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# **The suitability of estrogen and androgen bioassays for the measurement of endocrine activity in different water matrices**

By

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## Dedication

*“We must have perseverance and above all, confidence in ourselves. We must believe that we are gifted for something and this thing must be attained.”*

*~ Marie Curie*

I dedicate this work to my late sister Nompumelelo Patience Ngcobo

## Declaration

I, Silindile Ngcobo, hereby declare that this dissertation titled “The suitability of estrogen and androgen bioassays for the measurement of endocrine activity in different water matrices”, is my original work and has never been submitted for examination at the University of Pretoria or any other institution. In cases where other sources have been used, the original authors have been acknowledged.

Signature: .....

Date: .....

## Summary

Endocrine disrupting chemicals (EDCs) are ubiquitous in the environment and their presence in water bodies is documented. They discharge into surface water (SW) unmonitored, posing a threat to both aquatic and terrestrial lives. This is a challenge as not all populations have access to treated drinking water (TDW). The EDC contaminated serves as a route of exposure, together with ineffective treatment plants. Given the complexity of the endocrine system, EDCs may mimic or antagonise natural hormones or disrupt their synthesis, metabolism and excretion. The associated health effects include testicular dysgenesis syndrome, metabolic disorders and cancers. Policy and internationally standardised test methods are however still limited. This study therefore aimed to assess the suitability of two assays used for screening estrogenic activity and one for androgenic activity in different water sources.

The study consisted of two phases. In phase 1, water sample (tap, surface and treated wastewater) were collected from a catchment area in Pretoria. The samples and a spiked MilliQ laboratory water sample were extracted with solid phase extraction (SPE) and sent to Germany for distribution to participating laboratories. Samples (n=24) from six different countries were received to test for androgenic activity in the MDA-kb2 reporter gene assay. In phase 2, SW and TDW samples were collected from April 2015 until March 2016. The samples were filtered, extracted using SPE and assayed with the YES assay, T47D-KBluc reporter gene assay for estrogenic activity and MDA-kb2 reporter gene assay for androgenic activity.

In phase 1, androgenic activity was detected in 4 out of 24 (21%) samples and ranged from  $0.23 \pm 0.040$  ng/L to  $0.008 \pm 0.001$  ng/L DHTEqs. In phase 2, estrogenic activity was detected in 16 out of 24 (67%) SW samples in the T47D-KBluc reporter gene assay and ranged from  $0.31 \pm 0.05$  pg/L to  $10.51 \pm 5.74$  pg/L EEqs. It was below the detection limit (dl) in the YES assay. Androgenic activity was detected in 4 out of 24 (17%) SW samples, ranging from  $0.0033 \pm 0.0050$  ng/L to  $0.090 \pm 0.040$  ng/L DHTEqs. Androgenic and estrogenic activity was higher in pre-treatment samples compared to post-treatment in both treatment plants.

In phase 1, the MDA-kb2 reporter gene assay was successfully applied to water samples from different sources. Androgenic activity was highest in treated wastewater. In phase 2, treatment plants proved to be effective in removing estrogens detected in the SW samples, as the TDW samples were below the dl. Estrogenic activity is within the ranges reported in other studies. Positive samples were below the 0.7 ng/L proposed trigger value for health risk assessments. Detected androgenic activity was lower in TDW samples compared to the SW samples supplying the two treatment plants indicating that they were both effective in removing the androgenic activity detected. Few studies have reported androgenic activity in tap water.

This study strengthens the argument for using a battery of assays when monitoring endocrine activity as EDCs occur at low concentrations in mixtures.

**KEY WORDS:** Surface water, drinking water, endocrine disrupting chemicals (EDCs), estrogenic activity, androgenic activity, *in vitro* bioassay, YES assay, T47D-KBluc reporter gene assay, MDA-kb2 reporter gene assay, water monitoring

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## Abbreviations

|                    |  |
|--------------------|--|
| ADI                | Acceptable daily intake                        |
| AFP                | $\alpha$ -fucoprotein                          |
| AGD                | Anogenital distance                            |
| ANOVA              | Analysis of variance                           |
| AR                 | Androgen receptor                              |
| As                 | Arsenic  |
| ATCC               | American Type Culture Collection               |
| ATP                | Adenosine triphosphate                         |
| BM                 | Body mass                                      |
| BPA                | Bisphenol A                                    |
| BSA                | Bovine serum albumin                           |
| Ca <sup>2+</sup>   | Calcium ion                                    |
| CALUX              | Chemically activated luciferase expression     |
| Cd                 | Cadmium  |
| CO <sub>2</sub>    | Carbon dioxide                                 |
| CPRG               | Chlorophenol red- $\beta$ -D-galactopyranoside |
| DDE                | 1,1-Dichloro-2,2-bis(p-chlorophenyl) ethylene  |
| ddH <sub>2</sub> O | Double distilled water                         |
| DDT                | Dichlorodiphenyl trichloroethane               |
| DES                | Diethylstilbestrol                             |
| DHT                | Dihydrotestosterone                            |
| DHTEq              | Dihydrotestosterone equivalent                 |
| DI                 | Detection limit                                |
| DMSO               | Dimethyl sulfoxide                             |
| DNA                | Deoxyribonucleic acid                          |
| E <sub>1</sub>     | Estrone  |
| E <sub>2</sub>     | 17 $\beta$ -estradiol                          |
| E <sub>3</sub>     | Estriol  |
| EC <sub>50</sub>   | Half maximal effective concentration           |
| EDC                | Endocrine disrupting chemical                  |
| EEq                | Estradiol equivalent                           |

|                                 |  |
|---------------------------------|--|
| ER                              | Estrogen receptor                                      |
| ERE                             | Estrogen response element                              |
| FBS                             | Fetal bovine serum                                     |
| FSH                             | Follicle stimulating hormone                           |
| GnRH                            | Gonadotrophin releasing hormone                        |
| GPER                            | G-protein coupled estrogen receptor-1                  |
| G <sub>q</sub> /G <sub>11</sub> | G-protein alpha subunits Galphaq and Galpha11          |
| GWRC                            | Global Water Research Coalition                        |
| HBSS                            | Hank's balanced salt solution                          |
| HCl                             | Hydrochloric acid                                      |
| HEPES                           | 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| hER                             | Human estrogen receptor                                |
| Hg                              | Mercury  |
| HPG                             | Hypothalamic pituitary gonadal                         |
| Inc.                            | Incorporated   |
| kPa                             | Kilopascal   |
| L-15                            | Lebovitz's L-15 growth medium                          |
| LBR                             | Ligand binding region                                  |
| LDL                             | Low density lipoprotein                                |
| LH                              | Luteinizing hormone                                    |
| LOAEL                           | Lowest observed adverse effect level                   |
| MgCl <sub>2</sub>               | Magnesium chloride                                     |
| mM                              | Millimolar   |
| MMTV                            | Mammary tumor virus promoter                           |
| mRNA                            | Messenger ribonucleic acid                             |
| nM                              | Nanomolar  |
| NOAEL                           | No observed adverse effect level                       |
| NR                              | Nuclear receptor                                       |
| NR3C                            | Nuclear receptor subfamily 3 group C                   |
| OHF                             | Hydroxyflutamide                                       |
| Pb                              | Lead   |
| PBS                             | Phosphate buffered solution                            |
| PCB                             | Polychlorinated biphenyl                               |
| PCOS                            | Polycystic ovarian syndrome                            |

|       |  |
|-------|--|
| PFOA  | Perfluorooctanoic acid                     |
| pM    | Picomolar                                  |
| POF   | Premature ovarian failure                  |
| POPs  | Persistent organic pollutants              |
| RLU   | Relative light unit                        |
| RPMI  | Roswell Park Memorial Institute            |
| SA    | South Africa                               |
| scc   | Side chain cleavage                        |
| SD    | Standard deviation                         |
| SDGs  | Sustainable Development Goals              |
| SHBG  | Steroid hormone binding globulin           |
| SPE   | Solid phase extraction                     |
| SRM   | Selective receptor modulators              |
| SRY   | Sex-determining region of the Y-chromosome |
| StAR  | Steroidogenic acute regulatory protein     |
| SW    | Surface water                              |
| TBECH | Tetrabromoethylcyclonexane                 |
| TBT   | Tributyltin                                |
| TCDD  | 2,3,7,8-Tetrachlorodibenzo-p-dioxin        |
| TDS   | Testicular dysgenesis syndrome             |
| TDW   | Treated drinking water                     |
| TF    | Transcription factor                       |
| TZW   | Technologiezentrum Wasser                  |
| UK    | United Kingdom                             |
| UNEP  | United Nations Environmental Programme     |
| UP    | University of Pretoria                     |
| USA   | United States of America                   |
| VC    | Vehicle control                            |
| WHO   | World Health Organisation                  |
| YES   | Yeast estrogen screen                      |
| µL    | Microliter                                 |
| µM    | Micromolar                                 |
| 3βHSD | 3β-hydroxysteroid dehydrogen               |

# CHAPTER 1

## 1 Introduction

The presence of environmental chemical pollutants with endocrine activity date back as far as World War II (1939-1942).<sup>1</sup> Defined by the World Health Organisation (WHO) together with the United Nations Environmental Programme (UNEP), an endocrine disruptor/endocrine disrupting chemical (EDC) is ‘an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations’.<sup>2</sup> The term was first introduced in 1991 following a consensus reached by researchers at the Wingspread conference in the United States of America (USA). They agreed that EDCs were interfering with the reproductive health of both wildlife and humans.<sup>3</sup> Five years following this, the book *Our Stolen Future* presented a sequence of documented events on EDC exposure and resultant reproductive health consequences.<sup>4</sup> More research has been conducted since then and EDCs are now linked with a number of diseases and disorders.<sup>5</sup>

Given the complexity of the endocrine system, the mechanisms of disruption are various and are still under study. In general, EDC are known to:

- Bind to their respective receptors to either mimic or antagonise natural hormones,<sup>5</sup>
- Interfere with natural hormone synthesis, metabolism or excretion therefore altering plasma concentrations (bioavailability)<sup>5</sup> or
- Disrupt the signals between the components of the hypothalamus-pituitary endocrine gland axes.<sup>5</sup>

Important key characteristics can be highlighted by dissecting the definition of an EDC as given above. To begin with, endocrine disruptors are ubiquitous pollutants in the environment; with various sources which include pharmaceuticals and personal care products (e.g. 17 $\beta$ -ethinylestradiol),<sup>6-7</sup> industrial by-products (e.g. dioxins), pesticide residues in agricultural products (e.g. dichlorodiphenyl trichloroethane (DDT) and its metabolites), plastic toys and medical devices (e.g.

phthalates and bisphenol A (BPA)) as well as flame retardants (e.g. polybrominated diphenyl ethers).<sup>5,8</sup> They are introduced to endogenous physiological systems through multiple routes such as inhalation of gases and particles in the air, ingestion of contaminated water and food as well as absorption through the dermis.<sup>5,9</sup> Through the placenta, they're transferred from an exposed pregnant woman to her fetus and to an infant through breast feeding.<sup>9-11</sup> Both human and wildlife are therefore exposed to complex mixtures due to the multiple routes of exposure to various chemicals.<sup>5</sup>

Another characteristic that can be noted is that upon uptake, EDCs alter normal endocrine function resulting in adverse health effects, which are in relation to the pathway(s) disrupted.<sup>5,12-14</sup> Lifestyle and genetic predisposition alone are unable to account for the increase in disease prevalence over the past decades. This correlates well with the changes which have been occurring in the chemical environment, suggesting an interaction between the environment, lifestyle and genetics.<sup>15</sup> Animal experimental studies are in agreement that EDCs have contributed to the increased prevalence of metabolic diseases,<sup>16-19</sup> neurodevelopment disorders<sup>8,20</sup> and reproductive health problems such as cryptorchidism, hypospadias, reduced sperm counts, increased infertility rates, endometriosis like symptoms, disruption of fertility cycles, fibroids as well as cancers.<sup>5,21-23</sup> Historical evidence is traced back to the feminisation of male fish as well as cracking and thinning of bald eagles' eggs observed around the 1970s in the Great Lakes following large scale DDT spraying around the 1940s.<sup>1,4</sup> In humans, pregnant women who were prescribed the synthetic estrogen diethylstilbestrol (DES) for miscarriages between 1948-1971 were later found to have an increased risk for breast cancer.<sup>24</sup> Second generation *in utero* exposed daughters have also been linked to the development of adenocarcinomas, fertility problems and complications with pregnancies.<sup>24</sup> Furthermore, more evidence is accumulating from the Generation R study, an ongoing prospective cohort study which has aimed to 'identify early environmental and genetic causes and causal pathways leading to normal and abnormal growth, development and health during fetal life, childhood and adulthood' since the year 2012.<sup>25</sup>



In traditional toxicological studies, a substance must show adverse effects which increase proportionally with dose in order to be considered dangerous by classical toxicological standards.<sup>26</sup> This however is not the case with EDCs. At low doses (any dose below the lowest observed adverse effect level (LOAEL)), they are able to induce effects which are not predicted by effects at higher doses, challenging the “dose makes the poison” dogma.<sup>27</sup> Furthermore, exposure during critical windows of development (cell programming stages) may result in permanent alterations and through epigenetic mechanisms, these alterations may be transferred to subsequent generations.<sup>13,23</sup> The endocrine disrupting potential of a compound therefore extends far beyond actions at receptor level.<sup>26</sup> During non-critical windows, a key concern is chronic exposure over time. Effects may therefore not be observed until years after the initial exposure.<sup>12-14,26</sup>

Exposure through contaminated water sources gave rise to the research question addressed in this dissertation. Water is regarded as the bloodstream of the biosphere, however, its management is still a challenge.<sup>28</sup> This poses a burden to the water industry. South Africa (SA), in particular, is faced with the challenge of maintaining its water resources in good quality for potable water supply to its growing population.<sup>29</sup> Both estrogen and androgen disruptors are a threat to the water industry. They discharge into SW (surface water) unmonitored from sources such as industrial chemical leaks, agricultural runoffs<sup>30-31</sup> and effluents from sewage treatment works.<sup>32</sup> This is a concern as most SW is used to supply water treatment plants. Treatment plants may not be able to completely remove these contaminants, as they are highly stable and active at low concentrations.<sup>31,33</sup> Activated carbon filters are available however it has been reported that they may not efficiently remove all endocrine disruptors.<sup>34</sup> Furthermore, transporting pipes after treatment may contain epoxy resins (BPA based) that can leach into the water being transported<sup>35</sup> and not all treatment plants make use of activated carbon filters. Humans may therefore be exposed through drinking water.<sup>34</sup> Water treatment plants therefore need to be monitored constantly for estrogen and androgen disruptors as a strategy for water quality management, using reliable methods.

The complex mechanisms employed by EDCs require a multifaceted approach in assessing health risks with exposure. However, validated and internationally

standardised test methods still lack for a number of endocrine endpoints.<sup>5,36</sup> Test guidelines are in development from research data collected from around the world.<sup>36</sup> Thus far, a conceptual framework has been proposed by the Endocrine Disruptor Screening and Testing Advisory Committee.<sup>36</sup> It suggests evaluating EDCs through a tier approach. On the second tier of this framework is EDC activity screening through *in vitro* biological assays. These are assays engineered to rely on biological response of cultured cells upon chemical(s) exposure.<sup>36-37</sup> They are ideal as they test total endocrine activity and are sensitive to low concentrations.<sup>36-37</sup> This is important since EDCs occur in mixtures and induce effects at low concentrations. At this point however, they have not been validated beyond academic settings.<sup>38-39</sup> Nationally, SA also faces the challenge of limited regulatory framework on EDCs. Thus far, there is only a proposed trigger value of 0.7 ng estradiol equivalents (EEqs)/L for estrogenic activity in drinking water.<sup>40</sup> These are well researched quantitative values based on factors such as the acceptable daily intake (ADI) references, pharmacokinetics (absorption, distribution, metabolism and excretion) as well as endocrine potencies of specific reference compounds e.g. 17 $\beta$ -estradiol (E<sub>2</sub>) and DHT.<sup>41</sup> These values have not yet been incorporated into policy.

## 1.1 Research question

The research questions posed in this study are twofold; addressed in two phases:

1. Can the MDA-kb2 assay be applied as a screening tool for androgenic activity in water samples of different sources?
2. Can the Yeast estrogen screen (YES) assay, T47D-KBluc reporter gene assay and MDA-kb2 reporter gene assay be used as a battery to monitor estrogenic and androgenic activity in a water treatment plant?

## 1.2 Study rationale

In September 2015, SA committed itself to the Sustainable Development Goals (SDGs) under which section 6.2 of goal 6 aims that by 2030 water quality would be improved by 'reducing pollution, eliminating dumping, and minimizing the release of hazardous chemicals and materials, halving the proportion of untreated wastewater and substantially increasing recycling and safe reuse globally'.<sup>42</sup> This is also in line with the National Water Act; which aims to reduce and prevent pollution as well as

degradation of all water resources. Furthermore, the rights to health and clean water are basic human rights. The present study was therefore necessary, because research data on the usefulness of *in vitro* biological assays as monitoring tools was generated. This was with the hope that, in future, it will contribute towards the development of internationally standardised test methods and guidelines for estrogen and androgen disruptors in various water sources and ultimately their regulation through policy in SA. Ultimately, this will contribute towards the preservation of water resources as well as reducing water pollution together with its associated health effects.

## CHAPTER 2

### 2 Literature review

#### 2.1 The endocrine system

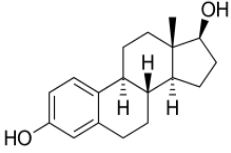
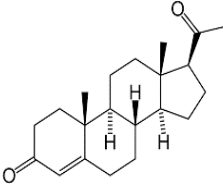
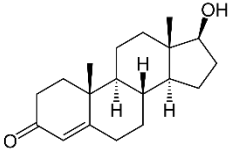
The endocrine system is one of the main systems involved in the regulation and maintenance of physiological processes such as energy metabolism, reproduction, growth, differentiation and development of tissue and organs in mammals.<sup>43-44</sup> To achieve these functions, it is organised into pathways/axes which are each programmed to interact at multiple levels and maintain homeostasis through the secretion of regulating signal molecules (hormones) by endocrine glands/cells.<sup>45</sup> These axes include the hypothalamic pituitary gonadal (HPG) axis, hypothalamic-pituitary adrenal axis and hypothalamic pituitary thyroid axis.<sup>43</sup> The HPG axis regulates the secretion of sex steroids.

Ernest Starling first coined the term “hormone” in 1905 and described it as ‘a chemical messenger which is able to speed from cell to cell in order to co-ordinate the activities of different parts of the body’.<sup>46</sup> Today, much more is known. It is known that upon their secretion by endocrine glands/cells, they are transported in the circulatory system to their target cells where they bind to their respective receptors and induce either an instant non-genomic response or a delayed genomic response in response to stimuli.<sup>47</sup> Their binding affinity is influenced by a number of biochemical factors which ultimately determine their potency.<sup>48</sup> High binding affinity is known to result in full agonistic effects (high potency) while low binding affinity results in partial agonistic effects (low potency).<sup>48</sup> Antagonists on the other hand block the action of agonists; they may however have mixed actions relative to the target receptor. Antagonists with such behaviour are known as selective receptor modulators (SRM) and induce partial agonistic behaviour, competing with agonists to achieve either reduced or no biological response.<sup>48</sup>

Various hormone classifications are known and include, but are not limited to, functional as well as chemical classes. Chemical classifications are the most common and include: amino acid derived hormones, protein/peptide hormones and steroid hormones.<sup>45</sup> Amino acid derived hormones are modified amino acids e.g.

melatonin from tryptophan; peptide hormones are linked amino acids e.g. cytokines and neurohormones and steroid hormones are derivatives of cholesterol.<sup>47,49</sup> Distinct differences exist between amino acid/peptide and steroid hormones. Peptides are hydrophilic and are able to dissolve with ease in the extracellular fluid for transportation while steroid hormones are lipophilic and rely on assistance from carrier proteins for transportation.<sup>45,49-50</sup> These differences are important as they determine their bioavailability and function. **Table 2.1** lists some endocrine hormones produced by mammals as well as their function(s).

**Table 2.1:** Tabulation of some known endocrine glands in mammals together with the hormones and function

| Hormone  | Endocrine Gland   | Function (s)  |
|--|---|---|
| Estrogen(s)<br>                 | Main: ovaries,<br>Other: adipocytes,<br>adrenal gland,<br>brain, placenta and<br>testes | <ul style="list-style-type: none"> <li>• <b>Biological:</b> Neurogenesis and development in the presence of <math>\alpha</math>-fetoprotein (AFP); adipogenesis; intracellular signalling and cell proliferation <sup>51-53</sup></li> <li>• <b>Reproductive:</b> Female secondary sex characteristics: mammary gland development, endometrial and uterine growth, pubic hair and Spermatogenesis <sup>51-52</sup></li> <li>• <b>Non-reproductive:</b> Regulation of innate and adaptive immune system; bone resorption; cardioprotective effects: activation of endothelial nitric oxide synthase leading to arterial vasodilatation as well as regulation of serum lipoproteins and triglyceride profiles and expression of coagulant and fibrinolytic proteins <sup>51-54</sup></li> </ul> |
| Progesterone<br>               | Main: Ovary<br>(corpus luteum),<br>adrenal cortex and<br>placenta                       | <ul style="list-style-type: none"> <li>• <b>Reproductive:</b> Promotes endometrial receptivity to an ovum for implantation by lining and thickening the uterus in each menstrual cycle; thickens the cervical mucus and vaginal epithelium; nourishes and maintains a fertilised ovum and mammary gland development together with estrogen <sup>55</sup></li> <li>• <b>Non-reproductive:</b> Promote oligodendrocyte synthesis thus myelination of peripheral nerves, conduction of nerve impulses, trophic support to axons <sup>55-57</sup></li> </ul>  |
| Androgens<br>Testosterone<br> | Main: testes<br>Ovaries and<br>Adrenals   | <ul style="list-style-type: none"> <li>• <b>Biological functions:</b> Spermatogenesis, myofibrillar protein synthesis, precursor for estradiol and dihydrotestosterone (DHT) <sup>58</sup></li> <li>• <b>Reproductive effects:</b> Prenatal development and maintenance of male reproductive organs such as penile differentiation, accessory glands (prostate, seminal vesicles) and ducts (epididymis, urethra, vas deferens), development of male secondary sex characteristics i.e. pubic hair, deepening of voice <sup>59-61</sup></li> <li>• <b>Non-reproductive functions:</b> Cognitive function and behavioural traits such as spatial and verbal memory, increased muscle mass and strength as well as bone maturation <sup>58,62</sup></li> </ul>                                  |
| Dopamine   | Hypothalamus  | <b>Neurocognitive function, Neurotransmitter</b> : controls & co-ordinates movement and modulates the reward system , vasodilator <sup>63-64</sup>  |
| Prolactin  | Anterior pituitary  | Stimulates milk production from the mammary glands <sup>65</sup>  |
| Trophic hormones   | Anterior pituitary  | Stimulate secondary glands to secrete hormones e.g. follicle stimulating hormone (FSH) stimulates maturation of follicles into ovum in females and sperm development in males <sup>66-68</sup>  |

Throughout the different stages of life, endocrine glands need to cautiously secrete their respective hormones according to their demand, without being interrupted.<sup>69</sup> For instance, each tissue/organ in the body has a defined window of development, that is, it develops over a set specific time with specific hormones released at precise concentrations.<sup>69-70</sup> This ensures that development and growth of tissue and organs is uninterrupted. Under tight regulatory mechanisms, the components of each pathway of the endocrine system thus work in harmony to maintain homeostasis.<sup>69-70</sup> Homeostasis however can be challenged by external environmental factors such as stress as well as foreign endocrine disruptors, which are the focus of this dissertation. These external factors interfere with hormone production and function leading to endocrine pathologies. They emerge as different vulnerabilities ranging from behavioural and neurological disorders to metabolic and reproductive health problems.<sup>5</sup>

### 2.1.1 Biosynthesis of estrogen and androgen sex steroids

Steroid hormones are a group of lipophilic hormones with closely related structures as they are all naturally synthesized from cholesterol in a process called steroidogenesis (**Figure 2-1**).<sup>71</sup> Synthetic forms are known and are commonly used in pharmaceuticals either as methods of hormone replacement therapies or as active ingredients in oral contraceptives or anabolic steroids.<sup>72</sup> These hormones are synthesized in the adrenal cortex, Leydig cells of the testes, theca cells of ovaries and as well as of the placenta during pregnancy.<sup>49,67</sup> A number of enzymes and co-factors mediate steroidogenesis of which the enzymes involved fall into two groups: the hydroxysteroid dehydrogenases and the cytochrome P450 enzymes, whose activities are modulated by other co-factors and posttranslational modifications.<sup>71</sup> The process therefore differs slightly from cell to cell depending on the presence or absence of the catalytic enzymes.<sup>71</sup>

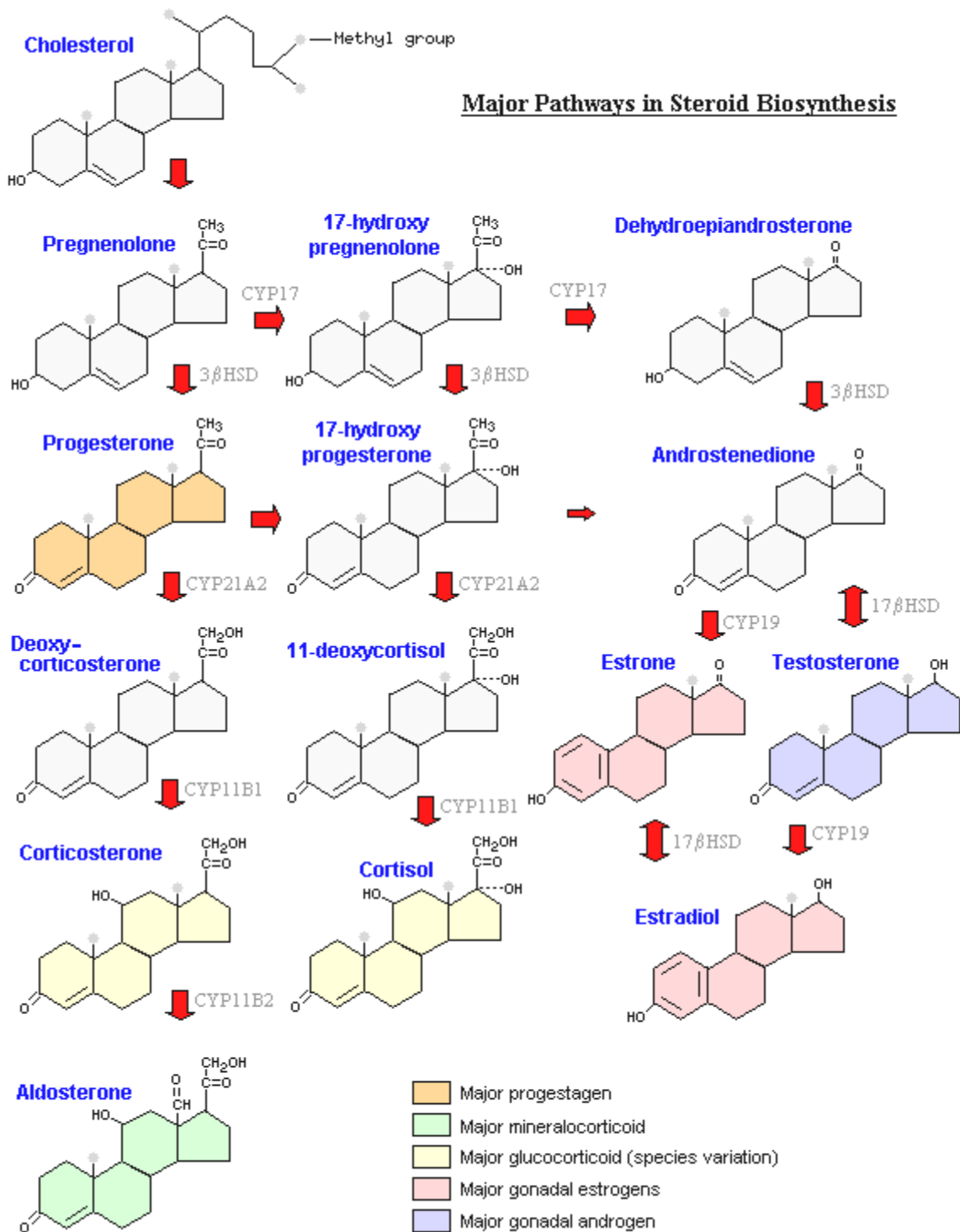
As indicated in **Figure 2-1**, the process begins with the synthesis of cholesterol. This occurs in the outer mitochondrial membrane, either *de novo* from acetate or from circulating plasma low density lipoproteins (LDLs) derived from the diet.<sup>71</sup> Facilitated by Steroidogenic Acute Regulatory protein (StAR), it enters the inner mitochondrial membrane where it is converted into pregnenolone through cleavage of the cholesterol side chain by the enzyme cytochrome P450 side chain cleavage (scc).<sup>71,73</sup> The placenta does not express StAR and only utilizes mitochondrial cytochrome P450scc for this.<sup>74</sup> Alternatively, it utilises carbon (C)<sub>19</sub> steroids from fetal adrenal gland and convert them directly to estrogens.<sup>74</sup> The conversion of cholesterol to pregnenolone is the rate limiting step of steroidogenesis.<sup>71,74</sup> Disturbance at these points can result into primary adrenal insufficiency, a condition whereby the adrenal glands fail to produce androgens.<sup>71</sup>

Following the production of pregnenolone from cholesterol, it is converted further into 17 $\alpha$ -hydroxypregnenolone by 17 $\alpha$ -hydroxylase or progesterone which is the first biologically significant steroid of the pathway. The 17 $\alpha$ -hydroxypregnenolone is then converted into 17 $\alpha$ -hydroxyprogesterone by the enzyme 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ HSD).<sup>71,73</sup> In humans, two forms of the enzyme are known (type 1 and 2) and are encoded by genes on chromosome 1p13.1.<sup>71,73,75</sup> Type 1 is active in the liver, breast, placenta as well as in other tissues. It is also the active isoform for



progesterone synthesis during pregnancy.<sup>71,74</sup> Type 2 is more active in the gonads and adrenals. Following its production, progesterone is either channelled to the production of mineralocorticosteroids or converted into 17 $\alpha$ -hydroxyprogesterone which is also either channelled into mineralocorticosteroid production or androstenedione, which is also produced from dehydroepiandrosterone channelled from 17 $\alpha$ -hydroxypregnenolone.<sup>71,73</sup> Dehydroepiandrosterone is the first androgen of the pathway and can produce either androstenedione or androstenediol. Both these products can convert into testosterone, which is further converted into potent DHT in males or E<sub>2</sub> by the enzyme 5 $\alpha$ -reductase in females. Androstenedione can also be converted into estrone (E<sub>1</sub>), also by 5 $\alpha$ -reductase. In the liver and placenta, estriol (E<sub>3</sub>) forms from either E<sub>2</sub> or E<sub>1</sub>.<sup>59,67,71,73</sup> Given the complexity and key roles of these enzymes, they are also targets for endocrine disruption.<sup>76</sup>

Following their biosynthesis, in the blood, they are distributed in three forms. The first is the bioavailable form. In this form, they are bound to low-affinity carrier proteins i.e. albumin for transportation.<sup>27</sup> The second is the inactive form where the hormones are bound to high-affinity proteins i.e. steroid binding proteins (SHB) and AFP.<sup>27</sup> Last is the free form; hormones are in an unconjugated/unbound form.<sup>27</sup> About 97% of testosterone and E<sub>2</sub> are transported bound to steroid hormone binding globulins (SHBG) and smaller fractions to either albumin or AFP. These proteins assist in solubility and give protection from degrading enzymes thus prolonging their half-life.<sup>43,77-78</sup>



**Figure 2-1: Steroidogenesis**

(Available from

<http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/basics/steroidogenesis.html>)

### 2.1.2 Role of sex steroids in male and female sexual differentiation

Reproductive structures begin to differentiate at about the 7<sup>th</sup> week of gestation.<sup>79-80</sup> Before that, the embryo has bipotential gonads which can develop either into testis or ovaries depending on the regression of one of the two sets of ducts in the gonads: Wolffian ducts or Müllerian ducts.<sup>49,79</sup> Additionally, in males, the gene *Sex-determining Region of the Y-chromosome* (*SRY*) is responsible for the male phenotype.<sup>49,80</sup> This gene expresses the SRY protein which directs the development of testis from gonads, independent of testosterone. Once the testis have differentiated, three hormones are secreted. These hormones are the anti-Müllerian hormone from the Sertoli cells which regresses the Müllerian ducts; testosterone and DHT by the Leydig cells of which testosterone converts the Wolffian ducts into accessory structures such as the seminal vesicles, vas deferens, epididymis as well as descending the testes from the abdomen into the scrotum at a later stage in life.<sup>49,79-80</sup> The hormone DHT is responsible for external genitalia development.<sup>59</sup> Excessive or deficient exposure to testosterone and its counterpart androgens during this period disrupts this process of sex differentiation, resulting in congenital malformations of the genitalia such as ambiguous genitalia, cryptorchidism, hypospadias and Müllerian agenesis.<sup>81-82</sup> Furthermore, because of the role androgens play in mammalian brain development this also interferes with the patterns of neural connectivity, cell death and survival as well as neurochemical characterisation.<sup>83</sup> This ultimately contributes to behavioural and cognitive alterations such as aggression in men and memory disorders later in life.<sup>83-84</sup>

In contrast to male embryos, female embryos lack the *SRY* gene, thus the gonadal cortex develops into ovarian tissue.<sup>49</sup> There is no inhibition from the anti-Müllerian hormone thus the Müllerian ducts develop into fallopian tubes, uterus and upper part of the vagina. The absence of testosterone allows the Wolffian ducts to degenerate and estrogen maintains the development of the differentiated structures.<sup>49,79-80</sup> Exposure of females to testosterone *in utero* can induce masculine characteristics such as increased AGD,<sup>85-86</sup> anovulation,<sup>87</sup> and agenesis of the lower vagina.<sup>88-89</sup>

## 2.2 The HPG axis

In 1955, Geoffrey Harris first proposed a model describing the neuronal control of the pituitary gland in secreting gonadal hormones.<sup>90</sup> More details have been added to the model since then. It is known today that gonadal function as well as the biosynthesis of sex steroids in other tissues are under neuro-endocrine control, mainly by the HPG axis.<sup>43</sup> This axis is an interaction of three components: gonadotrophin releasing hormone (GnRH) neurons of the hypothalamus, gonadotropes of the anterior pituitary gland and the somatic cells of the gonads, namely the granulosa and theca cells of the ovary as well as the Sertoli and Leydig cells of the testis.<sup>67-68</sup>

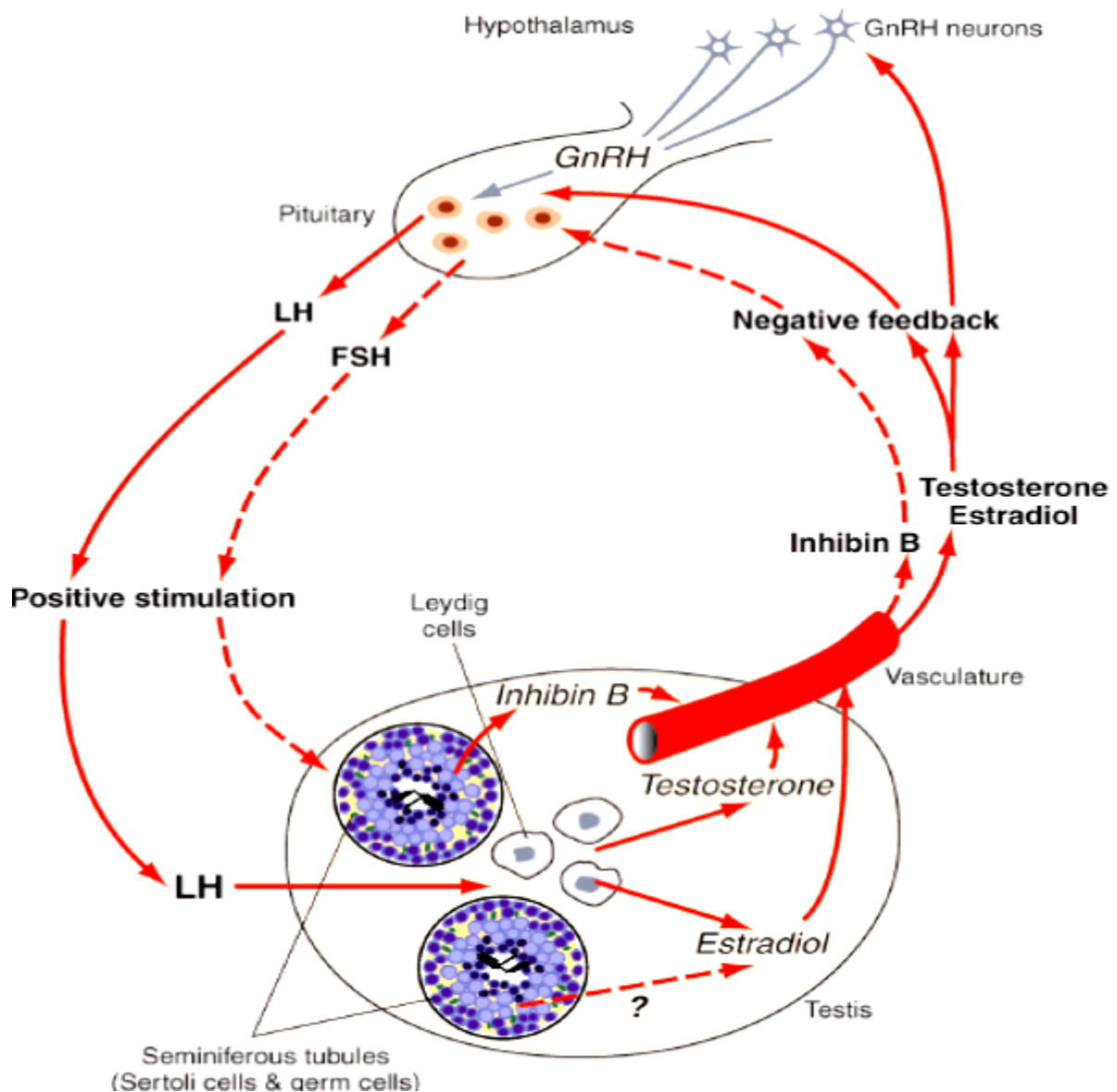
The pathway begins with the GnRH neurons secreting the GnRH into the hypophyseal portal circulation in a pulsative manner. The GnRH is encoded by the *GNRH<sub>1</sub>* gene and following its expression, the GnRH neurons store the product peptide for its release at later stages.<sup>91</sup> The *GNRH<sub>1</sub>* gene thus plays a role in the long term release of GnRH and the neurons for the moment to moment pulsative release.<sup>91</sup> Studies are also in agreement that the secretion of GnRH from neurons is under the influence of the protein kisspeptin, which has also been proposed to be part of the pulse generator.<sup>92-93</sup> Following its release, the hormone is transported to the gonadotropes in the anterior pituitary to stimulate the secretion of the FSH and luteinizing hormone (LH), collectively known as the gonadotropins.<sup>94</sup> The GnRH-action on the pituitary gonadotroph is mediated by the G-protein coupled receptors which signal through the G-protein alpha subunits Galphaq and Galpha11 (*G<sub>q</sub>/G<sub>11</sub>*) pathway in order to activate phospholipase-C leading to the mobilisation of calcium ion ( $\text{Ca}^{2+}$ ) for function.<sup>95-96</sup>

Following this, gonadotropins are directed to the gonads (LH to the theca and Leydig cells, FSH to the granulosa and Sertoli cells). The LH then acts on the Leydig cells of the testes as well as the theca cells of the ovaries to stimulate the production of androgens through steroidogenesis.<sup>43,94</sup> In the theca cells, testosterone is further converted to estrogen. The FSH on the other hand stimulates the maturation of follicular cells for ovulation as well as estrogen production. In the Sertoli cells, it stimulates spermatogenesis. The pattern of gonadotropin secretion in puberty is

approximately every two hours for males. In females, however, it varies with reference to the menstrual cycle, with an increased frequency closer to the follicular phase.<sup>43,73,97-98</sup>

### 2.2.1 Regulatory feedback mechanisms of the HPG axis

The LH as well as the peptide hormone inhibin (B form in males and A form in females), are able to provide negative feedback mechanisms by acting on the GnRH neurons and gonadotrophes to attenuate the production of GnRH, LH and FSH. Inhibin inhibits the FSH and the sex steroids attenuate LH and GnRH secretion, graphically depicted in **Figure 2-2**.<sup>99-100</sup> Studies have also shown that target cell response is also influenced by the sensitivity of the target receptor to stimuli. For instance, consistent and high frequency of GnRH pulses or its analogues lead to the down regulation of the GnRH receptors, making them less sensitive to their stimuli resulting in their 'desensitization'/reduced overall response.<sup>43,77</sup> Hormone metabolism is another noted point for hormone regulation. A decrease or increase in their catabolism determines circulating concentration levels.<sup>79</sup> Feedback mechanisms are therefore closed loop systems which fine tune the endocrine system, preventing the accumulation/deficit of hormone products therefore ensuring that the hormone concentrations are kept within the correct physiological ranges for function.<sup>100</sup> Endocrine disruptors may also interfere at this level, altering the synthesis and metabolism of hormones.<sup>101-102</sup>



**Figure 2-2:** A diagrammatic expression of the HPG axis function and its regulatory mechanism<sup>43</sup>

The GnRH neurons in the hypothalamus secrete the GnRH which acts on the gonadotrophs of the anterior pituitary to stimulate the secretion of the gonadotropins, FSH and LH, into the blood circulation to the target receptors of the gonads (testis illustrated). This triggers the Leydig cells to stimulate synthesis as well as secretion of testosterone. In the negative feedback mechanism, testosterone attenuates the synthesis and secretion of GnRH and LH in the hypothalamus and anterior pituitary. This is also true for  $E_2$  from the ovaries. The FSH stimulates Sertoli cells for inhibin B synthesis and secretion which inhibits FSH secretion. Other negative feedbacks of testosterone occur through  $E_2$ .<sup>43</sup>

### 2.2.1.1 Estrogens: Positive feedback mechanism

The positive feedback mechanism is unique and specific to estrogens.<sup>49</sup> It is triggered by high concentrations of circulating estradiol which in turn trigger a positive stimulation of the gonadotrophs to release the LH and FSH which ultimately cause an additional estrogen secretion in the gonads.<sup>103-104</sup> Recent studies have shown that estrogen receptor (ER)  $\beta$  is also expressed by the GnRH neurons thus a direct interaction between estradiol and GnRH neurons exists.<sup>105</sup> At the neurons, the estradiol has been proposed to either 1) directly influence the cell's membrane excitability or 2) regulate the synapses of the neurons or 3) control the glial cell function.<sup>105-107</sup> Studies have also indicated that exogenous estrogen agonists contribute to this positive feedback mechanism.<sup>108-109</sup>

This positive feedback is seen in the LH surge, which is necessary for ovulation in mammals. Circulating E<sub>2</sub> during the follicular phase of the menstrual cycle is highest around day 14 and triggers a sudden and increased LH secretion from the anterior pituitary.<sup>49,105,110</sup> This stimulates the release of a mature ovum from the ovary. This is an important time period, often referred to as a fertility window. Any form of disruption tempers not only with a successful menstrual cycle but also the ability to conceive. This is depicted in the polycystic ovarian syndrome (PCOS), common cause of infertility in women of reproductive age.<sup>111-112</sup> There is however still limited data on PCOS and fibroids with EDC exposure.<sup>5</sup>

### 2.2.1.2 Estrogens: Negative feedback mechanisms

The negative feedback loop exerts an inhibitory effect at the level of both the hypothalamus and anterior pituitary (**Figure 2-2**). This effect is triggered by very low levels of circulating estrogen.<sup>104,106</sup> It sends stimuli for a positive release of LH and FSH from the anterior pituitary (seen in the early follicular phase of the menstrual cycle) and as these hormones increase estrogen synthesis and production also gradually increases. This eventually results in the accumulation of the hormone which triggers a negative feedback response from the hypothalamus and anterior pituitary, attenuating the production of estrogen.<sup>105-106</sup> Extremely high concentrations further results into a positive feedback. Progesterone and the hormone inhibin A are also able to act in combination with estrogen to mediate negative feedbacks.<sup>99,110</sup>

### 2.2.1.3 Androgens: Negative feedback mechanisms

Unlike estrogens, only a negative feedback mechanism is employed by the androgens for homeostasis.<sup>43,49</sup> This is graphically depicted in **Figure 2-2**. It is triggered by excessive testosterone levels which attenuate GnRH production. This leads to a decreased GnRH signal directed to the anterior pituitary, thus a decrease in LH production, and ultimately an overall decrease in testosterone in proportion to LH.<sup>97,100</sup> Inhibin B from the Sertoli cells triggers the inhibition of FSH production and ultimately testosterone production in the testes.<sup>43</sup> Exogenous androgens have been shown to contribute to the accumulation of testosterone.<sup>28,113</sup>

## 2.3 Disruption of the endocrine system

Over 80 000 new chemicals, with some inherently bearing structures resembling natural hormones, have been synthesized in the past century and used in various ways resulting in harmful human exposure.<sup>114-115</sup> A large fraction of these are toxic due to their endocrine disrupting characteristics and include chemicals which were created intentionally for their then known health benefits in altering hormone signalling such as the drug DES as well as pesticides.<sup>116-117</sup> The WHO together with the UNEP defined an EDC as “an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or subpopulation”.<sup>2</sup> Disruption occurs at any point between the hypothalamus and the messenger ribonucleic acid (mRNA) hormone protein product. The most common and the central part of endocrine disruption is at cellular level through which endocrine disruptors bind to receptors, with varying affinities, to ultimately alter gene expression. Others include inhibition of the synthesis, metabolism and transport of hormone.<sup>118-120</sup> The feedback and regulatory mechanisms described in the previous sections act as compensatory mechanisms, however, with consistent exposure, it may be beyond the adaptive range of the endocrine system.<sup>43</sup>

### 2.3.1 Estrogen signalling and disruption

Estrogen molecular mechanisms are well studied compared to other hormone mechanisms thus research has given more attention to estrogen disruptors.<sup>121</sup> The mechanisms for estrogen signalling, thus disruption, are complex and depend on the



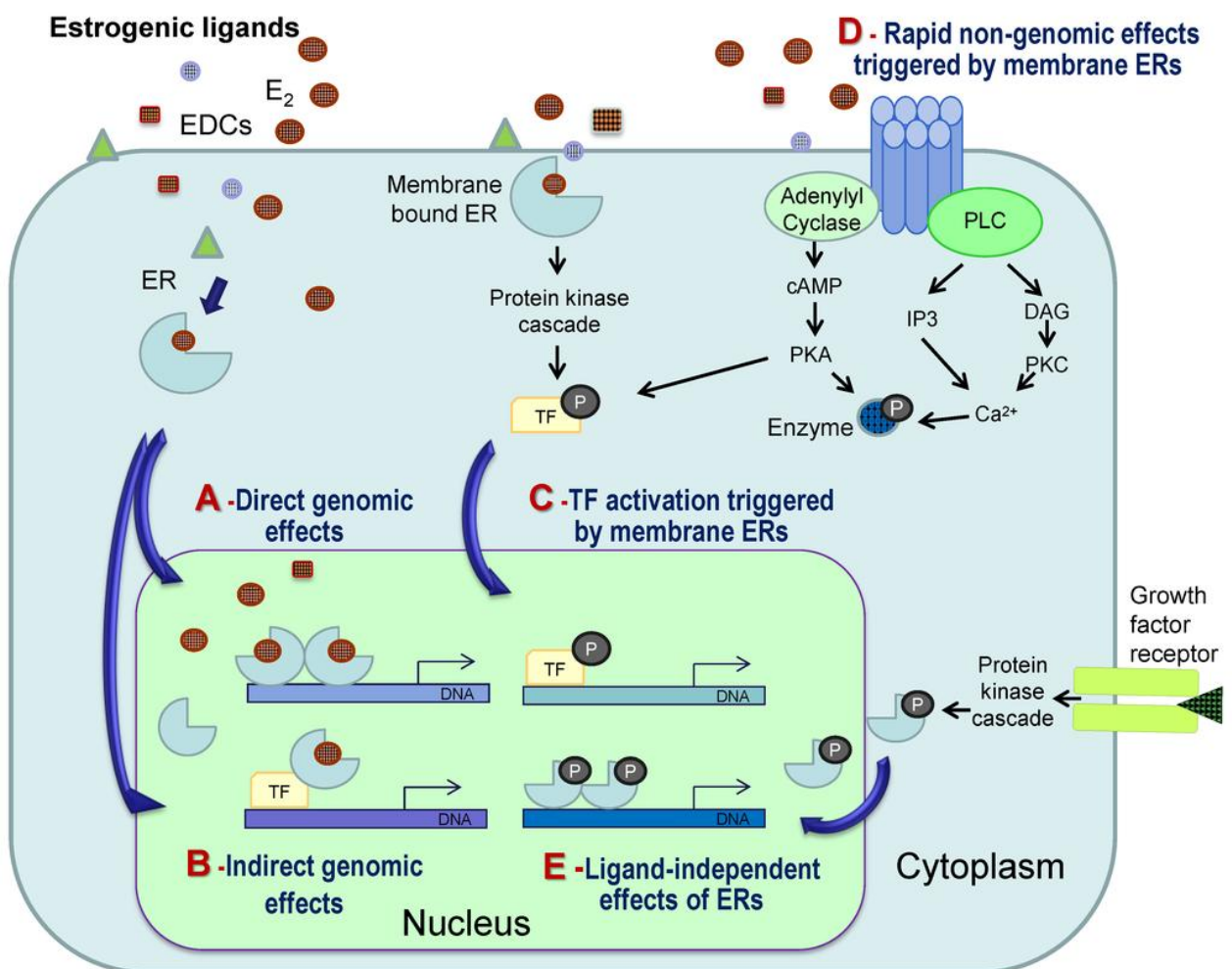
cellular contents of the target organ i.e. receptors and proteins. Different tissues therefore employ different mechanisms under different circumstances, that is, the effects are also tissue, species and cell specific.<sup>121</sup> The most common and well-studied mechanisms are through the classic receptors located in the nucleus: ER $\alpha$  and  $\beta$ . They are part of the nuclear receptor (NR) superfamily. In humans, 48 NRs have been identified and others are orphaned, meaning that their ligands are yet to be discovered.<sup>114,121</sup> Upon binding of a ligand (E<sub>2</sub>) to the receptor, a ligand-receptor complex forms resulting in a conformational change. This complex is then directed to the DNA where it binds to estrogen response element (ERE) located in the target cell's deoxyribonucleic acid (DNA), acting as a transcription factor (TF) to regulate gene expression thus resulting most often in mRNA synthesis.<sup>121-122</sup> Endogenous estrogen physiological levels are within the range of 10–900 pg/mL.<sup>27</sup> Two isoforms of target ERs are known, namely, ER $\alpha$  and ER $\beta$ . The ER $\alpha$  is widely distributed especially in various brain regions and ER $\beta$  has a more restricted distribution. The forms are structurally and functionally distinct. Estrogen disruption can therefore interfere with normal estrogen signalling by acting as either agonists or antagonists of these receptors.<sup>122-123</sup>

Alternative mechanisms have also been proposed including membrane signalling. Mammalian studies have revealed an indirect regulation of gene expression through ligand-ER complex interaction with other TFs, binding to membrane receptors (G-protein coupled receptors i.e. recently discovered GPER as well as protein kinase cascades activation or alterations of secondary messenger signalling cascades.<sup>121</sup> This ultimately leads to either gene expression or non-genomic activation specific enzymes. Furthermore, ERs have shown to regulate gene expression ligand-independently through phosphorylation response following binding growth factors to membrane receptors (**Figure 2-3**). These pathways are also prone to estrogen disruption. This remains an active area of research.<sup>121,124</sup>

Furthermore, in fish species (extensively used as models in EDC research), various mechanisms of E<sub>2</sub> action have been identified. These include direct vitellogenin (EDC biomarker) mRNA regulation, intracellular ER;<sup>125-126</sup> interfering with TFs through ER $\alpha$  directed pathways;<sup>127</sup> indirect regulation of the LH  $\beta$  gene through ER $\alpha$

interaction with other TFs and rapid GPER mediated effects characterized in fish gonads.<sup>66,127-128</sup>

For many EDCs such as DES, BPA, endosulfan and nonylphenol, disruption through the ER $\alpha$  and  $\beta$  are well documented.<sup>66,129-130</sup> These chemicals have been shown to also disrupt Ca<sup>2+</sup> channel activity and/or signalling in other cell types.<sup>125,131</sup> The EDCs and natural hormones can have hormonal activity by binding to nuclear hormone receptors as well as unexpected effects via receptor-mediated actions outside of the classical endocrine system.<sup>27</sup>



**Figure 2-3:** Mechanism of action for the estrogen signalling pathway<sup>47</sup>

cAMP=Cyclic adenosine monophosphate, DAG= Diacylglycerol, DNA= Deoxyribonucleic acid, EDC= Endocrine disrupting chemical, ER= Estrogen receptor, IP3= Inositol 1,4,5-triphosphate, P=Phosphate, PKA= Protein kinase A, PKC= Protein kinase C, PLC= Phospholipase C, TF= Transcription factor

### 2.3.2 Androgen signalling and disruption

Natural androgens occur in both males and females in varying ratios and are precursors to all estrogens.<sup>132</sup> The metabolite 5 $\alpha$ -DHT, is the most potent androgen, and is synthesized from testosterone. They elucidate their effects through a single androgen receptor (AR) occurring in all mammals.<sup>133</sup> This receptor is a member of the nuclear receptor subfamily 3 group C (NR3C) of the NR superfamily. On the AR nucleotide, the initial 30 residues are highly conserved. This is unique and critical for its interaction with the ligand binding region (LBR) on the receptor which is responsible for regulating agonistic effects and also, it is the primary site for androgen disruption.<sup>134-135</sup> The sensitivity and specificity of the receptors and signalling molecules make it possible for signalling molecules to co-exists without disrupting each other's signals.<sup>31</sup> Similar to estrogens, which are also part of the NR superfamily of TFs, they bind to response elements on the DNA to initiate transcription.<sup>134,136</sup>

A well-known anti-androgen is the fungicide vinclozolin which has shown, both in *in vivo* and *in vitro* assays, to block the AR preventing DHT from binding to it.<sup>134,136</sup> It has also been shown to be able to cross the blood brain barrier to reverse neurochemical phenotypes thus causing possible effects in neurodevelopment and behaviour with exposure.<sup>137</sup> The metabolite 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE), from DDT pesticide (still used for malaria control in a number of developing countries (including SA) because of the burden of the disease), is another known anti-androgen.<sup>134</sup> It has been shown *in vitro* to reduce DHT induced transcription as well as inhibit 5 $\alpha$ -reductase activity. This is a typical example of the ability of EDCs to disrupt at various sites other than the NRs.<sup>138</sup> Exposure of normozoospermic samples to *p,p'*-DDE increased Ca<sup>2+</sup> signalling resulting in compromised sperm function.<sup>139</sup> The flame retardant tetrabromoethylcylonehexane (TBECH) has been placed in the top 10% of compounds most hazardous to ecosystems. Its metabolites, TBECH  $\gamma$  and  $\delta$  have been reported *in vitro* to have agonistic effects with 22% of DHT's binding.<sup>140</sup>

## 2.4 Health effects associated with estrogenic and androgenic activity

Most of the early research on EDCs was limited to the effects of sexual reproduction disruption through the estrogen and androgen NRs. However, the accumulating evidence on other endpoints and mechanisms indicate that the entire endocrine system is vulnerable to disruption and therefore the health effects are broader than currently understood.<sup>114</sup> Generally, the function of sex hormones is broad and not limited to reproduction e.g. they play a role in the development and function of the central nervous system as well as in immune function.<sup>14</sup> The associated health effects are therefore also broad. Whilst this is the case a definitive safe level of exposure, no observed adverse effect level (NOAEL), is not known for these chemicals as they have been shown to induce effects at low concentrations.<sup>27,141</sup> For example, estrogen is biologically active at 10–900 pg/mL and testosterone at 300–10000 pg/mL.<sup>27</sup>

Epidemiological data is still limited for global trends for some health effects. Most available data is based on animal laboratory investigations. These investigations have supported in establishing the known associations between EDC exposure and resultant health effects. Epidemiological studies are challenging because some chemicals are rapidly degraded in the environment/ human body (e.g. do not bioaccumulate). Therefore, an exposure which may have caused an adverse health outcome is not detectable by the time clinical manifestations are evident in the phenotype.<sup>142-143</sup> However, in reality, we may be chronically exposed to EDCs due to their ubiquitous occurrence in the environment. Furthermore, transgenerational studies have shown that health effects may be transferred to subsequent generations through epigenetic mechanisms. Most cause-effect relationships have been drawn from EDCs that are ecologically and biologically persistent such as POPs, including dioxins and polychlorinated biphenyls (PCBs).<sup>5,43,144</sup> Although these chemicals are regulated, their persistent nature suggests that they may impact human health for decades to come.<sup>27</sup>

Various hypotheses have been proposed globally in studying human health effects. These include: *'The endometriosis in women is related to endocrine disruption mediated by exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and/or PCB'*; *'Global reductions in human semen quality over time are related to increasing*

*exposure to estrogenic, anti-androgenic or other as yet unidentified chemicals, during critical phases of testicular development* and *'Increased incidences of breast cancer are caused by exposure to organochlorine chemicals (e.g., PCBs, DDT, and metabolites) possessing estrogenic activity'*.<sup>43</sup> These are broken down into research questions and remain an active area of research.

#### **2.4.1 Testicular dysgenesis syndrome**

The notion that environmental chemicals affect reproduction is not new. It dates back to 1962 in Rachel Carson's *Silent Spring* where a link between wildlife male infertility was made with the EDC, DDT.<sup>145</sup> What is new however is the hypothesis that these chemicals might be the driving force behind the increased prevalence of reproductive health problems globally.<sup>146</sup> Sharpe and Skakkebaek therefore proposed a link between the environment and the increased incidence of testicular cancer; developmental events that result in decreased sperm quantity and quality; hypospadia as well as cryptorchidism, explaining that they arise from intrauterine disruption of testicular development and function.<sup>146-148</sup> This is collectively referred to as testicular dysgenesis syndrome (TDS).<sup>148</sup> Both epidemiological and laboratory investigations are in agreement to this.<sup>5</sup> Prostate cancer is the most prevalent male non-cutaneous cancer in SA.<sup>149</sup>

The main cellular trait of TDS is the dysfunction of the Leydig cells.<sup>150</sup> In mild cases, men present with low levels of testosterone, slightly decreased penile/testicular sizes and poor semen quality.<sup>151</sup> In more severe cases, there is also an increased risk of testicular cancer as well as hypospasia or cryptorchidism.<sup>152</sup> Cryptorchidism, which is the failure of one or both testicles to descend into the scrotal sac, is the most common congenital malformation in male children. It is currently the best characterised risk factor for infertility and testicular cancer in adulthood.<sup>153</sup> A prospective case control study assessing an incidence of cryptorchidism with fetal exposure to DDE and PCBs showed an increased risk for TDS features (hypospadias, cryptorchidism and compromised semen quality), with effects being more severe in individuals with a genetic predisposition.<sup>154</sup> Hypospadias on the other hand affect about 0.4% of males at birth and has been reported to have increased significantly over recent decades.<sup>155</sup> Endocrine disruptors such as

vinclozolin and phthalates are regarded as a contributing factor in inducing hypospadias *in vitro*.<sup>152</sup>

Disruption of the fetal testicular androgen production as well as disproportionate estrogenic exposure around the virilisation period (around 8-14 weeks into human fetal development) causes TDS.<sup>156-157</sup> A study found a linear correlation between maternal exposure to estrogens and lower testosterone levels and smaller penile length and higher incidences of reproductive anomalies e.g. hypopsadias in newborns.<sup>158</sup> Pregnant mice orally exposed to phthalates at 1 mg/kg body mass (BM) per day gave birth to male offspring with abnormalities such as cryptorchidism, testicular lesions, reproductive tract malformations and shorter anogenital distance (AGD).<sup>159</sup>

#### **2.4.2 Breast cancer**

*In utero* exposure to estrogens is well known to have long-term consequences in breast cancer development.<sup>160</sup> Scientists have hypothesized that fetal exposure to environmental estrogens is a possible underlying cause of the increased incidence of uterine leiomyoma, testicular cancer and breast cancer globally over the last 50 years. The long latency period between exposure and effect and an availability of birth estrogen records is however a challenge to establish epidemiological patterns.<sup>161-162</sup> The DES cohort women born to DES exposed mothers have provided evidence for a direct link between prenatal estrogen exposure and breast cancer development.<sup>163</sup> At 40 years and above, a DES exposed woman has a 2.5 fold increase in developing breast cancer compared to an unexposed woman of the same age.<sup>164</sup> Chemicals such as dioxins, most resultant from fuel combustion and incineration, the herbicide atrazine, as well as surfactant perfluorooctanoic acid (PFOA) have been found to delay mammary gland development in rodents due exposure during the gestational and lactation period.<sup>165</sup> To date, BPA is a well-studied estrogenic EDC and its link to cancer has been studied from fetal development to postnatal life.<sup>166</sup>

Inorganic arsenic (As) is an estrogen disruptor as well as a carcinogen to which humans may be exposed to primarily through drinking water, inhalation of coal mining dust and *in utero*.<sup>167-168</sup> Many countries still face a potential public health

crisis with As-contaminated drinking water.<sup>167</sup> Studies linking inorganic As directly to breast cancer are still inconsistent. In a case control study, López-Carrillo et al.,<sup>169</sup> associated methylation capacity of inorganic As with an increased breast cancer risk in a Northern Mexico population. Women with a higher capacity to methylate inorganic As to monomethylarsenic were at a higher risk to develop breast cancer while the capacity to methylate it to dimethylarsenic was associated with a lower breast cancer risk. In contrast, Smith et al.<sup>170</sup> found reductions in breast cancer mortality during high exposure to inorganic As in drinking water in northern Chile. A systemic review of epidemiologic studies by Khanjani et al.<sup>171</sup> found no significant effects and concluded that exposure may increase the risk of breast cancer however this relation can vary due to regional and individual differences.

### **2.4.3 Ovarian Dysgenesis Syndrome**

The ovarian dysgenesis syndrome has been proposed as the female form of TDS arising from an impaired ovarian development and function.<sup>172</sup> As with TDS in males, there is growing incidence of reproductive disorders in females including breast cancer, PCOS, premature ovarian failure (POF) and endometriosis.<sup>111,173</sup> The PCOS is a global concern in female fertility and affects about 5-8% of women of childbearing age with hyperandrogenemia and chronic anovulation.<sup>174</sup>

Up to 10% of women, also in childbearing age, get affected by endometriosis which causes infertility in about half these women. Women exposed to DES *in utero* may have an 80% higher risk of endometriosis than unexposed women.<sup>175</sup> Occupational exposure to estrogenic disruptors such as pesticides and plastics has been shown to be a risk factor for female infertility especially among women with high serum BPA concentrations (mixture effect).<sup>176-177</sup> Furthermore, theca cells as well as the granulosa cells have been shown to be the target with chronic exposure to TCDD at environmental levels (less than 1 ng/kg BW per day). The chemicals BPA and DES have also been found to contribute to uterine abnormalities by altering the Hox genes, responsible for regulating the development of the female reproductive phenotype.<sup>178</sup>

#### 2.4.4 Immune system dysfunction

Endocrine disruptors may also interrupt endocrine homeostasis by influencing the cytokine (interferons, tumor necrosis, chemokines, lymphokines and interleukins) signalling pathways. They have been proposed to do this by mimicking/interrupting cytokine activity thus leading to the attenuation of immunity (immunodeficiency) against infection or hyperactivity of immune responses.<sup>179-180</sup>

Immuno modulatory effects of EDCs may be classified as those that 1. decrease immunity, 2. induce inflammation and allergic diseases and 3. induce autoimmune diseases.<sup>181</sup> The National Health and Nutrition Examination Survey completed in the USA from 2003-2006 revealed that EDCs such as BPA and triclosan might negatively affect human immune function.<sup>182</sup> The study found that the BPA level is negatively associated with titer of the anticytomegalovirus antibody. This remains as an active area of research.

#### 2.4.5 Metabolic diseases: Obesity

The doubling of obesity since 1980 correlates well with the changes which have been taking place in the chemical environment since 40 years ago, e.g. agriculture and industry together with greenhouse gas accumulation.<sup>15</sup> This sudden change has drawn the attention of researchers and has led to the hypothesis that there are environmental chemical pollutants named EDCs which have the potential to interfere with body mass homeostasis hence contribute to the development of obesity.<sup>18,183</sup> The EDCs with such characteristics are referred to as obesogens.<sup>18</sup> About 20 chemicals have been identified as potential obesogens. Grün and Blumberg<sup>18</sup> defined an obesogen as “a molecule that inappropriately regulates lipid metabolism and adipogenesis to promote obesity”.<sup>18</sup> These chemicals are ubiquitous and interfere with normal body homeostasis through various NRs. Exposure occurs *in utero* and throughout life, with *in utero* exposure being most detrimental as its effects may be irreversible and result in inheritable epigenetic modifications.<sup>184</sup>

Evidence from experimental as well as epidemiological studies in support of this hypothesis is accumulating for the establishment of a definite consensus between exposure and risk of obesity. In mice, which were treated daily with 0.001 mg of DES, an increased mass, later in life, was observed. Also, *in utero* treatment of another set



of mice with tributyltin (TBT) led to an accumulation of lipids later in life.<sup>18</sup> Low birth mass, followed by obesity in adulthood resulted when these mice were treated with PFOA.<sup>20</sup> Moreover, DDE exposure to pre-adiposities *in vitro* promoted their differentiation to mature adipocytes with increased leptin production.<sup>185</sup>

## 2.5 Water pollution with endocrine disruptors

Drinking water quality is a great environmental determinant for a community's health, lifestyle as well as socio-economic status because all activities occurring in a community ultimately channel into the water systems.<sup>28,30,42,186</sup> Safe and accessible water resources are a necessity for a wholesome health of the public. Globally, close to 1 billion people still lack access to an improved water supply and about 2 billion deaths annually are attributed to poor water quality together with poor hygiene.<sup>186</sup> Poor management of waste disposal systems and practises have contributed greatly to contamination of water sources by EDCs.<sup>187-189</sup> Changing rainfall patterns and temperatures, and severe droughts reflective of climate change have exacerbated these effects. Mining activities and tailings have also been a source of heavy-metal contamination of untreated/wastewater.<sup>190</sup>

The WHO thus noted ten chemicals of major public health concern. They are pesticides, As, asbestos, benzene, cadmium (Cd), dioxins and dioxin-like substances, fluoride, lead (Pb) and mercury (Hg) respectively.<sup>191</sup> Dioxin and dioxin like substances, Cd, As, Pb, Hg and many pesticides are endocrine disruptive. In sub-Saharan Africa, SA stands as one of the highest pesticide users, in parallel to the country's dependence on agricultural activities.<sup>192</sup> Furthermore, groundwater (source for boreholes and some SW) may be contaminated with As.<sup>193</sup> Rural communities are more vulnerable to EDC contaminated water exposure as many depend on water sourced from either boreholes or untreated SW from rivers for drinking as well as day-to-day household activities. Many endocrine disruptors fall under a group of contaminants known as 'contaminants of emerging concern'.<sup>194</sup> These are chemicals with limited regulatory standards and not incorporated in routine monitoring programs for SW.<sup>194</sup>

Water pollution with estrogenic and androgenic chemicals has been reported in literature. Most of this endocrine activity reported ranges from being highest in

wastewaters to below the detection limit (dl) in TDW.<sup>195-198</sup> Using chemically activated luciferase expression (CALUX) based assays, Van der Linden et al.<sup>199</sup> detected estrogenic activity between 0.2-0.5 ng/L EEqs in SWs and no hormonal activity in drinking water samples in the Netherlands. Only 1 out of 8 samples had androgen activity. Conley et al.<sup>200</sup> in the USA detected estrogenic activity ranging from 0.044-0.47 ng/L EEqs in source water samples (16 of 24) to 0.037-0.078 ng/L EEqs in TDW samples (3 of 24) using the T47D-KBluc reporter gene assay.<sup>200</sup> Furthermore, the author found the treatment processes used in the study reduced estrogenic activity in the source water to below dl in drinking water. Aneck-Hahn et al.<sup>201</sup> in SA detected estrogenicity of 0.68 ng/L in tap water from a mine tap and 2.29 ng/L EEqs from spring water in a dry river bed using the YES assay. Studies using the MDA-kb2 reporter gene assay to test androgenic activity are limited. In SA a study by Pieters and Powrie<sup>202</sup> detected androgenic activity in SW sampled from agricultural areas.<sup>203</sup>

## 2.6 Aim

This study therefore aimed to assess the suitability of the MDA-kb2 reporter gene assay for androgenic activity and the YES assay and T47D-KBluc reporter gene assays for estrogenic activity in water from different sources.

## 2.7 Objectives

The objectives were divided into two phases

### Phase 1

- The objective was to apply the MDA-kb2 reporter gene assay to the Global Water Research Coalition (GWRC) water samples from different sources. Briefly, the GWRC is a platform for global cooperation for the exchange and generation of water knowledge achieved through research collaborations to support safe and sustainable water supply and sanitation for the protection of public health and aquatic environment. The coalition is formed by the: Canadian Water Network, Kia Water Research Institute, Public Utilities Board (Singapore), Foundation for Applied Water Management Research (Netherlands), International Research Center on Water and Environment (Spain), Water Technology Centre (German), Water Industry Research (United Kingdom),

Veolia Environment research and Innovation (France), Water Environment & Reuse Foundation (US), Water Research Australia Limited, Water Research Commission (SA), Water Research Foundation (US) and Water Services Association of Australia. This project was affiliated with the GWRC through the Water Research Commission (SA). It was part of the GWRC Toolbox II project which aimed to develop and validate methods that will test a range of other endocrine endpoints such as androgenic, thyroid, progestagen, glucocorticoid, retinoid and peroxisome proliferation activity for a variety of water samples.

## **Phase 2**

The objectives were to:

- Determine estrogenic activity in monthly surface and treated drinking water (TDW) samples for monitoring two treatment plants using the Recombinant YES and T47D-KBluc reporter gene assay
- Determine androgenic activity in monthly surface water and TDW samples for monitoring two water treatment plants using the MDA-kb2 reporter gene assays

## CHAPTER 3

### 3 Materials and Methods

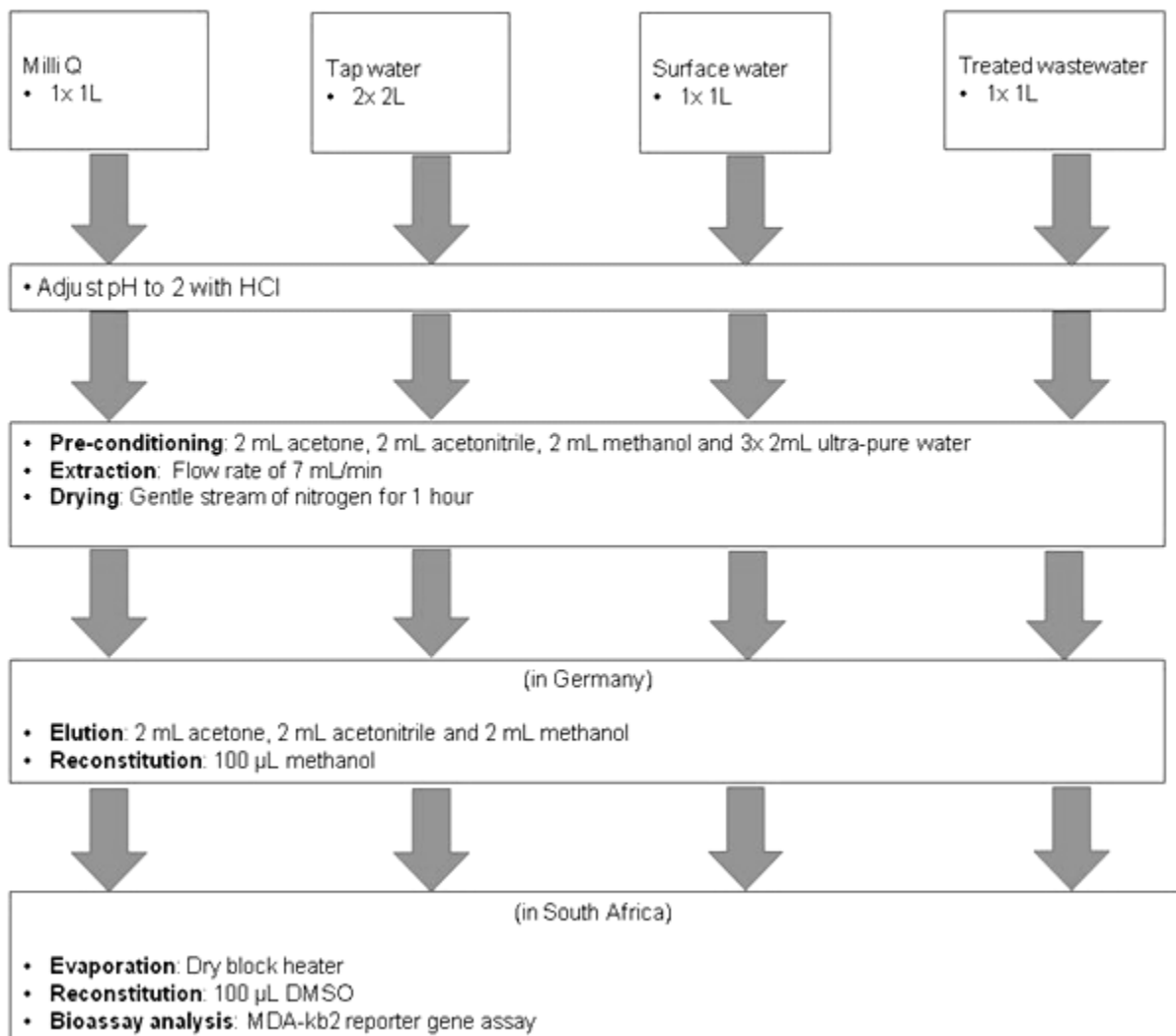
#### 3.1 Study design and setting

The study was a laboratory study conducted in the EDC laboratory at the University of Pretoria (UP).

#### 3.2 Ethical clearance

Ethical approval was granted by the Research ethics committee in the Faculty of Health Sciences, UP (Ethics Reference No.: 535/2015). A letter of clearance is attached in appendix A.

### 3.3 PHASE 1: The GWRC water samples



**Figure 3-1:** Overview of phase 1

#### 3.3.1 Water sampling

To address the aim in phase one, water samples were collected from a catchment area based in Pretoria using methanol rinsed glass Schott bottles (DURAN® Group, Hattenbergstraße, Germany). These samples were 2 L x2 tap water, 1 L x1 SW and 200 mL x1 treated wastewater respectively. A liter of ultrapure water (MilliQ) was also sampled from the EDC laboratory, and was spiked with DHT (Cat. No. A2570-000, Steroids Co.) for comparison of extraction efficiency (not the objective of this study). After collection, the pH of the samples was adjusted to two using 5N hydrochloric acid (HCl) for optimum analyte recoveries. The MilliQ system used in

this study is fitted with an activated carbon cartridge filter (Merck Millipore, Darmstadt, Germany, Cat. No. EDSPAK001) which is designed to remove EDCs such as phthalate esters, nonylphenol and BPA.

### **3.3.2 Solid Phase Extraction (SPE)**

The process, SPE, was carried out on 200 mg Strata™X SPE cartridges (Phenomenex®, Torrance, USA) at a flow rate of 7 mL/min. The cartridges were preconditioned with 2 mL of: 1) acetone (Sigma-Aldrich Pty. Ltd, St. Louis, Missouri, USA, Cat. No. 34850), 2) acetonitrile (Merck, Darmstadt, Germany, Cat. No. 100030) and 3) methanol (Merck, Darmstadt, Germany, Cat No. 1.06007.2500) and 3x 2 mL double distilled water (ddH<sub>2</sub>O) in a biohazard fume hood. The sorbents are bonded with C<sub>18</sub> hydrophobic alkyl groups that are solvated by these solvents. The broad spectrum of solvents also allows the alkyl groups to capture a wide range of EDCs which may be present in a sample. Following this, a litre of the sample was passed through the cartridges under a vacuum (Chromabond® Manifold, Cat. No. 730150); 5 mL loaded at a time using 10 mL serological pipettes (Corning Incorporated (Inc.), New York, USA, Cat. No. 4101). The pressure did not exceed 70 kPa. The cartridges were dried under a gentle stream of nitrogen for an hour and sent to Technologiezentrum Wasser (TZW), Karlsruhe, Germany for elution and distribution to the participating laboratories.

#### **3.3.2.1 Elution (in Germany)**

Cartridges were received by TZW-Germany for elution. A volume of 2 mL: 1) acetone, 2) acetonitrile and 3) methanol were added in each cartridge and allowed to gently percolate through the sorbent beds into test tubes. The resultant eluates were evaporated, reconstituted in methanol and aliquoted in amber vials (100 µL per vial) for distribution to the participating laboratories.

#### **3.3.2.2 Reconstitution**

Twenty-four samples in methanol, sourced from six different countries (Australia, Germany, Spain, France, Netherlands and SA), were received from TZW to be tested in the MDA-kb2 assay. This was evaporated using a dry block heater (Techne®, Staffordshire, United Kingdom (UK)), re-reconstituted in 100 µL dimethyl

sulfoxide (DMSO) (Sigma-Aldrich Pty. Ltd, St. Louis, Missouri, USA, Cat. No. D260) and stored at room temperature. Enrichment factors (concentration of the extracts) for the extracted samples were 200x for wastewater, 400x for tap water, 1000x for SW and 1000x for spiked MilliQ respectively.

### **3.3.3 Bioassay analysis**

#### **3.3.3.1 The MDA-kb2 reporter gene assay**

The MDA-kb2 reporter gene assay is an androgen-responsive transcriptional activation assay developed by Wilson and colleagues.<sup>204</sup> It uses the MDA-kb2 cell line transformed from MDA-MB-453 human breast cancer cells.<sup>204</sup> It was transformed with an androgen-responsive luciferase reporter plasmid which is driven by the mouse mammary tumor virus promoter (MMTV).<sup>204</sup> Both the nuclear AR and glucocorticoid receptor are expressed by the cell line and can both activate the MMTV reporter gene.

Briefly, upon binding of an agonist to the receptor, transcription is activated and the luciferase enzyme is produced.<sup>204</sup> This enzyme is directly proportional to the active ligand concentration. This is assayed using chemiluminescence, which measures light produced when exogenous luciferin substrate together with its co-factors are added.<sup>204-205</sup> Since the cell line expresses both the glucocorticoid receptor and the AR, which are both responsive to the MMTV, the source driving response must be distinguished by co-administering an AR antagonist, hydroxyflutamide (OHF).<sup>204</sup>

#### **3.3.3.2 General cell culture maintenance**

The cells were purchased from the American Type Culture Collection (ATCC) (CRL-2713™). They were grown and maintained in Lebovitz's L-15 growth medium (L-15) (Gibco®, Life Technologies Corporation, Paisley, UK, Cat. No. 41300-021) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, South Logan, Utah, USA, Cat. No. SH30071.03) and antibiotic solution (Gibco®, Life Technologies Corporation, Paisley, UK, Cat. No.15240-062) in a carbon dioxide (CO<sub>2</sub>) free incubator at 37°C. Antibiotic solution consisted of: 100 U/mL streptomycin, 100 µg/mL penicillin and 0.25 µg/mL amphotericin B.<sup>204</sup>

The cells were routinely maintained in sterile 75 cm<sup>2</sup> tissue culture flasks (Corning Inc., New York, USA, Cat. No. 430641) and sub-cultured at 80%-90% confluency (at least twice weekly). Briefly, the medium was removed by gently decanting it into a waste bottle. This was followed by rinsing each flask twice with 7 mL of 1x Hank's balanced salt solution (HBSS) (Gibco®, Life Technologies Corporation, Paisley, UK, Cat. No. 14185-045). A volume of 3 mL of 1x trypsin (Gibco®, Life Technologies Corporation, Paisley, UK, Cat. No. 15400-054) was added and incubated for 3 minutes to detach the cells from the flask as trypsin is a digestive enzyme that lyses peptide bonds attaching the cells to the flask. Following this, excess trypsin was gently decanted and the flask carefully tapped against the palm of one hand to assist the detachment process. It was neutralized with 9 mL L-15 medium and thereafter split in a 1:3 ratio into new flasks for growth. The flasks were labelled with the date, cell line, passage number and returned into the incubator for growth. Passage number for experiments never exceeded 20.

### **3.3.3.3 Assay procedure**

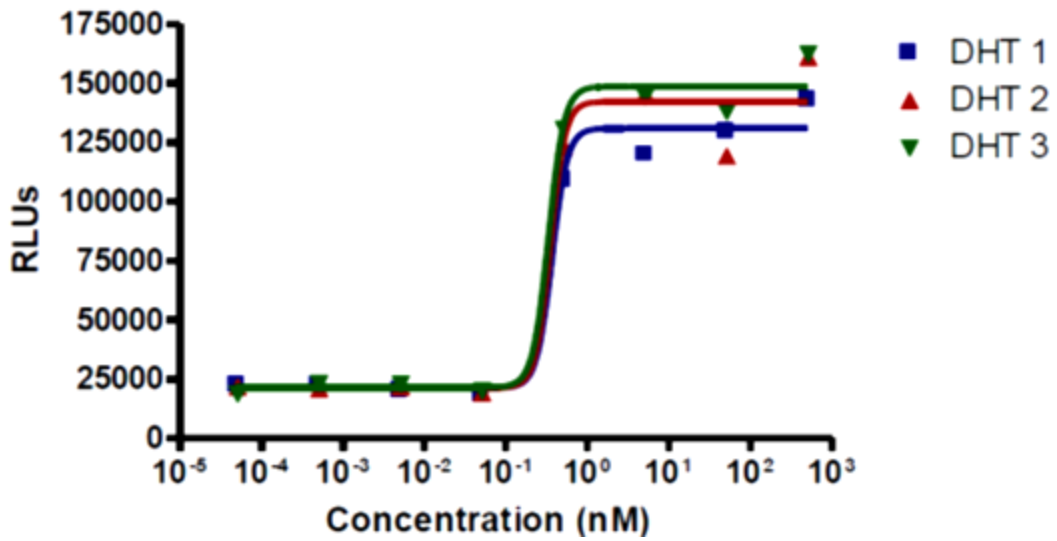
#### **3.3.3.3.1 Specificity and sensitivity of the assay**

The assay was performed according to the method described by Wilson et al.<sup>204</sup> with minor modifications. Briefly, to ensure that the assay was sensitive and specific to the receptor of interest, it was initially optimized. The cells were seeded at 5 x10<sup>4</sup> cells per well in 96 well luminometer plates (Corning Inc., New York, USA, Cat. No. 3610) and allowed to attach overnight in a CO<sub>2</sub> free incubator, at 37°C. Once attached the medium was removed and replaced with dosing solutions. The cells were dosed with different concentrations (nM) of the agonist (DHT) at: 500, 50, 5, 0.5, 0.05, 0.005, 0.0005, 0.00005. The stock solution was prepared at 200 µM in DMSO of which total DMSO concentration in each well did not exceed 0.5%. The initial concentration was prepared by transferring 5 µL of the stock into 995 µL of L-15 medium. Consecutive dosing concentrations were prepared in the vehicle control (VC); which was medium consisting of 0.5% DMSO. A volume of 50 µL of each preparation was subsequently transferred into each well. The final volume in a well was 100 µL.

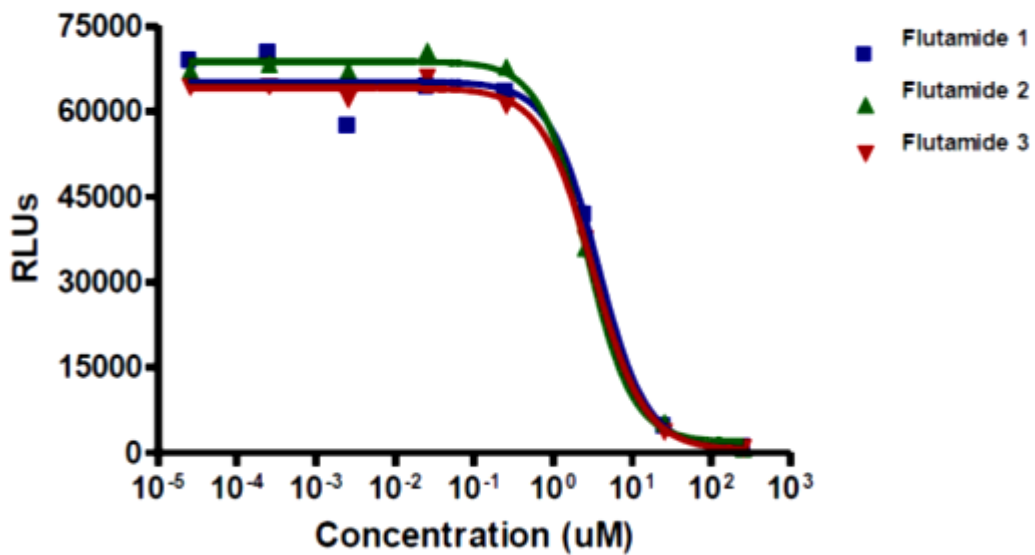
The maximum luciferase response was reached at 1 nM, which is within the linear range of DHT and is similar to the concentration (0.1 nM-1 nM) used by Wilson et al.<sup>204</sup> when the assay was developed. This is indicated in **Figure 3-1**. This



concentration was also suitable for screening anti-androgenic activity. A high concentration may increase the risk of over-looking weak antagonists while a lower concentration may compromise the dynamic range of the assay.<sup>206</sup> From literature<sup>204</sup>, flutamide is a potent androgen antagonist. When it was co-incubated with 1 nM DHT, it suppressed luciferase activity in a dose dependent manner (**Figure 3-3**). Flutamide (Sigma-Aldrich Pty. Ltd, St. Louis, Missouri, USA, Cat. No. 13311-84-7) stock solution was prepared at 100 mM. The controls included in each plate were: agonist positive control (1 nM DHT), VC, background control (VC and 10  $\mu$ M flutamide) as well as an antagonist control (10  $\mu$ M flutamide and 1 nM DHT). The ability of the MDA-kb2 cell line to bind DHT and be suppressed by flutamide (**Figure 3-3**) indicated the assays specificity for the AR receptor.



**Figure 3-2:** Standard curves showing DHT agonistic action on the MDA-kb2 cells



**Figure 3-3:** Dose dependent suppression of 1 nM DHT co-incubated with increasing flutamide concentrations on the MDA-kb2 cells

### 3.3.3.3.2 Testing the water sample extracts

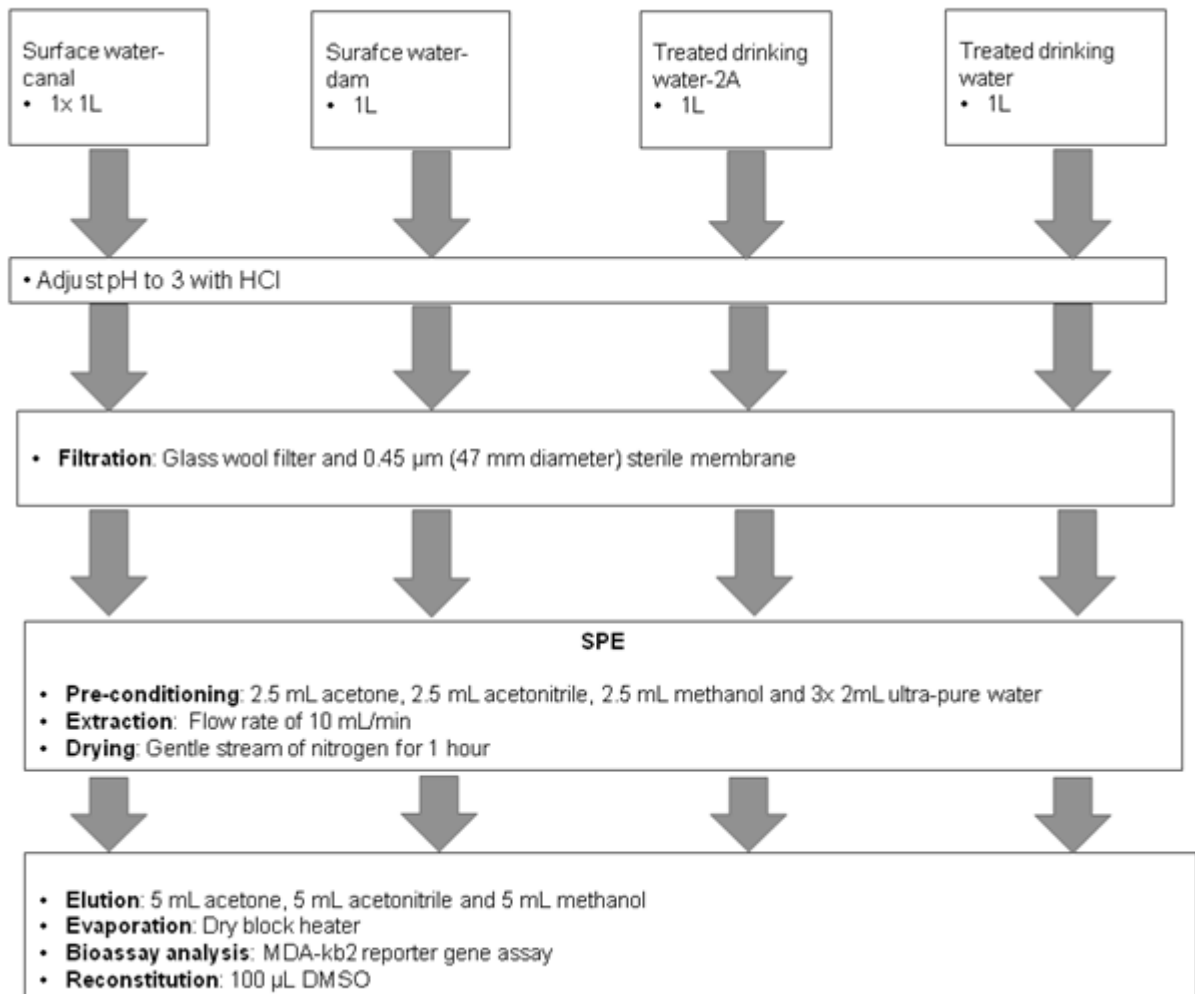
The assay was applied to the water sample extracts. Each sample was tested alone for androgenic activity and in the presence of 1 nM DHT for anti-androgenic activity. Initial concentrations of the sample extracts were prepared by adding 5  $\mu$ L into 995  $\mu$ L L-15 medium. Consecutive concentrations were prepared in the VC through a serial dilution of 1:10, with four points. The highest concentrations that the samples were tested at were 0.5x for wastewater, 1x for tap water and 2.5x for MilliQ and SW respectively.

The cells were seeded, dosed and returned into the incubator for 24 hours. Following this incubation period, they were observed for signs of cytotoxicity under a light microscope (Olympus Corporation, Tokyo, Japan). These included vacuolization, detachment, membrane degradation or lack of phase brightness.<sup>204</sup> The cells were then washed once with 100  $\mu$ L 1x phosphate buffered solution (PBS) (Gibco®, Life Technologies Corporation, Paisley, UK, Cat. No. 14080-048) in each well, discarding the PBS and adding 25  $\mu$ L reporter lysis buffer (Promega Corporation, Madison, Wisconsin, USA, Cat. No. E3971). The plates were thereafter frozen overnight at -80°C to activate the lysis buffer, after one freeze-thaw cycle. The lysis buffer breaks open the cells and releases its contents. Luciferase activity, in relative light units (RLUs), was read using LUMIstar OPTIMA luminometer (BMG Labtech, Offenburg, Germany) which dispensed 25  $\mu$ L reaction buffer followed by 1 mM D-luciferin after 5 seconds (s). The reaction buffer consisted of 15 mM magnesium chloride ( $MgCl_2$ ), 5 mM adenosine triphosphate (ATP) (Sigma-Aldrich Pty. Ltd, St. Louis, Missouri, USA, Cat. No. A7699) and 0.1 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich Pty. Ltd, St. Louis, Missouri, USA, Cat. No. A7906) at pH 7.8.

A screening approach was used to identify positive samples thereafter confirmed in triplicate runs with full dose response curves. The highest concentration of the positive samples was co-incubated with 10  $\mu$ M flutamide to confirm that the activity was through the AR receptor not the glucocorticoid receptor. Relative light units (RLUs) were converted to fold inductions. Triplicate plates were assayed for samples that induced luciferase activity above two-fold induction with reference to the VC. Standard curves were fitted (sigmoidal function, variable slope) using Graphpad Prism version 4 which interpolated the DHT equivalents (DHTEqs) from the DHT

standard curve and calculated the half maximal effective concentration ( $EC_{50}$ ), slope, minimum, maximum and 95% confidence limits. The interpolated values were corrected for dilution factors.

### 3.4 PHASE 2: Water samples from a Gauteng based catchment area



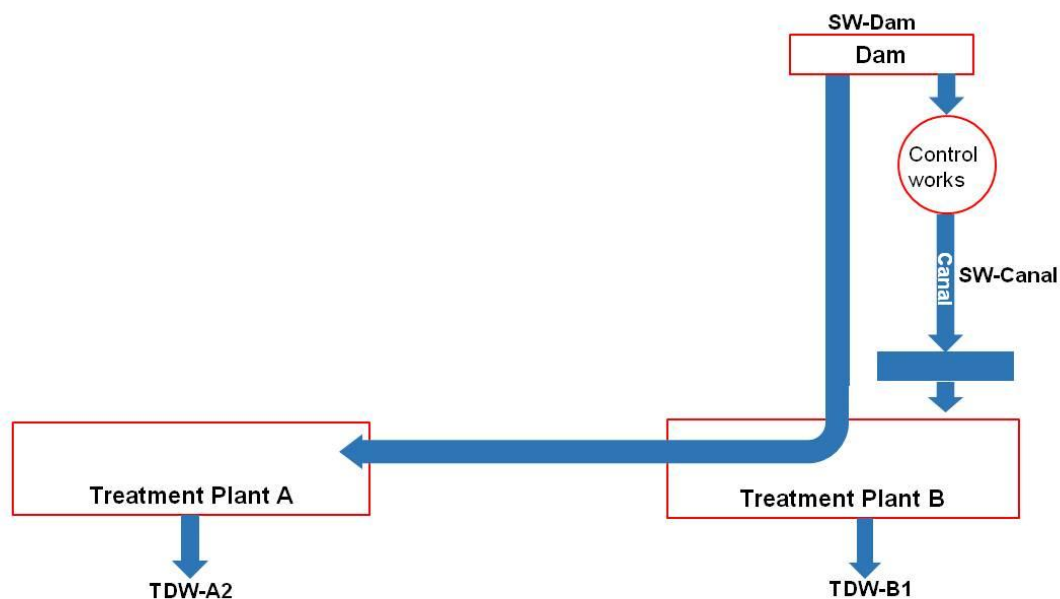
**Figure 3-4:** Overview of phase 2

#### 3.4.1 Water sampling

Water samples were collected in duplicates from a catchment area of one of SA largest dams. The dam spans four provinces: Gauteng, Mpumalanga, Free State and North West provinces. The samples were collected on a monthly basis from April 2015 until March 2016 using methanol rinsed Schott bottles. The samples

collected were: SW-Canal, sampled from a canal receiving water from the dam and goes into treatment plant B, coming out as TDW-B1 post treatment to supply the distribution network; SW-Dam, sampled near the dam wall; and TDW-A2 sampled from a post treatment line of treatment plant A. This is graphically depicted in **Figure 3-5**.

The treatment plants supply two of the largest metropolitan cities in SA with drinking water. The samples were selected to test if there was any estrogenic and (or) androgenic activity present in the SW, and if present, investigate if they are effectively removed by the treatment processes in the treatment plants. Upon arrival at the laboratory, the pH of the samples was adjusted to 3 using HCl and were stored at 4°C until extraction.



**Figure 3-5:** Graphical illustration of the four sampling sites in relation to the treatment plants

### 3.4.2 Sample filtration

The samples were filtered according to the protocol described by De Jager et al.<sup>205</sup> with slight modifications. Briefly, the 1 L water samples were each loaded into the

filtration reservoir, 250-300 mL at a time, under vacuum. Each sample was filtered through a glass wool filter (Macherey Nagel, Düren, Germany, Cat. No. 4110047) and a 0.45 µm (47 mm diameter) sterile membrane (Pall Corporation, New York, USA, Cat. No. 66234) to remove unwanted particulates which may interfere with the extraction process e.g. block the SPE cartridges. The filters were changed regularly within the same sample to prevent clogging.

### **3.4.3 Solid phase extraction**

Solid phase extraction was carried out according to the protocol described in the tool box by De Jager et al.<sup>205</sup> with minor modifications. In short, the water samples were extracted on pre-conditioned Oasis HLB reversed-phase SPE cartridges (Waters Corporation, Massachusetts, USA, Cat. No. 186000115) at a flow rate of 10 mL/min. The cartridges were preconditioned twice with 2.5 mL of: 1) acetone (Sigma-Aldrich Pty. Ltd, St. Louis, Missouri, USA, Cat. No. 34850), 2) acetonitrile (Merck, Darmstadt, Germany, Cat. No. 100030) and 3) methanol (Merck, Darmstadt, Germany, Cat No. 1.06007.2500) and 4) ddH<sub>2</sub>O in a biohazard fume hood. A litre of each sample was passed through the cartridge reservoir; 5 mL loaded at a time under a vacuum (Chromabond® Manifold, Cat. No. 730150) using 10 mL serological pipettes (Corning Inc., New York, USA, Cat. No. 4101). The pressure did not exceed 70 kPa. The cartridges were then dried under a gentle stream of nitrogen. For all extractions, an extraction (solvent) control was run with each batch in order to ensure that the extraction was successful, with no contamination.

### **3.4.4 Elution**

The cartridges were eluted with 5 mL of acetone, acetonitrile and methanol in glass test tubes (Hirschmann®, Eberstadt, Germany, Cat. No. 9270110) thereafter the eluates evaporated under a gentle stream of nitrogen at 37°C using a dry block heater (Techne®, Staffordshire, UK) and reconstituted in 1 mL DMSO in glass amber vials (Chromatography research supplies, Louisville, Kentucky, USA Cat. No.154515).

### **3.4.5 Bioassay analysis**

The samples were assayed in the YES assay, T47D-KBluc reporter gene assay and MDA-kb2 reporter gene assay

### 3.4.5.1 The YES assay

The cells were obtained from Professor J.P Sumpter's laboratory in the Department of Biology and Biochemistry, Brunel University, Uxbridge, UK. The assay makes use of the yeast strain, *Saccharomyces cerevisiae*. As developed by Routledge and Sumpter,<sup>207</sup> the yeast cells have been transfected with a human ER (hER)  $\alpha$  and a plasmid containing the *lac-Z* gene which encodes  $\beta$ -galactosidase enzyme.<sup>207</sup> Upon transcription, the receptor is expressed and active compounds bind to induce the expression of the *lac-Z* gene for the enzyme  $\beta$ -galactosidase.<sup>207</sup> The enzyme is secreted into the assay medium. The chromogenic substrate, chlorophenol red- $\beta$ -D-galactopyranoside (CPRG), added in the assay medium, is metabolised by  $\beta$ -galactosidase in the presence of an estrogenic ligand. This causes a colour change from yellow to red. The concentration of the  $\beta$ -galactosidase is directly proportional to the active ligand concentration.<sup>207</sup>

#### 3.4.5.1.1 Growth and assay medium preparation

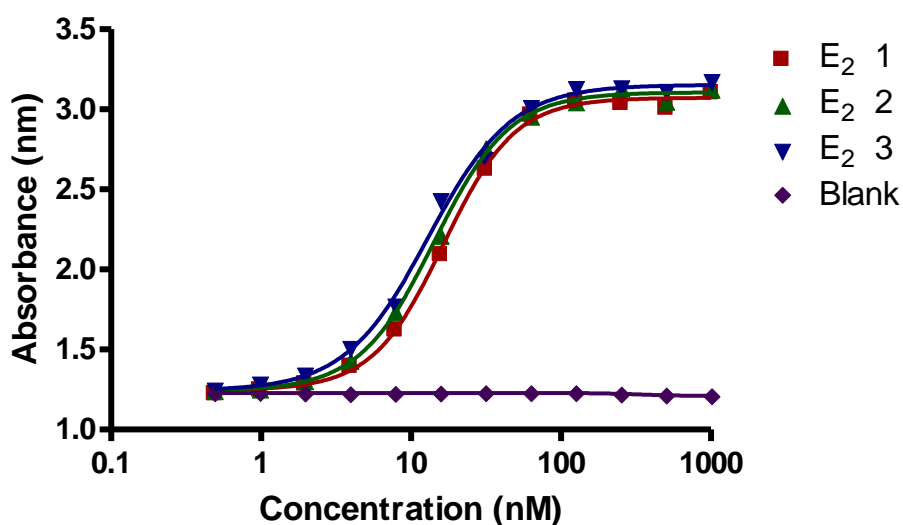
The assay components were prepared as described by Routledge and Sumpter.<sup>207</sup> Briefly, growth medium was prepared by adding 9 mL minimal medium, 1 mL of 0.2 g/mL glucose solution, 0.25 mL of 4 mg/mL of L-aspartic acid solution, 0.1 mL vitamin solution, 0.08 mL of 24 mg/mL L-threonine solution and 25  $\mu$ L of 3.2 mg/mL copper (II) sulphate solution and 100  $\mu$ L of the 10x concentrated yeast stock. This was incubated for 24 hours in a 150-155 rpm rotating water bath (Grant, Cambridge, UK) at 28°C.

For the assay medium, new growth medium was prepared (with adjustments for the number of experiments) and inoculated with the 24-hour yeast culture and 10 mg/mL of CPRG (Roche Diagnostics, Mannheim, Germany, Cat. No. 10884308001).

### 3.4.5.1.2 Assay procedure

The assay was performed as described by Routledge and Sumpter with minor modifications.<sup>207</sup> Briefly, using 96 well microtiter plates (Thermo Fisher Scientific™, Waltham USA, Cat. No. 168136), 200  $\mu$ L of the water sample extract, blank (VC), positive and extraction control were placed in the first well of each row of the dilution plates and serially diluted (1:2) in 100  $\mu$ L DMSO (Sigma-Aldrich Pty. Ltd, St. Louis, Missouri, USA, Cat. No. D2650) across the remaining 12 rows. The positive control used was E<sub>2</sub> of which the curve (**Figure 3-6**) ranged from 1000 nM-0.1 nM. The blank only consisted of DMSO in assay medium (2%). Following this, 4  $\mu$ L of each dilution was transferred into triplicate plates and thereafter 200  $\mu$ L of the assay medium was added.

The plates were then sealed with autoclave tape and incubated at 32 °C in a naturally ventilated incubator (Heraeus, Hanau, Germany, B290) for three-five days. After 3 days, absorbance was read using a plate reader (Thermo MultiSkan Spectrum plate reader 1500, Massachusetts, USA) at 540 nm for colour change and 620 nm for turbidity. Turbidity was corrected using the equation: Corrected value = test abs (540 nm) – [test abs (620 nm) – median blank abs (620 nm)]. Samples were considered positive if three or more consecutive observations were above the dl of the assay. The dl of the assay was calculated as: average of blank plus 3 times the standard deviation (SD).



**Figure 3-6:** E<sub>2</sub> agonistic action on the yeast cells



### **3.4.5.2 The T47D-KBluc reporter gene assay**

The cells were obtained from the ATCC (CRL-286™). The T47D-KBluc reporter gene assay uses the T47D human breast cancer cell line which naturally expresses both hER  $\alpha$  and  $\beta$  endogenous therefore an increased sensitivity compared to the YES assay which only has the  $\alpha$  receptor. The cells have been transfected with the ERE luciferase reporter gene, which activates the luciferase receptor gene for luciferase enzyme expression when estrogenic ligands bind to the receptor(s). The enzyme is directly proportional to ligand concentration and is assayed in the presence of luciferin substrate and other co-factors. An estrogen is defined as a chemical that induces a dose-dependent luciferase activity, which could be inhibited by the anti-estrogen ICI 182,780.<sup>208</sup>

#### **3.4.5.2.1 General cell culture maintenance**

The assay was performed according to the procedures described by Wilson et al.<sup>209</sup> and De Jager et al.<sup>205</sup> with minor modifications. The cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich Pty. Ltd, St. Louis, Missouri, USA, Cat. No. R8755), without phenol red, supplemented with 10% FBS, 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer) (Gibco®, Life Technologies Corporation, Paisley, UK, Cat. No. 15630-056), 2.5 g/L glucose (Merk, Cat. No. 8337), 100 mM sodium pyruvate (Gibco®, Life Technologies Corporation, Paisley, UK, Cat. No. 11360-039), 1.5 g/L NaHCO<sub>3</sub> (Sigma-Aldrich Pty. Ltd, St. Louis, Missouri, USA, Cat. No. S5761) and antibiotic solution (constituents described in section 3.3.3.2). A week prior to experiments, the cells received nutrient medium from which the hormones have been withdrawn by replacing 10% FBS with 10% dextran-charcoal treated FBS (Hyclone Laboratories, Cat. No. SH30068.03). The antibiotic was also excluded.

#### **3.4.5.2.2 Assay procedure**

The cells were seeded at  $5 \times 10^4$  cells per well in 96 well luminometer plates (Corning Inc. Cat. No. 3610) using 5% dextran-charcoal treated FBS medium and allowed to attach overnight in humidified air supplemented with 5% CO<sub>2</sub> incubator (NuAire, Plymouth, USA) at 37°C. Each sample extract was tested alone for estrogenic activity and in the presence of 1 nM E<sub>2</sub> for anti-estrogenic activity and DMSO concentration did not exceed 0.5%. Controls included in each plate were 1 nM E<sub>2</sub>

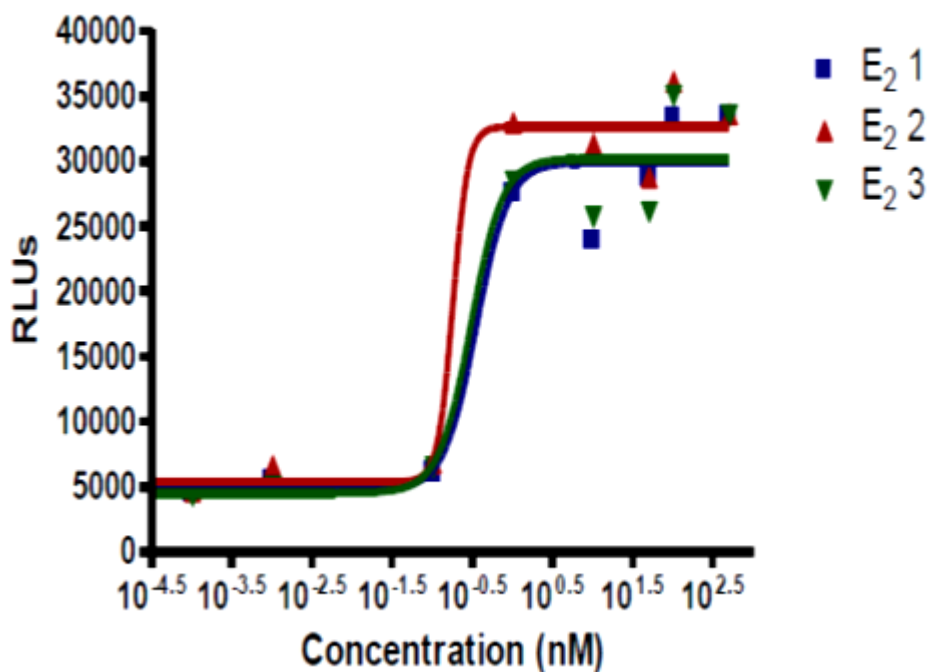
positive control, VC, background control (medium and 0.1  $\mu$ M ICI) as well as 0.1  $\mu$ M ICI and 1nM DHT as antagonist control. After a 24 hour incubation period, cells were observed under a light microscope (Olympus Corporation, Tokyo, Japan) for signs of cytotoxicity which included vacuolization, detachment, membrane degradation or lack of phase brightness.<sup>204</sup> Following this, the cells were washed once with 100  $\mu$ L 1x PBS (Gibco®, Life Technologies Corporation, Paisley, UK, Cat. No. 14080-048) in each well thereafter the PBS was discarded. A volume of 25  $\mu$ L reporter lysis buffer (Promega Corporation, Madison, Wisconsin, USA, Cat. No. E3971) was added in each well. This terminated the reactions. Luciferase activity, in RLU, was read after one freeze-thaw cycle using a LUMIstar OPTIMA luminometer (BMG Labtech, Offenburg, Germany) which dispensed 25  $\mu$ L reaction buffer followed by 1 mM D-luciferin after 5 seconds (s). Reaction buffer consisted of 15 mM  $MgCl_2$ , 5 mM ATP (Sigma-Aldrich Pty. Ltd, St. Louis, Missouri, USA, Cat. No. A7699) and 0.1 mg/mL BSA (Sigma-Aldrich Pty. Ltd, St. Louis, Missouri, USA Cat. No. A7906) at pH 7.8.

The EEqs were calculated for samples that induced luciferase activity above two-fold induction with reference to the VC and corrected with the appropriate dilution factor. A standard curve was fitted (sigmoidal function, variable slope) using Graphpad Prism version 4 which interpolated the EEqs from the  $E_2$  standard curve and calculated the  $EC_{50}$ , slope, minimum, maximum and 95% confidence limits. The interpolated values were corrected for dilution factors. The EEqs were reported as the average  $\pm$  the SD of three independent repeats. Passage number never exceeded 30.

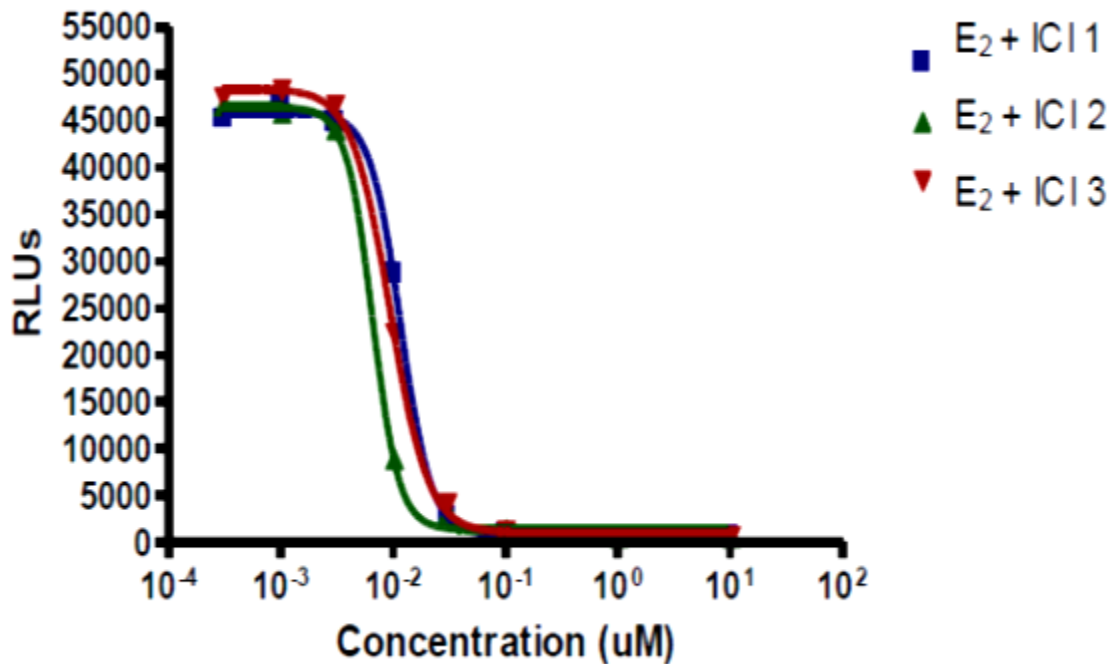
#### **3.4.5.2.2.1 Sensitivity and specificity of the assay**

All experiments were done in assay medium, which was 5% dextran-charcoal treated FBS medium made by mixing equal volumes of the 10% dextran-charcoal treated FBS and RPMI medium without FBS.<sup>205</sup> Cells were seeded at  $5 \times 10^4$  cells per well in 96 well luminometer plates (Corning Inc. New York, USA, Cat. No. 3610) and allowed to attach overnight in a 5%  $CO_2$  incubator (NuAire, Plymouth, USA) at 37°C. Prior to testing sample extracts, the assay's sensitivity and specificity were ensured by dosing the cells with increasing concentrations of the agonist  $E_2$  (Sigma-Aldrich Pty. Ltd, St. Louis, Missouri, USA, Cat. No. E8875) at concentrations (nM): 0.000005, 0.00005, 0.0005, 0.005, 0.05, 0.5, 5 and 50. The  $E_2$  stock was prepared in

DMSO at 200  $\mu$ M. The first dilution was prepared by transferring 5  $\mu$ L of the stock into 995  $\mu$ L assay medium. The DMSO concentration did not exceed 0.5%. Luciferase activity was increased at 1 nM and it was within the linear range of the curve (**Figure 3-7**). This concentration was chosen as the positive control as well as the co-incubation concentration for anti-estrogenic activity. The chemical ICI 182,780 (Tocris Biosciences, Ellisville, USA, Cat. No. 1047) was used as the antagonist. When it was co-incubated with 1 nM E<sub>2</sub>, it significantly suppressed luciferase activity in a dose dependant manner (**Figure 3-8**). The ability of the T47D-KBluc cell line to bind E<sub>2</sub> and be suppressed by ICI indicated the assays specificity for the ER receptors.



**Figure 3-7:** Standard curves E<sub>2</sub> agonist action on the T47D-KBluc cells



**Figure 3-8:** 1 nM 17β-estradiol suppression by ICI 182,780 on the T47D-KBluc cells

#### 3.4.5.2.2.2 Testing water sample extracts

Cells, which had been withdrawn for one week, were seeded and incubated at 37°C, 5% CO<sub>2</sub> overnight to allow the cells to attach. Following this, the medium was carefully removed and dosing solutions added. Initial sample concentrations were prepared by transferring 5 μL of the sample extract into 995 μL assay medium. Subsequent dilutions (1:10) were made in 360 μL VC (assay medium and 0.5% DMSO) by transferring 40 μL across each dilution. A volume of 50 μL of each dilution was transferred into the wells of the seeded plates. For estrogenic activity, this was co-incubated with 50 μL VC or 1 nM E<sub>2</sub> for anti-estrogenic activity. The highest concentration tested was at 2.5x. Each plate included, in triplicates, 1 nM E<sub>2</sub> positive control, VC, background control (medium and 0.1 μM ICI) as well as 0.1 μM ICI and 1 nM DHT as antagonist control.

The dosed plates were returned into the incubator for 24 hrs. Following this, the cells were observed under a light microscope for signs of any signs of cytotoxicity before reactions were terminated. Cells were rinsed once with PBS and 25 μL reporter lysis buffer was added into each well and frozen at -80. After one freeze thaw cycle,

luciferase activity was measured using LUMIstar OPTIMA luminometer (BMG Labtech, Offenburg, Germany) in RLUs, as described in section 3.4.5.2.2.

The RLUs were converted into fold inductions. To confirm activity, these were assayed in triplicates with full dose response curves. The highest concentration of the positive samples was co-incubated with 0.1  $\mu$ M ICI to confirm that activity was through the ERs. Graphpad Prism version 4 was used to interpolate the EEqs from the standard curve. The EC<sub>50</sub>, slope, minimum and maximum were also interpolated with 95% confidence limits. The interpolated values were corrected for dilution factors.

#### **3.4.5.3 The MDA-kb2 reporter gene assay.**

The assay was conducted as described in section 3.3.3.3.2. The sample extracts were tested at concentrations 2.5x, 0.25x, 0.025x and 0.0025x.

### **3.5 Statistical analysis**

A two-way analysis of variance (ANOVA) was used to analyse the data. It looked for significant differences between months, concentrations and interaction between the groups. A pairwise comparison was used to compare these different groups.

There was no evidence of significant differences between the above mentioned groups.

## CHAPTER 4

### 4 Results

#### 4.1 Phase 1: The GWRC water samples

Five out of 24 water samples (21%) tested positive for androgenic activity. The highest was in a treated wastewater sample from Netherlands ( $0.23 \pm 0.040$  ng/L DHTEqs) and lowest in tap water sample from TZW-Germany ( $0.008 \pm 0.001$  ng/L DHTEqs). It was also detected in TZW-Germany ( $0.028 \pm 0.006$  ng/L DHTEqs) and Cirsee-Spain ( $0.068 \pm 0.008$  ng/L DHTEqs) SW samples and in Cirsee-Spain treated wastewater ( $0.028 \pm 0.030$  ng/L DHTEqs). Androgenic activity for the other water samples was below the dl of the assay. This is indicated in **Table 4.1**. Cytotoxicity was not observed in any of the samples nor anti-androgenic activity detected.

**Table 4.1:** A summary of results for all GWRC samples screened in the MDA-kb2 reporter gene assay

| Water matrix              | Sample ID          | Androgenic activity (DHTEqs ng/L) |
|---------------------------|--------------------|-----------------------------------|
| <b>MilliQ</b>             |                    |                                   |
|                           | TZW-Germany        | <dl                               |
|                           | Griffith Australia | <dl                               |
|                           | Veri-France        | <dl                               |
|                           | UP-SA              | <dl                               |
|                           | Cirsee -pain       | <dl                               |
|                           | Netherlands        | <dl                               |
| <b>Tap water</b>          |                    |                                   |
|                           | TZW-Germany        | 0.008 ± 0.001                     |
|                           | Griffith Australia | <dl                               |
|                           | Veri-France        | <dl                               |
|                           | UP-SA              | <dl                               |
|                           | Cirsee Spain       | <dl                               |
|                           | Netherlands        | <dl                               |
| <b>SW</b>                 |                    |                                   |
|                           | TZW-Germany        | 0.028 ± 0.006                     |
|                           | Griffith Australia | <dl                               |
|                           | Veri-France        | <dl                               |
|                           | UP-SA              | <dl                               |
|                           | Cirsee -Spain      | 0.068 ± 0.008                     |
|                           | Netherlands        | <dl                               |
| <b>Treated wastewater</b> |                    |                                   |
|                           | TZW-Germany        | <dl                               |
|                           | Griffith Australia | <dl                               |
|                           | Veri-France        | <dl                               |
|                           | UP-SA              | <dl                               |
|                           | Cirsee-Spain       | 0.028 ± 0.030                     |
|                           | Netherlands        | 0.23 ± 0.040                      |

<dl=below detection limit, TZW= Technologiezentrum Wasser, SA=South Africa, UP=University of Pretoria, DHTEq= Dihydrotestosteronel equivalent, SD= standard deviations



## 4.2 Phase 2: Water samples from a Gauteng based treatment plant

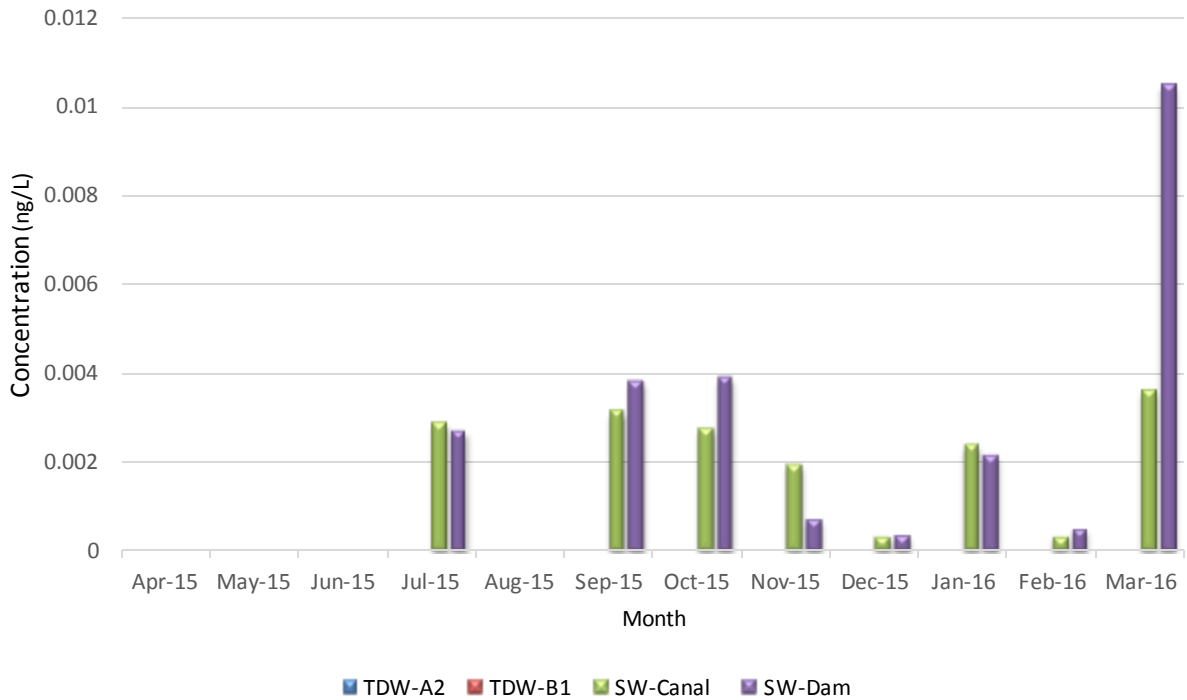
### 4.2.1 Estrogenic activity

In monitoring the water treatment plant, estrogenic activity in the YES assay was below the dl of the assay. The samples were consistently comparable to the blank (VC) used in the assay, with 95 % confidence interval. In the T47D-KBluc reporter gene assay, it ranged from below the dl to  $10.51 \pm 5.74$  pg/L (March 2016). It was higher in SW compared to the TDW. These results are shown in **Table 4.2**. No cytotoxicity was observed nor anti-estrogenic activity detected. **Figure 4-1** graphically depicts the estrogenic activity from SW and TDW samples collected from April 2015 to March 2016 and tested in the T47D-KBluc reporter gene assay.

**Table 4.2:** A summary of results from the treatment plant using the YES assay and T47D-KBluc reporter gene assay

| Month       | Estrogenic activity (EEq) |            |            |            |                              |                 |            |            |
|-------------|---------------------------|------------|------------|------------|------------------------------|-----------------|------------|------------|
|             | YES assay (pg/L ± SD)     |            |            |            | T47D-KBluc assay (pg/L ± SD) |                 |            |            |
|             | SW-<br>Canal              | SW-<br>Dam | TDW-<br>A2 | TDW-<br>B1 | SW-<br>Canal                 | SW-<br>Dam      | TDW-<br>A2 | TDW-<br>A2 |
| <b>2015</b> |                           |            |            |            |                              |                 |            |            |
| April       | <dl                       | <dl        | <dl        | <dl        | <dl                          | <dl             | <dl        | <dl        |
| May         | <dl                       | <dl        | <dl        | <dl        | <dl                          | <dl             | <dl        | <dl        |
| June        | <dl                       | <dl        | <dl        | <dl        | <dl                          | <dl             | <dl        | <dl        |
| July        | <dl                       | <dl        | <dl        | <dl        | 2.89 ±<br>1.28               | 2.70 ±<br>1.18  | <dl        | <dl        |
| August      | <dl                       | <dl        | <dl        | <dl        | <dl                          | <dl             | <dl        | <dl        |
| September   | <dl                       | <dl        | <dl        | <dl        | 3.13 ±<br>1.02               | 3.83 ±<br>0.75  | <dl        | <dl        |
| October     | <dl                       | <dl        | <dl        | <dl        | 2.75 ±<br>1.37               | 3.90 ±<br>3.07  | <dl        | <dl        |
| November    | <dl                       | <dl        | <dl        | <dl        | 1.94 ±<br>0.42               | 0.71 ±<br>0.15  | <dl        | <dl        |
| December    | <dl                       | <dl        | <dl        | <dl        | 0.31 ±<br>0.05               | 0.35 ±<br>0.42  | <dl        | <dl        |
| <b>2016</b> |                           |            |            |            |                              |                 |            |            |
| January     | <dl                       | <dl        | <dl        | <dl        | 2.38 ±<br>0.29               | 2.16 ±<br>1.34  | <dl        | <dl        |
| February    | <dl                       | <dl        | <dl        | <dl        | 0.32 ±<br>0.24               | 0.49 ±<br>0.14  | <dl        | <dl        |
| March       | <dl                       | <dl        | <dl        | <dl        | 3.62 ±<br>0.60               | 10.51 ±<br>5.74 | <dl        | <dl        |

<dl=below detection limit, Surface water= SW, TDW= treated drinking water, EEq= estradiol equivalent, SD= standard deviations



**Figure 4-1:** Estrogenic activity from surface and treated drinking water samples collected from April 2015 to March 2016 to monitor two treatment plants

#### 4.2.2 Androgenic activity

Androgenic activity was detected in four water samples, SW-Canal ( $0.024 \pm 0.026$  ng/L) and SW-Dam ( $0.090 \pm 0.040$  ng/L) collected in September 2015, as well as SW-Canal ( $0.031 \pm 0.030$  ng/L) and SW-Dam ( $0.0033 \pm 0.0050$  ng/L) collected in March 2016. In the TDW samples androgenic activity was below the dl. This is indicated in **Table 4.3**. Cytotoxicity was not observed in any of the samples nor anti-androgenic activity detected.

**Table 4.3:** A summary of results from the treatment plant using the MDA-kb2

|              | <b>Androgenic activity (DHTEq ng/L ± SD)</b> |                |               |               |
|--------------|--|----------------|---------------|---------------|
|              | <b>SW-Canal</b>                              | <b>SW-Dam</b>  | <b>TDW-A2</b> | <b>TDW-B1</b> |
| <b>Month</b> |  |                |               |               |
| <b>2015</b>  |  |                |               |               |
| April        | <dl  | <dl            | <dl           | <dl           |
| May          | <dl  | <dl            | <dl           | <dl           |
| June         | <dl  | <dl            | <dl           | <dl           |
| July         | <dl  | <dl            | <dl           | <dl           |
| August       | <dl  | <dl            | <dl           | <dl           |
| September    | 0.024± 0.026                                 | 0.090 ±0.040   | <dl           | <dl           |
| October      | <dl  | <dl            | <dl           | <dl           |
| November     | <dl  | <dl            | <dl           | <dl           |
| December     | <dl  | <dl            | <dl           | <dl           |
| <b>2016</b>  |  |                |               |               |
| January      | <dl  | <dl            | <dl           | <dl           |
| February     | <dl  | <dl            | <dl           | <dl           |
| March        | 0.031 ±0.030                                 | 0.0033 ±0.0050 | <dl           | <dl           |

<dl: below detection limit, DHTEq= dihydrotestosterone equivalent, Surface water= SW, TDW= treated drinking water, SD= standard deviations

## CHAPTER 5

### 5 Discussion

#### 5.1 Phase 1: The GWRC water samples

The MDA-kb2 reporter gene assay was successfully applied on 24 water samples from six different countries with four different water sources to measure androgenic activity. The results showed that 5 of the 24 (21%) water samples tested positive, with the highest activity in treated wastewater from the Netherlands (0.232 ng/L) followed by Spain (0.0283 ng/L). The SW from Germany had 0.028 ng/L DHTEqs. The lowest activity was in tap water from Germany with 0.008 ng/L. This is indicated in **Table 4.1**. These values were however below the proposed international trigger value of 11 ng/L DHTEqs.<sup>41</sup> Other sample extracts were below the dl of the assay. No anti-androgenic activity was detected.

A possible explanation for the higher levels of androgenic activity observed in the treated wastewater compared to the tap water may be that tap water is highly treated (therefore contains fewer contaminants). Wastewater may still contain residues from soaps, detergents and pharmaceuticals from our homes as well as industrial waste. The contamination in tap water may be attributed to either a transporting pipe after treatment that is coated with an androgenic chemical or a reduced efficiency of the treatment plant in removing EDCs e.g. not fitted with an activated carbon filter or an old filter not changed. Also, the chemical nature of the AR ligand could have prevented it from being removed by even effective water purification methods.<sup>210</sup> Further investigations will be beneficial.

## 5.2 Phase 2: Water samples from a Gauteng based treatment plant

### 5.2.1 Estrogenic activity

All samples were below the dl in the YES assay and 16 out of 24 (67%) SW samples tested positive over the 12 month period indicating the sensitivity of the T47D-KBluc cell line which has both the ER  $\alpha$  and  $\beta$  receptors compared to the yeast cells which only have the ER $\alpha$  receptor.<sup>205,209</sup> There were no discernible patterns observed with the same sample in different sampling periods. The YES assay, however, with its 3-5 day incubation period is good for screening slow reacting chemicals compared to the T47D-KBluc reporter gene which has a 24 hour incubation period.<sup>205</sup> This indicates that the two assays are complementary to each other and therefore should be used together when screening estrogenic activity in water samples. No cytotoxicity was observed.

In the TDW samples, estrogenic activity was below the dl of the assay. This indicates that: 1) Treatment plant B's treatment processes were effective in removing the estrogens detected in SW-Canal and SW-Dam as they supply the treatment plant, coming out as TDW-B1 2) Treatment plant A is also efficient in removing estrogen disruptors as disruptors which were detected in SW-Dam were below the dl in TDW-A2. The treatment plants are fitted with activated carbon filters which are able to remove EDC contaminants, in agreement with the results in this study.

The estrogenic activity reported in this study is within the ranges reported by Pieters and Powries<sup>202</sup> in South African SWs (Letsitele, Lomati and Vals & Renoster catchments), although it is more comparable to the lower ranges. This is explained by the fact that Pieters and Powrie's areas were agricultural areas with varying levels of pesticide use whilst the catchment area in this study is situated in an urban area. Other studies have also been successful in using the assay to detect estrogenic activity in SWs and TDW samples.<sup>198</sup>

In the 4 of the 8 months where estrogen activity was detected, SW-Dam had higher activity compared to SW-Canal (**Figure 4-1**). The DHTEqs (**Table 4.3**) were also slightly higher in SW-Dam compared to SW-Canal. This was however not statistically significant. A possible explanation for this is that there was more pollution with

estrogenic and (or) androgenic activity occurring near the dam wall. Estrogenic activity was however below the trigger value of 3.8 ng/L proposed by Brand et al.<sup>41</sup> and also below the stricter 0.7 ng/L proposed by Genthe et al.<sup>40</sup> for drinking drinking water.

The highest estrogenic activity was detected in SW-Dam ( $10.51 \pm 5.74$  pg/L) collected during March 2016 (**Figure 4-1**). There may have been a pollution spill of chemicals from industry or raw sewage. This was however below the trigger value that would warrant further investigation and a health risk assessment. It is worth mentioning that EDCs are known to induce health effects at low doses,<sup>5</sup> therefore chronic exposure at these low DHTEqs and EEqs levels may result in adverse health effects in the long run.

### 5.2.2 Androgenic activity

Androgenic activity was detected in 4 out of 24 (17%) SW samples collected in September 2015 (SW-Canal=  $0.024 \pm 0.026$  ng/L and SW-Dam=  $0.090 \pm 0.040$  ng/L) and in March 2016 (SW-Canal =  $0.031 \pm 0.030$  ng/L with and SW-Dam =  $0.0033 \pm 0.0050$  ng/L). These values are however below the trigger value of 11 ng/L DHTEqs proposed by Brand et al.<sup>41</sup> for health risk assessments. SW-Canal is water going into treatment plant B coming out as treated TDW-B1 and SW-Dam supplies treatment plant A and comes out as TDW-A2. Activity was below the dl in the TDW samples compared to the SW samples supplying the two treatment plants indicating that both treatment plant A and B were effective in removing androgenic activity detected in the SW samples (September 2015 and March 2016). In the other samples, androgenic activity was below the dl of the assay. Whilst Pieters and Powrie<sup>202</sup> detected androgenic activity in less SW samples compared to those which were tested, comparable to this study, other authors have seldom detected it in SW using the MDA-kb2 reporter gene assay.<sup>203,211</sup> Reported more often is anti-androgenic activity in SWs; however, none was detected in this study. Similar to the findings in this study, other authors have not been able to detect androgenic activity in drinking water using the assay.<sup>203</sup> This may indicate a much lower concentration of the EDCs in the drinking water, possibly needing sample enrichment (extract concentration). Most reported activity in literature is rather from wastewater and sediment samples, denoting its relevance in these types of samples.

According to Blake et al.<sup>212</sup> at high concentrations (threshold not determined) some estrogens (E<sub>2</sub>) are able to bind to the AR in the MDA-kb2 cell line, potentially interfering with the cell's response to androgens. Androgenic activity could therefore be underestimated due to the presence of estrogenic activity. This may explain undetected androgenic activity in months where estrogenic activity is present for SW samples. In cases where both antagonists and agonists are present, van der Linden<sup>38</sup> states that there is a possibility that the individual contributions could mask each other and activity may therefore be underestimated.

In the present study, cytotoxicity was checked under a microscope. Although none was detected at the highest concentrations tested, it will be beneficial in future to incorporate a cytotoxicity assay, that will give a better indication of cytotoxicity. Cytotoxicity may mask endocrine activity resulting in lower RLU readings thus interfering with the accuracy of the assay. Luciferase based assays are sensitive to cytotoxicity. Overall, the assay was rapid with a high throughput as it was conducted in 96 well plates allowing more samples to be processed in each run. Further studies are however still needed to strengthen its applicability in drinking water. This is supported by this study since in phase 1 the assay detected androgenic activity in only 1 out of 8 tap water samples (12.5%) (**Table 4.1**) and none (0%) in phase 2 (**Table 4.3**).



## CHAPTER 6

### 6 Conclusion

The ubiquitous presence of endocrine disruptors in the environment as well as the associated adverse health effects call for their constant monitoring using reliable and internationally standardized test methods. This is mostly true for water as it is the bloodstream of the biosphere. *In vitro* bioassays are ideal for this as they are sensitive to low concentrations and do not require prior knowledge of the chemical composition of the samples to be tested. They rely on their effect on receptors and/or biological response of the cell, based on the mechanism of action. In phase 1, the MDA-kb2 reporter gene assay proved to be applicable to water samples from different sources. Its applicability to drinking water however may not be as effective as in the other water sources because i.e. surface and wastewater. Factors which may improve the assay should be investigated. The water treatment plants investigated in phase 2 were also effective in removing endocrine activity, however, less sophisticated (especially those without activated carbon filters) treatment plants in SA should also be investigated.

The results of this study strengthen the argument for using a battery of assays when assessing endocrine activity as endocrine disruptors occur in water mostly as mixtures. This gives a more comprehensive assessment of the total endocrine activity present in a sample. This will ultimately lead to more sensitive monitoring and evaluation systems in the public health sector.

## CHAPTER 7

### 7 Recommendations

#### 7.1 Regarding policy

There is still limited regulatory framework on endocrine disruptors in SA. At present, there is a proposed trigger value of 0.7 ng/L for estrogenic activity in drinking water and 11 ng/L for androgenic activity. No policy is specifically dedicated to EDCs as water chemical pollutants; rather, they are broadly encompassed as chemical pollutants under the National Water Act. The EDCs are also not incorporated in routine monitoring programs in most water sectors.<sup>213</sup> SA is in the process of developing the Chemicals Management bill which will address the existing gaps for chemicals (i.e. chemical of emerging concern) not regulated by any existing legal infrastructure. This will contribute in meeting the aim of the Sustainable Goal 6.2 by 2030. In the meantime, it is recommended that the National Environmental Management: Water Act, 26 of 2014 be strengthened and implemented more stringently to ensure that good waste disposal systems, which are a major source for SW pollution, are fully in place.

A holistic and interdisciplinary collaborative approach from the government, industry as well as academic institutions is needed in generating and sharing research data, which will ultimately translate into effective policies. Furthermore, an increase in public awareness of EDCs is also recommended so that the general population is knowledgeable on the routes of exposure. Reducing human exposure and associated effects is a priority. This can be done generally by, among others, good waste disposal practices, avoiding the excessive use of personal care products and pharmaceuticals especially during pregnancy, choosing glass over plastic and constant monitoring of water sources.

#### 7.2 Future research recommendations

- Although none of the positive water samples were above the proposed trigger values, it will be beneficial to do a further chemical analysis test to identify the active chemicals so that they are monitored with both chemical and biological methods.

- Incorporating a cytotoxicity test along the assays. This will indicate cytotoxicity more accurately as compared to visualization for morphology changes under a microscope.
- For the MDA-kb2 reporter gene assay, substituting L-15 medium (with phenol red) which was used in this study with phenol red-free L-15 medium and compare if there are any significant differences which may improve the assay. Phenol-red is a weak estrogen and might interfere with AR-dependant luciferase induction.<sup>206</sup>
- Investigate less sophisticated treatment plants such as those found in non-urban areas and are not fitted with activated carbon filters.

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## Appendix A

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  
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Faculty of Health Sciences Research Ethics Committee

26/11/2015

### Approval Certificate New Application

**Ethics Reference No.:** 535/2015

**Title:** The suitability of estrogen and androgen bioassays for endocrine activity measurement in different water matrices

Dear Silindile Ngcobo

The **New Application** as supported by documents specified in your cover letter dated 6/08/2015 for your research received on the 2/11/2015, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 25/11/2015.

Please note the following about your ethics approval:

- Ethics Approval is valid for 2 years.
- Please remember to use your protocol number (**535/2015**) 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

**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 2015 (Department of Health).*

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