Extending the EDC Toolbox 1 to include thyroid and androgenic bioassays

A Report
to the Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND

Globally, population growth, increased urbanisation, industrialisation and changes in agricultural and other land uses have contributed to reducing water quality through naturally occurring and synthetic/man-made contamination. Endocrine disrupting chemicals (EDCs) are ubiquitous in our daily lives, eventually ending up in the aquatic environment, either as a parent compound or as breakdown products. Thus, we are exposed daily to EDCs, both natural and anthropogenic sources, which may cause adverse health effects. Present as either individual chemicals or mixtures, EDCs can trigger various endocrine system impairments; including testicular, breast and prostate cancers, declining sperm counts, pregnancy loss, pubertal abnormalities, reproductive organ deformities, neurological problems, diabetes and obesity, and has been reported to alter the reproductive sexual characteristics of fish by masculinisation or feminisation. Exposure early in life can trigger the onset of diseases in childhood, later in life and in subsequent generations. Compared to other methods, the use of bioassays as screening tools for indication indicator of biological activity of chemicals has become more important, particularly in the case of those less-studied endocrine endpoints, where the causative chemical(s) is/are often unknown. This project is a continuation from a previous joint Global Water Research Coalition (GWRC)[1] project, aimed at developing and validating methods to measure estrogenic activity in water to include a range of substantially less well-studied endocrine endpoints.

AIM AND OBJECTIVES OF THE PROJECT

The GWRC EDC Toolbox II inter-laboratory project was carried out in four inter-connected work packages (WP), developed to address knowledge gaps identified during the literature review stage of this project. WP1, involved reviewing literature data on bioassays for androgenic, progestagenic, glucocorticoid, thyroid and estrogenic activity (in both agonist and antagonist modes) and determining the suitability and sensitivity of various bioassays to detect endocrine activity in water samples. WP2, involved using a relatively simple method using commercially available solid phase extraction (SPE) sorbents (such as StrataX, C18 and Oasis HLB) and elution with acetone, acetonitrile and methanol to extract a wide range of endocrine active contaminants spiked into a water sample, with reasonable average recoveries.

This WRC project only reports on WPs 3 and 4, which was aimed at stablishing the suitability and application of particular thyroid and androgenic bioassays for the detection of EDC activity in water samples. The objectives were as follows:

1. To establish and optimise the GH3.TRE.Luc thyroid bioassay at the Environmental Chemical Pollution and Health Research Unit at the University of Pretoria
2. To participate in the GWRC international, inter-laboratory and inter-assay study to test the applicability of this specific thyroid assay (GWRC-WP3)

[1] The Water Research Commission is a member to the GWRC, a non-profit organisation that serves as a focal point for the global collaboration for research planning and execution on water and wastewater related issues.

3. To participate in the GWRC international, inter-laboratory and inter-assay study to test the applicability of the GH3.TRE.Luc thyroid assay and the MDA-kb2 androgen assay on surface, tap and treated waste water from six different countries including South Africa (GWRC-WP4).

METHOD

For GWRC WP3, the GH3.TRE.Luc thyroid assay was established in the laboratory. Nine reference compounds were analysed in Stage 1. For Stage 2, three environmental waters (surface, drinking and treated sewage) and blank water (spiked and unspiked) were prepared by Technologiezentrum Wasser (TZW), Germany, and sent to participating laboratories for thyroid activity analysis. In GWRC WP4, water samples from four matrices (tap, surface, treated waste water and spiked MilliQ water) were collected from six participating countries (Australia, Germany, Spain, France, The Netherlands and South Africa). Each country was responsible for their own sample extractions and the solid phase extraction cartridges were shipped to Germany for elution and further distribution for chemical and bioassay analysis. Our laboratory was required to test the samples in the MDA-kb2 reporter gene assay for (anti)-androgenic activity and GH3.TRE.Luc for (anti)-thyroid activity.

RESULTS AND DISCUSSION

During optimisation of the GH3.TRE.Luc thyroid assay, the use of a more cost-effective alternative to the Promega kit (E1500) proved to be effective. In order to enhance cell attachment to assay plates, the addition of fetuin to serum free medium was required. Further optimisation was done for WP4, and the method was adapted according to Mengeling et al. (2013), increasing the sensitivity of the assay. Thyroid activity was detected in all the model compounds analysed. The concentrations of the compounds provided in the stock solutions were too low to be able to detect antagonistic activity. Thyroid activity was detected in all the spiked samples but not in the unspiked environmental water extracts from WP3. Androgenic activity was detected in five environmental samples from WP4. No anti-androgenic activity or cytotoxicity was observed in the samples. Thyroid activity was detected in 12 of the WP4 samples.

Surface water and spiked MilliQ water from all the countries tested positive. No anti-thyroid activity was detected in any of the samples. Overall, the results in the larger GWRC study showed very low endocrine activity in the water samples. This may be due to sample preparation; for example, the wastewater samples were only concentrated 200 times and there was extensive splitting of the samples that were to be disseminated to the various laboratories. The number of samples was also limited, six samples from four matrices, but, despite this, the results obtained in the study are in good agreement with values previously reported in literature.

CONCLUSIONS

The objective of this study with regards to establishing and optimising the GH3.TRE.Luc thyroid assay has been achieved. The applicability of the assay has been successfully tested in the GWRC WP3 and WP4. The test compounds and environmental samples from WP3 tested positive for thyroid activity. The MilliQ (spiked) and surface water samples from WP 4 also tested positive. The applicability of the MDA-kb2 assay for androgenic activity was successfully applied to the GWRC water samples for WP4. The final outcome of all the assays applied in WP3 and WP4 of the larger project will be available on the GWRC website.

RECOMMENDATIONS FOR FUTURE RESEARCH

- Currently there is no trigger value available for thyroid activity in drinking water. Further research to determine this is recommended as this value is extremely important when doing a health risk assessment.
- Research has focused mainly on oestrogenic activity, but it is clear that EDCs also affect other pathways, including the hypothalamic pituitary thyroid axis. Studies have reported associations between exposure to thyroid disrupting chemicals and neurobehavioral disorders, obesity and reproductive abnormalities, among others. It is important to consider including thyroid activity in the EDC Toolbox.
- Although a trigger value of 11 ng dihydrotestosterone equivalent/L has been proposed by Brand et al. (2013), it may be necessary to adapt this value for South African conditions.

ACKNOWLEDGEMENTS

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Research Manager	Affiliation
Dr K Murray	Water Research Commission (2011- 2013)
Dr N Kalebaila	Water Research Commission

Collaborators	Affiliation
Dr F Leusch	Griffith University, Australia - GWRC Project leader
Dr M Schriks	KWR, The Netherlands - GWRC WP 3 leader
Dr A Hebert	Veolia, France - GWRC WP 4 leader

Other	Affiliation
Prof T Murk (Thyroid cell line)	Wageningen University, The Netherlands

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LIST OF ABBREVIATIONS

AH	Amiodarone hydrochloride
AR	Androgen receptor
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CO_2	Carbon dioxide
DHT	Dihydrotestosterone
DHTEq	Dihydrotestosterone equivalent
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EBT	Effects based trigger
ECPH Research Unit	Environmental Chemical Pollution and Health Research Unit
EDCs	Endocrine disrupting chemicals
ETU	Ethylene thiourea
FBS	Foetal bovine serum
GR	Glucocorticoid receptor
GWRC	Global Water Research Coalition
HCl	Hydrochloric acid
KWR	Kiwa Water Research
$MgCl_2$	Magnesium chloride
MMTV	Mouse mammary tumour virus
MTT	Memorandum of tissue transfer
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate buffered saline
PCP	Pentachlorophenol
PPAR	Peroxisome proliferator receptor
RGM	Regular growth medium
RP	Relative potency
RLU	Relative light unit
RFU	Relative fluorescence unit
RXR	Retinoid X receptor
SA	Sodium arsenite
SPE	Solid phase extraction
T3	Triiodothyronine
T4	Thyroxine
TEq	Thyroid equivalent
THBP	2,2,4,4-Tetrahydroxybenzophenone
THR	Thyroid hormone receptor
TTR	Transthyretin

TPO	Thyroid hormone peroxidase
TZW	Technologiezentrum Wasser
UP	University of Pretoria
WP	Work plan

CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Natural and anthropogenic (synthetic) compounds are produced and used in our daily lives and may eventually end up in the aquatic environment, either as a parent compound or as breakdown products (frequently unknown) (Van der Linden, 2013). Endocrine disrupting chemicals (EDCs) are ubiquitous in our daily lives, and exposure to these compounds, either as individual compounds or mixtures, can have effects on the endocrine system; for example, they can alter the reproductive sexual characteristics of fish by masculinisation or feminisation (Van der Linden et al., 2008). The water industry is faced with the challenge of ensuring a sustained and safe supply of drinking water from sources of varying quality, including the reuse of wastewater. As these compounds occur as mixtures in water, it is challenging and costly to identify individual chemicals using analytical methods. The detection of individual chemicals is not sufficient to safeguard water quality, unless the chemical composition of the water is known and chemicals are present at relatively high concentrations (Sumpter and Johnson, 2008; Van der Linden, 2013). Toxicological data is limited or non-existent for chemicals and their degradation products present in the aquatic environment, making the assessment of their impact on human and environmental health difficult and challenging (Van der Linden, 2013). Many of the compounds are found at low doses in the environment but may have a high potency. However, there is no threshold for EDC effects due to the presence of active hormone pathways, and EDCs are likely to have effects at low doses (Van der Linden, 2013; Bergman et al., 2013). It is also important to note that while individual compounds might not elicit effects at low concentrations, their combined presence may still be important, especially if they act on the same pathway (Silva et al., 2002).

Most research in South Africa, until recently, has focused on the effects of endocrine disrupting chemicals (EDCs) on the oestrogenic system (Freitas, 2012). However, the endocrine system is not limited to the reproductive axis; other hormone-driven signalling systems like progestogens, glucocorticoids, retinoids and thyroid hormones play a critical role in maintaining processes such as homeostasis (He et al., 2010), sexual development (Li and Kim, 2004), metabolism (Wiegratz and Kuhl, 2006), growth (Wightman et al., 2002) and behaviour (Mani and Oyola, 2012) throughout the different life-stages of a number of species (OECD, 2012; Christen et al., 2010, Freitas, 2012; Bergman et al., 2013). Assays have been developed to assess some of the modes of action, but only a limited number have been validated beyond an academic setting (Hartung, 2009; Hartung, 2010; Van der Linden, 2013) and even fewer in an environmental setting (Escher and Leusch, 2012; GWRC, 2012). In order to be able to apply a bioassay with reliability and a degree of accuracy, the test needs to be validated and standardised, usually by means of a ring test in an inter-laboratory quality control programme. The current project extended the Global Water Research Coalition (GWRC) Toolbox I that identified key bioassays (ER-CALUX, T47D-KBluc and E-screen assays) as reliable and repeatable platforms to evaluate oestrogenic activity in a variety of water samples.

The aim of the GWRC Toolbox II project was to develop and validate methods that will test a range of other endocrine endpoints such as androgenic, thyroid, progestogen, glucocorticoid, retinoid (RXR) and peroxisome proliferator (PPAR) activity for a variety of water samples. The project team developed a series of work plans (WPs) to achieve this goal.

WP1: To conduct a meta-analysis of available androgenic and progestogenic assays.

WP2: To develop and validate extraction methods to recover progestogenic, glucocorticoid, retinoid, PPAR and thyroid active compounds from water samples.

WP3: To conduct an inter-assay ring test between laboratories using the different thyroid assays to find the most reliable and repeatable assay.

WP4: To apply the GWRC battery of bioassays to measure endocrine activity in three different water matrices.

Several assays were reviewed in the first phase of the project in the form of a literature review (GWRC, 2012). A workshop, held at Kiwa Water Research (KWR) in Nieuwegein, the Netherlands, in 3-4 October 2011, discussed the findings of the literature review and concluded that each endpoint was at a different stage of development and validation:

- Androgenic and (to a lesser extent) progestogenic activity has undergone sound inter-laboratory and inter-assay validation with model compounds. While these comparisons did not test water samples, water data from other publications is available and a meta-analysis of all available literature would provide a broad understanding of the suitability of each assay to water testing.
- Several bioassays are available to measure thyroid activity, but it is unclear how relevant each assay is to water monitoring. Different assays measure thyroid interference at a different level, and this endpoint would greatly benefit from a methodical inter-assay comparison to determine the suitability of these assays to test thyroid activity in water.
- Little is known about glucocorticoid, RXR and PPAR activity in water. These endpoints have been identified as high priority by a recent Organisation for Economic Co-operation and Development (OECD) review on endocrine disruption. With causal links to metabolism and obesity, these endocrine endpoints need to be assessed in water sources.

A few of these assays have been applied patchily to measure endocrine activity in waste water. There is little information on non-oestrogenic endocrine activity in other waters, including drinking, surface and ground water. Together, the work plans will fill some of the data gaps identified from the Phase 1 literature review and enhance analytical capabilities within GWRC membership and in South Africa. The EDC Laboratory in the Environmental Chemical Pollution and Health (ECPH) Research Unit at the University of Pretoria has been selected by the GWRC as one of a number of international laboratories to take part in the project, specifically for WP3 and WP4.

1.2 AIM

To participate in the Global Water Research Coalition EDC Toolbox II inter-laboratory study to establish the suitability of certain thyroid and androgenic bioassays for the detection of EDC activity in water samples

1.3 OBJECTIVES

1. To establish and optimise the GH3.TRE.Luc thyroid bioassay at the ECPH Research Unit at the University of Pretoria (UP)
2. To participate in the GWRC international inter-laboratory and inter-assay study to test the applicability of this specific thyroid assay (GWRC-WP3)
3. To participate in the GWRC international inter-laboratory and inter-assay study to test the applicability of the GH3.TRE.Luc thyroid assay and the MDA-kb2 androgen assay on surface water, tap water and treated waste water from six different countries, including South Africa (GWRC-WP4)

1.4 LIMITATIONS

As indicated in the GWRC WP4 report (Hebert et al., 2016), budget constraints allowed for only limited sampling, i.e. only one grab sample per water matrix in each of the six countries. Therefore, only a brief 'snapshot' in time was provided. However, the results seem to compare favourably to other studies in literature which implies that the results do provide a meaningful 'snapshot'. Samples are designated by the country of origin, but the samples do not necessarily accurately represent the status of the water quality in the whole country.

CHAPTER 2: EXPERIMENTAL STUDIES

2.1 INTRODUCTION

The EDC Laboratory in the ECPH Research Unit at the University of Pretoria was selected by the GWRC as one of a number of international laboratories to take part in a project to develop and validate methods that will test a range of endocrine endpoints for a variety of water samples. The ECPH was specifically invited to participate in WP3 and WP4. WP 3 entails conducting an inter-assay ring test between laboratories using different thyroid assays to find the most reliable and repeatable assay. WP4 concerns the application of the GWRC battery of bioassays to measure endocrine activity in three different water matrices.

2.2 WORK PACKAGE 3

2.2.1 Optimisation of the GH3.TRE.Luc thyroid bioassay

The thyroid assay is a luciferase reporter gene assay that was developed based on the thyroid hormone responsive rat pituitary tumour GH3 cell line that constitutively expresses both thyroid hormone receptor (THR) isoforms. Stable transfection of the pGL4CP-SV40-2xtaDR4 construct into the GH3 cells resulted in a highly sensitive cell line (GH3.TRE.Luc), which was further optimised into an assay that allowed the detection of triiodothyronine (T3) and thyroxine (T4) concentrations in the picomolar range.

The ECPH Research Unit at the University of Pretoria signed a memorandum of tissue transfer agreement with Wageningen University in the Netherlands in 2013 that allowed the EDC laboratory to obtain the relevant cells to establish the thyroid assay in South Africa. The assay was set up according to the method described in Freitas et al. (2011).

Cells were maintained in regular growth medium (RGM) consisting of Dulbecco's Modified Eagle Medium (DMEM)/F12 (1:1) with 15 mM HEPES and supplemented with 10% foetal bovine serum (FBS). Before exposure, the cells were depleted of thyroid hormone by incubating them in serum free medium (PCM) for 24 hrs. PCM medium is DMEM/F12 (1:1) with 15 mM HEPES supplemented with 10 µg/mL bovine insulin, 10 µM ethanolamine, 10 ng/mL sodium selenite, 10 µg/mL human apotransferrin, 500 µg/mL bovine serum albumin (BSA) and 800 µg/mL fetuin. Stock solutions for the control and the test chemicals were 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 below 0.5%). Cells were incubated for 24 hrs in the presence (0.25 nM) or absence of T3, with or without the indicated test sample in DMSO. After rinsing with phosphate buffered saline (PBS), the cells were lysed by adding 25 µL lysis buffer to each well, followed by one freeze/thaw cycle. Luciferase activity was determined on lysed cells using a LUMIstar OPTIMA luminometer (BMG Labtech,

Offenburg, Germany). The luminometer was programmed to inject 25 µL reaction buffer (25 mM glycylglycine, 15 mM magnesium chloride (MgCl2), 5 mM adenosine triphosphate (ATP), 0.1 mg/mL BSA, pH 7.8) followed by 25 µL luciferin, 5 seconds later, into each well.

2.2.2 GWRC samples

The GWRC WP 3 lead laboratory (KWR, The Netherlands) sent out test samples in two stages to test in the assay: Stage 1 - bioassay analysis of model compounds in DMSO. Stage 2 - bioassay analysis of environmental water extracts.

2.2.2.1 *Stage 1*

Nine reference compounds in DMSO (a total of 2 mL in a 1 mM concentration) were prepared by Technologiezentrum Wasser (TZW) and sent to KWR (Table 2-1). KWR sent aliquots of the respective stock solutions to the participating laboratories for the generation of concentration response curves and the accompanying relative potencies (RP values).

Table 2-1: List of the nine selected compounds included in Stage 1

Sample ID	Compound	CAS no	TR[1]	TTR[2]	TPO[3]	*In vivo* priority	Use in Stage 2 spike
1	Triiodothyronine (T3)	6893-02-03	Agonist	Agonist			
2	Thyroxine (T4)	51-48-9	Agonist	Agonist		*YES*	*YES*
3	TRIAC (T3-like analogue)	51-24-1	Agonist	Agonist			
4	TETRAC (T4-like analogue)	67-30-1	Agonist	Agonist			
5	Amiodarone	19774-82-4	Antagonist			*YES*	
6	Pentachlorophenol (PCP)	87-86-5		Antagonist		*YES*	
7	Ethylene thiourea (ETU)	96-45-7			Antagonist		*YES*
8	2,2,4,4-Tetrahydrobenzo-phenone (THBP)	131-55-5			Antagonist		
9	Methimazole	60-56-0		Antagonist	Antagonist	*YES*	
10	Solvent only (negative control)						

[1] Thyroid hormone receptor (THR), [2]Transthyretin (TTR) and [3]Thyroid hormone peroxidase (TPO)

2.2.2.2 *Stage 2*

Three environmental waters (surface water, drinking water and treated sewage effluent) and a blank (milliQ water) were sampled and extracted by solid phase extraction (SPE) according to a protocol established by TZW. The extracts in organic phase were subsequently divided in two groups:

Group 1 - The first group (termed non-spiked environmental extracts) was split in two aliquots from which one part was utilised for chemical analysis by the project partners, while the other part was distributed to the various laboratories for bioassay testing;

Group 2 - The second group (termed spiked environmental extracts) was spiked with T4 and ethylene thiourea (ETU) after SPE extraction, then split into two aliquots from which one part was utilised for chemical analysis (TZW - Germany), while the other part was distributed to the various laboratories for bioassay testing.

2.3 WORK PACKAGE 4

2.3.1 Sampling and extraction of water

Water samples from four matrices were collected and extracted on 200 mg StrataX (phenomenex) SPE cartridges at the UP's EDC laboratory. These samples were 2 L x2 tap water; 1 L x1 surface water; 200 mL x1 treated waste water and 1 L x1 dihydrotestosterone (DHT) spiked MilliQ water from the EDC laboratory, for quality control. The pH of the samples was adjusted to 2 using hydrochloric acid (HCl) before extraction. The detailed extraction procedure and work flow is described in Figure 2-1. The dried cartridges were sent to TZW Karlsruhe in Germany for distribution to participating laboratories.

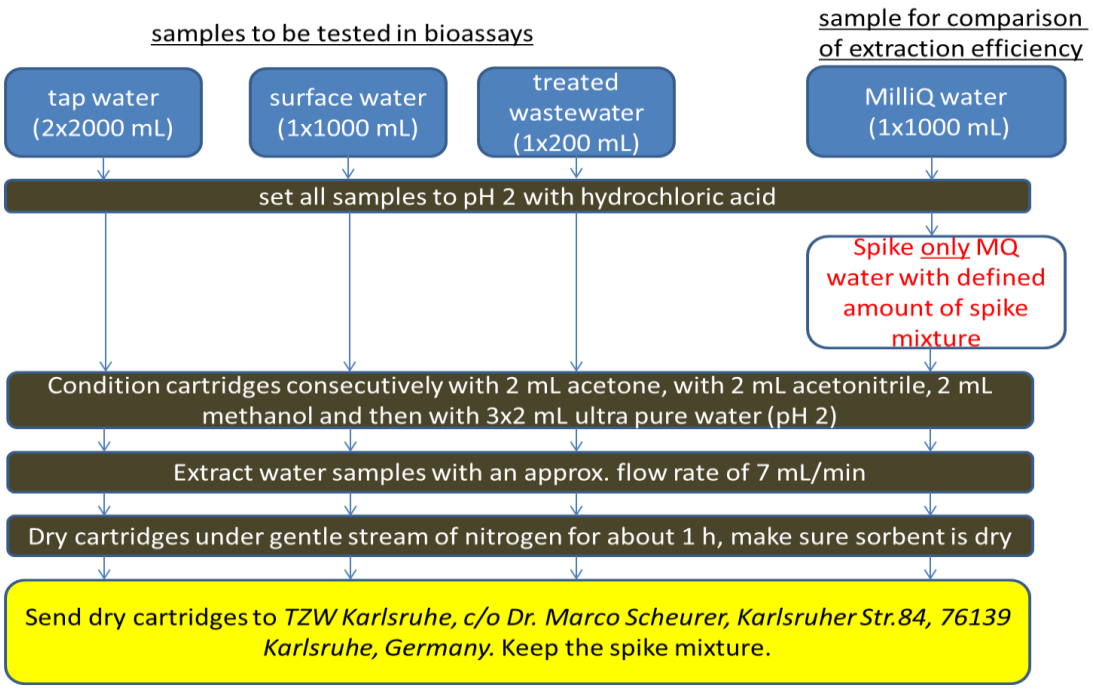

Figure 2-1: Extraction procedure and work flow for WP 4 samples

2.3.2 Reconstitution of the test samples

Twenty-four samples, sourced from six different countries (Australia, Germany, Spain, France, Netherlands and South Africa), were received from TZW Karlsruhe, Germany, to be tested in the MDA-kb2 and GH3.TRE.Luc thyroid bioassays. The samples had been reconstituted in 100 µL methanol. This was evaporated and the residues reconstituted in 100 µl DMSO. Enrichment factors were 200x for waste water, 400x for tap water and 1000x for MilliQ and surface water respectively.

2.3.3 MDA-kb2 reporter gene assay for (anti)-androgenic activity

In principle, the MDA-kb2 reporter gene assay is an androgen-responsive transcriptional activation assay (Wilson et al., 2002). It uses the MDA-kb2 cell line, transformed from MDA-MB-453 human breast cancer cells (Wilson et al., 2002). It was transformed with an androgen-responsive luciferase reporter plasmid which is driven by the mouse mammary tumour virus (MMTV) promoter (Wilson et al., 2002). Both the nuclear androgen receptor (AR) and glucocorticoid receptor (GR) are expressed by the cell line and can both activate the MMTV reporter gene. Briefly, upon binding of an agonist onto the receptor, transcription of the reporter gene is activated and the luciferase enzyme is produced (Wilson et al., 2002). This enzyme is directly proportional to the active ligand concentration. This is assayed using chemiluminescence, which measures the light produced when exogenous luciferin substrate together with its co-factors are added (Wilson et al., 2002; de Jager et al., 2011). Since the cell line expresses both the GR and the AR, which are both responsive to the MMTV, the source driving response must be distinguished. This is done by co-administering an AR antagonist (hydroxyflutamide) (Wilson et al., 2002).

The assay was conducted according to Wilson et al. (2002), with minor modifications. Briefly, the cells were seeded at 5 x104 cells per well (96 well plate) and incubated for 24 hrs at 37°C in a carbon dioxide (CO_2) free incubator before being exposed to samples and standards. A screening approach was used to first identify samples with androgenic/anti-androgenic activity. The activity was then confirmed with full dose response curves in triplicate. The seeded cells were either exposed to test samples alone (for androgenic activity) at four different concentrations, or test samples co-incubated with 1 nM DHT for anti-androgenic activity. Controls included in all plates were 1 nM DHT positive control for androgenic activity, vehicle control (0.5% DMSO in L-15 media) and 10 µM hydroxyflutamide as a control for anti-androgenic activity. Final DMSO concentration did not exceed 0.5% in the wells.

Following exposure, plates were returned to the incubator for 24 hrs, and thereafter the reactions were terminated by washing once with PBS, after observation for cytotoxicity under a light microscope. A volume of 25 µL reporter lysis buffer was then added to each well and luciferase activity (chemiluminescence) was read, after one freeze-thaw cycle using LUMIstar OPTIMA luminometer (BMG Labtech, Offenburg, Germany) which dispensed 25 µL of both reaction buffer and luciferin. Samples that showed a greater than two-fold induction above the vehicle control were considered positive, and triplicate plates with full dose response curves were assayed to quantify the activity.

2.3.4 GH3.TRE.Luc thyroid bioassay

The method for thyroid activity in WP4 was adapted from Freitas et al. (2011), with modification from Mengeling et al. (2015). Cells were seeded into a white, clear-bottom 96 well microplate at 5x104 cells per well (100 µL per well) and allowed to attach overnight. After 24 hrs, RGM was replaced with 100 µL/well of the PCM medium and incubated for a further 24 hrs to deprive cells of thyroid hormones.

Stock solutions for the control and the test chemicals were prepared in DMSO at least 200x the highest required final test concentration (to ensure that when diluted in PCM the DMSO concentration was below 0.5%). The following stock solutions were prepared in DMSO and stored at room temperature in the dark: 1000 nM T3 (Sigma cat no T2877), 1000 nM T4 (Sigma cat no T1775), 15 µM amiodarone hydrochloride (AH) (Sigma cat no A8423), 100 µM sodium arsenite (SA) (Sigma cat no S7400). All the test chemicals were diluted in PCM. To expose the cells, PCM was removed from the wells and 100 µL of the test chemical in PCM was transferred to each well. 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 (0.5% DMSO in PCM). Single plates were first tested as part of the screening process.

After analysis of the screening plates, triplicate plates were exposed for 24 hrs (full dose response curves), for samples with relative light unit (RLU)/relative fluorescence unit (RFU) above the detection limit. After a 24-hr exposure period, the cells were rinsed twice with PBS, before 20 µL of 1x lysis reagent was added to each well. The luciferase cell culture lysis reagent (Promega cat no E3971) was prepared by adding 4 volumes of water to 1 volume of 5x reporter lysis buffer. The 96 well plate was placed into a luminometer that has two injectors. The luminometer was programmed to add 25 µL luciferase assay reagent followed by 25 uL reaction buffer per well and to read immediately for 10 seconds. The reaction was measured in RLU. Cytotoxicity was determined using the resazurine cell proliferation assay. After adjustment for cytotoxicity, thyroid activity was determined. A sample was considered to have thyroid activity if it had an RLU/RFU value above 2 standard deviations from the control.

Cell proliferation and viability was determined by measuring the cells metabolic activity using resazurine dye. Resazurine is a non-toxic, water soluble and non-radioactive dye. It can be reduced by the GH3 cells to a pink fluorescent colour, from the original blue, making a resorufin complex. After 24 hrs exposure, 8 µL resazurine (400 µM) was added to each well and incubated in the dark for 4 hrs (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-sterilised 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 emissions. A chemical was considered cytotoxic if the fluorescence was less than the fluorescence of the vehicle control minus 3x the standard deviation. The resazurine assay does not require cell lysis, does not interfere with the luciferase activity measurements and can be done on the same plate.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 OPTIMISATION OF THE GH3.TRE.LUC THYROID BIOASSAY

In order to achieve objective 1, the method had to be optimised before sample analysis. The luciferase activity of cells exposed to a concentration range of T3 (0.0001–1000 nM) was determined using a Promega kit (E1500) and compared to an alternative method. Similar curve fits were obtained for both methods (Figure 3-1), but the alternative method was selected to be used for this project, as the method is more cost effective and better repeatability was obtained using this method.

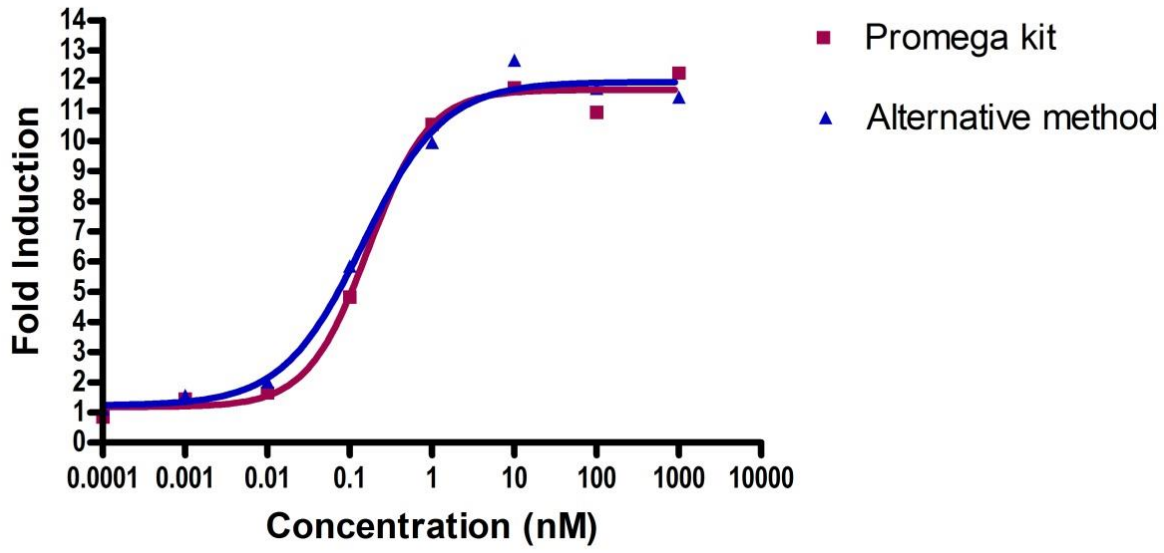

Figure 3-1: Curve fit for luciferase activity of T3 using a Promega kit and alternative method

The method also required the addition of fetuin to the serum free medium, as an additional growth factor to stimulate attachment, spreading and growth of the GH3 rat pituitary cells. By adding fetuin to the medium, fewer cells are lost when rinsing the cells with PBS before lysing, resulting in better repeatability (Figure 3-2 and 3-3).

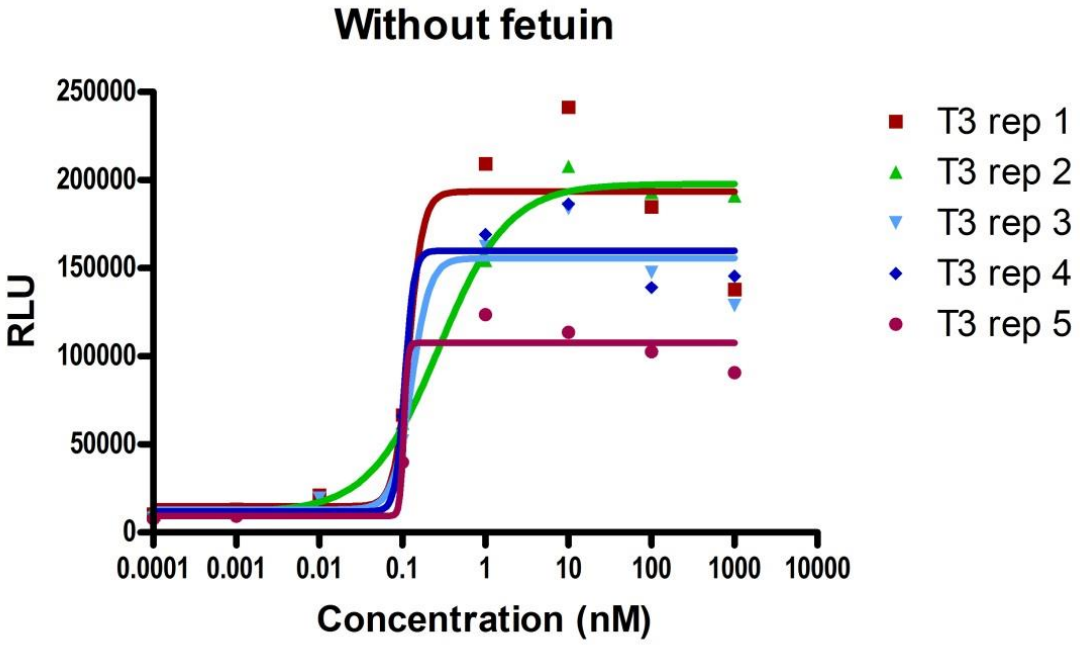

Figure 3-2: T3 curve fit for cells without the addition of fetuin to the serum free medium

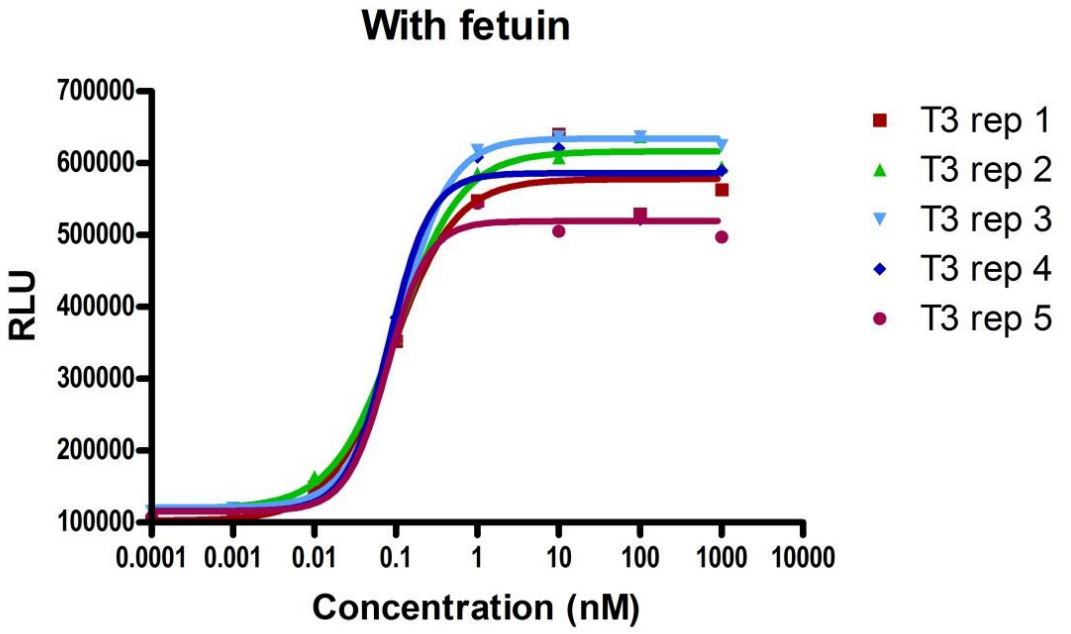

Figure 3-3: T3 curve fit for cells with the addition of fetuin to the serum free medium

3.2 GWRC – WP3

3.2.1 Stage 1- Bioassay analysis of model compounds

Model compounds provided by KWR were compared in the bioassay to a T3 control (Sigma-Aldrich, cat no T2877). Four of the compounds (T3, T4, TRIAC and TETRAC) displayed agonist activity (Figure 3-5). The EC50s and relative potencies of the compounds are given in Table 3-1.

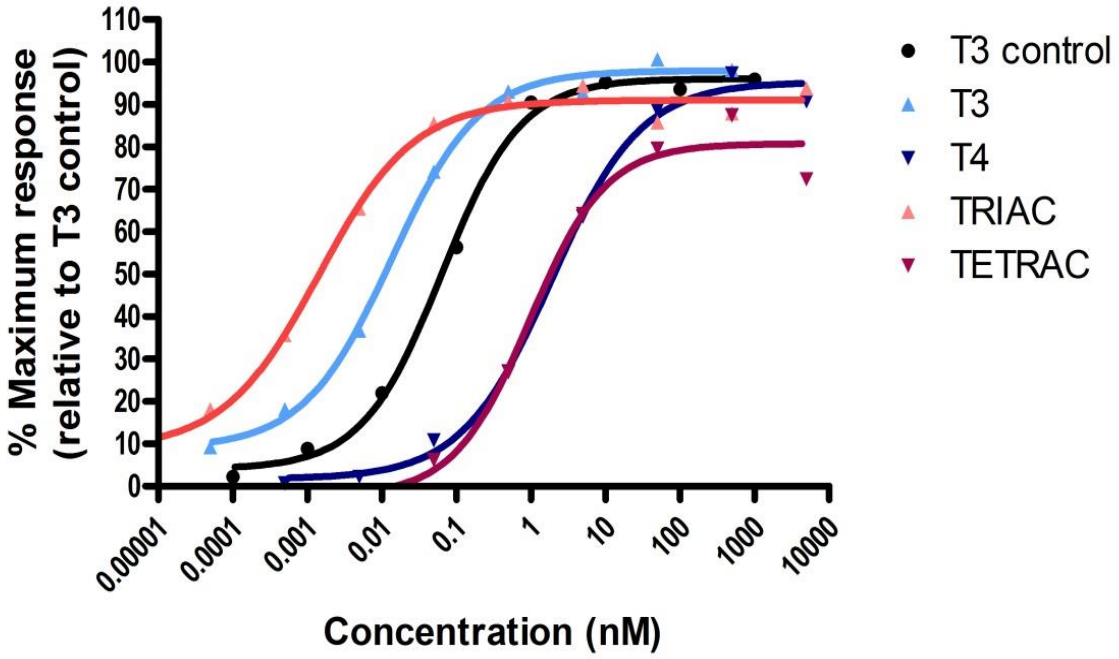

Figure 3-4: Agonist activity of model compounds

Table 3-1: EC50s and relative potencies of model compounds

Compound	EC50 ± SD (M)	RP
T3 control	6.5E-11 ± 1.1E-11	1
T3	1.4E-11 ± 3.1E-12	4.7
T4	1.9E-09 ± 5.6E-10	0.03
TRIAC	1.4E-12 ± 9.6E-14	46
TETRAC	1.1E-09 ± 3.5E-10	0.06

RP Relative potency = EC50 (T3 control) / EC50 (compound)

To test for antagonist activity, compounds were co-incubated with 0.25 nM T3 (Figure 3-6). A 1 mM concentration of each compound was provided by KWR to test in the assays. The dilution factor to test the samples for antagonist activity is 400x, therefore the highest test concentration was 2.5 µM for each compound. Only the highest concentration of pentachlorophenol (PCP) was able to inhibit T3 activity (13% reduction), but a higher stock concentration would be required in order to calculate the EC50. No inhibition was seen for the amiodarone compound provided. However, AH (Sigma, cat no A8423) was able to inhibit T3 activity from 5 µM in the assay. The stock concentrations provided were therefore not high enough to test for antagonist activity in this assay.

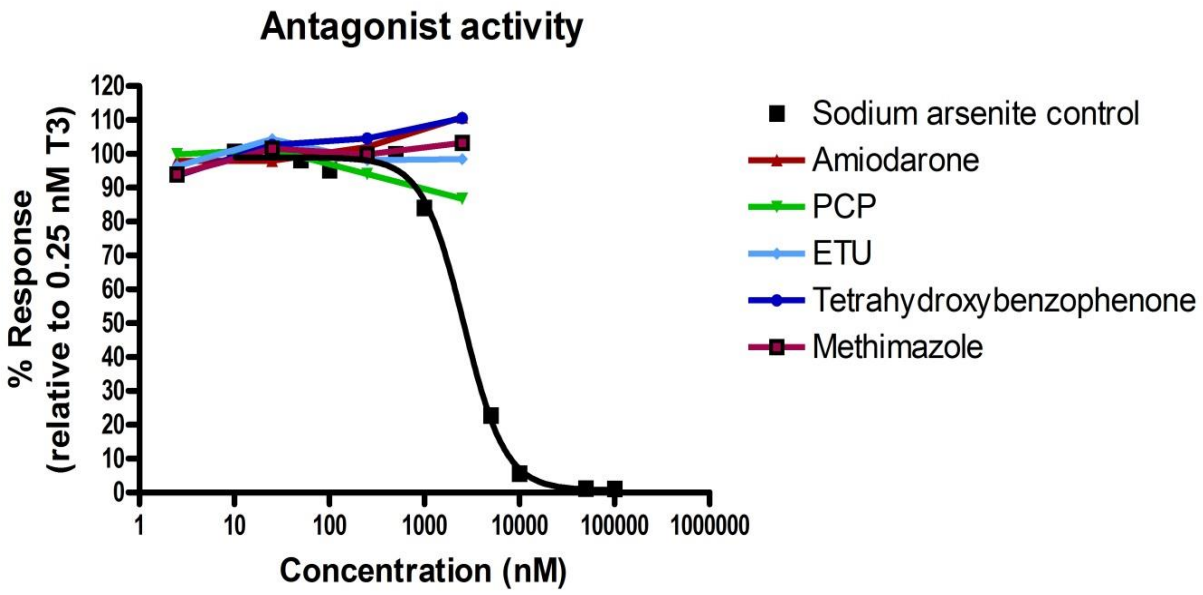

Figure 3-5: Antagonist activity of model compounds

3.2.2 Stage 2 - Bioassay analysis of environmental water extracts

The extracts were received from KWR in methanol. The methanol was evaporated and the samples were reconstituted in 100 µL DMSO for analysis in the bioassay. None of the unspiked environmental water extracts showed agonist activity. All the spiked samples showed agonist activity (Figure 3-7). The spiked surface water and spiked wastewater had sub-maximal responses that can be attributed to cytotoxicity observed at the highest test concentrations.

No antagonist activity was detected in any of the spiked or unspiked environmental extracts. Although a reduction in T3 activity was seen when co-incubated with the highest test concentrations of the spiked and unspiked waste and surface water, this could be attributed to the cytotoxicity at these concentrations, rather than antagonist activity. The T3 equivalent values for the spiked water samples are given in Table 3-2.

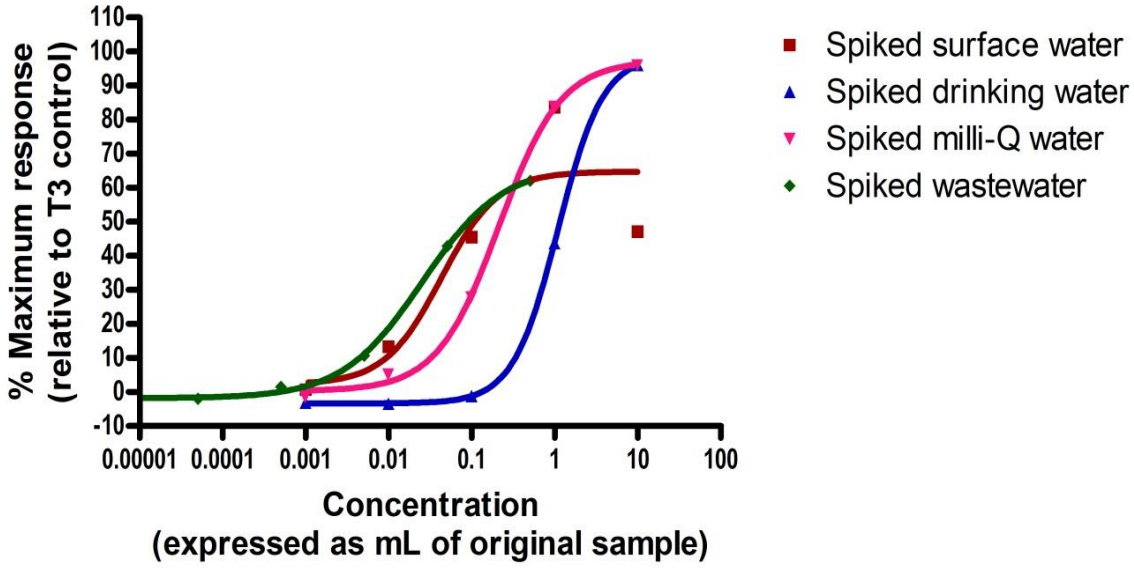

Figure 3-6: Agonist activity of spiked water samples

Table 3-2: T3 equivalent values for spiked water samples

Sample	T3 equivalent ± SD (M)
Spiked surface water	1.3E-10 ± 5.3E-12
Spiked drinking water	1.1E-11 ± 9.2E-13
Spiked milliQ	4.0E-11 ± 4.2E-12
Spiked wastewater	1.7E-10 ± 4.3E-11

3.3 GWRC – WP4

3.3.1 Androgenic activity

3.3.1.1 *Validation of the MDA-kb2 reporter gene assay for (anti)-androgenic activity*

Figure 3-8 depicts the DHT standard curves in RLU. Using the validation of the DHT standard curve, 1 nM DHT was selected as the positive control. The fold induction achieved at this concentration was 6, indicating a good measure of activity. Figure 3-9 depicts 1 nM DHT co-incubated with hydroxyflutamide. The 10 µM hydroxyflutamide concentration was selected as antagonist control.

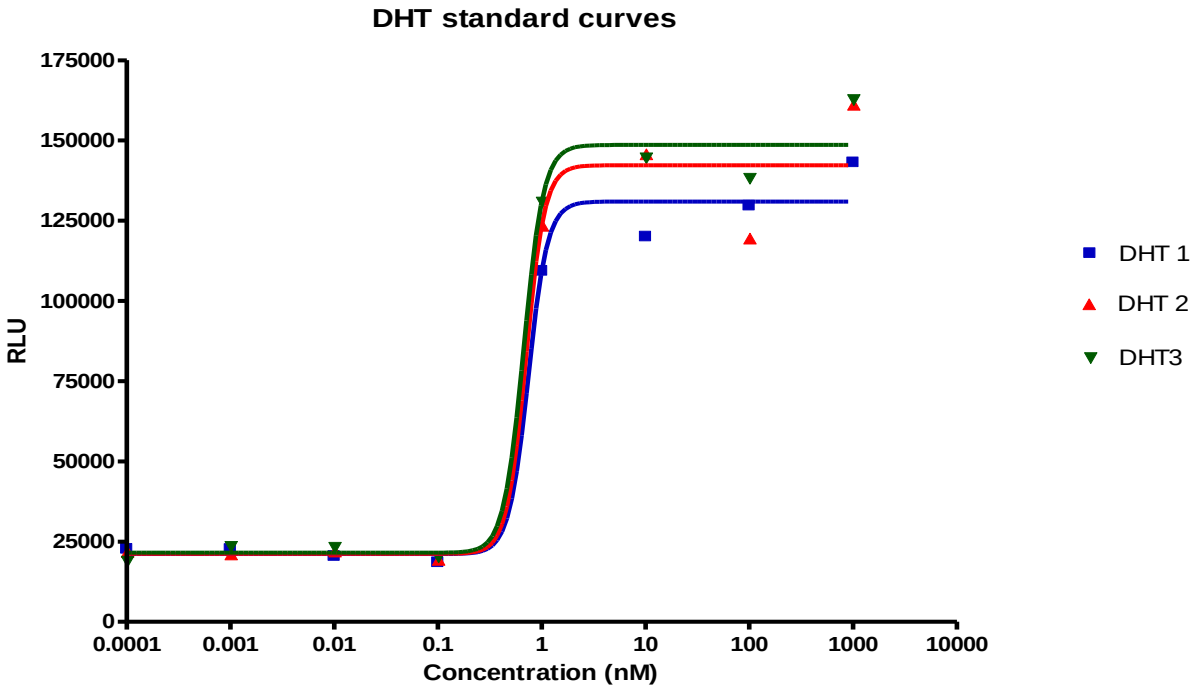

Figure 3-7: Standard curve for DHT in RLUs

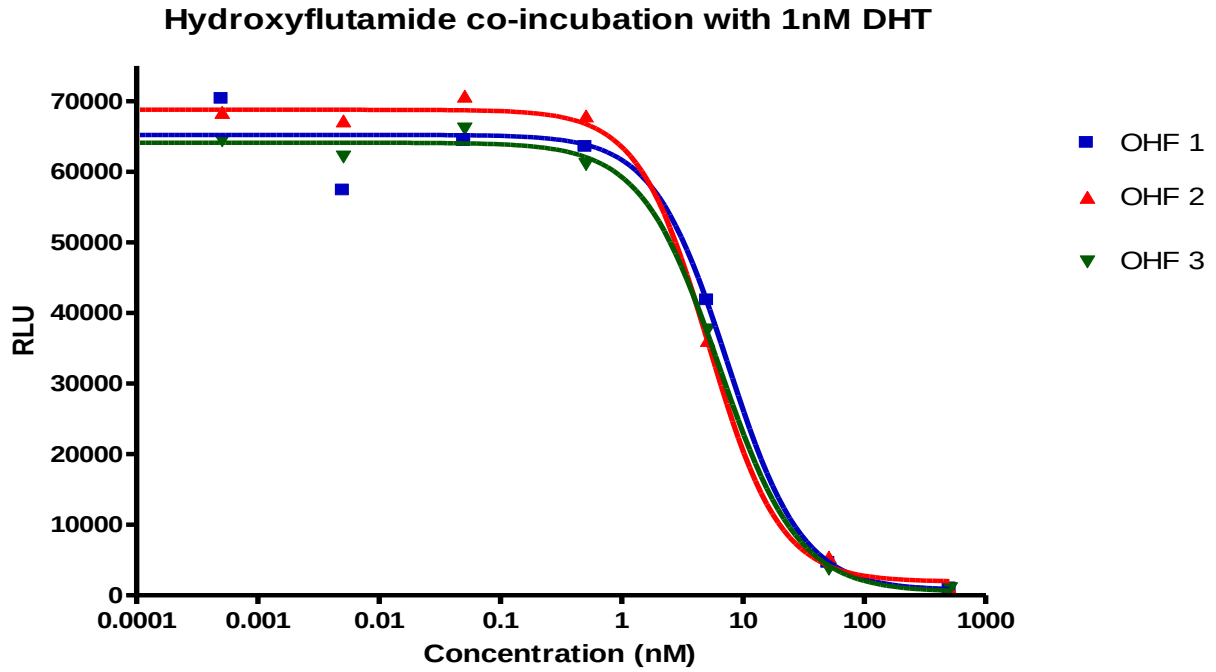

Figure 3-8: A graph indicating the dose dependent suppression of 1 nM DHT co-incubated with hydroxyflutamide

3.3.1.2 *GWRC samples – MDA-kb2 reporter gene assay*

Androgenic activity was detected in five samples, with dihydrotestosterone equivalent (DHTEq) values ranging from below the detection limit to 0.232 ng/L (Table 3-4). No anti-androgenic activity or cytotoxicity was detected in any of the samples.

Table 3-3: A summary of the inter-laboratory GWRC samples screening results in the MDA-kb2 androgen bioassay.

Water matrices	Sample ID	Cytotoxicity	Androgenic activity (DHTEq ng/L)	Anti-androgenic activity
MilliQ Water (spiked)	TZW - Germany	-	<dl	<dl
	Griffith University - Australia	-	<dl	<dl
	VERI - France	-	<dl	<dl
	UP – South Africa	-	<dl	<dl
	Cirsee - Spain	-	<dl	<dl
	KWR - Netherlands	-	<dl	<dl
Tap Water	TZW - Germany	-	**0.008 ± 0.0009**	<dl
	Griffith University - Australia	-	<dl	<dl
	VERI - France	-	<dl	<dl
	UP – South Africa	-	<dl	<dl
	Cirsee - Spain	-	<dl	<dl
	KWR - Netherlands	-	<dl	<dl
Surface Water	TZW - Germany	-	**0.028 ± 0.006**	<dl
	Griffith University - Australia	-	<dl	<dl
	VERI - France	-	<dl	<dl
	UP – South Africa	-	<dl	<dl
	Cirsee - Spain	-	**0.068 ± 0.008**	<dl
	KWR – Netherlands	-	<dl	<dl
Treated Waste Water	TZW – Germany	-	<dl	<dl
	Griffith University - Australia	-	<dl	<dl
	VERI - France	-	<dl	<dl
	UP – South Africa	-	<dl	<dl
	Cirsee - Spain	-	**0.028 ± 0.03**	<dl
	KWR – Netherlands	-	**0.232 ± 0.04**	<dl

-: not observed microscopically; <dl: below detection limit

3.3.2 Thyroid activity

3.3.2.1 *Optimisation and validation of the GH3.TRE.Luc thyroid bioassay*

For optimisation of the assay, T3 was used as the positive control. The capability of the assay was confirmed by its capability to detect T3 at the picomolar range. Results were comparable to those found in literature (Freitas et al., 2011). Figures 3-10 and 3-11 show the T3 curve (in triplicate) for thyroid activity and the results from the resazurine assay respectively. The results are expressed as a RLU/RFU (calculated from the RLU and RFU readings respectively), RLU for luciferase activity and RFU for the resazurine assay.

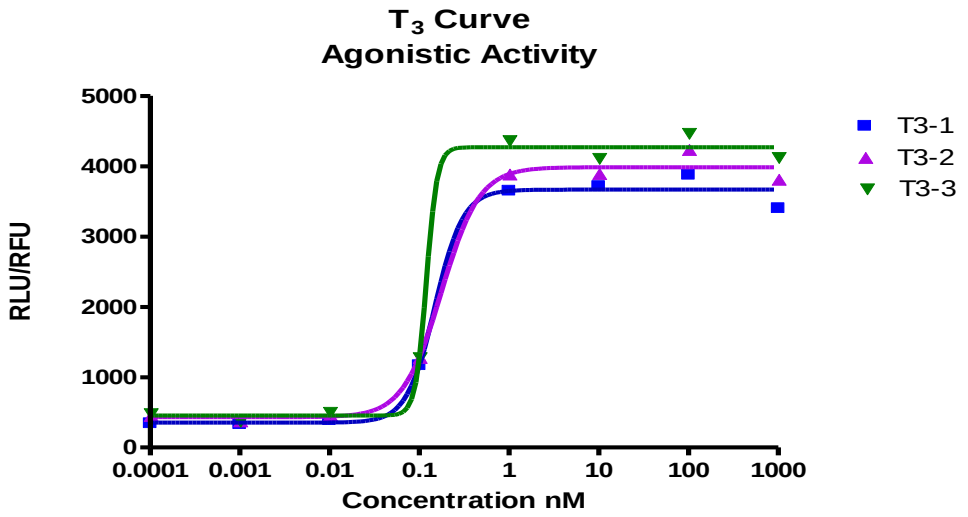

Figure 3-9: The T3 activity in the thyroid assay, with concentrations in nM ranging from 0.0001 to 1000

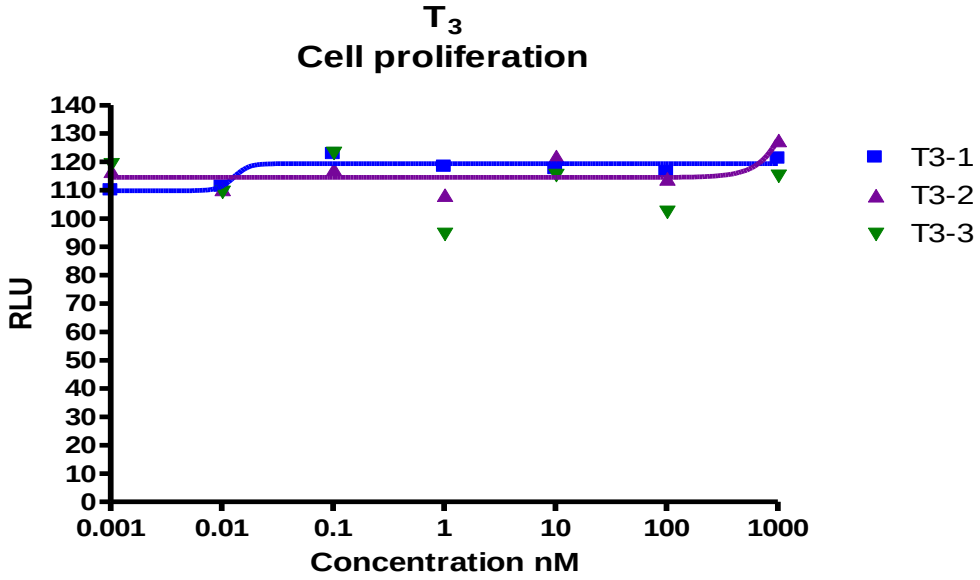

Figure 3-10: The T3 resazurine assay results, with concentrations in nM ranging from 0.0001 to 1000

A sigmoidal curve fit was obtained for the T3 curve, validating thyroid hormone activity. The results were comparable to those reported by Freitas et al. (2011). In the resazurine assay, all the tested T3 concentrations were within 3 standard deviations of the vehicle control, indicating no cytotoxicity.

SA and AH were evaluated in the assay as controls for anti-thyroid activity. These were co-incubated with 0.25 nM T3 and tested for thyroid activity and cytotoxicity. Figures 3-12 and 3-13 show graphical illustrations of the thyroid and the resazurine assays respectively.

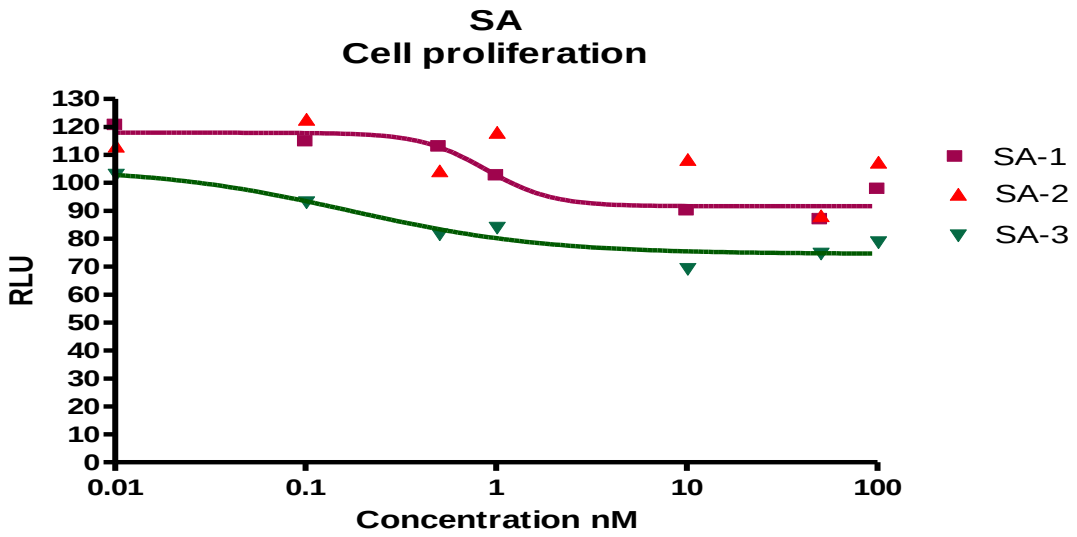

Figure 3-11: The effect on the GH3.TRE.Luc cells over a range of SA concentrations after 24-hour co-incubation with 0.25 nM T3

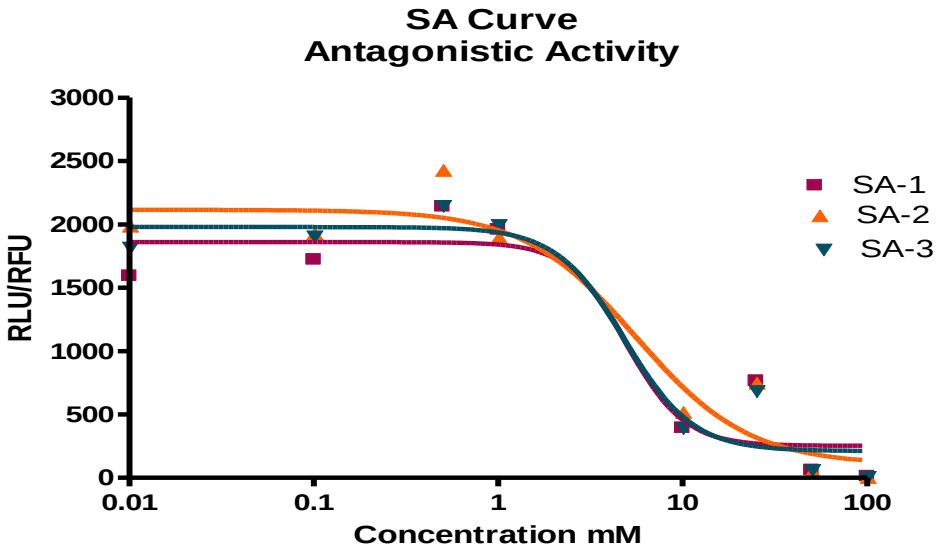

Figure 3-12: The effect on the GH3.TRE.Luc cells over a range of SA concentrations after 24-hour co-incubation with 0.25 nM T3

The high activity shown at the lower concentrations can be attributed to the 0.25 nM T3. As the concentrations of SA increase, its anti-thyroid properties are reflected through the decreased activity from 1 µM. No cytotoxicity was seen, indicating that the decrease in activity is due to SA's antagonist activity and not due to cytotoxicity. SA is therefore an appropriate control to use for antagonist activity. Figures 3-14 and 3-15 show the thyroid activity and resazurine assay results for AH respectively.

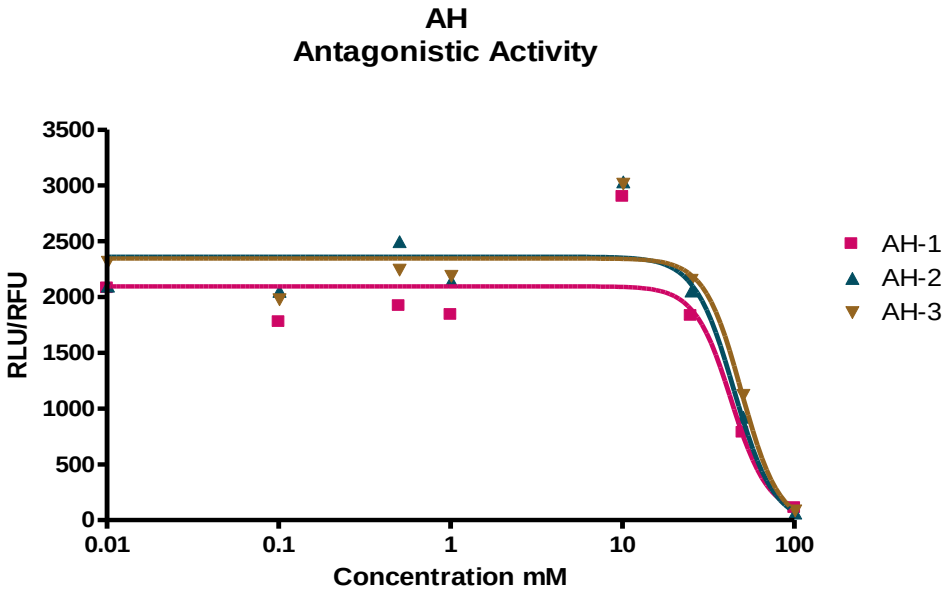

Figure 3-13: The graph illustrates the effect of AH on the GH3.TRE.Luc cells when incubated with 0.25 nM T3

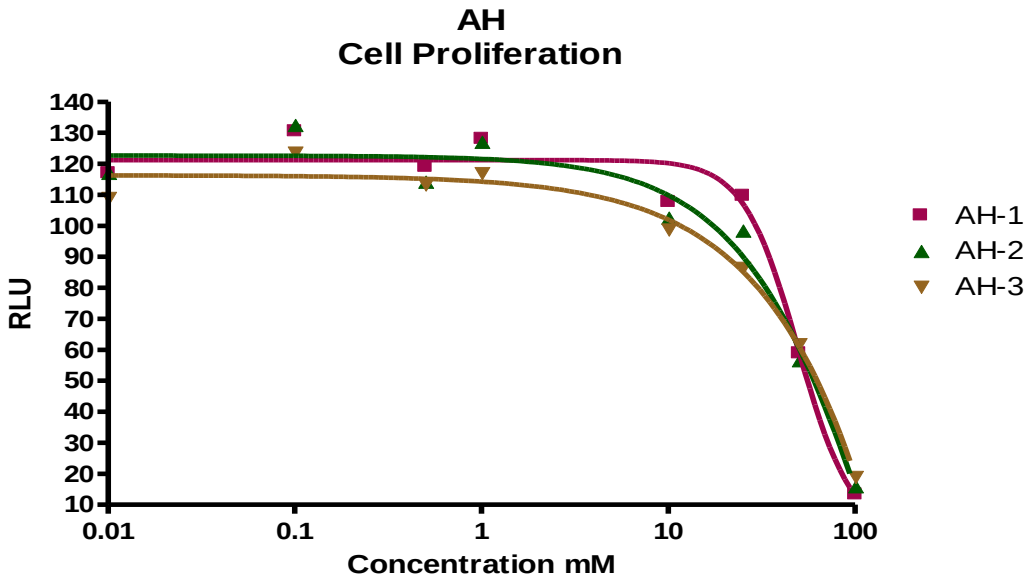

Figure 3-14: The graph illustrates the effect of AH on the GH3.TRE.Luc cells when incubated with 0.25 nM T3

The thyroid assay showed a decrease in thyroid activity from 10 nM. In the resazurine assay no cytotoxicity is detected until 25 µM where there is a clear decrease in cell viability. The resazurine results in this case indicate that the decrease in thyroid activity after 25 nM is as a result of cytotoxicity rather than anti-thyroid activity. Therefore, it was decided to rather use SA as a control for anti-thyroid activity.

3.3.2.2 *GWRC Samples - GH3.TRE.Luc thyroid bioassay*

Optimisation and validation using samples from the GWRC

The nine 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/anti-thyroid activity (AH, PCP, ETU, 2,2,4,4-Tetrahydrobenzo-phenone (THBP) and methimazole). Antagonistic activity was determined by the chemicals' ability to suppress the THR activity of 0.25 nM T3. Figure 3-16 shows the dose response curves obtained from the four GWRC agonists tested.

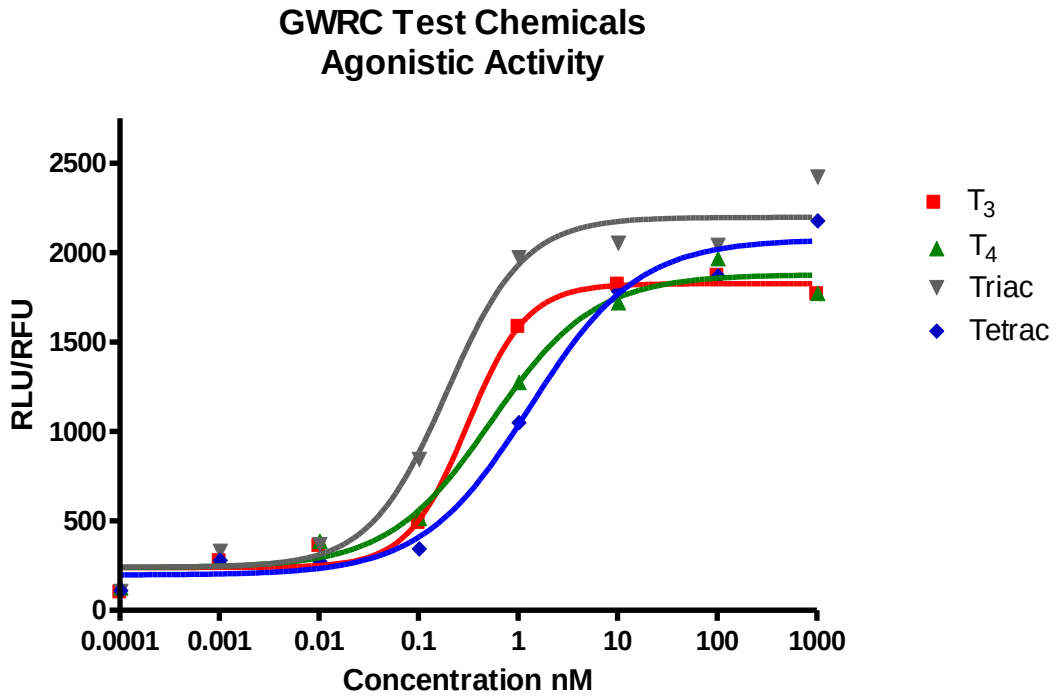

Figure 3-15: The graphs show GH3.TRE.Luc induction at concentration 0.0001 to 1000 nM of the agonists T3, T4, TRIAC (T3 analogue) and TETRAC (T4 analogue) after 24 hrs

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 RP of TETRAC, as reported in literature (Gutleb et al., 2015; Freitas et al., 2011). 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 are reported (Gutleb et al., 2005). Table 3-5 shows the EC50 and relative potency results from the agonists.

Table 3-4: Showing the EC50 and relative potency of the agonists

Compound	EC50 (nM)	Relative Potency (RP)
T_3	0.17	1
T_4	2.67	0.07
TETRAC	0.16	1.10
TRIAC	0.93	0.19

The five antagonists (AH, PCP, ETU, THBP and methimazole) were also tested for their ability to antagonise 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 supress THR activity. Only two chemicals were able to supress 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 of T3 mediated THR activity for the two chemicals are as follows: ethylene thiourea (39%) > PCP (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 four 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 3-6 shows the thyroid equivalents (TEq) obtained from the positive spiked samples in ng/L.

Table 3-5: 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

Application of the assay to GWRC samples WP4

The assay was then applied to the GWRC water samples according to WP4 guidelines. Figures 3-17 to 3-20 show the thyroid activity screening results for all of the water samples. Results are presented as RLU/RFU for the thyroid activity.

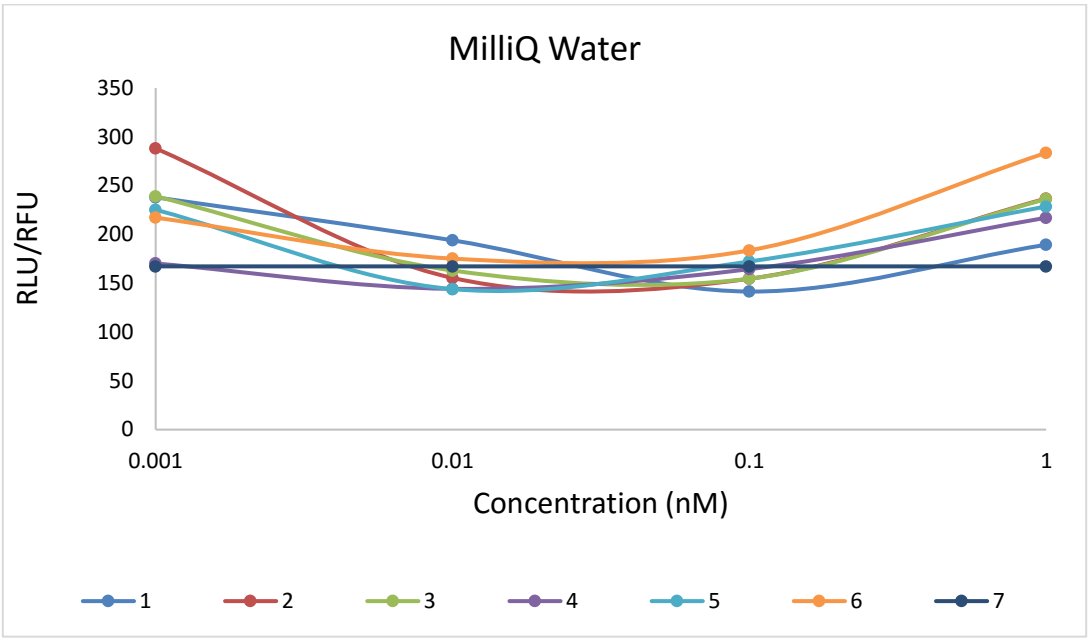

Figure 3-16: Graph showing the screening results (RLU/RFU) of MilliQ water from the respective laboratories. Key: 1-Cirsee - Spain; 2-TZW - Germany; 3-UP - South Africa; 4-VERI - France; 5-KWR - Netherlands; 6-Griffith University - Australia; 7-Vehicle Control (DMSO)

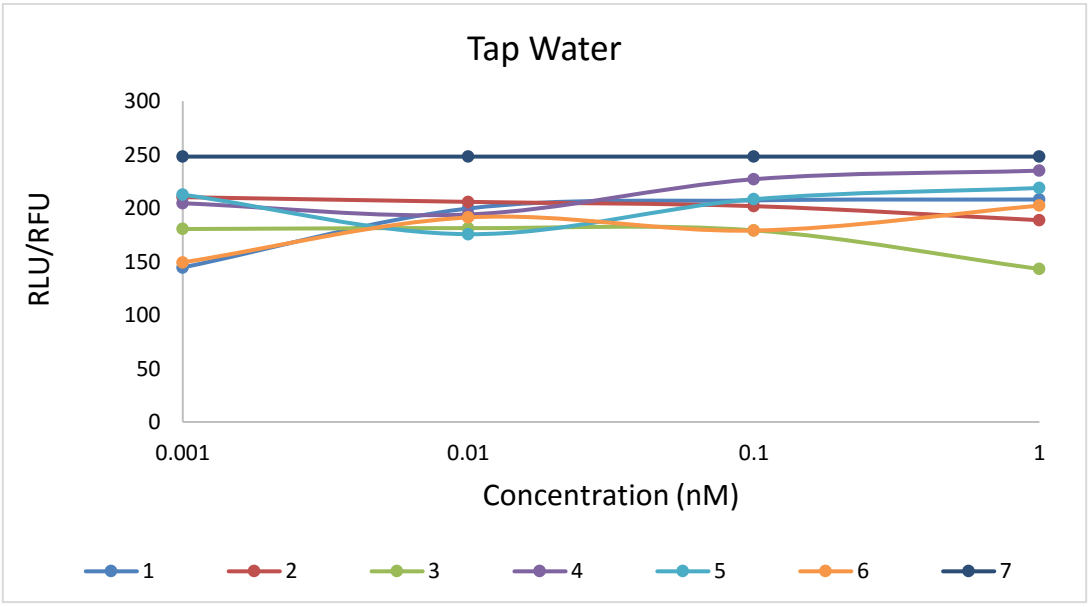

Figure 3-17: Graph showing the screening results (RLU/RFU) of tap water from the respective laboratories. Key: 1-Cirsee - Spain; 2-TZW - Germany; 3-UP – South Africa; 4-VERI - France; 5-KWR - Netherlands; 6-Griffith University - Australia; 7-Vehicle Control (DMSO)

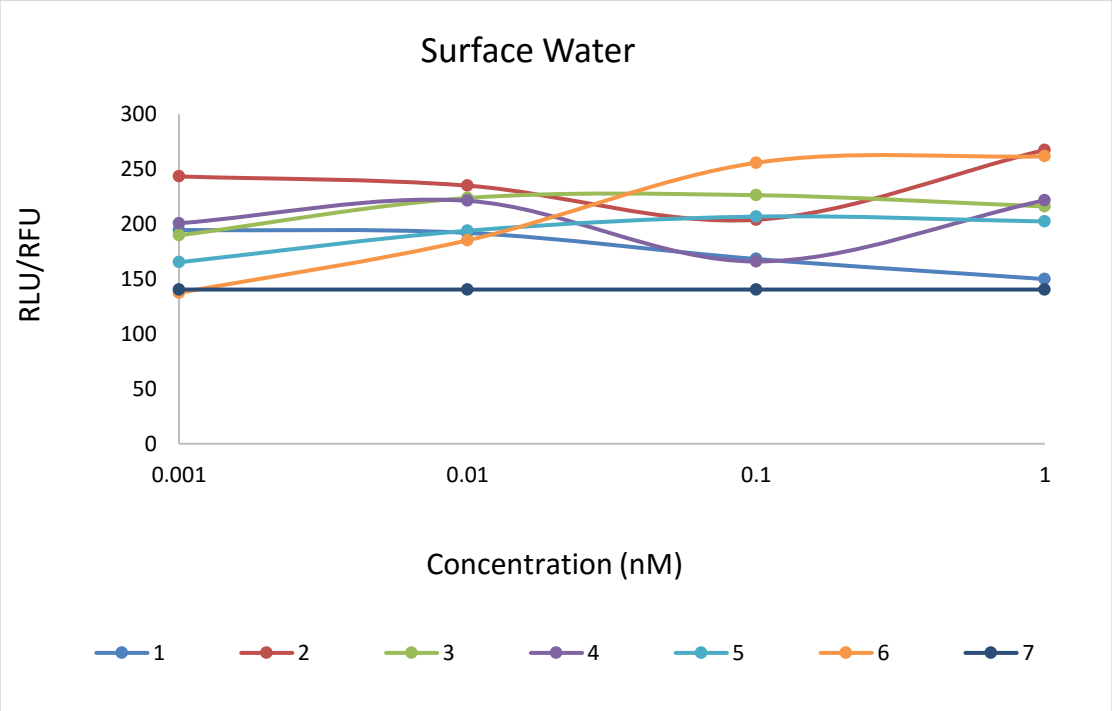

Figure 3-18: Graph showing the screening results (RLU/RFU) of surface water from the respective laboratories. Key: 1-Cirsee - Spain; 2-TZW - Germany; 3-UP - South Africa; 4-VERI - France; 5-KWR - Netherlands; 6-Griffith University - Australia; 7-Vehicle Control (DMSO)

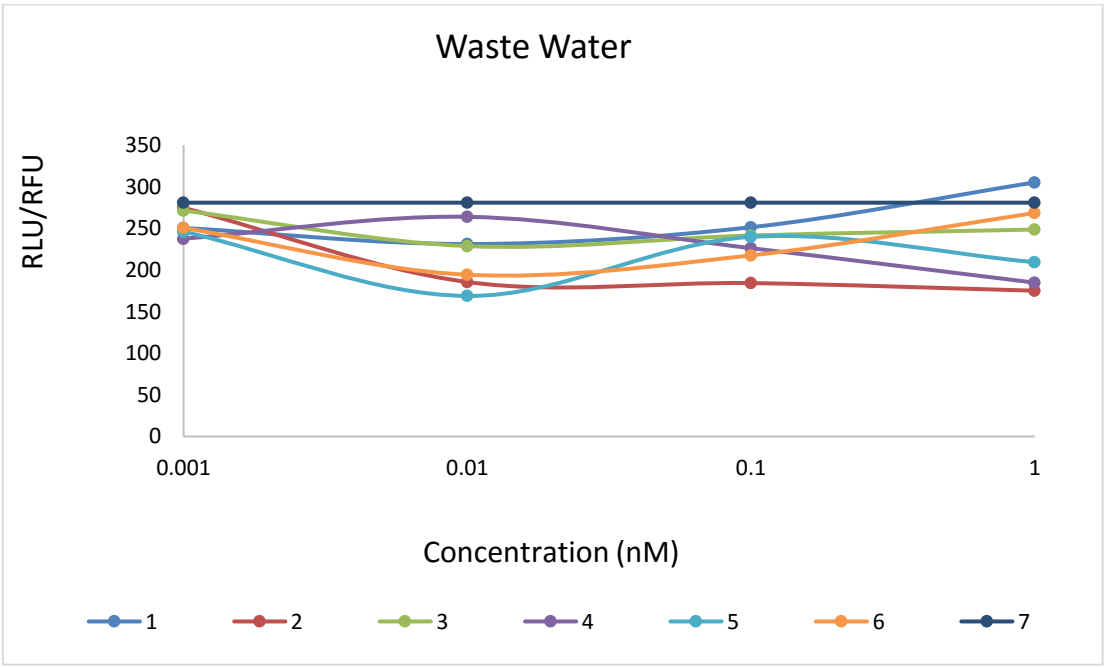

Figure 3-19: Graph showing the screening results (RLU/RFU) of waste water from the respective laboratories. Key: 1-Cirsee - Spain; 2-TZW - Germany; 3-UP-South Africa; 4-VERI - France; 5-KWR - Netherlands; 6-Griffith University - Australia; 7-Vehicle Control (DMSO)

The TEq values of the samples are given in Table 3-6. Twelve of the samples were above the detection limit. No anti-thyroid activity was detected in any of the samples. The resazurine assay showed that all samples were within 3 standard deviations of the vehicle control, indicating no cytotoxicity.

Table 3-6: A summary of the inter-laboratory GWRC sample results in the thyroid bioassay

Water matrices	Sample ID	TEq (ng/L)
MilliQ Water (spiked)	Vehicle Control	<dl
	Cirsee - Spain	**7.53 ± 0.005**
	TZW – Germany	**44.14 ± 0.04**
	UP - South Africa	**14.09 ± 0.01**
	VERI - France	**19.43 ± 0.02**
	KWR - Netherlands	**16.41 ± 0.02**
	Griffith University - Australia	**6.17 ± 0.005**
Tap Water	Vehicle Control	<dl
	Cirsee - Spain	<dl
	TZW – Germany	<dl
	UP - South Africa	<dl
	VERI - France	<dl
	KWR - Netherlands	<dl
	Griffith University - Australia	<dl
Surface Water	Vehicle Control	<dl
	Cirsee - Spain	**2.78 ± 0.002**
	TZW – Germany	**4.36 ± 0.003**
	UP - South Africa	**5.27 ± 0.004**
	VERI - France	**3.41 ± 0.002**
	KWR - Netherlands	**65.15 ± 0.07**
	Griffith University - Australia	**44.27 ± 0.07**
Treated Waste Water	Vehicle Control	<dl
	Cirsee - Spain	<dl
	TZW - Germany	<dl
	UP - South Africa	<dl
	VERI - France	<dl
	KWR - Netherlands	<dl
	Griffith University - Australia	<dl

TEq: T_3 equivalents; <dl: below detection limit

3.4 DISCUSSION

3.4.1 Thyroid activity

3.4.1.1 *GWRC - WP3*

Agonistic activity could be detected for the reference compounds and spiked environmental water samples. However, antagonistic activity was too low to detect. The cells did not attach optimally to the 96 well microplate. The recommended use of fetuin, which helps with cell attachment, resulted in an increase in background activity, possibly compromising the sensitivity of the assay. Options to reduce the high background were further investigated and applied in the GWRC-WP4.

3.4.1.2 *GWRC – WP4*

Modifications were made to the original assay by Freitas et al. (2011). Specific modifications to the exposure timeline solved the attachment problem and fetuin was no longer used (Mengeling et al., 2015). This increased the sensitivity and effectiveness of the assay.

The GH3.TRE.Luc reporter gene bioassay was successfully optimised and validated using known agonists and antagonists. The assay proved to be a sensitive and effective tool to identify and quantify thyroid activity in pure chemicals and in water samples containing complex environmental mixtures. It is a relatively rapid test, able to detect thyroid activity within 24 hrs of exposure; in addition, it has two thyroid receptor isoforms.

The method was applied to the 24 samples from six different countries in three different water matrices and spiked controls. The spiked MilliQ water and surface water samples from all six countries had thyroid activity, with TEq's ranging from 6.17 to 44.14 ng/L and from 2.78 to 65.15 ng/L respectively. The fact that the spiked MilliQ water had high activity indicates that the assay may be useful to detect thyroid activity in water samples.

3.4.2 Androgenic activity - GWRC – WP4

Achieving the objectives, the MDA-kb2 reporter gene bioassay was successfully applied on 24 samples from six different countries in three different water matrices and spiked controls (MilliQ) to measure androgenic/anti-androgenic activity. The results showed that five of the samples were above the detection limit. These were however below the trigger value (11 ng DHTEq/L) proposed by Brand et al. (2013). The assay may, in future, be a beneficial tool to monitor androgen disruptors in water systems as it is sufficiently sensitive to detect low concentrations of (anti)-androgenic activity.

3.5 SUMMARY

Four different water samples (waste water, surface water, drinking water and ultrapure control water), from six countries (Germany, Australia, France, South Africa, The Netherlands and Spain), were applied to *in vitro* bioassays targeting a range of nuclear receptors in the GWRC WP4, of which this study was SA's contribution. As part of the larger international study, the water samples were analysed in parallel by comprehensive chemical methods, which included high-resolution mass-spectrometry screening (results not shown, but can be found in the GWRC report) (Hebert et al., 2016).

Overall, the results in the larger GWRC study showed very low endocrine activity in water samples. This may be due to sample preparation, for example the wastewater samples were only concentrated 200 times, and extensive splitting of the samples that were to be disseminated to the various laboratories. The number of samples was also limited, six samples from four matrices, but despite this the results obtained in the study are in good agreement with values previously reported in literature (Hebert et al., 2016).

Bioassays for endocrine activity are extremely sensitive, therefore detection of activity in water does not immediately suggest a significant risk of endocrine disruption. Researchers have produced effects based trigger (EBT) values that indicate a human or ecological health risk. They are useful to use as a benchmark to indicate when endocrine activity may lead to adverse health effects (Hebert et al., 2016). When assessing endocrine activity in waste water and surface water in relation to the EBT values, the levels may exceed the trigger values, possibly by several orders of magnitude, and pose a risk to the aquatic environment (Hebert et al., 2016). In this current study, it appears that this is not the case for drinking water. The endocrine disruptive activity measured was below the relevant EBT values relevant to human health, suggesting a less significant health risk.

The bioassay results seemed to be in agreement with the predictions from the chemical analysis, often higher than predicted. This suggests the presence of additional chemicals that were possibly below the chemical detection limit or not monitored for. In cases where there was no detectable activity, the predicted activity in the chemical analysis was below detection limit. Bioassays and chemical analysis can be used as complementary tools to assess water quality (Hebert et al., 2016).

CHAPTER 4: CONCLUSIONS AND RECOMMENDATIONS

4.1 CONCLUSIONS

- The objective of the study with regards to establishing and optimising the GH3.TRE.Luc thyroid assay has been achieved.
- The applicability of the assay has been successfully tested in the GWRC WP3 and WP4. The test compounds and environmental samples from WP3 tested positive for thyroid activity. The MilliQ (spiked) and surface water samples from WP 4 were also positive.
- The applicability of the MDA-kb2 assay for androgenic activity was successfully applied to the GWRC water samples for WP4.
- The final outcome of all the assays applied in WP3 and WP4 of the larger project will be available on the GWRC website.

4.2 RECOMMENDATIONS

- Currently there is no trigger value available for thyroid activity in drinking water. Further research to determine this is recommended as this value is extremely important when doing a health risk assessment.
- Research has focused mainly on oestrogenic activity, but it is clear that EDCs also affect other pathways, including the hypothalamic pituitary thyroid axis. Studies have reported associations between exposure to thyroid disrupting chemicals and neurobehavioural disorders, obesity and reproductive abnormalities, among others. It is important to consider including thyroid activity in the EDC Toolbox.
- Although there is an international trigger value of 11 ng DHTEq/L, proposed by Brand et al. (2013), it may be necessary to adapt this value for South African conditions.

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