BIOCHEMICAL CHANGES IN TADPOLES EXPOSED TO EFFLUENTS FROM AUTOMOBILE WORKSHOPS IN EVBAREKA AND UWELU, BENIN CITY, EDO STATE.

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CERTIFICATION OF THESIS

We certify that this work was carried out by Samuel Ameh, GABRIEL in the Department of Biochemistry, University of Benin, Benin City, Nigeria.

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DEDICATION

I dedicate this work to my family for their prayers and support.

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ABSTRACT

This study aimed to determine metal analysis on the soil, weight changes, antioxidant defense systems, level of malondialdehyde, lipid content, the phospholipids profile, the activity for two weeks of energy and some enzymes in energy metabolism, and the lipid profile in tadpoles exposed to effluents from automobile workshops. Tadpoles were obtained from runoff water containing effluents used in automobile workshops from two different sites (Evbarake and Uwelu) in Benin City, Nigeria were used in this study. A total of 60 tadpoles were divided into three Groups with each group containing 20 tadpoles. Group 1 served as the control. Group 2 Evbarake site. Group 3 Uwelu site. Soil samples were collected from Capitol University of Benin Control group and automobile workshops (Exposed groups) respectively. The animals were maintained for the study period of two weeks with their requisite sample soil/water from the respective sites. Metal analysis on the soil, weight changes, antioxidant defense systems, level of malondialdehyde, lipid content, the phospholipids profile, the activity for two weeks of energy and some enzymes in energy metabolism, and the lipid profile were analyzed using standard methods.

The data from Table 4.1 show that the heavy metal analysis were significantly ($p < 0.05$) higher than the control. The malondyaldehyde levels were significantly ($p < 0.05$) higher in tadpoles from both sites compared to the control group. The Evbarake site showed a significant ($p < 0.05$) increase in catalase activity compared to the control, while the Uwelu site showed a slight decrease. The glutathione peroxidase activity was significantly increased in tadpoles from both test sites when compared to the control. Aldehyde oxidase activity was significantly higher in tadpoles from both contaminated sites compared to the control. The results demonstrated that the exposure led to a significant ($p < 0.05$) increase in total lipids, triacylglycerol, low density lipoproteins and high density lipoproteins in a site-dependent manner. The exposure led to a significant increase in glucose levels. It was also observed that the exposure of tadpoles within the test sites decreased the weight of tadpoles and final weight of head region in tadpoles from the Uwelu site showed a significant decrease when compared to those from the control site ($p < 0.05$).

Overall, this study provides important insights into the potential health risks associated with effluents exposure including alteration in glucose metabolism, antioxidant enzymes activity, and lipid peroxidation in the presence of effluents which may cause oxidative stress in the test sites. Further research is needed to fully elucidate the underlying mechanisms and potential longterm effect of this exposure.

CHAPTER ONE: INTRODUCTION

1.1 Background of the Study

The quality of the environment has continued to decline in many parts of the world due to human activity. Many metals are known to have negative effects on the biota associated with freshwater aquatic systems (Eriyamremu *et al*., 2008). Metals are released into aquatic systems in high concentrations by various industrial, agricultural and mining processes (Eriyamremu *et al.*, 2008). Degradation of habitat and pollution of the environment have garnered a lot of attention. Sadly, not much research has been done on the environmental pollution issues caused by small-scale industrial establishments like welding shops. An amazing amount of publications has been published in the literature on large-scale environmental pollution problems like acid rain and oil

pollution. Calcium carbide (CaC_2) is used by welders and panel beaters to produce acetylene (C_2H_2), which is the gas that is combined with oxygen to create the oxy-acetylene flame. The debris emerges from the welding as a white or greyish lump. Its natural state is alkaline. The waste that results from these welding operations is typically carelessly deposited on farms and water ways on the welding workshops' property, where it eventually becomes part of the soil and water.

According to Kinako and Amadi (1997), carbide waste significantly reduces the infiltration of water and accumulates plant biomass. They went on to say that the inward movement of fine carbide waste particles into the pore spaces of the soil is what causes the slowing down of water infiltration at a site polluted by carbide waste, and this always slows down plant growth. The hue of spent calcium carbide is pale or grey, and its pH is highly alkaline. According to Semikolennykh *et al.* (2012), it is mostly made up of calcium compounds, such as calcium hydroxide ($Ca(OH)_2$), which gradually combines with ambient carbon dioxide to generate non-toxic calcium carbonate. Studies on garbage exposed to air have demonstrated that it takes at least ten, if not more than thirteen years, for such waste to convert to calcium carbonate. Such waste has a toxicity since its pH is still very alkaline (Zhang *et al*., 2014). According to Semikolennykh *et al.* (2012), dilution at a waste-to-water ratio of 1:1000 is the only way to reduce pH to a level where the product is safe for the environment.

Although the welding sector produces toxic by-products that can damage the environment if illegally disposed of, it is essential for manufacturing, building, and repair operations. Spent carbide from tungsten or other hard metal welding electrode tips is one example of such waste material. Toxic heavy elements such chromium, cobalt, nickel, and others can be found in used carbide (Bringas, 2004). These welding wastes can have a major negative influence on water

quality and aquatic life when they are dumped directly into aquatic habitats or are released through surface runoff. It has been demonstrated that heavy metal pollution in water bodies causes sediments to build up and aquatic organisms to bioaccumulate (Kaviraj & Zamik, 2014). In species ranging from microbes to fish, this can result in developmental abnormalities, decreased growth, disease susceptibility, reproductive issues, and mortality. Because their eggs and larvae are permeable and they live in both aquatic and terrestrial settings, amphibians, such as frogs and salamanders, are especially vulnerable (Sparling *et al.,* 2010). An investigation has discovered connections between amphibian population decreases, malformations, and reproductive problems and heavy metal contamination (Hoskin *et al*., 2019). They are significant biological markers of the health of the environment because of their sensitivity. Tadpoles at contaminated sites may experience biochemical alterations compared to amphibians in uncontaminated conditions (Eriyamremu *et al*., 2008). The most common definition for oxidative stress is the existence of an imbalance between Reactive oxygen species (ROS) quantities in the body and antioxidant defenses in favor of ROS; called pro-oxidant state (Costantini, 2014). Reactive oxygen species (ROS) produced by heavy metals including chromium, nickel, and cobalt found in welding wastes can harm tadpole cells' proteins, lipids, and DNA (Hermes-Lima, 2004). Cadmium stands out as a potent toxicant that has been linked to alterations in cellular homeostasis and has been shown to enhance the production of several activated oxygen species designated ROS (Asagba and Eriyamremu, 2008). The antioxidant system, which includes enzymes like glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), and aldehyde oxidases, is activated by this oxidative stress. I therefore report the levels of Zn, Cu, Cd, Fe, Mn and Pb in the soil and the environmental stress using antioxidant enzyme system and

malondialdehyde formation level of the tadpoles present in effluent as surrogate biomarkers of aquatic pollution in Evbarake and Uwelu site, Benin City, Nigeria.

1.2 Statement of Research Problem

Concerns are raised over potential negative impacts on tadpoles and the ecosystem's overall health when effluents from automobile workshops, including a by-product of welding processes, is improperly disposed of in water bodies. Due to their high sensitivity to environmental pollutants, tadpoles may have developmental defects, decreased survival rates, and food chain disruptions if heavy metals and other pollutants are present in their habitat (Sparling *et al*., 2010). The purpose of this study is to look at how effluents from automobile workshops affect tadpoles in their natural environment. Through investigating the possible impacts on tadpole survival, growth, and development, this study will further our comprehension of the environmental ramifications of inappropriate waste management practices and provide guidance for the creation of suitable mitigation plans to safeguard aquatic environments.

1.3 Justification of the Study

Tadpoles contribute to the biodiversity of the ecosystem and are prone to biochemical changes in adverse conditions such as toxicity from chemicals. It will be interesting to access some biochemical changes in tadpoles in order to understand survival strategies of the animal during its development. Welding is an essential process in automobile workshops, and its by-products, such as effluents, require proper disposal to prevent environmental pollution. However, instances of improper disposal of effluents into water bodies have been reported, potentially impacting aquatic life, including tadpoles. Tadpoles are crucial components of aquatic ecosystems and play

a vital role in maintaining ecological balance (Hocking & Babbitt, 2014). The proposed study aims to investigate the effect of effluents from automobile workshops in water containing tadpoles. This research is necessary to understand the potential impact of effluents from automobile workshops on aquatic ecosystems

1.4 Aim and Objectives

The aim of this study was to assess biochemical changes and adaptability strategies in tadpoles that are exposed to effluents from automobile workshops in Evbareka and Uwelu.

The objectives of the study were to:

1. determine the heavy metal composition test on soil samples collected from the Evbarake and Uwelu sites
2. determine the weight changes of tadpoles exposed to effluents from automobile workshops for 2 weeks from the Evbarake and Uwelu sites.
3. evaluate the effect of effluents on superoxide dismutase, aldehyde oxidase, catalase activities as well as malondialdehyde level on the head and tail regions of tadpole infected with effluents from the Evbarake and Uwelu sites.
4. determine the lipids content of head and tail regions of tadpoles exposed to effluents for 2 weeks from the Evbarake and Uwelu sites.
5. determine the phospholipids profiles of head and tail regions of tadpoles exposed to effluents for 2 weeks from the Evbarake and Uwelu sites.
6. determine the concentration glucose activities of some enzymes of energy metabolism in tissues on the head and tail regions of tadpoles exposed to effluents for 2 weeks.

7. determine the triacylglycerols and cholesterol profiles on the head and tail regions of tadpoles exposed to effluents for 2 weeks from the Evbarake and Uwelu automobile workshops sites

CHAPTER TWO: LITERATURE REVIEW

2.1 Ecosystem

Ecosystems that function properly and the benefits they offer are essential to all life on earth. According to Nebel and Wright (1993), an ecosystem is an ecological system that is made up of its inhabitants, their habitat, and the interactions between them. To put it another way, an ecosystem is a group of living things alongside inanimate objects (Nebel and Wright, 1993).

The nonliving portion is made up of the physical environment, which includes elements like light, air, water, temperature, minerals, soil, and climate. An ecosystem's biotic components are its living elements, and its abiotic (nonbiotic) components are its nonliving ones.Topography (the landscape) and soil (edaphic elements) are examples of abiotic variables.

The ecosystem is maintained by the interaction of biotic and non-biotic elements.

The nonliving component of the ecosystem is explicitly referred to as the "environment."

A given ecosystem may contain a variety of conditions, microclimates, and habitats depending on geological features. This allows for complementary niches to support higher biodiversity, or a wider range of interdependent species (Chapin et al., 2011).

In addition to natural ecosystems like woods, lakes, or even animal bodies, artificial systems exist that either mimic the interdependencies of natural ecosystems (like ponds, aquaria, and sustainable gardens) or are themselves subject to ecosystem processes (like farmland that has been changed). The interactions between plant, animal, and microbial populations and the abiotic geological features and environmental characteristics give rise to ecosystem activities. Disturbances frequently occur in certain ecosystems, changing the system's composition over time and favoring a distinct community of species (Chapin et al., 2011). Pollution is the result of such an imbalance or disruption in the ecosystem.

2.2 Environmental Pollution

According to Tripati *et al,*. (2007) pollution is defined as the unintentional release of compounds by humans into the environment in amounts that endanger human health or the resource itself.

The use of chemicals in agriculture and industry is growing, and this is contributing to an increase in heavy metal related environmtal contamination. According to Huget *et al*. (2009), there is evidence of pollution in streams, lakes, and ground water, which is directly supplied by surface water. Over the past few decades, environmental contamination has grown to be a serious worry for emerging nations. Concern over the many chemicals utilised in different activities that are polluting our environment is becoming more and more urgent on a global scale (Palaniappan *et al*., 2009).Heavy metal pollution of water and soils is becoming a bigger issue in developed nations. Plant and animal survival has been significantly impacted by the advent of development through mining and smelting, metallurgical industries, sewage, warfare, and tanning (Xi *et al.,* 2009).

2.2.1 Types of Environmental Pollution

Numerous agronomic, ecological, biological, and hydrological research focus on soil, water, and biodiversity as essential components of ecosystems. Arable land treated with agrochemicals forms the upper layer of soil in a large majority of ecosystems. Significant amounts of chemical elements seep into the water that runs off the farmed soils, joining the food chain that feeds humans and other animals. There is an unbreakable link between the general state of the environment and the quality of life on Earth. Both the removal of hazardous substances that have been building up at dump sites in the soils and water systems over the last few decades and the disposal of massive amounts of waste that are continuously created are the two main pollution-related issues that exist today (Hsua *et al.,* 2006).

2.2.1.1 Air Pollution

A vital component of our health and well-being is the air we breathe. Regrettably, air pollution is widespread worldwide, particularly in wealthy nations since the 1960s. Particulate matter, lead, ground-level ozone, heavy metals, sulphur dioxide, benzene, carbon monoxide, and nitrogen dioxide are the principal contaminants in the air.

Air pollution levels are getting worse, according to Mishra (2003), as a result of growing industrialisation, fast urban population expansion, and increased energy and automobile needs. Other elements that exacerbate the issue, include lax environmental regulations, inefficient industrial technologies, clogged roads, and ageing and neglected automobiles. Natural and man-made forms of air pollution can lead to illness and death. Tobacco smoke, the burning of solid fuels for heating and cooking, the industries that produce pesticides and household cleansers, cars, power plants, lax environmental regulations, outdated production technology, clogged roads, and old, poorly maintained vehicles are the main man-made sources of ambient air pollution. Incinerators, the disposal of garbage, agricultural fires, and forest fires are examples of natural sources.

2.2.1.2 Water Pollution

Water is a necessary component of a healthy life and our well-being. Regrettably, contaminated air and water are widespread worldwide. According to the WHO, 1.1 billion people, or one-sixth of the world's population, do not have access to safe water, and 2.4 billion do not have access to basic sanitation. Water that has been contaminated by industrial discharged effluents, sewage water, rainwater pollution, and pollution from households or farms can harm the environment or human health (European Public Health Alliance, 2009, Ashraf *et al.,* 2010).

2.2.1.3 Land/Solid Waste Pollution

One of the primary contributors to environmental contamination is improper solid waste management (Kimani, 2007). One of the main environmental disasters that our planet is currently experiencing is land contamination (Khan, 2004). There have been reports of heavy metal industry wastes being deposited into landfills without taking extra safety procedures (Lenkova and Vargova, 1994). According to Rushbrook (1994), uranium and coal mines have caused major pollution issues, and a large portion of solid industrial waste including heavy metals is dumped in open dumps without any kind of preparation**.**

2.3 Heavy Metals

Generally speaking, heavy metals are defined as those metals that have a particular density greater than 5 g/cm3 and have a negative impact on both the environment and living things. It is only when a heavy metal's concentration in a plant or animal is beyond a specific threshold that the metal becomes toxic—as the saying goes, "it's the dose that makes the effect." Certain elements, also referred to as micronutrients or trace elements, are vital components of plant and animal cells. For Co, Cu, Fe, Mn, Mo, Ni, and Zn, this has been demonstrated. They are referred to as "heavy metals" because they only exhibit harmful effects when the internal concentration rises above a predetermined threshold (Jaishankar *et al.*, 2013). According to Jaishankar *et al.* (2013), heavy metals are important environmental contaminants, and there is growing concern about their toxicity for ecological, evolutionary, nutritional, and environmental reasons. One of the significant categories of pollutants present in the tissues and on the surface of fresh vegetables is heavy metals. According to Mapanda *et al*. (2005), heavy metals are one of the main pollutants of leafy greens. The body's many biological and biochemical processes may get disrupted if hazardous levels of heavy metals are consumed over an extended period of time.

Because they absorb these metals through their leaves, vegetables—especially leafy vegetables—growing in heavy metal-contaminated soils collect more metals than do vegetables produced in uncontaminated soils.

2.3.1 Heavy Metal Poisoning

There are numerous ways that metals can poison the environment in general. The durability of these materials allows them to potentially seep into environmental compartments. In certain situations, this might happen years after the initial deposition, causing pollution of soil and water systems. The weathering of disposed products can also cause pollution (Nordberg *et al*., 2005). Significant environmental contamination issues are caused by heavy metal buildup in plants and soil from both natural and anthropogenic sources, as well as the consequences that follow. This is one of the most important environmental concerns due of food safety and possible negative health effects. In trace amounts, certain heavy metals—like copper, zinc, manganese, cobalt, and molybdenum—act as micronutrients for animal and human growth, while others—like chromium, arsenic, and cadmium—act as carcinogens. Lead and mercury exposure are linked to the emergence of malformations in children. Renal, prostate, and ovarian cancers are brought on by prolonged cadmium use. Generally speaking, competition for sites with essential metabolites, replacement of essential ions, reactions with –SH groups, damage to cell membranes, and reactions with the phosphate groups are among the toxic effects of excess concentrations of heavy metals at the biochemical levels (Okoronkwo *et al*., 2005).

2.3.1.1 Routes of Heavy Metal Exposure

The two main ways that heavy metals enter the human body are by ingestion and breathing. For the human population, ingestion is the primary method of exposure to these components (Türkdogan *et al*., 2003). Another way that people are exposed to metals when they come into

contact with them is through their skin, which can happen in manufacturing, residential, industrial, or agricultural contexts. Adults are frequently exposed industrially exposure (Ngan, 2006). For children, ingestion is the most frequent mode of exposure. Youngsters may come into contact with hazardous levels through ordinary hand-to-mouth contact with polluted dirt or through ingesting non-food items (Dupler, 2001). Inadequate dosing or monitoring during intravenous feeding, radiographic procedures, and broken thermometers are less frequent sources of exposure (Smith *et al.,* 1997).

2.3.2 Classifications of Heavy Metal Exposure

Toxic heavy metal exposure is typically categorized as acute, lasting 14 days or fewer; intermediate, lasting 15–354 days; and chronic, lasting more than 365 days. Because heavy metals are difficult to biodegrade, they can build up in important human organs. Because there is no efficient way for the body to rid itself of heavy metals, chronic low-level intakes of these substances have negative consequences on humans and other animals. Lead, mercury, cadmium, and copper are examples of metals that accumulate poisonously. These metals are known to be extremely hazardous and to pose a risk to the environment. Repeated or continuous exposure to a hazardous chemical causes an accumulation of that substance in the body, which is known as chronic toxicity. Chronic exposure can be brought on by lead paint or solder-related activities, living close to a hazardous waste site, eating or drinking contaminated food, water, or dust, or by spending time in regions where lead paint is decaying. It can also occur from maternal transmission during pregnancy. Chronic exposure might happen at work or at home. Chronic poisoning symptoms might be difficult to identify since they frequently resemble those of numerous common disorders (Dupler, 2001).

2.3.3 Mechanism of Action of Heavy Metals

Proteins and heavy metal ions combine to create complexes involving thiol (-SH), amine (-NH2), and carboxylic acid (-COOH) groups. The cells malfunction or die as a result of these altered biological molecules' inability to operate correctly. Important enzyme systems are rendered inactive by metals binding to these groups, and protein structure is impacted, which is connected to the catalytic capabilities of enzymes. According to Neal and Guilarte (2012), this kind of toxin may also result in the production of radicals, which are harmful substances that oxidise biological molecules.

2.3.4 Heavy Metals Contamination of Soils

The following factors cause the heavy metals to become contaminants in soil environments:

i. They are produced at faster rates by man-made cycles than by natural ones; · they are transported from mines to random environmental locations where there is a higher potential for direct exposure;

ii. The concentrations of the metals in discarded products are relatively high compared to those in the receiving environment nevertheless, the receiving environment retains/accumulate the metals; and

iii. The metals may be more bioavailable depending on the chemical form (species) in which they are found in the receiving environmental system (D-Amore *et al.,* 2005).

The following is an expression of a basic mass balance of the heavy metals in the soil: In this equation, "M" stands for the heavy metal, "p" for the parent material, "a" for atmospheric deposition, "f" for fertiliser sources, "ag" for agrochemical sources, "ow" for organic waste sources, "ip" for other inorganic pollutants, "cr" for crop removal, and "l" for losses due to

leaching, volatilisation, and other factors. Mtotal = (Mp + Ma + Mf + Mag + Mow + Mip) – (Mcr + Ml) (1).

2.3.5 Sources of Heavy Metal Contamination of Soils

2.3.5.1 Fertilizer

The first significant human impact on the soil was agriculture (Scragg, 2006). Plants require both important micronutrients and macronutrients (N, P, K, S, Ca, and Mg) in order to grow and complete their life cycle. Certain soils lack important heavy metals (including Co, Cu, Fe, Mn, Mo, Ni, and Zn) that are necessary for strong plant growth; they can be added to the soil or sprayed on crops. Mn can also be added to cereal and root crops, and cereal crops cultivated on Cu-deficient soils are infrequently treated with Cu additions to the soil (Lasat, 2000). In intensive farming systems, significant amounts of fertilisers are routinely put to the soil in order to supply enough N, P, and K for crop growth. The compounds employed to supply these elements include trace levels of heavy metal impurities (such as Cd and Pb), which can considerably increase the quantity of these metals in the soil following continuous fertiliser application. It is known that metal like lead has no physiological function. Unintentionally adding Cd and other potentially harmful elements to the soil, such as F, Hg, and Pb, is a result of applying some phosphatic fertilisers (Jones and Jarvis, 1981).

2.3.5.2 Pesticides

Metal concentrations were present in a number of typical pesticides that were historically used quite a bit in horticulture and agriculture. For example, around 10% of the pesticides that the UK has licensed for use as fungicides and insecticides in the past are based on compounds that contain Cu, Hg, Mn, Pb, or Zn. These insecticides include copper-containing fungicidal sprays, like copper oxychloride and the 13 Bordeaux combination (copper sulphate) (Jones and Jarvis,

1981). This kind of contamination could be problematic, especially if the areas are used for uses other than agriculture. In contrast to fertilisers, these materials have only been used in certain locations or with specific crops (McLaughlin *et al*., 2000).

2.3.5.3 Biosolids and Manures

The unintentional application of several bio solids (such as composts, livestock manures, and municipal sewage sludge) to land causes heavy metals including As, Cd, Cr, Cu, Pb, Hg, Ni, Se, Mo, Zn, Tl, Sb, and so on to build up in the soil. Manures from poultry, cattle, and pigs are examples of animal wastes that are frequently applied to crops and pastures in the form of solids or slurries (Sumner, 2000). While most manures are considered excellent fertilisers, the pig and poultry industries also worry about the possibility of metal contamination of the soil due to the addition of Cu and Zn to diets as growth promoters and the As found in chicken health products (Sumner, 2000). Animals raised on such diets create manures that are high in As, Cu, and Zn. Sewage sludge, or biosolids, are essentially organic solid byproducts of wastewater treatment procedures that can be recovered for economic gain (USEPA, 1994). In many nations, it is customary to apply biosolids materials to land, allowing urban populations' waste to be reused (Weggler, 2004). Due to its widespread awareness and legislative definition, the phrase "sewage sludge" is frequently used in references. Nonetheless, the word "biosolids" is replacing "sewage sludge" more frequently since it is believed to more correctly capture the advantageous qualities that sewage sludge naturally possesses (Silveira, 2003). Over half of the 5.6 million dry tonnes of sewage sludge that are utilised or disposed of in the United States each year are thought to be applied to land, and biosolids are used for agriculture in all regions of the nation. More than thirty percent of the sewage sludge in the European Union is applied to farms as fertiliser (Silveira, 2003). The largest urban authorities in Australia generate more than 175,000 tonnes of

dry biosolids annually; at the moment, the majority of biosolids applied to agricultural land are employed in arable cropping conditions where they can be integrated into the soil (McLaughlin *et al*., 2000). Pb, Ni, Cd, Cr, Cu, and Zn are the heavy metals that are most frequently detected in biosolids. The type of method used during the biosolids treatment process, as well as the nature and intensity of the industrial activity, determine the metal concentrations**.**

2.3.5.4 Wastewater

Over 400 years ago, the practice of applying industrial, municipal, and related effluents to land was established, and it is still widely used today in many regions of the world. Waste water is reportedly used to irrigate 20 million hectares of agricultural land globally. Studies conducted in a number of Asian and African cities indicate that 50% of the vegetables supplied to metropolitan areas come from agriculture based on wastewater irrigation (Bjuhr, 2007). The main concern of farmers is increasing their yields and income; they are typically unconcerned about environmental advantages or hazards. Even while wastewater effluents typically have relatively modest metal concentrations, long-term irrigation of such land might eventually cause heavy metal deposition in the soil (Bjuhr, 2007).

2.3.5.5 Metal Mining and Milling Processes and Industrial Wastes

Numerous nations have been left with the legacy of widespread metal contamination in their soils due to the mining and milling of metal ores in conjunction with industry. Tailings—larger, heavier particles that settle at the bottom of the flotation cell—are directly dumped into natural depressions during mining, including wetlands on site, which causes concentrations to rise (DeVolder *et al.,* 2003). The extensive mining and smelting of lead and zinc ore has contaminated soil, endangering both human and environmental health. Numerous restoration techniques employed for these sites are time-consuming, costly, and might not be able to

increase soil productivity. Human exposure to soil heavy metals is correlated with their bioavailability. The consumption of plant material cultivated in a food chain (assimilation pathway) or the direct intake of contaminated soil (oral bioavailability) are two examples. Other materials, which vary greatly in composition, are produced by a wide range of sectors, including textile, tanning, petrochemicals from unintentional oil spills or the use of petroleum-based products, insecticides, and pharmaceutical facilities. Few of them are advantageous to forestry or agriculture, even though some are disposed of on land. Furthermore, a lot of them are rarely, if ever, applied to land and may be dangerous due to the presence of harmful organic compounds or heavy metals (Zn, Pb, and Cr). Others have no soil conditioning qualities or have very little plant nutrition (Sumner, 2000).

2.3.5.6 Air-Borne Sources

Metals can be found in the air due to fugitive emissions, such as dust from storage areas or garbage piles, and stack or duct emissions of air, gas, or vapour streams. Generally, metals produced from airborne sources end up as particles in the gas stream. Volatilisation of certain metals can also occur during high-temperature processing, including As, Cd, and Pb. If a reducing environment is not maintained, these metals will transform into oxides and condense as fine particles (Smith *et al.,* 1995).

2.3.6 The Fate of Heavy Metals in the Ecosystem

The localisation of elevated concentrations of heavy metals leads to an increase in their toxicity. Chimneys have been raised in some places in order to diffuse metal emissions so they don't fall in one concentrated region. Even said, there are occasions when this still has additional repercussions because acid rain is more likely to occur due to increased emissions. Although the earth is perceived as a single compartment, it is actually composed of numerous smaller

compartments, such as individual cells or organisms. Potential poisons on organisms have the ability to segregate into insoluble deposits, which stops them from interfering with vital metabolic processes that take place in the cytoplasm. Metals cannot be broken down since they are non-biodegradable and hence exist in the environment for a very long time. Until they are eluted to other compartments, heavy metals that are present in soils and sediments stay there for a long time. They may also generate or deteriorate into more hazardous forms in response to reactions with other components of the soil or sediment. One illustration of this is the way in which bacteria present in water, sediment, and soil combine to generate toxic methyl mercury from inorganic mercury (Walker *et al.,* 2012). In contaminated areas like abandoned mine sites or areas where metal-containing insecticides were historically used, anthropogenic activity has left an extremely high concentration of metals. Only strains that are tolerant of metals flourish in these locations, where there is little flora. In certain areas, capping—which entails covering the contaminated area with fresh soil and an impermeable layer—is occasionally used. By capping, heavy metals will be kept out of the flora and prevented from entering the groundwater by the falling water. Arsenic, copper, lead, and chromium were included in metal pesticides, and these elements may still be present in some places where the pesticide was applied (Walker *et al*., 2012). Sewage sludge is occasionally used by farmers and mixed into the soil; however, this may contain heavy metals, especially if the sludge was created by industries. High concentrations of heavy metals, including copper, zinc, lead, cadmium, and chromium, have been discovered in the soil of these agricultural areas. Smelting releases pollutants into the atmosphere, which settles on the ground and creates localised pollution. Certain regions where smelting takes place exhibit dead vegetation and a lack of organisms like woodlice and earthworms, which aid in the decomposition of vegetation. Lead shotgun pellets, lead fishing weights and petrol tainted with

high concentrations of lead all contributed to the presence of lead in our surroundings. Some have been outlawed in specific global regions. Lead has been discovered in wetlands as a result of shotgun pellets being ingested by birds and moving up the food chain. Higher levels of clay, organic matter, and pH bind metals more tightly to the soil. Less elemental elements have been discovered in more acidic soils because they dissolve more readily and seep into the earth where roots cannot reach, depriving plants of nutrients (Chibuike *et al.,* 2014).

2.3.7 Heavy Metals in the Environment

2.3.7.1 Cadmium

According to Leggett and Pagotto (1999), runoff water and sediment from roads contain an estimated 9.4 g/km/year of cadmium from atmospheric deposition and tyre wear on asphalt. The Paris metropolitan area in France had mean bulk cadmium levels in its rain that ranged from 0.09 to 2.4 µg/L during a seven-year period. The primary method of cadmium deposition was rainfall, which was followed by dry particle fallout. Urban air pollution from car exhaust, municipal dump sites, and waste incinerators (including medical waste) are possible sources of these particles (Garnaud *et al.* 1999). For amphibian eggs and larvae, early development exposure to cadmium is extremely hazardous. For instance, Herkovits *et al*. (1998) found that most African Clawed Frog (*Xenopus laevis*) embryos died after being exposed to 0.2 mg/L Cd^{2+}/L for 72 hours. Larvae of the American Toad (*Bufo americanus*) that were exposed to cadmium at values of 540 µg/L during their metamorphosis were fatal. Only 22.4% of these tadpoles survived at this dose. Increased death rates were also seen in Spanish Newt (*Pleurodeles waltl*) tadpoles exposed to 1–5 mg/L $CdCl_2$ (Flament *et al.*, 2003). Exposure of herpetofauna to cadmium has also been shown to cause sublethal consequences. Tadpoles of *Bufo americanus* that were exposed to 54 µg/L of cadmium over an extended period of time shed their tails earlier and with a higher weight than

those that received 5 µg/L of treatment. In a particular developmental stage, exposure to 1 mg/L cadmium prevented the metamorphosis of *Pleurodeles waltl* tadpoles (Flament *et al.*, 2003). Cadmium bioaccumulated from spiked soil substrate in fence lizard (*Sceloporus undulatus*) eggs led to acute death at the two highest doses (1480 and 14,800 µg Cd/g) and affected hatchlings' thyroid function in the 148 µg/g treatment (Brasfield *et al.* ,2004). In their livers, female Slider Turtles (*Trache myscripta*) bioaccumulated large levels of cadmium (mean of 3.57 µg/g dry mass) from a coal ash-affected site where their food, such as crayfish, were also considerably contaminated.

2.3.7.2 Copper

According to Hares and Ward (1999), copper is discovered on road surfaces as a result of brake lining erosion. This process can release 290 g/km of copper into streams annually through silt and runoff from pavement (Legret and Pagotto, 1999). Copper is also released into the environment by industrial processes like the manufacture of cement (Kalafatoglu *et al.*, 2001). Urban regions frequently see water coming into touch with copper sources due to the prevalence of copper-based home water pipes. In teleost fish, acute copper toxicity mostly affects the gill epithelium, but chronic low-level exposure frequently causes endocrine and physiological abnormalities (Handy 2003). Similar behaviours might be seen in amphibians at larval stages that are exposed to copper through similar pathways. For South American Toad (*Bufo arenarum*) embryos, Herkovits and Helguero (1998) determined a 24-168-hour LC_{50} of 0.085 mg/L; nevertheless, premetamorphic American Bullfrog (Rana catesbeiana) tadpoles subjected to the substance by Ferreira et al. (2004) discovered a 48-hour LC_{50} of 4.3 mg/L and a 96-hour LC_{50} of 2.4 mg/L. When establishing copper release limits, it is important to take into account the differences in the susceptibilities of various amphibian species and life stages to copper toxicity.

There is no research on the presence or effects of copper on reptiles living in urban areas, despite the fact that amounts of the metal have been found in some marine reptiles (Fletcher *et al*. 2008).

2.3.7.3 Lead

Particulate lead was discharged into the atmosphere from petrol exhaust until recently (Legret and Pagotto, 1999). Wetland sediments beside roadways are still being contaminated by residual quantities of leaded gasoline. Particulate matter can enter rivers through surface runoff during rainy events and settle on impermeable surfaces. This can lead to increases, with estimates of 660 g/km/year occurring in France (Legret and Pagotto, 1999). Research has shown that adult frogs can absorb lead from contaminated sites like skeet shooting ranges (Stansley and Roscoe, 1996) and industrial areas like La Plata City, Argentina (Arrieta *et al*., 2001). Argentine Toad (*Bufo arenarum*) larvae exposed for 120 hours to 16 mg/L of lead died 60% of the time, while the control group survived 100% of the time (Herkovits and Pérez-Coll, 1991).

There is heterogeneity in lead buildup in reptiles, but there is little information on toxicity. Burger (2002) discovered that wild-caught Diamond-backed Terrapin (*Malaclemys terrapin*) tissues in New Jersey, USA, have comparatively low lead levels (40–90 ng/g), including the egg, liver, and muscle. Burger (1992) discovered that Northern Pine Snake (Pituophis m. melanoleucus) hatchlings had a mean skin concentration of 2558 ng/g lead, compared to whole body concentrations of 596 ng/g at a sample site in Ocean County, NJ, close to an airport, train, and power line right-of-way. Lead amounts ranging from 6.8-13.3 μg/g were discovered by Loumbourdis (1997) in the livers of wild-caught Starred Agama (*Agama s. stellio*) lizards in northern Greece, together with samples of the entire body containing 12.8-15.6 μg/g of lead. In an urban area of Spain, Fletcher *et al*. (2008) found very high lead concentrations (mean = 30 ppm whole body concentration) in Moorish Wall Geckos (*Tarentola mauritanica*).

2.3.7.4 Zinc

Zinc can be found in urban contexts through corrosion from galvanised steel barriers, tyre wear and brake lining wear (Hares and Ward 1999; Legret and Pagotto 1999). The average zinc concentration in Bahrain's outdoor particulate matter was 67 mg/kg, with car exhaust being the most likely source (Madany *et al.*, 1994). For a section of French highway, Legret and Pagotto (1999) calculated zinc loading to be as high as 2420 g/km/year. According to Greenstein *et al.* (2004), storm water runoff from parking lots can also be a significant source of zinc contamination. Even though zinc is an essential microelement for biology, pollution containing zinc can harm reptiles and amphibians respectively. The survival of subsequent embryos at the branchial circulation stage was negatively impacted by a prolonged laboratory exposure of adult female Bufo arenarum to 4 μg/L zinc (from $ZnSO_4 \cdot H_2O$), even though no bioaccumulation was discovered in the adult ovarian tissues. Compared to the control group, the survival rate of exposed female embryos was lower. Females exposed to the environment exhibited reduced activity of glucose-6-phosphate dehydrogenase and elevated levels of endogenous glutathione in their ovaries. These modifications may have an impact on meiotic activity and cell energy production, which would lower these females' fecundity (Naab *et al.* 2001). Studies looking at zinc's effects on reptiles are scarce. Zinc whole body concentrations in Agama s. stellio lizards from a region close to Thessaloniki, Greece, were reported to reach 609 μg/g by Loumbourdis (1997), while the effects of this metal on the lizards were not mentioned. In order to effectively safeguard endangered species, additional ecotoxicological research on the effects of zinc and other metals on amphibians and reptiles is required.

2.3.8 Heavy Metals and Acidification

The incidence of heavy metals (and other contaminants) in surface waterways has increased due to intensive industrial and agricultural output as well as mine pollution, which may eventually have an impact on amphibian populations. Amphibians are impacted by a variety of metals, including manganese (Mn), molybdenum (Mo), antimony (Sb), aluminium (Al), lead (Pb), zinc (Zn), cadmium (Cd), mercury (Hg), silver (Ag), copper (Cu), arsenic (As), and copper (Cu). They may cause sublethal or lethal consequences, such as decreasing development and growth or changing behaviour (Raimondo *et al.,* 1998 and Blaustein *et al.*, 1997). Numerous studies have demonstrated the harmful effects of heavy metals from coal combustion and other sources on frogs. For instance, compared to larvae from areas without coal ash pollution, Rowe et al. (1998a) discovered a greater incidence of mouth malformations in bullfrog (Rana catesbeiana) larvae in a coal ash-polluted site. R. catesbeiana embryos were introduced to both an unpolluted and a location contaminated by coal combustion waste by Rowe *et al.* (1998a). In comparison to larvae at the unpolluted site, there was a higher incidence of oral malformations and a poorer rate of larval survival in the polluted site. Furthermore, compared to larvae obtained at an unpolluted site, those collected from a contaminated site showed higher metabolic rates. When compared to larvae brought to an unpolluted environment, those introduced to a contaminated site exhibited higher metabolic rates (Rowe *et al.,* 1998b). Elevated metabolic rates may suggest a physiological expense associated with exposure to pollutants. Southern toad (*Bufo terrestris*) larvae were obtained from a clean site and transplanted to a reference site and a site contaminated with heavy metals from coal combustion waste by Rowe *et al*. (2001). Southern

toads transplanted to polluted locations had a lower early larval survival rate than those at unpolluted sites. The polluted site's surviving larvae were either replaced at the polluted site or transplanted to the reference site midway through the larval phase. When transplanted from the contaminated site to the reference site, there was a much higher survival rate until metamorphosis than for any of the individuals reintroduced to the polluted site, who all perished before metamorphosis. Local amphibian extinctions could be attributed in part to acidification. According to Beebee *et al.* (1990), acid pollution appears to have contributed to the reduction of natterjack toads (*Bufo calamita*) in Britain. A variety of negative impacts on amphibians can result from acidification, either on its own or in conjunction with other causes (Dunson *et al.,* 1992). Because heavy metals can leach from soils in contact with acidic water, the effects of heavy metals on amphibian survivability are sometimes intimately tied to acidification. For instance, when the pH of water decreases, aluminium, a metal that is plentiful in soils, becomes more soluble. In amphibians, inorganic monomeric aluminium frequently works in concert with pH to trigger embryo death (Freda *et al*., 1990).

2.4 Stressors

The widespread risks to amphibian populations around the world are caused by a variety of common physiological stresses, such as disease, pollution, habitat loss, and combinations of these stressors (Foden *et al*., 2013; Grant *et al.,* 2016; Green *et al.,* 2020). At the organismal level, stressor effects can be identified prior to the long-term population loss becoming noticeable. Higher density resource competition that prevents certain species from undergoing metamorphosis, recruiting, or successful reproduction is a common observation of habitat restrictions (Harper and Semlitsch, 2007). The long-term consequences of these stressors are frequently recognised as systemic reactions inside the organism that come before effects that

become visible at the population level (Trudeau *et al*., 2020). Certain physiological indicators of biological function perturbations, such as oxidative stress, reduced immunity, endocrine disruption, and changed metabolic activity, can have phenotypic effects on individual fitness and have consequences for population dynamics.

2.5 Physiological Endpoints and Biomarkers in Amphibian Studies

Certain amphibian populations have been suggested to be declining due to environmental contamination and possible negative consequences of chemical exposure (Blaustein *et al.*, 1994). Amphibians exposed to toxins around the world are being evaluated for health using a variety of physiological endpoints and molecular biomarkers. Tadpole growth and survival rates, hatching rates, and other reproductive endpoints can provide important information about how long a population can remain viable in the face of environmental challenges. Biochemical biomarkers can offer valuable information on the health and fitness of individual animals as well as insight into how exposure affects survival potential.

2.5.1 Reproductive Endpoints

Exposure to environmental contaminants can impact amphibian reproductive success on multiple levels. Adult frogs exposed to contaminants may exhibit reduced gamete production, fertilisation, or offspring sex ratio (Carey and Bryant, 1995). Moreover, toxicants might impede the growth and development of larvae and embryos. Common indicators used to show how contamination affects the productivity of frog populations include hatching success, tadpole survival and growth, and time to metamorphosis.

2.5.2 Hatching Success

Amphibian eggs have a porous membrane that enables the developing embryo to absorb substances from the water. Unfavourable circumstances can reduce the rate at which amphibian

eggs hatch, including dissolved metals, low pH, and/or high quantities of organic carbon (Freda *et al*., 1990). Certain species' embryos can take several weeks to hatch, lengthening the time that they are exposed to any toxins in the water column. Low pH can promote thoracic enlargement in hatchlings of the Spotted (*Ambystoma maculatum*) and Jefferson (*Ambystoma jeffersonianum*) salamanders, according to studies conducted on these species. Curling malformations in the exposed embryo can also be caused by high cation concentrations, low pH, or high dissolved metal concentrations (Laposata and Dunson, 1998). The success of hatching and subsequent survival may be impacted by these abnormalities.

2.5.3 Tadpole Growth and Survival

Tadpole growth and survival can be impacted by changes in environmental conditions such as habitat desiccation, predation, resource constraint, and crowding. According to Shi (2000), these variables have the ability to either promote or restrict growth depending on when they occur during prometamorphosis or premetamorphosis. Larvae of anuran species can adjust to changes in water availability. They have two options: they can either stay in the water longer to maximize growth at the risk of desiccation, or they can get out of the drying conditions and shrink to less than ideal size. Small-sized metamorphosing juveniles are less able to endure desiccation and require more time to attain reproductive maturity (DiMauro and Hunter, 2002). The concentration of nonvolatile chemicals and metabolic wastes may rise as a result of pond evaporation, adding to the stress experienced by tadpoles as they develop. Because of the significant physiological and anatomical changes that occur during the final stage of transformation from an aquatic larval stage to a semi-aquatic adult, the majority of anurans do not consume food during metamorphosis. During these alterations, xenobiotic substances ingested in the larval stage may be either excreted or kept and reorganised inside the tissues.

Environmental pollutants that have been retained may become concentrated in tissues as a result of mass loss from starvation and the high energy demands of metamorphosis (Snodgrass *et al.*, 2003).

2.5.4 Thyroid Hormones

3,5,3'-Triiodothyronine (T3) and 3,5,3,5'-tetraiodothyronine (T4) are the two main thyroid hormones that control metabolism in vertebrates. The generic name for T4 is thyroxine. These hormones increase cellular respiration, oxygen intake, and metabolic rate by acting on the liver, kidney, heart, nervous system, and skeletal muscle. Thyroid hormones cause an increase in metabolism, which produces heat and is crucial for many vertebrates' ability to regulate their body temperature (Randall *et al.*, 2002). The hormone that the thyroid gland produces and circulates is called T4. In peripheral tissues, the enzyme type II iodothyronine deiodinase (D2) transforms it into the active hormone T3. A tissue's reaction to the hormone in circulation depends in part on the location of the enzyme D2 (Cai and Brown, 2004). In particular, thyroid hormones are crucial for the promotion of amphibian metamorphosis and are necessary for vertebrate development in general. In anurans, thyroid hormone levels typically increase dramatically during metamorphosis. For instance, research using Xenopus laevis shows that as the tadpoles grow and change morphologically, plasma thyroid hormone 17 concentrations rise continuously (White and Nicoll, 1981).

2.6 Body Condition Indices

An assessment of an amphibian's physical condition can forecast its overwintering survival and provide insight into its general fitness (Congdon *et al*., 2001). The metabolic rate of an amphibian may be directly or indirectly increased by contaminants. This can lead to biochemical alterations that can negatively impact the frog's capacity to store energy. Triacyglycerols are

primarily used to store fats, whereas glycogen is used to store carbohydrates (Nelson and Cox, 2005).

2.6.1 Triacylglycerols

Triacylglycerol (TAG) molecules, the building blocks of fat, usually gather in the fat vacuoles of specialised adipose cells in vertebrates. In lipid tissue, TAGs serve as the main source of energy storage. Because of the molecule's low oxygen content and comparatively high hydrogen and carbon content, they are extremely compact. As a result, when oxidised, 1g of triacylglycerols produces almost twice as much energy as 1 g of carbohydrates. Because TAGs are poorly soluble in water, the body can store large amounts of them (Randall *et al*., 2002). Anuran amphibians include lipids in their fat bodies, liver, muscle, gonads, subcutaneous tissue, and tail (Sheridan and Kao, 1998). Reduced triglyceride storage in adipose tissue can result from stress brought on by contaminant exposure, which can negatively impact vital energy-demanding processes like metamorphosis. Amphibians use TAGs not just for gamete synthesis during dormancy but also for metabolic maintenance. It has also been demonstrated that fat bodies are necessary for gonadal maintenance (Fitzpatrick, 1976). Measurement of whole body TAG concentration has been used as an index of body condition in small fish, and have been shown to be sensitive to the energetic demands of environmental stressors, including contaminants. For example, reduced TAG levels were measured in sailfin mollies (Poecilia latipinna) exposed to increasing concentrations of 1,1,1-trichorlo-2-(o-chlorophenyl)-2-(pchlorophenyl) ethane (o,p'-DDT), an agricultural pesticide (Benton *et al*., 1994). Triacylglycerol levels were also seen to be depleted

in the livers of cod (Gadus morhua) and winter flounder (Pseudopleuronectes americanus) from the Northwest Atlantic, following long-term exposure to crude petroleum (Dey *et al*., 1983).

2.7 Amphibian Model Species

A type of vertebrates known as amphibians typically go through a remarkable metamorphosis in which they change from aquatic larvae to terrestrial or semi-aquatic adults. Three orders comprise modern amphibians: Urodela (newts and salamanders), Anura (frogs and toads), and Caecilia (legless, wormlike animals) (Shi, 2000).

2.7.1 Amphibians as Indicator Species in Ecotoxicology Studies

Sentinel species that are highly helpful in assessing the effects of contaminants in wetland ecosystems are amphibians. They are crucial elements of numerous ecosystems across the globe. As tadpoles, many amphibians are herbivorous, and as adults, seven of them are carnivorous (Burger and Snodgrass, 1998). As a result, they are recognised as both a significant predator and a species of prey. For instance, frogs outnumber all other vertebrates in many forest ecosystems in terms of biomass and number (Stebbins and Cohen, 1995), therefore population decreases may have detrimental effects on the entire community. Because they often have both an aquatic and a terrestrial phase to their life cycle, amphibians are excellent models of wetland ecosystems. With the depositing of unshelled eggs in aquatic settings, a gill-respiring, swimming detritivore/herbivore larval stage, and a semi-aquatic hopping or climbing insectivorous adult stage, their life history is distinct among vertebrates. According to Wassersug (1997), this renders them potentially extremely susceptible to a variety of stresses in both aquatic and terrestrial habitats. Amphibians are susceptible to a wide range of contaminants, including pesticides like atrazine and DDT as well as metals like iron, zinc, lead, and cadmium. The susceptibility of a range of native amphibian species to fish species frequently employed in

toxicity testing was examined by Birge *et al.* (2000). In order to examine 50 metals, 13 organic compounds, and inorganic substances, a total of 694 amphibian/fish comparisons were conducted. The findings showed that frogs had lower LC50 values than fish in 64% of all tests. Because poisons are easily absorbed through the skin and through food, amphibians may also be more prone than other vertebrates to gain substantial body loads. Their extremely permeable skin allows for possible pollutant absorption as well as cutaneous respiration**.**

2.7.2 Amphibian (Anuran) Development

One of the earliest and most thoroughly researched hormone-regulated developmental processes is amphibian metamorphosis. Amphibian larvae rapidly develop into free-swimming tadpoles after emerging from eggs as embryos. The larvae subsequently go through a series of transformations to become frogs.

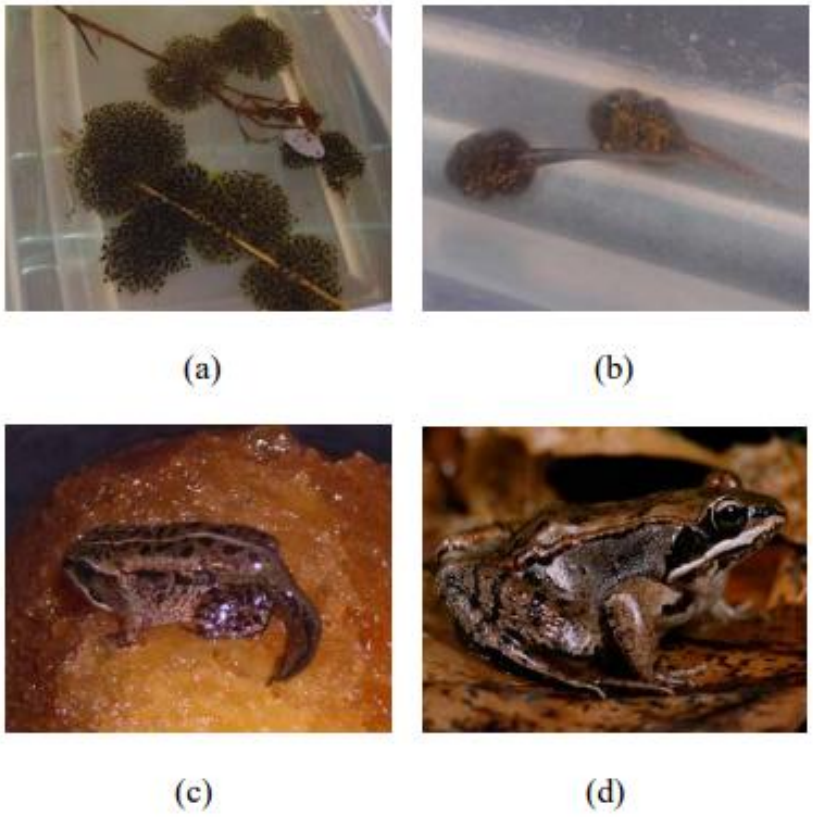


Figure 1.1: Life cycle of an amphibian: (a) clustered egg masses; (b) free-swimming tadpoles; (c) froglet with remnant tail; (d) fully metamorphosed frog.

Source: Niti Gupta *et al.,* (2009)

Premetamorphosis, prometamorphosis, and metamorphic climax are the three distinct stages of anuran transformation. Early tadpole growth and development, as well as embryogenesis, are included in premetamorphosis. During this stage, several morphological changes take place, such as the beginning of the hind limbs' development. The hind limbs expand rapidly and extensively during prometamorphosis. The stage known as the metamorphic climax is when the biggest and fastest morphological changes occur. These include the tadpole's entire tail resorption, the development of its forelimbs, and significant alterations to its internal organs (Shi, 2000).

2.7.3 Effects of Contaminant on Expected Physiological Pathways of Tadpole

Studies on other animals have shown that certain contaminants have an impact on dependable physiological mechanisms. For instance, herbicides containing carbamate and organophosphorus impede cholinesterase and interfere with the nervous system's regular operation, impairing neural control. Convulsions, lethargy, and loss of coordination are examples of overt symptoms of this disturbance. The information at hand indicates that these pesticides have comparable effects on frogs as they do on other animals. Low pH impacts ion balance, as do nitrate and ammonia molecules; heavy metals chelate with enzymes to disrupt physiological pathways and induce histological damage in some organs; aluminium binds with phosphorus and hinders its intake, resulting in rickets (Domingo, 1995). Amphibian responses in these situations ought to be qualitatively comparable to those of other vertebrates, particularly fish. The metabolism of cellular poisons and the hydrolysis of various organic pollutants, including some pesticides, PAHs, and PCBs, are processes carried out by mixed-function oxidases. Reduced capacity to metabolise the parent form of these chemicals may not always be detrimental, and amphibians' reactions to them may be different from those of homoeotherms due to the possibility that the degradates of these compounds are more poisonous than their parent form. Generally speaking,

anurans are less sensitive to PCBs than other vertebrates, particularly mammals. (Sparling, 2000) In other situations, scientists can anticipate that amphibians will react to pollutants similarly to fish and birds, and the impacts and bioindicators that are already understood can be applied to other situations**.**

2.7.4 Genotoxicity

At least in somatic cells, contaminants can cause DNA and chromosomal mutations. These alterations can reduce survival and change how cells function. There have been reports of herbicides, other pesticides, PCBs, wastewater, and PAHs having genotoxic effects on frogs. UV light irradiation can increase the effects of certain PAHs by a factor of 100 or more (Fernandez and Haridon, 1992).

2.7.5 Effects on Behavior and Development

Numerous laboratory investigations have demonstrated a decline in activity associated with pollutants. For instance, the organophosphorus pesticide temephos caused different diphasic activity cycles in response to dose and decreased the activity rate of R. sphenocephala tadpoles. The pesticide malathion disrupted the equilibrium maintenance in tadpole R. catesbeiana at the lowest measured concentration (500 µg/L). Chlorpyrifos (0.1 mg/L) reduced swimming speed and startle responses in Hyla versicolour when prodded. In a classical conditioning experiment, exposure to 750 µg Pb/L for 120 hours hampered the avoidance–preference responses of R. catesbeiana and R. clamitans, suggesting that tadpoles learnt and retained lead less well, (Steele and others, 1999) Additionally, the scientists observed that rats at 625 µg Pb/L or higher showed significant variance in locomotor activity, exhibiting spontaneous surges of activity not observed in controls. In reaction to atrazine, statistically reduced developmental times have been seen in H. versicolour, tiger salamanders (Ambystoma tigrinum), and other species. These delayed timings

could be attributed to a variety of physiological and behavioral variables. Similar effects on development are seen with aluminium, acidification, dioxins, triphenyltin, chromium, and a variety of other pollutants. Decreased rates of development could expose tadpoles to arid wetland conditions and have further effects on survival. Complete metamorphosis amphibians are potentially good candidates for research and chemical screening for endocrine disruptors, particularly thyroid-related ones (Tata, 1998). The two main hormones that control the climax stage of metamorphosis are tri-iodothyronine (T3) and thyroid stimulating hormone (TSH), while additional hormones may have an impact on how they function (Wolffe *et al.,* 1999). According to Check *et al.* (1999), exposure to various pollutants can modify the action of the thyroid, and metamorphosis can be a valuable marker for identifying possible disruptors of the thyroid. Like in other creatures, toxins can also have an impact on sex hormones in amphibians, and these changes may have a direct impact on reproduction. A study by Hayes *et al.* (2002) showed that atrazine may be a powerful endocrine disruptor; in Xenopus laevis, dosages as low as 0.1 ppb dramatically raise the prevalence of ovitestes.

2.7.6 Contaminants and Amphibian Malformations

Although amphibian malformations have garnered significant attention from researchers in recent years due to their increased prevalence and public awareness since their discovery by a group of students in 1995, the issue does not seem to be a brand-new occurrence. Undoubtedly, trematode parasitism is a contributing factor to the loss and excess of limbs as well as parts of limbs, (Johnson and others, 1999). But not all cases of high frequency of deformities can be attributed to parasitism; a significant body of research also suggests that waterborne toxins may be a contributing factor. Chlorinated hydrocarbon pesticides, boron, atrazine, ammonia, coal ash effluent, methyl-parathion and pirimicarb, methoprene derivatives, maneb, various medicines,

organic pollutants, and metals are among the contaminants that have been connected to deformities (Harfenist *et al*., 1989). At amounts that are realistic for the environment, several of these substances can result in deformities and shorten the lives of those who are impacted. In a past study, Kiesecker *et al.* (2002) discovered that, in comparison to trematodes alone, the interaction between trematode infestations and environmentally realistic doses of malathion dramatically increased the prevalence of deformities in native frogs. However, there are typically significant differences between the kinds of deformities created in laboratories and those observed in Minnesota's wetlands and other locations.

2.7.7 Effects on Energetics

According to research by Rowe *et al.* (1998), typical metabolic rates of R. catesbeiana tadpoles obtained from wetlands contaminated by coal ash holding pond effluent were 40–175% higher than those of tadpoles of a same age obtained from clean areas. Higher metabolic rates may leave less energy available for development or reproduction if an organism's energy allotment is finite and split between growth, storage, maintenance, and reproduction. On the other hand, tadpoles would have to devote more of their time to feeding and less to resting and avoiding predators. After being exposed to 200 mg/L Cd in solution for 30 days, adult R. ridibunda showed indications of metabolic stress in the liver for the first 10 days. This prompted the initiation of protective mechanisms, such as the synthesis of glutathione and metallothioneins, and by the end of the exposure period, most liver parameters had returned to normal. The toxicity of carbaryl and metabolism in R. clamitans tadpoles were found to be indirectly related. (Boone and associates, 1999). The liver glycogen concentrations of mudpuppies (*Necturus maculosus*), which inhabit streams contaminated with pesticides and various other organochlorines, were found to be lower than those of animals living in cleaner streams. This is because liver glycogen

is a major energy reserve for vertebrates and is influenced by corticosterone, which has been experimentally shown to be altered by PCB exposure. (Gendron and others, 1997)

2.7.8 Evidence for Interactions Between Contaminants and Other Factors Associated with Amphibian Population Decline.

Rarely does a single element limit a population's ability to develop or survive, with the exception of extremely simple ecosystems. A population may be impacted by multiple limitations across time, some of which may interact with one another, even though a factor may be limiting for a specific amount of time. Therefore, pollutants may increase the stress levels of individuals within a population, increasing their susceptibility to illness or parasites. Similar to this, a contaminant might change an animal's behaviour to make it less competitive or more susceptible to predators. Stress brought on by a herbicide's sublethal effects may raise metabolic needs while also reducing the availability of food. As such, it could be challenging to identify the true reasons behind population decreases. The combination between nonbiological elements such as habitat composition and solar radiation and biological factors like disease, predation, and competition may have a deleterious effect on amphibian populations.

2.8 Oxidative Stress

The most widely used definition of oxidative stress is a pro-oxidant state, which is defined as an imbalance between the body's ROS levels and antioxidant defences (Costantini, 2014). Nonetheless, alternative interpretations have surfaced lately (Costantini, 2014). For instance, contend that certain markers, like thiols, have the ability to "disrupt the redox-regulating mechanisms" when oxidised excessively (Costantini, 2014). However, there aren't enough studies on wild animals in this specific scenario (Costantini, 2014), which has led others to propose other markers as the primary indicator of oxidative stress. However, these efforts have

not yet yielded definitive results because, in addition to the implications ROS have at the cellular level (where their levels are easier to measure), the variations in ROS production and regulation among multicellular organisms make it nearly impossible to identify this particular marker for oxidative stress at a systemic level (Costantini, 2014). Taking into account additional environmental elements that could influence these reactions makes this much more challenging (Costantini, 2014,). Therefore, the basic description of ROS as a pro-oxidant imbalance will be taken to be accurate for the sake of simplicity (and because most, if not all, of the research evaluated here presume this interpretation). This is further supported by the fact that, as was already established, this definition is valid when looking at the impact ROS have on cells. Nonetheless, the origins and effects of oxidative stress are nearly always understood, independent of the markers that are thought to be the primary indicators of the condition.

2.8.1 Oxidative Response to Xenobiotics

Despite the wide variety of xenobiotics that exist, the Nrf2-Keap1 system is the sole cellular mechanism that participates in the oxidative response during their metabolism (Aita & Abdullah, 2014,; Casas-Grajales and Muriel, 2017). All vertebrates and the majority of marine invertebrates share this trait (Regoli and Giuliani, 2013; Casas-Grajales & Muriel, 2017). Figure 1.2 provides a more detailed view of its precise mechanism of operation.

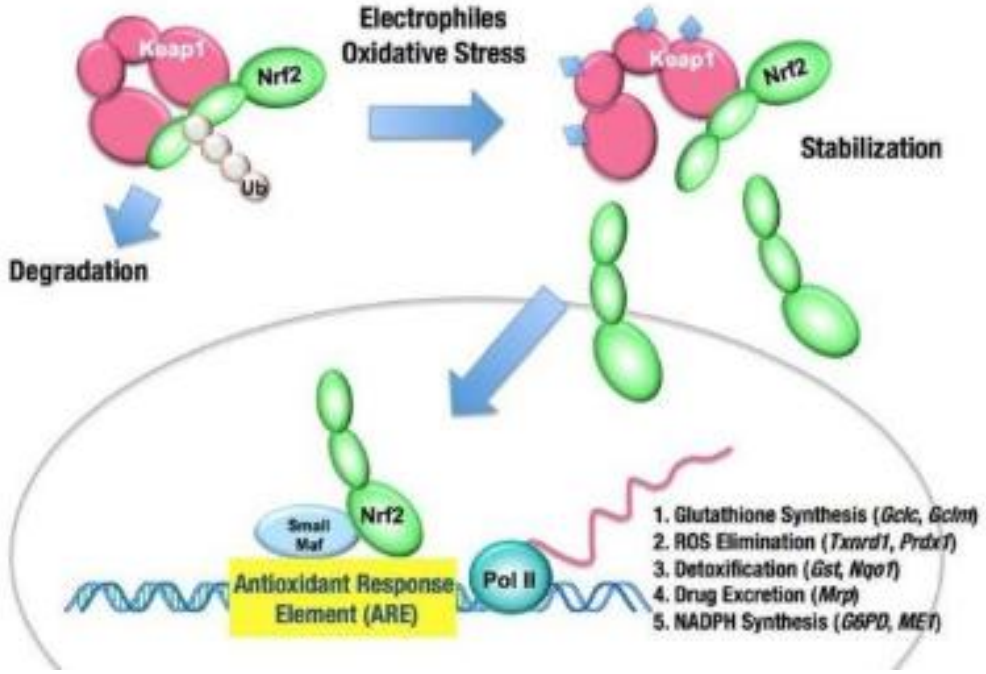


Figure 1.2: Action of the Nrf2-Keap1 system under normal conditions

Source: Mitsuishi *et al*., 2012. 38

Figure 1.2 illustrates how the Keap1-Nrf2 complex contains ubiquinone. However, when an oxidative stress signal is received, the ubiquinone breaks down and Nrf-2 enters the cell nucleus. This turns on the Antioxidant Response Element, or ARE, which in turn sets off the body's detoxification process and antioxidant response. As previously mentioned, this reaction starts in the liver of every animal (Casas-Grajales & Muriel, 2017) and sets off the detoxification process throughout the body. However, it is crucial to remember that, in spite of this typical cellular reaction, the overall organismic or systemic response might differ amongst various taxa, occasionally down to the species level (Costantini, 2014). Numerous factors, including phylogenetic and environmental ones, are mostly to blame for this, while size and nutrition also have an impact (Costantini, 2014).

2.8.2 Impact of Organic Compounds in the Oxidative Response in Reptiles and Amphibians

Heavy metal exposure can occur to animals through a number of routes and from a range of sources, such as "contaminated air, water, soil, and food" (Ercan *et al*., 2001). These are mostly the result of industrial waste and mining (Ercan *et al.,* 2001). But it's crucial to remember that, according to Valko *et al*. (2005), "a number of essential transition metals, including zinc, iron, copper, cobalt, and manganese, participate in the control of various ROS-mediated metabolic and signalling pathways." Accordingly, metals play a critical role in preserving cellular homeostasis processes (Valko *et al.,* 2005). However, excessive concentrations of these metals have been shown to cause oxidative stress in the body (Valko *et al*., 2005), which can lead to cancer and tissue damage. According to their effects on the body's oxidative balance, heavy metals fall into two categories: "Redox-active metals, like lead and copper, undergo redox cycling, whereas

redox-inactive metals, like lead, cadmium, and mercury, primarily deplete the organism's antioxidant defences" (Ercan *et al.,* 2001).

2.8.2.1 Anura

In addition, three distinct research (Wang & Jia, 2009; Zocche *et al.,* 2013; Taiwo *et al*., 2014) focus on anuran species (Hypsiboas faber, Rana nigromaculata, and Hoplobatrachus occipitalis, that is). Only one of the three concentrates on the impacts of a single metal. This one is related to Rana nigromaculata, or Pelophylax nigromaculatus as it is now recognised (Wang & Jia, 2009), the oxidative response to lead was evaluated by determining the levels of glutathione (GSH) and malondialdehyde (MDA) at various experimentally-manipulated Pb doses (Wang and Jia, 2009). The findings showed that MDA and GSH concentrations increased together with lead concentrations, which is unexpected considering that it was anticipated that GSH levels would drop as MDA levels increased (Wang and Jia, 2009). According to Vogiatzis and Loumbourdis (quoted by Wang and Jia, 2009), "lysosomal damage resulted after all of the −SH groups were occupied for action, which reduced the antioxidant cellular defences." It was acknowledged, although, that additional study is required to support this result (Wang and Jia, 2009). However lead binds to the SH group of the enzyme aminolevulinic acid dehydratase (ALAD) and inactivates it, leading to its enolisation (Ercan *et al*., 2001). This could account for this result. When this enzyme's enolized form comes into contact with oxygen, it produces superoxide anion (Ercan *et al*., 2001). Additionally, they verified that elevated levels of lead do cause damage to DNA (Wang and Jia, 2009). However, when comparing reference locations with contaminated ones, the results of the other two papers were comparable, e.g., TBARS assay levels did not vary between populations (Zocche *et al*., 2013; Taiwo *et al*., 2014). Nonetheless, there were notable variations in other outcomes. For example, Zocche *et al.* (2013) reported increased SOD activity

in the contaminated Hypsiboas faber frogs, which they linked to increased concentrations of Fe and Cu. On the other hand, Taiwo et al. (2014) found that Hoplobatrachus occipitalis SOD activity decreased in both polluted and unpolluted areas as heavy metal concentrations increased (generally). This may be because of high lead concentrations in this instance. The fact that Pb was discovered in greater concentrations in this instance confirms this (Taiwo *et al.,* 2014). Furthermore, Taiwo *et al.* (2014) support Wang & Jia (2009)'s findings that lead lowers GSH activity, indicating a shared reaction among members of the same taxonomic group.

The fact that none of the publications address whether these Anurans have the post-ischemic adaptations is significant since, even if they do, it appears that they have no bearing on their oxidative reactions to xenobiotics. However, based only on their environment, it is crucial to demonstrate that Hypsiboas faber, a tree frog found in the subtropical regions of Southern Brazil and Northern Argentina (Lavilla *et al.,* 2010), does not appear to have this adaptation. Since tree frogs are aquatic animals, the idea that this adaption developed as a result of diving is therefore rejected. But since it is known to hibernate, Rana nigromaculata—or Pelophylax nigromaculatus as it is currently known (Xu *et al*., 2015)—does appear to have this adaptation. Conversely, Hoplobatrachus occipitalis, also known as the crowned Bullfrog (Godome *et al*., 2018), is primarily found in tropical and subtropical regions of West Africa and the Sahel. Because of this, it lacks the adaption to withstand post-ischemic circumstances, much like H. faber. Its preference for shallow waters (Godome *et al.,* 2018) and lack of necessity for diving further support this. Overall, this indicates that although a common basic response appears to exist across Anuran species, post-ischemic adaptation does not mitigate the oxidative stress caused by heavy metals, at least not for the Anuran species surveyed here. However, experimental data is needed to support these assertions**.**

2.9 Welding

Welding is a common industrial procedure that joins metals together at temperatures that are far higher than necessary. Crucially, according to Antonini *et al*. (2004), the procedure generates metallic fumes and vapours that are thought to be potentially dangerous. Welding vapours resulting from component oxidation are recognised to pose biological risks. Increased body levels of welding fume metals cause harmful health effects. Wergeland and Iverson (2001) issued a warning that metal fume exposure from welding, cutting, or grinding may result in pneumonia and health hazards. The results inside the welding face shields were correlated with the concentration of particles from the breathing spaces (Kauppi *et al*., 2015). The inhalation of fumes from galvanised spot welding has caused acute lung damage, mostly because of the brief exposure to zinc-containing particles (Antonini *et al*., 2017). Exposure to hazardous metals poses dangers to human health that are typically correlated with a number of variables. Multimetallic pollution of the workroom, ambient air, and other areas of the environment, including foodstuffs produced in contaminated areas, is caused by technologies such as steel processing, electric arc welding, pyrometallurgy of heavy nonferrous metals, and electroplating (Minigalieva *et al.,* 2017). The vaporisation of the metals and oxides in an electrode or wire, which is utilised to produce fumes or dust clouds during the procedure, is the process of welding. One of the most popular forms of welding is arc welding, which is the act of joining two pieces of metal that were heated to a liquid state when electricity is transferred from one electrical conductor to another. Fumes are produced at the electrode tip during the process as a result of the fluxes and metals on the electrode evaporating. After coming into contact with air, these metal vapours condense and oxidise, forming microscopic particles that are made up of a complex mixture of metal oxides (Harris *et al*., 2002). The electrode composition and the material being welded play a major role

in determining the elemental composition of welding fume (Grishagin *et al.,* 2016). High fume levels in industrial locations are correlated with welding operations that use excessive heat. Metal oxide or dust particles that had condensed from vapour made up the fumes. Airflow can readily affect the particles, causing them to disperse outside of the working area and then be absorbed by the welder's body (Grishagin *et al*., 2016). The type of metals or materials used and the welding technique employed determine which metal complexes are produced. For instance, the elemental compositions of mild steel (MS) and stainless steel (SS), two of the most commonly used types of electrode wire, differ. The elements Cr and Ni found in SS emissions have been linked to lung disorders and have been shown to be cytotoxic to human pulmonary cells (Camner *et al*., 1992). All Fe, Mn, and Si dust particles—both nano and micro—that enter the human body can trigger the body's natural defence mechanism, activating macrophages and triggering a host of inflammatory processes that can lead to numerous illnesses. Apart from heavy metals, welding also releases ozone, carbon monoxide, carbon dioxide, and nitrogen oxides, which are all harmful pollutants. According to Leonard *et al.* (2010), one of the mechanisms for the acute harmful effects of welding fumes on health is the production of reactive oxygen species (ROS), with stainless steels containing nickel and chrome producing more ROS than mild steel.

2.10 Antioxidant Enzymes: First Line of Defense Against Oxidative Stress

Low-molecular-weight antioxidants (such as flavonoids, carotenoids, vitamins C and E, among others) have been shown to offer only a restricted level of defence against the deleterious effects of free radicals in biological systems (Halliwell 2022). Reaction rates between ROS and membrane lipids, proteins, and DNA are higher than those between ROS and low-molecular-weight antioxidants. For this reason, low-molecular-weight antioxidants have a limited capacity

to scavenge radicals. Superoxide dismutase (SODs) can effectively prevent biological harm by dismutating superoxide radicals into hydrogen peroxide. In the following stage, glutathione peroxidase and catalase eliminate hydrogen peroxide (Forman and Zhang 2021). Low-molecular-weight antioxidants do not react with cellular oxidants as quickly as antioxidant enzymes, which do so thousands or even millions of times faster. Extracellular space is the sole area where exogenous antioxidants and enzyme mimics can offer some effective protection against ROS because there are no antioxidant enzymes present there other than EC-SOD.

2.10.1 Superoxide Dismutase (SOD)

In 1969, Joe M. McCord and Irwin Fridovich identified the enzyme Cu,Zn-superoxide dismutase (McCord and Fridovich 1969). The most potent cellular antioxidant, superoxide dismutase (SOD), serves as an organism's first line of defence against biological oxidants. Enzymes containing transition metals (SODs) are found in all organisms that are oxygen-dependent. Two molecules of superoxide radical anions (O_2 •−) are converted by SODs into molecular oxygen (O_2) and hydrogen peroxide (H2O2). Mammals have three different types of SODs (Abreu and Cabelli 2010). One Cu and one Zn atom can be found in each subunit of the stable homodimer Cu,Zn-SOD (SOD1). SOD1 is mostly expressed in the cytoplasm of cells, the nucleus, and the space between the inner and outer membranes of mitochondria. The homotetrameric enzyme Mn-SOD (SOD_2) has a manganese atom in its active centre. The pH of the mitochondrial matrix, where SOD_2 is found, is roughly 7.8, which is higher than the pH of the intermembrane gap, which is between 7.0 and 7.4. Tetrameric Cu,Zn-SOD (SOD_3) is mostly found in the extracellular environment and is strongly expressed in the lung. The copper atom in SOD is engaged in catalytic electron transfer, whereas the zinc atom serves a structural purpose. Two near diffusion-limited processes drive the complicated catalytic dismutation of superoxide

radical (O_2 •−) (Perry *et al.* 2010). The initial reaction begins with the binding of the superoxide radical (O_2 •−) to the Cu(II) ions. This leads to the creation of the cuprous species Cu(I) in a trigonal planar configuration, an inner-sphere electron transfer process, and the oxidation of superoxide radicals to oxygen molecules. An anion-binding site created by partially protonated and reduction-ready Arg143 binds a second equivalent of superoxide. The second reaction takes place in the outer sphere and is typified by the simultaneous oxidation of Cu(I) back to the cupric species Cu(II) [Cu(II) has 9 d electrons, d9, with one unpaired electron] and the donation of a proton from His63 and an electron from Cu(I) [Cu(I) has 10 d electrons, d10, with all electrons paired] to superoxide to form hydrogen peroxide. (Quist *et al.* 2017). Hydrogen peroxide is thus created as a result of the coordinated action of protonation and electron reduction of superoxide radical anions. In the event where Zn and Cu are both present, pH has no bearing on either reaction step. Even at physiological pH levels, the protein's activity sharply declines in the absence of structurally significant zinc (Zn). All SOD isoforms are important and specific because of their various cellular locations, which keep cellular compartments' redox states within physiological bounds and promote cell survival and homeostasis**.**

2.10.1.1 Sods and Diseases

Numerous chronic illnesses, including cancer, heart disease, and neurological problems, have been related to changes in SOD expression and/or activity (Trist *et al*. 2021). Amyotrophic lateral sclerosis (ALS), a deadly neurological disease affecting motor neurones, is caused by the most common mutation in SOD1 (Cu,Zn-SOD), D90A (Trist *et al.* 2021). This illness is sometimes referred to as Lou Gehrig's disease, after the well-known baseball player who passed away in 1941 from ALS. The gradual degradation of nerve cells in the brain and spinal cord is a characteristic of ALS. Currently, familial (inherited) ALS has been linked to over 100 distinct

SOD1 mutations. A single amino acid position in the amino acid sequence is altered by mutations in SOD1. A4V is the mutation that occurs most commonly in the United States. Similar to other neurodegenerative conditions like Alzheimer's disease, ALS is typified by an aberrant protein build-up, some of which are mutant SOD1 proteins.

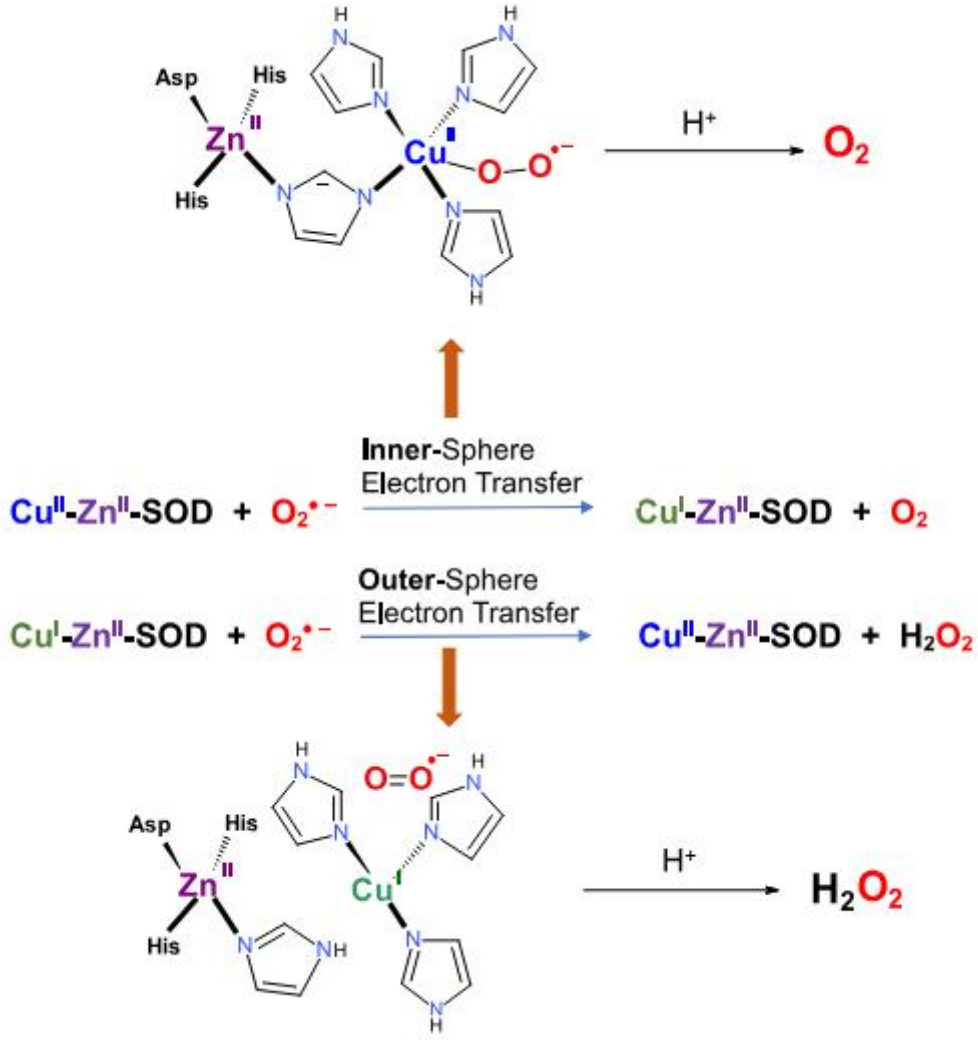


Fig.1.3: The mechanism of superoxide radical dismutation catalyzed by Cu,Zn-SOD showing inner- and outer-sphere electron transfers.

Source: Adapted from Quist *et al*. (2017).

The significance of reduced SOD2 (MnSOD) expression in cancer was first proposed by Oberley and Buettner in their groundbreaking research, which was published more than 40 years ago (Oberley and Buettner 1979). Additionally, their research indicated that cancer cells' redox balance was shifting in favour of a prooxidant condition. Many malignancies have also been reported to have changes in SOD1 expression. Nasopharyngeal carcinoma (NPC), breast cancer, and non-small cell lung cancer (NSCLC) have all been linked to overexpressed SOD1 (Li *et al*. 2020a, b). In these malignancies, elevated SOD1 expression is typically associated with the advancement of the disease (Li *et al*., 2018). According to recent findings, EC-SOD suppresses tumours in cancer, albeit the underlying mechanism is still unclear (Griess *et al.* 2017). Overexpressed EC-SOD has been connected to the suppression of both tumour growth and metastasis, whilst low expression of EC-SOD in cancer patients has been associated with a worse prognosis. It is well known that exposure to high oxygen pressure can result in hyperoxic lung damage, which is marked by an increase in the formation of ROS and the synthesis of pro-inflammatory cytokines including IL-8 and IL-1β. Enhanced ROS levels and enhanced transcription factor expression for AP-1 and IL-8 were observed in A549 cells subjected to high oxygen pressure, or hyperoxia. These effects were considerably reduced by overexpressing SOD2 or SOD1 (Liu *et al.* 2022). The advancement of cardiac fibrosis has been linked to SOD2 (Kwak *et al*. 2015). Mn-SOD overexpression has been shown to reduce cardiac fibrosis in elderly mice, whereas Mn-SOD-deficient mice show hypertrophy and fibrosis of the heart. Variations in EC-SOD expression are also linked to a number of illnesses. When angiotensin II (Ang II) was given to mice lacking EC-SOD, the incidence of hypertension was noticeably higher than when the control therapy was administered. Normal blood pressure was restored as a result of EC-SOD's enhancement of vascular relaxation and suppression of superoxide radical

levels (Gongora *et al*. 2006). According to reports, EC-SOD expression and activity in the lungs of wild-type mice are reduced by prolonged hypoxia; on the other hand, in EC-SOD transgenic mice, EC-SOD activity is roughly six times higher during hypoxia (Nozik-Grayck *et al.* 2008). The lungs and heart's arteries are impacted by pulmonary hypertension. According to Ahmed *et al.* (2012), overexpressed EC-SOD has a protective impact against pulmonary hypertension and pulmonary artery remodelling. Additionally, studies have demonstrated the significance of EC-SOD in reversing the progressive, chronic form of pulmonary arterial hypertension (Lopez *et al.* 2019).

2.10.2 Catalase

Oxidoreductases such as peroxidases and catalases, are found in almost all aerobic species. The majority of catalases are homotetramers, which have molecular weights ranging from 200 to 340 kDa and four prosthetic groups. These substances are crucial in avoiding oxidative stress brought on by aerobic metabolism's natural production of hydrogen peroxide (H_2O_2). According to Glorios and Calderon (2017), catalase is an efficient catalyst that cleaves the O − O bond of H_2O_2 into oxygen and water, avoiding the generation of ROS and preserving cellular redox equilibrium. Based on their structure and function, three classes of catalases are distinguished (Glorieux and Calderon, 2017). Catalases and catalase peroxidases that are conventional or real heme-containing enzymes are representative of the first two classes. Catalases that do not contain heme and contain manganese comprise the third group. The largest group consists of typical (genuine) catalases. Generally homodimers, catalase peroxidases are found in bacteria, fungi, and archaea. They range in size from 120 to 340 kDa. Compared to conventional catalases, these enzymes often have less effective catalytic activity. Only bacteria produce manganese-containing catalases, which use two Mn ions in the active site to generate oligomeric complexes that range

in size from 170 to 210 kDa. Their catalytic reaction mechanism differs greatly from that of common catalases and catalase-peroxidases. Among all the enzymes, catalase has the highest turnover rate and can break down millions of H_2O_2 molecules in a second. It is mostly found in peroxisomes, which are the sites of heme addition and tetramer formation where catalase monomers are imported (Lazarow and de Duve 1973). It has been documented that catalase exists in the cytosol in the tetrameric form, with varied abundances based on the type of cell (Middelkoop *et al*. 1993). Every human organ expresses catalase, however the liver, kidney, and erythrocytes have been shown to have the highest levels of activity. Erythrocytes produce a lot of hydrogen peroxide because of the oxygen transport system, and catalase is responsible for around half of the turnover of hydrogen peroxide (Mueller *et al.*1997).

2.10.3 Catalase Activity and Diseases

Genetic research has shown that the polymorphisms of the CAT gene vary significantly depending on the population and geographical area. To get a better knowledge of the function of the CAT gene in various disorders, more research including a global range of populations is required. By controlling the activity of this significant antioxidant enzyme, new therapeutic approaches may become apparent (Sumner and Dounce, 1937). Many diseases, including neurological conditions (Parkinson's disease, Alzheimer's disease), psychiatric conditions (bipolar disorder, schizophrenia), cardiovascular conditions (hypertension), metabolic conditions (diabetes mellitus, autoimmune diseases (vitiligo), Wilson's disease, and cancer, are caused by changes in catalase expression (Glorieux and Calderon, 2017) as seen in figure 1.4. It's interesting to note that individuals with acatalasemia, a genetic defect that causes limited catalase activity (Takahara disease), do not exhibit significant clinical symptoms. This is probably because other antioxidant enzymes that have catalase-like activity act as a compensatory factor.

According to Rochette *et al*. (2014), diabetes mellitus is a chronic metabolic disease characterised by increased blood glucose levels. Diabetes comes in a variety of kinds, with type 2 representing 90% of cases and being the most prevalent. Increased hydrogen peroxide levels have been linked to β-cell damage as a result of altered signalling pathways controlling the synthesis of insulin (Jörns *et al*. 1999).

The pancreas's poor expression of antioxidant enzymes limits the ability of β-cells to detoxify reactive oxygen species. According to recently published data, β-cells can detoxify micromolar amounts of hydrogen peroxide through a mechanism that is dependent on thioredoxin reductase (Stancill *et al*. 2019). Redox homeostasis is typically disrupted in tumour cells (Valko *et al*. 2006). ROS production is elevated in tumour cells, which promotes the growth of cancer cells and genetic instability. Different cancer tissues have been found to have different levels of catalase than healthy ones. While some writers (Rainis *et al.* 2007) found higher levels of catalase expression in tumours, other publications (Cullen *et al*. 2003) indicated lower levels of catalase expression.

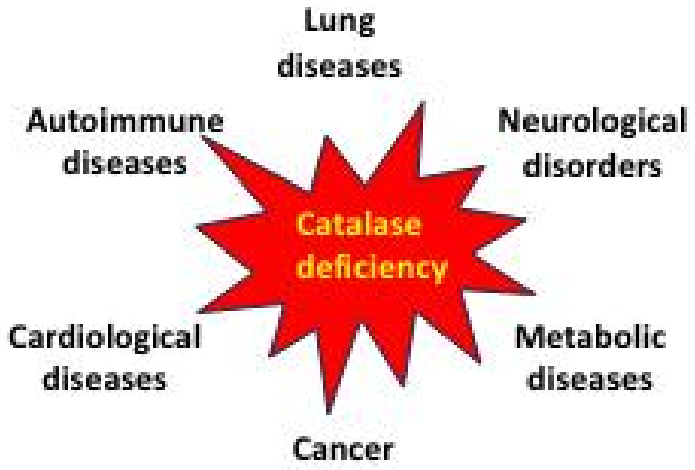


Fig 1.4: Catalase deficiency and human diseases

Source: Mueller *et al.*1997

The data obtained thus suggest that controlling the expression of catalase in cancer cells could be a novel prooxidant strategy to improve the efficacy of chemotherapy. Alzheimer's disease (AD) is a neurological condition marked by a progressive loss of thinking, memory, and other abilities. The aetiology of AD is complex, but one of its main features is the build-up of deadly senile plaques made of amyloid-β peptides in the brain. According to Milton (1999), amyloid-β has a strong affinity for catalase, which prevents hydrogen peroxide from breaking down. Furthermore, redox-active metals like copper (II) and iron(II) that catalyse the Fenton reaction—which breaks down hydrogen peroxide and produces hydroxyl radicals—tightly interact with amyloid-β. The oxidative damage seen in the brains of Alzheimer's patients is caused by hydroxyl radicals and other ROS (Valko *et al.,* 2005). As a result, catalase and the oxidative stress element of AD pathophysiology are indirectly related. Parkinson's disease (PD) is a neuropsychiatric and physical neurodegenerative illness that progresses over time (Meade *et al.,* 2019). A prominent characteristic and possible indicator of Parkinson's disease is the existence of brain aggregates containing misfolded α-synuclein protein. The release of dopamine into the cytoplasm and the inhibition of catalase expression and activity are caused by mutations in the gene producing the α-synuclein protein, which is linked to familial Parkinson disease (Day, 2009). Furthermore, Turnbull *et al*. (2001) reported that in vitro tests verified that α-synuclein is the catalyser for the creation of hydrogen peroxide. These investigations lead to the possibility that α-synuclein causes both the increased production of hydrogen peroxide and the decreased activity of catalase, which is supported by the elevated oxidative stress indicators in Parkinson's disease. An autoimmune disease called vitiligo is characterised by areas of skin colour loss. Numerous investigations have shown that vitiligo patients' epidermis contains less catalase than healthy controls. The reduced catalase levels lead to a higher concentration of hydrogen peroxide, which

can undergo photochemical breakdown to produce hydroxyl radicals (Schallreuter *et al*. 2006). In the epidermis, hydroxyl radicals can harm melanocytes and keratinocytes by oxidising membrane lipids. Furthermore, structural changes in keratinocytes or melanocytes may result from mutations in the CAT gene (Casp *et al.* 2002). However, these conclusions need more explanation because they conflict with current research conducted across population groupings.

2.10.4 Glutathione Peroxidase

All living things have the antioxidant enzyme family known as glutathione peroxidase (GPx), which was discovered in 1951. Numerous cellular processes are impacted by the diverse biological roles of the GPx protein family (Pei *et al*., 2023). Eight distinct GPx isoforms (GPx1–GPx8) have been identified in humans thus far. The most prevalent is glutathione peroxidase 1 (GPx1), which is particularly abundant in the heart and present in the cytoplasm of all organs. The four subunits of the tetrameric enzyme GPx1 are similar to one another. A distinct proteinogenic, redox-sensitive selenocysteine amino acid is present in each subunit. A key player in the enzymatic reduction of hydrogen peroxide and hydroperoxides, selocysteine is also referred to as the 21st amino acid in the genetic code (Lubos *et al.,* 2011). As the initial line of defence against ROS-induced oxidative stress, GPx collaborates with SOD and catalase. Hydroxyperoxides (like H_2O_2) can be reduced to H_2O by GPx, which does this by oxidising reduced glutathione (GSH) to its oxidised form (GSSH):

HOOH + 2GSH (21) GPx → 2HOH + GSSG(21)

or more generally

ROOH + 2GSH (22) GPx → ROH + GSSG + HOH.(22)

Glutathione peroxidases exhibit a nonsequential pingpong mechanism involving an oxidation reaction followed by a reduction step (Weaver and Skouta, 2022) as seen in fig. 1.5 below.

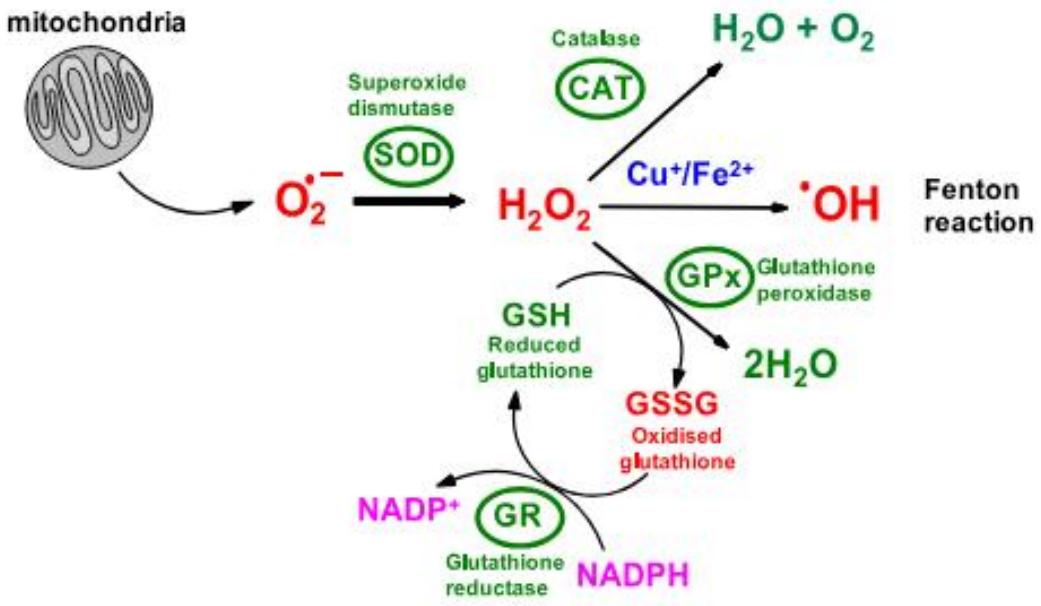


Fig 1.5: Concerted action of antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase.

Source: Weaver and Skouta 2022

The first stage involves hydroperoxides oxidising GPx. A sequence of reductions of the oxidised GPx by a reduced cofactor, such as glutathione (GSH), comprise the second step. In the first step, selenenic acid (GPx–Se–OH) or sulfenic acid (GPx–S–OH) is produced by oxidising the catalytic site of GPx, which is made up of either cysteine or selenocysteine (Flohe *et al.* 2022). Hydroperoxides (ROOHs) and hydrogen peroxide (H_2O_2) are transformed into their corresponding alcohols (ROHs) and water (H_2O) throughout this process. According to Brigelius-Flohé and Maiorino (2013), the oxidation step's rate constant is around 108 M−1s −1, making it one of the fastest enzymatic processes ever measured in biological systems. There are two steps in this reduction. Two equivalents of reduced glutathione (GSH) are used to reduce selenocysteine or cysteine from selenic or sulfenic acids, releasing oxidised glutathione (GSSG) in the process. The protein's active site is recovered as a result of this action. NADPH-dependent glutathione reductase is involved in the breakdown of oxidised glutathione (GSSG) (Pei *et al*. 2023). Almost all human cells have significant levels of GPx4 expression (Pei *et al*., 2023). Lipid hydroperoxides are preferred by GPx4, but hydrogen peroxide is highly preferred by GPx1. GPx3 is found in the extracellular area of plasma. GPx5, GPx7, and GPx8 have cysteine (Cys) residues in their active sites, whereas GPx1-4 and GPx6 have selenocysteine in the active centre. GPx1 mutant mice die from severe acute oxidative stress, whereas wild-type mice given selenium supplements live normally. Therefore, GPx1 is indispensable for other selenoproteins and has a significant role in reducing oxidative stress (Liu *et al.*, 2021).

The signalling pathway has been shown to target GPx2, which is involved in both normal physiological processes and embryonic development, as well as pathological processes like cancer (Zhao *et al*., 2022). The gastrointestinal tract is the primary site of GPx2 expression, which is thought to be the initial barrier preventing the absorption of hydroperoxides from

processed foods. Selenocysteine is present in the active site of the GPx family, and it has been suggested that GPx2's protective properties in reducing elevated oxidative stress may be more significant than those of the other GPxs. The Wnt pathway is activated and enhanced by the overexpression of GPx2, which also inhibits apoptosis, increases hydrogen peroxide dismutation, and reduces oxidative stress. The Wnt pathway is activated by GPx2, which has been linked to its actions in metastatic cervical cancer (Wang *et al.*, 2019a, b). Of a family of oxidoreductases, GPx3 is the only extracellular tetrameric GPx with antioxidant properties. GSHPx is another name for GPx3. GPx3 is a powerful hydroperoxide remover and a significant scavenger of free radicals in plasma. According to Chang *et al*. 2020, GPx3 plays two distinct roles in cancer: either it suppresses tumour growth or it promotes it. It has been suggested that GSH may be involved in the control of GPx3 function, which is consistent with the discovery that elevated proteins in cancer are known to transport GSH. The increased activity of GPx3 in tumours may be due to increased GSH efflux into the extracellular space. Therefore, focussing on GPx3 may be a useful tactic for treating cancer. The only enzyme that can directly degrade lipid hydroperoxides and big lipid molecules like cholesterol and phospholipids is GPx4, a membrane-bound enzyme. According to Bersuker *et al.* (2019), GPx4 is found in three distinct isoforms: mitochondrial (mGPx4), cytoplasmic (cGPx4), and sperm (sGPx4). The testis is the primary location of sperm GPx4. These results support the notion that GPx4 is critical for male sexual maturity. It was discovered that GPx4 is an essential glutathione peroxidase during embryonic development by using GPx1, GPx2, or GPx3 knockout mice. While GPx4 and GPx1 both have antioxidant and anti-inflammatory properties, only GPx4 is a selenoprotein that can lower peroxidised lipids that are anchored in biological membranes (Brigelius-Flohé and Flohé 2020). First identified by Brigelius-Flohé and Flohé (2020), GPx5 was extracted from epididymal (a

coiled, tubular structure sitting on the rear of testicles) mice tissue. The active region of GPx5 has cysteine residues instead of selenocysteine, unlike GPx1–GPx4, and it lacks the usual dismutation activity towards substrates like H_2O_2 (Taylor *et al.* 2013). Gpx5 is a significant antioxidant enzyme that regulates oxidative stress in the epididymis and is essential for the maturation, storage, and movement of sperm cells. Spermatozoa membranes are rich in polyunsaturated fatty acids that are reactive to reactive oxygen species (ROS). Only GPx5 offers defence against ROS since the sperm cytoplasm has a low antioxidant content (Taylor *et al.,* 2013). For the sake of preserving the male reproductive system, GPx5 thus provides protection against the process of sperm maturation. Selenocysteine is present in the active centre of GPx6, a tetramer (Brigelius-Flohé and Flohé, 2020). A near homolog of GPx3 is GPx6. GPx6 is mostly expressed in the olfactory (sensory) system, according to reports. It is thought that GPx6 contributes to the propagation and deterioration of signals connected to odour. According to reports, GPx6 is upregulated in environments with elevated oxidative stress, suggesting that GPx6 has antioxidant properties (Pei *et al.,* 2023). Because of its similarity to GPx4, GPx7 is referred to as a nonselenium cysteine. Nevertheless, in contrast to GPx4, GPx7 lacks a glutathione binding site (Pei *et al.,* 2023). Thus, GPx7 is not a GSH peroxidase but rather a protein disulphide isomerase (PDI) from a functional perspective. GPx7 is found in the endoplasmic reticulum lumen. Cysteine residues' thiol groups undergo oxidative stress, which can result in the formation of irreversible compounds such sulfinic acids (Cys–SO_2H) and sulfonic acids (Cys–SO_3H) or reversible intramolecular disulphide bonds (–S–S–) and cysteine sulfenic acids (Cys–SOH) (Kanemura *et al*. 2020). GPx7 is not involved in redox processes since it does not have a domain that is specific for binding GSH. Increased oxidative stress causes GPx7 to activate stress signalling and release proteins, including protein disulphide isomerase

and glucose regulating proteins (Wei *et al*., 2012a, b). The transmembrane protein GPx8 has peculiar structural properties. Like GPx7, GPx8's severely reduced GPx activity is mostly brought on by the absence of GSH-binding domains. The presence of a conserved N-terminal transmembrane domain and a C-terminal motif intended for endoplasmic reticulum localisation characterises GPx8 specifically. Yoboue *et al.* (2017) have revealed that there is a direct correlation between the expression of GPx8 and the regulation of calcium flux and storage in the endoplasmic reticulum. Hydrogen peroxide is thus produced in tandem with oxidative protein folding in the endoplasmic reticulum. There have been reports that this process is linked to the control of nuclear factor erythroid 2-related factor 2 (Nrf2). Through calcium ATPase, Nrf2 in turn controls GPx8 expression and impacts calcium regulation in the endoplasmic reticulum (Granatiero *et al.* 2019).

2.10.4.1 Glutathione Peroxidase and Diseases

The effect of selenium deficiency and the concomitant decrease of selenium protein expression in cancer has been investigated in a number of epidemiological experiments (Short and Williams, 2017). One selenium-related protein that is probably impacted by selenium levels is GPx1. Studies examining the function of selenium supplementation in chemoprevention were prompted by these findings (Vincenti *et al*., 2018). Selenium supplementation has been shown to have a positive impact on lowering the risk of cancer in a number of studies; however, positive effects have only been seen in certain people and in certain studies (Lippman *et al.*, 2009). The use of various types of selenium supplements is one of the causes of these inconsistent results. According to research done on experimental mice, selenium through GPx1 has a partially preventive impact on certain cancer forms (Diwadkar-Navsariwala *et al*., 2006). But keep in mind that a variety of dietary selenium concentrations and other variables affect the expression

of selenoproteins. Through a variety of ways, selenium supplementation is known to improve apoptotic signalling and cell growth inhibition. According to Brigelius-Flohé and Kipp (2009), GPx1's unclear function in cancer may be explained by its ability to control oxidative stress-induced inflammation and mutagenesis (DNA damage), as well as by preventing apoptotic cell death, which may prolong the lifetime of cancer cells. According to Li *et al*. (2020a, b), ferroptosis is a novel, distinct, and recently identified iron-dependent cell death pathway that is intimately linked to the pathogenic mechanisms of numerous illnesses, including cancer, blood disorders, and renal diseases. Glutathione peroxidases may be impacted directly or indirectly by factors that induce ferroptosis. Depletion of intracellular GSH and decreased GPx4 activity are hallmarks of ferroptosis. These events lead to the build-up of lipid peroxides, Fe(II)-catalyzed oxidation of lipids by the Fenton reaction, and increased production of reactive oxygen species (ROS). GPx is crucial for the pancreas' healthy operation and defence against damage brought on by oxidative stress. Hydrogen peroxide is required for the release of insulin in response to glucose, however there are many enzymes in the pancreatic islets that break down hydrogen peroxide, including GPxs. Reduced β cell mass, minor impairment of insulin production, and lower fasting insulin levels are indicative of mild pancreatitis in GPx1 knockout mice (Brigelius-Flohé and Flohé, 2020). Based on these findings, it appears that GPx1 is crucial in delaying the onset of diabetes. The elimination of hydrogen peroxide by GPx-1 is linked to the pancreas' antioxidant defence. Thus, the physiological level of insulin is guaranteed. Research using GPx1 knockout mice demonstrated that this enzyme plays a significant part in heart failure (Lubos *et al.,* 2011). An inadequately optimised dose of selenium may be the primary cause of discrepancies between clinical and experimental trials on the protection of cardiovascular illnesses. Selenium, which is mostly found in enzymes, was given as a daily supplement to

patients in the majority of the studies who had normal serum selenium levels. Nevertheless, the efficiency of selenium does not increase with dose. Superoxide radicals are created when selenium is overdosed and selenium-containing compounds undergo autooxidation (Spallholz, 2016). Serum selenium concentrations above 135 ng/ml are linked to cardiovascular incidents such as ischaemic stroke, myocardial infarction, and sudden cardiac death, despite trials confirming that selenium supplementation results in a quarterly reduction in the incidence of cardiovascular disease (Bleys *et al.,* 2009). GPx2 is essential for stem cell survival and is mostly located in the gastrointestinal tract, liver, kidney, skin, brain, and other organs (Kipp 2019). In Gpx2−/− mice, the gastrointestinal system is altered as one of the pathogenic effects. Ileocolitis, the most prevalent kind of Crohn's disease that is characterised by small intestine inflammation, develops in GPx2- and GPx1-knockout mice (Esworthy *et al*., 2001). According to Chu et al. (2004), Crohn's disease can progress to cause cancer, benign tumours of epithelial cells, or adenomas. These results validated GPx1 and GPx2's synergistic effects on gastrointestinal tract cancer and inflammation prevention. Most gastrointestinal tract, breast, and liver malignancies have elevated GPx2 levels. In cancer patients, increased expression of GPx2 is linked to growth, metastasis, and a poor prognosis (Peng *et al*., 2023). On the other hand, additional research demonstrated a correlation between GPx2 expression suppression and a lower chance of survival for cancer patients, so validating GPx2's tumor-suppressive role (Ren *et al.* 2022a, b). These investigations suggest that GPx2 is either an oncogene or a Janus tumour suppressor. As a result, the kind of tumour and illness stage determine GPx2's two-sided character. According to An *et al.* (2016), GPx3 has been shown to block the extracellular signal-regulated kinase (ERK)/nuclear factor-κB (NF-κB) pathway, thereby preventing lung and liver cancer cells from proliferating and spreading. Targeting GPx4 may be a viable tactic for promoting cancer cell death and

inducing ferroptosis in treatment-resistant malignancies, as the production and expression of GPx4 are governed by a number of biological mechanisms (Lee and Roh, 2023). In a Chinese hamster ovary cell line, overexpressed GPx5 was able to decrease lipid peroxidation and DNA damage. Histologically speaking, the sperm in GPx5 knockout young mice were normal, but the frequency of abnormalities in the progeny rose. Furthermore, in knockout mice older than a year old, the degree of DNA damage rises (Chabory et al., 2009). Studies on the connection between GPx6 and illnesses have been comparatively rare. GPx6 overexpression in the frontal cerebral cortex of aged mice with a Huntington's disease model reduced their motor deficits (Shema *et al.*, 2015). GPx7 deletion mice had a shorter life span and a higher incidence of cancer when compared to control mice. Uncertainty surrounds GPx8's expression and functions in the occurrence of cancer. According to recently published findings, GPx8 may be crucial to the prognosis of individuals with glioblastoma multiforme, an aggressive brain tumour that is growing quickly (Li *et al*. 2022). Treatment approaches that specifically target GPx8 might represent a novel therapeutic approach for this aggressive type of cancer.

2.11 Definition and Structure of Lipids

According to Fahy et al. (2011), lipids are a class of organic compounds that are soluble in non-polar organic solvents but insoluble in water. According to Waagbø (2001), lipids are essential for the structure of cell membranes, as well as for other non-polar molecule transportation, signal molecule precursory, and energy storage. Lipids are categorised based on their structure and function. Triacylglycerols are used in the transfer and storage of energy. They have a glycerol molecule joined to three fatty acids as their structural component as seen below in figure 1.6. Glycerophospholipids consist of a phosphate group attached to the third carbon atom of glycerol and two fatty acids linked to the molecule. After that, the phosphate is bound to an alcohol,

amino acid, or nitrogen base. These serve a number of objectives, including the formation of micelles during digestion and structural roles in the cell membrane. The most prevalent lipid in cell membranes is phosphatidyl choline (PC), which is followed by phosphatidyl-serine (PS), phosphatidyl-ethanolamine (PE), and phosphatidyl-inositol (PI). With its skeleton like that of steroids, cholesterol serves as the precursor to several hormones, including bile acid and vitamin D. Additionally present in cell membranes, cholesterol aids in their integrity by preventing the membrane from becoming overly fluid (Waagbø, 2001).

Fatty acids (FAs) are present in a few lipid classes, such as TAG and phospholipids. The general formula for FAs is CH3 (CH2) nCOOH. Differentiating between the three primary forms of FAs—polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), and saturated fatty acids (SFA)—is crucial. There are only single bonds between the carbon atoms in saturated fatty acids. MUFA contains a single double bond between two carbon atoms, while PUFA contains two or more. Certain well-known polyunsaturated fatty acids, or long-chained omega-3 fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are crucial for fish metabolism and for preserving the structural and functional integrity of cell membranes (Tocher, 2015). Additionally, EPA is a crucial precursor to the class of biologically active paracrine hormones known as eicosanoids. In the marine environment, EPA and DHA are both widely distributed (Valentine and Valentine, 2004). Because alternative oil sources of terrestrial origin are used, farmed Atlantic salmon has less EPA and DHA than it did in the past (Sprague et al., 2016).

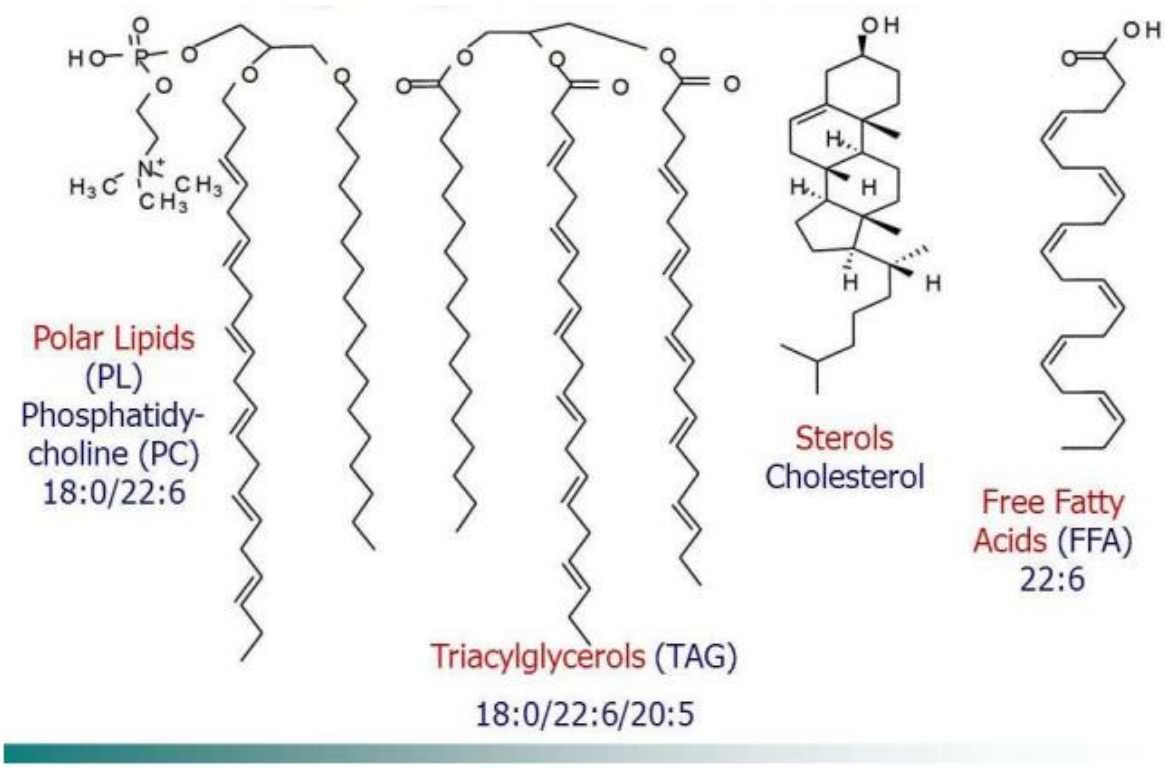


Fig 1.6: Structure of PC. TAG, Cholesterol and Free fatty acid

Source: Miller *et al* 2004

2.11.1 Digestion of Lipids

The process of digestion converts food into molecules that can go through the intestinal wall and be absorbed. Pellets are exposed to the stomach's acidic environment during feeding, when they undergo hydrolysis and solubilisation to break down. But fat does not begin to break down until it reaches the stomach's pylorus region. Cholecystokinin (CCK) is secreted into the bloodstream when stomach chyme enters the intestine due to a decrease in pH and an increase in fatty acids. This hormone will induce the gallbladder to contract and the pancreas to secrete more. Fatty acids will be emulsified by the bile acid, forming micelles with an outer layer of bile salts and polar lipids that are water soluble. By doing this, the chyme's lipid will be more evenly distributed, increasing the surface exposed to the digestive enzymes overall. The ester linkages in TAGs, phospholipids, wax esters, and cholesterol esters will be broken by lipase. Subsequent to the ester bond breaking, the micelles will contain cholesterol, phospholipids, diacylglycerols, and free fatty acids, among other substances. Due to their lower pH, the micelles will dissolve in the unstirred water layer, allowing the fatty alcohols from wax esters, monoacylglycerols, cholesterol, and free fatty acids to pass through the intestinal wall by both passive diffusion and active transport (Tocher, 2015). Although some lipid uptake takes place in the hindgut as well, the majority of fatty acid diffusion takes place in the front end of the midgut and near the pylorus (Tocher, 2015).

The lipids are carried by the blood after they have crossed the intestinal wall. The lipids are encapsulated in a polar apolipoprotein to form lipoproteins (LP) because the molecules are nonpolar. Hence, LP are lipids encased in a polar protein cape, which makes the particle soluble in water. Cholesterol esters and/or TAGs make up the protein-coated core. The polar side of phospholipids and cholesterols, along with proteins, make up the outer layer. Chylomicrons, very

low density LP (VLDL), intermediate density LP (IDL), low density LP (LDL), high density LP (HDL), and very high density LP (VHDL), are lipoproteins (LP) that are categorised by density and will decrease in size with increasing density. While some lipids are delivered to the liver for short-term storage and/or processing, others are carried straight to the fatty tissues and muscles. After being secreted into the bloodstream by the liver, VLDL undergoes lipoprotein lipase's breakdown of TAGs into free fatty acids and monoacylglycerols, which are then absorbed by various body tissues' cells. Monoacylglycerols and free fatty acids are linked to TAGs inside the cell and stored as lipid droplets if they are not used directly as an energy source (Waagbø, 2001).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment/Apparatus

The following equipments were used (500ml, 250ml, 2500ml beakers), (Pyrex, England), Micropipette (Miroclux), Stirring rod (Pyrex, England), Centrifuge (Gallenkamp 200),Test tubes (Pyrex, England), Masking tape,Dispensing bags,Sample bottles (Pyrex, England),Spatula (Pyrex, England), Syringes (Pyrex, England), Cuvette, Measuring cylinders and glassware, Hand gloves (Pyrex, England),pH meter (Pyrex, England),Plain tubes (Pyrex, England), Pipette (Pyrex, England),Pipette tips (Pyrex, England), Spectrophotometer (Haris Engl. Model 80.2)

3.1.2 Chemicals and Reagents

The chemicals and reagents used in this study were of analytical grade. anhydrous sodium carbonate (BDH Chemicals, Poole, England), concentrated sulphuric acid (May and Baker, Dagenham, England), disodium hydrogen phosphate (May and Baker, Dagenham, England), dipotassium hydrogen phosphate (May and Baker, Dagenham, England), epinephrine (BDH Chemicals, Poole, England), ethylene diaminetetracetic acid (EDTA) (BDH Chemicals, Poole, England), glacial acetic acid (BDH Chemicals, Poole, England), hydrochloric acid (May and Baker, Dagenham, England), hydrogen peroxide (May and Baker, Dagenham, England), potassium dihydrogen phosphate (BDH Chemicals, Poole, England), potassium permanganate (May and Baker, Dagenham, England), sodium chloride (BDH Chemicals, Poole, England), sodium dihydrogen phosphate (May and Baker, Dagenham, England), sodium hydrogen carbonate (BDH Chemicals, Poole, England),sodium hydroxide (Avondale Laboratories),and thiobarbituric acid (Merck).

3.2 Methods

3.2.1 Reagents Preparation

3.2.2 Preparation of Reagent for Superoxide Dismutase Assay

0.05 M Carbonate Buffer pH 10.2

Carbonate buffer was prepared by dissolving 2.014 g of sodium carbonate; 2.604 g Sodium hydrogen carbonate and 0.372 g EDTA in 900 mL of distilled water. The pH was adjusted to 10.2 by drop wise addition of 0.1M NaOH and made up to 1000 mL, by using distilled water.

0.1 M NaOH

The reagent was prepared by dissolving 0.4 g of NaOH in 200 mL of distilled water

0.03 mM Adrenalin

The Adrenalin solution was prepared by dissolving 27.5 mg in 500 mL of 0.005 M of HCl

0.05 M HCl

A concentration of 0.05 M HCl was prepared by dissolving 4.13 mL of conc. HCl in 1000 mL of distilled water.

0.005 M HCl

A solution of 0.005 M HCl was prepared by dissolving 100 mL of 0.05 M HCl and make up to 1000 mL with DH_2O.

3.2.3 Preparation of Reagents for Catalase Assay

0.05 M Phosphate Buffer pH 7.4

Phosphate buffer solution of 0.05 M was prepared by weighing 4.36 g of disodium hydrogen phosphate and 2.72 g potassium hydrogen carbonate, which was dissolved in 900 mL of DH_2O. The pH was adjusted as required using 0.1M HCl and then made up to 1000 mL of distilled water.

Phosphate Buffer Hydrogen Peroxide

Solution of Phosphate buffer hydrogen peroxide was prepared by measuring 0.34 mL of 30% H_2O_2 which was dissolved in 20 mL of 0.05M Phosphate buffer pH 7.4 and it was then, make up to 100 mL of the buffer solution.

6 M H_2SO_4

A solution of 6 M H_2SO_4 was prepared by adding a volume of 163.2 mL of H_2SO_4 to 600 mL of DH_2O. The volume of the solution was made up to 1000 mL with distilled water.

0.01 M $KMnO_4$

$KMnO_4$ solution of 0.01 M was prepared by weighing 0.158 g of $KMnO_4$, which was dissolved in 1000 mL of DH_2O.

3.2.4 Preparation of Reagents for Malondialdehyde (MDA) Assay

TBA 0.875%

A solution of thiobarbituric acid (TBA) was prepared, by weighing 0.875 g of TBA, which was dissolved in 100 mL of distilled water.

TCA 15%

Trichloroacetic acid solution was prepared by weighing 15 g of TCA which was dissolved in 10 mL of distilled water in volumetric flask. The solution was then made up to 100 mL

0.25 M HCl:

A solution of 0.25 M HCl was prepared by measuring 2.15 mL of conc. HCl, which was dissolved in 60 mL of distilled water and then made up to 100 mL of distilled water

Stock TCA-TBA-HCl reagent

Equal volumes of thiobarbituric acid 0.875% w/v, trichloroacetic acid 15% w/v, and hydrochloric acid 0.25 M reagent were measured and mixed together to give the stock.

3.2.5 Glutathione Peroxidase Assay Reagents Preparation

5% Pyrogallol Solution:

This was prepared by dissolving 5 g of crystal pyrogallol in 100 ml of distilled water (H_2O). This was prepared fresh.

0.147M H_2O_2 solution:

A volume of 1.67ml of 30% (w/v) H_2O_2 was diluted to 100ml with distilled water. This was prepared fresh.

0.1M phosphate buffer (pH 6.0):

This was prepared by drop-wise addition of dilute HCl to phosphate buffer of pH 7.0.

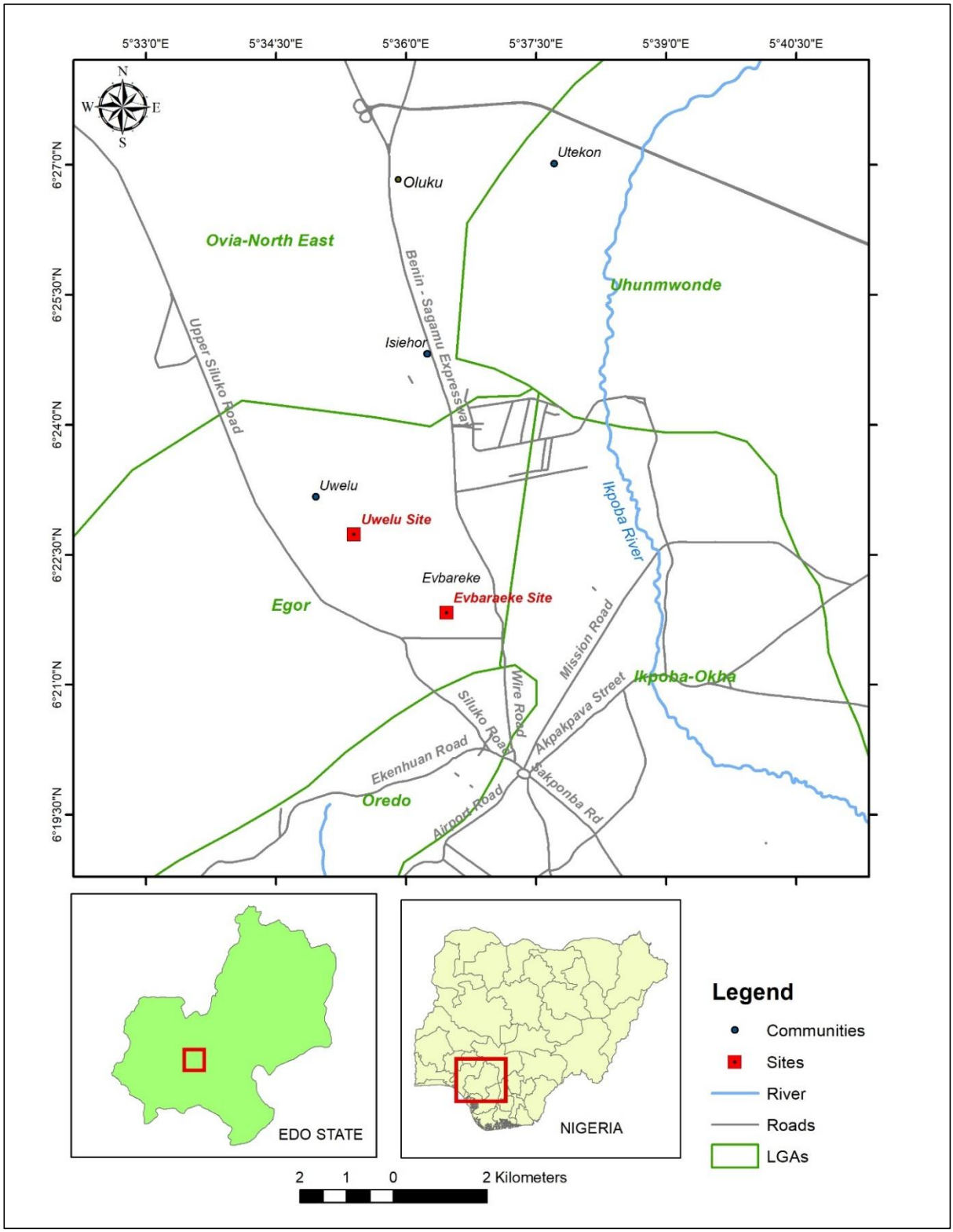
2.0N H_2SO_4 solution:

This was prepared by dilution of 6.0M H_2SO_4 solution with distilled water (H_2O) in ratio 1: 5 respectively.

3.3 Study Area

The experiment was conducted at Evbarake at 6.2150^o N, 5.3628^{o}E coordinate and Uwelu at 6. 2244^{o}N, 5.3524^{o}E coordinate Benin City, Nigeria between July and September 2023.

The detailed Coordinates and map are shown below.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E
S
Oluku
Utekon
Ovia-North East
Uhunmwonde
Benin - Sagamu Expressway
Upper Siluko Road
Isiehor
Uwelu
Uwelu Site
Ikpoba River
Evbareke
Evbaraeke Site
Egor
Mission Road
Wire Road
Siluko Road
Akpakpava Street
Ikpoba-Okha
Ekenhuan Road
Sakponba Rd
Oredo
Airport Road
EDO STATE
NIGERIA
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3.3.1 Experimental Animals

Tadpoles were obtained from stagnant water pools in automobile workshop containing calcium carbide used by welders for their welding work in vehicle maintenance shop from two different sites (Evbarake and Uwelu) in Benin City, Nigeria. Soil samples were also collected from these maintenance workshops. Control tadpoles were collected from Capitol area University of Benin City, Nigeria. Before initiating the experiment, the tadpoles were housed individually in plastic containers which contained sand and water so as to create an aquatic/terrestrial environment (mini-island) for these organisms.

3.3.2 Experimental Design

At the end of the five days of acclimatization, the tadpoles were weighed and assigned to a control group of 20 animals and two other test classes of 20 animals each. During the experiment the tadpoles were housed separately in outdoor mesocosms (polyethylene tanks) with about one-third of each container packed with the soil (50 g) soaked with 250 ml water from the test sites to stabilize the soil to simulated natural ponds and enhanced environmental realism relative to the laboratory. The water used for the soaking of the soil was obtained from the original habitat of the tadpoles which completely eliminated fatality. The tadpoles in each test group were further subdivided into four groups. The groupings were done such that the weight difference between all the study groups was about 0.01g. The tadpoles were maintained on daily rations of 0.5g lettuce that was boiled for 15min and chopped. The study lasted for 2weeks after which the tadpoles were weighed and then cut into head and tail regions (the two clearly distinct portions of the tadpoles)

3.3.3 Collection of Samples

The experiment of the tadpoles lasted for 12 days. The tadpole samples (20) weighed between 0.73 – 0.75 g. The tadpoles were dissected and the head and tail were quickly removed. The organs were washed in ice-cold 1.15%, KCl solution blotted and weighed. They were then homogenized in 4 volumes of homogenizing buffer (50 mM Tris - HCl mixed with 1.15% KCl and pH adjusted to 7.4), using Teflon Homogenizer. The resulting homogenate was centrifuged at 5000 g for 10 min in a Beckman L5-50B centrifuge at 0-4^{o}C. The supernatant was decanted and stored -20°C until analysis.

3.3.4 Preparation of Tissue Samples

Each region (head or tail) was weighed, and homogenized in ice-cold 0.9% normal saline to obtain 10% (W/V) homogenates. The homogenates were centrifuged at 5000 g for 10 min and the supernatant obtained were stored in a freezer for some biochemical assays. Analysis of lipid peroxidation, SOD, CAT, AO and GPx activities were done immediately.

3.4 Extraction of Head and Tail Total Lipids

The head and tail total lipid were extracted by a modification of the Bligh and Dyer (1959) method. 0.5 g of head and 1 g of tail samples were homogenised for 2 minutes at room temperature using a mixture of methanol; chloroform: water (2:1:0.8 v/v/v). The homogenate was centrifuged at l500 g and the supernatant decanted. The residue was re -extracted with a known-volume of methanol: chloroform: water (2/1/0. 8 v/v/v). After centrifugation the combined supernatants were diluted with equal volumes of chloroform and water (10 ml each) and the phases were separated in a Separating funnel. The lower chloroform layer was withdrawn and concentrated on a rotatory evaporator at 30- 35°C. The total weight of the lipid was determined gravimetrically. The lipid thus extracted was dissolved in chloroform and stored in refrigerator at -10°C.

3.5 Determination of Sample Total Cholesterol

The determination was based on the method of Zlatkis *et al.*, (1953). A purple color was formed when cholesterol in glacial acetic acid was treated with iron (III) chloride in 100% glacial acetic acid diluted with concentrated sulphuric acid. The color developed was quantitated at 560 nm. This method is sensitive and gives fairly accurate and reproducible results.

Materials:

i) Standard cholesterol solution (1.0 mg).

ii) Ferric chloride solution: 10.0 g of iron (III) chloride in 100 ml of 100% glacial acid.

iii) Colour reagent: 2.0 ml of ferric chloride solution above was diluted to 200 ml with concentrated sulphuric acid.

Procedure:

The tissue homogenate (0.1 ml) was pipetted into 3.0 ml of glacial acetic acid in a dry test tube. The color reagent (2.0 ml) was added and the content of each tube was mixed thoroughly. The tubes were allowed to cool down to room temperature. A standard cholesterol solution (1.0 mg/ml) and a blank were at the same time treated as above and the absorbance was read at 560 nm on a Coleman Jnr. Colorimeter. Cholesterol concentrations of sample homogenate were calculated using the equation below:

$$\frac{Optical\ density\ of\ test}{Optical\ density\ of\ standard} = \frac{Concentration\ of\ test}{Concentration\ of\ standard}$$

3.6 Determination of Sample High Density Lipoprotein Cholesterol

This determination was based on the method of Lopes-Virella, *et al*., (1977). This is a precipitation method in which a combination of phosphotungstate and; Mg^{2+} was used to

precipitate out very low density lipoprotein cholesterol which was then quantitated in terms of the amount of cholesterol remaining in the supernatant

Procedure:

An aliquot, 0.1 ml of 5% phosphotungstic acid reagent was added to 0.1 ml of the tissue homogenate and mixed. A 0.05ml, of magnesium chloride solution (2 M $MgCl_2$) was added and thoroughly mixed.

Centrifugation of the mixture was at 1500 g at 40°C for 30 minutes. The clear supernatant was carefully removed and cholesterol was determined in the supernatant.

3.7 Determination of Sample Total Triacylglycerols

This determination was based on the method of Gottfried and Rosenberg (1973) after a modification of Levy (1972). In this method serum samples were extracted with normal heptane. The triacylglycerols was hydrolysed to glycerol, which itself was oxidised with periodate. The resulting formaldehyde was condensed with acetyl acetone to form a yellow dehydrolutidine derivative. This was measured colorimetrically at 425 nm on a Coleman Jnr. Colorimeter. This method is very sensitive and gives a linear absorbance for samples containing up to 3 g triaclyglycerols/ litre.

Procedure:

Normal heptane (2.0 ml) was added to 0.5 ml of the tissue homogenate, 3.5 ml of isopropanol was added to the tube followed by 0.1 ml of concentrated H_2S0_4. The tubes were mixed with a vortex type mixer for 30 seconds and the phases allowed to separate without centrifugation. A standard triolein (100 mg/dl) solution and a blank were at the same time treated similarly. The

heptane extract (0.4 ml) was saponified by adding 2.0 ml of isopropanol and one drop of potassium hydroxide (6.25 mol/1). The mixture was well mixed, stoppered and incubated at 70^0C in a water bath for 10 minutes. Then, 0.2 ml of 0.06 M Sodium metaperiodate reagent and 1.0 ml of acetyl acetone reagent were added. The mixture was well mixed, stoppered and incubated for another 10 minutes at 70^0C.

The mixture was allowed to cool at room temperature for about, 3 minutes and the optical density was read at 425 nm against a blank. The concentration of triacylglycerols in the sample homogenate were calculated using the following equation:

$$\frac{Optical\ density\ of\ test}{Optical\ density\ of\ standard} = \frac{Concentration\ of\ test}{Concentration\ of\ standard}$$

3.8 Determination of Head and Tail Phospholipids.

Head and tail phospholipids were separated and quantitated by a combination of the methods of Bartlett (1959) and Cuzner and Davidson (1967)

3.8.1 Total Lipid Phosphorus Analysis

An aliquot, 1.0 ml of sample homogenate lipid was placed in a test tube and evaporated to dryness in an oven at 110^0C. The sample was digested on a sandbath with 0.5 ml 70% perchloric acid at about 200- 230^0C for 20 - 30 minutes. The tubes were well stoppered. The digest was brought to 10.0 ml with water. Then, 0.5 ml of 2.5% ammonium molybdate was added followed by 0.5 ml of 0.2% ascorbic acid. The solutions were well mixed and 10 minutes later, the blue colour was read colorimetrically at 625 nm. A blank containing10.0 ml of water was treated like the sample, except for digestion.

Standard Phosphorus: 1.0 ml of 0.001M KH_2PO_4 was put in a test tube and made up to 10 ml with water. The colour was then developed as in the test.

3.8.2 Individual Phospholipid Phosphorus Analysis

i) **Preparation of plates:** 50.0 g of sillica gel was added to 115.0 ml of 1mM Na_2CO_3 solution and mixed. The slurry was spread on thin layer chromatographic (TIC) plates at a thickness of 0.5 mm. The plates were allowed to dry at room temperature for one hour and heated in an oven for two hours at 110^oC and then stored.

ii) The plates were activated at 110^oC for one hour just before use.

iii) **Application of samples**: 0.5 ml of samples were applied to the thin-layer chromatographic plate with a capillary tube.

iv) **Chromatography**- Separation was effected with a Solvent system chloroform -methanol – ammonia (17:7:1 v/v/v). The development tank was lined with filter paper to improve equilibration and better resolution. The average running time was one hour. Individual phospholipid was identified by comparing the Rf values with that of a standard.

v) **Detection of spots**: The plates were dried at room temperature for 20 minutes. The spots were detected with iodine vapour and encircled with a fine dissecting needle. Most of the iodine was allowed to evaporate before removal of the spots.

vi) **Removal of spots**: The areas of sillica gel under the origin and above the solvent front were removed with a razor blade. Beginning with the origin of one running lane, the sillica gel area to be analysed was removed and transferred to a quick fit test tube. To ensure complete transfer of material, the remaining periphery of the spot was scrapped off the plate and the plate placed vertically and tapped to allow the scrapings to fall on the filter paper, which was combined with previously removed material.

3.8.3 Phosphorus Determination

Individual phospholipid phosphorus was determined by the method of Bartlett (1959). Each sample was digested with 0.5 ml of 70% perchloric acid as described previously (see total lipid phophorus analysis (3.8.2)). The phosphorus-containing solution was made up to 4. 1 ml with water and 0.5 ml of 10 N H_2S04, 0.2 ml of 2.5% ammonium molybdate, and 0.2 ml of the Fisle-Subbarow reagent were added in succession and mixed. The Solution was heated for seven minutes at 100^0C and the colour read at 830 nm. The blank contains 4. 1 ml of water and treated like the sample except for digestion. After color development, the sample was centrifuged to remove the silica gel.

Standard phosphorus: 0.5ml of 0.001M KH_2PO_4 was put in a test tube and treated as the blank.

Phosphorus was calculated using the following equation:

$$\frac{Optical\ density\ of\ test}{Optical\ density\ of\ standard} = \frac{Concentration\ of\ test}{Concentration\ of\ standard}$$

3.9 Determination of Heavy Metals in the Soil

The Bulk Scientific Atomic Absorption Spectrophotometer, AES, 2000 series, was used to measure the amounts of Cd, Zn, Pb, Mg, Fe, and Cu in the soil. The manufacturer's specifications were followed when configuring the device and setting its operating conditions. Analytical grade metal standard stock solutions (1 mg·dm−3) were used in triplicate to calibrate the device.

See appendix 1. for details of assay procedure

3.10 Assays of Enzymes of Energy Metabolism on the Head and Tail

3.10.1 Phosphofructokinase

The method of Bergmeyer (1974) was used for the determination. The activity of this enzyme was measured by a series of coupling reactions. The product of the enzyme was coupled to

aldolase, then triose-phosphate isomerase and finally the dihydroxy acetone phosphate (DHAP) produced was converted to glycerol phosphate by the action of glycero1 phosphate dehydrogenase. NADH oxidation in the process was measured and used to assign activity to this enzyme.

The assay mixture contained 0.90 ml of 100 mM Tris HCl buffer pH 8.0, 5 mM fructose-6-phosphabe, l.0 M ATP, 0.2 nW NADH $MgCl_2$, 50 mM KC1 and excess aldolase (2U), triosephosphate isomerase (10U), and alpha-glycerol--dehydrogenase (9U).

The reaction was started by the addition of the enzyme homogenate (0.0l ml).

3.10.2 Pyruvate Kinase

Pyruvate kinase was determined by the method of Bergmeyer (1974).

The pyruvate produced by this enzyme was acted upon by lactate dehydrogenase and the oxidation of NADH was used to assign activity to this enzyme.

The assay mixture contained 0.98 ml of l00 mM Tris-HCl buffer pH 7.5, 5 mM phosphoenol pyruvate, 5 mM ADP, 0.2 mM NADH, 10 mM, 100 mM $MgCl_2$ l00 mM KC1 and excess lactate dehydrogenase (14U). The reaction was started by the addition of the enzyme homogenate (0.01ml).

3.10.3 Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase was determined by the method of Storey and Bailey (1978). The NADPH produced by the action of the enzyme was measured and used to assign activity to the enzymes.

The assay mixture contained 0.98 ml of 100 mM Tris HC1, pH 7.4 1mM glucose 6 phosphate, 0. 4 mM $NADP^+$ and 5 mM $MgCl_2$. The reaction was started by the addition of 0.01ml enzyme homogenate.

3.10.4 Lactate Dehydrogenase.

This enzyme was assayed using the method of Bergmeyer (1974). The enzyme requires NADH. The enzyme activity is determined by the rate of oxidation of NADH (change in extinction at 340 nm).

The assay mixture contained 3.0ml of phosphate/pyruvate solution (50mM phosphate, pH 7.5: 0.63 mM pyruvate), and 0.05ml of 11.3mM NADH solution. 0.1ml of the enzyme extract was added to start the reaction.

3.10.5 Determination of Sugar

Reducing sugar released was measured by the Nelson-somogyi method (Nelson,1944: Somogyi,1952).

The sugar was heated with an alkaline solution of copper tartrate and cuprous oxide was produced, which reacts with arsenomolybdate to give molybdenum blue. The colour blue was measured in the colorimeter.

Materials

1. Reagent 1: Alkaline Copper tartrate solution, 25 g of anhydrous sodium carbonate, 2.5 g of potassium sodium tartrate, 2.5 g of sodium bicarbonate, 20 g of Na_2SO_4 were dissolved in about 80 ml of water before making it up to 1000 ml.
2. Reagent 2: Copper sulphate (3 g) was dissolved in 20ml distilled water and 4 drops of concentrated sulphuric acid was carefully added.
3. Reagent 3: Arsenomolybdate reagent: a. Ammonium molybdate (2.5 g) was dissloved in 40 ml of distilled water and followed by addition of 2.1 ml concentrated sulphuric acid.

   b. Sodium arsenate (0.3 g) was dissolved in 2.5 ml of distilled water and gradually and drop wisely added to solution (a), with constant stirring and finally diluted to 50 mls.

4. Reagent 4: Reagent 2 (1 ml) was mixed with 25 ml of reagent 1.

5. Standard glucose solution (100 ug/ml).

Procedure: The sample homogenate (1 ml) was added to 1ml of reagent 4, boiled for 20 minutes in a boiling water bath and cooled in a running tap. Reagent 3 (1 ml) was then added, shaken vigorously and allowed to stand for 10 minutes. Water (10 ml) was then added and the extinction of the blue color developed was read at 600 nm. Standard containing 20-100 ug were prepared by several dilutions of the standard stock. The blank contained 1ml of distilled water.

3.11 Estimation of Malondialdehyde

Principle:

Malondialdehyde (MDA) is a product of lipid peroxidation and when heated with thiobarbituric acid (TBA) under acid conditions, it forms a pink colored product which has a maximum absorbance at 532 nm.

Procedure:

The assay method of Hunter *et al.*, (1963), modified by Gutteridge and Wilkins (1980) was adopted. 1 ml of glacial acetic acid was added to the test tubes, followed by 1 ml of 1% TBA. 0.2 ml of the sample homogenate was added but the blank test tube contained 0.2 ml of distilled water. They were thoroughly mixed and incubated in a water bath at 95 ^{o}C for 15 min, then allowed to cool. Those with precipitates were centrifuged for 5 min and their supernatant was collected. The blank was used to zero the spectrophotometer and the absorbance of the test tubes were read at 532 nm.

Calculation

The MDA concentration of each sample was calculated using extinction co- efficient of $1.56x10^{5}M^{-1}$ in the formula:

$$\frac{OD \times V \times 1000}{a \times V \times I \times Y}$$

where, OD = absorbance of sample test at 532nm

a = molar extinction co-efficient of product

I = light path

V= volume of sample homogenate used

Y= mg of tissue in the sample used

3.12 Estimation of Superoxide Dismutase (SOD, EC. I.15.1.1)

This was determined by the method of Misra and Fridovich (1972).

Principle: The principle upon which this assay is based is as follows:

Adrenaline Auto: Oxidizes rapidly in aqueous solution to adrenochrome whose concentration can be determined spectrophotometrically at 420 nm. The auto – oxidation depends on the presence of superoxide anions (O_2). SOD inhibits this auto- oxidation by catalyzing the breakdown of superoxide anions. The degree of inhibition is thus an indication of the SOD activity. The amount of enzymes producing 50% inhibition is defined as one unit of the enzyme activity

Procedure: To the test tubes were added 2.5 ml of 0.05 M carbonate buffer of pH 10.2 and 0.2 ml of the sample homogenate. To the blank tube was added 0.2 ml of distilled water instead of the sample. 0.3 ml of 0.03 mM adrenaline solution was added to the test tubes to initiate the reaction.

To the reference tube was added 2.5 ml of carbonate buffer of pH 10.2, 0.2 ml of sample and 0.3 ml of distilled water. The increase in the absorbance at 480nm due to the adrenochrome formed was monitored every 30 seconds for 120 seconds.

Calculation

$$\% \text{ inhibition} = \frac{\text{OD test} - \text{OD reference}}{\text{OD test}} \times 100$$

However, 1 unit of SOD activity is taken as the amount of SOD required to cause 50% inhibition of the auto- oxidation of adrenaline to adrenochrom per minute.

Therefore, enzyme activity in units/ mg protein = % inhibition 50 x y

Where y = mg protein in the volume of sample.

3.13 Estimation of Catalase Activity (CAT, EC. 1.11.1.6)

Procedure:

The method of Cohen *et al.* (1970) was adopted. 0.5 ml aliquot of the homogenate samples were added into ice cold test tubes, while the blank tube contained 0.5 ml of distilled water. The reaction was initiated by the sequential addition at fixed intervals, 5 ml of cold 30 mM H_2O_2. It was mixed thoroughly by inversion and after 3 minutes, the reaction was stopped sequentially by the rapid addition of 1 ml of 6.0 M H_2SO_4 solution at fixed intervals. 7 ml of 0.01M $KMnO_4$ was added to the test samples and the blank test tubes. The absorbance was read at 480 nm after one minute interval.

Calculation

The activity of catalase in each sample was calculated by applying the formula:

$$\frac{\Delta 0D/\text{min} \times V \times 1000}{M \times v \times I \times y}$$

Where OD = absorbance of sample test at 480nm

V= total volume of the reaction mixture

M = molar extinction co- efficient of H_2O_2

l= light path

v = volume of sample homogenate used

y = mg of protein in tissue used

Expression of Result

The result is expressed in units/mg protein where 1 unit of enzymes activity is = 1 mole of H_2O_2 consumed per minute.

3.14 Estimation of Glutathione Peroxidase

Principle:

This was carried out by using Andy and Goodman method (1972), a modification of the Chance and Maehly method (1955).

$2\text{Pyrogallol} + 3\ H_2O_2 \longrightarrow 5H_2O + \text{Purpurogallin} + CO_2$

The appearance of Purpurogallin is measured at 420 nm using a spectrophotometer.

Procedure:

The reaction mixtures were prepared by adding 14.0 ml of distilled water, 2.0 ml of 5% pyrogallol solution, 1.0 ml of 0.147 M H_2O_2 solution and 2.0 ml of phosphate buffer (pH 7.0). The mixture was equilibrated a 20^0C for about 5 minutes. Next was the addition of 1.0 ml of the enzyme solution and mixed. This was followed by the addition of 2.0 N H_2SO_4 to stop the reaction after exactly 30 seconds and absorbance change was measured at 430 nm every 30 seconds for 2 minutes

Calculation

The peroxidase activity was calculated using extinction co-efficient of oxidase pyrogallol of $12M^{-1}$ cm $^{-1}$. The following formula was applied:

$$\frac{\Delta OD\ 430nM / 20\ secs \times Vt \times Df}{MxVxLxY}$$

Where OD = Absorbance of sample test at 430 nm

Vt= total volume of the reaction mixture

Df – dilution factor

M = molar extinction co- efficient of purpurogallin

L= light path

V = volume of sample homogenate used

Y= mg of protein in tissue used

Expression of result

The result is expressed in units/mg protein where I unit of enzyme activity is = 1 mole of pyrogallol oxidized per minute

3.15 Determination of Aldehyde Oxidase (AO)

Aldehyde oxidase activity assayed using the 'blank reversal technique' (Rajagopalan and Handler,1973). A Shimadzu spectrophotometer (UV 160 A) with matched 10 mm quartz cuvettes was used for the assay. The assay mixture contained 50 mM potassium phosphate buffer, pH 7.8, containing 0.005% EDTA, 300 µl of 0.01 M potassium ferricyanide solution, 50 µl of 0.3 M benzaldehyde and 50 µl of enzyme sample in final volume of 3 ml, in a 10 mm quartz cuvette. The blank cuvette consisted of all the components listed above except enzyme. The AO activity determination was initiated by addition of the enzyme and was monitored by analyzing the amount of potassium ferricyanide reduced at 420 nm at ambient temperature. The velocity of reaction was determined as µmol of potassium ferricyanide reduced per three ml per min using extinction coefficient (ε) of 2080 M^{-1} cm^{-1}. One unit of enzyme activity was defined as the

amount of enzyme that catalyzed the reduction of 1 µmol of potassium ferricyanide per min under the stated conditions (Krenitsky et al.,1972).

3.16 Inorganic Phosphate Determination

The supernatant (0.1 ml) was taken for inorganic phosphate (Pi) determination. A quantity, 1 ml of 2.5% ammonium molybdate was added followed by 1ml of 0.2% ascorbic acid. The colour developed was read immediately (within 1 minute) using the spectrophotometer at 625 nm.

The inorganic phosphate concentration of the sample homogenate was calculated using the equation below:

$$\frac{\text{Optical density of test}}{\text{Optical density of standard}} = \frac{\text{Concentration of test}}{\text{Concentration of standard}}$$

3.17 Protein Determination (Lowry's Method 1951)

Principle:

The principle behind he Lowry method of determining protein concentration lies in the reactivity of the peptide nitrogen(s) with the copper (II) ions under alkaline conditions and the subsequent reduction of the foline-ciocalteau phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper catalyzed oxidation of aromatic acids. Lowry's method is sensitive to pH changes and therefore the pH of the assay should be maintained at 10-10.5

The protein content was estimated on the supernatant.

The samples (0.1 ml) were added to the respective test tube, except the blank and made up to 4.5 ml with distilled water, 5 ml of alkaline reagent was added and the content of the tubes were mixed and allowed to stand for 10 minutes at room temperature. Folin-ciocalteau reagent (0.5 ml) was then added to the reaction mixtures and allowed to stand for 20 minutes at room temperature

for the colour to develop. The absorbance was read at 750 nm against reagent black without protein.

Concentration of protein was calculated using the formula:

$$\frac{\text{Concentration of test}}{\text{Concentration of standard}} = \frac{\text{absorbance of test}}{\text{absorbance of standard}}$$

3.18 Statistical Analysis

The data were presented as mean ± standard error of mean (SEM). Data were analyzed using one-way analysis of variance (ANOVA) and differences between means were determined using the least significant difference on Statistical Packages for Service Solutions (SPSS) version 20. The significance level was set at $p < 0.05$.

CHAPTER FOUR: RESULTS

4.1 Heavy Metal Composition of Soil Samples Collected from Evbarake and Uwelu Sites.

The data on some metal content of the test site (Uwelu and Evbarake) and Control site (Capitol, University of Benin) is presented in Table 4.1. The results showed that the lead, nickel, zinc, manganese, iron, copper, and cadmium contents in soil in the test groups were higher than those of the control site. Comparable levels of metals were observed in the test Sites. The data show that the test sites chosen for this study had high levels of lead, nickel, zinc, Magnesium, copper and cadmium.

4.2 Weight Changes of Tadpoles Exposed to effluents from the Test Sites for two weeks

Table 4.2 shows the weight changes in the head and tail regions of tadpoles exposed to effluents from automobile workshop. The statistical analysis of the data revealed that there was no significant difference in the initial weight of the tadpoles across groups when compared to the normal control ($p > 0.05$). However, there was a significant decrease in the final weight of tadpoles in Uwelu site when compared to the normal control ($p < 0.05$). Furthermore, the final weight of the head region in tadpoles from the Uwelu site showed a significant decrease when compared to those from the control site ($p < 0.05$). The drop in the final weight of the tadpoles observed did not reflect in weight changes in the tail region.

Table 4. 1: Heavy Metal composition of soil samples collected from the Evbarake and Uwelu Sites.

	Lead (ppm)	Nickel (ppm)	Zinc (ppm)	Manganese (ppm)	Iron (ppm)	Copper (ppm)	Cadmium (ppm)
Control	ND	ND	2.52	12.10	6.03	ND	ND
Evbarake Site	10.00	14.00	231.00	72.00	27.59	0.04	8.0
Uwelu Site	60.00	27.00	211.00	70.00	27.82	0.02	10.0

DT = Detection Limit for Lead = 0.08ppm; Nickel = 0.05ppm; Zinc = 0.005ppm; Manganese = 0.03ppm; iron = 0.05ppm; Copper = 0.005ppm; Cadmium = 0.01ppm.
*Accepted limit for consumption in biological systems (W.H.O) for Lead = 0.29ppm; Zinc = 5.00ppm; Copper = 0.50ppm; Nickel = 0.001ppm.

Table 4.2: Weight changes of tadpoles exposed to effluents from the test sites for 2 weeks.

Parameters	Control	Evbarake Site	Uwelu Site
Initial Weight	0.73 ± 0.11^{a}	0.75 ± 0.21^{a}	0.73 ± 0.20^{a}
Final Weight	2.32 ± 0.31^{a}	1.79 ± 0.41^{ab}	1.43 ± 0.43^{b}
Final Weight of the Head region	2.03 ± 0.33^{a}	1.31 ± 0.12^{ab}	1.01 ± 0.41^{b}
Final weight of the Tail region	0.61 ± 0.11^{a}	0.47 ± 0.14^{a}	0.42 ± 0.11^{a}

Values are means ± S.E.M, n=20. The weights of the animals are in grams. Means on the same row followed by different letters differ significantly ($P < 0.05$). Values represented with the same superscript are non-significantly different while those with different superscript differ significantly ($p < 0.05$).

4.3 Malondialdehyde, superoxide dismutase, catalase, glutathione peroxidase and aldehyde oxidase activities in the head region of tadpoles exposed to effluents from the test sites for two weeks

The data on malondialdehyde, superoxide dismutase, catalase, glutathione peroxidase and aldehyde oxidase activities in the head region of tadpoles grown from soils in the test sites is presented in Table 4.3. The statistical analysis of the data reveals that MDA level was significantly ($p < 0.05$) increased in the animals in the test sites when compared with the control. Also, tadpoles in Uwelu had the highest concentration of MDA and is significantly increased when compared to Evbarake ($p < 0.05$).

The activity of superoxide dismutase (SOD) in the test sites shown in Table 4.3 was significantly increased when compared to the control ($p < 0.05$).

The levels of catalase (CAT) in Evbarake was significantly increased when compared to the control ($p < 0.05$). However, there was a significant decrease in Uwelu site when compared to the control and Evbarake ($p < 0.05$).

The activity of glutathione peroxidase (GPx) in the test sites showed a significant increase when compared to the control ($p < 0.05$). The level of aldehyde oxidase in the test site was also significantly increased when compared to the control ($p < 0.05$)

4.4 Malondialdehyde, superoxide dismutase, catalase, glutathione peroxidase activities in the tail region of tadpoles exposed to effluents from the Test Sites for two weeks

From Table 4.4, the levels of MDA of tadpoles exposed to the effluents in Evbarake and Uwelu sites, showed a significant increase when compared to the normal control ($p < 0.05$). Also from the test sites, tadpoles in Uwelu had the highest level of MDA and is significantly increased when compared to Evbarake ($p < 0.05$).

The activity of superoxide dismutase (SOD) in the test sites as shown in the Table was significantly increased when compared to the normal control ($p < 0.05$).

The level of catalase (CAT) in Evbarake was significantly increased when compared to the normal control ($p < 0.05$). However, there was a significant decrease in Uwelu when compared to normal control and Evbarake ($p < 0.05$).

The activity of glutathione peroxidase (GPx) in the test sites showed a significant increase when compared to the control ($p < 0.05$). Also, the activity of aldehyde oxidase in the test site was significantly increased when compared to the control ($p < 0.05$).

Table 4.3: Levels of malondialdehyde (lipid peroxidation), superoxide dismutase, catalase, glutathione peroxidase activities and Aldehyde Oxidase in the Head region of tadpoles exposed to effluents for 2 weeks.

Parameters	Control	Evbarake Site	Uwelu Site
MDA	5.69 ± 0.55^{a}	11.42 ± 1.08^{b}	14.46 ± 1.33^{c}
SOD	3.26 ± 0.28^{a}	4.24 ± 0.13^{b}	4.52 ± 0.34^{b}
CAT	0.61 ± 0.05^{a}	0.94 ± 0.03^{b}	0.13 ± 0.07^{a}
GPx	24.13 ± 2.36^{a}	39.41 ± 3.53^{b}	39.11 ± 3.71^{b}
Aldehyde Oxidase	3.16 ± 0.52^{a}	5.16 ± 0.62^{b}	5.61 ± 0.74^{b}

Values expressed as mean ± SEM, (n = 20), Malondialdehyde (MDA) units are in mmole MDA g-1 tissue, superoxide dismutase (SOD) activity is in units g-1 tissue, catalase activity is in moles of H_2O_2 consumed/min, glutathione peroxidase (GPX) activity is in mmol/min/mg wet weight. Means of the same row followed by different letter(s) differ significantly ($p < 0.05$).

Table 4.4: levels of malondialdehyde, superoxide dismutase, catalase, glutathione peroxidase activities in the tail region of tadpoles exposed to effluents for 2 weeks.

Parameters	Control	Evbarake Site	Uwelu Site
MDA	6.00 ± 0.65^{a}	16.34 ± 1.02^{b}	19.27 ± 1.14^{c}
SOD	2.43 ± 0.27^{a}	3.09 ± 0.16^{b}	3.59 ± 0.35^{b}
CAT	0.47 ± 0.04^{a}	0.91 ± 0.02^{b}	0.06 ± 0.08^{a}
GPx	21.84 ± 2.60^{a}	36.81 ± 3.36^{b}	39.73 ± 2.87^{b}
Aldehyde Oxidase	2.11 ± 0.32^{a}	4.71 ± 0.42^{b}	4.04 ± 0.84^{b}

Values expressed as mean ± SEM, (n = 20), Malondialdehyde (MDA) units are in mmole MDA g-1 tissue, superoxide dismutase (SOD) activity is in units g-1 tissue, catalase activity is in moles of H_2O_2 consumed/min, glutathione peroxidase (GPX) activity is in mmol/min/mg wet weight. Means of the same row followed by different letter(s) differ significantly ($p<0.05$)

4.5 Lipid content of sections of tadpoles exposed to effluents for two weeks

Table 4.5 shows the total lipids and total phospholipids levels of the head and tail regions of tadpoles exposed to effluents. There was a significant decrease in total lipids in the test sites when compared to the normal control ($p< 0.05$). The total lipid from the head region of tadpoles showed a significant decrease in Uwelu site when compared to the normal control ($p < 0.05$).

Total lipids from the tail region of tadpoles showed no significant difference across groups exposed when compared to the normal control ($p< 0.05$). Total phospholipids in Uwelu site showed a significant decrease when compared to Evbarake site and normal control ($p < 0.05$). Statistical evaluation did not show any significant difference in the total phospholipid levels of all the head and tail regions of all the experimental groups.

Table 4. 5: Total lipids content of sections of tadpoles exposed to effluents

Parameters	Control	Evbarake Site	Uwelu Site
Total lipids	53.41 ± 1.86[a]	43.74 ± 4.56[b]	40.74 ± 3.76[b]
Total lipids of the Head region	32.42 ± 4.91[a]	27.12 ± 6.21 [ab]	22.52 ± 6.92[b]
Total lipids of the Tail region	22.22 ± 6.9[a]	18.22 ± 6.1[a]	18.22 ± 5.9[a]
Total phospholipids	25.30 ± 2.42[a]	20.42 ± 3.44[a]	18.40 ± 2.46[b]
Total phospholipids of the Head region	12.20 ± 3.12[a]	10.0 ± 3.42[a]	9.59 ± 3.52[a]
Total phospholipids of the Tail region	10.25 ± 3.02[a]	9.51 ± 3.51[a]	9.09 ± 3.15[a]

Values are Means ± S.E.M, n=20. Values are multiplied by 10^2. Means of the same row followed by different letters differ significantly ($P<0.05$).

4.6 The phospholipid profile of sections of tadpoles exposed to effluents from automobile workshop for two weeks.

The distribution of phospholipids among the phospholipid classes in the head and tail regions of tadpoles exposed to spent oil run off water is shown in table 4.6.

Phosphatidylethanolamine (PE) in test sites showed a significant decrease when compared to the normal control ($p < 0.05$). Phosphatidylcholine (PC) also showed a significant decrease in test sites when compared to the normal control ($P < 0.05$). Sphingomyelin (SGM) levels in test sites showed a significant increase when compared to the normal control ($p < 0.05$). The levels of Phoshatidylinositol (PI) and phosphatidylserine (PS) were significantly decreased in test sites when compared to the normal control ($p < 0.05$). There was no significant difference in test sites when comparing ratios of Phosphatidylethanolamine (PE) / Phoshatidyl choline (PC) levels compared to the normal control ($p < 0.05$). Also, there was no significant difference in sphingomyelin (SGM) / Phosphatidylcholine (PC) ratios across exposed groups when compared to the normal control ($p < 0.05$).

Phosphatidylethanolamine (PE) in tail region in the test sites showed a significant decrease when compared to the normal control ($p< 0.05$). Phosphatidylcholine (PC) also showed a significant decrease in test sites when compared to the normal control ($P < 0.05$). Sphingomyelin (SGM) levels in test sites showed a significant increase when compared to the normal control ($p < 0.05$). The levels of Phoshatidylinositol (PI) and phosphatidylserine (PS) were significantly decreased in test sites when compared to the normal control ($p < 0.05$).

There was no significant difference across sites in Phoshatidylethanolamine (PE)/Phoshatidylcholine (PC) ratios when compared to the normal control ($p < 0.05$). There was

no statistical difference between the sphingomyelin (SGM) / Phosphatidylcholine (PC) ratios of the experimental sites.

Table 4.6: The phospholipids profile of sections of tadpoles exposed to effluents for 2 weeks.

Experimental Group			
	Control	**Evbarake Site**	**Uwelu Site**
Parameter			
Head region			
PE	39.15 ± 6.45^{a}	28.45 ± 5.88^{b}	25.36 ± 6.21^{b}
PC	58.24 ± 5.38^{a}	41.55 ± 3.5^{b}	42.33 ± 4.11^{b}
SGM	1.87 ± 0.33^{a}	3.15 ± 0.44^{b}	3.74 ± 0.54^{b}
PI+PS	5.12 ± 0.55^{a}	3.27 ± 0.44^{b}	2.71 ± 0.47^{b}
PE/PC	0.67	0.68	0.60
SGM/PC	0.03	0.07	0.09
Tail region			
PE	34.42 ± 5.25^{a}	24.18 ± 3.55^{b}	23.16 ± 4.42^{b}
PC	47.26 ± 6.31^{a}	34.55 ± 3.50^{b}	33.41 ± 4.55^{b}
SGM	2.46 ± 0.33^{a}	3.82 ± 0.41^{b}	3.11 ± 0.31^{b}
PI+PS	5.48 ± 0.34^{a}	4.32 ± 0.21^{b}	3.90 ± 0.77^{b}
PE/PC	0.72	0.70	0.69
SGM/PC	0.05	0.11	0.09

Values are Means ± S.E.M, n=20. Values are multiplied by 10^2. Means of the same row followed by different letters differ significantly ($P<0.05$). PE = phosphatidylethanolamine, PC = phosphatidylcholine, SGM = Sphingomyelin, PI = phosphatidylinositol, PS = phosphatidylserine.

4.7 Concentration of glucose and the activities of some enzymes of engery metabolism in tissues of tadpoles exposed to effluents from automobile workshops for two weeks

Table 4.7 shows a profile of energy (glucose) and enzymes of energy metabolism in the head and tail regions of tadpoles exposed to effluents.

The levels of glucose in the test sites showed a significant increase when compared to the normal control ($p < 0.05$). There was no statistical difference ($p > 0.05$) in the activity of hexokinase between the test groups. The levels of this enzyme in the test sites were however significantly higher than the observed level in the control ($p < 0.05$). Statistical evaluation did not show significant difference ($p > 0.05$) in the activities of pyruvate kinase, lactate dehydrogenase, and glucose-6-phosphate in all the experimental sites

In the tail region, the levels of glucose in test sites showed a significant increase when compared to the normal control as shown in Table 4.7 ($p < 0.05$). Also, there was a significant increase in hexokinase levels in test sites when compared to the control ($p < 0.05$). Pyruvate kinase levels in test sites showed a significant increase when compared to the control ($p < 0.05$). For Lactate dehydrogenase there was a significant increase in test sites when compared to control ($p< 0.05$). Furthermore, glucose-6-phosphate dehydrogenase across test sites showed a significant increase when compared to normal control ($p < 0.05$).

Table 4.7: Activity of energy and some enzymes of energy metabolism in tissues of tadpoles exposed to effluents for 2 weeks

Parameters	Control	Evbarake Site	Uwelu Site
Head region			
Glucose	2.36 ± 0.97[a]	5.17 ± 1.56 [b]	5.63 ± 0.04[b]
Hexokinase	11.49 ± 1.47[a]	18.73 ± 3.76 [b]	16.40 ± 5.46 [b]
Pyruvate kinase	4.58 ± 0.59 [a]	3.33 ± 0.54 [b]	4.43 ± 0.46 [c]
LDH	1.09 ± 0.08 [a]	24.61 ± 5.69 [b]	32.73 ± 6.31 [b]
G6P-DH	17.54 ± 0.79 [a]	25.93 ± 4.09 [b]	28.04 ± 2.34 [b]
Tail region			
Glucose	1.40 ± 0.01 [a]	1.31 ± 0.06 [b]	1.33 ± 0.11 [b]
Hexokinase	4.80 ± 0.28 [a]	7.53 ± 0.64 [b]	9.33 ± 1.44 [b]
Pyruvate kinase	3.98 ± 0.48 [a]	4.84 ± 0.93 [b]	4.38 ± 1.73 [b]
LDH	23.34 ± 0.98 [a]	44.79 ± 3.81[b]	45.34 ± 2.45 [b]
G6P-DH	15.49 ± 0.79 [a]	34.80 ± 3.31[b]	33.23 ± 1.98 [b]

Values are Means ± S.E.M, n=20. Means of the same row followed by different letters differ significantly (P<0.05). Values represented with the same superscript are non-significantly different while those with different superscript differ significantly ($p < 0.05$).

4.8 Triacylglycerols and cholesterol levels of tadpoles exposed to effluents from automobile workshops

Table 4.8 shows the triacylglycerols and cholesterol in the head region of tadpoles exposed to spent oil run off water in various test sites.

The level of total cholesterol of tadpoles exposed to spent oil run off water in test sites, was significantly higher ($p < 0.05$) than the observed level in the normal control. Also, there was a significant decrease in total cholesterol levels in Evbarake relative to Uwelu as shown in the Table 4.8 ($p < 0.05$). HDL Cholesterol concentration was shown to be significantly increased across groups exposed when compared to the normal control ($p < 0.05$). LDL Cholesterol concentration was also shown to be significantly increased in test sites when compared to the normal control ($p < 0.05$). The Triacylglycerols level in the test groups was significantly higher ($p < 0.05$) than the levels in the control group. There was no significant difference in the value of HDL-Cholesterol in the tail region of tadpoles in the test groups, but the value in the test groups was significantly higher ($p < 0.05$) than the observed levels in the control site. LDL- Cholesterol concentration was shown to be significantly increased in test sites when compared to the normal control ($p < 0.05$). Triacylglycerols concentration across exposed groups was shown to be significantly increased when compared to normal control ($p < 0.05$). However, there was a significant decrease in triacylglycerol levels in Evbarake relative to Uwelu site ($p < 0.05$).

Table 4.8: Triacylglycerols and Cholesterol profile of tadpoles exposed to effluents.

Group/Assay	Control	Evbarake Site	Uwelu Site
Head region			
Total Cholesterol	182.72 ± 12.43[a]	386.30 ± 23.47[b]	3427.2 ± 34.33[c]
HDL-Cholesterol	45.55 ± 9.9[a]	67.83 ± 10.66[b]	61.34 ± 10.49[b]
LDL-Cholesterol	120.76 ± 13.3[a]	211.80 ± 14.6[b]	237.78± 14.10[b]
Triacylglycerol	217.28 ± 11.4[a]	433.33 ± 29.62[b]	445.64± 23.04[b]
Tail region			
Total Cholesterol	189.0 ± 13.1[a]	299.56 ± 22.01[b]	295.12± 24.42[b]
HDL-Cholesterol	17.2 ± 3.9[a]	42.23 ± 11.66[b]	43.52 ± 8.29[b]
LDL-Cholesterol	157.7 ± 13.5[a]	251.33 ± 10.56[b]	257.08± 12.53[b]
Triacylglycerol	250.8 ± 12.9[a]	450.00 ± 20.1[b]	497.42 ± 20.6[c]

Results are expressed as means ± SEM of determinations from the total samples. The lipids are expressed as µg/g tissue. Means of the same row followed by different letters differ significantly ($P<0.05$).

CHAPTER FIVE: DISCUSSION

The build-up of heavy metals and other toxic materials in agricultural soils and other water bodies is of increasing concern due to food safety problems and potential health risk as well as its detrimental effect on soil ecosystem (Erifeta *et al.,*2017). Studies have shown that the integrity of the cellular membranes of soil dwelling organisms are compromised when they are exposed to high content of toxicants (PAHS and nonessential trace elements like cadmium) present in crude oil (Jajer *et al*,2003; Erifeta *et al*,2017). High levels of lead, nickel, zinc, manganese, iron, copper, and cadmium detected in the test sites could be as a result of the vehicle maintainance and welding activities taking place in the study sites. A recent study by Liang *et al.* (2019) found similar elevated levels of these metals in soils near oil fields, associating them with potential ecological and health risks.

The concentration of heavy metals in these sites were all above the Desirable Contaminant Concentrations DCC and Acceptable Contaminant Concentration (ACC) thresholds as published by the National Water Quality Management Strategy (NWQMS) (2000). The elevated levels of nickel and zinc are consistent with findings by Ite *et al.* (2018), who reported high concentrations of these metals in soils from oil-polluted sites in Nigeria. These metals can accumulate in plants and enter the food chain, potentially affecting ecosystem health. The pattern of multi-metal contamination observed here is consistent with a comprehensive review by Chukwuk *et al.* (2023), which highlighted the complex nature of metal pollution in oil-impacted environments and its potential for long-term ecological effects.

Recent work by Akpor *et al*. (2022) demonstrated that such metal contamination can lead to reduced biodiversity and altered ecosystem functioning in oil-polluted areas. Given that these sites are near human habitation, the high levels of toxic metals like lead and cadmium raise

serious health concerns. A study by Ite *et al*. (2021) linked similar levels of metal contamination in oil-polluted areas to various health issues in local populations. Cadmium stands out as a potent toxicant that has been linked to alterations in cellular homeostasis and has been shown to enhance the production of several activated oxygen species designated ROS McLaughlin *et al.,* (2000).

Changes in body weight have often been used as an index of toxicity of chemicals (Timbrel, 1991). The weight loss in the tadpole induced by the exposure to the spent oil run off water (Table 4.2) is therefore not suprising and it corroborates the findings of Snodgrass et al., (2005) which also reported that tadpoles exposed to mine sediments of coal combustion waste suffered sub lethal effects in the form of reduced growth. As this study observed that the Evabarake Site has less effect on weight compared with the Uwelu site, it is conceivable that the Uwelu site may be more contaminated than the Evbareke site. This reduced growth in tadpoles exposed to spent oil runoff is consistent with recent studies on the effects of pollutants on amphibian development. For example, a study by Jones *et al*. (2021) found that exposure to petroleum hydrocarbons resulted in reduced growth rates and developmental abnormalities in wood frog tadpoles. This agrees with earlier studies which show that the degree of the exposure influences the sensitivity of amphibians to environmental pollutants (Snodgrass et al., 2005). Studies by Boone et al. (2005) reported that the combination of carbaryl and nitrate (interactive effect of multiple contaminants) had a negative effect on development and mass of tadpoles compared to the positive effect that either contaminant had alone. As the sites are exposed to multiple chemicals, due to the oil from car servicing and also spent carbide from welding activities which are potential contaminants, the observation of the effect of chemical effluents on weights of tadpoles may be the result of the interactive effects of these possible multiple contaminants. The more pronounced effect on head

weight could indicate potential neurotoxic effects of the oil contaminants. Most of the developmental activities occurred in the Head region of tadpoles and stands to reason that it is this region that is mostly affected compared with the tail region. This is supported by recent research by Regnault *et al*. (2020), who found that exposure to petroleum-related compounds led to neurodevelopmental abnormalities in Xenopus embryos, including reduced head size and altered brain morphology. The study reveals that weights of tadpoles are affected by environmental pollutant.

A product of autooxidation is malondialdehyde (MDA) and as a defense against reactive free radicals, the body produces antioxidant enzymes like, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and a metabolic enzyme like aldehyde oxidase. The study observed increased production of MDA in the tadpoles with differences in site exposure (Uwelu and Evbarake), indicating the increased chances of membrane lipid peroxidation and thus tissue damage. This increase in MDA indicates that exposure to spent oil runoff induces oxidative stress and lipid peroxidation in both the head and tail regions of tadpoles. Similar results have been observed in other studies. For instance, Oruç and Usta (2007) reported increased MDA levels in the liver, kidney, and gill tissues of fish exposed to 2,4-dichlorophenoxyacetic acid. SOD activity was significantly higher in tadpoles from both contaminated sites compared to the control. The observed increase in SOD and GPx activities of the head and tail regions after 2 weeks of exposure to effluent (Tables 4.3 and 4.4) may be an improved attempt by the tadpoles to mop up MDA produced and may be an adaptive feature. The interesting observation of a higher SOD and GPx activities in the head region compared with the tail region after two weeks exposure (comparing Tables 4.3 and 4.4) indicate that the adaptive mechanism is elaborated in the head region than the tail. It would be expected that majority of the metabolic processes for

the survival of the tadpole would occur in the head region and thus would not be easily compromised as the tail region which is mostly adapted to movement of the animal. A decrease in SOD activity in the tail region of the tadpoles exposed to the test sites (Table 4.4) would diminish the ability of this region to scavenge free radicals, which indicate that the tail will be more susceptible to damage by high MDA produced by the contaminants in the test sites. This increase in SOD activity suggest an adaptive response to increased oxidative stress. The body produces more SOD to combat the elevated levels of reactive oxygen species (ROS) generated due to oil exposure. These findings also align with a study by Achuba and Osakwe (2003), which reported increased SOD activity in fish exposed to petroleum hydrocarbons. SOD is a metalloenzyme requiring zinc for its structural stability and copper for its enzymatic activity (Gotz et al., 1994). Both sites contain cadmium and it has been reported that cadmium can induce inhibition of this enzyme by binding to and displacing these metals from the active site of the enzymes (Gotz et al., 1994). The decrease in the activity of CAT in both sites may in part be the result of the effect of cadmium in the environment. This study reports an increase in the activity of GPx after 2 weeks exposure of the tadpoles to the environment, with the tail region (Table4.4) displaying relatively lower activity compared with the head region (Table 4.3). This also may imply an attempt by the animals to not only scavenge free radicals but also improve the rate of detoxifying some of the toxic materials in the environment. This would have serious implication for the tadpoles as it would affect the biotransformation capability of hydrocarbons that could likely be present in the environment (Eriyamremu *et al.*, 2007). The result of this effect would further affect the propensity for tissue damage and would have contributed immensely to the observed weight losses in these tadpoles. Such compromise in the health of animals have also been demonstrated in the antarctic rock cod (Trematomus bernacchii) which

was exposed to model chemicals, including polycyclic aromatic hydrocarbons (benzo[a]pyr e-p-dioxin [TCDD]), cadmium, and a combination of cadmium and TCDD (Regoli et al. ,2005).

CAT activity showed different patterns between the two contaminated sites. The decrease in CAT activity at the Uwelu site might indicate enzyme inhibition or depletion due to severe oxidative stress. Similar variable responses in CAT activity have been reported by Farombi et al. (2007) in fish exposed to petroleum contamination. GPx activity was significantly increased in tadpoles from both contaminated sites compared to the control. This increase in GPx activity, like SOD, suggests an adaptive response to increased oxidative stress. These results are consistent with findings by Livingstone *et al*. (1992), who reported increased GPx activity in aquatic organisms exposed to organic pollutants. Aldehyde oxidase activity was significantly higher in tadpoles from both contaminated sites compared to the control. This increase suggests an up-regulation of xenobiotic metabolism pathways in response to oil exposure.

This study is consistent with the work of Eriyamremu *et al.*, (2007) that reported that some of the metabolic processes that may be compromised are those of free radical scavenging and detoxification processes and as earlier mentioned, the environmental effect was lesser in the head region compared with the tail region. In conclusion, the exposure to the test sites resulted in increase in MDA production, and the activities of SOD and GPx in the tadpoles increased in an attempt to handle the toxic chemicals in these Sites.

Biological membranes function in a lot of biochemical processes and are critically dependent on the fluidity of the membranes. As phospholipid composition plays a major role in the determination of membrane fluidity, they therefore affect many biochemical processes that affect the survival of the organism. The reduction in total lipids suggests that exposure to oil contaminants affects the overall lipid metabolism in tadpoles. This finding is consistent with a

study by Melvin *et al*. (2013), who found that exposure to petroleum hydrocarbons led to alterations in lipid metabolism in fish, including reduced total lipid content. The significant reduction in the total lipid as well as the total phospholipid contents of both the head and the tail region of the tadpoles exposed to spent oil runoff water as observed in this study (Table 4.5) is in agreement with an earlier finding. Bindesbol and his colleagues reported (2009) a significant decrease in the phospholipid content of Dendrobaena octaedra following exposure to copper. This reduction in phospholipids is particularly concerning as phospholipids are crucial components of cell membranes. A study by Sørensen *et al*. (2017) found similar reductions in phospholipid content in fish exposed to crude oil, which was associated with impaired membrane function and cellular stress.

Changes in individual phospholipids can affect membrane fluidity and transmembrane transport (Erifeta *et al.*, 2017). The real time analysis of individual phospholipids is important in determining the role of the phospholipids on this important membrane character and its contribution to metabolic processes. The lack of appropriate techniques for real time analysis makes the results obtained eventually subjective. However, within the confines of this drawback, we observed changes in the individual phospholipids following the environmental exposure (Tables 4.6). It can therefore be speculated that increase in phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SGM), observed in tadpoles exposed to spent oil runoff water may affect the animal's membrane character and eventually their metabolic processes. A change in the fluidity of the membrane has been associated with aging and other pathological conditions (Erifeta *et al*., 2017). The lytic activity is strongly dependent on the presence of SGM in the receptors of the target membranes (Lange *et al.*,1997). The tadpoles are able to suppress the production of SGM as an innate defence strategy to prevent

self-killing. The observed increase in the content of sphingomyelin, as shown in Tables 4.6, may be one of the probable mechanisms of toxicity of the sites. This research data is suggestive that these toxicants are able to compromise the tadpole's ability to suppress SGM synthesis. If that is the probable effect, then the elevated ratio of SGM to PC could be due to an increase in the synthesis of SGM from PC. In both head and tail regions, PE levels were significantly lower in tadpoles exposed to spent oil runoff from both sites compared to the control. This reduction in PE could affect membrane fluidity and cellular signaling processes. This finding is consistent with a study by Sørhus *et al*. (2016), who found that exposure to crude oil led to alterations in phospholipid composition, including reduced PE levels, in fish embryos. Similar to PE, PC levels were significantly lower in both head and tail regions of exposed tadpoles compared to the control. PC is a major component of cellular membranes, and its reduction could impact membrane integrity and function. Bui *et al.* (2017) reported similar decreases in PC levels in marine copepods exposed to oil. SGM levels increased significantly in both head and tail regions of exposed tadpoles compared to the control. This increase in SGM could be a compensatory response to the decrease in other phospholipids or a direct effect of the contaminants on sphingolipid metabolism. PI+PS levels decreased significantly in both head and tail regions of exposed tadpoles. These phospholipids play crucial roles in cell signaling and apoptosis, and their reduction could have significant implications for cellular function and development. The PE/PC ratio remained relatively stable across all groups in both head and tail regions, suggesting that while absolute levels of these phospholipids decreased, their relative proportions were maintained. The SGM/PC ratio increased in exposed tadpoles compared to the control, particularly in the tail region. This shift in phospholipid composition could affect membrane properties and cellular functions.

The glucose levels in the test tadpoles exposed to spent oil runoff water increased significantly (at $p < 0.05$) compared to the control. The elevations in sample glucose level, when compared to the control reported in this study, corroborated the report by Zhelev *et al*. (2021), who observed similar metabolic changes in frogs exposed to pollutants. This development is likely due to environmental stress from the toxicants resulting in a hyperglycaemic condition in the blood plasma causing disruption in the ability of the intestines to absorb soluble GLU (Ezike and Ufodike, 2008). The sample GLU level in the tadpoles collected from test sites was significantly ($p < 0.05$) greater than the control. The high level of GLU observed in test sites when related to the control suggests that during the period of contact with the environment, tadpoles were facing high stress. Stressful conditions caused by environmental toxicants may trigger the secretion of stress hormones and neurotransmitters such as cortisol and catecholamines, which result in hyperglycaemia (Teles et al., 2004). This finding is also similar to what was reported by Islam et al. (2020) wherein a common heavy metal in crude oil caused a significant increase in blood GLU in striped catfish and numerous erythrocytic abnormalities. Other studies have shown that oil pollution results in an elevated degradation of stored glycogen to GLU and its movement to other body tissues to take care of the energy problems and requirements of the fish (Pickering, 1993; Kanungo et al., 2018). Hexokinase and pyruvate kinase activities in the head and tail regions increased in both sites. The observed hexokinase activity may suggest increased energy production. Marchand *et al*. (2019) reported similar tissue-specific responses in fish exposed to oil pollution. Lactate dehydrogenase (LDH) activities in the head and tail region of tadpoles were observed to be higher when compared to the control. This is consistent with findings by Pasparakis *et al*. (2019), who observed increased anaerobic metabolism in fish embryos exposed to crude oil. The increase in LDH observed in this study may also be a reflection of a rise in

anaerobic metabolism, which is in agreement with the earlier study of Val and Almeid-Val (1999), and Adeyemo et al. (2022) on catfish. The rise in the activity of LDH is due to an anaerobic breakdown of GLU leading to the build-up of lactate. As the lactate accumulates, it undergoes rapid ionization leading to muscle acidification which eventually limits the period of energetic activities. The reaction that promotes lactate formation also produces NAD+ in the cytosol that may enter relevant pathways to control the redox potentials and stimulate substrate flux in glycolysis leading to ATP formation (Nelson and Cox, 2005). This, in effect, provides more energy. An observed rise in the mean LDH activity in the test groups compared to the control may be due to a rise in energy required to carry out various metabolic processes (Val and Almeid-Val, 1999). These findings suggest a high amount of lactic acid is produced as an alternate source of energy due to the oxygen depletion imposed on the environment by the environmental contaminants. This rise in the activity of LDH implies the likelihood of deviation in carbohydrate metabolic processes away from the aerobic oxidation, Kreb's cycle to favour the anaerobic segment, glycolysis (Skidmore, 1970). An earlier study revealed that the exposure of animals to crude oil produces reactive oxygen species and other free radicals which cause oxidative stress in biological systems (Khan et al., 2003). The observed rise in G-6-PDH activities after the experimental period when compared to the control may be an improved effort by the tadpoles to fight against free radicals formed and may also be an adaptation mechanism (Eriyamremu et al., 2007). Decreased G-6-PDH activity diminishes the ability of tadpoles to fight against free radicals, indicating a high susceptibility to damage by free radicals. The results for the test sites also revealed a rise in G-6-PDH activity when compared to the control showing that the tadpoles were also subjected to stressful environmental conditions resulting in the rise in the enzyme activity as a readiness to scavenge free radicals produced by the toxicant-induced

stress. Similar findings were reported by Velez-Zuazo *et al*. (2020) in marine organisms exposed to oil pollution. The alterations in enzyme activities and glucose levels suggest a complex response involving increased glucose utilization, a shift towards anaerobic metabolism, and potential oxidative stress.

Triacylglycerols and total cholesterol (TCHO) are considered as the important biomarkers for indicating any lipid metabolism disorders. TAGs constitute a prominent class of neutral lipids that supply most of the energy ingested by embryo throughout growth and development. Meanwhile, cholesterol, the most common tetracyclic hydrocarbon compound, exists as a crucial component of cell membranes (Simons and Ikonen, 1997) and provides protection of membrane lipids (Zhang et al., 2018). It is also essential for transport and signal transduction between cells (Simons and Toomre, 2000; Mu et al., 2015;) or it can exist in a neutral lipid storage form esterified to a fatty acid (Kojima et al., 2009). The experiment showed an appreciable increase in TCHO, TAGs and total lipid content in the test sites. Simons (1997) and earlier showed that difenoconazole exposure in marine medaka resulted in increased content of total lipids (Dong et al., 2016) and subsequently increased TAG level in embryonic zebra fish after 96 h (Hu et al., 2016). Similarly, environmental toxicants from the test sites might be inhibiting the uptake of LDL cholesterol and hence the increased level of LDL cholesterol in exposed tadpoles. However, HDL plays protective role against cardiovascular diseases and a decrease in its level could be associated with increased risk of diseases as observed in the study. Tadpoles from the Uwelu site had significantly higher total cholesterol levels in the head region compared to the control group and the Evbarake site. Similar trends were observed in the tail region. HDL-cholesterol levels were significantly higher in the head region of tadpoles from the Evbarake and Uwelu sites compared to the control group. The tail region showed a similar pattern. LDL-cholesterol levels

were significantly elevated in the head and tail regions of tadpoles from the Evbarake and Uwelu sites compared to the control group. Triacylglycerols levels were significantly higher in the head and tail regions of tadpoles from the Evbarake and Uwelu sites compared to the control group. These findings are consistent with previous studies that have reported similar disruptions in lipid profiles of aquatic organisms exposed to oil pollution (Nwani *et al.,* 2015; Okafor et al., 2018). The increase in HDL-cholesterol levels may be a compensatory mechanism to help transport excess cholesterol away from tissues, while the rise in LDL-cholesterol and triacylglycerols levels indicate impaired lipid homeostasis.

5.1 Conclusion

The finding from this study has shown that exposure to spent oil runoff water induces significant oxidative stress in tadpoles, as evidenced by increased lipid peroxidation (MDA levels) and altered antioxidant enzyme activities. The differential responses between the two sites suggest that the composition or concentration of contaminants may vary, leading to site-specific effects. There were slight decreases in phospholipid content in both head and tail regions for the exposed groups, these differences were not statistically significant. The elevated metal concentrations observed particularly of lead, cadmium, nickel, and zinc, pose significant risks to soil ecosystems and potentially to aquatic systems through runoff. The results indicate significant metal contamination at both the Evbarake and Uwelu sites, with concentrations of most metals far exceeding those in the control site and, in many cases, surpassing accepted limits for biological systems. The results presented in this study demonstrate that exposure to spent oil runoff water has significant impacts on the lipid metabolism of tadpoles. Compared to the control group, tadpoles from the Evbarake and Uwelu sites exhibited marked increases in total cholesterol,

HDL-cholesterol, LDL-cholesterol, and triacylglycerol levels in both the head and tail regions in this study.

5.2 Recommendations

The following are recommended for further study and application:

1. Further research is needed to elucidate the precise mechanisms by which spent oil runoff water is affecting tadpole lipid metabolism and to evaluate the long-term consequences of these alterations on tadpole growth, development, and overall fitness.
2. Evaluate the expression of genes and proteins regulating energy metabolism pathways to identify the specific molecular targets of spent oil runoff toxicity.
3. Investigate the long-term consequences of these metabolic disturbances on tadpole growth, development, and survival, as well as the potential for recovery upon removal from the polluted environment.
4. Environmental remediation efforts to mitigate the impact of oil pollution on aquatic ecosystems should also be a priority.

5.3 Contributions to Knowledge

The study has contributed to knowledge in the following ways:

1. This study contributes to ecotoxicology by revealing that automobile workshop effluents, even at sublethal levels, can cause significant biochemical disruptions in tadpoles. These include changes in oxidative stress markers, such as increased malondialdehyde (MDA) levels and decreased antioxidant enzyme activity (e.g., SOD, CAT, and GPx).
2. This study established evidence that exposure to heavy metals and hydrocarbons in effluents interferes with normal metabolic processes in tadpoles, including lipid

peroxidation, and altered protein synthesis. These changes are indicative of stress responses and may lead to stunted growth or delayed metamorphosis.

3. The study established reliable biochemical markers—such as altered enzyme activities and lipid profiles—that can be used for early detection of environmental contamination in freshwater ecosystems.
4. This study established that Tadpoles are crucial indicators of aquatic ecosystem health. By demonstrating the specific biochemical impacts of effluents, the research supports the use of tadpoles as sentinel organisms and strengthens the scientific basis for amphibian conservation in polluted environments.
5. This study established that there are urgent needs for better waste management practices in automobile workshops. The study offers scientific support for environmental regulations that restrict the release of untreated effluents into natural water bodies.
6. This study provides baseline biochemical data that can be compared with responses in other aquatic organisms (e.g., fish, snails), enriching the broader field of aquatic toxicology.
7. This study addressed a significant environmental issue in Evabareke and Uwelu areas where automobile workshops are common and often unregulated.

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APPENDIX I

Instrument Setup

1. **Power On**: The AES 2000 series spectrophotometer was turned on and allowed to warm up as per the manufacturer's instructions.
2. **Lamp Selection**: The appropriate hollow cathode lamps for each element were installed:
    i. Cd: 228.8 nm
    ii. Zn: 213.9 nm
    iii. Pb: 283.3 nm
    iv. Mg: 285.2 nm
    v. Fe: 248.3 nm
3. **Flame Type**: An air-acetylene flame was used for all elements.
4. **Background Correction**: Background correction was enabled using a deuterium (D2) lamp to minimize interference.

Sample and Standard Preparation

1. **Sample Digestion**: Samples were digested using an appropriate method, such as microwave digestion, to ensure complete dissolution of the metals.
2. **Standard Solutions**:
    i. Standard solutions for each element at known concentrations was prepared.
    ii. For example, for Pb, standards at 0, 1.0, 2.0, 3.0, 4.0, and 5.0 µg/mL were prepared by diluting a stock solution.
3. **Matrix Matching**: Calibration standards were prepared in the same matrix as the samples to account for matrix effects.

Analysis Procedure

1. **Blank Measurement**: The blank solution was aspired and the instrument was set to zero absorbance.
2. **Standard Curve**: Each standard solution was aspired and the absorbance was recorded. A calibration curve of absorbance versus concentration was plotted.
3. **Sample Analysis**: Each sample solution was aspired and the absorbance was recorded.
4. **Concentration Calculation**: The concentration of each metal in the sample was determined using the calibration curve.

Quality Control

1. **Replicates**: Each sample was analyzed in triplicate to ensure precision.
2. **Blanks**: A blank sample was included to account for any contamination.
3. **Standard Reference Materials (SRMs)**: SRMs was analyzed to validate the accuracy of the results.

APPENDIX II

Calculation of Standard Error of Means [SEM] And Analysis of Variance

Microsoft Excel[R] was used to calculate the standard deviations of all the observations. The various standard deviations were then divided by the square root of three.

The standard error in means [SEM] of the tail and head of the tadpoles in each of the groups after 2weeks are shown in the result.

An example of how all the SEM in this study were calculated, is shown below;

$$SEM = \frac{Standard\ deviation\ (SD)}{\sqrt{n}}$$

$$Where\ SD = \sqrt{\frac{\sum(X - x)^2}{n - 1}}$$

X = observation

x = mean of observations

n = number of animal in a group = 20