Determining the (anti)androgenic activity of agricultural pesticides in water systems with a luminescence bio-assay

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Dissertation submitted in fulfilment of the requirements for the degree *Magister Scientiae* in Environmental Sciences at the Potchefstroom Campus of the North-West University

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May 2016

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude towards the following people:

- My Creator and Bridegroom for blessing me with the opportunity, wisdom and courage to complete what You have started in me.
- My supervisor, Dr. Rialet Pieters. Thank you for your time, patience, high standards and teaching me not only to be a researcher but also equipping me with life skills.
- My co-supervisor, Dr. James Dabrowski for your time, patience, guidance and contributions.
- My family and friends (parents, grandparents, Bianca, Nicolene, Annerie, Maxine, Helga, Simóne, Arnè, Eileen, Silvia, Maryna, Simonè & Shannon) for your unconditional love and endless support, prayers and encouragement.

I would also like to thank the following institutions for their financial support towards this study:

- The Water Research Commission (WRC) of South Africa (K5/1956).
- The National Research Foundation (NRF) (grant-holder bursary).
- The North West University (post-graduate bursary).

Song of Solomon 2:16

My beloved is mine, and I am his.

ABSTRACT

South Africa has a large agricultural sector that produces a wide variety of crops on large scale for local use and exportation. This provides some explanation as to why the country has over 500 pesticide active ingredients registered for use. Studies have indicated that several current use pesticides have endocrine disrupting properties that may cause health effects if humans and animals are exposed to such chemicals. Distribution of pesticides into freshwater systems frequently occur which increases the potential of exposure since many South Africans use untreated water sources for domestic and agricultural purposes. This study aimed to determine the (anti)androgenic activity of several current use pesticides used in different agricultural practices in South Africa.

Surface and groundwater samples as well as sediment samples were collected from three different study areas in the country. In the Letsitele River catchment mangos, citrus and avocados are produced on large scale. The intensive cultivation of maize, sorghum and sunflower take place in the Renoster and Vals River catchments while sugarcane and citrus are produced in the Mzinti, Lomati and Ngweti River catchments. The samples were chemically screened for the presence of 291 pesticide active ingredients. The ten priority pesticides were selected based on their frequency of occurrence in each study area as well as on their intensity of use in the country. These pesticides were subjected to a chemical quantification analysis. The samples were also screened for their ability to activate the androgen receptor and/or to inhibit androgen binding to the androgen receptor. This was done with the MDA-kb2 reporter gene bio-assay. The assay also detects ligands capable of activating the glucocorticoid receptor. The chemical and biological analyses results were compared on a seasonal basis and the results of the samples from the different matrices were also compared to one another.

Several surface water and sediment samples elicited (anti)androgenic responses from the cells as well as glucocorticoid receptor activation. The most androgenic and/or glucocorticoid responses were caused by the sediment samples collected in the Renoster and Vals River catchments. The most anti-androgenic responses were collectively detected in the surface water and sediment samples from the Mzinti, Lomati and Ngweti River catchments. Atrazine and alachlor were the only priority pesticides that have been declared as definite endocrine disruptors while studies have indicated that atrazine, imazalil and propiconazole may have anti-androgenic properties. Comparison of the data showed no pattern between the samples from the different matrices from the same sampling event or between the samples from different sampling events. Interpretation of the results were further complicated since very

few regulatory guidelines exist for pesticides in aquatic ecosystems. This study can be used as the basis for future endocrine disrupting studies during which pesticide guidelines can be developed in a South African context.

Keywords: (Anti)androgenic activity; Endocrine disruption; Water and sediment quality guidelines; MDA-kb2 reporter gene bio-assay; Pesticides.

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LIST OF ABBREVIATIONS AND ACRONYMS

A	
ASE	Accelerated solvent extractor
ATCC	American Type Culture Collection
AR	Androgen receptor
B	
BAP	Bio-assay period
BC	Blank control
BEQ	Bio-assay equivalents
C	
cdtFBS	Charcoal dextran treated foetal bovine serum
CHO	Chinese hamster ovary
CV	Coefficient of variation
D	
DCM	Dichloromethane
DHT	Dihydrotestosterone
DWAF	Department of Water Affairs and Forestry
ddH_2O	Double distilled water
DMSO	Dimethyl sulphoxide
E	
EC	Effective concentration
ED	Endocrine disrupting
EDC	Endocrine disruptive compound
EDSP	Endocrine Disruptor Screening Program
EEP	Environmental exposure potential
EPI	Enhanced product ion
ER	Oestrogen receptor
EtOH	Ethanol
EU	European Union
F	
FBS	Foetal bovine serum

FI	Fold inhibition
G	
GPC	Gel permeation chromatography
GR	Glucocorticoid receptor
GV	Guideline value
H	
HEPA	High efficiency particulate air
HP	Hazard potential
HPLC	High performance liquid chromatography
I	
IDA	Independent data acquisition
L	
LOD	Limit of detection
M	
MAC	Maximum acceptable concentration
MCL	Maximum concentration level
MeOH	Methanol
MMTV	Mouse mammary tumour virus
MPC	Maximum permissible concentration
MRM	Multiple reaction monitoring
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
O	
OD	Optical density
P	
PBS	Phosphate buffered saline
PLE	Pressurized liquid extraction
Q	
QI	Quantity index
R	
REP	Relative potency value
RLU	Relative light unit
S	

SC	Solvent control
SPE	Solid phase extraction
SQG	Sediment quality guideline
T	
T	Testosterone
T-eq	Testosterone equivalent
TP	Toxicity potential
TV	Trigger value
TWQR	Target water quality range
U	
US EPA	United States Environmental Protection Agency
UV	Ultraviolet
W	
WHO	World Health Organization
WHP	Weighted hazard potential
WQG	Water quality guideline

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CHAPTER 1: INTRODUCTION

1.1. General overview and problem statement

South Africa has a variety of climatic conditions that result in regions ranging from semi-tropic to semi-arid. The country's agricultural sector is therefore able to produce an assortment of crops on large scale for local use and exportation. This provides some explanation as to why over 500 pesticide active ingredients are currently registered for use in the country (PAN, 2014). South Africa has also been ranked as one of the top users of pesticides in sub-Saharan Africa (Ansara-Ross *et* al., 2012; Dalvie *et al*., 2009; Quinn *et al*., 2011). Zhang *et al*. (2011) reported that South Africa accounts for approximately 2% of worldwide pesticide consumption that is expected to grow rapidly as the country's agricultural sector expands. Many pesticides that have been banned in some developed countries are still being used in South Africa due to the lack in awareness regarding the potential health effects of these pesticides, a shortage in the capacity to regulate pesticide applications and their low cost (Dalvie *et al*., 2009).

Concern has been raised regarding the widespread use of pesticides for animal disease control and plant protection as studies have indicated that several current use pesticides have endocrine disrupting (ED) properties (Aït-Aïssa *et al*., 2010; Burger & Nel, 2008; McKinlay *et al*., 2008; Preda *et al*., 2012). Chemicals with ED properties alter/disrupt the endocrine system of humans and wildlife and have therefore been termed as 'endocrine disrupting compounds' (EDC) by Colborn *et al*. in 1993. The United States Environmental Protection Agency (US EPA) and the World Health Organisation (WHO) basically define an EDC as an exogenous chemical substance or mixture that functionally modifies the endocrine system that may result in adverse health effects (Bergman *et* al., 2012). Mechanisms of action include mimicking, inducing or inhibiting endogenous hormones, altering hormone receptors and/or -metabolism and disrupting hormonal transport that may result in growth, development and reproductive complications (Aït-Aïssa *et al*., 2010; McKinley *et al*., 2008; Preda *et al*., 2012). Some of the health effects that have been associated with EDCs include reduced fertility and fecundity, polycystic ovary syndrome, abnormal reproductive organ function and morphology, spontaneous abortion, premature puberty and a variety of cancers (McKinley *et al*., 2008). Various chemicals have been declared as EDCs and include natural- (*e.g*. phytoestrogens and other plant sterols) (Young & Borch, 2009) and synthetic chemicals (*e.g*. pharmaceuticals, pesticides, synthetic hormones) (Blake *et al*., 2010).

EDCs often interfere with biochemical pathways and mechanisms that are regulated by sex steroid hormone receptors such as the androgen receptor (AR) and the oestrogen receptor (ER) (Odermatt *et al*., 2006). Androgens (*e.g*. testosterone) are testicular steroid hormones

that specifically interact with the AR and are responsible for the sexual differentiation of the male reproductive system (Mainwaring, 1977; US EPA, 2011; Wilson *et al*., 2002). Androgens bind to and activate the AR which results in the transcription of androgen-dependant genes (positive regulation) (Bergman *et al*., 2012). Chemicals that block the binding of androgens to the AR and inhibit normal androgen-dependant gene transcription (negative regulation) are called anti-androgens (*e.g*. flutamide) (Mnif *et al*., 2011). If any one of these two pathways are altered or disrupted (*e.g*. through xenobiotics that have (anti)androgenic activity) it may result in genital malformations, a decrease in semen quality and cancers of male reproductive organs (Ermler *et al*., 2010). The current study focused on the potential (anti)androgenic activity of certain pesticides that are currently being used in the South African agricultural sector.

Distribution of pesticides into freshwater systems (*e.g*. boreholes, rivers and dams) frequently takes place via non-point source pollution (*e.g*. leaching, spray drift and runoff) (Schulz, 2001). Due to a lack of access to treated water, many South African citizens from poor and/or rural areas often make use of untreated freshwater sources for domestic and agricultural purposes. Pesticide pollution may potentially have a great effect on their health (Aneck-Hahn *et al*., 2005; Dabrowski *et al.*, 2014; McKinley *et al*., 2008). Furthermore, wildlife and livestock exposure to the contaminated water sources may also influence environmental health (McKinley *et al*., 2008). The ED properties of agricultural pesticides are, however, not well understood and according to a scoping study conducted by Burger and Nel (2008) little information on the fate and behaviour of pesticides with ED properties in aquatic environments and within human and animal systems are available.

In this study water and sediment samples were collected from three study areas (Chapter 3) and initially screened for 291 pesticide active ingredients (Table A–1). The five active ingredients that were detected most frequently in both the water and sediment samples from each study area were subsequently subjected to a quantitative chemical analysis. A total of ten priority pesticides (alachlor, atrazine, carbofuran, diphenylamine, imazalil, imidacloprid, propiconazole, simazine, thiabendazole and terbuthylazine) were identified throughout all three study areas and will continually be discussed throughout this dissertation.

The chemical screening- and quantification analysis results were compared to national and international water and sediment quality guidelines (SQGs) (Tables 4.2–4.4). Empirical data for the effects of pesticides in water and sediment on human and environmental health are presently lacking for the majority of current use pesticides. This is primarily why no guideline values exist for most pesticides registered for use in South Africa even though the country is a major user of pesticides (DWAF, 1996a; London *et al*., 2005). The lack of comprehensive

quality guidelines for pesticides in water and sediment makes a thorough comparative study impossible and therefore further emphasizes the need for pesticide research, specifically in a South African context.

The MDA-kb2 reporter gene bio-assay was used as the androgen screening bio-assay to test the water and sediment samples for their ability to interfere with androgen-dependant gene transcription. The samples were screened for both androgenic- (AR activation) and anti-androgenic activity (AR inhibition). The MDA-kb2 cell line expresses high quantities of endogenous AR but also has glucocorticoid receptors (GRs) (Wilson *et al*., 2002). The GR is also a nuclear steroid receptor (such as the AR and ER) and modulated by glucocorticoids (*e.g*. cortisol) (Schaaf & Cidlowski, 2003). The MDA-kb2 cell line has been transformed with the firefly luciferase reporter plasmid and is driven by the mouse mammary tumour virus (MMTV) promoter (Chapter 2) that can be activated by both the AR and the GR (Wilson *et al*., 2002). The presence of both the AR and GR in the cells may therefore pose some difficulty in interpreting the androgenic (AR activation) responses subsequent to reporter gene expression since such responses can be the result of activation of any one of the two receptors. For this reason background information regarding the GR is briefly discussed in Chapter 2. The bio-assays were however conducted in such a manner that it indicated clear anti-androgenicity (AR inhibition).

Bio-assays, such as the MDA-kb2 reporter gene bio-assay, can only be used as a tool to detect the presence of ligands binding to the respective receptor(s) (Wilson *et al*., 2002). Bio-assays cannot be used to identify specific compounds responsible for reactions (*e.g*. (anti)androgenic reactions) but rather measures a specific mechanism of action that involves the receptor(s) (*e.g*. AR) and therefore supports the process of elimination if compared to the analytical chemistry results.

In this study androgen- as well as glucocorticoid dependant gene transcription (via the AR and/or GR) were measured and compared to the reaction elicited by the positive control testosterone. Currently no standards or guidelines for testosterone in water or sediment exist. There is therefore no regulatory way of knowing at what levels testosterone or testosterone-like compounds may have chronic health effects in humans and animals. None of the (anti)androgenic responses elicited throughout this study could therefore be discussed in terms of potential health effects. This situation emphasizes the great need for further EDC research, specifically for androgens and androgen-like compounds. In an attempt to explain the elicited responses, the MDA-kb2 bio-assay data were compared to detected pesticides in the relevant catchment that are known EDCs or that are considered as possible EDCs. Other land uses and/or industries in the various catchments were also considered as possible

culprits for the positive responses. Androgen receptor inhibition responses were expressed as a function of fold inhibition (FI) (Chapter 3)

1.2. Research aims and objectives

The current study formed part of a larger WRC project (K5/1956) during which the contamination of water resources by agricultural chemicals and their impact on environmental health was investigated. The project was additionally funded by the National Research Foundation (NRF). The aims and objectives for this specific study were based on the hypothesis that different agricultural practices introduce different agricultural pesticides, with varying (anti)androgenic capabilities, to the aquatic environment. The research aims to ultimately contribute information related to the ED properties of specific pesticides that may be used in risk assessment studies of human, animal and environmental health.

The first aim of this study was to determine the (anti)androgenic activity of water and sediment samples collected from three catchment areas with different agricultural practices.

The objectives were to:

- Apply appropriate extraction and clean-up procedures to water and sediment samples to collect target pesticide substances and/or compounds.
- Use the MDA-kb2 reporter gene bio-assay as a tool to screen the water and sediment extractions for (anti)androgenic activity.

The second aim of this study was to compare the (anti)androgenic activity of water and sediment from regions with different agricultural practices, on a seasonal basis.

The objectives were to:

- Target selected water systems that have been exposed to different types of large scale agricultural practices.
- Chemically screen the sampled water and sediment for the presence of the active ingredients of current use agricultural pesticides in South Africa and compare the chemical data with the bio-assay data retrieved on a seasonal basis.

The third aim of this study was to identify the agricultural pesticides' active ingredient(s) potentially responsible for (anti)androgenic responses through a literature review and comparison of the chemical- and bio-assay data.

CHAPTER 2: LITERATURE REVIEW

2.1. Introduction

The South African agricultural industry is responsible for the production of a large variety of crops and therefore explains why the country is one of the major users of pesticides in sub-Saharan Africa (Ansara-Ross *et al.*, 2012; Burger & Nel, 2008; Dalvie *et al*., 2009; PAN, 2014). Numerous studies have indicated that some pesticides may pose significant health effects to humans, animals and ecological systems due to their ED properties (Aït-Aïssa *et al*., 2010; Burger & Nel, 2008; Dalvie *et al*., 2009; McKinlay *et al*., 2008). This chapter gives an overview of the available literature regarding EDCs; pesticides as EDCs and their health effects; the (anti)androgenic effects of the priority pesticides that were analysed for in this study as well as a background on the MDA-kb2 reporter gene bio-assay that was used to screen for the presence of (anti)androgens in the water and sediment samples. A short background on the regulatory legislation that was used for comparison with the chemical analysis results is also provided.

2.2. Disruption of the vertebrate endocrine system

2.2.1. The vertebrate endocrine system

The endocrine system (or more commonly known as the hormone system) of vertebrates consists of three major components namely glands, hormones (that are chemical messengers produced by the glands and secreted into the bloodstream or interstitial fluid) as well as receptors in cells, organs and tissues that recognize and respond to specific hormones. These components collectively form physiological pathways through which the body can respond to internal and external stimuli. Subsequent to stimuli the appropriate hormones are secreted and bind to their receptors. These receptors belong to the nuclear receptor superfamily and are involved in various physiological processes (*e.g*. cell growth and proliferation, differentiation and maintaining homeostasis) in eukaryotic organisms. Binding of the hormone ligand to its receptor results in gene expression as the ligand-receptor complex is transferred into the nucleus where it binds to the corresponding responsive element on the DNA. Subsequent gene expression leads to protein synthesis and consequent reactions in the body (Burger & Nel, 2008; Diamanti-Kandarakis *et al*., 2009; Janosek *et al*., 2006; US EPA, 2011). The 48 nuclear receptors that have been identified so far have been classified into three classes. Hormones bind to Type 1 receptors (*e.g*. progestin receptor, ER, AR, GR and mineralocorticoid receptor) while Type 2 receptors (*e.g*. thyroid receptor, vitamin D receptor and the aryl hydrocarbon receptor) have various types of ligands. The ligands that bind to Type 3 receptors still have to be identified (Janosek *et al*., 2006). It has also been found that most nuclear receptors can be altered or regulated by more than one type of ligand and

similarly, any one type of ligand may have the ability to modulate several different nuclear receptors (Odermatt *et al*., 2006).

This study specifically focussed on pesticides that potentially mimic one of the three classes of steroid sex hormones called androgens (Young & Borch, 2009). Androgens (agonists of AR) are testicular steroid hormones and specifically responsible for sexual differentiation of the male reproductive system during foetal and neonatal development, affect male type behaviour and spermatogenesis and include hormones such as testosterone, dihydrotestosterone (DHT) and androstenedione (Janosek *et al*., 2006; Mainwaring, 1977; US EPA, 2011; Wilson *et al*., 2002; Xu *et al*., 2008). Androgens bind to nuclear ARs that regulate androgen-dependant gene expression (Kjeldsen *et al*., 2013; Mijamoto *et al*., 1998; Xu *et al*., 2008). The AR is produced in the testis but has also been detected in the prostate, adrenal glands, kidneys, brain and in the pituitary gland (Janosek *et al*., 2006). The disruption of androgen- and AR activity during developmental stages may lead to genital malformations, low sperm count and reproductive organ cancers (Ermler *et al*., 2011; Sharpe, 2006). Studies have also found androgens to play a role in adverse processes such as in the formation of prostate hyperplasia and carcinomas (Janosek *et al*., 2006). Anti-androgens (*e.g*. hydroxyflutamide) are a group of chemicals that prevents the binding of endogenous androgens to the AR, creating antagonistic action (Mnif *et al*., 2011). Since it disrupts AR activity it may increase the risk for the above mentioned, irreversible disorders (Ermler *et al*., 2011). Environmental pollutants may have androgen-like or anti-androgenic action (Janosek *et al*., 2006).

As mentioned in Chapter 1 the MDA-kb2 cell line (used as the bio-assay instrument during this study) not only expresses high levels of endogenous AR but also of the GR. Glucocorticoid (*e.g*. cortisol and dexamethasone) is a steroid hormone produced by the adrenal cortex. The hormone binds to and activates the GR that positively or negatively regulates specific GR gene expression. Glucocorticoids are involved in the regulation of several physiological processes (*e.g*. maintains blood pressure and electrolyte concentrations, modulates the immune system, regulates appropriate brain function as well as cell proliferation and differentiation (Bamberger *et al*., 1996; Odermatt *et al*., 2006; Schaaf & Cidlowski, 2003; Schäcke *et al*., 2007).

2.2.2. Endocrine disrupting compounds

Many of the receptor ligands have structural similarities with environmental pollutants and such xenobiotics may therefore interact with the nuclear receptor signalling pathways if the organism is exposed to the contamination. Hormone-like compounds can, for example, compete with endogenous hormones for binding to their receptors and cause abnormal

hormone dependant gene expression. Other pollutants may act as anti-hormones that compete with the endogenous hormones for the binding sites without activating the receptors and thereby preventing natural hormone activity (Janosek *et al*., 2006). The US EPA and the WHO defines an EDC as *"an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations"* (Bergman *et al*., 2012:11)

Compounds with ED properties have a tendency to interact with sex steroid hormone receptors (*e.g*. ER and/or AR) and may act through several different mechanisms (Blake *et al*., 2010; Mnif *et al*., 2011; Odermatt *et al*., 2006). Some of these mechanisms include modifying the normal synthesis and secretion of natural hormones, mimicking endogenous hormones and competing with it for binding to the ER or AR as agonists or antagonists, modifying the number of hormone receptors present in the cells or binding to and disabling the hormone transport proteins, binding to the hormones itself and causing antagonistic effects (Aït-Aïssa *et al*., 2010; Diamanti-Kandarakis *et al*., 2009; Hartig *et al*., 2007; Kojima *et al*., 2004; McKinlay *et al*., 2008; Mnif *et al*., 2011; Wilson *et al*., 2007). Any one of these mechanisms may lead to adverse health effects.

In general the alteration of the endocrine system may cause developmental, growth and reproduction problems in wildlife and humans or its progeny as a secondary result to changes in the endocrine function (Burger & Nel, 2008; McKinlay *et al*., 2008; Kjeldsen *et al*., 2013; Okubo *et al*., 2004). Some of the specific health disorders that have been associated with endocrine disruption include reduced fertility and fecundity, spontaneous abortion, polycystic ovary syndrome, reduced immune function, reduced sperm count, genotoxic effects, abnormal sperm morphology, several types of cancers and precocious puberty amongst others (McKinlay *et al*., 2008; Xu *et al*., 2008). Studies have shown that the health effects and the intensity thereof depend on different factors such as age, gender, diet and occupation (Mnif *et al*., 2011).

The ER and AR receptors are highly conserved across species (function and morphology are similar) (Hartig *et al*., 2007; Wilson *et al*., 2007) which raises concern in terms of human and wildlife exposure to chemicals in the environment that have ED properties. Various chemicals have been declared as EDCs and include natural substances (*e.g*. plant sterols and phytoestrogens) as well as synthetic substances (*e.g*. pharmaceuticals, pesticides, synthetic hormones, plastics and cleaning supplies) (Blake *et al*., 2010; Segner *et al*., 2003; Young & Borch, 2009). Exposure routes of EDCs in the environment include oral intake from polluted food and/or drinking water, via polluted air or through the skin from contaminated soil, dust and water (Odermatt *et al*., 2006).

2.3. Pesticides as endocrine disruptors

Pesticides are toxic substances or mixtures of substances used in agricultural and non-agricultural settings to prevent, destroy, repel or mitigate pests such as rodents, insects, fungi, weeds as well as plant, human and livestock disease vectors (Aït-Aïssa *et al*., 2010; Arias Estevez *et al*., 2008; Matisova & Hrouzkova, 2012; Mnif *et al*., 2011; Zhang *et al*., 2011). These substances have been divided into classes (*e.g*. herbicides, fungicides and rodenticides) based on their mode of action and period of action (Arias Estevez *et al*., 2008; Pletschke *et al*., 2011).

Exposure to pesticides in the environment can take place via different routes depending on the compartment (*e.g*. atmosphere, soil, surface freshwater, groundwater and ocean) in which the pesticides are present as well as the fate of the pesticides in each compartment (Aït-Aïssa *et al*., 2010; Warren *et al*., 2003). Runoff, leaching and spray drift (nonpoint source pollution) are the main routes of transport of pesticides from intended target areas into other ecological compartments (Arias Estevez *et al*., 2008; Schulz, 2001; Warren *et al*., 2003). Factors that influence runoff and leaching of pesticides into water systems include soil type, structure and temperature, precipitation volumes and sorption to colloids (Young & Borch, 2009).

Although pesticides are important for the growth of quality commercial crops and increase the security of food production (Ansara-Ross *et al*., 2012) they also cause severe health effects. Diseases and disorders such as cancer, neurological disorders, allergies, reproductive disorders, genotoxicity, mutagenicity and endocrine disruption have been linked to pesticide exposure (Dalvie *et al*., 2009; Mnif *et al*., 2011). McKinlay *et al*. (2008) listed 127 pesticides with ED properties that cause effects through several different modes of action in *in vitro* and *in vivo* systems. Some of the pesticides on this list (*e.g*. fenarimol, fipronil and vinclozolin) have the ability to inhibit binding of androgens and/or androgen-like compounds to the AR in the MDA-kb2 cell line (Aït-Aïssa *et al*., 2010). The endocrine disruption caused by pesticides can take place at concentrations below those described as toxic. Furthermore the mechanisms of action are not yet fully understood and they may have long term effects that may only be detected long after exposure (Burger & Nel, 2008; Pletschke *et al*., 2011).

The European Union (EU) (in collaboration with the US EPA and some other organisations) has done extensive research on EDCs since 1999 and has developed lists with priority substances that are continually being researched to determine their effects on endocrine systems. Suspected EDCs are listed based on available information and research regarding their potential ED properties. The US EPA also has the authority to test all manufacturing and processing chemicals for possible ED properties which aids in the modification of the lists (that are updated every few years) (EUC, 1999; Matisova & Hrouzkova, 2012). The most recent

list was published in November 2010 and includes 134 chemicals, of which many are pesticides. Each one of the chemicals has also been classified into a specific category based on their definite/potential ED properties. Pesticides classified as Category 1 EDCs have definite ED properties while pesticides with potential ED properties are considered a Category 2 EDC. If there is not enough evidence to prove the ED effects of a substance it is classified as a Category 3 EDC and subjected to further tests (Matisova & Hrouzkova, 2012).

2.4. (Anti)androgenic properties of the priority pesticides

Ten pesticides were selected as priority pesticides for this study based on the analytical chemistry results. The selection criteria were: (1) frequency of occurrence throughout the three study areas and (2) the intensity of their use in SA. In this section the focus was on the potential/definite (anti)androgenic properties of these priority pesticides and whether they could influence the AR's transcriptional activity. The reason for selecting this specific mechanism of action is that the reporter gene bio-assay that was used in this study to detect (anti)androgenic activity uses the principle of ligands binding to the AR (No literature could be found on any of the ten priority pesticides regarding their ability to activate the GR).

2.4.1. Alachlor

Alachlor is a herbicide in the chloroacetanilide chemical group (Matisova & Hrouzkova, 2012) and is used as a pre- and post-emergence application to control annual grasses and several types of broad-leaved weeds in maize, oilseed rape, peanuts, radish, soy beans and sugarcane. The pesticide is highly mobile and has been detected in surface and groundwater as well as in drinking water (WHO, 2011). Alachlor has been classified as a Category 1 EDC by the EU Commission (EUC, 1999; Matisova & Hrouzkova, 2012) as studies have shown the pesticide to bind to oestrogen and progesterone receptors and also to interfere with steroid hormone metabolism (Cocco, 2002; Klotz *et al*., 1997). In a study conducted by Kojima *et al*. (2004) alachlor inhibited AR transcriptional activity that was induced by 9.6×10^{-6} M DHT but no other literature could be found regarding the AR regulated (anti)androgenic activity of the pesticide. In another study that was done by Yi *et al*. (2007) alachlor caused a significant decrease in serum testosterone levels in Crucian Carp and also modified serum sex steroid levels in fish that was exposed to the pesticide (Klotz *et al*., 1996).

2.4.2. Atrazine

Atrazine is a herbicide in the triazine chemical group. It is typically used to control annual broadleaf and grassy weeds in corn and blueberries and may also be used for nonselective weed control (CCME, 1999a). According to the study conducted by Dabrowski *et al*. (2014), maize and sugarcane account for most of the atrazine used in the South African agricultural sector. It is a highly preferred pesticide in the country due to its efficacy and low cost (Du

Preez *et al*., 2005). Exposure to atrazine may take place via several pathways since it is a highly mobile pesticide. It has been detected in relatively high concentrations (ranging from 0.007 µg/ℓ to 25.3 µg/ℓ) in surface and groundwater sources around the world (Du Preez *et al*., 2005; Konstantinou *et al*., 2006; Sánchez-Camazano *et al*., 2005).

Atrazine has been classified as a definite endocrine disruptor (Category 1 EDC) (EUC, 1999; Matisova & Hrouzkova, 2012). The pesticide has been proven to increase oestrogen production through induction of aromatase activity (Sanderson *et al*., 2000), disrupt luteinising hormone and prolactin levels (Cooper *et al*., 2000), have weak oestrogenic activity and decrease DHT levels by inhibiting 5α-reductase (Thibaut & Porte, 2004). It has also been found to interfere with steroid hormone metabolism (Cocco, 2002). Very little literature could be found on the (anti)androgenic properties of atrazine with regards to androgen-dependant gene transcription. However, in a study conducted by Orton *et al*. (2009) atrazine was found to inhibit androgen binding in a yeast assay.

2.4.3. Carbofuran

Carbofuran is a carbamate insecticide and nematicide used to control pests such as worms and mites on vegetables, ornamentals, corn, alfalfa, sorghum, potatoes and sunflowers (CCME, 1999b). In South Africa 72.8% of the total quantity of carbofuran is used on maize while 4.8% thereof is used on potatoes (Dabrowski *et al*., 2014). Exposure to the pesticide is mainly through water sources (WHO, 2011) but may also be ingested as residue on food (CCME, 1999b). The pesticide has been ranked as a Category 2 EDC by the EU Commission, stating that it potentially has ED properties (EUC, 1999; Matisova & Hrouzkova, 2012). Several studies have proven carbofuran to be a potential endocrine disruptor such as the one done by Goad *et al*. (2004) during which carbofuran increased progesterone, cortisol and estradiol levels while it simultaneously decreased testosterone levels. Elayan *et al*. (2013) also found carbofuran to decrease testosterone levels by causing irreversible damage to the reproductive organs of male mice. No other literature could be found regarding the (anti)androgenic activity of carbofuran.

2.4.4. Diphenylamine

Diphenylamine is used as a plant growth regulator to control storage scald on apples and pears (García-Reyes *et al*., 2005; US EPA, 1998). Very little information is available regarding the potential ED effects of diphenylamine but it has been listed by the US EPA for further investigation and possible classification as an EDC (US EPA, 1998).

2.4.5. Imazalil

Imazalil is a systemic imidazole fungicide used as a post-harvest treatment on bananas, citrus and other fruit to control storage decay. It is also used as a barley and wheat seed treatment (US EPA, 2005). Imazalil has been classified as a Category 3 EDC by the EU Commission. This means that there is insufficient evidence available to accurately confirm its ED properties (EUC, 1999; Matisova & Hrouzkova, 2012). However, studies have shown that imazalil inhibits testosterone binding to the AR by 50% at a concentration of about 36 µM (Okubo *et al*., 2004). Orton *et al*. (2011) tested the (anti-)androgenic activity of imazalil with the MDA-kb2 reporter gene bio-assay and found that the fungicide inhibited binding of DHT by 20% at a concentration of 3.23 µM. A study done by Kojima *et al*. (2004) indicated that imazalil inhibited androgen-induced activation of the AR by 20% at a concentration of 4.20 µM.

2.4.6. Imidacloprid

Imidacloprid is a neonicotinoid insecticide used to control sucking insects on crops, in soil and as a seed treatment (Gervais *et al*., 2010). In South Africa citrus, maize, apples and wheat account for 68.2%, 11.5%, 5.8% and 1.0% of imidacloprid's total use respectively (Dabrowski *et al*., 2014). Several studies have linked imidacloprid to reproductive or teratogenic effects such as the study conducted by Bal *et al*. (2012) during which imidacloprid affected the reproductive organs of male rats. The pesticide decreased the mass of accessory sex organs, levels of testosterone and sperm concentration through several mechanisms. No information could be found regarding potential (anti-)androgenic activity of imidacloprid but the pesticide has been included in the final list of initial pesticide active ingredients to be screened under the US EPA Endocrine Disruptor Screening Program (EDSP) (US EPA, 2009a; Gervais *et al*., 2010).

2.4.7. Propiconazole

Propiconazole is one of the azole fungicides used in agricultural practices to control fungal growth on wheat and barley as well as on other vegetables and fruit (*e.g*. mangos) (Skolness *et al*., 2013). This pesticide has furthermore been classified by the EU Commission as a Category 3 EDC since there is not enough evidence to either substantiate or disapprove its potential ED properties (EUC, 1999; Matisova & Hrouzkova, 2012). However, studies conducted by both Kojima *et al*. (2004) and Kjærstad *et al*. (2010) indicated that propiconazole inhibited androgen-induced activation of the AR of Chinese hamster ovary (CHO) cells at concentrations of 6.20 µM and 15 µM respectively. The pesticide also showed to have additive anti-androgenicity when tested in combination with two other azole fungicides (*e.g*. epoxiconazole and tebuconazole) (Kjærstad *et al*., 2010). Studies have also proven that propiconazole inhibits testosterone production (Kjærstad *et al*., 2010; Skolness *et al*., 2013).

2.4.8. Simazine

Simazine is also included in the triazine chemical group along with atrazine (Section 2.4.2.) and used as a pre- and post-emergence herbicide to control annual grasses and broad-leaf weeds in citrus, pomefruits, grapes, chickpeas and canola crops (NHMRC, NRMMC, 2011). In South Africa simazine is mainly used on maize (68.2%), grapes (13.8%), citrus (4.2%) and apples (0.6%). The pesticide had also been found at relatively high levels, together with atrazine and terbuthylazine, in the country's freshwater sources, especially in areas where maize was cultivated on large scale (Dabrowski *et al*., 2014; Du Preez *et al*., 2005). Exposure to simazine is mainly due to residue on food and swimming pools when used as an algicide (NHMRC, NRMMC, 2011) but due to its relatively high mobility it also pollutes natural water sources (Dabrowski *et al*., 2014).

Simazine has been classified by the EU Commission as a Category 2 EDC because of its potential ED properties (EUC, 1999; Matisova & Hrouzkova, 2012). Studies reported simazine to induce aromatase activity in human adrenocortical carcinoma cells and thereby increase oestrogen production (Sanderson *et al*., 2000; Sanderson *et al*., 2001). In a study by Orton *et al*. (2009) simazine had a significant androgenic effect in the anti-androgenic test during which the pesticide increased the background testosterone response but did not activate the AR in the absence of testosterone. No more information regarding the (anti)androgenic activity of simazine was discovered.

2.4.9. Terbuthylazine

Terbuthylazine is another triazine and is a herbicide used as a pre- and post-emergent application to control weeds in a variety of crops and in the forestry industry. It can also be used as an algicide (NHMRC, NRMMC, 2011). In the South African context, terbuthylazine is mainly used on maize and to a lesser extent on grapes and sugar cane (Dabrowski *et al*., 2014). Terbuthylazine has been substituting atrazine that was banned in many international countries due to its negative health effects (Dolaptsoglou *et al*., 2007). Studies have shown terbuthylazine to have some ED properties such as the slight induction of aromatase activity in human JEG-3 cells (Kjeldsen *et al*., 2013) but no literature could be found on the (anti)androgenic activity of terbuthylazine with regards to AR agonism or antagonism.

2.4.10. Thiabendazole

Thiabendazole is registered as a systemic benzimidazole fungicide that is commonly used in agricultural practices to control various fungal diseases (*e.g*. mold, rot, blight, and stains) on fruits and vegetables. The pesticide can be used as a post-harvest treatment on citrus, mangos, avocados, apples, bananas and pears, amongst others (US EPA, 2002).

Thiabendazole has been reported to show oestrogenic activity (Kojima *et al*., 2004) but no studies were discovered regarding the potential (anti-)androgenic activity of thiabendazole.

2.5. MDA-kb2 reporter gene bio-assay

The development and basic principle of the MDA-kb2 reporter gene bio-assay are discussed in this section as well as the benefits and disadvantages of the assay as a screening tool.

2.5.1. Development and principle of the MDA-kb2 reporter gene bio-assay

The MDA-kb2 cell line is a stable cell line derived from the MDA-MD-453 human breast cancer cell line and expresses high quantities of the AR. During normal androgen dependant gene transcription the androgen hormone binds to the AR whereafter the AR-hormone complex moves to the nucleus and binds to the androgen responsive element on the DNA. Subsequent target gene expression and protein synthesis take place (Janosek *et al*., 2006). However, the MDA-kb2 cells have been transformed with the firefly luciferase reporter plasmid which is driven by the MMTV promoter (androgen-responsive and very robust) (Wilson *et al*., 2002). The firefly luciferase system of the *Photinus pyralis* firefly species is bioluminescent (emits light as a result of an enzyme catalysed chemical reaction), has been studied intensively, are effective and commercially available (Nguyen *et al*., 1988; Leitão & Da Silva, 2010).

Since the AR can activate the MMTV promoter the *in vitro* MDA-kb2 reporter gene bio-assay can be used to screen for and semi-quantify ligands of the AR. In the case where androgen-like compounds are present in a sample the compounds will enter the cell, bind to the AR which will activate the MMTV promoter. Expression of the luciferase enzyme will take place (positive response). Luciferase catalyse the ATP-dependent oxidation of the substrate, luciferin. The total reaction can be described as follows (Nguyen *et al*., 1988; Leitão & Da Silva, 2010):

$$\text{Luciferin} + \text{ATP} + O_2 \rightarrow \text{AMP} + \text{PPi} + CO_2 + \text{oxyluciferin} + h\nu$$

The emitted light is measured as relative light units (RLUs) with a luminometer. The RLUs of a sample can be compared to that of a positive control (*e.g*. testosterone) whereafter the results can be quantified (Chapter 3). Since this *in* vitro assay is based on competitive binding and the activation of the AR, antagonistic activity can be measured by initially exposing the cells to an androgen such as testosterone before adding the sample. Anti-androgens will then block the binding of the androgen and the decrease in light emission (RLUs) can be measured and quantified (negative response) (Ermler *et al*., 2010; Wilson *et al*., 2002).

2.5.2. Benefits and disadvantages of the MDA-kb2 reporter gene bio-assay as a screening tool

The *in* vitro MDA-kb2 reporter gene bio-assay can be used as a rapid, sensitive and cost effective screening tool to screen for the presence of low concentrations of androgen agonists and antagonists in complex chemical mixtures (Aït-Aïssa *et al*., 2010; Blake *et al*., 2010; Ermler *et al*., 2010; Nguyen *et al*., 1988; Wilson *et al*., 2002). The cell line is also stable and can be used to identify the mechanism of action of chemical compounds since it discriminates between androgen agonists and antagonists (Wilson *et al*., 2002). The bio-assay has also been refined over the past years. Magnesium has for example been added to the reagent buffer (containing the substrate luciferin) which prevents disruption of some nuclear structures (Nguyen *et al*., 1988) and act as a co-enzyme during the luminescent reaction (Ignowski *et al*., 2004). Co-enzyme A has been added to slow the light emission decay down since some of the reaction products (*e.g*. oxyluciferin and L-AMP) inhibits luciferase activity and subsequently inhibits light emission (Leitão & Da Silva, 2010). This biological assay can also be used to measure the response towards chemical mixtures which is not possible with chemical analysis alone (Blake *et al*., 2010).

The bio-assay can only be used as a screening tool to determine the presence of AR agonists or –antagonists in a mixture and not to identify the specific compounds. It must therefore be used in combination with analytical chemistry analysis and even with *in vivo* tests to identify, characterise and quantify potential toxic compounds (Blake *et al*., 2010). Risk assessments and human exposure can also only be done when the biological assays are integrated with analytical techniques (Elskens *et al*., 2011).

As mentioned earlier the MDA-kb2 cells also express high levels of endogenous GRs. The GR also has the ability to activate the MMTV promoter if GR agonists are present in a sample (Wilson *et al*., 2002). The presence of the GR may therefore pose some difficulties when screening for androgens but not when analysing samples for anti-androgens (Blake *et al*., 2010). Wilson *et al*. (2002) reported that hydroxyflutamide (AR antagonist) can be used to determine which steroid receptor was activated but in this study the AR inhibition (anti-androgenic) results of the reporter gene bio-assay was used as the control (Chapter 3).

2.6. Legislative quality guidelines

A short background is presented in this section regarding the water and sediment quality guidelines that were used in this study for comparison with the chemical analysis results.

2.6.1. Water quality guidelines for pesticides

Water is a basic life-sustaining necessity and therefore has the potential to influence human, animal and environmental health if the quality is not in a desirable state. According to the WHO the lifetime consumption of safe drinking water does not pose significant health risks, even during potentially sensitive life stages (WHO, 2011). It is the responsibility of every governing authority to develop and incorporate effective policies to ensure water quality that is safe for the intended use (*e.g*. domestic use or agricultural use). This may, in part, be accomplished with the development and implementation of water quality guidelines (or 'standards', 'indicators' or 'limits') that may vary according to each country's needs and capacity (WHO, 2011).

In 2005 London *et al*. (2005) reported that South Africa does not have suitable regulatory legislation in terms of pesticide pollution of the country's water resources. This is still the case since the drinking water quality guidelines as well as the water quality guidelines for the protection of aquatic ecosystems (developed by the Department of Water Affairs and Forestry; DWAF 1996a; DWAF 1996b) have only one standard for a pesticide and that's for atrazine. Since South Africa is one of the prolific users of pesticides in sub-Saharan Africa (Dalvie *et al*., 2009; London *et al*., 2005; Quinn *et al*., 2011) this poses a major concern as it complicates monitoring of pesticides in surface and groundwater. Furthermore, pesticide pollution may potentially lead to severe public health problems. Monitoring of the country's water resources is further complicated by factors such as the high costs associated with pesticide analyses and analytical equipment and a lack in laboratory skills, amongst others (London *et al*., 2005).

2.6.2. Sediment quality guidelines for pesticides

Sediment plays a significant role in the food web in terms of bioaccumulation and trophic transfer of contaminants (Burton, 2002) and may also aid in the re-suspension of contaminants back into the surface water (Long *et al*., 1998). Micro-organic contaminants (with a largely hydrophobic nature) as well as other nutrients, pathogens and metals can adsorb onto inorganic and organic particles which may lead to the subsequent accumulation of pollutants in bed sediment (Long *et al*., 1998; Öztürk *et al*., 2009; Praveena *et al*., 2007). Since sediments have a long retention time within water systems, they can be a useful tool in assessing the presence and toxicity of pollutants in aquatic ecosystems such as rivers and dams (Chakravarty & Patgiri, 2009). When contaminants accumulate in sediments the desorption-rates decrease and biotransformation is less likely to follow which is one of the reasons why sediments are now regarded as a diffuse source of pollution to surrounding ecosystems (Burton, 2002). Under the correct environmental conditions sediments may aid in the mobilization of contaminants back into other ecosystems (*e.g*. air, surface and ground

water, land and biota) since all of these compartments are interconnected (Burton, 2002; Öztürk *et al*., 2009, Warren *et al*., 2003).

The assessment of the toxicity of pollutants in sediment is a challenging task since several factors influence the pollutants' availability. Some compounds are only associated with the pore water (that is difficult to sample in sufficient volumes for analysis) while other compounds are sediment bound (Warren *et al*., 2003). For this study only bed sediment was sampled and analysed for the target compounds.

Only a few countries have been able to develop SQGs to assist regulators in managing sediment quality. The complexity of sediment and mixture interactions together with the high expenses in terms of money, effort and resources, associated with sediment quality management, confound the process of developing these criteria (Burton, 2002; MacDonald *et al*., 2000; WHO, 2011). The internationally available SQGs can be used as a screening tool in combination with other toxicity tests and environmental assessments in order to evaluate and determine sediment quality. Since several types of SQGs exist, choosing the correct one is difficult and the choice depends on the purpose of the assessment (MacDonald *et al*., 2000). South Africa does not have SQGs for the priority pesticides analysed in this study. The available international SQG values were compared to the detected priority pesticide levels in the sediment from all three study areas in order to get an idea of whether regulatory limits were exceeded even if these units were developed for a different country. The lack of applicable SQGs made a thorough comparative study impossible and therefore further emphasized the need for sediment quality regulatory research specifically in a South African context.

CHAPTER 3: MATERIALS AND METHODS

3.1. Introduction

Surface and borehole water as well as sediment were sampled in three catchments located in Limpopo, the Free State and Mpumalanga. Samples were collected and analysed approximately four times a year in order to take the influence of high and low flow seasons into consideration. The samples were chemically extracted according to accredited methods, whereafter chemical analyses were conducted to test for the presence of current use pesticides. The samples were tested using the MDA-kb2 reporter gene bio-assay to screen for (anti)androgenic activity.

3.2. Study areas and site selection

3.2.1. Letsitele River catchment

The Letsitele River catchment is situated in the Limpopo Province and forms part of the Great Letaba River catchment which is drained by the Great Letaba River and its major tributaries the Thabina, Letsitele, Politsi and Debengeni rivers. The Letsitele River arises in the Wolkberg Mountains and flows in a north easterly direction until it joins the Great Letaba River just upstream of the town of Letsitele (Figure 3.1). The upper reaches of the Letsitele River catchment primarily consist of large scale commercial forestry and are characterised by relatively steep slopes that may result in high runoff rates of pesticides. Intensive irrigated agriculture of fruits such as mangos, citrus and avocados occur farther downstream along the Letsitele River. The commercial farming occurs in close proximity to several human settlements, domestic livestock and subsistence farming which may all be exposed to potential spray drift and consequent increased health risk. The main urban areas adjacent to the Letsitele River are Mogoboya, Khujwana (which are both close to orchards), Dan, and Nkowakowa. The main soil type of the catchment is sandy clay loam with a relatively large percentage of sand, which increases potential leaching of agrochemicals (*e.g*. pesticides and fertilisers) (Arias-Estévez *et al*., 2008).

Initially 10 sites were selected in the Letsitele River catchment in a preliminary survey of agrochemical pollution (Figure 3.1; Table 3.1). Sites LT3, LT7, and LT9 were selected in the main stem of the Letsitele River since they were regarded as sites with potential high usage for domestic and agricultural purposes. Sites LT1 and LT10 are located in tributaries of the Letsitele River which are surrounded by large scale agricultural activities. Site LT8 was selected in the Great Letaba River upstream of the town of Letsitele. One site (LT2) was in the Murle Brook Dam and another three sites (LT4, LT5 and LT6) were from drinking water taps to determine if pesticides with ED properties occurred in the potable water supply. Site

LT4 is located at a primary school nearby the community of Mogoboya while sites LT5 and LT6 are taps in the Khujwana community. Site LT5 was not sampled again after the first sampling trip and therefore no results for the site are presented in this dissertation. Three borehole sites (BM, SL, and FM) were selected for the purpose of identifying pesticides in the groundwater used for domestic and agricultural purposes.

Table 3.1: Location and brief descriptions of sampling sites selected in the Letsitele River catchment.

Site	GPS co-ordinates	Site description
LT1	23°94'26''S 30°19'14''E	Tributary of the Letsitele River at the intersection with the Old Coach Road.
LT2	23°95'02''S 30°18'85''E	Murle Brook Dam.
LT3	23°97'46''S 30°16'51''E	Most upstream site in the Letsitele River.
LT4	23°97'46''S 30°16'48''E	Tap water at a primary school next to site LT3.
LT6	23°97'80''S 30°20'60''E	Tap water in the Khujwana community.
LT7	23°94'07''S 30°21'50''E	Middle site in the Letsitele River.
LT8	23°86'56''S 30°39'17''E	Site in the Great Letaba River.
LT9	23°92'46''S 30°26'71''E	Most downstream site in the Letsitele River.
LT10	23°91'58''S 30°22'22''E	Next to a fruit packing shed in a tributary of the Letsitele River.
BM	23°90'58''S 30°21'08''E	Borehole site near a tributary of the Letsitele River.
SL	23°91'56''S 30°22'09''E	Borehole site near a tributary of the Letsitele River.
FM	23°92'79''S 30°21'63''E	Borehole site near a tributary of the Letsitele River.

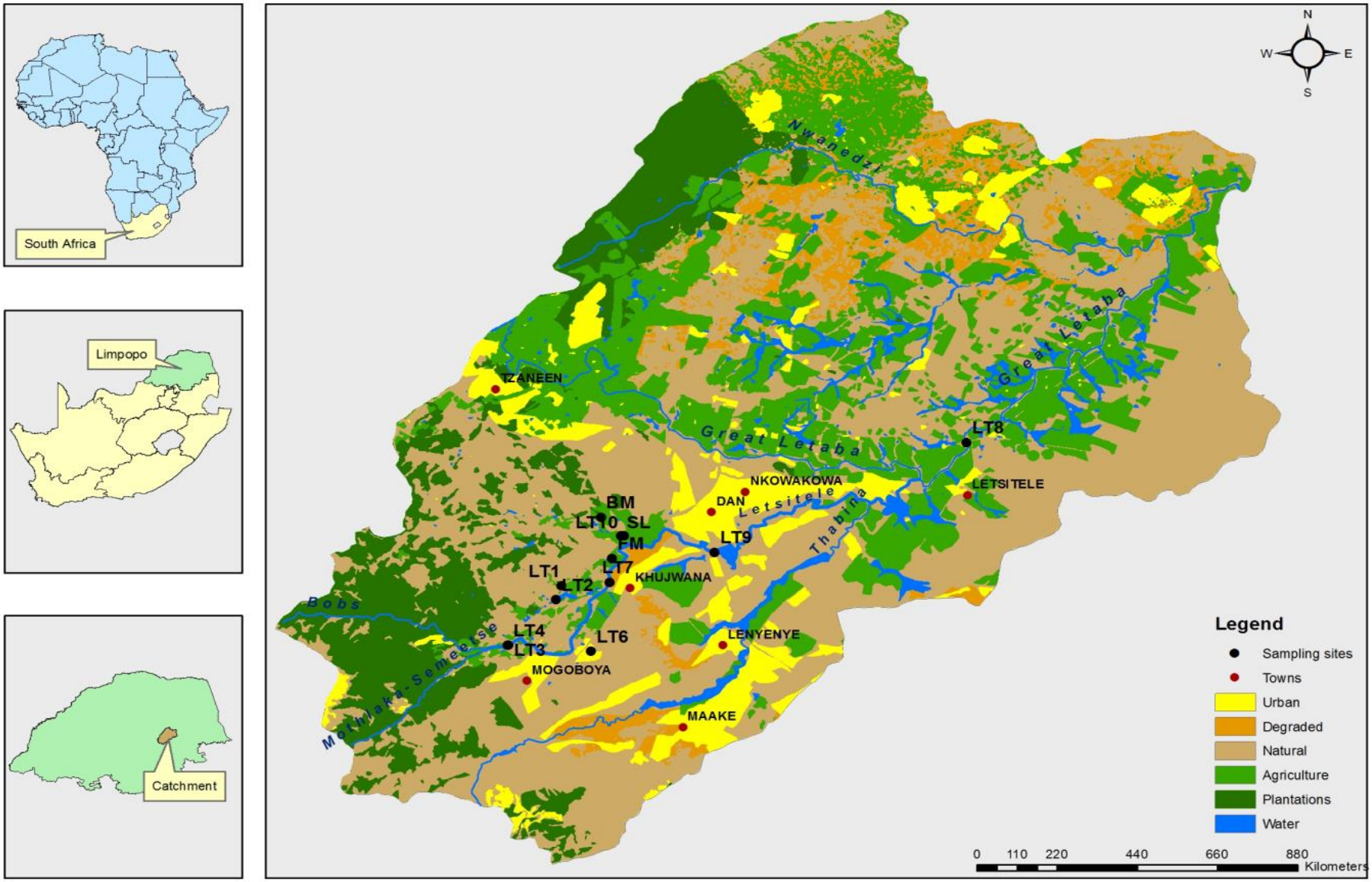

Figure 3.1: Land cover of the Letsitele River catchment and the locations of the sampling sites.

The first sampling event in July 2011 was a preliminary event for the purpose of site selection and chemical screening. The subsequent sampling events took place in November of 2011, August and November of 2012 as well as in February and September of 2013. The samples were chemically screened for the presence of the 291 pesticides (Table A–1) and the priority pesticides were also quantified through a chemical quantification analysis. The samples were then biologically analysed with the MDA-kb2 reporter gene bio-assay to test for (anti)androgenic activity (Table 3.2).

Table 3.2: Summary of sampling events as well as sites that were sampled and analysed in the Letsitele River catchment (indicated by 'X').

Site	Pesticide analysis				MDA-kb2 reporter gene bio-assay	
	Water		Sediment		Water	Sediment
	Screening	Quantification	Screening	Quantification		
July 2011						
LT1	X		X		X	X
LT2	X		X		X	X
LT3	X		X		X	X
LT4	X				X	
LT6	X				X	
LT7	X		X		X	X
LT8	X		X		X	X
LT9	X		X			X
LT10	X		X			x
November 2011						
LT1	X		X		X	X
LT2	X		X		X	X
LT3	X	X	X	X		X
LT4	X	X			X	
LT6	X	X			X	
LT7	X	X	X	X	X	X
LT8	X		X		X	X
LT9	X	X	X	X	X	X
LT10	X				X	
August and November 2012, February 2013						
LT2		X		X	X	X
LT3		X	X	X	X	X
LT4	X	X			X	X
LT9	X	X	X	X	X	X
LT10		X			X	
September 2013						
BM		X			X	
SL		X			X	
FM		X			X	

3.2.2. Renoster and Vals River catchments

The Renoster and Vals River catchments form part of the Vaal River system, falling mainly within the Middle Vaal Water Management Area, in the Free State. Predominant land-use in the catchments is the large scale cultivation of maize and sorghum, and sunflowers to a lesser extent. Land gradient is relatively flat, especially in the lower regions, and the soil has a high proportion of sand which increases potential leaching of agrochemicals (*e.g*. pesticides and fertilisers) (Arias-Estévez *et al*., 2008). Both the Renoster and Vals Rivers are used as the primary water sources for domestic and agricultural purposes for several urban settlements within the catchments.

The Renoster River originates in an intensive agricultural area between Heilbron and Petrus Steyn in the Free State and flows in a north westerly direction through Koppies Dam until it confluences with the Vaal River just downstream of the town of Viljoenskroon (Figure 3.2; Table 3.3). Three sites (RN1, RN3 and RN4) were selected in the main stem of the Renoster River. The Vals River originates in the south eastern regions of the Free State and flows in a westerly direction through Lindley, Kroonstad and past Khotsong and Bothaville before it joins the Vaal River downstream of its confluence with the Renoster River. Kroonstad is a relatively large city with several potential sources of pollution (*e.g*. sewage outlets and various industries). Two sites (VL2 and VL3) were selected in the main stem of the Vals River and one site (VL1) in the Blom Spruit tributary.

Only samples RN4 and VL3 were subjected to chemical screening as these sites are at the most downstream point in each of the rivers, therefore providing the most representative indication of pesticide use in the respective catchments. Water samples were also collected from three borehole sites (KSD, KOP and VJK) in October 2013. All of the samples were chemically and biologically analysed and quantified (Table 3.4).

Table 3.3: Location and brief descriptions of sampl-ing sites selected in the Renoster and Vals River catchments.

Site	GPS co-ordinates	Site description
RN1	27°29'38''S 27°78'97''E	Upstream of Koppies Dam in the Renoster River.
RN3	27°23'33''S 27°32'45''E	Downstream of Koppies Dam and the town of Koppies where the R721 crosses the Renoster River.
RN4	27°07'17''S 27°04'43''E	Site in the Renoster River approximately 16 km upstream from the Renoster-Vaal River confluence.
VL1	27°89'07''S 27°37'65''E	Site in Blom Spruit, a tributary of the Vals River, upstream of the town of Kroonstad.
VL2	27°61'09''S 27°10'20''E	Site in the main stem of the Vals River, downstream of the town of Kroonstad.
VL3	27°49'01''S 26°65'61''E	Site in the main stem of the Vals River, upstream of the town of Bothaville.
KSD	27°28'82''S 27°62'73''E	Borehole site in the Renoster River catchment.
KOP	27°23'59''S 27°23'59''E	Borehole site in the Renoster River catchment.
VJK	27°20'80''S 26°95'55''E	Borehole site in the Renoster River catchment.

Table 3.4: Summary of sampling events as well as the sites that were sampled and analysed in the Renoster and Vals River catchments (indicated by 'X').

Site	Pesticide analysis				MDA-kb2 reporter gene bio-assay	
	Water		Sediment		Water	Sediment
	Screening	Quantification	Screening	Quantification		
July and October 2012, January and April 2013						
RN1		X		X	X	X
RN3		X		X	X	X
RN4	X	X	X	X	X	X
VL1		X		X	X	X
VL2		X		X	X	X
VL3	X	X	X	X	X	X
October 2013						
KSD		X			X	
KOP		X			X	
VJK		X			X	

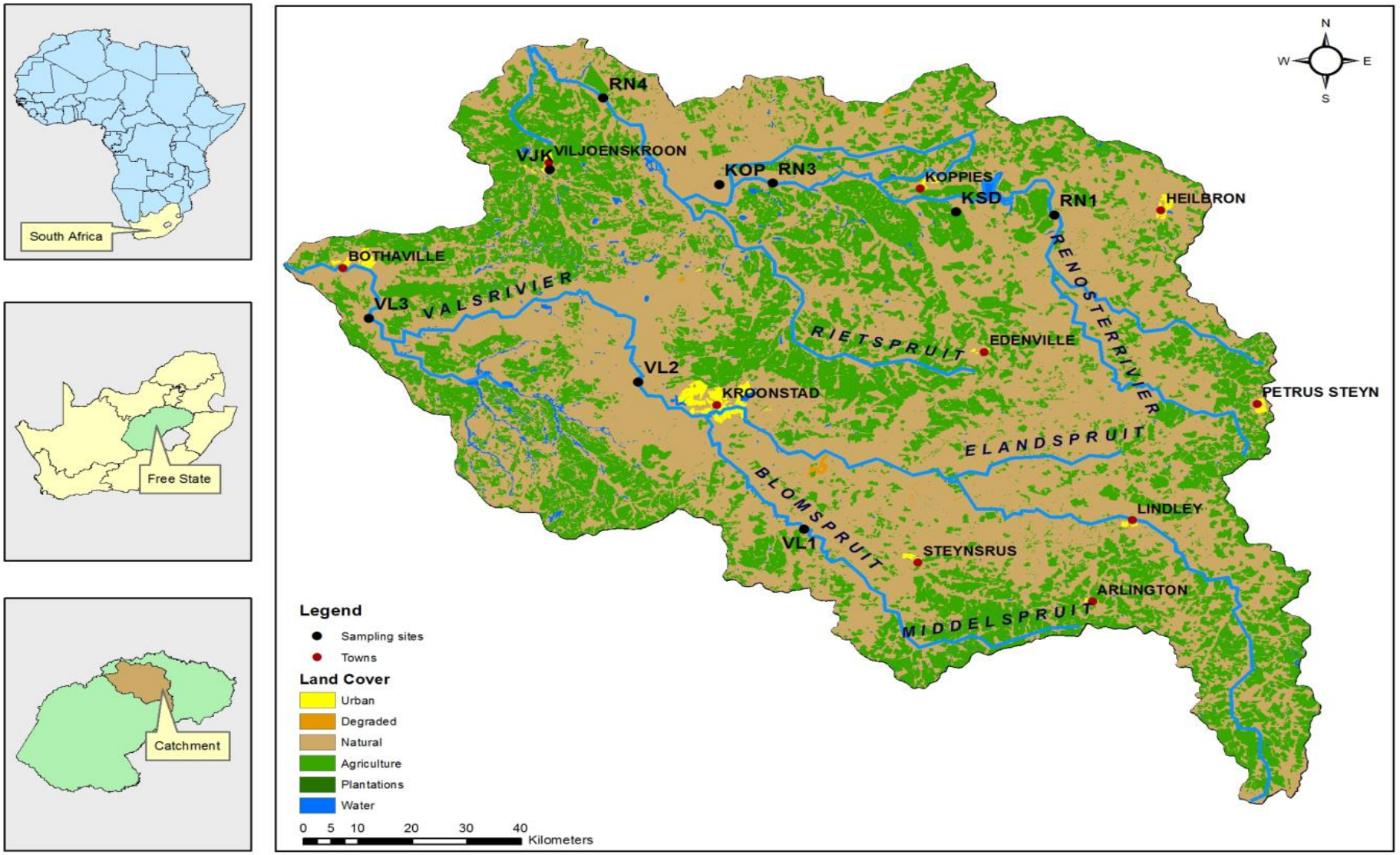

Figure 3.2: Land cover of the Renoster and Vals River catchments and locations of the sampling sites.

3.2.3. Mzinti, Lomati and Ngweti River catchments

The Komati River catchment forms part of the larger Inkomati River Basin which stretches over the eastern regions of Mpumalanga, northern parts of Swaziland and southern reaches of Mozambique. The Komati River originates in the Drakensberg mountain range west of the town of Carolina in Mpumalanga and flows in a north easterly direction until it joins the Crocodile River just upstream of the Mozambique border. The Komati River catchment is drained by several major tributaries including the Mzinti River, Lomati River (also known as the Mlumati River) and the Ngweti River (Figure 3.3). Intensive commercial and irrigated cultivation of sugarcane and citrus occurs on agricultural land which can generally be characterised by low to medium gradient slopes. The soil type in the catchment is primarily sandy which increases potential leaching of agrochemicals (*e.g*. pesticides and fertilisers) (Arias-Estévez *et al*., 2008). Other major crops such as bananas, litchis, mangoes, papaya as well as maize and wheat are also produced in the area on a smaller scale (Van der Laan *et al*., 2012).

The Lomati River also rises in the Drakensberg Mountains in the most northern reaches of Swaziland and flows in a north easterly direction through Driekoppies Dam after which it forms a confluence with the Komati River just downstream of the town of Phiva. Several human settlements occur adjacent to the river and in close proximity to agricultural activity. Four sampling sites (NK1, NK2, NK3, and NK6) were selected within the Lomati River catchment (Figure 3.3; Table 3.5). At site NK3 water samples were also taken from a tap at the Langeloop Water Purification Works, which purifies water from the Lomati River for distribution and subsequent domestic use within the town.

In the Ngweti River a site (NK4) was selected downstream of large scale agricultural activity. Site NK5 in the Mzinti River was expected to be the least impacted site with regards to agrochemicals because it is located downstream and directly outside of the Mahushe Shongwe Provincial Reserve. The borehole sites (RH, KC, and GW) were selected within a region with intensive agricultural activity. The water samples from these sites were used to identify potential pesticides that occurred in groundwater used for domestic and agricultural purposes. Sampling of water and sediment at all of the sites took place during high and low flow seasons (Table 3.6).

Table 3.5: Location and brief descriptions of sites selected in the Lomati, Mzinti and Ngweti River catchments.

Site	GPS co-ordinates	Site description
NK1	25°65'22''S 31°54'11''E	Site in the Mhlabanyatsi River, a tributary of the Lomati River.
NK2	25°68'45''S 31°53'86''E	Site in the upper Lomati River, downstream of Driekoppies Dam and the town of Schoemansdal.
NK3	25°67'15''S 31°63'99''E	Middle Lomati River, adjacent to the town of Langeloop at the intake point of Langeloop Water Purification Works.
NK3-TAP	25°67'22''S 31°63'94''E	Tap water at the Langeloop Water Purification Works.
NK4	25°44'99''S 31°94'44''E	Lower most site in the Ngweti River, downstream of intensive agricultural activity.
NK5	25°69'24''S 31°73'26''E	Site in the lower reach of the Mzinti River.
NK6	25°63'66''S 31°76'04''E	Lower most site in the Lomati River, at a weir just upstream of Phiva town.
RH	25°44'55''S 31°96'55''E	Borehole site in the Komati River catchment.
KC	25°44'66''S 31°96'40''E	Borehole site in the Komati River catchment.
GW	25°48'39''S 31°96'64''E	Borehole site in the Komati River catchment.

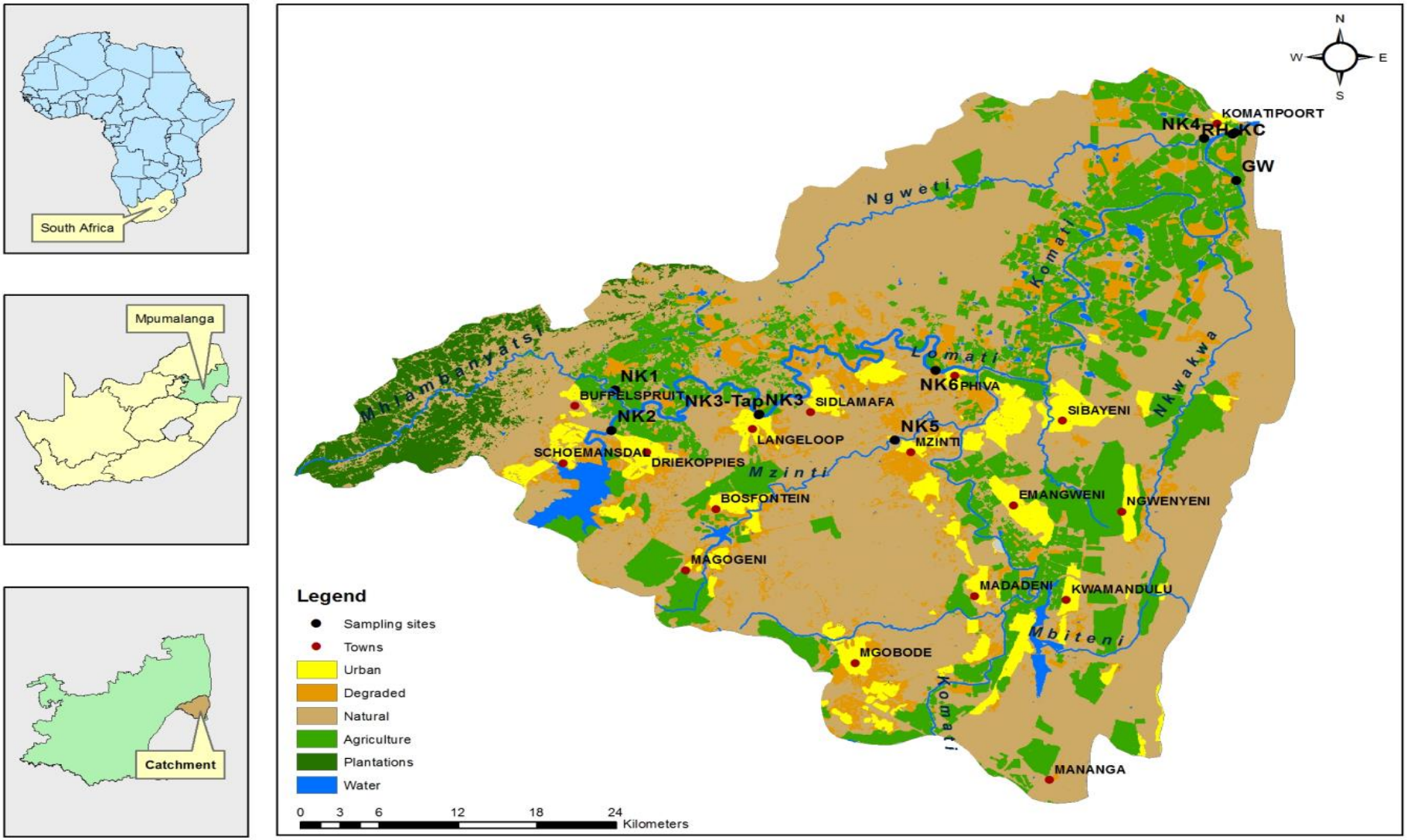

Figure 3.3: Land cover of the Mzinti, Lomati and Ngweti River catchments and locations of the sampling sites.

Table 3.6: Summary of sampling events as well as the sites that were sampled and analysed in the Mzinti, Lomati and Ngweti River catchments (indicated by 'X').

Site	Pesticide analysis				MDA-kb2 reporter gene bio-assay	
	Water		Sediment		Water	Sediment
	Screening	Quantification	Screening	Quantification		
June 2012						
NK1	X	X		X	X	X
NK2	X	X		X	X	X
NK3	X	X		X	X	X
NK3TAP	X	X			X	
NK4	X	X				
NK5	X	X		X	X	X
NK6	X	X	X	X	X	X
September and December 2012, March 2013						
NK1	X	X		X	X	X
NK2	X	X		X	X	X
NK3	X	X		X	X	X
NK3TAP	X	X			X	
NK4	X	X		X	X	X
NK5	X	X		X	X	X
NK6	X	X	X	X	X	X
November 2013						
RH		X			X	
KC		X			X	
GW		X			X	

3.3. Sampling and chemical analysis

Surface water and composite sediment samples were collected concurrently during each sampling event. Groundwater samples were only collected once from each study area (Tables 3.2, 3.4 and 3.6). Chemical extraction commenced as soon as was possible after initial collection. The chemical extraction, screening and quantification were conducted by LiquidTech Laboratories at the University of the Free State (see www.liquidtech.co.za for more information). An overview of the methods used is provided below.

3.3.1. Water sampling and extraction

Water samples were collected approximately 50 cm below surface in 1 ℓ glass Schott bottles which were wrapped in aluminium foil to prevent ultraviolet (UV) radiation breakdown of chemicals (Kuster *et al*., 2007). The samples were transported on ice and stored at -4°C (De Jager *et al*., 2011) until extraction could commence (which occurred within 48 hours of initial collection). The water samples were filtered through glass fibre filters to remove particulate matter where after they were cleaned-up on methanol (MeOH) conditioned C18 (6 mℓ) solid

phase extraction (SPE) cartridges (Strata, Phenomenex), set to a flow rate of 5 mℓ/min. Two millilitres of MeOH followed by 2 mℓ of ethyl acetate were used to slowly elute the bound samples from the dried cartridges. The eluent was vacuum-dried with the use of a Thermo Scientific Savant Speedvac and reconstituted in 1 mℓ purified water for use in further chemical analyses (method adapted from Method 3535A; US EPA, 2007b).

3.3.2. Sediment sampling and extraction

Composite sediment samples were collected in bulk at each site in zones where low flow causes sediment build-up. A stainless steel cup and dish were used for collection where after the homogenous sample mixture was transferred to a 250 mℓ high density polyethylene bottle (Nalgene, Thermo Scientific). The samples were transported on ice and stored at -20°C until chemical extraction and analyses could commence. The extraction method was adapted from a method approved by the US EPA (Method 3540C; US EPA, 1996). During extraction 5 g of freeze-dried sample was refluxed with 50 mℓ MeOH for 60 minutes in a cellulose thimble with a Soxhlet apparatus. The excess solvent was removed under reduced pressure and the sample extract was subsequently transferred to a vial and reconstituted in 1 mℓ MeOH.

3.3.3. Chemical analysis

The chemical analyses of the water and sediment samples were conducted with the use of an AB SCIEX 3200 QTRAP hybrid triple quadrupole ion trap mass spectrometer with an Agilent 1200 SL high performance liquid chromatography (HPLC) stack as front end. The samples were first screened for the presence of 291 pesticides (Table A–1) after which a targeted analysis was done during which selected detected pesticides were quantified (*C*hapter 4). The Analyst 1.5 (AB SCIEX) software was used to acquire and process the data. The screening and quantification methods that were used are similar to a method described in Zhang *et al*. (2012).

3.3.3.1. Screening analysis for unknown compounds

Initially a volume of 20 µℓ of each sample was separated on a C18 (150 mm x 4.6 mm, Gemini NX, Phenomenex) column at a flow rate of 300 µℓ/min. In positive ionisation mode a 30 minute gradient was used which included 5% of solvent A (H_2O/0.1% formic acid) to 95% of solvent B (MeOH/0.1% formic acid). In negative ionisation mode a 30 minute gradient from 5% solvent A (H_2O/10 mM NH_4OH) to 95% solvent B (MeOH/10 mM NH_4OH) was used. The total run time for each of the modes was 45 minutes which allowed for column re-equilibration. Electrospray in the TurboV ion source ionised the eluting analytes. Additional settings include a heater temperature of 500°C which ensured evaporation of excess solvent; 50 psi nebuliser gas, 50 psi heater gas, 25 psi curtain gas; an ion spray voltage of 5 500 V in positive ionisation mode and -4 500 V in negative ionisation mode.

The LC/MS/MS system that was used for the detection of unknown pesticides followed a multi-targeted MRM-IDA-EPI workflow. The pesticides that were screened for were divided into two groups which depended on their ionisation abilities. For each pesticide present in the sample one MRM (multiple reaction monitoring) was used to trigger fragmentation and fragment collection. The resultant fragmentation profiles were compared to a library containing the MS/MS fragmentation spectra of numerous current use pesticides.

3.3.3.2. Quantitative analysis

Quantification of selected pesticides that were detected during the unknown screening included 20 µℓ of each sample separated on a C18 (150 mm x 4.6 mm, Gemini NX, Phenomenex) column at a flow rate of 300 µℓ/min. A 2 minute gradient from 5% of solvent A (H_2O/0.1% formic acid) to 95% of solvent B (MeOH/0.1% formic acid) was used with a total run time of 9 minutes which allowed for column re-equilibration. The eluting analytes were electrospray ionised in the TurboV ion source; a heater temperature of 500°C ensured the evaporation of excess solvent; 50 psi nebuliser gas, 50 psi heater gas, 25 psi curtain gas and the set ion spray voltage was 5 500 V. Two MRM transitions per analyte were used for the targeted analyses. The first and most sensitive transition resulted in a peak area on the chromatogram that was used as the quantifier while the qualifier (which confirmed the presence of the analyte) was acquired from the second transition. The retention time for the quantifier and the qualifier had to be equal.

3.3.3.3. Quality control

To ensure quality control the water and sediment samples were alternated with blank runs and interspersed with quality control samples with known concentrations to validate the instruments performance. For each analyte a 4-point calibration curve was created which ranged in concentration from 1 ppm to 0.001 ppm. A linear fit through the origin produced a correlation coefficient (*r*-value) greater than 0.98.

3.4. Sampling, processing and storage for biological analysis

3.4.1. Water samples

Surface water samples were collected at each site in 1 ℓ, clear, glass Schott bottles and preserved by adjusting the pH to 3 with concentrated Hydrochloric acid (De Jager *et al*., 2011). The bottles were wrapped in aluminium foil to protect the target compounds from UV-radiation breakdown (Kuster *et al*., 2007) while the bottle caps were lined with aluminium foil to prevent contamination.

The samples were transported at 4°C and stored at -20°C to prevent thermal and bacterial breakdown of the analytes of interest (Kuster *et al*., 2007). Since the behaviour of androgens

in solution is unclear the chemical extraction was conducted as soon as was possible (usually within week of collection) at the University of Pretoria (Section 3.5.1). The glassware and laboratory equipment were pre-washed with 100% ethanol (EtOH) (Burdick and Jackson, Honeywell) before use (Aneck-Hahn *et al*., 2008). All of the solvents used in this study were of HPLC grade unless otherwise stated.

3.4.2. Sediment samples

Sediment samples for the bio-assay analysis were collected, stored and transported to the North-West University's laboratories in the same manner as were done for the chemical analysis discussed in section 3.3.2.

Processing of the samples, once they could be processed in the laboratory, was conducted according to a method approved by the US EPA (Method 3545A; US EPA, 2007a). Frozen samples were thawed and air dried at room temperature for approximately 48 hours while analytes of interest were protected from UV-radiation breakdown. The dried sediment was ground and sieved (0.5 mm mesh size) to obtain homogenous samples. The samples were stored in a dark and dry area until the chemical extraction process could commence.

All glassware and laboratory equipment was pre-washed with phosphate-free soap and rinsed with warm water as well as double distilled water after which it was left to air dry. The dry equipment was rinsed in triplicate with analytical grade acetone and hexane (Burdick and Jackson, Honeywell) to remove all potential polar and non-polar contaminants respectively.

3.5. Extraction and clean-up procedures

3.5.1. Water samples

The surface water samples that were collected for biological analysis were chemically extracted at the University of Pretoria before the final samples were analysed for (anti)androgenic activity at the North-West University (Potchefstroom Campus). The extraction protocol was compiled by De Jager *et al*. (2011). Turbid samples were pre-filtered through glass wool filters (Macherey-Nagel) under a vacuum to remove particulate matter. Subsequent vacuum-filtration of 250–300 mℓ water at a time took place through sterile 0.45 µm (47 mm diameter) filters (Microsep). A SPE was done with a SPE manifold through C18, end capped, SPE cartridges (6 mℓ/500 mg; Chromabond, Macherey-Nagel). The conditioning of the cartridges was done with an initial 5 mℓ H_2O (18 MΩ) followed by 5 mℓ MeOH and subsequently with a final volume of 5 mℓ ddH_2O under gravitational pull. Caution was taken to prevent the sorbent bed of the cartridges to run dry. Approximately 5 mℓ of sample was passed through a cartridge under vacuum (never exceeding a pressure of 20 mmHg) at a flow rate of about 10 mℓ/min until the full 1 ℓ water sample was extracted.

Percolation by gravity of 3–5 mℓ MeOH through each cartridge eluted the remaining sample analytes. The samples were evaporated to dryness with nitrogen gas in a Reacti-vap evaporator and reconstituted with 1 mℓ MeOH into 1.5 mℓ sterile amber glass vials. The final samples were stored at -80°C until the biological analysis could commence.

3.5.2. Sediment samples

The sediment sampled for the reporter gene bio-assay was extracted at the North-West University. A chemical extraction protocol for sediment, based on a pressurized liquid extraction (PLE) method approved by the US EPA (Method 3545A; US EPA, 2007a) was used. This method was used to extract all possible water insoluble or slightly water soluble organic pollutants such as organophosphate- and organochlorine pesticides using high temperature and pressure. The method also ensures the use of less solvent and saves time in contrast to the traditional Soxhlet extraction approach. Glassware and laboratory equipment were pre-cleaned as described above (Section 3.4.2).

A Dionex Accelerated Solvent Extractor (ASE) 100 was used for the extraction. The settings for the instrument included a static time of 5 minutes, the extraction cell was purged with nitrogen gas for 100 seconds, a flush volume of 60% and a two times repetition of the extraction cycle per sample. Approximately 20 g of dried homogenous sediment was mixed with pre-cleaned anhydrous sodium sulfate (Na_2SO_4, Merck) to remove trace water molecules. The sample was subsequently compacted between two 30 mm cellulose filters in a 40 mℓ stainless steel extraction cell. The extraction was done at a temperature of 40°C and under a pressure of 1 500 psi. A mixture (3:1) of dichloromethane (DCM) (Burdick and Jackson, Honeywell) and acetone was used as the solvent. The samples were collected in TurboVap flasks and evaporated to dryness with a gentle stream of nitrogen gas.

In order to remove insoluble particles the sample extracts were subsequently subjected to a SPE process (Method 3535A; USEPA 2007b) with the use of a SPE manifold. Each sample was reconstituted with 1 mℓ DCM while Florisil cartridges (Sep-Pak Florisil 3 cc: WAT020815, Waters) were conditioned with 2 mℓ MeOH (Burdick and Jackson, Honeywell). The samples were loaded, slowly filtered by gravitation and followed by 2 mℓ DCM as eluent. The final 5 mℓ sample volume was again evaporated to dryness with nitrogen gas.

The samples were subjected to a final clean-up process in order to remove the cytotoxic sulphur from the complex chemical mixture (Hilscherova *et al*., 2000). Gel permeation chromatography (GPC) was used for this purpose which is a HPLC system essentially based on the principle of size exclusion. The system consists of a Waters 717 plus auto-sampler, Waters 1515 isocratic HPLC pump, Waters 2487 dual λ absorbance detector, Waters fraction collector III as well as two Envirogel GPC clean-up columns (19 mm x 150 mm and 19 mm x

300 mm) which were connected in series and packed with high performance, porous polymeric gel particles (Munoz *et al*., 2012).

The GPC system was calibrated with a standard solution that contained corn oil (Sigma-Aldrich) as well as bis(2-ethylhexyl)phthalate, metoxychlor, perylene and sulphur (PESTANAL, Sigma-Aldrich). The mixture was made up to a final volume of 2 mℓ in DCM. Calibration was done to determine the retention time of each compound based on its known size which enabled the exclusion of sulphur from the sample extract

The development of the protocol was based on a GPC method approved by the US EPA (Method 3640A; US EPA, 1994). The dry sample extracts were reconstituted with 2 mℓ DCM where after it was filtered through 1 µm Acrodisc glass fibre syringe filters (Pall), which were attached to luer lock glass syringes, into recovery vials. The samples were injected into the GPC system and eluted with DCM at a flow rate of 5 mℓ/min for 30 minutes (collection time: 0–20 minutes). The collected samples were evaporated to dryness with nitrogen gas and reconstituted to 1 mℓ with MeOH which was used as the final sample extracts in the reporter gene bio-assay at the North-West University.

3.6. Biological analysis

3.6.1. Maintenance of the MDA-kb2 cell culture

The MDA-kb2 cell line (ATCC #CRL-2713) was a gift from Prof. J.P. Giesy, in 2003, then from the Michigan State University in the USA. The development of the protocol for initiation, maintenance, passaging and dosing of the MDA-kb2 cells was based on a method established by Wilson *et al*. (2002). Culturing and maintenance of the cells occurred in a sterile environment as described by Roberts (2011). All of the surfaces and instruments in the cell culture laboratory were cleaned with 70% EtOH prior to and after working with the cells. The incubator was fitted with a high efficiency particulate air (HEPA) filter to remove bacterial and viral cells from the inflow air. The interior surfaces of the laminar flow cabinet were sterilised at least once a week with UV-radiation.

The MDA-kb2 cells were cultured in 60.1 cm^2 tissue culture dishes (TPP, Separations). An aliquot of 1 mℓ thawed cells were initially added to 12 mℓ of L-15 (Leibovitz), phenol red containing medium (Gibco, Sigma Aldrich), which was supplemented with 20% foetal bovine serum (FBS) (HyClone, Separations). FBS contains essential minerals, lipids, hormones, transport proteins as well as attachment-, growth- and spreading factors necessary for the *in vitro* culturing of animal cells (Freshney, 2005; Van der Valk *et al*., 2004). The cells were maintained with L-15 medium supplemented with 10% FBS. Used media were replaced with fresh media 2–3 times per week. The cells were cultured at a temperature of 37°C in a

humidified atmosphere with no CO_2 supplementation. When the dishes were 70% confluent the cells were passaged. They were washed in triplicate with Dulbecco's phosphate buffered saline (PBS; Sigma Aldrich), which contained no added salts. This was done to remove cell debris before they were treated with 1.5 mℓ trypsin (HyClone, Separations) to separate them from the bottom of the plate allowing for sub-culturing.

3.6.2. MDA-kb2 reporter gene bio-assay

All of the sediment and water samples, chemically extracted for this purpose, were subjected to the 5-day, *in vitro* MDA-kb2 reporter gene bio-assay to screen them for their ability to activate the AR (androgenic activity) (Blake *et al*., 2010) or inhibit androgen binding to the AR (anti-androgenic activity) (Aït-Aïssa *et al*., 2010; Ermler *et al*., 2010).

To initiate the screening for androgenic activity the cultured MDA-kb2 cells were visually inspected under a phase contrast microscope for confluency and signs of cytotoxicity (*e.g*. detachment, vacuolisation and membrane degradation) (Wilson *et al*., 2002). MDA-kb2 cells were seeded at a density of 3×10^4 cells/well into the inner 60 wells of a white 96-well micro titre, clear bottomed plate (Bio-Greiner One, Lasec) in 250 µℓ dosing medium (L-15 medium supplemented with 10% charcoal dextran treated FBS (cdtFBS); HyClone, Separations). cdtFBS is hormone-free serum used to prevent false positive results. The outer 36 wells were filled with PBS to create a homogenous micro-environment across all cell-containing wells. The plates were incubated for 48 hr at 37°C with no CO_2 supplementation.

Serial dilutions (ratio 1:1) of the sample extracts were prepared in MeOH. Testosterone (positive control; minimum purity >99%, Sigma Aldrich) was also serially diluted but the concentrations of the positive control that was used in the test for AR activation varied between two bio-assay periods (BAPs) as well as the background testosterone concentration that was included in the medium during the test for AR inhibition. These differences were due to changes in the sensitivity of the MDA-kb2 cell line over the extended period of this project and are summarised in Table 3.7. The first BAP had a four times testosterone dilution series while the second BAP had a five times testosterone dilution series.

Sample dosing took place 48 hr after the initial seeding of the MDA-kb2 cells. Each 96-well plate was divided into dosing sections as illustrated in Figure 3.4. A volume of 2.5 µℓ of two separate sample extract dilution series as well as the same volume of the testosterone dilution series was dosed in the respective allocated sections. Each concentration in the dilution series was dosed in triplicate. Every 96-well plate also contained three wells dosed with 2.5 µℓ of 100% MeOH as the solvent (SC) to control for potential effects on the AR. A further three wells represented the blank control (BC) which consisted only of MDA-kb2 cells and dosing medium (Figure 3.4).

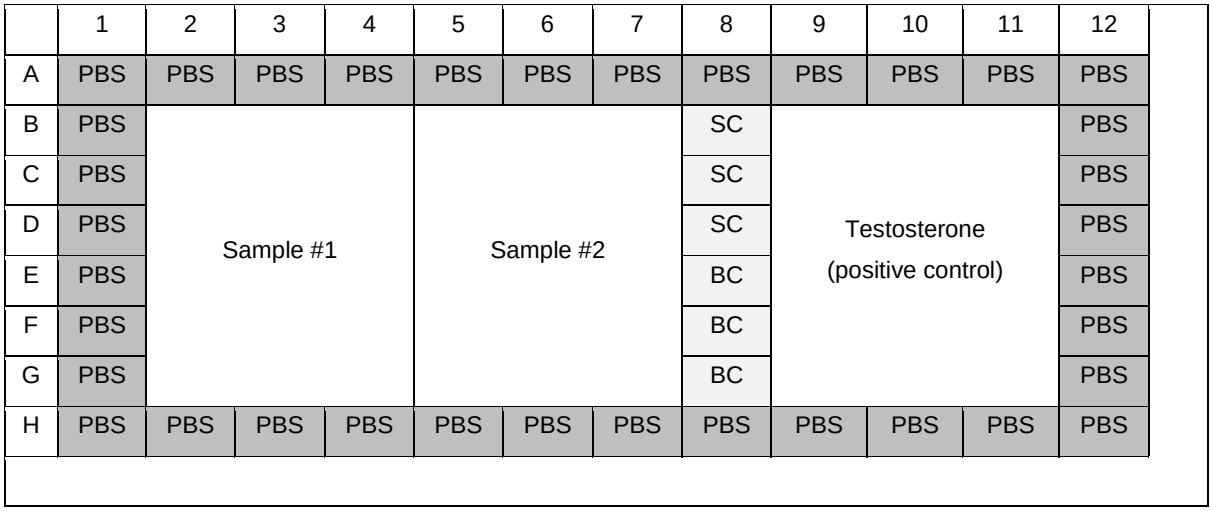

Figure 3.4: Layout of a 96-well plate. Sample extract dilution series, testosterone dilution series, MeOH (SC) and blank control (BC) were dosed in triplicate in the inner 60 wells. PBS filled the exterior 36 wells.

After the 48 hr incubation period the cells were inspected under a phase contrast microscope for confluency and signs of cytotoxicity. The dosing medium was removed and the cells were washed in triplicate with PBS supplemented with Mg^{2+} and Ca^{2+}. The cells were subsequently lysed with 25 µℓ lysis buffer (CelLytic, Sigma Aldrich) to ensure lysis of membrane proteins, where after the plates were stored in -80°C for at least 10 min. The rapid freezing of the cells further ensures rupturing of the cell membranes and the consequent release of cell content. A Berthold multimode micro plate reader (model LB941) was used to measure luciferase activity (luminescence). When thawed the plate reader injected 100 µℓ of a luciferin-containing reagent into each well. The reagent consisted of 20 mM tricine, 1.07 mM $Mg(CO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4 \bullet 7H_2O$, 0.1 mM EDTA, 270 µM coenzyme A, 530 µM ATP (everything supplied by Sigma Aldrich), 33.3 mM dithiothreitol and 470 µM beetle luciferin (Melford). The luminescence of each well was captured as RLUs which were used in further statistical analyses to express the samples responses quantitatively.

To measure anti-androgenic activity (AR inhibition), the same protocol was followed as described above, with two changes: the cells were seeded with a known background testosterone concentration in the media (Table 3.7) which resulted in cells with slightly activated ARs and the response from the SC wells were used as reference against which responses from samples could be compared to determine inhibition.

Table 3.7: Testosterone concentrations used during two bio-assay periods.

	Catchment	Sampling period	Matrix (W = Water; S = Sediment)	AR activation T dilution series (µg/mℓ)	AR inhibition T background (µg/mℓ)
Bio-assay period 1	**Letsitele**	July 2011	W & S	1. 23.1 2. 5.8 3. 1.4 4. 3.6×10^{-1} 5. 9.0×10^{-2} 6. 2.3×10^{-2}	8.6×10^{-5}
		November 2011	W & S		
		August 2012	W		
	Renoster & Vals	July 2012	W & S		
		October 2012	W		
	Mzinti, Lomati & Ngweti	June 2012	W & S		
		September 2012	W		
Bio-assay period 2	**Letsitele**	August 2012	S	1. 2.3×10^{-3} 2. 4.6×10^{-4} 3. 9.2×10^{-5} 4. 1.8×10^{-5} 5. 3.7×10^{-6} 6. 7.4×10^{-7}	3.91×10^{-5}
		November 2012	W & S		
		February 2013	W & S		
	Renoster & Vals	October 2012	S		
		January 2013	W & S		
		April 2013	W & S		
	Mzinti, Lomati & Ngweti	September 2012	S		
		December 2012	W & S		
		March 2013	W & S		

AR: Androgen receptor; **T:** Testosterone

3.7. Viability assay (MTT)

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay was first developed and described by Mosmann in 1983 and has since been used as a method to determine the viability of cells which have been exposed to toxic substances. The MTT viability assay was used to validate the reporter gene bio-assay data by preventing false negative responses since low responses (*i.e*. little or no luminescence) do not necessarily indicate the presence of antagonists (inhibits androgen binding to the AR) or absence of agonists (activates the AR and/or GR), but rather, may be due to cytotoxicity.

The MTT viability assay is based on an enzyme reaction during which the yellow, water soluble tetrazolium salt is transformed into a purple, insoluble formazan salt by succinate dehydrogenase (an enzyme present in active mitochondria and which cleaves the tetrazolium ring) (ATCC, 2011). The formazan salts are impermeable to cell membranes resulting in accumulation within the cells and can therefore be quantified spectrophotometrically (Fotakis *et al*., 2006; Mosmann, 1983).

The reporter gene bio-assay and MTT viability assay were done simultaneously which ensured that the same MDA-kb2 cell generations were used for the different assays. The first day of the MTT assay was identical to that described in Section 3.6.2 for the reporter gene bio-assay except for six extra wells that were also seeded with cells instead of being filled with PBS. These wells' cells were killed on day five which represented the negative control.

On day five of the MTT viability assay the wells were visually inspected and rinsed in triplicate with PBS as described in Section 3.6.2. The six negative control wells received 200 µℓ of 100% MeOH for 5 min. This step ensured 100% cell mortality. The MeOH was removed and all 66 wells received 100 µℓ of MTT solution (0.5 mg/mℓ MTT in L-15 stock media). The plates were subsequently incubated for 30 min at 37°C in a humidified atmosphere with no CO_2 supplementation to allow for the enzyme reaction to take place. The yellow MTT solution was removed and replaced with 200 µℓ of dimethyl sulphoxide (DMSO; Merck). DMSO dissolves the formazan crystals. The plates were left in the dark at room temperature for another 30 minutes before the OD of the solubilised formazan was measured at 560 nm with the plate reader.

The mean of the OD value of the MeOH killed cells (negative control) was subtracted from the mean OD of the cells that received sample. The resultant OD value for each well was subsequently expressed as a percentage of the mean OD of the positive control (SC) to indicate percentage viability. If the viability was lower than 80% the sample was considered to be cytotoxic which may have affected the reporter gene bio-assay responses.

3.8. Quality control in the MDA-kb2 reporter gene bio-assay

The principle of the MDA-kb2 reporter gene bio-assay is based on responses elicited by the MDA-kb2 cells. The responses of these live tissue culture cells may vary according to the environmental conditions (*e.g*. temperature and humidity) in which they are cultured. It is therefore important to include quality control methods in the bio-assay procedure to ensure repeatable and scientifically correct results with limited variation.

Culturing conditions in the laboratory were kept consistent as far as possible. Each 96-well plate was dosed with its own testosterone standard dilution series since the micro-environment of every plate in the same assay may also vary slightly. The extracted environmental samples as well as the testosterone standard were dosed in triplicate to ensure repeatable responses (Koh *et al*., 2004). A coefficient of variation (CV) value was determined for each sample which gave an indication on the precision with which the bio-assay was carried out (Whyte *et al*., 2004). The data was considered reliable if CV ≤ 20% (Tillitt *et al*., 1991; Whyte *et al*., 2004).

Every 96-well plate had three wells which contained only dosing media and MDA-kb2 cells. These wells represented the BC which was used to gauge the general well-being of the cells used for the assay (Windal *et al*., 2005). The SC wells measured the potential effects of the solvent (100% MeOH) on the receptors and also controlled for cytotoxicity caused by the solvent (Kraut & Kurtz, 2008). Finally, the MTT viability assay was conducted to ensure cell viability (≥ 80% live cells) in order to prevent false responses (Section 3.7).

3.9. Data processing and statistical analysis

Data processing and basic statistical analyses were conducted with Microsoft Excel to determine the testosterone equivalent (T-eq) value in the case of receptor activation responses. The sample RLU values were imported into an Excel spreadsheet after which the mean, standard deviation and CV values were calculated. The mean sample RLUs were expressed as a percentage of the maximum response induced by the testosterone reference (% T-max). Dose-response curves were plotted by using the sample volume logarithm (x-axis) and the % T-max values (y-axis). Dose-response curves were plotted for the testosterone reference by using the logarithm of the testosterone concentrations (x-axis) and the % T-max (y-axis) in order to create a regression equation. The data points (minimum of 3) on the linear part of the curves were used to calculate the slope and y-intercept in the straight line equation ($y = mx + c$) as well as the correlation coefficient (R^2).

These calculations were used to calculate the effective concentration (EC) which elicited a 20%, 50% and 80% response. The relative potency values (REPs) for each sample were calculated by dividing the testosterone reference's $EC_{20\text{-}80}$ by the samples' $EC_{20\text{-}80}$ (Villeneuve *et al*., 2000). The final $REP_{20\text{-}80}$ values for each sample were expressed as µg T-eq/g sediment or as µg T-eq/ℓ water (depending on the matrix used). The conversion to µg T-eq was done with a back calculation based on the initial quantity of sample extracted which were concentrated into a final dosing volume (*e.g*. 20 g sediment and 1 ℓ water were both concentrated into 1 mℓ samples which were used in the bio-assay).

The T-eq value enables quantification of complex chemical samples of which the exact composition are unknown. As mentioned the MDA-kb2 cells also express the GR and since the bio-assay recognised GR ligands in some instances the hormone-dependant gene transcription results (receptor activation) were rather reported as bio-assay equivalents (BEQs) instead because T-eq specifically implies activation of the testosterone receptor but the luminescence might very well have been caused by the GR).

Significant inhibition of the AR was determined by using the Mann-Whitney U Test (non-parametric test; SPSS Statistics). The sample RLU values were compared to the SC RLU

values. In the case of significant inhibition ($p<0.05$) the FI value (i.e. how many times the mean RLU of the exposed cells was less than that of the SC cells) was calculated and reported.

In Table 3.8 the limits of detection (LOD) for the positive control for both water and sediment matrices, during both of the BAPs (Table 3.7), are presented. The LOD is the lowest concentration of analyte that the analytical instrument (bio-assay in this case) can detect (Armbruster & Pry, 2008). The LOD values where calculated as follows: the EC_0 was determined for each testosterone standard graph, generated throughout the study, whereafter the mean and standard deviation values of the intercepts (n=25) were calculated. The final LOD value represented the concentration of the testosterone which will elicit a response from the cells significantly (95% confidence) different from zero.

The MDA-kb2 cells were more sensitive to the positive control (by a factor of 10 000) during BAP2 compared to their sensitivity in BAP1. This phenomenon is still unexplainable and will require further investigation. An advantage of the highly sensitive cells was that very low limits of receptor agonists (AR and/or GR) could be detected.

Table 3.8: Limits of detection values for bio-assay period 1 and bio-assay period 2.

	Limits of detection	
	Water (µg T/ℓ)	**Sediment (µg T/g)**
Bio-assay period 1	1.8×10^{-10}	9.0×10^{-3}
Bio-assay period 2	1.5×10^{-14}	8.0×10^{-7}

T: Testosterone

The fact that the MDA-kb2 cells also express GR (additionally to the AR) posed some difficulty in interpreting receptor activation results since the activation of any one of the two receptors cause the same reaction (light emission subsequent to activation of the MMTV promoter) when activated by its ligand (Wilson *et al*., 2002). The pesticide results that could potentially be observed are summarised in Table 3.9 as well as the deduction drawn from the observations. Both the test for activation and inhibition were included in the table. The following are examples of how the information in Table 3.9 can be used.

- If the extract contained AR agonists only (No. 1 in Table 3.9), the light would have been emitted during the activation assay, but also, when tested for inhibition (due to the background testosterone).
- If the extract contained AR antagonists and GR agonists (No. 6 in Table 3.9) then the activation assay would result in light emission, and depending on the concentration of

the relative ligands (as well as interaction between the compounds) there would be a decline in light emission compared to the SC wells if the AR antagonists response were stronger than the GR agonists. If they were equal, then there would be no net increase or decrease in light.

Table 3.9: Predictions of assay results when combinations of AR and GR agonists and antagonists are present in the extract

No.	If the extract contained	Result for activation assay	#Result for inhibition assay
1	AR agonists only	↑ or no response	NI or ↑* (Orton *et al*., 2009)
2	AR antagonists only	no response	↓
3	GR agonists only	↑	↑*
4	GR antagonists only	no response	NI
5	AR agonists + GR agonists	↑	↑*
6	AR antagonists + GR agonists	↑	↓ or NI$
7	AR agonists + GR antagonists	↑ or no response	NI or ↑* (Orton *et al*., 2009)
8	AR antagonist + GR antagonist	no response	↓

#: Results expressed in terms of the volume light emitted by SC wells which were already slightly activated via the AR ONLY

no response: No increase/decrease in light emission compared to background

↑: Light emitted

↓: Dampened light signal

$: Response depends on relative activation/inhibition by AR antagonists and GR agonists

***:** Significant increase in light emission compared to the SC

NI: No inhibition

It is important to note that testosterone can only act through the AR and not through the GR (Bergman *et al*., 2012) and therefore the GR cannot be activated in the inhibition test because of the background testosterone and thereby cause false positive responses. Another confounding effect is that even very small quantities (not detectable if the cells were not pre-activated) of androgens can potentially cause significantly more light emission compared to the SC in the receptor inhibition assay due to the testosterone background activation (No. 1 or 7 in Table 3.9). This was also seen in a study conducted by Orton *et al*. (2009) during which simazine caused androgenic responses in the AR inhibition test but did not elicit the same response in the absence of testosterone.

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Introduction

In this chapter the biological and chemical analyses results generated for each study area are presented. The chemical analysis results were compared to national and international water and sediment quality guidelines. Furthermore, if the samples elicited responses from the cells in the MDA-kb2 reporter gene bio-assay then the bio-assay results were compared to the analytical chemistry results in an attempt to identify the priority pesticide(s) mainly responsible for the (anti)androgenic activity but also for GR activity. It is important to keep the specific aims and objectives for this project in mind since it formed part of a larger WRC project (K5/1956) during which the contamination of water resources by agricultural chemicals were investigated as well as the impact thereof on environmental health.

4.2. Processing of results

4.2.1 Selection of priority pesticides

During this study all of the water and sediment samples that were collected from each study area were initially screened for 291 pesticide active ingredients (Table A–1). The five active ingredients which were detected most frequently in both the water and sediment samples from each study area were subjected to quantitative chemical analysis. Subsequently a total of ten priority pesticides (Table 4.1) were identified throughout all three study areas. This was done for the larger WRC project but results are included in this dissertation to help interpret the bio-assay results.

4.2.2 Water quality guidelines for pesticides

Surface water samples were primarily collected from untreated sources (*e.g*. rivers, dams and boreholes) while a few samples were taken from drinking water taps (Tables 3.1 and 3.5). Residents of the rural areas, located next to or in close proximity of the sampling sites, frequently make use of untreated freshwater sources for domestic and agricultural purposes as access to treated, piped water is often unreliable. For this reason the chemical analysis results for the water samples were compared to drinking water quality guidelines (Table 4.2) as well as to water quality guidelines for aquatic ecosystems (Table 4.3) (which are, in some cases, more strict compared to other water quality guidelines). These guidelines have been developed to give guidance in the ‘maximum permissible levels’ of contaminants (*e.g*. pesticides) which, in turn, aim at reducing human and environmental health risks (London *et al*., 2005).

Table 4.1: Pesticide active ingredients detected and quantified in the three study areas (indicated by 'X') as well as the crops associated with these areas.

PESTICIDE	LETSITELE	RENOSTER & VALS	MZINTI, LOMATI & NGWETI
	CROPS		
	Mangos, citrus & avocados	Maize, sorghum, sunflower	Sugarcane, citrus
Alachlor		X	
Atrazine		X	X
Carbofuran			X
Diphenylamine	X		
Imidacloprid	X	X	X
Imazalil	X		
Propiconazole	X		
Simazine		X	
Terbuthylazine		X	X
Thiabendazole	X		X

Water quality standards and legislation for pesticides in water used for domestic and agricultural purposes are currently lacking in South Africa (DWAF, 1996a) even though the country is a major user of pesticides (London *et al*., 2005). The South African water quality guidelines for domestic use (DWAF, 1996a) require that water quality constituents occur in levels which will not cause any effects either on the fitness of water for a specific use or on aquatic ecosystem health. For such constituents to have no effect it must occur within the Target water quality range (TWQR). The TWQR for atrazine in drinking water is 2 µg/ℓ (Table 4.2), which is comparable to the WHO guideline value (GV) for the same compound, and 10 µg/ℓ to ensure the protection of aquatic ecosystems (Table 4.3). None of the other priority pesticides analysed in this study have TWQR values.

The Australian as well as the WHO drinking water GVs, the maximum acceptable concentration (MAC) values used by Canadian regulatory agencies as well as the maximum concentration levels (MCLs) developed by the US EPA all represent the concentrations of constituents that do not pose a significant human health risk over a lifetime of consumption. They also aim to ensure water quality which is safe to drink during all stages of life (Health Canada, 2012; London *et al*., 2005; NHMRC, NRMMC, 2011; WHO, 2011). Many countries cannot afford to do their own risk assessments and therefore adopt the drinking water quality guidelines developed by the WHO. The WHO guideline values are derived for individual chemicals but take potential chemical interaction into consideration by integrating safety factors into the standards (WHO, 2011). The South African TWQR, Australian trigger value

(TV) as well as the Canadian water quality guideline (WQG) value attempt to ensure aquatic ecosystem health.

Table 4.2: Comparison of international drinking water quality guidelines for detected and quantified pesticides.

PESTICIDE	DRINKING WATER QUALITY GUIDELINES				
	SA DWAF (TWQR µg/ℓ)	**AUSTRALIA (GV µg/ℓ)**	**CANADA (MAC µg/ℓ)**	**US EPA (MCL µg/ℓ)**	**WHO (GV µg/ℓ)**
	DWAF, 1996a	NHMRC, NRMMC, 2011; *London *et al*., 2005	Health Canada, 2012	US EPA, 2009b	WHO, 2011
Alachlor	-	2*	-	2	20
Atrazine	2	20	5	3	2
Carbofuran	-	10	90	40	7
Diphenylamine	-	-	-	-	-
Imazalil	-	-	-	-	-
Imidacloprid	-	-	-	-	-
Propiconazole	-	100	-	-	-
Simazine	-	20	10	4	2
Terbuthylazine	-	10	-	-	7
Thiabendazole	-	-	-	-	-

DWAF: Department of Water Affairs and Forestry; **TWQR:** Target water quality range; **GV:** Guideline value; **MAC:** Maximum acceptable concentration; **US EPA:** United States Environmental Protection Agency; **MCL:** Maximum concentration level; **WHO:** World Health Organization

Table 4.3: Comparison of international water quality guidelines for aquatic ecosystems for detected and quantified pesticides.

PESTICIDE	WATER QUALITY GUIDELINES FOR AQUATIC ECOSYSTEMS		
	SA DWAF (TWQR µg/ℓ)	**AUSTRALIA (TV µg/ℓ)**	**CANADA (WQG µg/ℓ)**
	DWAF, 1996b	ANZECC, ARMCANZ, 2000	CCME, 2014
Alachlor	-	-	-
Atrazine	10	13	1.8
Carbofuran	-	0.06	1.8
Diphenylamine	-	-	-
Imazalil	-	-	-
Imidacloprid	-	-	0.23
Propiconazole	-	-	-
Simazine	-	3.2	10
Terbuthylazine	-	-	-
Thiabendazole	-	-	-

DWAF: Department of Water Affairs and Forestry; **TWQR:** Target water quality range; **TV:** Trigger value; **WQG:** Water Quality Guidelines

Empirical data for the effects of pesticides in water on human and environmental health are presently lacking for the majority of current use pesticides. This is primarily why no guideline

values exist for most pesticides (London *et al*., 2005). For this study no drinking water quality guideline could be found for the pesticides diphenylamine, imidacloprid, imazalil or thiabendazole (Table 4.2). Water quality guidelines for aquatic ecosystems only include atrazine, carbofuran, imidacloprid and simazine (Table 4.3).

4.2.3. Sediment quality guidelines for pesticides

South Africa does not have SQGs for the priority pesticides used in this study. The government of the Netherlands as well as the US EPA developed guideline values for at least three of the ten pesticides of interest (Table 4.4). The Netherlands' SQG values apply to sediment containing 10% organic carbon and 25% particles smaller than 2 µm (Warmer & Van Dokkum, 2002). The SQGs developed by the US EPA apply to dry-weight with 1% organic carbon (MacDonald *et al*., 2000). The available SQG values were compared to the detected priority pesticide levels in the sediment from all three study areas in order to determine whether regulatory limits were exceeded. The lack of applicable SQGs make a thorough comparative study impossible and therefore further emphasizes the need for sediment quality regulatory research specifically in a South African context.

Table 4.4: Comparison of sediment quality guidelines for detected and quantified pesticides.

PESTICIDE	SEDIMENT QUALITY GUIDELINES	
	NETHERLANDS (MPC µg/kg)	US EPA (µg/kg)
	Warmer & Van Dokkum, 2002	US EPA, 2006
Alachlor	-	-
Atrazine	26	6.62
Carbofuran	2	3.44
Diphenylamine	-	-
Imazalil	-	-
Imidacloprid	-	-
Propiconazole	-	-
Simazine	0.9	-
Terbuthylazine	-	-
Thiabendazole	-	-

MPC: Maximum permissible concentration; **US EPA:** United States Environmental Protection Agency

4.2.4. Prioritisation of pesticides in South Africa

The extensive use of a variety of pesticides in South Africa and the resultant pollution of aquatic and terrestrial ecosystems, as well as the potential health risks associated with pesticides, have led to the need to identify and prioritize current use pesticides in the country. Dabrowski *et al*. (2014) prioritized pesticides currently used in South Africa based on quantity of use and potential to cause chronic health problems in the case of exposure. The research also aimed to resolve the spatial distribution of priority pesticides by linking them with the crops

to which they were applied. This makes it possible to determine which human communities may potentially be exposed to toxic pesticides by identifying their proximity to specific crop types. At least six of the ten pesticides (*e.g*. alachlor, atrazine, carbofuran, imidacloprid, simazine and terbuthylazine) have been included in the final priority pesticide list and will be discussed in Sections 4.3–4.5 according to the parameters determined by Dabrowski *et al*. (2014).

The prioritisation was done in two phases. In the first phase 203 current use pesticide active ingredients were screened based on quantity of use and toxic properties. The quantity of use was determined by first screening, and later ranking, the pesticides according to mass used (kg). If less than 1000 kg of an active ingredient were sold per annum it was excluded from the priority list. Each of the remaining 152 compounds was subsequently screened for their potential to be endocrine disruptors, carcinogenic, teratogenic, mutagenic and/or neurotoxic.

In the second phase of the prioritization process, each one of the 152 remaining pesticides were ranked according to its quantity index (QI; the annual quantity of use of the pesticide in South Africa), toxicity potential (TP), environmental exposure potential (EEP; represents the mobility of the pesticide in the environment) and hazard potential (HP; indicates the exposure potential to highly toxic pesticides and is based on the toxicity potential of the pesticide as well as its EEP). Finally the hazard potential for each of the priority pesticides was determined per crop type (Dabrowski *et al*., 2014).

4.2.5. MDA-kb2 reporter gene bio-assay

The MDA-kb2 reporter gene bio-assay results are presented separately for each study area as well as for each matrix analysed (i.e. surface and groundwater or sediment). Androgen receptor (AR) activation responses as well as GR mediated responses were expressed as BEQs (Section 3.9) and AR inhibition as a function of FI. In the case where no receptor activation responses were detected the results were reported as below limit of detection (*e.g*. <LOD). The LOD values (Table 3.8) indicate the lowest levels of receptor ligands that the bio-assay could detect during the two BAPs.

Currently no environmental standards or guidelines for testosterone (or chemicals that mimic testosterone) in water or sediment exist. There is therefore no regulatory way of knowing at what levels androgens or androgen-like compounds may cause chronic health effects in humans and animals. None of the androgenic responses elicited throughout this study can therefore be discussed in terms of known health effects, only the possibility of effects. This emphasizes the need for further EDC research, specifically for androgens and androgen-like compounds. In an attempt to explain the elicited responses, the MDA-kb2 bio-assay data were compared to detected pesticides in the relevant catchment which have been declared

as EDCs or which are considered as possible EDCs. Other land uses and/or industries in the various catchments were also considered as possible culprits for the observed responses.

Activation responses mediated through the GR were also compared to the analytical chemistry results, but further interpretation was made difficult since no literature could be found regarding the GR activity of the priority pesticides. The GR mediated responses will have to be researched in future studies.

4.3. Letsitele River catchment

The large scale cultivation of mangos, citrus and avocados takes place in the Letsitele River catchment. The chemical screening analysis data (Tables A–2 and A–3) identified five frequently detected pesticides which were subsequently quantified. These were diphenylamine, imazalil, imidacloprid, propiconazole and thiabendazole, all of which are known for their use on one or more of the fruits cultivated in the study area. An overview of the sites, sampling programme and analyses conducted on each of the samples is presented in Table 3.2.

4.3.1. Chemical analysis

The chemical analysis results for the Letsitele River catchment are presented in Table 4.5 (surface and groundwater) and in Table 4.6 (sediment). This study area is the only one with data for 2011. The July 2011 sampling event was during the dry, low flow, winter season. During this season pesticide concentrations may be low (due to lower usage and rainfall which decreases runoff and leaching) or high (pesticides concentrate if it does not get flushed away by riverine flow). Samples collected in July 2011 were screened for the presence of 291 pesticides (Table A–1). Based on the screening results priority pesticides were selected for quantification during the following sampling events. According to the screening results of July 2011, diphenylamine was the most detected pesticide in surface water samples while propiconazole was detected in six of the seven sediment samples (Tables 4.5 and 4.6).

The November 2011 sampling trip was during late spring when the rainfall season started (which implies higher flow and possibly higher levels of pesticides in water systems due to runoff). Only the priority pesticides in samples collected from certain sites (LT3, LT4, LT6, LT7 and LT9) were quantified while all of the sites samples were screened again for the 291 pesticides to exclude the possibility of misidentification of pesticides during the July 2011 screening analysis. This also ensured consideration of seasonal differences. Based on the second screening analysis results, the final priority sites (LT2, LT3, LT4, LT9 and LT10) were selected in the main stem of the Letsitele River to be representative of the catchment. Only these sites were sampled and quantified from August 2012 and onwards.

Site LT4 is a tap at a primary school located right next to site LT3 in a community situated within an agricultural area (Figure 3.1). All of the priority pesticides were detected in the sample collected during November 2011 (Table 4.5), at higher concentrations compared to the levels detected at other sites. In contrast, only diphenylamine was detected in LT3's water sample while diphenylamine, imidacloprid and thiabendazole were detected in relatively low concentrations in the site's sediment sample (Table 4.6). Therefore the tap may possibly be linked to a borehole which potentially becomes polluted with pesticides on a seasonal basis. It raises concern as this is a direct source of drinking water used by the school and other local people. Fortunately none of the detected pesticides in sample LT4 exceeded regulatory guidelines (Tables 4.2–4.4).

Imazalil was the least commonly detected pesticide in both matrices sampled in November 2011 but with the highest concentration ($6x10^{-2}$ µg/ℓ) in the LT4 sample. Diphenylamine was the predominant pesticide detected in the surface water and was also detected in three of the six sediment samples. Pesticide concentrations in the sediment were 10–100 times lower compared to that detected in the surface water (Tables 4.5 and 4.6).

During the August 2012 to February 2013 sampling events only the sample from site LT9 was screened since this is the most downstream site in the Letsitele River (Table 3.1) and therefore regarded as most representative of pesticides used in the catchment. Site LT4 was screened as representative of tap water (Tables A–2 and A–3). Imidacloprid and thiabendazole were detected in all of the surface water samples collected in August 2012. This pattern occurred again in the February 2013 sediment samples (and in 50% of the surface water samples) as well as in the September 2013 groundwater samples (Tables 4.5 and 4.6). According to Dabrowski *et al.* (2014), citrus accounts for 68.2% of the total use of imidacloprid in South Africa while thiabendazole is commonly used as a postharvest treatment of several tropical fruit types including citrus, mangos and avocados (US EPA, 2002). This potentially explains why imidacloprid and thiabendazole (together with diphenylamine, which is used to control storage scald on certain tropical fruit; García-Reyes *et al*., 2005; US EPA, 1998) were the most commonly detected pesticides in the Letsitele River catchment (Figure 4.1).

Site LT10 is a surface water sampling site, located next to a fruit packing shed in a tributary of the Letsitele River. All of the pesticides except for propiconazole were detected in the August 2012 sample from this site. The February 2013 sample from LT10 rendered imazalil, imidacloprid and thiabendazole in concentrations 10–100 times greater compared to the August 2012 detections (Tables 4.5 and 4.6). Fruit washing typically takes place at site LT10 and may be the reason for the detection of higher pesticide levels. In a study conducted by Castillo *et al*. (2000) pesticide residues were measured in aquatic environments surrounding

banana plantation areas in Costa Rica. The pesticides imazalil, thiabendazole and propiconazole were detected most frequently in packing plants and surrounding streams. Imazalil and thiabendazole (both post-harvest fungicides) were detected in mean concentrations of 74 µg/ℓ and 126 µg/ℓ respectively in the effluent from the packing plants.

The groundwater sites (BM, FM and SL) are located in the same area as site LT10 and the same three pesticides (imazalil, imidacloprid and thiabendazole) were detected in these samples in September 2013. Imidacloprid was detected at higher concentrations compared to all of the samples analysed during all of the sampling events. The imidacloprid concentration in sample FM (1.9 µg/ℓ) was approximately 8 times higher than the Canadian WQG of 0.23 µg/ℓ for aquatic ecosystems (Table 4.3). Groundwater was only sampled once but the chemical analysis results support the possibility that the fruit packing shed may have a significant impact on the surrounding water quality in terms of pesticide pollution and therefore also on human, animal and environmental health.

In general the priority pesticides were detected in higher concentrations in the water samples compared to the sediment samples. It is possible that the priority pesticides do not have a high affinity for sediment binding due to their physical and chemical properties (Warren *et al*., 2003) which may have resulted in higher pesticide concentrations in the water samples.

Table 4.5: Chemical quantitative analysis results for the surface and groundwater samples from the Letsitele River catchment.

Sampling period	Site	Diphenylamine (µg/ℓ)	Imazalil (µg/ℓ)	Imidacloprid (µg/ℓ)	Propiconazole (µg/ℓ)	Thiabendazole (µg/ℓ)
July 2011	LT1	nd	nd	D	nd	nd
	LT2	D	nd	nd	nd	nd
	LT3	nd	nd	nd	nd	nd
	LT4	nd	nd	nd	nd	nd
	LT5	nd	nd	nd	nd	nd
	LT6	D	nd	nd	nd	nd
	LT7	D	nd	nd	nd	nd
	LT8	nd	D	nd	nd	D
November 2011	LT1	D	nd	D	nd	nd
	LT2	nd	nd	nd	nd	nd
	LT3	$1x10^{-2}$	nd	nd	nd	nd
	LT4	$3x10^{-2}$	$6x10^{-2}$	$3x10^{-3}$	$4x10^{-3}$	$4x10^{-2}$
	LT6	$6x10^{-4}$	nd	nd	nd	nd
	LT7	$2x10^{-3}$	nd	$2x10^{-3}$	nd	$8x10^{-4}$
	LT8	nd	nd	nd	D	nd
	LT9	$3x10^{-3}$	$8x10^{-4}$	$4x10^{-3}$	nd	$2x10^{-4}$
	LT10	D	nd	nd	D	nd
August 2012	LT2	nd	nd	$2x10^{-3}$	nd	$9x10^{-5}$
	LT3	nd	nd	$8x10^{-4}$	$9x10^{-4}$	$9x10^{-5}$
	LT4	nd	nd	$9x10^{-4}$	nd	$2x10^{-4}$
	LT9	nd	nd	$2x10^{-3}$	nd	$5x10^{-4}$
	LT10	$2x10^{-3}$	$6x10^{-3}$	$3x10^{-4}$	nd	$7x10^{-4}$
November 2012	LT2	nd	nd	nd	nd	nd
	LT3	nd	nd	$1x10^{-3}$	$4x10^{-3}$	nd
	LT4	nd	nd	nd	nd	nd
	LT9	nd	nd	$2x10^{-3}$	nd	nd
	LT10	nd	nd	$1x10^{-3}$	nd	nd
February 2013	LT3	nd	nd	nd	nd	nd
	LT4	nd	nd	nd	$4x10^{-3}$	nd
	LT9	nd	$3x10^{-4}$	$4x10^{-3}$	nd	$1x10^{-5}$
	LT10	nd	$6x10^{-2}$	$2x10^{-2}$	nd	$5x10^{-3}$
September 2013	BM	nd	nd	$3x10^{-1}$	nd	$2x10^{-4}$
	FM	nd	nd	1.9	nd	$3x10^{-4}$
	SL	nd	$3x10^{-3}$	$5x10^{-1}$	nd	$1x10^{-3}$

nd: not detected; **D:** Detected during screening analysis but not quantified

Table 4.6: Chemical quantitative analysis results for the sediment samples from the Letsitele River catchment.

Sampling period	Site	Diphenylamine (µg/kg)	Imazalil (µg/kg)	Imidacloprid (µg/kg)	Propiconazole (µg/kg)	Thiabendazole (µg/kg)
July 2011	LT1	nd	nd	nd	D	nd
	LT2	D	nd	nd	nd	nd
	LT3	D	nd	nd	D	nd
	LT7	nd	nd	nd	D	nd
	LT8	nd	D	nd	D	nd
	LT9	nd	nd	nd	D	nd
	LT10	nd	D	nd	D	nd
November 2011	LT1	nd	nd	nd	D	nd
	LT2	nd	nd	nd	D	nd
	LT3	**$3x10^{-6}$**	nd	**$6x10^{-6}$**	nd	**$2x10^{-5}$**
	LT7	nd	nd	nd	nd	**$1x10^{-6}$**
	LT8	D	D	nd	nd	D
	LT9	**$8x10^{-6}$**	nd	nd	nd	**$1x10^{-5}$**
August 2012	LT2	nd	nd	nd	nd	nd
	LT3	nd	nd	nd	nd	**$3x10^{-4}$**
	LT9	**$1x10^{-3}$**	nd	nd	nd	**$3x10^{-4}$**
November 2012	LT2	nd	nd	nd	nd	nd
	LT3	nd	nd	nd	nd	nd
	LT9	nd	nd	nd	nd	nd
February 2013	LT2	**$2x10^{-3}$**	nd	nd	**$3x10^{-4}$**	**$1x10^{-4}$**
	LT3	nd	nd	nd	nd	**$1x10^{-4}$**
	LT9	nd	nd	nd	nd	**$5x10^{-4}$**

nd: not detected; **D:** Detected but not quantified

Figure 4.1 illustrates the percentage of detections for each of the priority pesticides in both matrices. A total of 34 surface and groundwater samples were collected from 13 sites (Table 4.5) while 22 sediment samples were collected from a total of 7 sites (Table 4.6). The total number of detections for each pesticide was expressed as a percentage of the total sample value. Imidacloprid had the highest number of detections in the surface and groundwater samples and was detected at 53% of the sites at concentrations ranging from $3x10^{-4}$ µg/ℓ to 1.9 µg/ℓ. Thiabendazole was detected in equal proportions (41%) in both the water and sediment sampling sites. Propiconazole was also detected in 41% of the sediment samples.

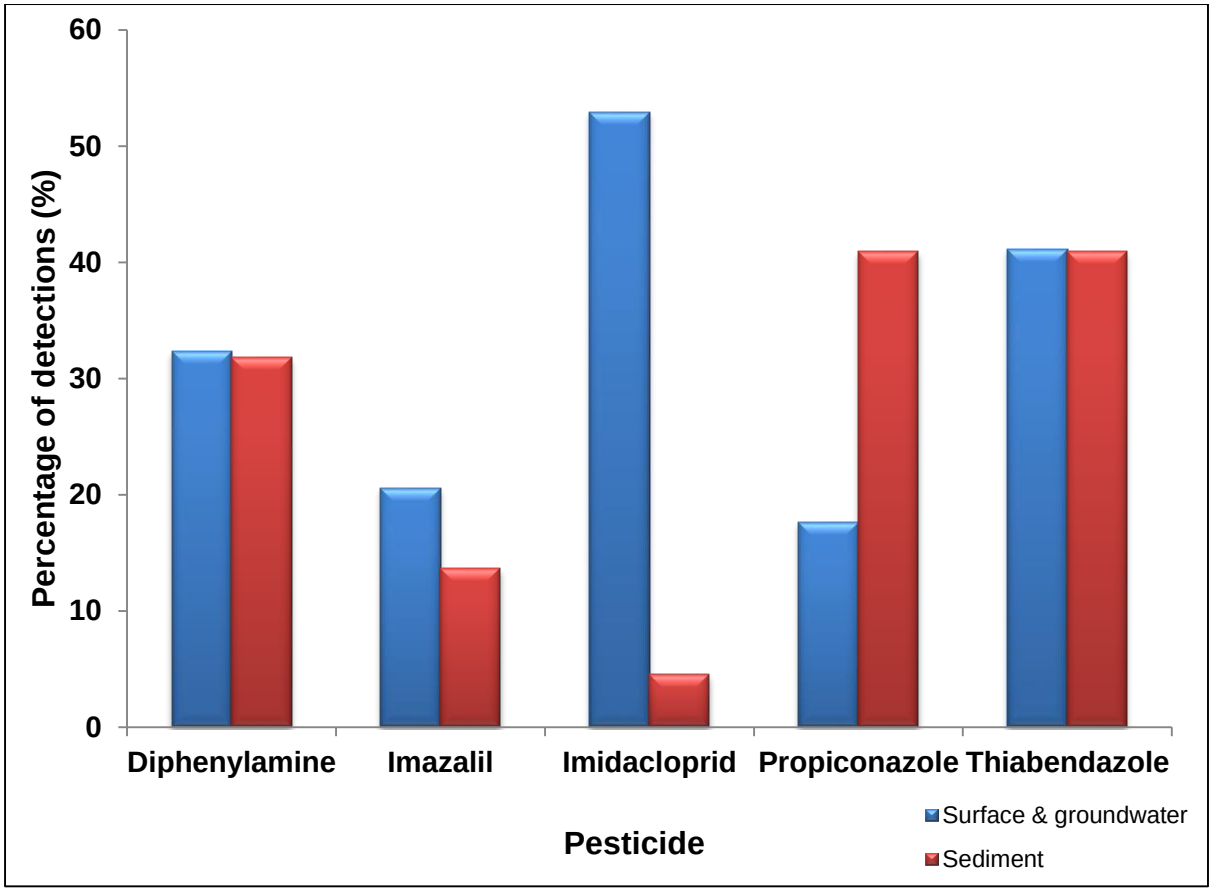

Figure 4.1: Percentage of priority pesticides detected in both the water and sediment matrices in the Letsitele River catchment. A total of 34 surface and groundwater samples and 22 sediment samples were collected.

4.3.2. MDA-kb2 reporter gene bio-assay

The surface water and sediment samples from the July 2011 and November 2011 sampling events as well as the August 2012 surface water samples were screened for (anti)androgenic activity during bio-assay period 1 (BAP1; Table 3.7). All of the detected (anti)androgenic and/or GR activity in both the water and sediment samples occurred during the first bio-assay period (Table 4.7 and 4.8).

Samples were regarded as cytotoxic if cell viability was less than 80%. A total of four samples (undiluted concentration; vial 1 in dilution series) caused cytotoxicity. The November 2012 LT10 surface water sample (site located next to the fruit packing shed) caused slight cytotoxicity (79% cell viability; Table 4.7). Only imidacloprid was detected at this site but since it doesn't seem like this pesticide caused cytotoxicity at other sites it is unlikely that it affected the cells in November 2012. Other chemicals used at the fruit packing shed, which were not screened for, may have been responsible for the cytotoxic effect.

Table 4.7: MDA-kb2 reporter gene bio-assay results for the surface and groundwater samples from the Letsitele River catchment. Reported FI values were all statistically significant.

Sampling period	Site	Activation (µg BEQ/ℓ water)	Inhibition (FI)	Viability (%)
July 2011	**LT1**	<LOD	**1.01**	100
	LT2	<LOD	NI	100
	LT3	<LOD	NI	100
	LT4	<LOD	NI	100
	LT5	<LOD	NI	100
	LT6	<LOD	NI	100
	LT7	<LOD	NI	100
	LT8	<LOD	NI	100
November 2011	**LT1**	<LOD	**1.47**	90
	LT2	<LOD	**1.36**	90
	LT4	<LOD	NI	100
	LT6	<LOD	NI	100
	LT7	<LOD	**1.39**	90
	LT8	<LOD	**1.30**	90
	LT9	<LOD	NI	100
	LT10	<LOD	NI	100
August 2012	**LT2**	<LOD	NI	100
	LT3	**$8.5x10^{-06} \pm 2.4x10^{-06}$**	↑*	95
	LT4	**$7.6x10^{-06} \pm 5.0x10^{-07}$**	↑*	95
	LT9	**$5.2x10^{-06} \pm 1.4x10^{-06}$**	↑*	95
	LT10	<LOD	NI	80
November 2012	**LT2**	<LOD	NI	97
	LT3	<LOD	NI	92
	LT4	<LOD	NI	86
	LT9	<LOD	NI	114
	LT10	<LOD	NI	**79**
February 2013	**LT3**	<LOD	NI	95
	LT4	<LOD	NI	100
	LT9	<LOD	NI	117
	LT10	<LOD	NI	117
September 2013	**BM**	<LOD	NI	97
	FM	<LOD	NI	109
	SL	<LOD	NI	112

<LOQ: Below Levels of Quantification; **↑*:** significant ($p<0.05$) activation response; **FI:** Fold Inhibition; **NI:** No inhibition; **Viability: %** of the cells which received undiluted sample extract

The sediment samples collected from sites LT3 (August 2012 and November 2012) and LT2 (November 2012) caused cytotoxicity (cell viability of 74%, 59% and 79%, respectively; Table 4.8). Only thiabendazole was detected at LT3 in August 2012. No pesticides were detected during November 2012. Site LT3 is the most upstream site in the Letsitele River and downstream from large scale forestry. This site is also situated next to a town (Mogoboya, Figure 3.1). Apart from natural occurring compounds, runoff from either the plantations or the town might have caused the samples to be cytotoxic.

Table 4.8: MDA-kb2 reporter gene bio-assay results for the sediment samples from the Letsitele River catchment. Reported FI values were all statistically significant.

Sampling period	Site	Activation (µg BEQ/g sediment)	Inhibition (FI)	Viability (%)
July 2011	**LT1**	<LOD	NI	100
	LT2	**2.1 ± 0.2**	**1.28**	100
	LT3	<LOD	**1.27**	100
	LT7	<LOD	**1.17**	100
	LT8	<LOD	**1.68**	100
	LT9	<LOD	NI	95
	LT10	<LOD	**1.34**	100
November 2011	**LT1**	<LOD	NI	100
	LT2	<LOD	NI	100
	LT3	<LOD	**1.72**	100
	LT7	<LOD	**1.46**	100
	LT8	<LOD	**1.93**	100
	LT9	<LOD	**1.57**	100
August 2012	**LT2**	<LOD	NI	92
	LT3	<LOD	NI	**74**
	LT9	<LOD	NI	85
November 2012	**LT2**	<LOD	NI	**79**
	LT3	<LOD	NI	**59**
	LT9	<LOD	NI	119
February 2013	**LT2**	<LOD	NI	93
	LT3	<LOD	NI	100
	LT9	<LOD	NI	100

<LOQ: Below Levels of Quantification; **FI:** Fold Inhibition; **NI:** No inhibition; **Viability: %** of the cells which received undiluted sample extract

Surface water samples collected from three sites (LT3, LT4 and LT9) during the August 2012 sampling event activated either the AR and/or the GR since these samples also caused significant activation in the AR inhibition test (Table 3.9; Table 4.7). The samples caused complete dose response curves, i.e. their maximum responses were comparable to the maximum response of the positive control (Figure 4.2; Table 4.7). The T-max values for the three samples were 146%, 117% and 136% respectively. Sites LT3 (most upstream site) and LT4 (tap at a primary school) are situated next to each other while site LT9 is the most downstream site in the Letsitele River. Imidacloprid and thiabendazole were detected in all three samples while propiconazole was also detected at site LT3. The isolated responses were potentially caused by other non-target pollutants with androgen and/or glucocorticoid-like activity since none of the other water or sediment samples, in which these pesticides were detected, elicited the same reactions from the cells. Furthermore, none of the mentioned pesticides have been proven to have definite androgenic or glucocorticoid-like activity. Imidacloprid has been included in the final list of initial pesticide active ingredients to be screened under the EDSP managed by the US EPA (Gervais *et al*., 2010; US EPA, 2009a).

Although propiconazole has been classified by the European Commission as a Category 3 EDC (there is insufficient evidence available to accurately confirm the ED properties of the pesticide; EUC, 1999; Matisova & Hrouzkova, 2012), studies have shown that propiconazole can inhibit androgen binding (Kjærstad *et al*, 2010; Okubo *et al*., 2004; Orton *et al*., 2011).

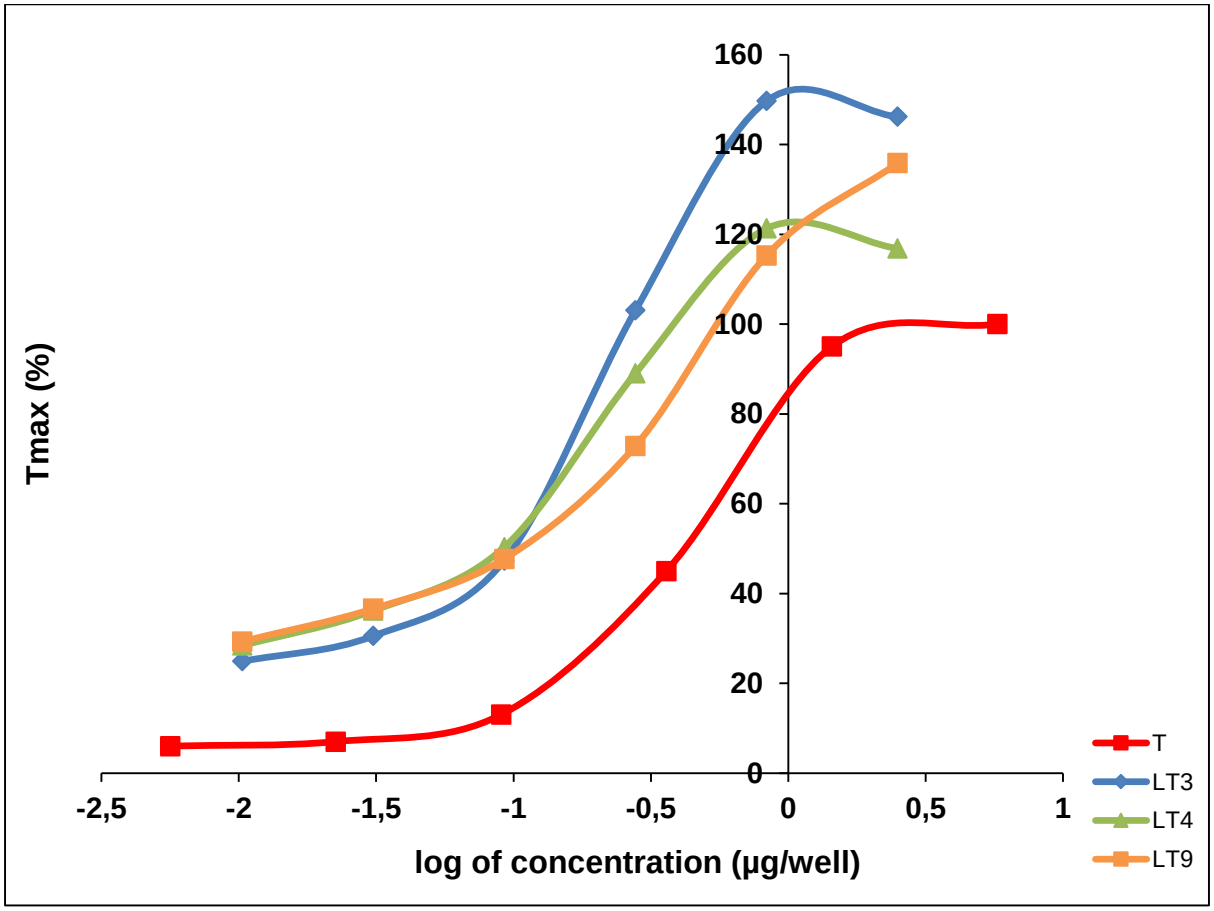

Figure 4.2: Activation of the AR and/or GR of the MDA-kb2 cells by surface water samples collected in the Letsitele River catchment in August 2012. Samples from sites LT3, LT4 and LT9 elicited responses comparable to the maximum response of the positive control, testosterone (T). Standard deviations are not indicated as they cause distortion of the typical dose response shape of the curve. For reference see Table A–8.

Only one sediment sample (LT2 from July 2011) elicited a luminescence response from the cells comparable to 20% of the maximum response of the positive control (T-max=18.9%; 2.1 ± 0.2 µg T/g; Table 4.8). The same July 2011 LT2 sediment sample also inhibited the androgen receptor statistically significantly (FI=1.28). It is possible that a GR agonist was present in the sample together with an AR antagonist (No. 6 in Table 3.9).

All of the July 2011 sediment samples, except for LT1 and LT9, inhibited androgen binding (Table 4.8). In November 2011 all of the sediment samples (except for LT1 and LT2) as well

as the surface water samples LT1, LT2, LT7 and LT8 (Table 4.7) inhibited androgen binding. Inhibition of AR only occurred during the 2011 sampling period. Pesticides which may have been responsible are imazalil and propiconazole since both have been proven to be anti-androgenic (Kjærstad *et al*, 2010; Okubo *et al*., 2004; Orton *et al*., 2011) even though they have only been declared as Category 3 EDCs by the European Commission (EUC, 1999; Matisova & Hrouzkova, 2012). In another study conducted by Kojima *et al*. (2004) pesticides were analysed for (anti)androgenic properties with the use of CHO cells in a reporter gene bio-assay. Results indicated that at a concentration of $4.2x10^{-6}$ M imazalil inhibited 20% of the androgen activity induced by $1x10^{-10}$ M DHT while propiconazole also inhibited 20% of the induced activity at a concentration of $6.2x10^{-6}$ M.

4.3.3. Summary: Letsitele River catchment

The priority pesticides (diphenylamine, imazalil, imidacloprid, propiconazole and thiabendazole), which were most frequently detected in the sample extracts, are known for their use on citrus, mangos and avocados (all of which are cultivated on a large scale in the Letsitele River catchment).

The MDA-kb2 reporter gene bio-assay results showed that three surface water samples from August 2012 activated either the AR or the GR (or both) and one sediment sample from July 2011 also caused a luminescence response. Inhibition of androgen receptors were caused by several surface water and sediment samples during the 2011 sampling events. None of the groundwater samples elicited responses from the cells.

Only imazalil and propiconazole have been classified as Category 3 EDCs by the European Commission since there is not enough evidence to confirm their potential ED properties (EUC, 1999; Matisova & Hrouzkova, 2012). However, these pesticides have been shown to be inhibitors of AR (Kojima *et al*., 2004; Kjærstad *et al*., 2010; Okubo *et al*., 2004; Orton *et al*., 2011) and may potentially have been responsible for some of the AR inhibition elicited by several of the surface water and sediment sample extracts. None of the other three priority pesticides have been proven to have either androgenic, anti-androgenic activity or glucocorticoid-like activity.

Imidacloprid was ranked in the 14th position as a pesticide used in high quantities in South Africa (QI=252; compared to glyphosate which has the highest QI of 3721) according to the study done by Dabrowski *et al*. (2014). This increases its potential for human exposure. The study also placed imidacloprid in the 9th position regarding its weighted hazard potential (WHP) of 0.80 (compared to atrazine which has the highest WHP of 3.63). The WHP indicates the pesticide's toxic effect on humans and the environment in terms of the total quantity used relative to the quantity of use of all of the pesticides which were included in the analysis. Figure

4.1 also illustrated that imidacloprid was the most frequently detected pesticide in the Letsitele River catchment. Even though this pesticide has not been classified as a definite EDC it deserves further research attention if its high usage and exposure potential is taken into consideration.

(Anti)androgenic and/or glucocorticoid-like responses elicited by some of the samples collected from the Letsitele River catchment may potentially have been caused by other non-target EDCs since no significant patterns (regarding the chemical and/or bio-assay data) occurred either between samples from different sampling events, between samples from the same sampling events or between the different matrices. The detected responses are ascribed to effects caused by compound mixtures (*e.g*. pesticide mixtures) (Orton *et al*., 2012) found in the extracts and unless a full chemical screen is conducted on such an extract, none of the responses can be contributed to a single compound.

4.4. Renoster and Vals River catchments

As mentioned in Chapter 3 intensive, commercial cultivation of maize, sorghum and sunflower takes place in the Renoster and Vals River catchments. In February 2012 a preliminary screening analysis was done on surface water and sediment samples collected from the two lower most sites in each of the rivers (*e.g*. RN4 and VL3). During the initial chemical screening analysis (Tables A–4 and A–5) eighteen pesticides were detected of which alachlor, atrazine, imidacloprid, simazine and terbuthylazine were selected as priority pesticides. Selection of the priority pesticides for quantification was based on the frequency of detection as well as on the priority of use of the pesticides on the crops cultivated in the area.

Throughout the rest of the study only samples from sites RN4 and VL3 were screened for the presence of the 291 pesticides (Table A–1) but all of the sites water and sediment samples were subjected to chemical quantification. The chemical quantification analysis results for the Renoster and Vals River catchments are presented in Table 4.9 (surface and groundwater) and Table 4.10 (sediment). Groundwater was only sampled in October 2013 from the Renoster River catchment. An overview of the sites, sampling events and analyses conducted on each of the samples is presented in Table 3.4.

4.4.1. Chemical analysis

The priority pesticide concentrations detected in the Renoster and Vals River catchments were noticeably higher compared to those detected in the Letsitele River catchment. None of the guideline values for any of the priority pesticides detected in the Renoster and Vals River catchments were exceeded during this study (Tables 4.2–4.4). All of the detected priority pesticides, except for imidacloprid, are pre- and post-emergence herbicides used to control

annual grasses as well as some broad-leaved weeds in crops such as maize, soybeans and sugarcane (Dabrowski *et al*., 2014; WHO, 2011). Imidacloprid is a systemic neonicotinoid insecticide used to control sucking insects, some chewing insects, soil insects and may also be used as a seed treatment for various crops (Gervais *et al*., 2010).

Atrazine and terbuthylazine were detected in every water sample (Figure 4.3; Table 4.9) in concentrations ranging from $5x10^{-4}$ µg/ℓ (KOP) to $4x10^{-1}$ µg/ℓ (VL2; January 2013) for atrazine and from $3x10^{-4}$ µg/ℓ (KOP) to $2x10^{-1}$ µg/ℓ (VL2; April 2013) for terbuthylazine. In the Renoster River the atrazine and terbuthylazine levels were higher at the downstream RN3 and RN4 sites than in RN1. In the Vals River this occurrence only appeared in January and April 2013 during which the levels for these two pesticides were markedly higher at the two downstream sites (VL2 and VL3). This makes sense since these sites in both of the rivers are located downstream of towns (Koppies and Kroonstad respectively) and also receives runoff water from heavily cultivated areas. Atrazine and terbuthylazine were also detected in more than 50% of the sediment samples (Figure 4.3; Table 4.10) in lower concentrations compared to those detected in the water samples.

Simazine was detected in fewer water and sediment samples but in concentrations comparable to those detected for atrazine and terbuthylazine (Tables 4.9 and 4.10). All of the above mentioned pesticides were detected in higher concentrations in the water samples during the January and April 2013 sampling events compared to the July and October 2012 events. This incidence was possibly due to increased pesticide application practices during the late summer months. Alachlor was detected less frequently while imidacloprid was only detected once in sample VL2 in April 2013.

Table 4.9: Chemical quantitative analysis results for the surface and groundwater samples from the Renoster and Vals River catchments.

Sampling period	Site	Alachlor (µg/ℓ)	Atrazine (µg/ℓ)	Imidacloprid (µg/ℓ)	Simazine (µg/ℓ)	Terbuthylazine (µg/ℓ)
July 2012	RN1	nd	$6x10^{-3}$	nd	nd	$8x10^{-3}$
	RN3	nd	$3x10^{-2}$	nd	nd	$5x10^{-2}$
	RN4	$2x10^{-3}$	$8x10^{-2}$	nd	nd	$8x10^{-2}$
	VL1	nd	$4x10^{-2}$	nd	nd	$4x10^{-2}$
	VL2	nd	$1x10^{-2}$	nd	$3x10^{-2}$	$2x10^{-2}$
	VL3	nd	$2x10^{-2}$	nd	$4x10^{-2}$	$1x10^{-2}$
October 2012	RN1	$5x10^{-3}$	$7x10^{-3}$	nd	nd	$1x10^{-2}$
	RN3	nd	$4x10^{-2}$	nd	nd	$6x10^{-2}$
	RN4	nd	$7x10^{-2}$	nd	nd	$5x10^{-2}$
	VL1	nd	$3x10^{-2}$	nd	nd	$2x10^{-2}$
	VL2	nd	$4x10^{-2}$	nd	$8x10^{-2}$	$3x10^{-2}$
	VL3	nd	$5x10^{-2}$	nd	$7x10^{-2}$	$4x10^{-2}$
January 2013	RN1	nd	$4x10^{-2}$	nd	$7x10^{-2}$	$2x10^{-2}$
	RN3	nd	$3x10^{-1}$	nd	$2x10^{-1}$	$1x10^{-1}$
	RN4	nd	$9x10^{-2}$	nd	$9x10^{-2}$	$5x10^{-2}$
	VL1	nd	$5x10^{-2}$	nd	$1x10^{-1}$	$4x10^{-2}$
	VL2	nd	$4x10^{-1}$	nd	$9x10^{-1}$	$2x10^{-1}$
	VL3	nd	$1x10^{-1}$	nd	$7x10^{-4}$	$4x10^{-2}$
April 2013	RN3	nd	$1x10^{-2}$	nd	nd	$7x10^{-2}$
	RN4	nd	$4x10^{-2}$	nd	nd	$4x10^{-2}$
	VL1	nd	$2x10^{-1}$	nd	nd	$1x10^{-1}$
	VL2	$2x10^{-3}$	$2x10^{-1}$	$2x10^{-2}$	$3x10^{-1}$	$2x10^{-1}$
	VL3	nd	$1x10^{-1}$	nd	$4x10^{-1}$	$9x10^{-2}$
October 2013	VJK	nd	$2x10^{-2}$	nd	$3x10^{-2}$	$1x10^{-3}$
	KOP	nd	$5x10^{-4}$	nd	nd	$3x10^{-4}$
	KSD	nd	$6x10^{-3}$	nd	nd	$6x10^{-4}$

nd: not detected

According to Dabrowski *et al*. (2014) atrazine and terbuthylazine are used in relatively high quantities in South Africa. The pesticides have respectively been ranked in the 4th and 5th position with regards to their QI. Furthermore, it has been found that maize (an important crop in terms of high priority pesticide application) accounts for 87.8%, 92.2% and 68.2% of the total quantities of atrazine, terbuthylazine and simazine used, respectively. Likewise, other studies conducted in South Africa have found that these three pesticides occur in high concentrations in surface and groundwater, especially in areas where maize is cultivated on a large scale (Burger & Nel, 2008; Du Preez *et al*., 2005; Hecker *et al*., 2004). This may account for the high frequency of detection of atrazine and terbuthylazine (and to a lesser extent, simazine) in the Renoster and Vals River catchments.

Table 4.10: Chemical quantitative analysis results for the sediment samples from the Renoster and Vals River catchments.

Sampling period	Site	Alachlor (µg/kg)	Atrazine (µg/kg)	Imidacloprid (µg/kg)	Simazine (µg/kg)	Terbuthylazine (µg/kg)
July 2012	RN1	nd	nd	nd	nd	nd
	RN3	nd	nd	nd	nd	$1x10^{-3}$
	RN4	nd	$4x10^{-3}$	nd	nd	$9x10^{-3}$
	VL1	nd	nd	nd	nd	nd
	VL2	nd	$1x10^{-3}$	nd	$1x10^{-3}$	$3x10^{-3}$
	VL3	nd	nd	nd	nd	nd
October 2012	RN1	nd	$1x10^{-3}$	nd	nd	$2x10^{-3}$
	RN3	nd	$1x10^{-3}$	nd	nd	$2x10^{-3}$
	RN4	nd	$1x10^{-3}$	nd	nd	$1x10^{-3}$
	VL1	nd	nd	nd	nd	nd
	VL2	nd	$1x10^{-3}$	nd	$1x10^{-3}$	$2x10^{-3}$
	VL3	nd	nd	nd	nd	nd
January 2013	RN1	nd	$4x10^{-4}$	nd	nd	$7x10^{-4}$
	RN3	nd	$5x10^{-5}$	nd	nd	$7x10^{-4}$
	RN4	nd	$2x10^{-4}$	nd	nd	$3x10^{-4}$
	VL1	nd	nd	nd	nd	$4x10^{-4}$
	VL2	nd	$6x10^{-4}$	nd	nd	$1x10^{-3}$
	VL3	nd	$2x10^{-4}$	nd	nd	$2x10^{-4}$
April 2013	RN1	nd	nd	nd	$5x10^{-4}$	$3x10^{-4}$
	RN3	$2x10^{-3}$	$5x10^{-4}$	nd	nd	$5x10^{-4}$
	RN4	nd	$2x10^{-4}$	nd	$1x10^{-3}$	$4x10^{-4}$
	VL1	nd	$3x10^{-4}$	nd	nd	$4x10^{-4}$
	VL2	nd	$5x10^{-4}$	nd	nd	$2x10^{-3}$
	VL3	nd	$1x10^{-3}$	nd	nd	$2x10^{-3}$

nd: not detected

Due to the widespread use of triazine herbicides such as atrazine, terbuthylazine and simazine, their high to medium mobility (Dabrowski *et al*., 2014), their persistence in aquatic environments (Matisova & Hrouzkova, 2012; Solomon *et al*., 1996), water solubility and relatively weak adsorption to organic carbon particles (Du Preez *et al*., 2005) (even though Guzzella *et al*. (2003) has found terbuthylazine to adsorb to soil more easily compared to atrazine) these pesticides have great potential to pollute surface and groundwater resources. Pathways of contamination, and subsequent potential exposure, typically include leaching, run-off and spray drift (Gfrerer *et al*., 2002). Since pesticides such as atrazine have been used extensively for decades in the Renoster and Vals River catchments it is also possible that the detected concentrations include residues which have accumulated over the years (Du Preez *et al*., 2005). As a final remark Dabrowski *et al*. (2014) also ranked atrazine in first position

with regards to its WHP of 3.63 which indicates the pesticides toxic effect on humans and the environment in terms of the total quantity used relative to the quantity of use of all of the pesticides which were included in the analysis.

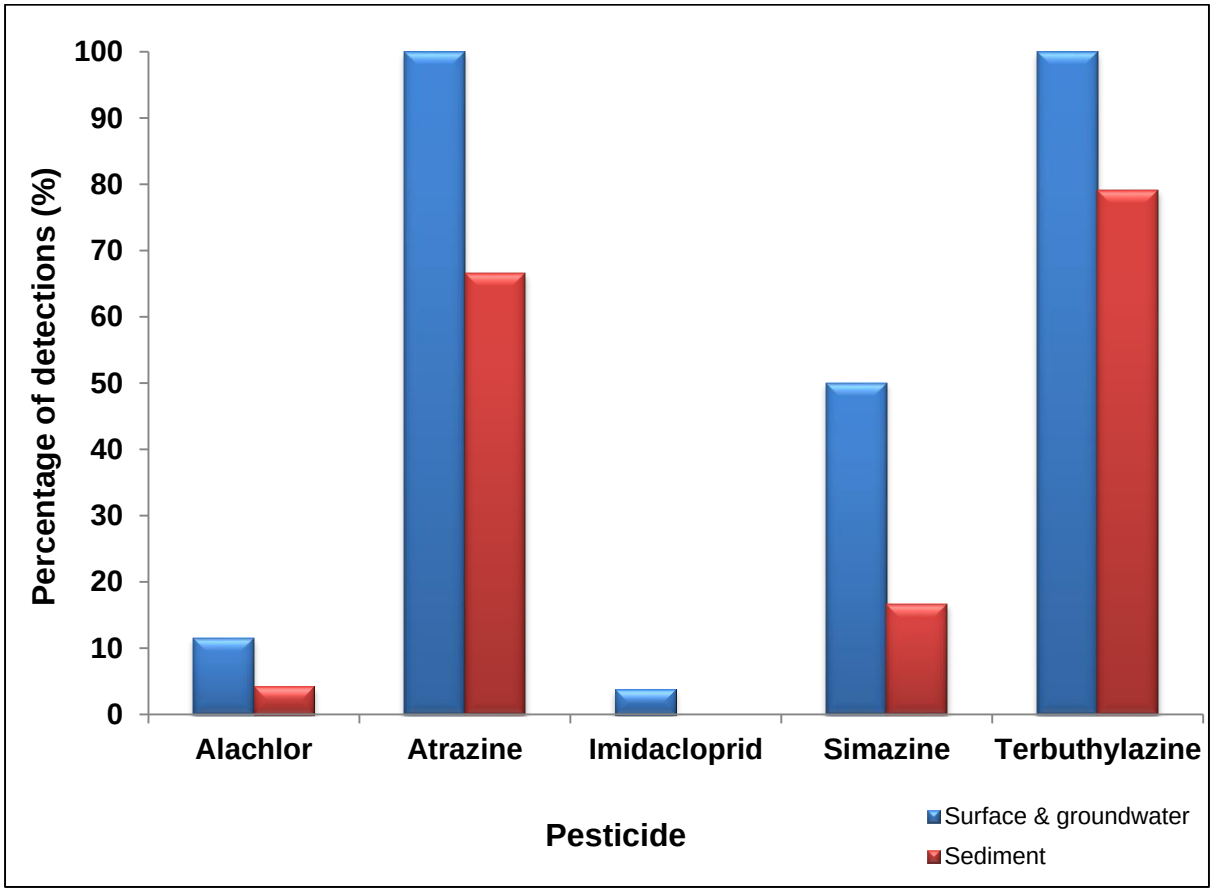

Figure 4.3: Percentage of priority pesticides detected in both the water and sediment matrices in the Renoster and Vals River catchments. A total of 26 surface and groundwater samples and 24 sediment samples were collected from both catchments.

4.4.2. MDA-kb2 reporter gene bio-assay

The surface water and sediment sampled in July 2012 as well as the sediment samples from October 2012 were screened for (anti)androgenic activity as well as for GR activation during bio-assay period 1 (BAP1; Table 3.7). The cell viability percentages reported in the bio-assay result tables represent the viability of the MDA-kb2 cells exposed to undiluted water and sediment sample extracts. Samples were regarded as cytotoxic if the cell viability was less than 80%.

All of the undiluted sediment samples as well as four of the six surface water sample extracts (RN3, VL1, VL2, VL3), collected in July 2012, had cytotoxic effects (Figure 4.4; Tables 4.11 and 4.12). Water sample VL3 (January 2013) as well as the sediment sample collected from the same site in April 2013 were toxic to the cells since only 42% and 55% of the cells

respectively survived the exposures. Sediment sample VL2 was slightly toxic in both January and April 2013 (cell viability 77%). As briefly mentioned in Section 4.4.1 the atrazine, simazine and terbuthylazine concentrations were markedly higher in the Vals River during January and April 2013 in both the water and sediment samples. However, the cytotoxicity results cannot definitely be ascribed to the presence of these priority pesticides since not all of the samples collected throughout the study, in which the pesticides were detected, caused cytotoxicity. The cell viability assay cannot be used to identify the cytotoxic compounds but indicates the presence of cytotoxic chemicals in the water system or at certain sampling sites.

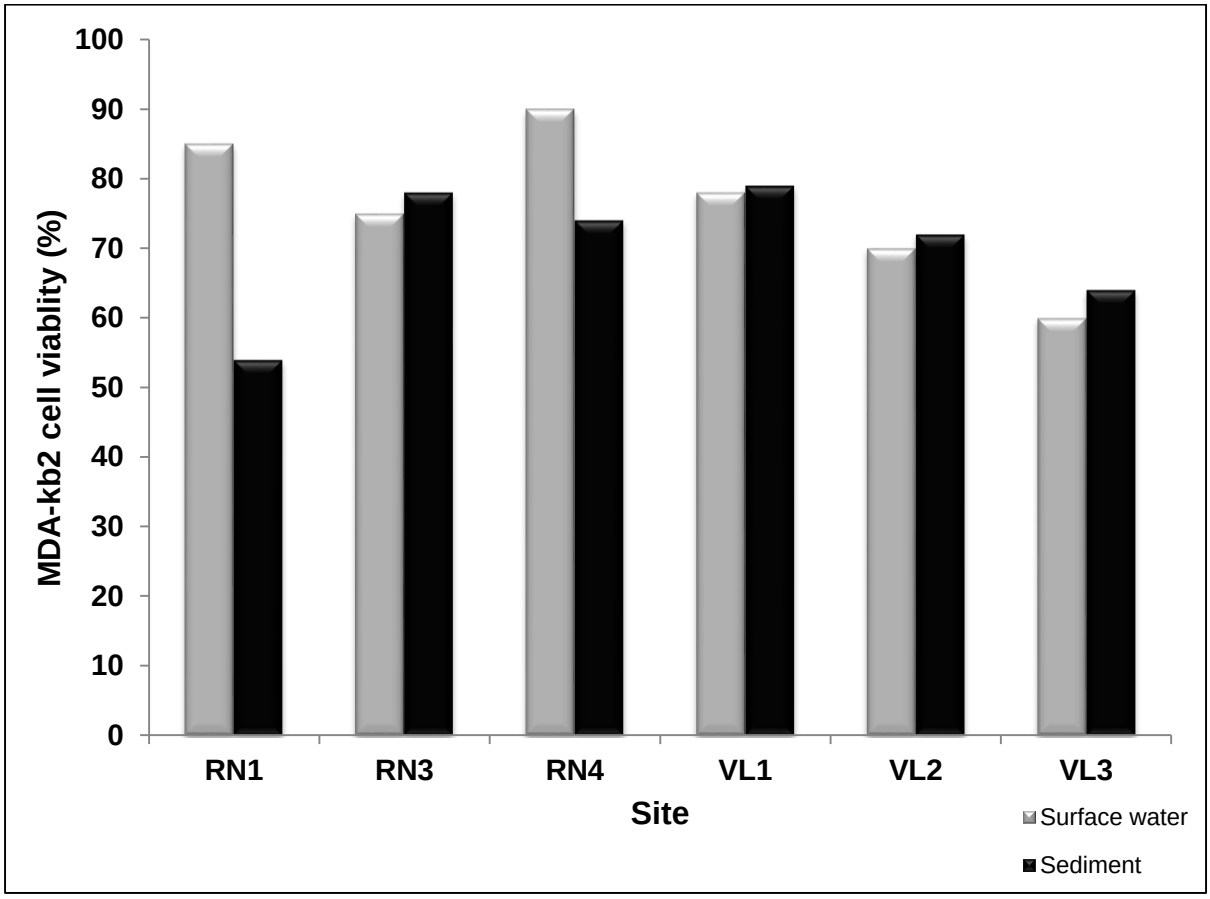

Figure 4.4: Cell viability of the MDA-kb2 cells exposed to the undiluted surface water and sediment sample extracts from July 2012.

None of the surface or groundwater samples elicited any (anti)androgenic or GR mediated responses from the cells even though the priority pesticides were detected in higher concentrations in the water samples compared to the sediment samples (Tables 4.9–4.11). The sediment samples, on the other hand, caused several (anti)androgenic and/or GR mediated reactions (Table 4.12). This potentially indicates that the priority pesticides were not responsible for the elicited reactions.

Table 4.11: MDA-kb2 reporter gene bio-assay results for the surface and groundwater samples from the Renoster and Vals River catchments.

Sampling period	Site	Activation (µg BEQ/ℓ water)	Inhibition (FI)	Viability (%)
July 2012	RN1	<LOD	NI	85
	RN3	<LOD	NI	**75**
	RN4	<LOD	NI	90
	VL1	<LOD	NI	**78**
	VL2	<LOD	NI	**70**
	VL3	<LOD	NI	**60**
October 2012	RN1	<LOD	NI	85
	RN3	<LOD	NI	90
	RN4	<LOD	NI	95
	VL1	<LOD	NI	100
	VL2	<LOD	NI	85
	VL3	<LOD	NI	95
January 2013	RN1	<LOD	NI	111
	RN3	<LOD	NI	93
	RN4	<LOD	NI	102
	VL1	<LOD	NI	112
	VL2	<LOD	NI	101
	VL3	<LOD	NI	**42**
April 2013	RN3	<LOD	NI	129
	RN4	<LOD	NI	126
	VL1	<LOD	NI	117
	VL2	<LOD	NI	106
	VL3	<LOD	NI	103
October 2013	VJK	<LOD	NI	123
	KOP	<LOD	NI	111
	KSD	<LOD	NI	106

<LOQ: Below Levels of Quantification; **FI:** Fold Inhibition; **NI:** No inhibition; **Viability: %** of the cells which received undiluted sample extract

All of the sediment samples collected in July 2012 caused androgen and/or glucocorticoid dependant gene transcription. Samples RN1, VL1 and VL3 activated the AR and/or GR since the samples also caused significant activation during the AR inhibition test (Table 3.9; Table 4.12). The light emission caused by samples RN3, RN4 and VL2 was probably due to the presence of an AR agonist since the samples didn't cause either inhibition or significant activation in the inhibition test (No. 1 or 7 in Table 3.9; Table 4.12). Samples RN1 and VL3 (most downstream site in the Vals River) also caused complete dose response curves, i.e. their maximum responses were comparable to the maximum response of the positive control (Figure 4.5; Table 4.12). The T-max values for these two samples were 91% and 78% respectively. Samples RN3 (T-max=51%), RN4 (T-max=45%) and VL1 (T-max=59%) elicited

responses comparable to 50% of the maximum response of the positive control while sample VL2 caused a response comparable to 20% of the maximum response of the positive control.

In October 2012 only sediment sample VL2 activated either the AR or the GR (or both; No. 5 in Table 3.9) and caused a response similar to one elicited by $4.8x10^{-3}$ µg/ℓ testosterone (T-max=21%; Table 4.12). In January 2013 all of the sediment samples collected in the Renoster River caused activation responses which could have been mediated by either the AR and/or the GR. Sample RN1 activated the AR and/or the GR (Table 4.12). Samples RN3 and RN4 most likely caused androgen dependant gene transcription since no significant activation were detected in the inhibition test. These three samples elicited responses from the cells comparable to 20% of the maximum response of the positive control.

The priority pesticides detected in the Renoster and Vals rivers have not been proven to activate AR (i.e. androgenic properties) or have glucocorticoid-like activity according to available literature. The activation responses were probably caused by another unidentified compound or mixture of compounds underlining that further research will have to be done to determine the source of the androgenicity and/or glucocorticoid-like activity in the Renoster and Vals River catchments. As a reminder the majority of the priority pesticides detected are herbicides which are highly water soluble and do not easily bind to organic carbon particles (Du Preez *et al*., 2005). If the pesticides were responsible for the androgenic reactions one would have expected these effects from the water samples, thus further emphasizing that something else potentially caused the reactions in the sediment samples.

The three Vals River samples collected in January 2013 caused significant activation of the AR during the inhibition test (Table 4.12) but no receptor activation was detected during the activation test. This may imply that small quantities of AR agonists were present in the samples and caused significant activation of the pre-activated MDA-kb2 cells (activation response was magnified) (Orton *et al*., 2009). These levels would potentially not have been detected if it wasn't for the already activated cells.

Only two sediment samples (RN4 and VL1) collected in October 2012 caused statistically significant inhibition of androgen binding (Table 4.12). Atrazine and terbuthylazine were the only pesticides detected and quantified in sample RN4, both at a concentration of $1x10^{-3}$ µg/kg which was relatively low compared to the levels detected in the water samples (Table 4.10).

Table 4.12: MDA-kb2 reporter gene bio-assay results for the sediment samples from the Renoster and Vals River catchments. Reported FI values were all statistically significant.

Sampling period	Site	Activation (µg BEQ/g sediment)	Inhibition (FI)	Viability (%)
July 2012	**RN1**	**22.6 ± 05.7**	↑*	**54**
	RN3	**3.7 ± 2.6**	NI	**78**
	RN4	**1.2 ± 0.6**	NI	**74**
	VL1	**4.2 ± 2.4**	↑*	**79**
	VL2	**44.7 ± 24.8**	NI	**72**
	VL3	**31.5 ± 03.9**	↑*	**64**
October 2012	**RN1**	<LOD	NI	105
	RN3	<LOD	NI	118
	RN4	<LOD	**1.46**	144
	VL1	<LOD	**1.15**	115
	VL2	**$4.8x10^{-3} \pm 5.6x10^{-3}$**	↑*	88
	VL3	<LOD	NI	97
January 2013	**RN1**	**$3.7x10^{-4} \pm 1.5x10^{-4}$**	↑*	125
	RN3	**$1.5x10^{-4} \pm 1.2x10^{-4}$**	NI	122
	RN4	**$1.7x10^{-4} \pm 2.8x10^{-5}$**	NI	123
	VL1	<LOD	↑*	85
	VL2	<LOD	↑*	**77**
	VL3	<LOD	↑*	93
April 2013	**RN1**	<LOD	NI	117
	RN3	<LOD	NI	105
	RN4	<LOD	NI	83
	VL1	<LOD	NI	97
	VL2	<LOD	NI	**77**
	VL3	<LOD	NI	**55**

<LOQ: Below Levels of Quantification; ↑***:** significant ($p<0.05$) activation response; **FI:** Fold Inhibition; **NI:** No inhibition; **Viability: %** of the cells which received undiluted sample extract

Atrazine and alachlor have been classified as pesticides with definite ED properties (Category 1 EDCs) by the European Commission while simazine is regarded as a Category 2 EDC (potentially an EDC; EUC, 1999; Matisova & Hrouzkova, 2012). In a study conducted by Kojima *et al*. (2004), during which 200 pesticides were tested for (anti)androgenicity in a reporter gene bio-assay using CHO cells, alachlor caused inhibition of AR binding. However, this pesticide was not detected in the samples that caused anti-androgenicity. Several studies have reported that the triazine pesticides (atrazine, simazine and terbuthylazine) are compounds that disrupt oestrogen activity and may also induce aromatase activity, which is a rate limiting enzyme in the reaction where androgens are converted to oestrogens (Kjeldsen *et al*., 2013; McKinley *et al*., 2008; Sanderson *et al*., 2001). Very little literature could be found on the (anti)androgenic properties of these pesticides. In a study done by Orton *et al*. (2009) atrazine and simazine were found to inhibit androgen binding in a yeast assay. In another

study done by Friedmann (2002) atrazine inhibited Leydig cell testosterone production in male rats which indicates the ability of the pesticide to interfere with androgen activity with mechanisms other than androgen-dependant gene transcription.

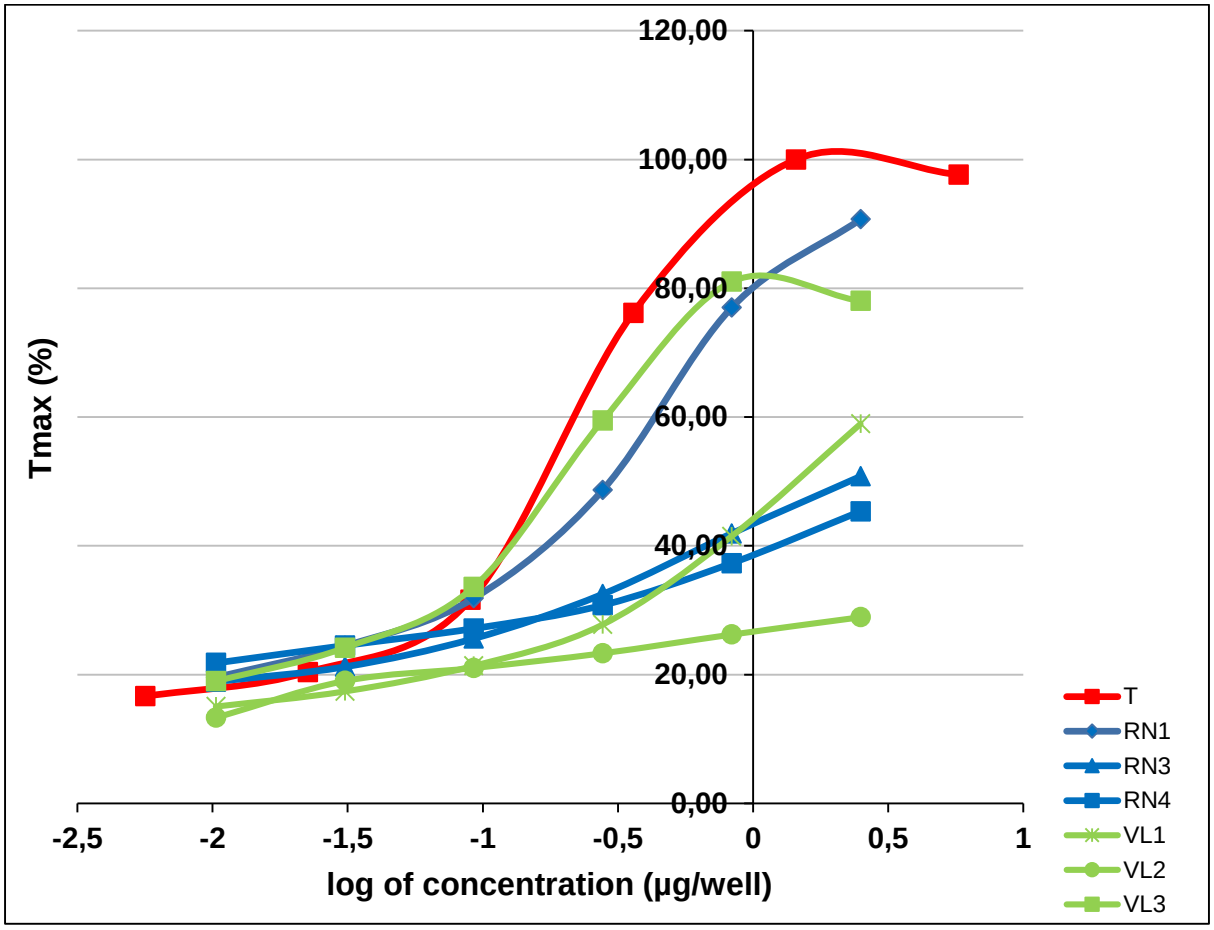

Figure 4.5: Activation of nuclear androgen receptors in the MDA-kb2 cells by sediment samples collected in the Renoster and Vals River catchments in July 2012. Samples from sites RN1 and VL3 elicited responses comparable to the maximum response of the positive control, testosterone (T). Standard deviations are not indicated as they cause distortion of the typical dose response shape of the curve. For reference see Table A–8.

4.4.3. Summary: Vals and Renoster River catchments

The priority pesticides atrazine, terbuthylazine and simazine were detected most frequently in the Renoster and Vals River catchments. The intensive cultivation of maize in these catchments explains why the triazine pesticides were detected in higher levels compared to other pesticides. Terbuthylazine was detected in a total of 45 water and sediment samples while atrazine was detected in 42 water and sediment samples. Simazine was placed in third position and was detected in 17 water and sediment samples.

The MDA-kb2 reporter gene bio-assay results indicated that the sediment samples collected during the July 2012 to January 2013 sampling events caused several instances of AR and/or GR activation responses. Some of the July 2012 sediment samples elicited relatively high responses from the cells even though the samples were cytotoxic. However, this phenomenon occurred only once. Sewage effluent originating from Kroonstad may have contributed to the positive as well as the cytotoxic responses elicited by the sediment samples collected at site VL2. The bio-assay data generated during this study could not be interpreted in terms of health effects (due to a shortage in regulatory guideline values for testosterone) therefore it was compared to the chemical analysis data. Even though atrazine and simazine have been classified as EDCs by the European Commission (EUC, 1999; Matisova & Hrouzkova, 2012), the (anti)androgen and/or glucocorticoid-like responses elicited from several samples cannot necessarily be ascribed to the presence of the pesticides since studies have shown the pesticides to mostly interfere with oestrogen pathways. Further investigation is therefore required to identify the compound(s) that may have been responsible for the responses.

4.5. Mzinti, Lomati and Ngweti River catchments

Extensive sugarcane and citrus production takes place in the Mzinti, Lomati and Ngweti River catchments as well as the small scale cultivation of various sub-tropical crops and grains (Section 3.2.3). All of the surface water samples collected during the June and September 2012 sampling trips were screened for the presence of the 291 pesticides (Table A–1). In December 2012 only water sample NK6 was screened since it is the most downstream site in the Lomati River and representative of the Lomati River's water quality. This site's sediment was also the only samples subjected to the chemical screening analysis throughout this study (Tables A–6 – A–7). The chemical quantification analysis results for the Mzinti, Lomati and Ngweti River catchments are presented in Table 4.13 (surface and groundwater) and Table 4.14 (sediment). Groundwater samples were only collected in November 2013 from areas surrounding the Komati River (Figure 3.3). An overview of the sites, sampling events and analyses conducted on each of the samples is presented in Table 3.5.

4.5.1. Chemical analysis

In June 2012 no pesticides were detected in the surface water samples while only ametryn and diphenylamine were detected in the sediment sample NK6 (Table A–7). The surface water sample results may potentially be due to the low flow, dry winter season during which sampling took place. Runoff and leaching are usually limited during that season. However, the September 2012 sampling event took place shortly after the rainfall season started and several pesticides were detected. Priority pesticides (atrazine, carbofuran, imidacloprid, terbuthylazine and thiabendazole) were selected for quantification. Although other pesticides

such as ametryn, diuron and hexazinone were also detected frequently (Table A–7), they couldn't be selected for quantification since no standards for chemical analysis were available at the time. However, their presence is expected since they are predominantly applied to sugar cane (Dabrowski *et al*., 2014) and they must therefore be included in future research.

Table 4.13: Chemical quantitative analysis results for the surface and groundwater samples from the Mzinti, Lomati and Ngweti River catchments.

Sampling period	Site	Atrazine (µg/ℓ)	Carbofuran (µg/ℓ)	Imidacloprid (µg/ℓ)	Terbuthylazine (µg/ℓ)	Thiabendazole (µg/ℓ)
June 2012	NK1	nd	nd	nd	nd	nd
	NK2	nd	nd	nd	nd	nd
	NK3	nd	nd	nd	nd	nd
	NK3TAP	nd	nd	nd	nd	nd
	NK5	nd	nd	nd	nd	nd
	NK6	nd	nd	nd	nd	nd
September 2012	NK1	$8x10^{-3}$	nd	nd	$4x10^{-3}$	nd
	NK2	$1x10^{-3}$	$3x10^{-4}$	nd	$4x10^{-3}$	nd
	NK3	$9x10^{-3}$	$5x10^{-3}$	$4x10^{-2}$	$4x10^{-3}$	$1x10^{-4}$
	NK3TAP	$8x10^{-3}$	$4x10^{-3}$	$4x10^{-2}$	$3x10^{-3}$	$1x10^{-4}$
	NK4	$9x10^{-2}$	$6x10^{-2}$	$1x10^{-2}$	$1x10^{-2}$	$7x10^{-3}$
	NK5	$3x10^{-3}$	nd	nd	$7x10^{-4}$	nd
	NK6	$2x10^{-2}$	$7x10^{-3}$	$7x10^{-2}$	$8x10^{-3}$	$1x10^{-4}$
December 2012	NK1	$2x10^{-2}$	$1x10^{-3}$	nd	$4x10^{-3}$	nd
	NK2	$3x10^{-3}$	$3x10^{-4}$	nd	$5x10^{-3}$	nd
	NK3	$1x10^{-2}$	$6x10^{-4}$	nd	$4x10^{-3}$	nd
	NK3TAP	$8x10^{-3}$	$3x10^{-4}$	nd	$2x10^{-3}$	nd
	NK4	$2x10^{-1}$	$8x10^{-4}$	nd	$2x10^{-2}$	nd
	NK5	$5x10^{-3}$	nd	nd	$3x10^{-3}$	nd
	NK6	$1x10^{-2}$	$3x10^{-4}$	nd	$4x10^{-3}$	nd
March 2013	NK1	$4x10^{-3}$	$1x10^{-4}$	$5x10^{-4}$	$7x10^{-4}$	nd
	NK2	$3x10^{-4}$	$3x10^{-4}$	$2x10^{-3}$	$1x10^{-3}$	nd
	NK3	$5x10^{-3}$	$4x10^{-4}$	$4x10^{-3}$	$3x10^{-3}$	$4x10^{-3}$
	NK3TAP	$5x10^{-3}$	$3x10^{-4}$	$3x10^{-3}$	$3x10^{-3}$	$1x10^{-4}$
	NK5	nd	$2x10^{-5}$	nd	$4x10^{-4}$	nd
	NK6	$4x10^{-3}$	$3x10^{-4}$	$2x10^{-3}$	$3x10^{-3}$	nd
November 2013	GW	nd	nd	nd	nd	nd
	KC	nd	nd	nd	nd	nd
	RH	nd	nd	nd	nd	nd

nd: not detected

Figure 4.6 illustrates the partitioning of each of the priority pesticides between the sediment and water matrices. The total number of detections for each pesticide was expressed as a percentage of the total sample value. A total of 29 surface and groundwater samples were collected from all three catchments as well as a total of 23 sediment samples. Atrazine, carbofuran and terbuthylazine were the most frequently detected pesticides in the surface

water samples (Table 4.13). Terbuthylazine was detected in 69% of the water samples while atrazine and carbofuran were detected in 66% and 59% of the water samples, respectively (Figure 4.6). Furthermore, atrazine and terbuthylazine were both detected in almost every surface water sample collected from September 2012 to March 2013 (atrazine was not detected in the March 2013 NK5 sample). Detected concentrations for these two pesticides ranged from the highest of $2x10^{-1}$ µg/ℓ for atrazine (NK4; December 2012) to the lowest of $4x10^{-4}$ µg/ℓ for terbuthylazine (NK5; March 2013). Atrazine was also the pesticide that was detected most frequently in the sediment samples (n=12).

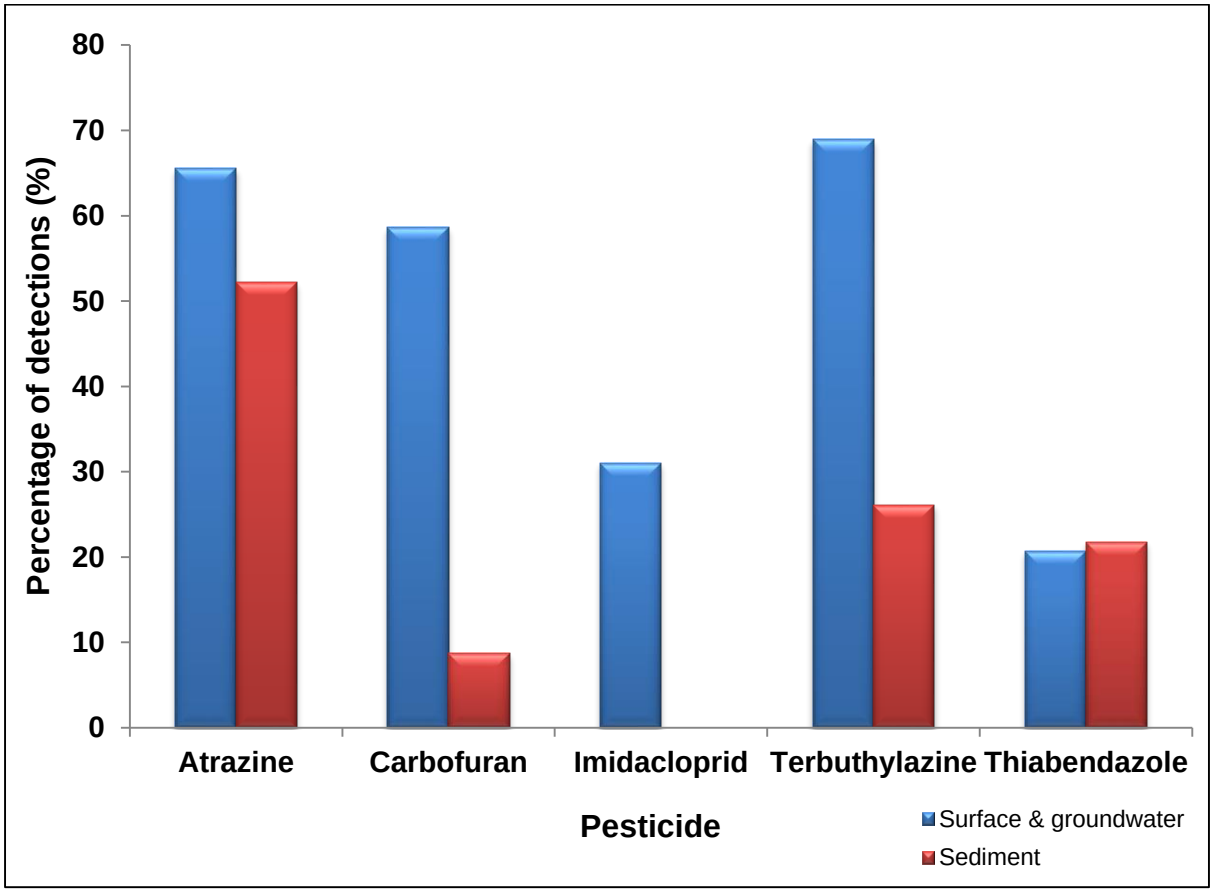

Figure 4.6: Percentage of priority pesticides detected in both the water and sediment matrices in the Mzinti, Lomati and Ngweti River catchments. A total of 29 surface and groundwater samples were collected as well as a total of 23 sediment samples.

Carbofuran was regularly detected in the surface water samples from September 2012 to March 2013 in concentrations comparable to those detected for atrazine and terbuthylazine. In September 2012 carbofuran was present in sample NK4 at a concentration of $6x10^{-2}$ µg/ℓ which is identical to the Australian TV of 0.06 µg/ℓ (Table 4.3). The TV attempts to ensure aquatic ecosystem health. Since this was an isolated occurrence no significant conclusion can be made regarding the health effects of carbofuran on the ecosystem at site NK4, situated

in the Ngweti River. None of the pesticide concentrations detected at any of the sites exceeded regulatory guidelines.

Surface water samples at NK3 were collected from the Lomati River at the intake point of the Langeloop water purification works. The same pesticides that were detected in this sample were also detected in the tap water sample collected on the premises of the purification works (NK3TAP). The concentrations of the pesticides in the tap water samples were furthermore only slightly lower compared to those detected in the river sample (Table 4.13). It therefore seems like the pesticides were barely removed during the purification process and distributed to the town of Langeloop where the water supply is used for domestic purposes.

Water sampled at site NK4 in September 2012 contained levels of the pesticides atrazine, carbofuran, terbuthylazine and thiabendazole that were between ten and a hundred times higher compared to the levels detected in the other samples from the same sampling period. This also occurred in December 2012 with regards to the atrazine and terbuthylazine levels. Site NK4 is the most downstream site in the Ngweti River just before its confluence with the Komati River. High volume pesticide use due to intensive agricultural practices and increased runoff and/or leaching potential may be the reason for the greater pesticide levels at this site during the rainfall season.

None of the priority pesticides were detected at quantifiable levels in any of the groundwater samples even though the sites are located in the vicinity of commercial crop cultivation. Further research may be required to determine if the pesticide applications in the area have a significant effect on the groundwater quality since sampling only occurred once.

With regards to the sediment samples the pesticides were detected less frequently and in lower concentrations compared to levels detected in the water samples. Only atrazine was detected at more than 50% of the sites (Figure 4.6). Imidacloprid wasn't present in any of the samples. During the September 2012 sampling trip, atrazine and terbuthylazine were detected in sample NK4 while carbofuran and thiabendazole were also found in December 2012 and March 2013. The high frequency of pesticide detection at this site in both matrices coincides with the intensive agricultural practices in the eastern parts of the Ngweti and Komati River catchments. It also deserves further investigation since human and animal exposure potential increase when medium to highly mobile pesticides are used in high quantities (Dabrowski *et al*., 2014).

Atrazine, carbofuran and terbuthylazine have been included as priority pesticides in the study conducted by Dabrowski *et al*. (2014). Only 7.5% of the total quantity of atrazine used in South Africa is used on sugar cane while this crop accounts for 0.8% of the total quantity of

terbuthylazine used. Imidacloprid and thiabendazole are commonly applied to citrus (Dabrowski *et al*., 2014; US EPA, 2002). Carbofuran is generally used on crops such as maize, potatoes, sunflowers and sorghum amongst others (CCME, 1999b) but not known for its use on sugar cane. The frequent detection of carbofuran therefore not only proves its high mobility but also suggests the large scale production of other crops apart from sugar cane and citrus.

Table 4.14: Chemical quantitative analysis results for the sediment samples from the Mzinti, Lomati and Ngweti River catchments.

nd: not detected

Sampling period	Site	Atrazine (µg/kg)	Carbofuran (µg/kg)	Imidacloprid (µg/kg)	Terbuthylazine (µg/kg)	Thiabendazole (µg/kg)
June 2012	NK1	**$1x10^{-3}$**	nd	nd	nd	nd
	NK2	nd	nd	nd	nd	nd
	NK3	nd	nd	nd	nd	nd
	NK5	nd	nd	nd	nd	nd
	NK6	nd	nd	nd	nd	nd
September 2012	NK1	**$1x10^{-3}$**	nd	nd	nd	nd
	NK2	nd	nd	nd	**$5x10^{-3}$**	nd
	NK3	**$1x10^{-3}$**	nd	nd	nd	**$2x10^{-4}$**
	NK4	**$3x10^{-3}$**	nd	nd	**$5x10^{-4}$**	nd
	NK5	nd	nd	nd	nd	nd
	NK6	**$1x10^{-3}$**	nd	nd	nd	nd
December 2012	NK1	**$2x10^{-4}$**	nd	nd	nd	nd
	NK2	nd	nd	nd	nd	**$1x10^{-4}$**
	NK3	**$4x10^{-4}$**	nd	nd	**$2x10^{-4}$**	nd
	NK4	**$1x10^{-3}$**	**$2x10^{-4}$**	nd	**$2x10^{-4}$**	**$2x10^{-4}$**
	NK5	nd	nd	nd	nd	nd
	NK6	nd	nd	nd	nd	nd
March 2013	NK1	nd	nd	nd	nd	nd
	NK2	**$1x10^{-4}$**	nd	nd	nd	nd
	NK3	**$3x10^{-4}$**	nd	nd	nd	nd
	NK4	**$1x10^{-3}$**	**$5x10^{-4}$**	nd	**$3x10^{-4}$**	**$3x10^{-4}$**
	NK5	nd	nd	nd	nd	nd
	NK6	**$5x10^{-4}$**	nd	nd	**$4x10^{-4}$**	**$1x10^{-4}$**

4.5.2. MDA-kb2 reporter gene bio-assay

The surface water and sediment samples collected in June 2012 as well as the surface water sampled in September 2012 were screened for (anti)androgenic activity as well as GR activation during bio-assay period 1 (BAP1; Table 3.7). All of the surface water samples from June 2012 were cytotoxic to some extent (Table 4.15). The tap water collected from site NK3 was the most cytotoxic compared to the other samples since only 58% of the cells survived exposure to the undiluted sample. As no pesticides were detected in the June 2012 water samples the cytotoxicity may have been the result of other unknown compounds in the sample

extract. Furthermore, none of the other water samples collected during the rest of the study caused any cytotoxicity.

Table 4.15: MDA-kb2 reporter gene bio-assay results for the surface and groundwater samples from the Mzinti, Lomati and Ngweti River catchments. Reported FI values were all statistically significant.

Sampling period	Site	Activation (µg BEQ/ℓ water)	Inhibition (FI)	Viability (%)
June 2012	**NK1**	<LOD	NI	**79**
	NK2	<LOD	NI	**72**
	NK3	<LOD	NI	**64**
	NK3TAP	<LOD	NI	**54**
	NK5	<LOD	NI	**78**
	NK6	<LOD	NI	**74**
September 2012	**NK1**	<LOD	NI	100
	NK2	<LOD	NI	87
	NK3	<LOD	NI	100
	NK3TAP	<LOD	NI	80
	NK4	<LOD	NI	100
	NK5	<LOD	NI	100
	NK6	<LOD	NI	83
December 2012	**NK1**	**$2.8x10^{-11} \pm 7.0x10^{-12}$**	↑*	103
	NK2	<LOD	NI	102
	NK3	<LOD	NI	112
	NK3TAP	<LOD	NI	108
	NK4	<LOD	NI	110
	NK5	<LOD	NI	108
	NK6	**$2.2x10^{-11} \pm 8.8x10^{-12}$**	NI	114
March 2013	**NK1**	<LOD	NI	124
	NK2	<LOD	NI	114
	NK3	<LOD	NI	112
	NK3TAP	<LOD	NI	129
	NK5	**$2.0x10^{-11} \pm 1.5x10^{-11}$**	↑*	111
	NK6	**$8.1x10^{-12} \pm 3.0x10^{-12}$**	NI	105
November 2013	**GW**	<LOD	NI	107
	KC	<LOD	NI	98
	RH	<LOD	NI	113

<LOQ: Below Levels of Quantification; ↑***:** significant ($p<0.05$) activation response; **FI:** Fold inhibition; **NI:** No inhibition; **Viability: %** of the cells which received undiluted sample extract

The sediment samples from sites NK3, NK5 and NK6 collected in June 2012 as well as sample NK3 from the March 2013 sampling event were also cytotoxic (Table 4.16). Sample NK3 caused complete cell death in June 2012 while only 44% of the cells survived exposure in March 2013. This occurrence was potentially due to the build-up of chemical compounds in the sediment during the dry, low flow winter seasons. Site NK3 is located downstream of

agricultural practices, several towns, Driekoppies Dam and situated adjacent to the town of Langeloop. Anthropogenic pollution (*e.g*. point source or diffuse) as well as agricultural runoff and/or leaching may possibly be the sources of cytotoxic compounds (*e.g*. that were not analysed for) at site NK3 in the Lomati River.

Only four surface water samples caused the cells to react positively and emit light. Samples NK1 (December 2012) and NK5 (March 2013) activated the AR and/or GR because the same samples significantly activated the cells in the inhibition test (Table 4.15). Sample NK6 activated the AR and/or GR in December 2012 and in March 2013 (No. 1 or 6 in Table 3.9). All of the responses were comparable to 20% of the maximum response of the positive control testosterone (Table 4.15) and relatively weak compared to the activation responses elicited by samples from the other study areas. These results were furthermore generated during the more sensitive BAP2 and therefore support the use of the MDA-kb2 reporter gene bio-assay as a sensitive screening tool for receptor mediated reactions.

Atrazine, carbofuran and terbuthylazine were predominantly detected in the samples that elicited responses from the cells. Carbofuran potentially has ED properties and has been classified as a Category 2 EDC by the European Commission (EUC, 1999; Matisova & Hrouzkova, 2012). Carbofuran was tested for its ability to activate AR and/or inhibited androgen binding but the pesticide didn't elicit any responses in the CHO cells (Kojima *et al*., 2004). No additional literature could be found regarding the ability of carbofuran to interfere with androgen-dependant gene transcription. However, studies have shown that carbofuran has an acute dwindling effect on testosterone levels. In a study using Sprague-Dawley rats, an acute dose (1.5 mg/kg) of carbofuran decreased testosterone levels by 88% (Goad *et al*., 2004). Neither atrazine nor terbuthylazine have been proven to activate the AR. As mentioned earlier no literature was discovered regarding glucocorticoid-like activity of any of the priority pesticides. The responses of the cells to the samples collected for this study can therefore not be ascribed to a specific compound.

The sediment sample collected at site NK6 in September 2012 as well as samples NK4 and NK5 (March 2013) activated the AR and/or GR (Table 3.9) because the samples also caused significant activation in the inhibition test (Table 4.16). Two other samples (NK1 and NK6 from March 2013) activated either the AR and/or the GR. The fact that there was no inhibition or significant activation (compared to the SC) in the inhibition test makes either deduction number 1 or 6 in Table 3.9 relevant. The BEQ values were comparable to those calculated for the surface water samples. Since none of the detected pesticides have been proven to activate the AR or GR in reporter gene bio-assays and no pattern of pesticide detection occurred in any of these samples it is difficult to link the MDA-kb2 reporter gene bio-assay data with the

chemical analysis data. Samples NK5 (September 2012) and NK2 (March 2013) inhibited androgen binding to the AR statistical significantly (Table 4.16).

Table 4.16: MDA-kb2 reporter gene bio-assay results for the sediment samples from the Mzinti, Lomati and Ngweti River catchments. Reported FI values were all statistically significant.

Sampling period	Site	Activation (µg BEQ/g sediment)	Inhibition (FI)	Viability (%)
June 2012	**NK1**	<LOD	NI	111
	NK2	<LOD	NI	118
	NK3	<LOD	NI	**0**
	NK5	<LOD	NI	**79**
	NK6	<LOD	NI	**74**
September 2012	**NK1**	<LOD	NI	96
	NK2	<LOD	NI	83
	NK3	<LOD	NI	81
	NK4	<LOD	NI	91
	NK5	<LOD	**1.26**	99
	NK6	**$8.8x10^{-5} \pm 2.3x10^{-5}$**	↑*	97
December 2012	**NK1**	<LOD	NI	102
	NK2	<LOD	NI	108
	NK3	<LOD	NI	133
	NK4	<LOD	NI	114
	NK5	<LOD	NI	118
	NK6	<LOD	NI	107
March 2013	**NK1**	**$6.6x10^{-22} \pm 7.4x10^{-22}$**	NI	92
	NK2	<LOD	**1.13**	106
	NK3	<LOD	NI	**44**
	NK4	**$1.2x10^{-09} \pm 2.0x10^{-09}$**	↑*	100
	NK5	**$3.8x10^{-09} \pm 6.6x10^{-09}$**	↑*	120
	NK6	**$9.7x10^{-20} \pm 1.6x10^{-19}$**	NI	100

<LOQ: Below Levels of Quantification; ↑***:** significant ($p<0.05$) activation response; **FI:** Fold Inhibition; **NI:** No inhibition; **Viability: %** of the cells which received undiluted sample extract

4.5.3. Summary: Mzinti, Lomati and Ngweti River catchments

In terms of anthropogenic pollution, fairly few problems have been reported in the literature with regards to the quality of the Lomati and Komati Rivers since there are limited industrial and urban areas in the catchments (Van der Laan *et al*., 2012). Agricultural activities are therefore considered to be the main source of chemical pollution. The priority pesticides atrazine, carbofuran, imidacloprid, thiabendazole and terbuthylazine were selected as priority pesticides in the Mzinti, Lomati and Ngweti River catchments. Atrazine was the most frequently detected pesticide and occurred in 31 surface water and sediment samples while terbuthylazine followed in second place with a total detection number of 26. Carbofuran was detected in 19 water and sediment samples collectively. The high frequency of detection

makes sense since these pesticides have been described as highly mobile in the environment (Dabrowski *et al*., 2014).

The MDA-kb2 bio-assay results indicated that several water and sediment samples caused weak androgenic and glucocorticoid-like responses (BEQ values were low compared to the responses elicited by samples from the other study areas). The responses could not be linked to specific detected pesticides but the bio-assay results did however support the suggestion that the MDA-kb2 reporter gene bio-assay can be used as a sensitive screening tool.

4.6. Link between the study areas, priority pesticides and MDA-kb2 reporter gene bio-assay results

More than 50% of the pesticides detected and selected for quantification during this study have been included in the list of agricultural priority pesticides used in South Africa (Dabrowski *et al*., 2014). In Table 4.1 the ten priority pesticides, as well as the catchment(s) in which they were detected, are summarized. Selection of the pesticides were mainly based on the frequency of detection, standards available for quantification and priority in terms of important crops to which it applies in South Africa, quantity of use and potential to cause possible human and environmental health effects.

Imidacloprid was the only pesticide that was detected in all three study areas. Since maize and citrus accounts for most of the total quantity used (11.5% and 68.2% respectively; Dabrowski *et al*., 2014) the frequent detection makes sense as one or both of these crops are cultivated in all of the catchments under study. The highest imidacloprid concentration of 1.9 µg/ℓ was detected in the groundwater sample, FM, in the Letsitele River catchment in September 2013. This concentration was approximately 8 times higher compared to the Canadian WQG of 0.23 µg/ℓ for imidacloprid in aquatic ecosystems (CCME, 2014).

More than half of the priority pesticides selected for this study have been ranked as an EDC by the European Commission based on the available evidence to accurately confirm the ED properties of the pesticide. Atrazine and alachlor have been proven as definite EDCs (Category 1 EDC), simazine and carbofuran has been described as potential EDCs (Category 2 EDC) while imazalil and propiconazole have been classified as Category 3 EDCs since there is not enough evidence to prove their ED properties (EUC, 1999; Matisova & Hrouzkova, 2012). The EDC categories assign definite or potential ED properties to the pesticides but not necessarily (anti)androgenic activity. Most of the priority pesticides which were screened for during this study have not been proven to be definite (anti)androgens with regards to the specific mechanism of action detectable by the MDA-kb2 cell line. However, studies have

shown propiconazole and imazalil to have anti-androgenic effects since it inhibited binding to the AR (Kjærstad *et al*., 2010; Kojima *et al*., 2004; Okubo *et al*., 2004; Orton *et al*. 2011).

The water samples collected in the Letsitele River catchment elicited the most combined (anti)androgenic and/or GR mediated responses (n=8) from the cells compared to the water samples from the other study areas. The sediment samples from this catchment also caused the most cases of anti-androgenicity (n=9). A total of 10 sediment samples collected from the Renoster and Vals River catchments elicited positive responses from the MDA-kb2 cells and either activated the AR or the GR, or both.

Overall the combined water and sediment samples from the Letsitele River catchment caused the most positive and negative responses (n=18). The Mzinti, Lomati and Ngweti River catchments water and sediment samples caused the lowest number of positive and negative responses (n=11). There was no response pattern between any of the sites in any of the study areas either on a seasonal basis or between sites from the same sampling period.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

The MDA-kb2 reporter gene bio-assay that was used in this study has shown to be a sensitive screening tool to measure the specific mechanism of receptor (AR and/or GR) dependant gene transcription. The bio-assay can measure the ability of a compound (or a mixture of compounds) to either activate the AR (androgenic response) or inhibit binding of androgens to the AR (anti-androgenic responses). The assay can also detect whether compounds (or a mixture of compounds) have glucocorticoid-like activity since the cells also express endogenous GRs. When the assay is used to screen for (anti)androgen activity the experimental method must include a control (*e.g*. hydroxyflutamide) that will distinguish between the two receptor mediated responses (Wilson *et al*., 2002).

Several surface water and sediment samples from each catchment under study (from different sampling occasions), elicited responses from the MDA-kb2 cells. In this case the bio-assay gave a total effect (and not the effects of single compounds) regarding the chemical mixtures that were screened for (anti)androgenic and GR activity. Since the cells were sensitive, low levels of androgen-like, glucocorticoid-like and/or anti-androgens could be detected. This is important when testing for EDCs that occur below the regulatory limits since small quantities of EDCs can have major health effects, especially at sensitive developing life stages (Bergman *et al*., 2012; McKinley *et al*., 2008; Pletsche *et al*., 2011).

The water and sediment samples were also subjected to analytical chemistry analyses. The selection of the priority pesticides were based on the frequency of occurrence throughout the three study areas as well as on the intensity of their use in SA. Only two of these pesticides (atrazine and alachlor) have been proven and classified as definite EDCs (Category 1; EUC, 1999; Matisova & Hrouzkova, 2012). None of the priority pesticides have been proven to have definite (anti)androgenic activity. However, studies have shown atrazine, imazalil and propiconazole to have anti-androgenic effects since it inhibited binding to the AR (Kjærstad *et al*., 2010; Kojima *et al*., 2004; Okubo *et al*., 2004; Orton *et al*. 2011). Yet, AR inhibition responses could not be linked to the presence of any of these pesticides since no obvious patterns occurred between samples from the same sampling events or between the different sampling events which occurred on a seasonal basis.

In general it was difficult to correlate the bio-assay and analytical chemistry results since no seasonal patterns between samples from the same sampling trip or between samples from different sampling events were evident. It was also challenging to compare the analytical chemistry results to current pesticide guidelines for water and sediment matrices since there is a lack in applicable and comprehensive regulatory guidelines, especially in a South African

context. There is also a lack in androgen dependant health risk assessment and applicable guidelines to make interpretation of bio-assay results possible. In this study the BEQ values could not be interpreted in terms of human and animal health. Fortunately the data is available for future EDC studies. The lack of applicable regulatory legislation possibly leaves South Africa without the capacity to address and regulate a potential public health problem (London *et al*., 2005).

As mentioned earlier in this dissertation South Africa is a major user of pesticides (PAN, 2014) since the country has a very large agricultural industry. The high intensity of use of pesticides contaminates South Africa's freshwater systems. Studies that have been done in the country have detected widespread pesticide pollution (although at low levels) of surface and groundwater (Du Preez *et al*., 2005; Schulz, 2001). If these pesticides have ED activity it may affect the health of humans and wildlife that use the water for drinking and recreational purposes (Burger & Nel, 2008; McKinley *et al*., 2008). However, at the present moment there are still many pesticides (and other chemical compounds) that have not been tested for their ability to disrupt endocrine systems (Burger & Nel, 2008; Bergman *et al*., 2012). Furthermore, studies and experience have indicated that the poor and marginalised people in a country are usually those that carry the most consequences as a result of environmental pollution. Therefore, they also benefit the most from access to safe, treated water which can be used as an intervention to alleviate poverty in a country (London *et al*., 2005; WHO, 2011).

Recommendations

- A major improvement to the bio-assay section of this study would be to include a way of distinguishing between AR and GR activation. This can be done by repeating the test for activation by adding an AR inhibiting ligand such as hydroxyflutamide. If light is created again after exposure to the same concentration of ligand in the sample extract, the conclusion can be made that the extract contained GR agonists only.
- South Africa is in urgent need of research regarding EDCs and specifically towards pesticides with ED activity. The development of context specific water and sediment guidelines will make regulation and public health crises prevention more feasible.
- The development of environmental guidelines for chemicals that mimic testosterone, as well as levels at which health effects can be expected, will make interpretation and application of (anti)androgen bio-assay results possible.
- Future chemical and bio-assay screening tests with regards to pesticides in water systems can also include adjuvants since these compounds occur in the pesticide formulation and may potentially contribute to ED related health effects.

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ANNEXURES

Table A–1: List of pesticides which were included in the qualitative screening analysis.

Abamectin	Butoxycarboxim	Diazinon	Fenazaquin	Hydroxycarbofuran 3-	Mevinphos	Propamocarb HCl	Terbuthialzine
Ac DON 3	Cadusafos	Dicamba	Fenbuconazole	Imazalil	Monocrotophos	Propanil	Terbuthialzine desethyl
Acephate	CAP	Dichlorvos	Fenhexamid	Imazamox	Monolinuron	Propaquizafop	Terbuthylazine
Acetamiprid	Carbaryl	Dicrotophos	Fenobucarb	Imazapyr	Monuron	Propargite	Terbutryn
Afla B1	Carbendazim	Dieldrin	Fenoprop (2,4,5-T)	Imidacloprid	Myclobutanil	Propazine	Tetrachlorvinphos
Afla B2	Carbetamide	Difenoconazole	Fenothiocarb	Indoxacarb	Naled	Propiconazole	Tetraconazole
Afla G1	Carbofuran	Diflubenzuron	Fenoxap-p-ethyl	Ioxynil	Nicosulfuron	Propoxur	Thiabendazole
Afla G2	Carbosulfan	Diflufenican	Fenoxycarb	Iprodione	Nitenpyram	Propyzamide	Thiacloprid
Alachlor	Carboxin	Dimefuron	Fenpropimorph	Iprovalicarb	NIV	Prosulfuron	Thiamethoxam
Alanycarb	Chlorfenvinphos	Dimepiperate	Fenpyroximate	Isofenfos	Omethoate	Pyriproxyfen	Thidiazuron
Aldicarb	Chloridazon	Dimethachlor	Fenthion	Isoprocarb	OTA	Pyraclostrobin	Thiobencarb
Aldicarb sulphone	Chloropropham	Dimethenamide	Fenuron	Isoproturon	Oxadixyl	Pyributicarb	Thiodicarb
Aldicarb-sulfoxide	Chlorotoluron	Dimethoate	Flazasulfuron	Isoxaflutole	Oxamyl	Pyridate	Thiofanox
Aldrin	Chlorpyrifos	Dimethomorph	Florosulam	Lenacil	Oxydemeton methyl	Pyrimethanil	Thiofanox-sulfone
Alpha-BHC	Chlorsulfuron	Dinoterb	Fluazifop	Linuron	Paclobutrazol	Quinmerac	Thiophanate methyl
Alpha-chlordane	Chlortoluron	Dioxacarb	Fluazifop-p-butyl	Lufenuron	Parathion	Quinoxyfen	Thiuram
Ametryn	Clofentezine	Diphenylamine	Fluazinam	Malaoxon	Parathion-methyl	Quizalofop-p-ethyl	Tokuthion
Aminocarb	Clomazone	Disulfoton	Fludioxonil	Malathion	PCP	Resmethrine	Tolylfluanid
Atrazine	Clopyralid	Diuron	Flufenacet	MCPA	Penconazole	Rimsulfuron	TP 2,4,5-
Azinphos methyl	Clothianidin	DON	Flufenoxuron	MCPP (Mecoprop)	Pencycuron	Sebuthylazine	Triadiminol
Azinphos ethyl	Coumaphos	DP 2,4-	Flumeturon	Mecarbam	Pendimethalin	Sethoxydim	Triallate
Azoxystrobin	Cyanazine	Endosulfan I (Alpha)	Fluometuron	Mepanipyrim	Phenmedipham	Siduron	Triazophos
Benalaxyl	Cymoxanil	Endosulfan II (Beta)	Fluroxypyr	Metalaxyl	Phenthoate	Simazine	Trichlorofon
Bendiacarb	Cyproconazole	Endosulfan sulfate	Furathiocarb	Metamitron	Phorate	Simetryn	Triclopyr
Benfuracarb	Cyprodinil	Endrin	FUS X	Metazachlor	Phorate sulfoxide	Spinosad A	Trifloxystrobin
Benomyl	D 2,4-	Endrin aldehyde	Gamma-BHC	Methabenzthiazuron	Phosalone	Spinosad D	Triflumuron
Bentazon	DAS	Endrin ketone	Gamma-chlordane	Methamidophos	Phoxim	Spiroxamin	Triforine
Beta-BHC	DB 2,4-	Epoxiconazole	Haloxyfop	Methiocarb	Picoxystrobin	Sulfotep	XMC
Bitertanol	DDD 4,4'-	EPTC	Haloxyfop methyl	Methiocarb-sulfone	Pirimicarb	Sulprofos	ZON
Boscalid	DDE 4,4'-	Esprocarb	Heptachlor	Methomyl	Pirimiphos-ethyl	T2	
Bromacil	DDT 4,4'-	Ethiofencarb	Heptachlor epoxide isomer B	Methoxychlor	Pirimiphos-methyl	Tebuconazole	
Bromoxynil	Delta-BHC	Ethiofencarb-sulfone	Heptenophos	Methoxyfenozide	Prochloraz	Tebuconazide	
Bromuconazole	Demeton-S-methyl	Ethiofencarb-sulfoxide	Hexaconazole	Metobromuron	Promecarb	Tebufenpyrad	
Bupirimate	Demeton-S-methyl-sulfone	Etofenprox	Hexaflumuron	Metolachlor	Prometon	Tebuthiuron	
Buprofezine	Desethyl atrazine	Etoxazole	Hexazinone	Metosulam	Prometryn	Teflubenzuron	
Butocarboxim	Desisopropyl atrazine	Famoxadone	Hexythiazox	Metoxuron	Propachlor	Tepraloxydim	
Butocarboxim-sulfoxide	Desmedipham	Famphur	HT 2	Metribuzin	Propamocarb	Terbucarb	

Table A–2: Results of pesticide screening analysis for surface water samples collected from the Letsitele River Catchment. The presence of the pesticide is indicated with an 'X'.

PESTICIDE/ SITE	Diphenylamine	Imazalil	Thiabendazole	Imidacloprid	Propiconazole	Carbendazim	Fenuron	Flusilazole	Prochloraz	Sulfmethazine
JULY 2011										
LT1				X						
LT2	X									
LT3										
LT4							X			
LT6	X									
LT7	X									
LT8		X	X							
LT9										
LT10				X						
NOVEMBER 2011										
LT1	X			X						
LT2										
LT3									X	
LT4	X	X	X		X	X		X		
LT6										X
LT7										
LT8					X					
LT9	X									
LT10	X				X					
AUGUST 2012										
LT4										
LT9										
NOVEMBER 2012										
LT4										
LT9										
FEBRUARY 2013										
LT4										
LT9										

Table A–3: Results of pesticide screening analysis for sediment samples collected from the Letsitele River Catchment. The presence of the pesticide is indicated with an 'X'.

PESTICIDE/ SITE	Diphenylamine	Imazalil	Thiabendazole	Imidacloprid	Propiconazole	Carbendazim	Fenuron	Flusilazole	Prochloraz	Sulfmethazine
JULY 2011										
LT1					X					
LT2	X									
LT3	X				X					
LT7					X					
LT8		X			X					
LT9					X					
LT10		X			X					
NOVEMBER 2011										
LT1					X					
LT2					X					
LT3	X									
LT7			X							
LT8	X	X	X							
LT9			X							
AUGUST 2012										
LT9										
NOVEMBER 2012										
LT9										

Table A–4: Results of pesticide screening analysis for surface water samples collected from the Renoster and Vals River Catchments. The presence of the pesticide is indicated with an 'X'.

PESTICIDE/ SITE	Alachlor	Atrazine	Imidacloprid	Simazine	Terbuthylazine	Popazyn-2-hydroxy	Tebuthiuron	Metolachlor	Terbutryn	Prometon
FEBRUARY 2012										
RN4	X		X	X	X					
VL3		X		X	X		X	X	X	X
JULY 2012										
RN4		X			X	X	X	X	X	
VL3		X		X	X		X		X	X
OCTOBER 2012										
RN4		X			X					
VL3				X	X		X			X
JANUARY 2013										
RN4		X			X		X			
VL3		X		X	X				X	
APRIL 2013										
RN4		X			X		X		X	
VL3		X		X	X				X	X

Table A–4 continued:

PESTICIDE/ SITE	Diphenylamine	Cyanazine	Ametryn	Carbendazim	Thiabendazole	Propazine	Atrazine-diesypropyl	Terbuthylazine-desethyl
FEBRUARY 2012								
RN4				X	X	X	X	X
VL3			X				X	X
JULY 2012								
RN4							X	
VL3								X
OCTOBER 2012								
RN4	X	X						
VL3	X							
JANUARY 2013								
RN4								
VL3								
APRIL 2013								
RN4								X
VL3							X	

Table A–5: Results of pesticide screening analysis for sediment samples collected from the Renoster and Vals River Catchments. The presence of the pesticide is indicated with an 'X'.

PESTICIDE/ SITE	Alachlor	Atrazine	Imidacloprid	Simazine	Terbuthylazine	Popazyn-2-hydroxy	Tebuthiuron	Metolachlor	Terbutryn	Prometon
JULY 2012										
RN4					X					
VL3										
OCTOBER 2012										
RN4					X					
VL3										
JANUARY 2013										
RN4										
VL3										
APRIL 2013										
RN4										
VL3										

Table A–5 continued:

PESTICIDE/ SITE	Diphenylamine	Cyanazine	Ametryn	Carbendazim	Thiabendazole	Propazine	Atrazine-diesypropyl	Terbuthylazine-desethyl
JULY 2012								
RN4	X							
VL3	X							
OCTOBER 2012								
RN4	X							
VL3	X							
JANUARY 2013								
RN4								
VL3								
APRIL 2013								
RN4								
VL3								

Table A–6: Results of pesticide screening analysis for surface water samples collected from the Mzinti, Lomati and Ngweti River Catchments. The presence of the pesticide is indicated with an 'X'.

PESTICIDE / SITE	Atrazine	Imidacloprid	Terbuthylazine	Thiabendazole	Carbofuran	Diuron	Hexazinone	Ametryn	Diphenylamine	Prometryn	Metribuzin	Metolachlor
JUNE 2012												
NK1												
NK2												
NK3												
NK3-TAP												
NK4												
NK5												
NK6												
SEPTEMBER 2012												
NK1	X		X									
NK2								X				
NK3		X	X			X	X	X		X		
NK3-TAP	X	X	X			X	X					
NK4	X	X	X		X	X		X		X	X	X
NK5	X		X									
NK6	X	X	X		X	X		X				
DECEMBER 2012												
NK6	X		X					X				
MARCH 2013												
NK3												
NK6												

Table A–7: Results of pesticide screening analysis for sediment samples collected from the Mzinti, Lomati and Ngweti River Catchments. The presence of the pesticide is indicated with an 'X'.

PESTICIDE/ SITE	Atrazine	Imidacloprid	Terbuthylazine	Thiabendazole	Carbofuran	Diuron	Hexazinone	Ametryn	Diphenylamine	Prometryn	Metribuzin	Metolachlor
JUNE 2012												
NK6								X	X			
SEPTEMBER 2012												
NK6								X				
DECEMBER 2012												
NK6												
MARCH 2013												
NK6								X				

Table A–8: Basic statistics for the water and sediment samples that elicited a luminescence response from the MDA-kb2 cells and for which dose response curves were reported in Chapter 4 (Figures 4.2 and 4.5).

Sample	Log (µℓ/well)	Mean RLU	Std Dev	Tmax
Letsitele River catchment Augustus 2012 Surface water				
LT3	0.40	631493.67	42482.67	146.20
	-0.08	646796.00	80137.96	149.74
	-0.56	445313.67	92137.75	103.10
	-1.03	204557.33	28851.60	47.36
	-1.51	131929.67	21875.27	30.54
	-1.99	107572.67	4704.49	24.90
LT4	0.40	504775.33	12491.54	116.86
	-0.08	524024.00	16186.74	121.32
	-0.56	384706.00	11812.28	89.07
	-1.03	217488.67	8470.69	50.35
	-1.51	156392.33	2399.48	36.21
	-1.99	122740.00	1677.00	28.42
LT9	0.40	586833.00	30147.56	135.86
	-0.08	498053.00	40645.81	115.31
	-0.56	314789.33	37445.99	72.88
	-1.03	206116.33	40337.09	47.72
	-1.51	158105.67	15117.00	36.60
	-1.99	126594.33	4378.02	29.31
Renoster & Vals River catchments July 2012 Sediment				
RN1	0.40	586833.00	30147.56	90.73
	-0.08	498053.00	40645.81	77.00
	-0.56	314789.33	37445.99	48.67
	-1.03	206116.33	40337.09	31.87
	-1.51	158105.67	15117.00	24.44
	-1.99	126594.33	4378.02	19.57
RN3	0.40	275223.67	41163.62	50.80
	-0.08	227102.33	37518.74	41.92
	-0.56	176037.67	39369.71	32.49
	-1.03	138627.67	24900.54	25.59
	-1.51	114868.67	17240.25	21.20
	-1.99	102439.33	21280.74	18.91
RN4	0.40	245752.00	7605.43	45.36
	-0.08	202046.00	25737.27	37.29
	-0.56	166721.00	3121.44	30.77
	-1.03	147116.33	12328.60	27.15
	-1.51	132844.00	8600.20	24.52
	-1.99	118132.67	7069.73	21.80

Table A–8 continued:

VL1	0.40	435015.00	37736.21	58.94
	-0.08	306168.33	29888.74	41.48
	-0.56	205005.00	30468.14	27.78
	-1.03	157709.00	15393.91	21.37
	-1.51	128445.00	11465.41	17.40
	-1.99	111068.00	3135.30	15.05
VL2	0.40	213504.67	14983.18	28.93
	-0.08	193595.00	2778.09	26.23
	-0.56	172148.00	2453.29	23.32
	-1.03	155262.67	8769.28	21.04
	-1.51	140362.00	5646.10	19.02
	-1.99	98166.33	3536.18	13.30
VL3	0.40	504775.33	12491.54	78.04
	-0.08	524024.00	16186.74	81.02
	-0.56	384706.00	11812.28	59.48
	-1.03	217488.67	8470.69	33.63
	-1.51	156392.33	2399.48	24.18
	-1.99	122740.00	1677.00	18.98