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HEPATOTOXIC AND NEPHROTOXIC EFFECTS OF ATRAZINE ON ADULT MALE *XENOPUS LAEVIS* FROGS. A LABORATORY STUDY

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A Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine

University of the Witwatersrand

Parktown, Johannesburg, South Africa

June 2017

DECLARATION

I Lynette Sena declare that this research report is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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Abstract

Atrazine, an extensively used herbicide is amongst the commonly detected herbicides in groundwater. Atrazine concentrations as low as 0.01µg/l have been implicated to affect frog populations, thus much attention has been placed on its use and safety. Several studies have examined atrazine effects on reproductive organs, immune systems and population fitness of adult *Xenopus laevis* species and we found no studies on the effects of atrazine on the liver and kidney. This study investigated biochemical and histopathological effects of chronic exposure to atrazine on livers and kidneys of adult *Xenopus laevis* frogs, post metamorphosis. Forty male frogs were randomly divided into four groups (A -D) of 10 frogs each, housed in stainless silver tanks with 60L of water and atrazine concentration of 0µg/l A: control, B: 0.01µg/l, C: 200µg/l and D: 500µg/l respectively, for 90 days. Liver (ALT, ALKp and AST) and kidney (urea, creatinine) biomarkers, malondialdehyde, an indicator of lipid peroxidation, histopathology, melanomacrophage percentage area and fibrosis were examined. Significant increases of ALT and creatinine were observed at 200 and 500µg/l ($P<0.05$). Malondialdehyde was significantly increased at 500µg/l ($P<0.05$). Histopathologically, the liver showed disorganization in the arrangement of hepatic cords, hypertrophied hepatocytes, hepatocyte vacuolization, vascular congestion and dilation, infiltration of inflammatory cells and apoptosis and/or necrosis, with the highest atrazine concentration causing the most adverse effects. The kidney showed glomerular atrophy and degeneration, tubular lumen dilation, vacuolization and degeneration of thick loop of Henle tubule epithelial cells. Melanomacrophage percentage areas were significantly decreased at 0.01µg/l and 500µg/l and significantly increased at 200µg/l ($P<0.05$). No significant fibrosis was observed in all treated groups. The results suggest that very low and high environmentally relevant doses of atrazine have the ability to adversely affect organs of amphibian species and potentially related aquatic organisms.

Keywords: Atrazine, Hepatotoxicity, Nephrotoxicity, Lipid peroxidation, Adult *Xenopus laevis*

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List of Abbreviations

ALKp	Alkaline Phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATZ	Atrazine
DAB	3,3′-Diaminobenzidine
HPLC	High performance liquid chromatography
IUCN	International Union for the Conservation of Nature
LPO	Lipid peroxidation
MDA	Malondialdehyde
MMC	Melanomacrophages
PBS	Phosphate buffered solution
PECs	Parietal epithelial cells
Ppb	Parts per billion
ROS	Reactive oxygen species
µg/l	Micrograms per litre
USEPA	United States Environmental Protection Agency

1. INTRODUCTION

1.1 The History of Herbicide Use

For decades, many environmental pollution issues have been raised and can be traced back to the industrial revolution in the 50s (Woster, 1994). Industrialization brought about the use of synthetic materials such as polychlorinated biphenyls, plastics and inorganic herbicides (Ross & Amter, 2010). Most of these synthetic materials proved beneficial to the society but not without their attendant unidentified shortcomings. These unforeseen effects of synthetic materials on human health and the environment only emerged years later.

Balancing the benefits and risks of man-made compounds in modern industrialised societies on humans and the environment (both aquatic and terrestrial) has always been an age old challenge, particularly extensive expansions over the years, in the agricultural sector (Food and Agriculture Organization, 2011). With the increase in global population comes the increase in food consumption and consequently the need to increase food production to sustain the growing world population. In the past, the primary solution was to use more land for agriculture. However, with the rapidly growing population and industrialization, productive agricultural land was and is presently being used for industrial growth, urbanization and technological advancement (Food and Agriculture Organization, 2011), further decreasing the possibility for increased food production. Consequently, alternative means of increasing food production to feed the population had to be developed and this led to the discovery and use of herbicides for farming (Godfrag, et al., 2010; Unsworth, 2010).

Herbicide use was implemented by farmers in Western European countries, the United States, South Korea, Canada, and Japan in the 1950s, to control unwanted plants/weeds and increase food productivity (Institute of Medicine (US), 1994; USEPA, 2003b; Hossain, 2015). Since then, the use of herbicides in farming processes not only gained recognition but has greatly increased globally. Presently billions of tonnes of herbicides are being globally produced per year, with approximately 70% being used in agriculture (Arya, 2005). The value of the global herbicide market grew by 39% between 2002 and 2011 (Hossain, 2015). It is likely that this trend will increase significantly in future as global pressure forces integrated weed management

and sustainability. In African countries, herbicides are being used on large-scale commercial farms (Zhang, 2003; Hossain, 2015), with South Africa on the lead in Sub-Saharan Africa.

South Africa forms about 60% of the herbicide market and has more than 500 registered herbicides (Naidoo & Buckley, 2003; Osibanjo, et al., 2002; Quinn, et al., 2011). Many of the herbicides registered for use in South Africa have been banned in many other countries due to their toxic effects on humans and the environment (Goldblatt, 2015). Although the usefulness of herbicides cannot be denied, the negative environmental and human health effects cannot be ignored. South Africa is a water poor country with water resources being utilized to their maximum capacity (Quinn, et al., 2011). Detectable levels of ATZ, endrin, terbuthylazine, simazine (Du Preez, et al., 2005), acetochlor, dichlorodiphenyltrichloroethane and its metabolite (Schultz, 2001), to name a few, have all been detected in South Africa waters.

Incidentally, these chemicals gradually gain access into human bodies either through food, drinking water, human interaction with the environment, direct or indirect contacts especially because these chemicals are not easily degraded in the environment (USEPA, 2002; USEPA, 2003b; Department of Agriculture, Forestry and Fisheries, 2010; International Union for the Conservation of Nature, 2011). Several of these herbicides have been shown to accumulate in the tissues of organs of various vertebrate and non-vertebrate animals (Vinggaard, et al., 2000; Kojima, et al., 2004; Hayes, et al., 2010). The above forms the backdrop of the current study which is aimed at investigating the effects of atrazine on the liver and kidneys of adult frogs in line with aquatic and underground water contamination.

1.2 *Xenopus Laevis* Liver and Kidney Gross Anatomy

The liver is the largest structure of the body cavity in *Xenopus laevis* frogs. It has a prism shape, with its base to the right and its apex to the left (Kapoo, 2015). It is red brown in colour and is divided into the right and relatively larger left lobe which are connected by a narrow bridge of liver tissue (Duellman & Trueb, 1986). It is highly vascular and easily friable. The microscopic anatomy of frogs is similar to that of humans. The surface of the liver is covered by visceral peritoneum (serosa), with a Glisson capsule underneath (Kapoo, 2015). At the porta hepatis, the Glisson capsule travels along the portal tracts (triads), carrying

branches of the hepatic artery, the portal vein, and the bile ducts into the liver substance (Kuntz & Kuntz, 2009).

The kidneys in adult frogs are red brown paired structures lying on either side of the dorsal aorta. They are highly vascularized from numerous venal arteries from the dorsal aorta which branch to form innumerable clusters of glomeruli. Each glomerulus is surrounded by an expanded end of a kidney tubule. The Bowmann's capsule if the major site of filtration (Hall & Guyton, 2011). The kidney tubules extend laterally through a capillary network and carry urine to the Wolffian duct (Duellman & Trueb, 1986).

1.3 Rationale

Atrazine (ATZ) has been reported in many cases to cause developmental and reproductive abnormalities in amphibians, mostly through the endocrine system (Tevera-Mendoza, et al., 2002; Hecker, et al., 2004; Hayes, et al., 2010; Zaya, et al., 2011). Very few studies on atrazine toxicity have been conducted on endocrine related organs such as the liver and kidneys. However, hepatic and renal toxicity of other herbicides has been extensively studied mostly in mammals, with very few studies done on amphibians (Zaya, et al., 2011; Jestadi, et al., 2014). Reproductive studies (Cooper, et al., 2000; Allran & Karasov, 2001; Hayes, et al., 2002; Jooste, et al., 2005) have indicated that amphibians, amongst other vertebrate species, are the most affected by ATZ concentrations as low as 0.1 parts per billion (ppb) (Hayes, et al., 2002).

The role of amphibians as predators and major herbivores suggests that frogs are important to the dynamics of the ecosystem and being sensitive to environmental contaminants makes them potential sentinel organisms for environmental health studies (Siddiqua, et al., 2010). Available literature has revealed that there exist knowledge gaps on ATZ toxicity in frogs, especially during the post-morphosis phase. Moreover, many studies have focused on the short-term exposure effects of ATZ on the development and function of reproductive organs, immune systems and population fitness of low ATZ concentrations on *Xenopus laevis* tadpoles and various other frog species.

It is therefore imperative to examine long term exposure effects of ATZ at both high and low concentrations and in different organs, in order to determine the magnitude of various toxic effects of ATZ in animals and actual threats to human health. Conclusions based on studies on a few species of frogs cannot adequately establish all the effects of chemical contaminants to frogs (due to species variation) and therefore, cannot be generalized. Previous studies have not been able to thoroughly characterize the long-term impacts of ATZ exposure on the liver and kidneys of amphibians. To our knowledge, studies have not been done on the effects of atrazine on the liver and kidneys of adult *Xenopus laevis* frogs at the histological and biochemical level. Thus, our study aimed to fill the void in knowledge on the effects of high and low concentrations of ATZ on the liver and kidneys of adult *Xenopus laevis*.

Outline of the Dissertation

Chapter one introduces the research area and outlines the rationale for the study. Chapter two (literature review) outlines the background to the study and reviews impacts of atrazine exposure on the African clawed frog (*Xenopus laevis*) and on various vertebral species. Chapter three describes laboratory procedures used during the 90days of ATZ exposure. It also highlights the various protocols used to process the tissues and analyse the data. Chapter 4 presents the general discussion on the hepatic and nephrotic effects observed. Chapter 5 summarizes the findings, outlines the limitations of this study and highlights research shortfalls which future studies can be focused on.

1.4 Aim

To investigate the effects of atrazine exposure on the structure and functions of the liver and kidneys of adult frogs (*Xenopus laevis*).

1.5 General Objective

To determine the toxic effects of atrazine exposure on the histological structure of the liver and kidneys in relation to function.

1.5.1 Specific Objectives for the Study

1. To determine the effects of atrazine on the serum levels of liver and kidney function biomarkers (aspartate aminotransferase (AST), alkaline phosphatase (ALKp) and alanine transaminase (ALT), creatinine and urea), using the colorimetric method.
2. To determine lipid peroxidation (LPO) by measuring the serum levels of malondialdehyde (MDA) in the *Xenopus laevis* frog, using High performance liquid chromatography (HPLC).
3. To determine the dose-dependent effects of atrazine on the cytoarchitecture of liver and kidneys of the *Xenopus laevis* frog using haematoxylin and eosin (H&E).
4. To determine the effects of atrazine on melanomacrophage (MMC) population in the liver tissues of the *Xenopus laevis* frog, using H&E.
5. To determine the effects on ATZ on the connective tissue content in the liver and kidneys of the *Xenopus laevis* frog, using the Van Gieson stain.
6. To determine changes in apoptosis in liver and kidney tissues of the *Xenopus laevis* frog, using activated caspase-3 immunohistochemistry.

2. LITERATURE REVIEW

2.1 Herbicide Use and Regulatory Policies

The use of herbicides for agricultural activities is constantly on the increase worldwide, with significant increases in food production (Nweke & Sanders, 2009). However, these herbicides are contributing to environmental contamination, adverse effects on human and animal health and leading to species extinction (Siddiqua, et al., 2010; Damalas & Eleftherohorinos, 2011; Sánchez-Bayo & Ortega, 2014). Despite these detrimental effects and limited understanding of the various mechanisms of herbicide toxicity at different levels of biological orientation, new herbicides are being manufactured regularly (Sánchez-Bayo & Richard, 2012).

The most common eco-toxicological effects exhibited by almost all harmful herbicides are their ability to resist bio-degradation and therefore, bio-accumulate in animals (Quinn, et al., 2011). Herbicides have been detected in ground water, soil and surface water (Burger, et al., 2008; Mariyono, 2008). Typical examples include organochlorine herbicides such as dichlorodiphenyltrichloroethane (Augustijn-Beckers, et al., 1994), triazine herbicides (Hayes, et al., 2002) and endosulfans (Weber, et al., 2010). For instance, Dalvie et al. (2003) reported endosulfan levels which exceeded the 'European drinking water standard of 0.1µg/l', in groundwater samples taken from agricultural locations in South Africa. Concentrations of triazine herbicides above 0.1µg/l have also been detected in various ground water sources (USEPA, 2001; Hayes, et al., 2003). According to Solomon et al. (1996), the biochemical properties of these herbicides aid in their persistence in the environment. Most of these herbicides end up bio-concentrating in several aquatic and terrestrial organisms including humans. Herbicide impacts are not immediately apparent within the ecosystem, making their negative consequences noticeable after a long time (Solomon, et al., 1996).

Though the negative consequences of these chemical are apparent, to peg their use in agricultural processes is increasingly difficult. Furthermore, while the toxicity of the old known herbicides/chemicals is still being investigated, chemical companies are constantly introducing new herbicides (USEPA, 2003b; Damalas & Eleftherohorinos, 2011) because of the increasing need to improve food production. Thus, countries have come up with regulatory policies and/or agencies to control the manufacturing and use of old and new herbicides. In South Africa, the Fertilisers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, which controls

pesticide use amongst other things, was approved by Parliament in 1947. Since then, the act has not been systematically revised and no re-evaluation on many of the more than 500 pesticide products approved for use in South Africa has been done. The safety of the pesticides therefore, has not been reviewed to bring them at parity with current stringent standards of risk assessment (Department of Agriculture, Forestry and Fisheries, 2010). The National Environmental Management Act (NEMA). 1998 (Act No. 107 of 1998) was passed to establish principles for decision making on issues affecting the environment (Department of Agriculture, Forestry and Fisheries, 2010). The South African Department of Water Affairs (DWAF) also initiated the National Toxicity Monitoring programme which monitored the levels of several pesticides in environmental water sources. It ensured that water pollution from pesticides did not threaten compliance with the National Water Act, 1998 (Act No. 36 of 1998) which protected water resources, including the prevention of pollution resulting from land and water activities (Department of Agriculture, Forestry and Fisheries, 2010).

Developing and implementing effective policies focused on agriculture is a challenge. To date, only a handful of African countries have regulatory policies and guidelines in place (Andanda, 2009). Additionally, public information on the quantities of herbicides used in agricultural activities in most African countries including South Africa, seem not to be available. This lack of information is generally the factor that limits our ability to prioritize, manage and predict environmental health risks posed by herbicides (Dabrowski, 2015). There are a limited number studies that have prioritized herbicide risks to human and environmental health in South Africa (Dabrowski, 2015). The fact that herbicides are still being detected in water bodies suggests loopholes in the regulatory policies and their inefficient to ensure end-user and environmental safety.

The European Union introduced a notion highlighting the health effects drinking herbicide contaminated water (USEPA, 2001; Sass & Colangelo, 2006). It further set a uniform limit of 0.1ppb for the residue of any herbicide in drinking water (Sass & Colangelo, 2006). The US Environmental Protection Agency (USEPA) strongly advised prioritizing, sorting out and thoroughly assessing herbicides and commercial chemicals for environmental risks (USEPA, 2003b), before their use.

2.2 Amphibians as a Vertebrate Model

Several ecological animal species are used to assess environmental conditions, delineate an ecosystem, find a disease outbreak and monitor pollution and climate changes (McDonough, et al., 2009). Amongst these species are amphibians. For the past decades, the ecology and eco-toxicology of amphibians has received great attention because of the global decline in the amphibian population (Houlahan, et al., 2000). Based on the lists of the International Union for the Conservation of Nature (IUCN), there are 787 endangered amphibian species and about 1900 species known to be threatened (IUCN, 2011). Studies have reported that amphibian development and survival can be adversely affected by exposure to agricultural chemicals such as herbicides (Hayes, et al., 2006a; Marcogliese, et al., 2009).

Amphibians have physiological traits common to all vertebrates, including mammals. They are found in aquatic and/or terrestrial environments and show diversified morphological and physiological adaptations to these environments (Burggren & Warburton, 2007). Toads and frogs which form the anuran group of amphibians constitute about 90% of all amphibians and are therefore regarded as an important link between human and ecosystem health (McDiarmid & Mitchell, 2000; Hayes, et al., 2002). They have a biphasic life cycle and are highly sensitive to environmental pollution due to their permeable skin. Therefore, they can easily be exposed to the harmful effects of environmental contaminants in their different life-stages (Blaustein, 1994; Solomon, et al., 1996). For these reasons, frogs as amphibian models are used as biological indicators of environmental herbicide pollution (Sheridan & Olson, 2003; Hayes, et al., 2010).

2.3 Atrazine as a Case Study in Pollution

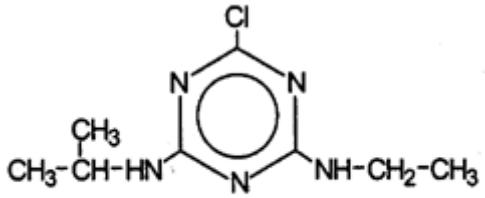
ATZ (2-chloro-4-ethylamino-6-isopropylamino-5-triazine) a broad spectrum triazine herbicide, is one of the widely used agricultural herbicides (Graymore, et al., 2001; Hayes, et al., 2010). Globally, ATZ is used to eliminate weeds during production of maize, sorghum, sugarcane, vines, fruit orchards, chemical fallows and grassland, with its biggest market in maize production (Graymore, et al., 2001; Ribeiro, et al., 2005). It has a wide range of trade names, such as Marksman, Atrazol, Vectal, Atrazina and Coyde (Kumar, et al., 2014). ATZ was first introduced as an herbicide in 1957 (USEPA, 2001), patented in Switzerland in 1958 and registered in USA in 1959, for commercial use (Giddings, et al., 2005). The main registrant of

ATZ is Syngenta Crop Protection, LLC but it is also manufactured and registered under license to several other agrochemical companies (Van Der Kraak, et al., 2014).

The use of atrazine has been a subject of significant concern because it is amongst the commonly detected herbicide contaminants of wells, rivers and streams. Atrazine can contaminate environmental water-bodies through various locations and pathways such as drainage/runoff of treated areas, herbicide water spills and precipitation, thus, making it a possible hazard to the ecosystem (USEPA, 2001). It has been detected in natural (Solomon, et al., 1996) and surface water sources at concentrations exceeding 0.1g/l (USEPA, 2001). Many European countries such as Germany, Sweden, Denmark, Norway and France have banned the use for ATZ because of its ground water contamination tendencies. In South Africa, ATZ was banned for industrial use in 1995 but surprisingly, it is being detected by scientists in the environment (Du Preez, et al., 2005; Hayes, et al., 2010). Research has shown that ATZ can bioaccumulate in various animal tissues and cause adverse health effects (Liu, et al., 2006; Lenkowski, et al., 2008; Jestadi, et al., 2014).

2.3.1 Structure and physiochemical properties.

The chemical properties of atrazine (Appendix 1) aid in its persistence in the environment following its application and its susceptibility to runoff from agriculture and urban application. The ring structural makeup of ATZ (Figure 2.1), a triazine ring with 5 nitrogen atoms and a chlorine atom results in strong covalent bonds which make ATZ resistant to degradation (Solomon, et al., 1996). According to Solomon et al. (1996), the vapour pressure and Henry's law constant of ATZ (Appendix 1.2) is considered relatively low and makes ATZ a relatively low risk for evaporating from surface water. This in turn would reduce contaminated rainfall and yet make it easy for ATZ to inundate environmental waters. They further reported that ATZ was stable to hydrolysis for 30 days at pH 5-9 (Appendix 1.2) , thus allowing it to be persistent in the environment.



atrazine

Figure 2.1: Chemical structural formula for atrazine.

2.3.2 Mode of action of atrazine

Plants absorb ATZ through foliage and the roots. Once absorbed, it accumulates in the growing tips and new leaves of plants (Marin-Morales, et al., 2013). In tolerant plants, it is metabolised. In targeted plants, it disrupts photosynthesis by inhibiting chloroplast –associated reactions through binding-to the-plastoquinone- binding-protein in-photosystem ll (Marin-Morales, et al., 2013). Photosystem ll (PS ll) is the first protein complex in the light-dependent reactions of photosynthesis involving oxygen. It is found in the thylakoid membrane of plants (Rochaix, 2011).

It has been suggested that due to a similarity in binding sites, ATZ binds-irreversibly to the-plastoquinone binding-sites of photosystem complex ll on thylakoid-membranes in chloroplasts, it may also bind to mitochondrial sites of electron transport chain complexes l and lll in animals (Lim, et al., 2009). Subsequently, this may result in the alteration of mitochondrial structure and functions such as adenosine triphosphate (ATP) production and uncontrolled reactive oxygen species (ROS). Using a transmission electron microscope, Lim et al. (2009) observed partial disorientation of the mitochondrial cristae in *Sprague Dawley* rats exposed to ATZ concentrations of 300µg/kg/day for 5 months. Mitochondrial alterations may induce oxidative stress, promote lipid accumulation, apoptosis and/or necrosis, leading to cytolysis and inflammation. However, the mechanisms of toxicity of the ATZ herbicide and its active metabolites are not yet fully understood.

2.4 Atrazine and Lipid Peroxidation

It is widely believed that environmental contaminants such as herbicides modulate antioxidant defensive systems and cause oxidative stress in aquatic animals by ROS production (Valavanidis, et al., 2006; Slaninova, et al., 2009; Nwani, et al., 2010). Oxidative stress occurs when there is an imbalance between free radicals and antioxidants ratios, a condition which leads to the generation of ROS, that is, chemically reactive chemical species containing oxygen (Livingstone, 2003; Slaninova, et al., 2009; Lushchak, 2014; Holmström & Finkel, 2014; Sies, 2015). Under normal conditions, a dynamic equilibrium exists between the production of ROSs such as hydrogen peroxide, hydroxyl radical, superoxide anion, singlet oxygen and the antioxidant capacity of the cell. Stress and injury to cells occurs when redox homeostasis is altered and ROS generation overrides the biochemical defences of the cell (Kehrer, 1993; Peña-Llopis, et al., 2003). The biochemical mechanisms involved in the cellular detoxification are particularly relevant in understanding the deleterious effects of agricultural chemicals, metals or other environmental pollutants (Banerjee, et al., 1999; Elia, et al., 2002; Valavanidis, et al., 2006; Blahová, et al., 2013).

Herbicides can induce oxidative stress which can result in the excess generation of free radicals and alterations in oxygen free radicals, antioxidant enzyme activities and lipid peroxidation (LPO) (Banerjee, et al., 2001; Nwani, et al., 2010). Antioxidant enzyme activities and the levels of lipid peroxidation products are often used as bioindicators in toxicological evaluations (Doyotte, et al., 1997; Banerjee, et al., 1999; Banerjee, et al., 2001; Oruc, et al., 2004; Nam, 2011; Dornelles & Oliveira, 2013; Yoshida, et al., 2013). Lipid peroxidation has been suggested as one of the molecular mechanisms involved in herbicide-induced toxicity (Bagchi, et al., 1995; Banerjee, et al., 1999). According to Yin et al. (2011), LPO is a chemical process in which unsaturated fatty acids of lipids are damaged by free radicals and ROS, resulting in lipoperoxides formation. Lipoperoxides are unstable compounds which decompose to form a wide range of intermediates and complex end products including aldehydes--malondialdehyde (MDA), reactive carbonyl compounds and 4-hydroxyl-2-nonenal (4-HNE) (Kuhn & Borchert, 2002; Ayala, et al., 2014). These products are found to be significantly modified in some pathological and clinical conditions. It is known that LPO products can damage cells by binding to free amino groups of amino acids of essential cell proteins, thus, altering cellular structure and function (Mylonas & Kouretas, 1999). Therefore, the degree of LPO is often used as a proxy for ROS-mediated damage (Mylonas & Kouretas, 1999; Kuhn & Borchert, 2002) and the

concentration of MDA in tissues and blood are commonly used as biomarkers of lipid peroxidation (Sehirli, et al., 2008; Nwani, et al., 2010).

In recent years, lipid peroxidation research has received renewed attention because LPO has been implicated as a primary event involved in mediating the toxicity of a broad spectrum of herbicides (Banerjee, et al., 1999; Dornelles & Oliveira, 2013). On account of its vast importance, a number of studies have been performed to measure the levels of lipid peroxidation products in various tissues. Above all, thiobarbituric reactive substances (TBARS) and MDA have been measured most frequently (Janero, 1990; Halliwell & Chirico, 1993). The TBARS method provides an overall measure of aldehyde levels, including MDA, but it is plagued with interferences from non-lipid derived aldehydes from sugars, amino acids, DNA and species resulting from chemical interaction of thiobarbituric acid with non-lipid molecules during the assay (Gutteridge, 1988; Janero, 1990). Therefore, TBARS method is not specific for free MDA. It is due to these methodological pitfalls that the high-performance liquid chromatographic (HPLC) method is, thus, preferred over the standard TBAS, as it improves the assay specificity and the sensitivity of MDA determination in tissues (Esterbauer, et al., 1984; Moselhy, et al., 2013).

During the past years, oxidative stress biomarkers have been determined in vertebral species to assess acute and chronic tissue damage (Nwani, et al., 2015). The use of biomarkers for investigating the effects of chemical compounds in vertebral species has generally increased but slowly progressing in amphibian studies. Generally, amphibians (and other anuran species) are not considered as standard test organisms and more often, the results from piscean and aquatic invertebrates, avian and mammals are used as proxies (Relyea, 2011) Taking into account the paucity of information, the present study was designed to investigate chronic effects of ATZ exposure on oxidative stress responses that is, quantifying the MDA content, a product LPO, in post-metamorphic *Xenopus laevis* frogs using a reliable, highly sensitive, HPLC method.

2.5 Atrazine and Cell Death

As mentioned earlier, damage to mitochondrial membranes may result in a decrease in the production of ATP, culminating in necrosis and/or release of proteins that trigger apoptosis

(Banerjee, et al., 1999; Nwani, et al., 2010). Mitochondria contains several proteins such as cytochrome c, which are capable of inducing apoptosis. Cytochrome c together with other co-factors, activates caspase-9 which further activates a cascade of caspases, resulting in cell death (Elservier, 2007). One of the caspases involved in this intrinsic pathway of apoptosis is caspase-3. Caspase-3, a member of the caspase family of proteases and main executioner of apoptosis is a cytoplasmic protein highly expressed in kidneys, spleen, heart, lungs and liver (Ceruti, et al., 2003). Caspase-3 is expressed in its inactive pro-form (pro-caspase-3). In apoptosis, the pro-caspase-3 is activated by initiator caspases such as caspase-9 and caspase-8, into its active form, which is a useful biomarker of apoptosis (Ceruti, et al., 2003). As caspase-3 is the principal executioner of apoptosis, immunohistochemistry of the active form of caspase-3 (active casp-3) is used to detect apoptosis in paraffin embedded sections of several tissues (Resendes, et al., 2004; Jakob, et al., 2008).

Commonly, studies on apoptosis utilize the *in situ* terminal deoxyribonucleotidyl transferase mediated deoxyuridine triphosphate nick end labelling (TUNEL) assay that detects DNA strand breaks in situ in tissue sections (Hughes & Gobe, 2007). However, localization of activated caspase-3 is now seen as an alternative to TUNEL (Glamoclija, et al., 2005; Hughes & Gobe, 2007). A large number of activated caspase-3 immuno-positive cells in the liver of *Xenopus laevis* tadpoles exposed to 400 µg/L of ATZ has been reported by Zaya et al. (2011). As ATZ has been reported by Lenkowski et al. (2008) to induce the activation of pro-caspase-3 cells into activated caspase-3 cells, in *Xenopus laevis* tadpoles, it is also crucial to investigate these apoptotic effects in adult *Xenopus laevis* frogs. Additionally, due to inter and intra-frog species diversity, the toxicity of ATZ may vary among species and the nature of the ecosystem. There is therefore a need to document the effects of ATZ on various species of adult frogs. The present study incorporates the use of this protease to detect ATZ induced apoptosis, if any, in the liver and kidney of adult *Xenopus laevis* frogs.

2.6 Biochemical Effects of Atrazine

The process of chemical injury to cells may involve not only gross lipid peroxidation but also plasma membrane permeability. Plasma membrane damage is associated with loss of osmotic balance and leakage of intracellular proteins (Elservier, 2007). Leakage of cellular contents provides a means of detecting tissue specific necrosis using blood or serum samples. In assessing and monitoring adverse effects of various chemicals, biochemical parameters may

have significant diagnostic value in understanding early signs of herbicide toxicity (Pant, et al., 1987).

A study done on *Wistar* rats treated with 300µg/kg body weight ATZ, showed significant increases in aspartate aminotransferase (AST), alkaline phosphatase (ALKp) and alanine transaminase (ALT) serum levels (Jestadi, et al., 2014). On the contrary, another study reported significant increases of AST and ALKp and non-significant increases of ALT serum levels in *Wistar* rats treated with 400mg/kg body weight ATZ, for 14 consecutive days (Campos-Pereira, et al., 2012). Studies on liver biomarkers in adult *Xenopus laevis* have not been conducted and need to be documented since there is great variability between these two animals (rats and frogs) especially relative to their environment which might affect their response to adverse effects of ATZ.

2.6.1 Atrazine and kidney biomarkers

Blood levels of urea and creatinine amongst other biomarkers, are useful parameters in evaluating kidney function. Most of the blood urea is synthesized in the liver from ammonia and most of the creatinine originates from the non-enzymatic conversion of creatine phosphate in muscle. The kidney is the major route of urea and creatinine excretion and an increase in these parameters may suggest pre-renal, renal and/or post-renal diseases (Hall & Guyton, 2011). Hence, these parameters have been extensively used as barometers of renal function. Very few studies on vertebrate species have been done to investigate the effects of ATZ on the serum levels of blood urea nitrogen and creatinine. Nevertheless, of the few studies conducted, elevations in serum levels of blood urea nitrogen and creatinine have been reported in *Wistar* rats treated with 300µg/kg body weight ATZ, for 15 days (Jestadi, et al., 2014). Liu et al. (2014) reported similar findings following treatment of 4 weeks old female *Wistar* rats with ATZ concentrations of 5, 25 and 125mg/kg /day, for 28 days by gavage.

It is hoped that the results of the present study will be critical in balancing the use of ATZ and the need for increased agricultural productivity, as well as the general and environmental health of our population.

3. MATERIALS AND METHODS

3.1 Chemicals

Atrazine [CAS Number 1912-24-9, technical grade, 98.9% purity] was bought from AccuStandard Inc. (New Haven, CT, USA). The reagent 1, 1, 3, 3-tetraethoxypropane (TEP) and HPLC-grade solvents were purchased from Sigma-Aldrich (Johannesburg, South Africa), KOH, KH_2PO_4, Perchloric acid ($HClO_4$) and HPLC-grade water (H_20) were purchased from Merck (Darmstadt, Germany). Serum biochemical analytical kits for liver and kidney function tests: alanine amino transferase (ALT), alkaline phosphate (ALKp) and aspartate amino transferase (AST); and kidney creatinine (CREA) and blood urea nitrogen (BUN) biomarkers kits were purchased from IDEXX Laboratories (Johannesburg, South Africa). Ponceau S, Celestine blue were purchased from Sigma-Aldrich (Johannesburg, South Africa). The rest of the products (Sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4), formaldehyde, sodium phosphate monobasic NaH_2PO_4, chloroform, 3-aminopropyl triethoxysilane, entellan blue, ammonium ferric sulphate ($NH_4Fe(SO4)_2.12H_2O$), saturated aqueous picric acid, glacial acetic acid and acetone) used in this study were purchased from Merck (Johannesburg, South Africa) unless stated otherwise.

3.2 Treatment Solutions and Dose Selection

Treatment solutions containing stock (standard) solution of 400mg/l of ATZ was prepared by dissolving 20mg of 98.9% pure ATZ (CAS Number 1912-24-9, AccuStandard, Inc, USA) in 150ml charcoal-filtered (Barnstead/Thermolyne Dubuque, IA, USA) dechlorinated tap water (pH 7.3). Further dilutions were accordingly made to obtain the following ATZ concentrations: 0.01µg/l, 200 µg/l and 500µg/l of ATZ, concentrations which were used in the present study. The choice of these regimens was based on doses used in a previous study of anuran reproductive susceptibility (Freeman & Rayburn, 2005). For instance, a low concentration of 0.01µg/l was chosen since a similar concentration was used by many other investigators who reported disruptions of the endocrine system in frogs (Cooper, et al., 2000; Hayes, et al., 2006a; Kloas, et al., 2009). Likewise, a 200µg/l concentration of ATZ used in another study, inconclusively mimicked the results as reported in 0.01µg/l ATZ concentration exposure (Zaya, et al., 2011). A further high concentration of 500µg/l of ATZ was included in the present study to eliminate the possibility of inconclusive results. Additionally, it is important in toxicological studies to have definite observations which can monitor future events, as ATZ concentrations as high as 500µg/l might be observed in cases, where a chemical disaster occurs due to spillage

of this herbicide, particularly in human populated areas and sources of water supply. Exposure to such high concentrations can be risky to the health of the population and the environment. Furthermore, concentrations higher than 500µg/l have been tested in several vertebral species in environmental risk assessment (Allran & Karasov, 2001; Diana, et al., 2000; Freeman, et al., 2005; Hussain, et al., 2012).

3.3 Animals and Housing

Forty (40) healthy, captive bred adult male African clawed *Xenopus laevis* frogs (of post-anuran metamorphosis age; measuring~7.5±9 cm in length), obtained from a local frog farm (African Xenopus Facility, Knysner, Western Cape, South Africa), were used for the study. The animals were immediately transported in polystyrene plastic boxes and housed at the Central Animal Services (CAS) of the University of the Witwatersrand. The animals were randomly distributed and placed in four stainless steel tanks (225cm x 24cm x 12.5cm) containing water and allowed to acclimatize in the tanks and environment for 30days prior to treatment. During this experimental period, the frogs were fed *ad libitum* with Kori TM Frog Brittle (Daro Pet Products, Johannesburg, South Africa) in the form of floating and sinking pellets and maintained on a 12:12 h light: dark cycle, a room temperature 22±2°C, oxygen saturation exceeding 70% and pH 6.5 according to methods of (Prokic´, et al., 2015). All experimental animal treatments were done according to the Ethical principles for Animal Research approved by the Animal Screening Ethics Committee (approval number 2014/14/D) of the University of the Witwatersrand and Gauteng Nature Conservation (permit numbers 0115 and 0120).

3.4 Experimental Design and Procedure

Four stainless steel test aquaria labelled A-D of dimensions: 225cm x 24cm x 12.5cm were cleaned and filled with 60 liters of water each, according to the dose concentrations of ATZ and control group. Forty (40) adult male *Xenopus laevis* frogs were randomly allocated to each of the tanks A to D (i.e. groups A-D); 10 frogs per tank as presented in Figure 3.1. Tank A contained zero ATZ concentration and the animals served as control, tank B with ATZ concentration 0.01µg/l housed group B, while tank C with 200µg/l of ATZ housed group C and tank D with 500µg/l of ATZ housed group D treated animals. Each of the groups C to D were exposed to the respective ATZ concentration for 90 days while food was provided *ad libitum* with Kori ^{TM} Frog Brittle (Daro Pet Products, Johannesburg, South Africa) in the form

of floating and sinking pellets. Tank water was replaced twice a week, in order to maintain a healthy environment with sufficient aeration, throughout the duration of the experiment. The atrazine concentration in the exposure tanks was monitored weekly by Gas Chromatography–Mass Spectrometry (GC-MS) to ensure that the concentration of ATZ was maintained within stipulated doses.

At the end of the experiment, the animals in each group were weighed and the weights were recorded. Thereafter, the animals were placed in a jar containing water with 0.02% benzocaine (Sigma, Johannesburg, South Africa). The animals were anaesthetized by benzocaine inhalation. Blood samples were collected by cardiac puncture into plain tubes and allowed to clot at room temperature and stored at -82°C, until analysis. Tissue samples (liver and kidney) were also harvested, weighed and the weights recorded. Liver and kidney tissues were appropriately sliced and immediately placed in labelled specimen bottles with 10% buffered formalin (Appendix 2.2) for fixation for subsequent histological analysis.

Experimental flow chart

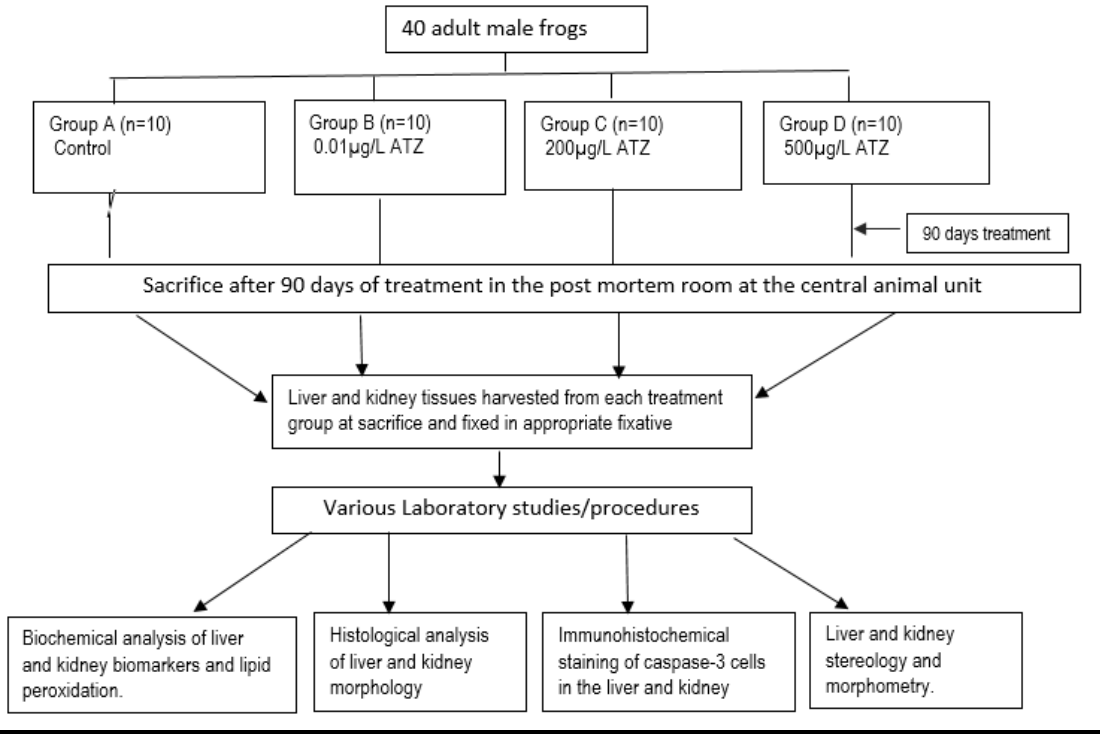


Figure 3.1: Experimental design

3.4.1 Preparation of samples for biochemical analysis

3.4.1.1 Measurement of serum biochemical enzyme parameters

The concentrations of liver (alanine amino transferase, alkaline phosphate and aspartate amino transferase) and kidney (urea and creatinine) biomarkers were determined using appropriate kits and according to the manufacturers' protocols. Blood samples were collected by cardiac puncture and put into plain heparin tubes. The blood samples were centrifuged at 4000x g at 4°C for 10 minutes, to collect sera for biochemical analysis. The sera were used for spectrophotometric determination of the serum levels of liver enzymes: ALT and AST, by the method of Reitman and Frankel (1957). ALKp was determined using an enzymatic colorimetric method according to Tietz et al. (1983). In addition, kidney parameters: creatinine (CREA) was measured according to (Larsen (1972) and the blood urea nitrogen (BUN) biomarkers according to Coulombe and Favreau (1963). The values were expressed as mg/dl.

3.4.1.2 Estimation of lipid peroxidation (LPO)

MDA, as an *in vitro* marker of lipid peroxidation, was assessed by using a sensitive High Performance Liquid Chromatography, according to the modified method adapted from Karatas et al. (2002). Briefly, the samples were analyzed on a Bischoff HPLC apparatus, with a UV detector set at 254 nm and the analytical Prontosil column, Bischoff Chromatography (Leonberg, Germany) (12.5 cm x 4.0 mm, 5 µm particle size), was used for identification and quantification of MDA. The mobile phase composition was acetonitrile-distilled water (50:50, v/v) where the flow rate was maintained at 1 ml/min in isocratic mode and the sample injection volume was 50 µl. MDA peaks were determined according to its retention time and confirmed by spiking with added exogenous standard. Concentrations of serum MDA were calculated from standard curve (Figure 3.2) prepared from 1, 1, 3, 3-tetraethoxypropane (TEP) and were expressed as µg/ml.

3.4.1.3 Preparation of malondialdehyde (MDA) standards

A 10µl volume of TEP was accurately diluted to 10ml with 0.1M HCl in a screw-capped test tube and placed in a boiling water bath for 5 minutes and thereafter, cooled with tap water (solution X). A working stock solution of MDA was prepared by pipetting 1ml of the hydrolyzed acetal (solution X) into a 100ml calibrated flask and diluted to volume with water. The working stock solution was 4.05 x 10-5 M acetal or 2.92µg/ ml MDA. The stock solution

was diluted to achieve nominal standard concentrations of 1.46, 0.73, 0.365, 0.183 and 0.0913 µg/ ml which were used to calibrate the graph.

A standard calibration graph was prepared by plotting peak area measurements at 254nm for various MDA standard concentrations. A typical fitting line is shown in Figure 3.2. From the equation y = 522.2x + 57.04, average MDA serum concentrations for the control and ATZ-treated groups were quantified, where x is the concentration of MDA and y is the peak area.

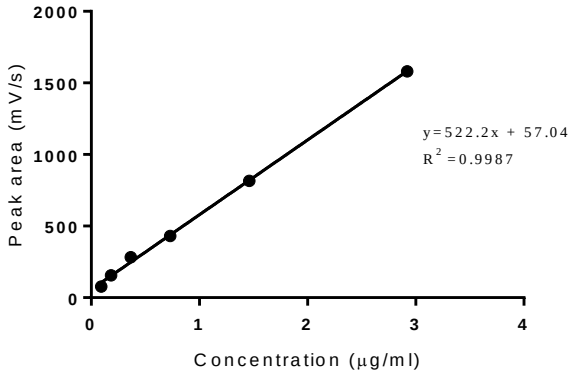


Figure 3.2: Calibration plot for the Malondialdehyde (MDA) at increasing concentration.

3.4.1.4 Sample preparation and assay procedure

An aliquot portion of 50µl frog serum was added to 250µl 0.1M $HClO_4$ and 700µl distilled water, in an Eppendorf tube. Perchloric acid was added to precipitate proteins and release the MDA bound to the amino groups of proteins and other amino (Draper & Hadley, 1990). Samples were shaken vigorously (vortexed) and thereafter centrifuged at 4000x g for 8 minutes. Samples were filtered using syringes with a 0.22µm nylon filtering tip attached and thereafter run on the HPLC machine. Low limit of detection (LOD) (54.8 µg/l) and limit of quantification (LOQ) (182.5 µg/l) were obtained for MDA analysis. The peak for MDA eluted early and was identified at the retention time of 1.08 minutes. The MDA peak areas from the sample chromatograms were obtained and used to determine MDA serum concentrations.

3.5 Organ and Tissue Parameters

3.5.1 Organ weights

At the end of the experiment and following anesthesia, a longitudinal incision was made to expose the internal organs (kidneys) and the rib cages were carefully transected to fully expose the liver. The liver and kidneys were harvested and weighed on an analytical balance (AS 160-R2, Radwag, Poland) and their wet weights recorded. Organ weights were later expressed as proportions of body weight for comparison purposes. The carcasses were disposed immediately by the Central Animal Services (CAS).

3.5.2 Preparation of samples for histological analysis

The fixed liver and kidney tissue samples were subjected to routine histological tissue processing (Appendix 3.1). Liver and kidney tissue blocks were processed overnight in an automated tissue processor (Shandon Citadel 1000) (Appendix 2.3) and thereafter embedded in paraffin wax. Tissue blocks were sectioned at 5µm thickness with a 2035 Biocut microtome (Leica, Germany). Tissue sections were placed on pre-cleaned glass slides and left overnight to adhere to slides. Thereafter, tissue sections were stained with Mayer's haematoxylin and eosin stain and cover-slipped for histo-architectural analysis.

3.5.2.1 Morphometry

In the liver, evaluation of tissue and histological measurements were done using a Leica ICC50HD-16142019 camera, Leica ICC50HD light microscope and the Leica application suite (version 3.0.0, Build:629, LAS EZ, Switzerland) imaging software. Images were captured on a computer screen and saved in jpeg format. Hepatocyte diameters were measured using the Fiji image analysis software (Schindelin, et al., 2012) line width option. Only hepatocytes with a visible complete outer lining were measured at 100X magnification. 20 hepatocytes per animal were measured. Furthermore, melanomacrophage percentage area was measured using Fiji's image thresholding function at 10X magnification.

In the kidney, glomerular tuft area, Bowman' space area, as well as luminal area, luminal perimeter and epithelial cell height of proximal convoluted and thick loop of Henle tubules, was measured at 40X magnification. Only Bowman's capsules with a complete parietal layer

were used for the analysis. Glomerular tuft area was measured using Fiji imaging software, by circling the tuft and selecting the area option under the set measurements tab. Bowman's space area was determined by subtracting corpuscle area (measured using Fiji imaging software) from the glomerular tuft area. Tubules with a distinct non-obstructed lumen were used to measure luminal area and perimeter using Fiji's imaging software freehand sketch tool. At least 20 kidney photomicrographs per animal were used for the analysis of the above-mentioned parameters.

3.5.2.2 Connective tissue analysis

Tissue slides were stained using the Van Gieson staining technique (Appendix 3.2), to allow for connective tissue analysis and quantification. Connective tissue area fraction in a single camera field (2048x 1536 pixels^2) was determined by superimposing a grid (area per point= 500pixels^2) onto the image sections at 40X magnification and using the cell count function of Fiji image analysis software to count the number of points hitting the red stained collagen. Eight (8) randomly selected sections per group were analysed and average counts were used for comparative analysis between groups.

3.5.3 Immunohistochemical labelling of caspase-3 cells

Slides for immunohistochemistry were coated with silane (Appendix 4) prior to tissue mounting to maximize cell adherence to slides. Fixed paraffin embedded liver and kidney tissue sections were de-paraffinized in xylene twice for 10 minutes. Sections were rehydrated through a series of graded alcohols followed by washing in 1M Phosphate buffer solution (pH 7.4) (Appendix 2.1) on a stirrer, for 5 minutes. Thereafter, sections were incubated in a solution containing 3% hydrogen peroxide in methanol for 30 minutes, with gentle agitation every few minutes. Tissue sections were washed in phosphate buffer solution (PBS) three times for 5 minutes each followed by incubation in 5% Normal goat serum for an hour. Thereafter, sections were incubated with anti-caspase-3 primary antibody (1:100 dilution, Ab 4059, Abcam) overnight at 4°C. Tissue sections were washed in PBS three times for 5 minutes and thereafter incubated with biotinylated goat anti-rabbit secondary antibody (1:1000 dilution, Vectastain, Vector Laboratories, Burlingame, CA) for 30 minutes. Sections were washed in PBS three times for 5 minutes, followed by incubation in Avidin-Biotin complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) for 30 minutes. Sections were washed in PBS three times for 5

minutes and then incubated with diaminobenzidine tetrachloride (DAB) working solution for another 5 minutes. Tissue sections were rinsed in running tap water for 5 minutes, counterstained in Hematoxylin for 3 minutes followed by washing in running tap water to remove the excess blue color. Tissue sections were subsequently hydrated in a series of graded alcohols, cleared in xylene and finally cover-slipped with Entellan New (Merck). To confirm the specificity of the anti-caspase-3 antibody, tonsil tissue was used as the positive control. The negative control included omission of the primary antibody and substituting it with PBS.

3.6 Statistical Analysis

Photomicrographs were taken using a Leica ICC50 HD video camera attached to a Leica DM500 light microscope and the Leica application suite (version 3.0.0, Build:629, LAS EZ, Switzerland) imaging software. Images were saved in jpeg format on the computer and analysed qualitatively and quantitatively. Fiji image analysis software was used to quantitatively analyse images. Statistical analysis was done using Statistica TM (StatSoft) and Graph Pad Prism version 7.0. All data were expressed as mean ± standard deviation (SD). One-way ANOVA was carried out, followed by a post-hoc Turkey's multiple tests, for statistical comparisons among the groups. The data obtained were presented in tables and graphs with Microsoft Excel and Graph Pad prism software for windows (version 7.0) respectively. $P < 0.05$ was considered as statistically significant.

4. RESULTS

4.1 Mortality

There was no incidence of mortality recorded in all the groups (A to D) throughout the 90 days exposure period.

4.2 Relative Mean Organ Weights

There were no significant differences observed in relative weights of the liver and kidneys of all treatment groups (Table 4.1).

Table 4.1: Effect of atrazine on the relative mean weights of the liver and kidneys of adult male *Xenopus laevis* frogs.

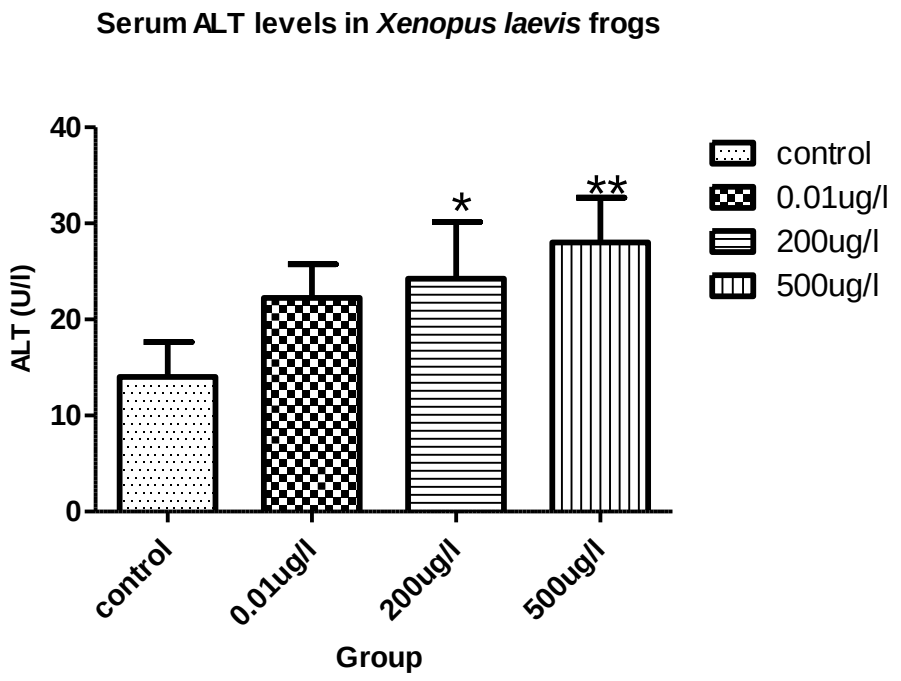
Organs	Control	(0.01µg/l)	(200µg/l)	(500µg/l)	P value
Liver	0.0142±0.008	0.0131±0.003	0.0157±0.005	0.0152±0.004	0.787
Kidney	0.0080±0.003	0.0091±0.002	0.0103±0.002	0.0095±0.002	0.247

The data are represented as mean ± SD (n = 10) and evaluated by one-way analysis of variance (ANOVA) confirmed by Turkey's test.

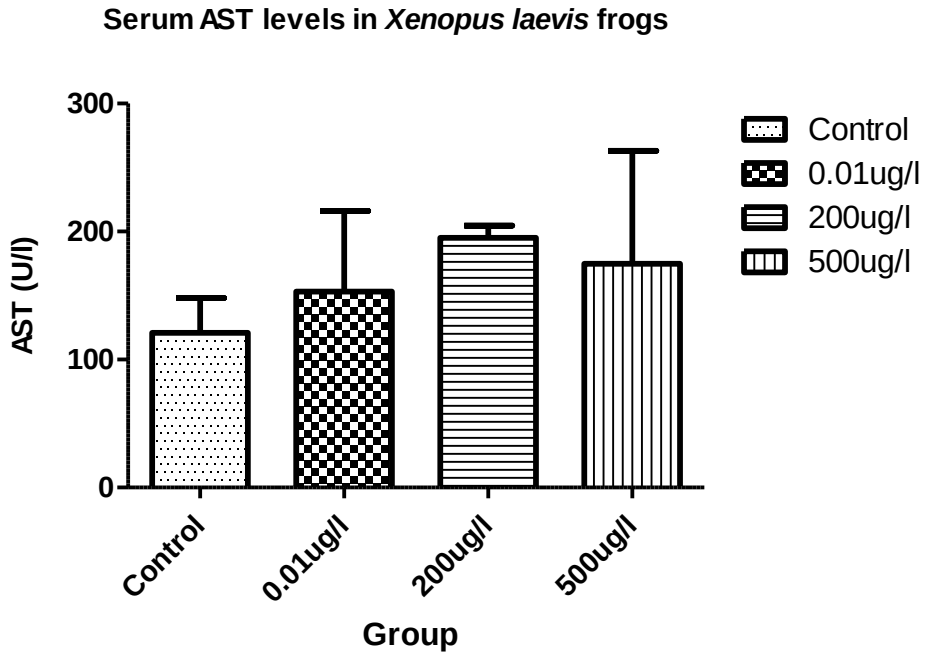
4.3 Serum Biochemical Parameters in Liver and Kidneys

The values of the biochemical analysis for the liver and kidney are presented in Figures 4.1 and 4.2 respectively. The serum levels of ALT in the frog groups exposed to ATZ concentrations of 200µg/l and 500µg/l were significantly higher ($P < 0.05$ and $P < 0.01$ respectively) compared with the control group (Figure 4.1a). However, there was no significant differences between frog groups exposed to ATZ concentrations of 200µg/l and 500µg/l ($P>0.05$). The levels of AST (Figure 4.1b) and ALKp (Figure 4.1c) non-significantly ($P> 0.05$) increased in all the treated groups when compared with the control group. There was a significant increase in the serum levels of creatinine in frog groups exposed to ATZ concentrations of 200µg/l ($P < 0.03$) and 500µg/l ($P < 0.002$) compared with the control group (Figure 4.2a). However, there was no significant difference between frog groups exposed to ATZ concentrations of 200µg/l and 500µg/l ($P> 0.05$). There was no significant difference in the serum levels of urea between all the frog groups treated with ATZ and the control group (Figure 4.2b).

(a)


Serum ALT levels in *Xenopus laevis* frogs
ALT (U/l)
40
30
20
10
0
*
**
control
0.01ug/l
200ug/l
500ug/l
Group
control
0.01ug/l
200ug/l
500ug/l

(b)


Serum AST levels in *Xenopus laevis* frogs
AST (U/l)
300
200
100
0
Control
0.01ug/l
200ug/l
500ug/l
Group
Control
0.01ug/l
200ug/l
500ug/l

(c)

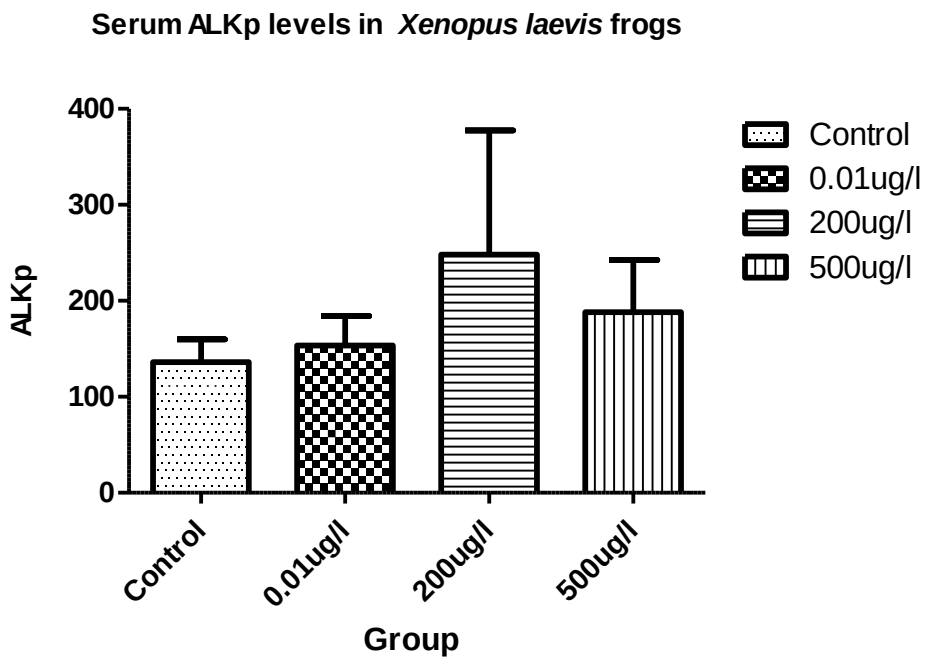
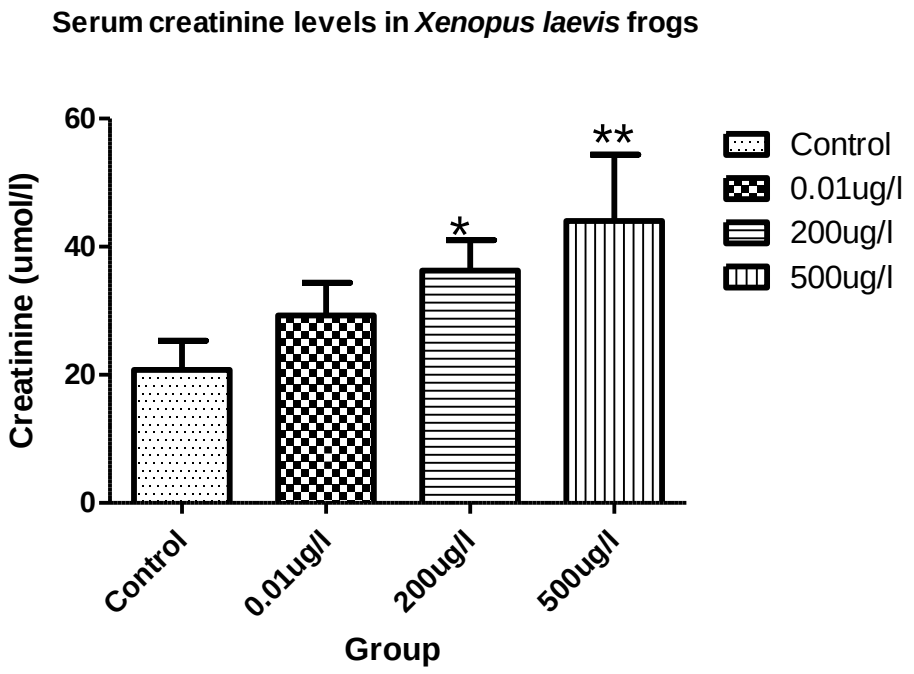


Figure 4.1: Effects of atrazine on serum levels of liver biomarkers (ALT, AST and ALKp) in adult male *Xenopus laevis* frogs.

The data are represented as mean ± SD (n=10). * means that the significance level was P< 0.05; **means that the significance level was P< 0.01. Absence of asterisk (*) indicates a non-significant difference between the control and the treated group. ALT-alanine aminotransferase, AST-aspartate aminotransferase, ALKp- alkaline phosphatase.

(a)



(b)

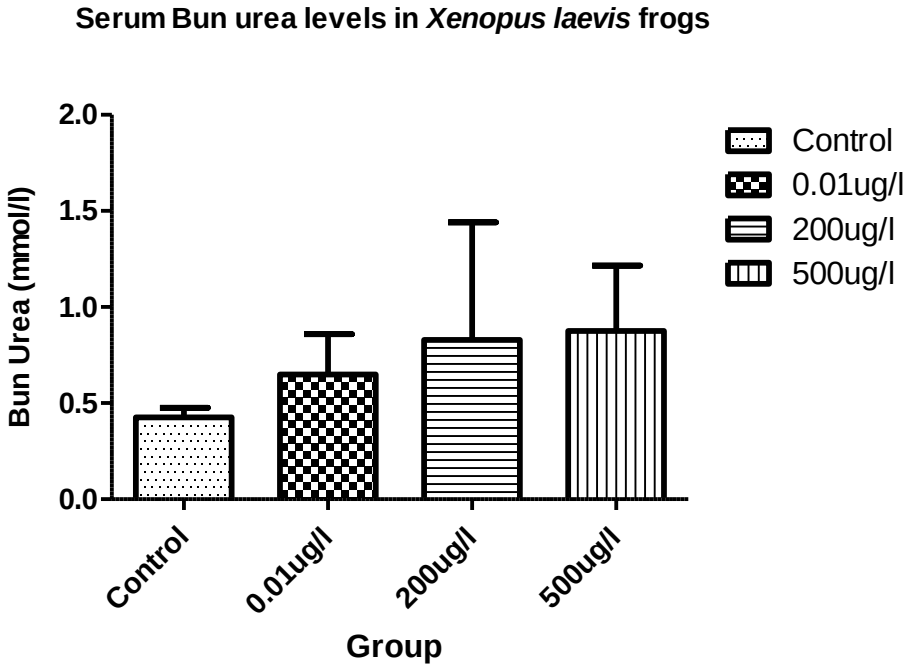


Figure 4.2: Effects of atrazine on serum levels of kidney biomarkers (creatinine and urea) in adult male *Xenopus laevis* frogs.

The data are represented as mean ± SD (n=10). * means that the significance level was P< 0.05. **means that the significance level was P< 0.01. Absence of asterisk (*) indicates a non-significant difference between the control and the treated group.

4.4 Lipid Peroxidation

Figure 4.3 shows the MDA peaks for the *Xenopus laevis* frog serum sample which were compared with MDA standard peaks in order to validate the protocol. A significant difference was observed ($P < 0.003$) between the serum levels of MDA in the frogs exposed to 500µg/l of ATZ and the control group (Figure 4.4).

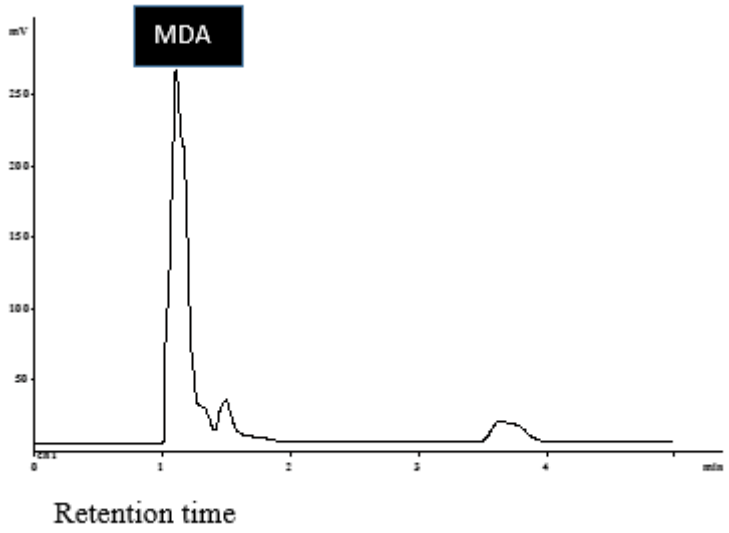


Figure 4.3: Chromatogram for the adult male *Xenopus laevis* frog serum sample.

Malondialdehyde peak (MDA) eluting at a retention time of 1.08 minutes after injection of serum sample.

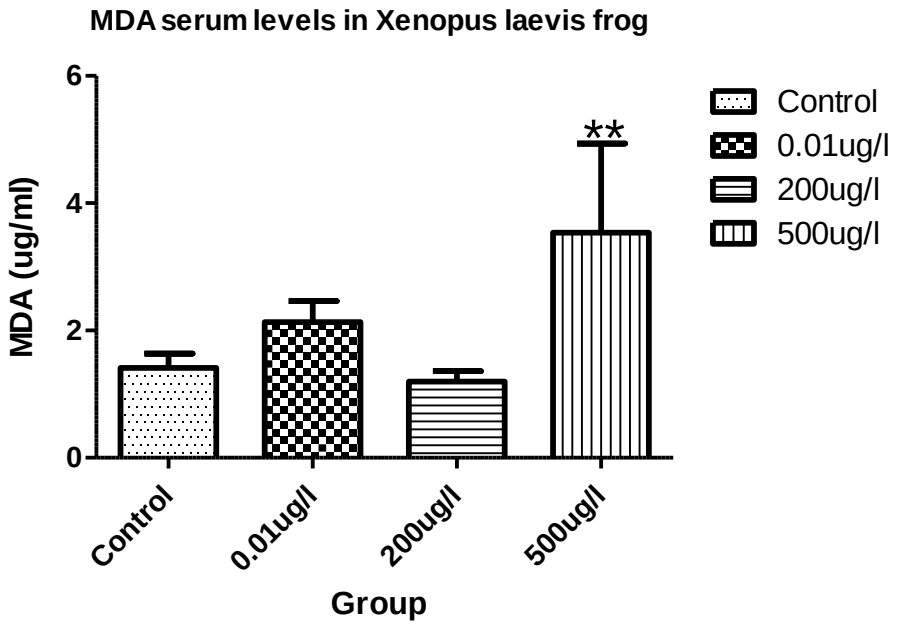


Figure 4.4: Effect of atrazine on lipid peroxidation (LPO) levels i.e. MDA content in adult male *Xenopus laevis* frogs.

The data are represented as mean ± SD (n=10). **means the significance level was $P < 0.01$. Absence of asterisk (*) indicates a non-significant difference between the control and the treated group.

4.5 Histopathological Findings

4.5.1 Liver morphology

The results of histological measurements in the liver are shown in Table 4.2. Compared to the control group, exposure of frogs to 200µg/l and 500µg/l of ATZ resulted in significant increases in hepatocyte width ($P < 0.0003$) in their livers.

Table 4.2: Morphometric evaluation of changes in liver histopathological parameters of adult male *Xenopus laevis* frogs.

Liver Parameter	Control	0.01µg/l	200µg/l	500µg/l	P value
Hepatocyte width/µm	267.7±68.92[a]	336.2±31.57[a]	411.8±39.39[b]	387±69.39[c]	0.0003
Connective tissue percentage area/µm^2	0.03±0.02[a]	0.12±0.14[a]	0.07±0.06[a]	0.09±0.06[a]	0.1621

Values are mean ± SD (n=10). Control values a, are significantly different from b and c (i.e. corresponding values in 200µg/l and 500 µg/l) of ATZ exposed groups respectively. TLOH-Thick loop of Henle.

Figures 4.5 and 4.7 show the photomicrographs of liver. In the control group, the liver parenchyma exhibited a normal microscopic structure (Figure 4.5, Figure 4.7A). Central veins (Cv) were surrounded by radiating anastomosing cords of hepatocytes (Figure 4.5A). Hepatocytes were clearly visible and contained one or two pale basophilic rounded nuclei and scanty cytoplasm (Figure 4.7A, red arrow). Hepatic cords were interspersed with narrow irregular hepatic sinusoids containing nucleated red blood cells (Figure 4.7 A, black arrow, insert). At the portal triad, branches of the hepatic artery, hepatic portal vein and bile duct lined by simple cuboidal epithelium were observed (Figure 4.5A, circled). Melanomacrophages (Figure 4.5A, red arrow) were distributed randomly throughout the interstitium.

Light microscopy revealed significant alterations in the livers of frogs exposed to atrazine. Hepatocyte cord arrangement was completely disorganized in the livers of frogs treated with 0.01µg/l (Figure 4.7B, insert) and 500µg/l of ATZ (Figure 4.7D). Hepatocyte hypertrophy was observed in the livers of frogs exposed to 200µg/l and 500µg/l of ATZ (Table 4.2, hepatocyte width; Figure 4.7C and 4.7D). Hepatocytes were slightly vacuolated in the livers of frogs treated with 200µg/l (Figure 4.7C, black arrow) of ATZ and highly vacuolated in the livers of frogs treated with 0.01µg/l (Figure 4.7B, black arrow) and 500 µg/l (Figure 4.7D, black arrow) of ATZ. Central veins in the livers of frogs treated with 0.01 µg/l (Figure 4.5B) and 500µg/l (Figure 4.5D) of ATZ were congested with blood. Central veins in the livers of frogs treated with 500µg/l ATZ were ruptured (Figure 4.6C). A decrease in sinusoids and dilated sinusoids (Figure 4.5C, black arrow) were observed in the livers of frogs treated with 200µg/l of ATZ. Sinusoidal congestion with blood and numerous haematopoietic cells (Figure 4.5D, insert) were observed in the livers of frogs treated with 500µg/l of ATZ. Portal neutrophil infiltration was observed in all the treated groups (Figure 4.5D, Figure 4.6A, B, red arrow). Infiltrating

inflammatory cells severely obscured some of the liver tissues of frogs treated with 500µg/l of ATZ (Figure 4.6D). Parenchyma necrotic areas were observed in the livers of frogs treated with 500µg/l ATZ (Figure 4.6C, circled).

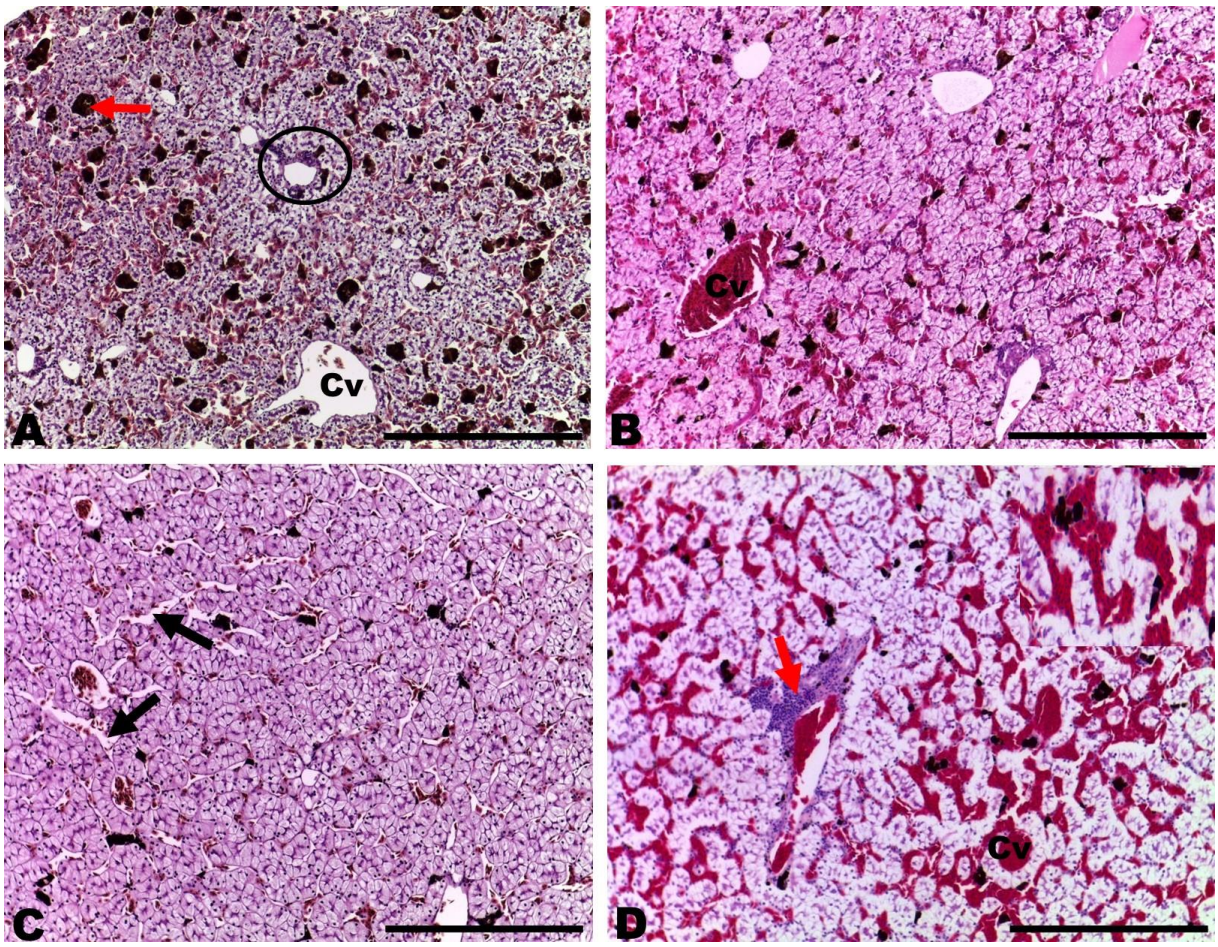


Figure 4.5: Representative photomicrographs of histopathological findings in frog liver following chronic exposure to atrazine.

(A) Control liver showing normal histological architecture. Normal central vein (Cv), portal triad (circle) and melanomacrophages (red arrow). (B) Liver sections of frogs treated with 0.01µg/l of ATZ. Congested central vein (Cv). (C) Liver sections of frogs treated with 200µg/l of ATZ. Dilated sinusoids (black arrow). (D) Liver sections of frogs treated with 500µg/l of ATZ. Central vein congestion (Cv), sinusoidal congestion (insert), portal inflammation (red arrow). Staining was performed on liver sections with the H&E stain. Scale bar in A to D= 240µm (10x magnification). Cv- Central vein.

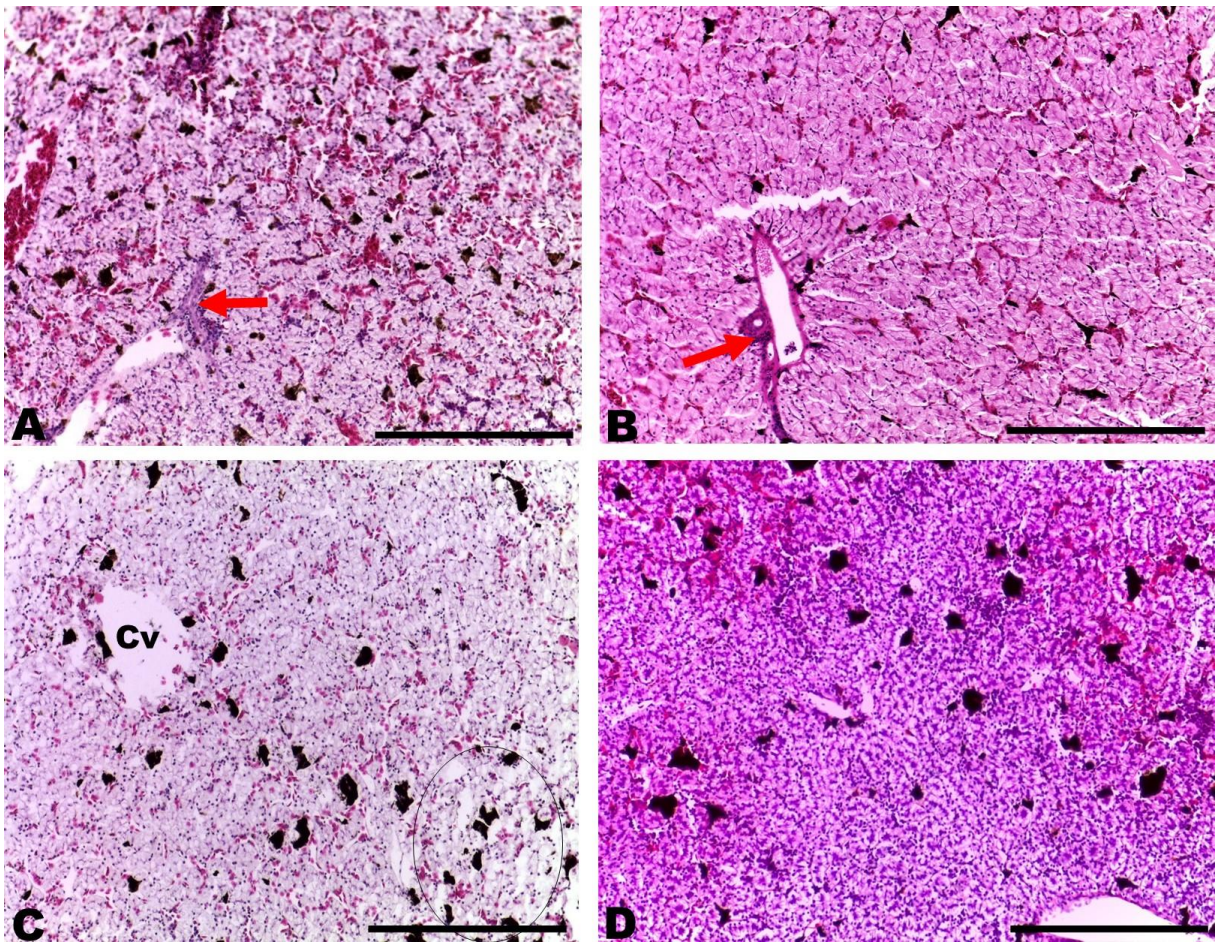


Figure 4.6: Representative photomicrographs of histopathologic findings in frog liver following chronic exposure to atrazine.

(A) Liver sections of frogs treated with 0.01µg/l of ATZ. Portal inflammation (red arrow). (B) Liver sections of frogs treated with 200µg/l. Portal inflammation (red arrow). (C) Liver sections of frogs treated with 500µg/l. Parenchymal (circle) and central vein (Cv) necrosis. (D) Liver sections of frogs treated with 500µg/l completely obscured with inflammatory cells. Staining was performed on liver sections with the H&E stain. Scale bar in A to D= 240µm (10x magnification). Cv- Central vein.

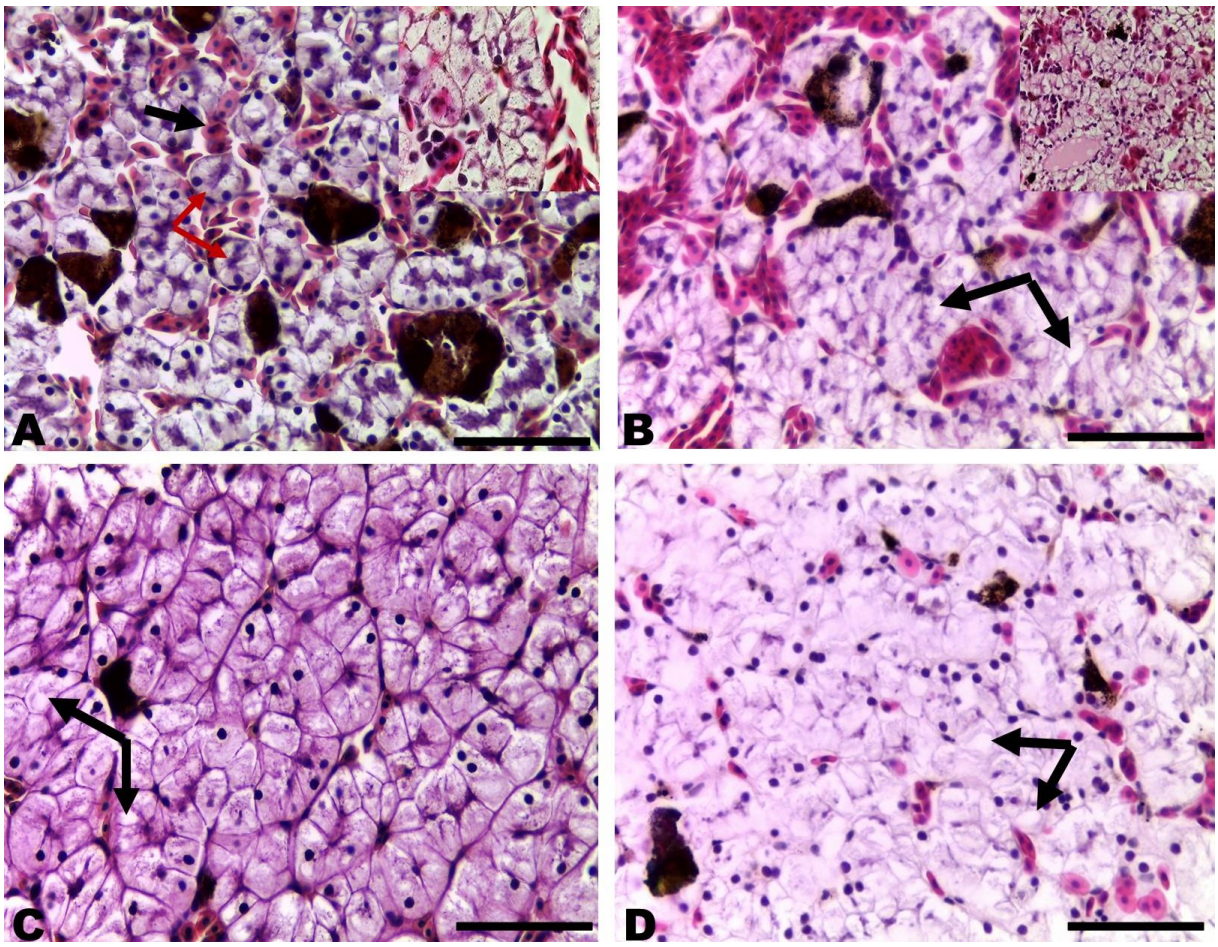


Figure 4.7: Representative photomicrographs of histopathologic findings in frog liver following chronic exposure to atrazine.

(A) Control liver showing normal histological architecture. Hepatocytes with one or two nuclei (red arrow) within cords alternating in arrangement with hepatic sinusoids (insert) with nucleated red blood cells (black arrow). (B) Liver sections of frogs treated with 0.01µg/l. Absence of normal hepatic cord arrangement (insert), vacuolated hepatocytes (black arrow). (C) Liver sections of frogs treated with 200µg/l. Hypertrophied and vacuolated hepatocytes (black arrow). (D) Liver sections of frogs treated with 500µg/l. Absence of hepatic cord arrangement. Highly vacuolated hepatocytes (black arrows). Staining was performed on liver sections with the H&E stain. Scale bar in A to D= 38µm (40x magnification).

4.5.2 Kidney morphology

The results of histological measurements in the kidney are shown in Table 4.3. Compared to the control group, significant increases in corpuscle perimeter in the kidney of frogs exposed to 500µg/l of ATZ was observed (P< 0.03). Epithelium height of proximal tubules in the 200µg/l ATZ exposed group and thick loop of Henle tubules in the 200µg/l and 500 µg/l ATZ exposed group was significantly decreased (P< 0.03 and P < 0.01 respectively) Non-significant alterations were found in the other parameters analysed.

Table 4.3: Morphometric evaluation of changes in kidney histopathological parameters of adult male *Xenopus laevis* frogs.

Kidney Parameter	Control	0.01 µg/l	200µg/l	500µg/l	P value
Glomerular count	12±9.84[a]	14.25±8.71[a]	9.13±8.98 [a]	5.5±3.42 [a]	0.1846
Corpuscle perimeter/µm	318.7±40.26 [a]	287.7±29.61 [a]	325.4±49.95[a]	260.4±30.79 [c]	0.0056
Bowman's tuft perimeter/ µm	252.3±80.73 [a]	234.8±21.18 [a]	257±37.65 [a]	208.9±38.43 [a]	0.2276
Bowman's tuft area/ µm^2	3957±1188 [a]	3814±906 [a]	3866±1079 [a]	3720±694 [a]	0.1034
Bowman's space area/ µm^2	2150±464 [a]	2563±1025 [a]	2325±826 [a]	1674±267 [a]	0.1053
Proximal epithelium height/ µm	10.85±1.86 [a]	7.986±0.90 [a]	7.038±2.63[b]	11.79±3.73 [a]	0.0244
Proximal luminal perimeter/ µm	46.78±1.67 [a]	38.21±8.16 [a]	39.51±7.33 [a]	42.92±10.27 [a]	0.1283
Proximal luminal area/ µm^2	146.2±21.45 [a]	104.2±49.86 [a]	113.4±41.15 [a]	133.6±58.24 [a]	0.2504
TLOH epithelium height/ µm	19.68±4.62 [a]	15.71±3.28 [a]	15.01±1.71[b]	13.88±0.94[c]	0.0036
TLOH luminal perimeter/ µm	92.74±15.51 [a]	76.06±10.97 [a]	74.17±14.17 [a]	79.2±23.56 [a]	0.1341
TLOH luminal area/ µm^2	561.7±243.60 [a]	397.1±97.80 [a]	382.2±163.30 [a]	436.4±275.30 [a]	0.3136

Values are mean ± SD (n=10). Control values a, are significantly different from b and c (i.e. corresponding values in 200µg/l and 500 µg/l) of ATZ exposed groups respectively. TLOH-Thick loop of Henle.

In the photomicrograph of the kidneys, the control group showed normal structures of renal corpuscles (Figure 4.8A, insert) and renal tubules (Figure 4.9A). Renal corpuscles, tufts of blood capillaries (the glomerulus) were surrounded by a Bowman's capsule. The Bowman's capsule comprised an inner visceral layer characterized by podocytes and an outer parietal layer consisting of squamous epithelial cells (Figure 4.8A, insert). The Bowman's space, that is, the space between the parietal and visceral layer, was well defined (Figure 4.8A, insert).

Proximal tubules (Figure 4.9, black arrow) and thick loop of Henle (Figure 4.9A) were lined by simple short columnar cells with distinct cell boundaries and spherical nuclei. The superficial region of the thick loop of Henle had well-developed brush borders that almost filled the lumen. Distal tubules were lined by simple cuboidal epithelial cells with spherical centrally placed nuclei (Figure 4.9A, red arrowhead). Collecting tubules consisted of stratified, cuboidal epithelial cells with oval to round centrally placed nuclei (Figure 4.9A, white arrow). Hematopoietic cells occupied inter-tubular space and were beset with numerous red nucleated blood corpuscles (Figure 4.9A, red arrow).

All the groups exposed to ATZ showed degeneration of the renal corpuscles (Figure 4.8B, C, D, black arrowhead). Glomerular atrophy was observed in all the treated groups (Figure 4.8B, C, D, black arrowhead). The lumen of tubules in the kidneys of frogs exposed to 500µg/l of ATZ were dilated (Figure 4.8D, black arrow). Tubular degeneration and loss of epithelial cell-cell border of the thick loop of Henle was observed in the kidneys of frogs treated with 200µg/l (Figure 4.9B, black arrowhead) and 500 µg/l (Figure 4.9C, black arrowhead) of ATZ. Vacuoles within the epithelial cells of tubules were noted in the kidney of frogs treated with 200µg/l of ATZ (Figure 4.9B, black arrow). The brush borders of thick loop of Henle tubules of frog kidneys exposed to 500µg/l were destructed (Figure 4.9D, arrowhead). Furthermore,

some tubules were replaced by eosinophilic material and the lumina of these tubules became relatively small (Figure 4.9C, asterisk). Mild peritubular haemorrhage in kidney tissues of frogs exposed to 0.01 µg/l (Figure 4.8B, H) and 200 µg/l (Figure 4.8C, H) of ATZ was observed.

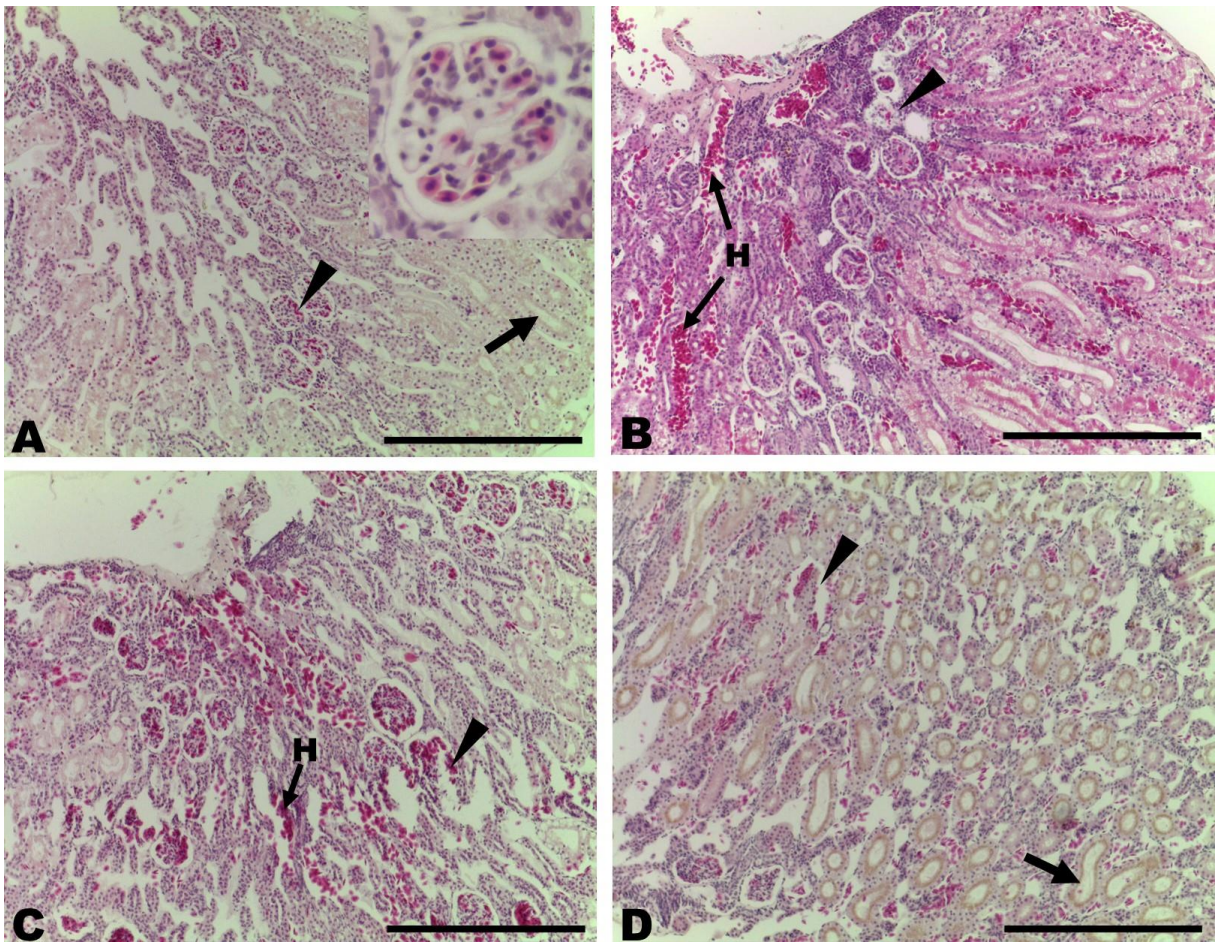


Figure 4.8: Representative photomicrographs of histopathologic findings in frog kidneys following chronic exposure to atrazine.

(A) Control kidney showing normal histological architecture. Intact glomeruli (insert), tubules (arrowhead). (B) Kidney sections of frogs treated with 0.01µg/l of ATZ. Glomeruli atrophy and necrosis (arrowhead), peritubular haemorrhage (H). (C) Kidney sections of frogs treated with 200µg/l of ATZ. Glomeruli atrophy and necrosis (arrowhead), peritubular haemorrhage (H). (D) Kidney sections of frogs treated with 500µg/l of ATZ. Mild glomerular atrophy (arrowhead). Dilated tubules (black arrow). Staining was performed on liver sections with the H&E stain. Scale bar in A to D= 240µm (10x magnification). H- haemorrhage.

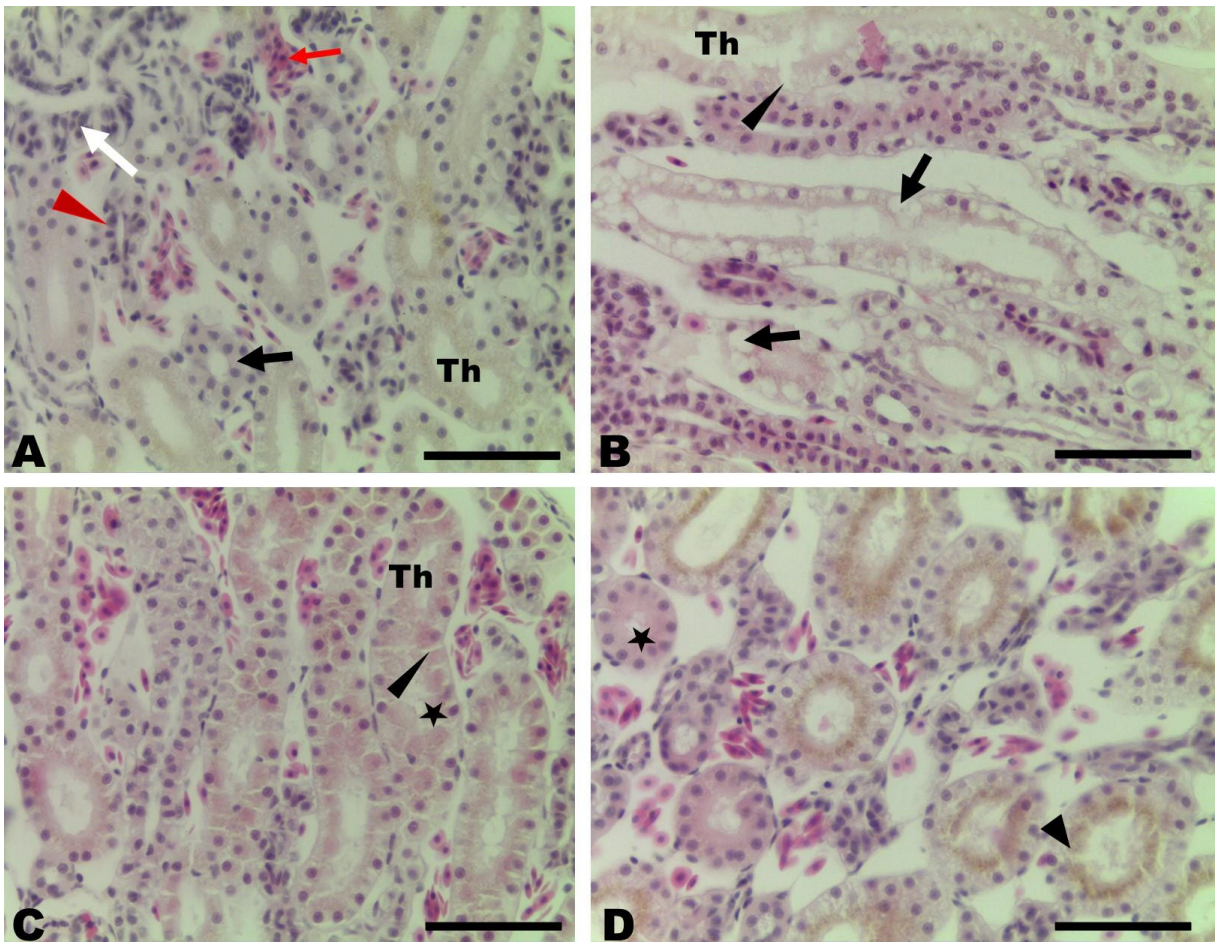


Figure 4.9: Representative photomicrographs of histopathologic findings in frog kidneys following chronic exposure to atrazine.

(A) Control kidney showing normal histological architecture. Proximal (black arrow), distal (red arrowhead), thick loop of Henle (Th) and collecting tubules (white arrow), inter-tubular space with nucleated red blood cell (red arrow). (B) Kidney sections of frogs treated with 200µg/l of ATZ. Thick loop of Henle tubule degeneration (Th), vacuolated tubules (black arrow head). (C) Kidney sections of frogs treated with 500µg/l. Thick loop of Henle epithelial cell-cell boarder loss (black arrowhead). Eosinophilic deposits (asterisk) inside the lumen of thick loop of Henle tubules (Th). (D) Kidney sections of frogs treated with 500µg/l. Loss of epithelial cell apical brush boarder (black arrowhead) and eosinophilic deposits (asterisk) inside the proximal tubules. Staining was performed on liver sections with the H&E stain. Scale bar in A to D= 38µm (40x magnification). Th- Thick loop of Henle.

4.6 Connective Tissue Analysis

4.6.1 Liver

There was no significant accumulation of collagen observed in the liver of all the treated groups when compared with the control group ($P > 0.05$). However, most of the collagen observed was found within the portal triads (Figure 4.10B, C, D: red arrow) and sinusoids (Figure 4.11B, C, D: red arrow) of the liver tissues from frogs exposed to ATZ. There were no collagen depositions within the hepatic lobules.

Table 4.4: Collagen area fractions in the livers of male adult *Xenopus laevis* frogs.

Parameter	Control	0.01µg/l	200µg/l	500µg/l	P value
Collagen area fraction	0.02±0.03	0.04±0.04	0.05±0.06	0.07±0.07	0.5644

The data obtained are presented as mean ± SD (n = 10). No significant differences observed in mean liver (p value= 0.5644).

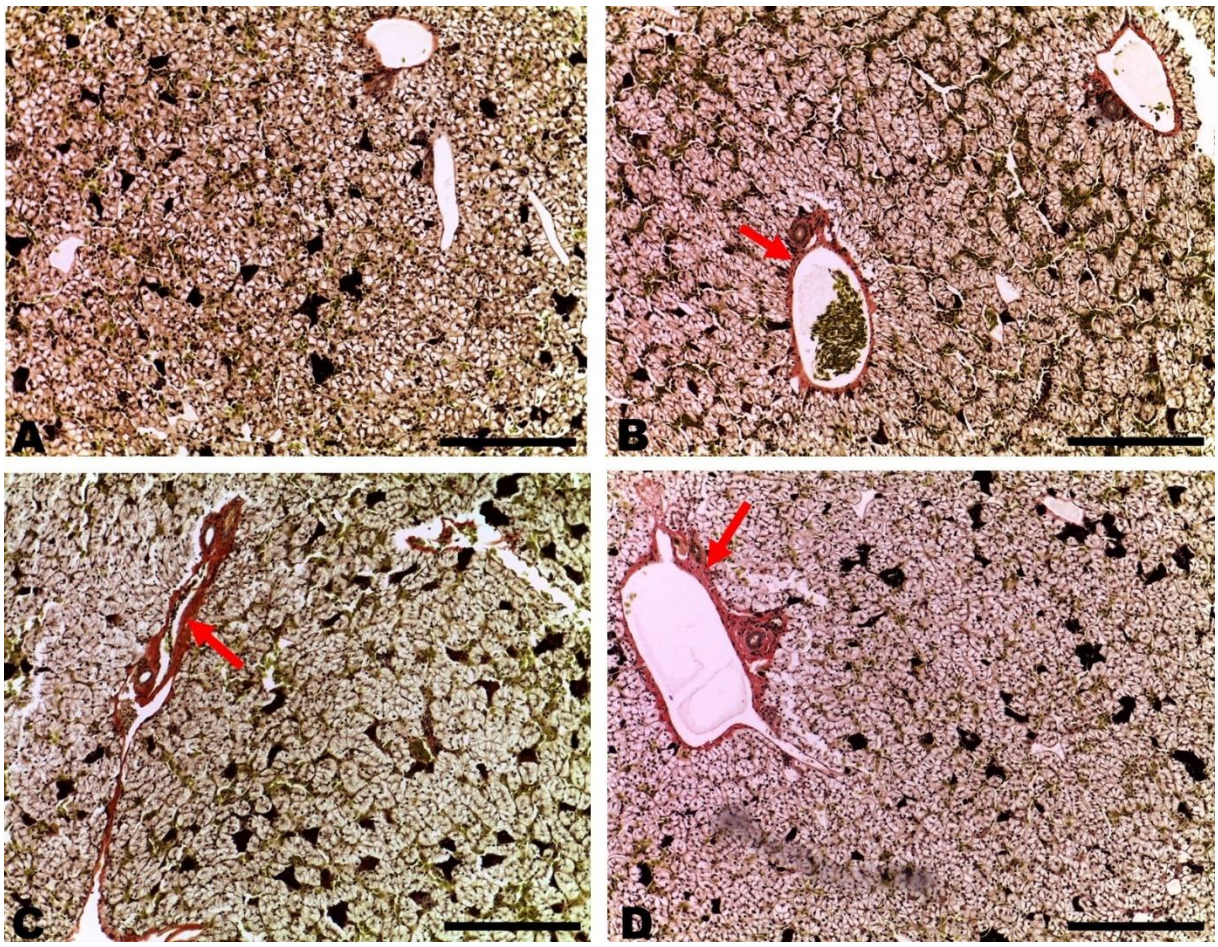


Figure 4.10: Representative photomicrographs of connective tissue accumulation in frog liver following exposure to atrazine.

(A) Control liver with normal connective tissue accumulation content. (B, C and D) Peri-portal fibrosis (red arrow)) in the liver of frogs treated with (B) 0.01µg/l), (C) 200µg/l and (D) 500µg/l of ATZ. Staining was performed on liver sections with the Van Gieson stain. Scale bar in A to D= 38µm (40x magnification).

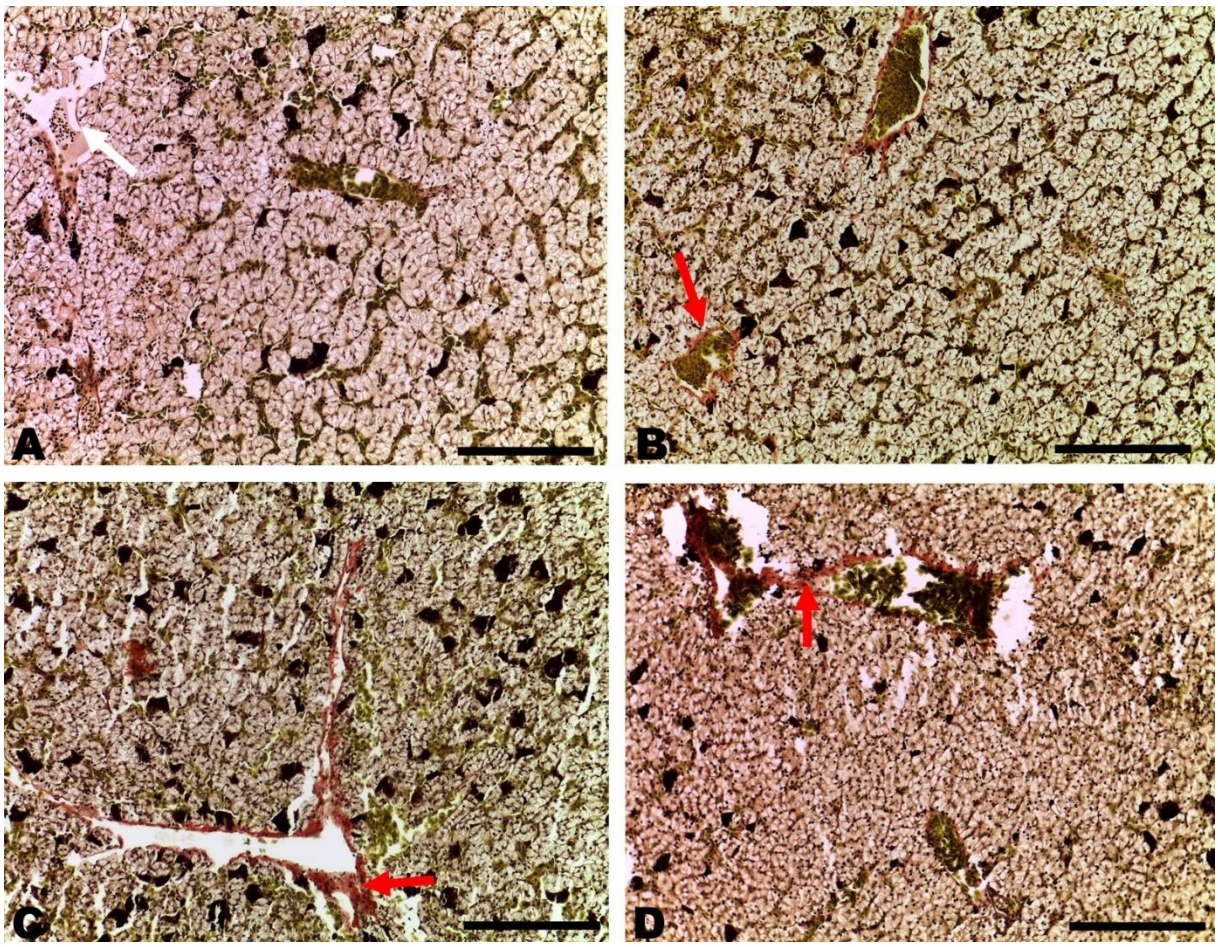


Figure 4.11: Representative photomicrographs of connective tissue accumulation in frog liver following exposure to atrazine.

(A) Control liver with normal connective tissue content. (B, C and D) Peri-sinusoidal fibrosis (red arrow)) in the liver of frogs treated with (B) 0.01µg/l), (C) 200µg/l and (D) 500µg/l of ATZ. Staining was performed on liver sections with the Van Gieson stain. Scale bar in A to D= 38µm (40x magnification).

4.6.2 Kidneys

Fibrosis in the kidney tissue was qualitatively analysed. There was no connective tissue accumulation observed within the interstitium, surrounding nephrotic tubules or within the renal corpuscles of all the frog groups (Figure 4.12B, C, D). Therefore, quantitative analysis was not conducted.

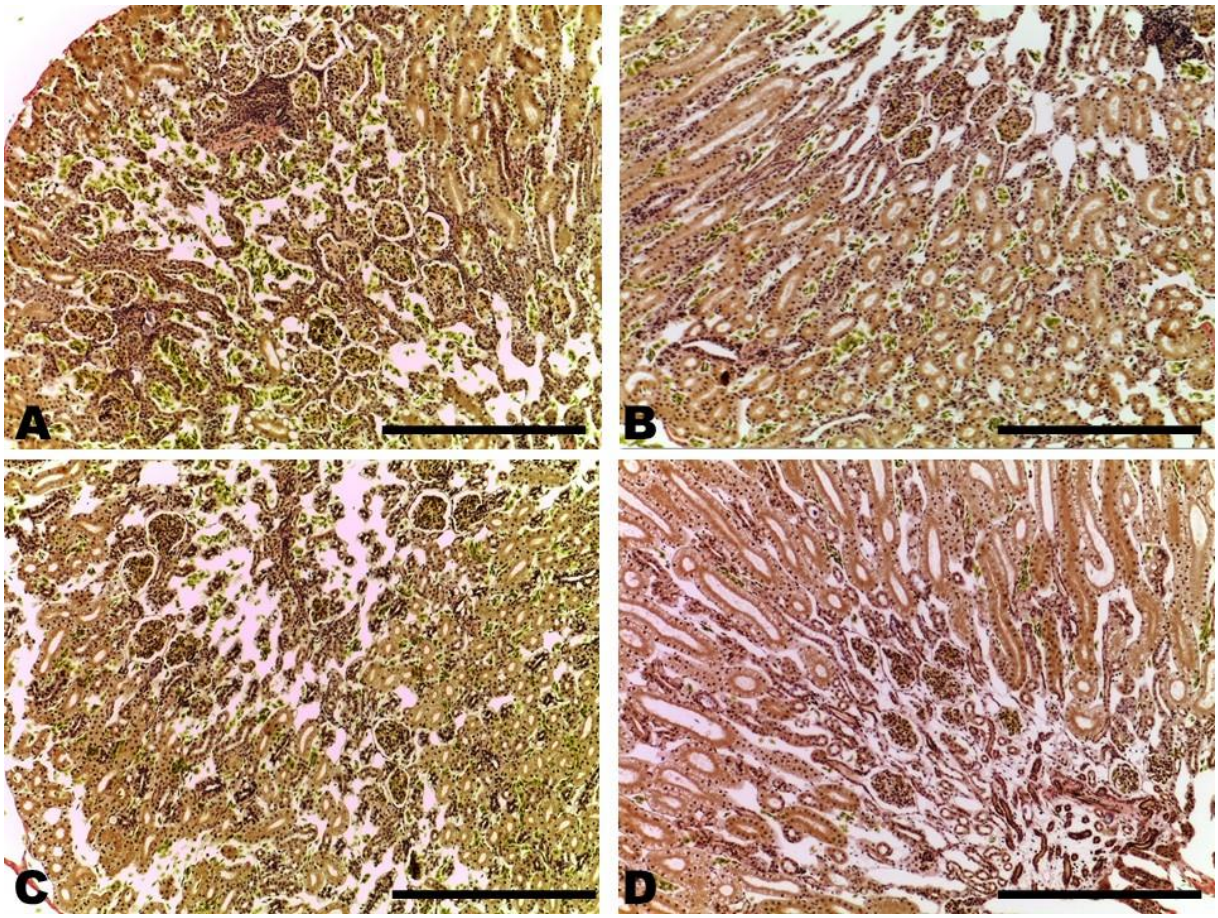


Figure 4.12: Representative photomicrographs of connective tissue accumulation findings in frog kidney following exposure to atrazine.

(A) Control liver with normal connective tissue content. (B) 0.01µg/l, (C) 200µg/l and (D) 500µg/l showing absence of connective tissue pathological accumulation within the interstitium. Staining was performed on liver sections with the Van Gieson stain. Scale bar in A to D= 240µm (10x magnification).

4.7 Immunohistochemical Findings

The immunoreactivity of caspase-3 was observed focally in the cytoplasm of cells in both the liver and kidney tissues. Results for negative controls showed no positive immuno-reaction (Figure 4.13A, Figure 4.15A, Figure 4.16A).

In the liver of frogs treated with ATZ, caspase-3 immuno-positive staining was seen within the peri-sinusoidal space, in Kupffer cells surrounding the hepatocytes (Figure 4.13B, C, D. red arrow) and in monocytes within the sinusoids (Figure 4.13B, D. black arrow) and sinusoidal endothelial cells (Figure 4.13B, C, D. blue arrowhead). In the liver of frogs exposed to 0.01µg/l (Figure 4.13E, arrowhead) and 500µg/l (Figure 4.13F, arrowhead) of ATZ, caspase-3 immuno-positive staining was seen in epithelial cells of bile ducts. There was no immuno-positive staining of caspase-3 in the cytoplasm of hepatocytes in all the groups. There was a significant decrease ($P < 0.0001$) in the number of melanomacrophages (MMCs) in the liver of frogs exposed to 0.01µg/l and 500µg/l of ATZ when compared with the control group (Figure 4.14). MMCs were significantly increased ($P < 0.03$) in the liver of frogs exposed to 200µg/l of ATZ compared to the control group.

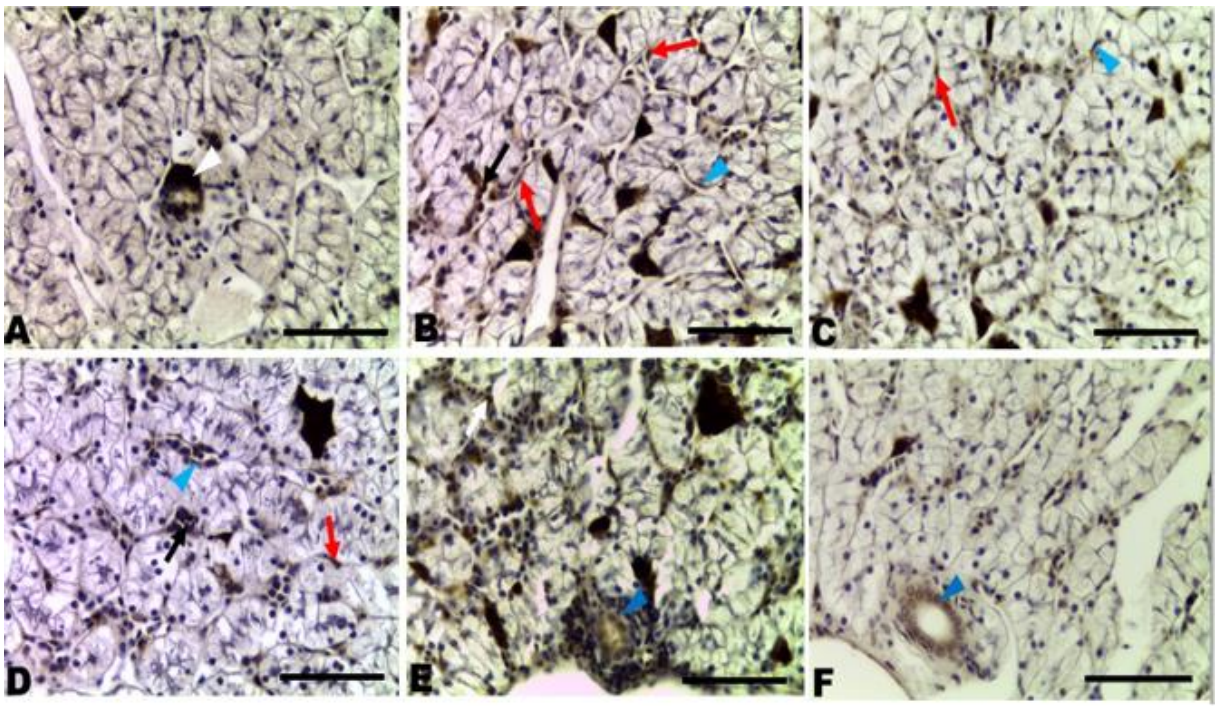


Figure 4.13: Representative photomicrographs of the cytoplasmic expression of caspase-3 in frog liver cells following chronic exposure to atrazine.

(A) Control liver tissue section showing melanomacrophages (arrowhead) and absence of positive caspase-3 expression. (B, C and D) Caspase-3 immuno-positive staining in monocytes in sinusoids (black arrow), Kupffer cells in the peri-sinusoidal space (red arrow) and flattened squamous endothelial cells lining sinusoids (blue arrowhead) in the liver of frogs treated with (B) 0.01µg/l), (C) 200µg/l and (D) 500µg/l of ATZ. (E and F) Caspase-3 immuno-positive staining in epithelial cells of bile ducts in the liver of frogs treated with (E) 0.01µg/l and (F) 500µg/l of ATZ. Staining was performed on liver sections with diaminobenzidine tetrachloride (DAB) stain. Scale bar in A to F= 38µm (40x magnification).

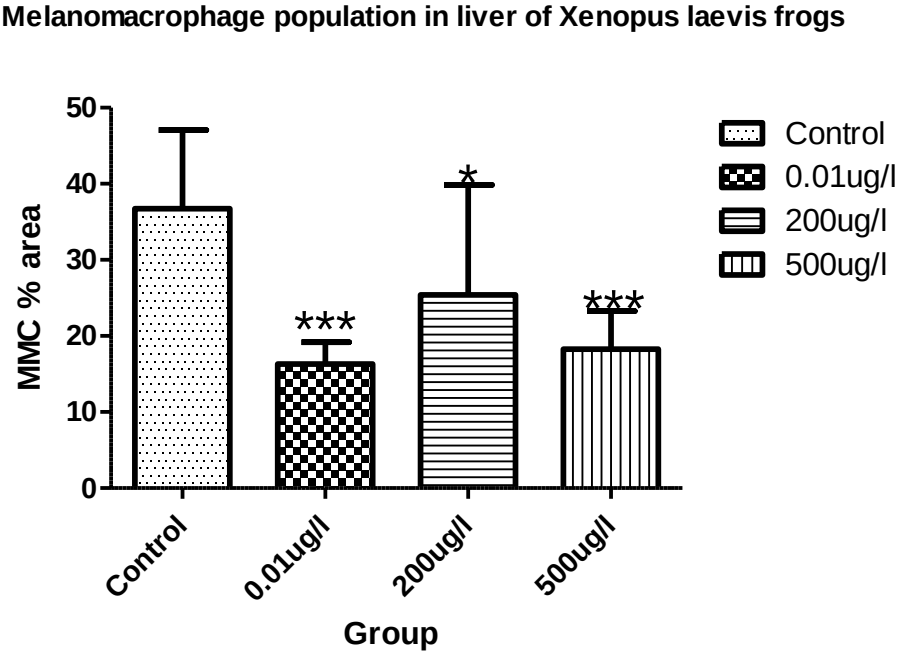


Figure 4.14: The effects of atrazine on the population (%) of melanomacrophages in frogs' exposure to atrazine compared to the controls.

Data presented as mean ± SD (n = 10). * means that the significance level was P< 0.05; ***means that the significance level was P< 0.001. Absence of asterisk (*) indicates a non-significant difference between the control and the treated group.

In kidneys of frog groups treated with ATZ, the expression of immuno-positively stained caspase-3 was seen in either the cortical and/or medullary regions (Figure 4.15B, C, D). Caspase-3 was intensely immuno-positively labelled in the epithelial cells of cortical proximal (Figure 4.16B, C, E. black arrow) distal (Figure 4.16B, C, E. red arrow) and collecting tubules (Figure 4.16B, C, E. white arrow) of all the treated groups. A very weak immuno-positive expression of caspase-3 was observed in the glomeruli of kidneys of frogs treated with 0.01µg/l of ATZ (Figure 4.16G). Intense caspase-3 immuno-positive expressions were observed in podocytes (black arrowhead) within the visceral layer, simple squamous epithelial cells (white arrowhead) of the parietal layer and within the macula densa cells (circle) at the vascular pole of glomeruli of kidneys from frogs treated with 200µg/ (Figure 4.16D) and 500µg/l (Figure 4.16E) of ATZ.

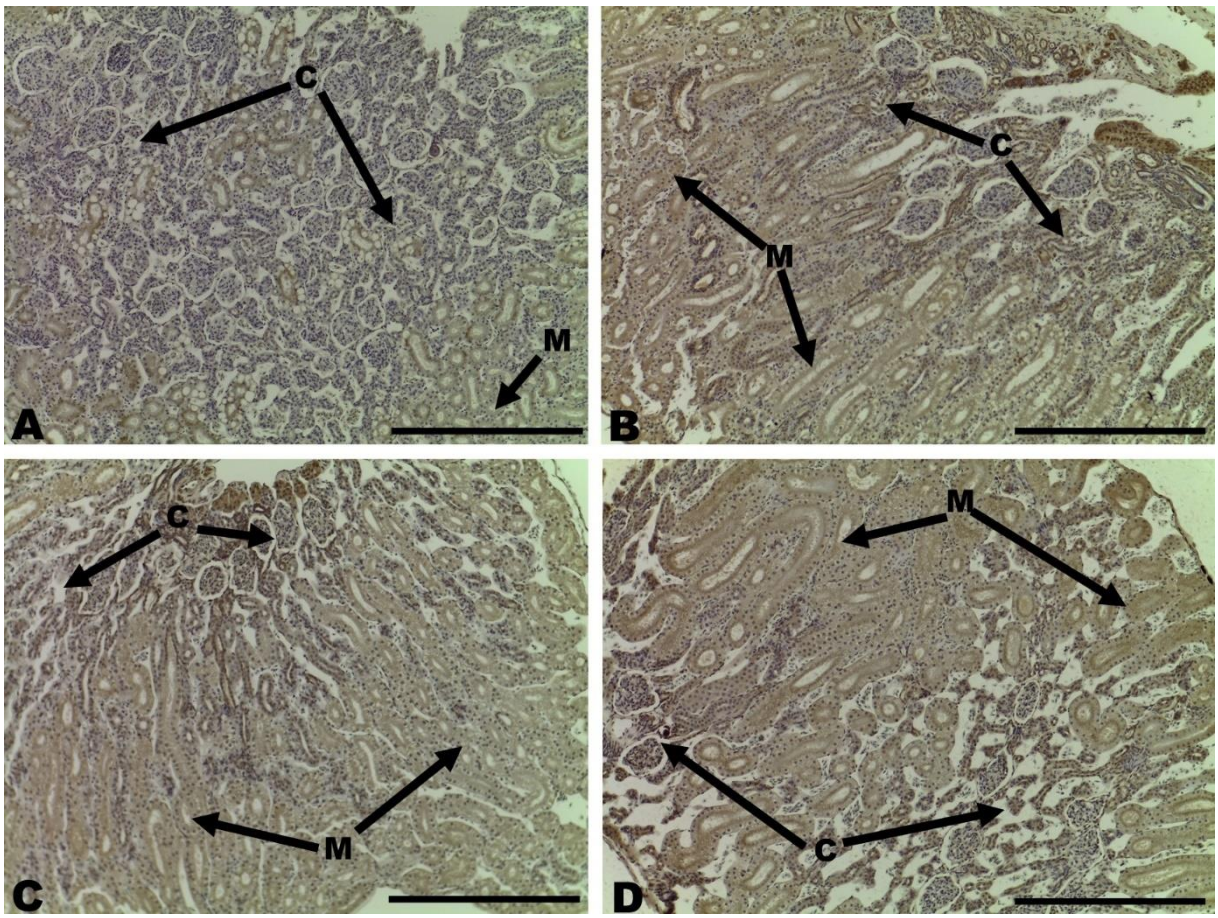


Figure 4.15: Representative photomicrographs of the cytoplasmic expression of caspase-3 in frog kidneys following chronic exposure to atrazine.

(A) Control kidney tissue section showing absence of immuno-positive caspase-3 expression. (B, C and D) Immuno-positive caspase-3 expression in the cortex and medulla of kidney tissues of frogs treated with (B) 0.01µg/l, (C) 200µg/l and (D) 500µg/l of ATZ. Staining was performed on liver sections with diaminobenzidine tetrachloride (DAB). Scale bar in A to D= 240µm (10x magnification). C- Cortex, M- Medulla.

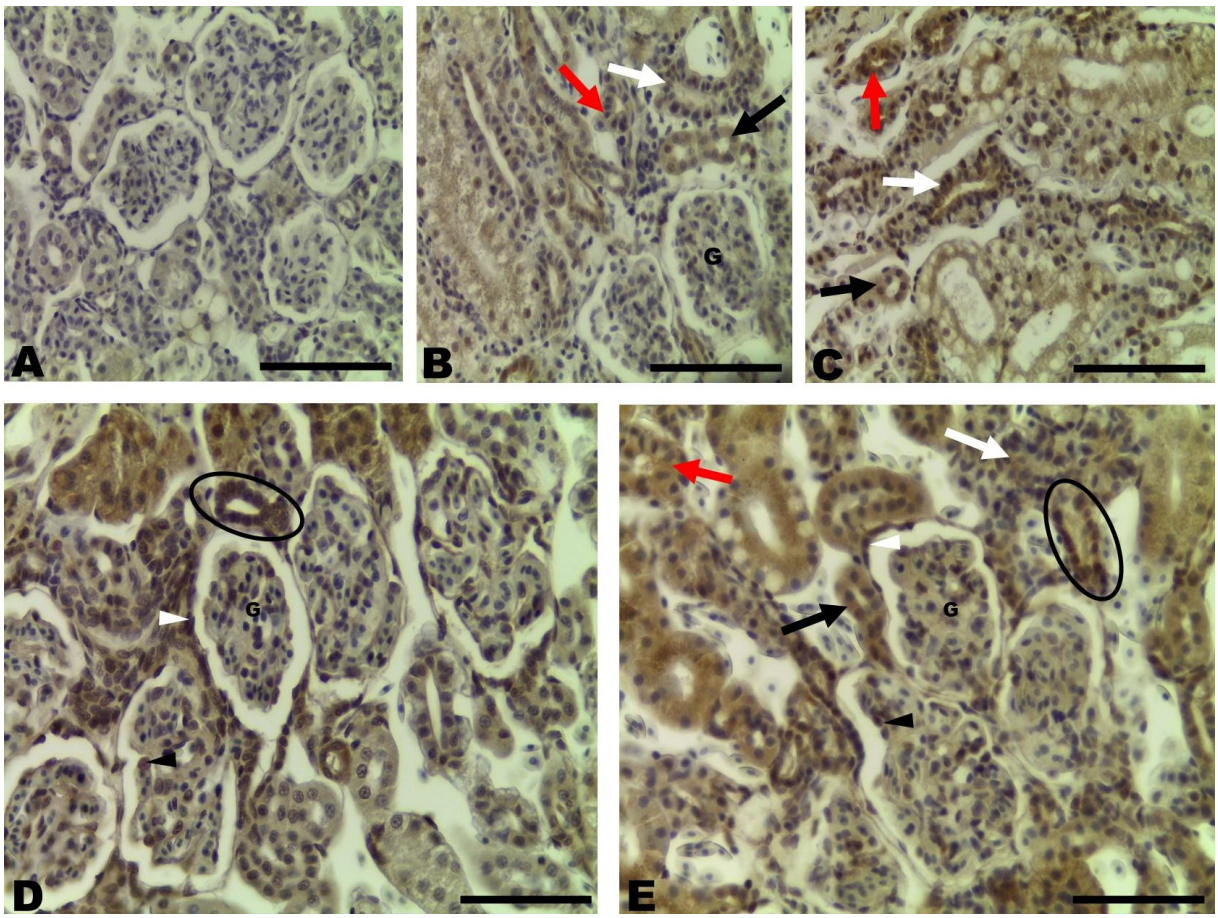


Figure 4.16: Representative photomicrographs of the cytoplasmic expression of caspase-3 in frog kidney following chronic exposure to atrazine.

(A) Control kidney tissue section showing absence of immuno-positive caspase-3 expression. (B, C and E) Immuno-positive caspase-3 expression in epithelial cells of proximal (black arrow), distal (red arrow) and collecting tubules (white arrow) in kidneys of frogs treated with (B) 0.01µg/l, (C) 200µg/l and (E) 500µg/l of ATZ. (D, E) Immuno-positive caspase-3 localization in macula densa cells (circle), podocytes (black arrowhead) and epithelial cells within the parietal layer (white arrowhead) of glomeruli of frogs treated with (D) 200µg/l and (E) 500µg/l (G). Staining was performed on liver sections with diaminobenzidine tetrachloride (DAB). Scale bar in A to E = 38µm (40x magnification).

5. DISCUSSION

In recent years, the extensive use of a wide variety of agricultural pesticides at lethal or sub-lethal concentrations has been cited as one of the factors contributing to environmental chemical pollution (Institute of Medicine (US), 1994; USEPA, 2002; Ross & Amter, 2010; IUCN, 2011; Sánchez-Bayo & Ortega, 2014). The indiscriminate use of pesticides in agricultural operations has resulted in an immense disruption of environmental and ecological balance and interference with physiological and biochemical parameters in non-targeted aquatic organisms, affecting functions such as growth, development and reproduction (Upasani & Balaraman, 2001; Venkataramana, et al., 2006). In *Xenopus laevis* frogs, studies have shown that atrazine has the potential to cause adverse effects on reproductive and endocrine systems (Hecker, et al., 2004; Jooste, et al., 2005; Hayes, et al., 2006a; Hayes, et al., 2010). Recent studies have indicated oxidative stress as a proxy in determining pesticide toxicity and evaluating biomarkers in tissues such as brain, liver and kidney (Abarikwu, 2014; Sinhorin, et al., 2014; Dornelles & Oliveira, 2015). This study therefore evaluated the potential adverse effects of chronic exposure to ATZ on hepatic and renal function of post-metamorphic *Xenopus laevis* frogs using the histo-architecture, biochemical and oxidative stress parameters.

5.1 Atrazine and Lipid Peroxidation

The mechanisms of pesticide toxicity are being linked to lipid peroxidation (Singh, et al., 2011) which is one of the hallmarks of oxidative stress in aquatic organisms (Valavanidis, et al., 2006; Nwani, et al., 2015). In the present study, the serum levels of MDA were significantly elevated in the group exposed to 500µg/l of ATZ when compared to the control group. Non-significant MDA increases were also observed in the group exposed to 0.01µg/l of ATZ. The present study is one of the very few amphibian studies where absolute levels of free MDA have been measured using the high-performance liquid chromatography method. Thus, results may differ from results from studies where tissue homogenates were used and where MDA levels were measured using the TBARS method. The increases in MDA levels suggest ATZ-induced lipid peroxidation in tissues of *Xenopus laevis* frogs. The results of the present study are similar to those reported on bullfrog tadpoles exposed to ATZ concentrations of 2.5 µg/l for 7 days, which showed an increase in lipid peroxidation (measured using TBARS method; TBA-reactive substances) in the liver, gills and muscle tissue (Dornelles & Oliveira, 2015). The discrepancies in dose-responses between the 500µg/l ATZ treated group and the 2.5µg/l ATZ treated bullfrog tadpoles could be due to differences in age at time of exposure and exposure

period. It seems that ATZ generally induce MDA increase irrespective of dose levels as seen in 0.01µg/l and 500µg/l exposed groups. However, the decrease in MBA level in 200µg/l treated group may be attributed to homeostatic adjustment by the animal following the chemical exposure. Toxicants are mostly dangerous if present during early animal development because organogenesis is carefully regulated during that time (Johansson, *et al*., 2006; F, 2008; David & Kartheek, 2015). The *Bullfrog* tadpoles could have exhibited a significant stage-specific sensitivity to acute atrazine exposure which could have led to oxidative stress and more pronounced levels of lipid peroxidation than in adult frogs (Abarikwu, et al., 2010).

Increases in MDA levels have been reported in rats (Abarikwu, 2014; Jestadi, et al., 2014), following acute exposure to ATZ. Generally, an increase in MDA content in tissues may be attributed to the increased oxidation of polyunsaturated fatty acids in animal (frog's) organ tissues due to increased ROS production following ATZ exposure. ROS are normal products of aerobic metabolism whose primary sources are the lipid rich structures such as mitochondria, plasma membrane, endoplasmic membrane and peroxisomes (Moldovan & Moldovan, 2004). However, in pathological conditions, an increase in ROS production can surpass the body's antioxidant defense system, resulting in a chain reaction of oxidation and consequently oxidative stress (Poli, et al., 1987; Jin, et al., 2010b; Dornelles & Oliveira, 2013), leading to disturbances of homeostatic balance.

However, a non-significant decrease in serum MDA levels observed in the group treated with 200µg/L of ATZ is in agreement with an earlier report on MDA level decreases in neonatal fish exposed to a low dose of ATZ (Santos & Martinez, 2012). Previous reports suggested that this reduction may be related to the removal of excess ROS by anti-oxidant enzymes. A number of studies have shown that various concentrations of ATZ can promote oxidative stress by increasing the concentration of ROS and products of oxidative damage and at the same time influence antioxidant enzymes activity (Elia, et al., 2002; Song, et al., 2009). Antioxidant defense enzymes such as SOD, GST, GSH and catalase are mobilized in order to remove ROS and free radicals that would have been generated during oxidation, thus, preventing lipid peroxidation and subsequently oxidative stress.

According to Santos and Martinez (2012), a decrease in ROS production and hence MDA levels, may be an indication of atrazine's capabilities to reduce metabolism and biotransformation processes in liver cells, ultimately impacting negatively on the overall body homeostasis. Reduced metabolism in liver cells would reduce ROS production, prevent or reduce lipid peroxidation, thus preventing increases in serum levels of MDA as a consequence of the ATZ exposure. Additionally, while there was no observed dose-response relationship in the present results, Nwani et al. (2010) and Liu et al. (2014) revealed in their reports a dose-dependant increase in MDA concentrations in rat kidney and fish liver respectively. The differences between these results may be species dependant and/or due to differences in the methods (HPLC versus the TBARS technique) used in each study.

5.2 Atrazine and Liver Biomarkers

The liver is the primary site of metabolism, detoxification and excretion of potentially toxic substances (Hall & Guyton, 2011). Several enzymatic liver biomarkers are being assessed in order to determine liver function and extent of injury (Regnault, et al., 2014; Mossaa, et al., 2015; Medina, et al., 2016). In the current study, the serum levels of ALT, AST and ALKp were determined in order to assess liver function and any ATZ induced injury. The significant increase in the serum levels of ALT observed in the groups exposed to 200µg/l and 500 µg/l of ATZ was inconsistent with the non-significant ALT increases reported by Campos-Pereira et al. (2012) following their exposure of 400µg/kg body weight ATZ to rats. The contrast could be due to differences in the experimental animal species used, modes of ATZ exposure and the timing of exposure. The blood levels ALT, AST and ALKp are used as markers of cell membrane integrity (Jestadi, et al., 2014; Hozyen, et al., 2015). ALT is a cytoplasmic enzyme present in high concentrations in the cytoplasm of hepatocytes (Hall & Guyton, 2011; Eguchi, et al., 2014). Therefore, its increase in serum is regarded as a sign of hepatic cell injury. The serum levels of AST and ALKp were non-significantly ($P > 0.05$) increased in all the exposed groups compared to the control group probably suggesting minimal adverse effects as against increased ALT levels. The overall result however, suggests (hepatotoxicity) disturbances of liver function following ATZ exposure. AST is present in tissues such as muscle, heart, liver and most of it is found in the cytoplasm and mitochondria of hepatocytes (Mauro, et al., 2006; Hall & Guyton, 2011). ALKp is an isoenzyme primarily found bound to cell membranes in almost all tissues and in bone (Mauro, et al., 2006; Hall & Guyton, 2011).

Mitochondria membranes in the liver contain unsaturated fatty acids in high proportion and are vulnerable to peroxidative attack by ROS (Poli, et al., 1987; Li, et al., 2015a). Moreover, the membranes contain electron transport system enzymes, making them capable of producing ROS (Slater, 1972; Poli, et al., 1987). Using transmission electron microscopy, Lim et al. (2009) observed partial disorientations of mitochondria cristae in skeletal muscle and liver of *Sprague Dawley* rats exposed to ATZ concentrations of 300µg/kg/day orally for 5 months. Based on their results, Lim et al. (2009) concluded that ATZ is capable of binding to sites of electron transport chain complexes I and III in the cytosol of mitochondria of skeletal muscle and liver of animals. Thus, chronic exposure to ATZ in our study, may have resulted in mitochondria disorientations, leading to uncontrolled production of ROS and consequently excess ROS accumulation in the liver of experimental animals. Unfortunately, this study did not determine ROS levels. ROSs are capable of binding covalently to macromolecules, inducing peroxidative degeneration and/or altering plasma membrane integrity (Sies, 1985; Dornelles & Oliveira, 2013). According to Poli et al. (1987), lipid peroxidation may interfere with the lipid composition of plasma membranes, alter their fluidity and permeability to intracellular components.

A study on the integrity and functionality of sperm membranes (plasma, acrosome and mitochondria) was conducted on capacitated bovine sperm isolated from the epididymis tail, following direct exposure to 0.1-10µM of ATZ for 4 hours. Damaged membranes (plasma membrane, acrosome and mitochondrial membrane) of sperm cells stained by fluorescence was observed mostly at the lowest concentration of ATZ. Their results highlight that ATZ disrupts membrane structure and impairs cellular function. Therefore, it is possible that ATZ could have indirectly affected plasma membranes via lipid peroxidation and/or directly adversely affected the structure of hepatocyte plasma membranes and mitochondria membranes. This could have resulted in subsequent release of membrane-bound ALKp and leakage of intracellular enzymes such as ALT and AST into the bloodstream, leading to elevated serum levels of these enzymes.

5.3 Atrazine and Kidney Biomarkers

Serum urea and creatinine are useful bio-indicators for evaluating renal function in both *in vitro* and *in vivo* studies. Although increased, urea serum levels observed in all the ATZ-treated groups were not significant, when compared to the control group, in the present study. Our results are inconsistent with those reported by Hussein et al. (1996) and Fisher-Scherl et al.

(1991) following acute exposure of *Chrysichthyes auratus* to 3000µg/l and 6000 µg/l of ATZ and rainbow trout to 1400 µg/l and 2800 µg/l of ATZ. They suggested that the increases in serum urea nirogen could have been due to necrosis of endothelial cells and renal hemopoietic tissue.

We observed a significant increase in the serum levels of creatinine in the ATZ-treated groups (i.e. 200µg/l and 500µ/l), when compared to the control group. Interestingly, alterations associated with kidney biomarkers due to sub-lethal or lethal concentrations of atrazine in post-metamorphic *Xenopus laevis* frogs have not been reported in the literature. In contrast, increases in creatinine levels following exposure to ATZ have been reported in *Wistar* rats (Jestadi, et al., 2014; Liu, et al., 2014). According to Ajeniyi and Solomon (2014), increases in urea and creatinine serum levels results amongst other reasons from impaired excretion and/or decreased urinary clearance by the kidneys. Normally, urea and creatinine are excreted primarily by the kidneys. Creatinine is freely filtered by glomeruli because it is a small molecule with a molecular weight of 113 Daltons (Ajeniyi & Solomon, 2014). Thus, serum increases of these parameters particularly creatinine, would reflect over production and /or renal impairment most probably at the level of the glomeruli. Significant increases in creatinine levels observed in this study may also imply that many waste products in the bloodstream of frogs treated with 500µg/l, were not filtered by the glomeruli. We can postulate that the frogs treated with the high-dosage of ATZ (500µg/l) concentrations may have suffered glomeruli dysfunctions rather than complete renal failure or tubular insufficiency partly because blood levels of creatinine depend largely on glomerular function. Moreover, the non-significant alterations in serum urea levels suggest that kidney morphological alterations in the present study were not as extensive as those observed in the liver. It is possible that ATZ concentrations used in the present study (0.01µg/l, 200µg/l and 500µg/l) and similar concentrations, are incapable of disrupting some kidney functions such as urea excretion through the kidneys, in *Xenopus laevis* adult frogs.

5.4 Atrazine and Liver Morphology

Liver damage in the treated groups was characterised by disorganization in the arrangement of hepatic cords, hypertrophied hepatocytes, hepatocyte vacuolization, vascular congestion and dilation, infiltration of inflammatory cells and apoptosis and/or necrosis. The most pronounced histological alterations were observed in the frogs exposed to high ATZ concentration. Our

findings are similar to those reported in freshwater fish (Wani & Vibhandik, 2011; Mela, et al., 2013) and mammals (Deshmukh & Ramteke, 2015) treated with ATZ and frog species exposed to Reldan 40EC (Paunescu, et al., 2010) and Dimethoate (Alarami, 2015). Research (Zaya, et al., 2011; Mela, et al., 2013; Dornelles & Oliveira, 2015; Rajini, et al., 2015) has shown that herbicides can suppress, alter or completely phase out liver functional properties in animals through alterations in histological structure. The histological results of this study are an indication that ATZ is capable of adversely altering liver tissue structure and inducing cell death.

According to Ziegler and Groscurth (2004), the early manifestation of almost all forms of injury to cells is cellular swelling as manifested in hepatocyte hypertrophy indicated by the significant increase in hepatocyte width observed in the livers of frogs exposed to high ATZ concentrations (200µg/l and 500µg/l). Kaware (2013) suggested that liver tissue morphological changes during exposure to a toxicant, could be adaptive mechanisms that allow animals to rapidly get rid of toxic compounds in the liver, through rapid metabolisation and excretion, as sustained insults may lead to possible irreversible damages. Therefore, the hypertrophy of hepatocytes observed could be an adaptive response to ATZ induced stress. The adaptive response may suggest increased activity of hepatocytes, in an attempt to eliminate ATZ toxic effects by metabolising it into its less toxic metabolites.

Hepatocyte vacuolization was observed in all the treated groups and was more pronounced at higher concentrations. In fish (Mela, et al., 2013) and mice (Ross, et al., 2009) exposed to ATZ, hepatic vacuoles have been observed. Other studies have also reported lipidosis in hepatocytes of animals exposed to different xenobiotics (Ribeiro, et al., 2005; Peebua, et al., 2008; Samanta, et al., 2015). According to Mela et al. (2007) cytoplasmic vacuolization of hepatocytes results from disturbances in lipid metabolism, a condition known as lipidosis. It is mainly encountered in cells actively involved in fat metabolism such as hepatocytes and myocardial cells (Elservier, 2007). Hepatocytes are involved in various aspects of intermediate metabolism of proteins, carbohydrate and lipids, and such effects as vacuolization may interfere with normal physiological functions of the liver.

Varying degree of vascular changes was observed in all the treated groups. Central vein and sinusoidal congestion was observed at the lowest and highest concentration of ATZ. Similar vascular changes have been reported from studies on other animals exposed to pesticides (Alarami, 2015; Rajini, et al., 2015; Rasgele, et al., 2015). It is possible that the central veins and sinusoids in livers of frogs exposed to 0.01µg/l and 500µg/l of ATZ were obstructed by increased immune cells migration and debri from cellular breakdown. The obstruction could have led to a reduction in blood flow due to cellular and debri overload.

In the livers of frogs treated with 200µg/l of ATZ, a reduction in vascularization was observed. Farber (1980) highlighted that extreme cases of hepatocyte hypertrophy can lead to compression of sinusoids, resulting in a reduction in sinusoidal area, thus, vascularization. Sinusoidal dilatation observed in the present study could have been a compensatory mechanism to reduce vascular flow resistance arising from sinusoidal compression, hence increasing blood supply to parenchymal cells and counteracting the reduction in vascularization.

Vascular haemorrhage as shown by the infiltration of red blood cells within and into the hepatocytes was observed in frogs treated with 500µg/l of ATZ. Our findings corroborate with findings reported on the liver of adult *Bufotes Variabili* frog exposed to carbaryl (Cakıcı, 2015). Red blood cell extravasation is a sign that sinusoidal endothelium has been compromised and may occur as a result of increased intra-sinusoidal pressure arising from vascular congestion (Kakar, et al., 2004). Red blood cell width is on average approximately 25% larger than the capillary diameter (Snyder & Sheafor, 1999), thus, we would not expect to find them in the interstitium. Endothelium breakdown in the present study was confirmed by the immune-positive staining of caspase- 3 in the cytoplasm of sinusoidal endothelial cells. This study is the first to immunohistochemically detect sinusoidal endothelium degeneration in the liver of adult frogs by targeting activated caspase-3 and our results show that exposure to high concentrations of ATZ can destroy the endothelium of sinusoids and cause internal animal bleeding. Moreover, endothelium breakdown could have resulted in a reduction in blood pressure and possibly leading to a decrease in renal flow and consequent accumulation of creatinine, as indicated by the increases in the serum levels of frogs exposed to 500µg/l of ATZ.

An infiltration of immune cells in tissues was observed in the livers of the treated groups. Inflammatory responses in the liver have been reported in male *Wistar* rats (Deshmukh & Ramteke, 2015) and fish (Mela, et al., 2013) exposed to ATZ and in frogs treated with Reldan 40EC (Paunescu, et al., 2010) and cadmium (Medina, et al., 2016). The majority of the inflammatory recruited cells in the present study were neutrophils in sinusoids and within the liver parenchyma. Neutrophils do not attack healthy cells but respond to distressed or dying cells (Jaeschke, 2006). The results show that exposure to ATZ concentrations as low as 0, 01µg/l can trigger inflammatory response in damaged tissues. Liver tissues of frogs treated with 500µg/l of ATZ (Figure 4.6D) were infiltrated with inflammatory cells, suggesting extensive liver injury. Hepatocellular necrosis in the 500µg/l ATZ treated group was confirmed by the significant elevated serum levels of ALT as well as the necrotic areas observed.

The immunity of amphibians to a range of various pathogens is based on innate immunologic and adaptive immune systems (Silva, et al., 2013). Few studies have reported effects of ATZ on immune responses in amphibians (Christin, et al., 2004; Brodkin, et al., 2007; Langerveld, et al., 2009) and overall, there is little evidence of adverse effects of ATZ on immune function in amphibians exposed to environmentally relevant concentrations of atrazine (Van Der Kraak, et al., 2014). Innate immune cells such as Kupffer cells, the resident macrophages in the liver, are critical for the rapid clearance of micro-organisms from the systemic circulation, by phagocytosis (Gallone, et al., 2002; Nagy, 2003). They also facilitate activation of more Kupffer cells (Sichel, et al., 2002; Silva, et al., 2013), generation of inflammatory responses that lead to the recruitment of inflammatory cells such as T and B lymphocytes, neutrophils, natural killer cells, as well as monocytes in injured tissues (Jaeschke, 2006; Ramaiah & Jaeschke, 2007). Interestingly, apoptosis confirmed by the cytoplasmic immuno-positive localization of caspase-3 in neutrophils and Kupffer cells was observed in our study. Our findings suggest that ATZ exposure could have either directly or indirectly via oxidative stress, suppressed phagocytic activity and recruitment of monocytes to sites of inflammation by inducing apoptosis in the respective immune cells.

On the other hand, according to Jaeschke et al. (2002), neutrophils and Kupffer cells are a source of chemokines, pro-inflammatory cytokines, reactive nitrogen and oxygen species which promote oxidative stress in injury induced by toxicants. Although responsible for removal of cell debris and being part of the host-defence system, these inflammatory cells under

appropriate conditions may release these substances and initiate additional liver injury. Thus, their apoptosis observed in the present study could have been an immune mechanism to reduce ROS production and therefore oxidative stress. In any case, the immune system of the animal may be weakened more, further predisposing the animal to toxic effects of this chemical insult.

The immune system of amphibians also includes melanomacrophages (MMCs). Melanomacrophages are focal accumulations of pigmented macrophages in the liver of frogs (Paunescu, et al., 2010; Regnault, et al., 2014). Their function is also linked to cellular storage, destruction, detoxification and iron recycling (Agius & Roberts, 2003). MMCs may contain four types of brown to black pigments (ceroid, melanin, hemosiderin/ferritin and lipofuscin) (Paunescu, et al., 2010; Jantawongsri, et al., 2015). Their proliferation varies according to natural factors (hibernation, aging, starvation) or pathological conditions such as pesticide exposure (Silva, et al., 2013). Increased densities of these cells have already been used as biomarkers of immune toxicity in fish (Mela, et al., 2007) and frogs (Loumbourdis, 2005; Paunescu, et al., 2010) and are often associated with degenerative necrotic conditions. These findings corroborate with the findings of this study were significant increases in the population of MMCs was observed in the frogs exposed to 200µg/l of ATZ.

On the other hand, a significant decrease in the population of MMCs in the livers of frogs exposed to the lowest and highest concentrations of ATZ was observed contrary to previous results (Loumbourdis, 2005; Silva, et al., 2013). A decrease in the number of MMCs has however, been reported in the liver of *Rana* (Pelophylax) *ridibunda* treated with Reldan 40EC at 22-24ºC (Paunescu, et al., 2010) and they emphasized that the decrease observed was temperature related not due to Reldan 40EC exposure. MMC population in frog liver has been reported to be at a minimum during the warmer period and at a maximum in cold months (Corsaro, et al., 1990; Barni, et al., 1999). If our findings were temperature related, we would have had similar MMC percentage areas between the treated groups and the control groups as the animals were housed under the same mean temperatures of 21°C. In fish, the size and number of MMCs is known to increase with age (Brown & George, 1985). These age-related increases might also be similar in frogs. Adult frogs of post-morphosis age were used in this current study, therefore, we would expect naturally increased populations of MMCs and further elevations of these cells particularly to counter the challenge following exposure to a toxicant.

Thus, results suggest that MMCs were unable to withstand ATZ toxicity resulting in reduction in their number which further points to reduced immunity of the animals.

In an eco-epidemiology study, aggregates of melanomacrophages in the livers of *L. pipiens* collected from wetlands were reported to be decreased with increased exposures to atrazine and its metabolite desethylatrazine (Rohr, et al., 2008).However, in this study, both low and high ATZ concentrations (0.01µg/l and 500µg/l) resulted in a decrease in MMCs. Since MMCs play roles in immunity (humoral and inflammatory responses) (Agius & Roberts, 2003) it is possible that the immune system of adult *Xenopus laevis* frogs may be disrupted by both low and high ATZ concentrations.

Peri-sinusoidal and peri-portal fibrosis was observed in some of the livers of the treated groups. Hepatic fibrosis is considered a common response to chronic liver injury and is characterized by excessive deposition of extracellular matrix components (Friedman, 1993). It has been shown that oxidative stress can stimulate fibroblast (Murrel, et al., 1990) and collagen synthesis (Fredman, 1995; Montosi, et al., 1996) both *in vitro* and *in vivo*. A quantitative analysis of the percentage area occupied by collagen showed non-significant increases in collagen content in all the treated groups, when compared to the control group, an indication that ATZ did not significantly induce or increase the extracellular matrix in these organs.

5.5 Atrazine and Kidney Morphology

In amphibians, as in higher vertebrates, kidneys serve as major routes for excretion of various xenobiotics and their metabolites thus, maintaining a stable internal environment relative to a highly variable external environment. This balance can be disturbed by the presence of xenobiotic substances which may affect the morphology (histological) of various organs tissues and in this case, in the kidney (Myers, et al., 1993; Păunescu, et al., 2012). The present results showed histopathological alterations such as glomerular atrophy and degeneration, tubular lumen dilation, vacuolization and degeneration of thick loop of Henle tubule epithelial cells.

Histopathological changes in the kidney at glomeruli and tubule level in aquatic species following exposure to pollutants including pesticides have been reported (Khan, et al., 2011;

Paunescu & Ponepal, 2011; Manjunatha, et al., 2015; Medina, et al., 2016). In this study, chronic exposure to low and high concentrations of ATZ induced pathologic alterations in glomeruli structure. Atrophied and completely degenerated glomeruli were observed in all the treated groups. Moreover, there were very few intact glomeruli in kidney tissues of frogs treated with 500µg/l of ATZ, as most of them had completely degenerated probably indirectly from the ATZ induced stress or directly as a consequence of ATZ exposure, suggesting higher toxicity at this concentration which is consistent with previous reports in fish (Fisher-Scherl, et al., 1991) and rats (Deshmukh & Ramteke, 2015).

Immunohistochemical examination of kidney tissues treated with 200µg/l and 500µg/l of ATZ showed positive immuno-localization of caspase-3 in the cytoplasm of podocytes, parietal epithelial cells (PECs) and macula densa cells. Similar results were reported by Uyanikgil et al. (2009), following exposure of adult male *Wistar* albino rats to 2, 4- dichlorophenoxyacetic acid (2,4-D), for 28 days. In this study, co-localization of activated caspase- 3 in the cytoplasm of cells suggest apoptosis. Probably, ATZ during its passage through the glomerular filtration membrane, directly interacted with filtration cellular components, causing induced cell death via activation of caspase-3 proteases, amongst other pathways. Such cellular changes could lead to glomerular filtration dysfunctions, such as increased or decreased glomerular filtration rate.

Podocytes together with glomerular endothelial cells and the glomerular basement membrane, form the glomerular filtration barrier in the kidney (Matovinovic, 2009; Hall & Guyton, 2011; Miceli, et al., 2014). Podocytes intimately surround the Bowman's tuft and form a cellular wall that restricts entry of large macromolecules and hematopoietic cells from leaving the blood circulation into the nephron (Hall & Guyton, 2011; Miceli, et al., 2014). Podocyte loss or damage is an early symptom of many kidney diseases presenting clinically with proteinuria with or without nephrotic syndrome and renal failure (Matovinovic, 2009).

Parietal epithelial cells (PECs) line the Bowman's capsule and are known as progenitor cells that enhance podocyte number (Appel, et al., 2009; Ohse, et al., 2009; Shankland, et al., 2013). According to Ohse et al. (2009), a balance between proliferation and loss of PECs is critical in maintaining the proper structure and function of glomeruli. They further speculated that PEC apoptosis could be a mechanism to normalize cell number within glomeruli. In this study, PEC

apoptosis could have been a positive adaptive mechanism of preventing glomerular hypertrophy that could have resulted from uncontrolled ATZ induced PEC proliferation and subsequent increases in podocyte cells. On the other hand, glomerular atrophy observed in this study could have been aided by apoptosis of PECs and podocytes.

Macula densa cells are closely packed specialized cells located in the distal convoluted tubule. They are part of the juxtaglomerular apparatus (Peti-Peterdi & Harris, 2010; Hall & Guyton, 2011). They are responsible for sensing changes in tubular fluid sodium concentrations (filtrate osmolarity), generating and conveying signals to the juxtaglomerular apparatus that controls renal blood flow and glomerular filtration rate (GFR) (Bell, et al., 2003; Schnermann & Levine, 2003). For example, when osmolarity (measure of solute concentration) decreases, macula densa cells cause afferent arterioles of kidneys to dilate, triggering an increase in pressure at the glomerulus which leads to increases in GFR (Bell, et al., 2003). Damage to macula densa cells would have an impact on blood flow to the kidneys because the afferent arterioles would not receive signals to dilate in response to a decrease in filtrate osmolarity, thus, pressure at the glomerulus would not be increased and GFR would remain low (Bell, et al., 2003). Exposure to high ATZ concentrations may result in the suppression of auto-regulatory responses to increases or decreases in filtrate osmolarity, through induction of macula densa cell death. Together, glomeruli atrophy and macula densa apoptosis could account for the significant increases in serum levels of creatinine observed in the kidneys of frogs exposed to high concentrations of ATZ.

Distinct alterations in the histological structure of thick loop of Henle tubules of kidneys of frogs treated with ATZ were observed. Of all the tubules of kidneys from the treated groups, thick loop of Henle tubules seemed to be the most affected. The epithelial cells of the thick loop of Henle tubules of kidneys from frogs treated with the highest concentration of ATZ were vacuolated and shrunken leading to loss of cellular structure integrity. Cellular shrinkage could have led to loss of epithelial cell-cell boarders as cells pulled away from each other. Cellular vacuolation and shrinkage further resulted in cellular flattening, causing a significant reduction in tubular epithelium height, leading to tubular lumen dilation.

The thick ascending limb is involved in the regulation of extracellular fluid volume, urine concentration, calcium, magnesium, bicarbonate and ammonium homeostasis and urine protein composition (Mount, 2014). Injury to the thick loop of Henle tubules may result in the disruption of any of these mechanisms. However, this is the first study that has targeted thick loop of Henle involving ATZ exposure, and no ATZ studies have been done to ascertain the interactive effects of this herbicide with this tubule.

Eosinophilic material characterizing hyaline deposits, observed in the lumen of thick loop of Henle (Figure 4.9C) and proximal tubules (Figure 4.9D) of kidneys of frogs treated with 500µg/l of ATZ almost completely blocked lumen and may have hindered the normal functioning of these tubules.

Proximal tubule maintains homeostasis by controlling renal acid-base regulation. This is achieved when chloride and sodium ions flux within the tubule, hydrogen carbonate ions and hydrogen ions are absorbed and secreted and when ammonium ions are trapped in the lumen (The International Programme on Chemical Safety , 1991; Hall & Guyton, 2011). According to Slack et al. (2010), intraluminal debris and obstructions from tubular cell damage may impair reabsorption and secretion, causing increased backpressure, fluid leakage and an imbalance in the acid-base status of an organism.

In other pesticide related aquatic toxicity studies, renal tubule morphological alterations in frog (Paunescu & Ponepal, 2011; Medina, et al., 2016) and fish kidney (Jiraungkoorskul, et al., 2002; Deivasigamani, 2015) have been restricted to the proximal and distal tubules. Proximal renal tubular cells are mostly susceptible to the toxic action of chemicals, due to their high-energy demand for reabsorption and secretion functions. The distal tubules are involved in both ion and water reabsorption but have a less significant role and require less energy than the proximal tubules (Valtin, 1973; Brenner & Rector, 1986; Hall & Guyton, 2011). In this study, there were no distinct morphological alterations observed in proximal and distal tubules of kidney tissues stained with the haematoxylin and eosin stain. Our findings varied with findings from previous studies on other amphibian species most probably because *Xenopus laevis* frogs are aquatic organisms whereas frog species from previous studies are either terrestrial or both aquatic and terrestrial inhabitants. Because of physiological variations in frog species which are adapted to

a wide array of habitats they occupy (i.e. ditches, ponds, and lakes) chronic effects of contaminants can be species specific (Berrill, et al., 1998; Bridges, 2000; Howe, et al., 2004).

However, immunohistochemical examination in the present study revealed positive cytoplasmic immunolocalization of caspase-3 in the epithelial cells of proximal, distal and collecting tubules of kidney sections from treated frogs, suggesting tubular cell death by apoptosis. The intensity of caspase-3 staining in these tubules increased with increasing concentrations of ATZ. No immuno-positive localization of caspase-3 cells was observed in the thick loop of Henle of all the treated groups. According to Ueda and Shah (2000), death pathways followed by cells are dependent on both nature and severity of stress, evolving from apoptotic to the necrotic form of cell death. Based on our results, it is possible that low concentrations of ATZ could have resulted in apoptosis whilst high concentrations of ATZ resulted in apoptosis of proximal and distal tubules and further induced necrosis in thick loop of Henle tubules. On the other hand, it can also be suggested that mechanisms other than the activation of caspase-3 such as activation of caspase 6 and 7 or extrinsic apoptotic pathways initiated by death receptors, including TNF-related apoptosis-inducing ligand (TRAIL) receptors, Fas and TNF receptor (R) 1 (Alkhouri, et al., 2011), could have mediated the ATZ induced degeneration of thick loop of Henle tubules.

This study is the first nephrotoxicity study involving caspase-3 immunolocalization in renal tubules of adult frogs. Overall, kidney tissues with glomeruli atrophy, of frogs exposed to ATZ were not adversely affected when compared to the liver tissues, as there was no sign of kidney inflammation or fibrosis. Regardless of the absence of kidney inflammation, the positive staining of activated caspase-3 in glomeruli and tubules from atrazine-treated frogs suggested that cells in these structures would likely become apoptotic and die. Degeneration of these nephrotic structures manifests in absorptive and secretory defects along the nephron, which in severe cases would exhibit phosphaturia, glucosuria, polyuria, aminoaciduria, elevated blood urea nitrogen and creatinine increased excretion of electrolytes (Bergeron, et al., 2000).

There was a correlation between the histopathological findings and alterations in the serum levels of urea and creatinine kidney biomarkers. The ability of ATZ to cause alterations in kidney morphology and serum biomarker level (urea and creatinine) could have been a

secondary event following oxidative damage as explained earlier. A "hyper-filtration injury" hypothesis proposed by Brenner (1893) and Brenner et al. (1978,1982) showed that when nephrons fail to function properly, the remaining nephrons tend to hypertrophy leading to an increase in flow rate per functioning nephron, thus increasing exposure to the toxic chemical. In this study, tubular hypertrophy was not observed. Instead, tubular dilatation was observed which probably ensued with consequent malfunctioning of the glomerular apparatus unit with attendant imbalances in ion exchange mechanisms as mentioned earlier. Tubular dilatation could also have been a secondary effect following a decrease in epithelial height as a consequence of the pathologic insult in the frogs exposed to 200 µg/l and 500µg/l. These effects could indicate that very high concentrations of ATZ induce increases in tubular lumen surface area, as a way of maximising its toxic effects or indirectly as a compensatory mechanism to counteract ion exchange imbalances.

Kidney damage in this study increased in a dose -dependant manner, and was characteristic of progressive organ failure if exposure was prolonged, particularly in glomeruli which are considered more sensitive to oxidative stress than other nephron parts (Yi, et al., 2011). Tubular susceptibility to nephro-toxins is related to the nature of normal tubular function. Studies on various models of nephrotoxicity induced by metals (Zhang & Lindup, 1996; Zalups, 2000; Zalups & Ahmad, 2003; Medina, et al., 2016) have shown that metal-induced overproduction of ROSs affects a number of nephrotic tubule cellular ion transport pathways through: (a) decreases in cellular ATP levels by inhibiting oxidative phosphorylation in mitochondria (b) alterations in transmembrane signalling by inhibiting regulatory enzymes (c) peroxidation of membrane phospholipids that might alter the microenvironment necessary for the maximum function of transporters and increases in membrane permeability for ions and (d) modulation of the activity of many ion transport proteins by altering the redox state of intracellular space and/or directly oxidizing their functionally-important SH groups. The application of this understanding provides a foundation upon which to study ATZ induced renal injury and a rational basis for the inference of animal toxicity data to man and risk assessment.

Areas of importance for further research would be immunological mechanisms, direct effects of ATZ on kidney ion mediation and receptor-mediated events and regulation of cellular metabolism in amphibians. Moreover, stereological quantification of the volume density of juxtaglomerular apparatus unit cells, renal tubules diameter, areas of renal tubules in damaged

tissues would provide further relevant information on the extent of ATZ effects on renal function in aquatic amphibians.

5.6 General Discussion

This study provides the first *in-vivo* evidence of histopathological and biochemical effects of chronic exposure of ATZ on the liver and kidneys, as well as the lipid peroxidation (MDA content) in the post-metamorphic *Xenopus laevis* frogs. The adverse histopathological alterations and extensive inflammation observed in the liver of exposed frogs reveal the inabilityof the liver to metabolize major proportion of ATZ and prevent ATZ bio-availability and therefore access to organs and tissues leading to toxicity. Pesticides gain entrance into the body systems of the adult frogs by passive diffusion mainly through the skin (Katagi & Ose, 2014). Thereafter, the pesticides are either metabolized by various enzymes in an organ or partitioned to a lipid phase inorder to reduce its level of toxicity (Katagi & Ose, 2014). Research has shown that ATZ is detoxified mainly in the liver and the remaining ATZ and its metabolites are excreted largely in the aqueous form in urine and bile (Rozman & Klaassen, 2001; National Academy of Sciences, 2004). Frog livers have higher lipid contents than other organs and therefore, lipophilic residues of pesticides have been detected therein (Licht, 1976; Keshavan & Trend, 1987). The metabolism of ATZ is a complex process and various human (Perry, et al., 2000; Catenacci, et al., 2002) and animal (Bakke, et al., 1972; Deshmukh & Ramteke, 2015) toxicity studies have presented varying reports.

Ideally, ATZ does not bio-concentrate in tissues of organisms or bio-magnify in the food chain because of its low log Kow (concentration in octanol/concentration in water) (2.56 – 2.61) and rapid metabolism in animals. However, studies have confirmed the bioaccumulation of ATZ in frog tissues (Hayes, et al., 2003; Hecker, et al., 2004; Jooste, et al., 2005), including the liver (Zaya, et al., 2011). A table of bio-concentration factors (BCF) for ATZ for several animal species has been previously reported by Giddings et al. (2005). The greater the BCF, the greater the levels of ATZ in the organisms' system. Reports have shown that ATZ has high BCF in the liver and ovaries of *Tilapia sparrmanii* banded tilapia fish after 72 hours of ATZ (16mg/l) exposure (Du Preez & van Vuren, 1992) as well as in moribund striped bass fish (Cashman & Maltby, 1992).

Bio-concentration factors seem to increase with an increase in ATZ exposure concentration. Though, BCF was not calculated in this study, histopathology results from the liver and kidneys of treated animals suggests that a high BCF may have been possible in the present study.

Varying exposure conditions can alter the rate of ATZ metabolism and influence its interaction with organs, potentially altering its toxicity level and direction on amphibian health. The effects can vary between and within species and can be influenced by a variety of experimental and husbandry factors such as duration of exposure, site of exposure, purity of treatment, rearing density and age (Buchholz & Hayes, 2005; Storrs & Semlitsch, 2008). Differences in species, concentration of atrazine, grade of atrazine or experimental setup may have caused the variations between the present study and other studies. For example, a commercial preparation containing 98.9% pure ATZ (which is the highest ATZ purity used in research to date) was used to create the atrazine concentrations for the present study, as against other grades (40.8% (Howe, et al., 1998), 85.5% (Storrs & Kiesecker, 2004) and 98% (Lenkowski, et al., 2008)) that have been previously used. Storrs & Kiesecker (2004) used commercial grade ATZ which contained inert ingredients and surfactants. Lenkowski et al. (2008) prepared their stock solution containing ATZ in ethanol or dimethyl sulfoxide. Inclusion of other constituents which could be possible contaminants could have impacted on their studies and could account for the differences noted between their study and the current study.

Furthermore, age has been documented to exacerbate bioaccumulation of xenobiotics in organisms. As organisms grow and age, the bioconcetration of toxicants in tissues often increases because the rate of toxicant uptake would have exceeded the rate of elimination (Gobas, 2001). Edginton and Rouleau (2005) exposed just-metamorphosed (stage 66) *Xenopus laevis* tadpoles to 410 pg/l 14C ATZ for 8 hours and ATZ was rapidly absorbed within the first hour of exposure and was efficiently eliminated, demonstrating a high metabolic capacity. In this study, post-metamorphic, adult *Xenopus laevis* frog were used. Age may have led to delayed elimination of ATZ and increased toxicity.

The results revealed that *Xenopus laevis* frogs are highly susceptive to adverse effects of all concentrations of ATZ as indicated by the histopathological and biochemical findings. Hepatocyte vacuolization, parenchymal tissue apoptosis and /or necrosis and inflammation

observed in the livers of exposed frogs may suggest direct effects of ATZ on the tissue or indirect secondary effects arising from oxidative stress.

ATZ has been documented to interact with hepatic enzymes such as glutathione – S transferase and cytochrome P450, enzymes that play a role in the metabolism of exogenous compounds , by competing for enzyme binding sites (Lang, et al., 1997; Islam, et al., 2002; Hanioka, et al., 1998), leading to a partial or complete disruption of its detoxification and promotion of its bioaccumulation in the liver.

As a result of impaired metabolism and hepatic injury, ATZ could have by-passed the liver without being fully metabolised, reaching the kidney through the blood circulation in its bio-toxic state, causing renal morphological alterations and dysfunctions. This is supported by a report by Matthew (2010) who exposed *Bufo melanostictus* frogs to sublethal concentrations of endosulfan and hinosan and reported bioaccumulation of endosulfan and hinosan in the kidney caused by their incomplete detoxification in the liver.

6. CONCLUSION

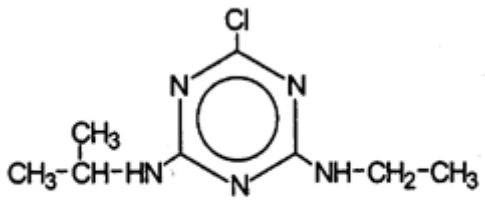
The results demonstrate clearly that ATZ's exposure interrupts the normal functioning of the liver and kidneys of adult *Xenopus laevis* frogs even at low concentrations. Though the results were consistent with some previous reports on different animals and amongst amphibian species, it slightly differed with others relative to age and probably experimental conditions. It further showed that ATZ can interfere with biochemical processes in the liver and kidneys as shown by the elevated levels of AST, ALT and ALKp and urea and creatinine respectively. The results further suggest that the generation of ROS following increased levels of MDA might have been the putative pathway of action. Moreover, it is possible that lipid peroxidation can be triggered in adult *Xenopus laevis* by an ATZ concentration threshold greater than 200µg/l.

Whether these effects occur through the oxidative stress axes or other mechanisms, liver and kidney damage could influence the future fitness of fresh water amphibians and thus the persistent levels of atrazine in the environment would remain a risk to *Xenopus laevis* frog populations in polluted environments.

7. APPENDICES

APPENDIX 1: Structure and Physiochemical Properties of Atrazine

1.1 Structural formula:



atrazine

1.2 Physiochemical Properties

Empirical formula: $C_8H_{14}N_5Cl$

Molecular weight: 215.7

Physical state: white crystalline

Melting point: 173-177°C

Ionization constant: (pK_a) 1.68

Density: 1.187 g/cm^3

Stability: Stable in neutral, slightly acidic/basic solutions; hydrolysed by alkali/ mineral acids at higher temperatures.

Vapour pressure: 2.89 X 10^{-7} mm at 25°C

Solubility: water ~33 parts per million (ppm), methanol 18 000 ppm, chloroform 52 000 ppm, dimethyl sulfoxide 183 000 ppm.

Henry's law constant: 2.49 x 10^{-9} atm^{-9} m^3 mol^{-1}

Octanol/Water coefficient: 418 ± 14 at 25°C; log $_{Kow}$ = 2.68 at 25°C

(WSSA, 1989; Solomon, et al., 1996)

APPENDIX 2: Solutions for Tissue Fixation

2.1 Phosphate buffered saline (PBS)

For 1000 ml

8 g of NaCl

2.6g of Na2HP04 anhydrous

0.2g of KH2PO4

0.2g of KCl

Make volume up to 1000ml with distilled water

Adjust to pH 7.4

2.2 Preparation of 10% phosphate buffered formalin

For 1000 ml,

4g of Sodium phosphate monobasic

6.5g of Sodium phosphate dibasic (anhydrous)

100 ml of Commercial formaldehyde (37-40%)

Make volume up to 1000ml with 900 ml of distilled water

pH to 7.2±0.5

Store at 4°C

2.3 Tissue processing

The tissue was processed using an automatic processor (Shandon Citadel 1000) as follows:

Dehydration

Immerse in 70% alcohol for 1hour

Immerse in 95% alcohol for 2hours

Immerse in 95% alcohol for 2 x $1^1/_2$ hours

Immerse in 100% ethanol for 1hour

Immerse in 100% ethanol for 2hour

Immerse in 100% ethanol for 1hour

Clearing

Immerse in chloroform for 1hour

Immerse in chloroform for 2 hours

Impregnation

Immerse in wax for 2x 2hours

2.4 Embedding

1. Warm the wax as well as the metal moulds and a pair of forceps making certain that the temperature does not exceed 58°C.
2. Once the wax has melted, fill the metal mould with wax.
3. Using the warmed forceps, gently orientate the specimen such that the intended cutting edge faces the floor of the metal mould.
4. Cool briefly, and then fit the plastic cassette accordingly.
5. Quickly top up with more melted wax and cool for approximately 15 minutes
6. The wax block can be easily separated from the metal mould while it remains attached to the plastic cassette.

APPENDIX 3: Staining Solutions

3.1: Haematoxylin and eosin stain

Staining protocol:

Hydration

1. Dewax in 2 changes of xylene, 5 minutes each

2. Dip 10 times in 2 changes of 100% alcohol

3. Dip 10 times in 95% alcohol

4. Dip 10 times in 70% alcohol

5. Wash in running water for 5 minutes

Nuclear staining

1. Stain with Meyer's haematoxylin for 5 minutes

2. Wash in water until blue stain disappears

3. Dip 3 times in 1% acid alcohol to remove haematoxylin from cytoplasm

4. Wash in running tap water for 5 minutes to remove acid alcohol

6. Wash in running tap water for 1 minute

Cytoplasm staining

1. Counterstain in Eosin-phloxine B solution for 2 minutes

2 Wash in running tap water for 1 minute

Dehydration and mounting

1. Dip 10 times in 70% alcohol

2. Dip 10 times in 95% alcohol

3. Dip 10 times in 100% alcohol

3. Dip 10 times in 2 changes of xylene

4. Mount in section with Entellan blue

Staining results:

- Nuclei: Blue-black
- Muscle fibres: deep pink red
- Cytoplasm: Varying shades of pink

3.2 Van Gieson staining

Reagents:

a) Celestine blue

100 ml of 5% ammonium ferric sulphate (iron alum)

0.5g of Celestine blue (Cl51050)

Add the Celestine blue to the ammonium ferric sulphate and boil for 3 minutes.

Filter when cool

Store refrigerated

b) 1% Ponceau S

1g of Ponceau S (Cl 27195)

100ml of distilled water

c) Curtis Stain

90ml of saturated aqueous picric acid

10 ml of 1% Ponceau S

10 ml of glacial acetic acid

Staining protocol:

1. Bring sections to distilled water
2. Stain nuclei with Celestine blue for 5 minutes
3. Rinse in distilled water for 5 minutes
4. Stain in haematoxylin for 5 minutes
5. Wash well in running tap water for 5 minutes
6. Flood with Curtis stain for 5 minutes
7. Blot
8. Dip 10 times in 70% alcohol
9. Dip 10 times in 95% alcohol
10. Dip 10 times in 100% alcohol
11. Dip 10 times in 2 changes of xylene
12. Mount in section with Entellan blue

Staining results:

- Nuclei: Blue
- Collagen: Bright red
- Cytoplasm, muscle, fibrin and red blood cells: Yellow

APPENDIX 4: Silane Coating of Slides for Immunohistochemistry

4.1 Reagent: 2% silane solution

200ml of acetone

20ml of xylene (3- aminopropyl triethoxysilane)

Coating protocol:

1. Immerse slides in silane for 30 minutes
2. Dip 10 times in 2 changes of acetone
3. Dip 5 times in 2 changes of distilled water
4. Put slides in incubator at 40°C overnight to allow them to dry.

APPENDIX 5: Ethical Clearance

5.1 Gauteng Nature Conservation Permit

PREMIER OF THE PROVINCE OF GAUTENG
NATURE CONSERVATION

CPF6 No. 0115

PERMIT TO HUNT AND/OR COLLECT AND CONVEY A WILD ANIMALS FOR SCIENTIFIC PURPOSES
Issued in terms of the provisions of the Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)

Name of permit holder: Chongetanji Cornelius Rimayi

Residential address: Dept of water Affairs
Roodeplaatdam
Moloto Road
Pretoria

DATE STAMP
2014 -10- 24
DIRECTORATE OF CONSERVATION

Name and address of institution or department on whose behalf shall be hunted and conveyed:

PARTICULARS OF WILD ANIMALS WHICH MAY BE HUNTED AND CONVEYED

Number	Species	Sex
360 (Three hundred & Sixty)	African Clawed Frogs (Xenopus laevis)	

In terms of and subject of the provisions of the **Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)** and the regulations framed thereunder, the abovementioned person is hereby authorised, subject to the conditions appearing on this permit to hunt and convey the wild animal/s referred to above during the period of validity of this permit on behalf of the institution or department referred to above.

PARTICULARS IN CONNECTION WITH THE HUNT

Number	Species	Sex	Date hunted and conveyed

Valid until 23/10/2015

SIGNED ON BEHALF OF THE PREMIER

SIGNATURE OF PERMIT HOLDER

(See conditions on reverse side)

the-blackhand 011 435 2800 (0054 - CPF6)

5.2 Johannesburg Animal Ethics Screening Committe

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2014/32/D

APPLICANT: Mr CC Rimayi

SCHOOL: Chemistry

LOCATION: Faculty of Sciences

PROJECT TITLE: *Frogs (Xenopus laevis) as an indicator for environmental organic pollution*

Number and Species

360 Frogs (180 adult and 180 tadpoles)

Approval was given for to the use of animals for the project described above at an AESC meeting held on 27 May 2014. This approval remains valid until 26 May 2016.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None.

Signed: _____ (Chairperson, AESC) Date: 3/11/2014

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 3rd Nov 2014

cc: Supervisor: Prof L Chimuka
Director: CAS

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