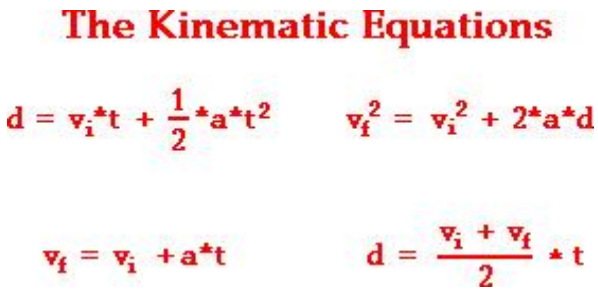
At the end of the exposure periods, a range of biomarkers was evaluated to assess the sublethal effects of 6PPD-q exposure. These included enzymes indicative of liver function such as Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST), Albumin; oxidative stress markers including Malondialdehyde (MDA), Glutathione Peroxidase (GPx), Glutathione (GSH), and Superoxide Dismutase (SOD); and lipid profile components such as total cholesterol (CHOL), triglycerides (TG), high-density lipoprotein (HDL), very-low-density lipoprotein (VLDL), and low-density lipoprotein cholesterol (LDL-C), to evaluate lipid peroxidation. Additionally, creatinine and urea levels were measured to assess renal and hepatic function, along with total protein to evaluate general physiological condition. These biomarkers were chosen for their relevance in detecting hepatotoxicity, oxidative stress, and systemic physiological changes in fish exposed to environmental contaminants like BPA.

3.6.1 Determination of swimming speed

An iPhone was used to record the movement of the fishes for at least 30 seconds. The videos were then imported into Kinovea software for analysis of swimming speed and swimming path.

3.6.2 Determination of kinematics

The four kinematic equations that describe an object's motion are:



There are a variety of symbols used in the above equations. Each symbol has its own specific meaning. The symbol d stands for the displacement of the object. The symbol t stands for the time for which the object moved. The symbol a stands for the acceleration of the object. And the symbol v stands for the velocity of the object; a subscript of i after the v (as in v_i) indicates that the velocity value is the initial velocity value and a subscript of f (as in v_f) indicates that the velocity value is the final velocity value.

3.6.3 Data Analysis

All values were expressed as the mean ± standard error of the mean (SEM). The difference between groups was analyzed with one-way ANOVA followed by Dunnett's multiple comparison test. The data were analyzed using GraphPad Prism version 8.01. The level of significance was set at $p< 0.05$. Graph analyses were plotted using the GraphPad Prism software and excel.

CHAPTER FOUR

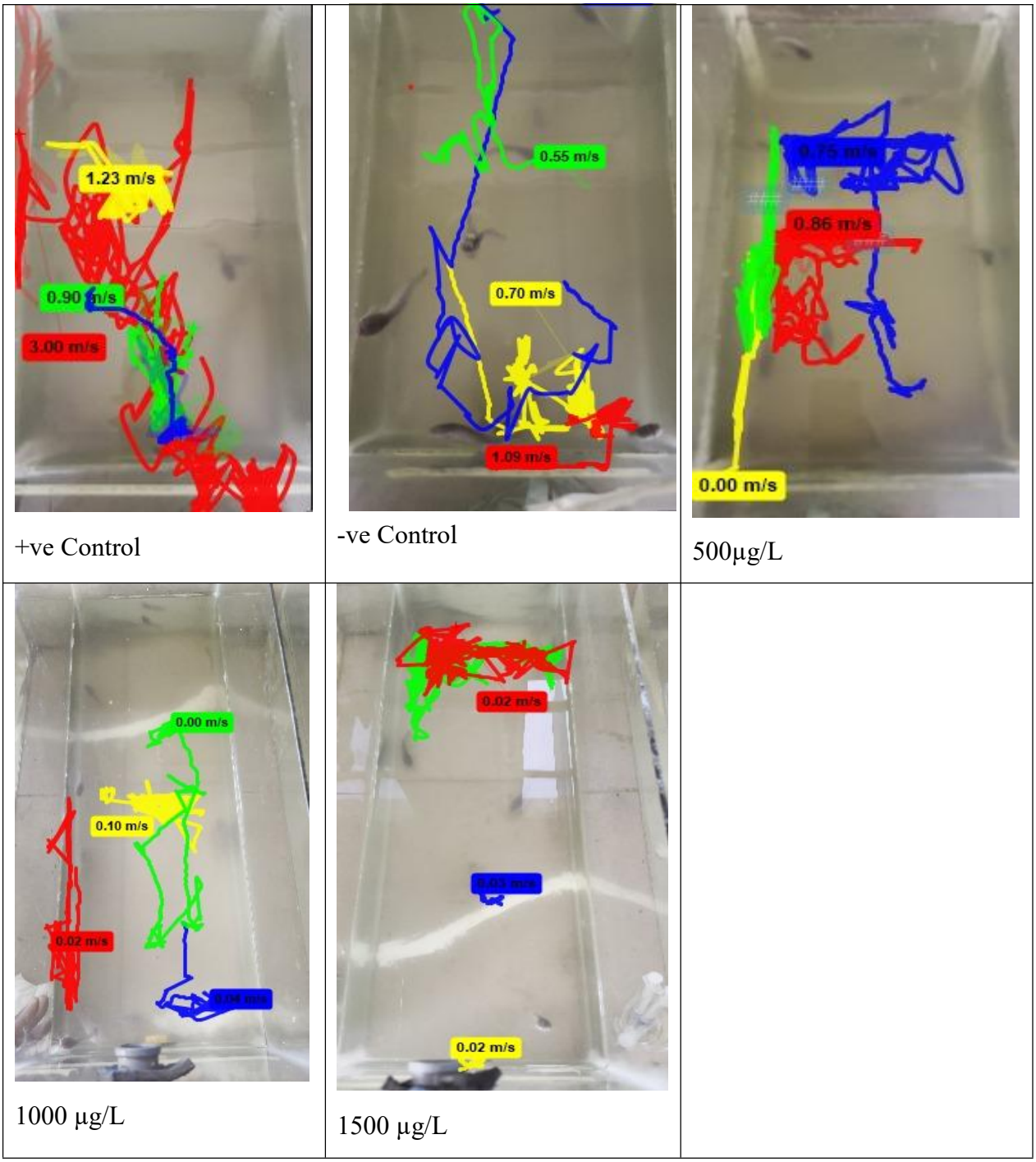
4.0 Swimming Speed

The effect of 6ppd-q on swimming speed in *Clarias gariepinus* juvenile showed a general concentration- and time-dependent decrease across the exposed groups compared to the positive control. The mean swimming speeds in *C. gariepinus* exposed to 0 µg/L 6PPD-Q (+ve control), 200 µg/L DMSO (-ve control) and 500, 1000 and 1500 µg/L 6PPD-Q were 2.115 ± 1.252, 0.8950 ± 0.2758, 0.8050 ± 0.07778, 0.1100 ± 0.02121, and 0.02500 ± 0.007071 m/s for 7 days; 4.110 ± 1.725, 0.4750 ± 0.1061, 0.06000 ± 0.02121, 0.06000 ± 0.02121, and 0.01500 ± 0.007071 m/s for 14 days; 0.8950 ± 0.2758, 0.6050 ± 0.07778, 0.1350 ± 0.02121, 0.05500 ± 0.007071, and 0.03500 ± 0.007071 m/s for 21 days; and 0.5800 ± 0.1838, 0.2500 ± 0.1556, 0.1650 ± 0.04950, , and 0.04000 ± 0.02121 m/s for 28 days (Figure X).

All 6PPD-Q treatment groups were statistically significantly different from the positive control ($p < 0.05$), except for fish exposed to 200 µg/L and the negative control on days 7, 21, and 28, which were not significantly different ($p > 0.05$). However, by day 21, all groups, including the negative control, were significantly lower than the positive control ($p < 0.05$).

There was a 57.7% reduction in swimming speed in the negative control group and reductions of 61.9%, 94.8%, and 98.8% in the 500, 1000, and 1500 µg/L 6PPD-Q groups, respectively, compared to the positive control on day 7. On day 21, reductions were 88.4% for the negative control and 98.5%, 98.5%, and 99.6% for the corresponding 6PPD-Q concentrations. Swimming speed reductions compared to the control on day 21 were 32.4% for the negative control and 84.9%, 93.9%, and 96.1% for the 500, 1000, and 1500 µg/L 6PPD-Q groups, respectively. By

day 28, reductions were 56.9% in the negative control group and 71.6%, 86.2%, and 93.1% in the 500, 1000, and 1500 μg/L 6PPD-Q-exposed groups, respectively.



+ve Control | -ve Control | 500µg/L

1000 µg/L | 1500 µg/L

Figure 4.1: Video tracks of representative *C. gariepinus* showing the swimming speed of juvenile exposed to 6PPD-Q. The blue, yellow, green and red lines represents the endpoints of each movement tracks at week one (7days).

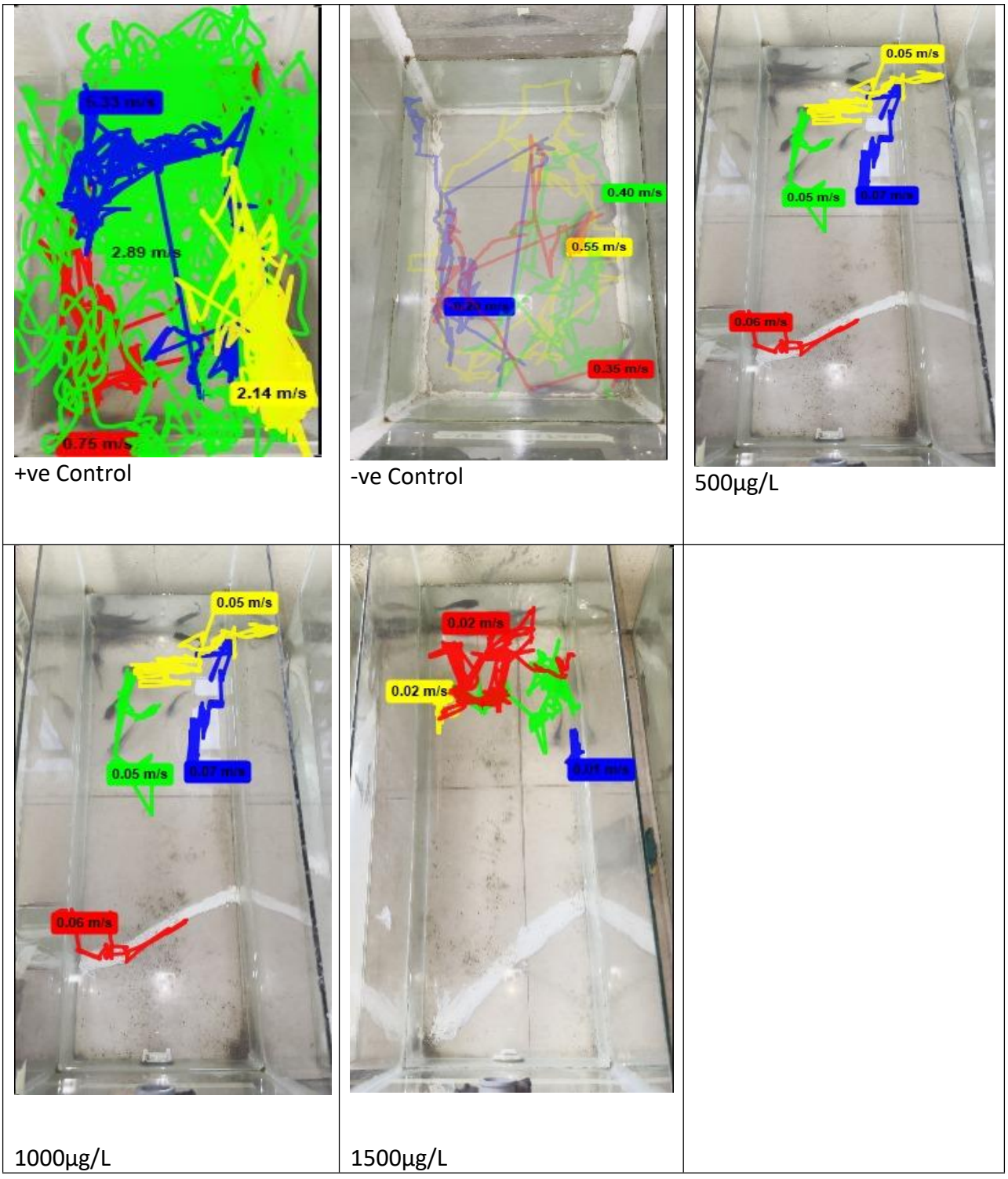
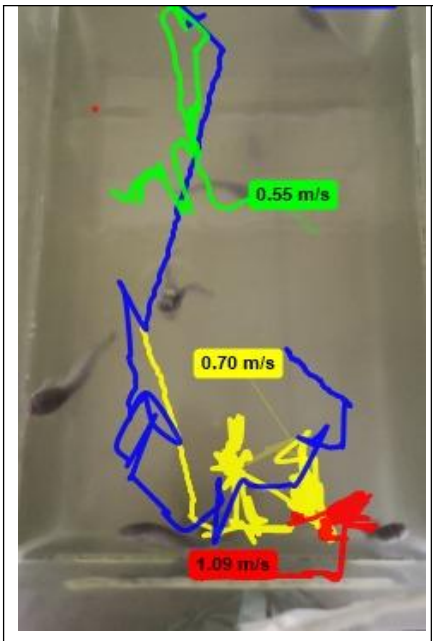
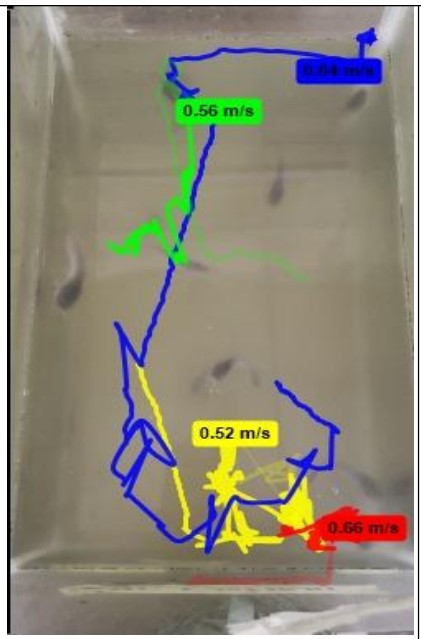


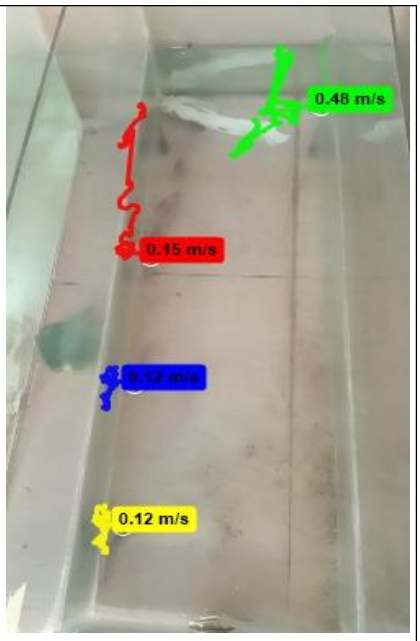
Figure 4.16: Video tracks of representative *C. gariepinus* showing the swimming speed of juvenile exposed to 6PPD-Q. The blue, yellow, green and red lines represents the endpoints of each movement tracks at week two (14days).



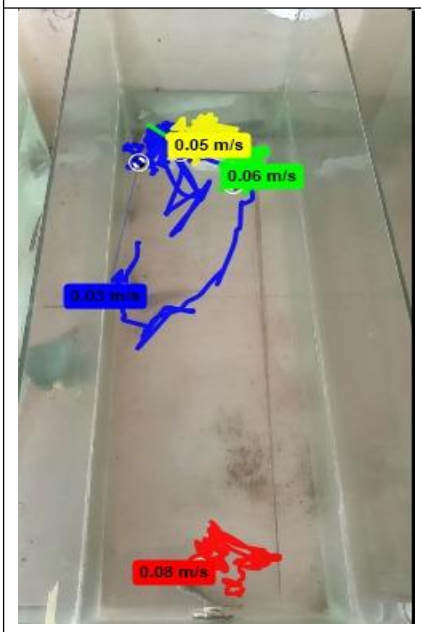
+ve Control



-ve Control



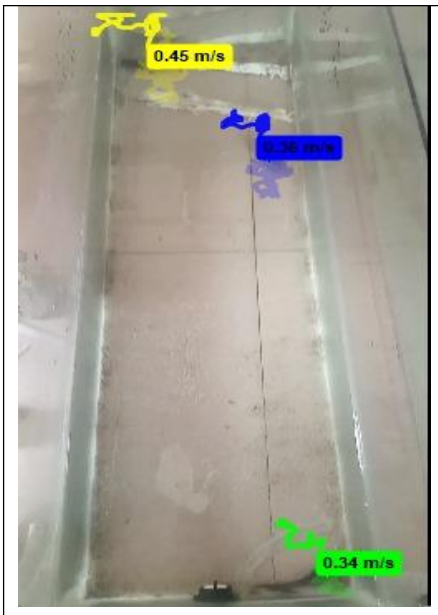
500µg/L



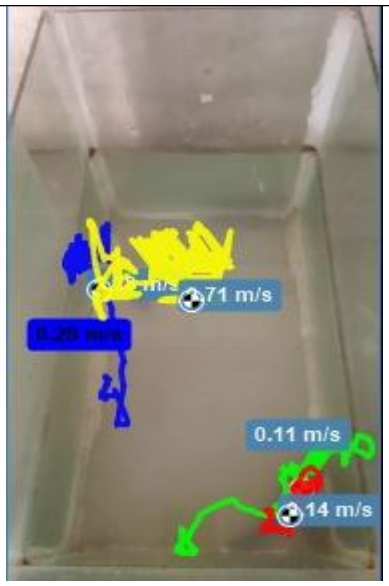
1000µg/L

1500µg/L

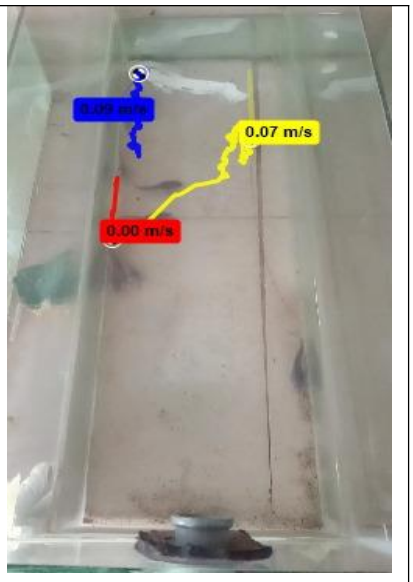
Figure 4.17: Video tracks of representative *C. gariepinus* showing the swimming speed of juvenile exposed to 6PPD-Q. The blue, yellow, green and red lines represents the endpoints of each movement tracks at week three (21days).



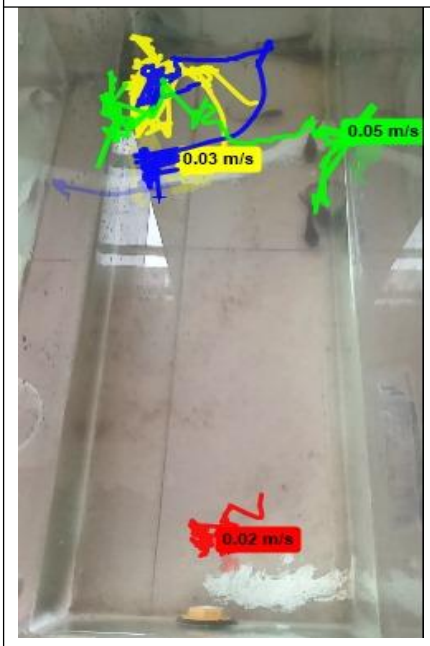
+ve Control



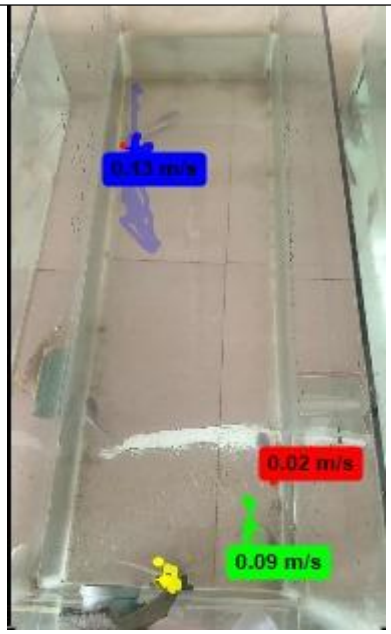
-ve Control



500 µg/L



1000 µg/L



1500µg/L

Figure 4.18: Video tracks of representative *C. gariepinus* showing the swimming speed of juvenile exposed to 6PPD-Q. The blue, yellow, green and red lines represents the endpoints of each movement tracks at week four (28 days).

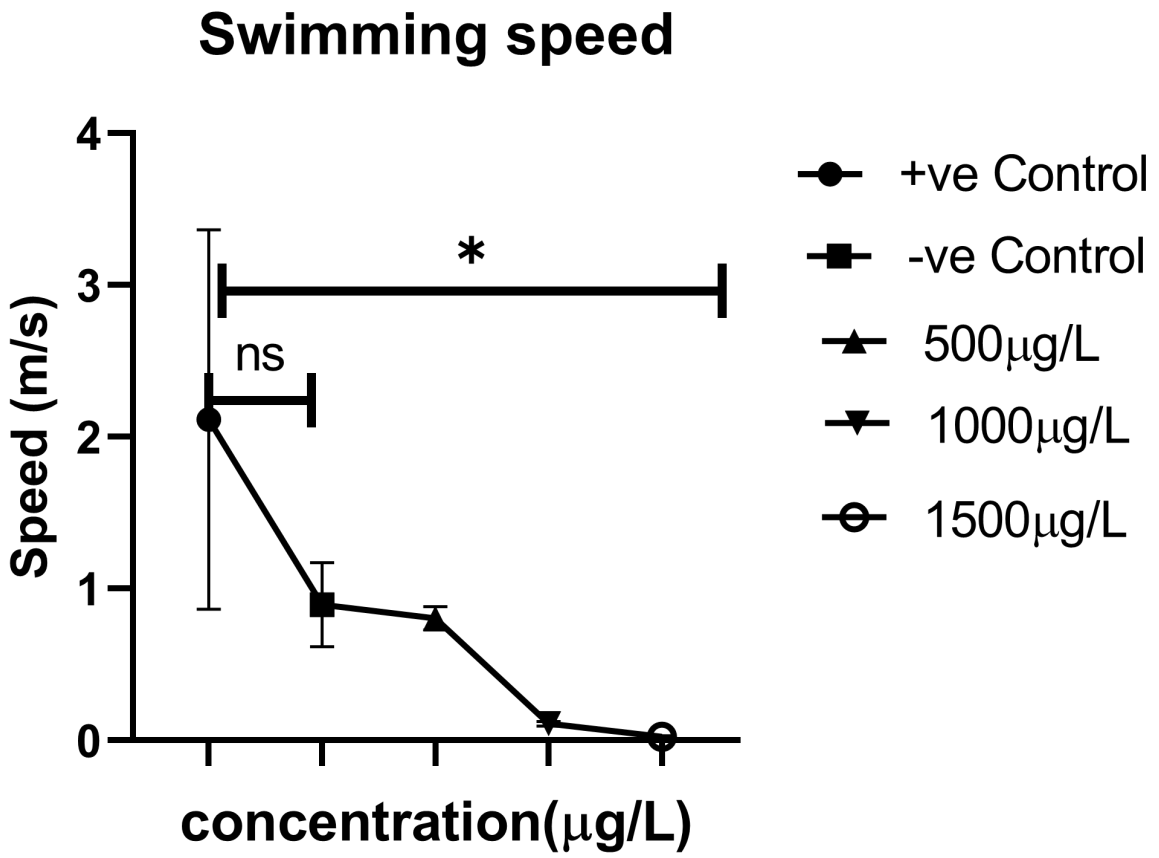


Figure 4.19: Mean swimming speed of *C. gariepinus* exposed to 6ppd-q line graph indicate mean± SD (n=7) on day 7

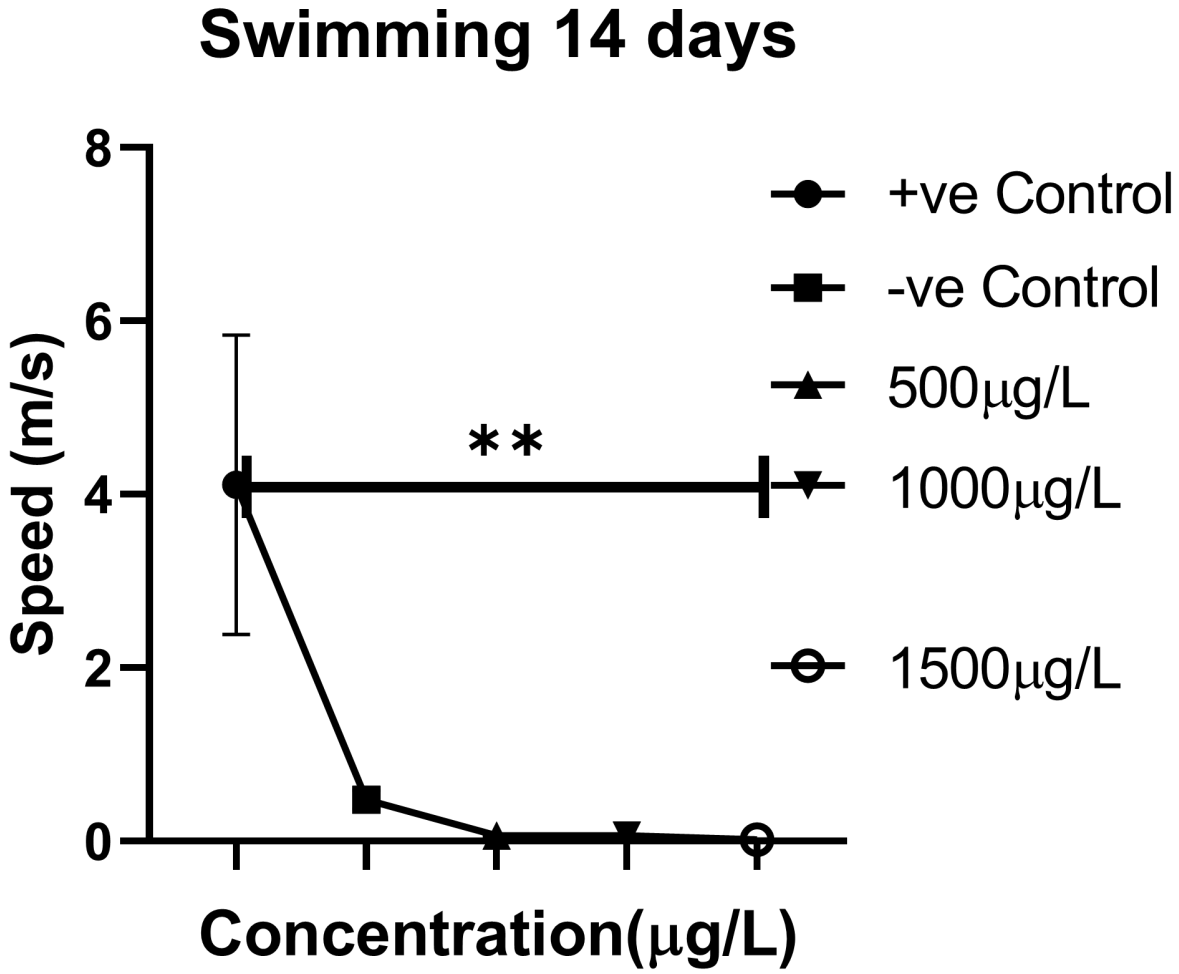


Figure 4.20: Mean swimming speed of *C. gariepinus* exposed to 6ppd-q line graph indicate mean± SD (n=7) on day 14.

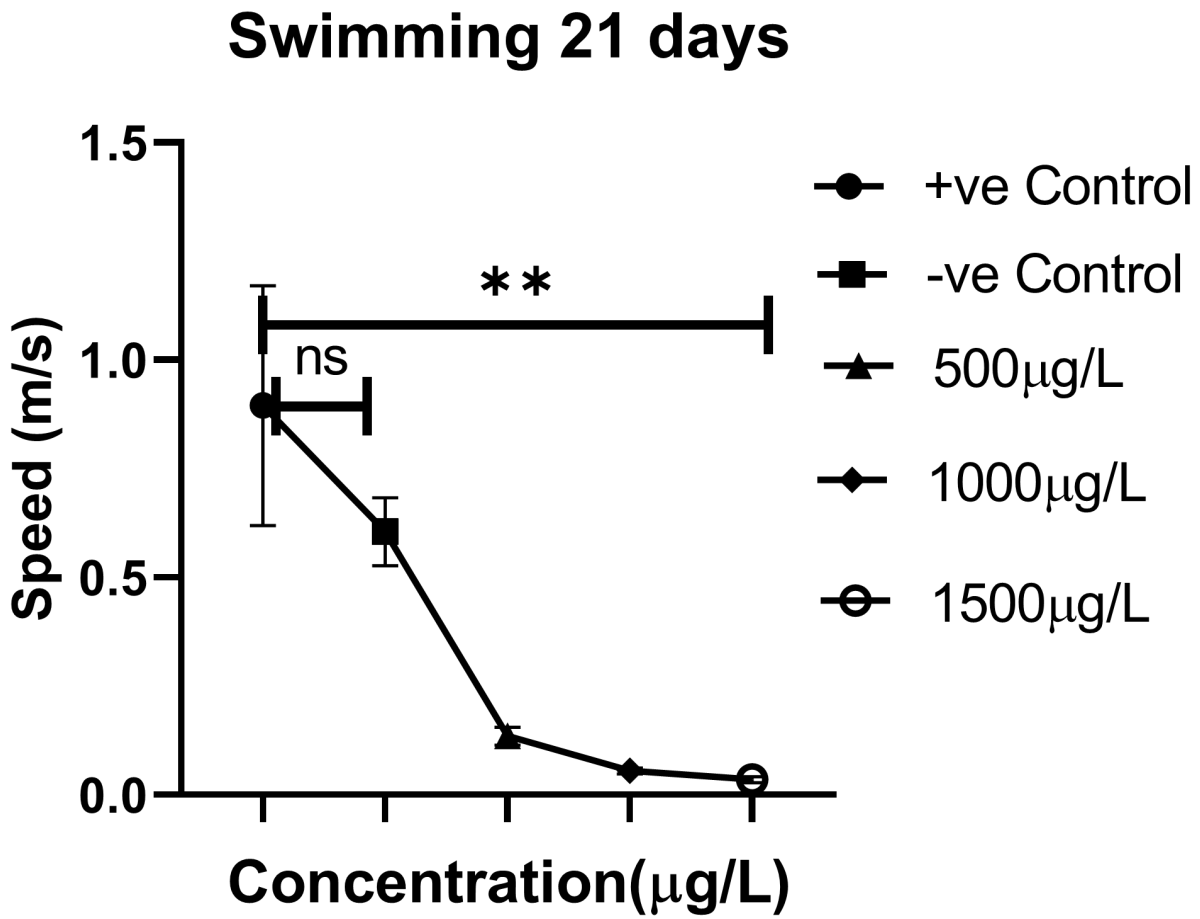


Figure 4.21: Mean swimming speed of *C. gariepinus* exposed to 6ppd-q line graph indicate mean± SD (n=7) on day 21

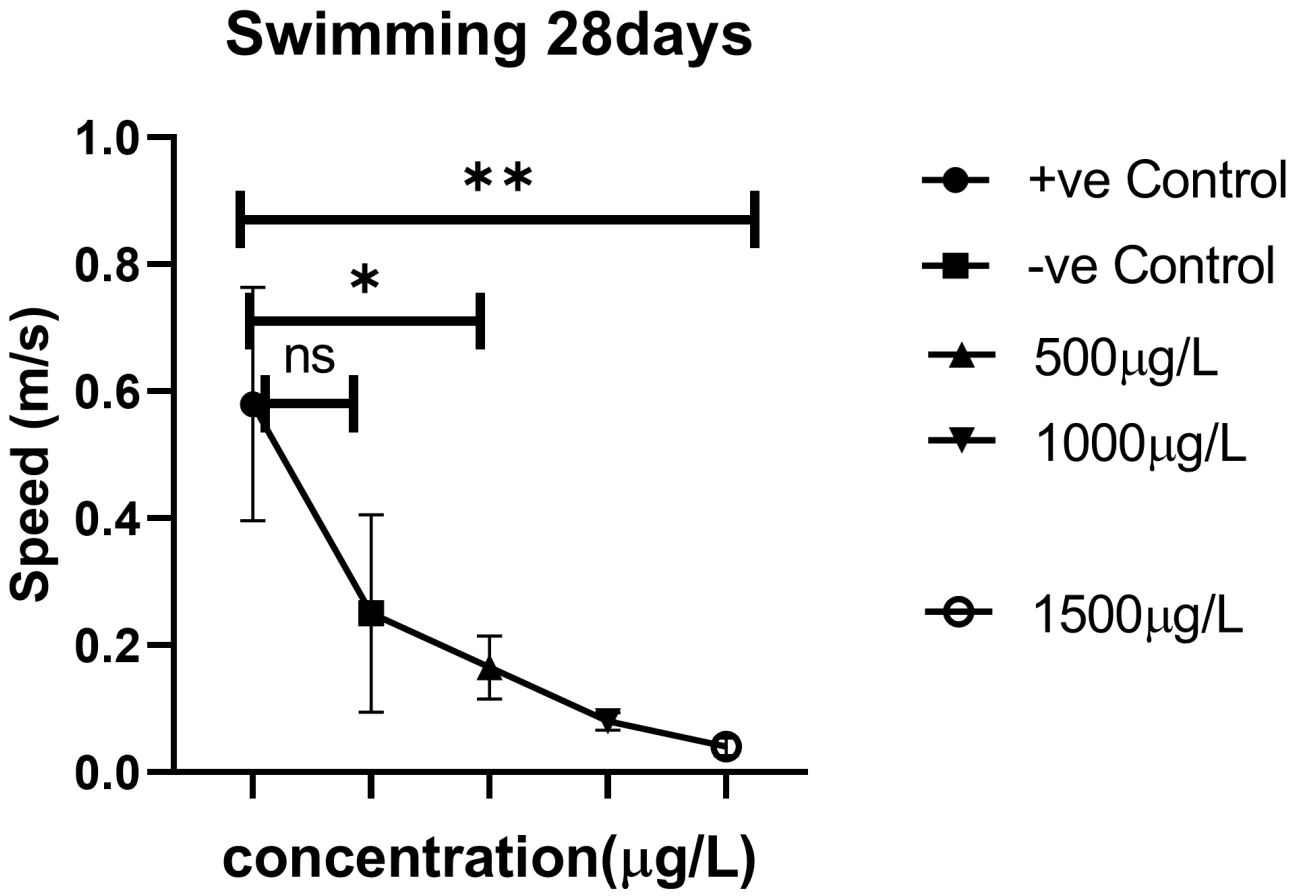


Figure 4.22: Mean swimming speed of *C. gariepinus* exposed to 6ppd-q line graph indicate mean± SD (n=7) on day 28

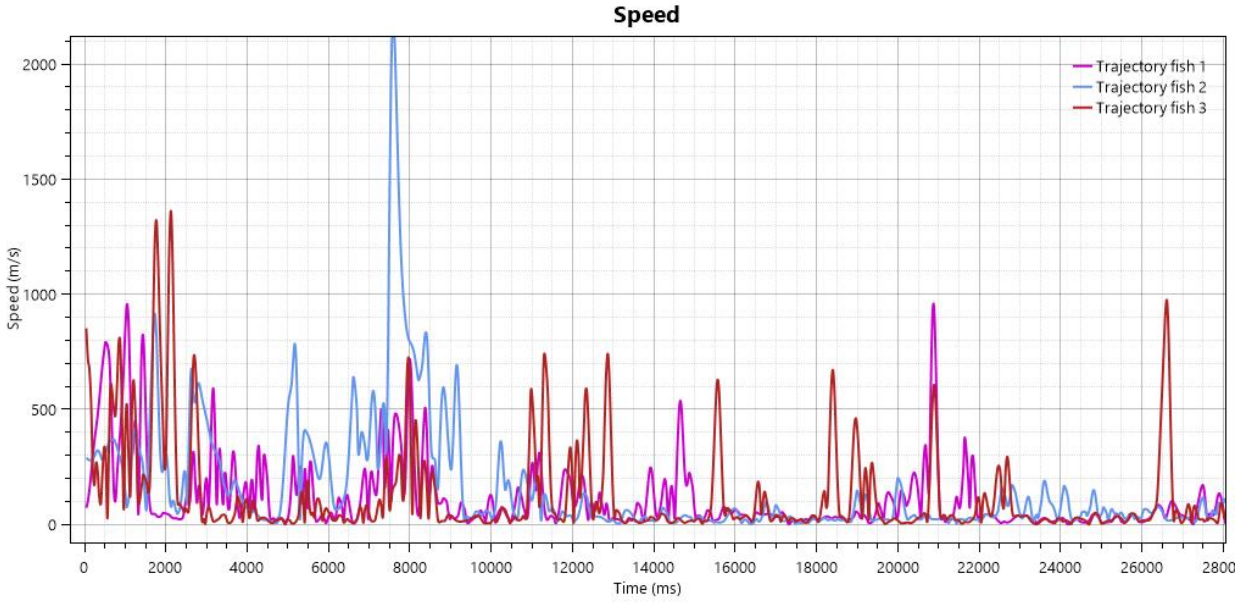


Figure 4.23: Average speed for catfish day 7(positive control)

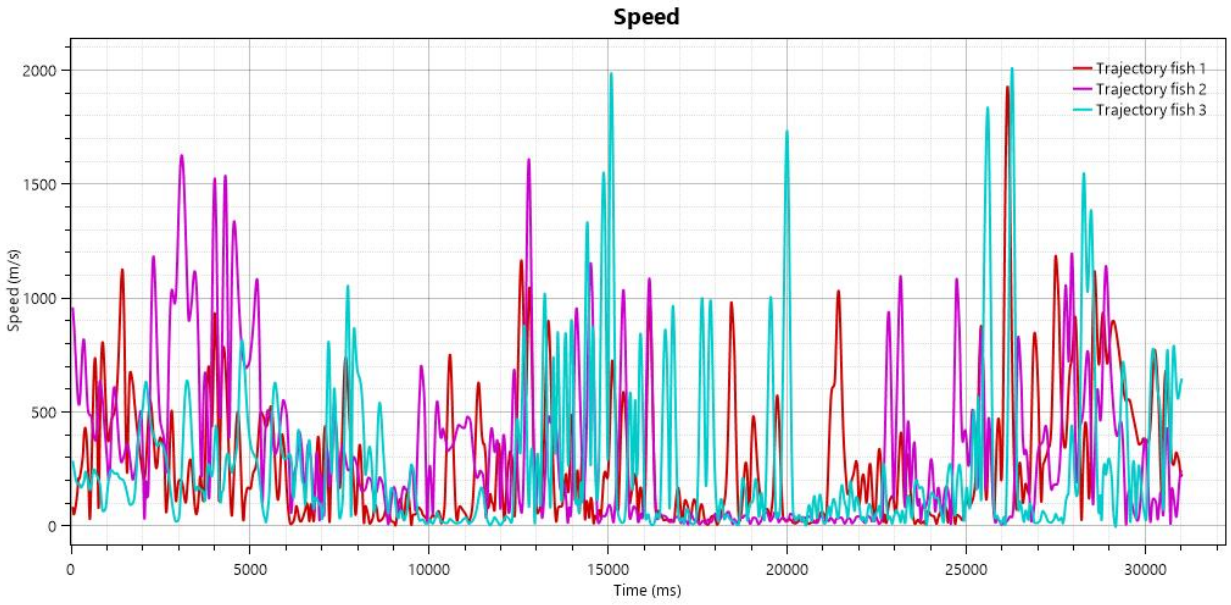


Figure 4.24 : Average speed of catfish day 7(negative control)

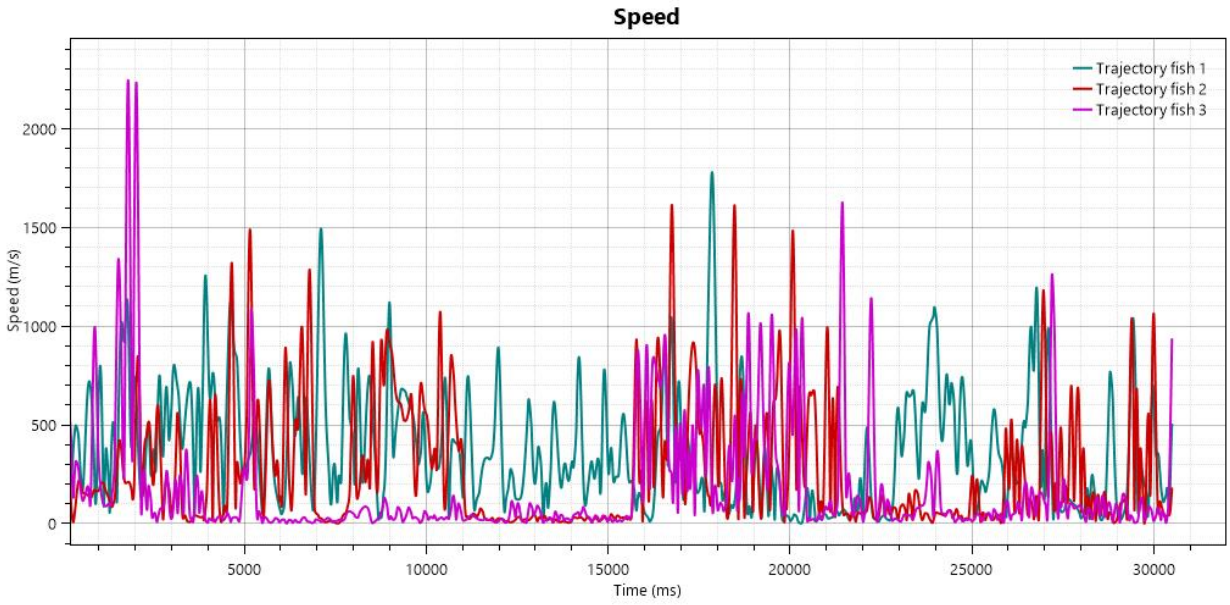


Figure 4.25:Average of catfish for day 7(500µL)

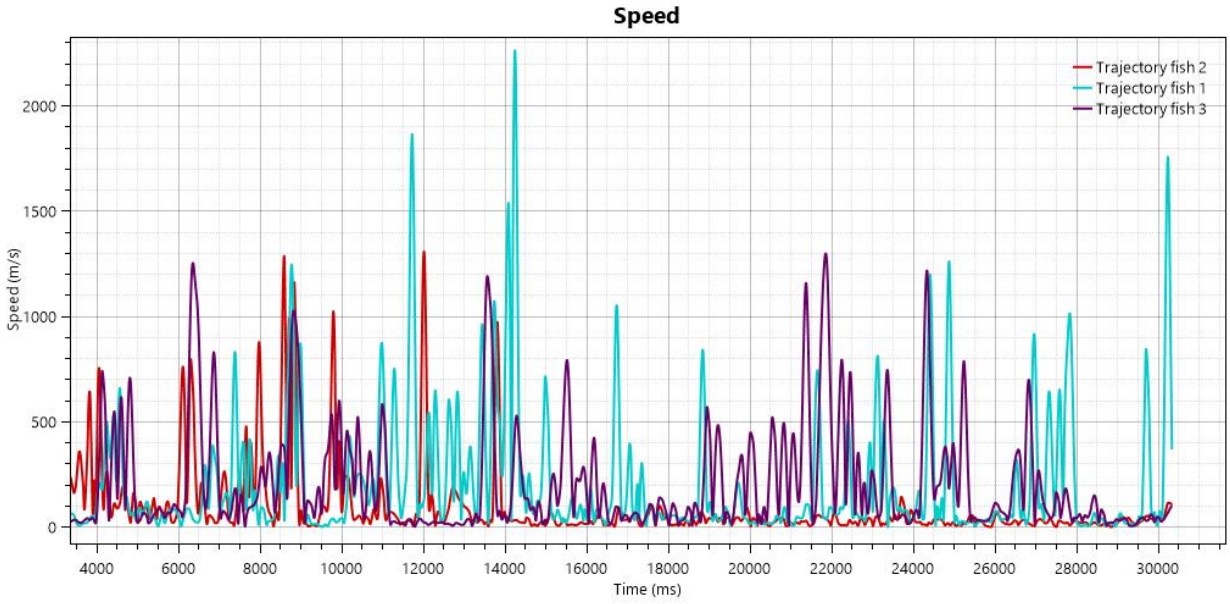


Figure 4.26 :Average speed of catfish day 7(1000µL)

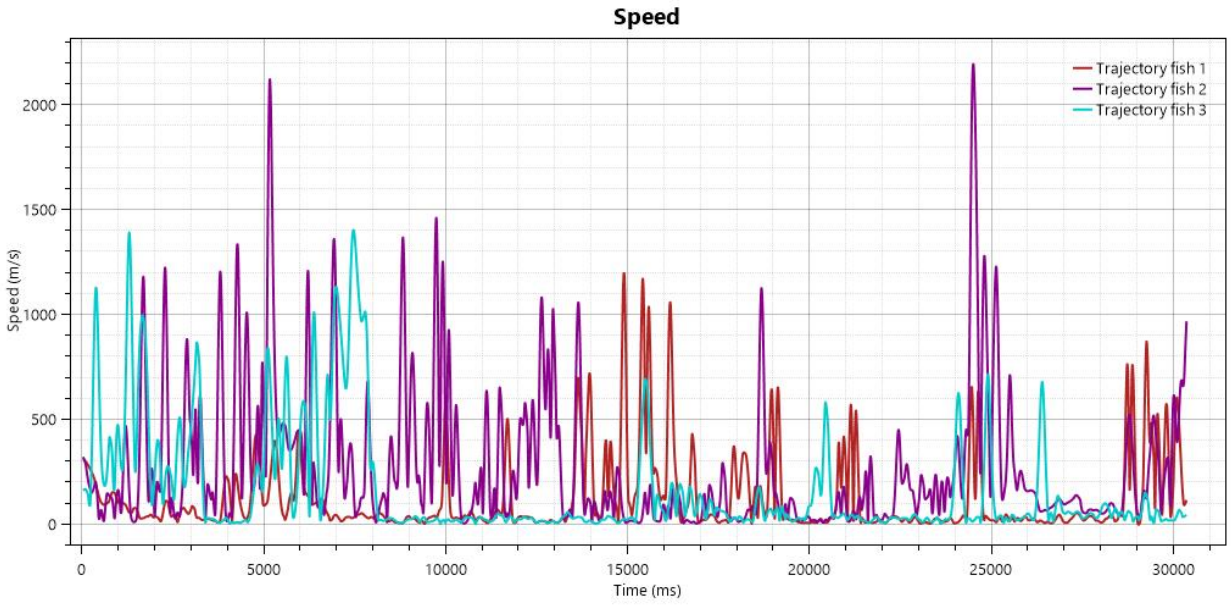


Figure 4.27 :Average speed of catfish day 7(1500µL)

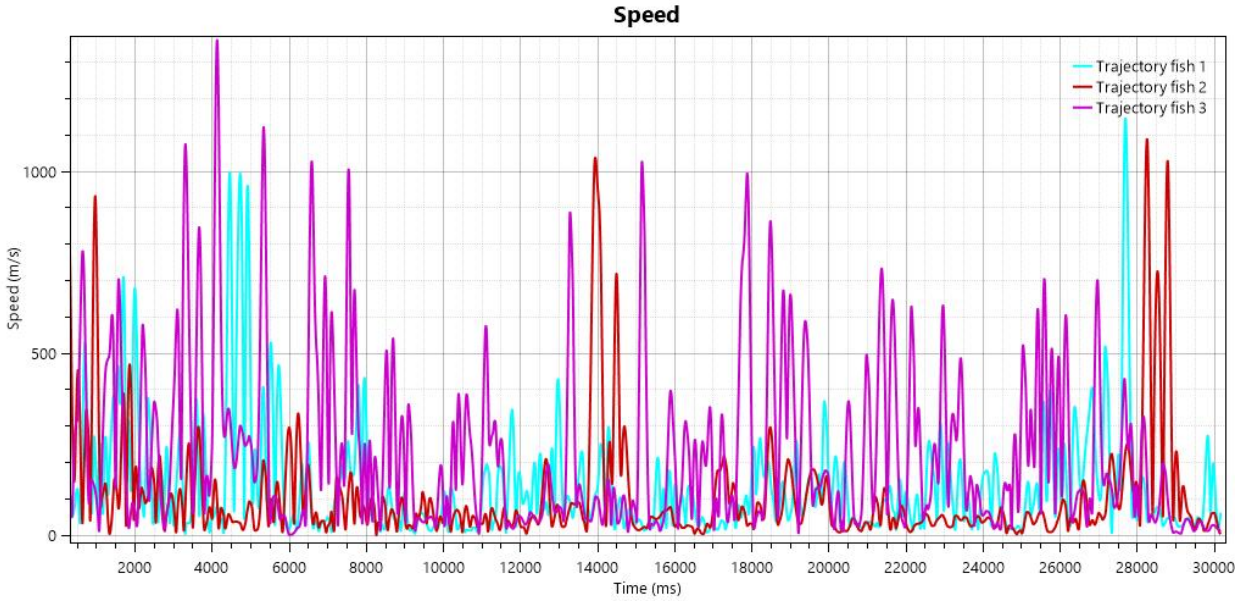


Figure 4.28 :Average speed of catfish day 14(positive control)

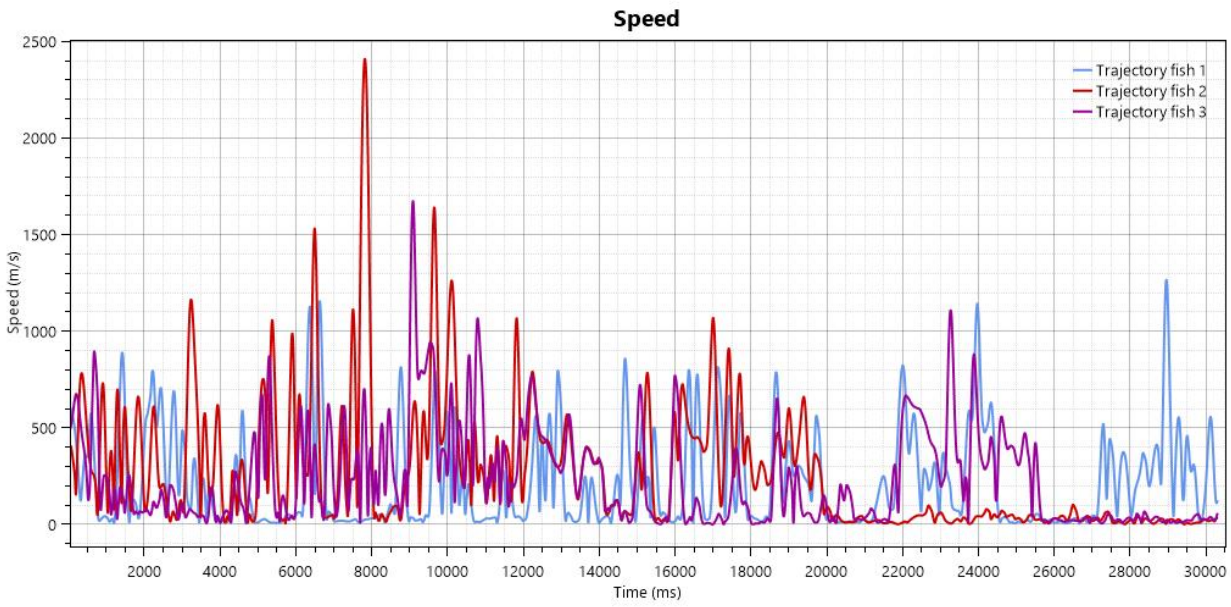


Figure 4.29 :Average speed of catfish day 14(negative control)

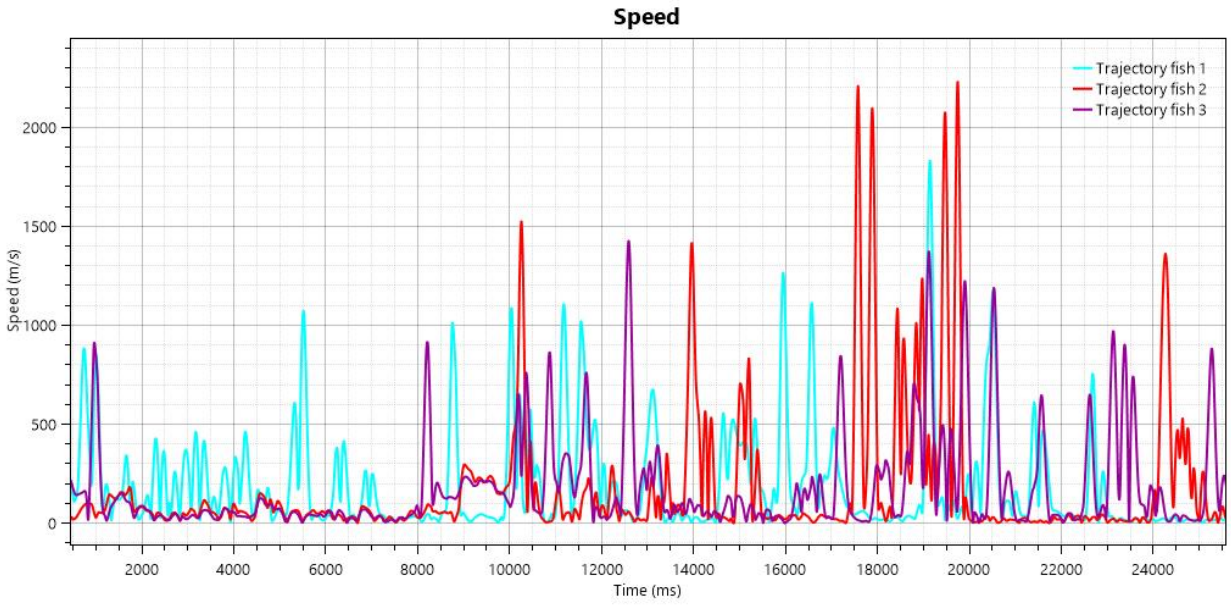


Figure 4.30 :Average speed of catfish day 14(500µL)

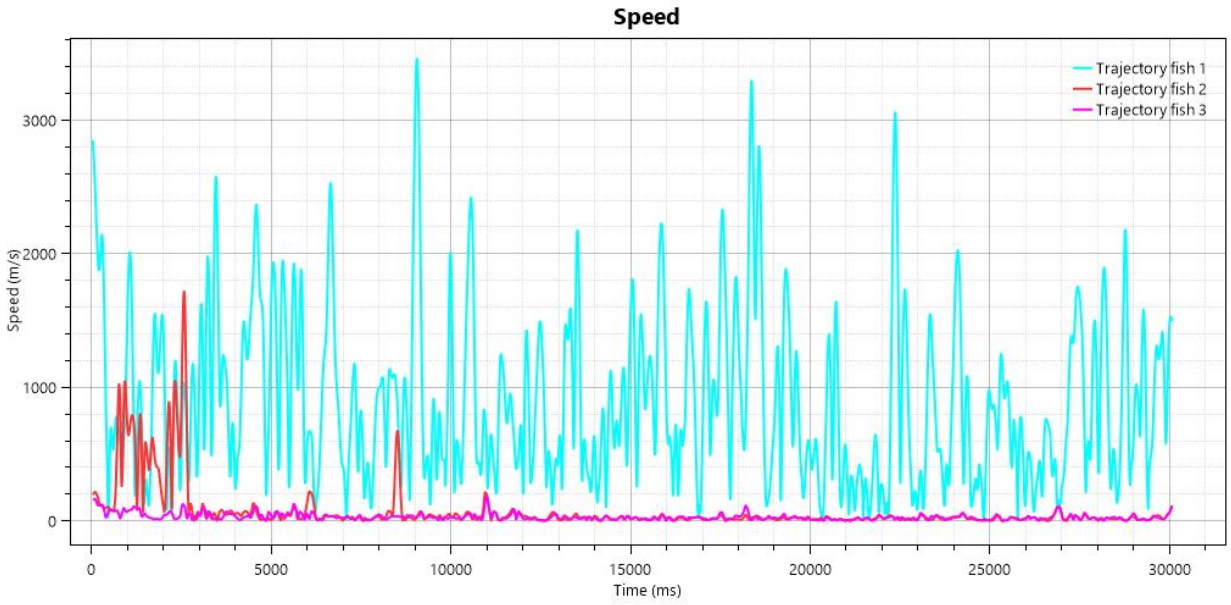


Figure 4.31:Average speed of catfish day 14(1000µL)

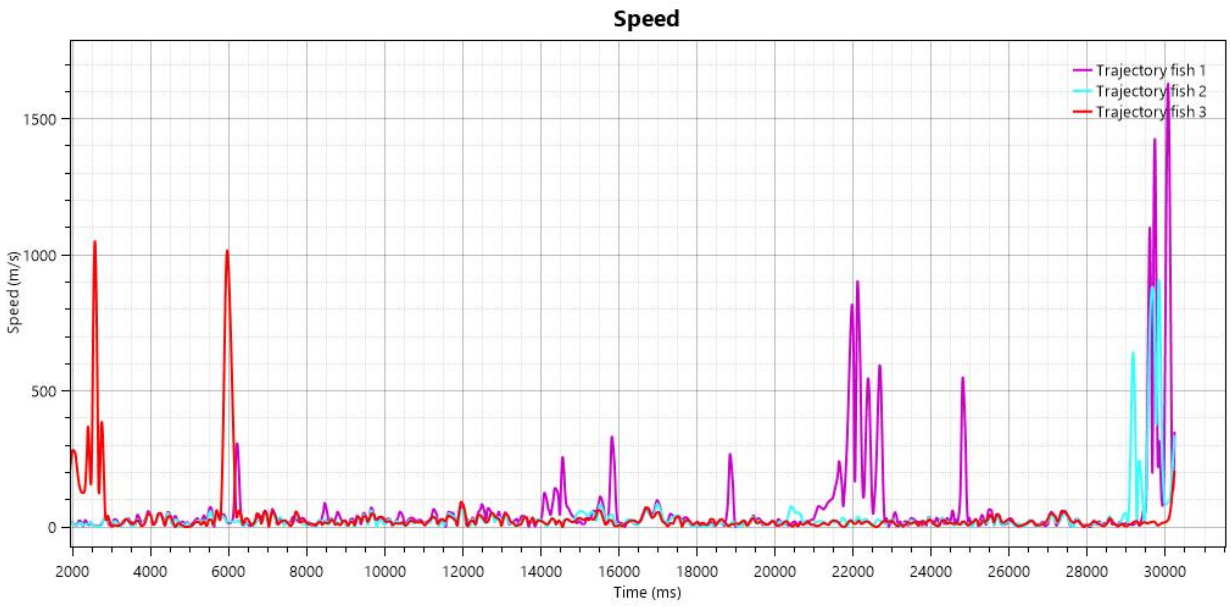


Figure 4.32:Average speed of catfish day 14(1500µL)

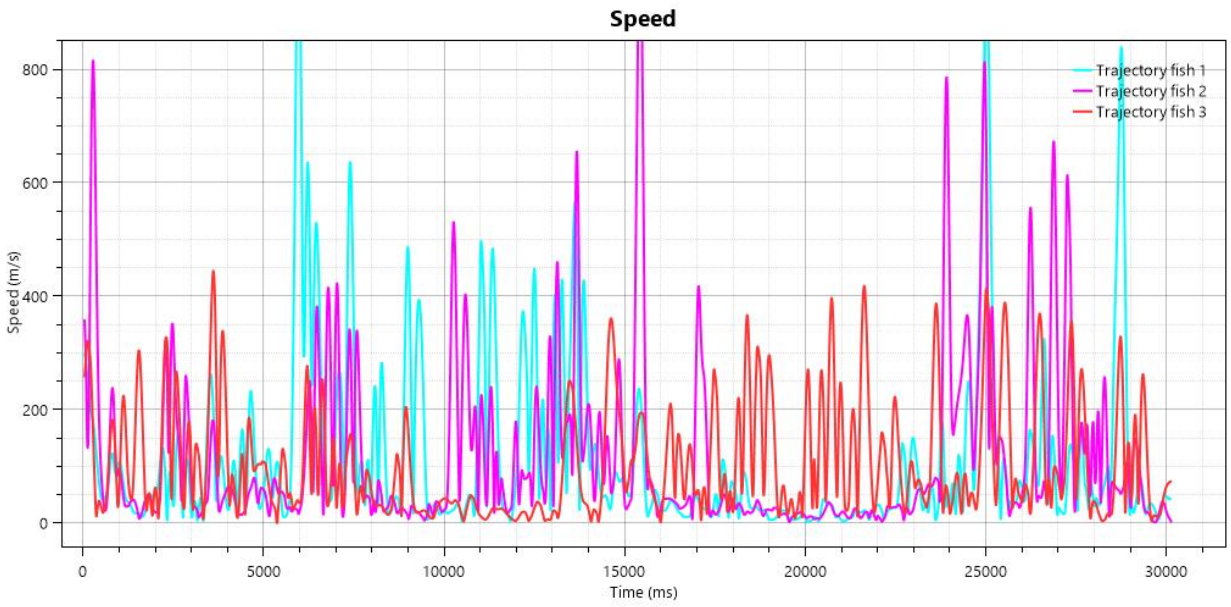


Figure 4.33:Average speed of catfish day 21(positive control)

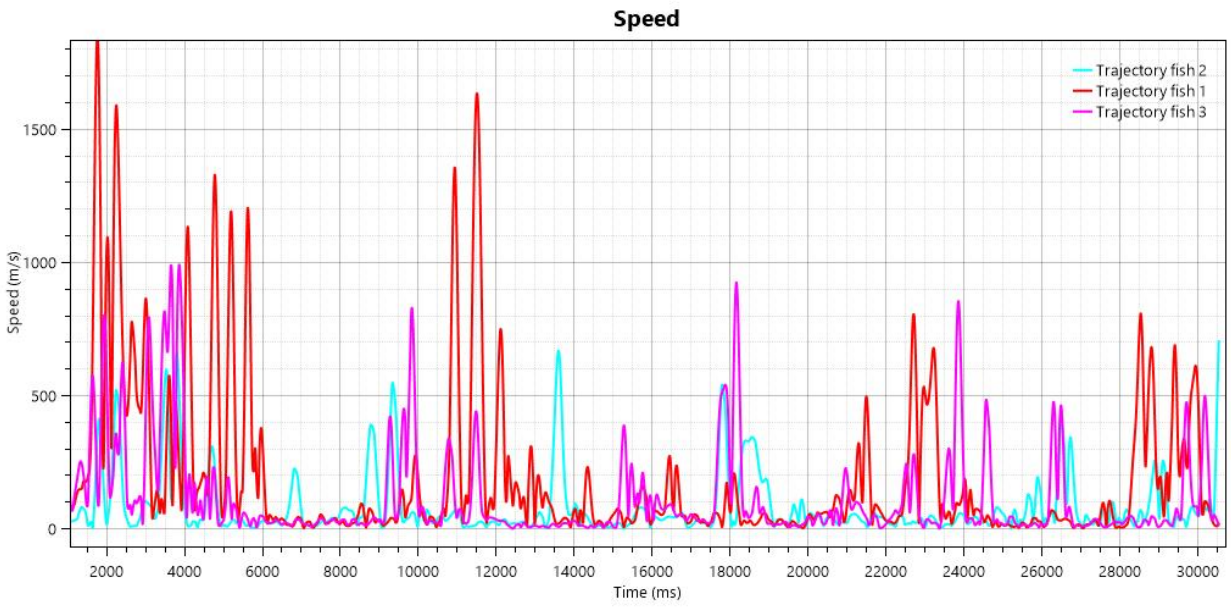


Figure 4.34:Average speed of catfish day 21(negative control)

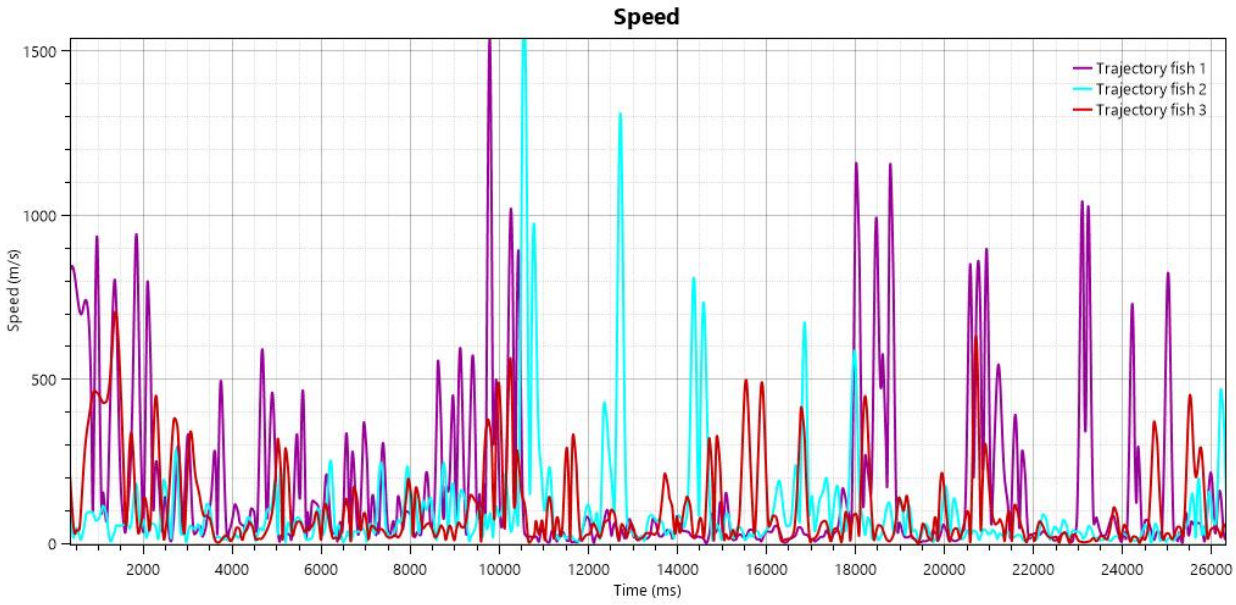


Figure 4.35:Average speed of catfish day 21(500µL)

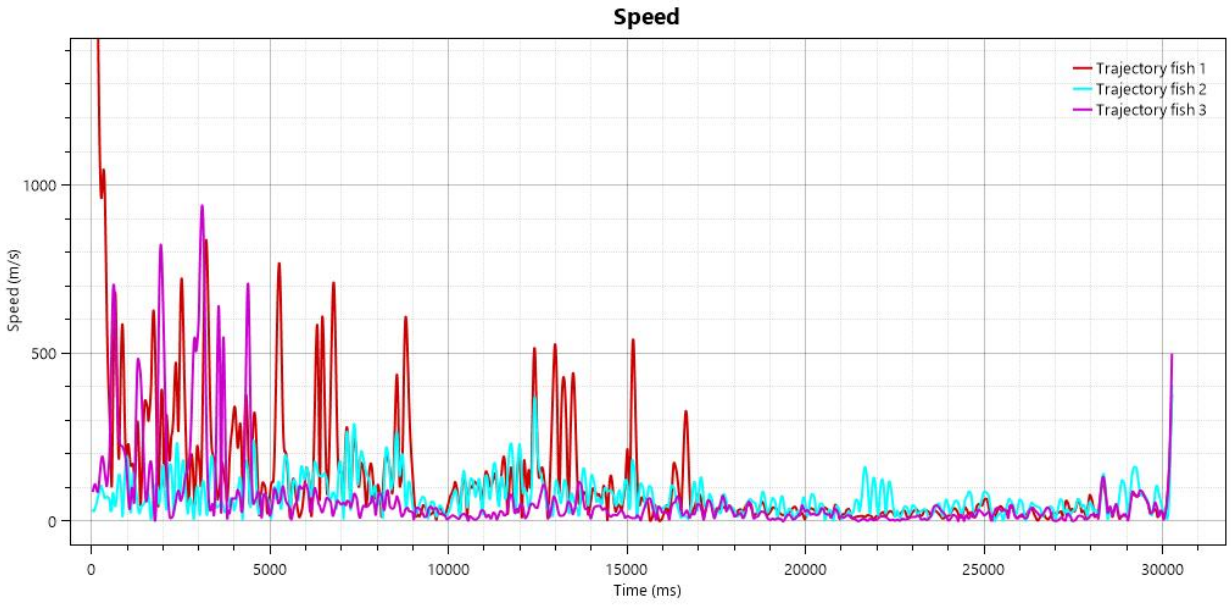


Figure 4.36:Average speed of catfish day 21(1000µL)

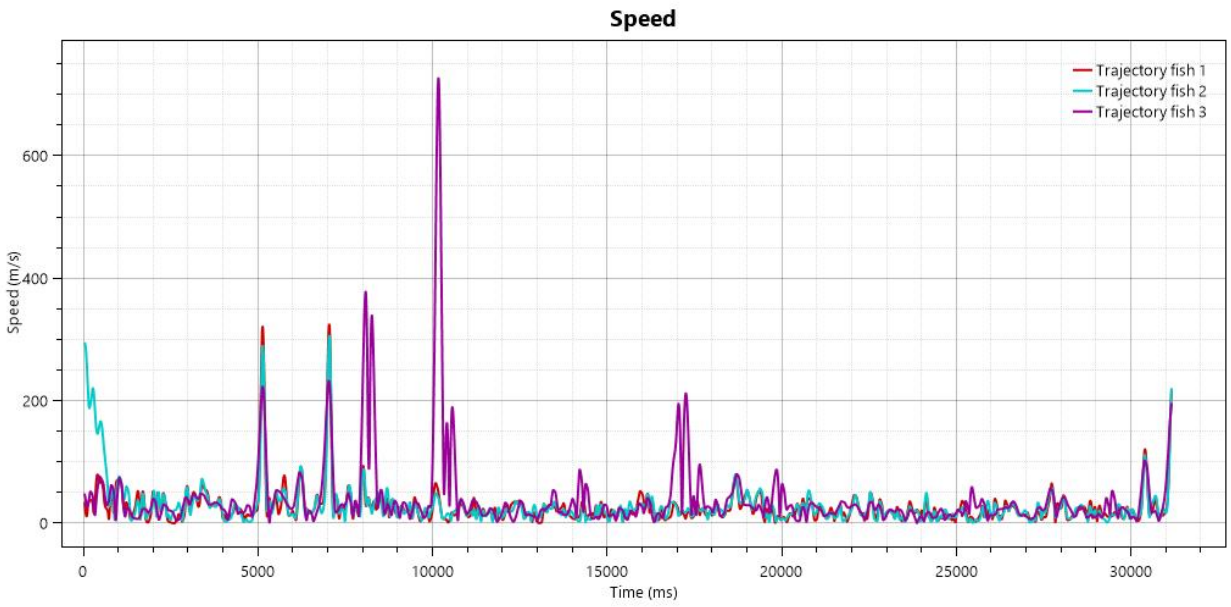


Figure 4.37 :Average speed of catfish day 21(1500µL)

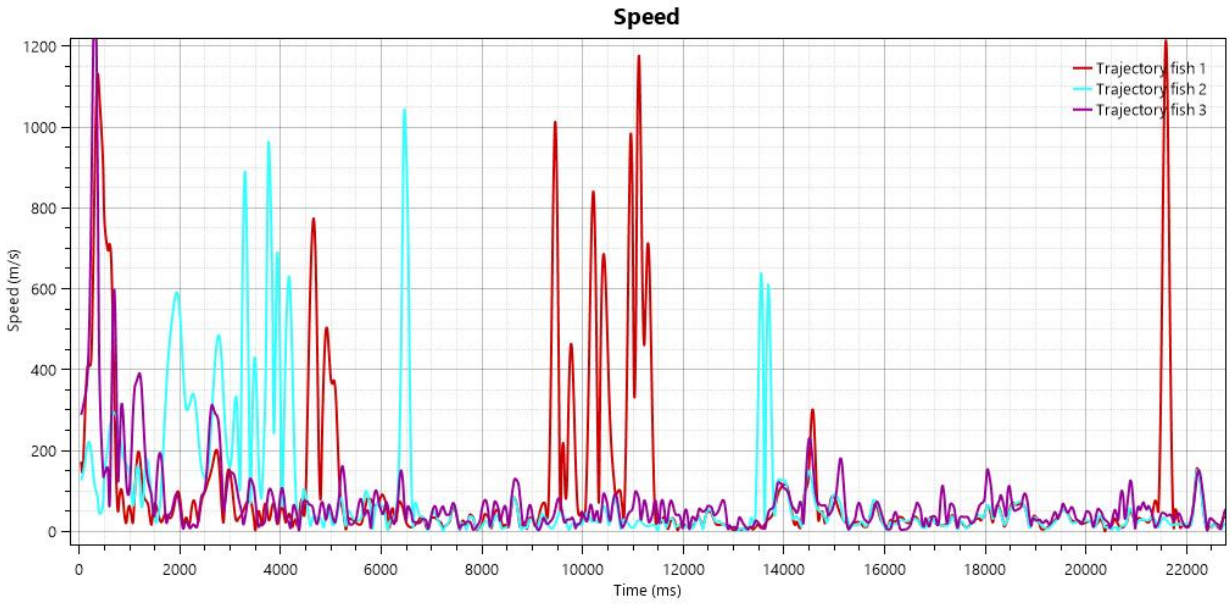


Figure 4.38 :Average speed of catfish day 28(positive control)

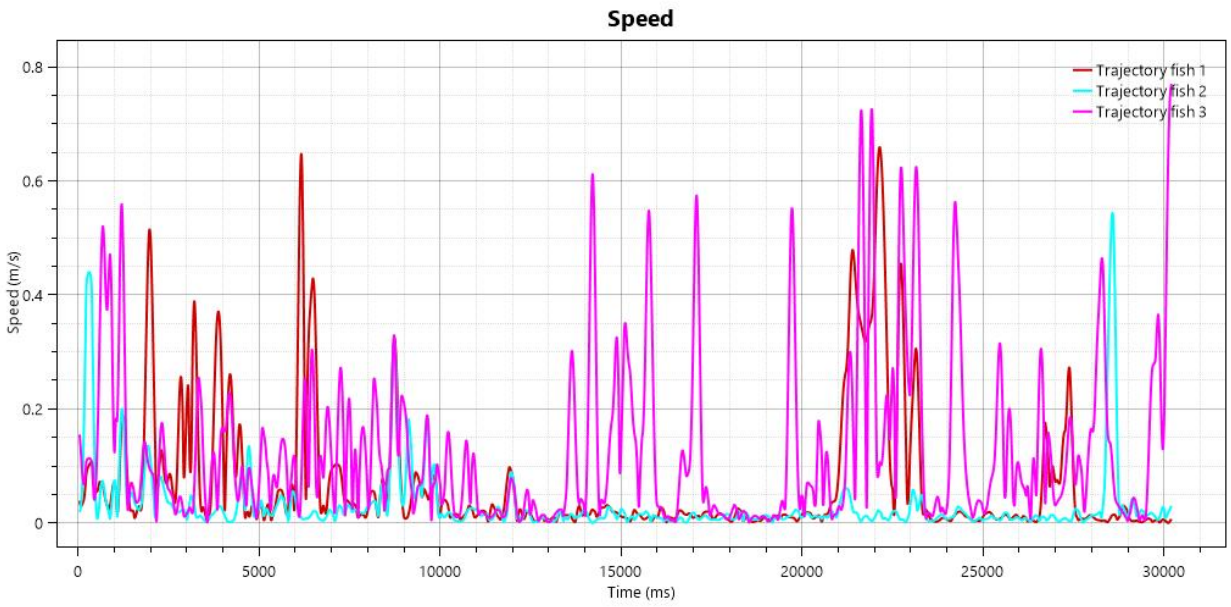


Figure 4.39 :Average speed of catfish day 28(negative control)

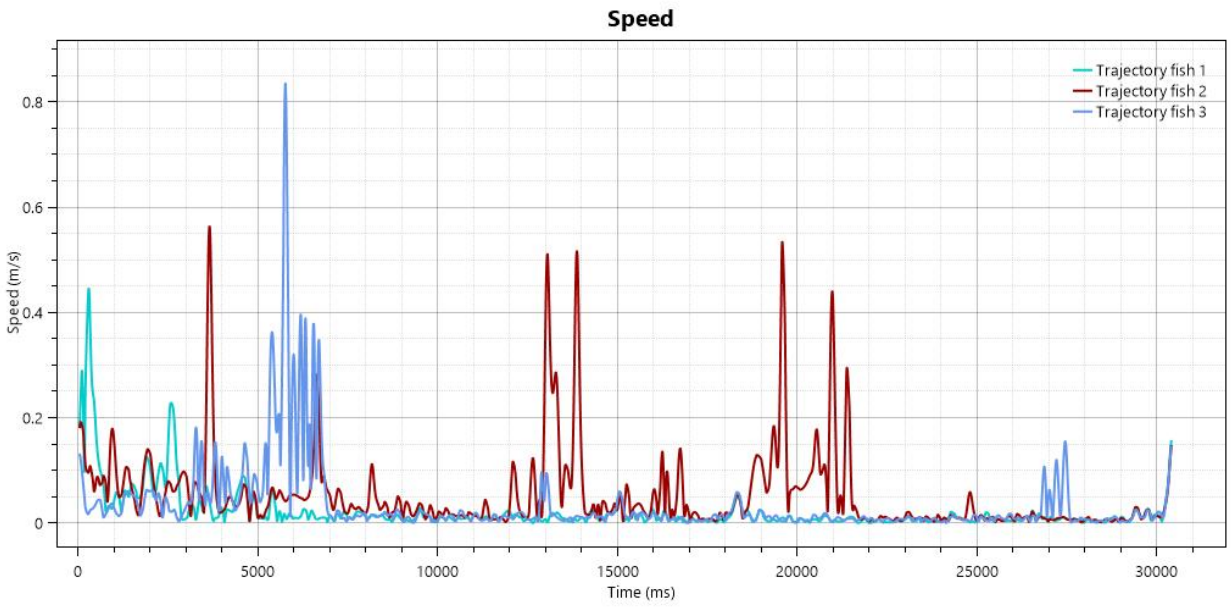


Figure 4.40 :Average speed of catfish day 28(500µg/L)

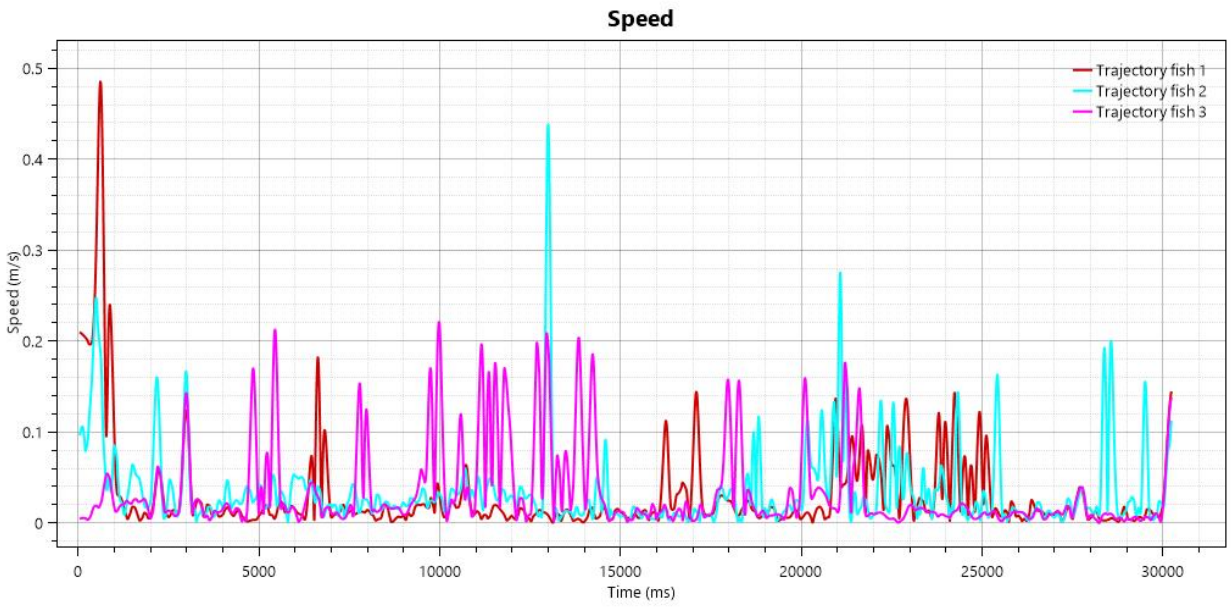


Figure 4.41 :Average speed of catfish day 28(1000µg/L)

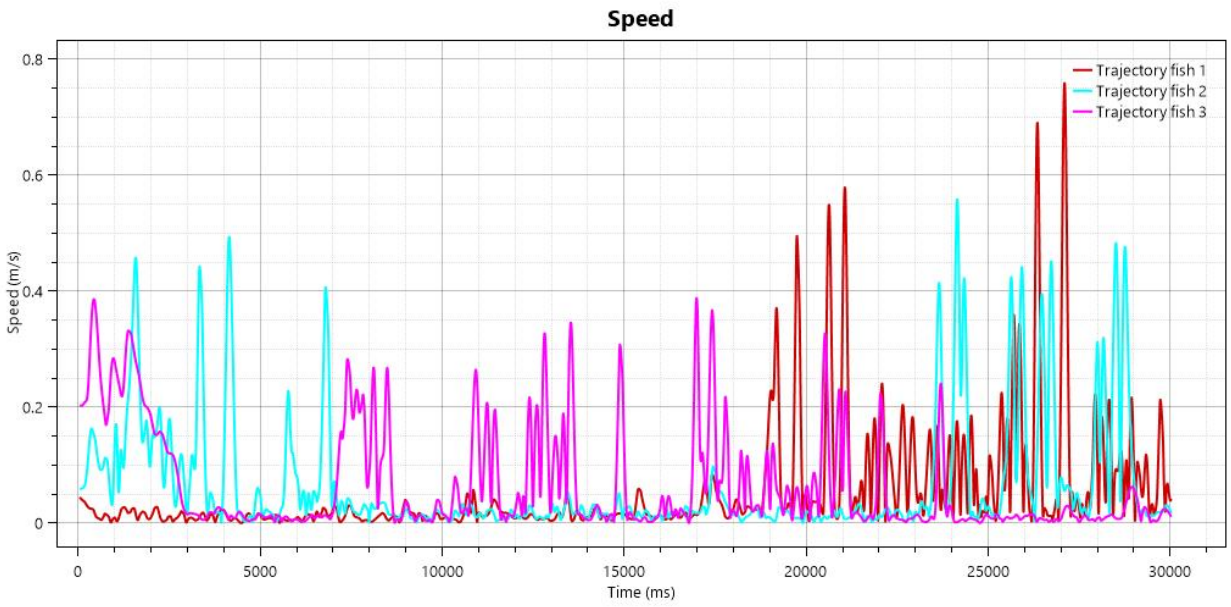


Figure 4.42 :Average speed of catfish day 28(1500µg/L)

CHAPTER FIVE

DISCUSSION

5.1 Discussion

The present study investigated the sublethal effects of N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine quinone (6PPD-Q) on the swimming kinetics of *Clarias gariepinus* juveniles. The results demonstrated a clear concentration- and time-dependent decline in swimming speed across exposed groups compared to the control. This reduction was statistically significant ($p < 0.05$) for nearly all treatment concentrations, indicating that 6PPD-Q exerts measurable behavioral and neuromotor impairment even at sublethal levels.

The observed decline in swimming velocity suggests that 6PPD-Q interferes with muscular performance and neuro-physiological coordination in *C. gariepinus.* Similar behavioral impairments have been reported in salmonids and other fish species exposed to 6PPD-Q, where erratic swimming, loss of equilibrium, and decreased endurance preceded mortality (Tian *et al.* 2021; Jaeger *et al.* 2024). These effects are consistent with hypotheses of vascular injury, oxidative stress, and disruption of the blood–brain and blood–gill barriers (Blair *et al.* 2025). The reduced swimming capacity may thus reflect compromised neuromuscular control resulting from oxidative damage and impaired oxygen transport efficiency.

At 500 µg/L, the reduction in swimming speed was moderate but significant, whereas at 1000 and 1500 µg/L, near-complete immobility was observed. This progressive decrease suggests a threshold effect where physiological compensatory mechanisms become overwhelmed at higher concentrations. The gradual decline across 7–28 days also implies cumulative toxicity, possibly

due to bioaccumulation or delayed physiological stress. Similar trends have been observed in zebrafish (Danio rerio), where chronic 6PPD-Q exposure resulted in altered swimming trajectories, reduced acceleration, and diminished burst swimming capability (Varshney *et al.* 2022).

The significant differences between the positive and negative controls also highlight the sensitivity of *C. gariepinus* behavior to solvent presence, though the DMSO concentration used (<0.001%) was below toxic thresholds. This minor reduction in swimming speed in the solvent control suggests a potential interaction between DMSO and the fish's sensory or motor systems, but its effect was negligible compared to the 6PPD-Q-treated groups.

Behaviorally, reduced swimming performance can have severe ecological implications. Swimming is essential for prey capture, predator avoidance, migration, and reproduction (Tierney, 2011). Therefore, sublethal impairment by 6PPD-Q could translate into decreased survival and reproductive fitness at the population level. The results thus reinforce growing evidence that 6PPD-Q contamination poses a significant ecological risk, even in the absence of acute lethality.

The mechanisms underlying the observed effects likely involve oxidative stress and mitochondrial dysfunction. Previous studies have reported that 6PPD-Q induces lipid peroxidation, alters antioxidant enzyme activity (e.g., superoxide dismutase and glutathione peroxidase), and interferes with cellular respiration (Mahoney *et al.* 2022). These biochemical disruptions could impair muscle contraction efficiency and neural signal transmission, leading to

the behavioral changes observed in this study. Additionally, vascular leakage and disruption of ion regulation may reduce the oxygenation of muscle tissue, further limiting endurance and speed.

The findings of this study confirm that *C. gariepinus*, a widely distributed tropical freshwater species, is susceptible to 6PPD-Q exposure at concentrations within the range of environmental relevance. This expands the known list of affected species beyond temperate salmonids, emphasizing the potential threat of tire-derived pollutants to tropical aquatic ecosystems. The results align with previous reports that 6PPD-Q can impair various non-salmonid species (Anderson-Bain *et al.* 2023; Ji *et al.* 2022), supporting its classification as an emerging global contaminant of concern.

Conclusion

The study demonstrated that exposure to N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine quinone (6PPD-Q) significantly affects the swimming kinetics of *Clarias gariepinus*. The observed concentration- and time-dependent reduction in swimming speed confirms that 6PPD-Q has pronounced sublethal effects on fish behavior and neuromotor function. These effects are indicative of physiological stress likely associated with oxidative damage, vascular disruption, and impaired neuromuscular coordination.

Given that swimming performance is a key determinant of ecological fitness, these findings suggest that chronic or repeated exposure to environmentally relevant concentrations of 6PPD-Q could impair fish populations and alter community dynamics in contaminated freshwater systems. This underscores the need for stricter regulation and monitoring of tire-derived contaminants in aquatic environments, particularly in urban catchments where runoff concentrations are likely

highest.

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