MANCOZEB IN NATURAL WATER SOURCES IN THE VHEMBE DISTRICT AND THE POSSIBLE ENDOCRINE DISRUPTING ACTIVITY/POTENTIAL THERE-OF

by

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Abstract

Many chemicals released into the environment are believed to disrupt normal endocrine functions in humans and animals. These endocrine disrupting chemicals (EDCs) affect reproductive health and development. A major group of EDCs that could be responsible for reproductive effects are those that mimic natural oestrogens, known as xeno-oestrogens. A number of *in vivo* and *in vitro* screening strategies are being developed to identify and classify xeno-oestrogens, in order to determine whether they pose a health risk to humans and animals. It is also important to be able to apply the assays to environmental samples for monitoring purposes. Oestrogens and androgens mediate their activity via intracellular receptors – directly in muscular tissue as well as indirectly via stimulation of growth hormones from the pituitary glands and other growth factors from liver plus several other organs. Mancozeb is a metal ethylenebisdithiocarbamate (EBDC) fungicide used to protect many fruits and vegetables and field crops against pathogenic fungal. It causes a variety of defects on the female reproductive system in experimental animals and is therefore considered a suspected EDC. This fungicide can also induce toxic effects in cells of the immune system and other non-immune cells leading to genotoxicity and apoptosis. The mechanisms of EDCs involve divergent pathways including (but not limited to) oestrogenic, antiandrogenic, thyroid receptors; that are highly conserved in wildlife and humans, and which can be modelled in laboratory *in vitro* and *in vivo* models. The endocrine disrupting properties of Mancozeb are not known as of yet and therefore the T47D-KBluc reporter gene assay, GH3.TRE-Luc and MDA-kb2 reporter gene assay were used determine the possible endocrine disrupting activity/potential there-of. No activity was detected in any of the assays and no mancozeb was detected in any of the dams either. Oestrogenic activity was detected in Albasini Dam, Nandoni Dam and Xikundu weir but all values were below 0.7 ng/ℓ trigger value for oestrogenic activity in drinking water.

Keywords: mancozeb, water, fungicide, endocrine disrupting chemicals, oestrogenicity, androgenicity, thyroid

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic
ADH	Antidiuretic hormone
ALAD	Aminolevulinic acid dehydratase
AR	Androgen Receptor
As	Arsenic
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BPA	Bisphenol A
Cd	Cadmium
CO_2	Carbon dioxide
DDT	Dichloro-diphenyl-trichloroethane
DHT	Dihydrotestosterone
dl	detection limit
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DWAF	Department of Water Affairs and Forestry
E2	17β-estradiol
EBDC	Ethylene bisthiocarbamate
EC50	Half maximum effective concentration
ECPH	Environmental Chemical Pollution and Health
EDCs	Endocrine Disruptive Chemicals
EDS-Pak cartridge	Endocrine disruptors pak cartridge
EDSTAC	Endocrine disruptor screening and testing advisory committee
EEq	Estradiol equivalent
ER	Oestrogen receptor
ERE	Oestrogen-responsive element
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
ETU	Ethylenethiourea
F	Flutamide
FBS	Foetal bovine serum
FI	Fold induction
FSL	Full supply level
FST	Follicle stimulating hormone
GC-TOF	Gas chromatograph mass spectrometry

GR	Glucocorticoid Receptor
GSH	Glutathione
GWRC	Global Water Research Coalition
HBSS	Hank's buffered salt solution
HCL	Hydrochloric acid
Hg	Mercury
HGH	Human growth hormone
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
ICCVAM	Interagency coordination Committee on the Validation of alternative methods
ICPMS	Inductively coupled plasma mass spectrometry
ICSH	Interstitial cell stimulating hormone
IPCS	International programme on chemical safety
IRS	Indoor residual spraying
LH	Luteinizing hormone
LOQ	Limit of quantification
LPS	Lipopolysaccharide
LWGS	Luvuvhu river government water scheme
Mn	Manganese
OHF	Hydroxyflutamide
OXT	Oxytocin hormone
Pb	Lead
PBS	Phosphate buffered saline
PCBs	Polychlorinated bisphenols
PFOS	Phenols and perfluorooctane sulfonate
POPs	Persistent organic pollutants
PRL	Prolactin
RIE	Relative inductive efficiency
RLU	Relative light unit
RP	Relative potency
RPMI	Roswell Park Memorial Institute
SA	Sodium arsenite / South Africa
SANAS	South African National Accreditation System
SD	Standard deviation
SPE	Solid Phase Extraction
STP	Sewage treatment plant

T3	Triiodothyronine
T4	Thyroxine
THs	Thyroxine
TNF	Tumor necrosis factor
TSH	Adrenocorticotrophic
TSH	Thyroid stimulating hormone
USA	United State of America
USEPA	Environmental Protection Agency in the United States
VC	Vehicle control
WRC	Water Research Commission
YAS	Yeast androgen screen
YES	Yeast oestrogen screen
Zn	Zinc

Measurements

mg/ℓ	milligrams per litre
ng/ℓ	nanograms per litre
µM	micrometre
µℓ	microliter
nm	nanometre
km	kilometre
pg/ℓ	picogram per litre
mℓ/minute	millilitre per minute
mm	millimetre
mℓ	millilitre
cm	centimetre

DECLARATION

I, Mokgadi F Seshoka, declare that this research proposal is my original work and has not been submitted for any degree at any other university or institution. The proposal does not contain other persons' writing unless specifically acknowledged and referenced accordingly.

Signed (Student): _____ Date: 21 February 2018

CHAPTER 1 - GENERAL INTRODUCTION

1.1 Introduction

Endocrine disrupting chemicals (EDCs) in freshwater sources in South Africa are very well discussed topic (London *et al*., 2000; Bornman *et al*., 2007; Burger and Nel, 2008; Aneck-Hahn *et al*., 2009; Barnhoorn *et al*., 2009; Fatoki *et al*., 2010, van Wyk *et al*., 2014; Bornman *et al*., 2017; Robson *et al*., 2017). Endocrine disruption is the result of hormone function interference by EDCs causing changes in development, growth, and reproduction in wildlife and humans (Toppari *et al*., 1996; Diamanti-Kandarakis *et al*., 2009). EDCs include various man-made substances such as pesticides, metals, additives or contaminants in food, and personal care products. A list of these chemicals in the South African context was compiled by Burger in 2005 (Table 1.1).

Table 1.1: Groups of endocrine disruption chemicals (EDCs) (adapted from Burger, 2005)

POPs (Persistent organic pollutants)		
Pesticides	**Industrial chemicals**	**Unintended by products**
Aldrin	Hexachlorobenzene (HCB)	Dioxins
Chlordane	Polychlorinated biphenyls (PCBs)	Furans
DDT		
Dieldrin		
Heptachlor		
Mirex		
Toxaphene		
EDCs apart from POPs		
Alkyl phenols and their ethoxylate	*p*-Nonylphenol (NP), *p*-Octylphenol (OP), *p*- Nonylphenol ethoxylate (NPn2EO) and *p*-Octyl phenol ethoxylate (OPnEO)	
Phthalate	Butyl benzyl phthalate (BBP), di-n-butyl phthalate (DBP) and di (2-ethyl hexyl) phthalate (DEHP)	
Plasticizers	Bisphenol (BPA)	
Herbicides	Atrazine, Simazine, 2,4-D	
Fungicides	Vinclozolin	
Organophosphate pesticides	Azinphos-methyl,Parathion	
Pharmaceuticals	Diethylstilbestrol (DES), Tamoxifen and Raloxifene	
Certain heavy metals	Cadmium, Arsenic, Lead and Mercury	
Flame retardants	Polybromobiphenyl ethers	
Natural and Synthetic hormones	17β-Oestradiol, Ethinyl-estradiol,estrone,estriol	
Other industrial chemicals	Benzene, Styrene	

There are also naturally occurring toxicants produced by fungi and plants (Ramesh, 2011). Endocrine disruption is both a developing multidisciplinary area of research, involving aspects of toxicology and endocrinology and a potential mechanism for many reproductive toxicants (Ramesh, 2011).

Some EDCs may mimic the working of the female hormone oestrogen and may be responsible for reproductive effects and are therefore sometimes referred to as synthetic/xeno-oestrogens (Aneck-Hahn *et al*., 2002). To be able to identify and classify xeno-oestrogens the recombinant yeast screen bioassay (YES) and ER-Calux® (Mertl *et al*., 2014) have been useful for screening oestrogenic activity of chemicals. On the other hand, some EDCs may show androgenic activity. Androgenic activity could result from interference of EDCs with the bioavailability of endogenous hormones, in this case testosterone, and/or their ability to activate or block hormone receptors within the cell (Brouwers *et al*., 2011). The MDA-kb2 reporter gene assay is used to measure the (anti-) androgenic activity of a chemical (Wilson, 2002). Thyroid hormones are an intrinsic part of animal and human growth, development, metabolic balance, as well as predominantly neurological cell networks and functions through life (Remaud *et al*., 2014). The most commonly used *in vitro* bioassay, is based on transcription analysis of reporter enzymes as a consequence of thyroid receptor activation in mammalian cells or yeasts (Freitas *et al*., 2011). In South Africa the thyroid screening assay used is a luciferase reporter gene assay and setup as described in Freitas *et al*. (2011).

Recently, after screening for organochlorine pesticides (OCs) against a comprehensive pesticide library, methoxychlor and aldrin were detected in more than half of the fish tissue samples collected from the Albasini Dam (Nibamureke *et al*., 2016). Nibamureke *et al*. (2016) also reported some heavy metals and OCs including *p,p'*-dichlorodiphenyltrichloroethane (DDT), *p,p'*-dichlorodiphenyldichloroethane (DDE), *p,p'*-dichlorodiphenylethylene (DDD), *cis*-chlordane, *trans*-chlordane, methoxychlor and heptachlor-epoxide in the water of the Albasini Dam. The aluminium (Al), copper (Cu), manganese (Mn) and zinc (Zn) levels were above the Target Water Quality Range (TWQR) for aquatic ecosystems (Nibamureke *et al*., 2016). Ethylene bisdithiocarbamate (EBDC) fungicides, mancozeb and maneb, constitute of 21% Mn by weight and may be a likely source of availability of Mn in the water systems (Gunier *et al*., 2014).

Mancozeb is used against fungi in guava, banana and mango farming in the Vhembe District of South Africa. This fungicide is considered to be an EDC and is widely used throughout the world for food production. Humans, especially farmers or those who work at agricultural sites exposed to this fungicide are mostly at risk imposed by this fungicide on their health. Mancozeb has been proven to be showing short term toxicity to humans and also induction of toxic effects in cells of the immune system and in other non-immune cells. Rossi *et al*. (2006) reported that the metabolite ethylenethiourea (ETU) of mancozeb may cause various toxic effects including thyroid and hepatic

neoplasms. Although mancozeb is considered an EDCs, not much is known about the endocrine disrupting properties thereof.

Water samples were collected in dams from the Vhembe District, Limpopo Province in South Africa. Most of the research conducted in the Luvuvhu River catchment included some of the limnological aspects of the main dams and tributaries as well as biomonitoring (Fouché *et al*., 2009; Barnhoorn *et al*., 2009, 2010; Bornman *et al*., 2009, 2010; Odiyo *et al*., 2012; Traoré *et al*., 2016). Since indoor residual spraying (IRS) of DDT is on-going, most of the epidemiology and ecological studies focused on the possible effects of DDT and its metabolites in aquatic biota and humans (Bornman *et al*., 2005, 2009; Aneck-Hahn *et al*., 2007; Barnhoorn *et al*., 2009, 2010; van Dyk *et al*., 2010).

1.2 Study motivation

The Environmental Protection Agency in the United States (USEPA) established a testing strategy for screening chemicals for endocrine activity (O'Connor, 1999). The importance of using a battery of bioassays is to detect endocrine-active compounds (O'Connor, 1999). More specifically, the aim of *in vitro* screening is to elucidate potential mechanisms of action as well as to determine the potency of the pesticides prior to *in vivo* testing (Ghisari *et al*., 2015).

In South Africa the need for a battery of bioassays was initially started in 2000 and compiled in a report to the Water Research Commission (WRC) of SA (de Jager *et al*., 2011). The human population of South Africa is growing gradually leading to a higher demand for natural resources such as food and clean water. Agricultural sectors tend to be forced to plough and plant more than they used to and their use of pesticides increased as a result. There is very limited data about EDCs in South Africa (Aneck-Hahn *et al*., 2009; Bornman *et al*., 2007; Bornman *et al*., 2010; Mahomed *et al*., 2008). The 2008 publication by the Global Water Research Coalition (GWRC) raised an awareness of the need to use a battery of assays. This led to the selection of oestrogen and anti-androgenic activity detection bioassays for the aquatic environment in South Africa (Dabrowski *et al*., 2015). Therefore, there seems to be an opportunity to use various bioassays to determine possible endocrine disrupting activity of a fungicide used in the agricultural setting of a rural area.

1.3 Aim and objectives

1.3.1 Aim

To determine the mancozeb, Mn and Zn concentrations in water from selected dams in the Luvuvhu River catchment and to determine the oestrogenicity/androgenicity and the thyroid activity thereof.

1.3.2 Objectives

- Measure mancozeb, Mn and Zn concentrations in the water from the Albasini-, Nandoni-, TateVondo dams and the Xikundu Weir.
- To determine the oestrogenicity, androgenicity and thyroid activity of mancozeb using the T47D-KBluc reporter gene assay, MDA-kb2 reporter gene bioassay and GH3.TRE-Luc assay.

1.4Hypotheses

1.4.1 There will be Mancozeb, manganese (Mn) and zinc (Zn) in the water from the Albasini-, Nandoni-, TateVondo dams and the Xikundu Weir.

1.4.2 Mancozeb will have one of the oestrogenic, androgenic or thyroid properties

1.5 Study area

The Luvuvhu River flows through an area where fruit farming and forestry comprise most of the landscape. Since mancozeb is used as a fungicide in the Vhembe district water samples were collected from the Albasini-, Nandoni-, and TateVondo dams as well as the Xikundu Weir (Figure 1.1). These impoundments are all situated in the Luvuvhu River and Mutshindudi River (TateVondo Dam) catchment in the Limpopo Province of South Africa. The Albasini Dam is a freshwater impoundment situated in the Luvuvhu River near the town of Louis Trichardt in the Vhembe District of the Limpopo Province of South Africa. In addition to the inflow from the Luvuvhu River, the dam also receives water from another river, the Doornspruit. The Albasini Dam was constructed as part of the Levubu irrigation scheme but also provides drinking water for the local municipality of Louis Trichardt (Department of Water Affairs and Forestry (DWAF), 2012). Commercial and subsistence farming practices are prominent in the surrounding areas. Further along the flow of the Luvuvhu River is the Nandoni Dam. The Nandoni Dam, which lies 16 km southeast of Thohoyandou, is part of the Luvuvhu River Government Water Scheme (LWGS) and the dam wall consists of a 38 m high composite concrete spillway section with earth flanks and at full supply level (FSL) the surface area of the Nandoni Dam is 1650ha with a gross storage capacity of 164 million m^3 (DWAF, 2001). The LWGS includes two water treatment plants, pumping stations and bulk water distribution pipelines. The scheme is designed to supply water for domestic use, irrigation and forestry. The Nandoni Dam is fed by the Mvudi-, Dzindi- and Luvuvhu rivers that receive agricultural and municipal effluent.

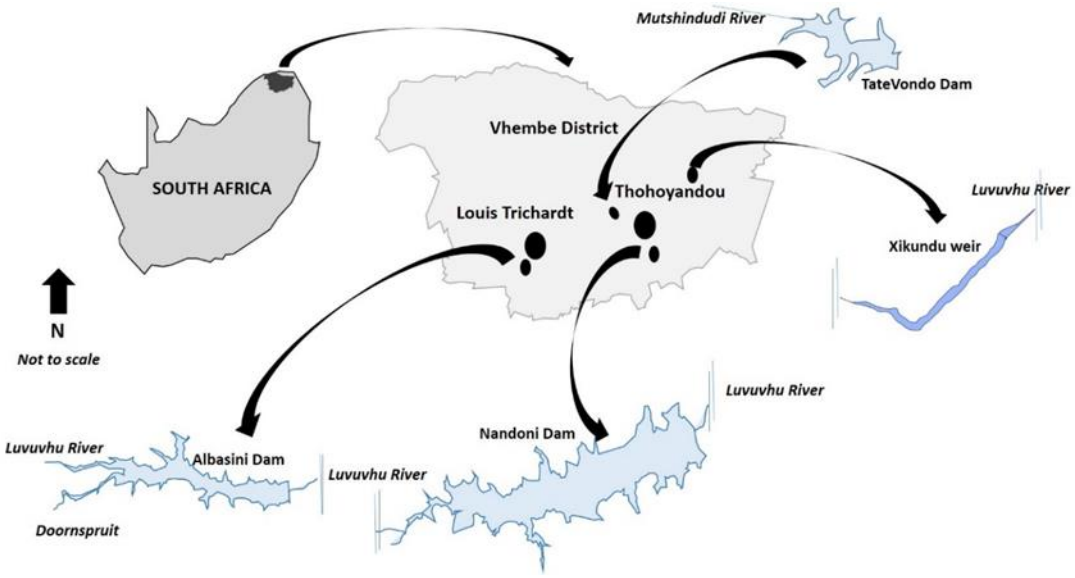

Figure 1.1: The Albasini-, Nandoni-, TateVondo dams and the Xikundu Weir situated in the Luvuvhu River and Mutshindudi River (TateVondo Dam) catchments in the Limpopo Province of South Africa.

The Xikundu Weir is about 20km downstream in the Luvuvhu River of the Nandoni Dam and is known for subsistence farming on the banks that is mostly replacing the riparian zone (Bornman *et al*., 2010). The fourth point of sampling is the TateVondo Dam situated in the Mutshindudi River catchment and in the area of tea plantations.

CHAPTER 2 - LITERATURE REVIEW

2.1 Introduction

Previous studies concerning pesticides in the Vhembe District of South Africa have mainly focused on DDT. Pesticides are defined as chemicals designed to kill or eliminate pest problems, protect crops, preserve food and materials and also prevent vector bone diseases as defined by Ghisari *et al*. (2015). There is evidence that they have been increasingly accumulating in the environmental waters in the ng/ℓ – µg/ℓ range and that many of them have been recently detected in natural streams (Campos-Manas *et al*., 2017). It has been reported that some pesticides are EDCs by Brouwers *et al*. (2011) and Mahomed *et al*. (2008). DDTs have been used in indoor residual spraying (IRS) in the Vhembe District and has been identified as an EDC as they mimic or antagonize the action of hormones (Kelce *et al*., 1995). As a result of on-going IRS most of the research study conducted in the Vhembe District focused on various aspects of DDT. It is therefore known that a pesticide such as DDT may interact with physiological systems and cause alterations in development, growth, and reproduction in wildlife and humans (Bornman *et al.*, 2009; Barnhoorn *et al*., 2010). Barnhoorn *et al*. (2010) reported that there is also concern about possible health effects in humans living in DDT-sprayed areas in the north of SA as high levels of DDT and metabolites were detected in human serum samples. A type of intersex called testis-ova which is formed when female gametes are distributed throughout the male gonadal tissue has been frequently observed and it has been reported to be consequence of oestrogenic EDCs (Guellard *et al*., 2015). Intersex is defined as presence of both male and female reproductive characteristics on same individual as a result of the embryo being exposed to EDCs (Barnhoorn *et al*., 2010) and in South Africa it was first reported by Barnhoorn *et al*. (2004).

According to the study by Hass *et al*. (2012) exposure to one endocrine disruptor to human health does not cause much effect but exposure to a mixture does, thus most studies recently are focusing on synergistic effects of EDCs. Another important aspect is the endocrine effects these EDCs have on an early life since there is much less research on early life exposure especially on body weight gain and other related metabolic imbalance (Bhaskar and Mohanty, 2014).

2.2. Endocrine system

Functionality of different cells in multicellular organisms requires maintenance and integration. There are two systems responsible for this, namely the nervous system and the endocrine system. The nervous system is used by the body for maintenance of the spinal cord, sensory organs, brain and all the nerves whereas the endocrine system is employed for the regulation and functions of glands that secrete hormones (Birkett and Lester, 2002). The glands include; the thyroid, pituitary, pineal body, pancreas, hypothalamus, adrenal glands and the reproductive glands. These glands

are essential as they are responsible for processes such as homeostasis, growth, reproduction, metabolism and maintenance. Each of the glands produces its specific hormone for a specific function (Birkett and Lester, 2002). However, if any EDCs are present, the metabolic clearance mechanism, driven by hormones, may not work. This may result in these chemicals hindering hormone performance and they may accumulate in the body (Birkett and Lester, 2002).

The hormones' functions and where they are produced are in indicated in Figure 2.1. Below a brief description of the glands and associated hormones (Adapted from wikibooks.org/w/index.php?human-physiology/The endocrine system&oldid):

- Thyroid gland - is a type of gland that is situated at the base of the throat and it is responsible for secretion of two hormones namely thyroxine (T4) and triiodothyronine (T3) which have similar function of tissue growth and differentiation.
- Pituitary gland – is also known as a 'master gland', because it controls all other glands of the endocrine system. It consists of an anterior lobe where follicle stimulating hormone (FSH), human growth hormone (HGH), prolactin (PRL), thyroid stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH), luteinizing hormone (LH), interstitial cell stimulating hormone (ICSH) and intermedin are produced. The posterior lobe produces antidiuretic hormone (ADH) and oxytocin hormone (OXT).
- Pineal gland - is a gland attached to the posterior wall of the third ventricle of the brain at the back of the skull. It secretes a hormone called melatonin which is responsible for informing the body on what is happening on a current situation.
- Pancreas - located behind the stomach. It secretes two hormones called insulin and glucagon. Insulin is responsible for the cellular uptake of glucose while glucagon is responsible for the conversion of glycogen to glucose.
- Hypothalamus - type of gland that is part of the brain which controls the centre of the endocrine system. It coordinates both the autonomic nervous system and the activity of the pituitary, controlling body temperature, thirst, hunger and other homeostatic systems.
- Adrenal glands - secretes three hormones namely adrenalin and noradrenalin responsible for preparation of the body for fight, fright, flights etc. and lastly corticosteroids responsible for utilization of carbohydrate, fat and protein.
- Reproductive glands - glands associates with ovaries and testes. Hormones secreted are oestrogen responsible for breaking down the uterine wall, progesterone responsible for maintaining and building up of the uterine wall and lastly testosterone for the development and function of the male sexual organ.

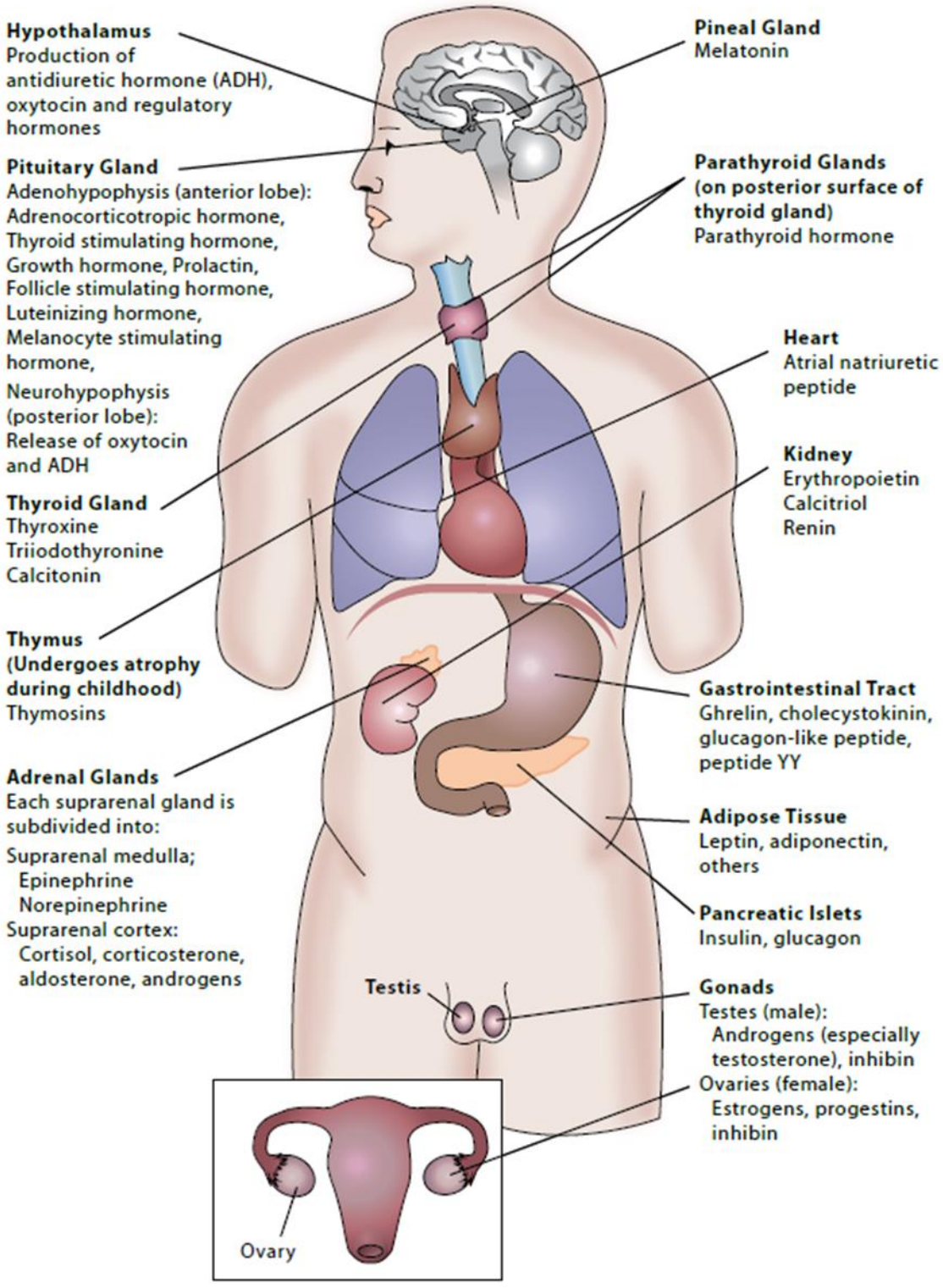

Figure 2.1: Diagram illustrating endocrine glands and all the hormones they secrete. Adapted from Bergman *et al*. (2013).

The hormones are transported from their glands of production to the target areas/organs through the bloodstream. For example; a steroid hormone attaches to the protein receptor to form a receptor complex where it passes the message and produce a new protein which later attach to the plasma membrane then the expected response is produced. For instance; insulin which is produced by specialized cells called beta cells. Glucose enters the beta cells through a specific protein transporter on the cell membrane and is converted to adenosine triphosphate (ATP). Insulin is then produced and travels to many different tissues and cells through blood resulting in taking up of glucose by those tissues (Bergman *et al*., 2013).

The moment there are some kind of interference with the hormone in questions normal physiological function it is generally called hormone disruption which in turn indicates endocrine disruption. The chemicals that may interfere with the normal function of hormones are called EDCs and are defined; as compounds or substances, either natural or synthetic that alters the hormonal and homeostatic system enabling the organism to respond and communicate with the environment (Diamanti-Kandarakis *et al*., 2009). There is a substantial list of EDCs which tend to cause disruption of the endocrine system (Diamanti-Kandarakis *et al*., 2009). Evidence from animal studies (Barnhoorn *et al*., 2004; Bhandari *et al*., 2015), human health studies (Damstra, 2002; Hotchkiss *et al*., 2008), human clinical observations (Balabanic *et al*., 2011; Kabir *et al*., 2015), university researchers etc showing the impact of EDCs on humans and wild life health are abundant. Most studies (Aneck-Hahn *et al*., 2009; Archer and Van Wyk., 2015; Barnhoorn *et al*., 2004; Mahomed *et al*., 2008; Medda *et al*., 2017; Mohanty *et al*., 2017; Pandey and Mohanty, 2015; Ksheerasagar *et al*., 2003; Runkle *et al*., 2017) have focused on the effects of EDCs on the thyroid hormones and the reproductive hormones.

2.3 Endocrine disruption and endocrine disrupting chemicals (EDCs)

Endocrine disruption is defined as the interference of normal hormonal functions (as described above) of living organisms by hormone mimicking chemicals causing adverse health effects (Van Wyk *et al*., 2014).These adverse physiological effects may include disturbances during reproduction (Bornman *et al*., 2010), neurological (Diamanti-Kandarakis *et al*., 2009) and immunological effects (Corsini *et al*., 2005), egg shell thinning in birds (Bowman *et al*., 2012), crocodiles (Bowman *et al*., 2014) and alligators (Guillette *et al*., 1994) and cancer in wildlife and human beings (Dabrowski *et al*.,2015; Kime., 2012; Swedenborg *et al*., 2009; Guillette *et al*., 2001).

EDCs are chemicals that mimic natural hormones and they affect the endocrine system in four possible ways (State of the Science of Endocrine Disrupting Chemicals – 2012):

- By mimicking the action of a naturally-produced hormone, such as oestrogen or testosterone, and thereby setting off similar chemical reactions in the body

- By blocking the receptors in cells receiving the hormones, thereby preventing the action of normal hormones
- By affecting the synthesis, transport, metabolism, and excretion of hormones, thus altering the concentrations of natural hormone
- Modify the creation and function of hormone receptors.

Throughout the last thirty six years a variety of organic pollutants caused adverse health effects in humans and animals as a result of hormone function interference (Damstra, 2002). The chemicals that interfere with hormone function are called EDCs and has been referred to by the International Programme on Chemical Safety (IPCS) in their 2002 report as "…*chemicals that have the potential to interfere with the endocrine system"; and also defied by them 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. A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations*." (IPCS, 2002).

Many of the so called EDCs are chemicals that humans come in contact with on a daily basis and they are commonly grouped as; persistent organohalogens, pesticides, phthalates, phenols and other chemicals as well as some metals. The list also includes the synthetic 17α- ethinylestradiol which is part of oral contraceptives.

These groups are presented below in table 2.1 adapted from;

http://www.ourstolenfuture.org/Basics/chemlist.htm.

Table 2.1: Table presenting the persistent organohalogens and the hormone system affected and the mechanism if known.

Compound(s)	Hormone system affected	Mechanism if known
Benzenehexachloride (BHC)	Thyroid	
1,2-dibromoethane	Reproductive	
Chloroform	Reproductive	
Dioxins and furans (in order of anti-oestrogenic potency : 2,3,7,8-tetrachlorodibenzo-p-dioxin > 2,3,7,8-tetrachlorodibenzofuran > 2,3,4,7,8-pentachlorodibenzo-furan > 1,2,3,7,9-pentachlorodibenzofuran > 1,3,6,8-tetrachlo-rodibenzofuran)	Oestrogen	work as anti-oestrogen through binding with Ah receptor, which then inhibits oestrogen receptor binding to oestrogen response elements, thereby inhibiting oestrogen action
Octachlorostyrene	Thyroid	
Polybrominated biphenyls (PBBs)	Oestrogen/ Thyroid	
Polychlorinated biphenyls (PCBs) (in order of anti-oestrogenic potency: 3,3'-pentachlorobiphenyl > 3,3,4,4,5,5'-hexachlorobiphenyl 3,3',4,4-tetrachlorobiphenyl > 2,3,3',4,4',5'-hexa, 2,3,3',4,4'- and 2,3,4,4',5-pentachlorobiphenyl > Aroclors 1221, 1232. 1248, 1254, and 1260 were inactive as antioestrogens at the highest concentrations used in this study (10-6 Ni)	Oestrogen/androgen/Thyroid Adverse outcomes in reproductive systems.	Inhibits oestrogen binding to the receptor; works as anti-oestrogen. anti-androgenic via Ah receptor interaction
PCB, hydroxylated	Thyroid	Binds to thyroid hormone binding protein, but not to the thyroid hormone receptor.
Polybrominated diphenyl ethers (PBDE)s	Thyroid	Interfere with thyroxine (T4) binding with transthyretin
Pentachlorophenol	Thyroid	Reduces thyroid hormone possibly through a direct effect on the thyroid gland.

(Table adapted from http://www.ourstolenfuture.org/Basics/chemlist.htm).

Polychlorinated bisphenols (PCBs) which are one of the EDCs listed in table 2.1 are more commonly known as research chemicals of exposure and effects studies. PCBs are chemicals used daily either as lubricants or industrial solvents. Even though they are of essential use some of them have been banned from manufacturing and use since the 1970s (Steinberg *et al*., 2008; Diamanti-Kandarakis *et al*., 2009). They have been identified as EDCs because of the adverse effects they

may have on humans and wildlife health (Grilo *et al*., 2013; Bodin *et al*., 2014; El Majidi *et al*., 2014). PCBs have been reported to cause a decrease in spermatogenesis and delayed puberty in rats (Diamanti-Kandarakis *et al*., 2009). They have also been reported to cause behavioral changes in early life forms of rodents (Steinberg *et al*., 2007; Steinberg *et al*., 2008), delayed growth development (Jacobson *et al*., 1997), low sperm parameters (Bornman *et al*., 2007) and depressed thyroid hormone (Morse *et al*., 1996). Diamanti-Kandarakis *et al*. (2009) reported that most of them have oestrogenic or antiandrogenic activity. In South Africa there is very little or no evidence on the bioaccumulation in tissue and sublethal effects in organisms but studies indicated levels in water and sediment during the 1990s (Batterman *et al*., 2009; Bouwman *et al*., 2008). Recently Rimayi *et al*. (2017) indicated high levels of PCBs in the Jukskei- and Klip rivers, and in another study by Garcia-Heras *et al*. (2017) reported that birds occupying nests next to electric transformers had high levels of PCBs (Northern Cape and Western Cape). PCBs are also considered persistent organic pollutants (POPs) (classified and listed by the Stockholm convention in 2004) because of the following characteristic (Bouwman 2004):

- They maintain their parent structure for very many years,
- they are distributed via natural processes involving soil, water and air,
- they are lipophilic and can therefore accumulate in the food chain, and
- they are toxic to both humans and wildlife.

Bouwman (2004) described the process behind the POPs as follows: “The Stockholm Convention on Persistent Organic Pollutants came into force on 17 May 2004. This action resulted from a process, initiated by the United Nations Environment Programme (UNEP) in 1997, which has led to a global, multilateral agreement with the aim of protecting human and environmental health from the effects of exposure to specific persistent organic pollutants (POPs). Restricting the use and production, or banning of these chemicals, will, when the measures of the convention are successfully implemented, reduce the hazards posed by these pollutants”.

Table 2.2: Pesticide classes and compounds that have been shown to disrupt hormone systems. Compounds in the right column may or may not be EDCs.

Pesticide class	Endocrine Disrupting Chemicals	Other Chemicals in the Group
Carbamates	Aldicarb, Carbaryl, Methomyl	Baygon(propoxur), Bendiocarb, Oxamyl
Chlorinated cyclodienes and camphenes	Aldrin, Chlordane, Endosulfan, Endrin Kepone, Mirex, Nonachlor	Oxychlordane
Dithiocarbamates	Mancozeb, Maneb, Thiram, Zineb, Ziram	Metam Natrium, Metiram, Nabam
Linuron, diuron, and derivatives/metabolites	Ethylene Thiourea (ETU), Linuron (Lorox)	1-(3,4-Dichlorophenyl)-3-methylurea, 1-(3,4-Dichlorophenyl)-3-methylurea, Diflubenzuron, Diuron
Organophosphates	Malathion, Ethylmalathion, Methylmalathion,Chlorpyrifos	Acephate, Chlorfenvinphos, , Cyclophosphamide, Demefion, Demeton-s-methyl, Diazinon, Dichlorvos, Dimethoate, Elsan (=Dimephenthoate), Ethylparathion, Fenitrothion, Fenthion, Formothion, Glufosinate, Metalodemeton, Mevinphos (=Phosdrin), Omethoate, Parathion, Phosphamidon, Quinalphos, Ronnel, Trichlorfon
Pyrethroids	Allethrin, Karate, Talstar, sumithrin, fenvalerate, *d-trans* allethrin, permethrin	Bifenthrin, Bioallethrin, Cyhalothrin, Cypermethrin, Deltamethrin, Esfenvalerate, Fenothrin, Fluvalinate, , Resmethrin
Pyrimidines and Pyridines		Bromacil, Fenarimol, Picloram
Triazines and triazoles	Amitrol, Atrazine	Biteranol, Cyanazine, Cyproconazole, Difenoconazole, Epiconazol, Epoxiconazole, Etridiazole, Febuconazole, Indole (3,2-b) carbazole (ICZ), Ketoconazol, Metribuzin, Penconazole, Propiconazole, Simazine, Tebuconazole, Terbutryn, Triadimefon, Triadimenol, Triazines

(Table adapted from http://www.ourstolenfuture.org/Basics/pesticides.htm).

As indicated in Table 2.2, there are quite a large variety of pesticides. Pesticides are very important chemicals especially in agricultural production as they are used to control pests or weeds. Many

farmers use them to protect fresh produce. Pesticides include; insecticides, herbicides, nematocides, molluscicides and termicides. Although these chemicals are important in agricultural production and domestic use they can be very harmful to human and wildlife health. Those who are exposed such as farmers, cleaners, people who produce them and the workers in the agricultural area end up accumulating them in their bodies either through inhaling, contact with the skin or they might accidentally ingest it via the food. It was reported by Conis (2010) that organophosphates have negative physiological effects in both humans and wildlife. They impose health effects such as low semen quality (Diamanti-Kandarakis *et al*., 2009) and hyperactivity disorders (Bergman *et al*., 2013). They have also been suspected to cause miscarriage, early delivery of infants and suppressed foetal growth (Bergman *et al*., 2013). Pesticides such as aldrin, chlordane, DDT, endosulfan, endrin and mirex, are considered as POPs (Bouwman, 2004).

Phthalates are a group of chemicals often used as plastic softeners but also as fragrance carriers. They can be found in products such as electronics, textile prints, flooring, toys and cosmetics (Hauser and Calafat, 2005). Many phthalates have shown anti-androgenic and oestrogenic properties (Table 2.3), and some have been classified as being toxic to reproduction like reduced anogenital distance observed on rats and human males (Foster *et al*., 2001; Heudorf *et al*., 2007; Diamanti-Kandarakis *et al*., 2009). Phthalates are also suspected to be potentially carcinogenic and impose other toxic effects to the exposed community in rural areas (Adeniyi *et al*., 2008; Fatoki *et al*., 2010). They have been reported in several aquatic systems in the Vhembe District (Fatoki *et al*., 2010), and several other rivers especially in the Cape Province (Olujimi *et al*., 2012).

Table 2.3: Table presenting the phthalates and the hormones affected and the mechanism if known.

Compound	Hormones affected	Mechanism
Butyl benzyl phthalate (BBP)	Oestrogen	Inhibits binding to the oestrogen receptor
Di-n-butyl phthalate (DBP)	Oestrogen Androgen	Inhibits binding to the oestrogen receptor. anti-androgenic
Di-ethylhexyl phthalate (DEHP)	Oestrogen Androgen	Inhibits binding to the oestrogen receptor. anti-androgenic
Diethyl Phthalate (DEP)	Oestrogen	

(Table adapted from http://www.ourstolenfuture.org/Basics/chemlist.htm).

Table 2.4: Table presenting the other compounds such as the phenols and perfluorooctane sulfonate (PFOS) and the hormones affected and the mechanism if known.

Compound	Hormones affected	Mechanism
Benzophenone	Oestrogen	Binds weakly to oestrogen receptors, roles of its metabolite remain to be clarified.
Bisphenol A	Oestrogen	Oestrogenic; binds to oestrogen receptor
Bisphenol F	Oestrogen	Oestrogenic; binds to oestrogen receptor
Benzo(a)pyrene	Androgen	anti-androgenic
Carbendazim	Reproductive	
Ethane Dimethane Sulphonate	Reproductive	
Perfluorooctane sulfonate (PFOS)	Thyroid, reproductive	suppression of T3,T4; mechanism unknown
Nonylphenol, octylphenol	Oestrogen	Oestrogen receptor agonists; reduces oestradiol binding to the oestrogen receptor.
Resorcinol	Thyroid	
Styrene dimers and trimers	Oestrogen	Oestrogen receptor agonists

(Table adapted from http://www.ourstolenfuture.org/Basics/chemlist.htm).

The phenols and perfluorooctane sulfonate (PFOS) are the most discussed chemicals from table 2.4. Bisphenol A (BPA) is the most produced and well-known of the bisphenols (Erler and Novak, 2010). It is one of the world's most widely manufactured and spread substance. BPA is oestrogenic and considered to be toxic to reproduction and also noted as a carcinogen by Bergman *et al*. (2012). BPA disrupts the gonadotropin-releasing hormone of fish at low levels as noted by Qin *et al*. (2013) and causes a decrease in DNA methylation of imprinted genes at an early age of mice (Bhandari *et al*., 2015). It is mostly used as a raw material in the manufacture of many products such as engineering plastics, dental sealants, polycarbonate plastics, food cans and cash receipts (Calafat *et al.*, 2009). Following the increased awareness of the hazardous properties of BPA the use of other bisphenols has increased. Bergman *et al*. (2012) also mentioned that there are several studies of the effects of bisphenol A in animals. As such more research is needed on human health effects imposed by these chemicals. People from many countries use bisphenols in almost every day of their lives. South Africa was the first country in Africa to put a stop on the use of these chemicals in 2011 (Baluka and Rumbeiha, 2016). Perfluorooctane sulfonate (PFOS) is a synthetic substance and is either partially or completely saturated with fluorine. PFOS and its precursors are primarily used as water, oil, soil and grease repellents for paper and packaging, and carpets and fabrics, as well as in aqueous film forming foam for fighting fuel fires (Stahl *et al*., 2011). Because of its chemical properties and use patterns, PFOS is typically found at higher concentrations in water than in air and it can travel to locations far from its point of release through river streams (Stahl *et al*., 2011). In addition, PFOS precursor compounds can travel through air and often end up in

isolated areas, where they degrade to PFOS (Geisy and Kannan, 2001). PFOS is an extremely persistent substance. It bioaccumulates, potentially harming many animals, including top predatory fish, birds and mammals, depending on their actual exposure (Hanekom *et al*., 2015). PFOS is one of the POPs and a known EDC that is restricted under the Stockholm Convention, a global treaty to protect human health and the environment. PFOS, according to Greenpeace International is also prohibited within Europe and in Canada for certain uses. In South Africa, levels of PFOS in African catfish and bird eggs are high compared to European levels and this is a concern (Orange-Senqu River Commission (ORASECOM), 2013).

Table 2.5: Table presenting the four so called endocrine disrupting metals and the hormones affected and the mechanism if known.

Compound	Hormones affected	Mechanism
Arsenic	Glucocorticoid	Selective inhibition of DNA transcription normally stimulated by the glucocorticoid-GR complex.
Cadmium	Oestrogenic	Activates oestrogen receptor through an interaction with the hormone-binding domain of the receptor.
Lead	Reproductive	
Mercury		

(Table adapted from http://www.ourstolenfuture.org/Basics/chemlist.htm).

Although cadmium (Cd), mercury (Hg), arsenic (As), lead (PB), Mn, and Zn are considered EDCs (Lavicoli *et al*., 2009) only the four in table 2.5 will be discussed. The metals in table 2.5 represents the so called endocrine disrupting metals and more information became available over recent years. Below a short discussion on each.

Arsenic (As)

Arsenic is an important element in our everyday life as it is mainly used to preserve wood (inorganic arsenic) and as pesticides on cotton plants (organic arsenic) (Bornman *et al*., 2007). There are many ways in which humans and wildlife can be exposed and include via food, ingestion by mistake, inhalation from the surrounding air and being in contact with the smoke coming from burning of wood while in the process of preserving wood (Agency for Toxic Substances and Disease Registry (ASTDR), 2000). Apart from being considered an EDC, As is also considered to be a carcinogen (Sun *et al*., 2016). It was suspected to be androgenic due to the stimulation of malignant transformation of prostate epithelial cells *in vitro* (Benbrahim-Tallaa *et al*., 2007). Arsenic may increase DNA damage and mutations indirectly, such as by altering DNA repair (Andrew *et al*., 2006), and may act as a co-carcinogen and/or tumour growth enhancer (Rossman, 2003).

Cadmium (Cd)

Cadmium is a very toxic, non-essential element that is soft and bluish-white in colour (Saeki *et al.,* 2000). According to Henson and Chedrese (2004) Cd has been linked to a wide range of detrimental effects on mammalian reproduction. They also noted that Cd either enhance or inhibit the biosynthesis of progesterone and decrease birth weights and cause premature birth. Animals exposed to high dosages experienced induced severe testicular interstitial haemorrhage with oedema, and increased incidence of foetal death and placental necrosis. Whereas low-dose exposure to Cd affects steroid synthesis in male and female reproductive organs (Takiguchi and Yoshihara, 2006). These authors also confirmed that Cd has potent oestrogen- and androgen-like activities *in vivo* and *in vitro*, by directly binding to oestrogen and androgen receptors. The effects of Cd on human and wildlife include kidney damage, low fecundity, testicular damage (Furness, 1996), and prostate cancer risk (Diamanti-Kandarakis *et al.,* 2009; Bergman *et al*., 2012). A study done on adult rodents indicated that Cd exposure induced high blood glucose levels which may also apply to humans (Edwards and Prozialeck, 2009).

Lead (Pb)

Lead is defined as a heavy, soft, gray solid metal. It is mainly used in industries for the production of batteries, cable covering and a coloring in ceramic glasses. Humans and wildlife get exposed to it through drinking water, old paints, food, soil and disposal of old batteries (ATSDR. 2000). Yamaguchi *et al*. (2007) reported that Pb may have inhibitory effects on spermatogenesis in catfish. Furthermore it may disrupt the function of the gonadal steroids (Ronis *et al*., 1996), adrenal steroids (Vyskocil *et al*., 1970) and thyroid hormones (Der *et al*., 1977) in animals. Pande and Flora (2002) reported that Pb induced inhibition of delta-aminolevulinic acid dehydratase (ALAD), reduction in glutathione (GSH) and an increased Zn protoporphyrin (ZPP) level in blood, indicating altered heme synthesis pathway in rats. It is also known that blood Pb levels below 10 µg/dℓ may cause cognitive dysfunction, neurobehavioral disorders, neurological damage, hypertension and renal impairment in children (Cory-Slechta , 1997; Lanphear *et al*., 2000; Canfield *et al*., 2003).

Mercury (Hg)

Humans are exposed to Hg generally by methylmercury (MeHg) via eating contaminated fish and seafood (Rice *et al*., 2014). Mostly MeHg toxicity is associated with nervous system damage in adults and impaired neurological development in infants and children. Whereas wildlife get exposed to mercury through ingestion of contaminated lower organisms in a food chain. It has been reported that Hg may induce alterations in male and female fertility, may affect the function of the hypothalamo-pituitary-thyroid axis or the hypothalamo-pituitary-adrenal axis, and disrupt biosynthesis of steroid hormones (Georgescu *et al*., 2011). Tan *et al.* (2009) noted that Hg is a

neurotoxicant and EDC that mimics oestrogen's effects with no direct action on hormones. However Lavicoli *et al*. (2009) stated that Hg might compromise endocrine function by reducing hormone-receptor binding or through the inhibition of one or more key enzymes or steps in hormone biosynthesis. Hormones that appear to be the most affected by Hg are insulin, oestrogen, testosterone and adrenaline.

2.4 The working of EDCs and oestrogenicity

Endocrine disrupting chemicals may interact with different hormone receptors, including the androgen receptor, oestrogen receptor (ER), and thyroid hormone receptor. They can act as agonists or antagonists. The natural hormone binds to its specific receptor to produce a natural signal, an agonistic response is when an EDC chemical binds to a receptor and activate a response which as a result mimics the response of a natural hormone and alter the function. An antagonistic chemical prevents the functioning of a natural hormone by binding to and blocking the hormone receptor, thereby preventing the natural hormone from binding. (Birkett and Lester, 2002). These different interactions are illustrated in Figure 2.2.

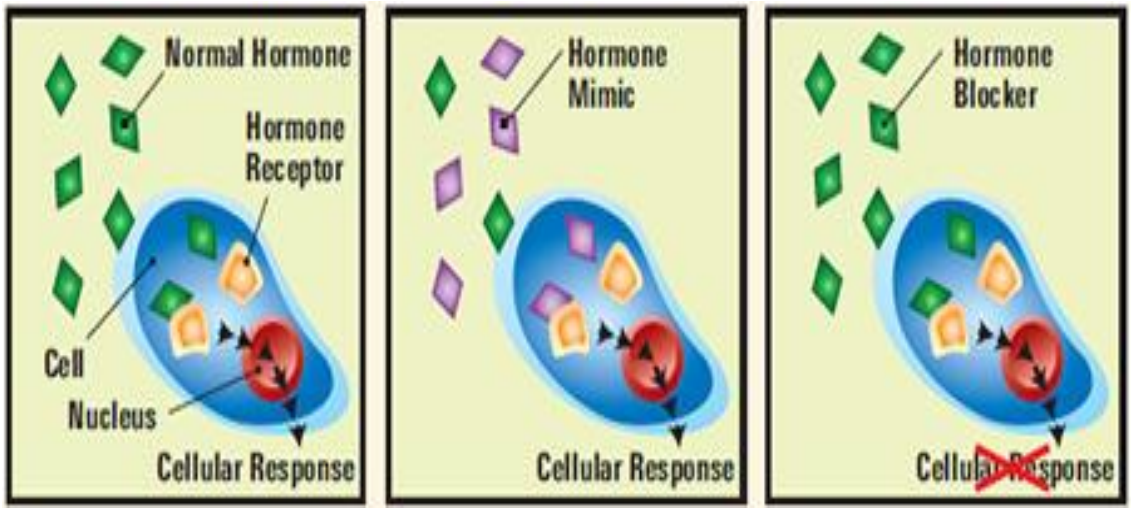

Figure 2.2: Illustration of natural (left), agonistic (middle) and antagonist (right) responses (Adapted from www.niehs.nih.gov/health/topics/agents/endocrine/index.cfm).

Oestrogenic activity is when the synthetic compounds mimic the oestrogen hormone (17β-oestradiol interfering with its physiologic activity (Aneck-Hahn *et al*., 2009). The EDCs enter the cell and attach to the receptor where the natural hormone is supposed to attach. They do so by binding to the respective endogenous receptors which bring a consequence of either agonistic or antagonistic responses (William *et al*., 2007; Aneck-Hahn *et al*., 2009). Studies on fish have shown various endocrine disrupting effects ranging from vitellogenin protein induction, altered sex ratios to

intersex, which is suspected to be induced by oestrogenic substances (William *et al*., 2007). Studies demonstrated the oestrogenic effects on alligators where female alligators had twice the plasma concentration of oestradiol which affected ovarian morphology. Male alligators had small phalli and abnormal testis and a reduction in testosterone levels (Guillette *et al*., 1994; Guillette *et al*., 1996; Harrison *et al*., 1997; William *et al*., 2007).

Status of endocrine disruption in SA

Reports of endocrine disruption in South African wildlife are limited. South Africa has been reported to have high number of pesticides but with little information on their effects on human and animal health (van Wyk *et al*., 2014). More than 500 active ingredients have been discovered in South Africa alone (Bornman *et al*., 2017) and most of these chemicals are known to have been used and abused by developed and developing countries (Olujimi *et al*., 2010). Intersex in wildlife was reported in South Africa for the first time by Barnhoorn *et al*. (2004) in sharptooth catfish from oestrogen polluted water. After this a variety of research reports have been published on endocrine disruption related studies. They included: Calcium-mediated aponecrosis plays a central role in the pathogenesis of oestrogenic chemical-induced neurotoxicity (Pretorius and Bornman, 2005), DDT and pyrethroid residues in human breast milk (Bouwman *et al*., 2006), oestrogenic activity in environmental water samples (Aneck-Hahn *et al*., 2007a) impaired semen quality associated with environmental DDT exposure in young men (Aneck-Hahn *et al*., 2007b), organochlorine contaminants in cormorant, darter, egret, and ibis eggs (Bouwman *et al*., 2008), DDT and urogenital malformations in newborn boys (Bornman *et al*., 2009), DDT residues in water, sediment, domestic and indigenous biota (Barnhoorn *et al*., 2009), histopathological changes in the reproductive system (ovaries and testes) of *Oreochromis mossambicus* following exposure to DDT (Mlambo *et al*., 2009), dioxin-like chemicals in soil and sediment (Nieuwoudt *et al*., 2009), testicular microlithiasis and neoplastic lesions in wild eland (*Tragelaphus oryx*) (Bornman *et al*., 2010), sperm motility and testicular histology as reproductive indicators of fish health of two feral fish species from a currently DDT sprayed area (Marchand *et al*., 2010), DDT contamination from indoor residual spraying for malaria control (van Dyk *et al*., 2010), chlorinated, brominated, and fluorinated organic pollutants in Nile crocodile eggs (Bowman *et al*., 2014) concentrations and implications of DDT residues in chicken eggs (Bouwman *et al*., 2015), the occurrence of anti-retroviral compounds used for HIV treatment in South African surface water (Wood *et al*., 2015), nonylphenol, an industrial EDC, affects root hair growth, shoot length and root length of germinating lettuce (de Bruin *et al*., 2016), the potential effects of efavirenz on *Oreochromis mossambicus* (Robson *et al*., 2017), to list a few. Endocrine disrupting related research is still ongoing.

2.5 Mancozeb

Mancozeb is a metal ethylenebisdithiocarbamate (EBDC) fungicide used to protect many fruits and vegetables and field crops against a variety of fungal conditions (Rossi *et al*., 2006; Paro *et al*., 2012). EBDC does not have a long half-life but the metabolite ethylenethiourea (UTA) is reported to have a long persistence in soil. A long persistence in soil is the reason mancozeb has been classified as an EDC as it causes concentration-dependent antiandrogenic effects (Kjeldsen *et al*., 2013). Mancozeb is composed of Mn and Zn of which Mn is an essential component important in multiple physiological processes such as somatic growth and bone formation (Mora *et al*., 2014). According to Mora *et al*. (2014), epidemiological studies found that both low and high Mn concentrations during pregnancy and early childhood were associated with impaired foetal growth and neurobehavioral deficits in children. A negative association has been shown between elevated Mn in drinking water and children's neurodevelopment, behaviour and academic achievement (Bouchard *et al*., 2011; de Joode *et al*., 2016). Heavy metals and pathogens are transported to the water through effluents which can be dangerous to the environment and human health (Gumbo *et al*., 2016). According to Grygo-Szymanko *et al*. (2016), exposure to Mn also occurs via inhalation and remains a serious concern to miners, welders, smelters or workers in dry cell battery factories. Animals and humans are exposed to mancozeb by a way of ingestion, inhalation or skin absorption. Symptoms of poisoning with EBDC compounds include; irritation of the skin, eyes and respiratory tract, skin sensitization and chronic skin disease (Kegley *et al*., 2000-2016).

A study by Rossi *et al*. (2006) indicated that female rodents show a significant decrease in size and number of healthy follicles and in embryo implantation. The metabolite UTA, inhibits thyroid peroxidase in rats and primates, and may cause thyroid cancers in animals (Bisson and Hontela, 2002; Pirozzi *et al*., 2016). This fungicide can also induce toxic effects in cells of the immune system and other non-immune cells (Pavlovic *et al*., 2016) leading to genotoxicity and apoptosis. Apoptosis obstructs the normal and controlled part of an organism's growth or development (Domico *et al*., 2006; Leiphon and Picklo, 2007). Gupta (2011) also indicated that mancozeb causes a variety of defects of the female reproductive system in experimental animals and is therefore considered a suspected EDC.

Corsini *et al*. (2005) found that mancozeb exerts slight immunomodulatory effects characterised by an increase in total leukocyte counts, increase in the percentage and absolute number of CD19+ cells, a reduction of CD2+ cells and a significant reduction in LPS-induced TNF-α release. Mancozeb was identified as the pesticide that has the highest endocrine disrupting potential compared to others.and is also capable of causing apoptosis in the human breast cancer line MCM-1 (Bisson and Hontela, 2002).

2.6 Bioassays for determination of endocrine disrupting activity.

In-vitro bioassays have been widely used to determine if chemicals have endocrine disrupting activity. Many of these assays however have limited usefulness and are not freely available as a scientific tool (Wilson *et al*., 2004). A suite of bioassays is therefore recommended when testing for endocrine disrupting activity (Beresford *et al*., 2000). The following bioassays were performed at the Environmental Chemical Pollution and Health (ECPH) Research Unit situated in the Faculty of Health Sciences at the University of Pretoria:

2.6.1 The T47D-KBluc reporter gene assay

Stable oestrogen-dependent gene expression assays have been recommended by advisory committees (EDSTAC, ICCVAM) to be developed for screening chemicals for oestrogenic activity because of its level of accuracy of the response and potential for use in a high-throughput mode (Wilson *et al*., 2004).

The USEPA developed an oestrogen-dependent stable cell line. The T47D human breast cancer cells, which contain both endogenous (oestrogen receptor) ERα and ERβ, were transfected with an oestrogen-responsive element (ERE) luciferase reporter gene construct. The reporter gene construct consists of three ERE upstream from TATA box that regulates the expression of a luciferase reporter gene. Stable transfection of this promoter-reporter construct into T47D cells leading to a sensitive, responsive clone. This provides an *in vitro* system that can be used to evaluate the ability of chemicals to modulate the activity of oestrogen-dependent gene transcription. In principle, compounds enter the cell; oestrogen receptor ligands bind to the ER; two ligand-bound receptors dimerize and bind coactivators; then the dimer binds to the ERE on the reporter gene construct and activates the luciferase reporter gene. The presence of the luciferase enzyme can then be assayed by measuring the light produced when the enzyme substrate, luciferin, and appropriate cofactors are added. The amount of light produced is relative to the degree of oestrogenic activity of the test chemical. When testing chemicals using the T47D-KBluc cells, an oestrogen is defined as a chemical that induces dose dependent luciferase activity, which could be specifically inhibited by the anti-oestrogen ICI182,780. Agonists stimulate luciferase expression and are compared to the vehicle control or to the relative response of their respective 17β-estradiol (E2) control. Anti-oestrogens block the E2-induced luciferase expression, which is compared to the E2 control (Wilson *et al*., 2004; Burger *et al*., 2005).

Wehmas *et al*. (2011) recommended T47D-KBluc assay as best compared to other *in vitro* screening methods because its reliability and sensitivity, Although latest study indicated that SPE elution with 100% methanol can interfere and alter with the results.

2.6.2 GH3.TRE-Luc assay

Living organisms have two thyroid hormones namely; thyroxine (T4) and triiodothyronine (T3) which have a very significant role in regulating growth and differentiating tissues, organs, energy homeostasis and metabolic pathways meaning that disruption of this hormones will lead to very lethal effects (Jugan *et al*., 2010). Assays have been developed after the concern of thyroid hormone disruption but did not give much help to high-throughput detection of compounds directly activating or inhibiting the thyroid hormone receptor (Marchesini *et al*., 2008; Freitas *et al*., 2011). As such a thyroid screening assay (GH3.TRE-Luc assay) has been developed and has been productive and giving reasonable results and has been reported to successfully predict the effects of some thyroid hormone disrupting chemicals (Freitas *et al*., 2011). The thyroid screening assay is a luciferase reporter gene assay that was developed based on the thyroid hormone responsive rat pituitary tumour GH3 cell line that constitutively expresses both thyroid hormone receptor isoforms. Stable transfection of the pGL4CP-SV40-2xtaDR4 construct into the GH3 cells resulted in a highly sensitive cell line (GH3.TRE-Luc), which was further optimized into an assay that allowed the detection of T3 and T4 concentrations in the pico molar range.

Although it has been taking the lead lately it has its own limitations, it is time consuming and is not always specific because of the effects on cell proliferation through non-thyroid receptor-mediated mechanisms which cannot be excluded (Freitas *et al*., 2011). A study has been done on a rat pituitary tumour and it has been found that rat pituitary cell lines cloned from the same rat pituitary tumour demonstrate an increase level of cell proliferation and growth hormone secretion (Freitas *et al*., 2011). The agonist control in this assay is T3 and the antagonist control is sodium arsenite.

2.6.3 MDA-kb2 assay

Androgens are commonly known as male hormones, their interaction with androgen receptors brings out sexual differentiation of male tissues during foetal development, accessory reproductive tract and reproductive tract (Wilson *et al*., 2002). Wilson *et al*. (2002) noted that a stable cell line could potentially be used to identify compounds that bind to the androgen receptor (AR) unlike standard receptor binding assays, have utility in exposing of androgen agonists from antagonists, thus aiding in defining mechanisms of action. Androgen agonist is activated by binding to either one of the androgenic hormones (testosterone or dihydrotestosterone) whereas antagonist blocks androgen receptor or inhibit androgen production. The stable cell line derived from MDA-MD-453 and designated MDA-kb2 is a useful tool for studying the activation of AR or glucocorticoid receptor (GR) by hormone agonists and antagonists. Since cells were derived from a single stable clone, inter- and intra-assay variability would be reduced, making the assay easier to standardize and validate (Wilson *et al*., 2002).

The breast cancer cell line, MDA-MB-453 contains both the GR and AR and was stably transformed with the MMTV.luciferase.neo reporter gene construct. In principle, when ligands bind to the receptors, the luciferase reporter gene is activated and luciferase is produced. The presence of the luciferase enzyme can then be assayed by measuring the light produced when the enzyme substrate, luciferin, and appropriate cofactors are added. The agonist positive control in this assay is dihydrotestosterone (DHT), the negative control is the solvent ethanol and the antagonist control is hydroxyflutamide (OHF). Samples are tested alone as well as in the presence of 0.1 nM DHT to test for anti-androgenic activity or with the anti-androgen, hydroxyflutamide (OHF) to distinguish between AR and GR mediated ligands.

CHAPTER 3 - MATERIALS AND METHODS

3.1. Water sampling

Water samples were collected once-off from the Albasini- (AD), Nandoni- (ND), TateVondo (TD) dams and the Xikundu Weir (XW) in 1 litre glass bottles for the bioassays and mancozeb analysis. A further 500mℓ was collected in plastic bottles for metal analyses (Bengu *et al*., 2017). The criteria of collection included points where the community utilise the water source. Surface water samples were taken about 50cm from the surface from the four sites (Table 3.1). Pre collection the bottles and lids were rinsed with methanol to avoid contamination. The original pH of the water for both mancozeb and metal analyses and for bioassay samples were measured using a pH strip and the pH of the water was adjusted to between 2 and 3 by adding HCl. The samples were stored in a refrigerator until extraction.

Table 3.1: Description of the sites where water samples were collected.

Locality name	Date	Description of the area where sample was collected
TateVondo Dam	18/05/2017	Edge of the dam, settled water.
Xikundu Weir	18/05/2017	Slow flowing water, just before the water channel.
Nandoni Dam	18/05/2017	Polluted site, dumping site for waste, washing of motors and brick making.
Albasini Dam	19/05/2017	Receives water from Muhohodi River which receives discharges from the Elim sewage treatment plant (Gumbo *et al*., 2016).

3.2 Bioassays

For the purpose of this study the focus was on the T47D-KBluc reporter gene assay, GH3.TRE-Luc assay and MDA-kb2 assay.

3.2.1 Materials

T47D-KBluc cells (cat. no. CRL-2865) and MDA-kb2 cells (cat no. CRL-2713) were purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA). GH3.TRE-Luc cells were a gift from Professor AJ Murk from Wageningen University (The Netherlands) to the University of Pretoria. Hank's buffered salt solution (HBSS, 10x, cat. no. 14185-045), trypsin (0.5% EDTA, 10x, cat. no. 15400-054) phosphate buffered saline (PBS, 10x, cat. no. 14080-048), Leibovitz's L-15

media powder (cat. no. 41300021),15 mM HEPES (cat no 31330095), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), buffer solution (1 M, cat. no. 15630-056), sodium pyruvate (100 mM, cat. no. 11360-039) and antibiotic/antimycotic solution (cat. no. 15240-062) were purchased from Gibco (Life Technologies Corporation, Paisley, UK). Bovine insulin (cat no I6634-100mg), ethanolamine (cat no E0135-100mℓ), sodium selenite (cat no S5261-10g), human apotransferrin (cat no T1147-500mg) and bovine serum albumin (cat no A7906-100g) which were used to prepare for PCM media, T3 (cat no T2877-250mg), resazurine (cat no R7017-5g) and sodium arsenite (cat no S7400-100g) were purchased from Sigma. MeOH (HPLC grade, cat. no.1.06007.2500), EtOH (HPLC grade, cat. no. 1.11727.2500) and D(+)-glucose (cat. no. 108342) were purchased from Merck (Darmstadt, Germany). Foetal bovine serum (cat no SH30071.03) and Charcoal/dextran treated FBS (c/d FBS, cat. no. SH30068.03) were purchased from Hyclone Laboratories. The above chemicals together with all other materials and apparatus such as Millipore Milli-Q synthesis ultrapure water system (Merck Millipore, Darmstadt, Germany) equipped with an EDS-Pak Cartridge (cat. no. EDSPAK001), Solid Phase Extraction (SPE)12-position vacuum manifold (Phenomenex, Torrance, California, USA) and LUMIstar OPTIMA luminometer (BMG Labtech, Offenburg, Germany) with two dispensers were all available at the ECPH Research Unit in the Faculty of Health Sciences at the University of Pretoria. The mancozeb fungicide was purchased from an agriculture store in the Limpopo Province. Reporter lysis buffer (5x, cat. no. PRE3971) and Beetle luciferin (cat. no. PRE1603) were purchased from Promega (Madison, Wisconsin, USA). RPMI 1640 powder (cat. no. R8755), sodium bicarbonate ($NaHCO_3$, cat. no. S5761), glycylglycine (1 M, cat. no. G7278), adenosine 5'-triphosphate (ATP, cat. no. A7699), bovine serum albumin (BSA, cat. no. A7906), magnesium chloride ($MgCl_2$) solution (1 M, cat. no. M1028), 17β- oestradiol (E_2, cat. no. E8875) and flutamide (cat. no. F9397) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). 10 mℓ disposable serological pipettes (cat. no. 4101), 75 cm^2 tissue culture flasks (cat. no. 430641), 96-well luminometer plates (cat. no. 3610), 50 mℓ centrifuge tubes (cat. no. 430828), 10 µℓ filter tips (cat. no. 4135S), 200 µℓ filter tips (cat. no. 4138S) and 1000 uℓ filter tips (cat. no. 4140S) were purchased from Corning Incorporated (Corning, New York, USA). 5α-dihydrotestosterone (DHT, cat. no. A2570-000) was purchased Steraloids (Newport, Rhode Island, USA). ICI 182,780 (cat. no. 1047) was purchases from Tocris biosciences (Ellisville, Missouri, USA). Oasis hydrophilic-lipophilic balance (HLB) SPE cartridges (5cc, 200 mg, cat. no. 186000683) was purchased from Waters (Milford, Massachusetts, USA).

3.2.2 General laboratory procedures

Nitrile gloves were worn throughout all the procedures. All glassware was washed using a phosphate free detergent (Liquinox, Alconox, NY, USA), rinsed 10 times using tap water, 10 times using Milli-Q water dispensed through EDS-Pak cartridge, rinsed twice in methanol and twice in

ethanol. Glassware were dried in oven and covered with foil. Glassware that had to be sterile, e.g. bottles to prepare cell culture media, were autoclaved (20 minutes, 121°C, 15 psi).

3.2.3 Filtration and extraction of water samples

The glass filtration unit was assembled and loaded with glass wool and 0.45 µm, membrane disk filters (47 mm diameter), then connected to the vacuum inlet. The sample (1ℓ) was then passed through the filtration unit under vacuum, 250 mℓ at a time. The filters were replaced after every 250 mℓ to prevent clogging of the filters (See figure 3.1). After filtration, the procedure continued with SPE (de Jager *et al*., 2011). The SPE cartridges were placed on the SPE manifold and pre-conditioned with 5 mℓ dd H_2O followed by 5 mℓ of methanol (HPLC grade) and 5 mℓ of dd H_2O, before the samples (1ℓ) were loaded (de Jager *et al*., 2011). Throughout the extraction procedure care was taken not to let the cartridge run dry until the whole sample volume passed through the cartridge. The flow rate never exceeded 10 mℓ/minute. Once all the sample volume passed through the cartridge, the cartridges were dried under vacuum.

Five (5) mℓ methanol was added to each cartridge reservoir and the solvent was allowed to filter through the sorbent bed. Elution was allowed to happen through gravity alone into methanol rinsed glass test tubes. The vacuum was turned on to elute the remaining solvent that did not elute with gravity. After all the solvent was eluted, the samples were removed from the manifold and placed in a sample concentrator with a heating block (Figure 3.2a) in a fume hood to be blown down.

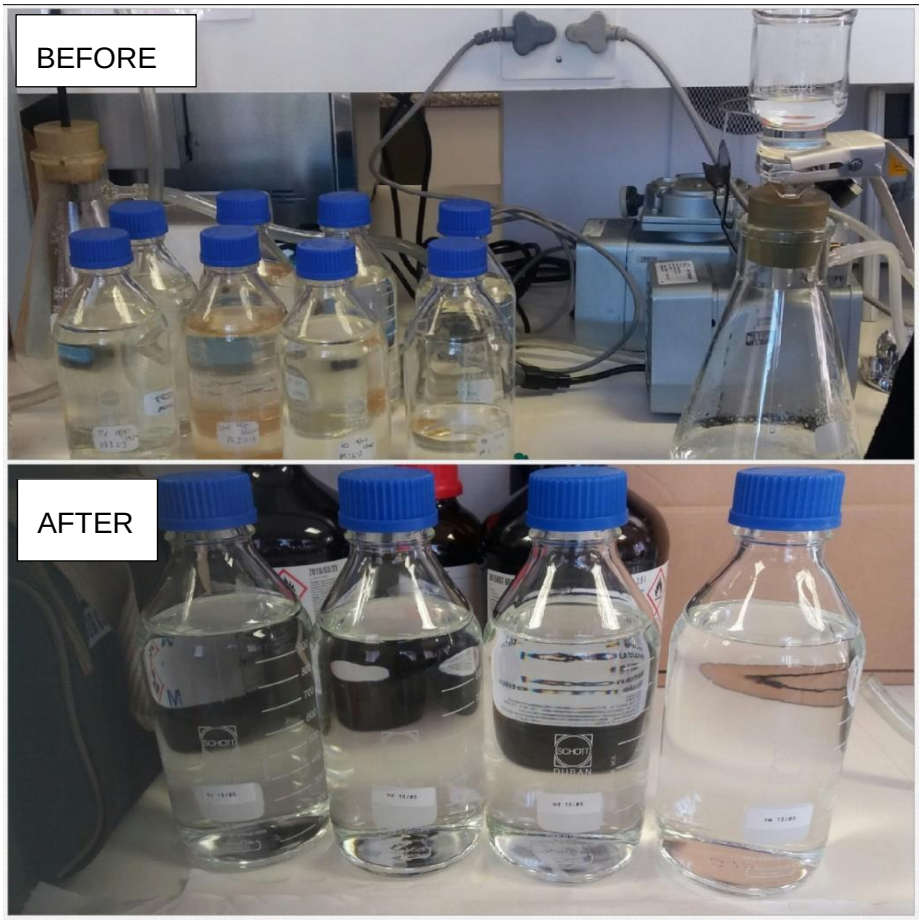

Figure 3.1: Filtration setup for water sample from the TateVondo-, Nandoni-, Albasini Dams and the Xikundu Weir before filtration and the results after filtration.

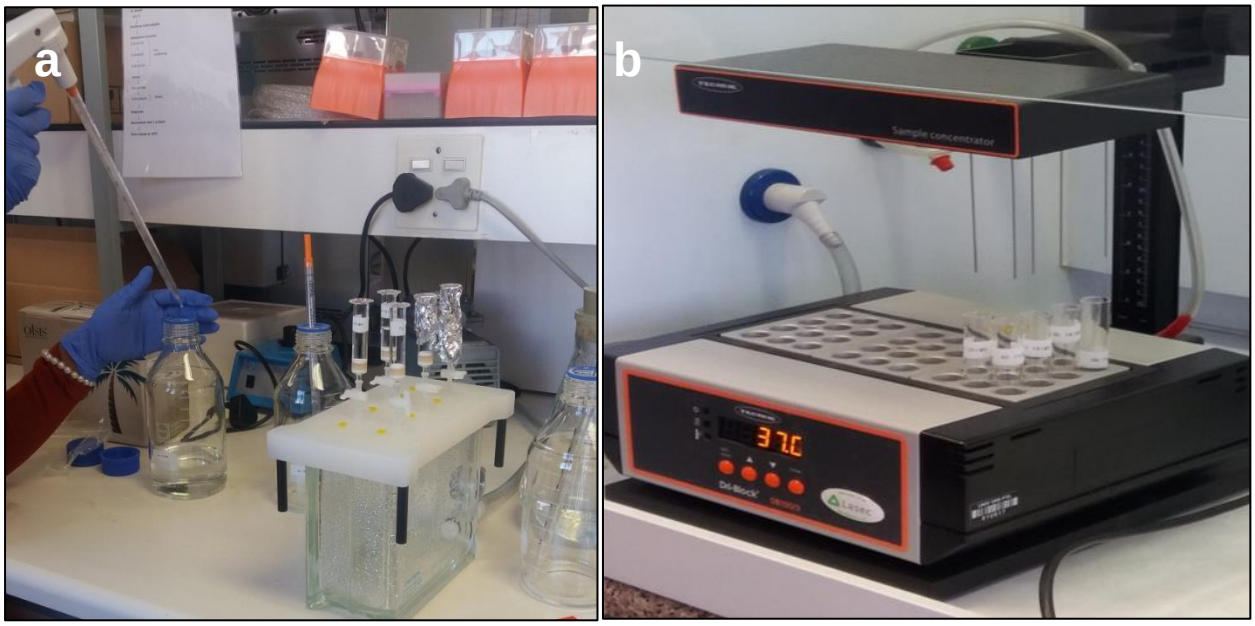

Figure 3.2: (a) Extraction of water samples using a solid phase extraction manifold (b) Evaporation setup of samples and extraction control.

The test tubes containing eluent were placed in a heating block (37°C) in a fume hood (Figure 3.2b), then the needles of the sample concentrator unit were lowered into the tubes and the nitrogen flow was turned on. The needles were lowered every 30 min in order to keep a constant flow on the surface of the samples. After approximately 1 h 30 minutes when the samples were completely dry the tubes were removed from the unit and reconstituted by adding 1 mℓ of ethanol in each tube (1000× concentration factor). The samples were vortexed thoroughly for mixing, before the reconstituted sample was placed into methanol rinsed glass amber vials (4 mℓ volume) and stored at -20°C for further analysis (de Jager *et al*., 2011).

3.2.4 T47D-KBluc reporter gene assay

a) Cell culturing

Cell culturing was performed in a Type II biohazard safety cabinet to reduce the possibility of contamination. All materials including nitrile gloves and reagents were wiped off by 70% ethanol before and the surface of the cabinet was wiped before and after working. Cells were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% foetal bovine serum (FBS). Thawing the cells, 10mℓ of RPMI maintenance media was placed in a 25 cm^2 tissue culture flask. The flask was placed in a CO_2 (5%) incubator for at least 15 minutes, in order for the media to reach 37°C and to equilibrate. Vials of frozen T47D-KBluc stock culture cells were thawed and the contents of the vial were transferred into the cell culture flask. The flask was labelled with the name of the cell line, the date and passage number. The flask was placed back in the CO_2 incubator and cells were allowed to attach to the surface overnight. The medium was discarded and replaced with fresh medium the following day. The cells were trypsinized and sub-cultured when confluent (3-4 day interval).

Media and HBSS were placed in the CO_2 incubator for at least 15 minutes before the cells were trypsinized in order for the media to reach 37˚C and its normal pH. The culture media from the flask were discarded and the cells were rinsed twice with HBSS (5-10 mℓ for a 75 cm^2 flask or 3-5mℓ for a 25 cm^2 flask). 3 mℓ of trypsin was added to a 75 cm^2 flask or 1 mℓ to a 25 cm^2 flask and cells were observed under an inverted microscope until cell layer started disassociating (approximately 2 minutes). The cells were detached by gently tapping the flask against the palm of the hand and 10 mℓ medium was added to a 75 cm^2 flask or 5 mℓ to a 25 cm^2 flask, then 1/3 or 1/4 was transferred to a new culture flask and maintenance or assay media (20 mℓ for 75 cm^2 flask or 10 mℓ for 25 cm^2 flask) was added. The new flasks were labelled, and passage number updated. The flasks were returned to incubator until cells reach 80-90% confluency. The media was changed twice a week.

b) Assay procedure

One week prior to the assay, cells were grown in RPMI supplemented with 10% charcoal/dextran treated FBS to withdraw the cells from steroids. Following the one-week withdrawal period, the cells were trypsinized and 10 mℓ medium containing 5% dextran/charcoal treated FBS was added. Cells from more than one flask was used so they were pooled into a 50 mℓ conical tube and mixed gently. The cells were counted using a haemocytometer. The concentration of the cells in the original suspension was calculated using the following formula:

cells/mℓ = average count per 1mm^2 square ×10 000 × dilution factor

Cells were seeded at 5 × 10^4 cells per well in 96-well luminometer plates (100 µℓ per well) and placed in the incubator to allow the cells to attach overnight.

The following stock concentrations were prepared in ethanol: 10Mm E2; 1mM ICI 182, 780; 200 mg/mℓ mancozeb.1000× concentrated dosing solutions were prepared for controls and test chemicals in HPLC grade ethanol in 2 mℓ Eppendorf tubes and were vortexed. A dilution series was prepared for samples and controls in ethanol of which 2 uℓ was transferred to 1mℓ medium to prepare the dosing solutions. The range of concentrations tested for each sample and controls were as follows; E2 100 pM - 0,03 pM (agonist control), ICI 10nM - 0,003 nM (antagonist control) and mancozeb 200 mg/ℓ - 2 pg/ℓ.

Fifty (50) µℓ of the dosing solutions was added to wells containing 50 µℓ vehicle control, 100 pM E2 or 10 nM ICI. The plates were incubated for 24 h in a 5% CO_2 incubator at 37°C. The plates were removed from the incubator and assessed under the microscope for any signs of cytotoxicity or any other abnormality. The dosing solution was removed by shaking the plates gently over a waste tray. The cells were then washed by filling each well with PBS at room temperature using 25 mℓ serological pipette then the PBS was discarded. In order to lyse the cells 25 µℓ of lyses buffer was added to each well using a multichannel pipette. The plates were placed in a freezer to allow the lyses buffer to be activated. The lysed cells were thawed at 37°C. The luciferase activity was determined using a luminometer with two dispensers programmed to inject 25 µℓ reaction buffer (25 mM Glycylglycine, 15 mM $MgCl_2$, 5 m M ATP, 0.1 mg/mℓ BSA, pH 7.8), followed by 25 µℓ 1 nM D-luciferin 5 s later. Luciferin activity was quantified as relative light units (RLU).

3.2.5 MDA kb2 reporter gene assay

a) Cell culturing

The MDA-kb2 reporter gene assay was used to determine androgenic activity in the samples, according to the method described by Wilson *et al*. (2002). In short; the cells of the MDA assay

procedure were grown in Leibovitz's L-15 media supplemented with 10% FBS. The cells were trypsinized and 10 mℓ medium containing 5% FBS was added. Cells from more than one flask was used so they were pooled into a 50 mℓ conical tube and mixed gently. The cells were counted using a haemocytometer.

Cells were seeded at 5 × 10^4 cells per well in 96-well luminometer plates (100 µℓ per well) and placed in the incubator to allow cells to attach overnight.

b) Assay procedure

Fifty (50) µℓ of the dosing solutions was added to the appropriate wells containing 50 µℓ vehicle control, 10 nM dihydrotestosterone (DHT) (agonist control) or 100 µM flutamide (F) (antagonist control). The plates were incubated for 24 h in an incubator at 37°C.

The stock concentration in the ethanol was 10 mM DHT; 1 mM F; 200 mg/mℓ mancozeb.1000× concentrated dosing solutions were prepared for controls and test chemicals in HPLC grade ethanol in 2 mℓ Eppendorf tubes and were vortexed. Concentrations and dilutions were as follows; DHT = 20 nM - 0.003 nM., *F* = 100 uM - 0.03 uM, mancozeb = same as in T47D-KBluc and GH3.TRE-Luc, water extracts = same as in T47D-KBluc. After the 24h exposure period, the cells were lysed and luciferase activity determined using the same method described for the T47D-KBluc assay above. The cells were terminated and plates read using the same procedure as for the T47D-KBluc reporter gene assay.

3.2.6 GH3.TRE-Luc reporter gene assay

a) Cell culturing

The GH3.TRE-Luc cell line was used to determine thyroid activity. Cells were maintained in regular growth medium (DMEM/F12 (1:1) with 15 mM HEPES supplemented with 10% FBS) in an incubator at 37 °C and 5 % CO_2. The cells were sub-cultured twice a week in a 75 cm^2 flask when confluent. The growth medium was discarded and cells were rinsed two times with HBSS, then HBSS was discarded. Trypsin (0.1%) was added into the flask to release the cells from the flask, and then excess trypsin was removed after 30s. The cells were then checked if they uprooted from bottom of flask and in order to neutralise trypsin, growth medium was added. Cells were then split into new flasks or seeded in 96-well plates for the luciferase assay.

Cells were seeded at 3 × 10^4 cells/well in clear bottom 96-well tissue culture plates (100 uℓ per well) and allowed to attach overnight. The growth medium was replaced with 100 µℓ serum-free PCM medium (DMEM/F12 (1:1) with 15 mM HEPES supplemented with 10 µg/mℓ bovine insulin, 10 µM ethanolamine, 10 µg/mℓ sodium selenite, 10 µg/mℓ human apotransferrin and 500 µg/mℓ BSA) to deplete the cells of thyroid hormones.

b) Assay procedure

The plates were incubated for 24h. After removing old PCM the cells were exposed to test chemicals, water extracts and controls, serially diluted in PCM (200 µℓ final volume). The tested concentration ranges were as follows; *T3 (agonist control)* = 10 nM - 0.00001 nM., *sodium arsenite (antagonist control)* = 200 µM - 0.1 µM., *mancozeb* = same as in T47D-KBluc. *Dilution series of water extracts* = 10x, 3x, 1x, 0.3x, 0.1.

Test chemicals were tested alone and in the presence of 1 nM T3 or 100 µM sodium arsenite. Each plate (of 3) had a T3 dose response curve and vehicle control and the triplicate plates were exposed for 24h. The cells were lysed and luciferase activity determined using the same method described for the T47D-KBluc reporter gene assay above.

Cytotoxicity was determined on a separate plate, using the resazurine cell proliferation assay. After a 24h exposure period, 8 µℓ of resazurine (400 µM in PBS, pH 7.4) was added to each well and the plates were incubated in the dark for 4h (37 °C, 5 % CO_2). The fluorescence was measured at 530 nm excitation and 590 emission. A sample was considered cytotoxic if the fluorescence was less than the fluorescence of the vehicle control minus 3x the standard deviation.

3.2.8 Data analysis (T47D-KBluc reporter gene, MDA-kb2 reporter gene and GH3.TRE-Luc reporter gene)

The calculations were done on Excel and Graph Pad Prism. Raw data were transferred to Excel and Relative Light Unit readings were converted to fold induction (FI) relative to the vehicle control for samples and positive control (de Jager *at al*., 2011). The FI values were expressed as percentage of the 0.1 nM agonist control maximum response. Data was transferred to Graph Prism version 4, with the test chemicals or agonist control concentrations as X-values and FI values (% max) as y values, x values were log transformed and test chemicals or agonist control curve was fitted (sigmoidal function, variable slope) to obtain the 50 % Effective Concentration (EC50) value.

The relative induction efficiency (RIE) was calculated as RIE = (Max RLU chemical/max RLU E2)*100 and relative potency (RP) as RP = (EC50 of E2/EC50 of test chemical)*100.

Equivalent values were calculated for samples that induced dose dependent luciferase activity, which could be inhibited by the antagonist. Sample concentrations were calculated from the agonist standard curve as unpaired Y-values and were corrected for the appropriate dilution factors to determine the equivalent value for the original sample. Equivalent values were reported as the average ± SD of triplicate samples. The antagonist control curve (co-incubated with 0.1 nM agonist) was fitted to quantify antagonist activity.

3.3 Mancozeb and metal analyses

In standard house method 039/2008 using gas chromatography electron capture detector spectrometry (GC-ECD) techniques were applied by a SANAS and ISO 17025 accredited laboratory for mancozeb analyses. The metals (Cd, Hg, Pd, As, Mn and Zn) were measured using inductively coupled plasma mass spectrometry (ICPMS) by a SANAS accredited laboratory (Bengu *et al*., 2017).

CHAPTER 4 - RESULTS

The T47D-KBluc cells were exposed to mancozeb from the highest concentration of 200 mg/ℓ to the lowest 2E-13 mg/ℓ in order to check for oestrogenic and anti-oestrogenic activity. Both oestrogenic and anti-oestrogenic activities were tested on the same plate. Mancozeb did not show any sign of oestrogenic characteristics (figure 4.1) but cytotoxicity was observed under a microscope on all cells in the wells containing the two highest concentrations (100 mg/ℓ and 200 mg/ℓ). All cells in the two wells were affected. It is important to note that cytotoxicity can mask oestrogenic activity.

4.1 T47D-KBluc

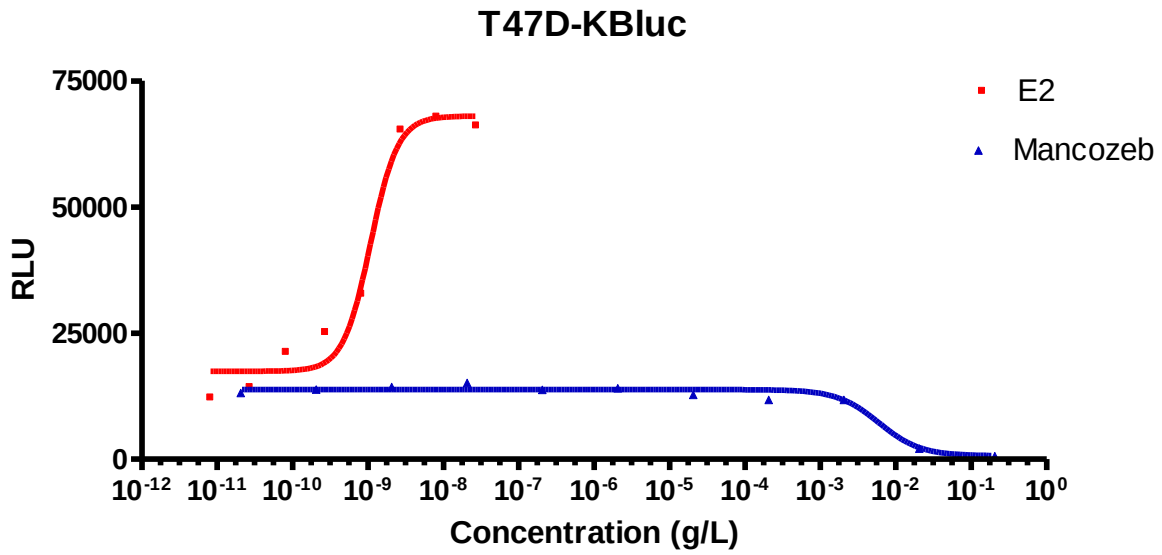

Figure 4.1: Dose-response curves for mancozeb and positive control 17β-oestradiol (E2) in T47D-KBluc cells.

The ICI and mancozeb were co-incubated with E2 and both suppressed the E2 activity (figure 4.2). ICI was able to suppress the E2 curve at lower concentrations. The response of E2 curve co-incubated with mancozeb as seen from the graph above was low because the cells were dead thus no activity was detected. This cytotoxicity was observed under a microscope on all cells in the wells within the two highest concentrations (100 mg/ℓ and 200 mg/ℓ).

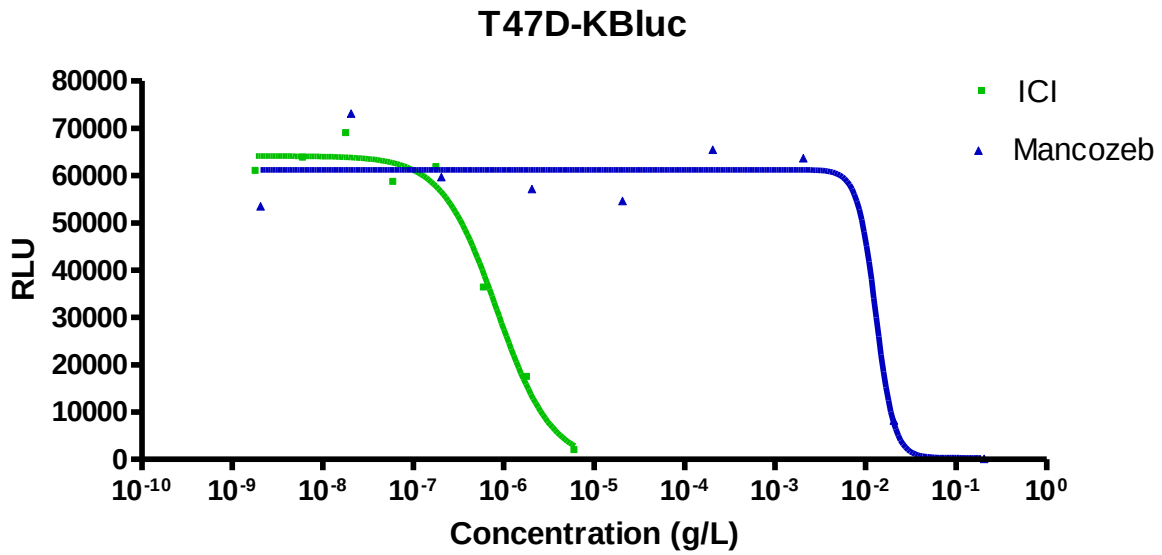

Figure 4.2: Dose-response curves for mancozeb and anti-oestrogen (ICI), co-incubated with 100 pM E2, in T47D-KBluc cells.

The level of quantification (loq) for the assay was defined as the EC10 of the E2 dose-response curve. No oestrogenic activity was detected in the extraction control, as all the values were well below the loq (Figure 4.3).

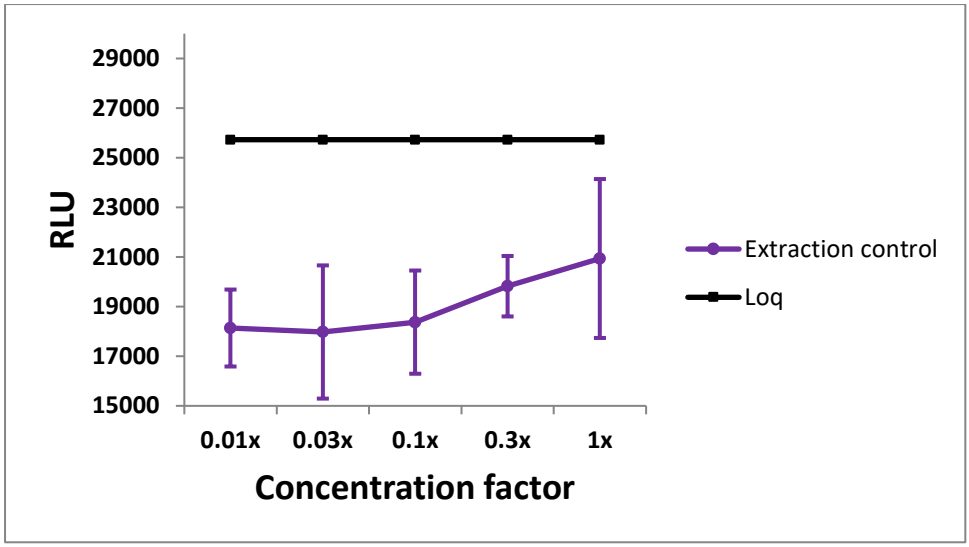

Figure 4.3: The response of the extraction control in the T47D-KBluc cells. HPLC grade ethanol was used as a control.

HPLC grade ethanol was used as a control. Water extracts were tested for oestrogenic activity using the T47D KBluc bioassay. Oestrogenic activity was detected in Nandoni Dam, Albasini Dam and Xikundu Weir (Table 4.1). Xikundu had the highest oestradiol equivalent as compared to other dams but it was still below the trigger value for drinking water which is 0.7ng/ℓ (Genthe *et al*., 2010). No oestrogenic activity was detected in the Tate Vondo Dam.

Table 4.1: Oestrogenic activity of extraction control and water samples expressed as oestradiol equivalents (EEq) in ng/ℓ.

Sample site & control	EEq (ng/ℓ) ± standard deviation
Extraction control	< dl
TateVondo Dam	< dl
Nandoni Dam	0.210 ± 0.059
Albasini Dam	0.226 ± 0.023
Xikundu Weir	0.237*

< dl Below the detection limit of the assay

* EEq value could only be calculated for one of the triplicate plates

4.2 MDA-kb2

The MDA-Kb2 cells were exposed to mancozeb from the highest concentration of 200 mg/ℓ to the lowest 2E-13 mg/ℓ in order to check for androgenic and anti-androgenic activity. Both androgenic and anti-androgenic activities were tested on the same plate. Mancozeb did not show any sign of androgenic characteristics (figure 4.4). Cytotoxicity was observed under a microscope in the wells with the two highest concentrations, which could mask the activity.

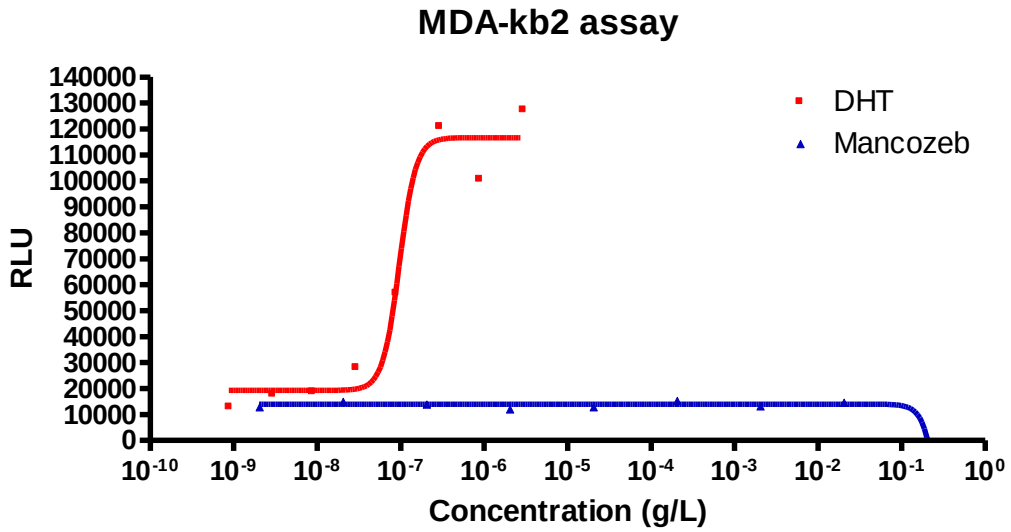

Figure 4.4: Dose-response curves for the mancozeb and dihydrotestosterone (DHT) in the MDA-kb2 cells.

After samples were exposed to dihydrotestosterone (DHT) in triplicates plates, none of the four samples showed any sign of anti-androgenic activity. Mancozeb did not show any sign of anti-androgenic activity, but cytotoxicity was observed in the well with the highest concentration (200 mg/ℓ) (figure 4.5). The F and mancozeb were co incubated with DHT and both suppressed the DHT activity. F was able to suppress the DHT curve at lower concentrations. The response of DHT curve co-incubated with mancozeb as seen from the graph above was low because the cells were dead thus no activity was detected. Cytotoxicity was seen under a microscope.

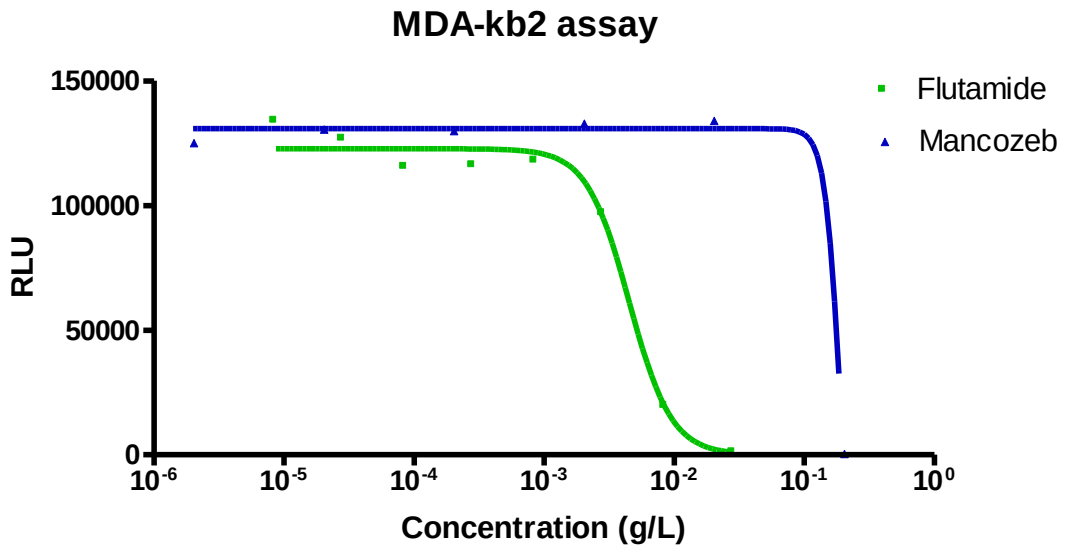

Figure 4.5: Dose-response curves for the mancozeb and flutamide (F), co-incubated with 10 nM DHT, in the MDA-kb2 cells.

There was no androgenic activity detected in the extraction control (Figure 4.6).

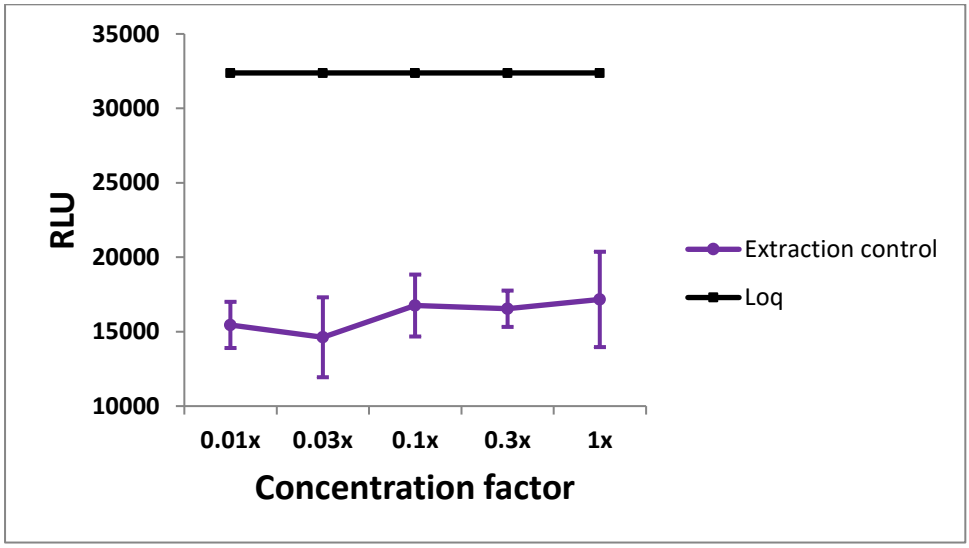

Figure 4.6: The response of the extraction control in the MDA-kb2 cells.

Androgenic activity was tested in all four dams using MDA-kb2 bioassay and all values were below detection limit (Table 4.2).

Table 4.2: Androgenic activity of extraction control and water samples expressed as dihydrotestosterone equivalents (DHT Eq).

Sample site & control	DHT Eq (ng/ℓ) and Standard deviation
Extraction control	< dl
Tate Vondo	< dl
Nandoni Dam	< dl
Albasini Dam	< dl
Xikundu Weir	< dl

<dl below detection limit of the assay

4.3 GH3.TRE-Luc

The GH3.TRE-Luc cells were exposed to mancozeb from the highest concentration of 1 mg/ℓ to the lowest 1E-15 mg/ℓ in order to check for thyroid activity. Mancozeb curves were compared to T3 standard curves. Mancozeb did not show any sign of thyroid activity at the tested concentrations (figure 4.7). At higher concentrations, mancozeb showed cytotoxicity. This was confirmed using the

resazurine assay, which indicated cytotoxicity at 20 mg/ℓ and higher concentrations. It should be kept in mind that cytotoxicity could mask thyroid activity if the activity is in the toxic range.

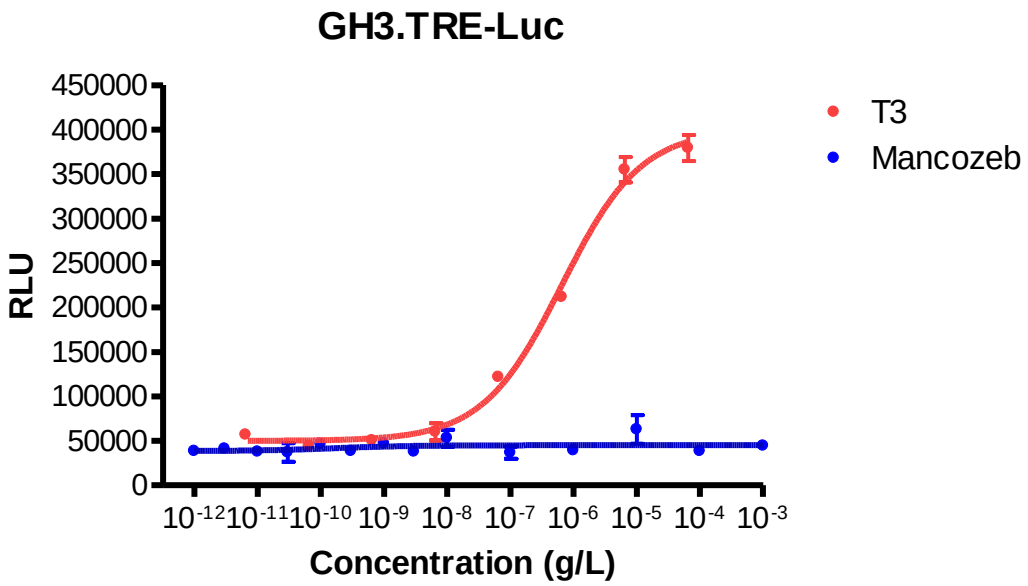

Figure 4.7: Dose-response curves for the mancozeb and positive control triiodothyronine (T3) in GH3.TRE-Luc assay cells.

The GH3.TRE-Luc cells were exposed to mancozeb from the highest concentration of 1 mg/ℓ to the lowest 1E-15 mg/ℓ in order to check for anti-thyroid activity. Mancozeb curves were compared to sodium arsenite standard curves when co-incubated with T3. Mancozeb did not show any sign of anti-thyroid activity (figure 4.8).

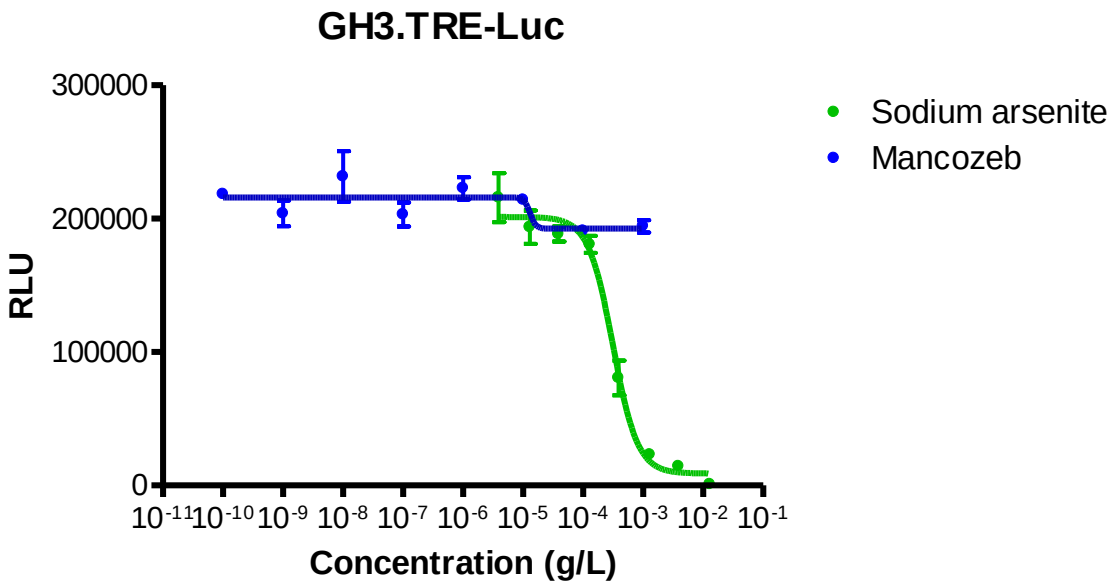

Figure 4.8: Dose-response curves and standard deviations for the mancozeb and antagonist control, co-incubated with 1 nM T3 in GH3.TRE-Luc cells.

Thyroid activity was below the level of quantification in all the water samples (Table 4.3). The resazurine assay revealed no cytotoxicity in any of the samples.

Table 4.3: Thyroid activity of the extraction control and water samples expressed as triiodothyronine equivalents (TEq).

Sample site & control	TEq (ng/ℓ) and standard deviation
Extraction control	< loq
TateVondo Dam	< loq
Nandoni Dam	< loq
Albasini Dam	< loq
Xikundu Weir	< loq

< loq Below the level of quantification, i.e. below the EC10 for the T3 standard curve

4.4 Water

4.4.1 Metals

The South African water quality guideline, field guide, indicates the guideline values of the concentrations of the chemicals in fresh water (Table 4.5). All dams were much lower than 0.01 mg/ℓ of Cd, Pb and Hg. The values of As fell within the safe level as they were all below 0.01 mg/ℓ.

All dams had the highest Mn value with Xikundu Weir toping which is of concern as it is above the Mn guideline value and poses a high risk for human health. Albasini Dam was the only Dam that had a Zn value but within the proposed of guideline values.

Table 4.5: Metal concentrations in the water collected from the four sites in the Vhembe District.

	Metal measured (mg/ℓ)					
Sampling sites	**Cd**	**Hg**	**Pb**	**As**	**Mn**	**Zn**
Guideline value (DWAF 1996)	0.00015	0.00004	0.0002	0.010	0.000180	0.002
Tate Vondo Dam	< 0.010	< 0.010	< 0.010	< 0.010	< 0.025	0.016
Nandoni Dam	< 0.010	< 0.010	< 0.010	< 0.010	< 0.025	0.010
Albasini Dam	< 0.010	< 0.010	< 0.010	< 0.0.10	< 0.025	< 0.010
Xikundu Weir	< 0.010	< 0.010	< 0.010	< 0.010	0.146	0.900

4.4.2 Mancozeb analyses

Mancozeb was not detected in any of the water samples collected from the four sites.

CHAPTER 5 - DISCUSSION

In vitro and *in vivo* studies reported on the endocrine disrupting properties of mancozeb (Runkle, 2017). After evaluating the endocrine disruptive properties of mancozeb using T47D-KBluc, MDA-kb2, and GH3-TRE-Luc bioassays, these properties could not be confirmed. In this study mancozeb did not show agonist activity or antagonist activity in any of the assays used. However, at the two highest test concentrations (100 mg/ℓ and 200 mg/ℓ), cytotoxicity was observed under the microscope and was confirmed with the resazurine assay. The cytotoxicity results are not surprising as it was reported before by Lin and Garry (2000) in MCF-7 breast cancer cells and by Ghisari *et al*. (2015) in GH3 rat pituitary tumour cells. Cytotoxicity can mask the oestrogenic, androgenic and thyroid activity under toxic concentrations. Therefore, although no oestrogenic, androgenic or thyroid activity was seen, there is a chance that any of the above activity could have been missed as a result of the cytotoxicity found.

Using the T47D-KBluc cell line, no oestrogenic or anti-oestrogenic properties were confirmed for mancozeb. This is similar to the study by Lin and Garry (2000) that reported no oestrogenic activity for mancozeb in the MCF-7 breast cancer cell line. However, this is in conflict with several in *vivo* studies that suggested that mancozeb may have oestrogenic effects. Gupta (1994) has indicated that mancozeb can cause decreases in the number of oestrous cycles and the duration of pro-oestrus, oestrus, and met-oestrus with a parallel increase in the di-oestrus phase. In rats mancozeb caused atretic follicles and a decrease in the number of healthy follicles (Baligar and Kaliwal, 2001). In parallel it may cause a decrease in uterus weight and inhibits implantation. Recent evidence includes a net delay of the breeding and an effect on sexual maturity in the tadpoles of the green frog (*Rana saharica*) treated with different mancozeb concentrations (Sana *et al*., 2015). Although this is not specifically for the female hormone it has to be assumed that breeding and sexual maturity is a characteristic held by both sexes. The discrepancy between the *in vitro* and *in vivo* studies may suggest that the oestrogenic effects of mancozeb might be non-receptor mediated.

Mancozeb is classified as an EDC and has anti-androgenic properties (Kjeldsen *et al*., 2013). A study by Archer and van Wyk (2015) confirmed the anti-androgenic properties of mancozeb in the recombinant yeast anti-androgen screen (anti-YAS) assay. Another report on the androgenic properties of mancozeb came from Kjeldsen *et al*. (2013) using carcinoma MVLN and hamster CHO-K1 cells. These authors noted that at low concentrations mancozeb has an inhibitory effect on AR activity. It was expected that mancozeb may show anti-androgenic activity using the MDA-kb2 assay, however this could not be confirmed. The MDA-kb2 is a well-known and used bioassay to investigate (anti-)androgenic properties of chemicals. It has been used by Wilson *et al*. (2002), Xu *et al*. (2008), Blake *et al*. (2010), Du *et al*. (2010), Ermler *et al*. (2010), Kugathas *et al.* (2016) and König *et al*. (2017) to show androgenic properties of chemicals. Ksheerasagar *et al*. (2010) exposed testes of mice to mancozeb for 30 days and detected toxicity in their testes, also a significant

decrease in size. Another study by Kugathas *et al*. (2016) exposed mouse sertoli cells to several pesticides and using an enzyme-linked immunosorbent assay (ELISA) they found that some of the pesticides antagonized the androgen receptor.

Although mancozeb is known to act as a thyroid inhibitor (Cocco, 2002; Pickford, 2010) no thyroid activity were found using the GH3-TRE-Luc cell line. In contrast, Ghisari *et al.* (2015) reported thyroid activity in the T-screen using GH3 cells. The activity was only detected at one concentration; the activity of mancozeb was within the maximum range of 38 - 7% compared to the activity induced by T3 and high concentrations were cytotoxic, similar to our study. The difference in the mancozeb between the two studies is that we used the commercial grade mancozeb and they used the reagent/analytical grade. Mancozeb contains Zn and Mn. A recent study of Li *et al*. (2016) exposed TR yeast cells to Cd, Zn, Hg, $CuSO_4$, and $MnSO_4$ and revealed that none of these elements exhibited TR-agonistic activities. They also exposed GH3 cells to the same metals and both TR yeast T-screen assays indicated the anti-thyroid activity on Zn, Hg and Cd ions.

Several studies focused on chemicals that have an effect on androgen and oestrogen hormones/receptors and less have researched about thyroid hormone, therefore more research on the effects of chemicals on thyroid receptors are needed (Kuster *et al*., 2010; Shi *et al*., 2012). Other chemicals that showed thyroid activity include *p,p'*-DDE, *trans*-nonachlor and oxychlordane exposure but the activity was very poor and they also changed the levels of T4 and TSH (Jain, 2014; Li, 2016) therefore we need to do more research on chemicals with thyroid activity. Mancozeb reduced T4 levels in dams in the study of Axelstad *et al*. (2011) and they also indicated that it may become a potential cause of the disruption in the thyroid of humans.

One of the challenges in the study of EDCs is that most of these chemicals react at low concentrations (Archer and van Wyk, 2015), which might be below the detection limit. This might be one of the reasons mancozeb could not be detected. A study by Archer and Wyk (2015), analysed several pesticides using the recombinant yeast androgen screen (YAS). Mancozeb was the most potent anti-androgen and showed activity from 1.95 µM whereas other pesticides only reacted from 1 mM.

Mancozeb is not the only concern in water, other EDCs (such as DDT) that are present in the water might have an additive effect. So even though the levels are below the level of quantification, when several chemicals with the same effect are added together, the resultant activity may have a detrimental effect (Archer *et al*., 2017).

Compared to this study the level of mancozeb in all four sites were below the level of quantification (10 µg/ℓ). In comparison, the maximum mancozeb concentration was 39 µg/ℓ in a simulation study that investigated point source contamination of pesticides from a vineyard farm in Italy (Fait *et al.,*

2007). Approximately 1 km form the source of point contamination, the mancozeb concentration exceeded 0.1 µg/ℓ.

Mancozeb falls under organophosphates and Rodriguez *et al*. (2006) detected organophosphates (with the concentrations of up to 10 ng/mℓ) using a SPE method too but when they sampled they kept the pH at 6.8 before extraction. They used OASIS cartridges which were conditioned with ethyl acetate methanol and water (2 mℓ each). In this study the pH was adjusted to 2-3 in the SPE extraction method for endocrine disruptors using Oasis HLB glass cartridges conditioned with 5 mℓ dd H_2O followed by 5 mℓ of methanol (HPLC grade) and 5 mℓ of dd H_2O but mancozeb was not detected. A different extraction method specific for organophosphates might be more appropriate to use.

Oestrogenic activity was only detected at three of the four sites at very low levels (Table 1.1). In contrast, oestrogenic activity was reported in the Stellenbosch region with the E2 equivalent values ranging between 0.082 and 0.029 µg/ℓ, their sampling was done in summer and the results indicated oestrogenic activity in two of the 10 sampling sites using the recombinant yeast oestrogen assay (YES) (van Wyk *et al*., 2014). The samples did not suppress the oestrogenic activity either. Although the three positive samples were below the trigger value of 0.7 ng/ℓ for oestrogenic activity of drinking water (Genthe *et al*., 2010), the fact that oestrogenic activity was detected is still a concern and needs to be monitored. Since mancozeb was not detected during the chemical analyses, the activity detected is probably from other sources such as other types of EDCs from the sewage effluent or pharmaceutical effluent from a nearby hospital. The activity from the Nandoni Dam might also be due to personal care products, household products and industrial products entering the dam via various effluent points from the area.

No oestrogenic activity was measured in the extraction controls, which were similar to Aneck-Hahn *et al*. (2008) thus excluding the possible contamination of the cartridges. Cell viability after exposure to the samples still looked more or less similar to the cell viability before exposure and to the control, meaning that the water samples were not toxic to the cells. The EEq values of extraction control and Tate Vondo Dam were below the limit of quantification whereas Nandoni Dam, Albasini Dam and Xikundu Weir had values above the limit of quantification but still very low (table 1.1). Gumbo *et al*. (2016) studied the similarities between upstream and downstream of sampling sites that are downstream of municipal sewage plants. Upstream of the Nandoni Dam are five sewage treatment plants (STPs) of which one of them is the Elim STP discharging its effluents into the Muhohodi River, possibly ending up into the Nandoni Dam lower down. The effluents/pollutants from the remaining four STPs (Vuwani oxidation ponds, Waterval STP, Thohoyandou and Vuwani STPs) also finally discharge in the Nandoni Dam (Gumbo *et al*., 2016). Effluent from STPs may contribute to the oestrogenic activity in water samples as most of these STPs are not fully functional.

Aneck-Hahn *et al*. (2009) did a study in the Limpopo province where they found positive oestrogenic activity in Molekane and Sekuruwe villages indicated by values above the detection limit of the assay. The EEq value of four sampling sites per area ranged between 0.68 and 2.29 ng/ℓ and 0.63 to 2.48 ng/ℓ respectively. The EEq of the four sampling sites (different dams) from this study were all below 1 ng/ℓ and ranged from 0.210 to 0.236 ng/ℓ (Table 4.1). The Nandoni Dam was expected to have a high EEq compared to other dams because the water was collected at the edge of the dam where people sometimes dump their used disposable nappies and general household garbage. Dzaga's unpublished masters study indicated that a number of schistosomiasis infections were discovered among villagers situated on the shoreline of Nandoni Dam (Dzaga, 2012 and Gumbo *et al*., 2016).

Unlike the Nandoni Dam and the Albasini Dam, the entrance of Tate Vondo Dam and Xikundu Weir is prohibited. This means that in terms of dumping they are safe thus they have very low levels of Oestrogen activity. They also serve as drinking area for animals and humans. The fact that water samples were collected when the water was settled might also be the reason activity was not detected. Water from the Tate Vondo Dam and the extraction control both had values of EEq less than the limit of quantification. The Albasini Dam receives run offs from a supposedly DDT free area, where there is no IRS (Barnhoorn *et al*., 2009). Therefore, we don't expect the water from the Albasini Dam to pose a threat to human and animal health. Aneck-Hahn *et al*. (2008) collected water samples during a rainy season in the Limpopo Province and detected oestrogenic activity in five out of seven sites with EEqs ranging from 0.3 ± 0.02 ng/ℓ to 2.1 ± 0.18 ng/ℓ. Two of the samples from this other area and the water from Tate Vondo Dam had no detectable oestrogenic activity with EEqs less than limit of quantification. The reason they were able to detect higher oestrogenic activity of the mancozeb as compared to this study might be that they sampled in the reserve's catchment area which is near industries, agricultural settlement and that the water was coming from the sewage treatment plant. They detected no oestrogenic activity at two sites because the water was coming from a household and was covered from contaminants (Aneck-Hahn *et al*., 2008). However, more research is needed from these sites to verify the results found.

Further studies are needed in natural water sources of the Vhembe Districts regarding the detection of oestrogenic activity. The results of this study are different from another study which was done in Pretoria, where oestrogen activity ranging from 0.000816 ng/ℓ to 2.44 ng/ℓ were detected from three different sampling sites (Mahomed *et al*.,2008).

When comparing the results from the Xikundu Weir which had the highest EEq (0.0237 ng/ℓ) to our other sites, it was evident that the site was more affected. This might be due to the fact that Xikundu Weir is situated within the DDT sprayed area (Brink *et al*., 2012; Bornman *et al*., 2009). The water samples did not show any signs of androgenic activity at all sites, but it cannot be concluded that these four dams do not have characteristics of androgen activity because the samples were taken

during the middle of May 2017 during a dry period. There were no run-off points to the dams since the environment was dry. This might be one of the reasons androgen activity was not detected. The water samples from the four dams had values similar to that of the value of the extraction controls. All four sites had very low levels of androgenic activity which were below detection limit. A study was done in summer in the Stellenbosch region, South Africa, in 10 dams and the results confirmed anti-androgenic activity in five of the ten sites using the YAS (van Wyk *et al*., 2014).

None of the samples showed activity using the GH3-TRE-Luc assay. In comparison with other studies anti-thyroid activity associated with phthalate esters was detected in water sources from Yangtze River Delta using TH reporter gene assay (Shi *et al*., 2012). Thyroid activity was also reported by Judan *et al*. (2009) in Sewage Treatment Plant effluents.

The structure of mancozeb consists of Mn and Zn, as such these metals were analysed for from the water extracts. However, the levels were below guideline values. The other reason these levels could not be detected might be because the life span of the mancozeb in soil is very short and it only persist up to 7 days (Geissan *et al*., 2010) meaning even if its transportation was through run-offs the amount will still be very low, or if there is no precipitation for that period and mancozeb degradation is higher at basic pH in the absence of light (López-Fernández *et al*., 2017). Another reason maybe that ETU which is mancozebs' metabolite might have decomposed while it was still on the fruit trees due to high temperature or sunlight since sampling was done during a dry season (Geissan *et al*., 2010). One of the major sources of natural water contamination is the discharge of partially-treated sewage effluent endangering aquatic animals, humans, wild life and livestock (Gumbo *et al*., 2016). The metal contamination of Nandoni Dam as indicated in table 4.5 might be coming from washing off of animal wastes and leaching of fertilizers from commercial agriculture in the tropical area (Gumbo *et al*., 2016) as well as littering. They might also be coming from the neighbouring mines. This can be avoided by placing waste receptacles at the entrance and exit points of the dam. Another thing is that the Nandoni Dam has no buffer vegetation meaning that all run-off from the rural areas end up in the dam (Van Riet and Louw Landscape architects, 2003).

CONCLUSION

Most studies examined the mode of action of mancozeb and detected anti-androgen and thyroid activity. However, no activity was detected in any of the bioassays in this study either oestrogenic, androgenic or thyroid. No mancozeb was detected at any of the sampling sites either. Mn and Zn were detected but only Xikundu Weir. Xikundu Weir had the highest Mn value which is of concern as it is above the Mn guideline value and poses a high risk for human health. Albasini Dam was the only site with Zn below detection but within the proposed of guideline values. Several studies are emphasising that mancozeb has health effects and most *in vivo* studies are reporting its oestrogenic

activity. Therefore, if any trace of mancozeb is found in water bodies it needs to be taken seriously, reported and mitigation plans put in place.

RECOMMENDATIONS

Since there is insufficient literature, further studies (*in vivo* and *in vitro*) are required to determine whether these results are consistent and to sample seasonally to see the effects, to determine the chemical(s) responsible for oestrogen, androgenic and thyroid activity and to characterise the potential effects on human, aquatic, and wildlife health (Aneck-Hahn *et al*., 2008) especially in the Vhembe District area. The sampling should not be once-off in order to quantify the significance of the results. One of the ways in which water contamination can be avoided is by placing waste receptacles at the entrance and exit points of the dam. Another thing is that the Nandoni Dam has no buffer vegetation meaning that all runoff from the rural areas end up in the dam (Van Riet and Louw Landscape architects, 2003).

CHAPTER 6 - REFERENCES

Archer, E., Wolfaardt, G.M. and van Wyk, J.H., 2017. Pharmaceutical and personal care products (PPCPs) as endocrine disrupting contaminants (EDCs) in South African surface waters. *Water SA*, *43*(4), pp.684-706.

Adeniyi, A., Dayomi, M., Siebe, P. and Okedeyi, O., 2008. An assessment of the levels of phthalate esters and metals in the Muledane open dump, Thohoyandou, Limpopo Province, South Africa. *Chemistry Central Joournal*, *2*(1), p.9.

Agency for Toxic Substances and Disease Registry (ATSDR). 2000.Toxicological profile for Arsenic. U.S. Department of Health and Human services, *Public Health Service*.

Andrew, A.S., Burgess, J.L., Meza, M.M., Demidenko, E., Waugh, M.G., Hamilton, J.W. and Karagas, M.R., 2006. Arsenic exposure is associated with decreased DNA repair *in vitro* and in individuals exposed to drinking water arsenic. *Environmental Health Perspectives*, *114*(8), p.1193.

Aneck-Hahn, N., Van Zijl, C., Barnhoorn, I. and Bornman, M., 2007a. Oestrogenic activity in environmental water samples from a nature reserve in an urban metropolis. *Toxicology Letters*, *172*, pp.S72-S73.

Aneck-Hahn, N.H., 2002. Screening for anti-oxidant pollutants and oestrogenicity in drinking water in poverty stricken areas of South Africa.

Aneck-Hahn, N.H., Bornman, M.S. and De Jager, C., 2008. Preliminary assessment of oestrogenic activity in water sources in Rietvlei Nature Reserve, Gauteng, South Africa. *African Journal of Aquatic Science*, *33*(3), pp.249-254.

Aneck-Hahn, N.H., Bornman, M.S., de Jager, C., 2009. Oestrogenic activity in drinking water from a rural area in the Waterberg District, Limpopo Province, South Africa. *Journal of Andrology*.35, 378-4738.

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

Aneck-Hahn, N.H., Schulenburg, G.W., Bornman, M.S., Farias, P., Jager, C., 2007b. "Impaired semen quality associated with environmental DDT exposure in young men living in a malaria area in the Limpopo Province, South Africa." *Journal of Andrology* 28, no. 3. 423-434.

Archer, E. and Van Wyk, J.H., 2015. The potential anti-androgenic effect of agricultural pesticides used in the Western Cape: *In vitro* investigation of mixture effects. *Water SA*, *41*(1), pp.129-138.

Axelstad, M., Boberg, J., Nellemann, C., Kiersgaard, M., Jacobsen, P.R., Christiansen, S., Hougaard, K.S. and Hass, U., 2011. Exposure to the widely used fungicide mancozeb causes thyroid hormone disruption in rat dams but no behavioral effects in the offspring. *Toxicological Sciences*, *120*(2), pp.439-446.

Balabanič, D., Rupnik, M. and Klemenčič, A.K., 2011. Negative impact of endocrine-disrupting compounds on human reproductive health. *Reproduction, Fertility and Development*, *23*(3), pp.403-416.

Baligar, P.N. and Kaliwal, B.B., 2001. Induction of gonadal toxicity to female rats after chronic exposure to mancozeb. *Industrial Health*, *39*(3), pp.235-243.

Baluka, S.A. and Rumbeiha, W.K., 2016. Bisphenol A and food safety: Lessons from developed to developing countries. *Food and Chemical Toxicology*, *92*, pp.58-63.

Barnhoorn, I.E.J., Bornman, M.S., Jansen van Rensburg, C., Bouwman, H., 2009. DDT residues in water, sediment, domestic and indigenous biota from a currently DDT-sprayed area. *Chemosphere*. 77, 1236 – 1241.

Barnhoorn, I.E.J., Bornman, M.S., Pieterse, G.M. and Van Vuren, J.H.J., 2004. Histological evidence of intersex in feral sharptooth catfish (Clarias gariepinus) from an estrogen-polluted water source in Gauteng, South Africa. *Environmental Toxicology*, *19*(6), pp.603-608.

Barnhoorn, I.E.J., Van Dyk, J.C., Pieterse, G.M., Bornman, M.N., 2010. Intersex in feral indigenous freshwater *Oreochromis Mossambicus*, from various parts in the Luvuvhu River, Limpopo Province, South Africa. *Ecotoxicology and Environmental Safety*. 73, 1537-1542.

Batterman, S., Chernyak, S., Gouden, Y., Hayes, J., Robins, T. and Chetty, S., 2009. PCBs in air, soil and milk in industrialized and urban areas of KwaZulu-Natal, South Africa. *Environmental Pollution*, *157*(2), pp.654-663.

Benbrahim-Tallaa, L., Webber, M.M., Waalkes, M.P., 2007. Mechanisms of acquired androgen independence during arsenic-induced malignant transformation of human prostate epithelial cells. *Environtal Health Perspectives* 115:243–247.

Bengu, T.S., du Plessis, J., Modley, L.S. and van Dyk, J.C., 2017. Health effects in fish from the polluted Orlando Dam and Klipspruit wetland system, Soweto, South Africa. *African Journal of Aquatic Science.* 42(2), 131-141.

Beresford, N., Routledge, R.J., Harris, C.A., Sumpter, J.P., 2000. Issues arising when interpreting results from an *in vitro* Assay for oestrogenic activity. *Toxicology and Applied Pharmacology*. 162, 22-33.

Bergman, Å., Heindel, J.J., Jobling, S., Kidd, K., Zoeller, T.R. and World Health Organization, 2013. State of the science of endocrine disrupting chemicals 2012: summary for decision-makers.

Bergman, Å., Heindel, J.J., Jobling, S., Kidd, K.A. and Zoeller, R.T., 2013. Endocrine disrupting chemicals-2012. *WHO Google Scholar*.

Bhandari, R.K., Deem, S.L., Holliday, D.K., Jandegian, C.M., Kassotis, C.D., Nagel, S.C., Tillitt, D.E., vom Saal, F.S. and Rosenfeld, C.S., 2015. Effects of the environmental oestrogenic contaminants bisphenol A and 17α-ethinyl estradiol on sexual development and adult behaviors in aquatic wildlife species. *General and Comparative Endocrinology*, *214*, pp.195-219.

Bhaskar, R. and Mohanty, B., 2014. Pesticides in mixture disrupt metabolic regulation: In silico and *in vivo* analysis of cumulative toxicity of mancozeb and imidacloprid on body weight of mice. *General and Comparative Endocrinology*, *205*, pp.226-234.

Birkett, J.W. and Lester, J.N. eds., 2002. *Endocrine disrupters in wastewater and sludge treatment processes*. IWA Publishing.

Bisson, M., Hontela, Z., 2002. Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan and Mancozeb in adrenocortical steroidogenic cells of Rainbow trout exposed *in Vitro*. *Toxicology and Applied Pharmacology*. 180, 110-117.

Blake, L.S., Martinovic, D., Gray, L.E., Jr., Wilson, V.S., Regal, R.R., Villeneuve, D.L., Ankley, G.T. (2010). Characterization of the androgen-sensitive MDAkb2 cell line for assessing complex environmental mixtures. *Environmental. Toxicology and Chemistry*. 29: 1367-1376.

Bodin, N., Tapie, N., Le Ménach, K., Chassot, E., Elie, P., Rochard, E. and Budzinski, H., 2014. PCB contamination in fish community from the Gironde Estuary (France): Blast from the past. *Chemosphere*, *98*, pp.66-72.

Bornman, R., De Jager, C., Worku, Z., Farias, P. and Reif, S., 2010. DDT and urogenital malformations in newborn boys in a malarial area. *BJU international*, *106*(3), pp.405-411.

Bornman, M.S and Pretorius, E., 2005. Calcium-mediated aponecrosis plays a central role in the pathogenesis of oestrogenic chemical-induced neurotoxicity. *Medical Hypotheses*. 65; 893-904.

Bornman, M.S., Aneck-Hahn, N.H., de Jager, C. 2017. Endocrine Disruptors and Health Effects in Africa : A call for action. *Environmental Health Perspective*.

Bornman, M.S., Barnhoorn, I.E.J., Aneck-Hahn, N.H., 2009. A pilot study on the occurrence of endocrine disruptive chemicals in a DDT-sprayed area. *WRC Report No. KV220-09*. Pretoria: *Water Research Commission*.

Bornman, M.S., Barnhoorn, I.E.J., de Jager, C., Veeramachaneni, D.N.R., 2010.Testicular microlithiasis and neoplastic lesions in wild eland pollutants. *Environmental Research*. 110, 327-333.

Bornman, M.S., van Vuren, J.H., Bouwman, H., de Jager, C., Gentle, B., Barnhoorn, I.E.J., 2007. The use of sentinel species to determine the use of endocrine disruptive activity in an urban nature reserve. *WRC Report No*. 1505/1/07.

Bornman, M.S., van Vuuren, J.H., Bouwman, H., de Jager, C., Genthe, B., Barnhoorn ,E.J. 2007. Endocrine disruptive activity and the potential health risk in an urban nature reserve. Water Research Commission Report No. 1505/1/07 290 pp. /http://.wrc.org.za/publications_reports2.htmlS.

Bouchard, M.F., Sauvé, S., Barbeau, B., Legrand, M., Brodeur, M.È., Bouffard, T., Limoges, E., Bellinger, D.C. and Mergler, D., 2011. Intellectual impairment in school-age children exposed to manganese from drinking water. *Environmental Health Perspectives*, *119*(1), p.138.

Bouwman, H., Booyens, P., Govender, D., Pienaar, D. and Polder, A., 2014. Chlorinated, brominated, and fluorinated organic pollutants in Nile crocodile eggs from the Kruger National Park, South Africa. *Ecotoxicology and Environmental Safety*, *104*, pp.393-402.

Bouwman, H., Kylin, H., Yive, N.S.C.K., Tatayah, V., Løken, K., Skaare, J.U. and Polder, A., 2012. First report of chlorinated and brominated hydrocarbon pollutants in marine bird eggs from an oceanic Indian Ocean island. *Environmental Research*, *118*, pp.53-64.

Bouwman, H., 2004. South Africa and the Stockholm Convention on persistent organic pollutants: science policy. *South African Journal of Science*, *100*(7-8), pp.323-328.

Bouwman, H., Bornman, R., Van Dyk, C. and Barnhoorn, I., 2015. First report of the concentrations and implications of DDT residues in chicken eggs from a malaria-controlled area. *Chemosphere*, *137*, pp.174-177.

Bouwman, H., Polder, A., Venter, B., Skaare, J.U., 2008. Organochlorine contaminants in cormorant, darter, egret and ibis eggs from South Africa. *Chemosphere*. 71, 227–241.

Bouwman, H., Sereda, B. and Meinhardt, H.M., 2006. Simultaneous presence of DDT and pyrethroid residues in human breast milk from a malaria endemic area in South Africa. *Environmental pollution*, *144*(3), pp.902-917.

Brink, K., van Vuren, J.J. and Bornman, R., 2012. The lack of endocrine disrupting effects in catfish (Clarias gariepinus) from a DDT sprayed area. *Ecotoxicology and Environmental Safety*, *79*, pp.256-263.

Brouwers, M.M., Besselink, H., Bretveld, R.W., Anzion, R., Scheepers, P.T., Brouwer, A. and Roeleveld, N., 2011. Oestrogenic and androgenic activities in total plasma measured with reporter-gene bioassays: relevant exposure measures for endocrine disruptors in epidemiologic studies? *Environment International*, *37*(3), pp.557-564.

Burger, A.E.C. and Nel, A., 2008. Scoping study to determine the potential impact of agricultural chemical substances (pesticides) with endocrine disruptor properties on the water resources of South Africa. Pretoria: *Water Research Commission*.

Burger, AEC., 2005. Strategic research plan for Endocrine Disrupters in South Africa water system.*WRC programme on endocrine disrupting compounds (EDCs) volume 1*.

Calafat, A.M., Weuve, J., Ye, X., Jia, L.T., Hu, H., Ringer, S., Huttner, K. and Hauser, R., 2009. Exposure to bisphenol A and other phenols in neonatal intensive care unit premature infants. *Environmental Health Perspectives*, *117*(4), p.639.

Campos-Mañas, M.C., Plaza-Bolaños, P., Sánchez-Pérez, J.A., Malato, S. and Agüera, A., 2017. Fast determination of pesticides and other contaminants of emerging concern in treated wastewater using direct injection coupled to highly sensitive ultra-high performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*.

Canfield, R.L., Henderson, C.R., Cory-Slechta, D.A., Cox, C., Jusko, T.A., Lanphear, B.P., 2003 Intellectual impairment in children with blood lead concentrations below 10 microg per deciliter. *N English Journal of Medicine.* 348 (16): 1517-1526.

Cocco, P., 2002. On the rumors about the silent spring: review of the scientific evidence linking occupational and environmental pesticide exposure to endocrine disruption health effects. *Cadernos de Saúde pública*, *18*(2), pp.379-402.

Conis, E., 2010. Debating the health effects of DDT: Thomas Jukes, Charles Wurster, and the fate of an environmental pollutant. *Public Health Reports*, *125*(2), pp.337-342.

Corsini, E., Birindelli, S., Fustinoni, S., De Paschale, G., Mammone, T., Visentin, S., Galli, C.L., Marinovich, M. and Colosio, C., 2005. Immunomodulatory effects of the fungicide Mancozeb in agricultural workers. *Toxicology and Applied Pharmacology*, 208(2), pp.178-185.

Cory-Slechta, D.A., 1997. Relationships between Pb-induced changes in neurotransmitter system function and behavioral toxicity. *Neurotoxicology*.18 (3): 673-688.

Dabrowski, J.M., Aneck-Hahn, N.H., Forbes, P.B.C., Genthe, B., Pieters, R., Meyer,J.R., 2015. Investigation of the Contamination of Water Resources by Agricultural Chemicals and the Impact on Environmental Health.Water Research Commision, Pretoria.Volume 1: *Risk Assessment of Agricultural Chemicals to Human and Animal Health.*

Damstra, T., 2002. Potential effects of certain persistent organic pollutants and endocrine disrupting chemicals on the health of children. *Journal of Toxicology: Clinical Toxicology*, *40*(4), pp.457-465.

de Bruin, W., Kritzinger, Q., Bornman, M.S. and Korsten, L., 2016. Nonylphenol, an industrial endocrine disrupter chemical, affects root hair growth, shoot length and root length of germinating cos lettuce (Lactuca sativa). *Seed Science and Technology*, *44*(1), pp.43-52.

de Jager, C., Aneck-Hahn, N.H., Barnhoorn, I.E.J., Bornman, M.S., Pieters, P., Van Wyk, J.H., Van Zijl, C., 2011. The compilation of toolbox of bioassays for detection of oestrogenic activity in water. *WRC Report No.1816/1/10*. *Water Research Commission*.

de Joode, B.V.W., Barbeau, B., Bouchard, M.F., Mora, A.M., Skytt, Å., Córdoba, L., Quesada, R., Lundh, T., Lindh, C.H. and Mergler, D., 2016. Manganese concentrations in drinking water from villages near banana plantations with aerial mancozeb spraying in Costa Rica: Results from the Infants' Environmental Health Study (ISA). *Environmental Pollution*, *215*, pp.247-257.

Der, R., Yousef, M., Fahim, Z., Fahim, M., 1977. Effects of lead and cadmium on adrenal and thyroid function in rats. *Research Communications in Chemical Pathology and Pharmacology* 17:237-253.Department of Water Affairs and Forestry (DWAF)., 1996. South African Water Quality Guidelines. Second edition. Volume 7: *Aquatic ecosystems*. Pretoria, South Africa.

DWAF (Department of Water Affairs and Forestry)., 2001. State of the rivers report: the Letaba and Luvuvhu River systems. *The River Health Programme*. Pretoria: Department of Water Affairs and Forestry.

Department of Water Affairs, South Africa., 2012. Development of a reconciliation strategy for the Luvuvhu and Letaba water supply system: Water quality assessment report. Prepared by: *Golder Associates Africa. Report No*. P WMA 02/B810/00/1412/8

Diamanti-Kandarakis, E., Bourguignon, J., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T. and Gore, A.C., 2009. Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. *Endocrine Reviews.* 30(4), 293-342.

Domico, L.M., Zeevalk, G.D., Bernard, L.P., Cooper, K.R., 2006. Acute neurotoxic effect of mancozeb and maneb in mesencephaic neuronal cultures are associated with mitochondrial dysfunction. *Neuro Toxicology*. 20, 816-825.

Du, G., Shen, O., Sun, H., Fei, J., Lu, C., Song, L., Xia, Y., Wang, S. and Wang, X., 2010. Assessing hormone receptor activities of pyrethroid insecticides and their metabolites in reporter gene assays. *Toxicological Sciences*, *116*(1), pp.58-66.

Dzaga, R.A., 2012. An assessment of the Impact of Catchment activities on water Quality: A Case study of Nandoni Dam,Vhembe District, Limpopo Province. Unpublished Master's Thesis, University of Venda, Thohoyandou, South Africa.

Edwards, J.R., Prozialeck, W.C., 2009. Cadmium, diabetes and chronic kidney disease. *Toxicology and Applied Pharmacology,* 238(3):289-293.

El Majidi, N., Bouchard, M. and Carrier, G., 2014. Systematic analysis of the relationship between standardized biological levels of polychlorinated biphenyls and thyroid function in pregnant women and newborns. *Chemosphere*, *98*, pp.1-17.

Erler, C. and Novak, J., 2010. Bisphenol A exposure: human risk and health policy. *Journal of Paediatric Nursing*, *25*(5), pp.400-407.

Ermler, S., Scholze, M. and Kortenkamp, A., 2010. The sensitivity of the MDA-kb2 cell *in vitro* assay in detecting anti-androgenic chemicals–identification of sources of variability and estimation of statistical power. *Toxicology In Vitro*, *24*(6), pp.1845-1853.

Fait, G., Nicelli, M., Fragoulis, G., Trevisan, M. and Capri, E., 2007. Reduction of point contamination sources of pesticide from a vineyard farm. *Environmental Science & Technology*, *41*(9), pp.3302-3308.

Fatoki, O.S., Bornman, M., Ravandhalala, L., Chimuka, L., Genthe, B. and Adeniyi, A., 2010. Phthalate ester plasticizers in freshwater systems of Venda, South Africa and potential health effects. *Water SA* 36(1), 117-126.

Flame retardants. 2017. National Institute of Environmental Health Services. https://www.niehs.nih.gov/health/topics/agents/flame_retardants/index.cfm.

Foster, P.M.D., Mylchreest, E., Gaido, K.W., Sar, M., 2001. Effects of phthalate ester on the developing reproductive tract of male rats. *Human Reproduction*, Vol, No.3.pp .231-235.

Freitas, J., Cano, P., Craig-Veit, C., Goodson, M.L., Furlow, J.D. and Murk, A.J., 2011. Detection of thyroid hormone receptor disruptors by a novel stable *in vitro* reporter gene assay. *Toxicology in Vitro*, *25*(1), pp.257-266.

Furness, R.W., 1996. Cadmium in birds. *Environmental contaminants in wildlife: interpreting tissue concentrations. Lewis, Boca Raton*, pp.389-404.

Garcia-Heras, M.S., Arroyo, B., Simmons, R.E., Camarero, P.R., Mateo, R. and Mougeot, F., 2017. Blood concentrations of PCBs and DDTs in an avian predator endemic to southern Africa: Associations with habitat, electrical transformers and diet. *Environmental Pollution*.

Genthe, B., Steyn, M., Aneck-Hahn, N.H., Van Zijl, C., De Jager, C., 2010. The feasibility of a health risk assessment framework to derive guidelines for oestrogen activity in treated drinking water. *WRC Report No. 1749/1/09. Water Research Commission of South Africa*.

Geissen, V., Ramos, F. Q., de J. Bastidas-Bastidas, P., Díaz-González, G., Bello-Mendoza, R., Huerta-Lwanga, E., & Ruiz-Suárez, L. E. (2010). Soil and Water Pollution in a Banana Production Region in Tropical Mexico. *Bulletin of Environmental Contamination and Toxicology*, *85*(4), 407–413. http://doi.org/10.1007/s00128-010-0077-y

Georgescu, B., Georgescu, C., Dărăban, S., Bouaru, A. and Paşcalău, S., 2011. Heavy metals acting as endocrine disrupters. *Scientific Papers Animal Science and Biotechnologies*, *44*(2), pp.89-93.

Ghisari, M., Long, M., Tabbo, A. and Bonefeld-Jørgensen, E.C., 2015. Effects of currently used pesticides and their mixtures on the function of thyroid hormone and aryl hydrocarbon receptor in cell culture. *Toxicology and Applied Pharmacology*, *284*(3), pp.292-303.

Giesy, J.P., Kannan, K., 2001. Global Distribution of Perfluorooctane Sulfonate in Wildlife. *Environmental Science Technology*, *35*(7): 1339–1342.

Grilo, T.F., Cardoso, P.G., Pato, P., Duarte, A.C. and Pardal, M.A., 2013. Organochlorine accumulation on a highly consumed bivalve (Scrobicularia plana) and its main implications for human health. *Science of The Total Environment*, *461*, pp.188-197.

Grygo-Szymanko, E., Tobiasz, A. and Walas, S., 2016. Speciation analysis and fractionation of manganese: A review. *TrAC Trends in Analytical Chemistry*, *80*, pp.112-124.

Guellard, T., Sokołowska, E. and Arciszewski, B., 2015. First report on intersex in invasive round goby Neogobius melanostomus from the Baltic Sea (Gulf of Gdańsk, Poland). *Oceanologia*, *57*(1), pp.102-106.

Guillette, L. J., Arnold, S. F. and Mclachlan, J. A. (1996). Eco estrogens and embryos - Is there a scientific basis for concern? *Animal Reproduction Science*. 42: 13-24.

Guillette, L. J., Gross, T. S., Masson, G. R., Matter, J. M., Percival, H. F. and Woodward, A. R. (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environmental Health Perspectives*. 102: 680-688.

Guillette, L.J. and Gunderson, M.P., 2001. Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. *Reproduction*, 122(6), pp.857-864.

Gumbo, J.R., Dzaga, R.A. and Nethengwe, N.S., 2016. Impact on Water Quality of Nandoni Water Reservoir Downstream of Municipal Sewage Plants in Vhembe District, South Africa. *Sustainability*, *8*(7), p.597.

Gunier, R.B., Jerrett, M., Smith, D.R., Jursa, T., Yousefi, P., Camacho, J., Hubbard, A., Eskenazi, B. and Bradman, A., 2014. Determinants of manganese levels in house dust samples from the CHAMACOS cohort. *Science of the Total Environment*, *497*, pp.360-368.

Gupta, P.K., 2011. Chapter 39. Herbicides and fungicides. In: *Reproductive and Developmental Toxicology*, R.C. Gupta (Ed). Academic Press, Elsevier, London, United Kingdom. Pages 503 – 521.

Gupta, V.K., Sharma, S.K. and Sharma, J.N., 1994. Persistence of mancozeb in apple. *Plant Disease Research*, pp.223-225.

Hanekom, L., Bouwman, H., Minnaar, K. and Swiegelaar, C., 2015, August. Effects of perfluorooctanesulfonic acid (PFOS) on the embryonic development of the freshwater mollusc Bulinus tropicus. In *7th International Toxicology Symposium in Africa* (Vol. 30, p. 99)

Harrison, P.T.C., Holmes, P. and Humfrey, C.D.N., 1997. Reproductive health in humans and wildlife: are adverse trends associated with environmental chemical exposure?. *Science of the Total Environment*, *205*(2-3), pp.97-106.

Hass, U., Boberg, J., Christiansen, S., Jacobsen, P.R., Vinggaard, A.M., Taxvig, C., Poulsen, M.E., Herrmann, S.S., Jensen, B.H., Petersen, A. and Clemmensen, L.H., 2012. Adverse effects on sexual development in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. *Reproductive Toxicology*, *34*(2), pp.261-274

Hauser, R. and Calafat, A.M., 2005. Phthalates and human health. *Occupational and environmental medicine*, *62*(11), pp.806-818.

Henson, M.C. and Chedrese, P.J., 2004. Endocrine disruption by cadmium, a common environmental toxicant with paradoxical effects on reproduction. *Experimental Biology and Medicine*, *229*(5), pp.383-392.

Heudorf, U., Mersch-Sundermann, V. and Angerer, J., 2007. Phthalates: toxicology and exposure. *International Journal of Hygiene and Environmental Health*, *210*(5), pp.623-634.

Hotchkiss, A.K., Rider, C.V., Blystone, C.R., Wilson, V.S., Hartig, P.C., Ankley, G.T., Foster, P.M., Gray, C.L. and Gray, L.E., 2008. Fifteen years after “Wingspread”—environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go. *Toxicological Sciences*, *105*(2), pp.235-259.

http://www.ourstolenfuture.org/Basics/chemlist.htm.

https://www.niehs.nih.gov/health/topics/agents/endocrine/index.cfm

https://www.whsc.on.ca/Files/Resources/Hazard-Resource-Lines/RL_Endocrine-Disruptors_V1_recreated-Aug-17.aspx

IPCS. 2002. *Global assessment of the state-of-the-science of endocrine disruptors*. Geneva, Switzerland.

Jacobson, J.L. and Jacobson, S.W., 1997. Evidence for PCBs as neurodevelopmental toxicants in humans. *Neurotoxicology*, *18*(2), pp.415-424.

Jain, R.B., 2014. Association between thyroid function and selected organochlorine pesticides: data from NHANES 2001–2002. *Science of The Total Environment*, *466*, pp.706-715.

Jugan, M.L., Levi, Y. and Blondeau, J.P., 2010. Endocrine disruptors and thyroid hormone physiology. *Biochemical Pharmacology*, *79*(7), pp.939-947.

Kabir, E.R., Rahman, M.S. and Rahman, I., 2015. A review on endocrine disruptors and their possible impacts on human health. *Environmental Toxicology and Pharmacology*, *40*(1), pp.241-258.

Kegley, S.E., Hill, B.R., Orme S., Choi A.H., 2000-2016.PAN Pesticide Database, Pesticide Action Network, North America (Oakland, CA, 2016), http:www.pesticideinfo.org. © 2000-2016 *Pesticide Action Network*, North America.

Kelce, W., Stone, C., Laws, S., *et al*., 1995. Persistent DDT metabolite *p,p'*-DDE is a potent androgen receptor antagonist. *Nature*. 375, 581–585.

Kime, D.E., 2012. *Endocrine disruption in fish*. Springer Science & Business Media.

Kjeldsen, L.S., Ghisari, M., Bonefeld-Jorgensen, E.C., 2013. Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity. *Toxicology. Applied and Pharmacology*. 272, 453–464.

König, M., Escher, B.I., Neale, P.A., Krauss, M., Hilscherová, K., Novák, J., Teodorović, I., Schulze, T., Seidensticker, S., Hashmi, M.A.K. and Ahlheim, J., 2017. Impact of untreated wastewater on a major European river evaluated with a combination of *in vitro* bioassays and chemical analysis. *Environmental Pollution*, *220*, pp.1220-1230.

Ksheerasagar, R.L. and Kaliwal, B.B., 2003. Temporal effects of mancozeb on testes, accessory reproductive organs and biochemical constituents in albino mice. *Environmental Toxicology and Pharmacology*, *15*(1), pp.9-17.

Ksheerasagar, R.L. and Kaliwal, B.B., 2010. Effect of mancozeb on thyroid, testis, accessory reproductive organs and biochemical constituents in albino mice. *Recent Research in Science and Technology*, *2*(8).

Kugathas, S., Audouze, K., Ermler, S., Orton, F., Rosivatz, E., Scholze, M. and Kortenkamp, A., 2016. Effects of common pesticides on prostaglandin D2 (PGD2) inhibition in SC5 mouse Sertoli cells, evidence of binding at the COX-2 active site, and implications for endocrine disruption. *Environmental Health Perspectives*, *124*(4), p.452.

Kuster, M., Díaz-Cruz, S., Rosell, M., de Alda, M.L. and Barceló, D., 2010. Fate of selected pesticides, estrogens, progestogens and volatile organic compounds during artificial aquifer recharge using surface waters. *Chemosphere*, *79*(8), pp.880-886.

Lanphear, B.P., Dietrich, K., Auinger, P., Cox, C., 2000. Cognitive deficits associated with blood lead concentrations <10 microg/dL in US children and adolescents. *Public Health Reports*. 115 (6): 521-529.

Lavicoli, I., Fontana, L. and Bergamaschi, A., 2009. The effects of metals as endocrine disruptors. *Journal of Toxicology and Environmental Health, Part B*, *12*(3), pp.206-223.

Leiphon, L.J., Picklo Sr, M.J, 2007. Inhibition of aldehyde detoxification in CNS mitochondria by fungicides. *Neuro Toxicology*. 28, 143-149.

Li, J., Liu, Y., Kong, D., Ren, S. and Li, N., 2016. T-screen and yeast assay for the detection of the thyroid-disrupting activities of cadmium, mercury, and zinc. *Environmental Science and Pollution Research*, *23*(10), pp.9843-9851.

Lin, N. and Garry, V.F., 2000. *In vitro* studies of cellular and molecular developmental toxicity of adjuvants, herbicides, and fungicides commonly used in Red River Valley, Minnesota. *Journal of Toxicology and Environmental Health Part A*, *60*(6), pp.423-439.

London, L., Dalvie, M.A., Cairncross, E., Solomons, A., 2000. The quality of surface and groundwater in the rural western cape with regard to pesticides. *WRC Report No*. 795/1/00.

López-Fernández, O., Barroso, M.F., Fernandes, D.M., Rial-Otero, R., Simal-Gándara, J., Morais, S., Nouws, H.P., Freire, C. and Delerue-Matos, C., 2015. Voltammetric analysis of mancozeb and its degradation product ethylenethiourea. *Journal of Electroanalytical Chemistry*, *758*, pp.54-58.

Mahomed, S.I., Voyi, K.W., Aneck-Hahn, N.H. and De Jager, C., 2008. Oestrogenicity and chemical target analysis of water from small-sized industries in Pretoria, South Africa. *Water SA*, *34*(3), pp.357-363.

Marchand, M.J., Pieterse, J.M., Barnhoorn, I.E.J., 2010. Sperm motility and testicular histology as reproductive indicators of fish health of two feral fish species from a currently DDT sprayed area, South Africa. Department of Urology, *J. Appl. Ichthyol*. 26 (2010), 707–714.

Marchesini, G.R., Meimaridou, A., Haasnoot, W., Meulenberg, E., Albertus, F., Mizuguchi, M., Takeuchi, M., Irth, H. and Murk, A.J., 2008. Biosensor discovery of thyroxine transport disrupting chemicals. *Toxicology and Applied Pharmacology*, *232*(1), pp.150-160.

Medda, E., Santini, F., De Angelis, S., Franzellin, F., Fiumalbi, C., Perico, A., Gilardi, E., Mechi, M.T., Marsili, A., Citroni, A. and Leandri, A., 2017. Iodine nutritional status and thyroid effects of exposure to ethylenebisdithiocarbamates. *Environmental Research*, *154*, pp.152-159.

Mertl, J., Kirchnawy, C., Osorio, V., Grininger, A., Richter, A., Bergmair, J., Pyerin, M., Washüttl, M. and Tacker, M., 2014. Characterization of estrogen and androgen activity of food contact materials by different *in vitro* bioassays (YES, YAS, ERα and AR CALUX) and chromatographic analysis (GC-MS, HPLC-MS). *PloS one*, *9*(7), p.e100952.

Mlambo, S.S., Van Vuren, J.H., Barnhoorn, I.E. and Bornman, M.S., 2009. Histopathological changes in the reproductive system (ovaries and testes) of Oreochromis mossambicus following exposure to DDT. *Environmental Toxicology and Pharmacology*, *28*(1), pp.133-139.

Mohanty, B., Pandey, S.P. and Tsutsui, K., 2017. Thyroid disrupting pesticides impair the hypothalamic-pituitary-testicular axis of a wildlife bird, Amandava amandava. *Reproductive Toxicology*, *71*, pp.32-41.

Mora, A.M., van Wendel de Joode, B., Mergler, D., Córdoba, L., Cano, C., Quesada, R., Smith, D.R., Menezes-Filho, J.A., Lundh, T., Lindh, C.H. and Bradman, A., 2014. Blood and hair Manganese concentrations in pregnant women from the infants' environmental health study (ISA) in Costa Rica. *Environmental Science & Technology*, 48(6), pp.3467-3476.

Morse, D.C., Wehler, E.K., Wesseling, W., Koeman, J.H. and Brouwer, A., 1996. Alterations in rat brain thyroid hormone status following pre-and postnatal exposure to polychlorinated biphenyls (Aroclor 1254). *Toxicology and Applied Pharmacology*, *136*(2), pp.269-279.

Nibamureke, U.M.C., Barnhoorn, I.E.J., Wagenaar, G.M., 2016. Health assessment of freshwater fish species from the Albasini Dam outside a DDT-sprayed area in Limpopo Province, South Africa. *African Journal of Aquatic Science.* DOI http://dx.doi.org/10.2989/16085914.2pul0016.1172198

Nieuwoudt, C., Quinn, L.P., Pieters, R., Jordaan, I., Visser, M., Kylin, H., Borgen, A.R., Giesy, J.P. and Bouwman, H., 2009. Dioxin-like chemicals in soil and sediment from residential and industrial areas in central South Africa. *Chemosphere*, *76*(6), pp.774-783.

O'Connor, J.C., Frame, S.R., Davis, L.G. and Cook, J.C., 1999. Detection of the environmental antiandrogen p, p-DDE in CD and long-evans rats using a tier I screening battery and a Hershberger assay. *Toxicological sciences: an official journal of the Society of Toxicology*, *51*(1), pp.44-53.

Odiyo, J.O., Phangisa, J.I., Makungo, R., 2012. Rainfall-runoff modelling for estimating Latonyanda River flow contributions to Luvuvhu River downstream of Albasini Dam. *Physics and Chemistry of the Earth*. 50-52, 5-13.

Olujimi, O.O., Fatoki, O.S., Odendaal, J.P. and Daso, A.P., 2012. Chemical monitoring and temporal variation in levels of endocrine disrupting chemicals (priority phenols and phthalate esters) from selected wastewater treatment plant and freshwater systems in Republic of South Africa. *Microchemical Journal*, *101*, pp.11-23.

Olujimi, O.O., Fatoki, O.S., Odendaal, J.P. and Okonkwo, J.O., 2010. Endocrine disrupting chemicals (phenol and phthalates) in the South African environment: a need for more monitoring. *Water SA*, *36*(5), pp.671-682.

Orange-Senqu River Commission (ORASECOM)., 2013. POPs, PAHs and elemental levels in sediment, fish and wild bird eggs in the Orange-Senqu River basin. Report 002/2013, prepared by Bouwman, H, Pieters, R, Genthe, B and Chamier, J for ORASECOM, Pretoria.

Pande, M. and Flora, S.J.S., 2002. Lead induced oxidative damage and its response to combined administration of α-lipoic acid and succimers in rats. *Toxicology*, *177*(2), pp.187-196.

Pandey, S.P. and Mohanty, B., 2015. The neonicotinoid pesticide imidacloprid and the dithiocarbamate fungicide mancozeb disrupt the pituitary–thyroid axis of a wildlife bird. *Chemosphere*, *122*, pp.227-234.

Paro, R., Tiboni, G.M., Buccione, R., Rossi, G., Cellini, V., Canipari, R., Cecconi, S., 2012. The fungicide mancozeb induces tpxic effects on mammalian granulosa cells. *Toxicology and Applied Pharmacology*.260, 155 – 161.

Pavlovic, V., Cekic, S., Circic, M., Krtinic, D., Jovanovic, J., 2016. Curcumin attenuates Mancozeb-induced toxicity in rat thymocytes through mitochondrial survival pathway. *Food and Chemical Toxicology*.88, 105-111.

Pickford, D.B., 2010. Screening chemicals for thyroid-disrupting activity: A critical comparison of mammalian and amphibian models. *Critical Reviews in Toxicology*, *40*(10), pp.845-892.

Pirozzi, A.V.A., Stellavato, A., La Gatta, A., Lamberti, M., Schiraldi, C., 2016. Mancozeb, a fungicide routinely used in agriculture,worsens non-alcoholic fatty liver disease in the human HepG2 cell model. *Toxicology Letters*. 249, 1-4.

Pretorius, E. and Bornman, M.S., 2005. Calcium-mediated aponecrosis plays a central role in the pathogenesis of oestrogenic chemical-induced neurotoxicity. *Medical hypotheses*, *65*(5), pp.893-904.

Qin, F., Wang, L., Wang, X., Liu, S., Xu, P., Wang, H., Wu, T., Zhang, Y., Zheng, Y., Li, M., Zhang, X., Yuan, C., Hu, G., Wang, Z., 2013. Bisphenol A affects gene expression of gonadotropin-releasing hormones and type I GnRH receptors in brains of adult rare minnow Gobiocypris rarus. Comparative. Biochemistry and Physiology. Part C: Toxicology and Pharmacology. 157, 192–202.

Ramesh, B.A., 2011. Ascher syndrome: Review of literature and case report. *Indian Journal of Plastic Surgery: Official Publication of the Association of Plastic Surgeons of India*, *44*(1), p.147.

Remaud, S., Gothié, J.D., Morvan-Dubois, G. and Demeneix, B.A., 2014. Thyroid hormone signaling and adult neurogenesis in mammals. *Frontiers in Endocrinology*, *5*.

Rice, K.M., Walker Jr, E.M., Wu, M., Gillette, C. and Blough, E.R., 2014. Environmental mercury and its toxic effects. *Journal of Preventive Medicine and Public Health*, *47*(2), p.74.

Rimayi, C., Chimuka, L., Odusanya, D., de Boer, J. and Weiss, J.M., 2017. Source characterisation and distribution of selected PCBs, PAHs and alkyl PAHs in sediments from the Klip and Jukskei Rivers, South Africa. *Environmental Monitoring and Assessment*, *189*(7), p.327.

Robson, L., Barnhoorn, I.E.J. and Wagenaar, G.M., 2017. The potential effects of efavirenz on Oreochromis mossambicus after acute exposure. *Environmental Toxicology and Pharmacology*, *56*, pp.225-232.

Rodriguez, I., Calvo, F., Quintana, J.B., Rubi, E., Rodil, R. and Cela, R., 2006. Suitability of solid-phase microextraction for the determination of organophosphate flame retardants and plasticizers in water samples. *Journal of Chromatography A*, *1108*(2), pp.158-165.

Ronis, M.J.J., Badger, T.M., Shema, S.J., Roberson, P.K., Shaikh, F., 1996. Reproductive toxicity and growth effects in rats exposed to lead at different periods during development. *Toxicology and Applied Pharmacology* 36:361-371.

Rossi, G., Buccione, R., Baldassarre, M., Macchiarelli, G., Palmerini, M.G., Cecconi, S., 2006. Mancozeb exposure *in vivo* impairs mouse oocyte fertilizability. *Reproductive Toxicology*. 21, 216-219.

Rossman, T.G., 2003. Mechanism of arsenic carcinogenesis: an integrated approach. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *533*(1), pp.37-65.

Runkle, J., Flocks, J., Economos, J. and Dunlop, A.L., 2017. A systematic review of Mancozeb as a reproductive and developmental hazard. *Environment International*, *99*, pp.29-42.

Saeki, K., Okabe, Y., Kim, E.Y., Tanabe, S., Fukuda, M. and Tatsukawa, R., 2000. Mercury and cadmium in common cormorants (Phalacrocorax carbo). *Environmental Pollution*, *108*(2), pp.249-255.

Sana, B., Reda, D.M., Houria, B., Amel, A., Marwa, B., Fateh, Z.M. and Rima, A., 2015. Toxicological impact of a mimetic estrogen specie: mancozeb on a tadpoles of the green frog (Rana saharica).

Shi, W., Zhang, F.X., Hu, G.J., Hao, Y.Q., Zhang, X.W., Liu, H.L., Wei, S., Wang, X.R., Giesy, J.P. and Yu, H.X., 2012. Thyroid hormone disrupting activities associated with phthalate esters in water sources from Yangtze River Delta. *Environment International*, *42*, pp.117-123.

Stahl, T., Mattern, D., Brunn, H., 2011. Toxicology of perfluorinated compounds. *Environmental Sciences Europe,* 23:38.

Steinberg, R.M., Juenger, T.E. and Gore, A.C., 2007. The effects of prenatal PCBs on adult female paced mating reproductive behaviors in rats. *Hormones and Behaviours*, *51*(3), pp.364-372.

Steinberg, RM, Walker, DM, Juenger, TE, Woller, MJ, Gore, AC., 2008. The effects of perinatal PCBs on adult female rat reproduction: development, reproductive physiology, and second generational effects. *Biological Reproduction* 78:1091–110.

Sun, H.J., Xiang, P., Luo, J., Hong, H., Lin, H., Li, H.B. and Ma, L.Q., 2016. Mechanisms of arsenic disruption on gonadal, adrenal and thyroid endocrine systems in humans: A review. *Environment International*, *95*, pp.61-68.

Swedenborg, E., Rüegg, J., Mäkelä, S. and Pongratz, I., 2009. Endocrine disruptive chemicals: mechanisms of action and involvement in metabolic disorders. *Journal of Molecular Endocrinology*, 43(1), pp.1-10.

Takiguchi, M. and Yoshihara, S.I., 2006. New aspects of cadmium as endocrine disruptor. *Environmental sciences: an International Journal of the Environmental Physiology and Toxicology*, *13*(2), pp.107-116.

Tan, S.W., Meiller, J.C. and Mahaffey, K.R., 2009. The endocrine effects of mercury in humans and wildlife. *Critical Reviews in Toxicology*, *39*(3), pp.228-269.

Toppari, J., Larsen, J.C., Christiansen, P., Giwercman, A., Grandjean, P., Guilette, L.J., Jegou, B., Jensen, T.K., Jouannet, P., Keiding, N., Leffers, H., Mclachlan, J.A., Meyer, O., Muller, J., Rajpert-De Meyts, E., Scheike, T., Sharpe, R., Sumpter, J. and Skakkebaek, N.E., 1996. Male reproductive health and environmental xenoestrogens. *Environmental Health Perspectives*. 104(4), 741-803.

Traoré, A.N., Mulaudzi, K., Chari, G.E.J., Foord, S.H., Mudau, L.S., Barnard, T.G. and Potgieter, N., 2016. The impact of human activities on microbial quality of rivers in the Vhembe District, South Africa. *International Journal of Environmental Research and Public Health*. 13, 817-830.

Van Dyk, J.C., Bouwman, H., Barnhoorn, I.E.J. and Bornman, M.S., 2010. DDT contamination from indoor residual spraying for malaria control. *Science of the Total Environment*, *408*(13), pp.2745-2752.

Van Riet and Louw Landscape Architects., 2003. Sustainable utilization plan for the Nandoni Dam in the Thohoyandou District of the Limpopo Province. *Department of Water Affairs and Forestry*.

Van Wyk, J.H., Archer, E., Babalola, O.O., Truter J.C., Jansen van Rensburg, E., & Dabrowski, J., 2014. Pesticides as endocrine disruptors in South Africa: Laboratory and field studies (Report No. 1932/1/14).

Vyskočil, A., Fiala, Z., Ettlerová, E. and Tenjnorová, I., 1990. Influence of chronic lead exposure on hormone levels in developing rats. *Journal of Applied Toxicology*, *10*(4), pp.301-302.

Wehmas, L.C., Cavallin, J.E., Durhan, E.J., Kahl, M.D., Martinovic, D., Mayasich, J., Tuominen, T., Villeneuve, D.L. and Ankley, G.T., 2011. Screening complex effluents for oestrogenic activity with the T47D-KBluc cell bioassay: Assay optimization and comparison with *in vivo* responses in fish. *Environmental Toxicology and Chemistry*, *30*(2), pp.439-445.

wikibooks.org/w/index.php.human-physiology/The endocrine system&oldid)

Williams, M., Woods, M., Kumar, A., Ying, G.G., Shareef, A., Karkkainen, M. and Kookana, R., 2007. Endocrine disrupting chemicals in the Australian riverine environment: a pilot study on oestrogenic compounds. *Land & Water Australia*, p.105.

Wilson, V.S., Bobseine, K., Gray, J.R., L.E., 2004. Development and characterisation of a cell line that stably expresses and oestrogen-responsive luciferase reporter for the detection of oestrogen receptor agonists and antagonists. *Toxicological Sciences* 81, 69-77.

Wilson, V.S., Bobseine, K., Lambright, C.R., Gray, L.E. Jr., 2002. A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. *Toxicological Sciences*. 66(1), 69-81.

Wood, T.P., Duvenage, C.S. and Rohwer, E., 2015. The occurrence of anti-retroviral compounds used for HIV treatment in South African surface water. *Environmental Pollution*, *199*, pp.235-243.

Xu, L.C., Liu, L., Ren, X.M., Zhang, M.R., Cong, N., Xu, A.Q., Shao, J.H., 2008. Evaluation of androgen receptor transcriptional activities of some pesticides in vitro. Toxicology 243, 59–65.

Yamaguchi, S., Miura, C., Ito, A., Agusa, T., Iwata, H., Tanabe, S., Tuyen, B.C. and Miura, T., 2007. Effects of lead, molybdenum, rubidium, arsenic and organochlorines on spermatogenesis in fish: monitoring at Mekong Delta area and in vitro experiment. *Aquatic Toxicology*, *83*(1), pp.43-51.

Annexure

Table 1: T47D-KBluc plate layout of extraction control and samples (Tate Vondo, Nandoni Dam, Albasini and Xikundu Weir) incubated together with E2 (17β estradiol) showing final concentrations in the wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		V Contr	V Contr	100	30	10	3	1	0.3	0.1	0.03	E2 (pM)
			0.1nM E2	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	
C		V Contr	V Contr	1x	0.3x	0.1x	0.03x	0.01x	1x	0.1x	0.01x	EC
			0.1nM E2	V Contr	V Contr	V Contr	V Contr	V Contr	0.1nM E2	0.1nM E2	0.1nM E2	
D		V Contr	V Contr	1x	0.3x	0.1x	0.03x	0.01x	1x	0.1x	0.01x	TV
			0.1nM E2	V Contr	V Contr	V Contr	V Contr	V Contr	0.1nM E2	0.1nM E2	0.1nM E2	
E		10 nM ICI	10 nM ICI	1x	0.3x	0.1x	0.03x	0.01x	1x	0.1x	0.01x	ND
		V Contr	0.1nM E2	V Contr	V Contr	V Contr	V Contr	V Contr	0.1nM E2	0.1nM E2	0.1nM E2	
F		10 nM ICI	10 nM ICI	1x	0.3x	0.1x	0.03x	0.01x	1x	0.1x	0.01x	AD
		V Contr	0.1nM E2	V Contr	V Contr	V Contr	V Contr	V Contr	0.1nM E2	0.1nM E2	0.1nM E2	
G		10 nM ICI	10 nM ICI	1x	0.3x	0.1x	0.03x	0.01x	1x	0.1x	0.01x	XW
		V Contr	0.1nM E2	V Contr	V Contr	V Contr	V Contr	V Contr	0.1nM E2	0.1nM E2	0.1nM E2	
H												
	Samples		Mancozeb		**Passage**			**Date**	30 May 2017		**Plate**	1-3

Table 2: T47D-KBluc plate layout of mancozeb incubated together with E2 (17β estradiol) showing final concentrations in the wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		V Contr	V Contr	100	30	10	3	1	0.3	0.1	0.03	E2 (pM)
			0.1nM E2	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	
C		V Contr	V Contr	200	20	2	0.2	0.02	0.002	0.0002	0.00002	Mancozeb
			0.1nM E2	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(mg/L)
D		V Contr	V Contr	0.000002	2E-07	2E-08	2E-09	2E-10	2E-11	2E-12	2E-13	Mancozeb
			0.1nM E2	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(mg/L)
E		10 nM ICI	10 nM ICI	10	3	1	0.3	0.1	0.03	0.01	0.003	ICI (nM)
		V Contr	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	
F		10 nM ICI	10 nM ICI	200	20	2	0.2	0.02	0.002	0.0002	0.00002	Mancozeb
		V Contr	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	(mg/L)
G		10 nM ICI	10 nM ICI	0.000002	2E-07	2E-08	2E-09	2E-10	2E-11	2E-12	2E-13	Mancozeb
		V Contr	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	0.1nM E2	(mg/L)
H												
	Samples		Mancozeb		**Passage**			**Date**	30 May 2017		**Plate**	4

Table 3: MDA-kb2 plate layout of extraction control and samples (Tate Vondo, Nandoni Dam, Albasini and Xikundu Weir) incubated together with dihydrotestosterone and flutamide (24 May 2017) showing final concentrations in the wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		V Contr	10 nM DHT	10	3	1	0.3	0.1	0.03	0.01	0.003	DHT
			V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(nM)
C		V Contr	10 nM DHT	1x	0.3x	0.1x	0.03x	0.01x	1x	0.1x	0.01x	EC
			V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	10 nM DHT	10 nM DHT	10 nM DHT	
D		V Contr	10 nM DHT	1x	0.3x	0.1x	0.03x	0.01x	1x	0.1x	0.01x	TV
			V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	10 nM DHT	10 nM DHT	10 nM DHT	
E		100 uM F	100 uM F	1x	0.3x	0.1x	0.03x	0.01x	1x	0.1x	0.01x	ND
		V Contr	10 nM DHT	V Contr	V Contr	V Contr	V Contr	V Contr	10 nM DHT	10 nM DHT	10 nM DHT	
F		100 uM F	100 uM F	1x	0.3x	0.1x	0.03x	0.01x	1x	0.1x	0.01x	AD
		V Contr	10 nM DHT	V Contr	V Contr	V Contr	V Contr	V Contr	10 nM DHT	10 nM DHT	10 nM DHT	
G		100 uM F	100 uM F	1x	0.3x	0.1x	0.03x	0.01x	1x	0.1x	0.01x	XW
		V Contr	10 nM DHT	V Contr	V Contr	V Contr	V Contr	V Contr	10 nM DHT	10 nM DHT	10 nM DHT	
H												
	Samples	Mancozeb			**Passage**			**Date**	31 May 2017		**Plate**	1

Table 4: MDA-kb2 plate layout of mancozeb incubated together with dihydrotestosterone and flutamide (24 May 2017) showing final concentration in the wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		V Contr	1nM DHT	1	0.3	0.1	0.03	0.01	0.003	0.001	0.0003	DHT
			V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(nM)
C		V Contr	1nM DHT	200	20	2	0.2	0.02	0.002	0.0002	0.00002	Mancozeb
			V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(mg/L)
D		V Contr	1nM DHT	0.000002	2E-07	2E-08	2E-09	2E-10	2E-11	2E-12	2E-13	Mancozeb
			V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(mg/L)
E		10 uM F	10 uM F	10	3	1	0.3	0.1	0.03	0.01	0.003	F
		V Contr	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	(uM)
F		10 uM F	10 uM F	200	20	2	0.2	0.02	0.002	0.0002	0.00002	Mancozeb
		V Contr	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	(mg/L)
G		10 uM F	10 uM F	0.000002	2E-07	2E-08	2E-09	2E-10	2E-11	2E-12	2E-13	Mancozeb
		V Contr	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	(mg/L)
H												
	Samples	Mancozeb			**Passage**			**Date**	24 May 2017		**Plate**	1

Table 5: MDA-kb2 plate layout of mancozeb incubated together with dihydrotestosterone and flutamide (31 May 2017) showing final concentrations in the wells.

	1	2	3	4	5	6	7	8	9	10	11	12	
A													
B		V Contr	1nM DHT	1	0.3	0.1	0.03	0.01	0.003	0.001	0.0003	DHT	
			V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(nM)	
C		V Contr	1nM DHT	200	20	2	0.2	0.02	0.002	0.0002	0.00002	Mancozeb	
			V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(mg/L)	
D		V Contr	1nM DHT	0.000002	2E-07	2E-08	2E-09	2E-10	2E-11	2E-12	2E-13	Mancozeb	
			V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(mg/L)	
E		10 uM F	10 uM F	10	3	1	0.3	0.1	0.03	0.01	0.003	F	
		V Contr	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	(uM)	
F		10 uM F	10 uM F	200	20	2	0.2	0.02	0.002	0.0002	0.00002	Mancozeb	
		V Contr	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	(mg/L)	
G		10 uM F	10 uM F	0.000002	2E-07	2E-08	2E-09	2E-10	2E-11	2E-12	2E-13	Mancozeb	
		V Contr	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	1nM DHT	(mg/L)	
H													
	Samples	Mancozeb			**Passage**			**Date**	24 May 2017		**Plate**	1	

Table 6: GH3.TRE-Luc plate layout of mancozeb incubated together with triiodothyronine and sodium arsenite (20 Sep 2017) showing final concentrations in the wells.

	1	2	3	4	5	6	7	8	9	10	11	12	
A													
B		V Contr	1 nM T_3	100	10	1	0.1	0.01	0.001	0.0001	0.00001	nM T3	
		V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(in MeOH)	
C		V Contr	1 nM T_3	1	1.00E-01	1.00E-02	1.00E-03	1.00E-04	1.00E-05	3.00E-06	1.00E-06	Mancozeb	
		V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(mg/L)	
D		V Contr	1 nM T_3	3.00E-07	1.00E-07	3.00E-08	1.00E-08	3E-09	1E-09	3E-10	1.00E-10	Mancozeb	
		V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(mg/L)	
E		100 uM SA	1 nM T_3	3E-11	1E-11	3E-12	1E-12	3E-13	1E-13	1E-14	1E-15	Mancozeb	
		V Contr	100 uM SA	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(mg/L)	
F		100 uM SA	1 nM T_3	100	30	10	3	1	0.3	0.1	0.03	uM SA	
		V Contr	100 uM SA	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3		
G		100 uM SA	1 nM T_3	1	0.1	0.01	0.001	0.0001	0.00001	0.000001	1E-07	Mancozeb	
		V Contr	100 uM SA	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	(mg/L)	
H													
	Samples	Flesher			**Passage**			**Date**	20 Sep 2017		**Plate**	4-6	

Table 7: GH3.TRE-Luc plate layout of extraction control and samples (Tate Vondo, Nandoni Dam, Albasini Dam and Xikundu Weir).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		V Contr	1 nM T_3	100	10	1	0.1	0.01	0.001	0.0001	0.00001	nM T3
		V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	(in MeOH)
C		V Contr	1 nM T_3	100x	30x	10x	3x	1x	0.1x	30x	30x	EC
		V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	1 nM T3	100 uM SA	
D		V Contr	1 nM T_3	100x	30x	10x	3x	1x	0.1x	30x	30x	TV
		V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	1 nM T3	100 uM SA	
E		100 uM SA	1 nM T_3	100x	30x	10x	3x	1x	0.1x	30x	30x	ND
		V Contr	100 uM SA	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	1 nM T3	100 uM SA	
F		100 uM SA	1 nM T_3	100x	30x	10x	3x	1x	0.1x	30x	30x	AD
		V Contr	100 uM SA	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	1 nM T3	100 uM SA	
G		100 uM SA	1 nM T_3	100x	30x	10x	3x	1x	0.1x	30x	30x	XW
		V Contr	100 uM SA	V Contr	V Contr	V Contr	V Contr	V Contr	V Contr	1 nM T3	100 uM SA	
H												
	Samples	Flesher			**Passage**			**Date**	20 Sep 2017		**Plate**	1-3