COMPARISON BETWEEN CHEMICAL AND TISSUE CULTURE METHODS TO MONITOR ENVIRONMENTAL ESTROGENS

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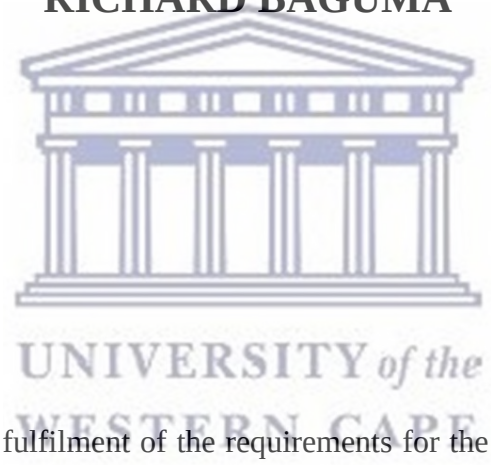

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KEY WORDS

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Steroidogenesis

Pollution

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Estrogens

Progesterone

ABSTRACT

COMPARISON BETWEEN CHEMICAL AND TISSUE CULTURE METHODS TO MONITOR ENVIRONMENTAL ESTROGENS

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Endocrine disrupting compounds (EDCs) are exogenous compounds/chemicals in the environment that interfere with the synthesis, secretion, distribution and function or elimination of natural hormones in the body. Environmental estrogens are a subclass of EDCs that may mimic or inhibit the effect of endogenous estrogen and can therefore influence developmental and reproductive health in humans and animals. EDCs have been reported to adversely affect the reproductive, immune, endocrine and nervous systems of wildlife and humans. The effects of EDCs include gonadal abnormalities, altered male/female sex ratios, reduced fertility and cancers of the male and female reproductive tract to mention a few. These effects are difficult to detect. Although it is essential to screen for EDCs in aqueous environmental samples, most countries have failed to implement this as part of their routine water quality monitoring programs due to various constraints such as the high cost of assays and the lack of infrastructure and skills required to do the assays. Therefore, there is a clear need for more user-friendly, more

economically viable and time saving assays that can be used for routine monitoring of environmental EDCs.

The aim of this study was to investigate the comparison between chemical and tissue culture methods to monitor environmental estrogens. 28 environmental water samples were collected from various sites around South Africa and analyzed for EDCs using a battery of rapid *in vitro* tests. Samples collected for the current study were selected based on various human impacts and also to give approximately 50% high and 50% low estrogen values. The 28 environmental water samples were separated into two groups based on the estradiol ELISA. The estradiol ELISA was chosen because estradiol is the principal estrogen found in all mammalian species during their reproductive years. For this separation, an estradiol level of 5 pg/ml was used as cut-off. Of the 28 samples investigated, 15 had estradiol levels higher than 5 pg/ml and were designated as high estradiol. The remaining 13 samples contained estradiol at 5 pg/ml or less and they were designated as low estradiol.

The first objective of this study was to compare different rapid ELISAs for EDC monitoring to determine if the data obtained with these assays are similar/identical. The data obtained from the estrogenic ELISAs was related/similar and showed good correlation with each other. This is because the different estrogens are very similar and also due to the fact that the same sub-group in the population (the reproductively active females) is secreting these hormones. Therefore, an estradiol rapid assay was proposed as a first screening system for estrogens in samples. Even though there was a positive correlation between the estradiol rapid assay and testosterone rapid assay, separation of samples based on estradiol levels wasn't a good predictor of testosterone levels in the samples. A testosterone rapid assay was therefore recommended as necessary to screen for androgens in samples. The positive correlation between the estradiol rapid assay and

progesterone rapid assay was expected because both estradiol and progesterone are secreted and excreted by the same population sub-group (reproductively active females). This study also demonstrated a good predictability of separating samples containing progesterone using the estradiol ELISA. Progesterone is secreted by pregnant women, a sub-group of the reproductively active females. It is advised that a progesterone rapid assay be included to screen samples for progestogens.

The second objective of this study was to compare estradiol rapid ELISAs with a bioassay for anti-androgenicity using mouse testicular cell cultures. The mouse testicular cell testosterone synthesis bioassay to monitor anti-androgenicity of the samples showed no correlation between the ELISA data for estrogens. This study shows that anti-androgenic effects need to be monitored independently because the data for estrogenic compounds cannot be used as a predictor for anti-androgenic effects. This demonstrated the need for the inclusion of a mouse testicular cell testosterone synthesis bioassay to screen for androgenicity and anti-androgenicity of water samples.

In summary, due to the different mechanisms of action of EDCs, this study recommended a battery of assays to monitor for EDCs. The battery of assays suggested is:

- Estradiol ELISA as a rapid assay to screen for estrogens.
- Testosterone ELISA as a rapid assay to screen for androgens.
- Progesterone ELISA as a rapid assay to screen for progestogens.
- Mouse testicular cell testosterone synthesis bioassay to screen for androgenicity and anti-androgenicity.

May 2012

DECLARATION

I, **Richard Baguma** declare that the thesis entitled *'Comparison between chemical and tissue culture methods to monitor environmental estrogens'* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: _____ Date: _____

Signed: _____

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This work is dedicated to the loving memory of my friend, Lumuli Mughogho (RIP), whose life was tragically cut short a few days before I completed this thesis.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

%	- percent
7-ER	- 7-ethoxyresorufin
^{0}C -	- degrees Celsius
µl	- microliters
AhR	- Aryl hydrocarbon receptor
ANOVA	- Analysis of variance
ATP	- adenosine triphosphate
BBP	- Butyl benzyl phthalate
BPA	- Bisphenol A
Ca^{2+}	- Calcium cation
CO	- Carbon monoxide
CO_2	- Carbon dioxide
CPRG	- chlorophenol red-β-D-galactopyranoside
CNS	- Central nervous system
CYP	- Cytochrome P450
Da	- Dalton
DDT	- dichlorodiphenyltrichloroethane
DES	- Diethylstilbestrol
DMSO	- Dimethyl sulfoxide
DNA	- deoxyribonucleic acid
E1	- Estrone

E2	- 17β-estradiol
E3	- Estrone
EC	- Electrochemical
EDCs	- Endocrine disrupting compounds
EDSTAC	- Endocrine Disruptor Screening and Testing Advisory Committee
EE2	- 17α-Ethinylestradiol
EI	- Electron impact
ELISA	- Enzyme-linked immunosorbent assay
EPA	- Environmental Protection Agency
ER	- Estrogen receptor
ERα	- Estrogen receptor alpha
ERβ	- Estrogen receptor beta
ERE	- Estrogen responsive element
EROD	- ethoxyresorufin O-deethylase
E-screen	- MCF-7 cell proliferation
FBS	- Fetal bovine serum
Fe^{3+}	- Iron cation
GC	- Gas chromatography
GC-MS	- Gas chromatography - mass spectrometry
GPC	- Gas permeation chromatography
GnRH	- Gonadotropin-releasing hormone
GtH I	- Gonadotropin hormone I
GtH II	- Gonadotropin hormone II

H_2SO_4	- Sulphuric acid
HPG	- Hypothalamus-pituitary-gonad
HPLC	- High-performance liquid chromatography
HSP	- Heat shock protein
HTS	- High throughput screening
HPT	- Hypothalamus-pituitary-thyroid
kDa	- kilodalton
L	- Litre
LC	- Liquid chromatography
LC-MS	- Liquid chromatography - mass spectrometry
LDH	- lactate dehydrogenase
LH	- Luteinizing hormone
LPS	- Lipopolysaccharides
M	- Molar
MES	- Mestranol
MetOH	- Methanol
Mg^{2+}	- Magnesium cation
MS	- Mass spectrometry
MS/MS	- tandem mass spectrometry
mg	- milligrams
ml	- millilitre
Mol/mg/min	- concentration per milligram per minute
mU	- milli units

MTs	- Metallothioneins
mRNA	- messenger ribonucleic acid
ng/L	- nanograms per litre
NCI	- negative chemical isolation
nm	- nanometres
NPs	- Nonylphenols
NPEs	- Nonylphenol ethoxylates
OD	- Optical density
OPs	- Octylphenols
PAHs	- polycyclic aromatic hydrocarbons
PBS	- Phosphate buffered saline
PCBs	- Polychlorinated biphenyls
PHH	- Phenylhydrazine hydrochloride
PCBs	- Polychlorinated biphenyls
pg/ml	- picograms per millilitre
POP	- Persistent organic pollutant
R^2	- correlation coefficient
rpm	- revolutions per minute
RPMI-1640	- Roswell Park Memorial Institute-1640
RT-PCR	- reverse transcription polymerase chain reaction
ROS	- Reactive oxygen species
SPE	- Solid Phase Extraction
TCDD	- 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin

TCDF	- 2, 3, 7, 8-tetrachlorodibenzofuran
TMB	- Tetramethylbenzidine
USA	- United States of America
VTG	- Vitellogenin
VN	- Vitellin
v/v	- volume to volume
Zn^{2+}	- Zinc cation

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

Literature review

An introduction to Endocrine Disrupting Compounds (EDCs)

1.1 Endocrine Disrupting Compounds (EDCs)

The U.S Environmental Protection Agency (EPA) defines EDCs as xenobiotics (agents foreign to an organism) that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for homeostasis, reproduction, development and/or behaviour (Crisp *et al*., 1998).

Studies have linked EDCs to adverse biological effects in animals, giving rise to concerns that low-level exposure might cause similar effects in humans (Colborn *et al.,* 1993). EDCs range in class from hormones, plant constituents, pesticides, compounds used in the plastics industry and in consumer products to industrial by-products and pollutants (WHO, 2002). Some EDCs are widely dispersed in the environment. Some are persistent organic pollutants (POPs), and can be transported long distances across national boundaries. POPs have been found in virtually all regions of the world. Others are rapidly degraded in the environment or human body or may be present for only short periods of time (Damstra *et al*., 2002). Health effects attributed to EDCs include a range of reproductive problems (male and female reproductive tract abnormalities, skewed male/female sex ratios, menstrual problems (Harrison *et al*., 1995)), decreased reproductive ability in men (Sharpe and Skakkebaek, 1993), premature puberty in females

(Herman-Giddens *et al*., 1997), increased rates of foetal death (Bell *et al*., 2001), changes in hormone levels, brain and behaviour problems/intellectual impairment (Jacobson and Jacobson, 1996), impaired immune functions and various cancers (McKinlay *et al.*, 2008; Barry, 2009).

1.2 Properties of EDCs

The effects of EDCs on physiological systems are dependent on their individual properties and these differ with chemical type (IEH, 1999). However, certain properties are common to many of the compounds. In general, the compounds are:

- Persistent. i.e. they are slow to degrade in the environment and are therefore present in the environment for long enough to enter the food chain. Examples of highly persistent compounds are the insecticide dichlorodiphenyltrichloroethane (DDT), which hasn't been manufactured or used in the developed world for more than 25 years but which remains in the environment in biologically significant concentrations (Pesticide News, 1998).
- Hydrophobic and lipophilic. Compounds with hydrophobic properties tend to come out of aqueous solution and become concentrated in organic matter, soil or silt. Lipophilic compounds associate with fat depots and are readily accumulated in the fat depots of animals (Nimrod and Benson, 1996).
- Biologically active at very low concentrations. It should be noted that these compounds act on physiological systems at concentrations which are well below toxic concentrations and are generally less than 10^{-9} M (Rhind, 2009).
- Weakly associated with steroid binding proteins in the blood of animals. Thus, although they may be present in animals in relatively low concentrations, most or all of the

compounds present may be biologically available, unlike endogenous steroids which are mostly attached to binding proteins and are not biologically available (Colborn *et al.*, 1993).

- Able to act additively, antagonistically or synergistically and effects may be species-dependent. Furthermore, responses observed *in vitro* are not always apparent when studied *in vivo*. These properties make it difficult to predict the likely effect of EDCs on animal physiology (Rhind, 2005).

1.3 Mechanisms of action of EDCs

The endocrine system is a complex system involving several central nervous system (CNS)-pituitary-target organ feedback pathways which are involved in regulating a multitude of functions and maintaining homeostasis (i.e. self-regulated feedback mechanisms within the body) (US EPA, 1997b). Given this, there are several target organ sites within the endocrine system whereby environmental chemicals could potentially interact and disrupt normal function (US EPA, 1997b). Due to the complexity of the cellular processes involved in hormone function, any of these systems may be involved in a chemical's influence on the endocrine system (Henley and Korach, 2006). Understanding of the specific mechanism of action of EDCs is also made difficult because of factors such as the type, duration and timing of exposure, cell/tissue type on which the EDC acts and the nutritional status, age and gender of the individual (Williams, 2007). Consequently, the mechanisms of action of EDCs can be divided into two main categories namely receptor mediated and other mechanisms.

- Receptor mediated mechanisms are based on hormones eliciting a response from their target tissue through direct and specific binding to its intracellular or membrane bound receptors (Tremblay *et al.,* 1998; Soverchia *et al.,* 2005).
- Other mechanisms include those which interfere with the synthesis (Almstrup *et al.,* 2002), release (Ando *et al.,* 2004), distribution and metabolism of hormones, secondary messenger systems (Gillesby and Zacharewski, 1998), translational and post-translational mechanisms (LaChapelle *et al*., 2007) and other systems under hormonal control including cytochrome P450 expression (Buhler *et al*., 2000; Montserrat *et al*., 2004).

Interaction with hormone receptors can result in either activation or inhibition of gene transcription by environmental hormones in the following ways:

- Binding of a compound to a steroidal receptor converts the receptor to an active state, which promotes gene expression (receptor agonist) (Brzozowski *et al.,* 1997).
- Binding of a compound to a steroidal receptor causes the receptor to be unavailable to participate in gene expression (receptor antagonist) (Brzozowski *et al.,* 1997; Tremblay *et al.,* 1998).
- Receptor phosphorylation, where activation of receptors occurs through processes not involving receptor binding (Daniel *et al.,* 2007).

1.4 Classes and sources of EDCs

EDCs are found in low doses in so many products and all people are exposed to compounds with endocrine disrupting effects in their daily life (AwwaRF, 2008). These compounds can be divided into three major classes:

- Natural steroids: These are required for a normally functioning endocrine system. Endogenous steroidal hormones include the estrogens such as 17β-estradiol (E2), estriol (E3) and estrone (E1), androgens like testosterone and phytoestrogens such as genestein, lignans and isoflavonoids. Phytoestrogens naturally occur in foods such as soy beans and flax seeds and they produce effects similar to those of the female sex hormone estrogen, although a person would probably need to consume copious amounts of them to affect the endocrine system (Safe and Gaido, 1998).
- Synthetic steroids: These are compounds that have been designed to target the endocrine system. They include 17α-ethinylestradiol (EE2), mestranol (MES), diethylstilbestrol (DES) and anti-estrogens like tamoxifen (Desbrow *et al.*, 1998).
- Non-steroidal synthetic chemicals: This group comprises a very wide range of compounds that have been designed for use in a variety of industries. They include alkylphenol ethoxylates, organohalogens, phenols, phthalates, pesticides, heavy metals and organotins. Table 1 below gives a few examples of key non-steroidal synthetic chemicals.

Classification	Compound	Reference
Alkylphenols	Nonylphenols (NPs) Nonylphenol ethoxylates (NPEs) Octylphenols (OPs)	Pojana *et al*., 2004
Phenols	Bisphenol A (BPA)	Pojana *et al*., 2004
Organohalogens	Dioxins, Polychlorinated biphenyls (PCBs)	DeRosa *et al*., 1998
Phthalates	Di-*n*-pentyl phthalate Butyl benzyl phthalate (BBP)	Harris *et al.,* 1997
Pesticides	DDT, Atrazine, Dicofol, Dieldrin, Endosulfan, Vinclozolin, Triclosan	DeRosa *et al*., 1998
Heavy Metals	Arsenic, Lead, Mercury, Cadmium	DeRosa *et al*., 1998
Organotins	Tributyltin	Nishikawa, 2006

Table 1. A few examples of key non-steroidal synthetic chemicals.

1.5 Effects of EDCs on ecosystems

The earliest and the best defined ecological effects of EDCs are on aquatic fauna. In 1980, Lake Apopka was the site of an insecticide spill containing DDT and during the last 40 years, it has received extensive agricultural pesticide (dicofol) and nutrient runoff. The impact on alligators and turtles was severe. Abnormal development of the reproductive system (morphological abnormalities of the testis and ovary), reduced egg hatching and low juvenile survival rates were recorded. Examination of the endocrine systems of hatchlings and juvenile alligators revealed alterations in testosterone, plasma 17β-estradiol, dihydrotestosterone and thyroxine concentrations (Guillette *et al*., 2000; US EPA, 1997b). Adverse effects on fish populations such as masculinization (development of male secondary sex characteristics) of female fish, are frequently recorded downstream of contaminant sources (Howell *et al*., 1980). It was also observed that the progeny of viviparous fish are biased towards male offspring (Larsson and Forlin, 2002). Perhaps the most powerful directly adverse effect on aquatic wildlife was that exerted by tributyltin, formerly widely used as an anti-fouling agent on ships' hulls (Matthiessen and Gibbs, 1998). This chemical tributyltin prevents the growth of marine organisms on submerged structures such as ships' hulls, buoys and oil rig supports. Unfortunately, contamination of ports by tributyltin resulted in the local extinction of some species of marine organisms and masculinization of others, a condition termed imposex. For example, female gastropod mollusks in marine environments heavily used by shipping developed a penis-like structure with an associated vas deferens resulting in infertility (Matthiessen, 2003). The opposite effect (feminization of male fish and mollusks due to estrogenic contamination) has been reported in fresh waters downstream of sewage treatment plants (Jobling and Sumpter, 1993). Feminization of reproductive ducts in male fish in which the testis forms an ovary-like

cavity, ovotestis (presence of oocytes in the testis) and the synthesis of the female egg protein vitellin (or its precursor vitellogenin) in male fish exposed to wastewater from sewage treatment plants have been recorded (Rodgers-Gray *et al*., 2001).

1.6 Effects of EDCs on humans

1.6.1 Pharmaceuticals

The case of the drug DES, a non-steroidal estrogen is one example of the consequences of exposing developing animals including humans, to hormonally active substances. In the past, large doses of DES were administered to women for pharmacological reasons such as prevention of miscarriage, the inhibition of lactation or stunting of growth in tall girls. This was later associated with several adverse effects (Swan, 2000; Milhan, 1992). It was discovered that DES exposure resulted in reproductive abnormalities in both male and female infants, and also vaginal cancer in females in later life (Herbst, 1981; Sharpe and Skakkebaek, 1993).

Experiments with animals have identified critical developmental time points *in utero* and days after birth when exposure to chemicals that interfere with or mimic hormones have adverse effects that persist into adulthood (Bigsby *et al*., 1999; Eriksson *et al*., 1991; Recabarren *et al*., 2008; Szabo *et al*., 2009).

1.6.2 Reproductive Hormones

The pharmacological effectiveness of estrogens and progestins has been studied for almost one hundred years, and these chemicals have been employed to both assist and block fertility (Glick, 1967). Estrogens and progestogens are naturally secreted in women as integral components of the menstrual cycle. Secreted residues of these are hydroxylated and conjugated to glucuronides before they are excreted in urine. These conjugated steroids are converted to active steroids by microbial action during sewage treatment (Panter *et al.,* 1999). The major recent pharmacological addition to the natural steroid hormone excretion in women has been the oral contraceptives, particularly the synthetic estrogen ethinylestradiol (EE2), which is similarly excreted in the urine (Falconer, 2006).

1.7 Biomarkers for EDCs

1.7.1 Vitellogenin (VTG) as a biomarker of exposure to EDCs

One of the most documented effects of xenoestrogens is the induction of vitellogenin (VTG) in oviparous (egg laying) animals. VTG is the precursor of the egg yolk protein, vitellin (VN), which provides energy reserves for the embryo. Vitellogenin is a large, glycolipophosphoprotein having calcium and zinc ligands (Wallace, 1985; Montorzi *et al*., 1994; Denslow *et al*., 1999). VTG is considered to have similar characteristics in vertebrates, such as fish and frogs, and invertebrates, particularly molluscs (Blaise *et al*., 1999). In addition to being a nutrient reserve and the major source of amino acids and lipids for the developing embryo, VTG also transports the bound cations (Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{3+}) to the oocyte for use during future

embryogenesis. It also binds steroid and thyroid hormones (Cyr and Eales, 1996; Scott *et al*., 1995; Tagawa *et al*., 2000) and may transport these into the egg to control embryo development.

Changes in environmental stimuli such as photoperiod, temperature or food availability activate the hypothalamus-pituitary-gonad (HPG) axis. The activated hypothalamus secretes gonadotropin releasing hormone (GnRH), which in turn stimulates the pituitary to synthesise and release two types of gonadotropin hormones; GtH I (similar to follicle stimulating hormone) and GtH II (similar to luteinizing hormone) (Kouril, 2009). In females, GtH I stimulates the follicular cells of the ovary to produce testosterone, which is then converted to 17β-estradiol (E2) by the enzyme aromatase (Redding and Patino, 1993). A rise in circulating endogenous estrogen (E2) stimulates VTG production in the liver of sexually mature female oviparous vertebrates. Within liver cells, E2 binds to the nuclear estrogen receptor (ER), which dimerises and binds to the estrogen responsive element (ERE) of the promoter region of E2-inducible genes, including VTG and the ER itself. The binding of the ER to the ERE thus results in increased mRNA transcription and consequent production of E2-inducible proteins (Ding, 2005).

In female oviparous animals, hepatically produced VTG is released into the bloodstream and transported to the ovary. It is then taken up through receptor-mediated endocytosis and stored in developing oocytes (Denslow *et al*., 1999). Here, it is sequestered by the developing oocytes and processed to form egg yolk. The incorporation of large amounts of VTG into the oocytes results in the characteristic increase in the size of the ovaries during sexual maturation. The VTG gene is also present in the livers of males but it is normally silent. Upon exposure to estrogen or to an estrogen mimic, the gene is turned on and VTG is synthesized and secreted (Jackson *et al*., 1977; Flouriot *et al*., 1995; Palmer and Palmer, 1995). It is then exported into the blood where, in males, it remains until it is degraded by plasma proteases or cleared out by the kidneys. VTG

proteins are degraded slowly and thus their levels can accumulate and remain high in the plasma for some time after exposure (Denslow *et al*., 1999). The presence of measurable amounts of VTG in the plasma of male or immature female fish, where it is not normally found, indicates exposure to chemicals that mimic the receptor binding properties of the female reproductive hormone 17β-estradiol (E2). This is considered a useful biomarker of exposure to estrogenic compounds in aquatic environments (Heppel *et al*., 1995; Hansen *et al*., 1998; Blaise *et al*., 1999). Low doses of estrogen can result in large increases in plasma VTG, making the determination of VTG in male fish an extremely sensitive marker for estrogen exposure (Sumpter and Jobling, 1995).

The degree to which an estrogenic compound is able to induce VTG is governed by its ability to bind to the hepatic estrogen receptor (ER). Many compounds for example alkylphenols, halogenated organic compounds, certain pesticides, some phthalate plasticizers, paraben preservatives and phytosterols are weakly estrogenic, whereas a few pharmaceutical compounds such as EE2 and DES may be more potent that E2 itself. Some organochlorine compounds including DDT, methoxychlor and certain polychlorinated biphenyl (PCB) congeners are themselves very weak ER agonists, but their hydrolysed metabolites are much stronger ER agonists (Bulger *et al*., 1978; Korach *et al*., 1988; Soontornchat *et al*., 1994).

ER agonists have the same mechanism of action as E2, and thus exposure to mixtures of exogenous estrogen mimics results in increased VTG response. The effects of exposure to multiple estrogenic compounds are sometimes additive, based upon the relative concentrations and potencies of the individual components of the mixture (Payne *et al*., 2000; Thorpe *et al*., 2003; Brian *et al*., 2005). Consequently, even when the individual compounds are each below the threshold concentration that causes VTG induction, the overall potency of the mixture may be

sufficient to induce VTG (Silva *et al.,* 2002). Some compounds block the binding of E2 to the ER and are thus anti-estrogenic. Others for example some planar organic compounds and certain metals have indirect anti-estrogenic effects. Many polycyclic aromatic hydrocarbons (PAHs) and other planar organic compounds that interact with the aryl hydrocarbon receptor (AhR) to cause induction of the cytochrome P450 1A (CYP1A) detoxification enzyme system, are also believed to be indirectly anti-estrogenic (Arukwe and Goksøyr, 2003). Female exposure to these compounds can lead to increased E2 metabolism and reduced plasma E2 titres, thus inhibiting VTG production, impairing oocyte development and reducing reproductive output (reviewed in Nicolas, 1999). The inhibition of estrogenic effects by AhR agonists may also be due to a reduction in ER expression (Bermanian *et al*., 2004).

1.7.2 Steroidogenesis Using Minced Testes Assay

Normal functioning of the reproductive system is essential for sexual development, behaviour and spermatogenesis. Under normal conditions, testosterone, the main sex hormone in males, is produced in the testes by the leydig cells through steroidogenesis upon stimulation by luteinizing hormone (LH) from the pituitary gland (Gail and Hedger, 1992; Kumar *et al.*, 2008). The complete steroidogenic pathway from signal transduction to end-hormone production is present in the testes. Modulation of this biochemical pathway can result in an inhibition or stimulation of steroid hormone synthesis, resulting in a hormonal imbalance which can cause adverse effects on the reproductive system (US EPA, 2005).

The minced testes assay can be used to identify substances that alter testosterone production due to direct effect on the enzymes or the endogenous components of the steroidogenic pathway

found in the testes. Anti-androgens and anti-estrogens act via a number of direct mechanisms in addition to those which directly involve the steroid hormone receptors. One prominent mechanism of anti-hormonal activity is inhibition of hormone synthesis by inhibiting the activity of P450 enzymes in the steroid pathway. Such activity could be detected in a fairly simple *in vitro* procedure with minced testicular tissue obtained from adult male rats. Leydig cell cultures could be used in place of the minced testes culture (US EPA, 1997a).

The minced testes assay is able to identify substances that either increase or decrease testosterone production. Thus, it can identify inhibitors or stimulants of the steroidogenic pathway (US EPA, 2005).

1.7.3 Breeding (nuptial) gland morphology

Adult males of many frog species exhibit patches of skin, nuptial pads, on the ventral side and digits of the forelegs. These nuptial patches may contain keratin hooks, which give the patch a darker appearance and simple saccular glands called breeding (nuptial) glands. Breeding (nuptial) glands are mostly recognized as a male secondary sex characteristic, restricted to the nuptial pads and digit pads in *Xenopus laevis* (african clawed frog) (Fujikura *et al.*, 1988).

The exact function of the secretions of these male breeding glands is still lacking, although several lines of evidence suggest that nuptial glands may be associated with sexual behaviour, specifically mating behaviour (Kurabuchi, 1993; Epstein and Blackburn, 1997; Emerson, *et al.*, 1999). The function of these breeding (nuptial) glands is also not well studied. It is however speculated, that they are used by males to clasp/grasp females during amplexus/mating

(Duellman and Trueb, 1986; Thomas *et al.*, 1993). Like the breeding glands, speculations are that these hooks assist with clasping during mating (Duellman and Trueb, 1986).

Several androgen replacement studies have shown that nuptial glands in amphibians are triggered by androgen hormones (Rastogi and Chieffi, 1971; Wetzel and Kelley, 1983; Thomas *et al.*, 1993; Lynch and Blackburn, 1995; Epstein and Blackburn, 1997; Emerson *et al.*, 1999). The sensitivity to and dependence on androgens makes nuptial glands potential biomarkers for environmental anti-androgens.

Several studies have indicated that gland epithelium heights and the gland cross-sectional area are sensitive biomarkers for androgenic receptor binding activity in frogs, including *X. laevis* (Thomas *et al.*, 1993; Lynch and Blackburn, 1995; Kelley and Pfaff, 1976; Epstein and Blackburn, 1997; Emerson *et al.*, 1999). It was reported that when female frogs were treated with androgens, breeding glands appeared (Kelley and Pfaff, 1976; Emerson, *et al.*, 1999). Several studies have indicated that estrogen inhibits breeding gland expression in both adult males and females (Emerson *et al.*, 1999).

Van Wyk *et al.*, (2003) investigated the potential of nuptial gland activity to be employed as a biomarker system for screening for anti-androgenic activity by certain EDCs. In their study, nuptial glands were not observed in female frogs. Their results indicated that flutamide, a pharmaceutic anti-androgen significantly affected the androgen-dependent breeding (nuptial) glands and plasma testosterone concentrations in male *X. laevis*. This study confirmed the potential of nuptial gland activity as a biomarker to screen chemicals and environmental samples for estrogenic and anti-androgenic activity using *X. laevis* males.

1.7.4 Dimorphism indicators: anal fin (gonopodium) in mosquitofish

The eastern mosquitofish (*Gambusia holbrooki*) is a sexually dimorphic species that is considered a sentinel species for the presence of EDCs (Batty and Lim, 1999; Angus *et al.,* 2002; Doyle *et al.,* 2003). Male mosquitofish are much smaller than female mosquitofish and possess a highly elongated and modified anal fin (the gonopodium) used as an intromittent organ during copulation. The development of the gonopodium occurs under androgenic stimulation from the testis in the final stages of sexual maturation (Turner, 1941). Gonopodium development can be inhibited by castration (Turner, 1947) and to a lesser extent by exposure to estrogenic chemicals (Doyle and Lim, 2002). However, the anal fins of female mosquitofish are also capable of responding to androgens and can be induced to develop into gonopodium-like structures by the administration of hormones (Turner, 1941; Turner, 1942a; Turner, 1942b). Laboratory exposure of juvenile female mosquitofish to androgenic stimulation results in gonopodium-like elongation of the anal fin typical of juvenile male mosquitofish (Angus *et al.*, 2001). Several studies done downstream of pulp and paper mills, (Bortone and Davis, 1994; Bortone and Cody, 1999 and Parks *et al.,* 2001) showed that there was an increase in the number of masculinized females, indicating that chemicals with androgenic activity are present in the mill effluents. The presence of modified anal fins in female mosquitofish is visible evidence of exposure to an androgenic substance.

Gonopodium elongation in mosquitofish has been used as an endpoint for endocrine impacts in mosquitofish exposed to sewage water in Australia (Batty and Lim, 1999). In that study, wild male *G. holbrooki* sampled in an industrial area downstream from a sewage and wastewater treatment plant in New South Wales had significantly shorter gonopodia than mosquitofish sampled at a reference site, suggesting the presence of estrogenic chemicals (Batty and Lim,

1999). This hormone-dependent morphological trait can thus be used as a biomarker for EDCs in fresh water systems.

1.7.5 Cytochrome P450 (CYP)

Cytochrome P450 is a diverse group of enzymes belonging to a super family of proteins. The cytochrome P450 heme-containing proteins are so named because of their cellular (cyto) location and spectrophotometric characteristics (chrome). When the reduced heme iron forms an adduct with carbon monoxide (CO), P450 enzymes absorb light at wavelengths near 450 nm, identifiable as a characteristic Soret peak (Mondal *et al.*, 2011). The function of this large and diverse group of enzymes is to oxidize, hydrolyze or reduce compounds through the insertion of an atom of atmospheric oxygen into an organic substrate while the other oxygen atom is reduced to water during the reaction cycle (Nebert *et al.*, 1993; Nelson *et al.*, 1996).

CYP enzymes have been identified in all kingdoms of life namely animals, plants, fungi, bacteria and archaea (Sigel *et al*., 2007). CYP enzymes are present in most tissues of the body, embedded within the smooth endoplasmic reticulum, where they synthesize cholesterol, metabolize/break down endogenous compounds such as steroidal hormones (estrogen and testosterone), fatty acids, cytokines, prostaglandins, vitamin D as well as exogenous compounds or potentially toxic substances like drugs (Oberdöster *et al*., 1998). CYPs are not only involved in the metabolism of the products of endogenous metabolism, for example bilirubin in the liver, but also drug metabolism and bioactivation, thus accounting for 75% of the total metabolism. By metabolizing different compounds, CYPs generally increase the water solubility of substrates, thereby

enhancing their elimination (Andersson and Förlin, 1992). In this way, cytochromes P450 such as CYP1A tend to detoxify xenobiotic chemicals.

CYP 1A is highly induced (increases in concentration) in the livers of aquatic organisms by very low levels of toxic compounds. This makes it a very good biomarker for exposure to xenoestrogens like PAHs, PCBs, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2, 3, 7, 8-tetrachlorodibenzofuran (TCDF) and DDT (Stegeman, 1993; Rattner *et al*., 1993; Bucheli and Fent, 1995). In the liver, the prominent expression and induction of CYP1A is consistent with the role of this organ in xenobiotic metabolism and excretion (Van Veld *et al*., 1997; Anulacion *et al*., 1998; Goksøyr and Husøy, 1998; Reinecke and Segner, 1998). Increased CYP1A expression may in a qualitative way, point to potential endocrine disruption activity that warrants further investigation.

CYP1A induction is mediated through the binding of xenobiotics to a cytosolic aryl hydrocarbon receptor (AhR). AhR ligands generally have isoteric configurations and are similar in structure to TCDD, a model CYP1A inducer. Receptor binding is followed by a series of molecular events leading to the expression of several genes (including CYP1A) known as the "Ah-gene battery" (Nebert *et al.*, 1993). The toxic effects of phenylhydrazine hydrochloride (PHH) and structurally similar compounds are thought to be mediated through the AhR, with induced proteins causing alterations in cellular homeostasis (DeVito and Birnbaum, 1994). In mammals, these effects include wasting syndrome, tumor promotion and thymic atrophy (Poland and Knutson, 1982). In fish, early life stages appear to be particularly sensitive to AhR ligands (Mehrle *et al.*, 1988; Walker and Peterson, 1991), and recent evidence indicates the involvement of CYP1A enzymes specifically in this toxic response (Cantrell *et al.*, 1996). The use of CYP1A induction as an assessment technique has increased in recent years, due mainly to the optimization of protocols

for the rapid and relatively inexpensive measurement of its associated enzymatic catalytic activity, the ethoxyresorufin O-deethylase (EROD) activity (Kennedy and Jones, 1994; Burke and Mayer, 1974; Pohl and Fouts, 1980).

EROD induction as a biomarker in teleost species has several advantages. By indicating the induction of CYP1A, EROD activity provides a fingerprint of the presence of AhR-active compounds. Induction of EROD is an extremely sensitive indicator of environmental alterations and is usually one of the first detectable, quantifiable responses to exposure (Stegeman, 1992). EROD activity describes the rate of the CYP1A mediated deethylation of the substrate 7-ethoxyresorufin (7-ER) to form the product resorufin. The catalytic activity towards this substrate is an indication of the amount of enzyme present and is measured as the concentration of resorufin produced per mg protein per minute (Mol/mg/min) (Kennedy and Jones, 1994). Because metabolism is generally highest in hepatic tissue, the assay is typically conducted using fish liver (Whyte *et al.,* 2000).

1.7.6 Metallothioneins (MTs)

Metallothioneins (MTs) are cysteine-rich, low molecular weight (500 to 14,000 Da) metal-binding proteins. Their function is not clear. MTs have the capacity to bind both physiological metals such as zinc, copper and selenium as well as xenobiotic toxic heavy metals such as cadmium, mercury, silver and arsenic (Sigel *et al*., 2009). Experimental data suggests that MTs may be involved in the regulation of homeostasis/cellular metabolism of these essential physiological metals, detoxification of heavy metals, cellular adaptation to stress and protection of cells against reactive oxygen species (ROS) and alkylating agents (Nordberg, 1998; Smirnov

et al., 2005). MTs likely participate in the uptake, transport and regulation of zinc in biological systems. By binding and releasing zinc, MTs may regulate zinc levels within the body. MTs are present in almost all forms of life ranging from prokaryotes, protozoa, invertebrates and vertebrates. Four main isoforms are expressed in humans and large quantities of MTs are synthesised primarily in the liver and kidneys (Thirumoorthy *et al.*, 2011). MT expression also occurs in the pancreas and intestine. Their production is dependent on availability of the dietary minerals zinc, copper and selenium and the amino acids histidine and cysteine.

Metallothionein gene expression can be induced by a large number of glucocorticoids, metal ion exposure (zinc, copper, cadmium, mercury), chatecholamines, ROS, lipopolysaccharides (LPS), physiological and physical stress (Penkowa, 2006; Klaassen *et al*., 1999). Elevated MT levels have frequently been associated with metal exposure and therefore MT induction is considered a response of an organism to physical or chemical stress. As such, it has been used as one of the biomarkers of exposure to heavy metal pollution (Amiard *et al*., 2006; Costa *et al*., 2007; Monserrat *et al*., 2007).

According to Rainbow (1988), the induction of metallothioneins is a common defence strategy in all organisms to protect against heavy metal exposure (Kägi, 1991; Webb, 1979). The binding by MTs, of nonessential metals, such as cadmium and mercury, most likely represents a sequestration function that aims to suppress their toxicity (Roesijadi, 1992).

MTs control oxidative stress by the cysteine residues capturing harmful oxidant radicals like the superoxide and hydroxyl radicals (Kumari *et al*., 1998). In this reaction, cysteine is oxidized to cystine, and the metal ions which were bound to cysteine are liberated to the media.

1.7.7 Heat Shock Proteins (HSPs)

Living organisms respond at the cellular level to unfavourable conditions such as temperature-shock and chemicals/toxins (ethanol, arsenic, trace metals and ultra violet light among others) by the rapid, vigorous and transient acceleration in the synthesis of a class of proteins known as heat shock proteins (HSPs) or stress proteins (Fincato *et al*., 1991). Other stressful situations include environmental pollution, pesticides, hormones, exercise, starvation, hypoxia, drugs, infection, inflammation or ischemia. First described in experiments in which sudden increases in temperature were used, hence their name, it is now well acknowledged that HSP synthesis can also be induced by other stress factors (Schlesinger, 1990; Nover, 1991; Sanders, 1993). The upregulation of heat shock proteins is sometimes described more generally as part of the stress response (Santoro, 2000).

Heat shock proteins (HSPs) are families of proteins that, when expressed, play an important role in the protection and maintenance of many vital cellular functions. They are normally localized in the cytoplasm and nucleus. In many of the model organisms studied, four major heat shock protein families were named based on the molecular mass (kDa) of the proteins. These protein families are HSP90 (85-90 kDa), HSP70 (68-73 kDa), HSP 60 and low-molecular-mass HSPs (16-24 kDa) (Forreiter and Nover, 1998). HSPs are highly conserved and found in both eukaryotes (plants and vertebrates, including humans) and prokaryotes (archaebacteria to eubacteria and yeasts) (Kelley and Schlesinger, 1982; Lindquist, 1986; Sanders, 1990; Ang *et al*., 1991). The low-molecular-mass HSPs have diverse functions and it has been proposed that they function as molecular chaperones, preventing irreversible protein aggregation (Derham and Harding, 1999). Extracellular and membrane bound heat shock proteins, especially HSP70 are involved in binding antigens and presenting them to the immune system (Nishikawa *et al*.,

2008). In humans, the major HSP group is the HSP70 family, which comprises constitutively expressed and inducible proteins and is known to assist in the folding of nascent polypeptide chains (Beckmann *et al.*, 1990; Fincato *et al*., 1991). This is done through adenosine triphosphate (ATP) - and cofactor-regulated binding and release cycles. They typically recognize hydrophobic amino acid side chains exposed by non-native proteins and may functionally cooperate with ATP-independent chaperones, such as the small HSPs, which function as 'holdases', buffering aggregation (Hartl *et al.*, 2011). Many HSPs also act as molecular chaperones by facilitating the folding of newly synthesized proteins to acquire their proper 3-dimensional conformation, organizing their transport out of the Golgi apparatus and mediating the repair (refolding/renaturing) or degradation of altered or denatured proteins in the proteasomes (Welch, 1992). This is a "housekeeping" role (Lindquist and Craig, 1988; Schlesinger, 1990; Becker and Craig, 1994; Morimoto *et al*., 1994; Hartl, 1996; Fink, 1999). HSP90 is active in supporting various components of the cytoskeleton, enzymes and steroid hormone receptors.

Because HSPs are activated very early in the cascade of cellular events that follow toxic exposure and at concentrations below the lethal dose, they are strong candidates for biomarkers of environmental pollution. Still, little is known about the induction of HSPs under different environmental conditions. Thus, analysis of the over expression and accumulation of these heat shock proteins may be useful in determining whether a particular environmental condition is perceived by the organism to be stressful or not. The use of stress protein response as a biomarker of environmental stress in aquatic organisms such as fish is also well documented (Köhler *et al*., 2001; Iwama *et al*., 1998; Sanders, 1993).

1.8 *In vitro* assays for screening EDCs

Analytical tools based on techniques such as gas or liquid chromatography (GC or LC) coupled to sensitive detection systems such as mass spectrometry (MS) or tandem mass spectrometry (MS/MS) have been applied to detect EDCs (Petrovic *et al.,* 2005; Mottaleb *et al.,* 2009). Unfortunately, most of these techniques are not only time consuming, but are also expensive due to the extensive cleanup and sample treatment methods. Treatment of samples very often requires derivatization procedures to aid detection (Bowden *et al.,* 2009). Alternatively, immunochemical techniques offer a large number of advantages. The most relevant advantages are the selectivity and sensitivity shown by the specific antibodies for the target analyte, the use of small sample volumes, the low cost and the simplicity of the methodologies (Estévez-Alberola and Marco, 2004). Moreover, they are easily adapted to automated systems and to development of high throughput screening (HTS) methods.

Several *in vitro* bioassays (Zacharewski, 1997) are being used as rapid primary screens to detect xenobiotic estrogenic activity (Odum *et al.*, 1998) in order to determine whether such chemicals are hazardous to human health. The *in vitro* bioassays available to assess estrogenic or androgenic activity of single compounds or complex mixtures range from simple competitive receptor binding assays, which rely entirely on the substance's ability to bind to the estrogen receptor (Jobling *et al.,* 1995; Shelby *et al.,* 1996), to more complex systems where the substance binds to and activates the receptor. Assay systems include direct immunoassay detection of single active compounds, reporter gene assays and cell proliferation assays such as the E-screen assay which involves the proliferation of the human breast cancer cell line (MCF-7) (Soto *et al.,* 1995; Soto *et al.,* 1994), vitellogenin gene expression in hepatocyte cultures (Jobling and

Sumpter, 1993) and yeast-based assays expressing either rainbow trout (Petit *et al.*, 1997) or human estrogen receptors (Routledge and Sumpter, 1996).

1.8.1 Immunoassays for specific EDCs

Immunoassay techniques, particularly colorimetric enzyme-linked immunosorbent assays (ELISAs), are currently very popular for the determination of trace amounts of environmental contaminants such as industrial pollutants like PCBs (Johnson and Van Emon, 1996), pesticides (Lee *et al.*, 1995; McAdam *et al.*, 1992; Wittmann *et al.*, 1996), herbicides (Wong and Ahmed, 1992), petroleum (Friedman and Allen, 1994) and heavy metals (Chakrabarti *et al.*, 1994). This is because of their robustness, high sensitivity, ease of use, smaller required sample volume and relative cost.

Compared to traditional analytical methods for environmental testing such as gas chromatography - mass spectrometry (GC-MS) and liquid chromatography - mass spectrometry (LC-MS), the ELISA technique is more cost effective and rapid, thus reducing the analysis time. The analysis time for an ELISA is typically one day and requires minimal cleanup, as compared to GC-MS, which is typically 2 to 3 weeks and requires extensive cleanup. ELISA kits are available commercially and are designed to be user friendly with easy to follow step-by-step instructions (Reddy *et al.*, 2005; Huang and Sedlak, 2001).

ELISAs are very specific and only measure the compound they are designed to detect. They employ antibodies as analytical reagents. The ELISA is based on the interaction of antigens and antibodies. Polyclonal or monoclonal antibodies (that have been raised against a particular analyte of interest) are used to detect the target analyte in a sample by specific antigen/antibody

interactions. The principle of immunoassay analytical detection is competition between an enzyme-labelled conjugate of the analyte and the particular analyte in the sample to bind with a limited number of antibody binding sites that coat the inside of a well (Plaza *et al.*, 2000). A colour change denotes reaction between the enzyme-labelled conjugate and a substrate and this colour change is indicative of the concentration of the analyte concentration in the sample. The colour change can then be recorded using a microtiter plate spectrophotometer. The extent of colour development is inversely proportional to the amount/concentration of chemical in the sample or standard. The higher the concentration of a specific steroid or other EDC chemical in the sample, the less colour reaction produced.

1.8.2 E-Screen assay

Both natural and synthetic environmental estrogens (ligands) are known to bind and activate the nuclear estrogen receptor (ER). Three receptor sub-types (ERα, ERβ1 and ERβ2), known to mediate the estrogen response are expressed in several body tissues (Esterhuyse, 2008; Swart and Pool, 2009; Fu *et al.*, 2008). The ligand binds to the nuclear ER, forming a complex, which also binds to a specific estrogen response element (ERE) sequence on DNA. This initiates mRNA transcription of a downstream gene and consequent production of specific E2-inducible proteins. Estrogens are important regulators of growth and differentiation of normal cells such as the mammary gland. However, altered expression of genes involved in the cell cycle could lead to the mitogenic effects resulting in breast cancer (Moggs, 2005). MCF-7 is a human breast cancer cell line that was derived from a patient with metastatic breast adenocarcinoma at the Michigan Cancer Foundation (Soule *et al.*, 1973). The MCF-7 cell line predominantly expresses ERα (also

some ERβ) (Bursztyka *et al.,* 2008) and has been extensively used to screen for EDCs with estrogenic activity (E-Screen) (Soto *et al.,* 1995; Soto *et al.,* 2006). This assay measures the ability of a sample to initiate the proliferation of the MCF-7 cells upon exposure. Quantification of the MCF-7 cell proliferation can be done by counting the cells, or by measuring secondary endpoints related to MCF-7 cell proliferation (Swart and Pool, 2009; Rasmussen and Nielsen, 2002). Two such secondary endpoints are the increase in cellular lactate dehydrogenase (LDH) and associated downregulation of ERα protein expression (Swart and Pool, 2009).

1.8.3 The Recombinant Yeast Screen Assay (YES)

The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) outlined a tiered screening/testing strategy for EDCs. Included in the Tier I testing scheme are *in vitro* transactivation assays such as yeast-based bioassays for screening xenobiotics that interact with the estrogen, androgen and thyroid hormone/receptor systems (US EPA, 1998). The advantages of using yeast cells are the cheap media components, they grow fast, are easy to handle and robust towards toxic effects of test chemicals or solvents. Therefore, yeast-based bioassays are a fast and easy tool for screening EDCs before performing more expensive, laborious and time consuming *in vivo* tests (Charles *et al.,* 2000).

Yeast cells do not normally contain endogenous steroid receptors. Mammalian steroid receptors are introduced along with a steroid responsive reporter gene into yeast cells by recombinant technology (Metzger *et al.,* 1988; Purvis *et al.,* 1991; Gaido *et al.,* 1998). The yeast cells contain expression plasmids carrying the reporter gene *lacZ,* which produces the enzyme β-galactosidase. This enzyme is used to measure the receptors' activity (Routledge and Sumpter,

1996; Beresford *et al.*, 2000). When a ligand binds the estrogen receptor, the reporter gene *lacZ* is expressed, producing β-galactosidase, which is secreted into the medium where it metabolises a yellow, chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG) into a red product that can be measured by absorbance at 540 nm. As a result, genetically modified yeast are useful tools for studying mammalian steroid receptor function in isolation of confounding factors found in mammalian cells. When a steroid responsive reporter gene is introduced into the yeast along with the steroid receptor, chemical interaction with that receptor can be determined by measuring the reporter gene product. A yeast based steroid receptor assay differs from a competitive binding assay in that it not only determines the ability of a chemical to bind to a receptor, but also to cause that receptor to dimerize and bind to the appropriate steroid responsive regions of the DNA to induce reporter gene activity. Other advantages of using yeast to study steroid receptor function include the ease of manipulation, rapid attainment of stable transformants, ability to process a large number of samples quickly and relatively inexpensively (Gaido *et al.*, 1998). A limitation of the yeast-based assay is that the permeability of the cells to some substances may generate false negative results and some strain specific effects have been reported. The yeast estrogenicity assay of Routledge and Sumpter (Routledge and Sumpter, 1996) has certain advantages over other yeast-based assays in that the colour can be monitored over a period of time.

1.8.4 High-performance liquid chromatography (HPLC) and gas chromatography (GC) for EDC determination

Chromatographic methods identify the chemical of interest and quantify the concentration. Sampling and handling are important to preserve the integrity of the sample and EDCs within. Complex matrices such as sewage or sediment often require extensive extraction and clean-up procedures before the sample is fit for determination (Petrovic *et al.,* 2002; Voulvoulis and Scrimshaw, 2003). For steroid estrogens, their determination requires concentration of the aqueous sample to reach trace levels (ng/L). Large volumes of typically 1L and up to 20L are collected, depending on the sensitivity and the selectivity of the chemical technique (Belfroid *et al.,* 1999). Upon collection of the aqueous sample, preservatives such as methanol (MetOH) (Desbrow *et al.,* 1998) and formaldehyde (1% v/v) (Baronti *et al.,* 2000), may be added to halt microbial activity whilst solid samples are refrigerated or stored at -18^{o}C (Ternes *et al.,* 2002). EDCs are mainly hydrophobic and tend to bind to sediment where it becomes concentrated (Bowman *et al.*, 2002).

Upon collection, aqueous samples are usually prepared by filtration to remove particles that may interfere with the extraction procedure. Any EDCs associated with the particles may be extracted by washing of the particles with a solvent such as MetOH. Extraction is generally carried out within 48 hours of collection and may even be carried out on site, negating the need for preservatives and ensuring that EDCs within are not adversely affected by storage conditions.

Solid Phase Extraction (SPE) is the main technique used to extract steroids from aqueous samples, while solid samples have to be freeze dried prior to ultrasonic extraction in solvent. SPE utilises either disks or more commonly, cartridges. Disks reduce sample clogging and have

a large surface area for sample contact compared to cartridges. Using dried sediment or sewage sludge samples, Soxhlet is the preferred extraction technique with duration periods of 4-24 hours (Petrovic *et al.*, 2002).

After extraction, sample clean-up utilizing mainly adsorption chromatography or SPE is necessary to remove further co-extractives (Voulvoulis and Scrimshaw, 2003). Further clean-up is not normally necessary for aqueous samples. For steroid estrogen monitoring in sediment and sludge samples, clean-up is a requirement and may be a multi-step procedure. Following extraction, steroid estrogens in sewage sludge are cleaned up by gel permeation chromatography (GPC) followed by silica gel (Ternes *et al.*, 2002). Some more intensive clean-up by HPLC fractionation is followed by elution over silica gel and (H_2SO_4) sulphuric acid treatment (De Boer *et al.*, 2000). Sulphuric acid is used for lipid removal although it may adversely affect the analyte. GPC, alumina and florisil columns are gentler ways to remove lipids. Sulphur present in sludge and sediment samples can interfere with gas chromatography (GC) determination. Removal of sulphur interferences can be achieved with the addition of copper either prior to extraction or during the clean-up stage (Eljarrat *et al.*, 2002).

The identification of the EDC of interest is dependent on an extraction procedure and an analytical technique which is selective for that compound. To allow investigation into the study of compounds and their interaction with their environment, column chromatography is able to achieve the objectives of separation, identification and quantification.

According to Richardson (2002), GC is the preferred analytical technique for the determination of persistent organic pollutants (POPs), having utilized GC with electrochemical (EC) detection and GC-MS with negative chemical isolation (NCI) or electron impact (EI) detection. A

prerequisite for analysis by GC is that the chemical of interest is volatile and thermally stable. When this is not the case, derivatization can be used to overcome this limitation (Blau and Halket, 1993). Traditionally, GC using derivatization has been used for estrogen monitoring. Disadvantages of derivatization are that it is labour intensive and can reduce analyte recovery. Apart from making chemicals amenable to GC-MS analysis, derivatization can also increase sensitivity using LC-MS.

1.8.5 Minced testes assay

With respect to the male reproductive system, several *in vitro* methods are used to screen for EDCs. These methods include whole testis used in simple incubation, perifusion, or perfusion assays. Other methods are testis cell line assays and sectioned or minced testes assays. Isolated and cultured crude or purified Leydig cells are other *in vitro* methods that can be used to screen for EDCs (US EPA, 2005). Of these *in vitro* methods, the sectioned or minced testes assay was selected as the most promising screening tool for identifying substances with steroidogenic-altering activity (US EPA, 2005). This method uses untreated animals as a source of testes. After removal of the whole testis from an anesthetized or euthanized animal, the testes are further processed into smaller sizes for use. A method to prepare the testes for measuring steroidogenesis was described in detail (Sikka *et al.,* 1985). It was also determined that this single assay would suffice as a screen for females as well, because the steroidogenic pathways of both males and females are very similar, the testes provide more organ for testing and organ isolation and preparation are technically easier to accomplish using the male organs. Other reasons for this assay's recommendation were its minimal cost, being quick and simple to

perform, relative sensitivity and specificity, use of standard and basic laboratory equipment and skills and its ease of preparation (Gray *et al.,* 1997). The organ remains viable for several hours and the assay also maintains the cyto-architecture of the organ, minimizes animal usage, has the ability to screen many samples, is relatively easy to standardize by optimization and has well-defined and multiple endpoints (US EPA, 2005).

Ebrahim (2010) optimized an *in vitro* minced mouse testicular cell culture assay that could be employed as a valuable screening tool for endocrine disrupting compounds in food, water and other environmental samples. Testosterone synthesis was used as an endpoint after luteinizing hormone (LH) stimulation of the testicular cells. Increased testosterone synthesis indicated the effect of androgenic compounds in a sample while a decrease in testosterone synthesis was indicative of the anti-androgenic effects of a sample.

1.8.6 Frog and fish *in vitro* liver assays

Monitoring the induction/synthesis of the estrogenic biomarker VTG in primary cultured hepatocytes and liver slices is a useful tool for screening the estrogenic potencies of chemicals and environmental samples. The genes for VTG are found in the livers of both females and males (Gronen *et al.,* 1999). Therefore, although males do not normally synthesize VTG, they possess the ability to synthesize VTG upon estrogen exposure (Green and Tata, 1976; Sumpter and Jobling, 1995). This ability of males to synthesize VTG after estrogen exposure has been exploited as a biomarker for measuring exposure of oviparous animals to environmental estrogens (Palmer and Palmer, 1995; Palmer *et al.,* 1998; Sumpter, 1995; Gronen *et al.,* 1999).

In vitro production of vitellogenin by hepatocytes of fish and amphibians has been used to study estrogenicity (Heppell *et al.,* 1995; Toomey *et al.,* 1999; Gagné *et al.,* 1999). Estrogenic compounds added to the hepatocyte culture medium activate the same receptor-mediated gene transcription mechanisms as endogenous estrogen, resulting in vitellogenin production.

A study done by Lutz and Kloas (1999) reports that in order to get a useful tool for investigating the binding of environmental samples to estrogen receptors in amphibians, a radio-receptor assay of estrogen receptors was established using cytosolic liver homogenates of *Xenopus laevis* (African clawed frog). Their results clearly indicated that liver cytosol of both male and female *X. laevis* can be used for determination of estrogen receptor binding because no sex-specific differences could be observed.

Lutz *et al.,* (1999) further described a novel semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) technique for screening estrogenicity by assaying messenger ribonucleic acid (mRNA) induction of the estrogenic biomarker vitellogenin. This *in vitro* method combined with classical exposure experiments *in vivo* demonstrated the biological significance of estrogenic endocrine disruptors on the amphibian *X. laevis*. The effects of endocrine disrupting chemicals were demonstrated at several levels of organization including binding to liver estrogen receptor and the induction of vitellogenin-mRNA by RT-PCR in primary cultured hepatocytes. According to Lutz *et al.,* (1999), both documented methods represented a comprehensive amphibian model to study potential estrogenic activities of endocrine disruptors.

A sensitive bioassay for detecting environmental estrogens and estrogen mimics was developed by Hurter *et al.,* (2002) using *X. laevis* liver slice culture. Vitellogenin synthesis by *X. laevis*

liver slice cultures was used as a biomarker for estrogenic activity of environmental water samples. It was found that not only uncontaminated males but also estrogen-pretreated males and females can successfully be used as bioindicators. Female liver tissue slices, being equally if not more sensitive than estrogen pre-exposed male liver tissue slices, can thus be used as an alternative to traditional studies that only used male animals to study the effects of estrogen and estrogen mimics on endocrine disruption (Hurter *et al.,* 2002).

Hurter *et al.,* (2002) optimized the *X. laevis* liver slice assay that could be used to detect man-made estrogen mimics that might pollute the environment. Only liver tissue slices were investigated because they were more representative of *in vivo* conditions than isolated hepatocytes. This is due to the fact that all the cell types are present and can interact as they would *in vivo*, as they are in the right spatial orientation with one another in the liver slice system (Hurter *et al.,* 2002). Hurter *et al.,* (2002) suggested that this assay, being more representative of *in vivo* conditions than the isolated hepatocyte assay and the yeast screen assay for estrogen, be included in the battery of tests for endocrine disruptors.

Williams *et al*., (1998) showed rainbow trout to be sensitive to xenoestrogens and their effects on responses such as modulation of cytochromes P450 (CYPs) and carcinogenesis. Since the use of rainbow trout (*Oncorhynchus mykiss*) liver slices to screen for estrogenic compounds offered a link between cultured cell models and *in vivo* studies, Shilling and Williams (2000) developed a rainbow trout liver slice assay to quantify the relative estrogenicity of chemicals by measuring VTG induction. Their model demonstrated the capacity to screen environmental and dietary compounds for estrogenicity. The results were consistent with those of other studies by Pelissero *et al.,* (1993) and Maitre *et al.,* (1986), which described similar *in vitro* techniques which utilized VTG induction by rainbow trout hepatocytes as a biomarker to assess the estrogenic activity of

chemicals. Jobling and Sumpter (1993) also reported the use of rainbow trout hepatocytes in an *in vitro* bioassay to detect the estrogenic potencies of detergent components (surfactants) in sewage effluent to fish. Slices are faster and easier to prepare than hepatocytes because no collagenase, isolation or culturing steps are required (Seglan, 1975). Another advantage is that unlike mammalian liver slices, rainbow trout slices can be maintained for several days, allowing for more extensive studies of induction and inhibition (Oganesian *et al.,* 1997a and b). Liver slices also allow for the preservation of the different types of cells and maintenance of cell-to-cell interactions (Guillouzo, 1998). That, and the retention of metabolic activity over a longer period of time (Vickers, 1994; Beamand *et al.,* 1993) make liver slices an ideal model to monitor estrogenicity of samples.

CHAPTER 2

Problem Statement and Aim

There is a growing concern over the occurrence of endocrine disrupting compounds (EDCs) in the environment (Kavlock *et al.,* 1996). Prolonged exposure to environmental estrogens (a sub-group of EDCs) is believed to be responsible for a variety of adverse health effects in humans and wildlife. These effects include gonadal abnormalities, reduced fertility, cancers of the male and female reproductive tract and altered male/female sex ratios among others (Sumpter, 1995; Sumpter and Jobling, 1995). These effects are difficult to detect. Although it is essential to screen for EDCs in aqueous environmental samples, most countries have failed to implement this as part of their routine water quality monitoring programs due to various constraints such as the high cost of assays and the lack of infrastructure and skills required to do the assays. Therefore, there is a clear need for more user-friendly, more economically viable and time saving assays that can be used for routine monitoring of environmental EDCs.

To this end, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was formed to make recommendations to the US Environmental Protection Agency (EPA) on how to develop screening strategies to monitor EDCs in the environment. EDSTAC recommended a two-tiered approach that involves *in vitro* and *in vivo* mammalian and eco-toxicological screens as a Tier 1 system and a set of *in vivo* tests as a Tier 2 system for the identification of endocrine effects of various environmental contaminants (US EPA, 1998).

The aim of this study is to compare different rapid ELISAs for EDC monitoring to determine if data obtained with these assays are similar/identical. The study also aims to compare the estradiol rapid ELISA with a bioassay for anti-androgenicity using mouse testicular cell cultures. In so doing, this research hopes to demonstrate how well estradiol levels can be used to predict the presence of other steroid hormones in a sample and also demonstrate how effective estradiol levels are at predicting/monitoring the anti-androgenicity of a sample. The eventual goal of the above is to select representative assays that can also be used to predict other likely contaminants in a sample. Assays giving identical/similar results will be replaced with a single assay that will be used for routine monitoring. Reduction of assays will have major cost and human resource benefits. These resources can then be redirected to develop and implement assays for other groups of EDCs, such as the hypothalamus-pituitary-thyroid (HPT) axis inhibitors, for which rapid assays are not yet available.

This research intends to evaluate data for various EDC screening tests and then propose a minimalist battery of screening assays to monitor for environmental estrogens, progestogens, androgens and anti-androgens in water samples.

CHAPTER 3

3.1 Introduction

The U.S Environmental Protection Agency (EPA) defines EDCs as xenobiotics (agents foreign to an organism) that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for homeostasis, reproduction, development and/or behaviour (Crisp *et al.*, 1998). Most EDCs are synthetic compounds produced as a result of industrial, agricultural and domestic activities (Desbrow *et al.*, 1998; Pojana *et al.*, 2004; DeRosa *et al.*, 1998, Harris *et al.*, 1997). Some EDCs are natural steroids. Examples of these are the endogenous steroid hormones such as the estrogens (estrone, 17β-estradiol and estriol), androgens like testosterone and phytoestrogens such as genestein and lignans (Safe and Gaido, 1998).

There is considerable concern over the occurrence of EDCs in the environment. They have been found in natural water resources, drinking water and in an accumulated form in the food of many human and animal populations, potentially posing a great health threat upon prolonged exposure (Soto *et al.*, 1995; Palmer *et al.*, 1998). EDCs have been reported to adversely affect the reproductive, immune, endocrine, nervous and metabolic systems in wildlife and human populations (Colborn, 1995).

EDCs have been shown to interfere with the normal functioning of the endocrine system by mimicking the effects of natural hormones. EDCs can mimic the binding of hormones to their receptors (for example binding the estrogen receptor leading to feminization), block the binding

of natural hormones to their receptors (for example the male hormone testosterone, leading to demasculinization of males) or modulate the effect of natural hormones by interfering with important cell signalling pathways (Kelce and Gray, 1999). The best studied environmental EDCs include chemicals that induce responses mimicking the effect of natural estrogen (Gillesby and Zacharewski, 1998; Toomey *et al.,* 1999; Soto *et al.,* 1995) or act as anti-estrogenic compounds (Kime *et al.,* 1999).

The effects of EDCs are among others, altered male/female sex ratios, feminization of males, reduced fertility and gonadal abnormalities to mention a few (Sumpter, 1995; Sumpter and Jobling, 1995; Gagné *et al.,* 1999; Kime *et al.,* 1999).

Biomarkers must be developed and implemented to screen for EDCs in the environment. Analytical tools based on traditional chemical monitoring techniques such as gas chromatography - mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) have been applied to detect EDCs (Snyder *et al.,* 1999; Snyder *et al.,* 2000). These techniques are reliable, but they are very expensive, require a very high level of skill to operate and are time consuming. Beresford *et al.,* (2000) concluded that due to the need for cost effective, rapid and simple methods that could also allow for screening of large numbers of samples, *in vitro* test methods were more appropriate.

Several *in vitro* bioassays (Zacharewski, 1997) are being used as rapid primary screens to detect xenobiotic estrogenic activity (Odum *et al.,* 1998). The *in vitro* bioassays available to assess estrogenic or androgenic activity of single compounds or complex mixtures range from simple competitive receptor binding assays, which rely entirely on the substance's ability to bind to the estrogen receptor (Jobling *et al.,* 1995; Shelby *et al.,* 1996), to more complex systems where the

substance binds to and activates the receptor. Assay systems include direct immunoassay detection of single active compounds, reporter gene assays and cell proliferation assays such as the E-screen assay which involves the proliferation of the human breast cancer cell line (MCF-7) (Soto *et al.*, 1995; Soto *et al.*, 1994), vitellogenin gene expression in hepatocyte cultures (Jobling and Sumpter, 1993) and yeast-based assays expressing either rainbow trout (Petit *et al.*, 1997) or human estrogen receptors (Routledge and Sumpter, 1996).

Other *ex vivo* bioassays are based on the quantitation of hormone-induced changes in the expression of endogenous proteins and genes. Examples of methods employing estrogen-induced changes in the expression of proteins include the *Xenopus laevis* (Hurter *et al.*, 2002) and rainbow trout (Shilling and Williams, 2000) liver slice assays. These assays monitor *in vitro* induced vitellogenin (VTG) as a biomarker endpoint. Another method, the minced testes assay is based on the steroidogenic activity of rat/mouse testicular cells. This assay monitors the potential of samples to interfere with steroid hormone biosynthesis or more directly alter testosterone levels (Gray *et al.*, 1995; Laskey *et al.*, 1995).

A study conducted in South Africa by Swart and Pool (2007), demonstrated the presence of significant amounts of steroid hormones released back into the environment from sewage treatment plants. Steroid hormones have the potential to cause endocrine disruption, not only in animals, but in humans as well (Swart and Pool, 2007). Research done by Swart *et al.* (2011) has shown that accurate evaluation of environmental water samples for estrogenic activity requires a battery of *in vitro* and *in vivo* bioassays. For this study, the environmental water samples collected were selected based on various human impacts and comparatively analysed using various biomarker assays.

The aim of this study was to investigate the comparison between chemical and tissue culture methods to monitor environmental estrogens. Specific objectives were to compare different rapid ELISAs for EDC monitoring and to compare the estradiol rapid ELISA with a bioassay for anti-androgenicity using mouse testicular cell cultures.

3.2 Materials and Methods

3.2.1 Reagents and chemicals

All chemicals, reagents and solvents were purchased from Sigma (USA), Merck (Germany) and Roche Diagnostics (South Africa). All reagents were of analytical grade.

3.2.2 Animals

After obtaining approval from the institutional animal ethics committee, male Balb/C mice were used for this study. Two month old, pathogen free mice were purchased from the University of Cape Town Animal Unit (Cape Town, South Africa). The mice were then housed in a well-ventilated animal house with a light/dark cycle of 12:12. The mice had free access to normal drinking water and were fed standard mouse feed (Medical Research Council, Cape Town, South Africa).

3.2.3 Water Sampling

Water samples were collected in clean 250 ml glass bottles. The bottles were cleaned by washing with soap, followed by rinsing with tap water. The bottles were then rinsed with distilled water, followed by analytical grade ethanol (Merck, Germany) and finally rinsed again with distilled water. The bottles were then allowed to dry upside down on a dry rack. Environmental water samples were collected from various sites around South Africa. Samples collected for the current study were selected based on various human impacts and also to give approximately 50% high and 50% low estrogen values. Samples were numerically labeled and given to the assay operator for an independent comparative analysis of various biomarker assays. The samples were taken to the laboratory and processed immediately. Normal laboratory tap water was used as a control.

3.2.4 Detection of steroid hormones in water

3.2.4.1 Solid Phase Extraction (SPE) of water samples

To prevent clogging the columns during extraction, particle-containing samples were centrifuged at 4000 rpm for 15 minutes. Water samples were then extracted using C-18 SPE columns (Sigma Aldrich, USA). Columns were conditioned by adding 2 ml of Phase B solution (45% methanol, 40% hexane and 15% 2-propanol), then charged by adding 2 ml ethanol and then washed with 4 ml distilled water. Care was taken to ensure that the sorbents do not dry between the different steps. After the wash step, 100 ml of water sample was passed through the column. The columns were then dried using a vacuum pump (PALL Life Sciences vacuum/pressure pump) until the column was completely dry. The hydrophobic analytes bound to the resin were eluted with 2 ml of Phase B solution. The eluates were dried under a stream of hot air. The dried sample was reconstituted in 100 µl dimethyl sulfoxide (DMSO) to make a 1000 times concentrated sample extract solution. The samples were stored at 4^0C until further use. Extracts were diluted appropriately for the different ELISAs.

3.2.4.2 Estrone (E1) ELISA analysis of water extracts

Concentrated (1000 times) water extracts were diluted 1/500 using wash solution. The diluted (2 times concentrated) extracts were assayed directly using E1 ELISA kits purchased from DRG Instruments GmbH, Germany. All the reagents required were supplied in the kit. The microtiter plate wells supplied with the kit were pre-coated with a polyclonal antibody directed towards an antigenic site on the E1 molecule. All assays were done in duplicate. Standard, control or samples (50 µl per well) were dispensed into appropriate wells on the anti-estrone coated plate. Thereafter, 100 µl of enzyme conjugate (estrone horseradish peroxidase) was added to each well.

The contents of the wells were mixed by tapping the plate. The mixture was incubated for 1 hour at room temperature on a plate shaker (Barloworld Scientific, Stuart Microtiter Plate Shaker, SSM5). After incubation, the wells were washed five times with 400 µl per well of wash solution and then tapped dry on adsorbent paper to remove residual droplets. Thereafter, 150 µl of tetramethylbenzidine (TMB) substrate was added to each well and incubated in the dark for 30 minutes at room temperature. The enzymatic reaction was stopped by adding 50 µl of stop solution (0.5 M H_2SO_4) to all wells. The absorbance/optical density (OD) of each well was then measured at 450 nm using a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). A standard curve was drawn using the OD readings obtained for the standards and the concentrations of the samples were read off this curve.

3.2.4.3 Estradiol (E2) analysis of water extracts

Concentrated (1000 times) water extracts were diluted 1/500 using wash solution. The diluted (2 times concentrated) extracts were assayed directly using E2 ELISA kits purchased from DRG Instruments GmbH, Germany. All the reagents required were supplied in the kit. The microtiter plate wells supplied with the kit were pre-coated with a polyclonal rabbit antibody directed towards an antigenic site on the E2 molecule. Assays were done in duplicate. Standard, control or samples (25 µl per well) were dispensed into appropriate wells on the anti-estradiol coated plate. Thereafter, 200 µl of enzyme conjugate (estradiol horseradish peroxidase) was added to each well. The contents of the wells were mixed by tapping the plate. The mixture was incubated for 2 hours at room temperature on a plate shaker (Barloworld Scientific, Stuart Microtiter Plate Shaker, SSM5). After incubation, the wells were washed four times with 400 µl per well of wash solution and then tapped dry on adsorbent paper to remove residual droplets. Thereafter, 100 µl

of tetramethylbenzidine (TMB) substrate was added to each well and incubated in the dark for 15 minutes at room temperature. The enzymatic reaction was stopped by adding 50 µl of stop solution (0.5 M H_2SO_4) to all wells. The absorbance/optical density (OD) of each well was then measured at 450 nm using a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). A standard curve was drawn using the OD readings obtained for the standards and the concentrations of the samples were read off this curve.

3.2.4.4 Testosterone ELISA analysis of water extracts

Concentrated (1000 times) water extracts were diluted 1/100 using wash solution. The diluted (10 times concentrated) extracts were assayed directly using testosterone ELISA kits purchased from DRG Instruments GmbH, Germany. All the reagents required were supplied in the kit. The microtiter plate wells supplied with the kit were pre-coated with a monoclonal mouse antibody directed towards a unique antigenic site on the testosterone molecule. All assays were done in duplicate. Standard, control or samples (25 µl per well) were dispensed into appropriate wells on the anti-testosterone coated plate. Thereafter, 200 µl of enzyme conjugate (testosterone horseradish peroxidase) was added to each well. The contents of the wells were mixed by tapping the plate. The mixture was incubated for 1 hour at room temperature on a plate shaker (Barloworld Scientific, Stuart Microtiter Plate Shaker, SSM5). After incubation, the wells were washed four times with 400 µl per well of wash solution and tapped dry on adsorbent paper to remove residual droplets. Thereafter, 200 µl of tetramethylbenzidine (TMB) substrate was added to each well and incubated in the dark for 15 minutes at room temperature. The enzymatic reaction was stopped by adding 100 µl of stop solution (0.5 M H_2SO_4) to all wells. The absorbance/optical density (OD) of each well was then measured at 450 nm using a microtiter

plate reader (Thermo Electron Corporation, Original Multiskan Ex). A standard curve was drawn using the OD readings obtained for the standards and the concentrations of the samples were read off this curve.

3.2.4.5 Progesterone ELISA analysis of water extracts

Concentrated (1000 times) water extracts were diluted 1/100 using wash solution. The diluted (10 times concentrated) extracts were assayed directly using progesterone ELISA kits purchased from DRG Instruments GmbH, Germany. All the reagents required were supplied in the kit. The microtiter plate wells supplied with the kit were pre-coated with a polyclonal antibody directed towards an antigenic site on the progesterone molecule. All assays were done in duplicate. Standard, control or samples (25 µl per well) were dispensed into appropriate wells on the anti-progesterone coated plate and incubated for 5 minutes at room temperature. Thereafter, 200 µl of enzyme conjugate (progesterone horseradish peroxidase) was added to each well. The contents of the wells were mixed by tapping the plate. The mixture was incubated for 1 hour at room temperature on a plate shaker (Barloworld Scientific, Stuart Microtiter Plate Shaker, SSM5). After incubation, the wells were washed four times with 400 µl per well of wash solution and tapped dry on adsorbent paper to remove residual droplets. Thereafter, 200 µl of tetramethylbenzidine (TMB) substrate was added to each well and incubated in the dark for 15 minutes at room temperature. The enzymatic reaction was stopped by adding 100 µl of stop solution (0.5 M H_2SO_4) to all wells. The absorbance/optical density (OD) of each well was then measured at 450 nm using a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). A standard curve was drawn using the OD readings obtained for the standards and the concentrations of the samples were read off this curve.

3.2.4.6 Estriol (E3) ELISA analysis of water extracts

Concentrated (1000 times) water extracts were diluted 1/40 using incubation buffer. The diluted (25 times concentrated) extracts were assayed directly using E3 ELISA kits purchased from DRG Instruments GmbH, Germany. All the reagents required were supplied in the kit. The microtiter plate wells supplied with the kit were pre-coated with antibody against the E3 molecule. All assays were done in duplicate. Before use, the standards were mixed for 2 minutes on a rotating mixer/vortex (Scientific Industries, Vortex Genie-2); whereas the enzyme conjugate (estriol horseradish peroxidase) was prepared immediately before use. Conjugate (10 µl) was added to 2 ml of incubation buffer and gently mixed on the rotating mixer for 5 minutes. Standard, control or samples (20 µl per well) were dispensed into appropriate wells on the anti-estriol coated plate. Thereafter, 200 µl of enzyme conjugate was added to each well. The contents of the wells were mixed by tapping the plate. The mixture was incubated for 1 hour at 37^0C. After incubation, the wells were washed three times with 300 µl per well of distilled water and tapped dry on adsorbent paper to remove residual droplets. Thereafter, 100 µl of tetramethylbenzidine (TMB) substrate was added to each well and incubated in the dark for 15 minutes at 22 - 28^0C. The enzymatic reaction was stopped by adding 100 µl of stop solution (0.15 M H_2SO_4) to all wells. The absorbance/optical density (OD) of each well was then measured at 450 nm using a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). A standard curve was drawn using the OD readings obtained for the standards and the concentrations of the samples were read off this curve.

3.2.4.7 Ethinylestradiol (EE2) ELISA analysis of water extracts

Concentrated (1000 times) water extracts were diluted 1/100 using diluent (40% (v/v) Methanol (MetOH) in distilled water). The diluted (10 times concentrated) extracts were assayed directly using EE2 ELISA kits purchased from R-Biopharm AG, Darmstadt, Germany. All the reagents required were supplied in the kit. The microtiter plate wells supplied with the kit were pre-coated with capture antibodies directed against anti-ethinylestradiol antibodies. All assays were done in duplicate. Before use, the ethinylestradiol enzyme conjugate and anti-ethinylestradiol antibody concentrates were diluted 1:11 in dilution buffer. Standard, control or samples (20 µl per well) were dispensed into appropriate duplicate wells on the anti-ethinylestradiol coated plate. Thereafter, 50 µl of diluted enzyme conjugate was added to the bottom of each well. 50 µl of diluted antibody solution was added to each well and the contents of the wells were mixed gently by shaking the plate manually. The mixture was incubated for 2 hours at room temperature (20 - 25^0C). After incubation, the wells were washed four times with 250 µl per well of distilled water and tapped dry on adsorbent paper to remove residual droplets. Thereafter, 50 µl of substrate (containing urea peroxide) and 50 µl of chromogen (containing tetramethylbenzidine) was added to each well and mixed gently by shaking the plate manually. The plate was then incubated in the dark for 30 minutes at room temperature. The enzymatic reaction was stopped by adding 100 µl of stop solution (0.5 M H_2SO_4) to each well. The absorbance/optical density (OD) of each well was then measured at 450 nm using a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). A standard curve was drawn using the OD readings obtained for the standards and the concentrations of the samples were read off this curve.

3.2.5 The effects of water extracts on testicular culture testosterone synthesis

3.2.5.1 Preparation of testicular cells

Mice were sacrificed by cervical dislocation. Their testes were then removed aseptically, finely minced and then transferred to a 50 ml tube (Greiner Bio-one) containing 10 ml serum-free medium (100 µl glutamax (Invitrogen), 100 µl Penicillin/Streptomycin/Fungizone mix (Sigma) and 9.8 ml RPMI-1640 medium (Sigma)). Debris was allowed to collect at the bottom of the tube and thereafter, the supernatant (containing testicular cells) was transferred to a new tube. Serum-free medium was then added to the cells resulting in a final volume of 20 ml. The cells were then incubated at 37^0C with 5% CO_2 for 1 hour. After incubation, the cells were centrifuged at 4000 rpm for 15 minutes on a Boeco C-28A centrifuge (Boeckel & Co.). The supernatant was then discarded and the cell pellet was resuspended in 15 ml fresh serum-free medium and incubated at 37^0C with 5% CO_2 for 30 minutes. The cells were centrifuged once again at 4000 rpm for 15 minutes and the supernatant obtained was again discarded. The cell pellet was then resuspended in 16 ml complete (enriched) medium containing 160 µl fetal bovine serum (FBS) (to give a concentration of 1.0×10^6 cells/ml). The complete medium also contained 160 µl Glutamax (Invitrogen), 160 µl Penicillin/Streptomycin/Fungizone mix (Sigma) and 15.52 ml RPMI-1640 medium (Sigma)). The cell suspensions were then used for testosterone production assays.

3.2.5.2 Testicular cell culture bioassays on water extracts

DMSO diluted 1/500 in RPMI-1640 medium (Sigma) was used as a control. Water extracts diluted 1/500 (2 times final concentration) in RPMI-1640 medium (Sigma) were added to the wells of a 96-well tissue culture plate (Nunc, Denmark) at a volume of 50 µl per well. Cell

suspension (50 µl per well) was then added to the water extracts in the tissue culture plate. The culture plate was then incubated overnight at 37^0C in 5% CO_2. Unstimulated cells then received 50 µl enriched medium, while stimulated cultures receive 50 µl enriched medium containing 10 mU per ml luteinizing hormone (LH). The cultures were then incubated for 4 hours at 37^0C in 5% CO_2. After the 4 hour incubation period, supernatants from LH-treated and non-treated cells were harvested and diluted 1/10 with phosphate buffered saline (PBS) containing 0.1% Tween 20. The diluted sample supernatant was screened for testosterone concentration using a commercially available ELISA kit (DRG Instruments GmbH, Germany). The ELISA kit contains all the necessary reagents for the assay and the assay was performed as per manufacturer's instructions (refer to 3.2.4.4).

3.2.5.3 Statistical analysis

Each experiment was performed in triplicate. The results were analyzed using one-way analysis of variance (ANOVA) followed by a pairwise multiple comparison procedure (Holm-Sidak method) to indicate significantly different groups ($P < 0.05$); using the SigmaStat 12.0 software package (Systat Software Inc., USA).

3.3 Results and Discussion

3.3.1 Steroid hormone detection in water samples using specific ELISAs

The standard curves for the ELISAs to determine the estrogen (estrone, estradiol, estriol and ethinylestradiol) concentrations in environmental water samples are shown in Figure 1. The correlation coefficients (R^2) for all the standard curves are between 0.939 and 0.991. This data is similar to results previously obtained by Swart and Pool (2007). The standard curves show good inverse correlations between the optical density and the estrogenic hormone concentration. The ELISAs have detection ranges between 15 - 2000 pg/ml, 9.7 - 2000 pg/ml, 2000 - 200000 pg/ml and 20 - 12800 pg/ml for the estrone, estradiol, estriol and ethinylestradiol ELISAs respectively. Estrone, estradiol, testosterone, progesterone, estriol and ethinylestradiol concentrations detected in environmental water samples are shown in Table 2. The levels of testosterone synthesized by mouse testicular cells exposed to the environmental water samples are also included in Table 2. The 28 environmental water samples were separated into two groups based on the estradiol ELISA. The estradiol ELISA was chosen because estradiol is the principal estrogen found in all mammalian species during their reproductive years (Duff and DeAvila, 2005). For this separation, an estradiol level of 5 pg/ml was used as cut-off. Valentini *et al.*, (2002) estimated the detection limit of 17β-estradiol in waste water to be 5 pg/ml. Of the 28 samples investigated, 15 had estradiol levels higher than 5 pg/ml and were designated as high estradiol. The remaining 13 samples contained estradiol at 5 pg/ml or less and they were designated as low estradiol.

3.3.2 Comparison between data obtained using the panel of estrogen ELISAs for monitoring water samples

The estrone, estriol and ethinylestradiol ELISAs for rapid screening of environmental water samples for EDCs show positive correlations of $R^2 = 0.539$, $R^2 = 0.720$ and $R^2 = 0.871$ respectively when compared with the estradiol ELISA (Figure 2). The estrone, estriol and ethinylestradiol levels of the low and high estradiol sets of environmental water samples were then compared to see how effective the estradiol levels were at predicting high and low levels of the other hormones within the samples (Figures 3, 4 and 5). Results of this show that separation of water samples based on estradiol levels was very effective at separating samples with high and low levels of estrone ($P < 0.001$), estriol ($P = 0.03$) and ethinylestradiol ($P = 0.006$). The similarity of results based on the estradiol assay may be due to the fact that these assays screen for hormones and pharmaceutical residues in the environment that are secreted mainly by the same population sub-group, namely reproductively active females.

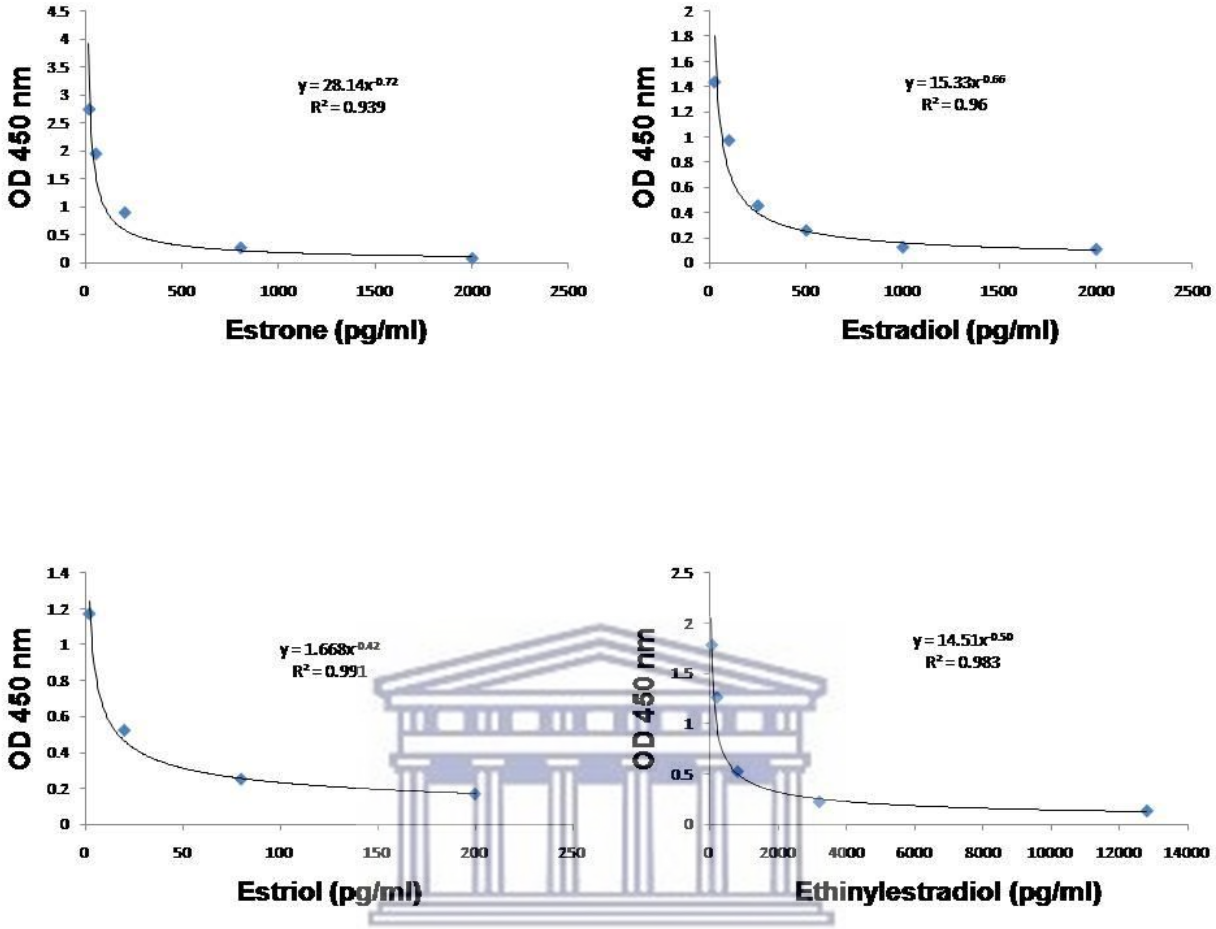


Figure 1. Standard curves for the ELISAs to determine the estrogenic hormone (estrone, estradiol, estriol and ethinylestradiol) concentrations in environmental water samples.

Sample number	Estradiol (pg/ml)		Estrone (pg/ml)		Estriol (pg/ml)		Ethinylestradiol (pg/ml)		Testosterone (pg/ml)		Progesterone (pg/ml)		Testosterone from mouse culture (pg/ml)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	14	0	18	1	0	0	2	0	0	0	10	3	77	15
2	41	1	34	0	0	0	3	0	0	0	7	1	83	8
3	3	0	3	0	0	0	2	0	0	1	0	0	93	11
4	34	1	13	1	0	0	2	0	0	0	0	1	84	16
5	5	0	5	0	0	0	2	0	0	0	4	1	100	10
6	22	1	10	1	0	0	3	1	0	1	5	2	96	16
7	7	0	34	1	0	0	3	0	0	0	18	1	84	12
8	11	1	29	0	0	0	4	1	0	0	22	7	92	8
9	6	0	11	1	0	0	4	1	0	0	11	1	0	2
10	3	0	3	1	0	0	2	0	0	1	3	4	43	12
11	3	0	4	1	0	0	3	0	0	0	0	1	0	1
12	43	2	42	4	2	0	10	0	96	9	94	6	0	1
13	93	10	47	9	3	0	19	0	334	30	119	4	0	1
14	78	8	26	0	1	0	20	0	351	15	56	4	0	1
15	2	0	4	1	0	0	2	0	0	3	6	3	84	13
16	2	0	1	0	0	0	0	0	0	16	5	1	101	4
17	2	1	2	0	0	0	1	0	0	3	3	3	103	5
18	71	2	40	4	2	0	17	1	412	55	204	25	9	21
19	4	1	5	0	0	0	2	0	0	5	6	0	95	16
20	4	0	6	0	0	0	1	0	0	7	5	1	110	1
21	2	0	4	0	0	0	1	0	0	5	3	3	93	9
22	44	5	28	0	2	0	12	0	110	36	112	3	0	3
23	2	0	4	0	0	0	0	1	7	1	4	5	0	1
24	1	1	4	0	0	0	1	2	16	3	5	3	0	2
25	2	1	7	1	0	0	0	0	11	3	5	3	0	0
26	2	0	28	2	0	0	2	2	17	3	8	0	93	3
27	7	0	24	1	0	0	2	2	17	1	10	0	0	1
28	7	0	22	0	0	0	2	1	17	3	9	0	0	4

Table 2. The concentrations and standard deviations of steroid hormones found in 28 environmental water samples investigated using rapid ELISAs.

Data published by Swart and Pool (2007), showed that the results obtained using rapid ELISAs for estrogens gave similar results. The same authors also showed that the rapid ELISAs for estrogens gave similar results to the estrogen bioassays done using juvenile tilapia fish *in vivo* and MCF-7 cell culture (Swart *et al.*, 2011). The current study showed similar data for the estrogen rapid ELISAs using a larger data set than the previous studies. Based on this, we recommend that only one of the estrogen rapid ELISAs be done for routine screening.

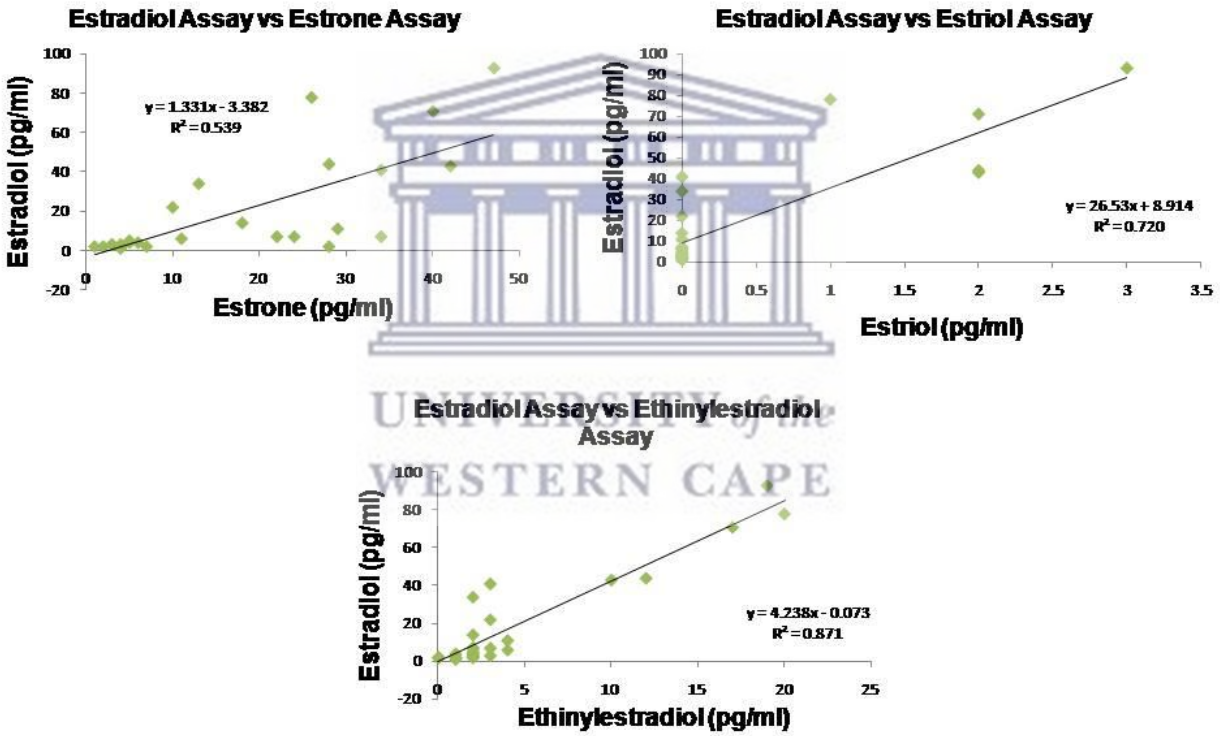


Figure 2. Correlation graphs between the estradiol ELISA and the estrone, estriol and ethinylestradiol ELISAs when rapidly screening environmental water samples for EDCs.

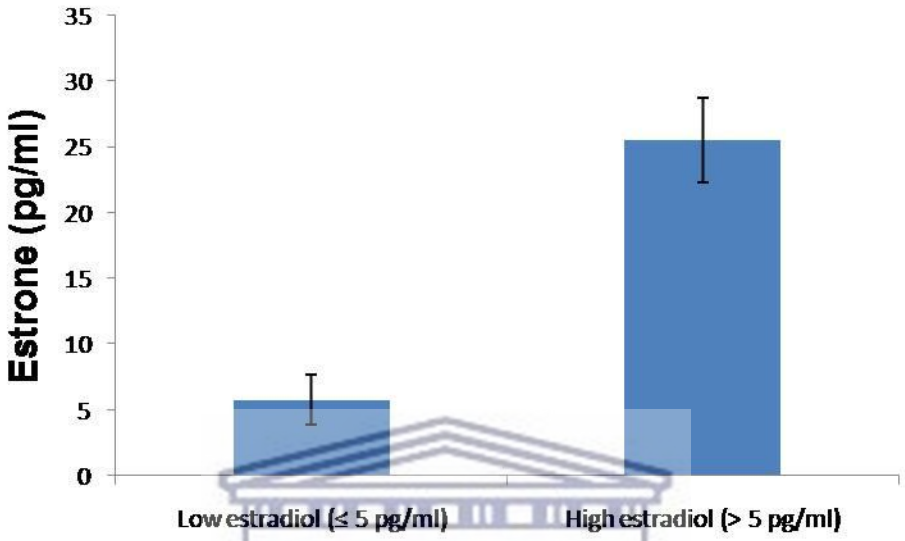


Figure 3. Bar graph of estrone levels of environmental water samples (N = 28) separated into high estradiol (> 5pg/ml; n = 15) and low estradiol (≤ 5 pg/ml; n = 13) groups. The estrone levels of the two groups are significantly different from each other (P < 0.001). Vertical bars represent the average estrone concentration, whereas error bars represent standard error of mean.

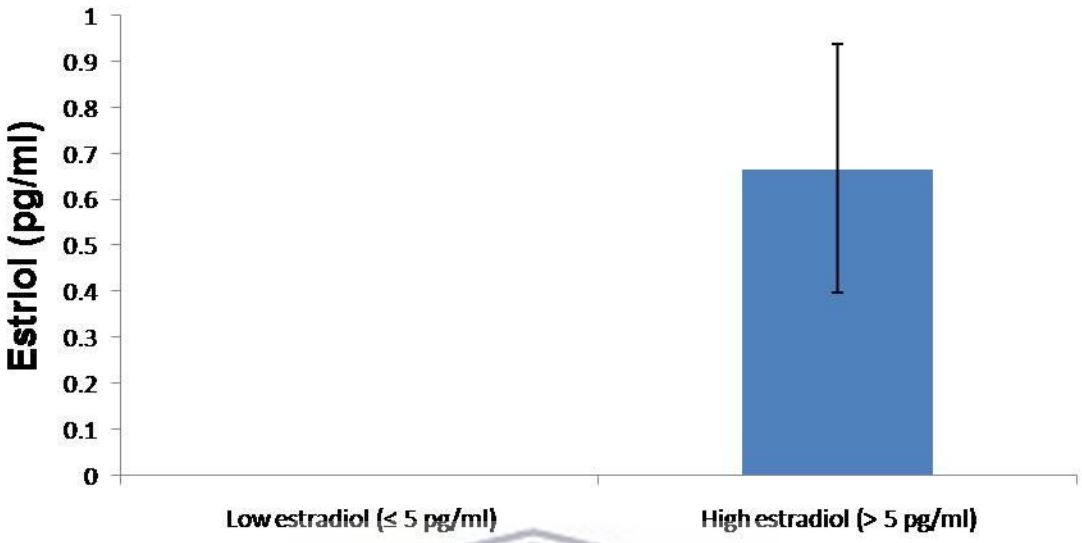


Figure 4. Bar graph of estriol levels of environmental water samples (N = 28) separated into high estradiol (> 5pg/ml; n = 15) and low estradiol (≤ 5 pg/ml; n = 13) groups. The estriol levels of the two groups are significantly different from each other (P = 0.03). Vertical bars represent the average estriol concentration, whereas error bars represent standard error of mean.

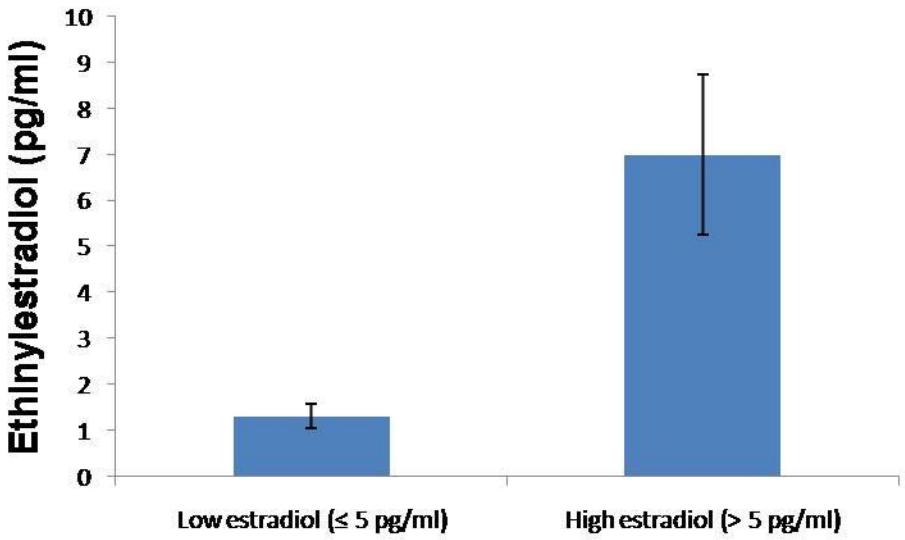


Figure 5. Bar graph of ethinylestradiol levels of environmental water samples (N = 28) separated into high estradiol (> 5pg/ml; n = 15) and low estradiol (≤ 5 pg/ml; n = 13) groups. The ethinylestradiol levels of the two groups are significantly different from each other (P = 0.006). Vertical bars represent the average ethinylestradiol concentration, whereas error bars represent standard error of mean.

3.3.3 Comparison between 17β-estradiol rapid assay and the rapid assays for other steroid hormones

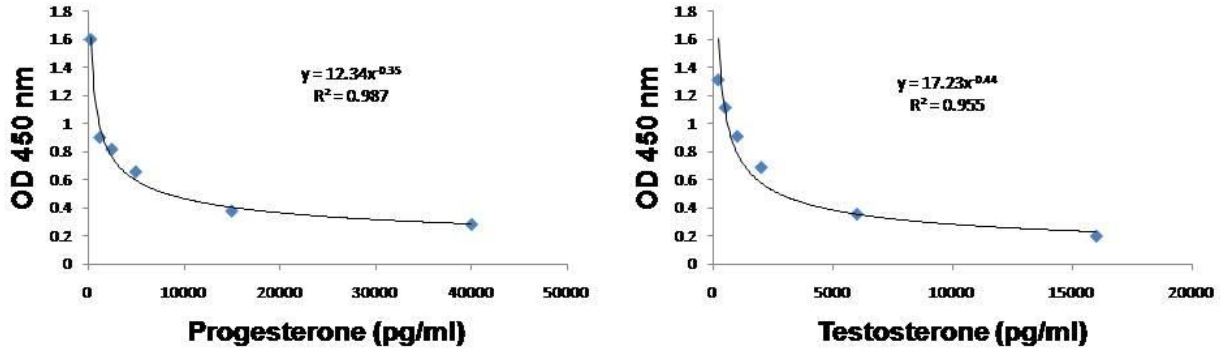
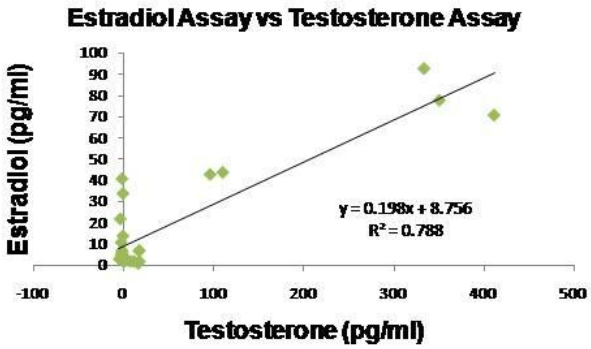


Figure 6. Standard curves for the ELISAs to determine other steroid hormone (progesterone and testosterone) concentrations in environmental water samples.

The standard curves for the ELISAs to determine other steroid hormone (progesterone and testosterone) concentrations in environmental water samples are displayed in Figure 6. The correlation coefficients (R^2) for these standard curves are 0.955 and 0.987 for testosterone and progesterone respectively. These standard curves show good inverse correlations between the optical density and the steroid hormone concentration. The testosterone and progesterone ELISAs have detection ranges between 83 - 16,000 pg/ml and 300 - 40,000 pg/ml respectively.

The current study showed that detectable levels of testosterone and progesterone are present in some of the environmental samples. The rapid ELISA kits are relatively expensive and due to this, it was investigated if estradiol concentration can be used to predict which of the high or low estradiol water samples had low or high levels of testosterone and progesterone. Figure 7 shows that there are positive correlations between the estradiol levels and the testosterone (R^2 = 0.788)

and progesterone (R^2 = 0.627) levels of the samples. The positive correlation between the estradiol and progesterone rapid assays was expected considering that both hormones are secreted and excreted by the same population sub-group (reproductively active females). The testosterone and progesterone levels of the low and high estradiol sets were then compared to see how effective the estradiol levels were at predicting high and low levels of testosterone and progesterone within the samples (Figures 8 and 9). Figure 8 shows that there is a significant difference between the progesterone levels of the group of samples with low estradiol levels and that with high levels of estradiol (P = 0.02). This indicates that separation of water samples based on estradiol levels is a good predictor of progesterone levels. This particular trend with progesterone is particularly akin to that displayed in Figures 3, 4 and 5 with the estrogens. Even though there is a positive correlation and good predictability using the estradiol ELISA, since progesterone is secreted by pregnant women, a sub-group of the reproductively active females, it is recommended to be assayed because it provides a different hormonal endpoint that can also be screened for in samples using the progesterone rapid assay. Figure 9 shows that there is no significant difference in the testosterone levels of the high and low estradiol groups (P = 0.051). This indicates that separation of samples based on estradiol levels is not a good predictor of testosterone levels. From this result, it can be concluded that a biomarker battery of tests must also include testosterone ELISA as positive and negative samples cannot be predicted by the estradiol ELISA data.



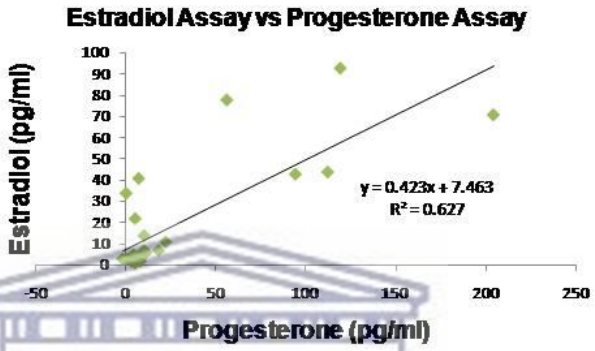


Figure 7. Correlation graphs between the estradiol ELISA and the testosterone and progesterone ELISAs when rapidly screening environmental water samples for EDCs.

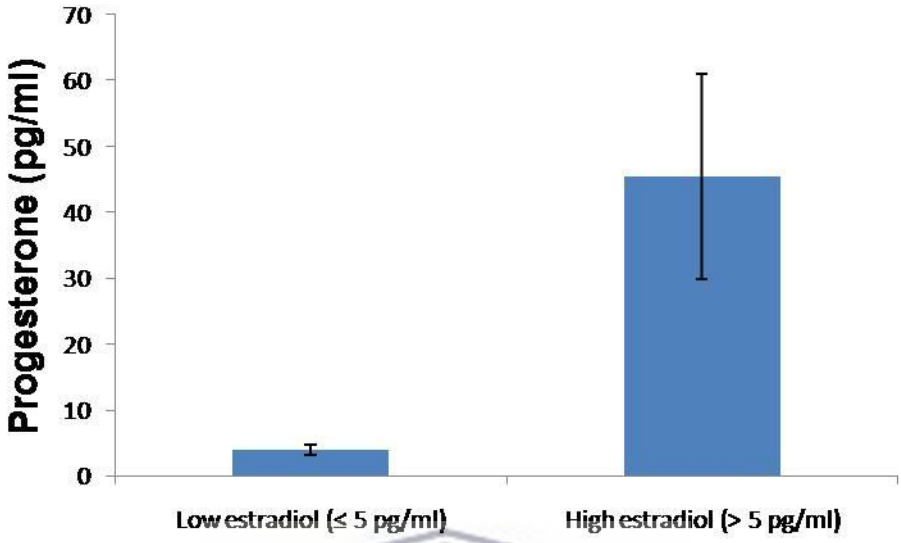


Figure 8. Bar graph of progesterone levels of environmental water samples (N = 28) separated into high estradiol (> 5pg/ml; n = 15) and low estradiol (≤ 5 pg/ml; n = 13) groups. The progesterone levels of the two groups are significantly different from each other (P = 0.02). Vertical bars represent the average progesterone concentration, whereas error bars represent standard error of mean.

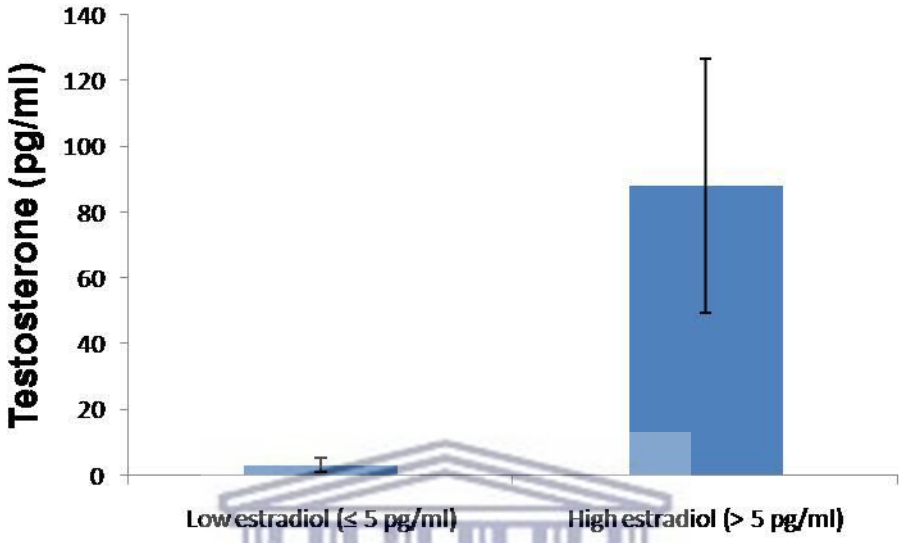


Figure 9. Bar graph of testosterone levels of environmental water samples (N = 28) separated into high estradiol (> 5pg/ml; n = 15) and low estradiol (≤ 5 pg/ml; n = 13) groups. The testosterone levels of the two groups are not significantly different from each other (P = 0.051). Vertical bars represent the average testosterone concentration, whereas error bars represent standard error of mean.

3.3.4 Comparison between 17β-estradiol rapid assay and the mouse testicular cell testosterone synthesis assay

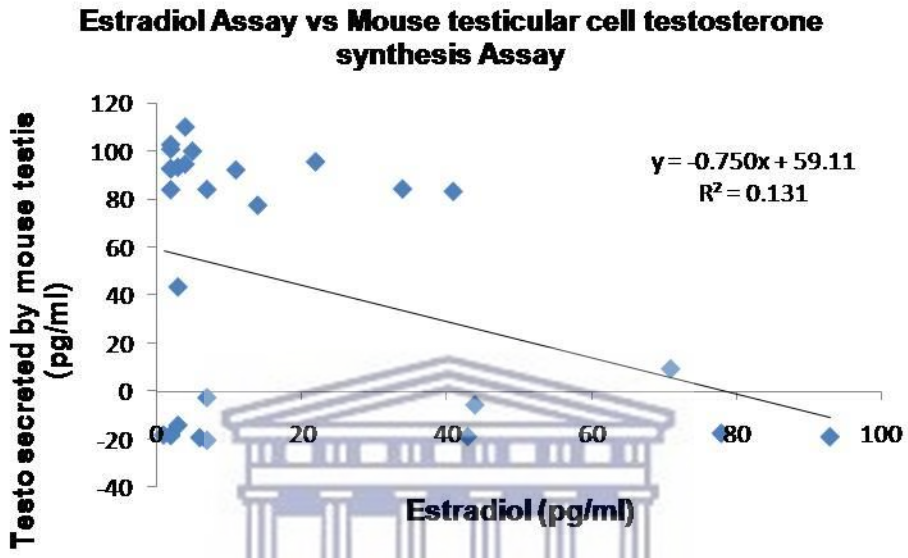


Figure 10. This graph shows a negative correlation (R^2 = 0.131) between the estradiol ELISA and the mouse testicular cell testosterone synthesis assay.

The graph in Figure 10 displays a R^2 of 0.131 between the 17β-estradiol rapid assay and the mouse testicular cell testosterone synthesis assay. This means that the 17β-estradiol rapid assay showed little or no relationship with the mouse testicular cell testosterone synthesis assay. Infact, the correlation coefficient suggests that only 13% of the variation in one of the assays is related to the variation in the other assay (only 13% of the variance is related). The current study investigates if the ELISA data for estrogens correlates with the anti-androgenic effects. This study shows that there is no correlation between the data obtained for the estrogenic compounds and the testosterone synthesis data. To determine how effective the estradiol levels were at predicting testosterone synthesized in mouse testicular cells exposed to different water samples, the group of samples containing low levels of estradiol was compared against that containing

high levels of estradiol. Results of this show no significant difference (P = 0.166) between the concentration of testosterone synthesized by mouse testicular cells exposed to low estradiol water samples and the concentration of testosterone synthesized by mouse testicular cells exposed to high estradiol water samples (Figure 11). The high variation of testosterone synthesized by testicular cells exposed to the water samples with low estradiol levels could be due to the presence of other anti-androgenic chemicals such as Bisphenol A (BPA), butyl benzyl phthalate (BBP) and dichlorodiphenyltrichloroethane (DDT). Anti-androgenic activity of the so-called 'environmental estrogens' BPA, BBP and DDT was reported by Sohoni & Sumpter (1998). Therefore, the estradiol assay is not a good predictor of the anti-androgenic effects of water samples. The anti-androgenic effects need to be monitored independently as part of an EDC monitoring battery of assays.

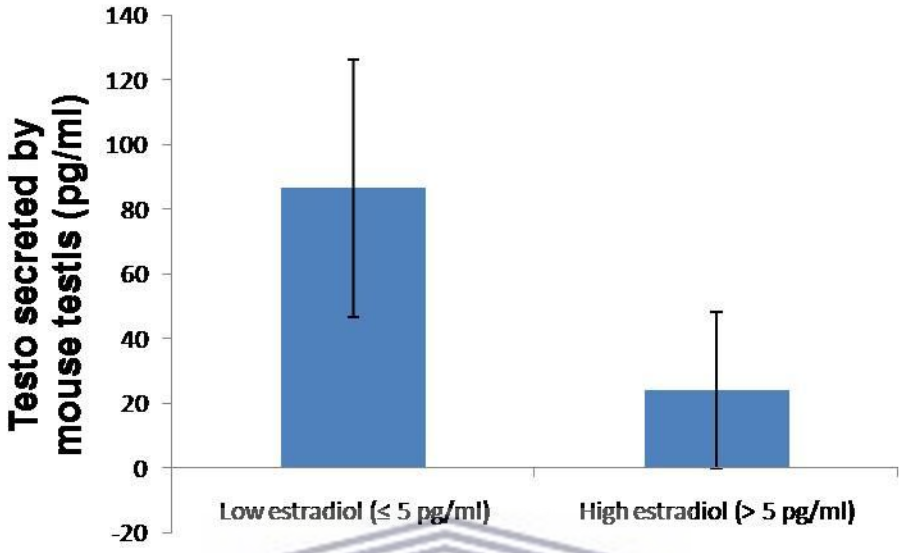


Figure 11. Bar graph of testosterone levels secreted by the mouse testicular cells exposed to low estradiol water samples (≤ 5 pg/ml; n = 13) and those exposed to high estradiol water samples (> 5 pg/ml; n = 15). The testosterone levels of the two groups are not significantly different from each other (P = 0.166). Vertical bars represent the average testosterone concentrations synthesized by the mouse testicular cells, whereas error bars represent standard error of mean.

3.4 Conclusion

Water is a very important natural resource and the availability of safe water is crucial to the life and good health of both humans and animals. However, there are major concerns that this very important natural resource is being contaminated by EDCs together with other pollutants such as pathogens and toxic chemicals (Mbazo, 2006). EDCs are not only altering normal hormone levels or activity in the body, but are also causing a number of adverse effects to human and animal health. The increased incidence of contamination of the environment with EDCs has necessitated the development of screening tools to detect and monitor EDCs (Snyder *et al.*, 2000). In this regard, the US EPA has set out to implement guidelines that can be used for monitoring and screening of EDCs.

Since *in vivo* methods for screening EDCs are time-consuming, expensive, laborious and require specialized skills and equipment, the US EPA recommends rapid screening methods as a first tier screen for EDCs. This is due to the fact that rapid screening tests are *in vitro*, relatively fast, inexpensive, simple to perform, very sensitive and can screen large numbers of samples at a time (US EPA, 2005).

The aim of this study was to investigate the comparison between chemical and tissue culture methods to monitor environmental estrogens. In order to achieve this aim, the first objective was to compare different rapid ELISAs for EDC monitoring. The second objective was to compare estradiol rapid ELISAs with a bioassay for anti-androgenicity using mouse testicular cell cultures. Both objectives were accomplished.

Environmental water samples were collected from various sites around South Africa and analyzed for EDCs using a battery of rapid *in vitro* tests.

This study confirmed earlier reports that estrogenic ELISAs are related/similar and show good correlations with each other (Swart and Pool, 2007). This is because the different estrogens are very similar and also due to the fact that the same sub-group in the population (the reproductively active females) is secreting these hormones. In an evaluation of commercial immunoassays for the detection of estrogens in water by comparison with high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS), Farré *et al.,* 2006 reported that the rapid estrogen ELISAs gave similar results to those from HPLC-MS/MS. Studies done by Swart *et al.,* (2011) showed that similar good correlations exist between the rapid estrogen ELISA and *in vivo* and *in vitro* bioassays for estrogens. Based on this good correlation, one estradiol rapid assay is recommended as a first screening system for estrogenicity in samples.

In this study, it was observed that even though there was a positive correlation between the estradiol rapid assay and testosterone rapid assay, separation of samples based on estradiol levels wasn't a good predictor of testosterone levels. It can hence be concluded that a testosterone rapid assay is necessary to screen for androgenicity in samples.

This study also indicated a positive correlation between the estradiol rapid assay and progesterone rapid assay. This result was expected because both estradiol and progesterone are secreted and excreted by the same population sub-group (reproductively active females). It also showed a good predictability of separating samples containing progesterone using the estradiol ELISA. Since progesterone is secreted by pregnant women, a sub-group of the reproductively active females, it is recommended that a progesterone rapid assay be included to screen for progestogens in samples.

Bioassay screening will help determine the endocrine disrupting potency/effects of environmental water samples and can also assess the cumulative effects of EDCs without having to quantify unknown chemicals (Bovee and Hoogenboom, 2009). The mouse testicular cell testosterone synthesis bioassay was used in this study to monitor anti-androgenicity. Anti-androgenicity is a function of the reduction of testosterone synthesis by these samples. The results for this study show no correlation between the ELISA data for estrogens and the data for testosterone synthesis. Many, but not all of the xenoestrogens possess anti-androgenic activity. Sohoni and Sumpter (1998) found that some other chemicals like BPA and DDT, which possess estrogenic activity, have been found to also possess anti-androgenic activity. By blocking androgen action, exposure to anti-androgens may cause changes similar to those associated with estrogenic exposure (Jobling *et al.*, 1995). Since the data for estrogenic compounds cannot be used as a predictor for anti-androgenic effects, the anti-androgenic effects need to be monitored independently. Therefore, there is a need to include a mouse testicular cell testosterone synthesis bioassay to screen for androgenicity and anti-androgenicity of the water samples.

Due to the different mechanisms of action of EDCs, it is favourable to use a battery of tests for monitoring (Shelby *et al.*, 1996). Based on data generated by this study, a single rapid ELISA for an estrogen (estrone, estradiol, estriol or ethinylestradiol), a progesterone and testosterone ELISAs as rapid assays plus the mouse testicular cell testosterone synthesis bioassay are recommended as part of a battery of tests for EDCs.

CHAPTER 4

Recommendations

All water resources intended for domestic, industrial, agricultural or recreational use must be routinely screened for EDCs. A US EPA report proposes a tiered strategy for screening and monitoring EDCs (Tyler *et al.*, 1998). This approach starts off with *in vitro* tests with endocrine endpoints followed by more extensive *in vivo* tests with developmental endpoints. The implementation of a battery of assays encompassing both *in vitro* and *in vivo* tests is essential for the comprehensive screening of EDCs and their effects. The implementation of the following guidelines can help mitigate the adverse health effects associated with exposure to environmental estrogens:

- Only one estrogenic ELISA is recommended in a battery of assays to monitor for environmental estrogens.
- To complete the battery of assays (in order to obtain a holistic picture inclusive of the potential effects of environmental estrogens), it is necessary to perform an estrogenic assay, an androgenic assay and an anti-androgenic assay.
- Therefore, the following assays are recommended as a first tier battery of assays for monitoring EDCs:
    - Estradiol, testosterone and progesterone ELISAs as rapid assays to screen for estrogens, androgens and progestogens respectively.

- Mouse testicular cell testosterone synthesis bioassay to screen for androgenicity and anti-androgenicity.

In order to monitor our environment, it is also recommended that further assays to comprehensively screen for EDCs be developed. It is also important that these rapid assays be affordable and use species relevant to a particular area. Very importantly, these *in vitro* assays need to be confirmed with further *in vivo* studies.

The media needs to be encouraged to highlight the growing problem of EDCs and sensitize the public. Government agencies need to implement guidelines that will safeguard the health of humans and wildlife from EDCs. Routine monitoring of water sources needs to be incorporated into standard routines by the water supply sector, to screen water for EDCs.

Lastly but certainly not least, as Marchese (2006) proposes, there are many actions we can take, both to reduce our personal risk and reduce the amount of dangerous chemicals that enter the environment to begin with. These actions comprise avoiding plastics as much as possible, eating fish low in mercury and fat, eating organic food whenever possible, using natural pest control instead of pesticides and herbicides, increasing awareness about EDCs and supporting efforts to increase EDC research and government regulation of EDCs to mention a few.

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