THE EFFECT OF NON-ALCOHOLIC WASTEWATER EFFLUENT ON THE GROWTH HORMONE GENE EXPRESSION IN AFRICAN CATFISH (*Clarias gariepinus*)

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AN UNDERGRADUATE DISSERTATION SUBMITTED TO THE DEPARTMENT OF ENVIRONMENTAL MANAGEMENT AND TOXICOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, EDO STATE, NIGERIA; IN PARTIAL FULFILMENT OF THE REQUREMENTS FOR AWARD OF BACHELOR OF SCIENCE(B.Sc) DEGREE IN ENVIRONMENTAL MANAGEMENT AND TOXICOLOGY.

APRIL, 2024.

CERTIFICATION

This is to certify that this research titled "**The effect of non-alcoholic wastewater effluent on the growth hormone gene expression in African catfish (*Clarias gariepinus*)**" was carried out by "**Adediji Ifeoluwa Blessing**" and presented to the Department of Environmental Management and Toxicology, Faculty of Life Sciences, University of Benin, Benin City; in partial fulfilment of the requirements for the award of Bachelor of Science (B.Sc) in Environmental Management and Toxicology. It was conducted under suitable conditions, was carefully supervised and subsequently approved as having met the requirements for the award of Bachelor of Science degree in Environmental Management and Toxicology.

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PROF. D.I. OLORUNFEMI **Date**

Project Supervisor

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PROF. A.A. ENUNEKU **Date**

Head of Department

DECLARATION

I "**Adediji Ifeoluwa Blessing**" declare that "**The effect of non-alcoholic wastewater effluent on the growth hormone gene expression in African catfish (*Clarias gariepinus*)**" is my own work and that all sources that I have used or quoted have been acknowledged by means of complete references and that this work has not been submitted for any other degree at any other University.

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ADEDIJI IFEOLUWA BLESSING

April, 2024

DEDICATION

I dedicate this project report to my ever loving, supportive, compassionate, and overall best parents in the world; Rev. and Mrs. S.G. Adediji. This report is also dedicated to my wonderful supervisor, Prof. D.I. Olorunfemi, for his support and assistance. Lastly, I'm dedicating this report to my lovely and very amazing siblings; Miss Opeyemi Adediji and Mr. Toluwase Adediji for their unwavering support.

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ABSTRACT

The discharge of industrial wastewater into aquatic ecosystems poses significant environmental challenges, including the contamination of water bodies with various pollutants and chemicals. This study investigates the effect of non-alcoholic wastewater effluent from a carbonated beverage manufacturing plant on the growth hormone (GH) gene expression of African catfish (*Clarias gariepinus*). The fish were exposed to concentration levels of 0.5%, 1.5%, 3.5% and 5.0% of the carbonated non-alcoholic wastewater effluent for 96 hours, and the liver was extracted for RNA isolation, DNA extraction, PCR amplification, and gel electrophoresis analysis. The results obtained revealed a dose-dependent effect of the non-alcoholic wastewater effluent on the expression of the GH gene in African catfish. The rise in gene expression in response to soft drink effluent exposure was statistically significant ($p < 0.05$) according to the statistically significant correlation between the gene's expression levels at different concentrations and the comparable control. The gene's increased expression indicates that the soft drink industry's effluent is having a detrimental effect on *Clarias gariepinus*. Therefore, Implementing more effective wastewater treatment processes in industries, conducting thorough Environmental Impact Assessments and increasing public awareness can help reduce the potential harmful effects of wastewater effluent on aquatic organisms.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

The global release of various pollutants into aquatic ecosystems via wastewater effluents, which is derived from human activities, is posing an increasing threat. The effects of non-alcoholic wastewater discharge on aquatic creatures' genetic and physiological integrity are especially concerning (Aladesanmi *et al.,* 2017). Heavy metals, organic chemicals, and nutrients are among the many contaminants that make up non-alcoholic wastewater discharge (Bashir *et al.,* 2020). These pollutants may cause changes in gene expression and growth patterns by upsetting aquatic organisms' endocrine systems. The African catfish *(Clarias gariepinus)* is one of these organisms that is particularly important for aquaculture operations as well as natural habitats (Anati *et al.,* 2021). In order to understand the molecular mechanisms underlying potential disruptions in the growth patterns of this commercially significant fish species, this study explores the complex link between non-alcoholic wastewater effluent and the growth gene expression of African catfish.

Wastewater effluent is a complex mixture of contaminants, including heavy metals, organic compounds, and nutrients. It is a result of home, agricultural, and industrial operations. The delicate balance of these ecosystems is seriously threatened by the uncontrolled release of such effluents into aquatic habitats (Akpor *et al.,* 2014). Natural water bodies being contaminated by industrial effluents has become a serious problem in emerging, highly populated nations like

Nigeria. The primary sources of drinking water in Nigeria are inland waterways and estuaries, both of which are frequently contaminated by the activity of nearby industrial facilities and people. Carbonated soft drinks are widely consumed, and their manufacture creates a considerable amount of wastewater (Fillaudeau *et al*., 2006). Effluents from soft drink manufacturing industries have a moderate concentration of pollutants because they are composed of washing waters from production lines which is derived from the ingredients used in the final production (Imoobe and Okoye., 2011).

Nearly half of the water used in the beverage and food processing industries is used for washing and rinsing. These washing and rinsing activities utilize water that contains caustics, detergents, discarded soft drinks and syrup, water from washing bottles and cans, and lubricants from their machinery, among other pollutants (Okoye,2011).

Although the detrimental effects of pollution on a variety of aquatic animals have been well studied in the past, there is still a significant lack of knowledge regarding the precise influence on the growth gene expression of African catfish.

As a member of the Clariidae family, the African catfish is essential to both commercial aquaculture and natural food webs (Kumari *et al.,* 2015). Due to its hardiness, capacity to adapt to a wide range of environmental conditions, and quick development, it is a species that is often used in aquaculture operations. Moreover, because it is a predator and scavenger in the food chain, it is especially vulnerable to the bioaccumulation of pollutants found in wastewater effluents. It is essential to comprehend how non-alcoholic wastewater effluent affects this species' growth gene expression in order to forecast possible cascade effects on ecosystem dynamics and the financial implications for aquaculture businesses.

In toxicological studies, the African catfish (*Clarias gariepinus*) is rapidly becoming one of the most extensively used sentinel model fish species (Karami *et al.,* 2015; Ayanda *et al.,* 2021) and this is because of its year-round availability, broad geographical distribution, and comparatively simple classification under laboratory circumstances (Nwani *et al.,* 2013). African catfish, or *Clarias gariepinus*, have been found to be incredibly sensitive when used for acute toxicity evaluation (Olorunfemi *et al*., 2019). As a result, they can be used as a useful indicator for pollutants in aquatic environments. Furthermore, the fish is mostly consumed in Nigeria and may be found in lakes, rivers, marshes, and streams (Olaniran *et al*., 2019). As a result, the study is aimed at utilising the African catfish (*Clarias gariepinus*) as a biomarker to assess the possible impact of the effluent obtained from a carbonated beverage industry in Benin city, Edo state, Nigeria on the aquatic ecosystem (especially fishes) of the receiving water body. *Clarias gariepinus* has been utilized as a modal organism to determine the effects of various contaminants such as pesticides, heavy metals, fertilizers and microplastics in the aquatic environment.

1.2 Statement of Research Problem

The discharge of industrial effluents from beverage industries into aquatic ecosystems has become a growing environmental concern, characterized by the release of complex mixtures of chemicals and contaminants. Of particular interest is the potential impact of these industrial effluents on the growth hormone gene expression in African catfish (*Clarias gariepinus*), a key species in freshwater environments. While the consequences of water pollution on aquatic organisms are widely acknowledged, there exists a critical gap in our understanding of the molecular responses of African catfish to the specific pollutants present in industrial effluents

from beverage manufacturing. Pathogenic organisms, total suspended solids (TSS), excessive nitrogen and phosphate compound loading, pH of the water, total alkalinity, biological oxygen demand (BOD), and chemical oxygen demand (COD) are the main problems with beverage industry effluent. The solid waste generated by the food and beverage industry includes both organic and packaging trash. Food grains, flavorings, and colorings are among the raw materials used in processing operations that result in organic wastes. Therefore, the research problem centers on elucidating the effect of industrial effluents from beverage industries on the growth hormone gene expression in African catfish, aiming to provide insights into the molecular mechanisms underlying the impact of industrial discharges on the physiological health of aquatic organisms. Addressing this research problem is essential for informing sustainable practices within the beverage industry, guiding environmental regulations, and safeguarding the integrity of aquatic ecosystems affected by industrial effluents.

1.3 Justification of the Study

Water pollution is a global environmental concern with far-reaching ecological consequences. Non-alcoholic wastewater effluents, often laden with various pollutants, pose a significant threat to aquatic ecosystems (Abowel and Sikoki, 2005; Ekubo and Abowel, 2011). African catfish (*Clarias gariepinus*), as a common inhabitant of freshwater environments, plays a crucial role in aquatic food webs and is particularly vulnerable to the impacts of water pollution. The discharge of non-alcoholic wastewater effluents into water bodies has been linked to the introduction of pollutants such as heavy metals, organic compounds, and pharmaceutical residues (Bhat *et al.,* 2017). Understanding the specific impacts of these contaminants on aquatic organisms is imperative for effective environmental management. African catfish, being a widely distributed

and economically important species, serves as a valuable indicator of ecosystem health. Investigating the ecotoxicological implications of non-alcoholic wastewater effluent from the beverage industry on the growth hormone gene expression in African catfish provides insights into the overall health of aquatic ecosystems (Adeogun, 2012). Given that African catfish is a common food source in many regions, studying the growth hormone gene expression becomes essential, as changes in gene regulation may have implications for the quality and safety of the fish as a human food source. Changes in gene expression serve as early indicators of stress or environmental disturbances in organisms (Adeogun, 2012). Studying the growth hormone gene expression in response to the non-alcoholic wastewater effluent exposure provides a potential molecular biomarker for assessing the health of aquatic ecosystems. Understanding how the non-alcoholic wastewater effluent affects the growth hormone gene expression in African catfish contributes to the conservation and management of this species. Such knowledge is vital for formulating effective strategies to mitigate the impacts of pollution and ensure the sustainability of aquatic ecosystems. Despite the increasing concern about water pollution, there is a notable gap in scientific literature regarding the specific molecular responses of African catfish to non-alcoholic wastewater effluents. This study aims to fill this knowledge gap and contribute to a more comprehensive understanding of the ecological impacts of water pollution.

1.4 Aim and Objectives of Research

The aim of this study is to determine the genotoxic effects of different concentrations of non-alcoholic beverage wastewater effluent on the liver of juvenile *Clarias gariepinus*.

The objectives of the study include:

1. To determine the acute toxicity on exposure to the wastewater effluent.
2. To extract total RNA of *Clarias gariepinus* liver cells exposed to effluent collected from 7-Up industry.
3. To denature the RNA of *Clarias gariepinus* liver cells exposed to wastewater effluent from 7-Up industry.
4. To amplify the genes using corresponding primers.
5. To observe RNA bands using blue light trans-illuminator
6. To quantify bands using graph pad prism.

CHAPTER TWO

LITERATURE REVIEW

2.1 SOFT CARBONATED BEVERAGE

Soft carbonated beverage is a non-alcoholic, sweet, light, flavored, water-based drink that have carbon dioxide added to them to make them bubbly or fizzy. The flavoring agents used may be artificial or natural, are often colored and can also contain fruit pulp, fruit juice or caffeine. The sweetener may be fruit juice, fructose corn syrup, sugar or sugar substitutes. These are also known as fizzy drink, soda pop, coke, pop etc. varying from region to region. It is generally accepted that the description of soft drinks excludes tea, coffee, dairy-based beverages and, until recently, alcohol. Soft carbonated beverages may contain alcohol but the content should not be more than 0.5% of the total volume (Chaudhary, 2018). Examples of soft carbonated beverages include: Pepsi, Coca cola, Marinda, Seven Up. The term soft drink was originated to differentiate the flavored drinks from hard liquor, or distilled spirits (Nawab *et al*., 2022).

2.2 BEVERAGE INDUSTRIES

Beverage industries are also known as drink industries, produces particularly ready to drink products. There are manly two categories of beverage industries: non-alcoholic and alcoholic. Non-alcoholic industries include soft drink, syrup and fruit juices etc. Whereas alcoholic industries include distilled sprites, wine and brewing etc. (Kumar *et al*., 2021).

With a global market of US$55 billion and a large market share in the food sector, the beverage industry continues to exhibit development potential (Sandhar *et al*., 2013). However, beverage

manufacturing presents some unique challenges in terms of effluent treatment. Beverage manufacturing facility effluent frequently contains the following chemicals: fruit juice concentrates, artificial sweeteners, maltose, lactose, glucose, fructose, preservatives, flavorings, colors, and mineral salts.

The discharge of effluents from beverage industries into water bodies can have detrimental effects on water quality. These effluents typically contain a complex mixture of pollutants, including organic compounds, sugars, additives, and potentially harmful chemicals used in the production process. The introduction of these contaminants can lead to increased nutrient levels, altered chemical composition, and changes in water pH. Additionally, the discharge may contribute to the depletion of dissolved oxygen levels in aquatic ecosystems. The cumulative impact of beverage effluents poses a threat to the overall health of water bodies, affecting aquatic organisms and potentially disrupting the balance of ecosystems. Regulatory measures and sustainable practices are essential to mitigate these adverse effects and preserve water quality (Ipeaiyeda and Onianwa, 2011).

2.2.1 Constituents of effluents from beverage industries

Wastewater discharged from soft drink industries usually is contaminated by suspended solid, organic substances including sugars, alcohols, organic acids, flavoring agents, and dyes. These compounds originate from ingredients used in beverage production and additives incorporated during processing. Chemical additives such as preservatives, stabilizers, antioxidants, and pH regulators are commonly used in beverage manufacturing to enhance product quality and shelf life. Residual amounts of these additives may be present in effluents (Arias *et al*., 2019). Cleaning agents, including detergents and sanitizers, are used to clean equipment and surfaces in

beverage production facilities. Residual amounts of these cleaning agents may be present in effluents discharged from cleaning operations. Inorganic compounds such as metals and salts may be present in effluents due to their use in processing or as ingredients. Metals like copper, zinc, and chromium can leach from equipment or be introduced through raw materials, pH regulators are used to adjust the acidity or alkalinity of beverages. Residual amounts of acids or bases may be present in effluents, potentially influencing the pH of receiving water bodies (Arias *et al*., 2020).

2.3 AFRICAN CATFISH (*Clarias gariepinus*)

African catfish (*Clarias gariepinus*) belongs to the family Clariidae. This omnivorous feeding habit fish is highly resistant to environmental stress and diseases (Adetuyi *et al*., 2014; Schram et al., 2014). *Clarias gariepinus* is widespread throughout numerous nations in Europe, Asia, and Africa (Saad *et al*., 2009). Africa's second-most important freshwater fish after tilapia is *Clarias gariepinus*. Except for Nigeria, where productivity of *Clarias gariepinus*, which makes up 70–80% of all freshwater fish production, exceeds that of tilapia. The fish is especially well-liked in these areas because of its mouth-watering flesh, unmatched toughness, rapid growth, and high market price. A native fish to Nigeria, the Clarias species can be discovered in freshwater habitats all around the nation. It's believed that *Clarias gariepinus* is the fish species that has spread the widest across Africa (Skelton, 2001).

A few of the watery habitats where North African catfish may be found are lakes, ponds, and pools. They are abundant in dams, rapids, and swift-moving rivers. African catfish are characterized by their streamlined bodies that are elongated and cylindrical. They have huge pectoral fins for movement and a single, long dorsal fin that runs along the back of the fish

(Eroldoğan *et al*., 2019). Males often grow larger than females, reaching lengths of up to 1.5 meters and weights surpassing 60 kilograms. This is an example of sexual dimorphism in action (Ketaren *et al*., 2018). African catfish vary in color. Juveniles typically have dark brown or black colors with pale undersides, while adults may have lighter coloration or mottled patterns (Arokoyu *et al*., 2021). They are quite adaptable to harsh environments and may survive in a pH range of 6.5 to 8.0. They can endure conditions that are extremely muddy and temperatures between 8 and 35 degrees Celsius. Their growth is best at temperatures between 28 and 30 degrees Celsius.

2.3.1 Taxonomic tree of African Catfish

- Domain: Eukaryota
- Kingdom: Animalia
- Phylum: Chordata
- Subphylum: Vertebrata
- Class: Teleostei
- Order: Siluriformes
- Family: Clariidae
- Genus: Clarias
- Species: *Clarias gariepinus* (Eschemeyer *et al*., 1998).

2.3.2 Ecosystem roles of African Catfish

The African catfish (*Clarias gariepinus*) plays various ecosystem roles, contributing to the structure and function of aquatic environments. Some of these roles include:

1. Top Predator: African catfish often occupy the upper trophic levels in freshwater ecosystems, acting as top predators that regulate prey populations and help maintain ecosystem balance (Agboola, 2016).

2. Influence on Community Structure: As apex predators, African catfish can influence the abundance and distribution of other aquatic organisms within their habitats, shaping the community structure and biodiversity (El-Sherif *et al*., 2020).

3. Biomanipulation: Introducing or enhancing populations of African catfish in aquatic ecosystems has been studied as a biomanipulation strategy to control undesirable prey species and improve water quality (Hernandez *et al*., 2017).

4. Nutrient Cycling: Through their feeding activities and excretion, African catfish contribute to nutrient cycling within aquatic ecosystems, redistributing nutrients and influencing nutrient availability for primary producers and other organisms (Magalhaes *et al*., 2019).

5. Ecosystem Engineer: African catfish, particularly in their burrowing behavior, can modify habitats and create microenvironments that influence sediment structure, oxygen levels, and nutrient dynamics, thereby serving as ecosystem engineers (Valenti *et al*., 2019).

2.4 TOXICITY TESTING

Toxicity is the degree to which a chemical substance or a particular mixture of substances can damage an organism. The substance that causes this biological impact is referred to as a toxicant.

There are two categories of materials that are released into the environment. Point discharges can originate from inadvertent spills, industrial waste streams, sewage discharges, and hazardous waste disposal sites. Characterizing point discharges in terms of materials released, release rates, and total volumes is rather simple. Conversely, materials originating from sources like air deposition, agricultural runoff, contaminated soils and aquatic sediments, and urban runoff are considered nonpoint discharges. Classification of nonpoint discharge is significantly more difficult. Nonpoint source discharges typically consist of complex mixtures with difficult-to-characterize toxicant levels, and their timing and rates are as unpredictable as the weather. The fact that the toxicological characteristics of the constituents may change is one of the most problematic aspects of nonpoint discharges. (Landis *et al*., 2003).

Assessing a chemical's toxicity entails determining if it has the capacity to function poisonously, determining the conditions under which this potential will manifest, and characterizing the behavior of the substance in issue. The goal of a toxicity test is to determine the quantities of a test material that, in a population exposed to controlled settings, might cause a particular response—typically an adverse one. Most toxicity testing is done on experimental animals, even though the primary goal of most toxicity research is to eliminate risks to humans. Since the quantitative structure-activity relationships (QSAR) data that are currently available do not allow for proper extrapolation to novel medications, this toxicity assessment is required. Although it is challenging to collect experimental human data due to ethical considerations, it is necessary to

understand adverse consequences such irritability, nausea, allergies, smell perception, and some higher nervous system functions (Hodgson *et al*., 2010).

2.4.1 Classifications of Toxicity Assessment

Toxicity assessment can be classified into various categories based on several factors, including the type of test organism, the duration of exposure, and the endpoints assessed. Here's an explanation of different classifications of toxicity assessment, supported by references:

1. Based on Test Organism

- In vitro Toxicity Assessment: In vitro assays involve using cells, tissues, or cellular components to evaluate the toxic effects of substances outside of living organisms. These assays provide valuable insights into cellular mechanisms of toxicity and are widely used in early-stage drug development and chemical safety assessment (Hartung, 2009).
- In vivo Toxicity Assessment: In vivo studies involve testing the toxicity of substances in living organisms, including animals such as rodents, fish, and birds. These studies provide information on the systemic effects of substances and their potential impacts on whole organisms (Leist *et al*., 2008).

2. Based on Duration of Exposure

- Acute Toxicity Assessment: Acute toxicity tests evaluate the immediate adverse effects of substances on organisms over a short duration, typically ranging from a few hours to a few days. These tests provide information on the potential hazards of high-concentration exposures (ECHA, 2012).

- Chronic Toxicity Assessment: Studies on chronic toxicity evaluate a substance's long-term effects on an organism during prolonged exposure times, usually weeks, months, or even years. These investigations shed light on the possible risks associated with long-term, low-level exposures.

3. Based on Endpoints Assessed

- Human Health Toxicity Assessment: Human health toxicity assessment focuses on evaluating the adverse effects of substances on human health, including acute and chronic effects, carcinogenicity, reproductive toxicity, and neurotoxicity. These assessments are crucial for assessing the risks associated with exposure to chemicals in occupational, environmental, and consumer settings (WHO, 2009).
- Ecotoxicity Assessment: Ecotoxicity assessment evaluates the effects of substances on ecosystems and ecological processes, including acute and chronic toxicity to aquatic and terrestrial organisms, bioaccumulation, and effects on ecosystem structure and function. These assessments help assess the risks posed by chemicals to environmental health and biodiversity (OECD, 2021).

2.4.2 Exposure scenarios

In aquatic test systems, for instance, exposure to a diluted stock effluent solution usually entails exposure to the entire body of the test organism. Through the skin, cell walls, respiratory system (gills, stomata), and ingestion, the toxicant (effluent) can enter the body. Toxicants are sometimes injected into aquatic species as part of toxicity studies to see what effects they have, though this is not particularly common. In land-based interactions, whole-body exposures are reduced. Typically, a xenobiotic dose is given by intramuscular, intraperitoneal, or vein injection

based on the weight of the toxicant per unit weight of the animal (intravenous). In some toxicity studies, the quantity of substance that enters the organism can be precisely controlled by administering a specific amount through a tube to the stomach (gavage). Feeding studies use a specific concentration of toxin mixed with food or water to ensure that the toxin is delivered appropriately. Upon encountering numerous substances that they find disagreeable, the test organisms cease to exist. Another way to be exposed to airborne pollutants is by inhalation. Frequently occurring dermal exposure may follow initial air exposure. An alternative method to guarantee inhalation exposure is to use an airtight or watertight seal that restricts exposure to the breathing apparatus. Rat's coat and paw contamination can be avoided by using nose-only exposures. Pollutants from polluted soils or atmospheric deposition are more easily absorbed when exposed to the skin. In toxicity testing, additional exposure pathways for organisms that live in plants, soil, and sediment may be used. Usually, plants encounter soil or atmospheric deposition. Soil invertebrates are often placed in standardized soil that has been infused with a particular concentration of the test medication. In sediment testing, contaminated sediments or substances added to a standard sediment are commonly utilized (Landis *et al*., 2003).

2.4.3 Test organisms

Two of the most crucial aspects of a toxicity test are the appropriateness and health of the test organisms, or, in the case of multispecies toxicity studies, the imported community. It's critical to understand the goals of the toxicity test. When protecting a specific economic resource, such a salmon fishery, it may be necessary to use a salmonid and its food sources as test species. Toxicological testing is done to determine the precise level of harm that a chemical causes to different animals. As a result, species used in labs are only regarded as typical of particular

classes or, frequently, entire phyla. When choosing a test species for toxicity testing, bear in mind some of the following factors:

1. The test organism should be easily obtained through laboratory culture, hatchery procurement, or field collection. Field collection is often required because it might be difficult to properly raise marine species in a laboratory.
2. The accessibility and simplicity of obtaining or cultivating the chosen species should be considered. Species that are easily obtained and able to be kept in well-regulated laboratory settings should be selected.
3. The species selected should be appropriate for the particular toxicity testing procedures and objectives under consideration.
4. The sensitivity of the test species must be typical of the phylum or class to which it belongs.
5. Species that show distinct and quantifiable reactions to exposure and are susceptible to the pollutants of concern and have well-characterized toxicant reactions, including biomarkers, sublethal endpoints, and acute and chronic impacts should be selected
6. It is critical to understand the culture's history, genetics, and genetic composition (Landis *et al*., 2003).

2.4.4 Acute Toxicity

Acute toxicity refers to the adverse effects that occur rapidly after exposure to a substance and typically within a short period, usually defined as hours to days. This type of toxicity is often assessed through acute toxicity tests, which measure the harmful effects of a substance on living organisms over a short duration. These effects can range from mild symptoms to severe illness or

death, depending on factors such as the dose of the substance, the route of exposure, and the susceptibility of the organism. Acute toxicity testing procedures assess the adverse effects that manifest soon after a single dosage of a test substance has been administered. Acute toxicity data can also be used to determine dose levels in repeated dose studies, help identify a substance's mode of toxic action, and help with the identification and management of adverse human reactions. Data from acute toxicity tests can also be used to evaluate the toxicity and dose-response of members of different chemical classes, which can help with the selection of new study subjects. Additionally, results from acute toxicity tests can be applied for a wide range of regulatory purposes. In many cases, the effects of the test substance happen swiftly and can be easily identified during these times. The "threshold" (the time at which there is no appreciable rise in mortality or impact) for some test materials won't be reached in 96 hours.

2.5 GENETIC MATERIALS

2.5.1 Gene

A gene is a DNA locus (or region) built on nucleotides that acts as the molecular unit of heredity (Susman, 2001). It is a fundamental unit of heredity that carries and transmits genetic information from one generation to the next. Genes are segments of DNA (deoxyribonucleic acid), which is the molecular code that contains the instructions necessary for building and maintaining an organism. DNA is organized into structures called chromosomes within the cell nucleus. The transmission of genes from one organism to its offspring is the basis for phenotypic inheritance. Polygenes (many different genes) and gene-environment interactions control the bulk of biological features. Mutations in the population's gene sequence can result in the development of distinct gene versions, or alleles. These alleles produce slightly varied

phenotypes of the identical protein that they gene for. With each new occurrence that is identified, the idea of a gene gets revised (Garickle *et al*., 2006).

2.5.2 Gene Expression

Gene expression refers to the process by which information encoded in a gene is used to synthesize a functional product, such as a protein or RNA molecule (Schena *et al*., 1995; Lodish *et al*., 2000). It involves the transcription of DNA into RNA, followed by the translation of RNA into a protein, although some genes encode functional RNA molecules that do not undergo translation.

Gene expression is tightly regulated at multiple levels to ensure that the right genes are expressed in the right cells, at the right time, and in the right amounts (Schena *et al*., 1995; Lodish *et al*., 2000). Regulation of gene expression occurs through various mechanisms, including transcriptional control, post-transcriptional modifications, and epigenetic regulation.

Transcriptional control involves the binding of regulatory proteins, such as transcription factors, to specific DNA sequences, known as enhancers or promoters, to either activate or repress gene transcription (Alberts *et al*., 2002). Post-transcriptional modifications, such as RNA splicing, editing, and stability, regulate the processing and stability of RNA molecules (Lodish *et al*., 2000). Epigenetic regulation involves chemical modifications to DNA or histone proteins, which can alter the accessibility of chromatin and affect gene expression (Alberts *et al*., 2002).

Gene expression plays a fundamental role in virtually all biological processes, including cell differentiation, development, response to environmental stimuli, and disease (Ptashne and Gann,

2002). Dysregulation of gene expression can lead to various disorders, including cancer, neurodegenerative diseases, and metabolic disorders.

2.5.3 Ribonucleic Acid (RNA)

RNA, or Ribonucleic Acid, is a molecule essential for various biological processes in living organisms. Structurally similar to DNA (Deoxyribonucleic Acid), RNA is composed of nucleotides, each containing a ribose sugar, a phosphate group, and one of four nitrogenous bases: adenine (A), cytosine (C), guanine (G), and uracil (U) instead of thymine (T) found in DNA (Alberts *et al*., 2002). The three main types of RNA present in cells are messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). mRNA carries genetic information from the DNA in the cell nucleus to the ribosomes in the cytoplasm, where it is translated into proteins through a process called protein synthesis. rRNA is a major component of ribosomes, the cellular organelles responsible for protein synthesis. Along with proteins, rRNA helps catalyze the assembly of amino acids into polypeptide chains according to the sequence of mRNA. tRNA molecules transport specific amino acids to the ribosome during protein synthesis. RNA molecules play critical roles in gene regulation, cell differentiation, and other cellular processes. These RNAs can modulate gene expression by interacting with mRNA molecules, affecting their stability or translation efficiency (Alberts *et al*., 2002). All of these RNA types are present in both prokaryotic and eukaryotic cells. Ribosomal RNA molecules make roughly 75% of all cellular RNA. The 28S (or 25S), 18S, and 5S in eukaryotic ribosomes and the 23S, 16S, and 5S in bacterial ribosomes are the three unique molecules that are present in ribosomes based on their sizes. With an estimated molecular weight of 25,000, transfer RNA is a smaller molecule that is responsible for supplying amino acids to ribosomes so they can synthesize proteins. Encoding proteins, messenger RNA accounts for only 1–5% of total cellular

RNA. All three types of RNA are important in the precise translation of genetic information (in the form of DNA) into protein in diverse ways. RNA is crucial for the functioning of cells and organisms, playing roles in protein synthesis, gene regulation, and other cellular processes. Its diverse functions make it a fundamental molecule in molecular biology and genetics.

2.5.4 Deoxyribonucleic Acid (DNA)

Deoxyribonucleic acid, also known as DNA, is a molecule that contains the genetic instructions necessary for all known living things as well as many viruses to form, function, grow, and reproduce. Its fundamental function in inheritance and heredity has led to it being referred to as the "molecule of life" frequently. With considerable advantages over protein analysis, DNA has been called the most appropriate molecule for identifying and locating fish species in processed food products. DNA is found in all sorts of tissues, is more stable at elevated temperatures, and the genetic code's variety allows closely related species to be distinguished (Lewis, 2005). The most common genetic material found in living things today is DNA. Still, this long polymer's popularity can be attributed to certain features. the structurally similar double-stranded RNA, on the other hand, is not. Watson and Crick's paper revealed the structure of DNA. Two of the polymer's most notable characteristics were the complementarity of the base sequences on the two strands and its double-helical structure (Alberts *et al*., 2002).

Recently, however, the fact that the two strands of DNA are entangled as a right-handed double helix is what distinguishes the molecule. "The double helix" is DNA. Simple straight ladder structures would serve these purposes just as well without the double helical feature, but they do confer important chemical and physical properties to the polymer. These characteristics are

largely responsible for DNA's biological activity. The genetic functions of DNA can thus be understood as the synergism of two properties:

• A tape holding the information store encoding the sequences of proteins and RNA molecules and

• A polymer existing as double-helical string enabling the packaging, accessibility and replication of the information store.

2.6 DNA DAMAGE

DNA damage refers to alterations or modifications to the structure of DNA molecules, leading to changes in the genetic code and potentially contributing to the development of diseases such as cancer and aging-related disorders [Ciccia and Elledge, 2010; Jackson and Bartek, 2009; Hoeijmakers, 2009]. Based on where it originates, DNA damage can be divided into two primary classes: endogenous and exogenous. The majority of the endogenous DNA damage that occurs naturally in cells is caused by chemically active DNA reacting hydrolytically and oxidatively with water and reactive oxygen species (ROS), respectively. Hereditary illnesses and spontaneous cancers are caused by these naturally occurring predisposed interactions of DNA with chemicals from its immediate surroundings. [Visconti and Grieco, 2009; Reuter *et al*., 2010; Perrone *et al*., 2016]. Exogenous DNA damage, on the other hand, occurs when environmental, physical and chemical agents damage the DNA. Examples include UV and ionizing radiation (IR), alkylating agents, and crosslinking agents (Chattergee and Walker, 2017).

2.6.1 Causes of DNA Damage

I. Ultraviolet Radiation:

UV radiation can directly damage DNA by inducing the formation of covalent bonds between adjacent pyrimidine bases on the same DNA strand, leading to the formation of pyrimidine (6-4) pyrimidine photoproducts (6-4PPs) and cyclobutane pyrimidine dimers (CPDs). These DNA lesions have the ability to disrupt transcription and DNA replication, which can result in mutations and perhaps lead to skin malignancies like melanoma. Furthermore, UV radiation can produce reactive oxygen species (ROS) in skin cells, which can damage DNA oxidatively and play a role in the development of skin cancer [Rastogi *et al*., 2010; Cadet and Douki, 2018].

II. Cytotoxic and Genotoxic Agents (Ionizing Radiation):

Any agent that damages live cells has the potential to damage the cell either generally (cytotoxic substances) or the DNA specifically (genotoxic agents). A contaminant may be mostly cytotoxic or predominantly genotoxic to cells, depending on the type of agent, the cells involved, and the duration of the damaging agent's action (Chacarov *et al.*, 2014). Ionizing radiation, which includes gamma and X-rays, can directly damage DNA by shattering the molecule's sugar-phosphate backbone, which can result in either double-strand breaks (DSBs) or single-strand breaks (SSBs). These fractures have the potential to cause cancer by upsetting the balance of the genome and resulting in chromosomal abnormalities. Furthermore, through radiolysis of water molecules, ionizing radiation can indirectly damage DNA by producing reactive oxygen species (ROS). ROS have the ability to oxidize DNA bases

and cause a range of DNA lesions, such as base alterations and breaks in DNA strands (Jeggo and Löbrich, 2007).

III. Chemical Agents:

Tobacco smoke, industrial chemicals, and environmental pollutants are examples of chemical agents that can interact directly with DNA molecules to cause different kinds of DNA damage. For instance, some substances have the ability to bind covalently to DNA bases to produce DNA adducts, which can deform DNA structurally. Furthermore, oxidative stress caused by some chemical agents can result in the production of reactive oxygen species (ROS) and oxidative DNA damage in cells. ROS have the ability to oxidize DNA bases, resulting in the creation of lesions such thymine glycol and 8-oxoguanine, which can be linked to the development of cancer and other disorders (Valavanidis and Fiotakis, 2009).

IV. Mutagens and Teratogens:

A "mutagen" is defined as an agent that modifies DNA, either directly or indirectly, to the point where the altered sequence or structure of the DNA eventually becomes potentially heritable. Since their damage can lead to neoplastic transformation, most mutagens have intrinsic carcinogenic properties. Teratogens are substances that, by definition, cause or raise the possibility of abnormal fetal development (Chacarov *et al.,* 2014).

V. Reactive Oxygen Species (ROS):

Reactive oxygen species (ROS) are extremely reactive molecules produced during regular cellular metabolism. Examples of ROS include superoxide radicals, hydroxyl radicals, and

hydrogen peroxide. ROS have the ability to oxidize DNA bases, which can result in base alterations, DNA strand breakage, and DNA-protein crosslinks, among other DNA defects. ROS-induced DNA damage can cause mutations, genomic instability, and cellular death by interfering with DNA replication, transcription, and repair mechanisms. Furthermore, oxidative DNA damage has been linked to the emergence of a number of illnesses, including as cancer, neurological diseases, and ailments associated with aging (Valko *et al*., 2007).

2.7 TECHNIQUES FOR DNA DAMAGE DETECTION

2.7.1 Polymerase Chain Reaction (PCR)-Based Assays:

PCR is a technique used in molecular biology to amplify a segment of DNA of interest or produce millions of copies (Ertl *et al*., 2014). PCR is simply used to produce millions of copies of a specific DNA sequence from an initially small sample. It is a crucial process for a range of genetic technologies and has enabled the development of a suite of new technologies. PCR-based assays can be used to quantify specific types of DNA damage or mutations.

2.7.2. Comet Assay (Single-Cell Gel Electrophoresis):

The comet assay is a sensitive technique used to detect DNA damage at the level of individual cells. In this assay, cells are embedded in agarose gel on a microscope slide, lysed to release DNA, and subjected to electrophoresis. Damaged DNA migrates away from the nucleus forming a "comet-like" tail, while undamaged DNA remains in the head (Kumari *et al*., 2008).

2.7.3. Loop-Mediated Isothermal Amplification (LAMP) Assay:

The LAMP assay is a powerful molecular biology technique used for the rapid and sensitive detection of DNA or RNA sequences. While the LAMP assay is not typically used specifically for DNA damage detection, it can indirectly aid in the assessment of DNA damage by detecting specific DNA sequences associated with damage or repair processes. It is a very sensitive, easy and time-efficient method. The LAMP reaction proceeds at a constant temperature using a strand displacement reaction. The LAMP assay is widely used for the detection of pathogens, genetic mutations, and DNA damage-associated sequences. It offers several advantages, including rapid amplification (within 30-60 minutes), high specificity, and robustness under isothermal conditions (Mori and Notomi, 2009).

2.7.4. Immunofluorescence Staining:

Immunofluorescence staining allows for the visualization of specific DNA lesions or repair proteins within cells using fluorescently labeled antibodies. For example, antibodies against 8-oxoguanine can be used to detect oxidative DNA damage, while antibodies against γ-H2AX can be used to visualize DNA double-strand breaks (Rogakou *et al*., 1998).

2.7.5. Comet-FISH (Fluorescence In Situ Hybridization Assay):

Comet-FISH combines the comet assay with fluorescence in situ hybridization to detect specific DNA lesions or chromosomal aberrations. After electrophoresis, the DNA in the comet tail can be visualized using fluorescently labeled DNA probes targeting specific genomic regions or DNA lesions (Olive and Banáth, 2006).

2.7.6. Enzyme-Linked Immunosorbent Assay (ELISA):

ELISA-based assays can quantify specific DNA lesions or repair proteins using antibodies that recognize the target molecules. For example, ELISA assays for 8-oxoguanine or thymidine dimers can quantify oxidative or UV-induced DNA damage, respectively (Dusinska *et al*.,2006).

2.7.7. Mass Spectrometry-Based Assays:

Mass spectrometry techniques, such as liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS), can identify and quantify DNA adducts or modifications induced by genotoxic agents. These methods provide high sensitivity and specificity for detecting DNA damage (Balbo *et al*., 2014).

2.8 MOLECULAR BIOLOGY TECHNIQUES RELEVANT TO THIS STUDY

2.8.1 Polymerase Chain Reaction

PCR or Polymerase Chain Reaction is a technique used in molecular biology to create several copies of a certain DNA segment. This technique was developed in 1983 by Kary Mullis, an American biochemist. PCR has made it possible to generate millions of copies of a small segment of DNA. It is a straightforward and affordable technology for amplifying a particular DNA segment, which is crucial for the detection and monitoring of genetic disorders, identifying offenders (in forensics), examining the function of the targeted segment, and other purposes (Bartlett and Stirling, 2003). The human β-globin gene was one of the first DNA sequences to be amplified using PCR (Saiki *et al*., 1988; Mullis, 1990).

Today, PCR is a commonly used and frequently necessary technique in medical and biological research labs for a wide range of applications, including the diagnosis of hereditary diseases, the identification of genetic fingerprints (used in forensic sciences and DNA paternity testing), the detection and diagnosis of infectious diseases, and DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes. Mullis and Michael Smith received the 1993 Chemistry Nobel Prize for their work on PCR. In order to induce DNA melting and enzymatic DNA replication, a process called thermal cycling entails periodically heating and cooling the reaction (Mullis, 1990). PCR technology has continued to evolve with the development of novel PCR variants, such as quantitative PCR (qPCR), reverse transcription PCR (RT-PCR), and digital PCR (dPCR). These advancements have enhanced the sensitivity, specificity, and throughput of PCR-based assays, expanding their applications in research and diagnostics (Huggett and Bustin, 2011).

2.8.2 Principles of PCR

Polymerase Chain Reaction (PCR) is a powerful and widely used technique that has greatly advanced our ability to analyze genes. Many thousands of genes are contained in the genomic DNA found in cells. Because of this, it is challenging to separate and examine any one gene. Through a straightforward enzyme reaction, PCR enables the replication of particular DNA sequences from genomic DNA, typically matching to genes or regions of genes. The only prerequisite is knowing a portion of the DNA sequence at each end of the area that needs to be replicated. Through PCR, DNA according to the desired sequence is replicated or amplified more than a million times, making it the dominant DNA molecule in the reaction. Enough DNA is extracted to allow for in-depth examination or modification of the truncated gene.

2.8.3 Components of PCR

I. **DNA Template:** The DNA template contains the target sequence to be amplified. It can be genomic DNA, complementary DNA (cDNA), plasmid DNA, or other sources containing the region of interest (Saiki *et al*., 1988)

II. **Oligonucleotide Primers:** These are short, single-stranded DNA oligonucleotides that anneal to complementary sequences flanking the target region. They serve as the starting points for DNA synthesis by the DNA polymerase (Higuch *et al*., 1992).

III. **DNA Polymerase:** A heat-stable DNA polymerase enzyme catalyzes the synthesis of new DNA strands using the DNA template and primers. Taq polymerase, derived from Thermus aquaticus, is commonly used in PCR due to its thermostability (Mullis, 1990).

IV. **Deoxynucleotides (dNTPS):** Deoxynucleotides (dATP, dCTP, dGTP, and dTTP) are the building blocks of DNA synthesis. They are added to the PCR reaction mixture to provide the nucleotides required for DNA polymerization (Ehrlich *et al*., 1991).

V. **Buffer Solution:** A buffer solution maintains the optimal pH and ionic conditions for DNA polymerase activity. It typically contains salts, such as Potassium Chloride and Magnesium Chloride, to stabilize the enzyme and facilitate DNA denaturation and annealing.

2.8.4 Types of PCR techniques

PCR is of the following types:

I. **Quantitative PCR (qPCR):** It uses the DNA amplification linearity to detect, characterize and quantify a known sequence in a sample.

II. **Reverse Transcription PCR (RT-PCR):** RT-PCR produces RNA amplification by synthesis of cDNA (DNA complementary to RNA), which is then amplified by PCR.

III. **Nested PCR:** Nested PCR improves sensitivity and specificity. They reduce the non-specific binding of products due to the amplification of unexpected primer binding sites.

IV. **Multiplex PCR:** This is used for the amplification of multiple targets in a single PCR experiment. It amplifies many different DNA sequences simultaneously (Ehtisham *et al*., 2016).

2.8.5 Stages in PCR amplification

A. Denaturation:

Taq polymerase-catalyzed PCRs reach denaturation at 94–95°C, the greatest temperature the enzyme can tolerate for 30 cycles or longer without experiencing damage. To guarantee that the lengthy template DNA molecules are completely denaturated, denaturation is carried out for five minutes during the first cycle. But occasionally, this kind of extended denaturation might be detrimental. It is advised to denaturate linear DNA templates for 45 seconds at 94–95°C when doing routine amplification on templates with 55% or less G+C. larger temperatures might be required for denaturation of templates with larger G+C contents. Moreover, the denaturation time needed increases with the length of the DNA templates. If the denaturation temperature is too low or the duration is too short, only the A-T rich sections of the template DNA will be denatured. When the denaturation temperature is reduced later in the PCR cycle, such DNA will re-anneal (Ukwubile, 2013).

B. Annealing:

Since complementary sequences have a chance to hybridize during annealing, the ideal temperature is determined by taking five degrees Celsius off the primers' predicted melting temperature (Tm). Depending on the length and sequence of the primer, annealing temperatures might range from 55 to 65°C. The amplified DNA is too low and the oligonucleotide primers anneal poorly at an excessively high temperature. Unwanted DNA segments may be amplified if the annealing temperature is too low because primers may not anneal precisely. To find the ideal annealing temperature, do a series of trial PCRs at room temperatures between 2 and 100°C below the melting points of oligonucleotide primers. A range of temperatures for annealing can also be used in a routine PCR (Ukwubile, 2013).

C. Extension/Elongation:

A temperature of 70–72°C is ideal for DNA polymerase activity, and primer extension can happen at up to 100 bases per second. The extension of oligonucleotide primers is catalyzed by DNA polymerase, which creates a new strand that is complementary to the template strand in sequence. The ideal temperature for DNA synthesis can vary slightly depending on the type of DNA polymerase that is employed. The optimal temperature range for Taq polymerase-mediated DNA synthesis is 72–78°C. Taq polymerase can insert about 2000 nucleotides per minute at this temperature (Ukwubile, 2013).

2.8.6 Selective DNA isolation

The principle of selective DNA isolation with PCR revolves around the selective amplification of target DNA sequences using sequence-specific oligonucleotide primers. Initially, genomic

DNA is extracted from the biological sample of interest using standard DNA extraction methods. Subsequently, specific primers are designed to flank the target DNA region, ensuring the selective amplification of the desired sequence. The PCR reaction mixture, comprising the extracted DNA template, target-specific primers, DNA polymerase enzyme, deoxynucleotides (dNTPs), and buffer solution, is subjected to multiple cycles of denaturation, annealing, and extension. This results in the exponential amplification of the target DNA fragment while minimizing nonspecific amplification. One of the key applications of selective DNA isolation with PCR is genotyping, wherein single nucleotide polymorphisms (SNPs), genetic variants, or mutations associated with diseases or traits are analyzed. By selectively amplifying and analyzing specific genomic regions, researchers can identify genetic variations and elucidate their implications in various biological processes and diseases. Furthermore, selective DNA isolation with PCR is widely used in gene expression analysis, enabling the quantification of mRNA transcripts to study gene expression patterns and regulatory mechanisms (Syvänen, 1999).

2.8.7 Electrophoresis

Thermal cycling, or varying the temperature of the PCR sample within a predefined range of increments, is employed in most PCR procedures. The first stage, known as DNA melting, physically splits the two strands of the DNA 24 double helix at a high temperature. The target DNA only is amplified in the second phase, which involves lowering the temperature and using the two DNA strands as templates for DNA polymerase. Using primers corresponding to the area of DNA that will be amplified under particular heat cycling conditions allows PCR to be highly selective. RNA, proteins, and other macromolecules can be separated via gel electrophoresis. The sizes of DNA fragments are used to separate them. Proteins can be divided into groups based on their charge and size. Below are some of the uses of gel electrophoresis:

- To analyze results of PCR
- In the separation of DNA fragments for DNA fingerprinting to investigate crime scenes
- In DNA profiling for taxonomy studies to distinguish different species
- In paternity testing using DNA fingerprinting
- In the analysis of antibiotic resistance
- In the study of evolutionary relationships by analyzing genetic similarity among populations or species
- To analyze genes associated with a particular illness.

In *Clarias gariepinus*, electrophoresis has been successfully used by many studies to check for the genetic variation (Popoola *et al*., 2014).

2.8.8 Amplification and quantification of DNA

Since PCR multiplies the targeted regions of DNA, it can evaluate minuscule amounts of sample. This is generally important for forensic analysis when there is only a small amount of DNA available as evidence. Tens of thousands of years' worth of ancient DNA can also be examined using PCR. These PCR-based methods have been applied to human DNA and animals, including a forty-thousand-year-old mammoth, for tasks like identifying Russian tsars and Egyptian mummies, as well as the body of English ruler Richard III. Quantitative PCR is a widely used technique to measure the levels of gene expression by estimating the amount of a certain sequence contained in a sample. There is a proven technique called quantitative PCR for figuring out the amount of DNA product produced (Ukwubile, 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 DESCRIPTION OF STUDY AREA

The study was conducted at the 7up bottling company (PLC) in Iguosa, Nigeria's Edo state, at Utekun village in Benin. The company is situated at Oluku Junction in Benin City, Edo State, which is near Oluku Junction on the Benin-Lagos route in the South-South area of Nigeria. The carbonated beverage industry's effluent was collected from the point of discharge at the Ise-uvbi river, which is quite a distance away from the facility. It is located in the community that was previously mentioned. Tall trees and rather tall grasses encircle the river. It also stands out due to its marshy surroundings.

The carbonated beverage effluent was stored at standard room temperature until the juvenile African Catfish (*Clarias gariepinus)* was exposed to the effluent.

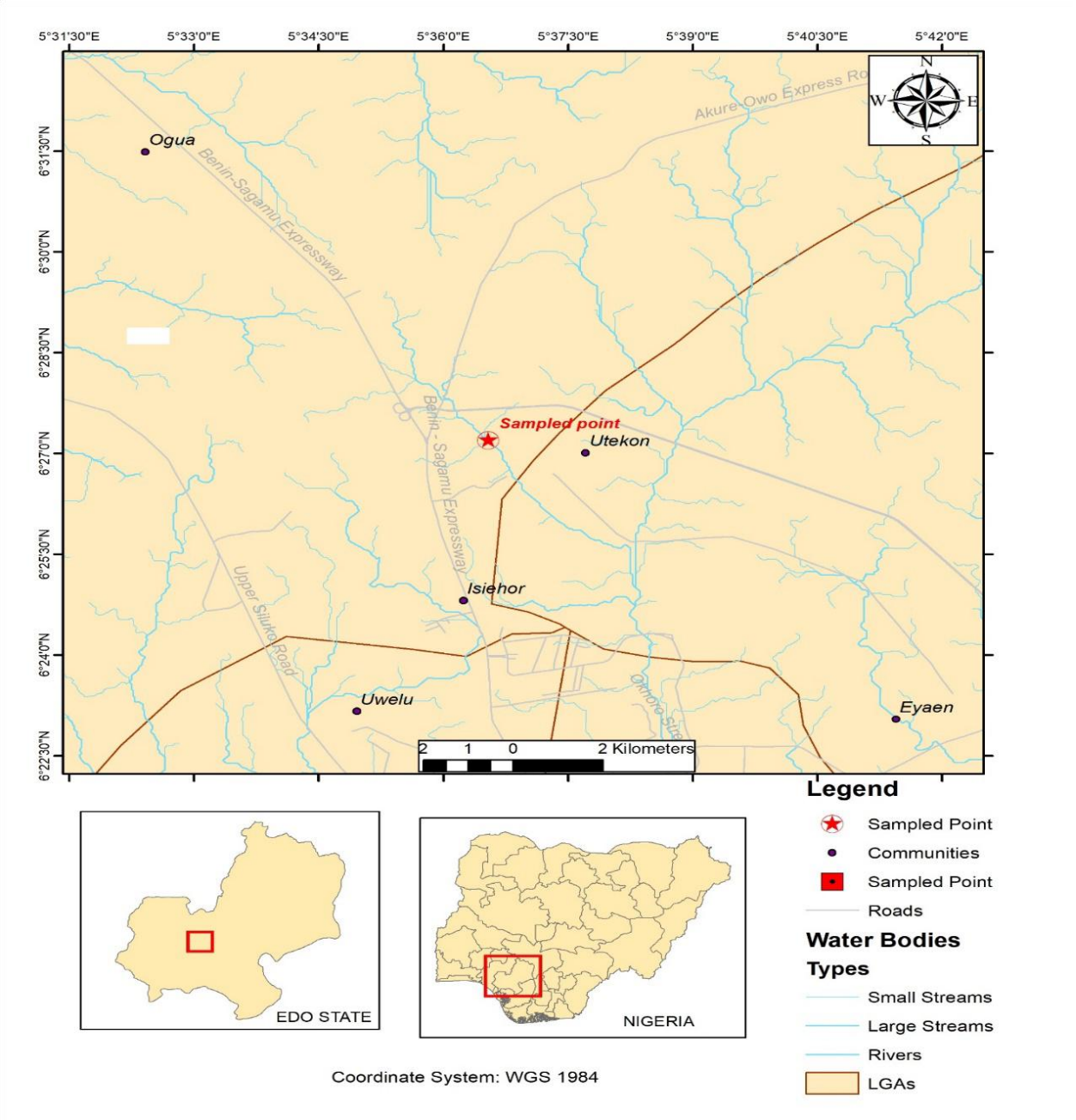


Figure 3.1 Map showing the collection point of the carbonated beverage effluent.

3.2 EFFLUENT COLLECTION AND DURATION

3.2.1 Effluent Collection

The study's sample was taken straight from the 7up Bottling Company's effluent discharge point in Oluku, Benin City, Edo State. Twenty (25) stiff plastic jerry cans with a capacity of one liter were used to collect the effluent. The samples were kept at room temperature and given the proper labels.

3.2.2 DURATION OF THE EXPERIMENTAL PROCESS

Two stages make up the experiment. A 96-hour acute toxicity investigation was the first phase, while DNA extraction was the second.

3.3 SAMPLE COLLECTION AND PREPARATION

3.3.1 African Catfish Collection

Juveniles of African Catfish (*Clarias gariepinus*), with a mean weight of 8-9g were purchased from a commercial fish farm located in Benin City, Nigeria. The fish samples were moved into an area designated for open storage. Water from a borehole was used to maintain the fish in an aquarium. During the acclimation period, all fish were fed fish meals that were readily accessible in stores. The fish were randomized into the plastic aquariums for the acute toxicity assay following the acclimation phase. Every 24 hours, the water in the plastic aquaculture tank was replaced to reduce the stress caused by contamination. The fish were fed 2 mm fish feed pellets twice a day. The feeding schedule for the test organism was stopped 24 hours prior to the exposure period.

3.3.2 Acute toxicity

There were five (5) concentrations, as follows: The control concentration was at 0%, while the other four (4) had sub-lethal amounts of 1.5%, 3.5%, 5.0%, and 0.5%. The zero percent consisted of ten (8) juvenile *Clarias gariepinus* and pure borehole water. A total of 72 *Clarias gariepinus* juveniles were examined across the various concentrations and their duplicates, along with the sub-lethal concentrations and their replicates, where the sub-lethal concentrations were designated as "A" of the concentration and its replicate as "B" of the concentrations. In order to reach the 3000 ml threshold, these were placed in their own containers, which were composed of the milliliter proportion of the effluent concentration to 1000 ml of water. This process was repeated four times. This experiment lasted for a period of 96 hours (4 days).

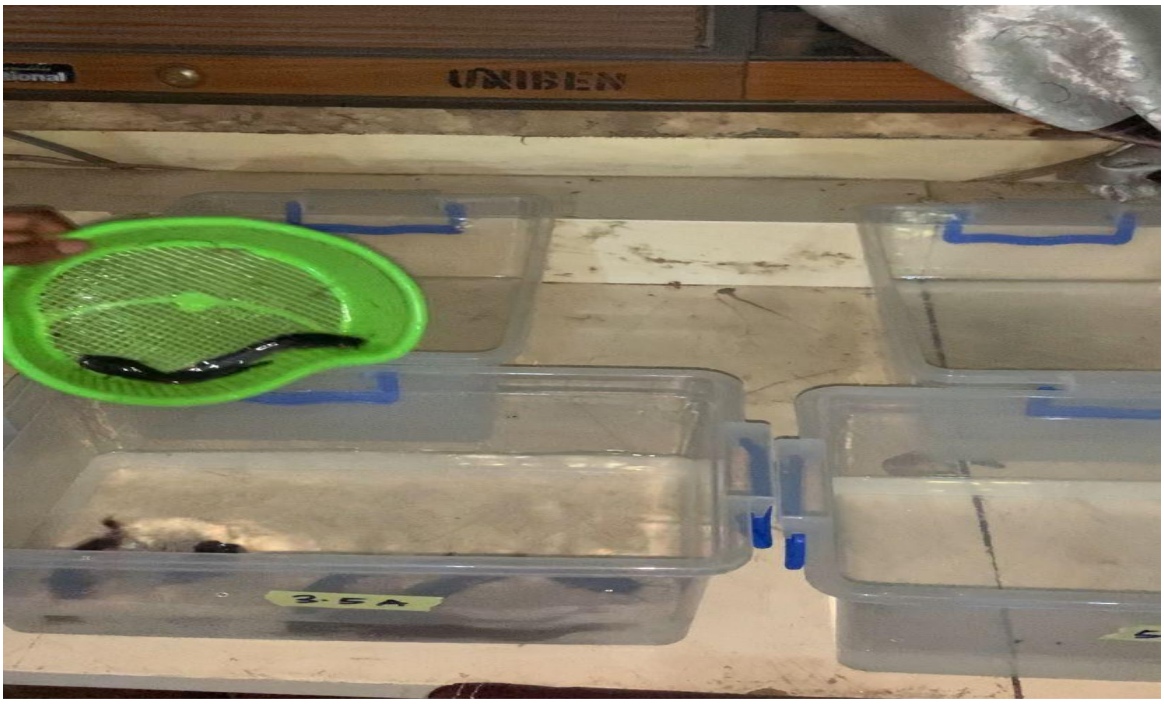


Plate 3.1: Samples of juvenile *Clarias gariepinus* exposed to treatments

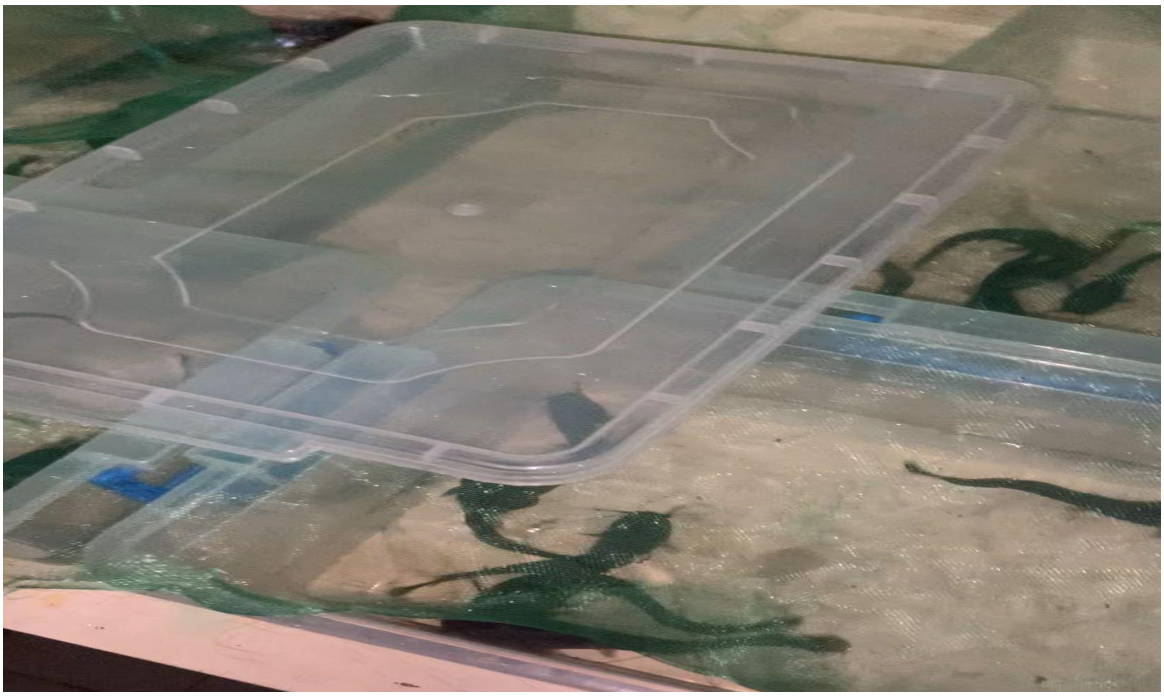


Plate 3.2: Juvenile *Clarias gariepinus*

3.4 EQUIPMENT

- Eppendorf tubes
- Micropipettes
- centrifuge machine
- UV spectrophotometer
- PCR machine
- Electrophoresis gel
- EDTA tubes
- Water bath

▪ Blue light transilluminator

3.5 REAGENTS AND SOLUTIONS

▪ 95µl Solid tissue buffer

▪ 10µl Proteinase K

▪ DNA Wash buffer

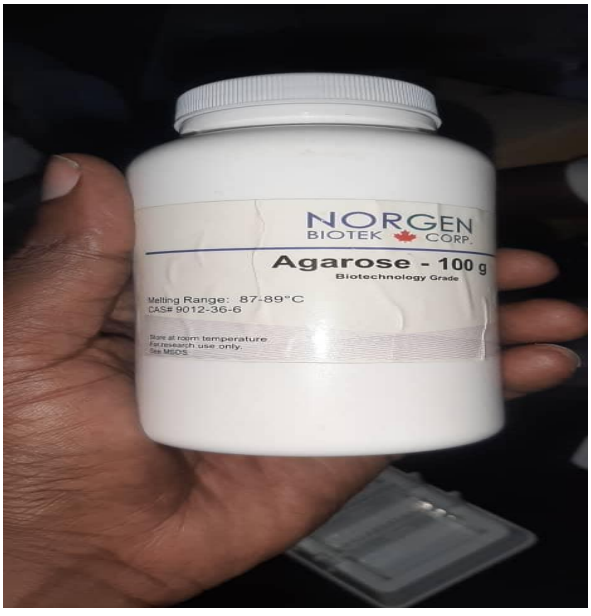
▪ DNA pre-wash buffer

▪ DNA Elution buffer

▪ Primers (forward and reverse)

▪ Taq polymerase (DNA polymerase) thermo-stable polymerase

▪ 95µl Nuclease free water



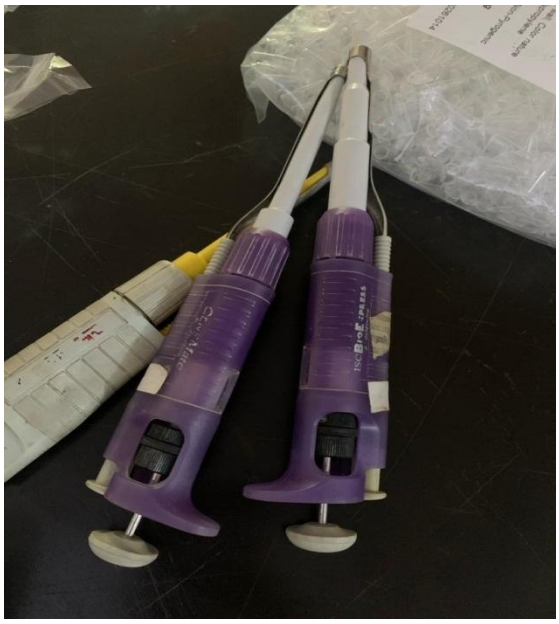


Plate 3.3 Agarose Powder

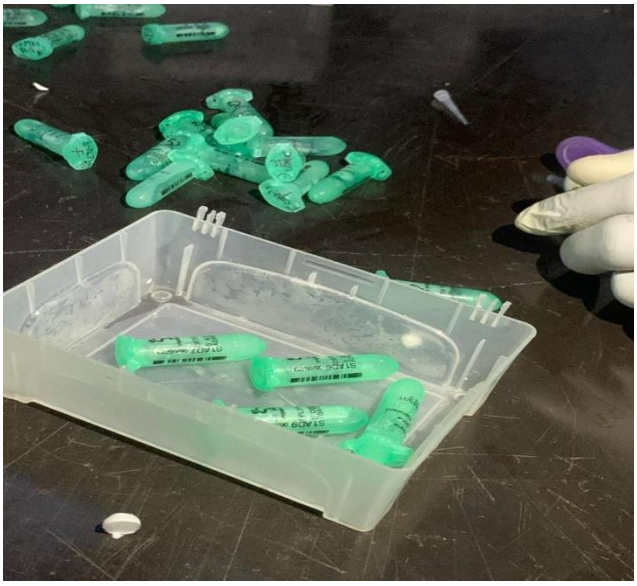


Plate 3.4 Micropipette

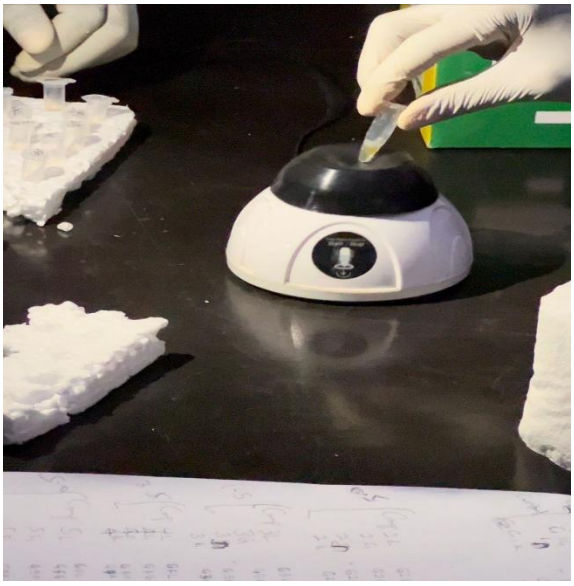


Plate 3.5 Primers

Plate 3.6 Centrifuge

Plate 3.7 PCR Machine

Plate 3.8 Water bath

3.6 TISSUE SAMPLE FROM JUVENILE FISH

The liver organ was extracted from the fish using sterile dissection tools, and the organ was stored in EDTA tubes after the fish was dissected. The liver organ was stored at freezing temperature to prevent denaturing. After that, the reverse transcriptase enzyme was used to convert the liver organ to cDNA, and PCR technology was used to measure gene expression to assess gene integrity. Since the liver is the primary organ of metabolism and is involved in numerous body functions, most notably the detoxification of chemical substances, it was utilized in compliance with the OECD's list of organs for toxicant exposure.

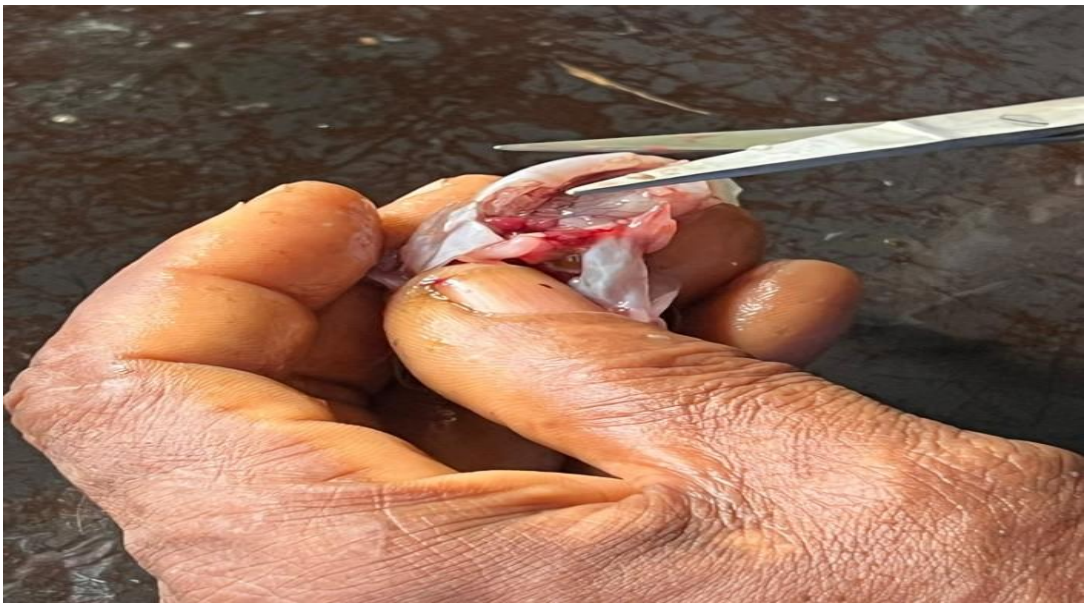


Plate 3.9: Dissection to remove liver organ from juvenile *Clarias gariepinus*

3.7 ISOLATION OF TOTAL RNA

The liver organ was removed from the sample and put into an Eppendorf tube. The cell nucleus was exposed by pipetting a solution containing 95µl water, 95µl solid tissue buffer, and 10µl proteinase K into the Eppednorf tube holding the organ. With the use of a centrifuge machine, the tissue's nuclear membrane and plasma were broken. After that, the Eppendorf tube was incubated for one to three hours at 55°C until the tissue became soluble. The tubes were vortexed one more for a minute following incubation. Following centrifugation, the cell material was separated into three phases: protein, DNA, and RNA. There is still some impurity and the supernatant contained. The sample was carefully pipetted into a new Eppendorf tube, then 2 volumes of Genome Binding Buffer was added to the supernatant and vortexed for 10-15 seconds. 400µl of DNA Pre-wash Buffer was added to the spin column in a new collection tube and was centrifuged at ≥12,000 x g for 1 minute, then the collection tube was emptied. After that, 700µl g-DNA wash buffer was added to the spin column and centrifuged at ≥12,000 x g for 1 minute, and the collection tube was emptied. 200µl g-DNA wash buffer was added to the spin column and centrifuged at ≥12,000 x g for 1 minute, then the collection tube was discarded with the flow through. The spin column was then transferred to a clean microcentrifuge tube. ≥50µl DNA elution buffer or water was added directly on the matrix and incubated for 5 minutes at room temperature, then centrifuged at maximum speed for 1 minute to elite the DNA. A set of forward and reverse primers that had been created and optimized was used to amp up the target genes. The matrix metallopeptidase 9 gene was also expressed, and the PCR Master Mix was used to catalyze the amplification for 30 cycles. Using the target DNA as a template, the DNA polymerase expands the primers by sequentially adding nucleotides to them.

3.7.1 DENATURATION AND QUANTIFICATION OF RNA STRANDS

The RNA wash buffer was added to the RNA in the tube (this is done to wash off any used solvent on the RNA) and centrifuged. Decantation was then done to remove the wash buffer and the RNA was left to dry. After drying, 50ml of nuclease free water was used to dissolve the RNA, after which it is placed under a UV spectrophotometer to quantify the amount of RNA isolated from the organ. After quantification, the sample was normalized, which was done by adding water to the sample. The RNA was then ready for conversion into cDNA.

3.7.2 cDNA CONVERSION

One (1 μg) of DNA-free RNA was converted to cDNA by reverse transcriptase reaction with the aid of cDNA synthesis kit based on ProtoScript II first-strand technology (New England BioLabs) in a condition of 3-step reaction: 65 °C for 5 min, 42 °C for 1 h, and 80 °C for 5 min (Elekofehinti *et al*., 2020).

3.7.3 PCR AMPLIFICATION

A set of forward and reverse primers that had been created and optimized was used to amp up the target genes. The matrix metallopeptidase 9 gene was also expressed, and the PCR Master Mix was used to catalyze the amplification for 30 cycles. Using the target DNA as a template, the DNA polymerase expands the primers by sequentially adding nucleotides to them. The PCR equipment was then used for amplification.

3.7.4 AGAROSE GEL ELECTROPHORESIS

The amplicons derived from RT-PCR products underwent a 1% agarose gel electrophoresis to aid in their migration from the anode to the cathode. An even voltage between 20 and 40 to 100

volts was used for the electrophoresis. The molecules travel more quickly as the voltage rises. A slower migration rate may make it easier to get definitive results. The following primers (Inqaba Biotec, Hatfield, South Africa) were used in OneTaqR2X Master Mix (NEB) polymerase chain reaction (PCR) for the amplification of the gene of interest. A 25µl reaction mixture including cDNA, primers (forward and reverse; as shown below), and Ready Mix Taq PCR master mix was used for the PCR amplification process. In the event that the following holds true: 30 cycles of amplification (denaturation at 95 ◦C for 30 s, annealing for 30 s, and extension at 72 ◦C for 60 s) preceded the first denaturation at 95 ◦C for 5 min. The final extension was conducted at 72 ◦C for 10 min. One percent agarose gel was used to resolve the amplicons. The relative amount of expression of each gene was normalized using the GAPDH gene, and band intensity was quantified using the "image J" program (Olumegbon *et al*., 2022).

3.7.5 PRIMER SEUENCES

ESR-α	5’- TGCAGGGAGAAGAGTTTGTG -3’	5’- GAGACTTCAAGGTGCTGGATAG -3’
ESR-β	5’- GATCACTAGAGCACACCTTACC -3’	5’- CCCTCTTTGCGTTTGGACTA -3’
GAPDH	5’GCAAGGATACTGAGAGCAAGAG-3’	5’- CATCTCCCTCACAATTCCATCC-3’

CHAPTER FOUR

4.0 RESULT

4.1 Acute toxicity responses of the African catfish to the industrial effluent

The mortality data for the African catfish exposed to the different dilution percentile (effluent in water ratio: 0.5, 1.5, 3.5 and 5.0) of the carbonated beverage industry effluent is presented in Table 4.1 and 4.2 below. The fishes exhibited behavioral responses including restlessness, erratic movements, pale skin, respiratory distress and gasping for breath.

Table 4.1: Mortality rate of African catfish (*Clarias. gariepinus*) juveniles exposed to different concentrations of effluents for A

Concentration	Mortality rate			
	24hrs	48hrs	72hrs	96hrs
Control	0	0	0	0
0.5	0	3	3	1
1.5	1	2	0	2
3.5	2	2	4	0
5.0	3	1	2	2

Table 4.2: Mortality rate of African catfish (*Clarias. gariepinus*) juveniles exposed to different concentrations of effluents for B

Concentration	Mortality rate			
	24hrs	48hrs	72hrs	96hrs
Control	0	0	0	0
0.5	3	0	3	1
1.5	3	1	2	2
3.5	3	2	3	0
5.0	0	0	3	3

4.2 Results Presentation

The effects of exposure of *Clarias gariepinus* to non-alcoholic carbonated beverage effluent were observed at different applied treatment levels; which were 0.5, 1.5, 3.5, and 5.0. The Growth Hormone gene expression (GH) was investigated during the study. Figure 4.1 shows the result for the changes in the expression levels for the Growth Hormone gene on exposure to the carbonated beverage effluent.

In order to assess the changes in GH gene expression, Figure 4.1 below presents the results obtained from this study. The graph displays the expression levels of the GH gene in response to exposure to the carbonated beverage effluent. According to the findings, it was observed that the

0.5 treatment level resulted in the largest increase in GH gene expression, as shown in Figure 4.1. Conversely, the lowest amount of GH gene expression was detected when the effluent concentration reached 5.0, originating from the beverage industry.

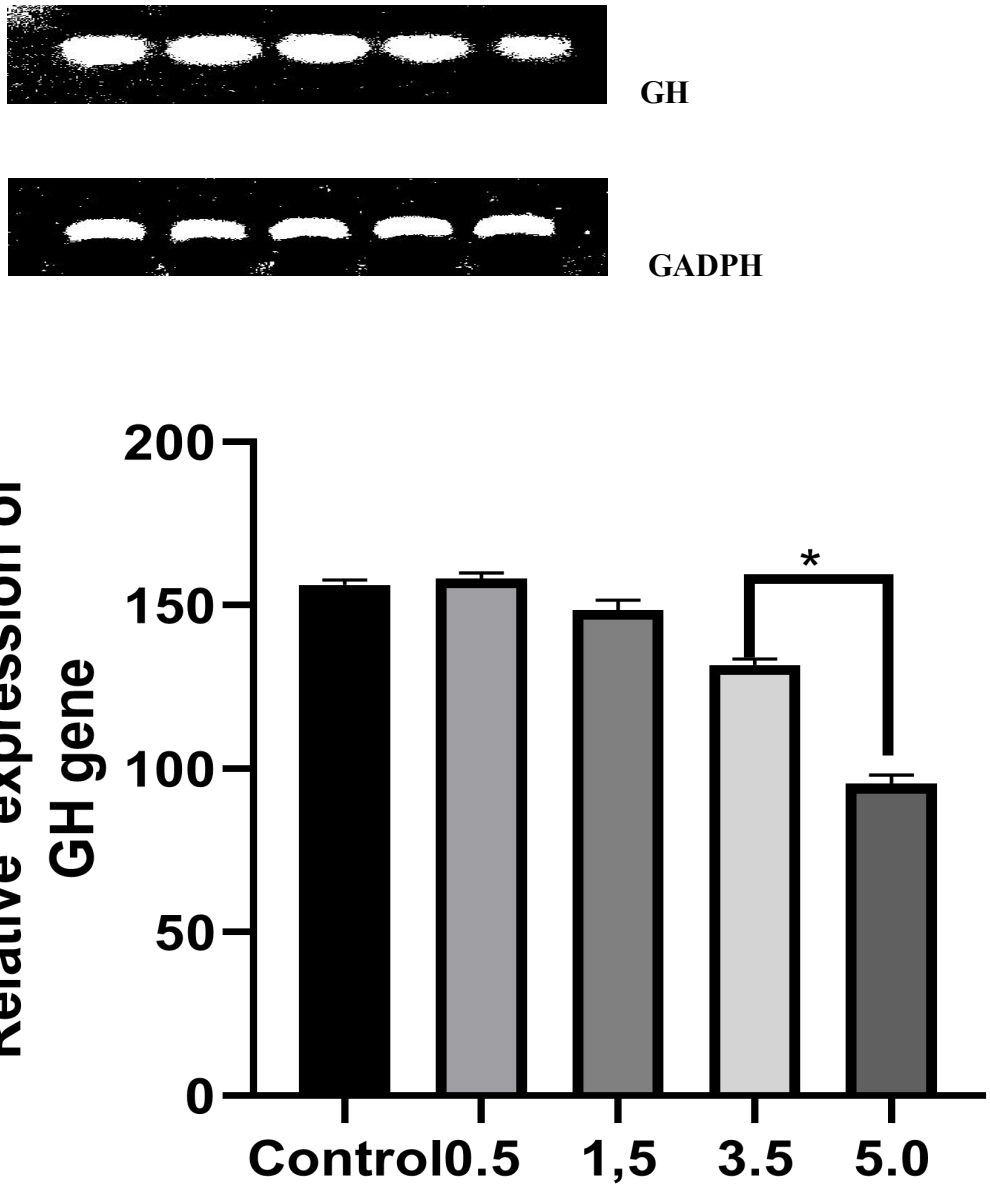


Figure 4.1: mRNA expression of growth hormone (GH) in *Clarias garienpinus* exposed to waste water. * Indicates statistical difference ($p < 0.05$) to control.

This result indicates that there is a dose-dependent relationship between the non-alcoholic carbonated beverage effluent and the expression of the GH gene in *Clarias gariepinus*. A higher treatment level of the effluent evidently suppressed the expression of the GH gene, leading to a decrease in its activity. On the other hand, when the fish were exposed to a lower treatment level of effluent (0.5), the GH gene exhibited a higher expression level.

The Growth Hormone gene is responsible for regulating various physiological processes, including growth, development, and overall metabolism. Therefore, the observed changes in the expression levels of this gene suggest potential impacts on the growth and development of *Clarias gariepinus* when exposed to the carbonated beverage effluent.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Discussion

Growth hormone (GH) plays a crucial role in regulating various physiological processes in vertebrates, including metabolism and growth. It exerts its effects through a wide range of targets in the body, influencing both cellular and systemic functions. One of the well-known effects of GH is its role as a potent growth enhancer, promoting the development of tissues and organs.

In addition to its role in growth and metabolism, GH has been shown to impact the behavior of vertebrates, including fish. Studies have demonstrated that GH can modulate fish behavior by influencing factors such as aggression, swimming activity, hunger, and responses to predators (Jonsson and Bjornsson, 2002). These behavioral changes are thought to be mediated by the actions of GH on the central nervous system and other relevant physiological pathways.

In a recent investigation focusing on the effects of wastewater exposure on fish, particularly *Clarias gariepinus*, the expression of GH gene was examined in response to different concentrations of wastewater from a 7up bottling company. The study found that exposure to wastewater led to alterations in the expression of the GH gene in the fish.

Specifically, the study revealed that at concentrations of 0.5 and 1.5, the gene was highly expressed, with 0.5 showing the highest level of expression among the treatment groups. Interestingly, downregulation of GH gene expression was observed at higher concentrations of wastewater (3.5 and 5.0), with 5.0 exhibiting the least expression compared to the control group.

These findings suggest that exposure to wastewater from the 7up bottling company can impact the expression of GH gene in *Clarias gariepinus*. The observed downregulation of GH gene expression at higher concentrations of wastewater is particularly noteworthy and indicates a potential disruption of GH signaling pathways in response to pollutant exposure. Importantly, statistical analysis (per $p<0.05$) confirmed the significance of these expression changes, highlighting the robustness of the findings.

Overall, the suppressed expression of GH gene in response to wastewater exposure underscores the potential adverse effects of environmental pollutants on the endocrine system and physiological processes in fish. Further research is needed to elucidate the mechanisms underlying these effects and their implications for fish health and ecosystem dynamics.

5.2 Conclusion

The aim of this study was to assess the impact of non-alcoholic wastewater effluent from the beverage industry on the expression integrity of genes in *Clarias gariepinus*, a species of freshwater fish commonly found in aquatic ecosystems. Through a comprehensive analysis, the study documented both the overexpression and downregulation of genes in response to exposure to wastewater effluent.

Gene expression serves as a fundamental mechanism by which cells regulate the production of proteins, acting as both an on/off switch and a volume control to modulate the synthesis of specific proteins in response to environmental stimuli. This tightly regulated process allows cells to adapt and respond dynamically to changes in their surroundings, ensuring proper functioning and homeostasis.

The findings of this research underscore the detrimental effects of environmental pollutants, originating from the beverage industry, particularly 7up bottling company, on *Clarias gariepinus*. The observed alterations in gene expression patterns highlight the sensitivity of aquatic organisms to chemical contaminants present in wastewater effluent. It is evident that before discharge into aquatic environments, wastewater from the beverage industry should undergo more rigorous treatment to minimize its adverse impacts on aquatic ecosystems and organisms.

Notably, the effluent generated by the carbonated beverage industry contains specific compounds that can disrupt gene expression in aquatic organisms. Further investigation is warranted to elucidate the specific mechanisms and compounds responsible for the observed changes in gene expression, particularly the expression of growth hormone (GH) gene, in *Clarias gariepinus*. Understanding these mechanisms is crucial for developing effective strategies to mitigate the ecological and environmental consequences of industrial wastewater discharge.

5.3 Recommendations

1. More effective wastewater treatment processes should be implemented at industrial facilities to reduce the concentration of pollutants and contaminants in the effluent before it is discharged into aquatic environments.
2. Regular monitoring programs should be established to assess the quality of wastewater effluent discharged into aquatic ecosystems.
3. Thorough Environmental Impact Assessments (EIAs) should be conducted prior to the establishment or expansion of industrial facilities.

4. Ecological restoration and habitat enhancement initiatives should be implemented in affected aquatic ecosystems to help mitigate the effects of wastewater pollution and support the recovery of aquatic biodiversity.
5. There should be increased public awareness and education about the environmental impacts of wastewater pollution and the importance of protecting aquatic ecosystems.
6. There should be continuous research and innovation in wastewater treatment technologies, pollution prevention strategies, and ecological monitoring techniques.

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