Ecotoxicological and potential endocrine effects of selected aquatic herbicides on life stages of the African clawed frog, *Xenopus laevis*.

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DECLARATION

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Dedication

To El-SHADAI, the I am that I am

The beginning and the end of all things

Never seen with naked eyes

Yet bigger than one could imagine

The God who never come late

But arrive at his own appointed time

I thank you once again, for proving to be my Alpha and Omega

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General Abstract

Many pesticides have shown health impacts in wildlife and humans, including endocrine system modulation. The potential health effects of the pesticides elicit concerns considering high volume being used in South Africa, especially herbicides in agriculture and alien plants eradication at the government "Working for Water" programme. These herbicides included Midstream (diquat dibromide), Basta (glufosinate ammonium), Arsenal (imazapyr), Roundup, Kilo-Max and Enviro-glyphosate (glyphosate) formulation. For most of these herbicides, toxicity and endocrine modulating potential are unknown. The present study therefore assessed the toxicity and endocrine modulating potential through teratogenicity, thyroidal and reproductive disruption, using the African Clawed frog, *X. laevis* as sentinel species. International validated protocols, including a 96-hour toxicity assay; frog embryo teratogenic assay (FETAX); Xenopus metamorphosis assay (XEMA), and semi-static exposure of adult male frogs, were used to evaluate relevant endpoints. The result showed that in 96-hour comparative toxicity study, the early tadpoles (NF-stage 48) in comparison to the embryos (NF-stage 8-11) and transitional tadpoles (NF-stage 60), were the most sensitive and vulnerable in the developmental stages of *X. laevis*. This present study confirmed a stage-dependent sensitivity to environmental herbicides during metamorphosis/developmental program. In the FETAX study, the 96-hour lethal concentration (LC50) for Midstream, Arsenal, Basta, Roundup, Kilo-Max and Enviro-glyphosate were 0.833, 35.99, 2.24, 1.052, 207.25 and 465.95 mg/L respectively. The 96-hour effective concentrations (EC50) were 0.241, 28.13, 2.01, 0.76, 150.8 and 287 mg/L, respectively. The LC50/EC50 ratio produced a positive teratogenic index (TI) for Midstream and Enviro-glyphosate with a TI of 3.5, 1.3, 1.1, 1.4, 1.4 and 1.6 for Midstream, Arsenal, Basta, Roundup, Kilo-Max and Enviro-glyphosate formulations, respectively. The minimum concentrations inhibiting growth were computed to be 0.5, 2.0, 2.0, 0.9, 190 and 440 mg/L for Midstream, Basta, Arsenal, Roundup, Kilo Max, Enviro-glyphosate formulations, respectively. Observed malformations from these formulations included edema (cardiac, abdominal and severe), gut, axial, blisters and eye malformations. The XEMA results (thyroidal modulation) revealed that Arsenal, Midstream, and Kilo-Max formulations resulted in thyroidal modulation as these significantly reduced the average developmental stages relative to the control tadpoles after 21-days exposure. Extending XEMA to completion of metamorphosis revealed a significantly skewed sex ratios at 0.14 and 280 mg/L of Midstream and Kilo-Max formulations, respectively. The gonadotoxicity assessment showed that the highest abnormality index ranged from 60%, 43%,

37.5%, 35%, 30% and 27.5% for Midstream, Kilo-Max, Enviro-Glyphosate, Arsenal, Roundup and Basta formulations, respectively. The observed gonadal malformations included folded gonads, segmented hypertrophy, hypoplasia, segmented aplasia and translucence. For adult exposure, no significant difference in the results, showing that adult, relative to other developmental stages, are less sensitive to exposure impacts of pesticides. In summary, different stages were differentially sensitives to the herbicide toxicity. The six formulations showed differential endocrine modulation, including teratogenicity (Midstream and Enviro-glyphosate), thyroid and growth disruption (all the six formulations), gonadotoxicity (all the six formulations), skewed sex ratios (Midstream and Kilo-Max) and estrogenicity/antiandrogen (Midstream). Finally, these herbicides have shown secondary effects on non-targets organisms, therefore their inclusion in aquatic weeds control should be investigated beyond lethal toxicity but also endocrine modulation.

"All meaningful and lasting changes starts from imagination…… and then works its way out. Imagination is more important than knowledge." ------ *Albert Einstein*

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LIST OF ABBREVIATIONS

DMSO	*Dimethyl Sulphoxide*
ED	*Endocrine Disruption*
EDC	*Endocrine Disrupting Contaminant*
EEC	*Expected Environmental Concentration*
EIA	*Environmental Impact Assessment*
ELISA	*Enzyme-Linked Immunosorbent Assay*
ER	*Oestrogen Receptor*
FETAX	*Frog Embryo Teratogenesis Assay-Xenopus*
GSI	*Gonadosomatic Index*
Hcg	*Human Chorionic Gonadotropin*
HPG	*Hypothalamic Pituitary Gonadal*
HPT	*Hypothalamic Pituitary Thyroid*
HSD	*Honest Significant Difference*
IC50	*Concentration Giving 50% Inhibition*
IPCS	*International Programme on Chemical Safety*
IR	*Intermediate Region*
LC50	*Lethal Concentration 50%*
MCIG	*Minimum Concentration Inhibiting Growth*
ND	*Not Detected*
NF	*Nieuwkoop and Faber Developmental Stage*
NOEL	*No Observed Effect Level*
NP	*Nuptial Pad*
OECD	*Organisation for Economic Co-operation and Development*
PAS	*Periodic Acid-Schiff*
PBS	*Phosphate Buffered Saline*
PNEC	*Predicted No-effect Concentration*
RO	*Reverse Osmosis*
RP	*Relative Potency*
SPC	*Spermatocyte*
SPG	*Spermatogonia*
SPT	*Spermatid*
SPZ	*Spermatozoa*

T	*Testosterone*
T3	*Triiodothyronine*
T4	*Thyroxine*
TDCs	*Thyroid-disrupting Contaminants*
TEDX	*The Endocrine Disruptor Exchange*
Trα	*Thyroid Receptor α*
Trβ	*Thyroid Receptor β*
Tshβ	*Thyroid Stimulating Hormone β*
USEPA	*United States Environmental Protection Agency*
VTG	*Vitellogenin*
WfW	*Working for Water*
WHO	*World Health Organisation*
WRC	*Water Research Commission*
XEMA	*Xenopus Metamorphosis Assay*

Chapter One

General Introduction

1.1 Introduction

Since the dawn of civilisation and advent of agriculture, mankind has continuously been engaged in improving its living conditions (Ansara-Ross *et al*., 2012). This is by increasing its food production through soil enrichment and reduction of pest attack through the application of numerous fertilizers and pesticides (Ansara-Ross *et al*., 2012). As human population grow, so does the demand for food, with concomitant increase in pest control activities, to reduce pest damages. This increasing pest control, coupled with industrial growth has led to unprecedented introduction of chemicals into the environment.

Man-made chemicals are an important part of modern life, and human and wildlife populations cannot avoid contact with chemicals that are employed in various human activities (Swedenborg *et al*., 2009; WHO, 2013). Today, human and wildlife are exposed to more than 100,000 of these anthropogenic substances (Soto *et al.*, 2007), aside from their unknown interactive, environmental by-products and metabolites that are also present as pollutants (Benachour and Seralini, 2009; Marllatt *et al*., 2012). Evidence of this persistent chemical exposure have been observed in samples of human blood, breathe, hair, tissue and body fluids and hence has aroused a strong interest in identifying their presence in food, drinking water and soil. These chemicals range from pesticides (herbicides, insecticides, fungicides, and bactericides), pharmaceuticals, industrial compounds and household, including beauty care products (WHO, 2013).

The annual global pesticides use is currently estimated at 11.2 billion kg, which are largely herbicides (Suntharasingham *et al*., 2010; Yadav *et al*., 2013). The global herbicide consumption for example, is as much as 48% of the total pesticide usage (Gupta, 2007). The increased in use of these herbicides, particularly in the aquatic ecosystem, has become a growing hazard (Petterson and

Ekelund, 2006, Mensah *et al*., 2013). The concern is premised on effects of these chemicals on non-target and at-risk aquatic organisms like fish and amphibians. Aside from direct application of these herbicides, their indiscriminate use, careless handling, accidental spillage, or discharge of untreated effluents and run-off into natural water-ways could and may contributes to long-term effects in the environment (Jiraungkooskuul *et al*., 2001; Glusczak *et al*., 2006; Chen *et al*., 2008; Govandarajulu, 2008; WHO, 2013), including reducing species diversity, changing community structures, modifying food chain, altering pattern of energy flow and nutrient recycling (Perez *et al*., 2011; Mensah *et al*., 2013).

Aside from their active ingredients, these various herbicide formulations also contain other chemical compounds generally referred to as surfactants, which facilitate emulsification, dispensing and wetting properties of the formulations (Hu *et al*., 2005; Wijnja, 2010). These surfactants are mostly classified into two groups: non-ionic forms that include alcohol ethoxylate (AEs), alkylphenol ethoxylate (APEs), alkylamine ethoxylate (ANEs), silico-based surfactants (organosilicones), and oils, as well as anionic forms which include phosphate ethoxylated esters (PE) (Wijnja, 2010). Despite their suggested inert tags, these surfactants may be biochemically active, and are only labelled inert because of their inert function in the formulated products (Cox and Surgan, 2006).

Many of these surfactants most often do not share the target specificity of the active ingredients (Mann *et al*., 2003), leading to higher toxicological risk. Hence, concerns have been raised about the potential risk that these surfactants and their degradation products may pose to aquatic organism, especially amphibians (Bakke, 2003; Wijnja, 2010). This is especially true for nonyl phenol ethoxylate (NPE) surfactants and their nonylphenol metabolites that have been identified as endocrine disruptors (Bakke, 2003; Trumbo, 2005; Yang *et al*., 2005; Othman, 2009; Mann *et al*., 2009).

One of the early victims of these widespread physiological disruptions are the amphibians that are globally declining at an alarming proportion (Khan and Law, 2005). The declining phenomenon was first observed as a global trend in the early 1990s (Hayes *et al*., 2010). Currently, over 60% of the known amphibian species are threatened or declining in numbers (Blausten, 1994; Perkins *et al*., 2000). This decline has now been documented in a variety of habitats across the six continents (Carey and Bryant, 1995), thus making some scientists to suggest that the current rate of amphibian extinction is greater than any known in the last 100,000 years (Wilson, 1992; Eldredge, 1998). In this amphibian decline, several possible hypotheses have been formulated, among which the anthropogenic alteration of the environment is the most implicated (Brunelli *et al*., 2009). This includes agrochemicals like herbicides, insecticides, fungicides, rodenticides and even fertilizers (Lavorato *et al*., 2013). In fact, with their dual life cycles and permeable skin, amphibians are particularly sensitive to environmental contaminants, so much so that environmental pollution is considered one of the causes of global amphibian declines (Reeder *et al*., 1998; Relyea, 2005: Johnson *et al*., 2006; Quassinti *et al*., 2009; Bruhl *et al*., 2013; Lavorato *et al*., 2013).

With about 32% of the worlds estimated 6600 amphibian species threatened with extinction, 43% experiencing declines, and another 22% with insufficient data, this phenomenon represents the earth's sixth mass extinction (Hayes *et al*., 2010) and 211 times the background amphibian extinction rate (Brunelli *et al*., 2009). The concern over this rapid global decline arise as frogs are considered as living barometers for the earth's environmental health (Blaustein, 1994; Blaustein and Wake, 1995; Perkins *et al*., 2000). By this barometer gauge, it means that the amphibian decline is a possible early signal of an impending crisis for all other living things.

Agricultural chemicals are receiving increasing attention as one of the potential causes for this amphibian decline (Mann *et al*., 2009; Porter *et al*., 2011; Lajmanoich *et al*., 2013; Gungordu, 2013), either acting singly or in combination with other stressors (Relyea and Mills, 2001). This is because surveys of natural populations have shown correlations between amphibian decline and

proximity to agricultural lands (Bishops *et al*., 1999; Edge *et al*., 2011). These surveys are further corroborated by many malformed amphibians. Decreased species richness has also been reported in agricultural areas where herbicides and insecticides are widely used (Mann *et al*., 2009; Brunelli *et al*., 2009; Lavorato *et al*., 2013).

Vulnerability of amphibians to pesticides may be attributed to their specific habitat requirements, including shallow temporary ponds, which is essential to their life cycle. These shallow or temporary water bodies are also areas where these agrochemicals mostly contaminate by spray drift and then accumulate, due to low dilution rate (Bishop and Pettit, 1992; Mann *et al*., 2003). Another factor for amphibian vulnerability is their various physiological uniqueness among the vertebrates which include; their eggs that are only covered by a layer of gelatinous materials, which makes the eggs easily exposed to the environmental chemicals (Vitt *et al*., 1990; Lavorato *et al*., 2013). The dual ecological mode of amphibian, where they alternate between aquatic and terrestrial habitat also exposed them to divergent forms of contaminants compared to fish that are permanently in water or reptiles that are permanently on land.

Accumulating evidence over the last two decades showed that wide range of anthropogenic chemicals have the ability to alter endocrine functions (interfere in hormonally controlled pathways) in humans and wildlife (Sparling *et al*., 2001; Jugan *et al*., 2009; Carr and Patino, 2010; Hinther *et al*., 2010; Marllatt *et al*., 2012; Heindel *et al*., 2013; WHO, 2013). The consequences of this interference are most often irreversible, in some cases leading to early death, but in others, not manifested until the individual reaches adulthood, with resultant loss of fertility and increased reproductive impairment (OECD, 2004). Through various subtle biochemical and physiological changes, many of these chemicals disrupt the normal pathways of hormones associated with a range of endocrine system (Figure 1.1).

The best studied interaction is receptor binding, eliciting a spectrum of downstream biological effects, either through organizational pathways during the development stage or during

adulthood (Orton *et al*., 2011). However, it is now known that endocrine modulation may also occur through interaction/modulation of other pathways associated with the life history of a hormone (for example, synthesis, transport, breaking down and uptake by target cells) but also affecting molecular expression of receptor and non-receptor proteins (Govindarajulu, 2008; Diamanti-Kandarakis *et al*., 2010; Bergman *et al*., 2013; Heindel *et al*., 2013). Chemicals modulating the endocrine system in some unnatural way, are collectively referred to as endocrine modulating substances or endocrine disrupting compounds (EDCs) (OECD, 2004; Li, 2007; OECD, 2008; Swedenborg *et al*., 2009; WHO, 2013).

1.2. Overview of the Endocrine System

The endocrine system is the assemblage of ductless glands that secrete hormones directly into the bloodstream, which regulate a wealth of biological processes (Chou, 2005; Watamura, 2008; Leblanc *et al*., 2011). The traditional organization of the endocrine system include hypothalamus, pituitary, pineal body, thyroid, parathyroid, adrenals, liver, kidney, gastrointestinal cells, ovaries, placenta, uterus as well as testes processes (Chou, 2005; Watamura, 2008). In addition, many organs not recognised as primary endocrine organs have secondary endocrine functions and also secrete hormones, for example heart, kidneys, liver, intestines and fat tissue (Diamanti-Kandarakis *et al*., 2010)**.** In general, these glands, in a single and complex interaction coordinate and regulate numerous physiological processes including development and growth, homeostasis and metabolism in general as well as the extended reproductive system (Diamanti-Kandarakis *et al*., 2010; Zoeller *et al*., 2012).

Ultimately, hormonal control is mediated through binding to hormone specific receptor-protein associated with the target tissues (Chou, 2005). Steroid hormones (cholesterol derivates) are mostly dependent on “transporter” proteins to be carried through the blood and can passively enter cells through the phospholipid-like cell membrane and bind to specific intracellular receptor

proteins (mostly nuclear-based). Protein hormones generally enter the cell passively and need to bind with membrane bound receptors and enter the cell by a secondary messenger system.

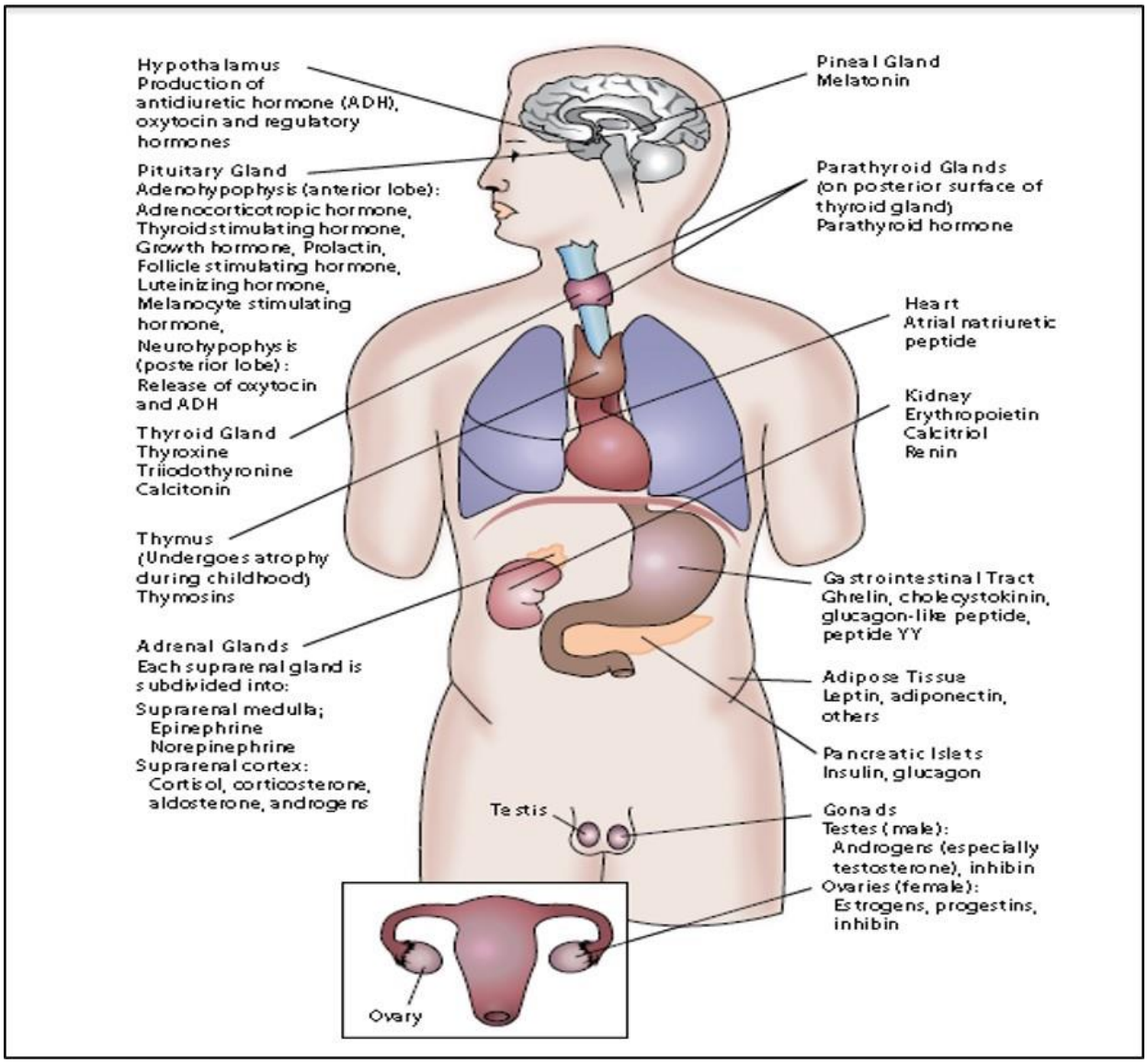

Figure 1.1: The human endocrine system showing the associated organ systems with the major glands and organs (WHO, 2013).

In addition to the classical models, where the thyroid hormone can enter the cell with the aid of specific transporter proteins and subsequently bind to intracellular receptor similar to steroid hormones, it is now known that steroid hormones may also bind to external membrane bound receptors (WHO, 2013). Nuclear receptors (steroid and thyroid hormones) form receptor-hormone

complexes that bind to specific regions of DNA, regulate gene transcription, mRNA synthesis and subsequently protein synthesis in the extra-nuclear environment (WHO, 2013). Adding to the complexity of the hormonal control system is the temporal and spatial differential expression of receptors resulting in target cell specific effects within certain temporal windows (WHO, 2013).

The window period is important because when disrupting chemicals are introduced during the developmental window, they affect the programming of cells and tissue development, in a way that the effects become permanent (WHO, 2013). According to the WHO, 2013 report, there are different effects (which could be transient) when the same endocrine disruptor is introduced later at childhood or during the adult phase. This varied sensitivity of endocrine disruptors during the life span has important implications which makes it important that the potential modulating effects of chemicals be studied using various developmental stages (Diamanti-Kandarakis *et al*., 2010; Heindel *et al*., 2013; WHO, 2013). Another implication of this varied sensitivity is that not all endpoints of hormone actions usually exhibit same sensitivity to chemicals exposure (WHO, 2013)

1.2.1 Endocrine modulation/disruption

Chemicals acting as endocrine disruptors interfere with hormone action in a way that can produce adverse effects on human and wildlife health, by interfering with the ability of cells, tissues and organs to communicate hormonally, resulting in numerous negative health effects including reduced fertility, skewed sex ratios of the offspring of the exposed parents, reduced viability of the offspring, genital deformities, birth defects and thyroid disorders etc. (Guillette *et al*., 2000; Baskin *et al*., 2001; Schreinemachens, 2003; Gary, 2004; Zala and Penn, 2004; Sikka and Wang, 2008; Swedenborg *et al*., 2009; WHO, 2013).

Endocrine disruptors, aside from mimicking the natural hormones, usually have two pathways by which they disrupt hormone action; a direct action on a hormone receptor protein complex, or a direct action on a specific protein that control aspect of hormone production (e.g.

aromatase), hormone transportation (e.g. sodium/iodide symporter) or a hormone carrier protein (e.g. cortisol binding protein) (Sikka and Wang, 2008; WHO, 2013). Although, hormone receptors usually have a high affinity for endocrine disruptors (Ruenitz *et al*., 1996; WHO, 2013), some exogenous chemicals have been shown to display an affinity similar to, or greater than that of natural ligands e.g. tributyltin (TBT), which has a high affinity for retinoid-X-receptor (RXR) and peroxisome proliferator activating receptor gamma (PPARϒ) (Grun and Blumberg, 2006). Essentially, the mechanism by which a chemical disrupt hormone action has a very large impact on the pattern of effects (WHO, 2013). However, the ability of endocrine disruptors to alter the normal hormonal control of development is perhaps the most significant consequence of exposure, as developmental effects will occur at lower doses than are required for effects in adults (Alonso-Magdalena *et al*., 2010).

Just like normal hormones that act at low doses due to the strong affinity for their corresponding receptors, some exogenous substances also have very high affinity for nuclear receptors, which also make them act at very low doses. But importantly, some endocrine disruptors can also act at low doses even if their affinity for hormone receptors is considerably lower than that of the natural hormones (WHO, 2013). This according to the WHO, (2013) report can happen because the impacts of the small changes in hormone action at the low end of the dose-response curve is much greater than at the high end of the curve. These endocrine disruptors that modulate hormone action at low doses can do so in a specific manner, such that traditional toxicological endpoints are not sufficient to preclude adverse outcome and these substances interact at dose responses that are nonlinear and potentially non-monotonic (Vandenberg *et al*., 2012). The overall effect of this low dose effects is that they pose a threat to human health (Kumar *et al*., 2008).

Initial concern about the threat posed by environmental contaminants, (including pesticides to human and wildlife health) was voiced in several books, including Silent Spring (Carson, 1962) and Our Stolen Future (Colborn *et al*., 1996). Although, the concept of endocrine disrupting activity

associated with man-made chemicals was suggested at a much earlier time (Krimsky, 2000; Burkhardt-Holm, 2010), this field of research exploded during the last decade with powerful weight-**of**-evidence showing all major aquatic wildlife groups are experiencing modulation of the endocrine system in a variety of contaminated sites (Hutchinson *et al*., 2006; Zoeller *et al*., 2012; Kidd *et al*., 2013). It is not surprising to find that aquatic organisms are the most affected, since more than 100,000 chemicals are thought to be discharged directly into the freshwater environment (Trudeau and Tyler, 2007).

Increased scientific evidence suggests that several man-made synthetic chemicals modulate several aspects of these endocrine pathways and processes, both in vertebrates as well as invertebrates (Knechtges *et al*., 2006), resulting in disruption of numerous physiological processes (vom Saal *et al*., 2008; Watamura, 2008). The effects of many of these chemicals on humans for examples, are highlighted by several keystone case-studies that accessioned the potential health hazards of these chemicals, including increasing incidence of cancer, reproductive and developmental anomalies like hypospadias and polycystic ovaries, cryptorchidism, diminishing sperm counts in the last fifty years, as well as developmental anomalies in reproductive organs (Harrison *et al*., 1997; Boettger-Tong *et al*., 1998; van Wyk *et al*., 2005; Orton *et al*., 2011).

1.3. The Human Wildlife Connection

In general, the endocrine systems of vertebrates conservatively share structural chemistry and molecular mechanisms associated with various hormone processes (Iguchi and Katsu, 2008), although the receptors are somehow different among the vertebrate species, which can influence the interaction of the exogenous chemicals (WHO, 2013). However, physiological consequences of several of these differentiated receptors may differ between vertebrate classes, especially in the area of sex determination and differentiation. The understanding of these species differences in hormone receptors will further help in protecting wildlife from endocrine disruption. Since humans

and wildlife share the same environment, wildlife sentinel species like amphibians and fish have the potential to serve as early warning signposts of potential modulation of specific physiological pathways by environmental chemicals either singly or in complex mixtures (Diamantis- Kandarakis *et al*., 2010). Although the design of high throughput screening tests based on in vitro exposures (mostly receptor binding approach) make sense at the tier 1 level, however, testing endocrine modulation hypotheses using whole animal (in vivo) exposures at different life stages will always be essential to fully understand the potential risks associated with potential EDCs (OECD, 2004; 2007).

In wildlife, abnormalities such as masculinisation in bivalves and gastropods; feminization of fish when exposed to sewage effluents; discontinuous gonads, reduced metamorphosis, delay growth and disrupts sex ratios in *Hoplobatrachus rugulosus*, hermaphroditism when exposed to Atrazine herbicide formulation (Kime, 1998; Hotchkiss *et al*., 2008). In addition to that, altered sex determination and differentiation in reptiles, including turtles and alligators, reduced fertility; hatchability and wasting syndrome in birds are reportedly caused by exposure to EDCs (Fry, 1995; Kime, 1998; Hotchkiss *et al*., 2008; Zaya *et al*., 2011; Tranchantong *et al*., 2013). There was also a significant increase in intersex animals due to certain herbicide formulations (Reeder *et al*., 1998; Larson *et al*., 1998; Hayes *et al*., 2002; Tavera-Mendosa *et al*., 2002 a, b; Carr *et al*., 2003; Trachantong *et al*., 2013). In addition, increase embryo lethality, malformation and growth inhibition as well as follicle hyperplasia testis weight reduction have been reported when *Xenopus laevis* were exposed to methoxychlor herbicide formulation (Fort *et al*., 2004).

1.4. Endocrine Mode of Action/Mechanism of Action

Hormones acts differently in different cell types, and they also acts differently in some cells at different developmental stages (WHO, 2013). This varied activities according to the WHO report, arises as multiple receptors types mediate the action of a single hormone. These different receptors

and varied activities also produces various mode of actions (MOAs), including estrogen and antiestrogen, androgen and antiandrogen, thyroid and antithyroidal etc.

1.4.1. (Anti) estrogenic activity (MOA)

Many pesticides have been shown to interact with estrogen receptors (ERs) (Kojima *et al*., 2010; WHO, 2013), by mimicking the natural estrogen hormone, leading to various estrogenic effects (Coster and Labereke, 2012). It is well-known that certain cells are naturally responsive to estrogens. This means that these cells naturally express estrogen-receptor proteins. These Receptor proteins may be located in the cytosol or nucleus of the response cell (genomic pathway) (Norris and Carr, 2006) or alternatively be incorporated in the cell membrane (non-genomic pathway) (Cornil *et al*., 2006). However, estrogenic effects include the control of other proteins associated with the estrogenic response (Wahlstrum, 2008). Some pesticides have also been shown to have antagonised effects on estrogen receptor binding or by affecting enzymatic hormone transformation pathways, for example inhibition of enzyme, aromatase (testosterone to estrogen transformation), therefore leading to antiestrogenic effects (Whitehead and Rice, 2006; Coster and Larebeke, 2012). Another important mode of action is the activities of certain pesticides that interfere with the binding of natural estrogens to estrogen receptors, thereby competing and preventing the estrogen receptor and hormone binding.

1.4.2 (Anti) androgenic activity (MOA)

Many pesticides have been identified with potential androgenic and anti-androgenic mode of action. These modulations are mostly achieved by upregulating expression of, or through competative binding to, the androgen receptor (AR) in one way or the other (Coster and Larebeke, 2012). It can also be by affecting upstream steroidogenesis pathways and transformation enzymes or transformation enzymes (5α Reductase Testosterone to Dihydrotestosterone) (Kojima *et al*., 2004; Lorenz *et al*., 2011; Wu *et al*., 2012). For example, antiandrogenic herbicides like chlornitrofen and

chlomithomyfes have been shown to have more potent inhibitory activity than known antiandrogenic pesticides, Viclozolin and p, p'- diclorodiphenyldichloro ethyne (DDE) (Kojima *et al*., 2004). Early life exposure to endocrine disrupting chemicals (EDCs) may be causative factors of numerous male human disorders including testicular dysgenesis syndrome, cryptorchidism, hypospadias, decreased penile length, reduced sperm quality, cancer from sources associated with chlordecone-producing companies (Waring and Harris, 2005), PCBs (Aneck-Hahn *et al*., 2007) and insecticides such as p, p'-DDE and Endosulfan (Kelce and Gray, 1999; Dalvie *et al*., 2004).

1.4.3. (Anti) thyroidal activity (MOA)

In addition to affecting the reproductive hormones, many pesticides and chemicals have been shown to interact with thyroid receptors, with both agonist and antagonist effects (Kojima *et al*., 2010; Coster and Labereke, 2012). Chemicals can also affects thyroid metabolism either through the hypothalamic-pituitary-axis or directly via nuclear receptors (Patrick, 2009). Thyroid hormone has two receptor classes, TRα and TRβ, each with different temporal and spatial patterns of expression (WHO, 2013). The spatial distribution of these receptor types varies widely and can influence the wider nature of the effects (WHO, 2013). It has been shown that only those chemicals with closely related structures to thyroid hormones can bind to ligand-binding pocket of TRs (Kojima *et al*., 2010), a good example include dioxin (Ahlborg *et al*., 1995; Nilsson *et al*., 2001).

Iodine is very essential in thyroid functioning. Through the pituitary and specific receptors binding, the TSH increases the uptake of iodine into the follicular cells of the thyroid, iodination of thyroglobulin (TG), synthesis and oxidation of thyroglobulin, uptake of TG, as well as synthesis and oxidation of thyroid hormones including the T3 and T4. (Norris and Carr, 2006; OECD, 2006). Both the T3 and T4 are iodinated derivatives of the amino acid tyrosine.

1.5. Pesticides as Endocrine Disruptors

Agricultural and environmental pesticides application has increased globally, and is now an important sector in the world economic (with more than 1000 active ingredients) mainly because this sector has grown exponentially alongside of growth in other related industries including agriculture (Plakas and Karabelas, 2012). These pesticides are now found in most compartments of the environment, and have been detected in ground water as well as surface water that are used as sources of drinking water (Plakas and Karabelas, 2012). It is therefore not surprising, that authorities all around the world are concerned about the potential health effects that pesticides may pose, even at very low concentrations (pg/L-ng/L range) (WHO, 2013). These pesticide chemicals, apart from their toxic effects that are often exhibited at high concentration, may also have other differential effects that may include teratogenic, thyroidal and reproductive effects, by acting through numerous pathways (Mckinlay *et al*., 2008; Mnif *et al*., 2011; Hamid and Eskicioglu, 2012). About 127 pesticides have been listed as having endocrine disruptive properties (Mckinlay *et al*., 2008). This has generated widespread concerns for their non-target impacts (Relyea, 2003; Govandarajulu, 2008; Mann *et al*., 2009; Piola *et al*., 2013; WHO, 2013).

1.6. Pesticides and South African Environment

South Africa is the highest produce-producing country in the African continent, and therefore has the largest pesticides market in the Sub-Saharan Africa (Bollmohr *et al*., 2008; Dalvie *et al*., 2009a; Ansara-Ross *et al*., 2012; Ansara-Ross *et al*., 2013; Dabrowski *et al*., 2014). Accordingly, there are approximately 180 pesticide active ingredients formulated within approximately 400 registered trade names currently available in South Africa (PAN, 2013). With the increase in demand for food productivity, due to population increase and increase in food demand, pesticide use in South Africa represent 60% of the pesticide market in Africa (Dabrowski *et al*., 2014). This plethora of pesticides has huge potential impacts consequences on the environment and ecological diversity, and not

going without public and government concern (Bollmohr *et al*., 2009; Ansara-Ross *et al*., 2012; Mensah *et al*., 2013; Dabrowski *et al*., 2014)

Ansara-Ross *et al*., (2012) reviewed all pesticides-related researches conducted in South Africa since 1970. These data showed that relative high levels of pesticides have been detected in various South African river catchments and man-made reservoirs. Both human and wildlife in South Africa environment are possibly exposed to these chemicals, the health implication of which is still largely unknown. Dabrowski *et al*. (2014) in a report categorized only 12% of total 152 pesticides active ingredients as potential EDCs.

1.6.1 Chemical control of invasive aquatic plants: Working for Water Programme (WfW) as a case study

The South African 'Working for Water' programme is an ecological conservation scheme, launched in 1995 by the South African Government, as a multi-departmental public project to control alien plants in river catchments. The project aims primarily to enhance water security, improve ecological integrity and restore the productive potential of land. The project identified invasive alien plants (Act No 10 of 2004) as one of major problems when considering the future of water conservation. Accordingly, alien plants waste about 7% of South Africa's water annually, thus impeding farming and irrigation, intensifying floods and fire, causing erosion, destroying rivers and promoting poor water quality (Working for Water, 2007). In attempt to eradicate these invasive plant species, particularly the aquatic weeds, one approach was to use chemical eradication by applying selected herbicide formulations. The herbicides applied include Glyphosate (Touchdown, Seismic, Glyph, Mamba and Roundup, Kilo Max and Muscle-up), Diquat dibromide (Midstream), Imazapyr (Chopper, Arsenal, Format and Hatchet) and Glufosinate ammonium (Basta) (Bold, 2007). The use of these herbicide formulations has elicited wide public and scientific concerns about the safety of non-target aquatic organisms like fish and amphibians (Radosevich *et al*., 2007; Nweke and Sanders, 2009; Ochoa-Heima *et al*., 2009).

1.7. Herbicides as Endocrine Disruptors

1.7.1 General overview

Herbicides are the leading group of pesticides in terms of annual production, total acreage size usage and total revenue sales (Mensal *et al*., 2013). Just like other groups of pesticides, herbicides are designed to interfere with living species and are necessarily characterised by variable level of toxicity (Collosio *et al*., 1999). Herbicides generally have relatively short half lives in soil and water, but can be found at relatively high concentration in the environment (Orton *et al*., 2009). Herbicide formulations have been assumed not to pose a great risk to non-target organisms (animals), but evidence are mounting showing that these chemicals or the associated constituents of the formulations can affect wildlife health in aquatic and terrestrial environment (Colborn *et al*., 1996; Relyea, 2005; Dinehart *et al*., 2009; Egea-Seranno *et al*., 2012). But apart from selected formulations (like atrazine and roundup), relatively few studies have focused specifically on the endocrine disruption potential of herbicides (McKinlay *et al*., 2008).

Even with the little attention that has been paid to the herbicides group as potential endocrine disruptors, about 21% of the total pesticides that has been implicated for endocrine disrupting activities or potentials are herbicides (Mnif, 2011). This is a pointer to the inherent endocrine disrupting capacity of the herbicidal groups. The fact that there is worldwide growing usage of herbicides makes these chemicals a growing environmental and health threat that deserve more attention. Moreover, given the widespread negative ecological impacts associated with herbicide formulation like atrazine in recent past, and the controversy generated by conflicting research reports (Kloas *et al*., 2009; Hayes *et al*., 2010), underline the importance of more research regarding an extended list of herbicides groups.

A closer look at the herbicidal groups, on the basis of their chemical structures revealed interesting pictures. The herbicides classification on the basis of chemical structures, consist of 40 groups, including an additional one unclassified group (Wood, 2011). Of a total 41 groups, 25 have

been suggested to have endocrine disrupting activity or predicted endocrine disrupting potential. These 25 herbicide chemicals represent 61% of herbicide groups, meaning that more than half of herbicide groups are already implicated as being endocrine disruptors ((Kojima *et al*, 2010; Mnief *et al*., 2011). The fact that the endocrine disrupting tendencies are widespread and not restricted to selected few, mean that the herbicidal groups just like insecticides possess a wide variety of chemical structures and are the most likely candidates for ligands against NRs and ARs (Kojima *et al*, 2010).

1.7.2 Herbicides; Modes of Actions

Mode of action is the biochemical pathways that a chemical acts through to interfere with the physiological processes or how a particular herbicide acts in affecting its target (Kaplard and Namuth, 2004). There are nine recognized modes of action within the herbicide groups (Kaplard and Namuth, 2004). According to these authors, the nine modes of action include amino acid synthesis inhibitors (including sulfonylureas and imidazolinones etc.), seedling growth inhibitors (including dinitroanilines carbaothiates and acetamides), growth regulators/ cell division inhibition (including phenoxies, benzoic acids, and carboxylic acids and the picolinic acids), photosynthesis inhibitor (including triazines, uracils phenylureas, benzothiadiazoles and nitriles etc), lipid synthesis inhibitors (aryloxyphenoxypropionates and cyclohexanediones), cell membrane disrupters (diphenylethers, arytriazolinones, phenypthalamides and bipyridilium etc.), pigment inhibitors (isoxazolidinones, isoxazoles and pyridazinones etc), photobleacher (diquat and paraquat), and one unknown mode of action (MSMA and Nortron) (see Table 1). Understanding these modes of action is very important since such knowledge can be used to predict potential endocrine disrupting pathways. However, such links are rarely made and more research are needed to assess the endocrine modulation risk associated with herbicides.

1.7.3 Herbicides; Site of Action

The site of action refers to the biochemical site that a particular herbicide acts upon in a plant to interfere with the plant growth and development (Kaplard and Namuth, 2004). There are currently twenty-two (22) known sites of action, as well as seven (7) unknown sites of action (Table 1.1).

Table 1.1 – Recognized sites-of-action of Herbicides (Adapted from Hartzler, 1998).

GP	Site of Action	Family	Active Ingredient
1	ACC-ase (Lipid synthesis) inhibitor	Aryloxyphenoxy propionate	diclofop, fenoxaprop, fluazifop, quizalofop-p
		Cyclohexanediones	clethodim, sethoxydim
2	Acetolactate synthase Inhibitors	Sulfonylurea	Prosulfuron, chlorimuron, metsulfuron, halosulfuron, nicosulfuron, primisulfuron, rimsulfuron, sulfometuron, thifensulfuron, foramsulam
		Imidazolinone	AC 299, 263, imazapyr, imazaquin, imazethapyr, flumetsulam chloransulam-methyl, pyrithiobac
3	Microtubule inhibitors	Dinitroanilines	benefin, ethalfluralin, oryzalin, pendimethalin, trifluralin
		Pyridazines	dithiopyr
4	Synthetic auxins	Phenoxys	2,4-D, 2,4-DP, dichlorprop; MCPA, MCPB, MCPP
		Benzoic acids	dicamba
		Carboxylic acids	clopyralid, picloram, triclopyr
		Quinoline carboxylic acid	quinclorac
5	Inhibition of photosynthesis at Photosystem II	Triazines	ametryne, atrazine, cyanazine, prometon, simazine
		Triazinones	hexazinone, metribuzin
		Uracils	bromacil, terbacil
6	Photosystem II inhibitor – different binding behavior than 5	Nitriles	bromoxynil
		Benzothidiazole	bentazon
		Phenyl-pyridazine	pyridate

7	Photosystem II inhibitor – different binding behavior than 5 & 6	Ureas	diuron, linuron, tebuthiuron
		Amide	propanil
8	Inhibition of lipid synthesis	Thiocarbamates	butylate, EPTC, vernolate
9	Inhibition of 5-enolpyruvyl-shikimate-3-phosphate Synthase	None	Glyphosate
10	Inhibition of glutamine synthetase	None	Glufosinate
11	Inhibition of carotenoid synthesis at unknown site (bleaching)	Triazole	Amitrole
12	Inhibition of carotenoid synthesis at phytoene desaturase (PDS)	Pyridazinone	norflurazon
		Others	fluridone
13	Inhibition of diterpenes (bleaching)	Isoxazolidinone	Clomazone
14	Inhibition of protoporphyrinogen oxidase (PPO)	Diphenylethers	acifluorfen, fomesafen, lactofen, oxyfluorfen
		N-phenylph thalamides	CGA-248757, flumiclorac
		Triazolinone	sulfentrazone, carfentrazone, flumioxazin
15	Inhibition of DHP	Carbamate	Asulam
16	Inhibition of indoleacetic acid action	Phthalamate	Naptalam
17	Inhibition of cellulose synthase	Nitrile	Diclobenil
18	Inhibition of cell wall synthesis site-B	Benzamide	Isoxaben
19	Inhibition of photosystem I – electron diversion	Bipyridyliums	diquat, paraquat
20	Inhibition of mitosis	Carbamates	chloropham, propham
21	Uncoupling – membrane disrupters	Dinitrophenol	Dinitrophenol
22	Inhibition of Carotene synthesis (4-HPPD)	Triketone	sulcotrione,
		Isoxazole	isoxaflutole
		Pyrazole	pyrazolynate
			mesotrione
23	Unknown	Chloroacetamides	acetochlor , alachlor, butachlor, metolachlor, pronamide, proplachlor, dimethenamid
		Oxyacetamides	bay foe 5043
		Benzofuran	Thofumesate
		Organoarsenicals	DSMA, MSMA

Arylaminopropionic acid	flamprop-methyl
None	TCA
Various	bromobutide, cinmethylin,dymron flupoxam

1.7.4 Herbicide Formulations and Developmental Toxicity

Herbicides, just like other member of pesticide group, have been linked to developmental malformations in amphibian including paraquat, mecoprop, amitrax, MCPA, 2, 4, 5-T, bromoxynil and picloram (Osano *et al*., 2002; Kojima *et al*., 2004; Mnief *et al*., 2011).

Osano *et al*. (2002) using FETAX protocol assessed the teratogenic effects of amitraz, 2, 4-demethylaniline, and paraquat. They noted that at concentration higher than 0.2 mg/L paraquat, all the embryos of *Xenopus laevis* were malformed, and growth reduction was apparent at concentration of ranging between 0.1-5 mg/L. The most common teratogenic effects recorded included flexures of the notochord. For Amitraz formulation, edema was the most common teratogenic effects. Osano *et al*. (2002) also noted that at 5 mg/L, 100% of surviving embryos were edematous. It was concluded that paraquat should be classified as teratogens and further application should be stopped.

Lenkowski *et al*. (2008) examined the perturbation of Atrazine on the organogenesis using *Xenopus laevis*. It was reported that at concentration of 10-35 mg/L, a dose dependent increase in percentage malformation incidence occurred, with various malformation tissue including main body axis, circulatory system, kidney, and digestive system as well as increased incidence of apoptotic cell in midbrain and kidney.

1.7.5 Herbicide formulations and impacts on reproductive system

Reproductive toxicity is regarded as one of the most serious ecological and health properties of a substance (Magnuusson and Brunstrom, 2007). Concerns are mounting as several studies are now

relating numerous environmental chemicals with observed reproductive toxicity in wildlife (Colborn *et al*., 1996; Gimeno *et al*., 1998; Jobling *et al*., 2002; Kidd *et al*., 2007: McDaniel *et al*., 2008; Bergman *et al*., 2013). The window of early development proved to be a sensitive stage, especially in the light of organizational effects that chemicals may have during developmental stages (Guillette *et al*., 1995; Petterson *et al*., 1996). This mean that the reproductive system would be easily perturbed as the development of vertebrate reproductive system differentiates during early life stages, when the hormonal milieu essential for sex differentiation and proper development of organ and brain are high (Berg *et al*., 2009).

Androgenic and anti-androgenic as well as estrogenic and anti-estrogenic effects of many pesticides have been demonstrated in many organisms (Sharpe and Skakkeback, 1993; Wise *et al*., 2011; Leblanc *et al*., 2011; Orton *et al*., 2012; Lavorato *et al*., 2013). Exposure related impacts of many pesticides have been shown on male sexual differentiation, through antagonising effects on androgen receptors (AR) (Orton *et al*., 2012). These chemicals that mimic or antagonise the activities of androgen and estrogen receptors, interfere with physiological functions, leading to impairment in sexual function and development (Ubatzka *et al*., 2007). Examples of herbicides with androgenic and anti-androgenic activities include diuron, methiocarb, nitrofen, linuron and pyridate (Andersen *et al*., 2002; Fang *et al*., 2003; Okubo *et al*., 2004; Kojima *et al*., 2004; Thibaut and Porte, 2004; Salazar *et al*., 2006; Schmwtzler *et al*., 2007). Herbicides with known estrogenic and anti-estrogenic activities include chlordane, methoxychlor, thenylchlor, oryzalin, and pendimethalin (Colborn *et al*., 1993; Sharpe *et al*., 1995; Hurley *et al*., 1998; Johnson *et al*., 2002; Kojima *et al*., 2004;). But in spite of evidence that selected herbicides are androgenic, anti-androgenic, estrogenic and anti-estrogenic, many commonly used herbicide formulations have not been assessed for their potential impacts on the reproductive system.

The increasing important of enzymes involvement in the disruption of steroid biosynthesis pathways are being recognised (Guillette *et al*., 2001; Sanderson 2006; Bretveld *et al*., 2006). The

interference of pesticides with the steroid biosynthesis necessary for normal cell functions, hormone synthesis and degradation, may altered the natural balance of circulating sex steroids, leading to impaired reproduction, alteration in sexual differentiation, growth and development (Guillette *et al*., 2001; Bretveld *et al*., 2006; Sanderson *et al*., 2006). Steroid hormone synthesis is controlled by the activity of several highly substrate-selective cytochrome P450 enzymes and a number of steroid dehydrogenases and reductases (Sanderson, 2006). For example, glyphosate herbicide have been noted to disrupt the P450 enzymes and amino acid biosynthesis (Samsel and Seneff, 2013). This glyphosate inhibition of cytochrome P450 enzymes is an overlooked component of its toxicity to animals including mammals (Samsel and Seneff, 2013). Many other herbicide formulations could share this activity which is still largely unknown and unexplored.

1.7.6. Herbicide formulations and impacts on thyroid endocrine system.

Many herbicide formulations have been suggested to be thyroid system toxicants, including tributylin, methoxychlor, amitrole, ethiozin, thiazophy and prodiamine (Hurley *et al*., 1998; Sanderson *et al*., 2000; Vos *et al*., 2000; Cocco *et al*., 2002; Fort *et al*., 2004; Kojima *et al*., 2004; Cauble and Wagner, 2005; Urbatzka *et al*., 2007; Mnief *et al*., 2011; Shi *et al*., 2012). These herbicides have been shown to affect the growth and development of many wildlife in a way that reduces their fitness

Shi *et al*. (2012) in a study on effects of tributylin herbicide on metamorphosis and gonadal differentiation on *X. laevis* at environmental relevant concentrations (for 19 days exposure), suggested that this formulation greatly retarded the development of the tadpoles, decreased the number of thyroid follicle, and induced thyroid follicle cell hyperplasia. Moreover, the Tributylin formulation induced many malformations including intersex phenotypes, segmented aplasia and multiple ovarian carvities, as well as male bias sex ratio. Fort *et al*. (2004) reported that methoxychlor herbicide impacts on *Xenopus tropicalis* reproduction and development. Exposure to methoxychlor (range of 1-100 µg), resulted in a delayed development, and tadpoles exposed to 10

µg methoxychlor for 30 days showed enlarged thyroid glands with follicular hyperplasia. A concentration dependent increase in external malformation after 90 days of exposure was also recorded (Fort *et al*., 2004). At a higher concentration (100 µg), the sex ratio was skewed towards female with decreased ovary mass and number of oocytes, as well as decreased testis mass and sperm count.

Cauble and Wagner, (2005) studied the sublethal effects of the herbicide glyphosate on amphibian metamorphosis and development. They reported that tadpoles of *Lithobates cascade* exposed to 1-2 mg/L of glyphosate for 43 days showed significantly reduced survivability, reduced rate of metamorphosis and low post metamorphosis mass. It was also noted that no individual survived from eggs to metamorphosis in the 2 mg/L glyphosate concentration exposure group.

Taken together, aquatic herbicides, just like other pesticide, although are designed to interfere with physiological processes of living plants species may disrupt the normal physiology and survivability of living, non-target animal species. Although, labels of many of the herbicides indicates non-toxic or low toxicity to aquatic organisms at recommended dosages, available evidences from several studies put their low health risk to animals in doubt (Boettger-Tong *et al*, 1998; Petterson and Ekelund, 2006; Govandarajulu, 2008). Some of the herbicides are already showing potential toxicity at relatively low concentrations (Wojtaszek *et al*., 2004; Relyea, 2005b), including potential endocrine effects (Walsh *et al*., 2000; Howe *et al*., 2004), as well as chromosomal and DNA damage (Clements *et al*., 1997) and teratogenic effects (Paganneli *et al*., 2010).

Even though herbicide like atrazine has received much attention internationally for its toxicity and endocrine disrupting effects, other herbicide groups have attracted less attention, both as an ecological threat on non-target organisms as well as health hazards (endocrine disruption) for mankind. The Atrazine case has shown the world that even at low environmental concentrations, some herbicides may potentially disrupt animal endocrine systems. Research should therefore be

extended to other herbicides to ensure effective application. Since herbicides are been applied to the environment, the health implication on both wildlife and human must be fully understood.

1.8 Research gaps in knowledge of diquat dibromide, imazapyr, glufosinate ammonium and glyphosate herbicide formulations

In addition to acute toxicity effects (at 96 hour exposures), particularly at different developmental stages, the various exposure impacts at sub-lethal concentrations including developmental toxicity, thyroidal toxicity, gonadal and reproductive toxicity of diquat dibromide (Midstream), imazapyr (Arsenal) glufosinate ammonium (Basta) and glyphosate herbicide formulations (Roundup, Kilo Max, Enviro Glyphosate) that are widely applied in the South African 'Working for Water' program are still largely unknown and undefined.

The broader aim of this study was therefore to contribute towards filling these identified knowledge gaps using the aquatic amphibian *X. laevis* as sentinel species**.**

1.9. Review of selected herbicide formulations used in 'Working for Water' (WfW) Program

Chemical control of invasive plants is one of the adopted options of managing alien plants by the 'Working for Water' programme of the South African government (Department of Environmental Affairs). The aquatic herbicide formulations that were used contained chemical components with differential physical and chemical properties (Table 1.2) that potentially could directly or indirectly affects the exposure impacts of these herbicide formulations.

Table 1.2: Physical and chemical properties of glyphosate, diquat dibromide and imazapyr active ingredients (selected WfW herbicide formulations)

	Glyphosate	Diquat	Glufosinate	Imazapyr
Physical state	White crystal Powder	NA	White to Light brown	White to tan Powder
Molar Mass		344 g	198.2 g/mol	261.3 g/L
Spec. Gravity	1.704 @20°C	1.61 g/cm^3	NA	NA
Vap. Press.	< 1x10^{-5} KPa @ 20°C	<10-9Kpa @25°C	<1x10^{-5} KPa @50°C	<1x10^{-7} mmHg @60°C
Solu. in water	10100 mg/L @20°C	718 g/L	7500 g/L @20°C	7490 mg @15°C

1.9.1 Glyphosate formulations (Roundup, Kilo Max and Enviro Glyphosate)

1.9.1.1. General aspects

Glyphosate *(N*-(phosphonomethyl) glycine) is a broad-spectrum, non-selective, post-emergence herbicide, with a molecular formula $C_3H_8NO_5P$ (WHO, 1994; WHO, 1996; Edge *et al*., 2011). It is a systemic herbicide that is active by plant translocation, and is first absorbed by foliage, then translocate throughout the plant via the phloem and is further transported to metabolic sinks such as meristems and roots (Laitinen *et al.*, 2009). Glyphosate is an organophosphonate with low mammalian toxicity, and does not inhibit cholinesterase in its natural form (WHO, 1996). It is perhaps the most important herbicide ever produced (Mann *et al*., 2009) (See Table 1.2 for the physical and chemical properties). Glyphosate Common formulations include Roundup, Kilo Max and Enviro Glyphosate which were included in the present study since they are widely used in WfW programme to control aquatic alien plants.

1.9.1.2. Roundup

Roundup is the most common glyphosate formulations. It consists of isopropylamine salt along with the surfactant polyethoxylated tallow amine (POEA), which has been identified in many

studies as basis of toxicity of the formulation (Perkins *et al.*, 2000). There are many conflicting reports about the toxicity of this formulation, particularly concerning amphibians. Internationally, some countries have already deregistering Roundup from the list of their approved herbicides (Relyea, 2005). While most studies suggest that it is the surfactant POEA rather than the active ingredient (isopropylamine salt of glyphosate) that is being responsible for the toxicity (Govandarajulu, 2008), others suggest otherwise (Relyea, 2005; Paganelli *et a*l., 2010). Recent studies however showed that amphibians may be one of the most sensitive vertebrate group to the toxicological effects of this herbicide (Bosch *et al*., 2011; Helbing, 2012).

Paganelli *et al*. (2010) in a study on glyphosate-based herbicides (GBH), reported that when *X. laevis* embryos were incubated with a 1/5000 dilution of a commercial GBH, the embryos were abnormal with marked alterations in cephalic and neural crest development as well as shortening of the anterior-posterior (A-P) axis. The alterations on neural crest markers were later correlated with deformities in the cranial cartilages at the tadpole stages. The embryos injected with pure glyphosate showed very similar phenotypes. This according to them suggests that glyphosate itself was responsible for the observed phenotypes, rather than just the surfactant or other components of the commercial formulations. Using a reporter gene assay, it was revealed that GBH treatment increased endogenous retinoic acid (RA) activity in *X. laevis* embryos and co-treatment with RA antagonist rescued the teratogenic effects of the GBH. Paganelli *et al*. (2010) therefore concluded that phenotypes produced by GBH are mainly consequence of the increase endogenous retinoic activity from the GBH exposure.

Benachour and Seralini (2009) reported on the toxicity of four glyphosate-based herbicides and Roundup formulations, using three different human cell types, at dilution levels that are far below agricultural recommendations. They compared the Roundup formulations to glyphosate alone and with its main metabolite amino-methyl-phosphonic acid (AMPA) or with one known adjuvant of Roundup formulations, POEA. The Roundup formulation induced apoptosis via

activation of enzymatic cysteine-aspartic proteases (caspases) 3/7 activity. According to them, the glyphosate provokes only apoptosis and growth media (HUVEC) are 100 times more sensitive overall at this level. They also pointed out that the deleterious effects are not proportional to glyphosate concentrations but rather depend on the nature of the adjuvants. They noted that AMPA and POEA separately and synergistically damage cell membranes like Roundup but at different concentrations. Their mixtures are generally even more harmful with glyphosate. In conclusion, the Roundup adjuvant POEA changed human cell's permeability and amplify toxicity induced already by glyphosate, through apoptosis and necrosis. These studies clearly confirm that the adjuvants in Roundup formulation are not inert, and that the glyphosate itself has its own toxicity contribution.

Jiraunghoor *et al.*, (2002) studied the acute toxicity of Roundup against Nile tilapia (*Oreochromis niloticus*). They reported that the values of 24 hour, 48 hour, 72 hour and 96 hour LC_{50} for young tilapia were lower than those determined for adult tilapia. This suggests that the adult tilapias are more tolerant to Roundup than the juveniles. Relyea, (2005) showed that Roundup completely eliminated two species (Leopard frogs (*Lithobates pipiens*) and Gray's tree frogs (*Hyla versicolor*) and nearly exterminated a third species (Wood frog (*Lithobates sylvatica*) resulting in a 70% decrease in the species richness of tadpoles, under ecologically relevant conditions.

Perkins *et al*. (2000) using the FETAX assay reported that Rodeo, the isopropylamine (ipa) salt of glyphosate (formulated without surfactant) had the least toxicity, with an LC_5 and LC_{50} of 3.8 and 5.4 mg/L (acid equivalent (a.e) respectively. For Roundup (with POEA surfactant), the LC_5 and LC_{50} was 6.4 and 9.4 mg/L (a.e) respectively, while the surfactant component POEA, have a LC_5 and LC_{50} of 2.2 and 2.7 mg/L respectively. Perkins *et al*. (2000) therefore concluded that the POEA surfactant is the main source of toxicity of glyphosate.

Dinehart *et al*. (2009) working on toxicity of several glyphosate based herbicides on juvenile amphibian of *Anaxyrus cognatus and Spea multiplicata* assessed the acute toxicity (48 hour) of "Roundup WeatherMax", "Roundup Weed" and "Grass Killer super concentrate", "Roundup

Weed" and "Grass killer Ready-to-use-plus", on two species of amphibians. They reported that survival of juvenile *Anaxyrus cognatus* and *Spea multiplicata* was reduced by exposure to "Roundup Weed" and "Grass killer Ready-to-use-plus". The survival of *A. cognatus* was also reduced by exposure to "Roundup Weed" and "Grass Killer super concentrate" on paper towels.

1.9.1.3. Kilo Max

According to "Material safety and data sheet" (MSDS), Kilo Max 700 is a glyphosate formulation containing sodium salt, with chemical formula of $C_6H_{17}N_2O_5P$. The 96 hour LC_{50} for trout, bluegill sunfish, and harlequin fish is 86 mg/L, 120 mg/L and 168 mg/L respectively. This formulation is one of the recent glyphosate formulations, hence no known studies exist in literatures.

1.9.1.4 Enviro Glyphosate

Enviro Glyphosate is a formulation of glyphosate that is non-volatile, and water soluble, with non-selective herbicidal activity against a wide range of annual and broadleaf weeds and grasses, in crop and non-crop situations. According to the herbicide MSDS, this formulation contain isopropyl-ammonium salt of glyphosate and polyethylene alkylamine as surfactant. Similar to Kilo Max, this formulation is also new and as such no known study has been published on it.

1.9.1.5 Diquat (Midstream formulation)

Diquat (9, 10-dihydro-8a, 10a-diazonia phenanthrene ion) is a post-emergent, non-selective contact herbicide and crop desiccant, which is also used in aquatic weeds control (Emmett, 2002; WHO, 2004). Diquat initially received U.S Federal registration for control of submersed and floating aquatic weeds in 1962 and completed the Registration Eligibility Decision (RED) process in February 2000 (Emmett, 2002). Diquat is widely used in the United State of America, Europe, Australia and Japan. These countries account for 90% of the diquat used for herbicidal purposes

(Emmett, 2002; WHO, 2004). Diquat contains nonyl phenol ethoxylate as surfactant which has been implicated as having estrogenic activities (Trumbo, 2005; Othman, 2009). Diquat is generally sold as Diquat bromide and commonly formulated as an aqueous solution (270 g of diquat ion per litre) (Emmett, 2002).

Although acute toxicity data of diquat for amphibians are largely unavailable, the available chronic toxicity data showed that for leopard frog (*Lithobates pipiens*) and the African clawed toad (*X. laevis*) the Maximum Allowable Toxic Conc (MATC) for development is 1.7 and 0.64 mg/L c.e (Cation Equivalent) respectively, while the chronic LC_{50} for leopard frog and X. laevis is < 5.4 mg/L c.e and 0.75 mg/L respectively (Emmett, 2002). It has been reported that when diquat was applied at field concentration of 1.0 mg/L, a reduction in growth rate was observed in *Lithobates temporia frog* few days after application, but they recovered their body mass back within 18 days post treatment (Emmett, 2002). However, additional work with amphibians will be important as the *X. laevis* frog and the endangered *Lithobates pipiens* have been shown to be acutely sensitive to diquat based on their chronic MATC for these species.

Greenlee et al. (2004) in a study using concentration of 0.0022 mg/L (equal to the Reference Dose (RfD) of the herbicide), showed that incubating mouse embryo with 0.0022mg/L led to significant increase in programmed cell death (apoptosis). This study concluded that embryos cleaving to blastocyst yet undergoing cellular death at a higher rate could result in embryos consisting of fewer cells, and could result in embryonic demise, implantation failures or alterations in physiological processes underlying maternal recognition of pregnancy (Greenlee et al., 2004).

1.9.1.6 Imazapyr (Arsenal formulation)

Imazapyr herbicide (2-(4-isoprpyl-4-methyl-5-oxo-2-imidazolin-2-yl) nicotini acid with molecular formula $C_{13}H_{15}N_3O_3$ belong to the chemical family of imidazolinone (Liu, 1992). This synthetic chemical compound was discovered in the 1970s, with the first U.S.A patent awarded in 1980 for

imazametha-benz-methyl, while imazapyr received U.S patent in 1989 (Hess *et a*l., 2001). Imazapyr is mainly in anionic form under normal environmental conditions, and the behaviour of the acid and salt forms are expected to be similar (WSDA, 2009). Imazapyr is a broad-spectrum herbicide formulation that is effective for non-crop uses that include total vegetation control on industrial sites, railroad, highways and forestry application (Hess *et al.*, 2001). Imazapyr degrade through photolysis in water with reported half-lives ranging between 2.5 and 5.3 days (WSDA, 2009). Some of its formulations include Format, Arsenal and Chopper. The most widely used of these imazapyr formulations in WfW is Arsenal.

According to Grisolia *et al*. (2004), Arsenal 250 NA formulation of imazapyr consists of 25 g/L imazapyr, 186 g/L of ammonium hydroxide, 18 g/L nonyphenol ethoxylate (with nine ethoxylated units) and water. For toxicity, 96 hour LC_{50} for rainbow trout, bluegill, Sunfish, Channel Catfish are all > 100 mg/L (WSSA, 1994).

1.9.1.7 Glufosinate ammonium (Basta formulation)

Basta consists of glufosinate ammonium (GA) (ammonium-DL- homoalanin-4-yl methyl) phosphate as active ingredient, and an anionic sodium polyoxyethylene alkyether sulphate (AES) as surfactant (Ebert *et al*., 1990; Koyama *et al*., 1997). The herbicidal action is related to the inhibition of glutamine synthetase (GS), an enzyme that plays an important role in ammonia detoxification and amino acid metabolism in plants (Ebert *et al*., 1990). Hence the herbicidal toxicity is caused by cytotoxicity of increased ammonia concentration and the lack of amino donors for the transamination of glyoxylate to glycine (Hack *et al*., 1994: Fabian *et al*., 2011). The commercial formulations have been noted to be more toxic than the technical grade glufosinate, for example, for the aqueous formulation, the LC50s for the fish tested were between 12.3 and 79 mg/l and for the active ingredient they were between 320 and 1000 mg/l.47 (Wolterink *et al*., 2012).

1.10. South African Knowledge Gap

For all these listed herbicide formulations, there have not been any known studies regarding effects on developmental, thyroidal, gonadal malformation as well as reproductive disruption that have been carried out in South Africa. Review of published literatures on pesticides and endocrine studies in South Africa showed only limited aspect of endocrine disruption. The focus areas include studies linking some pesticides and waste water to specific endocrine mode of actions (van Wyk *et al*., 2003; 2005; and 2013) and identification of endocrine activity in waste water (Swart and Pool, 2007; Swart and Pool, 2009a). It also include possible exposure impact assessment of pesticides on human health (Dalvie *et al*., 2004). Majority of other studies are in the areas of environmental and field assessment (London *et al*., 2000; Dalvie *et al*., 2003; Bollmohr *et al*., 2008; Bollmohr and Schuls, 2009; Ansara-Ross *et al*.,2012) and risk assessment of pesticides residues (Dalvie and London, 2009) as well as guidelines setting and prioritising of pesticides (Burger and Nel, 2008; Mensah *et al*., 2013). All indications are that there is a serious knowledge gap regarding the possible exposure impacts of locally used herbicides in South Africa, particularly on the aquatic organisms like amphibians.

1.11. Xenopus laevis as Sentinel species to study endocrine disruption potential of aquatic herbicides.

The African clawed frog, *X. laevis,* a local aquatic species, with a widespread distribution in Southern Africa (Du Preez and Caruthers, 2009), has been used and well validated as an animal model for environmental quality assessment (Mann and Bidwell, 2000; Paggeti *et al*., 2006; Kloas *et al*., 2009), toxicological testing (Bantle *et al*., 1989; Yu *et al*., 2013b) and endocrine disrupting research (Kloas and Lutz, 2006; Lutz *et al*.,2008; Opitz and Kloas, 2010) and recently validated for thyroid disruption using the XEMA protocol (van Wyk, 2013). *Xenopus laevis* are well adapted and can breed in the laboratory throughout the year, which makes them easily adaptable for all year

research. *Xenopus laevis* allow relatively quick assessment while providing very sensitive endpoints (Paggeti *et al*., 2006; Kloas *et al*., 2009; Lavorato *et al*., 2013). This fully aquatic amphibian species present a promising model to investigate toxicity of hormonally active chemicals for several reasons: gonadal differentiation is modulated by hormonally active substances, metamorphosis processes are under control of the thyroid hormonal system and susceptible to numerous environmental chemicals, and the organisation and component of hypothalamic-pituitary-thyroid (HPT), and hypothalamic-pituitary- gonad (HPG) are similar to that of higher vertebrates (Kloas *et al*., 1999; Hurter *et al*., 2002a, b; Kloas and Lutz, 2006; OECD, 2006; Optiz *et al*., 2008; Quassinti *et al*., 2009; Berg *et al*., 2009; Heindel *et al*., 2013; Boggs *et al*., 2013). Xenopus males can be used to test for estrogenicity as they have the potential to produce the yolk precursor, vitellogenin (estrogen controlled synthesis in the liver) when exposed to exogenous estrogenic substances (Kloas *et al*., 1999; Kloas and Lutz, 2006; Kloas *et al*., 2009). Their skin at all developmental stages are permeable to varieties of ions and cations (Vitt *et al*., 1990). *Xenopus laevis* also have a well-defined developmental atlas for tadpoles' stage development (Kloas *et al*., 1999; Kloas *et al*., 2009).

1.12. The exposure rationales of selected protocols

The value of *Xenopus laevis* has been realised internationally, and several validated toxicological assays have been developed as assessment tools to evaluate environmental/man-made chemicals for potential wildlife and human health effects.

The Frog Embryo Teratogenic Assay-Xenopus is a 4-day (96 hours) bioassay for assessment of developmental effects (Bernardini *et al*., 1994; Mann and Bidwell, 2000, Mann *et al*. 2011). It essentially assesses the effect of environmental impacts during embryogenesis normally highly conserved across amphibians and mammals (NICEATM, 2000). Its endpoints include mortality, incidence of malformation and growth inhibition during the embryonic developmental phase in the life cycle of an organism.

The Xenopus Metamorphosis Assay (XEMA), essentially assesses the agonist and antagonist effects of chemicals on the thyroid axis as well as associated physiology, for example, iodine metabolism, during metamorphosis using *X. laevis* tadpoles (Optiz *et al*., 2005). XEMA is a 21 day bioassay that has several advantages over other animal or cell-based in vivo assays, as it displays a temporal coupling of TH exposure to subsequent observable and measurable morphological outcome (Helbing *et al*., 2010). The XEMA assay endpoints include developmental stage, morphological effects, for example, hind limb length, front limb length snout-vent length, wet body mass as well as thyroid gland histopathology, which are all assessed following a 21-day exposure period relative to a control group (Degitz *et al*., 2005; OECD, 2007; Miyata and Ose 2012).

In the case of reproduction and sexual differentiation, the XEMA assay can be extended to completion of metamorphosis (a 28 day bioassay), and have been widely used in assessing several reproductive related endpoints, for example, gonadal development, sex ratio and gonadal histopathology (Kloas *et al*., 1999; Hurter *et al*., 2002 a, b; Kloas and Lutz, 2006; Urbatzka *et al*. 2007; Kloas *et al*., 2009).

For adult *Xenopus laevis* exposure, especially male frogs, they have been widely used to assess estrogenic (Chang and Witschi, 1955; Kloas *et al*., 1999; Hurter *et al*., 2002 (a & b); Kloas and Lutz, 2006; Kloas *et al*., 2009), as well as anti-androgenic (Hurter *et al*., 2002; van Wyk *et al*., 2003; Hayes *et al., 2002; Wise et al., 2012*) endocrine effects. Wild caught males have been used in 21-day semi-static exposures followed by assessment of functional endpoints, including VTG determination, as well as circulating hormone levels (testosterone, and thyroxine hormone) and histological endpoints (testicular, skin breeding glands, vocal cords muscles and thyroid histology) (Hurter *et al*., 2002; van Wyk *et al*., 2003; Hayes *et al., 2002;* Wise *et al., 2012*).

1.13. General Aims of the Study

The general aims of this study was to assess the toxicity and endocrine disrupting potential (aspects of the reproductive and thyroidal systems) of six herbicide formulations (known to be used in aquatic systems) including diquat dibromide (Midstream formulation), glufosinate ammonium (Basta), imazapyr (Arsenal), and glyphosate (Roundup, Kilo Max and Enviro Glyphosate formulations) through the developmental, hypothalamic-pituitary-thyroid (HPT), and hypothalamic-pituitary-gonad (HPG) pathways, using the aquatic amphibian the African clawed frog *X. laevis* as sentinel species

1.14 Specific Objectives

1. **Chapter One**- Introductory chapter giving the background to the study and reviewing aspects of the toxicology and current knowledge of the potential non-lethal effects, including endocrine disruption of herbicides on aquatic organisms. This chapter also introduced the sentinel amphibian species, the African Clawed frog *Xenopus laevis* and the Xenopus-based assays used in the present studies

   The following Chapters aimed- To assessed the exposure impacts of the six selected herbicide formulations including Diquat dibromide (Midstream formulation), Glufosinate ammonium (Basta), Imazapyr (Arsenal), and Glyphosate (Roundup, Kilo Max and Enviro Glyphosate formulations at different levels of organization using *Xenopus laevis* as model system

References

Ahlborg, U. G; Lipworth, L.; Titusernstoff, L. H.; Hsieh, C. C.; Hanberg A.; Baron, J.; Tridiopoulois, D.; Adanni H. O. (1995). Organochlorine compound in relation to breast-cancer, endometrial cancer and endometriosis- an assessment of the biological and epidemiological evidence. *Critical Reviews in Toxicology* **25(6)***:* 463-531.

Alonso-Magdalena P.; Ropero A. B.; Carrera M. P.; Cederroth C. R.; Baquie M.; Gauthier B. R.; Nef S.; Stefani E.; Nadal A. (2008). Pancreatic insulin content regulation by the estrogen receptor ER alpha. *PLoS One* **3**(4*):* e2069.

American Society for Testing and Materials (ASTM) (1998). *Standard Guide for Conducting the Frog Embryo Teratogenesis Assay – Xenopus* (FETAX). E1439-98. In: Annual book of ASTM standards, American Society for Testing and Materials, Philadelphia: 825-836.

American Society for Testing and Materials (ASTM) (1997). *Standard Guide for Conducting the Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macro-invertebrates, and Amphibians*. E1192-97. In: Annual book of ASTM standards. American Society for Testing and Materials, Philadelphia: 218-229.

Andersen, H. R.; Vinggaard, A. M.; Rasmussen, T. H.; Gjermandsen, I. M.; Bonefeld-Jorgensen, E.C (2002). Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. *Toxicology and Applied Pharmacology*. **179**:1-12.

Aneck-Hahn, N. H.; Schulenburg, G.W.; Bornman, M. S.; Farias, P.; de Jager, C (2007). Impaired semen quality associated with environmental DDT exposure in young men living in a malaria area in the Limpopo Province, South Africa. *Journal of Andrology* **28**: 423-434.

Ansara-Ross, T. M.; Wepener, V.; van den Brink, P. J.; Ross, M. J. (2012). Pesticides in South African fresh waters. *African Journal of Aquatic Science* **37**: 1-16.

Anguiano, O. L.; Montagna, C. M.; Chifflet de Liamas, M.; Ganna, L.; Pechen de D Angelo, A.M. *(1994).* Comparative toxicity of parathion in early embryos and larvae of the toad, *Anaxyrus arenarum Hensel*. *Bulletin of Environmental Contamination and Toxicology* **52**: 649-655.

Bantle, J.; Dumont, J.; Finch, R.; Linder, G. (1990). *Atlas of Abnormalities: A Guide for the Performance of FETAX*. Oklahoma State Publications Department, Printing Services, Stillwater, OK, USA. P.74.

Bantle, J. A.; Fort, D.J.; James, B.J. (1989) Identification of Developmental Toxicants using the Frog Embryo Teratogenesis Assay-Xenopus (FETAX). Hydrobiologia **188/189**: 577-585.

Bancroft, J. D.; Stevens, A. (1977). Theory and Practice of Histological Techniques. Churchill Livingstone, Edinburg.

Baskin, L.S.; Himes, K.; Colborn, T. (2001). Hypospadias and endocrine disruption: Is there a connection? *Environmental Health Perspectives* **109***:* 1175-1183.

Bergman, A.; Heindel, J. J.; Jobling, S.; Kidd, K. A.; Zoeller, R. T. (2013). *State of the Science of Endocrine Disrupting Chemicals – 2012*. An assessment of the State of the Science of Endocrine Disruptors Prepared by a Group of Experts for the United Nations Environment Programme and World Health Organization, Geneva, p. 258 Available at: http://www.who.int/ceh/publications/endocrine/en/. Accessed on 10th July 2013.

Berg, C.; Gyllenhammar, I.; Kvarnryd, M. (2009). *Xenopus tropicalis* as a test system for developmental and reproductive toxicity. *Journal of Toxicology and Environmental Health Part A* ***72****:* 219-225.

Bernardini, G.; Vismara, C.; Boracchi, P.; M, Camatini (1994). Lethality, teratogenicity and growth inhibition of heptanol in *Xenopus* assayed by a modified frog embryo teratogenesis assay-Xenopus (FETAX) procedure. *The Science of the Environment* **151***: 1-8.*

Berrill, M.; Coulson, D.; Mcgillivray, L.; Pauli, B. (1998). Toxicity of endosulfan to aquatic stages of anuran Amphibians. *Environmental Toxicology and Chemistry* **17(9)***:* 1738–1744.

Benachour, N.; Seralini, G.E. (2009). Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. *Chemical Research and Toxicology* **22**: 97–105.

Bishop, C. A.; Mahony, N. A.; Strugger, J.; Ng, P.; Pettit, K.E (1999). Anuran development density and diversity in relation to agricultural activity in the Holland Rivers watershed, Ontario, Canada. *Environmental Monitoring and Assessment.* **57***: 21-43.*

Bishop C. A.; Brooks R. J.; Carey J. H.; Ng, P.; Norstrom R. J.; Lean D.R.S (1991). The case for a cause-effect linkage between environmental contamination and development in eggs of the common snapping turtle *(Chelydras serpentina*) from Ontario, *Canada. Journal of Toxicology and Environmental Health* **33**: 521-547.

Bishop, C. A.; Pettit, K. E. (1992). Declines in Canadian Amphibian Populations: Designing a National Monitoring Strategy. Canadian Wildlife Services Occas. p.76

Blaustein, A. R.; Wake, D. B.; Sousa, W. P. (1994). Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. *Conservation. Biology* **8**: 60-71.

Blaustein, A. R; Wake, D. B (1995). The puzzle of declining amphibian populations. ***Sci. Am***. **272**: 52–57.

Boettger- Tong, H.; Murthy, L.; Chiappetta, C.; Kirkland, J.L.; Goodwin, B.; Adlercreutz, H.; Stancel, G.M.; Makela, S. (1998). A case of a laboratory animal feed with high estrogenic activity and its impact on in-vivo responses to exogenously administered estrogens. *Environmental Health Perspectives* **106***(7): 369-373.*

Bollmohr, S.; Schulz, R. (2009). Seasonal changes of macro-invertebrate communities in a Western Cape river, South Africa, receiving nonpoint-source insecticide pollution. *Environment Toxicology and.Chemistry* **28**: 809-817.

Bollmohr, S.; Thwala, M.; Jooste, S.; Havemann, A. (2008). *An Assessment of Agricultural Pesticides in the Upper Olifants Catchment*. Report Resource Quality Services, Pretoria, South Africa, p. 45.

Bold, T (2007*). Management Treatments Summary Guide- Aquatics.* Working for Waters National Office, p.7. www.wfw.co.za. Accessed on 31th Ocober, 2011.

Bosch, B.; Mañas, F.; Gorla, N.; Aiassa, D. (2011). Micronucleus test in post metamorphic *Odontophrynus cordobae* and *Rhinella arenarum* (Amphibia: Anura) for environmental monitoring. *Journal of Toxicology and Environmental Health Sciences* **3**(6): 155-163.

Boggs A. S. P.; Lowers R. H.; Cloy-McCoy J. A.; Guillette L. J. Jr (2013). Organizational changes to thyroid regulation in *Alligator mississippiensis*: evidence for predictive adaptive responses. *PLoS ONE* **8**(1*):* e55515.

Bridges, C. M (2000). Long-term effects of pesticide exposure at various life stages of the southern leopard frog (*Lithobates sphenocephala*). *Archives of Environmental Contamination and Toxicology* **39**: 91–96.

Brühl, C. A.; Pieper, S.; Weber, B. (2011). Amphibians at risk? Susceptibility of terrestrial amphibian life stages to pesticides. *Environment Toxicology and Chemistry.* **30**: 2465–2472.

Brühl, C. A.; Schmidt, T.; Pieper, S.; Alscher, A. (2013). Terrestrial pesticide exposure of amphibians: an underestimated cause of global decline? *Science Report.* **3**: 1135.

Brunelli, E.; Bernabo, I.; Berg, C.; Lundstedt-Enkel, K.; Bonacci, A.; Tripepi, S. (2009). Environmental relevant concentrations of endosulfan impair development, metamorphosis and behaviour in Anaxyrus tadpoles. *Aquatic Toxicology* **91**: 135-142.

Burger, A.E.C.; Nel, A. (2008). Report on the scoping study to determine the potential impact of agricultural substances with endocrine disruptor properties on the water resources of South Africa. WRC Project. Water Research Commission of South Africa, Pretoria.

Burkhardt-Holm, P. (2010). Endocrine disruptors and water quality: A state-of-the-art review. *International Journal of Water Resources Development* **26**: 477-493.

Cauble, K.; Wagner, R. S (2004). Sub-lethal effects of Glyphosate herbicide on amphibian metamorphosis and development. *Bulletin of Environmental Contamination and Toxicology* **75**: 429–435.

Carey, C.; Bryant, C. J. (1995). Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. *Environmental Health Perspectives* **103** (4)*:* 13-17.

Carr, J. A.; Gentles, A.; Smith, E. E.; Goleman, L. W.; Urquidi, L. J.; Thuett, K.; Kendall, R. J.; Giesy, J. P.; Gross, T. S.; Solomon, K. R.; Van Der Kraak G. (2003). Response of larval *Xenopus laevis* to Atrazine: assessment of growth, metamorphosis, gonadal and laryngeal morphology. *Environment Toxicology and Chemistry* **22**: 396-405.

Carson, R. (1962). Silent Spring. Houghton Mifflin Publishers, Boston, USA.

Cauble, K.; Wagner, R. S. (2005). Sub-lethal effects of Glyphosate Herbicide on amphibian metamorphosis and development**.** *Bulletin Environmental Contamination and Toxicology* **75**:429–435.

Chang, C.Y.; Witsch, E. (1955). Breeding of Sex-reversed males of *Xenopus laevis* Daudin. *Proceedings of Society of Experimental Biology and Medicine* **89**(1): 150-152.

Chen, C. Y.; Hathaway, K. M.; Thompson, D. G.; Folt, C. L (2008). Multiple stressor effects of herbicides, pH, and food on wetland zooplankton and a larval amphibian. *Ecotoxicology and Environmental Safety*. **71**: 209-218.

Chou, K (2005). Endocrine System: Encyclopaedia of Toxicology 2nd Edition, Philip Wexler (Ed). Elsevier Inc., London. P.170-174.

Clements, C.; Stevens, R.; Petras, M. (1997). Genotoxicity of select herbicides in *Lithobates catesbeiana* tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay. *Environmental and Molecular Mutagenesis.* **29**: 277-288.

Coady, K. K.; Murphy, M. B.; Villeneuve, D. L.; Hecker, M.; Carr, J. A.; Solomon, K. R.; Smith, E. E.; Van Der Kraak, G.; Kendall, R. J.; Giesy, J. P.(2005). Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, sex steroid hormones, and

aromatase activity in juvenile *Xenopus laevis. Ecotoxicology and Environmental Safety* **62** (2*)*:160-173.

Cocco, P. (2002). On the rumours about the Silent Spring. Review of the scientific evidence linking occupational and environmental pesticide exposure to endocrine disruption health effects. *Cadernos de Saúde Pública* **18***:* 379-402.

Colborn, T.; Dumanowski, D.; Meyers, P. (1996). Our Stolen Future. Penguin Books, Washington, USA.

Colborn, T.; vom Saal, F.S.; Soto, A.M. (1993). Development effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives* **1101**: 378-384.

Colosio, C.; Corsini E.; Barcellini W.; Maroni, M. (1999). Immune parameters in biological monitoring of pesticide exposure: current knowledge and perspectives. *Toxicology Letter*. **108**: 285-295.

Cornil, C.A.; Ball, G.F.; Balthazart, J. (2006). Functional significance of the rapid regulation of brain estrogen action: Where do the estrogens come from? Brain Research. **1126**: 2-26.

Cothran, R.D; Brown, J M.; Relyea, R. A. (2013). Proximity to agriculture is correlated with pesticide tolerance: evidence for the evolution of amphibian resistance to modern pesticides. *Evolutionary Applications* ***6(5)*:** 832-941.

Cox, C.; Surgan, M. (2006). Unidentified inert ingredients in pesticides: implications for human and environmental health. *Environmental Health Perspectives* **114**: 1803-1806.

Dabrowski, J. M.; Shadung J. M.; Wepener, V. (2014). Prioritizing agricultural pesticides used in South Africa based on their environmental mobility and potential human health effects. *Environment International* **62**: 31-40.

Dabrowski, J.; Oberholster, P. J.; Dabrowski, J. M. (2014). Water quality of Flag Boshielo Dam, Olifants River, South Africa: historical trends and the impact of drought. *Water SA 40* DOI: http://dx.doi.org/10.4314/wsa.v40i2.17.

Dalvie, M. A.; Africa, A.; London, L. (2009). Change in the quantity and acute toxicity of pesticides sold in South African crop sectors, 1994-99. *Environment International* **35**: 683-687.

Dalvie, M. A.; Cairncross, E.; Solomon, A.; London, L. (2003). Contamination of rural surface and ground water by endosulfan in farming areas of the Western Cape, South Africa. *Environmental Health*. **2**: 1-15.

Dalvie, M. A.; London, L. (2009). Risk assessment of pesticide residues in South African raw wheat. *Crop Protection* **28**: 864-869.

Dalvie, M. A.; Myers, J. E.; Thompson, M. L.; Robins, T. G.; Dyer, S.; Riebow, J.; Molekwa, J.; Jeebhay, M.; Millar, R.; Kruger, P. (2004). The long-term effects of DDT exposure on semen, fertility, and sexual function of malaria vector-control workers in Limpopo Province, South Africa. *Environmental Research.* **96**: 1-8.

De Coster, S.; van Larebeke, N. (2012). Endocrine-disrupting chemicals: Associated disorders and mechanisms of action. *Journal of Environmental and Public Health.* **2012**: 1-53.

Degitz, S. J.; Holcombe, G. W.; Flynn, K. M.; Kosian, P. A.; Korte, J. J.; Tietge J. E. (2005) Progress towards development of an amphibian-based thyroid screening assay using *Xenopus laevis*. Organismal and thyroidal responses to the model compounds 6-Propylthiouracil, Methimazole, and Thyroxine. *Toxicological Science* ***87(***2***)*****:** 353–364.

Diamanti-Kandarakis, E.; Palioura, E.; Kandarakis, S.A.; Koutsilieris, M. (2010). The impact of endocrine disruptors on endocrine targets. *Hormone and Metabolite Research* **42** (8): 543-552.

Dinehart, S. K.; Smith, L. M.; McMurry, S. T.; Anderson, T. A.; Smith P. N.; Haukos, D. A (2009). Toxicity of a glufosinate and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plain, USA. *Science.of Total Environment.* **407**: 1065-1071.

Dutta, S. K.; Mohanty-Hejmadi, P. (1978). Life history and pesticide susceptible embryonic stages of the Indian bull frog *Lithobates tigrina*. *Indian Journal of Experimental Biology* **16**: 727-729.

Du Preez, L. H.; Carruthers, V. (2009). *Complete Guide to the Frogs of Southern Africa*. Struik Publishers, Cape Town, South Africa.

Ebert, E.; Leist, K. H.; Mayer, D. (1990). Summary of safety evaluation toxicity studies of glufosinate ammonium. *Food and Chemical Toxicology* **28**: 339-349.

Edington, A. N.; Sheridan, P. M.; Stephenson, G. R.; Thompson, D. G.; Boermans, H. J (2003). Comparative effects of pH and Vision herbicide on two life stages of four anuran amphibian species. *Environmental Toxicology and Chemistry* **23** (4): 815–822.

Edge, C. B.; Gahl, M. K.; Pauli, B. D.; Thompson, D. G.; Houlahan, J. E. (2011). Exposure of juvenile green frogs (*Lithobates clamitans*) in littoral enclosures to a glyphosate-based herbicide. *Ecotoxicology and Environmental Safety* **74** (5*)*: 136–139.

Egea-Serrano, A.; Relyea, R. A.; Tejedo, M.; Torralva, M. (2012). Understanding of the impact of chemicals on amphibians: a meta-analytic review. *Ecology and Evolution* **2(**7**):** *1382-1397.*

Eldredge, N. (1998). *Life in the Balance: Humanity and the Biodiversity Crisis*. Princeton University Press, Princeton.

Emmett, K. (2002). Final Risk Assessment for Diquat Bromide. The Water Quality Program of the Washington State Department of Ecology. Washington State Department of Ecology Publication 02-10-046.www.wsde.org. Accessed 13 March 2012.

Fabian, D.; Bystriansky. J; Burkuš, *J.*; Rehak. *P.*; Legath. J.; Koppel, J. (2011). The effect of herbicide Basta 15 on the development of mouse pre-implantation embryos in-vivo and invitro. *Toxicology in Vitro* **25**: 73-79.

Fang, H.; Tong, W.; Branham, W. S.; Moland, C. L.; Dial, S. L.; Hong, H.; Xie, Q.; Perkins, R.; Owens, W.; Sheehan, D.M. (2003). Study of 202 natural synthetic and environmental chemicals for binding to the androgen receptor. *Chemical Research in Toxicology* **16**: 1338-1358.

Farah, M. A.; Ateeq, B.; Ali, M. N; Sabir, R.; Ahmad, W. (2004). Studies on lethal concentrations and toxicity stress of some xenobiotics on aquatic organisms. *Chemosphere.* **55**: 257-265.

Fort D .J; Guiney P. D; Weeks J. A; Thomas J. H; Rogers R .L; Noll A. M.; Spaulding, C.D (2004). Effect of methoxychlor on various life stages of *Xenopus laevis*. *Toxicological Sciences* **81**: 454–466.

Fry, D. M. (1995). Reproductive effects in birds exposed to pesticides and industrial chemicals. Environmental Health Perspectives ***103*****(7)**: 165-171.

Garry, V. V (2004). Pesticides and children. *Toxicology and Applied Pharmacology* ***198*****:** 152-163.

Gimeno, S.; Komen, H.; Jobling, S.; Sumpter, J.; Bowmer, T. (1998). Demasculinisation of sexually mature male common carp, *Cyprinus carpio*, exposed to 4-*tert*-pentylphenol during spermatogenesis. *Aquatic Toxicology* ***43***: 93–109.

Glusczak, L.; Miron, D. S.; Crestani, M.; Fonseca, M. B.; Pedron, F. A.; Duarte, M. F; Viera, V. L. (2006). Effects of glyphosate herbicides on acetylcholinesterase activity and metabolic and haematological parameters in Piava (*Leporinus obtusidens*). *Ecotoxicology and Environmental Safety* **65**: 237-241.

Govandarajulu, P. P. (2008). *Review of Impacts of Glyphosate Herbicide on Amphibians: What Risks can the Silvicultural Use of this Herbicide Pose for Amphibians in B.C. British Columbia.* Ministry of Environment. Wildlife Report No R-28.

Goldman J. M.; Stoker T. E.; Cooper R. L.; McElroy W. K.; Hein, J. F (1994). Blockade of ovulation in the rat by the fungicide sodium N-methyldithiocarbamate: relationship

between effects on the luteinizing hormone surge and alterations in hypothalamic catecholamines. *Neurotoxicology and Teratology*. **16**:257-268.

Gray, L. E Jr.; Kavlock, R. J.; Chernoff, N.; Ostby, J.; Ferrel, J. (1983). Postnatal developmental alterations following prenatal exposure to the herbicide 2, 4-dichlorophenyl-p-nitrophenyl ether: A dose response evaluation in the mouse. *Toxicology and Applied Pharmacology*. **67(**1*)*: 1-14.

Greenlee, A.R.; Ellis, T.M.; Berg, R.L. (2004). Low-Dose Agrochemicals and Lawn-care pesticides induced Developmental Toxicity in Mouse Preimplatation Embryos. *Environmental Health Perspectives* **112**(6): 703-709.

Greulich, K.; Pflugmacher, S. (2003). Differences in susceptibility of various life stages of amphibians to pesticides exposure. *Aquatic Toxicology* **65**: 329-336.

Grisolia, C. K.; Bilich, M. R; Formigli, L. M (2004). A comparative toxicologic and genotoxic study of the herbicide arsenal, its active ingredient imazapyr, and the surfactant nonylphenol ethoxylate. *Ecotoxicology and Environmental Safety* **59**:123–126.

Guillette, L. J.; Crain, D. A.; Rooney, A. A.; Pickford, D. B. (1995). Organization versus activation: The role of endocrine-disrupting contaminants (EDC's) during embryonic development in Wildlife. *Environmental Health Perspectives* **103**: 157-164.

Guillette, L. J. Jr; Crain, D. A.; Gunderson, M. P.; Kools, S. A. E.; Milnes, M. R.; Orlando, E. F.; Rooney, A. A.; Woodward, A. R. (2000). Alligators and endocrine disrupting contaminants: A current perspective. *American Zoology **40*:** 438–452.

Guillette, L. J Jr; Gunderson, M. P (2001). Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. *Reproduction* **122**: 857–864.

Gungordu, A. (2013). Comparative toxicity of methidathion and glyphosate on early life stages of three amphibian species: *Pelophylax ridibundus*, *Pseudepidalea viridis*, and *Xenopus laevis*. *Aquatic Toxicology **140– 141***: 220– 228.

Gupta, R. C. (2007). *Rotenone.* In Veterinary Toxicology (Ed) Basic and Clinical Principles Gupta, R.C. Academic Press, New York, USA. pp. 499-506.

Hack, R; Ebert, E.; Ehling, G.; Leist, K. H (1994). Glufosinate-ammonium- some aspects of its mode of action in mammals. *Food and Chemical Toxicology* **32**(5*)*: 461-470.

Hamid, H.; Eskicioglu, C. (2012). Fate of estrogenic hormones in wastewater and sludge treatment: A review of properties and analytical detection techniques in sludge matrix. *Water Research.* **46**: 5813-5833.

Harrison, P. T. C.; Holmes, P.; Humphrey, C. D. N (1997). Reproductive health in humans and wildlife: are adverse trends associated with environmental exposure. *The Science of Total Environment* **205**: 97-106.

Hayes, T. B.; Falso, P.; Gallipean, S.; Stice, M. (2010).The cause of global amphibian declines: A developmental endocrinologist perspective. The Journal of Experimental Biology **213***: 921-933.*

Hayes, T. B.; Collins, A.; Mendoza, M.; Noriega, N.; Stuart, A. A.; Vonk, A (2002). Hermaphroditic, demasculinized frogs exposure to the herbicide atrazine at low ecology relevant doses. *Proceedings of the National Academy of Sciences, USA*, **99***:* 5476-5480.

Hayes, T. B.; Collins, A.; Mendoza, M.; Noriega, N.; Stuart, A. A.; Vonk, A. (2003). Atrazine induced hermaphroditism at 0.1 ppb in American leopard frogs *(Lithobates Pipiens):* laboratory and field evidence. *Environmental Health Perspectives* **3**: 568-575.

Hess, F. G; Harris, J. E.; Pendino, K.; Ponnock, K. (2001). *Imidazolinones.* Hayes' Handbook of Pesticide Toxicology, 2 (2). Robert Krieger (Eds). Academic Press, USA, p.1543-58.

Hess, R. A.; Bunick, D.; Bahr, J. (2001). Oestrogen, its receptors and function in the male reproductive tract- A review. *Molecular and Cellular Endocrinology* **178** (1-2): 29-38.

Helbing, C. C.; Propper, C. R.; Veldhoen, N. (2011). Triclosan affects the thyroid axis of amphibians. *Toxicological Sciences* **123**(2*):* 601–602.

Helbing, C. C.; Maher, S. K.; Han, J.; Gunderson, M. P.; Borchers, C. (2010). Peering into molecular mechanisms of action with frogSCOPE. *General and Comparative Endocrinology* **168**: 190-198.

Helbing, C. C (2012). The metamorphosis of amphibian toxicogenomics. *Front Genetics* **3**: 1-6

Heindel J. J.; Zoeller, R.; Jobling, S.; Iguchi, T.; Vandenburg, M. J.; Woodruff, T. J (2013). What is Endocrine Disruption All About? In : Bergman A, Hindel JJ, Jobling S, Kidd VA, Zoeller R, eds. State of the Science; Endocrine Disrupting Chemicals- Geneva; WHO & UNEP. 1-22. Available at: http://www.who.int/ceh/publications/endocrine/en/. Accessed on 10th July 2013.

Hinther, A.; Vawda, S.; Skirrow, R. C.; Veldhoen, N.; Collins, P.; Cullen, J. T.; van Aggelen, G.; Helbing, C. C (2010). Nanometals induce stress and alter thyroid hormone action in amphibian at or below North American water quality guidelines. *Environment Science and Technology* **44**: 8314–8321.

Holmes, A. M.; Creton, S.; Chapman, K. (2010) Working in partnership to advance the 3Rs in toxicity testing. *Toxicology* **267**: 14–19.

Hoke, R. A.; Ankley, G. T (2005). Application of frog embryo teratogenesis assay-Xenopus to ecological risk assessment. *Environmental Toxicology and Chemistry* **24(**10*)*: 2677-2690.

Hotchkiss, A. K.; Rider, C. V.; Blystone, C. R.; Wilson, V. S.; Hartig, P. C.; Ankley, G. T.; Foster, P. M.; Gray, C. L.; Gray, L. E (2008). Fifteen years after 'wingspread'-environmental endocrine disrupters and human and wildlife health: Where we are today and where we need to go. *Toxicological Sciences* **105(**2*)*: 235-359.

Howe, C. M.; Berrilli, M.; Pauli, B. D.; Hebing, C. C.; Werry, .K; Vedhoen, N. (2004). Toxicity of glyphosate-based pesticides to four North America frog species. *Environental Toxicology and Chemistry* **23**: 1928-1938.

Hurter, E.; Pool, E. J.; Van Wyk, J. H. (2002). Validation of an *ex vivo Xenopus* liver slice bioassay for environmental estrogens and estrogen mimics. *Ecotoxicology and Environmental Safety* **53***:* 178-187.

Hutchinson, T. H.; Ankley, G. T.; Segner, H.; Tyler, C. R. (2006). Screening and testing for endocrine disruption in fish-biomarkers as "signposts", not "trafic lights" in risk assessment. *Environmental Health Perspectives* **114**: 106-114.

Hurley, P. M; Hill, R. N; Whiting, R. J. (1998). Mode of carcinogenic action of pesticides inducing thyroid follicular cell tumors in rodents. *Environmental Health Perspectives* ***106(***8*)*:437-445.

Hynes, S. V.; Wilson, S.; Byrne, M. (2009). *Frogs as Bio-indicators of Chemical Usage and Farm Practices in An Irrigated Agricultural Areas*. Final Report to Land and Waters Australia. PN30152, Land & Water Australia, Canberra, Australia.

Iguchi, T.; Katsu, Y. (2008). Commonality in signalling of endocrine disruption from snail to human. *Bioscience* **58**(11):1061-1067.

Ishihara, A.; Nishiyama, N.; Sugiyama, S.; Yamauchi, K (2003). The effect of endocrine disrupting chemicals on thyroid hormone binding to Japanese quail transthyretin and thyroid hormone receptor. *General and Comparative Endocrinology* **134**: 36-43.

Jiraungkoorsku, W.; Upatham, E. S.; Kruatrachue, M.; Sahaphong, S.; Vichasri-Grams, S.; Pokethitiyook, P. (2001). Histopathological effects of Roundup, a glyphosate herbicide, on Nile tilapia. *Science Asia* **28**: 121-127.

Jobling, S.; Beresford, N.; Nolan, M.; Rodgers-Gray, T.; Brightly, G. C.; Sumpter, J. P.; Tyler, C. R. (2002a). Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus)* living in rivers that receive treated sewage effluents. *Biology of Reproduction* **66**: 272–281.

Jobling, S.; Coey, S.; Whitmore, J. G.; Kime, D.E.; Van Look, K. J. W.; McAllister, B. G.; Beresford, N.; Henshaw, A. C.; Brightly, G.; Tyler, C .R.; Sumpter, J. P. (2002b). Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biology of Reproduction* **67**: 515–524.

Johansson, M.; Piha Kylin, H.; J, Merila (2006). Toxicity of six pesticides to common frog (*Lithobates temporaria)* tadpoles. *Environmental Toxicology and Chemistry* **25 (**12*)*: 3164–3170.

Johnson, M. L.; Hall, L. C (2002). The Estrogenicity of Selected Herbicides and Adjuvants endocrine disruption capabilities of Surflan and Oryzalin. A report prepared for the Division of Environmental Analysis, California Department of Transportation Interagency. http://www.dot.ca.gov/hq/env/stormwater/pdf/CTSW-RT. Assessed 10 Oct 2013.

Jugan, M. L.; Oziol, L.; Bimbot, M.; Hunteau, U.; Tmisier-Karolak, S.; Blondeau, J. P.; Y, Levi (2009). In vitro assessment of thyroid and estrogenic endocrine disruptors in wastewater treatment plants, rivers and drinking water supplies in the greater Paris area (France).*Science of the Total Environment* **407**: 3579-3587.

Kappler B.; D, Namuth (2004). *Herbicide Classification*. Plant and Soil Science eLibrary. www.passel.unl.edu/pages/printinformationmodule. Assessed on 24-08-2013.

Khan, M. Z.; Law, F. C. P (2005). Adverse effects of pesticides and related chemicals on enzymes and hormone systems of fish, amphibians and reptiles: *A review. Proceding of Pakistan Academic of Science* **42*(4)***: 315-323.

Kidd, K. A.; Blanchfield, P. J.; Mills, K. H.; Palace, V. P.; Evans, R. E.; Lazorchak, J. M.; Flick, R. W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceding of National Academic of Science, USA.* ***104*****:** 8897-8901.

Kidd, K. A.; Becher, G.; Bergman, A.; Muir, D. C. G.; Woodruff, T. J. (2013). Human and wildlife exposures to EDCs, In: Bergman, A., Heindel, J., Jobling, S., Kidd, K. A., Zoeller, R. T. (Eds.), State of the Science of Endocrine Disrupting Chemicals -2012. WHO and UNEP, Geneva, 189-209.

Kime, D. E. (1998). *Endocrine Disruption in Fish*. Kluwe Academic Publishers, London, UK.

Kloas, W.; Lutz, I. (2006). Amphibians as model to study endocrine disrupters. *Journal of. Chromatography A* **1130**: 16-27.

Kloas, W.; Opitz, R.; Lutz, I. (2003). XEMA - Standard Operating Procedure for the conduct of the Xenopus Metamorphosis Assay (XEMA). Department of Inland Fisheries, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany, Berlin, Germany.

Kloas, W.; Lutz, I.; Einspanier, R. (1999). Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals *in vitro* and *in vivo*. *Science of Total Environment* **225***:* 59-68.

Kloas, W.; Urbatzka, R.; Opitz, R.; Wurtz, S.; Behrends, T.; Hermelink, B.; Hoffmann, F.; Jagnytsch, O.; Kroupova, H.; Lorenz, C.; Neumann, N.; Pietsch, C.; Trubiroha, A.; Ballegooy, C.; Wiedermann, C.; Lutz, I. (2009*). Endocrine Disruption in Aquatic Vertebrates.* Trends in Comparative Endocrinology and Neurobiology: *Annual New.York Academic of Science* **1163**: 187-200.

Koyama, K.; Koyama K.; Goto, K. (1997). Cardiovascular effects of an herbicides containing glufosinate and a surfactant: in vitro and in vivo analysis in rat. *Toxicology And Applied. Pharmacology* **145**: 409-414.

Kojima, H.; Katsura, E.; Takeuchi, S.; Niiyama, k.; Kobayashi, K. (2004). Screening for estrogen and androgen receptor activities in 200 pesticides by *in vitro* reporter gene assays using Chinese hamster ovary cells. *Environmental Health Perspectives* **112**: 524-531.

Kortenkamp, A.; Martin, O.; Faust, M.; Evans, R.; McKinlay, R.; Orton, F.; Rosivatz, E. (2011). State of the art assessment of endocrine disrupters- Final report project contract number 070307/2009/550687/SER/D3.

Krimsky, S. (2000). Hormonal Chaos: The Scientific and Social Origins of the Environmental endocrine Hypothesis. The John Hopkins University Press, Baltimore.

Kumar, V.; Chakraborty, A; Viswanathl G; Roy, P (2008). Androgenic endocrine disruptors in wastewater treatment plant effluents in India: Their influence on reproductive processes and systemic toxicity in male rats. *Toxicology and Applied Pharmacology* **226***:* 60–73.

Laitinen, P.; Ramo, S.; Nikunen, U.; Jauhiainen, L.; Siimes, K.; Turtola, E. (2009). Glyphosate and Phosphorus leaching and residues in Boreal sandy Soil. *Plant Soil.* **323**: 267-283.

Lajmanovich, R. C.; Junges, C. M.; Attademo, A. M.; Peltzer, P. M.; Cabagna-Zenklusen, M. C.; Basso, A. (2013). Individual and mixture toxicity of commercial formulations containing Glyphosate, Metsulfuron-methyl, Bispyribac-sodium, and Picloram on *Rhinella arenarum* tadpoles. *Water. Air. Soil Pollution* **224**:1404.

Lamoreaux, R. J. (1994). Herbicide discovery and development: emphasis on groundwater protection. *Crop Protection.* **13**(7*):* 483-487.

Larson, D. L.; Mcdonald, A. J.; Fivizzani, W. E.; Newton; Halmilton, S. J. (1998). Effects of the herbicide atrazine on *Ambystoma tigrinum* metamorphosis: during larval growth and hormonal response. *Physiological Zoology.* **71**: 671-679.

Lavorato, M. I.; Bernabò Antonio Crescente Mathieu Denoël; Sandro Tripepi Elvira Brunelli (2013). Endosulfan effects on *Lithobates dalmatina* tadpoles: Quantitative developmental and behavioural analysis. *Archives of Environmental Contamination and Toxicology*. ***64*:** 253–262.

Lemaire, G.; Mnif, W.; Pascussi, J. M.; Pillon, A.; Rabenoelina, F.; Fenet, H.; Gomez, E.; Casellas, C.; Nicolas, J. C.; Cavailles, V.; Duchesne, M. J.; Balaguer, P. (2006). Identification of new human pregnane X receptor ligands among pesticides using a stable reporter cell system. *Toxicological Science* **91**(**2**): 501-509.

Lenkowski, J. R.; Reed, J. M.; Deininger, L.; McLaughlin, K. A. (2008). Perturbation of organogenesis by the herbicide atrazine in the amphibian Xenopus *laevis*. *Environmental Health Perspectives* **116**: 223–230.

Li, J.; Ma, M.; Wang, Z. (2008). A two-hybrid yeast assay to quantify the effects of xenobiotics on thyroid hormone-mediated gene expression. *Environmental Toxicology and Chemistry* **27(1***)*: 159-167.

Liu, W.; Pusino, A., Gessa, C. (1992). High-perfomance liquid chromatographic determination of the herbicide imazapyr residues in water and soil. *The Science of the Total Environment*. **123/124**: 39-43.

London, L.; Dalvie, M.A.; Cairncross, C.; Solomons, A. (2000). The Quality of Surface and Groundwater in the Rural Western Cape with regard to Pesticides. WRC Report. Water Research Commission, Pretoria, South Africa.

Lorenz, C.; Contardo-Jara, V.; Trubiroha, A.; Kruger, A.; Viehmann, V.; Wiegand, C.; Pflugmacher, S.; Nutzmann, G.; Lutz, I.; Kloas, W (2011). The synthetic gestagen Levonorgestrel disrupts sexual development in *Xenopus laevis* by affecting gene expression of pituitary gonadotropins and gonadal steroidogenic enzymes. *Toxicological Science*. **124**: 311-319.

Lutz, I.; Kloas, W.; Springer, T. A.; Holden, L. R.; Wolf, J. C.; Krueger, H. O.; Hosmer, A. J. (2008). Development, standardization and refinement of procedures for evaluating effects of endocrine active compounds on development and sexual differentiation of *Xenopus laevis*. *Analytical and Bioanalytical Chemistry* **390**: 2031-2048.

Mann, R. M.; Bidwell, J. R. (2000). Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. *Aquatic Toxicology* **51**: 19-29.

Mann, R. M.; Hyne R. V.; Choung, C. B.; Wilson, S. P. (2009). Amphibians and agricultural chemicals: review of the risks in a complex environment. *Environmental Pollution* **157**: 2903-2927.

Mann, R. M.; Bidwell, J. R.; Tyler, M. J (2003). Toxicity of herbicide formulations to frogs and the implications for product registration: A case study from Western Australia. *Applied Herpertology* **1**: 13-22.

Marlatt, V.L.; Gerrie, E.; Wiens, S.; Jackson, F.; Moon, T.W.; Trudeau, V.KL. (2012). Estradiol and triiodothyronine differentially modulate reproductive and thyroidal genes in male goldfish. *Fish Physiology and Biochemistry* **38**:283–296.

Marlatt, V.L.; Veldhoen, N.; Lo, B.P.; Bakker, D.; Rehaume, V.; Vallée, K.; Haberl, M.; Shang, D.; van Aggelen, G.C.; Skirrow, R.C.; Elphick, J.R.; Helbing, C.C.(2013). Triclosan exposure alters postembryonic development in a Pacific tree frog (*Pseudacris regilla*) Amphibian Metamorphosis Assay (TREEMA) *Aquatic Toxicology* **126**: 85– 94.

Magnuusson, U.; Brunstrom, B. (Ed) (2007). Reproductive Toxicology in Environmental Research. Swedish Environmental Protection Agency Report 5729. www.sepa.org/ report. Accessed on 12th October, 2011.

McDaniel, T.V.; Martin, P. A.; Struger, J.; Sherry J.; Marvin C. H.; McMaster M. E., Clarence S.; Tetreault, G. (2008). Potential endocrine disruption of sexual development in free ranging male northern leopard frogs (*R. pipiens*) and green frogs (*R. clamitans*) from areas of intensive row crop agriculture. *Aquatic Toxicology* ***88***: 230–242.

McKinlay, R.; Plant, J.A.; Bell, J.N.B.; Voulvoulis, N. (2008). Endocrine disrupting pesticides: Implications for risk assessment. *Environment International* **34**: 168-183.

Mensah, P. K.; Palmer, C. G.; Muller, W.J (2013). Derivation of South African water quality guidelines for Roundups using species sensitivity distribution. *Ecotoxicology and Environmental Safety* **96***:* 24–31.

Miyata, K.; Ose, K. (2012). Thyroid hormone-disrupting effects and the amphibian metamorphosis assay. *Journal of Toxicology and Pathology* **25**: 1–9.

Mnif, W.; Hassine, A. I.; Bouaziz, A.; Bartegi, A.; Thomas, O.; Roig, B. (2011). Effect of endocrine disruptor pesticides: a review. *International Journal of Environmental Research and Public Health* ***8*:** 2265-2303.

Muths E; Gallant A. L; Grant E. H. C; Battaglin W. A; Green D. E; Staiger J. S; Walls S. C; Gunzburger M. S; Kearney R. F (2006). The Amphibian Research and Monitoring Initiative (ARMI): 5-year report: US Geological Survey Scientific Investigations Report 2006–5224, p.77.

National Toxicology Program Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM) (2000). Frog Embryo Teratogenesis Assay- Xenopus (FETAX) -

Background Review Document. National Institute of Environmental Health Sciences (NIEHS) Research Triangle Park, Report NC 27709.

Nilsson, S.; Makela, S.; Treuter, E.; Tujague, M.; Thomsen, J.; Andersson, G.; Enmark, E.; Petterson, K.; Warner, M.; Gusta, J.A (2001). Mechanisms of estrogen action. *Physiology Reviews* **81** (4): 1535-1565.

Norris, D. O.; Carr, J. A. (2006). Endocrine Disruption: Biological Bases for Health Effects in Wildlife and Humans. Oxford University Press, Oxford.

Nweke O. C.; Sanders, W. H (2009). Modern environmental health hazards: a public health issue of increasing significance in Africa. *Environmental Health Perspective* **117**(6): 863–70.

Ochoa-Acuña, H.; Frankenberger, J.; Hahn, L.; Carbajo, C. (2009). Drinking-water herbicide exposure in Indiana and prevalence of small-for-gestational-age and preterm delivery. *Environmental Health Perspective* **117(**10*)*:1619–24.

Okubo, T.; Yokoyama, Y.; Kano, K.; Soya, Y.; Kano, I. (2004). Estimation of estrogenic and antiestrogenic activities of selected pesticides by MCF-7 cell proliferation assay. *Archives of Environmental Contamination and Toxicology* **46(**4*)*:445–453.

Opitz, R.; Schmidt, F.; Braunbeck, T.; Wuertz, S.; Kloas, W. (2009). Perchlorate and Ethylenethiourea induce different histological and molecular alterations in a non-mammalian vertebrate model of thyroid goitrogenesis, *Molecular and Cellular Endocrinology* **298**(2*)*: 101-114.

Opitz, R.; Braunbeck, T.; Bögi, C.; Pickford, D. B.; Nentwig, G.; Oehlmann, J.; Tooi, O.; Lutz, I.; Kloas, W. (2005). Description and initial evaluation of a *Xenopus* metamorphosis assay for detection of thyroid system-disrupting activities of environmental compounds. *Environmental Toxicology and Chemistry* **24:** 653-664.

Organisation for Economic Cooperation and Development (OECD) (2004). Environment Health and Safety Publication Series on Testing and Assessment No 46. ENJ/JM/MONO 17. Available at: www.oecd.org/officialdocuments/. Accessed on 11 May 2011

Organisation for Economic Cooperation and Development (OECD) (2007).Validation of the amphibian metamorphosis assay as a screen for thyroid-active chemicals: integrated summary report. AMA integrated summary report. Available at: www.oecd.org/officialdocuments/. Accessed on 11 May 2011.

Organisation for Economic Cooperation and Development (OECD) (2006) Current approaches in the statistical analysis of ecotoxicity data. A guidance to application. OECD series on testing and assessment No 54. ENV/JM/ MONO (2006) 18. Available at: www.oecd.org/officialdocuments/displaydocument. Accessed Jan 2013.

Organisation for Economic Co-operation and Development (OECD) (2008). Series on Testing and Assessment. No. 91. Report of the Validation of the Amphibian Metamorphosis Assay

(PHASE 3) ENV/JM/MONO (2008)18 Available at: www.oecd.org/officialdocuments/displaydocument.

Orton, F.; Rosivatz, E.; Scholze, M.; Kortenkamp, A. (2011). Widely used pesticides with previously unknown endocrine-activity revealed as *in vitro* anti-androgens. *Environmental Health Perspectives.* **119**: 794-800.

Orton, F.; Rosivatz, E.; Scholze, M.; Kortenkamp, A. (2012). Competitive androgen receptor antagonism as a factor determining the predictability of cumulative antiandrogenic effects of widely used pesticides. *Environmental Health Perspectives* **120:** 1578-1584.

Orton, F; Lutz, I.; Kloas, W.; Routledge, E. J. (2009). Endocrine disrupting effects of herbicides and pentachlorophenol: In vitro and in vivo evidence. *Environment Science and Technology* **43***:* 2144-2150.

Ortiz-Santaliestra, M. E.; Marco, A.; Fernandez, M. J.; Lizana, M. (2006). Influence of developmental stage on sensitivity to Ammonium nitrate on aquatic stages of amphibians. *Environmental Toxicology and Chemistry* **25**(1*)*: 105–111.

Osano, O.; Oladimeji, A. A.; Kraak, M. H. S.; Admiral, W. (2002). Teratogenic effects of Amitraz, 2, 4-Dimethylaniline, and Paraquat on developing frog (*Xenopus*) embryos**.** *Archives of Environmental Contamination and Toxicology* **43**: 42–49.

Othman, M; Ding, L; Jiao, Y (2009). 'Effect of anionic and non-ionic surfactants on activated sludge oxygen uptake rate nitrification', In W*orld Academy of Science, Engineering and Technology Issue* **58***:* 1199-1205.

Patrick, L. (2009). Thyroid disruption: Mechanisms and clinical implications in human health. *Alternative Medicine Review* **14 (**4*)*: 326-346.

Paggetti, E.; Biaggini, M.; Corti, C.; Lebboroni, M.; R, Berti (2006). Amphibians and reptiles as indicators in Mediterranean agro-ecosystems: A preliminary study. Proceedings of the 13th Congress of the Societas Europaea Herpetologica. 107-110.

Paganelli, A.; Gnazzo, V.; Acosta H.; Lopez, S. L.; Carrasco, A. E. (2010). Glyphosate-Based herbicides produce tetratogenic effects on vertebrates by impairing retinoic acid signalling. *Chemical Research in Toxicology* **23***:* 1586-1595.

Pašková, V.; Hilscherová, K.; Bláha, L. (2011). Teratogenicity and embryotoxicity in aquatic organisms after pesticide exposure and the role of oxidative stress. In D.M. Whitacre (ed.), *Reviews of Environmental Contamination and Toxicology* **211**: 25-61.

Perkins, P. J.; Boermans, H. J.; Stephenson. G. R. (2000). Toxicity of glyphosate and triclopyrusing the frog embryo teratogenesis assay-*Xenopus*. *Environmental Toxicology and Chemistry* **19**: 940–945.

Petterson, M.; Ekelund, N. G. A (2006). Effects of the herbicides Roundup and Avans on *Euglena gracilis*. *Archives of Environmetal Contamination and Toxicology* **50**: 175-181.

Pickford, D. B.; Morris, I. D. (2003). Inhibition of gonadotropin-induced oviposition and ovarian steroidogenesis in the African clawed frog *(X. laevis*) by the pesticides methoxychlor *Aquatic Toxicology* **62**: 179-194.

Piha, J.; Kylin, M; Merila, H. J, (2006). Toxicity of six pesticides to common frog (*Lithobates temporaria)* Tadpoles. *Environmental Toxicology and Chemistry **25** (12)*: 3164–3170.

Piola, L.; Fuchs, J.; Oneto, M. L; Basack, S; Kesten, E; Casabe, N. (2013). Comparative toxicity of two glyphosate-based formulations to *Eisenia andrei* under laboratory conditions. *Chemosphere* **91**: 545-551.

Plakas, K. V.; Karabelas, A. J. (2012). Removal of pesticides from water by NF and RO membranes - A review. *Desalination.* **287**: 255-265.

Przewlocka, B.; Sarnek, J.; Szmigielski, A.; Niewiadomska, A. (1975). The effect of some dithiocarbamic acids on dopamine-beta-hydroxylase and catecholamines level in rat's brain. *Poland Journal Pharmacology and Pharmacy* **27:** 555-559.

Quassinti, L.; Maccari, E.; Murri, O.; Bramucci, M. (2009). Effects of Paraquat and Glyphosate on steroidogenesis in gonads of the frog *Lithobates esculenta* in vitro. *Pesticide Biochemistry and Physiology* **93**: 91-95.

Radosevich, S. R.; Holt J. S.; Ghersa C. M. (2007). Ecology of Weeds and Invasive Plants: Relationship to Agriculture and Natural Resource Management (3rd Ed.), Wiley-Inter-science, Hoboken, USA.

Reeder, A.L., Foley, G.L., Nichols, D.K., Hansen, L.G., Wikoff, B., Faeh, S., Eisold, J., Wheeler, M.B., Warner, R., Murphy, J.E., Beasley, V.R., 1998. Forms and prevalence of intersexuality and effects of environmental contaminants on sexuality in cricket frogs (Acris crepitans). *Environmental Health Perspectives* **106**: 261–266.

Reinert, K. H.; Giddings, J. M.; L, Judd (2002). Effects analysis of time-varying or repeated exposures in aquatic ecological risk assessment of agrochemicals. *Environmental Toxicology and Chemistry* **21**: 1977–1992.

Reini, W. B.; Thomas, C. M. G.; Scheepers, P. T. J.; Zielhuis, G. A.; Roeleveld, N. (2006). Pesticide exposure: the hormonal function of the female. *Reproductive Biology and Endocrinology **4*(30):**1-14.

Relyea, R. A.; Mills, N. (2001). Predator-Induced Stress Make the Pesticide Carbaryl more deadly to Gray Treefrogs Tadpoles. *Proceeding of the National Academic Of Sciences of USA* **98:** *2491-2496.*

Relyea, R. A. (2009). A Corktail of contaminants: how mixtures of pesticides at low concentrations affects aquatic communities. *Oecologia **159**: 363-376.*

Relyea, R. A. (2005). The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecological Applications* **15(**2*): 618-627.*

Ruenitz, P.C.; Bourne, C.S.; Sullivan K.J.; Moore, S.A. (1996). Estrogenic Triarylethylene Acetic Acids: Effects of Structural Variation on Estrogen Receptor Affinity and Estrogenic Potency and Efficacy in MCF-7 Cells. *Journal of Medical Chemistry* **39**: 4853-4859.

Salazar, K. D.; Schafer, R.; Barnett, J. B.; Miller, M. R. (2006). Evidence for a novel endocrine disruptor, the pesticide propanil requires the ovaries and steroid synthesis to enhance humoral immunity. *Toxicological Sciences* **93:** 62-74.

Samsel, A.; Seneff, S, (2013). Glyphosate's suppression of cytochrome P450 enzymes and amino acid biosynthesis by the gut microbiome: Pathways to modern diseases entropy 2013 (15): 1416-1463; doi:10.3390/e15041416.

Sanderson, J. T (2006). The Steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. *Toxicological Sciences* **94**(1*):* 3–21 doi:10.1093/toxsci/kfl051.

Sanderson, J. T.; Seinen, W.; Giesy, J. P; van den Berg, M. (2000). 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells, a novel mechanism for estrogenicity? *Toxicological Sciences* **54**: 121-127.

Schmutzler, C.; Gotthardt, I.; Hofmann, P.J.; Radovic, B.; Kovacs, G.; Stemmler, L.; Nobis, I.; Bacinski, A.; Mentrup, B.; Ambrugger, P (2007). Endocrine disruptors and the thyroid gland—A combined *in vitro* and *in vivo* analysis of potential new biomarkers. *Environmental Health Perspectives* **115**: 77-83.

Schreinemachers, D. D (2003). Birth malformations and other adverse perinatal outcomes in four US wheat-producing states. *Environmental Health Perspectives* **111**: 1259-1264.

Sharpe, R. M (1993). Commentary. Declining sperm counts in men: is there an endocrine cause? *Journal of Endocrinology* **136**: 357-360.

Sharpe, R. M.; Skakkebaek N.E (1993). Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* **341**: 1392-1395.

Sharpe, R. M.; Fisher J. S.; Millar M. M.; Jobling S.; Sumpter J. P (1995). Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. *Environmental Health Perspectives* **103**:1136-1143.

Shi, H.; Zhu, P.; Guo, S. (2012). Effects of tributyltin on metamorphosis and gonadal differentiation of *Xenopus laevis* at environmentally relevant concentrations. *Toxicology and Industrial Health* 1–7.

Soyano, K.; Aoki, J.; Itashiki, Y.; Park, C.; Nagae, M.; Takao, Y.; Lee, Y.; Yeo, I.; Zhong, J. (2010). Contaminations by endocrine disrupting chemicals in coastal waters of the East China Sea**.** Coastal Environmental and Ecosystem Issues of the East China Sea, Eds., A. Ishimatsu, H.-***J. Lie*** 215–226.

Sparling, D.W.; Linder, G.; Bishop, C.A (2000). Ecotoxicology of Amphibian and Reptiles. SETAC Press, Pensaiola, Florida, USA.

Sparling D. W.; Fellers G. M.; McConnell L. S (2001). Pesticides and amphibian population declines in California USA. *Environmental Toxicology and Chemistry* **20:** 1581-1595.

Suntharasingham, C.; Oyiliagu, C. E.; Cao, X.; Gouin, T.; Wania, F.; Lee, S. C.; Pozo, K.; Harner, T.; Muir, C.G (2010). Spatial and temporal pattern of pesticides in the global atmosphere. *Journal of Environmental Monitoring* **12**: 1650–1657.

Swart, J. C.; Pool, E. J. (2007). Rapid detection of selected hormones from sewage effluents using an ELISA in the Kluis River water catchment area, South Africa. *Journal of Immunoassay and Immunochemistry* **28**: 395-408.

Swart, J. C.; Pool, E. J. (2009a). The development and validation of a quantitative ELISA for in vivo and in vitro synthesized vitellogenin from mossambicus tilapia (*Oreochromis mossambicus*). *Journal of Immunoassay and Immunochemistry* **30**: 208-223.

Swedenborg, E. J.; Egg, lle R.U.; Ma¨kela, S.; Pongratz, I. (2009). Endocrine disruptive chemicals: mechanisms of action and involvement in metabolic disorders. *Journal of Molecular Endocrinology*. **43**: 1–10.

Takahashi, M. (2007). Oviposition site selection: Pesticide avoidance by Gray Treefrogs. *Environmental Toxicology and Chemistry* **26** (7***)*:**1476-1480.

Tavera-Mendoza, L.; Ruby, S.; Brousseau, P.; Fourier, M.; Cyr, D.; Marcogliese, D. (2002a). Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. *Environmental Toxicology and Chemistry* **21**: 527–531.

Tavera-Mendoza, L.; Ruby, S.; Brousseau, P.; Fourier, M.; Cyr, D.; Marcogliese, D. (2002b). Response of the amphibian tadpole *Xenopus laevis* to atrazine during sexual differentiation of the ovary. *Environmental Toxicology Chemistry* **21**: 1264–1267.

Thibaut, R.; Porte, C. (2004). Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish. *Journal of Steroid Biochemistry and Molecular Biology* **92**: 485-494.

Trachantong, W.; Promya, J.; Saenphet, S.; Saenphet, K. (2013). Effects of atrazine herbicide on metamorphosis and gonadal development of *Hoplobatrachus rugulosus. Maejo International Journal of Science and. Technology* ***7*** (Special Issue): 60-71.

Trudeau, V.; Tyler, C. (2007). Endocrine disruption. *General and Comparative Endocrinology* **153**: 13-14.

Trumbo, J. (2005). An assessment of the hazard of a mixture of the herbicide Rodeo and the non-ionic surfactant R-11to aquatic invertebrates and larval amphibians**.** California Fish and Game **91**(1):38-46.

Urbatzka, R.; van Cauwenberge, A.; Maggioni, S.; Vigano, L.; Mandich, A.; Benfenati, E.; Lutz, I.; Kloas, W. (**2007**). Androgenic and anti-androgenic activities in water and sediment samples from the river Lambro, Italy, detected by yeast androgen screen and chemical analyses. *Chemosphere* **67**: 1080–1087.

USDA Forest Service (2003). Human and ecological risk assessment of nonyl-phenol polyethoxylate-based (NPE) surfactants in Forest Service herbicide applications. Unpublished Report, Pacific Southwest Region Pesticide-Use Specialist, p.182.

Vandenberg, L. N.; Colborn T.; Hayes T. B.; Heindel J. J.; Jacobs D. R.; Lee D. H.; Shioda, T.; Soto, A. M.; vom Saal, F. S.; Welshons, W. V.; Zoeller, R. T.; Myers, J. P (2012). Hormones and endocrine-disrupting chemicals: Low-dose effects and non-monotonic dose responses. *Endocrine Reviews* **33(**3): 378-455.

Van Wyk, J. H.; Pool E. J.; Leslie, A. J. (2003). The effects of anti-androgenic and estrogenic disrupting contaminants on breeding gland (nuptial pad) morphology, plasma testosterone levels, and plasma vitellogenin levels in male *Xenopus laevis*. *Archives of. Environmental. Contamination and.Toxicology* **44**: 247-256.

Van Wyk, J. H.; Pool, E. J.; Hurter, E.; Leslie, A. J (2005). The development and validation of bioassays to detect estrogenic and antiadrogenic activity using selected wildlife species. WRC Report No 926 & 1253/1/05.

Van Saal, F.S.; Guillette Jnr L. J.; Myers, J.P.; Swan, S.H (2008). Endocrine Disruptors: Effects in Wildlife and laboratory Animals. *Encyclopaedia of Ecology* 1265-1269.

Venturino, A; E Rosenbaum; A Caballero De Castro; O. L Anguiano; L. Gauna; T. F, De Schroeder; De D'Agelo M.P (2003). Biomarkers of effect in toads and frogs. *Biomakers* ***8 (3-4)*****:** 167-186.

Vitt, L. J.; Caldwell, J. P.; Wilbur, H. M.; Smith, D. C (1990). Amphibians as harbingers of decay. *Bioscience* **40**: 418.

Vos, J. G.; Dybing, E.; Greim, H. A.; Ladefoged, O.; Lambré, C.; Tarazona, J. V.; Brandt, I.; Vethaak, A. D (2000). Health effects of endocrine-disrupting chemicals on wildlife. *Critical Review in.Toxicology **30*:** 71-133.

Wahlstrum, D (2008). Molecular mechanism of ARH mediated endocrine disruption of estrogen and retinoic acid signalling pathways. National Institute of Environmental Risk of Assessment. Karolinka Institute, Stockholm, Sweden.

Wagner, N.; Wolfram, R.; Hanka, T.; Beatrix, T.; Stefan, L. (2013). Questions concerning the potential impact of glyphosate-based herbicides on amphibians. *Environmental Toxicology and Chemistry*. **32:** 1688–1700.

Walsh, L. P.; McCormick, C.; Martin, C.; Stocco, D. M (2000). Roundup inhibits steroidogenesis by disrupting stereoidogenic acute regulatory (StAR) protein expression. *Environmental Health Perspectives* **108**: 769-776.

Waring, R. H.; Harris, R. M. (2005). Endocrine disrupters: a human risk? *Molecular and Cellular Endocrinology* **244**: 2-9.

Washington State Department of Agriculture (WSSA) (1994). *Herbicide handbook.* Weed Society of America. Champaign, Illinois, p.352.

Washington State Dept. of *Agriculture (WSDA) (2009). Human health and ecological effects Imazapyr risk assessment, Washington State," .www.ecy.wa.gov. Accessed on 24 October 2010.*

Watamura, S. E (2008). Endocrine System. Encyclopaedia of Infant and Early Childhood Development. 450-459.

Wilson, E. O (1992). The diversity of life. Cambridge, MA: Harvard University Press.

Whitehead, S. A.; Rice, S. (2006). Endocrine-disrupting chemicals as modulators of sex steroid synthesis. *Best Practice and Research* **20**(1*)*: 45–61.

Wijnja, H. (2010). Ecotoxicological Risk Assessment of Surfactants Associated with Herbicides in Right-of ways Areas. Division of Crop and Pest Services. Massachussets Dept of Agricultural Resources.

Wise, A.; Brien, K, O.; Woodruff, T. (2011). Are oral contraceptives a significant contributor to the estrogenicity of drinking water? *Environment Science and Technology* **45***:*51–60.

Wojtaszek, B. F.; Staznik, B.; Chartrand, D. T.; Stephenson, G. R; Thompson, D. G (2004). Effects of vision herbicide on mortality, avoidance response, and growth of amphibian larvae in two forest wetlands. *Environmental Toxicology.and Chemistry* **23**: 832–842.

Wood, A. (2011). Classification of Herbicides According to Chemical Structure. www.alanwood.net. (Accessed on 7th August 2011).

Wolterink, G.; Mahieu, C. M.; Davies, L. (2012). Glufosinate Ammonium. Centre for Substances and Integrated Risk Assessment, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands.

World Health Organisation (WHO) (1990). Public health Impacts of pesticides used in agriculture. World Health Organisation, Geneva. www.who.com/publications.

World Health Organisation (WHO) (1994). Glyphosate. Environmental health criteria 159. World Health Organisation, Geneva. www.who.com/publications.

World Health Organisation (WHO) (1996). Glyphosate World Health Organisation, Geneva. www.who.com/publications.

World Health Organisation (WHO) (2004). Diquat in Drinking Water. Background Document for Development of WHO Guidelines for Drinking-water Quality. World Health Organisation. www.who.com/publications.

World Health Organisation (WHO) (2013). State of the Science of Endocrine Disrupting Chemicals. United Nations Environmental Programme and World Health Organization. www.who.com/publications.

Wu, F. C. W (1997). Endocrine aspects of anabolic steroids. *Clinical Chemistry* **43(**7**):** 1289–1292.

Wu, W.; Shen, O.; Qin, Y.; Lu, J.; Niu, X.; Zhou, Z.; Lu, C.; Xia, Y.; Wang, S.; Wang, X. (2012). Methylenetetrahydrofolate reductase C677T polymorphism and the risk of male infertility: a meta-analysis. *International Journal of Andrology* **35**: 18-24.

Yadav, S.; Giri, U.; Singha, F.; Boro, A. G (2013). Toxic and genotoxic effects of Roundup on tadpoles of the Indian skittering frog (Euflictis cyanophlyctis) in the presence and absence of predator stress. *Aquatic Toxicology* **132–133**: 1– 8.

Yang, F. X.; Xu, Y.; Wen, S. (2005). Endocrine-disrupting effects of nonylphenol, bisphenol A, and p, p'-DDE on *Lithobates nigromaculata* tadpoles. *Bulletin of Environmental Contamination and Toxicology* **75**: 1168–1175.

Yu, S.; Wages, M.R.; Cai, Q.; Maul, J.D.; Cobb, G.P (2013). Lethal and sub-lethal effects of three insecticides on two developmental stages of *Xenopus laevis* and comparison with other amphibians. *Environmental Toxicology and Chemistry* **32**: 2056-2064.

Zala, S.; Penn, D. J. (2004). Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. *Animal Behaviour* **68**: 649-664.

Zaya, R. M.; Z Amini, Z; Whitaker, A.S; Kohler, S. L; Ide, C.F (2011). Atrazine exposure affects growth, body condition and liver health in *Xenopus laevis* tadpoles. *Aquatic Toxicology* **104***: 243– 253.*

Zoeller, R.T.; Brown, T. R.; Doan, L.L.; Gore, A.C.; Skakkebaek, N.E.; Soto, A.M.; Woodruff, T.J.; Vom Saal, F.S. (2012). Endocrine-disrupting chemicals and public health protection: a statement of principles from The Endocrine Society. *Endocrinology* **153**: 4097-4110.

Chapter Two

Comparative toxicity and identification of the most sensitive developmental stage of *Xenopus laevis* to various herbicide formulations

2.1 Introduction

Agro-pesticides including insecticides, fungicides, molluscides and herbicides have become integral part of agriculture, not only in the developed countries, but also increasingly in the developing countries (WHO, 1990; 2013). Because of their advantages in enhancing agricultural outputs, new formulations are continually developed (Farah *et al*., 2004). The intensive use of the agricultural pesticides has led to their widespread presence in all compartment of the environment, which has exposed wildlife and human to these arrays of chemicals (Holmes *et al*., 2010; Lavorati *et al*., 2013). The ubiquity of these anthropogenic chemicals in nature presents a challenge to understanding how these chemicals impact ecological communities (Relyea, 2009; Lavorati *et al*., 2013).

The widespread nature of these agrochemicals have been suggested as one of the several hypotheses put forward for the global declined of amphibian populations (Bishop *et al*., 1999; Muths *et al*., 2006; Brunelli *et al*., 2009; Paskova *et al*., 2011; Egea-Seranno *et al*., 2012). Several surveys of natural populations have shown positive correlations between amphibian malformities (Brunelli *et al*., 2009), population declines and proximity to agricultural lands and the presence of agrochemicals in water sources (Bishop *et al*., 1999). This potential chemical contaminants link has been stimulating increased research focus to assess and understand the ecological impacts of agrochemicals on amphibians. Among the global pesticides, herbicides are the most commonly applied (Takahasi**,** 2007). Herbicides generally have shorter half-lives, but are found at much higher concentrations in the environment compared to the other pesticide groups (Orton *et al*., 2009).

In spite of widespread concern about the role of pesticides in the global decline of amphibians populations, current pesticides risk assessment programmes do not specifically consider amphibians as test organism (Bruhl *et al*., 2011). This is very surprising, given that amphibian will be exposed across both terrestrial and aquatic systems at different stages of their life cycle, which could produce different physiological challenges to their survival (Pickford and Morris, 2003). Since anthropogenic environmental contamination induced changes may be a powerful and ubiquitous evolutionary force (Conthran *et al*., 2013), but the impacts for amphibians

may be largely different to that of other aquatic and terrestrial organisms. It is clear therefore that current pesticides risk assessment tools needs to be extended to include detailed amphibian studies, particularly given the current global status of amphibian population.

Agrochemicals inflow into aquatic environment often occurs intermittently rather than continuous flow (Dabrowski *et al*., 2002; Reinert *et al*., 2002), and high concentrations may be present immediately after reaching the nearest water body. The aquatic herbicides that are directly applied in water bodies (to control alien water plants) on the other hand, are usually present at high concentration during and after such applications. Aquatic organisms are therefore often get exposed to very high concentrations of these agrochemicals, before the effects of dilution, interaction and degradation set in (Reinert *et al*., 2002). The possible effects of these occasional high concentration peaks on aquatic amphibians, particularly on different developmental stages are still largely unknown.

Variances in acute susceptibility to chemicals and pesticides have been observed among developmental stage of amphibians (Pauli *et al*., 1999; Grelich and Pflugmacher, 2003; Ortiz-Santaliestra *et al*., 2006). For example, Edginton *et al.* (2004) in a study on the comparative effects of an herbicide formulation and pH on two life stages of four amphibian species showed that the larvae *of Anaxyrus americanus* and *Lithobates clamitans* were 1.5 to 3.8 times more sensitive than their corresponding embryo. They also pointed out that *Xenopus laevis* and *Lithobates pipiens* larvae were 6.8 to 8.9 times more sensitive than their embryos. In another study, Siddiqua *et al*. (2013), using atrazine herbicide on various developmental stages including hatching, premetamorphosis and prometamorphosis larvae of *Rhinella marina* and *Limnodynastes peroni* showed that late larva stages were more sensitive than early stages, and that different premetarmorphic stages showed variation in sensitivity in both test species.

Duttan and Mohanty-Hejmadi (1978), in a study on susceptibility of the Indian bull frogs *Lithobates tigrina* pointed out that when the eggs, cleaving embryos and pre-limb larvae were treated with 0.001 to 0.009% of Rogor formulation (dimethoate Insecticides), developmental arrest in eggs and embryos occurred at 0.008 % within 24 hours of treatment, while developmental arrest took place within 4 ½ hours of exposure at 0.005% treatment in pre-limb larvae. The authors showed that pre-limb stages were more susceptible to the Rogor formulation than the eggs and cleaving embryos. Anguiano *et al.* (1994), in a study on comparative toxicity of parathion in embryos and larvae of *Anaxylus arenarum,* pointed out that young embryos were 4.4 times more tolerant to the acute lethal toxicity of the chemical than their larvae. This varied response according

to them, could be attributed to kinetics of cholinesterase inhibition, as the activity of larvae cholinesterase declined faster than the embryonic enzymes after parathion exposure.

Greulich and Pflugmacher (2003), in a study on differential susceptibility of life stages of amphibians to pesticide exposure, pointed out that the eggs were significantly harmed at different concentrations, while the embryos hatched with apparent abnormalities. They also observed that the individuals exposed at early life stages metamorphosed earlier than corresponding control tadpoles/larvae, with a significant reduction in length and increase in weight, as a physiological resistance to the adverse impacts of the chemicals. They suggested that these impacts could negatively affect the viability of the larvae in the long term.

Although the hypothesis that the larval stages of amphibians being more sensitive than other developmental stages is now getting established (Anguiano *et al*., 1994; Berrill *et al.*, 1998; Pauli *et al*., 1999; Greulich and Pflugmacher 2003; Edginton *et al*., 2004; Ortiz-Santaliestra *et al*., 2006), variation in sensitivity among stages and species are still high. The use of single developmental stage as opposed to a range of stages needs more study and well establish laboratory model *Xenopus laevis* is well suited to clear up the current doubt on the stage sensitivity within a developmental series.

Even though many studies have showed differential larva stages sensitivity of amphibian larvae to environmental chemicals, the wide range of stages spinning between four days to 80 days, and up to 2 years in Bullfrog species; remains a daunting task to determine the most vulnerable stage for a particular species. Therefore selection of specific developmental stage representing different phases within the metamorphosis programme (for example corresponding to the activity of the thyroid gland controlling metamorphosis) will be important. This will help in reducing the need for assessment of toxicity across the developmental stages of amphibian life cycle.

The African clawed frog (*X. laevis)* is an amphibian species with a fully aquatic life cycle and is recognized as an international model species in environmental quality assessment studies (from both ecotoxicology and endocrine disruption perspectives) (Vitt *et al*., 1990; Mann and Bidwell, 2000; Paggeti *et al*., 2006; Kloas *et al*., 2009). These frogs are known to survive in poor water quality environments, are prolific breeders in captivity and provide severally validated endpoints (Kloas *et al*., 2009). Their integument at all developmental stages is permeable to many electrolytes, and their eggs are covered only by a layer of gelatinous material and are always directly exposed to the environment (Vitt *et al*., 1990).

In the present study, 96-hour acute toxicity at three *Xenopus laevis* developmental tadpoles stages (Nieuwkoop and Faber, 1994) (NF-stage 8-11 (embryo, premetamorphic phase) NF-stage 48 (larval, prometamorphic phase) and NF-stage 60 Larval, metamorphic phase). This was assessed against three commercial formulations of glyphosate herbicides (Roundup, Kilo Max and Enviro glyphosate) as well as a commercial formulation each of the Arsenal (imazapyr), Basta (glufosinate ammonium) and Midstream (diquat dibromide) in order to compare the differential toxicity to the selected developmental stages. And to compare the effects of change in surfactants from POEA in Roundup formulation to ammonium sulphate and polyrthylene alkylamine in Kilo Max and Environ glyphosate formulations of Glyphosate. The selected herbicides are those included in the Working for Water (WfW) programme of Department of Environmental Affairs in South Africa to eradicate alien plants from river catchments (Bold, 2007). NF-Stage 8-11 is the cleaving stage, which is the mid-blastula to early gastrula embryo. NF-stage 48 correspond to the developmental stage where the yolk mass was completely absent from the tadpole's alimentary canal, and the point where first taste bud appear in the roof of oro-pharygeal cavity with the commencement of full feeding. NF-Stage 60 tadpoles represent the transition phase where the greater strips of larval skin get remodelled to adult skin, and the thyroid activity is at the peak (Shi, 2000).

2.2 Materials and Methods

Test herbicides

Herbicide formulations: Roundup (360 g a.e/L, Monsanto, Ltd., South Africa), Enviro Glyphosate (360 g a.e /L, Enviro Industries Ltd., South Africa), Kilo Max (700 g /kg a.e glyphosate, Volcano Agroscience Ltd., South Africa), Midstream (373 g/L, diquat dibromide, Syngenta Ltd., South Africa), Basta (200 g/L, glufosinate ammonium, Bayer Crop Science AG Ltd, Germany) and Arsenal (250 g/L, imazapyr, BASF Chemical Ltd., South Africa) were either locally sourced or supplied by Working for Water Organization.

2.2.1 *Xenopus laevis* care and breeding of tadpoles.

Prior to breeding, sexually mature *X. laevis* males and females were maintained separately in 5 L glass tanks containing carbon filtered water, and were fed three times per week with fish pellets (Aqua-Nutro, RSA). Breeding induction was performed according to ASTM, (1998) protocol. Males were primed with 100 IU human chorionic gonadotropin (hCG) injection(Merck Ltd, Germany), injected into their dorsal lymph sac, four days prior to the commencement of mating and again just prior to the mating, by another 100IU and 200 IU hCG to males and females

respectively (usually in late afternoon). Male and female pairs were placed together in a 15 L exposure tanks lined with plastic netting (to separate the eggs from the adults), and positioned in a well-ventilated dark place. Amplexus occurred within four hours and eggs were deposited within 12 hours after injection. All the eggs and tadpoles staging were performed using a normal developmental atlas by Nieuwkoop and Faber (1994) (referred to as NF-stages).

For the exposure studies, NF-stage 8-11, freshly fertilised eggs were harvested immediately after laying, and de-jellied following the OECD, (2008) guidelines and subjected for exposure. NF-stage 48 (premetamorphic phase) and NF-stage 60 tadpoles were obtained by maintaining developing larvae tanks at density of 40/10L of reversed osmosis oral water (supplement with NaCl), where they were fed with sera micron (Heimberg, Germany) until reaching the appropriate stages were reached and were then transferred to the exposure tanks. All breeding and general maintenance procedures were approved by the Animal Ethics Committe of the Stellenbosch University (Approval no- SU-ACUM 12-00013**).**

2.2.2 Exposure set-up: chemical concentrations

For the selected herbicides, range-finding pre-exposures were carried out to determine the possible lethal concentration range (data not presented here), since there is no information from the literature available on most of these formulations, except for Roundup. Five concentrations were then established based on the results of the range-finding exposure trials.

In other to confirm the experimental concentrations, one sample was randomly collected per concentration from the exposures, because of the large exposure concentrations that resulted from several replicates. Unfortunately, only one laboratory could handle glyphosate analysis and none of the laboratories could handle diquat, imazapyr and glufosinate at the time of exposure. The glyphosate sampled concentrations were analyses at Synexa Analytical laboratory in Cape Town, South Africa. The laboratory used liquid chromatography tandem mass spectrometry (LC/MSMS) method which has a high validity, throughput and sensitivity. The analytical results showed very low variations compared to the predicted nominal concentrations (Table 2.1).

Table 2.1- The nominal herbicide concentrations, selected after the range finding exposures and observed concentrations as determined using LC/MSMS (Synexa laboratory in Cape Town).

Formulation	Exposed Conc. (mg/L a.e.)	Observed Conc. (mg/L a.e.)
Roundup	1.0	1.2
	1.4	1.4
	1.8	1.5
	2.2	2.1
Kilo Max	320	355
	380	387
	440	448
	500	480
Enviro Glyphosate	520	541
	580	599
	640	604
	700	629

2.3. Exposure procedure

Following the ASTM (1997) acute toxicity protocol guidelines, twenty tadpoles were introduced into the exposure glass. Chemicals in the exposure glass were replicated twice at each concentration. The exposures were performed in a controlled climate room following the setting recommended by OECD (2008) guidelines. In brief, these include; water temperature of 22 ± 1 ^{o}c, pH of 6.5 – 7.2, dissolve oxygen of > 3.5 mg/L and 12 hours light and 12 hours dark photoperiod ($L_{12}D_{12}$). The exposure medium was completely replaced every 24 hour. The exposed larvae were not feed throughout the 96 hours duration of the exposure.

2.3.1. Mortality/Survival as Endpoint

Mortality/survival records were obtained every six hours. At 96-hour, the assay was terminated and the final dataset used to determine the 96 hour lethal concentrations at three different levels (LC_5, LC_{50} and LC_{95}) for each of the test substances, using US EPA (1996) Probit Software. The

remaining surviving tadpoles were euthanized using buffered MS 222 (Tricaine methane sulfonate) (200 mg/L and buffered with sodium bicarbonate at 0.42 - 1.05 g / liter) (OECD, 2007). All the tadpoles collected were fixed and preserved in buffered formalin (4% formaldehyde) (Bancroft and Stevens, 1977) and preserved for future reference. The experimental protocol was approved by the Animal Ethics C0mmitte of the Stellenbosch University (Approval no- SU-ACUM 12-00014).

2.4. Results

2.4.1 Glyphosate herbicides

2.4.1.1 Roundup formulation

For Roundup, at NF-stage 8 - 11, six exposure concentrations between 0.3 - 1.3 mg/L at 96 hour produced 5 – 70 % mortality range (Table 2.2), resulting in a 1.05 mg/L LC_{50} (Table 2.3, Fig 2.1). At NF-stage 48, six exposure concentrations ranging between 0.8 - 1.2 mg / L for 96 hours produced 20 - 100 % mortality (Table 2.2), resulting in a 0.89 mg/L LC_{50} (Table 2.3, Fig 2.1). At NF-stage 60, five exposure concentrations ranging between 1 - 6 mg/L for 96 hours produced 5 – 100 % mortality (Table 2.2), resulting in a 2.75 mg/L LC_{50} (Table 2.3, Fig 2.1).

Table 2.2: Percentage (%) mortality in three different developmental stages (NF 8-11, NF 48 and NF 60) of *X. laevis* embryos and tadpoles following a 96-hour exposure to a range of concentrations of six herbicide formulations

Herbicides	NF 8-11 Conc.	96 hour % Mortality	NF 48 Conc.	96 hour %Mortality	NF 60 Conc	96 hour %Mortality
Roundup	0.3	5	0.8	20	1	5
	0.5	5	0.85	40	3	45
	0.7	20	0.9	50	4	70
	0.9	35	0.95	80	5	100
	1.1	55	1	90	6	100
	1.3	70				
Kilo Max	130	0	20	20	400	20
	160	5	40	30	450	40

	190	25	60	40	500	70
	220	70	80	60	550	100
	250	85	100	60	600	100
	280	100	120	100		
Enviro glyphosate	360	20	100	0	4800	25
	400	30	120	30	5000	30
	440	30	140	70	5200	40
	480	40	160	70	5400	60
	520	60	180	100	5600	65
	540	90				
Midstream	0.5	40	0.09	20	6	0
	1	50	0.1	20	8	10
	1.5	60	0.2	30	12	45
	2	80	0.3	70	16	90
	2.5	80	0.4	90	20	100
	3	90				
Basta	1.2	10	0.1	20	15	0
	1.4	20	0.5	30	20	35
	1.6	25	1	60	25	45
	2	40	1.5	65	30	65
	2.5	55	2	80	35	95
	3	75	2.5	100		
Arsenal	20	5	30	20	150	0
	25	15	33	40	160	30
	30	20	36	90	170	35
	35	35	39	100	180	65
	40	60	42	100	190	85
	45	90				

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Table 2.3: *X. laevis* tadpoles NF-Stages, exposure concentrations and calculated 96-hour LC_5, LC_{50} and LC_{95} for the selected herbicide formulations. (LC50 values in bold, upper and lower range in brackets).

Herbicide	NF Life Stage	Exposure Conc. (mg/L)	96hr LC_5 (95% CI) (mg/L)	96hr LC_{50} (95% CI) (mg/L)	96hr LC_{95} (95% CI)(mg/L)
Roundup	8-11	0.3, 0.5, 0.7, 0.9, 1.1, 1.3	**0.4** (0.24-0.53)	**1.05** (0.91-1.32)	**2.67** (1.88-5.99)
	48	0.8, 0.85, 0.9, 0.95, 1.0, 1.2	**0.74** (0.66-0.78)	**0.89** (0.85-0.91)	**1.04** (0.99-1.15)
	60	1, 3, 4, 5, 6	**1.3** (0.65-1.8)	**2.75** (2.1-3.2)	**5.77** (4.8-8.2)
Kilo Max	8-11	130, 160, 190, 220, 250, 280	**162.8** (143-175)	**207** (197-218)	**264** (247-296)
	48	20, 40, 60 80, 100, 120	**12.9** (4.3-21.1)	**58.1** (44.8-73.0)	**261.7** (166.6-714)
	60	400, 450, 500 550, 600	**375** (332-400)	**455** (435-473)	**552** (523-607)
Enviro glyphosate	8-11	320,360, 400, 440, 480, 520, 540	**277** (196-322)	**466** (434-512)	**784** (655-1198)
	48	100, 120, 140, 160, 180	**102** (88-111)	**134.6** (127-142)	**177** (164-202)
	60	4800, 5000, 5200 5400, 5600	**4379** (3557-4672)	**5257** (5098-5466)	**6311** (5875-7952)
Midstream	8-11	0.5, 1, 1.5, 2, 2.5, 3	**0.11** (0.008-0.264)	**0.83** (0.431-1.14)	**6.34** (3.62-30.0)
	48	0.09, 0.1, 0.2, 0.3 & 0.4	**0.06** (0.03-0.08)	**0.2** (0.16-0.26)	**0.72** (0.48-1.62)
	60	6, 8, 12, 16, 20	**7.8** (6.1-9.0)	**11.8** (10.6-13)	**17.7** (15.7-21.8)
Basta	8-11	1.0, 1.2, 1.4, 1.6, 2, 2.5, 3	**0.918** (0.6-1.14)	**2.24** (1.97-2.71)	**5.46** (4-10.5)

	48	0.1, 0.5, 1.0, 1.5, 2 & 2.5	**0.053** (0.009-0.12)	**0.59** (0.36-0.85)	**6.57** (3.5-23.6)
	60	15, 20, 25, 30, 35	**15.7** (12.05-18.03)	**24.9** (22.7-27.1)	**39.4** (34.4-50.7)
Arsenal	8-11	20, 25, 30, 35, 40, 45	**22** (17.2-25.2)	**36** (33.3-39.5)	**58.5** (50-79)
	48	30, 33, 36, 39, 42	**28.7** (26.3-30.1)	**32.8** (31.7-33.8)	**37.4** (36-40)
	60	150, 160, 170 180, 190	**150** (138.5-156.4)	**173.5** (169-178.5)	**200.7** (191.6-219.5)

2.4.1.2 Kilo Max formulation

For Kilo Max formulation, at NF-stage 8 - 11, six exposure concentrations ranging between 130 - 280 mg/L for 96 hours produced 0 - 100 % mortality (Table 2.2), resulting in a 207 mg/L LC_{50} (Table 2.3, Fig 2.1). At NF-stage 48, six exposure concentrations ranging between 20 – 120 mg/L for 96 hours produced 20 – 100 % mortality (Table 2.2) that resulted in a 58.1 mg/L LC_{50} (Table 2.3, Fig 2.1). In addition, at NF-stage 60, six exposure concentrations ranging between 400 - 600 mg/L for 96-hours produced 20 - 100 % mortality (Table 2.2), resulting in a 455 mg/L LC_{50} (Table 2.3, Fig 2.1).

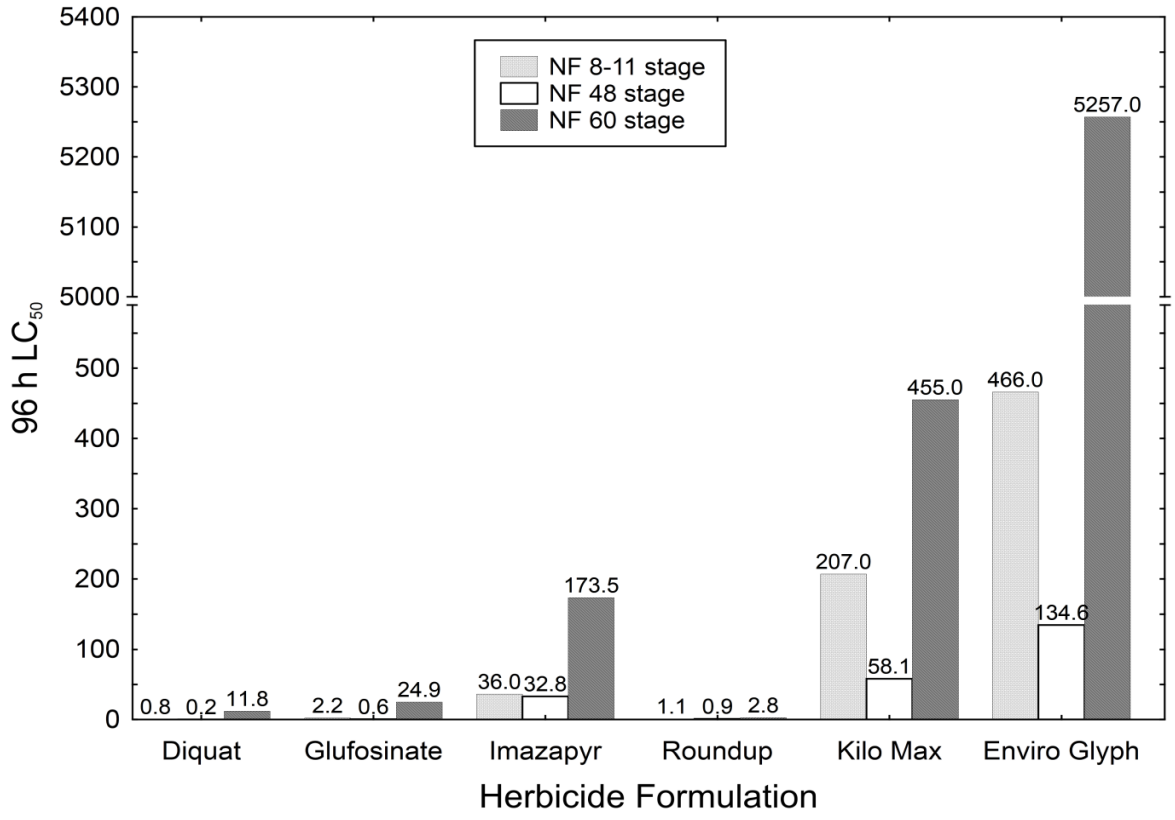

Figure 2.1: The 96-hours LC50 indexes determined for three different *X. laevis* developmental stages exposed to six herbicides formulations. The 96-hours LC_{50} comparison suggests marked variation among exposed chemicals as well as among developmental stage in the *X. laevis* developmental life cycle.

2.4.1.3 Enviro glyphosate formulation

At NF-stage 8 - 11, seven exposure concentrations ranging from 320 – 540 mg/L for 96-hours produced 20 – 90 % mortality (Table 2.2), resulting in a 466 mg/L LC_{50} (Table 2.3, Fig 2.1). At NF-stage 48, five exposure concentrations ranging from 100 - 180 mg/L for 96-hours produced 0 – 100 % mortality (Table 2.2), resulting in a 134.6 mg / L LC_{50} (Table 2.3, Fig 2.1). Also at NF-stage 60, five exposure concentrations ranging from 4800 – 5600 mg/L for 96-hours produced 25 – 65 % mortality (Table 2.2), resulting in a 5257 mg / L LC_{50} (Table 2.3, Fig 2.1).

2.4.1.4 Midstream formulation (diquat dibromide)

For the Midstream formulation, at NF-stage 8 - 11, six exposure concentrations ranging from 0.5 - 3 mg/L for 96-hours produced 40 -90 % mortality (Table 2.2), resulting in a 0.833 mg/L LC_{50} (Table 2.3, Fig 2.1). At NF-stage 48, five exposure concentrations ranging from 0.09 - 0.4 mg/L for 96-hours produced 20 - 90 % mortality (Table 2.2), resulting in a 0.2 mg / L LC_{50} (Table 2.3, Fig 2.1).

At NF-stage 60, the five exposure concentrations between 6 - 20 mg / L for 96-hours produced 0 - 100 % mortality (Table 2.2), resulting in 11.8 mg/L LC_{50} (Table 2.3, Fig 2.1).

2.4.1.5 Basta formulation (glufosinate ammonium)

For this formulation, at NF-stage 8-11, seven exposure concentrations ranging from 1 - 3 mg/L for 96-hours produced 10 – 75 % mortality (Table 2.2), resulting in a 2.24 mg/L LC_{50} (Table 2.3, Fig 2.1). At NF-stage 48, five exposure concentrations between 0.1 - 2.5 mg/L at 96-hours produced 20 – 100 % mortality (Table 2.2), resulting in a 0.589 mg/L LC_{50} (Table 2.3, Fig 2.1). Also, at NF-stage 60, five exposure concentrations range of 15 - 35 mg/L for 96-hours produced 0 – 95 % mortality (Table 2.2), resulting in a 24.9 mg/L LC_{50} (Table 2.3, Fig 2.1).

2.4.1.6 Arsenal formulation (imazapyr)

At NF-stage 8 - 11, a six point concentrations range of 20 - 45 mg/L for 96-hours produced 5 – 90 % mortality range (Table 2.2), resulting in a 36 mg/L LC_{50} (Table 2.3, Fig 2.1). At NF-stage 48, five exposure concentrations range of 30 - 42 mg/L for 96-hours produced 20 – 100 % mortality range (Table 2.2), resulted to 32.8 mg/L LC_{50} (Table 2.3, Fig 2.1). At NF stage 60, five exposure concentrations range between 150 – 190 mg/L at 96-hours produced 0 – 85% mortality range (Table 2.2), resulted to 173.5 mg/L LC50 (Table 2.3, Fig 2.1).

2.5 Discussion

Pesticides have been suggested to be one of the contributing factors leading to the declines in amphibian populations around the world (Perkins *et al*., 2000; Gungordu, 2013). Glyphosate herbicide are used widely in agriculture but also used in South Africa to eradicate aliens from valuable river catchments (WfW programme) and may pose direct and indirect hazards to non-target organisms including aquatic stages in the amphibian life cycle. *Xenopus laevis* occurs naturally in water bodies throughout South Africa but is also globally recognized as a laboratory model organism and used for Frog Embryo Teratogenesis Assay- Xenopus (FETAX) as well as general toxicity testing for environmental assessment (Paggeti et al., 2006; Kloas *et al*., 2009). It has recently been recognized that different developmental stages in the life cycle of an aquatic species like *X. laevis* may show differential sensitivity to environmental chemicals (Wagner *et al*., 2013; Saddiqua *et al*., 2013). Since little is known about the effects of new glyphosate formulations (relative to old Roundup) as well as imazapyr, glufosinate ammonium as well as diquat dibromide herbicides on the survival of premetamorphic amphibian stages, in this study tadpoles from three different developmental stages were exposed to commonly used formulations of these herbicides.

These herbicides were selected since they are globally used in and around the natural habitat of *X. laevis*. In the present study it was confirmed that both developmental stages and the herbicides formulation were factors affecting toxic sensitivity in *X. laevis.*

Data from this study showed that there is a stage-dependent differential toxicity response, which cut across all the six herbicide formulations including the three glyphosates (Roundup, Kilo Max and Enviro glyphosate), Midstream, Basta and Arsenal formulations. In each of the formulations, a U-shape response pattern of 96-hours LC_{50} Index among NF-stage 8-11, NF-stage 48 and NF-stage 60 showed that NF-stage 48 were the most vulnerable stage among the stages evaluated within the life cycle of the *X. laevis*.

This result was consistent with the findings of several studies suggesting that young tadpole stages are more sensitive to environmental chemicals than their corresponding embryos. For example, Dutta and Mohanty-Hejmadi, (1978), showed that the pre-limb tadpole stages of *Lithobates tigrina* were more susceptible to Rogor (Dimethoate insecticide) formulation than their eggs and cleaving embryos. The findings in this study are also consistent with the results of Herkovit and Perez-Coll (1993), who noted that early larva stages (Gosner 16) of *A. arenarum* were more sensitive to cadmium than those of the embryos (Gosner 3 and 12). The results are also consistent with the findings of Anguiano *et al*. (1994), who reported that early embryos were 4.4 times more tolerant to the acute lethal effects of parathion than their corresponding larvae. Similar results was reported by Berryll *et al*. (1998), who noted that newly hatched tadpoles have a lower mortality rate (40%) compared to two weeks old tadpoles (50%) for three species of amphibians including *Lithobates sylvatica, L. clamitians* and A. *anenarium* when expose to endosulfan for 96 hours. In another study, Bridges *et al*. (2000) also noted that they are quite resistant to acute exposure when compared to tadpoles. Edington *et al*. (2004) similarly reported that larvae of several amphibian species including *A. americanus L. clamitians, X. laevis* and *L. pipiens* were more sensitive than their corresponding embryos exposed to Vision (glyphosate) herbicide formulation under different pH conditions. Oritz-Santaliestra *et al*. (2006) also noted that after four days of exposure to 225.8 mg of ammonium nitrate, Gosner stage 19 of *P. cultripes* were more sensitive than those of Gosner 13 with 100% and 7.3% mortality respectively. They also noted that late developmental stage Gosner 21 of *B. calamita* were more sensitive than those early Gosner stage 13 and 19. Similarly, Aronzon *et al*. (2009) in a study also noted that when six different developmental stages of *B. arenarium* (from embryos to young metamorphs) were exposed to 2, 4-D active ingredients as well as commercial formulations, the Gosner larva stage-21, which they called open mouth stage was the most sensitive than their corresponding embryos. However, the

results from this study and many other mentioned above are in contrast to the findings of Ezenmoye and Tongo (2009) who noted that using graded concentration of atrazine on tadpoles of *Ptyychadena bibroni* between one to four weeks post hatch developmental stages for 96-hour experiment, percentage mortality decreased with increasing maturity. Similarly, Sparling *et al*. (2001), noted that differences in an organism biological adjustment and behavioural responses to changes in water chemistry and osmotic condition depends on developmental stages.

Several reasons can be responsible for the high sensitivity of this early larva stage relative to other developmental stages; the most important being the complete change in feeding behaviour with the disappearance of embryonic yolk, and the development of taste bud (Nieuwkoop and Faber, 1994). Fresh food intake ability could therefore expose this stage to high levels of environmental toxins, and could be an important additional route of entry for toxic substances relative to the embryos. In addition, metabolic detoxifying mechanisms already developed in the older larva stage relative to the younger larva stage may explain the variation in sensitivity that was observed (Bucciarrelli *et al*., 1999). Another important reason according to Gungordu (2013) could be the differential binding rate of some chemical detoxifying enzymes, including glutathione-s-transterace (GST), oxidative stress enzymes (glutathione reductase) and metabolic enzymes (acetycholinesterase (AST). According to Gungordu (2013), the GST enzymes played an important role in cellular detoxification of various xenobiotic chemicals and that differential expression of these enzymes alongside AST enzymes at different developmental stages could be responsible for the susceptibility of the early larva stages.

That this observed susceptible early larva stage cut across many environmental chemicals and heavy metals including pesticides (glyphosate, endosulfan and parathion) (Anguiano et al., 1994; Berryll *et a*l., 1998; Edginton *et al*., 2004) and heavy metals (Lead and Cadmium) (Perez-Coll and Herkovit, 1990; 1993) as well as other environmental chemicals like ammonium nitrate means that the susceptible early larva stage might be a weak link across all chemical groups. It has also been shown to be across many amphibian species including B. arenarium, and B. calamila (Perez-Coll & Henkovit, 1990; Anguaino *et al*., 1994; Berryll *et al*., 1998; Oritz-Santaliestra *et al*., 2008), *L. pipiens, L.sylivatica, L. tigrina* and *L. marina* (Duttan and Mohenty, 1978; Berryll *et al*., 1998; Edginton *et al*., 2004; Siddiqua *et al*., 2013), X. laevis (Edginton *et al*., 2004) as well as Lmonnyndynate peroni (Siddiqua *et al*., 2013). This means that the susceptible early larva stage phenomenon might be common among the amphibian groups.

Importantly, for some of the formulations including Roundup, Midstream and Basta, the 96-hour LC_{50}s at NF-stage 48 were below their respective expected environmental concentration (EEC) at the approved application rate. For example, in the case of Roundup, the 96-hour LC50 of 0.89 mg/L at NF-stage 48 was less than the EEC of 2.85 mg a.e/L (Peterson *et al*., 1994) and even lower than the highest environmental reported concentration of 1.7 mg a.e /L (Homer, 1990). Roundup is therefore a highly toxic and noteworthy ecotoxicological risk for amphibians, and requires serious attention. For the Midstream formulation, the 96-hours LC_{50} of 0.2 mg/L at NF-stage 48 is also relatively low compared to the EEC of 0.733 mg/L of the Midstream formulation (Peterson *et al*., 1994). The implication being that at the EEC of 0.733 mg/L, more than 85 % of the NF-stage 48 will be eliminated. This will negatively impact the local population of the exposed amphibian and could lead to serious population decline in long term. It may even be worst for the exposed population considering the half-life of this formulation in the environment, given that a concentration of 1.7 mg/L have been detected in water sediment four years post application of this formulation, and at the low rate of 0.35 kg/ha (William *et al*., 1996). Amphibians are known to be bottom dwellers, particularly when escaping from predators. In the case of Basta formulation, the 96-hours LC_{50} of 0.59 mg/L at NF-stage 48 is also relatively low compared to the EEC of 1.0 mg/L of the glufosinate ammonium formulation (Dinehart *et al*., 2010). The implication being that at the EEC of 1.0 mg/L, more than 65 % of the NF-stage 48 will be eliminated, and which will have severe consequences on the local population dynamics of the organism.

Evidence from this study showed that the toxicity of some of the herbicide formulations might be underestimated for *X. laevis*. For example, the 96-hour LC_{50} of 1.05 mg a.e /L at NF-stage 8-11 was lower than the result of Perkins *et al*. (2000), who reported a 96-hour LC_{50} of 9.4 mg a.e /L. The 9.4 mg a.e/L from Perkins et al. (2000), seems very strange and isolated, since several other studies have shown Roundup toxicity against many other amphibian tadpoles to be very high. Mann and Bidwell (1999) observed a LC_{50} 3.6 mg a.e /L for *Crinia insignifera* and Litoria *moorei* as 2.9 mg a.e/L after only 48 hour exposure. The fact that the Mann and Bidwell (1999)'s LC50 values are at 48-hour exposure means that the toxicity will be much higher at 96-hour. Moreover, Gungordu, (2013) recently reported 96-hour LC_{50} of 3.8 mg a.e /L for *X. laevis* at NF-stage 46, which showed that the Roundup formulation on amphibian larvae is moderately toxic. The result of Relyea et al., (2009), where 13 species including (including *L. sylvatica, L. pipiens, L. cascadae, L. clamitans, L. catesbeiana, A. americanas, B. boreas, Hyla versicolor, Pseudacris crucifer, Ambyston gracile, A. maculatium, A. laterale,* and *Notophthalinus viridesiens*) testes against Roundup formulation showed LC_{50} that ranged from 0.8-3.2 mg a.e/L, further proved the high

toxicity/ moderate toxicity nature of the Roundup formulation. This Relyea *et al*. (2009) study showed that the Roundup toxicity cut across very large group of amphibians**.** But in general, water source could also be a factor in the current variation of the lethal concentrations observed among studies. As noted by Mann and Bidwell (1999), when diluted lake water was used compared to the aged tap water, the 48-hour LC_{50} changed from 11.6 mg/L to 2.9 mg/L for the same *Litoria moorei* species.

Compared to other aquatic organisms like fish, Roundup formulation seem to be more toxic to some larva stages at 96-hour LC_{50} range of 0.89-1.05 mg/L compared to young tilapia (*Orechromis niloticus*) species with a 96- hour LC_{50} of 16.8 mg/L. Although, based on this current result, and according to Ayoola (2008), both juvenile tilapia, *Orechromis niloticus* and *X. laevis* embryos shared the same 96 hour LC_{50} of 1.05 mg/L.

The observed differential toxicity in the three Glyphosate formulations between Roundup formulation and the new Kilo Max and Environ glyphosate formulations suggested the involvement of their varied surfactants. The high /moderate toxicity of Roundup formulation relative to the Kilo Max and Environ Glyphosate formulations in this current study confirmed various reports linking the POEA surfactant to the high toxicity of Roundup (Folmar *et al*., 1979; Mitcheal *et al*., 1987). The result of this current study also showed that both ammonium sulphate surfactant in Kilo Max and Polyethylene alkylamine surfactant in Environ Glyphosate formulations appearerd far less toxic relative to the POEA in Roundup. This result is similar to the findings of Folmar *et al*., 1979, where the POEA surfactant was showed having a higher toxicity compared to the glyphosate technical grade. This results also supports the findings of Wan *et al*., 1989, where they noted a reduction in toxicity of Roundup formulation, when the percentage of POEA surfactant was reduced.

For the Midstream formulation, some of the larva stages of *X. laevis* also showed more susceptibility than the early stages of some fish. As noted by Paul *et al*. (1994), the 96-hour LC_{50} for the early life stages of walleye (*Stizostedion vitreum*) range from 0.74-4.9 mg/L which when compared to 0.2 -0.8 mg/L for the early larva stages of *X. laevis* in the present study showed the increased sensitivity of the *X laevis* larva stages. But compared to the adult stage, some adult fish as shown by Paul *et al*. (1994), might be more susceptible than the *X. laevis* adults. For example, the 96-hour LC_{50} for *Micropterus dolomieu* was 3.9 mg/L, and 4.9 mg/L for *Micropterus salmoides* (Paul *et al*., 1994). These LC_{50} compared to 96-hour LC_{50} for NF-stage 60 of *X. laevis* calculated at 11.8 mg/L showed that the xenopus adult might be more resistance to the acute toxicity effects

of Midstream formulation than the fish. This is consistent with the finding of Bridges *et al*. (2002), who noted that fish are more susceptible than the amphibians to many chemicals.

Not much research has been done on the acute toxicity of Arsenal formulation, apart from the LC_{50} of imazapyr to adult rainbow trout as > 100 mg /L according to the imazapyr material data safety sheet (MSDS) (Imazapyr MSDS, 2013). This compared favourably with the result of this study, particularly as from NF-stage 60 where the 96-hour LC_{50} was 173.5 mg/L. This suggested that this formulation has a low acute toxicity on adult fish and amphibians.

2.6 Conclusion

The result of this study revealed the high sensitivity of premetamorphic NF-stage 48 of *X. laevis* to all the tested herbicide formulations relative to early embryos (NF-stage 8-11) and tadpoles undergoing metamorphosis (NF-stage 60). The result furthermore showed that varied response in the acute toxicity to different glyphosate formulations (real world scenario) affected survival differentially, and with Enviro glyphosate having the lowest toxicity among the glyphosate**.** The 96-hour LC_{50}s of the premetamorphic larva (NF-stage 48) for the Roundup, Midstream and Basta formulations were found to be lower than the expected environmental concentration (EEC) of those formulations. These formulations can therefore potentially affect survival rates and long term amphibian population dynamics. Although the LC_{50} concentrations of the embryos were generally higher, these pre-hatch stages may not be adequately protected. When considering the susceptibility of premetamorphic larva, it is clear that the protection of one developmental stage will be ecological inadequate if another developmental stage is seriously vulnerable to the same chemical. The fact that similar trends have also been identified in different anuran species including *R. clamitans, R. pipiens, B. arenarum and B. americanus* suggest that premetamorphic tadpoles represent a vulnerable life stage that could be an important factor in understanding the global amphibian declines.

This study therefore confirmed the importance of using premetamorphic tadpoles (for example NF-stage 48) in exposure studies to evaluate the potential threat that pesticide formulations reaching the aquatic environment, directly or indirectly, may hold for non-target amphibian species. This may prove to be an important consideration for aquatic alien plant eradication programmes using chemical means, since the mobility of glyphosate for example is known to be slow in the soil but may pose a greater risk in aquatic systems when applied directly.

References

American Society for Testing and Materials (ASTM) (1997). Standard guide for conducting the acute toxicity tests on aqueous ambient samples and effluents with fishes, macro-invertebrates and amphibians. E1192-97. In: Annual book of ASTM standards. American Society for Testing and Materials, Philadelphia: 220-240.

American Society for Testing and Materials (ASTM) (1998). Standard guide for conducting the frog embryo teratogenesis assay – Xenopus (FETAX). E1439-98. In: Annual book of ASTM standards, Vol 11.5. American Society for Testing and Materials, Philadelphia: 825-836.

Anguiano, O. L.; Montagna, C. M.; Chifflet de Liamas, M.; Ganna, L.; Pechen de D Angelo, A. M. (1994). Comparative toxicity of parathion in early embryos and larvae of the toad, *Anaxyrus arenarum Hensel*. *Bull. Environ. Contam. Toxicol.* **52***:* 649-655.

Aronzon, C. M.; Sandoval M. T.; Herkovits, J.; Perez-Coll, C. S. (2009). Stage-Dependent toxicity of 2, 4-dichlorophenoxyacetic on the embryonic development of a South American toad, *L*. arenarum. *Environmental Toxicology* 373-380.

Ayoola, S. O (2008).Toxicity of glyphosate herbicide on Nile tilapia (*O. niloticus*) juvenile**.** *African Journal of Agricultural Research* **3** (12)*:* 825-834.

Berrill, M.; Coulson, D.; McGillivray, L.; Pauli, B. (1998). Toxicity of endosulfan to aquatic stages of anuran amphibians. *Environmental Toxicology and Chemistry* **17**(9)*:* 1738–1744.

Berrill, M.; Bertram, S.; McGillivary, L.; Kolohan, M.; Paul, B. (1994). Effects of low concentrations of forest use pesticides on frog's embryo and tadpoles. *Environmental Toxicology and Chemistry* **18***:* 657-664.

Bishop, C. A.; Mahony, N. A.; Strugger J.; Ng P.; Pettit, K. E. (1999). Anuran development density and diversity in relation to agricultural activity in the Holland Rivers watershed, Ontario. Canada. Environ. Monitor. Assess. **57***: 21-43***.**

Blaustein, A. R.; Wake, D. B.; Sousa, W. P. (1994). Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. *Conservation Biology* **8***: 60*–71.

Bold, T. (2007). Management treatments summary guide- aquatics. Working for Waters National Office. 7Pp. www.wfw.co.za.

Bridges, C. M. (2000). Long-Term effects of pesticide exposure at various life stages of the Southern Leopard Frog (*R. sphenocephala*). *Archives of Environmental Contamination and Toxicology* **39**: 91–96.

Bridges, C. M.; Dwyer, F. J.; Hardesty, D. K.; Whites, D. W. (2002). Comparative contaminant toxicity: are amphibian larvae more sensitive than fish. *Bulletin of Environmental Contamination and Toxicology* **69**:562–569.

Brunelli, E.; Bernabo, I.; Berg, C.; Lundstedt-Enkel, K.; Bonacci, A.; Tripepi, S. (2009). Environmental relevant concentrations of endosulfan impair development, metamorphosis and behaviour in *Anaxyrus anaxyrus* tadpoles. *Aquatic Toxicology* **91:** 135-142.

Bru¨hl, C. A.; Pieper, S.; Weber, B. (2011). Amphibians at risk? Susceptibility of terrestrial amphibian life stages to pesticides. *Environmental Toxicology and Chemistry* **30**: 2465–2472.

Bucciarelli, T.; Sacchetta, P.; Pennelli, A.; Cornelio, L.; Romagnoli, R.; Merino, S.; Petruzelli, R.; Di Ilio, C. (1999). Characterization of toad glutathione transferase. *Biochimica. Biophysica Acta* **1431:**189–198.

Cothran, R. D.; Jenise, M. B.; Relyea, A. R. (2013). Proximity to agriculture is correlated with pesticide tolerance: evidence for the evolution of amphibian resistance to modern pesticides. *Evolutionary Applications* 1752-4571.

Dabrowski, J. M.; Peall, S. K. C.; Reinecke, A. J.; Liess, M.; Schulz, R. (2002). Runoff-related pesticide input into the lourens river, South Africa: Basic data for exposure assessment and risk mitigation at the catchment scale. *Water, Air, and Soil Pollution* **135**: 265-283.

Dutta, S. K.; Mohanty-Hejmadi, P. (1978). Life history and pesticide susceptible embryonic stages of the Indian Bull Frog *L. tigrina*. *Indian Journal of Experimental Biology* **16**: 727-729.

Dinehart, S. K.; Smith, L. M.; McMurrya, S. T.; Smith, P. N.; Anderson, T. A.; Hauko, D. A. (2010). Acute and chronic toxicity of Roundup Weathermax_ and Ignite_ 280 SL to larval *Spea multiplicata* and *Spea bombifrons* from the Southern High Plains, USA. *Environmental Pollution* **158**: 2610-2617.

Dinehart, S. K.; Smith, L. M.; McMurrya, S. T.; Smith, P. N.; Anderson, T. A.; Hauko, D. A. (2009). Toxicity of a glufosinat and several glyphosate herbicides to juvenile amphibians from Southern High Plains, USA. *Science of the Total Environment* **4 0 7**: 1 0 6 5 – 1 0 7 1.

Ebert, E.; Leist, K. H.; Mayer, D. (1990). Summary of safety evaluation toxicity studies of glufosinate ammonium. *Food and Chemical Toxicology* **28**: 339-349.

Edginton, A. N.; Sheridan, P. M.; Stephenson, G. R.; Thompson, D.G.; Boermans, H. J. (2004). Comparative effects of pH and vision herbicide on two life stages of four anuran amphibian species. *Environmental Toxicology and Chemistry **23*** (4): 815–822.

Egea-Serrano, A.; Relyea, R. A.; Tejedo, M.; Torralva, M. (2012).Understanding of the impact of chemicals on amphibians: a meta-analytic review. *Ecology and Evolution* **2(7)**:1382–1397.

Ezemonye, L. N.; Tongo, I. (2009). Lethal and sub-lethal effects of atrazine to amphibian larvae. *Jordan Journal of Biological Sciences* **2** (1*): 29-36.*

Emmett, K. (2002). Final risk assessment for diquat bromide. The water quality program of the Washington State Department of Ecology. 02-10-046. www.wsde.org.

Farah, M. A.; Ateeq, B.; Ali, M. N.; Sabir, R.; Ahmad, W. (2004). Studies on lethal concentrations and toxicity stress of some xenobiotics on aquatic organisms. *Chemosphere* **55**: 257–265.

Greulich, K.; Pflugmacher, S. (2003). Differences in susceptibility of various life stages of amphibians to pesticides exposure. *Aquatic Toxicology*. **65**: 329-336.

Gungordu, A. (2013). Comparative toxicity of methidathion and glyphosate on early life stages of three amphibian species: Pelophylax ridibundus, Pseudepidalea viridis, and *Xenopus laevis*. *Aquatic Toxicology* **140– 141**: 220–228.

Herkovit, J.; Perez-Coll, C.S. (1993). Stage dependent susceptibility of Anaxyrus arenarum embryos to Cadmium. Bulletin of EnvironmentalToxicology **50***: 608-611.*

Herkovits, J.; Cardellini, P.; Pavanati, C.; Perez-Coll, C. S. (1997). Susceptibility of early life stages of *Xenopus laevis to* cadmium. *Environmental Toxicology and Chemistry **16(***2**)**:* 312–316.

Hayes, T. B.; Falso, P.; Gallipean, S.; Stice, M. (2010). The Cause of global amphibian declines: A developmental endocrinologist perspective. The Journal of Experimental Biology **213***:* 921-933.

Holmes, A. M.; Creton, S.; Chapman, K. (2010). Working in partnership to advance the 3Rs in toxicity testing. *Toxicology* **267**: 14–19.

Hynes, S.V.; Wilson, S.; Byrne, M. (2009). Frogs as bioindicators of chemical usage and farm practices in an irrigated agricultural areas. Final Report to Land and Waters Australia.

Kloas, W.; Urbatzka, R.; Opitz, R.; Wurtz, S.; Behrends, T.; Hermelink, B.; Hoffmann, F.; Jagnytsch, O.; Kroupova, H.; Lorenz, C.; Neumann, N.; Pietsch, C.; Trubiroha, A.; Ballegooy, C.; Wiedermann, C.; Lutz, I. (2009). Endocrine disruption in aquatic vertebrates. Trends in Comparative Endocrinology and Neurobiology: *Ann N.Y. Acad. Sci.* **1163:** 187-200.

Koyama, K.; Koyama, K.; Goto, K. (1997). Cardiovascular effects of an herbicide containing glufosinate and a surfactant: in vitro and in vivo analysis in rat. *Toxicology and Applied Pharmacology* **145**: 409-414.

Lavorato, M. I. B.; Antonio, C. M.; Denoël, S. T.; Brunelli, E. (2013). Endosulfan effects on *Lithobates dalmatina* tadpoles: quantitative developmental and behavioural analysis. *Archives of Environmental Contamination and Toxicology* **64**: 253–262.

Mann, R. M.; Bidwell, J. R. (1999b). The toxicity of glyphosate and several glyphosate formulations to four species of south western Australian frogs. *Archives of Environmental Contamination and Toxicology* **36**:193–199.

Mann, R. M.; Bidwell, J. R. (2000). Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. Aquatic Toxicology **51**: 19-29.

Muths, E.; Gallant, A. L.; Grant, E. H. C.; Battaglin, W. A.; Green, D. E.; Staiger, J. S.; Walls, S. C.; Gunzburger, M. S.; Kearney, R. F. (2006). The Amphibian Research and Monitoring Initiative (ARMI): 5-year report: *US Geological Survey Scientific Investigations Report 2006–5224*. p.77.

Nieuwkoop, P. D.; Faber, J. (1994). *Normal Table of Xenopus laevis (Daudin)*, 3rd ed. Garland, New York, NY, USA.

Ortiz-Santaliestra, M. E.; Marco, A.; Fernandez, M. J.; Lizana, M. (2006). Influence of developmental stage on sensitivity to ammonium nitrate of aquatic stages of amphibians. *Environmental Toxicology and Chemistry* **25**(1): 105–111.

Orton, F.; Lutz, I.; Kloas, W.; Routledge, E. W. (2009). Endocrine disrupting effects of herbicides and pentachlorophenol: In vitro and in vivo evidence. *Environmental Science and Technology* **43**: 2144-2150.

Organisation for Economic Cooperation and Development (OECD) (2004). Environment Health and Safety Publication Series on Testing and Assessment 46. ENJ/JM/MONO 17. www.oecd.org/document.

Organisation for Economic Cooperation and Development (OECD) (2007).Validation of the Amphibian Metamorphosis Assay as a Screen for Thyroid-active Chemicals: Integrated Summary Report. AMA integrated summary report. www.oecd.org/document.

Organisation for Economic Co-operation and Development (OECD) (2008). Series on Testing and Assessment No. 91. Report of the validation of the amphibian metamorphosis Assay (PHASE 3) ENV/JM/MONO 18.www.oecd.org/document

Paggetti, E.; Biaggini, M.; Corti, C.; Lebboroni, M.; Berti, R. (2006). Amphibians and Reptiles as Indicators in Mediterranean Agro-ecosystems: A Preliminary Study. Proceedings of the 13th Congress of the Societas Europaea Herpetologica 107-110.

Pašková, V.; Hilscherová, K.; Bláha, L. (2011). Teratogenicity and embryotoxicity in aquatic organisms after pesticide exposure and the role of oxidative stress. In D.M. Whitacre (ed.), *Reviews of Environmental Contamination and Toxicology* **211**: 25-61.

Pauli, B. D.; Perrault, J. A.; Money, S. L. (2000). RATL: A Database of Reptile and Amphibian Toxicology Literature. Technical Report Number 357. Pp 494.

Paul, E.; Howard, A.; Simonin, A.; Symula, J. (1994). The toxicity of diquat, endothall, and fluridone to the early life stages of Fish. *Journal of Freshwater Ecology* **9** (3): 229-239.

Perez-Coll, C. S.; Herkovits, J. (1990). Stage dependent susceptibility to Lead *in Anaxyrus anerarum* embryos. *Environmental Pollution* **63**: 239-245.

Perkins, P. J.; Boermans, H. J.; Stephenson, G. R. (2000). Toxicity of glyphosate and triclopyr using the frog embryo teratogenesis assay-*Xenopus*. *Environmental Toxicology and Chemistry* **19***:* 940–945.

Peterson, H. G.; Boutin, G.; Martin, P. A.; Freemark, K. E.; Ruecker, N. J.; Moo.dy, M. J. (1994). Aquatic phyto-toxicity of 23 pesticides applied at expected environmental concentrations. *Aquatic Toxicology.* **28***:* 275-292.

Pickford, D. B.; Morris, I. D. (2003). Inhibition of gonadotropin-induced oviposition and ovarian steroidogenesis in the African clawed frog (*X. laevis*) by the pesticide methoxychlor. *Aquatic Toxicology* **62**: 179-194.

Reinert, K. H.; Giddings, J. M.; Judd, L. (2002). Effects analysis of time-varying or repeated exposures in aquatic ecological risk assessment of agrochemicals. *Environmental Toxicology and Chemistry* **21**: 1977–1992.

Relyea, R. A. (2009). A cocktail of contaminants: how mixtures of pesticides at low concentrations affects aquatic communities. *Oecologia* **159***: 363-376.*

Servizi, J. A.; Gordon, R. W.; Martens, D. W. (1987). Acute toxicity of garlon 4 and roundup herbicides to salmon, daphnia and trout. *Bulletin of Environmental Contamination and Toxicology* **39**: 15–22.

Shi, Y. B. (2000). Amphibian Metamorphosis. Wiley-Liss, New York, Chichester, Weinheim, Singapore, Toronto.

Siddiqua, K. A.; Alquezar, R.; *Wilson, S.P. (2013).* Comparative sensitivities of larval stages of the cane toad, *Rhinella marina*, and the striped marsh frog, *Limnodynastes peronii*, to atrazine. *Australian Journal of Zoology* **61**: 320–327.

Sparling, D. W.; Fellers, G. M.; McConnell, L. S. (2001). Pesticides and amphibian population declines in California USA. *Environmental Toxicology and Chemistry* **20:** 1581-1595.

Takahashi, M. (2007). Oviposition site selection: Pesticide avoidance by Gray Treefrogs. *Environmental Toxicology and Chemistry* **26** (7): 1476-1480.

United State Environmental Protection Agency (US EPA) (1999). LC_{50} Software Program, Version 1.00.Center for Exposure Assessment Modelling (CEAM) Distribution Centre. www.usepa.org/software.

Venturino, A.; Rosenbaum, E.; Caballero De Castro, A.; Anguiano, O. L.; Gauna, O.; De Schroeder, T. F.; De D'Agelo, M. F. (2003). Biomarkers of effect in toads and frogs. *Biomarkers* **8** (3-4): 167-186.

Vitt, L. J.; Caldwell, J. P.; Wilbur, H. M.; Smith, D. C. (1990). Amphibians as harbingers of decay. *Bioscience* **40***:* 418-422.

Wagner, N.; Wolfram, R.; Hanka, T.; Beatrix, T.; Stefan, L. (2013). Questions concerning the potential impact of glyphosate-based herbicides on amphibians. *Environmental Toxicology and Chemistry* **32**: 1688–1700.

World Health Organization (WHO) (1990). Public health impacts of pesticides used in agriculture. World Health Organisation, Geneva. www.who.publications.

World Health Organisation (WHO) (1994). Glyphosate. Environmental health criteria 159. World Health Organisation, Geneva. www.who.publications.

World Health Organisation WHO (2004). Diquat in drinking water. Background document for development of WHO guidelines for drinking-water quality. World Health Organisation, WHO/SDE/WSH/03.04/91. www.who.publications.

World Health Organisation (WHO) (2013). State of the science of endocrine disrupting chemicals 2012. United Nations Environmental Programme and World Health Organisation. ISBN: 978-92-807-3274-0 (UNEP) and 978 92 4 150503 1 (WHO).

Chapter Three

Lethal and teratogenic impacts of six herbicides formulations using Frog Embryo Teratogenesis Assay- Xenopus (FETAX)

3.1 Introduction

Pesticides play an important role in global agricultural development, contributing to reduction in pest attacks and weeds infestation, thereby improving general agricultural yields. Global pesticide application is extensive and increasingly escalating over the years (Bjorling-Poulsen *et al*., 2008; Mensah *et al*., 2013). For example, annual global agrochemicals usage is estimated at 11.2 billion kg, most of which are herbicides (Suntharasingham *et al*., 2010; Yadav *et al*., 2013). Despite these positive contributions, pesticides have however also contributed to the deterioration of water and soil quality (Hovart *et al*., 2005). Pesticides as environmental chemicals therefore raise concerns about their non-target ecological impacts (Dirzo and Raven 2003; Relyea, 2003; Govandarajulu, 2008; Mann *et al*., 2009; Piola *et al*., 2013). Herbicides formulations have been assumed not to pose a great risk to non-target organisms, however evidence are mounting showing that these chemicals and or associated chemicals in the formulation can affect wildlife health in aquatic and terrestrial habitats (Relyea, 2005; Dinehart *et al*., 2009; Egea-Seranno *et al*., 2012).

The freshwater habitats are experiencing rapid biodiversity modifications largely due to agricultural practices (Downing *et al*., 2008), as agrochemicals have become major sources of water pollution, with devastating health impacts on humans and wildlife (WFW, 2010). Estimates of freshwater biodiversity loss are suggesting that the current rate of extinction is the most profound in the last 100,000 years (Wilson, 1992; Eldredge, 1998). The concern about the global amphibians decline problem for example (Dinehart *et al*., 2009; Gongordu, 2013; Lajmanoich *et al*., 2013), highlight the need to assess the potential impact of agricultural chemicals on these organisms. Amphibian vulnerability to agrochemicals has been linked to their specific ecological/aquatic requirements linking them to permanent or temporary water-bodies to complete their life cycle. However, it is these very same water pools and shallow water-bodies ponds where agrochemicals easily contaminate and accumulate (Mann *et al*., 2003). Although it is known that many agrochemicals bind to the sediment the assumption that these chemicals pose relatively little risk to non-target, have been questioned (Relyea, 2005) and is it therefore more realistic to assume direct exposure of early life stages in the aquatic environment. Apart from these early stages being sensitive to the chemical component of the environment, early organizational effects may have long term health effects (Guillette, 1995).

The global use and presence of the agrichemicals in aquatic systems have been proposed as a contributing factor to the hypothesized declines in amphibian populations (Bishop *et al*., 1999: Brunelli *et al*., 2009; Paskova *et al*., 2011; Egea-Seranno *et al*., 2012; Gungordu, 2013; Lajmanoich *et al*., 2013). Environmental surveys have also showed an association between amphibian population declines and proximity to agricultural lands (Bishop *et al*. 1999; Edge *et al*., 2011). But despite the proposed causal links to pesticide exposures, research emanating from developing countries is mostly absent or largely inadequate (Ansara-Ross *et al*., 2012).

For most pesticides, ecotoxicology studies report LC_{50} concentrations for aquatic organisms, mostly with reference to fish species, while information regarding toxicity to amphibians are generally scanty (Relyea *et al*., 2005; Johansson *et al*., 2006; Relyea, 2009; Mann *et al*., 2009; Bruhl *et al*., 2011; Wagner *et al*., 2013). Since it has been shown that amphibians may be just as sensitive to environmental contaminants than fish, due to their permeable skin, therefore suggestions that exposure to agricultural chemicals could be linked to global amphibian declines (Relyea, *et al*., 2005; Edge 2011; Bruhl *et al*., 2013), and calls for more amphibian related studies have increased (Relyea and Jones, 2009; Mann *et al*., 2009; Bruhl *et al*., 2011; Plotnet and Matschke 2012; Wagner *et al*., 2013). Amphibians are generally regarded as good models to understand the mechanism of action for pesticides (including insecticides, fungicides and herbicides) when affecting physiological functioning (Lajmanovich *et al*., 2013). But in general, relatively few studies used amphibians as model organisms (Mann *et al*., 2009; Bruhl *et al*., 2011).

Herbicides are the leading group of pesticides in terms of annual production, total acreage size usage, and total revenue sales (Mensal *et al*., 2013). They generally have relatively short half-lives in soil and water, but are found at relatively high concentration in the environment (Orton *et al*., 2009). Diverse ranges of herbicide active ingredients have been shown to have differential effects in living organisms, even when present at relatively low environmental concentrations. In this regard, specifically organizational effects during embryonic development and juvenile life cycle phases are a growing concern (Guillette *et al*., 1995; Petterson *et al*., 1996). And with the continuous widespread of amphibian malformations currently observed globally, developmental and teratogenic impacts from herbicide formulations are of major concerns.

Herbicide formulations (i.e. active ingredient and inert compounds) have increased exponentially in the last decade and new formulations are added regularly to compensate for resistance (Mensah *et al*., 2013). Herbicides, according to Kappler and Namuth, (2004) are classified into eight modes of actions, 22 specific sites action in biochemical pathways. But

generalisations regarding mode of action or biological endpoints associated with the resulting herbicides families, specifically in amphibians, have not been well-studied (Relyea, 2006). This makes it difficult to assess the exposure impacts of these herbicides in the environment. Several of these herbicide formulations are applied in the aquatic environment for management of aquatic weeds and aliens plants. These herbicide formulations include Roundup, Kilo Max, Enviro glyphosate, Midstream, Basta and Arsenal.

One of the widely used herbicide families includes the glyphosates (Kappler and Namuth, 2004; Lanctot *et al*., 2012; Sihtmae *et al*., 2013), used as broad-spectrum herbicides to inhibit post emergent growth through pathway generally thought not to be associated with animal (Sihtmae *et al*., 2013) Typically, all glyphosate-based herbicide formulations contain two major ingredients, isopropylamine (IPA) salt of glyphosate as active ingredient, and surfactant that vary from one formulation to another (Dinehart *et al*., 2009; Lanctot *et al*., 2012).

Roundup formulation, one of the best-studied herbicides for example contains glyphosate, and a polyethoxylated tallow amine (POEA) surfactant that facilitate plant cuticle penetration by polar compound. It has been suggested that the POEA surfactant is more toxic than active glyphosate (Giesy *et al*., 2000; Sihtmae *et al*., 2013). Notwithstanding, exposure to glyphosate formulations or POEA in amphibians have been reported to affect amphibians physiology at several levels but need more study (Mann *et al*., 2009; Williams and Semilitsch, 2010; Jones *et al*., 2010; 2011). Researchers have shown that Roundup could be very toxic to amphibians (Relyea and Jones, 2009), and also cause growth abnormality in growing tadpoles when exposed to relatively low, non-lethal concentrations (Lajmanovich *et al*., 2002). But apart from a few key studies reporting some evidence for non-lethal physiological endocrine modulation, for example, by glyphosate in amphibians (Howe *et al*., 2004; Kloas *et al*., 2005), mammalian cell cultures (Richards *et al*, 2005), and teratogenicity in birds (Paganneli *et al*., 2010), evidence of disrupting pattern of glyphosate in amphibians particularly regarding the development of malformations, following early life cycle exposure is not well characterised (Govindarajulu, 2008). Kilo Max is another glyphosate formulation with sodium salt and ammonium sulphate as surfactant. Enviro glyphosate is also a formulation of glyphosate; contain isopropyl ammonium salt of glyphosate and polyethylene alkylamine as surfactant. The kilo Max and Enviro glyphosate are relatively new glyphosate formulations which have not been particularly tested for developmental toxicity and endocrine disruption in general as there is no data on them currently in any literature.

Several non-glyphosate herbicides including diquat dibromide, imazapyr, and glufosinate ammonium are also used in aquatic systems and present some concern for non-target aquatic organisms. Diquat dibromide (9, 10-dihydro-8a, 10a-diazonia phenanthrene ion) is a post-emergent, non-selective contact herbicide and crop desiccant that is also used in aquatic weeds control (Emmett, 2002; WHO, 2004). Diquat dibromide is widely used in the United State, North America, Europe, Australia and Japan (Emmett, 2002; WHO, 2004). The herbicide contains nonyl phenol ethoxylate as surfactant, which has been implicated as having estrogenic activities (Trumbo, 2005; Othman, 2009). There have been some controversies about the impacts of this herbicide on amphibians and wildlife in general. Anderson and Prahlad, (1976) using concentration of 0.0075 to 0.002 mg /L discovered that Diquat formulation inhibited general body growth and pigmentation as well as result in distortion in body shape at concentrations ranging from 0.0015 to 0.002 mg /L. Bimber and Mitchell (1978), using *Lithobates pipiens* at high concentration of 0.1 mg/L, reported increased rates of exogastrulation and mortality. Selypes *et al.* (1980), using nullipara mice that were exposed to 11 mg/kg of Reglone formulation of diquat on the 9^{th} day of gravidity, pointed out that the death of foetus increased with concentration, and that the average embryonic weight decreased as the number of embryos retarded in weight increased. According to these authors, Diquat formulation even caused retardation in the embryos of females repeatedly treated with smaller doses, with changes in the skull, vertebrae, sternum and the limbs observed. But, Dial and Dial, (1987), using Diquat formulation on *Lithobates pipiens* at concentration between 2.0 to 10 mg/L observed that their eggs were resistant to Diquat and exposure at both early and late gastrula produced no abnormalities.

Imazapyr herbicide belongs to the chemical family of imidazolinone (Liu, 1992), and it usually degraded through photolysis in water with half-life ranging between 2.5 and 5.3 days (WSDA, 2009). According to Grisolia *et al*. (2004), Arsenal, one of its formulations consists of 25 g/L Imazapyr, 186 g/L of ammonium hydroxide, 18 g/L nonyl-phenol ethoxylate (with nine ethoxylated units) and water. There is scarcity of literature on the teratogenicity and developmental toxicity of this herbicide. But various reviews, including USEPA 2006 (Registration Eligibility Decision (RED), and Washington State Department of Agriculture (WSDA, 2003), rated this herbicide active ingredient as having no developmental toxicity and teratogenicity. And PAN, (2012) also put the developmental and teratogenicity as well as endocrine disruption as unknown.

Glufosinate ammonium (GA) (ammonium-D,L- homoalanin-4-yl methyl) phosphate, one of its formulations known as Basta, contains an anionic sodium polyoxyethylene alkyether sulphate (AES), which constitute 30% as surfactant, a wetting agent (alkylether-sulphate), solvent

(propylene glycol ether, defoamer and a blue dyestuff (Ebert *et al*., 1990: Koyama *et al*., 1997). Even though the herbicide and its analogues are now registered for used in more than forty countries worldwide (Qian *et al*., 2008), not much is known about its teratogenicity and endocrine disrupting potential. Watanabe and Iwase (1996) in a study examined its developmental and dysmorphogenic effects on mouse embryos in culture and observed that eight (8) days old embryos cultured for 48 hour showed a significant overall embryonic growth retardation and increase embryotoxicity (37.5% at 10 mg/L). Accordingly, all embryos in the treated group exhibited specific morphological defects including blisters in the lateral head (100%), hypoplasia of the prosencephalon (forebrain) (100%) and visceral arches (100%). Using the micromass cell culture method, GA also inhibited the differentiation of midbrain cells in Day 12 embryos, with 50% inhibition occurring at 0.00055 mg/L. The ratios of 50 % inhibition of concentration for cell proliferation to differentiation in limb bud cell were 0.76 and 1.52 in day 11 and 12 embryos. It was concluded that glufosinate ammonium was embryotoxic in vitro (Watanabe and Iwase, 1996).

South Africa is the largest pesticides user in sub-Saharan African, with about 60% of the pesticides market in Africa (Ansara-Ross *et al*., 2012). Currently, approximately 180 different pesticides active ingredients are commercially available in South Africa, with approximately 400 registered trade names (Menhardi, 2008). The use of several herbicides and other pesticides in South Africa has increased in recent years, not only by farmers using herbicides extensively in all sectors of commercial farming, but also for domestic use and in government programs including the Working for Water Program (WfW) of National Department of Water Affairs, towards the control of alien plant species in natural catchment areas of major rivers as well as large reservoirs (Bold, 2007; Ansara-Ross *et al*., 2012; Mensah *et al*., 2013).

But despite this increase herbicide formulations application in South Africa, scientific information about their fate as well as impacts in the local aquatic ecosystems is very scanty (Ansara-Ross *et al*. 2012). This, in spite of relative high concentrations of glyphosate previously reported in intensive farming areas like Hex River Valley, in Western Cape Province, Oliphant's River project and Groblersdal project (Dalvie *et al*. 2011; Mensah *et al*., 2013). There is also no known national guideline and permissible limit in the aquatic ecosystem, to assess the impacts of these formulations on the indigenous species, including amphibian, in order to protect the local biota (Mensah *et al*., 2013).

The Frog Embryo Teratogenesis Assay-Xenopus (FETAX) is a standardised 96-hour whole-embryo assay used for the assessment of potential developmental and teratogenic effects in humans

(Bernardini *et al*., 1994; Mann and Bidwell, 2000; Mann *et al*., 2011; Leconte and Mounche, 2013; Yu *et al*., 2013). FETAX has been widely used in aquatic toxicity testing and is well-suited for testing environmental samples or complex mixtures (industrial and wastewater effluents) (Bantle *et al*., 1999). This assay essentially assesses environmental impacts during embryogenesis, which is may be highly conserved across vertebrates (NICEATM, 2000). FETAX is a four (4)-day (96-hour) protocol for predicting potential developmental toxicants and teratogens (ASTM, 1998). Endpoints include mortality, malformation and growth inhibition. Recently, the FETAX protocol using *X. laevis* have been criticised as been less sensitive than when using other amphibians. However, Yu *et al.* (2013) confirmed the sensitivity of Xenopus embryo and its value as robust laboratory species.

The FETAX assay has been widely used to assess embryotoxicity of various environmental chemicals including; Nonylphenol ethoxylate (Mann and Bidwell, 2000), heptanol (Bernardini *et al*., 1994), nicotine and cotinine (Dawson *et al*., 1988), acidic mine water (Dawson *et al*., 1985), aqueous soil extracts (Fort *et al*., 1995), atrazine herbicides (Morgan *et al*., 1996), organophosphate, plant growth regulators (Boga *et al*., 2009) and organochlorine insecticides (Schuytema *et al*., 1994). Moreover, Bantle *et al*. (1999) and Leconte and Mouche (2013) reported a predictivity of 75% and 81% respectively for mammals or human teratogenic potential. Leconte and Mouche (2013) correlated FETAX data that span 12 years of screening pharmaceutical compounds and linking results to mammalian embryotoxicity studies.

The aim of this study was to select herbicide formulations including Midstream, Basta, Arsenal and three (3) glyphosate-based herbicide formulations (including Enviro glyphosate and Kilo Max) used to control alien plants in local aquatic systems and screen for developmental toxicity and teratogenic potential, using the African clawed frog, *X. laevis* as model organism.

3.2.Materials and methods

3.2.1. Test chemicals

The herbicides-formulations include Roundup (360g/L a.e, Monsanto, South Africa), Enviro Glyphosate (360g/L a.e, Enviro Industries Ltd, South Africa), Kilo Max (700g/kg a.e Glyphosate, Volcano Agroscience Ltd, South Africa), Midstream (373g/L, diquat dibromide, Syngenta S.A Ltd), Basta (200g/L, glufosinate ammonium, Bayer Crop Science AG Ltd, Germany) and Arsenal (250g/L, imazapyr, Base Chemical Ltd). The FETAX solution used

containing 625mg NaCl, 96mg $NaHCO_3$, 30mg KCl, 15mg $CaCl_2$, 60 mg $CaSO_4.2H_2O$ and 70mg $MgSO_4$ per litre of distilled water (ASTM, 1998).

3.2.2. Exposure concentrations

Based on the list of aquatic herbicides in South Africa, six most widely-used formulations were selected for the study. Following the initial pilot studies and some acute toxicity results from chapter one, six concentrations were selected for each of the six herbicide formulations (Table 3.1).

Table 3.1: Exposure concentrations for the six herbicide formulations

Treatment	Exposure Concentrations (mg a.e/L)
Roundup	0, 0.5, 0.7, 0.9, 1.1, 1.3
Kilo Max	0, 130, 160, 190, 220, 250, 280
Enviro Glyphosate	0, 320, 360, 400, 440, 480, 520, 560.
Midstream	0, 0.5, 1.0, 2.0, 2.5, 3.0
Arsenal	0, 20, 25, 30, 35, 40 and 45
Basta	0, 1.6, 2.0, 2.5 and 3.0

3.2.3. Analytical assessment of experimental concentrations

In **order** to confirm the experimental concentrations, four random concentrations per formulation were sampled from the exposure tanks. The sampled concentrations were independently (blind) analysed at Synexa Analytical laboratory in Cape Town, in South Africa. The laboratory used liquid chromatography tandem mass spectrometry (LC/MSMS) method which has a high validity and sensitivity. The variation between the measured concentrations and nominal concentrations were low (between 0-5% across the measured samples) (Table 3.2), except for one sample that showed a high 10 % variation. These variations are all within the acceptable limit of USEPA, (1996) toxicity guidelines.

Table 3.2- The exposed nominal concentrations and observed concentration

Formulation	Exposed Concentration (mg a.e./L)	Observed Concentration (mg a.e./L)
Roundup	1	1.19
	1.4	1.41
	1.8	1.50
	2.2	2.10
Kilo Max	320	354.80
	380	386.50
	440	448
	500	480
Enviro glyphosate	520	540.80
	580	599.20
	640	603.50
	700	629.30

3.2.4. *Xenopus laevis* care and breeding of tadpoles.

The Africa clawed frog (*X. laevis)* is a local aquatic species that has globally been used as a model species to study environmental quality, but also widely used to specifically study the effects of environmental contamination on amphibians (Vitt *et al*., 1990; Mann and Bidwell, 2000; Paggeti *et al*., 2006; Kloas *et al*., 2009). Prior to breeding, four sexually mature males and females *X. laevis* were maintained separately in 15 L glass tanks containing reverse osmosis water, and were fed three times per week with fish pellets (Aqua-Nutro, RSA). Breeding induction was performed according to ASTM, (1998) protocol. Males were primed with 200 IU human chorionic gonadotropin (hCG) (Merck Ltd, Germany) injection, injected into their dorsal lymph sac, four days prior to the commencement of mating and again just prior to the mating, by another 100IU and 200 IU hCG to males and females respectively (usually in late afternoon). Male and female pairs were placed together in a 15 L exposure tanks lined with plastic netting (to separate the eggs from the adults), and place in a well-ventilated dark place. Amplexus occurred within four hours and eggs were deposited within 12 hours after injection. All the eggs staging were done using a normal developmental atlas by Nieuwkoop and Faber (1994) (referred to as NF-stages).

3.2.5. FETAX bioassay

Frog embryo teratogenesis assay-Xenopus (FETAX) was performed following the basic guidelines as described by American Society for Testing and Materials (ASTM, 1998). Fertilised

eggs were collected immediately after spawning and de-jellied by gentle swirling in 2% L- cystein (Sigma, GE) (prepared in FETAX solution and adjusted to pH 8.1 with NaOH)(ASTM, 1998) for three minutes. Normal cleaving embryos were separated from the pool of de-jellied eggs with the aid of stereomicroscope. All the tadpole staging were done using a developmental atlas according to Nieuwkoop and Faber, (1994) (NF-stages). NF stages 8-11 (mid-blastula to early gastrula embryos), were used for all exposures. The breeding, housing and experimental procedures were approved by the Animal Ethical Committee of the Stellenbosch University (Approval: SU-ACUM12-00014).

3.2.6. Exposure set-up

Each exposure cup (500 ml) contained twenty (20) selected embryos, replicated twice for each concentration. Exposure tanks were set-up in a controlled climate room for the duration of the experiment - under the following physical conditions (OECD, 2008); water temperature 23 ± 1 ºC, pH of 6.5 – 7.4, dissolved oxygen of >3.5 mg/L and 12 hours light and dark photoperiod ($L_{12}D_{12}$). In order to standardize the exposure of herbicide concentrations, all the herbicide stocks were freshly prepared daily, to avoid any breakdown by light, heat or other environmental factors. For this study, a semi-static exposure approach was adopted, where the exposure medium was changed every 24 hours. The experiments were repeated twice.

3.2.7. Mortality

Mortality incidences were recorded every six (6) hours for the duration of the exposure. After 96 hours, the final data were used to define the 96-hour LC_5, LC_{10} and LC_{50} for each of the test substances. The remaining tadpoles were euthanized using MS 222 (Tricaine methane sulfonate) (200 mg/l buffered with sodium bicarbonate at 0.42-1.05 g/l) (OECD, 2007). All the tadpoles collected were fixed and preserved in buffered formalin (4% formaldehyde) for observation and measurement.

3.2.8. Body length and growth Inhibition

The minimum concentration to inhibit growth (MCIG) was determined for all the substances by statistically comparing the mean 96-hour head-to-tail length of the treated embryos at each treatment concentration to that of the control embryos using Kruskal-Wallis ANOVA test.

3.2.9. Malformations

Developmental malformations, (defined according to Atlas of Abnormalities by Bantle *et al*. (1998)) including facial as well as axial malformations (tail, and notochord abnormalities). The incidences of abnormalities (%) were used to determine the 96-hour malformation (EC_{50}) index.

3.2.10. Teratogenic Index (TI)

TI is the ratio of the 50% embryolethality (LC_{50}) to the concentration causing 50% malformation (EC_{50}) (Leconte and Mouche, 2013). Based on the mortality and malformation incidence, data obtained over the range of concentrations were used to calculate 96-hour 50% lethal concentration (LC_{50}) and 50% effective concentration (EC_{50}) for malformation. The LC_{50} and EC_{50} were used to calculate the teratogenic index (TI) where TI= LC/EC) (NICEATM, 2000; ASTM, 2004). A substance is considered teratogenic when $TI \geq 1.5$ (ASTM, 1998).

3.2.11. Data analysis

Mortality and malformation data were used to generate LC_{50} and EC_{50}, using the USA-EPA Probit analysis software program (USEPA, 1998). The teratogenic indexes (TI) for the selected herbicides formulations were derived from the 96-hour LC_{50} and EC_{50} values. The variation in body length between different concentrations and the control data were used to derive the minimum concentration to inhibit growth (MCIG). Normality of the data was assessed using Shapiro-Wilk test, and treated samples were analysed using Kruskal-Wallis ANOVA test at $\alpha = 0.05$ for significance difference.

3.3. Results

3.3.1. Embryotoxicity/ Mortality

The 96-hour LC_{50} for Midstream, Arsenal, Basta, Roundup, Kilo Max and Environ Glyphosate were 0.833, 36, 2.24, 1.05, 207.25 and 465.95 mg/L respectively (Table 3.3).

Table 3.3: The exposure concentrations (mg/L), LC_{50}, EC_{50}, Teratogenic Index (TI), and minimum concentration that inhibit growth (MCIG) for the herbicide formulations (95% CI in bracket).

Treatment	Exposure Concentrations	LC_{50} (95%CI)	EC_{50} (95%CI)	TI	MCIG
Roundup	0, 0.5, 0.7, 0.9, 1.1, 1.3.	1.052 (0.91-1.32)	0.76	1.4	0.9
Kilo Max	0, 130, 160, 190, 220, 250, 280	207.25 (196.6-217.6)	150.8 (139-160)	1.4	190
Enviro Glyphosate	0, 320, 360, 400, 440, 480, 520	465.95 (434-51)	287 (142.7-320)	1.6	440
Midstream	0, 0.5, 1.0, 2.0, 2.5 and 3.0	0.833 (0.43-1.14)	0.24	3.5	1.0
Arsenal	0, 20, 25, 30, 35, 40 and 45	36 (33.3-39.5)	28.13 (26.2-29.96)	1.3	30
Basta	0, 1.6, 2.0, 2.5 and 3.0	2.240 (1.968-2.71)	2.01 (1.78-2.31)	1.1	2.0

3.3.2. Growth Effects

For Roundup formulation, using Kruskal-Wallis ANOVA test, there was significant variation in length within exposure concentrations ($P< 0.05$). The reduction in length was significantly different from concentrations of 0.9 mg/L to 1.3 mg/L (Kruskal-Wallis, $P < 0.05$) relative to the control (Figure 3.1).

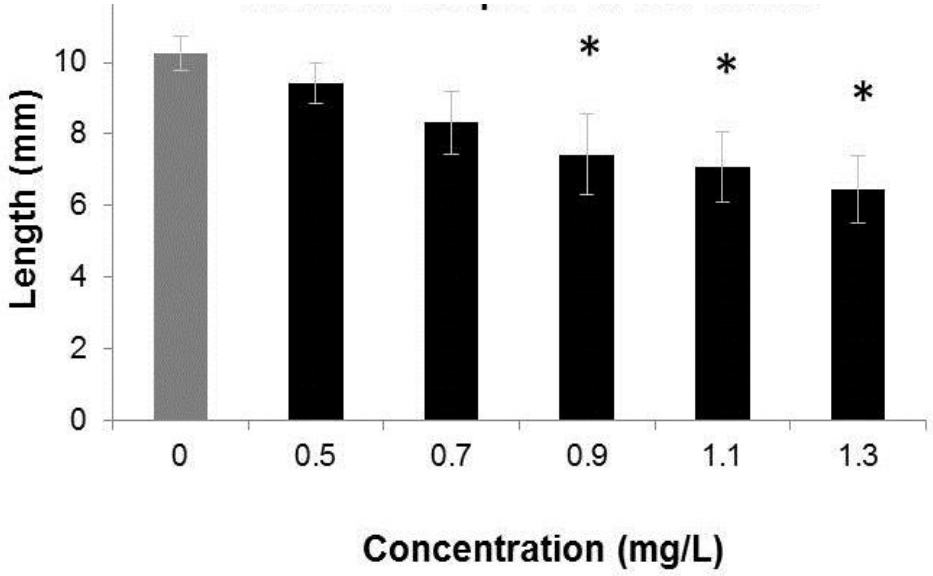

Figure 3.1: Length (mean ± SD) of tadpoles exposed to Roundup formulation significantly varied among the exposure concentrations (Kruskal-Wallis, P< 0.05). The reduction in length was significantly different from concentrations of 0.9-1.3 mg/L compared to the control (Kruskal-Wallis, P < 0.05), confirming the inhibiting effects of Roundup formulation on *X. laevis* embryo.

For Kilo Max formulation, using Kruskal-Wallis ANOVA test, there was a significant and concentration dependent reduction in length (P< 0.05) among the exposed tadpoles. The reduction in length was significantly different from concentration of 190 to 280 mg/L compared to the control (Kruskal-Wallis, P< 0.05) (Figure 3.2).

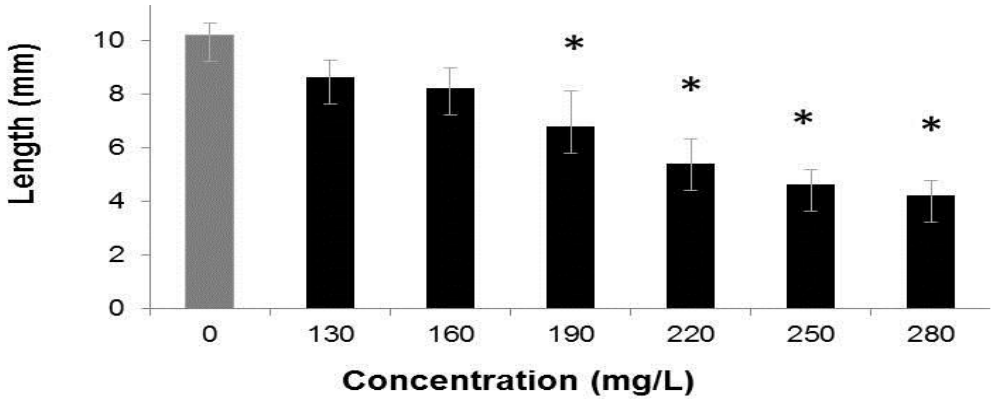

Figure 3.2: The length (mean ± SD) of tadpoles exposed to Kilo Max formulation varied significantly among the concentrations (Kruskal-Wallis, P < 0.05), and the mean length reduced in concentrations dependent manner relative to the control.

For Enviro glyphosate formulation, using the same Kruskal-Wallis ANOVA test, there were variations in length of exposed tadpoles among the concentrations, which was only significantly different ($P < 0.05$) at concentration of 400 mg/L compared to the control (Figure 3.3).

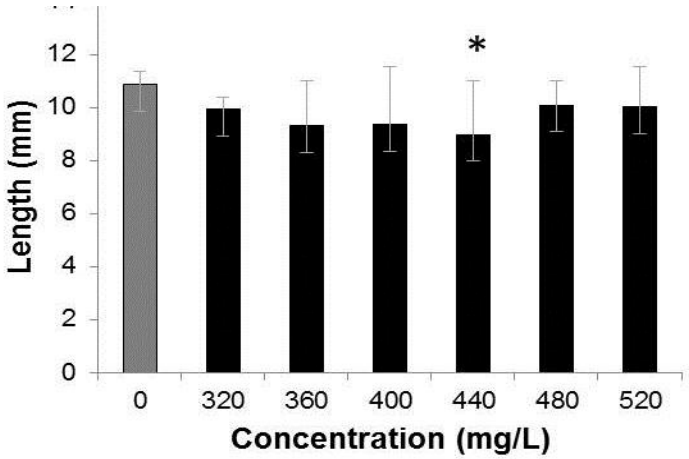

Figure 3.3: Total length (mean ± SD) of tadpoles exposed to Enviro glyphosate formulation varied widely within the exposure concentrations.

For the Midstream formulation, a generalised and significant decrease in length (Kruskal-Wallis, $P < 0.05$) from concentration of 1 mg/L to 3 mg/L was observed relative to the control. There was no significance difference in length reduction from the lowest exposure concentration of 0.5 mg/L to the highest of 3 mg/L (Kruskal-Wallis, $P > 0.05$) (Figure 3.4).

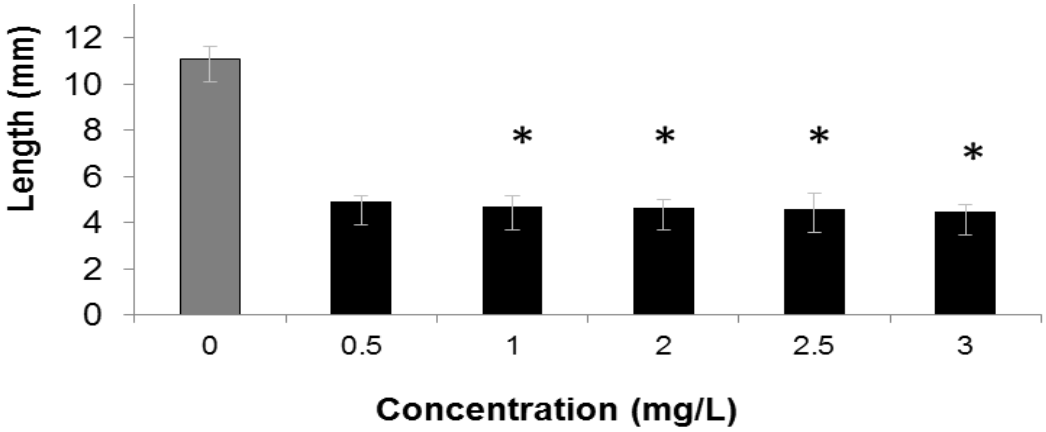

Figure 3.4: Total length (mean ± SD) of tadpoles exposed to Midstream formulation. There was significant different in length between the exposed tadpoles and the control (Kruskal-Wallis, $P < 0.05$).

For Arsenal formulation, significant variation occurred within the exposure concentrations (Kruskal-Wallis, $P > 0.05$). The total length of all exposed tadpoles were reduced in concentration

dependent manner, but only significantly different at concentration of 30- 45 mg/L relative to the control ($P < 0.05$) (Figure- 3.5).

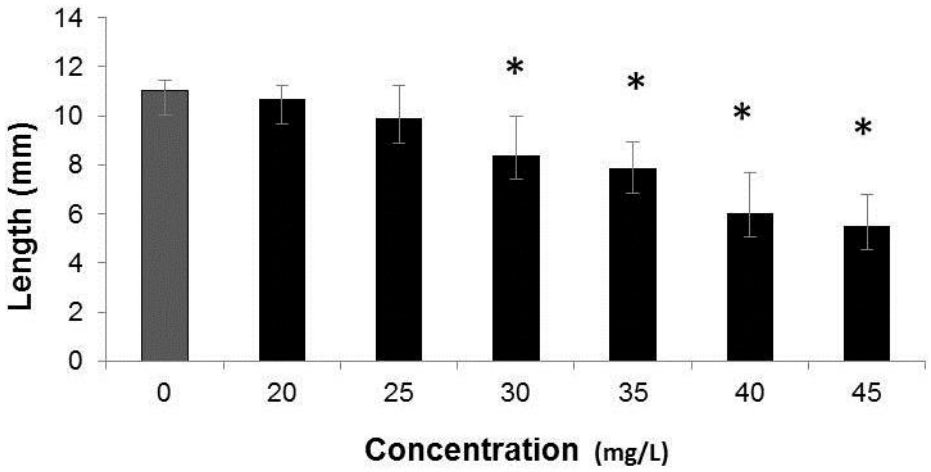

Figure 3.5- Total length (mean ± SD) of tadpoles exposed to Arsenal formulation varied significantly within the exposure concentrations (Kruskal-Wallis, P<0.05) and in concentration dependent manner relative to the control.

The Basta formulation showed a significant variation and concentration dependent decreased in length within the exposed tadpoles (Kruskal-Wallis, $P < 0.05$) (Figure 3.6). The reduction in length was only significant different (Kruskal-Wallis, P<0.05) from concentration of 2 to 3 mg/L compared to the control.

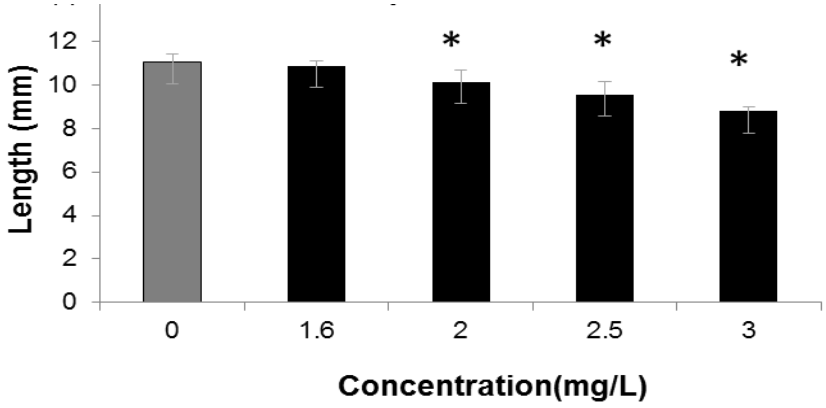

Figure 3.6: Total length (mean ± SD) of tadpoles exposed to Basta formulation varied significantly (Kruskal-Wallis, $P < 0.05$) within the exposure concentrations compared to the control.

3.3.3. Malformations (MI) and teratogenic Index (TI)

The 96-hour EC_{50} malformation index obtained for these herbicides were 0.241, 28.13, 2.01, 0.76, 150.76 and 287 mg a.e./L for Midstream, Arsenal, Basta, Roundup, Kilo Max, and Enviro Glyphosate respectively (Table 3.3). The TIs obtained were 3.5, 1.3, 1.1 1.4, 1.4, and 1.6 for Midstream, Arsenal, Basta, Roundup formulation, Kilo Max and Enviro glyphosate respectively (Table 3.3).

3.3.3.1. Observed malformation

Roundup: - the observed malformations associated with this formulation included gut abnormalities (Figure 3.7b), edema (abdominal, cardiac and severe generalised (Figure 3.7c)), blistering and eye malformations (Figure 3.7f). Some of the malformations occurred together in a single organism. The percentage incidence of various malformation categories for this formulation showed the following order; gut abnormalities (42.5%), generalised edema (22.6%), blistering (22.2%), abdominal edema (5.4%), cardiac edema (3.6%), head (2.3%) and eye (1.4%).

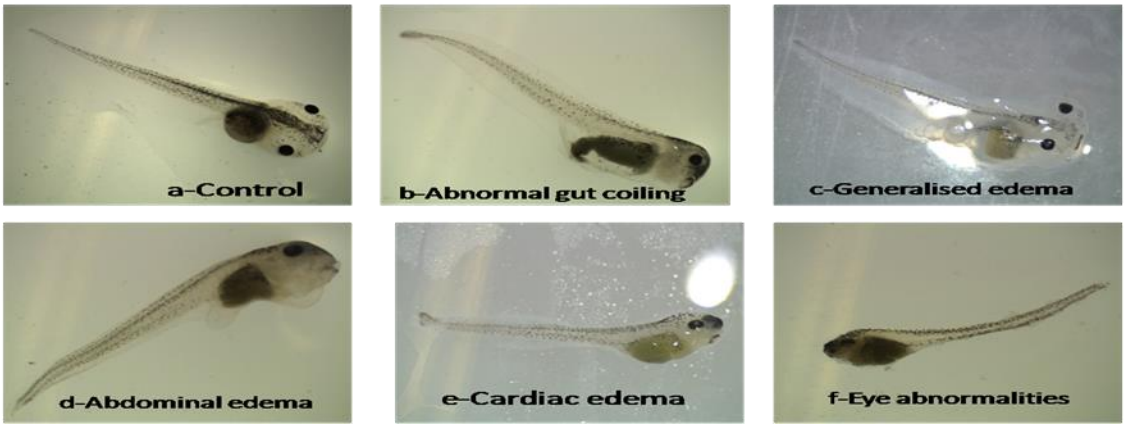

Figure 3.7: Examples of the malformations produced by the Roundup formulation included gut abnormalities (abnormal gut coiling (Figure 3.7b), edema (including generalised edema (Figure 3.7c), abdominal edema (Figure 3.7d) and cardiac edema (Figure 3.7e).

Kilo Max: the observed malformations included axial malformations (Figure 3.8d), edema (abdominal, cardiac and severe generalised (Figure 3.8f), gut abnormalities (Figure 3.8c), head and eye malformations (Figure 3.8). While some of the malformations occurred together in a single organism, the percentage incidence of various malformation categories showed the following order;

gut abnormalities (32.5%), generalised edema (29%), axial abnormalities (22.5%), abdominal edema (8 %), blistering (4%), eye (2.2 %), head (1.3 %) and cardiac edema (0.9%).

Figure 3.8: Malformations associated with Kilo Max formulation ranged from gut abnormalities (Figure 3.8c), edema including generalised edema (Figure 3.8f), abdominal edema (Figure 3.8b), and axial malformation including curved tail (Figure 3.8d).

Enviro glyphosate: the observed malformations in this formulation included gut abnormalities (Figure 3.9b), axial malformations (including wavy tails (Figure 3.9e & f) and curve tails (Figure 3.9d)), edema (abdominal, cardiac and severe generalised), head and eye malformations. While some of the malformations occurred together in a single organism, the percentage incidence of various malformation showed the following order; gut abnormalities (figure 3.9b) (34 %), abdominal edema (31.6 %), cardiac edema (20.9 %), axial abnormalities (wavy and curved tail) (Figure 3.9d-f) (6.4%), head (2.1%) and eye (1.6 %).

Figure 3.9: Malformations associated with Enviro glyphosate formulation include generalised edema (Figure 3.9c), wavy tail (Figure 3.9e), curved tail (Figure 3.9d) and cardiac edema (Figure 3.9h), all compared to the control tadpole (Figure 3.9a)

Midstream formulation: malformations such as edema (Figure 3.10f), blistering, extreme head abnormalities, gut abnormalities, wavy tails (Figure 3.10e), tail flexures and eye malformations (Figure 3.10) were observed. The most common malformation for this herbicide formulation is the generalised edema. But the most unique and unusual malformation is the two headed and multiple tails tadpole (Figure 3.10d). The percentage incidence of various malformations showed the

following order; edema (generalised, cardiac and abdominal) (43%), gut abnormalities (11.4%), blistering (14%), axial malformation (wavy and curved tail) (10.04%), eye abnormalities (2.9%), and head (1.4%).

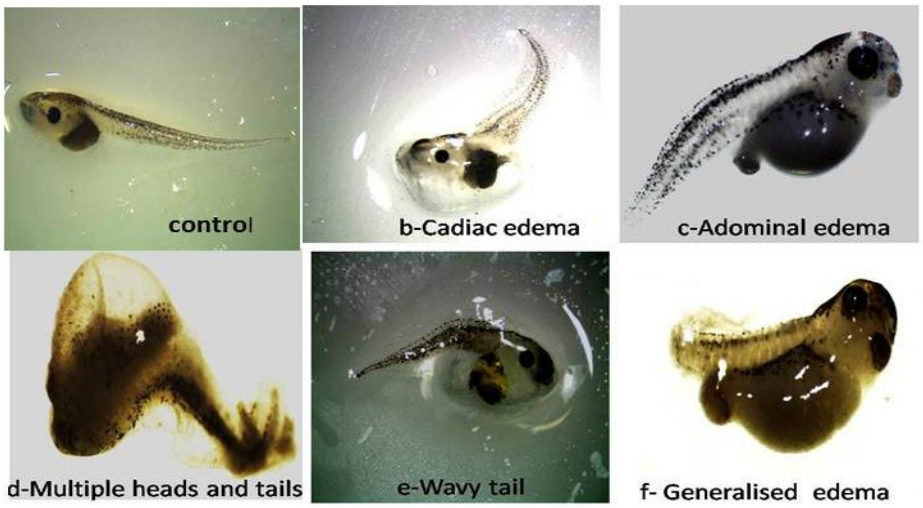

Figure 3.10: Malformations associated with Midstream formulation include cardiac edema (Figure 3.10b), abdominal edema (Figure 3.10c), generalised edema (Figure 3.10f), wavy tail (figure 3.10e) and multiple heads and tail embryo (figure 3.10d), all compared to the control (figure 3.10a).

Arsenal formulation: the observed malformations included edema (Figure 3.11d), blistering, gut (Figure 3.11b) and eye malformations, as well as improper head (figure 3.11c) and body formation. The percentage incidence of various malformations showed the following order; edema (generalised, cardiac and abdominal) (58.1 %), gut abnormalities (47.6 %), blistering (8.1 %), head (2.3 %), and eye (1.2).

Figure 3.11- Malformations associated with Arsenal formulation including generalised edema (Figure 3.11d), cardiac edema (Figure 3.11e), abdominal edema (Figure 3.11f) and gut abnormality Figure 3.11b), all compared to the control (Figure 3.11a).

Basta formulation: the observed malformations included axial malformation (wavy tails) (Figure 3.12d), edema and gut abnormalities (Figure 3.12c) and edema (abdominal edema) (figure 3.12b). The percentage incidence of various malformations showed the following order; edema (generalised, abdominal and cardiac) (50.4%), axial malformation (wavy and curved tail) (38.8%), gut abnormalities (15.4%), as well as eye and head abnormalities with (3.8%) each.

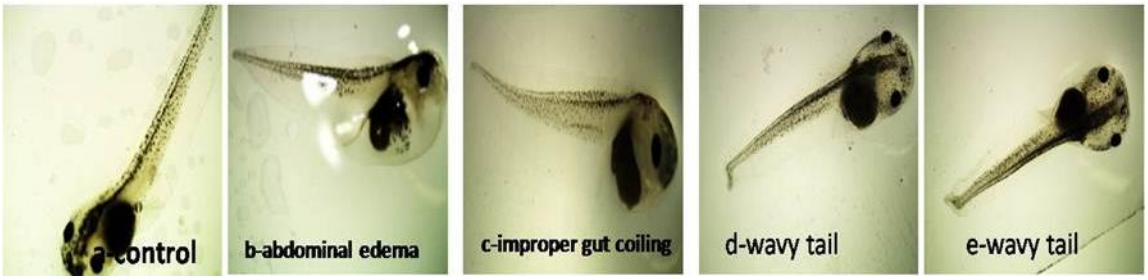

Figure 3.12.-Malformations associated with Basta formulation included abdominal edema (Figure 3.12b), improper gut coiling (Figure 3.12c) and wavy tails, all compared to the control (Figure 3.12a).

3.4. Discussion

The global rises in volume of agro-environmental herbicides continue to raise health and environmental concern on non-target organisms. Aside from human health, this concern is also premise on the basis of alleged contribution of pesticides to the suspected global amphibian population declines via developmental toxicity, thyroid and reproductive disruption (Brunelli *et al*., 2009; Egea-Seranno *et al*., 2012; Lajmanoich *et al*., 2013)**.** The aquatic herbicides link to such a decline in particular has generated increased attention on exposure impacts of various herbicides formulations on different stages of the amphibian life cycle**.** The present study examined the lethal effects along with non-lethal, developmental effects including body size effects, teratogenic (dysmorphogenic) potential and malformation-type, following the exposure to a concentration series of six selected herbicide formulations including Midstream, Basta, and Arsenal as well as

Roundup glyphosate formulation, together with two new glyphosate formulations including Kilo Max and Enviro glyphosate. These herbicide formulations are used extensively in the commercial farming as well as controlling alien plants in South African aquatic system through an alien eradication programme, managed by the WfW Program of the Department of Water Affairs (Bold, 2007; Mensah *et al*., 2013).

The present study confirmed that Roundup (glyphosate formulation), Midstream and Basta formulation had differential toxic effects but also differentially affected early growth of *Xenopus laevis* embryos. The incidence of malformation revealed a range of different malformation-types in all exposure groups. Teratogenic potential for the selected herbicides were mostly positive (>1), two (Midstream and Enviro glyphosate formulations) of the selected six herbicides exceeding TI values of 1.5 (Bantle *et al*, 1999; ASTM, 1998) but only one (Basta formulation) had a TI below 1.2 (Laconte and Mouche, 2013 guideline).

Out of the three glyphosate formulations, only Roundup showed a moderatel toxicity at 96 hour LC_{50} of 1.052 mg a.e. /L This 96-hour LC_{50} is below the expected environmental concentration (EEC) of-1.43 a.e mg/L of this formulation at the recommended aquatic application rate (Govandarajulu, 2008). This result is consistent with the findings of Mann & Bidwell (1999), who pointed out that Roundup is moderately toxic to amphibian at LC_{50} of 2.9 mg a.e. /L for *Litoria moorei* tadpoles, and 3.6 mg a.e. /L for *Crinia insignifera* tadpoles at 48 hour. It also support the conclusion of Govadarajulu (2008), who pointed out that amphibians are one of the most sensitive vertebrate groups to this herbicide formulation. Based on this result, it seems that at an expected environmental concentration of 1.43 mg a.e /L, compared to the current 96-hour LC_{50} of 1.052 mg/L, more than 80% mortality will be recorded in the field. This supports the claims of Lanctot *et al*. (2013) and Gungordu, (2013) that Roundup formulation is not suitable for use in aquatic habitats, especially where amphibians and other sensitive biota are expected to occur.

For Kilo Max and Enviro glyphosate, with 96-hour LC_{50} of 150 mg/ a.e. L and 465.95 mg a.e. /L respectively, the embryos will be well-protected from acute toxicity at the current application rate, although studies regarding impacts of chronic toxicity and sub-lethal concentration are still lacking. Present result were consistent with the suggestion of Dinehart *et al*. (2009) and Shihtmae *et al*. (2013) that toxicity of some glyphosate-based herbicides to non-target aquatic organisms may be varied, not posing a threat to some amphibian species. Comparing the toxicity of the three glyphosate-based formulations based on the current toxicity results; it seems that the glyphosate active ingredient played little or no role in the toxicity of these formulations on the embryos. The

fact that enviro glyphosate and Kilo Max formulations at 96 hour produced an LC_{50} 465 and 207 mg a.e/L respectively with high amount of active glyphosate compared to 1.052 mg/L in Roundup, showed that the toxicity may largely be caused by the surfactant in the Roundup formulation. This result therefore is consistent with the findings of Howe *et al*. (2004) and Lanctot *et al*. (2013) who noted that the surfactant POEA was the basis of Roundup toxicity. Clearly, more studies are needed to confirm the toxicity of the unknown surfactant used in the respective formulations.

Midstream formulation showed a high toxicity with 96-hour LC_{50} of 0.83 mg/l. This support the report of Anderson and Prahlad (1976), who reported that diquat formulation at concentration of 0.001- 0.002 mg/L, was highly embryotoxic to *Xenopus laevis*. This 96-hour LC50 is very close to the expected environmental concentration (EEC) of 0.733 mg/l of this herbicide at the recommended application rate of 0.1-2.0 mg/L (Dial and Dial, 1987; Petterson *et al*., 1994). This means that based on this result, at the EEC concentration of 0.733 mg/L, more than 30% mortality is expected. Similarly related quaternary ammonium paraquat herbicide has also been reported with high toxicity on *Xenopus laevis* embryos at 96-hour LC_{50} of 0.670 mg/L (Osaro *et al*., 2002). It therefore seems that this herbicide is not appropriate for aquatic environment, where amphibians and other equally sensitive aquatic organisms are meant to reside.

The Arsenal formulation, was found to be slightly toxic, with 96-hour LC_{50} of 36 mg/L. But when compared to the expected environmental concentration of 0.083 mg/a.e L (US EPA, 2010), toxicity is unlikely to reach that level under normal application rates**.** The amphibian eggs will therefore be protected from acute toxicity of this herbicide at this application dosage.

In the case of Basta formulation (Glufosinate ammonium), with 96-hour LC_{50} of 2.24 mg/L, showed the moderate embyotoxicity of this herbicide to *X. laevis.*The result was consistent with the findings of Ebert *et al*. (1990), who reported that glufosinate ammonium was only slightly toxic following oral exposure in rats and dogs. And with the EEC of 1.0 mg/L, the Basta formulation, if applied correctly in aquatic habitat does not guarantee no effect at 96-hour LC_{50} of 2.24 mg/L, as concentrations spikes just after application may exceed the LC_{50} concentration. This therefore calls for concern on the application of this formulation on the safety of the sensitive embryos of aquatic organisms like amphibians.

Although the MCIG (minimum concentration inhibiting growth) endpoint has been known to vary (Bantle *et al*., 1999), the present study confirmed decreasing growth as concentration increased, for several of the herbicides. Of the three glyphosate-based formulations, only Kilo Max and Roundup showed a significant concentration dependent growth inhibition. This result support

the findings of Edginton *et al*. (2004a), who noted a concentration dependent decrease in growth rate of *X. laevis* exposed to Vision formulation of Glyphosate at concentration between 1.4 to 3.3 mg a.e /L. The Roundup and Kilo Max formulations can therefore be classified as growth disruptor. Enviro glyphosate, did not show much evidence for growth inhibition, even at very high concentrations. This result is consistent with the submission of Lanctot *et al*. (2013), that some glyphosate-based herbicide formulations present little evidence for major effects on growth and development of tadpoles.

The Midstream formulation, on the other hand showed rather strong inhibition of growth at all exposure concentrations. The control tadpoles was more than twice the average length (11.07 vs 4.98) of the exposed tadpoles, even at the lowest exposure concentration. This inhibition result support the finding of Anderson and Prahland, (1976), who noted that Diquat formulation inhibited general body growth in amphibian embryos at concentration of 0.0015 mg/L (Present MCIG = 1.0 mg/L). The fact that midstream formulation inhibited more than 100% body growth rate, at concentration below its expected environmental concentration of 1.0 mg/L (Peterson et al., 1994), suggest that this formulation is a strong growth disruptor (Anderson and Prahlad 1976; Selypes *et al*., 1980). Clearly, more research is needed to understand the mechanism of action behind the inhibition as well as investigate the potential disruption of thyroid endocrine system, known to control development in tadpoles (Kloas *et al*., 2003; Opitz *et al*., 2005; Coady *et al*., 2009).

The Arsenal formulation, in the exposure range of 30-45 mg/L revealed a concentrated dependent significant growth reduction, which showed the inhibiting potential of this formulation**.** The Arsenal formulation can therefore be classified as growth disruptor. In the case of Basta formulation (Glufosinate ammonium), there was a significant reduction in the embryo exposed to this herbicide, particularly through the range from 2.0 mg/l to 3.0 mg/l. The fact that this herbicide has an expected environmental concentration of 1.0 mg/L (Dinehart *et al*., 2010) and resulted in significant growth inhibition from 2 mg/L concentration, makes it a strong growth disruptor. Watanabe and Iwase, (1996) reported that Basta cause growth retardation at 0.1 mg/L in mice embryo cultures. Further study is needed and needs to be extended to the thyroid endocrine system, given the impacts on growth at relative low concentrations of the formulation. For this formulation, the observed malformations included axial abnormalities that encompass curve tail as well as wavy tail. There was also the incident of severe edema, and gut abnormalities.

The teratogenic potential has been widely used as one of the FETAX endpoints (Bantle *et al*., 1999; Leconte and Mouche, 2013). Bantle *et al*. (1999) also showed that it is a less variable

endpoint for non-lethal toxic effects. The fact that Leconte and Mouche (2013) showed a good correlation (85 %) with human malformations, add value to this endpoint. Bantle *et al*. (1999) suggested that a teratogenic index value larger than 1.5 would indicate positive teratogenic potential. On the other hand, Laconte and Mouche (2013) regarded TI values > 1.2 as positive dysmophogenic. In the present study, of the three glyphosate-based formulations, only exposure to enviro glyphosate (TI of 1.6) suggested positive teratogenic potential. In terms of the Laconte and Mouche, (2013) threshold TI value of 1.2, Roundup and Kilo Max could also be regarded as teratogenic at TI of 1.4 each. The marginal or low teratogenic potential of Roundup found in this study is in support of the findings of Lewinski *et al*. (2010), who reported that a concentrations range of 0.25-5 mg/L of Roundup did not induced any serious malformations during organ morphogenesis. The associated malformations observed in the three glyphosate formulation were very similar, with gut abnormalities and edema as the most dominant malformations across the three formulations. This result confirmed the findings of Lajmanovich *et al*. (2002), who reported that larval exposed to similar GLY-F formulation of glyphosate exhibited same pattern of abnormalities that include abdominal, craniofacial, eye abnormalities and bent/curved tail**.**

The teratogenic index (TI) for Midstream formulation (Diquat) was 3.5, which would be considered relatively high (TI > 1.5) (Bantle *et al*. 2013). This TI result is consistent with the findings of Osano *et al*., (2002) who reported a TI of 3.72 for Paraquat, a similarly related quaternary ammonium member. The high teratogenicity of these two herbicides may then be a trait for the members of quaternary ammonium. Midstream formulation also exhibited widespread occurrence of malformations with severe generalised edema, as the most common abnormalities. These edema abnormalities according to Osano *et al*. (2002) may be due to a disruption of osmoregulation from cell membrane lipid layer disruption. There were also occurrences of blistering, gut abnormalities, wavy tails, tail flexure and eye malformation. But the most surprising of abnormalities was the occurrence of double headed and multiple tails embryo, which according Bantle *et al*. (1990) is probably the most unusual embryo abnormalities produced in FETAX testing till date. This could be a signal of high teratogenic potency of this formulation.

Lastly, the Arsenal formulation, revealed a TI of 1.3, which is below the 1.5, Bantle *et al.* (1999) threshold but above the Leconte and Mouche (2013) TI value of 1.2, indicating a positive dymorphogenic potential. The main abnormalities observed in this herbicide included gut malformation that showed slightly improper gut coiling as well as complex improper gut formation.

Severe generalised edema was also frequently observed and may be linked to disruption in osmoregulation cause by cell membrane lipid layer disruption (Osaro *et al*., 2002).

3.5. Conclusion

In conclusion, this present study showed that Roundup, Midstream and Basta formulations are highly/moderately embryotoxic to the *Xenopus laevis*, while Arsenal, Enviro glyphosate and Kilo Max showed relative low toxicity to the *X. laevis* embryo. Kilo max, Roundup, Basta, and Arsenal formulation revealed significant growth disruption. In terms of teratogenicity, Midstream formulation showed a strong teratogenic potential, while Enviro glyphosate showed positive teratogenicity at relative high exposure concentrations.

This result showed that the alleged contribution of pesticides/chemicals contamination to the global declines of amphibian cannot be ruled out, as many of these formulations revealed toxicity, growth inhibition and teratogenicity to the challenges facing amphibians, particularly at the sensitive embryonic stages. The importance of embryotoxicity assessment at the first tier stage testing must not be underestimated, especially screening the formulation as they are applied in practice, and not just the active ingredient. This information will be valuable when planning investigation including other endpoint, for example testing the endocrine disruption hypothesis. As was the case with Roundup, if there is high toxicity or teratogenicity potential additional experimentation to investigate the effects of formulation ingredients (if known) must follow.

In South Africa, the advantages of extensive assessment of relatively large pesticides including herbicides currently been used, in the intensive agriculture practices as well as terrestrial and aquatic alien plant control, for example the Working for Water and Work for Wetlands programmes coordinated by government agencies cannot be over emphasised. Herbicide formulation selection could be done on a scientific basis and ensure limited impacts on the wildlife, especially aquatic organisms. More importantly, amphibians are not standard test animals and usually official testing only include fish and aquatic invertebrates as representative for aquatic organisms. Transferring these data to amphibians remains questionable (Wagner *et al*., 2013).

References

Anderson R. J.; Prahlad, K. V (1976). The deleterious effects of fungicides and herbicides on *Xenopus laevis* embryos. *Archives of Environmental Contamination and Toxicology* **4**(3):312-323.

American Society for Testing and Materials (ASTM) (1997). Standard guide for conducting the acute toxicity tests on aqueous ambient samples and effluents with fishes, macro-invertebrates, and amphibians. E1192-97. In: Annual book of ASTM standards. American Society for Testing and Materials, Philadelphia: 218-229.

American Society for Testing and Materials (ASTM) (1998). Standard guide for conducting the frog embryo teratogenesis assay – Xenopus (FETAX). E1439-98. In: Annual book of ASTM standards, 11.5. American Society for Testing and Materials, Philadelphia: 825-836.

Ansara-Ross, T. M.; Wepener, V.; van den Brink, P.J.; Ross, M.J (2012). Pesticides in South African fresh waters. *African Journal of Aquatic Science* **37** (1): 1-16.

Antoniou, M.; Habib M. M.; Howard C. V.; Jennings R .C.; Leifert C.; Nodari R. O.; Robinson C. J.; Fagan, J. (2012). Teratogenic effects of glyphosate-based herbicides: Divergence of regulatory decisions from scientific evidence. *J. Environ. Anal. Toxicol.* S4:006. doi:10.4172/2161-0525.

Bantle, J.; Dumont J.; Finch, R.; Linder, G. (1990). Atlas of abnormalities: A guide for the performance of FETAX. Oklahoma State Publications Department, Stillwater, OK, USA.

Bantle JA.; Finch, R.A.; Fort, D.J.; Stover, E.L.; Hull, M.; Kumsher-king, M.; Gudet-hull, A.M (1999). Phase III interlaboratory study of FETAX, Part 3: FETAX validation using 12 compounds with and without an exogenous metabolic activation system. *Journal of Applied Toxicology* **19**:447–472.

Barakat, A. O. (2004) Assessment of persistent toxic substances in the environment of Egypt. *Environment International* **30**: 309– 322.

Bernardini, G.; Vismara, C.; Boracchi P.; Camatini, M. (1994). Lethality, teratogenicity and growth inhibition of heptanol in Xenopus assayed by a modified frog embryo teratogenesis assay-Xenopus (FETAX) procedure. *The Science of the Environment* **151**: 1-8.

Bimber, D. L.; Mitchell, R. A. (1978). Effects of diquat on amphibian embryo development. *Ohio Journal of Science* **78**(1): 50-51.

Bishop, C. A.; Mahony N. A.; Strugger, J.; Ng, P.; Pettit, K. E. (1999). Anuran development density and diversity in relation to agricultural activity in the Holland Rivers watershed, Ontario, Canada. *Environmental. Monitoring and. Assessment* **57**: 21-43**.**

Blaustein, A. R.; Wake, D. B; Sousa, W. P. (1994). Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. *Conservation Biology* **8***: 60*–71.

Bru¨hl, C. A.; Pieper, S.; Weber, B. (2011). Amphibians at risk? Susceptibility of terrestrial life stages to pesticides. *Environmental Toxicology and Chemistry* **30**(11*)*: 2465–2472.

Brühl, C. A.; Schmidt, T.; Pieper, S.; Alscher, A. (2013). Terrestrial pesticide exposure of amphibians: an underestimated cause of global decline? *Science. Report.* **3**: 1135.

Brunelli, E.; Bernabo, I.; Berg, C.; Lundstedt-Enkel, K.; Bonacci, A.; Tripepi, S. (2009). Environmental relevant concentrations of endosulfan impair development, metamorphosis and behavior in Anaxyrus tadpoles. *Aquatic Toxicology* **91***:* 135-142.

Bold, T. (2007). Management Treatments Summary Guide- Aquatics. Working for Waters National Office, Cape Town. P. 7. www.wfw.co.za.

Boga, A.; Binokay, S.; Sertdemir, Y. (2009).The toxicity and teratogenicity of gibberellic acid (GA3) based on the frog embryo teratogenesis assay-Xenopus (FETAX). *Turkish Journal of Biology* ***33***: 181-188.

Boyer, R.; Grue, C. E. (1995). The need for water quality criteria for frogs. *Environmental Health Perspectives* **103**: 352–357.

Bjørling-Poulsen, M. H.; Andersen, R.; Grandjean, P. (2008). Potential developmental neurotoxicity of pesticides used in Europe. *Environmental Health* **7 (50)**: 1-22.

Dallegrave, E.; Mantese, F. D.; Coelho, R. S.; Pereira, J. D.; Dalsenter, P .R.; Langeloh, A. (2003). The teratogenic potential of the herbicide glyphosate- Roundup in Wistar rats. *Toxicology Letters* ***142***(1-2): 45-52.

Davis, K. R; Schultz, T. W; Dumont, J.N (1981). Toxic and teratogenic effects of selected aromatic amines on the embryos of the amphibian *Xenopus laevis*. *Archives of Environmental Contamination Toxicology* **10**: 371-391.

Dawson, D. A.; Fort D. J.; Smith, G .J.; Newell, D .L.; Bantle, J. A. (1988). Evaluation of developmental toxicity of nicotine and cotinine with Frog Embryo Teratogenesis Assay-Xenopus. *Teratogen Carcinogen Mutagen* **8**: 329-338.

Dawson, D. A.; Mcmcormick, C A.; Bantle, J.A. (1985). Detection of teratogenic substances in acidic mine water samples using the Frog Embryo Teratogenesis Assay-Xenopus (FETAX). *Journal of Applied Toxicology* **5**: 234-244.

Dhaouadi, A.; Monser, L.; Adhoum, N. (2010). Removal of rotenone insecticide by adsorption unto chemically modified activated Carbons. *Journal of Hazardous Materials* **181**: 692-699.

Dial, N. A.; Cherly, A.; Dial, B. (1987). Lethal effects of diquat and paraquat on developing frog embryos and 15 day-old tadpoles, *Lithobates pipien*. *Bulletin Environmental Contamination Toxicology* **38**: 1006-1011.

Diamanti-Kandarakis, E.; Bourguignon, J.; Giudice, L. C.; Hauser, R.; Prins, G. S.; Soto, A. M.; Zoeller, R. T.; Gore, A. C. (2009). Endocrine-disrupting chemicals. An endocrine society scientific statement. *Endocrine Reviews* **30**(4): 293-342.

Dirzo, R.; Raven, P. H. (2003). Global state of biodiversity and loss. Annual Review of the *Environmental and Resources* **28**: 137–167.

Dinehart, S. K.; Smith, L .M.; McMurry, S. T.; Anderson, T. A.; Smith, P. N.; Haukos, D.A. (2009). Toxicity of a glufosinate and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plain, USA. *Science of Total Environment* **407**: 1065-1071.

Downing, J. A.; Cole J. J.; Middelburg, J .J.; Striegl, R .G.; Duarte, C. M.; Kortelainen, P.; Prairie, Y. T.; Laube, K. A. (2008). Sediment organic carbon burial in agriculturally eutrophic impoundments over the last century. *Global Biogeochemical Cycles* **22**: GB1018, doi: 10.1029/2006GB002854.

Ebert, E.; Leist, K. H.; Mayer, D. (1990). Summary of safety evaluation toxicity studies of glufosinate ammonium. *Food and Chemical Toxicology* **28**: 339-349.

Edginton, A .N.; Sheridan, P. M.; Stephenson, G .R.; Thompson, D .G.; Boermans, H. J. (2004a). Comparative effects of pH and Vision herbicide on two life stages of four anuran amphibian species. *Environmental Toxicology and Chemistry* **23**(4)**:** 815–822.

Edge, C. B.; Gahl, M. K.; Pauli, B. D.; Thompson, D. G.; Houlahan, J. E. (2011). Exposure of juvenile green frogs (*Lithobates clamitans*) in littoral enclosures to a glyphosate-based herbicide. *Ecotoxicology and Environmental Safety* **74** (5): 136–139.

Egea-Serrano, A.; Relyea R. A.; Tejedo, M.; Torralva, M. (2012). Understanding of the impact of chemicals on amphibians: a meta-analytic review. *Ecology and Evolution* **2**(7):1382–1397.

Eldredge, N. (1998). Life in the Balance: Humanity and the Biodiversity Crisis. Princeton, NJ: Princeton University Press.

Emmett, K. (2002). Final Risk Assessment for Diquat Bromide. The Water Quality Program of the Washington State Department of Ecology. 02-10-046. www.wsde.org.

European Chemical Bureau, (2005). Final conclusions from the meeting of the commission working group of specialised experts in the fields of reprotoxicity- Glufosinate ammonium. European Commission Directorate General JRC Joint Research Centre Institute for Health and Consumer Protections, Unit: Toxicology and Chemical Substances. ECBI/OO2/05. www.ecb.org/research.

European Environment Agency (2011). Safe water and health water Services in a changing environment. EEA Technical Report No7, ISSN 1725-2237. Luxembourg: Publications Office of the European Union. 66pp.

Fabian, D.; Bystriansky, L.; Burkuš, J.; Rehak, B.; Legath, J.; Koppel, J. (2011). The effect of herbicide Basta 15 on the development of mouse pre-implantation embryos in-vivo and in-vitro. *Toxicology in Vitro* **25**: 73-79.

Fort, D. J.; Stower, E. L.; Norton, D. (1995). Ecological hazard assessment of aqueous soil extracts using FETAX. *Journal Applied Toxicology* **15**: 183-191.

Giesy, J. P.; Dobson, S.; Solomon, K.R. (2000). Ecotoxicological risk assessment for Roundup herbicide. *Reviews of Environmental Contamination and Toxicology* **167**: 35-120.

Govidarajulu, P. P. (2008). Review of impacts of glyphosate herbicide on amphibians: What risks can the silvicultural use of this herbicide pose for amphibians in B.C. British Columbia Ministry of Environment? Wildlife Report. No R-28.

Greulich, K.; Pflugmacher, S. (2003). Differences in susceptibility of various life stages of amphibians to pesticides exposure. *Aquatic Toxicology* **65**: 329-336.

Grisolia, C. K.; Bilich, M. R.; Formigli, L.M. (2004). A comparative toxicologic and genotoxic study of the herbicide Arsenal, its active ingredient imazapyr, and the surfactant nonylphenol ethoxylate. *Ecotoxicology and Environmental Safety* **59**:123–126.

Guillette, L. J.; Crain D. A.; Rooney, A. A.; Pickford, D. B. (1995). Organization versus activation: The role of endocrine-disrupting contaminants (EDC's) during embryonic development in wildlife. *Environmental Health Perspectives* **103**: 157-164.

Güngördü, A. (2013). Comparative toxicity of methidathion and glyphosate on early life stages of three amphibian species: *Pelophylax ridibundus*, *Pseudepidalea viridis*, and *Xenopus laevis*. *Aquatic Toxicology* **140-141**: 220-228.

Hack, R. E.; Ebert, G.; Ehling, K.; Leist, H. (1994). Glufosinate-ammonium; some aspects of its mode of action in mammals. *Food and Chemical Toxicology* **32**(5*)*: 461-470.

Hermelink, B; Urbatzka, R; Wiegand, C; Pflugmacher, S; Lutz, I; Kloas, W (2010). Aqueous leaf extracts display endocrine activities in vitro and disrupt sexual differentiation of male *Xenopus laevis* tadpoles in vivo. *General and Comparative Endocrinology* **168**: 245-255.

Hess, F. G.; Harris, J.E.; Pendino, K.; Ponnock, K. (2001). Imidazolinones. Hayes' Handbook of Pesticide Toxicology. 1853-1863.

Holmes, A. M.; Creton, S.; Chapman, K. (2010). Working in partnership to advance the 3Rs in toxicity testing. *Toxicology* **267**: 14–19.

Horvat, T.; Kalafati, M.; Kopjar, N.; Kovaˇcevi, G (2005). Toxicity testing of herbicide norflurazon on an aquatic bioindicator species – the planarian *Polycelis felina (*Daly). *Aquatic Toxicology* **73:** 342–352.

Hotchkiss, A. K.; Rider, C. V.; Blystone, C.; Wilson, V. S.; Hartig, P. C.; Ankley, G .T.; Foster, P.M.; Gray, L. E. (2008). Fifteen years after 'wingspread'- environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go. *Toxicological Sciences* **105**(2): 235-359.

Howe, C. M.; Berrilli, M.; Pauli, B. D.; Hebing, C .C.; Werry, K.; Vedhoen, N. (2004). Toxicity of glyphosate-based pesticides to four North America frog species. *Environmental Toxicology and Chemistry* **23**: 1928-1938.

Hynes, S. V.; Wilson, S.; Byrne, M. (2009). Frogs as bioindicators of chemical usage and farm practices in an irrigated agricultural area. Final report to land and water in Australia.

Johansson, M.; Piha Kylin, H.; Merila, J. (2006). Toxicity of six pesticides to common frog (*R. temporaria)* tadpoles. *Environmental Toxicology and Chemistry* **25** *(12)*: 3164–3170.

Jones, D.K.; Hammond, J.I.; Relyea, R.A. (2010). Roundup and amphibians; the importance of concentration, application time and stratification. *Environmental Toxicology and Chemistry* **29:** 2016-2025.

Jones, D.K.; Hammond, J.I.; Relyea, R.A. (2011). Competitive stress can make the herbicide Roundup more deadly to larval amphibians. *Environmental toxicology and Chemistry* **30**: 446-454.

Kloas, W.; Opitz, R.; Lutz, I. (2003). Ringtest: effects of pesticides and other chemicals on thyroid system in the amphibian *Xenopus laevis*. Federal Environmental Agency Research Report 20067409, Berlin, Germany.

Kloas, W.; Urbatzka, R.; Opitz, R.; Wurtz, S.; Behrends, T.; Hermelink, B.; Hoffmann, F.; Jagnytsch, O.; Kroupova, H.; Lorenz, C.; Neumann, N.; Pietsch, C.; Trubiroha, A.; Ballegooy, C.; Wiedermann, C.; Lutz, I. (2009). Endocrine disruption in aquatic vertebrates. Trends in comparative endocrinology and neurobiology: *Annual NewYork Academic of Science **1163***: 187-200.

Kortenkamp, A.; Martin, O.; Faust, M.; Evans, R.; McKinlay, R.; Orton, F.; Rosivatz, E. (2011). State of the art assessment of endocrine disrupters- Final report project contract number 070307/2009/550687/SER/D3.

Koyama, K.; Koyama, K.; Goto, K. (1997). Cardiovascular effects of an herbicide containing glufosinate and a surfactant: in vitro and in vivo analysis in rat. ***Tox. And App. Pharm***. **145**: 409-414.

Lanctôt, C.; Robertson, C.; Navarro-Martín, L.; Edge, C.; Melvin, S. D.; Houlahan, J.; Trudeau, V. L. (2012). Effects of the glyphosate-based herbicide Roundup WeatherMax on metamorphosis of wood frogs *(Lithobates sylvaticus*) in natural wetlands. *Aquatic Toxicology* **141**: 48– 57.

Lajmanovich, R. C.; Sandoval, M. T.; Peltzer, P. M. (2003). Induction of mortality and malformation in *Scinax nasicus* tadpoles exposed to glyphosate formulations. *Bulletin of Environmental Contamination and Toxicology* ***70***:612–618.

Lajmanovich, R .C.; Junges, C. M.; Attademo, A. M.; Peltzer, P. M.; Cabagna-Zenklusen, M. C.; Basso, A. (2013). Individual and mixture toxicity of commercial formulations containing glyphosate, metsulfuron-methyl, bispyribac-sodium, and picloram on *Rhinella arenarum* tadpoles. *Water. Air and Soil Pollution* **224**:1404.

Leconte, I.; Mouche, I. (2013). Frog embryo teratogenesis assay on *Xenopus* and predictivity compared with *in vivo* mammalian studies. *Methods in Molecular Biology* **947**: 403-421.

Liu, W.; Pusino, A.; Gessa, C. (1992). High-Perfomance liquid chromatographic determination of the herbicide imazapyr residues in water and soil. *The Science of the Total Environment* **123/124**: 39-43.

Lushchak, O .V.; Kubrak, O .I.; Storey, J. M.; Storey, K. B.; Lushchak, V. I. (2009). Low toxic herbicide Roundup induced mild oxidative stress in Goldfish tissues. *Chemosphere* ***76***: 932-937.

Mann, R. M.; Hyne, R. V.; Choung, C. B.; Wilson, S. P. (2009). Amphibians and agricultural chemicals: review of the risks in a complex environment. *Environmental Pollution* **157***:* 2903-2927.

Mann, R. M.; Bidwell, J.R (1999). The toxicity of glyphosate and several glyphosate formulations to four species of South Western Australian frogs. *Archives of Environmental Contamination and Toxicology* **36**: 193–199.

Mann, R. M.; Bidwell, J. R. (2000). Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *X. laevis* and two Australian frogs. *Aquatic Toxicology* **51**: 19-29.

Mann, R. M.; Bidwell, J. R.; Tyler, M. J. (2003). Toxicity of herbicide formulations to frogs and the implications for product registration: A case study from Western Australia. *Applied Herpetology* **1**: 13-22.

Mensah, P. K; Palmer, C. G; W .J Muller (2013). Derivation of South African water quality guidelines for Roundups using species sensitivity distribution. *Ecotoxicology and Environmental Safety* **96**: 24–31.

Mesnage, R.; Clair, E.; Spiroux de Vendomois, J.; Seralini, G. E. (2010). Two cases of birth defects overlapping the Stratton-Parker syndrome after multiple pesticide exposure. *Occupational and Environmental Medicine* **67**(5*)*: 359.

Meinhardt, H. R. (2008). Evaluation of predictive models for pesticide behaviour in South African soils.Unpblished PhD thesis, University of the North-West, South Africa.

Morgan, M. K.; Scheuerman, P. R.; Bishop, C. S.; Pyles, R. A. (1996). Teratogenic potential of atrazine and 2, 4-D using FETAX. *Journal of Toxicology and Environmental Health* **48**: 151-168.

National Toxicology Program Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM) (2000). Frog Embryo Teratogenesis Assay- Xenopus (FETAX) - Background Review Document.

Nieuwkoop, P.D.; Faber, J. (1994). *Normal Table of Xenopus laevis (Daudin)*, 3rd ed. Garland, New York, NY, USA.

Opitz, R.; Braunbeck, T.; Bögi, C.; Pickford, D .B.; Nentwig, G.; Oehlmann, J.; Tooi, O.; Lutz, I.; Kloas, W. (2005). Description and initial evaluation of a Xenopus metamorphosis assay for detection of thyroid system-disrupting activities of environmental compounds. *Environmental Toxicology and Chemistry* **24***:* 653-664.

Organisation for Economic Cooperation and Development (OECD) (2004). Environment Health and Safety Publication Series on Testing and Assessment No 46. ENJ/JM/MONO 17. www.oecd.org/document.

Organisation for Economic Cooperation and Development (OECD) (2007).Validation of the amphibian metamorphosis assay as a screen for thyroid-active chemicals: Integrated summary report. AMA integrated summary report. www.oecd.org/document.

Organisation for Economic Co-operation and Development (OECD) (2008). Series on Testing and Assessment. No. 91. Report of the Validation of the Amphibian Metamorphosis Assay (PHASE 3) ENV/JM/MONO/18. www.oecd.org/document.

Orton, F.; Lutz, I.; Kloas, W.; Routledge, E. J. (2009). Endocrine disrupting effects of herbicides and pentachlorophenol: In-vitro and in-vivo evidence. *Environmental Science and Technology* **43**: 2144-2150.

Osano, O.; Oladimeji, A. A.; Kraak, M. H. S.; Admiral, W. (2002). Teratogenic effects of Amitraz, 2, 4-dimethylaniline, and Paraquat on developing frog (*Xenopus*) embryos**.** *Archives of Environmental Contamination Toxicology* **43**: 42–49.

Othman, M. Z.; Ding, L.; Jiao, Y. (2009). Effect of anionic and non-ionic surfactants on activated sludge oxygen uptake rate and nitrification. World Academy of Science, Engineering and Technology 58. www.was.org.

Paganelli, A.; Gnazzo, V.; Acosta, H.; Lopez, S. L.; Carrasco, A. E. (2010). Glyphosate-Based herbicides produce tetratogenic effects on vertebrates by impairing retinoic acid signalling. *Chemical Research in Toxicology* **23**: 1586-1595.

Paggetti, E.; Biaggini, M.; Corti, C.; Lebboroni, M.; Berti, R. (2006). Amphibians and reptiles as indicators in mediterranean agro-ecosystems: A preliminary study. Proceedings of the 13th Congress of the Societas Europaea Herpetologica. 107-110.

Pašková, V.; Hilscherová, K.; Bláha, L. (2011). Teratogenicity and embryotoxicity in aquatic organisms after pesticide exposure and the role of oxidative stress. In D.M. Whitacre (ed.), *Reviews of Environmental Contamination and Toxicology* **211**: 25-61.

Peterson, H. G.; Boutin, G.; Martin, P. A.; Freemark, K. E.; Ruecker, N. J.; Moody, M. J. (1994). Aquatic phyto-toxicity of 23 pesticides applied at expected environmental concentrations. *AquaticToxicology* **28**: 275-292.

Piola, L.; Fuchs, J.; Oneto, M. L; Basack, S; Kesten, E; Casabe, N. (2013). Comparative toxicity of two glyphosate-based formulations to *Eisenia andrei* under laboratory conditions. *Chemosphere* **91**: 545-551.

Qian, H.; Chen, W.; Sheng, G.D; Xu, X.; Liu, W.; Fu, Z. (2008). Effects of glufosinate on antioxidant enzymes, subcellular structure, and gene expression in the unicellular green alga *Chlorella vulgaris*. *Aquatic Toxicology* **88**: 301–307.

Relyea, R. A.; Devin, K. J. (2009). The toxicity of Roundup Original Max to 13 species of amphibians larval. *Environmental Toxicology and Chemistry* **28**(9): 2004–2008.

Relyea, R.A. (2003). Predator cues and pesticides: a double dose of danger for amphibians. *Ecological Applications* **13**: 1515–1521.

Relyea, R.A. (2006). The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities – response. *Ecological Applications* **16**:2027–2034.

Richard, S.; Moslemi, S.; Sipahutar, H.; Benachour, N.; Seralini, G.E (2005). Differential effects of glyphosate and Roundup on human placental cells and aromatase. *Environmental Health Perspective* **113**:716–720.

Savitz, D. A.; Arbuckle, T.; Kaczor, D.; Curtis K .M (1997). Male pesticide exposure and pregnancy outcome. *American Journal of Epidemiology* **146**(12): 1025-1036.

Selypes, A.; Nagymojtenyi, L.; Berensi, G. (1980). Mutagenic and embryotoxic effects of Paraquat and Diquat. *Bulletin of Environmental Contamination and Toxicology* **25***:* 513-517.

Sewalk, C. J.; Brewer, G. L.; Hoffman, D. J. (2000). Effects of Diquat, an aquatic herbicide, on the development of mallard embryos. *Journal of Toxicology and Environmental Health Part A,* **62** (1*): 33-45.*

Schreiber, A .M.; Das, B.; Huang, H.; Marsh-Amstrong, N.; Brown, D. D. (2001). Diverse developmental program of *Xenopus laevis* metamorphosis are inhibited by a dominant negative thyroid hormone receptor. *Developmental Biology* **98**(19*): 10739-10744.*

Schuytema, G. S.; Nebeker, A. V.; Griffis, W. L. (1994). Toxicity of guithion and guthion 2S to *X. laevis* embryos. *Archives Environmental Contamination and Toxicology* **27***:* 250-255.

Sihtmäe, M.; Blinova, I.; Künnis-Beres, K.; Kanarbik, L.; Heinlaan, M.; Kahr, A. Ecotoxicological effects of different glyphosate formulations. *Applied Soil Ecology* **72**: 215–224.

Takahashi, M. (2007). Oviposition site selection: Pesticide avoidance by gray tree-frogs. *Environmental Toxicology and Chemistry **26*** (7*)*: 1476-1480.

Tebourbi, O.; Sakly, M.; Rhouma, K.B. (2011). Molecular mechanisms of pesticide toxicity**.** Pesticides in the modern world– pest control and pesticides exposure and toxicity assessment. In "Pesticides in the modern world pests control and pesticides exposure and toxicity assessment" (Stoytcheva, M., Ed.). Tech Publishing, Croatia, 297-332.

Tietge, J. E. G.; Ankley, T.; Defoe, D. L.; Holcombe, G. W.; Jensen, K. M. (2000). Effects of water quality on development of *Xenopus laevis*: A frog embryo teratogenesis assay—*Xenopus* assessment of surface water associated with malformation in native anurans. *Environmental Toxicology and Chemistry* **19** (8*):* 2114–2121.

Tomlin, C. D. (2006). *The pesticide manual: A world compendium* (14th Ed.). Hampshire, UK: British Crop Protection Council.

Trumbo, J. (2005). An Assessment of the hazard of a mixture of the herbicide Rodeo and the non-ionic surfactant R-11 to aquatic invertebrates and larval amphibians**.** *California Fish and Game* **91**(1*)*: 38-46.

United State Environmental Agency (USEPA), (1999). LC50 Software Program, Version 1.00. Center for Exposure Assessment Modelling (CEAM) Distribution Center. USA EPA, Washington, USA. PA, http://www.epa.gov/ncea/bmds/index.html. Accessed April 2013.

United State Environmental Agency USEPA, (2014). Probit software 3.3. USA EPA, Washington, USA. PA, http://www.epa.gov/ncea/bmds/index.html. Accessed April 2013.

Venturino, A.; Rosenbaum, E.; Caballero De Castro, A.; Anguiano, O. L.; Gauna. L.; De Schroeder, T. F.; De D'Agelo, M. P. (2003). Biomarkers of effect in toads and frogs. *Biomarkers* **8**(3-4)*:* 167-186.

Vitt, L. J.; Caldwell, J. P.; Wilbur, H. M.; Smith, D.C. (1990). Amphibians as harbingers of decay. *Bioscience* **40**: 418.

Wagner, N.; Wolfram, R.; Hanka, T.; Beatrix, T.; Stefan, L. (2013). Questions concerning the potential impact of glyphosate-based herbicides on amphibians. *Environmental Toxicology and Chemistry* **32**: 1688–1700.

Washington State Department of Agriculture (WSDA) (2003). Ecological risk assessment of the proposed use of the herbicide imazapyr to control invasive cordgrass (*Spartina spp) www.ecy.wa.gov. Accessed on 24 October 2010.*

Washington State Dept. of Agriculture (WSDA) (2009). Human health and ecological effects Imazapyr risk assessment, Washington State. www.ecy.wa.gov. Accessed on 24 October 2010.

Watanabe, T.; Iwase, T. (1996). Developmental and dysmorphogenic effects of glufosinate ammonium on mouse embryo in culture. *Teratogenesis, Carcinogenesis and Mutagenesis* **16**: 287-299.

World Health Organization (WHO), (2004). Diquat in drinking water. Background document for development of WHO guidelines for drinking-water quality. World Health Organisation, WHO/SDE/WSH/03.04/91. www.who.org/document.

Williams, B.K.; Semilitsch, R, D (2010). Larval responses of three Mid-western anurans to chronic, low dose exposure of four herbicides. *Archives of Environmental Contamination and Toxicology* **58:** 819-827.

Wilson, E. O (1992). The diversity of life. Cambridge, MA: Harvard University Press.

WWF-CHEM Trust (2010). Protecting future generations by reducing exposure to endocrine disruptors. CHEM Trust and WWF-EPO proposals for the regulation of chemicals with endocrine disrupting properties under REACH (EC 1907/2006) and under the Plant Protection Products Regulation (EC No 1107/2009).

Yadav, S.S.; Giri, S.; Singha, U.; Boro, F.; Giri, A. (2013). Toxic and genotoxic effects of Roundup on tadpoles of the Indian skittering frog (*Euflictis cyanophlyctis*) in the presence and absence of predator stress. *Aquatic Toxicology* **132– 133**: 1– 8.

Yu, S.; Wages, M. R.; Cai, Q.; Maul, J. D.; Cobb, G. P. (2013). Lethal and sublethal effects of three insecticides on two developmental stages of *Xenopus laevis* and comparison with other amphibians. *Environmental Toxicology and Chemistry* **32**: 2056-2064.

Chapter Four

Potential thyroid system disruption by six selected herbicide formulations using the Xenopus Metamorphosis Assay (XEMA).

4.1 Introduction

Accumulating evidence is showing the increasing contribution of agrichemicals to endocrine disrupting activities in receiving aquatic environments (Mnief, 2011; Bergman *et al*., 2013), which cut across the agrichemicals including insecticides, herbicides and fungicides. Many of these pesticides along with other man-made chemicals are today encountered in various compartments in the environment, including ground and surface water, widely used for wildlife and human consumption (Plakas and Karabelas, 2012).

In reaction to the concerns raised about the possibility of environmental chemicals acting as disruptors of endocrine pathways or interactions of endogenous hormones with their respective receptors, organizations like USEPA (USE-PA, 2009) OECD (OECD, 2006) suggested a two tiered

screening and testing programme. The Tier 1 screening battery consist of eleven separate bioassays, mostly single cell (in vitro) assays, but also including two assays using tadpoles or fish (in vivo) tests. Through weight of evidence, following testing at this level, a chemical may continue to be tested at the tier 2 level, mostly including in vivo testing. The Xenopus Metamorphosis Assay (XEMA or AMA) is performed in an aquatic situation as a 21-day exposure assay to evaluate environmental chemicals specifically for potential interaction with the hypothalamus-pituitary-thyroid (HPT) pathway.

Although the initial focus of the endocrine disruptor screening activity was on potential interaction with the reproductive system, including both females and male steroidogenic pathways and target-tissue receptors, recently, increasing concern that many of these pesticides and chemicals may interfere with the normal function of the hypothalamic-pituitary-thyroid (HPT) axis (OECD, 2006; Bergman *et al*., 2013; Heindel *et al*., 2013). Currently, several researches link exposure impacts of the many pesticides and several industrial chemicals to thyroid hormone (TH) disruption (Cauble and Wagner, 2004; Opitz *et al*., 2009; Brande-Lavridsen *et al*., 2010; Helbing *et al*., 2011; Boggs *et al*., 2013; WHO, 2013).

Thyroid hormone (THs) are essential for normal growth and development in all vertebrates (OECD, 2006; Jobling *et al*., 2013). More than any other hormone, THs influence the activity of wider varieties of tissues and biological functions including osmoregulation, metabolism, and post-hatch metamorphosis in fish and amphibian (Blanton and Specker, 2007). The synthesis and regulation of THs release, delivery to tissues and cells as well as transformation to different forms and interaction with target tissue receptors, during development and in the adult, represent a complex system including an extended network of feedback systems (Norris and Carr, 2006; Fort *et al*., 2007; Jobling *et al*., 2013). In amphibians, THs are required for necessary remodelling in transforming a basic aquatic organism into a terrestrial one (metamorphosis). In fact, it is now well known that the role and importance of thyroid hormones in amphibian development is unparalleled in any other vertebrate group (Norris and Carr, 2006). It is therefore not surprising, that metamorphosis received so much attention as biomarker system for thyroid system disruption in a fully aquatic species like the Africa clawed frog, *Xenopus laevis*.

The fact that environmental chemicals could affect thyroid axis at multiple sites including; the central nervous system (CNS), pituitary, the thyroid glands, during TH transport, during TH metabolism and elimination as well as in peripheral tissues (OECD, 2007), makes thyroid disruption crucial to the development and survival of the organisms (Kortenkarp *et al*., 2011). The realization

of the pivotal regulatory role of THs during amphibian metamorphosis, led to the selection of the amphibian thyroid system as an in vivo model to screen for thyroid disrupting activity (OECD, 2006; Fort *et al*., 2007). The aim being to capture the integration of numerous potential points of modulations within the thyroid endocrine system that may be disrupted during exposure to thyroid toxicants. The USA-EPA's DRP reviewed several anuran species candidate and recommended that the African Clawed Frog *X. laevis* be used in metamorphosis assay (Xenopus Metamorphosis Assay) (Degitz *et al*., 2005; Fort *et al*., 2007).

Unlike the insecticide and fungicide pesticides, less attention has been relatively paid to the herbicides, as endocrine disruptors. Yet, more than 21% of the pesticides that have been implicated as endocrine modulators are herbicides (Mnif, 2011). The atrazine experience, particularly in relation to thyroid, reproductive and endocrine disruption generally, also increased the research attention on the herbicidal group. The atrazine has generated widespread controversy based on conflicting scientific report from ecological and physiological impact (Hayes *et al*., 2002; Hayes *et al*., 2006; Kloas *et al.*, 2009). In this conflicting scientific reports, several researchers have shown numerous endocrine disrupting properties (Hayes *et al*., 2003; Coady *et al*., 2005; Hotchkiss *et* al., 2008; Lenkwoski *et al*., 2008), while some others reports have shown that atrazine have not shown consistent endocrine disrupting properties (Preez *et al*., 2008; Kloas *et al*., 2009). Another herbicide with conflicting scientific results is Roundup. While several researchers on Roundup have shown high toxicity as well as endocrine disrupting potential (Benachour and Seratoni, 2009; Paganelli *et al*., 2010), some other reports have shown inconsistent results. The general lesson from these two herbicide formulations has taught scientists to always be on the side of caution as regards the ecological and endocrine disrupting properties of pesticide substances.

Several studies have linked herbicides formulations to thyroid disruption: including tributylin (Fort *et al*., 2004; Shi *et al*., 2012), Glyphosate (Cauble and Wagner, 2005**),** atrazine (Hayes *et al*., 2003; Hayes *et al*., 2006; Rohr and McCoy, 2010). Case studies coming specifically from mammals as well as non-mammalian vertebrates are also growing exponentially (Kidd *et al*., 2013).

Although atrazine has been focus in some studies in South Africa, but the health and ecological impact of several other commonly applied herbicide formulations, particularly used locally in South Africa are still unknown, particularly on the thyroid system. Similar to the other part of the world, several herbicide formulations are used in agricultural, forestry and garden across the country, as well as eradication of alien plants in rivers catchment zones (Bold, 2007).

Linked to continuous deterioration of aquatic conditions, amphibian global population are thought to be declining at a rapid rate (Stuart *et al*., 2004; Marllatt *et al*., 2012). This hypothesized global amphibian decline have been widely linked to increasing volumes of pesticides in the environment (Oka *et al*., 2008; Shi *et al*., 2012), potentially, through disruption of several physiological systems including the thyroid and reproductive systems. Because amphibians undergo complex and well-orchestrated morphological changes during their metamorphosis, that are controlled by hormones, thyroidal and adrenal, respectively (Marllatt *et al*., 2012), insult on these thyroid gland results in disruption of metamorphosis.

South Africa is recognised as one of the main food-producing countries in Africa, and as such, also recognized as the pesticides market hub in the sub-Saharan African region (Bollmohr *et al*., 2008; Ansara-Ross *et al*., 2013; Dabroski *et al*., 2014). Moreover, a governmental employing programme aimed at the eradication of alien plants from River catchment areas, the Working for Water (WfW) program, utilize several herbicide formulations as part of the chemical control program, for example, glyphosate formulations (Roundup, Kilo Max, Enviro glyphosate), diquat dibromide (Midstream), glufosinate ammonium (Basta) and imazapyr (Arsenal) among others **(**Bold, 2007)**.** Although toxicological thresholds for these chemicals are known, potential endocrine modulation effects on aquatic vertebrates, happening at low, mostly non-lethal concentrations remains largely unknown.

Imazapyr is one of the most widely used herbicide active ingredients in the world. Imazapyr is essentially stable to hydrolysis, aerobic and anaerobic soil degradation, as well as aerobic and anaerobic aquatic metabolism (EPA, 1995). Hence, it is very mobile and persistent, with half-life of around 50 months on the soil surface (Vizantinopoulon and Lolos, 1994). Tatum *et al*. (2010) reported the expected environmental concentration to be 0.92mg/L, but according to Washington State Department of Agriculture (WSDA) (WSDA, 2003), concentration of up to 5.7 mg/L have been measured in surface water and treated sediment. WSDA, (2003) concluded that Imazapyr has not been thoroughly tested for chronic or sub-lethal effects on wide variety of aquatic organisms. Well-known formulations of Imazapyr include Arsenal, Chopper and Format. The Arsenal formulation, selected for the present study, consists of 25 g/L Imazapyr, 186 g/L of ammonium hydroxide, 18 g/L of nonyl phenol ethoxylate (9 ethoxylated ring) and water (Grisolia *et al*., 2004).

Diquat dibromide (9, 10-dihydro-8a, 10a-diazonia phenanthrene ion) is used as a post emergent herbicide. It is a non-selective contact herbicide and crop desiccant that is also used in aquatic weeds control programmes (Emmett, 2002; WHO, 2004). It is widely used in the United

State, North America, Europe, Australia, Japan and Africa (Emmett, 2002; WHO, 2004). The expected environmental concentration of diquat dibromide is 0.073 mg/L (Peterson *et al*., 1994). One of its commonly known formulations is Midstream containing nonyl phenol ethoxylate as surfactant, also implicated to have estrogenic activity (Trumbo, 2005; Othman, 2009).

Glufosinate ammonium (N-phosphonomethyl) glycine) is a broad spectrum and systemic herbicide (Ebert *et al*., 1990; Hack *et al*., 1994). Owing to the structural analogy of glufosinate ammonium to glutamate, it acts as an irreversible inhibitor of gluthamine synthatase activity in different tissues that often lead to slight increases of gluthamate and ammonia (Hack *et al*., 1994). The expected environmental concentration of glufosinate is 1.0 mg/L (Dinehart *et al*., 2010). Glufosinate ammonium formulations include Basta, Rely, Finale and Challenges. Basta formulation used in the present study, contains 18.5% glufosinate ammonium and 30% of sodium polyoxyethylene alkylether sulphate (AES) surfactant (Koyama *et al*., 1997).

Glyphosate *(N*-(phosphonomethyl) glycine) is a broad-spectrum, non-selective, post-emergence herbicide (WHO, 1994; 1996). It is perhaps the most important and best-selling herbicide ever produced (Mann *et al*., 2009; Gungordu *et al*., 2013; Wagner *et al*., 2013; Yadav *et al*., 2013). The increasing global use of glyphosate herbicide formulations has generated widespread concern on their potential adverse effects in aquatic ecosystem (Perez *et al*., 2011; Mensah *et al*., 2013; Yadav *et al*., 2013; Lanctot *et al*., 2013). The expected environmental concentration of glyphosate is 2.85 mg/L, but the highest reported environmental concentration is 1.7 mg/L (Homer, 1990; Govindarajulu, 2008). Most glyphosate-based herbicides are manufactured under varying trade names such as Roundup Original, Roundup WeatherMax, Vision, VisionMax, Rodeo, Touchdown and Glyfos etc. (Lanctot *et al*., 2013). These Glyphosate based-herbicides are either applied with or without surfactant. Roundup (included in the present study) is the most common glyphosate formulation consist of isopropylamine salt along with the surfactant polyethoxylated tallow amine (POEA). For Kilo Max (used in the present study), according to the MSDS, is a glyphosate formulation with sodium salt and an undisclosed surfactant. Another Glyphosate formulation in the present study is Enviro glyphosate, which contains isopropyl ammonium salt and polyethylene alkylamine as surfactant. Research evidence are mounting suggesting that the increased use of glyphosate may be linked to high mortality, gonadal malformation, disruption in growth and developmental rate in amphibians (tadpoles) (Williams and Semlitsch, 2010; Jones *et al*., 2010; 2011, Relyea 2012), resulting in alteration of aquatic

biodiversity as well as ecosystem functions and services (Valencia- Aguilara *et al*., 2012; Yadav *et al*., 2013).

The present study therefore assessed the potential impact of the six selected herbicide formulations including Midstream (diquat dibromide), Basta (glufosinate ammonium) and Arsenal (imazapyr) as well as new glyphosate formulations including Kilo Max and Enviro Glyphosate alongside the established Roundup formulation on the thyroid endocrine system, using the established amphibian metamorphosis assay that utilise African clawed frog, *Xenopus laevis* as model species (XEMA protocol).

4.2 Materials and Methods

4.2.1 *Test chemicals*

The herbicide formulations include the following: Arsenal (imazapyr) (BASF Chemical Ltd., South Africa), Basta (glufosinate ammonium) (Bayer Crop Science AG Ltd., Germany), Midstream (diquat dibromide) (Syngenta Ltd., South Africa), Roundup (glyphosate) (Monsanto Ltd., South Africa), Enviro Glyphosate (glyphosate) (Enviro Industries Ltd., South Africa) and Kilo Max (glyphosate) (Volcano Agro-science Ltd., South Africa).

4.2.2 *Xenopus laevis* breeding and tadpole care

From a *X. laevis* breeding stock, mature adult males and females were maintained separately in 15 L glass tanks containing buffered (2.5 g sea salt/10 L) reverse osmosis water, and were fed three times per week with fish pellets (Aqua-Nutro, South Africa). Tanks were cleaned and refilled with clean water after feeding. Breeding induction was performed according to ASTM, (1998) protocol. Males and females were primed with 100 IU human Chorionic Gonadotropin (hCG) (Merck Ltd Germany), injected into their dorsal lymph sac, four days prior to mating, followed by a second treatment just prior to the mating, of 100 IU and 300 IU hCG to males and females respectively. Single male and female pairs were placed together in 15 L exposure tanks lined with plastic netting (to ensure separation of the eggs from the adults during oviposition), and place in a well-ventilated dark place. Theoretically, amplexus occurred within four hours and eggs were deposited within 12 hours after injection. All the eggs and tadpoles staging were done using a normal developmental

atlas by Nieuwkoop and Faber (1994) (referred to as NF-stages). The eggs were collected and then spread out into several well aerated 15 L tanks at the density of 50 tadpoles per 10 L water. Feeding commenced once the tadpoles reached free swimming NF-stage 47-48, and they were fed two times per day until they reached NF-stage 51. Breeding and tadpoles maintenance operating procedures were approved by the Animal Research Ethical Committee (AREC) of the Stellenbosch University (Approval no- SU-ACUM 12-00015).

4.2.3 *Test procedure*

4.2.3.1. Exposure set-up

Newly hatched tadpoles were distributed into several 15 L tanks at density of 40 tadpoles per tank, to reduce growth effects of overcrowding. Tadpoles were fed a powdered algae mixture, Sera Micro (Sera, Heinsberg, Germany), until they attained NF stage 51. At NF-stage 51, twenty premetamorphic tadpoles were randomly selected from holding glass tanks and transferred to 15 L exposure tanks. Individual exposure tanks were replicated twice at each of the selected concentrations. The exposures were done under controlled climate conditions according to the XEMA experimental protocol (OECD, 2008), the following physical conditions were applied including water temperature at 23 ± 1 ºC, pH ranging between 7.5 - 8.5, ensured dissolve oxygen of >3.5 mg/L exposed to a 12 hours of light and dark photoperiod ($L_{12}D_{12}$) regime. The tadpoles were fed Sera Micron (Sera Heinsberg, Germany) from 30 mg/animal/day initially and later increased to 50 mg/animal/day in other to account for the increased in growth (OECD, 2008).

4.2.3.2. *Exposure Concentrations*

The selected herbicide exposure concentrations were centred on relevant and low environmental relevance concentration (ERC) or 96-hour LC_{50} for NF-stage 48 or NF-stage 60 of the *X. laevis* tadpoles (see Table 2.2 in Chapter two). Mortality in tanks was monitored every day while the exposure medium was completely replaced every third day (Monday, Wednesday and Friday). Only mortality incidence less than 10% in the control group was accepted for the experiment to continue (OECD, 2008).

Table 4.1: The selected exposure concentrations (centred on the 96-hour LC_{50} for NF-stage 48 / 60 of *X. laevis* tadpoles) of the six selected herbicide formulations.

Formulation	Exposure conc. (mg/a.e./L)	Based on LC50 /ERC
Arsenal	0, 0.5, 2.0, 3.5	Low ERC of 5.77 mg/L
Basta	0, 0.05, 0.15 and 0.25	Low ERC and LC_{50} of 0.59 mg/L at NF-stage 48
Midstream	0, 0.05, 0.11 and 0.14	Low ERC and LC_{50} of 0.2 mg/L at NF-stage 48
Roundup	0, 0.2, 0.4 and 0.6	Low ERC and LC_{50} of 0.89 mg/L at NF-stage 48
Environ Glyphosate	0, 9, 19 and 28	LC_{50} of 102 mg/L at NF-stage 48
Kilo Max	0, 90, 190 and 280	LC_{50} of 455 mg/L at NF- stage 60

1

4.2.4. Autopsy procedure and morphometric measurements.

At the end of the 21-days period, the exposures were terminated. The tadpoles were carefully collected and euthanized in 0.1 % benzocaine. Tadpoles were blotted dry and individually weighed (to nearest 0.01 g), and snout–vent length (SVL) (to nearest 0.1 mm) recorded and fixed in Davidson's solution for 72 hours prior to being transferred to, and preserved in 4 % neutral buffered formalin (OECD, 2007**;** Shi *et al*., 2012). The fore-limb length (FLL) and hind-limb length (HLL) were measured using Leica EZ4D stereo microscope (Leica Microscope Ltd, Germany) (to nearest 0.1 mm). Digital photographs of the tadpoles were used to determine hind-limb length by using the metric trace ruler which has the capacity to measure both straight line and curved line using traced lines. The heads of tadpoles containing the lower jaws, and the thyroid glands, were carefully severed transversely using a sharp blade, just posterior to the eye, and subjected to routine (paraffin wax imbedding) histological procedures (Bancroft and Steven, 1977). Sectioning, mounting and staining then followed (see 4.2.6).

4.2.5. Developmental Stage (NF-stage) determination

Five tadpoles per tank (at similar stage) were selected at each concentration, and were compared to the median developmental stage of the control for histopathological examination of the thyroid gland.

4.2.6. Histological Procedures.

The lower jaw samples containing the thyroid glands were removed from the formalin, washed in running tap water and processed for routine paraffin wax-based histology (Bancroft and Steven, 1977). The lower jaws were dehydrated in a series of graded alcohol and embedded (in frontal plane to facilitate the caudal surface of the tissue first) in histowax (Histolab Product, Sweden). The embedded tissue were sectioned at 7-8 µm using Reichert-Jung microtome (Cambridge Instrument, Germany), the sections mounted on clean, albumin coated glass slides and oven-dried (40 ^{0}C) overnight. The section were subsequently dewaxed, stained with haematoxylin and eosin (H & E) (Bancroft and Stevens, 1977), cleared in xylene before mounting glass cover slips using a resin-based mounting medium (DPX, Sigma Ltd).

4.2.7 Histological measurement of the thyroid.

Using the right-side thyroid, the thyroid image (Leica DMLB microscope equipped with digital camera (Leica Microscope Ltd, Germany) was used to measure the epithelia cell heights by taking measurement from the base to the apical edge of the cell. The epithelia cell height of four cells were randomly measured, producing 60 cell-height measurement per individual. A mean value was then calculated per individual and used with other individual group members to calculate a group mean for follicle cell height. Follicular cross sectional area (follicle lumen area), as well as thyroid cross sectional area were also measured and calculated (using image analysis software (Sigmascan, Systat Software Inc.) using the right thyroid gland side of each tadpoles by measuring cross sectional area of all the serial sections and then summed. Ten follicles in each section were measured in each section, making ten thyroid follicle in each tadpoles. The data was then combined for all of the tadpoles in each exposure group. The averages of sum total of the cross-sectional areas give the gland area for each exposure group.

4.2.8 Data analysis

The non-parametric Kruskal-Wallis test was used to assess variation in median NF-stage among exposure groups (since developmental stage constitutes ordinal data), followed by Dunn's multiple comparison test (DMCt) to identify significant pairwise differences in stages (Shi *et al*., 2012). Normality and homogeneity of variance in wet body mass (WBM), whole body length (WBL) and snout to vent length (SVL) data were analysed using Shapiro-Wilk's and Levene's tests respectively. One way ANOVA or Kruskal-Wallis ANOVA test (K-W ANOVA) for non-parametric data was subsequently used to analyse variations among exposure groups. Front limb

length (FLL) and hind limb length (HLL) were normalized to snout vent length in order to correct for the effect of growth (or size related NF stages) (Coady *et al*., 2010). Normality of the normalized FLL and HLL was evaluated using residuals' normal probability plots and the Shapiro-Wilks test, whereas Levene's test was applied to test for homogeneity of variance. The effect of treatment (i.e. specific pesticide concentration), developmental stage and the treatment stage interaction on FLL and HLL was tested using mixed model ANOVA, with individual tadpole as random factor. Pairwise differences in WBM, WBL, SVL and normalized FLL and HLL between pesticide treatments and the control groups were assessed using the Tukey HSD test with Spjotfoll/Stoline correction for parametric data or the Dunn's test for non-parametric data. Significant differences between treatments were taken at $P<0.05$. All statistical analyses were performed using Statistica V12 (Statsoft Inc., USA).

4.3. Results

4.3.1 Arsenal formulation (imazapyr)

4.3.1.1. Mortality

There was no incidence of mortality recorded in all the exposure tanks as well as the control tanks, throughout the 21-days exposure period.

4.3.1.2. Variation in Developmental Stages

After 21-day exposure, the NF developmental stages of the tadpoles exposed to this formulation ranged between NF-stage 55 through 65 (Table 4.2). The frequency distribution of the NF stages recorded after exposure (Figure 4.1) showed varied response in development compared to the control (Figure 4.1 a-d). The control exposure for Arsenal formulation showed relative wide frequency distribution comprised of NF-stage 58 through NF-stage 65, with a median stage of NF-stage 60. Relative to the control, even though the median stage remain at the same NF-stage 60 at all exposure concentrations, the development was totally arrested at under NF-stage 61 at all concentrations except in concentration 2 mg/L.

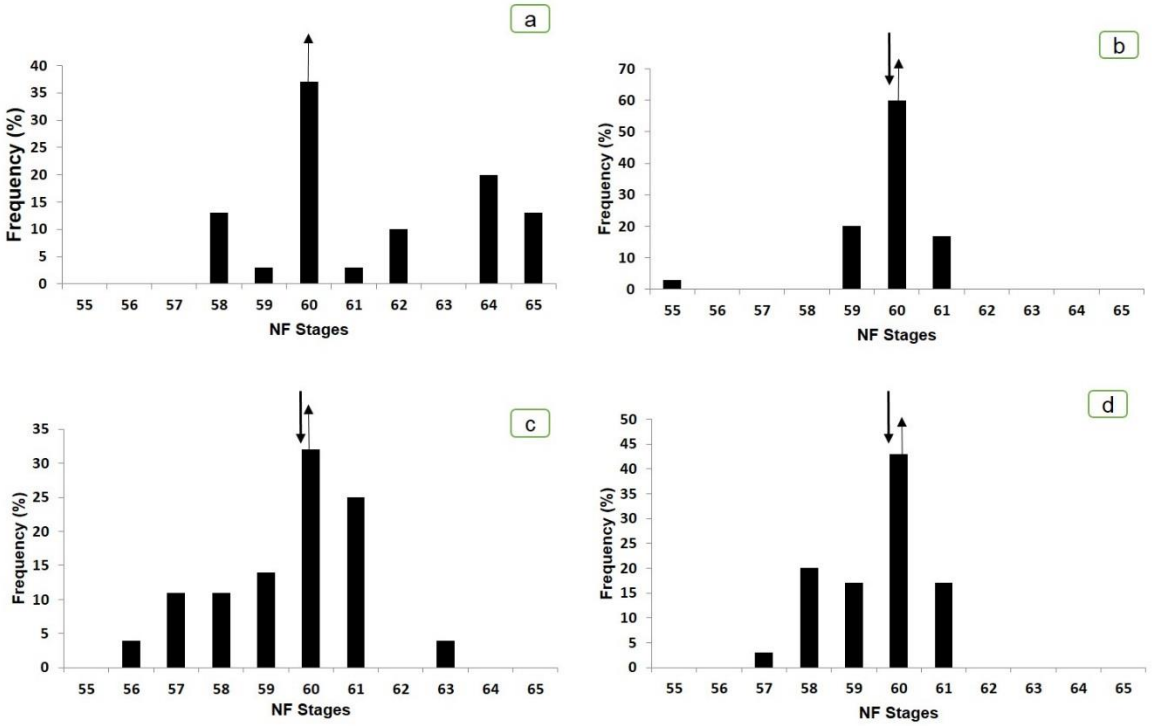

Figure 4.1**:** The frequency distributions (n= 30) of developmental stages (Nieuwkoop and Faber, 1958) attained by *X. laevis* tadpoles exposed after a 21-day exposure period to clean water (control) (a) and graduated concentrations of Arsenal formulation from Concentration 0.5 mg/L (b), 2 mg/L (c), and 3.5 mg/L (d). The upward arrow show the median at the control relative to downward arrow show the median attained in the various concentrations.

Kruskal-Wallis ANOVA test confirmed variation in NF-stage development among the exposed tadpoles. Multi-comparison analysis showed that, relative to the control treatment, the delay in development was significant at the highest exposure concentration of 3.5 mg/L (DMCt; $P < 0.05$) relative to the control treatment (Fig. 4.2).

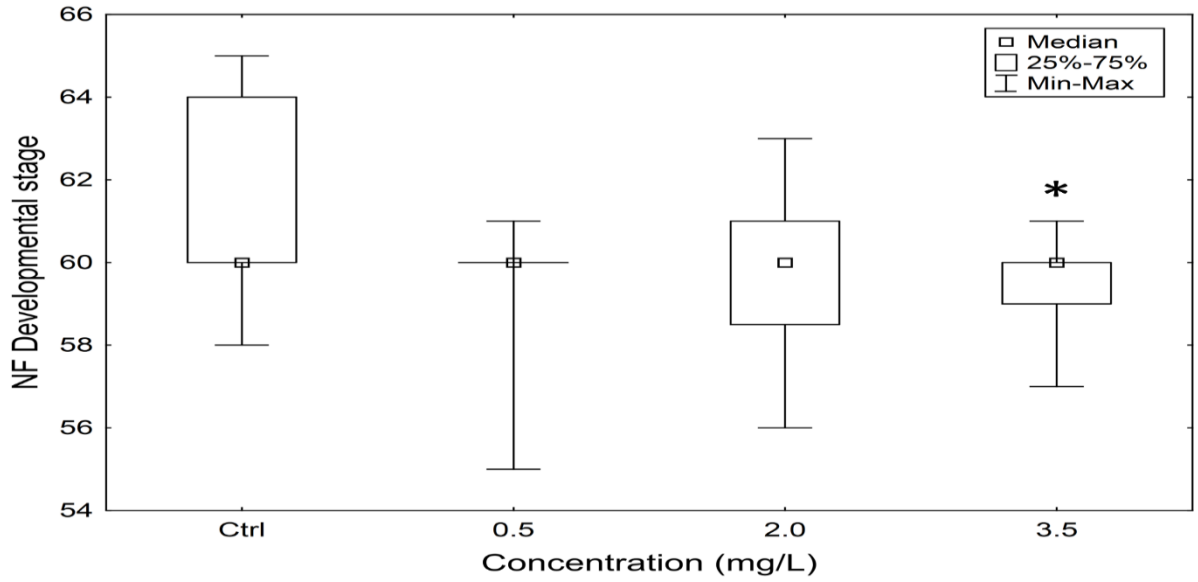

Figure 4.2: The NF-stage differentiation of *X. laevis* after 21-days exposure to a range of concentrations of the Arsenal formulation compared to a control (Ctrl) group. Asterisks indicate significant difference (DMCt, $P < 0.05$) from the control.

4.3.1.3. Whole body length (WBL) and Snout-vent length (SVL)

The exposure impact of the Arsenal formulation showed an increasing mean WBL trend (Fig 4.3a) and SVL (Fig 4.3b) of the treated tadpoles relative to their control group. However, Kruskal-Wallis ANOVA test showed no significant variation (K-W; $P>0.05$) in WBL and SVL among all exposure concentrations as compared to the tadpoles in the control group.

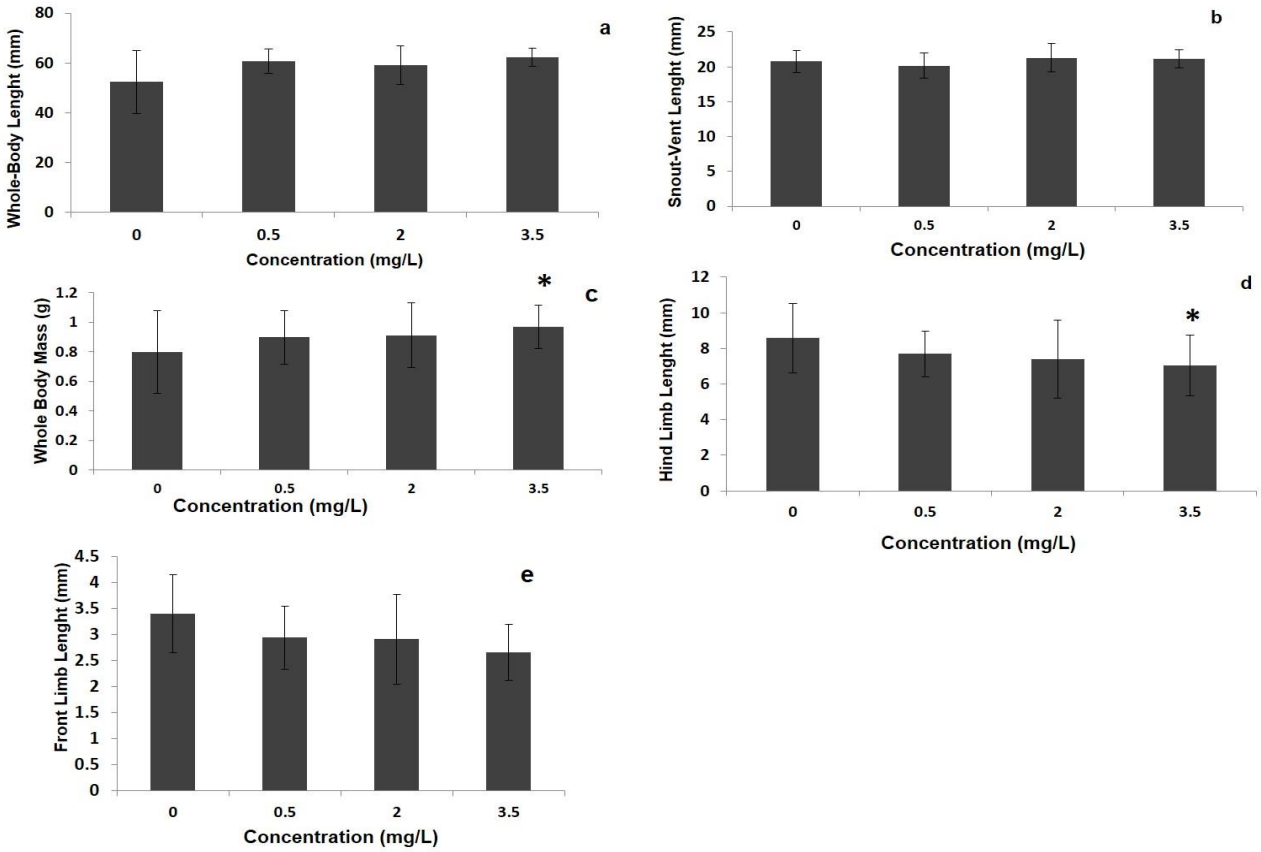

Figure 4.3: Exposure impacts of Arsenal formulation on treated *X. laevis tadpoles:* (a) Whole Body Length, (b) Snout-Vent Length (c) Whole body Mass, (d) Hind Limb Length, (e) Front Limb Length. Means ± SD are indicated. Asterisks indicate significant difference (DMCt, $P< 0.05$) from the control.

4.3.1.4. Whole body mass (WBM)

The exposure impact of Arsenal showed concentration dependence increased in WBM compared to the control. Kruskal-Wallis ANOVA test followed by Tukey HSD multiple comparison test confirmed a significant increase in WBM of tadpoles exposed at highest exposure concentration of 3.5 mg/L compared to the control group (Tukey HSD; $P>0.05$) (Fig 4.3 c).

4.3.1.5. Hind limb length (HLL)

The exposure impact of this Arsenal formulation resulted in a reduced mean HLL (normalised) of the treated tadpoles in a concentration dependent manner, relative to the control group (Figure 4.3d). Kruskal-Wallis ANOVA test, followed by Tukey HSD multiple comparison test, showed that the reduced HLL (normalized) of the treated tadpoles was significantly different at exposure concentrations of only 3.5 mg/L (Tukey HSD test; $P < 0.05$) relative to the control.

4.3.1.6. Front limb length (FLL)

The exposure impact of this Arsenal formulation on the treated tadpoles showed reduction in mean FLL (normalized) at all exposure concentrations, relative to the control (Figure 4.3 e). The reduction in normalized FLL of the treated tadpoles was found not to be significantly different (KW ANOVA test; P>0.05) compared to the control group.

4.3.1.7. Histopathological Endpoints

The impact the Arsenal formulation on the developing tadpoles showed that the colloidal (luminal) area was significantly ($P < 0.05$) reduced at concentrations of 0.5 and 2 mg/l compared to the colloidal area in the control tadpoles (Table 4.2). The follicle epithelium of the exposed tadpoles also significantly increased in height ($P < 0.05$) for the treated tadpoles at all exposure concentrations compared to the control (Table 4.2). For the gland area, the exposure impact of formulation led to a general reduction in overall gland area, but which was only found to be significantly different ($P < 0.05$) in the 2 mg/L concentration group when compared to the control tadpoles (Table 4.2; Fig 4.4).

Figure 4.4: Cross-sections of the *X. laevis* tadpoles paired thyroid glands exposed to Arsenal formulation (A) the control, (B) Arsenal at 2 mg/L. The gland area reduction was only statistically significant at concentrations of 2 mg/L relative to the control (Table 4.2). Micrographs were taken at 100X magnification

Table 4.2: Histo-morphometric data following a 21-day XEMA exposure to graded concentrations of Arsenal formulation (ARS). Values represent the mean ± SD. The zero concentration (ARS) represents the control values for each herbicide treatment. The asterisk signified significant difference ($P<0.05$) relative to the control

TREATMENT Mg/L	Mean Follicle Epithelium (SD)	Mean colloidal Area (SD)	Mean Gland Area (SD)
ARS 0	7.85 (1.48)	4798.35 (3012.1)	119157.5 (33814.9)
ARS 0.05	**10.93 (1.52)***	**3040.02(2122.6)***	92922.8 (10403.5)
ARS 2.0	**11.34 (1.50)***	**3189.13 (1783.1)***	**83186.2 (25250.6)***
ARS 3.5	**11.15 (1.46)***	3588.1 (1964.8)	93683.0 (21768.9)

4.3.2. Midstream formulation (diquat dibromide)

4.3.2.1. Mortality

There was no incidence of mortality in any of the exposure tanks as well or the control tanks, throughout the 21 days exposure period.

4.3.2.2. Variation in Developmental Stages

The exposure impacts of Midstream formulation on the developing tadpoles showed that the frequency distribution ranged from NF-stage 57 through NF-stage 61. Compared to the median of NF-stage 60 at the control, the two highest exposure concentrations of 0.11 and 0.14 mg/L produced tadpoles with their median developmental stage at NF-stage 57 (Fig 4.5 a-d).

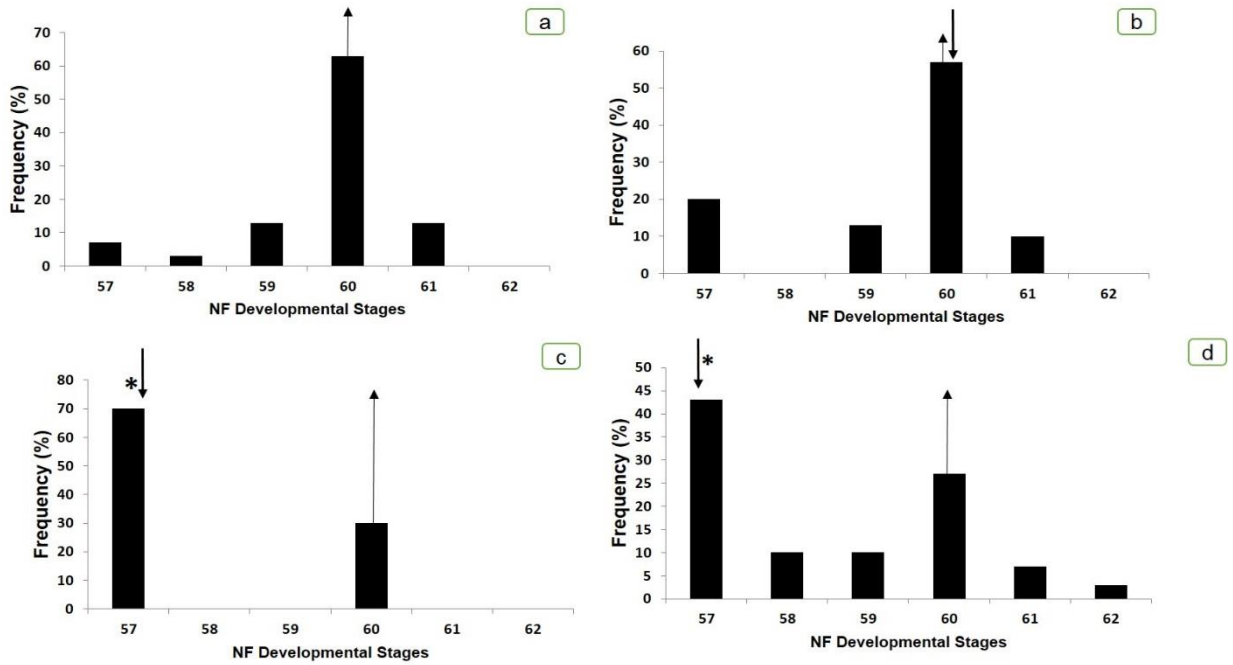

Figure 4.5: The frequency distributions (n=30) of developmental stages (Nieuwkoop and Faber, 1958) attained by *X. laevis* tadpoles after the 21-days exposure to graduated concentrations of Midstream formulation relative to the control (Fig 4.5a). The upward arrow show the median NF-stage at the control relative to downward arrow that show the median at the various concentrations. The asterisk showed significant difference ($P<0.05$) relative to the control

Kruskal-Wallis ANOVA, followed by Dunn's Multi-comparison analysis confirmed significant variation (a delay) in NF-stage development between the control and Midstream concentrations of 0.11 mg/L and 0.14 mg/L (Fig 4.6) (DMCt; $P < 0.05$).

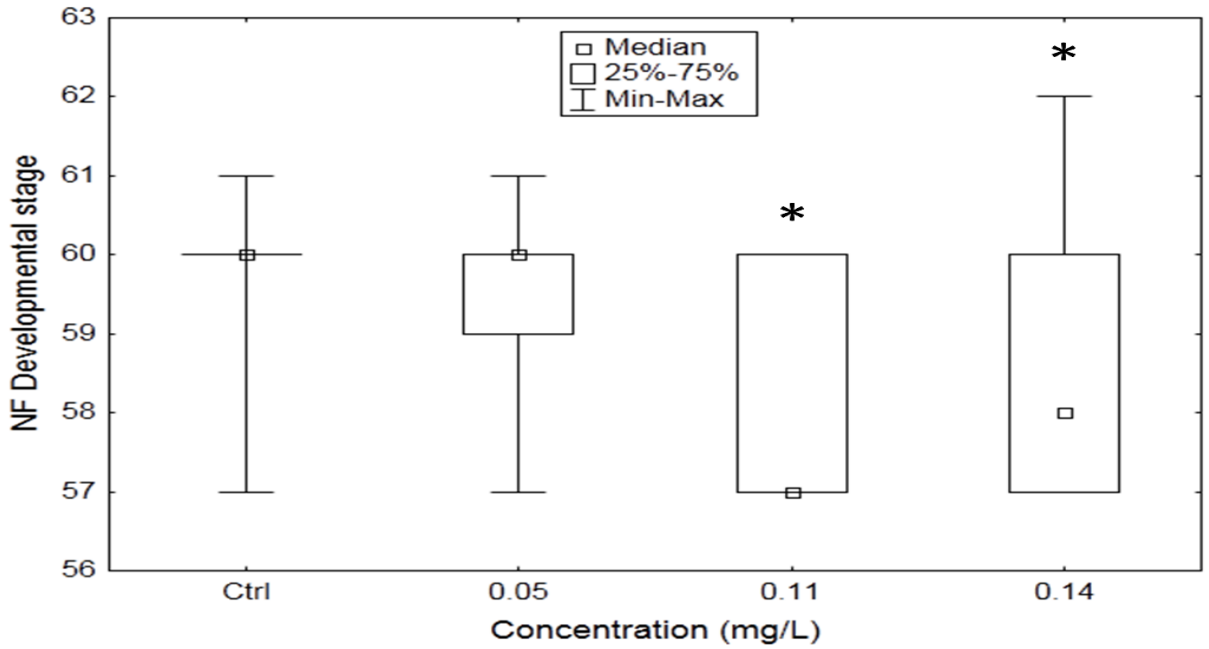

Figure 4.6: NF-stage differentiation following 21-days exposure to graded concentrations of the Midstream formulation compared to the control (Ctrl). Asterisks indicate significant difference (DMCt; $P < 0.05$) from the control.

4.3.2.3. Whole body length (WBL) and Snout-vent length (SVL)

The exposure to Midstream formulation resulted in slight reduction in mean WBL (Fig 4.7a) alongside slight increase in mean SVL (Fig 4.7 b). However, Kruskal-Wallis ANOVA test confirmed that these variations were not significant (K-W ANOVA test, $P > 0.05$).

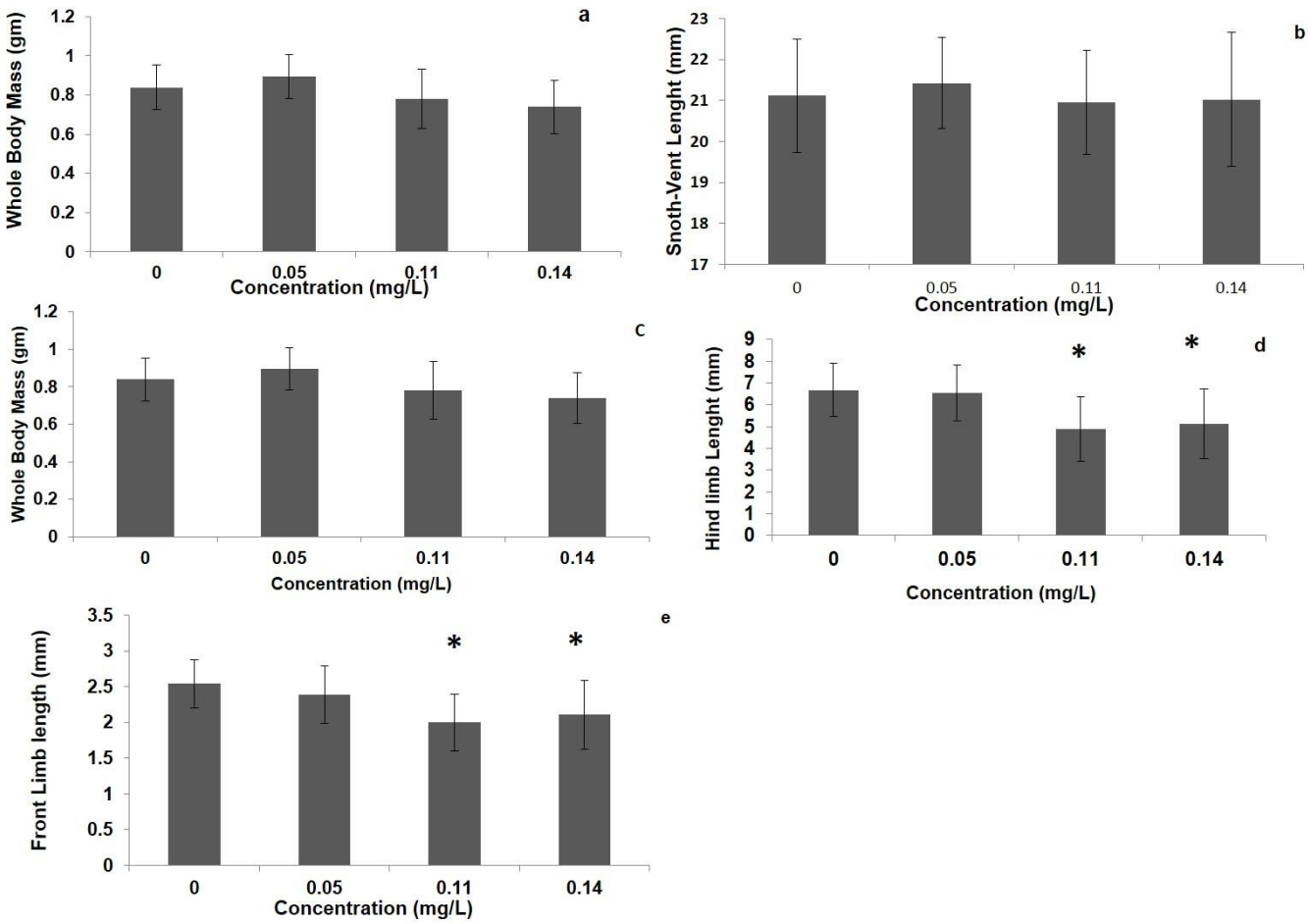

Figure 4.7: Exposure impacts of Midstream formulation on treated *Xenopus laevis tadpoles*; (a) Whole Body Length, (b) Snout-Vent Length (c) Whole body Mass, (d) Hind Limb Length, (e) Front Limb Length. Asterisks indicate significant difference (DMC, P< 0.05) from the control

4.3.2.4. Whole body mass (WBM)

Midstream formulation produced varied responses in mean WBM of the treated tadpoles (Fig 4.7 c). However, Kruskal Wallis ANOVA test showed that this variation in mean WBM was not significant (K-W ANOVA Test; P> 0.05) at all exposure concentrations relative to the control.

4.3.2.5. Hind limb length (HLL)

The mean HLL (normalized) of the tadpoles exposed to Midstream formulation was found to be significantly reduced following Kruskal-Wallis ANOVA and Tukey HSD test (T HSD; P< 0.05) at concentrations of 0.11 mg/L and 0.14 mg/L (Fig 4.7d) relative to the control.

4.3.2.6. Front limb length (FLL)

The exposure of tadpoles to the Midstream formulation resulted in reduction in mean FLL (normalized) of the treated tadpoles relative to the control (Fig 4.7e). Using Tukey HSD test, significant difference in reduction was confirmed (T HSD; $P< 0.05$) for the two highest concentrations of 0.11 mg/L and 0.14 mg/L relative to the control group, respectively.

4.3.2.7. Histopathological Endpoints

The thyroid colloidal (luminal) area in the tadpoles exposed to Midstream formulation was found to be significantly reduced at the highest exposure concentration of 0.14 mg/L ($P<0.05$) compared to the control (Table 4.3). The mean height of the thyroid follicle epithelium showed a significant increased at only the lowest exposure concentration of 0.05 mg/L compared to the control (KW ANOVA Test; $P<0.05$). Mean thyroid gland area, on the other hand, showed no significantly difference ($P>0.05$) at all the exposure concentrations compared to the control.

Table 4.3: Histo-morphometric data following a 21-days XEMA exposure to graded concentrations of Midstream formulation (MID). Values represent the mean ± SD. The zero concentration (MID 0) represents the control values for each herbicide treatment. Asterisk represent significant difference relative to the control.

TREATMENT Mg/L	Mean Follicle Epithelium (SD)	Mean colloidal Area (SD)	Mean Gland Area (SD)
MID 0	7.82 (1.65)	3410.64 (2299.2)	53903.80 (27630.7)
MID 0.05	**8.92 (1.68)***	3146.04 (1793.6)	69585.69 (12328.7)
MID 0.11	8.27 (1.22)	2413.78 (1195.1)	59964.97 (15335.3)
MID 0.14	8.28 (1.33)	**1832.86 (999.8)***	39340.26 (10961.2)

4.3.3. Basta formulation (glufosinate ammonium)

4.3.3.1. Mortality

There was no incidence of mortality in any of the exposure tanks as well as the control tanks, throughout the 21 days exposure period.

4.3.3.2. Variation in Developmental Stages

In the exposure to the Basta formulation, the developmental stages reached after the 21-days exposure ranged from NF-stage 58 through NF-stage 63. The median of the frequency distribution

in the control and lower Basta concentration (0.05 mg/L) group centred around NF-stage 58 whereas in the higher Basta concentration (0.15 mg/L and 0.25 mg/L) the median was higher (NF-stage 60). However, the advance median NF-stages were not found to be significant (Fig 4.8) (K-W ANOVA test; $P > 0.05$).

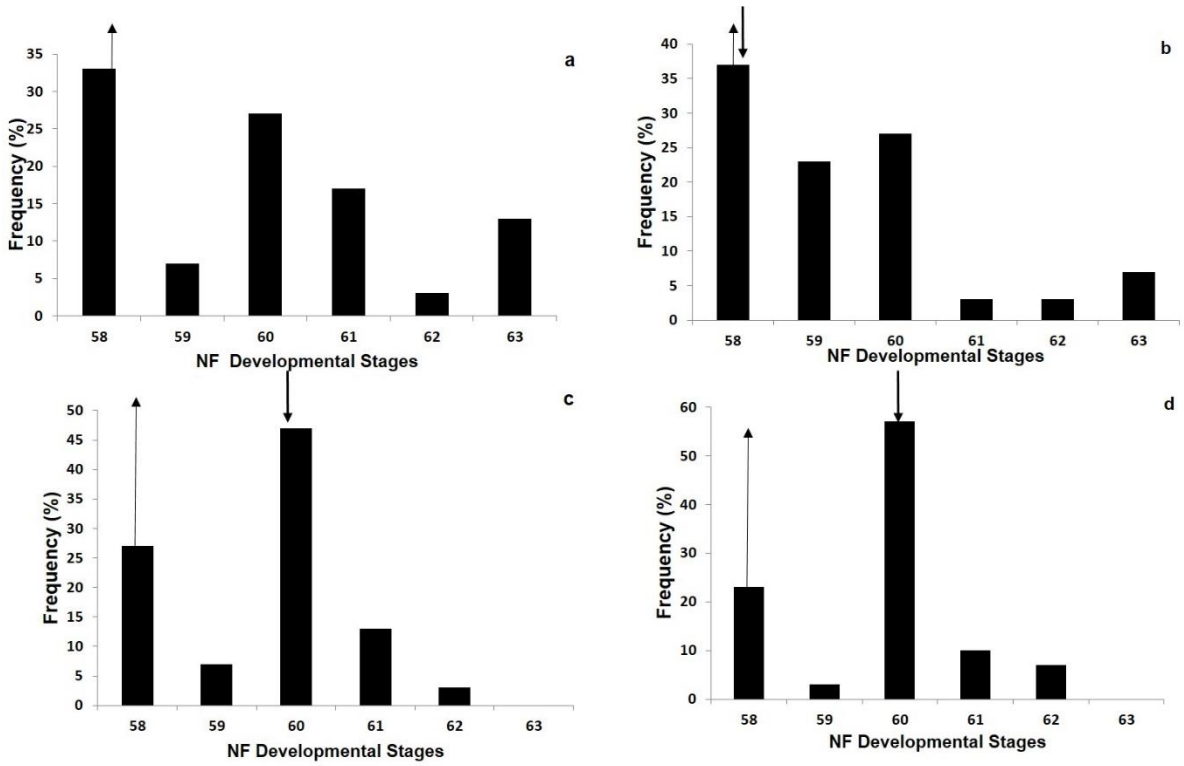

Figure 4.8: The frequency distributions (n=30) of developmental stages (Nieuwkoop and Faber, 1958) attained by *X. laevis* tadpoles after 21-days exposure clean water (a) and graduated concentrations of Basta formulation (glufosinate ammonium) (b) 0.05 mg/L, (c) 0.15 mg/L, and (d) 0.25 mg/L. The upward arrow showed the median NF-stage at the control relative to downward arrow that showed the median at the various concentrations.

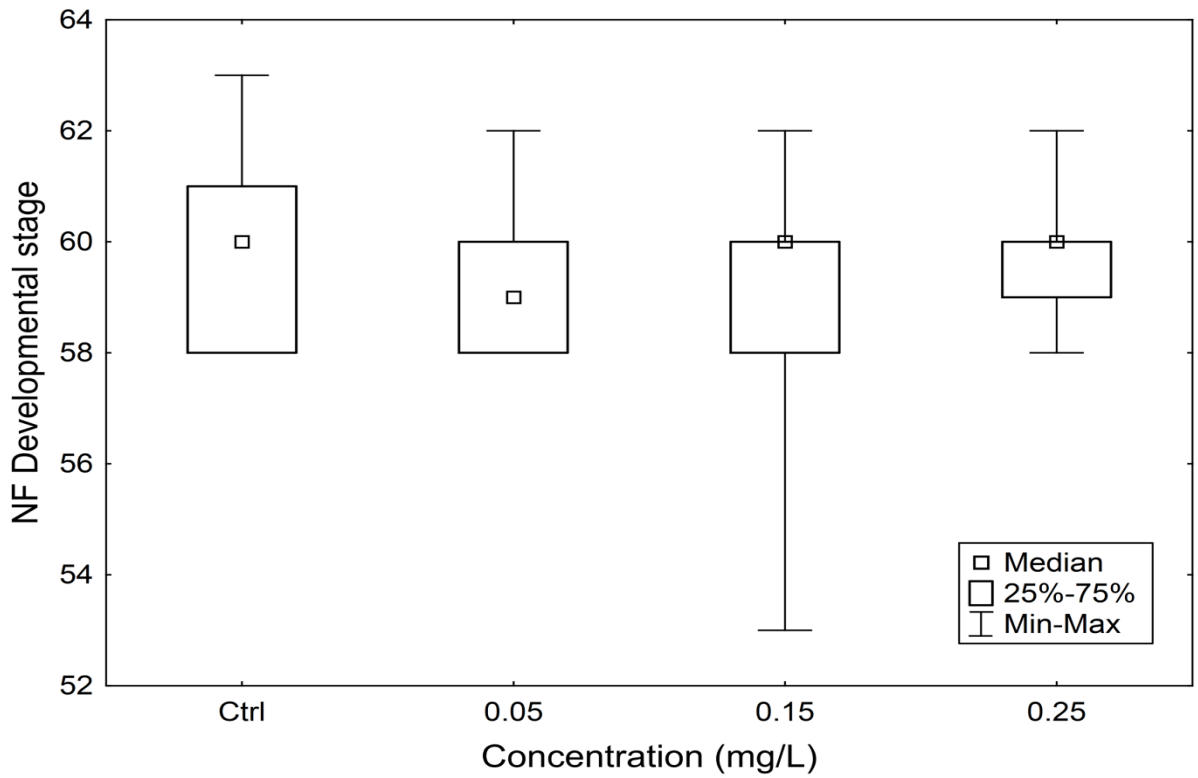

Figure 4.9: NF-Stage differentiation following 21-days exposure to graduated concentrations of the Basta formulation (glufosinate ammonium). The medians of the developmental stages of exposed tadpoles (Basta concentrations) did not vary significantly different from the tadpoles in the control (K-W ANOVA Test; $P > 0.05$).

4.3.3.3. Whole body length (WBL) and Snout-vent length (SVL)

For this Basta formulation, the exposure of tadpoles led to an increasing trend in mean WBL and mean SVL relative to the control. However, a Kruskal-Wallis ANOVA, could not confirm that significant variation existed in size variable, mean WBL (Fig 4.10a) and mean SVL (Fig 4.10b) (K-W ANOVA Test, $P>0.05$) at all exposure concentrations, when compared to the control group.

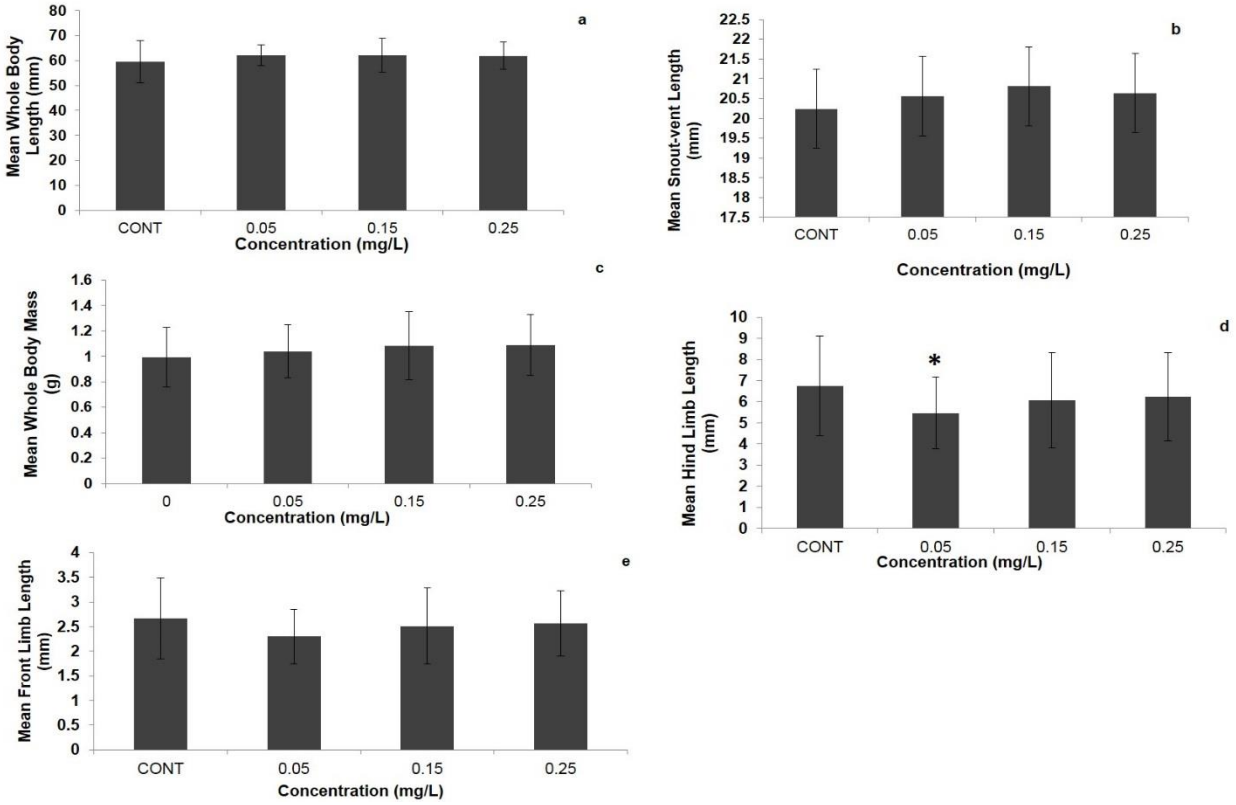

Figure 4.10: Exposure impacts of Basta formulation on treated *X. laevis tadpoles;* (a) Whole Body Length, (b) Snout-Vent Length (c) Whole body Mass, (d) Hind Limb Length, (e) Front Limb Length. Asterisks indicate significant difference ($P < 0.05$) relative to the control.

4.3.3.4. Whole body mass (WBM)

Similarly, WBM was unaffected by exposure to Basta formulation (Fig 4.10 c), (K-W ANOVA test; $P > 0.05$) when compared to the control group.

4.3.3.5. Hind limb length (HLL)

Tadpoles exposed to the Basta formulation (0.05 mg/L) showed a significant decrease mean HLL (normalized) compared to the control (Fig 4.10d) (Tukey HSD test; $P < 0.05$).

4.3.3.6. Front limb length (FLL)

The mean FLL (normalized) in Basta treated tadpoles relative to the control (Fig 4.10 e) did not vary significantly among experimental groups (K-W ANOVA Test; $P > 0.05$).

4.3.3.7. Histopathological Endpoints

In tadpoles exposed to Basta formulation, the mean thyroid colloidal (luminal) area did not vary significantly among tadpoles and the control (K-W ANOVA Test; $P > 0.05$). The mean follicle epithelium height and mean thyroid gland area, however, increased significantly in the 0.15 and 0.25 mg/L concentrations ($P < 0.05$) (Fig 4.11; Table 4.4).

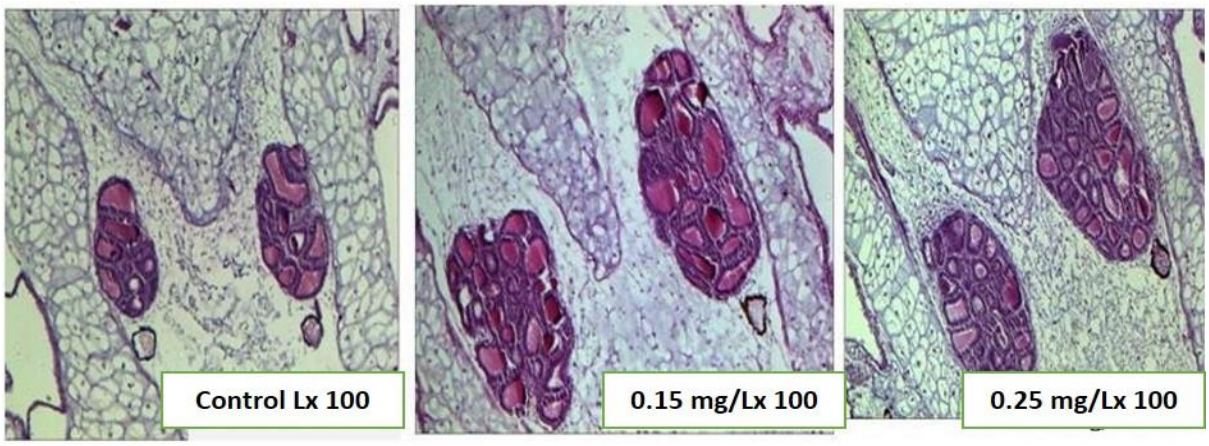

Figure 4.11: Cross-sections of the paired thyroid glands (stained with H & E) of tadpoles exposed to Basta formulation at (a) Control (b) 0.15 and (c) 0.25 mg/L

Table 4.4: Histo-morphometric data following a 21-days XEMA exposure to graded concentrations of Basta formulation. Values represent the mean ± SD. The zero concentration represents the control values for each herbicide treatment.

TREATMENT Mg/L	Mean Follicle Epithelium (SD)	Mean colloidal Area (SD)	Mean Gland Area (SD)
Basta 0	7.30 (1.08)	1910.06 (1055.5)	47014.7 (6771.12)
Basta 0.05	**8.05 (1.11)***	1711.86 (1013.1)	43919.03 (8161.9)
Basta 0.15	**8.02 (1.18)***	1916.95 (1078.6)	**86800.98 (17907.1)***
Basta 0.25	**10.02 (1.86)***	1843.37 (793.4)	**78414.85 (16871.5)***

4.3.4. Kilo max (Glyphosate)

4.3.4.1. Mortality

There was no incidence of mortality in any of the exposure tanks as well as the control tanks, throughout the 21-days exposure period.

4.3.4.2. Variation in Developmental Stages

Following the 21-day exposure to the Kilo Max formulation, the frequency distribution of developmental stages ranged from NF-stage 54 through NF-stage 64 (Fig 4.12). At both the control group and concentration of 90 mg/L, the median was NF-stage 60. The exposure at 190 mg/L and 280 mg/L showed a significant delay in development with a median NF-stage 58 (Fig 4.12). The exposure impact of the Kilo Max delayed the NF developmental stages of the exposed in dose dependent manner at all exposure concentrations.

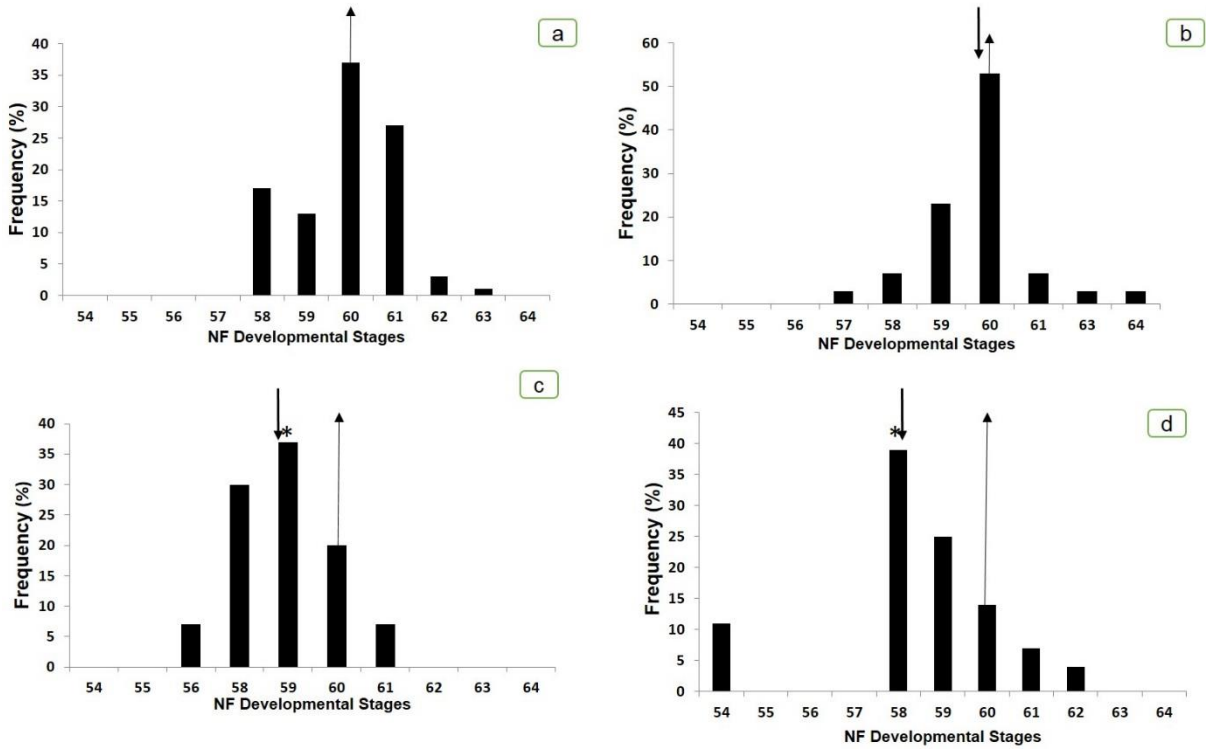

Figure 4.12: The frequency distributions (n= 30) of developmental stages (Nieuwkoop and Faber, 1958) attained by *X. laevis* tadpoles exposed to clean water (a) control and graduated concentrations of Kilo Max formulation (b) 90 mg/L, (c) 190 mg/L and (d) 280 mg/L. The asterisk indicated the significant difference (Post -HOC; $P< 0.05$) relative to the median stage in the control. The upward arrow showed the median at the control relative to downward arrow that showed the median distribution of developmental stage at the various concentrations.

Comparing the NF developmental stages medians between the Kilo Max treated and the tadpoles in the control group, the Kruskal-Wallis ANOVA test showed significant variation in median of developmental stages and multi-comparison analysis confirmed a significant delayed in

developmental median (Fig 4.13) at concentrations of 190 mg/L and 280 mg/L relative to the tadpoles in the control treatment (Fig. 4.13).

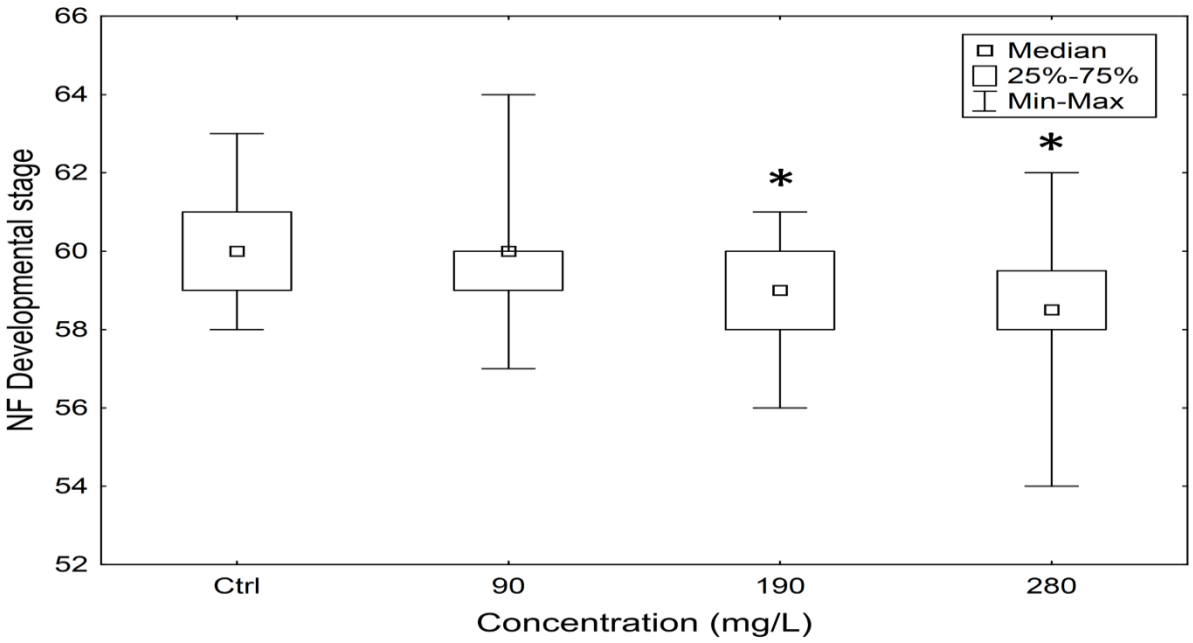

Figure 4.13: NF-stage differentiation following 21-days exposure to graded concentrations of the Kilo Max formulation compared to the control. Asterisks indicate significant difference (DMCt; P< 0.05) from the control.

4.3.4.3. Whole body length (WBL) and Snout-vent length (SVL)

The exposure of tadpoles to Kilo Max led to lower mean WBL and SVL relative to the control group. The Kruskal-Wallis ANOVA test showed reduction in the mean WBL (Fig. 4.14a) and SVL (Fig. 4.14b) of the treated tadpoles. The Tukey HSD test confirmed the significant reduction in the mean WBL and SVL of the treated tadpoles at the two highest exposure concentrations of 190 mg/L and 280 mg/L compared to the control (Tukey HSD Test; $P < 0.05$).

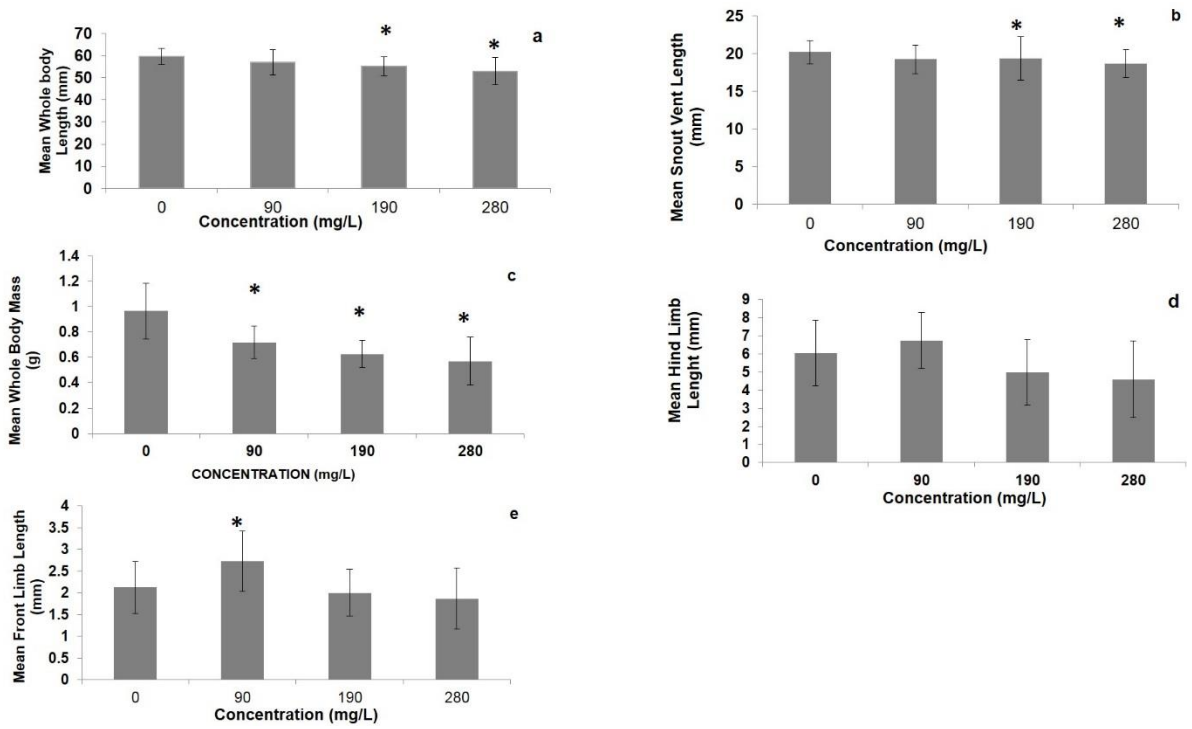

Figure 4.14: Exposure impacts of Kilo Max formulation on treated *Xenopus laevis tadpoles;* (a) Whole Body Length, (b) Snout-Vent Length (c) Whole body Mass, (d) Hind Limb Length, (e) Front Limb Length. Asterisks indicate significant difference (P< 0.05) from the control.

4.3.4.4. Whole body mass (WBM)

The exposure to Kilo Max formulation showed a concentration dependent reduction in WBM relative to the control (Fig. 4.14c). This reduction in mean WBM was significantly different (Tukey HSD; P < 0.05) at the three exposure concentrations of 90 mg/L, 190mg/L and 280 mg/L compared to the control group.

4.3.4.5. Hind limb length (HLL)

Exposure of the tadpoles to Kilo Max formulation produced mean HLL (normalized) that was not significant ((K-W ANOVA Test; P< 0.05) at all exposure concentrations relative to the control group (Fig. 4.14d).

4.3.4.6. Front limb length (FLL)

For the Kilo Max treated tadpoles, the mean FLL (normalized) showed increased pattern relative to the control (Fig. 4.14 e). The difference was confirmed by the Kruskal-Wallis test. Tukey HSD test also confirmed significant increase in tadpoles exposed to the lowest exposure concentration of 90 mg/L compared to the control (Tukey HSD Test; P < 0.05).

4.3.4.7. Histopathological Endpoints

The exposure of developing tadpoles to Kilo Max formulation showed the reduction in the gland area and the colloidal (luminal) area of the treated tadpoles, that was not significantly different (K-W ANOVA Test; P> 0.05) compared to the control (Fig 4.15; Table 4.5). But the follicle epithelium of the treated tadpoles was significantly increased (K-W ANOVA Test; P< 0.05) at all exposure concentrations compared to the control.

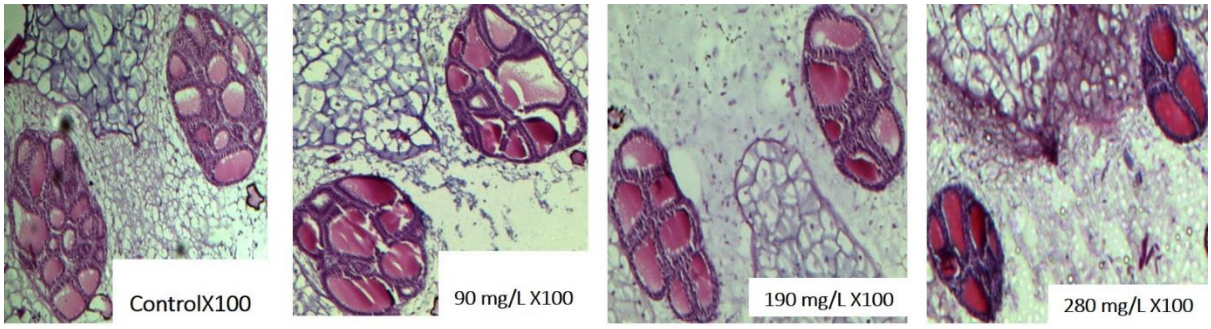

Figure 4.15: Histological cross-sections of the *X. laevis* thyroid gland of the Kilo Max treated tadpoles (A) the control, (B) 90 mg/L., (c) 190 mg/L, and (D) 280 mg/L (Micrographs were taken at 100X magnification).

Table 4.5: Histo-morphometric data following a 21-days XEMA exposure to graded concentrations of Kilo Max formulation. Values represent the average measurement ± one standard deviation. The zero concentration represents the control values for each herbicide treatment.

TREATMENT Mg/L	Mean Follicle Epithelium (SD)	Mean colloidal Area (SD)	Mean Gland Area (SD)
KILO 0	7.48 (1.02)	2968.36 (2455.5)	68737.3 (15494.6)
KILO 90	**10.15 (1.77)***	3499.79 (2387.8)	76658.08 (26164.1)
KILO 190	**9.46 (2.42)***	2179.34 (1795.7)	56645.62 (21408.0)
KILO 280	**9.21(2.25)***	2830.88 (2183.0)	47363.29 (24470.8)

4.3.5. Roundup (Glyphosate)

4.3.5.1. Mortality

There was no incidence of mortality in any of the exposure tanks or the control tanks throughout the 21 days exposure period.

4.3.5.2. Variation in Developmental Stages

The exposure impact of the Roundup formulation on the treated tadpoles produced a varied rate of development that ranged from NF-stage 57 through NF- stage 64 (Fig. 4.16). The median of developmental stages shifted from NF-stage 60 at both control and concentration of 0.2 mg/L to NF-stage 61 at both concentrations of 0.4 mg/L and 0.6 mg/L (Fig 4.16). However, Kruskal-Wallis ANOVA test confirmed that this observed shift were not significant compared to the control ($P > 0.05$) (Fig 4.17).

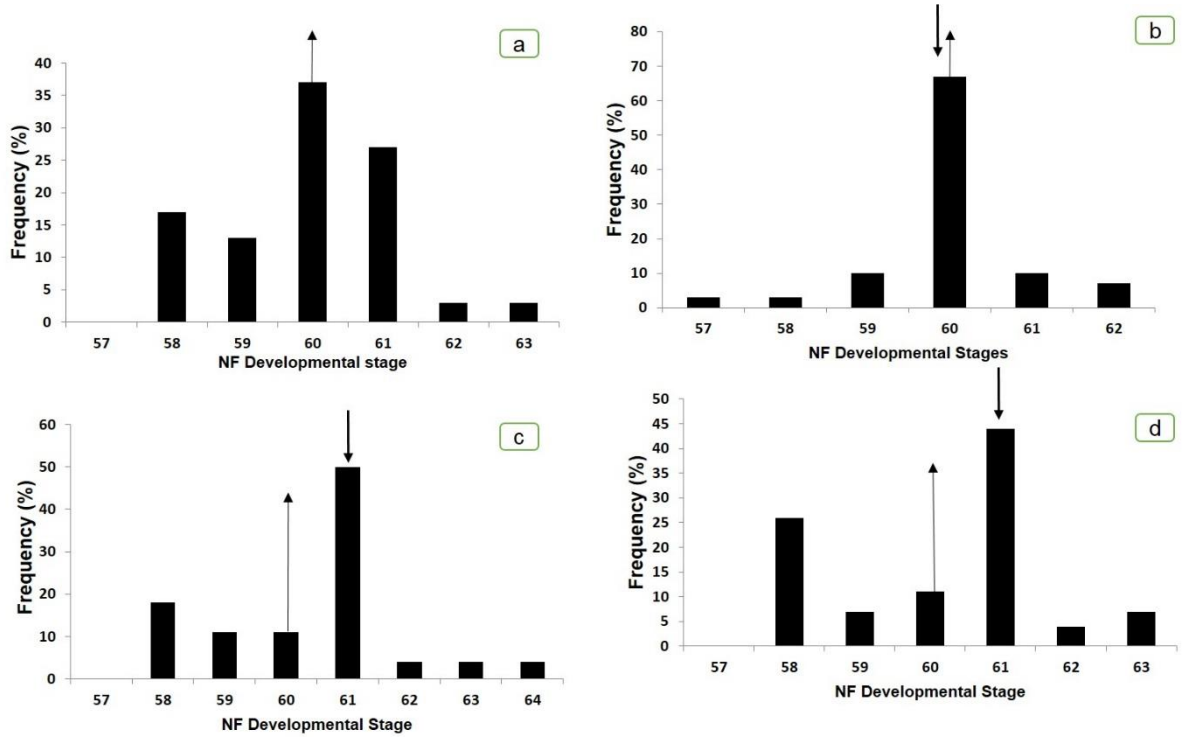

Figure 4.16: The frequency distributions (n=30) of developmental stages (Nieuwkoop and Faber, 1958) attained by *X. laevis* tadpoles exposed for 21-day to clean water (a) control, and graduated concentrations of Roundup formulation (glyphosate) (b) 0.2 mg/L, (c) 0.4 mg/L (d) 0.6 mg/L. The upward arrow showed the median at the control relative to downward arrow that showed the median of developmental stage at the various concentrations.

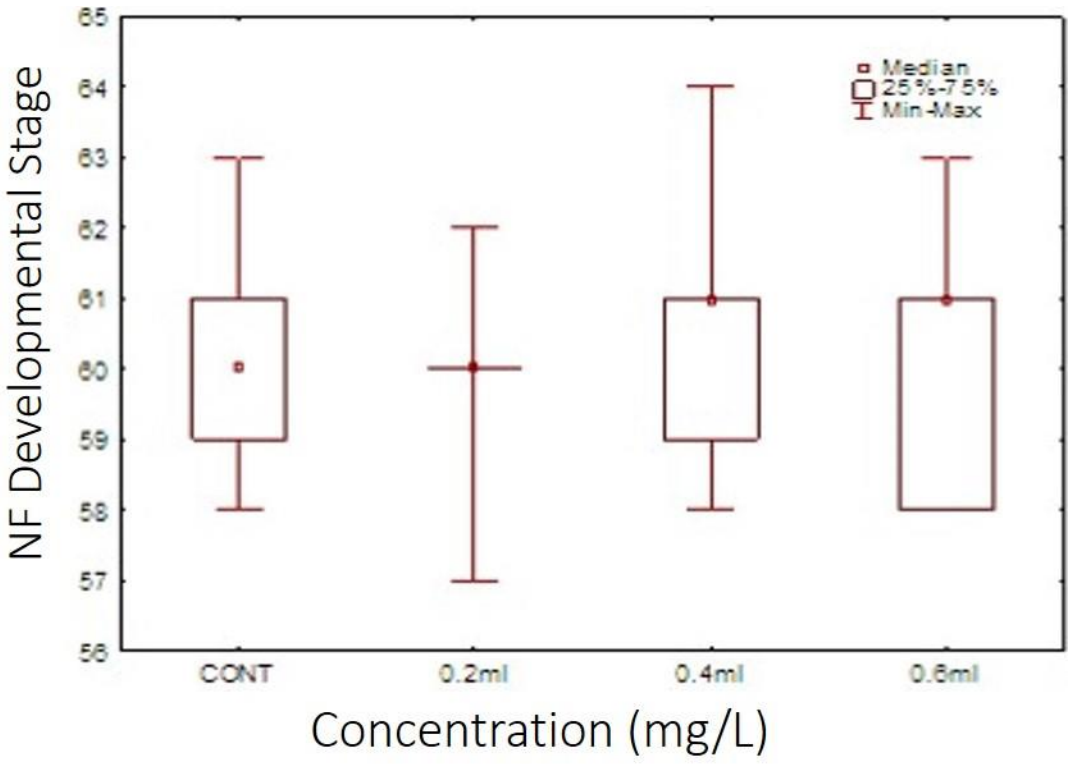

Figure 4.17: Stage differentiation following 21-days exposure to different concentrations of the Roundup formulation compared to the control (Ctrl).

4.3.5.3. Whole body length (WBL) and Snout-vent length (SVL)

The exposure of the tadpoles to Roundup formulation resulted in higher mean WBL (Fig. 4.17 a) and SVL (Fig. 4.18 b) relative to the control. Using the Kruskal-Wallis ANOVA test, followed by Tukey HSD multiple comparison test, only the mean WBL was significantly different (Tukey HSD ANOVA test; $P< 0.05$) at the lowest exposure concentration of 0.2 mg/L compared to the control.

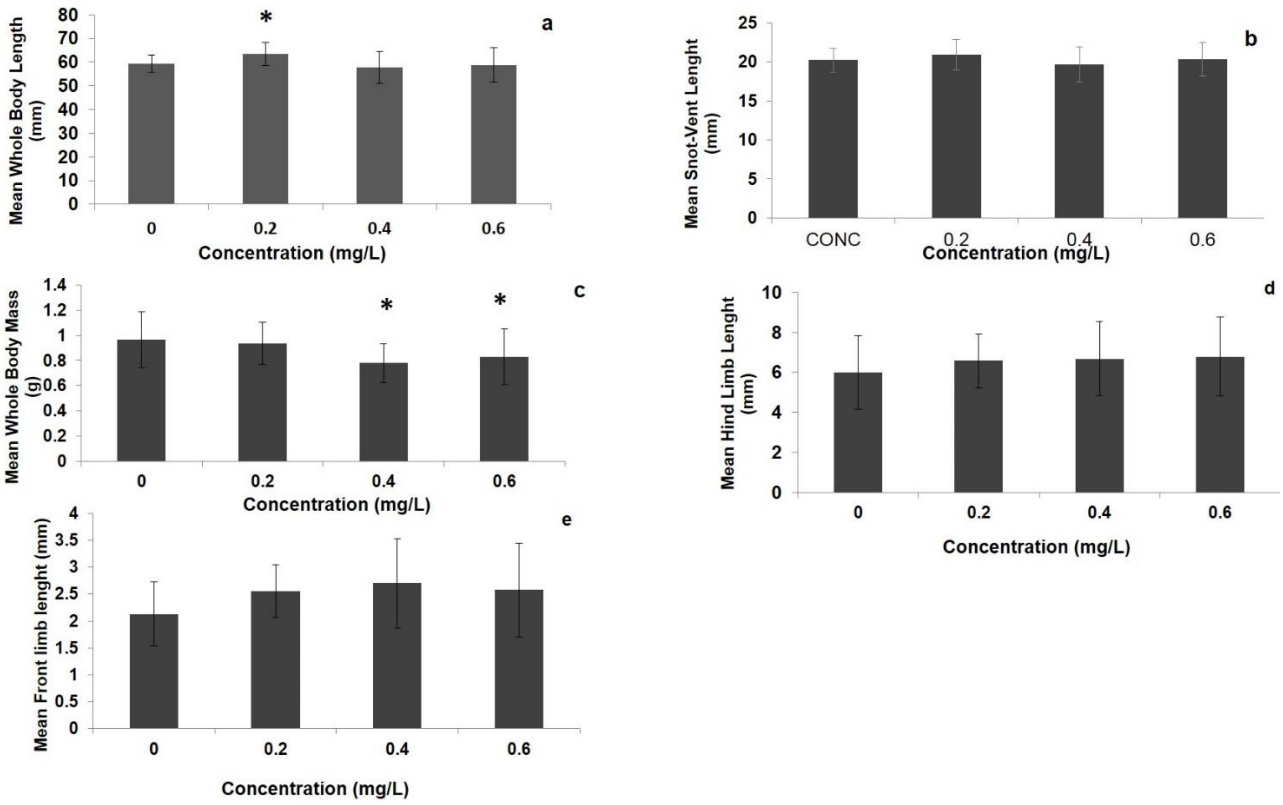

Figure 4.18: Exposure impacts of Roundup formulation on treated *Xenopus laevis tadpoles* on (a) Whole Body Length, (b) Snout-Vent Length (c) Whole body Mass, (d) Hind Limb Length, (e) Front Limb Length. Asterisks indicate significant difference (P< 0.05) from the control

4.3.5.4. Whole body mass (WBM)

The Roundup treated tadpoles showed reduction in mean WBM with increasing concentrations, relative to the control group (Fig. 4.18 c). However, the Kruskal-Wallis ANOVA test followed by Tukey HSD multiple comparison test showed that the mean WBM were significantly reduced at concentrations of 0.4 mg/L and 0.6 mg/L (Tukey-HSD ANOVA Test; $P < 0.05$) compared to the control.

4.3.5.5. Hind limb length (HLL)

The exposure impact of Roundup formulation showed a higher mean HLL (normalized) relative to the control group (Fig. 4.18 d). However, the Kruskal-Wallis ANOVA test showed this variation to be non-significant (K-W ANOVA Test; $P>0.05$) relative to tadpoles in the control group.

4.3.5.6. Front limb length (FLL)

The treated tadpoles showed an increasing mean FLL (normalized) pattern compared to the control (Fig 4.18 e). However, the Kruskal-Wallis ANOVA test followed by Tukey HSD multiple comparison test, showed no significant difference (Tukey HSD Test; $P>0.05$) in mean FLL of the treated tadpoles relative to that of the control group.

4.3.5.7. Histopathological Endpoints

Following the exposure to the Roundup formulation, the cross-sectional gland area (Fig 4.19) and colloidal (luminal) area of the thyroid gland of the treated tadpoles significantly increased ($P< 0.05$) at the highest exposure concentration of 0.6 mg/L compared to the control (Table 4.6). The height of the follicle epithelium also significantly increased ($P< 0.05$) at all exposure concentrations compared to the control (Table 4.6).

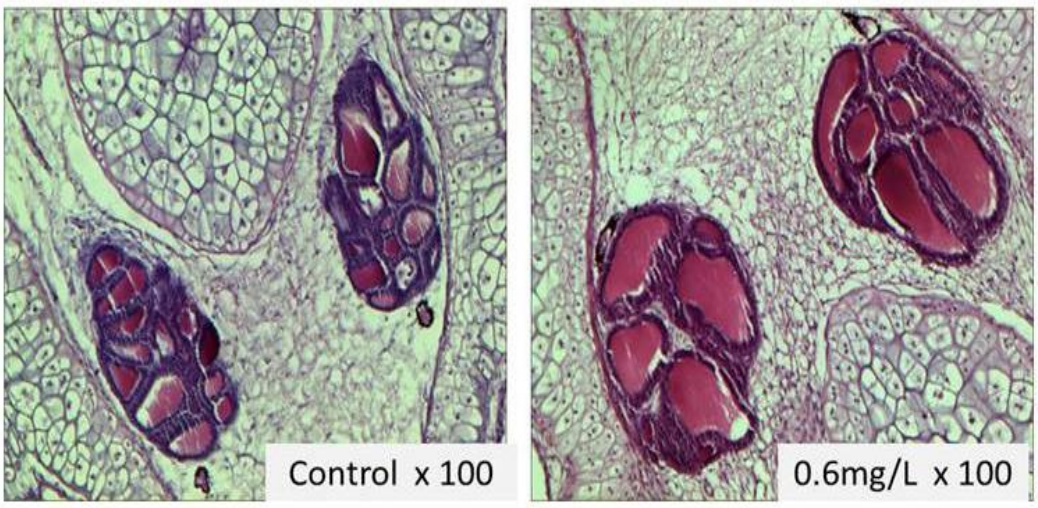

Figure 4.19: The cross-sections of the *X. laevis* thyroid gland of the tadpoles exposed to the Roundup formulation (A) the control, (B) gland of the tadpoles at concentration of 0.6 mg/L. (Micrographs were taken at 100X magnification).

Table 4.6: Histo-morphometric data following a 21-days XEMA exposure to graded concentrations of Roundup formulation (RDP). Values represent the average measurement ± one standard deviation. The zero concentration (RDP) represents the control values for each herbicide treatment.

TREATMENT Mg/L	Mean Follicle Epithelium (SD)	Mean colloidal Area (SD)	Mean Gland Area (SD)
RDP 0	7.48(1.02)	2968.36(2455.5)	68737.3 (15494.6)
RDP 0.2	**9.81 (1.65)***	2508.87 (1755.7)	79514.1 (26146.0)
RDP 0.4	**8.23 (1.33)***	3016.78 (1704.8)	78167.2 (21408. 0)
RDP 0.6	**11.31(1.77)***	**8501.64 (4354.3)***	**100907.3 (24470.8)***

4.3.6. Enviro Glyphosate (Glyphosate)

4.3.6.1. Mortality

There was no incidence of mortality in any of the exposure tanks or the control tanks, throughout the 21 days exposure period.

4.3.6.2. Variation in Developmental Stages

For Enviro glyphosate treated tadpoles, the rate of development varied between NF-stage 57 through NF-stage 62 (Fig. 4.20). The median of developmental stages remain constant at all the exposure concentrations as well as the control exposure.

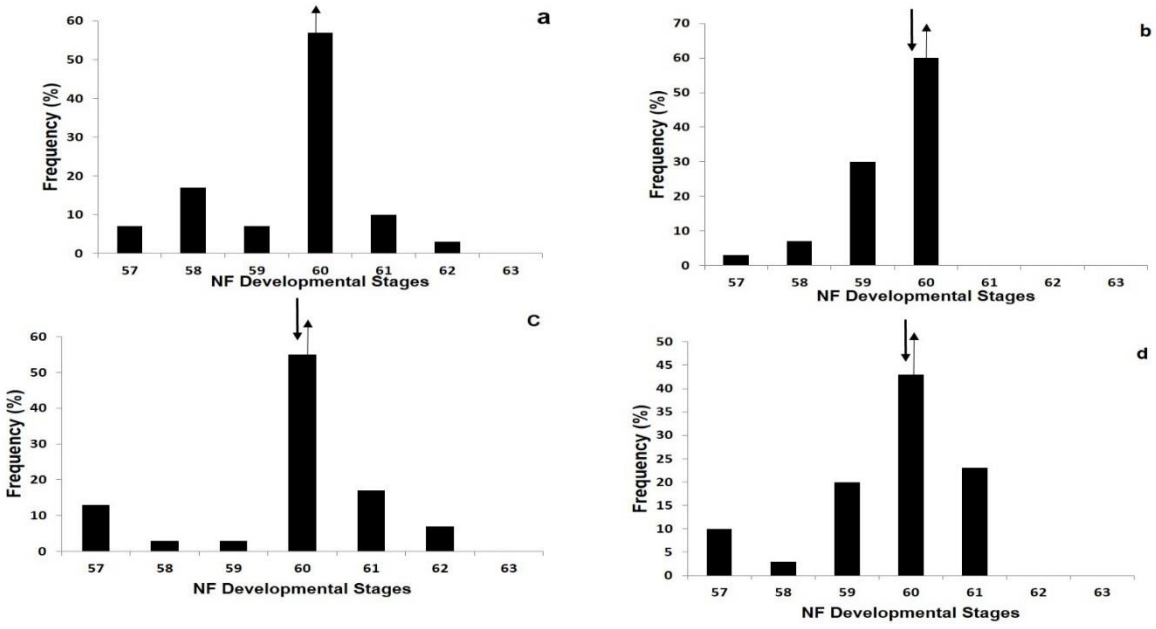

Figure 4.20: The frequencies distributions (n=30) of developmental stage by *X. laevis* tadpoles exposed for 21 days to clean water (a) Control and graduated concentrations of Enviro Glyphosate formulation (b) 9 mg/L, (c) 19 mg/L, (d) 28 mg/L. The upward arrow showed the median at the control relative to downward arrow at the various concentrations.

Using the Kruskal-Wallis ANOVA test and Dunn's multi-comparison test confirmed the lack of significant variation in the developmental stage distribution of the treated tadpoles (Fig 4.21) compared to the control group (DMCt; $P > 0.05$). (Fig 4.21)

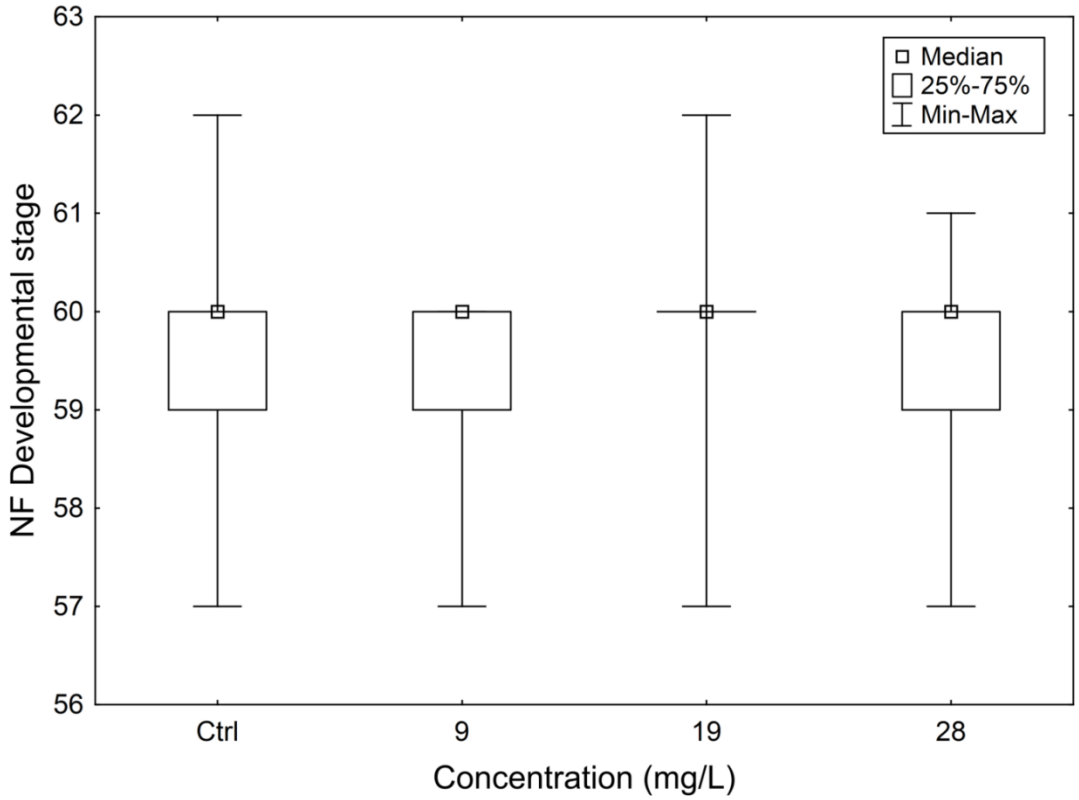

Figure 4.21: Stage differentiation following 21-days exposure to different concentrations of the Enviro Glyphosate formulation compared to the control (Ctrl).

4.3.6.3. Whole body length (WBL) and Snout-vent length (SVL)

The mean WBL (Fig 4.22 a) and SVL (Fig 4.22 b) of the Enviro Glyphosate treated tadpoles was lower compared to the respective control. Using the Kruskal-Wallis ANOVA test, followed by Tukey HSD multiple comparison test, significant reduction in mean WBL and SVL was confirmed at only the highest exposure concentration of 28 mg/L compared to the control (Tukey HSD test; $P < 0.05$)

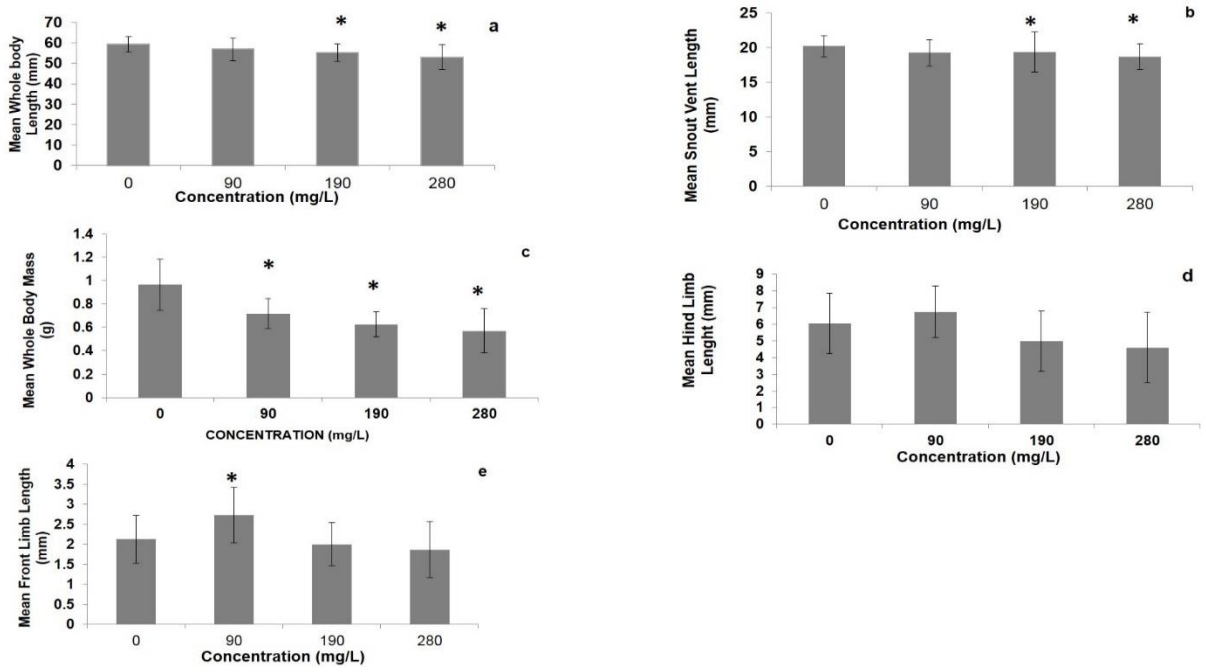

Figure 4.22: Exposure impacts of Enviro glyphosate formulation on treated *Xenopus laevis tadpoles* (a) Whole Body Length, (b) Snout-Vent Length (c) Whole body Mass, (d) Hind Limb Length, (e) Front Limb Length. Asterisks indicate significant difference ($P < 0.05$) from the control.

4.3.6.4. Whole body mass (WBM)

The exposure impact of Enviro Glyphosate formulation on the treated tadpoles showed a concentration dependent reduction in mean WBM relative to the control (Fig 4.22 c). Using Kruskal-Wallis ANOVA test followed by Tukey HSD multiple comparison test, the reduction in mean WBM was confirmed at the highest exposure concentration of 28 mg/L (Tukey HSD Test, $P < 0.05$) compared to the control.

4.3.6.5. Hind limb length (HLL)

The exposure to Enviro glyphosate formulation caused an increased mean HLL (normalized) on the treated tadpoles relative to the control group (Fig 4.22d). The Kruskal-Wallis ANOVA test showed no significant difference in normalized HLL at all exposure concentrations and those of the control (K-W ANOVA Test; $P > 0.05$).

4.3.6.6. Front limb length (FLL)

The exposure impact of Enviro glyphosate showed increased mean FLL (normalized) on the treated tadpoles relative to the control (Fig 4.22 e). However, using the Kruskal-Wallis ANOVA test, there was no significant difference (K-W ANOVA Test; $P>0.05$) between the mean FLL of the treated tadpoles and that of the control group.

4.3.6.7. Histopathological Endpoints

The exposure of tadpoles to Enviro Glyphosate formulation led to significant reduction in the colloidal (luminal) area of the exposed tadpoles (K-W ANOVA Test; $P< 0.05$) at concentrations of 9 and 19 mg/L compared to the control (Table 4.7). The follicle epithelium significantly increased (K-W ANOVA Test; $P< 0.05$) at the two highest exposure concentrations of 19 and 28 mg/L compared to the control. For the gland area, there was no significant difference in the treated tadpoles (K-W ANOVA Test; $P> 0.05$ relative to the control (Fig 4.23).

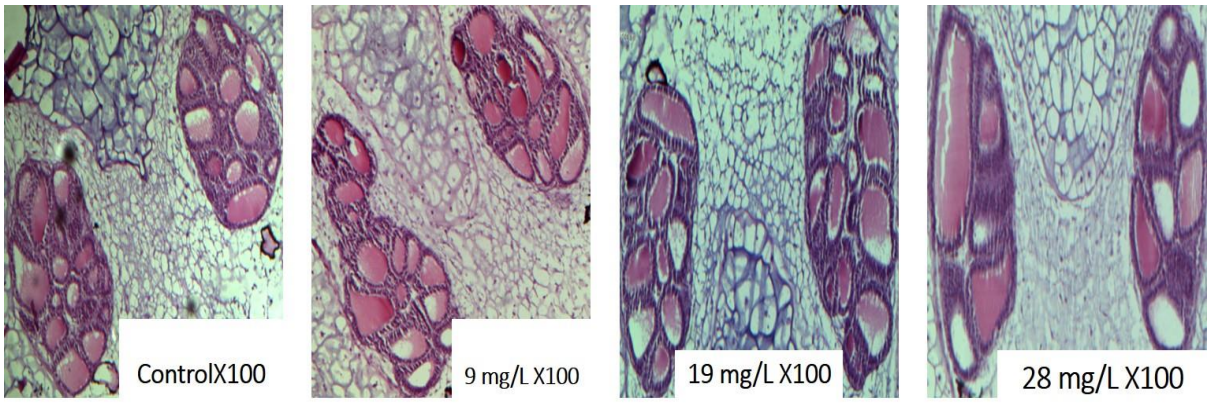

Figure 4.23.: The cross-sections of the *X. laevis* thyroid gland of the tadpoles exposed to the Enviro Glyphosate formulation (A) the control, (B) gland of the tadpoles at 9 mg/L., (c) gland of the tadpoles at 19 mg/L, and, (D) gland of the tadpoles at 28 mg/L (Micrographs were taken at 100X magnification).

Table 4.7: Histo-morphometric data following a 21-days XEMA exposure to graded concentrations of Enviro glyphosate formulation (ENVIRO). Values represent the mean ± one standard deviation. The zero concentration (ENVIRO 0) represents the control values for each herbicide treatment.

TREATMENT Mg/L	Mean Follicle Epithelium (SD)	Mean colloidal Area (SD)	Mean Gland Area (SD)
ENVIRO 0	8.44 (1.43)	4925.20 (2626.2)	92515.93 (31110.2)
ENVIRO 9	8.69 (1.26)	**3026.5 (1622.6)***	89325.43 (35154.5)
ENVIRO 19	**9.25 (1.21)***	**3264.5 (1742.7)***	104426.8(19727.6)
ENVIRO 28	**11.8 (0.78)***	4666.8 (3915.5)	81688.8 (16599.9)

4.4 Discussion

The reality of global amphibian population decline is no longer in doubt, but what is still challenging is the search for the underlying environmental factors that are responsible for the declines (Stuart *et al*., 2004). Widespread concern regarding the increasing use of herbicides and their adverse health and ecological effects in the aquatic ecosystems is voiced internationally (Edge *et al*., 2013). Disruption of the thyroid and reproductive pathways through chemical/agrochemicals contamination, have been listed amongst the potential contributing factors (Miyata and Ose, 2012). The fact that thyroid hormone coordinate growth, development as well as reproductive makes the HPT axis a very important pathway to consider when dealing with environmental chemicals potentially disrupting the endocrine system in wildlife and humans. It is also well-recognized that early development represent a sensitive window to endocrine modulation effects, since organogenesis and development of functional physiological systems is regulated during this time (Lenkowski *et al*. 2008; Diamanti-Kandarakis *et al*., 2009; WHO, 2012**;** Bergman *et al*., 2013)

The present study, showed that four out of the six herbicide formulations, including Arsenal (imazapyr), Midstream (diquat dibromide), and the glyphosate formulations (Roundup and Kilo Max), affected the development of *X laevis* during the developmental window (NF-stages 47-60) associated with thyroid activity**.** They inhibited the development of *X. laevis* tadpoles during the stages associated with thyroid activity**.**

The tadpoles in the control groups in the present study were healthy, and passed through the processes of metamorphosis at the expected rate. This was consistent with the OECD pre-validation test guidelines, which recommended that control tadpoles should reach a minimum median developmental stage of NF-stage 57 at the test termination (OECD, 2007). The tadpoles in the exposure groups showed varied developmental rates and were distributed between NF-stage 58- 65

for Arsenal, NF-stage 57-62 for Midstream, NF-57-62 for Enviro Glyphosate and NF-58-63 for Basta, Roundup and Kilo Max formulation. The exposure concentration of the selected herbicide formulations used in this present study (most of which were within predicted environmental concentrations) did not show any toxicity on the survival of the *X. laevis* tadpoles, as none of the exposed tadpoles died during the course of the exposure. The overall mean body mass and snout-vent length (±SD) of the control tadpoles, after 21-day exposure compared favourably with OECD's phase 1 pre-validation studies with stage 57 tadpoles, suggesting a mean body mass target of 0.944 g and a mean snout-vent length of 19.5 mm respectively.

Arsenal Formulation

The facts that the Arsenal formulation significantly delayed the developmental stages of the exposed tadpoles at environmental relevant concentrations of 2.0 mg/L (Patter, 2003) suggests the potential inhibitory properties of this formulation on the thyroid system of *X. laevis*. Although, no thyroid related study has been done on Arsenal formulation using XEMA, the result was consistent with the OECD, (2008) report, linking thyroid modulation to delayed development. The present result is also generally consistent with the findings in several studies, reporting that the inhibition of developmental stages may be linked to thyroid disruption (Snawder and Chamber, 1990; Richard and Kendall, 2003; Balch *et al*., 2006; Brunelli *et al*., 2009; Shi *et al*., 2012; Tranchantong *et al*., 2013). The thyroid gland histology in the Arsenal exposed tadpoles showed a significant decrease in colloidal area at concentrations of 0.5 and 2 mg/L as well as significant increase in follicular epithelium height at all exposure concentrations; alongside the reduction pattern in gland area which was significantly different at 2 mg/L compared to the control. These results are consistent with several studies, suggesting that a decreased in thyroid gland size is an important indication of the thyroid system disruption (Khan *et al*., 1999; Degitz *et al*., 2005; Carlson and Norrgreen, 2007; Brunelli *et al*., 2009). The potential modulation of thyroid action at the target tissue level was confirmed with the significant reduction in mean HLL (normalized to SVL) at concentration of 3.5 mg/L, as well as significant increase in body mass and SVL at concentration of 3.5 mg/L. This reduction in HLL has been described to be sensitive to changes in circulating TH (Goleman *et al*., 2002; Mitsui *et al*., 2006; Shi *et al*., 2012). That this Arsenal formulation mainly inhibited the sensitive and thyroid dependent HLL, without generally affecting the overall somatic growth (as evidence in concentration dependent increase in WBL and wet body mass), confirms the anti-thyroidal effects of this formulation on the Xenopus laevis tadpoles. The increased in body mass might also be a response to the inhibiting activities of the formulation. Coady *et al*. (2010) suggested that an inhibition of metamorphosis during a 21-day XEMA, at which time the tadpoles would

normally be approaching metamorphic climax, would likely result in increased body mass. As was also noted by Brande-Larviden *et al*. (2010), that whenever chronic TH deficiency retarded or prevent metamorphosis, oversized larvae resulted. The increased body size therefore may be linked to a chronic impact of TH deficiency due to Arsenal formulation exposure.

Ecotoxicological studies suggested that toxicity of formulations may be attributed to the inclusion of surfactants (Edge *et al.*, 2013). The Arsenal formulation is known to contain nonyl phenol as surfactant (Grisolia *et al*., 2004), and several studies have reported estrogenic potential (Trumbo, 2005; Othman, 2009), thyroid inhibitory activity (Chritensen *et al*., 2005; Yang *et al*., 2005; Mann *et al*., 2009) as well as narcosis (Mann and Bidwell, 2001) associated with nonyl phenol. The surfactant's contribution to the thyroid inhibition in this formulation, therefore, needs confirmation through further studies.

Midstream formulation

Similar to the Arsenal formulation, exposure to the Midstream formulation resulted in a significant reduction in mean developmental stage at the higher concentration of 0.11 and 0.14 mg/L. However, histological observation showed that the gland area was not significantly different when compared to the control tadpoles, although a significant reduction in colloidal area (at concentration of 0.14 mg/L) alongside a slight increase in follicular epithelium (significant at 0.05 mg/L) occured. The dissociation of developmental effects and thyroid histopathology, could imply a target tissue effect (extra-thyroidal pathways) (Opitz *et al*., 2005; OECD, 2008; Shi *et al*., 2012**)** or the inhibition of T4 to T3 transformation (Miyata and Ose, 2012**)**, rather than HPT regulatory effects. Saka *et al*. (2013) reported a similar result for Simetryn herbicide, and concluded that the delay in development may be due to non-thyroidal effects**.**

As noted in several studies, the active ingredient of Midstream formulation, diquat dibromide, have been linked to high oxidative mode of action (Hook *et al*., 2006). This oxidative mode of action usually results to selective accumulation of herbicide in fish liver, and then undergo redox cycling that produced superoxide, leading to stress condition (Yadav *et al*., 2013). The inhibition of developmental and other thyroid controlled endpoints as well as growth endpoints by the Midstream formulation at concentration below 0.733 mg/L which is the expected environmental concentration of this formulation (Peterson *et al*., 1994) is a serious ecological highpoint that deserved further attention.

Basta formulation

In contrary to the Arsenal and Midstream formulations, exposure to Basta formulation did not show any significant differences in developmental rate compared to the control. However, histological evidence showed that at concentration of 0.15 and 0.25 mg/L significant increases in follicle epithelium and gland area were evident. Although no known thyroid activities studies using XEMA have been done with the Basta formulation, histopathological effects on thyroid glands histology have been demonstrated in the absence of developmental effects (OECD, 2008) and the thyroid gland histology has been noted as the most sensitive parameter for the detection of the thyroidal effects (Brande-Larviden *et al*., 2010). The lack of developmental effects could be due to physiological thyroid compensatory mechanism. The observed enlarged thyroid gland following Basta formulation exposure, therefore suggested potential modulation in the thyroid hormone related pathways in exposed tadpoles. The enlarged gland area and increased follicle epithelium is also consistent with the results of several other related studies (Opitz *et al*., 2005; Mitsui *et al*., 2006; Carlson and Norrgreen, 2007; Mirata and Ose, 2012). These studies noted similar effects in Propylthioural (PTU) (a known thyroid inhibitor) on the exposed tadpoles, pointing towards enlarged thyroid gland (goitre effect) This therefore means that Basta formulation may be upregulating T4 production, or directly lower or inhibit circulating thyroid hormone, resulting in the overstimulation of the HPT axis (Degitz *et al*., 2005). The Basta formulation may also modulate the transformation of T4 to T3, hence the normal stage development but increased T4 production. The thyroid disrupting potential of the Basta formulation at concentrations far below its expected environmental concentration of 1.0 mg/L (Dinehart *et al*., 2010) should be regarded as an ecological concern in the aquatic environment.

Although the Basta formulation did not significantly affected tadpole development, the mean hind limb length (HLL; normalised to SVL) (thyroid hormone sensitive) did not show a reduction in the lower concentration used in the exposed tadpoles. The fact that no significant variation occurred in size related endpoints corroborate similar findings by Degitz *et al*. (2005) and Coady *et al*. (2010), that chemical alteration of thyroid axis do not necessarily affect the growth of tadpoles as the growth is not directly under the thyroid hormones. Tata, (1999) also noted that thyroidectomised tadpoles continue to grow (under the influence of growth hormone and other growth- promoting factors) but without final metamorphosis into the adult phenotypes.

Kilo Max formulation

Similar to the Midstream exposures, the Kilo Max formulation significantly inhibited developmental stages at 190 and 280 mg/L without impacting on the histopathology of the thyroid glands. Although, Saka *et al*. (2013) suggested non-thyroidal pathways affected the development programme of the tadpoles exposed to the herbicide Simetryn. Kilo Max formulation could also disrupt interaction at the target tissue level, be disrupting T4 transportation to target tissues, or the transformation of T4 to T3 (diodinase activity) in the target tissues (Balch *et al*., 2006). The reduced tadpoles size, for example, endpoints like body mass and whole body length, suggest that aside from possible endocrine disrupting activities, there is possible extra-thyroidal toxic activities involved. As pointed out by Optiz et al. (2005), total blockage of TH synthesis and thus complete inhibition of metamorphosis does not necessarily inhibits growth of the tadpoles,

Roundup formulation

Development of Roundup-exposed tadpoles was not significantly affected when compared to that of the tadpoles in the control group. However, evidence of thyroid inhibiting activities was noted in histopathology of the thyroid glands. The significant increase in follicle epithelium height at all exposure concentrations, as well as increased in colloidal area (goitre phenotype), at a concentration of 0.6 mg/L was recorded. This goitre phenotype, may be an indication of thyroid hormone synthesis, either by modulating iodine uptake or biosynthesis of TH and through hyperthyroidism (Mitsui *et al*., 2006; Opitz *et al*., 2006; Carlson and Norrgreen, 2007; Brande-Lavriden *et al*., 2010; Mirata and Ose, 2012). As noted by several researchers, low level of thyroid hormone (TH) usually activates the HPT for increase secretion of TSH from the pituitary gland, resulting in excessive stimulation, and an increase in thyroid gland size (Capen, 1997; OECD, 2008). However, it is expected that low circulating free TH, could result in the arrest of the developmental programme in tadpoles. This is consistent with the result of Howe *et al*. (2004), who noted the inhibitory impacts of Roundup formulation on the *R. pipiens* tadpoles.

The tadpoles exposed to Roundup also showed a significant reduction in mean body mass at concentrations of 0.4 and 0.6 mg/L. This findings is consistent with the result of Howe *et al*. (2004), who noted that Roundup exposed *R. pipiens* tadpoles were significantly smaller compared to the control tadpoles. This body mass reduction might be a physiological response to high toxicity effects of the Roundup formulation, since the same effects was observed in tadpoles exposed to POEA surfactant (Howe *et al*., 2004). This reduction in body mass impact at concentration below the Roundup formulation's expected environmental concentration of 1.43 mg/L (Govandarajulu,

2008) could have serious implication on the development of the tadpoles. Several researchers have noted the numerous implications of the size reduction, including increasing chance of predation, and possible influence for lower survival rate and later reproductive fitness (Semlitch 1989; Sullwan and Spencer 2003; Howe *et al*., 2004; Gupta, 2012). The size reduction may also have strong downstream effects on adult phenotype and fitness (Dmitriew and Rowe, 2011). This means that reduction in *X. laevis* body mass resulted by exposure to the Roundup formulation will have negative impact on the reproductive fitness and subsequently have wider effects on the population.

Enviro glyphosate

Both the developmental stages and histological evidence showed that there was no difference between the treated tadpoles and the control development stages of the exposed tadpoles compared to the control. However, the reduction in body mass, SVL and WBL at concentration of 28 mg/L, suggested the possible involvement of toxic properties of the formulation rather than the thyroidal activities (Opitz *et al*., 2005; Opitz *et al*., 2006; OECD, 2008).

The amphibian (Xenopus) metamorphosis assay (AMA OR XEMA) has been shown to be a valuable tool to assess thyroid-dependent developmental modulations caused by man-made chemicals (Tietge *et al*., 2013). In terms of comparison with the results of the present study, not many published studies have dealt with thyroid response following chronic exposure of developing tadpoles to herbicides. Saka *et al*. (2013) studied three rice paddy herbicide formulations using the closely related *Silurana tropicalis* as indicator species. Similar to the present studies, varied endpoint responses were obtained, for example, Simetryn (triazine) resulted in delayed development and smaller body sizes, but had no histopathological effects on the thyroid gland, therefore suggesting a non-thyroidal modulation of development and growth. The other herbicides, Mefenacet (acid-amine) and Thiobencarb (carbamate) had no developmental, growth or thyroid histopathological effects.

4.5. Conclusion

The present study assessed the chronic toxicity of six herbicide formulations (Arsenal, Midstream, Basta, Roundup, Kilo Max and Enviro glyphosate) used to control invasive alien plants along and in waterways using the Xenopus metamorphosis assay (XEMA) under semi-static renewal conditions. The herbicides were tested for 21 days, using different dilutions centred on the environmental relevant concentrations **(**Midstream, Basta, Roundup and Arsenal Formulations) or the 15, 30 and 45% of the 96-hr LC50 values at NF-stage 48 (Kilo Max and Environ Glyphosate

formulations). Using morphometric, as well as histopathological endpoints, the outcome of the exposure showed that the selected herbicide formulations differentially inhibited the developmental stages, body sizes and histopathology of the thyroid in *X. laevis* (Table 4.8)

Table 4.8: Result summary of exposure chronic toxicity exposure impacts of the six herbicide formulations on *X. laevis.*

Herbicide ERC ot 15, 30, 45% of 96 Hr LC_{50}	Developmental Stage affected	Body size affected	Front limb affected	Hind limb Affected	Thyroid histology Affected
Arsenal (ERC)	Yes	No	No	Yes	Yes
Midstream (ERC)	Yes	No	Yes	Yes	No
Basta (ERC)	No	No	No	Yes	Yes
Kilo Max (96 Hr LC_{50} @ NF 48)	Yes	Yes	Yes	No	No
Roundup (ERC)	No	Yes	No	No	Yes
Enviro Glyphosate(96 Hr LC_{50} @ NF 48)	No	Yes	No	No	No

In South Africa, because of large volumes (and diversity) of pesticides been used, attention must therefore be directed in assessing the exposure impacts of these pesticide formulations on human as well as wildlife. Clearly, chronic exposures including environmental relevant dilutions, needs more attention. In particular, potential thyroid disruption needs more attention, especially in light of the varied responses reported in the present study. Moreover, additional confirmation research is needed to understand/model the potential population effects that herbicides may have on local amphibian species.

References

Allen, M.A. (1929).The influence of the thyroid gland and hypophysis upon growth and development of amphibian larvae. *The Quarterly Review of Biology* **4 (3)***:* 325-352.

American Society for Testing and Materials (ASTM) (1998). Standard guide for conducting the frog embryo teratogenesis assay – Xenopus (FETAX). E1439-98. In: Annual book of ASTM standards, Vol 11.06. American Society for Testing and Materials, Philadelphia: 825-836.

Balch, G.C.; Luis, A.; Ve´lez-Espino, C. S.; Alaee, M.; Metcalfe, C.D. (2006). Inhibition of metamorphosis in tadpoles of *Xenopus laevis* exposed to polybrominated diphenyl ethers (PBDEs). *Chemosphere* **64**: 328–338.

Bancroft, J. D.; Stevens, A. (1977). Theory and Practice of Histological Techniques. Churchill Livingstone, Edinburg.

Benachour, N.; Seralini, G.E. (2009). Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. *Chemical Research in Toxicology* **22**: 97–105.

Bergman, A.; Andersson, A.; Becher, G.; van den Berg, M.; Blumberg, B.; Bjerregaard, P.; Bornehag, C.; Bornman, R.; Brandt, I.; Brian, J.V.; Casey, S.C.; Fowler, P.A.; Frouin, H.; Giudice, L.C.; Iguchi, T.; Hass, U.; Jobling, S.; Juu, A.; Kidd, K.A.; Kortenkamp, A.; Lind, M., Martin, O.V.; Muir, D.; Ochieng, R.; Olea, N.; Norrgren, L,; Ropstad, R.E.; Ross, P.S.; Rudén, C.; Scheringer, M.; Skakkebaek, N.E.; Söder, O.; Sonnenschein, C.; Soto, A.; Swan, S.; Toppari, J,; Tyler, C.R.; Vandenberg, L.R.; Vinggaard, A.M.; Wiberg. K.; Zoeller. R.T. (2013). Science and policy on endocrine disrupters must not be mixed: a reply to a "common sense" intervention by toxicology journal editors. *Environmental Health* **12**: 69.

Bo¨gi, C.; Schwaiger, J.; Ferling, H.; Mallow, U.; Steineck, C.; Sinowatz, F.; Kalbfus, W.; Negele, R.D.; Lutz, I.; Kloas, W. (2003). Endocrine effects of environmental pollution on *Xenopus laevis* and *Lithobates temporaria*. *Environmental Research* **93**: 195–201.

Boas, M.; Feldt-Rasmussen, U.; Skakkebæk N.E.; Main, K.M. (2006). Environmental chemicals and thyroid function. *European Journal of Endocrinology* **154***:* 599–611.

Boggs, A. S. P.; Lowers, R. H.; Cloy-McCoy, J. A.; Gillette, L. J. Jr (2013). Organizational changes to thyroid regulation in alligator mississippiensis: Evidence for predictive adaptive responses. *PLoS ONE **8**(1): e55515*.

Bollmohr, S.; Thwala, M.; Jooste, S.; Havemann, A. (2008). *An Assessment of Agricultural Pesticides in the Upper Olifants Catchment*. Report Resource Quality Services, Pretoria, South Africa, p. 45.

Brande-Lavridsen, N.; Christensen-Dalsgaard, J.; Korsgaard, B. (2010). Effects of ethinylestradiol and the fungicide prochloraz on metamorphosis and thyroid gland morphology in *Lithobates temporaria. The Open Zoology Journal* **3***:* 7-16.

Brown, A. L. (1970). The African Clawed Frog. Butterwoth & Co Publishers, London.

Brunelli, E.; Bernabo, I.; Berg, C.; Lundstedt-Enkel, K.; Bonacci, A.; Tripepi, S. (2009). Environmental relevant concentrations of endosulfan impair development, metamorphosis and behavior in Anaxyrus tadpoles. *Aquatic Toxicology* **91**: 135-142.

Cabrera-Guzma´n, E.; Crossland, M. R.; Brown, G. P.; Shine, R. (2013). Larger body size at metamorphosis enhances survival, growth and performance of young Cane toads (*Rhinella marina*). *PLoS ONE **8**(7)*: e70121.

Carlsson, G.; Norrgren, L. (2007). The impact of the goitrogen 6-propylthiouracil (PTU) on West-African clawed frog (*X. tropicalis*) exposed during metamorphosis. *Aquatic Toxicology* **82**: 55–62.

Carey, C.; Bryant, C. J. (1995). Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. *Environmental Health Perspectives* **103**:13–17.

Cauble, K.; Wagner, R. S. (2005). Sub-lethal effects of Glyphosate Herbicide on amphibian metamorphosis and development**.** *Bulletin of Environmental Contamination and Toxicology* **75**:429–435.

Coady, K. K.; Murphy, M. B.; Villeneuve, D. L.; Hecker, M.; Carr, J. A.; Solomon, K. R.; Smith, E. E.; Van Der Kraak, G.; Kendall, R. J.; Giesy, J. P.(2005). Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, sex steroid hormones, and aromatase activity in juvenile *X. laevis. Ecotoxicology and Environmental Safety* **62 *(2)***:160-173.

Coady, k.; Marino, T.; Thomas, J.; Currie, R.; Hancock, G.; Crofoot, J.; Mcnalley, L.; Mcfadden, L.; Geter, D.; Klecka, G. (2010). Evaluation of the amphibian metamorphosis assay: exposure to the goitrogen methimazole and the endogenous thyroid hormone-thyroxine. *Environmental Toxicology and Chemistry* **29(4*)***: 869–880.

Collins, J. P. (1975). A Comparative Study of the Life History Strategies in a Community of Frogs. Ph.D. dissertation. University of Michigan, Ann Arbor U.S.A.

Collins, J. P.; Storfer, A. (2003). Global amphibian declines: Sorting the hypotheses. *Diversity and Distribution* **9**: 89–98.

Dabrowski, J. M.; Shadung J. M.; Wepener, V. (2014). Prioritizing agricultural pesticides used in South Africa based on their environmental mobility and potential human health effects. *Environment International.* **62**: 31-40.

Dabrowski, J.; Oberholster, P. J.; Dabrowski, J. M. (2014). Water quality of Flag Boshielo Dam, Olifants River, South Africa: historical trends and the impact of drought. *Water SA 40* DOI: http://dx.doi.org/10.4314/wsa.v40i2.17.

Degitz, S. J.; Holcombe, G. W.; Flynn, K. M.; Kosian, P. A.; Korte, J. J.; Tietge, J. E. (2005). Progress towards development of an amphibian-based thyroid screening assay using *Xenopus laevis*. Organismal and thyroidal responses to the model compounds 6-propylthiouracil, methimazole, and thyroxine. *Toxicological Sciences* **87(2***)*: 353–364.

Doerge, D. R.; Takazawa, R. S. (1990). Mechanism of thyroid peroxidase inhibition by Ethylenethiourea**.** *Chemical Research inToxicology.* **3**: 98-101.

Diamanti-Kandarakis, E.; Bourguignon, J.; Giudice, L. C.; Hauser, R.; Prins, G. S.; Soto, A. M.; Zoeller, R. T.; Gore, A. C. (2009). Endocrine disrupting chemicals. An endocrine society scientific statement. *Endocrine Reviews* **30***(4)*: 293-342.

Dinehart, S. K.; Smith, L. M.; McMurry, S. T.; Anderson, T. A.; Smith, P. N.; Haukos, D. A. (2009). Toxicity of a glufosinate and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plain, USA. *Science of Total Environment* **407**: 1065-1071.

Dinehart, S. K.; Smith, L. M.; McMurrya, S. T.; Smith, P. N.; Anderson, T. A.; Hauko, D. A. (2010). Acute and chronic toxicity of Roundup Weathermax_ and Ignite_ 280 SL to larval *Spea multiplicata* and *Spea bombifrons* from the Southern High Plains, USA. *Environmental Pollution* **158**: 2610-2617.

Dmitriew, C.; Rowe, L. (2011). The effects of larval nutrition on reproductive performance in a food-limited adult environment. *PLoS ONE* **6**(**3***)*: e17399.

Ebert, E.; Leist, K. H.; Mayer, D. (1990). Summary of safety evaluation toxicity studies of glufosinate ammonium. *Food and Chemical Toxicology* **28**: 339-349.

Edge, C. B., Gahl, M. K., Thompson, D. G., Houlahan, J. E (2013). Laboratory and field exposure of two species of juvenile amphibians to a glyphosate-based herbicide and Batrachochytrium dendrobatidis. *Science of the Total Environment* **444**: 145–152.

Emmett, K. (2002). Final risk assessment for diquat bromide. The water quality program of the Washington State department of ecology. 02-10-046. www.wsde.org/document. Assessed on 12 March 2013.

Fort, D. J.; Stower, E. L.; Norton, D. (1995). Ecological hazard assessment of aqueous soil extracts using FETAX. *Journal of Applied Toxicology* **15**: 183-191.

Fort, D. J.; Guiney, P .D.; Weeks, J. A.; Thomas, J. H.; Rogers, R. L.; Noll A M: Sparling, C. D. (2004). Effect of methoxychlor on various life stages of *Xenopus laevis*. *Toxicological Sciences* **81**: 454–466.

Fort, D. J.; Thomas, J. H.; Rogers, R. L.; Noll, A.; Spaulding, C. D.; Guiney, P. D.; Weeks, J. A. (2004). Evaluation of the developmental and reproductive toxicity of methoxychlor using an anuran (*X. tropicalis*) chronic exposure model. *Toxicological Sciences* **81**: 443–453.

Giesy, J. P.; Dobson, S.; Solomon, K. R. (2000). Ecotoxicological risk assessment for Roundup herbicide. *Reviews of Environmental Contamination and Toxicology* **167**: 35-120.

Goleman, W. L.; Urquidi, L. J.; Anderson, T. A.; Smith, E. E.; Kendall, R. J.; Carr, J. A. (2002). Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *X. laevis*. *Environment Toxicology and Chemistry* ***21***:424–430.

Govindarajulu, P. (2008). Review of impacts of glyphosate herbicide on amphibians: What risks can the silvicultural use of this herbicide pose for amphibians in B.C. British Columbia Ministry of Environment? Wildlife Report No R-28. www.organiclandcare.org. (Accessed March 14, 2012).

Grisolia, C. K.; Bilich, M. R.; Formigli, L. M. (2004). A comparative toxicological and genotoxic study of the herbicide arsenal, its active ingredient imazapyr, and the surfactant nonylphenol ethoxylate. *Ecotoxicology and Environmental Safety* **59**:123–126.

Gutleb, A. C.; Appelman, J.; Bronkhorst, M.; van den Berg, J. H.; Murk, A. J. (2000). Effects of oral exposure to polychlorinated biphenyls (PCBs) on the development and metamorphosis of two amphibian species (*X. laevis* and *R. temporaria*). *Science of Total Environment* **262**: 147–157.

Gungordu, A. (2013). Comparative toxicity of methidathion and glyphosate on early life stages of three amphibian species: *Pelophylax ridibundus*, *Pseudepidalea viridis*, and *Xenopus laevis*. *Aquatic Toxicology* **140– 141**: 220– 228.

Hack, R.; Ebert, E.; Ehling, G.; Leist, K. H. (1994). Glufosinate ammonium - some aspects of its mode of action in mammals. *Food and Chemical Toxicology* **32 (5)**: 461-470.

Hayes, T.; Tsui, M.; Hoang, A.; Haeffele, C.; Vonk, A. (2003). Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs *(Lithobates pipiens*); laboratory and field evidence. *Environmental Health Perspectives* **111**: 568-575.

Hayes, T.; Haston, K.; Tsui, M.; Hoang, A.; Haeffele, C.; Aaron Vonk (2002a). Feminization of male frogs in the wild. *Nature **419***: 895-896.

Hayes, T.B.; Collins, A.; Lee, M.; Mendoza, M.; Noriega, N.; Stuart, A.A.; Vonk, A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proc. Natl. Acad. Sci. U S A **99**: 5476-5480.

Hayes, T. B.; Collins, A.; Mendoza, M.; Noriega, N.; Stuart, A. A.; Vonk, A. (2003). Atrazine induced hermaphroditism at 0.1 ppb in American leopard frogs *(Lithobates Pipiens):* laboratory and field evidence. *Environmental Health Perspectives* **3**: 568-575.

Hayes, T. B.; Case, P.; Chui, S.; Chung, D.; Haeffele, C.; Haston, K.; Lee, M.; Mai, V.P.; Marjuoa, Y.; and Tsui, M. (2006). Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? *Environmental Health Perspective* **114**:40–50.

Hermelink, B.; Urbatzka, R.; Wiegand, C.; Pflugmacher, S.; Lutz, I.; Kloas, W. (2010). Aqueous leaves extract display endocrine activities in vitro and disrupt sexual differentiation of male *X. laevis* tadpoles in vivo. *General and Comparative Endocrinology* **16***:* 245-255.

Helbing, C. C.; Maher, S. K.; Han, J.; Gunderson, M. P.; Borchers, C. (2010). Peering into molecular mechanisms of action with FrogSCOPE. *General and Comparative Endocrinology* **168**: 190–198.

Helbing, C. C.; Propper, C. R.; Veldhoen, N. (2011).Triclosan affects the thyroid axis of amphibians. *Toxicological Sciences* **123(2)**: 601–602.

Heindel, J. J.; Zoeller, R.; Jobling, S.; Iguchi, T.; Vandenburg, M. J.; Woodruff, T. J. (2013). What is endocrine disruption all about? In : Bergman A, Hindel J. J.; Jobling S.; Kidd V.A.; and Zoeller R, eds. State of the science; endocrine disrupting chemicals- Geneva; WHO & UNEP. 1- 22.

Hook, S.E.; Skillman, A.D.; Small, J.A.; Schultz, I.R. (2006). Gene expression partterns in rainbow trout, Oncorhynchus mykiss, exposed to a suite of model toxicants. *Aquatic Toxicology* **77**: 372-385.

Houlahan, J. E.; Findlay, C. S.; Schmidt, B. R.; Meyer, A. H.; Kuzmin, S. L. (2000). Quantitative evidence for global amphibian population declines. *Nature **404***:752–755.

Howe, C. M.; Berrill, M.; Pauli, B. D.; Helbing, C.; Werry, K.; Veldhoen, N. (2004).Toxicity of glyphosate-based pesticides to four North American frog species. *Environmental Toxicology and Chemistry* **23**: 1928–1938.

Jiraungkoorsku, W.; Upatham, E. S.; Kruatrachue, M.; Sahaphong, S.; Vichasri-Grams, S.; Pokethitiyook, P. (2002). Histopathological effects of Roundup, a glyphosate herbicide, on Nile tilapia. *Science Asia* **28**: 121-127.

Jobling, S.; Bjerregaard, P.; Blumberg, B.; Brandt, I.; Brain, J.V.; Casey, S.C.; Frouin, H.; Giudice, L. C.; Heindal, J. J.; Igushi, T.; Kidd, K. A.; Kortenkamp, A.; Lind, M.; Ropstad, E.; Ross, P. S.; Shakkebaek, N.; Toppari, J.; Woodruff, T. J.; Zoeller, R. (2013). Evidence for endocrine disruption in human and wildlife. In: Bergman A, et al (2013). State of the science of endocrine disrupting chemicals- Geneva: WHO & UNEP, 23-186.

Jones, D. K.; Hammond, J. I.; Relyea, R. A. (2010). Roundup and amphibians: the importance of concentration, application time, and stratification. *Environmental Toxicology and Chemistry* **29***:* 2016–2025.

Jones, D. K.; Hammond, J. I.; Relyea, R. A. (2011). Competitive stress can make the herbicide Roundup more deadly to larval amphibians. *Environmental Toxicology and Chemistry* **30**:446–454.

Khan, M. A.; Davis, C. A.; Foley, G. L.; Friedman, M. A.; Hansen, L. G. (1999). Changes in thyroid gland morphology after acute acrylamide exposure. *Toxicological Sciences* **47***:* 151-157.

Kidd, K. A.; Becher, G.; Bergman, A.; Muir, D. C. G.; Woodruff, T. J. (2013). Human and wildlife exposures to EDCs, In: Bergman, A., Heindel, J., Jobling, S., Kidd, K. A., Zoeller, R. T. (Eds.), State of the Science of Endocrine Disrupting Chemicals - 2012. WHO and UNEP, Geneva, 189-209.

Kloas, W.; Lutz, I.; Einspanier, R. (1999). Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals *in vitro* and *in vivo*. *Science of the Total Environment* **225**: 59-68.

Kloas, W.; Urbatzka, R.; Opitz, R.; Wurtz, S.; Behrends, T.; Hermelink, B.; Hoffmann, F.; Jagnytsch, O.; Kroupova, H.; Lorenz, C.; Neumann, N.; Pietsch, C.; Trubiroha, A.; Ballegooy, C.; Wiedermann, C.; Lutz, I. (2009). Endocrine disruption in aquatic vertebrates. Trends in comparative endocrinology and neurobiology: *Annual NewYork Academic Science* **1163**: 187-200.

Kortenkarp, A.; Martin, O.; Faust, M.; Evans, R.; McKinlay, R.; Orton, F.; Rosivatz, E. (2011). State of the art assessment of endocrine disrupters- Final report project Contract Number 070307/2009/550687/SER/D3.

Koyama, K.; Koyama, K.; Goto, K. (1997). Cardiovascular effects of an herbicide containing glufosinate and a surfactant: In vitro and in vivo analyses in Rat. *Toxicology and Applied Pharmacology* **145***:* 409-414.

Lanctôt, C.; Robertson, C.; Navarro-Martín, L.; Edge, C.; Melvin, C.D.; Houlahan, J.; Trudeau, V. L (2013). Effects of the glyphosate-based herbicide Roundup WeatherMax on metamorphosis of wood frogs (*Lithobates sylvaticus*) in natural wetlands. *Aquatic Toxicology* **140– 141***:* 48– 57.

Lenkowski, J. R.; Reed, J. M.; Deininger, L.; McLaughlin, K. A. (2008). Perturbation of organogenesis by the herbicide atrazine in the amphibian Xenopus *laevis*. *Environmental Health Perspectives* **116**: 223–230.

Lushchak, O V.; Kubrak, O. I.; Storey, J. M.; Storey, K. B.; Lushchak, V .I (2009). Low toxic herbicide Roundup induced mild oxidative stress in Goldfish tissues. *Chemosphere* **76***:* 932-937.

Marlatt, V.L.; Gerrie, E.; Wiens, S.; Jackson, F.; Moon, T.W.; Trudeau, V.K.L. (2012). Estradiol and triiodothyronine differentially modulate reproductive and thyroidal genes in male goldfish. *Fish Physiology and Biochemistry*.**38**:283–296.

Marlatt, V.L.; Veldhoen, N.; Lo, B.P.; Bakker, D.; Rehaume, V.; Vallée, K.; Haberl, M.; Shang, D.; van Aggelen, G.C.; Skirrow, R.C.; Elphick, J.R.; Helbing, C.C.(2013). Triclosan exposure alters postembryonic development in a Pacific tree frog (*Pseudacris regilla*) Amphibian Metamorphosis Assay (TREEMA). *Aquatic Toxicology* **126**: 85– 94.

Mann, R. M.; Bidwell, J. R. (2000). Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. *Aquatic Toxicology* **51:** 19-29.

Mann, R. M.; Hyne, R. V.; Choung, C. B.; Wilson, S. P (2009). Amphibians and agricultural chemicals: review of the risks in a complex environment. *Environmental Pollution* **157**: 2903-2927.

Mensah, P. K.; Palmer, C. G.; Muller, W. J. (2013). Derivation of South African water quality guidelines for Roundups using species sensitivity distribution. *Ecotoxicology and Environmental Safety* **96**: 24–31.

Mesnage, R.; Clair, E.; Spiroux de Vendomois, J.; Seralini, G. E (2009). Two cases of birth defects overlapping the Stratton-Parker syndrome after multiple pesticide exposure. *Occupational and Environmental Medicine* **67(5)**: 359-362.

Mnif, W., Hassine, A.I., Bouaziz, A., Bartegi, A., Thomas, O., Roig, B (2011). Effect of endocrine disruptor pesticides: a review. *International Journal of Environmental Research and Public Health* **8**: 2265-2303.

Mitsui, N.; Fujii, T.; Miyahara, M.; Oka, T.; Kashiwagi, A.; Kashiwagi, K.; Hanada, H.; Urushitani, H.; Santo, N.; Tooi, O.; Iguchi, T. (2006). Development of metamorphosis assay using *S. tropicalis* for the detection of thyroid system-disrupting chemicals. *Ecotoxicology and Environmental Safety* **64**: 281– 287.

Miyata, K.; Ose, K. (2012). Thyroid hormone-disrupting effects and the amphibian metamorphosis assay. *Journal of Toxicology and Pathology* **25***: 1–9.*

Nieuwkoop P.D., Faber J (1994). Normal table of *Xenopus laevis* (Daudin). North-Holland Publishing Co. Amsterdam

Norris, D.O., Carr, J.A. (2006). Endocrine Disruption: Biological bases for health effects in wildlife and humans. Oxford University Press, Oxford.

O'Connor, J. C.; Frame S. R.; Davis L. G.; Cook J. C. (1999). Detection of thyroid toxicants in a tier I screening battery and alterations in thyroid endpoints over 28 days of exposure. *Toxicological Sciences* **51**: 54-70.

Oka, T.; Tooi, O.; Mitsui, N.; Miyahara, M.; Ohnishi, Y.; Takase, M.; Kashiwagi, A.; Santo, N.; Iguchi, T. (2008). Effect of atrazine on metamorphosis and sexual differentiation in *Xenopus laevis*. *Aquatic Toxicology* **87**: 215–226.

Opitz, R.; Braunbeck, T.; Bo¨gi, C.; Pickford, D. B.; Nentwig, G. (2005). Description and initial evaluation of a *Xenopus* metamorphosis assay for detection of thyroid system disrupting activities of environmental compounds. *Environmental Toxicology and Chemistry* **24(3)**: 653–664.

Opitz, R.; Hartmann, S.; Blank, T.; Braunbeck, T.; Lutz, I.; Kloas, W. (2006). Evaluation of histological and molecular endpoints for enhanced detection of thyroid system disruption in *Xenopus laevis* tadpoles. *Toxicological Sciences* ***90***: 337–348.

Opitz, R.; Schmidt, F.; Braunbeck, T.; Wuertz, S.; Kloas, W. (2009). Perchlorate and ethylenethiourea induce different histological and molecular alterations in a non-mammalian vertebrate model of thyroid. *Goitrogenesis, Molecular and Cellular Endocrinology* **298**: 101-114.

Organisation for Economic Cooperation and Development (OECD) (2006) Current approaches in the statistical analysis of ecotoxicity data. A guidance to application. OECD series on testing and assessment No 54. ENV/JM/ MONO (2006) 18. http://www.oecd.org/officialdocuments. (Accessed Jan 2013).

Organisation for Economic Cooperation and Development (OECD), (2007).Validation of the Amphibian Metamorphosis Assay as a Screen for thyroid-active chemicals: integrated summary report. AMA integrated summary report. http://www.oecd.org/officialdocuments. (Accessed March, 2013).

Organisation for Economic Co-operation and Development, (OECD) (2008). Series on testing and assessment. No. 91. Report of the validation of the amphibian metamorphosis assay (PHASE 3) ENV/JM/MON (2008)18. http://www.oecd.org/officialdocuments. (Accessed Jan, 2013).

Orton, F.; Lutz, I.; Kloas, W.; Routledge, E. J. (2009). Endocrine disrupting effects of herbicides and pentachlorophenol: In vitro and in vivo evidence. *Environmental Science and Technology*.**43***:* 2144-2150.

Osano, O.; Oladimeji, A.; Kraak, M. H. S.; Admiraal, W. (2002). Teratogenic effects of amitraz, 2, 4-dimethylaniline, and paraquat on developing frog (*Xenopus*) embryos**.** *Archives of Environmental Contamination and Toxicology* **43**: 42–49.

Othman, M. Z.; Ding, L.; Jiao, Y. (2009). Effect of anionic and non-ionic surfactants on activated sludge oxygen uptake rate and nitrification. World Academy of Science, Engineering and Technology 58. Available at: www.was.org/publication. (Assessed March, 2013).

Paganelli, A.; Gnazzo, V.; Acosta H.; Lopez, S. L.; Carrasco, A. E. (2010). Glyphosate-Based herbicides produce tetratogenic effects on vertebrates by impairing retinoic acid signalling. *Chemical Research in Toxicology.* **23***:*1586-1595.

Paggetti, E.; Biaggini, M.; Corti, C.; Lebboroni, M.; Berti, R. (2006). Amphibians and reptiles as indicators in Mediterranean agro-ecosystems: A preliminary study. Proceedings of the 13th Congress of the Societas Europaea Herpetologica 107-110.

Patten, K. (2003). Persistence and non-target impact of imazapyr associated with smooth cordgrass control in an estuary. *Journal Aquatic Plant Management* **41**:1-6.

Plakas, K.V., Karabelas, A.J. (2012). Removal of pesticides from water by NF and RO membranes — A review. *Desalination* **287**: 255-265.

Peterson, H. G.; Boutin, G.; Martin, P. A; Freemark, K. E.; Ruecker, N. J.; Moody, M. J. (1994). Aquatic phyto-toxicity of 23 pesticides applied at expected environmental concentrations. *Aquatic Toxicology* **28***: 275-292.*

Preez, L. H.; Nisile Kunene, N.; Everson, G. J.; Carr, J.A.; Giesy J. P.; Gross, T. S.; Hosmer, A. J.; Kendall, R. J.; Smith, E. E.; Solomon, K. R.; Van Der Kraak, G.J (2008). Reproduction, larval growth, and reproductive development in African clawed frogs (*Xenopus laevis*) exposed to atrazine. *Chemosphere* **71**: 546–552.

Relyea, R. A.; Devin, K. J. (2009). The toxicity of Roundup original max to 13 species of larval amphibians. *Environmental Toxicology and Chemistry* **28(9)**: 2004–2008.

Relyea, R. A. (2012). New effects of Roundup on amphibians: predators reduce herbicide mortality; herbicides induce antipredator morphology. *Ecological Applications* **22**: 634–647.

Richard, S.; Moslemi, S.; Sipahutar, H.; Benachour, N.; Seralini, S. E. (2005). Differential effects of glyphosate and Roundup on human placental cells and aromatase. *Environmental Health Perspectives* **113**:716–720.

Richards, S. M.; Kendall, R. J. (2003). Physical effects of Chlorpyrifos on two stages of *Xenopus laevis*. *Journal of Toxicology and Environmental Health* **66**: 75–91.

Rohr, J. R.; McCoy, K. (2010). A qualitative metal analysis reveals consistent effects of atrazine on freshwater fish and amphibians. *Environmental Health Perspectives* **118**:20-32.

Saka, M.; Tada, N.; Kamata, Y. (2013). Application of an amphibian (*Silurana tropicalis*) metamorphosis assay to the testing of the chronic toxicity of three rice paddy herbicides: Simetryn, mefenacet and thiobencarb. *Ecotoxicology and Environmental Safety* **92**; 135–143.

Salthe, S. N. (1969). Reproductive modes and the number and sizes of ova in the urodeles. *American Midland Naturalist.* **81**:467-490.

Schuytema, G. S.; Nebeker, A. V.; Griffis, W. L. (1994). Toxicity of guithion and guthion 2S to *Xenopus laevis* embryos. *Archives of Environmental Contamination and Toxicology* **27**: 250-255.

Schreiber, A. M.; Das, B.; Huang, H.; Marsh-Amstrong, N.; Brown, D. D. (2001). Diverse developmental program of *X. laevis* metamorphosis are inhibited by a dominant negative thyroid hormone receptor. *Developmental Biology* **98 (19)***:* 10739-10744.

Semlitsch, R. D. (1989). Effects of body size, sibship, and tail injury on the susceptibility of tadpoles to dragonfly predation. *Canadian Journal of Zoology* **68**:1027–1030.

Shi, Y. B. (2000). Amphibian Metamorphosis. Wiley-Liss, New York, Chichester, Weinheim, Brisbane, Toronto.

Shi, H.; Zhu, P.; Guo, S. (2012). Effects of tributyltin on metamorphosis and gonadal differentiation of *Xenopus laevis* at environmentally relevant concentrations. *Toxicology and Industrial Health* 1–7.

Snawder, J. E.; Chambers, J. E (1990). Critical time periods and the effect of tryptophan in malathion induced developmental defects in *Xenopus* embryos. *Life Science* **46:**1635–1642.

Stoker, T. E.; Ferrell, J. M.; Laws, S. C.; Cooper, R. L.; Buckale, A. (2006). Evaluation of ammonium perchlorate in the endocrine disruptor screening and testing program's male pubertal protocol: Ability to detect effects on thyroid endpoints. *Toxicology* **228**: 58–65.

Stuart, S. N.; Chanson, J. S.; Cox N. A.; Young B. E.; Rodrigues, A. S. L.; Fischman, D. L.; Waller, R. W (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* **306**: 1783.

Sullivan, K.; Spence, K. (2003). Effects of sub-lethal concentrations of atrazine and nitrate on metamorphosis of the African clawed frog. *Environment Toxicology and Chemistry* **22***:* 627-635.

Tietge, J.E.; Degitz, S.J.; Haselman, J.T.; Butterworth, B.C.; Korte, J.J.; Kosian, P.A.; Lindberg-Livingston A.J.; Burgess, E.M.; Blackshear, P.E.; Hornung, M.W (2013). **I**nhibition of the thyroid hormone pathway in Xenopus laevis by 2-mercaptobenzothiazole. *Aquatic Toxicology* **126**: 128-136.

Tomlin, C. D (2006). *The Pesticide Manual: A World Compendium.* British Crop Protection Council (14th Ed.). Hampshire, UK.

Trachantong, W.; Promya, J.; Saenphet, S.; Saenphet, K. (2013). Effects of atrazine herbicide on metamorphosis and gonadal development of Hoplobatrachus rugulosus. *Maejo International Journal of Science and Technology* **7(**Special Issue), 60-71.

Trumbo, J. (2005). An assessment of the hazard of a mixture of the herbicide Rodeo and the non-Ionic surfactant R-11 to aquatic invertebrates and larval amphibians**.** *California Fish and Game* **91(1):**38-46.

Vitt, L. J.; Caldwell, J. P.; Wilbur, H. M.; Smith, D.C. (1990). Amphibians as harbingers of decay. *Bioscience* **40**: 418.

Vizantinopoulos, S.; Lolos, P. (1994). Persistence and leaching of the herbicide imazapyr in soil. Bulletin of Environmental *Contamination and Toxicology.* **52**:404-410.

Wagner, N.; Wolfram, R.; Hanka, T.; Beatrix, T.; Stefan, L. (2013). Questions concerning the potential impact of glyphosate-based herbicides on amphibians. *Environmental Toxicology and Chemistry* **32(8***):* 1688–1700.

World Health Organisation (WHO) (1990). Public health Impacts of pesticides used in agriculture. World Health Organisation, Geneva. www.who.com/publications

World Health Organisation (WHO) (1994). Glyphosate. Environmental health criteria 159. World Health Organisation, Geneva. www.who.com/publications.

World Health Organisation (WHO) (1996). Glyphosate World Health Organisation, Geneva. www.who.com/publications.

World Health Organisation (WHO) (2004). Diquat in Drinking Water. Background Document for Development of WHO Guidelines for Drinking-water Quality. World Health Organisation. www.who.com/publications.

World Health Organisation (WHO) (2013). State of the Science of Endocrine Disrupting Chemicals. United Nations Environmental Programme and World Health Organization. www.who.org/publications. Assessed on 10th, April, 2014.

Washington State Department of Agriculture (WSDA), (2003). Ecological risk assessment of the proposed use of the herbicide imazapyr to Control Invasive Cordgrass (*Spartina spp) www.ecy.wa.gov. (Accessed October, 2010).*

Williams, B. K.; Semlitsch, R. D. (2010). Larval responses of three Midwestern anurans to chronic, low-dose exposures of four herbicides. *Archives of Environmental Contamination and Toxicology* **58***:* 819–827.

Wilson, E. O (1992). The Diversity of Life. Harvard University Press, Cambridge, MA.

Yadav, S. S.; Giri, S.; Singha, U.; Boro, F.; Giri, A. (2013). Toxic and genotoxic effects of Roundup on tadpoles of the Indian skittering frog (*Euflictis cyanophlyctis*) in the presence and absence of predator stress. *Aquatic Toxicology* **132–133**: 1–8.

Yang, F. X.; Xu,Y.; Wen, S. (2005). Endocrine-disrupting effects of nonylphenol, bisphenol A, and p, p'-DDE on *Lithobates nigromaculata* tadpoles. *Bulletin of Environmental Contamination and Toxicology* **75**: 1168–1175.

Zoeller, R. T.; Tyl, R. W.; Tan, S. W. (2007). Current and potential rodent screens and tests for thyroid toxicants. *Critical Reviews in Toxicology* **37**:55-95.

Zoeller, R. T. (2007). Environmental chemical impacting the thyroid: targets and consequences. *Thyroid* **17 (9)***:* 811-817.

Chapter Five

Impacts of six herbicide formulations on amphibian gonadal (reproductive) development using *Xenopus laevis* as sentinel

5.1. Introduction

There are increasing concerns about the endocrine disrupting potential of anthropogenic chemicals in the environment, particularly having modulatory effects on the reproductive system (Quellet *et al*., 1997). This is because many studies are now linking environmental chemicals to developmental and reproductive disorders in fish (Jobling *et al*., 1998, 2002; Gimen *et al*., 1998; Belt *et al*., 2001; Aerle *et al*., 2001; Jobling *et al*., 2002; Kidd et al., 2007; Urbatzka *et al*., 2007), amphibians (Fort *et al*., 2001; Hayes *et al*., 2002a, Hayes 2005; McDaniel *et al*., 2008), reptiles (Guillette *et al*., 1994; Crain *et al*., 1997; Crain 1997; Crain *et al*., 1998), birds (Giesy *et al*., 1994), mammals (D'Souza, 2004), and humans (WHO, 2013). These substances adversely affect the reproductive biology through inhibition of spermatogenesis, induction of sex reversal and disruption of gonadal development (Guillette *et al*., 1994; Urbatzka *et al*., 2007) as well as numerous reproductive abnormalities (Soyano *et al*. 2010). Exposure to EDCs, which alter normal hormonal signalling during embryonic development could permanently change adult reproductive system morphology and function as well as reproductive behaviour (Guillette *et al*., 1995). The fact also that reproductive disorder is one of the most serious properties of any chemical makes environmental impacts on reproductive system highly important (Magnusson and Brunstrom, 2007).

Today, more than ever before, there are increasing challenges on the ability of organisms to reproduce and develop properly **(**Kortenkamp *et al*., 2011). For example, pesticides and other chemical contaminants have been suggested as contributing factor in the global amphibian declines (Hayes *et al*., 2002a; Hayes 2005; McDaniel *et al*., 2008; Brunnel *et al*., 2009). In fact, surveys of natural amphibian populations have shown correlations between population declines and proximity to agricultural land and activities (Bishop *et al*., 1999). As noted by Guillette *et al*. (2000), these potential endocrine disrupting pesticides can cause organizational effects on developing embryos, which in turn can permanently modify the organizational function in a manner that will impair the future function of the reproductive system, thus leading to possible serious effects on natural populations.

Even in the absence of toxicants, ontogeny period is a very vulnerable time in the life cycle of amphibians (Carey and Bryant, 1995), just like all other animals. According to Carey and Bryant,

(1995), the amphibian reproductive success could be impaired by environmental interference that could lead to inhibition of breeding behaviour, disruption in gamete production, or fertilization. Growth, metabolism and reproductive fitness are interwoven. Boggs *et al*. (2011) suggested that exogenous effects on thyroid hormones can alter reproduction by altering growth and metabolism. They noted that during juvenile life stages, a large percentage of the energy budget is channelled towards growth, hence reduced growth rate due to hormonal imbalances could negatively impacts sexual maturation, clutch and egg sizes, in a manner that could lead to reduce hatching size and survival. Body size at metamorphosis influences body size at first reproduction (Collins, 1979), while reduced size at metamorphosis is likely to be followed by reduced survival and impaired reproductive fitness (Gupta, 2012).

Globally, herbicides are extensively used in agriculture and environmental management. South Africa has the largest pesticides market in sub-Saharan African, accounting for about 60% of the pesticides used in Africa (Ansara-Ross *et al*., 2012). According to Ansara-Ross *et al*. (2012), there has been an increase in application of glyphosate-based herbicides in South Africa in recent years. For example, large numbers of farmers are applying herbicides in their intensive wheat, corn and fruits farming practises, aside from being widely used in gardening. In South Africa, the Working for Water Program of the National Department of Water Affairs also uses large volumes of various herbicides in managing alien vegetation in valuable water catchment areas including glyphosate-based formulations, glufosinate ammonium formulations, Imazapyr and diquat dibromide formulations among many others.

Imazapyr is one of the most widely used herbicides in the world. It is very mobile and persistent, with a half-life of around 50 months on soil surface (Vizantinopoulon and Lolos, 1994). Imazapyr formulations include Arsenal, Chopper and Format. The Arsenal formulation of Imazapyr consists of 25 g/L Imazapyr, 186 g/L of ammonium hydroxide, 18 g/L of nonyl phenol ethoxylate (9 ethoxylated ring) and water (Grisolia *et al*., 2004). Even though the expected environmental concentration is 0.92 mg/L (Tatum *et al*., 2010), according to the Washington State Department of Agriculture (2003), concentrations of up to 5.7 mg/L have been measured in surface water and treated sediment with no overlying canopy.

Diquat dibromide (9, 10-dihydro-8a, 10a-diazonia phenanthrene ion) is a post emergent herbicide. Diquat dibromide formulations include Midstream, Aquacide and Dextrone. It is a non-selective contact herbicide and crop desiccant that is also used in aquatic weeds control (Emmett, 2002; WHO, 2004). The Midstream formulation of diquat is widely used in the United State, North

America, Europe, Australia and Japan (Emmett, 2002; WHO, 2004). Midstream formulation contains nonyl phenol ethoxylate as surfactant, which has been implicated with estrogenic activities in several studies (Trumbo, 2005; Othman, 2009).

Glufosinate ammonium (N-phosphonomethyl) glycine) is a phosphinic acid, which is a broad spectrum and systemic herbicide (Ebert *et al*., 1990; Hack *et al*., 1994). Owing to the structural analogy of glufosinate ammonium to glutamate, glufosinate ammonium usually acts as an irreversible inhibitor of gluthamine synthatase activity in different tissues that often lead to slight increases of gluthamate and ammonia (Hack *et al*., 1994). Glufosinate ammonium formulations include Basta, Rely, Finale and Challenges. Basta formulation contains 18.5% glufosinate ammonium and 30% of sodium polyoxyethylene alkylether sulphate (AES) surfactant (Koyama *et al*., 1997).

Glyphosate *(N*-(phosphonomethyl) glycine) is a broad-spectrum, non-selective, post-emergence herbicide, with a molecular formula $C_3H_8NO_5P$ (WHO, 1994; 1996). It is perhaps the most important and best-selling herbicide ever produced (Mann *et al*., 2009; Gungordu *et al*., 2013; Wagner *et al*., 2013; Yadav *et al*., 2013). The increasing global use of this herbicide has generated widespread concern on their adverse effects on aquatic ecosystem (Mensah *et al*., 2013; Yadav *et al*., 2013; Lanctot *et al*., 2013).

But in spite of the wealth of knowledge regarding the endocrine disrupting effects of many pesticides, very little is known about exposure impacts of many of these herbicides, particularly on the reproductive system. There is also much controversy about whether reproductive abnormalities observed in wild populations are endocrine disrupting effects of the agro-pesticides and not other natural processes (Kloas *et al*., 2009; Hayes *et al*., 2010). According to Wagner *et al*. (2013) it is still unclear whether some glyphosate-based herbicides for example could affect the sexual development of frogs, thereby affecting the reproductive capacity of their population. The high adverse effects of reproductive toxicity on the maintenance of biodiversity, makes the assessment of reproductive fitness essential in environmental monitoring (Magnusson and Brunstrom, 2007).

In order to add to the emerging body of knowledge on the impact of pesticides on wildlife reproductive systems, this study assessed the impacts of six selected herbicide formulations including Midstream (diquat dibromide), Basta (glufosinate ammonium), Arsenal (imazapyr), and glyphosate formulations including Roundup, Kilo Max and Enviro Glyphosate on sexual development and reproductive health, using *X. laevis* as sentinel species.

5.2. Materials and methods

5.2.1. **Test chemicals**

The herbicide formulations used for the study include: Midstream (diquat dibromide) (Syngenta, Ltd., South Africa), Basta (glufosinate ammonium) (Bayer Crop Science AG Ltd, Germany) and Arsenal (imazapyr)(BASF Chemical Ltd., South Africa), Roundup (Monsanto, Ltd., South Africa), Enviro Glyphosate (Enviro Industries Ltd., South Africa), Kilo Max (Volcano Agro-science Ltd., South Africa).

5.2.2. Test organisms

Wild capture adult African clawed frog (*X. laevis)* frogs were sourced from a local licensed collector, Loesch Paddas Klapmuts, Western Cape Province. The *X. laevis* is a Southern African frog species that has been globally used as experimental model for environmental monitoring and assessment (Vitt *et al*., 1990; Mann and Bidwell, 2000; Paggeti *et al*., 2006; Kloas *et al*., 2009).

5.2.3 Breeding and rearing of tadpoles

Sexually mature males and females frogs (two pairs) were maintained separately in 15 L glass tanks containing buffered reverse osmosis (RO) water, and fed with fish pellets three times per week (Aqua-Nutro, RSA). Breeding induction was performed following the ASTM, (1998) protocol. In brief, the males were primed with 100 IU of human chorionic gonadotropin (hCG) (Merck Ltd, Germany) into the dorsal lymph sac, four days prior to the commencement of mating. The male and female received further 100 and 200 IU respectively just prior to mating. The male and female pairs were placed together in separate 15 L tanks lined with plastic netting (see further description in section 3.2.4 of this thesis). All the procedures were approved by the Animal Ethics Committee of the Stellenbosch University, Stellenbosch, South Africa (Approval no- SU-ACUM 12-00015**).**

5.2.4. **Test procedure**

5.2.4.1. Exposure set-up

The tadpoles were distributed into several 15 L tanks containing 10 L buffered RO water at a density of 40 tadpoles per tank. They were fed with Sera Micron (Sera, Heinsberg, DE), until they attained NF-stage 51. The tadpoles were fed 30 mg/animal/day initially which was later increased to 50 mg/animal/day in order to account for the increased in sizes (OECD, 2008). At NF stage 51, randomly selected tadpoles (n=20) were allocated from holding tanks and transferred into 15 L

exposure tanks containing 10 L of RO water, replicated twice at each of the selected concentrations. The exposure experiment was done in a controlled climate room according to the XEMA experimental protocol including the following physical conditions (OECD, 2008); water temperature of 24 ± 1 ° C, pH ranging between 7.5 - 8.5, dissolve oxygen concentration of >3.5 mg/L and a 12 hours of light and dark photoperiod ($L_{12}D_{12}$). The exposure concentrations were centred on 15, 30 and 45 % of 96 hour LC50 of the herbicides at NF 48 of *X. laevis* (Table 5.1; see Chapter 2). The exposure medium was completely changed every Monday, Wednesday and Friday. At the end of the exposure, the young metamorphs were collected, weight, dissected and assessed for various reproductive malformations, before the carcasses were preserved for future references.

Table 5.1. The selected exposure concentrations (based on the 15, 30 and 45% of 96 hour LC_{50} at NF stage 48) (see Chapter 2) for the six herbicide formulations.

Formulation	Concentration (mg a.e./L)
Midstream (ERC)	0, 0.05, 0.11, and 0.14
Arsenal (ERC)	0, 0.5, 2.0, and 3.5
Basta (ERC)	0, 0.05, 0.15 and 0.25
Roundup (ERC)	0, 0.2, 0.4, and 0.6
Enviro Glyphosate (15, 30 and 45 OF 96 Hr LC_{50} @ NF stage 48)	0, 9 , 19, and 28
Kilo Max (15, 30 and 45 of 96 Hr LC_{50} @ NF stage 48)	0, 90, 190 and 280

5.2.5. Survival and Developmental Disruption

Embryonic survival rate was determined at 96 hours post hatching. Growth (size) disruption was assessed by determined body mass (to the nearest gram) measuring weight at prometamorphic NF-stage 55 and at metamorphosis NF-stage 66. Gonadal sex ratio was determined by microscopic inspection. Histological analysis was performed with a Leica DMLB light microscope and image analysing software. Sex ratios of juvenile frogs exposed to the different herbicide formulations (concentration series) were statistically compared to the sex ratios of the control groups. Gonadal morphological malformations (after Lutz *et al*., 2008) were studied by dissecting the young frogs at the pleuroperitoneal cavity, to expose the visceral organs. Using a dissecting stereo microscope, gonads were assessed for gross morphology abnormalities and categorised as testes, ovaries or

recorded as improper formation of part(s) of the organs using the following characteristics adapted from Lutz *et al*. (2008):

a. Adhesion- Gonads that join to other abdominal tissues.
b. Aplasia (agenesis)- Total lack of gonads development.
c. Segmented aplasia- Longitudinal discontinuous gonads (e.g. tissue separation, extraneous gonadal tissue).
d. Bifurcation- where the division of gonads oriented longitudinally (e.g. protuberance and symmetric).
e. Angular deformity- Gonads that bends to an excessive degree (e.g. gonad folded).
f. Displaced gonads- Gonads or section of it not typically located (e.g. lateral, or medially displacement).
g. Fused- The fusion of left and right gonads to a varying degree.
h. Hypertrophy- enlargement of gonads (e.g. wider, thick or enlarged).
i. Segmented hypertrophy- excessive enlargement of one or more areas of the gonads (e.g. mass enlargement, pearling and partly thick).
j. Hypoplasia- decrease in gonad's size (e.g. narrow, slightly narrow, and truncated, slightly truncated, thin and margin entire).
k. Segmented hypoplasia- gonad where one or more areas are excessively reduced, attenuated, or poorly developed but not separated (e.g. partly narrow, partly thin, margin slightly, margin partially entire and pearling).
l. Intersex- gonads where ovary and testicular tissues are present in separate structures.
m. Mixed sex- gonads where ovary and testicular tissues are present in the same structure.
n. Translucent- gonads that appear not so dense (e.g. slightly translucent).
o. Segmental translucent- where section(s) of the gonads appears not so dense.

The percentage abnormality was expressed as a malformation index (Lutz *et al*., 2008).

5.2.6. Histological Sex Validation

Microscopic views of testes and ovaries were validated by histological examination of 10 selected males and females. The females were identified based on the presence of an ovarian cavity and the growth of germ cell in the cortical region, while the males were identified by the absence of ovarian cavity as well as growth of germ cells (in seminiferous tubules) in the medullar region (Fig. 5.1).

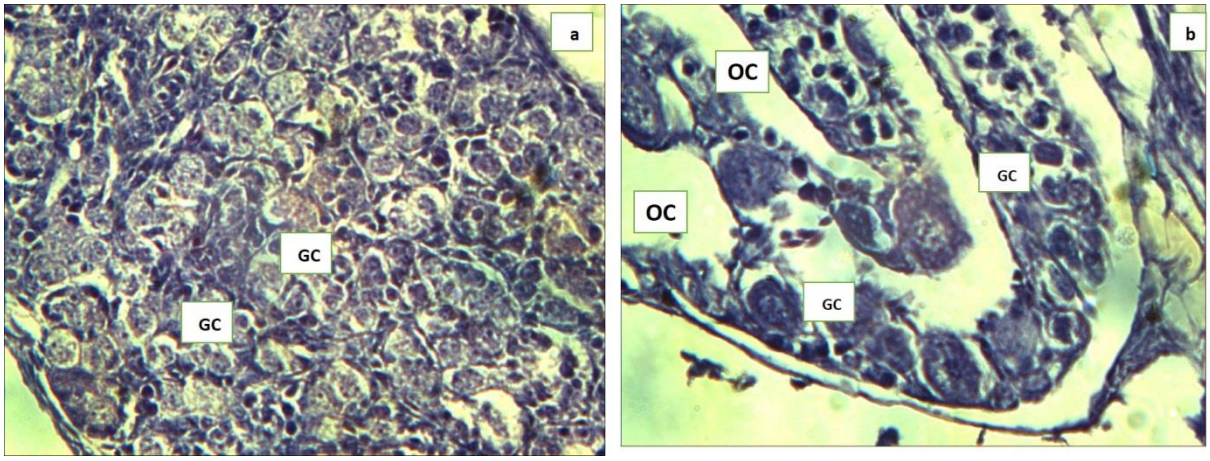

Figure 5.1: Histology (400X) of validated testis (a) and ovary (b) for sex ratio determination of *X. laevis*. The ovaries were identified with the presence of an ovarian cavity (OC) and the growth of germ cell (GC) in the cortical. The testes were identified by the absence of ovarian cavity (OC) as well as growth of germ cells (GC) in the medullar region.

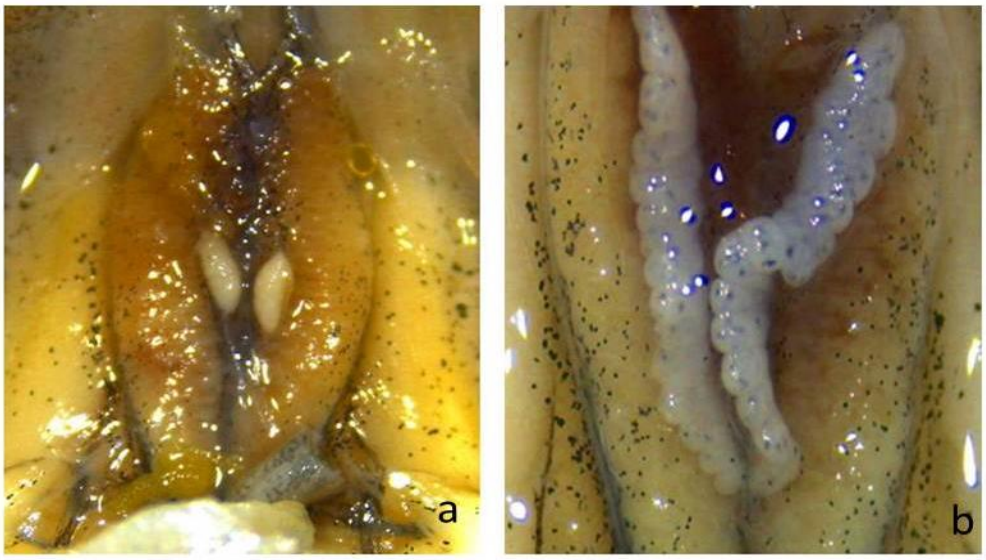

Figure 5.2: sample of male (a) and female (b) morphological gonads (100X) of *X. laevis* showing the testis (a) and the ovary (b).

5.2.7. Data Analysis

Normality and homogeneity of variance was evaluated using the respective Shapiro-wilk's and Levene's tests. Mean body mass at prometamorphosis and at the completion of metamorphosis were compared to the respective untreated controls using one way ANOVA, which was followed by Tukey HSD pos hoc test for parametric data and Kruskal-Wallis ANOVA followed by DUNN'S multiple comparison of mean rank test for non-parametric data. Differences in gonadal sex ratio at

each of the concentrations and control were analysed with Chi (X^2) square test using Graphpad online software (Graphpad Software Inc., USA).

5.3. Results

5.3.1. Survival

The survival rate of tadpoles in each of the breeding pairs ranged between 94 and 96 %.

5.3.2. Body Mass Effects

Midstream formulation: the mean body mass (MBM) at the *X. laevis* prometamorphic NF-stage 55 tadpoles varied, although not significant (P>0.05) when compared to the control tadpoles (Table 5.2). The MBM initially increased at concentration of 0.05 mg/L rising up to 0.89 g, but was reduced between concentrations of 0.11 to o.14 mg/L, moving from 0.78 g at 0.11 mg/L to 0.74 g at 0.14 mg/L compared to control. The reduction in mass was statistically significant (P< 0.05) at concentrations of 0.11 mg/L and 0.14 mg/L compared to the lower exposure concentration of 0.05 mg/L but not significantly different to the Control (Table 5.2). At metamorphosis (NF stage 66), the MBM was reduced in a dose dependent manner from lowest to the highest exposure concentrations compared to the control (Table 5.2). The decreased in body mass was statistically significant (P< 0.05) at concentrations of 0.11 mg/L and 0.14 mg/L compared to the Control.

Table 5.2: The impacts of the selected herbicide formulations on mean body mass (MBM) at both NF-stage 55 (prometamorphic) and NF-stage 66 (metamorphic stages) of *X. laevis*. Asterisks indicate significant difference from the control treatment

Herbicide Formulation	Concentration (mg/L)	MBM ± SD @ Prometamorphic (NF 55) (g)	MBM ± SD @ Metamorphosis (NF 66) (g)
Midstream	0	0.84 ± 0.11	0.72 ± 0.10
	0.05	0.89 ± 0.11	0.69 ± 0.07
	0.11	0.78 ± 0.15	**0.62 ± 0.02***
	0.14	0.74 ± 0.13	**0.55 ± 0.04***
Basta	0	0.99 ± 0.23	1.27 ± 0.22
	0.05	1.04 ± 0.21	1.32 ± 0.14
	0.15	1.09 ± 0.27	1.4 ± 0.12
	0.25	1.09 ± 0.24	**1.4 ± 0.15***
Arsenal	0	0.8 ± 0.3	1.03 ± 0.15
	0.5	0.9 ± 0.18	1.05 ± 0.17
	2.0	0.97 ± 0.14	1.07 ± 0.22
	3.5	**0.91 ± 0.22***	1.13 ± 0.18

Kilo Max	0	0.96 ± 0.22	0.84 ± 0.11
	90	**0.72 ± 0.13***	0.72 ± 0.10
	190	**0.62 ± 0.12***	**0.66 ± 0.11***
	280	**0.57 ± 0.19***	**0.64 ± 0.12***
Roundup	0	0.96 ± 0.22	0.84 ± 0.11
	0.2	0.94 ± 0.17	0.82 ± 0.12
	0.4	**0.78 ± 0.15***	0.81 ± 0.11
	0.6	**0.83 ± 0.22***	0.79 ± 0.11
Enviro Glyphosate	0	0.90 ± 0.12	0.82 ± 0.10
	9	0.93 ± 0.12	0.76 ± 0.09
	19	0.80 ± 0.17	**0.75 ± 0.10***
	28	**0.68 ± 0.16***	**0.68 ± 0.16***

Basta formulation: the MBM of *X. laevis* exposed to this formulation at NF-stage 55 (prometamorphic) gradually increased from concentration of 0.05 to 0.25 mg/L. It moved from 1.04 g to 1.09 g compared to 0.99 g at Control, but the increased MBM was not statistically significant compared to the Control group. At NF-stage 66 (metamorphosis), the MBM increased from 1.32 g at 0.05 mg/L to 1.4 g at 0.25 mg/L compared to 1.27 g at the Control (Table 5.2). The increase in body mass was statistically significant ($P < 0.05$) at concentration of 0.25 mg/L compared to the Control.

Arsenal formulation: the MBM of the *X. laevis* exposed to this formulation at NF-stage 55 (prometamorphosis) fluctuated with increased concentrations. The MBW increased from 0.9 g at a concentration of 0.5 mg/L to 0.97 g at a concentration of 2.0 mg/L, then moved down to the 0.91 g at 3.5 mg/L compared to the 0.8 g of the Control (Table 5.2). The reduction in body mass was statistically significant ($P < 0.05$) at a concentration of 3.5 mg/L compared to the Control. At NF-stage 66 (metamorphosis), the MBM increased in a dose dependent manner from the lowest to the highest exposure concentrations, but the increases in mass were not statistically significant at all the exposed concentrations compared to the Control (Table 5.2).

Kilo Max: at NF-stage 55 (prometamorphic) the MBM of the exposed *X. laevis* reduced between exposure concentrations of 90 to 280 mg/L in a dose dependent manner. The body mass moved down from 0.72 g at concentration of 90 mg/L to the 0.57 g at a concentration of 280 mg/L compared to the Control at 0.96 g. The reduction in body mass was statistically significant ($P < 0.05$) at all exposure concentrations of 90 mg/L to 280 mg/L compared to the Control. At NF-stage 66 (metamorphosis), the MBM of the *X. laevis* was also reduced in a dose dependent manner. It moved down from 0.72 g at 90 mg/L to 0.64 g at 280 mg/L compared to 0.84 g in the Control (Table

5.2). The reduction in weight was statistically significant (P< 0.05) at concentrations of 190 and 280 mg/L compared to the Control.

Roundup formulation: the MBM of the exposed *X. laevis* at NF-stage 55 (prometamorphic) fluctuated with increased concentrations. The MBM initially reduced from 0.94 g at 0.2 mg/L to 0.78 g at 0.4 mg/L, and then moved up to 0.83 g at 0.6 mg/L compared to Control at 0.96 g. The reduction in body mass was statistically significant (P< 0.05) at concentrations of 0.4 and 0.6 mg/L relative to the Control (Table 5.2). At NF-stage 66 (metamorphosis), the MBM slightly decreased from 0.82 g at 0.2 mg/L to 0.79 g at 0.6 mg/L compared to the Control at 0.84 g (Table 5.2). The decreased in body mass were not statistically significant at all exposure concentrations.

Enviro Glyphosate formulation: the MBM of *X. laevis* at NF-stage 55 (prometamorphic stage) initially increased at 9 mg/L. It moved up to 0.93 g compared to 0.9 g at Control. The MBM then decreased from 0.80 g at concentration of 19 mg/L to 0.68 g at 28 mg/L compared to the Control at 0.9 g. The reduction in body mass was statistically significant (P< 0.05) at a concentration of 28 mg/L) compared to the Control. At NF-stage 66 (metamorphosis), the MBM reduced in a concentration dependent manner. It moved down from 0.76 g at 9 mg/L to 0.73 g at 28 mg/L compared to 0.82 g at the Control (Table 5.2). The reduction in mean body mass was statistically significant (P< 0.05) at concentrations of 19 and 28 mg/L compared to the Control.

5.3.3. **Sex Ratio Effects**

Midstream formulation: the percentage sex ratio of *X. laevis* exposed to Midstream formulation increased in favour of female in a dose dependent manner. When compared with the ratio 50:50 (F: M) in the Control, there was no difference in ratio 50:50 (F: M) at a concentration of 0.05 mg/L, and the ratio of 55:45 (F: M) at a concentration of 0.11 mg/L. The sex ratio was statistically significant (X^2 test; P< 0.05) in the 0.14 mg/L treatment, with 60:40 (F: M) relative to the Control. This pattern of sex ratio showed a direct correlation with increasing dose (Fig. 5.3).

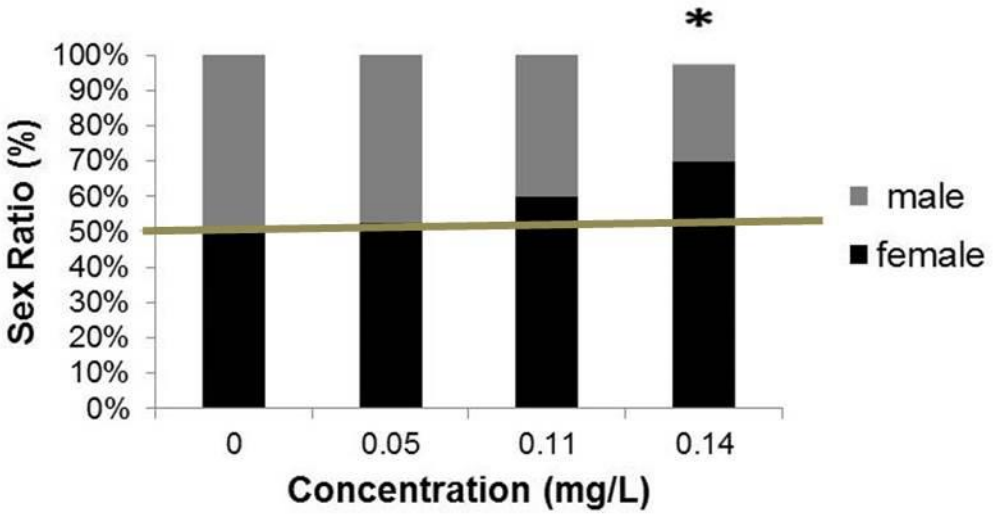

Figure 5.3: The percentage sex ratio of *X. laevis exposed to* Midstream formulation compared to a negative control. The horizontal line is the 50% average mark point. Asterisks indicate significant difference from the control treatment (X^2 Test, $P< 0.05$).

Basta formulation: the percentage sex ratio of *X. laevis* exposed to Basta formulation increased from 50:50 (F: M) at a concentration of 0.05 mg/L to 52.5:47.5 (F: M) at a concentration of 0.15 mg/L and 55:45 (F: M) at a concentration of 0.25 mg/L when compared to 50:50 at the Control. The increasing sex ratio was however not statistically significant at all the concentrations (Fig. 5.4).

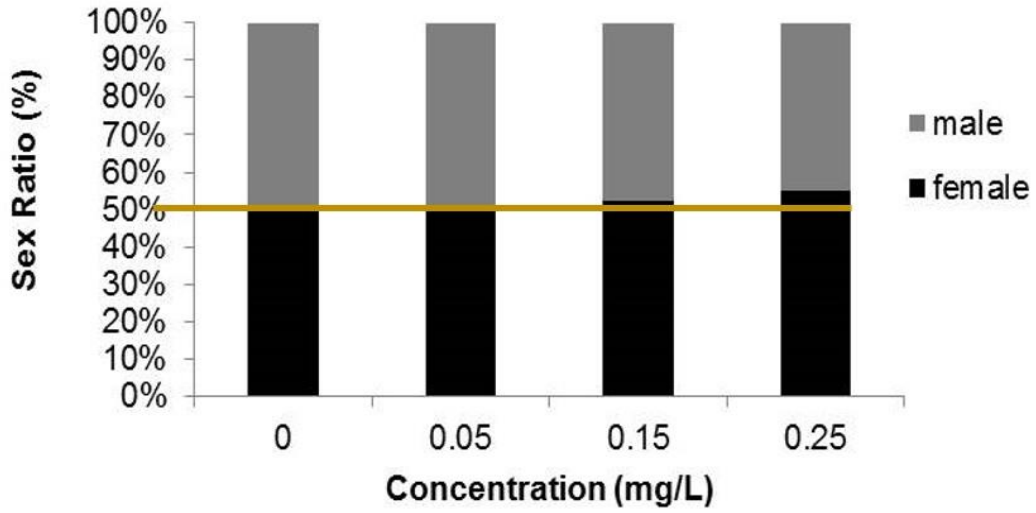

Figure 5.4: The sex ratio of *X. laevis* metamorphs following exposure to Basta formulation compared to the control. The horizontal line is the 50% average mark point

Arsenal formulation: the percentage sex ratio of *X. laevis* following exposure to Arsenal formulation only showed a marginal increase. The ratio moved from 47.5:52.5 (F: M) at a concentrations of 0.5 and 2.0 mg/L down to ratio of 45:55 (F: M) at a concentration of 3.5 mg/L compared to ratio of 47.5:52.5 (F: M) in the Control. All the ratios were not significantly different compared to the Control (Fig. 5.5).

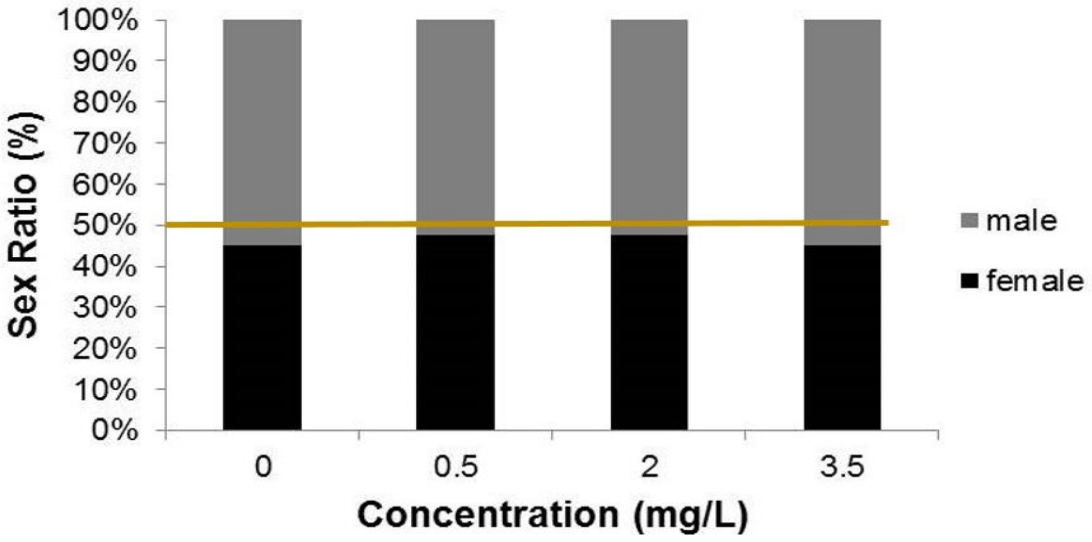

Figure 5.5: Percentages of sex ratio of Arsenal formulation exposed *X. laevis* compared to the Control. The horizontal line is the 50% average mark point.

Kilo Max formulation: the percentage sex ratio of *X. laevis* following exposure to Kilo Max formulation increased in favour of female in a dose dependent manner. The ratio moved from 47.5:52.5 (F: M) at 90 mg/L to ratio of 52.5:47.5 (F: M) at 190 mg/L and ratio 68:32 (F: M) at concentration of 280 mg/L compared to 50:50 (F: M) at the Control. The sex ratio was only statistically significant (X^2 Test, $P< 0.05$) at a concentration of 280 mg/L compared to the Control (Fig. 5.6).

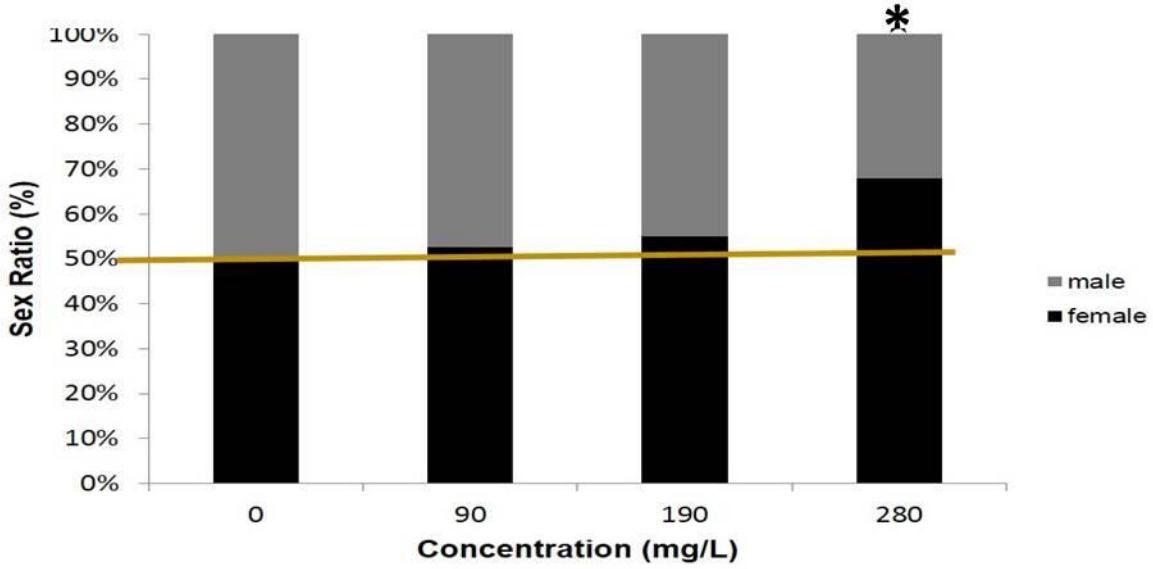

Figure 5.6: Percentages of sex ratio of *X. laevis* exposed to Kilo Max formulation compared to the Control. Asterisks indicate significant difference from the Control treatment (X^2 square Test, $P<0.05$). The horizontal line is the 50% average mark point.

Roundup formulation: the percentage sex ratio of *X. laevis* following exposure to Roundup formulation showed a u-shaped pattern in favour of female. The ratio increased from 45:55 (F:M) at concentrations of 0.2 and 0.4 mg/L to 47.5:52.5 (F:M) at concentration of 0.6 mg/L relative to 50:50 (F:M) at Control. The sex ratios were not significantly different at all the exposed concentrations in this study compared to the Control (Fig. 5.7).

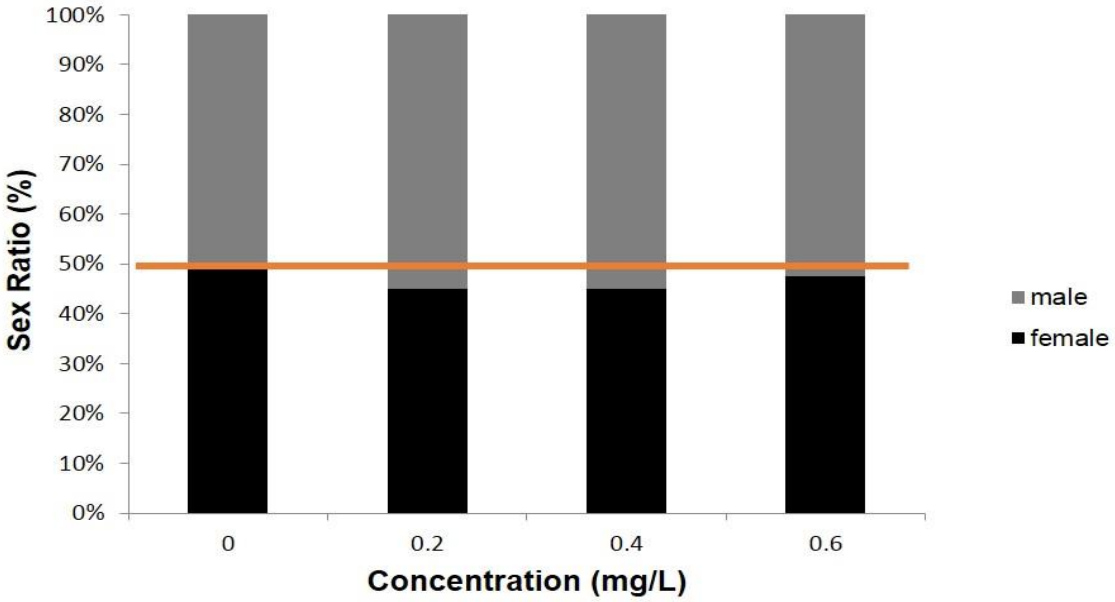

Figure 5.7: Percentages of sex ratio of *X. laevis* metamorphs following exposure to Roundup formulation compared to the Control. The horizontal line is the 50% average mark point.

Enviro Glyphosate formulation: the percentage sex ratio of the exposed X. *laevis metamorphs* showed a little bias towards the female. The sex ratio moved from a ratio of 52:47.5 (F:M) at concentrations of both 9 and 19 mg/L, to a ratio of 55:45 (F:M) at a concentration of 28 mg/L compared to a ratio of 52:47.5 (F:M) at the Control. None of the sex ratios at all the exposed concentrations were significantly different compared to the Control (Fig. 5.8).

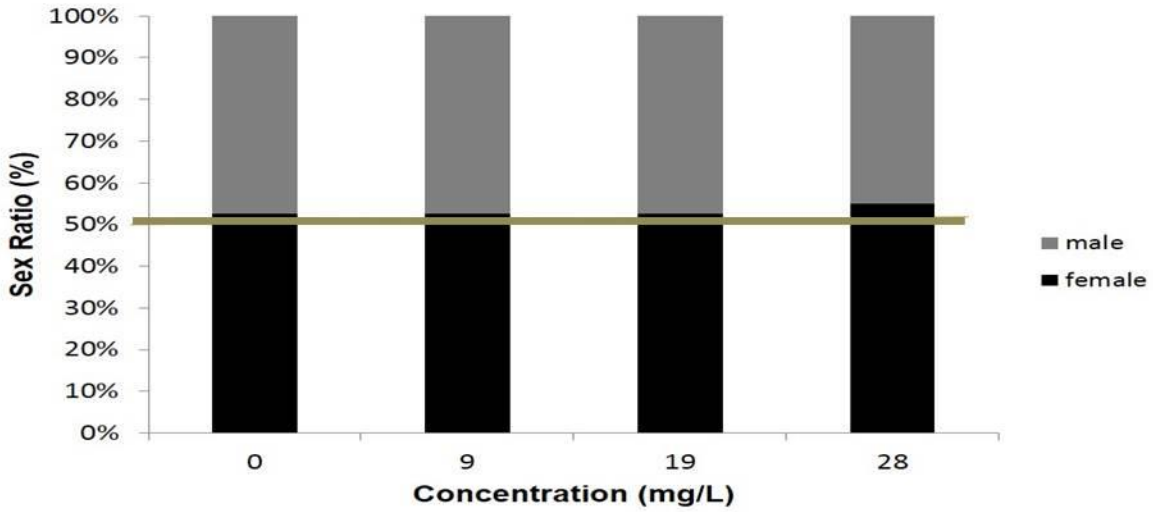

Figure 5.8: Percentages of sex ratios of *X laevis* exposed to Enviro glyphosate compared to the Control. The horizontal line is the 50% average mark point.

5.4 Intersex

No occurrence of intersex was observed across all the exposure in the six herbicides formulations.

5.5. Reproductive Malformation Effects

Midstream formulation: widespread morphological malformations were recorded in the Midstream formulation exposed juveniles. At a concentration of 0.05 mg/L, the Midstream formulation resulted in an abnormality index of 32.5 % (Table 5.3). The malformations recorded included segmented hypoplasia (the most widespread), segmented aplasia and slight translucent abnormalities (Fig. 5.9). At a concentration of 0.11 mg/L, the abnormality index was 37.5 % (Table 5.3) and the observed abnormalities included slightly translucent, which was the most widespread,

enlarged hypertrophy, segmented hypertrophy, translucence, gonad folded and mixed sex respectively. At a concentration of 0.14 mg/L, abnormality index was 60 % (Table 5.3), and the observed abnormalities included gonad folded, the most widespread, followed by slightly translucent, enlarged hypertrophy, segmented aplasia, segmented hypertrophy, segmented hypoplasia and partly narrow segmented hypoplasia (Fig. 5.9). The rate of abnormalities upon exposure to Midstream formulation increased in dose dependent manner (Table 5.3).

Table 5.3- Types of morphological malformations, Abnormalities Index and % Sex Ratio at various graded concentrations of the six herbicide formulations

Herbicide	Conc (mg/L)	No of Morphological malformation	Abnormality /Malformation Index (%)	% Sex ratio F:M
Midstream	0	Narrow hypoplasia (2), slightly translucent (1)	7.5	50:50
	0.05	Segmented hypoplasia (8), Slightly translucent (2), segmental aplasia (3)	32.5	52.5:47.5
	0.11	Segmented hypertrophy (2), slightly translucent (3), mixed sex (1), translucent (2), enlarged hypertrophy (5), gonad folded (2)	37.5	55:45
	0.14	Segmented hypertrophy (2), gonad folded (9), segmental aplasia (3), slightly translucent (4), enlarge hypertrophy (4), segmented hypoplasia (1), partly narrow segmented hypoplasia (1)	60	60:40
Basta	0	Hypoplasia (4), folded gonad (1)	12.5	50:50
	0.05	Gonad folded (2), adhesion (2), angular deformity (2)	15	50:50
	0.15	Gonad folded (4), enlarged hypertrophy (2), segmental aplasia (1)	17.5	52.5:47.5
	0.25	Gonad folded (6), slightly translucent (2), angular deformity (1), hypoplasia (2)	27.5	55:45
Arsenal	0	Segmented aplasia (1), narrow hypoplasia(1), slightly translucent (1)	7.5	47.5:52.5
	0.5	Aplasia (1), angular deformity (1), segmental aplasia (2), tissue separation (2), partly narrow segmented(1)	17.5	47.5:52.5
	2.0	Aplasia (2), angular deformity (1), segmental aplasia (1), tissue separation (3), hypoplasia (2), slightly translucent (1)	25	47.5:52.5
	3.5	Gonadal folded (6), segmented hypoplasia (2), aplasia (4), segmental aplasia (1), narrow hypoplasia (1).	35	45:55
Kilo Max	0	Narrow hypoplasia (3), hypoplasia (1)	10	50:50

	90	Aplasia (2), segmented aplasia (2), segmented hypoplasia (1), slightly translucent (4)	22.5	47.5:52.5
	190	Tissue separation (2), folded gonadal (2), protuberances (2), translucent (2), segmented aplasia (1), segmented hypoplasia (1)	25	52.5:47.5
	280	Aplasia(4), segmented bifurcation(2), segmented hypertrophy (2), translucent (8), segmental aplasia (1)	43	68:32
Roundup	0	Aplasia (2), hypoplasia (1)	7.5	50:50
	0.2	Aplasia (1), segmented aplasia (1), narrow hypoplasia (1), partly narrow hypoplasia(1), segmented hypoplasia (1), folded gonadal (1), angular deformity (1), displaced (1)	20	45:55
	0.4	Aplasia (1), segmented aplasia (1), narrow hypoplasia (2), segmented hypoplasia (1), folded gonadal (2), angular deformity (2).	22.5	45:55
	0.6	Translucence (4), segmented hypoplasia (2), narrow hypoplasia (2), aplasia (1), adhesion (1), segmented aplasia (1), hypoplasia (1).	30	47.5:52.5
Enviro	0	Hypoplasia (3)	7.5	52.5:47.5
Glyphosate	9	Slightly translucent (2), segmental aplasia (2), segmented hypoplasia (1), aplasia (2)	17.5	52.5:47.5
	19	Slightly translucent (2), hypoplasia (1), segmental aplasia (2), aplasia (4)	22.5	52.5:47.5
	28	Folded gonadal (6), segmented hypoplasia (1), aplasia (4), segmental aplasia (2), narrow hypoplasia (2)	37.5	55:45

Basta formulation: numerous abnormalities were observed in the juveniles exposed to this formulation. At a concentration of 0.05 mg/L, the abnormality index recorded was 15 %. The observed abnormalities included gonad folded, adhesion and angular deformity. At 0.15 mg/L, the abnormality index recorded was 17.5 % (Table 5.3), with abnormalities including gonad folded, the most widespread, hypoplasia, slightly translucent and angular deformity (Fig. 5.9). At 0.25 mg/L, the observed abnormalities included gonad folded, the most widespread, hypoplasia, angular deformity and slightly translucent respectively. The abnormality index recorded at 0.25 mg/L was 27.5 % (Table 5.3). The rate of abnormalities upon exposure to Basta formulation increased in a dose dependent manner (Table 5.3).

Arsenal formulation: the Arsenal formulation also resulted in numerous morphological malformations in the exposed juveniles. At 0.5 mg/L, the recorded abnormality index was 17.5 %. The observed abnormalities included tissue separation, segmented aplasia, aplasia and angular deformity, with tissue separation being the most widespread (Fig. 5.9). At a concentration of 2.0 mg/L, the abnormality index recorded was 25 % with various abnormalities that included tissue separation, segmented aplasia, aplasia, hypoplasia and angular deformity. The most widespread abnormalities was tissue separation (Table 5.3). At 3.5 mg/L, the abnormality index observed was 35 % for the formulation. The observed abnormalities included folded gonads, being the most widespread, aplasia, segmented hypoplasia, segmented aplasia and narrow hypoplasia respectively (Fig. 5.9).

Kilo Max formulation: numerous abnormalities which were concentration dependent were observed in the juvenile exposed to this formulation. At a concentration of 90 mg/L, the abnormalities index recorded was 22.5 %, with various abnormalities that included slightly translucent, which was the most widespread, aplasia, segmented aplasia, and segmented hypoplasia respectively (Fig. 5.9). At a concentration of 190 mg/L, 25 % was calculated as the abnormality index. The observed abnormalities at this concentration included gonad folded, tissue separation, protuberance, translucence, segmented hypoplasia, and segmented aplasia. At 280 mg/L, the abnormality index recorded was 43 % (Table 5.3). The abnormalities observed included translucence, which was the most widespread, aplasia, segmented hypertrophy, segmented aplasia and segmented bifurcation respectively (Fig. 5.9).

Roundup formulation: Roundup formulation showed various abnormalities on the exposed juveniles. At a concentration of 0.2 mg/L, the abnormality index recorded was 20 %, with numerous abnormalities that included segmented aplasia, narrow hypoplasia, partly narrow hypoplasia, segmented hypoplasia, gonad folded, angular deformity and displaced (Fig. 5.9). At a concentration of 0.4 mg/L, the abnormality index recorded was 22.5 % (Table 5.3), and the observed abnormalities included segmented aplasia, which was the most widespread, angular deformity, aplasia, narrow hypoplasia, segmented hypoplasia and gonad folded respectively. At a concentration of 0.6 mg/L, the abnormality index recorded was 30 % (Table 5.3), with observed abnormalities that included translucence, segmented hypoplasia, segmented aplasia, narrow hypoplasia, aplasia and adhesion. The most widespread being the translucence (Fig. 5.9).

Enviro Glyphosate formulation: Enviro Glyphosate formulation showed numerous abnormalities on the exposed juveniles. At a concentration of 9 mg/L, abnormality index recorded

was 17.5 % (Table 5.3), with observed abnormalities that included slightly translucent, segmented aplasia, and segmented hypoplasia. At a concentration of 19 mg/L, 22.5 % was the recorded abnormality index, with various abnormalities that included aplasia, being the most widespread, segmented aplasia, hypoplasia and slightly translucent (Fig. 5.9). At a concentration of 28 mg/L, the abnormality index recorded was 37.5% (Table 5.3). The observed abnormalities included folded gonads, aplasia, segmented hypoplasia, segmented aplasia and narrow hypoplasia respectively (Fig. 5.9).

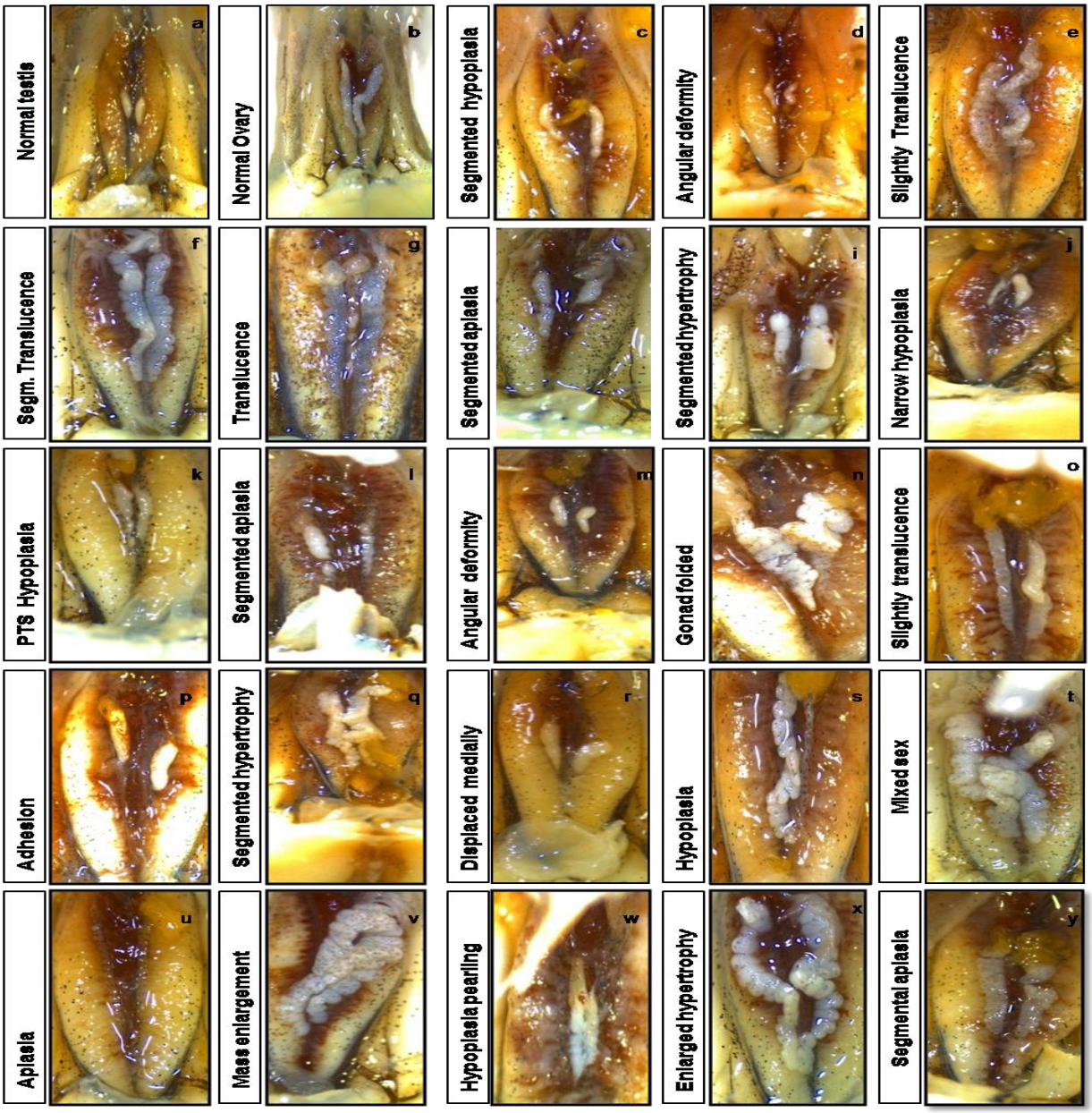

Figure 5.9: Various morphological abnormalities in reproductive gonads of *X. laevis* juveniles exposed to six herbicide formulations including Midstream, Basta, Arsenal, Roundup, Kilo Max

and Enviro Glyphosate formulations. Description of abnormalities were taken after Lutz *et al.* (2008)

5.6. Discussion

Increasing use of pesticides including herbicides, insecticides, fungicides etc. continue to generate serious health and ecological concerns, not only for human beings, but also on non-target organisms **(**Diamanti-Kandarakis *et al*., 2009**)**. The reprotoxicity impacts of these pesticides are particularly disturbing giving the direct impacts of reproduction on biodiversity. The exposure impacts of various aquatic herbicides on reproductive systems and organs of the non-target organisms like amphibians remains highly controversial and unclear (Hayes *et al*., 2002; Carr *et al*., 2003; Taylor *et al*., 2005; Oka *et al*., 2008). This present study examined the reproductive impacts of selected aquatic herbicides including Roundup, Kilo Max, Enviro Glyphosate (glyphosate-based formulation), Basta (glufosinate ammonium formulations), Arsenal (imazapyr) and Midstream (diquat dibromide formulations) on reproductive system of amphibians using *X. laevis* as sentinel. The results showed that many of these selected aquatic formulations have negative impacts on the reproductive organs causing widespread abnormalities on the *X. laevis* reproductive organs.

Impacts on body mass.

Midstream formulation

In a very typical endocrine disrupting chemicals pattern, which usually produce an inverted u-shaped response (Calabrese, 2001; Labereke *et al*., 2008**), where the mass increased at lower concentration before decreasing at higher concentrations**. The observed body mass reduction from the prometamorphic and the metamorphosis stage may be the consequence of the high toxicity of this formulation to the test organism. Various scholars have highlighted the numerous implications of size reduction in amphibians, including increases predation risk, as well as lower survival rate and future reproductive fitness (Sathe, 1969, Collins 1975, Howard, 1978; Semlitch, 1989; and Sullwan and Spencer 2003; Howe *et al*., 2004; Gupta, 2012). Furthermore, size may also have strong downstream effects on adult phenotype and fitness (Dmitriew and Rowe, 2011). According to Cabera-Guzman *et al*. (2013), larger body size enhance metamorphs survival and growth rate, with better locomotory performance, as well as being more adapted to catching and consuming prey. This means that the weight reduction observed in the frogs exposed to the Midstream formulation will negatively impact the developmental and reproductive fitness for the exposed amphibians in a way that could have wider effects on the population.

Basta formulation

In Basta formulation, the observed increased body mass in the treated metamorphs may be a physiological response to the toxic and inhibitory property of this formulation. The increased in body mass observed in this study is similar to the observation of Greulich and Pflugmacher (2003), where they noted that metamorphs treated with Cypermethrin become distinguished by their more compact physique with increased body mass compared to the Control. Greulich and Pflugmacher (2003) concluded that the increased body mass must be a physiological response to resist the adverse toxic effects of the chemical. The impacts of such increased body mass on the physiology and reproductive fitness are still largely unknown, and might be detrimental to the frogs.

Arsenal formulation

Similar to occurrence in Midstream exposure, Arsenal formulation also produced an inverted-u-shaped response pattern in prometamorphic tadpoles, with body mass increasing at lower concentrations, but reduced at highest exposure concentration, all relative to the Control. At metamorphosis stage, the body mass was normalised with tadpoles not differing in body size when compared to those of the Control group. The fact that the frogs can adequately recover from the exposure at adult stage suggests that at these concentrations, the formulation might not have any serious irreversible exposure impacts on the growth rate of the frogs. However, since concentrations of 5.78 mg/L have been measured in surface water and treated sediment with no overlying canopy (WSDA, 2003), suggesting that the frog could be facing bigger toxicity challenges from the Arsenal formulation in the environment, compared to the current exposure concentration range of 0.5 mg/L to 3.5 mg/L.

Glyphosate formulations (Kilo Max, Roundup and Enviro Glyphosate)

For the Glyphosate formulations, the three formulations reduced the body mass of the treated *X. laevis.* While the three formulations reduced the body mass at prometamorphic stage, the reduction was sustained till metamorphic stage in both Kilo Max and Enviro Glyphosate formulations except in Roundup formulation, all relative to the Control. The reduction in body mass for the three glyphosate formulations is consistent with the several scientific findings (Edington *et al*., 2004; Howe *et al*., 2004). As noted by Edington *et al*., 2004, significant reduction in body size was observed in *X laevis*, leopard frogs, and Green frogs after 96hour exposure to formulations of glyphosate herbicide. Growth rate and smaller size at metamorphosis would affect survival (Shenoy *et al*., 2009), immune-competence (Carey *et al*., 1999) and territorial defence (Bridges, 1999). The organisms are also at risk of decrease egg clutch size and potential fewer breeding

attempts over the life of the animals (Howard, 1978; Semilitch *et al*., 1988; Smith, 1987; Berven, 1990), which could have a larger impacts on growth and sexual development.

5.12. Sex Ratio

The significant shift towards female-biased sex ratios in the Midstream and Kilo Max formulations 60:40 and 68:32 (F: M) respectively, relative to a ratio of 50:50 (F: M) in the Control group showed the feminization potential of these formulations on *X. laevis.* For the Midstream formulation, even though no known studies have examined its exposure impacts on sex ratios in amphibians, the 60:40 ratio at concentration of 0.14 mg/L, which is quite lower than the 0.733 mg/L, the expected environmental concentration (EEC) (Peterson *et al*., 1994) of this formulation showed that this formulation could have a serious feminization effect at the EEC. This result showed that complete feminization is possible at a slightly higher concentration, even below the EEC. For Kilo Max formulation, the slightly high concentration used in this study (relative to the EEC) means the amphibians and other aquatic organisms would not normally be exposed to this concentration in the natural environment and hence the feminization level. But the potential feminization impacts of Midstream and Kilo Max at lower concentrations could negatively impact the aquatic organisms and have serious ecological effects on the exposed populations. As noted by Langlois *et al*. (2010), the female-biased ratio is an important physiological consequence of chemical exposure which could potentially alter amphibian population fitness.

For Roundup and Environ Glyphosate formulations, the observed slight tendencies for masculinization and feminization impact of these two glyphosate formulation respectively showed the increasing contribution of their different surfactants. For Roundup formulation, the non-significant shift in male-biased sex ratio (47.5:52.5) confirmed the findings of Howe *et al*. (2004), who reported that the exposure of *Lithobates pipiens* and *Lithobates clamitan* to Roundup formulation produced a male biased sex ratio that were not significantly different to the Control group. In the case of Environ Glyphosate formulation, the low sex ratio deviation relative to the Control showed the low feminization potential of this formulation. This is consistent with the findings of Howe *et al*. (2004), who noted that some formulations of glyphosate did not cause any disruption in sex ratio. The authors also showed that the technical glyphosate did not cause any sex ratio disruption. This further showed the contribution of the different surfactants as the real cause of slight feminization and masculinization in these glyphosate formulations.

For Arsenal formulation, although the sex ratio showed no serious threat at a concentration of 3.5 mg /L, further study at EEC of 5.7 mg/L (WSDA, 2003), will be very important to clear any doubt at of the potential feminization at relevant environmental concentrations. In the case of Basta formulation, the non-significant result even at concentration of 0.25 mg/ L showed that this formulation low feminization potential, but further study at the EEC 1.0 mg/L of this formulation (Dinehart *et al*., 2010), will further increase our ecological knowledge of this formulation.

5.13. **Gonadal Malformations**

One of the hypotheses for the global amphibian declines is the issue of chemical/pesticides contamination in the environment. Today, the contribution of pesticides to reproductive toxicity is no doubt a serious raging debate. As noted by several authors, malformations of all kinds occur naturally in wild amphibian populations (Hanken, 1983; Read and Tyler, 1994). The severities of malformations only become worrisome when the gonadal malformations become dose dependent as found in this study. The increased abnormality index from the highest at Midstream (33-60 %), Kilo Max (22.5-43 %), Enviro Glyphosate (17.5-37.5 %), Arsenal (18-35 %), Roundup (20-30 %) and then Basta (13-28 %) in that order, showed high reproductive toxicity potentials for these formulations.

To my knowledge, no evidence of gonadal impairment has been reported for Midstream formulation (diquat dibromide), Basta formulation (glufosinate ammonium) as well as Arsenal formulation (imazapyr), this study represent the first account thereof. For Midstream formulation, the high teratogenic index of 3.5 (recorded for FETAX in Chapter 3) and thyroid disruption potential (for XEMA in Chapter 4) is certainly a pointer to the possible hidden potential reproductive toxicity of this formulation, since thyroid disrupting substances have been shown to affects the sex steroids (Hayes, 1998, Bogi *et al*., 2011). The fact that Midstream formulation produced a malformation index of up to 60 % at a concentration below the expected environmental concentration shows the serious reproductive impacts of this formulation. More attention should be focused on the impacts of this formulation, as the formulation could potentially impact any exposed organism population and not just of amphibians. For Arsenal formulation, the occurrence of up to 35 % at a concentration of 3.5 mg/L, which is below the current environmental relevant concentration of 5.78 mg/L, makes this formulation a potential threat in any aquatic environment.

For the glyphosate formulations, although all the three formulations showed a dose dependent increase in gonadal abnormalities, Roundup emerged as the most reprotoxic of the three,

as it produced more morphological malformations at the lowest concentration. For Roundup formulation, these results confirmed the observations of Howe *et al*. (2004) who found the occurrence of gonadal malformities in metamorphs exposed to Roundup formulation. But since Howe *et al*. (2004) also noted that the technical glyphosate produced no significant gonadal malformation, it means that the observed malformations are therefore the impacts of the various surfactants in each of these formulations.

In general, as noted by several authors, gonadal abnormalities as observed in this study could lead directly to reproductive dysfunction (Qin *et al*., 2005) and are likely to reduce the reproductive success of exposed individuals (Quellet *et al*., 1997; Howe *et al*., 2004; Qin *et al*., 2005; McCoy *et al*., 2008; McDaniel *et al*., 2008). The widespread nature of these pesticide induced amphibian gonadal malformations could also be a contributing factor to the global decline that is currently being witnessed (Qin *et al.*, 2005).

5.14. Conclusions

The results of this study showed the potential capacities of Midstream (diquat dibromide), Basta (glufosinate ammonium), Arsenal (imazapyr) and glyphosate formulations (Roundup, Kilo Max and Environ Glyphosate) to negatively impact the reproductive fitness of *X. laevis* in particular but also amphibians in general. This effects include the reduced growth rate as demonstrated following exposures to Midstream, Kilo Max, Roundup, and Enviro Glyphosate formulations. The herbicide formulations also resulted in sexual developmental effects as demonstrated by the feminisation following exposure to Midstream, and Kilo Max formulations. Apart from the sex ratios modulation, the herbicide formulations also differentially impaired gonadal development as observed following exposures of *X. laevis* tadpoles to graded concentrations of the six selected formulations.

Even though the physiological and reproductive impact of gonadal malformations are still largely unknown, particularly as it may affects reproductive success within the larger population, more attention is required to assess the real reproductive impact of many pesticides known to induce malformations in amphibian species like *X. laevis*. Since endocrine modulation studies using herbicides are mostly focused on a handful of widely used chemicals, more attention should be given to the other herbicide formulations, since it is clear that these compounds may have more reprotoxic potential than previously thought, when compared to other groups of pesticides including fungicides and insecticides. Environmental assessment at the population level in aquatic systems potentially being contaminated by herbicide formulations directly or indirectly should be monitored

for developmental and reproductive effects on a regular basis. In terms of the regional South African aquatic environment, this study has shown that herbicide may well be a threat to endemic aquatic biodiversity and field studies including aquatic species, (like fish and amphibians) needs urgent attention. Moreover, when studying pesticide effects in aquatic ecosystems, clearly herbicides, individually or in mixtures should be included. The assessment should be used not only to re-evaluate the impacts of old pesticides, but to also show the potential effects of newly developed ones before they are applied into the environment.

References

Aerle, R.; Nolon, T. M.; Jobling, S.; Christiansen, L. B.; Sumpter, J. P.; Tyler, C. R. (2001). Sexual disruption in a second species of wild cyprinid fish (the gudgeon, *Gobio gobio*) in United Kingdom freshwaters. *Environmental Toxicology and Chemistry* **20***:* 2841–2847.

Ansara-Ross, T. M.; Wepener, V.; van den Brink, P. J.; Ross, M. J. (2012). Pesticides in South African fresh waters. *African Journal of Aquatic Science* **37**: 1-16.

Belt, Van den, K.; Verheyen, R.; Witters, H. (2001). Reproductive effects of ethynylestradiol and 4t-octylphenol on the zebrafish (*D. rerio*). *Archives of Environmental Contamination and Toxicology* **41**: 458–467.

Berven, K. A. (1990). Factors affecting population fluctuations in larval and adult stage of the Wood Frog *Lithobates sylvatica. Ecology* **71**:1599–1608.

Bishop, C. A.; Mahony, N. A.; Strugger, J.; Ng, P.; Pettit, K.E. (1999). Anuran development density and diversity in relation to agricultural activity in the Holland rivers watershed, Ontario, Canada. *Environmental Monitoring and Assessment* **57**: 21-43.

Brunelli, E.; Bernabo, I.; Berg, C.; Lundstedt-Enkel, K.; Bonacci, A.; Tripepi, S. (2009). Environmental relevant concentrations of endosulfan impaired development, metamorphosis and behaviour in *Anaxyrus anaxyrus* tadpoles. *Aquatic Toxicology* **91**: 135-142.

Cabrera-Guzmán E.; Crossland M. R.; Shine R. (2013) Competing tadpoles: Australian native frogs affect invasive cane toads (*Rhinella marina*) in natural waterbodies. *Australian Ecology*. DOI: 10.1111/aec.12029.

Cabrera-Guzmán E. (2013) Interactions between Invasive Cane Toads and Native Fauna in Tropical Australia. Unpublished PhD Thesis, University of Sydney, Sydney.

Calabrese, E.D. (2001). Estrogen and related compounds: biphasic dose responses. *Critical Review in Toxicology* **31**: 503–515.

Carr, J. A.; Gentles, A.; Smith, E. E.; Goleman, W. L.; Urouidi, L. J.; Thuett, K.; Kendall, R. J.; Giesy, J. P.; Gross, T. S.; Solomon, K. R.; Kraak, G.V. (2003). Response of larval *Xenopus laevis* to Atrazine: Assessment of growth, metamorphosis, and gonadal and larygeal morphology. *Environmental Toxicology and Chemistry* **22**(2): 396-405.

Carey, C.; Bryant, C. J. (1995). Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. *Environmental Health Perspective* **103** (4): 13-17.

Cevasco, A.; Urbatzka, R.; Bottero, S.; Massari, A.; Pedemonte, F.; Kloas, W.; Mandich, A. (2008). Endocrine disrupting chemicals with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of *X. laevis*: II. Effects on gonads histomorphology. *Comparative Biochemistry and Physiology Part C* **147**: 241-251.

Collins, J. P. (1975). A comparative study of the life history strategies in a community of frogs. Unpublished Ph.D. Dissertation, University of Michigan, USA.

Collins, J.P. (1979). Intrapopulation variation in the body size at metamorphosis and timing of metamorphosis in the bullfrog, *Lithobates catesbeiana*. *Ecology* **60**: 738–749.

Crain, D. A. (1997). Effects of endocrine-disrupting contaminants on reproduction in the American alligator, Alligator mississippiensis. Unpublished Ph.D Thesis, University of Florida, USA.

Crain, D. A.; Guillette, L.J.; Pickford, D.B.; Percival, H.F.; Woodward, A.R (1998). Sex-steroid and thyroid hormone concentrations in juvenile alligators (Alligator mississippiensis) from contaminated and reference lakes in Florida. *Environmental Toxicology and Chemistry* **17:**446–452.

Crain, D. A.; Guillette, L. J.; Rooney, A. A.; Pickford, D. B (1997). Alterations in steroidogenesis in alligators (Alligator mississippiensis) exposed naturally and experimentally to environmental contaminants. *Environmental Health Perspective* **105**: 528-533.

Diana, S. G.; Resetarits, W. J.; Schaeffer, D.J.; Beckmen, K.B.; Beasley, V.R. (2000). Effects of Atrazine on amphibian growth and survival in artificial aquatic communities. *Environmental Toxicology and Chemistry* **19**(12): 2961–2967.

Diamanti-Kandarakis, E.; Bourguignon, J.; Giudice, L. C.; Hauser, R.; Prins, G. S.; Soto, A. M.; Zoeller, R. T.; Gore, A.C. (2009). Endocrine-disrupting chemicals. An endocrine society scientific statement. *Endocrine Reviews* **30**(4): 293-342.

Dinehart, S. K.; Smith, L. M.; McMurrya, S. T.; Smith, P. N.; Anderson, T. A.; Hauko, D. A. (2010). Acute and chronic toxicity of Roundup Weathermax_ and Ignite_ 280 SL to larval *Spea multiplicata* and *Spea bombifrons* from the Southern High Plains, USA. *Environmental Pollution **158***: 2610-2617.

Dmitriew, C.; Rowe, L. (2011). The effects of larval nutrition on reproductive performance in a food-limited adult environment. *PLoS ONE* **6**(3): e17399.

D'Souza, U. (2004). Effects of tamoxifen on spermatogenesis and tubular morphology in rats. *Asian Journal of Andrology* **6:** 223-226.

Ebert, E.; Leist, K. H.; Mayer, D. (1990). Summary of safety evaluation toxicity studies of glufosinate ammonium. *Food and Chemical Toxicology* **28**: 339-349.

Edginton, A. N.; Sheridan, P.M.; Stephenson, G.R.; Thompson, D.G.; Boermans, H. J (2004a). Comparative effects of pH and Vision herbicide on two life stages of four anuran amphibian species. *Environmental Toxicology and Chemistry* **23**(4): 815–822.

Emmett, K. (2002). Final risk assessment for diquat bromide. The water quality program of the Washington State Department of Ecology. 02-10-046. www.wsde.org. Accessed on 4th of Dec., 2013.

Fort, D. J.; Stover, E. L.; Bantle, J. A.; Dumont, J. N.; Finch, R. A (2001). Evaluation of a reproductive toxicity assay using *Xenopus laevis*: Boric acid, cadmium, and ethylene glycol monomethyl ether. *Journal of Applied Toxicology* **21**: 41–52.

Giesy, J.; Ludwig, J.; Tillitt, D (1994). Deformities in birds of the great lakes region; assigning causality. *Environment Science and Technology* **28:** 128-135.

Gungordu, A. (2013). Comparative toxicity of methidathion and glyphosate on early life stages of three amphibian species: Pelophylax ridibundus, Pseudepidalea viridis, and *Xenopus laevis*. *Aquatic Toxicology* **140– 141:** 220–228.

Guillette, L. J.; Gross, T. S.; Masson, G. R.; Matter, J. M.; Percival, H. F.; Woodward, A. R. (1994). Developmental abnormalities of the gonad and abnormal sex-hormones concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environmental Health Perspective* **102** (8): 680-688.

Guillette, L. J.; Crain, D. A.; Rooney, A. A.; Pickford, D. B. (1995). Organization versus activation: The role of endocrine-disrupting contaminants (EDC's) during embryonic development in Wildlife. *Environmental Health Perspectives* **103**: 157-164.

Guillette, L. J. Jr.; Crain, D. A. (eds.) (2000). Endocrine disrupting contaminants: An evolutionary perspective, Francis and Taylor, Inc., Philadelphia p.355.

Gimeno, S.; Komen, H.; Jobling, S.; Sumpter, J. P.; Bowmer, T. (1998). Demasculinisation of sexually mature male common carp, Cyprinus carpio, exposed to 4-tert-pentylphenol during spermatogenesis. *Aquatic Toxicology* **43***:* 93 -109**.**

Greulich, K.; Hoque, E.; Pflugmacher, S. (2002). Uptake, metabolism, and effects on detoxication enzymes of isoproturon in spawn and tadpoles of amphibians. *Ecotoxicology and Environmental Safety* **52**: 256–266.

Greulich, K.; Pflugmacher, S. (2003). Differences in susceptibility of various life stages of amphibians to pesticides exposure. *Aquatic Toxicology* **65**: 329-336.

Grisolia, C. K.; Bilich, M. R; Formigli, L. M (2004). A comparative toxicologic and genotoxic study of the herbicide arsenal, its active ingredient imazapyr, and the surfactant nonylphenol ethoxylate. *Ecotoxicology and Environmental Safety* **59**:123–126.

Hack, R. E.; Ebert, G.; Ehling, K.; Leist, H. (1994). Glufosinate-ammonium; some aspects of its mode of action in mammals. *Food and Chemical Toxicology* **32**(5*)*: 461-470.

Hanken, J. (1983). High incidence of limb skeletal variants in a peripheral population of thered-backed salamander, *Plethodonchereus* from Nova Scotia**.** *Canadian Journal of Zoology*. **61**:1925-1931.

Hayes, T. (2005). Welcome to the evolution: Integrated biology and assessing the impacts of endocrine disruptors on environmental and public health. *Integrated and Comparative Biology*. **45**: 321-329.

Hayes, T.; Haston, K.; Tsui, M.; Hoang, A.; Haeffele, C.; Aaron Vonk (2002a) Feminization of male frogs in the wild. *Nature* **419**: 895-896.

Hayes, T.B.; Collins, A.; Lee, M.; Mendoza, M.; Noriega, N.; Stuart, A.A.; Vonk, A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceding of National Academic of Science U S A **99**: 5476-5480.

Hayes, T. B. (1998). Sex determination and primary sex differentiation in amphibians: Genetic and developmental mechanisms. *Journal of Experimental Zoology* **281**:373–399.

Hayes, T. B.; Falso, P.; Gallipean, S.; Stice, M. (2010).The cause of global amphibian declines: A developmental endocrinologist perspective. *The Journal of Experimental Biology* **213***: 921-933.*

Howe, S. R.; Borodinsky, L. (1998). Potential exposure to biosphenol A from food-contact use of polycarbonate resins. *Food Additives and Contaminants* **15***:* 370-375.

Howe, C. M.; Berrill, M.; Pauli, B. D.; Helbing, C. C.; Werry, K.; Veldhoen, N. (2004). Toxicity of glyphosate-based pesticides to four North American frog species. *Environmental Toxicology and Chemistry* **23**: 1928–1938.

Howard, R. D (1978). The evolution of mating strategies in bullfrogs, *Lithobates catesbeiana*. *Evolution* **32***:*850-871.

Jayawardena, U. A.; Rajakaruna, R. S.; Navaratne, A. N.; Amerasinghe, P. H. (2010). Toxicity of agrochemicals to common hourglass tree frog (*Polypedate scruciger*) in acute and chronic exposure. *International Journal of Agricultural Biology* **12**: 641-648.

Jobling, S.; Nolan, M.; Tyler, C. R.; Brightly, G.; Sumpter, J. P (1998). Widespread sexual disruption in wild fish. *Environment Science and Technology* **32**: 2498–2506.

Jobling, S.; Beresford, N.; Nolan, M.; Rodgers-Gray, T.; Brightly, G. C.; Sumpter, J. P.; Tyler, C. R (2002a). Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus)* living in rivers that receive treated sewage effluents. *Biology of Reproduction.* **66**: 272–281.

Jobling, S.; Coey, S.; Whitmore, J. G.; Kime, D. E.; Van Look, K. J. W.; McAllister, B .G.; Beresford, N.; Henshaw, A. C.; Brightly, G.; Tyler, C. R.; Sumpter, J. P (2002b). Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biology of Reproduction.* **67:** 515–524.

Kidd, K. A.; Blanchfield, P. J.; Mills, K. H.; Palace, V. P.; Evans, R .E.; Lazorchak, J. M.; Flick, R. W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceeding of National Academic of Science. USA* **104**: 8897-8901.

Kloas, W.; Urbatzka, R.; Opitz, R.; Wurtz, S.; Behrends, T.; Hermelink, B.; Hoffmann, F.; Jagnytsch, O.; Kroupova, H.; Lorenz, C.; Neumann, N.; Pietsch, C.; Trubiroha, A.; Ballegooy, C.; Wiedermann, C.; Lutz, I. (2009). Endocrine disruption in aquatic vertebrates. Trends in comparative endocrinology and neurobiology: *Annual NewYork Academic of Science*. **1163**: 187-200.

Koyama, K.; Koyama, K.; Goto, K. (1997). Cardiovascular effects of herbicides containing glufosinate and a surfactant: in vitro and in vivo analysis in rat. *Toxicology and Applied Pharmacology* **145***:* 409-414.

Langlois, V.S.; *Carew,* A.C.**;** *Pauli,* B.D.**;** Wade**,** *M. G.; Cooke, G. M.; Trudeau***,** V. L. (2010**).** Low Levels of the Herbicide Atrazine Alter Sex Ratios and Reduce Metamorphic

Success in *Lithobates pipiens* Tadpoles Raised in Outdoor Mesocosms. *Environmental Health Perspective* **118:**552–557.

Larebeke, N. A. V.; Sasco, J. A.; Brophy, J. T.; Keith, M. M.; Gilbertson, M.; Watterson, A. (2008). Sex ratio changes as sentinel health events of endocrine disruption. *International Journal of Occupational and EnvironmentalHealth* **14***:*138–143.

Lenkowski, J. R.; Reed, J. M.; Deininger, L.; McLaughlin, K. A. (2008). Perturbation of organogenesis by the herbicide atrazine in the amphibian *Xenopus laevis*. *Environmental Health Perspective* **116**: 223–230.

Lutz, I.; Kloas, W.; Springer, T.A.; Holden, L.R.; Wolf, J.C.; Krueger, H.O.; Hosmer, A.J. (2008). Development, standardization and refinement of procedures for evaluating effects of endocrine active compounds on development and sexual differentiation of Xenopus laevis. *Analytical and Bioanalytical Chemistry* **390**:2031–2048.

Magnuusson, U.; Brunstrom, B. (Ed) (2007). Reproductive Toxicology in Environmental Research. Swedish Environmental Protection Agency Report 5729.

Mann, R. M.; Bidwell, J. R. (2000). Application of the FETAX protocol to assess the developmental toxicity of nonyl phenol ethoxylate to *Xenopus laevis* and two Australian frogs. *Aquatic Toxicology* **51**: 19-29.

Mann, R. M.; Hyne R. V.; Choung, C. B.; Wilson, S. P. (2009). Amphibians and agricultural chemicals: review of the risks in a complex environment. *Environmental Pollution.* **157**: 2903-2927.

McCoy, K. A.; Bortnick, L. J.; Campbell, C. M.; Louis, H. J.; Guillette Jr, J.; St. Mary, C. M. (2008). Agriculture alters gonadal form and function in the toad *Anaxyrus marinus Environmental Health Perspective* **116**:1526–1532.

McDaniel, T. V.; Martin, P. A.; Struger, J.; Sherry, J.; Marvin, C. H.; McMaster, M. E.; Clarence, S.; Tetreault, G. (2008). Potential endocrine disruption of sexual development in free ranging male northern leopard frogs (*L. pipiens*) and green frogs (*L. clamitans*) from areas of intensive row crop agriculture. *Aquatic Toxicology* **88***:* 230–242.

Mensah, P. K.; Palmer, C. G.; Muller, W. J (2013). Derivation of South African water quality guidelines for Roundups using species sensitivity distribution. *Ecotoxicology and Environmental Safety* **96***:* 24–31.

Othman, M; Ding, L; Jiao, Y (2009). 'Effect of anionic and non-ionic surfactants on activated sludge oxygen uptake rate nitrification'.International *Journal of Chemical, Molecular, Nuclear, Materials and Metallurgical Engineering* **3**(10)**:** 596-602.

Oka, T.; Tooi, O.; Mitsui, N.; Miyahara, M.; Ohnishi, Y.; Takase, M.; Kashiwagi, A.; Santo, A.; Iguchi, T. *(2008).*Effect of atrazine on metamorphosis and sexual differentiation in *Xenopus laevis. Aquatic Toxicology* **87**(4)**:**215-26.

Organisation for Economic Co-operation and Development (OECD) (2008). Series on Testing and Assessment. No. 91. Report of the Validation of the Amphibian Metamorphosis Assay (PHASE 3) ENV/JM/MONO (2008)18 Available at: www.oecd.org/officialdocuments

Paggetti, E.; Biaggini, M.; Corti, C.; Lebboroni, M.; Berti, R. (2006). Amphibians and reptiles as indicators in Mediterranean agro-ecosystems: A preliminary study. Proceedings of the 13th Congress of the Societas Europaea Herpetologica 107-110.

Peterson, H. G.; Boutin, G.; Martin, P. A.; Freemark, K. E.; Ruecker, N. J.; Moo.dy, M. J. (1994). Aquatic phyto-toxicity of 23 pesticides applied at expected environmental concentrations. *Aquatic Toxicology* **28***: 275-292.*

Qin, Z. F.; Zhou, J. M.; Cong, L.; Xu, X. B. (2005). Potential ecotoxic effects of polychlorinated biphenyls on *Xenopus laevis*. *Environmental Toxicology and Chemistry* **24**: 2573–2578.

Quellet, M.; Bonin, J.; Rodrigue, J.; Desgranges, J. L.; Lair, S. (1997). Hind limb deformities (*Ectromelia, ectrodactyly*) in free-living anurans from agricultural habitats. *Journal of Wildlife Distribution* **33**(1*)*: 95-104.

Read, J. L.; Tyler, M. L. (1994). Natural level of abnormalities in the trilling frog (*Neobatrachus centralis*) at the Olympic Dam mine. *Bulletin Environmental Contamination and Toxicology* **53**: 25-31.

Salthe, S. N. (1969).Reproductive modes and the number and sizes of ova in the Urodeles. The American Midland Naturalist. **81**:467-490.

Semlitsch, R. D.; Scott, D. E.; Pechmann, J. H. K (1988). Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. *Ecology* ***69***(1): 184-192.

Semilitch, R. D.; Bodie, R. (1998). Are small, isolated wetlands expendable? *Conservation Biology* **12**: 1129-1133.

Semlitsch, R. D. (1989). Effects of body size, sibship, and tail injury on the susceptibility of tadpoles to dragonfly predation. *Canadian Journal of Zoology***. 68**:1027–1030.

Sharma, B.; Patino, R. (2009). Effects of Cadmium on growth, metamorphosis and gonadal sex differentiation in tadpoles of the African Clawed Frog, *Xenopus laevis*. *Chemosphere* **76**: 1048-1055.

Sherry, J.; Gamble, A.; Fielden, M.; Hodson, P.; Burnison, B.; Solomon, K. R. (1999). An ELISA for brown trout (*Salmo trutta*) vitellogenin and its use in bioassays for environmental estrogens. *Science of Total Environment* **225**: 13–31.

Soyano, K.; Aoki, J.; Itashiki, Y.; Park, C.; Nagae, M.; Takao, Y.; Lee, Y.; Yeo, I.; Zhong, J. (2010). Contaminations by Endocrine Disrupting Chemicals in Coastal Waters of the East China Sea**.** *Coastal Environmental and Ecosystem Issues of the East China Sea*, Eds., A. Ishimatsu and H.-J. Lie, 215–226.

Sparling, D. W.; Linder, G.; Bishop, C. A (2000).Ecotoxicology of Amphibian and Reptiles. SETAC Press, Pensaiola, Florida, USA.

Sullivan, K.; Spence, K. (2003). Effects of sublethal concentrations of atrazine and nitrate on metamorphosis of the African clawed frog. *Environmental Toxicology and Chemistry* **22**: 627-635.

Taylor, B.; Skelly, D.; Demarchis, L. K.; Slade, M. D.; Galusha, D.; Rabinowitz, P. M. (2005). Proximity to pollution sources and risk of amphibian limb malformation. *Environmental Health Perspectives* ***113***(11): 1497–1501.

Urbatzka, R.; van Cauwenberge, A.; Maggioni, S.; Vigano, L.; Mandich, A.; Benfenati, E.; Lutz, I.; Kloas, W. (2007a). Androgenic and antiandrogenic activities in water and sediment samples from the river Lambro, Italy, detected by yeast androgen screen and chemical analyses. *Chemosphere* **67**: 1080–1087.

Vitt, L. J.; Caldwell, J. P.; Wilbur, H. M.; Smith, D.C. (1990). Amphibians as harbingers of decay. *Bioscience* **40**: 418.

Vizantinopoulos, S.; Lolos, P. (1994). Persistence and leaching of the herbicide imazapyr in soil. Bulletin of Environmental *Contamination and Toxicology* **52**:404-410.

Washington State Department of Agriculture (WSDA) (2003). Ecological risk assessment of the proposed use of the herbicide Imazapyr to Control Invasive Cordgrass (*Spartina spp) www.ecy.wa.gov. Accessed on 24 October 2010.*

Chapter Six

Estrogenicity and androgenicity potential of Midstream (diquat dibromide) and Arsenal (imazapyr) formulations on Adult male Amphibian using *Xenopus laevis* as sentinel species

6.1. Introduction

Despite the increased growth in the non-agricultural sectors of the global economy, agricultural activities still dominate the use of freshwater (OECD, 2012). This has led to widespread contamination and pollution of aquatic environment with various chemicals and agro-pesticides, including herbicides, fungicides and insecticides (Lavorato *et al*., 2013). The presence of these pesticides residues in food, soils and sediments as well as in runoff water are ubiquitous environmental toxicants that pose a risk to human and animal health (Sharma *et al*., 2011). Pesticides have been identified as one of the major factors affecting biological diversity (Bouetard *et al*., 2013). In fact, internationally, pesticides have been recognised as a potential contributing factor to the global amphibian decline (Gibbons *et al*., 2000; Stuart *et al*., 2004; Hayes 2005; McDaniel *et al*., 2008; Lance *et al*., 2012; Levarato *et al*., 2013). Many of these pesticides and other environmental contaminants are alleged to mimic or antagonising the action of hormones, in a way that might disrupt the development and reproductive systems of the organism (Urbatzka *et al*., 2007; Cevasco *et al*., 2008; Flint *et al*., 2012).

These commonly called endocrine disrupting compounds (EDCs) have been reported to be present in aquatic systems that are receiving effluent from wide ranging sources. Accumulation of EDCs in surface waters and sediments makes permanent and occasional aquatic inhabitants like fish and amphibians face an increase ecological risk (Cevasco *et al*., 2008). For example, several EDCs have been reported to interfere with the androgenic system and shown to modulate the normal functions of tissues and organs associated with the male reproductive system (Jugan, 2009; Orton *et al*., 2012). The exposure-impacts of many these chemicals may be directed at the endocrine system which may result in several health alterations including loss of fertility and fecundity, result in developmental abnormalities, immune-suppression and mortality (Zaya *et al*., 2011).

Not surprising, exposure of humans and wildlife to chemical contaminants, sharing the aquatic sources of the world is practically inevitable. Hence the exposure impacts of these pesticides to human and wildlife health usually depends on levels and duration of exposure. It has been

hypothesised that many of the alterations in reproductive phynotype and functions are due to the endocrine disruptive effects of various pesticide and chemical contaminants (Guillette *et al*., 2000; Hecker *et al*., 2005). And because many organs are targeted by sex steroids including the hypothalamic-pituitary-gonadal system, breast, uterus, cervix, vagina, brain, and non-reproductive tissues such as bone, muscle and skin (Diamanti-Kandarakis *et al*., 2009), these organs may be vulnerable to endocrine disruption.

Recent increased in report of incidence of intersex in fish and other aquatic organisms inhabiting global waterway has further raised the concern about threat of endocrine disrupting compounds in the aquatic environment (Wise *et al*., 2011). Many studies are now linking environmental pesticides to reprotoxic incidences in the wild, including fish (Jobling *et al*., 1998, 2002; Gimeno *et al*., 1998; Aerle *et al*., 2001; Urbatzka *et al*., 2007; Kidd *et al*., 2007), amphibians (Fort *et al*., 2001; Hayes *et al*., 2002a, Hayes 2005; McDaniel *et al*., 2008), reptiles (Guillette *et al*., 1994; Crain *et al*., 1997; Crain 1997; Crain *et al*., 1998), birds (Giesy *et al*., 1994), and mammals (D'Souza, 2004). Most of the documented health impacts of the EDCs both in human and wildlife have been linked to estrogenic effects (Krisnan *et al*., 1993; Nimrod and Benson, 1996). According to Diamanti-Kandarakis *et al*. (2009), low level and chronic exposure to endocrine disruptors, especially estrogenic substances may be contributing to adverse human health effects. It is also known that amphibian larvae exposed to estrogenic compounds develops gonadal differentiation towards female biased sex ratios (Kloas *et al*., 1999; Mackenzie *et al*., 2003).

Androgenic, anti-androgenic as well as estrogenic and anti-estrogenic effects of substances has been demonstrated in many organisms (Wise *et al*., 2011; Orton *et al*., 2012; Lavarato *et al*., 2013). For example, many pesticides are known to disrupt male sexual differentiation in vivo by antagonising the androgen hormone binding to its intracellular receptor (AR) (Orton *et al*., 2012). Androgens are male reproductive hormones, synthesised by the Leidig cells in the testes (Bauer *et al*., 1998), and controlling spermatogenesis and secondary sexual traits (Kumar *et al*., 2008; Streck, 2009). The testes contain paracrine and autocrine regulations in various compartments that are under the endocrine influence from the pituitary and hypothalamus. These numerous compartments as well as other receptors make the testes highly susceptible to endocrine disruption (Sikka and Wang, 2008). The AR upon ligand binding translocate to the nucleus where it binds to the regulatory regions of androgen-responsive gene, and subsequently stimulates their transcription (Kumar *et al*., 2008). Chemicals that mimic androgens or antagonizing binding of androgen to the androgen receptor, could therefore interfere with androgen functions, leading to impairment in sexual functions and development (Bauer *et al*., 1998; Ubatzka *et al*., 2007).

On the other hand, feminizations of males following exposure to estrogen mimics substances have been widely reported in wildlife species (Du Preez *et al*., 2005; Hayes *et al*., 2006; Tranchantong *et al*., 2013). A global example is the Atrazine herbicide, which has been shown to feminize male frogs (Tavera-Mendoza *et al*., 2002; Du Preez *et al*., 2005). The expression of estrogen-induced proteins like the yolk precursor, vitellogenin (Hurter *et al*.,2002) and anti-androgenic effects of estrogens on male breeding glands in amphibian skin (van Wyk *et al*., 2003) have been reported as biomarkers of exposures.

According to Sikka and Wang (2008), the normal functioning of the male reproductive system may therefore be at risk when adult males are exposed to reproductive toxic environmental pollutants including numerous pesticides, particularly herbicides, fungicides and insecticides. Thyroid hormones modulation have also been noted to have a role in the reproductive system disturbance. According to Crain *et al.* (1998), thyroid hormones play important roles in growth and reproductive capacity. They noted that removal of thyroid in lizards and geckos can result in the inhibition of spermatogenesis, which can be restored with the admnistration of thyroxine. Crain *et al.* (1998) further pointed out that by exposing some organism with an intact thyroid to triiodothyronine also resulted in the suppression of spermatogenesis, suggesting that the normal functioning of reproductive organs is dependent on a specific range of thyroid hormones level. This mean that pesticides that affect thyroid system could also have a serious impacts on the reproductive system of organisms.

Globally, herbicides are extensively used in agriculture and environmental management programmes to control unwanted and alien plants. Currently, about 180 different pesticides active ingredients are available with about 400 registered trade names (Menhardi, 2008). South Africa is the largest pesticides market in sub-Saharan African, with about 60% of the pesticides market in Africa (Ansara-Ross *et al*., 2012). According to Ansara-Ross *et al*. (2012), there is increasing application of glyphosate-based herbicides in South Africa in recent years for example, as large numbers of farmers are applying herbicides to herbicide-resistant genetically modified wheat, corn, and canola crops. However, herbicides are also widely used in other agricultural sectors, for example pasture, vegetable, fruit and grapes farming, but also for domestic use (Bold, 2007; Ansara-Ross *et al.*, 2012; Mensah *et al*., 2013). The Working for Water Program launched and managed by the National Department of Water Affairs also uses large volumes of various herbicides including glyphosate-based formulations, glufosinate ammonium formulations, imazapyr and diquat dibromide formulations, among many others, in eradicating alien vegetation in valuable water catchment areas (Bold, 2007; Ansara-Ross *et al.*, 2012).

Several of these commonly used herbicide formulations have not been assessed for androgenic, anti-androgenic, estrogenic as well as anti-estrogenic properties. The present study aimed to assess the exposure impacts of Midstream formulation (diquat dibromide) and Arsenal formulation (imazapyr) to specifically answer questions about the estrogenicity (feminization) and (anti-) androgenicity potential on adult male *Xenopus laevis*. In previous chapters 3-5, Midstream and Arsenal formulations exposure resulted in sex ratios disruption (60:40 and 45:55 F: M ratios for Midstream and Arsenal respectively), high gonadal abnormality ratios (60 % and 35 % for Midstream and Arsenal respectively) and varying high degree of developmental and thyroid gland disruption. The Midstream formulation equally showed high teratogenic index value (at TI of 3.5). These two herbicide formulations were therefore selected for adult stage exposures.

Midstream formulation (diquat dibromide (9, 10-dihydro-8a, 10a-diazoni a phenanthrene ion) is a post-emergent, non-selective contact herbicide and crop desiccant that is equally used in aquatic weeds control (WHO, 2004; Emmett, 2002). It is widely used in the United State, North America, Europe, Australia and Japan (WHO, 2004; Emmett, 2002). One of the most common formulations of diquat dibromide is Midstream formulation that contains nonyl phenol ethoxylate as surfactant that has been implicated as having estrogenic activities (Trumbo, 2005; Othman, 2009). The expected environmental concentration is 0.733 mg/L (Williams *et al*., 1996)

Arsenal formulation (imazapyr) on the other hand, belongs to the chemical family of imidazolinone (Liu, 1992). It is a systemic, non-selective, pre- and post-emergent herbicide used for the control of a broad range of terrestrial and aquatic weeds, and controls plant growth (EPA, 2006 According to Grisolia *et al*. (2004), the Arsenal formulation is made up of 25 g/L imazapyr, 186 g/L of ammonium hydroxide, 18 g/L nonyphenol ethoxylate (with nine ethoxylated units) and water. The expected environment concentration is 5.77 mg/L (WSDA, 2003)

The Africa clawed frog (*Xenopus laevis)* is an aquatic species that has been used globally as an animal model for environmental quality assessment (Mann and Bidwell, 2000; Paggeti *et al*., 2006; Kloas *et al*., 2009; Vitt *et al*., 1990), adapted for in-vitro research (Kloas *et al*., 1999), in vivo studies (Ubatzka *et al*., 2007). As sentinel species allow for relatively quick surveys (Paggeti *et al*., 2006) while providing very sensitive endpoints (Kloas *et al*., 2009; Lavorato *et al*., 2013). Adult male *X. laevis* are good models for estrogenicity assessment since they have the potential to produced vitellogenin when exposed to exogenous estrogenic substances (Kloas *et al*., 1999; Hurter *et al*., 2002 (a & b); Kloas and Lutz, 2006; Kloas *et al*., 2009). The males are also good models for

antiandrogenic screening as their breeding glands and /or vocal cords are sensitive to androgenic substances (Hayes *et al 2002;* Hurter *et al*., 2002; van Wyk *et al*., 2003).

6.2. Materials and Methods

6.2.1 Chemicals

Midstream (diquat dibromide), Syngenta S.A Ltd) was purchased from a local agricultural dealer in Stellenbosch, South Africa. Arsenal (Imazapyr herbicide, 2-(4-isoprpyl-4-methyl-5-oxo-2-imidazolin-2-yl) nicotini acid) (Base Chemical S.A Ltd) was obtained from Working for Water, South Africa.

6.2.2. Test organism

Wild caught adults male Africa Clawed frogs (*X. laevis)* were obtained from an accredited supplier, Loesch Paddas, Klapmuts Western Cape Province, South Africa.

6.2.3. Exposure concentrations

Exposure concentrations for this study were non-lethal and largely environmental relevant, based on 15, 30 and 45 % of 96 hour NF stage 48 of *X. laevis* (see Chapter 2).

6.2.4. Exposure methodology

Adult males were acclimatized in the laboratory using charcoal filtered water for 10 days under regulated climatic conditions (temp of 25 ±1 ^{0}C and 1 hour of light and dark (12:12 L:D). The frogs were fed commercial fish pellet (Aqua-Nutro RSA.) twice a week prior to tank cleaning and water replacement.

After the 10 days acclimatization period, five (5) frogs were transferred to each non-aerated 15 L glass tanks containing reverse osmosis water, buffered with 2.5 g marine salt (Levy et al., 2004). The frogs were subsequently exposed to three (3) graded concentrations of Midstream and Arsenal formulations for 28 days (Table 6.1).

Table 6.1. Commercially available Midstream (diquat dibromide) and Arsenal (imazapyr) formulations and the selected graded exposure concentrations. The zero concentration represents the control group.

	Formulation	Concentration (mg/L)
a	Midstream (diquat dibromide)	0, 0.25, 0.45, 0.65
b	Arsenal (imazapyr)	0, 2, 4, 8

The herbicide formulations were directly spiked into the exposure water which was replicated twice, with the exposure medium renewed every 48 hours (Monday, Wednesday and Friday). The exposed frogs were fed fish pellet on Monday and Friday before replacing the water. The exposure study was performed under the following physical conditions; pH of 6.5-8, dissolved oxygen of 3.5 mg/L and 12 hours of light and dark (12:12 L:D). All the procedures were performed according to the ethical protocol and principles as approved by the central Ethical Committee of the Stellenbosch University (Protocol no SU-ACUM13-00017).

6.2.5. Sampling Procedure

At the end of 28 exposure days, all the exposed frogs were sacrificed by decapitation followed by spinal pithing after ten (10) minutes anaesthesia in MS222 (200 mg/L). The frogs were dissected and approximately 1 mL whole blood was sampled from each frog, via cardiac puncture using heparinised syringes and needles. The blood samples obtained were subsequently centrifuged at 800 rpm for two minutes for plasma separation, and stored at -80 ^{0}C until hormonal assays were conducted.

The frogs were dissected and the testes and liver were collected and weighed, prior to the testes being individually labelled and fixed in Bouin's fluid for 24 hours. The testes were removed from the fixative, rinsed in water and stored in 70 % ethanol until routine histological processes followed. The following endpoints were assessed; (a) body size, mass organ for calculating gonadosomatic index (GSI) (gonad weight/wet mass of the frog X 100 (Gong *et al*., 2014), (b) hepatosomatic index (HSI), (c) testes histopathology, (d) blood plasma vitellogenin (VTG) concentration (e) blood plasma testosterone (T) concentration (to assess (anti)-androgenic activity) (f) blood plasma thyroid hormone (T4) concentration.

6.2.5.1. Plasma testosterone determination

A commercially available testosterone ELISA kit was used to determine plasma total T concentrations in adult male frogs as described by the manufacture (DRG, Germany). In summary, unextracted plasma samples were diluted 40 fold with PBS containing 0.1% human serum albumin (HSA) and put through the steps as directed by the ELISA kit in duplicate. Validation of the assay revealed sensitivity for testosterone of 0.069 ng /mL, with intra-assay coefficient of variance (CV) of 1.8 % and 10 % respectively. Spiked sample indicated recoveries that ranged between 90-110 %.

6.2.5.2. Total Plasma Thyroxine (T4) concentration

Total plasma thyroxine was determined as described by the manufacturer (DRG, Germany). In summary, 25 µl of each standard, control and undiluted samples was dispensed into appropriate wells followed by the addition of 100 µl working conjugate reagent. The enzymatic reaction was stopped with the addition of 100 µl of stop solution and the absorbance (O.D) was measured at 450 nm on a Labsystems MultiscanMS plate reader. According to the manufacturer, the accuracy of this standard is 100 ± 5%, and the minimum detectable for the assay was 0.5µg/dL, while the specificity of this assay for T4 is very high.

6.2.5.3. Plasma Vitellogenin determination

Plasma vitellogenin (VTG) was assessed using a validated in-house ELISA following the Hurter *et al*., (2002) protocol. In brief, the procedure involved samples been assayed in duplicate using a 96-well ELISA plate (Maxisorp, Nunc, Denmark). Each plate included a positive and negative control. The positive control was a sample with a known VTG concentration, while the negative control consisted of a sample containing no VTG. Wells were coated with 50 µl of sample diluted at 1/50 in phosphate buffered saline (BPS) and incubated for two hours, before been washed with 0.9% v/m NaCl. Subsequently, 50 µl mouse anti-Xenopus VTG diluted 1/1000 with PBS was added to each well and incubated for two hours at room temperature. The plate was washed followed by the addition of 50 µl/ well of peroxidise linked goat anti-mouse immunoglobulin conjugate complex (Amersham, South Africa) and incubated for an hour before been washed again with 0.9% v/m NaCl. Substrate (POD substrate, Roche, Germany) (100 µl) was added to each well and the reaction was stopped through the addition of 50 µl 0.5 M H_2SO_4 per well. The optical densities were read on a Labsystems MultiscanMS plate reader at 450 nm. VTG concentration was calculated from a standard curve prepared using samples of known VTG concentration on each plate.

6.2.6. Histological Preparation and Assessment

Testes samples preserved in 70 % ethanol were taken and subjected to routine paraffin wax (57 ^{o}C) histological (Bancroft and Steve, 1977) procedure. Sections were taken from wax blocks at 7µm thickness using Reichert Jung microtome (Cambridge Instrument GmgH, Germany). The mounted sections were dewaxed (xylene) and hydrated in graded series of ethanol and stained with haematoxylin and counter-stained with eosin (H&E) ((Bancroft and Stevens, 1977), followed by dehydrated in a dilution ethanol series, and cleared in xylene before coverlid were mounted with Entellen (Sigma, Ltd) mounting medium.

6.2.7 Spermatogenesis evaluation

Spermatogenic activity and testicular histopathology was evaluated relative to the control frogs according to Kaplan and Murathanoglu, (2008)**.** Anurans have a cystic-type of testis (Kaplan and Murathanoglu, 2008). Their cysts are randomly organised in the seminiferous tubules of the testis, where each cyst include Seroli cells and germ cells of the same developmental stage (spermatogonia (SPG), spermatocysts (SPC) or spermatids (SPT)). The cysts were classified as SPG when it contain large cells having solid oval nuclei and surrounded by a membrane. The SPC was identified as having cells with dispersed chromatin and no clear nuclear envelope. For the SPT, they were characterised as having condensed nuclei, smaller in size than SPCs and darkly stained. Some SPT cell stages were also identified as having elongated cell size leading to spermatozoa formation, which was identified as having cresent-shaped tail flagella and situated towards the lumen of the seminiferous tubules. The incidence of germ cell stages were compared between treatment concentrations by calculating percentage ratio of cysts bearing the germ cell types. The average percentage of SPZ per tubule area was determined by calculating the area of SPZ relative to the area of the tubule. Ten seminiferous tubules per testis sample were examined under 100x magnification to observe the stages of germ cells present in each cyst. Spermatogenic activity (status) of the frogs in the different exposure groups were determined by comparing the ratio of germ cell-types (% SPG, % SPC, % SPT) of each cyst within the seminiferous tubules and comparing it to the spermatogenic activity of the frog testes in the control group. Imaging was done using Leica DMLB microscope equipped with digital camera (Leica Microscope Ltd, Wetzlar GmbH).

6.2.8. Data Analysis

Normality and homogeneity of variance was evaluated using the respective Shapiro-Wilk's and Levene's tests. Total body mass, testes mass, HSI, GSI, as well as variation in histological

spermatogenic cells were compared to the respective untreated controls using one way ANOVA followed by Tukey HSD post hoc test for parametric data and Kruskal-Wallis ANOVA followed by Dunn's multiple comparison of mean rank test for non-parametric data. P-values < 0.05 were considered significant.

6.3. Results

6.3.1. Mortality and Gross Abnormality

Throughout the 28 days exposure period, no mortalities were recorded in all of the exposure tanks. There was also no incidence of gross abnormality during the exposure period.

Table 6.2: Morphometrics of adult *X. laevis* exposed to concentration series of Midstream (MID) and Arsenal (ARS) formulations. Hepatosomatic index (HSI) and gonadosomatic index (GSI) is expressed as percentage of body mass relative to liver and testes respectively. Values given as mean ± SD (N= 5 per group)

Conc. Mg/L	Body mass (SD)	TESTIS mass (SD)	TESTIS length (SD)	TESTIS width (SD)	LIVER mass (SD)	HSI (SD)	GSI (SD)
0 (control)	42.8 (7.4)	0.1 (0.03)	6.6 (0.87)	3.09 (0.28)	2.24 (0.91)	5.51 (1.95)	0.21 (0.08)
MID 0.5	38.2 (6.8)	0.1 (0.02)	7.1 (0.77)	2.8 (0.51)	1.97 (0.84)	5.05 (1.68)	0.27 (0.07)
MID 0.11	41.6 (8.2)	0.1 (0.05)	6.89 (0.85)	2.96 (0.46)	2.46 (0.91)	5.29 (1.85)	0.26 (0.11)
MID 0.14	41.6 (8.9)	0.1 (0.04)	6.85 (0.9)	2.77 (0.34)	2.43 (0.61)	5.91 (1.49)	0.23 (0.08)
ARS 0.5	53.3 (7.92)	0.11 (0.03)	7.9 (0.9)	3.27 (0.5)	2.99 (0.8)	5.6 (1.1)	0.2 (0.1)
ARS 2.0	49.9 (8.25)	0.11 (0.03)	7 (1.0)	3.15 (0.4)	2.77 (0.6)	5.6 (1.0)	0.22 (0.03)
ARS 3.5	44.1 (9.46)	0.1 (0.02)	7.4 (1.1)	2.79 (0.4)	2.48 (1.0)	5.51 (1.5)	0.23 (0.1)

6.3.2. Total body mass

The mean body mass of the adult frogs following exposure to Midstream formulation showed little variation compared to 42.8 g at the Control (Table 6.2). The reduction in body mass using Kruskal-Wallis ANOVA test, however, proved to be not significantly different compared to the Control group.

For the frogs exposed to Arsenal formulation, the mean body mass which was 53.3 g at 2 mg/L dropped to 49.9 g at 4 mg/L and then to 44.1 g at 8 mg/L compared to the 42.8 g at the Control group (Table 6.2). The increased body mass using Kruskal-Wallis ANOVA test was however not significantly different compared to the Control at all exposure concentrations.

6.3.3. Testicular morphometric

6.3.3.1. Testes length

The mean testes length did not vary significantly (P>0.05) among exposure groups compared to the Control group (Table 6.2). Using Unequal N HSD test, the increased length was however not statistically significant at all exposure concentration of the two formulations compared to the Control group.

6.3.3.2. Testes width

The mean testes width following exposure to both Midstream and arsenal formulation did not vary significantly (P>0.05) among the exposed groups relative to the Control group (Table 6.2; Figure 6.2).

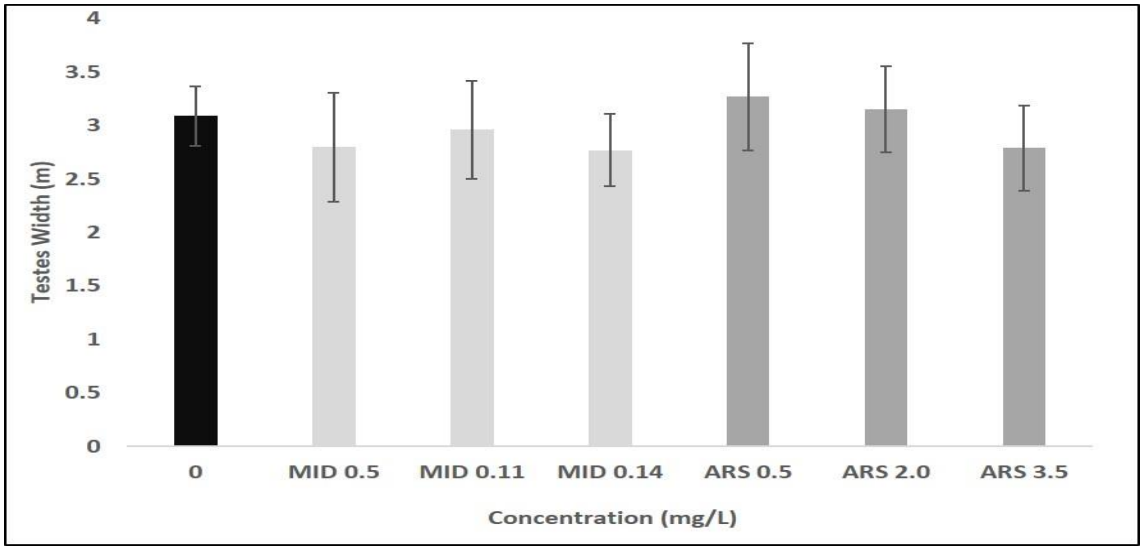

Figure 6.2: Mean testes width of the adult (male) *X. laevis* following exposure to Midstream and Arsenal formulation compared to the control

Using Unequal N HSD test, the reduction was not significantly different at all exposure concentrations compared to the control.

For Arsenal formulation, the mean testes width showed no slightly increased at lower exposure concentrations of 0.5 and 2.0 mg/L compared to the control. (Table 6.2; Figure 6.2). Using Unequal N HSD test, the increased was however not significantly different at all exposure concentrations compared to the Control group.

6.3.4. Gonadosomatic index

The mean gonadotropic Index (GSI) of the frogs exposed to Midstream using Kruskal-Wallis ANOVA test was however found not to be statistically significant (P>0.05) at all exposure concentrations relative to the Control group.

For frogs exposed to Arsenal formulation, the mean gonadosomatic Index (GSI) using Kruskal-Wallis ANOVA test was not statistically significant (P>0.05) at all exposure concentrations compared to the Control group.

6.3.5. Hepato-somatic Index (HSI)

For the HSI of the adult male frog following exposure to both the Midstream and Arsenal formulations, using Kruskal-Wallis test did not significantly vary (P>0.05) relative to the Control group (Table 6.2). Using Kruskal-Wallis test, HSI in the two formulations were found not to be significantly different (P>0.05) at all exposure concentrations compared to the Control group.

6.3.6. Spermatogenic activity of testes

Table 6.3. Spermatogenic activities of adults *Xenopus laevis* following exposure to concentration series of Midstream (MID) and Arsenal (ARS) formulations. Different spermatogenic cells, Spermatogonia (SPG), spermatid (SPT) and Spermatocytes (SPC) were expressed as mean of ten cross sections of seminiferous tubules diameter per testes.

Conc. (mg/L)	SPG (SD)	SPT (SD)	SPC (SD)
0 (control)	**37.5 (16.2)**	**45.3 (16)**	**13 (16.2)**
MID 0.25	**32.0 (15.8)**	**52.0 (13.4)**	**22.4 (20.1)**
MID 0.45	**37.2 (14.9)**	**42.5 (10.2)**	**20.6 (18.7)**
MID 0.65	**38.6 (16.5)**	**44.0 (10.2)**	**17.8 (13)**
ARS 2	**33.9 (17.3)**	**46.4 (14.3)**	**13.2 (11.5)**
ARS 4	**37.5 (18.3)**	**45.1 (16.2)**	**13.2 (13.6)**
ARS 8	**34.3 (15.3)**	**43.8 (17.5)**	**7.4 (8.8)**

6.3.6.1. Midstream formulation

Spermatogonia (SPG) in lumen

The mean percentage of spermatogonia cells in the frogs exposed to Midstream formulation slightly increased relative to the control (Table 6.3; Figure 6.3). Using Kruskal-Wallis ANOVA test, however showed that the variation was not significantly when compared with the Control group at all concentrations.

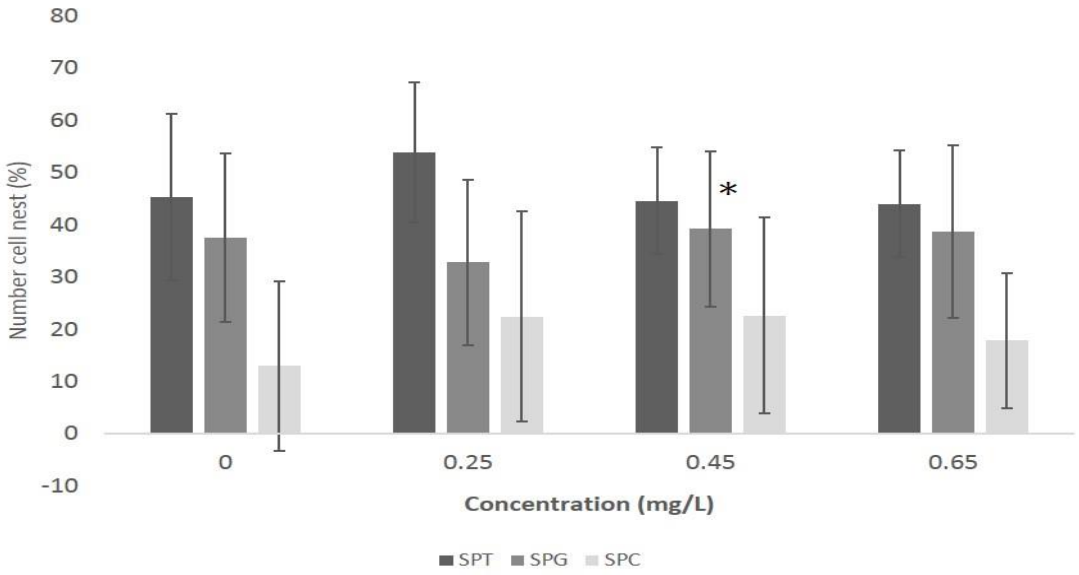

Figure 6.3: Variation in Spermatogenic activity, mean percentage volume of Spermatid (SPT), Spermatogonia (SPG) and Spermatocyte (SPC) of adult male *Xenopus laevis* following exposure to Midstream formulation compared to the Control group. (Asterisk indicated significant difference ($P<0.05$) relative to the control).

Spermatids (SPT) in lumen

The mean percentage of spermatid cells released into the lumen in the frogs following exposure to Midstream formulation showed no difference relative to the Control group (Table 6.3: Figure 6.3). Using Kruskal-Wallis test, showed that the decrease was not significant at all exposure concentration compared to the Control group.

Spermatocytes (SPC) in lumen

The mean percentage SPC of the frogs exposed to Midstream formulation increased across the exposure concentrations compared to the Control group. The mean percentage spermatocytes increased from 22.4 % at 0.25 mg/L to 22.6% at 0.45 mg/L and decreased to 17.8% at 0.65 mg/L compared to the 13% at the Control group (Table 6.3; Figure 6.3). However, Kruskal-Wallis ANOVA test confirmed that the increased percentage spermatocytes was only significant ($P<0.05$) at concentration of 0.45 mg/L compared to the Control group.

6.3.6.2 Arsenal formulation

Spermatogonia (SPG) in lumen

The mean percentage SPG of the frogs exposed to Arsenal formulation decreased in an n-shaped pattern. (Table 6.3; Figure 6.4). Using Kruskal-Wallis ANOVA test, confirmed that there was no significant different ($P>0.05$) between the Control group and the exposed frogs at all exposure concentrations (Figure 6.4).

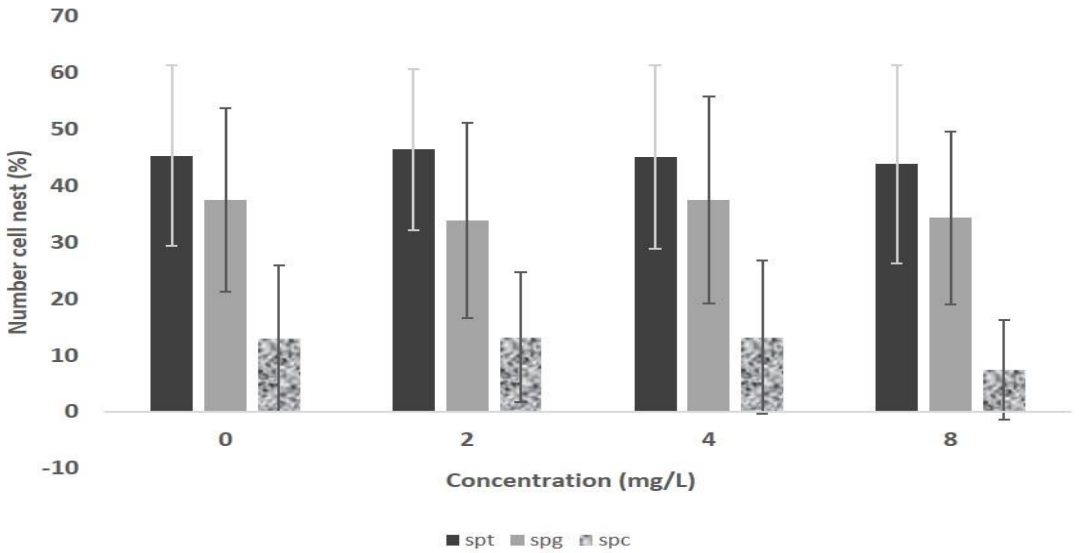

Figure 6.4: Percentage volume of Spermatogonia (SPG), Spermatid (SPT), and Spermatocyte (SPC) of adult *Xenopus laevis* male following exposure to Arsenal formulation compared to the Control group

Spermatids (SPT) in lumen

The mean percentage SPT released into the lumen in the frogs exposed to Arsenal formulation reduced in concentration dependent manner. (Table 6.3; Fig 6.4). Using Kruskal-Wallis ANOVA test, there was no significant difference (P>0.05) at all exposure concentrations compared to the Control group (Fig. 6.4).

Spermatocytes (SPC) in lumen

The mean percentage SPC of the frogs exposed to Arsenal formulation showed slightly decreased across the exposure concentrations compared to the Control group (Table 6.3; Figure 6.4). Using Kruskal-Wallis ANOVA test, showed that there was no significant difference in percentage of SPC relative to the control.

6.3.7. Variation in plasma Testosterone, Thyroxine (T4), and Vitellogenin (VTG) concentrations

6.3.7.1. Plasma Testosterone concentration

The mean plasma testosterone (T) concentration for both Midstream and Arsenal formulations did not significantly different (P>0.05) between the exposed frogs and the Control group following the 28-day exposure period. And using Kruskal-Wallis ANOVA test showed that there was no significant difference (P>0.05) at all exposure concentrations in the two formulations relative to the Control group.

Table 6.4: Variation in mean plasma Testosterone (T), Vitellogenin (VTG), and Thyroxine (T4) concentrations in adult male *Xenopus laevis* frogs following exposure to the herbicide formulations (Midstream (MID) and Arsenal (ARS)).

Conc. (mg/L)	Testosterone (SD) (ng/mL)	Vitellogenin (SD) (ng/mL)	Thyroxine (SD) (ng/mL)
Control (0)	42.4 (8.6)	1.O (0.6)	20.3 (5.8)
MID 0.25	43.5 (7.8)	1.88 (1.3)	26.8 (7.2)
MID 0.45	42.3 (6.3)	1.60 (0.4)	24.2 (5.0)
MID 0.65	44.2 (13.1)	2.53 (1.6)	21.2 (2.9)
ASR 2	43.6 (7.7)	1.39 (1.0)	22.1 (7.6)
ASR 4	41.9 (6.3)	3.00 (2.6)	18.4 (5.4)
ASR 8	40.8 (4.8)	1.23 (0.5)	22.0 (6.2)

6.3.7.2. Plasma Vitellogenin (VTG) concentration

The mean plasma VTG concentration for both the Midstream and Arsenal formulation showed no significantly different between the exposure and the control (P>0.05). Using Kruskal-Wallis ANOVA test confirmed that there was no significant difference at all exposure concentration compared to the Control group.

For frogs exposed to Arsenal formulation, the mean VTG concentrations showed no variation in relation to the control. And using Kruskal-Wallis ANOVA test showed that there was no significant difference (P>0.05) in the increased VTG concentrations at all exposure concentrations compared to the Control.

6.3.7.3. Plasma Thyroxine (T4) concentration

The mean plasma T4 concentration following exposure to the Midstream formulation showed no variation relative to the control frogs. (P>0.05). Using Kruskal Wallis ANOVA test showed that there was no significant difference (P>0.05) at all exposure concenytrations crelative to the Control groups of the Midstream and Arsenal formulations (Table 6.4).

6.4. Discussion

Several man-made pesticides that are currently found in the environment have been shown to induce wide range of reproductive abnormalities through their endocrine disrupting activities, particularly when exposed during the developmental phase (Bouetard *et al*., 2013). However, modulation of the

endocrine system of adult through chronic exposures have not been well-studied in amphibians. Many pesticides have been suggested to have (anti)estrogenic, (anti)androgen effects on wildlife and humans, disrupting reproductive capacity and success (Ubatzka *et al*., 2007; Cevasco *et al*., 2008; Levorato *et al*., 2013). For example, testosterone and dihydrotestosterone are crucial, not only for the differentiation of foetal male reproductive system, but also for the maintenance of reproductive functions in mature adults. Similarly, thyroid hormones play an important role in reproduction in amphibians.

The global amphibian population decline for example, has been widely linked with pesticide pollution (Hecker *et al*., 2005). Recent research focus on herbicide formulations have resulted in conflicting data regarding the potential to disrupt the endocrine system of aquatic organisms like amphibians (Ghosh *et al*., 1991; Mosselman *et al*., 1996; Qin *et al*., 2003; Ge *et al.*, 2008; McDaniel *et al*., 2008; Panupong *et al.*, 2012**).** This has shifted research focus to pesticides, and the understanding of potential links to population declines. It is in line with this objective that the study examined the impacts of Midstream and Arsenal formulations on the reproductive system of adult male *Xenopus laevis*.

The exposure of adult male *X laevis* frogs to the herbicide formulations, Midstream and Arsenal, however, showed no convincing endocrine modulation effects after 28-day in situ exposure to environmental relevant concentration ranges. Almost all testes morphological parameters assessed, including GSI, testes length, testes width, and spermatogenic activity were not significantly different to the clean water control frogs. The same was true for variation in physiological endpoints, for example, testosterone, thyroid hormone (thyroxine) and vitellogenin, the estrogen controlled yolk precursor.

The variation in body mass following the exposure of the adult males to Midstream and Arsenal formulation respectively, proved to be non-significant. Although it has been suggested that environmental chemicals may result in decresed fat body mass and that such loss in energy source (stored in fat bodies) could be detrimental to the maintenance of reproductive activities (testicular or ovarian) (Goetz *et al*., 1979; Gomez *et al.* 2012). The observed no-effect of the formulations on body size in current study is similar to the observation of Allran and Karasov, (2001), where they noted no significant variation in body mass of frogs after exposure to atrazine. Clearly, in the light of the variation in body mass noted in the present study, more research is needed to understand the effects of herbicides (at environmental relevant concentrations) on metabolic pathways, fat storage and body mass.

Similar to the body size effects, the testes of the treated frogs showed no significant difference in mean mass, width and length, across all the exposure concentrations of both the Midstream and Arsenal formulation, relative to the control frogs. This means that the environmental relevant concentrations used in the current study is either too low to elicit a response, or some of the parameters including testes mass and length might not be responsive to the formulations. For example, as noted by Lee *et al*. (2005), the lack of sensitivity of relative testis-kidney mass makes it unattractive in evaluation of testicular impacts of chemical exposure. This current result is consistence with the findings of Panupong *et al*. (2012), where it was reported that there was no significant difference in testes mass between the exposed frogs from agricultural farmland and control. On the other hand, the result from the present study are in contrary to the observation by Ge *et al* (2008), that the exposure of the mice to high levels of estrogenic chemicals like Methoxychlor resulted in lower gonad size**.** The current result on the testes also correlate with the non-significant impact on GSI of frogs treated with both Midstream and Arsenal formulations. The GSI has been noted to correlate with increased with testosterone production and reproductive activities (Artchariya *et al*., 2003: Juberg *et al*., 2013). Although the slight increase in testes sizes shown by the two formulations could suggest androgenic activities, on the treated frogs, but more studies will be needed to further understand the exposure impacts.

For both the Midstream and Arsenal formulations, the spermatogenesis of the exposed frogs showed no variations relative to the control. In the frogs treated with Midstream formulation, out of the three testicular cell-types, only spermatogonia and spermatocytes increased relative to the control. That the two higher stages of spermatogenesis (except spermatids) were reduced may be indicative that the Midstream formulation could be disrupting the testicular activities. For example, the male frogs exposed to the Midstream formulation showed pattern of increase in plasma Testosterone (T) concentration, all of which may be consistent with a response to antiandrogenic activity. The rationale being that plasma T will increase if binding of T to its target cell receptor is inhibited or if the transformation of T to DHT is inhibited (by decreasing the activity of 5 alpha reductase (van Wyk *et al*., 2003; Behrends *et al*., 2010).

In the case of Arsenal formulation exposure, the initial increase at lower concentration (non-significant) in numbers of spermatocyte, spermatida and spermatozoa showed the increased spermatogenic activities in the treated frogs, suggesting possible androgenic influence of the Arsenal formulation. The reduction in plasma Testosterone may corroborate this suggestion since T is mobolized into target cells for increasing binding capacity to its receptos. However, in the present study, the Plasma T did not vary significantly. This current result is similar to the

observation of Lee *et al*., (2005), where they noted that there was no significant change in the distribution of several spermatogenic cell-types in adult male frogs exposed to Di-n-butyl phthalate (DPB). McDaniel *et al*. (2008) and Sanchez *et al*. (2014), also noted that there was no significant variation in the spermatogenesis stages observed in exposed frogs from agricultural farmlands relative to those from reference control site. But the result is contrary to the observations of many other reports (Ghosh *et al*., 1991; Kerr *et al*., 1993; Mosselman *et al*., 1996; Qin *et al*., 2003; Cevasco *et al*., 2007; Ge *et al*., 2008), where they all noted significant difference in spermatogenesis activities between the treated and the control frogs..

The result of the present study is also contrary to the findings of Archer, (2014), where it was noted that even when there was no significant variation in plasma T concentration between the treated frogs and the control, there was lower incidence of androgen receptors immunolabelled in the nuptial pad integument and altered ultrastructure of breeding gland secretory cells, suggesting that Plasma T is not a good indicator of receptor interaction or target tissue response. It therefore means that the exposure impacts of the Midstream and Arsenal formulations on spermatogenesis could still be critical on the exposed frogs, even though there was no significant difference in plasma testosterone. The use of plasma testosterone as endpoint in assessing male reproductive system disruption should be re-examined in the light of current evidence.

Both the HSI and Plasma Vitellogenin of the adult male frogs exposed to Midstream and Arsenal formulations were not significantly different to the control. Changes in the HSI, as noted by Shalaby *et al*. (2007), is an indication of changes in body nutrient contents and disruption in normal body metabolism and absorption. In the present study, the reduction in body mass in Midstream formulation treated frogs could also mean a change in the enzymatic activities within the liver which could have been compromised by the Midstream formulation. The increase in liver mass as well as increase (non-significant) in HSI for Midstream formulation, could be as a result of the toxic impacts due to the oxidative redox reaction synonymous with its active substance. As noted by several researchers, diquat formulation usually accumulates in the liver, and undergoes redox cycling reaction to produce superoxides, which lead to stress (Hook *et al*., 2006; Yadav *et al*., 2013). But generally, the non-significant difference of the HSI at the two formulations could mean that the adult Xenopus is able to metabolise the formulations without seriously compromising their liver system. But the impacts of increased liver mass needs further assessment in the future.

In the case of Vitellogenin, produced in the liver, both Midstream and Arsenal formulations also showed non-significant effects in VTG levels in the plasma, neither did the hepatic mass index

(HSI) vary significantly. This current result is contrary to findings in several studies (Palmer and Palmer, 1995; Jones *et al*., 2000; Pickford and Morris, 2002; Arukwe and Goksory, 2003; Matozzo *et al*., 2008; Oka *et al*., 2008; Peters *et al*., 2009), where they all showed estrogenic activities of various pesticides through the induction of VTG in male *Xenopus laevis*. The result is similar to the findings of Spanno *et al*. (2004), where they observed that there was no different in Vitellogenin induction and hence estrogenic effects in males goldfish treated with atrazine. McDaniel *et al*. (2008) and Hecker *et al*. (2004) also similarly observed that there was no significant different in Plasma Testosterone and Estradiol concentration of frogs exposed to corn grown agricultural site compared to the non-agricultural site. Also similar to the current result is the findings of Lee *et al*. (2005), where it was noted that there was no significant variation in mean testosterone observed in Di-n-butyl phthalate treated group compared to the control. Therefore, based on the results obtained in the present study, the two selected formulations could not be shown to have estrogenic properties. More study is needed using other indicator organisms, including fresh water fish species to validate this findings.

For the thyroxine hormone, It is in this pituitary gland that thyroid stimulating hormone (TSH), liteinizing hormone-releasing hormone (LHRH) as well as gonadotropic hormones are produced (Denver *et al*., 1988; Jacob *et al*., 1988). But the non-significant difference in concentration of thyroxine in the current study showed that there was no serious reproductive disruption by the two formulations. Just like the other endpoints, it is either the current environmental relevant concentrations is too low to elicit serious negative reaction or the two formulations have no serious impacts on the reproductive system of the frogs. Further studies to understand the long term impacts of these formulations on the pituitary gland, thyroid gland and the reproductive system generally will therefore be very important.

Although, contrary to the negative findings in the present study, the results obtained with similar concentration ranges applied against both embryos and developing larvae (Chapter 3, 4 & 5), produced numerous significant negative response (including teratogenicity, growth disruption, thyroid disruption and reproductive malformation). This result corroborate the idea of differential response associate with different life stages, as have been suggested in several studies (Harfenist *et al.*, 1989; Bridges, 2000; Bridges and Semilitch, 2000; Greanlich and Pflumacher, 2003; Ortiz-Sandaliestra *et al*., 2006).

The importance of testing all life stages (embryo, larvae and adults), because of the wide range of sensitivity associated with different life stages to the same toxicant (Kobayashi, 1980;

Harfenist *et al*., 1989; Vitt *et al*., 1990; Gutleb *et al*., 1999; Alltran and Karasiv, 2001). Adult resistance, according to Smith *et al*. (2007), could be due to increased detoxifying ability, as well as various physical adaptive and developmental features. In general, compared to the adults, the incomplete development of the nervous system, tissue and organ differentiation may also protect embryos and larva from those chemical affecting nervous system (Ortiz-Santaliestra *et al*., 2006).

The exposure period of 28 days in this study might not be adequate enough to produce appropriate physiological response, particularly where the endocrine activity of the formulation is not very strong (Qin *et al*., 2007). The importance of this is that future study should also consider exposure time as possible factor, since increase exposure time will also lead to increase bioaccumulation of the chemicals.

In general, the wider implications of the observed low sensitivity at adult stage of *Xenopus laevis* relative to the eggs, embryos and larvae stages is that at the various low concentrations (that is still less toxic to adults), only few eggs, embryos and larvae will be able to survive. This will mean a serious negative effect on the exposed population. As shown by the teratogenicity study on Midstream and Arsenal formulations in Chapter 3 of this study, where similar concentrations were used, majority of those eggs and embryos that will survive from the exposure will develop numerous developmental malformations that could be difficult to survive from**.** As also shown in the thyroid and gonadal studies in Chapter 4 and 5 of this study, similar concentrations caused disruption in thyroid system and numerous gonadal malformations. This could be one of the reasons why there is increasing widespread of malformed adults amphibian population globally.

6.5. Conclusion

Both formulations did not disrupt the reproductive or thyroid endpoints in adult *X. laevis* in the present study. Since the concentrations applied in this study are all environmental relevant, and similar range had produced developmental, thyroidal and gonadal effects at embryo and larvae stages, but no significant effects at adult stage, this findings was suprising. The result suggests that in adults detoxification and possible selective uptake through the skin, may prevent harmful effects.

The significance of this observation is that adult amphibian may be poor indicators, compared to the egg, embryos and larval developmental stages, when studying potential endocrine disruptive effects of pesticides. Further studies will therefore be necessary to further understand the

factors responsible for the high resistance of the adult stage relative to the lower developmental stages

The importance of this differential response cannot be overemphasised as this could lead to serious negative effects on the population dynamics. The organism will not be protected if a developmental stage is seriously susceptible to pesticides. It will be important to know if the differential response is true for all pesticides or only occurs in some selected groups, for example herbicides.

In South Africa, the fact that *Xenopus laevis* are locally endemic amphibian, may be at risk (especially during metamorphosis) and therefore makes it important for continued assessment/research of pesticides impacts including herbicides, using at least three developmental stages.

References

Aerle, R.; Nolon, T. M.; Jobling, S.; Christiansen, L. B.; Sumpter, J. P.; Tyler, C. R.; (2001). Sexual disruption in a second species of wild cyprinid fish (the gudgeon, *Gobio gobio*) in United Kingdom freshwaters. *Environmental Toxicology and Chemistry* **20:** 2841–2847.

Allran, J. W.; Karasov, W. H. (2001). Effects of atrazine on embryos, larvae and adults of anuran amphibians. *Environmental Toxicology and Chemistry* **20**: 761- 775.

Ansara-Ross, T. M (2010). An ecological risk assessment of pesticides using a probabilistic model and its implementation on the Crocodile and Magalies rivers, South Africa. Unpublished PhD thesis, University of Johannesburg, South Africa.

Ansara-Ross, T. M.; Wepener, V.; van den Brink, P. J.; Ross, M. J (2008). Probabilistic risk assessment of the environmental impacts of pesticides in the Crocodile (west) Marico catchment, North-West province. *Water SA* **34**: 637–644.

Ansara-Ross, T. M.; Wepener, V.; van den Brink, P. J.; Ross, M. J (2009). Application of a direct toxicity assessment approach to assess the hazard of potential pesticide exposure at selected sites on the Crocodile and Magalies rivers, South Africa. *African Journal of Aquatic Science* **34**: 207–217.

Ansara-Ross, T. M.; Wepener, V.; van den Brink, P. J.; Ross, M. J. (2012). Pesticides in South African fresh waters. *African Journal of Aquatic Science* **37**: 1-16.

Archer, E. (2014). Androgen controlled secondary sexual characters in the male clawed frog, Xenopus laevis, as potential biomarkers for endocrine disruptor contaminants (with special reference to fungicides) in aquatic systems. Unpublished Msc thesis of the Stellenbosch University, Stellenbosch, South Africa.

Artchariya, C., Prakong, T., Putsatee, P. Kingkaew, W., (2003). Effects of Methylparathion on the Reproductive System in Male Frogs, *Hoplobaprachus rugulosus.* Journal of Science. Research *Chula University* **28** (1): 28-40.

Arukwe, A.; Goksøyr, A. (2003). Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comparative Hepatology* 2003, **2**(4): 1-21.

Bauer, H., Meier, A., Hild, M., Stachel, S., Economides, A., Hazelett, D., Harland, R.M., and Hammerschmidt, M. (1998). Follistatin and Noggin are excluded from the zebrafish organizer *Developmental Biology* **204**: 488-507.

Behrends, T.; Urbatzka, R.; Krackow, S.; Elepfandt, A.; Kloas, W. (2010). Mate calling behavior of male South African clawed frogs (*Xenopus laevis*) is suppressed by the antiandrogenic endocrine disrupting compound flutamide. *General and Comparative Endocrinology* **168**:269–274.

Berg, C..; Gyllenhammar, I.; Kvarnryd, M. (2009). *Xenopus tropicalis* as a test system for developmental and reproductive toxicity**.** *Journal of Toxicology and Environmental Health.* Part A: *Current Issues* **72** (3-4)**:** 219-225.

Bhende A. M.; Sitre S. R. (2014). Alterations in the Gonads of *Labeo rohita* Exposed to Endosulfan At Sublethal Dose in Long Duration Experiments. *Online International Interdisciplinary Research Journal **IV**: Special Issue*. 232-240.

Blaustein, A. R.; Wake, D. B.; Sousa, W. P (1994). Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. *Conservation Biology* **8**: 60–71.

Blaustein, A. R; Wake, D. B (1995). The puzzle of declining amphibian populations. ***Sci. Am***. **272**: 52–57.

Bold, T (2007). Management Treatments Summary Guide- Aquatics. Working for Waters National Office. p.7. www.wfw.co.za.

Bouétard, A.; Besnard, A.; Vassaux, D.; Lagadic, L.; Coutellec, M. (2013). Impact of the redox-cycling herbicide Diquat on transcript expression and antioxidant enzymatic activities of the freshwater snail *Lymnaea stagnalis*. *Aquatic Toxicology* **126***: 256– 265.*

Bretveld, R.W., C.M.J. Thomas, P.T.J. Scheepers, G.A. Zielhuis and N. Roeleveld (2006). Pesticide exposure: The hormonal function of the female reproductive system disrupted? *Reproductive Biology and Endocrinology* **(4)**: 30-36.

Bridges, C. M. (2000) Long-term effects of pesticide exposure at various life stages of the Southern Leopard Frog (*R. sphenocephala). Archives of Environmental Contamination and Toxicology* **39**:91–96.

Bridges C. M.; Semlitsch, R. D (2000) Variation in pesticide tolerance of tadpoles among and within species of Ranidae and patterns of amphibian decline. *Conservation Biology* **14**:1490–1499.

Bruhl, C. A.; Pieper, S.; Weber, B (2013). Amphibians at risk? Susceptibility of terrestrial amphibian life stages to pesticides. *Environmental Toxicology and Chemistry* **30**: 2465–2472.

Brunelli, E.; Bernabo, I.; Berg, C.; Lundstedt-Enkel, K.; Bonacci, A.; Tripepi, S. (2009). Environmental relevant concentrations of endosulfan impair development, metamorphosis and behavior in Anaxyrus tadpoles. *Aquatic Toxicology* **91**: 135-142.

Carr, J.A.; Patino, R. (2010). The hypothalamus-pituitary-thyroid axis in teleost and amphibians. Endocrine disruption and its consequences to natural populations. *General and Comparative Endocrinology* **290**: 299-312.

Carr, J. A.; Gentles, A.; Smith, E. E.; Goleman, W. L.; Urquidi, L. J.; Thuett, K.; Kendall, R. J.; Giesy, J. P.; Gross, T. S.; Solomon, K. R.; Van Der Kraak, G. (2003). Response of larval *Xenopus laevis* to atrazine: assessment of gonadal and laryngeal morphology. *Environmental Toxicology and Chemistry* **22**: 396–405.

Cevasco, A.; Urbatzka, R.; Bottero, S.; Massari. A.; Pedemonte, F.; Kloas, W.; Mandich, A. (2008). Endocrine disrupting chemicals (EDC) with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of *Xenopus laevis*: II. Effects on gonads histomorphology. Comparative Biochemistry and Physiology, Part C **147**: 241-251.

Chang, C.Y; Witsch, E. (1955). Breeding of sex-reversed males of Xenopus laevis (Daudin). Proceed of Society of *Experimental Biology and Medicine* **89**(1): 150-152.

Coady, K. K.; Murphy, M. B.; Villeneuve, D. L.; Hecker, M.; Carr, J. A.; Solomon, K. R.; Smith, E. E.; Van Der Kraak, G.; Kendall, R. J.; Giesy, J. P (2005). Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, sex steroid hormones, and aromatase activity in juvenile *Xenopus laevis. Ecotoxicology and Environmental Safety* **62***: 160-173*.

Coady, K. K.; Murphy, M. B.; Villeneuve, D. L.; Hecker, M.; Carr, J. A.; Solomon, K. R.; Smith, E. E.; Van Der Kraak, G.; Kendall, R. J.; Giesy, J. P (2004). Effects of atrazine on metamorphosis, growth, and gonadal development in the green frog (*Lithobates clamitans*). *Journal of Toxicology and Environmental Health, Part A: Current. Issues*. **67**: 941–957.

Cong, L.; Zhan-Fen Qin; Xiang-Ning Jing; L. Yang; Jing-Ming Zhou; Xiao-Bai Xu (2006). *Xenopus laevis* is a potential alternative model animal species to study reproductive toxicity of phytoestrogens. *Aquatic Toxicology* **77***:* 250–256.

Costa, C L. S. (1992). Desenvolvimento do aparelho reprodutor e fatores associados ao ciclo reprodutivo da rã-touro no sistema anfigranja. Viçosa: Univers. Federal de Viçosa, 98p.

Crain, D. A. (1997). Effects of endocrine-disrupting contaminants on reproduction in the American alligator, Alligator mississippiensis. Unpublished Ph.D. Dissertation. University of Florida.

Crain, D. A.; Guillette, Jr. L.J.; Pickford, D.B.; Percival. H.F.; Woodward, A.R. (1998). Sex-steroid and thyroid hormone concentrations in juvenile alligators (Alligator mississippiensis) from contaminated and reference lakes in Florida. *Environmental. Toxicology and Chemistry* **17**: 446–452.

Crain, D. A.; Guillette, L. J.; Rooney, A. A.; Pickford, D. B (1997). Alterations in steroidogenesis in alligators (*A. mississippiensis*) exposed naturally and experimentally to environmental contaminants. *Environmental Health Perspectives* **105**: 528-533.

Denver, R. J. (1988). Several hypothalamic peptides stimulate *in vitro* thyrotropin secretion by pituitaries of anuran amphibians. *General and Comparative Endocrinology* **72**: 383-393.

Desforges, J W.; Peachey, B.D.L.; Sanderson, P.M.; White, P.A.; Blais, J.M. (2010). Plasma vitellogenin in male teleost fish from 43 rivers worldwide is correlated with upstream human population size *Environmental Pollution* **158**: 3279-3284.

Diamanti-Kandarakis, E.; Bourguignon, J.; Giudice, L. C.; Hauser, R.; Prins, G. S.; Soto, A. M.; Zoeller, R. T.; Gore A. C. (2009). Endocrine-disrupting chemicals. An endocrine society scientific statement. *Endocrine Reviews* **30**(4**):** 293-342.

D'Souza, U (2004). Effects of Tamoxifen on spermatogenesis and tubular morphology in rats. *Asian Jounal of Andrology* **6**: 223-226.

Du Preez, L.H.; Jansen van Rensburg, P.J.; Jooste, A.M.; Carr, J.A.; Giesy, J.P.; Gross, T.S.; Kendall, R.J.; Smith, E.E.; Van Der Kraak, G.; Solomon, K.R. (2005a). Seasonal exposures to triazine and other pesticides in surface waters in the western Highveld corn-production region in South Africa. *Environmental Pollution* **135**, 131–141.

Du Preez, L.H.; Solomon, K.R.; Carr, J.A.; Giesy, J.P.; Gross, T.S.; Kendall, R.J.; Smith, E.E.; Van Der Kraak, G.J.; Weldon, C. (2005b). Population structure of the African clawed frog (*Xenopus laevis*) in maize-growing areas with atrazine application versus non-maize growing areas in South Africa. *African Journal of Herpetology* **54**: 61–68.

Emmett, K. (2002). Final Risk Assessment for Diquat Bromide. The Water Quality Program of the Washington State Department of Ecology. 02-10-046. www.wsda.org. Accessed on 3rd Nov 2013.

Fort, D. J.; Rogers, R. L.; Paul, R. R.; Miller, M. F.; Clark, P.; Stover, E. L.; Yoshioko, J.; Rolland, R.; Quimby, F.; Sower, S. A.; Reed, K. L.; Babbitt, K. J. (2001). Effects of pond water, sediment, and sediment extract samples from New Hampshire USA, on early Xenopus development and metamorphosis: Comparison to native species. *Journal of Applied Toxicology* **21**: 199–209.

Fort, D. J.; Guiney, P. D.; Weeks, J. A.; Thomas, J. H.; Rogers, R. L.; Noll, A. M.; Spaulding, C.D (2004). Effect of methoxychlor on various life stages of *Xenopus laevis*. *Toxicological Sciences* **81**: 454–466.

Ge, R., Chen, G. and Hardy, M.P. (2008). The Role of the Leydig Cell in Spermatogenic Function. Molecular and Spermatogenesis. In Cheng C.Y (2008) (Edited). Landes Bioscience and Springer Science Business Media.

Ghosh, S.; Sinha-Hikim, A.O.; Russell, L.D. (1991). Further observations of stage-specific effects seen after short-term hypophysectomy in the rat. *Tissue Cell* **23**: 613-630.

Gibbons, J. W.; Scott, D. E.; Ryan, T. J.; Buhlman, K. A.; Tuberville, T. D.; Metts, B. S.; Greene, J. L.; Mills, T.; Leiden, Y.; Poppy, S.; Winne, C. T (2000). The global decline of reptiles, déjà vu amphibians. *BioScience* **5***:* 653–666.

Giesy, J.; Ludwig, J.; Tillitt, D (1994).Deformities in birds of the great lakes region; assigning causality. *Environment, Science and Technology* **28**: 128-135.

Gimeno, S.; Komen, H.; Jobling, S.; Sumpter, J. P.; Bowmer, T (1998). Demasculinisation of sexually mature male common carp, *Cyprinus carpio*, exposed to 4-tert-pentylphenol during spermatogenesis. *Aquatic Toxicology* **43**: 93 -109**.**

Goetz, F.W., Donaldson, E.M., Hunter, G.A and Dye, H.M (1979). Effects of Estradiol-17β and 17α-methyl-testosterone on gonadal differentiation in the Cohoe Salmon, *Oncorhychue Kisutch. Aquaculture* **17***: 267-278.*

Gomes, M.L.M.; Matta, S.L.P.; Ribeiro-Filho, O.P.; Monteiro, J.C. (2012).Stereological analyses of the annual variation of captive bullfrog adult testes (Lithobates catesbeianus, Shaw 1802). *Journal of Morphological Science* **29**(3): 182-186.

Gong, Y.; Tian, H.; Wang, L.; Yu, S.; Ru, S. (2014). An integrated approach combining chemical analysis and an in vivo bioassay to assess the estrogenic potency of a municipal solid waste landfill leachate in Qingdao. *PLoS ONE* **9**(4*)*: e95597.

Guillette, L. J.; Gross, T. S.; Masson, G. R.; Matter, J. M.; Percival, H. F.; Woodward, A. R. (1994). Developmental abnormalities of the gonad and abnormal sex-hormones concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environmental Health Perspective* **102**(8): 680-688.

Guillette, L. J. Jr.; Crain, D.A (Eds.) (2000). Endocrine Disrupting Contaminants: An Evolutionary Perspective. Francis and Taylor, Inc., Philadelphia, p. 355.

Gutleb, A. C.; Appelman, J.; Bronkhorst, M.; van den Berg, J. H. J.; Spenkelink, A.; Brouwer, A.; Murk, A. J. (1999). Delayed effects of pre- and early-life time exposure to

polychlorinated biphenyls (PCBs) on tadpoles of two amphibian species (*Xenopus laevis* and *Lithobates temporaria*). Environmental Toxicology and Pharmacology **8**: 1-14.

Greulich, K.; Pflugmacher, S. (2003). Differences in susceptibility of various life stages of amphibians to pesticide exposure. *Aquatic Toxicology*. **65**:329–336.

Grisolia, C. K.; Bilich, M.R.; Formigli, L. M (2004). A comparative toxicologic and genotoxic study of the herbicide arsenal, its active ingredient imazapyr, and the surfactant nonylphenol ethoxylate. *Ecotoxicology and Environmental Safety* **59**:123–126.

Gyllenhammar, I.; Holm, L.; Eklund, R.; Berg, C. (2009). Reproductive toxicity in *Xenopus tropicalis* after developmental exposure to environmental concentrations of ethynylestradiol *Aquatic Toxicology* **91**: 171–178.

Harfenist, A.; Power, T.; Clark, K.L.; Peakali, D. B. (1989). A review and evaluation of the amphibian toxicological literature. Series 61. Technical Report of Canadian Wildlife service, Ottawa, ON.

Hayes, T (2005). Welcome to the evolution: Integrated biology and assessing the impacts of endocrine disruptors on environmental and public health. *Integrated Comparative Biology* **45**: 321-29.

Hayes, T.; Haston, K.; Tsui, M.; Hoang, A.; Haeffele, C.; Vonk, A (2002a) Feminization of male frogs in the wild. *Nature* **419**: 895-896.

Hayes, T. B.; Collins, A.; Lee, M.; Mendoza, M.; Noriega, N.; Stuart, A. A.; Vonk, A (2002b). Hermaphroditic, demasculinized frogs after exposure to the herbicide, atrazine, at low ecologically relevant doses. Proceeding of National Academic of Science U.S.A. **99**: 5476–5480.

Hayes, T. B.; Haston, K.; Tsui, M.; Hoang, A.; Haeffele, C.; Vonk, A. (2002b). Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Lithobates pipiens*): Laboratory and field evidence. *Environmental Health Perspectives* **111**: 568–575.

Hayes, T. B.; Case, P.; Chui, S.; Chung, D.; Haeffele, C.; Haston, K.; Lee, M.; Mai, V.P.; Marjuoa, Y.; and Tsui, M. (2006). Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? *Environmental Health Perspective* **114**:40–50.

Hecker, M.; Kim, W.; Park, J. W.; Murphy, M.; Villeneuve, D.; Coady, K.; Jones, P.D.; Solomon, K. R.; Van Der Kraak, G.; Carr, J. A.; Smith, E.E.; du Preez, L.; Kendall, R.J.; Giesy L.J (2005). Plasma concentrations of estradiol and testosterone, gonadal aromatase activity and ultrastructure of the testis in *Xenopus laevis* exposed to estradiol or atrazine. *Aquatic Toxicology (Amsterdam)* **72**: 383–396**.**

Hecker, M. J., Giesy, J. P., Jones, P., Jooste, A. M., Carr, J., Solomon, K. R (2004). Plasma sex steroid concentrations and gonadal aromatase activities in African clawed frogs (*X. laevis*) from South Africa. *Environmental Toxicology and Chemistry* **23**:1996–2007.

Hook, S.E.; Skillman, A.D.; Small, J.A.; Schultz, I.R. (2006). Gene expression partterns in rainbow trout, Oncorhynchus mykiss, exposed to a suite of model toxicants. *Aquatic Toxicology* **77**: 372-385.

Howe, G.E.; Gills, R.; Mowbray, R.C (1998). Effect of chemical synergy and larvae stage on the toxicity of atrazine and alachlor to amphibian larvae. *Environmental Toxicology and Chemistry* **17**: 519-525.

Hurter, E.; Pool, E.J.; Van Wyk, J.H. (2002a). Validation of an *ex vivo Xenopus* liver slice bioassay for environmental estrogens and estrogen mimics. *Ecotoxicology and Environmental Safety* **53**: 178-187.

Hurter, E.; Pool, E.J.; Van Wyk, J.H. (2002b). Description and validation of a sensitive *in vitro* bioassay for detecting environmental estrogenicity using *ex vivo Xenopus laevis* liver slices. *Ecotoxicology and Environmental Safety* **53**:178–187.

Hynes, S.V.; Wilson, S.; Byrne, M (2009). Frogs as bio-indicators of chemical usage and farm practices in an irrigated agricultural areas. Final Report to Land and Waters Australia.

Jacobs, G. F. M.; Goyvaerts, M. P.; Vandorpe, G.; Quaghebeur, A. M. L.; Kuhn, E. R (1988). Luteinizing hormone-releasing hormone as a potent stimulator of the thyroidal axis in Ranid frogs. *General and Comparative Endocrinology* **70**: 274-283.

Jobling, S.; Nolan, M.; Tyler, C. R.; Brightly, G.; Sumpter, J. P (1998). Widespread sexual disruption in wild fish. *Environment Science and Technology* **32**: 2498–2506.

Jobling, S.; Coey, S.; Whitmore, J. G.; Kime, D.E.; Van Look, K. J. W.; McAllister, B. G.; Beresford, N.; Henshaw, A. C.; Brightly, G.; Tyler, C. R.; Sumpter, J. P (2002b). Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biology of Reproduction.* **67**: 515–524.

Jobling, S.; Tyler, C.; Nolan, M.; Sumpter, J (1998). The identification of oestrogenic effects in wild fish. Research and development Technical Report W 119.

Jones, P.D; Tremblay, L.A.; Coen, W.M.; Giesy, J.P. (2003). Vitellogenin as a biomarker for environmental estrogen. *Australasian Journal of Ecotoxicology* **6**: 45-48.

Jugan, M.L.; Oziol, L.; Bimbot, M.; Hunteau, U.; Tmisier-Karolak, S.; Blondeau, J. P.; Levi, Y (2009). In vitro assessment of thyroid and estrogenic endocrine disruptors in wastewater

treatment plants, rivers and drinking water supplies in the greater Paris area (France). *Science of the Total Environment* **407***:* 3579-3587.

Juberg, D.R.; Gehen, S.C.; Coady,K.K.; LeBaron, M.J.; Kramer,V.J.; Lu,H.; Marty, M.S. (2013). Chlorpyrifos: Weight of evidence evaluation of potential interaction with the estrogen, androgen, or thyroid pathways. *Regulatory Toxicology and Pharmacology* **66** (2013) 249–263.

Juberg, D.R., Borghoff, S.J., Becker, R.A., Casey, W., Hartung, T., Holsapple, M.P., Marty, M.S., Mihaich, E.M., Van Der Kraak G, Wade, M.G., Willett, C.E., Andersen, M.E., Borgert, C.J., Coady, K.K., Dourson, M.L., Fowle, J.R., Gray, L.E., Lamb, J.C., Ortego, L.S., Schug, T.T., Toole, C.M., Zorrilla, L.M., Kroner, O.L., Patterson, J., Rinckel, L.A., Jones, B.R. (2014). T4 workshop report: Lessons Learned, Challenges, and Opportunities: The U.S. Endocrine Disruptor Screening Program. ALTEX **31**:63-78.

Kaptan, E.; Murathanog˘lu, O. (2008). Annual Morphological Cycles of Testis and Thumb Pad of the Male Frog (*Lithobates ridibunda*). *The Anatomical Record* **291**:1106–1114.

Kelley, D.; Sassoon, D.; Segil, N.; Scudder M. (1989). Development and hormone regulation if Androgen receptor levels in the sexually Dimorphic Laryx of *Xenopus laevis*. *Developmental Biology* **181**: 112-118.

Kerr, J. B.; Millar, M.; Maddocks, S.; Sharpe, R.M. (1993). Stage-dependent changes in spermatogenesis and Sertoni cells in relation to the onset of spermatogenic failure following withdrawal of testosterone. *Anatomical Record* **235** (4): 547-559.

Kidd, K. A.; Blanchfield, P. J.; Mills, K. H.; Palace, V. P.; Evans, R. E.; Lazorchak, J. M.; Flick, R. W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceeding of National Academic of Science, USA.* **104**: 8897-8901.

Kloas, W.; Lutz, I.; Einspanier, R. (1999). Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals *in vitro* and *in vivo*. *Science of Total Environment* **225**: 59-68.

Kloas, W.; Lutz, I. (2006). Amphibians as model to study endocrine disrupters. *Journal of. Chromatography A* 1130: 16-27.

Kloas, W.; Urbatzka, R.; Opitz, R.; Wurtz, S.; Behrends, T.; Hermelink, B.; Hoffmann, F.; Jagnytsch, O.; Kroupova, H.; Lorenz, C.; Neumann, N.; Pietsch, C.; Trubiroha, A.; Ballegooy, C.; Wiedermann, C.; Lutz, I (2009). Endocrine disruption in aquatic vertebrates. *Trends in Comparative Endocrinology and Neurobiology*: *Ann N.Y. Acad. Sci.* **1163**: 187-200.

Knechtges, P.L.; Sprando, R.L.; Brennan, L.M.; Miller, M.F.; Kumsher, D.M.; Dennis, W.E.; Brown, C.C.; Clegg, E.D. (2006). Assessment of Protocol Designed to Detect Endocrine Disrupting Effects of Flutamide in *Xenopus Tropicalis*. USACEHR-TR-0601 ed. DTIC, 1-45.

Kobayashai, N. (1980). Comparative sensitivity of various developmental stages of Sea Urchins to some chemicals. *Marine Biology* **58**(3): 163-171.

Krishnan, A.V., Stathis, P., Permuth, S.F., Tokes, L., and Feldman, D. (1993). Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132**(6):2279-86.

Kumar, V.; Chakraborty, A.; Viswanath, G.; Roy, P. (2008). Androgenic endocrine disruptors in wastewater treatment plant effluents in India: Their influence on reproductive processes and systemic toxicity in male rats. *Toxicology and Applied Pharmacology* **226**: 60–73.

Lance, S.L.; Erickson, M.R.; Flynn, R.W.; Mills, G.L.; Tuberville, T.D.; Scott, D.E (2012). Effects of chronic copper exposure on development and the survival in the southern leopard frog (*L. sphenocephalus*). *Environmental Toxicology and Chemistry* **31**(7): 1587–1594.

Lavorato, M.; Bernabò, I.; Crescente, A.; Denoël, M.; Tripepi, S.; Brunelli, E. (2013). Endosulfan effects on *Lithobates dalmatina* tadpoles: Quantitative developmental and behavioural Analysis**.** *Archives of Environmental Contamination and Toxicology* **64**: 253–262.

Lee, S. K.; Owens, G. A.; Veeramachaneni, D. N. (2005). Exposure to low concentrations of di-*n*-butyl phthalate during embryogenesis reduces survivability and impairs development of *Xenopus laevis* frogs. *Journal of Toxicology and Environmental Health A* **68**: 763–772.

Lenkowski, J.R.; Reed, J.M.; Deininger, L.; McLaughlin, K.A. (2008). Perturbation of organogenesis by the herbicide atrazine in the amphibian *Xenopus laevis*. *Environmental Health Perspectives.* **116**: 223–230.

Levy, G.; Lutz, I.; Kru¨ger, A.; Kloas, W. (2004). Bisphenol A induces feminization in *Xenopus laevis* tadpoles. *Environmental Research* **94**: 102–111.

Li, X.L.; Luan, T. G.; Liang, Y.; Wong, M.H.; Lan, C.Y. (2007). Distribution patterns of octylphenol and nonylphenol in the aquatic system at mai po marshes nature reserve, a subtropical estuarine wetland in Hong Kong. *Journal of Environmental Science (China)* **19**: 657-662.

Liu, W.; Pusino, A.; Gessa, C. (1992). High-Performance liquid chromatographic determination of the herbicide Imazapyr residues in water and soil. *The Science of the Total Environment* **123/124:** 39-43.

Mackenzie, C. A.; Berrill, M.; Metcalfe, C.; Pauli, B. D. (2003). Gonadal differentiation in frogs exposed to estrogenic and antiestrogenic compounds. *Environmental Toxicology and Chemistry* **22:** 2466-75.

Mann, R.M.; Bidwell, J.R. (1999b). The toxicity of glyphosate and several glyphosate formulations to four species of south western Australian frogs. *Archives of Environmental Contamination and Toxicology* **36**: 193–199.

Mann, R. M.; Bidwell, J.R (2000). Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. *Aquatic Toxicology* **51**: 19-29.

Marin, M. G.; Matozzo, V. (2004) Vitellogenin induction as a biomarker of exposure to estrogenic compounds in aquatic environments. *Marine Pollution Bulletin **48***: 835–839.

Matozzo, V.; Gagné, F.; Marin, M.G.; Ricciardi, F.; Blaise, C (2008). Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review. *Environment International* **34**: 531–545.

McDaniel, T.V.; Martin, P.A.; Struger, J.; Sherry, J.; Marvin, C. H.; McMaster, M.E.; Clarence, S.; Tetreault, G (2008). Potential endocrine disruption of sexual development in free ranging male northern leopard frogs (*R. pipiens*) and green frogs (*R. clamitans*) from areas of intensive row crop agriculture. *Aquatic Toxicology* **88**: 230–242.

Meinhardt, H. R. (2008). Evaluation of predictive models for pesticide behaviour in South African soils. Unpublished PhD thesis, University of the North-West, South Africa.

Mensah, P. K.; Palmer, C. G.; Muller, W.J. (2013). Derivation of South African water quality guidelines for Roundups using species sensitivity distribution. *Ecotoxicology and Environmental Safety* **96**: 24–31.

Milnes, M. R.; Bermudez, D.S.; Bryan, T.A.; Edwards, T.M.; Gunderson, M.P.; Larkin, I.V.L.; Moore, B.C.; Guillette Jr, L. J (2006). Contaminant-induced feminization and demasculinization of non-mammalian vertebrate males in aquatic environments. *Environmental Research* **100**: 3–17.

Mosselmam, S.; Polman, J.; Dijkema, R (1996). ER β Identification and characterization of a novel human estrogen receptor. *FEBS Letters* **392**: 49-53.

Nieuwkoop, P.D.; Faber, J (1994). *Normal Table of* Xenopus laevis *(Daudin)*, 3rd ed. Garland, New York, NY, USA

Nimrod, A. C. and W. H. Benson. (1996). Estrogenic responses to xenobiotics in channel catfish (*Ictalurus punctatus*). *Marine Environmental Research* **42**:155– 160.

Oka, T.; Tooi, O.; Mitsui, N.; Miyahara, M.; Ohnishi, Y.; Takase, M.; Kashiwagi, A.; Santo, N.; Iguchi, T. (2008). Effect of atrazine on metamorphosis and sexual differentiation in *Xenopus laevis*. *Aquatic Toxicology* **87**: 215–226.

Othman, M.Z.; Ding, L.; Jiao, Y. (2009). Effect of anionic and non-ionic surfactants on activated sludge oxygen uptake rate and nitrification. *World Academy of Science, Engineering and Technology* 58.

Organisation for Economic Co-operation and Development (OECD) (2012). Detailed Review Paper on the State of the Science on novel In vitro and In vivo Screening and Testing Methods and endpoints for Evaluating Endocrine Disruptors. OECD Environment, Health and Safety Publications, Series on Testing and Assessment, No. 178.

Orton, F.; Rosivatz, E.; Scholze, M.; Kortenkamp, A (2011). Widely used pesticides with previously unknown endocrine activity revealed as *in Vitro* antiandrogens. *Environmental Health Perspectives* **119**:794–800.

Orton, F.; Rosivatz, E.; Scholze, M.; Kortenkamp, A. (2012). Competitive Androgen Receptor Antagonism as a Factor Determining the Predictability of Cumulative Antiandrogenic Effects of Widely Used Pesticides. *Environmental Health Perspective* **120**:1578–1584.

Ortiz-Santaliestra, M. E.; Marco, A; Fernandez, M. J; Lizana, M (2006). Influence of developmental stage on sensitivity to ammonium nitrate of aquatic stages of amphibians. *Environmental Toxicology and Chemistry* **25**(1): 105–111.

Paggetti, E.; Biaggini, M.; Corti, C.; Lebboroni, M.; Berti, R. (2006). Amphibians and reptiles as indicators in Mediterranean agro-ecosystems: A preliminary study. Proceedings of the 13th Congress of the Societas Europaea Herpetologica. 107-110.

Palmer, B.D., Palmer, S.K. (1995). Vitellogenin induction by xenobiotic estrogens in the red-eared turtle and African clawed frog. *Environmental Health Perspective* **103** (4): 19–25.

Panupong, T., Wichase, K., Jirarach, K., Pakorn V., Noppadon K., (2012). Morphometric and Gravimetric Parameters of the Rice Frog *Fejervarya limnocharis* living in Areas with Different Agricultural Activity. *Journal of Environmental Protection* **2012**(3): 1403-1408.

Peters, R.E.M.; Courtenay, S.C.; Hewitt, M.L; MacLatchy, D.L. (2009). Effects of 17α-ethynylestradiol on early-life development, sex differentiation and vitellogenin induction in mummichog (*Fundulus heteroclitus*). Marine Environmental Research 223-226.

Pickford, D. B.; Morris, I. D. (2003). Inhibition of gonadotropin-induced oviposition and ovarian steroidogenesis in the African clawed frog *(X. laevis*) by the pesticides methoxychlor *Aquatic Toxicology* **62**: 179-194.

Qin, Z.; Qin, X.; Yang, L.; Han-Ting, L.; Zhao, X.; Xu, X. (2007). Feminizing/demasculinizing effects of polychlorinated biphenyls on the secondary sexual development of *Xenopus laevis. Aquatic Toxicology* **84**:321–327.

Qin, Z.; Zhou, J.; Chu, S.; Xu, X. *(2003).* Effects of Chinese domestic polychlorinated biphenyls (PCBs) on gonadal differentiation in *Xenopus laevis. Environmental Health Perspectives* **111**:553–556.

Quassinti, L.; Maccari, E.; Murri, O.; Bramucci, M. (2009). Effects of paraquat and glyphosate on steroidogenesis in gonads of the frog *Lithobates* esculenta in vitro. *Pesticide Biochemistry and Physiology* **93**: 91–95.

Russel, L. D.; Clemont Y (1977). Degeneration of germ cells in normal, hypophysectomied and hormonal treated hypophysectomised rats. *Anat Rec **187***: 347-366.

Sanchez, L. C.; Lajmanovich, R. C.; Peltzer, P. M.; Manzano, A. S.; Junges, C. M.; Attademo, A. M. (2014). First evidence of the effects of agricultural activities on gonadal form and function in *Rhinella fernandezae* and *Dendropsophus sanborni* (Amphibia: Anura) from Entre Ríos Province, Argentina. Acta Herpetologica **9**(1): 75-88.

Servos, M. R. (1999). Review of the aquatic toxicity, estrogenic responses and bioaccumulation of alkylphenols and alkylphenol polyethoxylates. *Water Quality Research Journal of Canada* **34**:123-77.

Shalaby A. M.; Mousa, M. A. A.; El-Dian, H. A. T (2007). Toxicological effect of Butataf herbicide on some physiological aspects and the reproductive performance of Nile tilapia *Orechromis niloticus. Egyptian Journal of Aquatic Biology & Fish* **11** (2)**:** *145-163.*

Sharma, R. K.; Chauhan, P. R.; Fulia, A. (2011) Atrazine induced morphological alteration in spermatocytes of Goat in Vitro. *Journal Medical Science.* **11**(4)**:** 177-184.

Sikka, S.C.; Wang, R. (2008). Endocrine disruptors and estrogenic effects on male reproductive axis. *Asian Journal of Andrology* **10** (1)**:** 134–145.

Smith P. N.; Cobb G. P.; Godard-Codding C.; Hoff, D.; McMurry S.T.; Rainwater T.R.; Reynolds K. D. (2007). Contaminant exposure in terrestrial vertebrates. *Environmental Pollution* **150**:41–64.

Spanò, L.; Tyler, C. R.; Aerle, R.; Devos, P.; Mandiki, S.N.M.; Silvestre, F.; Thomé, J.; Kestemont, P.(2004). Effects of atrazine on sex steroid dynamics, plasma vitellogenin

concentration and gonad development in adult goldfish (*Carassius auratus*). *Aquatic Toxicology* **66**: 369–379.

Streck, G. (2009). Chemical and biological analysis of estrogenic, progestagenic and androgenic steroids in the environment. *Trends in Analytical Chemistry* **28** (6)**:** 635-652.

Stuart, S. N.; Chanson J. S.; Cox N. A.; Young B. E.; Rodrigues A. S. L.; Fischman D. L.; Waller R. W (2004). Status and Trends of amphibian declines and extinctions worldwide. *Science* **306**: 1783.

Tavera-Mendoza, L.; Ruby, S.; Brousseau, P.; Fournier, M.; Cyr, D.; Marcogliese, D (2002a). Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. *Environmental Toxicology and Chemistry* **21**:527–531.

Tomohiro Oka, T.; Tooi, O.; Mitsui, N.; Miyahara, M.; Ohnishi, Y.; Takase, M.; Kashiwagi, A.; Shinkai, T.; Santo, N.; Iguchi, T. (2008). Effect of atrazine on metamorphosis and sexual differentiation in *Xenopus laevis. Aquatic Toxicology* **87**: 215–226.

Toyama, Y.; Hosoi, I.; Ichikawa, S.; Maruoka, M.; Yashiro, E.; Ito, H.; Yuasa, S (2001). Estradiol 3-benzoate affects spermatogenesis in the adult mouse. *Molecular and Cellular Endocrinology*. **178**: 161–168.

Trachantong, W.; Promya, J.; Saenphet, S.; Saenphet, K (2013). Effects of atrazine herbicide on metamorphosis and gonadal development of *Hoplobatrachus rugulosus. Maejo International Journal of Science and Technology* **7 (**Special Issue): 60-71.

Trumbo, J. (2005). An assessment of the hazard of a mixture of the herbicide Rodeo and the non-Ionic surfactant R-11to aquatic invertebrates and larval amphibians**.** *California Fish and Game* **91**(1): 38-46.

Tyler, C. R.; van Aerle, R.; Hutchinson, T. H.; Maddix, S.; Trip, H (1999). An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. *Environmental Toxicology and Chemistry* **18**: 337–347.

Urbatzka, R.; van Cauwenberge, A.; Maggioni, S.; Vigano, L.; Mandich, A.; Benfenati, E.; Lutz, I.; Kloas, W (2007). Androgenic and antiandrogenic activities in water and sediment samples from the river Lambro, Italy, detected by yeast androgen screen and chemical analyses. *Chemosphere* **67**: 1080–1087.

USEPA. (2006). Decision Documents for Atrazine. Washington, DC: United States Environmental Protection Agency, Office of Prevention, Pesticides, and Toxic Substances. 323 p. www.epa.gov/oppsrrd1/REDs/atrazine.

Van den Belt, K.; Verheyen, R.; Witters, H. (2001). Reproductive effects of ethynylestradiol and 4t-octylphenol on the zebrafish (*Danio rerio*). *Archives of Environmental Contamination and Toxicology* **41**, 458–467.

van Wyk, J. H.; Pool, E. J.; Leslie, A. J. (2003).The effects of anti-androgenic and estrogenic disrupting contaminants on breeding gland (nuptial pad) morphology, plasma testosterone levels, and plasma vitellogenin levels in male *X. laevis* (African clawed frog). *Archives Environmental Contamination and Toxicology*. **44**: 247–256.

van Aerle, R.; Pounds, N.; Hutchinson, T. H.; Maddix, S.; Tyler, C. R (2002). Window of sensitivity for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow, *Pimephales promelas. Ecotoxicology **11***: 423-34.

Venturino, A.; Rosenbaum, E.; Caballero De Castro, A.; Anguiano, O.L.; Gauna, L.; De Schroeder, T.F.; De D'Agelo, M.P (2003). Biomarkers of effect in toads and frogs. *Biomarkers* **8** (3-4)**:** 167-186.

Veeramacheneni, D. N. R (2014). Anti-androgenic Pesticides: Impact on Male Reproduction. Research Proposal. Animal Reproduction and Biotechnology Laboratory Colorado State University, Fort Collins, CO.

Vitt, L. J.; Caldwell, J. P.; Wilbur, H. M.; Smith, D.C (1990). Amphibians as harbingers of decay. *Bioscience* **40**: 418.

Wise, A.; Brien, K, O.; Woodruff, T. (2011). Are oral contraceptives a significant contributor to the estrogenicity of drinking water? *Environmental Science and Technology* **45***:* 51–60.

World Health Organisation WHO (2004). Diquat in Drinking Water. Background Document for Development of WHO Guidelines for Drinking-water Quality. World Health Organisation, WHO/SDE/WSH/03.04/91.

Yadav, S.; Giri, U.; Singha, F.; Boro, A. G (2013). Toxic and genotoxic effects of Roundup on tadpoles of the Indian skittering frog (*Euflictis cyanophlyctis*) in the presence and absence of predator stress. *Aquatic Toxicology* **132–133**: 1– 8.

Zaya, R. M.; Amini, Z.; Whitaker, A. S.; Kohler, S. L.; Ide, C. F (2011). Atrazine exposure affects growth, body condition and liver health in *Xenopus laevis* tadpoles. *Aquatic Toxicology* **104***: 243– 253*.

Chapter Seven

General Summary and Conclusion

7.1 Introduction

Anthropogenic chemicals including agrichemicals, for example pesticides and fertilizers are integral reality of modern life, and human as well as wildlife cannot avoid contacts, and in cases even long-term chronic exposure (WHO, 1990; Mathias *et al*., 2012)**.** Pesticides, include insecticides, herbicides, rodenticides, fungicides but recently fertilizer have also been identified as potential environmental toxicants (WHO, 1990; 2013)**.** Pesticides (active ingredients and surfactants) are characterised by their toxic properties since these chemicals are designed to kill those unwanted plants and animals (WHO, 1990; 2013; Colosio *et al*., 1999). According to Magnarelli and Fonovich, (2013), pesticides may be considered as one of the contributing factors with when considering environmental contamination of today's world, pointing out that estimated 95% of applied herbicides formulations reach a destination other than their target organisms (plants). Aside from their toxic effects, many of these anthropogenic chemicals have been shown to have non-lethal health and ecological effects, both on human and wildlife. Documented effects include developmental abnormalities, thyroidal effects, gonadal and reproductive malformation among other effects (Relyea, 2005; Dinehart *et al*., 2009; Egea-Seranno *et al*., 2012).

Herbicides are widely used in South Africa, particular in agricultural and domestic environmental activities. In 1995, the South African Government (Department of Environmental Affairs) initiated a plan of action (Working for Water programme) to eradicate alien plants in water catchments using a variety of methods, including herbicides application. In light of the international concern that herbicides may have subtle toxic effects, including teratogenic and endocrine disrupting effects on non-target aquatic organisms, the use of herbicides close to or in aquatic ecosystems raises a health and ecological questions. This voiced concern are premised on the controversial ecological and

health antecedent of herbicides, like Atrazine that has been linked to widespread reported incidence of intersex and discontinuous gonads, disruption in sexual development and gonadal differentiation in wildlife (Hayes *et al*., 2002; 2006; Tevera-Mendoza *et al*., 2002a,b; Carr *et al*., 2003; Trachantong *et al*., 2013). In human, numerous health effects including cancer, bone marrow effects, reproductive effects, cytogenetic, neurotoxicity, immune system, skin effects have been linked to exposure to herbicides (WHO, 1990; WHO, 2013).

Arising from these non-target health and ecological impacts linked to herbicides, this study aimed to assess the ecotoxicity, potential endocrine disrupting (developmental abnormalities, thyroidal, gonadal and reproductive) as well as effects on adult reproduction in the aquatic African Clawed frog (*Xenopus laevis*). The focus of this study was to assess herbicide formulations, including diquat dibromide (Midstream), glufosinate ammonium (Basta), imazapyr (Arsenal) and glyphosate (Roundup, Kilo Max, Enviro Glyphosate) currently used in the South African "Working for Water" alien plants removal programme.

7.2. Summary of Result

In **Chapter Two**, comparative toxicity at different developmental stages of *Xenopus laevis* to the six selected herbicide formulations was assessed. To evaluate differential sensitivity among different developmental stages, the toxicity was assessed using tadpoles at three different developmental stages including embryos (NF-stage 8-11), premetamorphic larva stage (NF-stage 48) and transitional tadpole stage (NF- stage 60). The result showed that:

- Midstream formulation (diquat dibromide) was found to be toxic to *Xenopus laevis* (amphibian larva) with a 96-hour LC50 of 0.83 mg/L, 0.7 mg/L and 11.8 mg/L at NF stage 8-11, NF stage 48 and NF stage 60 respectively.

- Basta formulation (glufosinate ammonium) was toxic to *Xenopus laevis* larva with 96-hour LC50 of 2.24 mg/L, 0.59 mg/L and 24.9 mg/l at NF stage 8-11, NF stage 48 and NF stage 60 respectively
- Arsenal formulation (imazapyr) was slightly toxic to *Xenopus laevis* larva with 96 hour LC50 of 36 mg/L and 32.8 mg/L at NF stage 8-11 and NF stage 48 respectively
- Roundup formulation (glyphosate) was generally toxic to *Xenopus laevis* larva at 96-hour LC 50 of 1.052 mg/L, 0.89 mg/L and 2.75 mg/L at NF stage 8-11, NF stage 48 and NF stage 60 respectively.
- Kilo Max formulation (glyphosate) showed low toxicity to *Xenopus laevis* larva with 96-hour LC 50 of 207.3 mg/L, 58.1 and 455 mg/L at NF stage 8-11, NF stage 48 and NF stage 60 respectively
- Enviro Glyphosate formulation (glyphosate) also showed a relatively low toxicity to *Xenopus laevis* larva with 96-hour LC 50 of 464 mg/L, 134.6 mg/l and 5297 mg/L at NF stage 8-11, NF stage 48 and NF stage 60 respectively.
- This study showed that the 96-hour LC_{50} of many of the herbicide formulations including Midstream, Basta and Roundup were below the expected environmental concentrations (EEC) of these formulations. And the exposure to these herbicide formulations at these toxicity level will eliminate all the exposed larva, in a way that could disrupt the population dynamics
- Therefore this study established/confirmed the differential toxicity response of *Xenopus laevis* to herbicide formulations. The premetamorphic larva stage (NF-stage 48) was observed to be more susceptible to all the herbicide formulations than even the embryos (NF stage 8-11) as well as the tadpoles undergoing final metamorphosis (NF stage 60). This implies that the vulnerable NF-stage 48 tadpoles may not be protected from the exposure

impacts of these herbicides, and this could seriously negate the population dynamics of the exposed *Xenopus laevis*.

In **chapter three**, using Frog Embryo Teratogenic Assay (FETAX), this study examined the lethal effects, along with non-lethal, developmental effects including types and incidence of developmental malformations, teratogenic index, and growth disruption following the exposure of *Xenopus laevis* embryo (NF stage 8-11) to diquat dibromide (Midstream), glufosinate ammonium (Basta), imazapyr (Arsenal) and glyphosate (Roundup, Kilo Max, Enviro Glyphosate) formulations. The result showed that:

- Midstream, Roundup and Basta formulations were embryotoxic to the *Xenopus laevis* embryos, with 96-hour LC_{50} of 0.83 mg/L, 1.052 mg/L and 2.24 mg/L respectively
- Arsenal formulation was found to be slightly embryotoxic with 96-hour LC_{50} of 36 mg/L
- Kilo Max, and Enviro Glyphosate were not regarded as embryotoxic, with 96-hour LC_{50} of 207.3 mg/L and 464 mg/L respectively.

For growth inhibition and disruption, the results showed that

- Midstream formulation was a generalised growth disruptor in *Xenopus laevis* with a minimum concentration inhibiting growth at 1.0 mg/L. The Midstream formulation inhibited more than 100% body growth rate at concentration below its EEC of 1.0 mg/L
- Basta, Roundup formulations could be classified as growth disruptor with minimum concentrations inhibiting growth at 2.0 mg/L and 0.9 mg/L respectively. The two formulations significantly inhibited growth rates of the embryos at relevant environmental concentrations relative to the control group.
- Arsenal formulation could also be classified as growth disruptor as the formulation showed significant and concentration dependent growth inhibition at concentration of 30-45 mg/L compared to the control group.

- Kilo Max formulation was a potential growth disruptor with minimum concentration inhibiting growth at 190 mg/L, which is quite above relevant environmental concentration.
- Environ Glyphosate formulation was not regarded as growth disruptor as the formulation did not result in consistent growth inhibition at all exposure concentrations used.

For malformation incidence and teratogenic index, the result of this study showed that:

- Midstream formulation could be regarded as a teratogenic herbicide with a teratogenic index (TI) of 3.5, (which is above 1.5 for teratogenic index). The observed malformation included severe generalised edema, abdominal and cardiac edema, blistering, gut and head abnormalities, wavy tails, and tail flexus and eye malformation.
- Basta formulation showed a relatively low teratogenic potential with a teratogenic index (TI) of 1.1, (which is below the international teratogenic index of 1.5). The observed malformation included axial malformation (wavy tails), cardiac and abdominal edema and gut abnormalities.
- Arsenal formulation had a relatively low teratogenic potential with a teratogenic index of 1.3 (Below the international teratogenic index of 1.5 standard). The observed malformation included edema, blistering, gut and eye malformations, as well as improper head and body formation.
- Both Roundup and Kilo Max formulation have relatively low teratogenic potential with a teratogenic index of 1.4 each (slightly below the international teratogenic index of 1.5 standard). The observed malformation incidence included gut abnormalities, edema (abdominal, cardiac and severe generalised), blistering and eye malformations.
- Environ Glyphosate formulation could be regarded as a teratogenic herbicide with a teratogenic index of 1.6 (Above the international teratogenic index of 1.5). The observed malformation incidence included gut abnormalities, edema (abdominal, cardiac and severe generalised), blistering and eye malformations.

- Therefore, this present study confirmed the embryotoxicity of Midstream, Basta and Roundup formulations to *Xenopus laevis*. This study also confirmed that Midstream, Basta and Arsenal formulations are growth disruptor to *Xenopus laevis*. Moreover, it also showed that Midstream and Enviro glyphosate formulations are teratogenic to *Xenopus laevis.* It therefore mean that continuous use of these herbicide formulations (and other untested formulations) will potentially results in embryotoxicity and teratogenic effects in aquatic organisms like *Xenopus laevis*

In Chapter Four Impacts of Roundup, Kilo Max and Enviro Glyphosate formulations, Arsenal formulation (imazapyr), Midstream formulation (diquat dibromide) and Basta formulation (glufosinate ammonium) on metamorphosis was assessed using Xenopus Metamorphosis Assay (XEMA).

The results showed that:

- the Midstream (diquat dibromide) formulation can be regarded as a thyroid disruptor that significantly inhibited development of NF-stage 51 larva at concentration of 0.11 mg/L and 0.14 mg/L relative to the expected environmental concentration (EEC) of 0.733 mg/L
- the Arsenal (imazapyr) formulation can also be regarded to be a thyroid disruptor that significantly inhibited development of NF stage 51 larva at concentration of 3.5 mg/L relative to the Control group
- the Kilo Max formulation can be regarded as thyroid disruptor that significantly inhibited NF stage 51 of the *Xenopus laevis* at concentration of 190 mg/L and 280 mg/L (Although these concentrations are above relevance environmental concentrations of this formulation).
- Basta (glufosinate ammonium) formulation may be regarded as a potential thyroid disruptor that significantly increased the thyroid gland area of tadpoles at concentration of 0.15 mg/L and 0.25 mg/L relative to the control and in comparison to the expected environmental

concentration (EEC) at 1.0mg/L, but developmental stages was not significantly different to the control. Although sign of thyroid disruption were recorded, more research is needed to confirm the Basta formulation as regard thyroid disruption

- Roundup formulation (glyphosate) is a potential thyroid disruptor, that significantly increased the gland area at concentration of 0.6mg/L compared to the control group and relative to the expected environmental concentration at 1.43 mg/L
- Enviro Glyphosate formulation did not affect developmental programme but can be regarded as a potential thyroid disruptor since the average thyroid gland area was reduced as well as whole body length and snout vent length at concentration of 28mg/L. More research is needed to confirm the thyroid disrupting action of this formulation.

The present study therefore established that the Midstream, Kilo Max and Arsenal disrupted the HPT axis in developing *Xenopus laevis* tadpoles. However, for the Basta, Roundup and Environ Glyphosate formulations, the potential as thyroid disruptors cannot be ignored since differential impacts on the thyroid glands of Xenopus laevis were recorded. Therefore the continuous use of these herbicide formulations will exposed aquatic organisms to thyroid disruptors and potentially affect their normal development.

In Chapter five, the impacts of Midstream (diquat dibromide), Basta (glufosinate ammonium), Arsenal (imazapyr), and glyphosate including Roundup, Kilo Max and Enviro Glyphosate herbicides formulations, applied during premetamorphosis through metamorphosis on gonadal development post metamorphosis was assessed.

The result showed that:

- the Midstream formulation exposure resulted in high incident of gonadal toxicity with the malformation index ranging between 32.5 and 60% at concentration of 0.05 through 0.14 mg/L compared to the malformation index of 7.5% in the Control group. The observed

gonadal malformations included segmented hypoplasia, slightly translucence, segmented hypertrophy, aplasia, mixed sex, enlarged hypertrophy and folded gonad.

- the Basta formulation exposure led to some gonadal toxicity with the malformation index ranging between 15% and 27.5% at concentration of 0.05mg/L to 0.25 mg/L compared to malformation index of 12.5% in the Control group. The observed malformation included folded gonad, adhesion, angular deformity, enlarged hypertrophy, segmented aplasia, slightly translucence, angular deformity and hypoplasia.
- the Arsenal formulation similarly showed some evidence of gonadal toxicity with the malformation index ranging from 17.5% to 35% at concentrations of 0.5 mg/L to 3.5 mg/L compared to 7.5% in the Control group. The observed malformation included segmented aplasia, narrow hypoplasia, slightly translucence, aplasia, angular deformity, tissue separation, partly narrow segmented hypoplasia, slightly translucence, folded gonads.
- the Kilo Max formulation on the other hand showed a relatively high gonadal toxicity with malformation index ranging from 22.5% through 43% compared to the 10% in the Control group. The observed malformation included aplasia, segmented aplasia, segmented hypoplasia, slightly translucence, tissue separation, folded gonads, protuberances, segmented bifurcation and segmented hypertrophy.
- the Roundup formulation showed relatively high gonadal toxicity with malformation index ranging between 20% and 30% at concentration of 0.2 mg/L to 0.6 mg/L compared to 7.5% in the Control group. The observed malformations included aplasia, segmented aplasia, narrow hypoplasia, partly narrow hypoplasia, segmented hypoplasia, folded gonads, angular deformity, translucence, adhesion and hypoplasia.
- the Environ Glyphosate formulation resulted in high gonadal toxicity with the malformation index ranging from 17.5% and 37.5% at concentration of 9 mg/L to 28 mg/L compared to

7.5% in the Control group. The observed malformation included slightly translucence, segmented aplasia, segmented hypoplasia, aplasia, folded gonadal and narrow hypoplasia**.**

For Sex Ratio effects, the result showed that:

- the Midstream formulation exposure resulted in a significant skewed sex ratio at concentration of 0.14 mg/L. This formulation therefore has the potential to be regarded as an estrogenic/antiandrogenic substance that would feminized the exposed tadpoles, and lead to a population skewed towards females in the wild.
- the Arsenal formulation showed a different to the sex ratio in the Control group, the low androgenic/antiestrogenic potential of this formulation will require further research.
- the Kilo Max formulation showed some trend towards increased female sex ratios in concentration dependent manner. The sex ratio was only significantly skewed compared to the Control group at a concentration of 280 mg/L. Clearly, the estrogenic/antiandrogenic potential of Kilo Max and its effects on sex ratio outcome in a population needs further study.
- the Basta, Roundup and Enviro Glyphosate formulations showed non-significant variations skewed when compared to the Control group. The estrogenic/antiandrogenic potential of these herbicide formulations deserves further study.

Therefore, this study showed that these herbicide formulations have the potential to cause differential gonadal toxicity and lead to various gonadal malformations and disruption in the phenotypic sex ratio. Functional implications of these reproductive disruptions at the population level awaits further study.

In chapter six, wild-caught, adult male *Xenopus laevis* frogs were exposed (30-day semi-static set-up) to two selected formulations, Midstream (diquat dibromide) and Arsenal (imazapyr)

formulations on adult *Xenopus laevis* to assessed the effects on the mature male reproductive system. The results showed that

- The adult *Xenopus laevis* have higher resistance and are less sensitive to the potential exposure impacts of both Midstream and Arsenal formulations at relevant environmental concentrations.
- There is wide differential response to pesticides between the adult stage relative to the eggs, embryos and larval developmental stages.
- The adult developmental stage could be a poor indicator of pesticides contamination and pollution in environment relative to the eggs, embryos and larval stages.

Therefore, exposure impacts of pesticides should focus more on the eggs, embryos and larval developmental stages rather than adults, as the impacts could be more severe at that stages.

7.3. General Conclusion

From the results obtained in the present study, evidence showed that all six herbicide formulations considered possessed varied toxicity and endocrine disrupting activities in different developmental stages of *Xenopus laevis*. Data obtained confirmed the embryotoxicity effects following exposure to Midstream, Basta and Roundup formulations. Teratogenic activities were linked to exposure to Midstream and Enviro Glyphosate formulations. Different degrees of the thyroid disrupting activities were observed following exposure to Midstream, Basta, Arsenal and Roundup formulations. Kilo Max and Enviro Glyphosate formulation on the other hand showed very low thyroid disrupting potential with the endpoints considered. The gonadotoxicity and reproductive disruption in juvenile *Xenopus laevis* frogs, including differential skewed sex ratios were recorded following exposure to Midstream, Kilo Max, Basta, Arsenal and Roundup formulations. For the

adult stage, the exposure to Midstream and Arsenal formulations showed that the adult are less sensitive, relative to eggs, embryos and larval stages, particularly at relevant environmental concentrations.

In general with the results obtained in the present study, calls for cautions against the continued exposure of aquatic organisms like amphibians and other sensitive and endangered wildlife organisms, to herbicides. Moreover, this study confirmed that sensitivity towards environmental toxicants may vary in developing (metamorphic) stages and that the FETAX assay should be extended to include different developmental stages. Importantly, those formulations to be used in the aquatic ecosystems, with LC50s that are less than the published expected environmental concentrations (EEC), should be prioritized for more detailed studies at the chronic exposure level.

The present study (six herbicide formulations) also confirmed that herbicide formulations might be a contributing factor to reckon with (through the toxicity, teratogenicity, and disruption of thyroidal pathways) when considering the global amphibian decline. Clearly, the potential contribution of pesticide/chemical contamination to a so called global decline of amphibians, cannot be ruled out. The differential vulnerability of premetamorphic larva stages (for example NF-stage 48 vs NF-stage 8-11 embryos) corroborate similar suggestions published for several anuran species, including *Lithobates clamitans*, *Lithobates pipiens*, *Anaxyrus arenarum* and *Anaxyrus americanus.* Importantly, sensitive premetamorphic larva stage (NF-stage 48) should be included or repeated using a modified FETAX assay as standard Amphibian toxicity testing. It is therefore important that aquatic vertebrate testing be extended to include amphibians since most official testing usually only include fish and aquatic invertebrates as representative for aquatic organisms. Although in this study, focus was only on the aquatic amphibian, *Xenopus laevis,* varied response among developmental stages suggest that other amphibian species should also be included, but importantly also extending similar assays to other aquatic vertebrates, for example fish.

In South Africa, due to large diversity (number) and volumes of pesticides (mostly same formulation) widely applied, the lack of chronic exposure knowledge remains a concern. Moreover, the large volume of herbicides that are applied in present day agriculture as well as aquatic control programmes initiated to eradicate alien plants in river catchments (RSA government WfW programme) and other natural areas calls for extensive screening programmes using non-target organisms. This will lead to intelligent herbicide formulations selection, specifically aimed at aquatic application, considering the potential chronic, non-lethal physiological disrupting effects, in addition to the traditional toxicological endpoints. The fact that *Xenopus laevis* are locally endemic amphibian, may be at risk (especially during metamorphosis) and therefore makes it important for continued assessment/research of pesticides impacts including herbicides, using at least three developmental stages. This will help to extend the toxicity data-base regarding non-lethal effects of pesticides used in high volumes to formulate environmentally friendly management policies that will go a long way in policy formulation on pesticides impacts and management

7.4. Recommendations

- Extensive and robust experimental researches are needed for most locally applied herbicide formulations in other to establish their potential health risk to wildlife and humans
- Improve capacity building and skill development are needed in in-vitro and in vivo screening protocols using a tiered screening programme to identify problematic herbicide formulations.
- General toxicity testing, including determination of LC 50 should be applied for a series of developmental stages when using aquatic sentinel species to screen locally used herbicides

- The use of standardized FETAX protocol should be encouraged widely as it is a four day (96 hour) bioassay, with clear endpoints linked to mortality, embryotoxicity, teratogenicity and growth disruption.
- The internationally accepted XEMA protocol for developmental effects (thyroidal disruption) should also be extensively encouraged, as it is very reliable, consistent, with easy to follow methodology and clear endpoints.
- The adult developmental stage could be a poor indicator of environmental contamination and pollution, hence egg-larval developmental stages should be focused for studies on exposure impacts of pesticides.
- Potentially linked endocrine disrupting activity of pesticides including herbicides, needs to be researched more widely, especially with focus on specific mechanism of actions.
- Long-term chronic exposure programmes including multi-generational studies should also be used more widely to establish and characterise the generational (and epigenetic) impacts of the pesticides
- Precautionary principle should be applied when selecting herbicide formulation for environmental application, especially for aquatic use, if impacts in terms of toxicity, developmental, thyroidal and reproductive disruptions are not known.
- A Central African databank must be established to share data to assist with interpretation and critical assessment of data obtained during past experiments as well as provide inter-laboratory validations.

References

Carr, J. A.; Gentles, A.; Smith, E. E.; Goleman, W. L.; Urquidi, L. J.; Thuett, K.; Kendall, R. J.; Giesy, J. P.; Gross, T. S.; Solomon, K. R.; Van Der Kraak, G (2003). Response of larval *Xenopus laevis* to atrazine: assessment of gonadal and laryngeal morphology. *Environmental Toxicology and Chemistry* **22**: 396–405.

Colosio C.; Corsini E.; Barcellini W.; Maroni, M. (1999). Immune parameters in biological monitoring of pesticide exposure: current knowledge and perspectives. *Toxicological Letter* **108**: 285-295.

Dinehart, S. K.; Smith, L. M.; McMurry, S. T.; Anderson, T. A.; Smith, P. N.; Haukos, D. A (2009). Toxicity of a Glufosinate and Several Glyphosate-based herbicides to Juvenile Amphibians from the Southern High Plain, USA. *Science of Total Environment* **407***:* 1065-1071.

Egea-Serrano, A.; Relyea, R. A; Tejedo, M.; Torralva, M. (2012). Understanding of the impact of chemicals on amphibians: a meta-analytic review. *Ecology and Evolution* **2***(7)*:1382–1397

Hayes, T.; Haston, K; Tsui, M.; Hoang, A.; Haeffele, C.; Vonk, A. (2002a) Feminization of male frogs in the wild. *Nature* **419**: 895-896.

Hayes, T. B.; Collins, A.; Lee, M.; Mendoza, M.; Noriega, N.; Stuart, A. A.; Vonk, A (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine, at low ecologically relevant doses. *Proceedings of National Academic Science, U.S.A.* **99**: 5476–5480.

Hayes T .B.; Haston K.; Tsui M.; Hoang A.; Haeffele C.; Vonk A. (2002b). Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Lithobates pipiens*): Laboratory and field evidence. *Environmental Health Perspective* **111**: 568–575.

Hayes, T.B.; Stuart, A.A.; Mendoza, M.; Collins, A.; Noriega, N.; Vonk, A.; Johnston, G.; Liu, R.; Kpodzo, D (2006). Characterization of Atrazine-Induced gonadal malformations in African clawed frogs *(Xenopus laevis)* and comparisons with effects of an androgen

antagonist (Cyproterone Acetate) and exogenous estrogen (17β-estradiol): Support for the demasculinization/feminization hypothesis. *Environmental Health Perspective* **114** (suppl 1):134–141 doi:10.1289/ehp.8067

Relyea, R. A. (2005). The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecological Applications **15***(2)*:* 618-627.

Tavera-Mendoza, L.; Ruby, S.; Brousseau, P.; Fourier, M.; Cyr, D.; Marcogliese, D. (2002a). Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. *Environmental Toxicology and Chemistry* **21**:527–531.

Tavera-Mendoza, L.; Ruby, S.; Brousseau, P.; Fourier, M.; Cyr, D.; Marcogliese, D. (2002b). Response of the amphibian tadpole *Xenopus laevis* to atrazine during sexual differentiation of the ovary. *Environmental Toxicology and Chemistry* **21**: 1264–1267.

Trachantong, W.; Promya, J.; Saenphet, S.; Saenphet, K. (2013). Effects of atrazine herbicide on metamorphosis and gonadal development of *Hoplobatrachus rugulosus. Maejo International Journal of Science and Technology* **7**(Special Issue): 60-71.

World Health Organisation (WHO) (1990). Public health Impacts of pesticides used in agriculture. World Health Organisation, Geneva. www.who.org/publications. Assessed on 16 October, 2012.

World Health Organisation (WHO) (2013). State of the Science of Endocrine Disrupting Chemicals. United Nations Environmental Programme and World Health Organization. www.who.org/publications. Assessed on 10th, April, 2014.