TOXIC EFFECTS OF GLYPHOSATE AND PARAQUAT TO JUVENILES OF THE AFRICAN CLARIID CATFISH *CLARIAS GARIEPINUS* (TEUGELS, 1986)

BY

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DECLARATION

I declare that the work in this dissertation entitled 'TOXIC EFFECTS OF GLYPHOSATE AND PARAQUAT TO JUVENILES OF THE AFRICAN CLARIID CATFISH *CLARIAS GARIEPINUS* (TEUGELS, 1986)' has been performed by me in the Department of Biological Sciences.' 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

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CERTIFICATION

This dissertation entitled 'TOXIC EFFECTS OF GLYPHOSATE AND PARAQUAT TO JUVENILES OF THE AFRICAN CLARIID CATFISH *CLARIAS GARIEPINUS* (TEUGELS, 1986)' by Opeyemi Isaac AYANDA meets the regulations governing the award of the degree of Doctor of Philosophy (Fisheries) of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to my unborn child who will arrive to know his daddy as a Doctor

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ABSTRACT

The impact of short and long term exposure to the commonly used herbicides, glyphosate and paraquat on *Clarias gariepinus* juveniles was evaluated using standard methods that assessed fish behaviour, mortality, biochemical, histological, cytotoxic and genotoxic parameters. Fish exhibited some behavioural abnormalities such as hyperactivity, hitting of tails against the walls of the holding medium and abnormal swimming. The herbicides caused mortality of fish in a concentration dependent manner for the duration of exposure to acute concentrations of both herbicides for 96hrs. The LC_{50} value for paraquat by probit method/analyses was 0.07mg/L while that of glyphosate was 0.53mg/L, hence paraquat was more toxic than glyphosate. Fractions, 0.0035, 0.007, 0.014mg/L paraquat and 0.0265, 0.053 and 0.106mg/L glyphosate representing 1/20, 1/10 and 1/5 respectively of these LC_{50} values were used for a 56day chronic exposure study following a static renewal protocol. Samples for biochemical analysis, pathology, cytotoxicity and genotoxicity were collected at intervals of 14days respectively. Assessment of biochemical parameters revealed significant increases ($p \leq 0.05$) in the activities of stimulating liver enzymes, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Lactate Dehydrogenase (LDH) and Alkaline Phosphatase (ALP) in all experimental groups compared with control exposures. There were also significant increases ($p \leq 0.05$) in the activities of the antioxidant enzymes Catalase (CAT), Superoxide dismutase (SOD), Glutathione Peroxidase (GPx) and in lipid peroxidation which was measured by thiobarbituric reactive substances (TBARS). These increases were either dose dependent, time dependent, or both. The increase in the activities of these enzymes was slightly more in paraquat-treated than in glyphosate-treated fishes indicating that paraquat is more toxic to *C. gariepinus*. Histological analysis of the fish organs examined revealed varying degrees of pathological alterations/degerations to the gills, liver and the brain in both the short and long term study. The gills of fish showed alterations like sloughing off and thickening of lamella. The liver exhibited changes such as vacuolar degeneration of hepatocytes, hepatocyte swelling and necrosis. In the brain, neuronal degeneration of neutrophils, gliosis amongst others were observed. The blood cells, following herbicide treatment showed the presence of

micronucleus, fragmented apoptotic cells, bean-shaped cells and lobed nucleus. The percentages (0.56% in control and 1.80% in the highest concentration for glyphosate; 0.61% in control and 1.57% in the highest concentration for paraquat in the acute study) of these different alterations increased with increasing concentration and with time in both the short and long term exposure experiments. The genotoxic analysis showed damage to fish DNA, particularly in fish exposed to acute concentrations of both herbides. The damage to fish DNA observed during the short term exposure was greater compared to damage in the long term study. The study therefore revealed that exposing *Clarias gariepinus* juveniles to acute and sublethal concentrations of glyphosate and paraquat upset metabolic, cytological and nuclear morphology in *Clarias gariepinus* and implies poor environmental fitness for fish exposed to herbicides in natural environments.

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ABBREVIATIONS, DEFINITIONS, GLOSSARIES AND SYMBOLS

Symbol	Definition
FAO	Food and Agricultural Organization
LC_{50}	Median lethal concentration
H_o	Null hypothesis
D.O	Dissolved Oxygen
pH	Hydrogen ion concentration
DNA	Deoxyribonucleic acid
PAH	Poly Aromatic Hydrocarbons
NA	Naphthenic Acid
MFO	Mixed-Function Oxygenase
ATP	Adenosine triphosphate
POP	Persistent Organic Pollutants
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
OH	Hydroxyl ion
TBARS	Thiobarbituric Acid Reactive Substances
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase

LDH	Lactate dehydrogenase
ALP	Alkaline Phosphatase
CAT	Catalase
GPx	Glutathione peroxidase
LPO	Lipid peroxidation
GR	Glutathione Reductase
O_2^-	Superoxide anion radical
GST	Glutathione transferase
OP	Organophosphate
GPT	Glutamic Pyruvic Transaminase
Na/K	Sodium/Potassium
GSSG	Reduced glutathione
CA	Chromosomal Aberration
SCE	Sister Chromatid Exchanges
MN	Micronucleus
UV	Ultraviolet
BN	Binucleated cells
NB	Nuclear Buds
FAC	Fragmented Apoptotic Cells

MNE	Micronucleated erythrocytes
$NaAsO_2$	Sodium arsenite
SCGE	Single cell gel electrophoresis
OC	Organochlorine
PCB	Polychlorinated biphenyls
HBCD	Hexabromocyclododecanes
Ht	Haematocrit
Hb	Haemoglobin
TPP	Total Plasma Protein
MCV	Mean Corpuscular Volume
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
EEZ	Exclusive Economic Zone
NSPFS	National Special Programme for Food Security
B.O.D	Biological Oxygen Demand
E.D.T.A	Ethylenediaminetetraacetic acid
Mg/L	Milligram per liter
APHA	American Public Health Association
H & E Stain	Haematoxylin and Eosin Stain

%	Percentage
OECD	Organization for Economic Cooperation and Development
AGI	Air Gulping Index
TMR	Tail fin Movement Rate
OVC	Opercular Ventilation Count
NADH	Reduced form Nicotinamide adenine dinucleotide
IU	International Unit
NADPH	Nicotinamide adenine dinucleotide phosphate
MDA	Malondialdehyde
TBA	Thiobarbituric Acid
HCl	Hydrochloric acid
PBS	Phosphate Buffered Saline
LMA	Low Melting Agarose
ANOVA	Analysis of variance
DMRT	Duncan Multiple Range Test
Conc.	Concentration
g	Grams
S.E.	Standard Error

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

The environment is under constant threat largely due to anthropogenic activities resulting from expanding human population, industrialization, urbanization and intensive agriculture causing tremendous stress and damage to our environment. As such, several authors have concluded that effluents from these anthropogenic activities have negative implications for environmental health and sustenance (Bhatia, 2002). The population density in the environment has put the survival of man in jeopardy, hence, preservation of the environment is *sine-qua non* for the existence of human beings (Bhatia, 2002).

The increasing industrial and agricultural activities arising from man's sophisticated technology have introduced many synthetic and organic wastes into the aquatic environment (Ogbueibu and Ezenara, 2002) with pollution from land-based discharges, coastal industries, municipal outflows, agricultural activities, oil and gas industry (drilling and transportation) and transport activities, resulting in enormous chemical discharges into the aquatic environment (Jha, 2004; 2008). A large proportion of these compounds are potentially toxic, genotoxic and carcinogenic to aquatic organisms (Jha, 2004; 2008). The interaction of genotoxic contaminants with DNA causes various genetic disturbances, which often are irreversible and can be transmitted to the next generations (Depledge and Fossi, 1994; Dixon *et al*., 1999; Jha, 2004). The analysis of environmental samples for genotoxicity provides early warning signs of adverse long-term effects of contamination (Hafez, 2009).

In order to assess, monitor and maintain the health status of an aquatic environment, the aquatic ecosystem in question should be monitored biologically and chemically (Roux, 2001). The measurement of only physical and chemical water quality variables cannot provide an accurate

account of the overall condition of an aquatic ecosystem. Chemical monitoring alone is insufficient to detect, for example, the cumulative and/or synergistic effects of multiple stressors on aquatic ecosystems and biota. Effective management of aquatic ecosystems must therefore address the cumulative effect of various changes in the system including pollution (Roux, 2001).

In response to the need to protect both humans and the environment more effectively, an integrated approach to risk assessment that addresses real-life situations of multi-chemicals, multi-media, multi-route and multi-species exposures is of utmost importance. The UNEP/ILO/WHO, International Programme on Chemical Safety (IPCS), in collaboration with the U.S. Environmental Protection Agency (USEPA), European Commission and other international and national organizations, have developed a working partnership to foster the integration of assessment approaches to evaluate human health and ecological risks (Sekizawa and Tanabe, 2005).

Most chemical pollutants find their way into the environment via various products and processes. Once in the environment, they can persist for long periods of time or break down into other more or less toxic chemicals with their associated environmental risks (UNEP, 2003). They may also produce potential adverse effects on the environment and human health when they act together with other natural or manufactured chemicals that are already in the environment. Effective risk management for chemicals therefore depend on tracking the pathways, fate and exposure implications of chemicals, yet, data on such pathways, emissions, environmental fate and exposure for risk assessment are only available for very few chemicals and species (whether terrestrial or aquatic) (UNEP, 2002).

The state and health of fish communities have been reported to give a reliable indication of

short and long-term stress on aquatic systems (Adams *et al*., 1993; Allan and Flecker, 1993; Barbour *et al*., 1999). Fish communities possess various characteristics that render them important in the assessment of river health. Fish occupy positions throughout the aquatic food web, and are typically present in all but the most polluted of waters; because fish often range over considerable distances, and have the potential to integrate diverse aspects of relatively large-scale habitats (Karr *et al*., 1986). As such, fish can provide an integrative view of watershed conditions. Compared to other aquatic organisms, fish are relatively long-lived, and are therefore useful in providing a temporal dimension of aquatic environmental conditions and are also relatively easy to handle, identify, and after data is gathered, they can be released again into the environment (Karr *et al*., 1986).

The aquatic environment (ponds, lakes, rivers, streams, and oceans) and its resources are exceptionally valuable natural assets enjoyed by millions of people by providing citizens with generous long-term benefits in return for minimal care and protection. These benefits can be direct financial ones that provide employment, profit, and savings. For example, the seafood industry provides jobs for commercial fishers, wholesalers, and retailers; indirectly, the benefits of fish and aquatic ecosystems include recreational boating, sport fishing, swimming, relaxation, and natural beauty (Roux, 2001).

Appreciation of fisheries and aquatic systems has been accompanied by increasing concern about the effects of growing human populations and human activities on aquatic life and water quality. Forty percent of hazardous wastes on the Environmental Protection Agency's (EPA) National Priority List (NPL) are co-contaminated with pollutants that pose health hazards to humans and wildlife (Sekizawa and Tanabe 2005). Yousafzai and Shakoori (2011) reported derangement in liver architecture ranging from visible dermal and liver neoplasms, chronic degenerative inflammation of the internal organs, non-specific anaemia, aplasia or hypoplasia

of the RBC's and low leucocyte levels to serious pathological liver conditions, particularly shrinkage of liver cells which spread with advancing age, and also accompanied by the dissolution of the well organized liver structure, blood clots and necrosis in freshwater fish *Tur pittutora* sampled from river Kabul in Pakistan.

The environmental impact of pesticides is often greater than the target intended by those who use them. Over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, including non-target species, air, water, bottom sediments, and food (Miller and Miller, 2004). Pesticide-contaminated water may have undesirable effects in fish and other aquatic biota. Pesticide-surface runoff into rivers and streams can be highly lethal to aquatic life, sometimes killing all the fish in a particular stream (Toughill, 1999).

There are five potential routes for a pollutant to be incorporated into fish: via the food, non-food particles, gills, oral consumption of water and the skin. Once the pollutant is absorbed, it is transported by the blood either to a storage point (fat) or to the liver for transformation (Heath, 1991). If the pollutant is transformed by the liver, it may be stored there, excreted in the bile or passed back into the blood for possible excretion through the gills, kidneys or stored in fat which is an extra-hepatic tissue (Heath, 1991). Therefore, the concentrations found in different tissues after environmental exposure for a specific time depends on several dynamic processes all taking place concurrently. Changes taking place at the molecular level in fish can provide an early warning that pollution is reaching harmful levels (Heath, 1991).

1.2 Statement of Problem

The use of herbicides to control weeds has been recognized as a part of agricultural practice throughout the world. Unfortunately, the indiscriminate use of these herbicides to improve agricultural production and yield may have negative impacts on non-target organisms,

especially aquatic life forms and their environment. Herbicides widely used for the control of water plants may impede the flow of aquatic life and may contribute long term deleterious effects in the environment (Annune *et al*., 1994). The constant flow of agricultural effluents into fresh water often leads to a variety of pollutant accumulation, which becomes apparent when considering toxic pollution (Mason, 1991). Pesticide-surface runoff into rivers and streams can be highly lethal to aquatic life. Herbicides can accumulate in waterbodies at levels that kill zooplankton, the main source of food for young fish. Accidental spills and dumpsites also account for a part of the environmental pesticide input (Doherty *et al*., 2011). Pesticides are manufactured to be harmful to specific target organisms, or groups of organisms, and their toxic properties are essential to give the pesticides a satisfactory function (Akerblom, 2004). Due to pesticides' toxic properties, there is an obvious risk that non-target organisms are affected, either at the application site, or due to unintentional spreading, at nearby, or even distant areas (Akerblom, 2004). The indiscriminate use of herbicide, careless handling, accidental spillage or discharge of herbicide laden untreated effluents into natural water ways have harmful effects on the fish populations and other aquatic organisms and may contribute to short, as well as long term effects in the environment. Sublethal effects with biochemical and histopathological alteration of fish tissues may occur with long term exposure to high levels of herbicides (Neškovic *et al*., 1993).

The toxicity of some chemicals may also be enhanced or mitigated in the presence of other chemicals (Wilson, 2006). In addition to killing the organisms, some pesticides can have negative but non-lethal effects on individual organisms and populations, such as reduced reproduction, reduced mobility to escape predation, or alterations in behaviour (Wilson, 2006).

It is well established that pollution lowers the quality of life in various aspects and affects health and life span (Grover and Kaur, 1999). Besides the direct health effects, the subtle

danger of pollutants lies in the fact that they may be mutagenic (or toxic) and lead to several human diseases like cancer, atherosclerosis, cardiovascular diseases and premature ageing (Grover and Kaur, 1999).

Many environmental contaminants exert their effects via genotoxic and metabolically toxic mechanisms simultaneously causing carcinogenesis, embryotoxicity and teratogenic with long term alterations in organisms' body by being active through several generations (Jha *et al*., 2000). Different methods have been developed for the detection of both double- and single-strand breaks of DNA, DNA-adducts, micronuclei formation, and chromosome aberrations. *Alium cepa* assay and micronucleus (MN) assay are two of the most popular and promising assays in cytogenetic studies. The latter is a marker of cytogenetic damage usually caused by clastogenic or aneugenic compounds. The assessment of cytogenetic damage has been presented as a very important assay for identification of pollution hazards in marine environment (Dixon *et al*., 2002).

1.3 Justification

Pesticides at high concentration are known to reduce the survival, growth and reproduction of fish, and to produce many visible effects on fish (Rahman *et al*., 2002). Toxicity testing of chemicals on animals has been used for a long time to detect the potential hazards posed by chemicals to humans (Rahman *et al*., 2002). Bioassay technique has been the cornerstone of programmes on environmental health and chemical safety (Oshode *et al*., 2008). Aquatic bioassays are necessary in water pollution control to determine whether a potential toxicant is dangerous to aquatic life and to find the relationship between toxicant concentration and effect on aquatic animals (Olaifa *et al*., 2003). The application of environmental toxicology studies on non-mammalian vertebrates particularly fish is rapidly expanding, for the evaluation of the effects of environmental contamination by noxious compounds (Ayoola, 2008).

Fishes are the most useful bioindicators of environmental quality and fish erythrocytes are a potential biomarker for *in situ* monitoring of water quality of an aquatic ecosystem because of their close contact with water (De Flora *et al*., 1993). Genotoxic evaluation of pollutants in fish is of great concern because of their potential adverse effects on human health after consumption. There is also a great susceptibility of the liver of fish to be damaged as a result of its primary role of metabolism of these foreign substances. Liver damage alters lactate dehydrogenase and glutamic oxaloacetic transaminase activities which are two of the most important liver function test enzymes (Bigoniya *et al*., 2009). Thus toxicity tests or studies are essential for determining sensitivity of animals to toxicants and also useful for evaluating the degree of damage to target organs and the consequent physiological, biochemical and behavioral disorders. Thus, to supplement risk assessment studies of these herbicides, it is important to obtain information on their toxicity and effects on some local species.

Clarias gariepinus is a popular species in warm water aquaculture and it is indigenous to Africa. It is widely distributed and accepted by many farmers in Africa because of its fast growth, large size, low bone content, hardiness, high yield, tolerance to poor water quality, omnivorous feeding habit, fine flavour, adaptability to overcrowding, high market value and has been successfully propagated artificially thereby making its fry and fingerlings easily available (Osman *et al*., 2006).

For sustanaible fish production in Nigeria, the ecotoxicological monitoring programmes need to incorporate proper management programmes for pesticide use and disposal in aquatic habitat. This study was therefore aimed at determining if glyphosate and paraquat are toxic to juveniles of *Clarias gariepinus*.

1.4 Aim and Objectives

1.4.1 Aim:

The aim of this study was to determine the toxicity of glyphosate and paraquat to juveniles of *Clarias gariepinus*

1.4.2 Specific objectives

The specific objectives are:

(i) To determine the acute toxicity of glyphosate and paraquat on some behavioural parameters of *Clarias gariepinus*

(ii) To determine the 96-hr lethal concentration (LC_{50}) of glyphosate and paraquat to *Clarias gariepinus*

(iii) To determine the acute and sublethal toxic effects of glyphosate and paraquat on some biochemical and histological parameters of *Clarias gariepinus*

(iv) To determine the cytotoxic and genotoxic effects of acute and sublethal concentrations of glyphosate and paraquat on *Clarias gariepinus*

1.5 Statement of Research Hypotheses

(i) Ho**:** There is no effect between control and treated fish exposed to glyphosate and paraquat

(ii) Ho**:** There is no significant difference ($p \geq 0.05$) between behavioural parameters of control and exposed fish to acute concentrations of glyphosate and paraquat in 96hrs

(iii) Ho**:** There is no difference between the 96-hr lethal concentration (LC_{50}) of glyphosate and paraquat to *Clarias gariepinus*

(iv) Ho**:** There is no significant difference ($p \geq 0.05$) between biochemical parameters of control and exposed fish to acute and sublethal concentrations of glyphosate and paraquat in 96hrs

(v) Ho**:** There is no significant difference ($p \geq 0.05$) between the cytotoxic parameters of control and exposed fish to acute and sublethal concentrations of glyphosate and paraquat in 96hrs

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Pesticides

Pesticides are chemical compounds that are used to eradicate pests, including insects, rodents, fungi and unwanted plants (weeds) according to the World Health Organization (WHO, 1990). Pesticides are used in public health to eradicate vectors of disease, such as mosquitoes, and in agriculture, to kill pests that damage crops. There are human benefits to the use of pesticides though by their nature, they are potentially toxic to other non target organisms, including humans. As such, advocacy has centred on a safe use and proper disposal practices for pesticides (WHO, 1990). According to the Stockholm Convention on Persistent Organic Pollutants (2001), 9 of the 12 most dangerous and persistent organic chemicals are pesticides. Pesticides are categorized into four main substituent chemicals: herbicides; fungicides; insecticides and bactericides (Gilden *et al*., 2010). Many tonnes of pesticides are used annually in agriculture and horticulture. Consequently, wastewater from greenhouses and runoff from agricultural land are nearly always contaminated with pesticides (Schulz and Leiss, 1999). Due to their toxicity, pesticides affect the ecology of the receiving bodies of water and contaminate drinking water supplies (Fernández-Alba *et al*., 2000). Some pesticides bioaccumulate, affecting fish, birds, other animals and in human food sources (Fernández-Alba *et al*., 2000). Depending on the local cultivation practices, a water body may receive a single pesticide or a varying cocktail of compounds (Fernández-Alba *et al*., 2000). Toxicity of pesticide-contaminated effluent depends on the amounts and types of the individual pesticides present; however, even for pure compounds, concentration–toxicity relationships are generally nonlinear. Cocktails of compounds pose bigger problems because toxicity of a mixture is not easily linked to individual toxicities of components in the mixture (Readman *et al*., 1992).

Pesticides are included in a broad range of organic micro pollutants that have negative ecological impacts (Dhakal, 2012). Different categories of pesticides have different types of effects on living organisms; therefore generalization is difficult (Dhakal, 2012). Although terrestrial impacts by pesticides do occur, the principal pathway that causes ecological impacts is that of water contaminated by pesticide runoff. The two principal mechanisms are bioconcentration and biomagnification (Sivaperumal and Sankar, 2013). Bioconcentration is the movement of a chemical from the surrounding medium into an organism. The primary "sink" for some pesticides is fatty tissue ("lipids"). Some pesticides, such as DDT, are "lipophilic", meaning that they are soluble in, and accumulate in, fatty tissue such as edible fish tissue and human fatty tissue. Other pesticides such as glyphosate are metabolized and excreted. Biomagnification is the term that describes the increasing concentration of a chemical as food energy is transformed within the food chain (Jonsson *et al*., 1990; Torstensson, 1990). As smaller organisms are eaten by larger organisms, the concentration of pesticides and other chemicals are increasingly magnified in tissue and other organs. Very high concentrations can therefore be observed in top predators, including humans (Sivaperumal and Sankar, 2013).

The ecological effects of pesticides (and other organic contaminants) are varied and are often inter-related (Torstensson, 1990). Effects on the organism or at the ecological level are usually considered to be an early warning indicator of potential human health impacts. Such effects vary depending on the organism under investigation and the type of pesticide used. Different pesticides have markedly different effects on aquatic life which makes generalization very difficult (Dhakal, 2012). The important point is that many of these effects are chronic (not lethal), are often not noticed by casual observers, yet have consequences for the entire food chain – ranging from death of the organism, cancers, tumours and lesions on fish and animals, reproductive inhibition or failure, suppression of immune system and disruption of endocrine (hormonal) system. In addition, other effects include - cellular and DNA damage, teratogenic

effects (physical deformities such as hooked beaks on birds), poor fish health marked by low red to white blood cell ratio, excessive slime on fish scales and gills and intergenerational effects (not apparent until subsequent generations of the organism) (Dhakal, 2012). Other physiological effects such as egg shell thinning were also observed as major effects of pesticides (Torstensson, 1990). These effects are not necessarily caused solely by exposure to pesticides or other organic contaminants, but may be associated with a combination of environmental stresses such as eutrophication and pathogens. These associated stresses need not be large to have a synergistic effect with organic micro pollutants. ((Torstensson, 1990))

2.1.1 Environmental fate of pesticides

When a pesticide is introduced into the environment by application, dispersal or a spill, it is influenced by many processes. These processes determine a pesticide's persistence, movement and ultimately, its fate (Kearney *et al*., 1977). The fate can have both positive and negative influences on a pesticide's effectiveness or its impact on the environment (Navarro *et al*., 2007). They can move a pesticide to the target area or destroy its potentially harmful residues. They can be detrimental, leading to reduced control of a target pest, injury to non-target plants and animals, and environmental damage (Navarro *et al*., 2007). Different soil and climatic factors and different handling practices can prevent or promote each of these processes. An understanding of the fate processes can ensure that applications are not only effective, but are also environmentally safe. Fate processes could be categorized into four major groups: degradation, transfer, adsorption and bioaccumulation/biomagnification. These physical and chemical processes of pesticides determine their environmental risk (Navarro *et al*., 2007).

Pesticide degradation refers to the breakdown of pesticides in the environment. The rate at which degradation occurs is measured by the pesticides half-life. A pesticide with a long half-life is described as persistent. Persistence is good for long-term pest control but it is also

undesirable because the pesticide can cause environmental damage after a long time period (Williams, 2011). Pesticide degradation is usually beneficial as pesticide-destroying reactions change most pesticide residues in the environment to non-toxic or harmless compounds. However, degradation is detrimental when a pesticide is destroyed before the target pest can be controlled. The rate of pesticide degradation is affected by many environmental factors including temperature, moisture and pH. The three types of pesticide degradation are microbial, chemical and photo degradation (Williams, 2011).

Pesticide transfer is sometimes essential for pest control as some pesticides need to circulate for effective utilization. The mechanism for dispersion of a pesticide could result in movement away from a target pest, leading to reduced pest control, contamination of surface and groundwater and injury to non-target species including humans (Dustin, 2011). Pesticides can be transferred through natural processes such as volatilization, run-off, leaching, adsorption and crop removal. Volatilization is the conversion of a solid or liquid to gas. Once volatilized, a pesticide can move in air currents away from the treated surface. Vapour pressure is an important factor in determining pesticide volatility. The higher the vapour pressure, the more volatile the pesticide is. Environmental factors such as high temperature, low relative humidity and air movement tend to increase volatilization. A pesticide tightly adsorbed to soil particles is less likely to volatilize (Dustin, 2011). Soil conditions such as texture, organic matter content and moisture content can thus influence pesticide volatilization. Volatilization can result in reduced control of the target pest because less pesticide remains at the target site. Vapour drift, the movement of vapour or gases in the atmosphere can lead to injury of non-target species. (Dustin, 2011).

Adsorption is the movement of pesticides into plants and animals or structures such as soil and wood. Absorption of pesticides by target and non-target organisms is influenced by

environmental conditions, physical and chemical properties of the pesticide and the soil (Ikpesu and Ariyo, 2013). Once absorbed by plants, pesticides may be broken down or remain in the plant until tissue decay or harvest. Similarly, desorption is the release of pesticides from soil, wood or other substances (Ikpesu and Ariyo, 2013). Crop removal transfers pesticides and their breakdown products from the treatment site to areas far away from the application site and sometimes end up in the aquatic environment. Most harvested food varieties are subjected to washing and processing procedures that remove or degrade much of the remaining pesticide residue. Pesticides can be transferred during such operations as tree and shrub pruning and turf grass mowing. Pesticide adsorption is the binding of pesticides to soil particles and organic matter. Positively charged pesticide molecules, for example, are attracted and bound to negatively charged clay particles. The amount of adsorption in the soil depends on the type of the soil, the conditions (temperature, pH, moisture content etc) and the characteristics of the pesticide (Ikpesu and Ariyo, 2013).

Bioaccumulation is the ability of some chemicals to build up in the body tissue of animals (Advaiti *et al*., 2013). Pesticide build up can cause long term damage or death. It can also build up in the food chain – a process called biomagnification. Biomagnification result in much greater exposure in organisms at the top of the food chain (EXTONET, 1993). Persistent pesticides in food chains for example organochlorine pesticides such as dichlorodiphenyltrichloroethane were banned from agricultural use due its ability to persist in the environment. Bioaccumulation and bio magnification also occur in aquatic systems. Fishes for example are affected when their water habitat or food sources are contaminated. The extent of damage to fish depends not only on the properties of the pesticide but also on the species of fish, its age, size and its position in the food chain (van der Oost *et al*., 2003).

2.2 Herbicides

A herbicide is a pesticide, described by The United States Environmental Protection Agency (USEPA) as an agent used to prevent, destroy, repel or mitigate any pest ranging from insects, animals and weeds to microorganisms such as fungi, molds, bacteria and viruses. A herbicide, then, is any compound capable of killing or severely injuring plants and may be used for the elimination of plant growth or the killing off of plant parts (Amdur *et al*., 1991).

2.2.1 Herbicide classification systems

Herbicides can be classified in several ways such as usage, translocation patterns, mode of action, toxicology and chemical structure. Systemic herbicides are translocated to sites that use high amount of energy such as the root and shoot growing points and reproductive structures. Herbicides are translocated in the xylem (apoplast) or phloem (symplast). Apoplasm is the non-living portion of the plant cell walls and xylem. Herbicides applied to foliage can move into plants through cracks in the leaf, open stomata and leaf cuticle. (DiTomaso, 2000).

2.2.1.1 Toxicology

Several toxicological tests can be conducted on each herbicide. From these tests, a variety of hazard indicator values are derived. The most commonly used of these include an Oral LD_{50}, Inhalation LC_{50} and Dermal LD_{50}. A LD_{50} or LC_{50} value is the lethal dose or concentration of a herbicide which will kill 50% of the test animals based on mg of chemical per kg of body weight. These values are used to classify herbicides and specific formulations into toxicity categories. The signal words “DANGER”, “WARNING”, or “CAUTION” normally appear on labels of herbicides, depending upon their toxicity profile. Changing laws, rules, and regulations, as well as new trends in herbicide development over the past couple of decades have led to a higher percentage of registered herbicides with a CAUTION Signal Word (least toxic) compared to herbicides available in 1970 or earlier (DiTomaso, 2010).

2.2.1.2 Chemistry

Like plants, insects and animals, chemicals share various properties that enable them to be classified into specific families or classes. In most cases, herbicides in the same chemical family will inhibit plant growth by similar mechanisms. This is not always the case, however. There are examples of insecticides, fungicides, and other pesticides that belong to the same family as herbicides. This is because they share similar important chemical characteristics. Some herbicides are unique and are, therefore, considered to be either unclassified or in their own family. Occasionally a quality of a family may make it easy to visually recognize. The dinitroanilines are always yellow or orange in color, both as solid and liquid forms (DiTomaso, 2010).

2.2.1.3 Mode of action

All herbicides exert their actions in one or more of the following ways - Plant-growth regulators, Photosynthetic inhibitors, Bleaching Agents (Pigment Inhibitors), Lipid Synthesis Inhibitors, Cell Wall Synthesis Inhibitors, Cell Division Inhibitors, Amino acid Inhibitors, EPSP Inhibitors, Energy-Production Inhibitors, Cell Membrane Destructors, General Cell Toxicants and Herbicides with Unknown Mode of Action (DiTomaso, 2010).

2.3 Glyphosate

Glyphosate-based herbicides are among the most widely used broad-spectrum herbicides in the world because they are highly efficacious, cost effective, relatively non-toxic, and degrade readily in the environment (Giesy *et al*., 2000; Williams *et al*., 2000). Glyphosate is soluble in water and tends to bind tightly to sediment, suspended particulates, organic matter and soil, becoming essentially unavailable to plants or other aquatic organisms. Glyphosate does not bioaccumulate in terrestrial or aquatic animals (Giesy *et al*., 2000; Williams *et al*., 2000). Herbicidal effects are therefore limited to foliar contact, cut stump or stem injected applications

on plants. Glyphosate rapidly dissipates from surface waters, and soil microflora quickly biodegrade glyphosate into AMPA and CO_2 (Gardner and Grue, 1996). Formulations of glyphosate including Rodeo®, Roundup®, and Aquamaster® have been extensively investigated for their potential to produce adverse effects in non-target organisms. Governmental regulatory agencies, international organizations, and others have reviewed and assessed the available scientific data for glyphosate formulations and independently judged them to be of minimal risk to the environment (Agriculture Canada 1991; USEPA 1993; WHO 1994).

Since glyphosate's development in the 1970's, there have been no documented cases of adverse effects on fish or aquatic invertebrates associated with its use for the control of aquatic weeds (Giesy *et al*., 2000). No measurable increases in effects on density, abundance, or survival of aquatic invertebrates have been reported from the direct effects of glyphosate in field studies (Gardner and Grue, 1996; Simenstad *et al*., 1996; Linz *et al*., 1999). The International Union of Pure and Applied Chemistry (IUPAC) nomenclature for glyphosate is N-(phosphonomethyl) glycine and the Chemical Abstracts Service (CAS) registry number is 1071-83-6 (Tomlin, 2006). Its empirical formula is $C_3H_8NO_5P$ (Fig 2.1a). It is a widely used herbicide for 3 main reasons:

1) The target site is meristematic tissue, 2) Its ability to translocate in plants and 3) The inability of plants to rapidly detoxify it. Formulations of glyphosate include an acid, monoammonium salt, diammonium salt, isopropylamine salt, potassium salt, sodium salt, and trimethylsulfonium or trimesium salt (Tomlin, 2006; Vencill, 2002). Technical grade glyphosate is used in formulated products, as are the isopropylamine, sodium, and monoammonium salts. Of these, the isopropylamine salt is most commonly used in formulated products.

2.3.1 Glyphosate mode of action

In order for glyphosate to act in the plant it has to be taken up by the aerial parts, leaves and stems i.e. translocated throughout the plant (Tomlin, 2006; Roberts, 1998). It is then transported from the place of uptake to its site of action - the meristem tissue (Franz *et al*., 1997). Uptake is crucial, and unfortunately, glyphosate is not the easiest molecule to be readily taken up by plants. This makes glyphosate prone to being washed off from the plant by rainfall within four to six hours after spraying. The amount of applied glyphosate to plants not washed from the leaves is considered the effective amount. The efficacy of glyphosate is dependent upon the concentration in the plant, or more precisely at the meristem which is the site of action of the compound. Glyphosate is a systemic herbicide, which means that it moves from place of uptake to the site of action in the actively growing tissues. Plants exposed to glyphosate display stunted growth, loss of green coloration, leaf wrinkling or malformation, and tissue death and eventually plant death which may take from 4 to 20 days to occur (Vencill, 2002; Franz *et al*., 1997).

Glyphosate kills plants by inhibiting the activity of the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSP). EPSPS is a key enzyme in the shikimate biosynthetic pathway which is necessary for the production of the aromatic amino acids- tyrosine, tryptophan, and phenylalanine. These amino acids are important in the synthesis of proteins that link primary and secondary metabolism (Carlisle and Trevors, 1988). It also catalyses the production of auxin, phytoalexins, folic acid, lignin, plastoquinones and many other secondary products. Over 30% of the carbon fixed by plants passes through this pathway. Inhibition of EPSPS by glyphosate deregulates the pathway, leading to even more carbon flowing through the pathway with accumulation of shikimate and shikimate-3-phosphate. Glyphosate occupies the binding site on EPSPS for phosphoenol pyruvate, a substrate of EPSPS, by mimicking an intermediate state of the enzyme-substrates complex (Shaner, 2006). There are two forms of EPSPS in

nature, EPSPS I, which is found in plants, fungi, and most bacteria, and is sensitive to glyphosate, and EPSP II, which is found in glyphosate resistant bacteria and is not inhibited by glyphosate. It is the gene for an EPSPS II that has been used to genetically engineer resistance in crops. The shikimate pathway is most active in meristematic tissue. Hence, glyphosate has to translocate to the meristematic tissue to be effective. Glyphosate translocates in the plant from a source to sink direction. Up to 70% of absorbed glyphosate can translocate out of the treated leaves to the root and shoot apices. However, glyphosate translocation is self-limiting and only occurs for the first 48-72 h after application. The reason for this self-limiting phenomenon is not clear, but is related to the site of action of the herbicide, since there is greater translocation in glyphosate resistant crops compared to susceptible plants. Glyphosate's ability to translocate readily in plants results in it controlling not only annual, but also perennial weeds. EPSPs are present in the chloroplast of most plant species, but are not present in animals (Sprankle *et al*., 1975). Animals need tyrosine, tryptophan, and phenylalanine, but obtain them by eating plants or other animals. Glyphosate is therefore, relatively non-toxic to animals. Certain surfactants or other ingredients that are added to some glyphosate formulations are toxic to fish and other aquatic species (EXTONET, 1996). It can also act as a competitive inhibitor of phosphoenolpyruvate (PEP), which is one of the precursors to aromatic amino acid synthesis. It also affects other biochemical processes, and, although these effects are considered secondary, they may be important in the total lethal action of glyphosate (Steinrücken and Amrhein, 1980; Bode *et al*., 1984a; Rubin *et al*., 1984).

2.3.2 Environmental fate of glyphosate:

Glyphosate is moderately persistent in soil, with an estimated half-life of 47 days. Reported field half-lives range from 1 to 174 days (Wauchope *et al*., 1992). It is strongly adsorbed to most soils, even those with lower organic and clay content. Thus, even though it is highly

soluble in water, field and laboratory studies show it does not leach appreciably, and has low potential for runoff (except as adsorbed to colloidal matter) (Wauchope *et al*., 1992). One estimate indicated that less than 2% of the applied chemical is lost to runoff (Wauchope *et al*., 1992). Microbes are primarily responsible for the breakdown of the product, and volatilization or photodegradation losses will be negligible. In water, glyphosate is strongly adsorbed to suspended organic and mineral matter and is broken down primarily by microorganisms. Its half-life in pond water ranges from 12 days to 10 weeks (Wauchope *et al*., 1992).

2.4 Paraquat

Paraquat was introduced in 1962 for agricultural use, but was originally synthesized by Weidel and Russo in 1882 (WHO, 1984). Paraquat is a quaternary ammonium salt which does not occur naturally. There are a number of methods available for the synthesis of paraquat dichloride; the most common method used is the treatment of 4,4'-bipyridine with an excess of methyl chloride . The reaction begins with the formation of free radicals at the four positions of the pyridine rings. Two rings are coupled at the four positions to form the bipyridyl anion. This is then oxidized with two moles of methyl chloride to create paraquat dichloride (WHO, 1984). This method yields an aqueous solution of the herbicide with greater than 95% purity (Summers, 1980).

Paraquat (1,1-dimethyl-4,4-bipyridinium dichloride) also known as methyl viologen is an important member of bipyridylium family of non-selective herbicide developed by plant protection division of Imperial Chemical Company in 1958 and first marketed for agricultural purposes in the United Kingdom in 1962. Its empirical formula is $C_{12}H_{14}Cl_2N_2$ (fig 2.1b). It is a herbicide widely used for broadleaf weed control (Chia *et al*., 1982). Paraquat (PQ) is probably the most effective herbicide that exists and one of the world's worst poisons (Suntres, 2002).

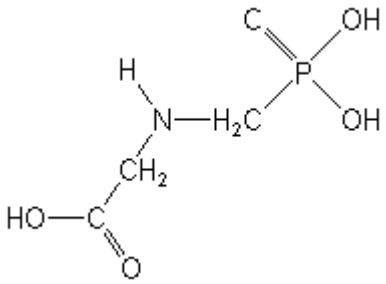


Figure 2.1a**:** Chemical structure of Glyphosate

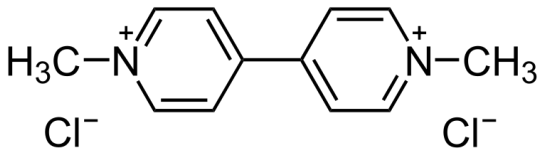


Figure 2.2b**:** Chemical structure of Paraquat

Paraquat dichloride is one of the most widely used herbicides in the world (Suntres, 2002). It is quick-acting and non selective, killing green plant tissue on contact and becomes biologically inactive when it comes in contact with soil and rain within minutes of application. Paraquat is moderately to highly toxic to many species of aquatic life including rainbow trout, bluegill, and channel catfish. Its toxicity to fish varies with the species, size, and the softness or hardness of water (Madeley, 2002). At high levels, paraquat inhibits the photosynthesis of some algae in stream water (Kenneth, 1990). It is also toxic to human beings when swallowed. Paraquat applied to water for aquatic weed control purposes quickly disappears due to uptake by weeds and absorption by soil, silt and particulate suspended matter. However, some quantities of this herbicide end up in the body of fish and other aquatic life.

Repeated exposure to sub-lethal doses of some pesticides can cause physiological and behavioral changes in fish that reduce populations, such as abandonment of nests and broods, decreased immunity to disease, and increased failure to avoid predators (Helfrich *et al*., 1996).

2.4.1 Paraquat mode of action

Paraquat reacts as an electron acceptor in radical reactions. As a herbicide, paraquat acts by inhibiting photosynthesis. In light-exposed plants, it accepts electrons from photosystem I (more specifically Fd, which is presented with electrons from PS I) and transfers them to molecular oxygen. In this manner, destructive reactive oxygen species are produced. In forming these reactive oxygen species, an oxidized form of paraquat is regenerated, and is again available to shunt electrons from photosystem I to start the cycle again (Summers, 1980).

Paraquat is a non-selective, contact herbicide that is absorbed by the foliage. It destroys plant tissue by disrupting photosynthesis and rupturing cell membranes, which allows water to escape leading to rapid desiccation of foliage (Dinis-Olivera *et al*., 2006). It can also be translocated within the plant, increasing the likelihood of residues. Paraquat, originating from

agricultural activity enters the aquatic environment through atmospheric deposition, surface run-off or leaching and frequently accumulates in soft-bottom sediments and aquatic organisms (Fryer *et al*., 1975). Paraquat is often used to catalyze the formation of reactive oxygen species (ROS), more specifically, the superoxide free radical (Dinis-Olivera *et al*., 2006). Paraquat undergoes redox cycling *in vivo*, being reduced by an electron donor such as "NADPH", before being oxidized by an electron receptor such as dioxygen to produce superoxide, a major ROS.

2.4.2 Environmental distribution, transformation and effects of paraquat

Paraquat deposits on plant surfaces undergo photochemical degradation into compounds that have a lower order of toxicity than the parent compound. On reaching the soil, paraquat becomes rapidly and strongly adsorbed to the clay minerals present. This process inactivates the herbicidal activity of the compound (Zain, 2007). While free paraquat is degraded by a range of soil microorganisms, degradation of strongly-adsorbed paraquat is relatively slow. In long-term studies, degradation rates were 5-10% per year (Zain, 2007).

Strongly bound paraquat has no adverse effects on soil microfauna or soil microbial process. Paraquat residues disappear rapidly from water by adsorption on aquatic weeds and by strong adsorption to the bottom mud (Zain, 2007). Normal applications of paraquat for aquatic weed control are not harmful to aquatic organisms. However, care should be taken when applying paraquat to water containing heavy weed growth to treat only a part of the growth, since oxygen consumed by subsequent weed decay may decrease dissolved oxygen levels to an extent that may be dangerous for fish (Zain, 2007).

Acute effects of paraquat on fish include abnormal stress behaviour such as excessive gulping of air, erratic swimming, restlessness, loss of movement, loss of equilibrium, increased beating of the gill operculum, excessive secretion of mucus, swimming on the back, and paralysis

(Omitoyin *et al*., 2006). Sublethal effects on fish include adverse effects on the immune system, with the effect enhanced by elevated temperatures within the environment (Salazar-Lugo *et al*., 2009); alterations to gonads likely to affect reproductive activity particularly in males (Figueiredo-Fernandes *et al*., 2006); and oxidative stress (Stephensen *et al*., 2002). Moreso, oxidative stress occurs when the production of 'reactive oxygen species', such as free radicals and hydrogen peroxide, exceeds the body's ability to neutralize and eliminate them, overwhelming antioxidant defenses such as glutathione, and resulting in DNA damage, cell and tissue death (Mc Cord, 2000). Oxidative stress is involved in many human diseases including Parkinson's disease, cancer, Alzheimer's disease, diabetes, and heart failure (Stefanis et al., 1997). Commonly used measures of the extent of oxidative stress in laboratory studies are the levels of glutathione and associated enzymes of the antioxidant system such as glutathione reductase, glucose-6-phosphate dehydrogenase, glutathione-S-transferase (GST), glutathione peroxidise (GPx), catalase (CAT) and superoxide dismutase (SOD). Alterations in the components of the antioxidant system can be used as biomarkers for paraquat poisoning (Ray *et al*., 2007).

2.5 Histopathological Effects of Herbicides in Major Fish Organs

Histopathological alterations can be used as indicators of the effects of various anthropogenic pollutants on organisms and are a reflection of an overall health of the entire population in the ecosystem (Velkova-Jordanoska and Kostoski, 2005). These histological changes are closely related to other biomarkers of stress since many pollutants have to undergo metabolic activation in order to be able to provoke cellular change in the affected organism. For example, the mechanism of action of several xenobiotics could initiate the formation of a specific enzyme that causes changes in metabolism, further leading to cellular intoxication and death at a cellular level, manifesting as necrosis, at the tissue level (Velkova-Jordanoska and Kostoski, 2005). Toxic chemicals cause tissue damage and histophathological degradations as the fish

show haematological responses to toxicants. Generally, such degradation of histological origin occurs in the gills, liver, heart, kidney and epidermis of animals (Velkova-Jordanoska and Kostoski, 2005).

Histopathology is a higher-level response, reflecting prior alteration in physiological and/or biochemical function (Hilton *et al*., 1992). The liver is exposed to toxic substances because it is the organ in charge of transforming them (Boeger *et al*., 2003). Previous studies of *in vitro* chronic exposure of rainbow trout gill and liver cell lines showed a reduction in cell membrane integrity, mitochondrial activity and lysosomal function at high levels of extracted naphthenic acids (100mg/L) (Lee *et al*., 2000).

Cellular swelling occurs either directly by denaturation of volume-regulating ATPases or indirectly by disruption of cellular energy transfer processes required for ionic regulation (Hinton and Lauren, 1990). Authors also reported that vacuolation of hepatocytes are associated with the inhibition of protein synthesis, energy depletion, disaggregation of microtubules or shifts in substrate utilization. Hyalinization is the result of disturbances of protein synthesis and protein inclusion bodies are common in metal toxicities (Cheville, 1994).

van den Heuvel *et al*. (2000), reported that an increased prevalence of skin pathologies (caudal fin erosion) and proliferative changes (epithelial and chloride cells) to the gills of yellow perch were associated with long-term exposure (3-10 months) to reclaimed sites containing elevated levels of oil-sands constituents (Naphthenic acids [NA] and polycyclic aromatic hydrocarbons [PAHs]). The authors also observed that exposed yellow perch had increased liver size and hepatic mixed-function oxygenase (MFO) activity. Mucous cell proliferation of yellow perch from oil sands reclaimed environments following longer residency periods was also observed (Van den Heuvel *et al*., 2000).

Olojo *et al*. (2005) investigated the effect of sub-lethal concentration of heavy metal lead (Pb) on the gill and liver of *Clarias gariepinus* and reported a dose and time dependent degeneration of both organs. The cyto-architectural distortions in the gill include: overlapping of primary and secondary lamella, occlusion of inter-lamella spaces, thin core and shrinkage of cartilaginous supporting mass resulting in decrease in size of gills. At higher concentrations, the epithelium was completely disrupted owing to the lysis of the cells.

Cengiz *et al*. (2001) reported the following histopathological alterations in the liver of mosquito fish (*Gambusia affinis*) after exposure to sub-lethal concentrations of endosulfan: hypertrophy, sinusoid enlargement, haemorrhage, pyknosis, vacuolization of cell cytoplasm, infiltration of mononuclear lymphocytes and congestion, and these alterations were time and dose dependent. Similarly Edson *et al*. (2001) reported that after acute (24hr) exposure of juveniles of *Prochilodus lineatus* to organophosphate pesticide, Dipterex 500 (R) (Trichlorfon), the hepatocytes became tumified with vacuolation. Additional alterations were cytoplasmic granulation, nuclear lateralization and the nuclei had various diameters and densities and condensed chromatin in the central region with pyknosis.

Denton *et al*. (2005) exposed larval fathead minnows (*Pimephales promelas*) to acute concentrations of diazinon and esfenvalerate singly and in combinations and reported liver glycogen depletion as the only histopathological effect.

Olurin *et al*. (2006) exposed *Clarias gariepinus* fingerlings to sub lethal concentrations of the herbicide, glyphosate over a 42 day period. After the exposure period, the gills showed marked alterations in the epithelia in response to glyphosate treatment. There was fusion in adjacent secondary lamellae resulting in hyperplasia, with profound oedematous changes, characterised by epithelial detachment. In the liver, the enlargement of the hepatocytes was related to the

concentration and duration of exposure to glyphosate. There were also large vacuoles in the hepatocytes, with pyknotic nuclei, and cytolysis that increased with concentration. Focal necrosis was also observed in the hepatocytes. It was concluded that glyphosate had a deleterious effect on the organs of *C. gariepinus*.

Van Dyk *et al*. (2007) reported that sublethal levels of metal mixtures of cadmium and zinc resulted in hyalinization of hepatocytes, increased vacuolation associated with lipid accumulation, congestion of blood vessels and cellular swelling in the liver of *Oreochromis mossambicus* after short and long term exposure.

Atif *et al*. (2009) investigated the bioaccumulation of some heavy metals and examined histopathological alterations in the liver of *Oreochromis niloticus* at different localities along the River Nile in Egypt. The histological study indicated that the liver of *O. niloticus* living in the studied stations showed several pathological alterations such as degeneration of hepatocytes, fatty degeneration, necrosis and edema, congestion, branching (anastimosis), haemorrhage, haemolysis and haemosiderin.

In a recent study by Fatma (2009) on *Tilapia zillii* and *Solea vulgaris* from Lake Qarun, in Egypt, the liver showed vacuolar degeneration of the hepatocytes, focal areas of necrosis and fibrosis, aggregations of inflammatory cells between the hepatocytes, dilation and congestion in blood sinusoids and thrombosis formation in the central veins. In the gills, pathological alterations included proliferative, degenerative and necrotic changes in the epithelium of gill filaments and secondary lamellae, oedema in secondary lamellae, dilation and congestion in blood vessels of gill filaments and mucous cell proliferation.

Ogundiran *et al*. (2010) studied the toxicological impact of detergent effluents on African

catfish and reported that the liver of the control fish showed normal parenchyma appearance of hepatocyte with normal staining patterns of the cell. In the treated fishes, there was congestion of central vein, vacuolation of hepatocytes, oedema, cellular infiltration and cellular necrosis.

In a 96hr exposure of *Clarias gariepinus* to lethal concentrations of paraquat dichloride by Doherty *et al*. (2011), histopathology of the gills after 96 hr period revealed cell proliferation, lamellar fusion, lamellar cell hyperplasia, and epithelial lifting. In the liver, there was vacuolation of hepatocytes and necrosis. The histological alterations observed in gills and liver of *Corydoras paleatus* after exposure to sub-lethal doses of the organophosphate methyl parathion included: epithelial hyperplasia in the respiratory lamellae, edema and detachment of the epithelial cells. The liver exhibited cloudy swelling, bile stagnation, focal necrosis, atrophy and vacuolization (Fanta *et al*., 2003).

2.6 Generation of Reactive Oxygen Species in fish exposed to herbicides and effects on Biochemical Parameters

2.6.1 Reactive oxygen species

Oxidative stress, a pathological process relating to over-production of reactive oxygen species (ROS) in tissues is one important general toxicity mechanism for many xenobiotics. Oxidative stress was shown to be induced by anthropogenic contaminants such as persistent organic pollutants (POPs), heavy metals, and also by toxins produced during massive blooms of cyanobacteria (Ding *et al*., 1998; van der Oost *et al*., 2003). Many organisms including fishes have evolved mechanisms to counteract the impact of ROS. These include various antioxidant defense enzymes such as superoxide dimutase, catalase and glutathione S- transferase.

Many water contaminants can stimulate the production of ROS and result in oxidative damage to aquatic organisms (Sturve *et al*., 2008). Under normal conditions, ROS and other pro-

oxidants are continually detoxified and removed in cells by antioxidant systems (Li *et al*., 2009). Some of the most important antioxidant enzymes are superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase (Li *et al*., 2010a, d). These systems can prevent the formation of ROS, which can react with susceptible biological macromolecules and produce lipid peroxidation, resulting in oxidative stress (Zhang *et al*., 2008; Li *et al*., 2010b). Many antioxidant responses, including oxidative stress biomarkers and antioxidant enzyme activities, are used in environmental risk assessment (Song *et al*., 2006).

Antioxidant enzymes of fishes, that play a crucial role in maintaining cell homeostasis, have received much attention in ecotoxicology since oxidative damage was considered a mechanism of toxicity in aquatic organisms exposed to environmental contaminants in general (Santos *et al*., 2004).

Studies carried out on various fish species have revealed that heavy metals may alter biochemical parameters both in tissues and in the blood (Usha, 2000; Ashraf, 2006). Aerobic organisms generate reactive oxygen species (ROS), such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), because of oxidative metabolism. The hydroxyl radicals can initiate lipid peroxidation (LPO) in tissues. To attenuate the negative effects of ROS, fish possess an antioxidant defense system like other vertebrates that utilizes enzymatic and non-enzymatic mechanisms. The most important antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione-S-transferase (GST). The non-enzymatic defense system includes Vitamins E, C, and A, glutathione, carotenes and ubiquinol10 (Filho, 1996).

There is substantial evidence that glutathione reductase (GSH) is a powerful antioxidant as it binds to many different toxicants, inactivating them (Kelly *et al*., 1998). The nonoxidant

fluoride ions cause oxidative stress indirectly leading to an increase in lipid peroxide levels (Anuradha *et al*., 2000). Chronic exposure to Selenite has been shown to deplete GSH and decrease the ratio of GSH to oxidized GSSG in rainbow trout fed Selenite-methionine in the laboratory (Holm, 2002). Dorval *et al*. (2005) reported that GSH reserves also decreased with exposure to sublethal endosulfan. Lipid peroxidation may decrease with increased Selenite exposure because Selenite is a constituent of antioxidants glutathione peroxidise and thioredoxin reductase (Steinbrenner *et al*., 2006) and at lower levels protects fish from oxidative damage. Yuanyual *et al*. (2006) investigated the biochemical processes in liver of freshwater gold fish *Carassius auratus*. It was shown that the hepatic antioxidant defense parameters of goldfish, including the contents of reduced glutathione (GSH) and activities of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) were highly sensitive to HC Orange No 1 exposure, accompanied by changes of HC Orange No 1 accumulation in the liver. Changes of these parameters indicated that there was an increase in the production of oxyradicals by goldfish and the presence of oxidative stress. With these findings, the authors were of the opinion that glutathione as well as other antioxidant enzymes function in the protection against HC Orange No 1 toxicity and that these antioxidants provide a first line of defense against HC Orange No 1, before the induction of any other detoxification mechanism.

Yaunyuan *et al*. (2006) also reported that the activities of hepatic SOD were significantly induced at 2, 4 and 7 days and then were inhibited gradually at 10 and 16 days after exposure to HC Orange No 1. The authors said induction of SOD could occur during high production of superoxide anion radicals. Therefore an increase in SOD activity indicates that there is O_2^- generation and generation of O_2^- within the SOD elimination capacity. However when superoxide anion radical (O_2^-) generation increases to an extent that exceeds the function of

SOD elimination, O_2^- as well as other oxyradicals can inversely insult the enzyme to make it inactivated. This means a severe oxidative stress may suppress SOD activity due to loss of adaptive mechanisms. GSH, as an oxyradical scavenger is important in the antioxidant defense. GSH also act as a reactant in conjugation with electrophilic substances. Thus a change in GSH level may be a very important indicator of the detoxification ability of an organism. GSH is often the first line of defense against oxidative stress. Variations in GSH levels under laboratory exposure to a variety of contaminants have been observed and responses were variable for different species of goldfish and contaminants. A decrease of hepatic GSH content was found in *Carassius auratus* exposed to 2, 4-dichlorophenol for 40 days (Zhang *et al*., 2004).

Miller *et al*. (2007) reported a significant decrease in the hepatic oxidative stress indicators; hepatic gluthathione levels, lipid peroxidation levels and glutathione peroxidise activities when rainbow trout was exposed to selenite for 96 hours.

Antioxidants protect an organism against oxyradical damage, such as DNA strand breaks, protein oxidation and the induction of lipid peroxidation (Winzer *et al*., 2000). The increase in the activated form of molecular oxygen species due to overproduction and/or to the inability to destroy them may lead to damage in the DNA structure and thus may cause mutations, chromosomal aberrations, and carcinogenesis. A shift to a more oxidative state or any imbalance between production and degradation of reactive oxygen species (ROS) in animal tissues may cause lipid peroxidation, plasma membrane alterations, and inactivation of enzymes (Anand *et al*., 2000). The use of the biochemical approach has been advocated to provide an early warning of potentially damaging changes in stressed fish. The use of oxidative stress biomarkers for stress evaluation have rapidly increased in the field of ecotoxicology, therefore, it has been suggested that they could be used in environmental monitoring systems (Pandey *et al*., 2003).

2.6.2 Alteration of biochemical parameters

Biochemical characteristics of blood are among the important indices of the status of internal environment of the fish organism (Edsall, 1999). Glutamate oxaloacetate (AST) and glutamate pyruvate (ALT) transaminases are enzymes frequently used in the diagnosis of damage caused by pollutants in various tissues such as the liver, muscle and gills (de la Torre *et al.*, 1999). Of these two enzymes, ALT is specific for liver damage because it predominates in organs with intensive glycogenesis (Ulrich, 1994; Torre *et al* 2000). AST is present in myocardium, skeletal muscle, kidney and brain and increased plasma activity may be indicative of degenerative changes in these organs. Increased plasma ALT and AST activities are a measurable biochemical symptom of cytolysis as aminotransferases play vital roles in carbohydrate-protein metabolism in fish (Eze, 1983). They occupy a central position in amino acid metabolism in that they help in retaining amino groups during degradation of amino acid and are involved in the biochemical regulation of intracellular amino acid group. They also provide necessary intermediates for gluconeogenesis as such elevated levels in plasma may have an adverse effect on amino acid metabolism (*Murray et al*., 1996). An increased activity of these transaminases is an indication of amplified transamination process (Kori-Siakpere *et al*., 2010). Also alterations in the metabolism of proteins and carbohydrates are used for a similar purpose. The treatment of *Clarias batrachus* with carbaryl led to a marked increase in the activities of liver transaminases (Sharma, 1999).

Cells contain enzymes that are necessary to their function. When the integrity of a cell is disrupted, enzymes escape into plasma/serum, where their activity can be measured as a useful index of cell integrity (Coppo *et al*., 2002). Changes in the biochemical blood profile indicates changes in metabolism and biochemical processes of the organism, resulting from the effect of various pollutants and they make it possible to study the mechanisms of the effects of various pollutants (Luskova *et al*., 2002).

Chronic hepatic disorders and excessive steroids results in increase plasma alkaline phosphatise (ALP) in most animal. During normal bone growth in young animals, a large amount of ALP is found in plasma; also ostheopathies result in increase of plasma ALP (Coppo *et al*., 2002). According to Sevigler *et al*. (2004), alkaline phosphatase an enzyme involved in bone growth and liver function, may be affected by low level fluoride intake. Fluoride switches off the enzyme cytochrome c oxidase, an oxygen-carrying respiratory enzyme by oxidation (Sevigler *et al*., 2004). The generation of free radicals, lipid peroxidation and altered antixodant status are considered as important factors in the toxic effects of fluoride.

A few intercellular enzymes of intermediary glucose metabolism like lactate dehydrogenase (LDH) are widely distributed in tissues of the body (liver, heart and muscle) and their appearance in the plasma in measurable quantities have been used to identify specific tissue damage (Coles, 1989). The liver has been documented as one of the richest sources of LDH and the leakage of enzyme from even small mass of damaged liver tissue can increase the observed level to a significant extent. This is because the liver is the main organ for enzymatic decontamination process, vitellogenin production and storage of glycogen as energy reserves (Jenkins, 2004).

Omoregie *et al*. (1990) reported that *Tilapia sp* showed marked hyperglycaemic response to stressed environmental conditions as a result of incomplete metabolism of blood sugar due to impaired osmoregulation. Das and Mukherjee (2000) reported progressive accumulation of blood glucose due to exposure of rohu (*Labeo rohita*) to sublethal concentration of quinalphos (an organophosphate pesticide). Shalaby *et al*. (2006) reported significant elevated plasma glucose in fish administered with doses of chloramphenicol, compared with control and suggested that this antibiotic affects glucose dynamics in *Oreochromis niloticus*. They posited that the increase in glucose levels was in order to obtain more energy to withstand and

overcome the existing stress condition.

Miller *et al*. (2007) exposed rainbow trout to waterborne acute and chronic sodium selenite and reported that acute exposure to sodium selenite significantly increased plasma cortisol and glucose levels but gill Na/K ATPase activities, plasma T3 and T4 levels were comparable to control. The 30 days sub-chronic exposure increased plasma cortisol, thyroxine (T3) and more active triiodothyronine (T4) but no effect on plasma glucose levels, gills Na/K- ATPase activity.

Abalaka *et al*. (2011) reported elevations in ALP activity of *C. gariepinus* exposed to aqueous and ethanolic extracts of *Parkia biglobossa* and concluded that such elevations may be due to cholestasis. Kavitha *et al*. (2012) observed increases in the activities of AST and ALT in *Cyprinus carpio* exposed to extracts of *Moringa oleifera* and concluded that these responses indicate liver necrosis. Adeogun *et al*. (2012b) reported significant increases in the plasma levels of ALT, AST, ALP and LDH after exposing *Clarias gariepinus* to sublethal concentrations of *Raphia hookeri* for 56 days. They concluded that increase in the activities of these enzymes was suggestive of liver damage.

2.7 Genotoxic Evaluations of Herbicides in Marine Organisms

Cytogenetic techniques have already been applied for monitoring fish or mussel exposured to pollutants under field conditions or for assessing water quality under laboratory conditions. A number of laboratory studies aimed at developing and validating cytogenetic techniques in aquatic organisms exposed to genotoxicants have been reported (Hyashi *et al*., 1998). Cytogenetic analyses can detect gross genomic alterations after exposure to genotoxicants. In spite of certain technical difficulties, the assessment of chromosomal aberrations, (CA) sister chromatid exchanges (SCE) and micronuclei (MN) has also been applied to marine vertebrates

and invertebrates (Ayllon *et al*., 2000).

It is a reasonable assumption that increases of the genotoxic load in ecosystems by the release of genotoxic chemicals and the increased levels of radiation (UV) has an impact on mutation frequencies as well as on fertility of organisms. These adverse effects may lead to a decline of population size and an eventual extinction of species. These deleterious effects may affect the sustainability of ecosystems. During the past few years specific molecular methods have been developed that can be used to generate data for mathematical modelling to assess the effects of mutagen exposure on population levels (Belfiore and Anderson, 2001). It is also well documented that the incidence of cancer is higher in organisms living in environment that are prone to contamination with genotoxins (Silberhorn *et al*., 1990; Tchounwou *et al*., 1996; van Noorden *et al*., 1997). Typical examples are the elevated cancer rates in fish exposed to polycyclic aromatic hydrocarbons (PAHs) (Stalker *et al*., 1991).

Fish micronucleus assays have been shown to be a useful *in vivo* technique for in situ monitoring of water quality. Al-Sabti and Metcalfe (1995) reviewed literature on the clastogenic effects of chemical and physical agents on fish cells with emphasis on the induction of micronucleus in teleosts. Michelmore and Chipman (1998) reported the use of DNA strand breakage as an endpoint for environmental pollution monitoring in aquatic organisms. Studies with aquatic organisms exposed to polluted waters or sediments in the field supported the use of the comet assay and other DNA strand breakage methods as rapid sensitive screens for genotoxic pollutant exposure (Belpaeme *et al*., 1996).

Bivalve mussels (*Mytilus edulis* and *M. galloprovincialis*) seem to be ideal bioindicators for contaminant evaluation/assessment because of their ability to concentrate local pollutants. Micronucleus (MN) and sister chromatid exchange (SCE) frequency in the gill tissue and

developing eggs of *M. galloprovincialis* and chromosomal aberration (CA) and SCE frequency in gill tissue of *M. edulis* have been reported (Jha *et al*., 2000). Examination of the gill cell population of mussels and fish species shows that only a small fraction of it undergoes mitosis. Developing eggs in mussels are an appropriate target for SCE induction because they contain a population of actively proliferating cells (De Flora *et al*., 1993).

Hafez (2009) reported various genotoxic indexes such as chromosomal aberrations (CAs), micronuclei (MN), nuclear buds (NB), binucleate cells (BN), and fragmented apoptotic cells (FAC) in the blood, kidney and gill cells of flathead mullet (*M. cephalus*) collected from four different locations along Abu-Qir Bay, in Egypt contaminated by various environmental pollutants.

In studying the genotoxic effects of heavy metals on fish, Bagdonas and Vosyliene (2006) exposed rainbow trout *Oncorhynchus mykiss* to different concentrations of Copper and Zinc and reported an increase in the frequency of micronucleated erythrocytes (MNE) after exposure to these heavy metals and their mixtures at all the concentrations studied but that there were no significant differences in MNE levels among the concentrations studied. After a 96h recovery (in clean water) of the exposed fish, the levels of MNE significantly decreased at the highest and lowest concentrations studied and they suggested that cell repair might have occurred during recovery.

Ahmed *et al*. (2011) conducted an experiment to study the genotoxic potentials of sodium arsenite ($NaAsO_2$) in the freshwater fish *Oreochromis mossambicus* using the alkaline comet assay and micronucleus (MN) test. Fish were exposed to three different concentrations (3, 28 and 56 ppm) of arsenic and gill, liver and blood samples were collected after 48 h, 96 h and 192 h of exposure. Arsenic exposure induced DNA damage in all tissues examined in a

concentration dependent manner. A significant ($p \leq 0.05$) increase in the comet tail DNA (%) of exposed fish liver, gill, and blood was observed after 48 h and 96 h of exposure, but a decline in DNA damage was recorded in all the tissues at all the three concentrations studied after 192 h of exposure. They concluded that arsenic is genotoxic to fish and that it provides a basis for further studies in aquatic toxicology.

The mutagenic and genotoxic effects of 'Rasayanzine', an atrazine herbicide, was observed in the fish *Channa punctatus* using micronucleus and single cell gel electrophoresis (SCGE) assays by Nwani *et al*. (2010b). Three sub-lethal concentrations of the test material were calculated using LC_{50} value and the fish specimens were exposed to these concentrations. Erythrocyte and gill cells were sampled on day 0, 1, 3, 5, 7, 14, 21, 28 and 35 of exposure for assessment of micronuclei induction in blood erythrocytes and DNA damage using SCGE assay in both blood erythrocytes and gill cells. Significant effects ($p \leq 0.01$) for both concentration and time of exposure were observed in treated fish. Micronuclei induction in erythrocytes was highest on day 7 of exposure. The highest level of DNA damage in the SCGE was observed in both tissues on day 5 at all concentrations followed by gradual non-linear decline.

Cavas and Konen (2007) studied the genotoxic effects of glyphosate on the freshwater goldfish *Carassius auratus* exposed to three doses of glyphosate formulation (5, 10 and 15 ppm). Analysis of micronuclei, nuclear abnormalities and DNA damage were performed on peripheral erythrocytes sampled at intervals of 48, 96 and 144 h post-treatment. They reported significant dose-dependent increases in the frequencies of micronuclei, nuclear abnormalities as well as DNA strand breaks. Their findings also confirmed that the alkaline comet assay and nuclear deformations in addition to micronucleus test on fish erythrocytes *in vivo* are useful tools in determining the potential genotoxicity of commercial herbicides.

2.8 Bioaccumulation in Fish

Fish biomarkers may be useful tools in several steps of the risk assessment process: effect, exposure level and hazard assessment, risk characterization or classification, and monitoring the environmental quality of aquatic ecosystems. In time these biomarkers will thus become a routine, well-characterized and scientifically and legally defensible tool for monitoring and assessing environmental pollution (McCarthy and Shugart, 1990). Based on the magnitude and pattern of biomarker responses, aquatic species offer the potential of serving as sentinels demonstrating the presence of bioavailable contaminants and the extent of exposure; surrogates indicating potential human exposure and effects and predictors of long-term effects on the health of populations or the integrity of the ecosystem (McCarthy and Shugart, 1990).

Agricultural and industrial activities are the most important sources of chlorinated compounds. In addition, organochlorine pesticides (OCPs) are atmospheric pollutants that are transported over long ranges and bioaccumulate in the food chain (Negoita *et al*., 2003).

The presence of a xenobiotic compound in a segment of an aquatic ecosystem does not, by itself, indicate injurious effects. Connections must be established between external levels of exposure, internal levels of tissue contamination and early adverse effects (Van Der Oost *et al*., 2003). Many of the hydrophobic organic compounds and their metabolites, which contaminate aquatic ecosystems, have yet to be identified and their impact on aquatic life is yet to be determined (Damasio, 2010). Therefore, the exposure, fate and effects of chemical contaminants or pollutants in the aquatic ecosystem have been extensively studied by environmental toxicologists. The widespread use and environmental release of persistent organic pollutants (POPs) through disposal and spillage have resulted in both the contamination of fresh water and the accumulation of these toxicants in aquatic organisms through different mechanisms: bioconcentration by direct uptake from water through the gills and skin, particle ingestion, or biomagnification through the consumption of contaminated food

(Miranda *et al*., 2008). Even without detectable acute or chronic effects in standard ecotoxicity tests, bioaccumulation should be regarded as a hazard criterion in itself, since some effects may only be recognized in a later phase of life, are multi-generation effects or manifest only in higher members of a food-web, e.g. impact of PCBs on the hatching success of eggs (Tillitt *et al*., 1992).

Bioaccumulation of chemicals in biota may be a prerequisite for adverse effects on ecosystems (Franke *et al*., 1994). Miranda *et al*. (2008) assessed the impact of aquatic pollutants such as organochlorine pesticides, polychlorinated biphenyls, chlorotriazines (atrazine, simazine) and chlorinated phenylureas (diuron), on the Ponta Grossa lake South of Brazil. Ten freshwater trahira fish (*Hoplias malabaricus*) were collected. The results showed detectable amounts of persistent organic pollutants (POPs) in the liver and muscle, and bioaccumulation was higher in the liver than in the muscle. The presence of some banned pesticides, such as hexachlorobenzene and dichlorodiphenyltrichloroethane in the liver suggests acute exposure to these compounds. They also reported that some physiological disturbances and morphological damages found in the liver of *Hoplias malabaricus* were associated with chlorinated-compound bioaccumulation.

Wang *et al*. (2013) investigated the presence of Hexabromocyclododecanes (HBCDs) in 79 wild fish from high mountain lakes and rivers of the Tibetan Plateau, China. The ΣHBCDs in fish muscles ranged from non detectable levels to 13.7 ng/g lipid weight (lw) (mean value of 2.12 ng/g lw) with a high detection frequency of 65.8%; α-HBCD dominated among the isomers and accounted for 78.2% of the total burden. Concentrations of ΣHBCDs in the fish were significantly correlated with the lipid content and a decreasing trend was observed between α-HBCD and trophic level meaning that fishes in water found on the higher altitudes of the plateau has less of the α-HBCD.

2.9 Effects of Herbicides on Physico-Chemical Parameters of Water

Water means “life” and it is one of the abundantly available substances in nature, which man has exploited more than any other resources for the substance of life. Water covers about 70% of the earth’s surface out of which only 2.7% of the total water if freshwater of which 1% is ice free water in the rivers, lakes and atmosphere as biological water. It has been estimated that only 0.00192% of the total water on earth is available for human consumption (Trivedy R.K., 1988). In developing countries, about 1.8 million people mostly children’s die every year as a result of water related diseases (WHO, 2004).

Analysis of water quality and monitoring for physico-chemical parameters are essential to preserve and protect the natural ecosystem. Water quality index (WQI) is very useful and efficient method for assessing the suitability of water quality. It is also a very useful tool for communicating the information on overall quality of water (Asadi *et al*., 2007) to the concerned citizens and policy makers. The limnology play an important role in decision making process for problems like dam construction, pollution control, fish and aquaculture practices (Muly and Gaikwad, 1999).

The study of different water parameters is very important for understanding of the metabolic events in the aquatic ecosystem. The parameters such as temperature, pH, dissolved oxygen, alkalinity, hardness etc. influence each other and also the sediments parameters as they govern the abundance and distribution of the flora and fauna (Balaji, 2015).

Water quality is the suitability of water for the survival and growth of aquatic organisms like fish (Boyd, 1982). Water quality is defined by the physical, chemical and biological characteristics of water which include Temperature, turbidity, Dissolved oxygen content, Biochemical oxygen demand, pH, alkalinity, Hardness, Nutrient (nitrate and phosphate) content, ammonia and nitrite contents, faecal coliform content etc (Balaji, 2015).

2.9.1 Temperature

Temperature of aquatic environment is important for ensuring survival, distribution and normal metabolism of fish. Failure to adapt to temperature fluctuations is generally ascribed to the inability of fish to respond physiologically with resultant mortality, which is related to changes in the metabolic pathways (Adeyemo *et al*., 2003). When water is heated, much energy, oxygen and vapour is released into the air, leaving behind a high concentration of carbon dioxide which makes the water more acidic. The result is a collapse in osmoregulatory functions during temperature extremes (Gubbins *et al*., 2000). The normal range of temperature in the tropics to which fish are adapted is 22-35˚C (Howerton, 2001).

Wilson and Taylor (1993), observed reduced oxygen consumption in common carp (*Cyprinus carpio*) at 28-32˚C. Some fish species including *Sarotherodon mosambicus*, *Cyprinus carpio* and *Salmo gairdneri* were acclimatized at temperatures of 15, 20 and 25˚C in order to study physiological responses of their blood to temperature fluctuations in the laboratory. *Cyprinus carpio* exhibited greater ability to survive at these temperatures. *Sarotherodon mosambicus* experienced osmoregulatory collapse at 15˚C, a similar phenomenon occurred in trout at 25˚C. Furthermore, a decrease in haematocrit (Ht), haemoglobin (Hb) and total plasma protein (TPP) was observed at 23±1˚C and 41±1˚C relative to control (29±1˚C). Adeyemo *et al*., (2003) that HCO_3^-, Na^+, K^+, Cl^- osmolality and some blood parameters such as MCV, MCH and MCHC were temperature dependent in *Clarias gariepinus*. They concluded that *Clarias gariepinus* seem to have ability to conserve osmolality over a wide or higher range of temperatures and that the fish species has a high adaptive ability.

Temperature is known to affect the behaviour, feeding, growth and reproduction of fish. In general, it has a pronounced effect on chemical and biological processes. Rates of chemical and biological reactions double for every 10˚C increase in temperature because fishes are known to have poor tolerance to sudden changes in temperature (Boyd and Lichkoppler, 1979). As such

an optimal temperature range of 25-32˚C should be maintained all times and fish differ in their tolerance to extremes in temperature depending on the species involved, stage of development, environmental temperature, dissolved oxygen, pollution, seasons etc.

2.9.2 Hydrogen-ion concentration (pH)

There is now abundant evidence that acidification, particularly of soft water has a damaging effect on fish and a water body that feature (naturally or artificially) episodic or chronic pH levels of about 4.0-4.2 or less are devoid of fish (Odunze, 2004). Stress and death due to acidification are not simple physiologically because acidification is invariably associated with water of low conductivity (e.g. low calcium content) and elevated trace metal levels. Acidified water may impair physiological processes including gas transfer, ionic regulation and acid-base balance (McDonald, 1983), available information on acid-stressed fish suggests that survival at low pH demands considerable (and presumably energy-expensive) physiological adaptation that will compromise physiological capacity in other directions. Exposure of freshwater species to alkaline water (pH 9.5) inhibits ammonia excretion (Wilkie *et al*., 1999), although this inhibition is more dramatic in alkaline soft water than alkaline hard water. Clearly in water of pH 9.10, the excretion of ammonia is a physiological challenge and fish may need to utilize special mechanisms to maintain nitrogen excretion in severely alkaline environments (Yesaki and Inuwa, 1992). The physical and chemical characteristics of water may play an important role in copper toxicity for aquatic animals. Copper speciation is directly affected by water pH and the free cupric ion concentration is higher in water with low pH while copper hydroxide complexes prevail in water with high pH (Tao *et al*., 2001). Hydrogen ion concentration (pH) is important as a measure of acidity of the fish pond water as pH affects many chemical and biological processes in the water and is known to influence the toxicity of certain chemicals like ammonia, sulphides and cyanides in water (Dupree and Huner, 1984). An un-ionized sulphide (e.g. H_2S) is extremely toxic to fish at concentrations that may occur in natural water

(Badiru, 2005). Bioassay of several species of fish suggests that any detectable concentration of H_2S should be considered detrimental to fish production (Boyd, 1981). In acidic waters, crustaceans and fish have suffered retarded growth and skeletal deformities (Haines, 1981) and impaired ionic regulation (Morgan and McMahon, 1982). The effect of ambient pH on urea-N excretion *Pardanthus chinensis* was more pronounced at high pH than at low pH (Chen and Lin, 1995). In aquaculture ponds, pH reductions can be exacerbated during periods of heavy rain as acidic soils in pond dykes erode into ponds (Boyd, 1989). Many aquaculture research scientists agreed that freshwaters with alkaline pH have the potential to be productive and suitable for fish culture (Boyd, 1979; Adeniji, 1986). Acidic waters (pH below 6.5) will not have good plankton growth and so will not be productive. Generally freshwater fish cannot survive in waters below pH 4 and above pH 11 for long periods. The optimum pH for fish is between 6.5 and 9.

Table 2.1: Response of fish to different pH values

pH Range	**Effect**
4- below	Fish die because of acidity
4 - 5	No reproduction
5 - 6.5	Slow growth because of fish food production
6.5 - 9.0	Fish thrive well and grow fast
11 - above	Fish die becaue of alkalinity

Adapted from Swingle, 1969

2.9.3 Dissolved oxygen

Maintenance of sufficient dissolved oxygen in the fish pond at all times is without doubt the most essential water quality management tasks performed by fish farmers. Its presence in good quality in fish pond will improve the water quality by oxidizing poisonous gases such as

ammonia, carbon dioxide etc into their non-poisonous forms - ammonium salts, carbonates and bicarbonates etc. Low dissolved oxygen levels can be lethal, resulting in acute fish anoxia and reduces fish fecundity thereby preventing spawning and egg hatchability. It also retards growth of embryo, juveniles and leads to eventual mortality (Badiru, 2005). Safe recommended concentration of DO is 4 mg/L for fish, however, most species are distressed when it falls between 2 and 4 mg/l. Low level of DO such as less than 2 mg/l can cause fish mortality (McNeil and Closs, 2007). According to Boyd (1981) and Badiru (2005), warm water fish survive at dissolve oxygen levels as low as 1mg/L, but growth is slowed down by prolonged exposure. A developed dissolved oxygen scale for warm water fish as presented by in table 2.1

Table 2.2: Response of fish to different dissolved oxygen concentrations

D.O< 0.3mg/L	Fish die after short-term exposure
D.O 0.3-1mg/L	Lethal for long-term exposure
D.O 1mg – 5mg/L	Fish survive but growth is slow for long-term exposure
D.O > 5mg/L	Minimum for warm water fish (fast growth).
	Adapted from Boyd (1981).

Some causes of oxygen deficiency in ponds are plankton bloom, decaying or dead fish and decomposed organic matter. Most prominent of these, however, is heavy application of organic fertilizer in ponds since decaying organic matter absorb oxygen and give off carbon dioxide (2kano, 2011).

2.9.4 Total alkalinity and total hardness

In natural waters, total alkalinity (mg/L) usually refers to the total concentration of bases which are primarily carbonate and bicarbonate ions. Rocks and soils, salts, certain plant activities and certain industrial wastewater discharges usually influence alkalinity in water (Badiru, 2005).

Waters with high alkalinities have a higher buffering capacity than waters with low alkalinities. Alkalinities of 30-150mg/L are preferred in fish culture operations (Dupree and Huner, 1984). Total hardness in fish pond usually refers to the total concentration of divalent metal ions (primarily calcium and magnesium), expressed in mg/L of equivalent calcium carbonate (Badiru, 2005). Hardness is not as critical to fish pond as alkalinity but it is desirable to have water with a total hardness greater than 20mg/L. The preferred range extends as high as 300mg/L (Boyd and Lichtkoppler, 1979). Total alkalinity and total hardness values are normally similar in magnitude because calcium, magnesium, bicarbonate and carbonate ions in water are derived in equivalent quantities from the solution of limestone in geological deposits (Badiru, 2005).

2.10 Fisheries Resources and Production in Nigeria

Nigeria is located in West Africa bordering the Gulf of Guinea between Benin and Cameroon. It is a coastal state with a coastline of around 853km and a 200 nautical miles Exclusive Economic Zone (EEZ), in which it has exclusive rights to the fish and other natural resources. The brackish and coastal waters of Nigeria support harvests at the artisanal level, while the industrial sector operates only outside the 5 nautical miles (Ibeun, 2006). Nigeria has a total land area of 923,768 sq. km. and 13,000 sq. km. of inland water bodies. Generally, the climate varies from South to the North ranging from equatorial in the South, tropical in the centre and arid in the North (Ibeun, 2006). The population of the country is put at about 160 million. In addition to the marine and brackish water resources, Nigeria has massive freshwater systems, including lakes, rivers, reservoirs, dams and floodplains which support extensive artisanal fisheries. The River Niger which arises in Sierra Leone and has a total length of 4,184 kilometers, flows through West Africa, enters Nigeria in the northwest and runs southwards to join the River Benue at Lokoja, before traveling the remaining 547 kilometers to the sea. These two major rivers and the many smaller rivers support large freshwater artisanal fisheries in the

country. Ita *et al*. (1985) has identified about 365 lakes and reservoirs and 687 ponds and floodplains totalling over 13 million hectares of water bodies. Despite this potential, the current annual demand for fish is 1.5 million tonnes, whereas local production stands at about 0.4 million tonnes. A study by the National Special Programme for Food Security (NSPFS) (2004) identified about 2,658 fish farms and 937 Dams and Reservoirs in Nigeria. In 2000, the fish import bill exceeded N30 billion naira i.e. US$241.1m (Dada, 2004). This relatively low production and the impact on the economy of high imports, is of concern and a challenge to all in the fisheries sector (Ibeun, 2006).

The collection of accurate data is most difficult for inland fisheries but there is thought to be great potential for increased fish production in freshwaters. The artisanal nature and the rural location of the inland fishermen and the rivers has caused most of the freshwater catches are not accounted for. For example Lake Chad has been well studied for the past five years and production statistics kept. Thus it has been established that the potential yield of the lake is 200,000 m.t. while current actual production is only half of that. The Nigerian side of the Lake produces 60,000 m.t. annually, with a monetary value in excess of $22 million (ca. 2.95 billion Naira). Lake Chad currently produces about 33% of Nigerian freshwater fish. Unfortunately, this type of data is not available for other water bodies (Ibeun, 2006).

Ezenwa (1994) asserted that accurate estimates for aquaculture production in Nigeria are difficult to obtain due to logistic reasons. Many private fish farmers do not keep statistics and, when they do, are not always ready to give actual production figures. However, the national production figures show a yearly increase, which suggests that the government is putting some emphasis on aquaculture. Madu (1995) showed that more private individuals are becoming involved in fish farming and government policy is to empower them through assistance from relevant government departments.

Nigeria is rich in a variety of fish species. The landing locations of various species can be classified based on the natural habitat of the fishes. On this basis, we have:

Inland waters (*Tilapia* and *Clarias*)

Inshore waters (*Musil* and *Arius*)

Offshore waters (Tuna)

Aquaculture (*Tilapia* and *Clarias*)

Miscellaneous sources (Ajayi and Talabi, 1984)

Inland waters consist of rivers, tributaries, flood plains, lakes, coastal and brackish waters. The total fishery resource potential of this source has been put at 288,500 tonnes (Ajayi and Talabi, 1984). The various species found in this habitat include *Lates niloticus*, *Clarias* spp, *Heterotis niloticus*, *Tilapia* spp. etc. The African catfish, *Clarias gariepinus* is a member of the family Clariidae. Apart from the adult stage, there are three distinct stages in the life cycle of *C. gariepinus*; the larval stage which comprises the yolk-sac fry stage (non-feeding period) a stage which starts from immediately after hatching to the beginning of exogenous feeding, the swim-up larvae or early fry stage and the advanced fry or fingerling stage. The fingerling looks more or less like the adult but smaller in size. Fish have an important role in the food chain therefore investigation of the effects of pesticides on fish has a relevant significance in evaluation of adverse effects of pesticides to human health (Begum and Vijayaraghavan, 1996).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection and Maintenance of Fish

Juveniles of *Clarias gariepinus* were purchased from a fish farm in Ota, Ogun State, Nigeria. The length and weight of the fish were taken to indicate age. The *Clarias* species averaging 12.14 ± 1.76cm standard length and body weight of 4.45 ± 1.37g were used for the study. The fish were conveyed in a well aerated container containing water from the fish farm to the holding units at Covenant University, Ota. Ten fish were held in containers of 25L capacity and acclimatized for two weeks in dechlorinated water. Eighteen 25L capacity containers were used in all. During this period, the fishes were fed with commercial feed containing 35% crude protein twice per day at 4% body weight. The experimental setup is shown in Plate 1.

3.2 Physico-Chemical Parameters of Water

Pipeborne water was dechlorinated by allowing it to stand for 72hr in an overhead tank during which it was aerated. Temperature, pH, alkalinity, hardness, conductivity and dissolved oxygen levels of control and other treatment exposures were determined and the readings taken at 12hr, 48hr and 96hr intervals in three replicates.

3.2.1 Determination of temperature

Temperature was determined using the mercury-in-glass thermometer, which was inserted in water and the temperature (°C) reading was taken after four minutes.

3.2.2 Determination of hydrogen ion concentration

pH was determined using a Jenway® type pH meter (Model 3015). The probe was first inserted in the buffer for 5 minutes to standardize the meter to pH 7, thereafter, it was dipped into the water and the static pH was read 60 seconds later.



Plate I: Experimental Set up with the plastic tanks

3.2.3 Determination of dissolved oxygen

DO was measured by Winklers method (APHA, 2005). Duplicate water samples were taken in 250ml B.O.D stopper bottles and 2mls of manganese sulphate solution were added to each. 2mls of alkali-azide were added below the surface of the sample. The bottle was carefully cocked to expel air-bubble and to discard excess liquid. The bottle was inverted to mix the content. As the resulting precipitate settled, the bottle was inverted again and left to settle. 2mls of concentrated tetraoxosulphate VI acid was added and the bottle was cocked to discard excess liquid. This was mixed gently by inverting the bottle. Appropriate volume was pipetted out of the bottle (to correct for loss of sample during addition of reagents) and poured into a conical flask and titrated with 0.025N of sodium thiosulphate until a pale straw colour was achieved. 1ml of starch solution was added and titration continued until the blue colour became colourless. The volume of the thiosulphate used is equal to dissolved oxygen per litre (APHA, 2005) i.e. one ml of 0.025N sodium thiosulphate is equivalent to one mgL^{-1} of oxygen. The concentration of DO in the sample was calculated using the following formula:

mg/L DO = (mL titrant x normality of titrant x 8000)/equivalent volume of sample titrated (APHA, 2005).

3.2.4 Determination of total hardness

Ethylenediaminetetraacetic acid (EDTA) titration method (APHA, 2005) was used to determine water hardness. Water samples were mixed thoroughly and 25mL was taken and diluted to 50mL with distilled water. Then 25mL of buffer solution (0.05N borax, 0.1N sodium hydroxide and 0.025N sodium sulphide) were added, followed by 2 drops of Eriochrome black indicator. The samples were then titrated with 0.02N EDTA solution within 5 minutes interval. In the presence of calcium and magnesium, the colour changed from red to blue. The calculation was based on the equation

Total hardness in mg/l $CaCO_3$ = $\frac{A \times B \times 1000}{\text{ml of sample}}$

where A = ml titration and B = mg $CaCO_3$ equivalent to 1.00ml EDTA titrant (APHA, 2005).

3.2.5 Determination of total alkalinity

Total alkalinity was determined as described by APHA (2005); 100ml of sample was taken into a conical flask and 3 drops of phenolphthalein indicator were added to it. Three (3) more drops of methyl orange indicator were added as the sample did not change colour. As the sample turned yellow, 0.02N tetraoxosulphate VI acid was added from a burette until the colour changed to orange. The volume (ml) of acid used was then recorded. Total alkalinity was obtained from the following calculation

Phenolphthalein alkalinity = $\frac{A \times N \times 50{,}000 \text{ (as mg/l } CaCO_3)}{\text{ml of sample}}$

Total alkalinity/acidity as $CaCO_3$ = $\frac{B \times N \times 50{,}000 \text{ (as mg/l } CaCO_3)}{\text{ml of sample}}$

Where A = ml of titration for sample to reach phenolphthalein endpoint
B = ml of titration for sample to reach methyl orange endpoint
C = Normality of acid (0.02N) (APHA, 2005).

3.3 Preparation of Test Solutions

Two herbicides were used in the investigation. Both herbicides were purchased from a commercial outlet in Lagos. Out of 360g/L glyphosate, a stock solution (5mg/L) of the toxicant was prepared by adding 1mL of the toxicant to 999mls of water (Reish and Oshida, 1987). Similarly, out of 276g/L paraquat, stock solution of 1mg/L was prepared in the same way as glyphosate. The stock solutions were used for preparing different concentrations of the test solutions by diluting measured volumes (i.e. 0.36mL in 999.64mL of dechlorinated tap water for 0.36mg/L concentration). The dechlorinated tap water used had the same physical and chemical properties with the one used in acclimatizing the fish. The control solutions were made up of only dechlorinated tap water.

3.4 Test Procedure for Range Finding Test (Pilot Study)

For each test concentration of the herbicides, 8 large tanks of 150L were used. Smaller holding containers of 25L each were connected to each of these large tanks. The test solutions and the controls were run in triplicates. For each triplicate, the test solutions and controls were prepared and labelled, thoroughly mixed and allowed to stand for about 5 minutes before use. For the range finding tests, the concentrations of toxicants in the five holding tanks were different from one another based on a uniform logarithm ratio (0.001, 0.01, 1 and 10mg/L) (Parrish, 1988). Ten juvenile fish (12.14 ± 1.76cm standard length and body weight of 4.45 ± 1.37g) were introduced into each test concentration and the control in triplicates. Prior to the addition of each concentration of the toxicant into each holding tank, an equal volume of water was drawn out and then the toxicant introduced to make the test solution 25L. The fishes were not fed during the 96hr experimental period.

3.5 Procedure for Definitive Test to Determine Exposure Concentrations for Acute Toxicity Bioassay

The test concentrations were fine-tuned until a range of test concentrations in which no fish died in the lowest concentrations and all fishes died in the highest concentration in one treatment was obtained (OECD 1992). The final concentrations for glyphosate were 0.36, 0.48, 0.60, 0.72, 0.84 and 0.00 (control); while that of paraquat were 0.012, 0.037, 0.055, 0.110, 0.221 and 0.00mg/L (control).

3.6 Exposure of Fish to Acute Concentrations of Glyphosate and Paraquat to Determine Fish Behavioural Parameters and 96hr LC_{50} values

Static bioassay was conducted in the laboratory between March and July, 2012 following OECD (1992) guidelines to determine the toxicity of glyphosate and paraquat to *C. gariepinus*. From freshly prepared stock solutions, five concentrations of 0.36, 0.48, 0.60, 0.72, 0.84 for

glyphosate and 0.012, 0.037, 0.055, 0.110, 0.221mg/L for paraquat were dispensed with a 100mL measuring cylinder and 5mL syringe respectively into 150L tanks (for each concentration) containing dechlorinated water connected to 25L tanks and control. Ten fishes were randomly distributed into each test tank in 3 replicates. The physicochemical parameters of the diluting water (temperature, pH, dissolved oxygen, total hardness, total alkalinity and conductivity) during the acute test were measured. Air gulping index (AGI) was determined as the frequency of air gulping activity of fish per tank per minute, divided by the number of fish or surviving fish in the exposed groups. The opercular ventilation count (OVC) and tail fin movement rates (TMR) were counted manually per minute, for three minutes and the mean recorded using a stop watch. The AGI, OVC and TMR were observed and recorded at 12, 24, 48, 72 and 96hr. Survival and mortality were noted during this period. Fishes were considered dead when the opercular movement ceased and there was no response to gentle probing. This was used as a measure of mortality. The LC_{50} was determined by plotting a graph of the different concentrations of the toxicants against the number of dead fishes using the probit method. One fish species (*C. gariepinus*) was used for the study. Each of the herbicides was administered at five levels of concentration and a control. That is, 6 treatments, three replicates 18 experimental set ups or tanks for each herbicide

3.7 Determination of Sublethal Concentrations of Herbicides

From the result of the acute bioassay, fractions, 1/5, 1/10 and 1/20 of the 96h LC_{50} were used to determine sublethal concentrations for the herbicides as recommended by Oladimeji and Ologunmeta (1987) and Mohammed (1995) using a flow through experiment where the water in the tanks were renewed every 12h. Four 25L tanks of same size were used in three replicates for the toxicants and a control. Appropriate volumes of stock solution were dispensed using syringe and measuring cylinder into 150L of dechlorinated tap water in each of the tanks except the control. The juveniles were exposed to nominal concentrations of toxicants for

8weeks. The concentrations used for chronic study of the two toxicants were 0.0265, 0.053, 0.106 and control (0.00mg/L) for glyphosate and 0.0035, 0.007, 0.014 and control (0.00mg/L) for paraquat. Each treatment was in triplicate. This means four treatments for each herbicide i.e. 12 experimental set-ups or tanks for each herbicide. The fishes were randomly assigned to give 10 fish per tank. Fishes were fed 4% body weight with 35% crude protein level pelleted diet. The natural 12:12 day/night photoperiod was maintained. The 150L tanks were filled with water and the appropriate concentrations of the toxicants each time it was empty. This maintained toxicant strength and the level of dissolved oxygen.

3.8 Histological Examination of Fish Tissues.

Fish were anesthesized on ice, excised and fresh specimens of liver, gills and brain were collected from both control and herbicides-exposed fish for histological studies. The specimens were rapidly fixed in 10% phosphate buffered saline for at least 24hr. The fixed specimens were processed by passing through graded series of alcohol; (70%, 95% and absolute ethanol). They were further passed through xylene and infiltrated with paraffin. From the prepared paraffin blocks, 5µm thick sections were obtained and sections were further processes as described by Culling (1983).

3.9 Biochemical Profiling of Fish Tissues

Commercial kits were purchased form Germany for the determination of the levels of the activities of the liver enzymes. These include Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Lactate dehydrogenase (LDH) and Alkaline phosphatase (ALP).

3.9.1 Processing of fish tissue

0.18g of fish liver was mixed with 2mls of sucrose and homogenized. It was then centrifuged at 12,000 x g for 10 minutes. The supernatant was carefully recovered and the pellet discarded. The supernatant was used as the sample according to Nwani *et al*., (2010a).

3.9.2 Determination of alkaline phosphatase (ALP)

This was determined as described by the manufacturer's recommendation (Cypress diagnostics, Germany, 10.2004).

Reagent composition:

Reagent 1 (Buffer) is a mixture of Diethanolamine buffer pH 10.4 1 mmol/L and Magnesium chloride 0.5 mmol/l. Reagent 2 (Substrate) is p-nitrophenolphosphate 10 mmol/L. One tablet R2 was dissolved in 15 mL of buffer Reagent R1, capped and mixed gently to dissolve the contents. This composition was the working reagent. At room temperature, 20µL of sample was pipetted into a 1cm light path cuvette and added to 1.2mL of the working reagent. It was mixed and allowed to stand for one minute. The spectrophotometer was adjusted to zero using distilled water. Initial absorbance was read at 405 nm and further read every minute for three minutes. Differences between absorbance and average absorbance per minute were calculated as (Δ abs/min). ALP (lU) = Δ Abs/min x 3300. One international unit (IU) is the amount of enzyme that transforms 1 µmol of substrate per minute, in standard conditions. The concentration was expressed in units per litre of sample (IU).

3.9.3 Determination of aspartate aminotransferase (AST)

This was determined as described by the manufacturer's recommendation (Cypress diagnostics, Germany, 07.2004).

Reagent composition:

Reagent 1 (Buffer) is a mixture of Tris buffer pH 7.8, 80mmol/l and L - Aspartate 200mmol/l. Reagent 2 (Substrate) is NADH 0.18mmol/l, LDH 800 U/I, MDH 600 U/I, α - ketoglutarate 12mmol/l. One tablet R2 was dissolved in 15 mL of buffer Reagent R1, capped and mixed gently to dissolve the contents. This composition was the working reagent. At room temperature, 0.1ml of sample was pipetted into a 1cm light path cuvette and added to 1.0ml of the working reagent. This was mixed and allowed to stand for one minute. Spectrophotometer

was adjusted to zero using distilled water. Initial absorbance was read at 340 nm and further read every minute for three minutes. Differences between absorbances and average absorbance per minute were calculated as (abs/min). ALP (IU) = Δ Abs/min x 1750. One international unit (IU) is the amount of enzyme that transforms 1 μmol of substrate per minute, in standard conditions. The concentration was expressed in units per litre of sample (lU).

3.9.4 Determination of alanine aminotransferase (ALT)

This was determined as described by the manufacturer's recommendation (Cypress diagnostics, Germany, 07.2004).

Reagent composition:

Reagent 1 (Buffer) is a mixture of Tris buffer pH 7.8, 100mmol/l and L - Alanine 500mmol/l. Reagent 2 (Substrate) is NADH 0.18mmol/l, LDH 1200 U/I, α - ketoglutarate 15mmol/l. One tablet R2 was dissolved in 15 mL of buffer Reagent R1, capped and mixed gently to dissolve the contents. This composition was the working reagent. At room temperature, 0.1ml of sample was pipetted into a 1cm light path cuvette and added to 1.0ml of the working reagent. It was mixed and allowed to stand for one minute. Spectrophotometer was adjusted to zero using distilled water. Initial absorbance was read at 340 nm and further read every minute for three minutes. Differences between absorbances and average absorbance per minute were calculated as (abs/min). ALP (lU) = Δ Abs/min x 1750. One international unit (IU) is the amount of enzyme that transforms 1 μmol of substrate per minute, in standard conditions. The concentration was expressed in units per litre of sample (lU).

3.9.5 Determination of lactate dehydrogenase (LDH)

This was determined as described by the manufacturer's recommendation (Cypress diagnostics, Germany, 12.2004).

Reagent composition:

Reagent 1 (Buffer) is a mixture of Imidazole 65mmol/l and Pyruvate 0.6mmol/l. Reagent 2 (Substrate) is NADH 0.18mmol/l. One tablet R2 was dissolved in 15 ml of buffer Reagent R1, capped and mixed gently to dissolve the contents. This composition was the working reagent.

At room temperature, 100µl of sample was pipetted into a 1cm light path cuvette and added to 3.0ml of the working reagent. It was mixed and allowed to stand for one minute. Spectrophotometer was adjusted to zero using distilled water. Initial absorbance was read at 340 nm and further read every minute for three minutes. Differences between absorbances and average absorbance per minute were calculated as (Δ abs/min). ALP (U/l) = Δ Abs/min x 4925. One international unit (IU) is the amount of enzyme that transforms 1 µmol of substrate per minute, in standard conditions. The concentration was expressed in units per litre of sample (lU).

3.9.6 Determination of glutathione peroxidase

Glutathione peroxidase (GPx) activity was assayed as described by Paglia and Valentine (1967) with modifications according to Lawrence and Burke (1978). The reaction mixture contained 50mM potassium phosphate buffer (pH 8.3), 1mM EDTA, 1mM sodium azide, 0.2mM nicotinamide adenine dinucleotide phosphate (NADPH), and 1 U/mL glutathione reductase. The reaction was initiated with the addition of 1.5mM cumene hydroperoxide. The enzyme activity was estimated from the rate of oxidation of NADPH. The reagents were mixed and the absorbance measured at 340 nm. Enzyme activity was expressed in mmol/minute/milligram protein

3.9.7 Determination of catalase activity

Catalase (CAT) activity was assayed as described by Clairborne (1985). This method was based on the disappearance of H_2O_2 at 240 nm. The reaction mixture consisted of 1 ml of

0.05M H_2O_2 in 0.1M sodium phosphate buffer (pH 7.4), at 25˚C, 1.9 ml of distilled water and 0.1 ml of liver homogenate. Change in absorbance was recorded at 240 nm. Catalase activity was calculated in terms of micromole H_2O_2 consumed per minute per milligram of protein.

3.9.8 Determination of superoxide dismutase activity

Superoxide dismutase (SOD) activity was assayed as described by Misra and Fridovich (1972). The assay was based on the ability of SOD to inhibit the autoxidation of epinephrine at an alkaline pH. 0.5ml of tissue homogenate was diluted with 0.5ml of distilled water, to which 0.25ml of ice-cold ethanol and 0.15ml of ice-cold chloroform was added. The mixture was properly mixed using a cyclo mixer for 5 minutes and centrifuged at 2500 rpm. To 0.5ml of supernatant, 1.5ml of carbonate buffer (0.05M, pH 10.2) and 0.5ml of EDTA solution (0.49M) were added. The reaction was initiated by the addition of 0.4ml of epinephrine (3mM) and the change in optical density/minute was measured at 480nm against reagent blank. SOD activity was expressed as units/mg protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme was taken as the enzyme unit.

3.9.9 Determination of lipid peroxidation

The malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reactive substances (TBARS) using the method of Buege and Aust (1978). 1.0mL of the sample was added to 2mL of (15 % (w/v) trichloroacetic acid, 0.375 % (w/v) thiobarbituric acid (TBA), 0.25 M HCl). The solution containing plasma and reagent was heated in a boiling water bath for 15 min and then cooled. Precipitates were removed by centrifuging at 1000 rpm for 10 minutes. The supernatant was removed and the absorbance read at 535nm against a blank. MDA was calculated using the molar extinction coefficient ε for MDA 1.56×10^5 M^{-1} cm^{-1}. Results were expressed as μ mol^{-1}

3.10 Micronucleus (MN) Assay

Blood samples from each group were collected by piercing the tails of the fish and taking blood in a heparinized microcapillary tube. The micronucleus assay of erythrocytes was conducted following a modified version of Udroiu (2006). The peripheral blood erythrocytes from each fish were dropped onto three clean slides that were flattened by other slides to produce an evenly distributed blood smear, treated with a fixative (methanol) for 15 min at room temperature, air-dried, stained with 10% Giemsa in a phosphate buffer (PBS), washed twice with PBS and mounted. Micronucleus scoring was conducted on the cells that had been spread onto clean slides and air-dried. For micronuclei analysis, approximately 5000-8000 erythrocytes per concentration were observed at a 1000X magnification using an Olympus B50 fluorescence microscope. The frequencies of clearly outlined and typically shaped micronuclei in the peripheral blood erythrocytes were observed. This criteria, introduced by Fenech (2000), specified that scorable cells should be separate, easily distinguished and of approximately equal size. Moreover, only micronucleus (MN) isolated from the main nucleus and stained in the same color as the main nucleus with an area less than 1/3 of the nucleus was scored.

3.11 Comet Assay

Alkaline Comet Assay was used to determine the genotoxicity of glyphosate and paraquat to fish using a CometAssay Reagent Kit for Single Cell Gel Electrophoresis Assay (Trevigen).

Fish liver was macerated into pieces (1–2 mm^3), allowed to settle for 5 minutes then aspirated to discard medium. 1–2 ml of ice cold 20 mM EDTA was added in 1X phosphate buffered saline PBS (Ca^{++} and Mg^{++} free), tissue was minced into very small pieces and allowed to stand for 5 minutes. The cell suspension was recovered, avoiding transfer of debris. Cells were counted, pelleted and suspended at 1 x 105 cells/ml in ice cold 1X PBS (Ca^{++} and Mg^{++} free). Cells were combined at 1 x 10^5/ml with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v) i.e. 50 µl of cells in suspension at 1 x 10^5/ml and 500 µl of molten agarose. From this, 50 µl

was immediately pipetted onto a CometSlide. Where necessary, pipette tip was used to spread agarose/cells over sample area to ensure complete coverage of the sample area. Slides were placed at 4°C in the refrigerator for 10 minutes. Thereafter, slides were immersed in 4°C Lysis Solution overnight at 4°C. Excess buffer was drained from slides and immersed in freshly prepared Alkaline Unwinding Solution, pH>13. The slides were allowed to stand in the Alkaline Unwinding Solution for 1hr at 4°C. The slides were placed in electrophoresis solution and made to undergo electrophoresis for 30 minutes at 21 volts. Again excess electrophoresis solution was gently drained from slides, gently immersed twice in dH_2O for 5 minutes each, then in 70% ethanol for 5 minutes. Samples were dried at 37°C for 10-15 minutes. 100 µl of diluted SYBR was placed onto each circle of dried agarose and stained for 30 minutes (room temperature) in the dark. Slides were gently tapped to remove excess SYBR solution and rinsed briefly in water. They were then allowed to dry completely at 37°C. Slides were viewed by epifluorescence microscopy (Olympus B50) at an excitation/emission of 496 nm/522 nm. Pictures were taken using an image system attached to the microscope.

3.12 Statistical Analysis

Probit analysis (Finney, 1971) was used to calculate LC_{50} values for the two pesticides. The data gathered from the analysis of biochemical parameters and the cytotoxic tests were subjected to analysis of variance (ANOVA) using GenStat 4.2 (2006) software; Duncan Multiple Range Test (DMRT) was used to test for differences between levels of treatment where applicable (Duncan, 1955). Data were considered significant at $p< 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Physico-Chemical Parameters of Diluting Water Used in Acute Toxicity Test for Glyphosate and Paraquat.

Results of the physico-chemical analysis of the water used for exposure concentrations of the administered pesticide concentrations in the laboratory are presented in Tables 4.1 and 4.2. The ranges were within the recommended values for *Clarias gariepinus*.

4.2 Effects of Acute Concentrations of Glyphosate and Paraquat on Some Fish Behavioural Parameters

Glyphosate

The different concentrations of glyphosate and the durations of exposure had significant effects on the rate of opercular ventilation of *C. gariepinus*. Across the exposure times, the opercular ventilation count in exposed fish were lower ($p<0.05$) when compared with the control except in the first 12hrs of exposure (Table 4.3). Within concentrations, apart from concentration of 0.36mg/L glyphosate, fish in all other concentrations had lower opercular ventilation rates than the control and the 0.36mg/L concentration (Table 4.4). The highest opercular ventilation count was observed in the first period of observation i.e. 12hrs after exposure. Significant reduction was observed in opercular ventilation count by the 24^{th} hr, a duration which represents the lowest opercular ventilation count. The opercular ventilation count peaked again by the 48^{th} hour with a slight increase by the 96^{th} hr (Table 4.5).

Tail movement rates were significantly affected by acute concentrations of glyphosate. Interplay between concentration and time revealed that tail fin movement rate was highest in the first 12 hrs of exposure to glyphosate (Table 4.6). Compared with the control, the tail movement rates in exposed fish were significantly lower from 24 to 96 hrs of exposure. Tail fin

Table 4.1: Physicochemical Parameters of water used for exposure concentrations of Glyphosate

Parameters	**Range**	**Mean ± SE**
Temperature (˚C)	28.2 – 30.8	29.52 ± 0.06
pH	6.04 – 7.14	06.59 ± 0.09
Dissolved Oxygen (mg/L)	3.7 – 5.7	03.98 ± 0.18
Conductivity (μscm^{-1})	138 – 190	160.00 ± 9.44
Hardness (mgL^{-1} $CaCO_3$)	30 – 44	36.60 ± 0.78
Alkalinity (mL^{-1})	10 – 76	40.00 ± 3.38

Table 4.2: Physicochemical Parameters of water used for exposure concentrations of Paraquat

Parameters	**Range**	**Mean ± SE**
Temperature (˚C)	29.40 – 32.2	29.68 ± 0.18
pH	6.24 – 6.86	06.52 ± 0.34
Dissolved Oxygen (mg/L)	3.0 – 6.98	05.94 ± 0.22
Conductivity (μscm^{-1})	134 – 164	152.00 ± 8.88
Hardness (mgL^{-1} $CaCO_3$)	32 – 54	42.40 ± 0.82
Alkalinity (mL^{-1})	18 – 54	33.60 ± 3.32

Table 4.3: Interaction Effects of Acute Concentrations of Glyphosate with Time on *C. gariepinus* Opercular Ventilation Count

	Time (hrs) and Opercular Ventilation Count				
Conc (mg/L)	12	24	48	72	96
0.00	48.00±1.00^{A}	50.00±1.00^{A}	49.00±1.00^{A}	50.00±0.58^{A}	50.00±1.00^{A}
0.36	46.00±1.15^{A}	52.00±1.15^{A}	50.00±1.06^{A}	54.00±1.15^{A}	58.00±0.58^{A}
0.48	47.00±1.15^{A}	40.00±0.58^{B}	48.00±1.03^{A}	50.00±1.15^{B}	51.00±1.15^{B}
0.60	60.00±0.58^{B}	35.00±0.58^{C}	47.00±1.00^{A}	48.00±1.15^{B}	50.00±0.58^{B}
0.72	61.33±1.15^{B}	38.00±0.58^{C}	44.00±1.00^{B}	45.00±0.58^{C}	50.00±0.58^{B}
0.84	62.00±1.15^{B}	38.00±0.58^{C}	45.00±1.02^{B}	47.00±1.15^{B}	49.00±1.15^{B}

Means with the same superscript along same columns are not significantly different ($p \geq 0.05$). (Mean values ± SE) n =3

Table 4.4: The Effects of Acute Concentration of Glyphosate on some Behavioural Parameters of *C. gariepinus* at 96hrs

Conc (mg/L)	Opercular Ventilation Count	Tail Fin Movement Rate	Air Gulping Index
0.00	51.60±0.94[a]	105.40±1.23[a]	0.39±0.03[a]
0.36	52.00±1.15[a]	88.00±6.52[b]	0.42±0.02[a]
0.48	47.20±1.12[b]	80.20±4.09[c]	0.82±0.02[b]
0.60	48.00±2.30[b]	92.00±5.51[d]	0.66±0.04[c]
0.72	47.60±2.23[b]	91.80±5.85[d]	0.62±0.07[c]
0.84	48.20±2.26[b]	95.80±4.61[e]	0.88±0.08[d]

Means with the same superscript along same columns are not significantly different ($p \geq 0.05$).
(Mean values ± SE) n =15

Table 4.5: Time Effects of Acute Concentrations of Glyphosate on some Behavioural Parameters of *C. gariepinus* at 96hrs

Time (hrs)	Opercular Ventilation Count	Tail Fin Movement Rate	Air Gulping Index
12	54.00±1.82[a]	121.00±3.12[a]	0.72±0.08[a]
24	42.17±1.66[b]	74.67±3.55[b]	0.73±0.07[a]
48	47.17±0.55[c]	86.17±2.47[c]	0.67±0.03[b]
72	49.83±0.94[c]	86.00±2.18[c]	0.48±0.05[c]
96	52.33±0.88[a]	93.17±1.75[d]	0.55±0.05[d]

Means with the same superscript along same columns are not significantly different ($p \geq 0.05$). (Mean values ± SE) n =18

Table 4.6: Interaction Effects of Acute Concentrations of Glyphosate with Time on *C. gariepinus* Tail Fin Movement Rate

	Time (hrs) and Tail Fin Movement Rate				
Conc (mg/L)	12	24	48	72	96
0.00	102.00 ± 1.00^{A}	100.00 ± 0.58^{A}	103.00 ± 1.00^{A}	102.00 ± 1.00^{A}	103.00 ± 0.58^{A}
0.36	130.00 ± 0.58^{B}	66.00 ± 1.15^{B}	75.00 ± 1.15^{B}	77.00 ± 1.15^{B}	92.00 ± 0.58^{B}
0.48	98.00 ± 0.58^{C}	56.00 ± 1.15^{C}	78.00 ± 1.15^{B}	79.00 ± 0.58^{B}	90.00 ± 0.58^{B}
0.60	128.00 ± 1.15^{B}	71.33 ± 1.15^{D}	85.00 ± 1.15^{C}	87.00 ± 0.58^{C}	89.00 ± 1.15^{B}
0.72	131.00 ± 0.58^{B}	73.67 ± 0.58^{D}	86.00 ± 1.15^{C}	81.00 ± 1.15^{D}	88.00 ± 0.58^{B}
0.84	127.00 ± 1.15^{B}	82.00 ± 1.15^{E}	88.00 ± 0.58^{C}	90.00 ± 0.58^{C}	92.00 ± 1.15^{B}

Means with the same superscript along same columns are not significantly different ($p\geq0.05$). (Mean values ± SE) n =3

movement rates also significantly reduced with increasing concentrations of glyphosate when compared with the control (Table 4.4). Considering the time of exposure, tail fin movement rate was highest in the first 12 hrs of exposure of fish to glyphosate. It dropped significantly after 24 hrs and then rose significantly until the 96th hr of exposure (Table 4.5).

Acute concentrations of glyphosate showed significant effects on the air gulping index of *C. gariepinus*. The interaction between duration and concentration effects of this toxicant is presented in Table 4.7. The control fish showed lower air gulping index when compared to the exposed fish throughout the exposure period. The highest concentration also showed the highest air gulping index at 96 hrs ($p<0.05$) (Table 4.4). The air gulping index of exposed fish was highest within the 12th and 24th hr of exposure after which there was a significant decline in the higher concentrations till the 96th hr (Table 4.5 and 4.7).

Paraquat

Exposure of fish to acute concentrations of paraquat also had significant effects on opercular ventilation counts. In all exposure concentrations, opercular ventilation counts were lowest within the 12th and 24th hr. After these periods, opercular ventilation count rose significantly till the 96th hr of exposure (Table 4.8). The control fishes had the highest frequency of opercular ventilation counts across concentrations while the highest concentration had the lowest opercular ventilation count at 96 hrs (Table 4.9). Considering different exposure times, the opercular ventilation counts were very low in the first two periods of observation i.e. the 12th and 24th hrs and significantly higher ventilation rates were observed between 48-96 hrs (Table 4.10).

Tail fin movement rates changed significantly with duration of exposure and with concentration of the herbicide in fish exposed to paraquat. Interaction between concentrations

Table 4.7: Interaction Effects of Acute Concentrations of Glyphosate with Time on *C. gariepinus* Air Gulping Index

	Time (hrs) and Air Gulping Index				
Conc (mg/L)	12	24	48	72	96
0.00	0.30±0.05[a]	0.40±0.05[a]	0.30±0.06[a]	0.40±0.05[a]	0.40±0.05[a]
0.36	0.50±0.05[b]	0.50±0.05[b]	0.50±0.05[a]	0.30±0.05[a]	0.30±0.06[b]
0.48	0.70±0.05[b]	0.80±0.12[c]	0.80±0.06[b]	0.90±0.05[b]	0.90±0.12[b]
0.60	0.90±0.06[c]	0.70±0.05[c]	0.70±0.05[b]	0.50±0.06[c]	0.50±0.05[a]
0.72	0.80±0.05[c]	0.90±0.05[c]	0.70±0.12[b]	0.30±0.05[a]	0.40±0.05[a]
0.84	1.20±0.05[d]	1.20±0.06[d]	0.80±0.12[b]	0.50±0.05[c]	0.70±0.05[c]

Means with the same superscript along same columns are not significantly different ($p \geq 0.05$). (Mean values ± SE) n =3

Table 4.8: Interaction Effects of Acute Concentrations of Paraquat with Time on *C. gariepinus* Opercular Ventilation Count

	Time (hrs) and Opercular Ventilation Count				
Conc (mg/L)	12	24	48	72	96
0.00	46.00 ± 1.00^{A}	49.00 ± 1.15^{A}	49.00 ± 1.00^{A}	50.00 ± 0.58^{A}	50.00 ± 1.00^{A}
0.012	12.00 ± 0.58^{B}	35.33 ± 1.15^{B}	69.00 ± 1.15^{B}	67.33 ± 0.58^{B}	60.00 ± 1.00^{A}
0.037	11.33 ± 0.58^{B}	24.00 ± 1.15^{C}	69.33 ± 1.15^{B}	70.00 ± 1.00^{B}	68.67 ± 1.15^{B}
0.055	10.00 ± 1.00^{B}	21.67 ± 0.58^{C}	49.67 ± 0.58^{A}	60.00 ± 1.00^{C}	50.00 ± 1.00^{C}
0.110	10.00 ± 1.00^{B}	11.00 ± 1.15^{D}	75.33 ± 1.15^{C}	79.00 ± 1.15^{D}	70.00 ± 1.00^{B}
0.221	10.00 ± 1.00^{B}	10.00 ± 1.00^{D}	50.00 ± 1.00^{A}	57.33 ± 0.58^{A}	49.67 ± 0.58^{C}

Means with the same superscript along same columns are not significantly different ($p\geq0.05$). (Mean values ± SE) n =3

Table 4.9: The Effects of Acute Concentrations of Paraquat on some Behavioural Parameters of *C. gariepinus* at 96hrs

Conc (mg/L)	Opercular Ventilation Count	Tail Fin Movement Rate	Air Gulping Index
0.00	51.00±1.10[a]	105.60±1.37[a]	0.42±0.02[a]
0.012	48.73±6.34[b]	107.20±3.68[a]	0.68±0.04[b]
0.037	48.67±7.40[b]	106.80±3.56[a]	0.50±0.07[c]
0.055	38.27±5.50[c]	107.60±3.47[a]	0.68±0.09[b]
0.110	47.07±9.88[b]	105.20±2.65[a]	0.55±0.12[d]
0.221	33.40±6.80[d]	107.60±4.74[a]	0.63±0.09[b]

Means with the same superscript along same columns are not significantly different ($p \geq 0.05$). (Mean values ± SE) n =15

Table 4.10: Time Effects of Acute Concentrations of Paraquat on some Behavioural Parameters of *C. gariepinus* in 96hrs

Time (hrs)	Opercular Ventilation Count	Tail Fin Movement Rate	Air Gulping Index
12	13.22 ± 4.00^{a}	123.00 ± 1.87^{a}	0.88 ± 0.07^{a}
24	25.17 ± 3.52^{b}	89.33 ± 1.47^{b}	0.82 ± 0.08^{a}
48	60.39 ± 2.85^{c}	108.17 ± 1.12^{c}	0.48 ± 0.05^{b}
72	64.78 ± 2.13^{c}	109.50 ± 1.40^{c}	0.50 ± 0.04^{b}
96	59.06 ± 2.09^{cd}	102.83 ± 0.57^{c}	0.33 ± 0.03^{c}

Means with the same superscript along same columns are not significantly different ($p\geq0.05$). (Mean values ± SE) n =18

and time showed that all the concentrations had the lowest tail fin movement rate in the 24th hr of exposure (Table 4.11). Within concentration, tail fin movement rate was lowest in the control fishes compared with the different concentrations (Table 4.9). Furthermore, the first 12 hours showed the highest rate of tail fin movement, thereafter, dropping significantly in the 24th hour to increase till the 96th hour (Table 4.10).

Similar pattern of effects as were observed in the fishes exposed to glyphosate were observed in the air gulping index of fish exposed to acute concentrations of paraquat. The interaction between the effects of paraquat in time and concentration is as presented in Table 4.12. A 3-fold increase was observed in air gulping index between control and the highest exposure at 12hrs. This decreased to 0.3 fold increase by 96 hrs (4.12). The control fish showed lower air gulping index as compared with the exposed fish. Fish exposed to two of the highest concentrations showed the highest air gulping index (Table 4.9). Fish also showed the highest air gulping index within the first two periods of observation i.e. 12th and 24th hr after exposure (Table 4.10). Other fish behaviours noticed in the course of the acute study included restlessness, hitting of tails against the walls of the holding medium, leading to haemorrhage and loss of some portions of their tails, abnormal swimming and hyperactivity.

4.3 Mortality/96-hr LC_{50} Values

Mortality was observed to increase with increasing concentrations of both herbicides, and the highest mortality was recorded with the highest concentrations. The mortality and probit values for fishes exposed to acute concentrations of glyphosate are shown in Table 4.13 while Table 4.14 shows the mortality and probit values for fishes exposed to acute concentrations of paraquat. The 96hr LC_{50} values for both herbicides were calculated based on these values and was found to be 0.53mg/L for glyphosate and 0.07mg/L for paraquat (Figs 4.1 and 4.2).

Table 4.11: Interaction Effects of Acute Concentrations of Paraquat with Time on *C. gariepinus* Tail Fin Movement Rate

	Time (hrs) and Tail Fin Movement Rate				
Conc (mg/L)	12	24	48	72	96
0.00	104.00 ± 1.10^{A}	102.00 ± 0.58^{A}	104.00 ± 1.00^{A}	103.00 ± 0.58^{A}	105.00 ± 1.00^{A}
0.012	129.00 ± 0.58^{B}	90.00 ± 1.00^{B}	110.00 ± 1.00^{BC}	105.00 ± 1.15^{A}	102.00 ± 1.15^{BC}
0.037	125.00 ± 1.15^{C}	87.00 ± 1.15^{B}	112.00 ± 1.15^{C}	106.00 ± 1.15^{A}	104.00 ± 0.58^{C}
0.055	128.00 ± 1.15^{B}	92.00 ± 0.58^{C}	108.00 ± 1.15^{B}	110.00 ± 1.00^{B}	100.00 ± 1.15^{B}
0.110	112.00 ± 0.58^{A}	89.00 ± 0.58^{B}	108.00 ± 0.58^{B}	115.00 ± 1.15^{C}	102.00 ± 1.15^{BC}
0.221	130.00 ± 1.00^{B}	81.00 ± 0.58^{D}	107.00 ± 1.15^{B}	118.00 ± 0.58^{D}	102.00 ± 0.58^{BC}

Means with the same superscript along same columns are not significantly different ($p\geq0.05$). (Mean values ± SE) n =3

Table 4.12: Interaction Effects of Acute Concentrations of Paraquat with Time on *C. gariepinus* Air Gulping Index

	Time (hrs) and Air Gulping Index				
Conc (mg/L)	12	24	48	72	96
0.00	0.40 ± 0.05^{a}	0.30 ± 0.05^{a}	0.40 ± 0.00^{a}	0.40 ± 0.05^{a}	0.30 ± 0.00^{a}
0.012	0.70 ± 0.05^{b}	0.80 ± 0.05^{b}	0.80 ± 0.05^{b}	0.70 ± 0.12^{b}	0.40 ± 0.06^{b}
0.037	0.80 ± 0.05^{b}	0.70 ± 0.05^{b}	0.50 ± 0.06^{a}	0.30 ± 0.06^{a}	0.20 ± 0.05^{a}
0.055	1.00 ± 0.05^{c}	1.10 ± 0.12^{c}	0.40 ± 0.06^{a}	0.50 ± 0.05^{c}	0.40 ± 0.06^{b}
0.110	1.20 ± 0.05^{d}	1.30 ± 0.05^{c}	0.50 ± 0.05^{a}	0.40 ± 0.05^{a}	0.33 ± 0.00^{a}
0.221	1.20 ± 0.05^{d}	0.70 ± 0.06^{b}	0.20 ± 0.05^{b}	0.70 ± 0.05^{b}	0.33 ± 0.00^{a}

Means with the same superscript along same columns are not significantly different ($p\geq0.05$). (Mean values ± SE) n =3

Table 4.13: Mortality and probit values of *Clarias gariepinus* exposed to Acute concentrations of Glyphosate at 12, 24, 48 and 96 hrs

Conc. (mg/L)	**Log $_{10}$ Conc.**	**Total number of fish Exposed**	**Number of Dead Fish**				**% Mortality**				**Probit Value (96hrs)**
			12	**24**	**48**	**96**	**12**	**24**	**48**	**96**	
0.00	0	30	0	0	0	0	0	0	0	0	0
0.36	-0.444	30	1	2	4	5	3.33	6.67	13.33	16.67	4.05
0.48	-0.319	30	3	5	7	10	10.00	16.67	23.33	33.33	4.56
0.60	-0.222	30	10	12	15	20	33.33	40.00	50.00	66.67	5.44
0.72	-0.143	30	10	14	20	24	33.33	46.67	66.67	80.00	5.84
0.84	-0.076	30	15	18	25	26	50.00	60.00	83.33	86.67	6.13

Table 4.14: Mortality and probit values of *Clarias gariepinus* exposed to Acute concentrations of Paraquat at 12, 24, 48 and 96 hrs

Conc. (mg/L)	**Log $_{10}$ Conc.**	**Total number of fish Exposed**	**Number of Dead Fish**				**% Mortality**				**Probit Value (96hrs)**
			12	**24**	**48**	**96**	**12**	**24**	**48**	**96**	
0.00	0	30	0	0	0	0	0	0	0	0	0
0.018	-1.744	30	0	1	1	2	0	3.33	3.33	6.67	3.12
0.037	-1.432	30	1	2	3	5	3.33	6.67	10.00	16.67	3.87
0.055	-1.260	30	4	6	9	10	13.33	20.00	30.00	33.33	5.44
0.110	-0.959	30	12	16	22	24	40.00	53.33	73.33	80.00	5.84
0.221	-0.656	30	14	20	24	27	46.67	66.67	73.33	90.00	6.28

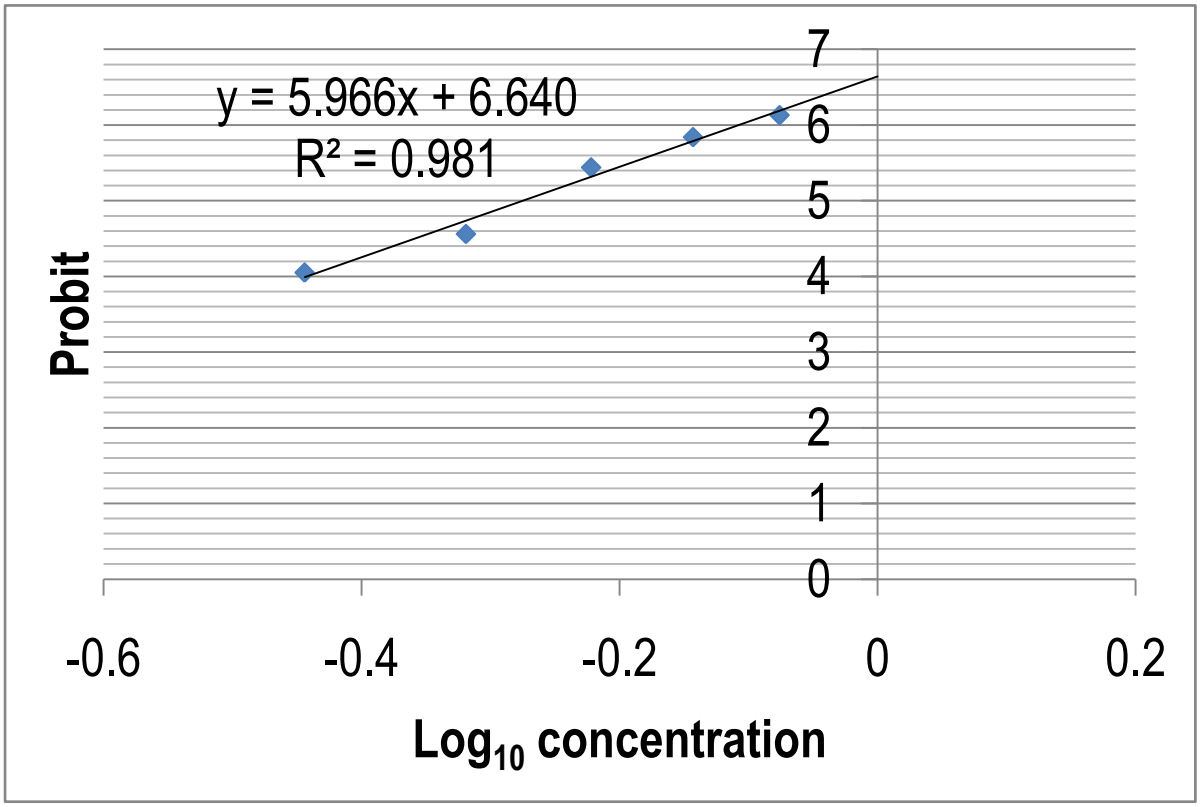


Figure 4.1: Determination of median lethal concentration (LC_{50}) of *Clarias gariepinus* exposed to glyphosate for 96 hrs by probit method

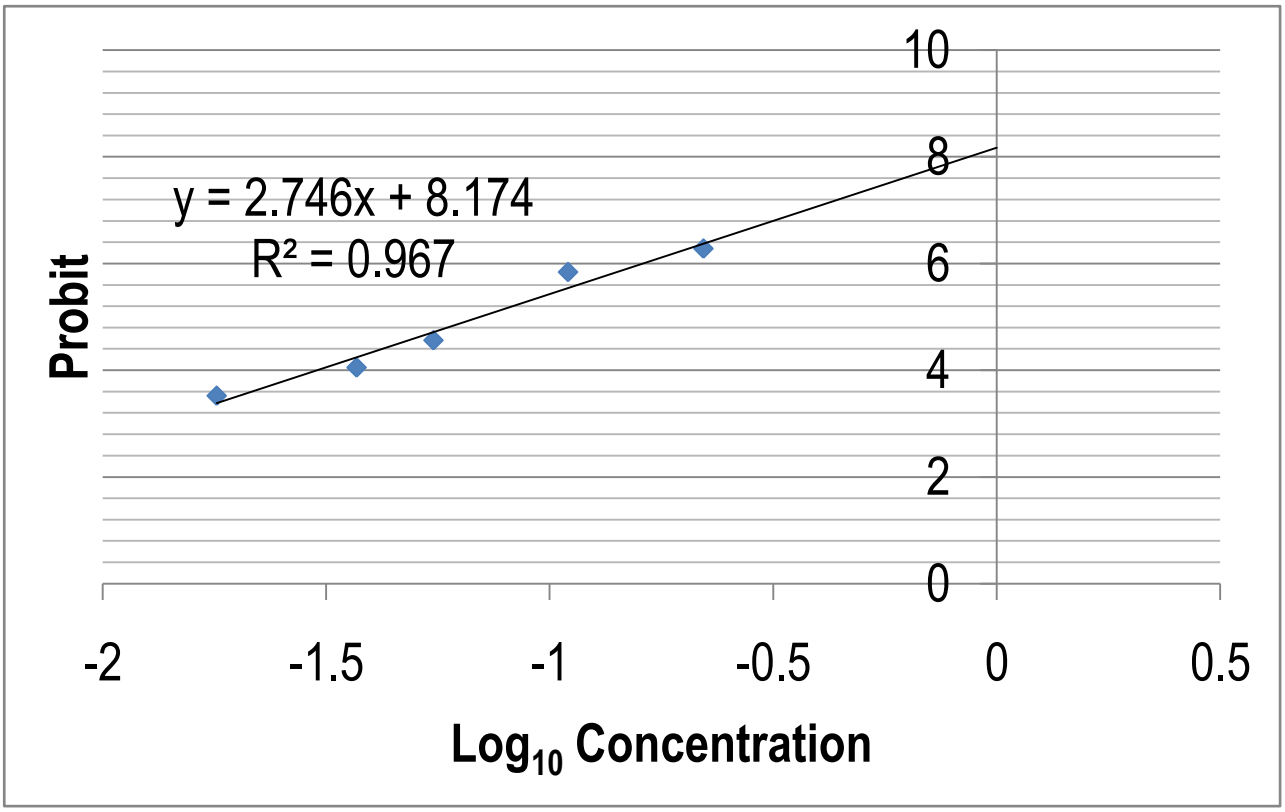


Figure 4.2: Determination of median lethal concentration (LC_{50}) of *Clarias gariepinus* exposed to glyphosate for 96 hrs by probit method

4.4 Effects of Acute Concentrations of Glyphosate and Paraquat on Fish Biochemical Parameters after 96 hrs.

Activities of the stimulated liver enzymes of fish exposed to acute concentrations of glyphosate and paraquat are presented in Tables 4.15 and 4.16 respectively. There was an increase in the activity of all the enzymes as the concentrations of glyphosate increased and these increases were significant ($p \leq 0.05$) in the activities of all the enzymes. Comparing the control and the highest concentration, there was 1.50, 1.56, 1.44 and 1.53% fold increase in the activities of ALT, AST, LDH and ALP respectively. AST has the highest % fold increase (Table 4.15).

A similar observation was observed in the liver of fish exposed to acute concentrations of paraquat (Table 4.16). Increase in enzyme activities was noticed to be concentration dependent. These increases in the activity of all the enzymes were also significant ($p \leq 0.05$). The % fold increase in the activities of the enzymes was 1.49, 1.44, 1.36 and 1.58% for ALT, AST, LDH and ALP respectively. ALP has the highest % fold increase (Table 4.16).

Activities of antioxidant enzymes in fish exposed to acute concentrations of glyphosate are presented in table 4.17. All of these enzymes increased with increasing concentration of glyphosate. Increases in the activities of these enzymes were significant ($p \leq 0.05$). The % fold increases for CAT, SOD, MDA and GPx are 1.34, 1.95, 1.72 and 1.70% respectively between control and the highest concentration. SOD showed the highest % fold increase. Similarly, table 4.18 shows the result of antioxidant enzymes exposed to acute concentrations of paraquat. There were also significant increases ($p \leq 0.05$) in the activities of all of these enzymes. Between control and the highest concentration, % fold increase was 1.27, 2.07, 1.10 and 1.76% for CAT, SOD, MDA and GPx respectively. Again, SOD was observed to have the highest % fold increase.

Table 4.15: Activities of stimulated liver enzymes in *C. gariepinus* after exposure to Acute concentrations of Glyphosate

Concentrations (mg/L)	**ALT (IU)**	**AST (IU)**	**LDH (IU)**	**ALP (IU)**
0.00	60.32 ± 0.87^{a}	59.19 ± 1.59^{a}	70.57 ± 1.11^{a}	21.89 ± 0.84^{a}
0.36	73.12 ± 1.88^{a}	74.04 ± 1.67^{b}	88.63 ± 0.89^{b}	24.50 ± 1.09^{b}
0.48	78.50 ± 1.42^{b}	80.06 ± 1.06^{c}	96.75 ± 1.00^{c}	26.74 ± 0.35^{c}
0.60	82.08 ± 1.70^{c}	85.32 ± 1.91^{d}	98.30 ± 1.60^{d}	27.65 ± 1.08^{c}
0.72	87.38 ± 1.19^{d}	87.86 ± 1.76^{e}	99.18 ± 0.92^{d}	31.23 ± 0.64^{d}
0.84	90.20 ± 1.21^{e}	92.27 ± 1.88^{f}	101.51 ± 0.84^{e}	33.65 ± 0.86^{e}
% Fold Increase	1.50	1.56	1.44	1.53

Values with the same superscript in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 4 fishes)

ALT – Alanine aminotransferase
AST – Aspartate aminotransferase
LDH – Lactate dehydrogenase
ALP – Alkaline phosphatase

Table 4.16: Activities of stimulated liver enzymes in *C. gariepinus* after exposure to Acute concentrations of Paraquat

Concentrations (mg/L)	**ALT (IU)**	**AST (IU)**	**LDH (IU)**	**ALP (IU)**
0.00	61.71 ± 1.23^{a}	63.08 ± 1.03^{a}	72.81 ± 0.91^{a}	20.43 ± 1.12^{a}
0.012	76.22 ± 1.17^{b}	75.11 ± 1.15^{b}	85.31 ± 1.18^{b}	22.74 ± 1.46^{b}
0.037	79.22 ± 0.92^{c}	82.29 ± 0.97^{c}	87.44 ± 0.85^{c}	23.55 ± 1.10^{b}
0.055	83.08 ± 1.26^{d}	86.24 ± 1.12^{d}	90.42 ± 1.13^{d}	26.84 ± 1.23^{c}
0.110	86.53 ± 0.92^{e}	88.95 ± 0.72^{d}	95.20 ± 0.86^{e}	28.56 ± 1.31^{c}
0.221	91.71 ± 1.16^{f}	90.81 ± 1.24^{e}	99.08 ± 0.99^{f}	32.25 ± 0.94^{d}
% Fold Increase	1.49	1.44	1.36	1.58

Values with the same superscript in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 3 fishes)

ALT – Alanine aminotransferase
AST – Aspartate aminotransferase
LDH – Lactate dehydrogenase
ALP – Alkaline phosphatase

Table 4.17: Activities of antioxidant enzymes after exposure to Acute concentrations of Glyphosate

Concentration (mg/L)	CAT (Unit/mg protein)	SOD (Unit/mg protein)	MDA(nmole/mg protein)	GPx (mmol/min/mg protein)
0.00	117.88±0.97[a]	18.17±0.92[a]	1.26±0.04[a]	1.97±0.09[a]
0.36	134.27±1.19[b]	25.18±0.73[b]	1.54±0.06[b]	3.20±0.08[b]
0.48	136.63±1.06[c]	27.19±0.68[c]	1.89±0.08[c]	3.27±0.05[bc]
0.60	150.99±1.08[d]	30.29±0.83[c]	2.11±0.09[d]	3.34±0.10[cd]
0.72	155.56±0.96[e]	31.40±1.09[d]	2.17±0.06[d]	3.38±0.06[d]
0.84	158.56±1.22[f]	35.39±0.58[e]	2.17±0.05[d]	3.34±0.07[d]
% Fold Increase	1.34	1.95	1.72	1.70

Values with the same superscript in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 4 fishes)

CAT – Catalase
SOD – Superoxide dismutase
MDA – Malondialdehyde
GPx – Glutathione peroxidase

Table 4.18: Activities of antioxidant enzymes after exposure to Acute concentrations of Paraquat.

Concentrations (mg/L)	**CAT (Unit/mg protein)**	**SOD (Unit/mg protein)**	**MDA(nmole/mg protein)**	**GPx mmol/min/mg protein)**
0.00	122.16±0.78^{a}	17.18±1.29^{a}	1.35±0.02^{a}	1.97±0.03^{a}
0.012	136.40±1.17^{b}	22.33±1.02^{a}	1.36±0.02^{a}	3.14±0.09^{b}
0.037	140.49±1.20^{c}	24.95±1.19^{b}	1.39±0.01^{b}	3.36±0.09^{c}
0.055	146.83±2.19^{d}	30.23±1.17^{c}	1.40±0.01^{b}	3.36±0.05^{c}
0.110	151.90±1.81^{e}	32.18±1.38^{d}	1.43±0.03^{b}	3.39±0.08^{c}
0.221	154.87±1.42^{f}	35.56±1.08^{e}	1.49±0.03^{c}	3.46±0.06^{c}
% Fold Increase	1.27	2.07	1.10	1.76

Values with the same superscript in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 3 fishes)

CAT – Catalase
SOD – Superoxide dismutase
MDA – Malondialdehyde
GPx – Glutathione peroxidase

4.5 Activities of Liver Enzymes in Fish Exposed to Long Term Sublethal Concentrations of Glyphosate and Paraquat

Glyphosate

Liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were significantly elevated correspondingly with increasing concentrations of glyphosate (Tables 4.19 – 4.22). The increase in the activities of these enzymes was also time dependent. In the liver of fish exposed to glyphosate, there was a significant increase in ALT levels only in the highest concentration in the first two weeks. All three concentrations (excluding control) showed significant increases ($p \leq 0.05$) in ALT activity compared with the control values during the eight weeks exposure period. In week 2, there 1.08% fold increase, 1.12, 1.13 and 1.16% fold increases at weeks 4, 6 and 8 respectively were observed between the control and the highest concentrations (Table 4.19). There was no significant increase in AST activity for all concentrations in weeks 2 and 4; even though there was 1.16 and 1.45 % fold increase respectively in AST activity in this period. Also, there was 1.14 and 1.37 % fold increase in weeks 6 and 8 respectively (Table 4.20). Furthermore, only at week did the highest concentration show a significant increase in AST activity with time.

For LDH, significant increase ($p<0.05$) was observed only at week 8 for concentration 0.0265mg/L throughout the eight week period. The LDH levels in the liver of control fish were also not significant during the period. However, there was 1.01, 1.10, 1.15 and 1.21 % fold increase at weeks 2, 4, 6 and 8 respectively between the control and the highest concentration (Table 4.21).

For ALP, its activity increased significantly with time only in the highest concentration throughout the eight week period. However, there was a significant increase in ALP levels within concentrations. There was 1.07, 1.08, 1.11 and 1.10 % fold increase at weeks 2, 4, 6 and 8 respectively between the control and the highest concentration (Table 4.22).

Table 4.19: Activities of alanine aminotransferase ALT (IU) in *C. gariepinus* exposed to Sublethal concentrations of Glyphosate in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	59.20±2.49 ^{aA}	58.78±1.21 ^{aA}	59.56±2.16 ^{aA}	60.23±2.72 ^{aA}
0.0265	59.08±2.89 ^{aA}	59.22±2.08 ^{abA}	62.21±3.06 ^{bcA}	64.35±2.10 ^{cB}
0.053	60.04±2.76 ^{aA}	61.21±3.05 ^{aA}	65.23±2.51 ^{bB}	66.77±2.20 ^{bB}
0.106	63.87±3.27 ^{aB}	65.88±3.82 ^{abB}	67.31±2.59 ^{bC}	69.99±3.53 ^{bC}
% Fold Increase	1.08	1.12	1.13	1.16

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Table 4.20: Activities of aspartate aminotransferase AST (IU) in *C. gariepinus* exposed to Sublethal concentrations of Glyphosate in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	58.41±2.06 ^{aA}	59.94±1.06 ^{aA}	60.70±3.17 ^{aA}	58.10±2.03 ^{aA}
0.0265	62.95±2.24 ^{aA}	63.95±2.48 ^{aA}	66.62±3.62 ^{aB}	67.29±2.40 ^{aB}
0.053	65.62±2.93 ^{aA}	66.30±1.43 ^{aA}	68.96±2.19 ^{aB}	67.88±2.55 ^{aB}
0.106	67.76±2.03 ^{aA}	68.61±3.42 ^{aA}	69.31±2.49 ^{aB}	79.87±2.92 ^{bC}
% Fold Increase	1.16	1.45	1.14	1.37

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Table 4.21: Activities of lactate dehydrogenase LDH (IU) in *C. gariepinus* exposed to Sublethal concentrations of Glyphosate in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	71.55±3.15 ^{aA}	71.13±2.23 ^{aA}	72.18±3.70 ^{aA}	70.66±2.06 ^{aA}
0.0265	70.03±2.34 ^{aA}	72.49±2.81 ^{aA}	74.01±2.40 ^{aA}	80.14±2.85 ^{bB}
0.053	72.99±2.75 ^{aA}	75.82±2.58 ^{aAB}	83.57±3.49 ^{aB}	84.11±3.37 ^{aB}
0.106	72.45±3.80 ^{aA}	78.12±3.18 ^{aB}	82.85±3.01 ^{aB}	85.15±3.36 ^{aB}
% Fold Increase	1.01	1.10	1.15	1.21

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Table 4.22: Activities of alkaline phosphatase ALP (IU) in *C. gariepinus* exposed to Sublethal concentrations of Glyphosate in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	20.53±1.25 ^{aA}	20.73±1.03 ^{aA}	20.86±2.73 ^{aA}	21.46±2.46 ^{aA}
0.0265	20.08±1.82 ^{aAB}	20.18±2.05 ^{aAB}	21.68±2.92 ^{aAB}	21.69±2.14 ^{aAB}
0.053	20.13±2.25 ^{aB}	22.05±2.99 ^{aB}	22.35±1.91 ^{aB}	21.77±2.62 ^{aAB}
0.106	21.89±2.17 ^{aAB}	22.35±3.20 ^{bB}	23.19±1.14 ^{bB}	23.56±2.27 ^{bB}
% Fold Increase	1.07	1.08	1.11	1.10

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Paraquat

In the liver of fishes exposed to paraquat, the control and the lowest concentration did not show any significant increase in ALT levels throughout the exposure period (Table 4.23). The two highest concentrations showed significant rise in the levels of ALT over the exposure period (Table 4.23). Also, there was no significant increase in ALT levels in week 2 within concentration but there was 1.08% fold increase between control and the highest concentration. Also, there was 1.15, 1.22 and 1.26 % fold increase at weeks 4, 6 and 8 respectively.

For AST, there was no significant increase in AST levels of the control fish. AST levels increased significantly ($p<0.05$) with time in all concentrations throughout the exposure period. There was 1.05, 1.06, 1.11 and 1.15 % fold increase at weeks 2, 4, 6 and 8 respectively between control and the highest concentration (Table 4.24).

The increase in levels of LDH with time was significant ($p<0.05$) in all the three concentrations; whereas, there was no significant increase ($p>0.05$) in LDH levels within concentration throughout the exposure period (Table 4.25). However, there was 1.05, 1.07, 1.06 and 1.06 % fold increase at weeks 2, 4, 6 and 8 respectively between the control and the highest concentration.

The levels of ALP increased significantly ($p<0.05$) with time in the two highest concentrations only. ALP levels increased significantly ($p<0.05$) within concentration only between weeks 4 and 8. However, there was 1.01, 1.06, 1.12 and 1.18 % fold increase in weeks 2, 4, 6 and 8 respectively between control and highest concentration (Table 4.26).

4.6 Antioxidant Enzyme Activities in Liver of Fish Exposed to Long Term Sublethal Concentrations of Glyphosate

Antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activity and malondialdehyde (MDA) content in the liver tissue of *Clarias gariepinus* in 56 days of exposure to glyphosate are presented in the Tables 4.27 to 4.30. The activity of SOD

Table 4.23: Activities of alanine aminotransferase ALT (IU) in *C. gariepinus* exposed to Sublethal concentrations of Paraquat in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	60.42±2.06^{aA}	61.79±2.30 ^{aA}	61.06±2.05 ^{aA}	59.99±1.65 ^{aA}
0.0035	63.65±2.52 ^{aA}	65.43±2.26 ^{aB}	65.86±3.85 ^{aA}	69.16±3.74 ^{aB}
0.007	63.93±2.32 ^{aA}	67.26±3.74 ^{abB}	72.35±3.22 ^{bcB}	74.38±4.09 ^{cB}
0.014	65.53±3.64 ^{aA}	70.87±3.10 ^{abB}	74.23±3.72 ^{bB}	75.67±4.77 ^{bB}
% Fold Increase	1.08	1.15	1.22	1.26

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Table 4.24: Activities of aspartate aminotransferase AST (IU) in *C. gariepinus* exposed to Sublethal concentrations of Paraquat in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	64.17±2.82 ^{aA}	62.03±2.26 ^{aA}	62.25±2.94 ^{aA}	63.65±3.51 ^{aA}
0.0035	62.02±2.66 ^{aAB}	63.28±2.29 ^{aA}	66.41±1.98 ^{abAB}	72.23±2.85 ^{bB}
0.007	65.50±3.92 ^{aA}	63.73±2.08 ^{aA}	67.52±2.52 ^{abB}	72.58±2.36 ^{bB}
0.014	67.61±2.17 ^{aAC}	65.50±2.87 ^{abA}	69.22±2.34 ^{aB}	73.14±2.13 ^{acB}
% Fold Increase	1.05	1.06	1.11	1.15

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Table 4.25: Activities of lactate dehydrogenase LDH (IU) in *C. gariepinus* exposed to Sublethal concentrations of Paraquat in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	70.21±2.30 ^{aA}	71.04±2.64 ^{aA}	72.52±2.89 ^{aA}	72.19±2.13 ^{aA}
0.0035	68.86±2.46 ^{aA}	71.49±2.66 ^{abA}	71.23±2.18 ^{abA}	72.70±1.01 ^{bA}
0.007	70.20±2.49 ^{aA}	73.58±2.23 ^{abA}	74.87±2.25 ^{bA}	75.54±2.12 ^{bA}
0.014	73.72±2.28 ^{aA}	75.89±2.34 ^{abA}	76.69±1.23 ^{bA}	76.83±2.01 ^{bA}
% Fold Increase	1.05	1.07	1.06	1.06

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Table 4.26: Activities of alkaline phosphatase ALP (IU) in *C. gariepinus* exposed to Sublethal concentrations of Paraquat in eight weeks

	WEEKS			
Concentrations (mg/L)	**2**	**4**	**6**	**8**
0.00	19.57±1.43 ^{aA}	19.01±1.96 ^{aA}	18.53±2.18 ^{aA}	18.32±1.14 ^{aA}
0.0035	18.49±3.06 ^{aA}	19.63±1.43 ^{aA}	19.75±1.02 ^{aAB}	19.96±1.68 ^{aAB}
0.007	19.52±1.61 ^{aA}	19.99±2.49 ^{aA}	19.98±1.52 ^{abBC}	21.33±2.08 ^{bBC}
0.014	19.87±1.33 ^{aA}	20.15±1.89 ^{abA}	20.83±1.57 ^{bcC}	21.69±1.70 ^{cC}
% Fold Increase	1.01	1.06	1.12	1.18

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Table 4.27: Activities of superoxide dismutase (Unit/mg protein) in *C. gariepinus* exposed to Sublethal concentrations of Glyphosate in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	18.11±0.15 ^{aA}	18.05±0.14 ^{aA}	17.97±0.26 ^{aA}	18.01±0.11 ^{aA}
0.0265	18.21±0.26 ^{aAB}	20.23±0.26 ^{aB}	20.46±0.62 ^{bB}	21.97±1.04 ^{bB}
0.053	19.43±0.11 ^{aB}	21.01±0.21 ^{abB}	22.21±0.94 ^{bB}	23.45±1.78 ^{bB}
0.106	19.64±0.12 ^{aB}	22.25±0.22 ^{bB}	23.09±0.90 ^{bB}	23.92±0.87 ^{bB}
% Fold Increase	1.08	1.23	1.28	1.33

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Table 4.28: Activities of catalase (Unit/mg protein) in *C. gariepinus* exposed to Sublethal concentrations of Glyphosate in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	117.30±3.35 ^{aA}	117.27±3.88 ^{aA}	115.53±3.11 ^{aA}	116.99±3.88 ^{aA}
0.0265	119.08±3.26 ^{aA}	122.11±4.23 ^{abA}	124.01±4.07 ^{abB}	125.35±5.03 ^{bB}
0.053	120.51±3.92 ^{aA}	122.16±4.38 ^{abA}	123.05±4.59 ^{abB}	126.93±5.08 ^{bB}
0.106	122.06±3.28 ^{aA}	123.47±4.59 ^{abA}	128.73±4.16 ^{abB}	130.09±5.11 ^{bB}
% Fold Increase	1.04	1.05	1.11	1.11

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Table 4.29: Activities of malondialdehyde (nmole/mg protein) in *C. gariepinus* exposed to Sublethal concentrations of Glyphosate in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	1.26±0.01 ^{aA}	1.27±0.10 ^{aA}	1.26±0.02 ^{aA}	1.28±0.02 ^{aA}
0.0265	1.28±0.06 ^{aA}	1.30±0.06 ^{bA}	1.38±0.01 ^{bB}	1.39±0.01 ^{bB}
0.053	1.29±0.02 ^{aA}	1.31±0.02 ^{bA}	1.41±0.03 ^{bB}	1.45±0.03 ^{bAB}
0.106	1.31±0.01 ^{aA}	1.33±0.01 ^{abA}	1.39±0.03 ^{bB}	1.45±0.03 ^{bB}
% Fold Increase	1.04	1.05	1.10	1.13

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Table 4.30: Activities of glutathione peroxidase (mmol/min/mg protein) in *C. gariepinus* exposed to Sublethal concentrations of Glyphosate in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	1.89±0.08 ^{aA}	2.15±0.10 ^{aA}	2.14±0.14 ^{aA}	1.91±0.09 ^{aA}
0.0265	2.57±0.14 ^{aB}	2.81±0.17 ^{abB}	3.26±0.14 ^{cB}	3.15±0.16 ^{bcB}
0.053	2.97±0.16 ^{aC}	2.99±0.19 ^{aB}	3.04±0.17 ^{aB}	3.15±0.14 ^{aB}
0.106	2.59±0.17 ^{aB}	3.09±0.18 ^{bB}	3.10±0.19 ^{bB}	3.01±0.18 ^{bB}
% Fold Increase	1.37	1.44	1.45	1.58

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

in the liver increased in a concentration and time dependent manner at the end of the 56 day exposure period (Table 4.27) with a significant increase ($p < 0.05$) in SOD levels in all exposure concentrations between week 2 and week 8. Furthermore, there is 1.08, 1.23, 1.28 and 1.33 % fold increase at weeks 2, 4, 6 and 8 respectively between the control and the highest concentration.

Activity of CAT was also concentration and time dependent over the 56 day exposure period. No significant increase ($p>0.05$) in activities of catalase in fishes in the control group. There was a significant increase ($p<0.05$) in CAT levels in all of the concentrations between week 2 and week 8. However, significant increase in CAT levels was noticed only in weeks six and eight though there is 1.04, 1.05, 1.11 and 1.11 % fold increase at weeks 2, 4, 6 and 8 respectively between control and highest concentration (Table 4.28).

Pattern of increase in MDA levels in the liver was also similar. MDA levels in the liver increased significantly ($p < 0.05$) in all of the concentrations between week 2 and week 8. However, significant increase ($p < 0.05$) in MDA levels within concentration was recorded only beginning from week 6. The % fold increase for MDA was 1.04, 1.05, 1.10 and 1.13 at weeks two, four, six and eight respectively between control and the highest concentration. There was no significant increase ($p > 0.05$) in activities of MDA in fishes in the control group (Table 4.29).

Glutathione peroxidase levels in the liver also increased significantly ($p < 0.05$). Table 4.30 shows that apart from the control and concentration of 0.053mg/l, all other concentrations showed significant increases in GPx levels ($p < 0.05$) from week 2 to week 8. Also, there was significant increase ($p<0.05$) within concentration in each of the period under observation. GPx levels showed 1.37, 1.44, 1.45 and 1.58 % fold increase at weeks 2, 4, 6 and 8 respectively between control and the highest concentration. There was no significant increase ($p > 0.05$) in activities of GPx in liver of fish in the control group (Table 4.30).

4.7 Antioxidant Enzyme Activities in Liver of Fish Exposed to Long Term Sublethal Concentrations of Paraquat

Antioxidant enzymes (SOD, CAT and GPx) activities and malondialdehyde content in the liver tissues of *Clarias gariepinus* after 56days of exposure to paraquat are summarised in Tables 4.31 to 4.34.

Similar to what was noticed in glyphosate, Table 4.31 shows that SOD activities in fish liver increased significantly ($p<0.05$) as the concentration and as time of exposure increased. Significant increase ($p<0.05$) in SOD levels was not recorded until week six in all concentrations excluding control. Also, there was a significant increase in SOD levels in each of the period under observation within concentration. The % fold increase is 1.11, 1.14, 1.18 and 1.28 % at weeks 2, 4, 6 and 8 respectively between control and highest concentration. There was no significant increase ($p > 0.05$) in the activity of SOD in fishes in the control group.

CAT levels increased with concentration and time, and were significant ($p < 0.05$). There was a significant increase in CAT levels within the concentration starting from week six to the eighth week. However, significant increase in CAT levels was observed beginning from week 6 for all the concentrations. There was 1.15, 1.19, 1.31 and 1.38 % fold increase at weeks 2, 4, 6 and 8 respectively between control and the highest concentration. In the control group, there was no significant difference ($p > 0.05$) in the increase (Table 4.32).

Table 4.33 shows that GPx levels increased significantly ($p < 0.05$) in a concentration dependent manner; however, significant increase within concentration was noticed from the fourth to the eighth week. There was no significant increase ($p > 0.05$) in exposure concentrations throughout exposure period. There was 1.25, 1.60, 1.08 and 1.58 % fold increase at weeks 2, 4, 6 and 8 respectively between control and highest concentration. Furthermore, there was no significant difference ($p > 0.05$) in the increase in GPx levels in the control.

Table 4.31: Activities of superoxide dismutase (Unit/mg protein) in *C. gariepinus* exposed to Sublethal concentrations of Paraquat in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	17.50±0.84 ^{aA}	17.96±0.56 ^{aA}	17.56±0.33 ^{aA}	17.23±0.31 ^{aA}
0.0035	18.06±1.20 ^{aA}	18.98±0.82 ^{aA}	19.29±0.53 ^{abB}	20.89±1.22 ^{bB}
0.007	18.13±0.77 ^{aA}	19.87±1.12 ^{aA}	20.57±0.35 ^{bB}	21.21±1.51 ^{cB}
0.014	19.45±0.89 ^{aB}	20.41±0.67 ^{aB}	20.65±1.39 ^{bB}	21.32±0.98 ^{bB}
% Fold Increase	1.11	1.14	1.18	1.24

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Table 4.32: Activities of catalase (Unit/mg protein) in *C. gariepinus* exposed to Sublethal concentrations of Paraquat in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	90.77±3.66 ^{aA}	91.00±3.41 ^{aA}	89.22±3.19 ^{aA}	88.34±2.10 ^{aA}
0.0035	100.36±4.40 ^{aA}	105.61±3.73 ^{abA}	110.45±3.25 ^{abB}	118.66±3.08 ^{bB}
0.007	101.41±4.78 ^{aA}	106.78±3.40 ^{aA}	114.26±3.54 ^{abB}	120.65±3.25 ^{bB}
0.014	104.61±4.15 ^{aA}	108.09±4.62 ^{aA}	116.97±3.64 ^{abB}	122.13±3.130 ^{bB}
% Fold Increase	1.15	1.19	1.31	1.38

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Table 4.33: Activities of glutathione peroxidase (mmol/min/mg protein) in *C. gariepinus* exposed to Sublethal concentrations of Paraquat in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	1.61±0.11 ^{aA}	1.60±0.11 ^{aA}	1.62±0.14 ^{aA}	1.60±0.12 ^{aA}
0.0035	1.80±0.13 ^{aA}	2.19±0.14 ^{aB}	2.10±0.14 ^{aAB}	2.24±0.16 ^{aAB}
0.007	1.95±0.16 ^{aA}	2.25±0.16 ^{aB}	2.36±0.19 ^{aB}	2.59±0.20 ^{aB}
0.014	2.01±0.18 ^{aA}	2.56±0.18 ^{aB}	1.75±0.17 ^{aAB}	2.52±0.19 ^{aB}
% Fold Increase	1.25	1.60	1.08	1.58

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

Table 4.34: Activities of malondialdehyde (nmole/mg protein) in *C. gariepinus* exposed to Sublethal concentrations of Paraquat in eight weeks

Concentrations	**WEEKS**			
(mg/L)	**2**	**4**	**6**	**8**
0.00	1.29±0.01 ^{aA}	1.28±0.01 ^{aA}	1.28±0.01 ^{aA}	1.28±0.01 ^{aA}
0.0035	1.28±0.01 ^{aA}	1.29±0.01 ^{abA}	1.32±0.02 ^{bB}	1.31±0.01 ^{abAB}
0.007	1.29±0.01 ^{aA}	1.30±0.01 ^{aA}	1.32±0.01 ^{aB}	1.32±0.01 ^{aB}
0.014	1.29±0.01 ^{aA}	1.31±0.01 ^{abA}	1.33±0.01 ^{bB}	1.34±0.01 ^{bB}
% Fold Increase	1.00	1.02	1.04	1.05

Values with the same small letter superscript (a) in the same row, and values with the same capital letter superscript (A) in the same column are not significant ($p \geq 0.05$). (Mean values ± SE are for 6 fishes)

MDA levels also increased significantly ($p<0.05$) with concentration and time. Also, significant increase was noticed within the concentrations only in weeks 6 and 8. % fold increase was 1.00, 1.02, 1.04 and 1.05 % at weeks 2, 4, 6 and 8 respectively between control and the highest concentration. There was no significant difference ($p > 0.05$) in the increase in fish in the control group (Table 4.34).

4.8 Histological Analysis of Liver, Brain and Gill Tissues Exposed to Acute Concentrations of Glyphosate and Paraquat

The different pathological observations in the gills, brain and liver of fish after exposure to acute concentrations to glyphosate and paraquat are presented in Tables 4.35 and 4.36 respectively. All fish organs showed architectural damage to exposure with both herbicides. There were no pathological changes to the gills of control fish (Plate II). The gills of fish exposed to acute concentrations of glyphosate showed thickening of secondary lamella, massive sloughing off of lamella and loss of epithelial cells (Plate II). The gills of fish of exposed to acute concentrations of paraquat showed cellular infiltration, marked thickening of the gills with fibrous metaplasia of the covering epithelium, epithelial swelling of secondary lamella and swelling of epithelial cells (Plate III).

There were no pathological changes to the brain of control fish. The brain of fishes exposed to glyphosate showed vacoules in neurons and blood cells, accumulation of glial cells (gliosis) as well as multiple foci of severe spongiform encephalopathy and extensive foci of spongiform encephalopathy as well as necrosis of a few neurons (Plate IV). Brain of fishes exposed to paraquat also showed numerous large vacoules in neutrophils (i.e. blood cells), moderate congestion of blood vessels and multiple foci of liquefied necrosis (Plate V).

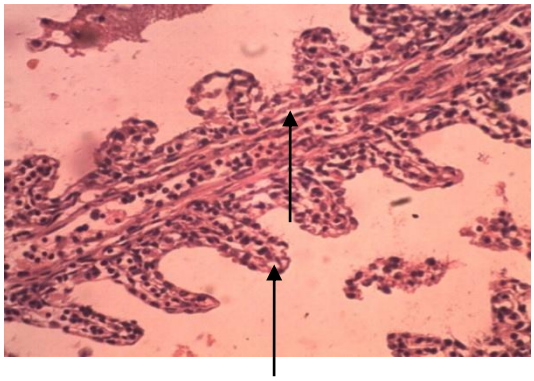
Table 4.35: Pathological changes in different fish organs exposed to Acute concentrations of Glyphosate

Organs	Concentrations (mg/L)	Pathological Observations
Gills		
	0.00	Nil
	0.36	Clubbing of gills
	0.48	Thickening of secondary lamella
	0.60	Oedema
	0.72	Massive sloughing off of gill lamella
	0.84	Loss of epithelial cells
Liver		
	0.00	Nil
	0.36	Vacuolation of hepatocytes
	0.48	Vacuolation of hepatocytes
	0.60	Vacuolation of hepatocytes
	0.72	Vacuolation of hepatocytes
	0.84	Vacuolar degeneration of hepatocytes
Brain		
	0.00	Nil
	0.36	Neuronal degeneration
	0.48	Neuronal degeneration
	0.60	Spongiform encephalopathy
	0.72	Gliosis
	0.84	Neuronal necrosis

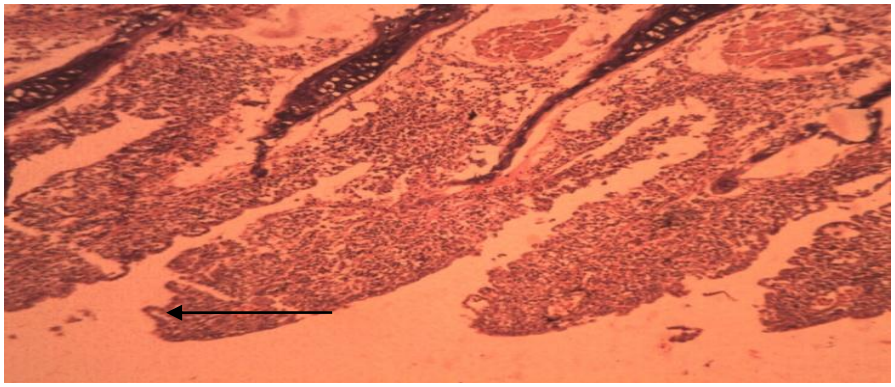
Table 4.36: Pathological changes in different fish organs exposed to Acute concentrations of Paraquat

Organs	Concentrations (mg/L)	Pathological Observations
Gills		
	0.00	Nil
	0.012	Thickening of gills with fibrous metaplasia of covering epithelium
	0.037	Epithelial swelling of secondary lamella
	0.055	Cellular infiltration
	0.110	Loss of gill lamella
	0.221	Cellular infiltration
Liver		
	0.00	Nil
	0.012	Vacuolar degeneration of hepatocytes
	0.037	Vacuolar degeneration of hepatocytes
	0.055	Vacuolar degeneration of hepatocytes
	0.110	Vacuolar degeneration of hepatocytes
	0.221	Vacuolation of hepatocytes
Brain		
	0.00	Nil
	0.012	Large cytoplasmic vacuoles in heterophils
	0.037	Congestion of blood vessels
	0.055	Moderate congestion of blood vessels
	0.110	Multiple foci of liquefactive necrosis
	0.221	Gliosis

a



b



c

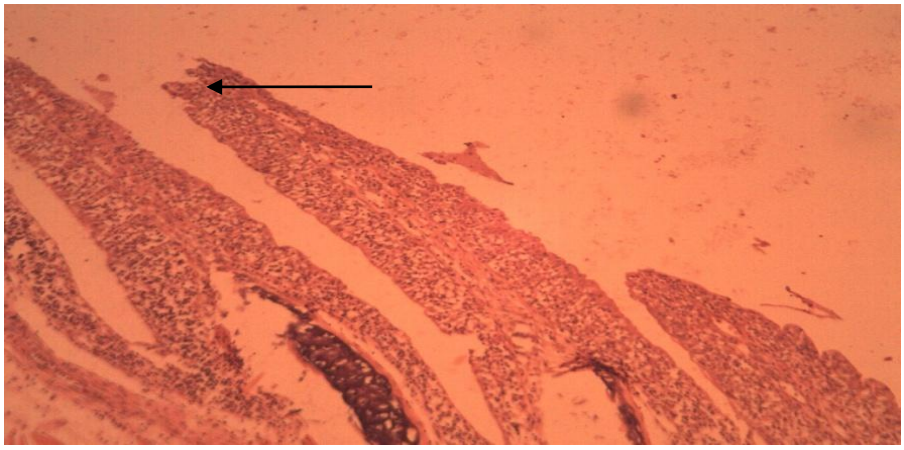


Plate II: Photomicrograph of the gills of *C. gariepinus* after exposure to glyphosate for 96 hrs showing (a) Normal fish gill structure with gill filaments and villi in control fish, H & E Stain X100 (b) Thickening of secondary lamella in gills of fish at 0.48mg/L, (c) Loss of epithelial cells at 0.84mg/L. H & E Stain X400

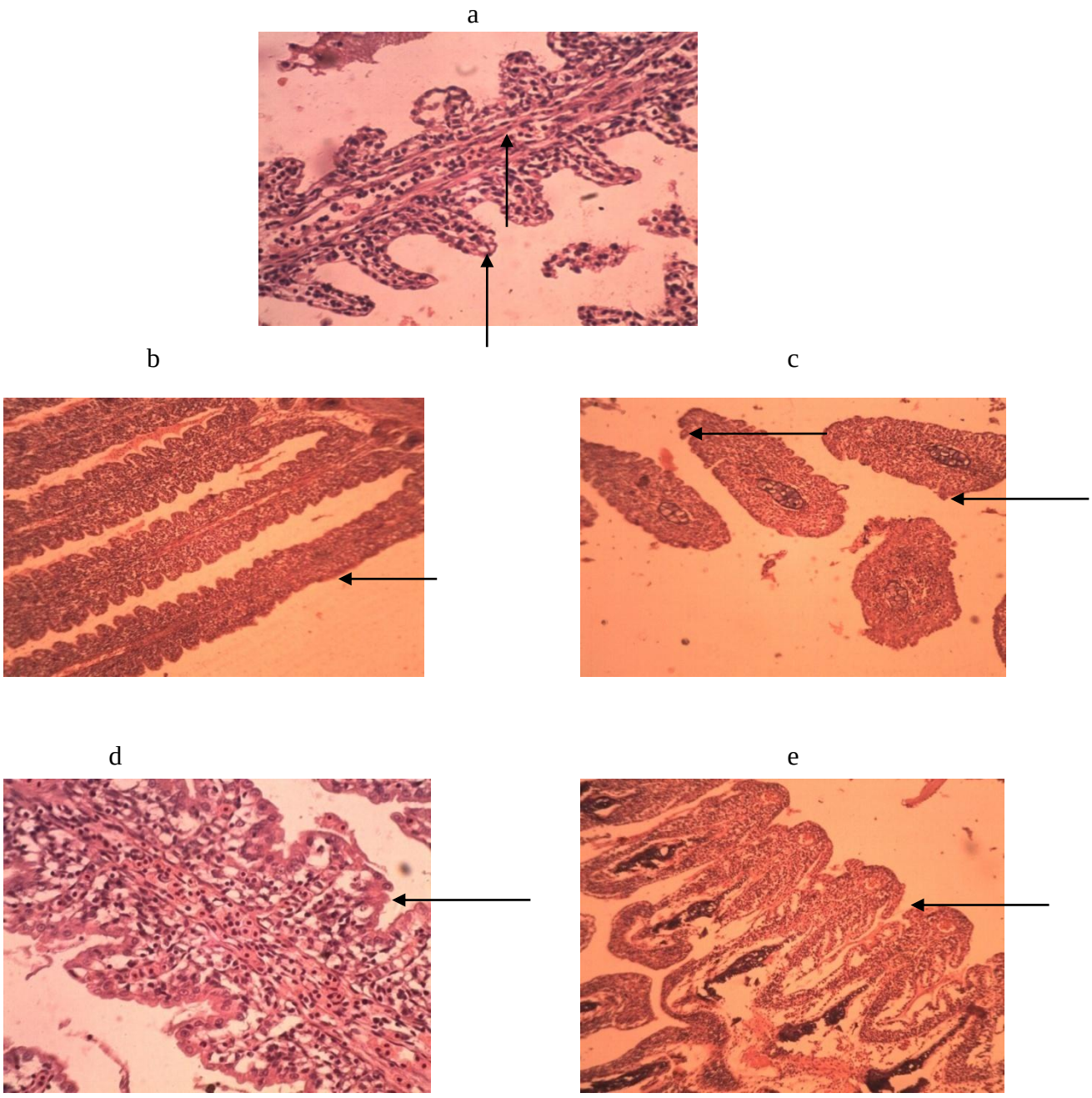
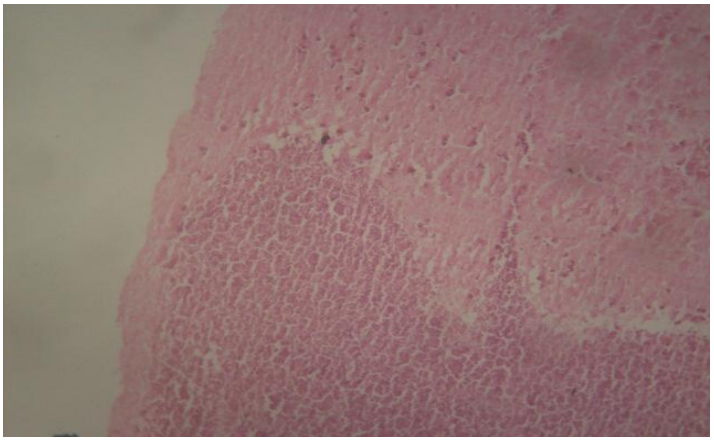
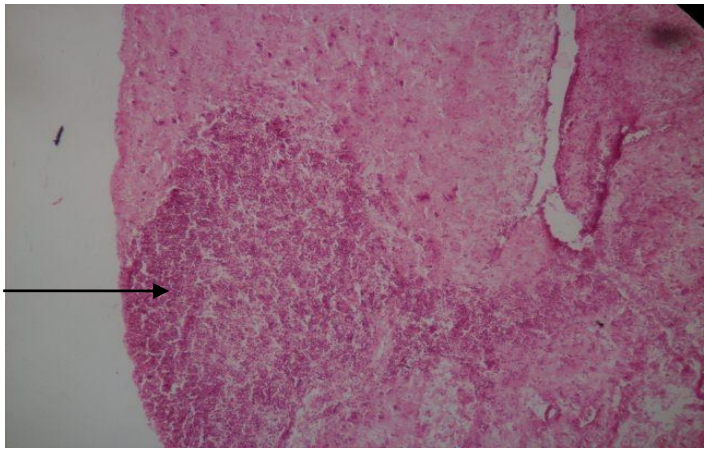


Plate III: Photomicrograph of the gills of *C. gariepinus* after exposure to paraquat for 96 hrs showing (a) Normal fish gill structure with gill filaments and villi in control fish, H & E Stain X100 (b) Marked thickening of the gills with fibrous metaplasia of the covering epithelium at 0.012mg/L, (c) Epithelial swelling of secondary lamella at 0.037mg/L, (d) Cellular infiltration at 0.055mg/L, (e) Loss of gill lamella at 0.110mg/L after exposure to paraquat for 96 hours. H & E Stain X400

a



b



c

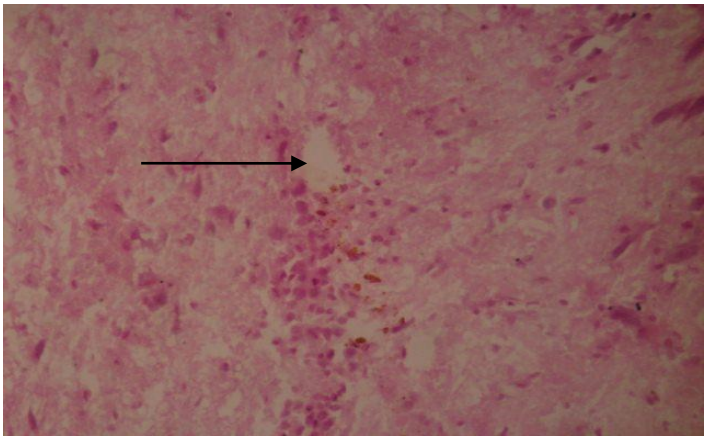
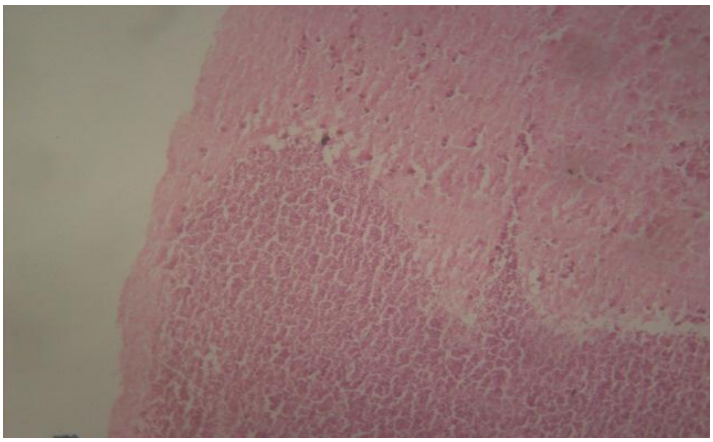
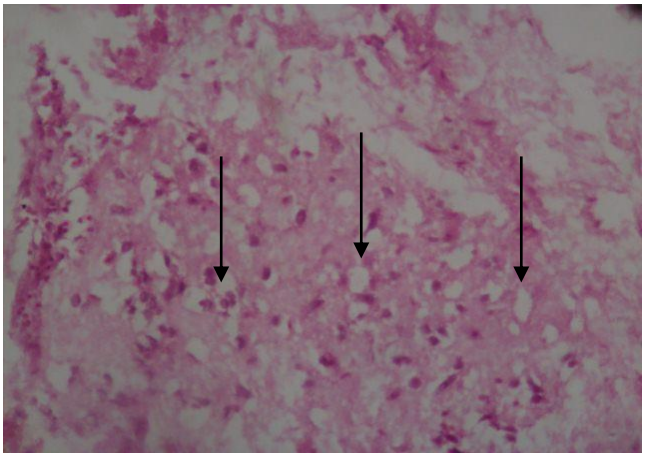


Plate IV: Photomicrograph of the brain of *C. gariepinus* after exposure to glyphosate for 96 hrs showing (a) Normal fish brain structure in control fish, H & E Stain X100 (b) Gliosis at 0.72mg/L, (c) Necrosis of a few neurons at 0.84mg/L. H & E Stain X400.

a



b



c

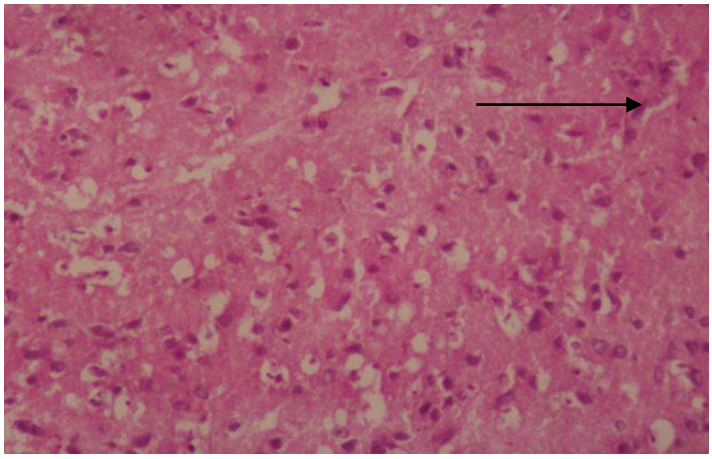


Plate V: Photomicrograph of the brain of *C. gariepinus* after exposure to paraquat for 96 hrs showing (a) Normal fish brain structure in control fish, H & E Stain X100 (b) Numerous large cytoplasmic vacuoles in heterophils at 0.012mg/L, H & E Stain X400 (c) Moderate congestion of blood vessels at 0.055mg/L, H & E Stain X400

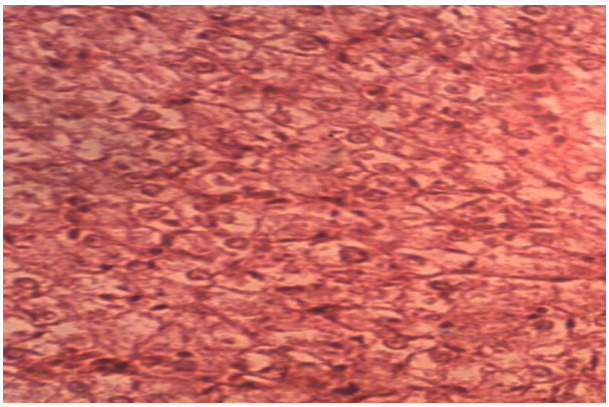
There were no pathological changes to the liver of control fish. The liver of fish exposed to glyphosate showed numerous vacuolation of hepatocytes. Liver of fish exposed to acute concentrations of paraquat also showed vacuolar degeneration of hepatocytes (Plate VI).

4.9 Histological Analysis of Liver, Brain and Gill Tissues Exposed to Sublethal Concentrations of Glyphosate and Paraquat

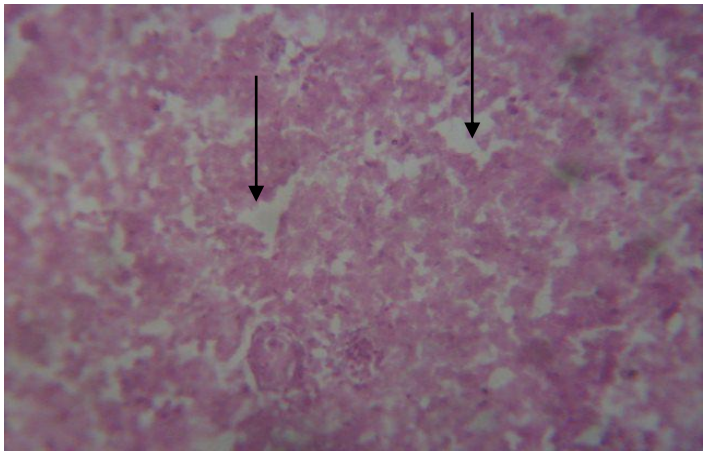
4.9.1 Gill

All pathological changes observed in the different concentrations for the different exposure periods and herbicides are presented in Tables 4.37 to 4.44. No pathological change was observed in the gills of the control fish, the structural details of the gill of control fish showed uniform arrangement of primary and secondary lamellae and inter lamellar spaces. Gills of fish exposed to concentrations of 0.265 and 0.530 mg/L of glyphosate did not exhibit alterations to the gills throughout the exposure period. Similarly, fishes exposed to concentrations of 0.0035 and 0.007mg/L of paraquat did not show alteration in the gill architecture. Only the highest concentrations with both herbicides, 0.106mg/L (glyphosate) and 0.014mg/L (paraquat) showed alterations in the structure of the gills. After 2 weeks of exposure to glyphosate, the gills showed mild to moderate proliferative thickening of the gill lamellae. By week 4, stunted gill lamellae (Plate VIIa) was observed. Thickening of gill lamella and loss of epithelial cells were observed at weeks 6 and 8 of exposure respectively (Tables 4.37 and 4.40). A similar trend was recorded in fish exposed to paraquat. In addition, the fish gills showed moderate sloughing off and loss of epithelium of the gill lamellae by the end of the second week of exposure; only sloughing off was seen after 4 weeks of exposure (Tables 4.41 and 4.42). Thickening of gill lamella and loss of epithelial cells were observed at weeks 6 and 8 of exposure respectively (Tables 4.43 and 4.44).

a



b



c

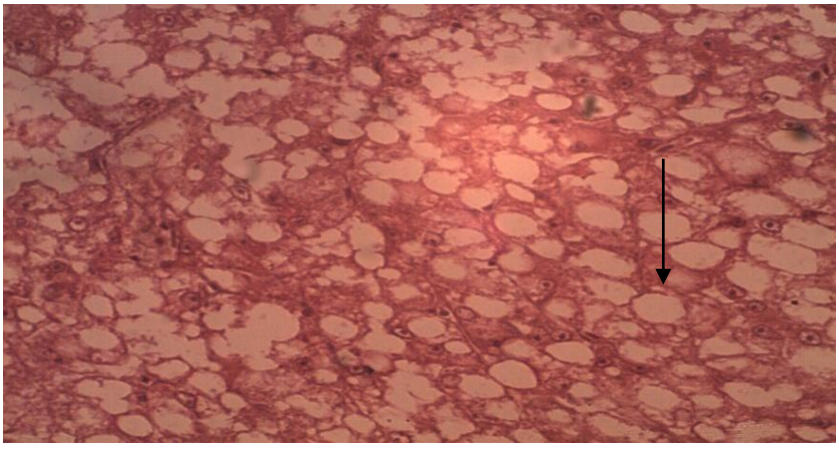


Plate VI**:** Photomicrograph of the liver of *C. gariepinus* showing (a) normal liver structure in control fish, H & E Stain X400 (b) Vacuolation of hepatocytes at concentration 0.36mg/L glyphosate, (c) Vacuolation of hepatocytes and several foci of aggregates of heterophils in the hepatic parenchyma at concentration 0.110mg/L paraquat. H & E Stain X400

Table 4.37: Pathological changes in fish organs exposed to Sublethal concentrations of Glyphosate at week two

Organs	Concentrations (mg/L)	Pathological Observations
Gills	0.00	Nil
	0.0265	Nil
	0.530	Nil
	0.106	Proliferative thickening of gill lamella
Liver		
	0.00	Nil
	0.0265	Nil
	0.530	Vacuolation of hepatocytes
	0.106	Vacuolation of hepatocytes
Brain		
	0.00	Nil
	0.0265	Nil
	0.530	Nil
	0.106	Congestion of blood vessels

Table 4.38: Pathological changes in fish organs exposed to Sublethal concentrations of Glyphosate at week four

Organs	Concentrations (mg/L)	Pathological Observations
Gills	0.00	Nil
	0.0265	Nil
	0.530	Nil
	0.106	Sloughing off of gill lamella
Liver	0.00	Nil
	0.0265	Hepatocytes with numerous large vacuoles
	0.530	Vacuolation of hepatocytes
	0.106	Vacuolation of hepatocytes
Brain	0.00	Nil
	0.0265	Nil
	0.530	Nil
	0.106	Vacoules in neurons

Table 4.39: Pathological changes in fish organs exposed to Sublethal concentrations of Glyphosate at week six

Organs	Concentrations (mg/L)	Pathological Observations
Gills	0.00	Nil
	0.0265	Nil
	0.530	Nil
	0.106	Loss of epithelial cells
Liver	0.00	Nil
	0.0265	Hepatocytes with numerous large vacuoles
	0.530	Vacuolation of hepatocytes
	0.106	Vacuolar degeneration of hepatocytes
Brain	0.00	Nil
	0.0265	Nil
	0.530	Congestion of blood vessels
	0.106	Vacuoles in neurons

Table 4.40: Pathological changes in fish organs exposed to Sublethal concentrations of Glyphosate at week eight

Organs	Concentrations (mg/L)	Pathological Observations
Gills	0.00	Nil
	0.0265	Nil
	0.530	Nil
	0.106	Thickening of gill lamella
Liver		
	0.00	Nil
	0.0265	Degeneration of hepatocytes
	0.530	Vacuolation of hepatocytes
	0.106	Degeneration of hepatocytes
Brain		
	0.00	Nil
	0.0265	Gliosis
	0.530	Cytoplasmic vacuoles in neurons
	0.106	Cytoplasmic vacuoles in neurons

Table 4.41: Pathological changes in fish organs exposed to Sublethal concentrations of Paraquat at week two

Organs	Concentrations (mg/L)	Pathological Observations
Gills	0.00	Nil
	0.0035	Nil
	0.007	Oedema
	0.014	Loss of epithelial cells
Liver	0.00	Nil
	0.0035	Nil
	0.007	Nil
	0.014	Multiple foci of hepatocytes with moderate vacuolar change
Brain	0.00	Nil
	0.0035	Spongiform vacuoles in neurons
	0.007	Gliosis
	0.014	Spongiform vacuoles in neurons

Table 4.42: Pathological changes in fish organs exposed to Sublethal concentrations of Paraquat at week four

Organs	Concentrations (mg/L)	Pathological Observations
Gills	0.00	Nil
	0.0035	Nil
	0.007	Nil
	0.014	Clubbing of gill lamella
Liver	0.00	Nil
	0.0035	Nil
	0.007	Cellular degeneration of hepatocytes
	0.014	Vacuolar degeneration of hepatocytes
Brain	0.00	Nil
	0.0035	Spongiform vacuoles in neutrophils
	0.007	Spongiform vacuoles in neutrophils
	0.014	Spongiform vacuoles in neutrophils

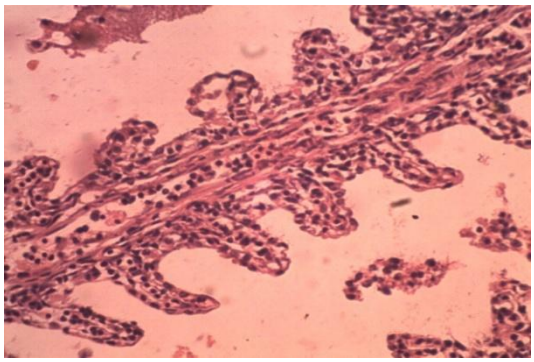
Table 4.43: Pathological changes in fish organs exposed to Sublethal concentrations of Paraquat at week six

Organs	Concentrations (mg/L)	Pathological Observations
Gills	0.00	Nil
	0.0035	Nil
	0.007	Nil
	0.014	Thickening of gill lamella
Liver	0.00	Nil
	0.0035	Hepatocyte swelling
	0.007	Vacuolar degeneration of hepatocytes
	0.014	Hepatocyte swelling
Brain	0.00	Nil
	0.0035	Gliosis
	0.007	Congestion of blood vessels
	0.014	Gliosis

Table 4.44: Pathological changes in fish organs exposed to Sublethal concentrations of Paraquat at week eight

Organs	Concentrations (mg/L)	Pathological Observations
Gills	0.00	Nil
	0.0035	Nil
	0.007	Nil
	0.014	Loss of epithelial cells
Liver	0.00	Nil
	0.0035	Nil
	0.007	Vacuolar degeneration of hepatocytes
	0.014	Kuppfer cell hyperplasia
Brain	0.00	Nil
	0.0035	Nil
	0.007	Spongiform vacuoles in neutrophils
	0.014	Mild to moderate congestion of blood vessels

a



b

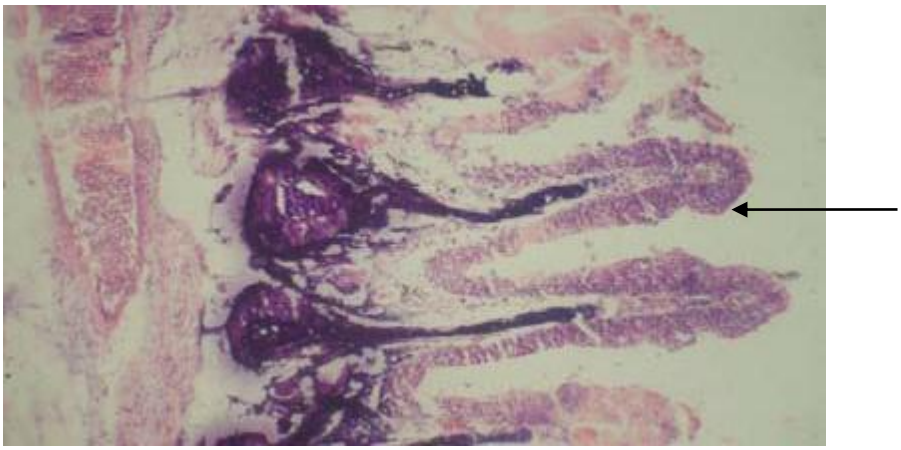


Plate VII**:** Photomicrograph of the gills of *C. gariepinus* showing (a) normal gill structure in control fish, H & E Stain X100 (b) Proliferative thickening of the gill lamellae at week two of exposure to 0.106mg/L glyphosate. H & E Stain X400

4.9.2 Liver

The structure of liver samples from control group revealed normal cytoarchitecture (Plate VIIIa). As at the second week of exposure to glyphosate, liver of fish showed no visible lesions. At week 4, fish exposed to glyphosate at concentration of 0.0265mg/L showed vacuolar degeneration of hepatocytes. Vacuolar degeneration of hepatocytes was also the only noticeable feature in week 6 in liver of fish exposed to the same concentration of glyphosate (Plates VIIIb, c). Degeneration of hepatocytes was observed after 8 weeks in liver of fish exposured to glyphosate at concentration of 0.106mg/L (Plate VIIId).

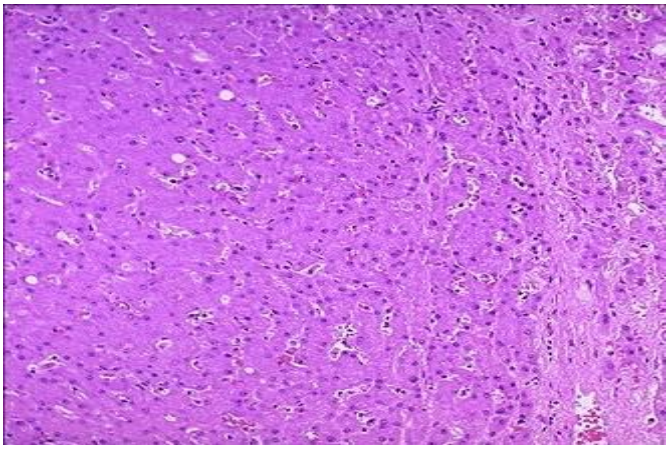
The structure of liver samples from control group revealed normal cytoarchitecture (Plate IXa) Liver of fish exposed to paraquat showed a few multiple foci of hepatocytes with moderate vacuolar degeneration after 2 weeks (Plate IXb). Cellular degeneration in hepatocytes and hepatocyte swelling was observed in weeks 4 and 6 respectively (Plates IXc, d). Kuppfer cells hyperplasia in hepatocytes was noticed in liver of fish at week 8 of exposure (Plate IXe). These changes were not concentration dependent.

4.9.3 Brain

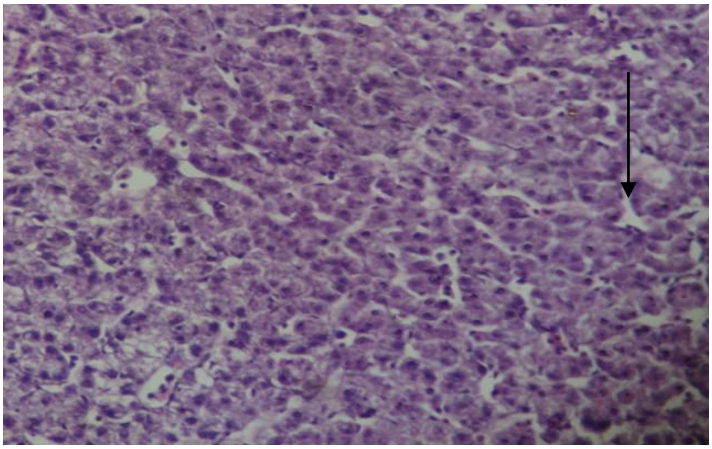
Fish in control media showed normal cytoarchitecture (Plate Xa). There was no noticeable visible lesion in the brain of fish exposed to glyphosate in the first 4 weeks. There was congestion of blood vessels in the brain of fish exposed to glyphosate at concentration of 0.053mg/L in week 6 and few foci of moderate gliosis in the 8th week at concentration of 0.0265mg/L of glyphosate (Plates Xb, c).

Fish in control media showed normal cytoarchitecture (Plate XIa). Brain of fish exposed to paraquat showed numerous spongiform vacuoles in neurons after the second week at concentration 0.0035mg/L (Plate XIb). There were also numerous foci of moderate spongiform vacuolations of the neutrophils in the fourth week in the brain of fish exposed at concentration of 0.007mg/L. There was gliosis and congestion of blood vessels in

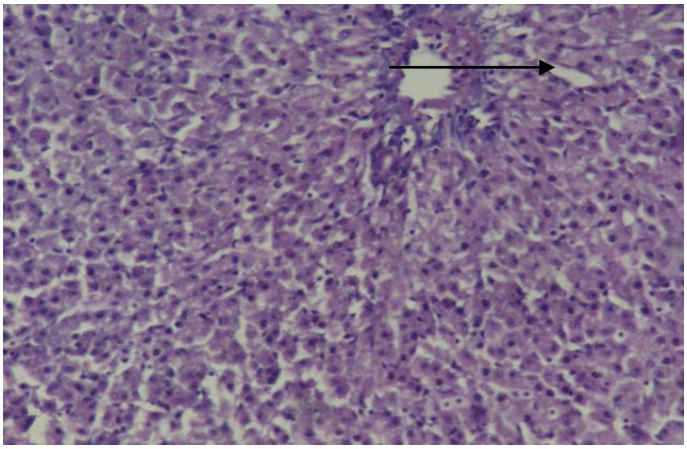
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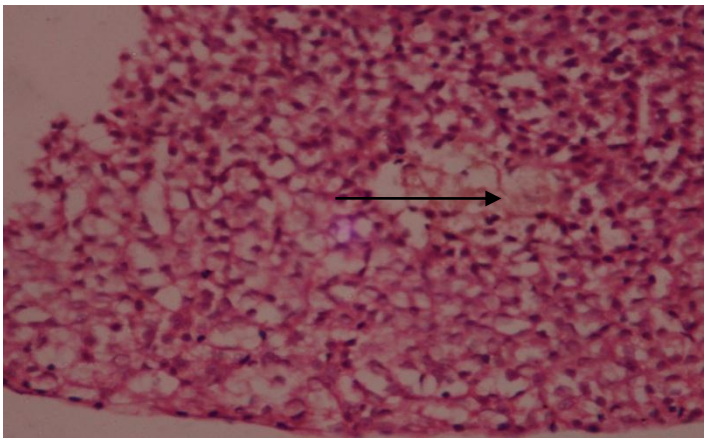
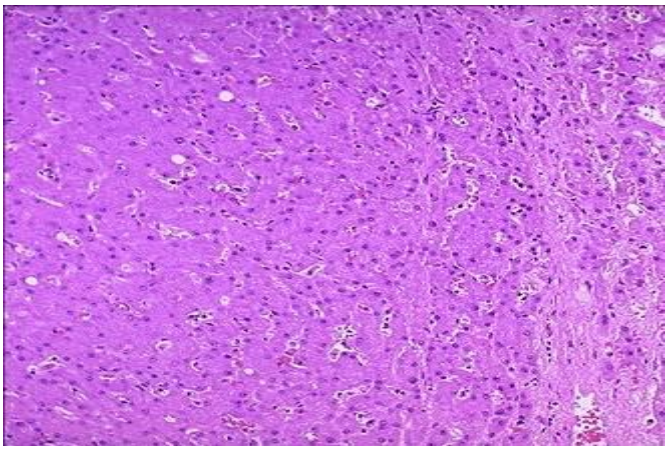
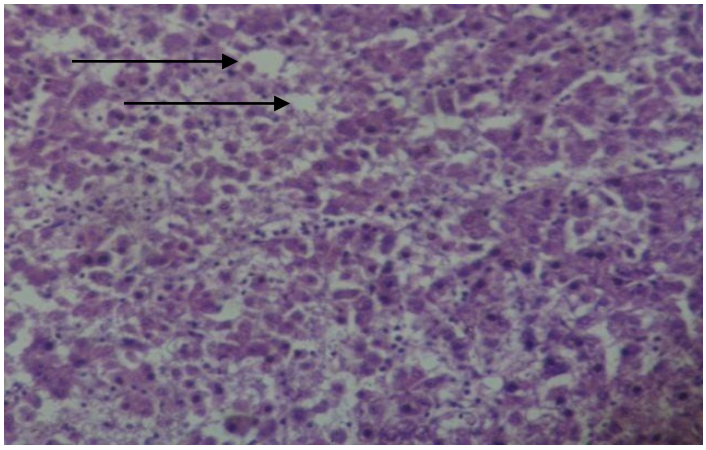


Plate VIII**:** Photomicrograph of the liver of *C. gariepinus* showing (a) normal fish liver cells of control fish at week eight, H & E Stain X100 (b) Hepatocytes containing numerous large vacuoles at concentration 0.0265mg/L glyphosate at week four, (c) Hepatocytes containing large vacuoles at concentration 0.0265mg/L glyphosate at week six, (d) Degeneration of hepatocyte in fish liver at concentration 0.106mg/L glyphosate at week eight. H & E Stain X400

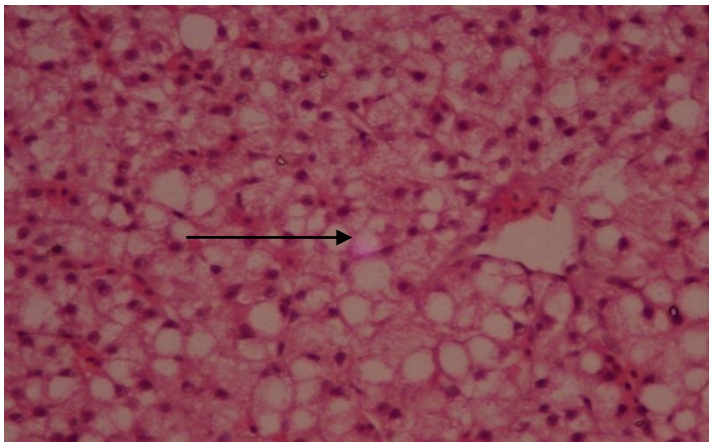
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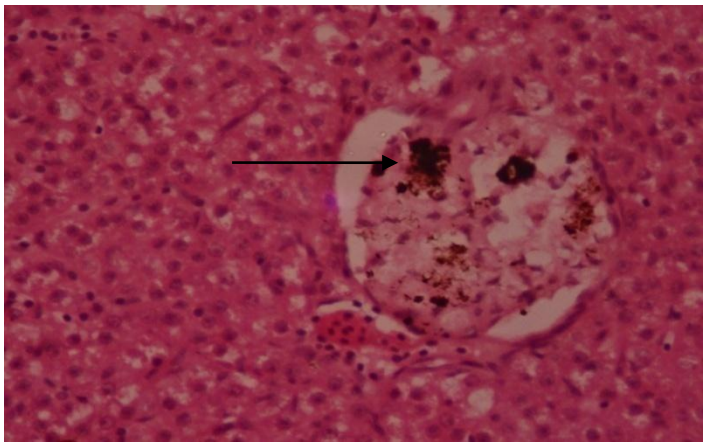
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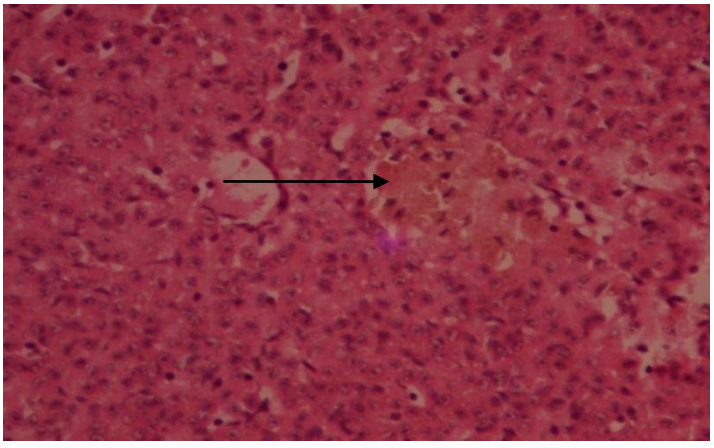
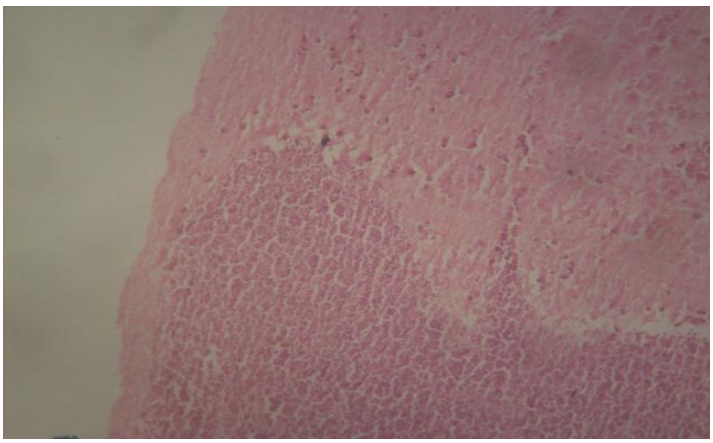
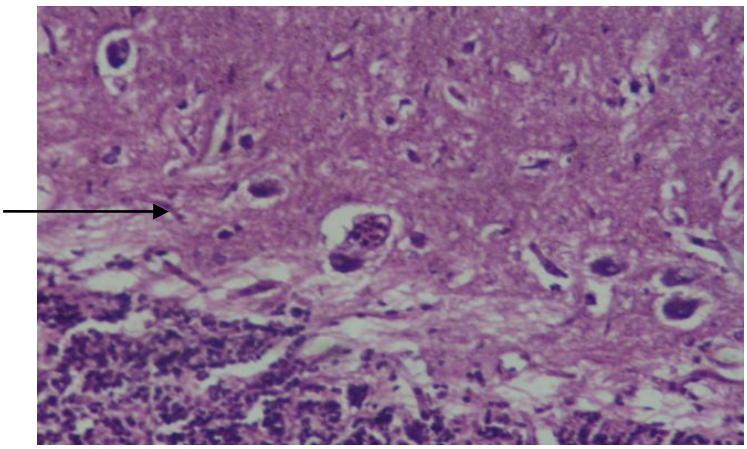


Plate IX**:** Photomicrograph of the liver of *C. gariepinus* showing (a) normal fish liver cells of control fish at week eight, H & E Stain X100 (b) A few multiple foci of hepatocytes with moderate vacuolar degeneration at concentration 0.014mg/L paraquat at week two, (c) Cellular degeneration of hepatocytes at concentration 0.007mg/L paraquat at week four, (d) Hepatocyte swelling at concentration 0.0035mg/L paraquat at week six, (e) Kuppfer cells hyperplasia in hepatocytes at concentration 0.014mg/L paraquat at week eight. H & E Stain X400

a



b



c

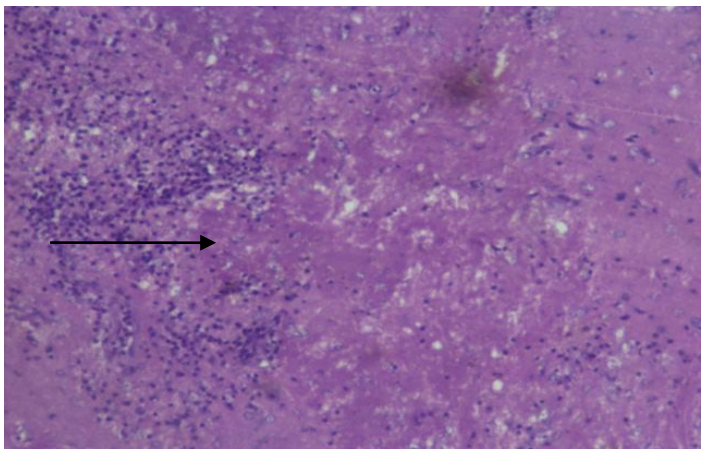
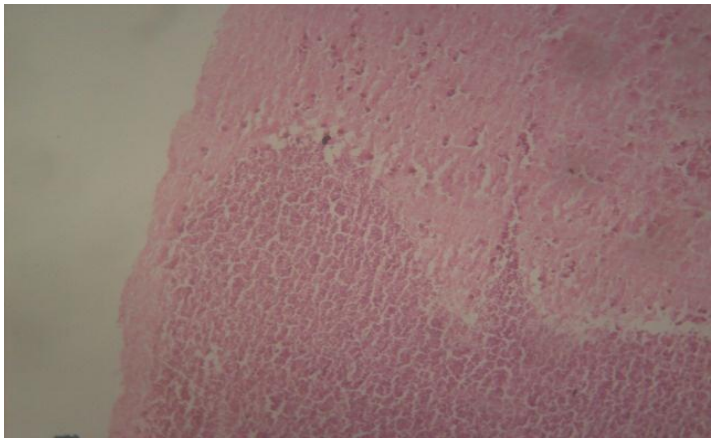


Plate X**:** Photomicrograph of the brain of *C. gariepinus* showing (a) normal fish brain cells of control fish at week eight (b) Congestion of blood vessels at concentration 0.053mg/L glyphosate at week six, (c) a few foci of moderate gliosis g, at concentration 0.0265 mg/L glyphosate at week eight. H & E Stain X400

a



b

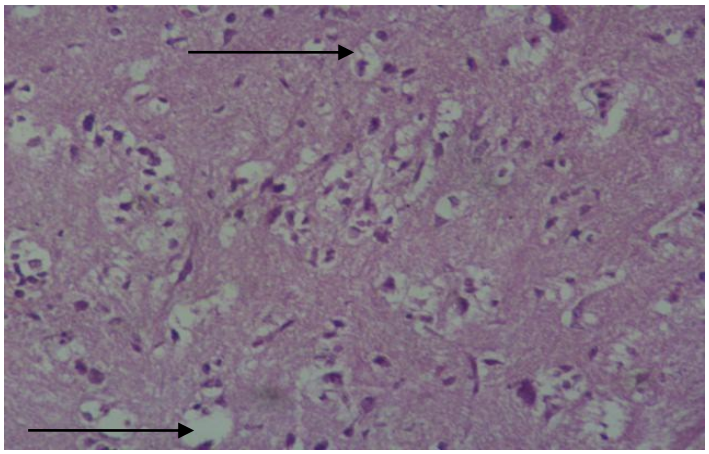


Plate XI**:** Photomicrograph of the brain of *C. gariepinus* showing (a) normal fish brain cells of control fish at week eight, (b) Numerous spongiform vacoules in neurons at concentration 0.0035mg/L paraquat at week two. H & E Stain X400

week 6 of exposure (Table 4.43). In the eighth week, there was moderate congestion of blood vessels at concentration of 0.014mg/L (Plate XII). These changes were not dependent on concentration.

4.10 Cytotoxic Effects of Acute Concentrations of Glyphosate and Paraquat in Fish Blood

Table 4.45 summarizes the capacity of acute concentrations of glyphosate in fish blood to induce micronucleus and other nuclear abnormalities while Table 4.46 summarizes the capacity of acute concentrations of paraquat to induce similar responses. Apart from micronucleus, other nuclear abnormalities noticed included bean-shaped cells, fragmented apoptotic cells and lobed nuclei. Significant increases, compared with the control, were observed in the number of micronucleus and other nuclear abnormalities for both herbicides. The frequency of occurrence of other cell abnormalities was more than the frequency of occurrence of micronucleus for both herbicides. The result also showed a time and concentration dependent increase in the frequencies of these cell abnormalities. In the blood of the glyphosate-treated fishes, there was 0.78% frequency of altered cells in the lowest concentration and this gradually increased to 1.80% in the highest concentration after the 96hr period. Similarly, the frequency of altered cells in the blood of fish exposed to the lowest concentration of paraquat was 0.71% and this increased gradually to 1.57% in the highest concentration within the same treatment period.

4.11 Cytotoxic Effects of Sublethal Concentrations of Glyphosate and Paraquat in Fish Blood

Frequency of micronucleus and other altered erythrocytes for both herbicides is presented in Tables 4.47 and 4.48. These alterations and the unaltered cells in control fish are shown in Plates XIII and XIV. Number of micronucleus increased in all exposure concentrations over the exposure period for both herbicides. The other nuclear abnormalities and the total number

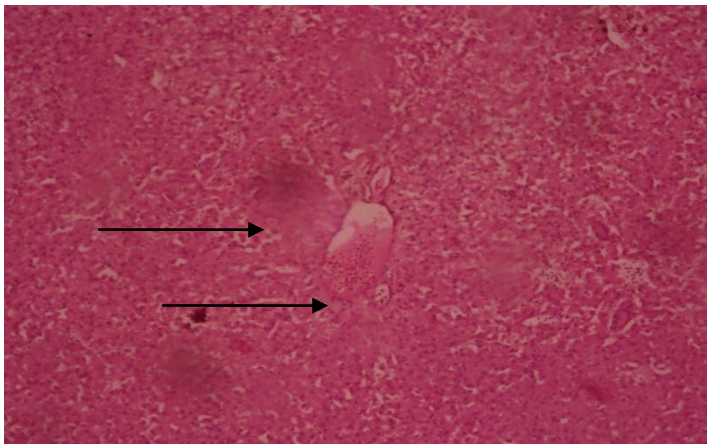


Plate XII**:** Photomicrograph of the brain of *C. gariepinus* showing mild to moderate congestion of blood vessels at concentration 0.014mg/L paraquat at week eight. H & E Stain X400

Table 4.45: Numbers and frequencies of different cell alterations caused by cytotoxic effects of acute concentrations of glyphosate in *C. gariepinus*

Conc (mg/L)	Total cells scored	Alterations				Total number of Altered Cells	Frequency of Altered Cells (%) ± S.E
		MN	LN	FAC	B-SC		
0.00	5723	12	11	0	9	32	0.56±0.03[a]
0.36	6505	16	12	12	11	51	0.78±0.02[b]
0.48	5902	24	15	14	18	71	1.20±0.03[c]
0.60	5722	22	20	18	16	76	1.33±0.02[d]
0.72	6950	32	25	24	20	101	1.50±0.05[e]
0.84	5290	30	22	2	21	95	1.80±0.02[f]

Values in the same column with different superscript are significant ($p \leq 0.05$). (Mean values ± SE are for n = 8)

KEYS:
MN: Micronucleus
LN: Lobed nucleus
FAC: Fragmented Apoptotic Cell
BSC: Bean-shaped cell

Table 4.46: Numbers and frequencies of different cell alterations caused by cytotoxic effects of acute concentrations of paraquat in *C. gariepinus*

Conc (mg/L)	Total cells scored	Alterations				Total number of Altered Cells	Frequency of Altered Cells (%) ± S.E
		MN	LN	FAC	B-SC		
0.00	6020	10	13	2	12	37	0.61 ± 0.01^{a}
0.012	6750	13	11	14	10	48	0.71 ± 0.02^{b}
0.037	6732	15	15	21	24	75	1.11 ± 0.01^{c}
0.055	6900	10	30	32	25	97	1.41 ± 0.01^{d}
0.110	6090	18	21	25	28	94	1.54 ± 0.01^{e}
0.221	6550	18	27	28	30	103	1.57 ± 0.01^{f}

Values on the same column with different superscript are significant ($p \leq 0.05$). (Mean values ± SE are for n = 6)

KEYS:
MN: Micronucleus
LN: Lobed nucleus
FAC: Fragmented Apoptotic Cell
BSC: Bean-shaped cell

Table 4.47: Time course numbers and frequencies of different cell alterations caused by cytotoxic effects of sublethal concentrations of glyphosate in *C. gariepinus*

Time	Conc (mg/L)	Total number of cells scored	Alterations				Total number of Altered Cells	Frequency of Altered Cells (%) ± S.E
			MN	LN	FAC	B-SC		
	0.00	6556	13	16	23	12	64	1.02 ± 0.025^{Aa}
	0.0265	6494	13	28	14	26	81	1.25 ± 0.035^{Ba}
	0.053	7633	16	23	33	42	114	1.49 ± 0.028^{Ca}
	0.106	6735	25	32	37	21	115	1.71 ± 0.022^{Da}
Wk 2								
	0.00	5779	12	11	24	13	60	1.04 ± 0.013^{Aa}
	0.0265	6782	20	40	27	25	112	1.65 ± 0.028^{Bb}
	0.053	7540	15	33	27	48	123	1.63 ± 0.028^{Cb}
	0.106	6987	21	35	58	28	142	2.03 ± 0.022^{Db}
Wk 4								
	0.00	6200	13	12	20	13	58	0.95 ± 0.029^{Aa}
	0.0265	7509	20	44	38	29	131	1.74 ± 0.027^{Bc}
	0.053	6844	24	45	29	38	136	1.99 ± 0.028^{Cc}
	0.106	6787	25	42	42	37	146	2.15 ± 0.019^{Dc}
Wk 6								
	0.00	6989	11	19	20	24	74	1.06 ± 0.008^{Aa}
	0.0265	7254	17	45	32	38	132	1.82 ± 0.017^{Bd}
	0.053	6856	20	54	36	46	156	2.28 ± 0.017^{Cd}
	0.106	6788	19	56	38	53	170	2.50 ± 0.021^{Dd}
Wk 8								

Values with the same capital letter superscript, within the same week/fortnight are not significant while values with the same small letter superscript, against the same concentration are not significant ($p \geq 0.05$). (Mean values ± SE are for n = 12)

KEYS:
MN: Micronucleus
LN: Lobed nucleus
FAC: Fragmented Apoptotic Cell
BSC: Bean-shaped cell

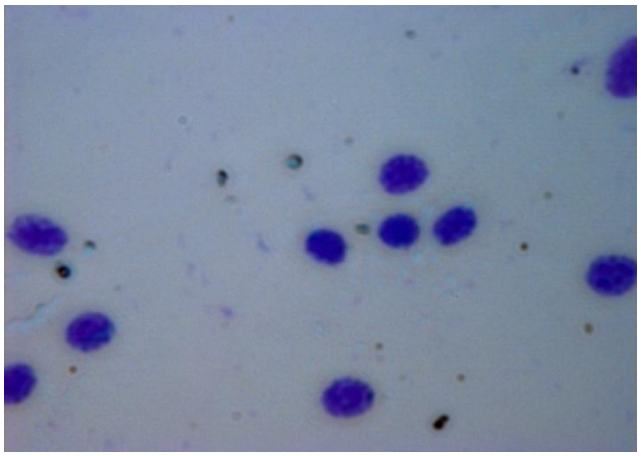
Table 4.48: Time course numbers and frequencies of different cell alterations caused by cytotoxic effects of sublethal concentrations of paraquat in *C. gariepinus*

Time	Conc (mg/L)	Total number of cells scored	Alterations				Total number of Altered Cells	Frequency of Altered Cells (%) ± S.E
			MN	LN	FAC	B-SC		
	0.00	6513	10	14	16	20	60	0.92 ± 0.026^{Aa}
	0.0035	6798	15	23	23	21	82	1.21 ± 0.019^{Ba}
	0.007	7120	14	27	30	41	112	1.57 ± 0.036^{Ca}
	0.014	6907	20	37	31	26	114	1.65 ± 0.025^{Da}
Wk 2								
	0.00	6645	11	12	15	23	61	0.91 ± 0.017^{Aa}
	0.0035	7343	16	24	30	48	118	1.61 ± 0.033^{Bb}
	0.007	6578	28	28	29	27	112	1.70 ± 0.028^{Cb}
	0.014	6998	20	32	46	32	130	1.86 ± 0.018^{Db}
Wk 4								
	0.00	6809	12	13	18	19	62	0.91 ± 0.031^{Aa}
	0.0035	6787	20	30	37	34	121	1.78 ± 0.040^{Bc}
	0.007	7155	20	43	42	35	140	1.96 ± 0.060^{Cc}
	0.014	7483	24	46	37	44	151	2.02 ± 0.023^{Cc}
Wk 6								
	0.00	6850	12	12	16	20	60	0.87 ± 0.022^{Aa}
	0.0035	7140	34	38	31	31	134	1.88 ± 0.013^{Bd}
	0.007	7151	32	54	36	42	166	2.32 ± 0.018^{Cd}
	0.014	6583	30	54	51	42	167	2.54 ± 0.030^{Dd}
Wk 8								

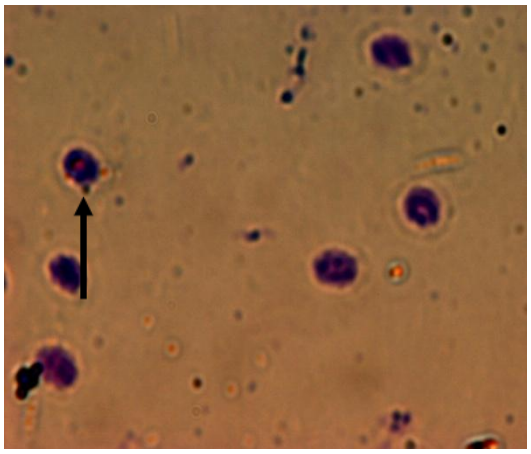
Values with the same capital letter superscript, within the same week/fortnight are not significant while values with the same small letter superscript, against the same concentration are not significant ($p \geq 0.05$). (Mean values ± SE are for n = 12)

KEYS:
MN: Micronucleus
LN: Lobed nucleus
FAC: Fragmented Apoptotic Cell
BSC: Bean-shaped cell

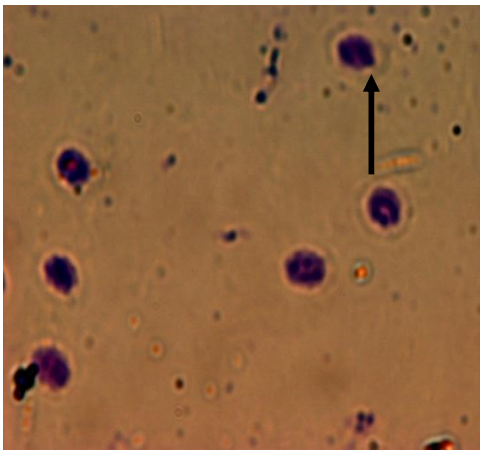
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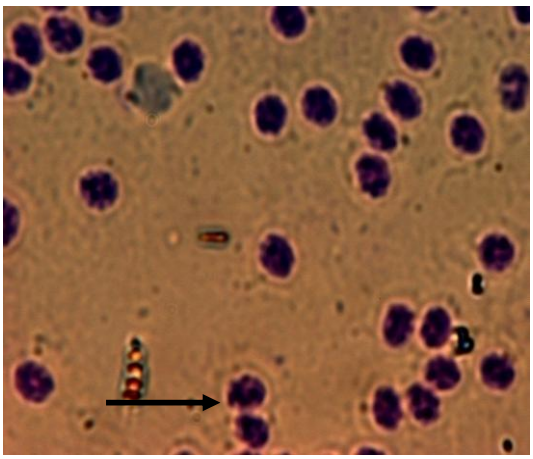
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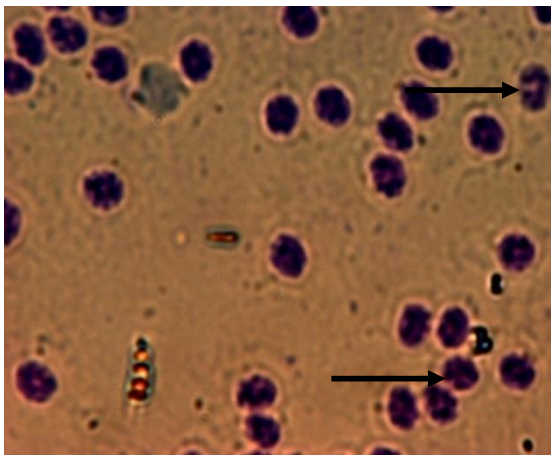
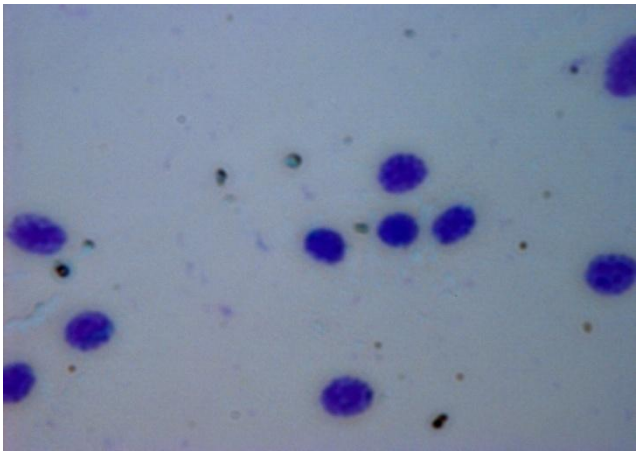


Plate XIII**:** (a) Unaltered cells in control fish flood (b) Micronucleus (c, d) Lobed Nuclei (e) Bean Shaped cell in fish blood exposed to concentrations of both herbicides. X1000

a



b

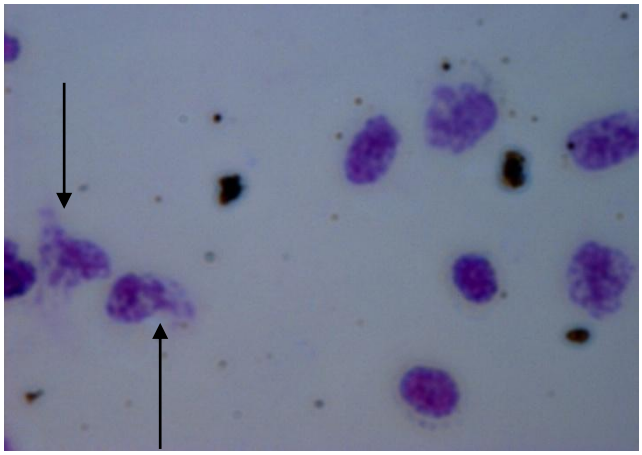


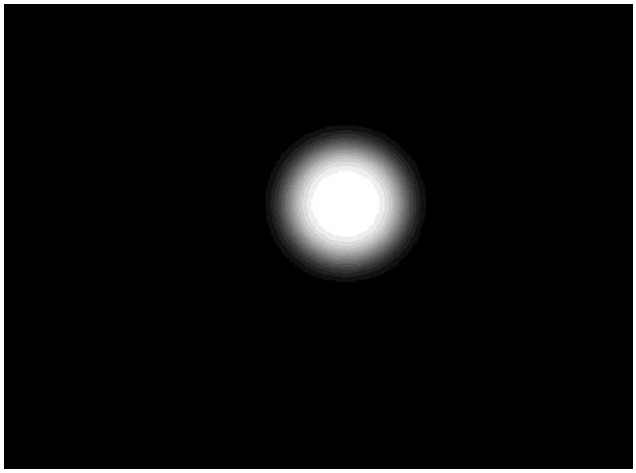
Plate XIV**:** (a) Unaltered Cells in control fish blood X1000 (b) Fragmented Apoptotic cells in fish blood exposed to concentrations of both herbicides

of altered cells also increased over the exposure period. Also, there was a dose and time dependent increase in the MN and other abnormalities for both herbicides. For the fishes exposed to 0.0265mg/L of glyphosate, there was an increase in frequency of total altered cell from 1.25% in week two, to 1.65% in week 4, to 1.74% in week 6 and 1.82% in week 8. In fishes exposed to 0.053mg/L of glyphosate, there was consistence increase from 1.49% in week 2 to 1.63% in week 4, to 1.99% in week 6 and 2.28% in week 8. The highest concentration of glyphosate, 0.106mg/L showed total cell alteration frequency of 1.71% in week 2, 2.03% in week 4, 2.15% in week 6 and 2.50% in week 8. A similar trend was observed in the fishes exposed to paraquat. The lowest concentration of 0.0035mg/L induced a 1.21% frequency of cell alterations in week 2, 1.61% in week 4, 1.78% in week 6 and 1.88% in week 8. The concentration of 0.007mg/L revealed a 1.57% frequency of altered cell in week 2, 1.70% in week 4, 1.96% in week 6 and 2.32% in week 8. The highest concentration of 0.014mg/L caused an altered erythrocyte frequency of 1.65% in week 2, 1.86% in week 4, 2.02% in week 6 and 2.54% in week 8. These increases were significant ($p \leq 0.05$). There was only one micronucleus per cell, no case of multiple micronuclei was observed. There was a similar pattern of increase in the frequency of altered cells caused by both herbicides.

4.12 Genotoxic Effects of Acute Concentrations of Glyphosate and Paraquat in *Clarias gariepinus*

Fish specimens exposed to acute concentrations of both herbicides showed DNA damage in the liver in comparison with the control samples (Plates XV and XVI). Induction however was not concentration dependent. Only one exposure concentration (0.84mg/L) was positive for DNA damage in glyphosate (Plate XVb) while three concentrations induced DNA damage in paraquat (Plates XVI b, c, d). However the highest degree of damage was shown by fishes

a



b

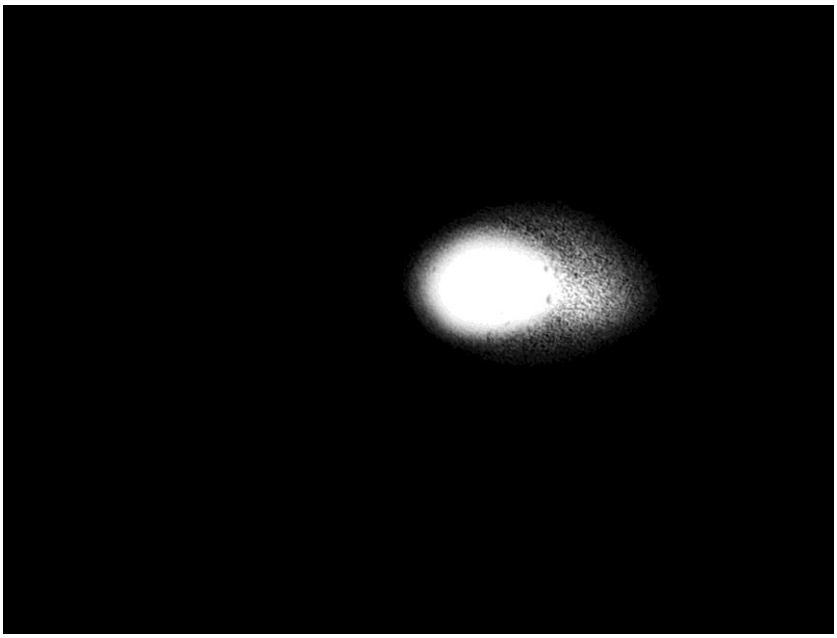
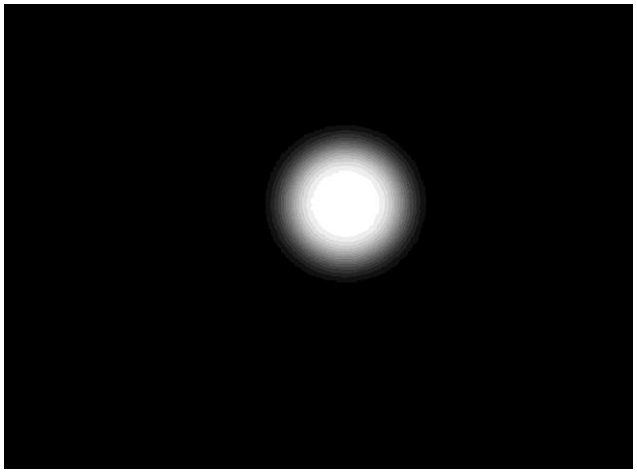
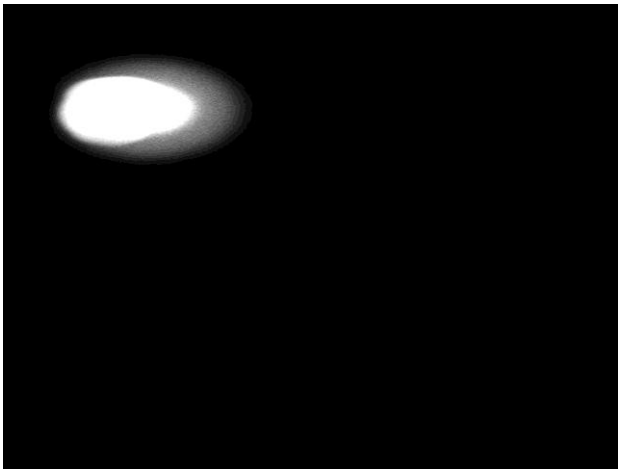


Plate XV: (a) DNA in control fish liver (b) DNA of fish liver exposed to 0.84mg/L concentration of glyphosate after 96 hours. X1000

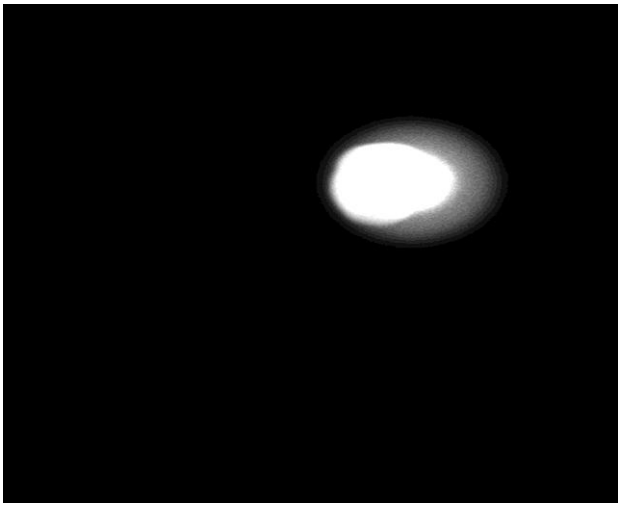
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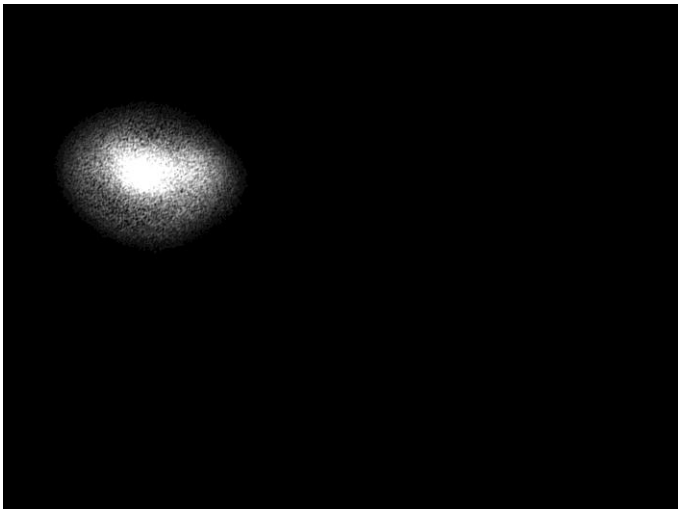


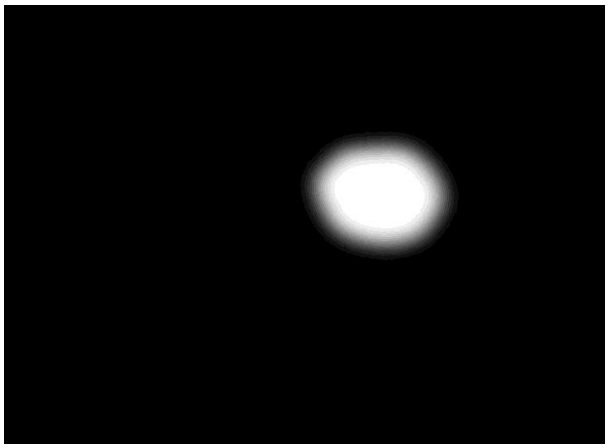
Plate XVI**:** (a) DNA in control fish liver (b) DNA of fish liver exposed to 0.037mg/L paraquat, (c) DNA of fish liver exposed to 0.055mg/L paraquat (d) DNA of fish liver exposed to 0.221mg/L paraquat after 96 hours. X1000

exposed to the highest concentration of glyphosate. The result obtained for fish samples exposed to acute concentrations of paraquat were similar to that recorded in glyphosate-exposed fish. The highest concentration of paraquat also showed the highest degree of DNA damage and as glyphosate, all concentrations did not reveal DNA damage though the induction frequency was higher in paraquat. Induction of DNA damage in the liver of *Clarias gariepinus* exposed to acute concentrations of both glyphosate and paraquat indicated potential genotoxic properties.

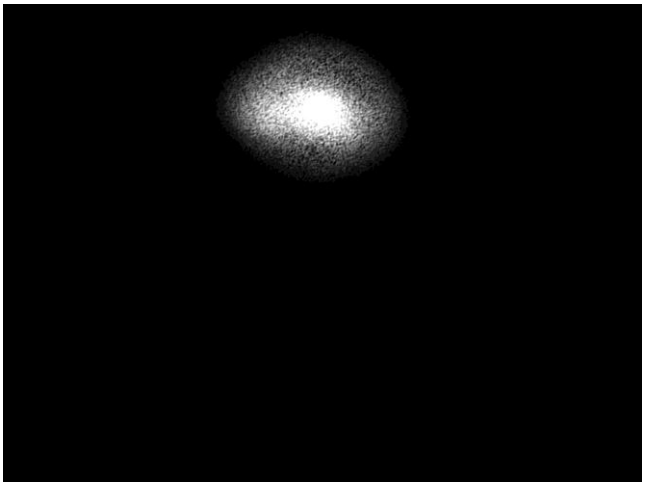
4.13 Genotoxic Effects of Sublethal Concentrations of Glyphosate and Paraquat in *Clarias gariepinus*

The control fishes also showed no damage to DNA (Plate XVIIa). Damage to the DNA observed after exposure of fish to sublethal concentrations of glyphosate and paraquat was minimal compared with acute exposures. Damage was observed only after two weeks of exposure (Plates XVIIb, c). All three exposure concentrations starting from week four did not induce damage to fish liver. DNA damage was not concentration dependent as the highest exposure concentrations did not show damage in both herbicides. The level of DNA damage after week two appeared similar in both herbicides at different concentration levels. The DNA damage observed after exposure of fish to sublethal concentrations of glyphosate and paraquat showed slight damage. The genotoxic potentials of these concentrations seemed weak.

a



b



c

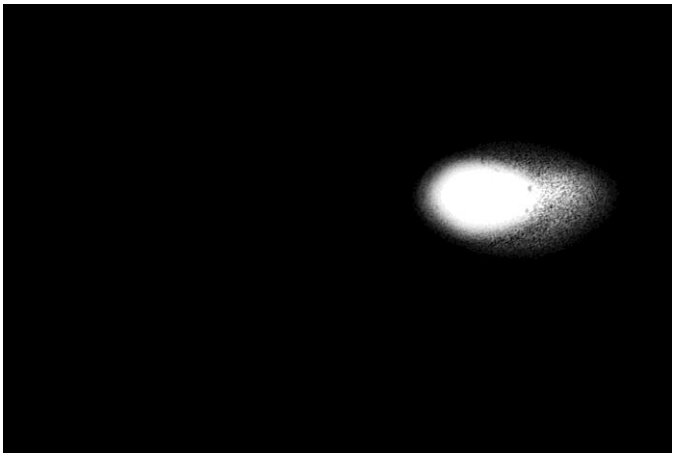


Plate XVII**:** (a) DNA in control fish liver (b) DNA of fish liver exposed to 0.053mg/L glyphosate (c) DNA of fish liver exposed to 0.014mg/L paraquat after 2 weeks. X1000

CHAPTER FIVE

DISCUSSION

Physico-chemical Parameters of Diluting Water

Although herbicides cause changes in the quality of water in and around sprayed areas and decrease the dissolved oxygen in the water, along with an increase in temperature which may pose a threat to the survival of fish species, the result of the present study indicates that glyphosate and paraquat application does not result in significant changes in the physicochemical parameter to a point that is capable of causing visually observable deleterious effects in fish. This is probably because the concentrations studied here are of lower magnitude compared to those applied in agricultural practices. Also the water quality parameters were within the recommended range for the culture of tropical fishes (Olaifa, *et al*., 2003; Omitoyin *et al*., 2006). Boyd (1979) recommended a pH range of 6.5 - 9 and Davis and Parker (1990) recommended a temperature range of 25^0C-32^0C. Similar findings were also reported by Adigun (2005) and Kolo *et al*. (2009).

Effects of Acute Concentrations of Glyphosate on Fish Behavioural Parameters

The results of the effects of acute concentrations of glyphosate on fish behavioural parameters showed that both herbicides have significant effects on the opercular ventilation count of fish, tail fin movement rate and air gulping index. The impairment of respiration due to the toxic effect of glyphosate herbicide on the gills has been reported by Omitoyin *et al*. (2006). The highest opercular ventilation rate observed within the first 12 hrs of exposure might be suggestive of a physiological response to dissolved oxygen stress. Opercular ventilation rate reduced by the 24^{th} hr which is an indication that the fish are gradually assuming a new homeostatic balance to a polluted environment. A similar explanation may probably be made for the increase in tail movement rate by the 12^{th} hr and reduction by the 24^{th} hr. Ogueji *et al*.

(2013) reported that surviving fish was maximally intoxicated at this period due to maximum bioconcentration and bioaccumulation of toxicants. Also, the marked increase in opercular ventilation and tail fin beats per minute may be that the exposed fish needed more oxygen for increased metabolic rate especially within 12 hrs of exposure. This behaviour suggests respiratory impairment, due to changes in gill pathology resulting in reduced oxygen exchange at the gills thereby leading to a hypoxic condition within the fish internal environment. Lloyd (1992) reported that an increase in oxygen consumption may be associated with additional energy requirements for detoxification or it may be caused by the extra activity necessary for an avoidance reaction to the toxicant and also, an attempt to escape from the toxicant environment. The reduction in opercular and tail fin beats with time of exposure suggests decrease in oxygen consumption and reduction of respiratory and metabolic rates. The reduction in respiratory and metabolic rates are pointers to the onset of fatigue due to several attempts to escape from the toxic medium or frequent surfacing to facilitate more oxygen intake. Similar findings were observed by Auta (2002) after exposing Dimethoate to juveniles of *Oreochromis niloticus* and *Clarias gariepinus*. Furthermore, the fact that some of the fish often hit their tails against the edges of the holding medium, resulting in loss of some portions of their tail fins and even haemorrhage, might also be a contributing factor to the reduction in the tail fin movement over time.

Air gulping index was lower in the control fishes in comparison with exposed fish. This is an indication that the fish required increased supply of oxygen and had to swim to the surface to gulp air. This activity was observed to be at its highest within 12 to 24 hrs after exposure. This period coincided with a period of reduction in opercular ventilation, stressed cellular respiration and hence the need for an alternative oxygen source. Air gulping index significantly reduced after 48 hrs, which suggests physical fatigue due to swimming and other cumulative

physiological effects of the toxicants on fish. Ogueji *et al*. (2013) also reported increased air gulping activity within 24 hrs of exposure to lambda cyhalothrin.

Effects of Acute Concentrations of Paraquat on Fish Behavioural Parameters

The opercular ventilation rate, the tail fin movement rate and air gulping index of *C. gariepinus* all showed toxic responses when exposed to acute concentrations of paraquat. The higher concentrations had the lowest opercular ventilation count while the lower concentrations had rapid counts. There were rapid opercular movements because of improper ventilation or impairment in the mechanism of exchange of oxygen from the environment. In the control fish, the opercular movements were normal when compared with experimental fish. Opercular ventilation counts were very low in the first two periods of observation i.e. at the 12^{th} and 24^{th} hrs, such decreases probably helps in reducing absorption of pesticide through gills (Venkata and Nagaraju, 2013).

Opercular ventilation was very much reduced in the two highest concentrations (0.11mg/L and 0.221mg/L) in the first 12 hrs of exposure due to the colour of paraquat which rendered water to be very turbid at these two concentrations. Observation from the base of the holding tanks revealed very slow opercular movement during these periods. Thereafter, opercular ventilation count rose significantly, probably as the effect of the toxicant became limited with time and the turbidity of water reduced as a result of exposure to atmospheric oxygen. Furthermore, it could be associated with the inhibitory action of the toxicant on respiration as well as malfunctioning of some vital organs which may reduce the available energy for respiration. The fish could have increased ventilation rates in an attempt to make up for the loss in oxygen content in the gill. Opercular ventilation rate increased from 12 and 24 hrs then further increased from 48^{th} to 72^{nd} hr and then a sharp drop as death approached at about the 96^{th} hr. This indicates hyperventilation towards the 96^{th} hr and a decrease or seizure as death approached (Babatunde

and Oladimeji 2014). It is likely that the high opercular ventilation rate observed between the 48th and 72nd hr is due to thickened mucus layer as a result of continuous mucus production, which subsequently hinders gas exchange, which might be compensated for by elevating opercular ventilation count. Mucus production has been reported as a response to many toxicants for providing a barrier that prevents toxin interaction with epithelial cells (Mallatt, 1985; Mcdonald and Wood, 1993, Venkata and Nagaraju, 2013).

Tail fin movement changed significantly with duration of exposure only. The first 12 hrs showed the highest rate of tail fin movement. After this, it dropped significantly in the 24th hr and thereafter increased till the 96th hr. High tail movement rate is due to avoidance symdrome/increased escape reflex and general restlessness. The significant reduction in tail fin movement by the 24th hr may be due to exertion and lethargy in fish (Adeogun, 2012a).

The air gulping index was also observed to increase significantly in the fish exposed to paraquat. Peak of this activity was within the first 24 hrs of exposure. This period coincides with a period of reduction in opercular ventilation in which case the fishes were avoiding contact with the herbicide. The observed respiratory distress may have been due to both decreasing dissolved oxygen contents of reconstituted extract with decreasing ability of the exposed fish to respire (Ojutiku *et al*., 2013).

Other abnormal behaviours elicited by fish include abnormal swimming, hyperactivity, hitting of tails against the walls of the holding tank and restlessness. These behaviours points to an environmental stressor (the toxicant), hence fish provides important indices for ecosystem assessment (Robinson, 2009). In addition, these abnormal behaviours may be caused by the neurotoxic effects of the herbicides and also by irritation to perceptive system of the body due to accumulation of acetylcholine at synaptic junctions through the inactivation of

acetylcholinesterase and stimulation of peripheral nervous system leading to higher metabolic rate (Rao *et al*., 2005).

Mortality/96-hr LC_{50} values

Mortality was observed to be concentration and time dependent in this study. A threshold is attained above which there is no survival of animals and below this threshold, animals attain a tolerance zone. The mortality pattern was between 17% and 87% for glyphosate, and 6% and 90% for paraquat which is similar to the report by Rand and Pectrocelli (1985) that there should be less than 35% mortality in the lowest concentration and at least more than 65% mortality in the highest concentration. These were similarly observed by Ateeq *et al*. (2005), Olurin *et al*. (2006). As the physico-chemical parameters of water were found to be within range of *C. gariepinus* culture in this study, death could therefore have occurred either by direct poisoning or indirectly by making the medium uncomfortable for the fishes or even by both.

The present study shows that the 96hr LC_{50} value of glyphosate was 0.530mg/L. This is in contrast to previous studies by Akinsorotan *et al*. (2013), Okomoda and Ataguba (2011) and Ayoola (2008) who all reported LC_{50} values of 43.65mg/L, 17.5mg/L and 1.05mg/L respectively after exposing *C. gariepinus* fingerlings to acute concentrations of glyphosate. The 96hr LC_{50} value of paraquat as observed in this study was 0.07mg/L, which is also at variance with previous studies. Doherty *et al*. (2011) reported a value of 1.75mg/L while Omitoyin *et al*, (2006) reported a value of 18mg/L of paraquat to *C. gariepinus* fingerlings. The LC_{50} values depend on fish species and the test conditions as well as herbicide formulations (WHO, 1994). This variation may also be due to age of the experimental fish. Fish used in this study were juveniles averaging between 12.14 ± 1.76cm standard length and body weight of 4.45 ± 1.37g

while fish used by Akinsorotan *et al*. (2013) were adults measuring between 29.3-32.6cm standard length and weighed between 180g and 250g. Rand and Petrocelli (1985) observed that physiological advancement, related to a more developed immune system offers resistance to stress which may also explain differences in values between this study and others. Neibor and Richardson (1980) reported that the level of toxicity of any pesticide depends on its bioaccumulation, the different chemistries of the compound forming the pesticide and the reactions of the organisms receiving the toxicant.

Effects of acute concentrations of glyphosate and paraquat on liver enzymes activities in fish

The acute concentrations of glyphosate and paraquat caused significant changes in the activities of liver enzymes in *Clarias gariepinus*, the former showing more toxicity than the latter. There were significant increases in all hepatotoxic parameters such as in alkaline phosphatase (ALP), lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) activities on exposure to acute concentrations of glyphosate and paraquat. These increases connotes that the fishes responded to the toxic condition by producing more of these metabolic enzymes in a bid to adapt to the new condition. Similar increase in the activities of these enzymes was reported by Atamaniuk *et al*. (2013) and Gholami-Seyedkolaei (2013) after exposing goldfish to 2,4-dichlorophenoxyacetic acid and common Carp to glyphosate respectively.

The increase in the activities of ALT and AST (1.50 and 1.56 % fold increase respectively in glyphosate; 1.49 and 1.44 % fold increase respectively in paraquat) in liver of fish exposed to both herbicides in this study could be due to incorporation of keto acids into tricarboxylic acid (TCA) cycle via generation of glutamate through tissue transamination. These enzymes may thereafter be converted to α -ketoglutarate through oxidative deamination to produce energy

through different energy-producing pathways (Prashanth and Neelagund, 2008). Activities of transaminases (aminotransferases), such as aspartate transaminase (AST) and alanine transaminase (ALT) are considered to be important, because they form a strategic link between carbohydrate and amino acid metabolism (Harper *et al*., 1978). There is also the possibility of increase in the level of metabolites due to herbicidal stress resulting in elevation of activities of transaminases. Stress conditions, in general, induce elevation in the transamination pathway (Awasthi *et al*. 1984).

Alkaline phosphatase activity was enhanced in fish liver. Activity of ALP was higher in paraquat (1.58% fold increase) than in glyphosate (1.53% fold increase) during exposure of fish to these herbicides in the present study. This may be due to cellular damage in tissue system which may indicate higher metabolic activities to meet the high energy demand to cope with herbicidal stress. The strong toxic action of toxicant probably ruptures the cellular and lysosomal membrane that contains hydrolytic enzymes, resulting in its increase. Alkaline phosphatase, a brush border enzyme, splits various phosphate esters at an alkaline pH and mediates membrane transport (Bhattacharya and Anilava, 2009). The results were in agreement with the findings of Al-Attar (2005) and Samanta *et al*. (2013) who also reported increase in activities of ALP after exposure of fish to toxicants.

The observed elevation of lactate dehydrogenase may be in response to stress or hypoxic condition in which case the fish has to compensate for more oxygen demand in order to overcome the stressed condition. An increase of lactate levels in the liver indicates metabolic disorders and a severe respiratory stress in fish tissues (Begum and Vijayaraghavan, 1999; Glusczak *et al*., 2007; Pretto *et al*., 2011). Increased LDH activity levels have been observed in conditions of stress when high levels of energy are required (Oliveira *et al*., 2009). Acute concentrations of glyphosate and paraquat have probably resulted in stress, and detoxification

mechanisms were no longer able to overcome toxic effects, hence induction of LDH activity. Specifically, pesticides, such as paraquat and glyphosate, have been reported to modulate the activity of LDH (Hernandez *et al*., 2006).

Effects of acute concentrations of glyphosate and paraquat on antioxidant enzymes activities in liver of fish

In the current study, a general increasing trend in the activities of antioxidant enzymes observed in the liver of *Clarias gariepinus* exposed to both herbicides; all the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and glutathione peroxidase (GPx) increased in the liver. SOD had the highest activity of all the antioxidant enzymes in both herbicides; with a 2.07% fold increase in paraquat and 1.95% increase in glyphosate. These results agree with other reports pointing out increase of antioxidant enzyme activities in fish exposed to environmental pollutants (Monteiro *et al*., 2006; Sturve *et al*., 2008; Li *et al*., 2010h, 2011). These results suggest that the herbicides, at the concentrations used, may be enzyme activators thus promoting hepatic stress. The stress on the liver is further supported by the vacuolation observed in the hepatocytes of liver in the histopathology experiment.

The CAT-SOD system provides the first defense line against oxygen toxicity and usually used as a biomarker indicating reactive oxygen species (ROS) production (Ballesteros *et al*., 2009). In this study, the antioxidant enzyme catalase showed an increase in activity in the liver with increasing herbicide concentration. Activity of catalase was higher in glyphosate (1.34% fold increase) than in paraquat (1.27% fold increase). The enhancement of catalase activity in fish exposed to glyphosate and paraquat may be due to herbicide-mediated oxyradical production. Catalase seems to be important as an antioxidant defense against possible lipid damage generated by the herbicides. Thus, oxidative stress generated by water containing these herbicides may suppress the antioxidant defense of fish, leading to a loss of this compensatory

mechanism. Glusczak *et al*. (2011) also reported increase in catalase activity after exposure of fish to glyphosate.

In this study, there was a significant rise in the activity of SOD, the % fold increase in paraquat being higher than in glyphosate meaning that paraquat was more toxic. In essence, these herbicides enhanced the synthesis of this enzyme thereby leading to increased liver cell metabolism caused by these herbicides. Overall, it further emphasizes the important role of liver in detoxifying of xenobiotics.

Hepatic tissues showed an increase in TBARS levels at all tested concentrations for both herbicides; the % fold increase being 1.72% in glyphosate and 1.10% in paraquat. In the liver, elevation in TBARS suggests participation of free-radical-induced oxidative cell injury caused by glyphosate toxicity. Apparently, glyphosate caused lipid peroxidation in liver tissues, and changes in TBARS vary depending on the tissue considered. The peroxidation of lipids, which is measured in form of thiobarbituric acid substances (TBARS), is basically damaging because the formation of LPO products generates a cascade of free-radical reactions (Catala, 2006). As a result, LPO can greatly alter the physicochemical properties of membrane lipid bilayers, resulting in severe cellular dysfunction. In addition, LPO products could react with some amino-acid side-chains of proteins or with reducing sugars or their oxidation products (Stadtman and Levine, 2000). Recently, glyphosate exposure was reported to cause lipid peroxidation and impair oxidative defenses in *Prochilodus lineatus* (Modesto and Martinez, 2010).

Also, there is a significant increase in the enzyme activities of the reduced form of glutathione (GSH), glutathione peroxidase (GPx) in the liver when comparing treated with control fish. Paraquat induced more enzyme activity (1.76% fold increase) than glyphosate (1.70% fold

increase). This may suggest that the herbicides induced anti-oxidative components in the liver in order to cope with the chemical insult imposed by glyphosate and paraquat. It is known that fishes react in response to oxidative chemical stressors by up- regulating numerous detoxifying enzymes such as cytochrome P450, phase II conjugation enzymes and anti-oxidative enzymes (Nwani *et al*., 2010a).

Comparing the two herbicides, the response (evaluated by % fold increase) elicited by fish liver vis-à-vis all the enzymes assayed is close. Paraquat induced more activity in SOD and GPx while glyphosate induced more activity in CAT and MDA. A plausible explanation for this may be that the detoxification pathway in the liver for these two herbicides is similar. This will probably mean that the fishes just responded to a toxic medium rather than them responding specifically to the each herbicide.

Effects of long term sub-lethal concentrations of glyphosate and paraquat on liver enzyme activities in fish

The increased levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the liver of exposed fish are an indication of damage to liver architecture. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are important in the diagnosis of heart and liver damage (Crook, 2006). Moreso, it has been observed that ALT is particularly specific as an indicator of liver dysfunction (Sunmonu and Oloyede, 2007; Adeyemi *et al*., 2009). Ogueji *et al*. (2012) also reported increases over time in activities of AST and ALT following exposure of *Clarias gariepinus* to sub-lethal concentrations of Chlorpyrifos and lambda-cyhalothrin. However, Adeyemi *et al*. (2009), reported decreases in hepatic activities of AST and ALT after exposing *Clarias gariepinus* to sublethal concentrations of phthalate, benzene and cyclohexane for 56 days. They concluded that these enzymes are distributed differently in different body tissues which probably might be a function of the health status of

the fish. It may also be that lower concentrations that elicited less toxic effects were used. Surface/body weight ratio, extraction medium, age of fish and toxicant type are all factors that can affect the magnitude of toxic effects of a toxicant. Furthermore, there may be inhibition.

Activities of alkaline phosphatase (ALP) also increased over from week two to week eight in all exposure concentrations for both herbicides during the sub-lethal experiment. The elevated enzyme activity was concentration dependent and could be considered to be manifestation of oxidative stress caused by glyphosate and paraquat. This increase may be due to their leakage from the cytosol across damaged plasma membrane into the general blood circulation or an increase in their synthesis as a result of the organ dysfunction. According to Rao, (2006), elevation in alkaline phosphatase (ALP) suggests an increase in the lysosomal mobilization and cell necrosis due to pesticide toxicity. Vutukuru *et al*. (2007) also reported that when the liver cell is damaged, tissue specific enzymes are released into intracellular spaces resulting in their increase in plasma.

Lactate dehydrogenase (LDH) level was increased with increasing concentrations of glyphosate and paraquat. These findings also suggest that fish were getting additional energy from the anaerobic pathway in an attempt to support the processes (e.g. detoxification mechanisms) needed to overcome chemical insult. Lactate dehydrogenase (LDH) is also a marker of tissue damage and its increased level is reported in liver necrosis (Ramesh *et al*., 1993). This is corroborated by the liver damage, in the form of degeneration of hepatocytes observed in the histological examination of the fish liver.

Even though the response of fish to both herbicides in relation to the stimulating liver enzymes are similar, result shows that the values for the activities of these enzymes are lower in the fish exposed to sublethal concentrations than those exposed to acute concentrations of both

herbicides. An explanation for this may be that the higher acute concentrations caused more toxicity in the fish than the lower sublethal concentrations. Ogueji *et al*. (2012) reported more toxic response from fishes exposed to acute concentrations of chlorpyrifos and lambda cyhalothrin than those exposed to sublethal concentrations of the same chemicals.

Effects of long term sub-lethal concentrations of glyphosate and paraquat on antioxidant enzyme activities in liver of fish

Antioxidant enzymes, Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and lipid peroxidation, which was measured in form of thiobarbituric reactive substances (TBARS or MDA), all increased significantly during the eight weeks exposure period. The elevated levels of lipid peroxidation in the liver of *C. gariepinus* in response to exposure to glyphosate and paraquat in the present study suggest that there is increased production of reactive oxygen species (ROS). Increased ROS production may be associated with the metabolism of the herbicide by fish leading to the peroxidation of membrane lipids of the liver. Induction of ROS', enhances the oxidation of polyunsaturated fatty acids which can lead to lipid peroxidation (Valavanidis *et al*., 2006; Liu *et al*., 2008). Significant differences were recorded in malondialdehyde (MDA) levels with concentration and time, the overall percentage change between the highest concentration and control being 13% in glyphosate, 4% in paraquat indicating that glyphosate was probably more potent.

There were % fold increases in MDA levels within concentration in each of the period under observation. According to Cossu *et al*. (2000), organisms with lowered antioxidant status could be more sensitive to lipid peroxidation, exhibiting higher levels of MDA. This may have been responsible for the increase in levels of MDA recorded in this study. This will mean that reactive oxygen species are generated in fish by these herbicides leading to peroxidation of lipid, the products of which is measured in form of TBARS. The destruction of the lipid bilayer

of cells is a very seious threat to their continued existence as cells become weak and unprotected from other substances injurious to them.

Fishes treated with paraquat concentration of 0.007mg/L showed no significant difference in MDA levels throughout the eight week period. The percentage increase in MDA levels, comparing the control and the highest concentration is just about 4%, even lower in fish treated with 0.007mg/L of paraquat, hence, the fishes in this group just about coped for the whole eight week period correspondingly with the little percentage increase in MDA levels. Interplay or any one of factors such as genetic makeup, health status, strength of immune system and physiology of the fishes might have played a key role for better adaptability within the eight weeks period.

Activities of SOD, CAT and GPx also significantly increased throughout the exposure period. Similar observation was reported by Vasanth *et al*. (2012) after exposure of *Labeo rohita* to sub-lethal concentrations of athracene. According to Nwani *et al*. (2013), organisms are equipped with independent cascades of enzymes to alleviate oxidative stress and repair damaged macro-molecules produced during normal metabolism or due to exposure to xenobiotics. In this cascade, SOD and CAT are major enzymes in eliminating reactive oxygen species (ROS), and the induction of SOD/CAT provides a first line of defense against ROS. SOD converts the superoxide anion radical to hydrogen, while CAT converts hydrogen peroxide to water and molecular oxygen (Shao *et al*., 2012). As there were only elevations, instead of a decrease in the levels of antioxidant enzymes in the present study, it can be said that the fishes responded very well to the oxidative stress insult by the herbicides. This implies that antioxidant enzymes were constantly produced and were regularly clearing the ROS generated. Part of the functions of these antioxidant enzymes is repair of damage and adaptive response. Either or both of these appear to have come into play in the present study. This also support the reports of Livingstone (2001) that ROS produced in biological systems are

detoxified and purportedly held back in check by antioxidant defences, which are generally ubiquitous in animal species and different tissue types. The increase in the activity of SOD, and CAT in this study reflects the development of a compensatory mechanism in response to increased oxidative stress.

The level of glutathione peroxidase in the liver in the present study was found to be significantly increased during the eight weeks exposure period. This indicates the protective role of the enzyme against lipid peroxidation. This probably reflects an adaptation to the oxidative conditions to which the fish have been exposed (Lenartova *et al*., 1997). Abdel-Tawwab and Wafeek (2008) reported similar findings in *Oreochromis niloticus* after exposure to cadmium. Parthasarathy and Joseph (2011) reported a significant ($p \leq 0.05$) decline in the activities of GSH-dependent antioxidant enzymes, GPx and GST, in the liver tissue of *Oreochromis mossambicus* after exposure to lambda cyhalothrin and concluded that it reflected an increased oxidative stress in λ cyhalothrin induced fishes.

The increase in LPO, CAT, GPx and SOD activities in the liver of *C. gariepinus* supports the hypothesis that sublethal concentrations of glyphosate and paraquat induced oxidative stress in *C. gariepinus* and this could be an adaptive response to protect the fish from the herbicide-induced free radical toxicity.

Increase in the activities of these enzymes was lower than what was observed in the result for the acute toxicity test. This implies that the higher concentrations in the acute study exert more toxic responses in the fish as compared with the concentrations used for the sublethal study. The overall increases between the control and the highest concentrations in the activities of all of these enzymes were more in paraquat than glyphosate. It may imply that paraquat was more potent than glyphosate. This is supported by the lower LC_{50} value of paraquat (0.07 mg/L) as against 0.530 mg/L for glyphosate.

Histological effects of acute concentrations of glyphosate and paraquat on major fish organs

Gills

This study shows that acute concentrations of glyphosate and paraquat are both toxic to *Clarias gariepinus*. This is in agreement with previous researchers (Doherty *et al*., 2011; Ayoola, 2008; Okayi *et al*., 2010) who observed that either or both of these herbicides are toxic to fish. Pathological changes in fish gills lead to an overall reduction in the efficiency of gill filaments to aid in diffusion of oxygen across the gill lamellae resulting in the development of a hypoxic condition within the fish (Elahee and Bhagwant, 2007) and further confirm the deleterious effects some environmental pollutants may have on major fish organs.

The gills of fish exposed to acute concentrations of glyphosate showed thickening of secondary lamella, massive sloughing off of lamella and loss of epithelial cells. The degree of pathological alterations in fish gill were less severe compared to the findings of Ayoola (2008) who reported severe damage to the gills of *Clarias gariepinus* treated with acute concentrations of glyphosate. The overall toxicity of commercial formulations of glyphosate partly depends on the toxicity of its associated compounds, especially surfactants (Amarante *et al*., 2002; Diamond and Durkin, 1997; Peixoto, 2005). There is a slight probability that the percentage of the poly ethoxylated tallow amine (POEA) surfactants used in the herbicide formulations in this study differs from the report of Ayoola (2008) which was more toxic.

The gills of fish exposed to acute concentrations of paraquat showed loss of gill lamella, marked thickening of the gills with fibrous metaplasia of the covering epithelium, heterophilic cells, epithelial swelling of secondary lamella and swelling of epithelial cells. The thickening of the secondary lamella may likely be a defensive response (in form of an allergic reaction) to the toxicant. Cengiz and Unlu, (2003) had reported that alterations such as hyperplasia,

thickened epithelial layers and fusion of adjacent secondary lamellae serves as a defense mechanism, leading to decreased respiratory surface and increased toxicant–blood diffusion distance. Some authors have also indicated that gill lesions do not only indicate possibilities of impaired respiratory functions but impaired osmo-regulatory functions too (Mallatt, 1985; Au, 2004; Tang and Au, 2004). Doherty *et al*. (2011) reported necrosis, inflammation and lesions in the gills of *C. gariepinus* after exposure to acute concentrations of paraquat. Banee *et al*. (2013) reported different degrees of alterations such as hypertrophy, epithelial increase of gill filament, and oedema of secondary gill lamella of *Trichogaster trichopterus* exposed to paraquat.

The alterations in the gills of fish exposed to glyphosate are lower than in the gills of those exposed to paraquat. Fernandez *et al*., (2012) had earlier reported that the gills of fish exposed to glyphosate formulations exhibited low frequency of histopathology, suggesting that they have a high tolerance to glyphosate and associated compounds. This also supports earlier assertion that paraquat was more potent that glyphosate as it also stimulated more antioxidant enzymes activities than glyphosate.

Liver

Pathological changes in liver indicate that glyphosate and paraquat are both hepatotoxic to *C. gariepinus*. No pathological alteration were recorded in liver of control fish, indicating that observed damage can only have occurred as a result of exposure of fish to contaminated water. Vacuolar degeneration of hepatocytes is the major alterations observed in fish liver in this study. Vacuoles in the cytoplasm of the hepatocytes contain lipids and glycogen, which is related to the normal metabolic function of the liver. Thus, vacuolar degeneration will result in a depletion of the glycogen reserves in the hepatocytes (Hinton and Laurén, 1990; Wilhelm - Filho *et al.*, 2001). Vacuolar degeneration will result in stress to fish because glycogen acts as a reserve of glucose to supply higher energetic demand occurring in such situations (Panepucci *et*

al., 2001). Also, vital processes like detoxification and biotransformation are performed by the liver and 80% of these functions are carried out by the hepatocytes. This implies that if the integrity of the hepatocytes is compromised, the liver becomes inefficient to carry out these vital processes. Furthermore, Pacheco and Santos (2002) reported that increased vacuolisation of hepatocytes in fish exposed to contaminated water is a sign of degenerative process which itself suggests metabolic damage. Some other studies (Hued *et al*., 2012; Altinok *et al*., 2010; Ayoola, 2008) have reported liver necrosis as a result of exposure of fish to acute concentrations of pollutants. The similarity in the damage caused by both herbicides in the liver may mean there is a resemblance in the mechanism by which they exert their toxic actions in fish or rather in the manner in which the liver tries to detoxify them.

Brain

The brain of fish revealed proliferation of glial cells after exposure to acute concentratons of the herbicides. Gliosis is a tumor (composed of glial cells) of the brain. Diotel *et al*. (2010) reported that there is strong evidence that aromatase B is expressed virtually only in radial glial cells in fish. The cytochrome P450 aromatase is the only enzyme permitting the conversion of C19 androgens, such as testosterone (T) or androstenedione, into C18 estrogens, estradiol (E2) and estrone, respectively. Estradiol is involved in triggering and maintaining of ovarian differentiation and vitellogenin production in females. These are very important hormones in the reproduction of fish. That glyphosate has the ability to negatively influence the production pathway of these hormones will consequently mean reproductive impairment in fish. This is not encouraging in the present world where global fish production has not attained the required level. The spectrum of estrogenic actions of estradiol is constantly enlarging and now covers effects on the cardiovascular system, the mineralization of the bones, the adipose tissue, the immune system or the central nervous system (Diotel *et al*., 2010). Since estradiol also plays a

key role in other physiological systems of fish as the cumulative effect of the toxic action of this pollutant on fish is of reproductive significance.

Another damage to fish brain as a response to the herbicides is spongiform encephalopathy. Babatunde *et al*. (2014) reported encephalopathy in the brain of fish after exposure to paraquat. They concluded that pathological changes in the brain of the fish clearly shows that paraquat has neurotoxic effects and can adversely affect normal fish brain functioning. The brain also showed severe congestion that indicates brain damage. Congestion of blood vessels will literarily impede the flow of blood in the brain and consequently, oxygen supply to the brain will be low. These findings agree with those of Ayoola (2008) and Omitoyin *et al*. (2006) who reported that glyphosate caused pathological alterations in the brain of fish. Necrosis is the other brain alteration observed in exposed fish. This implies that some portions of the brain tissues are dead. This will ultimately reduce the functions of the brain as such those physiological activities requiring nervous signals from the brain are impeded. Necrosis has previously been reported by Ayoola (2008).

Histological effects of sublethal concentrations of glyphosate and paraquat on major fish organs

Gills

Histopathological changes were observed in the gills of fish after exposure to sublethal concentrations of glyphosate and paraquat. The alterations recorded in the gills of fish exposed to glyphosate included proliferative thickening of gill lamella and stunted gill lamella. Lower concentrations did not reveal any major damage to the gills during the exposure period. This may mean that the concentrations were not high enough to negatively affect the fish gills and so the fish were not negatively impacted by it. Thickening of gill lamella and stunted gill lamella may be a defensive response to the toxicants. Features like stunted lamella and

thickening of lamella (oedema) observed in the gills of glyphosate-treated fishes have been reported (Nandan and Nimila, 2012) to be defense response to toxicants.

These histopathological changes were limited to the second and fourth weeks alone. This may mean that there was little or no ionoregulatory and/or osmoregulatory dysfunction in response to the toxicants during the period when no damage was observed. It is also possible that there was increased mucus secretion during the sixth and eighth weeks of exposure. According to Mallatt, (1985) increased mucus secretion helps to reduce pollutant uptake. Furthermore, Ibarz *et al*. (2010) reported that the presence of fat tissue in fish could act as barriers to protect the gills, decreasing the respiratory surface and increasing the pollutant blood diffusion distance. The protective capability of the gills described above might also have played a role in ensuring pathological damage did not extend to the sixth and eigth weeks of exposure. Ogueji *et al*. (2008) had also reported that there was no pathological damage to fish gills after four weeks of exposure to lambda cyhalothrin and chlorpyrifos.

In the paraquat-treated fishes, sloughing off occurred in the gill epithelium, which means the inner healthy endothelial cells may be impaired with time. Gills are the first target of waterborne pollutants due to the constant contact with the external environment, as well as the main place for pollutant uptake due to high redox potential of this tissue. Pollutants enter cells through polyamine transporters present in the cellular membrane of the epithelium, and interfere with some gill functions such as respiration and osmoregulation (Boetslerli, 2007). This effect may account for the damage in the epithelial cells of the gills in the paraquat-treated fishes. It is also very likely that the noticeable damages are not detrimental and may not compromise the overall function of the gills as the gills were able to overcome the alteration by the sixth and eighth weeks. Poleksic and Mitrovic- Tutundzic (1994) reported that minor

lesions are repairable with water amelioration; however, if a large area of the gill is damaged and the contaminant levels increase or if other environmental events contribute to reduce the quality of the water, the existing histopathological changes may affect gill function and could even progress to become fatal lesions, since none of the factors highlighted above occurred during the treatment period, it is likely that the lesions observed in weeks 2 and 4 have been repaired by the sixth and eighth weeks.

Liver

In the present study, the main architectural changes shown by the liver tissues on exposure to both toxicants are swelling and vacuolation of hepatocytes and congestion of blood vessels. No visible lesions were observed in glyphosate-treated fish in week two of exposure. In the paraquat-treated fishes, there were alterations in fish liver from week 2 to 8. There was also no major damage in the liver of control fish.

Fish commonly accumulate greater concentrations of toxicants in liver than other parts of their body (Al-Yousuf *et al*., 2000). The aberrant hepatocytes reported in this study could disturb the normal metabolism of the organisms, thereby inducing diseases or even death. Vacuolation of hepatocytes are also associated with the inhibition of protein synthesis, energy depletion, disaggregation of microtubules or shifts in substrate utilization (Hinton and Lauren, 1990). The vacuolization of hepatocytes might also indicate an imbalance between the rate of synthesis of substances in the parenchymal cells and the rate of their release into the systemic circulation (Gingerich, 1982). In addition, contaminants have the capacity of inducing biological derangement of the molecular organization of the cell membranes leading to their degradation (Byzkowski, 1976). Vacuolation of hepatocytes has been reported by John *et al*. (1993), Ogueji *et al*. (2008) and Ayoola (2008).

The swelling of hepatocytes observed in this study was considered by Mollendroff (1973) as a cellular defense mechanism against substances injurious to hepatocytes and this mechanism responsible for collecting the injurious elements and preventing them from interfering with the biological activities of these cells.

The histopathological alterations observed in this study did not suggest a linear relationship between the concentrations of pesticides and the degenerative changes in various organs of *Clarias gariepinus.* According to Lindstoma-Seppa *et al*. (1981), changes in the liver tissues are linked with histological abnormalities in the kidneys and gills. Once the absorbed toxicant is transported via blood circulation to the liver for transformation and storage, it may be excreted through the bile or be passed back into the bloodstream for possible excretion by the kidneys or gills.

Brain

The sublethal concentrations of glyphosate and paraquat caused alterations to the brain structure of *Clarias gariepinus*, as evidenced by the congestion of blood vessels, spongiform vacuoles in neutrophils and gliosis. Damage to the fish brain, as evidenced by a series of lesions in the brain tissue, may subsequently influence normal physiological activities. The pathological changes observed in the brain of exposed fish may be associated with the hypoxic condition developed within the fish. Adeogun *et al*. (2012c) also reported histopathological damage to brain of *C. gariepinus* exposed to sublethal concentrations of *Raphia hookeri* extratcs.

Cytotoxic effects of acute concentrations of glyphosate and paraquat on fish

The shape of cells in the control fish looked normal with no form of abnormalities. High concentrations of glyphosate and paraquat were able to induce micronuclei formation (MN) and various other cell abnormalities like apoptosis, bean-shaped cell and lobed nuclei in fish.

This corroborates the findings of previous researchers (Cavas and Konen, 2007; Grisolia, 2002) who have reported cytogenetic effects of glyphosate in fish. This study also reveals that the higher the concentration, the higher the frequency of nuclear abnormalities. A dose dependent increase in the induction of micronuclei in peripheral blood of *Heteropneustus fossilis* has been reported by Das and Nanda (1986), in response to mitomycin C and paper mill effluents. Micronuclei frequency in *Channa punctata* (Bloch) was also reported to increase with increasing concentrations of chlorypyrifos after acute exposure (Ali *et al*., 2008). This implies that the different abnormalities observed in the exposed fish could have been as a result of the toxic action of the herbicides to fish.

Lobed nucleus is an indicator of abnormal cell division due to blocking of cytokinesis. This abnormal cell division would result in genetic imbalance in the cells, which may also lead to carcinogenesis (Rodilla, 1993).

The bean-shaped erythrocytes recorded in this study, may be due to their cellular outlines that are necrotic (Bushra *et al*., 2002). Erythrocytes are very important for oxygen transport and oxygen is important to fish during respiration occurring via the gills. Erythrocyte deformations causecould result in low oxygen levels, affecting the integrity of circulating erythrocytes thereby causing respiratory dysfunction. As such, respiratory stress may alter fish erythrocyte number and morphology (Brown, 1993).

Based on numbers alone, there were more of the other nuclear abnormalities i.e. apoptosis, bean-shaped cell and lobed nuclei than MN. It has been reported that the MN is a weak indicator of cytotoxicity compared to these other cell abnormalities. Carrasco *et al*. (1990) postulated the low sensitivity of the micronucleus test for fish, which could be related to a very low frequency of occurrence of micronucleated cells in fish. Some of the other cell nuclear abnormalities (apoptosis, bean-shaped cell and lobed nuclei) reported in this study has been

reported by Martinez *et al*. (2011). These changes in nuclear morphology are known to originate from genotoxic events as a result of exposure to xenobiotic contaminants (Pacheco and Santos, 1997). Winter *et al*. (2007) and Martinez *et al*. (2011) also reported very low induction of micronuclei in fish erythrocytes after exposure to different environmental contaminants.

The difference in the percentage frequency of altered cells between the lowest and highest concentrations is higher in glyphosate than in paraquat. These differences in the erythrocyte micronucleus frequencies are generally thought to be related to cell kinetics and replacement level (Cavas and Ergene-Gozukara, 2003).

Cytotoxic effects of sublethal concentrations of glyphosate and paraquat on fish

The sublethal concentrations of glyphosate and paraquat are toxic to the catfish *C. gariepinus*, the latter, showing more potency. The induction of micronucleated erythrocytes and other cell abnormalities following exposure to sublethal concentrations of both herbicides indicate a potential clastogenic effect of cypermethrin in fish. Rios *et al*. (1995), Ortiz *et al*. (2000), Palmisano *et al*. (2007) all reported the clastogenic potential of paraquat, using the micronucleus test. The clastogenic effect of glyphosate was also reported by Cavas and Konen (2007) using the same test.

The occurrence of a greater number of micronucleus and other nuclear abnormalities in the treated fishes compared with the control in this study provides evidence that the fishes had been under toxic stress. Since micronuclei are normally extruded along with the main nucleus, their presence would suggest their origin at a more recent cell cycle (Chandra and Khuda-Bukhsh, 2004). According to Jerbi *et al*. (2011), the presence of micronucleated cells may indicate aneugenic and/or clastogenic activities, because the formation of micronucleus can be

related to entire chromosomes, due to a poor functioning of the spindle, or with chromosome fragments, derived from chromosome breakage.

Toxicity of these herbicides on *C. gariepinus* increased with increasing concentrations in this study; again paraquat showed more potency. Hooftman and de Raat (1982) and Das and Nanda (1986) also reported a concentration and time-dependent increase in micronuceli induction in the fish erythrocytes, an effect corroborated by the present work. However, it is in contrast to some studies (de la Torre *et al*., 1999; Cavas *et al*., 2005) that reported decrease in the number of micronucleus in fish erythrocytes after 14 -21 days of exposure. Interplay between differences in fish age and species, to toxicant and dose administered might be responsible for these differences.

Other erythrocyte abnormalities like apoptosis, lobed nuclei and bean-shaped cells were also observed. This cytotoxic effect may be the direct result of the chromosomal damage and the destruction of cellular genetic material attributed to the test agents. Deoxyribonucleic acid alterations are known mechanisms responsible for cell death (Midander and Revesz, 1980). Furthermore, abnormality of erythrocytes has been ascribed to pathological conditions in fishes exposed to toxicants (Buckley *et al*., 1976). It has been reported that high or sublethal concentrations exposure of fish to pesticides (Adedeji *et al*., 2009) and to heavy metals such as nickel (Ololade and Oginni, 2010), lead (Adeyemo, 2007) cause decrease in erythrocyte integrity.

The overall differences between the percentage frequency of altered cells between the lowest and highest concentrations is higher in paraquat than in glyphosate in the sublethal exposures. It may be an indication that paraquat is more toxic. It is important also to note that differences such as these in the erythrocyte micronucleus frequencies are sometimes thought to be related

to cell kinetics and replacement (Cavas and Ergene-Gozukara, 2003). Comparing the acute and sublethal experiments, the frequency of altered cells is higher in the acute study than in the long term treatment. This may be because of the higher concentrations of the toxicant in the acute study which probably caused more toxicity to the fish erythrocytes.

Genotoxic effects of acute concentrations of glyphosate and paraquat in *Clarias gariepinus*

In the present study, glyphosate and paraquat have genotoxic potential in the liver cells of *Clarias gariepinus*. This is demonstrated by observation of DNA damage or DNA strand breakage as detected in this study. This could be considered as a kind of lesion which may be potentially pre-mutagenic (Kamman *et al*., 2001), to mutagenic and genotoxic properties of these pesticides (Frenzilli *et al*., 2000). According to Fairbairn *et al*. (1995), DNA damage could originate from DNA single-strand breaks, DNA double strand breaks, DNA adducts formations and DNA–DNA and DNA–protein cross-links resulting from the interaction of the pesticide or its metabolites with DNA. Roundup® and glyphosate-based products could affect cellular function and interfere with regulation of the cell cycle regulation, which can lead to genomic instability and the subsequent development of cancerous cells (Molinari, 2000; Stewart *et al*., 2003; Marc *et al*., 2004).

In mitochondria, methyl groups, which are components of herbicides used in this study, in a process called methylation, can bind to crucial enzymes and respiratory protein complexes uncoupling oxidative phosphorylation, leading to the generation of reactive oxygen species (ROS) (Di Giulio *et al*., 1995). The presence of indicators positive for the generation of reactive oxygen species as reported in the present study (increased lipid peroxidation products) might also be responsible for the damage to the DNA. Several investigations have demonstrated that the generation of reactive oxygen species could be associated with DNA

damage resulting in an increase of lesions at chromosomal level (Emerit *et al*., 1982; Soloneski *et al*., 2001, 2003).

The result of this study agrees with previous studies (Nwani *et al*., 2013) that investigated the genotoxic effects of acute concentrations of glyphosate in fish, similarly paraquat has also shown genotoxic potentials. Comparing both herbicides, paraquat caused more damage to the DNA than glyphosate and may be more toxic than glyphosate as indicated by LC_{50}.

Genotoxic effects of sublethal concentrations of glyphosate and paraquat in *Clarias gariepinus*

The induction of DNA damage in liver cells of *Clarias gariepinus* by glyphosate and paraquat exposure at different concentrations indicates potential genotoxic and mutagenic properties. The control fishes had intact DNAs and thus DNA damage can be correlated with the clastogenecity of the herbicides. An increase in both micronuclei and DNA migration due to environmental mutagen has been reported in fish (Russo *et al*., 2004).

Usually, the first mechanism of action of genotoxic agents is to promote DNA damage, which can have three possible outcomes: the damage can be repaired, the damage can become irreversible, or the damage may lead to cell death (Vicari *et al*., 2012). According to Akcha *et al*. (2003), the formation of DNA strand breaks in erythrocytes could result from the absorption and the biotransformation of genotoxic environmental pollutants. Furthermore, the damage to fish DNA caused by herbicides used in the present study might also have occurred as a result of the production of reactive oxygen species (ROS). Reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH^-) have been shown to produce damage such as DNA strand breaks, enzyme inactivation and even apoptosis (Peña-

Llopis *et al*., 2003; Banudevi *et al*., 2006). Thus, it is possible that glyphosate and paraquat could cause alterations in DNA of *C. gariepinus* resulting in formation of comets.

The disappearance of the comets in DNA as observed in the tissues of fishes exposed to different concentrations of glyphosate and paraquat after 2 weeks may indicate repair of damaged DNA, loss of heavily damaged cells, or both (Banu *et al*., 2001). The long-term genotoxicity studies can be an important approach for achieving greater insight into the organisms DNA repair ability and other protective mechanisms for excreting the toxic chemicals. Furthermore, the absence in tail DNA at longer exposure as observed in this study could also be due to other mechanisms like toxicity of the contaminant preventing the enzymatic process of DNA damage (Rank and Jensen, 2003).

Comparing the acute and sublethal toxicity test results, the former caused more damage to fish DNA. This is most likely because of the higher concentrations of the herbicides in the acute study thereby resulting in more DNA damage. Also, as in acute study, the damage recorded in the DNA of exposed fish to paraquat was more as evidenced in the tail length. This probably further indicates that paraquat may be more toxic to fish than glyphosate.

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

Glyphosate and paraquat were toxic to juveniles of *Clarias gariepinus* with paraquat showing more toxicity than glyphosate. The LC_{50} of the former is 0.042mg/L and that of the latter, 0.530mg/L. These values may probably be useful guides in determining the quantity of these chemicals that may have deleterious effects on aquatic organisms. As this study comprehensively looked into how these two commonly used herbicides affect aspects of fish physiology like behavioural and biochemical parameters, histotology of major fish organs, cytotoxicity and genotoxicity. It is believed that the present study has provided further information on the threats these chemicals can pose to aquatic organisms. Both acute and sublethal exposure of *Clarias gariepinus* to these two chemicals elicited changes in the behavioural and biochemical parameters, histology of organs, cellular and molecular indexes of the fish. These changes were either dose dependent, time dependent, or both. The changes observed in the histopathological and biochemical analyses, comparing both herbicides are similar. Behavioural features like opercular ventilation count, tail fin movement rate and air gulping index were all negatively impacted by acute concentrations of both herbicides. Both herbicides caused no major lesion in the gills. The implication of this is that the gills being the first contact organ for these chemicals possess mechanisms to get rid of these chemicals. This may however not be the same in fishes that are immuno-compromised. Both herbicides also caused swelling and vacuolation of hepatocyte in fish liver. It is only in the brain that an alteration (gliosis) caused by glyphosate was not caused by paraquat. It is also safe to conclude that both herbicides showed oxidative/oxidant potentials as this can be easily inferred from the anti-oxidant responses shown by the fish liver. The values obtained from these anti-oxidant capabilities of the fish liver are very similar in both herbicides. However, the responses shown

by the fishes to the cellular and molecular indexes are greater in those exposed to paraquat than those exposed to glyphosate. This is evident in more DNA damage recorded in the former than the latter. From the result of all the toxic indeces evaluated in fish after exposure to the herbicides, if these chemicals find their ways into freshwater bodies, their cumulative effects in fish populations might have important implications. Juveniles will probably experience reduced competitive ability, vulnerability to predation, decreased ability to feed, compromised immune system, decreased mating success or even outright infertility. Further studies should be carried out to either authenticate the result of the current study or look at other aspects of the fish physiology where these chemicals may be harmful. Adequate data build-up on the investigated parameters of our local fish species may be helpful in the possible early diagnosis of a disease before it becomes epidemic, and also in the assessment of possible toxic effects of various environments when the toxins are in acute and sublethal concentrations. In summary, the result indicate that several biochemical parameters, related to both anti-oxidant potential, histology, cytotoxicity and genotoxicity may be useful indicators in monitoring glyphosate and paraquat exposure at sublethal levels in fishes.

6.2 Conclusions

1. In conclusion, glyphosate and paraquat were found to be toxic to juveniles of *Clarias gariepinus*.

2. The LC_{50} of the herbicides to juveniles of *C. gariepinus* was determined and paraquat was found to be more toxic.

3. Acute concentrations of both herbicides caused behavioural abnormalities in fish. The results of the biochemical parameters assayed also showed that paraquat was more toxic to fish while the acute toxicity tests elicited more toxic response in fish than the sublethal tests in both herbicides.

4. Histopathological observation further revealed that acute and sublethal concentrations of these herbicides damaged the gills, liver and brain of fish.

5. Mutagenicity of the acute and sublethal concentrations of glyphosate and paraquat to juveniles of *C. gariepinus* was observed in this study via different cell abnormalities and DNA damage that were observed during this study.

6. As major fish organs, such as gills, brain and liver were adversely affected after exposure to these two herbicides, it may be recommended that:

6.3 Recommendations

i. Further studies are required to investigate the immunotoxicological mechanisms of action of these herbicides in *C. gariepinus* and other fish species.

ii. Fish bioassay as a regulatory test should be supplemented by toxicity tests on other aquatic food chain organisms to give a picture of cumulative toxicity at the community level in the ecosystem.

iii. Biomonitoring technology should be used to predict the effects of new chemical substances likely to reach aquatic ecosystems. In addition, the use of biomonitoring tools to predict the future of ecological effects of chemical substances is necessary to prevent these substances from reaching hazardous concentrations in the ecosystem.

iv. The DNA repair capacities of fish (particularly spermatozoa), should be investigated to assess the effects of these herbicides on reproduction.

v. Further research should focus on the prospects of microbial degradation and phytoremidiation of these herbicides

vi. It is also recommended that investigations for natural herbicides from plants, potent on target organisms but with short half life with little or no effect on fish are worth researching for.

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APPENDIX

APPENDIX A

RESULTS OF THE ANALYSIS OF VARIANCE FOR BEHAVIOURAL PARAMETERS OF FISH XPOSED TO GLYPHOSATE

Opercular Ventilation Count

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	74.55742	4	18.63936	0.618531	0.655708	3.006917
Columns	603.5734	4	150.8934	5.00727	0.00824	3.006917
Error	482.1577	16	30.13486			
Total	1160.289	24				

Tail Fin Movement Rate

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	704.4436	4	176.1109	4.001416	0.019506	3.006917
Columns	7963.6	4	1990.9	45.23525	1.58E-08	3.006917
Error	704.1942	16	44.01214			
Total	9372.238	24				

Air Gulping Index

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.656	4	0.164	6.368932	0.002901	3.006917
Columns	0.432	4	0.108	4.194175	0.016427	3.006917
Error	0.412	16	0.02575			
Total	1.5	24				

APPENDIX B

RESULTS OF THE ANALYSIS OF VARIANCE FOR BEHAVIOURAL PARAMETERS OF FISH XPOSED TO PARAQUAT

Opercular Ventilation Count

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1065.934	4	266.4834	5.16709	0.011782	3.259167
Columns	6938.082	3	2312.694	44.84293	8.53E-07	3.490295
Error	618.8785	12	51.57321			
Total	8622.894	19				

Tail Fin Movement Rate

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	7.3	4	1.825	0.102193	0.979593	3.259167
Columns	1637.2	3	545.7333	30.55903	6.69E-06	3.490295
Error	214.3	12	17.85833			
Total	1858.8	19				

Air Gulping Index

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.17707	4	0.044268	1.171072	0.371634	3.259167
Columns	0.94774	3	0.315913	8.357311	0.002866	3.490295
Error	0.45361	12	0.037801			
Total	1.57842	19				

APPENDIX C

RESULTS OF THE ANALYSIS OF VARIANCE FOR BIOCHEMICAL PARAMETERS OF FISH IN ACUTE STUDY WITH GLYPHOSATE

Variate: ALT

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	12865.56	1	12865.56	548.016	1.18E-08	5.317655
Within Groups	187.813	8	23.47662			
Total	13053.38	9				

Variate: AST

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	13529.82	1	13529.82	563.6388	1.05E-08	5.317655
Within Groups	192.0353	8	24.00441			
Total	13721.85	9				

Variate: LDH

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	18650.48	1	18650.48	1490.264	2.23E-10	5.317655
Within Groups	100.119	8	12.51488			
Total	18750.6	9				

Variate: ALP

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1981.225	1	1981.225	295.5619	1.33E-07	5.317655
Within Groups	53.626	8	6.703251			
Total	2034.851	9				

Variate: CAT

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	53728.61	1	53728.61	873.2936	1.86E-09	5.317655
Within Groups	492.1928	8	61.5241			
Total	54220.8	9				

Variate: SOD

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2086.667	1	2086.667	213.2686	4.74E-07	5.317655
Within Groups	78.27377	8	9.784221			
Total	2164.941	9				

Variate: MDA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.725188	1	4.725188	87.03349	1.42E-05	5.317655
Within Groups	0.434333	8	0.054292			
Total	5.15952	9				

Variate: GPx

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	18.29797	1	18.29797	887.48	1.75E-09	5.317655
Within Groups	0.164943	8	0.020618			
Total	18.46292	9				

APPENDIX D

RESULTS OF THE ANALYSIS OF VARIANCE FOR BIOCHEMICAL PARAMETERS OF FISH IN ACUTE STUDY WITH PARAQUAT

Variate: ALT

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	13289.31	1	13289.31	547.3818	1.18E-08	5.317655
Within Groups	194.2236	8	24.27795			
Total	13483.53	9				

Variate: AST

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	13831.26	1	13831.26	766.6296	3.12E-09	5.317655
Within Groups	144.3332	8	18.04164			
Total	13975.59	9				

Variate: LDH

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	16646.73	1	16646.73	985.3471	1.15E-09	5.317655
Within Groups	135.1542	8	16.89428			
Total	16781.88	9				

Variate: ALP

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1781.985	1	1781.985	238.1245	3.09E-07	5.317655
Within Groups	59.86734	8	7.483417			
Total	1841.852	9				

Variate: CAT

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	53298.47	1	53298.47	1802.464	1.04E-10	5.317655
Within Groups	236.5583	8	29.56978			
Total	53535.03	9				

Variate: SOD

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2067.844	1	2067.844	127.3939	3.41E-06	5.317655
Within Groups	129.8551	8	16.23189			
Total	2197.699	9				

Variate: MDA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.387738	1	4.387738	920.1794	1.51E-09	5.317655
Within Groups	0.038147	8	0.004768			
Total	4.425884	9				

Variate: GPx

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	26.48431	1	26.48431	2427.671	3.19E-11	5.317655
Within Groups	0.087275	8	0.010909			
Total	26.57158	9				

APPENDIX E

RESULTS OF THE ANALYSIS OF VARIANCE FOR BIOCHEMICAL PARAMETERS OF FISH EXPOSED TO SUBLETHAL CONCENTRATIONS OF GLYPHOSATE

Variate: ALT

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	898.77	3	299.59	10.42523	0.002756	3.862548
Columns	194.0251	3	64.67503	2.250582	0.151607	3.862548
Error	258.6333	9	28.73703			
Total	1351.428	15				

Variate: AST

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	899.6287	3	299.8762	25.3266	0.000101	3.862548
Columns	424.8212	3	141.6071	11.95969	0.001713	3.862548
Error	106.5633	9	11.84037			
Total	1431.013	15				

Variate: LDH

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1295.096	3	431.6988	6.503423	0.012424	3.862548
Columns	284.7468	3	94.9156	1.429877	0.297197	3.862548
Error	597.4222	9	66.38025			
Total	2177.265	15				

Variate: ALP

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	52.76318	3	17.58773	31.8911	4E-05	3.862548
Columns	18.97787	3	6.325957	11.4706	0.001982	3.862548
Error	4.963439	9	0.551493			
Total	76.70449	15				

Variate: CAT

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1077.214	3	359.0713	16.28525	0.000558	3.862548
Columns	463.8214	3	154.6071	7.012024	0.009913	3.862548
Error	198.4397	9	22.04886			
Total	1739.475	15				

Variate: SOD

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	8.13E-05	3	2.71E-05	6.210191	0.014226	3.862548
Columns	5.53E-05	3	1.84E-05	4.22293	0.040269	3.862548
Error	3.93E-05	9	4.36E-06			
Total	0.000176	15				

Variate: MDA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.6259	3	0.208633	23.22449	0.000143	3.862548
Columns	0.12445	3	0.041483	4.617811	0.032117	3.862548
Error	0.08085	9	0.008983			
Total	0.8312	15				

Variate: GPx

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Rows	2.756507	3	0.918836	35.80049	2.49E-05	3.862548
Columns	0.320136	3	0.106712	4.15781	0.041845	3.862548
Error	0.230989	9	0.025665			
Total	3.307632	15				

APPENDIX F

RESULTS OF THE ANALYSIS OF VARIANCE FOR BIOCHEMICAL PARAMETERS OF FISH EXPOSED TO SUBLETHAL CONCENTRATIONS OF PARAQUAT

Variate: ALT

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1509.995	3	503.3316	174.5283	2.72E-08	3.862548
Columns	136.1351	3	45.37838	15.73478	0.000634	3.862548
Error	25.95559	9	2.883954			
Total	1672.086	15				

Variate: AST

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	55.95689	3	18.6523	8.151041	0.006205	3.862548
Columns	130.5319	3	43.51065	19.01412	0.00031	3.862548
Error	20.595	9	2.288333			
Total	207.0838	15				

Variate: LDH

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	42.08807	3	14.02936	2.575204	0.118731	3.862548
Columns	327.816	3	109.272	20.05778	0.000253	3.862548
Error	49.03076	9	5.447863			
Total	418.9349	15				

Variate: ALP

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	11.77436	3	3.924785	3.363338	0.068664	3.862548
Columns	19.46562	3	6.48854	5.560342	0.019496	3.862548
Error	10.50238	9	1.166932			
Total	41.74236	15				

Variate: CAT

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1295.553	3	431.8512	17.54871	0.000421	3.862548
Columns	376.9582	3	125.6527	5.106025	0.024634	3.862548
Error	221.4784	9	24.60872			
Total	1893.99	15				

Variate: SOD

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.000226	3	7.52E-05	4.028635	0.045197	3.862548
Columns	0.000398	3	0.000133	7.107847	0.009512	3.862548
Error	0.000168	9	1.87E-05			
Total	0.000792	15				

Variate: MDA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.002619	3	0.000873	6.014354	0.015608	3.862548
Columns	0.001919	3	0.00064	4.406699	0.036196	3.862548
Error	0.001306	9	0.000145			
Total	0.005844	15				

Variate: GPx

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1.120103	3	0.373368	8.186551	0.00612	3.862548
Columns	0.394845	3	0.131615	2.88582	0.094978	3.862548
Error	0.410467	9	0.045607			
Total	1.925415	15				

APPENDIX G

RESULT OF THE ANALYSIS OF VARIANCE OF FREQUENCY OF ALTERED CELLS IN FISH EXPOSED TO ACUTE CONCENTRATIONS OF GLYPHOSATE

Variate: Frequency of altered cells

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.30321	1	1.30321	14.62473	0.005059	5.317655
Within Groups	0.71288	8	0.08911			
Total	2.01609	9				

APPENDIX H

RESULT OF THE ANALYSIS OF VARIANCE OF FREQUENCY OF ALTERED CELLS IN FISH EXPOSED TO ACUTE CONCENTRATIONS OF PARAQUAT

Variate: Frequency of altered cells

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.476282	1	3.476282	50.74597	9.96E-05	5.317655
Within Groups	0.548029	8	0.068504			
Total	4.02431	9				

APPENDIX I

RESULT OF THE ANALYSIS OF VARIANCE OF FREQUENCY OF ALTERED CELLS IN FISH EXPOSED TO SUBLETHAL CONCENTRATIONS OF GLYPHOSATE

Variate: Frequency of altered cells

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.465817	2	0.232908	20.59617	0.002055	5.143253
Columns	0.8262	3	0.2754	24.35372	0.000928	4.757063
Error	0.06785	6	0.011308			
Total	1.359867	11				

APPENDIX J

RESULT OF THE ANALYSIS OF VARIANCE OF FREQUENCY OF ALTERED CELLS IN FISH EXPOSED TO SUBLETHAL CONCENTRATIONS OF PARAQUAT

Variate: Frequency of altered cells

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.328617	2	0.164308	14.00687	0.005489	5.143253
Columns	0.952167	3	0.317389	27.05659	0.000695	4.757063
Error	0.070383	6	0.011731			
Total	1.351167	11				