

Optimisation and application of the GH3.TRE.Luc Reporter Gene Bioassay to assess thyroid activity in drinking and source water

Hannah Simba

11098393

Submitted in Partial fulfilment of the degree MSc in Environmental Health in the faculty of Health Sciences, University of Pretoria

Supervisor: Dr NH Aneck-Hahn

Co-Supervisor: Prof C de Jager

20/02/2017

Declaration of originality

I, Hannah Simba, declare that the dissertation, “Optimisation and application of the GH3.TRE.Luc Reporter Gene Bioassay to assess thyroid activity in drinking and source water” which I hereby submit for the degree MSc Environmental Health at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature

Date

___/___/_____

Summary

The endocrine system is vulnerable to a range of chemicals in the environment. Endocrine disrupting chemicals (EDCs) are exogenous agents that can induce responses on the endocrine system because of their hormone-like activity and toxicity. Specific to this study are thyroid disrupting chemicals (TDCs), these are EDCs that specifically disrupt the thyroid hormone signalling pathway, and this may result in adverse health effects. Thyroid hormones play a crucial part in metabolism, growth, maintenance of brain function and fertility; hence disruption of the thyroid signalling axis implicates human health. We are exposed to TDCs regularly, and studies have shown an association between TDC exposure and neurobehavioural disorders, reproductive abnormalities and obesity. There is a lack of data associated to thyroid hormone receptor activity in surface and drinking water. Hence, the potential human health risks posed by thyroid disruption may therefore be underestimated.

The aim of the study was to optimise and validate the GH3.TRE.Luc reporter gene bioassay that can measure thyroid hormone receptor mediated activity and cytotoxicity in drinking and source water, with relevance to water monitoring.

The GH3.TRE.Luc reporter gene bioassay was established, optimized and validated to detect thyroid hormone receptor activity. The luciferase assay was used to test for metabolic activity and the resazurine cell proliferation assay was used to assess cell viability. The assay was applied to compounds with agonistic and antagonistic properties; triiodothyronine (T_3), thyroxine (T_4), triac, tetrac, amiodarone, sodium arsenite, pentachlorophenol (PCP), ethylene thiourea, 2,2,4,4-tetrahydroxybenzophenone (THBP) and methimazole. It was also applied to environmental and drinking water samples from the Global Water Research Coalition (GWRC). Finally, the assay was applied to 48 water samples from a water treatment plant in South Africa, collected over a period of 12 months. Every month, four samples were collected. Two samples were source water samples, with one going into the treatment plant and coming out as 2 distribution pipelines (drinking water).

For optimisation and validation, the dose response curves obtained for T_3 , T_4 , tetrac and triac (agonists) were comparable to literature. Antagonistic behaviour was seen in sodium arsenite, amiodarone, PCP and methimazole. Spiked water samples from

the GWRC showed thyroid hormone receptor activity. Sixteen of the 48 water samples collected from the water treatment plant were positive for thyroid hormone disruptor activity. Highest activity was seen in the winter season, accounting for seasonal variations. High TDCs activity reported in the source water may be due to activities occurring near the dam. The water treatment plant seemed effective for only one of the distribution pipelines, and not the other.

This study confirms that GH3.TRE.Luc Reporter Gene Bioassay is a sensitive and effective tool to identify and quantify TDC activity in pure chemicals and in complex environmental mixtures present in water. Further monitoring of water sources for TDCs is recommended to ensure water quality and safety.

Keywords: GH3.TRE.Luc reporter gene assay, thyroid hormones, thyroid hormone receptor disruptor, thyroid disrupting chemicals, endocrine disrupting chemicals, water quality, thyroid equivalents

Acknowledgements

I would like to acknowledge and express gratitude to my supervisors, Dr N Aneck-Hahn and Professor C de Jager for their support, guidance and contribution to the dissertation. Their doors were always open and I benefited greatly over and above this MSc from their immense knowledge.

I would like to acknowledge Professor M Taylor, from the Department of Urology, University of Pretoria, for her mentorship, encouragement and continuous support from the beginning of my study. Her significant contribution to the project has made the writing of this thesis possible.

I would also like to thank my lab mates, Ms Silindile Ngcobo and Mrs Catherina van Zijl, the long hours in the lab and the discussions which greatly added to my research and laboratory skills.

Thanks are due to Rand Water for the collection of water samples and permission to use these data for my project. A MSc bursary from the Rand Water Chair in Public Health is gratefully acknowledged

Lastly, I would like to thank my family, my parents and brother, for their patience and support throughout this journey.

.

Table of Contents

Declaration of originality	ii
Summary	iii
Acknowledgements	v
List of Figures	viii
List of Tables	x
List of Abbreviations	xi
Chapter 1: Introduction	1
1.2 Endocrine disrupting chemicals (EDCs).....	1
1.3 The endocrine system.....	3
1.3.1 Natural hormones vs EDCs	4
1.3.2 Key characteristics of endocrine disruptors	5
1.3.3 Endocrine pathways	8
1.3.4 Hypothalamic pituitary thyroid (HPT) axis	13
1.3.5 Thyroid disrupting chemicals (TDCs).....	19
1.3 One health	34
1.4 Sources and routes of exposure	36
Chapter 2: Motivation, aims and objectives	41
2.1 Background.....	41
2.2 Problem statement.....	42
2.3 Aims and objectives	44
2.4 Hypothesis	44
Chapter 3: Materials and methods	45
3.1 Establishment of the assay	45
3.1.1 Luciferase reporter gene assay using GH3.TRE.Luc cell line.....	45
3.2 Phase 1: Optimisation and Validation	49
3.3 Phase 2: GWRC water samples	50



3.4 Phase 3: Water Samples from a water treatment plant - Gauteng.....	50
3.5 Data analysis	53
Chapter 4: Results	55
4.1 Modification of the assay.....	55
4.2 Phase 1: Optimisation and validation.....	61
4.3 Phase 2: GWRC water samples	72
4.4 Phase 3: Water samples from a water treatment plant - Gauteng	76
Chapter 5: Discussion	85
Chapter 6: Conclusion	90
6.1 Recommendations	91
Chapter 7: References	93
Appendix	107
Ethics Approval Letter.....	107

List of Figures

Figure 1: Mechanism of action of EDCs.....	2
Figure 2: Structures of thyroid hormones.....	15
Figure 3: Simplified depiction of the HPT axis.....	17
Figure 4: Illustration of how TDCs interfere with the HPT axis	28
Figure 5: A diagrammatic representation of the one health concept.....	35
Figure 6: Flow diagram of the extraction process for water samples.....	52
Figure 7: Graph showing GH3.TRE.Luc induction following T ₃ exposure for 24 hours (0.0001 – 1000 nM) using the original method.....	56
Figure 8: Graph showing GH3.TRE.Luc induction following T ₃ exposure for 24 hours (0.0001 – 1000 nM) using the original method and the incorporation of fetuin.....	58
Figure 9: Graph showing GH3.TRE.Luc induction of T ₃ and T ₄ using new method.....	60
Figure 10: Graphs showing the T ₃ activity in the luciferase and resazurine assays, with concentrations in nM ranging from 0.0001 to 1000.....	62
Figure 11: Graphs showing the T ₄ activity in the luciferase assay (Induction) and resazurine assays (cell proliferation), with concentrations in nM ranging from 0.0001 to 1000.....	64
Figure 12: The graphs shows the effect on the GH3.TRE.Luc cells over a range of sodium arsenite (SA) concentrations (0,01 to 100mM) after a 24 hour co-inubation with 0.25 nM T ₃	66
Figure 13: The graphs illustrate the effect of amiodarone hydrochloride on the GH3.TRE.Luc cells when incubated with 0.25 nM T ₃	68
Figure 14: The graphs show GH3.TRE_Luc induction at concentration 0.0001 to 1000 nM of the agonists T ₃ , T ₄ , triac (T ₃ analogue) and tetrac (T ₄ analogue) after 24 hours.....	70

Figure 15; Graphs A to D for the luciferase reporter gene assay results in RLU/RFU values73

Figure 16: Graphs for the April 2015 to March 2016 samples using the GH3.TRE.Luc Assay to test for THR activity (agonistic and antagonistic) and cytotoxicity.....77

Figure 17: Graphical representation of the results from the water treatment plant over the 12-month period.....82

List of Tables

Table 1: TDCs that interrupt the thyroid signalling pathways and their sources.....	20
Table 2: Template for the 96 well plate for both the luciferase and resazurine Assays.....	47
Table 3: Showing the EC50 and relative potency of the agonists.....	71
Table 4: Thyroid Equivalentents (TEq) from the GWRC spiked water extracts.....	72
Table 5: TEq values for the GWRC samples that were positive for TDC activity.....	75
Table 6: Samples from the water treatment plant positive for THR activity.....	81

List of Abbreviations

ACTH	Adrenocorticotropin Hormone
ADHD	Attention Deficit Hyperactive Disorder
AH	Amiodarone Hydrochloride
AR	Androgen Receptor
AVP	Arginine Vasopressin
BFR	Brominated Flame Retardants
CaO	Hydrated Lime
Cl ₂	Chlorine
CO ₂	Carbon Dioxide
CEC	Chemicals of Emerging Concern
CRH	Corticotropin Releasing Hormone
D	Deiodinase
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
Dha-	Dehydroalanine
DIT	Di-iodinated Tyrosine
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DWA	Department of Water Affairs
DWAF	Department of Water Affairs and Forestry
EDC	Endocrine Disrupting Chemical
ER	Oestrogen Receptor
ETU	Ethylene Thiourea
E-waste	Electronic Waste

Fe ³⁺	Ferric
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
GR	Glucocorticoid Receptor
GWRC	Global Water Research Coalition
HBCD	Hexabromocyclododecane
HOME	Health Outcomes and Measures of the Environment
HPA axis	Hypothalamic Pituitary Adrenocortical axis
HPG axis	Hypothalamic Pituitary Gonadal axis
HPT axis	Hypothalamic Pituitary Thyroid axis
IGF	Insulin-like Growth Factor
JNK-c	Jun N-terminal Kinases
LH	Lutenizing Hormone
MCT8	Monocarboxylate Transporter 8
MIT	Mono-iodinated Tyrosine
Na ₂ O ₃ Si	Sodium Silicate
NADPH	Nicotinamide Adenine Dinucleotide
NIS	Sodium Iodide Symporter
NTMP	National Toxicity Monitoring Program
OATP1C1	Organic anion transporter peptide 1C1
PBB	Polybrominated biphenyls
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyls
PCP	Pentachlorophenol



POP	Persistent Organic Pollutants
PR	Progestogenic Receptor
PVN	Paraventricular Nuclei
RA	Retinoic Acid
RAR	Retinoid Acid Receptor
RXR	Retinoid X Receptor
T ₃	Triiodothyronine
T ₄	Thyroxine
TBBPA	Tetrabromobisphenol-A
TBG	Thyroxin Binding Globulin
TBT	Tributyltin Chloride
TDC	Thyroid Disrupting Chemicals
TH	Thyroid Hormone
THBP	2,2,4,4-tetrahydroxybenzophenone
THR	Thyroid Hormone Receptor
TPO	Thyropoxidase
TR	Thyroid Receptor
TRH	Thyroid Releasing Hormone
TRHR	Thyrotropin Releasing Hormone Receptor
TSH	Thyroid Stimulating Hormone
TTR	Transtheyretin
VDR	Vitamin D Receptor



Chapter 1: Introduction

1.1 Endocrine disrupting chemicals (EDCs)

Environmental contamination and human exposure to chemicals are a major public health concern¹. As chemical production and usage continue to grow globally, the implications to human and wildlife health are also receiving a lot of attention from both the scientific world and the public. Chemical pollution dates back to early human history². However, the industrial revolution became a defining era with regards to the increase in chemical pollution, and continues to be a major source of chemical pollutants³. Until the end of the 20th century, the main groups of environmental contaminants with significance to human and wildlife health were carcinogens, teratogens and lethal chemicals³. There has been increasing concern over a group of environmental chemicals that are able to interact with the endocrine (hormonal) system, interrupting its normal function^{4,5}. Apprehension over the effect of these chemicals, termed endocrine disrupting chemicals (EDCs), on human and wildlife is global, and has received attention from a range of entities, including international organisations, government, public interest societies, chemical industries and researchers. Over the past few decades, increasing scientific evidence has established that there may be a causal relationship between exposure to EDCs, and adverse health effects⁶⁻⁸.

An EDC is an exogenous agent, or a mixture of chemicals, that disrupts and modifies the functionality of the endocrine system, by interfering with the mechanism of action of the natural hormones. This results in adverse health effects in an organism or its progeny⁹. EDCs encompass a range of substances which include pesticides, fungicides, metals, pharmaceuticals, personal care products, industrial chemicals, agricultural products, natural substances and chemicals used in plastic production or plasticizers¹⁰. Some of these chemicals may be found in cosmetics and sunscreens, food packaging material and food, plastic bottles, toys, detergents and flame retardants. Through a number of signalling pathways, EDCs can mimic (agonists) and block (antagonists) natural hormones, causing signals that can be misinterpreted by the body resulting in disruption of the key endocrine pathways. Figure 1 shows how EDCs elicit their effect through receptor mediated action. The potency of an EDC and the extent of its effect may be dependent on the dose, the duration of exposure, and the time of exposure (critical windows of development)⁹.

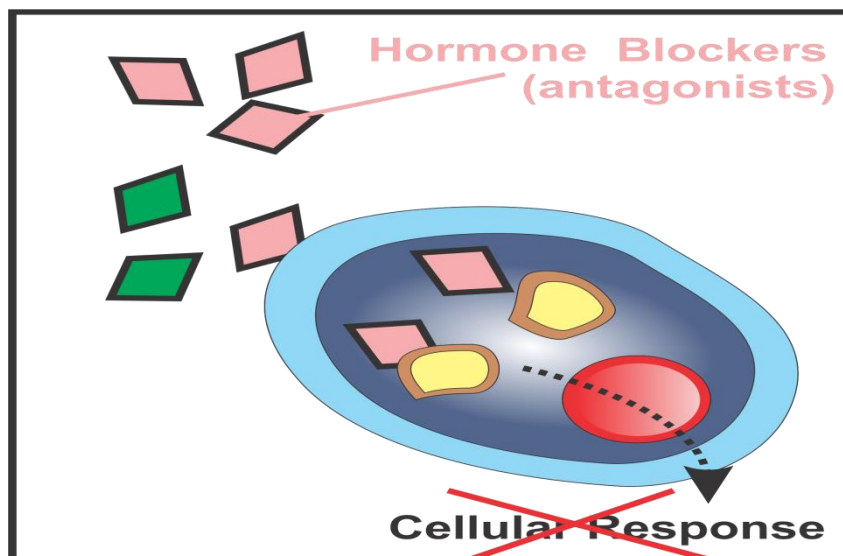
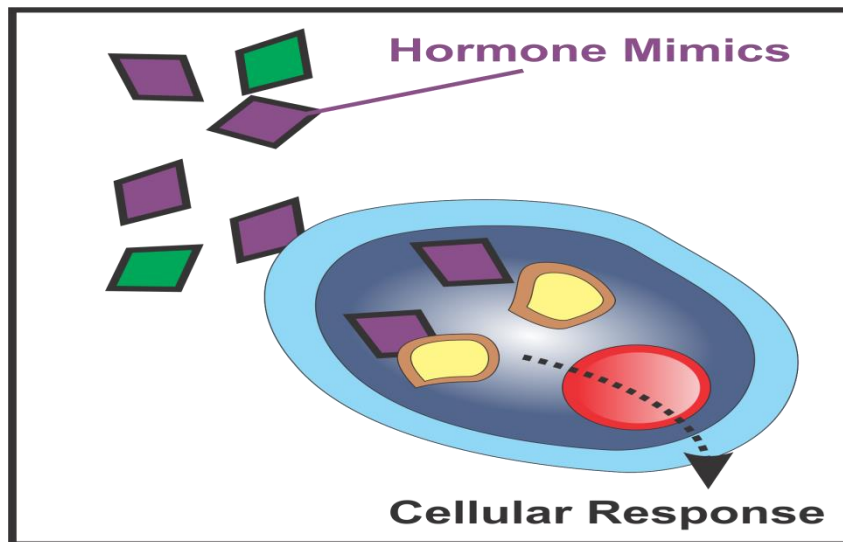
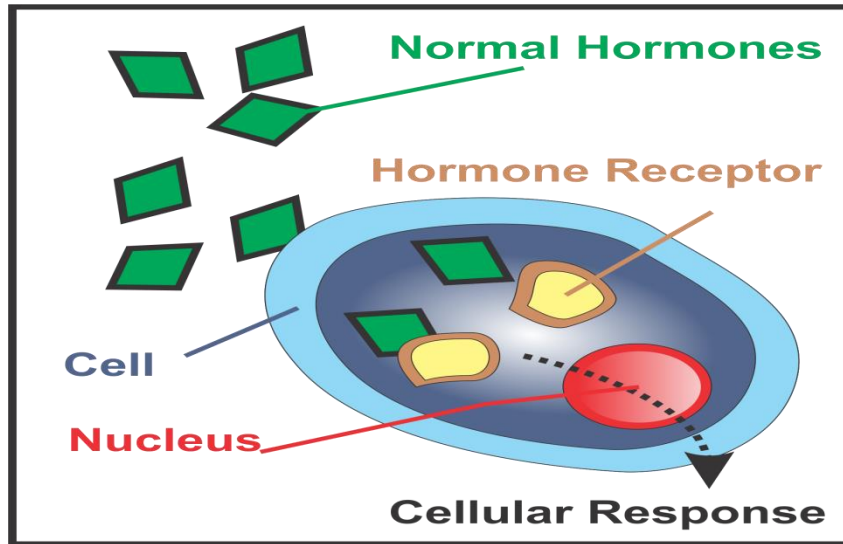


Figure 1: Mechanism of action of EDCs. Adapted from <http://www.niehs.nih.gov/health/topics/agents/endocrine> with modifications

The question of whether or not there is a connection between environmental chemical exposure to human and wildlife disease is not new. Over five decades ago, scientists began to study and hypothesise this possible interaction^{11,12}. One of the pioneers of this work was Rachel Carson, who advocated for environmental awareness and brought attention to the dangers and possible implications of pesticide exposure. In her book, *Silent Spring* (1962), Rachel Carson alluded to the possible detrimental effects that pesticides have on the environment and to human health¹¹. Although the term endocrine disruptors had not been coined yet, research has since shown that a number of pesticides discussed in the book were EDCs. Rachel Carson's work spawned environmental awareness, activism and also scepticism¹². Years that followed saw an increase in knowledge with regards to the biological and physiological effects from exposure to a wider range of chemical contaminants. In 1991, Theo Colborn devised the term endocrine disruptors. Through her ground-breaking work, our current understanding of the interactions between EDCs and human and wildlife health has been determined¹³. In the last decade, a wealth of scientific data has emerged and continues to emerge, aimed at filling in knowledge gaps pertaining to the health effects of EDC exposure through disruption of the endocrine system^{5,8}.

1.2 The endocrine system

The endocrine system is an assemblage of glands whose purpose is to secrete hormones into circulation, thereby regulating metabolic and developmental functions in target cells, tissues and organs^{5,14,15}. The glands form pathways with specific functions in different organs of the body. These glands include the hypothalamus, pituitary, thyroid, parathyroid, pancreas, adrenals, ovaries and testes. The glands have hormone secreting cells which control the signalling pathways allowing for communication between organs¹⁵. Homeostasis of the endocrine pathways is regulated by positive and inhibitory feedback loops (through hormones secreted by the glands), and through neural control from the brain's synaptic signals¹⁵. The endocrine pathways have a major role in early developmental processes, puberty, and adult metabolism while working under strict hormonal control^{5,14}.

1.2.1 Natural hormones vs EDCs

A healthy endocrine system is necessary for humans and wildlife to physiologically develop, reproduce and maintain a stable metabolic state⁵. The endocrine system produces over 50 hormones, which function in different endocrine signalling pathways. These hormones mediate and maintain normal body functions from cellular level to organ level. A hormone is defined as a molecule produced by an endocrine gland, which is secreted into circulation and causes an effect in other cells through genomic (hormone receptors) or non-genomic mechanisms (protein interaction)⁵. There are over 800 EDCs that have been discovered, which have the ability to interrupt natural hormone signalling pathways⁵. For the most part, this is made possible by the structural similarities between EDCs and hormones. There are functional similarities in the action and effect between natural hormones and EDCs.

Similar to natural hormones, EDCs induce effects via hormone receptors, and also mediate non-genomic response through interaction with protein molecules^{5,16}. However, responses mediated by EDCs are disruptive to the normal hormone signalling, causing alterations in the endocrine status. The low dose effect seen from natural hormones is also seen from EDCs⁵.

EDCs have been shown to induce effects at very low concentrations, similar to natural hormones¹⁷. However, whilst EDCs have a propensity to bio-accumulate, natural hormones do not⁵. Both natural hormones and EDCs have nonlinear dose-response relationships⁵. This means that responses can be non-monotonic, and there can be saturation at certain doses⁵. Similar to hormones, high dose effects of EDCs cannot give you an idea of how low dose effects will look like, in relation to biological effects⁵. This means that low doses can induce high responses. Temporal and spatial specificity with regards to hormonal induction have been observed in both natural hormones and EDCs⁵. The effect on the normal developmental process caused by natural hormones and EDCs is permanent. Endocrine disruption can lead to aberrant development of organs and tissue⁵. Both natural hormones and EDCs exhibit variance in sensitivity of different end points⁵. It is, therefore, critical to take this information into consideration when assessing the effects of EDCs on public health.

1.2.2 Key characteristics of endocrine disruptors

There are a number of key endocrine characteristics, which have allowed us to understand the mechanistic pathways of EDCs as we do now. These characteristics are in part as a result of the structural and functional similarity between EDCs and natural hormones, which have been discussed. They are as follows;

i) Causal relationship between exposure and disease.

EDCs have been implicated in adverse health effects in animal studies. In humans, whilst this relationship is not well established, due to a number of constraints, increasing evidence shows that there may be an association between exposure and health effects^{5,6,18-22}. Animal models have been used extensively in EDC research to predict human health effects, and have been an invaluable resource in theorising the exposure effect relationship^{23,24}. These effects include disease conditions linked to developmental malformations, reproductive and, neurodevelopmental anomalies^{20,21}.

ii) Critical windows of development

For both natural hormones and EDCs, the timing of action and exposure on a specific organ or tissue in the body is important as it can have an influence on the magnitude or extent of the biological response⁵. During organ or tissue development, sensitivity to hormones or EDCs is very high²⁵. This occurs mainly because many genes are active, hence numerous targets are available for the disruption of normal hormone and the endocrine system²⁶. Some sensitive developmental stages include foetal development, pregnancy, early childhood, puberty and, reproductive age^{5,27}. Therefore if exposure occurs during these sensitive developmental stages, permanent defects in physiological growth and development may occur. Particularly for EDCs, the hormonal imbalance caused can result in disruption of the normal hormonal pathways, leading to permanent structural and functional abnormalities⁵. The effects of disruption during early development may not be immediately apparent, but may manifest later in life, causing adverse health effects^{5,21}. This phenomenon is called the foetal basis of disease and forms the foundation of developmental programming^{5,21,27}. An example is the effect of trans-placental exposure of

polybrominated diphenyl ether to fetuses¹⁸. This exposure has been linked to neurodevelopmental conditions after birth in the child until adulthood¹⁸. It is important to note that intrauterine exposure to EDCs is possible due to the ability of EDCs to cross the placental barrier²¹. This trans-placental characteristic of EDCs is important in understanding the foetal basis of adult disease.

iii) Trans-generational effect

The era of genomic medicine has allowed for the determination of the link between gene variants and endocrine disorders. Genotypes can be passed on to the next generations resulting in shared traits¹⁵. One of the best evidence of this phenomenon is the diethylstilboestrol (DES) case^{22,28,29}. Transgenerational effect/epigenetic imprinting refers to the ability to alter deoxyribonucleic acid (DNA) by causing mutations in the germ line which can be passed on to future generations^{21,26,30}. A number of EDCs have been shown to cause epigenetic imprinting by altering DNA methylation and causing histone modifications^{21,31,32}. It is uncommon for toxins to have the ability to alter DNA sequences, because of inherent mechanisms that ensure a level of DNA stability and fidelity²⁶. However, a number of EDCs have this ability.

iv) Bioaccumulation and bio-magnification

Bioaccumulation and bio-magnification of some EDCs is a major issue as it results in increased exposure effects. This process usually occurs in the aquatic environment, where EDCs are present in solution. Aquatic organisms are exposed via gills, skin, and through ingestion³³. Whilst bio-magnification occurs through ingestion of food only, bioaccumulation involves ingestion of both food and water. The resultant effect is referred to as ecological magnification, which entails cumulative concentrations of chemicals in the food chain³³. The effect of bioaccumulation and bio-magnification of EDCs poses a danger for predators and humans who may feed on these aquatic species, exposing themselves to high levels of EDCs³³.

v) Additivity and synergism

It is apparent that exposure to multiple EDCs can occur simultaneously, because of their presence in the environment. This leads to combined

exposure and effects³⁴⁻³⁶. Laboratory studies looking at combinations of exposures have reported that EDCs have additive and synergistic properties³⁴. *In vivo* and *in vitro* studies have shown that mixtures of EDCs can elicit synergistic and additive effects, leading to increased health effects^{21,35}.

vi) Low dose effects

It has been established that EDCs, similar to natural hormones, can cause a response at low doses^{17,26,37}. It is known that natural hormones induce effects at low concentrations (pico-molar to nano-molar range). Low dose effects can be defined as biological changes happening at a specific dose, that is below the traditional toxicology threshold³⁷. Many EDCs are found in low doses in the environment. Scientific evidence has shown that they may be highly potent and cause significant endocrine effects. This defies the conventional concept in toxicology which states that “the dose makes the poison”^{26,38}. Endogenous hormones can act at low doses, therefore the presence of EDCs even at low concentrations, can cause biological effects¹⁶. An example is the chemical BPA, which has been shown to elicit biological effects at the picomolar levels, similar to the endogenous oestrogens.³⁷

To date, there have been a number of studies that have confirmed the existence and effects of EDCs on the neurological, reproductive, developmental and immune systems of both humans and wildlife^{5,18,19,39}. The mechanism of action of a number of the EDCs is still unknown, requiring more research.

There has been an increase in endocrine related disorders over the past few decades^{5,14,32}. The disorders include metabolic conditions, obesity, diabetes, developmental conditions, thyroid diseases, neurodevelopmental disease, sexual disruption and other hormone related complications^{5,14}. It has been theorized that EDCs may be involved in this increase in endocrine related diseases⁵. Scientific evidence has shown the ability of EDCs to interact and disrupt endocrine pathways/axes^{14,32}.

1.2.3 Endocrine pathways

Hypothalamic pituitary adrenocortical (HPA) axis

There are seven main endocrine pathways. The hypothalamic pituitary adrenocortical (HPA) axis comprises of the hypothalamus, pituitary, and adrenal glands. The main functions of this axis are to regulate stressors to body homeostasis, body metabolism, the immune system, growth, and to regulate reproductive function and development^{14,15,40}. The metabolic functions include neuroendocrine, cardiovascular, and ionic regulation. Principal hormones secreted by the HPA axis, include corticotropin releasing hormone (CRH), arginine vasopressin (AVP), adrenocorticotropin hormone (ACTH), and glucocorticoids^{14,40,41}. HPA axis malfunction disrupts body metabolism, resulting in obesity, diabetes, reduced stress response, disrupted osmotic balance, cancer, and reproductive disorders^{14,41,42}. A number of EDCs have been shown to have agonistic and antagonistic effects of the HPA axis, causing an imbalance to the normal concentrations and functioning of the endogenous hormones^{14,43}. Animal studies are beginning to show an association between EDC exposure and HPA axis malfunction⁴⁴. Merlo *et al* (2016) recently demonstrated that exposure to tributyltin chloride (TBT) results in disruption of the HPA axis leading to abnormalities in the adrenal and pituitary glands. However, not much research about the human health effects has been done¹⁴.

Hypothalamus pituitary gonadal (HPG) axis

The hypothalamus pituitary gonadal (HPG) axis is composed of the hypothalamus, the pituitary gland, and the gonads (ovaries and testes). The role of the HPG axis is to regulate and foster sexual and reproductive growth, development and function^{14,45}. Gonadotropin-releasing hormone (GnRH), gonadotropins [follicle stimulating hormone (FSH) and luteinizing hormone (LH)], oestrogens, androgens, and gestagens are the main hormones of the HPG axis^{14,45}. Disruption of the HPG axis due to EDCs has detrimental effects, and these effects are dependent on when exposure occurs, dosage, and the mechanism of action of the EDCs¹⁴. The EDCs can be antagonistic, agonistic, modulatory, or interfere in the transportation and biotransformation of the natural hormones¹⁴.

The oestrogenic pathway of EDCs is one of the well-studied topics in endocrine disruption^{5,14,46}. The mechanisms of action are better documented in both wildlife and humans than other endocrine pathways, particularly the genomic pathway^{5,9,14,39,47-49}. Activation and disruption of the HPG axis can be mediated through nuclear receptors, which are ligand dependent transcription factors. Studies have shown that mammals have one androgen receptor, and two oestrogen receptors (ER α and ER β)^{14,49}. The effect of EDCs on oestrogenic activity in animal studies (fish, amphibians) have shown that exposure to xenoestrogens results in disruption in reproductive structure and function^{14,39}. Reports have indicated that male animals exposed to EDCs present with intersex (feminization) characteristics, and changes in the sex ratios, both in experimental studies and in the wild^{14,39}. Animal studies have shown that the female oestrogenic signalling is also susceptible to xenoestrogens, resulting in perturbed sexual development and function. This includes sex reversal, ovarian regression and low fertility^{14,39,50}. Human studies have shown some association but the overall causal relationship is still controversial, particularly in males^{14,50}. The global rise in reproductive disorders have been linked to exposure to xenoestrogens during early development¹⁴. Androgenic exposure has also been reported to cause masculinization and feminization in animal populations¹⁴. Because the sex hormones play a role in muscle growth and the immune system, the disruption may also cause effects in the growth and immune systems.

Somatotropic axis

The somatotropic axis consists of the hypothalamus, pituitary and liver glands. The function of this axis is the secretion of growth hormones and insulin-like growth factors (IGF) whose roles are to modulate metabolism and growth^{14,51}. The effects of suppression and excitation of the somatotropic axis have been reported from studies using gene knockout techniques and transgenic insertion in experimental animals¹⁴. In mammals, suppression results in elevated body fat, atypical lipid profile, impaired cardiac function, reduced muscle mass, atherosclerosis, insulin resistance and immunodeficiency in mammals^{14,15}. Excitation results in increased body mass,

thyroid dysfunction and sea-water tolerance in fish, as well as hypertension, heart disease and abnormal menstruation in mammals^{14,15}.

Exposure to oestrogens has been reported to suppress the somatotrophic axis in experimental studies using mammals and fish^{14,52}. This results in a reduction in the expression of IGF in the liver and in serum and this has been reported to cause reduced growth and smoltification in fish¹⁴. Thyroid hormone exposure has been shown to cause excitation of the somatotrophic axis in birds, fish and mammals^{14,53-55}. Corticosteroids have also been reported to cause suppression of the axis in experimental fish and mammals¹⁴. The effect of specific EDCs on the axis is not fully known as little research has been done.

Retinoid signalling pathway

The retinoid signalling pathway functions by switching the retinoic acid receptor (RAR) and retinoid x receptor (RXR) from suppressors to transcription factors⁵⁶. This occurs through the action of retinoic acid, all-*trans*-retinoic acid and 9-*cis*-isomer respectively, as a ligand for the nuclear receptors^{56,57}. Retinoic acid (RA) is a derivative of vitamin A (dietary sources) and is metabolized through the action of retinol dehydrogenases⁵⁶. Regulation of this signalling pathway is achieved through the dehydrogenases (synthesis) and through inactivation of the cytochrome P450 enzymes^{14,56}. RA has pleiotropic effects on vertebrate development and differentiation⁵⁶. Above or below normal concentrations of RA during early development (trans-placental) may result in developmental deformities^{14,56}. The RXR plays a role in sexual development as well as embryo and foetal development in mammals^{14,58}. Lack of RXR expression in mice results in infertility, whilst deficiency of vitamin A during embryonic development results in malformations of the brain due to reduced expression of RXR and RAR^{14,56}. RXR also mediates lipid metabolism and homeostasis in the body¹⁴.

A number of studies have reported an association between the environmental exposures with disruption in the retinoid signalling pathway in wildlife^{14,59-62}. A wide range of EDCs have been implicated in the alteration of the normal functioning of the axis^{14,63,64}.

Vitamin D signalling pathway

The vitamin D signalling pathway comprises of the steroid hormone vitamin D, vitamin D receptor VDR and RXR¹⁴. Vitamin D can be accessed (dermally) from ultra violet light and from dietary intake^{15,65,66}. It is metabolized into 25 hydroxyvitamin D and 1,25 dihydroxyvitamin D₃ in the in the liver and kidney respectively¹⁵. 1,25 dihydroxyvitamin D₃ has a greater affinity to bind on the nuclear hormone receptor vitamin D receptor (VDR)¹⁴. The vitamin D endocrine axis is thereby regulated by binding of 1,25 dihydroxyvitamin D₃ to the VDR⁶⁵. This action mediates gene transcription of the target gene as the binding results in conformational change of the VDR⁶⁵. After binding, heterodimerization with RXR occurs followed by a cascade of processes at the promoter region, which results in initiation of transcription^{14,65}.

Vitamin D's role in skeletal development and maintenance is well-documented^{14,67,68}. Deficiency results in rickets in children and osteomalacia in adults. Vitamin D has been reported to play a role in the functioning of the immune system and a lack thereof has been linked to autoimmune disorders such as rheumatoid arthritis, type 1 diabetes, and multiple sclerosis^{14,65,69,70}. It has been shown that VDR and vitamin D have an inverse correlation with the occurrence of certain cancers (i.e. colo-rectal, prostate and breast cancers)⁷¹. Hence, insufficiency of vitamin D has been linked to cancer incidences whilst the activation of the VDR by 1,25 dihydroxyvitamin D₃ causes suppression of tumorous cell proliferation and induction of cancer cell apoptosis^{14,71}. Vitamin D also plays a role in neurodevelopment and normal cardiac function^{14,72}.

Studies looking at the consequences of disruption of the vitamin D signalling pathway have been mainly in knockout mouse models and humans^{14,73,74}. Not much research has been dedicated on wildlife. The knockout mouse models have been used as animal models for human pathology. Results from these VDR knock out experimental studies have shown skeletal malformations, muscle, motor and cognitive abnormalities, and weakened immune defence¹⁴. Because of the strict substrate control of the VDR, EDCs are less likely to cause disruption at the receptor level¹⁴. However, experimental evidence have shown that exposure to EDCs can alter the vitamin D concentrations in serum and cause bone malformations, reduced

growth and development, perturbed cardiovascular function, and brain deformities^{14,75-77}.

Peroxisome proliferator-activated receptor (PPAR) signalling pathway

The peroxisome proliferator-activated receptors (PPARs) are nuclear receptors belonging to the nuclear hormone receptor superfamily⁷⁸⁻⁸⁰. They function in regulating biological and metabolic processes⁸⁰. The nuclear hormone superfamily includes thyroid hormone receptors, steroid hormone receptors, vitamin D₃ receptors, retinoid acid receptors and PPARs^{78,79}. The PPARs are made up of three isoforms PPAR α (*NR1C1*), PPAR β (*NR1C2*), and PPAR γ (*NR1C3*)^{14,79}. The three PPARs are ligand activated transcription factors and regulate the signalling pathway through transcription of target genes. Transcription is initiated through heterodimerization with RXR^{14,79}. Activation of the PPARs occurs through fatty acids, xenobiotics and pharmacological ligands resulting in regulation of transcription in genes responsible for proliferation, fatty acid metabolism and inflammation¹⁴.

Each PPAR has specific functions and these functions in vertebrates have been well studied¹⁴. Some of the major functions of each PPAR are as follows: PPAR α – mediates proper functioning of energy homeostasis by increasing fatty acid catabolism, gluconeogenesis, and cholesterol levels; PPAR β - regulates energy homeostasis through target genes in the liver, heart, and skeletal muscle which control fatty acid catabolism and adaptive energy expenditure; PPAR γ - mediates differentiation and function of adipocytes, and plays a role in glucose uptake^{14,79,80}.

Because of the PPARs role in lipid and glucose metabolism, disruption of the receptors by environmental chemicals may have a role in lipid disorders, glucose intolerance, insulin resistance and obesity¹⁴. Weight gain due to EDC exposure has been hypothesized by a number of scientists. This class of EDCs is called obesogens, and they disrupt the lipid and energy homeostasis^{14,81}. Animal models have shown that exposure to obesogens cause weight gain and obesity⁸². A number of EDCs have been reported to interact and disrupt the PPAR signalling pathway and they include hypolipidemic drugs, pharmaceuticals (e.g glitzones), and anti-inflammatory drugs (e.g ibuprofen and indomethacin)^{14,83,84}.

The final endocrine axis to be discussed is the hypothalamus pituitary thyroid axis (HPT), and is significant to this study. Adverse effects of EDCs on the hypothalamic pituitary thyroid axis are of major concern because the thyroid hormones triiodothyronine and thyroxine (T_3 and T_4) have an important role in growth and development (brain, tissue, muscle, kidney, and ear), homeostasis, energy metabolism, thermo regulation, osmo regulation, regulation of other endocrine systems, cardiovascular function, fertility, and reproductive behaviour⁸⁵⁻⁸⁷.

1.2.4 Hypothalamic pituitary thyroid (HPT) axis

The (HPT) axis is made up of the hypothalamus, pituitary and the thyroid glands. Each of these glands secretes specific hormones which allow for the proper functioning of the axis. The overall function of the axis is to maintain a stable physiological state of thyroid hormone production from the thyroid gland⁸⁸.

1.2.4.1 Description of hormonal secretion in the HPT axis

The cascade of events starts in the hypothalamus, where the thyroid releasing hormone (TRH) is synthesized by hypophysiotropic neurons in the paraventricular nuclei (PVN)^{89,90}. TRH is then transported to the posterior pituitary by the hypothalamic-pituitary portal system where it mediates the biosynthesis and release of thyroid stimulating hormone (TSH)/thyrotropin⁸⁹. TRH effects this secretion by binding to the thyrotropin releasing hormone receptor (TRHR), a G-coupled protein receptor, in the thyrotroph cells of the posterior pituitary¹⁴. TRH also stimulates glycosylation of TSH, a post translational modification process, thereby activating it¹⁴. There is currently no evidence of TSH causing a negative feedback on the synthesis of TRH in the hypothalamus^{88,89}. TSH is a heterodimer (α and β). TSH then binds to receptors on the follicle cells of the thyroid gland, causing an increase in uptake of iodine into the gland followed by biosynthesis and release of thyroid hormones, T_3 and T_4 , into circulation^{88,89}.

TH, through negative and positive feedback on the posterior pituitary and hypothalamus, maintain stable concentrations of thyroid hormones in circulation^{88,89}. TH can facilitate the production of TRH from the hypothalamus through positive feedback. There is, therefore, a negative correlation between TSH and TH levels,

with normal thyroid signalling. This correlation serves as a reference which is used to establish normal thyroid function and pathology states⁵. However, emerging evidence has shown that even minimal alterations to TH and TSH levels can cause thyroid disease, and this has been a topic of recent debate⁹¹. Other mechanisms that facilitate TRH synthesis from the hypothalamus include body thermoregulation, leptin and melanocortin signalling (regulation of dietary consumption and energy expenditure) and cardiovascular function¹⁴. Each of these mechanisms targets the hypophysiotropic neurons of the hypothalamus, and through integration of multiple inputs and TRH is produced.

1.2.4.2 The thyroid and thyroid hormone (TH) synthesis

The thyroid gland is one of the largest glands of the endocrine system. It is found in the neck on the trachea⁵. It has two lobes connected by the isthmus⁸⁹. The function of the thyroid gland is to synthesise and secrete the hormones T₃ and T₄. The process of thyroid hormone production is initiated by the binding of TSH on the G-coupled receptors on the cell membrane of the thyrocytes¹⁴. The activation of the receptor results in the uptake of dietary iodine from circulation into the thyroid follicle cells through the sodium/iodide symporter (NIS)⁹². The action of the NIS is coupled with the transport of sodium ions into the follicle cells through the Na⁺/K⁺ATPase resulting in a concentration gradient which allows iodine to be concentrated up to 40 fold in the thyroid gland¹⁴. Once in the follicle cells, the iodine is further transported into follicle colloid through the pendrin transporter⁹². The follicle colloid is the inner part of the thyroid gland. There, in the follicle colloid, the iodine molecule undergoes a peroxidation process in the presence of hydrogen peroxide and thyroperoxidase enzyme forming activated iodine species^{14,89}. The glycoprotein thyroglobulin, which is transported from the follicle cells into the follicle colloid through exocytosis, is then combined with the activated iodine species through an iodination process forming mono-iodinated tyrosine (MIT) and di-iodinated tyrosine (DIT) molecules¹⁴. The tyrosine rings on the thyroglobulin are the substrates for the iodination process. Phenolic coupling of these tyrosine molecules then results in T₄ and T₃⁹³.

The formation of either of the TH is dependent on which tyrosine rings conjugate. One MIT (one iodine molecule attached) and one DIT (two iodine molecules

attached) molecule will form T_3 , whilst two DIT molecules will form T_4 ^{14,93}. Therefore, T_4 differs from T_3 because of the presence of an extra iodine molecule located at the 5' position of the first tyrosine ring as shown in figure 2.

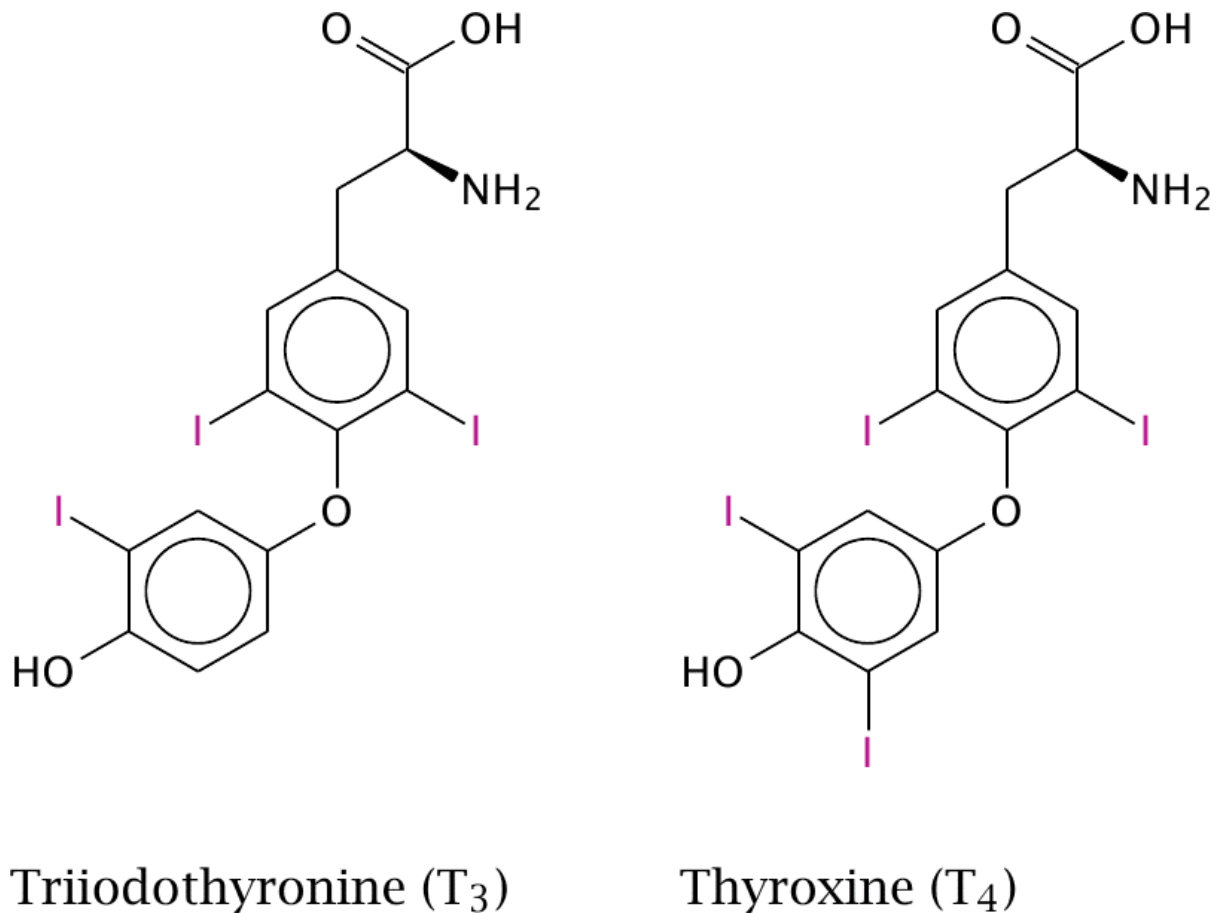


Figure 2: Structures of thyroid hormones. Adapted from <http://watcut.uwaterloo.ca/webnotes/Pharmacology/graphics/t3-slide-pic-98827.png>

The hormones are stored in the colloid still bound to the thyroglobulin until release through activation of the TSH receptor by TSH¹⁴. The T_4 and T_3 are separated through lysosomal proteolysis and transported into circulation through the monocarboxylate transporter 8 (MCT8) transporter⁹³. The ratio of the T_3 secreted from the thyroid gland compared to T_4 is 20% : 80%⁹⁰.

1.2.4.3 Transportation and peripheral biotransformation of thyroid hormones

In the plasma, the thyroid hormones are bound to plasma proteins, because of their low water solubility. A small proportion of the hormones circulates unbound in the plasma (~1%)⁹⁰. The main proteins they bind to include transthyretin (TTR), thyroxin

binding globulin (TBG) and globulin⁹⁴. These carrier proteins also provide a stable pool of thyroid hormones from the free active hormones which are released for cellular uptake⁹⁴. The protein then transports THs to their target sites where they bind to receptors on the cell membranes and are transported into the cells^{14,90,92}. A number of membrane transporters are responsible for this uptake, they include the organic anion transporter peptide 1c1 (OATP1C1) which is present in the brain capillaries, astrocytes and choroid plexus, and the MCT8 and 10 transporters, which are located in various organs of the body^{14,92}.

Once in the target cell, TH undergoes peripheral bio-modification. There are enzymes, called iodothyronine deiodinases, that are responsible for this bio-modification, deiodinase 1, 2 and 3 (D1,2 and 3)^{14,90,92}. D1 functions by converting T₄ to T₃ and also in the breakdown of reverse T₃ (r T₃)^{90,92}. D1 is found in the thyroid, liver, pituitary gland and the kidney and can deiodinate the inner and outer rings of T₄. D2 is responsible for the transformation of T₄ to T₃ and conversion of rT₃ to 3,3'-diiodothyronine (T₂)^{90,92}. D2 expression is in the thyroid, skeletal muscle, brain, anterior pituitary, brown adipose tissue and placenta^{90,92}. D3 is completely an inactivating enzyme. It is present in the brain, placenta (during gestation), foetal tissue and skin^{90,92}. It mediates the inactivation of T₄ to rT₃, and T₃ to 3,3'-diiodothyronine (T₂)^{90,92}. Overall, about 80% of T₄ is converted to T₃ by deiodinases⁸⁹. D3 is particularly important in facilitating the homeostasis of TH levels by ensuring there is no excess TH in tissue^{90,92}. In the liver, TH are also cleared from circulation in the liver, through glucuronidation and sulfonation, then removed through biliary excretion^{14,89}. Availability of TH in tissue is highly dependent on well-functioning and well-regulated membrane transporters, deiodinases and liver. Figure 3 shows a diagrammatic depiction of the HPT axis, TH synthesis, biotransformation and action in the target tissues.

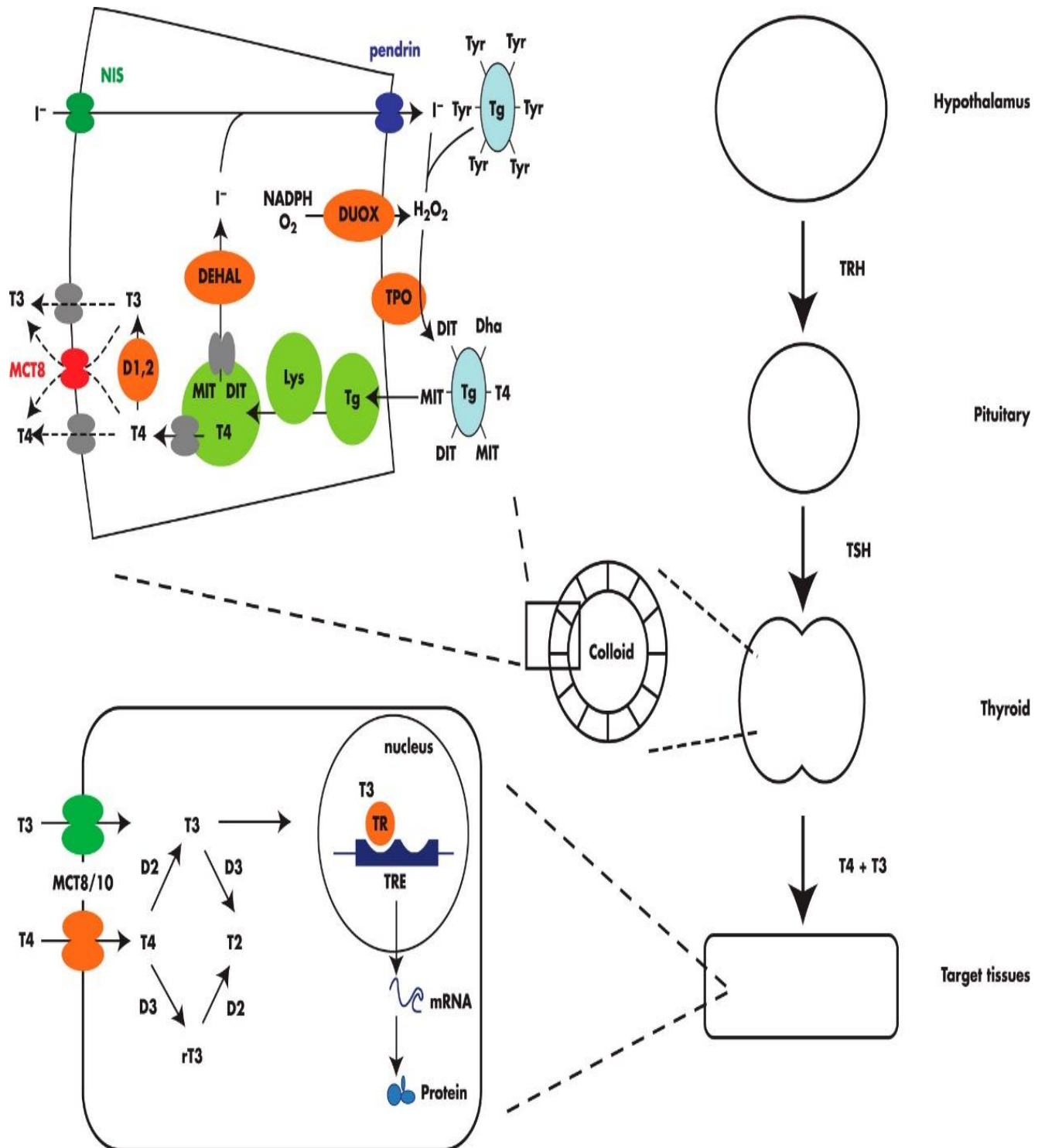


Figure 3: Simplified depiction of the HPT axis, TH synthesis and biotransformation and action in target cells. (Dha- dehydroalanine; Lys-lysosome; TRE-TH response element, Tg-thyroglobulin, DUOX-dual oxidases, DEHAL-dehalogenase, NADPH-nicotinamide adenine dinucleotide), Adapted from Medici et al 2015⁹².

1.2.4.4 Thyroid hormones (THs) mechanism of action

T₃ is assumed to be the more biologically active TH and is reported to bind to thyroid receptors (TR) in the nucleus of target cells with greater affinity than T₄ (50 fold). In the target cell, only T₃ is transported into the nucleus. TH receptors are nuclear receptors of the ligand dependent transcription factor superfamily⁸⁹. They act on the target DNA sequence of the target gene by either suppressing or initiating transcription⁸⁵. In humans, they are encoded by two genes, *TRα* and *TRβ*, which are on chromosome 17 and 3 respectively. Through alternative splicing, four receptors are formed from the two genes, and these include; TRα1, TRα2, TRβ1, and TRβ2⁸⁹. There is an extensive expression of TRs in the body^{89,92}. In the nucleus, they form a heterodimer with retinoid X receptor (RXR) and bind to the promoter region of the TH-responsive gene regulating transcription²⁷. When T₃ binds to the TR and RXR heterodimer, a series of events occur, including conformational change of the receptor, which leads to the transcription of the TH-responsive gene^{27,89}. Without T₃ binding to the TR and RXR heterodimer on the gene, transcription will not occur²⁷.

Thyroid hormones are able to elicit multiple effects throughout the body. These pleiotropic effects are made possible by the regulated expression of TR, and provide an insight into the mechanistic pathways of THs. It is important to note that these effects are still not clearly understood. The first of these effects is the tissue/organ specificity and time specificity that TR exert, with regards to their expression⁸⁹. Evidence has shown that TR can be expressed in a certain body tissue, organ or system, only for a specific time period (spatial and temporal specificity). This allows for TH effects only in specific tissues for a period of time⁸⁹. Secondly, TRs are expressed differentially in the body⁸⁹. This differential expression in the brain is key to regulating transcription of certain genes in the brain. For instance, whilst TRH containing neurons are present in all parts of the brain, regulation of the TRH genes only occurs in the PVN region of the hypothalamus. The PVN region is the primary site for TRH synthesis⁸⁹. This effect occurs through THs binding selectively to TRs in the PVN region. Lastly, the ability of the TR to heterodimerize with RXR nuclear receptors is also another way that TRs are able to exert its genomic response, and this has been discussed (section 1.2.2.3). However, in these TR/RXR heterodimer complexes, the RXR has also been shown to be the silent cofactor in some response genes, whilst in some, it has been shown to be responsive to its specific ligands

hence being an active cofactor⁸⁹. All these are factors that make pleiotropic genomic responses of TH action possible.

Molecular responses of THs are also non-genomic. This occurs when THs or their metabolites interact with proteins from the cytosol or the cell membrane²⁷. Some of these interactions result in increased Na-K-ATPase activity when T₃ interacts with phosphatidylinositol 3-kinase (P13K)²⁷. The enzyme P13K is responsible for cellular metabolism, including proliferation and apoptosis and cell motility⁹⁵. THs have also been reported to have effects on the mitochondria by having a role in energy consumption and oxidative phosphorylation⁸⁹. It has been suggested that this effect may be through T₃'s ability to activate adenine nucleotide translocase, a mitochondrial protein transporter⁸⁹. T₄ is reported to play a role in actin polymerization in astrocytes, thereby regulating growth and motility⁸⁹. Thyroid hormones T₄ and rT₃ have specifically been linked to this effect⁸⁹. Finally, studies have shown that T₃ contributes significantly to the rapid increase in cellular glucose uptake when protein synthesis is inhibited and also by ¹⁴C-2-deoxy-glucose by heart muscle.²⁷ Genomic responses to glucose uptake have also been reported to be affected by THs, i.e. expression of glucose transporters GLUT4 and GLUT1. Disruption of TR implicates glucose uptake in the brain⁸⁹. In this study, EDCs which disrupt the thyroid signalling pathway will be described as thyroid disrupting chemicals (TDC) or thyroid hormone receptor disruptors (THRD).

It is evident that the thyroid signalling pathway is complex, with a regulatory system that is multifaceted, and effecting a number of body mechanisms and functions. Disruption of this pathway is a major concern, particularly with EDCs as they can mimic or antagonize the effects of natural hormones, causing disruption in the normal functioning of the body. Understanding the mechanism of action of the TH both genomic and non-genomic sheds some light on the possible pathways that thyroid disruption may occur and thus allow for the development of assays and tests to assess and analyse thyroid disruption effects.

1.2.5 Thyroid disrupting chemicals (TDCs)

A number of EDCs, to which humans are exposed regularly, voluntarily or involuntarily, are able to interrupt the normal TH signalling axis⁸⁵. Table 1 shows the

various TDCs that can interrupt the thyroid signalling axis. Sources of these chemicals include industrial chemicals and by-products, plastic and plasticizers, pesticides, fungicides, cosmetics, heavy metals and polyphenols⁹⁶.

Table 1: TDCs that interrupt the thyroid signalling pathways and their sources⁹⁶.

Industrial chemicals and by-products (solvents/lubricants)	Polychlorinated dioxins and furans (PCDD/Fs), perchlorate, polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs)
Plastics and plasticizers	Bisphenol A (BPA), tetrabromo-bisphenol A (TBBPA) and phthalates
Pesticides	Alachlor, dicamba, carbamate, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT), fibronil Endosulfan, heptachlor, lindane, toxaphene
Fungicides	Amitriole, vinclozolin, mancozeb
Sunscreen-Cosmetics	Benzophenone2 & benzophenone3, homosalate (HMS), 2-ethylhexyl 4-dimethylaminobenzoate (OD-PABA) and 4-aminobenzoic acid (PABA)
Heavy metals	Cadmium, mercury, lead
Polyphenols	Isoflavones (soy), flavonoids (catechin, quercetin)

Studies on the Great Lakes' wildlife (Canada) in the early 1990s provided a turning point in showing evidence that exposure to a certain group of EDCs can disrupt the

thyroid signalling pathway⁹⁷. The researchers linked polychlorinated biphenyls (PCBs) and specific organochlorine pesticide exposure to thyroid abnormalities other endocrine defects in the herring gulls⁹⁷. More studies on the Great Lakes showed that top predator fish had thyroid glands that were severely enlarged⁹⁷. Since this study, there have been on-going studies looking at thyroid disruption, both epidemiology and laboratory studies. Recently, a clinical trial was done looking at the effects of PCB and pesticide exposure to 50 Laotian women who are directly exposed to these chemicals⁹⁸. The study was looking at the reproductive effects of the chemicals; the results from this trial have not been published as yet. It has however not been easy to establish direct linkages between exposure to the the compounds and adverse health effects, just as it has not been easy with all other EDCs¹⁴. Special focus on TDCs have been the effect of exposure to pregnant women and children, and there have been associations between exposure to TDCs during foetal life to disruption of brain development and function⁹¹.

1.2.5.1 Industrial chemicals in the environment

PCDD/Fs, furans, PCBs and perchlorate are some of the well-known industrial chemicals which have the ability to disrupt thyroid hormones signalling. Although PCBs are not widely used anymore, the chemical is still being detected in the environment, and in human tissue⁹⁶. PCBs have a particularly high structural similarity to THs⁹⁹. PCB exposure in humans has been linked to low IQ, neurobehavioural and motor deficits due to their ability to induce a state of hypothyroidism^{5,96,99}. It has also been reported to be neurotoxic to the developing brain causing structural abnormalities to the brain⁹⁶. Some studies, although still controversial, have reported an association between perchlorate exposure and reduced thyroid function⁵. Dioxins and furans are by-products of a number of industrial processes and have been shown to disrupt thyroid function in a number of *in vivo* and *in vitro* studies^{99,100}. These industrial processes include production of pesticides, waste combustion, and paper bleaching using chlorine.⁹⁹ Dioxins are highly persistent in the environment and have a long half-life in human tissue (7 years)^{99,100}.

1.2.5.2 Plastic and plasticizers in the environment

A lot of the plastics produced in industry contain EDCs in variable concentrations, from food packaging material, plastic bottles and containers, food cans and toys. Some of the most common EDCs are BPA (4, 4' isopropylidenediphenol) and phthalates. BPA is added to plastics usually to harden them. Examples include an epoxy lining in food and beverage containers, dental sealants and polycarbonate plastics^{96,99}. Average human consumption of BPA is reported to be 6.6 µg per day, with high concentrations being found in serum blood of pregnant women, and in the fetuses' amniotic fluid^{96,99}. BPA is a TH antagonist and studies have shown an association between BPA exposure and attention deficit hyperactive disorder ADHD and thyroid resistance syndrome in animals⁹⁶.

Phthalates are a group of EDCs that have been categorized as emerging TDCs. They are added to plastics to make the plastic flexible and their use in industry has been increasing^{99,100}. Although exposure potential is broad, the particularly vulnerable group are hospitalized individuals and neonates as medical devices including feeding tubes, contain phthalates^{99,100}. Animal studies have shown structural malformations of the thyroid gland after exposure to phthalates. There is a lack of data with regards to the effects of human exposure. However, recent studies have reported an association between exposure and disruption of TH levels^{99,100}.

1.2.5.3 Pesticides

There a number of pesticides that can disrupt the HPT axis. Most common dichlorodiphenyltrichloroethane/1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) and alachlor, have both been shown to disrupt TH levels¹⁴.

DDT was introduced primarily for the eradication of malaria in the late 1940s and gained its worldwide use. Whilst DDT is a xenoestrogen, binding to the oestrogen receptor as an agonist, it is also a TDC^{14,101,102}. It is highly persistent in the environment and has a long half-life¹⁰². Almost immediately after its wide spread use as a pesticide, its deleterious effect on the environment started being reported on^{6,20}. Exposure to both animals and humans is through inhalation and ingestion (contaminated food sources). After the introduction of DDT in the environment, the population of the white-tailed eagle in Sweden began to deteriorate⁶. Studies

revealed that the eagles were feeding on prey that had high levels of DDT in their tissue, and this resulted in bioaccumulation of the pesticide in the eagles. The high levels of DDT in the birds significantly disrupted reproductive behaviours^{6,20}.

In 1980, a chemical spill, containing 15% DDT and its metabolites, in one of the lakes in Florida USA (Lake Apopka) resulted in disrupted reproductive health amongst the alligator population²⁴. A study done on the exposed alligators reported that exposure to DDT results in defective embryonic reproductive development in both structure and function²⁴. The authors also raised the question of the effect on other animal populations and the human population as well who were exposed to the spill²⁴.

Although the Stockholm Conventions on POPs banned the use of DDT, exceptions were made for countries which need DDT for vector control pending the development of new cost effective methods which can combat the malaria vector (*Anopheles mosquito*)⁶. DDT use still occurs here in South Africa in malaria endemic areas, and is used for vector control. Large populations of people are still exposed as DDT continues to contaminate the environment. A number of studies looking into the effect of exposure to DDT still focus on the oestrogenic pathway, hence information on its effect on the thyroid signalling pathway are still lacking^{103,104}.

1.2.5.4 Brominated flame retardants

Over the past couple of years, a group of chemicals has become a target of scientific enquiry with regards to their endocrine disrupting potential. These chemicals called brominated flame retardants (BFRs) are added onto industrial and consumer products (electrical devices/equipment, furniture, textiles, construction materials, car seats etc.) to increase their resistance to flammability^{18,105,106}. Studies have demonstrated that BFRs are persistent and bio-accumulative in the environment and humans, and recent evidence has shown that they may be endocrine disruptors¹⁰⁶. Exposure to BFRs can occur through inhalation, ingestion (dietary) and dermal. BFRs can be grouped into five major classes: brominated bisphenols, diphenyl ethers, phenols, cyclo-dodecanes, phthalic acid derivatives and phenols¹⁰⁵. Of these, the first three have the highest production rate, with tetrabromobisphenol-A

(TBBPA), polybrominated diphenyl ethers (PBDEs), and hexabromocyclododecane (HBCD) having the highest consumption rate globally¹⁰⁶.

TBBPA, HBDCs, and PBDEs have been reported to interact with the thyroid axis^{94,106}. These two BFRs have structural similarities with the thyroid hormone thyroxine (T₄), and hence their ability to induce TH like effects on the thyroid axis^{94,106}. T₄ is a precursor of T₃, the active form of the thyroid hormones¹⁰⁶. An *in vitro* study reported that TBBPA and PBDEs compete for binding sites with T₄ on the human transthyretin protein, responsible for transporting T₄ in plasma. This results in low T₄ plasma concentrations and it has been shown that low levels of T₄ can be associated with neurotoxicity during development and behavioural effects in experimental animals^{106,107}. More studies on experimental animals have also shown reduced cognitive functions and hyperactivity upon exposure to PBDEs¹⁸.

In 2001, a human prenatal cohort study was done in the USA to determine the effect of PBDEs on IQ and hyperactivity in children born after the World Trade Centre attack in New York¹⁰⁸. Another study, completed 2013, also looked at the effect of prenatal exposure to PBDEs in a migrant population and followed up the children up to 7 years of age¹⁰⁹. Both the studies reported that prenatal exposure to PBDEs resulted in behavioural problems and reduced IQ in the children^{18,108,109}. One of the most comprehensive studies on prenatal exposure to PBDEs to date is the Health Outcomes and Measures of the Environment (HOME) by Chen *et al*¹⁸. It was a birth cohort study looking at the effect of PBDEs on neurodevelopment in children exposed *in utero* from 2003 (March) to 2006 (February)¹⁸. 309 mothers took part in the study, and were assessed for PDBE serum levels whilst pregnant¹⁸. The children were followed up from birth up to five years of age and were assessed for neurobehaviour, physical growth and health¹⁸. The authors reported that *in utero* exposure to brominated flame retardants, specifically PBDEs, is linked to low IQ and hyperactive disorders in children¹⁸. Whilst the health effects reported in the HOME study were in the children, it has also been reported that the PBDE exposure causes thyroid hormone disruption in the pregnant women¹⁰⁷.

There are over 75 known BFRs produced globally, but toxicological data is only available for half of them¹⁰⁵. More research needs to be done to assess the toxicology and health effects of these BFRs in order to address this literature gap.

One of the sources of BFRs is electronic waste (e-waste). E-waste is discarded and/or non-functioning TVs, cell phones, computers, household electronics, office electronics and lighting equipment¹¹⁰. An estimated 20-25 million tonnes of e-waste are produced annually, with USA, Europe and Australia being the major producers¹¹¹. Whilst most of the developed countries have the resources to handle, recycle and dispose of e-waste, developing countries at times lack in this regards. The expense of proper disposal of e-waste has resulted in a large quantity of the e-waste being shipped to poorer countries with China, Pakistan, and Nigeria being major destinations^{111,112}. Over half of the e-waste produced in developed countries ends up in developing countries¹¹⁰. E-waste, whilst it contains precious metals, also has toxic metals and chemicals which have been shown to negatively impact the environment and affect human health^{110,111}. These metals include mercury, lead, chromium and cadmium¹¹⁰. Some of these metals, mercury, cadmium, and lead have been reported to have a role in endocrine disruption¹¹³. E-waste also contains amounts of brominated flame retardants with high concentrations of polybrominated diphenylethers PBDEs, and polybrominated biphenyls (PBB)¹¹⁰.

Developing countries lack the capacity to handle e-waste, and this situation is worsened by the presence of informal recycling sectors¹¹². Handling of e-waste is important because some of the individuals dismantling the e-waste can get exposed through dermal contact and inhalation. Most of the e-waste is disposed of in landfills¹¹¹. This poses a danger of leaching into the soil, and into the water bodies, causing exposure to animals and humans¹¹¹. Some of the e-waste is also burnt resulting in brominated flame retardants in the air and increasing exposure. A study done in an informal recycling centre in China showed that e-waste resulted in contamination of the soil, air, and water, causing chemical exposure to the environment and to humans¹¹¹. Studies looking at the impact of e-waste on the environment and on human health in Africa are lacking. There is no doubt that the issue of EDCs is global affecting both developed and developing countries.

1.2.5.5 Thyroid disruptors and the HPT axis

Disruption of the thyroid metabolism occurs throughout the HPT axis; at the pre-receptor synthesis, transport, metabolism, cellular uptake and feedback mechanism

of the hormones, and the TR, hence influencing transcription of target genes⁸⁵. Laboratory studies have established that TDCs can have agonistic, antagonistic or modulatory effects on TR, influencing various mechanisms. Figure 4 illustrates how TDCs interrupts the HPT signalling pathway. The mechanisms through which TDCs induce their effects will be discussed. It is important to reiterate that these effects are dependent on the dose, and the duration of exposure.

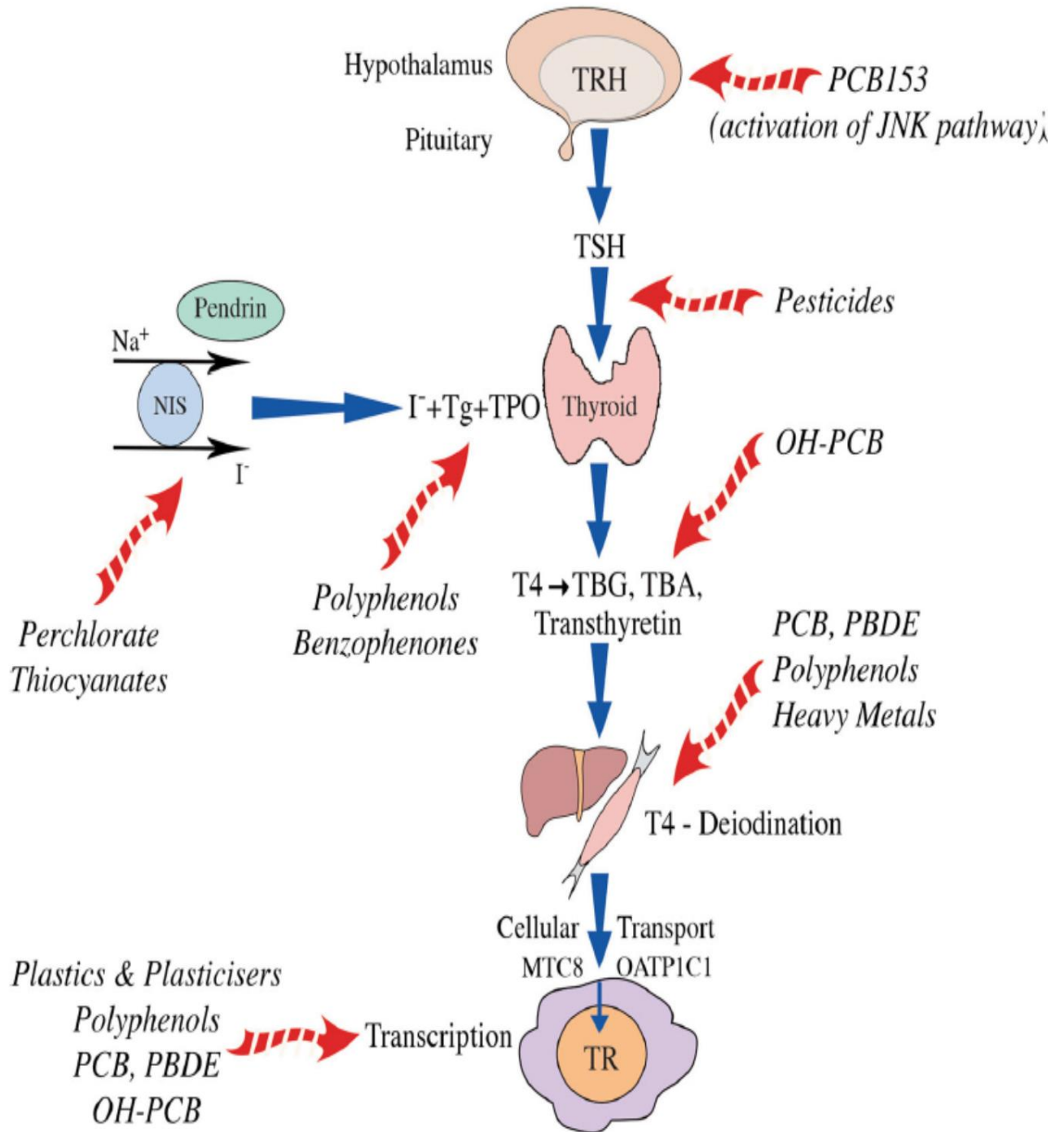


Figure 4: Illustration of how TDCs interfere with the HPT axis from synthesis, secretion, metabolism and action of TH. (JNK- c-Jun N-terminal kinase) Figure adapted from Duntas 2015⁹⁶.

Polychlorinated biphenyls (PCBs) are TDCs as they have been implicated in TH disruption. Polychlorinated biphenyl (PCB) 153 is able to interact with the c-Jun N-terminal kinases (JNKs) in the hypothalamus causing decreased levels of TRH, and TH^{96,114}. This effect has been theorized to be caused by activation of the JNKs by PCB 153, which causes interaction of the JNKs with the TRH receptor and reduction of TRH^{96,114}. Some TDCs have been reported to be able to interact with the NIS, found on the thyrocyte membrane, by competing (antagonistic) with iodine for the binding site^{96,97}. This causes a decrease in the synthesis of TH. These TDCs are perchlorate, phthalates, thiocyanate, bromates and nitrates^{96,97}. The enzyme TPO (thyroperoxidase) plays a role in the formation of TH, without which, TH synthesis would not occur. There are particular TDCs which have been shown to obstruct TPO production and hence decreasing the production of TH. These TDS are polyphenols, benzophenones (sunscreen), methimazole, amitrole (herbicide), mancozeb, (fungicide) and soy isoflavones^{96,97}. Transportation of the thyroid hormones to target cells by carrier proteins is an essential process that allows the hormones to be able to elicit their effects. However, PCBs, flame retardants, phthalates, and pentachlorophenol have been shown to competitively bind to the protein TTR, hence reducing the amount of TH bound to proteins and their access to target cells^{96,97}. Because of the structural similarities of some of the compounds to TH, they may be able to interact with the receptors on target cells, causing disruption. Low foetal brain levels of T₄, when exposed to these chemicals, have been attributed to this mechanism⁹⁷. At the target cells, TH enters the cells through transporters and induce effects through the nuclear receptors.

A group of TDCs has been reported to disrupt this process through either inhibition or activation of cellular uptake. These TDCs are flame retardants, PCBs, bisphenol A, and dioxins⁹⁷. The resulting effect is an increase in TH biliary secretion through enhanced hepatic metabolism⁹⁷. This also affects the TR activity leading to altered transcription of the target gene⁹⁷. Receptor inhibition of TSH and TRH has reportedly been caused by these TDCs as well⁹⁷. DDT and PCBs have been reported to specifically inhibit TSH receptor, leading to low TH levels⁹⁷. In the target cell TH undergo transformations through the action of deiodinases. Metals, lead and cadmium, PCBs, FD&C red dye #3, octyl_methoxycinnamate (sunscreens) and methoxychlor interrupt deiodinase activity resulting in low levels of T₃ produced

peripherally⁹⁷. This reduces the T₃ levels available for transcription of target genes in the nuclei. These specific TDCs, flame retardants, PCBs, bisphenol A, and hexachlorobenzene are able to interfere the THs binding to the TRs inside the cells. This affects gene transcription^{96,97}.

1.2.5.6 Thyroid disorders and thyroid disrupting chemicals (TDCs): Could there be a link?

The past several decades have seen an increase in the disease burden of thyroid disorders⁵. An alarming two billion people worldwide are said to have thyroid disorders⁵. Human thyroid disorders can be broadly divided into these categories; congenital and adult, hypothyroidism, hyperthyroidism, autoimmune thyroid disorder, goitres and thyroid cancer⁴. In Africa, the most reported thyroid disorders reported are hypothyroidism, hyperthyroidism (particularly thyrotoxicosis), thyroid cancer and iodine deficiency disorders¹¹⁵. The commonest form reported is iodine deficiency, although not as prevalent as it used to be due to iodization programs^{115,116}. In both children and adults iodine deficiency has been reported to cause goitre, hypothyroidism, impaired mental function, and retarded mental and physical development in children¹¹⁵. It is important to note that there have been reports of endemic iodine deficiency being linked to exposure to goitrogens (chemicals that inhibit iodine uptake at the thyroid) in some parts of Africa.¹¹⁵ These goitrogens interrupt the uptake of iodine and include thiocyanates which are present in the diet¹¹⁵.

Genetic and environmental factors have influenced the prevalence of thyroid disorders. However, increasing evidence from animal studies, laboratory assays, human studies and the existing human health trends, suggest that exposure to TDCs may be associated^{4,14,18-20,23,96,97,105,115}. Because TDC exposure alters serum TH levels, it is possible that they may cause thyroid disease.

1.2.5.6.1 Thyroid disrupting chemicals (TDCs) and sexual development

The effect of thyroid hormones on sexual development has not been well studied. However, more studies are starting to look into the role played by TH in sexual development and function, and the information from those studies will be discussed.

Hypothyroidism and hyperthyroidism in both males and females have been shown to affect sexual development, particularly in the pubertal stage²⁷. Puberty is a complex process which involves rapid hormonal changes and impacting sexual development, and hence very sensitive to any hormonal interruption. Studies looking at thyroid dysfunction and sexual development have mainly been in animal models. In the female rodents, induction of neonatal hypothyroidism has shown a delay in sexual maturation and vaginal opening, small ovaries and immature uterine and vaginal tissue²⁷. Induction of post-pubertal hypothyroidism in rodents has been reported to affect the menstrual cycle²⁷. These results have been in concordance with human effects. However, the mechanistic pathways with which this occurs is still being researched on²⁷. In women, hypothyroidism has been associated with menstrual irregularities, particularly oligomenorrhea²⁷. Hyperthyroidism has been shown to cause delayed sexual maturation and onset of menstrual cycle, also menstrual irregularities (hypomenorrhea and polymenorrhea)²⁷. However there are still gaps with regards to the effect of TH on the female reproductive development.

In the male, rodent studies have shown that neonatally induced hypothyroidism results in impairment of fertility and sperm motility²⁷. However, these effects are reversible in the human as treatment of neonatal exposed men results in normal sexual development²⁷. Pre-pubertal hypothyroidism in both male rodents and humans has been reported to cause testicular atrophy, delayed sexual development, altered gonadotropin secretion and gonadal function²⁷. If this state is prolonged in humans, up to adulthood, the resulting effect is reduced testicular mass, impaired fertility, and hypogonadotropic hypogonadism²⁷. Hyperthyroidism in males causes alteration of testosterone levels²⁷. Hyper- and hypothyroidism states in the human male have been linked to the following conditions: erectile dysfunction, delayed and/or premature ejaculation and decreased libido²⁷. However, with treatment, these symptoms can greatly improve. TH targets in the male reproductive pathway are relatively more established than in the female²⁷.

The role of EDCs in reproductive anomalies has recently become an area of scientific scrutiny. Epidemiologic studies have shown that there has been major changes in pubertal timing and also increases in gonadal maldevelopment²⁷. It is still not clear if these changes can be attributed to exposure to thyroid disruptors. A growing body literature has confirmed that the disruptors of pubertal sexual

development are well-known thyroid disruptors which interfere with thyroid hormone synthesis, metabolism and action. These include phthalates, bisphenol A and PCBs²⁷. Theoretically, any chemical with TDC properties should essentially interfere with the TH targets for sexual development. However, as it has been explained, the magnitude of disruption (that can cause an impact) and windows of development are an important factor in endocrine disruption²⁷. There is an apparent lack of scientific data with regards to the exact timing and extent of TDC exposure that would result in sexual development abnormalities during puberty²⁷. Overall, the effect of TDC on sexual development and function is not well established.

1.2.5.6.2 Thyroid disrupting chemicals (TDCs) and neurodevelopment

Thyroid hormones play an essential part in brain development, maturation and maintenance from foetal through to adult life. They exert their effects through specific time windows and influence major developmental processes in the brain¹¹⁷. These include neurogenesis, cell migration, neuronal and glial cell differentiation, myelination, and synaptogenesis^{117,118}. Neurogenesis is the production of new neuronal cells. TH are involved in the later stages of neurogenesis¹¹⁷. Adult hypothyroidism affects neurogenesis, resulting in cognitive deficits, depression and mental illness¹¹⁷. Cell migration, defined as the orchestrated movement of cells, is also aided by TH, particularly in the cerebellum, cerebral cortex and hippocampus¹¹⁷. Deficiency in the process results in structural malformations in the brain, which can have significant impact on function¹¹⁷. Neuronal and glial cell differentiation is an important process for specification into functional neuron types. This process is also aided by TH through the control in the expression of genes that regulates differentiation¹¹⁷. Nerve fibres have a myelin sheath wrapped around them. The myelin sheath is an insulating layer wrapped around nerve fibres, important for neuronal communication in the brain. Without the myelin sheath, communication in the brain is disrupted. Hypothyroidism disrupts the myelination process, causing reduced deposition of myelin. Synaptogenesis is the formation of connections and functional circuits for transmission of messages between neurons. TH have a role in the formation of a synapse¹¹⁷.

THs are able to exert their effects in the brain because of their ability to cross the blood brain barrier (BBB), through the use of specific trans-membrane transporters MCT8 and OATP1C1^{117,119}. Once in the brain they can induce genomic responses through TR α and TR β which are expressed throughout the brain¹¹⁷. Mutations of these transporters and receptors also cause neurological impairment, likely due to decreased TH induction¹¹⁷. There have also been reports of non-genomic effects of TH in the brain¹¹⁸.

Production of the TH begins at about 11 weeks of the gestational period, after which it increases with maturation of the hypothalamus-pituitary- thyroid axis⁸⁶. The role of TH in the overall human body have been mentioned but they also play a particularly significant role in the perinatal stage of human development⁸⁶. In the developing foetus, the source of thyroid hormones is the mother. The thyroid hormones that the mother provide are transported via the placenta to the foetus and are important for neurodevelopment⁸⁶. It has been noted in mothers that have hypothyroidism that their children are born with impaired psychomotor development, mental retardation, deaf-mutism, behavioural issues, decreased IQ and visual-spatial processing impairment^{86,118}. Because of the extent of impairment, most of these effects are non-reversible. TH imbalance hence results in impaired development⁸⁶.

TH imbalance does not only cause impairments in the developing brain. The adolescent and adult brain is also susceptible to this imbalance¹¹⁹. Both hypo- and hyperthyroidism can lead to mood changes, dementia, and confusion. Hypothyroidism can lead to bipolar disorders, depression, cognitive defects, lethargy and poor motor coordination¹¹⁷. Hyperthyroidism has been reported to cause anxiety, irritability and hyperreflexia¹¹⁷.

There is still debate on whether EDCs targeting the thyroid hormone system are the cause of the current rise in these neurodevelopmental disorders and others including autism and attention-deficit-hyperactive disorder (ADHD)⁹⁶. However, since neurodevelopment is highly dependent on thyroid hormones it is highly likely that it will be sensitive to thyroid hormone disruption by EDCs during the perinatal period resulting in long-term detrimental health effects^{86,96}. Exposure to TDCs *in utero* has been associated with neurobehavioural deficits, particularly brominated flame retardants, and this has been discussed previously. Alteration in the thyroid hormone

signalling system by TDCs has been implicated in a number of mental disease including Alzheimer's disease, bipolar disorder, and major depressive disorder at a later stage in life⁸⁶.

1.2.5.6.3 Thyroid disrupting chemicals (TDCs) and body metabolism

Thyroid hormones regulate metabolic functions in growth, development and in the maintenance of a stable metabolism in the adult¹²⁰. The local transformation of T₄ to T₃ by D2 is essential in TH regulation of metabolism¹²⁰. D2's activity is expressed in the hypothalamus, white fat, brown fat (brown adipose tissue), skeletal muscle, liver, and pancreas, and these are the sites for key metabolic regulation by TH¹²⁰. D2 is the main enzyme responsible for the activation of T₄ to T₃¹²⁰. In these tissues, TH regulates energy expenditure through maintaining basal metabolic rate, aiding adaptive thermogenesis, controlling appetite and food intake thereby regulating body mass¹²⁰. Lipid metabolism is also regulated by TH, specifically through the action of T₃ in the liver¹²⁰. Here T₃ is responsible for regulating cholesterol synthesis and activation of the fatty acid metabolism processes: lipolysis and lipogenesis.¹²⁰ Finally, THs are involved in carbohydrate metabolism. The TH T₃ has been reported to stimulate gluconeogenesis. This occurs through an upregulation of genes that control gluconeogenesis in the liver¹²⁰. TH imbalances have an effect on glucose homeostasis. THs have been reported to an effect on insulin synthesis and action. Scientific reports have shown that TH action is associated with the pancreatic islet cell development and function¹²⁰. The thyroid receptors TR α and TR β are expressed in the pancreatic islets. THs can, therefore, bind and activate the transcription. Through this transcription, transition of the pancreatic islet cells to glucose receptive and insulin-producing cells is achieved¹²⁰.

Hyperthyroidism has been reported to interrupt metabolic pathways by causing a hypermetabolic state. The resultant effects of a hypermetabolic state include high resting energy expenditure, weight loss, low cholesterol levels, increased lipolysis, and gluconeogenesis¹²⁰. Inversely, hypothyroidism causes a hypo-metabolic state, causing low resting energy expenditure, weight gain, high cholesterol levels, reduced lipolysis, and reduced gluconeogenesis¹²⁰. It no surprise therefore that TH

imbalances have been linked with metabolic conditions such as obesity and diabetes^{26,92,120-122}.

1.3 One health

The notion that the state of the environment affects human health is not new. The one health concept recognises that human health and animal health are connected, and both are dependent on the state of the environment¹²³. Human activities have been implicated in a number of environmental hazards including climate change, loss of biodiversity and animal habitats and pollution^{123,124}. These hazards directly impact the well-being of humans and wildlife. One health is important as it looks at this multifaceted problem and recognises the need for a holistic and inter-disciplinary approach to solving the problem. Figure 5 shows a diagrammatic representation of the one health concept, showing the interdependence of human health, animal health and environment health. EDCs are a one health issue, as the pollutants from human activities directly affect animal health and contaminate the environment. The contaminated environment also acts as a route of exposure to the toxic chemicals for both humans and wildlife.



Figure 5: A diagrammatic representation of the one health concept. Adapted from <http://alaska.edu/blast/biomedicine-subsistence-o>

Access to a safe environment is an equality issue, and providing an equal environment to all people has become a global matter. It is well known that larger populations from poorer countries or communities are more likely to be exposed to toxic chemicals that are present in the air, soil, and water. The inequalities are distinct between and within countries. Within countries, the distinction may be visible between social groups, wealth classes, rural and urban settings and gender.¹²⁵ It is apparent that the effects induced by chemical exposures are exacerbated by poor nutrition and poverty, hence causing an increase in morbidity¹²⁴. In 2002, the global community, with the aim to address these disproportions, made protecting vulnerable populations from the dangers of a toxic environment one of the Millennium Development Goals⁴. These vulnerable groups include women, children, the elderly, disabled and the poor.

Over the past two decades, access to improved water services has improved globally. However, Sub-Saharan Africa has been lagging behind; evident in the statistics previously discussed (Section 1.3). This clearly shows the influence and resulting effects of interventions that do not have an equality focus. Without special focus on marginalized and disadvantaged populations, inequalities within populations will continue to increase¹²⁴.

One of the environmental rights in the South African constitution (Section 24) states that “Everyone has the right to an environment that is not harmful to health or wellbeing, and that it is the government’s obligation to safeguard the environment by passing laws which prevent pollution”¹²⁶. Whilst great strides have been made to honour this obligation, large populations of South Africans who live in rural areas and/or who are poor still lack access to treated piped water and use source water for drinking and household activities¹²⁷. This increases the risk of chemical exposure from pollutants present in the water bodies. In addition, there are number of municipalities providing treated piped water with questionable quality, as was evident from the 2011 water quality assessment done by the Department of Water Affairs (DWA)¹²⁸. Again, this causes an increased risk of chemical exposure from water and related health impacts.

1.4 Sources and routes of exposure

The chemical industry is one of the major drivers of chemical pollution. Whilst it is now clear that industrial production will not likely slow down, emphasis should rather be directed towards new ideas for the management of chemicals and waste, and also adopting sustainable renewable energy options. A major point of concern is that, previously, developed countries were at the forefront of chemical production. However, the past decade has shown a significant increase in production of old and new chemicals in the developing countries¹²⁴. China has become the biggest consumer of textile chemicals, taking about 42% of the global consumption¹²⁴. In South Africa, the money spent on pesticides has increased to about 60% since the 1990s¹²⁴.

The chemical pollution that is produced by the chemical industry causes contamination of the three vital life support systems: soil, air and water. The

increased populations, urbanisation, industrialisation and modern agricultural practices worldwide have resulted in chemicals, including EDCs contaminating the air, the soil and subsequently water sources. They have hence found their way into the human body through inhalation, ingestion, dermal contact and drinking water¹²⁹.

The contamination of water sources occurs in multiple pathways. It can occur through leaching from landfills into ground or surface water, deposits from air pollutants, incineration of products containing contaminants and hydraulic fracturing^{124,130}. It can also occur through legal, illegal or accidental spillage. The use/production of pharmaceuticals by industry, agriculture and householders can lead to the contamination of water sources.

One of the most recent water contamination issues in South Africa has been on the subject of fracking¹³¹. An oil company was given the permission to start hydraulic fracturing/fracking in Kwa-Zulu Natal¹³¹. Fracking is a process of releasing natural gas from underground through drilling into the ground, then injecting large amounts of fluid, full of chemicals, into the ground under high pressure¹³². This has spawned a lot of environmental debate over the fate of the chemicals used in the process, and the environmental and human consequences. The process of fracking involves the use of chemicals in every step and these chemicals end up in the air, soil, ground water and hence contaminate water sources¹³²⁻¹³⁴. Some of these chemicals have been associated with adverse health impacts, and it is not surprising that a good proportion of these chemical pollutants are EDCs^{132,134}. A study was done in the US to test endocrine disruptor activity on a surface water body that had been used as a fracking waste disposal site. The results showed significant level of endocrine activity, including oestrogenic, androgenic, progesterone, glucocorticoid, and thyroid active compounds^{132,134}.

In 2002, the Department of Water Affairs and Forestry (DWAF) embarked on the National Toxicity Monitoring Program (NTMP) with the aim of assessing the health effects of toxic pollutants in the environment and to address the lack of scientific data with regards to toxic substances in South African water sources¹³⁵. The NTMP has so far managed to test for only 14 organic pesticides in selected sites as part of the phase three plan but the implementation of the monitoring program is yet to occur¹³⁵.

Whilst some of the pesticides are known EDCs, the program did not include the EDC criterion in their testing.

In the past few years the water industry in South Africa has also grown aware of water contaminants of the endocrine disruptor nature being present in water. In particular, the role of agricultural pesticides in contaminating water sources, causing a reduction in water quality and potentially affecting human health^{128,136}. As of 2012, there are 8256 pesticides registered in South Africa, and a large proportion of them have been identified as EDCs¹³⁷. Although there is a lack of pesticide monitoring programs in the country, it is clear that the use of pesticides causes contamination of the ground and surface water. These chemicals are able to contaminate the water sources through leaching, spray drift and runoff¹²⁸. This is particularly concerning because many communities do not have access to treated water and use collected source water for household-use including drinking. Also, there is poor management of some municipality water treatment facilities, causing risk of exposure from pipeline distribution¹²⁸.

A study was done in 2014 by the Water Research Commission (SAWRC) with the aim of addressing the lack of agricultural pesticide management^{128,136}. In the study water and sediment were analysed for pesticide contaminants and prioritising these pesticides for better monitoring purposes. In total 69 pesticides, were added onto this priority list^{128,136}. The criteria for pesticides to be selected for this priority list were endocrine disruption, carcinogenicity, teratogenicity, mutagenicity and neurotoxicity^{128,136}. It is important to point out that only oestrogenic and androgenic activity were tested for the EDC component. Prior to this study, a study was done in 2009 which looked at oestrogenic activity in drinking water from two rural communities³⁹. The authors reported presence of oestrogenic activity in the water and suggested that exposure to these EDCs may have been the cause for health effects that were seen in the community³⁹.

A number of assays have also been developed in the past 6 years with the aim of filling in the data gap that is apparent. In 2010 a toolbox of assays targeting the oestrogenic pathway was developed for South Africa⁴⁶. These include the YES assay T47D-Kbluc MCF7 proliferation assay (E-Screen) and the Juvenile fish VTG assay. Three were *in vitro* and one was an *in vivo* assay. In 2013, an *in vivo* assay

was developed focusing on thyroid disruption, called the *Xenopus* Metamorphosis Assay (XEMA)¹³⁸.

1.4.1 Chemicals of emerging concern

There is a group of micro-pollutants, which contaminate water sources at very low concentrations. They cannot be removed by traditional treatment plants, which are equipped to remove EDCs. These chemicals are called chemicals of emerging concern (CEC). These chemicals include persistent organic pollutants (POPs), BFRs, nanomaterials, veterinary medicine and pharmaceuticals and personal care products (PPCP)¹³⁹. Many of the CECs are endocrine disruptors.

PPCP include prescribed and non-prescribed drugs (pharmaceuticals) and soaps, cosmetics and sunscreens (personal care products). The main source of pollution of PPCPs is through wastewater discharge¹³⁹. Other routes of source and groundwater contamination include disposal of unused and expired drugs in landfills, runoff, agricultural effluent, pharmaceutical company discharge, and household discharge. Contamination of the water bodies have resulted PPCPs being found in drinking water¹³⁹. Both humans and animals are therefore at risk of being exposed to PPCPs. The effect of CECs is already being seen especially in developing countries where aquatic organisms are reported to be exhibiting endocrine disorders, and also the presence of these CECs is being reported¹⁴⁰. The lack of adequate treatment plants in developing countries also exacerbates this situation and implicates water quality.

Because pharmaceuticals are developed to elicit effects in the body, exposure to the chemicals in water, therefore, have a potential to cause a biological effect. The human health effects of PPCPs have not been well studied, but it is reported that they are bio-accumulative, bioactive and persistent¹⁴⁰. Although PPCPs are found at very low concentrations, EDCs have been shown to elicit effects at very low levels, and also their effects may be synergistic or additive if present in mixtures. There has also been concern over their chemicals' persistence and causing microbial resistance¹⁴⁰. There are a number of PPCPs that are TDCs and these include amitrole, mancozeb, benzophenone 2 and benzophenone 3.

Unfortunately, many people are not aware of the potential health risk to their endocrine system that may come from exposure to CECs and still continue to dispose of drugs in sinks and toilets.

Chapter 2: Motivation, aims and objectives

2.1 Background

The quality of drinking water is a major environmental health determinant.¹⁴¹ The water crisis is undeniable, an estimated 868 million people across the globe do not have access to clean and improved water supplies (basic water sanitation systems with no contamination)¹²⁵. About half of that population is in Sub-Saharan Africa^{125,141,142}. In Africa, this population translates to two out of five people without access to improved water supplies. Almost 185 million people worldwide rely on surface water for drinking water^{141,142}. Globally, the supply of safe water has been impeded by a number of factors which include the lack of implementation sustainable water supplies and sanitation, and water contamination¹⁴¹. The accessibility of safe drinking water reduces the associated health risks which may arise due to the contamination of water by infectious, toxic and radiological agents¹⁴². The contamination of water by toxic chemicals has been of high concern in the past decade and the potential adverse health risks thereof^{39,142-144}. Of particular concern, and for purposes of this study, is the presence of EDCs in drinking and surface water¹⁴⁴.

The contamination of water by EDCs implicates water quality. Because water is a widely used resource, contamination makes it a direct source, and at times a continuous source of exposure. The presence of EDCs in water is not a new occurrence, as some of the earliest studies on EDCs analysed exposure to aquatic animals^{19,20}. Since then, more studies have investigated the presence of EDCs in surface water, waste water, treated waste water and drinking water^{39,102,132,144,145}. These studies have determined the concentration and chemical composition of EDCs present, whilst others have analysed their effects on wildlife and humans. It is clear that South Africa is also not exempt from this contamination of water sources by EDCs^{39,102}. However, the full extent and effect of this contamination is not known due limited data.

2.1.1 Testing for TDCs in water

There are a number of assays which have been developed to assess TDCs in water. Some of these assays (both *in vivo* and *in vitro*) have been included in the GWRC toolbox II (Work Plan 3)⁴⁶. *In vivo* assays which can detect TDCs include the

watchfrog transgenic amphibian metamorphosis assay and the amphibian metamorphosis assays^{146,147}. These *in vivo* assays are able to give a holistic picture of the extent of TDCs because of the multiple endpoints which include development of vital organs and body systems (neurological, reproductive and metabolic), thyroid gland histology, limb and tissue growth¹⁴⁶. Amphibians e.g. *Xenopus laevis* tadpoles are exposed to different TDCs at varying concentrations for periods of time, then the effect of exposure is evaluated^{146,148}. However, because of legislation pushing for less *in vivo* studies due to ethical issues¹⁴⁹, there has been development of more *in vitro* studies.

In vitro studies which test for TDCs can be classified according to the endpoint. The first class targets TH biosynthesis e.g. thyroperoxidase (TPO) inhibition assay. This is a new *in vivo* assay looking at how TDCs can disrupt the formation of TH by interfering with the enzyme TPO¹⁵⁰. This disruption results in TH insufficiency and subsequent health effects¹⁵⁰. The second class targets TH transport. TDCs can bind to the transport protein e.g. TTR, because of their structural similarity to THs, hence causing competitive displacement of TH from the protein¹⁵¹. Several assays have been developed to analyse competitive displacement of TH on TTR protein by TDCs¹⁵¹. The TTR displacement assays in the GWRC Toolbox II⁴⁶ consist of three variations; ANSA, fluo- probe and luc-based. These assays measure the potency of TDCs in displacing the natural THs from TTR¹⁵¹.

The final class is the TR mediated action endpoint. Assays developed to test this endpoint assess the TDCs' ability to bind on TR causing agonistic or antagonistic effects^{85,152,153}. The resultant effect being either activation of the reporter gene or suppression in the respective cells⁸⁵. These assays include TR- β CALUX, TR- β GeneBLazer, T-screen and GH3.TRE.Luc^{85,153-155}. For this study, the GH3.TRE.Luc Reporter gene assay was used.

2.2 Problem statement

Human beings are exposed to complex mixtures of EDCs, voluntarily or involuntarily, throughout their life time²⁸. There is, therefore, a need to identify and test for potential endocrine disruptors in drinking water and water sources, and to determine the potential risk posed by these chemicals to humans and the environment²⁸. There

are several classes of EDCs, and this study focussed on thyroid disrupting chemicals (TDCs), which are EDCs that interrupt the thyroid hormone signalling pathway.

There is an apparent gap in literature with regards to the toxicological information on TDCs⁸⁵. Whilst the mechanism of action of some of the thyroid disruptors is well documented, the specific doses at which they induce biological effects have not been widely researched⁸⁵. There is currently no trigger value for TDCs in water which has been developed worldwide.

Over the years, research has focussed on the influence of EDCs on oestrogenic activity. As a result, the majority of the screening undertaken in South Africa is on compounds that disrupt the oestrogenic pathway. There is limited information available on thyroid activity in drinking ground and surface water. Information is available on the health effects due to the disruption of the oestrogenic pathway^{19,23}. However, there is a dearth of information on the disruption of the thyroid pathway, which may have related adverse health risks as the thyroid system is as equally vulnerable to EDCs. Several bioassays available on the thyroid activity do not clearly show relevance to water monitoring, and none have been validated outside the academic setting^{85,156,157}. Hence there is need for to validate and optimize an assay that is suitable to test for thyroid disruptor activity in water with relevance to water monitoring.

In the South African context, information pertaining to TDCs in source and drinking water is scarce. Some of the traditional water treatment plants are not equipped to remove EDCs from drinking water. Investigations need to be carried out to determine the efficiency of water treatment plants in removing thyroid disruptors, if present in the source and surface waters.

2.3 Aims and objectives

Aims

The main aim of this research study is to establish and optimise a thyroid assay, which can be used to assess thyroid activity in treated drinking and source water.

Objectives

1. To establish, optimize and validate the GH3.TRE.Luc thyroid bioassay to measure thyroid activity in water samples (Phase 1)
2. To apply the assay to water samples from the Global Water Research Coalition (GWRC) Toolbox II and III (Phase 2).
3. To use the optimised GH3.TRE.Luc thyroid bioassay to assess thyroid activity in field samples of source and treated water samples from a Water Treatment Plant in Gauteng (Phase 3).

2.4 Hypothesis

It was hypothesised that the GH3.TRE.Luc reporter gene assay would be able to detect thyroid activity from reference compounds in a dose responsive manner as reported in literature, and that thyroid activity would be detected in water extracts.

Chapter 3: Materials and methods

Whilst there are several assays targeting several endpoints at the thyroid signalling pathway, for this study, the GH3.TRE.Luc reporter gene assay was used. It is both a transfection and gene regulation *in vitro* assay expressing the TR α and TR β . The assay was chosen because the main mechanism of action for TH and EDCs reported thus far is the regulation/interference of gene expression through the TR. The pituitary is also the central gland in the HPT axis which controls the production of THs by the thyroid gland and their interaction with other hormones; hence the pituitary cell line was the best for detecting thyroid hormone receptor disruptors.

Reporter gene bioassays have the ability to detect low levels of hormonal activity due to environmental stimuli. A reporter gene assay was hence the best tool for the establishment, optimisation and validation of an assay that is sensitive for thyroid activity.

3.1 Establishment of the assay

3.1.1 Luciferase reporter gene assay using GH3.TRE.Luc cell line

Rat pituitary cells (GH3) were used for the assay. The thyroid hormone responsive rat pituitary tumour GH3 cell line was a kind gift from Professor AJ Murk from Wageningen University (The Netherlands). The cell line was developed by a stable transfection of the luciferase reporter pGL4CP-SV40-2xtaDR4 construct into the GH3 cells from the sensitive GH3.TRE.Luc cell line.⁸⁵ The transfect has two thyroid receptors TR α and TR β , making it more robust⁸⁵.

The method for the assay was adapted from Freitas et al (2011), with modifications from Mengeling et al^{85,153}. The use of the original method and the modifications allowed for the establishment of a working assay. The cells were grown in a regular growth medium (RGM). The RGM was (1:1) (v/v) of Ham's F12 nutrient medium and Dulbecco's Modified Eagle's Medium (DMEM/F12), (Life Technologies cat no. 31330095). The DMEM/F12 was supplemented by 10% foetal calf serum, which gave the cells enough nutrients to grow. Culture conditions were as follows: 37°C, 5% (v/v) CO₂ and humid atmosphere. 75 cm² flasks was used for sub-culturing thyroid cells, and every four days cells were released from the flask by adding 0.1% (w/v) trypsin. The cells were observed under a microscope for minutes as they

dislodged and after 3 minutes the excess trypsin was removed. Trypsin dislodged the cells from the flask by breaking down adherence proteins which facilitate attached of cells to the flask. Cells were dislodged by gently tapping the flask against the palm of the hand a few times, then regular growth medium was added into the flask to neutralize trypsin before splitting the cells into new flasks.

Forty-eight (48) hours prior to plating into the 96 well plate the cells were seeded at 80% confluency (4/5 of a 100% confluent flask) in a 75 cm² culture flask. Regular growth medium was added into the flask and cells were incubated. At 80% confluency, cells were seeded into a white clear bottom 96 well plate at 5x10⁴ cells per well (100 µl per well). Prior to seeding the cells, RPM was put in the 96 well at 100 µl/well. After 24 hours RGM was replaced with 100 µl/well of the PCM medium and incubated for a further 24 hours to deprive cells of thyroid hormones. The serum free perfusion medium (PCM) was prepared by making 1000x stock concentrations of bovine insulin, ethanolamine, sodium selenite, human apotransferrin, bovine serum albumin and fetuin in double distilled water, and diluting the stock concentrations in DMEM/F12 (1:1) with 15 mM HEPES to obtain the following final concentrations: 10 µg/mL bovine insulin (Sigma cat no I6634-100mg), 10 µM ethanolamine (Sigma cat no E0135-100ml), 10 ng/mL sodium selenite (Sigma cat no S5261-10g), 10 µg/mL human apotransferrin (Sigma cat no T1147-500mg), 500 µg/mL bovine serum albumin (Sigma cat no A7906-100g), 800 µg/mL fetuin (Sigma cat no F3385-1g) (only added if the cells struggle to attach/grow). The medium was filter sterilized with 0.2 µm filters.

The stock solution for the controls and the test chemicals (listed below) was prepared in dimethyl sulfoxide (DMSO) at least 200x the highest required final test concentration (to ensure that when diluted in PCM the DMSO concentration was above 0.5%). The test chemicals were, T₃ (Sigma cat no T2877-250mg) 1 000 nM, T₄ (Sigma cat no T1775-100mg) 1 000 nM, amiodarone hydrochloride (Sigma cat no A8423-1g) 15 µM, Sodium arsenite (Sigma cat no S7400-100g) 100 µM. Stock solutions were stored at room temperature in the dark to avoid light sensitivity reactions.

All the test chemicals (prepared in DMSO) were diluted in PCM and transferred to each well at 100 µl (100 µL final volume). PCM was used because it contains no

FBS which may have endocrine activity. The DMSO concentration must never exceed 0.5% to avoid cytotoxicity to cells. Test chemicals were tested alone and in the presence of 0.25 nM T₃. Each plate contained a full T₃ standard curve (ranging from 0.001 – 1000 nM) and vehicle control. Single plates were first tested as part of the screening process for 24 hours. After analysis of the screening plates, samples with relative light units/relative fluorescence units (RLU/RFU) above the detection limit were exposed in triplicates for 24h (full dose response curves). Table 2 shows an example of a template for the 96 well plates, and how the samples were diluted and exposed in the plate. The plate included three vehicle control samples and a standard curve for T₃ with seven to eight dilutions. It included the samples which were tested, in five serial dilutions, to test for THRD activity. To test for anti-THRD activity, the same samples were co-incubated with 0.25 nM T₃, to assess their ability to suppress T₃ induction of the cells. The T₃ concentration (0.25 nM) was maintained whilst the samples were tested in five serial dilutions.

Table 2: An example of a template of the 96 well plate for both the luciferase and resazurine assays

Controls			T ₃ Standard Curve (nM)						
Vehicle Control	Vehicle Control	Vehicle Control	0.001	0.01	0.1	1	10	100	1000
Testing for THRD activity (Samples)					Testing for anti-THRD activity (Samples + 0.25 nM T ₃)				
S1 (1)	S1 (1:10)	S1 (1:100)	S1 (1:1000)	S1 (1:10000)	S1+T ₃ (1)	S1+T ₃ (1:10)	S1+T ₃ (1:100)	S3+T ₃ (1:1000)	S4+T ₃ (1:10000)
S2 (1)	S2 (1:10)	S2 (1:100)	S2 (1:1000)	S2 (1:10000)	S2+T ₃ (1)	S2+T ₃ (1:10)	S2+T ₃ (1:100)	S2+T ₃ (1:1000)	S2+T ₃ (1:10000)
S3 (1)	S3 (1:10)	S3 (1:100)	S3 (1:1000)	S3 (1:10000)	S3+T ₃ (1)	S3+T ₃ (1:10)	S3+T ₃ (1:100)	S3+T ₃ (1:1000)	S3+T ₃ (1:10000)
S4 (1)	S4 (1:10)	S4 (1:100)	S4 (1:1000)	S4 (1:10000)	S4+T ₃ (1)	S4+T ₃ (1:10)	S4+T ₃ (1:100)	S4+T ₃ (1:1000)	S4+T ₃ (1:10000)
S5 (1)	S5 (1:10)	S5 (1:100)	S5 (1:1000)	S5 (1:10000)	S5+T ₃ (1)	S5+T ₃ (1:10)	S5+T ₃ (1:100)	S5+T ₃ (1:1000)	S5+T ₃ (1:10000)

Key S = Sample

The two assays, luciferase and the resazurine, were done on the same plate, as the resazurine assay does not require cell lysis and does not interfere with the luciferase activity measurements.

Luciferase activity

To test for metabolic activity (gene response), the luciferase reporter gene assay was using the luciferin reagent, beetle luciferin, potassium salt (Anatech cat no. PRE1603). The prepared luciferin (159.21 mg in 500 mL distilled water) reagent was divided into aliquots and stored at -70°C , covered in foil and protected from light. For use, luciferin reagent was removed from the freezer and equilibrated to room temperature. The cells were rinsed twice with PBS, 25 μL of 1x lysis reagent was added to each well in order to lyse the cells before adding the luciferin reagent. The lysis reagent, a reporter lysis buffer (Anatech cat no. PRE3971) was prepared by adding 4 volumes of water to 1 volume of 5x reporter lysis buffer. After lysis, the 96 well plate was placed into a luminometer that has an injector. The luminometer (LUMIstar OPTIMA luminometer, BMG Labtech, Offenburg, Germany) was programmed to add 25 μL luciferase assay reagent and 25 μL reaction buffer per well and to read immediately for 10s. Relative light units (RLUs) were obtained from the luminometer, and after cytotoxic adjustment (RLU/RFU), were used to determine thyroid activity. A sample was considered to have thyroid activity if it had an RLU/RFU (measure of TDC activity after cytotoxic adjustment) value above 2 standard deviations from the control (DMSO).

Resazurine cell proliferation assay for cytotoxicity

Cell proliferation and viability was determined by measuring the cells metabolic activity using resazurine dye. Resazurine is non-toxic, water soluble and non-radioactive dye. It can be reduced by the viable GH3 cells to a pink fluorescent colour, from the original blue, making a resorufin complex. After 24h exposure, 8 μL resazurine (400 μM) was added to each well and incubated in the dark for 4h (at 37°C , 5 % CO_2). To prepare the resazurine (Sigma cat no R7017-5g), a 400 μM solution in PBS (pH 7.4) was prepared. The resazurine solution was then filter-

sterilized through a 0.2 μm filter and aliquoted in Eppendorf tubes, covered with foil to protect it from light and stored at -20°C . Fluorescence was measured at 530 nm excitation and 590 nm emission. If the cells were exposed to a cytotoxic chemical, the resazurine was not reduced to resofurin, and remained in its blue colour. The extent of its cytotoxicity was then measured by the Flouroskan[®]. A chemical was considered cytotoxic if the fluorescence is less than the fluorescence of the vehicle control minus 3x the standard deviation.

3.2 Phase 1: Optimisation and Validation

Four reference chemicals from the laboratory and eighteen (18) GWRC water samples were used for the optimisation and validation of the GH3.TRE.Luc reporter gene assay. The four reference chemicals were T₃, T₄, amiodarone and sodium arsenite. Of the 18 GRWC samples 9 were reference chemicals [(T₃ (Sigma cat no. 6893-02-03), T₄ (Sigma cat no. 51-48-9), triac (Sigma cat no. 521-2-1), tetrac (Sigma cat no. 67-30-1), amiodarone (Sigma cat no. 19774-82-4), pentachlorophenol (Sigma cat no. 87-86-5), ethylene thiourea (Sigma cat no. 96-45-7), 2,2,4,4-tetrahydroxybenzophenone (Sigma cat no. 131-55-5) and methimazole (Sigma cat no. 60-56-0)], which are known thyroid hormones, antagonists and agonists. The agonists were diluted into concentrations of 0.0001 – 1000 nM, and added into the 96 well plates using the protocol discussed under 3.1. The antagonists were diluted into concentrations of 0.01 – 100 mM and co-incubated with 0.25 nM T₃. These samples were used to optimize and validate the assay, by comparing the responses to the ones in literature. The agonists used were T₃ and T₄ natural hormones with their acetic acid derivatives and structural analogues triiodothyroacetic acid (triac), (Sigma cat no. 51-24-1) and tetraiodothyroacetic acid (tetrac), respectively. Triac and tetrac are both pharmaceuticals that have been used in hormone replacement therapy⁸⁵.

Eight (8) water extracts from the GWRC were used to check the suitability and sensitivity of the assay to detect TDCs in water samples. The water samples were paired samples of surface, waste, milliQ (double distilled water), tap water samples, and a control. Of each pair of the eight samples, one was spiked with T₄ (20 mM) and ethylene thiourea (20 mM) before we received it at the laboratory, making the

analysis a blind test, as the ID of the spiked sample was not shared during testing. This was done to remove bias.

3.3 Phase 2: GWRC water samples

Twenty-four (24) GWRC water samples were additionally tested for thyroid activity using the GH3.TRE.Luc reporter gene assay and the resazurine assay. After the thyroid cells were seeded into the 96 well plates, the water extracts were added into the plate at varying concentrations and following the luciferase and resazurine protocol, were analysed for THR activity. The countries which provided water samples for the study were South Africa, Germany, Netherlands, France, Spain and Australia. The water samples were grouped into milliQ water, tap water, surface water, and treated waste water, and some of the samples were spiked blindly. The GWRC water samples were tested as part of the GWRC toolbox II and III which is an extension of the toolbox I⁴⁶. The EDC toolbox II, was set out by the Global Water Research Coalition to address this lack of information. The overall aim of the toolboxes is to introduce and validate other biological methods that test for androgenic, progestagenic, glucocorticoid, retinoid (RXR), peroxisome proliferator (PPAR) and thyroid activity in environmental waters. This also allowed for analysis of different matrices of water samples on a global scale.

3.4 Phase 3: Water Samples from a water treatment plant - Gauteng

The water samples used were from the Rand Water catchment area, including the Vaal Dam. The dam is one of the largest in South Africa bordered by three provinces Gauteng, Free State and Mpumalanga. Four sampling sites were used all in Gauteng at a water treatment plant (WTP). The drinking water (DW) A and B sites were treated drinking water coming from the plant into the distribution network. Source water (SW) A and B were source/surface water sites. SW A is a site at the dam wall close to the treatment plant, whilst SW B goes into the WTP and comes out as DW A or B once treated.

The reason for the selection of these sites was to have source and treated water samples from the same water treatment plant, in order to determine if there were thyroid disruptors in the source water, and if the water treatment plant was able to

remove these thyroid disruptors. The sampling was done monthly over a period of 12 months, in order to monitor if there are any trends due to seasonal patterns.

Sample Collection

The water samples were collected in 1 L Schott bottles, and were delivered to the laboratory on the same day. After collection the pH was adjusted to pH 3 using hydrochloric acid and then filtered using a 0.45 µm filter and glass wool. The pH was adjusted in order to stop any biological activity in the water samples, which may include small water organisms or plant/algae growth. The 0.45 µm filter removes any materials that may clog the filters during the extraction procedure. All the samples were stored at 4°C in the dark until use.

Water extraction

The water extraction method was according to the GWRC guidelines from Toolbox 2⁴⁶. All 1 litre samples were concentrated using the solid phase extraction (SPE) method. The Oasis HLB reversed-phase SPE cartridges (Oasis HLB glass cartridge 5cc/200mg LP, Microsep) were preconditioned using 2 X 2.5 mL methanol, acetonitrile, acetone and water consecutively. This was done to activate the sorbent material in the cartridges so they could retain the EDCs. This SPE method ensures the adsorption of polar and organic chemicals which include EDCs into the cartridges¹⁵⁸. After the preconditioning the water samples passed through the cartridges on a vacuum manifold set with a flow rate of about 7mL/minute. If EDCs were present they would remain in the cartridges as water passed through. The cartridges were then eluted to collect the EDCs from the cartridges. Elution (into glass tubes) was done by passing 3 mL of each of the methanol, acetonitrile and acetone consecutively. The eluate was then concentrated by evaporating the solvents using a steady stream of nitrogen gas. DMSO (500 µL) was used to reconstitute the sample for analysis. Figure 6 is a flow diagram of the extraction method.

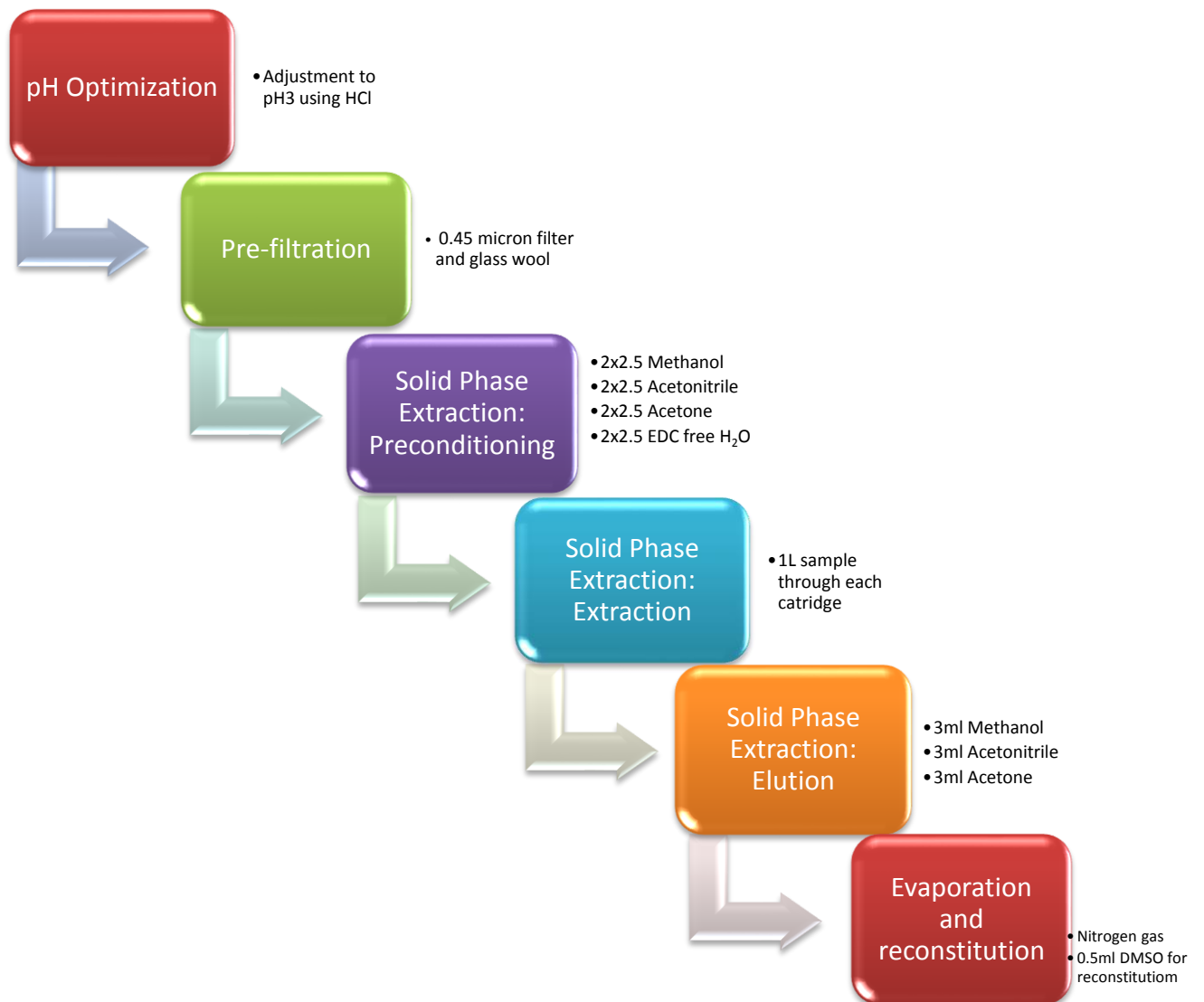


Figure 6: Flow diagram of the extraction process for water samples from the WTP

After reconstitution the samples were diluted with PCM into 4 concentration ratios and applied to the cells in the 96 well plates following the protocol under 3.1. Analysis of THR activity and cytotoxicity was done using the luciferase and resazurine assays.

3.5 Data analysis

A T_3 standard curve was constructed for each assay. Cell proliferation was expressed relative to the maximum response observed at 10nM T_3 , set at 100% induction. The response for the solvent control was set at 0%. The EC50 or IC50 for the compound was calculated determining the concentration at which 50% of the maximum cell proliferation occurs. The cumulative fit equation was used to reach 50% of the maximum decrease of cell growth in the presence of T_3 . The relative potency (RP) of compounds was calculated by dividing the EC50 of T_3 by EC50 of the test compound.

For environmental samples concentrations were calculated from the T_3 standard curve as unpaired Y-values and corrected for the appropriate dilution factors to determine the equivalent value for the original sample TEq in ng/L. Graphpad Prism Software® was used to obtain dose response curves and extrapolate RLU/RFU values from the standard curves. The main statistical tests done were Analysis of Variance (ANOVA) and general linear regression models. For the test chemicals, the ANOVA was used to determine if there was any significance difference between the chemicals, concentrations of individual chemicals and also to test the interaction between the chemicals and its concentrations. A regression model was also done on these 3 variables. For the GWRC, both the ANOVA and the linear regression model were done on the water source, country and concentration variables. The interaction variables tested were source and country, source and concentration and, country and concentration. The water samples from the WTP were tested using the ANOVA and the regression model on the following variables: concentration, months, site and interaction variable between concentration and site. Only those variables which either showed a significant difference ($p \leq 0.05$) or were worth explaining with relevance to the study were reported.

3.5.1 Quality control and quality assurance

Before the commencement of the study, training was done on how to handle the samples and run the assays. Water samples were collected and pH was adjusted on the same day.

To reduce assay errors, all the assays were run concurrently with an extraction control and a vehicle control (DMSO only). An extraction control is a control sample that was only preconditions and eluted with no water sample run through it. This was done to test the cartridges, and the solvents for any contamination. The vehicle control was a DMSO only sample to test for any contamination in the DMSO.

The positive samples we repeated three times on different plates to reduce error, increase accuracy and test for repeatability. Statistical analysis was done on the final results correct for errors in the final interpretation.

Equipment calibration

Equipment used the project was checked and was functioning properly prior to starting the project. All instruments (Flouroskan®, LUMIstar Omega® and the MilliQ® water purification system) used on the assay were calibrated before the project was started.

Chapter 4: Results

4.1 Modification of the assay

The method that was initially used to culture the cells and expose them was according to Freitas et al⁸⁵. However, the dose-response-curves obtained for GH3.TRE.Luc induction were not optimum or repeatable. Some dose response curves were not obtainable using Graphpad Prism[®]. After troubleshooting, it was discovered that this was because the cells were not attaching in the wells after seeding and were lost during the lysis step when washed with PBS. Figure 7 shows one of the curves for thyroid hormone receptor (THR) activity induced by exogenous T₃ using the original method.

T₃ Curve Agonistic Activity

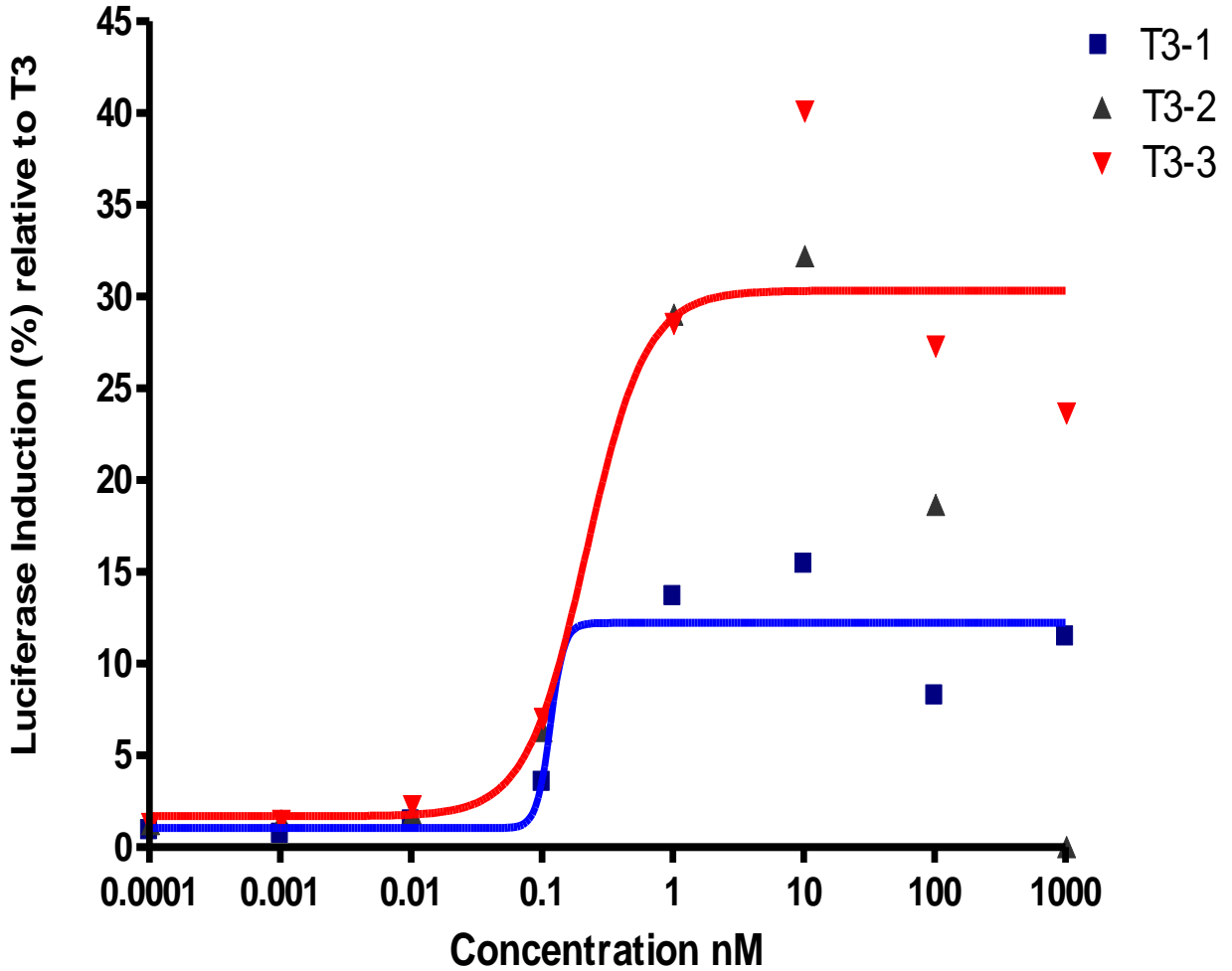


Figure 7: Graph showing GH3.TRE.Luc induction following T₃ exposure for 24 hours (0.0001 – 1000nM) using the original method. The curves were not producing optimum induction and were not repeatable.

After troubleshooting, fetuin (used in the laboratory to assist in the attachment of cells to the plate) was incorporated into the method to aid in the attaching of the cells in the 96 well plates. The addition of fetuin resulted in more cells attaching and less cells being lost during the lysis step when the cells were washed with PBS. Figure 8 shows the T_3 curves for thyroid hormone receptor (THR) activity after 24 hours of exposure with the incorporation of fetuin.

T₃ Curve Agonistic Activity (with fetuin)

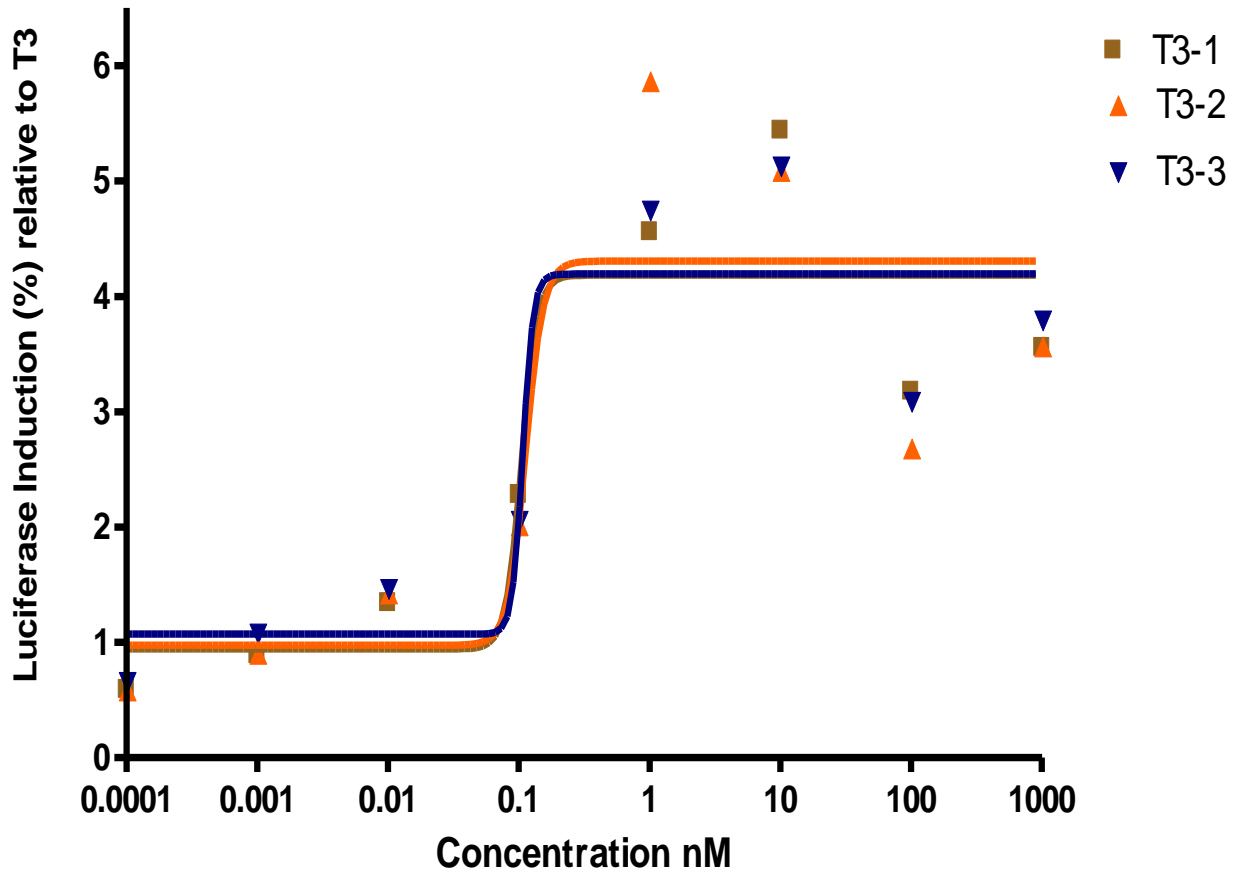


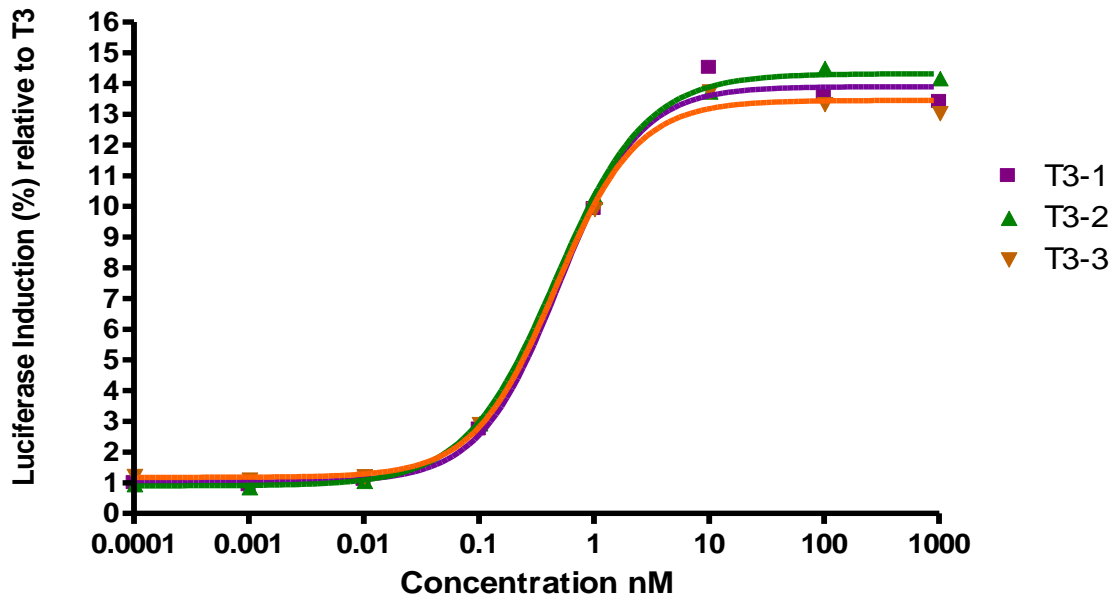
Figure 8: Graph showing GH3.TRE.Luc induction following T₃ exposure for 24 hours (0.0001 – 1000nM) using the original method and the incorporation of fetuin. Repeatability increased.

Whilst repeatability increased upon addition of fetuin, the dose response curves still did not show optimum GH3.TRE_Luc induction and there was high background activity as a result of using the fetuin, as seen from figure 8.

Further troubleshooting was done, and modifications to the original method were done using information from Mengeling et al¹⁵³ and from a number of trials done in the laboratory. The modifications were as follows; Instead of using a cell scraper to harvest the cells for seeding, the trypsin was used, as the cells scraper may have damaged the cells, resulting in failure of the cells to attach in the 96 well plate. The cells were seeded using the RGM to increase their chance of attaching. The withdrawal step using PCM was done 24 hours after the cells had been seeded (in RGM), in the 96 well plates. After seeding, every step in the method maintained a 24-hour period. The cells seeded per well were increased from 30000/well to 50000/well. After this the original method was followed, and a Luminometer was used to read the plates. The use of fetuin was discontinued, due to the high background that was observed. Figure 9 shows the dose response curves obtained after these modifications.

A

T₃ Curve Agonistic Activity Modified Method



B

T₄ Curve Agonistic Activity Modified Method

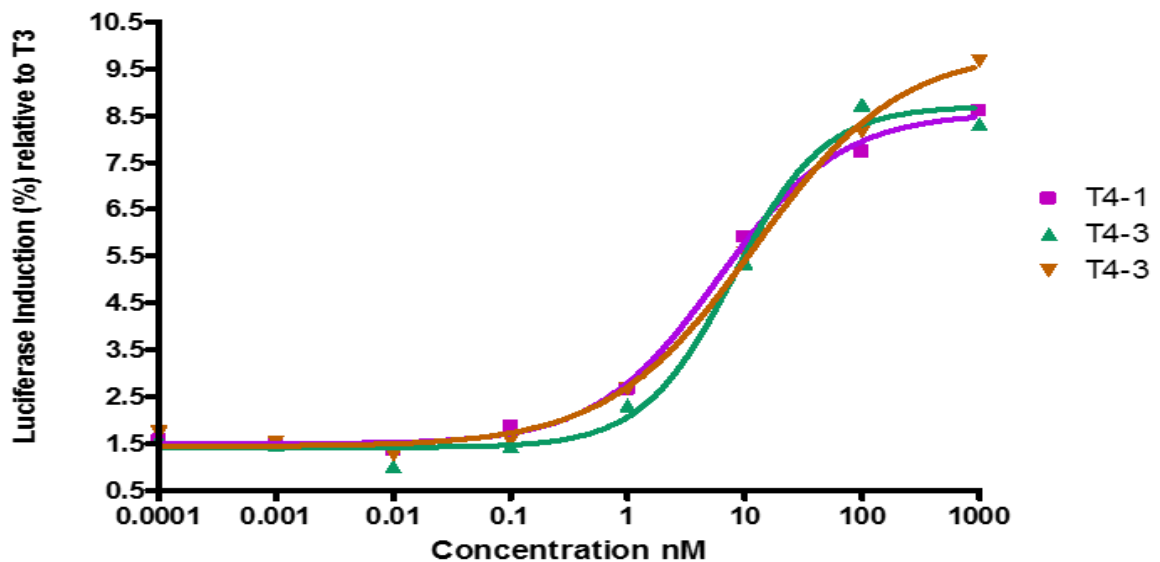


Figure 9: Graph showing GH3.TRE_Luc induction following A) T₃ exposure for 24 hours (0.0001 – 1000nM) using the modified method B) T₄ exposure for 24 hours (0.0001 – 1000nM). Both graphs had increased repeatability and optimum induction. The fold induction was more than 10 for both graphs, showing low background levels.

The modification method resulted in dose response curves which were comparable to literature and repeatable. The original method used the Promega kit (E1500) for luciferase activity, however after comparing with the laboratory luciferase kit, there was no differences between the dose response curves. The laboratory kit was therefore used in place of the Promega kit as it was more cost effective whilst producing the same results.

The luciferase assay was used to test for metabolic activity, which was reported as thyroid hormone receptor activity (THR activity), and the resazurine assay was used to test for cytotoxicity, which was reported as cell proliferation. For THR activity, the results were expressed as a RLU/RFU, calculated from the relative light unit (RLU) and relative fluorescence unit (RFU) readings, for luciferase assay and for the resazurine assay respectively. This was done to normalize for viable cell number. Cytotoxicity/cell proliferation using the resazurine assay was measured using RFUs.

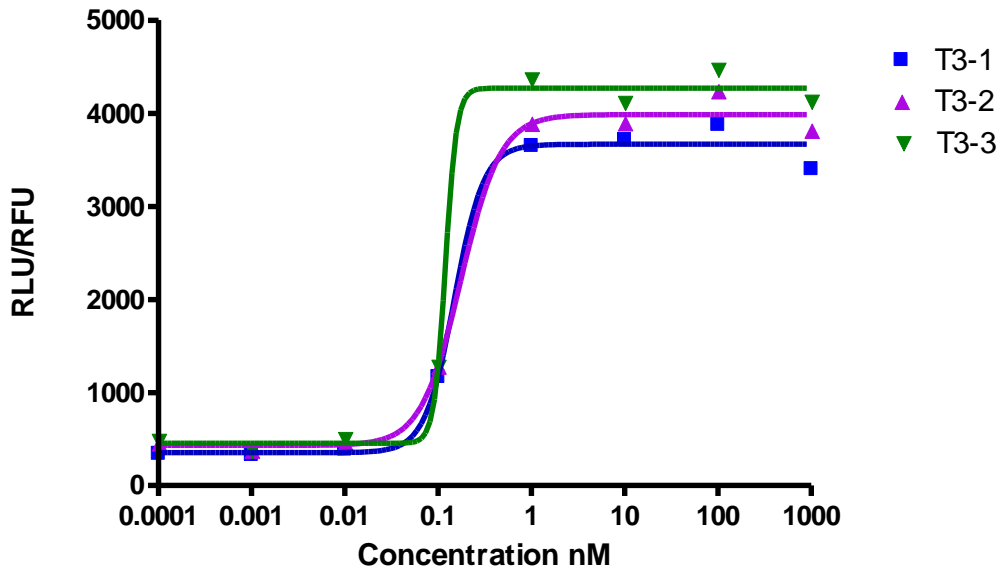
4.2 Phase 1: Optimisation and validation

Test compounds

For optimisation of the assay, exogenous natural hormone triiodothyronine (T_3) was used as the positive control. The capability of the assay was confirmed by its capability to detect T_3 at the picomolar range, with dose response curves comparable to those found in literature⁸⁵. Figure 10 shows the T_3 curve (in triplicates) for THR activity and the results from the resazurine assay respectively.

A

T₃ Curve Agonistic Activity



B

T₃ Cell proliferation

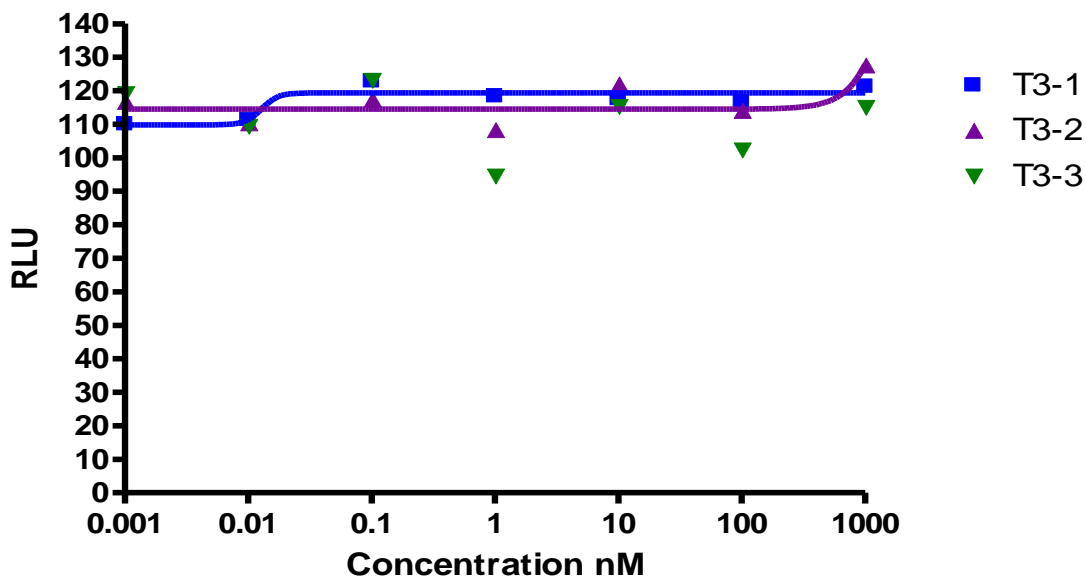


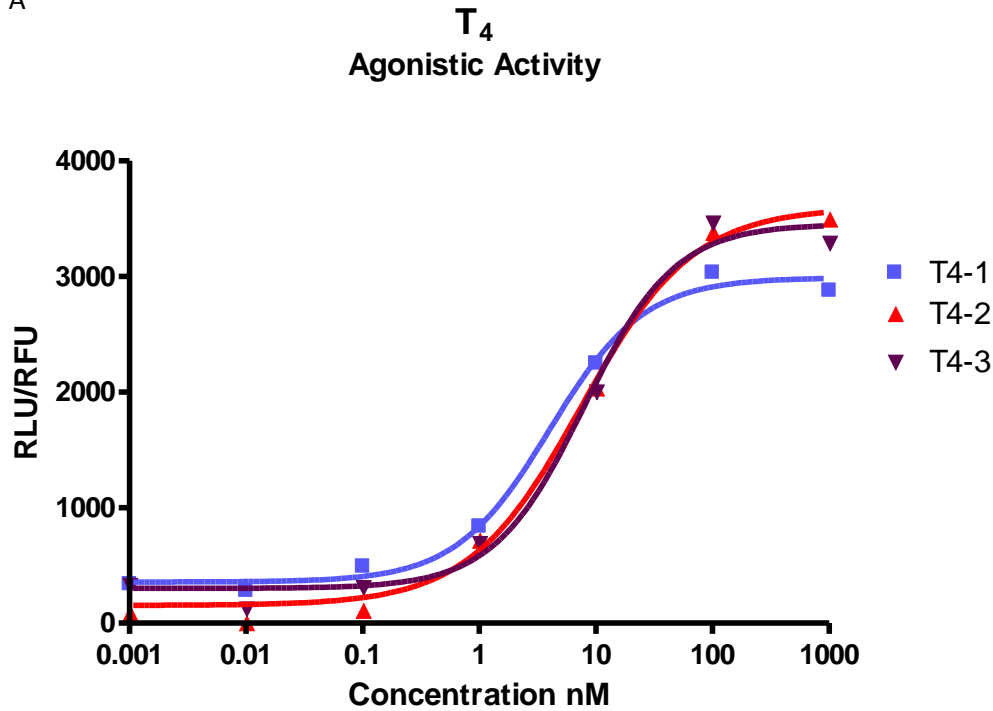
Figure 10: Graphs showing the T₃ activity in the luciferase and resazurine assays, with concentrations in nM ranging from 0.0001 to 1000. A) The graph illustrates GH3.TRE.Luc induction of cells after 24-hour exposure to T₃. B) Graph showing the effect of T₃ on the cell proliferation after incubation with resazurine to test for cytotoxicity.

A sigmoidal curve fit was obtained for the T₃ curve, validating thyroid hormone activity. The results were comparable to those found by Freitas *et al* (2011)⁸⁵. The average EC₅₀ from the triplicate T₃ tests was 0.15. The relative fluorescence Units (RFU) was within 3 standard deviations of the vehicle control (DMSO)

In the resazurine assay (cell proliferation), all the tested T₃ concentrations were within 3 standard deviations of the vehicle control (DMSO), indicating no cytotoxicity.

The agonistic behaviour of exogenous T₄ was also tested and results were comparable to literature. Figure 11 shows the luciferase and resazurine assay results.

A



B

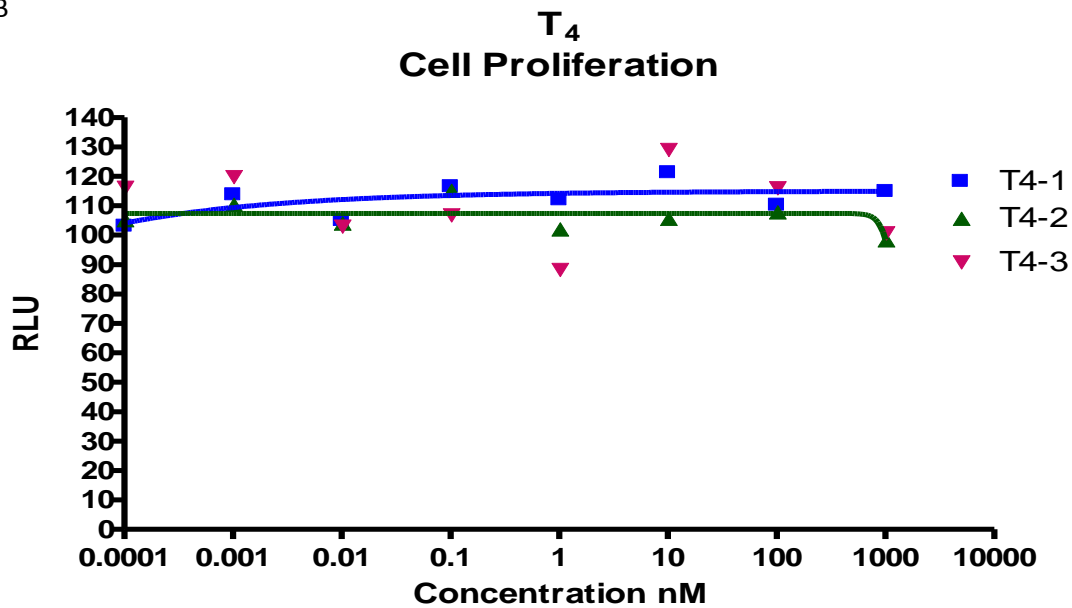
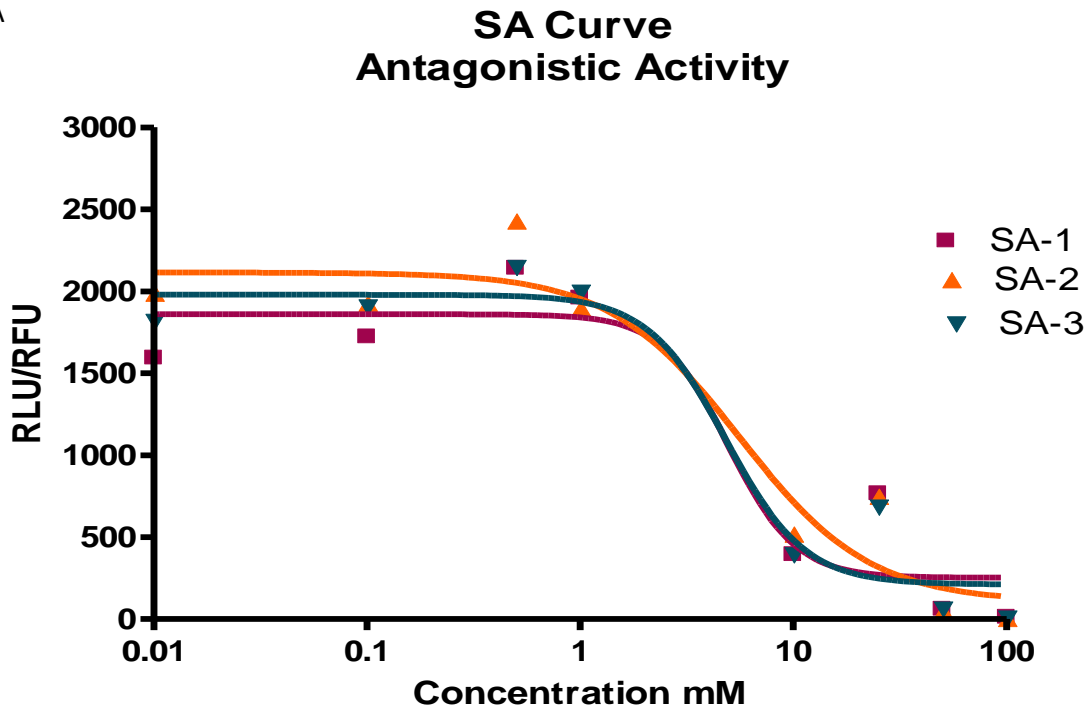


Figure 11: Graphs showing the T₄ activity in the luciferase assay (Induction) and resazurine assays (cell proliferation), with concentrations in nM ranging from 0.0001 to 1000. A) The graph illustrates the effect of T₄ to the GH3.TRE.Luc cells after 24-hour exposure. B) Graph showing the effect of T₄ on the cells after incubation with resazurine to test for cytotoxicity.

Sigmoidal dose response curves were also obtained for T₄ curve. Average EC₅₀ from the triplicates tests was 6.4 nM. There was no cytotoxicity as the T₄ concentrations were within 3 standard deviations of the vehicle control (DMSO).

As controls for anti-thyroid activity (antagonists), the two proposed chemicals were sodium arsenite (SA) and amiodarone hydrochloride (AH). These were co-incubated with 0.25 nM T₃ for 24 hours and tested for antagonistic activity (suppression of T₃ THR activity) and cytotoxicity. Figure 12 and 13 shows the graphical illustrations of the luciferase and the resazurine assays

A



B

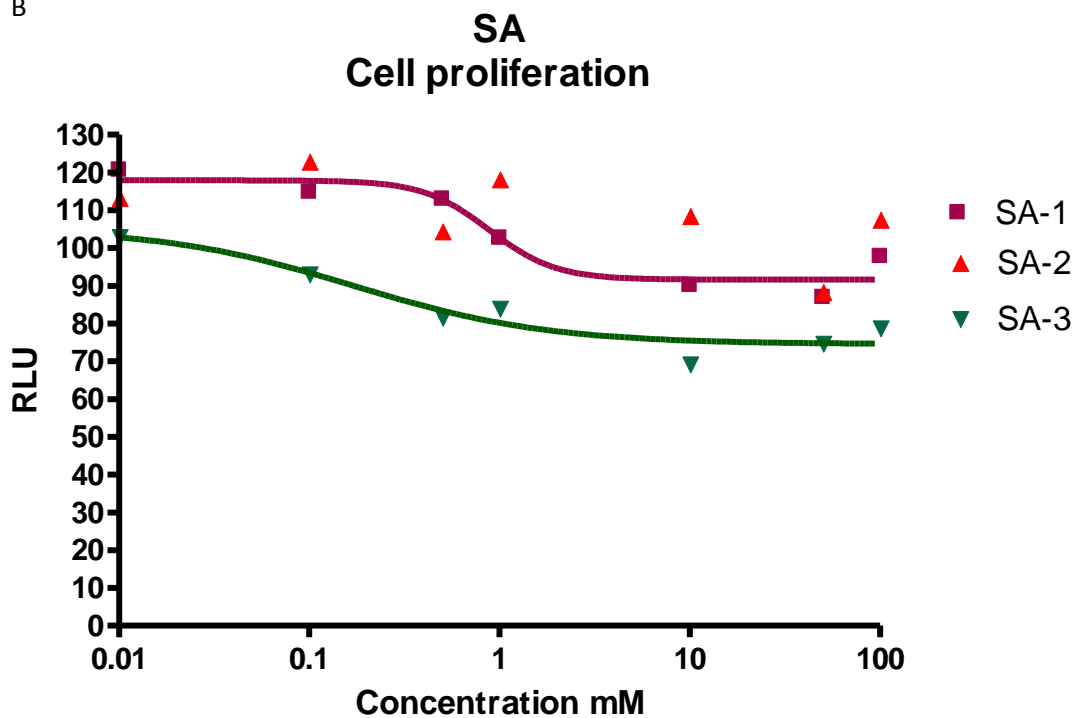


Figure 12; The graphs shows the effect on the GH3.TRE.Luc cells over a range of sodium arsenite (SA) concentrations (0,01 to 100mM) after a 24 hour co-inubation with 0.25nM T₃. A) High THR activity is visible at the lower concentrations and a reduction in activity at 1 uM. B) The resazurine assay shows all the values are within 3 standard deviations of the vehicle control (VC).

The high activity detected at the lower concentrations can be attributed to the 0.25nM T₃, as the concentrations of the SA increases, its anti-thyroid properties are reflected through the decreased THR activity. The average EC₅₀ of SA from the curves was 5.13 mM. No cytotoxicity was seen. Figure 13 shows the luciferase and resazurine assay results for AH.

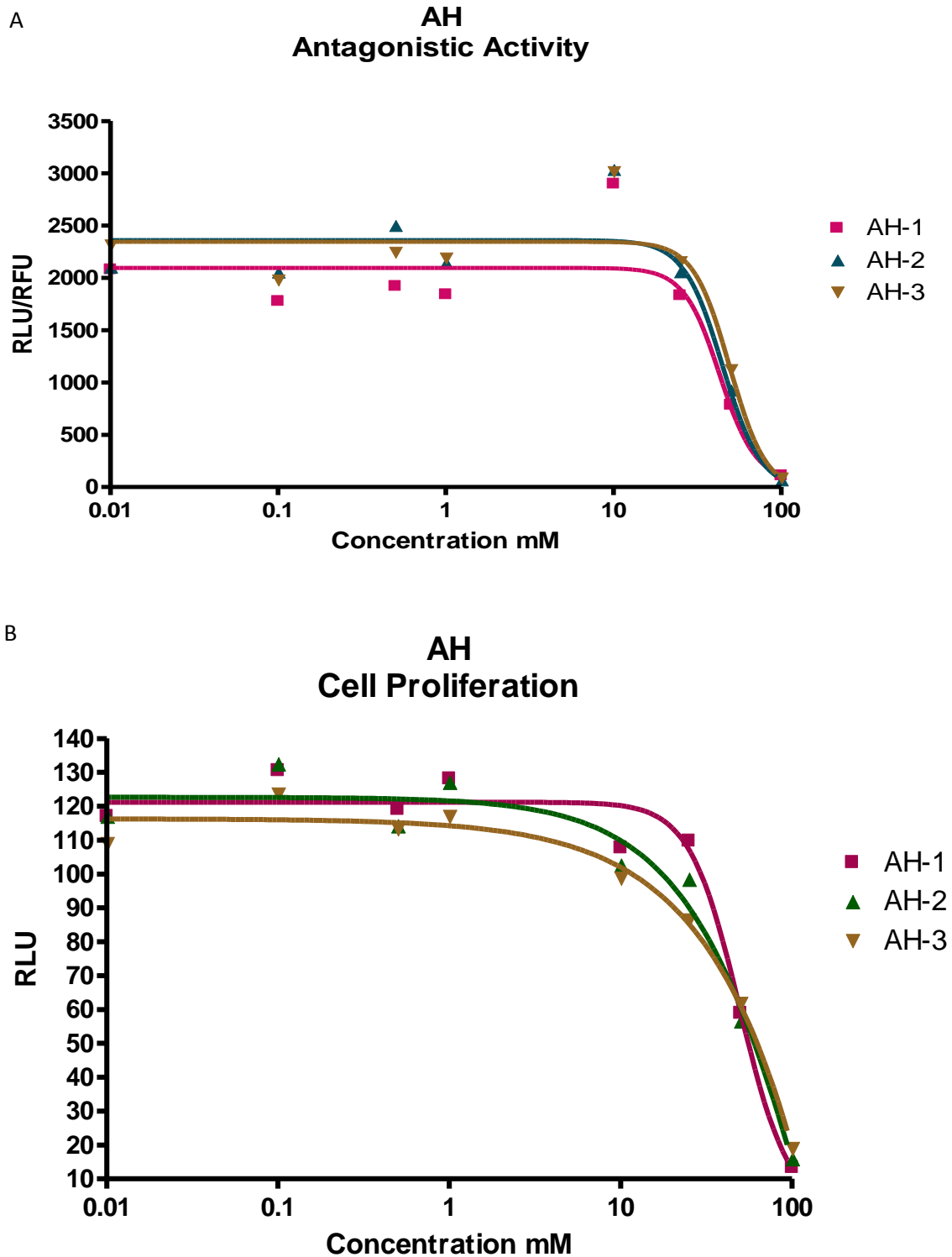


Figure 13: The graphs illustrate the effect of amidarone hydrochloride on the GH3.TRE.Luc cells when incubated with 0.25nM T₃. A) The graph shows a decreased THR activity from 10uM of AH. B) The resazurine assay showed no cytotoxicity from 0.01uM to 25uM, after which there is clear loss off cell viability, indicating cytotoxicity.

The luciferase assay showed induction of the GH3.TRE.Luc cells from AH concentrations of 0.01 to 100mM, this can be attributed to the 0.25nM T₃. After 10mM activity started to decrease. On the resazurine assay no cytotoxicity was detected until at 25uM where there is a clear decrease in cell viability. The resazurine in this case showed that the decrease in activity in the luciferase assay after 25 nM was as a result of cytotoxicity rather than anti-thyroid hormone receptor activity. Hence it was decided to rather use SA as a control for anti-thyroid hormone receptor activity, and the importance of the resazurine assay became apparent. The average EC50 for AH was 45.69 mM.

Optimisation and validation using samples from GWRC

The nine (9) test chemicals from the GWRC were also used to optimise the assay. Four of the samples were tested for agonistic activity; T₃, T₄, triac (T₃ analogue) and tetrac (T₄ analogue). Five chemicals were tested for antagonistic/antithyroid activity and there were; amiodarone hydrochloride (AH), pentachlorophenol (PCP), ethylene thiourea (ETU), 2,2,4,4-tetrahydroxybenzophenone (THBP) and methimazole. Antagonistic activity was determined by the chemicals' ability to suppress the THR activity of 0.25 nM T₃. Figure 13 shows the dose response curves obtained from the four GWRC agonists tested.

GWRC Test Chemicals Agonistic Activity

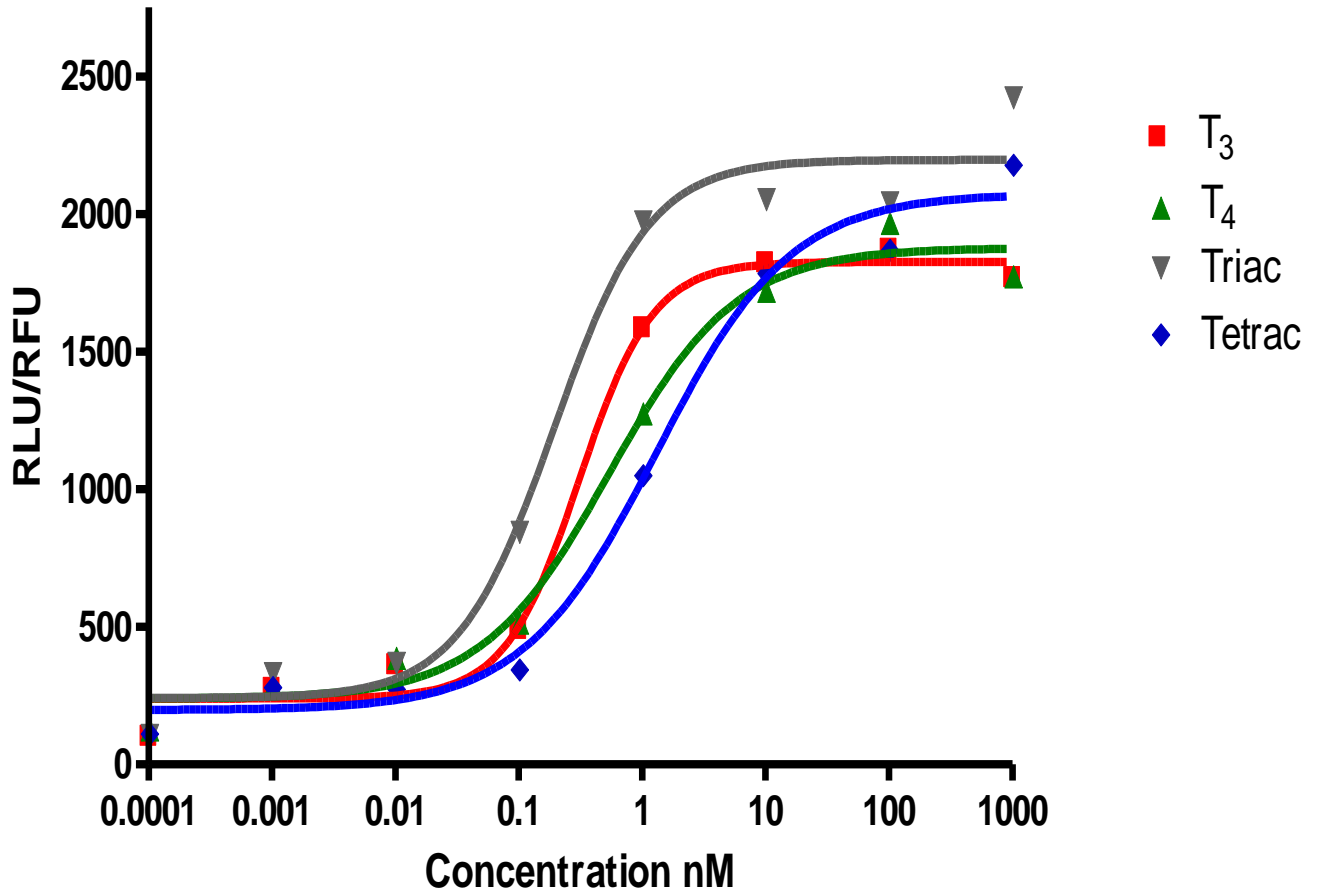


Figure 14: The graphs show GH3.TRE.Luc induction at concentration 0.0001 to 1000 nM of the agonists T₃, T₄, triac (T₃ analogue) and tetrac (T₄ analogue) after 24 hours.

The agonists were able to induce THR receptor activity. For the natural hormones the curves were comparable to the curves obtained from the laboratory reference chemicals. All four agonists had dose response curves comparable to literature, with some difference with regards to the EC50 and relative potency (RP) of tetrac, as reported in literature^{85,154}. EC50 and RP found for tetrac was 0.15 and 1.10 respectively, and in literature a 2.4 nM EC50 and 0.07 RP a reported¹⁵⁴. Table 3 shows the EC50 and relative potency results from the agonists.

Table 3: Showing the EC50 and relative potency of the agonists

Compound	EC50 (nM)	Relative Potency (RP)
T₃	0.17	1
T₄	2.67	0.07
Tetrac	0.16	1.10
Triac	0.93	0.19

The five antagonists (amiodarone, pentachlorophenol, Ethylene thiourea, 2,2,4,4-tetrahydroxybenzophenone and methimazole) were also tested for their ability to antagonize THR activity. The samples received were not enough to do full dose response curves, and to make higher concentration dilutions, similar to the ones done using the laboratory chemicals. The highest concentration possible was 0.005 mM which was significantly lower than the concentration previously shown to suppress THR activity. Only two chemicals, were able to suppress T₃ (0.25 nM) induction of the GH3.TRE.Luc cells at 0.005 mM. This was the highest concentration possible. The results in percentage (%) suppression if T₃ mediated THR activity are as for the two chemicals are as follows; Ethylene thiourea (39%) > Pentachlorophenol (29%). Statistical analysis from ANOVA and the regression linear model showed significant variation between all the chemicals (p=0.0), between all the different concentrations tested for each chemical (p=0.0) and for the chemical vs concentration interaction (p=0.0).

GWRC spiked water extracts

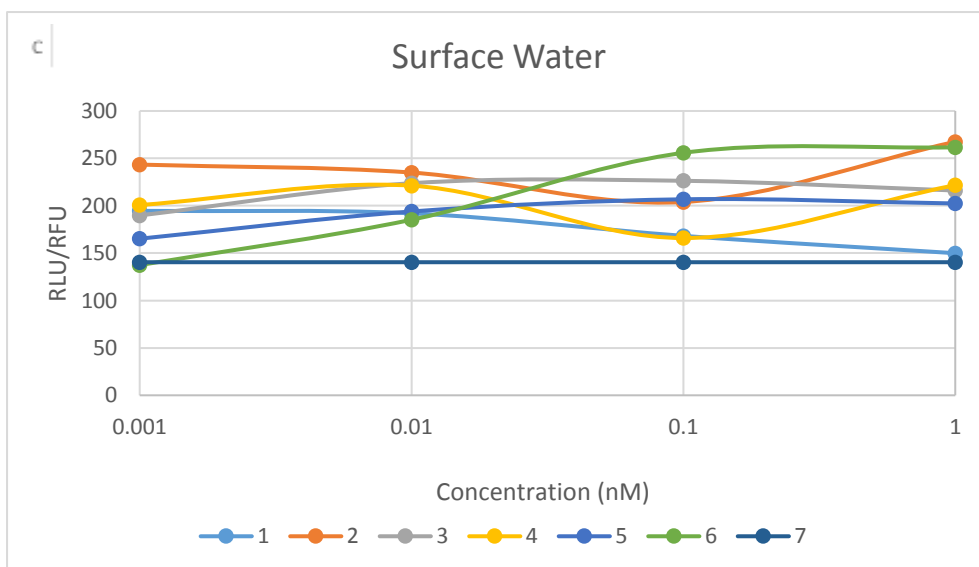
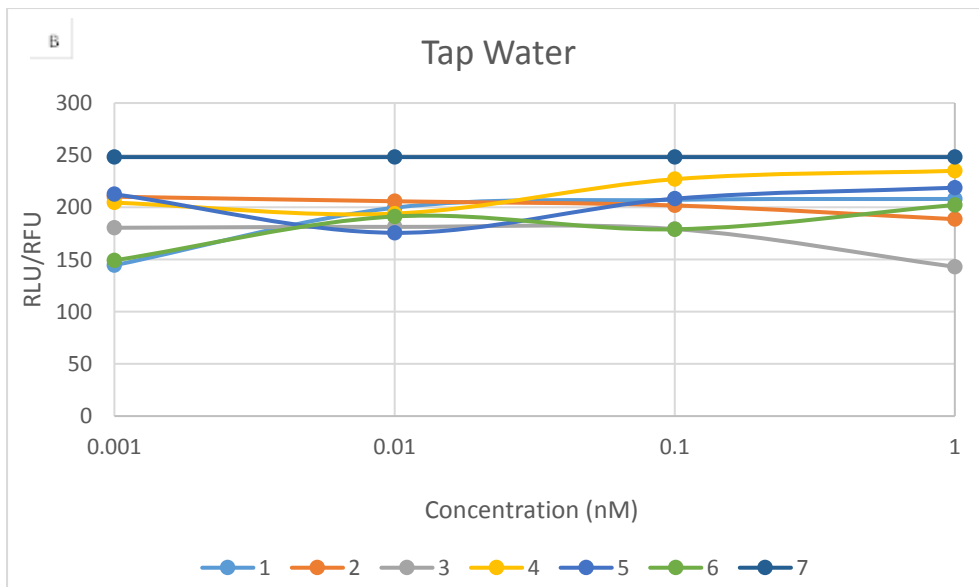
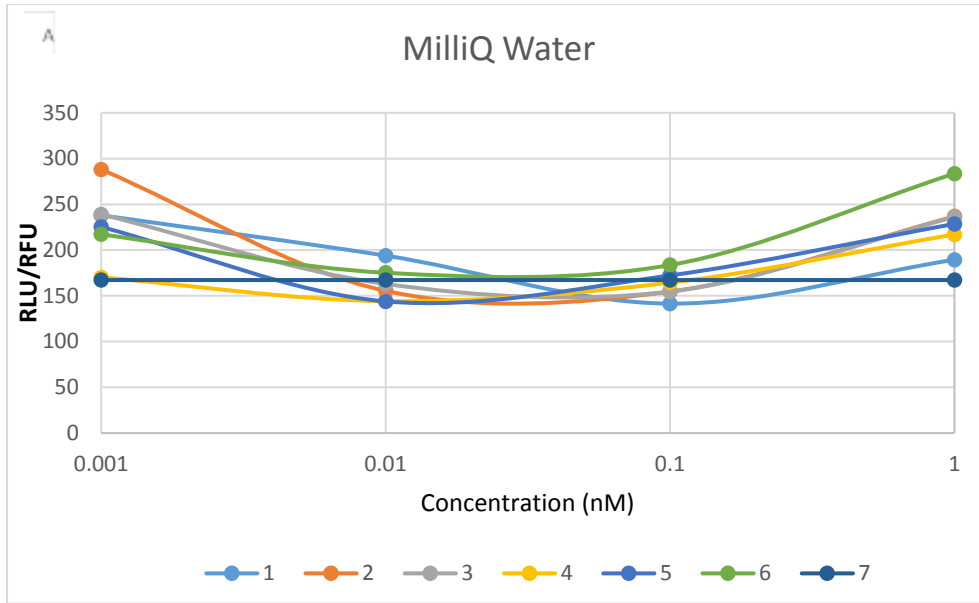
Eight spiked water samples were also tested for THR activity (agonistic and antagonistic) and cytotoxicity. The results showed THR agonistic activity in the 4 spiked water extracts. The non-spiked samples showed neither agonistic nor antagonistic activity. Some of the spiked samples showed THR activity which was higher than the standard curve and hence their highest RLU/RFU values could not be extrapolated using graphpad prism®. This was true for surface and waste water. No antagonistic activity or cytotoxicity was detected. Table 4 shows the Thyroid equivalents (TEq) obtained from the positive spiked samples in n g/L.

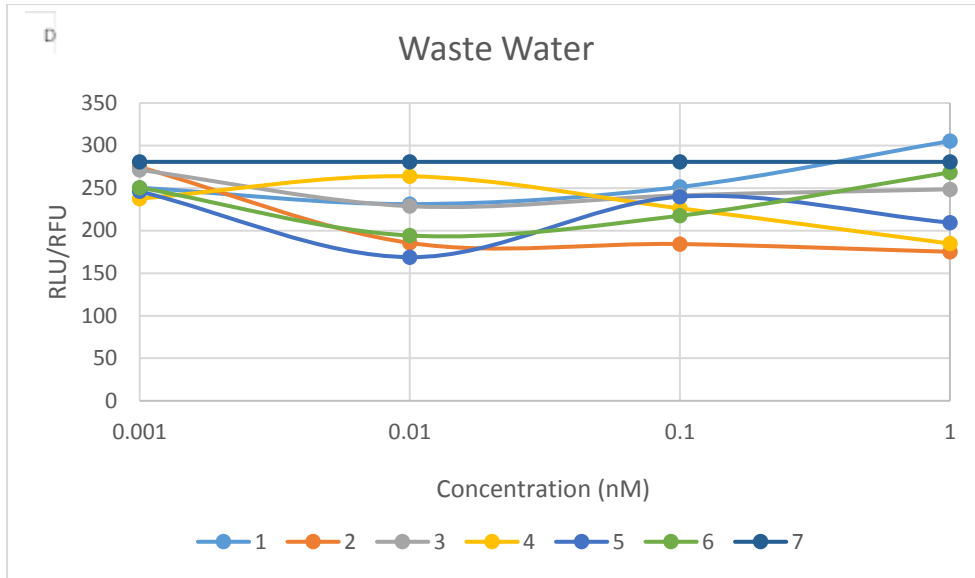
Table 4: Thyroid Equivalents (TEq) from the GWRC spiked water extracts

Sample ID	Thyroid Equivalents (TEq) ng/L	Standard Deviation(+/-)
Spiked Drinking Water	78.42	0.18
Spike MilliQ Water	2522.84	4.46
Spiked Waste Water	450.35	1.11
Spiked Surface Water	1237.59	3.29

4.3 Phase 2: GWRC water samples

The GH3.TRE.Luc was applied to 24 water samples from the GWRC as part of the Phase 2. Figure 15 (A-D) shows the luciferase reporter gene assay results for all of the water samples. Results were presented as RLU/RFU for the thyroid activity.





Key 1- Spain 2-Germany 3-South Africa 4-France 5-Netherlands 6-Australia 7-Vehicle Control (DMSO)

Figure 15; Graphs A to D for the luciferase reporter gene assay results in RLU/RFU values. All samples were tested in the presence of a 0.25 nM T₃ positive control, with RLU/RFU values of A) 1426.3 B)1057.4 C)1120.9 D)1238.

All the samples that had RLU/RFU values above the detection limit (vehicle control + 2 standard deviations) were tested again in triplicates to confirm thyroid hormone disruptor activity, and their thyroid equivalents (TEq) calculated with the aid of Graphpad Prism Software® (Version 4). Table 5 shows the GWRC samples that were further tested, in TEq (ng/L).

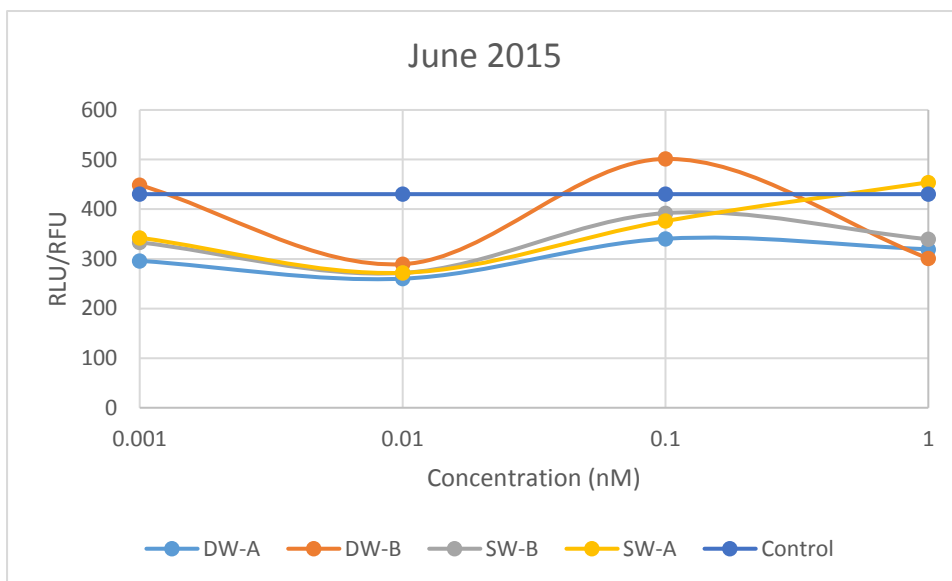
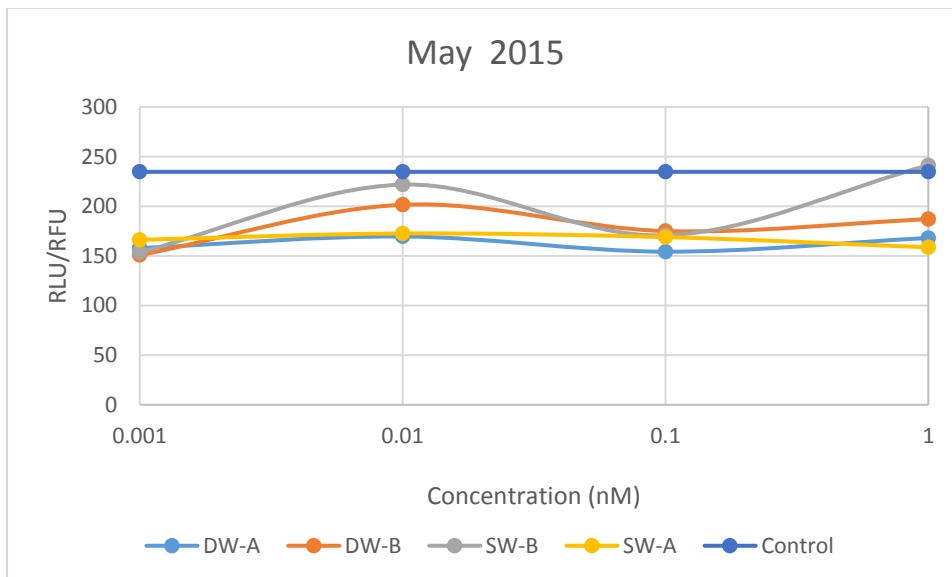
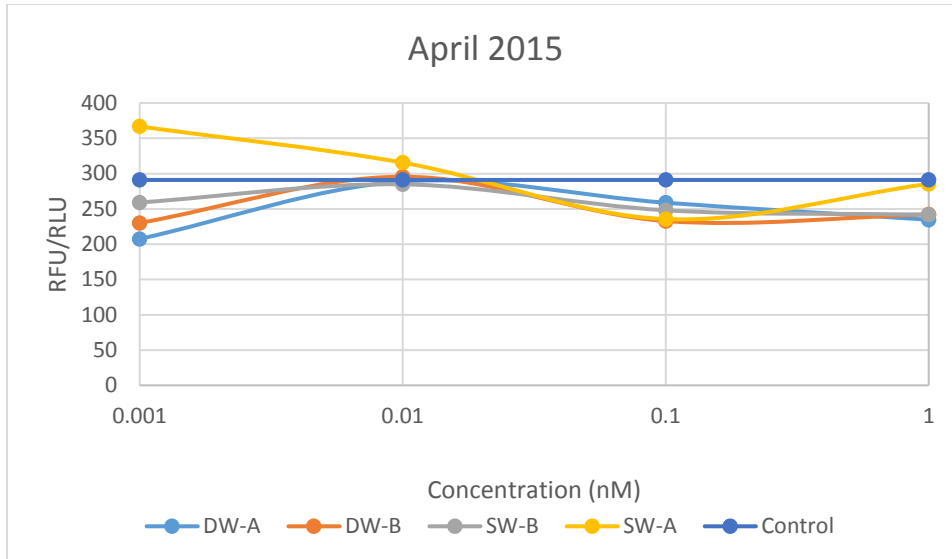
Table 5: TEq values for the GWRC water samples that were positive for TDC activity

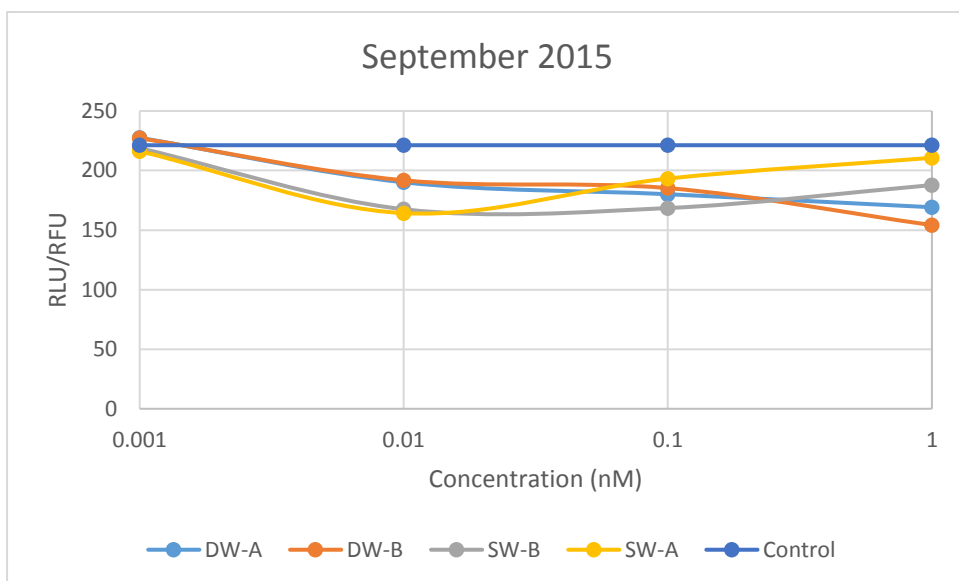
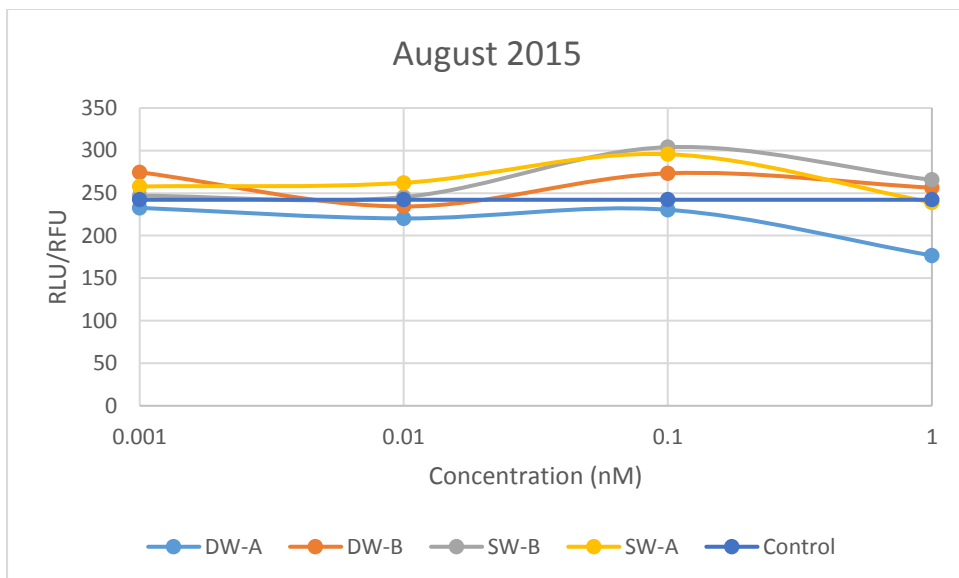
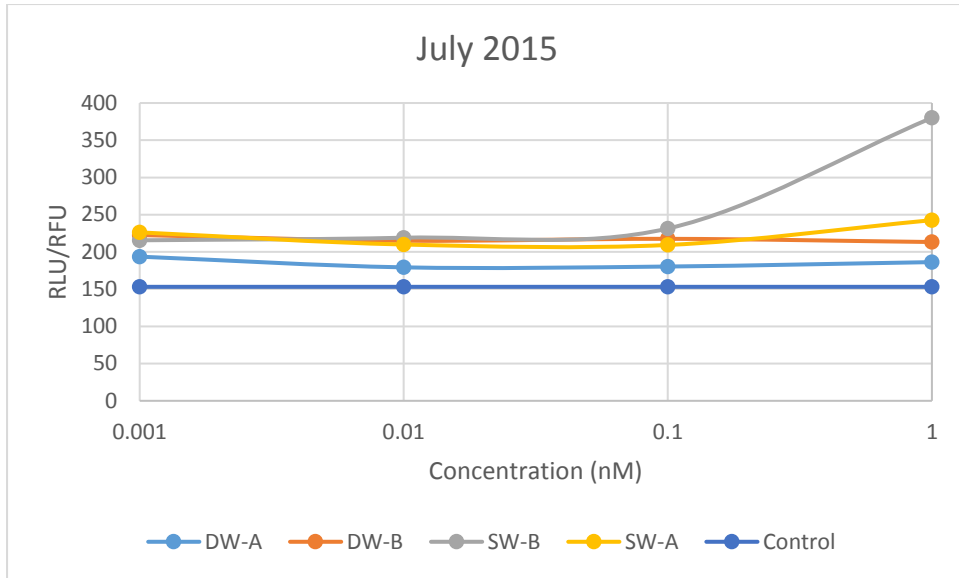
Sample	Sample ID	TEq (ng/L)	Standard Deviation (+/-)
MilliQ Water	Spain	7.53	4.91E-03
	Germany	44.14	4.32E-02
	South Africa	14.09	1.2E-02
	France	19.43	2.1E-02
	Netherlands	16.41	1.7E-02
	Australia	6.17	4.7E-03
Surface Water	Spain	2.78	1.5E-03
	Germany	4.36	3.1E-03
	South Africa	5.27	4.2E-03
	France	3.41	2.4E-03
	Netherlands	65.15	6.8E-02
	Australia	44.26	6.7E-02

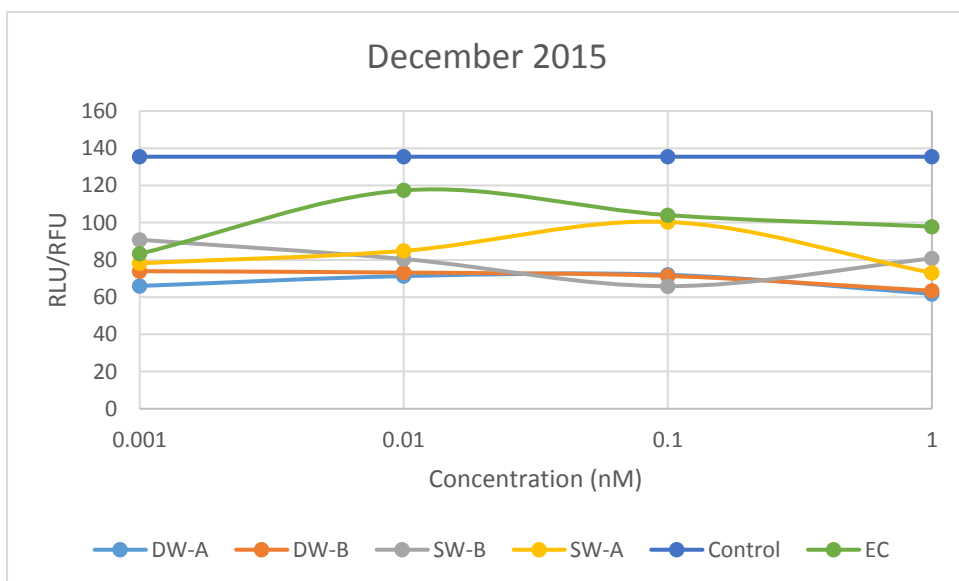
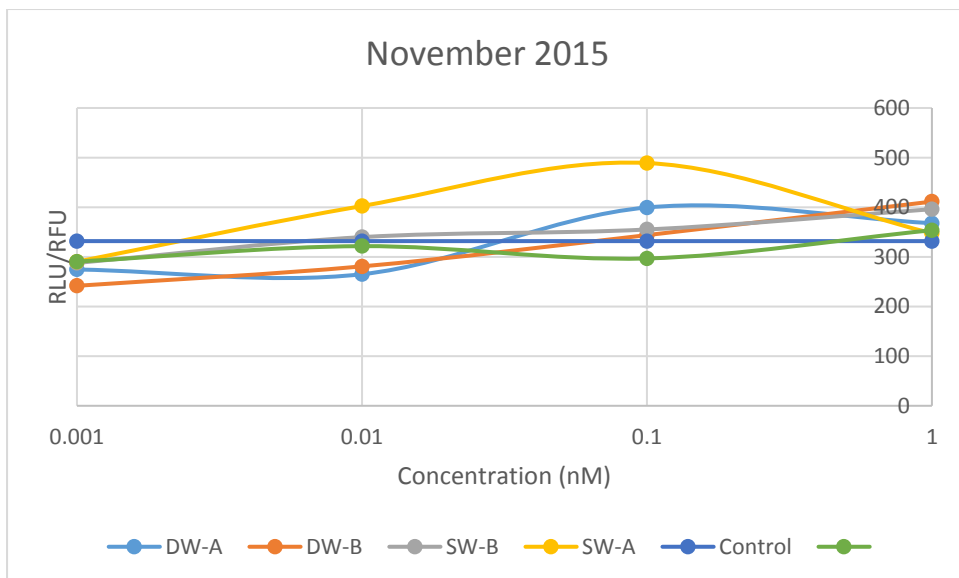
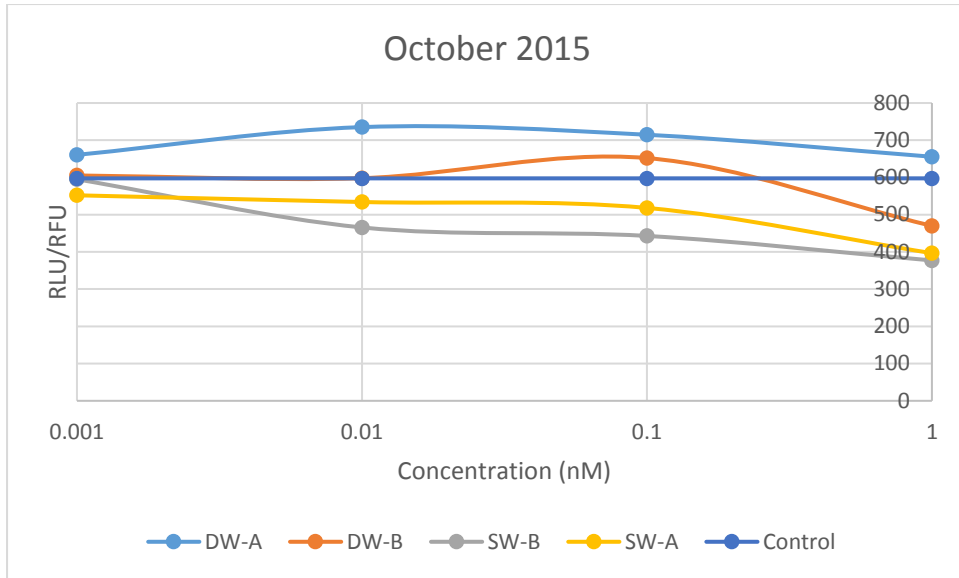
There was no anti-thyroid hormone activity detected in any the samples. The resazurine assay showed that all samples were within 3 standard deviations of the vehicle control, indicating no cytotoxicity. Statistical analysis using ANOVA showed significant variation between the different sources of the water ($p=0.0004$).

4.4 Phase 3: Water samples from a water treatment plant - Gauteng

Water samples from the treatment plant shown went through the screening April (2015 to March 2016). Samples that were above the detection limit were repeated to confirm activity using full dose response curves. Figure 16 shows the graphical results reported as (RLU/RFU) units from the screening tests for all the samples from April 2015 to March 2016).







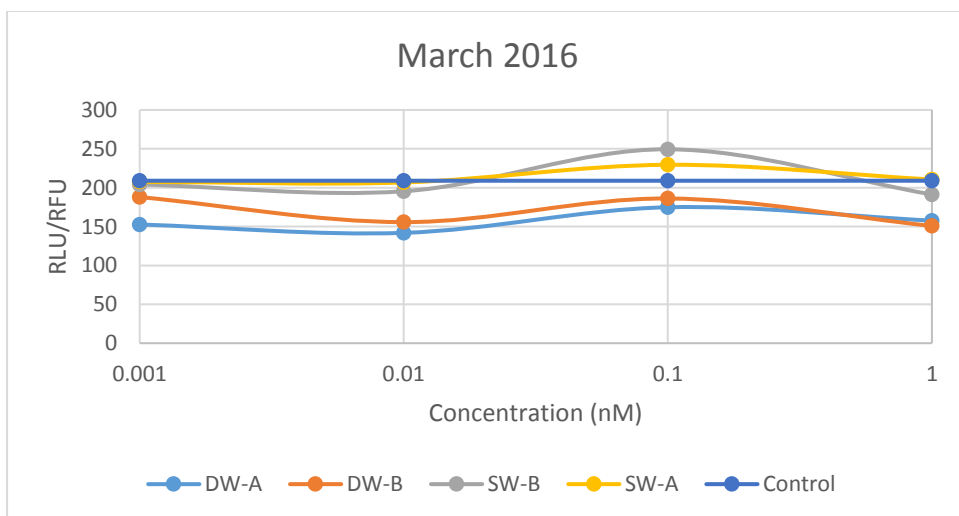
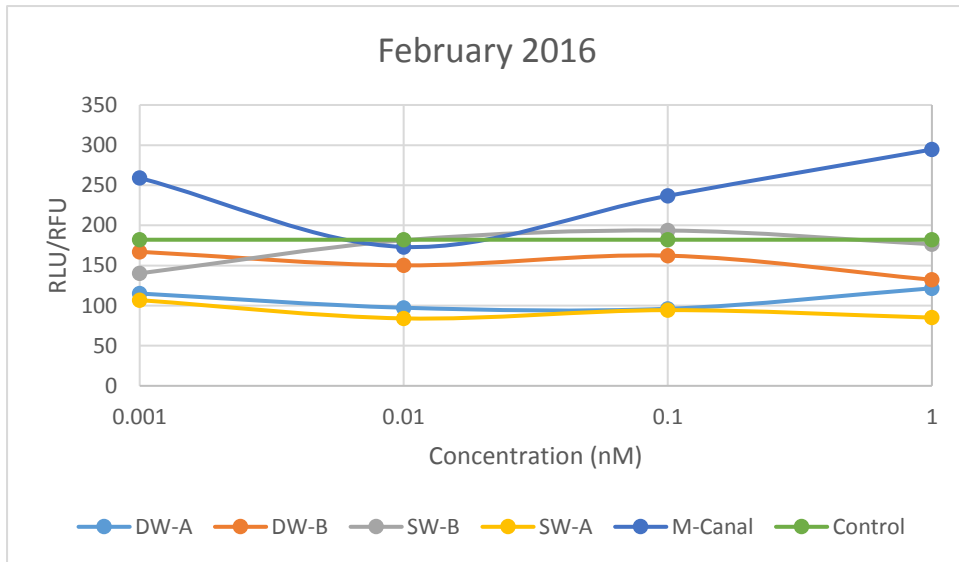
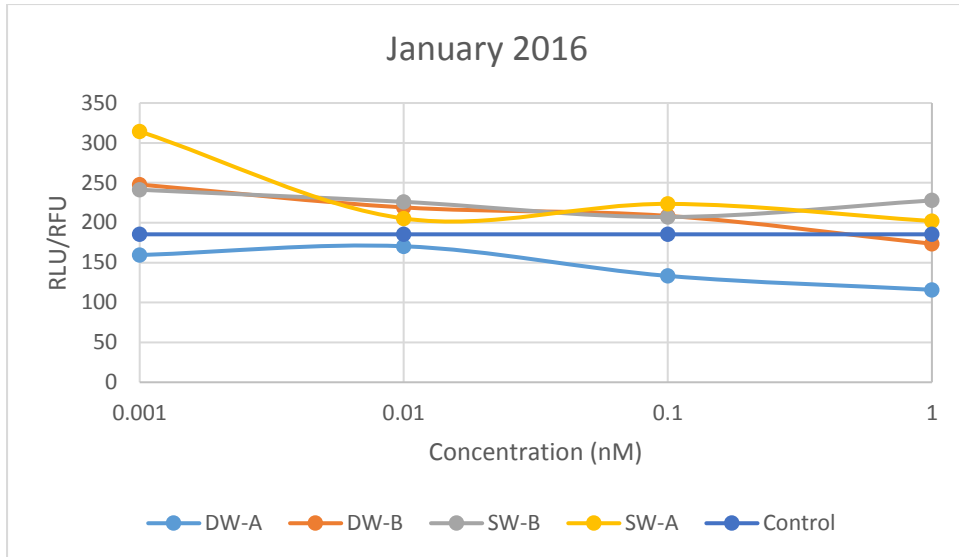


Figure 16: Graphs for the April 2015 to March 2016 samples using the GH3.TRE.Luc assay to test for THR activity (agonistic and antagonistic) and cytotoxicity. All

samples were tested in the presence of a 0.25 nM T3 positive control, with RLU/RFU values of April (1825.9), May (1840.7), June (1521.7), July (1088.5), August (1504), September (2692), October (1500), November (1739.7), December (1400), January (1019.9), February (1875.2), March (1133.4).

Of the 48 samples from the WTP that were analysed, 16 had RLU/RFU values above the detection limit (vehicle control + 2 standard deviations), hence positive for THR activity. These samples were repeated in triplicates to confirm activity and to quantify the activity as TEq in ng/L. Table 6 shows results from the positive samples. There was no antagonistic activity detected. Slight toxicity was observed from the source water samples. Figure 17 shows a graphical representation of the positive results over the 12-month period.

Table 6: Water samples from the treatment plant which tested positive for THR activity

Month	Sample ID	TEq ng/L	Standard Deviation (+/-)
April	SW A	87.59	1.6E-01
June	DW B	164.32	1.6E-01
July	DW B	195.39	1.5E-01
	SW B	76.32	1.3E-01
	SW A	91.77	1.3E-01
August	DW B	214.62	1.3E-01
	SW B	134.97	9.2E-02
	SW A	148.26	7.8E-02
October	DW A	184.61	1.1E-01
	DW B	105.55	8.3E-02
	SW A	81.51	2.7E-02
January	DW B	39.54	5.3E-02
	SW B	150.68	9.8E-02
	SW A	32.05	2.2E-02
February	SW B	49.73	5.0E-02
March	SW B	52.32	5.8E-02

Key Surface Water –SW Drinking Water - DW

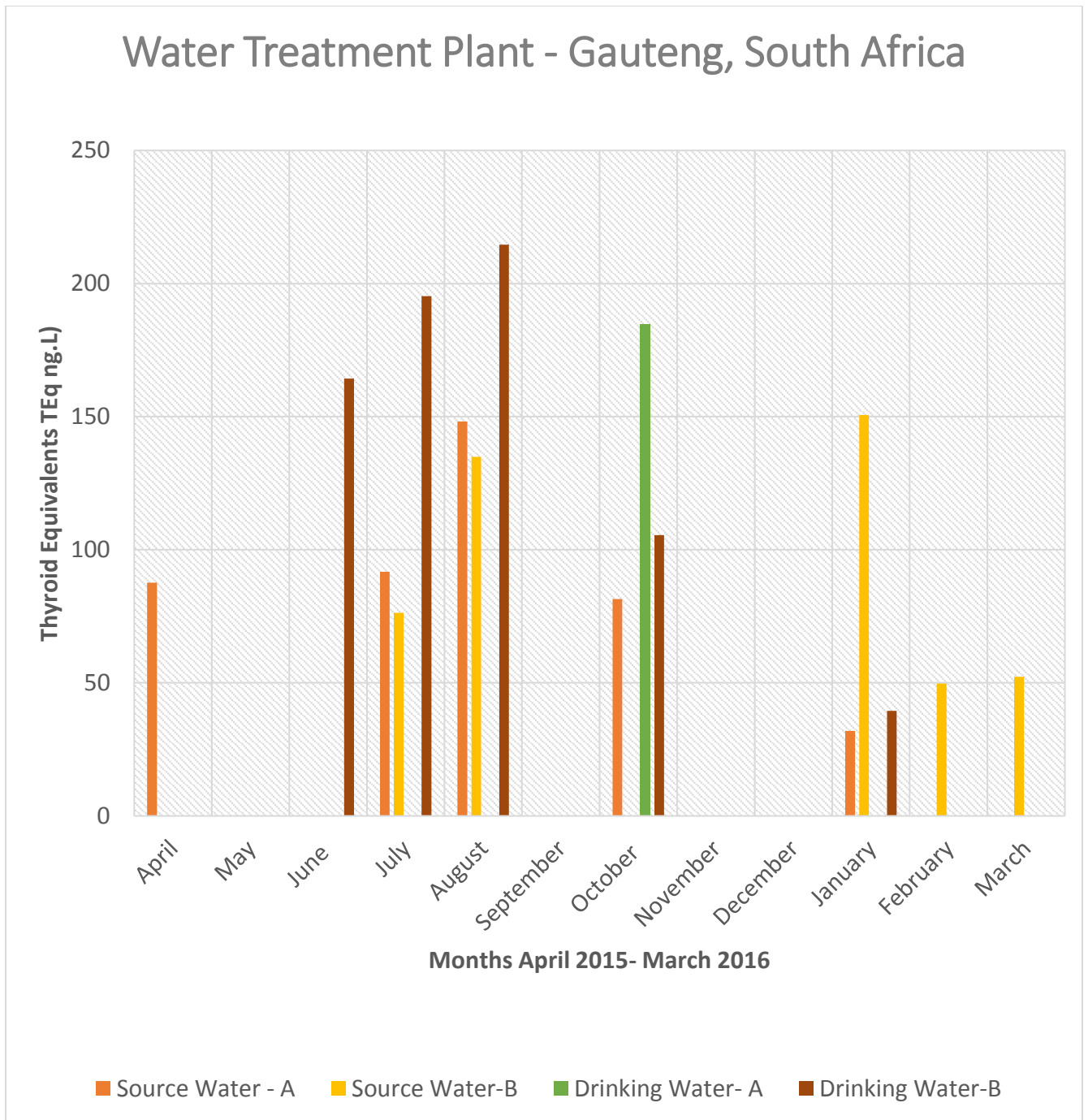


Figure 17: Graphical representation of the results from the water treatment plant over the 12-month period.

There was relatively higher THR disruption during the June to July period, which is a winter period in South Africa. There was also higher THR disruption in October. Of the 16 positive samples, 10 were source water samples. The highest TEq value was detected in September, from DW B (214 ng/L).

Statistical analysis using ANOVA and linear regression model showed a significant variation ($p=0.0000$) between the 12 months. There was also significant variation between the four sampling sites ($p=0.000$).

Chapter 5: Discussion

The GH3.TRE.Luc reporter gene bioassay was successfully optimised and validated using known agonists and antagonists. The GH3.TRE.Luc reporter gene bioassay is a sensitive and effective tool to identify and quantify thyroid activity in pure chemicals and in complex environmental mixtures present in water. It is also rapid, being able to detect THR activity within 24 hours of exposure, and robust, by having the TH receptor isoforms α and β . Modifications, suggested by Mengeling *et al*¹⁵³ were done on the original assay by Freitas *et al*⁸⁵, and this increased the sensitivity and effectiveness of the assay. The main issue was with the attachment of the cells in 96 well plates. Fetuin, which helps with cell attachment, increased background activity. We suspect therefore that fetuin may have some EDC characteristics. The specific modifications on the exposure timeline solved the attachment problem and we discontinued the use of fetuin.

Both the Luciferase (metabolic activity) and the resazurine (cell viability) assays were done on the same plate. This was possible because the resazurine dye does not interfere with the luminometric measurements and also because the resazurine assay is quick and does not require cell lysis. This greatly reduced the time needed to do the assay as both assays were done at once. The luciferase measurements were reported as RLU/RFU values normalised for cell viability using the resazurine measurements.

For optimisation and validation assay, the THR activity obtained from the agonists and antagonists were comparable to literature. The antagonists T₃ and T₄ had maximum induction factors averaging 14 (10nM) and 10 (1000nM) respectively (Figure 8). This is relatively high inducibility as it is recommended to have a fold induction of 6 or more for dose response curves. Induction factors of 23 for T₃ have been obtained in other studies using this cell line, which has a stable transfection of the expression vector and reporter plasmid.⁸⁵ Other studies using the GH3 cell line, with transient transfection, have reported a maximum induction of 4.¹⁵⁹ Freitas *et al*⁸⁵ reports that the high inducibility may be because of the use of the destabilized luciferase in the GH3.TRE.Luc assay, which decreases the background effect after 24 hours⁸⁵. The agonists T₃, T₄ triac and tetrac (GWRC samples) were also tested, the dose response curves was comparable to literature, but the EC₅₀ values for tetrac were not similar to those previously reported¹⁵⁴. In a study done by Gutleb *et*

al (2005), the EC₅₀ for tetrac was 2.45 nM, whilst in our study the EC₅₀ was 0.16 nM. Triac and tetrac are structural analogues of T₃ and T₄ respectively, and are both pharmaceuticals that have been used in hormone replacement therapy. The dose response curves and EC₅₀ for T₃ and triac were similar and close to each other, and the same applied for tetrac and T₄ (Figure 13). The relative potencies (RP) and EC₅₀ for the three agonists (except tetrac) were similar to those reported in literature¹⁵⁴.

Antagonistic activity tested using sodium arsenite and amiodarone hydrochloride, which were the antagonists of choice, resulted in dose response curves similar to those in literature (Figure 11 and 12). Sodium arsenite and amiodarone hydrochloride were able to suppress T₃ induction on the GH3.TRE.Luc cells from 1mM and 10mM respectively. However, amiodarone showed cytotoxicity at concentrations greater than 25 mM, which could have easily been mistaken for anti-thyroid hormone receptor activity if the resazurine assay had not been done. It was noted that a decrease in metabolic activity in the assay may be attributable to cytotoxicity, rather than antagonism. The antagonists from the GWRC; amiodarone hydrochloride, pentachlorophenol (PCP), ethylene thiourea (ETU), 2,2,4,4-tetrahydroxybenzophenone (THBP) and methimazole were received in volumes that could not be tested at the concentration that suppresses THR activity induced by T₃. The highest concentration that could be obtained was 0.005 mM. There was however suppression detected from ethylene thiourea (39%) and pentachlorophenol (29%) and this may be due to these chemicals having a higher antagonistic potency than the rest of the chemicals. The ability of the assay to detect THR activity at the nM and mM range showed the assay is sensitive and effective.

As part of the validation process, 8 water spiked water extracts were also tested, and the assay was able to detect THR activity in all the spiked samples. The samples were spiked with both T₄ and ETU at 20mM. The high THR activity detected showed that at 20 mM T₄ had higher potential to induce agonistic responses than ETU had to induce antagonistic response at the same concentration. The ability of the assay to detect THR activity in these samples exhibited the assay's ability to detect thyroid hormone receptor disruption in water extracts. At this point, there was high confidence that the assay had successfully been optimised and validated. The THR disruption values were reported as TEq in ng/L

To further test if the assay could be applied to water samples, 24 samples from the GWRC from six different countries were tested (South Africa, Germany, Netherlands, France, Spain and Australia). The assay was able to detect THR disruption in 12 of the samples, which were milliQ water and surface water. Because this was a blind test, the spiked samples are still not known. We suspect that these samples were positive because they were spiked, due to the uniformity of the samples that were positive. The THR disruption was reported as TEq in ng/L (Table 5). There was no significant cytotoxicity detected.

The variation of responses at specific concentrations of the screening tests, (figure 15 and 16) may have been due to a number of reasons which include but not limited to i) the contaminants having an effect on the cells at a specific concentration where there is a higher response, ii) where there is a lower response, the chemicals in the water samples may have been slightly cytotoxic at that specific concentration hence causing a reduced response.

In phase 3 of the study we assessed a water treatment plant in Gauteng, South Africa. The objective was to analyse if TDCs were present in the source water and if the treatment process was effective enough to remove them if present in the source water. SW A was a source water site near the dam wall, SW B was a source water site feeding into the treatment plant and producing DW A and B for distribution. It was found that 16 out of 48 samples (collected over 12 months) from the treatment plant tested positive (33%). Ten of the positive samples were source water samples, which were divided equally between SW A and B. There was relatively higher TDC activity during the June to August period. Seven of the 16 positive samples were in these 3 months, with the highest recorded TEq value of 214 ng/L in August (DW B). This is a winter season in South Africa and a dry period in Gauteng, therefore we suspect that the contaminants were more concentrated in the water during this time. There was also high TDC activity in October, and the highest activity in that month was from SW A. This was the only time there was TDC activity from SW A, we suspect that there might have been an issue with the treatment process for SW A during that month.

The results indicated that the water treatment process was effective in removing TDCs for SW A than for SW B, as we found TDC activity in SW B. This may be because of some treatment chemicals that may be adding into the treatment process that may have THR disruption activity. However, to the best of our knowledge, the treatment plant uses six chemicals in the treatment process, which are; hydrated lime (CaO), sodium silicate (Na₂O₃Si), polyelectrolyte, carbon dioxide (CO₂), chlorine (Cl₂) and ferric (Fe³⁺). None of these chemicals have been classified as EDCs. Therefore, the more likely reason is that the source water is contaminated and the treatment plant is not effective enough in removing some of the TDCs. However, in our future work we will incorporate the testing of these chemicals as individuals and as mixtures to verify that they do not induce TDC activity. We noticed that in some months where we found TDC activity is the drinking water; there was no TDC activity in the source water feeding into the plant. This may have been attributable to the slight toxicity that we detected in the source water, resulting in low induction of the THR activity on the cells.

We highly suspect that the TDC activity detected in the source water is due to activities occurring near the source water. This includes;

- Human settlements
- Illegal sewage dumping from municipalities
- Farming
- Mining
- Industry

All these activities may result in TDCs making their way into the water sources, and subsequently into the water supply. The water treatment plant employs a seven step purification process; coagulation; flocculation; sedimentation; stabilisation; filtration; disinfection and chloramination. Purification processes which include coagulation, flocculation and lime softening have been reported to be ineffective in removing some EDCs, particularly CECs, which include EDCs like PPCPs.¹⁶⁰ It is advisable for water treatment plants to incorporate the use of activated carbon and advanced oxidation, using ozone or UV, in order to increase chances of removing EDCs and

PPCPs. The activated carbon has the ability to remove most of the organic and non-polar compounds.¹⁶⁰

It is important to mention that, although there were samples positive for TDC activity, with TEq values ranging from 32 to 214 ng/L, we cannot say if these values will result in a human health risk. This is because there has not been development to a thyroid equivalents trigger value. Hence human health risk assessment cannot be performed. Trigger values for other endocrine pathways such as oestrogenic receptor (ER), androgenic receptor (AR), glucocorticoid receptor (GR) and progestogenic receptor (PR) have been developed for EDC activity in water using the CALUX bioassay, and are as follows;

$ER\alpha(3.8) < AR(11) < GR(21) < PR(333) \text{ ng/L}^{161}$

Unfortunately, there have been no studies done in South Africa on quantification of TDCs in water samples, using a THR based assay, therefore we cannot compare our results to any in South Africa. There have been few studies done of the presence of EDCs particularly TDCs in the water and their effects. Internationally, most of the studies done on the effect of TDCs have been on the presence of TDCs in source water, and their impact on aquatic life. An interassay laboratory ring test with other laboratories using different thyroid assays would be of great benefit.

Chapter 6: Conclusion

It is clear the issue of EDCs is global, with both developed and developing countries at a risk from disrupted environmental, wildlife and human health. Worryingly, EDC research in South Africa is still a low priority concern. There has been a lot of research done on EDCs in the developed world, with Africa lagging behind. The lack of research means a lack of data, and therefore the extent of contamination is not known, putting large populations at risk of being exposed unknowingly. The lack of scientific data also makes it difficult to address this EDC issue, at public and government level, and to find solutions that are relevant to the African and South African context.

For every body, organ, and tissue in the human body to be in a healthy condition there needs to be achievement of a euthyroid state and a HPT axis functioning at its optimum. An interruption thereof would result in adverse effects of the neural and physical growth (particularly in children), reproductive anomalies, and negative effects on the homeostasis of the body. It is therefore imperative that thyroid assays which can detect TDC activity be established and optimised, in order for potential sources of exposure to be assessed, and for thyroid hormone disruptor activity to be reported.

Of the research that has been done, both globally and locally, focus has been on the oestrogenic pathway. However, it is clear that EDCs also affect other pathways, including the HPT axis, and studies have reported associations between TDC exposure and neurobehavioral disorders, obesity and reproductive abnormalities, among others. It is therefore important that assays which can detect TDCs be developed and validated.

In this study, the GH3.TRE.Luc Reporter Gene Bioassay was successfully optimised, validated and applied to test chemicals and water extracts. The assay is effective in detecting TDCs present in water extracts, with relevance to water monitoring. The assay is rapid, robust and sensitive; therefore assessment of water samples has a short turnover time. It is possible for the water industry to adopt this assay as a routine test for water quality assessments.

6.1 Recommendations

Policy makers and stakeholders in the water industry

We recommend:

- i) Development of legislation and policy to protect the public from being exposed to EDCs. Currently, there are no water quality guidelines for EDCs
- ii) Further monitoring of water sources for TDCs to ensure water quality and safety.
- iii) Awareness of sources and routes of exposure e.g. at home or at work. There are a number of sources of exposure, and the public needs to be aware and be educated about these sources and routes of exposure.
- iv) Reduction of the EDC load as it may lead to mixture effects. We may be exposed to a number of EDCs at the same time, and this may result in synergistic or additive effects of EDCs in the body. This may cause increased adverse health effects. We also recommend that the public minimise activities that may result in an increase of the EDC load in the environment, especially with regards to PPCPs.
- v) Being smart consumers. The public needs to be aware of the chemicals that are in the products they buy, to avoid exposure to EDCs

Research community

We recommend:

- vii) Chemical analysis of the chemicals which may be causing TDCs activity in the water in South Africa
- viii) Development of a trigger TEq value. This is important so that human health risk assessment can be done to determine TDC activity values which will affect health
- ix) More research on EDCs as a whole, and on other EDC endpoints and sources. There are still a number of endocrine pathways and sources that have not been tested in South Africa.

- x) More collaboration between disciplines as EDCs are a One Health issue, and needs an interdisciplinary approach. Collaboration between developing and developed countries as well, so there can be sharing of knowledge.

Overall, more research on EDCs in Africa needs to be conducted, in order for us to be able to “find African solutions to African problems.” An EDC free environment should be advocated for, by all citizens.

Chapter 7: References

1. Sharpe RM, Irvine DS. How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health? *British Medical Journal*. 2004;328(7437):447-51.
2. Steffen P, Moravchik B. Nonpoint source pollution 2008 [updated 25 March 2008; cited 2016 19 October]. Available from: <http://oceanservice.noaa.gov/education/kits/pollution/02history.html>.
3. Guillette Jr LJ, Crain DA, Rooney AA, Pickford DB. Organization versus activation: the role of endocrine-disrupting contaminants (EDCs) during embryonic development in wildlife. *Environmental Health Perspectives*. 1995;103:105-57.
4. Bergman A, Heindel JJ, Kasten T, Kidd KA, Jobling S, Neira M, et al. The impact of endocrine disruption a consensus statement on the state of the science. *Environmental Health Perspectives*. 2013;121(4):104-6.
5. Bergman Å, Heindel J, Jobling S, Kidd K, Zoeller RT. State-of-the-science of endocrine disrupting chemicals, 2012. *Toxicology Letters*. 2012;211:1-38.
6. Bouwman H, Bornman R, van den Berg H, Kylin H. DDT: fifty years since Silent Spring. 2013.
7. Gore A, Crews D, Doan L, La Merrill M, Patisaul H, Zota A. Introduction to endocrine disrupting chemicals (EDCs)—a guide for public interest organizations and policy makers. *Endocrine Society Reports and White Papers*. 2014:1-76.
8. Damstra T, Barlow S, Bergman A, Kavlock R, Van Der Kraak G. Global assessment of the state-of-the-science of endocrine disruptors. Geneva: World Health Organization. 2002.
9. Zoeller RT, Brown T, Doan L, Gore A, Skakkebaek N, Soto A, et al. Endocrine-disrupting chemicals and public health protection: a statement of principles from The Endocrine Society. *Endocrinology*. 2012;153(9):4097-110.
10. Gore AC. *Endocrine-disrupting chemicals: from basic research to clinical practice*: Springer Science & Business Media; 2007.
11. Carson R. *Silent spring*: Houghton Mifflin Harcourt; 2002.
12. Langston N. Rachel Carson's legacy: Endocrine disrupting chemicals and gender concerns. *GAIA-Ecological Perspectives for Science and Society*. 2012;21(3):225-9.

13. Grossman E, Vandenberg LN, Thayer K, Birnbaum LS. Theodora (Theo) Colborn: 1927–2014. *Environmental Health Perspectives*. 2015;123(3):54.
14. LeBlanc GA, Norris DO, Kloas W, Kullman SW, Baldwin WS, Greally JM. Draft detailed review paper state of the science on novel *in vitro* and *in vivo* screening and testing methods and endpoints for evaluating endocrine disruptors. Organisation for Economic Cooperation and Development, Paris, France. 2011.
15. Melmed S, Polonsky KS, Larsen PR, Kronenberg HM. *Williams Textbook of Endocrinology*: Elsevier Health Sciences; 2015.
16. Vandenberg LN, Gerona RR, Kannan K, Taylor JA, van Breemen RB, Dickenson CA, et al. A round robin approach to the analysis of bisphenol a (BPA) in human blood samples. *Environmental Health*. 2014;13(1):1-25.
17. Vandenberg LN. Non-monotonic dose responses in studies of endocrine disrupting chemicals: bisphenol a as a case study. *Dose-response*. 2014;12(2):259-76.
18. Chen A, Yolton K, Rauch SA, Webster GM, Hornung R, Sjödin A, et al. Prenatal polybrominated diphenyl ether exposures and neurodevelopment in US children through 5 years of age: the HOME study. *Environmental Health Perspectives* 2014;122(8):856.
19. Colborn T. Clues from wildlife to create an assay for thyroid system disruption. *Environmental Health Perspectives*. 2002;110(3):363-7.
20. Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives*. 1993;101(5):378-84.
21. Muncke J. Exposure to endocrine disrupting compounds via the food chain: Is packaging a relevant source? *Science of the Total Environment*. 2009;407(16):4549-59.
22. Schrage S, Potter BE. Diethylstilbestrol exposure. *American Family Physician*. 2004;69(10):2401-2.
23. Baumann L, Ros A, Rehberger K, Neuhauss SC, Segner H. Thyroid disruption in zebrafish (*Danio rerio*) larvae: Different molecular response patterns lead to impaired eye development and visual functions. *Aquatic Toxicology*. 2016;172:44-55.
24. Guillette Jr LJ, Gross TS, Masson GR, Matter JM, Percival HF, Woodward AR. Developmental abnormalities of the gonad and abnormal sex hormone

- concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environmental Health Perspectives*. 1994;102(8):650-80.
25. Walker DM, Gore AC. Transgenerational neuroendocrine disruption of reproduction. *Nature Reviews Endocrinology*. 2011;7(4):197-207.
 26. Schug TT, Janesick A, Blumberg B, Heindel JJ. Endocrine disrupting chemicals and disease susceptibility. *The Journal of Steroid Biochemistry and Molecular Biology*. 2011;127(3):204-15.
 27. Diamanti-Kandarakis E, Gore AC. Introduction to endocrine disruptors and puberty. *Endocrine Disruptors and Puberty*: Springer; 2012. p. 1-8.
 28. Gore AC, Patisaul HB. Neuroendocrine Disruption: Historical Roots, Current Progress, Questions for the Future. *Frontiers in Neuroendocrinology*. 2010;31(4):395-9.
 29. Reed CE, Fenton SE. Exposure to Diethylstilbestrol during Sensitive Life Stages: A legacy of heritable health effects. *Embryo Today* 2013;99(2):1-23.
 30. Anway MD, Cupp AS, Uzumcu M, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*. 2005;308(5727):1466-9.
 31. Crews D, McLachlan JA. Epigenetics, evolution, endocrine disruption, health, and disease. *Endocrinology*. 2006;147(6):s4-s10.
 32. De Coster S, van Larebeke N. Endocrine-disrupting chemicals: associated disorders and mechanisms of action. *Journal of Environmental and Public Health*. 2012;2012.
 33. Geyer HJ, Rimkus GG, Scheunert I, Kaune A, Schramm K-W, Ketttrup A, et al. Bioaccumulation and occurrence of endocrine-disrupting chemicals (EDCs), persistent organic pollutants (POPs), and other organic compounds in fish and other organisms including humans. *Bioaccumulation—New Aspects and Developments*: Springer; 2000. p. 1-166.
 34. Crofton KM, Craft ES, Hedge JM, Gennings C, Simmons JE, Carchman RA, et al. Thyroid-hormone-disrupting chemicals: evidence for dose-dependent additivity or synergism. *Environmental Health Perspectives*. 2005:1549-54.
 35. Chen H, Liu Z, Zhang X, Jia X, Li Q, Su Q, et al. Assessment of synergistic thyroid disrupting effects of a mixture of EDCs in ovariectomized rats using factorial analysis and dose addition. *Toxicology Research*. 2016;6(5):1585-93.

36. Pop A, Drugan T, Gutleb AC, Lupu D, Cherfan J, Loghin F, et al. Individual and combined in vitro (anti) androgenic effects of certain food additives and cosmetic preservatives. *Toxicology In Vitro*. 2016;32:269-77.
37. Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs Jr DR, Lee D-H, et al. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocrine reviews*. 2012;33(3):378-455.
38. Wang Y, Zhou J. Endocrine disrupting chemicals in aquatic environments: A potential reason for organism extinction? *Aquatic Ecosystem Health & Management*. 2013;16(1):88-93.
39. Aneck-Hahn NH, Bornman M, De Jager C. Oestrogenic activity in drinking waters from a rural area in the Waterberg District, Limpopo Province, South Africa. *Water SA*. 2009;35(3):245-51.
40. Laryea G, Muglia L, Arnett M, Muglia LJ. Dissection of glucocorticoid receptor-mediated inhibition of the hypothalamic–pituitary–adrenal axis by gene targeting in mice. *Frontiers in Neuroendocrinology*. 2015;36:150-64.
41. Castro-Vale I, van Rossum EF, Machado JC, Mota-Cardoso R, Carvalho D. Genetics of glucocorticoid regulation and posttraumatic stress disorder—What do we know? *Neuroscience & Biobehavioral Reviews*. 2016;63:143-57.
42. Myers B, McKlveen JM, Herman JP. Glucocorticoid actions on synapses, circuits, and behavior: implications for the energetics of stress. *Frontiers in Neuroendocrinology*. 2014;35(2):180-96.
43. Raff H. Do the Effects of the Triorganotin Tributyltin on the Hypothalamic-Pituitary-Adrenal Axis *In Vivo* Contribute to Its Environmental Toxicity? : Endocrine Society Washington, DC; 2016.
44. Merlo E, Podratz PL, Sena GC, de Araújo JF, Lima LC, Alves IS, et al. The Environmental Pollutant Tributyltin Chloride Disrupts the Hypothalamic-Pituitary-Adrenal Axis at Different Levels in Female Rats. *Endocrinology*. 2016;157(8):2978-95.
45. Thackray VG, Mellon PL, Coss D. Hormones in synergy: regulation of the pituitary gonadotropin genes. *Molecular and Cellular Endocrinology*. 2010;314(2):192-203.
46. De Jager C, Aneck-Hahn N, Van Wyk J, Moolman A. A toolbox of bio-assays for detection of estrogenic activity in water. 2010.

47. Li Y, Luh CJ, Burns KA, Arao Y, Jiang Z, Teng CT, et al. Endocrine-disrupting chemicals (EDCs): in vitro mechanism of estrogenic activation and differential effects on ER target genes.

Environmental Health Perspectives. 2013;121(4):459.

48. Dabrowski JM, Shadung JM, Wepener V. Prioritizing agricultural pesticides used in South Africa based on their environmental mobility and potential human health effects. Environ Int. 2014;62:31-40.

49. Barrett JR. EDCs and estrogen receptor activity: a pathway to safer chemical design? Environmental Health Perspectives. 2014;122(12):295-339.

50. Zama AM, Bhurke A, Uzumcu M. Effects of Endocrine-disrupting Chemicals on Female Reproductive Health. The Open Biotechnology Journal. 2016;10(1).

51. Renaville R, Hammadi M, Portetelle D. Role of the somatotrophic axis in the mammalian metabolism. Domestic Animal Endocrinology. 2002;23(1):351-60.

52. Filby AL, Thorpe KL, Tyler CR. Multiple molecular effect pathways of an environmental oestrogen in fish. Journal of Molecular Endocrinology. 2006;37(1):121-34.

53. Schmid AC, Lutz I, Kloas W, Reinecke M. Thyroid hormone stimulates hepatic IGF-I mRNA expression in a bony fish, the tilapia *Oreochromis mossambicus*, *in vitro* and *in vivo*. General and Comparative Endocrinology. 2003;130(2):129-34.

54. Tsukada A, Ohkubo T, Sakaguchi K, Tanaka M, Nakashima K, Hayashida Y, et al. Thyroid hormones are involved in insulin-like growth factor-I (IGF-I) production by stimulating hepatic growth hormone receptor (GHR) gene expression in the chicken. Growth Hormone & IGF Research. 1998;8(3):235-42.

55. Leung AM, Brent GA. The Influence of Thyroid Hormone on Growth Hormone Secretion and Action. Growth Hormone Deficiency: Springer; 2016. p. 29-46.

56. Rhinn M, Dollé P. Retinoic acid signalling during development. Development. 2012;139(5):843-58.

57. Di Masi A, Leboffe L, De Marinis E, Pagano F, Cicconi L, Rochette-Egly C, et al. Retinoic acid receptors: from molecular mechanisms to cancer therapy. Molecular Aspects of Medicine. 2015;41:1-115.

58. Das BC, Thapa P, Karki R, Das S, Mahapatra S, Liu T-C, et al. Retinoic acid signaling pathways in development and diseases. Bioorganic & Medicinal Chemistry. 2014;22(2):673-83.

59. Elmazar MM, Rühl R, Nau H. Synergistic teratogenic effects induced by retinoids in mice by coadministration of a RAR α -or RAR γ -selective agonist with a RXR-selective agonist. *Toxicology and Applied Pharmacology*. 2001;170(1):2-9.
60. Zile MH. Vitamin A homeostasis endangered by environmental pollutants. *Experimental Biology and Medicine*. 1992;201(2):141-53.
61. Novák J, Beníšek M, Hilscherová K. Disruption of retinoid transport, metabolism and signaling by environmental pollutants. *Environment International*. 2008;34(6):898-913.
62. Wang Y, Chen J, Du C, Li C, Huang C, Dong Q. Characterization of retinoic acid-induced neurobehavioral effects in developing zebrafish. *Environmental Toxicology and Chemistry*. 2014;33(2):431-7.
63. Skaare JU, Bernhoft A, Wiig Ø, Norum KR, Haug E, Eide DM, et al. Relationships between plasma levels of organochlorines, retinol and thyroid hormones from polar bears (*Ursus maritimus*) at Svalbard. *Journal of Toxicology and Environmental Health*. 2001;62(4):227-41.
64. Hallgren S, Sinjari T, Håkansson H, Darnerud P. Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. *Archives of Toxicology*. 2001;75(4):200-8.
65. Ding C, Gao D, Wilding J, Trayhurn P, Bing C. Vitamin D signalling in adipose tissue. *British Journal of Nutrition*. 2012;108(11):1915-23.
66. Norman AW. From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. *The American Journal of Clinical Nutrition*. 2008;88(2):491-9.
67. Ceglia L. Vitamin D and skeletal muscle tissue and function. *Molecular Aspects of Medicine*. 2008;29(6):407-14.
68. Girgis CM, Clifton-Bligh RJ, Hamrick MW, Holick MF, Gunton JE. The roles of vitamin D in skeletal muscle: form, function, and metabolism. *Endocrine Reviews*. 2012;34(1):33-83.
69. Souberbielle J-C, Body J-J, Lappe JM, Plebani M, Shoenfeld Y, Wang TJ, et al. Vitamin D and musculoskeletal health, cardiovascular disease, autoimmunity and cancer: recommendations for clinical practice. *Autoimmunity Reviews*. 2010;9(11):709-15.
70. Prietl B, Treiber G, Pieber TR, Amrein K. Vitamin D and immune function. *Nutrients*. 2013;5(7):2502-21.

71. Thorne J, Campbell MJ. The vitamin D receptor in cancer. *Proceedings of the Nutrition Society*. 2008;67(02):115-27.
72. Kočovská E, Fernell E, Billstedt E, Minnis H, Gillberg C. Vitamin D and autism: clinical review. *Research in Developmental Disabilities*. 2012;33(5):1541-50.
73. Pludowski P, Holick MF, Pilz S, Wagner CL, Hollis BW, Grant WB, et al. Vitamin D effects on musculoskeletal health, immunity, autoimmunity, cardiovascular disease, cancer, fertility, pregnancy, dementia and mortality—a review of recent evidence. *Autoimmunity Reviews*. 2013;12(10):976-89.
74. Eyles DW, Burne TH, McGrath JJ. Vitamin D, effects on brain development, adult brain function and the links between low levels of vitamin D and neuropsychiatric disease. *Frontiers in Neuroendocrinology*. 2013;34(1):47-64.
75. Lilienthal H, Fastabend A, Hany J, Kaya H, Roth-Härer A, Dunemann L, et al. Reduced levels of 1, 25-dihydroxyvitamin D₃ in rat dams and offspring after exposure to a reconstituted PCB mixture. *Toxicological Sciences*. 2000;57(2):292-301.
76. Nishimura N, Nishimura H, Ito T, Miyata C, Izumi K, Fujimaki H, et al. Dioxin-induced up-regulation of the active form of vitamin D is the main cause for its inhibitory action on osteoblast activities, leading to developmental bone toxicity. *Toxicology and Applied Pharmacology*. 2009;236(3):301-9.
77. Haynes JM, Wellman ST, Beckett KJ, Pagano JJ, Fitzgerald SD, Bursian SJ. Histological lesions in mink jaws are a highly sensitive biomarker of effect after exposure to TCDD-like chemicals: field and literature-based confirmations. *Archives of Environmental Contamination and Toxicology*. 2009;57(4):803-7.
78. Grygiel-Górniak B. Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications—a review. *Nutrition Journal*. 2014;13(1):1.
79. Neels JG, Grimaldi PA. Physiological functions of peroxisome proliferator-activated receptor β . *Physiological Reviews*. 2014;94(3):795-858.
80. Monsalve FA, Pyarasani RD, Delgado-Lopez F, Moore-Carrasco R. Peroxisome proliferator-activated receptor targets for the treatment of metabolic diseases. *Mediators of Inflammation*. 2013;2013.
81. Stel J, Legler J. The role of epigenetics in the latent effects of early life exposure to obesogenic endocrine disrupting chemicals. *Endocrinology*. 2015;156(10):3466-72.

82. Holtcamp W. Obesogens: an environmental link to obesity. *Environmental Health Perspectives*. 2012;120(2):62-8.
83. Riu A, Grimaldi M, le Maire A, Bey G, Phillips K, Boulahtouf A, et al. Peroxisome Proliferator-Activated Receptor [gamma] Is a Target for Halogenated Analogs of Bisphenol A. *Environmental Health Perspectives*. 2011;119(9):1227.
84. Ernst J, Jann J-C, Biemann R, Koch HM, Fischer B. Effects of the environmental contaminants DEHP and TCDD on estradiol synthesis and aryl hydrocarbon receptor and peroxisome proliferator-activated receptor signalling in the human granulosa cell line KGN. *Molecular Human Reproduction*. 2014;20(9):919-28.
85. Freitas J, Cano P, Craig-Veit C, Goodson ML, Furlow JD, Murk AJ. Detection of thyroid hormone receptor disruptors by a novel stable *in vitro* reporter gene assay. *Toxicology In Vitro*. 2011;25(1):257-66.
86. Murk AJ, Rijntjes E, Blaauboer BJ, Clewell R, Crofton KM, Dingemans MM, et al. Mechanism-based testing strategy using *in vitro* approaches for identification of thyroid hormone disrupting chemicals. *Toxicology in vitro*. 2013;27(4):1320-46.
87. Reinehr T. Obesity and thyroid function. *Molecular and cellular endocrinology*. 2010;316(2):165-71.
88. Ortiga-Carvalho TM, Chiamolera MI, Pazos-Moura CC, Wondisford FE. Hypothalamus-Pituitary-Thyroid Axis. *Comprehensive Physiology*. 2016.
89. Zoeller RT, Tan SW, Tyl RW. General background on the hypothalamic-pituitary-thyroid (HPT) axis. *Critical Reviews in Toxicology*. 2007;37(1-2):11-53.
90. Fliers E, Kalsbeek A, Boelen A. Mechanisms of Endocrinology: Beyond the fixed setpoint of the hypothalamus–pituitary–thyroid axis. *European Journal of Endocrinology*. 2014;171(5):197-208.
91. Boas M, Feldt-Rasmussen U, Main KM. Thyroid effects of endocrine disrupting chemicals. *Molecular and Cellular Endocrinology*. 2012;355(2):240-8.
92. Medici M, Visser WE, Visser TJ, Peeters RP. Genetic determination of the hypothalamic-pituitary-thyroid axis: where do we stand? *Endocrine Reviews*. 2015;36(2):214-44.
93. Boron WF, Boulpaep EL. Chapter 49, "Synthesis of Thyroid Hormones. *Medical Physiology* (2nd ed) Elsevier/Saunders 2012. p. 1352.
94. Schriks M. Novel *in vitro*, *ex vivo* and *in vivo* assays elucidating the effects of endocrine disrupting compounds (EDCs) on thyroid hormone action: WUR Wageningen UR; 2006.

95. Cao X, Kambe F, Yamauchi M, Seo H. Thyroid-hormone-dependent activation of the phosphoinositide 3-kinase/Akt cascade requires Src and enhances neuronal survival. *Biochemical Journal*. 2009;424(2):201-9.
96. Duntas LH. Chemical contamination and the thyroid. *Endocrine*. 2015;48(1):53-64.
97. Patrick L. Thyroid disruption: mechanisms and clinical implications in human health. *Alternative Medicine Review*. 2009;14(4):326-47.
98. NIEHS. Endocrine Disruptors 2016 [updated 15 July 2016. Available from: <http://www.niehs.nih.gov/health/topics/agents/endocrine/>.
99. Massart F, Saggese G, Ferrara P. Environmental thyroid disruptors and human endocrine health. *Intech Open Access*. 2012;2:1-25.
100. Boas M, Feldt-Rasmussen U, Skakkebaek N, Main KM. Environmental chemicals and thyroid function. *European Journal of Endocrinology*. 2006;154(5):599-611.
101. Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC, Hauser R, Prins GS, Soto AM, et al. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocrine Reviews*. 2009;30(4):293-342.
102. Aneck-Hahn NH, De Jager C, Bornman MS, Du Toit D. Oestrogenic activity using a recombinant yeast screen assay (RCBA) in South African laboratory water sources. *Water SA*. 2005;31(2):253-6.
103. Aneck-Hahn NH, Schulenburg GW, Bornman MS, Farias P, Jager C. 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*. 2007;28(3):423-34.
104. De Jager C, Aneck-Hahn NH, Bornman M, Farias P, Leter G, Eleuteri P, et al. Sperm chromatin integrity in DDT-exposed young men living in a malaria area in the Limpopo Province, South Africa. *Human Reproduction*. 2009;24(10):2429-38.
105. Birnbaum LS, Staskal DF. Brominated flame retardants: cause for concern? *Environmental Health Perspectives*. 2004;112(1):9.
106. Hamers T, Kamstra JH, Sonneveld E, Murk AJ, Kester MH, Andersson PL, et al. In vitro profiling of the endocrine-disrupting potency of brominated flame retardants. *Toxicological Sciences*. 2006;92(1):157-73.
107. Zota AR, Linderholm L, Park J-S, Petreas M, Guo T, Privalsky ML, et al. Temporal comparison of PBDEs, OH-PBDEs, PCBs, and OH-PCBs in the serum of

second trimester pregnant women recruited from San Francisco General Hospital, California. *Environmental Science & Technology*. 2013;47(20):11776-84.

108. Herbstman JB, Sjödin A, Kurzon M, Lederman SA, Jones RS, Rauh V, et al. Prenatal exposure to PBDEs and neurodevelopment. *Everyday Environmental Toxins: Children's Exposure Risks*. 2015:223.

109. Eskenazi B, Chevrier J, Rauch SA, Kogut K, Harley KG, Johnson C, et al. In utero and childhood polybrominated diphenyl ether (PBDE) exposures and neurodevelopment in the CHAMACOS study. *Environmental Hazards and Neurodevelopment: Where Ecology and Well-Being Connect*. 2015:285-304.

110. Sthiannopkao S, Wong MH. Handling e-waste in developed and developing countries: Initiatives, practices, and consequences. *Science of The Total Environment*. 2013;463–464:1147-53.

111. Robinson BH. E-waste: an assessment of global production and environmental impacts. *Science of the Total Environment*. 2009;408(2):183-91.

112. Schmidt CW. Unfair trade: e-waste in Africa. *Environmental Health Perspectives*. 2006;114(4):232-35.

113. Iavicoli I, Fontana L, Bergamaschi A. The effects of metals as endocrine disruptors. *Journal of Toxicology and Environmental Health*. 2009;12(3):206-23.

114. Liu C, Ha M, Cui Y, Wang C, Yan M, Fu W, et al. JNK pathway decreases thyroid hormones via TRH receptor: a novel mechanism for disturbance of thyroid hormone homeostasis by PCB153. *Toxicology*. 2012;302(1):68-76.

115. Ogbera AO, Kuku SF. Epidemiology of thyroid diseases in Africa. *Indian Journal of Endocrinology and Metabolism*. 2011;15(6):82.

116. Tsegaye B, Ergete W. Histopathologic pattern of thyroid disease. *East African Medical Journal*. 2003;80(10):525-8.

117. Bernal J. Thyroid hormones in brain development. *Vitamins and Hormones*. 2005;31:95-112.

118. Horn S, Heuer H. Thyroid hormone action during brain development: more questions than answers. *Molecular and cellular endocrinology*. 2010;315(1):19-26.

119. Wirth EK, Schweizer U, Köhrle J. Transport of Thyroid Hormone in Brain. *Frontiers in Endocrinology*. 2014;5:1-98.

120. Mullur R, Liu Y-Y, Brent GA. Thyroid Hormone Regulation of Metabolism. *Physiological reviews*. 2014;94(2):355-82.

121. de Jesús Garduño-García J, Romero EC, Ochoa AL, Romero-Figueroa S, Bravo GH, García RT, et al. Thyroid function is associated with insulin resistance markers in healthy adolescents with risk factors to develop diabetes. *Diabetology & Metabolic Syndrome*. 2015;7(1):16.
122. Knudsen N, Laurberg P, Rasmussen LB, Bülow I, Perrild H, Ovesen L, et al. Small differences in thyroid function may be important for body mass index and the occurrence of obesity in the population. *The Journal of Clinical Endocrinology & Metabolism*. 2005;90(7):4019-24.
123. Aguirre AA, Ostfeld RS, Tabor GM, House C, Pearl MC. *Conservation medicine: ecological health in practice*: Oxford University Press; 2002.
124. Kemf E. *Global Chemicals Outlook: Towards Sound Management of Chemicals*. United Nations Environment Programme: Washington, DC, USA. 2013.
125. WHO/UNICEF. *Progress on drinking water and sanitation: 2014 update*. Sanitation Monitoring Programme. World Health Organization: World Health Organization; 2014.
126. Republic of South Africa. *Constitution of the Republic of South Africa, Act 108 of 1996*. Government Gazette. 1996;378.
127. Statistics South Africa. *Census 2011, Statistical release (Revised) P0301*. 4. Statistics South Africa, Pretoria, from <http://www.statssa.gov.za/Publications/P2012>.
128. Dabrowski JM, Shadung JM, Wepener V. Prioritizing agricultural pesticides used in South Africa based on their environmental mobility and potential human health effects. *Environmental International*. 2014;62:31-40.
129. Falconer IR, Chapman HF, Moore MR, Ranmuthugala G. Endocrine-disrupting compounds: a review of their challenge to sustainable and safe water supply and water reuse. *Environmental toxicology*. 2006;21(2):181-91.
130. Van Wyk S. *The effects of growth stimulants used at cattle feedlots, on reproductive health and thyroid function of Sprague-Dawley rats*: University of Pretoria; 2011.
131. Ingle M, Atkinson D. Can the circle be squared? An enquiry into shale gas mining in South Africa's Karoo. *Development Southern Africa*. 2015;32(5):539-54.
132. Kassotis CD, Iwanowicz LR, Akob DM, Cozzarelli IM, Mumford AC, Orem WH, et al. Endocrine disrupting activities of surface water associated with a West Virginia oil and gas industry wastewater disposal site. *Science of The Total Environment*. 2016;557:901-10.

133. Colborn T, Schultz K, Herrick L, Kwiatkowski C. An exploratory study of air quality near natural gas operations. *Human and Ecological Risk Assessment: An International Journal*. 2014;20(1):86-105.
134. Mrdjen I, Lee J. High volume hydraulic fracturing operations: potential impacts on surface water and human health. *International Journal of Environmental Health Research*. 2015:1-23.
135. Jooste S, Bollmohr S, Thwala M. National toxicity monitoring program: report on phase 3: pilot implementation and testing of the design. Report No. N/0000/REQ1008. Resource Quality Services, Department of Water Affairs and Forestry, Pretoria, South Africa.[Links], 2008.
136. Shadung JM. Prioritizing pesticides in South Africa based on their environmental mobility and potential to cause human health effects. Johannesburg, South Africa: University of Johannesburg; 2014.
137. Dabrowski JM. Investigation of the contamination of water resources by agricultural chemicals and the impact on environmental health. Volume 1: Risk assessment of agricultural chemicals to human and animal health South Africa: University of Pretoria/WRC/CSIR, CSIR; 2015 October 2015. Report No.: 1956/1/5 Contract No.: 1956/1/15.
138. Van Wyk J. Thyroid-disrupting activity in the South African aquatic environment. Cape Town: Stellenbosch University; 2013.
139. Ankley G, Erickson R, Hoff D, Mount D, Lazorchak J, Beaman J. Draft white paper: aquatic life criteria for contaminants of emerging concern, part i, general challenges and recommendations. Prepared by the Office of Water and Office of Research and Development Emerging Contaminants Workgroup Washington, DC: US Environmental Protection Agency. 2008.
140. Tijani JO, Fatoba OO, Petrik LF. A review of pharmaceuticals and endocrine-disrupting compounds: sources, effects, removal, and detections. *Water, Air, & Soil Pollution*. 2013;224(11):1-29.
141. Moe CL, Rheingans RD. Global challenges in water, sanitation and health. *Journal of water and health*. 2006;4:41.
142. WHO. Health Topics -Drinking Water 2015 [updated February 2015; cited 2015 12 March]. Available from: http://www.who.int/topics/drinking_water/en/.

143. Organization WH. Guidelines for drinking-water quality: recommendations: World Health Organization; 2004 [cited 2016 10 November 2016]. Available from: http://www.who.int/watersanitation_health/dwq.
144. Pawlowski S, Ternes T, Bonerz M, Rastall A, Erdinger L, Braunbeck T. Estrogenicity of solid phase-extracted water samples from two municipal sewage treatment plant effluents and river Rhine water using the yeast estrogen screen. *Toxicology in Vitro*. 2004;18(1):129-38.
145. Broberg K, Concha G, Engström K, Lindvall M, Grandér M, Vahter M. Lithium in Drinking Water and Thyroid Function. *Environmental Health Perspectives*. 2011;119(6):827-30.
146. EP-W E. Peer Review Results for the Amphibian Metamorphosis Assay. 2007.
147. Fini J-B, Le Mével S, Turque N, Palmier K, Zalko D, Cravedi J-P, et al. An *in vivo* multiwell-based fluorescent screen for monitoring vertebrate thyroid hormone disruption. *Environmental Science & Technology*. 2007;41(16):5908-14.
148. Miyata K, Ose K. Thyroid hormone-disrupting effects and the amphibian metamorphosis assay. *Journal of Toxicologic Pathology*. 2012;25(1):1-9.
149. Scholz S, Renner P, Belanger S, Busquet F, Davi R, Demeneix B, et al. Alternatives to *in vivo* tests to detect endocrine disrupting chemicals (EDCs) in fish and amphibians—screening for estrogen, androgen and thyroid hormone disruption. *Critical Reviews in Toxicology*. 2013;43(1):45-72.
150. Marty MS, Consult TER. AOP Title Xenobiotic Induced Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals. 2015.
151. Montañó M, Cocco E, Guignard C, Marsh G, Hoffmann L, Bergman Å, et al. New approaches assess the TTR binding capacity of bio-activated thyroid hormone disruptors. *Toxicological Sciences*. 2012;130(1):94-105.
152. Freitas J, Miller N, Mengeling BJ, Xia M, Huang R, Houck K, et al. Identification of Thyroid Hormone Receptor Active Compounds Using a Quantitative High-Throughput Screening Platform. *Current Chemical Genomics and Translational Medicine*. 2014;8:36-46.
153. Mengeling BJ, Furlow JD. Pituitary specific retinoid-X receptor ligand interactions with thyroid hormone receptor signaling revealed by high throughput reporter and endogenous gene responses. *Toxicology in Vitro*. 2015;29(7):1609-18.

154. Gutleb AC, Meerts IA, Bergsma JH, Schriks M, Murk AJ. T-Screen as a tool to identify thyroid hormone receptor active compounds. *Environmental Toxicology and Pharmacology*. 2005;19(2):231-8.
155. van der Linden SC, von Bergh AR, van Vught-Lussenburg BM, Jonker LR, Teunis M, Krul CA, et al. Development of a panel of high-throughput reporter-gene assays to detect genotoxicity and oxidative stress. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 2014;760:23-32.
156. Furlow JD, Dobrawa LN, Wei Y, Streekstra M, Neff ES, Mengeling BJ, et al. *In Vitro to In Vivo* Screening for Thyroid Hormone Receptor Active Compounds. *Metabolic Effects of Nuclear Receptors and Coregulators*. p. SUN-0408-SUN-.
157. Gutleb A, Cambier S, Serchi T. Impact of Endocrine Disruptors on the Thyroid Hormone System. *Hormone Research in Paediatrics*. 2016;86(4):1-8.
158. Thurman E, Mills M. *Soild phase extraction: principles and practise*. New York: Wiley; 1998.
159. Davey JC, Nomikos AP, Wungjiranirun M, Sherman JR, Ingram L, Batki C, et al. Arsenic as an endocrine disruptor: arsenic disrupts retinoic acid receptor-and thyroid hormone receptor-mediated gene regulation and thyroid hormone-mediated amphibian tail metamorphosis. *Environmental Health Perspectives*. 2008;116(2):165.
160. Bolong N, Ismail A, Salim MR, Matsuura T. A review of the effects of emerging contaminants in wastewater and options for their removal. *Desalination*. 2009;239(1):229-46.
161. Brand W, de Jongh C, Van der Linden SC, Mennes W, Puijier L, van Leeuwen C, et al. Trigger values for investigation of hormonal activity in drinking water and its sources using CALUX bioassays. *Environmental International*. 2013;55:109-18.



Appendix

Ethics Approval Letter

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

1/08/2016

Approval Certificate New Application

Ethics Reference No.: 533/2015

Title: Optimisation and application of the GH3.TRE.Luc Reporter Gene Bioassay to assess thyroid activity in drinking and source water

Dear Miss Hannah Simba

The **New Application** as supported by documents specified in your cover letter dated 5/07/2016 for your research received on the 1/08/2016, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 1/08/2016.

Please note the following about your ethics approval:

- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (533/2015) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:

- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

*** Kindly collect your original signed approval certificate from our offices, Faculty of Health Sciences, Research Ethics Committee, Tswelopele Building, Room 4.59 / 4.60.*

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

☎ 012 354 1677 📠 0866516047 ✉ deepeka.behari@up.ac.za 🌐 <http://www.healthethics-up.co.za>
✉ Private Bag X323, Arcadia, 0007 - 31 Bophelo Road, HW Snyman South Building, Level 2, Room 2.33, Gezina, Pretoria