EFFECTS OF GLYPHOSATE AND 2, 4-DICHLOROPHENOXYACETIC ACID ON THE HATCHABILITY AND LARVAL DEVELOPMENT OF AFRICAN CATFISH *(Clarias gariepinus*).

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CERTIFICATION

This is to certify that this work, "Effects of Glyphosate and 2, 4-Dichlorophenoxyacetic acid on the Hatchability and Larval Development of the African Catfish (*Clarias gariepinus*)", was carried out by STEPHEN NNADOZIE ALOZIE (20154944338) in partial fulfilment for the award of the degree of M.Sc. in Biotechnology in the Department of Biotechnology of the Federal University of Technology, Owerri.

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DEDICATION

This thesis is dedicated to my lovely mum, Mrs Chinyere Anna Alozie, whose love for education is unequalled.Her earnest desire is for her children to attain the greatest height in education, even though she could not, as a result of being orphaned early in life.

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ABSTRACT

The effects of the exposure of glyphosate and 2, 4-dichlorophenoxyacetic acid, on the egg hatchability and larvae development ofCatfish (*Clarias gariepinus)*were evaluated using standard methods. Acute toxicity,hatchability of eggs, survivability of larvae, biochemical andhistological parameters of reproductive organs and skin were evaluated. The acute lethal study of glyphosate and 2, 4-D on catfish fingerlings showed that no fish died within 24 hrs. However, for the mixture of glyphosate and 2, 4-D, at higher concentrations death occurred, and the $LD_{50}$was less than 2500mg/kg (2154.07mg/kg). There was a change inwater quality resulting from application of the toxicants compared to the control. There wasalso decrease in the hatchability of the eggsand reproductive success for the mixture of glyphosate and 2, 4-D treatments. The highest percentage mortalityand least percentage survivability(after fourteen days) were recorded in the mixture of glyphosate and 2, 4-D-treated eggs (72±60 and 20±16.67 respectively). The mean weight gain value and the instantaneous growth rate, expressed as Specific Growth Rate (SGR), werelow in the mixture of glyphosate and 2, 4-D (0.75 ± 0.08g and 0.71 respectively) compared with control (2.24±0.22). Physical examination showed the larvae of the mixture of glyphosate and 2, 4-D to be smaller and lighter (tail region appeared transparent) relative to those from other treatments and control. Biochemical parameterson the liverrevealed elevation in the activities of stimulating liver enzymes (GSH and GST)in all experimental groups (1.06/6.88E-06, 1.67/5.72E-06, 1.73/5.88E-06) compared with control (1.53/4.28E-06). There werealso elevation in the activities of the antioxidant enzymes such as Catalase (CAT), Superoxide dismutase (SOD), Glutathione Peroxidase (GPx), and lipid peroxidation compared with control. There was reduction in the total protein level for the glyphosate treatment (15.46g/l) compared to control (25.60g/l). The histological changes observed in this experiment revealed that glyphosate, 2, 4-D and their mixtures caused negativeeffects on the skin, testes and ova of *Clarias gariepinus* brood stocks. The fish organs examined showed varying degrees of pathological alterations/degenerations to the skin, testes and ova in all the treatments compared to the control. These results show that the herbicides, glyphosate and 2, 4-D, have toxic effects on the hatchability and survivability of *Clarias gariepinus*. These toxic effects were more deleterious when the two herbicides were mixed, as the synergistic effects greatly impeded fish reproduction.

Key words: Toxicity, Hatchability, Herbicide, Histology, *Clarias gariepinus*

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND INFORMATION

Fish has long been providing food or animal protein for millions of poor people in Nigeria. To meet the increasing domestic and foreign demands of fish and fishery products, aquaculture has expanded tremendously in Nigeria over the last two decades. The increase in aquaculture production now is really impressive. For this higher production, aquaculture practice has been moved from traditional to improved or semi-intensive system and with the expansion and intensification of aquaculture in Nigeria there has been increasing trend in using chemicals in aquatic animal health management (Adekoya*et al.,* 2004). Now, the use of chemicals and drugs has become vital input in aquaculture for effective farming and high production, as intensification make farmed animals susceptible to a variety of diseases and health problems (Adekoya*et al.,* 2004).

Chemicals used in aquaculture can be classified according to purpose of use, the type of organism under culture, the lifecycle state for which they are used, the culture method and intensity of culture, and the type of people who are using them (Hossain*et al.,* 2013). Chemicals and antibiotics are important apparatus in health management of aquatic animals, pond construction, soil and water management, improve aquatic productivity, transportation of live fish, feed formulation, manipulation of reproduction, growth promotion, (Subasinghe*etal.,* 1996; Gesamp, 1997). Commonly used chemicals for aquaculture are Lime, Rotenone, Fertilizers, Phostoxin, Salt, Dipterex, Antimicrobial, Potassium permanganate, Copper sulphate, Formalin, Sumithion, Malachite green, Methylene Blue, Malathion and bleaching powder. (Plumb, 1992; Frank *et al.,* 2004; Brown and Brooks, 2002;

Khan *et al.,* 2011). In most cases, the farmers have poor or no knowledge regarding the appropriate chemicals and those they should use. Intensive culture of fish requires chemicals that control disease, enhance the growth of cultured species, reduce handling trauma to organisms, improve water quality, disinfect water and control aquatic vegetation. These aquaculture chemicals help in eliminating harmful bacteria and pathogens which lead to complete purification of water used in agricultural and aquaculture industries. Many chemicals tend to persist for many months or years in aquatic systems. Some antibacterials, notably Oxytetracycline, Oxolinic acid, and Flumequine, can be found in sediments at least six (6) months following treatment (Weston,1996). Indiscriminate use of aqua drugs and chemicals often lead to problems like drug resistance, tissue residues, adverse effects on species biodiversity etc. which ultimately affect the cultured species, world local species, human and environment. Antibiotic-resistant bacteria in the aquatic ecosystem have the potential of reaching terrestrial animals by gene transfer. Several of these aspects have been documented (Spranggard*et al.*, 1993; Herwig and Gray, 1997; Anderson and Levin, 1999; Tendecia and De la Pena, 2001).

Fish seed production is an important aspect of aquaculture that has witnessed continuous research and innovation for increased fish production. Artificial propagation method constitutes the major practicable means of providing enough quality seed for rearing in confined fish enclosure waters such as fish ponds, reservoirs and lakes (Charo and Oirere, 2000). Catfish seed production is not left out as it represents a valuable fish species most especially in Africa. This fish exhibits a seasonal gonadal maturation which is frequently triggered by rainy season. The maturation process of *Clariasgariepinus*in nature is generally influenced by annual changes in water temperature and photoperiodicity and the

final triggering of spawning is usually caused by rise in water level due to rainfall (De Graaf*et al*., 1995). In captivity, Catfish does not spawn by itself, except the environment is simulated to mimic its natural habitat, which is a cumbersome process and uneconomical (De Graaf*et al.,* 1995). Meanwhile, in its natural environment, several studies have reported an average rate of 59.1% in the rainy season for *C. gariepinus* in the republic of Congo while Macharia *et al.*(2005) reported a rate as low as 40% for *C. gariepinus* eggs incubated on a nylon substrate. As a result of this, several researches have been conducted to improve hatchability and survival of catfish larvae in captivity (Huisman and Ritcher, 1987; Olubisi*et al.*, 2005; Phelps, 2010; Ude*et al.* 2005; and Olanike *et al*., 2011).

Despite all these researches, there still exist a wide gap between fish seed demands and supply. However, the insufficiency of supply and relatively high cost of fingerlings of *C. gariepinus* (Ofor, 2007), resulting from low output per breeding attempt, indicates the need to widen the study regarding the scope of factors affecting the low output. As a result of this backdrop, this project is aimed at studying the effects of Glyphosate and 2, 4-Dichlorophenoxyacetic acid (herbicides) on the hatchability and larvae development of African Catfish (*Clariasgariepinus*).

1.2 PROBLEM STATEMENT

Appreciation of fisheries and aquatic systems has been accompanied by increasing concern about effects of growing human population and human activities on aquatic life and water quality. The environmental impact of pesticides is often more 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 on fish and other aquatic life biota. Pesticide runoff into rivers and streams can be highly lethal to aquatic life, sometimes killing all the fish in a particular stream (Toughill, 1999). Herbicides can accumulate in water bodies 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 pesticides input (Doherty *et al.*, 2011).

The indiscriminate use of herbicide, careless handling, accidental discharge of herbicide laden untreated effluent into natural water ways has harmful effects on the fish populations and other aquatic organisms and may contribute to short, as well as long term effects in the environment. Sub-lethal effects with biochemical and histopathological alteration of fish tissues may occur with long term exposure to high levels of herbicides (Neskovic*et al.*, 1993)

The toxicity of some chemicals can 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 alteration in behavior (Wilson, 2006). 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, arteriosclerosis, 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 effects with long term alterations in organism's body by being active through several generations (Jha*et al.*, 2000).

1.3 AIMAND OBJECTIVES

AIM:

The aim of this study is to determine the effects of glyphosate and 2, 4-Dichlorophenoxyacetic acid on the hatchability and larvae development of *Clariasgariepinus.*

OBJECTIVES:

1. To determine theindividual effects of glyphosate, 2, 4-Dichlorophenoxyacetic acid, and the mixture of glyphosate and 2, 4-D on the hatchability and survival rate of Catfish (*Clariasgariepinus).*
2. To determine the effects of glyphosate, 2, 4-Dichlorophenoxyacetic acid, and the mixture of glyphosate and 2, 4-Dichlorophenoxyacetic acid on some biochemical parameters of the liver of brood stocks of Catfish (*Clarias gariepinus).*
3. To determine the effects of glyphosate, 2, 4-Dichlorophenoxyacetic acid, and the mixture of glyphosate and 2, 4-Dichlorophenoxyacetic acid on some histological parameters of the eggs, testes and skin of Catfish (*Clarias gariepinus).*
4. To determine the water quality parameters for both the treatments and the control of the water used for housing the African catfish brood stocks and the fingerlings exposed to glyphosate, 2, 4-Dichlorophenoxyacetic acid, and the mixture of glyphosate and 2, 4-D.
5. To determine the acute toxicity (LD_{50}) of glyphosate, 2, 4-Dichlorophenoxyacetic acid, and the mixture of glyphosate and 2, 4-Dichlorophenoxyacetic acid on the fingerlings of Catfish (*Clarias gariepinus).*

1.4RESEARCH HYPOTHESIS

I. H_o: There is no effect between control and treatments ofCatfish brood stocks exposed to Glyphosate, 2, 4-D, and the mixture of glyphosate and 2, 4-D herbicides.

II. H_o: There is no significant difference ($P>0.05$) between the hatchability and survival rates of the control and exposed Catfish broodstocks to concentrations of glyphosate, 2, 4-D, and the mixture of glyphosate and 2, 4-D.

III. H_o: There is no significant difference ($P>0.05$) between the biochemical profiles of control and exposed Catfish brood stocks to concentrations of glyphosate, 2, 4-D, and the mixture of glyphosate and 2, 4-D.

IV. H_o: There is no significant difference ($P>0.05$) between the histological parameters of the eggs, testes and skin of control and exposed Catfish broodstocks to concentrations of glyphosate, 2, 4-D, and the mixture of glyphosate and 2, 4-D.

V. H_o: There is no significant difference ($P>0.05$) in the water quality parameters between the control and the treatments at the start and towards the end of the experiment.

VI. H_o: There is no toxic effect of the herbicides on the fingerlings of Catfish (*Clarias gariepinus)*.

1.5JUSTIFICATION OF STUDY

- 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 programmers on environmental health and chemical safety (Oshode *et al.*, 2008).
- 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). Aquatic bioassay is 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 effect of environmental contamination by noxious compounds (Ayoola, 2008). Fishes are the most useful bio-indicators 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).
- *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 habits, 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, Opeyemi, 2015).

- For sustainable fish production in Nigeria, the ecotoxicological monitoring programmes need to incoperate proper management programmes for pesticides use and disposal in aquatic habitat. This study was therefore aimed at determining if glyphosate and 2, 4-D havetoxic effects on the hatchability and larvae development (survival rate) of *Clarias gariepinus.*

CHAPTER TWO

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 diseases, such as mosquitoes, and in agriculture, to kill pests that damage crops (Opeyemi, 2015). 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 practicesfor pesticides (WHO, 1990). According to the Stockholm Convention on Persistent Organic Pollutants in 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 (Golden *et al.,* 2010). Many tonnes of pesticides are used annually in agriculture and horticulture. Consequently, water from green houses and runoff from agricultural land are nearly always contaminated with pesticides (Schalz and Leiss, 1999). Due to their toxicity, pesticides affect the ecology of the receiving bodies of water and contaminated drinking water supplies(Fernandez-Alba *et al.,* 2000). Some pesticides bioaccumulate, affecting fish, birds other animals and human food source (Fernandez-Alba *et al.*, 2000). Depending on the local cultivation practices, a water body may receive a single pesticides or a varying cocktail of compounds (Fernandez-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 non-linear. Cocktails of compounds pose bigger problems because toxicity of a mixture is not easily linked to individual toxicities of compounds 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 category 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 bio-concentration and biomagnifications (Sivaperumal and Sanker, 2013). Bio-concentration is the movement of a chemical from the surrounding medium into an organism. The primary "Sink" for some pesticides is fatty tissues (Iipids). Some pesticides such as DDT, are "Lipophilic", meaning that they are soluble in and accumulated in fatty tissues 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 large organisms, the concentration of pesticides and other chemicals are increasingly magnified in tissues and other organisms. Very high concentration can therefore be observed in top predators, including humans (Sivaperumal and Sanker, 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 pesticides 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, cancer, tumors and lesions on fish and animals, reproductive inhibition or failure, suppression of immune system and disruption of endocrine (hormonal) system (Opeyemi, 2015). 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 whiteblood cell ratio, excessive slime on fish scales and gills and inter-generational 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).

2.2 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 influence 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

theseprocesses. 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 five major groups: degradation, transfer, adsorption, bioaccumulation and 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 pesticide's 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 pesticides 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 ground water and injury to non- target species including humans (Dustin, 2011). Pesticides can be transferred through natural processes such as volatilization, runoff, 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 determiningpesticide 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).

Absorption is the movement of pesticides into plants and animals or structures such as soil and wood. Absorption of pesticidesby target and non- target organisms is influenced by environmental conditions, physical and chemical properties of the pesticides and the soil (Ikpesu and Ariyo, 2013). Once absorbed by plants, pesticides may be broken down or remain in the plant until tissues 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 endup in the aquatic environment. Most harvested food varieties are subjected to washing and processing procedures that remove or degrade much of the remaining pesticide residues. 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 pesticides (Ikpesu and Ariyo, 2013).

Bioaccumulation is the ability of some chemicals to build up in the body tissues 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 bio-magnification. Bio-magnification results in much greater exposure in organisms at the top of the

food chain (EXTONET, 1993). Persistent pesticides in food chains, for example organo-chlorine pesticides such as dichlorophenyl trichloroethane, were banned from agricultural use due to 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 pesticides but also on the species of fish, its age, size and its position in the food chain (Van der Oost *et al.*, 2003).

2.3 HERBICIDES

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 to micro-organisms such as fungi moulds, 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 of plant parts (Amdur *et al*., 1991).

2.4 HERBICIDE CLASSIFICATION SYSTEMS

Herbicides can be classified in several ways such as usage, translocation patterns, modeof 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 wall and xylem. Herbicides applied to foliage can move into plants through cracks in the leaf, open stomata and leaf cuticle (Ditomaso, 2000).

2.5 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}. An LD_{50} or LC_{50} Value is the lethal dose or concentration of herbicide which will kill 50% of the test animals based on mg of chemical per kg of body weight. These formulations 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 herbicides 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). 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 colour, both as solid and liquid forms (Ditomaso, 2010).

2.6 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, EPSP inhibitors, energy production inhibitors, cell membrane destructors, general cell toxicants and herbicides with unknown mode of action (Ditomaso, 2010).

2.7 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 (Glesy *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 (Glesy *et al*., 2000; Williams *et al*., 2000). Herbicidal effects are therefore limited to foliar contact, cutstump or stem injected application 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. Government regulatory agencies, international organization, and others have reviewed and assessed the available scientific data for glyphosate formulation 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 (Glesy *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; Simentad *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 $C_3H_8NO_5P$. (Figure 2.1).

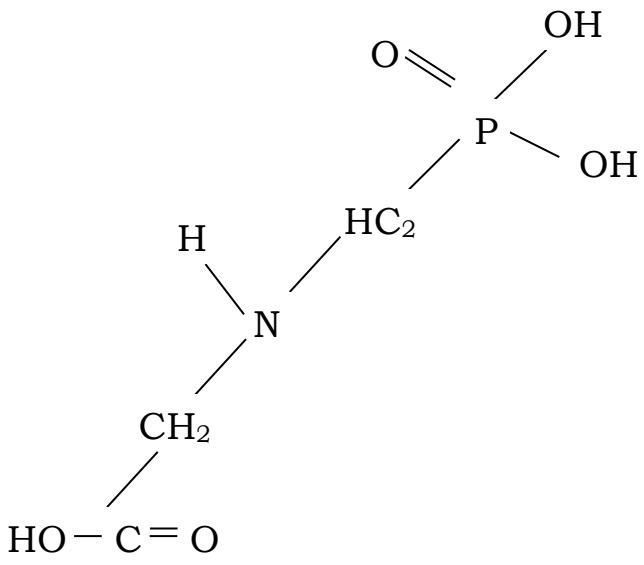


Figure 2.1: Chemical Structure of Glyphosate

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, iso-propylamine salt, potassium salt, sodium salt, and trimethyl sulfonium or

trimesium salt (Tomlin, 2006; Vencil, 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.

2.7.1 GLYPHOSATE MODE OF ACTION

ForceUp® was used. ForceUp® is used to control the emerged weeds. It inhibits 5-enol pyruvylshikimate-3-phosphate (EPSP), an acid biosynthetic pathway. This prevents synthesis of essential aromatic amino acids needed for protein biosynthesis in plants. ForceUp® is a non-selective systemic foliar-applied, total agricultural herbicide with rapid translocation throughout the plant for the control of most stubborn annual and perennial grasses, broad-leaved weeds and sedges in arable and plantation crops, under pre-plant, post-emergence directed conditions, reduced or zero tillage systems.

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 meristematic 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 plants, 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

colouration, leaf wrinkling or malformation and tissue death, and eventually plant death, which may take from 4 to 20 days to occur (Franz *et al.*, 1997; Vencill, 2002).

Glyphosate kills plants by inhibiting the activity of the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS). 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 glyphosatederegulates 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 1, which is found in plants, fungi and most bacteria, and is sensitive to glyphosate, and EPSPS 11 which is found in glyphosate-resistant bacteria and is not inhibited by glyphosate. It is the gene for an EPSPS 11 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 first 48-72h after application. The reason for this self-limiting phenomenon is not clear, but is related to the site of action of the herbicides, since there is grated translocation in

glyphosate resistant crops compared to susceptible plants. Glyphosate's ability to translocate readily in plants results in it controlling not only animal 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,1993). It can also act as a competitive inhibitor of phosphonenol pyruvate (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 (Steinrucken and Amrhein, 1980; Bode *et al*., 1984; Rubin *et al*., 1984).

2.7.2 ENVIRONMENTAL FATE OF GLYPHOSATE

Glyphosate is moderately persistent in soil with 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 soil 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). Microbes are primarily responsible for the breakdown of the products, 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).

2.8 2, 4-DICHLOROPHENOXYACETIC ACID (2,4-D)

2,4-D is the common name of the herbicide, 2,4- dichlorophenoxyacetic acid. Its Chemical Abstracts Service (CAS) registry number is 94-75-7 and the Chemical family is the phenoxyacetic acid compounds (Registration Eligibility Decision (RED), 2005).

2.8.1 CHEMICAL CLASS AND TYPE

Cotamine® 2, 4-D Amine herbicide is an herbicide used for the control of broadleaf weeds in crops such as maize, rice, sugarcane, sorghum, groundnut, palm, banana, plantations and for control of some aquatic weeds.

2,4,-Dichlorophenoxylacetic acid is a herbicide, and secondarily a plant growth regulator (Tomlin, 2006). Formulations include esters, acids and several salts, which vary in their chemical properties, environmental behaviour and to a lesser extent, toxicity (WHO, 1989; RED, 2005). The salt and ester forms are derivatives of the parent acid (WHO, 1989). The dimethyl amine salt (DMA) and 2-ethylexyl ester (EHE) forms account for approximately 90-95% of the total global use (Charles *et al.*, 2001). The acid form is low in solubility and herbicide formulations consist of more soluble forms of the chemical (WHO, 1989). Products containing 2,4-D frequently contain other herbicides as well (Carlo *et al*., 1992).

Agent Orange, the herbicide widely used during the Vietnam war, contained 2,4-D. However, the controversy regarding health effects centered round the 2,4,5-T component of the herbicide and its contaminant, dioxin (Kamrin, 1997; Munro *et al.*, 1992). 2,4-Dichlorophenoxyl acetic acid has been used in the United States since the 1940s, and it was evaluated for re-registration in 2005 by the United State Environmental Protection Agency (USEPA)(RED, 2005). The U.S. EPA

determined that 2,4-D was eligible for re-registration, but required certain changes to labelled uses to mitigate risk (RED, 2005).

2.8.2MODE OF ACTION

2.8.2.1 Target Organisms

2,4-Dichlorophenoxyl acetic acid (2, 4-D) is used on a wide variety of terrestrial and aquatic broad leaf weeds. It has little effect on grasses (Herbicides Hand book, 2002). It appears to work by causing uncontrolled cell division in vascular tissue. Abnormal increase in cell wall plasticity, biosynthesis of proteins and production of ethylene occur in plant tissue following exposure and these processes are responsible for uncontrolled cell division. (Herbicides Handbook, 2002; RED, 2005). The ester forms of 2,4-D penetrate foliage, whereas plant roots absorb the salt to be similar in action to other auxin-type herbicides (Herbicides Hand book, 2002).

2.8.2.2 NON-TARGET ORGANISMS

The modes of toxicity to animals, from the acid ester and salt forms of 2,4-D are similar. The primary exception is that the salt and acid forms can be extreme eye irritants (RED, 2005). 2,4-Dichlorophenoxyl acetic acid is actively secreted by the proximal tubules of the kidney, and toxicity appears to result when renal clearance capacity is exceeded (RED, 2005). Dose dependent toxic effects include damage to the eye, thyroid, kidney, adrenals and ovaries or testes. In addition, researchers have observed neurotoxicity, reproductive toxicity and developmental toxicity (RED, 2005). Chlorophenoxy herbicides exhibit a variety of mechanisms of toxicity, including dose dependent cell membrane damage leading to central nervous system toxicity, interference with cellular metabolism involving acetyl-coenzymeA(Brad berry *etal.*, 2004).

2.8.3USES OF 2,4-D

2,4-Dichlorophenoxylacetic acid is used for broadleaf weed control in agricultural and non-agricultural settings and it is registered for use in both terrestrial and aquatic environments. Major sites include pasture and range land, residential lawns, roadways, and cropland. Corps treated with 2,4-D include: field corn, soybeans, spring wheat, hazelnuts, sugar cane, and barley (RED,2005). Uses for products containing 2,4-D vary widely. Signal words for products containing 2,4-D may range from CAUTION to DANGER (Pesticides Products, 2007). The signal word reflects the combined toxicity of the active ingredient and other ingredients in the product(Jervais *et al*., 2008).

2 Table 2.1. Toxicity classification of 2, 4-D

	High Toxicity	**Moderate Toxicity**	**Low Toxicity**	**Very Low Toxicity**
Acute Oral LD50	Upto and including 50mg/kg (≤50mg/kg)	Greater than 50 through 500mg/kg (>50-500mg/kg)	Greater than 500 through 500mg/kg (>500-5000mg/kg)	Greater than 5000mg/kg (>5000mg)
Inhalation LC50	Upto and including 0.05mg/l (≤0.05mg/l)	Greater than 0.05 through 0.5mg/l (>0.05-0.5mg/l)	Greater than 0.5 through 2.0mg/l (>0.5-2.0mg/l)	Greater than 2.0mg/l (>2.0mg/l)
Dermal LD50	Upto and including 200mg/kg	Greater than 200 through 2000mg/kg (>200-2000mg/kg)	Greater than 2000 through 5000mg/kg (>2000-5000mg/kg)	Greater than 5000mg/kg (>5000mg/kg)
Primary Eye Irritation	Corrosive (irreversible destruction of ocular tissue or corneal involvement or irritation	Corneal involvement or other eye irritation clearing in 8-21 days	Corneal involvement or other eye irritation clearing in 7 days or less (Ester)	Minimal effects clearing in less than 24 hours (Ester)

	persisting for more than 21 days (Acid, Ester)			
Primary skin irritation	Corrosive (tissue destruction into the dermis and or scaring	Severe irritation at 72 hours (severe erythema or edema)	Moderation irritation at 72 hours (Moderate erythema)	Mild or slight irritation at 72 hours (no erythema) Ester Salt.

Source:(Jervais *etal.*,2008).

2.8.4Signs of Toxicity-Animals

Dogs fed 2,4-D exhibited myotonia, vomiting, and weakness; dog are more sensitive to chlorophenoxy acid herbicides than other animals (Peterson and Talcatt, 2006). In addition, dogs and cats have displayed inappetance, anorexia, ataxia, salivation, diarrhea, lethargy and convulsions following exposure to 2,4-D, which may include: eating treated grass (Campbell and Chapman, 2000) although the potential for this is unclear (Arnold *et al.*, 1991). Rats demonstrated in coordination, central nervous system depression and muscular weakness following acute oral dosing (Paulino *et al*., 1996; RED, 2005).

Biochemical analysis of rat tissue suggested hepatic and muscle damage following acute subchronic and chronic oral exposures (Paulino *et al.*, 1996).

2.8.5 Signs of Toxicity-Humans

No occupational studies were found reporting signs or symptoms following exposure to 2,4-D under normal usage. Symptoms of acute oral exposure to 2,4-D include:vomiting, diarrhoea, headache, convulsion, aggressive or bizarre behaviour. A peculiar odour is sometimes noted on the breath, skeletal muscle injury and renal failure may also occur (Reigart and Roberts, 1999). Systemic toxicity is mainly associated with suicide attempts (Reigart and Roberts 1999). Researchers compiled the medical cases of 69 people who ingested 2,4-D and other chlorophenoxy herbicides, 23 of these patients died (Bradberry *et al*., 2004). Ingestion led to vomiting, abdominal pain, diarrhoea, and development of hypotension (Brad berry *et al*., 2004).Peripheral neuromuscular effects including muscle troitching, weakness and loss of tendon reflexes have been reported (Brad berry *et al.*, 2004). Effects of 2,4-D on human health and the environment depend on how much 2,4-D is present and the length and frequency of exposure. Effects also depend on the health of a person and or certain environmental factors. Because 2,4-D has demonstrated toxic effects on the thyroid and gonads

following exposure there is concern over potential endocrine disrupting effects (RED, 2005).

2.9 REPRODUCTIVE OR TERATOGENIC EFFECTS

Teratogenic effects were not observed in mice, rats, and rabbits unless the excretion capacity of the mother was overwhelmed following oral exposure to 2,4-D or its salt and ester forms (Charles *et al.,* 2001; Garabant and Philbert, 2002). Reduced fetal viability was observed in hamsters following maternal dosing at 40 mg/kg day during pregnancy, although effects did not follow a dose-response relationship (Collins and Williams, 1971). In humans, there are some reports of reproductive effects following occupational exposure to chlorophenoxy herbicides (Munro*et al.,* 1992) including reduced sperm motility and viability following occupational exposure.

2.10 ENVIRONMENTAL FATE OF 2,4-D

In soil, 2,4-D amine salts and esters are not persistent under most environmental conditions (RED, 2005). Typically the ester and amine forms of 2,4-D are expected to degrade rapidly to the acid form (RED, 2005). Soil half-lifevalues have been estimated at 10days for the acid, diethylamine salt, and ester forms (Vogue *et al.,* 2004; Jervais *et al*., 2008). Another study estimate a soil half-life for the ester form EHE ranging from 1-14 days with a median half-life of 2-9 days (RED, 2005). Microbial degradation of 2,4-D in soil involves hydroxylation, cleavage of the acid side-chain, decarboxylation and ring opening (Tomlin, 2006). The ethyl hexyl form of the compounds is rapidly hydrolyzed in soil and water to form the 2,4-D acid (Tomlin, 2006).

2,4-Dichlorophenoxylacetic acid has a low binding affinity in mineral soils and sediment, and in those conditions is considered intermediately to highly mobile(RED, 2005). Although 2,4-D is highly mobile, rapid mineralization rates may reduce the potential of 2,4-D to affect groundwater (Boivin *et al*., 2005).

Microbes may have a major role in degradation (WHO, 1989). Breakdown products of 2,4-D detected in laboratory experiments included 1,2,4-benzenetriol, 2,4- dichlorophenol (2,4-DCP); 2,4- dichloroanisole (2,4-DCA); 4-chlorophenol; chlorohydroquinone (CHQ), volatile organics, bound residues and carbon dioxide. These degradates are expected to be of low occurence in the environment, of low toxicity, or both (RED, 2005).

In water, the half-life of 2,4-D in aerobic aquatic environments was estimated to be 15 days and in anaerobic aquatic laboratory studies, 41-333 days (RED,2005). A granular formulation of the water column in alkaline conditions but was present in sediment for 186 days (RED, 2005). The ethyl hexyl form is rapidly hydrolyzed in water to 2,4-D acid with a degradation half-life (DT50) of less than one day (Tomlin, 2006). Ester forms of 2,4-D hydrolyze at rates that are pH dependent, the hydrolysis half-life of the butoxy ester increased from 9 hours at pH 8 to more than one year in more acidic conditions with a pHof 5.38. The acid form of 2,4-D is very resistant to a biotic hydrolysis (RED, 2005). 2,4-D has been detected in streams and shallow ground water at low concentrations in both rural and urban areas (OSHA, 1999; RED, 2005).

In air, volatility for most forms of 2,4-D is low. However the vapour pressure of some ester forms range from $1.1x10^{-3}$ to $2.3x10^{-3}$ $mmHg^2$, indicating that these forms readily volatilize. The Henry's law constant for 2,4-D acid is $3.5x10^{-4}$ at PH 7 (NAWQA, 1998), indicating low potential for movement from water to air. No data were found regarding the degradation of 2,4-D in the atmosphere.

In plants, the ester forms of 2,4-D penetrates foliage, whereas plant roots absorb the salt forms (Herbicide Handbook,2002). Accumulation occurs primarily at the hexyl ester form of 2,4-D degraded slowly on foliage and in leaf litter (RED, 2005). Residues of an ester form of 2,4-D were detected in samples of dead birch leaves for up to three years post application (McPherson *et al*., 2003; Jervais *et al*., 2008).

2.11 ECO-TOXICITY STUDIES

Toxicity to fish and aquatic invertebrates varies widely depending on chemical form, with esters being the most toxic (Tomlin, 2006; WHO, 1989). Acid and amine salt LC_{50}range from greater than 80 to 2244mg acid equivalents per litre whereas the ester range from less than 1.0 to 14.5mgacid equivalents per litre (RED, 2005). The greater toxicity generally of the ester in fish is likely due to the greater absorption rates of the esters through the gills where they are hydrolyzed to the acid form (WHO, 1989). Daphnia exposed to the acid form for 21 days exhibited an LC_{50} of 235mg/L when exposed to 2,4-D acid for 21 days and an LC_{50} of 5.2 mg/L when exposed to the ethyl hexyl form for 48 hours (Tomlin, 2006). Therefore the acid form is practically non-toxic to Daphnia but the ethyl hexyl form is moderately toxic. As with fish, esters are more toxic than acid or amine salt forms to fresh water aquatic invertebrates, with LC_{50} values ranging from 25 to 643 mg ac/L for esters (RED, 2005). The relative toxicities for acids and salts are slightly toxic to practically non-toxic, whereas the esters are moderately to slightly toxic.

Researchers have estimated a No Observed Effects Concentration (NOEC) of 16.1 mg ac/L for the DEA ester and 79.0mg ac/L for the acid form based on survival and reproduction for DEA and number of young produced for the acid form. The fresh water aquatic invertebrate NOEC for the BEE ester was estimated at 0.2 mg ac/L based on survival and reproduction (RED, 2005). 2,4-D is marked for controlling aquatic plants. Therefore, the lethal concentrations are reported as effective concentrations for killing half the target population (EC_{50}). Researchers estimated an EC_{50} of 0.58 mg/L for duckweed (*Lemna gibba*). A variety of algal species exhibited LC_{50} values ranging between 0.23 and greater than 30 mg/L for the ethyl hexyl form (Tomlin, 2006). The EC_{50} for the dimethyl amine salt form against *Selenastrumcapricornutum* was estimated at 51.2 mg/L

(Tomlin, 2006). No effects were recorded for 19 genera of algae exposed to 2,4-D at concentrations of up to 222 mg/L (WHO, 1989).

Bioavailability and uptake of 2,4-D by organisms are strongly influenced by pH, temperature and other environmental factors (WHO, 1989). The sensitivity of aquatic invertebrates to 2,4-D increased with temperature concentrations below those associated with short term toxic effects, impaired reproduction when ambient temperature was elevated (WHO, 1989). Fish appear to avoid 2,4-D in a dose dependent manner until the onset of toxic effects (WHO, 1989). Toxicity of 2,4-D was increased when fish were simultaneously exposed to 2,4-D and carbaryl or picloram (WHO, 1989).

2.12 REGULATORY GUIDELINES

The reference dose (RFD) for 2,4-D is 0.01 mg/kg day (Rice *et al*., 1997). The U.S. EPA has classified 2,4-D as 'Group D-not classifiable with regard to human carcinogenicity' in 2004 (RED, 2005). IARC had not assigned 2,4-D a cancer rating as of December 2016. However, the chlorophenoxyl herbicides as a group were classified in Group 2B, meaning that they are considered to be possibly carcinogenic to humans by IARC in 1987 (U.S. EPA, 2008). The threshold limit value or TLV, for 2,4-D is 10mg/m3 for an 8-hour time weighed average exposure (ACGIH, 2003; IARC, 1987). This limit is based on results of animal feeding experiments (OSHA 1999). This same dose was selected by the occupational safety and health administration (OSHA) for the permissible exposure limit (PEL) for an 8 hour time weighted average exposure safety and health (NIOSH) for the recommended exposure limit (REL) for a 10 hour workday and a 40-hour workweek (OSHA, 1999).

The MCL for 2,4-D in drinking water is 0.07 mg/L (U.S. EPA, 2008; Jervais *et al*., 2008) MCL is Maximum Contaminant Level.

2.13 FISHERIES RESOURCES AND PRODUCTION IN NIGERIA

Nigeria is located in West Africa, bordering the Gulf of Guinea betweenBenin 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 right to the fish and other natural resources(Opeyemi, 2015). The brackish and coastal waters of Nigeria support harvest at the artisanal level, while the industrial sector operates only outside the 5 nautical miles (Ibeun,2006). Nigeria has a total land area of 923,768sq.km, and 13,000sq.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 fresh water systems, including lakes, rivers, reservoirs, dams and flood plains which support extensive artisanal fisheries. The river Niger which arises in Siera Leone and has a total length of 4,184 km, flows through West Africa, enter Nigeria in the North West and runs outwards 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 fishrivers in the country (Opeyemi, 2015). Ita *et al.*, (1985) has identified about 365 lakes and reservoirs and 687 ponds and flood plains totaling 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 Program for Food Security (NSPFS) (2004) identified about 2,658 fish farms and 937 Dams and reserviors in Nigeria (Opeyemi, 2015). In 2000, the fish import bill exceeded 30 billion naira, i.e, U.S £241.1 million (Dada, 2004). This relatively low production and the impact on the economy of high imports are 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 fresh waters. The artisanal nature and the rural location of the inland fishermen and the rivers have caused most of the fresh water catches to be unaccounted 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 mt. while current actual production is only half of that in the Nigeria side of the lake, producing 60,000mt annually, with a monetary value in excess of £22 million (ca. 2.95 billion Naira). Lake Chad currently produces about 33% of Nigeria freshwater fish. Unfortunately this type of data is not available for other water bodies (Ibeun, 2006; Opeyemi 2015)

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 fish. On this basis we have: Inland waters (Tilapia and *Clarias*); In-shore water (*Musil* and *Arius*); offshore waters (Tuna); Aquaculture (Tilapia and *Clarias*); and miscellaneous sources (Ajayi and Talabi, 1984). Inland waters consist of rivers, tributaries, flood plains, lakes, coastal and brackish water. The total fishery resource potential of these sources has been put at 288,500 tonnes (Ajayi and Talabi, 1984). The various species found in this habitat include *Lates*, *Niloticus, Clarias, SPP, Hetrotis*, *Niloticus*, *Tilapia species*, etc. In the African

catfish, apart from the adult stage, there are three distinct stages 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 has a relevant significance in evaluation of adverse effects of pesticides to human health (Begun and Vijayaraghavan, 1996).

2.14 BIOACCUMULATION IN FISH

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

Agricultural and industrial activities are the most important sources of chlorinated compound. 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, indicateinjurious effects. Connection 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 ecosystem, are yet to be determined (Damasio, 2010). Therefore, the exposure to fat and effects of chemical contaminants or pollutant in the aquatic ecosystem have been extensively studied by environmental toxicologists. The wide spread 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 biomagnifications through the consumption of contaminated food (Miranda *et al.,* 2008). Even without detectable acute or chronic effects in standard ecotoxicity tests, bioacuumulation should be regarded as a hazard criterion in itself, since some effects may only be recognized in a later phase of life and multi-generation effects or manifest only in higher members of a food web, e.g., impact of PCBS on the hatching success of eggs (Tillit *et al*., 1992).

Bioaccumulation of chemicals in biota may be a prerequisite for adverse effects on ecosystem (Franke *et al*., 1994). Miranda *et al*., (2008) assessed the impact of aquatic pollutants such as organo-chlorine pesticides, polychlorinated biphenyls, chlorotriazines (atrazine simazine) and chloroinates phenylureas (diuron), on the *ponta grossa* lake south of Brazil. Ten freshwater trahira fish (*Hoplias malabaricus*) were collected. The result showed detected amounts of persistent organic bioaccumulation was higher in the liver than in the muscle. The presence of some banned pesticides such as hexachlorobenzene and dichlorodipheryl trichloroethane in the liver, suggest acute exposure to these compounds. They also reported that some physiological disturbance and morphological damages found in the liver of *Hoplias malabaricus* were associated with chlorinated compound bioaccumulation.

Wang*et al.*, (2013) investigated the presence of Hexabromocyclodecanes (HBCDS) in 79 wild fish from high mountain lakes and rivers of the Tibetan plateau, China. The EHBCDS in fish muscles ranged from non-detected levels to 13.7 ng/g lipid weight (lw) (mean value of2.12ng/g lw) with a high detected frequency of 65.8%; and HBCD dominated among the isomers and accounted for 78.2% of the total burden. Concentration of EHBCDs in the fish were significantly corrected with the lipid content and HBCD and trophic level nearing that fishes in water found on the higher altitudes of the plateau has less of the HBCD.

2.15RISKS INDUCED BY PESTICIDES ON FISH REPRODUCTION

Pollution of water resources with agricultural water drainage has a great risk on fish reproduction. Organophosphrous pesticides such as malathion and dimethaote are frequently used due to their highly effectiveness for controlling agricultural pests (El- Gawad *et al.*,2012). These pesticides were found that have endocrine disrupting effects on fish reproduction through lowering sex steroid hormones (estradiol and testosterone). Endocrine disrupting pesticides also have been implicated in the impairment of fish fecundity, semen quality, hatchability and survivability. Organo-somatic index and histopathology are considered as biomarker tools used for assessing disrupting effects of pesticides on fish (El-Gawad *et al*., 2012). There has been concern that uncontrolled uses of pesticides that reach the environment exert great and harmful effects on wildlife and human health since the publication of ‘a silent spring’ book by Rachel Carson in 1962. The term “endocrine disruptor” was introduced since publication of book entitled “Our Stolen Future, are we threatening our fertility, intelligence and survival?” by Colborn *et al.*(1996). In this book, she recorded that environmental chemicals disrupt development of the endocrine system, and exposure during development is often permanent.

Endocrine disruption chemicals (EDCS) are defined as chemical substance that alter the normalendocrine function (McKinlay *etal.,* 2008) including either naturally occuring chemicals as phytoestrogen or synthetic chemicals such as pesticides, plasticizers, polychlorinated biphenyls (PCBS) and alkylphenolic compounds. EDC'S exert their effects either through mimicking (act like a natural hormone) such as methoxychlor pesticides, certain polychlorinated bipheryls (PCBS) and bisphenol A (BPA) or antagonizing endogenous hormones such as tamoxifen or disrupt the synthesis and metabolism of hormones or interactwith the hormone receptors (Somenschein and Soto 1998).

These chemicals can be found in environment (air, water, soil), food products, household products, pesticides, plastics (bisphenol A, phthalates), pharmaceutical drugs (birth control pills, DES, cimetidine), industrial chemicals and heavy metals. Pesticides are used for controlling agricultural pests, flies in homes, garden and on livestocks (Srivastava *et al.,* 2010). The types and quantities of pesticides used vary partly on types of crop (Matheous, 1999). The water resources are polluted with these pesticides from agricultural runoff or industrial effluents, and their concentration in the ecosystem affected by many factors, stop microbial degradation which anaylzes organic pesticides as part of their food or mineralization of pesticides to carbon dioxide, ammonia water and inorganic salts (Muller and Korte, 1975) or photodegradation by (high temperatures, oxygen and hydrogen peroxide) which accelerate pesticides degradation (Muszkat *et al*., 2002) in addition high density of phytoplankton in water could absorb a high quality of most pesticides in the water and so decreases its concentrations (El-Nemaki *et al.,* 2008).

2.16HORMONAL CONTROL OF FISH REPRODUCTION

Normal reproduction in vertebrate is controlled by two factors; extrinsic factors such as temperature and/or photoperiod orrainfall and intrinsic factors; the hypothalamic-pituitary gonadal (HPG) axis. These external stimuli act on hypothalamus resulted in secreted of gonadotrophin releasing hormone (GNRH) which in turn act on anterior pituitary that release gonadotrophin hormone (GTH) (Brooder *et al*., 1991) which stimulate gonads to secrete steroid hormone. 11-ketotestoserone is the major androgen hormone in fish secreted from sertoli cells and responsible for sperm maturation (Kime, 1993). While 17B-estradiol is secreted from the follicular cells (Nagler and Idler 1992) andstimulate liver to synthesize the egg yolk protein precursor (Vitellogenin). This protein is transported to the ovaries and incooperated into the oocyte, where it is rapidly cleaved to form the yolk protein lipovitellin andphovitin (Tyler *et al*., 1988). Once Oogenesis and spermatogenesis is completed, the sex steroid hormone ceases and progesterone is secreted which induce the final maturation of oocytes and sperm.

2.17EFFECTS OF PESTICIDES ON GONADAL HORMONES

The hormonal level of both testosterone and estradiol are different according to the reproductive cycle of fish (Rothbard *etal*., 1987); during normal sexual ontogenesis in fish, both testosterone and estradiol increase. Concentration of plasma sex steroid are considered as an indicator of gonadal status. In ovary, estradiol must be synthesized in sufficient amount to stimulate liver to produce vitellogenin. Incase of Oocyte atresia, the estratiol hormone decrease lead to impaired vitellogenesis (Ankley *et al*., 2002).

Exposure of *Heteropneusts fossilis* to sublethal concentration of malathioan for 72hrs decreased the estradiol hormone (Dutta *etal*., 1994) and also showed low level of testosterone and estradiol with Y-hexachlorohexane for 4 weeks. Sigh and Canario, 2004 and Barber *et al*., 2007, mentioned that estradiol and 11-KT in

largemouth bass, *Micropterus salmoides* decreased after feeding on treated diet contain 46µg/g P,P-DDE and 0.8µg/g dieldrin for 4months. Nile tilapia also showed decrease in sex steroid hormones upon exposure to organochlorine pesticides hexachlorobenzene (HCB) (Rodas-Ortiz *et al.,* 2008); acute dose (1.05 ppm) chronic dose (0.21ppm) of butachlor (Ghada, 2009) and chlorpyrifos-ethyl for 15 days (Ozcan Oruc, 2010). Eman *etal.,* (2011) mentioned that *O. niloticus* fed on diet incorporated with malathion for 4 months exert endocrine disrupting effects on males than females through decreasing testosterone hormone.

2.18EFFECTS OF PESTICIDES ON FECUNDITY

There are marked differences among fish species which reflects often different reproductive strategies. Even within species, fecundity may vary as a result of adaptation of environmental habitat (Witthames *et al*., 1995). Absolute fecundity means total number of ripened eggs per female, while number of eggs in relation to weight or length of fish means relative fecundity.

Decline in fish fecundity may be due to changed environmental conditions such as temperature or contamination with pesticides. Khallaf *et al*., (2003) mentioned that *O. niloticus* collected from polluted Shanawan drainage canal, Al-Miriufuya Governorate showed lower fecundity of range between 1234to3893 eggs from female with total length 12 to 23cm, and Ghada (2009) found that *O. niloticus* exposure to 1.05 and 0.21ppm of butachlor for 6 days and 6 weeks showed highly significant decreases in absolute fecundity. Inaddition, absolute fecundity in *O. niloticus* was significantly decreased after treatment with Dimethaote and Malathion for the control (245.25 +23.69 eggs/female) (Eman *et al.,* 2011) due to Oocyte atresia and decreased estradiol hormone.

2.19EFFECTS OF PESTICIDES ON SPERM

Teleosts are different from mammals that have immotile sperm and it attains motility only on contact with water for only a few minutes and it enters theeggs

via the testis during spermatogensis resulted in malformed sperm and abnormal sperm motility in *Heteropneutes fossilis* after 45days exposure (Singh and Singh 2008 a) and *O. niloticus* exposure to butachlor and malathion (Ghada, 2009 and Musa, 2010) respectively. It was found that malathion and dimethoate have great effects on semen quality of *O. niloticus* after 120 days treatment. They significantly decreased sperm cell concentration and sperm motility with significant increase in tail deformity (Eman *etal.,* 2011).

Significant decrease of individual sperm motility may be due to the effects of organophoshorus pesticides on mitochrondria that alter ATP production or may be due to oxidative stress which lead to production of lipid peroxidation in spermatozoa affecting its motility (Pina-Guzman *et al*., 2006). Sperm cell concentration and sperm quality decrease due to degradative changes and lacking of germ cell lining to seminferous tubules associated with organophosphrous pesticide treatment and deformities of sperm resulted from a decrease in acetylcholine terase inhibitors activity that impaired function of caput of epididymis (Okamura *et al.,* 2009).

Our aquaculture are at great risk from these endocrine disrupting pesticides which drain to water either directly or indirectly affecting the reproductive performance of fish through decreased sex steroid hormones, fecundating and containing endocrine disrupting pesticides must be avoided to use in rearing brood stock.

2.20HISTOPATHOLOGY OF FISH REPRODUCTION TISSUES

Histopathology has received increased interest as an endpoint in EDC research in aquatic organisms, because histopathological changes are often the result of the integration of a large number of interactive physiological processes. Sub-lethalexposure to persistent organic compounds may cause reproductive effects by reducing fecundity and population recruitment (Patina *et al*., 1999). The hypothalamic pituitary gonadal (HPG) axis of teleost fishes, whose principal

components are the hypothalamus and pituitary gland in the brain, the gonads and the liver is generally similar to that of other oviparous vertebrates (Ankley and Johnson, 2004). The gonads are the primary organs of reproduction and as such, will reflect substantive disturbance to the HPG axis (USEPA, 2006). Histopathology helps to identify target organs of toxicity and mechanism of action (West *et al.*, 2002). As a tool for assessing endocrine disruption effects in fish, histopathology has also been applied in other studies such as endocrine disruption in the ovaries and testes of Zebrafish (Vander Ven *et al*., 2003) and in the adult fathead minnow (Leino *et al.*, 2005).

Toxicological histopathology is used to identify and describe morphologic differences between unexposed and compound exposed animals.

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

2.21.1 Reactive Oxygen Species (ROS).

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 dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST). 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.*, 2010). 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*., 2009). 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 non-oxidant 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 peroxidase 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 goldfish 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), and 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 inthe 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 O2- generation and generation of O2- within the SOD elimination capacity. However when superoxide anion radical (O2-) generation increases to an extent that exceeds the function of SOD elimination, O2- 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).

2.21.2. 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). Aspartate transaminase (AST) and Alanine transaminase (ALT) 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 *Clariasbatrachus* 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 theenzyme 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*. (2012a) 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.

2.22. 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 is freshwater. 1% of this is ice-freewater 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, 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 a 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). Conductivity shows significant correlation with ten parameters such as temperature, pH value, alkalinity, total hardness, calcium, total solids, total dissolved solids, chemical oxygen demand, chloride and iron concentration of water. The underground drinking water quality of study area can be checked effectively by controlling conductivity of water, and this may also be applied to water quality management of other study areas. Carbon dioxide is the end product of organic carbon degradation in almost all aquatic environments, and its variation is often a measure of net ecosystem metabolism. Therefore, in aquatic biogeochemical studies, it is desirable to measure parameters that define the carbon dioxide system. CO_2 is also the most important green-house gas on earth. Its fluxes across the air-water or sediment-water interface are among the most important concerns in global change studies and are often a measure of the net ecosystem production/metabolism of the aquatic system. There are various readily measurable parameters of aquatic carbon dioxide system: such as pH (pCO_2), total dissolved inorganic carbon (DIC) and total alkalinity (TA). Surface water pCO_2 can be measured by photometric method and DIC CO_2 is measured by coulometer or by an infrared CO_2 analyzer (Dickson, 1994).

2.22.1Temperature

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

HCO3- , 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.

2.22.2 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 Hunner, 1984). An un-ionized sulphide (e.g. H2S) 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.

2.22.3 Dissolved Oxygen (DO).

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 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 because of alkalinity 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 below:

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 (Kano, 2011).

2.22.4 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).

CHAPTER THREE

MATERIALS AND METHODS

3.0 MATERIALS

3.1 TEST SAMPLES

3.1.1. GLYPHOSATE HERBICIDE

ForceUp® glyphosate herbicide was used. This was purchased from a chemical shop at Aba, Abia State and taken to the lab for stock preparations. Active ingredients include: 360g of glyphosate/L (in the form of 480g/L Glyphosate-isopropylamine salt).

3.1.2. 2, 4-DICHLOROPHENOXYLACETIC ACID (2, 4-D) HERBICIDE

Cotamine® 2, 4-D Amine herbicide was used. This was also purchased from a local chemical shop at Aba, Abia State and taken to the lab for stock preparstions.

Composition: 2, 4-Dimethylamine salt® 720g/L, 72.0% w/v; inert ingredients 28.0% w/v. 60% acid equivalence.

It was formulated by Springfield Agro Ltd. 122-132 Oshodi Apapa express way, Isolo, Lagos state.

3.2. TEST ORGANISMS

Eight (8) domesticated catfish brood stocks (4 males and 4 females) were purchased from Decfiro Standard Link farm at No. 7 Wogu Street, Oyigbo, Rivers State. This same farm was used for acclimation of the fishes; sampling/exposure of the broodstocks to the herbicides; and acute toxicity testing of the fingerlings.

3.3. EXPERIMENTAL DESIGN/METHODS

3.3.1. Preparation of stock solutions of the herbicides used.

The concentration of the herbicides prepared was as used by Olanike *et al*. (2011).

The 720g/L of the Cotamine® 2, 4-D Amine herbicide was scaled down mathematically to 20mg/ml by collecting ≅27.8ml of the 720 g/l into a 1000ml measuring cylinder and making it up with distilled water. Also, the 360g/l of the glyphosate herbicide was scaled down to 20mg/ml by collecting 56ml of the ForceUp® herbicide into a 1000ml measuring cylinder and making it up with distilled water. Finally, a mixture of glyphosate and 2, 4-D was prepared by collecting 14ml of the Cotamine® herbicide and 28ml of the ForceUp® herbicide into a 1000ml measuring cylinder and then making up with distilled water. These stock solutionsprepared were stored separately in three white 2-Litre plastic containers until used.

3.3.2. Sampling/Exposure of Broodstocks to the Herbicides

The method used was as described by Olanike *et al.*, 2011.

Four male brood stocks, aged three years, had average mean weight of 4.2 ± 1.0kg, while the four female broodstocks, aged three years, with average mean weight of 3.5 ± 1.0kg, and mean length 19.2 ± 0.87cm, were used. Male brood-stocks were examined for rigid and reddish infusion of the genital papillae and female genital orifice were examined for reddish infusion, distension of the belly and release of eggs when gentle pressure was applied on the abdomen. Before stocking, the brood-stocks were disinfected with 0.5 % salt bath (5 g NaCl/1 litre water) at temperature of 27°C. Bathing was done by dipping the fish into the solution for fifteen minutes. They were later acclimatized for two weeks in

holding indoor 1000L plastic tanks.During the time of acclimatisation, the broodstocks were fedcommercially prepared feed pellets (10mm “Coppens” feed pellets at 3% body weight using 10mm, once a day (by broad cast method), after which they were subjected to treatment. Feeding was stopped 24hours before treatment to minimize excretion of waste which may contaminate the water.

One male and one female broodstocks were put in separate 20-litre black bowls and exposed to the herbicide–glyphosate at 1ml of stock solution per 20L well aerated water, and allowed to stand for 45min and then removed and taken back to the well aerated water. A wire gauge was used to cover the bowls to avoid the fishes escaping. They were observed closely throughout the 45mins. Also, one male and one female broodstocks were exposed to the herbicide- 2, 4-D at 1ml of stock solution of well aerated water for 45mins and then removed and taken back to the well aerated water. Another male and female broodstock wereexposed to a mixture of the herbicides (glyphosate and 2, 4-D) at 1ml of stock solution of well aerated water for 45min and then removed and taken back to the well aerated water.

This procedure was repeated every other day for the three treatments. A set of male and female broodstocks were not exposed to any chemicaland were regarded as the control for the experiment.

3.3.3. Assessment of Water Quality: Water quality assessmentswere carried out for both the treatments and the control at the Day1 and Day3 of the experiment.

The water quality parameters determined included: Alkalinity, Ammonia, Carbon dioxide, Chloride, Electrical conductivity, Dissolved Oxygen (DO), Nitrate, pH and Hardness.

3.3.4. Artificial Spawning of Broodstocks

Spawning was induced in the females (both treatments and control) using Ovaprim™ at 1.8ml/kg as recommended by the manufacturers. Ovaprim™ is a potent ovulating/spermiating hormone which promotes and synchronizes ovulation in many species of fish. The hormone was drawn with a 2ml syringe and administered intra-muscularly at 45 degree in the dorsal muscle. Each female brood stock was injected 1.8ml of the hormone per kg body weight while each male brood stock was likewise injected 0.5ml of the hormone.

Twelve hours after injection, the males were dissected to access their testes and avoid delay in administering the milt on the eggs. Any delay would lead to the closure of the egg micropile, making fertilization of the eggs impossible. The females were stripped of eggs into a dry, sterile bowl 12 hours after administration of Ovaprim™ and weighed. Approximately, 1000 eggs were collected from each female brood stock by estimation (Aluko and Ali, 2001; Jensen, 1996) for fertilization.Egg samples were also collected using sample bottles and preserved in Boiun's fluid obtained for histological assessment. The remaining eggs weremixed with the milt from corresponding male broodstocks, and fertilization was activated with distilled water.

In four separate flow-through hatching systems (one each for control and treatments), fertilized eggs were spread on three carcabans (mosquito nets, of 1mm in diameter) at a constant flow rate of 3.5L per min. The setup was allowed to run for twenty four hours to allow for hatching of fertilized eggs. The flow-through system was allowed to run for four days, while regression of yolk sac, growth rate, and abnormalities in hatchlings were monitored daily using a digitalcamera.

Immediately after fertilization occurred, new development commenced. The eggs absorbed water and stuck to the net. Also, red spots were observed on the green

colour of the eggs, indicating life. Healthy developing eggs were transparent greenish-brown in colour, while the white coloured eggs were those not hatched. Hatching commenced at around the 24th hour, and continued till the 48th hour after fertilization. The newly hatched fries escaped through the 1mm gauge into the tank underneath while the unhatched eggs remained on the net.

3.3.5. Fries Management/Feeding

To prevent water pollution, un-hatched eggs were removed from the hatching net by siphoning. Feeding commenced on the 4th day for the treatments and 5th day for the control, after their yolk sacs have been completely absorbed. They were fed with small quantity of processed Artemia salina *ad libitum*. Excess feed and waste were siphoned out using 1mm diameter hose.

Ten (10) fingerlings were randomly selectedfrom each treatment weekly and weighed, using a sensitive weighing balance, to adjust the feeding rate per their body weight. Also, at the end of the culture period (after four weeks), final weight (g), final length (cm), survival rate, for each treatment were recorded. Final mean weight, mean weight gain (g), final mean length, mean daily weight gain (g), specific growth rate (percent/day), survival rate (%) for each treatment were estimated as follows:

Weight gain (g): W_1-W_o, Where, W_1= Final weight and W_o= Initial weight

Mean daily weight gain (g/day): W_1-W_o/t, where, W_1 = Mean final body weight (g), W_o = Initial mean body weight (g) and t = Culture period (days)

Specific growth rate (percent/day):

$$\text{Specific growth rate} = \frac{\text{Log}_e W_1 - \text{Log}_e W_o}{t} \times 100$$

Where, W_o = Mean initial body weight of fish (g), W_1 = Mean final body weight of fish (g), Log_e = Natural logarithm and t = Culture period (days) (Adewolu *et al*., 2008).

3.3.6. Determination of Eggs hatchability

Gamete quality in female *C. gariepinus* was determined by fecundity/Gonado-Somatic Index Ratio (GIS). Hatchability rate of the eggs was determined on the basis of the percentage of the un-hatched as used by Aluko and Ali, 2001. An estimation which assumed hatching rate of flow-through water system to be calculated on live/dead ratio of incubated eggs as follows:

% Hatchability = (Number of hatched eggs/ Total number of eggs) x 100%

Survival rate = (Number of hatchlings alive up to larvae stage/ Total number of hatchlings) x 100%

Survival rate was determined based on Jensen (1996) method. The normal healthy larvae were estimated on percentage basis of dead and deformed hatchlings.

% of deformity, marked by curved tail and shortened body, was noted in each case as suggested by De Leeuw *et al*. (1985).

3.3.7. Determination of Growth performance

Growth responses were determined as described by Olvera-Novoa *et al*., 1990.

3.3.8. Biochemical Profiling of Fish Liver

After sacrificing the males to obtain milt, testes, and skin, the liver was also obtained from both treatments and control. These were put in sample collection bottles and placed inside a flask filled with ice blocks, and immediately transported to the lab for analysis.Determination of the levels of the activities of the liver enzymes was carried out. The enzyme activities determined included Superoxide dismutase activity (SOD), Catalase activity (CAT), Glutathione perioxidase (GPx), Glutathion-s-transferase activity (GST). Oxidative stress markers (Glutathione (GSH), lipid peroxidation (MDA) and Total protein) and liver function tests were carried out also.Commercial kits were purchased for the determination of the levels of the activities of the liver enzymes.

3.3.8.1. Preparation of Homogenate

Liver tissues were homogenized in phosphate buffer (p^H 7.4) in 4 parts of homogenizing buffer, i.e, 1:4 ratio, and centrifuged at 12,000×g for 20 mins at 2□C. The supernatant was collected and kept in the freezer at 4□C and used to assay for oxidative stress parameters (thiobarbituric acid reactive substances, Catalase, Superoxide dismutase, Glutathione, Glutathione-S-transferase, Glutathione peroxidase), Total protein, were also estimated in the sample homogenates.

3.3.8.2. Determination of Aspartate Aminotransferase (AST)

This was determined as described by the manufacturer‘s recommendation (Cypress diagnostics, Germany, 07, 2004).

Principle: The determination is based on the absorbance of hydrazones of 2-oxoglutarate and pyruvate in an alkaline medium. AST catalyses the transfer of

an amino group from L-aspartate to 2-oxoglutarate to form oxaloacetate and L-glutamate. Oxaloacetate spontaneously decarboxylates to form pyruvate under the strongly acidic conditions. An increase in pyruvate concentration corresponds with the levels of AST activity. The pyruvate concentration is determined spectrophotometrically in the form of hydrazone, which is produced by reaction with 2,4-dinitrophenylhydrazine in an alkaline medium. The pyruvate hydrazone absorbs at 510 nm more than 2-oxoglutarate hydrazone.

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) = 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).

Procedure: The AST reagent is used to measure aspartate aminotransferase activity by an enzymatic rate method.In the assay reaction, the AST catalyzes the reversible transamination of L-aspartate and α-ketoglutarate to oxaloacetate and L-glutamate. The oxaloacetate is then reduced to malate in the presence of malate dehydrogenase (MDH) with the concurrent oxidation of β-Nicotinamide Adenine Dinucleotide (reduced form) (NADH) to β-Nicotinamide Adenine Dinucleotide (NAD). The SYNCHRON® System(s) automatically proportions the appropriate

sample and reagent volumes intoa cuvette. The ratio used is one part sample to 11 parts reagent. The system monitors the rate of changein absorbance at 340 nanometers over a fixed-time interval. This rate of change in absorbance is directly proportional to the activity of AST in the sample and is used by the SYNCHRON® System(s) to calculate and express the AST activity.

3.3.8.3. Determination of Glutathione Peroxidase (GPx)

Glutathione peroxidase (GPx) activity was assayed as described by Paglia and Valentine (1967) with modifications according to Lawrence and Burke (1978).

Principle: Glutathione Peroxidase (GSH-PX) can promote the reaction of hydrogen peroxide (H_2O_2) and reduced glutathione to produce $H_2O$and oxidized glutathione (GSSG). The activity of glutathione peroxidase can be expressed by the rate of enzymatic reaction. The activity of glutathione can be calculated by measuring the consumption of reduced glutathione. Hydrogen peroxide (H_2O_2) and reduced glutathione can react without catalysis of GSH-PX, so the portion of GSH reduction by non-enzymatic reaction should be subtracted. GSH can react with dinitrobenzoic acid to produce 5-thio-dinitrobenzoic acid anion, which showed a stable yellow color. Measure the absorbance at 412nm, and calculate the amount of GSH.

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH-PX}} 2H_2O + GSSH$$

$$GSH + DTNB \longrightarrow GSSH + TNB$$

Procedure: 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.

3.3.8.4. Determination of Catalase activity (CAT)

The catalase enzyme activity in tissues was assayed following the procedure of Sinha (1998).

Principle: The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of $H_2O_2$with the formation of perchromic acid as an unstable intermediate. The chromic acetate thus produced is measured calorimetrically at 570-610nm. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromicacetate colorimetrically after heating the reaction mixture.

Procedure: Tissue homogenate (0.1mL) was incubated with H_2O_2 (5mM, 2.0 mL), in the presence of 2.5ml 0.01 M phosphate buffer (pH 7.4). A 1.0 ml portion of the reaction mixture was withdrawn and added into 2 ml of dichromate/acetic acid reagent at 60 sec intervals. Samples were incubated in boiling water for 15 min. After cooling at room temperature the volume of the reaction mixture was made 3 ml with Phosphate buffer and the optical density was measured at 570nm. The upper layer of the mixture was taken and the absorbance read at 570 nm.

The rate of decomposition of H_2O_2 was measured using the equation for a first-order reaction. $K = 1/t \, Log_{10}SO/S$

Where SO is the initial H_2O_2 concentration and S is the H_2O_2concentration at a particular time interval given as t (minutes). The values of K are plotted against t, and the velocity constant K (0) at 0 minute determined by extrapolation (that is

the intercept on the vertical axis). The catalase activity of the sample was expressed in terms of catalase unit per g protein

Cat. = K (0) / g protein = Unit/g.protein.

3.3.8.5. Determination of Superoxide Dismutase activity (SOD)

Superoxide dismutase (SOD) activity was assayed as described by Misra and Fridovich (1972) and modified by Packer and Glazer (1990).

Principle:The assay was based on the ability of SOD to inhibit the autoxidation of epinephrine at an alkaline pH.

Procedure: 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.

3.3.8.6. Assessment of Lipid peroxidation

Principles: Lipid peroxidation in the supernatant fractions of the test materials was determined spectrophotometrically by assessing the concentration of thiobarbituric acid reactive substances (TBARS) as described by Liu *et al.* (2008. The results were expressed in malondiadehyde (MDA) formed relative to an extinction coefficient of 1.56 x 10^6mol/cm. Small quantities of MDA are produced during lipid peroxidation. These react with Thiobarbituric acid (TBA) to generate a pink coloured complex which in acid solution absorb light at 532nm

and flourescences at 532nm and is readily extractable into organic solvents such as butan-1-ol.

Procedure: This was estimated by the method of Liu *et al*. (2008). Acetic acid 1.5mL (20%; pH 3.5), 1.5mL of thiobarbituric acid (0.8%) and 0.2mL of sodium dodecyl sulphate (8.1%) was added to 0.1mLof liver homogenate and heated at 100 ◦C for 60 min. Mixture was cooled and 5mL of *n*-butanol-pyridine (15:1) mixture, 1mL of distilled water was added and vortexed vigorously. After centrifugation at 1200×*g* for 10 min, the organic layer was separated and absorbance measured at 532 nm using a spectrophotometer Malondialdehyde (MDA) was calculated using a molar extinction coefficient of $1.56\times10^{5}M^{-1}$ $cm^{-1}$and expressed as nanomoles of MDA.

3.3.8.7. Determination of Glutathione concentration

Principles: Glutathione (Reduced) was measured according to the method of Ellman (1995) as described by Raja *et.al.* (2007). Reduced glutathione (GSH) forms the bulk of non-protein sulfhydryl groups. This method is based on the formation of relatively stable yellow colour when Ellman's reagent is added to a sulfhydryl compound. 2-nitro-5-thiobenzoic acid, the chromophoric product resulting from the reaction of Elmman's reagent with reduced glutathione.

Procedure: Equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.1ml of the supernatant, 2ml of Ellman's reagent (5, 5-dithio, bis (2-nitrobenzoic acid)) was added. Mixture was vortexed and the absorbance of mixture read at 412 nm within 15 min.

Calculations: Absorbance (concentration) of glutathione was calculated from a standard calibration curve (y = Mx) prepared by plotting absorbance of standard glutathione concentrations against their standard (known) concentrations when subjected to the same experimental conditions.

3.3.8.8. Determination of Total Protein Concentration

The Biuret method as described by Tietz, 1995 was employed for the determination of protein concentration.

Principle: Copper (II) ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a coloured complex. The test was carried out using a protein test-kit (Randox, UK) that utilizes the Biuret method for protein determination.

Procedure: The reagents were brought to room temperature then pipetted into labelled test tubes as follows:

Reagents	Reagent Blank	Standard	Sample
Distilled water	0.02ml	-	-
Standard (66g/l bovine serum albumin)	-	0.02ml	-
Sample (Serum)	-	-	0.02ml
Solution1(Biuret Reagent) 6mmol/l of copper (II) acetate, 12mmol/l of potassium iodide, 1.15mmol/l of sodium hydroxide, and detergent	1.0ml	1.0ml	1.0ml

The test tubes were mixed thoroughly and incubated for 30mins at room temperature

- The absorbance (A) of samples or standard was read against the reagent blank at 546nm in a spectrophotometer.

Calculations: The total protein concentration in the sample was calculated as follows:

$$C_{sample} = \frac{A_{sample} \times C_{standard}}{A_{standard}}$$

3.3.9. Histological Assessment

After sacrificing the male broodstocks to obtain milt, necropsy was done and samples of skin and testes were harvested and preserved in Bouin's fluid for 24hrs, after which tissues were fixed in 10% phosphate-buffered formalin until processing.

Processing involved dehydrating tissues, putting them into a xylene phase and impregnating them with paraffin wax under vacuum. Following this process, the tissues wereembedded in wax and cut on a microtome into 5µm sections. Selected sections were floated and stretched on a hot-water bath, mounted on a clean glass slides and placed on a warming tray to dry and adhere. Following staining with haematoxylin and eosin, sections were covered with a coverslip and mounted on a light microscope for evaluation by the pathologist (Kiernan, 1990). Abnormalities were documented daily using a digital camera.

3.3.10. Physico-chemical Parameters of Water

Some amount of water were collected using sterile plastic white containers from the tanks housing the catfish brood stocks before treatment commenced and towards the end of the treatments, i.e., on days 0 and 3 (before treatment for that day). These were kept in the freezer maintained at 4^{o}C until they were taken to the lab for physico-chemical assessments. The parameters assessed included:

Alkalinity, Ammonia, Carbon dioxide, Chloride, Electrical conductivity, Dissolved Oxygen (DO), Nitrate, pH and Hardness.

3.3.10.1. Determination of Temperature

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

3.3.10.2. Determination of Hydrogen ion concentration (pH)

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 dippedinto the water and the static pH was read 60 seconds later.

3.3.10.3.Determination of Dissolved Oxygen (DO)

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).

3.3.10.4. 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:

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

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

3.3.10.5. 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:

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

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

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).

3.3.10.6. Electrical Conductivity (EC)

It was measured with the help of EC meter, which measured the resistance offered by the water between two platinized electrodes. The instrument was standardized with known values of conductance observed with standard KCl solution. The EC meter was dipped in the sample water and observed for 4mins before the measurement was taken.

3.3.10.7. Carbon dioxide (CO_2)

Total alkalinity CO_2 was determined. This was determined by HCl titration of the water sample to the CO_2 equivalence point. And measurement taken.

3.3.10.8. Ammonia (Nitrogen)

It was measured spectroscopically at 425 nm radiation by making a colour complex with Nessler's reagent. The conditions of reaction are alkaline and cause severe interference from hardness in water.The sample is buffered at a pH of 9.5 with a borate buffer in order to decrease hydrolysis of cyanates and organic nitrogen compounds, and is distilled into a solution of boric acid. Alkaline phenol and hypochlorite react with ammonia to form indophenol blue that is proportional to the ammonia concentration. The blue color formed is intensified with sodium nitroprusside and measured colorimetrically.

3.3.10.9. Chloride (Cl)

It was measured by titrating the known volume of sample with standardized silver nitrate solution using potassium chromate solution in water and eosin/fluorescein solution in alcohol as indicator. And result recorded. The latter indicator is an adsorption indicator while the former makes a red colored compound with silver as soon as the chlorides are precipitated from solution.

3.4. Acute toxicity of glyphosate and 2, 4-D herbicides on *Clarias gariepinus* fingerlings

Fifty fingerlings of *Clarias gariepinus*, of mean weight 5.8 ± 0.2g and mean length 4.2 ± 0.3cm, aged 10weeks, collected from Decfiro Standard Link farm, 7 Wogu street, Oyigbo city, PH, Rivers State, were used for the investigation. The fingerlings were acclimatized for seven days in plasticblack bowls containing de-chlorinated and aerated tap water, at room temperature of 28.43 ± 0^{o}C, following the method of Hoque *et al*. (1993). During the acclimation period, fingerlings

were examined for pathogens and diseases. There was no mortality during the acclimation period. Water was changed at three days interval to prevent the build-up of metabolic wastes, and was aerated to increase oxygen supply. Fingerlings were fed twice daily with 2mm feed pellets at 3% body weight. Feeding was stopped 24 hours prior to and during exposure period that lasted for 48 hours. This was necessary because feeding increases the rate of respiration and excretory products, which may influence the toxicity of test solution.

The method used to determine acute toxicity was asdescribed by Lorke (1983). The study was conducted in two phases.

3.4.1. Glyphosate herbicide

Phase 1: Nine fingerlings were used during this phase. Nine fingerlings were divided into three groups of three fingerlings each. These were put in three different 30-litre capacity black bowls, labelled A, B and C, based on the weight of the fingerlings. Each group of fingerlings were administered different doses (10, 100 and 1000mg/kg of body weight) of the glyphosate herbicide, according to Lorke, 1983, and then topped up with tap water to the 20-litre mark. The fingerlings were placed under close observation for 1hr, 12hrs, 18hrs and 24hours to monitor their behaviour as well as if mortality will occur. Observations were noted.

Phase 2: During this phase, three fingerlings were used. Three fingerlings were distributed into three groups of one fingerling each, based on the weight of the fingerlings. The fingerlings were administered higher doses (1600, 2900, and 5000mg/kg) of the glyphosate herbicide, according to Lorke, 1983, and then observed for another 24hours for signs of toxicity, abnormal behaviour as well as mortality. From the data obtained, LD_{50} was determined.

The LD_{50} was calculated using the formula:

$LD_{50} = \sqrt{(D_0 \times D_{100})}$.

Where D_0 = Higher dose that gave no mortality,

D_{100} = Lowest dose that produced mortality.

3.4.2. 2, 4-D herbicide

Phase 1: Nine fingerlings were used during this phase too. Nine fingerlings were divided into three groups of three fingerlings each. These were put in three different 30-litre capacity black bowls, labelled A, B and C. Each group of fingerlings were administered different doses (10, 100 and 1000mg/kg of body weight) of the 2, 4-D herbicide and then topped up with tap water to the 20-litre mark. The fingerlings were placed under close observation for 1hr, 12hrs, 18hrs and 24hours to monitor their behaviour as well as if mortality will occur.

Phase 2: During this phase, three fingerlings were used. Three fingerlings were distributed into three groups of one fingerling each. The fingerlings were administered higher doses (1600, 2900, and 5000mg/kg) of the 2, 4-D herbicide and then observed for another 24hours for abnormal behaviour as well as mortality.

Then the LD_{50} was calculated using the formula:

$LD_{50} = \sqrt{(D_0 \times D_{100})}$.

Where D_0 = Higher dose that gave no mortality,

D_{100} = Lowest dose that produced mortality.

3.4.3. Mixture of Glyphosate and 2, 4-D herbicides

Phase 1: Nine fingerlings were also used during this phase. Nine fingerlings were divided into three groups of three fingerlings each. These were put in three different 30-litre capacity bowls, labelled A, B and C. Each group of fingerlings were administered different doses (10, 100 and 1000mg/kg) of the mixture of glyphosate/2, 4-D herbicide (1:1 v/v) and then topped up with tap water to the 20-litre mark. The fingerlings were placed under close observation for 1hr, 12hrs, 18hrs and 24hours to monitor their behaviour as well as if mortality will occur.

Phase 2: During this phase, three fingerlings were used. Three fingerlings were distributed into three groups of one fingerling each. The fingerlings were administered higher doses (1600, 2900, and 5000mg/kg) of the mixture of glyphosate and 2, 4-D herbicide, and then observed for another 24hours for behaviour as well as mortality.

Then the LD50 was calculated using the formula:

$LD50 = \sqrt{(D_0 \times D_{100})}$.

Where D_0 = Highest dose that gave no mortality,

D_{100} = Lowest dose that produced mortality.

3.4.4. Control

Three fingerlings were not exposed to any herbicide for each treatment (12 fingerlings in all were used), but rather maintained in well-aerated and de-chlorinated water. This was regarded as the control of the experiment.

Temperature condition was kept at room temperature, and all bowls were exposed to equal amount of natural light. Fish were examined for abnormal behaviours and mortality for 1 hour, 12 hours, 18hours, and 24 hours during the period. The 24 hour LD_{50} toxicity for each glyphosate, 2, 4-D, and glyphosate/2, 4-D mixture

concentrations were determined as a summary of percentage mortality data following the methods of Lorke (1983). Which were immediately removed and counted in every bowl at each observation time during the exposure periods. The carbon dioxide, chloride, total alkalinity, hardness, pH, dissolved oxygen and ammonia were monitored 24 hours using methods described by APHA (1985).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1RESULTS

4.1.1. Effects of the herbicides on exposure of the brood stocks

Throughout the exposure of the catfish to glyphosate- and 2, 4-D-treated water, the skin of fish was stained slightly especially at the head and dorsal regions. This white stain disappeared immediately after they were returned to fresh water. Fish were calm with no observable discomfort.

Table 4.1. Percentage (%) hatchability of incubated eggs

Herbicides	No. of eggs stripped and incubated.	No. of hatched and un-hatched eggs.		% hatchability.
		Hatched	Un-hatched	
Control	1000	935	65	93.5
Glyphosate	1000	820	180	82
2, 4-D	1000	640	360	64
Mixture of glyphosate and 2, 4-D.	1000*	120	880	12

***Obtained from the weight of the eggs collected, when weighed with a weighing balance.**

4.1.2. Effects of glyphosate on the reproductive success

Glyphosate slightly affected reproductive success as observed in Table 4.1 above, where we recorded about 180 un-hatched eggs compared to the control (65 un-hatched). These un-hatched eggs could be as a result of some reasons ranging from unripe or immature eggs, deformed or dead eggs to enucleated eggs. In the male, the milt collected from the brood stocks exposed to Glyphosate was watery, whitish to creamy in colour and reduced in quantity compared to that from the control which was watery, whitish to creamy in colour but much in quantity.

4.1.3. Effects of 2, 4-D on the reproductive success

2, 4-D significantly affected reproductive success as observed in Table 4.1 above, where we recorded about 360 un-hatched eggs compared to the control (65 un-hatched eggs). In the male, the milt collected was watery, deeply cream in colour and greatly reduced in quantity when compared with that from the control which was watery, whitish to creamy in colour but much in quantity.

4.1.4. Effects of the mixture of glyphosate and 2 4-D on the reproductive success

The mixture of glyphosate and 2, 4-D greatly affected reproductive success as seen in Table 4.1 above, where we recorded about 880 un-hatched eggs compared to the control (65 un-hatched eggs). In the male, the milt collected was watery, brownish-cream in colour and greatly reduced in quantity when compared with that from the control which was watery, whitish to creamy in colour but much in quantity.

Table 4.2. Number and % mortality and survivability of fish fry after fourteen days (2wks) of hatching

Herbicides	No. of hatched eggs.	No. and % of live fish larvae.	No. and % of deformed fish larvae.	No. and % of dead fish larvae.
Control	935	885 (94.65)	15 (1.60)	35 (3.74)
Glyphosate	820	712 (86.83)	66 (8.05)	42 (5.12)
2, 4-D	640	530 (82.81)	52 (8.13)	58 (9.06)
Mixture of glyphosate and 2, 4-D.	120	20 (16.67)	28 (23.33)	72 (60)

Table 4.3. Growth response of *Clarias gariepinus* larvae in two weeks and four weeks after hatching.

Herbicides	Growth parameters			
	Aver. mean weight (g) at 2 wks old (W_0).	Aver. mean weight (g) at 4 wks old (W_0).	Aver. mean weight gain (g).	SGR (%/day).
Control	2.24 ± 0.22	4.98 ± 0.50	2.74 ± 0.27	1.24*
Glyphosate	1.98 ± 0.20	3.32 ± 0.33	1.34 ± 0.13	0.80
2, 4-D	1.82 ± 0.18	3.02 ± 0.30	1.20 ± 0.12	0.79
Mixture of glyphosate and 2,4-D.	1.29 ± 0.13	2.04 ± 0.20	0.75 ± 0.08	0.71

*10 fries were weighed for each treatment and control, and average weight taken; time was 28days.

Table 4.4. Relative fecundity of both treatments and control of the female brood stocks of Clarias gariepinus.

Herbicides	No. of stripped eggs.	Body weight of female (kg).	Relative fecundity.
Control	1000	3.52	284.09
Glyphosate	1000	3.84	260.42
2, 4-D	1000	3.65	273.97
Mixture of glyphosate and 2, 4-D.	1000	3.55	281.69

The result of percentage hatchability of eggs in both treatments and control is shown in Table 4.1. Numerical estimation for both the hatched and un-hatched eggs was cautiously and manually done, and percentage hatchability calculated. From Table 4.1, untreated (control) had the highest hatchability rate of 93.5% followed by glyphosate-treated eggs (82%), 2, 4-D-treated eggs, and the least was seen in eggs fromthe mixture of glyphosate and 2, 4-D (12%). After fourteen days of the hatching, the live, deformed and dead fish fries were estimated, and the percentage mortality and survivability were calculated as shown in Table 4.2. The highest percentage mortalities were recorded in 2, 4-D-treated eggs and also on the mixture of glyphosate and 2, 4-D-treated eggs, which were58±9.06% and 72±60% respectively (Table 4.2). It was observed that most of the dead fish fries were not seen, may be as a result of cannibalism among the fish fries. Control (un-treated) had the highest percentage survivability (94.65%) after fourteen days, while the least value was recorded in the mixture of glyphosate and 2, 4-D (16.67%) (Table 4.2). The percentage of deformed fish fries was highest (23.33%) in the mixture of glyphosate and 2, 4-D and least in the control (1.60%) (Table 4.2). The growth response of the fish larvae under the treatments and control in two weeks and four weeks after hatching, is presented in Table 4.3. There were differences in growth performance of *C. gariepinus* larvae. The mean weight gain value was highest in control (2.74 ± 0.27g), followed by that of the glyphosate-treated eggs (1.34 ± 0.13g) and least in that of the mixture of glyphosate/2, 4-D (0.75 ± 0.08g) (Table 4.3). The instantaneous growth rate, expressed as Specific Growth Rate (SGR) was highest in control (1.24) and least in the mixture of glyphosate and 2, 4-D (0.71). This shows that the herbicides (glyphosate and 2, 4-D have detrimental effects on the growth rate of *Clarias gariepinus* frys as seen in the present study. These results also goes to show that the herbicides, glyphosate and 2, 4-D, have toxic effects on the hatchability and survivability of *Clarias gariepinus*. And the toxic effects are more deleterious

when these two herbicides are mixed, as the synergistic effects greatly impeded fish reproduction.

Plate 4.1 (Control)

Plate 4.2

Plate 4.3

Plate 4.4 (Control)

Plate 4.5

Plate 4.6

*** Plates 4.1 to 4.3 show the photomicrographs of some of the larvae for Control and both treatments (glyphosate and 2, 4-D) at day 3, after hatching. (The larvae from control (plate 4.1) looked healthy, well pigmented, with well-developed yolk sacs, while the larvae from glyphosate (plate 4.2) and 2, 4-D (plate 4.3) treatments looked unhealthy, less pigmented, with reduced yolk sacs).**

*** Plates 4.4 to 4.6 show the photomicrographs of the larvae at day 5, after hatching.**

Plate 4.7 (Control)

Plate 4.8

Plate 4.9

Plate 4.10 (Control)

Plate 4.11

Plate 4.12

*** Plates 4.7 to 4.9 and 4.10 to 4.12 show the size (weight) of the larvae at day 7 and 10 respectively, after hatching (The larvae of 2, 4-D (plates 4.9 and 4.12) looked smaller and lighter in weight, with tail region appearing transparent, while those from the control (plates 4.7 and 4.10) looked bigger and healthier.**

Plates 4.1 to 4.3 show the photomicrographs of some of the larvae for both treatments (glyphosate and 2, 4-D) and control at day 3, after hatching. The larvae in the control looked healthy, well pigmented, with well-developed yolk sacs (Plate 4.1), while the larvaefrom glyphosate treatment looked unhealthy, less pigmented, and with reduced yolk sacs (Plate 4.2). Plate 4.3 shows the larvae from 2, 4-D-treatment looking smaller (compared to control), greatly reduced pigmentation, with shrivelled yolk sacs. These effects (small size, reduction in pigmentation and poor yolk sac development or faster yolk regression) could be due to the toxic effects of the herbicides on the gametes of the broodstocks. Plates 4.4 to 4.6 show the photomicrographs of the larvae at day 5, after hatching. At this stage, it is expected that there would be total regression of the yolk sacs and an insatiable need for external supply of food. It was observed that larvae of the control still retained some amount of yolk sac (Plate 4.4), while there was some degree of yolk sac regression in the larvae from the glyphosate treatment (Plate 4.5). The larvae from the 2, 4-D treatmentlooked smaller, with total regression of the yolk sacs, and were swimming slowly due to weakness (Plate 4.6). Plates 4.7 to 4.9 and 4.10 to 4.12 show the size (weight) of the larvae at day 7 and 10 respectively, after hatching. From the physical examination, the larvae of the 2, 4-D looked smaller and lighter (tail region appeared transparent) (Plates 4.9 and 4.12) compared with those from the control that looked bigger and healthy (Plate 4.7 and 4.10), while those from the glyphosate treatment were average in size and outlook (Plates 4.8 and 4.11).

Table 4.5. Biochemical profiling of fish liver for both treatments and control.

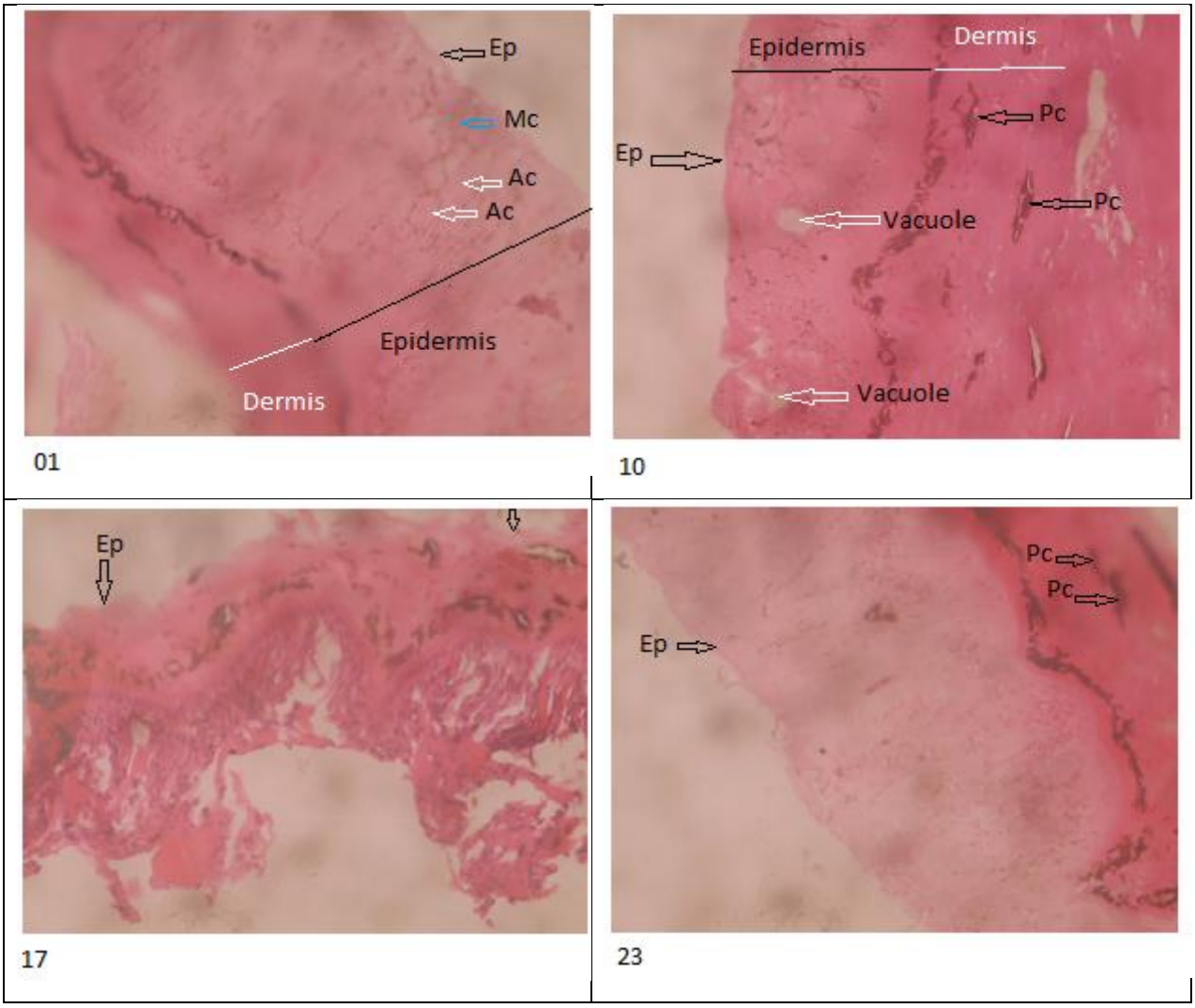
Parameter	Total Protein (g/l)	GSH (mg/g protein)	SOD (IU/mg protein)	MDA (Nmol/ mg protein)	GST (µmol GSH-CDNB/min/mg)	GPx (Mg GSH/min/mg protein)	CAT (µm H_2O_2/min/mg protein)
Control	25.60	1.53	4.41E-05	0.58	4.28E-06	4.45	1.75E-05
Glyphosate	15.45	1.73	8.79E-05	0.91	5.88E-06	2.85	2.74E-05
2, 4-D	24.63	1.67	6.03E-05	0.68	5.72E-06	2.49	1.98E-05
Gly. & 2, 4-D	23.67	1.06	6.29E-05	0.54	6.88E-06	4.63	1.82E-05

4.1.6. Effects of Glyphosate, 2, 4-D and Glyphosate/2, 4-D on Fish Biochemical Parameters.

Activities of the stimulated liver enzymes of fish exposed to glyphosate, 2, 4-D and mixture of Glyphosate and 2, 4-D are presented in Tables 4.5. There was an increase in the activities of all the enzymes.The total protein levels of catfish brood stock liver exposed to glyphosate, 2, 4-D, and mixture of glyphosate and 2, 4-D were 15.46 g/l, 24.63 g/l, and 23.67 g/l respectively compared to the control (25.60 g/l). This shows that there was drastic reduction of the total protein level of the glyphosate treatment. The GSH levels of catfish brood stock liver exposed to glyphosate, 2, 4-D and mixture of glyphosate and 2, 4-D were 1.73 mg/g protein, 1.66 mg/g protein, and 1.05 mg/g protein respectively compared with the control (1.53 mg/g protein). This indicates that glyphosate treatment had elevated level of GSH.The SOD levels of catfish brood stock liver exposed to glyphosate, 2, 4-D and mixture of glyphosate and 2, 4-D were 8.79 IU/mg protein, 6.03 IU/mg protein, and 6.29 IU/mg protein respectively, compared with the control (4.81 IU/mg protein). Glyphosate treatment has the highest level of SOD. This high values of glyphosate-treatment could be as a result of its complete metabolism by the liver as studied by Glesy *et al*., 2000 and Williams *et al*., 2000. The lipid peroxidation (MDA) levels of catfish brood stock liver exposed to glyphosate, 2, 4-D and mixture of glyphosate and 2, 4-D were 0.09 nmol/mg protein, 0.11 nmol/mg protein, and 0.08 nmol/mg protein respectively, compared to the control (0.10 nmol/mg protein). They were all within the range of level for control. GST O.D was calculated at 0 min, 1 min, 2mins, and 3mins. The GST levels of catfish brood stock liver exposed to glyphosate, 2, 4-D and mixture of glyphosate and 2, 4-D were 5.88 µmol GSH-CDNB/min/mg protein, 5.72 µmol GSH-CDNB/min/mg protein, and 6.88 µmol GSH-CDNB/min/mg proteinrespectively, compared with the control (4.28 µmol GSH-CDNB/min/mg protein). The mixture of glyphosate and 2, 4-D had the highest level of GST. The GPx levels of catfish

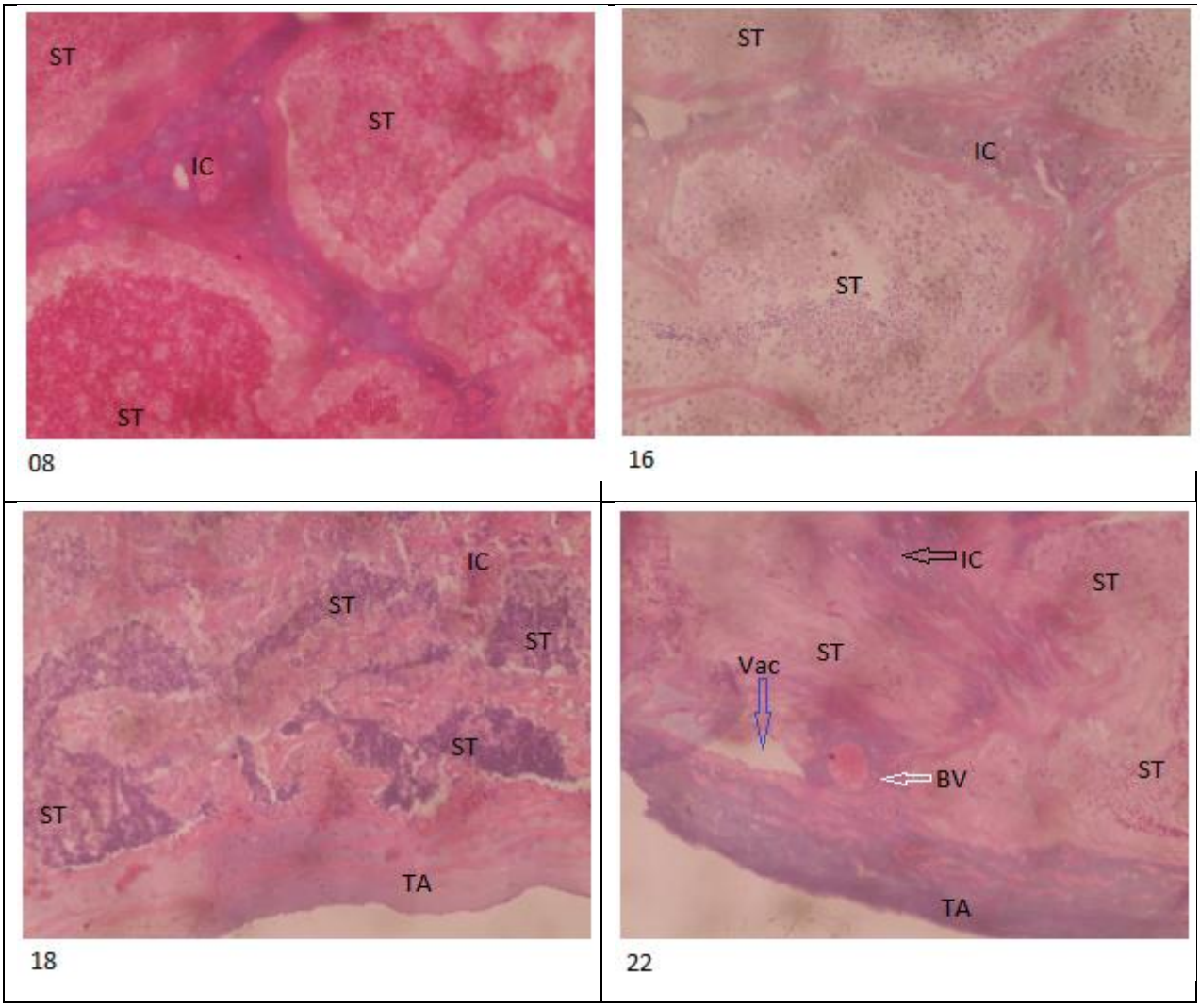
brood stock liver exposed toglyphosate, 2, 4-D and mixture of glyphosate and 2, 4-D were 2.85 mg GSH/min/mg protein, 2.49 mg GSH/min/mg protein, and 4.63 mg GSH/min/mg protein respectively, compared to the control (4.45 mg GSH/min/mg protein). It is obvious that there was a slight elevation of GPx in the mixture of glyphosate and 2, 4-D treatment, and so no significant difference between the mixture and control, as elevation is not significant. There was a significant decrease in the levels of GPx in glyphosate- and 2, 4-D-treatments. The CAT levels of catfish brood stock liver exposed to glyphosate, 2, 4-D and mixture of glyphosate and 2, 4-D were 2.74 µm H_2O_2/mg protein, 1.98 µm H_2O_2/mg protein, and 1.82 µm H_2O_2/mg protein respectively as compared to control (1.75 µm H_2O_2/mg protein). Glyphosate-treated had the highest elevated CAT.

4.1.7. Histological Assessments of the skin, testes and ova from the brood stocks of *Clarias gariepinus* (for both treatments and control).



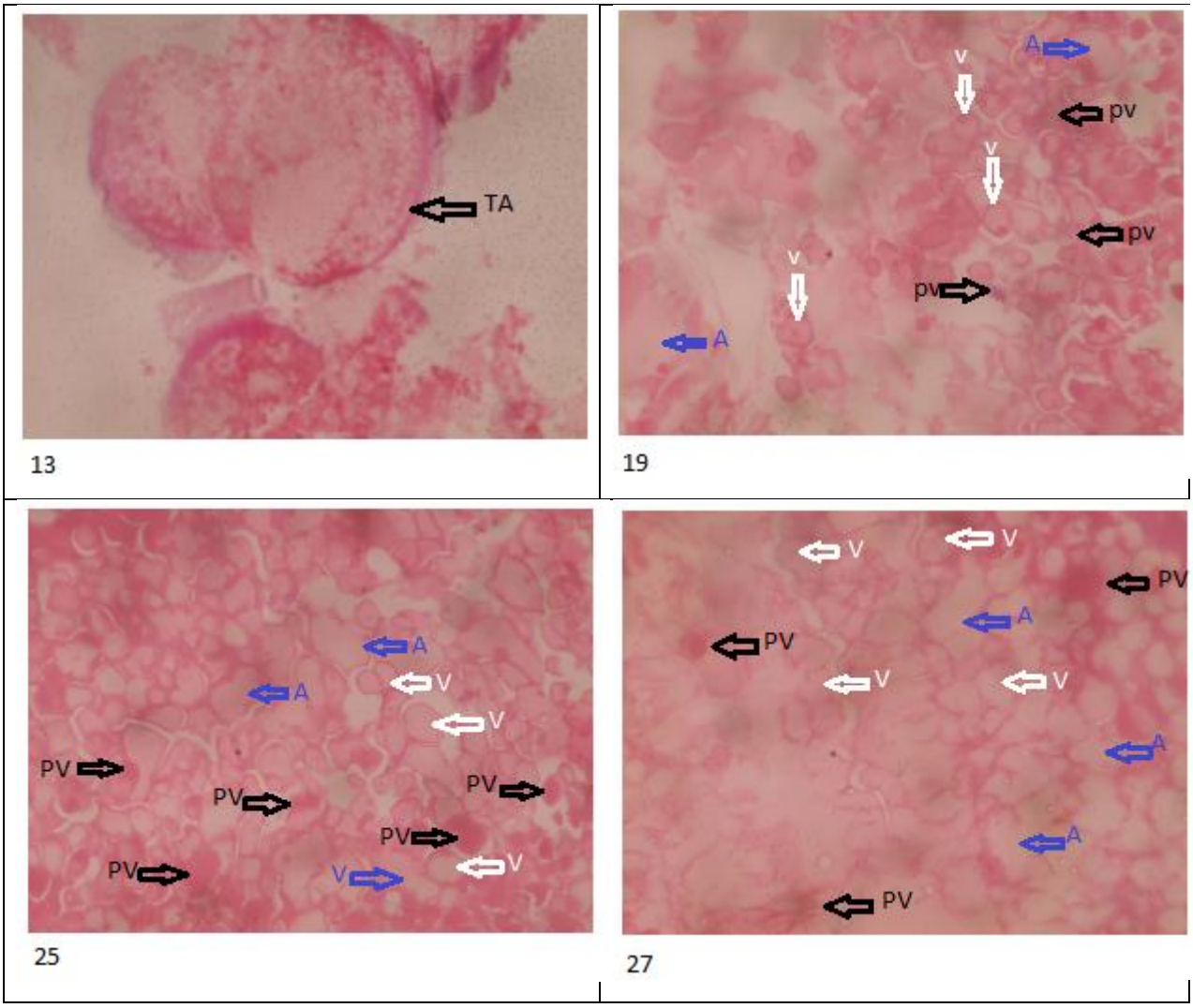
Plates 01, 10, 17 and 23 show the photomicrographs of the histological assessments of the skin from the brood stocks of *Clarias gariepinus* (for both treatments (10, 17 and 23) and control (01)).Magnification: x400 H & E.

Plates 01 and 23 (Control and Glyphosate treatment respectively) showed histologically normal skin sections, while plate 10 (2, 4-D treatment) had closely delineated epidermis and dermis, with vacuoles (Vac) in the epidermis. So, histologically distorted skin section. Plate 17 (mixture of glyphosate and 2, 4-D treatment) had thickened epidermis and discontinuous epithelial lining.So, histologically distorted skin section.



Plates 08, 16, 18 and 22 show the photomicrographs of the histological assessments of the testes from the brood stocks of *Clarias gariepinus* (for both treatments (16, 18 and 22) and control (08)). Magnification: x400 H & E.

Plates 08 and 16 (control and glyphosate respectively) showed normal histological testes, while plate 18 (mixture of glyphosate and 2, 4-D) had thickened Tunica albuginea and thickened connective tissue septo that extends and surrounds the seminiferous tubules (ST). So, it is histologically distorted. Plate 22 (2, 4-D) showed vacuolation (Vac) and blood vessels in the interstitial spaces. So, mildly distorted testicular histology.



Plates 13, 19, 25 and 27 show the photomicrographs of the histological assessments of the ova from the brood stocks of *Clarias gariepinus* (for both treatments (13, 25 and 27) and control (19)). Magnification: x400 H & E.

Plates 19 (Control), 25 (glyphosate) and 27 (2, 4-D) showed histologically normal ovarian sections, while plate 13 (mixture of glyphosate/2, 4-D) had thickened Tunica albuginea (TA) surrounding the ovary. So, it is a histologically distorted ovarian section.

4.1.7.a.i. The Photomicrograph results for the histology of catfish Skin; Magnification: 400 H &E; for both treatments and control are as follows:

Slide 01: Photomicrograph of the skin of *C. gariepinus* brood stock that was not exposed to any herbicide (control) showing dermis and epidermis.Superficial part of the epidermis is covered by the epithelium (Ep). Deep into the epidermis, the mucosal cell (Mc) and alert cells (Ac) are found.

Impression: These cells are histologically normal.

Slide 10: Photomicrograph of the skin of *C. gariepinus* brood stock exposed to 2, 4-D herbicide. The dermis and epidermis are closely delineated. Epithelium clearly green (Ep) and pigment cells (Pc) seen. However, some vacuoles were in the epidermis.

Impression: Histologically distorted catfish skin section.

Slide 17: Photomicrograph of the skin section of*C. gariepinus* brood stock exposed to the mixture of glyphosate and 2, 4-D herbicides. This shows reduced thickness of the epidermis and discontinuous epithelial lining.

Impression: Histologically distorted *C. gariepinus* skin section.

Slide 23: Photomicrograph of the skin section of *C. gariepinus*brood stock exposed to Glyphosate herbicide. This slide shows clearcut epidermis and dermis. The epithelium (Ep) is continuous.

Impression: Histologically normal catfish skin.

4.1.7.b.i.The photomicrograph results for the histology of catfish Testes; Magnification: 400 H & E; for both treatments and control are as follows:

Slide 08: shows the photomicrograph of the testis of *C. gariepinus*brood stock that was not exposed to any herbicide (control). The interstitial spaces are filled with interstitial cells (Ic) of *leydig*. Seminiferous tubules (St) contain

spermatogenic cells and spermatozoa. The arrangement of spermatogenic cells is such that spermatogonia are close to the basement membrane; and towards the luminal border are: primary and secondary spermatocytes, spermatids and spermatozoa respectively.

Impression: Histologically normal testis.

Slide 16: shows the photomicrograph of the testis of *C. gariepinus*brood stock exposed to the Glyphosate herbicide. There are clusters of interstitial cells (Ic) of *leydig* in the interstitial spaces. The seminiferous tubules (ST) are filled with spermatogenic cells and spermatozoa.

Impression: Histologically normal testis.

Slide 18: shows the photomicrograph of the testis of *C. gariepinus* brood stock exposed to the mixture of glyphosate/2, 4-D herbicides. This shows the following features: *Tunica albuginea* that is thickened. Thickened connective tissue septo that extends and surround the seminiferous tubules (ST). Seminiferous tubules contain spermatogenic cells that are not clearly in layers extending from spermatogonia to mature spermatozoa. And interstitial spaces and poorly delineated.

Impression: Histologically distorted catfish testicular section.

Slide 22: shows the photomicrograph of the testis of *C. gariepinus* brood stockexposed to 2, 4-D herbicide. This shows clusters of interstitial cells (Ic) of *leydig* in the interstitial spaces. Seminiferous tubules filled sertoli cells, spermatogenic cells and mature spermatozoa. Vacuolation (Vac) and blood vessels in the interstitial spaces.

Impression: mildly distorted testicular histology (Reason is vacuolation otherwise it is histologically normal).

4.1.7.c. The photomicrograph results for the histology of catfish Ova; Magnification: □ 400 H & E; for both treatments and control are as follows:

Slide 19: shows the photomicrograph of the ova of *C. gariepinus* brood stockthat was not exposed to the herbicides. This slide featured: a.) pre-vitellogenic follicles (Pv), and b.)few post-vitellogenic cells (A).

Impression: Histologically normal ovarian section.

Slide 25: shows the photomicrograph of the ova of *C. gariepinus* brood stockexposed to glyphosate herbicide. This slide shows pre-vitellogenic follicles (Pv); vitellogenic follicles (V) are numerous while post-vitellogenic follicles (A) are few in number.

Impression: Histologically normal ovarian section.

Slide 27: shows the photomicrograph of the ova of *C. gariepinus*brood stock exposed to 2, 4-D herbicide. This slide shows pre-vitellogenic (Pv) and vitellogenic (V) follicles in large numbers. Post-vitelogenic follicles are scanty.

Impression: Histologically normal ovarian section.

Slide 13: shows the photomicrograph of the ova of *C. gariepinus* brood stock exposed to the mixture of glyphosate and 2, 4-D herbicides. This slide shows thickened *Tunica albuginea* (TA) surrounding the ovary. The ovary contains follicles at different stages of development.

Impression: Histologically distorted ovarian section.

4.1.8. Water quality assessment of the treatments and control before (Day 1) and towards the end (Day 3) of the exposure.

Table 4.6. Physico-chemical parameters of the treatment water and control before (Day 0) and towards the end (Day 3) of the exposure.

Parameters	Control	Before 2,4-D treatment	After 2,4-D treatment	Before glyphosate treatment	After glyphosate treatment	Before glyphosate/2,4-D treatment	After glyphosate/2,4-D treatment
pH	4.59	4.81	6.54	4.36	7.23	4.69	4.56
Electrical conductivity (µS/cm)	81.4	46.70	68.40	46.30	48.90	68.30	63.30
Dissolved oxygen (DO) (mg/l)	5.90	5.77	8.14	6.30	7.30	6.26	5.99
Ammonia (mg/l)	0.180	0.263	0.214	0.259	0.226	0.210	0.270
Nitrate (mg/l)	0.792	1.157	0.942	1.140	0.994	0.924	1.188
Alkalinity (mg/l)	8.20	3.60	11.60	3.40	4.60	7.00	12.80
Chloride (mg/l)	14.00	17.60	15.20	17.80	16.20	14.80	19.80

Carbon dioxide (mg/l)	1.8	1.8	2.0	1.0	1.0	1.8	2.2
Total hardness (mg/l)	18.00	42.00	30.00	24.00	20.00	28.00	28.00

4.1.9 ACUTE TOXICITY TESTS.

Table 4.7. Physico-chemical parametersof the experimental water and control for phase-1 and phase-2 water for acute toxicity tests.

Parameters	Control	Glyphosate	2, 4-D	Mixture of Gly. and 2, 4-D
Temperature (^{0}C)	25	28	29.8	31
Alkalinity (m/L)	12	10	12	14
Ammonia	0.5	0.4	0.5	0.7
Carbon dioxide	3.0	3.5	4.0	4.4
Chloride	4.5	4.5	4.8	5.1
Dissolved Oxygen (mg/L)	5.0	4.7	4.5	3.2
pH	7.0	6.04	6.08	6.30
Hardness (mg/L CaCo3)	35.0	30	35	38

Table 4.8: Acute lethal effect of Glyphosate administered to fingerlings of *Clarias gariepinus*.

Experiment	Dose(mg/kg b.w.)	No. of dead fingerlings after 12 hrs.	No. of dead fingerlings after 24 hrs.
Phase-1	10	0/3	0/3
	100	0/3	0/3
	1000	0/3	0/3
Control	0	0/3	0/3
Phase-2	1600	0/1	0/1
	2900	0/1	0/1
	5000	0/1	0/1*

***LD_{50}>5000mg/kg b.w.**

Table 4.9: Acute lethal effect of 2, 4-D administered to fingerlings of *Clarias gariepinus*.

Experiment	Dose(mg/kg b.w.)	No. of dead fingerlings after 12 hrs.	No. of dead fingerlings after 24 hrs.
Phase-1	10	0/3	0/3
	100	0/3	0/3
	1000	0/3	0/3
Control	0	0/3	0/3
Phase-2	1600	0/1	0/1
	2900	0/1	0/1
	5000	0/1	0/1*

***LD_{50}>5000mg/kg b.w.**

Table 4.10: Acute lethal effect of a mixture of Glyphosate/2,4-D administered to fingerlings of *Clarias gariepinus*.

Experiment	Dose(mg/kg b.w.)	No. of dead fingerlings after 12 hrs.	No. of dead fingerlings after 24 hrs.
Phase-1	10	0/3	0/3
	100	0/3	0/3
	1000	0/3	0/3
Control	0	0/3	0/3
Phase-2	1600	0/1	0/1
	2900	0/1	1/1*
	5000	1/1	1/1

***LD_{50}<2900mg/kg b.w.**

The acute lethal study of glyphosate and 2, 4-D (Tables 4.8 and 4.9) on catfish fingerlings showed no animal died within 24 hrs. The LD_{50} was therefore greater than 5000mg/kg b.w. But for the mixture of glyphosate and 2, 4-D, at higher concentrations (2900mg/kg and 5000mg/kg), during the phase-2 of the herbicide mixtures (glyphosate and 2, 4-D) exposed to the fingerlings, death occurred. So, the LD_{50}, calculated from the formula stated above, was less than 2900mg/kg (2154.07mg/kg).Several abnormal behaviours such as restlessness, erratic swimming, air gulping, respiratory distress, loss ofequilibrium, and resting motionless at the bottom of the bowl (for those exposed to the mixture of glyphosate and 2, 4-D) were observed at higher concentrations of the toxicants, similar to Lovely (1998) observations. Avoaja and Oti (1997) reported these abnormal behavioural responses in fish exposed to toxicants.

4.2 DISCUSSION

4.2.1. Effects of glyphosate, 2, 4-D and a mixture of glyphosate and 2, 4-D herbicides on the hatchability and larvae development of *Clarias gariepinus*

In order to establish aquaculture as a successful and efficient agricultural activity, there is a need to find out the impediments to reproduction rate, so as to obtain high-quality seed after hatching and produce juveniles for grow-out without the need to obtain them from the wild.In this study, untreated (control) eggs incubated had the highest percentage (%) hatchability (93.5%), while the lowest percentage (%) hatchability (12%) was observed in the group administered with mixture of glyphosate and 2, 4-D-treated eggs. The glyphosate-treated eggs hada higher percentage (%) hatchability rate (82%) compared to that of the 2, 4-D-treated eggs (64%). This was in line with the works of Glesy *et al*., 2000 and Williams *et al*., 2000 who reported that glyphosate may be relatively non-toxic to non-target animals in the aquatic environment (Glesy *et al*., 2000; Williams *et al.,* 2000). The low hatchability rate of 2, 4-D is a confirmation of its toxicity to the reproductive cells of the fish. RED (2005) stated that dose-dependent toxic effects of 2, 4-D could include damage to the eye, thyroid, kidney, adrenals, ovaries or testes, and a drastic reduction in reproduction rate. In addition, researchers have observed neurotoxicity, reproductive toxicity and developmental toxicity of 2, 4-D on non-target organisms in the environment (RED, 2005). The very low hatchability of the mixture of glyphosate and 2, 4-D confirmed the synergistic effects of the combination of a non-toxic herbicide (glyphosate in this case) and a toxic herbicide (2, 4-D). The rate of toxicity seems to drastically increase due to this combination.Also, the very high toxicity of the herbicide (mixture of glyphosate and 2, 4-D) could probably be attributed to some possible synergistic effects likely to be produced by the active ingredients in the herbicides, of which is the compound that exists separately as herbicide and is likely to be equally toxic to fish.

Absolutefecundity means total number of ripened eggs per female, while number of eggs in relation to weight or length of fish means **relative fecundity**. In this study there seems to be a decline in relative fecundity of the fishes exposed to the herbicides compared to the control, though each of the fishes produced almost the same average number of eggs (Table 4.2). Decline in fish fecundity may be due to changed environmental conditions such as temperature or contamination with pesticides. Khallaf *et al*., (2003) mentioned that *O. nilotius* collected from polluted Shanawan drainage canal showed high decrease in fecundity, Al-Miriufuya Governorate showed lower fecundity of range between 1234to 3893 eggs for female with total length 12 to 23cm; and Ghada (2009) found that *O. niloticus* exposure to 1.05 and 0.21ppm of butachlor for 6 weeks and 6 days showed highly significant decreases in absolute fecundity. In addition, absolute fecundity in *O. niloticus* was significantly decreased after treatment with Dimethaote and Malathion for the control (245.25 ± 23.69 eggs/female) (Eman *et al.,* 2011) due to Oocyte atresia and decreased estradiol hormone.

4.2.2. Effects of glyphosate, 2, 4-D and mixture of glyphosate and 2, 4-D on liver enzymes activities infish

The glyphosate-, 2, 4-D- and a mixture of glyphosate and 2, 4-D-treatments caused significant changes in the activities of liver enzymes in *Clarias gariepinus*brood stocks, the glyphosate-treatment showing more toxicity than the other treatments (2, 4-D and a mixture of glyphosate and 2, 4-D). There were significant increases in all enzyme activities on exposure to glyphosate-, 2, 4-D- and mixture of glyphosate and 2, 4-D-treatments. 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 GST and GSH (5.88 and 1.73 respectively in glyphosate; 5.72 and 1.66 respectively in 2, 4-D) in the 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 aminotransferases, such as glutathione (GSH) and glutathione-S-transferases (GST) 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 aminotransferases. Stress conditions, in general, induce elevation in the transamination pathway (Awasthi *et al*. 1984). Superoxide dismutase (SOD) activity was enhanced in fish liver. Activity of SOD was higher in Glyphosate (8.79) than in 2, 4-D (6.03) 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.

Also, in the current study, a general increasing trend in the activities of antioxidant enzymes was observed in the liver of *Clarias gariepinus* exposed to both herbicides and their mixture; 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 (Table 4.5); with 8.79 in glyphosate-treatment, 6.03 in 2, 4-D-treatment and 6.29 in the mixture of glyphosate/2, 4-D-treatments. 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*., 2009). These results suggest that the herbicides, at the concentrations used, may be enzyme activators thus promoting hepatic stress. 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 (CAT) showed an increase in activity in the liver with the glyphosate-treatment (2.74mg/l) when compared to the control (1.75mg/l). Activity of catalase was higher in 2, 4-D-treatment (1.98mg/l) than in the mixture of glyphosate and 2, 4-D-treatment (1.82mg/l). The enhancement of catalase activity in fish exposed to glyphosate and 2, 4-D 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 compensatorymechanism. 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, being higher in glyphosate-treatment than in 2, 4-D- and the mixture of glyphosate and 2, 4-D-treatments, meaning that glyphosate was more toxic to the liver. 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 for both herbicides; the 0.91mg/l in glyphosate-treatment, 0.68mg/l in 2, 4-D-treatment, and 0.54mg/l in mixture of glyphosate/2, 4-D-treatment. 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)and glutathione peroxidase (GPx) in the liver when comparing treatment with control fish. Glyphosate induced more enzyme activity (1.73mg/l) than 2, 4-D (1.67mg/l) and mixture of glyphosate and 2, 4-D (1.05mg/l) for GSH. But the mixture of glyphosate/2, 4-D induced more enzyme activity (4.62mg/l) than glyphosate (2.84mg/l) and 2, 4-D (2.49mg/l) for GPx.

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 2, 4-D. 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 elicited by fish liver vis-à-vis all the enzymes assayed is close. Glyphosate induced more activity in SOD and GSH while the mixture of glyphosate and 2, 4-D induced more activity in GST and GPx. 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 responding specifically to each of the herbicides.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. Vutukuru *et al*. (2007) reported that when the liver cell is damaged,

tissue specific enzymes are released into intracellular spaces resulting in their increase in plasma.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 three days exposure. The elevated levels of lipid peroxidation (MDA) in the liver of *C. gariepinus* in response to exposure to glyphosate and 2, 4-D 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). There were increases in MDA levels in all the herbicide treatments. 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 serious threat to their continued existence as cells become weak and unprotected from other substances injurious to them. Interplay or any one of the 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 experimental 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 defence 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 experimental 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).

4.2.3. Effects of glyphosate, 2, 4-D and the mixture of glyphosate/2, 4-D on the histological parameters of catfish skin, testes and ova

According to Stentiford *et al.* (2003) and Zimmerli *et al.* (2007), histological analysis represents a useful tool to assess the degree of pollution, particularly for sub-lethal and chronic effects. The histological changes observed in this experiment revealed that glyphosate and 2, 4-D caused mild, but not disasterous effects to the skin, testes and ova of *Clarias gariepinus*brood stockswhile the mixture of glyphosate and 2, 4-D caused detrimental effects to the skin, testes and ova. The pathological lesions and thickened *Tunica albuginea,*with the connective tissue septae that extends and surrounds the seminiferous tubules in the testes of a mixture of glyphosate and 2, 4-D treatments compared to control (Slide 18) were necrosis, disruption and depletion of the seminiferous tubules (Slide 08). This showed that the mixture of glyphosate and 2, 4-D was extremely harmful to the testes of C. gariepinus brood stocks. Additionally, compared with the skin of the control (Slide 01), 2, 4-D and a mixture of glyphosate and 2, 4-D treatments had some level of vacuolation and reduced thickness of the epidermis, with discontinuous epithelial lining respectively (Slides 10 and 17). This was in agreement with the work of Fayhaa (2015) on Histopathological effect of heavy metal on different organs of fresh water fish tissues. The epidermis is a fragile layer, which is regularly sloughed off and regenerated. The scales covering the skin of African catfish act as an additional, physical barrier separating the skin and underlying tissues from the flowing water currents (Helfman *et al*.,2009). The dermis lies beneath the epidermis. This layer contains blood vessels, nerves, connective tissues and sense organs. The dermis is well supplied by blood vessels; hence, it also provides nourishment to the epidermis. Also, there was a focal localized degeneration of the skin (Slide 17). This is in agreement with the degenerative changes, reported to have occurred in the gonads of catfish following acute and chronic exposures to sub-acute and sub-lethal concentrations

of Malachite green (Srivastava *etal*., 1998). The skin is an outer wrapper of fish; hence, it is in-full-force of environmental fluctuations. As a consequence, the skin plays a key role as the first line of defense in physical and chemical means (Vernerey and Barthelat, 2014). As observed in the skin of the catfish brood stocks in the present study, vacuolar degenerations of the liver was similarly reported in *Oreochromis niloticus* subsequent to exposure to copper sulphate, another fish herbicide (Osman *et al*., 2009) and in the liver of *Clarias lazera* exposed to untreated dyestuff effluent (Abdel-Moneim *et al*., 2008). In the present study, histopathological changes were observed in the testes and ova of African catfish (Slides 13, 18 and 22). According to Tayel *et al.* (2007), this may be due to their exposure to sewage, domestic, and agricultural wastes. Similar histopathological changes (lesions) have been reported in fish exposed to pollutants (Gaber *et al.*, 2013). Mohamed (2001&2003) recognized histopathological alterations in fish testis and ovary suggesting that this may reduce the ability of fish to reproduce. It is well known that water pollution has a serious inhibitory effect on fish reproduction (Mohamed, 2001 & 2003) resulting in a decrease in their abundance and, consequently, a decline in fish species diversity. In the present study, pollution effects appeared as disruption in gonadal development. It comes in agreement with other studies for fish inhabiting polluted water (Balch *et al*., 2000). Also, oogenesis and spermatogenesis were influenced by exposure to sewage effluents (Kiparissis *et al*., 2003).

Finally, this study also showed an apparent reduction, albeit not significant, in reproductive rate in all treatments. Therefore, the potential for adverse effects of glyphosate, 2, 4-D and their mixtures on reproductive output and impact on wild populations cannot be ruled out. A number of potential mechanisms may contribute to the observed effect of these herbicides on reproductive rate, including disruption of normal progression through oogenesis of the eggs, inhibition of ovulation and increased rate of oocyte atresia or even poisoning of the mature spermatozoa. To explore this, hence the need to carry out histological

analysis of the gonads of exposed males and females. Ovarian follicle atresia is an apoptotic process leading to reabsorption of maturing oocytes rather than ovulation. It is a highly regulated, natural process thought to have a role in maintaining ovarian homeostasis; however various environmental stressors, as well as disruption of the hormonal control of oogenesis and ovulation, have been shown to increase atresia (Lubzens *et al.*, 2010). Atretic vitellogenic oocytes was found in the ova of catfish brood stocks exposed to the mixture of glyphosate/2, 4-D treatment (Slide 13).

4.2.4. Water quality assessment of the treatments and control before (Day 1) and towards the end (Day 3) of the exposure

Water quality was monitored in the fish throughout the experimental period (before (i.e. Day 1) and towards the end (i.e. Day 3) of the exposure), and the results are presented as Table 4.6. Water quality assessment indices are also used to identify the relative health of cultured fish species (Adeyemo and Babalobi, 2008). Glyphosateadministered at therapeutic level did not significantly affect the quality of water compared with control (Table 4.6). The no-effect of glyphosateon water quality agrees with the report of Omitoyin *et al*. (2006) following exposure of *Clarias gariepinus* juveniles to glyphosate herbicide. Although there were slight fluctuations in the pH for the mixture of glyphosate and 2, 4-D (4.56) and 2, 4-D (6.54) after the various exposures compared with glyphosate (7.23).These pH values reflect that the water has little pollution. It was claimed that the pH of surface waters is an important indicator of its quality and the extent of pollution in the watershed area. Normally, unpolluted waters show a pH of about 7.00 and 8.00 (WHO, 2008). The values for total hardness, 24mg/l, 30mg/l and 28mg/l for samples from glyphosate, 2, 4-D and a mixture of glyphosate/2, 4-D, respectively (Table 4.6), are both below the desirable drinking water standards indicated by WHO 150.0-500.0 mg/l. Some reported studies of surface water have found very low values for the water hardness parameter: Kusti

(Sudan) with a range of 55.0-59.0 mg/l (Ibrahim *et al*., 2015), in Kontagora (Nigeria) with 56.0 mg/l for dry and 49.0 mg/l for rainy seasons (Ibrahim *et al*., 2009). Some of these reported values are compatible to that found in the present study. It has been claimed that hardness is not considered of health concern at levels found in drinking water (WHO, 2011). Generally, increased water hardness is attributed to increased amounts of dissolved chlorides or sulphates of calcium and magnesium, although positively charged divalent ions, such as Iron (Fe), Serenium (Sr) and Manganese (Mn) can also contribute to water hardness (Meena *et al*., 2012). The values for the electrical conductivity measurements found in the current studyare much lower than the standard drinking water guideline value of 2,500 µS/cm quoted by EU guidelines. It could also be noticed that generally electrical conductivity values for 2, 4-D (68.40µS/cm) and the mixture of glyphosate/2, 4-D (63.3 µS/cm) are slightly higher than that of glyphosate (48.90 µS/cm), due to the increased water hardness of these herbicide treatments.

Parameters such as conductivity, water hardness, total dissolved salts and availability of high levels of ions which have divalent cations are all inter-related (Heston, 2015; Xylem Inc., 2011). The alkalinity values agreed with the range value documented by Moyle (1946) and Boyd (1981) for natural water. The alkalinity is higher in 2, 4-D treatment (11.60mg/l) and the mixture of glyphosate/2, 4-D (12.80mg/l), but greatly lower in glyphosate treatment (4.60mg/l) and control water (8.20mg/l). The high value could be due to high concentration of salts, and the lower value could be due to dilution. The different values obtained may be an indication that alkalinity of water increases with decreasing water purity. Similar observations have been made by Holden and Green (1960) on River Sokoto. Also, the higher nitrate-nitrogen ($N0_3$-N) concentration observed in the mixture of glyphosate and 2, 4-D (1.188mg/l) compared with the value of the control water (0.792mg/l) could be due to decomposition of organic matter. Comin*et al*., (1983), stated that high nitrate

concentrations in water is related to inputs from agricultural lands. Finally, the values of the other parameters (Chloride, Carbon dioxide and Ammonia) determined were within the acceptable ranges for domestic water purposes and fish production.

4.2.5. Physico-chemical parameters of water from the acute toxicity tests

Water quality attributes are prime factors that influence fish survival, reproduction, growth performance, and overall biological production (King, 1998; King and Jonathan, 2003). They affect aquatic biotic integrity by directly causing mortality and/or shifting the equilibrium among species due to subtle influences such as reduced reproductive rates or alternations in competitive ability. This inverse relationship is interesting and indicative of higher demand for oxygen prompted by condition of hyperactivity as observed and explained by Ofojekwu *et al*., 2001andOti, 2002.Physico-chemical parameters measured (Tables 4.7) seemed to be within optimum range for fish culture as reported by Omitoyin *et al.,* 2006 and Olaifa *et al*., 2003. There was a significant change in water quality resulting from application of the toxicants compared to the control. This observation was in line with Okoli-Anunobi *et al*., 2002 who investigated the lethal effect of the ElephantBlue® detergent on the Nile Tilapia *Oreochronis niloticus*.

In the case of dissolved oxygen, there was a slight decrease in the range for glyphosate- (4.7mg/l) and 2, 4- D-treated water (4.5mg/l) (Table 4.7), while there was much decrease in the dissolved oxygen for the mixture of glyphosate/2, 4-D water (3.2mg/l) (Table 4.7), compared with the control (5.0mg/l). This decline can be attributed to a situation referred to as oxygen sag, which is characterized by high mortality within short time. Death recorded in the phase two of the acute toxicity test of the mixture of glyphosate and 2, 4-D (Table 4.10) could therefore have occurred because of this. Warren, 1977 had earlier reported that the introduction of a toxicant into an aquatic system might decrease the dissolved

oxygen concentration, which will impair respiration, leading to asphyxiation. This was probably why the fishes exposed to higher concentrations of a mixture of glyphosate and 2,4-D were stressed progressively with time before death. The pH fluctuated slightly from the treatment water compared with the control water, but was not upto the alkaline death point as recorded by Okoli-Anunobi *et al*., 2002. The water hardness for glyphosate decreased slightly (30mg/l) compared with the control (35mg/l) (Table 4.7) and increased slightly for 2, 4-D (35mg/l) and the mixture of glyphosate and 2, 4-D (38mg/l). This could be as a result of the high toxicity of these toxicants. This will invariably affect the optimum growth and development of the cultured fish. Bacteria and other germs will thrive with bad water quality. But the temperature, ammonia, chloride and carbon dioxide values were almost the same with the value of the control (Table 4.7).

The physico-chemical parameters recorded were within the permissive limits fixed by WHO which are 6.5 and 6.5- 8.0 for dissolved oxygen and pH respectively. Although the presence ofherbicides causessome changes in the quality of water in and around sprayed areas and decrease the dissolved oxygen in water, along with an increase in temperature, which may pose a threat to the survival of fish species, the results of the present study indicates that 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).The erratic swimming, restlessness, gulping of air, and eventual resting motionless at the bottom of bowl (for higher concentrations of the mixture of glyphosate/2, 4-D-treatments) observed in this investigation are indications that

mortality of the exposed fish is not only due to impaired metabolism, but could in addition be due to nervous disorder. This is similar to the findings of Oti (2000) and Annune, *et al* (1994), who reported these abnormal behavioural responses in fingerlings of the hybrid, *Hetero-clarias* exposed to toxicants at 96 hours period.

4.2.6. Effects of Acute lethal concentrations ofglyphosate, 2, 4-D and a mixture of glyphosate and 2, 4-D on catfish fingerlings.

The no toxic effects (no mortality) seen in the resultsof the acute toxicity tests of the glyphosate-treated and 2, 4-D-treated fingerlings showed the LD_{50} was greater than 5000mg/kg. This could be because the acid forms of these herbicides were used in this study instead of the ester forms.Thiswas in line with the findings of Tomlin, 2006. He asserted that thetoxicity to fish and aquatic invertebrates varies widely depending on chemical form, with esters being the most toxic (Tomlin, 2006; WHO, 1989). Acid and amine salts LC_{50} range from greater than 80 to 2244mg acid equivalents per litre, whereas the ester forms range from less than 1.0 to 14.5mg acid equivalents per litre (RED, 2005). The greater toxicity generally of the ester in fish is likely due to the greater absorption rates of the esters through the gills where they are hydrolysed to the acid form (WHO, 1989). As with fish, esters are more toxic than acid or amine salt forms to fresh water aquatic invertebrates, with LC_{50} values ranging from 25 to 643 mg ac/L (for esters) (RED 2005). The relative toxicities for acids and salts are slightly toxic to practically non-toxic, whereas the esters are moderately to slightly toxic.The acute lethal concentrations of a mixture of glyphosate and 2, 4-D on the catfish fingerlings showed that the herbicides have significant effects on thephysiological parameters of the fingerlings.

Several abnormal behaviours such as restlessness, erratic swimming, air gulping, respiratory distress, loss of equilibrium, and the fingerlings resting motionless at the bottom of the bowl (dead) were observed. The impairment of respiration due to the toxic effects of glyphosate herbicide on the gills has been reported by

Omitoyin *et al*. (2006). At higher concentrations of the mixture (2900mg/kg and 5000mg/kg), there was an increase in the opercular ventilation rate within the first 12 hours of exposure. This might be suggestive of a physiological response to dissolved oxygen stress. Opercular ventilation rate reduced by the 24th hour. This is an indication that the fingerlings were gradually assuming a new homeostatic balance to a polluted environment, before death occurred. A similar explanation may probably be made for the increase in tail movement rate observed by the 12th hour and reduction by the 24th hour. Ogueji *et al*., (2013) reported that surviving fish were maximally intoxicated at this period due to maximum bioconcentration and bioaccumulation of toxicants. Also, there was marked increase in opercular ventilation and tail fin beats per minute. This may be because the exposed fish needed more oxygen for increased metabolic rate, especially within 12 hours 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.

There was reduction in opercular and tail fin beats at the 24th hour. This suggests a 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 the fish often hit their tails against the edges of the holding medium, resulting in loss of some portions of their tail fins and haemorrhage, might also be a contributing factor to the toxicity.Air

gulping was lower in the control fishes in comparison with exposed fishes at higher concentrations (2900mg/kg and 5000mg/kg) at the phase-2 of the mixture of glyphosate/2, 4-D. 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 hours 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 significantly reduced after 24 hours, 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 hours of exposure to lambda cyhalothrin.

Although the physicochemical parameters of water fluctuated slightly during the bioassay, this fluctuation was not enough to have caused the mortality. Death of test fish exposed to the mixture of glyphosate and 2, 4-D herbicides at higher concentrations may be attributed to the destruction of such organs as the gills, liver, kidney, brain, blood system and the pancreas. Annune *et al*., (1994) also reported that gill tissues are the most sensitive to water pollutants, since gills are the primary sites for osmoregulation and respiration. They are highly vulnerable to lesions due to their immediate contact with aquatic pollutants. While some pollutants enter the body, there is evidence that some of them exert their effects on the external surface of the fish especially the gills. The very high toxicity of the herbicides (mixture of glyphosate and 2, 4-D) could probably be attributed to some possible synergistic effects likely to be produced by the active ingredients in the herbicides, of which is the compound that exist separately as herbicide and likely to be equally toxic to fish. The herbicide mixture is acutely toxic to catfish (*Clarias gariepinus)*.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Glyphosate and 2, 4-Dichlorophenoxyacetic acid were found to be toxic to brood stocks of *Clariasgariepinus*as these herbicides inhibited hatchability to some extent and also affected the development of the larvae.The LD_{50} of the herbicides to fingerlings of *C. gariepinus* was determined, although the herbicides (glyphosate and 2, 4-D) did not affect the catfish fingerlings even at higher doses but when they were mixed, there were deleterious effects, especially at higher doses. So, the mixture of glyphosate and 2, 4-D was found to have an LD_{50} less than 2,900mg/kg, and so was more toxic.The results of the biochemical parameters assayed showed that glyphosate and the mixture of glyphosate/2, 4-D were more toxic to fish, with more effects when mixed. Histopathological observation further revealed that acute concentrations of these herbicidesdamaged the skin, testes and eggs of catfish, with the greatest damage being from their mixtures. This could be the reason for the number of unhatched eggs and low survivability of the fries after two weeks, observed in the treatments. Hence, the use of glyphosate and 2, 4-D herbicides in agricultural farms should be monitored to avoid continuous leaching into water bodies.

5.2Recommendations

1. Further studies are required to investigate the immune-toxicological mechanisms of action of these herbicides in *C. gariepinus* and other fish species.
2. 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.
3. Bio-monitoring technology should be used to predict the effects of new chemical substances likely to reach aquatic ecosystems. In addition, the use of bio-monitoring tools to predict the future of ecological effects of chemical substances is necessary to prevent these substances from reaching hazardous concentrations in the ecosystem.
4. The DNA repair capacities of fish (particularly spermatozoa), should be investigated to assess the effects of these herbicides on reproduction.
5. Further research should focus on the prospects of microbial degradation and phytoremediation of these herbicides.
6. 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 into.
7. Finally, further research should also be geared towards finding out the genotoxic effects of these herbicides on the organs of catfish brood stocks.

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APPENDIX A

Table: Biochemical profiling of fish liver for both treatments and control.

Gly.&2, 4-D	2, 4-D	Glyphosate	Control	Parameter
0.049	0.051	0.032	0.053	**Total protein O.D**
23.66621	24.63218	15.45548	25.59815	**Total protein (g/l)**
0.014	0.023	0.015	0.022	**GSH O.D**
1.056358	1.667389	1.733088	1.534709	**GSH (mg/g protein)**
0.137	0.138	0.172	0.206	**SOD O.D**
6.29E-05	6.03E-05	8.79E-05	4.81E-05	**SOD (IU/mg protein)**
0.082	0.107	0.09	0.095	**MDA O.D**
0.540517	0.67765	0.908415	0.578948	**MDA (Nmol/mg protein)**
0.564	0.56	0.38	0.563	**GST O.D 0min**
0.587	0.586	0.386	0.58	**GST O.D 1min**
0.604	0.597	0.394	0.591	**GST O.D 2mins**
0.616	0.605	0.409	0.598	**GST O.D 3mins**
6.88E-06	5.72E-06	5.88E-06	4.28E-06	**GST (µmol GSH-CDNB/min/mg**
0.026	0.014	0.016	0.025	**GPx O.D**
4.626335	2.491103	2.846975	4.448399	**GPx (Mg GSH/min/mg protein)**
0.068	0.077	0.067	0.071	**CAT O.D**
1.82E-05	1.98E-05	2.74E-05	1.75E-05	**CAT (µm H_2O_2/min/mg protein**

Appendix B

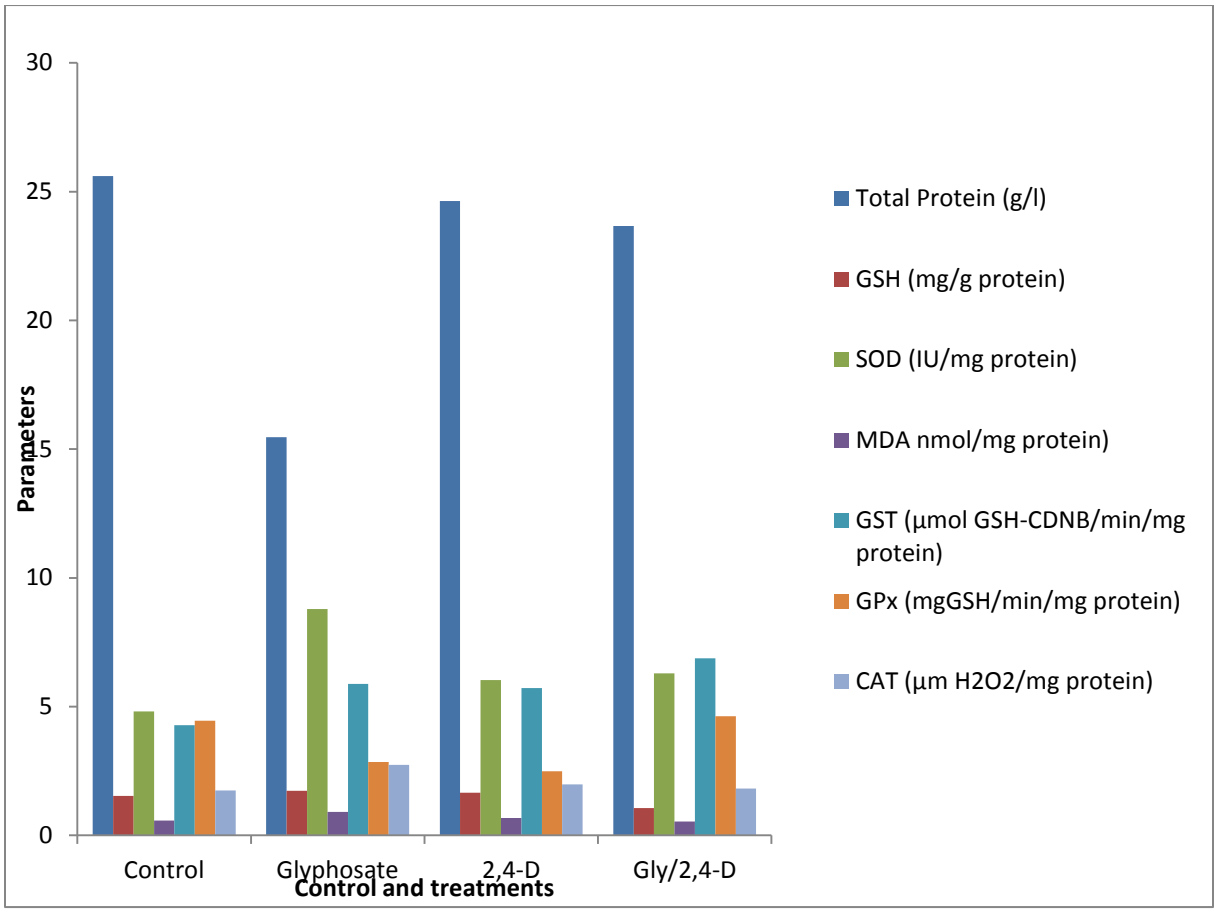

Figure B: Biochemical profiling of fish liver for both treatments and control.

Appendix C

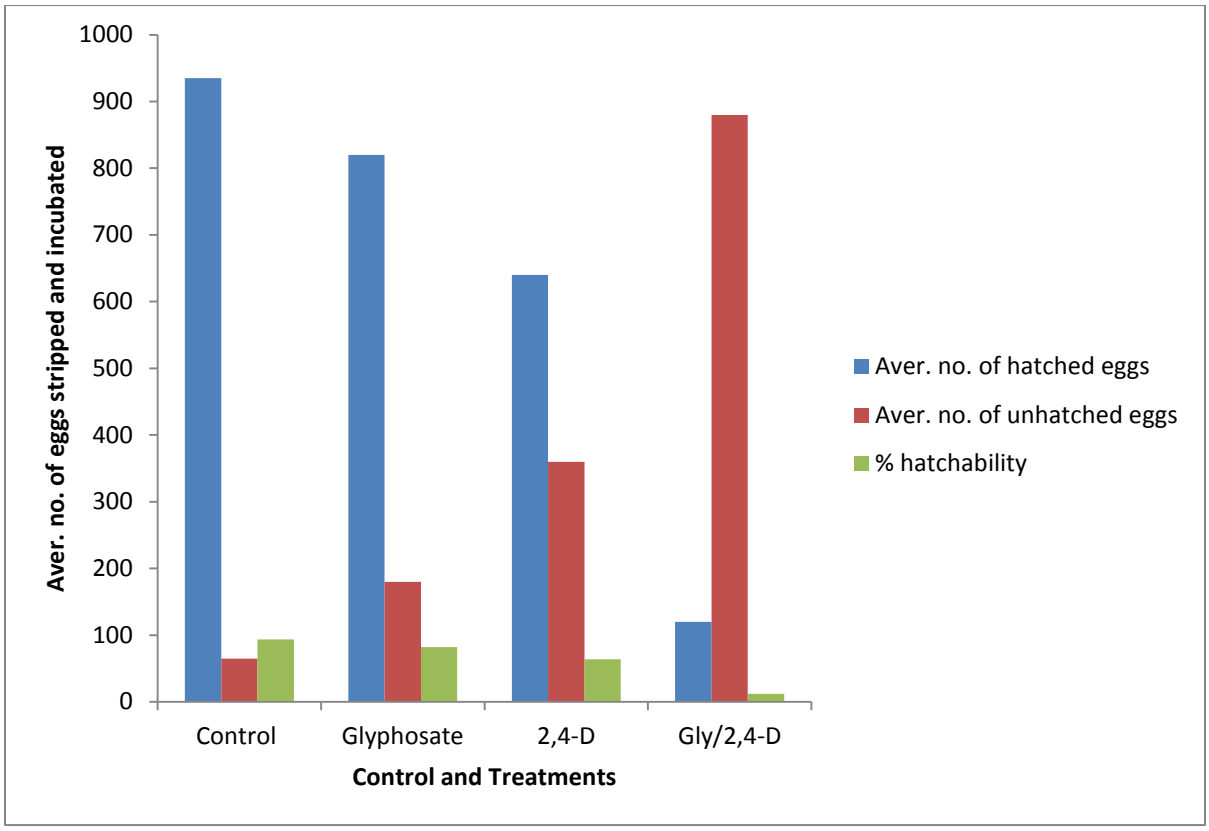

Figure C: Percentage (%) hatchability of incubated eggs.

Appendix D

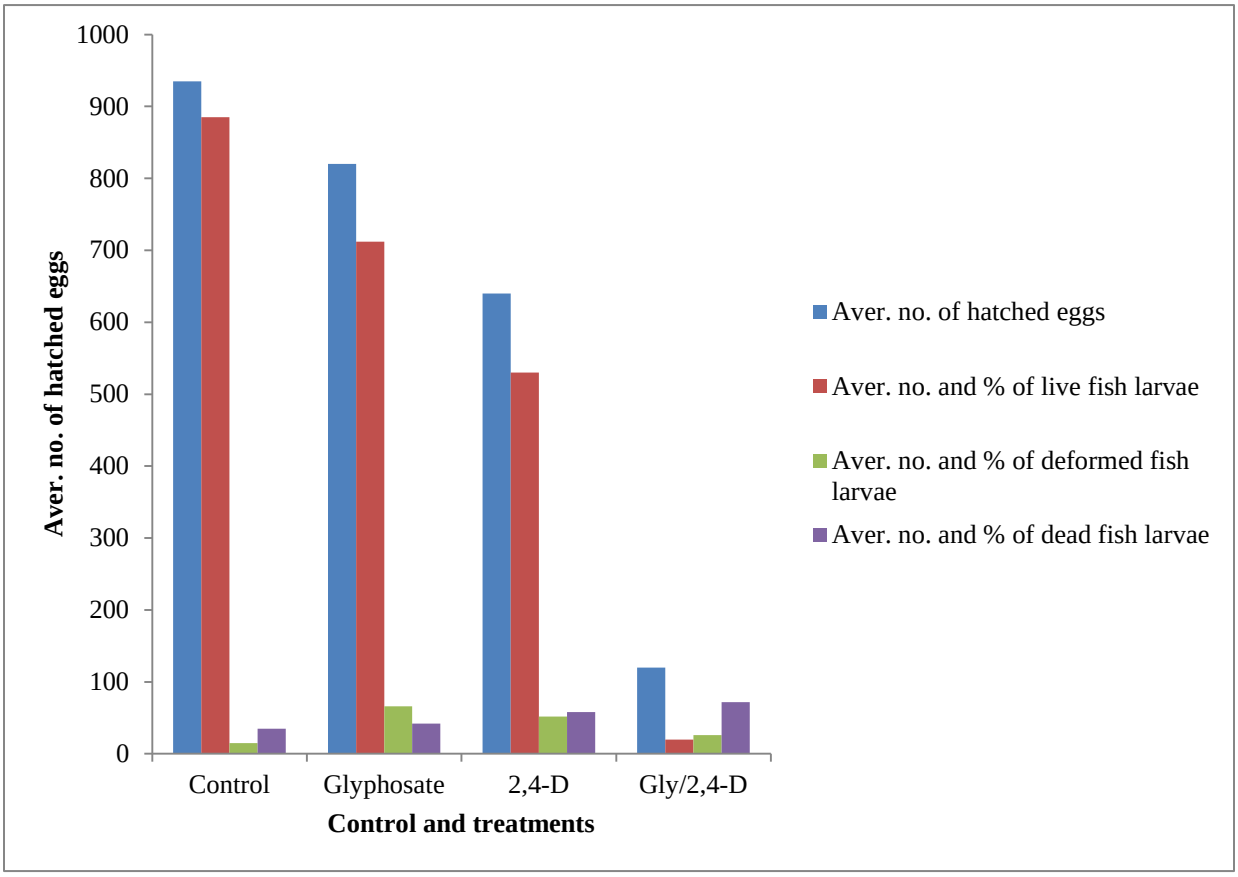

Figure D: Average number and % mortality and survivability of fish fry after fourteen days (2 wks) of hatching.

Appendix E

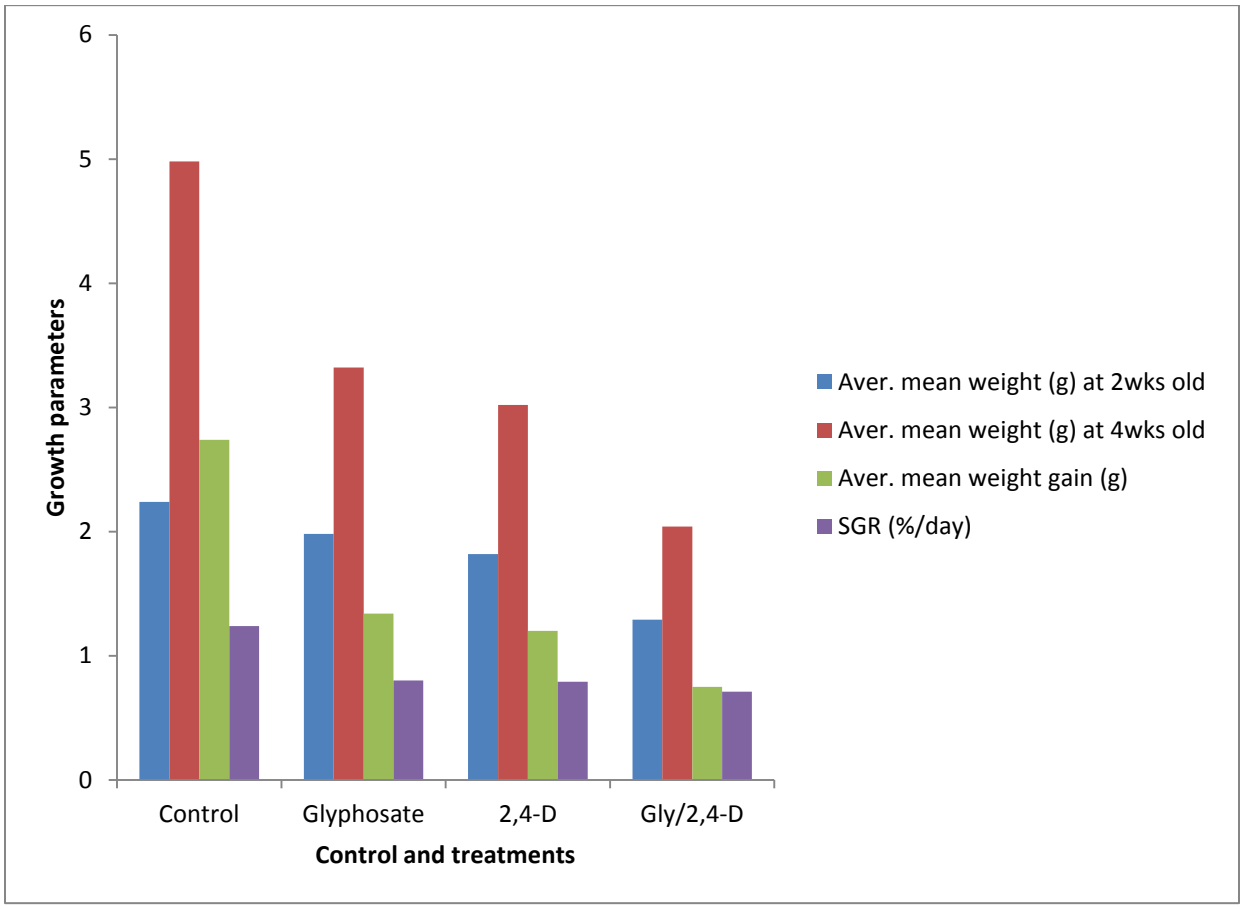

Figure E: Growth response of *Clarias gariepinus* larvae in two weeks and four weeks after hatching.

Appendix F

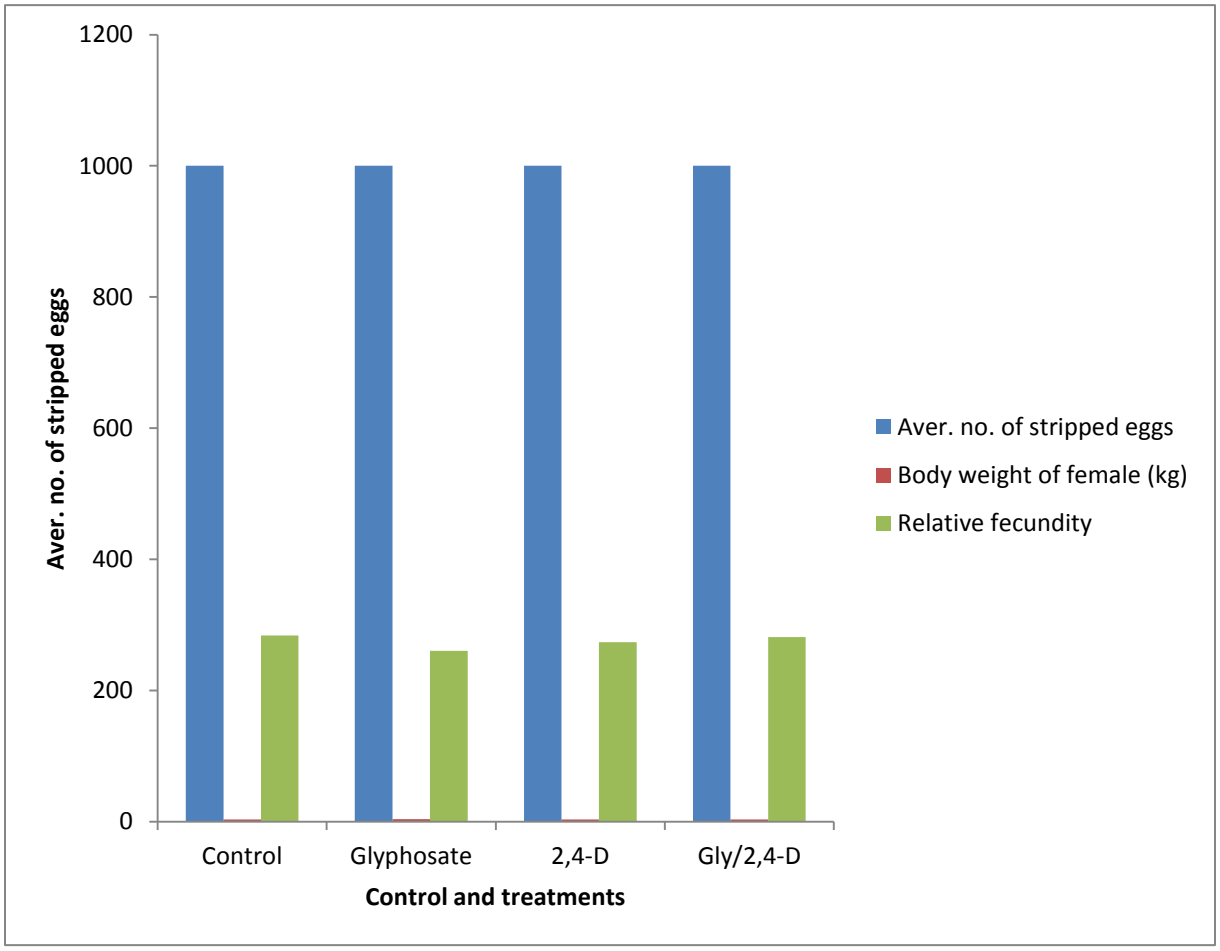

Figure F: Relative fecundity of both treatments and control of the female brood stocks of *Clarias gariepinus.*

Appendix G

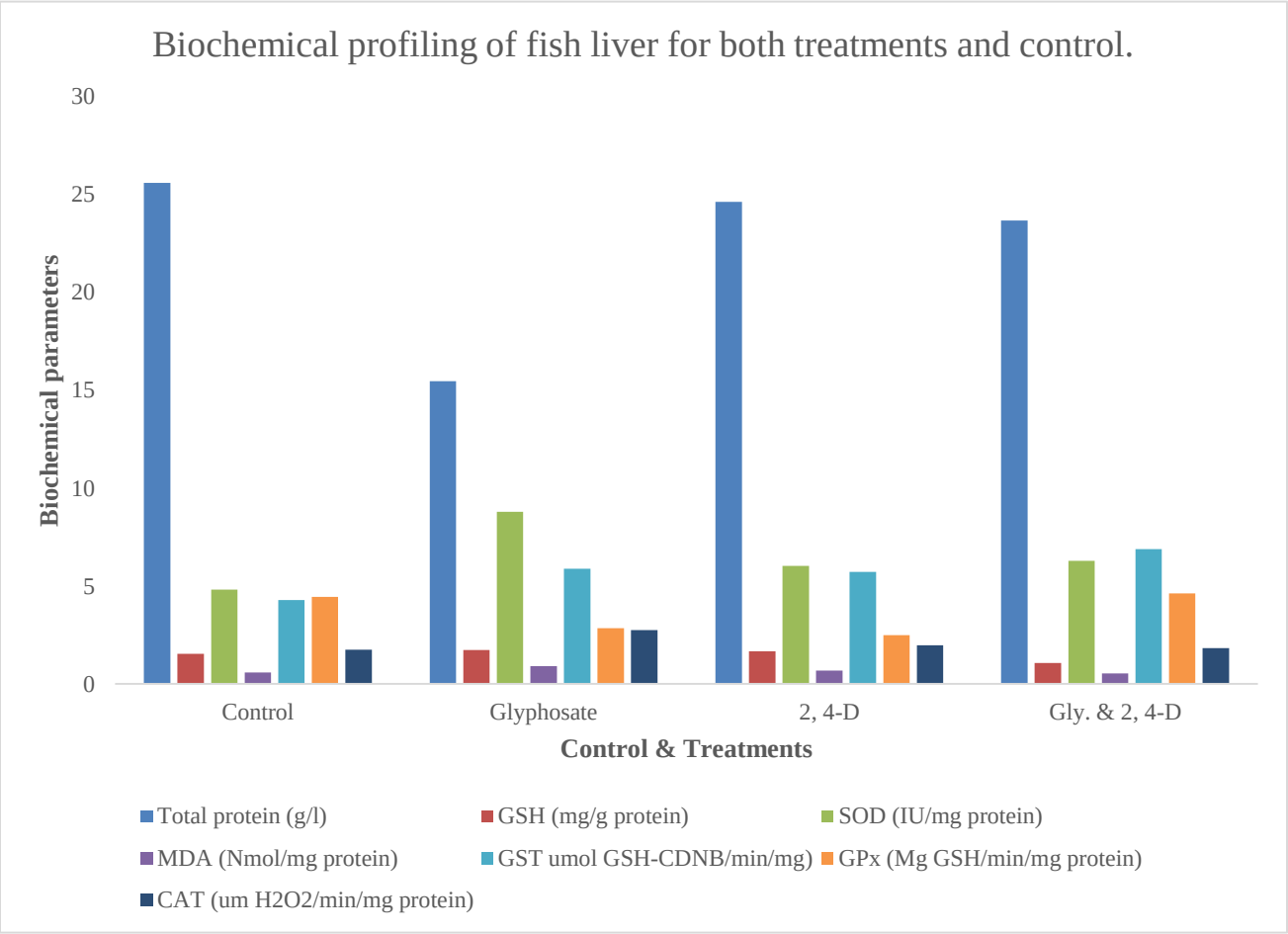

Figure G: Biochemical profiling of fish liver for both treatments and control.

Appendix H

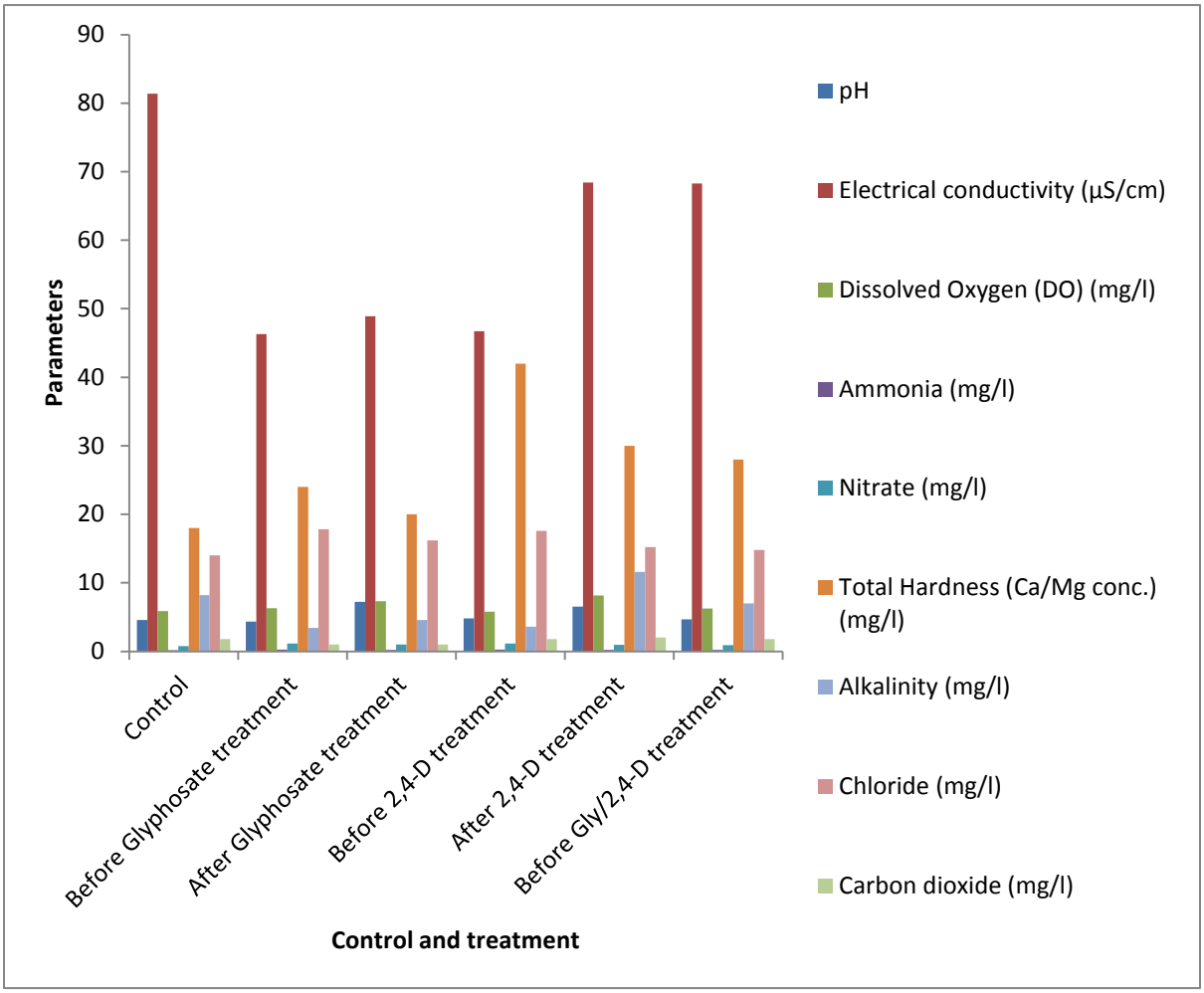

Figure H: Physico-chemical parameters of the treatment water and control before (Day 1) and towards the end (Day 3) of the exposure.