EFFECT OF SUB LETHAL CONCENTRATION OF GLYPHOSATE BASED HERBICIDE ON SOME BIOCHEMICAL PARAMETERS OF MALE *Clarias gariepinus*

BY

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CERTIFICATION

We hereby certify that this thesis "**Effect of sub lethal concentration of glyphosate on some biochemical parameters of male *Clarias gariepinus*.**" Was carried out by EZEMA JOSHUA CHIBUEZE (20144912608) and accepted in partial fulfillment for the award of Masters (M.Sc) degree in Industrial and Toxicological Biochemistry in the Department of Biochemistry, Federal University of Technology, Owerri.

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DEDICATION

Dedicated to God almighty the source of knowledge and to my parent Pastor & Deac Emmanuel O. Ezema.

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ABSTRACT

The effect of sublethal concentration of glyphosate on some biochemical parameters of male *Clarias gariepinus* was investigated. Healthy matured (4 months) male *Clarias gariepinus* were used as test and control organism. The effect on male reproductive hormone (testosterone), thyroid hormone, oxidative stress biomarkers, liver function parameters and haematological parameters were determined. Results obtained revealed that testosterone concentration in exposed groups was significantly ($p<0.05$) lower than that of *Clarias gariepinus* in control group. Thyroid stimulating hormone (TSH) and thyroxin (T4) concentration in exposed groups were significantly ($p<0.05$) higher than that of the control group where as triiodothyronine (T3) concentration in exposed groups were significantly ($p<0.05$) lower than that of the control group. Malondialdehyde (MDA) concentration of the liver, kidney and gill of exposed fish were significantly ($p<0.05$) higher than the control group. Glutathione peroxidase (GPx) and superoxide dismutase (SOD) activity in exposed groups were significantly ($p<0.05$) lower than that of the control group while catalase (CAT) activity was significantly ($p<0.05$) higher in exposed fish. Lactate dehydrogenase (LDH) activity of the gill of exposed fish was significantly ($p<0.05$) higher than that of the control group. The exposure of *Clarias gariepinus* to glyphosate also caused a significant increase in the activity of alkaline phosphatase (ALP), alanine amino transferase (ALT), aspartate amino transferase (AST) and serum bilirubin. The haematological response of *Clarias gariepinus* exposed to the concentration of glyphosate showed a significant increase in white blood cell count (WBC) and a reduction of erythrocyte count (RBC), haemoglobin (HGB) level, platelet (PLT) and procalcitonin (PCT) concentration of the blood. No significant difference ($p<0.05$) was observed on LY, MID, GRAM, MCV, HCT, MCH, MCHC, RDWSD, RDWCV, MPV and PDW. This study shows that glyphosate may induce hormonal changes, oxidative stress, hepatoxicity and haematological changes in *Clarias gariepinus* and therefore should not be used indiscriminately.

Keywords: *Clarias gariepinus*, glyphosate, endocrine disruption, oxidative stress, biomarkers.

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

In the last few decades colossal human population, increase in urbanization, industrialization and technology have resulted to a higher rate of pollution of the environment. Agriculture, industries and domestic activities are the major sources of these hazardous pollutants (Negga, Stuart, Machen, Salva, Lizek, Richardson, Osborne, Mirallas, McVey and Fitsanakis, 2012). These pollutants include domestic waste, untreated waste released from industries and even the pesticides used in the Agricultural sector to enhance the production of food (Cattani,de Liz Oliveira, Cavalli, Heinz Rieg, Domingues, Dal-Cim and Tasca, 2014).

Gross increase in Agricultural activities has resulted to an increase in the use of agrochemicals and pesticides to eradicate pest and enable increased yield of crops and other agricultural products. These pesticides are likely to be washed into water bodies, thereby affecting aquatic organisms (Mohammad, Ahmad, Masoud and Aliakbar, 2018).

Glyphosate is one of the widely used herbicide which is considered to be persistent and mobile in soil and water. It is known to be one of the most common terrestrial and aquatic contaminants which portray a tendency of resulting to endocrine disruption (Negga *et al.,* 2012).

Endocrine disruptors are substances that interfere with the synthesis, secretion, transport, binding or elimination of natural hormones in the body. Hormones are responsible for the maintenance of homeostasis, reproduction, development and or behavior. Many endocrine disruptors are thought to mimic hormones (Chang, Simcik and Capel, 2011). They have chemical properties similar to

hormones and that allows their binding to hormone specific receptors on the cells of target organs. Endocrine disrupting pesticides or their residues can interfere with production, transport, metabolism, reception and alteration of hormones which regulates the homeostasis of the organism.The endocrine system is also involved in the regulation of the sexual and reproduction processes (Reyes, Giuseppe, Galindo, Del Carmen and Foday, 2014).

Endocrine disruptors include dioxins, polychlorinated biphenyls (PCB), heavy metals, phenols, furan and aromatic hydrocarbon (Reyes *et al*., 2014). Health effects attributed to endocrine disrupting compounds include a range of reproductive problems (reduced fertility, reproductive tract abnormalities), changes in hormone levels, early puberty, brain and behavior problems, impaired immune functions and various cancers (Chang *et al.*, 2011). Endocrine disruptors interfere with endocrine function and thyroid hormone production. Environmental exposures, specifically to pesticides, should be considered potential risk factors for the thyroid hormone. Evidence have shown that they are harmful to thyroid synthesis. Certain insecticides, herbicides, and fungicides have been previously reported to be endocrine disruptors and, more specifically, thyroid disruptors acting through diverse mechanisms (Koller, Fürhacker, Nersesyan, Mišík, Eisenbauer, and Knasmueller, 2012). Some of these mechanisms include inhibition of thyroidal iodine uptake, interference at the thyroid hormone receptor, binding to transport proteins, interference with iodothyronine deiodinases, increased clearance of thyroid hormones, interference with cellular uptake of thyroid hormones, and interference with thyroid hormone gene expression (Koller *et al.*,2012).

Hormones travel throughout the body and act as chemical messengers. They interface with cells that contain matching receptors in or on their surfaces by binding to the receptors. Hormones are secreted by the endocrine system in response to environmental stimuli and to orchestrate

development and reproductive changes. The adjustments brought on by the endocrine system are biochemical, involving changing the cell chemistry to bring about a long term change in the body. These systems work together to maintain the proper functioning of the body through its entire life cycle and a change of the endocrine system through the activities of toxicants will affect the release of hormone (Klassen and Hood, 2001).

Environmental pollutants such as pesticides induce oxidative stress in aquatic organisms when exposed, by frequently activating the endogenous production of reactive oxygen species (Tsui and Chu, 2004). These reactive oxygen species are continuously generated by the activities of these pesticides and eliminated by the activity of antioxidants within the organism. Reactive oxygen species cause oxidative stress, a situation when steady-state reactive oxygen species concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation and damaging cellular constituents (Ujowundu, Nwaoguikpe, Okwu, and Ene, 2014). Most of these reactive oxygen species are generated as side products of tissue respiration. Reactive oxygen species can initiate cellular tissue damage by modifying lipids, proteins and DNA, thereby resulting to a change in oxidative stress biomarkers, which can seriously compromise cell health. These reactive oxygen species are mainly produced in the mitochondria. In fish tissues like kidney, liver, and gills which tends to be the first tissue in contact with the xenobiotic are very necessary in detecting the effect of reactive oxygen species released by the xenobiotic (Ujowundu *et al.*, 2014). The integrity of the liver could be affected by the activities of glyphosate and this could be observed by assaying for the activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate transaminase (AST), gamma-glutamyl transpeptidase (GGT). Also determining the changes in prothrombin time (also expressed as

international normalized ratio (INR)), serum bilirubin, urine bilirubin, albumin and alpha-1 antitrypsin are important in determining the integrity of the liver (Kier and Kirkland, 2013).

Fish haematology is increasingly gaining importance as a means of determining the health status of fish (Antoniou, Habib, Howard, Jecmings, Leifert, Nodari, Robinson and Fagan, 2012). Environmental and physiological factors are known to influence fish haematology and these include stress due to capturing, transportation, sampling, age and sex. The knowledge of haematological characteristics of fish is important in toxicological studies (Aderolu, Ayoola, and Otitoloju, 2010). The exposure of fish to several types of chemical agents may induce changes in several haematological variables, which are frequently used to assess fish health (Martinez and Souza, 2002; Aderolu *et al.,* 2010). The study of blood parameters in fish have been widely used for the detection of physiopathological alterations in different conditions of stress (Antoniou *et al*., 2012). Haematological parameters such as heamatocrit, haemoglobin, red blood cell count and white blood cells count can be used as indicators of toxicity with a wide potential for application in environmental monitoring and toxicity studies in aquatic animals (Adeyemo, 2005). Knowledge of the haematological characteristics is an important tool that can serve as an effective and sensitive index to monitor physiological and pathological changes in fishes cultured in glyphosate polluted water (Aderolu *et al.,* 2010).

1.2 Statement of Problem

The presence of toxic chemicals in both aquatic and terrestrial ecosystems have become an important issue globally. Daily usage of pesticides and other agrochemical has turned out a threat to the survival of some organisms in the ecosystem including fish. Growing research based

evidence shows that pesticides, metals and many industrial chemicals interfere with the health and normal functioning of the endocrine systems of a wide range of organisms. Glyphosate a commonly used pesticide and other agrochemicals are not only hormone disruptors but also induce oxidative stress in organism exposed to them. Aquatic organisms exposed to these xenobiotics often accumulate them in their tissues causing a general reduction in their health status which manifest in the deterioration of haematological indices and organ integrity. When these aquatic organisms, such as fish, are ingested by humans as food, these xenobiotics and their attendant health consequences are also transferred to humans.

1.3 Justification of Study

Due the heavy use of glyphosate as a strong herbicide and its risks to farmers, other organisms and the environment, the present study will investigate and create awareness on possible hazards to humans who depend on African catfish as one of the main sources of protein especially when it is commercially produced in bodies of water which could be polluted by glyphosate in order to provide information to users of this herbicide about its proper user guide and safety issues. The result of this study on *Clarias gariepinus* will not only describe the effect of pesticides to the environment but also as a model of what may happen to human exposed to these chemicals.

1.4 Aim

The aim of this study was to determine the effect of sub-lethal concentration of glyphosate on some biochemical parameters of male African cat fish (*Clarias gariepinus)*

1.5 Specific objectives of the study

The specific objectives of this study were:

- To determine the effect of glyphosate on the male reproductive hormone (testosterone) of *Clarias gariepinus.*
- To determine the effect of glyphosate on thyroid hormones; thyroid stimulating hormone (TSH), thyroxine (T4) and triiodothyronine (T3) of *Clarias gariepinus.*
- To determine the effect of glyphosate on lipid peroxidation and the antioxidant enzymes glutathione peroxidase, superoxide dismutase and catalase in the kidney, liver and gill of *Clarias gariepinus*.
- To determine the effect of glyphosate on the liver function parameters ALP, ALT, AST and bilirubin of *Clarias gariepinus.*
- To determine the effect of glyphosate on hematological parameters white blood cell count, lymphocytes, mid-range, gram, red blood cell count, hemoglobin, mean corpuscular volume, hematocrit, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, red blood cell distribution width standard deviation, red blood cell distribution width coefficient of varience, platelet, mean platelet volume, procalcitonin and platelet distribution width of *Clarias gariepinus.*

CHAPTER TWO

LITERATURE REVIEW

2.1 African catfish (*Clarias gariepinus*)

African sharptooth catfish (*Clarias gariepinus*) is a typical air-breathing catfish with a scale-less, bony elongated body with long dorsal and anal fins, and a helmet like head (Figure 2.1). The catfish genus can be defined as displaying an eel shape, having an elongated cylindrical body with dorsal and anal fins being extremely long (nearly reaching or reaching the caudal fin) both fins containing only soft fin rays as seen in Figure 2.1 (FAO, 2000) . The male and females of *Clarias gariepinus* can be easily recognized as the male has a distinct sexual papilla, located just behind the anus. This sexual papilla is absent in females (Chen *et al.,* 2013; Mohammad *et al.,* 2018).

Colour varies dorsally from dark to light brown and is often mottled with shades of olive and grey while the underside is a pale cream to white (Chen, Cao, Jiang, and Zhu, 2013). It can grow very large with a maximum reported length of 170 cm and weight of 60 kg (Chen *et al.,* 2013; Chaufan *et al.,* 2014).

Clarias gariepinus is widely considered to be one of the most important tropical catfish species for aquaculture, has a Pan-African distribution, from Nile to West Africa and from Algeria to South Africa (Antoniou *et al.,* 2012; Chaufan *et al.,* 2014). The African catfish has a high growth rate; it is very resistant to handling stress and is very well appreciated in a wide number of African countries including Nigeria. It is commonly used as an experimental fish since it possesses necessary breathing organs, which enables them to tolerate adverse aquatic and environmental conditions where other cultivate fish species cannot survive, and it is hardy and

does not easily succumb to death (Chen *et al.,* 2013). It inhabits calm waters such as lakes, streams, rivers, swamps and floodplains, some of which are subject to seasonal drying. It is very tolerant to muddy waters and conditions of low dissolved oxygen. They also occur in Minor-Asia (Israel, Syria and South of Turkey) (Chen *et al.,* 2013; Mohammad *et al.,* 2018).

2.2 Mode of Feeding

Clarias gariepinus is considered to be omnivorous, displaying both scavenging and predatory behavior (Antoniou *et al.,* 2012). It is known to have an extremely varied diet, consuming fruits and seeds, all types of aquatic invertebrates and small vertebrates, small mammals and even plankton (Chaufan *et al.,* 2014). They prefer inactive foods, which they detect with there sensory barbells before securing with there array of very fine teeth prior to gulping (Antoniou *et al.,* 2012; Chaufan *et al.,* 2014).

Alternatively, it can be an efficient predator and even hunt in 'packs' where it may herd shoals of small fish against submerged aquatic vegetation before devouring them. Social hunting and coordinated pack-hunting, foraging behaviours and even feeding migrations have all been observed (Antoniou *et al.,* 2012).

2.3 African Catfish Tolerance

Clarias gariepinus can tolerate extremely harsh conditions (Dai, Hu, Tang, Li, and Li, 2016). It is also able to tolerate very low oxygen concentrations and even survive for considerable periods out of water, via the use of a specialized organ on the body (Antoniou *et al.,* 2012). The suprabranchial organ is a large paired chamber with branches above the gill arches specifically adapted for air breathing (Antoniou *et al.,* 2012). This organ allows it to move over land even when not forced to do so by drought (Dai *et al*., 2016). Water temperatures between 8 and 35°C,

salinities of 0 to 10‰ and a wide pH range are all tolerated by the African catfish. The ability of the fish to tolerate these extreme conditions allows it to survive even in moist sand or in borrows (which contain a very low amount of water) with an air-water interface (Dai *et al*., 2016).

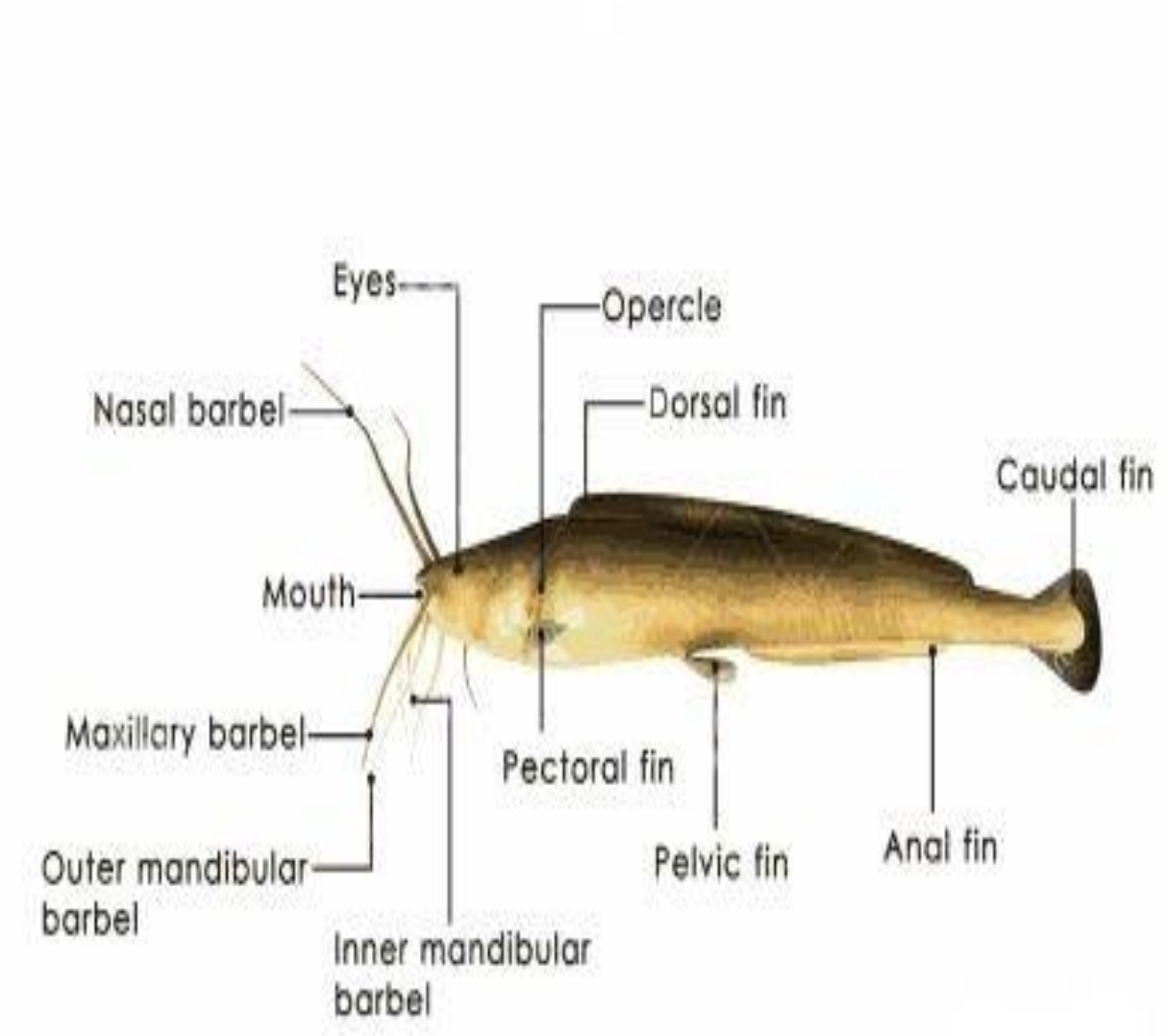


Figure 2.1 Lateral view of African Catfish *Clarias gariepinus (*Source*:* FAO 2013*).*

2.4 Pollution

Pollution is the introduction of harmful substances or products into the environment. It is also described as the introduction of substances naturally and artificially that makes a resource unfit

for a specific purpose (Dai *et al*., 2016). Environmental pollution agency defines pollution as the presence of a substance in the environment that because of its chemical composition or quantity prevents the functioning of natural processes and produces undesirable environmental and health effects (EPA, 2015). Under the Clean water act, for example, the term has been defined as the man-made or man-induced alteration of the physical, biological, chemical, and radiological integrity of water and other media (Ayanda, Olasehinde, and Ajayi, 2018).

The agents which cause environmental pollution are called pollutants (Antoniou *et al.*, 2012).A pollutant may be defined as a physical, chemical or biological substance unintentionally released into the environment which is directly or indirectly harmful to humans and other living organisms (EPA, 2015).

A toxicant is referred to as any chemical that can injure or kill humans, animals or plants. Toxin is usually used when referring to a toxic substance produced naturally. Toxicant is usually used when referring to a toxic substance that is produced by, or a by-product of, man-made activities (Ayanda *et al*., 2018).

2.5 Water Pollution

Water Pollution is described as physical, chemical, biological changes in water quality that adversely affect living organisms.Water pollution may also be defined as the contamination of streams, lakes, seas, underground water or oceans by the entrance of substances, which are harmful to living beings (EPA, 2015). If the concentration of substances naturally present in water increases then also the water is said to be polluted (Antoniou *et al.*, 2012).

Water pollutants are substances which are capable of causing any physical, chemical or biological change in the water body, resulting in undesirable effect on living organisms (EPA,

2015).Water used for domestic, agricultural and industrial purposes is discharged with some undesirable impurities in it, which may result to pollution. They include organic and inorganic pollutants (Nwani, Nagpure, Ravindra, Basdeo, Kushwahab and Lakra, 2013).

Inorganic pollutants are naturally found in the environment but due to human development these pollutants are often released. They include cadmium, copper, zinc, nitrogen, phosphorous, lead (Pb) in gasoline, radionuclides and other heavy metals released as a result of industrial activities. The additional phosphate in the environment grows excess algae resulting to oxygen depletion (EPA, 2015).

Organic Pollutants are generated from organic matters including easily degradable household waste, manure, sewage, emission from cars, pesticides and herbicides. Organic pollutants can increase water turbidity and cause eutrophication, with decrease in the quantity of dissolved oxygen. Some organic pollutants are highly biodegradable e.g carbamates, while some others are persistent e.g dioxins (EPA, 2015).

The sources of water pollution include the point sources and the non-point sources. Point source pollution is the type of pollution that can be easily tracked back to its source. They are the easiest to be identified and control (Ayanda *et al*., 2018). Point Source pollution is pollution caused from a stationary location or fixed facility where pollutants are discharged. Some point sources of water pollution include factories, sewage system, power plants, underground coalmines, oil wells e.t.c (EPA, 2015)

Non-point source pollution is the type of pollution that cannot be easily tracked back to its source. This type of pollution happens every day in all communities (EPA, 2015). Non-point source pollution is more frequent than point source pollution and is the type of pollution that is

responsible for harming most of the animals in the ocean. The pollutants are generally carried off the land by storm water (Ayanda *et al*., 2018). The term non-point source encompasses a large range of sources such as: when rain or snow moves through the ground and picks up pollutants as it moves towards a major body of water, the runoff of fertilizers from farm animals and crop land, air pollutants getting washed or deposited to earth, storm water drainage from lawns, parking lots, and streets (EPA, 2015).

2.6 Pesticides

Pesticides are toxic chemical agents (mainly organic compounds) that are deliberately released into the environment to combat crop pests and disease vectors. The need to produce a greater quantity and quality of food by pest control resulted in intensive use of pesticides over the last 50 years (Cattani, de Liz Oliveira, Heinz Rieg, Domingues, Dal-Cim, and Tasca, 2014). Pesticides are chemicals used to control agricultural pests and their correct application is very much required for effective and maximum crop production (Nwani *et al.,* 2013; Mohammad *et al.,* 2018).

Pesticide use is known to cause serious environmental problems, especially in the dry season, because during this period the dilution capacity of the water systems is low, thus increasing the risk of high concentrations of toxic chemicals. Moreover, the dry season is often the critical period for many animals, especially fish and birds. Fish stocks suffer from natural mortality and high fishing pressure at the end of the dry season. Contamination of water by pesticides either directly or indirectly can lead to fish deaths, reduced fish productivity or elevated concentrations of undesirable chemicals in edible fish tissue which can affect the health of humans eating these fish (Cattani *et al*., 2014).

2.7 Classificationof Pesticides

Pesticides can be classified based on their chemical structures and based on the target organism to mitigate.

2.7.1 Classification based on their chemical structure

2.7.1.1 Organochlorine

Organochlorines are a class of pesticides containing moieties of chlorine. They persist in the environment and in the bodies of humans and other animals long after their use. These pesticides include Dichlorodiphenyltrichloroethane (DDT), and Polychlorinated biphenyls (PCBs) among others (Nwani *et al.*, 2013; Cattani *et al*., 2014; Mohammad *et al.*, 2018).

Stable compounds are too persistent in the environment and tend to accumulate in fatty tissue (Waliszewski, Gómez-Arroyo, Infanzón, Villalobos-Pietrini and Maxwell, 2003). In humans these substances or their metabolites act primarily at the level of central nervous system altering the electrophysiological properties and enzymatic neuronal membranes. It causes alterations in the kinetics of the flow of Na^+ and K^+ through the membrane of the nerve cell, resulting in the spread of multiple action potentials for each stimulus, causing symptoms such as seizures and acute poisoning death from respiratory arrest (Greim, Saltmiras, Mostert, and Strupp, 2015). Figure 2.2 shows some examples of organochlorine pesticides which include lindane, dieldrin, methoxychlor, crichlorfon and chlorpropharm.

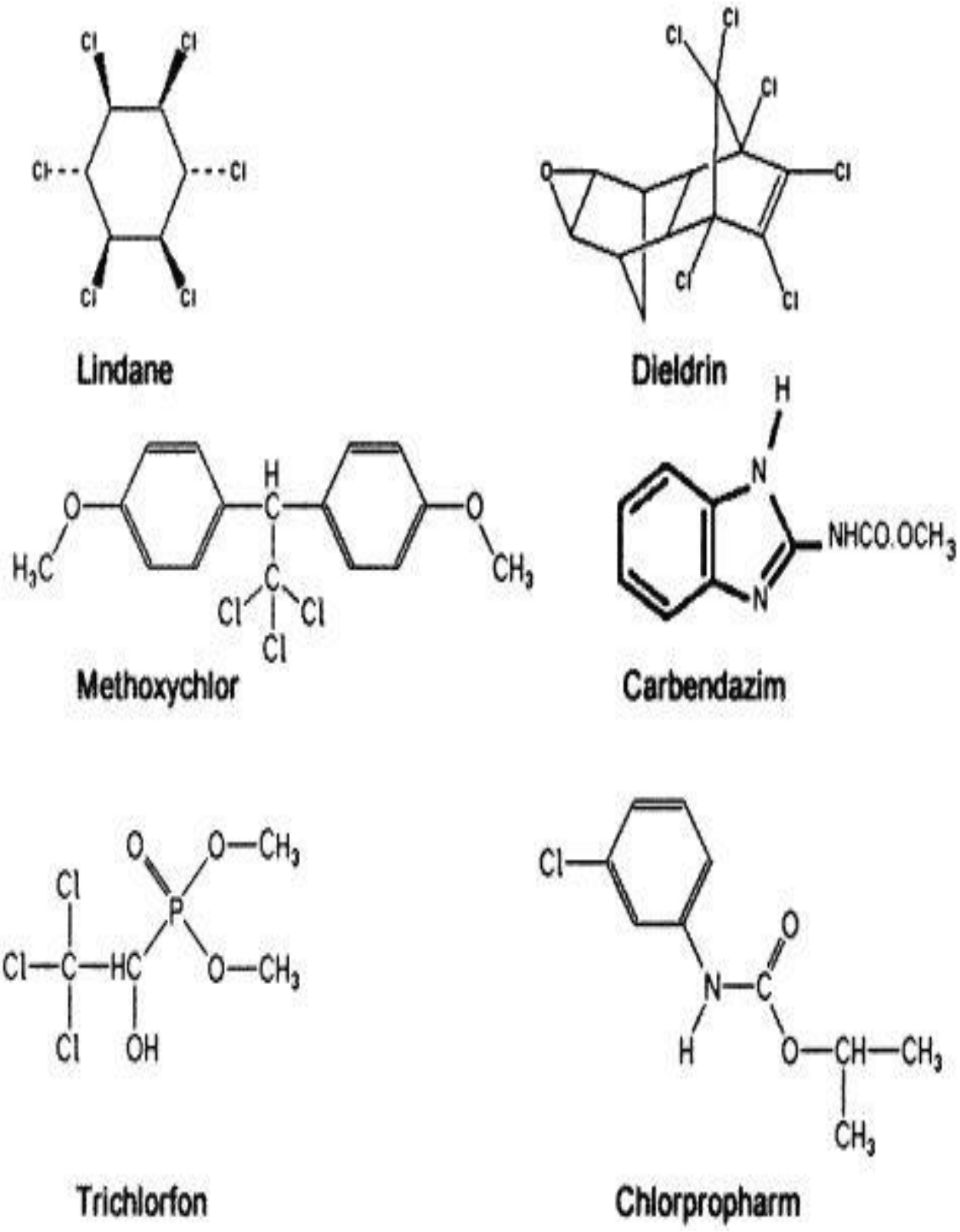


Figure 2.2 Oganochloride pesticides (Cattani *et al*., 2014).

2.9.1.2 Organophosphates

Organophosphates are esters of phosphoric acid (Figure 2.3) (Dai *et al*., 2016). Organophosphates act on the central nervous system by inhibiting acetyl cholinesterase, the enzyme that modulates the amount and levels of the neurotransmitter acetylcholine. Organophosphates disrupt the nerve impulse by serine phosphorylation of the hydroxyl group in the active site of the enzyme acetyl cholinesterase (Dai *et al*., 2016). Most organophosphates are insecticides and some are very poisonous. However, organophosphates are usually not persistent in the environment.

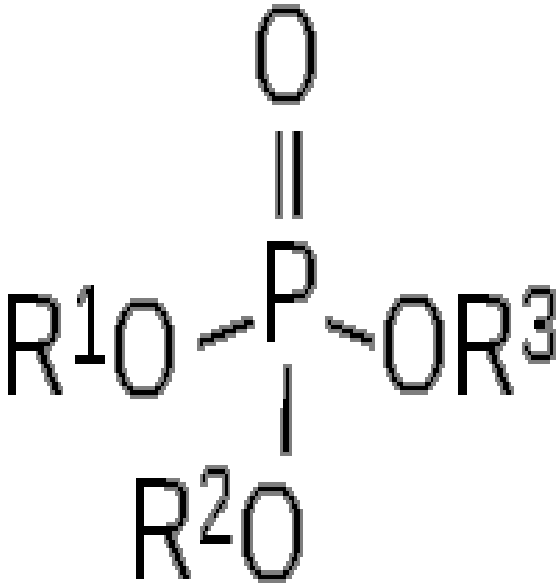


Figure 2.3 General chemical structure of an organophosphate (Dai *et al*., 2016).

An organophosphate (sometimes abbreviated OP) or phosphate ester is the general name for esters of phosphoric acid. Many of the most important biochemical compounds are organophosphates. They include DNA and RNA, as well as many of the cofactors essential for

life. Organophosphates are the basis of many insecticides, herbicides, and nerve agents. Organophosphorus compounds commonly used in agriculture are insecticides and miticides, and target organisms exposed to organophosphates are affected when they ingest and contact it (Dai *et al*., 2016).

2.7.1.3 Carbamates

A carbamate is an organic compound derived from carbamic acid (NH_2COOH). The chemical structure is as shown in Figure 2.4. .A carbamate group, carbamate ester and carbamic acids are functional groups that are interrelated structurally and often are interconverted chemically. Carbamate esters are also called urethanes. Dimethyl N-methyl carbamic acids are used as insecticides, herbicides, fungicides and nematicides (Gholami-Seyedkolaei, Mirvaghefi, Farahmand and Kosari, 2013).

Figure 2.5 shows some examples of carbamates which include methomyl, fenobucard and carbendazim (Gholami-Seyedkolaei *et al*., 2013; Greim *et al.,* 2015). Carbamates are less persistent than organochlorines and organophosphates, and likewise the latter inhibit acetyl cholinesterase. However, in the case of carbamates action is fast and the kinetics of blocking is through the carbamylation of the enzyme by the covalent attachment of the electrophilic group at the steric carbamoyl sites of the enzyme. Carbamate pesticides affect the nervous system by disrupting acetylcholine regulation by acetyl cholinesterase. The enzyme effects are usually reversible (Gholami-Seyedkolaei *et al*., 2013; Greim *et al.,* 2015).

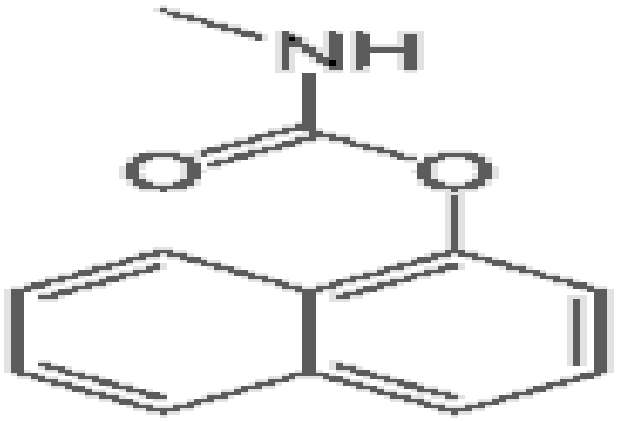
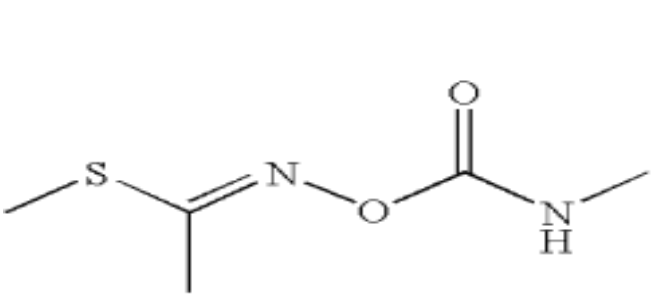
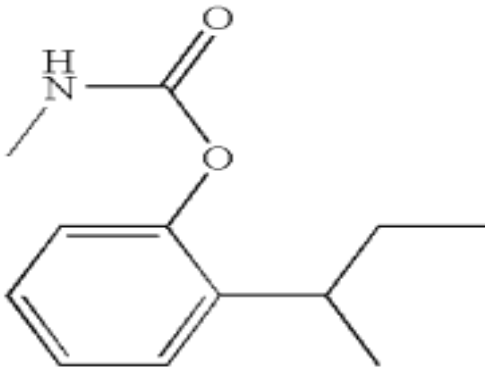


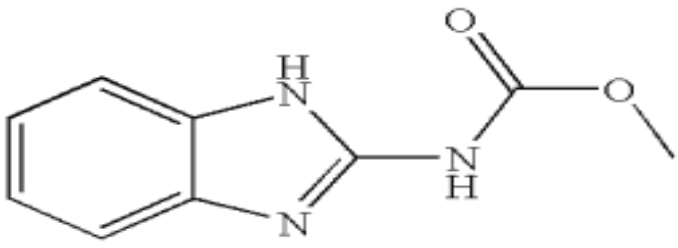
Figure 2.4 Chemical structure of carbamate showing the functional group (Greim *et al.,* 2015).



Methomyl



Fenobucard



Carbendazim

Figure 2.5 Examples of carbamate (Gholami-Seyedkolaei *et al*., 2013).

2.7.1.4 Pyrethroids

Pyrethroid pesticides were developed as a synthetic version of the naturally occurring pesticide pyrethrin, which is found in chrysanthemum. They have been modified to increase their stability in the environment. Some synthetic pyrethroids are toxic to the nervous system (Gholami-Seyedkolaei *et al*., 2013).They originate from natural insecticide derived from pyrethrum extract derived from chrysanthemum flowers, known as pyrethrins. Subsequently pyrethroids were obtained synthetically and are presently about 100 different commercial products have been manufactured. The examples of pyrethroids are shown in Figure 2.6 (Gholami-Seyedkolaei *et al*., 2013). They act on the central nervous system and produce changes in the dynamics of the Na^+ channels in the membrane of the nerve cell, thereby causing it to increase its opening time and prolong sodium current across the membrane in both insects and vertebrates. These events can lead to neuronal hyperexcitation (Greim *et al.*, 2015).

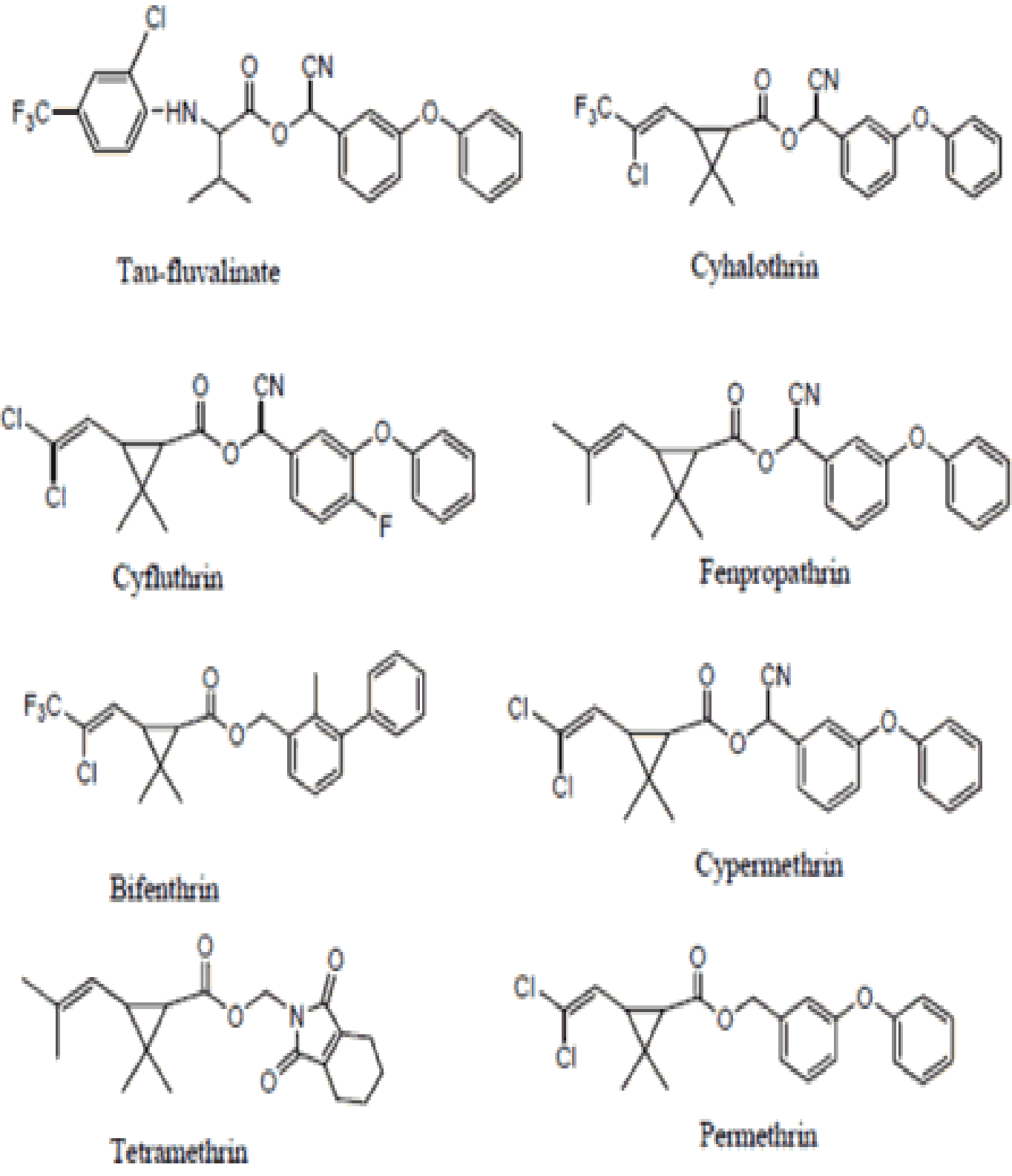


Figure 2.6: Examples of Pyrethroids (Greim *et al.*, 2015).

Other available pesticides include triazine herbicides, ureic, hormonal, amides, nitro compounds, benzimidazoles, bipyridyl compounds, ethylene dibromide, sulfur containing compounds, copper or mercury, among others.

2.8 Classification of Pesticides Based on Type of Pest it Controls

Fungicide: Fungicides are biocidal chemical compounds or biological organisms used to kill fungi (including blights, mildews, and molds) or inhibit fungal growth (Greim *et al.,* 2015).

Insecticides: are pesticides that are formulated to kill, harm, repel or mitigate one or more species of insect. Insecticides work in different ways. Some insecticides disrupt the nervous system, whereas others may damage their exoskeletons, some others repel them or control them by some other means. They can also be packaged in various forms including sprays, dusts, gels, and baits (Greim *et al.,* 2015).

Miticides: mitigate mites that feed on plants and animals

Molluscicides: kill snails and slugs

Nematicides: control nematodes (microscopic, worm-like organisms that feed on plant roots)

Ovicides: kill eggs of insects and mites

Algicides: control algae in lakes, canals, swimming pools, water tanks, and other sites (Greim *et al.,* 2015).

Herbicides: Herbicides kill weeds and other plants that grow where they are not wanted. Herbicides, also commonly known as weed killers, are chemical substances used to control unwanted plants. Selective herbicides control specific weed species, while leaving the desired

crop relatively unharmed, while non-selective herbicides can be used to clear waste ground, industrial and construction sites, railways and railway embankments as they kill all plant material with which they come into contact (Greim *et al.*, 2015). Herbicides are actively used in terrestrial and aquatic ecosystems to control unwanted weeds, and their use has generated serious concerns about their potential adverse effects on the environment and human health (Greim *et al.*, 2015). Glyphosate is a typical example of herbicides (Okomoda and Ataguba, 2011).

2.9 Glyphosate

Glyphosate is a broad-spectrum non-selective herbicide used for the control of the growth and spread of unwanted weeds and grasses in agricultural, industrial, urban, forestry and aquatic environment. It is widely used for the control of annual, perennial, brush and woody weeds because it is are highly efficacious, cost effective, relatively non-toxic, and degrade readily in the environment (Okomoda and Ataguba, 2011).

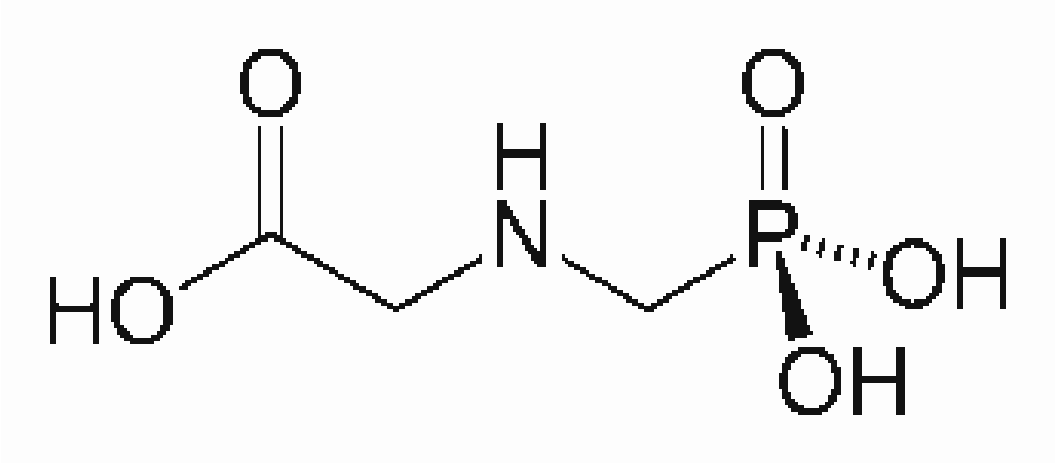


Figure 2.7 structure of glyphosate (Okomoda and Ataguba, 2011).

Glyphosate herbicide is one of the most frequently applied pesticides in agriculture for the control of a great variety of annual, biennial and perennial grasses, sedges, broad leaved weeds

and woody shrubs (Nwani *et al.,* 2013). It is also used for aquatic weed control in fish ponds, lakes, canals and slow running water (Okomoda and Ataguba, 2011). It is widely used in the rural communities and it is perhaps the most commonly used important herbicide ever developed (Genazzani, Stomati, Morittu, Bernardi, Monteleone, Casarosa, Gallo, Salvestroni and Luisi, 2000). Glyphosate is moderately to very slightly toxic to aquatic animals and shows high water solubility (EPA, 2015). The half-life of glyphosate range from 7 to 14 days and has low vapor pressure which suggest that loss to the atmosphere from treated surfaces will be small (Genazzani, Stomati, Morittu, Bernardi, Monteleone, Casarosa, Gallo, Salvestroni and Luisi, 2000).

It is absorbed by plant foliage and green stems and moves through the plant from the point of contact to and into the root system.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 (Genazzani *et al.,* 2000; Williams *et al.,* 2000). It controls weeds by inhibiting the synthesis of aromatic amino acids necessary for protein formation in susceptible plants. Glyphosate is strongly adsorbed to soil particles, which prevents it from excessive leaching or from being taken-up from the soil by non-target plants. Glyphosate has little or no herbicidal activity ("killing power") once it touches soil because it rapidly binds to soils, (Genazzani *et al.,* 2000). Glyphosate can also be inactivated by adsorption if mixed with muddy water. Adsorption prevents glyphosate from being mobile in the environment except when the soil particles themselves are washed away (Okomoda and Ataguba, 2011). It is degraded primarily by microbial metabolism, but strong adsorption to soil can prevent microbial metabolism and slow degradation. The half-life of glyphosate ranges from several weeks to years, but averages two months (Majewski, Coupe, Foreman, and Capel, 2014). In water,

glyphosate is rapidly dissipated through adsorption to suspended and bottom sediments, and has a half-life of twelve days to ten weeks. Glyphosate and its degradation product, aminomethylphosphonic acid (AMPA) have been detected in air, rain (Majewski *et al.*, 2014), groundwater, surface water, soil and sea water (Majewski *et al*, 2014). This shows that glyphosate and AMPA persist in the soil and water and the amounts detected are increasing over time with increasing agricultural use; Glyphosate bioaccumulates in organs and tissue (Majewski *et al*, 2014).

2.9.1 Mode of action of glyphosate

Glyphosate is a broad-spectrum, systemic, post-emergence herbicide that is phloem mobile. Uptake is crucial, and unfortunately, glyphosate is not so 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 taken up by the plant, or more precisely at the meristem which is the site of action of the compound (McCourt, Pang, King-Scott, Guddat and Duggleby, 2006).

The absorbed glyphosate are readily translocated throughout the plant (Genazzani *et al.,* 2000; Williams *et al.,* 2000). From the leaf surface, glyphosate molecules are absorbed into the plant cells were they are translocated to meristematic tissues (McCourt *et al.,* 2006). Glyphosate kills plants by inhibiting the activity of the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSP), a chloroplast-localized enzyme in the shikimic acid pathway of plants (Figure 2.7) (Genazzani *et al.,* 2000; Williams *et al.,* 2000; Gasnier *et al.,* 2009; Gaafar *et al*., 2010). This prevents the production of chorismate which is necessary for the formation 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 (Genazzani *et al.,* 2000; Williams *et al.,* 2000; Gasnier *et al.,* 2009; Gaafar *et al*., 2010). These acids are used by plants in protein synthesis and to produce many secondary plant products such as growth promoters, growth inhibitors, phenolics, and lignin (Genazzani *et al.,* 2000; Williams *et al.,* 2000; Gasnier *et al.,* 2009; Gaafar *et al*., 2010). The enzyme,5-enolpyruvylshikimate-3-phosphate synthase are present in the chloroplast of most plant species, but are not present in animals. Animals need these three amino but they obtain them either by eating plants or other animals (McCourt *et al.,* 2006).

Glyphosate is a systemic herbicide, which means that it moves from the 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's death which may take from 4 to 20 days to occur (Gaafar *et al*., 2010; Genazzani *et al.,* 2000; Gasnier *et al.,* 2009; Williams *et al.,* 2000).

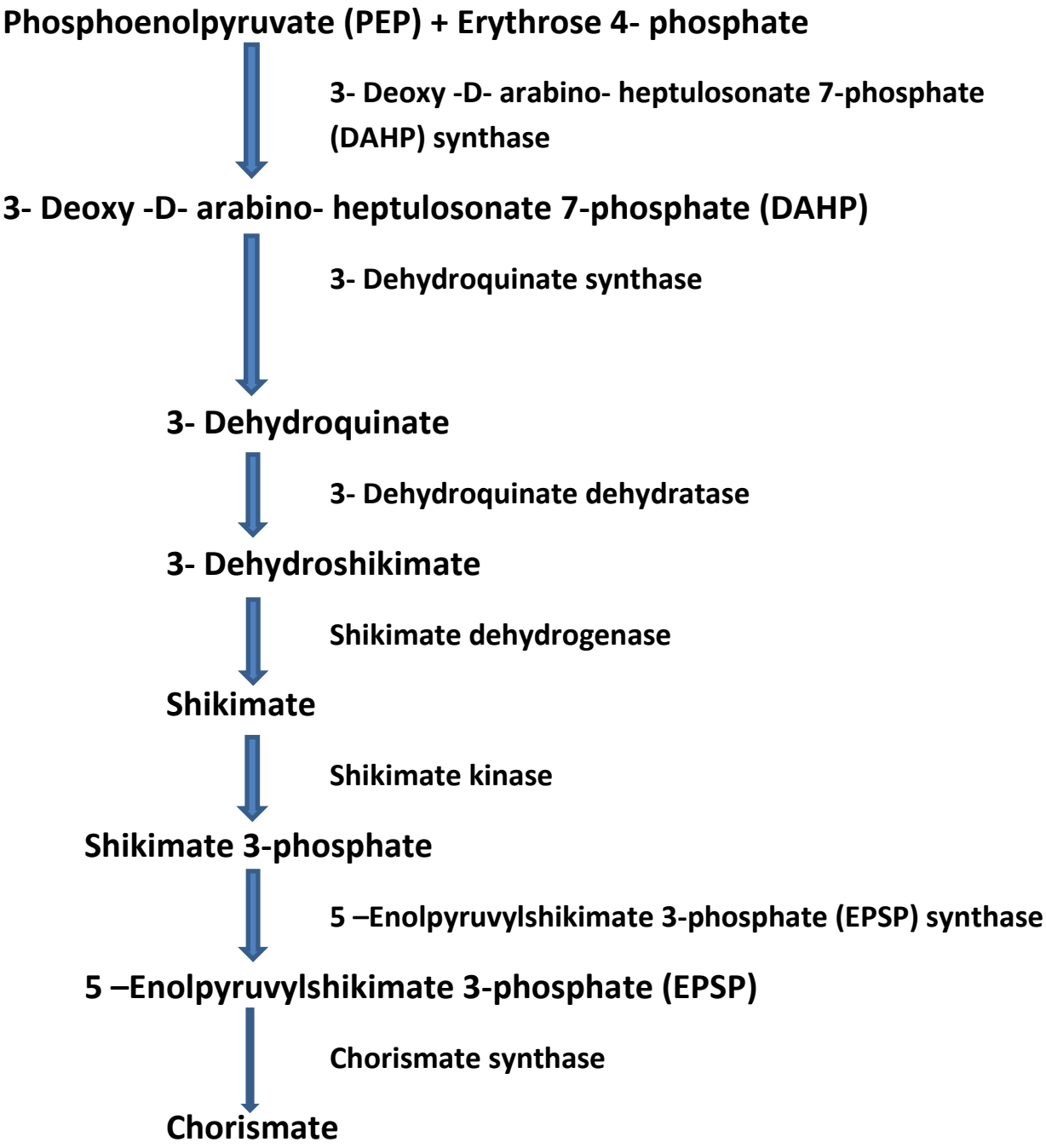


Figure 2.8 The shikimic acid pathway (Balasch. 2003).

Glyphosate is therefore, relatively non-toxic to animals. However, certain surfactants or other ingredients that are added to some glyphosate formulations are toxic to fish and other aquatic species (EPA, 2015).

Glyphosate can also act as a competitive inhibitor of phosphoenolpyruvate (PEP), which is one of the precursors to aromatic amino acid synthesis (Figure 2.8). It also affects other biochemical processes, and, although these effects are considered secondary, they may be important in the total lethal action of glyphosate (McCourt *et al.*, 2006).

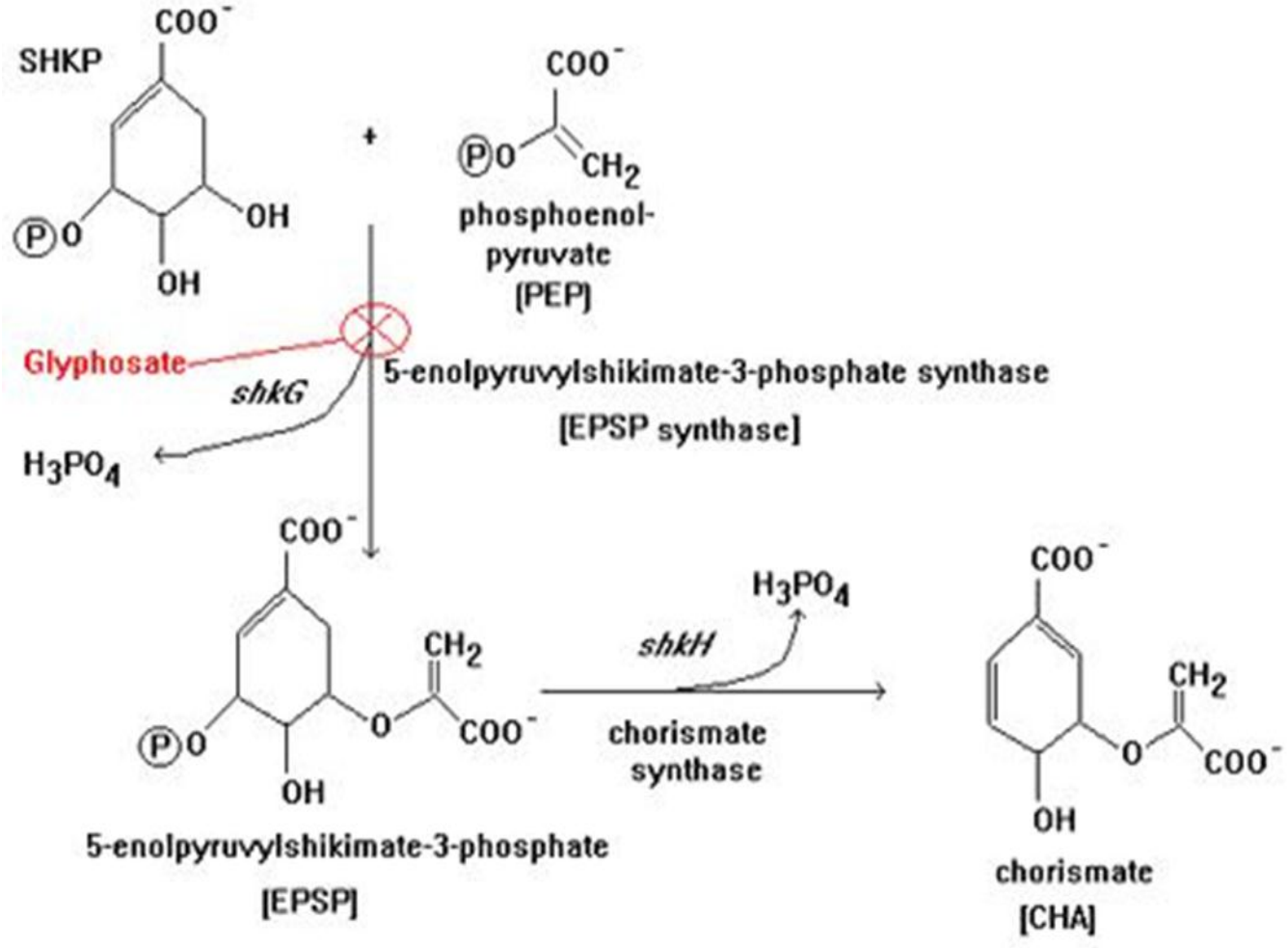


Figure 2.9 Mode of action of glyphosate on the shikimic acid pathway (Balasch. 2003).

2.9.2 Dissipation mechanism of glyphosate

Glyphosate is degraded primarily by microbial metabolism. Glyphosate is also believed to be susceptible to photo-degradation (Balasch. 2003; Eriksson *et al.,* 2008; McCourt *et al.,* 2006), but the extent to which this occurs is uncertain. Glyphosate is not significantly degraded by other chemical mechanisms in the field (Williams *et al.,*2000). Glyphosate is strongly adsorbed to soil, which can slow microbial metabolism but prevents excessive movement in the environment (Williams *et al.,* 2000; Gasnier *et al.,* 2009; Gaafar *et al*., 2010).

Although originally thought to be unaffected by sunlight, later studies found glyphosate to be susceptible to photo-degradation(Balasch. 2003; Eriksson *et al.,* 2008; Gasnier *et al.,* 2009; Gaafar *et al*., 2010; McCourt *et al.,* 2006).

Glyphosate is degraded primarily by microbial metabolism. Two steady rates of degradation have been identified (Balasch. 2003; Eriksson *et al.,* 2008; Dai *et al.,* 2016). It has been hypothesized that the more rapid rate of degradation represents the metabolism of unbound glyphosate molecules, while the slower rate represents the metabolism of glyphosate molecules bound to soil particles (De Cock *et al.,* 2012; Gholami-Seyedkolaei *et al.,* 2013). The degradation of glyphosate is slower in soils with a higher adsorption capacity. Degradation rate was also affected by the particular microbial community of each soil. The soil microflora quickly biodegrade glyphosate into AMPA and CO_2 (De Cock *et al.,* 2012; Gholami-Seyedkolaei *et al.,* 2013).Glyphosate is broken down to amino methyl phosphonic acid (AMPA) and sarcosine which is further degraded to glycine by the enzyme sarcosineoxidase (Figure.2.9). In plants, glyphosate is slowly metabolized (Williams *et al.,* 2000).

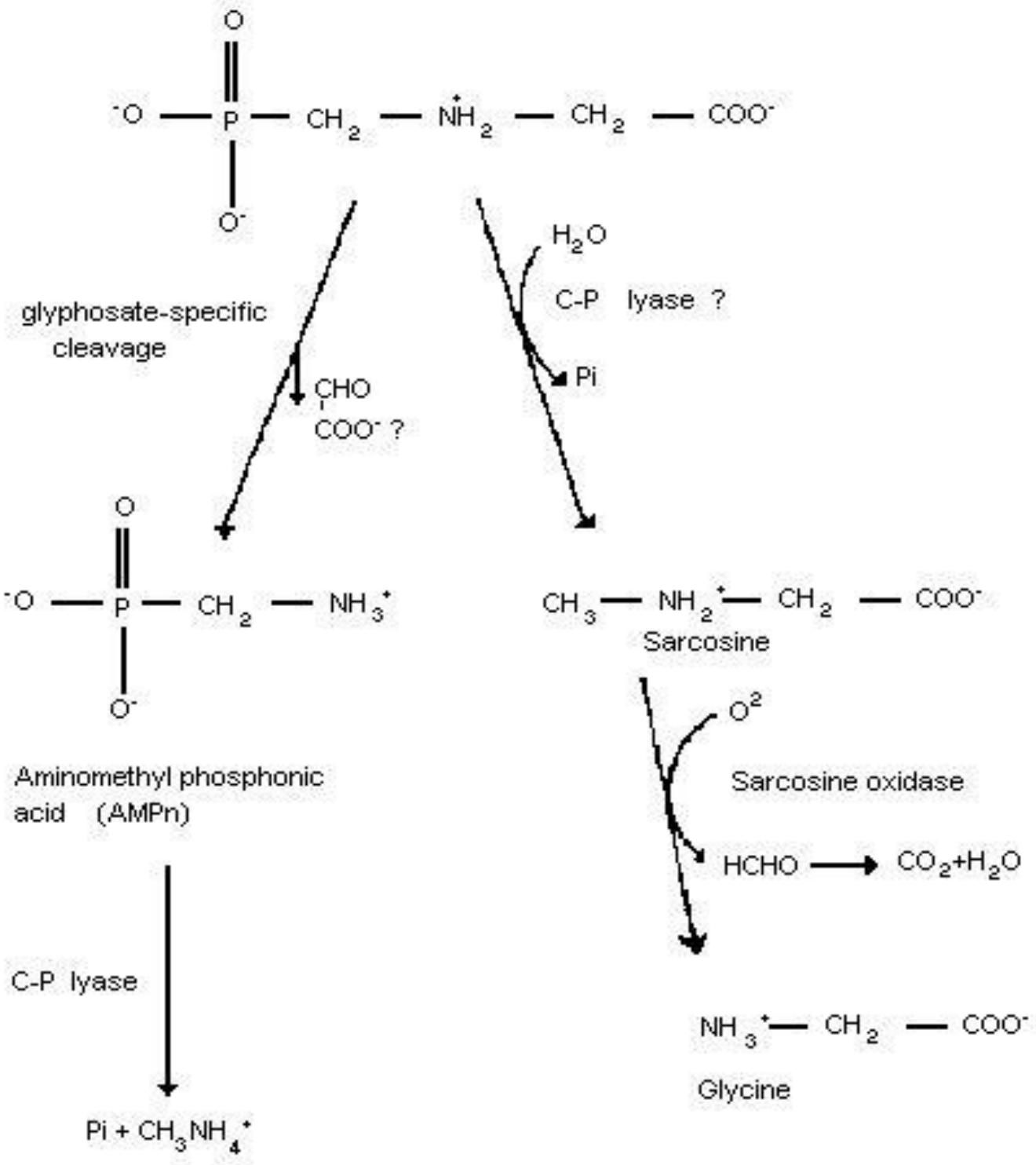


Figure 2.10; Degradation routes of glyphosate in soil (McCourt *et al.*, 2006).

2.9.3 Environmental Toxicity of Glyphosate

Birds and mammals are indirectly exposed to glyphosate from polluted water or exposed food. Glyphosate is of relatively low toxicity to birds and mammals (De Cock*et al.*,2012; Gholami-Seyedkolaei *et al.*, 2013).. The LD50 of glyphosate for rats is 5,600 mg/kg and for bobwhite quail, >4,640 mg/kg (EPA 2011). Environment protection agency (EPA) Reregistration eligibility decision states that blood and pancreatic effects and weight gain were noted during sub-chronic feeding studies with rats and mice (EPA 2011).

Glyphosate is of moderate toxicity to fish. The 96-hour LC50 of technical grade glyphosate for bluegill sunfish and rainbow trout are 120 mg/L and 86 mg/L, respectively. Fish exposed to 5 mg/L of glyphosate for two weeks were found to have gill damage; and liver damage was observed at glyphosate concentrations of 10 mg/L (De Cock *et al.*, 2012; Gholami-Seyedkolaei *et al.*, 2013). The technical grade of glyphosate is of moderate toxicity to aquatic species, and the toxicity of different glyphosate formulations can vary considerably (De Cock *et al.*, 2012; Gholami-Seyedkolaei *et al.*, 2013).

2.10 Glyphosate and Endocrine Disruption

In recent decades, scientific concern has been growing about chemicals that interfere with hormones in the body at very low doses, called endocrine disrupting chemicals (Reyes *et al.*, 2014). At particular life stages, such as during pregnancy or puberty, endocrine disrupting chemicals may cause irreversible effects even though there are no obvious signs of toxicity at the time. Glyphosate is an endocrine disruptor (Gasnier *et al*., 2009; Paganelli *et al*., 2010; Antoniou *et al*., 2012; Thongprakaisang *et al.*, 2013); There are no safe levels of endocrine disruptors (Vandenberg *et al.*, 2012). Investigations into glyphosate suggest it may show endocrine

disrupting effects, particularly on reproductive development (Williams *et al*., 2000). For example, in one study pregnant female rats were given a glyphosate-containing herbicide at high doses, but not enough to affect their health or their pregnancies (Gasnier *et al.,* 2009; Paganelli *et al.,* 2010; Antoniou *et al.,* 2012; Thongprakaisang *et al*., 2013). The reproductive development of their male offspring was altered compared to normal, including lower testosterone levels and reduced sperm production in adults (Williams *et al.,* 2000).

Evidence from cell culture (*in vitro*) studies show that glyphosate blocks receptors for male sex hormones, while glyphosate-containing herbicides reduce testosterone production in male reproductive cells and inhibit the production of other hormones (Paganelli *et al.,* 2010).

2.11Endocrine System and endocrine disruptors

The endocrine system is one of the body's main communication networks and is responsible for controlling and coordinating numerous body functions. Hormones are first produced by the endocrine tissues, such as the ovaries, testes, adrenal, pituitary, thyroid, and pancreas, and then secreted into the blood to act as the body's chemical messengers where they direct communication and coordination among other tissues throughout the body (Gasnier *et al.,* 2009; Paganelli *et al*., 2010; Antoniou *et al.,* 2012; Thongprakaisang *et al.,* 2013). The endocrine system consists of a set of glands (thyroid, gonads, adrenal and pituitary) and the hormones they produce (thyroxin, estrogen, testosterone and adrenaline), which help to guide the development, growth, reproduction, and behaviour of animals, including humans. Hormones are signaling molecules, which travel through the bloodstream and elicit responses in other parts of the body (El-shenaw, 2009). The endocrine system keeps our bodies in balance, maintaining homeostasis and guiding proper growth and development. Endocrine disrupting chemicals may interfere with

the body's own hormone signals because of their structure and activity (Gasnier *et al.,* 2009; Paganelli *et al.,* 2010; Antoniou *et al*., 2012; Thongprakaisang *et al.,* 2013)

Endocrine disruptors are naturally occurring compounds or man-made substances that may mimic or interfere with the function of hormones in the body. They may turn on, shut off, or modify signals that hormones carry, which may affect the normal functions of tissues and organs (Gasnier *et al*., 2009). Most of these substances have been linked with developmental, reproductive, neural, immune, and other problems in wildlife and laboratory animals (Gasnier *et al.,* 2009; Gaafar *et al*., 2010).

Endocrine disruptors function by: mimicking the action of a naturally-produced hormone, such as estrogen or testosterone, thereby setting off similar chemical reactions in the body; blocking hormone receptors in cells, thereby preventing the action of normal hormones. They also affect the synthesis, transport, metabolism and excretion of hormones, thus altering the concentrations of natural hormones. Exposure may occur in the womb, through the air we breathe at home, in the work- place or at school, or through residues in our food and water (Gasnier *et al*., 2009).

Endocrine disruptors have been linked to attention deficit, hyperactivity disorder (ADHD), parkinsons, alzheimers, diabetes, cardiovascular disease, obesity, early puberty, infertility and other reproductive disorders, childhood and adult cancers (Gasnier *et al*., 2009). Scientists believe that neurological disorders observed in children, such as ADHD and autism, may be related to the prenatal chemical disruption of the thyroid system (Kavlock *et al.,* 1996). Certain pesticides are believed to alter thyroid function, interfere with brain development and cause deficits in cognitive functions in the developing foetus. Other effects include physical and mental retardation, alterations of the cardiovascular system and musculoskeletal defects, alterations of

the menstrual cycle, obesity, and failure to develop secondary sex characteristics (McCourt *et al.*, 2006).

2.11.1 Testosterone

Testosterone is a steroid hormone from the androgen group found in human and other vertebrates. The structure is shown in figure 2.10. It is primarily secreted though the testicles of the male. It plays a key role in the promotion of sexual characteristics which include maturation of the sex organs mostly the penis, formation of the scrotum in foetus, muscle increase and bone mass elevation. Fish make a slightly different form known as 11-ketotestosterone.There are three basic androgens, which include testosterone, androstenedione, and dehydroepiandrosterone (DHEA) (El-shanaw, 2000; Harding and Velotta, 2011). Men produce about 6 to 8 milligrams of testosterone per day in their youthful age, while women produce about one twentieth of that-(approximately 300 micrograms). Men produce testosterone in their testicles and adrenal glands, and cannot naturally overproduce this hormone. There is no such condition as “hypergonadism” in men. The only way men can have an excessive level, is by taking some type of testosterone supplement (Asklund, Jensen, Main, Sobotka, Skakkebaek, and Jorgensen, 2009). Even then, the male body will only allow so much blood testosterone, and then turns any excess into estradiol and estrone. This is done by “aromatization” using the enzyme aromatase (El-shanaw *et al.*, 2000). Approximately 50% of testosterone circulating in plasma is bound to sex hormone binding globulin (SHBG), a ß-globulin with high affinity; but limited binding capacity for testosterone and the remaining 50% is bound to albumin, which has a low-affinity for testosterone but a high binding capacity (Asklund, Jensen, Main, Sobotka, Skakkebaek, and Jorgensen, 2009).

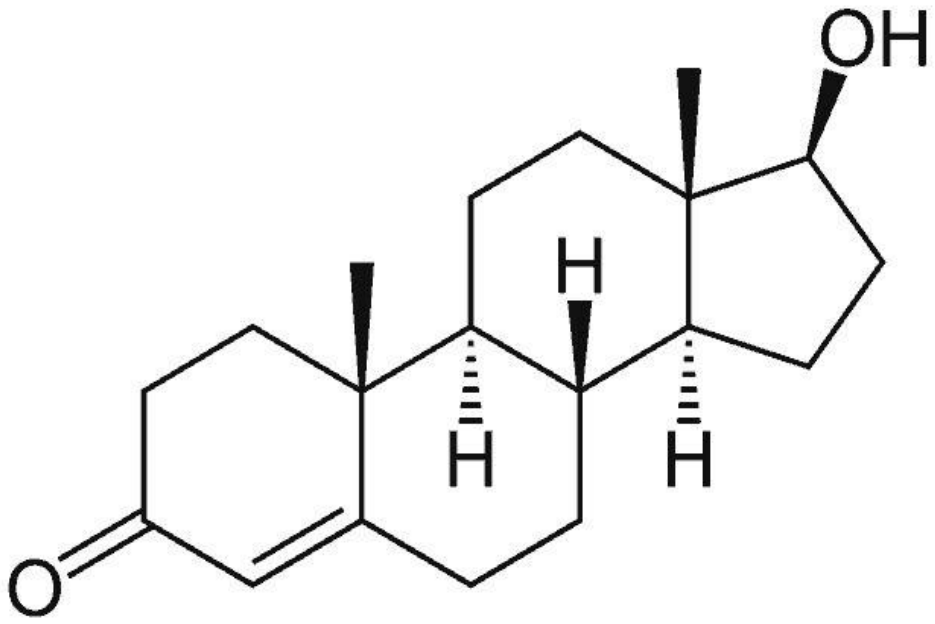


Figure 2.11 Chemical structure of testosterone (Lenz *et al.*, 2010).

2.11.1.1Testosterone deficiency

Testosterone deficiency in men is manifested typically by symptoms of hypogonadism, including decreases in erectile function and libido. One quarter of men over 65 years have subnormal testosterone levels (Lenz and McCarthy, 2010). Testosterone also has an important role in the regulation of normal growth, bone metabolism, and body composition. Men with testosterone deficiency have significant decreases in bone density, particularly in the trabecular bone compartment (Lenz *et al.*, 2010). Testosterone deficiency has been reported in over half of elderly men with a history of hip fracture (Asklund, Jensen, Main, Sobotka, Skakkebaek, and Jorgensen, 2009). Men with testosterone deficiency also have alterations in body composition, which includes an increase in body fat. Because truncal fat correlates with glucose intolerance and cardiovascular risk, hypogonadism may have important implications with regard to overall health and mortality (Lenz *et al.*, 2010). Therefore, testosterone deficiency is associated with an

enhanced risk for osteoporosis, altered body composition including increases in truncal fat, and, possibly, decreases in muscle performance (Bagnyukova, Chahrak and Lushchak, 2006).

Figure 2.12: Biosynthesis of testosterone and progesterone (Genazzani *et al*., 2000).

2.11.2 Thyroid Hormone

The thyroid gland is a butterfly-shaped endocrine gland that is normally located in the lower front of the neck. The thyroid's job is to make thyroid hormones, which are secreted into the blood and then carried to every tissue in the body. Thyroid Hormones are Thyroxin {3, 5, 3', 5' – Tetra-Iodothyronine} and Tri-Iodothyronine: {3, 5, 3' – Tri-Iodothyronine}. Thyroid hormones are unique because they contain the trace element iodine for biological activity. Thyroxin is also called T4 because it contains four iodine atoms. To exert its effects, T4 is converted to triiodothyronine (T3) by the removal of an iodine atom. This occurs mainly in the liver and in certain tissues where T3 acts, such as in the brain. T3 is Biological active form of Thyroid hormones, because it binds to receptors and trigger end-organ effects. The amount of T4 produced by the thyroid gland is controlled by another hormone, which is made in the pituitary gland located at the base of the brain, called thyroid stimulating hormone (abbreviated TSH) (Klassen *et al*., 2001). The amount of TSH that the pituitary sends into the blood stream depends on the amount of T4 that the pituitary sees (Melesse, Maak, Schmidt, and Von Lengerken, 2011).

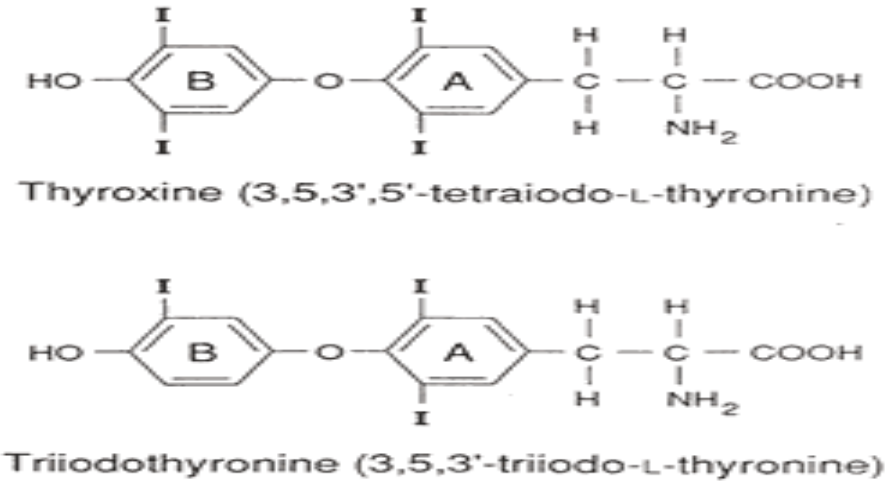


Figure 2.13: Structure of thyroxin and triiodothyronine (Melesse *et al*., 2011).

2.11.2.1 Synthesis of thyroid hormone

Tyrosine and iodine are essential for the synthesis of thyroid hormones as shown in Figure 2.14. Tyrosine is provided from a large glycoprotein scaffold called thyroglobulin. Iodine is avidly taken up from blood by thyroid epithelial cells, which have on their outer plasma membrane a sodium-iodide symporter. Once inside the cell, iodide is transported into the lumen of the follicle along with thyroglobulin (Melesse *et al*., 2011). The synthesis of thyroid hormone is conducted by the enzyme thyroid peroxidase, an integral membrane protein present in the apical plasma membrane of thyroid epithelial cells. Thyroid peroxidase catalyzes the Iodination of tyrosine on thyroglobulin which results in either a mono-iodinated tyrosine (MIT) or di-iodinated tyrosine (DIT). Thyroid peroxidase also catalyzes the Synthesis of thyroxin or tri-iodothyronine from two iodotyrosines. The coupling of two di-iodotyrosine molecules would result to the formation of thyroxin (T4), also the coupling of a di-iodotyrosine and a mono-iodotyrosine would result to the formation of tri-iodothyronine (T3). Thyroid hormones are excised from their thyroglobulin scaffold by digestion in lysosomes of thyroid epithelial cells which contain hydrolytic enzymes. The liberated free thyroid hormones diffuse out of lysosomes, through the basal plasma membrane of the cell into blood where they quickly bind to carrier proteins for transport to target cells (Melesse *et al*., 2011).

2.11.2.2 Regulation of thyroid metabolism

The hypothalamus (a pea-sized gland in the brain) regulates the levels of thyroid hormone in the body by producing thyrotropin releasing hormone (TRH). TRH acts on the anterior pituitary (directly below the hypothalamus, but outside of the blood-brain barrier) to produce thyrotropin, also refered to as thyroid stimulating hormone (TSH). TSH acts on the thyroid gland, which produces thyroxine (T4) and triiodothyronine (T3), the primary circulating thyroid hormones.

The thyroid produces T4 in significantly greater quantities (in a ratio of 17:1) than T3, which is approximately 5 times more biologically active than T4. T4 is converted into the more active T3 by the deiodinase system (D1, D2, D3) in multiple tissues and organs, but especially in the liver, gut, skeletal muscle, brain and the thyroid gland itself. D3 converts T3 into an inactive form of thyroid hormone in the liver. Transport proteins produced by the liver which include thyroid binding globulin (TBG), transthretin and albumin, carry T4 and T3 to the tissues where they are cleaved from their protein-carriers to become free T4 and free T3 which bind to thyroid hormone receptors (THRs) and exert their metabolic effect (Melesse *et al*., 2011).

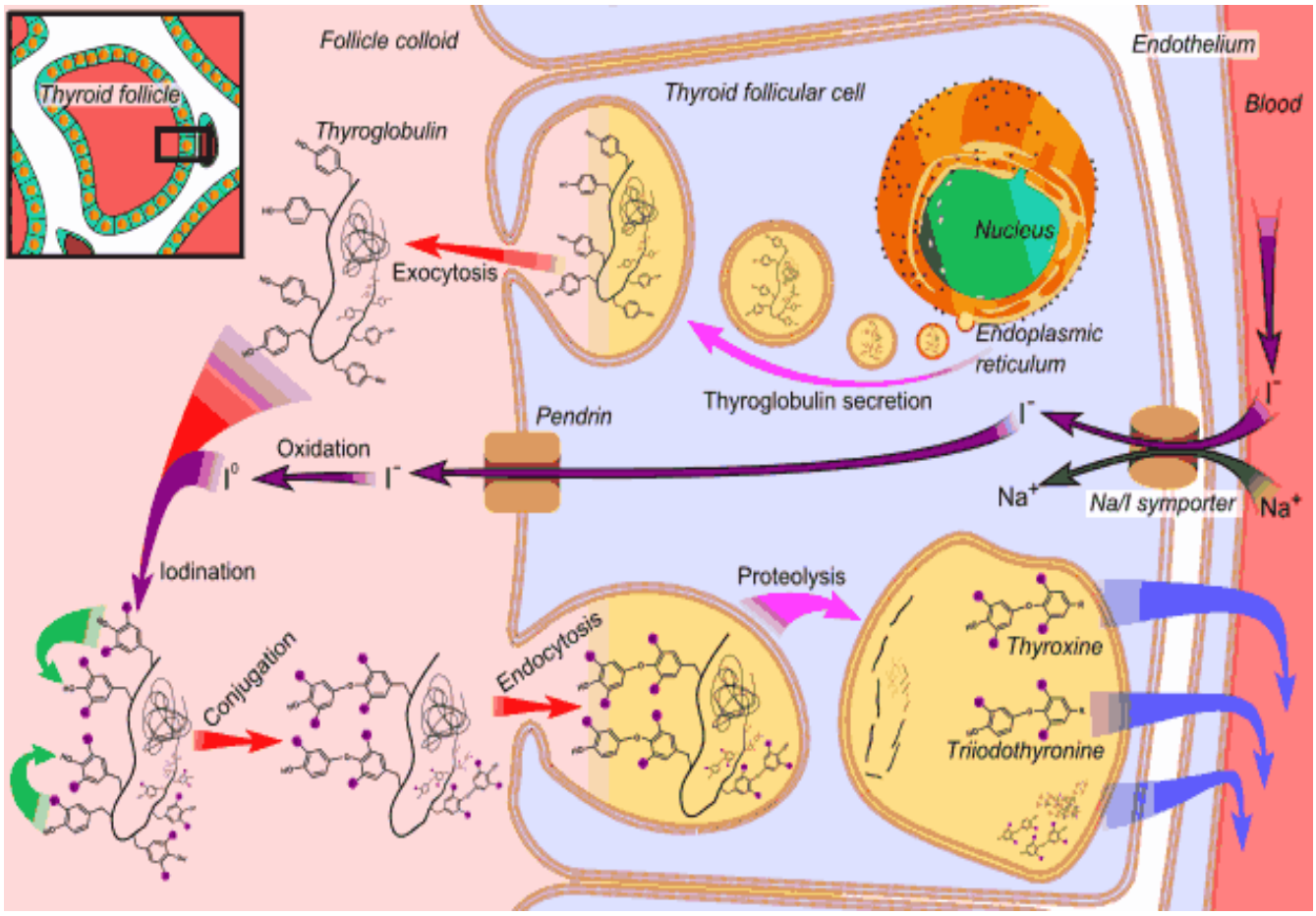


Figure 2.14: synthesis of thyroid hormones (Melesse *et al*., 2011).

2.11.2.3 Functions of thyroid hormone

The actions of thyroid hormones on whole body metabolism include: increase basal metabolic Rate (BMR); increase oxygen consumption; increase thermogenesis (heat production in the body); activate Na^+ -K^+-ATPase in cells; increase number of mitochondria in cells; increase mobilization of endogenous: carbohydrate, fat and protein as substrates for energy metabolism; increase glycolysis, glycogenolysis, gluconeogenesis; increase lipolysis and protein degradation; decrease muscle mass; decrease adipose tissue; increase beta-adrenergic receptors, which leads to increased cardiac output; increase systolic blood pressure only; increase ventilation rate; required for maturation of ovary and testis; required for actions of growth hormone (GH) to promote linear growth / bone formation; required for development of central nervous system(CNS) in foetus (Melesse *et al*., 2011). Figure 2.13 shows some functions of thyroid hormone which include growth and development. It also shows the stimulation of the thyroid gland by the thyroid stimulating hormone released from the anterior pituitary gland (Straus and Hsueh, 2001; Lenz *et al.*, 2010).

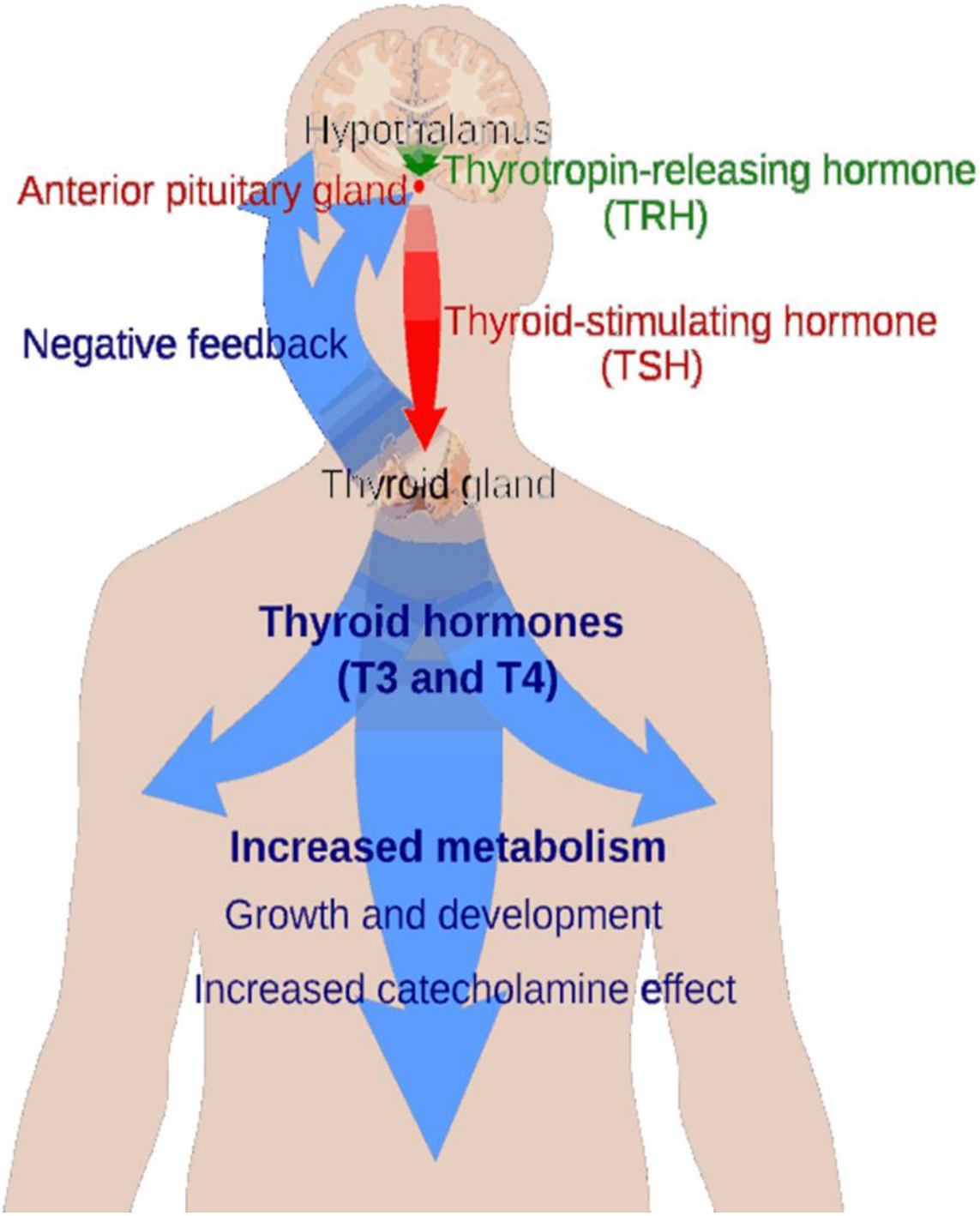


Figure 2.15 Function of thyroid hormone (Melesse *et al*., 2011).

2.12 Oxidative stress

Oxidative stress is defined as a "state in which oxidants exceed the antioxidant systems in the body secondary to a loss of the balance between them." It is also defined as a situation when steady-state ROS concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation and damaging cellular constituents (Lushchak *et al.*, 2009). It does not only cause hazardous events such as lipid peroxidation and oxidative DNA damage, but also physiologic adaptation phenomena and regulation of intracellular signal transduction. Oxidative stress induce overproduction of reactive oxygen species which can damage the most important cellular molecules such as nucleic acids, proteins and lipids, resulting in serious diseases and dysfunction of living organisms (Bagnyukova, Chahrak and Lushchak, 2006). Oxidative stress-induced peroxidation of membrane lipids can be very damaging because it leads to alterations in the biological properties of the membrane, such as the degree of fluidity, and can lead to inactivation of membrane-bound receptors or enzymes, which in turn may impair normal cellular function and increase tissue permeability (Ujowundu, Ogbede, Igwe, and Nwaoguikpe, 2016).

Figure 2.16 shows the effect of reactive oxygen species on mitochondria dysfunction, apoptosis, and necrosis, resulting to various diseases and ageing. It also illustrates the ability of reactive oxygen species to cause mutation and deletion of the mitochondria DNA (mDNA), lipid peroxidation and other defects (Balasch, 2003; Lushchak, Kubrak, Storey and Lushchak, 2009).

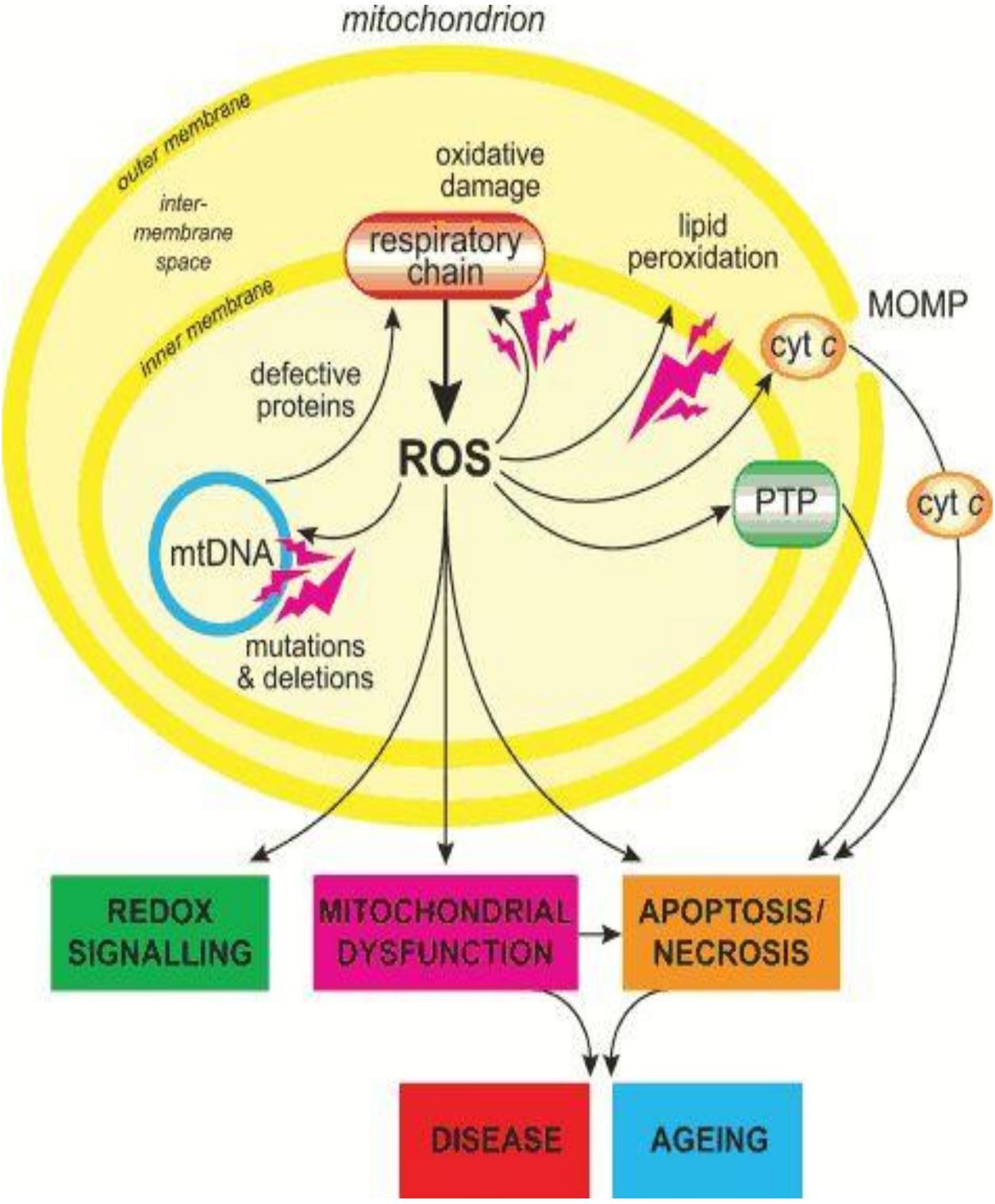


Figure 2.16 Effects of reactive oxygen species (Lushchak *et al.*, 2009).

2.12.1 Free radicals

A free radical could be described as an atom or group of atoms possessing one or more atoms with one or more unpaired electrons (Figure 2.17) (Ayanda, Olasehinde, and Ajayi, 2018). Free radicals are very unstable and react quickly with other compounds, as they try to capture the needed electron to gain stability (Ayanda, Olasehinde, and Ajayi, 2018), thereby starting a chain reaction. Once the process is started, it can cascade, and finally results in the disruption of a living cell. Free radicals are also known as reactive oxygen species (ROS) or reactive nitrogen species (RNS). Free radicals attack cellular macromolecules such as lipids, proteins and DNA (Ayanda, Olasehinde, and Ajayi, 2018).

Lipids: Peroxidation of lipids in cell membranes can damage cell membranes by disrupting fluidity and permeability, leading to proximal membrane damage, thereby interfering with the cell activity. Lipid peroxidation can also adversely affect the function of membrane bound proteins such as enzymes and receptors (Ayanda, Olasehinde, and Ajayi, 2018).

Proteins: Direct damage to proteins can be caused by free radicals. This can affect many kinds of protein, thereby interfering with enzyme activity and the function of structural proteins (Ayanda, Olasehinde, and Ajayi, 2018).

Deoxyribonucleic acid (DNA): Free radicals cause damage to deoxyribonucleic acid by attacking the nitrogenous base, interfering with the binding of the nitrogenous base. This can cause cancer or leukaemia within the body (Ayanda, Olasehinde, and Ajayi, 2018).

The site of tissue damage by free radicals is dependent on the tissue and the reactive species involved. Extensive damage can lead to death of the cell; this may be by necrosis or apoptosis depending on the type of cellular damage (Ayanda, Olasehinde, and Ajayi, 2018; Gaafar, El-

Manakhly, Soliman, Soufy, Mona, Mohamed, and Hassan, 2010). When a cell membrane or an organelle membrane is damaged by free radicals, it loses its protective properties. This puts the health of the entire cell at risk of damaging effects (Ayanda, Olasehinde, and Ajayi, 2018).

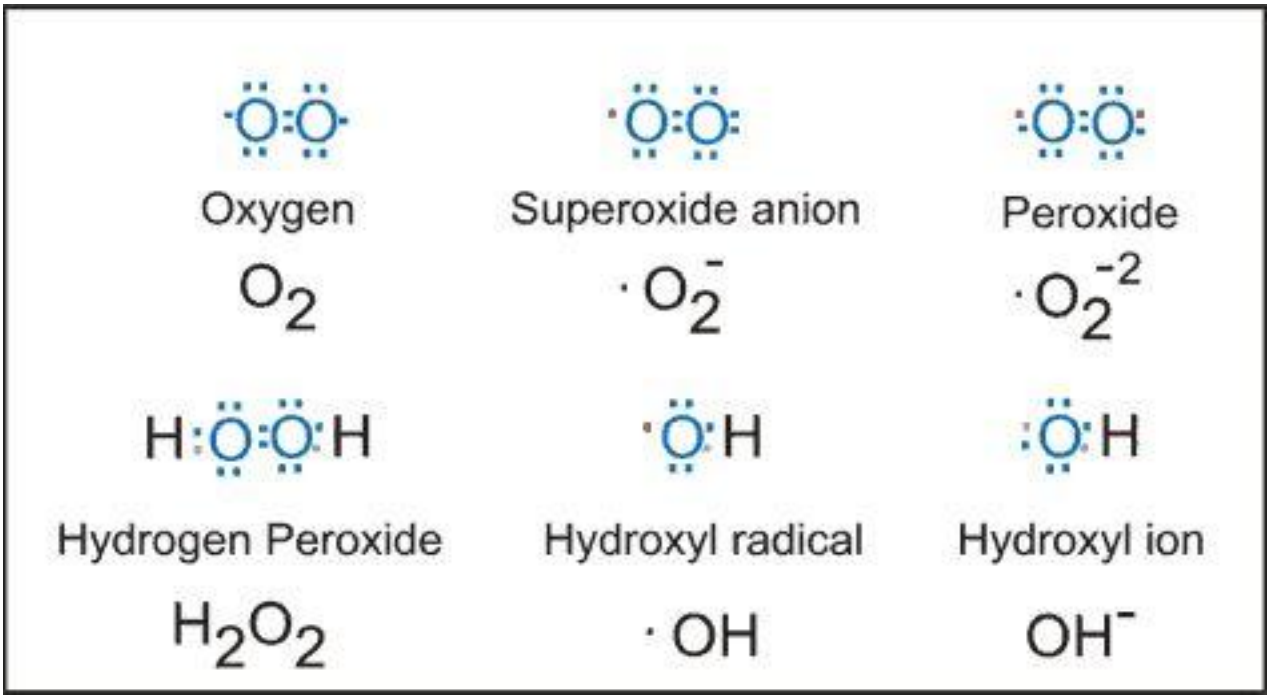


Figure 2.17: Major types of free radicals (Ayanda, Olasehinde, and Ajayi, 2018).

2.12.1.1 Reactive oxygen species (ROS)

Oxygen-centered free radicals are those in which an unpaired electron is on an oxygen atom and contain two unpaired electrons in the outer shell. When free radicals "steal" an electron from a surrounding compound or molecule a new free radical is formed in its place. In turn, the newly formed radical then looks to return to its ground state by stealing electrons with antiparallel spins from cellular structures or molecules. Thus the chain reaction continues and can be "thousands of events long" (Bagnyukova, Chahrak and Lushchak, 2006). Radicals derived from oxygen represent the most important class of radical species generated by organisms. Any free

radical involving oxygen can be referred to as reactive oxygen species (ROS). A major consequence of oxidative stress is damage to nucleic acid bases, lipids, and proteins, which can severely compromise cell functioning and viability or induce a variety of cellular responses through generation of secondary reactive species, ultimately leading to cell death by necrosis or apoptosis (Bagnyukova, Chahrak and Lushchak, 2006).

2.12.1.2 Superoxide anion (O_2^-)

Molecular oxygen has a unique electronic configuration and is itself a di-radical with two such unpaired electrons (Bagnyukova, Chahrak and Lushchak, 2006). If a single electron is added to the ground-state oxygen (O_2) molecule, it must enter one of the π antibonding orbitals. The product is called superoxide anion and the production of super oxideanion (O_2^-) occurs mostly within the mitochondria of a cell (Balasch, 2003; Bagnyukova, Chahrak and Lushchak, 2006) compared with other free radicals. Superoxide anion has a relatively long half-life that enables diffusion within the cell thereby increasing the number of potential targets. Beside super oxide anion (O_2^-), some other biologically relevant free radicals derived from oxygen are the perhydroxyl radical, the hydroxyl radical (•OH), and free radical nitric oxide (NO•). With only one unpaired electron, superoxide is less of a radical than is oxygen (O_2) itself, despite its "super" name. superoxide anion (O_2^-), arising either through metabolic processes or following oxygen activation by physical irradiation, is considered the primary ROS and can further interact with other molecules to generate secondary ROS such as lipid radicals, either directly or prevalently through enzyme or metal-catalyzed processes (Balasch, 2003; Bagnyukova, Chahrak and Lushchak, 2006). As a redox-active species, superoxide anion (O_2^-) can reduce some biological materials (e.g., cytochrome c) and oxidize others such as ascorbate. During energy transduction, a small number of electrons "leak" to oxygen prematurely, forming the oxygen free

radical O_2^-(Balasch, 2003; Bagnyukova, Chahrak and Lushchak, 2006). The tissue toxicity of superoxide anion (O_2^-) generated extracellularly seems to be based on its direct reactivity with numerous types of biological molecules (lipid, DNA, RNA, catecholamines, steroids, etc.) and from its dismutation to form H_2O_2 and the concomitant reduction of ferric ion (Fe_3^+) to ferrous ion (Fe_2^+); reaction of these two products yields the highly toxic hydroxyl radical that may cleave covalent bonds in proteins and carbohydrates, cause lipid peroxidation, and destroy cell membranes (Balasch, 2003; Bagnyukova, Chahrak and Lushchak, 2006). There are three strategies available to "detoxify" or to prevent formation of locally produced oxygen radicals: to deliver superoxide dismutase (SOD) or a superoxide dismutase mimetic (SODm) to the area, to deliver catalase or a related peroxide scavenger or, to chelate the trace iron that catalyzes the reaction (Balasch, 2003; Bagnyukova, Chahrak and Lushchak, 2006).

2.12.1.3 Hydroxyl radical (•OH)

Hydroxyl radical is the neutral form of the hydroxide ionbut reacts very rapidly with almost every type of molecule found in living cells: sugars, amino acids, phospholipids, DNA, and organic acids (Isibor, 2017). Indeed, •OH is the most reactive oxygen radical known, with a highly positive reduction potential of +2310 mV (Isibor, 2017). •OH can be generated in biologically relevant systems by multiple reactions including: Fenton reaction and Haber-Weiss reaction.

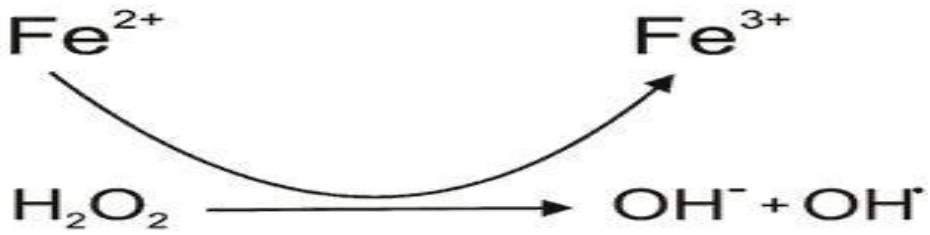


Figure 2.18: Fenton chemistry (Gaafar *et al*., 2010)

Fenton chemistry is a prime example of damaging free radical reactions catalyzed by transition metals. A mixture of H_2O_2 with a Fe^{+}_2 salt oxidizes many different organic molecules and can provoke a whole series of radical reaction. *In vivo*, •OH are most likely generated from superoxide anions via an iron-catalyzed Fenton reaction (Isibor, 2017; Gaafar *et al*., 2010; Gasnier *et al.*, 2009). Electron paramagnetic resonance spectroscopy (EPR) studies demonstrated the ability of iron bound to transferrin to catalyze •OH formation in the presence of O_2^-. However, these results have subsequently been questioned (Lushchak *et al.*, 2009; Bagnyukova *et al.*, 2006). Another source of iron is the intracellular pool, where iron is principally bound to ferritin. It has been shown *in vitro* that O_2^- is capable of releasing iron from ferritin, thereby allowing the formation of •OH. Alternatively, the presence of low molecular weight iron chelates has been hypothesized (Isibor, 2017; Gaafar *et al*., 2010; Gasnier *et al.*, 2009).

Haber-Weiss reaction (free radical formed from O_2^- and H_2O_2) might provide a means to generate more toxic radicals. Although the basic reaction has a second order rate constant of zero in aqueous solution and thus it cannot take place under physiological conditions, the ability of iron salts to serve as catalysts was discussed by those authors. Because transition metal ions, particularly iron, are present at low levels in biological systems, this pathway has been widely postulated to account for the in vivo generation of the highly reactive •OH. If •OH radicals meet

each other, they can form dimers, thus yielding hydrogen peroxide (Isibor, 2017; Gaafar *et al*., 2010; Gasnier *et al.*, 2009).

2.12.1.4 Peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals

Peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$) are good oxidizing agents, since they have a tendency to accept electrons thereby undergoing reduction themselves having highly positive E°′ values, although alkoxyl formed in biological systems often undergoes rapid molecular rearrangement to other radical species. Indeed, $HO_2^{\bullet}$ which is the protonated form of O_2^{-} and is usually termed either hydroperoxyl radical or perhydroxyl radical can be regarded as the simplest $RO_2^{\bullet}$. For example, $RO_2^{\bullet}$ radicals oxidize ascorbate and NADH, the latter leading to O_2^{-} formation in the presence of oxygen

$$RO_2^{\bullet} + NADH \rightarrow RO_2H + NAD^{\bullet}$$

$$NAD^{\bullet} + O_2 \rightarrow NAD^{+} + O_2^{-}$$

Aromatic alkoxyl and peroxyl radicals tend to be less reactive, since electrons can be delocalized into the benzene ring. It has been demonstrated that RO_2• initiates fatty acid peroxidation by two parallel pathways: fatty acid hydroperoxide (LOOH)-independent and LOOH-dependent (Isibor, 2017; Gaafar *et al*., 2010; Gasnier *et al.*, 2009). These reactions account for much of the stimulation of lipid peroxidation by transition-metal ions in biological systems. The carbon-centered radicals are capable of reacting directly with certain biological molecules including DNA and albumin -SH-groups. RO_2• derived from azo-initiators can induce peroxidation of lipids and can damage proteins, e.g. they inactivate the enzyme lysozyme. The ability of various antioxidants to prevent azo-initiator-induced lipid peroxidation or protein

damage is frequently used to assess antioxidant activity (Isibor, 2017; Gaafar *et al*., 2010; Gasnier *et al.,* 2009).

2.12.1.5 Singlet oxygen ($^1O_2$)

Singlet oxygen ($^1O_2$) was first observed in 1924 and then defined as a more reactive form of oxygen (Halliwell & Gutteridge, 2000). It is the most important in biological systems with ample higher energy state molecular oxygen species but is not a radical since it contains no unpaired electrons (Lushchak *et al.,* 2009; Bagnyukova *et al.,* 2006). Although not a free radial, it can be formed in some radical reactions and also can lead to others since it is one of the most active intermediates involved in chemical and biochemical reactions (Canonica *et al.,* 2003). It has been demonstrated that $^1O_2$ can react with many kinds of biological molecules such as DNA, proteins and lipids. Since oxygen is ubiquitous and efficiently quenches electronically excited states, $^1O_2$ is likely to be formed following irradiation in countless situations and involved in various chemical and biological processes as well as in several disease processes which can lead to excessive singlet oxygen formation, especially many different porphyrias. Both physical and chemical methods can generate singlet oxygen. One well-established reaction used in the laboratory to generate singlet oxygen is the reaction of H_2O_2 and the hypochlorite ion, the ionized form of hypochlorous acid (Jiri, Tazvivinga, Greenfield, and van Vuren, 2018).

$OCl^- + H_2O_2 \rightarrow Cl^- + H_2O + {}^1O_2$ (singlet)

2.12.1.6 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is liquid and is toxic to most cells in the 10–100 μM range but is not a free radical, although it can be formed from two $HO^{\bullet}$ radicals. Although this reaction has a high rate constant, it is unlikely to occur *in vivo* since the steady-state concentration of •OH is effectively

zero. Several enzymes can generate hydrogen peroxide (H_2O_2) *in vivo* e.g., xanthine oxidase, urate oxidase, and D-amino acid oxidase. In addition, any biological system that generates superoxide anion (O_2^-) will also produce hydrogen peroxide (H_2O_2) by superoxide anion (O_2^-) dismutation. However, hydrogen peroxide (H_2O_2) is only a weak oxidizing and reducing agent and is generally poorly reactive at physiological levels but is capable of inactivating several enzymes and oxidized keto-acids such as pyruvate and oxoglutarate. It can react with iron and possibly copper to form much more damaging species such as •OH. It is important to know that H_2O_2 can degrade haem proteins including myoglobin, haemoglobin and cytochrome c (Lushchak *et al.,* 2009; Bagnyukova *et al.,* 2006). It can affect the proliferation of cells and facilitate phagocyte adherence to endothelium by up-regulating expression of such adhesion molecules as E-selectin, ICAM-1 and VCAM-1 (Jiri *et al.,* 2018). It also leads to oxidative DNA damage by oxo-copper complexes. Hydrogen peroxidase (H_2O_2) production rates by cells and organelles are often in the range of a few nmoles per minute (Canonica *et al.,* 2003). Therefore, methods for measuring H_2O_2 in the biological material should be sufficiently sensitive. These methods include reaction with dichlorofluorescin diacetate, polarographic detection (O_2 electrode) and histochemical staining methods (Jiri *et al.,* 2018).

2.13 Lipid peroxidation

Lipid peroxidation is described as an oxidative alteration of polyunsaturated fatty acids or oxidative deterioration of polyunsaturated fatty acids. It is initiated by hydrogen abstraction or addition of oxygen radical. Polyunsaturated fatty acids are abundant in cellular membranes and in low-density lipoproteins (LDL). The polyunsaturated fatty acids allow for fluidity of cellular membranes. The membranes that surround cells and cell organelles contain large amounts of polyunsaturated fatty acid side-chains (Jiri *et al.,* 2018). Lipid peroxidation is a complex process

known to occur in both plants and animals. It involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids and the eventual destruction of membrane lipids, with the production of a variety of breakdown products, including alcohols, ketones, alkanes, aldehydes and ethers (Canonica *et al.,* 2003). Membrane lipids are generally amphipathic molecules, i.e. they contain hydrocarbon regions that tend to cluster together away from water, together with polar parts that like to interact with water. In animal cell membranes the dominant lipids are phospholipids, esters based on the alcohol glycerol (Lushchak *et al.,* 2009). Some membranes, particularly plasma membranes, contain significant proportions of sphingolipids and of the hydrophobic molecule cholesterol. The commonest phospholipid in animal cell membranes is lecithin (phosphatidylcholine). Free-radical processes are particularly prone to proceed via efficient chain reactions in which the initiating active radical is generated only in very low concentrations (Canonica *et al.,* 2003; Jiri *et al.,* 2018). Lipid peroxidation causes a decrease in membrane fluidity and in the barrier functions of the membranes. The many products of lipid peroxidation such as hydroperoxides or their aldehyde derivatives inhibit protein synthesis, blood macrophage actions and alter chemotactic signals and enzyme activity (Lushchak *et al.,* 2009; Bagnyukova *et al.,* 2006). Lipid peroxidation of polyunsaturated fatty acids may be enzymatic and non-enzymatic. Enzymatic lipid peroxidation is catalyzed by the lipoxygenases family, a family of lipid peroxidation enzymes that oxygenates free and esterified PUFA generating as a consequence, peroxyl radicals. Non-enzymatic lipid peroxidation and formation of lipid peroxides are initiated by the presence of molecular oxygen and is facilitated by Fe_2^+ ions (Lushchak *et al.,* 2009; Dai *et al.,* 2018).

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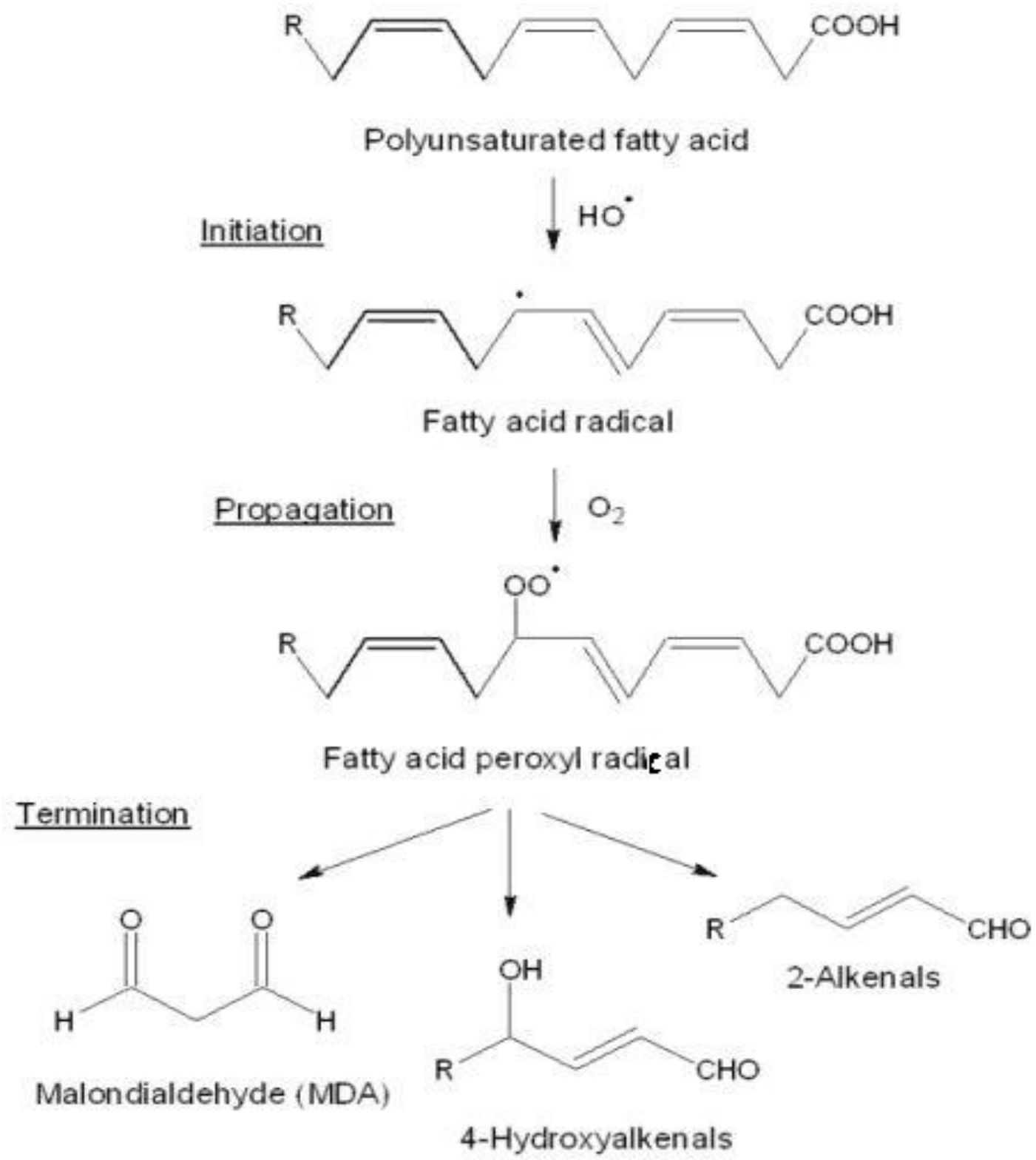


Figure 2.19: Lipid peroxidation (Lushchak *et al.*, 2009).

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals as shown in Figure 2.19. Lipid peroxidation is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds (Lushchak *et al.,* 2009; Dai *et al.,* 2018; Jiri *et al.,* 2018). Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) upon decomposition. Measurement of malondialdehyde and 4-hydroxyalkenals has been used as an indicator of lipid peroxidation (Lushchak *et al.,* 2009; Bagnyukova *et al.,* 2006). Oxidative damage in liver is associated with hepatic lipid metabolism, and may be affecting the absorption and transport mechanisms of tocopherol in this organ. In the liver, the morphological damage is could be as a result to the lipid peroxidation and the consumption of antioxidants could ameliorate it. In kidney and heart, indeed, lipid peroxidation and oxidative damage preceded necrosis. Products of lipid peroxidation such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 2-propenal (acrolein) and isoprostanes are commonly used as biomarkers of oxidative damage (Lushchak *et al.,* 2009; Dai *et al.,* 2018; Jiri *et al.,* 2018).

2.14 Antioxidants

Antioxidants are free radical scavengers and other reactive oxygen species (ROS) within the body, hence hindering the process of oxidation. Antioxidant enzymes are included in the environmental pollution assessment because of their inducibility under conditions of mild oxidative stress and their potential role in adaptation to pollutant-induced stress (Gasnier *et al.,* 2009; Gaafar *et al*., 2010). It is expected that they may be more sensitive at detecting of initial insults than such markers as histopathologies, changes in growth rates, etc. (Lushchak *et al.,* 2009; Bagnyukova *et al.,* 2006).

Cells normally defend themselves against ROS damage through the use of enzymes such as superoxide dismutase and catalase. Small molecule antioxidants such as ascorbic acid (vitamin C), uric acid, and glutathione also play important roles as cellular antioxidants. Similarly, polyphenol antioxidants assist in preventing ROS damage by scavenging free radicals. The negative effects of ROS on cell metabolism include roles in programmed cell death (apoptosis) whereas positive effects include induction of host defense genes and mobilization of ion transport systems. In particular, platelets involved in wound repair and blood homeostasis release ROS to recruit additional platelets to sites of injury. These also provide a link to the adaptive immune system via the recruitment of leukocytes (Lushchak *et al.,* 2009; Bagnyukova *et al.,* 2006).

The various roles of enzymatic antioxidants (SOD, catalase, glutathione peroxidase) and non-enzymatic antioxidants (vitamins C and E, carotenoids, lipoic acid and others) in the protection against oxidative stress can be found in numerous papers asshown in Figure 2.18 (Lushchak *et al.,* 2009; Dai *et al.,* 2018; Jiri *et al.,* 2018).

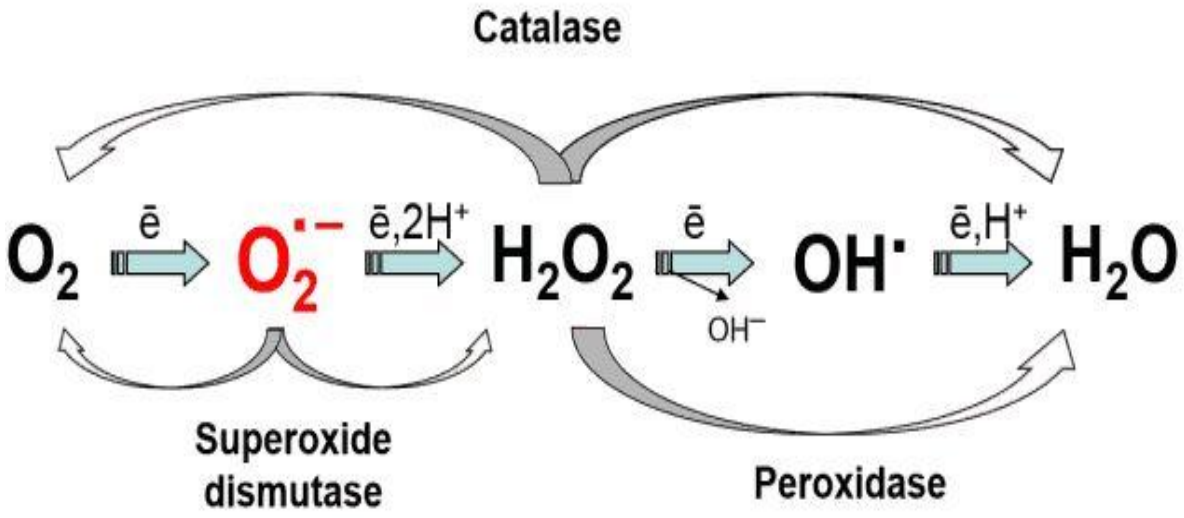


Figure 2.20: Role of catalase, peroxidase and superoxide dismutase (Dai *et al.,* 2018).

2.14.1 Catalase

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in reproductive reactions. Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second. Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long. It contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide. Hydrogen peroxide (H_2O_2) is a potent oxidizing agent that can wreak havoc in a cell. Found extensively in organisms that live in the presence of oxygen, catalase prevents the accumulation of and protects cellular organelles and tissues from damage by peroxide, which is continuously produced by numerous metabolic reactions. In mammals, catalase is found predominantly in the liver (McCourt *et al.*, 2006; Dai *et al.*, 2018).

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

All known animals use catalase in every organ, with particularly high concentrations occurring in the liver.

2.14.2 Glutathione peroxidase

Glutathione peroxidase is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage (Maser *et al.*, 1994). The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. The main reaction that glutathione peroxidase catalyzesis:

$$2GSH + H_2O_2 \xrightarrow{\text{glutathione peroxidase}} GS–SG + 2H_2O$$

GSH represents reduced monomeric glutathione, and GS–SG represents glutathione disulfide. The mechanism involves oxidation of the selenol of a selenocysteine residue by hydrogen peroxide (Maser *et al.*, 1994).

2.14.3 Superoxide dismutases

Superoxide dismutases are metalloproteins which catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. The enzyme is believed to be present in all oxygen-metabolizing cells but lacking in most obligate anaerobes, presumably because it's physiological function is to provide a defense against the potentially damaging activities of the superoxide radical generated by aerobic metabolic reactions. Thus, they are an important antioxidant defense in nearly all cells exposed to oxygen. Superoxide is one of the main reactive oxygen species in the cell. Consequently, SOD serves a key antioxidant role. Simply stated, SOD out-competes damaging reactions of superoxide, thus protecting the cell from superoxide toxicity (McCourt *et al.*, 2006; Dai *et al.*, 2018).

The functions of superoxide dismutase, catalase, glutathione peroxidase, glutathione and other antioxidants illustrated in figure 2.20 are shown to minimize the effect of xenobiotic on DNA, protein and lipid.

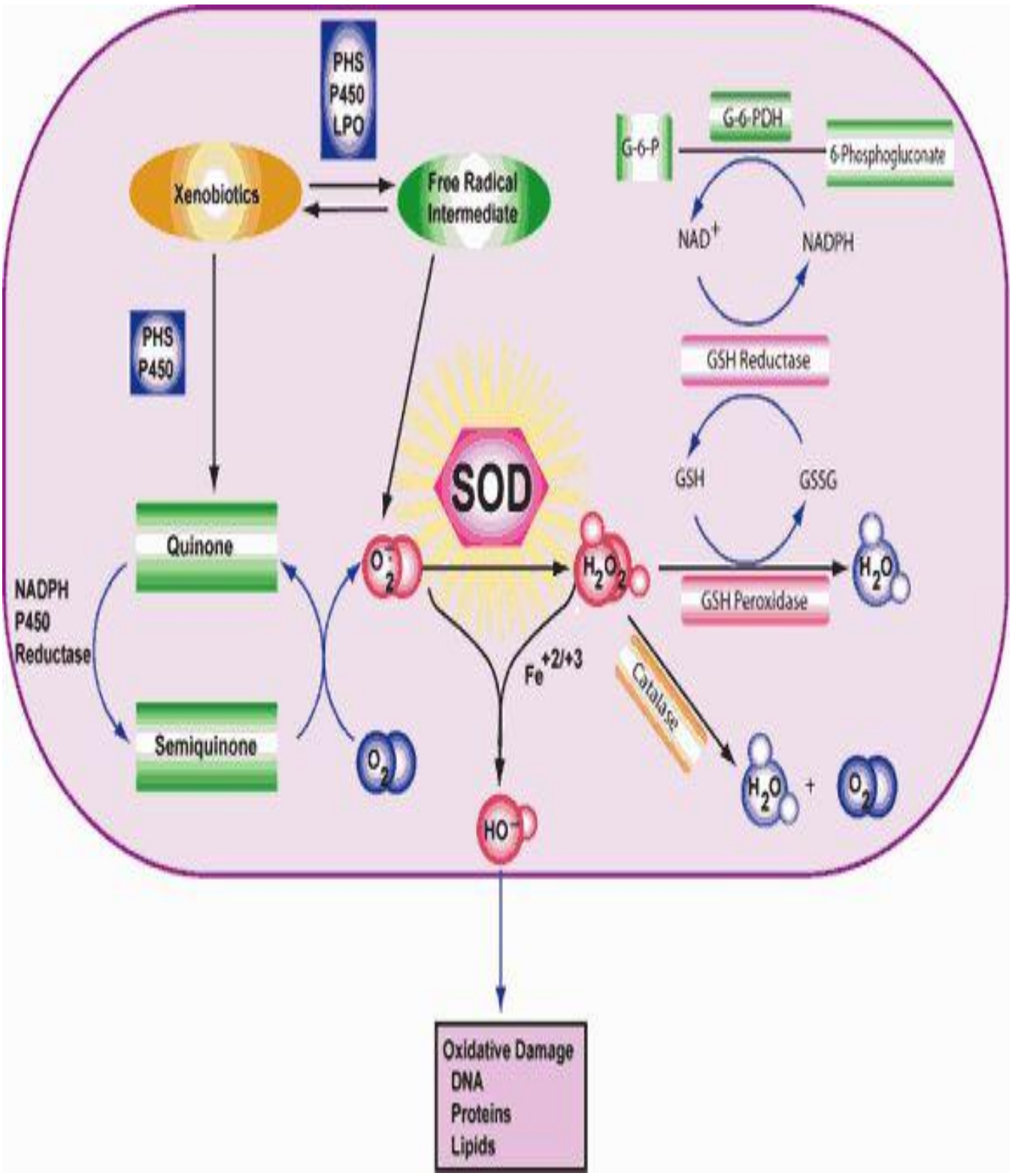


Figure 2.21: function of superoxide dismutases, catalase, (McCourt *et al.*, 2006).

Enzymatic antioxidants	Enzyme code	Reaction catalyzed	Subcellular location
Superoxide dismutase (SOD)	1.15.1.1	$O_2^- + O_2^- + 2H^+ \longrightarrow 2H_2O_2 + O_2$	Peroxisomes, mitochondria, cytosol and chloroplast
Catalase (CAT)	1.11.1.6	$2H_2O_2 \longrightarrow O_2 + 2H_2O$	Peroxisomes and mitochondria
Ascorbate peroxidase (APX)	1.11.1.11	H_2O_2 + AA $\longrightarrow$ $2H_2O$ + DHA	Peroxisomes, mitochondria, cytosol and chloroplast
Monodehydroscorbate reductase (MDHAR)	1.6.5.4	2MDHA + NADH $\longrightarrow$ 2AA+NAD	Mitochondria, cytoplasm and chloroplast
Dehydroascorbate reductase (DHAR)	1.8.5.1	DHA + 2GSH $\longrightarrow$ AA + GSSG	Mitochondria, cytoplasm and chloroplast
Glutathione reductase (GR)	1.6.4.2	GSSG + NADPH $\longrightarrow$ 2GSH + $NADP^+$	Mitochondria, cytoplasm and chloroplast
Glutathione peroxidase (GPX)	1.11.1.7	H_2O_2+DHA $\longrightarrow$ $2H_2O$+GSSG	Mitochondria, cytoplasm, chloroplast and ER

Table 2.1 Enzymatic antioxidants

Non-enzymatic Antioxidants	Function	Subcellular location
Ascorbic Acid (AA)	Detoxifies H_2O_2 via action of APX	Cytosol, chloroplast, mitochondria, peroxisome, vacuole, and apoplast
Reduced Glutathione (GSH)	Acts as a detoxifying co-substrate for enzymes like peroxidase, GR, GST	Cytosol, chloroplast, mitochondria, peroxisome, vacuole, and apoplast
Tocopherol	Guards against and detoxifies products of membrane lipid peroxidation	mostly in membrane
Carotenoids	Quenches excess energy from the photosystems, LHCS	chloroplast
Flavonoids	Direct scavengers of hydrogen peroxidase, hydroxyl and singlet oxygen	vacuole
Proline	Efficient scavenger of hydroxyl and singlet oxygen, prevent damage due to lipid peroxidation	Mitochondria, cytosol, and chloroplast

Table 2.2 Non-enzymatic antioxidants (McCourt *et al.*, 2006).

2.14.4 Lactate dehydrogenase

Lactate dehydrogenase (LDH, EC 1.1.1.27) is an enzyme that catalyses the oxidation of L-lactate to pyruvate with nicotinamide-adenine dinucleotide (NAD) $^{+}$ as hydrogen acceptor. The conversion of lactate to pyruvate is an important step in energy production in cells. Lactate dehydrogenase converts pyruvate, the final product of glycolysis, to lactate when oxygen is absent or in short supply and it performs the reverse reaction during the Cori cycle in the liver. At high concentrations of lactate, the enzyme exhibits feedback inhibition, and the rate of conversion of pyruvate to lactate is decreased. Tissue breakdown releases LDH, therefore LDH can be measured as a surrogate for tissue breakdown. Some of the organs relatively rich in LDH are the heart, kidney, liver, and muscle. As cells die, their LDH is released and finds its way into the blood (Majewski *et al.*, 2014).

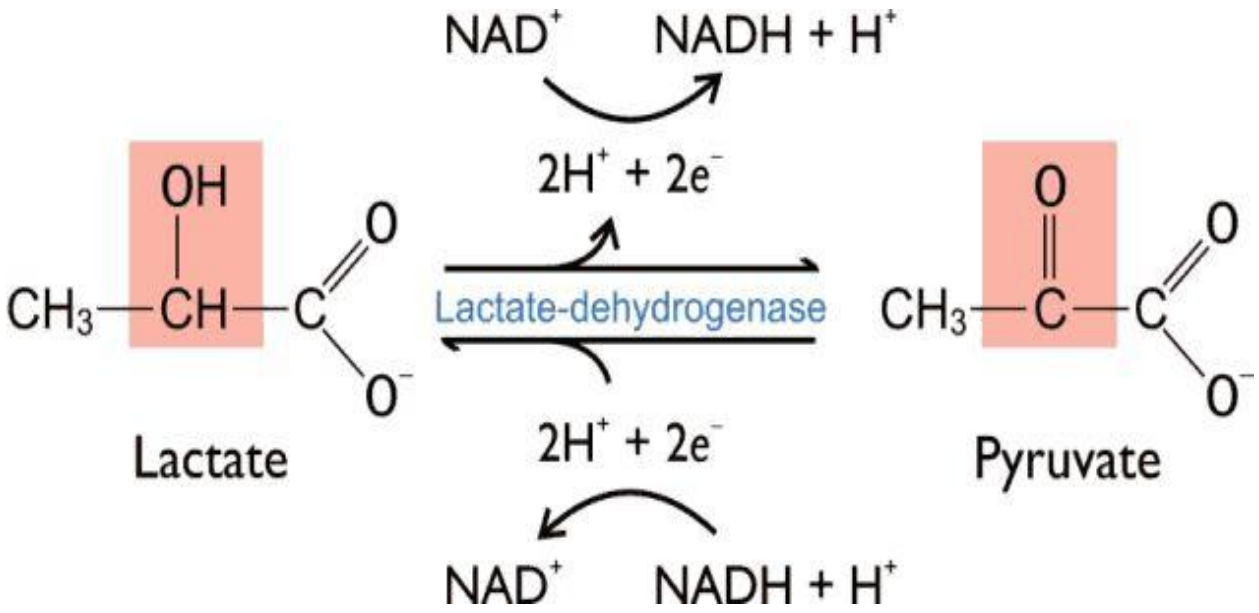


Figure 2.22: Reaction of lactate dehydrogenase (Majewski *et al.*, 2014).

2.15 Liver function test

Liver function tests are common tests that are used to detect damage and diseases of the liver and to determine how well the liver is working. Liver function test (LFT) is a group of blood tests that measure certain enzymes or proteins in the blood. They are used to evaluate and monitor liver diseases and damages on the liver by toxicants. Some liver function test parameters may not necessary prove that the liver is damaged. This isbecause those parameters could as well be detected while checking other organs, like the evaluation the enzymes released by the liver (Paetoe *et al., 2012*).

Liver function test include the following: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate transaminase (AST) (Ujowundu *et al.,* 2014), gamma-glutamyl transpeptidase (GGT).Prothrombin time, also expressed as international normalized ratio (INR), serum bilirubin, urine bilirubinalbumin, alpha-1 antitrypsin and so on (Paetoe *et al., 2012*).

2.15.1 Alkaline phosphatase

Alkaline phosphatase (ALP) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. As the name suggests, alkaline phosphatases are most effective in an alkaline environment. Abnormal levels of alkaline phosphatase in the blood most often indicate a problem with the liver, gall bladder, or bones (Ujowundu *et al.,* 2014). However, they may also indicate malnutrition, kidney tumors, or a serious infection. Alkaline phosphatase test helps to detect liver disease or bone disorders. Damaged liver cells release increased amounts of ALP into the blood. This test is often used to detect blocked bile ducts because ALP is especially high in the edges of cells that join to form bile ducts (Paetoe *et al., 2012*; Roustan *et al*., 2014; Sinhorin *et al*., 2014).

2.15.2 Alanine aminotransferase (ALT)

Alanine aminotransferase (ALT) is a cytosolic enzyme primarily localized in the liver and small amount in the kidney. It is also called alanine transaminase. Alanine transaminase (ALT) catalyzes the transfer of the amino group from alanine to α-ketoglutarate leading to the formation of pyruvate and glutamate. Increase in activity of the enzyme in serum or plasma could come as a result of damaged cells (Paetoe *et al., 2012*; Sinhorin *et al*., 2014). Alanine aminotransferase is measured to determine if the liver is damaged or diseased. Low levels of ALT are normally found in the blood. But when the liver is damaged or diseased, it releases ALT into the bloodstream, which makes ALT levels go up such that most increase in ALT level is caused by liver damage. Alanine aminotransferase was originally referred to as serum glutamic pyruvic transaminase (SGPT) (Paetoe *et al., 2012*; Sinhorin *et al*., 2014).

α-ketoglutarate + alanine ⇌glutamate + pyruvate

2.15.3 Aspartate transaminase

Aspartate transaminase (AST) catalyzes the reversible transfer of an α-amino group between aspartate and glutamate and, as such, is an important enzyme in amino acid metabolism. Aspartate transaminase catalyzes the conversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate. Aspartate transaminase is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells. An aspartate aminotransferase (AST) test measures the amount of this enzyme in the blood Low levels of AST are normally found in the blood. Serum AST level are commonly measured clinically as biomarkers for liver health (Ujowundu *et al.,* 2014). When body tissue or an organ such as the heart or liver is diseased or damaged, additional AST is released into the bloodstream. The amount of aspartate transaminase in the blood is directly

related to the extent of the tissue damage (Lushchak *et al.*, 2009; Al-Shebly and Mansour, 2012; Dai *et al.*, 2018; Jiri *et al.*, 2018).

2.15.4 Bilirubin

Bilirubin is the yellow breakdown product of normal haem catabolism and it is produced when the liver breaks down old red blood cells which contain haemoglobin as seen in Figure 2.24. Bilirubin is excreted in bile and urine, and elevated levels may indicate certain diseases. It is responsible for the yellow colour of bruises and the yellow discoloration in jaundice. It is also responsible for the brown colour of faeces, through its conversion to stercobilin, and the background straw-yellow colour of urine through its breakdown product urobilin. Bilirubin is an orange-yellow pigment, a waste product primarily produced by the normal breakdown of haem. Haem is a component of hemoglobin, which is found in red blood cells (RBCs). Bilirubin is ultimately processed by the liver to allow its elimination from the body. Any condition that accelerates the breakdown of RBCs or affects the processing and elimination of bilirubin may cause an elevated blood level. Bilirubin test measures the amount of bilirubin in a blood sample. Total bilirubin and direct bilirubin levels are measured directly in the blood, whereas indirect bilirubin levels are derived from the total and direct bilirubin measurements (Lushchak *et al.*, 2009; Al-Shebly and Mansour, 2012; Dai *et al.*, 2018; Jiri *et al.*, 2018).

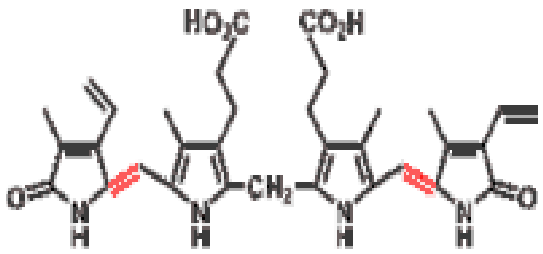


Figure 2.23 Chemical structure of bilirubin (Mengel et al., 2005).

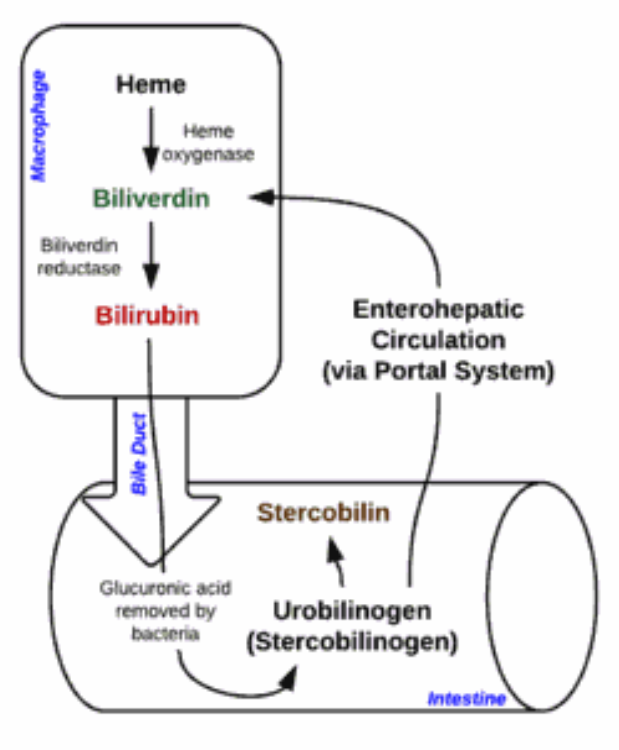


Figure 2.24: showing haem catabolism to form bilirubin (Mengel *et al*., 2005).

2.16 HAEMATOLOGY

Hematology is the branch of medicine concerned with the study, diagnosis, treatment, and prevention of diseases related to blood. Hematology refers to the study of the morphology of the cellular elements of the blood the red cells (erythrocytes), white cells (leucocytes), and the platelets (thrombocytes) and the use of these results in the diagnosis and monitoring of disease (Paetoe *et al., 2012*; Roustan *et al*., 2014; Sinhorin *et al*., 2014). Hematological studies are useful in the diagnosis of many diseases as well as investigation of the extent of damage to blood (Okomoda *et al*., 2010). Hematological studies are of ecological and physiological interest in helping to understand the relationship of blood characteristics to the environment (Okomoda *et al*., 2010).

2.17 Blood

Blood is a specialized body fluid. It is composed of pale yellow fluid called plasma in which are suspended red cells (erythrocytes), white cells (leukocytes), and platelets (thrombocytes). Plasma forms about 55% of the blood volumes and contains water (95%) and many solutes, including proteins, and waste products for excretion. Blood flows through every organ of the body, providing effective communication between tissues (Okomoda*et al*., 2010). It is kept in continuous circulation by the pumping action of the heart, flowing through arteries which carry the oxygenated (bright red) blood from the heart to all parts of the body, and veins which carry the deoxygenated (dark red) blood from different parts of the body back to the heart and to the lungs. The same amount of blood that pumped out of the heart returns to it (Buckley *et al.,* 1976).

2.18 Haematological parameters

This includes red blood cell count, white blood cell count, haemoglobin and so on. Hematological parameters are good indicators of the physiological status of animals (Okomoda *et al*., 2010). Hematological parameters are those parameters that are related to the blood and blood forming organs (Waugh *et al.,* 2001).

2.18.1 White blood cells (WBC) Count

White blood cell count is the number of leukocytes measured directly, multiplied by the calibration constant, and expressed as n x 10^3 cells/µL. White blood cells (WBCs) or leukocytes are important to body defense against disease form a protective, movable army that helps defend the body against damage by bacteria, viruses, parasites, and tumor cells (Okomoda *et al*., 2010). When the white blood cell count is low, it is easier to get an infection and harder to get over it.

They are far less numerous than red blood cells. White blood cells are the only complete cells in blood; that is; they contain nuclei and the usual organelles. White blood cells are able to slip into and out of the blood vessels (by process called diapedesis). The circulatory system is simply their means of transportation to areas of the body where their services are needed for inflammatory or immune responses. In addition, WBCs can locate areas of tissue damage and infection in the body by responding to certain chemicals that diffuse from the damaged cells. This capability is called positive chemotaxis. Once they have "caught the scent," the WBCs move through the tissue spaces by ameboid motion (forming of cytoplasmic extensions that help move along). By following the diffusion gradient, they pinpoint areas of tissue damage and rally round in large numbers to destroy microorganisms or dead cells. Whenever WBCs mobilize for action, the body tends to speed up their production (Buckley *et al.*, 1976).

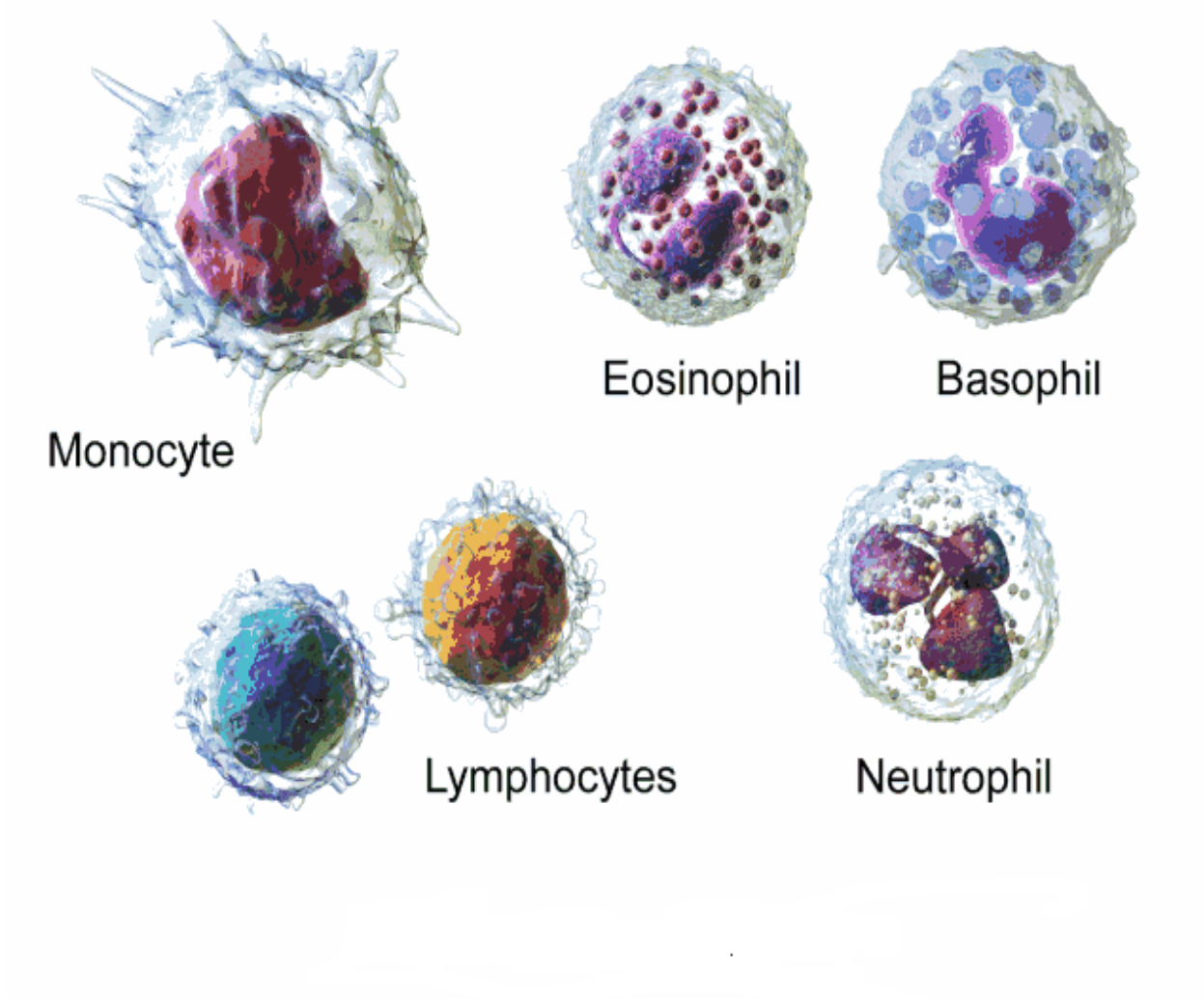


Figure 2.25 five kinds of white blood cell (Cazenave *et al.*, 2005).

There are usually five kinds of white blood cells found in the blood stream as shown in Figure 2.25. In a normal adult about three-fourths (¾) are neutrophils, about one-fourth (¼) are lymphocytes, and there will be a few of the other three (monocytes, eosinophils and basophils). Most analyzers group everything that is not either a neutrophil or a lymphocyte into a category called "**MID**" meaning "mid-range" these cells are between neutrophils and lymphocytes in size (Cazenave *et al.*, 2005; Boveris *et al.*, 2008; Lushchak *et al.*, 2009; Al-Shebly and Mansour, 2012; Dai *et al.*, 2018; Jiri *et al.*, 2018).

2.18.2 Red blood cell (RBC) count

Red blood cell count is the number of erythrocytes measured directly, multiplied by the calibration constant, and expressed as n x 10^6 cells/µL. Red blood cells carry oxygen and remove waste from the body. The number of red blood cells is a measure of the blood's oxygen-carrying capacity. Mature red cells are non- nucleated and are shaped like flattened, bilaterally indented spheres, a shape often referred to as ”biconcave disc” with a diameter 7.0-8.0µm and thickness of 1.7-2.4µm. These cells also have a protein in them called haemoglobin, which is what makes red blood cells red. Red blood cell production occurs in the red bone marrow. A deficiency in red blood cells or quantity of hemoglobin results in anemia (Okomoda *et al*., 2010).

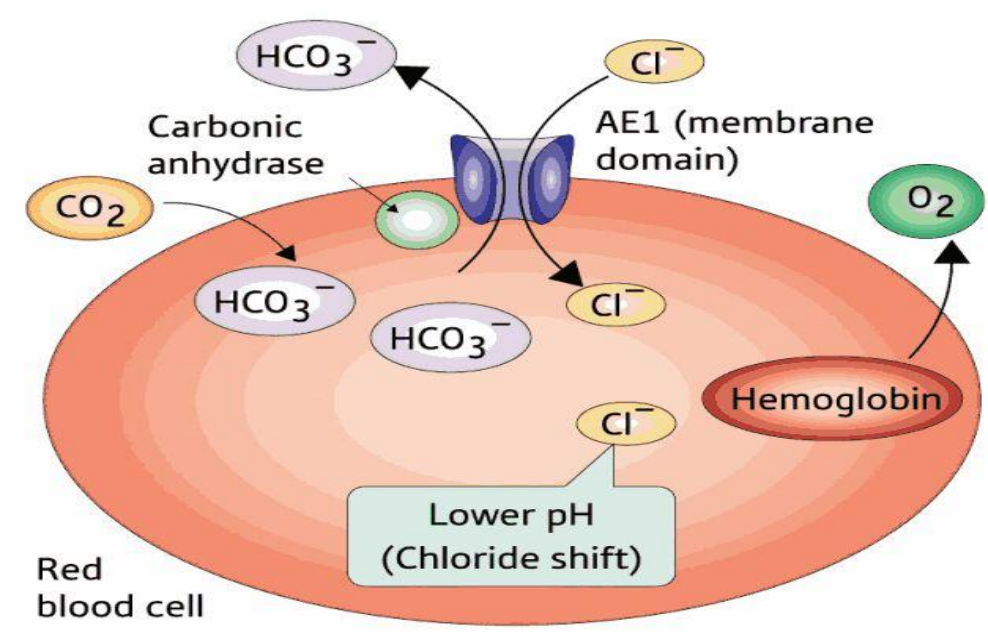


Figure 2.26 showing the structure of the red blood cell and oxygen transfer (Cazenave *et al.*, 2005).

2.18.3 Haemoglobin

Haemoglobin (Hb), the main component of the red blood cell, is a conjugated protein that serves as the vehicle for the transportation of oxygen and carbondioxide. When fully saturated, each gram of haemoglobin holds 1.34ml of oxygen. A spheroidal haem protein having four subunits each consisting of a globular protein non-covalently bound, with an embedded haem group. The globular protein unit of Hb is made up of two identical pairs of polypeptide chains. The functional properties of haemoglobin molecules are primarily determined by the characteristic folds of the amino acid chains of the globin proteins. The functions of haemoglobin include: Transport of O_2 from the lungs to the tissues and of CO_2 in the reverse direction. Figure 2.25 illustrates the role of haemoglobin in oxygen transfer. Figure 2.24 also shows the function of haemoglobin (Cazenave *et al.*, 2005).

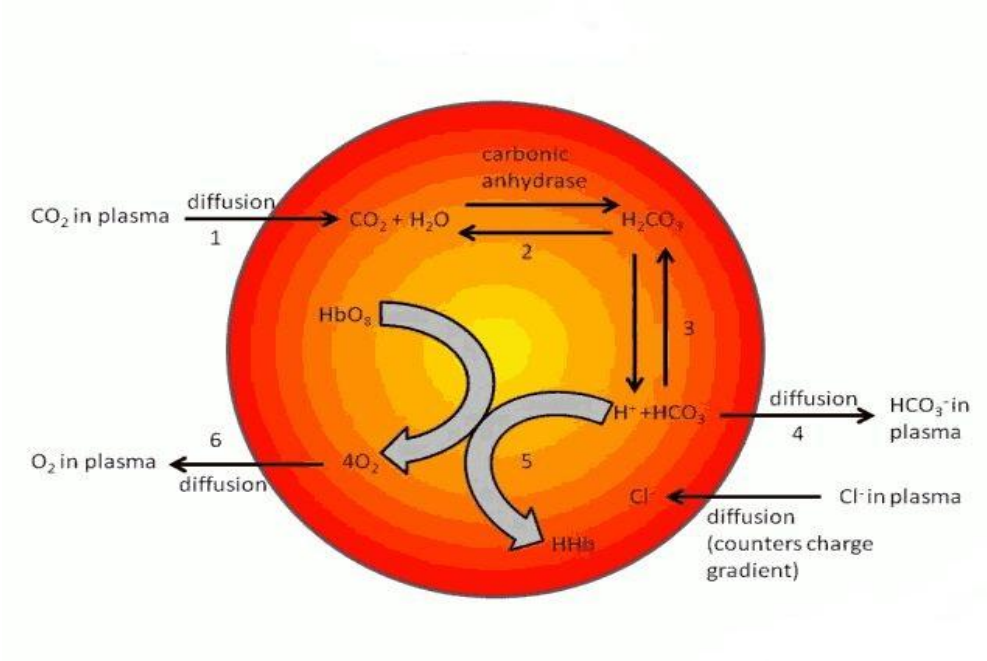


Figure 2.27 Role of hemoglobin (Cazenave *et al.*, 2005).

2.18.4 Platelet

These are small, non-nucleated, round/oval cells/cell fragments that stain pale blue and contain many pink granules. Platelet count is the number of thrombocytes derived from the platelet histogram and multiplied by a calibration constant. This number is expressed as: n x 10^3 cells/µL. Platelets also called thrombocyte are parts of the blood that help the blood clot. It is produced from megakaryocytes in the bone marrow. Their primary functions are related to coagulation of blood. Because of their adhesion and aggregation capabilities they can occlude small breaks in blood vessels and prevent escape of blood. They are smaller than red and white blood cells. Platelet the smallest of the formed elements in blood, a disk-shaped, non-nucleated blood element with a fragile membrane, formed in the redbone marrow. The rate of platelet formation seems to be governed by the amount of oxygen in the blood and the presence of nucleic acid derivatives from injured tissue. A platelet count is a lab test to measure the number of platelets present in the blood. A normal platelet count range is 140 to 400 K/uL (Cazenave *et al.,* 2005).

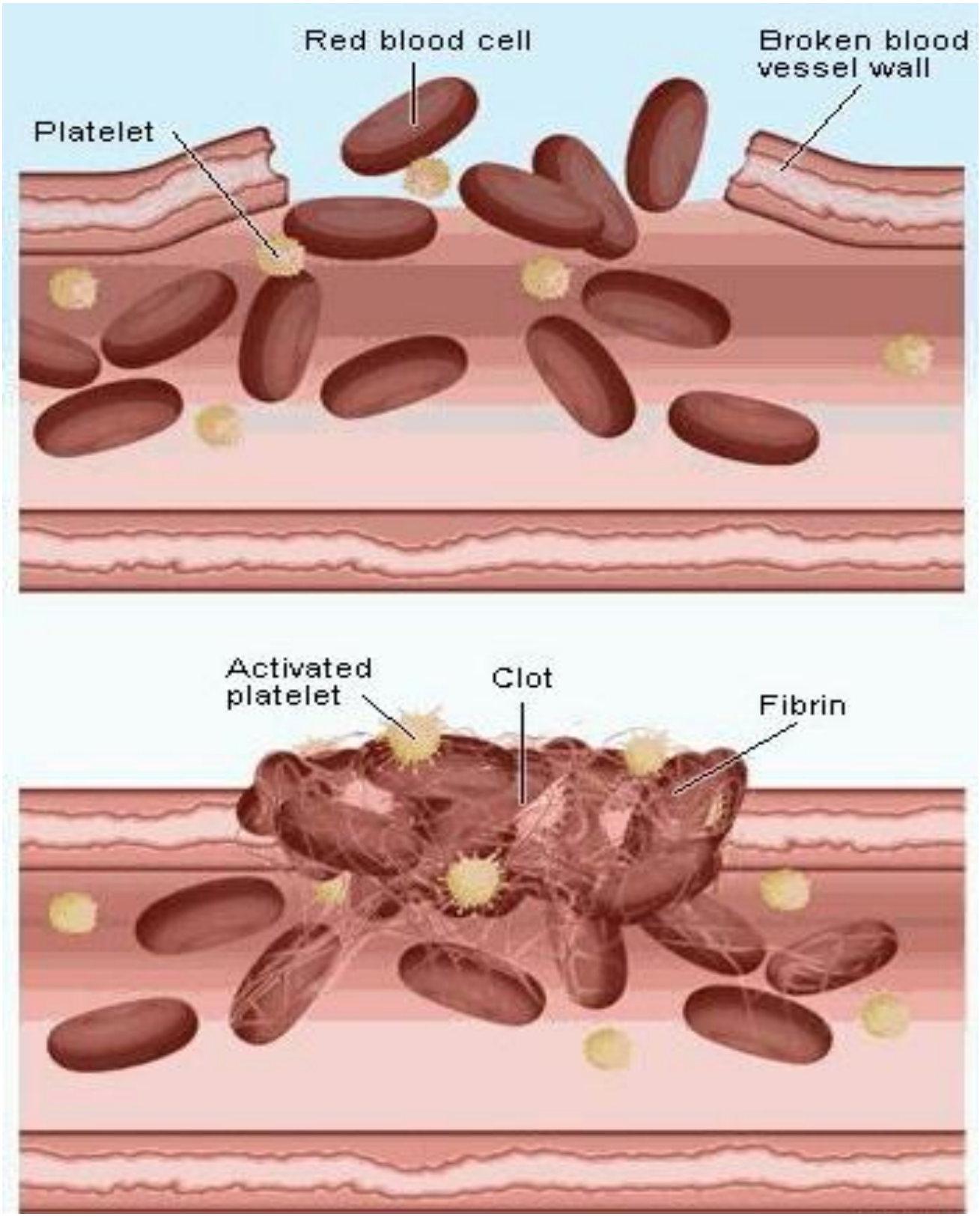


Figure 2.28: Blood clotting (Schuetz *et al.*, 2009)

2.18.5 Procalcitonin

Procalcitonin (PCT) the precursor of the hormone calcitonin is produced by C-cells of the thyroid gland or neuroendocrine cells in the lung or intestine (Schuetz *et al.,* 2009). The advantages of PCT over older markers include: specificity for bacterial infection (verses inflammation in general), the rapidity of its rise after an insult (6 hours), the rapid decline with immune control on infection (half-life of 24 hours), excellent correlation with severity of illness (higher levels in more severely ill), and the lack of impact of anti- inflammatory and immunosuppressive states on production (Okomoda *et al*., 2010).Procalcitonin (PCT) may be useful in differentiation of bacterial verses viral respiratory tract infection, determination of antibiotic treatment length in respiratory infections, diagnosis, risk stratification, and monitoring of sepsis and septic shock, monitoring response to antibacterial therapy, levels of PCT rise dramatically during bacterial infections, whereas low levels were detected during viral infections or non-infectious febrile conditions (Stolz *et al.*, 2009; Okomoda *et al*., 2010).

2.18.6 Hematocrit

The hematocrit (HCT) also called the packed cell volume (PCV), measures how much of total blood count is made up of red blood cells i.e. Hematocrit is the relative volume of packed erythrocytes to whole blood. It is one of the simplest, most accurate and most valuable of all hematological investigations. It is of greater reliability and usefulness than the red cell count that is performed manually. In conjunction with estimation of hemoglobin and RBC count, knowledge of PCV enables the calculation of the red cell indices (absolute values that indicate red cell volume, hemoglobin content and concentration) that are widely used in the classification of anemia. computed as:

Hct (%) = RBC × MCV/10

It is considered an integral part of a complete blood count result, along with hemoglobin concentration, white blood cell count, and platelet count. Because the purpose of red blood cells is to transfer oxygen from the lungs to body tissues, a blood sample's hematocrit—the red blood cell volume percentage—can become a point of reference of its capability of delivering oxygen. Typically, a higher hematocrit level signifies the blood sample's ability to transport oxygen. A low hematocrit level is a sign of a low red blood cell count. A low hematocrit with a low mean corpuscular volume (MCV) with a high RDW suggests a chronic iron-deficient anemia resulting in abnormal hemoglobin synthesis during erythropoiesis. The PCV is also used to screen for anemia when it is not possible to measure hemoglobin, and to diagnose polycythemia vera and to monitor its treatment (Schuetz *et al.*, 2009).

2.18.7 Mean corpuscular volume

Mean corpuscular volume (MCV) is the average volume of red cells in a specimen. It can be directly measured by automated hematology analyzer, or it can be calculated from hematocrit (Hct) and the red blood cell count (RBC) as follows: It is given by:

MCV (fl) = PCV (l/l) No. of RBC/l

MCV is elevated or decreased in accordance with average red cell size; i.e low MCV indicates small average RBC size (microcytic anemia), normal MCV indicates normal average RBC size, and high MCV indicates large average RBC size (macrocytic anemia). It is useful in the morphological characterization of anemia. MCV is increased in macrocytic anemia and decreased in iron deficiency anemia, thalassemia and microcytic anemia (Stolz *et al.*, 2009.)

2.18.8 Mean Corpuscular Haemoglobin

Mean corpuscular haemoglobin (MCH) or mean cell haemoglobin is the weight of hemoglobin in the average erythrocyte count, computed as:

MCH = Hgb/RBC x 10

It is a measurement of the average amount of oxygen-carrying haemoglobin inside a red blood cell. Macrocytic RBCs are large so tend to have a higher MCH, while microcytic red cells would have a lower value. A complete blood count can be used to monitor MCH levels in blood. MCH is increased in macrocytic anemia and is decreased in microcytic anemia and iron deficiency anemia (Schuetz *et al.*, 2009).

2.18.9 Mean corpuscular hemoglobin concentration

Mean cell haemoglobin concentration or mean corpuscular haemoglobin concentration (MCHC) is the average weight of haemoglobin in a measured dilution, It is calculated by dividing the hemoglobin by the hematocrit, It is conventionally expressed in femtoliters per red cell, computed as:

$$\text{MCHC (g/l)} = \frac{\text{Hb (g/l)}}{\text{PCV (l/l)}}$$

Mean corpuscular hemoglobin concentration (MCHC) is the average concentration of hemoglobin in a given volume of packed red blood cells, or in other words, the ratio of hemoglobin mass to the volume of red cells. It is reported as part of a standard complete blood count. MCHC is diminished ("hypochromic") in microcytic anemia, normal (" normochromic ") in macrocytic anemia and elevated in hereditary spherocytosis, sickle cell disease and homozygous hemoglobin C disease. The MCHC is calculated by multiplying the amount of

hemoglobin by 100 and dividing that number by the amount of packed red blood cells. The number of packed red blood cells is also known as the hematocrit (Schuetz *et al.,* 2009; Okomoda *et al*., 2010).

2.18.10 Mean platelet volume

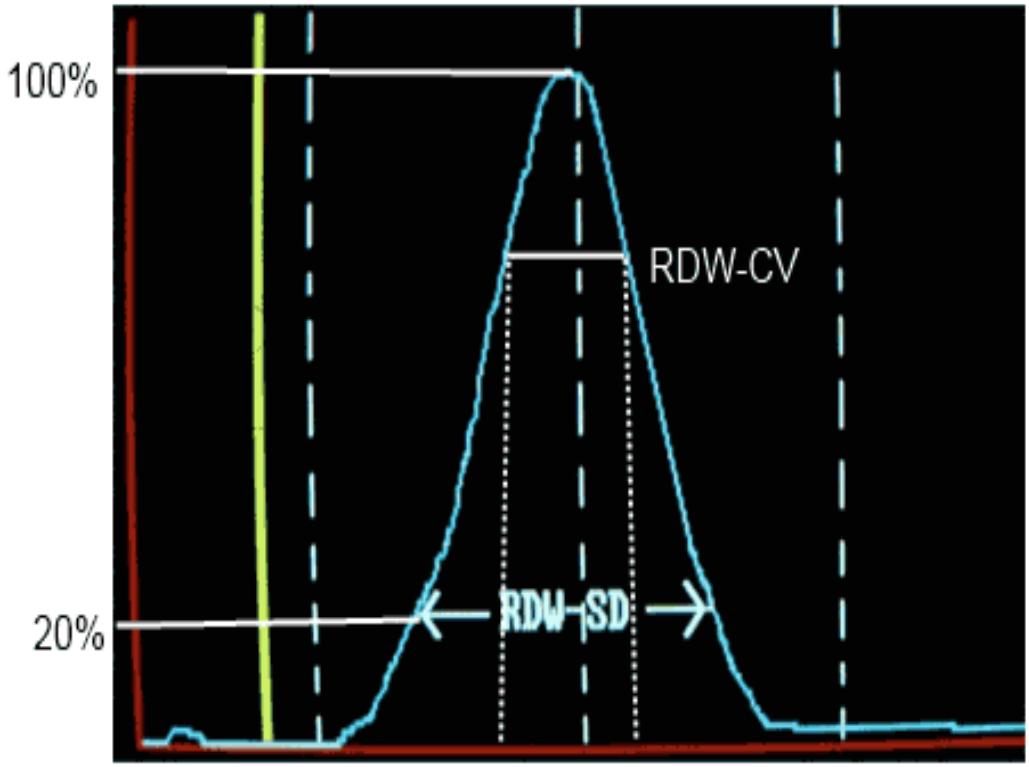
Mean platelet volume (MPV) is a machine-calculated measurement of the average size of platelets found in blood and is typically included in blood tests as part of the CBC. Mean Platelet Volume is the average volume of individual platelets derived from the PLT histogram. It represents the mean volume of the PLT population under the fitted PLT curve multiplied by a calibration constant, and expressed in femtoliters. Since the average platelet size is larger when the body is producing increased numbers of platelets, the MPV test results can be used to make inferences about platelet production in bone marrow or platelet destruction problems. MPV is higher when there is destruction of platelets. A typical range of platelet volumes is 9.7–12.8 fl (femtolitre) (Schuetz *et al.*, 2009).

2.18.11 Red Blood Cell Distribution Width (RDW)

Red blood cell distribution width (RDW) is a measurement derived from the red blood cell distribution curves generated on automated hematology analyzers and is an indicator of variation in red blood cell (RBC) size within a blood sample. The RDW is used along with the indices (MCV, MCH, MCHC) to describe a population of RBCs. The RDW measures the deviation of the RBC width, not the actual width or size of individual cells. The two RDW measurements currently in use are the red cell distribution width - coefficient of variation (RDW-CV) and the red cell distribution width standard deviation (RDW-SD).The RDW-CV is a calculation based on both the width of the distribution curve and the mean cell size. It is calculated by dividing the

standard deviation of the mean cell size by the MCV of the red cells and multiplying by 100 to convert to a percentage. A normal range for the RDW-CV is approximately 11.0 - 15.0%. Because it is a calculation, the RDW-CV is dependent not only on the width of the distribution curve but also the MCV of the red cell population and may not always reflect the actual variation in red cell size. The RDW-SD is an actual measurement of the width of the red cell distribution curve in femtoliters (fL). The width of the distribution curve is measured at the point that is 20% above the baseline. Since the RDW-SD is an actual measurement, it is not influenced by the MCV and more accurately reflects the red cell size variance. The normal RDW-SD range for adults is 40.0 - 55.0fL (Okomoda *et al*., 2010).

PDW -- Platelet Distribution Width



RDW-CV is a calculation based on both the width of the distribution curve and the mean cell size.

$$\text{RDW-CV} = \frac{\text{1 SD}}{\text{MCV}} \times 100$$

RDW-SD is an actual measurement of the width of the red cell distribution curve in femtoliters (fL) at the point that is 20% above the baseline.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

The chemicals used in this study were

Chemical	Manufacturer
EDTA	
NADP	FLUKA, St. Louis, U.S.A
Hydrogen Peroxide	LOBACHEM, Kenilworth, U.S.A
Ascorbic Acid	BDH, Darmstadt, Germany
Iron (III) Chloride	BDH, Darmstadt, Germany
Thiobabituric acid	FLUKA, St. Louis, U.S.A
Sodium Hydroxide	LOBACHEM, Kenilworth, U.S.A
Sulphanilic Acid	FLUKA, St. Louis, U.S.A
Nitrite	FLUKA, St. Louis, U.S.A
Tertrate	FLUKA, St. Louis, U.S.A

Herbicide Uproot (glyphosate 360g/L) purchased from the Ministry of Agriculture Abia State Nigeria.Other reagents for the assay were Commercial kits and products of randox laboratories ltd, United Kingdom, Biosystems S.A. Barcelona Spain and Teco diagnostics, lakeview Avenue Anaheim.

3.1.2 Equipment

The equipment used in this study were

Equipment	Manufacturer
Biochemical analyzer	LABTECH, Kenilworth, U.S.A
Electronic weighing balance	METTLER PT320, P160, Princeton, U.S.A
Spectrophotometer	TURNER, 390, U.S.A

Digital pH meter	LABTECH, Kenilworth, U.S.A
Bench Centrifuge	UNISCOPE, U.S.A
Rotary Evaporator	NYC (U.S.A)
Water Bath	UNISCOPE, U.S.A
Electric Heater	UNISCOPE, U.S.A
Stainless Metal Incubator	TURNER, 390, U.S.A

3.1.3 Experimental animals

Twenty (20) adult male *Clarias gariepinus* weighing 425–430g were purchased from Fishery and Aquaculture Department of the Federal University of Technology Owerri (FUTO). The sex of the fish was identified by Mr Ezeafulukwe from Fishery and Aquaculture Department of the Federal University of Technology Owerri, by observing the genital papilla of the fishes. The male *Clarias gariepinus* have a distinct sexual papilla located above the anus. All animals received professional humane care in compliance with the guidelines of Ethical Animal Handling (Oliveira *et al.,* 2002). They were housed in aquarium and left to acclimatize for 16 days with laboratory condition at the Department of Biochemistry animal house. The animals were maintained on standard fish feed.

3.2 Methods

3.2.1 Experimental design

Groups	A (300 mg/l)	B (60 mg/l)	C (10 mg/l)	D (control)
Number of fish	5 fish	5 fish	5fish	5 fish

The study was designed as shown in Table 3. Twenty (20) fishes were weighed separated into four groups of five fishes, each were placed into 100 liter aquarium filled with water and allowed to acclimatize for 16days before exposing it to glyphosate for 9 days. Prior to sacrifice the fish was anaesthetized using cool water temperature. A 5ml syringe was inserted under the skin of the ventral midline below the anal opening, the needle was pushed towards the vertebral column until it reaches the base and then the plunger was pulled to to draw the blood. Afterwards the test tube containing the clothed blood was centrifuged at 500 rpm for five minutes to enable complete separation of the serum. Subsequently they were sacrificed to obtain the liver, kidney and gill samples.

Also the hepatic tissue, kidney and gill samples obtained were excised, weighed and homogenized respectively in KCL (10 mM) phosphate buffer (1.15 %) with ethylene diamine tetra acetic acid (EDTA, Ph 7.4) and centrifuged at 2,000 xg for 60 minutes to obtain the supernatant used for the enzyme assay (catalase, superoxide dismutase, glutathione peroxidase and malondialdehyde).

3.2.2 Biochemical studies

3.2.2.1 Hormonal analysis

3.2.2.1.1 Determination of testosterone

Principle

The essential reagent required for enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing biotinylated antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and enzyme-antigen conjugate for a limited number of antibody binding sites.

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs.

$AgAb_{Btn}$ + $^{Enz}AgAb_{Btn\_}$+ $Streptavidin_{cw}$ => Immobilized $complexstreptavidin_{cw}$ = Streptavidin immobilized on well

Immobilized complex = sandwich complex bound to the solid surface.

The enzyme activity in the antibody is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve is generated from which the antigen concentration of unknown is ascertained.

Procedure

The microplates' well was formatted for each serum reference, control and fish specimen to be assayed in duplicate. Unused microwell strips were placed back into the aluminum bag, seal and stored at $8^{o}C$.

Then, 0.010 ml (10 µl) of the appropriate serum reference, control or specimen was pipetted into the assigned well.

Also 0.050 ml (50µl) of the working testosterone enzyme reagent was added to all wells.

The microplates were swirled gently for 30 seconds to mix.

Then, 0.050ml (50 µl) of testosterone biotin reagent was added to all wells.

The microplates were swirled gently for 30 seconds to mix.

It was covered and incubated for 60 mins at room temperature.

The contents of the microplate were discarded by decantation and the plate blotted dry with absorbent paper.

Also 350µl of wash buffer was added to it before decanting. It was repeated two (2) additional times for a total of three (3) washes. It was decanted then washed and repeated two (2) additional times.

Furthermore 0.100 ml (100 ul) of working substrate solution was added to all wells before incubating at room temperature (27 ^{o}C) for 15 mins.

Then, 0.050 ml (50 µl) of stop solution was added to each well and it was gently mixed for 15 seconds.

Absorbance in each well was read at 450 nm in a microplate reader. The result was read within 30 mins of adding the stop solution. The concentration of the sample was later extrapolated from the table on the manual in the reagent.

3.2.2.1.2 Determination of thyroid stimulating hormones (TSH)

The Determination of thyroid stimulating hormones (TSH) was done following the ELISA method descrbed by Pierce (1971) for the quantitative *in-vitro* determination of thyroid stimulating hormones concentration in serum using teco diagnostic ELISA test kit (Anaheim, CA).

Principle

In the test the target antigen TSH, in the sample is immobilized through binding between the streptavidin coated onto the walls of the micro plate and the biotin molecules that is part of the biotinylated monoclonal antibody added in the enzyme-conjugate, the target antigen and the biotinylated antibody forms a sandwich complex on the walls of the micro plate after a period of incubation.

After a passing through a washing step that removes any unbound molecules, a substrate is added to react with the enzyme-conjugate antibody and develop a blue colour during the incubation period. The intensity of the resulted colour is directly proportional to the concentration of the target antigen, TSH, present in the serum sample. After the addition of the stop solution, the concentration of TSH can be quantified using this correlation.

Procedure

The microplates' well was formatted for each serum reference, control and fish specimen to be assayed in duplicate. Unused microwell strips were placed back into the aluminum bag, seal and stored at $8^{o}C$.

Then, 50 µl of T_3 standard solution, sample and control reagent were pipette into appropriate wells.

Furthermore, 100 µl of working conjugate reagent was added into each wells, mixed thoroughly for 30 seconds and incubated at room temperature for 60 minutes

The incubated mixture was discarded by decantation and the plate blotted dry with absorbed paper.

Also, 300 µl of working wash solution was pipette and decanted into the microplates' this was repeated for a total of three washes.

Then, 100 µl Tetramethylbenzidine (TMB) solution was added into each well, gently mixed for 10 seconds and incubated at room temperature in the dark for 15 minutes without shaking.

The reaction was stopped by adding 50 µl of the stop solution to each well and gently mixed for 10 -20 seconds.

The absorbance was read at 450 nm with a micro titter reader within 30 minutes and the concentration of TSH from the manual (leaflet) in the test kit.

3.2.2.1.3 Determination of thyroxin (T_4)

The determination of thyroxin (T_4) was done by Skelly *et al*, (1973) ELISA method for the quantitative *in-vitro* determination of total thyroxin concentration is serum using teco diagnostics ELISA test kit (Anaheim, CA).

Principle

The ELISA utilizes the high affinity between streptavidin and biotin to achieve solid phase immobilization of target molecule. The microplate is coated with streptavidin while the monoclonal anti-T_4 antibody is modified with biotin. Because T_4 only contains one epitope, sample serum and the streptavidin – coated micro plate are incubated together at room temperature for 60 minutes. The T_4 in the serum sample binds to the biotinylated antibody to form a complex which is immobilized by the bond between the streptavidin and biotin. A T_4 analogue competes with the T_4 present in serum sample for the binding on the biotinylated anti-T_4 antibodies. The micro plate is then washed to remove any unbound molecules

TMB solution is added and incubated for 20 minutes to develop a blue colour in the samples. The reaction is terminated with the addition of stops solution. The absorbance can then be measured by a spectrophotometer at 450 nm wavelength. Because the labelled T_4 analogue and T_4 in serum sample compete for the biotinyplate antibody, the intensity of the resulted colour is inversely proportional to the concentration of T_4 present in the serum sample. The concentration can then be determined by extrapolating from the standard curve.

Procedure

The microplates' well was formatted for each serum reference, control and fish specimen to be assayed in duplicate. Unused microwell strips were placed back into the aluminum bag, seal and stored at 8^oC.

To start 50 µl of T_4 standard solution, sample and control reagent were pipetted into appropriate wells.

Then, 50 µl of working conjugate reagent was added into each well, mixed thoroughly for 30 seconds

Also, 50 µl of the antibody reagents was dispensed into each well, mixed thoroughly for 30 seconds, and incubated at room temperature for 60 minutes.

The incubated mixture was removed by flicking plate content into a waste container, and 300 µl of working wash solution was pipetted. This was repeated for total of three washes.

The well was sharply stroked onto absorbent paper to remove residual water droplets.

Furthermore, 100 µl TMB solutions was dispensed into each well, gently mixed for 5 seconds and incubated at room temperature in the dark for 15 minutes without shaking.

The reaction was stopped by adding 50 µl of the stop solution to each well and gently mixed for 30 seconds

The optical density was read at 450 nm with a micro titter reader within 30 minutes and the concentration of T_4 in the serum samples were determined by interpolating on the standard curve in the manual (leaflet) in the test kit.

3.2.2.1.4 Determination of triiodothyronine (T_3)

The determination of triiodothyronine (T_3) was done by Utiger (1974) ELISA method for the quantitative in-vitro determination of total triiodothyronime concentration is serum using teco diagnostic ELISA test kit (Anaheim, CA).

Principle

The microplate is coated with monoclonal anti-T_3 antibody. The serum sample and the antibody – coated micro plate are incubated together at room temperature for 60 minutes. The T_3 in the serum sample binds to the antibody to a form complex which is immobilized onto the plate. A T_3 analogy conjugated with horseradish peroxidise is also added to the mixture during incubation. This T3 analogy completes with T_3 present sample for the binding on the anti-T_3 antibodies. The micro plate is then washed to remove any unbound molecules.

TMB solution is added and incubated for 20 minutes to develop a blue colour in the samples. The reaction is terminated with the addition of stop solution. The absorbance can then be measured by a spectrophotometer at 450 nm wavelength. Because the labelled T_3 analogy and T_3 in serum sample compete for the antibody coated onto micro plate, the intensity of the resulted colour is inversely proportional to the concentration of T_3 present in the serum sample. The concentration can then be determined by interpolating on the standard curve.

Procedure

The microplates' well was formatted for each serum reference, control and fish specimen to be assayed in duplicate. Unused microwell strips were placed back into the aluminum bag, seal and stored at 8^oC.

To start, 50 µl of T_3 standard solution, samples and control reagent were pipette into appropriate wells

Then, 100 µl of working conjugate reagent was added into each wells, mixed thoroughly for 30 seconds and incubated at room temperature for 60 minutes.

Later, the incubated mixture was removed by flicked 3 times with working wash solution

The wells were sharply stroked onto absorbent paper to remove residual water droplets

Afterwards, 100 µl TMB solutions was dispensed into each well, gently mixed for 5 seconds and incubated at room temperature in the dark for 15minutes without shaking.

The reaction was stopped by adding 50 µl of the dark stop solution to each well and gently mixed for 30seconds.

The Optical Density was read at 45 nm with a micro titter reader within 30 minutes and the concentration of T_3 in the serum sample determined by interpolating on the standard curve in the manual (leaflet) in the test kit.

Following the same principle and instructions on the other test kits the serum thyroxine (T_4) and thyroid stimulating hormone (TSH) were similarly determined.

3.2.2.2 Determination of oxidative stress

3.2.2.2.1 Determination of Lipid peroxidation

Lipid peroxidation is determined by spectrophotometrically measuring the level of the lipid peroxidation product – malondialdehyde (MDA). The estimation of malondialdehyde concentration was done using Wall *et al*, (1993) method for *in-vitro* determination of malondialdehyde concentration in serum.

Principle

Malondialdehyde (MDA) reacts with thiobarbituric acid to form a red or pink coloured complex which in acid solution, absorbs maximally at 532 nm

MDA + 2 TBA $\longrightarrow$ MDA: TBA adduct + H_2O

Procedure

Test tubes was prepared and aligned in rack.

Then, 0.1ml of sample and 0.9ml distilled water and 0.5ml of 25% TCA and 0.5ml of 1% TBA in 0.3% NaOH were pipetted into test tubes. The mixture was incubated for 40 mins and cooled in water. Afterwards, 0.1ml of 20% sodium dodecyl sulphate was added to the mixture. The absorbance of the mixture was determined at 532 nm against a blank.

The concentration of malondialdehyde was determined from the absorbance's read from the mixture. The concentration of the sample was later extrapolated from the table on the manual in the reagent.

3.2.2.2.2 Determination of glutathione peroxidase (GPX)

Glutathione peroxidase (GPX) activity was determined based on the method of Paglia and Valentine (1967) for the *in-vitro* determination of glutathione peroxidase activity in serum.

Principle

Glutathione peroxidase catalyses the oxidation of glutathione in the presence of glutathione reductase (GRX) and NADPH. The oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured in a spectrophotometer..

Reaction principle

$2GSH + ROOH \xrightarrow{GPX} ROH + GSSG + H_20$

$GSSH + NADPH + H^+ \xrightarrow{GPX} NADP + 2\ GSH$

Procedure

Test tubes were prepared and aligned in test tube rack.

Then, 3 ml of phosphate buffer and 0.05 ml of guaiacol and 0.03 ml of sample were added into the respective test tube and appropriately mixed.

The absorbance of the mixture was taken at 436 nm for 2 mins at 30 seconds interval.

The glutathione peroxidase activity was determined from the absorbance reading taken. The concentration of the sample was later extrapolated from the table on the manual in the reagent.

3.2.2.2.3 Determination of superoxide dismutase (SOD)

This was determined using Xin *et al*, (1991) method for the *in-vitro* determination of superoxide dismutase activity in serum.

Principle

Superoxide dismutase (SOD) reduces superoxide anion to hydrogen peroxide. The theory of this is based on the competition between SOD activity and idonitrazolium violet in reacting with superoxide, which is generated by xanthine oxidase (XOD) reaction.

$O_2 + O_2 + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$

$O_2 + \text{violet SOD} \longrightarrow \text{oxidized detector}$

Procedure

Test tubes were prepared and aligned in rack.

Then, 0.9 ml of distilled water and 0.1 ml of the samples were pipetted into a test tube.

Furthermore, 0.1 ml of the sample was mixed with 0.9ml of carbonate buffer and 75 µl of xanthine oxidase added.

The absorbance was determined at 500 ml for 3mins at 20 second interval.

The changing rate of absorbance was used to determine the superoxide dismutase activity. The concentration of the sample was later extrapolated from the table on the manual in the reagent. The result was expressed in (Units/1)

3.2.2.2.4 Determination of catalase

This was done according to the method of Aebi (1983) methods for the *in-vitro* determination of catalase activity in serum.

Principle

The ultraviolet absorption of hydrogen peroxide can be easily measured at 240 nm. On the decomposition of hydrogen peroxide (H_2O_2) with catalase the absorption decrease catalase activity can be measured.

Procedure

Test tubes were prepared and placed in the rack

To start with, 2.5 ml of phosphate buffer and 2 ml of H_2O_2 and 0.5 ml of sample were pipetted into test tube. To 1 ml portion of the reaction aliquot from test tube, 2 ml of dichromate acetic acid reagent was added. The absorbance of the mixture was determined at 240 nm at a minute interval into 4 places. The concentration of the sample was later extrapolated from the table on the manual in the reagent.

3.2.2.2.5 Assay for Lactate dehydrogenase activity

Lactate dehydrogenase activity was assayed because differences in lactate dehydrogenase activities could be indicative of anaerobic respiration in *Clariase gariepinus* exposed to glyphosate as a result of oxidative stress.

Lactate dehydrogenase activity was assayed using colorimetric method of Kaplan, (1983) from Span Diagnostics reagent kit.

Principle:

Lactate dehydrogenase catalyses the conversion of pyruvate to lactate with simultaneous oxidation of reduced Nicotinamide Adenine Dinucleotide (NADH) to oxidized Nicotinamide Adenine Dinucleotide (NAD^+). The rate of decrease in absorbance due to formation of NAD^+ is measured at 340 nm and is proportional to the lactate dehydrogenase activity in the sample.

3.2.2.3 Liver function test

3.2.2.3.1 Estimation of alkaline phosphatase

Principle

p-nitrophenylphosphate + H_2O $\xrightarrow{ALP}$ phosphate + p-nitrophenol

Procedure

The estimation of the alkaline phosphate activity was done by reitman and frankel, (1957) method for the quantitative in-vitro determination of alkaline phosphate in serum using randox laboratory test kit (Antrim UK).

Fresh double distilled water was aspirated and a new Gain Calibration was performed in flow cell mode. ALP was selected in the run test screen and water blank was used as instructed.

0.01ml of sample and 0.5ml of reagent were pipetted respectively, then mixed and aspirated into the Rx Monza. The concentration of the sample was later extrapolated from the table on the manual in the reagent. The result is expressed in U/L.

3.2.2.3.2 Estimation of serum alanine aminotransferase

The concentration of alanine aminotransferase in serum of the test animals were assayed using the Randox test kits. This followed the Reitman and Frankel method (1957).

Principle

This measures the concentration of pyruvate hydrazine, and oxaloacetate hydrazone, respectively, formed when the test solution reacts with 2, 4-dinitrophenylhydrazine.

a-oxoglutarate+ L-alanine $\xrightarrow{\text{ALT}}$ L-glutamate + pyruvate

Procedure

Test tube were prepared and labeled reagent, blank and sample

To start, 0.1 ml of the samples were pipetted into the test tubes labeled sample

Then, 0.5 ml of the ALT reagent containing phosphate buffer, L-Alanine and oxoglutarate was added to all test tubes.

Also, 0.1 ml of distilled water was pipette into the test tube labeled reagent blank then mixed and allowed for 30 mins at 37 ^{0}C.

Afterwards, 0.5 ml of ALT reagent II containing 2,4-dinitrophenythydrazine was added to all test tubes, appropriately mixed and allowed to stand for further 20 minutes at 25^{0}C.

Later 5.0 ml of NaOH was added to all the test tubes and properly mixed, then the absorbence of the reagent blank was read after 5 minutes. The ALT concentration of the sample was later extrapolated from the table on the manual in the reagent. The result is expressed in U/L.

Calculations

The activity of ALT in the serum is obtained from the table below;

Absorbance	**U/1**	**Absorbance**	**U/1**
0.025	4	0.275	48
0.050	8	0.300	52
0.075	12	0.035	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77

0.200	34	0.450	83
0.225	39	0.475	88
0.250	43	0.500	94

3.2.2.3.3 Estimation of serum aspartate aminotransferase

The concentration of aspartate aminotransferase in serum of the test animals were assayed using the Randox test kits. This followed the Reitman and Frankel method (1957).

Principle

This measures the concentration of pyruvate hydrazine, and oxaloacetate hydrazone, respectively, formed when the test solution reacts with 2, 4-dinitrophenylhydrazine.

a-oxoglutarate + L- asparate $\xrightarrow{AST}$ L- glutamate + oxaloacetate

Procedure

Test tube were prepared and labeled reagent, blank and sample

To start, 0.1 ml of the samples were pipetted into the test tubes labeled sample

Then, 0.5 ml of the AST reagent containing phosphate buffer, L-Alanine and oxoglutarate was added to all test tubes.

Also, 0.1 ml of distilled water was pipette into the test tube labeled reagent blank then mixed and allowed for 30 mins at 37 ^{0}C.

Afterwards, 0.5 ml of AST reagent II containing 2,4-dinitrophenythydrazine was added to all test tubes, appropriately mixed and allowed to stand for further 20 minutes at 25^0C.

Later 5.0 ml of NaOH was added to all the test tubes and properly mixed, and then the absorbence of the reagent blank was read after 5 minutes. The AST concentration of the sample was later extrapolated from the table on the manual in the reagent. The result is expressed in U/L.

Calculations

The activity of AST in the serum is obtained from the table below;

Absorbance	**U/I**	**Absorbance**	**U/I**
0.020	7	0.100	36
0.030	10	0.110	41
0.040	13	0.120	47
0.050	16	0.130	52
0.060	19	0.140	59
0.070	23	0.150	67
0.080	27	0.160	76
0.090	31	0.170	89

3.2.2.3.4 Estimation of bilirubin

Principle

Colorimetric method based on methods described by Jendrassik and Grof (1938). Direct (conjugated) bilirubin reacts with diazotized sulphanilic acid in alkaline medium to form a blue colored complex. Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction with diazotized sulphanilic acid.

Total bilirubin

Procedure

Fresh double distilled H_2O was used to perform a new Gain Calibration in cuvette mode. TBIL was selected in the Run Test screen and water blank was carried out as instructed.

Test tubes were prepared and labeled: Sample Blank and Sample

200ul of bilirubin reagent 1 containing sulphanilic acid were added to all test tubes respectively.

1 drop (50ul) of bilirubin reagent 2 containing nitrite was added to the test tubes labeled sample.

1000ul of bilirubin reagent 3 containing caffeine was added to al test tubes and the 200ul of sample was added to all test tubes. The test tubes were appropriately mixed and incubated for 10 minutes at 20-25^{0}C.

Afterwards 1000 ul of bilirubin reagent 4 containing tartrate was added to all test tubes appropriately mixed and incubated further 5-30 minutes at 25^{0}C. The cuvette was inserted into the Rx Monza flowcell holder and Read. The result was expressed in micro mole per liter.

Manual calculation

Total Bilibrubin (umol/l) = $185 \times A_{TB}$ (578nm)

Total Bilirubin (mg/dl) = $10.8 \times A_{TB}$ (578nm)

3.2.2.4 Hematological parameters

3.2.2.4.1 Determination of White Blood Cell Count

Red and white blood cell counts can be performed in the laboratory using a hemacytometer and appropriately diluted blood. By properly diluting blood, counting all cells in specified squares, and multiplying by the proper conversion factor, the number of cells per cubic millimeter can be determined (Stolz *et al.,* 2009).

Procedure:

Blood was aspirated from the sample probe into the sample rotor valve. Then, 6µl of blood measured by the sample rotor valve is transferred to the WBC transducer chamber along with 1.994 ml of diluent. At the same time, 1.0 ml of WBC/HGB lyse was added to prepare 1:500 dilution sample. When the solution is made to react in this status for approximately 10 seconds, RBC is haemolysed and platelets shrink, with WBC membrane held as they are. At the same time, haemoglobin is converted into red colored methaemoglobin. Of the hemolysed sample in the WBC transducer chamber, approximately 1 ml was transferred to the hemoglobin flow cell.

Then, 500 µL of sample in the WBC transducer was aspirated through the aperture. The pulses of the blood cell when passing through the aperture were counted by the DC detection method.

3.2.2.4.2 Determination of Red Blood Cell (RBC) Count and Platelet Count

Red and white blood cell counts can be performed in the laboratory using a haemacytometer and appropriately diluted blood. By properly diluting blood, counting all cells in specified squares, and multiplying by the proper conversion factor, the number of cells per cubic millimeter can be determined (Stolz *et al.,* 2009).

Procedure:

Blood wass aspirated from the sample probe into the sample rotor valve.

Four micro litre (4.0 µL) of blood measured by the sample rotor valve was diluted into 1:500 with 1.996 mL of diluent and brought to the mixing chamber as diluted sample.

Out of the 1:500 dilution sample, 40 µL was measured by the sample rotor valve, diluted into 1:25000 with 1.960 mL of diluent, then transferred to the RBC/PLT transducer chamber.

Furthermore, 250 µl of the sample in the RBC and PLT transducer chamber was aspirated through the aperture. At this time, RBC and PLT are counted by the DC detection method. At this time, HCT (hematocrit value) wass calculated by RBC pulse height detection method.

3.2.2.4.3 Haematocrit (HCT)

The haematocrit is also called packed cell volume (PCV). It was determined by the microhaematocrit method (Okomoda et al., 2013). Haematocrit was computed as:

Hct (%) = RBC × Mean Corpuscular Volume / 10

3.2.2.4.4 Mean Corpuscular Volume (M.C.V)

To calculate the MCV, expressed in femtometers (fL, or 10-15L), the following formula is used:

$$\text{MCV (fL)} = \frac{\text{HCT (\%)} \times 10}{\text{RBC } (\times 10^6/\mu\text{L})}$$

3.2.2.4.5 Mean corpuscular Haemoglobin concentration (M.C.H.C)

This is the amount of haemoglobin relative to the size of the cell (haemoglobin concentration) per red blood cell. It implies the percentage of Hb in 100ml of red cell as opposed to percentage of Hb in 100 ml of whole blood. Calculation was done as follows:

$$\text{MCHC (g/dL)} = \frac{\text{Haemaglobin Concentration (g/dL)} \times 100}{\text{HCT(\%)}}$$

3.2.2.4.6 Mean Corpuscular Haemoglobin (MCH)

This represents the amount of microgram of haemoglobin per blood cell. It is the average haemoglobin content expressed in microgram (ug. ug) of a single red cell. The normal range is 27-32 microgram or pictograms/cell**)** (Schuetz *et al.,* 2009). Mean Corpuscular Haemoglobin (MCH) or Mean Cell Hemoglobin is the weight of hemoglobin in the average erythrocyte count, computed as:

$$\text{MCH (pg)} = \frac{\text{HGB (g/dL)} \times 10}{\text{RBC } (\times 10^6/\mu\text{L})}$$

3.2.2.4.7 Mean platelet volume (MPV)

Mean platelet volume is a machine-calculated measurement of the average size of platelets found in blood and is typically included in blood tests as part of the CBC. Mean Platelet Volume is the

average volume of individual platelets derived from the PLT histogram. It represents the mean volume of the PLT population under the fitted PLT curve multiplied by a calibration constant, and expressed in femtoliters (femtolitre) (Schuetz *et al.*, 2009). Calculated according to the formular PCT = Platelet count x MPV/ 10,000 (25-27).

3.2.2.4.8 Red Blood Cell Distribution Width (RDW)

The two RDW measurements currently in use are the red cell distribution width - coefficient of variation (RDW-CV) and the red cell distribution width - standard deviation (RDW-SD).The RDW-CV is a calculation based on both the width of the distribution curve and the mean cell size. It is calculated by dividing the standard deviation of the mean cell size by the MCV of the red cells and multiplying by 100 to convert to a percentage. Because it is a calculation, the RDW-CV is dependent not only on the width of the distribution curve but also the MCV of the red cell population and may not always reflect the actual variation in red cell size. The RDW-SD is an actual measurement of the width of the red cell distribution curve in femtoliters (fL). The width of the distribution curve is measured at the point that is 20% above the baseline. Since the RDW-SD is an actual measurement, it is not influenced by the MCV and more accurately reflects the red cell size variance.

3.7 Statistical Analysis

The results were expressed as mean $\pm$ standard deviation and test of statistical significance were carried out using one way ANOVA. The statistical package used was statistical package for Microsoft office version 2007. $P < 0.05$ was considered statistically significant and was indicated by dissimilar alphabets a, b, c and d.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Result

4.1.1 Hormonal Analysis

4.1.1.1 Effect of glyphosate on testosterone of African cat fish

Testosterone concentration in African Cat fish from control group and glyphosate polluted groups were analyzed and the result is as shown in Figure 4.1. From the result of the analysis, the 300 mg/l polluted group shows a significant decrease ($p<0.05$) in testosterone concentration compared to the control. The 60mg/l and 10mg/l polluted groups showed no significant difference ($p < 0.05$) in testosterone concentration compared to the control group.

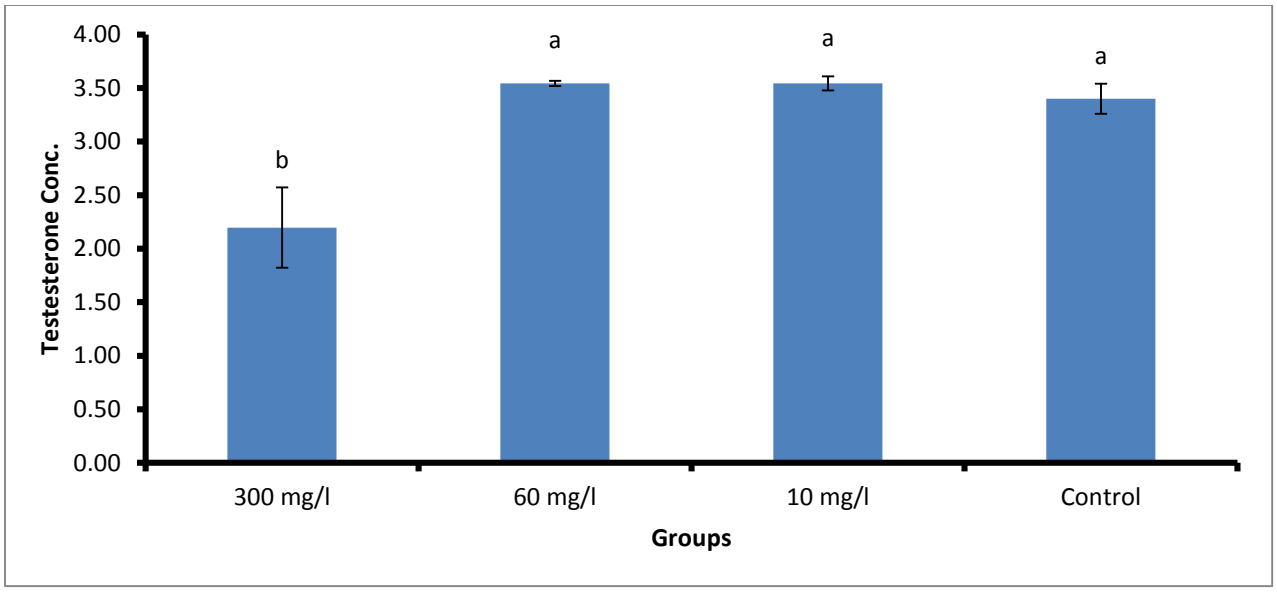


Figure 4.1 Concentration of Testosterone in African cat fish from control group and glyphosate polluted group. Bars are presented mean ± standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

4.1.1.2 Effect of glyphosate on thyroid stimulating hormone of African cat fish

The result TSH concentration presented in Figure 4.2. There was a significant increase ($p < 0.05$) of the thyroid stimulating hormone concentration in 300mg/l polluted group compared to the control group. The concentration of TSH in 10mg/l polluted group was significantly higher ($p < 0.05$) than that of 60mg/l polluted group and the control group but none significantly lower ($p < 0.05$) than that of the group polluted with 300mg/l of glyphosate. Increase in the 60 mg/l group, a little below expection could be due to experimental error.

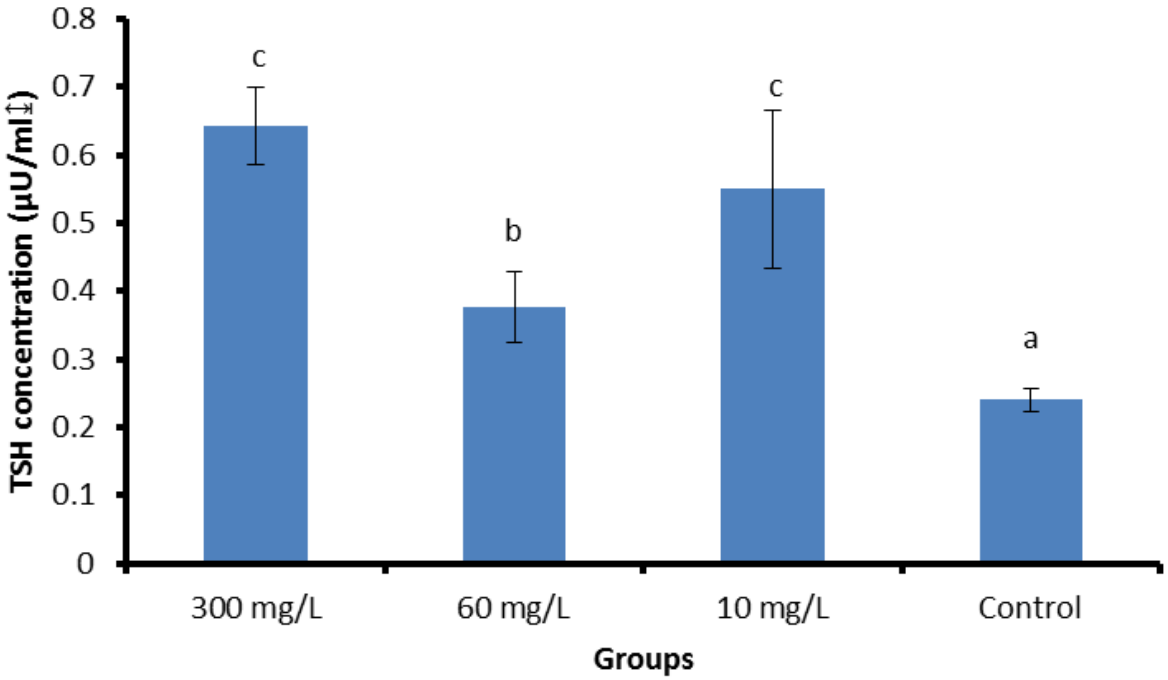


Figure 4.2 Thyroid stimulating hormone (TSH) concentration in African cat fish from control group and glyphosate polluted group. Bars are presented mean ± standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

4.1.1.3 Effect of glyphosate on thyroxin of African cat fish

The result expressed in Figure 4.3 shows the effect of glyphosate on T4 concentration in African cat fish. The polluted groups (300 mg/l, 60 mg/l and 10 mg/l) compared to the control group show a significant increase ($p < 0.05$) in T4 concentration compared to the control group. There is no significant difference when comparing the different concentrations of glyphosate.

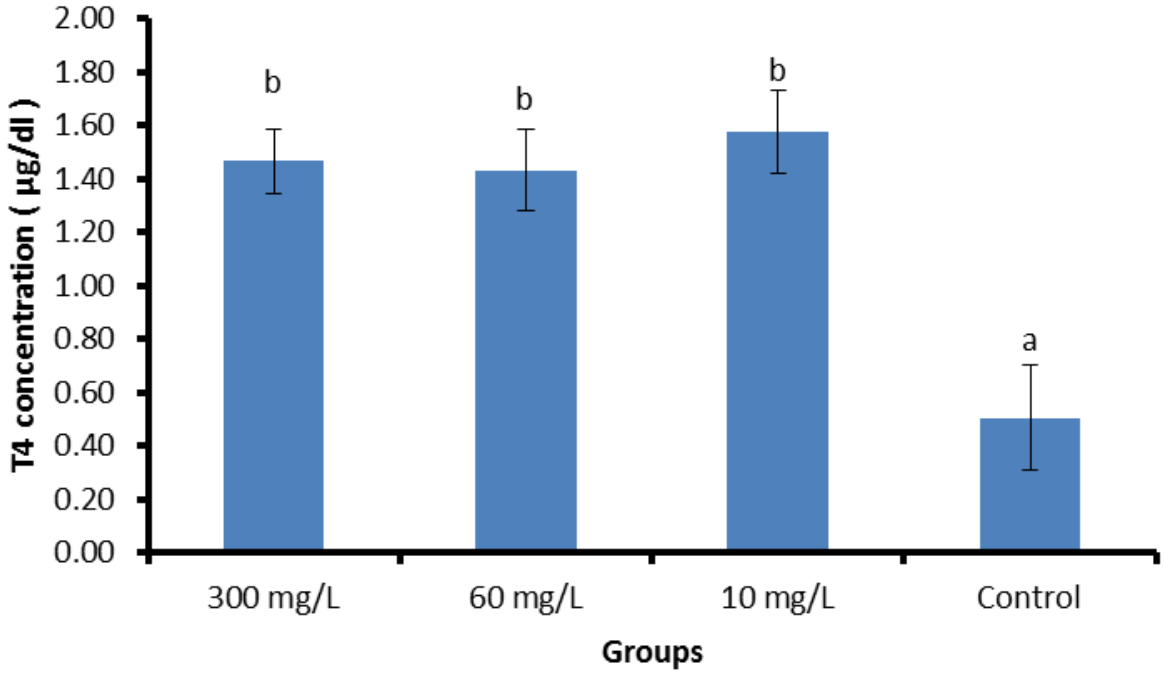


Figure 4.3 Thyroxin concentrations in African cat fish from control group and glyphosate polluted group. Bars are presented mean ± standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

4.1.1.4 Effect of glyphosate on Triiodothyronin (T3) of African cat fish

The result in Figure 4.4 presents the concentration of T3 in the serum of the African cat fish from control group and glyphosate polluted group. The glyphosate polluted groups show a significant reduction ($p<0.05$) in the concentration of T3 compared to the control group.

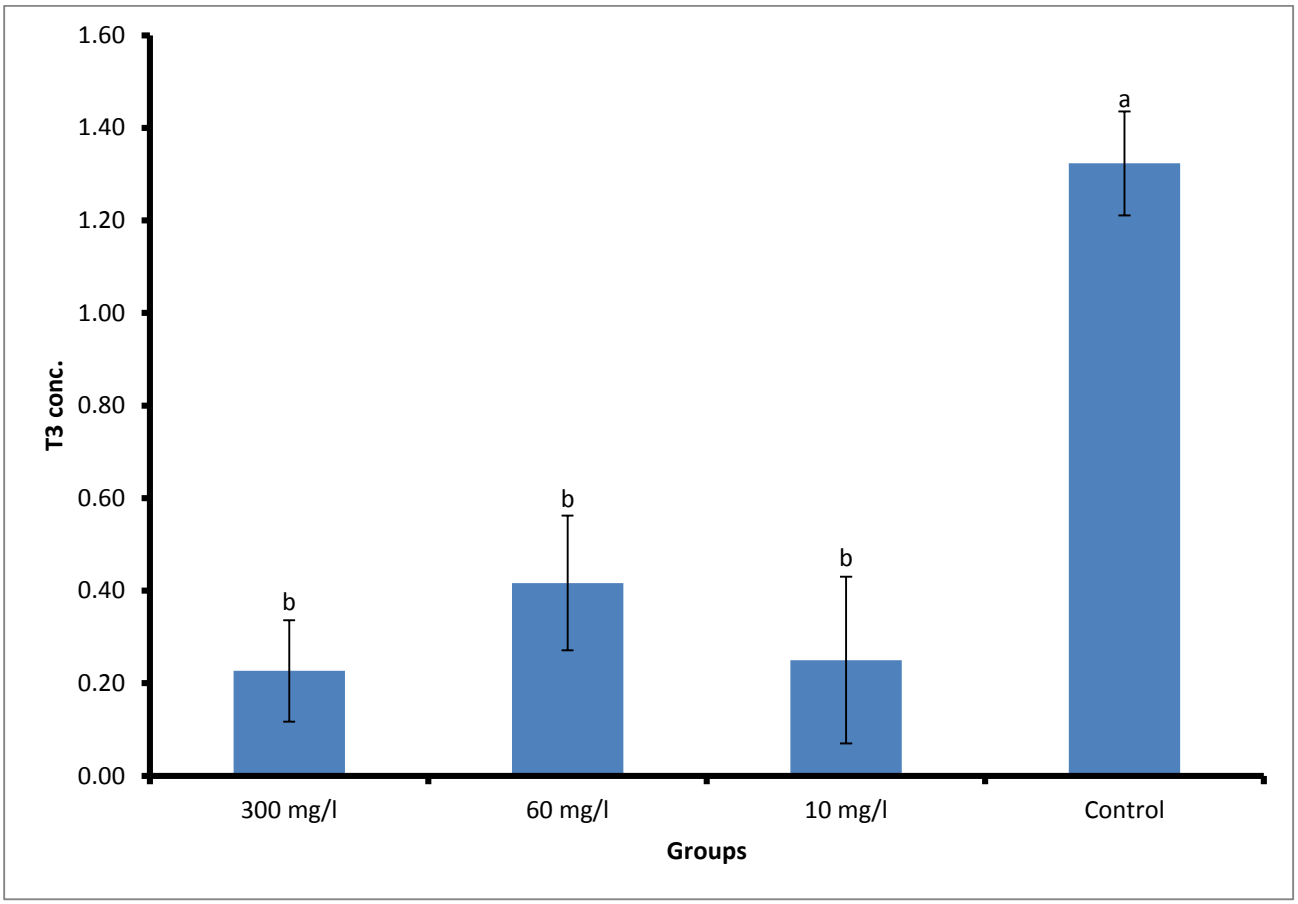


Figure 4.4: Triiodothyronine (T3) concentration of African cat fish from control group and glyphosate polluted group. Bars are presented mean $\pm$ standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

4.1.2 Effect of glyphosate on oxidative stress bio-markers

The result of Malondialdehyde (MDA) concentration in the kidney of control group and polluted group is presented in Figure 4.5. The result presented above shows that the 300 mg/l group shows a significant increase ($p < 0.05$) in the concentration of MDA compared to the control group and the lower concentration groups.

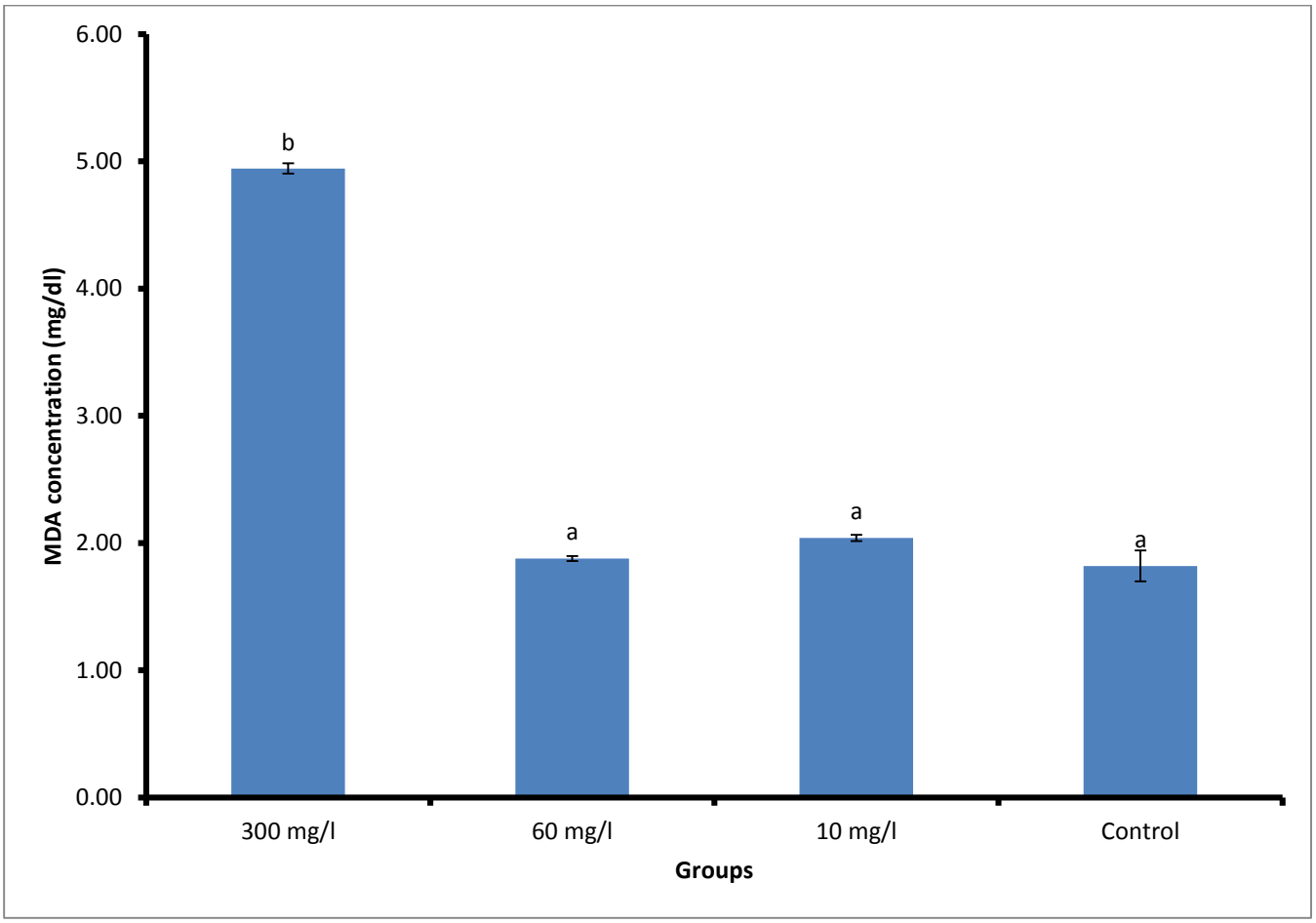


Figure 4.5: Malondialdehyde concentration in the kidney of African cat fish from control group and glyphosate polluted group. Bars are presented mean ± standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

The result of Malondialdehyde (MDA) concentration in the gill of control group and polluted group is presented in Figure 4.6. Malondialdehyde concentration was used as a maker of lipid peroxidation. The result represented above shows that the 300 mg/l, 60 mg/l and 10 mg/l groups, produced a significant increase ($p < 0.05$) in the concentration of MDA compared to the control group.

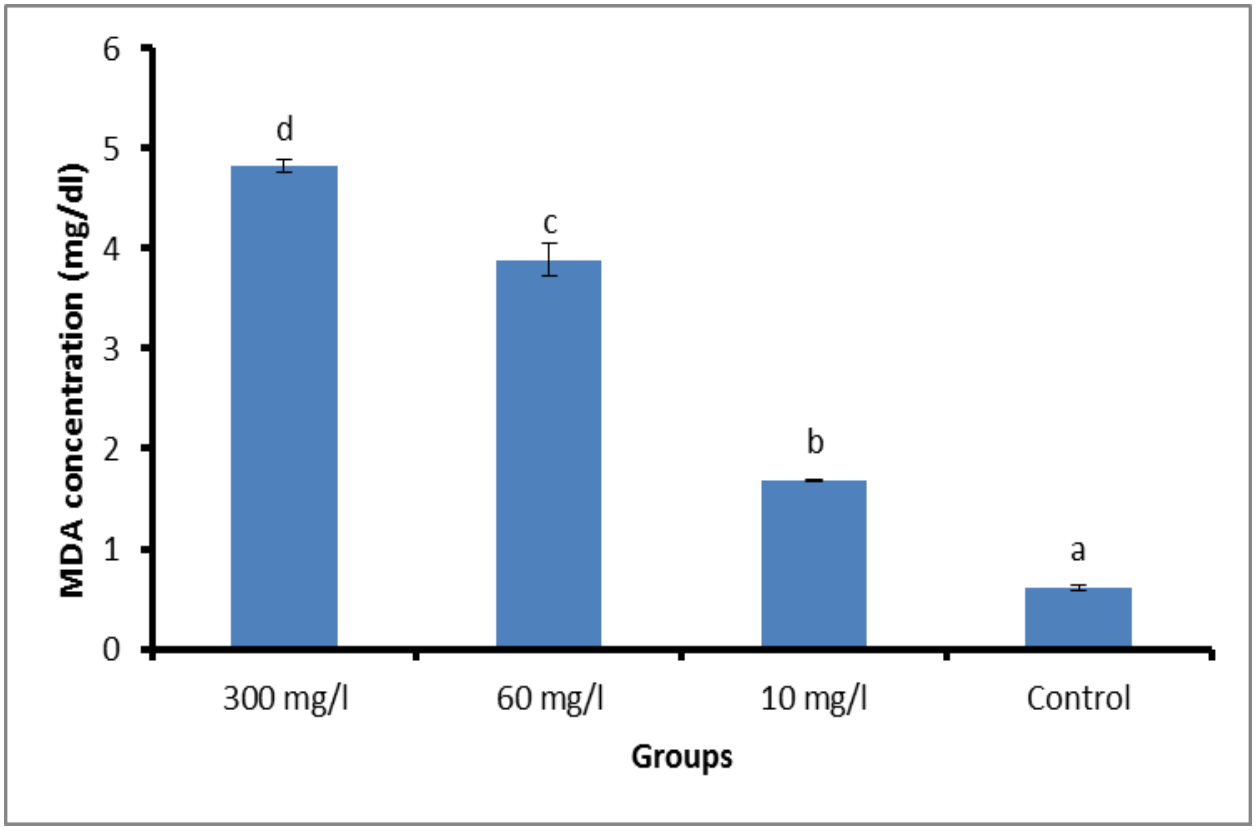


Figure 4.6: Malondialdehyde concentration in the gill of African cat fish from control group and glyphosate polluted group. Bars are presented mean $\pm$ standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

The result of superoxide dismutase activity in the kidney of control group and polluted group is presented in Figure 4.7. Superoxide dismutase was used as a maker of oxidative stress. The results show that superoxide dismutase activity in 300 mg/l group showed a significant reduction ($p< 0.05$) compared to the control group. There was no significant deference ($P>0.05$) comparing 60 mg/l and 10 mg/l groups to the control group.

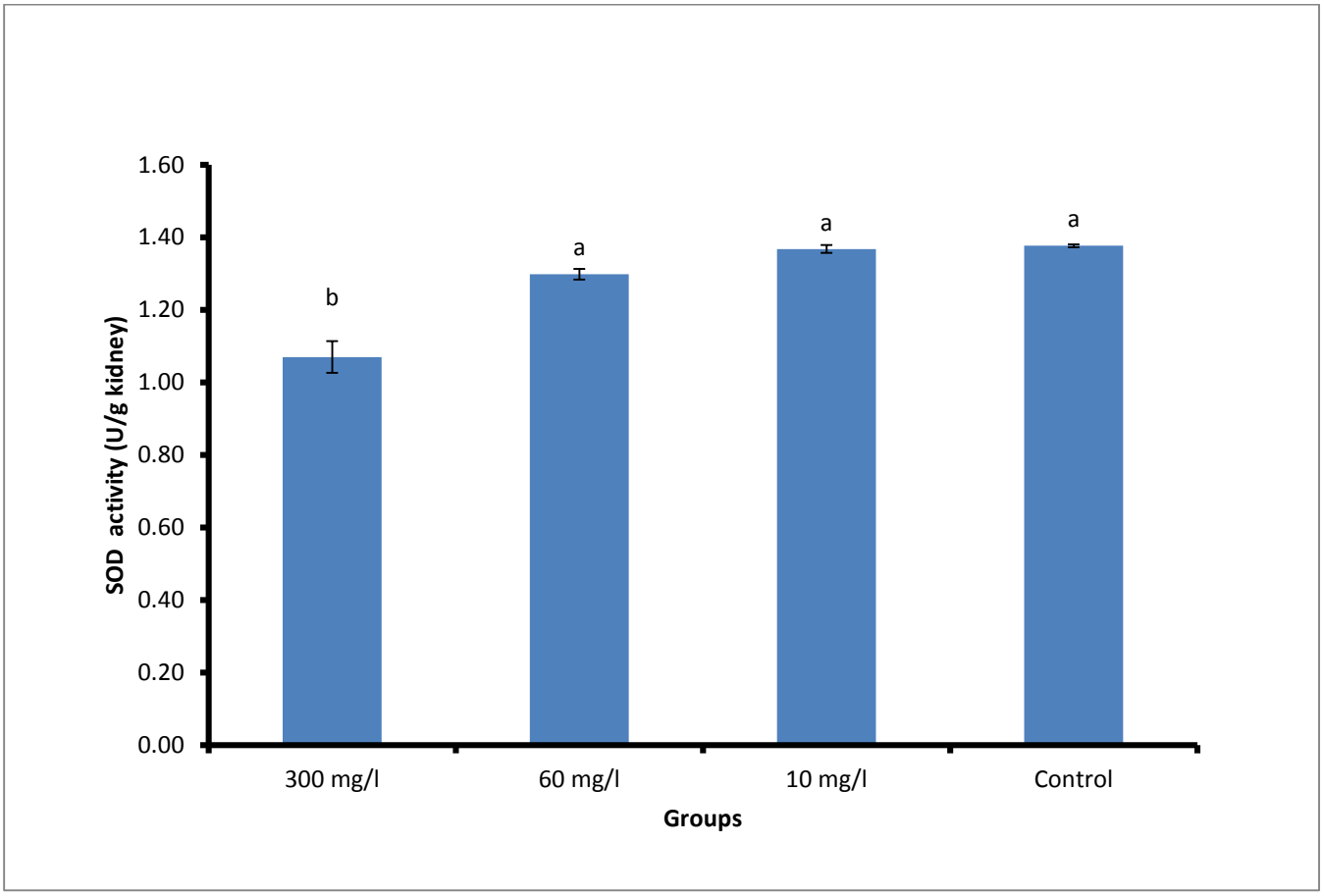


Figure 4.7: Superoxide dismutase activity in the kidney of African cat fish from control group and glyphosate polluted group. Bars are presented mean $\pm$ standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

The result in Figure 4.8 above presents the effect of glyphosate on liver superoxide activity. The results showed that superoxide dismutase activities in 300 mg/l, 60 mg/l and 10 mg/l groups were significantly reducted ($p < 0.05$) compared to the control group. There wasno significant deference comparing 10 mg/l group to the 60 mg/l group. Therefore the 300 mg/l polluted group presents the significantly lowest activity of superoxide dismutase.

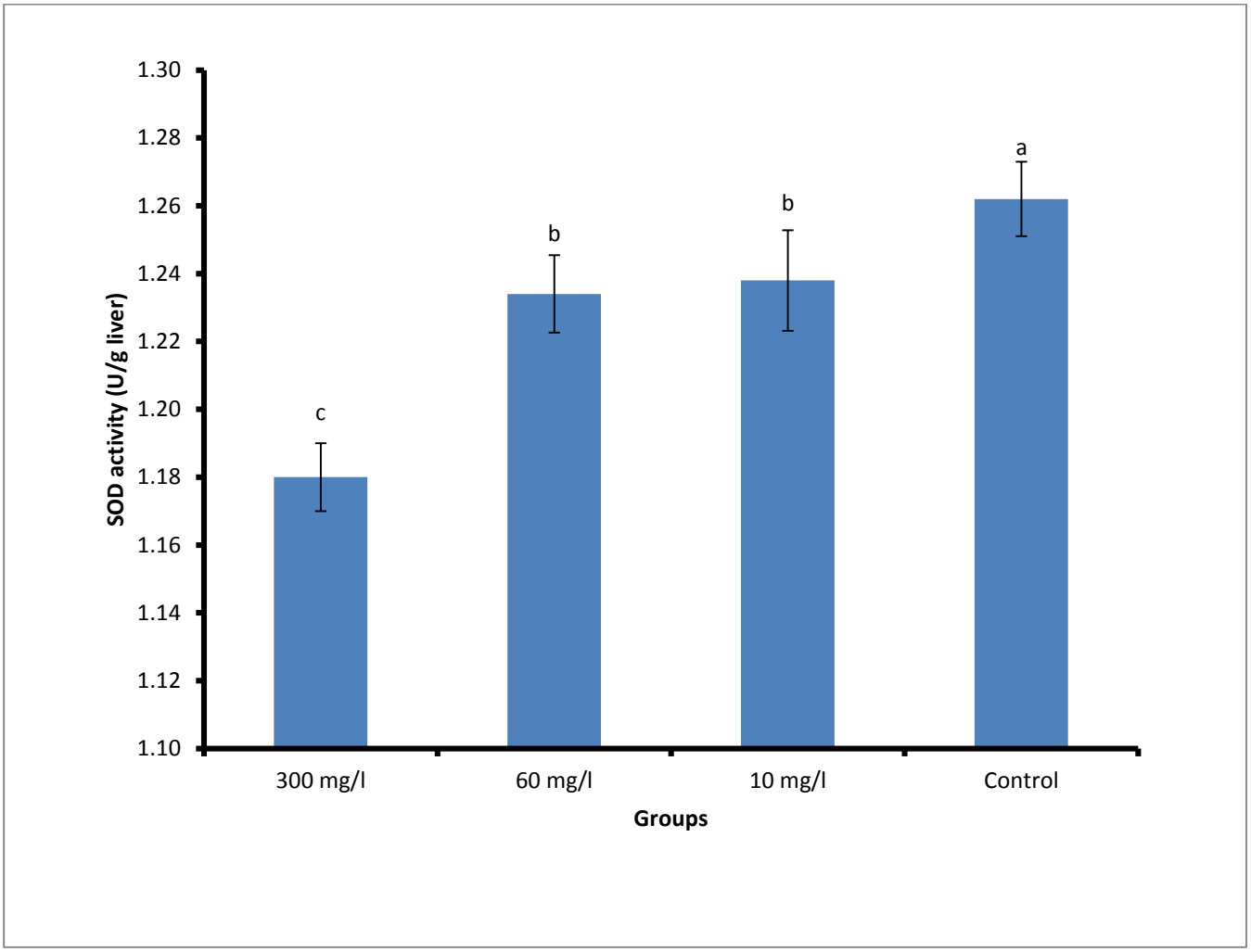


Figure 4.8: Superoxide dismutase activity in the liver of African cat fish from control group and glyphosate polluted group. Bars are presented mean $\pm$ standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

The result in Figure 4.9 above presents the effect of glyphosate on superoxide activity of the gill of *Clarias gariepinus*. The results showed that superoxide dismutase activities in 300 mg/l and 10 mg/l groups were significantly shows reduced ($p < 0.05$) compared to the control group. However, there was no significant deference comparing 60 mg/l and 10 mg/l groups to the control group. Therefore the 300 mg/l polluted group presented the significant lowest activity of superoxide dismutase.

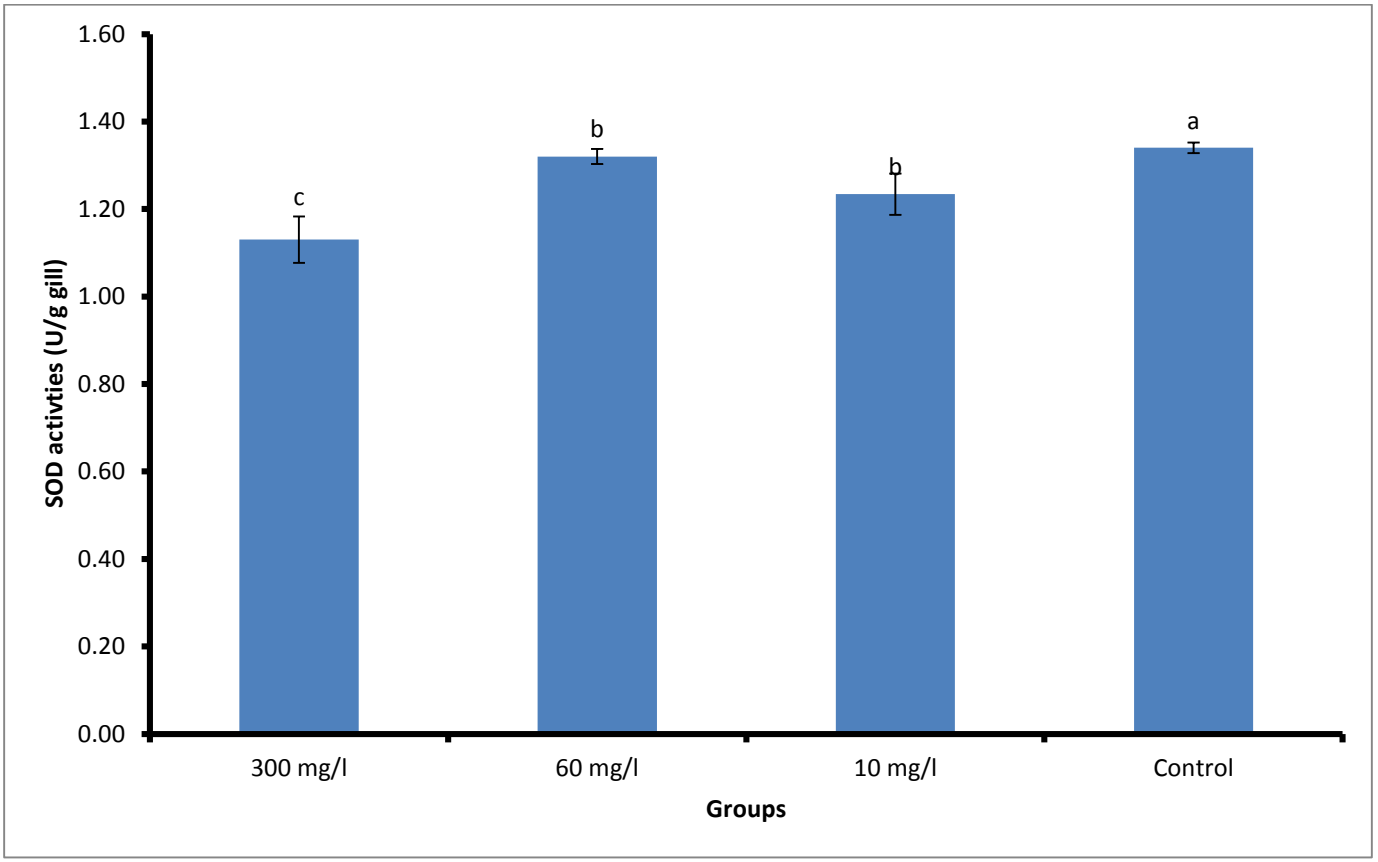


Figure 4.9: superoxide dismutase activity in the gill of African cat fish from control group and glyphosate polluted group. Bars are presented mean $\pm$ standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

The result of catalase activity in the kidney of control group and polluted group is presented in Figure 4.10. From the result catalase activity was used as marker of oxidative stress. From the result represented there wass a significant increase ($p < 0.05$) in the activity of catalase in 300 mg/l group compared to the control group. On the other hand 60 mg/l group and 10 mg/l showed no significant ($p<0.05$) difference in catalase activity compared to the control group. Therefore the 300 mg/l polluted group presents the significantly highest activity of catalase.

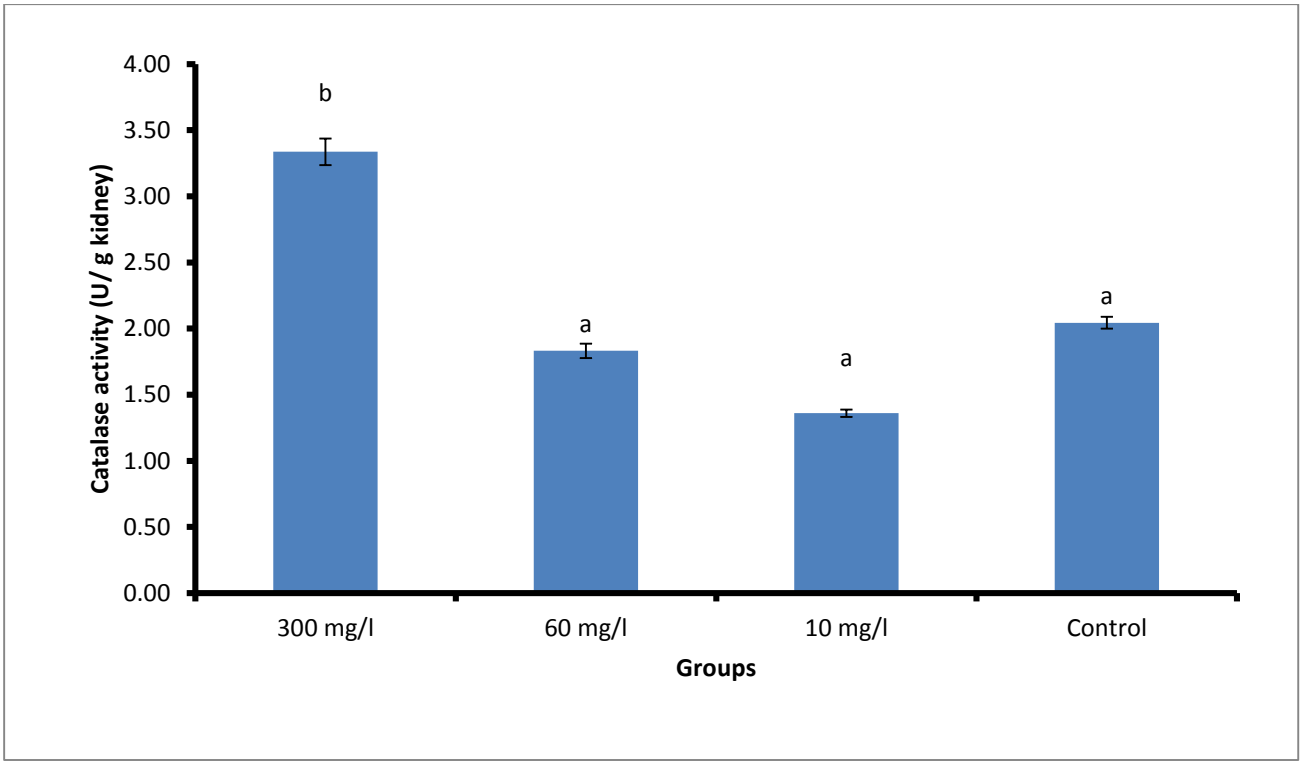


Figure 4.10: Catalase activity in the kidney of African cat fish from control group and glyphosate polluted group. Bars are presented mean ± standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

The result of catalase activity in the liver of control group and polluted group is presented in Figure 4.11. From the result catalase was used as a maker of oxidative stress. From the result represented above, there wasa significant increase ($p < 0.05$) in the activity of catalase in 300 mg/l, 60 mg/l and 10 mg/l groups compared to the control group. Therefore the control group presented the significantly lowest activity of catalase

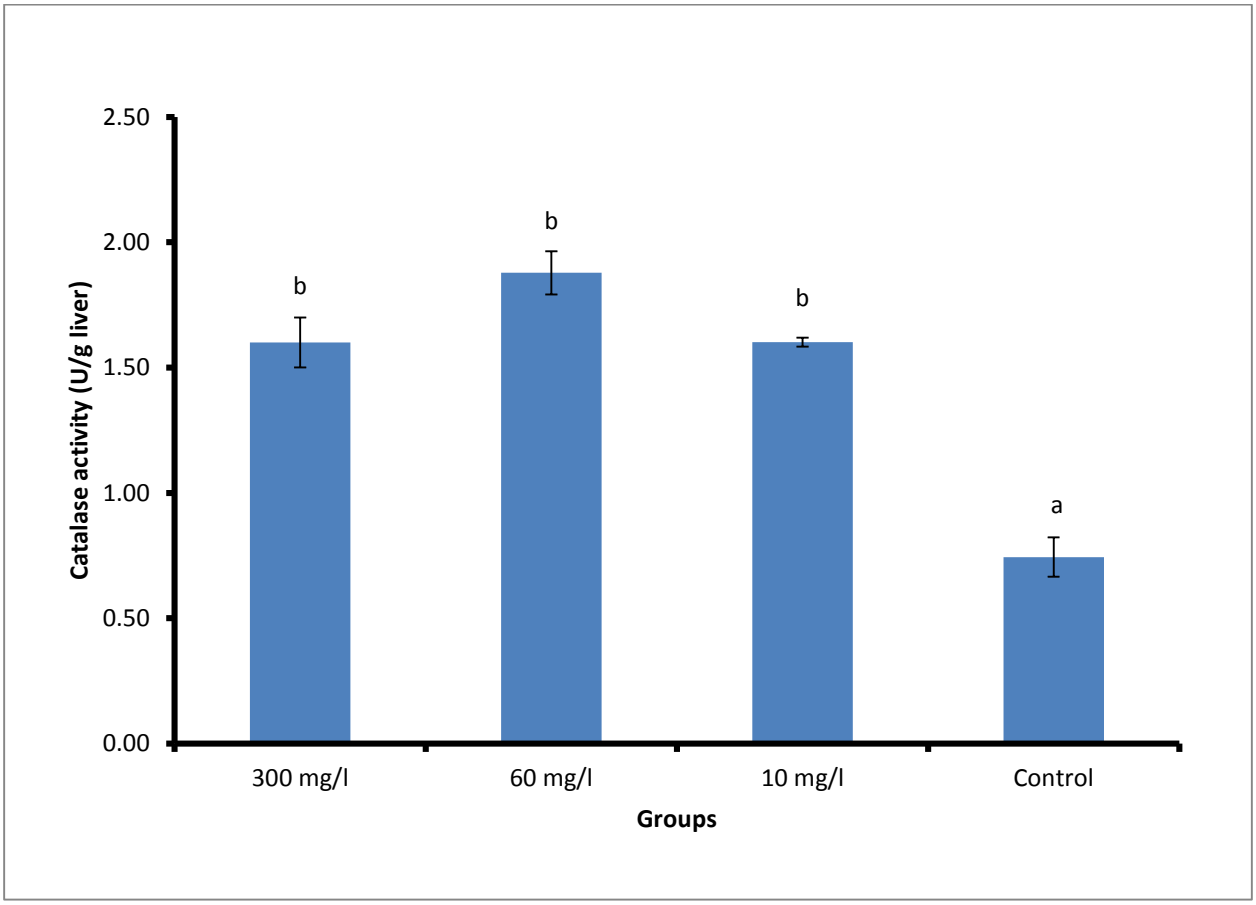


Figure 4.11: Catalase activity in the liver of African cat fish from control group and glyphosate polluted group. Bars are presented mean $\pm$ standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

The result in Figure 4.12 below shows the effect glyphosate on the catalase activity in the gill of the African Cat fish. From the result presented above, there was a significant increase ($p < 0.05$) in the activity of catalase in 300 mg/l group compared to the control group. On the other hand the 10mg/l and 60 mg/l groups showed no significant increase ($p > 0.05$) in catalase activity when compared to the control group. Therefore the 300 mg/l polluted group showed the significantly highest activity of catalase.

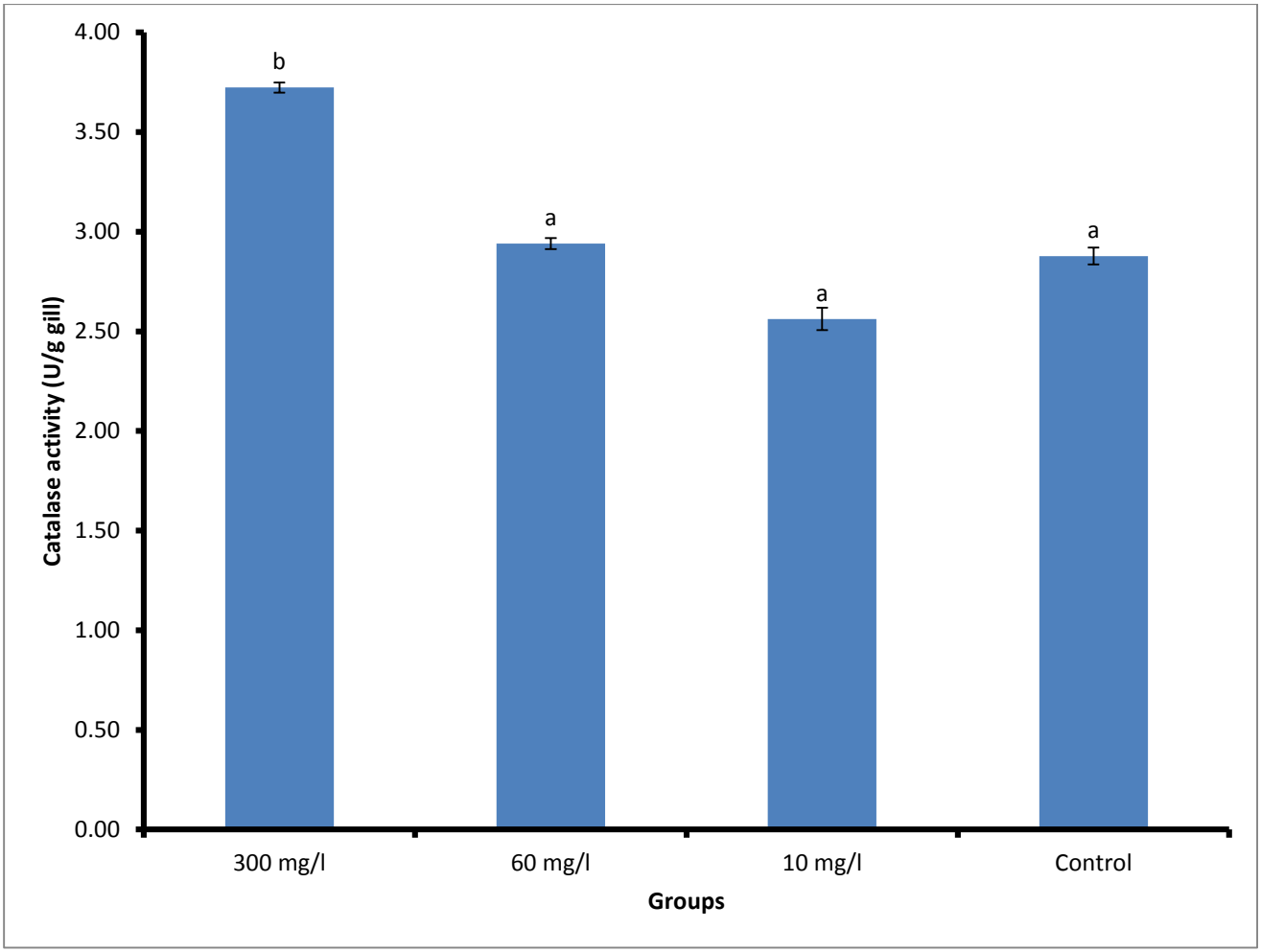


Figure 4.12: Catalase activity in the gill of African cat fish from control group and glyphosate polluted group. Bars are presented mean $\pm$ standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

The result of glutathione peroxidase in the liver of control group and polluted group is presented in Figure 4.13. From the result glutathione peroxidase was used as a maker of oxidative stress. The result showed a significant rise ($p<0.05$) in the activity of glutathione peroxidase activity in the liver of 60 mg/l when compared to the control group. This could be due to experimental error. The 300 mg/l polluted group showed a significant decrease ($p<0.05$) in the activity of Glutathione peroxidase when compared to the control. Therefore the 300 mg/l polluted group presents the significantly lowest activity of Glutathione peroxidase.

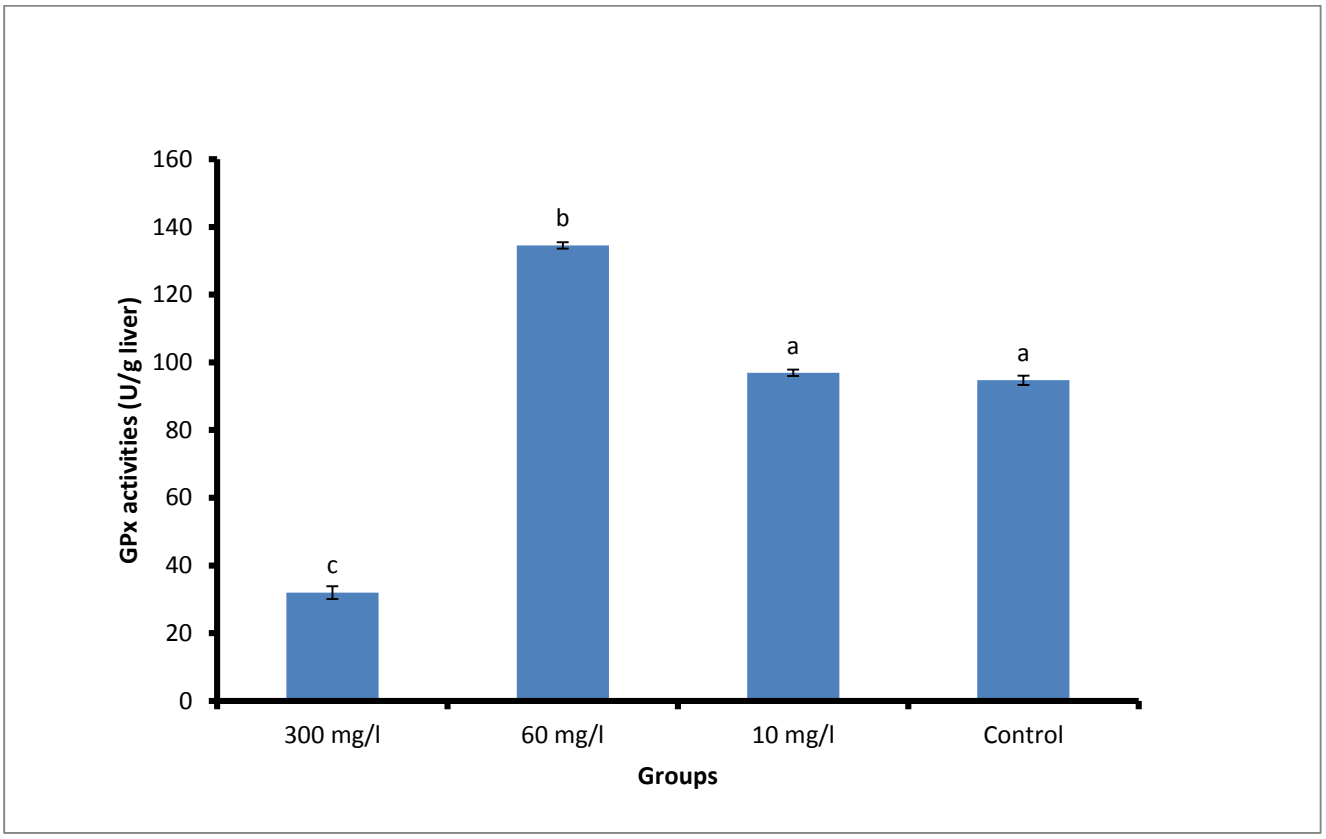


Figure 4.13: Glutathione peroxidase activity in the liver of African cat fish from control group and glyphosate polluted group. Bars are presented mean $\pm$ standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

The result in Figure 4.14 shows the effect of Glyphosate induced oxidative toxicity on kidney of African cat fish. The result showed a significant reduction ($p < 0.05$) in the activity of glutathione peroxidase activity in the gill of 300mg/l, 60 mg/l and 10 mg/l groups when compared to the control group.

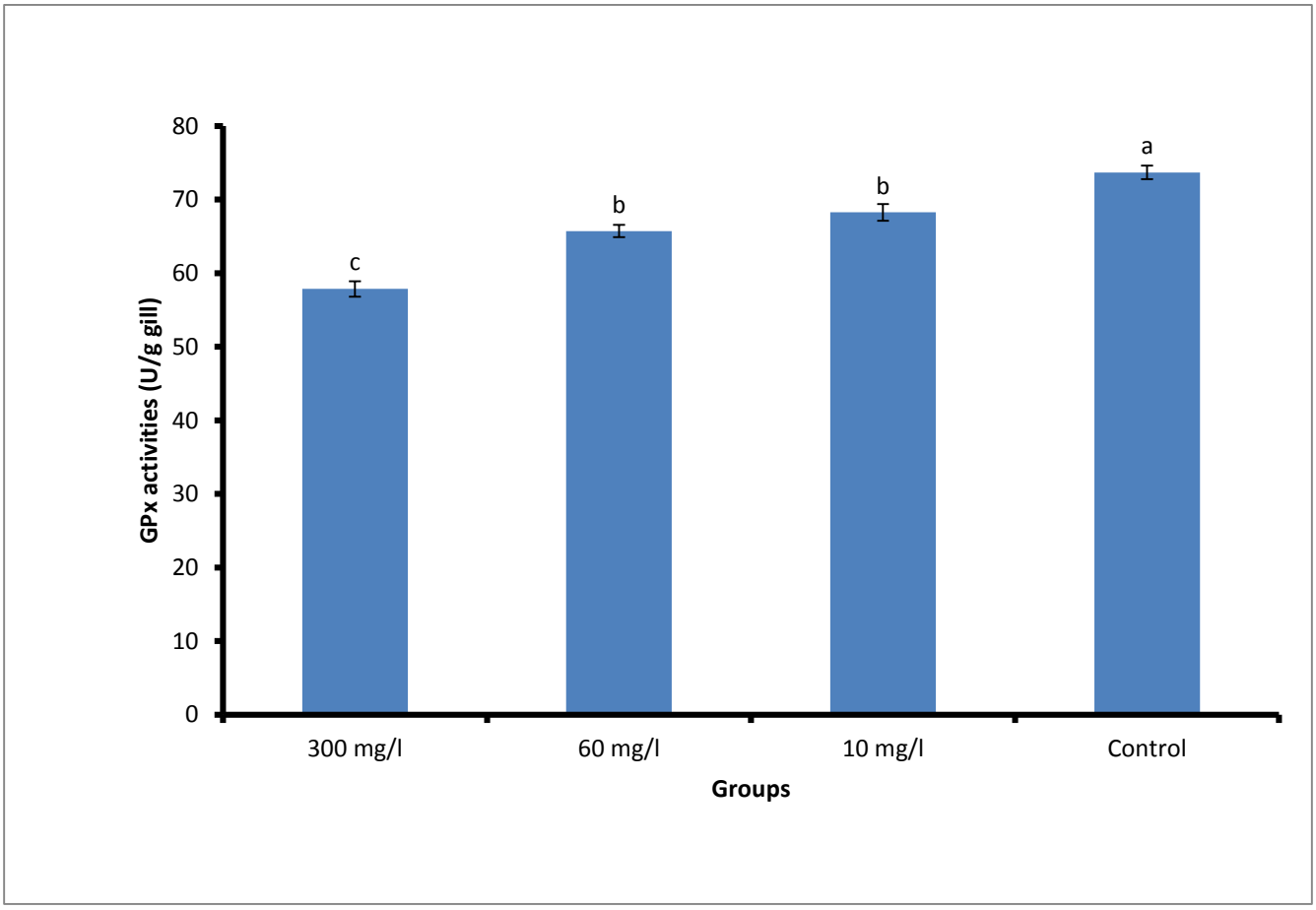


Figure 4.14: Glutathione peroxidase activity in the gill of African cat fish from control group and glyphosate polluted group. Bars are presented mean ± standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

The result of Lactate dehydrogenase in the gill of control group and polluted group is presented in Figure 4.15. Lactate dehydrogenase was used as a maker of oxidative stress. The result presented in Figure 4.15 showed that there was a significant increase ($p < 0.05$) in lactate dehydrogenase activity of 300 mg/l, 60 mg/l and 10 mg/l groups compared to the control group. The control group presented the significantly lowest activity of lactate dehydrogenase.

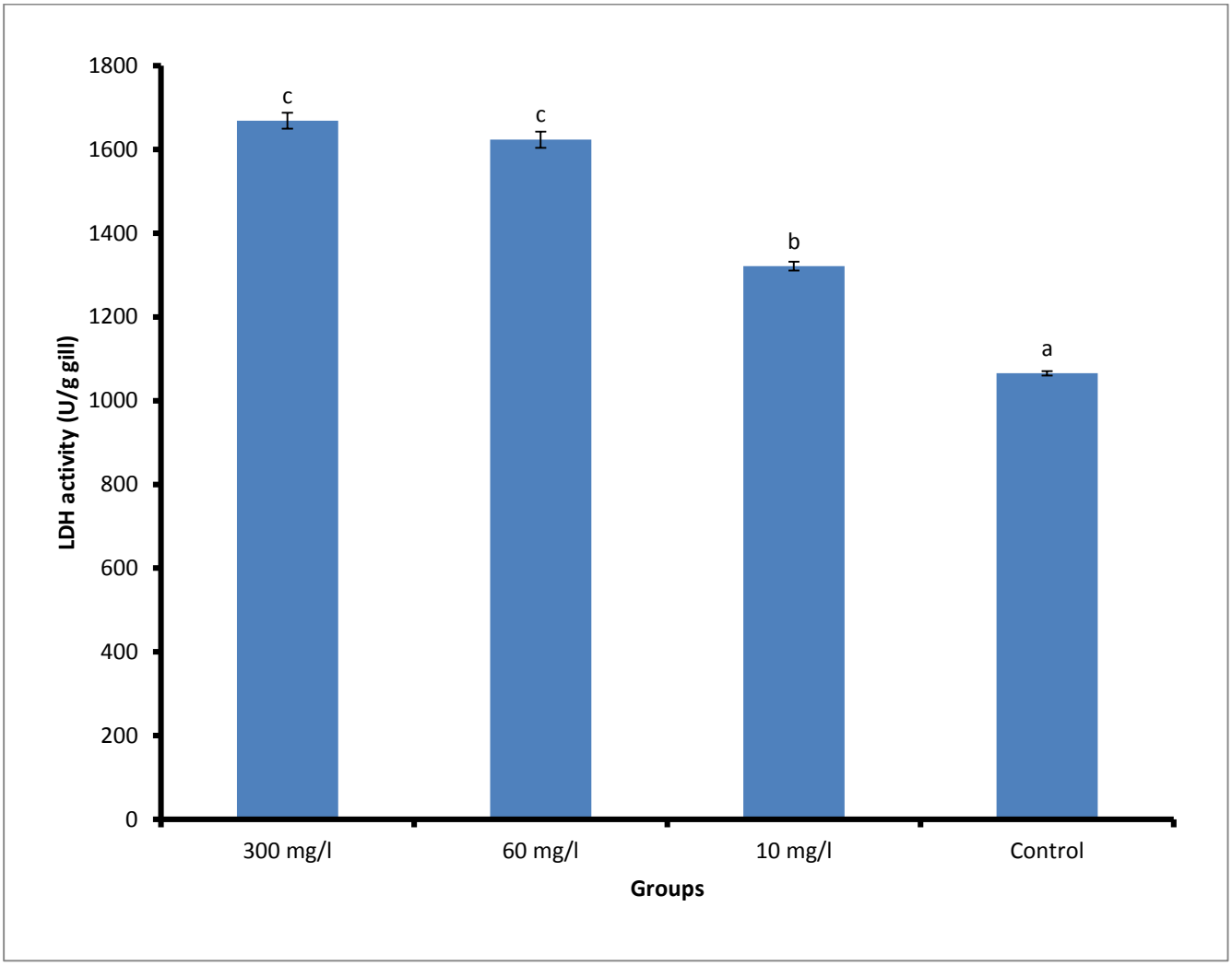


Figure 4.15: Lactate dehydrogenase (LDH) activity in the gill of African cat fish from control group and glyphosate polluted group. Bars are presented mean $\pm$ standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

4.1.3 Effect of glyphosate on liver function

The result in Figure 4.16 shows the effect of the different concentration of glyphosate on serum ALP activity. From the result, 300 mg/lgroup and 60 mg/l group showed significant ($p < 0.05$) increase in the activity of ALP than control group. The 10 mg/lgroup did not show any significant increase ($p < 0.05$) in the activity of ALP compared to the control.

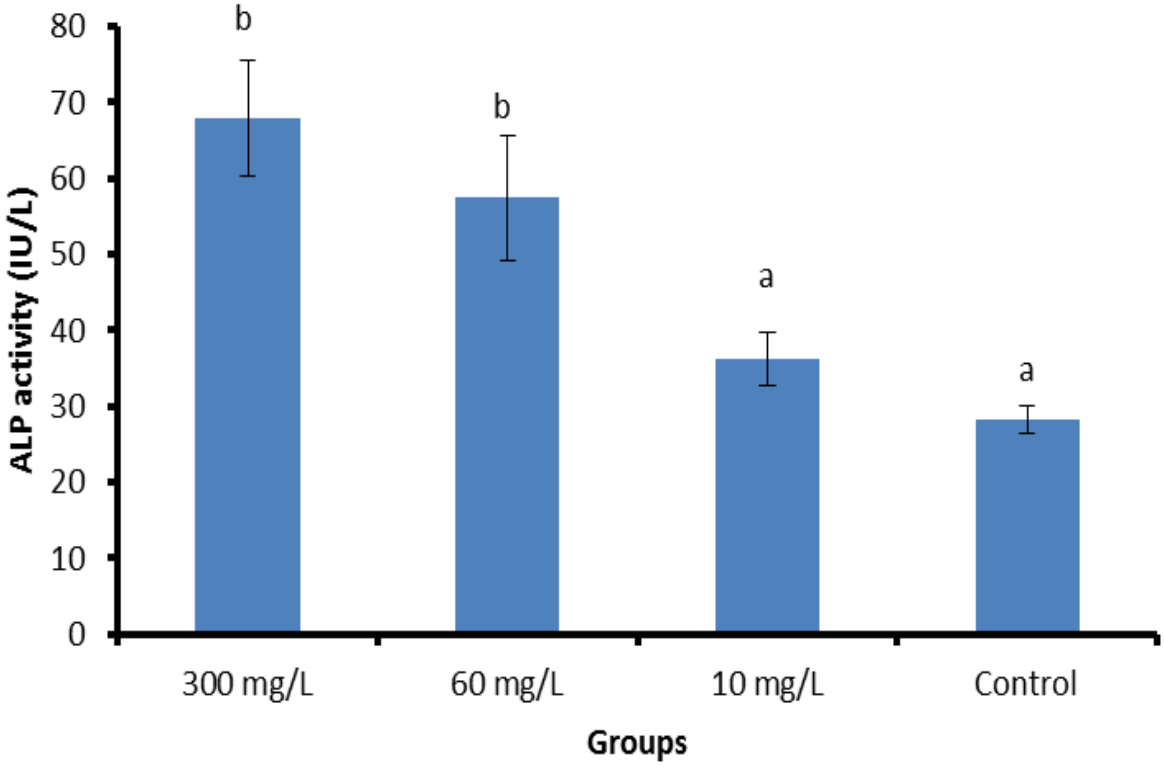


Figure 4.16: Serum ALP activity of African cat fish from control group and glyphosate polluted group. Bars are presented mean ± standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

The result in Figure 4.17 shows the effect of the different concentration of glyphosate on serum ALT activity. From the result groups 300mg/l and 60mg/l showed a significant increase ($p < 0.05$) in ALT activity than the control. Group 10mg/l seams to be unusually higher than expected which could be due to technical error. However the lowest activity of ALT was observed in the control group.

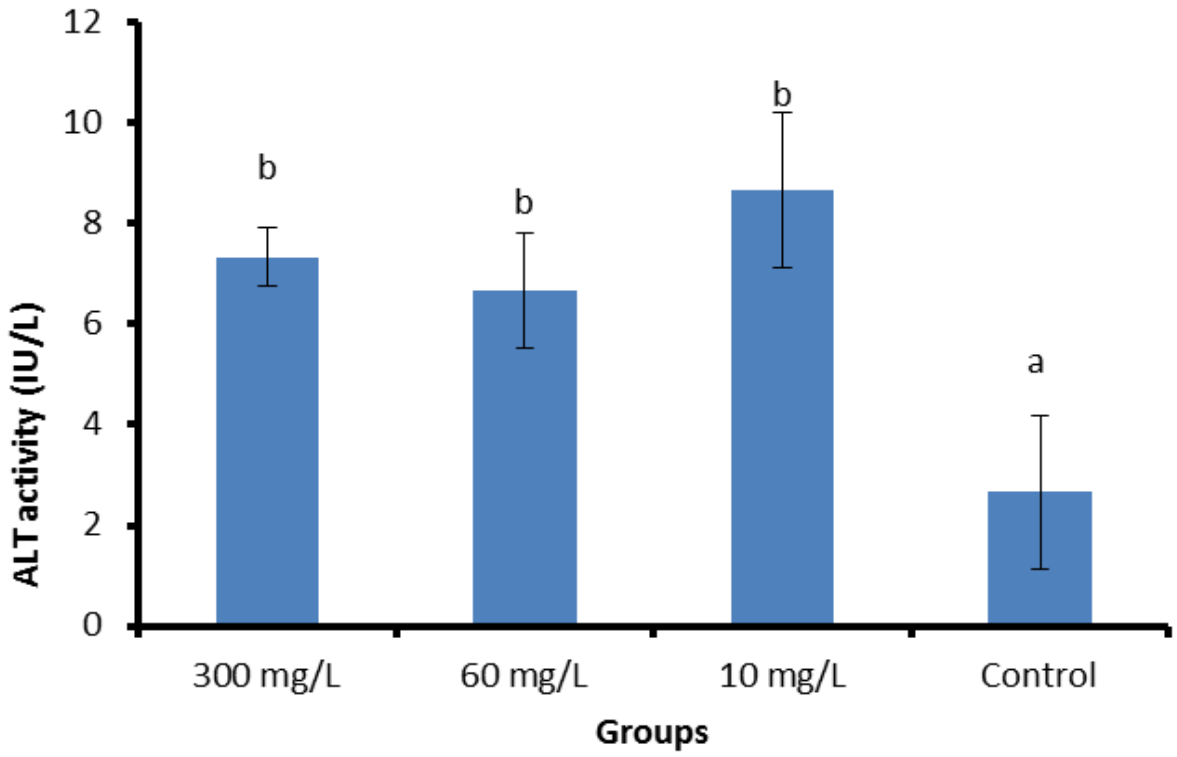


Figure 4.17: Serum ALT activity of African cat fish from control group and glyphosate polluted group. Bars are presented mean ± standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

The result in Figure 4.18 shows the effect of the different concentration of glyphosate on serum Aspartate transaminase activity. From the result group 300mg/l shows a more significant increase ($p < 0.05$) in AST activity than group 60mg/l and group 10mg/l compared to the control. The control group shows the significantly lowest activity of AST.

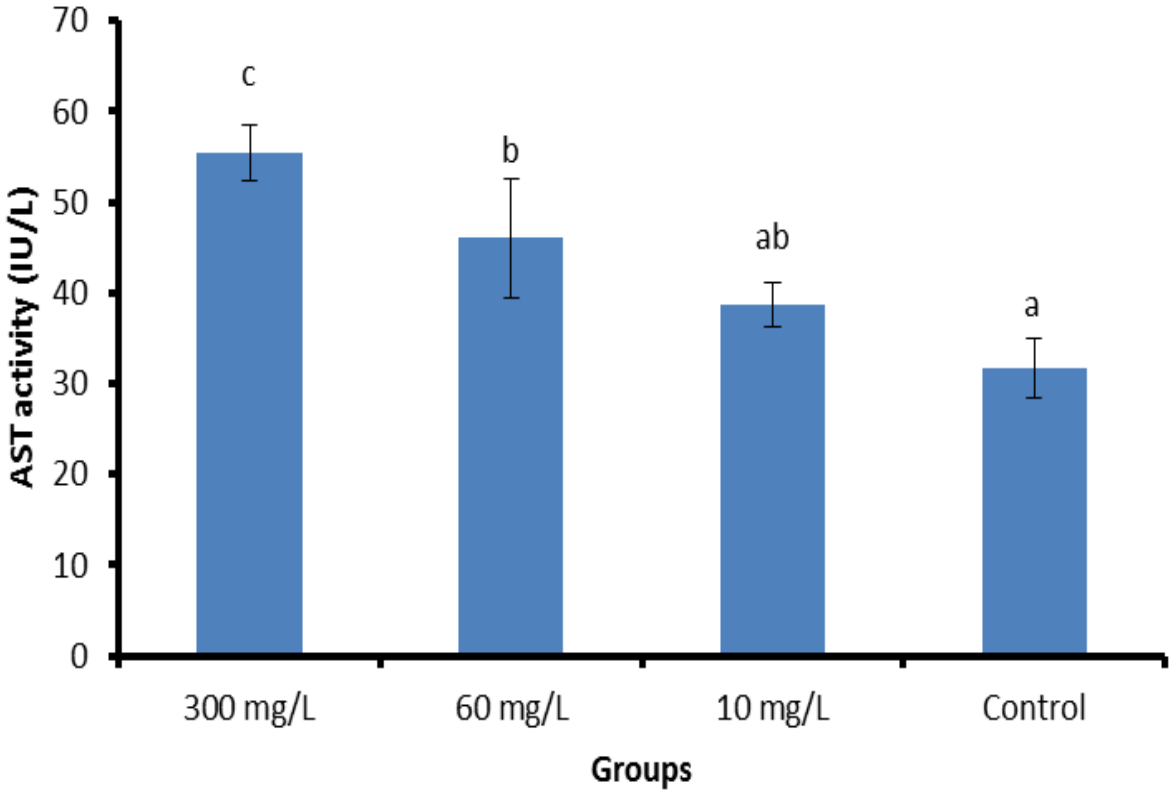


Figure 4.18: Serum AST activity of African cat fish from control group and glyphosate polluted group. Bars are presented mean ± standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

The result in Figure 4.19 shows the effect of the different concentration of glyphosate on Total bilirubin concentration in the serum as a liver function parameter. From the result group 300mg/l showed significant increase ($p < 0.05$) in total bilirubin concentration than group 60mg/l and group 10mg/l compared to the control.

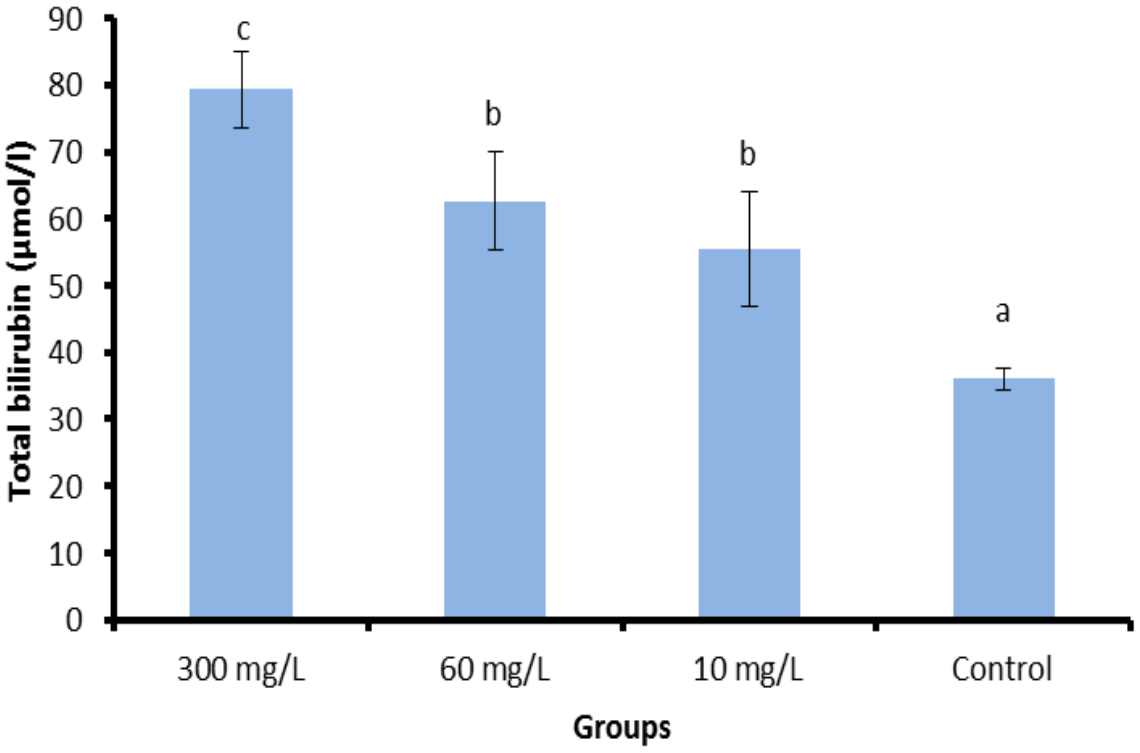


Figure 4.19: concentration of bilirubin in the serum of African cat fish from control group and glyphosate polluted group. Bars are presented mean ± standard deviation (SD) of the determination (5) and bars with different alphabets are statistically significant ($P<0.05$).

4.1.4 Effect of glyphosate on heamatological parameters

Table 4.1 presents the effect of glyphosate on hematological parameters (WBC, LY, MID, GRAM, RBC, HGB, MCV, HCT and MCH) in African cat fish. The result of WBC, RBC and HGB showed a significant increase ($P<0.05$) when compared to the control.While the result of LY, MID, GRAM, MCV, HCT and MCH show no significant difference ($P>0.05$) when compared to the control.

Table 4.2 presents the effect of glyphosate on hematological parameters (MCHC, RDWSD, RDWCV, PLT, MPV, PCT and PDW) of African cat fish. The result for MCHC, RDWSD, RDWCV, MPV and PDW showed no significant difference ($P>0.05$) when compared to the control. PLT and PCT showed a significant decrease ($P<0.05$) in exposed group when compared to the control.

Table 4.1: Effect of glyphosate on hematological parameters (WBC, LY, MID, GRAM, RBC , HGB,MCV, HCT and MCH) in African cat fish.

Groups	WBC	LY	MID	GRAM	RBC	HGB	MCV	HCT	MCH
	(10^3mm^{-3})		(%)		10^3mm^3		($10^{-3}mm^{-3}$)	(g/dl)	(fl)	(%)	(pg)
300mg/l	7.53 ± 0.45^{c}	3.86 ± 0.56^{a}	0.82 ± 0.87^{a}	2.18 ± 1.06^{a}	4.66 ± 0.34^{a}	123.67 ± 6.81^{a}	81.67 ± 1.53^{a}	0.38 ± 0.03^{a}	25.20 ± 3.34^{a}
60mg/l	6.43 ± 0.54^{ab}	3.81 ± 1.63^{a}	0.63 ± 0.40^{a}	2.25 ± 0.89^{a}	4.85 ± 0.75^{a}	133.67 ± 3.21^{a}	79.33 ±7.64^{a}	0.40 ± 0.01^{a}	25.13 ± 1.1o^{a}
10mg/l	6.82 ± 0.44^{bc}	2.61 ± 0.47^{a}	0.50 ± 0.51^{a}	3.72 ± 2.12^{a}	5.08 ± 0.10^{a}	126.33 ± 4.93^{a}	77.00 ± 4.00^{a}	0.38 ± 0.02^{a}	24.37 ± 1.98^{a}
Control	5.77 ± 0.20^{a}	2.08 ± 0.57^{a}	0.89 ± 0.69^{a}	2.17 ± 0.99^{a}	6.65 ± 0.40^{b}	154.33 ±12.50^{b}	76.00 ± 200^{a}	0.41± 0.06^{a}	24.43 ± 2.24^{a}

Values = means ± standard deviation of five determination (column having different superscript are significantly different ($P < 0.05$)).

Table 4.2: Effect of glyphosate on hematological parameters (MCHC, RDWSD, RDWCV, PLT, MPV, PCT and PDW) of African cat fish

Group	MCHC (g/dL)	RDWSD (fL)	RDWCV (%)	PLT (10^3/µL)	MPV (fL)	PCT µg/L	PDW .
300mg/l	307.67 ± 36.40[a]	35.73 ± 1.22[a]	13.23 ∓ 0.35[a]	325.33 ± 64.76[a]	8.57 ± 0.90[a]	0.33 ± 0.08[a]	12.69 ± 0.54[a]
60mg/l	318.00 ± 18.36[a]	37.07 ± 1.25[a]	14.23 ± 1.18[a]	445.67 ± 69.04[b]	7.79 ± 0.51[a]	0.36 ± 0.08[a]	12.60 ± 0.54[a]
10mg/l	314.67 ± 10.02[a]	35.93 ± 1.50[a]	14.10 ± 0.10[a]	618.67 ± 27.79[c]	7.77 ± 0.49[a]	0.51 ± 0.02[b]	12.74 ± 0.51[a]
Control	322.33 ± 23.16[a]	35.73 ± 2.31[a]	14.30± 0.65 [a]	749.33 ±70.52[d]	8.07 ± 0.35[a]	0.52 ± 0.12[b]	12.74 ± 0.29[a]

Values = means ± standard deviation of five determination (column having different superscript are significantly different ($P < 0.05$)).

4.2 Discussion

Glyphosate is one of the widely used herbicides and it is considered to be persistent and mobile in soil and water. It is also known to be one of the most common terrestrial and aquatic contaminants (Ayoola *et al*., 2008) and it is used extensively in agricultural lands and wetlands (Ejike *et al.*, 2011) Glyphosate is an endocrine disruptor (Gasnier *et al*., 2009; Paganelli *et al.*, 2010; Antoniou *et al*., 2012; Thongprakaisang *et al.*, 2013). Fish and aquatic invertebrates have been considered to be efficient and cost effective model systems for studying the toxic, mutagenic and carcinogenic potential of pollutants due to their ability to metabolize, concentrate and store water-borne pollutants. There are no safe levels of endocrine disruptors (Vandenberg *et al.*, 2012; Ayanda *et al.,* 2018). As a result of the repeated applications of the herbicicide in most developing countries, the concentration of the herbicide in the aquatic ecosystem may be high, thus, necessitating the need to monitor and assess its impacts in the environment (Glusczak *et al*., 2007; Modesto and Martinez, 2010; Delfina *et al.,* 2014)

The result obtained from the study has shown that the herbicide, glyphosate, can produce a reduction in testosterone concentration of *Clarias gariepinus*. The result of this study supports the research of Reyes (2014) on bioaccumulation and evidence of hormonal disruptions in fish exposed to Sub-lethal Concentrations of pesticides. This study also supports the observation that prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology (Romano *et al.,* 2010). The significantly decreased testosterone in male African cat fish may be as a result of direct damage of the herbicide glyphosate on the leydig cells (Harding and Velotta, 2011; Delfina *et al.,* 2014; Ayanda *et al*., 2018; Ogunwole *et al*., 2018). There is already evidence that glyphosate may act as an endocrine disruptor for both

males and females by altering aromatase activity, oestrogen regulated genes, and testosterone levels in rats (Delfina *et al.*, 2014; Ayanda *et al*., 2018; Ogunwole *et al*., 2018; Dai *et al.*, 2018).

Testosterone deficiency is associated with an enhanced risk for osteoporosis, altered body composition including decrease in muscle performance (Dai *et al.*, 2018). Glyphosate is an endocrine disruptor, and imbalances and malfunctions of the endocrine system can lead to diabetes, hypertension, obesity, kidney disease, cancers of the breast, prostate, liver, brain, thyroid, non-Hodgkin's lymphoma (Delfina *et al.*, 2014; Ayanda *et al*., 2018; Ogunwole *et al*., 2018), osteoporosis, cushing's syndrome, hypo- and hyperthyroidism, infertility, birth defects, erectile dysfunction, sexual development problems and neurological disorders such as: learning disabilities, attention deficit disorder (de Cock *et al*., 2012), autism (Schulkin, 2007), dementia (Ghosh, 2010), Alzheimer's (Merlo *et al*., 2010). Several pesticides have been identified by previous studies as endocrine disruptors in other vertebrate fish and these include atrazine, diazinon, diuron, endosulfan, fenthrothion, lindane, parathion and permethrine (Harding and Velotta, 2011; Vandenberg *et al.*, 2012; Isibor *et al.*, 2017; Ayanda *et al*., 2018; Dai *et al.*, 2018).

Results of the present work confirm the observations of Schweizer (2014) who observed that herbicides lead to decrease in tri-iodothyronine (T3) and an increase in thyroid stimulating hormone (schweizer *et al.*, 2014). The present work shows a significant increase in thyroid stimulating hormone (TSH) and thyroxine (T4). The result of this study corresponds to the report of Kusakebe *et al.* (2016) on the effect of glyphosate on human cell line. The significant decrease in tri-iodothyronine (T3) is believed to be as a result of the inhibition of deiodinase which catalyzes the deiodination of T4 to T3 which is the active form (schweizer *et al.*, 2014). Studies have shown that Selenium deficiency has an implication on the fall of T3 levels. Lactobacillus which converts inorganic selenium into more bioavailable forms such as

selenocysteine and selenomethionine are negatively impacted by glyphosate, which in turn lead to a depletion of selenomethionine and selenocysteine resulting to the fall of T3 (Tania *et al.,* 2014).

Glyphosate has been reported to cause oxidative stress leading to lipid peroxidation indifferent tissues of other fish species (Glusczak *et al*., 2007; Langiano and Martinez, 2008; Modesto and Martinez, 2010; Delfina *et al.,* 2014). Major biomarkers of oxidative stress include changes in antioxidant enzyme activity and accumulation of oxidative damage products as indicated by *Clarias gariepinus* exposed to glyphosate in this study. Malondialdehyde (MDA) is one of the oxidative damage products of lipid peroxidation. Its presence in tissues indicates oxidative stress. According to Hazarika *et al*. (2003) and Kavitha and Rao (2007), lipoperoxidation can occur through the direct interaction of organophosphorus compounds with the cytoplasmic membrane, resulting in systemic damage. This is the principal molecular mechanism associated with the toxicity of several pesticides (Delfina *et al.,* 2014; Isibor *et al.,* 2017; Ayanda *et al*., 2018).

Results from this study showed that MDA increased in the kidney and gill after 9 days of exposureof *Clarias gariepinus* to different concentrations of glyphosate. Oxidative stress due to the toxic effect of pollutants is usually indicated by increased levels of products of oxidative damage (MDA). The observed increase in MDA corresponds with the study by Kathya *et al.* (2010) on the effect glyphosate on the liver, muscle and brain of prochiloduslineatus.There was a subsequent increase in the defense enzyme catalase (CAT) in response to oxidative stress on the kidney, liver and gill of *Clarias gariepinus*. This finding is in line with a previous investigation made by Guilherme *et al.,* (2012) who reported an increase in catalase activity indicating overproduction of H_2O_2 which is considered to be the most toxic ROS (Guilherme *et al.,* 2012). Moraes *et al*. (2007) found increased CAT activity in the liver of L. obtusidens exposed to the

herbicides clomazoneand propanil. Pereira Maduenho and Martinez (2008) also found increased CAT in the liver of P. lineatus exposed to diflubenzuron (Delfina *et al.,* 2014).

Glyphosate caused decrease in superoxide dismutase activity in the kidney, liver and gill of *Clarias gariepinus*. Decreased SOD was also observed by Modesto and Martinez (2010) in the liver from prochilodus lineatus exposed to glyphosate. This could be explained by the accumulation of hydrogen peroxide which has been reported to inhibit enzyematic activity (Luschak *et al.,* 2009; Delfina *et al.,* 2014; Isibor *et al.,* 2017; Ayanda *et al*., 2018) Superoxide dismutase (SOD) can be inactivated by hydrogen peroxide Kathya *et al.,* (2010). This could also be related with the increase of protein carbonyl which causes change on the protein and reduction of the enzymatic activity. Decrease in SOD and glutathione peroxidase corresponds to the report of Guilherme *et al.,* (2012). The reduction in glutathione peroxidase activity would be explained by a decrease in the availability of the tripeptide GSH which acts as a substrate to glutathione peroxidase. Shortage of GSH would result to reduction of GPx activity (Kathya *et al.,* 2010).

The result also showed an increase in the lactate dehydrogenase level in the gill of *Clarias gariepinus* exposed to glyphosate. The result is in line with a previous study by Ayanda (2015) on the effect of long-term exposure of *Clarias gariepinus* juveniles to glyphosate. Shiwanand (2014) also reported an increase in LDH activity of the gill of *Clarias gariepinus* exposed to glyphosate. The result also corresponds with the study of Haskovic *et al.*, (2016) on the effect of glyphosate on Rat. In fish, the gills provide a large interface between the external and internal environments and as such are the first organs to get in contact with xenobiotics (Melesse *et al.,* 2011).The gills are particularly sensitive to changes in environmental conditions and perform

such vital functions as ion osmoregulation, gas exchange and nitrogen excretion (Melesse *et al.*, 2011).

The liver is the main organ involved in the biotransformation of xenobiotics. The liver tissue consequently shows high enzyme activity, although such activity does not appear to have been sufficient to avoid the damage promoted by glyphosate based herbicide. Among the different types of enzyme, both AST and ALT were proven to play an essential role in the metabolism of proteins and carbohydrates (Gholami-Seyedkolaei *et al*., 2013). In the present study, the increase in plasma transaminases concentration indicates liver damage under glyphosate exposure. In this context, they appear to be reliable in demonstrating liver damage caused by pesticides (Gholami-Seyedkolaei *et al*., 2013). Elevation of plasma AST, ALT and ALP shows liver damage which may be hepatitis or necrosis of cells (Chen *et al., 2013;* Haskovic *et al.*, 2016; Isibor *et al.,* 2017; Ayanda *et al*., 2018). Increase in AST and ALT indicates active transamination, so as to maintain energy cycle (Gholami-Seyedkolaei *et al*., 2013). ALP in the cellular external membrane plays the major role in phosphate metabolism and it prevents the external membrane from being damaged. Its increase may be due to phosphate ingestion by the fish. The increase in this enzyme implies overproduction of phosphate bound esters, which helps in the maintenance of cell integrity (Chen *et al., 2013;* Isibor *et al.,* 2017; Ayanda *et al*., 2018).

In the present study, an increase was observed in serum mean ALP, AST and ALT.Other authors have demonstrated increased AST and ALT activity when *Cyprinus carpio* were exposed to glyphosate (Gholami-Seyedkolaei *et al*., 2013). The results of this study also correspond with the observation of Haskovic *et al.* (2016) on the effect of glyphosate on *Rattus norvegicus* (Haskovic *et al.*, 2016). An increase in these enzymatic activities (ALT and AST) in the extracellular fluid or plasma may be a sensible indicator of cellular liver damage caused by glyphosate exposure.

The increase in the plasma enzymes could be due to toxic injury caused by glyphosate which may have stimulated tissue repair through protein turnover and increased respiration. Assessment of plasma and liver enzyme activities can be considered as diagnostic tool to determine the physiological status of cells or tissue (Kier *et al.,* 2013; Chen *et al., 2013*). Alteration in plasma enzyme activities of fish resulting from toxicants or contaminant affecting various cells, immune system, tissues and organs of fish was also reported by Gabriel (Isibor *et al.,* 2017; Ayanda *et al*., 2018).

The values obtained show an elevation in serum concentration of total bilirubin and conjugated bilirubin in the *Clarias gariepinus*. This corresponds with the study of Gholami-Seyedkolaei (Gholami-Seyedkolaei *et al*., 2013). This may be consequence of the disruption of the hepatic architecture by glyphosate such that the conjugation of bilirubin and excretion of bilirubin is altered. Elevations in serum total and conjugated bilirubin concentrations are attributable to liver and/or biliary tract disease, while elevations in ALT and AST concentrations in serum are usually due to disruption of hepatic cells as a result of necrosis or altered membrane permeability, and elevations in serum ALP concentration is often due to cholestasis (Ejike *et al*., 2008).

Blood parameters could be a useful tool for understanding and describing the impact of pesticides in several fish species, owing to the sensibility and indication of physiopathological alterations under different conditions of stress (Modesto & Martinez, 2010; Kreutz *et al.,* 2011; Isibor *et al.,* 2017; Ayanda *et al*., 2018) Hematological parameters are useful factors in the assessmentof fish health status in a numerous fish species to determine physiological changes due to impact of exposure to varying stressful condition such as handling, pollutants, pesticides metals, hypoxia, anesthetics and acclimation (Ayuba *et al*., 2018). Fish are known to be in close

relationship with the aqueous environment, therefore, the conditions within the body of the fish long before there is any visible manifestation of disease would be revealed by the blood (Okomoda *et al.,* 2013).The production of ROS induced by glyphosate could be the cause of the haematological alterations observed in this study. The changes in the haematological parameters suggest that the herbicide caused an anemic syndrome in the animals treated.

The white blood cells in fish respond to various stressors including infections and chemical irritants. Differential White Blood cell profiles are sensitive to physiological changes and health status in animals (Safahieh *et al.,* 2010; Paetoe *et al., 2012*; Sinhorin *et al*., 2014). White blood cell numbers can also be affected by a variety of physiological and environmental factors and the responses normally found when fish are subjected to an array of toxicants are lower percent lymphocytes and higher percent neutrophils (Witeska, 2005). In the present study there was an elevation in the white blood cell of *Clarias gariepinus* exposed to various concentrations of glyphosate. Thus increasing or decreasing numbers of white blood cells are a normal reaction used to reveal the exposure of toxicants (Kori-Siakpere, 2006).White Blood Cells (WBC) count increase with increasing level of the toxicant, this increase is likely due to heightened immune mechanism of the experimental fish species stimulated to fight against the toxicant.

Erythrocytes are produced in the haematopoietic tissue, which is situated in the spleen and head kidney. Erythrocytes possess limited antioxidant defenses, which renders the cells more sensitive to changes in the antioxidant/pro-oxidant balance. The result of this study shows a reduction in the red blood cell count of *Clarias gariepinus* exposed to glyphosate. This result supports the report of Ikpesu (2010) on *Clarias gariepinus* exposed to endosulfan (Safahieh *et al*., 2010).The reduced quantity and quality of erythrocytes and a decreased haemoglobin concentration as seen in the present study may lead to a deteriorated oxygen supply. In addition to transport of oxygen,

erythrocytes have other functional tasks in the body; therefore an insufficient quantity and quality of erythrocytes would consequently have several additional effects on metabolism beyond the simple oxygen supply for tissue metabolism. Prolonged reduction in haemoglobin content has been reported to be deleterious to oxygen transport and degeneration of the red blood cells could be referred to as pathological conditions in fishes exposed to toxicants. The significant difference in erythrocyte count of African Catfish exposed to various concentration of glyphosate herbicide compared to the control in this study agree with the findings of Ikpesu, (2010) of *Clarias gariepinus* exposed to Endosufan. This finding also agrees with the results reported by Glusczak *et al*. (2006), who observed a decrease in number of erythrocytes and haemoglobin of *Leporinus obtusidens* exposed to different concentrations of Round-up original.

The present study shows a reduction in the haemoglobin content of the exposed fish. This corresponds to the study of Gaafar *et al*. (2010) who reported that prolonged reduction in haemoglobin content is deleterious to oxygen transport and degeneration of the erythrocytes could be due to pathological condition in fish exposed to toxicants. Similar results had been reported on fish exposed to different toxicants under laboratory conditions by Aderolu *et al*. (2010), and Okomoda *et al.* (2010).The significant reduction in these parameters is an indication of severe anaemia caused by destruction of erythrocytes (Kori-Siakpere *et al*., 2009), Heamodilution (Adeyemo, 2005 and Ayuba 2008) resulting from impaired osmoregulation across the gill epithelium and according to Okomoda *et al*. (2010) could be as a result of the destruction of intestinal cells.It is well known that a reduced quantity and quality of erythrocytes and a decreased haemoglobin level as seen in the present study led to a deteriorated oxygen supply. In addition to transport of oxygen, erythrocytes have other functional tasks in the body; therefore an insufficient quantity and quality of erythrocytes would consequently have several

additional effects on metabolism beyond the simple oxygen supply for tissue metabolism. Prolonged reduction in haemoglobin content has been reported to be deleterious to oxygen transport (Safahieh *et al*., 2010).

The rate of platelet formation seems to be governed by the amount of oxygen in the blood and the presence of nucleic acid derivatives from injured tissue. The result of the study shows a decrease in the platelet count for *Clarias gariepinus* exposed to glyphosate. This could be as a result of lowered number of red blood cells or reduced haemoglobin content. The reduction in platelet count of the fish is assumed to affect blood clothing this would result to internal bleeding, excessive bleeding at the slightest cut. On a more serious case blood will be detected in urine and faeces (Kori-Siakpere *et al*., 2009; Safahieh *et al*., 2010).

The result of the present study shows that haematological response of *Clarias gariepinus* exposed to the concentration of glyphosate herbicides showed a reduction of Erythrocyte count (RBC), Haemoglobin (HGB) level, platelet (PLT) and procalcitonin (PCT) concentration of the blood. No significant difference ($P<0.05$) was observed on LY, MID, GRAM, MCV, HCT, MCH, MCHC, RDWSD, RDWCV, MPV and PDW.The calculated hematological indices, MCV, MCHC, MCH, and HCT have a particular importance in the diagnosis of anemia in most animals (Kori-Siakpere *et al*., 2009). Low number of red blood cells or insufficient amount of their hemoglobin content could influence energy balance of the body. In this case fish may suffer from oxygen deficiency, which ultimately prohibits its normal growth (Kori-Siakpere *et al*., 2009; Safahieh *et al*., 2010).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Based on the objectives of the research, glyphosate has been successfully confirmed to be toxic. The results obtained in this study on the effect of sublethal concentrations of glyphosate indicate that:

- Glyphosate caused decrease in the testosterone concentration of male *Clarias gariepinus*.
- Glyphosate caused increase in TSH and T4 concentration and a decrease in T3 concentration on the exposed fish.
- Exposure to glyphosate induced oxidative stress on *Clarias gariepinus.* The product of oxidative stress MDA increased in the kidney and gill of *Clarias gariepinus* indicating lipid peroxidation. The antioxidant enzyme catalase increased in the kidney, liver and gill of *Clarias gariepinus,* superoxide dismutase decreased in the kidney, liver and gill and glutathione peroxidase decreased in the liver and gill of the exposed fish.
- Exposure to glyphosate caused an increase in the liver function parameters ALP, ALT, AST and bilirubin of *Clarias gariepinus.*
- Furthermore this study shows that glyphosate induces haematological changes therefore should not be used indiscriminately.

5.2 Recommendation

Fish farmers are now better positioned, scientifically, to make informed decision on the use of glyphosate for weed control around the fish farm.

More evidence-based toxicity studies will be needed to confirm the effect of glyphosate on lipid peroxidation and antioxidant enzymes.There is also need for a molecular organ to organ tracing to find out which organ is most affected by glyphosate and the specific pathway it inhibits.

Biochemical studies can also be done so as to give information of the effect on humans and other organism which might use the fish as food. There is also need to determine the extent of bioaccumulation of glyphosate in fish.

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