TOXICITY OF ATRAZINE (HERBICIDE) TO JUVENILES OF THE AFRICAN CATFISH, *CLARIAS GARIEPINUS* (BÜRCHELL, 1822)

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AHMADU BELLO UNIVERSITY, ZARIA

NIGERIA.

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BY

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DEPARTMENT OF BIOLOGY

FACULTY OF LIFE SCIENCES

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FEBRUARY, 2017

DECLARATION

I declare that the work in this dissertation entitled "TOXICITY OF ATRAZINE (HERBICIDE) TO JUVENILES OF THE AFRICAN CATFISH, *CLARIAS GARIEPINUS* (BÜRCHELL, 1822)" has been carried out by me in the Department of Biology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

_____ _____

Bayero, Umar Date

CERTIFICATION

This dissertation entitled "TOXICITY OF ATRAZINE (HERBICIDE) TO JUVENILES OF THE AFRICAN CATFISH, *CLARIAS GARIEPINUS* (BÜRCHELL, 1822)" by Umar BAYERO meets the regulations governing the award of the degree of Masters of Science in Biology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This research work is dedicated to the memory of my late father Alhaji (Engr.) Abubakar Bayero and to my mother Hajiya Maryam Shuraihu. May the Almighty have mercy on you all.

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I praise Almighty Allah who gave me the health, means and ability to complete this M.Sc. program. It is indeed His grace that kept me going even when all hope seems lost. May His blessings and salutations be upon His noble prophet Muhammad (SAW), his household and all those who follow his guidance till the day of reckoning. AlhamduLILLAH!

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ABSTRACT

In the present study, juveniles of *Clarias gariepinus* were exposed to the herbicide atrazine in order to determine the herbicide's toxicity to the species at both acute and sub-lethal exposures. Fish were exposed to acute concentrations of 9, 10, 11, 12 and 13mg/L of atrazine, as well as sub-lethal doses of 0.59, 1.19 and 2.38mg/L of atrazine. Both experiments had a control that served as a reference point. Effects of the herbicide were measured using behavioural, haematological, biochemical, histopathological as well as growth indices (as suggested by previous authors). Results of the acute toxicity test showed that atrazine was toxic to the species in a dose-dependent manner. The LC_{50} value of atrazine established for the species was 11.89mg/L. Most importantly, atrazine induced hyperactivity at acute levels of exposure. Significant ($p<0.05$) haematological changes observed at both acute and sub-lethal exposures included anaemia, leukocytosis, proteinaemia, haemoglobinaemia, neutrophilia as well as lymphocytosis. Acute and sub-lethal concentrations of atrazine also induced an increase in the generation of reactive oxygen species. Histopathological changes observed in the gills included attenuation and clubbing at the tips of the primary lamellae, gradual degeneration of the primary lamellae, total dislocation of the primary lamellae from its position as well as mild congestion in the cartilage. Changes in the liver of exposed fish included perivascular cuffing around the central vein, mild congestion of the sinusoids, pyknosis of the nuclei, mild necrotic damage, congestion of the central vein as well as mild coagulative necrosis. Atrazine also had effect on the growth parameters (weight gain, percentage live weight gain and specific growth rate) as well as nutrient utilization parameters (feed conversion efficicency, feeed conversion ratio, gross feed conversion efficiency and nitrogen metabolism) although some of these effects were not statistically significant ($p>0.05$). The study revealed that atrazine was moderately toxic to *C. gariepinus* juveniles and

affected the behaviour, haematology, biochemistry, histology as well as growth of the fish. Hence, use of the herbicide should be with caution especially near water bodies.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

The ever-increasing world population and the attendant increase in food demand necessitated that new ways of increasing agricultural output be sought. In an attempt to increase agricultural output, man relies heavily on the use of chemicals to protect crops from pests, right from the time of dressing of seeds before planting, through fighting weeds and other pests on the farm, to the preservation of already harvested products. On one side, benefits derived from the use of pesticides in agriculture are immense, but on the other side, environmental pollution and/or degradation is one major problem that is linked to their application (Olusegun, 2001).

The competition for survival between humans and other organisms in the environment dates back to the beginning of history. Insects, rodents and generally pests have taken a serious liking for cultivated crops (Olusegun, 2001), or the conducive environments in which they are grown. This competition became more intense as humans continued to modify the environment, leading to the evolution of an organized pattern of pest control through the development of pesticides (McEwen and Stephenson, 1979).

At its onset, the development of pesticides was actually slow and limited in application. In such instances, their use was highly restricted to small acreage, hence small and restricted impacts on the environment (McEwen and Stephenson, 1979). The increase in the world population led to the increase in the use of pesticides, resulting in higher and more pronounced impacts on the environment.

The presence of pesticides in the environment has caused significant social and scientific development anxiety worldwide, as their all-over-the-world extensive usage can create

potential risks to the environment and human health, and easily pollute bodies of water thereby resulting in extensive damage to non-target species, including fish (Moreno *et al.,* 2010). Be it by intentional or unintentional application, water is contaminated through direct application into the aquatic system, drifts during spray, atmospheric fallout as rain and dust, soil erosion, sewage, industrial effluent and occasionally by spillage (McEwen and Stephenson, 1979).

The aquatic environment is particularly one vulnerable area as it is the ultimate recipient of pollutants due to basin drainage. The aquatic ecosystems have been known to receive a wide spectrum of pollutants, which may be introduced to them directly or indirectly. The indiscriminate use of chemicals has resulted in large scale reduction in aquatic productivity. Pesticides have different diverse impacts on aquatic animals especially fishes which are of economic importance and high value from the point of biological conservation (Mekkawy *et al.,* 2013). Environmental pollution by pesticides has become a serious problem in terms of global conservation and animal and human health (Katsumata *et al.,* 2005; Velisek *et al.* 2010). Besides overexploitation and habitat loss, pollution is ranked third on the list of main causes of fish species loss (West and Biney, 1991).

Fish is highly nutritious, easily digestible and a much sought after food. Nutritional value of fish depends on their biochemical composition, which is affected by water pollution (Prado *et al*., 2009). The African catfish, *Clarias gariepinus* is one of the richest source of animal protein to man.

1.2 Statement of Research Problem

Herbicide use in the Northern part of Nigeria where grains are cultivated most dates back to more than half a century (Mbagwu and Ita, 1994). Incidentally, over 80% of dams and reservoirs are constructed over seasonal and perennial rivers in the same region (Olusegun, 2001). This makes pollution of aquatic environments by agricultural activities a major source of concern.

Herbicides are indiscriminately used with little or no regulation, and they persist in the environment for a long time. They are applied with the sole aim of controlling weeds, but they end up in aquatic environments thereby affecting non-target organisms including fish. They may cause fish kill, affect fish behaviour, feeding, growth and ultimately reduce fish productivity (Yaji, 2012). Over time, these chemicals may accumulate in fish tissues and are biomagnified in the food chain. Often, information on how herbicides affect fish is limited.

There is limited baseline information on the toxicity of herbicides that will guide regulators in setting out environmental limits of herbicides. For example, the guidelines and standards for environmental pollution control by FEPA (1991) (now Ministry of environment) specifies a Maximum Contaminant Level (MCL) of less than 0.01mg/L on all types and classes of pesticides. Hence, in Nigeria, regulations relating to the importation, sales, use and disposal of herbicides are based on the label information provided by manufacturers which are in most cases from foreign nationalities.

Atrazine and simazine are stable in pure solution, with an estimated half-life for hydrolysis of atrazine in sterile, neutral water of 1800 years (Armstrong and Chesters, 1968). Atrazine is persistent in water and together with its metabolites (which are often more harzadous than the parent product) can accumulate in drinking water resources downstream from farms (Kolpin *et al*., 1997). As of 2001, it is the most commonly

detected pesticide contaminating drinking water in the US with 75% of surface water and 84% of all groundwater in agricultural areas tested by the US Geological Survey contaminated with atrazine (Gilliom *et al*., 2006). Atrazine in soil breaks down through interaction with environmental compounds, particularly soil bacteria. Because the metabolizing bacteria are rare or nonexistent in surface water and groundwater, atrazine can persist for months to as long as a year (Rohr and McCoy, 2010) once it enters surface or ground water.

Furthermore, atrazine has been associated with adverse health effects in humans. It stimulates aromatase activity in human ovarian cancer cells (Albanito *et al*., 2008), and increases birth defects and infant mortality (Mattix *et al*., 2007, Winchester *et al*., 2009). In animals, it causes sexual abnormalities in male frogs at levels commonly found in rivers, streams and even rain at 30 times below the level allowed in drinking water (Sanders, 2002).

Sub-lethal effects with biochemical and histopathological alteration of fish tissues may occur with long term exposure to levels of less than 2 mg/L of atrazine (Neškovic *et al.,* 1993). In addition, atrazine is not readily broken down under alkaline soil conditions. It has a carryover effect, a generally undesirable property of herbicides.

1.3 Justification

The reduction in water quality cannot be ruled out as a major contributing factor to declining aquatic productivity and fish catches. The increase in the use and application of pesticides in agriculture in Nigeria today, which contributes greatly to aquatic pollution and hence reduction in water quality is mainly due to the increase in agricultural activities. Fish are one of the most widely distributed organisms in an aquatic environment and being susceptible to environmental contamination may reflect the extent

of the biological effects of environmental pollution in waters (Ramesh *et al.*, 2009). Fish are widely used to evaluate the health of aquatic ecosystems, and biochemical changes observed in fish serve as biomarkers of environmental pollution (Schlenk and Di-Giulio, 2002).

Fish are often used as sentinel organisms for ecotoxicological studies because they play a number of roles in the trophic web, accumulate toxic substances and respond to low concentrations of mutagens (Cavas and Ergene-Gözükara, 2005). Therefore, the use of fish biomarkers as indices of the effects of pollution are of increasing importance and can permit early detection of aquatic environmental problems (Lopez-Barea, 1996; Van Der Oost *et al*., 2003).

The importance of fish to man cannot be overemphasized. It constitutes one of the cheapest sources of animal protein as it contains biological values of essential amino acids. As the world population is expected to reach 9 billion by 2050 (New Nigerian, 2003), the demand for protein will equally be expected to increase.

Atrazine (2-Chloro-4-ethylamino-6-Isopropylamino-s-triazine) is one of the most currently and widely used herbicides in this part of the world and several studies have detected its presence in water bodies at levels above the limits determined by local and international authorities (for example the Maximun Contaminat Level of 3µg/L by USEPA). Due to its potential to bioaccumulate in organisms and its proposed negative effects, the application of atrazine has been banned in many countries (in the EU region by European Commission Directive No. 2004/248/EC). Hence, the tolerance limit of this herbicide on this promising species needs to be investigated.

Toxicants accumulated in tissues of animals catalyze redox reactions that generate ROS which may lead to oxidative stress and therefore cause haematological, biochemical and histopathological alterations. Knowledge on the nature of these alterations would be

pivotal to accurate diagnosis of pesticide toxicity especially in an environment that is becoming increasingly prone to pesticide contamination, and hence the possibility of increased incidence of pesticide poisoning. The nature of the haematological, biochemical, morphological and histopathological alterations associated with atrazine poisoning remains to be clearly defined in aquatic animals, hence the need for this study. Fish are widely used to evaluate the health of aquatic ecosystems, and biochemical changes observed in fish serve as biomarkers of environmental pollution (Schlenk and Di-Giulio, 2002).

Mortality or bioassay experiments in general present the most preferred way to evaluate the ecological influence of toxic compounds as their effects on fish and ecological risks cannot be determined by chemical analysis (Baser *et al*., 2003; Svobodova *et al*., 2003). Acute toxicity tests provide basis for understanding the limiting effects of various chemicals on organisms. Similarly, knowledge of the sub-lethal effects of toxic compounds at the biochemical, genetic and histopathological levels is very important for delineating fish health status and for understanding future ecological impact. The findings from this study will provide baseline information that will be of relevance in setting out environmental limits as regards use of atrazine.

1.4 Aim

The aim of this study was to evaluate the toxicity of atrazine to the juveniles of the African catfish, *Clarias gariepinus* (Bürchell, 1822).

1.5 Objectives

To determine the:

i. Acute toxicity of atrazine to *C. gariepinus* juveniles

ii. Acute and sub-lethal effect(s) of atrazine on certain haematological and oxidative stress response parameters of *C. gariepinus* juveniles

iii. Acute and sub-lethal effect(s) of atrazine on the histology of the liver and gills of *C. gariepinus* juveniles

iv. Sub-lethal effect(s) of atrazine on the growth of *C. gariepinus* juveniles

1.6 Hypotheses

i. Atrazine has no toxic effect on *C. gariepinus* juveniles

ii. Acute and sub-lethal concentrations of atrazine have no effect on the haematological and oxidative stress response parameters of *C. gariepinus* juveniles

iii. Acute and sub-lethal concentrations of atrazine have no effect on the histology of the liver and gills of *C. gariepinus* juveniles

iv. Sub-lethal concentrations of atrazine have no effect on the growth of *C. gariepinus* juveniles

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Presence and Effects of Pesticides in the Ecosystem

Pesticides are synthetic organic and inorganic compounds used to kill or contain the activities of pests. They may be chemical substances, biological agents (such as bacteria, viruses etc. as in biological control), or devices used against pests. At the beginning of the 20th century, two classes of pesticides were primarily used: botanical, which comprises naturally derived plant materials such as pyrethroids, rotenoids, nicotinoids, etc; and inorganic salts which are formulated and used as fungicides, herbicides, insecticides, molluscicides, acaricides, nematicides and algicides. Pesticides are generally applied to soil, plants, water bodies and in human settlements either as liquids, dusts or granules using man-mounted equipment such as tractors, knapsacks or aircrafts (Ndagi, 2014). With increasing industrialization and agricultural expansion, humans are continuously disturbing the delicate ecological balance in aquatic ecosystems (Kotb *et al.*, 2013).

According to Khan *et al.* (2003), pesticides affect all members of an ecosystem from the smallest vertebrates to birds and humans, and their toxicities to both urban and agricultural settings are responsible for the death of many fishes, birds and smaller aquatic animals that fish depend on for food. In their separate studies, Omitoyin *et al.* (2006) and Velmurugan *et al.* (2007) showed that aquatic contamination by pesticides cause acute and chronic poisoning of fish as well as severe damage to vital organs. It has also been shown that humans are at risk of exposure to pesticides either through skin, oral consumption or respiration (Hofelt, 2004). There is evidence of bioaccumulation of pesticides at higher trophic levels with possible deleterious effects, and also an indication

that humans are at risk of pesticide contamination through fish consumption (Giddings *et al.,* 2000).

Vigário and Sabóia-Morais (2014) reported that only a small portion of the large amounts of pesticides applied to crops reach their target. The other portions can have various effects in the ecosystem as outlined in numerous studies on wildlife, insects, soil microorganisms and aquatic organisms such as reduced biodiversity, reproductive and behavioral alterations, increased disease susceptibility, and accumulation of toxic substances that can reach humans through the food chain (Ozmen *et al.,* 2008).

A number of authors have linked pesticide contamination to changes in the chemistry of aquatic environments. Oruc *et al.* (2004) suggested that alterations of the chemical composition of natural aquatic environments can affect all fauna present, especially fish. However, Fent (2003, 2004) noted that pesticide toxicity is highly dependent on concentration, frequency, intensity of exposure, and target organism susceptibility, which in turn, is influenced by age, sex, health status and genetic attributes.

Herbicides are mainly synthetic organic compounds that are deliberately introduced into the environment to control weeds. They are a distinctive group of pesticides, which are considered selective chemical weed killers; hence they have been intensively used to destroy unwanted plants, especially in agricultural settings (Dutta and Meijer, 2003). However, there is a high risk of non-target organisms being affected due to drift sprays or run-offs.

Several authors have reported that herbicides originating from agricultural activity enter the aquatic environment through atmospheric deposition, surface run-of or leaching and frequently accumulate in soft bottom sediments as well as in aquatic organisms (Miles and Pfeuffer, 1997; Lehotay *et al.,* 1998; Kreuger *et al.,* 1999). Tilak *et al.* (2004) also reported that due to their persistence in the aquatic environment, herbicides accumulate

in fish causing toxicity. Maroni and Bersani (1994) reported that aquatic herbicides widely used for controlling undesirable weeds ended up polluting water bodies.

Wiegand *et al.* (2000) noted that herbicides are used to control aquatic weeds in fish management, especially in rice fields and some fish farms. Despite being the most widely used toxic chemicals for various purposes in gardens and agriculture where water often serves as a sink for these chemicals as reported by Houlahan *et al.* (2000), herbicides are often not entirely specific to their target organisms, hence non-target species such as aquatic biota are affected. There is a large amount of circumstantial evidence linking decline in population level (Houlahan *et al.*, 2000), developmental impairments, behavioural disturbances (Alvarez and Fuiman, 2005), deformities (Klump *et al.*, 2002) and various diseases in aquatic organisms to the use of herbicides.

2.2 Atrazine

Atrazine is a herbicide of the triazine class, which have been used extensively or selectively primarily to control broadleaf and some grassy weeds in agriculture and in non-agricultural areas worldwide for more than 50 years (Moretti *et al.*, 2002; Arufe *et al.*, 2004; Breckenridge *et al.*, 2008). Atrazine is used to prevent pre and post-emergence broadleaf weeds in crops such as maize (corn) and sugarcane and on turf, such as golf courses and residential lawn. It is chemically called 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine. Due to the low persistence of atrazine herbicide, repeated applications are practiced for the control of weeds in agricultural fields, and as a result, large quantities of the herbicide find their way into water bodies (Nwani *et al.*, 2010). Atrazine was manufactured in 1958 in the Geigy laboratories as the second of a series of 1, 3, 5-triazines (Wolfgang, 2007). In its press release of 4th April, 2002, the University of California through its public relations officer described it as a top-selling weed killer.

The herbicide is sold in Nigeria under the trade names Atraz 50, AtraForce®, Delzin®, Atranex, Actazin etc.

Solomon *et al*. (1996) reported that atrazine is a pre-emergent herbicide first approved for use in the US in 1958, where it is used primarily on corn, sorghum and sugar cane. According to Scrubner *et al.* (2005), Battaglin *et al.* (2007) and Battaglin *et al.* (2008), it is rated as moderately toxic to aquatic species; it is mobile in the environment and is among the most detected pesticides in streams, rivers, ponds, reservoirs and ground water.

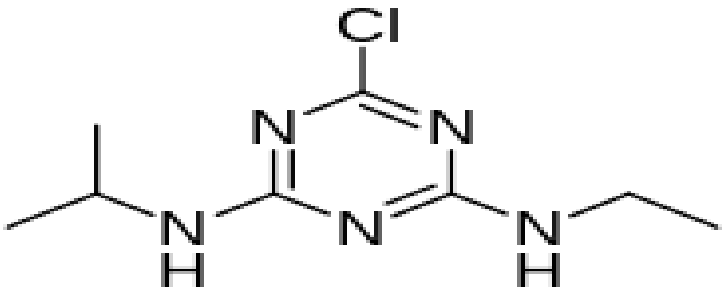


Figure 2.1: Chemical structure of Atrazine

IUPAC name: 1-Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine

Other names:

Atrazine
2-Chloro-4-ethylamino-6-isopropylamino-s-triazine
6-Chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine

Table 2.1: Properties of Atrazine at Standard Temperature and Pressure

Chemical formula	$C_8H_{14}ClN_5$
Molar mass	215.69 g·mol^{-1}
Appearance	colorless solid
Density	1.187 g/cm^3
Melting point	175°C (347°F; 448 K)
Boiling point	200°C (392°F; 473 K)
Solubility in water	7 mg/100 Ml

2.2.1 Mode of action

Atrazine shows high degree of crop tolerance and soil activity due to its high solubility in water, though lower than other s-triazines. Its uptake is through plants roots as well as young leaves; and has high crop selectivity. It functions by binding to the plastoquinone-binding protein in photosystem II, which animals lack. As a result, plants die from starvation and oxidative damage caused by breakdown in the electron transport process. The oxidative damage is quickened in high light intensity (Appleby *et al.,* 2002). Atrazine is also absorbed through leaves but has 0% transference rate (Abdali *et al.,* 2011).

When atrazine was first released for agricultural use, it was thought that since photosynthesis is limited to plants, animals and humans would be immune to any effects of atrazine. However, it was soon suspected that atrazine might have non-target action in animals including clastogenic (Yoder *et al.,* 1973), biochemical (Weigand *et al.,* 2001) and genotoxic (De Ventura *et al.,* 2008).

2.2.2 Environmental fate

Atrazine is a pervasive environmental contaminant (Cox, 2001). It is strongly persistent and is one of the most significant water pollutants in rain, surface, marine, and ground water (Wiegand *et al.,* 2001). Its persistence (it has a half-life of 125 days in sandy soils (WHO, 1990)) and mobility in some types of soils because it is not easily absorbed by soil particles, means that it often causes contamination of surface and ground waters. In the US for example, it has been found in the groundwater of all 36 river basins studied by the US Geological Survey (USGS) (Cox, 2001) and the USGS estimates that persistence in deep lakes may exceed 10 years.

Abdali *et al.* (2011) reported the half-life of atrazine to be 4 days within the soil (but normally the volume of the product may remain in the soil for up to 385 days in dry and sandy areas); three days in pure water; 30 days in seawater; 35 days on the sea bed and 72 days in vertebrate animals. According to Orme and Kegley (2004), atrazine has a hydrolysis half-life of 30 days and relatively high water solubility (32 mg/L), which aids in its infiltration into ground water. Atrazine concentrations of 20μg/L have been commonly detected in surface water runoff, while concentrations as high as 700μg/L have been reported (Perry, 1990; Solomon *et al.,* 1996; Selim, 2003).

Gamble *et al*. (1983) reported that atrazine or triazine based herbicides are not degraded by microbial or hydrolytic process after reaching the environment. However, a WHO (1996) report pointed out that atrazine can be degraded in surface water by photolysis and microorganisms and the half-lives of 20-50 days at 20-25°C have been found under laboratory conditions and increasing at lower temperatures (USEPA, 1988). Similarly, another report by USEPA (2003) points out that atrazine degrades in soil primarily by the action of microbes, with its half-life in soil ranging from 13 to 261 days. Atrazine was

banned in the European Union in 2004 because of its persistent groundwater contamination (Ackerman, 2007).

In their report, Rohr and McCoy (2010) reported that atrazine in soil breaks down through interaction with environmental compounds, particularly soil bacteria. Because the metabolizing bacteria are rare to nonexistent in surface water and groundwater, atrazine can persist for months and even to as long as a year once it enters surface or ground water. Approximately 75% of surface water and 40% of all groundwater in agricultural areas tested by the U.S. Geological Survey were contaminated with atrazine (Gilliom *et al*., 2006).

The USEPA (2003) reported that atrazine degrades in soil primarily by the action of microbes which occurs in two known pathways:

i. Hydrlysis of the C-Cl bond is followed by the ethyl and isopropyl groups, catalyzed by the hydrolase enzymes called AtzA, AtzB, and AtzC. The end product of this process is cyanuric acid, itself unstable with respect to ammonia and carbon dioxide. The best characterized organisms that use this pathway are *Pseudomonas* sp. strain ADP.

ii. Dealkylation of the amino groups gives 2-chloro-4-hydroxy-6-amino-1,3,5-triazine, the degradation of which is unknown. This path also occurs in *Pseudomonas* species, as well as a number of bacteria (Wackett *et al.,* 2002*;* Zeng *et al*., 2004).

2.2.3 Effect on humans

Atrazine is slightly to moderately toxic to humans and other animals. It can be absorbed into the bloodstream through oral, dermal and inhalation exposure. Symptoms of poisoning include abdominal pain, diarrhea and vomiting, eye irritation, irritation of

mucous membranes, and possible skin reactions (Hallenbeck and Cunningham-Burns, 1985).

Atrazine's effects in humans and animals primarily involve the endocrine system. Studies suggest that atrazine is an endocrine disruptor that can cause hormone imbalance (USEPA, 2007). Atrazine has been found to act as an agonist of the G protein-coupled estrogen receptor 1 (Prossnitz and Barton, 2014).

Little information is available regarding the effects of atrazine in children. Maternal exposure to atrazine through drinking water has been associated with low fetal weight and heart, urinary, and limb defects in humans (ATSDR, 2003). A study by Albanito *et al.* (2008) found that atrazine stimulates aromatase activity in human ovarian cancer cells, confirming an earlier study by Fan *et al*. (2007) which reported the same findings. Aromatase, an enzyme that converts testosterone into estrogen, is produced by the adrenal glands and by some cancer cells. Cancer patients with estrogen-promoted cancers (primarily breast, prostate, and ovarian cancers) are treated with aromatase inhibitors, a class of drugs designed specifically to block the production of aromatase, thereby decreasing levels of estrogen in patients (Duke, 2010).

A number of epidemiological studies found evidence linking atrazine exposure to adverse health effects in humans, For example, a study of Iowa farm communities found that increased exposure to atrazine nearly doubled women's risk of having low birth weight and premature deliveries (Munger *et al.*, 1997). More recent studies have found an increase in birth defects, the leading cause of infant mortality, among infants conceived during periods of peak atrazine use (Mattix *et al.*, 2007; Winchester *et al*., 2009).

2.2.4 Effect on other animals

A 2011 review of the mammalian reproductive toxicology of atrazine jointly conducted by the World Health Organization and the Food and Agricultural Organization of the United Nations concluded that atrazine was not teratogenic. Reproductive effects in rats and rabbits were only seen at doses that were toxic to the mother. Observed adverse effects in rats included fetal resorption (at doses >50mg/kg per day), delays in sexual development in female rats (at doses >30mg/kg per day), and decreased birth weight (at doses >3.6mg/kg per day) (WHO, 2011). At very high doses, rats showed excitation followed by depression, slowed breathing, incoordination, muscle spasms, and hypothermia (Hayes and Laws, 1990). After consuming a large oral dose, rats exhibited muscular weakness, hypoactivity, breathing difficulty, prostration, convulsions and death.

Lee (2003), Hayes *et al.* (2003) both reported that atrazine has been a suspected teratogen, with some studies reporting demasculinization in male northern leopard frogs even at low concentrations. Mizota and Ueda (2006) termed it an estrogen disruptor that can alter the natural hormonal balance. Koprivnikar *et al.* (2007) examined the relative importance of environmentally relevant concentrations of atrazine on trematode cercariae and tadpole defense against infection. Its principal finding was that susceptibility of wood frog tadpoles to infection by *Echinostoma trivolvis* was increased only when hosts were exposed to an atrazine concentration of 30ng/L and not to concentration 3ng/L. Sanders (2002) also reported that atrazine disrupts the sexual development of frogs at concentrations 30 times lower than levels allowed by the Environmental Protection Agency (EPA, 2007), raising concerns about heavy use of the herbicide on corn, soybeans and other crops in the Midwest and other parts of the world.

Lenkowski *et al.* (2008) reported that tadpoles developed deformed hearts and impaired kidneys and digestive systems when chronically exposed to atrazine concentrations of

10,000ppb in their early stages of life. Tissue malformation may have been induced by ectopic programmed cell death, although the mechanism was not identified. Similarly, a study conducted by Hayes *et al.* (2010) concluded that atrazine rendered 75% of male frogs sterile and turned one out of 10 into females. However in the same year, the Australian Pesticides and Veterinary Medicines Authority, APVMA (2010) tentatively concluded that environmental atrazine "at existing levels of exposure" was not affecting amphibian populations in Australia consistent with the USEPA (2007) findings. APVMA responded to Hayes' (2010) published paper, that his findings "do not provide sufficient evidence to justify a reconsideration of current regulations which are based on a very extensive dataset."

2.2.5 Acute and sub-lethal toxicity of atrazine to fish

Extensive use of atrazine has brought about focus of studies on its environmental effects. Studies on the toxic effects of atrazine on fish have shown varied responses according to the type of species and dosages, but the deadly concentration of the product has been assigned as 3 to 45mg/L (Elia *et al.*, 2002). Atrazine belongs to the toxicological class III, considered moderately toxic, used as a selective herbicide of pre and post emergence in different cultures.

Atrazine has been found to affect a variety of physiological processes in aquatic animals (Du Preez and van Vuren, 1992). Hostovsky *et al.* (2014) evaluated the overall effects of triazine herbicides on the physiology of fish. The study indicated that acute exposure of fish to triazines affects reproductive development in fish while long term exposure to low concentrations did not affect fish behaviour. Hussein *et al.* (1996) reported the LC_{50} of atrazine to be 9.35mg/L for the species *Oreochromis niloticus*. Aaronson *et al.* (1980) reported that rainbow trout (*Onchorhynchus mykiss*) died in pools containing 1000µg/L of atrazine. Ada *et al.* (2012) evaluated the effects of atrazine on some biological and

haematological responses of *Oreochromis niloticus* juveniles and observed some behavioural responses such as loss of reflex, air gulping, discolouration, haemorrhage and molting.

Ramesh *et al*. (2009) studied the terminal toxicity effects of atrazine on the blood factors of common carp (*Cyprinus carpio*) and concluded that the studied blood parameter levels were significantly affected by atrazine toxicity.The red blood cell count, haemoglobin, plasma glucose and plasma protein decreased whereas white blood cell count was enhanced upon exposure. The authors reported the 24-hour LC_{50} value of atrazine for the species to be 18.5ppm. Agbon *et al.* (2014) investigated the effects of atrazine on Nile Tilapia, using static renewal bioassay and reported the 96-hour LC_{50} value to be 6.977mg/L for the species. Sub-lethal exposure resulted in anaemia and increased white blood cell count.

Ada *et al.* (2012) reported an increase in the packed cell volume (PCV) and red blood cell count (RBCC), a decrease in haemoglobin (Hb), erythrocyte sedimentation rate (ESR) and mean cell haemoglobin (MCH), while there was no significant change in the white blood cell count (WBCC) and mean cell volume (MCV) values of *Oreochromis niloticus* juveniles exposed to nominal concentrations of atrazine. The authors attributed the decrease in number of cells to damage made to the cell nuclei by the herbicide, while increase in red bllod cells and multiplication of blood cell number may be an attempt to make more surface area available for oxygen binding. Atrazine was also found to cause a significant increase ($P<0.05$) in serum glucose, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) on one hand, and a decline ($P<0.05$) in serum total protein, total lipid, blood haemoglobin total erythrocyte count, packed cell volume and mean corpuscular haemoglobin concentration in the blood of *Clarias gariepinus* (Mekkawy *et al.,* 2013).

Nwani *et al.* (2010) studied the toxic effects of the herbicide atrazine on lipid peroxidation and antioxidant enzyme activity of *Channa punctatus* (Bloch). Results of the study indicated evidence of oxidative stress induction in the liver of the freshwater fish as shown by increased lipid peroxidation levels; antioxidants superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR), responded positively in a concentration dependent pattern.

In a study by Mekkawy *et al.* (2013), atrazine was found to cause a significant increase in lipid peroxidation in liver of *Clarias gariepinus* with increasing concentration. Manjunatha *et al.* (2015) observed an increase in antioxidant enzyme activity (catalase, superoxide dismutase, and lipid peroxidation) in the freshwater fish *Labeo rohita* during atrazine exposure. According to Kadry *et al.* (2012), atrazine exposure was associated with a marked induction of oxidative damage in the liver tissue of *Clarias gariepinus* as evidenced by increased level of lipid peroxidation (LPO) and reduced glutathione (GSH) content. Atrazine exposure also lead to a significant ($P<0.05$) increase in the activities of catalase (CAT) and suproxide dismutase (SOD).

Weignad *et al.* (2001) studied the changes induced by the toxicity of atrazine on Zebra fish embryos and concluded that it caused damage and disorder in the growth stages of embryos. Du Preez and van Vuren (1992) demonstrated that clustering of atrazine occurred in the kidney (at 40µg/g) and in the egg sack (at 50µg/g) of Tilapia fish after 72 hours of exposure. Ortiz *et al*. (2002) studied the effects of environmental chemicals on the bodies of various fishes and observed that fish had high capacities to bioaccumulation of the toxin in their fatty components which is transferred to the body through the gills and skin.

Warring and Moore, (2004) studied the effects of atrazine on Atlantic salmon smolt in fresh water and after transfer to salt water. They observed that it caused considerable

damage to the gill cells. Kadry *et al.* (2012) also carried out histopathological examinations of the liver of atrazine-exposed *Clarias gariepinus* and observed dilatation and congestion of blood vessels, fatty degeneration, necrosis and pyknotic nuclei of hepatocytes. Major histopathological effects of atrazine on the gills of *Rutilus frisii kutum* fry were found to be hyperplasia and thickening of the filaments, separation of the pavement cells of the lamellae epithelium from the pillar cells and swelling of the epithelial cells (Khoshnood *et al.,* 2014). Furthermore atrazine can cause damage to the gill epithelium and kidneys, increase the renal excretion of sodium, chloride and proteins in the rainbow trout (*Oncorhynchus mykiss*) (Fisher-Scherl *et al.* 1991) and common carp (*Cyprinus carpio*) (Neskovic *et al.*, 1993).

2.3 Pesticide Toxicity to Fish

2.3.1 Susceptibility of fish

Fent (2003, 2004) noted that pesticide toxicity is highly dependent on concentration, frequency, intensity of exposure, and target organism susceptibility, which in turn, is influenced by age, sex, health status and genetic attributes. Several studies have reported that the size and species of fish affects toxicity of pollutants. Mohammed (1995) demonstrated that larger fishes proportionately remove higher amounts of pesticides than smaller ones suggesting that susceptibility decreases with age and size. Auta *et al*. (2000) also observed that larger fishes are less susceptible to toxicants than smaller ones. Auta *et al.* (2002) exposed juveniles of *Oreochromis niloticus* and *Clarias gariepinus* to dimethoate and observed that symptoms of toxicosis varied in the two species. Differences in susceptibility to certain industrial wastes have also been established for *Oreochromis niloticus* and *Clarias garipinus* (Gbem *et al.,* 1997; Gbem, 1998).

Susceptibility of fish also depends on the time of the year. In Nigeria for example, susceptibility is higher during the dry season at a time when the dilution capacity of the

water systems is low thereby increasing the effects of the pesticides, making the dry season the critical period for many animals especially fish and birds (Ita, 1993).

Ndagi (2014) reported that the length of time a pesticide remains in the environment determines its capacity to harm organisms. This depends on how quickly it breaks down which is largely a function of its chemical composition and the prevailing environmental conditions. The mode or process of degradation also comes into play as chemicals broken down strictly by microbial processes may remain longer in periods or climates were such organisms are rare, absent or non-existent.

2.3.2 Pesticide toxicity and fish behaviour

The behavioural and morphological change in fish, a diagnostic end point for screening and differentiating chemicals according to their mode of action has been considered a promising tool in ecotoxicology (Dummond *et al.,* 1986). Little (1990) stated that behavioural responses are effective indicators of contamination and reflect sub-lethal toxicity. Chemicals are categorized to correspond to three general modes of action syndrome namely hyperactivity, hypoactivity and physical deformity and each sign of stress is indicative of a different mode of action (Drummond and Russom, 1990).

Symptoms of toxicosis such as hyperactivity and agitated swimming, loss of equilibrium, air gulping, period of quiescence and death as well as accumulation of mucus on the gills and body surface were observed in *Oreochromis niloticus* and *Clarias garipinus* after exposure to dimethoate for 96 hours (Auta *et al.*, 2002). The behavioural responses of *Oreochromis niloticus* and *Clarias garipinus* to cassava mill effluent, cassava leaf extract, chlorpyrifos and detergent included increase in opercular beat frequency, tail beat frequency, erratic swimming, frequent attempts to jump out of the aquaria, loss of balance excess mucus secretion (towards the 96th hour), darker skin at death, skin of fish becomes

dry and losses mucus lining and haemorrhaging of gill filaments were also observed (Oti, 2002; Chindah *et al.*, 2004; Ogundele *et al.*, 2004; Onuoha and Ohaturonye, 2007). Auta *et al.* (2002) reported that acute concentrations of cypermethrin cause behavioural abnormalities such as irregular and erratic movement and air gulping.

Similarly, Nwani *et al.* (2013a) evaluated the acute toxicity of butachlor and its effects on the behaviour of the freshwater fish *Tilapia zilli*. Findings indicated the 96-hour LC_{50} value for the species to be 1.25mg/L of the herbicide. Stress signs in form of hyperactivity, erratic and jerky swimming, attempt to jump out of water, frequent surfacing and gulping of air, decreased opercular movement and secretions of mucus on the body and gills, skin discolouration, vigorous jerks of the body followed by exhaustion and death were also observed. Ikele *et al.* (2011) reported the 96-hour LC_{50} value for *C. gariepinus* to be 1.87µg/L, and during exposure to diethyl phthalate, fish were restless, swam erratically, and haemorrhaging of the gill filament was evident. Okayi *et al.* (2012) recorded a 96-hour LC_{50} value of 0.0097ml/L of propanil for fingerlings of *O. niloticus* under laboratory conditions. Fish showed abnormal behaviour upon exposure to propanil such as erratic swimming and tendency to jump out of the test bowl, restlessness, uncoordinated movement, vertical swimming, air gulping, equilibrium loss, a period of quiescence and eventually death.

Nwani *et al.* (2013b) evaluated the acute toxicity and behavioural changes in *C. gariepinus* exposed to the herbicide Termifos and reported behavioural changes such as uncoordinated behaviour, erratic and jerky swimming, attempt to jump out of water, frequent surfacing and gulping of air, decreased opercular movement and secretions of mucus on the body and gills, followed by exhaustion and death. The symptoms of toxicity of pollutants and toxicants reported by various authors seem to be independent of the

toxicant type. These symptoms include loss of balance, air gulping, agitated swimming, sudden darting, swimming in circles, profuse secretion of mucus on bodies, swellings of abdomens, exfoliation of the skin and in some cases gill haemorrhage (Olusegun, 2001).

2.3.3 Pesticide toxicity and fish respiration

In fish, gills are vital for respiration and osmoregulatory functions, and respiratory distress is one of the early signs of pesticide intoxication (Jayachandran and Pugazhendy, 2009). The first site of toxicant action in fish is probably the gill, being the primary site for respiration and osmoregulation. Damage of the gill architecture may trigger physiological responses as it tries to offset the balances by breathing faster, causing more toxicant to be passed over the gills (Balinth *et al.,* 1997). Since fishes breathe in the water in which they live, changes in its chemical properties may be reflected in the fish's ventilation activity particularly if the environmental factors affect respiratory gas exchange (Mushigeri, 2003). The fluctuated response in respiration may be attributed to respiratory distress as a consequence of the impairment of oxidative metabolism,

The opercular ventilation and tail fin beat of *Oreochromis niloticus* exposed to Gammalin 20 and actellic 25EC initially increased sharply in a directly proportional relationship with toxicant concentration followed by a drop first to the status quo around the 48th hour and thereafter below it (Ufodike and Omoregie, 1990). Oropesa *et al.* (2009) reported rapid ventilation, increased rate of gill cover movement and floating at the surface of water as a response to respiratory distress in common carp after exposure to simazine. The increase was positively correlated to the toxicant concentration.

2.3.4 Pesticide toxicity and fish haematology

Haematological parameters are important physiological indices of changes in the external conditions of an organism. Blood reflects pathophysiological status of the body as it's

conditions at a point in time depends on internal and external variables; hence it can serve important roles in diagnosis of the structural and functional status of fish exposed to toxicants (Ololade and Oginni, 2010). Also, being a supplier of essential nutrients, ions, gases and endocrine factors, coupled with its function as a reservoir of excretory products of metabolism implies that alterations in blood parameters are often reflective of the overall toxic impacts of environmental contaminants (Dietrich *et al.,* 2006).

Lindalva *et al.* (2013) in their study on the haematological and biochemical alterations in the fish caused by the herbicide clamazone reported that exposure of the fish *Prochilodus lineatus* to the herbicide for 96 hours at concentrations of 5 and 10mg/L led to important alterations in the haematological and biochemical parameters of *Prochilodus lineatus* as well as neurotoxic effects on the species. Jayaprakash and Shettu (2013) observed a decrease in the haemoglobin content, total erythrocyte count, packed cell volume, mean corpuscular volume and mean corpuscular concentration in *Channa punctatus* exposed to deltamethrin. These changes were attributed to stress and hypoxia caused by the toxicant or the swelling of erythrocytes causing lysing of the red blood cells.

Significantly higher values ($P<0.01$) of erythrocyte count, haematocrit and haemoglobin were reported by Velisek *et al.* (2008). However, they observed a significant decrease ($P<0.01$) in both relative and absolute lymphocyte count and a significant increase ($P<0.01$) in both relative and absolute count of neutrophil granulocytes in rainbow trout (*Oncorhynchus mykiss*) exposed to the herbicide metribuzin. Ajani and Awogbade (2012) observed a significant decrease in total protein and albumin levels in the blood of *Clarias gariepinus* exposed to higher concentrations of Diuron, while fish exposed to a working dilution of $1/10LC_{50}$ (0.003g/L) of the herbicide showed no significant differences from the control. The authors attributed the decrease observed in total protein values at half the

LC_{50} to the possible toxic effects of diuron on the immune system and/or the haemodilution effect. The 96-hour LC_{50} for the species was 0.03g/L of Diuron.

Elbialy *et al.* (2015) reported a significant decrease in red blood cell count, packed cell volume, haemoglobin, neutrophilia, lymphonia and monocytosis in Nile Tilapia (*Oreochromis niloticus*) exposed to different levels of organochloride and organophosphate pesticides. Obiezue *et al.* (2014) reported no significant differences in the erythrocyte, lymphocyte, neutrophil, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and haemoglobin levels of *Clarias gariepinus* between diethyl phthalate treated group and the control. However, the packed cell volume, mean corpuscular volume and leucocytes count showed statistically significant values between the control and treated groups. The authors attributed the observed decrease in the PCV, Hb and erythrocyte count of *C. gariepinus* after 30 day exposure to be indicative of heamodilution due to erythrocyte sequesteration. In the same vein, reduction in the total erythrocyte count of tannery effluent exposed *Clarias garieinus*, as reported by Gbem *et al.* (2003) was attributed to the possibility of inhibition in erythrocyte production as well as a possible increase in the rate of erythrocyte destruction or haemodilution. The authors also associated the increase in packed cell volume and haemoglobin concentrations in tannery effluent treated group of the fish to haemo-concentration since changes occurred in erythrocyte numbers.

2.3.5 Pesticide toxicity and fish histology

Histopathology reflects a higher level response as a manifestation of prior alterations in the physiological and/or the biochemical function of organisms (Hinton *et al.*, 1992). The structure and function of tissues may be affected by toxic substances in aquatic environments. Velisek *et al.* (2009) observed that exposure to toxicants causes severe alterations in the tissue biochemistry and histology of fishes. Omoregie and Ufodike

(1991) evaluated the toxicological effects of acetellic 25EC on the gills, liver and kidneys of *Oreochromis niloticus* fingerlings using the continuous water flow system and observed that structural damages were more pronounced in the gills while the liver and kidney had minor necrotic damages. The authors concluded that the major sites of histopathological damages in fish are the liver, gills and kidneys.

Oulmi *et al*. (1995) studied the deadly effects of various atrazine concentrations in Rainbow Trout (between 10 to 160µg/L) and observed effects on the kidney including endoplasmic softening. Palikova *et al.* (2004) observed vacuolar dystrophy of hepatocytes with damage of nuclei (pyknosis, karylosis) in the liver as well as changes in the kidney of *Cyprinus carpio* after long term exposure to low concentrations of cyanobacteria. Yildrim *et al.* (2006) reported that exposure of *O. niloticus* to 5µg/L of deltamethrin caused lamellar fusion in the gills. Histopathological changes in the liver and kidney of diethyl exposed *Clarias gariepinus* revealed pyknotic nuclei, fusion of tubules, condensation of the glomerulli and severe destruction of the tubule in the kidney tissue of fish, while cellular proliferation, congestion, necrosis, sinusoid enlargement, paranchymatous degeneration and fatty or glycogen degeneration were observed in the liver tissue of fish (Ikele *et al.* 2011). They attributed changes in the liver to the fact that it is a major site for biotransformation of toxic chemicals which usually makes them less toxic and more easily excreted; necrosis of the liver tissues observed probably resulted from the excessive work required by the fish to get rid of the toxicant from its body during the process of detoxification by the liver. The inability of fish to regenerate new liver cells may also have led to necrosis.

Fanta *et al.* (2003) reported that the histopathological alterations observed in the gills of *Corydoras paleatus* after exposure to sub-lethal doses of methyl parathion included epithelial hyperplasia in the respiratory lamella, oedema and detachment of the epithelial

cells while changes observed in the liver were cloudy swelling, bile stagnation, focal necrosis, atrophy and vacuolization. Denton *et al.* (2005) reported glycogen depletion as the only histological effect after exposure of head minnows (*Pimephales promelas*) to acute concentrations of diazinon and fenvalerate singly and in combination. Chromcova *et al.* (2015) observed histopathological changes such as oedema of the gill lamellae, focal separation of gill lamellar surface and aggregation of mononuclear cells compared with the control in the gills of *Cyprinus carpio* exposed to NeemAzal while no histopatthological changes were observed in the kidney, liver and skin.

The 96-hour LC_{50} value of 34.64 microliter per liter of the herbicide 2, 4-D for the species *Poecilia vivipara* was reported by Vigario and Saboia-Morais (2014). Histopathological changes observed in the fish exposed to the highest concentration of 40µl/L of the herbicide include normal hepatocytes as well as changed ones, many Ito cells, as well as micronuclei and nuclear swelling. Similarly many histopathological changes including degeneration and necrosis, disorganised myofibres with chromophobis cytoplasm, haemocytic infiltration, oedema and inflammations were noticed in nimbecidine-treated Nile Tilapia (Mutawie and Hegazi, 2011).

Velisek *et al.* (2008) exposed Rainbow trout (*Oncorhynchus mykiss*) to the herbicide metribuzin. Histopathological examinations revealed mild proliferations of goblet cells of the respiratory epithelium of secondary gill lamellae and hyaline degeneration of epithelial cells of the renal tubules in the kidney of exposed fish. Obiezue e*t al.* (2014) reported gill damages in *Clarias gariepinus* exposed to diethyl phthalate with raised lamella, oedema of the lamella epithelia, loss of lamellar epithelium, mild oedema and raising of the filament. Liver damage showed focal necrosis and vacuolization as well as hepatocyte degeneration.

2.3.6 Pesticide toxicity and fish biochemical properties

According to Coppo *et al.* (2002), cells contain enzymes that are vital in executing their functions. When the integrity of the cell is disrupted, enzymes escape into plasma serum, where their activity is measured as a useful index of cell integrity. Luskova *et al*. (2002) reported that changes in the biochemical blood profile indicate changes in metabolism and processes of an organism resulting from the effects of various pollutants and makes it possible to study the mechanism of the effects of such pollutants. Ram *et al.* (2003) observed that the rate of protein synthesis may be affected due to impaired incorporation of amino acids into polypeptide chains. Plasma protein can vary with sex, size and maturity and is influenced by temperature and food availability. Prado *et al.* (2009) reported that the nutritional value of fish depends on their biochemical composition which is affected by water pollution.

Loro *et al.* (2015) investigated the effects of glyphosate-based herbicide on some biochemical parameters in *Rhamdia quelen* and *Leporinus obtusidens* and reported an increase in the transaminase activities (ALT and AST). A significant increase in alanine aminotransferase (ALT), aspartate aminotransferase, urea and creatinine levels was observed in Nile Tilapia exposed to different concentrations of organochloride and organophosphate herbicides (Elbialy *et al.*, 2015). Ogueji and Auta (2007) reported that sub-lethal concentrations of chlorpyrifos ethyl resulted in a highly significant ($P<0.001$) dose dependent inhibition in protein, cholesterol, triglycerides, glutamic oxaloacetic transaminase GOT, alkaline phosphatase ALP and highly significant ($P<0.001$) increase in glucose and glutamic pyruvic acid transaminase GPT. Sarikaya (2009) observed that sub-lethal dose of cypermethrin (1/10 and 1/10 of LC_{50} at 0.13ppm) altered the biochemical and haematological parameters, as well as enzymes of organs and exerted stress in Indian major carp (*Labeo rohita*) fingerlings.

Velisek *et al.* (2008) investigated the effects of metribuzin on rainbow trout (*Oncorhynus mykiss*). Exposed fish also showed lower levels ($P<0.01$) of plasma total protein, triacylglycerols, asprtate aminotransferase, ammonia, calcium, lactate and alkaline phosphatase compared to the control. Al-Kahtani (2011) evaluated the effects of the insecticide abamectin on some biochemical characteristics of the Tilapia fish *Oreochromis niloticus*. Data he obtained showed a decline in the rate of oxygen consumption during all the exposure periods. On the other hand, all biochemical parameters (total protein, carbohydrate and cholesterol in liver, muscle, kidney and gills) were found to decrease in all tissues in comparison with the control. El-Shebly and El-Kady (2008) evaluated changes in serum concentrations of growth hormone (GH) and muscle protein content in *Oreochromis niloticus* treated with different concentrations of glyphosate herbicide. Control fish exhibited higher serum growth hormone levels ($P<0.05$) compared to treated fish. Growth hormone decreased significantly with increase in glyphosate concentration.

2.3.7 Pesticide toxicity and oxidative stress induction and anti-oxidant enzyme activity

Oxidative stress represents an imbalance between the production of free radicals and the biological system's ability to readily detoxify the reactive intermediaries or to repair the resulting damage (Flora, 2011). Oxidative stress occurs when there is a disruption in the balance between the production and removal of reactive oxygen species ROS and other free radicals (Santos *et al.,* 2004; Ortiz-Ordonez *et al.,* 2011). The highly susceptible targets of oxidative stress are polyunsaturated fatty acids and proteins of the cell membrane (Sevgiler *et al.,* 2007).

Oxidative stress in aquatic organisms, principally fish, has great importance for environmental and aquatic toxicology. Because oxidative stress is induced by many

chemicals, including some pesticides, these contaminants may stimulate reactive oxygen species and alteration in antioxidant systems. Pro-oxidant factor actions in fish can be used to assess pollution of specific areas or worldwide marine pollution (Üner *et al.,* 2006 and Slaninova *et al.,* 2009). Herbicides are able to stimulate the production of reactive oxygen species through diverse different mechanisms (van der Oost *et al.,* 2003). Reactive oxygen species play an important role in the toxicity caused by herbicides and other environmental chemical contaminants (Uner *et al.,* 2005).

Reactive oxygen species are removed by cellular defenses such as catalase CAT, superoxide dismutase SOD, reduced glutathione GSH, or glutathione peroxidase GPX. Non-enzymatic antioxidants such as α-tocopherol (vitamin E), ascorbate (vitamin C), β-carotene (vitamin A), flavonoids (quercetin, rutin, etc.) and selenium can also act to overcome the oxidative stress, being a part of total antioxidant system (Sies *et al*., 1992). However, when ROS become too numerous or are produced beyond the natural system's ability to get rid of them, damage to membranes, proteins and DNA results. Reactive oxygen species, free radicals and reactive metabolites are produced during metabolism of toxins through mixed oxidase systems including cytochrome P450 (Yaji, 2012).

A number of authors have recorded antioxidant activity in toxicant exposed fish, thus suggesting the use of these antioxidants as potential biomarkers of toxicity associated with toxicant exposure in freshwater fishes. Kotb *et al.* (2013) recorded noticeable changes in antioxidant biomarkers with a reduction in SH-protein levels and an elevation in lipid peroxidation in plasma after treatment with two concentrations of diclofop-methyl herbicide when they studied the biochemical effects of the herbicide and its bioaccumulation in the freshwater fish *Oreochromis niloticus.* In their study, Chromcova *et al.* (2015) reported a significant increase in glutathione peroxidise and glutathione S-transferase ($P<0.05$) in common carp (*Cyprinus carpio* L.) exposed to NeemAzal.

Exposure of Nile Tilapia to Nimbecidin induced significant decrease in the activity of glutathione, catalase and total protein content of muscle of the species in a concentration dependent manner (Mutawie and Hegazi, 2011). Stara *et al.* (2012) evaluated the effects of chronic exposure to prometryne on oxidative stress and antioxidant response in the early life stages of common carp *Cyprinus carpio*. The authors reported no significant effect on the levels of oxidative damage on fish of exposed group. Franco-Bernardes *et al.* (2014) evaluated the biochemical and genotoxic effects of a commercial formulation of the herbicide tebuthiuron in *Oreochromis niloticus* of different sizes. They reported alterations in antioxidant parameters only in the liver of treated fish compared to the control group. Catalase (CAT) decreased in large fish and glutathione increased in small fish. However no lipid peroxidation was shown when melanoldehyde analysis was carried out. Nwani *et al* (2015) evaluated the oxidative stress and biochemical responses in the tissues of African catfish *Clarias gariepinus* exposed to primextra herbicide. Results of the study indicted that superoxide dismutase (SOD) levels in the treated groups were comparable to that of the control except on day 15 of the study in the liver when it declined. The study concluded that the herbicide induced toxic stress even at sub-lethal concentrations.

2.3.8 Pesticide toxicity and fish growth and reproduction

The reproductive potential of fish is affected when reared in water containing pesticide residues (Moore and Warring, 2001). Gbem (1998) observed that toxicants suppress growth of fish, the intensity of this effect being dose dependent. Growth can be affected by toxins directly or indirectly through effects on feeding due to the fact that these processes are linked (Olusegun, 2001). An indirect link is that reduced physical activities could affect feeding (Gbem, 1998), and invariably growth. Notably, fish tend to increase their metabolic activities towards excretion of toxicants, hence making more energy

available for homeostatic maintenance rather than storing which could be geared towards growth (Gbem *et al.*, 2003).

In cases where toxicants destroy the gills and also affect the erythrocyte counts or cause histopathological damage of the gut, inefficient transportation and utilization of assimilated food may result leading to reduced growth (Oladimeji and Ologunmeta, 1987; Gbem, 1998). A decline in the growth of *Cyprinus carpio* was observed when exposed to sub-lethal concentrations of dipterex (Chindah *et al.*, 2004). The rate of feeding in Heteroclarias was found to reduce considerably with increasing concentrations of the pesticides thiodon, malathion and acarbaryl (Avoja and Oti, 1997). Ponmani *et al.* (1997) observed a significant reduction in feeding rates, consumption, absorption, metabolism and growth, as well as conversion efficiencies of *Cyprinus carpio* exposed to sub-lethal concentrations of monocrotophos. Aguigwo (2002) observed growth parameters such as specific growth rate (SGR), feed conversion efficiency (FCE) and protein efficiency ratio (PER) to decrease as the concentration of cymbush pesticide increases in sub-lethal tests.

Auta and Ogueji (2006) observed marked changes in the feeding, behaviour, feed utilization and growth of *Oreochromis niloticus* after long term exposure to dimethoate. This was evident in the significant reduction in specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), apparent net protein utilization (APP NPU) and productive protein value when compared with the control. Shallangwa and Auta (2008) reported a reduction in the growth of *Clarias gariepinus* exposed to sub-lethal doses of 2, 4-Dichlorophenoxy-acetic acid and attributed this to lower feeding rates, unsuitability of the feed caused by the toxicant or an increased expenditure of energy on chemical detoxification and tissue repair. In addition, comparison of the length-weight and condition factor of Caspian kutum fry exposed to atrazine showed no

significant differences between atrazine exposed fish and the control group (Khoshnood *et al.*, 2014).

Ramachandra (2000) has associated lower doses of malathion with a reduction in the ovarian weight and growth inhibition of the vitellogenic oocytes. Giri *et al.* (2000) observed that the insecticide basathrin induced histoanatomical insult of the ovarian tissues of *Heteropneustes fossilis.* They also observed marked damages in the germinal epithelium, artesia of oocytes, stromal haemorrhage, vacuolization of oocytes and general inflammation

Sprague (1970) advocated routine growth measurement in all chronic toxicity tests because it is easy to measure. However, he went further to caution that growth is not always a sensitive indicator of toxicity. Food availability, consumption and efficiency of utilization reflect growth. Growth is also a reflection of an organisms' stage of development, maturation and size.

2.3.9 Bioaccumulation of pesticide residues in fish tissues

Several studies have reported bioconcentration of pesticides in higher trophic levels implying that human beings are at risk of pesticide poisoning through fish consumption (Giddings *et al.*, 2000). Pesticide accumulation in fish tissues may be attributed to their lipophilic nature. They become biomagnified in the food chain which is a source of concern since they are fed upon by humans (Tilak *et al.*, 2003).

Ahmed *et al.* (2001) reported residues of endosulfan, heptachlor and dicofol in the gills of *Mugil cephalus.* Tilak *et al.* (2003) observed uptake of fenvalerate in tissues (liver, gill, brain, kidney and muscle) of *Channa punctatus*. Lamai (2003) evaluated the accumulation of dieldrin in muscle, liver and brain of *Clarias gariepinus.* Yaji (2012)

reported significant ($P<0.05$) values of propanil and cypermethrin herbicide residues in the tissues of *Oreochromis niloticus* juveniles.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Fish and Atrazine Sources

Juveniles of *Clarias gariepinus* of mixed sexes and fairly uniform sizes were obtained from a fish farm in Zaria, Kaduna State, Nigeria, and transported in plastic containers to the outdoor holding ponds in the Department of Biological Sciences, Ahmadu Bello University, Zaria. Fish were acclimated in the pond for a period of two (2) weeks in dechlorinated tap water under prevailing weather conditions and natural photoperiods. During acclimation, fish were fed with a commercial feed (Coppens 2mm) at 4% body weight daily in two rations i.e morning and evening (Ajani *et al.,* 2007). Feeding was stopped 24 hours prior to the commencement of the experiment. Atrazine was obtained

as a commercially available herbicide AtraForce® 50% SC from an agrochemical outlet in Zaria.

3.2 Preparation of Test Solution

AtraForce® 50% SC was obtained at a concentration of 500g/L in a one liter container. From the 500g/L, a stock solution was prepared by adding 1ml of the herbicide to 999ml of water (Reish and Oshida, 1987). The stock solution was then used to prepare different nominal concentrations of the toxicant by diluting measured volumes of the toxicant with dechlorinated tap water. The control solutions had only dechlorinated tap water without the toxicant.

3.3 Range Finding Test for the Determination of Atrazine Concentrations (Pilot Studies)

Based on available literature on acute and sub-lethal toxicity on tropical freshwater fish species, range-finding tests were conducted to determine the concentrations of atrazine that were used in the definitive tests. This was done by placing six concentrations of the herbicide in separate plastic aquaria containing 20 liters of dechlorinated tap water. Mortality of fish was determined at 12, 24, 48, 72 and 96 hours. The concentrations of the herbicide were fine-tuned using lower ranges until about 80 to 90% mortality was recorded in the highest concentration and 20 to 30% in the lowest concentration. The five concentrations used in the acute test were then geometrically ranged between these highest and lowest concentrations and made into triplicates.

3.4 Experimental Design

Healthy fish were randomly selected, weighed and distributed into eighteen (18) plastic aquarium tanks (42.5 × 30.5 × 22.5cm) containing 20 liters of dechlorinated water. 10 juveniles of *Clarias gariepinus* (of mean weight 9.13±2.12g and standard length 10.00±0.88cm) regardless of sex were plaed in each tank, (totaling one hundred and eighty (180) fish), for the acute toxicity test i.e. six (6) treatments, including control in three (3) replications. Similarly, 10 fishes were randomly placed in each test aquarium tank for the sub-lethal toxicity bioassay consisting of four treatments (including a control) in three replications, (totaling one hundred and twenty (120) fish). The experiment was set out in 6 × 3 factorial in a completely randomized design for the acute toxicity test and 4 × 3 in the sub-lethal bioassay.

3.5 Exposure of Fish to Atrazine

3.5.1 Acute toxicity bioassay

A four-day static renewal toxicity bioassay was conducted in the laboratory to determine the toxicity of atrazine to juveniles of *Clarias gariepinus* as described by ASTM (2014) Sprague (1973) and APHA (2005). From the results of the pilot studies, five concentrations (0, 9, 10, 11, 12, and 13mg/L) were selected and used for the acute toxicity. These concentrations were dispensed using a pipette into each aquarium tank and allowed to stand for 10 minutes for the toxicant to be evenly distributed by dilution. The control had only dechlorinated tap water. Ten fish specimens were selected randomly and stocked in each aquarium tank (APHA, 1981; Cengiz *et al.,* 2001; Adeyemo, 2005; Ayoola, 2008). These were replicated three times (Ayoola 2008) for each concentration. Water quality properties (temperature, pH, total dissolved solids, electrical conductivity and dissolved oxygen) of the test solution and the control were

monitored every 24 hours (APHA, 2005), to ensure they are within suitable range for the growth of the fish. Fish were not fed during the experiment.

3.5.2 Behavioural Studies

Observations on behavioural responses were made at 3, 6, 12, 24, 48, 72 and 96 hours post exposure (Agbon *et al.,* 2014; ASTM, 2012). To provide a reference point for determining changes in morphological or behavioural features, the toxicant exposed fish were monitored and compared with the control. Changes in toxicant-exposed fish different from that of the control were recorded if they occurred in at least 10% of the fish in each test aquarium.

The opercular ventilation frequency and tail fin beat rates were observed as beats per minute using a stopwatch. The opercular ventilation frequency/count and tail fin movement were determined twice daily at predetermined time intervals (Olusegun, 2001). Observations were made for three fishes in each aquarium and the mean recorded. The results were pooled and daily averages determined.

3.5.3 Mortality

Examination of the experimental set-up for fish mortality was made at the end of 1, 2, 4, 6 and 12 hours post exposure and then twice daily before termination of the experiment (Olusegun, 2001). Juveniles of *Clarias gariepinus* were certified dead when there was cessation in opercular movement and a gentle prodding elicited no response. The number of dead fish was recorded against the time of death in a tabular form in each treatment (Sprague, 1973). Dead fish were immediately removed to avoid dissolved oxygen depletion (Schreck and Brouna, 1975).

3.5.4 Sub-lethal toxicity bioassay

Based on the results of the acute toxicity bioassay, the 96-hours LC_{50} was calculated and fractions of 1/5, 1/10, 1/20 of the 96 hour LC_{50} were used as the concentrations of the toxicant used during the sub-lethal toxicity bioassay as recommended by Oladimeji and Ologunmenta (1987) and Mohammed (1995) in a static experiment. Following the same pattern of randomization as in the acute toxicity test, fresh specimens were exposed to concentrations of 0, 0.59, 1.19 and 2.38mg/L of the toxicant in triplicates, at a stocking density of 10 fish per aquarium tank. A total of one hundred and twenty fishes were used i.e. four treatments (including a control) in three replications. 12 plastic aquaria were used for the sub-lethal experiment which lasted for eight (8) weeks. Fish were fed with pelleted commercial feed at 3% body weight daily, which was split into two rations. Water quality properties (temperature, pH, electrical conductivity and dissolved oxygen) were monitored on a weekly basis throughout the period of the experiment following methods described by APHA (2005).

3.6 Haematological Analysis

3.6.1 Blood sampling

At the end of both acute and sub-lethal toxicity bioassays, blood samples were collected from fish using 2ml syringes from the dorsal blood vessel lying below the vertebral column (Lewbert 2001; Ada *et al*., 2012). The blood was then quickly transferred into EDTA bottles to avoid clotting. From each replicate, composite samples were obtained from 3-5 fishes to get sufficient blood for haematological analysis. Part of the blood samples was put into plain sample bottles to obtain serum after coagulating for total protein determination.

Indices used to evaluate the haematological profile of *Clarias gariepinus* from control and toxicant exposed groups include the total erythrocyte count (RBC), haemoglobin concentration (Hb), haematocrit (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), leukocyte count (WBC) and the differential leukocyte count (DLC).

3.6.2 Packed cell volume, PCV (haematocrit)

PCV of fish exposed to the toxicant as well as that from the control in both tests was determined by the Unified Methods for Haematological Examination of Fish as described by Svobodova *et al*. (1991). Blood sample was drawn into micro-capillary tubes. The tubes were then centrifuged for five minutes and the reading taken with the aid of a haematocrit reader and expressed as the volume of erythrocytes per $100cm^3$.

3.6.3 Total leucocyte count (TLC)

The number of leucocytes was determined according to the Haemocytometer method of Hesser (1960), using Shaw's solution which is made up of two diluting fluids A (neutral red 25mg; sodium chloride 0.9g; distilled water 100mls) and B (crystal violet 12mg; sodium citrate 3.8g; formaldehyde 0.4mls; distilled water 100mls). For leucocyte counts, Shaw's solution developed for counting avian leucocytes was found to be satisfactory. This is because in fish, both leucocytes and erythrocytes are nucleated. As a result, the solutions which would normally lyse human erythrocytes cannot lyse that of fish (Hesser, 1960). Blood was drawn to the 0.5 mark on the stem of a white cell pipette. Solution A was drawn until the bulb of the pipette was filled half way and filled with solution B to the 101 mark and shaken. Few drops were dispensed into the haemocytometer. The cells in the four large squares of the chamber were counted with the microscope at ×40. The

number of cells counted was multiplied by 500 to obtain the total number of leucocytes per cubic millimeter (mm^3) of blood (Hesser, 1960).

3.6.4 Total erythrocyte count (TEC)

For erythrocytes count, Hendricks diluting solution was used. The standard RBC diluting pipette was used and a 1:200 dilution was made. This was done by drawing blood up to the 0.5 mark and then filling the pipette up to the 101 mark with the diluting fluid. The pipette was shaken to ensure thorough mixing. The diluted suspension of cells was drawn into the counting chamber of the improved Neubauer's haemocytometer by capillary action. The haemocytometer was then placed under the microscope and the number of cells counted and multiplied by 10 which gave the total number of cells per cubic millimeter of blood (Hesser, 1960).

3.6.5 Differential leucocyte count (DLC)

The slide method was used to determine the DLC, employing Giemsa staining technique. 2 drops of the blood sample were placed on a slide and made into a thin-film smear. After drying, the smear was fixed in absolute methanol, stained with Giemsa stain and rinsed with buffered distilled water. The slide was allowed to stand for 30 minutes, rinsed again with buffered distilled water and allowed to air-dry. After drying, a microscope was used to count the number of cells (Blaxhall and Diasely, 1973).

3.6.6 Determination of absolute values

The absolute values of mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were obtained from the results of the RBCC, WBCC and PCV using the formulae by Dacie and Lewis (1991) as expressed below:

$$\text{MCV } (\mu m^3) = \frac{\text{Ht}(\%) \times 10}{\text{RBCC (cells/mm}^3\text{)}}$$

$$\text{MCH (Pg/cell)} = \frac{\text{Hb (g/100ml)} \times 10}{\text{RBCC (cells/mm}^3\text{)}}$$

$$\text{MCHC (g/100ml)} = \frac{\text{Hb (g/100ml)} \times 100}{\text{Ht (\%)}}$$

Where Ht is the haematocrit or PCV value; RBCC is the erythrocyte or red blood cell count; Hb is the haemoglobin value and pg = picogram.

3.6.7 Total protein determination

Serum total protein was determined according to the method of Lowry *et al.* (1951). This method is based on the principle of the Biuret reaction: copper in alkaline solution reacts with the peptide bonds in proteins, producing a violet colour which is proportional to the amount of protein present. Reagents used for the test were the Biuret solution, the serum and a standard solution. Stock solution of the Biuret reagent was prepared by dissolving 45g of sodium potassium tartrate in 500ml of 0.2N sodium hydroxide. 15g of copper sulphate ($CuSO_4.5H_2O$) was then added to the resulting mixture and stirred. 5g of potassium iodide was added; the volume made up to 1 litre with 0.2N NaOH and stored in a bottle away from light. The working solution (0.2N alkaline iodide) was prepared by diluting 200ml of the stock solution with 0.2N NaOH (8g/L) containing 5g potassium iodide to 1 litre, while the standard solution used was bovine serum albumin diluted with saline in a ratio of 1:5. Serum total protein was calculated using the formula below:

$$Serum\ Total\ Protein = \frac{Absorbance\ of\ Test \times Standard\ Concentration}{Absorbance\ of\ Standard}$$

3.7 Determination of Antioxidant Enzyme Activity

3.7.1 Processing of fish tissue

Fish organs (gills and liver) were collected and homogenized in phosphate buffer (1g of sample to 5ml of phosphate - saline buffer pH 7.4). The resulting homogenate was centrifuged at 2500 rpm for 15mins and the supernatant decanted and stored at -20°C for further processing (Atawodi *et al*., 2011).

3.7.2. Lipid peroxidation (LPO)

Lipid peroxidation as an index of oxidative stress was determined as thiobarbituric acid reactive substance (TBARS) according to the method of Ohkawa *et al.* (1979) with slight modifications by Atawodi *et al.* (2011), using trichloroacetic acid (TCA) and thiobarbituric acid (TBA). This method is based on the principle that lipid peroxidation generates peroxide intermediaries which upon cleavage releases malondialdehyde (MDA), a product which reacts with thiobarbituric acid. The product of the reaction is a coloured complex which absorbs light at 535nm and can hence be measured.

Exactly 2ml of 15% trichloroacetic acid were measured into a test tube followed by the addition of 2ml thiobarbituric acid and then 100µl of the tissue homogenate. The mixture was incubated at 80^0C for 30 minutes in a water bath. The mixture was allowed to cool and then centrifuged at 3000rpm for 10 minutes. A clear supernatant was collected and its absorbance determined at 535nm using a spectrophotometer. TBARS concentration was expressed in nmol/mg of protein calculated as:

$$Concentration\ nmol/mg\ protein\ = \frac{\text{Absorbance of sample}}{1.56 \times 10^{-5}\ \text{protein concentration (mg)}}$$

3.7.3 Superoxide dismutase (SOD)

The Superoxide Dismutase assay is based on the ability of SOD to inhibit auto-oxidation of adrenalin at pH 10.2 according to Fridovich (1989). Reagents used for the assay include 0.5M carbonate buffer (14.3g of Na_2CO_3 and 4.2g were dissolved in distilled water by adding up to 100ml in a volumetric flask; the buffer was adjusted to pH 10.2) and 0.3mM adrenaline (0.01g of adrenaline was dissolved in 17ml of distilled water, prepared fresh).

Tissue homogenate of 0.1ml was diluted in 0.9ml of distilled water to make 1:10 dilution of microsome. An aliquant mixture of 0.2ml of the diluted microsome was added to 2.5ml of 0.05M carbonate buffer. The reaction was started with the addition of 0.3ml of 0.3mM adrenaline. The reference mixture contained 2.5ml of 0.05M carbonate buffer, 0.3ml of 0.3mM adrenaline and 0.2ml of distilled water. The Absorbance was measured over 30 seconds up to 150 seconds at 480nm

$$\text{Increase in absorbance per minute} = \frac{(\text{A5} - \text{A1})}{2.5}$$

$$\%\ inhibition = 100 - \ \left(\frac{increase\ in\ absorbance\ of\ sample}{increase\ in\ absorbance\ of\ blank}\ \times 100\right)$$

1 unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the oxidation of Adrenaline to adenochrome in 1 minute.

3.7.4 Catalase activity (CAT)

Catalase activity was measured using Abei's method (1974). Exactly 10µl tissue homogenate supernatant was added to a test tube containing 2.80ml of 50mM potassium phosphate buffer (pH 7.0). The reaction was initiated by adding 0.1ml of freshly prepared 30mM H_2O_2 and the decomposition rate of H_2O_2 was measured at 240nm for 5 minutes

on a spectrophotometer. A molar extinction coefficient (E) of 0.041mM^{-1}cm^{-1} was used to calculate the Catalase activity.

$$Catalase\ concentration = \frac{\text{Absorbance of sample}}{\text{Molar extinction coeficient (E)}}$$

1 unit of catalase activity is the amount of catalase that decomposes 1Nmol of H_2O_2 per minute at pH 7.

3.8 Histological Analysis

The gills and liver of fish from the control and exposed groups were studied and compared for histopathological changes. These organs were collected from fish and immediately fixed in 10% formalin for 24 hours (Velisek *et al*., 2008; Khoshnood *et al*., 2014). At the end of 24 hours, tissue samples were washed in running water to remove traces of formalin. Specimens were processed further by passing through graded series of alcohol (30%, 50%, 70%, 95% and absolute ethanol) for two hours each. This was the dehydration process. Specimens were later passed through xylene (clearing agent) and embedded in paraffin wax of 60^{0}C melting point. From the preserved paraffin blocks, 3µm thick sections were obtained using a rotary microtome (LEICA RM2125RT) and sections were further processed. The sections were floated on a water bath maintained at 2 to 3°C below the melting point of paraffin wax. They were placed on a hot plate thermostatically maintained at a temperature of 2 to 3°C above the melting point of paraffin wax. When properly dried (15 to 30 min), they were stained with Harry's haematoxylin and eosin (H and E), dehydrated, cleared and mounted on clean glass slides, avoiding air bubbles and observed under a light microscope (Obiezue *et al.*, 2014).

Haematoxylin and Eosin staining was used for the demonstration of general tissue structures in various colours. The nuclei as well as some calcium salts and ureates take

up blue colour. Other tissue structures appear red, pink or orange in color (eosinophilic) (Obiezue *et al.*, 2014). These slides were subsequently made into permanent slides using a mountant. The permanent slides prepared were mounted one after the other and viewed at different magnifications of the microscope. Photomicrographs of the slides were taken and the results presented in plates. To show the effects of the toxicant at different concentrations on cell structure and organ morphology, tissues from the toxicant exposed groups were compared with that of the control group.

3.9 Analysis of Fish Growth and Nutrient Utilization

The total length, standard length as well as weight of fish were measured at the start of the experiment and same after exposure to sub-lethal concentrations of the toxicant (Shalaby *et al.*, 2006). The data were used to analyze fish growth and nutrient utilization using the appropriate indices.

3.9.1 Feeding of fish

Juveniles of *Clarias gariepinus* were fed on a commercial feed (Coppens® 2mm) at 4% body weight daily, during the period of acclimation. During sub-lethal toxicity bioassay, fish were fed at 3% body weight daily. Daily ration was divided into two and fish were fed twice daily (9:00am and 5:00pm) as suggested by Ajani *et al.* (2007). Feeding at two rations per day has higher digestibility coefficient and higher feed conversion efficiency (Mills 1986).

3.9.2 Fish weight determination

The measurement of fish fresh weight was done by using an electric weighing balance (Satorious CP 8201) at the end of every two weeks. Fish in each tank were brought out using a hand net and weighed. The average weight of fish in each tank was obtained by

dividing the total weight of all the fishes by their number. Mean and percentage cumulative weights were calculated at the end of the experiment. The standard and total lengths of fish were also taken using a measuring board. This was done by gently placing fish on the board and the corresponding length on the scaled rule part taken.

3.9.3 Weight gain (WG)

Fish weight gain was calculated as the difference between the final weight of fish at the end of the experiment and the initial weight in grams (Akinwole and Faturoti 2007).

$$WG\ (g)\ =\ W_t\ -\ W_0$$

Where: W_t is the final weight and W_0 the initial weight of fish.

3.9.4 Percentage live weight gain (%LWG)

The percentage live weight gain (**%**LWG) was calculated as the difference between the initial and final fish weight of fish divided by the initial weight expressed as a percentage (Wannigamma *et. al.*, 1985).

$$\%LWG = \frac{W_t\ -\ W_0}{W_0} \times 100$$

Where: W_0 = Initial Fish weight (g)

W_t = Weight at time 't' days (g)

3.9.5 Specific growth rate (SGR)

The SGR was calculated as described by the formula below reported by Bwala and Omoregie (2009).

$$SGR\ = \frac{l_n W_t\ -\ l_n W_0}{D}$$

Where: W_t = Weight at time of observation (g)
W_0 = Initial weight
D = the period under study in days
l_n = The natural logarithm

3.9.6 Feed conversion ratio (FCR)

The feed conversion ratio, FCR, was calculated as the dry weight of feed offered divided by the net weight gain of fish (Stickney, 1979; Hepher, 1988; Shalaby *et al*., 2006):

$$FCR = \frac{\text{Weight of feed offerd (g)}}{\text{Net weight gain of fish (g)}}$$

3.9.7 Gross feed conversion efficiency (GFCE)

This was calculated as the reciprocal of the FCR expressed as a percentage (Stickney, 1979; Shalaby *et al*., 2006)

$$GFCE = \frac{1}{FCR} \times 100$$

3.9.8 Feed efficiency

Feed efficiency was computed as the ratio of the fish weight gain to the quantity of feed as reported by (Yaji, 2012).

$$FE = \frac{\text{Weight gain (g)}}{\text{Feed fed (g)}}$$

3.9.9 Nitrogen metabolism

Nitrogen metabolism was calculated using the formular described by Dabroski (1977) and Shalaby *et. al.*, (2006):

$$NM = \frac{(0.549)(\text{b}-\text{a})\text{h}}{2}$$

where:

a = initial weight of fish (g)
b = final weight of fish (g)
h = period of experiment (days)

3.10 Water Quality Determination

During the experiment, physico-chemical properties of the test water namely temperature, hydrogen-ion concentration (pH), total dissolved solids (TDS), electrical conductivity (EC) and dissolved oxygen (DO) were monitored. These were measured daily during the acute toxicity test and on a weekly basis during the sub-lethal toxicity bioassay.

3.10.1 Temperature, pH, electrical conductivity and total dissolved solids

These parameters were measured with a hand-held Hanna Combo instrument (HI 981 29). The pH and temperature were measured by setting the pH mode on the instrument. The probe was submerged in the water for about one minute and the reading taken when the stability symbol on the top left corner of the LCD disappeared. The pH value was displayed on the primary LCD of the instrument while the secondary LCD displayed the temperature.

The electrical conductivity and total dissolved solids were also measured by setting the EC or TDS mode on the instrument using the set/hold button. The probe was submerged in water and the readings were taken as in the case of pH above. The primary LCD showed readings for both EC and TDS while the secondary LCD always displayed the temperature of the sample.

3.10.2 Dissolved oxygen (DO)

Dissolved oxygen of the diluting water was measured according to the Winkler's method described by APHA (2005). This is described in the steps below:

- Water sample was poured into a 300ml biological oxygen demand (BOD) bottle. 2ml $MnSO_4$ solution was added followed by the addition of 2ml alkali-iodide azide reagent and the bottle stoppered with care to exclude air bubbles
- The sample was then mixed gently by inverting the bottle a number of times until a clear supernatant was obtained
- The sample was then allowed to settle for two minutes followed by the addition of 2ml H_2SO_4, allowing the acid to run down the neck of the bottle
- The bottle was stoppered again and gently inverted until dissolution was complete
- 100ml of the prepared solution was poured into a conical flask and titrated with 0.0125N $Na_2S_2O_3.5H_2O$ solution to a pale straw/yellow colour
- 3ml of freshly prepared starch solution was added, making the solution become blue in colour
- Titration was continued by adding the thiosulphate drop-wise until the blue coloured disappeared. The amount of thiosulphate used in the titration represented the amount of dissolved oxygen in the sample

3.11 Data Analyses

At the end of the acute toxicity test, regression coefficient between the probit kill and log of concentration of the herbicide was determined by the probit model developed by Finney (1971), using Minitab software version 17. Mean values of haematological parameters, opercular and tail fin beats, growth parameters, biochemical activities and physico-chemical properties of the test water were analyzed for any significant difference using Analysis of Variance (ANOVA). Differences between means were partitioned using the Duncan's Multiple Range Test (Duncan, 1995). The Statistical Package for Social Sciences (SPSS) version 21 was used.

CHAPTER FOUR

4.0 RESULTS

4.1 Physico-chemical Parameters of Test Water

4.1.1 Acute toxicity bioassay

The physicochemical parameters of the test water measured daily during acute toxicity bioassay are presented in Table 4.1. Dissolved oxygen ranged from 4.1-4.8mg/L ($\bar{x}$ = 1.27 ± 0.05); hydrogen ion concentration (pH) ranged from 8.76-9.06 ($\bar{x}$ = 8.48 ± 0.02); temperature ranged from 25.2-28.5°C ($\bar{x}$ = 26.76 ± 0.08); electrical conductivity ranged from 177-1267µS/cm ($\bar{x}$ = of 911.36 ± 27.37); while total dissolved solids ranged from 86-636ppm ($\bar{x}$ = of 456.39 ± 13.74).

4.1.2 Sub-lethal toxicity bioassay

The physicochemical parameters of the test water measured daily during sub-lethal toxicity bioassay are presented in Table 4.2. Dissolved oxygen ranged from 4.3-5.2mg/L ($\bar{x}$ = 1.62 ± 0.08); hydrogen ion concentration (pH) ranged from 7.97-9.26 ($\bar{x}$= 8.36 ± 0.02); temperature ranged from 24.3-30.4°C ($\bar{x}$ = 27.17 ± 1.86); electrical conductivity ranged from 831-1547µS/cm ($\bar{x}$= 1125.49 ± 18.57); while total dissolved solids ranged from 415-774ppm ($\bar{x}$ = 561.56 ± 9.28).

Table 4.1: Physicochemical parameters of the test water during acute toxicity bioassay

Parameter	Range	Mean ± SE
Dissolved Oxygen (DO) (mg/L)	4.1-4.8	1.27 ± 0.05
Hydrogen Ion Concentration (pH)	8.76-9.06	8.48 ± 0.02
Temperature (°C)	25.2-28.5	26.76 ± 0.08
Electrical Conductivity (µS/cm)	177-1267	911.36 ± 27.37
Total Dissolved Solids (ppm)	86-636	456.39 ± 13.74

Table 4.2: Physicochemical parameters of the test water during sub-lethal toxicity bioassay

Parameter	Range	Mean ± SE
Dissolved Oxygen (DO) (mg/L)	4.3-5.2	1.62 ± 0.08
Hydrogen Ion Concentration (pH)	7.92-9.26	8.36 ± 0.02
Temperature (°C)	24.3-30.4	27.17 ± 1.86
Electrical Conductivity (µS/cm)	831-1547	1125.49 ± 18.57
Total Dissolved Solids (ppm)	415-774	561.56 ± 9.28

4.2 Symptoms of Toxicity

4.2.1 Behavioural and morphological responses of *C. gariepinus* juveniles exposed to acute concentrations of atrazine

At the onset of the experiment (12-24 hours post exposure), behavioural changes in fish were rather rapid (Table 4.3). There was an immediate burst of activity (hyperactivity) in all the toxicant-exposed groups. This was characterized by abnormal or agitated/erratic swimming, colliding and hitting of tails against wall of the aquarium, sudden or quick movements as well as general restlessness. With progression of exposure time (48-72 hours post exposure), activity of fish in the exposed groups decreased (hypoactivity). There was air gulping (rapid opercular movement), loss of balance or equilibrium characterized by fish swimming backwards or in circles, free fall, as well as vertical positioning. Fish exhibited startle or panic responses to stimulus with sudden darts of energy and holding out their pelvic and pectoral fins (Plate I). At the time of quiescence, fish exhibited highly reduced activity during which they remained vertically still (Plate II) with much reduced faint and irregular opercular beats. This period was followed by death.

Morphological changes observed in the toxicant exposed groups included execcisve mucus secretion in the opercular and head regions, and this was dose dependent. Swollen abdomens were also observed in the exposed groups (Plate III). In addition, discolouration of the normal dark pigmentation on the lateral and dorsal parts of fish was observed in the exposed groups (Plate IV). These changes were conspicuously absent in the control group.

Table 4.3: Behavioural and morphological responses of *C. gariepinus* juveniles exposed to acute concentrations of atrazine

Behavioural and morphological symptoms	Diagnosis
Loss of equilibrium	Yes (occasional)[a]
General activity	Hyperactive to hypoactive[b]
	Vertical positioning[c]
	Posturing of pectoral and pelvic fins[d]
Startle response	Over-reactive to under-reactive[e]
Haemorrhage	None[f]
Deformities	Lateral flexure[g]

a. Loss of equilibrium	Fish rolled over on side or back and hanged vertically or move in a reverse manner.
b. Hyperactive	Fish swam faster than the control fish, dart around tank without being provoked at initial exposure time.
b. Hypoactive	Fish swam slower than the control fish with increased exposure period of more than 48 hours.
c. Vertical positioning	Fish occasionally assumed a vertical position before death.
d. Posturing of pectoral and pelvic fins	Distinguishable behavioural/morphological change or sign of stress.
e. Over-reactive	Fish were hyper-excitable; dart away from stimulus faster than the control fish at initial period of exposure.
e. under-reactive	Fish dart away from stimulus slower than the control fish with increased exposure time.
f. Haemorrhage	No haemorrhage was observed
g. Lateral flexture	No lateral flexure observed

Adapted from Drummond *et al.,* 1986



Plate I: Fish in exposed group holding out pectoral and pelvic fins (arrowed)

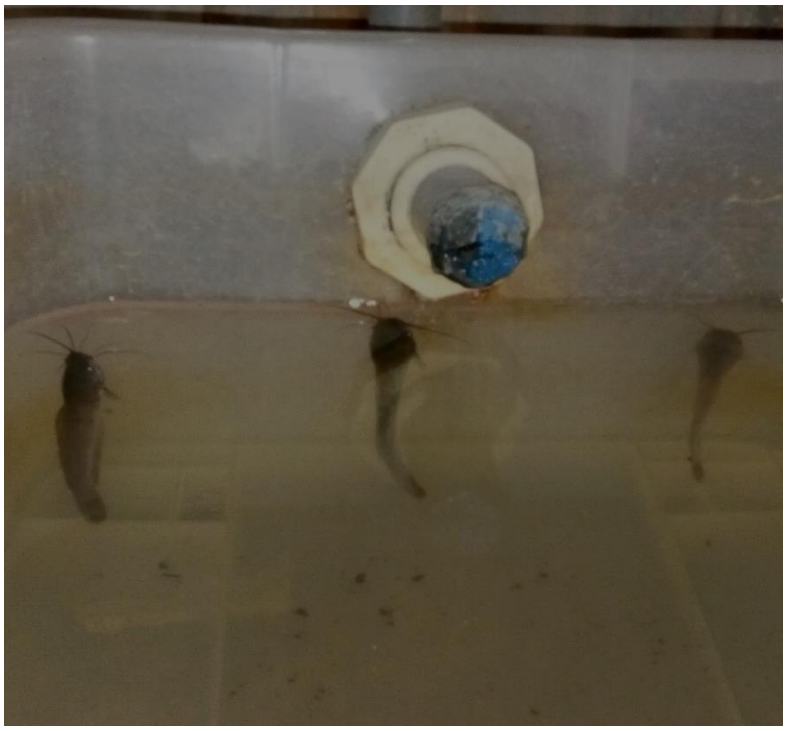


Plate II: Vertical positioning in fish of exposed group

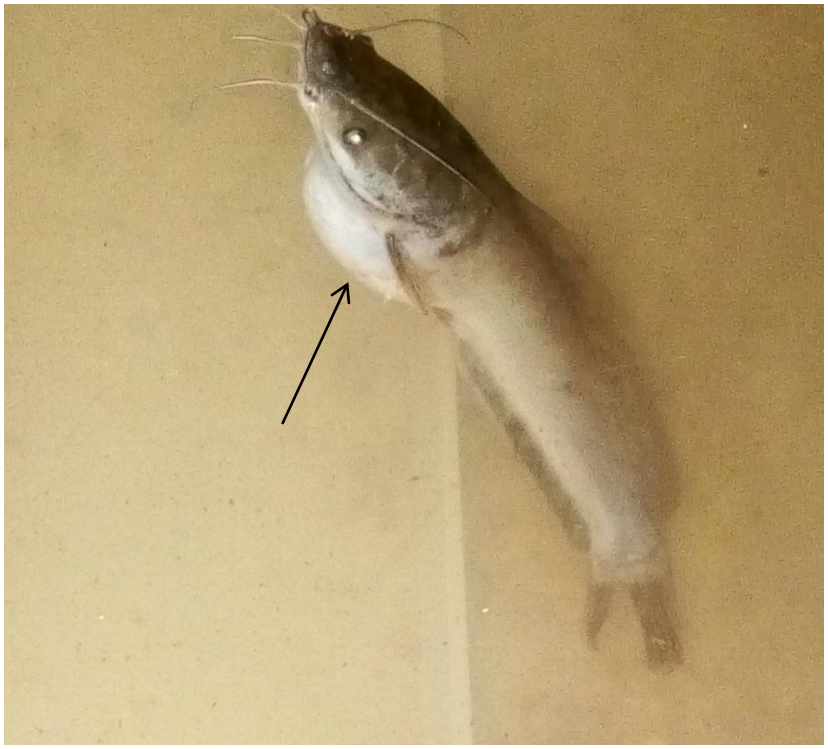


Plate III: Swollen abdomen (arrowed) in fish exposed to atrazine



Plate IV: Discolouration in fish of exposed group

4.2.2 Opercular ventilation counts

The results of opercular ventilation counts are presented in Figures 4.1 and 4.2. Mean values showed that the opercular ventilation counts (per minute) were significantly higher ($p<0.05$) in the 12th and 24th hours of exposure. This was followed by a significant ($p<0.05$) time-dependent decrease in opercular ventilation counts in the 48th, 72nd and 96th hours of exposure, with the 96th hour recording the least opercular ventilation count (Figure 4.1). However, there were no significant differences in the opercular ventilation counts of fish between the treatment groups (Figure 4.2).

4.2.3 Tail fin beat frequency

The results of tail fin beat rates are presented in Figures 4.3 and 4.4. Mean values showed that the tail fin beat rates per minute were significantly higher ($p<0.05$) in the 12th hour of exposure. This was followed by a significant ($p<0.05$) time-dependent decrease in the 24th, 48th, 72nd and 96th hours of exposure, with the 96th hour recording the least tail fin beat frequency (Figure 4.3). However, there were no significant differences in the tail fin beat frequency of fish between the treatment groups (Figure 4.4).

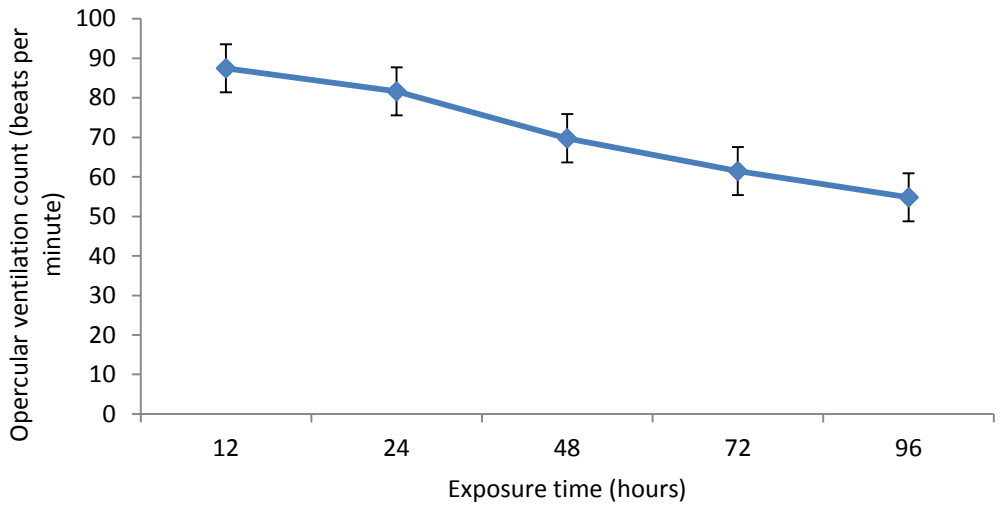


Figure 4.1: Time-course effect of acute concentrations of atrazine on the opercular ventilation count of *C. gariepinus* juveniles

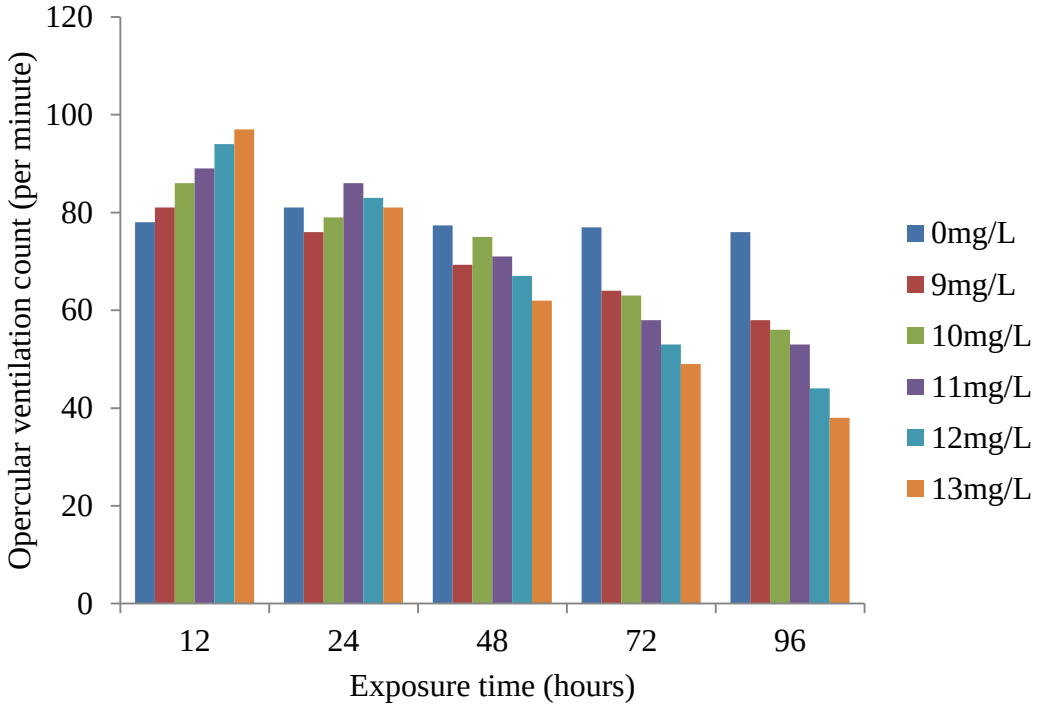


Figure 4.2: Acute effects of atrazine on the opercular ventilation count of *C. gariepinus* juveniles

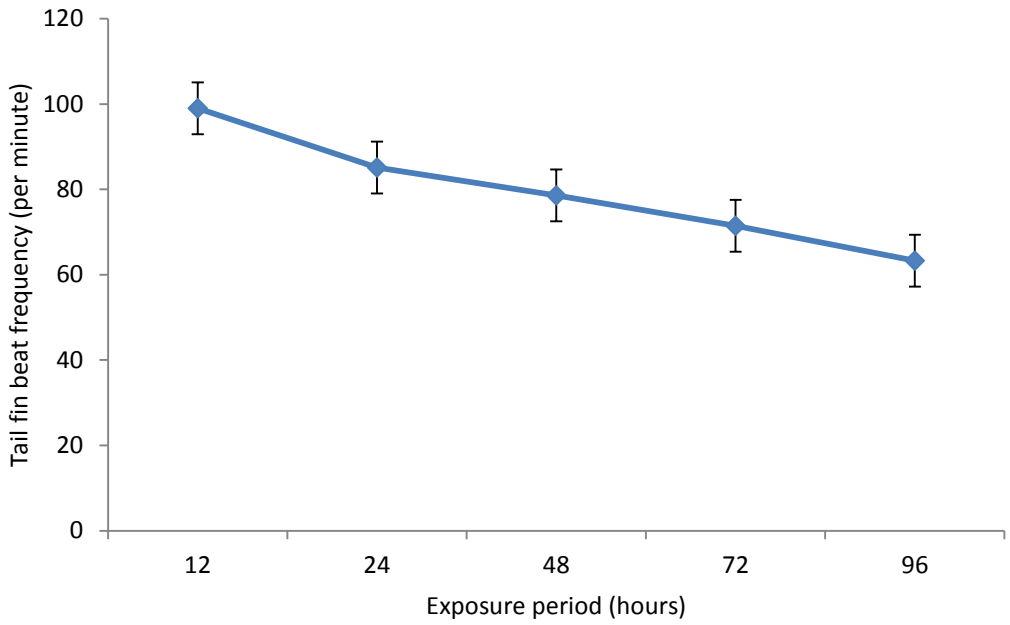


Figure 4.3: Time-course effect of acute concentrations of atrazine on the tail fin beat frequency of *C. gariepinus* juveniles

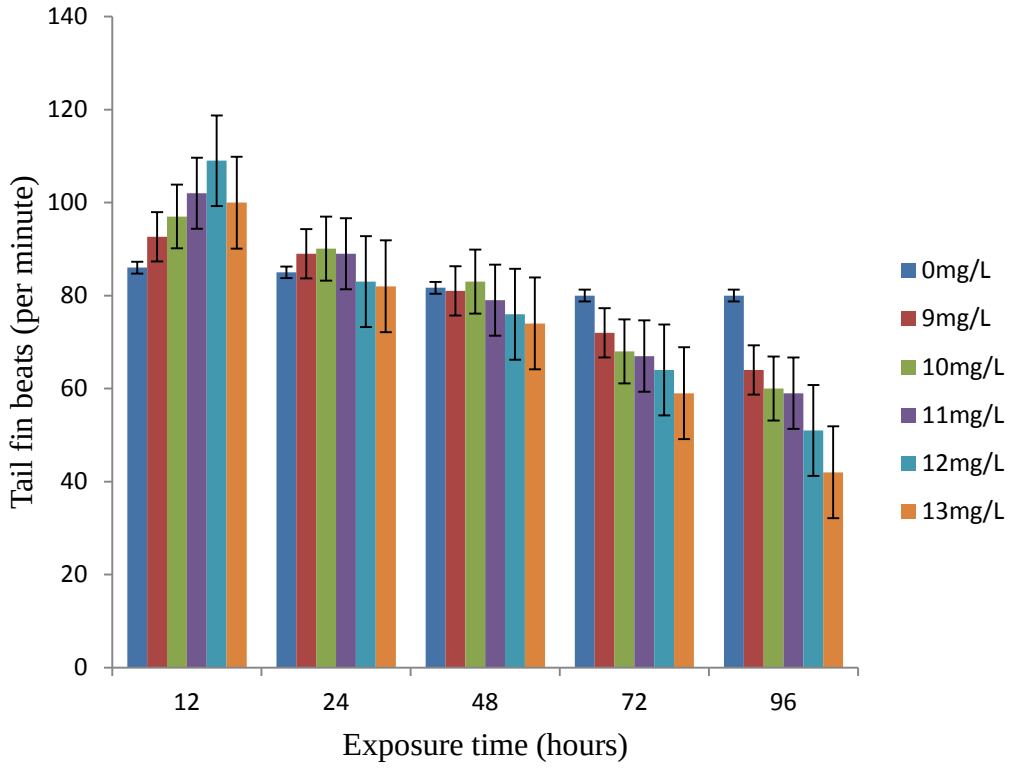


Figure 4.4: Acute effects of atrazine on the tail fin beat frequency of *C. gariepinus* juveniles

4.2.4 Mortality rates

The results of the acute toxicity bioassay showing mean mortality of *C. gariepinus* are presented in Table 4.4. Mortality of fish was observed in all the treatment groups except the control. Mortality was first observed at twelve hours in the 10, 12 and 13mg/L concentrations. The highest mortality of 17 was recorded in the highest concentration (13mg/L) of atrazine while the least mortality of 6 was observed in the 9mg/L treatment.

Similarly, the total mortality, percentage mortalities and probit kill values of *C. gariepinus* exposed to acute nominal concentrations of atrazine are presented in Table 4.4. The percentage mortality was highest in the 13mg/L treatment and lowest in the 9mg/L treatment, while no mortality occurred in the control group. Mean mortality was observed to be concentration-dependent. The 96-hour median lethal concentration (LC_{50}) of atrazine for *C. gariepinus* using the probit method was found to be 11.89mg/L (Figure 4.5).

Table 4.4: Mortality rates, percentage mortality and probit kill values of *C.gariepinus* juveniles exposed to acute concentrations of atrazine

Conc (mg/L)	Log of conc.	Total number of fish exposed	Mortality	% Mortality	Probit kill value
0	0	30	0	0.00	0.00
9	0.95	30	6	20.00	4.16
10	1.00	30	7	23.33	4.26
11	1.04	30	14	46.67	4.92
12	1.08	30	16	53.33	5.08
13	1.11	30	17	56.67	5.18

conc. = Concentration

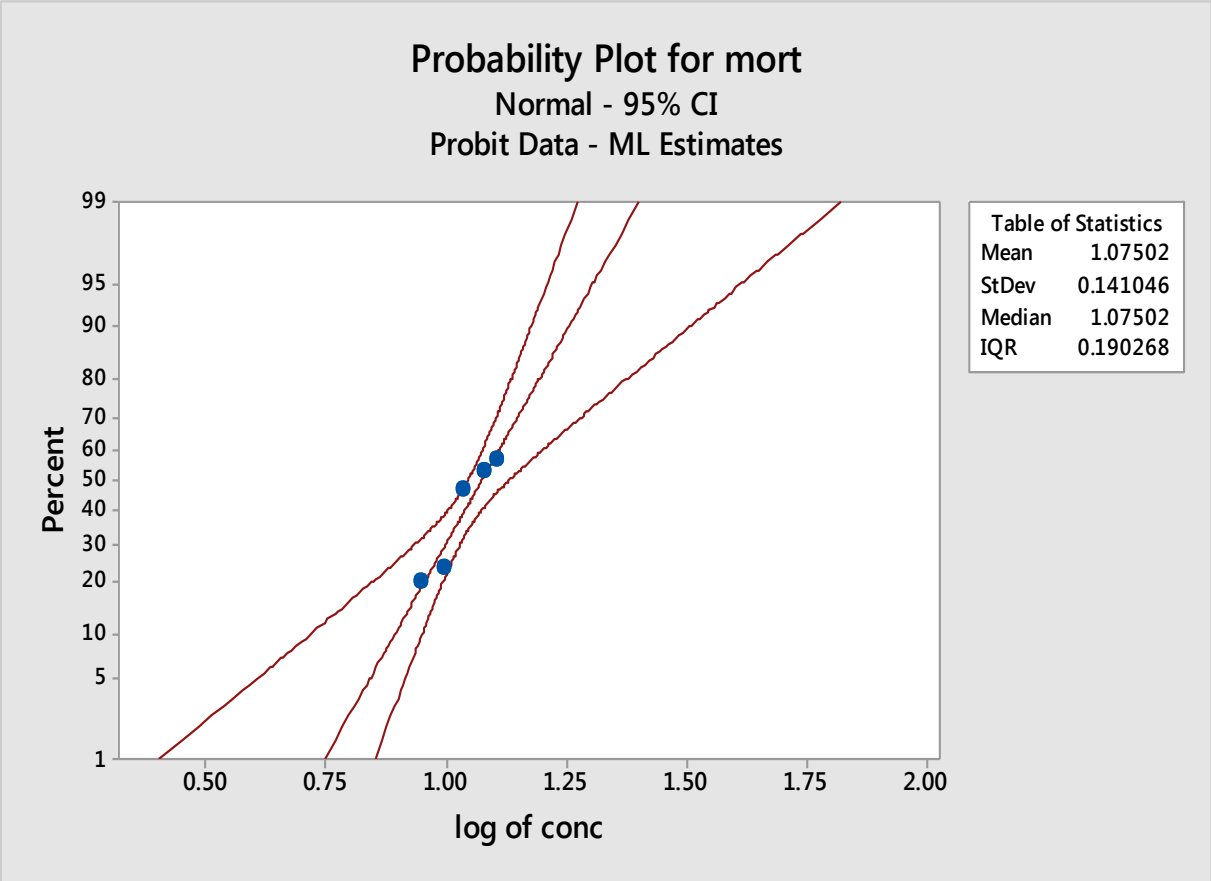


Figure 4.5: Median lethal concentration (LC_{50}) of atrazine for the species *C. gariepinus* at 96 hours of exposure

4.3 Effects of Atrazine on the Haematological Parameters of *C. gariepinus* Juveniles

4.3.1 Acute toxicity bioassay

Juveniles of *Clarias gariepinus* exposed to acute concentrations of atrazine for 96 hours showed significantly lower values ($p<0.05$) of packed cell volume (PCV), blood haemoglobin (Hg), total protein (TP) and total erythrocyte counts (TEC) with increase in concentration of the toxicant than in the control; while total leucocyte counts (TLC) values increased significantly ($p<0.05$) with increase in concentration, and were higher than that of the control group. The highest mean value (38.00 ± 1.15%) of PCV was recorded in the control group while the lowest value (14.67 ± 1.45) was recorded in the group exposed to highest concentration (13mg/L) of atrazine. Haemoglobin recorded the highest value (12.30 ± 0.23) in the control group and the least value (6.70 ± 0.17) was recorded in the highest concentration of 13mg/L. Total protein recorded the highest value (6.80 ± 0.21) in the control while the highest concentration had the least value (1.27 ± 0.12). Total erythrocyte count (TEC) decreased from 6.63 ± 0.15 in the control to 3.03 ± 0.09 in the highest concentration. Total leucocyte count (TLC) however recorded the highest value in the highest concentration (11.80 ± 0.15) and the least value was recorded in the control group (8.40 ± 0.26) (Table 4.5).

Mean corpuscular haemoglobin concentration (MCHC) values recorded a mixed trend with mean values of 10, 11, 12 and 13mg/L being significantly different from other groups. The highest value (46.47 ± 4.02) was however recorded in the highest concentration of atrazine and the least in the control group (33.45 ± 1.42). Mean values of mean corpuscular haemoglobin (MCH) recorded the least value (17.98 ± 1.02) in the 9mg/L group while the highest value (22.16 ± 1.20) was observed in the highest concentration of atrazine, being significantly higher than all other groups. However,

statistical analysis did not show any significant difference in the control, 10 and 11mg/L concentrations, but were all significantly different from that of the 9mg/L treatment. Similarly, statistical analysis did not show any significant difference between the mean values of mean corpuscular volume (MCV) of the control and all treatment groups with the highest value (57.39 ± 2.63) being recorded in the control group and the least value (48.63 ± 5.75) observed in the highest concentration of 13mg/L of atrazine (Table 4.5).

Mean values of neutrophils showed a mixed trend. They increased from the control, in the 9mg/L group and then subsequently decreased in a concentration dependent manner. The highest value (14.00 ± 1.00) was recorded in the 9mg/L group, while the least value (4.33 ± 0.33) was recorded in the highest concentration of atrazine. Statistical analysis showed mean values of the control to be significantly higher ($p<0.05$) than those of 10, 11, 12 and 13mg/L treatment, but not significant compared to that of 9mg/L. The least mean value (86.00 ± 1.53) of lymphocytes was recorded in the control group while the highest value (96.67 ± 1.20) was observed in the highest concentration of atrazine. Lymphocytes values increased in a concentration-dependent manner with mean values of the control being significantly lower ($p<0.05$) than those of all other groups. However, monocytes, eosinophils, basophils and band cells were not detected during the assay (Table 4.5).

Table 4.5: Acute effects of atrazine on some haematological parameters of *C. gariepinus* juveniles

Parameters	Concentration (mg/L)					
	0	9	10	11	12	13
PCV (%)	38.00 ± 1.15^{a}	30.00 ± 1.00^{b}	24.33 ± 1.20^{c}	20.33 ± 0.88^{d}	18.67 ± 0.88^{d}	14.67 ± 1.45^{e}
Hg (g/dl)	12.30 ± 0.23^{a}	10.07 ± 0.58^{b}	9.27 ± 0.27^{bc}	8.50 ± 0.15^{c}	7.20 ± 0.17^{d}	6.70 ± 0.17^{d}
Tp (g/dl)	6.80 ± 0.21^{a}	5.33 ± 0.20^{b}	4.33 ± 0.08^{c}	3.47 ± 0.26^{d}	2.40 ± 0.21^{e}	1.27 ± 0.12^{f}
TLC (x10^{9}/l)	8.40 ± 0.26^{e}	9.10 ± 0.21^{d}	9.90 ± 0.15^{c}	10.33 ± 0.15^{c}	10.93 ± 0.15^{b}	11.80 ± 0.15^{a}
TEC (x10^{12}/l)	6.63 ± 0.15^{a}	5.60 ± 0.15^{b}	4.90 ± 0.44^{b}	4.10 ± 0.21^{c}	3.40 ± 0.21^{cd}	3.03 ± 0.09^{d}
MCV (μm^{3})	57.39 ± 2.63^{a}	53.68 ± 2.61^{a}	50.32 ± 4.60^{a}	49.73 ± 2.16^{a}	55.61 ± 5.79^{a}	48.63 ± 5.75^{a}
MCH (pgcell)	18.55 ± 0.07^{ab}	17.98 ± 1.02^{b}	19.23 ± 1.84^{ab}	20.81 ± 0.76^{ab}	21.29 ± 1.03^{ab}	22.16 ± 1.20^{a}
MCHC (g/100ml)	33.45 ± 1.42^{b}	33.53 ± 1.27^{b}	38.39 ± 3.14^{ab}	41.90 ± 1.18^{a}	38.81 ± 2.65^{ab}	46.47 ± 4.02^{a}
Neutrophils (%)	12.00 ± 2.65^{a}	14.00 ± 1.00^{a}	9.67 ± 1.20^{ab}	9.00 ± 1.53^{abc}	6.00 ± 1.53^{bc}	4.33 ± 0.33^{c}
Lymphocytes (%)	86.00 ± 1.53^{c}	88.00 ± 1.00^{bc}	89.00 ± 2.08^{bc}	92.00 ± 1.53^{ab}	94.00 ± 1.53^{a}	96.67 ± 1.20^{a}
Monocytes (%)	ND	ND	ND	ND	ND	ND
Eosinophils (%)	ND	ND	ND	ND	ND	ND
Basophils (%)	ND	ND	ND	ND	ND	ND
Band (%)	ND	ND	ND	ND	ND	ND

Means with different superscripts along rows are significantly different (p>0.05)

Conc. = Concentration, PCV = packed cell volume, Hg = haemoglobin, TP = total protein, TLC = total leucocyte count, TEC = total erythrocyte count, MCV = mean corpuscular volume, MCH = mean corpuscular haemoglobin, MCHC = mean corpuscular haemoglobin concentration

4.3.2 Sub-lethal toxicity bioassay

The results of the haematological parameters of *C. gariepinus* exposed to sub-lethal concentrations of atrazine are shown in Table 4.6. Juveniles of *C. gariepinus* exposed to sub-lethal concentrations of atrazine showed significantly lower values ($p<0.05$) of packed cell volume (PCV), blood haemoglobin (Hg), total protein (TP) and total erythrocyte counts (TEC) than the control. Total leucocyte counts values increased with increase in concentration, and were higher than that of the control group although statistical analysis showed no significant differences among treatment groups. The highest mean value (32.33 ± 2.03%) of PCV was recorded in the control group while the least value (20.33 ± 1.20) was recorded in the highest concentration (2.38mg/L). Haemoglobin recorded the highest value (10.30 ± 0.52) in the control group and the least (6.67 ± 0.92) was recorded in the highest concentration of 2.38mg/L. Total protein recorded the highest value (3.87 ± 0.24) in the control while the highest concentration had the least value (2.77 ± 0.15). Total erythrocyte count decreased from 4.97 ± 0.20 in the control to 3.47 ± 0.20 in the highest concentration. Total leucocyte count however recorded the highest value (13.70 ± 0.46) in the highest concentration while the least value was recorded in the control group (8.07 ± 0.73).

Mean corpuscular volume (MCV), mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration (MCHC) values recorded no significant differences from the control in all the treatment groups with a mixed trend observed in their mean values. A decrease was observed in mean values of MCV from 65.16 ± 3.61 in the control to 57.49 ± 2.73 in the 1.19mg/L treatment, and then followed by an increase again in the last treatment of 2.38mg/L with a value of 59.45 ± 6.81. MCH increased from 20.86 ± 1.75 in the control group to 22.22 ± 3.18 in the 0.59mg/L treatment, decreased to 19.11 ± 0.73 in the 1.19mg/L group and then increased again to 19.23 ± 2.34 in the highest

concentration of 2.38mg/L. MCHC values recorded a slight increase from 32.01 ± 1.86 in the control to 36.06 ± 1.72, decreased again to 33.28 ± 0.32 in the 1.19mg/L treatment and then slightly increased to 33.47 ± 6.53 in the highest concentration of 2.38mg/L treatment (Table 4.6).

Mean values of neutrophils showed a dose-dependent decrease with increase in concentration although this was not statistically significant ($p>0.05$) when compared to that of the control. The highest value of neutrophils (12.67 ± 2.40) was recorded in the control while the least value (10.33 ± 2.03) was observed in the highest concentration. Lymphocyte values showed a dose dependent increase with increase in concentration. Mean value of lymphocytes of control fish was found to be significantly lower ($p<0.05$) than those exposed to all concentrations of the toxicant. However, monocytes, eosinophils, basophils and band cells were not detected in blood of fish during the assay (Table 4.6).

Table 4.6: Sub-lethal effects of atrazine on some haematological parameters of *C. gariepinus* juveniles

Parameters	Concentration (mg/L)			
	0	0.59	1.19	2.38
PCV (%)	32.33 ± 2.03^{a}	24.33 ± 2.19^{b}	21.67 ± 2.33^{b}	20.33 ± 1.20^{b}
Hg (g/dl)	10.30 ± 0.52^{a}	8.57 ± 1.02^{ab}	7.20 ± 0.72^{b}	6.67 ± 0.92^{b}
Tp (g/dl)	3.87 ± 0.24^{a}	3.60 ± 0.12^{ab}	3.17 ± 0.20^{bc}	2.77 ± 0.15^{c}
TLC ($x10^{9}$/l)	8.07 ± 0.73^{a}	11.27 ± 2.70^{a}	11.77 ± 2.24^{a}	13.70 ± 0.46^{a}
TEC ($x10^{12}$/l)	4.97 ± 0.20^{a}	3.90 ± 0.25^{b}	3.77 ± 0.34^{b}	3.47 ± 0.20^{b}
MCV (μm^{3})	65.16 ± 3.61^{a}	63.37 ± 8.87^{a}	57.49 ± 2.73^{a}	59.45 ± 6.81^{a}
MCH (pgcell)	20.86 ± 1.75^{a}	22.22 ± 3.18^{a}	19.11 ± 0.73^{a}	19.23 ± 2.34^{a}
MCHC (g/100ml)	32.01 ± 1.86^{a}	35.06 ± 1.72^{a}	33.28 ± 0.32^{a}	33.47 ± 6.53^{a}
Neutrophils (%)	12.67 ± 2.40^{a}	11.67 ± 3.48^{a}	11.00 ± 1.53^{a}	10.33 ± 2.03^{a}
Lymphocytes (%)	78.33 ± 2.33^{b}	84.00 ± 0.58^{ab}	86.67 ± 2.33^{a}	89.33 ± 2.40^{a}
Monocytes (%)	ND	ND	ND	ND
Eosinophils (%)	ND	ND	ND	ND
Basophils (%)	ND	ND	ND	ND
Band (%)	ND	ND	ND	ND

Means with different superscripts along rows are significantly different ($p>0.05$)

Conc. = Concentration, PCV = packed cell volume, Hg = haemoglobin, TP = total protein, TLC = total leucocyte count, TEC = total erythrocyte count, MCV = mean corpuscular volume, MCH = mean corpuscular haemoglobin, MCHC = mean corpuscular haemoglobin concentration

4.4 Effects of Atrazine on the Oxidative Stress Response Parameters of *C. gariepinus* juveniles

4.4.1 Acute toxicity bioassay

Mean values of oxidative stress response parameters in various organs of *C. gariepinus* exposed to acute concentrations of atrazine showed a significant ($p<0.05$) dose-dependent increase in catalase and superoxide dismutase activities in gill and liver of fish, while a dose dependent decrease in lipid peroxidation was observed in both organs (Table 4.7). The highest value of catalase in liver (91.22 ± 0.28) of fish was recorded in the highest concentration of the toxicant while the least value (16.34 ± 0.50) was observed in liver of the control fish. A similar trend was observed in catalase values assayed in gill of fish with the highest value (49.27 ± 0.68) recorded in fish exposed to the highest concentration of the toxicant while the least value (18.54 ± 0.23) was observed in the gill of control fish. Superoxide dismutase value in liver of fish was highest (70.97 ± 0.06) in that of fish exposed to the highest concentration of the toxicant while the least value (12.91 ± 0.18) was observed in the control. Superoxide dismutase values in the gill of control fish were significantly lower than those of fish in all exposed groups. This value peaked in gill of fish (163.15 ± 1.01) exposed to the highest concentration of the toxicant and was least (116.13 ± 1.81) in that of the control fish. Whereas higher values of catalase activity were observed in the liver of exposed fish as compared to the gill, an opposite trend was observed in the case of the enzyme superoxide dismutase.

Mean values of lipid peroxidation in both liver (347.47 ± 1.29) and gill (341.93 ± 0.16) of control fish were found to be significantly higher than those of the exposed groups. The least value in liver (115.89 ± 0.53) and gill (162.13 ± 0.69) were all observed in that of fish exposed to the highest concentration of the toxicant (Table 4.7).

Table 4.7: Effects of atrazine on some oxidative stress response parameters in organs of *C. gariepinus* juveniles after 96 hours of exposure

Concentration (mg/L)	Catalase activity (µg/g tissue)		Superoxide dismutase (µg/g tissue)		Lipid peroxidation (nmol/mg tissue)	
	Liver	Gill	Liver	Gill	Liver	Gill
0	16.34 ± 0.50^{f}	18.54 ± 0.23^{f}	12.91 ± 0.18^{f}	116.13 ± 1.81^{f}	347.47 ± 1.29^{a}	341.93 ± 0.16^{a}
9	21.22 ± 0.27^{e}	27.02 ± 1.13^{e}	35.48 ± 0.13^{e}	122.58 ± 0.63^{e}	334.04 ± 1.13^{b}	307.00 ± 1.06^{b}
10	40.01 ± 0.35^{d}	32.20 ± 0.40^{d}	38.71 ± 0.63^{d}	141.94 ± 0.76^{d}	282.07 ± 0.62^{c}	281.70 ± 0.43^{c}
11	46.44 ± 0.82^{c}	38.66 ± 0.18^{c}	58.06 ± 0.60^{c}	148.39 ± 0.39^{c}	143.53 ± 0.32^{d}	205.46 ± 0.17^{d}
12	67.32 ± 0.12^{b}	44.04 ± 0.33^{b}	64.52 ± 0.10^{b}	154.84 ± 2.01^{b}	135.86 ± 0.08^{e}	166.18 ± 0.12^{e}
13	91.22 ± 0.28^{a}	49.27 ± 0.68^{a}	70.97 ± 0.06^{a}	163.15 ± 1.01^{a}	115.89 ± 0.53^{f}	162.13 ± 0.69^{f}

Means with different superscripts along columns are significantly different ($p>0.05$)

4.4.2 Sub-lethal toxicity bioassay

Mean values of oxidative stress response parameters in various organs of *C. gariepinus* exposed to sub-lethal concentrations of atrazine for 8 weeks showed a dose dependent increase in gill and liver of fish for catalase and superoxide dismutase values, while a dose dependent decrease in lipid peroxidation was observed in both organs (Table 4.8). Mean values of catalase in liver and gill of control fish were significantly lower ($p<0.05$) than those of the exposed groups. The highest value of catalase (8.03 ± 0.06) in liver of fish was recorded in the highest concentration of the toxicant while the least value (2.12 ± 0.02) was observed in liver of the control fish. A similar trend was observed in catalase values in gill of fish with the highest value (12.09 ± 0.01) recorded in fish exposed to the highest concentration of the toxicant while the least value (4.59 ± 0.04) was observed in the gill of control fish. Superoxide dismutase value in liver of fish was highest (5.93 ± 0.02) in the fish exposed to the highest concentration of the toxicant while the least value (4.06 ± 0.01) was observed in the control fish. Like that in the liver, superoxide values in gill of control fish were significantly lower than those of fish in all exposed groups. This value peaked in gill of fish (6.67 ± 0.01) exposed to the highest concentration of the toxicant and was least (4.81 ± 0.04) in the gill of the control fish. A trend observed was that higher values of both catalase activity and superoxide dismutase were noticed in the gills when compared to the liver.

Mean values of lipid peroxidation in both liver (684.30 ± 1.91) and gill (1859.40 ± 1.21) of control fish were found to be significantly higher than those of the exposed groups. The least value in liver (243.00 ± 3.58) and gill (1111.50 ± 1.04) were both observed in that of fish exposed to the highest concentration of the toxicant (Table 4.8).

Table 4.8: Sub-lethal effects of atrazine on some oxidative stress response parameters in organs of *C. gariepinus* juveniles

Concentration (mg/L)	Catalase activity (µg/g tissue)		Superoxide dismutase (µg/g tissue)		Lipid peroxidation (nmol/mg tissue)	
	Liver	Gill	Liver	Gill	Liver	Gill
0	2.12 ± 0.02^{d}	4.59 ± 0.04^{d}	4.06 ± 0.01^{d}	4.81 ± 0.04^{d}	684.30 ± 1.91^{a}	1859.40 ± 1.21^{a}
0.59	2.68 ± 0.01^{c}	8.37 ± 0.09^{c}	4.44 ± 0.03^{c}	5.26 ± 0.13^{c}	439.40 ± 0.69^{b}	1685.10 ± 0.75^{b}
1.19	7.18 ± 0.01^{b}	9.59 ± 0.05^{b}	5.19 ± 0.01^{b}	5.93 ± 0.03^{b}	288.50 ± 0.75^{c}	1624.20 ± 0.23^{c}
2.38	8.03 ± 0.06^{a}	12.09 ± 0.01^{a}	5.93 ± 0.02^{a}	6.67 ± 0.01^{a}	243.00 ± 3.58^{d}	1111.50 ± 1.04^{d}

Means with different superscripts along columns are significantly different ($p>0.05$)

4.5 Histopathology of the Liver and Gill of Fish Exposed to Atrazine

4.5.1 Histology of the gills of *C. gariepinus* juveniles from control fish

The gills of control fish comprised of a curved bony gill arch from which radiate double rows of paired long, thin primary lamellar or filaments (Plate V). Running across the primary filament, on both sides, are secondary lamellae arranged in a perpendicular fashion to the primary lamellae.

4.5.2 Histopathology of the gills of *C. gariepinus* exposed to acute and sub-lethal concentrations of atrazine

Results of the histopathological studies of the gills of *C. gariepinus* juveniles exposed to acute and sub-lethal concentrations of atrazine revealed changes especially in the architecture of the primary lamella. Such changes included hyperplasia, attenuation and clubbing at the tips of the primary lamellae (Plate VI), gradual degeneration of the primary lamellae (Plate VII), mild congestion in the cartilage (Plate VIII) as well as total dislocation of the primary lamellae from its position (Plate IX)

4.5.3 Histology of the liver of *C. gariepinus* in control group

The histology of liver from control fish revealed the typical parenchymatous appearance of the liver. The liver was primarily composed of polyhedral hepatocytes typically with central nuclei with densely stained chromatin margins and a prominent nucleolus (Plate X). Central veins were found randomly throughout the hepatic parenchyma of fish. Between the cords of hepatocytes is a three-dimensional network of cylindrical blood sinusoids. The sinusoid is an irregularly dilated vessel whose caliber is larger than the diameter of regular capillaries. Vacuolization of hepatocytes resulting from glycogen and fat deposits may be a source of histological variability.

4.5.4 Histopathology of the liver of *C. gariepinus* juveniles exposed to acute and sub-lethal concentrations of atrazine

Results of the histopathological studies of the liver of *C. gariepinus* exposed to acute and sub-lethal concentrations of atrazine revealed prominent changes in the liver such as perivascular cuffing around the central vein and mild congestion of the sinusoids (Plate XI); pyknosis of the nuclei and mild necrotic damage (Plate XII); congestion of the central vein as well as mild coagulative necrosis (Plate XIII).

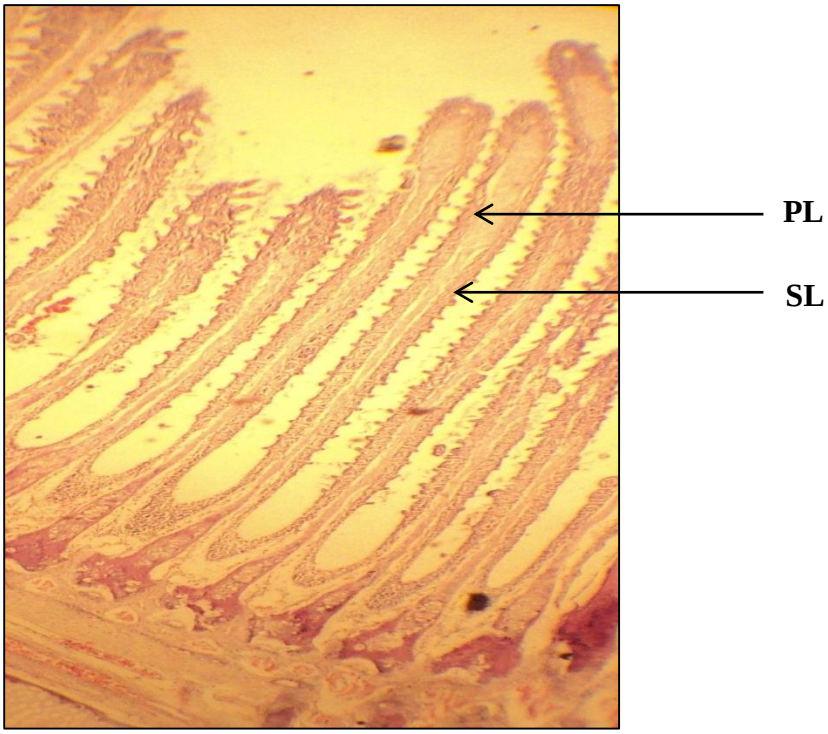


Plate V: Section of the gills of control fish showing normal arrangement of primary (PL) and secondary lamellae (SL) (H and E ×100)

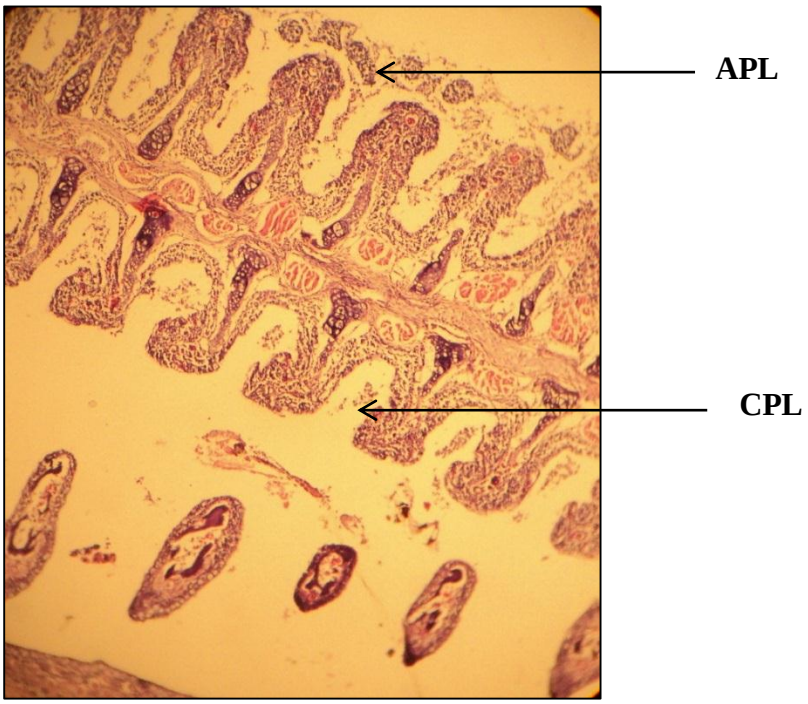


Plate VI: Section of the gill of fish exposed to acute (11mg/L) of atrazine showing hyperplasia, attenuation (APL) as well as clubbing (CPL) of the primary lamella (H and E ×100)



Plate VII: Section of the gill of fish exposed to sub-lethal (0.59mg/L) concentration of atrazine showing degeneration of the primary lamellae (DPL) (H and E ×100)

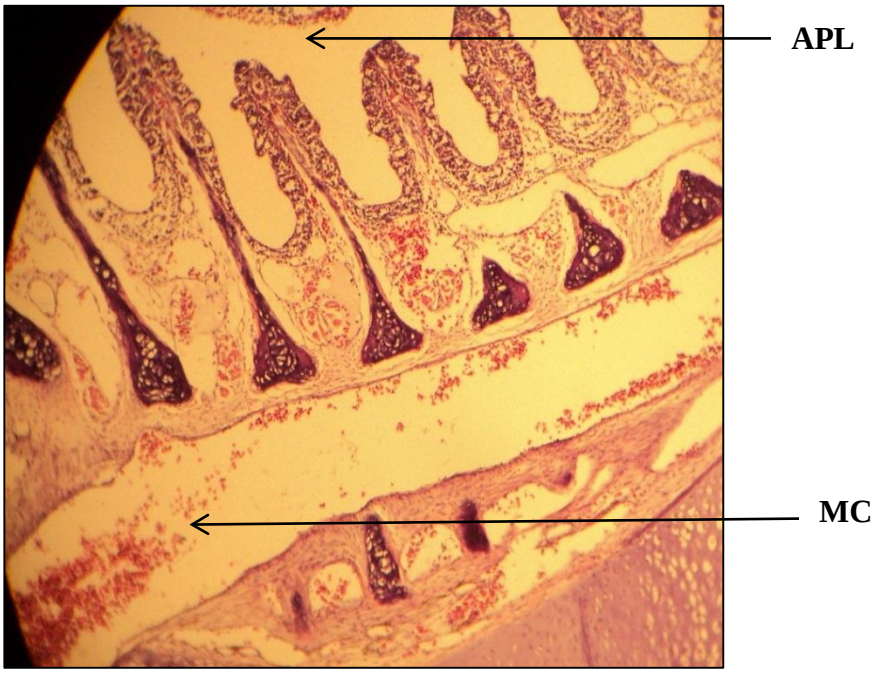


Plate VIII: Section of the gill of fish exposed to acute (10mg/L) concentration of atrazine showing attenuation and swelling of the primary lamellae (APL), as well as mild congestion (MC) in the cartilage (H and E ×100)

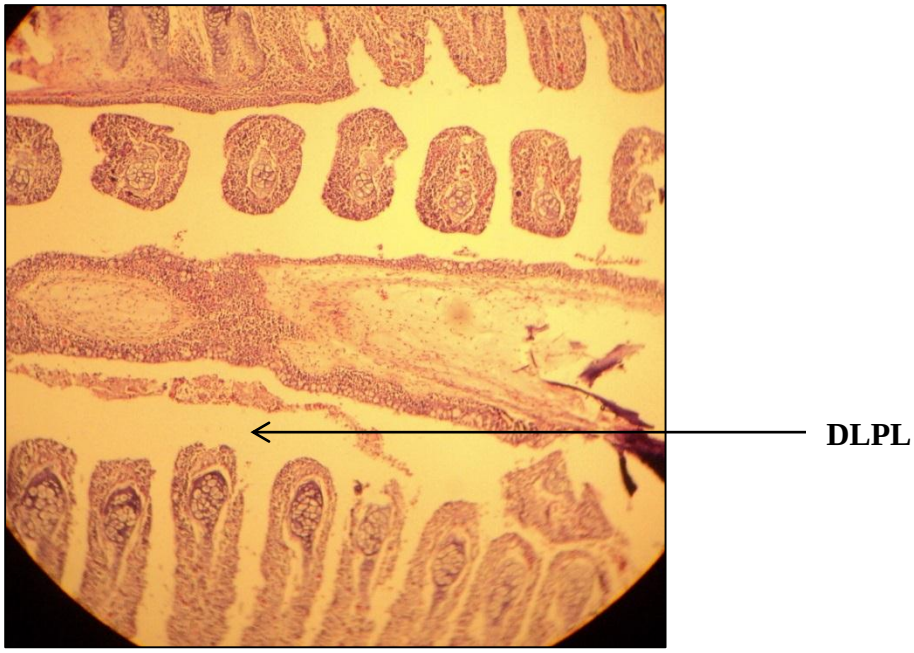


Plate IX: Section of the gill of fish exposed to acute (12mg/L) concentration of atrazine showing dislocation (DLPL) of the primary lamellae (H and E ×100)

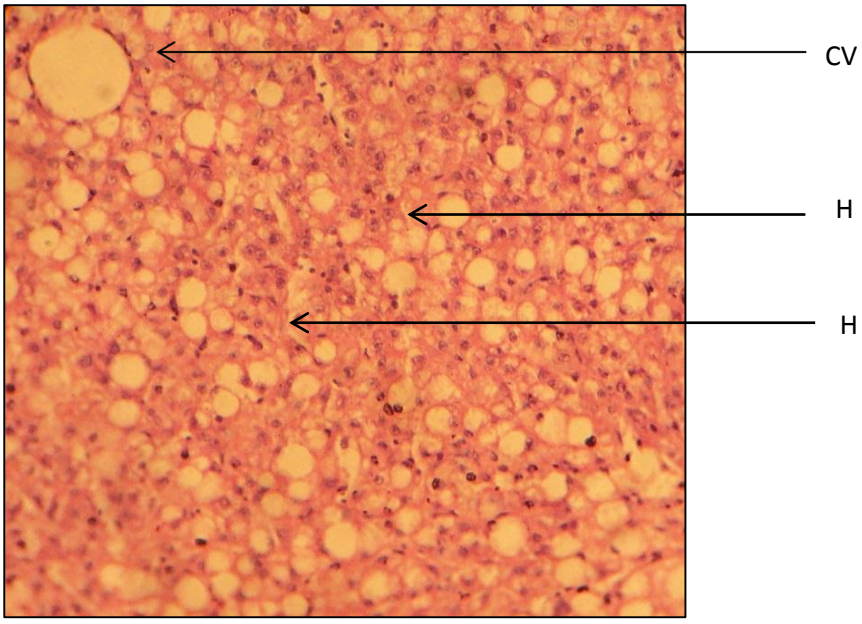


Plate X: Section of the liver of control fish showing a clear central vein (CV) and hepatocytes (H) with normal spherical nuclei (H and E ×100)

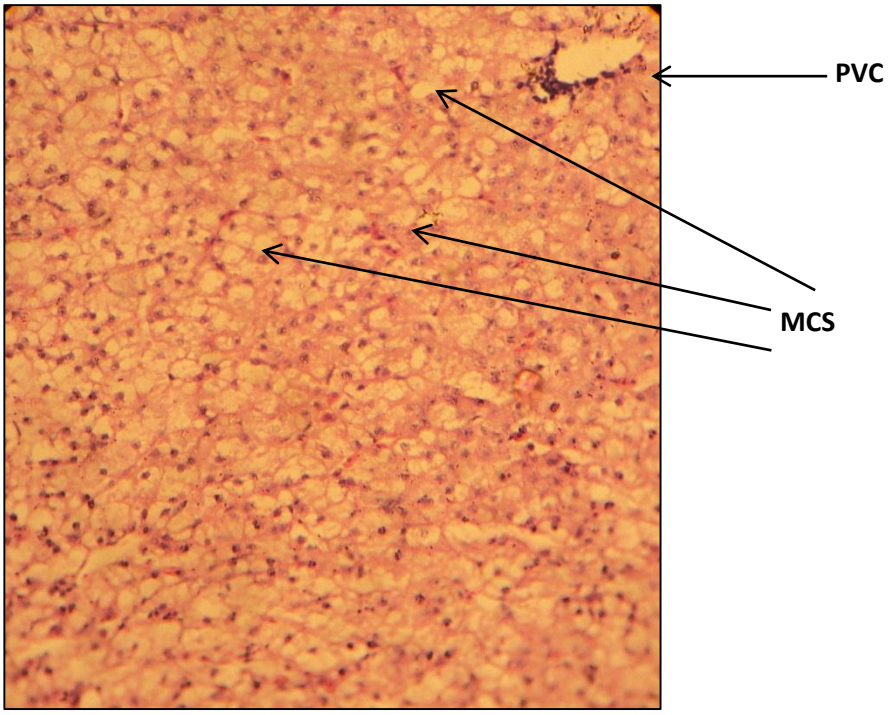


Plate XI: Section of the liver of fish exposed to sub-lethal (1.19mg/L) concentration of atrazine showing perivascular cuffing (PVC) around the central vein and mild congestion of the sinusoids (MCS) (H and E ×100)

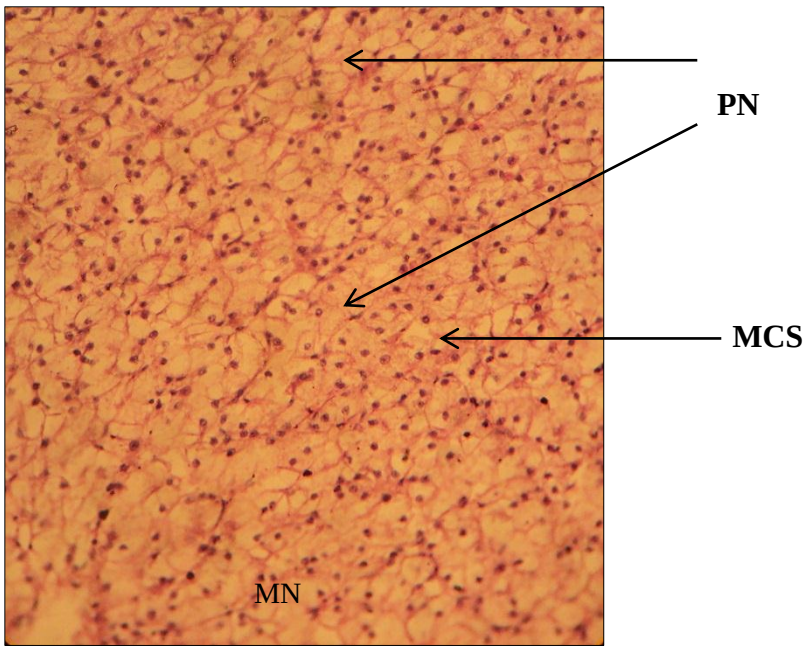


Plate XII: Section of the liver of fish exposed to sub-lethal (0.59mg/L) of atrazine showing pyknosis of the nuclei (PN), mild congestion of the sinusoids (MCS) and area of mild necrosis (MN) (H and E ×100)

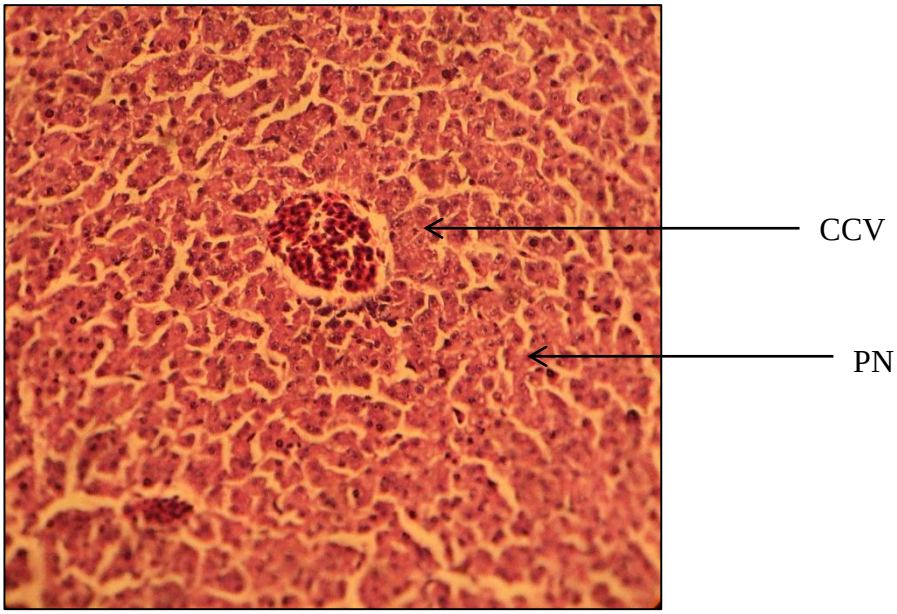


Plate XIII: Section of the liver of fish exposed to acute (10mg/L) concentration of atrazine showing congested central vein (CCV), pyknosis of the nuclei (PN) and mild coagulative necrosis (H and E ×100)

4.6 Sublethat Effects of Atrazine on the Growtht of *C. gariepinus* Juveniles

Observations on the growth of *C. gariepinus* juveniles are presented in Table 4.9. Fish in the control group had the highest final mean weight of 40.03g while fish in the exposed groups of 0.59, 1.19 and 2.38mg/L had final mean body weights of 35.83, 33.78 and 34.73g respectively. Similarly, the weight gain of control fish (31.98g) was higher than the values of 26.98, 24.98 and 23.63g of fish exposed in 0.59, 1.19 and 2.38 respectively.

The percentage live weight gain (%LWG) of the control fish (403.03) was significantly higher ($p<0.05$) than those of fish in the exposed groups (Table 4.10). The specific growth rate (SGR) of the control fish (0.0293) was significantly higher than those of fish in the exposed groups (0.0254, 0.0241 and 0.0207 respectively).

Table 4.9: Sub-lethal effects of atrazine on the growth of *C. gariepinus* juveniles.

Parameters	Concentration (mg/L)

	0	0.59	1.19	2.38
Number Of Fish	10	10	10	10
Percentage Mortality	0	3	7	13.33
Mean Initial Weight (g)	8.05	8.85	8.8	11.1
Mean Final Weight (g)	40.03	35.83	33.78	34.73
Weight Gain (g)	31.98^{a}	26.98^{a}	24.98^{a}	23.63^{a}
Specific Growth Rate	0.0293^{a}	0.0254^{ab}	0.0241^{ab}	0.0207^{b}

Means with different superscripts along rows are significantly different ($p>0.05$)

4.7 Sub-lethal Effects of Atrazine on the Nutrient Utilization of *C. gariepinus* Juveniles

The nutrient utilization indices of *C. garueinus* exposed to sub-lethal doses of atrazine for 8 weeks are presented in Table 4.10. Mean values of the control for feed conversion efficiency FCE (0.11), gross feed conversion efficiency GFCE (62.79) as well as nitrogen metabolism (481.31) were higher than those of the exposed groups although they were not statistically significant. All these values decreased in a concentration dependent manner. However, mean value of the control for feed conversion ratio (FCR) (1.61) was lower than those of the exposed groups, but this value increased with increase in the concentration of the toxicant (Table 4.10).

Table 4.10: Sub-lethal effects of atrazine on the nutrient utilization of *C. gariepinus* juveniles

Concentration (mg/L)	FCE	FCR	GFCE	NM	%LWG

0	0.11[a]	1.61[a]	62.79[a]	481.31[a]	403.03[a]
0.59	0.09[a]	1.9[a]	52.97[a]	407.38[a]	308.59[ab]
1.19	0.08[a]	2.17[a]	49.44[a]	377.19[a]	283.74[b]
2.38	0.08[a]	2.19[a]	46.39[a]	356.8[a]	215.73[b]

Means with different superscripts along columns are significantly different ($p>0.05$)

FCE = feed conversion efficiency, FCR = feed conversion ratio, GFCE = gross feed conversion efficiency, NM = nitrogen metabolism, %LWG =percentage live weight gain

CHAPTER FIVE

5.0 DISCUSSION

The physico-chemical parameters of the test water measured during both acute and sub-lethal toxicity bioassay were within suitable ranges for the survival and normal growth of *C. gariepinus*. Hence changes in fish behaviour and subsequently death could not have arisen from poor water quality of the test water. On the optimum pH scale for fish growth developed by Badiru (2005), the range of pH for this study (7.92-9.26) corresponds to the desirable range (6.5-9) for fish production. However, dissolved oxygen range for this study (4.1-5.2mg/L) spans the range for slow growth following long term exposure (1-5mg/L) of the dissolved oxygen scale for warm water fishes by Badiru (2005). Similarly, the temperature range for this study (24.3-30.4) is within the normal range of temperature in the tropics to which fish are adapted (22-35°C) as reported by Howerton (2001).

Changes in behaviour observed in this study are similar to those reported by several authors. Hyperactivity of fish in exposed groups during 12-24 hours could be attributed to an attempt to escape the toxic environment. Hyperactivity of fish on introduction to an unfavourable environment has been suggested as the primary and principal sign of nervous system failure due to pesticide poisoning which affects physiological and biochemical activities. Ramesh *et al*. (2009) reported similar behavioral responses of common carp to atrazine exposure which include increased opercular movement, mucous secretion, jerky movement, floating on the sides and hypersensitivity showing violent erratic and fast swimming, and opined that the abnormal behaviour of the fish indicates the toxic effect of atrazine on the central nerves system (CNS) and cardiovascular system. Mekkawy *et al*. (2013) also reported hyperactivity in *C. gariepinus* exposed to atrazine which was characterized by rapid and erratic swimming or darting, partial loss of equilibrium, rapid pectoral fins and opercular movements, reduction in the feeding activity, fins haemorrhage and loss of some skin parts. In the course of metribuzin poisoning in rainbow trout, Velisek *et al* (2008) reported similar clinical symptoms such

as accelerated respiration, loss of movement coordination, fish lying on their flanks and moving in this position.

Swollen abdomen and discolouration of the skin were also observed in fish exposed to the toxicant. This is attributable to necrotic damage to the gut of fish and suggests that toxicity of atrazine is not restricted only to the outside. Annune *et al*. (1994) and Olusegun (2001) reported similar findings. Nwani *et al*. (2013a) also reported skin discolouration in *Tilapia zilli* exposed to the chloroacetanilide herbicide butachlor. Ikele *et al*. (2011) similarly observed that the *C. gariepinus* normal darkly pigmentation in the dorsal and lateral parts was changed to very light pigmentation when exposed to diethyl phthalate. Chromcova *et al.* (2015) also observed a high number of pigmentation changes by day 6 in early stages of Common carp exposed to NeemAzal T/S compared with the control. Generally, it is argued that behavioural studies gives a direct picture of response of the fish to pesticides and related chemicals and the behavioural activity as well as morphological responses of organisms represents the final integrated result of a diversified biochemical and physiological processes.

Mucus secretion observed in gill area of fish may be due to increased activity of mucus cells as a form of protection against atrazine exposure. This may interfere with gaseous exchange and osmoregulation with possible results of death as reported by Gbem *et al*. (1997). The initial increase in opercular ventilation count in atrazine exposed groups may be due to impairment of respiration as a result of mucus secretion or an attempt by the fish to counteract or offset effect of the toxicant by breathing faster. This is also attributable to an increase in oxygen demand needed for the increased metabolic activity as an attempt to metabolize the toxicant. This finding is similar to that of Nwani *et al* (2013b) where faster opercular movements in *C. gariepinus* exposed to termifos was recorded. Obiezue *et al*. (2014) observed rapid opercular movement in *C. gariepinus*

exposed to diethyl. A plausible reason for this as well as other abnormal behaviours observed suggests nervous disorder. Secretion of mucus by the gills and their possible destruction could have inhibited osmoregulatory activity of exposed fish. Physiologically, fish reacts by increasing rate of respiration in an attempt to pass more water and invariably oxygen over the gills surface. However, fish end up passing more toxicant over the gills leading to more intoxication, and a decline in opercular ventilation counts with increase in exposure period. The decline in opercular ventilation count observed in this study may be attributed to a blockage in the electron transport chain during synthesis of ATP. This results in the accumulation of oxygen at the end of the chain hence reduced or no further intake. Gbem *et al*. (1997), Auta *et al*. (2002, 2005) reported a decrease in opercular ventilation count with increased exposure time. However, this is in contrast to the findings of Yaji (2012) where an increase in the opercular ventilation count of *Oreochromis niloticus* exposed to acute concentrations of cypermethrin in static assay at the 96th hour was observed.

Initial increase in tail beat frequency of fish in exposed groups can be linked to an increased metabolic activity by the attempt of fish to swim faster and escape the polluted environment. Ufodike and Omeregie (1990) suggested that increase in opercular ventilation counts and tail fin beats within initial periods of toxicant exposure was an avoidance syndrome exhibited by fish. However as this effort became futile, fish became fatigued and a decline was observed in the tail fin beat frequency with increase in exposure time. Tail fin movement is a mechanical activity and requires the use of energy. Hence this decline in tail fin movement rates can be attributed to disruption in energy synthesis possibly through blockage of the electron transport chain. This is similar to the reports of Chindah *et al.* (2004), Ogundele *et al.* (2004) as well as Onuoha and Ohaturonye (2007). Yaji (2012) reported a different finding in *O. niloticus* exposed to

cypermethrin for 96 hours in both static and flow through experiments. There was an increased tail movement rate in the 96th hour of exposure.

A slight change in opercular ventilation count and tail fin beat frequency of the control group showed stabilization after agitation probably as a result of introduction into a new environment of the test tank. This was seen to stabilize in subsequent periods. It could be asserted that the primary mode of toxicity of atrazine would be the disruption of gaseous exchange and salt-water balance due to necrotic damage particularly to the gills.

The fact that mortality of fish during acute and sub-lethal bioassays was concentration dependent could be directly linked with the direct effect of the toxicant. This is similar to the findings of Obiezue *et al*. (2014) in which a direct relationship between mortality in *C. gariepinus* and concentration of diethyl phthalate was recorded. Khoshnood *et* al. (2014) reported a dose and time-dependent decrease in mortality rate, such that as the exposure time increased from 12 to 96 hours, the median concentration was reduced. In contrast, the highest cumulative percentage mortality was observed in the 10mg/L group of NeemAzal treated early stages of common carp, while higher concentrations of 30 and 60mg/L showed lower mortalities (Chromcova *et al*., 2015).

The 96-hour LC_{50} of atrazine (11.89mg/L) for the fish species *C. gariepinus* from this study was higher than the 96-hour value of 7.2 mg/L for the fish species *O. niloticus* reported by Ada *et al*. (2012) and also the 96-hour value of 6.977mg/L of the same toxicant for the fish species *O. niloticus* as reported by Agbon *et al*. (2014) as well as the 24-hour value of 18.5mg/L for the fish species *Cyprinus carpio* (Ramesh *et al*., 2009). However, the value was lower than the 96-hour value of 24.95mg/L of atrazine for the Caspian Kutum fry (Khoshnood *et al*., 2014) and the 96-hour value of 89.3 mg/L of metribuzin for rainbow trout (Velisek *et al*., 2008), as well as the 96-hour value of 42.381mg/L of atrazine for the freshwater fish *Channa punctatus* (Bloch) (Nwani *et al*.,

2010). Abdali *et al*. (2011) reported high value of 80mg/L of for Grass Carp (*Ctenopharhyngoden idella*) using atrazine. Toxicity of chemicals to aquatic organisms however has been shown to be affected by age, size and health of the species (Abdul-Farah *et al*., 2004). Physiological parameters, and water quality parameters (temperature, pH, dissolved oxygen and turbidity), the amount and kind of aquatic vegetation, concentration and formulation of the chemical and its exposure also greatly influence such studies (Young, 2000). According to Jiraungkoorskul *et al*. (2002), toxicity of any poison is species and environmental factors related. Hence, it is expected that toxicity of toxicants may vary based on the above factors and different species may have differing values of LC_{50}.

The decrease in total erythrocyte count observed in both acute and sub-lethal exposures is attributable to a possible inhibition of erythrocyte production through destruction of stem cells in the bone marrow which are the progenitor cells (absolute anaemia) and/or an increase in their destruction or haemodilution (relative anaemia). The same can be said for observed reduction in packed cell volume values as the volume of blood cells is a function of their numbers. Similarly, the decrease in haemoglobin values could be due to the decreased number of erythrocytes, as erythrocytes are the haemoglobin carrires. Also, this reduction may be indirectly attributed to pesticide-induced decrease in growth and other food utilization parameters which resulted in severe anemia (James and Sampath, 1999). Hypoproteinemia observed may be due to direct effect of the utilization of body protein as an energy supply to meet the increasing physiological demands in overcoming the stress induced by the polluted medium. This was similarly reported by Abd El-Salam *et al.*, 1994, El-Sayed *et al*., 1996 and Fontana *et al*., 1998). Hypoproteinemia may also be attributed to several pathological processes including plasma dissolution, renal damage and elimination in the urine, decreased liver protein synthesis, alteration in

hepatic blood flow and/or hemorrhage into the peritoneal cavity and intestine as observed by Keith and Weber, (1979). Results of the haematological parameters from this study were also in agreement with those of Mekkawy *et al*. (2013) which revealed atrazine-induced decrease in erythrocyte counts, haemoglobin, total protein and packed cell volume of *C. gariepinus.* This was attributed to the reduction of these parameters at sub-lethal levels to the destruction of matured red blood cells and the inhibition of erythrocyte production due to reduction of haemsynthesis that is affected by the pollutant. The results were also in consonance to that of Jayaprakash and Shettu (2013) which showed a decrease in the haemoglobin content, total erythrocyte count, packed cell volume, mean corpuscular volume and mean corpuscular concentration in *Channa punctatus* exposed to deltamethrin. The authors attributed these changes to stress and hypoxia caused by the toxicant or the swelling of erythrocytes causing lysing of the red blood cells. Heath (1987) and Abo-Hegab *et al.* (1993) interpreted stress-induced decrease in the haemoglobin and haematocrit values in terms of heam dilution of blood and elimination of red blood cells as well as disequilibrium of the osmotic pressure inside and outside the blood cell. The findings are also in line with that of Elbialy *et al.* (2015) which reported a significant decrease in red blood cell count, packed cell volume, haemoglobin, neutrophilia and lymphonia and monocytosis in Nile Tilapia (*Oreochromis niloticus*) exposed to different levels of organochloride and organophosphate pesticides. However, the results are in contrast to the report of Velisek *et al.* (2008) which reported significantly higher values ($P<0.01$) of erythrocyte count, haematocrit and haemoglobin in rainbow trout (*Oncorhynchus mykiss*) exposed to the herbicide metribuzin. The findings from this study also did not agree with that of Ajani and Awogbade (2012) that revealed increased values of packed cell volume in *C. gariepinus* juveniles exposed to Diuron. Chindah *et al*. (2004)

also reported a reduction in the leucocyte and erythrocyte numbers of *Tilapia guineensis* exposed to chloropyrifos.

In the present study, the increase in values of leucocyte counts observed with increase in atrazine concentration is attributable to an attempt or response to combat detected foreign substance in the body which prompted the production of more antibodies hence the increase in number of leucocyte to withstand the effect of the toxicant. Leucocytes are involved in the regulation of immunological function and their numbers increase as protective response in fish to stress. Such an increase in total leucocyte count (TLC) occurs by the increase in lymphopoiesis and/or enhanced release of lymphocytes from lymphoid tissues. This is in consonance with the report of Kori-Siakpere *et al*. (2007) which revealed a significant increase ($p< 0.05$) in the total leucocyte counts in the blood of *C. gariepinus* exposed to paraquat herbicide. However, this is in contrast to the findings by Ada *et al*. (2012) that reported an unexpected decrease in the white blood cell count with increase in concentration of atrazine in *O. niloticus*. Ajani *et al*. (2007) also reported a decrease in the number of white blood cells in *C gariepinus* exposed to nitrite. This was attributed to the toxicant which may have eliminated the red blood cells suddenly without the chance to produce young ones to replace the older cells.

The atrazine-induced changes towards increase or decrease in MCV, MCH and MCHC values were reported in the present work. As these are absolute values derived from total erythrocyte counts, haemoglobin and PCV, considerable decrease in total erythrocyte counts could directly affect MCV and MCH values. This indicates that the toxicant interfered with the normal physiology of erythrocytes. MCV has been reported to provide information on the size and status of erythrocytes (Nussey *et al*. 1995). Adeyemo (2007) reported significant increase in the mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration values of *Clarias*

gariepinus exposed to lead. Siang *et al*. (2007) reported a decrease in mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration and an increase in mean corpuscular volume values of *Monopterus albus* exposed to endosulfan. Shah (2006) reported that chemical-induced alterations in MCV, MCH and MCHC were attributed to direct or feedback responses of structural damage to red blood cell membranes resulting in haemolysis and impairment in hemoglobin synthesis, stress-related release of red blood cells from the spleen and hypoxia. Erythrocyte count, MCV, MCH and MCHC decreased in *Heteropneutes* sp. treated with insecticides with concomitant decline in oxygen transport (Thakur and Bais 2000).

The leucocyte differential counts of *C. gariepinus* in the present study in both acute and sub-lethal exposures revealed a dose-dependent inhibition in neutrophil number (neutropenia) and an elevation in lymphocyte values (lymphocytosis) with increase in concentration of the toxicant. The lymphocytosis observed may have been due to antigenic response to a foreign substance (atrazine) or may be associated with pollutant induced tissue damage. This contrasts the report of Ogueji and Auta (2008) on a dose-dependent lymphopenia in *C. gariepinus* exposed to sub-lethal concentration of chlorpyrifos-ethyl. Blahova *et al*. (2014) also reported several fold significant decrease ($p<0.05$) in lymphocytes in experimental groups (15, 20, and 30mg/L) during 96h toxicity test of atrazine on common carp. Svobodova *et al*. (2003) also reported a decline in lymphocyte counts in common carp following deltamethrin exposure. However, this finding is in accord with that of Jayaprakash and Shettu (2013) which reported a significant increase in the populations of lymphocytes, in fish of 30 and 45 day test period when they studied the toxicity of deltamethrin to *Channa punctatus* (Bloch). The increase in lymphocytes number was attributed to immune response by the fish system, while the

increase in neutrophils in the blood was attributed to non-specific response to a variety of stress stimuli in fishes.

Neutropenia observed may be due to the possible direct effect of the toxicant on the number of neutrophils. In contrast, Blahova *et al*. (2014) reported a several fold significant increase ($p<0.05$) in the number of monocytes, segmented and band neutrophile granulocytes, metamyelocytes, and myelocytes in common carp exposed to atrazine for 96 hours. Similarly, Svobodova *et al*. (2003) reported an increase in neutrophil number in deltamethrin-exposed group of common carp when compared with the control.

Activities of anti-oxidant enzymes in tissues (gills and liver) of *C. gariepinus* indicated a significant dose-dependent increase in catalase as well as superoxide dismutase activities and the highest activity was recorded in the fish exposed to the highest concentration of the toxicant. Antioxidant enzyme activites have been used as an early warning sign of environmental pollution (Rosety *et al*., 2005). In vertebrates, superoxide dismutase is one of the most important antioxidant enzymes that detoxify superoxide anion radical (O_2^-) while catalase (CAT) reduces hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2). Thus CAT and SOD provide the first line of defense against stress. Both enzymes (CAT and SOD) are inducible and may have been produced in response to the toxicity of atrazine.

Increase in catalase activity observed in this study in both the gill and liver of atrazine exposed fish could be attributed to the production of hydrogen peroxide (H_2O_2). As catalase is responsible for its detoxification to water and oxygen, more activity of catalase is observed. Superoxide dismutase catalyzes the dismutation of the superoxide anion radical to water and hydrogen peroxide, which is detoxified by catalase. The increase in superoxide dismutase activity after atrazine administration appears to be an adaptive

response to increased generation of reactive oxygen species. Ullah *et al*. (2014) had reported elevated levels of catalase activity in muscles and brain of *Tor putitora* exposed to cypermethrin. Nwani *et al*. (2014) also reported elevated levels of catalase activity in primextra exposed *C. gariepinus*, with 4.80%–23.76% and 6.20%–37.81% increase in the muscle and liver tissues respectively at different exposure levels. Mirvaghefi *et al*. (2015) also observed that catalase activity was significantly higher in diazinon-exposed groups compared to the control group of Rainbow trout. Manjunatha *et al*. (2015) reported increased levels of catalase and superoxide dismutase in *Labeo rohita* during atrazine exposure. Kadry *et al*. (2012) similarly reported that atrazine exposure also led to a significant increase in the activities of catalase (CAT) and superoxide dismutase (SOD) in *C. gariepinus*. Nwani *et al*. (2010) also reported a concentration-dependent increase in the antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) thus suggesting the use of these antioxidants as potential biomarkers of toxicity associated with contamination in freshwater fishes.

However, the results of antioxidant enzymes observed in this study is in contrast to the report of Stara *et al*. (2012) which showed no differences with controls in superoxide dismutase and catalase activity of exposed groups, in the study of chronic exposure of common carp to prometryne effects on oxidative stress and antioxidant response. Mirvaghefi *et al*. (2015) also reported contrasting findings of superoxide dismutase activity being significantly lower in diazinon-exposed fish than in the control group in Rainbow trout. The findings from this study is also in contrast with that of Nwani *et al*. (2014) which reported that the values of superoxide dismutase and protein in tissues of primextra exposed *C. gariepinus* were comparable to the control except on day 15 in the liver where the values significantly declined. Hamed (2016) also observed a significant decline in the activities of superoxide dismutase, catalase and glutathione reductase in the

liver, kidney and gill tissue samples from *C. gariepinus* groups treated with deltamethrin. The depletion in level of catalase activity might be due to an increased production of the superoxide radical (O2−), as an excess of this anion is known to inhibit catalase activity (Bainy *et al.,* 1996). Similarly, Winkaler *et al.* (2007) reported that fish exposed to sublethal concentrations of neem extract showed significant reduction in hepatic catalase activity which is likely to affect the capacity of liver cells to defend themselves and respond to contaminant-induced oxidative stress. In Tilapia, a significant reduction in catalase activity in the muscle of *O. niloticus* obtained downstream of Kizilirmak River, Turkey, due to exposure to petrochemical industry effluent contamination was observed (Avei *et al.*, 2005). Antioxidant enzyme activities in fish depends on species, exposure time and dose of pollutant (Elia *et al*., 2002; Nwani *et al*., 2010), whereas feeding behaviour and habitat also influence their activities (Ahmad *et al*., 2000). Therefore, variations in antioxidant enzyme activities in different tissues of various fish species have been reported (Ullah *et al*., 2014).

The decrease in lipid peroxidation observed with increase in concentration as evidenced by reduced formation of malondialdehyde is attributable to an attempt by the defense system of the organism to remove generated reactive oxygen species. This is in line with the report of Moraes *et al.* (2009) on significant decrease in thiobarbituric acid reactive substances (TBARS) levels in brain, muscle and liver tissues of the teleost fish, *Leporinus obtusidens* exposed to clomazone and propanil for 90 days. A study by Stara *et al*., (2012) depicted a similar trend, where no elevated levels of TBARS in response to the exposure to prometryne in common carp (*Cyprinus carpo* was observed. Lushchak *et al.* (2009) also did not record elevation of lipid peroxidation in the brain and liver of goldfish *Crassius auratus* exposed to sub-lethal concentrations of Roundup®. However, this contrasts to the findings of other authors. Ullah *et al*. (2014) reported decreased lipid

peroxidation levels in cypermethrin exposed *Tor putitora*. Nwani *et al*. (2014) also reported that exposure to sub-lethal concentrations of primextra significantly increased levels of lipid peroxidation in a time-and concentration–dependent manner, in liver and muscles of the African catfish *C. gariepinus* juveniles, thus reflecting increased oxidative stress and lipoperoxidation. Manjunatha *et al*. (2015) also reported increase in lipid peroxidation with increase in atrazine concentrations in the fresh water fish *Labeo rohita*. Increase in lipid peroxidation suggests that there was increased production of reactive oxygen species as suggested by Nwani *et al*. (2010) in *Channa punctatus* in response to atrazine exposure. Increase in malondialdehyde levels reflects the higher production of hydroxyl radicals (OH-) subsequent to exposure to a poison as reported by Mirvaghefi *et al*. (2015) in Rainbow trout following Diazinon exposure. Hamed (2016) also reported significant increase in malondialdehyde (MDA) in liver, kidney and gills of the African Catfish; *C. gariepinus* following Deltamethrin exposure. On the whole, responses to oxidative stress may differ with respect to species, age, habitat, feeding, behavior, duration of exposure, particular tissues, and concentration of the herbicide tested (Stara *et al*., 2012).

Histopathological findings from this study are in line with that of Ramah (2011) which reported that light microscopic study of the gills of *Ctenopharygodan idella* (grass carp) exposed to herbicides (butachlor, oxyfluorfen and thiobencarb) revealed several pathological changes such as cartilaginous hyperplasia of gill rays, proliferation of lamellar epithelium and vacuolation of cytoplasm of lining epithelium, focal loss of lamellar epithelium and congestion of blood spaces. This study agrees with that of Peebua *et al*. (2008) which reported telangiectatic secondary lamella and primary lamella hypertrophy, extensive aneurysms with some ruptures in many secondary lamellae and necrosis of the primary epithelial cells, extensive hypertrophy and hyperplasia of primary

epithelial cells and slight clubbing at the tip of the secondary lamellae in gills of the Nile Tilapia upon acute exposure to alachlor. In the sub-chronic exposure, gills showed a slight thickening of the primary lamellar epithelium and several areas of edema extensive hypertrophy and hyperplasia of epithelial cells and accumulation of erythrocytes. There were severe clubbings at the tips of the secondary lamellae. Winkaler *et al*. (2007) had described frequent histological alterations in the gills (lamellar aneurism, rupture of the lamellar epithelium) and in the kidney (reduction of Bowman's space, cytoplasmic vacuolation, granular degeneration, and the narrowing of tubular lumen) of fish exposed to neem extracts of 5g/l and 7.5g/l.

Damages observed in the gill architecture in this study may have been responsible for impairment of the respiratory and regulatory functions of the gills and hence resulted in death. Hyperplasia is considered as a protective mechanism from environmental irritant which works by decreasing the respiratory surface and increasing the toxicant–blood diffusion distance (Meissner and Diamandopoulos, 1977). Its intensification could result in the thickness of epithelial layers, which could be supported by the increase in epidermal thickness and lamellar width. Thus, all the lesions found in the present study had probably inhibited the respiratory, secretory and excretory functions in the gill of fish as suggested by Khoshnood *et al*. (2014).

The liver also plays a central role in osmoregulation. Hence, attempt in detoxification of toxicants may compromise its integrity and affect its functions. Changes observed in the liver of exposed fish in this study might have occurred as a result of the constant influx of the toxicant to the liver at a time when the detoxification ability of the liver was exceeded. A similar study by Jiraungkoorskul *et al*. (2003) depicted that *O. niloticus* exposed to sub-lethal concentrations of the commercial glyphosate herbicide Roundup®, showed histopathological changes in the liver namely vacuolation of hepatocytes and

nuclear pyknosis. Ramah (2011) observed that pathological changes observed in the liver tissues of grass carp exposed to different herbicides were congestion of sinusoids congestion of central vein and proliferation of bile ductular epithelium. The results of the study by Peebua *et al*. (2008) which was similar to this showed hydropic swelling, accumulation of lipid vacuoles and focal necrosis in some liver area of *O. niloticus* exposed to alachlor. In teleosts, the liver is the primary organ for biotransformation of organic xenobiotics, and probably also for the excretion of harmful trace metals, food digestion and storage, and metabolism of sex hormones (Heath, 1995; Hinton *et al*., 2001). Liver of fish is sensitive to environmental contaminants because many contaminants tend to accumulate in the liver thereby exposing it to much higher levels than in the environment or in other organs (Heath, 1995). Hence, observed damages to liver of fish could be used as an index of environmental pollution.

The decrease observed in the weight gain of fish as well as the specific growth rate with increase in concentration of atrazine is attributable to the channeling of energy for homeostatic processes and tissue damage repair to offsetting the effect of the toxicant, rather than for storage and growth. This is in line with the work of Auta and Ogueji (2006) which reported a significant dose-dependent decrease in the growth and food utilization of *O. niloticus* exposed to sub-lethal concentrations of dimethoate, and that this decrease in weight gain and growth might have been due to the suppressive effect of the toxicant on food consumption of the fish. The report further stated that a reduced growth rate may be due to an increased activity associated with attempt to avoid the contaminated water or an increased expenditure of energy on chemical detoxification and tissue repair.

The decrease observed in the nutrient utilization parameters by fish exposed to atrazine was similar to the work of Yaji (2012). Yaji (2012) reported a decrease in percentage live weight gain, weight gain, specific growth rate, gross feed conversion efficiency, feed

efficiency and nitrogen metabolism, and that these might have been caused by the loss of appetite in the exposed fish, which triggered the behavioural and morphological symptoms, and reduction in the number of red blood cell count, which is an evidence of oxidative stress. Similarly, Alvarez and Fuiman (2005) reported a significant decrease in the growth rate of Red drum larvae by 7.9–9.8% after exposure to environmental levels of atrazine. Shallangwa and Auta (2008) also reported a reduction in the growth of *C. gariepinus* exposed to sub-lethal effects of 2, 4-Dichlorophenoxyacetic acid, and that this might have been due to lower feeding rates, or that the toxicant made the feed unsuitable for consumption; or it could have been due to the increased expenditure of energy on chemical detoxification and tissue repair.

Generally, it is probable that increase in concentration of the toxicant led to an increase in oxygen tension which reduced metabolic activity such as synthesis of cellular material, ATP, etc. This also affected synthesis of new cells which in turn led to reduced or no growth at all. Reduced growth will therefore translate to reduced or no increase in weight of the fish.

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS, RECOMMENDATIONS AND CONTRIBUTIONS TO KNOWLEDGE

6.1 Summary

From the study, atrazine was found to be moderately toxic to *C. gariepinus* juveniles with an LC_{50} value of 11.89mg/L. Changes in behaviour and morphology observed in the species included hyperactivity, startled responses, rapid opercular movement, loss of

balance, skin discolouration, holding out pectoral and pelvic fins, swollen abdomens, mucus secretion and a period of quiescence prior to death. Exposure of the fish to acute and sub-lethal concentrations of the toxicant resulted in a number of significant changes in the haematology, histopathology as well as the oxidative stress response of the species. Changes in haematology included anaemia, leukocytosis, proteinaemia, haemoglobinaemia, neutrophilia as well as lymphocytosis. Acute and sub-lethal concentrations of atrazine also induced an increase in the generation of ROS as evidenced in the enzymes assayed. Histopathology of the gills and liver of fish exposed to acute and sub-lethal concentrations of atrazine showed marked changes in both tissues. Changes in the gills included attenuation and clubbing at the tips of the primary lamellae, gradual degeneration of the primary lamellae, total dislocation of the primary lamellae from its position as well as mild congestion in the cartilage. Changes in the liver of exposed fish included perivascular cuffing around the central vein, mild congestion of the sinusoids, pyknosis of the nuclei, congestion of the central vein as well as mild coagulative necrosis. Atrazine was also found to affect growth parameters (weight gai, percentage live weight gain, specific growth rate) as well as nutrient utilization parameters (feed conversion efficiency, feed conversion ratio, gross feed conversion efficiency, and nitrogen metabolism) although some of these effects were not statistically significant.

6.2 Conclusions

- At the end of the study, it was concluded that atrazine is toxic to juveniles of *C. gariepinus*. The 96-hour LC_{50} (median lethal concentration) value for juveniles of *C. gariepinus* was 11.89mg/L.
- Atrazine significantly induced anaemia (from $6.63\pm0.15x10^{12}$ cells/L in the control to $3.03\pm0.09x10^{12}$ cells/L), leukocytosis (from $8.40\pm0.26x10^{9}$ cells/L in the control to $11.80\pm0.15x10^{9}$ cells/L), and lymphocytosis (from 86.00±1.53% in

the control to 96.67±1.20%) in *C. gariepinus* juveniles in acute exposure. A similar trend was observed in sub-lethal exposure. Atrazine induced oxidative stress in the species (catalase activity peaked 91.22±0.28µg/g from 16.34±0.50 µg/g in the control, in liver of fish in acute exposure) in a dose-dependent manner.

- Atrazine caused histopathological changes in the liver (perivascular cuffing, mild congestion of the sinusoids, pyknosis, congestion of the central vein as well as mild coagulative necrosis), and gills (attenuation and clubbing at the tips of the primary lamellae, gradual degeneration of the primary lamellae, total dislocation of the primary lamellae from its position as well as mild congestion in the cartilage) of *C. gariepinus* juveniles.
- Atrazine also inhibited the growth and nutrient utilization of *C. gariepinus* juveniles. Specific growth rate dropped from 0.03 in the control to 0.02 in 2.38mg/L exposure. Feed conversion efficiency dropped from 0.11 in the control to 0.08 in 2.38mg/L sub-lethal exposure.

6.3 Recommendations

- Due to the significant use of pesticides, it is recommended that toxicity studies be continuous involving various fish species and invertebrates. New formulations of herbicides should also be used under different environmental conditions in order to test all variables that can be used as a source of reference for toxicity studies.
- The indiscriminate use of pesticides as well as their use near water bodies should be discouraged. Instead, more environmental friendly approaches to pest control (such as biocontrol) should be explored.

- Since the outright discontinuation of the use of pesticides is almost impossible, the use of non-persistent, highly selective pesticides should be advocated in place of the highly persistent and non-selective ones.
- Further studies should be carried out in the field (natural environment), with other aspects such as fecundity and genetics included in it.
- There is the need for the relevant authorities to intensify efforts at making environmental policies that will keep the use of pesticides in check as well as a periodic review of such policies based on the emergence of new facts.
- Farmers should also be made aware of the dangers of the indiscriminate use of herbicides. Consumers should also be sensitized on the health risks associated with consuming pesticide-contaminated fish.
- Bioaccumulationo of atrazine should be assessed in *C. gariepinus*, in other fish species as well as other invertebrates.

6.4 Contributions to Knowledge

- The present study revealed that the median lethal concentration (LC_{50} of atrazine to juveniles of *C. gariepinus* is 11.89mg/L.
- Atrazine is toxic to juveniles of *C. gariepinus*, and this by extension is likely to be the same in other aquatic animals and invariably humans.
- Atrazine caused changes in various haematological, biochemical, histopathological, growth and nutrient utilization paramaters which can be used as indices to pesticide toxicity in the environment.

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APPENDICES

Appendix I: Mortality of *C. gariepinus* exposed to acute nominal concentrations of atrazine

Conc. (mg/L)	Rep	Time (Hours)					Mortality	Mean Mortality
		12	24	48	72	96		
0	R1	0	0	0	0	0	$\frac{0}{10}$	
0	R2	0	0	0	0	0	$\frac{0}{10}$	$\frac{0}{10}$
0	R3	0	0	0	0	0	$\frac{0}{10}$	
9	R1	0	0	0	1	0	$\frac{1}{10}$	
9	R2	0	0	0	0	1	$\frac{1}{10}$	$\frac{6}{10}$
9	R3	0	0	1	1	2	$\frac{4}{10}$	
10	R1	0	0	1	0	0	$\frac{1}{10}$	$\frac{7}{10}$
10	R2	1	0	1	0	0	$\frac{2}{10}$	
10	R3	0	0	3	0	1	$\frac{4}{10}$	
11	R1	0	0	4	0	0	$\frac{4}{10}$	
11	R2	0	0	0	3	1	$\frac{4}{10}$	$\frac{14}{10}$
11	R3	0	0	0	5	1	$\frac{6}{10}$	
12	R1	1	0	2	3	0	$\frac{6}{10}$	
12	R2	0	0	2	0	0	$\frac{2}{10}$	$\frac{16}{10}$
12	R3	1	1	3	3	0	$\frac{8}{10}$	
13	R1	0	2	3	2	1	$\frac{8}{10}$	$\frac{17}{10}$
13	R2	1	0	1	1	1	$\frac{4}{10}$	

13	R3	0	0	1	2	2	$\frac{5}{10}$

Conc. = Concentration Rep. = Replicate