The development and implementation of biomarker assays for estrogenic endocrine disruptors

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in the thesis is my own original work, and that I have not previously in it's entirely or in part submitted it at any university for a degree.

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LIST OF ABBREVIATIONS

17KSR	17-ketosteroid reductase
°C	centigrade
17β-HSD	17β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase
aa	amino acids
ANOVA	analysis of variance
APEs	alkylphenol polyethoxylates
AV–HRP	avadin horse radish peroxidase
BBA	biological based assay
BPA	bisphenol A
BSA	biovine serum albumin
CB-ER	*in vitro* competitive receptor binding assay
CV	coefficient of variation
Da	Dalton
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyl trichloroethane
DES	diethylstilbestrol
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
DPF	days post fertilization
E2	17β-estradiol
EA	estrogen ligands
EDCs	endocrine disrupting chemicals
EDSP	Endocrine Disruptor Screening Program
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EDTA	ethylene diamine tetra-acetic acid
EE2	17α-ethynylestradiol
e-EDCs	estrogenic endocrine disrupting chemicals
EGF	epidermal growth factor
E-KRCA	Eerste River - Kuilsriver catchment area
ELISA	enzyme linked immunosorbent assay

EPA	Environmental Protection Agency
ER	Estrogen receptor
ERE	estrogen response element
E-Screen	MCF-7 cell proliferation
FBS	fetal bovine serum
FQPA	Food Quality Protection Act
FSH	follicle-stimulating hormone
GnRH	gonadotrophin-releasing hormone
GS	gas chromatography
GTH	gonadotropic hormones
HDL	high density protein
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal axis
HPLC	high performance liquid chromatography
HPT	hypothalamic-pituitary-thyroid
HRE	hormone response elements
IgG	immunoglobulin G
IPCS	International Programme on Chemical Safety
IQ	intelligence quotient
LC	liquid
LDH	lactate dehydrogenase
LDL	low density protein
LH	luteinizing hormone
LOEC	lowest observed effect concentration
MS	mass spectometry
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NP	nonylphenol
OD	optic density
OECD	Organization for Economic Cooperation and Development
P450c17	cytochrome P450 17α – hydroxylase/C17 – 20 lyase
P450scc	cytochrome P450 cholesterol side-chain cleavage enzyme
PBS	phosphate buffered saline
PCB	polychlorinated biphenyls

RER	in vitro recombinant receptor-reporter cell bioassay
RIA	radio immuno assay
RIPA	radio-immuno-precipitation-assay-buffer
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDWA	Safe Drinking Water Act
SHBG	sex hormone-binding globulin
StAR	steroid acute regulatory
STW	sewage treatement works
T3	triiodothyronine
T4	throxine
TBT	tributyltin
TF	transcription factor
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
TSH	thyroid stimulating hormone
VMGs	Validation Management Groups
VTG	vitellogenin
x g	gravitational force
YES	yeast based recombinant ER-reporter assay

ABSTRACT

Endocrine disrupting chemicals (EDCs) are compounds found in the environment that have the potential to disrupt normal endocrine function. Estrogenic EDCs (e-EDCs) is a subclass of EDCs and is defined as substances contaminating the environment that may mimic or inhibit the effect of endogenous estrogen and therefore may influence developmental and reproductive health in humans and animals. The aim of this study was to develop, validate and implement a battery of *in vitro* and *in vivo* screening assays for e-EDCs.

The first objective of this study was the validation of commercially available Enzyme linked Immunosorbent Assay (ELISA) kits (designed for hormone quantification in human serum) to detect natural estrogenic hormones. These ELISA kits were validated to quantify estrone, estriol and 17β-estradiol (E2) in complex environmental water extracts. These ELISAs were proved to be very sensitive and reliable with detection limits of 0.3 ng/ml, 15 pg/ml and 25 pg/ml for estriol, estrone and E2 respectively. The ELISAs showed inter- and intra assay variations of less than 10 %.

The second objective was the development of a multi biomarker whole cell bioassay that can be used as a rapid, sensitive and inexpensive screen for total environmental estrogenic activity as well as cytotoxicity. The human breast cancer cell line, MCF-7 was used to develop this assay. Total lactate dehydrogenase (LDH) activity, total protein yield and mitochondrial activity (XTT activity) were evaluated as proliferation biomarkers for estrogenic endocrine disruption. The current study shows that the total cellular LDH assay is the most sensitive assay for E2 dependent proliferation monitoring. The MCF-7 total LDH assay can be used to detect estrogens over the range from 0.1-1000 nM E2. It was also showed that the well know E-screen assay can be performed in culture medium containing serum replacement factor instead of fetal bovine serum.

The third objective of this study was to develop a direct ELISA on cultured MCF-7 cells to quantify ERα levels as a biomarker for estrogenicity. This biomarker had a detection range between 0.1 - 1000 nM for E2. ERα levels were suppressed in the presence of estrogen. Results obtained with the ERα ELISA for estrogenicity showed a good correlation with the total cellular LDH assay for estrogenicity in MCF-7 cells. This ELISA was successfully employed to assess environmental water extracts for estrogenicity.

The forth objective of this study was to setup and validate a competition ELISA to quantify vitellogenin (VTG) from *Oreochromis mossambicus* (tilapia). A competition ELISA for tilapia VTG using commercially available antibodies was developed. This tilapia VTG competition ELISA has a broad detection range between 80 ng/ml – 5.4 µg/ml VTG and is able to detect *in vitro* and *in vivo* synthesized VTG.

The fifth objective of the study entailed using this quantitative ELISA to validate the use of tilapia juveniles (less than 70 days post fertilization (DPF)) as a short term *in vivo* screen for estrogenicity. Tilapia juveniles between 35 and 45 (DPF) exhibited the maximum ability to induce VTG upon estrogen exposure. At this age, 200 ng/l of diethylstilbestrol (DES) was the lowest concentration of estrogen that was able to induce significant VTG levels after 10 days of exposure. Analysis of the natural VTG levels of a mixed sex population of juveniles at 5 day intervals between 15 and 70 DPF showed a peak at 50 DPF which coincided with a peak in natural whole body homogenate E2 concentrations.

This study was concluded by implementing this battery of assays to assess the Eerste River, South Africa at three sampling sites, namely Jonkershoek, Stellenbosch sewage treatment works (STW) effluent and Spier for e-EDCs. The control site, Jonkershoek contained very low levels of estrone. Water from this site showed no estrogenic activity when the E-screen and the ERα induction in MCF-7 cells. Some of the water samples collected at this site tested positive for estrogenicity when analysed with the juvenile tilapia VTG assay, whereas the rest were negative. The estrone levels in the sewage effluent extracts as well as Spier were significantly higher. The assay using ERα protein induction by the MCF-7 cell line, the MCF-7 proliferation assay and the tilapia *in vivo* screen for estrogenicity showed that these samples are estrogenic. Results obtained for estrogenicity at the three different sampling sites for each of the assays in the battery were comparable.

In this study we developed, validated and also implemented a battery of assays encompassing both *in vitro* and *in vivo* assays, based on different biological mechanisms, to detect estrogenic EDCs. To our knowledge, this is the first study that has used a battery of bio-assays to specifically assess a South Africa river for estrogenicity.

CHAPTER 1

1.1. Background

During the past few decades, a rapid increase in the number of reproductive and developmental deviations has been observed in humans and wildlife exposed to environmental compounds (WHO/IPCS, 2002). This observation has heightened the concerns about the potential of some xenobiotics to interfere with normal endocrine function (Kavlock, 1996). Endocrine disrupting chemicals (EDCs) define a broad group of substances that are found in the environment and have the potential to elicit negative effects on the endocrine systems of human and wildlife. The US Environmental Protection Agency (EPA) defines EDCs as: An exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour (USEPA, 1997).

Most of the documented adverse health effects caused by EDCs in humans and wildlife are due to estrogenic EDCs (e-EDCs) [Gellert 1978a; Gellert 1978b; Hammond et al., 1979; Kupfer and Bulger, 1987; Krishnan et al., 1993; White et al., 1994; Newbold 1995; Katzenellenbogen 1995; Nimrod and Benson, 1996]. This broad class of chemicals includes both natural and synthetic estrogens. Some specific examples of e-EDCs include pesticides like atrazine, dieldrin and toxaphene (Ramamoorthy et al., 1997; Hayes et al., 2002). Surfactants such as alkylphenol-ethoxalates (Folmar et al., 2002; Legler et al., 2002a; Ying et al., 2002) are other major source of e-EDCs in the environment. Natural hormones and pharmaceutical estrogens such as 17β-estradiol and 17α-ethynylestradiol (Folmar et al., 2000; Legler et al., 2002a) are also regarded as e-EDCs. Phytoestrogens including isoflavonoids and coumestrol (Bacaloni et al., 2005; Stopper et al., 2005) as well as other industrial compounds for example bisphenol A (Mocarelli et al., 1996; Ramamoorthy et al., 1997; Howdeshell et al., 1999) are all e-EDCs. Many of theses e-EDCs have the potential to influence normal endocrine function at very low concentrations (parts per billion to parts per trillion) and as a result may cause adverse health effects in humans and wildlife (Campbell et al., 2006). The concern is that e-EDCs have been found in wastewater, surface waters, sediments, groundwater and even drinking water (Benfenati et al., 2003; Petrović et al., 2003; Snyder et al., 2003; Petrović et al., 2004).

For this reason the development of new assays and screens to monitor e-EDCs in our environment has received considerable attention over the last few decades. The US EPA has been given the mandate, under the Food Quality Protection Act of 1996, to assess the endocrine disruptive activity of over 87,000 chemicals (US EPA, 1998). To this end, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) were formed to make recommendations to the EPA on how to develop screening strategies. EDSTAC presented its final report in 1998, laying out a recommended approach to the problem (US EPA, 1998). This program's aim is to develop a two-tiered approach, e.g. *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for the identification and characterization of endocrine effects of pesticides, industrial substances and environmental contaminants.

The aim of this study was to develop and validate a battery of screening assays encompassing both *in vitro* and *in vivo* assays to detect e-EDCs. This study was concluded by implementing this battery of screening assays to assess the Eerste River, South Africa for environmental e-EDCs.

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CHAPTER 2

Literature Review

2.1 Introduction to EDCs

During the last few decades, international concern has been raised regarding the possible harmful effects of exposure to certain chemicals in the environment. This concern has initially centred on chemicals that may mimic the action of natural female hormones and were thus termed "environmental estrogens" (Allen and Doisy, 1923; Burlington and Lindeman, 1950). However, this issue has since broadened and now encompass various groups of chemicals with the ability to interact and disrupt normal animal and human wellbeing on cellular, metabolic and physiological level (Colborn et al., 1993). Some chemicals can also affect the progeny of previously exposed parents (Colborn et al., 1993). Moreover, every year hundreds of newly developed chemicals are released into the environment. The toxicity and health effects of these chemicals on human and animal wildlife are unknown (Rodriquez-Mozaz et al., 2004). These chemicals are now referred to as endocrine disrupting chemicals or EDCs. EDCs represent a wide range of chemicals that are found in the environment and have the potential to elicit negative effects on the endocrine systems of humans and wildlife. Various definitions have been proposed for EDCs. The US Environmental Protection Agency (EPA) defines EDCs as: an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour (USEPA, 1997).

Some EDCs have structures similar to the natural hormone that they mimic or inhibit, while other EDCs have no resemblance at all. Therefore, EDCs belong to a class of substances which is defined by biological effect rather than chemical nature. For instance, various natural and synthetic chemical compounds including pharmaceuticals, pesticides, industrial chemicals and heavy metals have been identified to induce estrogen-like responses (Giesy et al., 2002). A wide spectrum of pollutants can therefore collectively be referred to as estrogenic EDCs (e-EDCs) (Lopez de Alda and Barcelo, 2001). Because of the vast amount of literature available on EDCs, this chapter will only focus on e-EDCs.

2.2 Estrogenic EDCs

Estrogenic endocrine disrupters are ubiquitously found throughout the environment and in broad terms are grouped into two major groups (Keith, 1998; Baker, 2001). The one group consist of natural compounds such as phytoestrogens, bioflavanoids and mycoestrogens that

are naturally found in the environment (Kuiper et al., 1998; Giesy et al., 2002). The other group consist of synthetic compounds that are designed for several uses such as pesticides, herbicides, plastics etc., which is now suspected to unintentionally disrupt endocrine systems of humans and wildlife (Kuiper et al., 1998). This group also includes synthetic hormones such as contraceptives that are designed to intentionally disrupt the endocrine system (Isselbacher et al., 1994). EDCs can be found in soil, water, air and food as well as in commonly used industrial and household products (WHO/IPCS, 2002). Giesy et al, (2002) compiled a list of natural (Table 2.1) and synthetic compounds (Table 2.2) that are capable of disrupting the endocrine system. This list of EDCs includes:

- Natural products (e.g. Phytoestrogens, Bioflavanoids, Mycoestrogens)
- Pharmaceuticals (e.g. Ethynylestradiol, Flutamide)
- Additives (e.g. Parabenes)
- Insecticides (e.g. Endosulfan, Dieldrin, Lindane)
- Fungicides (e.g. Vinclozolin)
- Herbicides (e.g. Atrazine)
- Industrial chemicals (e.g. Bisphenol A, Phthalates, PCBs)
- Heavy metals (e.g. Lead, Cadmium)

Some of the best-known man-made endocrine disrupters are products that most of us use every day. Specific examples of EDCs include synthetic hormonal drugs, such as birth control pills and diethylstilbestrol (DES), dioxins (by-product of waste incineration and industrial processes e.g., the production of some pesticides) as well as the bleaching of paper pulp (Huisman et al., 1996; Colton and Greenberg, 1993). Polychlorinated biphenyls (PCBs), which were once used world wide in electrical equipment, adhesives and plasticizers (Jacobson and Jacobson, 1996), is another example of EDCs. Other well known synthetic compounds that disrupt hormone activity are alkylphenol and phtalates, which are widely used in industrial and household products and some pesticides, fungicides and insecticides (Sonnenschein and Soto, 1998). Phytoestrogens occur naturally in the environment and are present in grains, legumes, herbs, nuts and a variety of fruits and vegetables (Cassidy, 1988; Barnes, 1998). Phytoestrogens induce a weaker estrogenic response than the endogenous estrogens (i.e., they do not bind as well to hormone receptors) and are quickly excreted or broken down into other compounds and do not accumulated in the body.

EDCs that contaminate the aquatic environment originate from point and non-point sources. Point source refers to specific sources of contamination for instances sewage treatment plants, pulp mills and industrial effluent. Non-point refers to urban and agricultural runoffs (Folmar et al., 2002). Both of these two sources may include natural and pharmaceutical chemicals from domestic, human and animal origin. Waste water treatment facilities have been widely identified as a major source of e-EDCs (Sumpter, 1995a; Kolpin et al., 2002; Legler et al 2002a) and are classified as a point source of contamination. The actual sources of contamination are upstream discharges that end up in these treatment facilities. A few examples of the upstream sources include natural hormones and pharmaceutical estrogens which are flushed down home toilets. Household cleaning products containing nonylphenol (NP), industrial processes that use cleaning reagents containing NP, plastics containing bisphenol A (BPA) or agrochemicals containing alkylphenol and nonylphenol ethoxylate surfactants are all sources of e-EDCs (Staples et al., 1998; Ying et al., 2002 a,b; Snyder et al., 2003). Wastewater treatment facilities serve as a focal point where treatment is possible if source mitigation is impractical (e.g. removal of e-EDCs from product formulation or reducing pharmaceutical estrogens in household waste). Agricultural activity has been identified as a nonpoint sources for e-EDC which includes waste waters from dairies and aquaculture (Kolodziej et al., 2004). The spawning of fish in aquaculture farms may locally increase the concentrations of estrogens in river water (Kolodziej et al., 2004). Livestock feed lots have previously also been demonstrated to be potential sources of estrogenic compounds from excretion of hormones in manure and urine (Hanselman et al., 2003; Tashiro et al., 2003; Soto et al., 2004). Another potential source of e-EDCs from agricultural activity is runoffs containing pesticides and fertilizers which may contain estrogenic surfactants (e.g. nonylphenol ethoxylates) that make up the chemical formulation (Staples et al., 1998; Ying et al., 2002).

In order to have a better understanding of how EDCs may inflict an estrogenic response in humans and wildlife, it is necessary to briefly review the basics of endocrinology and the pathways involved in estrogen biosynthesis.

Table 2.1. Examples of endocrine disrupting compounds: natural products (Giesy et al., 2002)

Compound	Mode of action	Assay	Reference
Phytoestrogens			
Indole-3-carbinol	ER agonist.	RER (MCF-7-luc), YES.	Villeneuve et al., 1998; Gaido et al., 1997a
β-Sitosterol	ER agonist, androgenic after metabolization.	YES, *in vivo* fish.	Gaido et al., 1997a; Stahlschmidt- Allner et al., 1997
Coumestrol	ER agonist.	RER (MCF-7-luc), YES.	Villeneuve et al., 1998; Gaido et al., 1997a
		In vitro ER mediated PAP induction.	Markiewicz et al., 1993
Enterolactone, enterodiol	Decrease aromatase enzyme activity.	*In vitro* human cell culture system.	Wang et al., 1994
Bioflavanoids			
Genistein	ER agonist.	RER (ER-CALUX).	Markiewicz et al., 1993; Legler et al., 1998
	Estrogenic.	*In vitro* and *in vivo* vitellogenin production.	Nimrod and Benson, 1997
Biochanin A, daidzein, equol	ER agonists, estrogenic.	*In vitro* and *in vivo* vitellogenin production.	Nimrod and Benson, 1997
		In vitro ER mediated PAP induction.	Markiewicz et al., 1993
Quercetin, naringenin, luteolin apigenin, chrysin,	Estrogenic, antiestro-genic, ER agonists.	CB-ER, RER, RER (MVLN).	Kuiper et al., 1998, Safe and Gaido, 1998; Le Bail et al., 1998
Mycoestrogens			
Zearalenone	ER agonist.	CB-ER, RER, VTG *in vitro.*	Kuiper et al., 1998; Celius et al., 1999

YES, yeast based recombinant ER-reporter assay; E-screen, MCF-7 cell proliferation; CB-ER, *in vitro* competitive receptor binding assay; RER, *in vitro* recombinant receptor-reporter cell bio-assay; VTG-*in vitro*, *in vitro* vitellogenin synthesis in cultured male trout hepatocytes.

Table 2.2. Examples of endocrine disrupting compounds: synthetic compounds (Giesy et al., 2002)

Compound	Mode of action	Assay	Reference
Pharmaceuticals			
Flutamide	Anti-androgenic activity.	YES.	Sohoni and Soto, 1998
Tamoxifen	Anti-estrogenic drug binding to ER, antagonist.	*In vitro* cell line tests, *in vivo* E-screen and other effects.	Taylor et al., 1984; Ramkumar and Alder, 1995; Shelby et al., 1996; Favoni and Cupis (1998)
Hydroxytamoxifen	Anti-estrogenic.	YES, E-screen and other effects.	Sohoni and Soto, 1998; Favoni and Cupis, 1998
Nafoxidine, clomiphene	ER agonist.	YES.	Gaido et al., 1997a
Ethynylestradiol	ER agonist.	*In vitro, in vivo*.	Nimrod and Benson, 1997; Stahlschmidt-Allner et al., 1997
Additives			
Parabens	ER agonists.	CB-ER, YES, *in vivo* uterotrophic response.	Routledge et al. (1998)
t-Butylhydroxyanisol	Estrogenic.	E-screen.	Soto et al., 1995
Insecticides			
o,p´-DDT	ER agonist, anti-androgenic activity.	YES, RER (ER-CALUX), VTG-*in vitro*.	Sohoni and Soto, 1998; Gaido et al., 1997a; Legler et al., 1998; Sumpter and Jobling, 1995
o,p´-DDD, o,p´-DDE	ER agonists.	YES.	Gaido et al., 1997a
p,p´-DDE	Androgen receptor antagonist, weak ER and androgen receptor	CB-androgen receptor, *in vivo* mice study.	Kelce et al., 1995
	agonist. Anti-adrogenic and weak anti-estrogenic activity.	YES.	Sohoni and Soto, 1998
p,p´-DDD	ER agonist.	YES, CB-ER, RER (MCF-7-luc).	Klotz et al., 1996
p,p´-DDT	ER agonist, estrogenic.	E-screen.	Nimrod and Benson, 1997
Kepone	ER agonist, estrogenic after metabolization.	RER (ER-CALUX), E-screen, *in vitro* and *in vivo.*	Legler et al., 1998; Nimrod and Benson, 1997; Shelby et al., 1996.
Endosulfan, Dieldrin, Lindane	ER agonist.	RER (ER-CALUX).	Legler et al., 1998

Table 2.2 ***Continued***

Compound	Mode of action	Assay	Reference
Toxaphene	Estrogenic.	E-screen.	Soto et al., 1994
Methyl parathion	Estrogenic.	YES, VTG - *in vitro.*	Petit et al., 1997
		In vivo effects on estrus cycle in mice.	Asmathbanu and Kaliwal, 1997
Chlordecone	Estrogenic.	YES, VTG - *in vitro.*	Petit et al., 1997
Chlordane	ER agonist.	RER (ER-CALUX).	Legler et al., 1998
		In vivo - effects on endocrine function in mice.	Cranmer et al., 1984
Methoxychlor	ER agonist – after metabolization.	RER (ER-CALUX) *in vitro* and *in vivo.*	Legler et al., 1998; Shelby et al., 1996
Carbamate insecticides (Aldicarb, Bendiocarb, Cabaryl, Methomyl, Oxamyl)	Endocrine modulators, non-ligand binding.	*In vitro* modulation of estrogen and progesterone receptor in human breast and endometrial cancer cells.	Klotz et al., 1996
Pyrethroid insecticides (Sumithrin, Fenvalerate, D-trans Allethrin, Permethrin)	Estrogenic (different mechanisms).	*In vitro* pS2 gene expression E-screen.	Go et al., 1999
Fungicides			
Vinclozolin	Anti-androgen.	*In vitro* androgen receptor binding assay, YES.	Kelce et al., 1994; Sohoni and Soto, 1998
Dodemorph, Triadimefon, Biphenyl	Estrogenic.	YES, VTG *in vitro.*	Soto et al., 1994
Herbicides			
Atrazine	Anti-estrogen	RER (MCF-7-luc), *in vivo.*	Villeneuve et al., 1998
Simazine	Anti-estrogen.	*In vivo.*	Tennant et al., 1994

Table 2.2 ***Continued***

Compound	Mode of action	Assay	Reference
Alachlor,Nonachlor, Tributyltins	ER agonists, Androgenic.	YES, CB-ER, RER (MCF-7-luc)	Klotz et al., 1996; Stahlschmidt-Allner et al., 1997; Matthiessen and Gibbs, 1998
Industrial chemicals			
Phthalates Butylbenzylpthalate	ER agonist, antiandrogenic activity.	*In vitro* and *in vivo*, E-screen, YES.	Sohoni and Soto, 1998; Stahlschmidt-Allner et al., 1997; Soto et al., 1995; Jobling et al., 1995
Dibutylpthalate	ER agonist.	*In vitro* and *in vivo.*	Stahlschmidt-Allner et al., 1997; Jobling et al., 1995
Alkylphenols Nonylphenol	ER agonist, estrogenic.	RER (MCF-7-luc, ER-CALUX), YES, number of *in vitro* and *in vivo* assays, E-screen, VTG- *in vitro.*	Villeneuve et al., 1998; Gaido et al., 1997a; Legler et al., 1998; Servos, 1999; Nimrod and Benson, 1997; Shelby et al., 1996; Soto et al., 1995
Octylphenol	ER agonist.	RER (MCF-7-luc). Number of *in vitro* and *in vivo* assays.	Servos, 1999; Soto et al., 1995
Butylphenol, Pentylphenol	Estrogenic.	E-screen.	Nimrod and Benson, 1997; Soto et al., 1995
Nonylphenol, polyethoxylates, and polyethoxycarboxylates	ER agonists.	Number of *in vitro* and *in vivo* assays.	Servos, 1999
Pentachlorophenol	Decrease in blood testosterone concentration.	*In vivo* ewes feeding study.	Beard et al., 1999
Bisphenol A	ER agonist.	RER (MCF-7-luc, ER-CALUX), YES, VTG *in vitro.* YES.	Villeneuve et al., 1998; Gaido et al., 1997a; Legler et al., 1998; Soto et al., 1995.

Tabel 2.2 ***Continued***

Compound	Mode of Action	Assay	Refernce
PCBs	ER agonists or antagonists or other mechanism - depending on the substitution.	RER (transient MCF-7-luc), E-screen, *in vivo* - vaginal cell cornification in mice.	Joyeux et al., 1997; Soto et al., 1995
Arochlor 1260 (PCBs mixture), Arochlor 1260	Estrogenic, effect on sexual differentiation, gonadal abnormalities.	VTG *in vitro*, *in vivo* trout study.	Soto et al., 1995; Matta et al. 1998
Hydroxy-PCBs	ER agonists or antagonists.	RER (MCF-7-luc), E-screen, CB-ER, *in vivo* - vaginal cell cornification in mice.	Joyeux et al., 1997; Soto et al., 1995; Beard et al., 1999; Kramer et al., 1997
PAHs	ER agonists - estrogenic, Anti-estrogenic - different mechanisms.	YES, E-screen RER (MCF-7-luc).	Tran et al., 1996a; Chaloupka et al., 1992; Santodonato, 1997; Clemons et al., 1998.
6-hydroxy chrysene	Anti-estrogenic.	YES.	Tran et al., 1996b
Heavy metals			
Cations of cadmium, cobalt, copper, mercury, nickel, zinc	Depression or increases in testosterone production.	*In vitro* substrate stimulated testosterone production by Leydig cells.	Laskey and Phelps, 1991
Cadmium	Decrease in plasma testosterone and cortisol Modification of pituitary hormone secretion.	*In vivo* juvenile rainbow trout exposure.	Ricard et al., 1998
		In vivo rat feeding exposure.	Lafuente et al., 1997
Lead	Delayed sexual maturation, suppression of sex steroid biosynthesis.	*In vivo* rat feeding study.	Ronis et al., 1998

2.3 General Endocrinology

The endocrine system is also referred to as the hormone system. It regulates chemical signalling and gene expression from the time of conception, through development and maturation. The endocrine system is one of three very important control systems in vertebrates. The nervous system and the immune system are the other two control systems. Endocrine systems are found in mammals, non-mammalian vertebrates (e.g., fish, amphibians, reptiles and birds), and invertebrates (snails, lobster, insects and other species). In vertebrates the function of the endocrine system involves the regulation of a wide range of biological processes. Vertebrate endocrine systems consist of three major axis, namely the hypothalamic-pituitary-thyroid (HPT), hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axis. These three axes are responsible for a multitude of biological processes for example blood sugar level stability, which is regulated through the hormone insulin secreted from the pancreas. Metabolism is regulated by the hormones cortisol from the adrenal glands and thyroxin from the thyroid gland. The development and functioning of the reproductive systems is regulated by the hormones estradiol and testosterone and related compounds from the testis and ovaries. The development of the brain and the rest of the nervous system are also regulated through estrogen as well as thyroid hormones. The endocrine system regulates the development and sustainment of an organism from conception through adulthood and old age. The normal function of the endocrine system therefore enables animals to control and regulate reproduction, development and behaviour (Payne and Dale, 2004).

2.3.1 Overview of the HPG Axis in mammals

The HPG axis (Fig. 2.1) involves three major components. The first component is gonadotrophin-releasing hormone (GnRH) neurons projecting from the hypothalamus of the brain. The second major component is the secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by gonadotropes in the anterior pituitary gland (adenohypophysis). The third component is the somatic cells of the gonads (theca and granulosa cells in the ovary, Leydig and Sertoli cells in the testis). GnRH is secreted in pulses by the GnRH neurons (Kimura and Funabashi, 1998; Terasawa, 1998) and acts on the gonadotropes to release LH and FSH. These two gonadotropins enter the blood stream where they then act on their respective target cells in the gonads (LH on theca/Leydig cells; FSH on granulosa/Sertoli cells). The secretion of GnRH can be modified by other neurons (Crowley,

1999), and the actions of GnRH on gonadotropin release may be modified by other hypothalamic or pituitary peptides (Evans, 1999). Consequently, gonadal sex steroids

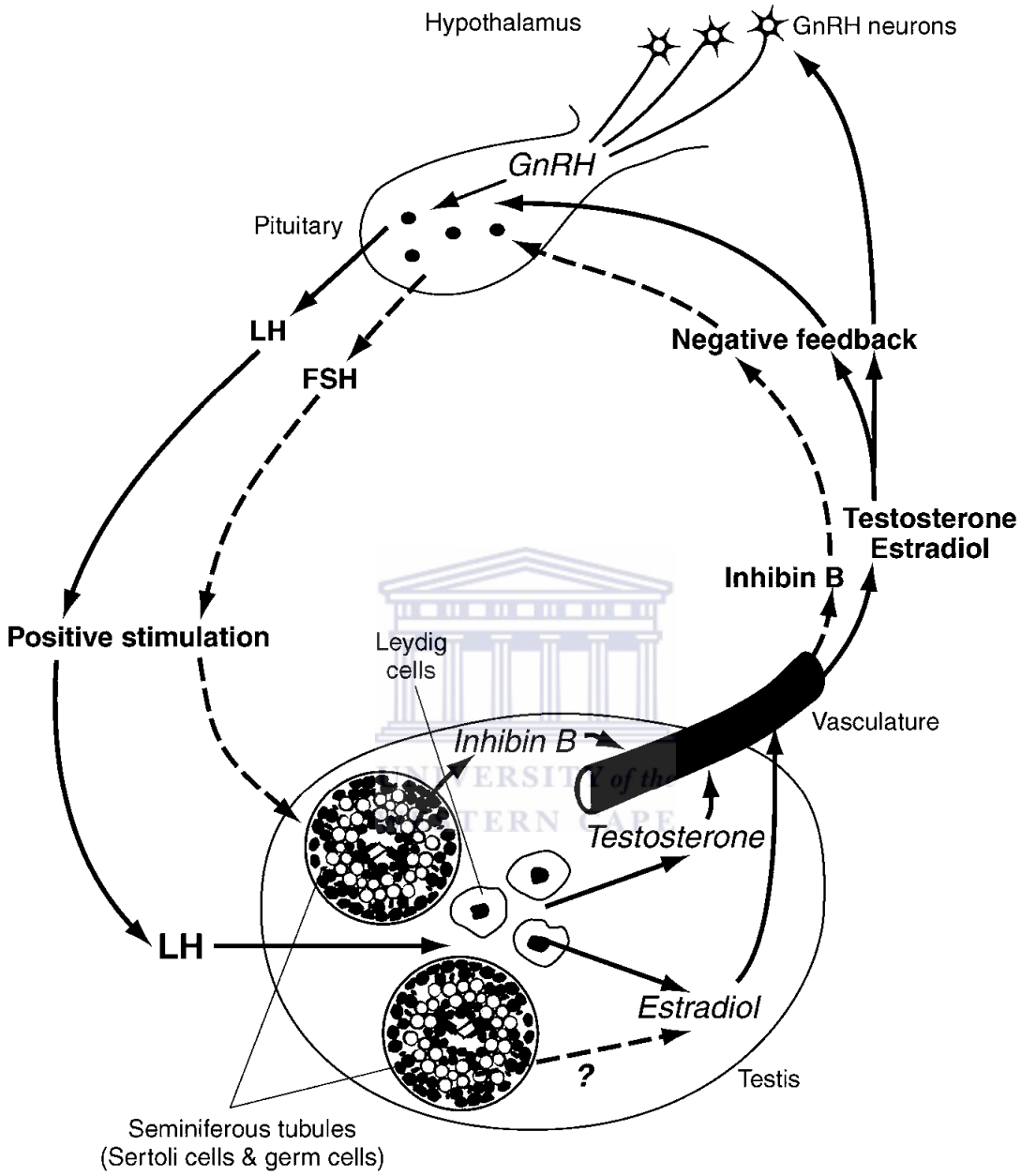

Figure 2.1 Diagrammatic representation of the main components of the mammalian HPG axis (IPCS Global Assessment of EDCs).

stimulated by LH and the protein hormone inhibin (the A form in females, the B form in males) stimulated by FSH are released back into the bloodstream. Inhibin and the sex steroids provide feedback to the hypothalamus and pituitary gonadotropes to reduce the secretion of GnRH, LH and FSH (Crowley et al., 1991). Figure 2.1 is a simple diagrammatic representation of this system.

Nonmammalian vertebrates differ from mammals and also one another in their modes of reproduction, with patterns of sequential and simultaneous hermaphroditism, parthenogenesis, viviparity and gonochorism found in many species (van Tienhoven, 1983). The HPG axes of different animals are surprisingly similar in their operation, in the pattern of feedback mechanisms, and in the hormones involved to that described for mammals (Norris, 1997; Bentley 1998). Differences in the HPG axes in nonmammalian species will however not be discussed in this section.

2.3.2. Steroidogenesis

In order to understand how estrogenic xenoestrogens may disrupt normal endocrine function, it is necessary to give a brief overview of how steroid hormones are synthesized using mammals as model. Steroidogenesis is a complex process which involves the conversion of cholesterol into biologically active steroid hormones.

2.3.2.1. Sites of steroidogenesis

The gonads, adrenals and the brain are the major centres for steroid hormone biosynthesis (Kime, 1987; Nagahama, 2000; Schumacher et al., 2003). The male steroidogenic pathways are found mainly in the testis and to a much lesser extend in the adrenal glands. Within the testes, steroidogenesis occurs in the Leydig cells (Chen et al., 1996). Several other peripheral tissues are involved in testosterone's role as a pro-hormone (Fig 2.2). Examples of testosterone as a pro-hormone is for instance in the liver and brain (hypothalamus) where its converted to estradiol and in the liver, brain, prostate and external genitalia where it is converted to dihydrotestosterone. The biosynthesis of female steroid hormones occur in the ovary (Carr and Wilson, 1994). Several cell types participate in estrogen synthesis within the ovary. These are the follicular-, theca-, interstitial-, and luteal cells. Different cell types have the ability to synthesize different amounts of a specific steroid hormone. This is a result of the fact that the different cell types contain varying amounts of a given steroidogenic enzyme.

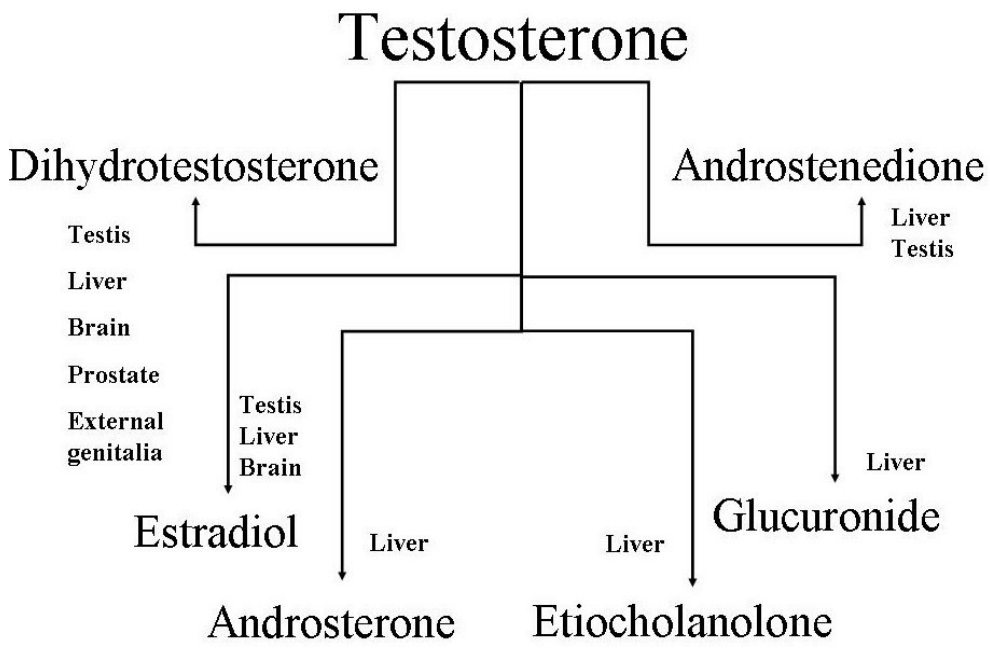

Figure 2.2. Testosterone conversion in peripheral tissue (Federman, 1981).

2.3.2.2. Steroidogenic enzyme reactions

The common precursor for biosynthesis of all gonadal steroid hormones is cholesterol (Miller, 1988). The primary source for cellular cholesterol is serum. Cholesterol is transported to the cell by the serum protein carriers, high density lipoproteins (HDL) and low density lipoproteins (LDL). Another source of cholesterol is *de novo* synthesis through acetate. Once cholesterol enters the cell, it can be stored, e.g. lipid droplets or immediately utilized. Upon LH-induced stimulation, mobilization of newly synthesized and stored cholesterol (through enzymatic hydrolysis of cholesterol esters) in lipid droplets occurs. Cholesterol is then transported from the cytosol to the mitochondria. Within the mitochondria cholesterol is transported from the outer to the inner membrane. The transport of cholesterol across the mitochondrial membranes requires a transport protein and this is the rate limiting step in steroid biosynthesis. Upon LH stimulation of steroidogenic cells, these cells initiate the *de novo* synthesis of the cholesterol transport protein. Since this cholesterol transport protein mediate the rate limiting step in steroidogenesis, it is referred to as the steroid acute regulatory (StAR) protein (Stocco and Clark, 1996). StAR protein is rapidly synthesized, cycloheximide-sensitive (dependent on *de novo* protein synthesis), and highly labile (short-life). The StAR protein is synthesized in the cytoplasm as a precursor molecule from where it is transported to the mitochondria and cleaved to its active form. In the mitochondria, the StAR protein is responsible for the transport of cholesterol to the inner mitochondrial membrane, where the

first cytochrome P450 enzymatic conversion of cholesterol takes place (Stocco and Clark, 1996).

The enzymatic conversion of cholesterol to pregnenolone is the first step in a series of enzymatic conversions that culminate in the production of hormones. Figure 2.3 illustrates the enzymatic pathway for the conversion of cholesterol to steroid hormones. The first enzymatic reaction in this final stage of steroidogenic biosynthesis is mediated by cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) to convert cholesterol to pregnenolone (Boyd and Simpson, 1968; Burstein and Gut, 1976). P450scc activity is also regarded as a rate-limiting step of steroid biosynthesis. This reaction occurs in the mitochondrial inner membrane and involves three sequential oxidation reactions, each requiring molecular oxygen and NADPH. This reaction adds a hydroxyl group at C_{20} and C_{22} followed by the cleavage between the two hydroxyl groups. Cholesterol, which is a 27- carbon sterol, is thereby cleaved of its 6-carbon group side-chain and results in the 21- carbon steroid, pregnenolone (Kagawa and Waterman, 1995).

The second enzymatic reaction in the steroidogenic biosynthesis pathway is the conversion of pregnenolone to progesterone. This reaction is catalysed by the enzyme 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD) (Thomas et al., 1989; Thomas et al., 1995; Thomas et al., 2003). This reaction together with P450scc is believed to occur in the mitochondrial inner membrane. It is speculated that the StAR protein may interact with the mitochondrial inner membrane and thereby initiate the formation of P450scc and 3β-HSD. These reactions result in the rapid conversion of cholesterol to pregnenolone and then to progesterone (Stocco, 1999). 3β-HSD is responsible for the dehydrogenation and isomeration of pregnenolone to progesterone. This reaction converts Δ^5-3β-hydroxysteroid to a Δ^4-3-ketosteroid, which is the active form of steroid hormones. The conversion of pregnenolone to progesterone by 3β-HSD can also occur in the cytosol. Thus, the steroidogenic pathway splits into a Δ^5 – hydroxysteroid pathway (starting with pregnenolone) and a Δ^4- ketosteroid pathway (starting with progesterone). Therefore, although the same enzymes use different substrates along the parallel pathways, both pathways converge and result in the production of androstenedione. The Δ^5 – hydroxysteroid pathway substrates, 17α – hydroxypregnenolone and dehydroepiandrosterone (DHEA) are converted by 3β-HSD into their respective Δ^4-ketosteroids namely, 17α – hydroxyprogesterone and androstenedione, respectively.

The third enzymatic reaction of the steroid biosynthesis pathway is mediated by cytochrome P450 17α – hydroxylase/C_{17-20} lyase (P450c17) (Robichaud et al., 2004). This enzyme requires molecular oxygen and NADPH. It performs two chemical reactions namely, hydroxylation and cleavage (conversion of the steroid from a 21- to a 19-carbon molecule). The hydroxylation products are considered as intermediates thus, P450c17 initially catalyses the conversion of pregnenolone to 17α - hydroxypregnenolone which is then converted to dehydroepiandrosterone (DHEA) in the Δ5 – hydroxysteroid pathway. It was mentioned earlier that DHEA is converted to androstenedione by 3β-HSD. Similar, for the Δ^4-ketosteroids, P450c17 converts progesterone to 17α – hydroprogesterone which is then converted to androstenedione by 3β-HSD.

The lyase activity of P450c17 differs for the intermediate substrates among species. In humans P450c17 converts 17α – hydroxypregnenolone to DHEA (Δ^5 – hydroxysteroid pathway) whereas in rats, P450c17 converts the intermediates of both the Δ^5 – hydroxysteroid and Δ^4 – ketosteroid pathways equally. P450c17 lyase activity differences between species may explain some of the species dependent differences in response to substances that alter steroidogenesis.

The following enzyme reaction is responsible for the conversion of androstenedione to testosterone. This reaction is mediated by 17-ketosteroid reductase (17KSR) which is also known as 17β-hydroxysteroid dehydrogenase (17β-HSD) (Wang and Tuohimaa, 2007). Testosterone is considered as an end-point or end-hormone product whereas, a second possible reaction involving androstenedione occurs in females. In females androstenedione is converted to estrone by aromatase. 17KSR can mediate either the reduction reaction (testosterone synthesis) or the oxidation reaction (androstenedione synthesis). This reversible reaction is dependent on product concentrations. In males testosterone is further converted to dihydrotestosterone (DHT) by 5α-reductase. This enzyme is localized in the cellular membranes, nuclear envelope and endoplasmic reticulum. DHT is considered to be significantly more potent than testosterone as an androgen and is also a steroidogenesis end-point. DHT is primarily produced in peripheral tissues but it is also found in the testis.

The last reaction of steroid biosynthesis is mediated by an enzyme named aromatase (Bulun et al., 2001). This enzyme is responsible for the conversion of androgens to estrogens. In males, aromatase converts testosterone to estradiol, whereas in females androstenedione is converted

to estrone. DHT, estradiol and estrone are all considered as steroidogenesis end-points. Aromatase is an enzyme complex that consists of two cytochrome P450 enzymes: a reductase and an aromatase bound to the endoplasmic reticulum. This enzyme complex catalyses two hydroxylation steps and the aromatization of Ring A in the steroid nucleus that results in the loss of the C-19 carbon atom and thereby producing a C-18 molecule characteristic of estrogens. Aromatase is found in many male and female peripheral gonodal tissues. The activity of this enzyme varies with species and age.

2.3.2.3 Hormonal control of steroidogenesis

Cholesterol is the common precursor for the synthesis of steroid hormones. A series of enzymatic actions is responsible for the conversion of cholesterol to end hormone products namely testosterone, DHT, estradiol and estrone. The steroidogenic pathway is under regulation of gonadotropins and end product hormones. Alteration to the regulatory mechanisms, steroidogenic enzymes and/or the substrates and products can effect end hormone production and result in reproductive system toxicity. An example of an enzyme under gonadotropin regulation is P450scc. Both P450scc and P450c17 are regulated by LH. In males FSH stimulates the release of a Sertoli cell factor that increases the effect of LH on 3β-HSD activity, whereas in females FSH increases aromatase activity and enhances the conversion of androstenedione to estrone. In addition to the regulatory effects of gonadotropins, gonodal hormones exert feedback regulation loops on steroidogenic enzymes. Examples of this are for instance testosterone that inhibits P450c17 activity, which occurs via effects on the second messenger cAMP pathway. Testosterone also suppresses 3β-HSD through inhibitory effects on the cAMP-mediated 3β-HSD mRNA transcription.

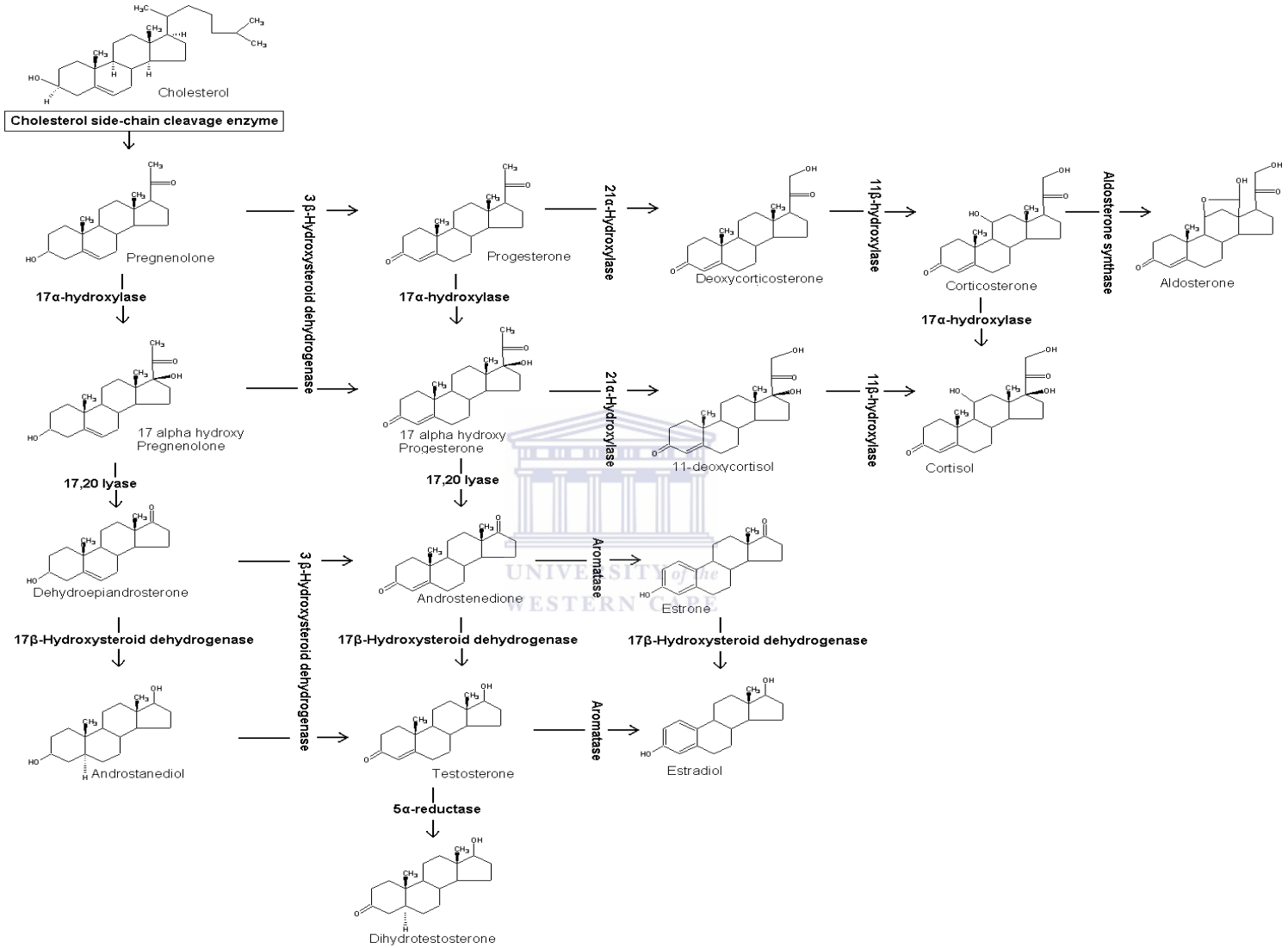

Figure 2.3. Enzymatic conversions of cholesterol and intermediate/end-product hormones (EPA, 2005)

2.4 Mechanisms of estrogenic endocrine disruptors

The ability of pesticides to act as estrogen agonists have been known for over 40 years (Bitman et al., 1968). The estrogenicity of anthropogenic chemicals, for example, bisphenol A and diethylstilbestrol (DES), were first described in 1938 (Dodds and Lawson, 1938). EDCs typically influence reproductive health of vertebrates by more than one mechanism. Several target organs can be impacted, although not necessarily at the same dose or developmental stage of life. Considerable homology exists in the endocrine systems of vertebrates. Toxicants that alter endocrine function in one species are very likely to produce similar adverse effects in another. The endocrine system is very complex and a number of mechanisms have been documented whereby EDCs interact and disrupt normal endocrine function (Reviewed in IPCS, WHO, 2002). In a summarized version: EDCs can mimic the effect of natural hormones (e.g. estradiol) by binding to the hormone receptor, by antagonising the effects of natural hormones, by blocking the binding to the hormone receptor (e.g. tamoxifen), by reacting directly/indirectly with the hormone structure to alter it, by interfering with hormone synthesis; by altering hormone receptor levels and by interfering with the transport and elimination of hormones. Figure 2.4 illustrates a schematic diagram depicting several key steps of steroid hormone synthesis as well as steps that may be sensitive to disruption by environmental chemicals (Reviewed in WHO/IPCS, 2002).

1.) Gonodal cells synthesize steroid hormones such as 17β-estradriol (E2), testosterone and progesterone which are collectively referred to as estrogenic ligands (EA). Chemicals that inhibit Cyp 450 enzymes, including drugs and pesticides may act at this location.

2.) Following the synthesis of hormones in the gonads, it is secreted into the blood stream. Hormones in the blood stream are available to target cells through diffusion or it may be transported bound to sex hormone-binding globulin (SHBG). The amounts of free and bound hormone depend on several mechanisms. Bound hormone dissociates from SHBG at a rate depending on the affinity of the steroid for SHBG protein. Toxicants may alter blood SHBG and it has been reported that some hormone mimics do not bind SHBG as well as natural hormones. This could result that hormone mimics may be more available for both the target cell and for liver metabolism in comparison with natural hormones.

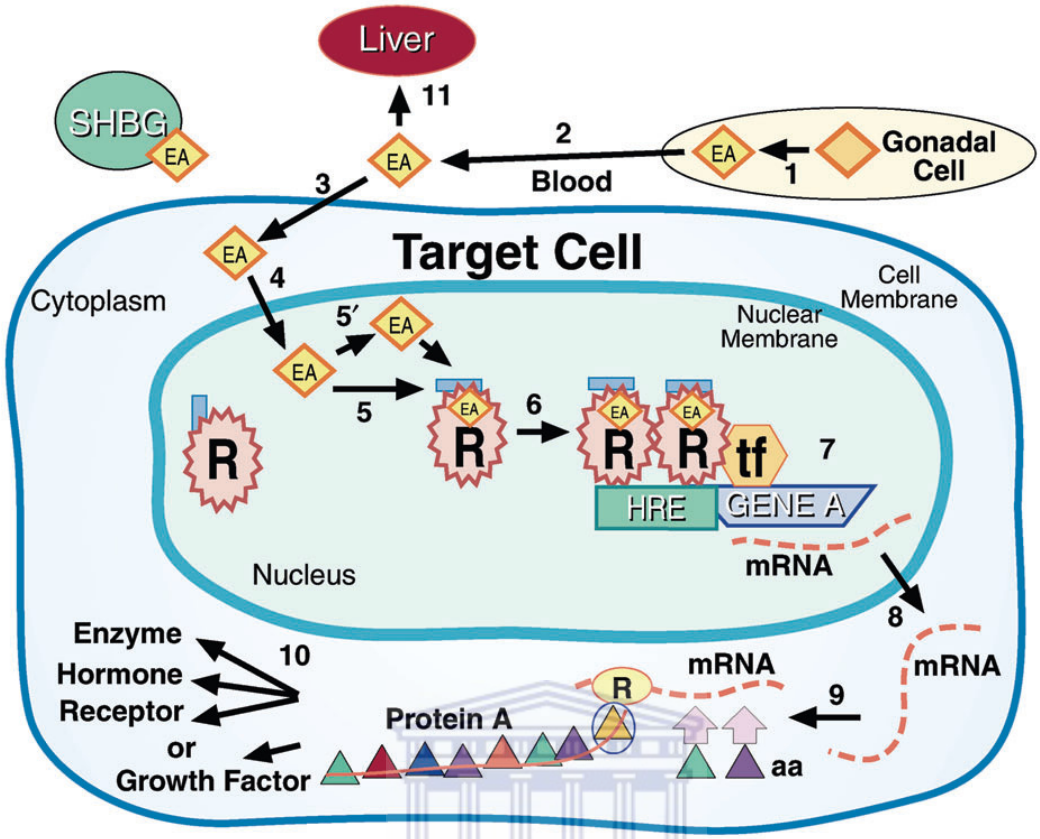

Figure 2.4. Schematic diagram depicting several key steps of steroid hormone synthesis as well as steps that may be sensitive to disruption by environmental chemicals (Reviewed in WHO/IPCS, 2002). Abbreviations used: Estrogenic ligands (EA); sex hormone-binding globulin (SHBG); hormone receptor (R); transcriptional factores (tf); hormone response elements (HRE); amino acids (aa).

3.) Steroid hormones diffuse into the cell.

4.) The hormone diffuses into the perinuclear region, where unoccupied receptors (R) are located.

5.) At this step, hormone or hormone mimics may bind to the receptor. Many xenobiotics have been shown to bind the ER. It has also been reported that the same chemical secreted into the blood stream may be a prohormone, which is then metabolized in the cell. For example, in some tissues, testosterone is metabolized by aromatase to E2, whereas in others the enzyme 5α-reductase converts it to dihydrotestosterone. In some tissues such as muscle, testosterone itself is the active hormone. EDCs may inhibit the conversion of the prohormone to active hormone in the target tissue.

6.) The receptor (R) which is now bound to a natural or synthetic ligand, undergoes a conformational change, exposing key protein binding sites and forms homodimers.

7.) The homodimers are responsible for the accumulation of transcriptional factors (tf), which forms a transcriptional complex. This complex will bind to specific sequences on the DNA of hormone dependent genes, known as hormone response elements (HRE). The transcriptional complex then initiates mRNA synthesis (mRNA). Some chemicals found in the environment known as anti-hormones interfere with DNA binding.

8.) mRNA is transported out of the cell into the cytoplasm.

9.) At this step proteins (the circles on a "string") are synthesized from the RNA template. Initially, amino acids (aa) will bind to specific tRNAs (the thick arrows) which again will then bind to ribosomes where translation will occur.

10.) The protein being synthesized (a marker of endocrine action), could be an enzyme, a protein hormone or growth factor, or a structural component of the cell. An example of a hormone dependent biomarker is vitellogenin, an estrogen-sensitive protein produced by oviparous vertebrates.

11.) Toxicants may disrupt endocrine function by altering liver function. It has been shown that toxicants can either increase or decrease metabolism of a specific hormone in such a way that serum levels are altered. An example of this is some PCBs that stimulate metabolism of T4 resulting in dramatic reduction in serum T4 levels. Several pesticides have been shown to stimulate the liver and reduce serum steroid hormone levels.

2.4.1. Specific examples of e-EDCs and their mode of action

2.4.1.1. Estrogen Receptor mediated chemicals

Several chemicals bind hormone receptors and then mimic or block the actions of natural hormones. Table 2.1 and 2.2 displays a list of chemicals that mediate endocrine disruption through interacting with the ER. Such compounds include some naturally occurring chemicals, such as coumestrol and genistein, pharmaceuticals such as diethylstibestrol, 17α-

ethinylestradiol and tamoxifen, and industrial chemicals such as dichlorodiphenyl trichloroethane (DDT), bisphenol-A, nonylphenol and other alkylphenol polyethoxylates (APEs) (CMA, 1993; DFG and Eisenbrand, 1996; Shäfer et al., 1996). Methoxychlor, chlordecone (kepone) some PCBs and alkylphenols have also been shown to disrupt estrogen receptor function (Mueller and Kim, 1978; White et al., 1994). Several instances of endocrine disruption via other steroid receptors have also been reported. Metabolites of the fungicide vinclozin and the DDT metabolite, DDE have been found to bind to the androgen receptor and block testosterone-induced cellular responses *in vitro*. DDT and chloredecone inhibit ligand binding to the estrogen and progesterone receptors (Laws et al., 1995).

2.4.1.2. Chemicals that can interact with specific steroidogenic enzymes

Several classes of fungicides have been developed to inhibit fungal membrane synthesis and growth by specifically inhibiting CYP450 enzymes. However, the process of steroidogenesis is sufficiently conserved that these chemicals can also inhibit mammalian steroidogenesis. There are several CYP450 enzymes in the steroid pathway. The binding affinity of each of these enzymes varies depending on the chemical used. In general, at relatively high concentrations, these fungicides are nonspecific inhibitors of CYP450 enzymes. For instance, the antifungal imidazole derivative, ketoconazole inhibits various enzymes that belong to the CYP450-dependent mono-oxygenases in rodent and humans. Ketoconazole inhibits side chain cleavage of cholesterol and 11β-dyroxylase in the adrenal gland and 17α-hydroxylase and C_{17-20} lyase in rat and human testes (Schurmeyer and Nieschlag, 1984; Pepper et al., 1990). When ketoconazole is administered to adult rodents, it can have dramatic effects even after a single dose (Bhasi et al., 1986; Heckman et al., 1992; Waller et al., 1990). A number of pharmaceuticals have also been developed that inhibit aromatase enzyme activity. These pharmaceuticals are used for the treatment of postmenopausal breast cancer (Brodie et al., 1999). This P450 enzyme is also highly conserved in a variety of tissues and many species. Accidental inhibition of aromatase activity in wildlife may result in adverse effects such as abnormal sexual behaviour, fertility problems and extinction. The lists of pharmaceutical chemicals that have been developed to inhibit specific steroidogenic enzymes are endless. These chemicals may end up in the environment and cause adverse effects on different species. Table 2.3 displays a list of substances and the reported steroidogenic enzyme that it influences.

Table 2.3. Substance that directly alter steroidogenesis (EPA, 2005)

Site of chemical action	Reference
cyclic-AMP Second Messenger System	
Bisphenol A/octyphenols	Nikula et al., 1999
Nitrate/nitric oxide	Panesar, 1999
Glucocorticoids	Orr et al., 1994
Indomethacin	Lopez-Ruiz et al., 1992
Chloroquine	Lopez-Ruiz et al., 1992
Ethane dimethanesulfonate	Klinefelter et al., 1991
Dibromoacetic acid	Goldman and Murr, 2002
Nicotine	Patterson et al., 1990
Cotinine	Patterson et al., 1990
Tylosin	Meisel et al., 1993
Gossypol	Pearce et al., 1986
Lindane	Ronco et al., 2001
StAR Protein	
Barbiturates	Gocze and Freeman, 1999
Lindane	Walsh et al., 2000a
Dibromoacetic acid	Goldman and Murr, 2002
Dimethoate	Walsh et al., 2000a
Diethylumbelliferyl phosphate	Choi et al., 1995
DMSO	Stocco et al., 1995
P450SCC	
Lead	Huang et al., 2002
Ketoconazole	Kan et al., 1985
Mibolerone	Fanjul et al., 1989
Aminoglutethimide	Uzgiris et al., 1977
Taxol	Rainey et al., 1985
Cis-platinum	Maines et al., 1990
Vitamin A deficiency	Jayaram et al., 1973
3β-HSD	
Daidzein/genistein/biochanin A	Ohno et al., 2002
Lithium chloride	Ghosh et al., 1991
Mibolerone	Fanjul et al., 1989
Danazol (ethinyltestosterone)	Barbieri et al., 1977
Cyproterone acetate	Lambert et al., 1987
Ethionine.	Goldberg et al., 1969
Cyanoketone (WIN-19578)	Goldman et al., 1965
Mitomycin C	Deb et al., 1980
Aflatoxin	Verma and Nair, 2002
P450c17 (17"-hydroxylase/C17-20 lyase)	
Ethanol (17"-hydroxylase)	Murono, 1984
Bromocriptine	Kovacevic and Sarac, 1993
Mibolerone	Fanjul et al., 1989
Danazol	Barbieri et al., 1977
Cyproterone acetate	Ayub and Levell, 1987

Table 2.3. ***continued***

Site of chemical action	Reference
Cyclosporin A	Seethalakshmi et al., 1992
Nicotine	Kasson and Hsueh, 1985
Flutamide	Ayub and Levell, 1987
17KSR	
Cotinine	Yeh et al., 1989
Danazol	Barbieri et al., 1977
Cyclosporin A	Kasson and Hsueh, 1985
Lithium chloride	Ghosh et al., 1991
5α-Reductase	
Finasteride	Morris, 1996
Aromatase	
Aminoglutethimide	Johnston, 1997
MEHP	Thomas, 1996
Fenarimol	Vinggaard et al., 2000
Fadrazole	Yue and Brodie, 1997
Letrozole	Bhatnagar et al., 2001
Anastrozole	Bhatnagar et al., 2001
Arimidex	Johnston, 1997
Flavenoids	Saarinen et al., 2001
Prochloraz	Andersen et al., 2002
Enconazole/miconazole/ketoconazole	Doody et al., 1990
Imizolil	Doody et al., 1990
4-hydroxyandrostenedione	Doody et al., 1990
10-propargylestr-4-ene-3,17-dione	Doody et al., 1990

2.5 Effect of e-EDCs on health

The hypothesis that chemicals found in the environment may induce estrogenic effects in experimental animals and humans is not new at all (Allen and Doisy, 1923; Burlington and Lindeman, 1950). The level of concern regarding estrogenic EDCs has intensified during the last two decades following a drastic increase in reports of adverse effects in human and wildlife reproductive health that is speculated to be due to exposure to endocrine-disrupting chemicals. Reported abnormalities in laboratory animals, wildlife and humans exposed to endocrine-disrupting chemicals include, feminization of males, abnormal sexual behaviour, birth defects, altered sex ratios, decreased sperm density, decreased size of testes, breast cancer, testicular cancer, reproductive failure and also thyroid dysfunction (Table 2.4) (Crisp et al., 1998; Vom Saal et al., 1998).

2.5.1 Wildlife health

The most consistent evidence for endocrine disruption due to water contaminants has been reported from wildlife (Sumpter, 2005). A report by Matthiessen and Gibbs (1998) indicates masculinisation (imposex) of female snails exposed to tributyltin (TBT – an antifouling agent in paints). Masculinisation of female snails may result in a decline or extinction of snail populations. Alligators with impaired sexual development and function were observed in Lake Apopka, Florida after a major pesticide spill in 1980. The observed effects were hypothesised to have been linked to DDT contamination (Guillette et al., 1994). Reproductive defects have also been reported in various fish species in association with sewage effluents, paper industry and industrial chemical pollution (Purdom et al., 1994; Sumpter and Jobling, 1995). Reproductive defects, including reduced fertility (Bortone and Davis, 1994), masculinisation of female fish (Denton et al., 1985) and feminisation of male fish (Gimeno et al., 1998a, 1998b; Batty and Lim, 1999), as well as variable spawning time (Kramer et al., 1998) have been reported. Alkyl phenols (e.g., nonylphenol and octylphenol) are breakdown products of alkylphenol polyethoxylates, which are used as industrial surfactants and bases for household products. The alkylphenols are estrogenic and have been linked to reproductive defects in fish (Jobling et al., 1996). Impaired reproduction in Baltic grey, ringed seals (Helle, 1980) and field studies on harbour seals has been linked to PCBs and its metabolites in their food chain.

In birds, egg shell thinning has been linked to exposure to DDE (DDT derivative) (Cooke 1973). Egg shell thinning, resulting in accidental breakage of eggs, has resulted in population declines in a number of species of predatory birds in Europe and North America. Agricultural chemicals which are currently used and suspected of having endocrine-disruptive activity are atrazine and related herbicides such as simazine, endosulfan, methoxychlor and fenitrothion (pesticides) and the fungicides vinclozolin and ketoconazole (WHO/IPCS, 2002). Atrazine, which is a herbicide, has been shown to have neuroendocrine effects on reproduction in rodents and fish (WHO/IPCS, 2002). Simazine has been demonstrated to impair reproduction in the water flea, *Daphnia pulex* at 4 – 20 mg/L (Fitzmayer et al., 1982). Chemicals like fenitrothion, vinclozolin and ketoconazole exhibit anti-androgen effects in mammals by inhibiting key enzymes of androgen synthesis (WHO/IPCS, 2002). Methoxychlor is an organochlorine pesticide which is used against a range of pests, and it is believed to have estrogenic effects on mice (US EPA, 1997).

Table 2.4. Examples of the health effects of endocrine disrupters in wildlife and humans (Solomon and Schettler, 2000)

Chemical	Use	Mechanism	Health Effect	Reference
DES	Synthetic estrogen	Estrogen receptor agonist.	Humans (prenatal exposure): vaginal cancer, reproductive tract abnormalities (females). Cryptorchidism, hypospadias, semen abnormalities (males).	Giusti et al., 1995
Methozychl	Insecticide	Metabolite is an estrogen receptor agonist.	Rodents: accelerated puberty, abnormal ovarian cycling (females). Aggressive behaviour (males).	Swartz and Corken, 1992; Vom Saal et al., 1995
DDT	Insecticide	Metabolite (DDE) is an androgen receptor antagonist.	Rodents (males): delayed puberty, reduced sex accessory gland size, altered sex differentiation.	Gray, 1998
Vinclozolin	Fungicide	Androgen receptor antagonist.	Rodents (males): feminization, nipple development, hypospadias.	Gray et al., 1999a
PCBs	No longer manufactured; still in electrical transformers, capacitors, toxic waste sites, food chain	Accelerated T4 metabolism, decreased T4 levels, elevated TSH levels (high doses: thyromimetic).	Humans (in utero exposure): delayed neurological development and IQ deficits.	Jacobson and Jacobson, 1996; Zoeller et al., 2000
Atrazine	Herbicide	Reduces gonadotropin-releasing hormone from hypothalamus, reduces pituitary LH levels, interferes with metabolism of estradiol, blocks estrogen receptor binding.	Rodents (females): mammary tumours, abnormal ovarian cycling. Humans: some evidence of breast and ovarian tumours.	Bradlow et al., 1995; Tran et al., 1996b; Cooper et al., 1999; Donna et al., 1989; Kettles et al., 1996
Dioxin	By-product of industrial processes including waste incineration; food contaminant	Aryl hydrocarbon receptor agonist; increases estrogen metabolism, decreases estrogen mediated gene transcription, decreases estrogen levels, decreases testosterone levels by interfering with HPG axis.	Rodents (in utero exposure): delayed puberty, increased susceptibility to mammary cancer (females). Decreased testosterone, hypospadias, hypospermia, delayed testicular descent, feminized sexual behaviour (males). Humans: decreased T3 and T4 levels, decreased testosterone levels.	Brown et al., 1998; Mably et al., 1992; Egeland et al., 1994; Nagayama et al., 1998; Steenland et al., 1999

Note: DES = diethylstilbestrol, DDT = dichlorodiphenyltrichloroethane, PCBs = polychlorinated biphenyls, T4 = thyroxine, TSH = thyroid stimulating hormone, IQ = intelligence quotient, LH = luteinizing hormone, HPG axis = hypothalamic–pituitary–gonadal axis, T3 = triiodothyronine.

2.5.2 Human health

Current human epidemiological data is inadequate for drawing solid conclusions regarding correlations between endocrine disrupting compounds and human health. Most of the evidence obtained for this possible link is derived from cases of pharmacological dosing, accidental exposure and occupational exposure. The first evidence that e-EDCs influence human health were found in aviation crop-dusters handling DDT, who had reduced sperm counts (Singer, 1949). Following this incident back in 1949, various reports have indicated that e-EDCs may have a negative impact on human health, although reports are very inconclusive. Table 2.5 lists some trends in human health effects potentially related to endocrine disruption (Solomon and Schettler, 2000).

2.5.2.1 Developmental defects

There appear to be an increase in sexual development defects in recent years and some of these may be linked to exposure of e-EDCs. For example, more baby boys have to undergo operations to correct undescended testicles (cryptorchidism) now than 30 years ago. The numbers affected by cryptochidism have increased 2-3 fold during the past 30 years (Keith 1998). The incidence of the birth defect called “hypospadias” in which the male urinary canal is open underneath the penis is also increasing (Keith 1998). Boys exposed to higher levels of toxic chemicals during embryonic development have shorter penises than the average, similar to Lake Apopka's alligators in Florida (Keith 1998). Boys born from women who were exposed to PCB-poisoned rice bran cooking oil in 1978-79 in central Taiwan, the socalled "Yucheng" boys, were found to have significantly shorter than normal penis lengths at ages l l to 14 (Guo et al., 1993).

2.5.2.2. Decreased sperm production in men

It has been speculated that *in utero* exposure to environmental estrogens may be responsible for the decline in sperm production levels seen during the last 50 years (Sharp and Skakkebaek, 1993). A meta-analysis of 61 studies that includes about 15 000 men found a substantial decrease in sperm concentration from 113 x 10^6/ml in 1938 to 66 x 10^6/ml in 1990 (Carlsen et al., 1992). A follow-up study conducted by Shanna Swan et al. (2000) also demonstrated a reduction in sperm count. Decreased sperm counts may result in fertility problems as 50 % of all fertility problems can be related to the male reproductive system (Swerdloff, 1985).

Table 2.5. Trends in human health effects potentially related to endocrine function

End point	Region	Trend	Degree of change	Reference
Hypospadias	US	Increasing incidence	4.3 % per year	Paulozzi, 1999
	Canada		3.3 % per year	
Cryptorchidism	US	Increasing incidence	3.5 % per year	Burlington and Lindeman, 1950
	Canada		1.6 % per year	
Sperm Count	Canada	Decrease	– 0.7 %/mL per year*	Younglai et al., 1998
			– 3 %/mL per year	Swan et al., 1997
			– 5.3 %/mL per year	Swan et al., 1997
Testicular cancer	Canada	Increasing incidence	2.1 % per year	Liu et al., 1999
	US		2.3 % per year	McKiernan et al., 1999
	Europe		2.3 % – 5.2 % per year†	Bergstrom et al., 1996
Prostate cancer	Canada	Increasing incidence	3 % per year	Levy et al., 1998
	US		5.3 % per year	Haas and Sakr, 1997
Breast cancer	Saskatchewan, US	Increasing incidence	3.3 % per year	Wang and Cao, 1996
			1.9 % per year	Wolff et al., 1996
Sex ratio	Canada	Shift towards females	– 1.0 males/10 000 per year	Allan et al., 1997
	US		– 0.5 males/10 000 per year	
Age at breast development	US	Shifting earlier	11.2 – 9.96 years in white population	Herman-Giddens et al., 1997

*This trend disappears when data from before 1984 are included. †Range is dependent on country, with Sweden at the lower and the former East Germany at the upper end of the range. ‡International trends in prostate cancer are complicated by the introduction of the prostate specific antigen screen, but prostate cancer mortality also increased (by about 1 % per year through 1995 in the US and Canada), implying that improved diagnosis may not fully explain the rising incidence trends.

2.5.2.3. Testicular- and prostate cancer in men

The incidence of testicular cancer in men has drastically increased during the last decades (Forman and Moller, 1994). Testicular cancer is the most common malignancy among men at age 25-34 year in Denmark, while in Finland the incidence is much lower (Adami et al., 1994). It has not yet been concluded whether the apparent increase in testicular cancer in many countries is due to hormonal substances (EDCs), change in life-style conditions or other causes. Cancer of the prostate is the second leading cancer cause of death in the USA. Death due to prostate cancer has increased 17 % over the last 30 years. More over, there are race differences in prostate cancer susceptibility. The prevalence is very rare in Orientals, 20-30 times higher in Caucasians and even higher in Afro-American males (Crisp et al., 1998). A meta-analysis found a positive association between prostate cancer and farming occupation (Keller-Byrne et al., 1997). Little is known about the causes of prostate cancer, but age, genetics, diet and environmental risk factors have been proposed.

2.5.2.4. Breast cancer

Breast cancer is the most frequent tumour in women world wide. The incidence of breast cancer in Europe has increased steadily over the last few decades (Amaral Mendes, 2002). Several factors have been identified as potential risks, but their mechanisms are still unknown. One of these proposed factors are exposure to estrogenic chemicals whereas the other is high fat diets (Schmidt and Wayne, 1997). This hypothesis is supported by several case studies where a correlation between exposures to organochlorine chemicals such as DDT and certain PCB congeners and breast cancer incidences have been drawn (Dewailly et al., 1994). However, the association between breast cancer and exposure to DDT and its most prevalent breakdown product, dichlorodiphenyldichloroethylene (DDE) is also not yet considered conclusive (Snedeker, 2001).

2.5.2.5. Endometrioses

Another disease that can possibly be linked to e-EDCs is endometrioses. There is evidence suggesting an increased frequency of endometrioses in female offspring exposed *in utero* to DES (Berger and Alper, 1986). An association between endometrioses and high serum levels of PCB have also been reported (Gerhard and Runnebaum, 1992). However, the World Health Organization considers the link between endometriosis in females and exposure to organochlorines, as well as dioxins, as non conclusive (WHO/IPCS, 2002).

2.5.2.6. Male to female sex ratio of new borns

Male to female sex ratio of newborns has been used as an indicator of environmental effects on human reproduction. Evidence, indicating a change in male/female ratios due to e-EDCs were seen in Seveso, Italy after dioxin exposure of the local population. Following this accident there was an increase in the percentage of females born in the period April 1977 to December 1984 (Mocarelli et al., 1996). The percentage female births declined again from 1985 to 1994 and were thereafter similar to before the spill. Reports also indicate a decline in males born in Denmark (Moller, 1996), The Netherlands (Van der Pal-de Bruin et al., 1997), England and Wales (Dickinson and Parker, 1996) and Canada (Allan et al., 1997) over the last decade. Recent research supports the idea that environmental pollution leads to a decrease in the percentage of male births (Hood, 2005; Mackenzie et al., 2005).

2.5.2.7. Effects caused by natural compounds

Naturally occurring compounds such as phytoestrogens (naturally occurring plant compounds are structurally related to steroid hormones) have also been shown to affect normal endocrine function. One such case was illustrated by the lactation of castrated male sheep and infertility in female sheep grazing on phytoestrogens-rich pasture species (Adams, 1998). Numerous plant extracts used in herbal remedies have estrogenic activity. An example is terpenoids, which is found in products like neem. This is a pharmacologically active extract derived from an Indian tree (*Azadirachta indica*) that is used medicinally in India as a contraceptive and also to induce abortions (National Research Development Corporation of India, 2003). The evidence to date for endocrine disruption in humans from exposure to environmental chemicals is limited, and the issue remains controversial (Degen and Bolt, 2000; Safe, 2000).

2.6. Development of test systems for estrogenic EDCs

In order to assess the potential health risk (estrogenic characteristics) water sources, such as waste, ground and surface water may pose, assays are required to measure adverse effects. The choice and also development of appropriate test systems to identify e-EDCs are very complicated. Endocrine disrupters may act via a number of mechanisms including receptor dependent and independent processes. In addition, they can demonstrate species, tissue- and cell specific effects, as well as being influenced by metabolism. More over, many potential endocrine disruptors exist as complex mixtures in the environment that may possibly act synergistically. As a result EDCs can produce a wide range of responses. In the last few decades increasing attention has been given to the evaluation and development of appropriate

test systems for a wide range of endocrine disrupting chemicals (EDCs) and the adverse health effects that it may cause. The passage of two laws in 1996, the Food Quality Protection Act (FQPA) and Amendments to the Safe Drinking Water Act (SDWA) were the first to mandate the United States Environmental Protection Agency (U.S. EPA) to screen pesticides for endocrine disrupting characteristics. These two laws also authorized the U.S. EPA to screen chemicals found in drinking water to determine whether they are estrogenic or possess other endocrine activity (Federal Register, 1998a, 1998b). In order to provide recommendations regarding a strategy for the development of a testing paradigm for compounds that may interfere with naturally-occurring hormones, the U.S. EPA established the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC). The U.S. EPA established the Endocrine Disruptor Screening Program (EDSP) upon recommendations made by EDSTAC in its final report (EDSTAC, 1998). The aim of EDSTAC is to develop a screening program to determine whether substances found in the environment have endocrine disrupting effects. This screening program must be appropriately validated and be supported by relevant scientific information (FQPA, 1996). This program's aim is to develop a two-tiered approach, e.g. *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for the identification and characterization of endocrine effects of pesticides, industrial substances and environmental contaminants. The development of new test guidelines and the revision of existing test guidelines for the screening and testing of potential endocrine disrupters were initiated as a high-priority activity in 1997 by the Organization for Economic Cooperation and Development (OECD). OECD assessments and test guidelines is managed by three Validation Management Groups (VMGs) and contain mammalian, ecotoxicological and non-animal methods.

2.7 Current assays for e-EDC analysis

2.7.1. Analytical mass based assays

The use of mass based analytical test methods, including high performance liquid chromatography (HPLC), gas chromatography mass spectrometry (GC/MS), GC MS/MS and liquid (LC) MS/MS (Petrović and Barceló, 2000; Huang and Sedlak, 2001; Petrović et al., 2002) have been very popular for the identification of specific EDCs in the environment. These methods have been presented in detail by various studies (Huang and Sedlak 2001; Heisterkamp et al., 2004; Wozei, 2004; Zhang et al., 2004; Fan et al., 2005). These analytical techniques are very sensitive and provide excellent precision for monitoring e-EDC mass. However, these techniques do not provide any data on estrogenic effects, synergistic or anti-

estrogenic influences from multiple estrogenic compounds found in environmental matrixes. Mass based analytical techniques are applicable when the objective is to determine the fate and transport of specific e-EDCs in the environment.

2.7.2 Bio-assays

Bio-assays provide an attractive alternative towards traditional analytical mass based assays. Bio-assays may provide useful information regarding the mode of action of specific contaminants (Geisy et al., 2002) as well as the integrated possible action of various contaminants from different environmental matrices. Bio-assays include various *in vitro* and *in vivo* assays based on a number of mechanisms including cell proliferation, ligand binding, vitellogenin induction, luciferase induction, or antigen-antibody interaction. Bio-assays may provide either qualitative or quantitative responses. Bio-assays, which include both *in vitro* and *in vivo* assays, may use whole organisms, whole cells, or biological materials like antibodies or estrogen receptors.

2.7.2.1 In Vitro bio-assays

A large number of *in vitro* tests have been developed for the screening of environmental EDCs (ECETOC, 1996; Holmes et al., 1998). In general, *in vitro* assays are designed to be sensitive and rapid to facilitate the screening of large numbers of chemicals. The rapid response and lower equipment requirements, resulted in *in vitro* bio-assays being widely used as alternative to conventional analytical techniques for environmental monitoring, particularly when measuring relative increases in total estrogenic activity is the monitoring objective. The most widely used *in vitro* bio-assays for the prediction of estrogenic endocrine disruption are listed in table 2.6.

2.7.2.2 Receptor Binding Assays

Steroid hormones, such as 17β-estradiol act on their specific target cells by binding to a high affinity hormone receptor within the cell nucleus (Fig. 2.4). Receptor binding assays have been developed to assess the ability of a substance, or environmental chemical mixture, to bind directly to the hormone receptor (Jensen and Jacobson, 1960; Ireland et al., 1980; Baker et al., 1999 a, b, 2000).

Table 2.6. Main types of *in vitro* test systems for the detection of endocrine disrupters

Assay Type	Endpoint Measured
Receptor Binding Assays	Binding affinity of a substance to a hormone receptor
Cell proliferation assays	Ability of a substance to stimulate growth of hormone responsive cells (e.g. MCF-7, E-screen)
Reporter gene Assays	Ability of a substance to activate transcription of a reporter gene construct in cells (mammalian/yeast)
Analysis of hormone-sensitive gene expression	Ability of a substance to induce expression of hormone-sensitive genes (e.g. pS2)
Non-cellular bio-assays	A range of assays not requiring viable cells i.e. ELISA, RIA

Receptor binding assays have been widely used because it is rapid, easy to use and also relatively cheap. These assays are very appropriate to use when screening a large number of samples. However, these assays have some limitations. They cannot distinguish between agonistic and antagonistic effects, as binding to the receptor do not necessarily result in transcriptional activation. They are also only suitable for the detection of hormone-receptor mediated effects and cannot detect pro-estrogens (absence of metabolism in cell-free systems).

2.7.2.3 Cell proliferation assays.

Cell proliferation assays to monitor estrogenicity are well known and used by several research groups. These assays are based on the ability of a test substance to stimulate growth of an estrogen dependent cell line (Soto et al., 1992, 1995). The most commonly used cancer cell lines for cell proliferation assays are MCF-7 and T47D cells. In these assays breast cancer cells are exposed to both positive (17β-estradiol) and negative controls (vehicle control) as well as to the environmental sample being analysed. The cell proliferation of the environmental sample compared to the controls provides the basis for demonstrating estrogenic responses. Cell proliferation assays have the advantage over ER binding assays in that they take cellular, biological and metabolic responses into account. These assays are therefore useful to determine both synergistic and antagonistic estrogenic characteristics of complicated biological mixtures. Cell proliferation assays also have some disadvantages. Various studies have reported inter laboratory variation of the MCF-7 proliferation assay. This is mainly due to differences in culture conditions, as well as different MCF-7 strains (Osborne et al., 1987; Villalobos et al., 1995; Jones et al., 1997, 1998; Odum et al., 1998). Moreover, some studies have reported that MCF-7 cells can be stimulated to proliferate by a

range of non-oestrogenic substances including epidermal growth factor (EGF), progesterone, dihydrotestosterone, insulin-like growth factors, lithium, chloride and ethanol (Hackenburg et al., 1988; Osborne et al., 1990; Savouret et al., 1990; Jones et al., 1998).

2.7.2.4 Reporter gene assays

Reporter gene assays measure the ability of a substance to activate the transcription of a hormone (estrogen)-sensitive promoter in eukaryotic cells (e.g. yeast and mammalian cells). These assays are established using eukaryotic cells that are transfected with an expression vector encoding the human estrogen receptor, as well as estrogen response elements, which is in reading frame with a reporter gene (e.g. luciferase and β-galactosidase that can be readily detected and quantified). When single chemicals or complex environmental mixtures that contain e-EDCs are exposed to these cells, it will activate the receptor and response elements to stimulate expression of the reporter gene. A large number of reporter gene assays have been developed for a range of steroid hormone receptors using both yeast and mammalian cells. The use of yeast cells in these assays (Routledge et al., 1998; Gaido et al., 1997a,b) offer a number of advantages. Yeast cells are fairly easy to culture, ideal for genetic manipulation and also contain some degree of metabolic competence. The yeast reporter gene screens also have some limitations as reported by Joyeux et al (1997). The sensitivity of the system can depend on the number of receptor protein being expressed, the response element and also the reporter gene used. Yeast cell membranes have also been reported to be impermeable to some test substances and may result in false negatives (Gray et al., 1997).

2.7.2.5 Non-cellular bio-assays

Non-cellular bio-assays do not require viable cells in order to test for environmental endocrine disruptors. These assays have the advantage of not having difficulties with membrane permeability, cell function, and organism life stage, as well as toxicity responses to a given sample. Many of these assays are quantitative and provide reasonable detection limits for measurement of e-EDCs. Enzyme linked Immunosorbent Assay (ELISA) and Radio Immuno Assay (RIA) kits have been developed for many environmentally relevant surfactants and estrogenic compounds, as well as for some pesticides, antibiotics, and other personal care products (Gascón et al., 1997; Neogen Corp Lexington, KY; ALPCO Diagnostics Salem, NH; Assay Designs Inc. Ann Arbor, MI; Bio-Quant Inc., San Diego, CA; BioSource Internacional, Camarillo, CA; Cayman Chemical Company, Ann Arbor, MI; Immuno-Biological Laboratories, Inc., Minneapolis, MN; Envirologix. Portland, Me: York Nutritional

Laboratory, Osbaldwick, York, UK). In addition to these immunosorbent assays, advances have been made in electrochemical sensors, fluorescent indicators, and microarray relative binding assays (Zhihong et al., 1999; Murata et al., 2001; Awais et al., 2004).

2.7.3 *In vivo* assays for e-EDC detection

The use of i*n vivo* assays for the analyses of specific compounds or environmental samples for estrogenicity have the advantage that it measures the true effect and impact of the substance being analyzed on the target specie. One of the most widely and oldest assays used for the detection of estrogenic substances is the uterotrophic assay (Dorfman and Dorfman, 1954). The endpoint of this assay measures uterine weight changes in immature or ovariectomised rodents (to minimize the effects of endogenous hormones). The compound of interest is usually administered for 3-4 days. This assay however requires standardization of protocols that specify species, strain, age and route of administration of the test compound (Odum et al., 1997). Another widely used short-term test designed to investigate reproductive effects in males is the Hershberger assay (Hershberger et al., 1953). This assay detects the ability of a compound to elicit agonistic or antagonistic effects at the androgen receptor. The assay is traditionally conducted using castrated rats and measures an increase in weight of the ventral prostate and semical vesicles.

The use of animals for example amphibians, fish, birds and insects are very popular to determine endocrine disruption. The US EPA has developed various fish assays for e-EDC determination. Fish species being used are rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), sheephead minnow (*Cyprinodon variegates*) and zebrafish (*Brachydanio rerio*) (Fenner-Crisp et al., 2000; Folmar et al., 2000; Legler et al., 2002a). There are various approaches to determine estrogenic responses in these organisms, including morphological deformities, reproductive deficiencies, egg and offspring development, and serum protein production like vitellogenin (Table 2.7). Various fish species have been genetically engineered to respond to e-EDCs. Zebrafish has been bioengineered with luciferase expression coordinated to vitellogenin production (Legler et al., 2002a) and medaka fish (*Oryzias latipes*) was bioengineered to express a green fluorescence protein in response to vitellogenin production (Kurauchi et al., 2005).

In vivo tests, like most other tests for e-EDC assays have a number of limitations. One example is that rodents do not synthesize sex-hormone binding globulin after parturition. This

protein is important for regulating the bioavailability of hormones and drugs that bind to them and is involved in their metabolic clearance. Rodent models are therefore often oversensitive to e-EDCS and some species and strain variation in sensitivity have been reported (Hisaw, 1959). Large numbers of animals are often required to ensure statistically significant results (e.g. vaginal cornification). The uterotrophic assay has also been shown to respond to non-estrogenic substances such as progesterone, testosterone and epidermal growth factor (Korach and McLachlan, 1995).

Table 2.7. Examples of whole organism studies as *in vivo* assay for e-EDCs

Species	Common name	EDC effect	Reference
Chrysemys picta	Painted turtle	Vitellogenin induction.	Irwin et al., 2001
Oncorhynchus mykiss	Rainbow trout	Reproductive deficiencies, egg and offspring development, and vitellogenin induction.	Fenner-Crisp et al., 2000, Folmar et al., 2000, Anderson et al., 1996
Pimephales promelas	Fathead minnow	Gonad development; reproductive deficiencies, development; vitellogenin induction.	Fenner-Crisp et al., 2000, Folmar et al., 2000
Cyprinodon variegates	Sheephead minnow	Vitellogenin induction.	Legler et al., 2002a, Fenner-Crisp et al., 2000, Folmar et al., 2000
Danio rerio *Brachydanio rerio*	Zebrafish	Gonad development; physiological development; vitellogenin induction, luciferine (luminescence).	Maack et al., 1999, Legler et al., 2002a
Oryzias latipes	Medaka fish	Gonadal development; reproductive success; green fluorescence protein (GFP).	Gray et al., 1999b, Kurauchi et al., 2005
Platichthys flesus	Flounder	Vitellogenin induction; gonad development; physiological development.	Allen et al., 1999
Salmo salar	Atlantic	Salmon Zona radiata protein and vitellogenin induction.	Arukwe et al., 2000
Halineetus leucocephalus	Bald eagle	Reproductive and teratogenic effects.	Bowerman et al., 2000
Coturnix coturnix japonica *Colinus virginianus*	Japanese quail and bobwhite quail	sexual behavior; embryo development; egg shell thickness.	Lien et al., 1985, Berg et al., 1999
Gallus domesticus	Domestic chicken	Embryo development; egg shell thickness.	Berg et al., 1999
Daphnia magna	Water flea	Physiological and biochemical disruption.	Baldwin et al., 1997
Tisbe battagliai	Marine copepod	Fecundity, longevity, and rate of development.	Bechmann, 1999
Rana pipiens	Leopard frogs	Gonadal abnormalities.	Hayes, 1998, Hayes et al., 2002)

2.8 Objectives of the current study

Contaminants in the environment may pose a health threat for humans and wildlife (USEPA, 1997; Neubert, 1997). EDCs are widely distributed in the air, water, soil, as well as food that we eat, and have the potential to interfere with normal endocrine function (Ying and Kookana, 2002). Endocrine disruptors can act via a wide range of mechanisms, including receptor dependent and independent processes. Moreover, EDCs can demonstrate species, tissue- and cell-specific effects, as well as being influenced by metabolism (WHO/IPCS, 2002). Yang (1994), made an observation that > 95 % of the resources in toxicological research are devoted to the study of single chemicals, with the almost complete neglect of complex mixture studies. In the last 10 years, many articles have been published on the combination effects of EDCs (Kortenkamp, 2007). EDCs found in the environment can have antagonistic or synergistic effect on a biological system. In other words, the combination effect of different chemicals may be smaller or larger than the sum of the individual effects of all components (Kortenkamp, 2007).

Due to this, mass-based analytical techniques for example GCMS and HPLC, do not provide any data on estrogenic activity. *In vitro* test assays are powerful to use when screening a large number of chemicals/samples and can be used to characterize the mechanistic action and potency of specific compounds (Anderson et al., 1999; Beresford et al., 2000). However, *in vitro* assays do not always reflect the outcome of *in vivo* assays. This is due to differences in metabolic capabilities of the test systems (and lack of pharmacokinetics and pharmacodynamics) and the diverse range of mechanisms by which EDCs may act (Anderson et al., 1999; Beresford et al., 2000). In addition, no single *in vitro* assay is able to detect all properties of a hormonal active substance (Scrimshaw and Lester, 2004). Therefore, complementary *in vitro* and *in vivo* assays that assess both receptor and non-receptor mediated mechanisms seem to be the most appropriate way to assess the potential endocrine disrupting activities of a substance or environmental mixture.

The aim of this study was the development, validation and implementation of a battery of *in vitro* and *in vivo* screens for environmental estrogenicity. In order to achieve the aim of this study, several objectives had to be reached. The first objective of this study was the validation of commercially available ELISA kits (designed for hormone quantification in human serum) to detect natural estrogenic hormones in water samples. The second objective was the

development of a multi biomarker cell culture assay that can be used as a rapid, sensitive and inexpensive screen for total environmental estrogenic activity as well as cytotoxicity. The third objective of this study was to development a direct ELISA on cultured MCF-7 cells to quantify ERα protein levels as a biomarker for estrogenicity. The forth objective of this study was to set up and validate a competition ELISA to quantify VTG from *Oreochromis mossambicus* (tilapia). The fifth objective of the study entailed using this quantitative ELISA for VTG to validate the use of tilapia juveniles (less than 70 days post fertilization) as a short term *in vivo* screen for estrogenicity. The sixth objective was the implementation of the battery of assays as a first tier screen to assess the Eerste River, South Africa for environmental e-EDCs.

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Chapter 3

The rapid detection of selected steroid hormones in sewage effluent

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3.1. Abstract

Steroid hormones are naturally synthesized by both humans and animals and released into the environment. Significant levels of steroid hormones have been detected in sewage effluent around the world. The potential problem is that these hormones may interfere with the normal function of the endocrine systems, thus effecting reproduction and development in wildlife. Due to the major shortage of water in the Western Cape, South Africa there is a great need to recycle water by either direct or indirect methods. The treated sewage effluent – natural surface water mixture found in the Kuils and Eerste Rivers is used directly for irrigation of agricultural areas. Sewage effluent were collected from four sites (Jonkershoek, Belville, Zandvliet and Macassar) and subjected to C18 solid phase extraction. Commercially available rapid ELISA kits were validated for the quantification of estrogens in these sewage effluent samples. Analysis of estrone, estradiol and estriol levels showed a significant difference between the control site (Jonkershoek) and sewage effluent from the three sewage treatment works. Steroid hormone concentrations detected in these sewage effluents were similar to reports from Brittan, Italy, Germany, Canada and Netherlands.

3.2. Introduction

Steroid hormones are a group of biological active compounds that are synthesized from cholesterol and have a cyclopentan-o-perhydrophenanthrene ring in common (Guang-Guo et al., 2002). The steroids include progestogens, glucocorticoids, mineralocorticoids, androgens and estrogens (Raven and Jonhson, 1999). Natural steroids are secreted by the adrenal cortex, testis, ovary and placenta in both humans and animals. Estrogens (estriol, estradiol and estrone) are predominantly female hormones and are responsible for the maintenance of reproductive organs/tissue, breasts, skin and brain. Androgens are predominantly male hormones and are responsible for tissue regeneration, especially the skin, muscle and brain. Progesterone can be thought of as a hormonal balance, especially of estrogens. Glucocorticoids (cortisol) are produced by adrenal glands in response to stressors such as emotional upheaval, exercise, illness, surgery or starvation (Guang-Guo et al., 2002).

All humans and animals excrete hormones through their bodies, which can end up in the environment through sewage discharge or animal waste disposal (Lintelmann et al., 2003; Shore and Shemish, 2003; Wenzel et al., 1998). Many of these hormones are peptides and are rapidly destroyed. However, steroid hormones are chemically very stable and are excreted in the free form or as conjugates. Steroid conjugates very readily biotransform to the free form (Wenzel et al., 1998; Panter et al., 1999). Steroids have been detected in effluents from sewage treatment plants and surface water before (Desbrow et al., 1998; Kuch and Ballschmitter, 2001; Ternes et al., 1999a). The potential problem of these natural steroids ending up in the environment is that they may interfere with the normal function of the endocrine systems, thus affecting reproduction and development in wildlife (Jobling et al., 1998). The steroids of major concern in the aquatic environment due to their endocrine disrupting potential are mainly estrogens (Guang-Guo et al., 2002). However, little research has been performed on androgens in the aquatic environment and their potential endocrine disrupting properties. It has only been recently that androgens were found in treated sewage (Kirk et al., 2002) and river water (Thomas et al., 2002).

Several studies in the United Kingdom have shown that wild fish exposed to treated sewage water exhibit reproductive abnormalities consistent with exposure to estrogen and estrogen mimics (Jobling et al., 1998; Harries et al., 1996; Jobling and Tyler, 2003; Purdom et al., 1994). Toxicity identification revealed that natural and synthetic hormones excreted by humans, as well as some alkylphenolic industrial chemicals in sewage effluent, were

responsible for the majority of estrogenic activity (Desbrow et al., 1998; Routledge et al., 1998). This problem is not confined to the United Kingdom. Studies in continental Europe, Japan and North America confirmed that their sewage treated water also contains estrogenic chemicals (Körner et al., 2001; Onda et al., 2002; Solé et al., 2002) and that these may be impacting a wide range of fish species (Christiansen et al., 2002; Folmar et al., 2001; Hashimoto et al., 2000). The concentration of 17β-estradiol in sewage effluent range from 2.7 – 48 ng/l (United Kingdom) (Desbrow et al., 1998), < limit of detection (LOD) – 3 ng/l (Germany) (Ternes et al., 1999a), < LOD – 64 ng/l (Canada) (Ternes et al., 1999a), 3.2 – 55 ng/l (Japan) (Nasu et al., 2000).

In British sewage treatment works (STWs), the concentrations of estrone in the effluents vary widely from 1.4 to 76 ng/l (Desbrow et al., 1998). Estrone has also been detected in sewage effluent in Italy (2.5 – 82.1 ng/l) (Baronti et al., 2000), Germany (< LOD – 70 ng/l) (Ternes et al., 1999a), Canada (< LOD-48 ng/l) (Ternes et al., 1999a) and Netherlands (<0.4 – 47 ng/l) (Belfroid et al., 1999). Estriol was only recently reported in Italian STW influents and effluents (0.43-18 ng/l) (Baronti et al., 2000). A survey of 139 polluted streams and rivers in the US found the following concentrations of steroids (in ng/l; maximum, median; LOD 5 ng/l): testosterone (214, 111), estradiol (200, 160), estrone (112, 27), ethinylestradiol (831, 73) and estriol (51, 31) (Kolpin et al., 2002).

Estrogenic hormones in water matrix are usually quantified by techniques such as gas chromatography, mass spectrometry (GC–MS), GC–MS/MS, high performance liquid chromatography (HPLC), HPLC–MS and HPLC–MS/MS (Snyder et al., 1999; Snyder et al., 2001). These methods are reliable but have several potential drawbacks such as expensive instrumentation, complex derivatization, extensive clean-up and purification, and also require a very high level technical expertise for operation. High cost and low throughput limit the use of these techniques. Thus there is a strong need for rapid, simple and cost-effective methods for quantitative analysis of estrogenic hormones, such as enzyme linked immunosorbent assay (ELISA). ELISA kits are commercially available for the quantification of estrogenic and androgenic hormones. Large numbers of samples can be analysed simultaneously and machines that do the readings are relatively cheap and also available in portable format that can be used in field studies.

Due to the major shortage of water in the Western Cape, South Africa there is a great need to recycle water by either direct or indirect methods. The treated sewage effluent – natural surface water mixture found in the Kuils and Eerste Rivers is used directly for irrigation of agricultural areas. The Western Cape has a high rainfall in winter with very low/no rain in summer. During summer most of the water in these rivers is treated sewage effluent. According to a recent report of the Cape Metropolitan Council (www.capetown.gov.co.za), Cape Town, South Africa has 19 wastewater treatment works, of which only 20 % comply with the quality requirements specified in their permits issued by the Department of Water Affairs and Forestry (www.cndv.co.za). Problems encountered by most of these plants include inadequate sludge disposal, maintenance of the plants and bacteriological quality of the effluents (www.capetown.gov.co.za).

Three sewage treatment works were incorporated in this study for analysis of samples collected from respective sites, i.e. Belville, Zandvliet and Macassar. Effluents from these plants enter the Eerste River – Kuils River system. Jonkershoek was used as a negative control for it is situated near the origin of the Eerste Rivier, up in the Stellenbosch mountains. The aim of this study was firstly to validate rapid ELISAs for screening natural steroid hormones using commercially available ELISA test kits. Secondly, to use the validated ELISAs to determine the level of estrogens in treated sewage effluent.

3.3. Methods and Materials

3.3.1. Collection of samples.

Water samples were collected from three sewage plants on the Eerste River - Kuilsriver catchment area (E-KRCA) in March 2005 at the end of the dry season and again in April 2005 after the first winter rains (Fig. 3.1). A control sample was collected at Jonkershoek. The control site is near the origin of the river and upstream from all human activity. The water was collected in clean 2,5 liter glass containers and immediately transported to the laboratory for extractions. Extractions were done within 24 hours after sample collection. Samples were stored at 4 °C until extraction.

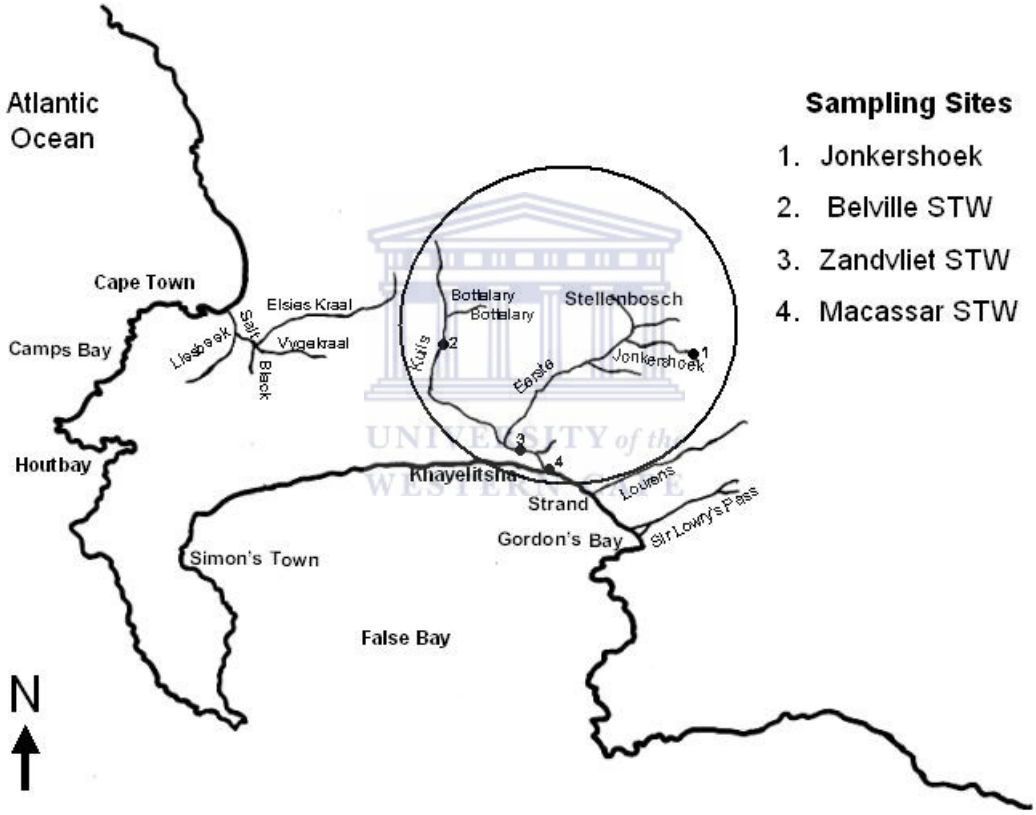

Figure 3.1. Water was collected from four sites along the Eerste- and Kuilsriver water catcment area: (1) Jonkershoek; (2) Belville STW effluent; (3) Zandvliet STW effluent; (4) Macassar STW effluent.

3.3.2. Extraction of water samples.

Water samples were extracted on C18 SPE columns (Anatech) using our in-house extraction procedure. In brief: C18 columns were pre-washed with 4 ml of solvent mixture (40 % hexane, 45 % methanol and 15 %, 2-propanol), followed by another wash with 4 ml of ethanol. The column was then washed with one column volume of reverse osmosis water

after which the water sample was applied onto the column. The column was then air-dried. The bound hydrophobic substances were eluted with solvent mixture. The eluate was dried under air and then reconstituted to 1/1000 th of the original sample volume with ethanol. The samples were stored at –20 °C.

3.3.3. ELISA Reagents

All reagents required for the assays are supplied with the kits.

3.3.4. ELISA for Estrone

Working conjugate solution was prepared by mixing 100 µl estrone-biotin and 100 µl avidin peroxidase conjugate and 9.8 ml assay buffer. The working conjugate were mixed and incubated at room temperature for at least 20 minutes prior to addition to the ELISA plate.

Concentrated (1000 x) water extracts were diluted 1/10 using 0.1 % (w/v) bovine serum albumin in 0.9 % NaCl. The diluted (100 x concentrated) extracts were assayed directly on the estrone ELISA kit (cat number DB52051, IBL, Germany) using the manufacturer's instruction manual. In brief: microtitre plate strips precoated with rabbit anti-estrone was removed from the strip holder and firmly fixed in the ELISA plate. All assays were done in duplicate. Samples and standards were transferred to the wells (25 µl per well), followed by the addition of working conjugate solution (100 µl per well). The contents of the wells were mixed by tapping the plate. The ELISA plate was then incubated for one hour at room temperature, followed by washing the plate four times with wash buffer (300 µl/well). TMB substrate was dispensed at 150 µl per well after which the plate was incubated for 15 minutes at room temperature. The reaction was stopped by the addition of stop solution (50 µl per well). The optic density (OD) was measured at 450 nm using a plate reader. A standard curve was drawn using the OD readings obtained for the standards and the concentrations for the samples were read off this curve.

3.3.5. ELISA for 17β-estradiol

Concentrated (1000 x) water extracts were diluted 1/10 using 0.1 % (w/v) bovine serum albumin in 0.9 % NaCl. The diluted (100 x concentrated) extracts were assayed directly on the estradiol ELISA kit (catalogue number RE52041, IBL, Germany) using the manufacturer's instruction manual. In brief: microtitre plate strips precoated with rabbit anti-estradiol was

removed from the strip holder and fixed firmly in the ELISA plate. All assays were done in duplicate. Samples and standards were transferred to the wells (25 µl/well). Estradiol – horseradish peroxidase conjugate was added to all the wells (200 µl/well). The solutions were mixed by gently tapping the plate, where after it was incubated for 120 minutes at room temperature. At the end of the incubation period the solutions in the wells were decanted after which the wells were washed three times with 300 µl/well of was solution. Substrate was then dispensed at 100 µl per well after which the plate was incubated for 15 minutes at room temperature. The reaction was stropped by addition of stop solution (100 µl/well). The OD was determined at 450 nm using a plate reader. A standard curve was drawn using the readings obtained for the standards and the concentration of the samples was read off this curve.

3.3.6. ELISA for Estriol

Concentrated (1000 x) water extracts were diluted 1/10 using 0.1 % (w/v) bovine serum albumin in 0.9 % (w/v) NaCl. The diluted (100 x concentrated) extracts were assayed directly on the estriol ELISA kit (cat number BM52011, IBL, Germany) using the manufacturer's instruction manual. In brief: microtitre plate strips precoated with rabbit anti-estriol was removed from the strip holder and fixed firmly in the ELISA plate. All assays were done in duplicate. Samples and standards were transferred to the wells (10 µl/well) followed by the addition of 100 µl estriol – horseradish peroxidase conjugate. The solutions was mixed by gently tapping the plate after which it was incubated for 1 hour at room temperature. The contents of the wells were decanted and the plates were washed four times with wash buffer (300 µl/well). TMB substrate was then added and the plates were incubated for 30 minutes at room temperature. The enzyme reaction was stopped by the addition of stop solution. The OD was determined at 450 nm using a plate reader. A standard curve was drawn using the reading obtained for the standards and the concentration of the samples was read off this curve.

3.3.7. Validation of assays

Kits were assayed for accuracy as follows: A dilution series of a sample containing high steroid hormone concentrations was prepared using 0.1 % (w/v) bovine serum albumin in 0.9 % (w/v) NaCl. The diluted samples were then assayed using the kit and the data obtained was plotted on the same graph as the standard curve to determine if the curves were parallel. Kit standard steroid hormones were also titrated with ethanol to determine the recovery of the ELISA assay on samples containing ethanol. The kits were assayed for intra-assay

reproducibility by assaying replicates of the same sample on a single assay plate. The kits were also assayed for inter-assay reproducibility by assaying the same sample on several plates.

3.4. Results

3.4.1. Validation of assays

Typical standard curve data for the estriol, estrone and estradiol ELISAs are presented in figure 3.2. The correlation coefficients for all three of the curves are between 0.97 and 0.99. The estriol ELISA has a detection range between 0.3 and 40 ng/ml. The estrone ELISA has range between 15 and 2000 pg/ml whereas the estradiol ELISA has a detection range between 25 and 2000 pg/ml. Sensitivity (minimum detection limit) was determined by the supplier.

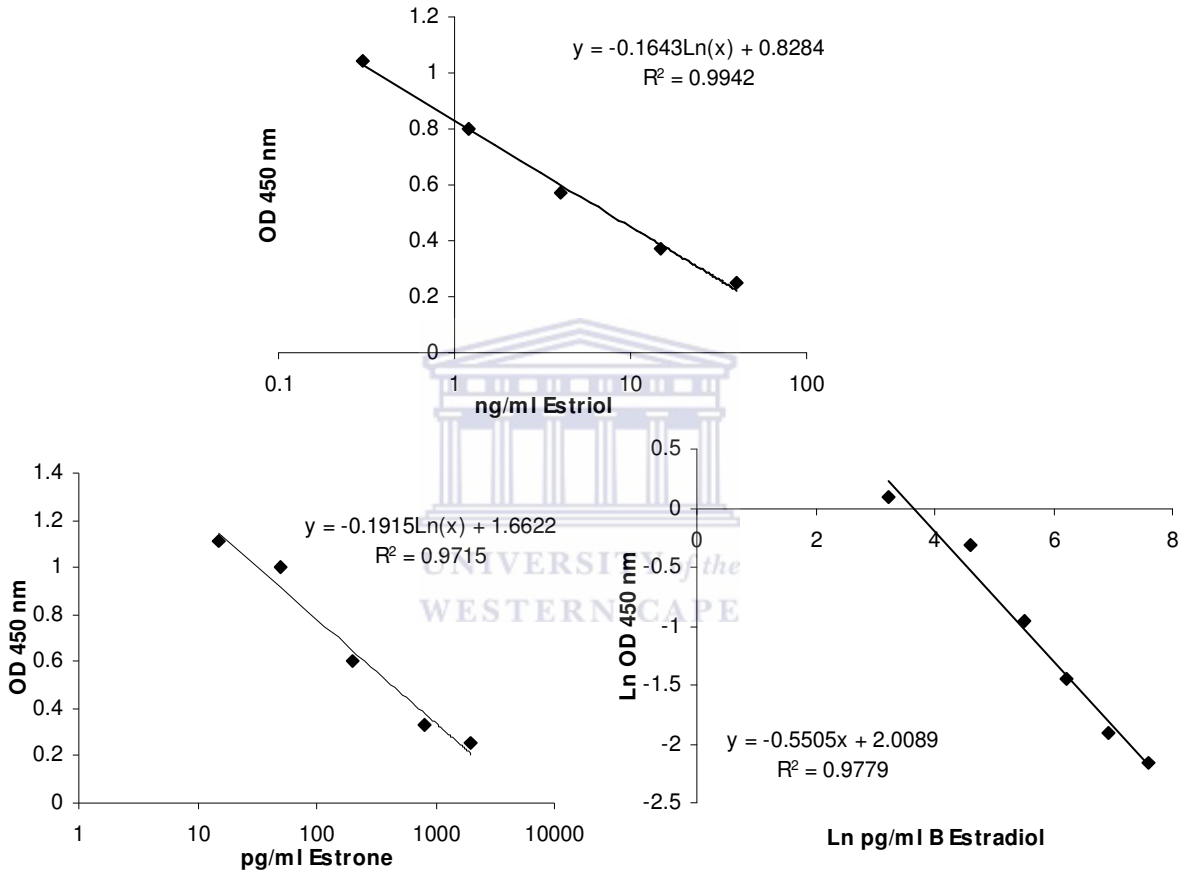

Figure 3.2. Standard curves for the ELISAs to determine estriol, estradiol and estrone concentrations in sewage effluent.

Parallelism of the ELISA kits standard curves and environmental samples was established using Macassar sewage effluent after the first rains as an environmental sample. Curves produced by assaying environmental samples at various dilutions showed parallelism with the standard curve produced for estrone and estradiol (Fig. 3.3). Parallelism between the standard curve of the estriol ELISA kit and the Macassar sewage effluent after the first rains could not be establish because estriol concentrations in the C18 extraction were already near the detection limit of 0.3 ng/ml.

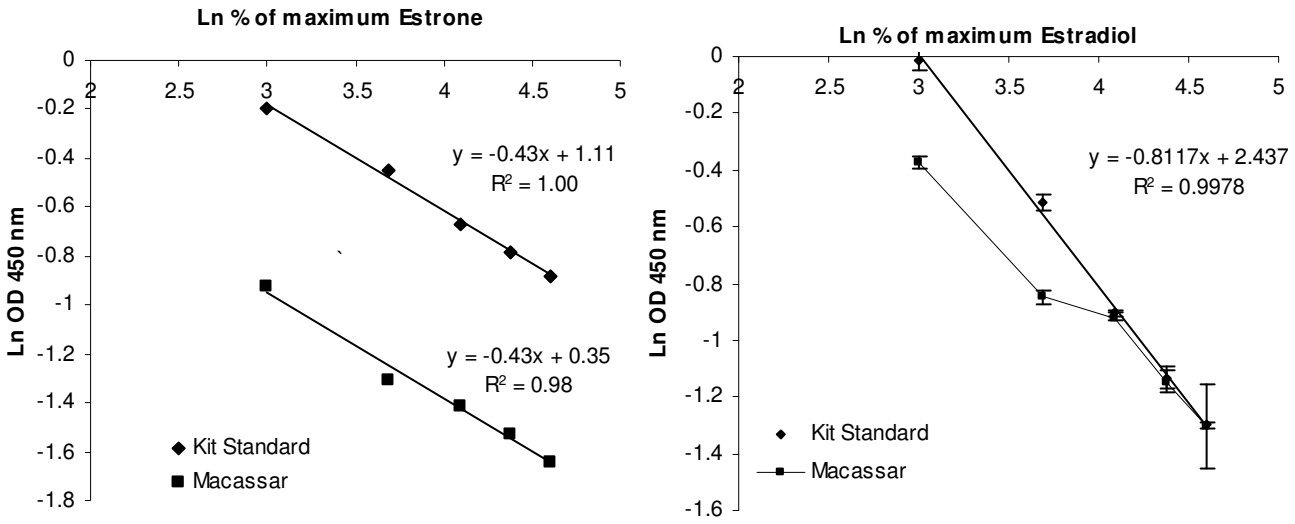

Figure 3.3. Parallelism between kit standard and sewage effluent sample.

Sewage effluent was subjected to C18 solid phase extractions and finally dissolved in analytical grade ethanol as described in the section, *methods and materials.* The supplier optimized the ELISA kits for the analysis of the specific steroid hormone in human serum. The effect of ethanol on the recovery of the ELISA assay was analyzed by assaying 10 % of the kit's maximum standard steroid hormone at 0 %, 10 % and 20 % ethanol. The estriol ELISA assay had a recovery of 98.3 ± 7.1 % at 10 % ethanol, whereas 87.7 ± 2.4 % could be established at 20 % ethanol (Table 3.1). There were no significant difference between the percentage recoveries (P = 0.125). Ethanol had no effect on the estrone ELISA (P = 0.576) with recoveries of 105.3 ± 7.2 % at 10 % ethanol and 102.1 ± 6.5 % at 20 % ethanol (Table 3.2). The estradiol ELISA had a recovery of 88.3 ± 3.9 % at 10 % ethanol and 98.6 ± 14.5 % at 20 % ethanol (Table 3.3). Ethanol again had no significant influence on the recovery of the estradiol ELISA (P = 0.346).

Intra assay variation was less than 2.5 % at 0, 10 and 20 % ethanol for the estriol ELISA, whereas inter assay variation was 5.6 ± 0.3 % at 10 % ethanol (Table 3.1). Intra assay variation was less than 7 % at 0, 10 and 20 % ethanol, whereas intra assay variation was 8.2 ± 0.7 % at 10 % ethanol for the estrone ELISA (Table 3.2). The estradiol assay had an intra assay variation of less than 8 % at 0 and 10 % ethanol. Intra assay variation is 13.9 ± 2.1 % at 20 % ethanol. Inter assay variation is 3.9 ± 0.1 % at 10 % ethanol (Table 3.3).

Table 3.1. Estriol ELISA validation. The effect of ethanol on recovery and intra assay variation as well as the inter assay variation at 10% ethanol on the estriol ELISA kit.

n	% Ethanol	% Recovery	% Intra assay variation	% Inter assay variation
3	0	100.6 ± 9.2	2.5 ± 0.2	
3	10	98.3 ± 7.1	1.9 ± 0.1	5.6 ± 0.3
3	20	87.7 ± 2.4	0.6 ± 0.02	

Table 3.2. Estrone ELISA validation. The effect of ethanol on recovery and intra assay variation as well as the inter assay variation at 10% ethanol on the estrone ELISA kit

n	% Ethanol	% Recovery	% Intra assay variation	% Inter assay variation
3	0	100 ± 3.3	3.3 ± 0.1	
3	10	105.3 ± 7.2	6.9 ± 0.5	8.2 ± 0.7
3	20	102.1 ± 6.5	6.4 ± 0.4	

Table 3.3. 17β-estradiol ELISA validation. The effect of ethanol on recovery and intra assay variation as well as the inter assay variation at 10% ethanol on the estradiol ELISA kit

n	% Ethanol	% Recovery	% Intra assay variation	% Inter assay variation
3	0	100.5 ± 8.7	8.4 ± 0.7	
3	10	88.3 ± 3.9	4.5 ± 0.2	3.9 ± 0.1
3	20	98.6 ± 14.5	13.9 ± 2.1	

3.4.2. The detection of estriol in sewage effluent

Very low concentrations of estriol were detected in sewage effluent from all the STWs. All the samples tested had levels lower than 1.1 ng/l of estriol (Fig. 3.4). These values were near to the lower detection limit of the ELISA kit. Samples collected in April and March, at all the STWs, show significant higher levels of estriol ($P = < 0.001$) in comparison with the control site, except for Macassar in March.

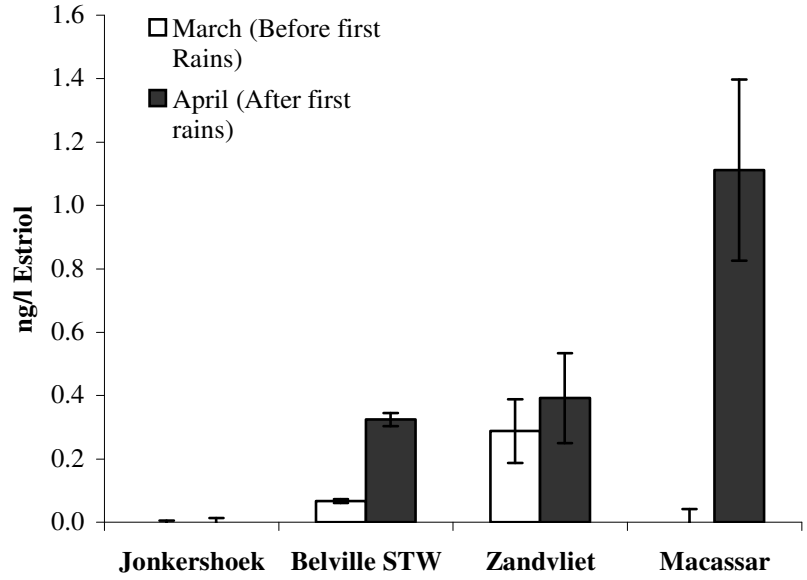

Figure 3.4. Estriol quantification in water extracts. Water samples were collected from sites along the Kuils and Eerste Rivers in March and April. The samples were extracted using C18 chromatography and then assayed using the estriol ELISA kit

3.4.3. The detection of estrone in sewage effluent

Less than 0.2 ng/l estrone were detected in the Jonkershoek samples (Fig. 3.5).

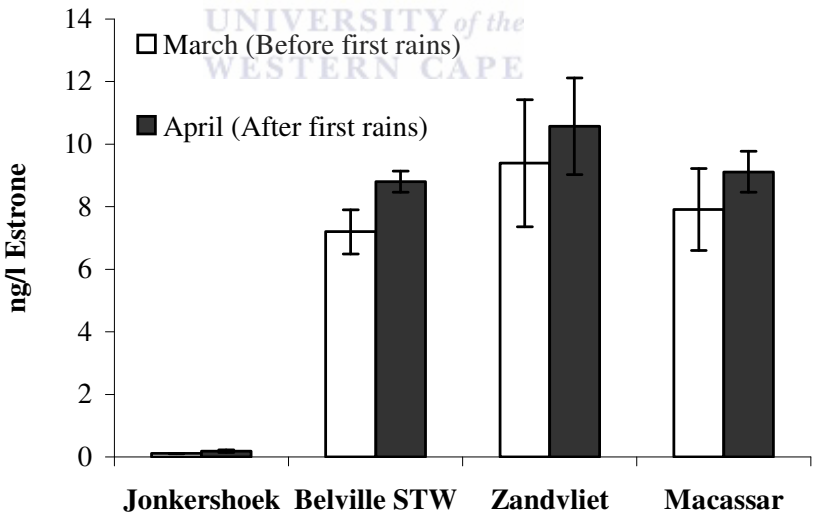

Figure 3.5. Estrone quantification in water extracts. Water samples were collected from sites along the Kuils and Eerste Rivers in March and April. The samples were extracted using C18 chromatography and then assayed using the estrone ELISA kit.

The levels of estrone was significant higher in the sewage effluents in comparison with the control site ($P = < 0.001$). Zandvliet had the highest level of estrone in both March and April, 9.4 and 10.6 ng/l respectively. Effluent collect from Belville in March had the lowest level of

estrone (7.2 ng/l). Generally samples taken in April have higher levels of estrone in comparison with March, although statistically insignificant (P = > 0.001).

3.4.4. The detection of estradiol in sewage effluent

Very low levels of estradiol were detected in samples obtained from the control site in Jonkershoek (Fig. 3.6). There were significant higher levels of estradiol detected from the sewage effluents in comparison with the control site (P = < 0.001). Estradiol levels ranged between 0.8 ng/l (Macassar, April) and 4.7 ng/l (Zandvliet, April). At both Zandvliet and Macassar estradiol levels were higher in April then in March.

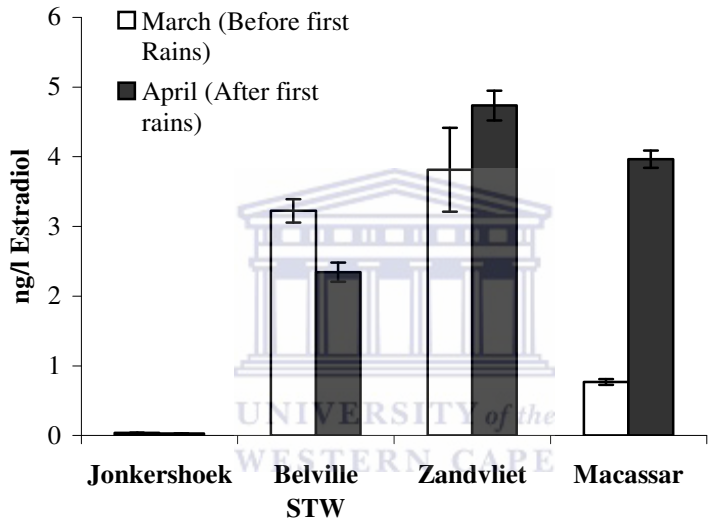

Figure 3.6. 17β-estradiol quantification in water extracts. Water samples were collected from sites along the Kuils and Eerste Rivers in March and April. The samples were extracted using C18 chromatography and then assayed using the estradiol ELISA kit.

3.5. Discussion

As stated in the introduction, the purpose of this work was to validate commercially available rapid ELISA kits for the quantification of estrogens in sewage effluent. One of the major potential obstacles to overcome was the fact that these ELISA kits are optimized for the quantification of estrogens in blood serum. Sewage effluents samples subjected to C18 solid phase chromatography extraction is ultimately reconstituted in ethanol before assayed using the ELISA kits in this study. Recovery analysis showed that ethanol had no significant effect on the sensitivity of the ELISA kits towards the specific steroid hormone assayed (Table 3.1, 3.2, 3.3). One way analysis of variance (ANOVA) on the hormone recovery of the ELISA kits showed that no significant difference exist between samples containing 0, 10 and 20 % ethanol for the estriol, estrone nor estradiol kits. Moreover, intra- and inter assay variability was less than 5.6 %, 8.2 % and 4.5 % for the estriol, estrone estradiol ELISA kits respectively at 10 % ethanol in the sample (Table 3.1, 3.2, 3.3). This data illustrates that these ELISA kits are highly repetitive with minimal inter- and intra assay ELISA kit interference. The validation of these ELISA kits used to determine steroid hormone levels in sewage effluent are further supported by good parallelism between dilution curves of the kit standards and sewage effluent samples (Fig. 3.3).

Analysis of estrone, estradiol and estriol levels showed a significant difference between the control site (Jonkershoek) and sewage effluent from three STWs (Bellville, Zandvliet and Macassar) (Fig. 3.4, 3.5, 3.6). The control site contained very low or less than the lowest observable level of estrone, estradiol and estriol. This was to be expected because this sample site is situated in the mountains, near the origin of the Eerste River and is not exposed to any human activity except for occasional hikers. Estriol, estrone and estradiol concentrations were higher in April than in March, although this is not statistically significant. April samples were taken shortly after the first heavy winter rains. A possible explanation may be that the sewage plants were not able to handle the increased influent volumes of water. Therefore some overflowing or contamination of sewage effluent with raw sewage water may have occurred.

Estriol had the lowest levels, less than 1.1 ng/l, of the three steroid hormones analyzed in this study. A study previously conducted by Baronti et al. (2000) showed the same trend where estriol was detected at much lower levels than estradiol and estrone. Zandvliet showed the highest levels of both estrone and estradiol. Estrone levels were between 7.2 and 10.6 ng/l, whereas estradiol levels range between 0.8 and 4.7 ng/l. These values are similar to the lower

range of estrone and estradiol concentrations detected in sewage effluent from Brittan (Desbrow et al., 1998), Italy (Baronti et al., 2000), Germany (Ternes et al., 1999a), Canada and Netherlands (Belfroid et al., 1999). Although the steroid hormone levels in our study may seem low, it is still quite concerning due to the fact that this water is used for agricultural and horticultural irrigation, drank by farm animals, farm workers as well as people from informal settlements along river banks. Concentrations as low as 1 ng/l of 17β-estradiol (natural estrogen) led to induction of vitellogenin in male trout (Hansen et al., 1998). It was also observed that ova formed in the testis of Japanese medaka at concentrations as low as 4 ng/l 17β-estradiol (Metcalfe et al., 2001). Futhermore, Alfalfa plants irrigated with sewage effluent containing steroid hormones, display elevated levels of phytoestrogens (Shore et al., 1995).

To our knowledge, this is the first study conducted in South Africa to demonstrate the presence of significant amounts of steroid hormones released back into the environment from sewage treatment plants. Detailed surveys are necessary to understand the distribution of steroid hormones in the environment. Significant levels of these steroid hormones do have the potential to cause endocrine disruption, not only in animals but in humans as well. Future work must be directed to test whether these samples are biologically active through *in vivo* and *ex vivo* studies.

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Chapter 4

MCF-7 total LDH, XTT and total protein as biomarkers for estrogenicity

Key words: MCF-7, LDH, XTT, estrogenicity, bio-monitoring, EDCs.

4.1. Abstract

The occurrence of environmental chemicals with estrogenic activities are a major concern internationally. Studies have shown that these chemicals, or estrogenic endocrine disrupting chemicals (e-EDCs), can pose a health risk to both males and females. The aim of this study was to compare various biomarkers of the MCF-7 cell line as end-points for estrogenic activity. The MCF-7 cell line is an estrogen dependent cell line and has been used previously in the E-screen for estrogens. The biomarkers that we used were total cellular LDH, total cell protein and mitochondrial activity using the XTT assay. The current study shows that the total cellular LDH assay is the most sensitive assay for 17β-estradiol (E2) monitoring. The sensitivity of the total LDH assay with the MCF-7 cell line was 0.1-1000 nM E2.

4.2. Introduction

Estrogens play an important role in the growth, differentiation and functions of many target organs. These organs include both male and female reproductive systems such as the mammary gland, uterus, vagina, ovary, testis, epididymis and prostate (Korach et al., 1995; Couse et al., 1999). Every year a large number of diverse synthetic chemicals (xenobiotics) are released into the environment. This is due to increased agricultural and industrial activity. These chemicals include pesticides, insecticides, fungicides, herbicides, solvents, detergents styrenes, polychlorinated biphenyls, and penta- to nonylphenols that accumulate in the air, water and food chain (Colborn and Clement 1992; Colborn et al., 1993). The problem is that despite the structural diversity among these chemicals, they all have the ability to bind to estrogen receptors (ER) in cells of the body and can initiate (agonist) or inhibit (antagonist) estrogen-like actions (Soto et al., 1992; Eckert and Katzenellenbogen, 1982). In doing so, they may disrupt normal endocrine function that may lead to reproductive failure in wildlife and humans (Sharpe and Skakkebaek, 1993; Hoyer et al., 1998). The chemicals that mimic the effect of natural estrogens are collectively referred to as estrogenic endocrine disruptors (e-EDCs). It has been reported that e-EDCs may be responsible for congenital malformations of the reproductive tract and increased incidences of cancer in estrogen responsive tissues (Colborn et al., 1993; Davis et al., 1993). Concerns regarding the adverse effects of e-EDCs on human health include reports of increased incidences of testicular (Osterlind, 1986), prostate and breast (Wolff and Toniolo, 1995) cancer.

The incidence of testicular cancer has significantly increased over the last few decades (Forman and Moller, 1994). The tumors are primarily from germ cell origin. Testicular cancer is the most common malignancy among men of age between 25-34 years in Denmark, while in Finland the incidence is much lower (Adami et al., 1994). Cancer of the prostate is the second leading cause of cancer deaths in males in the USA. A meta-analysis found a positive correlation between prostate cancer and farming occupation (Keller-Byrne et al., 1997). Cancer of the breast is the most frequent tumor in women in the world. The increased risk of breast cancer has been suggested to be related to exposure to e-EDCs. Several case-control studies published in the last two decades have raised the issue that women exposed to organochlorine chemicals such as DDT and certain PCB congeners may have higher incidences of breast cancer than non-exposed women (Dewailly et al., 1994). Endometrioses is characterized by growth of endometrial cells

outside the uterus. The etiology of this disease is unknown. Exposure to estrogen-like compounds has been hypothesized to be involved in the disease process (Hill, 1992). An association between endometriosis and high levels of PCB in plasma has been reported (Gerhard and Runnebaum, 1992).

Estrogen responsive cancer tissues have been widely used for the development of *in vitro* assays to detect e-EDCs. The most common estrogen dependent human cell lines which are used for the development of estrogenic test assays are breast cancer cell lines such as MCF-7 (E-Screen) or T47D (Soto et al., 1992, 1995). In all of these *in vitro* systems, the ability of a specific chemical, or complex environmental mixture, to stimulate the growth of an estrogen dependent cell lines is measured (Soto et al., 1992, 1995). *In vitro* cell based bio-assays offer a rapid, sensitive and relatively inexpensive solution to some of the limitations of mass based chemical instrumental analysis such as HPLC, GC/MS, GC–MS/MS, and LC–MS/MS (Petrović and Barcelo, 2000; Huang and Sedlak, 2001; Petrović et al., 2002). They enable estimation of total biological activity of all compounds that acts through the same mode of action present in extracts of environmental medium. Bio-assays also integrate possible interactions among chemicals (Giesy et al., 2002). *In vitro* whole cell bio-assays for estrogenicity are based on genetically engineered eukaryotic or wild type cells. The cells are used to asses the ability of individual chemicals or extracts of complex environmental mixtures to cause an ER mediated proliferation effect. The MCF-7 cell line is a human breast cancer cell line that has been derived from a patient with metastatic breast adenocarcinoma at the Michigan Cancer Foundation (Soule et al., 1973). Lipman and co-workers discovered estrogen-responsive cell growth of MCF-7 cells in 1976 (Lippman et al., 1976). In the E-screen assay developed by Soto et al. (1992), proliferation of MCF-7 cells as a response to estrogen is measured. The E-screen is based on the following three premises: (i) factors in human serum inhibit the proliferation of MCF-7 cells, (ii) estrogens induce cell proliferation by negating this inhibitory effect, and (iii) non-estrogenic steroids and growth factors do not neutralize the inhibitory signal present in human serum (Soto et al., 1992, 1995; Sonnenschein et al., 1996; Zacharewski, 1997).

The MCF-7 cell line has been extensively used to study the molecular interactions of estrogens and anti-estrogens with the ER (Brooks et al., 1973). Cellular bio-assays such as the MCF-7

breast cancer cell proliferation assay (E-Screen) measure the potency of a sample to induce cell proliferation (Soto et al., 1992 and Soto et al., 1995). Several methods have been developed to quantify cellular proliferation. Examples include the capability of the cells to incorporate a radioactively labeled substance ([^{3}H] – thymidine). Total cellular protein synthesis and total cellular DNA synthesis have also been used as proliferation endpoints. An alternative assay is the XTT assay, which is based on the cleavage of the yellow tetrazolium salt XTT to form an orange fomazan dye by metabolically active cells (Gerlier and Thomasset, 1986). This conversion only occurs in viable cells and is therefore a sensitive assay to determine metabolically active cells. Another alternative assay for proliferation, as well as cytotoxicity, is the detection of the cytoplasmic enzyme, lactated dehydrogenase (LDH). LDH is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane and therefore a valuable marker for cytotoxicity. Alternatively, when the cell culture supernatant is discarded and the remaining cells thoroughly washed and then lysed, LDH levels can be used as a alternative indicator of cell number (similar to total DNA/protein). Breast cancer proliferation assays provide a sensitive measure of estrogenicity and complement receptor-binding assays by providing a further dimension to estrogenicity evaluation.

The aim of this study was to compare and evaluate multiple endpoints of the MCF-7 E-Screen assay as biomarkers for environmental estrogens and cytotoxicity. Total protein, LDH and XTT were evaluated as measurements of estrogen dependent proliferation effect. LDH activity released into the cell culture medium was also used as an indicator of cytotoxicity. In this study the E-screen assay was also simplified by establishing MCF-7 cultures a 96-well format without fetal bovine serum.

4.3. Methods and Materials

4.3.1. Hydrophobic phase extraction of water samples

The hydrophobic content of environmental water samples were extracted on C18 SPE columns (Anatech) using a method previously described by Swart and Pool (2007). In brief: C18 columns were pre-washed with 4 ml of solvent mixture (40 % hexane, 45 % methanol and 15 %, 2-propanol), followed by another wash with 4 ml of ethanol. The column was then washed with one column volume of HPLC grade water after which the water sample was applied onto the column. The column was then air-dried. The bound hydrophobic substances were eluted with solvent mixture. The eluate was dried under air and then reconstituted to 1/1000 th of the original sample volume with dimethyl sulfoxide (DMSO). The samples were stored at –20 °C until further use.

4.3.2. Cell culture reagents

RPMI-1640 with L-glutamine and phenol red and RPMI-1640 modified without L-glutamine and phenol red were supplied from Sigma Aldrich. Glutamax (L-glutamine), fetal bovine serum (FBS), antibiotic-antimycotic solution, phosphate buffered saline (PBS) solution, serum replacement solution, trypsin ethylene diamine tetra-acetic acid (EDTA) solution and 17β-estradiol were also supplied from Sigma Aldrich. Estradiol was prepared as a 1mg/ml stock solution in Dimethyl sulfoxide (DMSO). Estradiol (E2) and environmental extracts were diluted 1/1000 (v/v) in culture medium for all assays.

4.3.3. MCF-7 experimental cell culture

MCF-7 cells were maintained and harvested as previously described by Soto et al. (1992, 1995). Confluent growing MCF-7 cells (70 %) were suspended in *full medium* (RPMI-1640 medium containing phenol red and L-glutamine and supplemented with 10 % (v/v) FBS and 1/100 diluted antibiotic-antimycotic solution) to a concentration of 5 x 10^5 cells/ml. The cell suspension were then dispensed at 200 µl per well in 96 well flat bottom tissue culture plates (Nunc, AEC-Amersham). Cells were allowed to attach for a minimum of 5 hours before the medium was decanted and the wells rinsed twice with PBS pre-heated to 37 ˚C. The wells were then filled with 200 µl *estrogen deprived medium* (RPMI-1640 modified without L-glutamine and phenol red and supplemented with 1/100 diluted antibiotic-antimycotic solution, 1/100 diluted glutamax

and 1/50 diluted serum replacement). Cells were allowed to grow for 48 hours in order to deplete estrogen levels. Medium was then replaced with estrogen deprived medium containing the appropriate concentration of 17β-estradiol, environmental water extract diluents or dimethyl sulfoxide (DMSO) vehicle control. Cells were cultured for another 48 hours before any procedures were done.

4.3.4. Lactate dehydrogenase (LDH) detection

4.3.4.1. Culture supernatant LDH for cytotoxicity

Cell death was determined by measuring lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. LDH was measured using the instructions of a cytotoxicity detection kit supplied from Roche Applied Science. In short: Cells were grown in a in a 96 well format as described in the previous section. Following the final 48 hour culture period, culture supernatants were transferred to an optically clear 96-well flat bottom plate. LDH reaction mixture was prepared according to the manufacturer's instructions immediately before use. To determine the LDH activity, 100 µl of LDH reaction mixture were added to 100 µl of 1:2 diluted culture medium (diluted with estrogen deprived medium) and gently tapped to mix. The absorbance was immediately measured at 492 nm to obtain a background reading. The plate were now incubated in the dark for 30 min at room temperature, where after the absorbance were again measured.

4.3.4.2. Total cellular LDH indicative of cell number

In order to obtain total LDH levels, culture supernatant was removed from the exposed cells after the last 48 hour incubation period. Estrogen deprived medium containing 2 % (v/v) Triton X-100 was added at 200 µl/well. The plate was incubated on a shaker for 10 min at 300 rpm after which the cell lysates were assayed for LDH as described above.

4.3.5. Total protein yield by MCF-7 culture determinations

Following the last 48 hour culture period of the MCF-7 cells, culture supernatants were replaced with 100 µl of a 1 M NaOH solution. The 96 well plates were allowed to gently shake at room temperature for 30 minutes. A multi pipet was used to gently detach all cells after which the appropriate volume of cell lysate was transferred to clear 96 well plates. Total protein yield of the

cell lysate was determined according to the method of Bradford (1976) using Biovine Serum Albumin (BSA) as a standard protein (Sigma).

4.3.6. XTT determinations

The cell proliferation assay (XTT) was performed using a kit which was supplied from Roche Applied Sciences, according to the manufacturer's instructions. In short: Cells were grown in a 96 well format as described in the previous section. XTT reaction mixture was prepared according to the manufacturer's instructions immediately before use. To determine the XTT activity, 50 µl of XTT reaction mixture were added to the each well after the final cell culture incubation period. The absorbance was immediately measured at 492 nm to obtain a background reading. The plate were now incubated in the dark for 2 hours, where after the absorbance was again measured.

4.3.7. Cell number determination

Cell counts were performed using a hemacytometer under a inverted phase microscope.

4.3.8. Statistical analysis

Differences in response of MCF-7 cells between controls groups and treatments were analyzed using analysis of variance (ANOVA). Tuckey's HSD test was used for all pair wise multiple comparisons.

4.4. Results

4.4.1 The effect of estrogen on MCF-7 cell growth

MCF-7 cells were cultured for 48 hours in full medium as explained in the methods and materials section. Following the initial 48 hour culture, MCF-7 cells received three different medium treatments. The control treatment again received full medium (containing FBS and phenol red RPMI medium). The two other treatments were cultured in estrogen deprived medium complimented with the vehicle control or 10 nM E2 (cultures received commercially available serum replacement solution). After the 48 hour culture period, the number of cells (4.1A), total protein yield (Fig. 4.1B), total LDH (Fig. 4.1C) and XTT (Fig. 4.1D) were determined for each of the culture medium treatments.

No significant differences in the cell number were observed between cells cultured in full medium and estrogen deprived medium complimented with 10 nM E2 (Fig. 4.1A.). Significantly lower cell numbers ($P < 0.01$) were observed for cells cultured in estrogen deprived medium. Similar results were observed when the total protein yield, total LDH- and XTT activity were determined for the three different cell culture treatments (Fig. 4.1 B,C,D.). Total LDH-, XTT activity and total protein were significantly lower for cells cultured in estrogen deprived medium compared to cells cultured in full medium or estrogen deprived medium complimented with 10 nM E2. Cells cultured in full media resulted in similar total protein yield, total LDH- and XTT activity compared to cells cultured in estrogen deprived medium supplemented with 10 nM E2.

4.4.2. E2 dose response on MCF-7 proliferation

MCF-7 cells were cultured in estrogen deprived medium complimented with a 10 fold dilution series of E2. The same volume of the vehicle control (DMSO) was used with all E2 concentrations so that the latter were the only variant. At the end of the culture period, culture supernatant LDH (results not shown), total cellular LDH (Fig 4.2A), total protein (Fig 4.2B), and XTT (Fig 4.2C), activity were determined for cells at each of the E2 concentration exposures. Statistical analysis shows that E2 does not have an effect on LDH release into the culture medium. The proliferation effect of estrogen dependent MCF-7 cells can be observed when total LDH, total protein yield and XTT activity were determined for the same cultures.

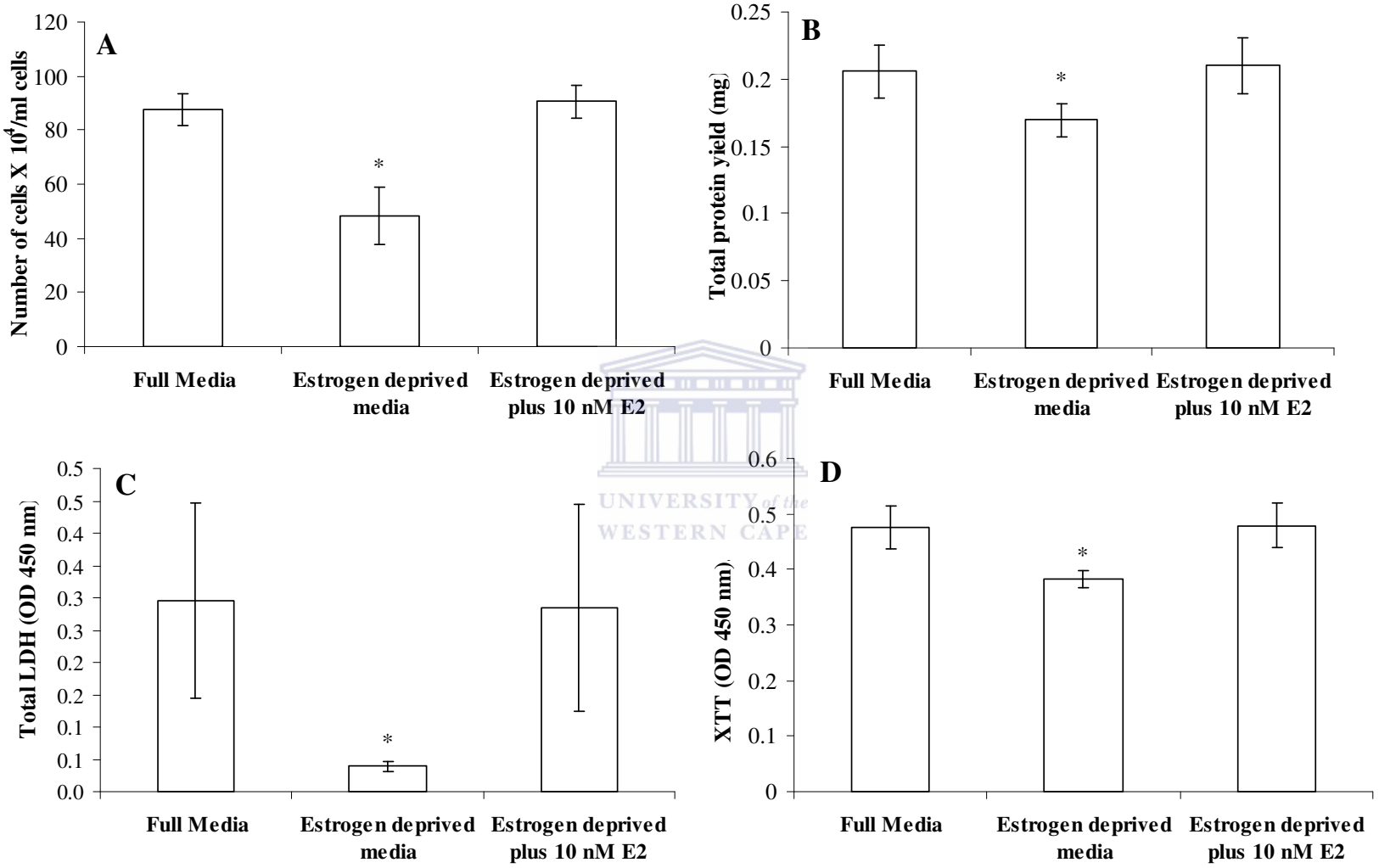

Figure 4.1. Culture of MCF-7 cells with serum replacement solution. MCF-7 cells were cultured in three different mediums. After 96 hours of culture, the number of cells was counted using a hemacytometer (A); total protein yield was determined (B); total LDH activity (C); and XTT activity measured (D). Vertical error bars represent the standard deviation of the mean.

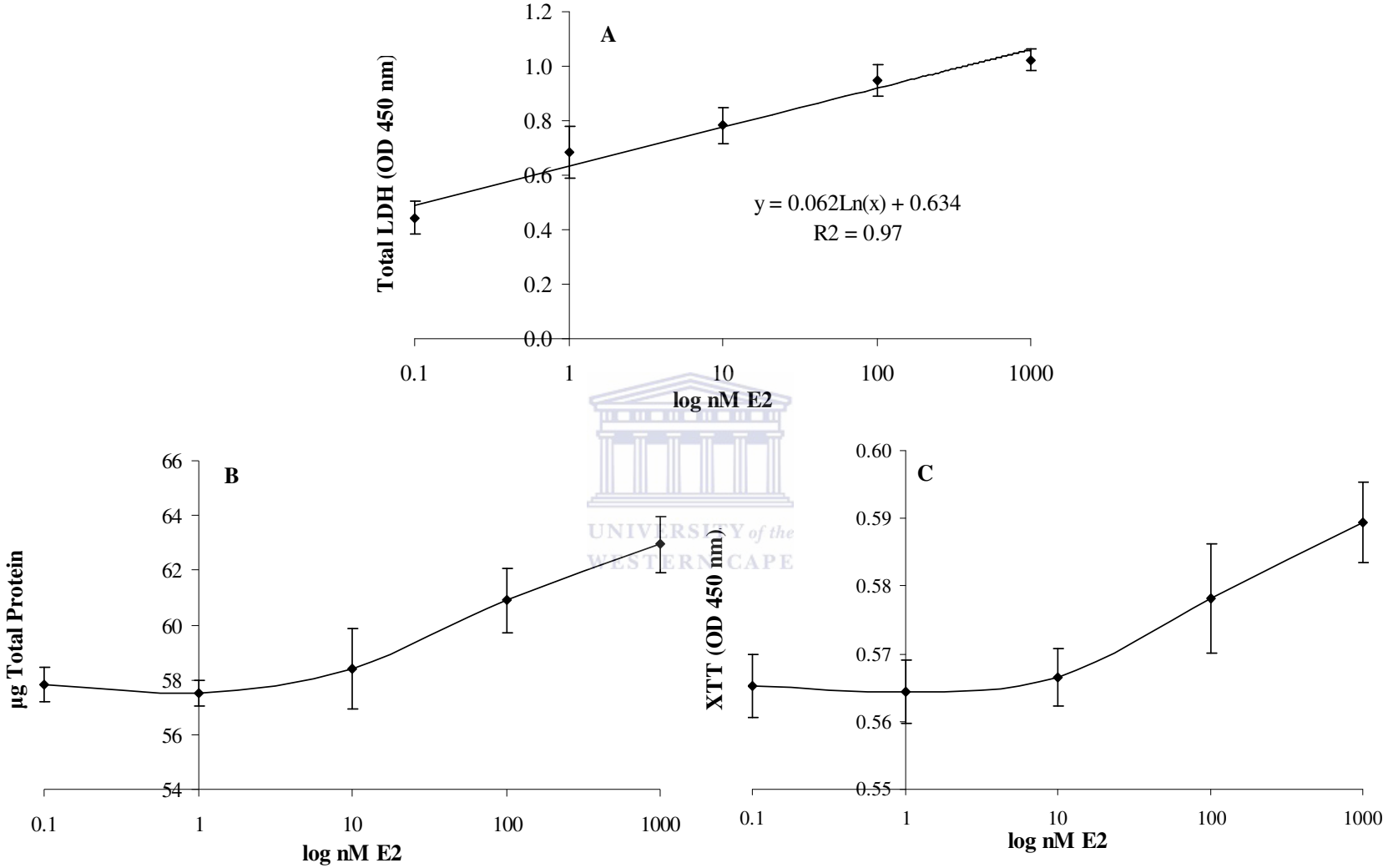

Figure 4.2. MCF-7 E2 dose responsiveness. MCF-7 cells were cultured in estrogen deprived medium after which it was exposed to a dilution series of E2. Total LDH (A), total protein (B), and XTT activity (C) were determined. Vertical error bars represents the standard deviation of the mean for ten replicates.

A direct linear correlation with an R^2 value of 0.9 exists between the concentration of E2 exposed MCF-7 cells and total LDH levels. The linear range for the total LDH assay was between 0.1 – 1000 nM E2. This is wider than the detection range obtained for total protein and XTT activities (10 – 1000 nM E2).

4.4.3. Correlation of total LDH with total protein and XTT

The total LDH levels obtained for MCF-7 cells exposed to a dilution series of E2 between 1 and 1000 nM E2 were correlated with XTT activity (Fig. 4.3A) and total protein yield (Fig. 4.3B). A good correlation between total LDH and XTT activity with an R^2 value of 0.93 were obtained. A good correlation between total LDH and total protein yield were also obtained with an R^2 value of 0.97.

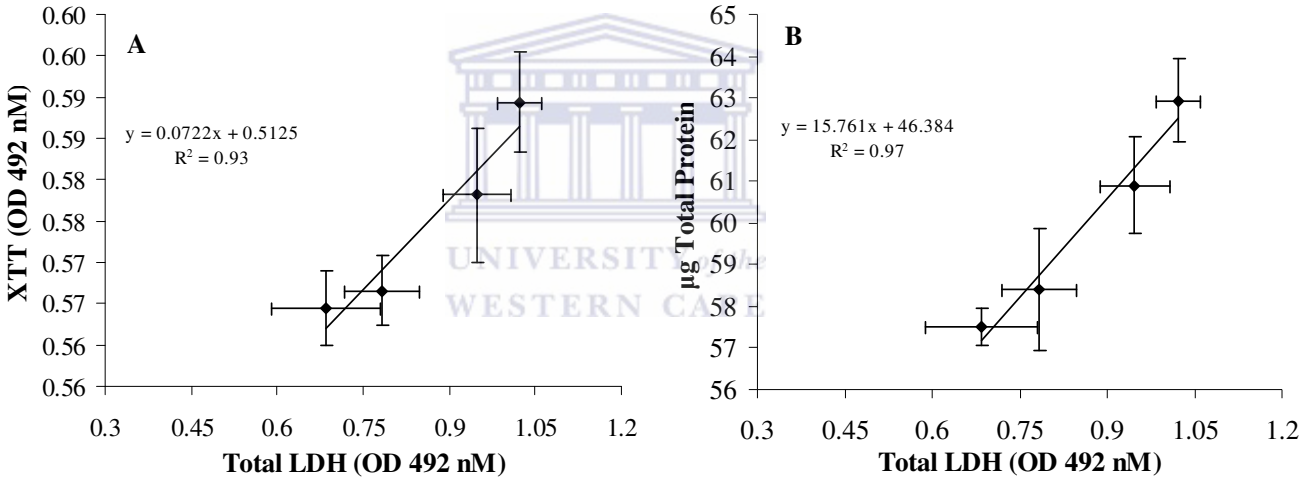

Fig. 4.3. Correlation of total LDH activity with XTT and total protein yield. MCF-7 cells were exposed to a 10 fold concentration series between 1 and 1000 nM of E2. Total LDH activity are plotted again XTT activity (A) and total protein yield (B). Vertical and horizontal error bars represent the standard deviation of the mean for ten replicates.

4.4.4. Analysis of environmental water extracts for estrogenicity and cytotoxicity

Water samples were collected from a pristine site, which is situated in the mountains, and sewage effluent from a local town. The hydrophobic content of the water samples were extracted as explained in the methods and materials. MCF-7 culture supernatant LDH activity was measured in order to assess if the water extracts were cytotoxic (Fig. 4.4). Total LDH activity (Fig. 4.5A), total protein yield (Fig. 4.5B) as well as XTT activity (Fig. 4.5C) were used as endpoints to assess the estrogen dependent proliferation of MCF-7 cells exposed to these water extracts.

4.4.4.1 Cytotoxicity of environmental water extracts

No significant differences in the culture supernatant LDH levels could be observed when comparing results obtained from cultures exposed to 0.1 nM E2 and 1000 nM E2 (Fig. 4.4). LDH levels obtained from MCF-7 cells exposed to water from the pristine site also showed no significant differences with the E2 standard and control. Medium LDH levels from cultures exposed to sewage effluent were significantly higher (42 %) than that of the pristine site.

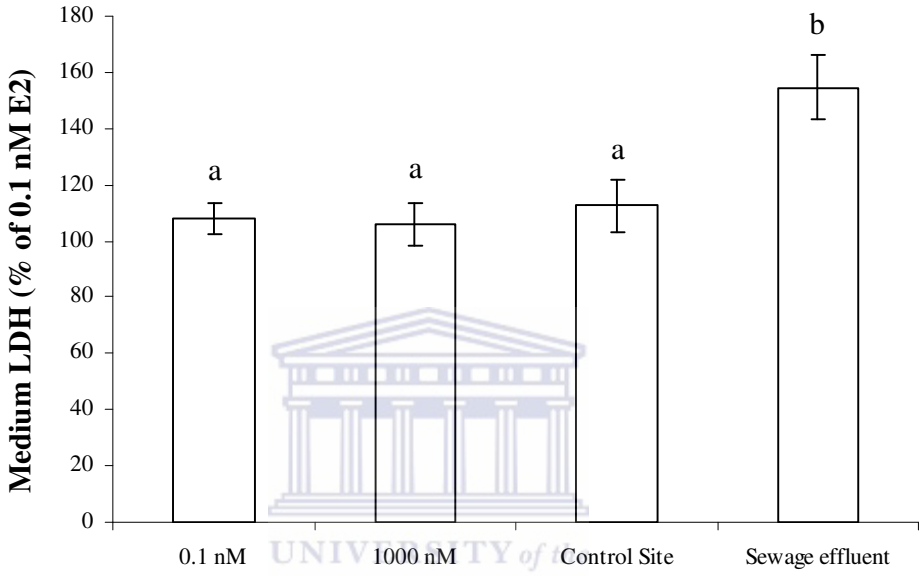

Fig. 4.4. Cytotoxicity of water samples. Water samples collected from a pristine site and sewage effluent were subjected to the MCF-7 screen for estrogenicity and cytotoxicity. Culture supernatant LDH activity was determined in order to assess cytotoxicity. Vertical error bars represents the standard error of the mean of six assays.

4.4.4.2 Estrogenicity of environmental water extracts

A significant difference ($P < 0.001$) between the 0.1 and 1000 nM E2 standards were observed for total LDH (Fig. 4.5A), total protein (Fig. 4.5B) and XTT (Fig. 4.5C) activity. For each of these endpoints determined, no significant differences could also be observed between the 0.1 nM E2 standard and the pristine site. However, total LDH, total protein and XTT activity of MCF-7 cells exposed to sewage effluent, were statically similar to that of MCF-7 cells exposed to the 1000 nM E2 control. Total LDH activity and total protein yield of the MCF-7 cells exposed to water extracts from the pristine site were statistically similar to cells exposed to the sewage effluent. Total LDH and total protein yield of the pristine sites were however significantly lower than that of cells exposed to 1000 nM E2.

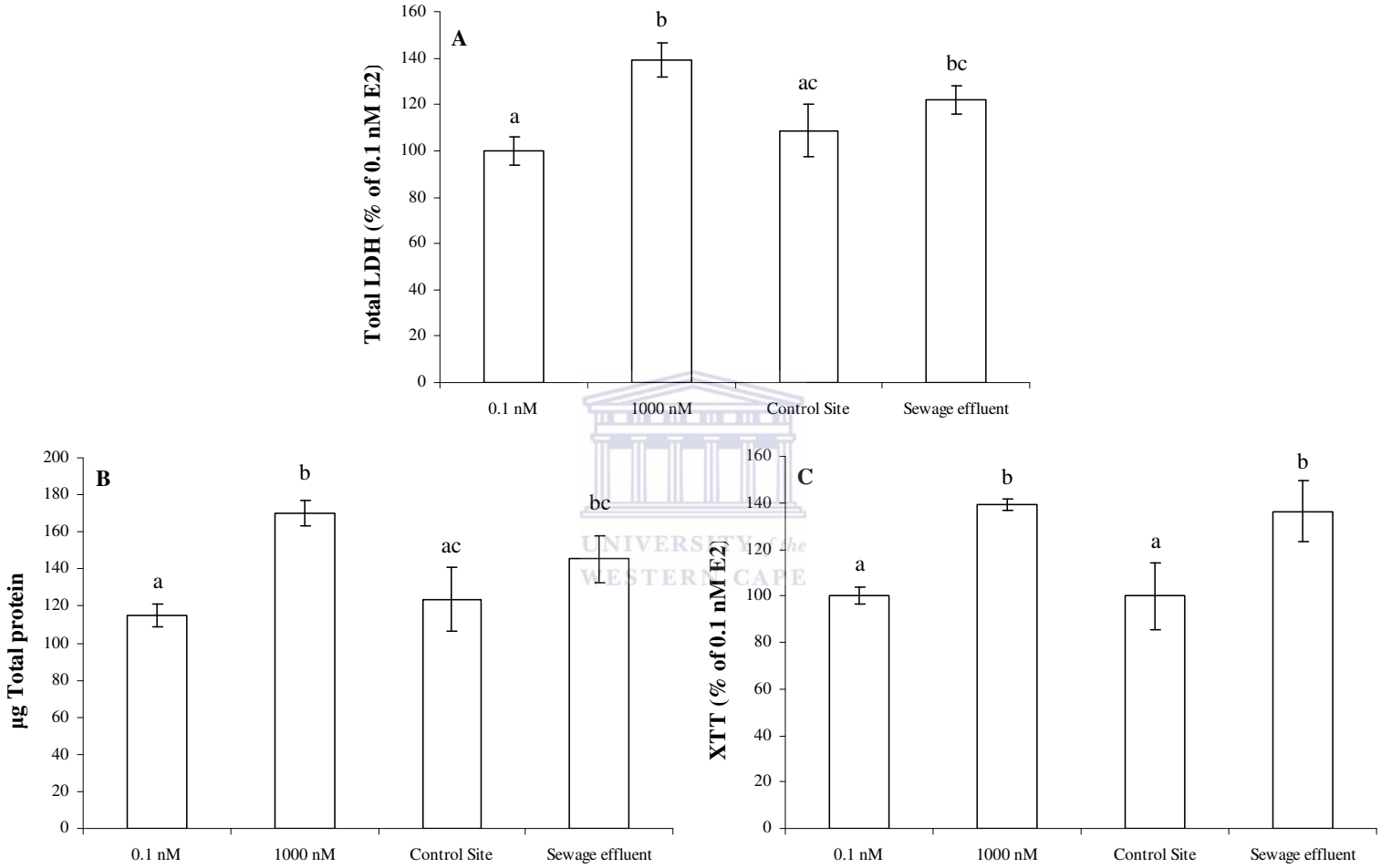

Figure 4.5. Estrogen induced proliferation of MCF-7 cells exposed to environmental water samples. Water samples collected from a pristine site and sewage effluent were subjected to the MCF-7 screen for estrogenicity and cytotoxicity. Total LDH levels (A), total protein yield (B) and XTT activity (C) was determined as indicators of MCF-7 estrogen dependent proliferation. Vertical error bars represents the standard error of the mean of six assays.

4.5. Discussion

A large number of *in vitro* assays have been developed over the years for the screening of estrogenic EDCs (ECETOC, 1996; Holmes et al., 1998; Soto et al., 1992). The most recent literature regarding estrogenic EDCs have focused mainly on the development and evaluation of different detection methods (Soto et al., 2006). Bio-assays are useful alternatives (Campbell et al., 2006) for traditional mass based analytical assays such as HPLC, GC/MS, GC–MS/MS, and LC–MS/MS (Petrović and Barcelo, 2000; Huang and Sedlak, 2001; Petrović et al., 2002). The MCF-7 human breast cancer cell line has been extensively used to study the molecular interactions of estrogens and anti-estrogens with the ER (Brooks et al., 1973). This cell line has also been used in various *in vitro* cellular bio-assays, of which the cell proliferation assay (E-Screen) first described by Soto et al., (1992) is probably the most common of them all. The aim of the present study was to evaluate several endpoints of the E-Screen assay as biomarkers for estrogenicity and cytotoxicity. Total LDH activity, total protein yield as well as XTT activity were evaluated as proliferation endpoints and biomarkers for estrogenicity. This study was concluded by screening environmental samples for estrogenicity and cytotoxic using the different validated biomarkers for estrogenicity and cytotoxicity.

One of the aims of this study was to introduce some modifications and simplifications to the well known E-Screen. The culture of MCF-7 cells in a 96-well format using chemically defined serum replacement factors instead of FBS was validated. All of the E-screen protocols normally involve the maintenance of MCF-7 cells in medium containing human serum or FBS. In order then to use these cells in a screen for estrogens, medium has to be changed to estrogen deprived medium which consist of medium without phenol red and FBS that has been charcoal dextran treated (stripping FBS of hormones). In the current study we made use of an estrogen deprived medium that contains no FBS. In stead of going through all the effort, time and costs involved of stripping FBS with charcoal and dextran, we validated the use of a commercially available serum replacement solution in our estrogen deprived medium. MCF-7 cells were cultured as previously described by Soto et al., (1995). The results show that MCF-7 cells that were cultured in estrogen deprived medium (containg no FBS but serum replacement solution and phenol red free RPMI) resulted in 44 % reduction in cell number (Fig. 4.1A), 18 % reduction in total protein yield (Fig.4.1B), 86 % reduction in total LDH activity (Fig. 4.1C) and 19 % reduction in XTT activity

(Fig. 4.1D) in comparison with cells cultured in maintenance medium (containing FBS and normal RPMI with phenol red). Cells that were cultured in estrogen deprived medium but complemented with 10 nM E2 again were able to yield the same number of cells, total protein, total LDH and also XTT activity as for cells cultured in full medium (Fig. 4.1A, B, C, D). It has been established many years ago that MCF-7 cells are dependent on estrogen for proliferation (Brooks et al., 1973; Soule et al., 1973). The differences observed in the cell number and total protein yield when MCF-7 cells are cultured in full medium, estrogen deprived medium and estrogen deprived medium complemented with E2 can be explained as a result of available estrogen to the MCF-7 cells. These results show that the use of commercially available serum replacement solution can be successfully used as an alternative to charcoal stripped FBS in the E-screen assay. Unlike FBS that may vary from batch to batch, depending on herds that it was collected from, serum replacement solution is a chemically defined additive. This makes inter-laboratory comparisons of this assay easier as the reagents can thus be standardized.

The sensitivity of this E-Screen assay was assessed by exposing MCF-7 cells to a 10 fold E2 concentration series. Total LDH, total protein yield as well as XTT activity were evaluated as biomarkers for estrogenic responses. Total LDH activity showed a direct linear correlation (R^2 = 0.97) with the E2 concentration that the cells were exposed to. The linear range for the total LDH assay was between 0.1-1000 nM E2. This is wider than the range obtained for total protein and XTT activity (10-1000 nM E2). Therefore, it appears that total LDH is 100 times more sensitive than total protein or XTT activity as a biomarker for estrogenicity in our E-Screen assay. Furthermore, a good correlation between total LDH activity and total protein (R^2 = 0.93) and LDH with XTT activity (R^2 = 0.97) where observed between 1 and 1000 nM E2 exposed MCF-7 cells. Thus, similar to the use of total DNA, total protein or XTT activity previously used as indicators of cell number in the E-Screen, the total amount of LDH activity from viable cells can also be used as an indicator of cell number. It is important to note that total LDH refer to the amount of LDH measured after the culture supernatant has been discarded and the remaining cells have been lysed. Measuring LDH in this way therefore gives you an indication of the amount of protein or activity of a single protein from a well rather than the total protein or DNA as indicators of cell number from a well.

The use of LDH as a biomarker for estrogenicity in the E-screen assay therefore has a detection range for E2 between 100 pM E2 and 1000 nM E2. Previous investigations have found that the detection limit of the E-Screen varies between laboratories and detection limits ranging between 30 pM (Soto et al., 1992) and 1 pM (Körner et al., 1998) have been reported. Various factors such as differences between cell line clones, culture conditions, receptor level differences, differences in serum cell density and clone heterogeneity may influence the proliferation of MCF-7 cells and therefore the sensitivity of the E-Screen, including: (Zacharewski, 1997).

The different biomarkers that we evaluated were used to screen environmental water extracts for estrogenicity and cytotoxicity. Water samples were obtained from a pristine site, previously described by Swart and Pool (2007) as well as sewage treatment work effluent from a local town. No significant differences in total LDH (Fig 4.5A), total protein (Fig 4.5B) or XTT activity (Fig 4.5C) were detected when comparing the 0.1 nM E2 standard and pristine site data. Exposure of MCF-7 cells to water extracts from the sewage effluent resulted in significantly higher LDH, total protein and XTT activity in comparison with the 0.1 nM E2 standard. This data therefore suggests that water collected from the sewage effluent contains estrogenic properties. All three of the biomarkers analyzed showed statistically similar values compared to MCF-7 cells exposed to 1000 nM E2. Analysis of the culture supernatant of MCF-7 cells exposed to the water extracts suggest that none of the samples are cytotoxic except for the sewage treatment work effluents samples that contained 46 % higher medium LDH compared to the other samples.

In this study we showed that the classic E-screen for estrogenicity using MCF-7 cells can be successfully performed without the use of any serum. We showed that total LDH activity as a biomarker for estrogenicity in the E-Screen assay is much more sensitive than using total protein or XTT activity. These three biomarkers were successfully employed to assess two environmental water extracts for estrogenicity and cytotoxicity. The E-Screen assay that we have simplified and validated in this study is cost effective, easy to perform and large numbers of samples can easily be analyzed for both estrogenicity and cytotoxicity.

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Chapter 5

Development of a bio-assay for estrogens using estrogen receptor alpha gene expression by MCF7 cells as biomarker.

Journal of Immunoassay & Immunochemistry. Accepted for publication.

Key words: MCF-7, ERα, EDC, bio-monitoring, bio-marker

5.1. Abstract

Estrogenic endocrine disruptors (e-EDCs) have been identified in soil, food, air and water, and may produce adverse health effects in both humans and wildlife. Various *in vitro* assays, including the E-screen that measures estrogen dependent proliferation of the MCF-7 human breast cancer cell line, have been developed and implemented to screen for environmental estrogenic EDCs. This study describes a new amendment to the well known E-screen. A direct ELISA to quantify ERα protein levels on MCF-7 cells cultured in a high through put 96-well format were validated as a biomarker for estrogenicity. The ELISA shows that there is an inverse correlation between ERα levels and 17β-estradiol (E2) concentration (R^2 = 1). The detection range of the assay is between 1 and 1000 nM for E2. Results obtained with the ERα ELISA showed a good inverse correlation with total cellular LDH levels that is conventionally used to quantify MCF-7 cell proliferation. This ELISA was employed to assess environmental water extracts for estrogenicity.

5.2. Introduction

There is an increased concern regarding the potential adverse effects of pollutants found in the environment and also food on human and wildlife health. In the environment, these compounds occur as complex mixtures containing different congeners and isomers of both natural and anthropogenic chemicals. Some of these compounds exert their adverse effects by disrupting the natural hormone balance of the animal (Phillips and Forster, 2008). These chemicals are collectively named endocrine disrupting chemicals (EDCs). Most of the known adverse effects of these EDCs have been attributed to environmental estrogens (xenoestrogens) (Gellert , 1978a; Gellert, 1978b; Hammond et al., 1979; Kupfer and Bulger, 1987; Krishnan et al., 1993; White et al., 1994; Newbold 1995; Katzenellenbogen1995; Nimrod and Benson, 1996). Some of the reported diverse range of effects/defects that estrogenic EDCs may have on human and animal health include reduced fertility, congenital malformations of the reproductive tract and increased incidence of cancer in estrogen responsive tissues (Colborn et al., 1993; Davis et al., 1993).

The molecular structure of exogenous natural and synthetic estrogens may be similar or extremely different from endogenous natural estrogens (Jordan et al., 1985; McLachlan, 1993; Safe 1995). Despite the vast structural diversity among environmental estrogens, estrogenic compounds can be characterized by their ability to bind to and activate the estrogen receptor (ER). Ingestion of xeno-estrogens results in either initiation (agonist) or inhibition (antagonist) of estrogenic responses (Soto et al., 1992; Eckert and Katzenellenbogen 1982). Upon binding of an estrogenic compound to the ligand binding domain of the ER (located predominantly in the nucleus of cells), the associated heat shock protein complex, which masks the DNA binding domain dissociates. The ligand occupied receptor undergoes conformational changes that allow the ER-hormone complex to bind as a homo- or heterodimer (Cowley et al., 1997) to specific sites on the DNA called the estrogen response element (ERE) (Walker et al., 1984; Gronemeyer, 1992). Once bound to the DNA, this ER-hormone complex modulates the transcription of the specific target genes (Jensen, 1996; Tsai and O'Malley, 1995). ER complexes bound to an ERE may recruit additional transcription factors, leading to increased or decreased gene transcription and synthesis of proteins (Joyeux et al., 1997; Fielden et al., 1997). Other modes of actions of estrogenic EDCs include the binding of these chemicals to numerous other nuclear receptors and/or signal transduction pathways resulting in the modulation of steroidogenesis and

catabolism of active steroid hormones (Machala and Vondracek, 1998). To date, at least two subtypes of ERs have been identified and described namely ERα [NR3A1] and ERβ [NR3A2] (Nuclear Receptors Nomenclature Committee, 1999). Another subtype, ERγ, has been identified in fish (Drummond et al., 2002). ERα has been well characterized, whereas ERβ was later discovered in the rat (Kuiper et al., 1996), mouse (Tremblay et al., 1997) and human (Mosselman et al., 1996).

The development of *in vitro* models for screening environmental samples for endocrine disrupting characteristics as a first screen has drawn continued interest over the past years (Scrimshaw and Leser, 2004). Many approaches have been used to identify EDCs using a variety of biological screening assays. There are four main categories of *in vitro* bio-assays available to assess estrogenic or androgenic activity of single compounds or complex mixtures, namely, competitive receptor binding assays, cell proliferation assays and reporter gene assays, and direct immunoassay detection of single active compounds (Kinnberg, 2003; Körner et al., 1998). As an alternative, *in vitro* assays could be based on the quantitation of estrogen-induced changes in the expression levels of endogenous genes and proteins. Examples of estrogen-induced changes in the expression levels of endogenous genes and proteins as biomarker for EDCs include the induction of vitellogenin (VTG) by hepatocyte cultures of oviparous animals (Navas and Segner, 2006).

The MCF-7 cell line is a human breast cancer cell line that has been derived from a patient with metastatic breast adenocarcinoma at the Michigan Cancer Foundation (Soule et al., 1973). The MCF-7 cell line has been extensively used to study the molecular interactions of estrogens and anti-estrogens with the ER (Brooks et al., 1973). Cellular bio-assays such as the MCF-7 breast cancer cell proliferation assay (E-Screen), which measure the potency of a sample to induce cell proliferation, has been used as a screening assay for estrogenicity (Soto et al., 1992 and Soto et al., 1995). However, the quantification of ERα protein levels using a direct ELISA on cultured MCF-7 cells in a high throughput 96-well format has not been validated as a biomarker for estrogenicity before. The aim of this study was to development a direct ELISA to quantify ERα levels of cultured MCF-7 cells as a biomarker for estrogenicity.

5.3. Methods and Materials

5.3.1. Reagents

RPMI-1640 with L-glutamine and phenol red as well as RPMI-1640 modified without L-glutamine and phenol red were supplied by Sigma Aldrich. Glutamax (L-glutamine), fetal bovine serum (FBS), antibiotic-antimycotic solution, phosphate buffered saline (PBS) tablets, serum replacement solution, trypsin in ethylenediaminetetraacetic acid (trypsin-EDTA) solution and 17β-estradiol (E2) were also supplied from Sigma Aldrich. Estradiol was prepared as stock solution (1mg/ml) in dimethyl sulfoxide (DMSO). MCF-7 maintenance medium was prepared by adding 10 ml FBS and 1 ml antibiotic-antimycotic solution to 100 ml RPMI-1640 medium containing phenol red and L-glutamine. MCF-7 serum deprived medium were prepared by adding 1 ml antibiotic-antimycotic solution, 1 ml glutamax and 2ml serum replacement solution to 100 ml RPMI-1640 without L-glutamine and phenol red. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a SDS-PAGE gel preparation kit supplied from Sigma Aldrich. Nitrocellulose membranes were supplied from AEC-Amersham International. All other protein blotting detection reagents, solvents and biochemicals were supplied by Roche International. The standard protein molecular mass markers were from Sigma Chemical Company.

5.3.2. Solid phase extraction of hydrophobic molecules from water samples

Water samples were collected from a pristine site and also from a sewage treatment plant outlet. The pristine site is a river site near the origin of the river and not impacted by human activity. Sewage water is from a sewage treatment plant servicing a population of approximately 40 000 inhabitants. The hydrophobic content of environmental water samples were extracted on C18 SPE columns (Anatech) using a method previously described by Swart and Pool (2007). In brief: C18 columns were pre-washed with 4 ml of solvent mixture (40 % hexane, 45 % methanol and 15 %, 2-propanol), followed by another wash with 4 ml of ethanol. The column was then washed with one column volume of reverse osmosis water after which the water sample was applied onto the column. The column was then air-dried. The bound hydrophobic substances were eluted with solvent mixture. The eluate was dried under air and then reconstituted to 1/1000 of the original sample volume with DMSO. The samples were stored at –20 °C until further use.

5.3.3. MCF-7 cell culture

MCF-7 cells were maintained and harvested as previously described by Soto et al. (1992, 1995). MCF-7 cells were suspended in maintenance medium to a concentration of 5 x 10^5 cells/ml. The cell suspension were then dispensed at 200 µl per well in a 96 well flat bottom tissue culture plate (Greiner, AEC-Amersham). Cells were allowed to adhere to the wells for a minimum of 5 hours before the medium was decanted. The wells were then rinsed twice with PBS pre-heated to 37 ˚C. This was followed by the addition of 200 µl estrogen deprived medium per well. Cells were cultured for 48 hours in order to deplete estrogen levels. Medium was then replaced with 200 µl per well estrogen deprived medium containing 1 % v/v of the E2 standards, environmental water extracts or DMSO controls. The cells were cultured for another 48 hours before analysis.

5.3.4. SDS PAGE and Western blotting of ERα

MCF-7 cell lysates were prepared according to a method described by Anitbodycam International (www.antibodycam.com/technical). Lysis buffer contains 150 mM NaCl, 1 % v/v Triton X-100, 0.5 % w/v sodium deoxycholate, 0.1 % w/v SDS and 50 mM trishydroxymethylaminomethane (Tris) pH 8. A 10X concentrated stock of lysis buffer was prepared. Cells were harvested as described by Soto et al. (1992, 1995) and then suspended in PBS to yield 1 x 10^7 cells/ml. Concentrated lysis buffer was added to the cell suspension (100 µl lysis buffer for every 900 µl cell suspension). The suspension was gently agitated for 30 min at 4 ˚C after which it was centrifuged for 10 min 10 000 x g to remove cell rests. Supernatants were removed and aliquots of the supernatant were frozen at -80 ˚C until further use. Protein content of cell lysates were determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard protein (Sigma). SDS-PAGE gels and buffers were prepared according to the kit manufacturer's instructions. MCF-7 protein preparations (5µg) were resolved in 10 % w/v polyacrylamide gels. Polyacrylamide gels were run at 120 V in a Hoefer Mighty Small II slab electrophoresis unit.

Separated polypeptides were transblotted onto nitrocellulose in Towbin buffer (25 mM Tris-HCl, 192 mM glycine and 20 % (v/v) methanol) at 15 Volts for 1 hour. Following transfer, the blots were stained in Ponceau-S (0.2 % w/v Ponceau-S, 3 % w/v trichloroacetic acid). The blots were then thoroughly rinsed in saline and blocked for 1 hour at room temperature in blocking solution

containing 3 % w/v low fat milk powder in saline. All subsequent steps in the procedure were carried out at room temperature. Blots were incubated overnight in saline containing 0.3 % w/v low fat milk powder, 0.01 % v/v tween and 1/500 anti-ERα (Santa Cruz Biotechnology, INC.). The following day unbound antibody was removed by washing the nitrocellulose membranes four times for five minutes each in wash solution (saline and 0.01 % v/v tween). The nitrocellulose was then incubated for 1 hour in saline containing 1/2500 v/v horseradish peroxidase conjugated anti-mouse immunoglobulin and 0.1 % w/v serum albumin. The nitrocellulose was washed as before and finally it was stained using BM Blue precipitating peroxidase substrate. After staining the nitrocellulose, it was washed with distilled water, dried and stored in an aluminum foil envelope.

5.3.5. ERα ELISA

Following the culture of MCF-7 cells, medium was carefully removed from the wells and cells fixated to the tissue culture plate as previously described by Maggiolinie et al. (2002). In short, 200 µl of 2 % v/v paraformaldehyde in PBS were dispensed in all the wells and the plate was then incubated for 45 minutes at room temperature. The paraformaldehyde solution was then replaced with 200 µl of 3 % v/v H_2O_2 in methanol and incubated for another 45 minutes. After fixation wells were washed twice with PBS. Protein adsorption sites in the well were then blocked with 3 % w/v low fat milk powder in PBS for 1 hour at room temperature with gently shaking. Anti-ERα was diluted 1/200 in saline containing 0.3 % w/v milk powder and dispensed at 50 µl per well. The plate was then incubated for 2 hours at room temperature. The wells were then washed 4 times with 200 µl PBS. Horseradish peroxidase conjugated anti-mouse immunoglobulin (AEC-Amersham International) was diluted 1/2500 with PBS containing 1 % w/v human serum albumin and 0.01 % v/v Tween and dispensed at 50 µl per well. Plates were incubated for another hour and the same wash procedure was followed. BM Blue soluble peroxidase substrate was heated to 37°C and dispensed at 50 µl per well. Plates were incubated at room temperature for 30 minutes followed by the addition of 50 µl per well of stop solution (0.5 M H_2SO_4). The optical density was lastly determined at 450 nm. All ELISA readings were corrected for background. Background controls are wells that receive all procedures except that the anti-ERα step is replaced by an incubation step with 0.3 % (w/v) milk powder in saline. Results were expressed as ERα OD/mg cell protein which was calculated using the formula: ERα

OD/mg cell protein = ($OD_{\text{experimental well}} - OD_{\text{background control}}$) / cellular protein (mg). In order to compare the results of multiple plates with each other, ELISA results of the experimental wells being analyzed were expressed as a percentage of the ERα OD/mg protein obtained for the 0.1 nM E2 exposure control used on all 96 well plates.

5.3.6. Determination of total cellular protein

Following the last 48 hour culture period of the MCF-7 cells, cultures were washed twice with PBS. Each well then received 100 µl of a 1 M NaOH solution. The plates were allowed to gently shake (300 rpmi) at room temperature for 30 minutes. A multi-pipet was used to gently suspend all particulates by slowly pipetting well contents up and down. Total protein concentration of the cell hydrolysates were determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard protein (Sigma).

5.3.7. Determination of total cellular lactate dehydrogenase (LDH)

Total LDH activity was determined as an indicator of total cell number or proliferation. LDH was measured using the instructions of a cytotoxicity detection kit supplied from Roche Applied Science. In short: Cells were grown in a 96 well format as described in the previous section. In order to obtain total LDH levels, culture medium was removed from the exposed cells after the last 48 hour incubation period. Estrogen deprived medium containing 2 % (v/v) Triton X-100 was then added at 200 µl/well. The plate was incubated on a shaker for 10 min at 300 rpm, after which the cell lysates were assayed for LDH. LDH reaction mixture was prepared according to the manufacturer's instructions immediately before use. To determine the LDH activity, 100 µl of LDH reaction mixture was added to 100 µl of 1:10 cell lysate preparation in an optically clear 96-well flat bottom plate. The absorbance was immediately measured at 492 nm to obtain a background reading. The plate was then incubated in the dark for 30 min at room temperature, where after the absorbance was again measured. Absorbance readings were corrected by subtracting the background reading for the specific sample.

5.3.8. Statistical analysis

Differences in response of MCF-7 cells between controls groups and treatments were analyzed using analysis of variance (ANOVA). Tuckey's HSD test was used for all pair wise multiple comparisons.

5.4. Results

5.4.1. Anti ERα specificity

In order to use the anti-ERα antibody in an ELISA system, the antibody specificity was first checked by protein blotting (Fig. 5.1).

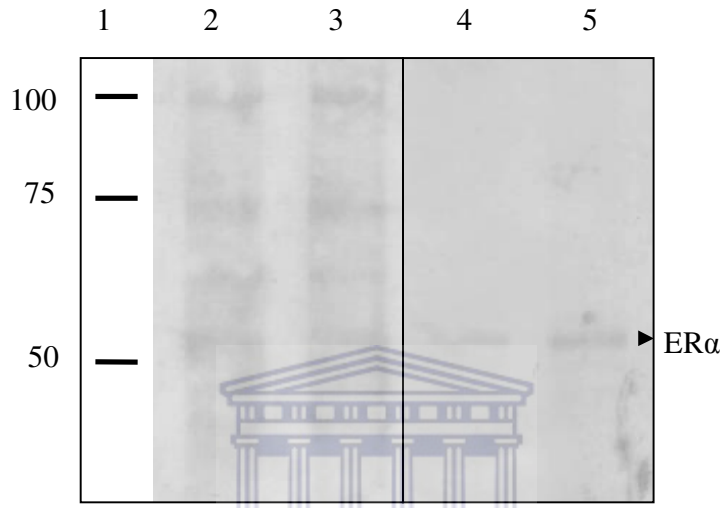

Figure 5.1. SDS-Page and protein blotting of MCF-7 whole cell lysates. Commercially available anti-ERα antiserum was checked for specificity. MCF-7 cells were cultured in maintenance- and estrogen deprived medium where after their whole cell lysates were subjected to SDS-Page and protein blotting. Lanes 1, 2 and 3 are a SDS-Page gel stained with Coomassie, whereas lanes 4 and 5 are protein blot with anti-ERα. Lane 1, Molecular weight marker; lane 2, maintenance medium whole cell lysate; lane 3, estrogen deprive whole cell lysate; lane 4, maintenance medium whole cell lysate; lane 5, estrogen deprive whole cell lysate.

The Coomassie stain of cells cultured in maintenance medium (lane 2) and estrogen deprived medium (lane 3) shows that equal amounts (10 μg) of protein were loaded onto lanes of the SDS-Page gel. In lanes 4 and 5 it can be observed that the anti-ERα antibody recognized a single peptide with an apparent molecular weight of around 55 kDa. It can also be observed that the ERα protein levels are higher in cells cultured in estrogen deprived medium (lane 5) compared to cells cultured in medium containing FBS and phenol red (lane 4).

5.4.2. ERα ELISA validation

The optimum anti-ERα titer was determined using a direct ELISA as discussed in the materials and methods section. In order to obtain the optimum antiserum dilution, anti-ERα was added in a

two fold dilution series between dilutions ranging from 1/100 to 1/3200. An antiserum dilution of 1/200 gave an OD response midway in the linear region of the titration graph (results not shown). This antiserum dilution factor was chosen to perform all direct ELISAs on the MCF-7 cells using ERα as the primary antibody.

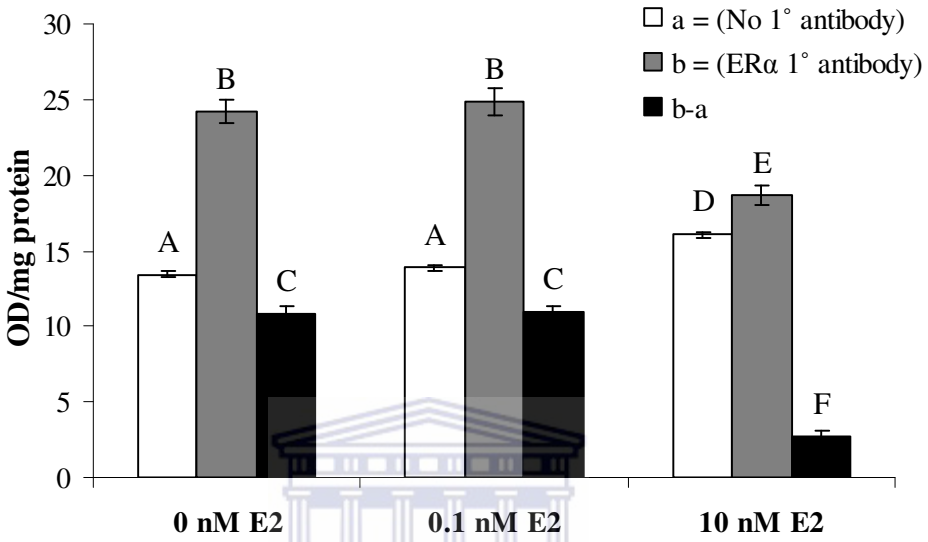

Figure 5.2. Anti-ERα ELISA validation. The ERα antibody was employed in a direct ELISA on the cultured and then fixated MCF-7 cells. MCF-7 cells were exposed to the DMSO vehicle control (0 nM E2), 0.1 nM E2 and 10 nM E2. White bars represent the OD/mg protein obtained when the ELISA was performed with no 1˚ antibody (background OD/mg protein), grey bars with the ERα antiserum (1/200 diluted) as the 1˚ antibody (assay OD/mg protein) and black bars are the corrected OD/mg protein obtained using the formula described in the methods section. Each data bar represents three replicates. Vertical error bars represent standard deviations of the mean. Letters above the data bars of each treatment represent significant differences ($P < 0.001$).

Figure 5.2 displays the results obtained (expressed as OD/mg protein) when the ELISA was performed with no 1˚ antibody (background OD/mg protein), 1/200 diluted anti-ERα antiserum (assay OD/mg protein) as well as the differences in OD/mg protein observed between the two antibody controls (corrected OD/mg protein). This ELISA was performed on MCF-7 cells exposed to estrogen deprived medium complemented with DMSO vehicle control (0 nM E2), 0.1 and 10 nM E2. No significant differences in the background OD/mg protein (ELISA performed with no 1˚ antibody) can be observed between the three different treatments (0 nM E2, 0.1 E2 and 10 nM E2). When this ELISA is performed with 1/200 anti-ERα antiserum, cells exposed to 10 nM E2 displays significantly lower OD/mg protein compared to cells exposed to 0.1 and 0 nM E2. There was no statistical difference in the OD/mg protein for 0 nM and 0.1 nM E2 exposures.

5.4.3. The effect of E2 concentration on the ERα level

MCF-7 cells were cultured in estrogen deprived medium supplemented with various concentrations of E2. The same volume of the vehicle control (DMSO) was used with all E2 concentrations so that the E2 concentration was the only variant. ERα-ELISA was done as described in the methods. The ERα OD/mg protein shows a good inverse correlation (R^2 = 0.97) with the log of the E2 concentration to which the MCF-7 cells were exposed to (Fig. 5.3). The lowest concentration of E2 that result in a significant reduction in ERα OD/mg protein compared with the control (0 nM E2) was 1 nM E2. The range of the assay was between 1-1000 nM E2.

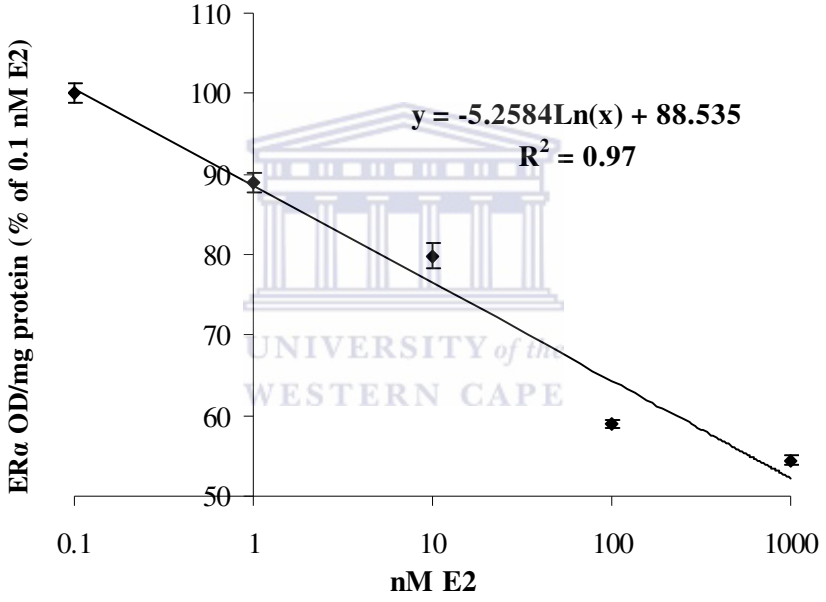

Figure 5.3. ERα-ELISA and E2 dose responsiveness. MCF-7 cells were cultured in estrogen deprived medium after which it was exposed to a dilution series of E2. ERα protein levels were determined and is expressed as the ERα OD/mg protein as a percentage of the 0.1 nM E2 exposure. Vertical error bars represents the standard deviation of the mean for ten replicates.

5.4.4. Correlation of ERα and total LDH levels

The total LDH levels obtained for MCF-7 cells exposed to the dilution series of E2 between 1 and 1000 nM E2 were correlated with the ERα ELISA OD/mg protein (Fig. 5.4.). There is an inverse correlation between the ERα ELISA and total LDH activity (R^2 = 1).

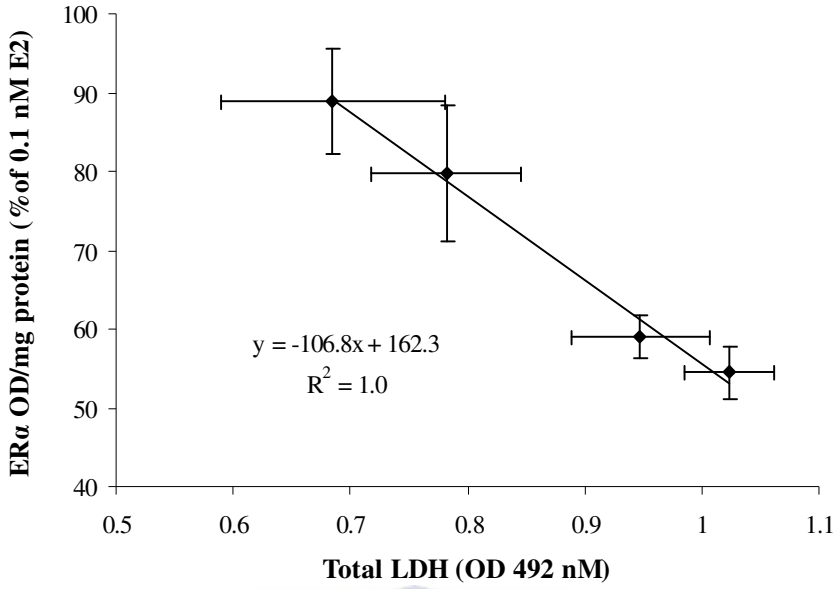

Fig. 5.4. Correlation of ERα ELISA and total LDH activity. MCF-7 cells were exposed to a 10 fold concentration series between 1 and 1000 nM of E2. Total LDH activity is plotted against ERα OD/mg protein. Vertical and horizontal error bars represent the standard deviation of the mean for ten replicates.

5.4.5. Analysis of environmental water extracts for estrogenicity

ERα levels of MCF-7 cells exposed to extracts of environmental samples were measured as biomarkers for estrogenicity of the sample (Fig. 5.5). Analysis of the ERα levels showed a highly significant ($P < 0.001$) decrease in ERα levels in the presence of 1000 nM E2 standard (55.01 % ± 3.15) compared with the 0.1 nM E2 standard (100 % ± 7.39). There is no significant difference between the ERα levels induced by the 0.1 nM E2 standard and the pristine site. However, extracts of the sewage effluent resulted in a significantly lower ERα level compared to the 0.1 nM E2 standard ($P < 0.001$) and also the 1000 nM E2 standard ($P < 0.001$).

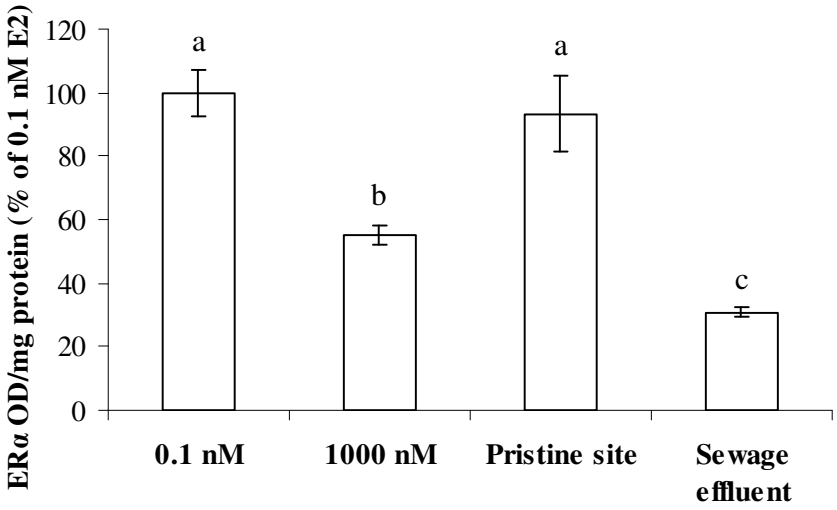

Figure 5.5. ERα protein levels of MCF-7 cells exposed to environmental water samples. Water samples collected from a pristine site and sewage effluent were subjected to the MCF-7 screen for estrogenicity and cytotoxicity. ERα protein levels were determined with the ERα-ELISA. Vertical error bars represents the standard error of the mean of six assays. Different letters indicate significant differences among treatments ($P < 0.001$).

5.5. Discussion

Several characterized EDCs have been reported to act in an estrogen-like manner in living organisms (Soto et al., 1995). When estradiol or estrogenic substances enter a target cells, it rapidly complexes with the ER, translocates into the nucleus where it binds to chromatin and induces gene activation and an acceleration of biosynthetic processes (O'Malley et al., 1974). Estrogenic EDCs such as phytoestrogens, mycoestrogens and xenoestrogens have the ability to interact with the ER which can then initiate (agonist) or inhibit (antagonist) estrogen-like actions (Eckert and Katzenellenbogen 1982; Soto et al., 1992). Various *in vitro* assays have been developed, based on the quantitation of estrogen-induced changes in the expression levels of endogenous genes and proteins in estrogen responsive tissues (Inoue et al., 2002). The human breast cancer cell line, MCF-7, has been well characterized as an *in vitro* screen for estrogenicity (E-Screen) (Soto et al., 1992, 1995). The E-Screen assay is based on the dose-response relationship between the proliferation of the cells and the concentration of estrogen to which the cells are exposed to (Soto et al., 1995). Previous studies have reported that the molecular weight of ERα is in the region of 65 kDa (Green and Chambon, 1991). The antibody used for this study reacted with a single peptide with an apparent molecular weight of ± 60 kDa, similar to previous reports using this specific antibody (Morelli, 2003; Likhite, 2004). More over, protein blot analysis showed that ERα levels were elevated in estrogen deprived medium and suppressed in maintenance medium (containing E2). Previous studies have shown that ERα expression is upregulated by estrogen deprivation (Villalobos et al., 1995).

This antibody was used for the development of an ELISA to directly monitor ERα expression by MCF-7 cells cultured and subsequently fixed in 96 well trays. When MFC-7 cells were cultured in serum free medium high levels of ERα was expressed. The levels of ERα expression is down-regulated by the addition of estrogen to the culture medium. There is an inverse relationship between the ERα expression and log of the estrogen concentration ($R^2 = 0.97$). Previous studies using ERα expression by MCF-7 cells have employed ELISAs on cell homogenates of the cultured cells and obtained similar results (Villalobos et al., 1995).

Results obtained using ERα protein levels as biomarker showed a good correlation ($R^2 = 1$) with total LDH as biomarker for estrogenicity when MCF-7 cells were exposed to the same E2

dilution series. Previous investigations have found that the detection limit of the E-Screen varies widely and reported values are 0.03 nM (Soto et al., 1992) and 0.001 nM (Körner et al., 1999). Various factors may influence the sensitivity of MCF-7 for E2 including: differences between cell line clones; culture conditions; receptor level differences; differences in cell density and clone heterogeneity (Zacharewski, 1997).

The newly developed ELISA was used to monitor ERα levels as a biomarker to assess the estrogenicity of environmental water extracts. Water samples were obtained from a pristine site, previously described by Swart et al., (2007) as well as sewage treatment work effluent from a local town. The level of ERα expression by MCF-7 cells cultured in the presence of hydrophic extracts of water collected from the pristine site was similar to the ERα expression by MCF-7 cells cultured in the presence of the 0.1 nM E2 control ($P > 0.05$). This indicates that either the water from the pristine site does not contain estrogen or if this sample contains E2 or estrogenic compounds, it is well below the detection limit of the current assay. However as expected, the sewage treatment effluents resulted in significantly lower ERα levels compared to the E2 negative control ($P < 0.001$). More over, the sewage treatment effluent also resulted in significant lower ERα levels compared with the 1000 nM E2 standard ($P < 0.001$). These results therefore suggest that the pristine site shows no signs of estrogenicity, whereas the sewage effluent samples induced estrogenic responses greater than that observed for MCF-7 cells exposed to 1000 nM E2.

In this study we extended the range of end-points of the classical E-Screen by using ERα expression as a biomarker for estrogenicity. We validated the use of a commercially available anti-ERα antibody and showed that this antibody can be successfully employed in a direct ELISA on MCF-7 cells fixed to the 96-well culture tray. This assay has a broad detection range between 1 and 1000 nM for E2. This detection range is similar and lower then the detection range of the other *in vitro* and *in* vivo assays evaluated in this thesis (Chapters 3, 4 and 7). Previous studies have also investigated the use of ERα as a biomarker for estrogenicity using the MCF-7 cell line. Results obtained in our study corroborate the results obtained by Villabos et al (1995). However, the ELISA described by us is performed on cultured and fixated cells in a 96 well format and does not require protein preparation of cultured cells. The assay described by us is therefore less

time consuming, easy to use, sensitive and can be used as a high throughput *in vitro* screen for environmental estrogens.

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Chapter 6

The development and validation of a quantitative ELISA for *in vivo* and *in vitro* synthesized vitellogenin from Mossambicus tilapia (*Oreochromis mossambicus*)

Keywords: *Oreochromis mossambicus*, Vitellogenin, ELISA, EDC, estrogenicity.

6.1. Abstract

The induction of vitellogenin (VTG), an egg yolk precursor found in most oviparious vertebrates, has been well established as a valuable biomarker for assessing estrogenic endocrine disruption. *Oreochromis mossambicus* is a tilapiine species indigenous to Southern Africa and therefore represents a potential African bio-indicator for the development of *in vitro* and *in vivo* screens for estrogenicity using VTG as biomarker. However, few ELISAs have been developed to quantify tilapia VTG protein levels. In the present study commercially available VTG antiserum that shows cross reactivity with tilapia VTG was used to setup and validate a quantitative competition ELISA for tilapia VTG. This ELISA has a broad detection range between 80 ng/ml – 5.4 µg/ml VTG and is able to detect both *in vivo* and *in vitro* synthesized VTG. This ELISA is highly repeatable with intra- and interassay variations less than 3.4 % at the lowest detection limit.

6.2. Introduction

Several toxicants found in the environment act by disrupting the endocrine system of animals and humans. Traditional chemical monitoring techniques have become very expensive, are time consuming and requires a high level of skill. Moreover, since it is not yet known which of the myriad of substances in the environment and combinations of them may cause endocrine problems, the measurement of biological effects seems to be the more appropriate way to go. A number of studies have concluded that in terms of cost effectiveness and to allow for screening of a large number of compounds, *in vitro* test methods are more appropriate (Beresford et al., 2000; Andersen et al., 1999). Vitellogenin (VTG) has become a very popular biomarker for environmental estrogen induced toxicity and is used in both *in vitro* and *in vivo* screening assays in oviparous vertebrates.

VTG is a high molecular weight phospholipoglycoprotein (250-600 kDa) that is produced and secreted by the liver of oviparious vertebrates as the precursor to several egg yolk proteins (Wallace and Selman, 1985; Ho et al., 1980, Specker et al., 1994). During sexual maturation, the female fish synthesizes 17β-estradiol (E2) in the gonads, and the circulating E2 is subsequently taken up by hepatocytes where it binds to estrogen receptors (ER) leading to the transcription of the VTG gene. The VTG protein is then secreted from the hepatocytes, enters blood circulation and is taken up by growing oocyte through receptor-mediated endocytosis (Matozzo et al., 2007). Within the oocyte VTG is degraded to the yolk proteins, lipovitellin 1, lipovitellin 2 and phosphitin (Parks et al., 1999; Brion et al., 2000). These yolk proteins serve as a nutrient store for developing embryos (Matsubara et al., 1999).

Investigations have shown that although VTG is normally expressed in female fish only, E2 and a number of synthetic chemicals (e.g., ethinyl estradiol, nonylphenols) are capable of inducing VTG production in male fish (Purdom et al., 1994; Jobling et al., 1995; Sumpter, 1995). This endocrine disruption phenomenon is due to the fact that these xenoestrogens may interact with estrogen receptors by mimicking natural estrogens (Stahlschmidt-Allner et al., 1997). Exposure of fish to estrogenic sewage effluent may lead to reproductive impairment (Lomax et al., 1998; Gronen et al.,1999). Spermatogenesis is inhibited by estrogens and some exposed male fish have testisova (Gronen et al., 1999). Because of the sensitivity of this response, VTG detection in blood and/or liver from male oviparous verterbrates has become a common biomarker for monitoring environmental estrogen contamination (Gimeno et al., 1997; Harries et al., 1997; Jobling et al., 1998; Cheek et al., 2001; Jobling and Tyler, 2003).

A number of methods have been developed for the quantification of VTG in blood plasma, liver tissue, or whole-body homogenates from several fish species. These methods include: radioimmunoassay (RIA), enzyme linked immuno sorbent assay (ELISA), single radial immunodiffusion (Specker et al., 1994) and real time PCR (Celius et al., 2000). These various methods differ in sensitivity, specificity, and technical difficulty. Currently, the most popular approach to measure VTG is some form of an ELISA, although RIAs may occasionally be employed (OECD, 2004). Fish VTG was quantified for the first time by ELISA in *Solea vulgaris* (Nuñez Rodriguez et al., 1989) and since then in various other species. The anti-VTG antibodies are very specific for each individual fish species and often require the production of specific antibodies for the selected species (Hansen et al., 1998). These antibody based procedures for VTG quantification may use antisera prepared against VTG from the fish species being studied. Although antisera can cross react with VTG from multiple fish species, the affinity can vary substantially (Silversand et al., 1993).

The development of screening and testing programmes for endocrine disrupting effects of new chemicals are one of the priority focus areas of major organizations such as the Organization for Economic Cooperation and Development (OECD, 2004). The focus of these are use of small fish test species such as fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), stickleback (*Gasterosteus aculeatus*) and Japanese medaka (*Oryzias latipes*) as bio-indicators. These fish species share several characteristics that make them ideal test species for reproductive toxicity such as small size at maturity, relatively short generation times, asynchronous spawning, and overall ease of culture. Southern Africa, like most areas in the world, is also facing major aquatic pollution problems and these are posing a threat to human and animal health. Tilapia (*Oreochromis mossambicus*) also shares the same characteristics as the above mentioned fish species. Tilapia is found in the natural environment throughout Southern Africa and can potentially be used as an indigenous indicator species for this region. The aim of this study was to use commercially available antibodies to setup, validate and implement a competition ELISA to quantify *Oreochromis mossambicus* VTG.

6.3. Methods and Materials

6.3.1. Experimental animals

Juvenile and adult Tilapia were obtained from the breeding stock of the Welgevallen experimental farm from the University of Stellenbosch, Stellenbosch, South Africa. Tilapia juveniles were four weeks old, whereas adult fish used weighed in the order of 100g.

6.3.2. Chemicals

All auxiliary enzymes, cofactors and substrates used in this study were from either Sigma Chemical Company (St Louis, MO, USA) or Roche (South Africa). Sea Bream Vitellogenin polyclonal antibody (Anti-VTG) were supplied by Cayman Chemical Co. Anti-rabbit IgG (whole molecule) horseradish peroxidase conjugate (HRP) and Hybond-C Extra nitrocellulose were supplied by Amersham International. All other protein blotting detection reagents were supplied by Roche International. The standard protein molecular mass markers were from Sigma Chemical Company. All solvents and biochemicals were of analytical grade.

6.3.3. Juvenile diethylstilbestrol (DES) exposure

Four-week-old juvenile Tilapia fishes were obtained from the Welgevallen experimental farm and brought back to the lab. Juvenile tilapias were exposed to DES for seven days under static conditions in two-liter glass containers containing one liter of water. Water was not changed during the exposure period. DES was dissolved in dimethyl sulfoxide (DMSO) and did not exceed 0.1 ml per liter as per OECD, 2004 protocol agreement. Two treatment groups received 25 ng/l DES (n=20) and 250 ng/l DES (n=20) respectively. An additional group of 20 fish were kept without DES and served as a control group.

6.3.4. Tissue culture

The medium used for tissue culture was RPMI 1640 containing L-glutamine, $NaHCO_3$ and 25 mM Hepes (Highveld Biologicals, South Africa). A mixture of penicillin, streptomycin, and fungizone (Highveld Biologicals) was added to the RPMI medium according to manufacturer's instructions. The RPMI medium containing the antibiotics will subsequently be referred to simply as “medium."

Adult male fish were brought back to laboratory after which they were decapitated and placed in 70 % ethanol for three minutes in order to decontaminate the skin. All subsequent procedures were carried out aseptically in a laminar flow cabinet. Cubes of liver tissue,

measuring approximately 1mm^3, were prepared and put into high quality 1.5 ml eppendorph tubes at one cube per tube. Four cubes were prepared for each DES treatment exposure. Cubes were covered with medium an incubated in the dark at a constant 27°C in a water bath. Cubes were incubated for four days and the medium was not changed during the exposure time. Aspirant medium, for the determination of VTG concentration, were stored at -80°C until use.

6.3.5. Tissue sampling

Adult Tilapias were anesthetized using 100 mg/l Benzocaine. Organs used for experiments were dissected out and placed in extraction buffer (saline containing 0.01 % (w/v) phenylmethylsulphonylfluoride) with a weight to volume ratio of 1:10. Samples were then sonicated at 14 watts (Virsonic-60) for six bursts of ten seconds each. Samples were allowed to stand for one-minute intervals on ice between bursts. Samples were then centrifuged at 12 000 × g for 10 min at 4°C. Blood was collected using capillary tubes and dispensed in 1.5 ml tubes. After clotting the samples were centrifuged at 12 000 × g for 10 minutes at 4°C. The supernatants from both the blood and organ samples were, aliquoted and stored at -80°C for analysis.

6.3.6. Protein determinations

Protein contents of samples were measured according to the method of Bradford, (1976) using biovine serum albumin (BSA) as a standard protein (Sigma).

6.3.7. SDS PAGE and protein blotting

Proteins were resolved in a 10 % (w/v) polyacrylamide gel. Gels were prepared using a SDS-PAGE gel preparation kit supplied by Sigma Chemical Company (St Louis, MO, USA). Gels and solutions were prepared according to the manufacturers instructions. Polyacrylamide gels were run at 120 V in a slab electrophoresis unit.

Separated polypeptides were transblotted onto nitrocellulose in Towbin Buffer (25 mM Tris-HCl, 192 mM glycine and 20 % (v/v) methanol) at 15 Volts for 1 hour and dried between filter paper. Following transfer, the blots were stained in Ponceau-S (0.2 % [m/v] Ponceau-S, 3 % [m/v] trichloroacetic acid), to be able to mark the standard molecular weight bands. The blots were then thoroughly rinsed in saline and blocked for 1 hour at room temperature in blocking solution containing 1 % (w/v) human serum albumin (HSA) in saline. All

subsequent steps in the procedure were carried out at room temperature. Blots were incubated in saline containing 0.1 % (w/v) BSA, 0.01 % (v/v) Tween and 1/2000 anti-VTG. Unbound antibody was removed by washing the nitrocellulose 4 times for 5 minutes in wash solution containing saline and 0.01 % (v/v) Tween. The nitrocellulose was then incubated for 1 hour in wash solution containing anti-rabbit horse radish peroxidase conjugate (1/2500), 0.1 % BSA and saline. The nitrocellulose was washed as before and finally it was stained for horseradish peroxidase binding using BM Blue POD precipitating substrate. After staining the nitrocellulose it was washed with distilled water, dried and stored in an aluminium foil envelope.

6.3.8. Tilapia vitellogenin competition ELISA

Nunc-Immuno Maxisorp® plates (Nalge Nunc, Denmark) were used for all ELISA assays. Plates were coated overnight at 4°C with 50 µl per well of 1/2000 diluted ovary homogenate in saline. At the end of the incubation period plates were washed four times with Saline. Following the wash procedure, remaining adsorption sites on the plate were blocked by dispensing 0.2 ml of block solution (1 % w/v HSA in Saline) per well and incubating the plate for one hour at room temperature. The same wash procedure was followed where after samples for analysis and the VTG standards were dispensed at 50 µl per well. Anti-VTG was diluted 1/1000 with Saline containing 1 % (w/v) HSA and were also dispensed at 50 µl per well. Samples were incubated for three hours at room temperature and the same wash procedure was followed again. Anti-rabbit horse radish peroxidase conjugate was diluted 1/2500 with Saline containing 1 % (w/v) HSA and dispensed at 50 µl per well. Plates were incubated for one hour at room temperature where after it was washed eight times with Saline. BM Blue POD soluble substrate was heated to 37°C and dispensed at 50 µl per well. Plates were incubated at room temperature for thirty minutes followed by addition of 50 µl per well of stop solution (0.5 M H_2SO_4). The optical density was lastly determined at 450 nm.

6.3.9. Expression of results and statistical analysis

The percentage of maximum (percentage binding) in each standard or sample dilution was calculated using the following relationship:

% of maximum = (standard or sample absorbance / maximal binding absorbance) x 100. Results were statistically analysed using analysis of variance (ANOVA). Tuckey's HSD test was used for all pair wise multiple comparisons.

6.4. Results

6.4.1. Specificity of the Anti-VTG

Polyclonal anti-VTG was bought from Cayman Chemical Co (catalog number: 170150). This antibody was produced from purified VTG of E2 treated Sea Bream (*Sparus aurata*). According to the manufacturer, this antibody recognized VTG from several fish species, including (*Oreochromis niloticus*). The specificity of this antibody was tested on *Oreochromis mossambicus* using protein blotting (Fig. 6.1).

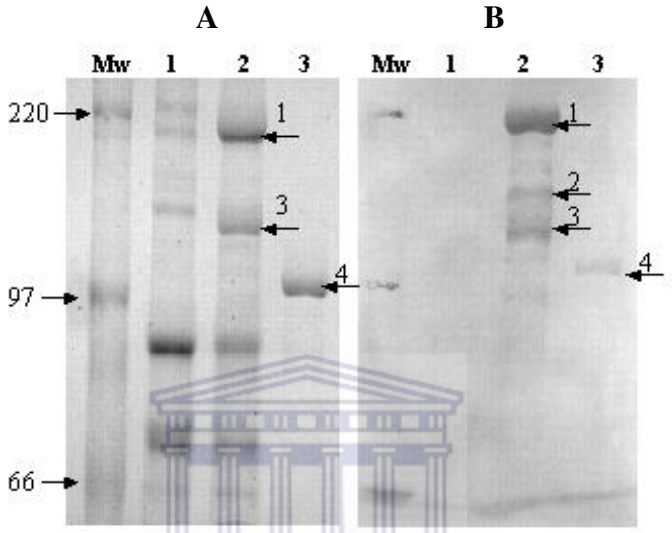

Figure. 6.1. Specificity of the Anti-VTG antiserum. SDS-Page (A) and Protein Blotting (B) of: Male plasma (1), DES treated female plasma (2) and Ovaries (3). SDS-Page gel was stained with Coomassie Blue. Plasma samples were diluted 1:100 and 20 µl of sample were used for SDS-Page and protein blotting.

The antiserum immunostained both the plasma of DES treated females as well as ovary extracts, while there was no reaction with the control male plasma. The anti-VTG recognized peptides with apparent molecular weight of approximately 200 kDa (protein 1), 140 kDa (protein 2) and 130 kDa (protein 3) in the treated female serum. In the ovary extracts a protein with an apparent molecular weight of 110 kDa (protein 3) was immunodetected.

6.4.2. ELISA Validation

6.4.2.1. Anti-VTG and VTG antigen titration

The optimal assay concentrations for VTG antigen and antibody were determined to obtain a maximum absorbance value near 1, since most plate readers have a linear response in the range 0.1-1.1 optical density units. The two-way titration of the VTG antiserum and antigen is displayed in figure 6.2. A double dilution series of both VTG antiserum and antigen were prepared to obtain optimal ELISA assay concentrations. The VTG coating antigen dilution

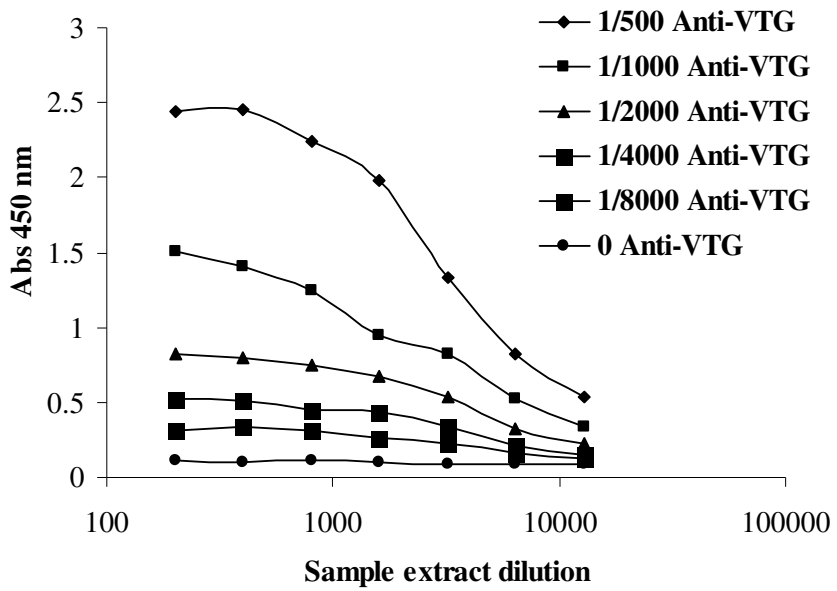

Figure 6.2. Determination of optimal concentrations of tilapia vitellogenin and antiserum for the validation of the tilapia ELISA. Tilapia ovary extracts were used as the VTG antigen, whereas seabream anti-VTG was used as the primary antibody. Abs = absorbance.

started at 1:200, whereas VTG antiserum started at 1:500. VTG antigen dilution of 1:2000 and VTG antiserum dilution of 1:1000 were chosen for setting up the competition ELISA. These assay concentrations gave an absorbance value near 1, which is midway between the linear and plato area of the graph. At these assay concentrations very low background readings were obtained (Abs = 0.097).

6.4.2.2. VTG antiserum specificity

Blood from DES treated female tilapia and control untreated males were screened for VTG induction using the optimal ELISA assay concentration (Fig. 6.3). At a blood dilution factor of 1:2000 exposed females showed absorbance values of 1.6, whereas unexposed males showed very low background readings of 0.1. Blood from the exposed females was stored at -80°C as an in-house standard for VTG.

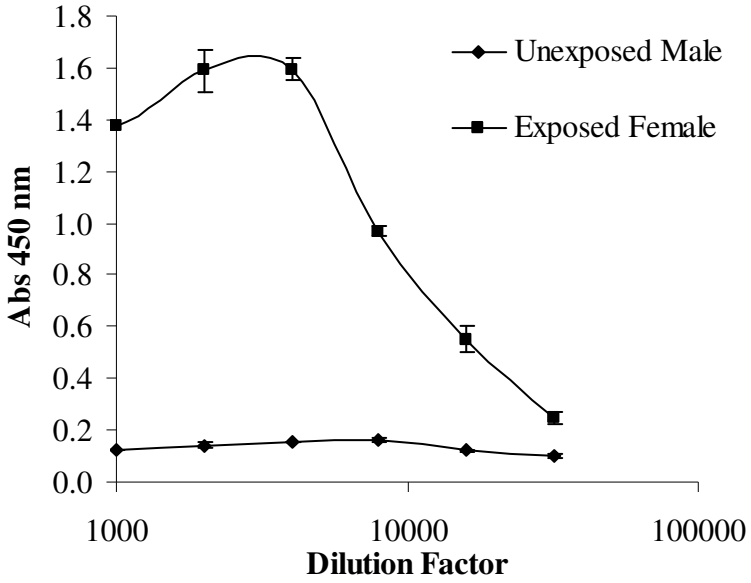

Figure 6.3. Screen for VTG induction in sexually mature female tilapia blood exposed to DES as well as untreated males. Female, sexually mature female tilapia fish were exposed for 7 days to 60 ng/l DES. Male tilapias were kept under the same conditions without DES treatment. Blood were screened for VTG using the optimal ELISA assay concentrations.

6.4.3. VTG competition ELISA

A typical tilapia VTG competition ELISA standard curve is displayed in figure 6.4. The standard curve was linearized by plotting percentage of maximum values on a logarithmic Y-axis.

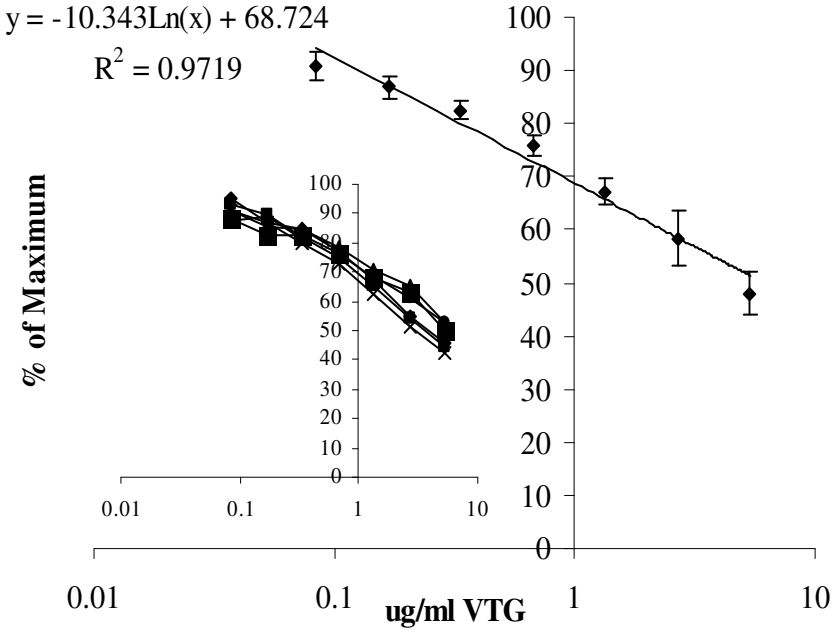

Figure 6.4. A typical tilapia VTG competition ELISA standard curve. In-house VTG standard were diluted from 84.1 ng/ml to 5.4 µg/ml VTG. The competition ELISA procedure was followed as explained in the methods and materials section.

The sensitivity of the ELISA (the amount of VTG which gave 90 % of binding) was 80 ng/ml VTG with a practical operating range betweem 80 ng/ml and 5.4 ug/ml VTG. The standard curve displayed in figure 6.4 is the result of six standard curves over a period of six months and the ELISA precision (intra- and interassay variation) was calculated from this (Table 6.1). Intra-assay variation was less than 3.4 %, whereas interassay variation was 8.4 % at 50 % binding and less then 2.9 % at 90 % and 80 %.

Table 6.1. Characteristics of the tilapia VTG competition ELISA[a]

	Binding (Percentage of Maximum)		
	90 %	80 %	50 %
CV[b] intra-assay (%)	3.4	1.2	1.1
CV interassay (%)	2.9	2.2	8.4

[a] For routine measurement 1:2000 VTG antigen and 1:1000 antibody dilution was selected.
[b] CV = coefficient of variation.

6.4.4. Implementation of ELISA for *in vivo* induced VTG

To validate the applicability of the competition ELISA for quantification of *in* vivo induced tilapia VTG, groups of 20 four-week-old tilapia fish were exposed to DES at various concentrations for 7 days and VTG concentrations determined using the ELISA (Fig. 6.5).

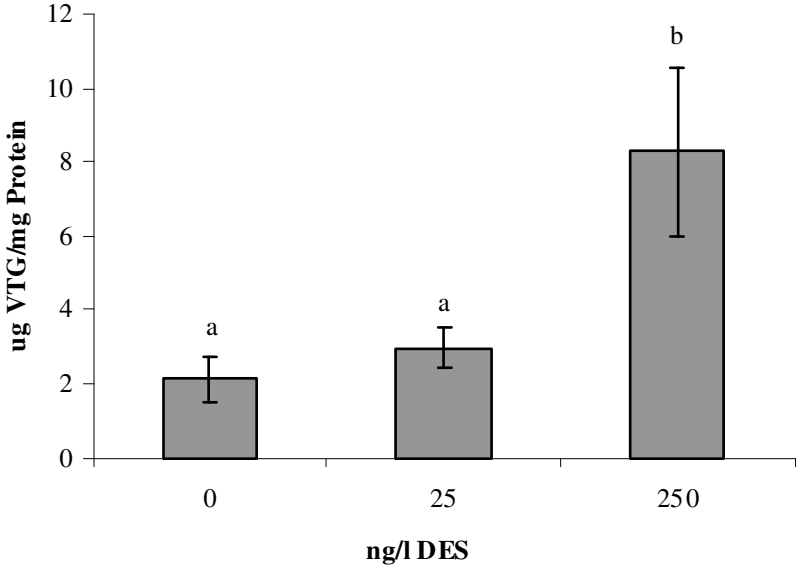

Figure 6.5. VTG concentration of four-week-old juvenile tilapia whole body homogenates. Fish were exposed to different concentrations of DES over a period of seven days. Vertical bars are the average concentration obtained for six individual fish, whereas error bars represent standard deviations. Groups marked with different letter are significantly different ($p < 0.001$).

Exposure to DES did not significantly influence the mortality of exposed juveniles, suggesting that neither the solvents nor the tested concentrations of DES had an acute toxicity effect under our test conditions. Figure 6.5 displays VTG concentrations of the whole body homogenates measured by the competition ELISA after 7 days of exposure. In the control group, the measured VTG concentration was 2.1 µg VTG/mg protein. Exposure of juveniles to 25 ng/l and 250 ng/l DES resulted in VTG concentrations of 3 µg/ml and 9 µg/ml respectively. There was no significant difference in VTG concentration between the control and 25 ng/l DES groups. However, the 250 ng/l DES exposed tilapia have significantly higher VTG compared to the control ($P < 0.001$) and the 25 ng/l DES exposed group ($P < 0.001$).

6.4.5. Implementation of ELISA for *in vitro* induced VTG

Liver slice cultures from sexually mature male tilapia was used to validate the competition ELISA for *in vitro* VTG synthesis (Fig. 6.6). Liver slices were exposed to 0.5 µg/ml DES for a total of four days. VTG concentrations measured in the medium from the control samples did not change over the culture period ($p > 0.01$). After three days exposure to 0.5 µg/ml DES, VTG concentrations increased significantly from 1.3 µg/ml (control) to 4.3 µg/ml VTG ($p < 0.001$). VTG concentrations increased from 4.3 µg/ml to 14.6 µg/ml VTG after four days in culture.

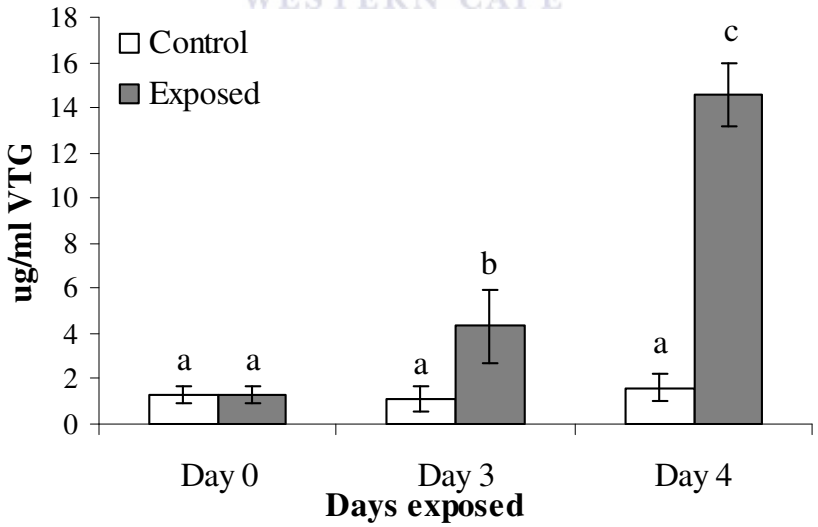

Figure 6.6. Vitellogenin synthesis by sliced liver cultures exposed to 0.5 µg/ml DES over a period of four days. Vertical bars represent the mean of four independent experiments, whereas errors bars represent standard deviations. Different letters above the data bars indicate significant differences ($p < 0.001$).

6.5. Discussion

In this study commercially available anti-seabream VTG was used to set up, validate and implement a competition ELISA which can be used for the quantification of *in vitro* and *in vivo* synthesized *Oreochromis mossambicus* VTG. Previous investigations have shown that anti-VTG is very species specific and therefore very often requires the production of species specific anti-VTG antiserum (Hansen et al., 1998). It is evident from the protein blot analysis (Fig. 6.1) that the anti-seabream VTG recognize tilapia VTG. The antibody reacted with peptides in DES treated plasma, whereas no reaction was visible with untreated male plasma. These results are supported by the ELISA on serum from DES exposed females and unexposed males (Fig. 6.3). The antibody reacted with the DES exposed serum whereas no crossreactivity is observed with the unexposed male fish. In a previous study conducted by Kishida and Specker, (1993) VTG were isolated by DEAE agarose ion-exchange chromatography from plasma of the tilapia, *Oreochromis mossambicus*. The monomers had apparent molecular masses of 200 and 130 kDa. SDS-PAGE of the oocyte extract showed a major protein band at 106.6 kDa. The anti-seabream VTG used for this study similarly detected three bands at molecular weight 200 kDa, 140 kDa and 130 kDa respectively. The 140 kDa protein of tilapia may be a degradation product of a larger VTG molecule. Previous investigations have shown that VTG in many fish is rather unstable, and even the addition of protease inhibitors to a plasma sample cannot fully prevent its proteolysis (Tyler and Sumpter, 1990; Silversand et al., 1993). A protein with a molecular weigh of approximately 110 kDa was detected in ovary extracts.

The validated ELISA is a competitive-binding assay in which antibodies are preincubated together with the sample of analysis (or standards). The immobilized VTG on the plate and the soluble VTG in the sample or standard competes for antibody binding. The tilapia ELISA validated in the present study is sensitive and has a detection limit of 80 ng/ml VTG, which is comparable to radioimmunoassays for VTG (Tyler and Sumpter, 1990; Norberg and Haux, 1988; Benfey et al., 1989). The working range of the ELISA is very broad (80 ng/ml - 5.4 ug/ml VTG), which makes it suitable for VTG quantification when studying very weak as well as very potent estrogens. The ELISA is also very reliable and repeatable with intra- and interassay variation less than 3.4 % at the detection limit (Table 6.1) and is comparable to VTG ELISAs for other teleosts (Tyler et al., 1999; Bon et al., 1997; Kishida et al., 1992).

To test the applicability of the validated ELISA for *in vivo* analysis of VTG induction, four weak old tilapia juveniles were exposed to different concentrations of DES for seven days (Fig. 6.5). Due to the sensitivity of the validated ELISA, VTG concentration can be accurately determined in fish weighing as little as 10 mg. Exposure of juveniles to 250 ng/l DES induced significantly higher concentrations of VTG compared to control fish. A dose responsive effect to DES exposure can be observed where the juveniles induced higher VTG concentrations to higher DES levels. A similar induction of VTG has previously been shown for fathead minnow (*P. promelas*) exposed to various concentrations of estradiol-17β until 30 days post hatching (Tyler et al., 1999). Control juveniles showed VTG concentrations of 2.1 µg VTG/mg protein. This baseline level of VTG may be because of estrogenic substances in the food or small amounts of natural estrogens secreted by female fish in the breeding tanks. The ELISA was tested for the quantification of *in vitro* synthesized VTG using tilapia liver cultures as model culture system. VTG was detectable after exposing liver slices for three days to 0.5 µg/ml DES. After four days of DES exposure, an 11.2-fold increase in VTG (from 1.3 to 14.6 µg/ml VTG) was observed.

In conclusion, a tilapia competition VTG ELISA was validated using commercially available anti-seabream VTG as the detecting antibody to quantify tilapia VTG. This ELISA is very specific for VTG, has a wide detection range, is very sensitive and has low inter- and intra-assay variations. We showed that the ELISA can be used for both *in vivo* and *in vitro* quantification of VTG. Our results show that the validated competition ELISA can be used as a useful tool to detect environmental estrogen contamination and endocrine disruption using tilapia VTG as a biomarker.

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Chapter 7

The use of Mossambicus tilapia (*Oreochromis mossambicus*) juveniles as bio-indicators for environmental estrogenicity

Key words: *Oreochromis Mossambicus*, Tilapia, Vitellogenin, ELISA, EDC, Bio-monitoring.

7.1. Abstract

Millions of tons of chemicals are released into the environment every year. Some of these may cause adverse health effects through disrupting normal endocrine function of humans and wildlife. Previous investigations have indicated that most of the adverse health effects observed are due to compounds found in the environment that mimic natural endogenous estrogens or inhibit the actions of estrogen. Several *in vitro* and *in vivo* bio-assays have been developed and validated to screen for environmental estrogens. Vitellogenin (VTG) is a well-known estrogen induced yolk-precursor and is synthesized in the liver of oviparous animals. The induction of vitellogenin in many fish species is a sensitive biomarker for estrogenic activity. Because the use of adult fish may be problematic in terms of endogenous VTG levels as well as sensitivity to induce VTG upon estrogen exposure, the use of juvenile fish as a bio indicator needs investigation. The objective of this study was to develop VTG induction by juvenile tilapia (*Oreochromis mossambicus*) as a rapid *in vivo* bio-assay for estrogenicity. Natural as well as estrogen induced VTG protein levels were determined using a competition ELISA. Analysis of the natural VTG levels of an un-sexed population ranging from 15 to 70 days post fertilization (dpf) juveniles revealed a sudden significant 5 fold increase in natural VTG levels at 50 dpf fish. This increase coincided with a significant 2 fold increase in natural 17β-estradiol levels. No difference in total protein or albumin levels could be detected. It was also evident from this study that juvenile tilapias have the ability to induce VTG upon estrogen exposure from a very early stage (25 dpf). Juveniles exhibited the maximum ability to induce VTG, upon estrogen exposure between 35 and 45 dpf. At this age 200 ng/l of DES was the lowest observable concentration of estrogen that could induce significant VTG levels after 10 days of exposure.

7.2. Introduction

Human and animal health is dependent on the state of the environment that we live in. Every year hundreds of new chemicals with unknown toxicity or subtle disruptive effects on human health are released into the environment. Scientific evidence increasingly suggest that exposure to some of these chemicals, both natural and synthetic have the potential of interfering (either by modulation or disruption) with an organism's normal endocrine function (Kelce et al., 1995; Oehlmann et al., 1996; Milligan et al., 1998). These chemicals may be responsible for some of the physiological and anatomical defects seen in both wildlife and humans (Reviewed by Solomon and Schettler, 2000). Chemicals that potentially disrupt the functioning of the endocrine system are categorized as endocrine disrupting chemicals (EDCs) (Krimsky, 2000). EDCs enter the aquatic environment as either point and/or non-point sources of contamination. Point sources of contamination refer to specific sources, for instance sewage treatment plants, pulp mills and industrial effluent (Folmar et al., 2002). Non-point sources of contamination are for instance urban and agricultural runoffs (Folmar et al., 2002). Both of these two sources of contamination may include natural and pharmaceutical hormones excreted in the urine of humans and domestic livestock (Giesy et al., 2002). It may also include a wide spectrum of chemicals like organochlorine pesticides, polychlorinated biphenyls, polynuclear aromatic hydrocarbons, dioxins plasticizers and surfactants that can interact with hormone receptors or affect steroidogenesis (Ying et al., 2002). Water bodies, including rivers and dams may act as a sink for chemicals which are discharged via different routes into the environment. As a result much of the evidence for endocrine disruption has come from organisms living in the aquatic environment (Jobling et al., 1998; Rodgers-Gray et al., 2000).

The majority of the reported cases of endocrine disruption have been estrogenic effects caused by substances that mimic endogenous estrogen responses or affecting estrogen action (Panter et al., 2002). Therefore, substances with estrogenic activity have received a lot of attention during the last two decades (Aherne and Briggs, 1989; Shore, 1993; Stumpf et al., 1996, Andersen et al., 2003). The European scientific community, the Organization for Economic Co-operation and Development (OECD) and the United States Environmental Protection Agency (US EPA) are in the process of validating test assays to be incorporated in a screening and testing program to detect certain types of EDCs with special emphasis on estrogen-mimicking chemicals (U.S. EPA 1998a). Various *in vitro* and *in vivo* test assays have been validated to screen for environmental estrogens or associated biological estrogenic responses which

includes whole organism assays, cellular- and non-cellular assays (Reviewed by Campbell et al., 2006). *In vitro* systems such as recombinant yeast assays and several estrogen-responsive cell lines have proved to be valuable assay systems to determine possible estrogenic compounds as well as assessing their modes of action (Soto et al., 1995; White et al., 1994; Zacharewski et al., 1995; Beresford et al., 2000). The shortfall of in vitro assays is that they do not take into account bioaccumulation and bio-metabolism of the compound. Moreover, they exclude all metabolic effects that are not mediated via the estrogen receptor. *In vivo*/whole animal studies includes these parameters. The mouse uterotrophic assay is one of the oldest and most widely used *in vivo* assays for estrogenic activity. This assay is sensitive and can account for additive activity of multiple estrogens but exposure to androgens or progestins can also produce false positive results (Kupfer, 1988).

Several aquatic species have been studied and proposed as bio-indicators for low concentration EDCs (Bruton & Boltt, 1975; Nagahama, 1990; Helbing et al., 1992; Nagahama, 1994; Kloas, 2002; Noaksson et al., 2003; Hahlbeck et al., 2004; Sharpe et al., 2004; Zhong et al., 2005; Piferrer et al., 2005; Kuhl et al., 2005; Tao et al., 2006; Zhang et al., 2006; Filby et al., 2007; Cheshenko et al., 2008). The OECD fish screening assays suggests the use of three core endpoints or biomarkers as indicators of estrogenic endocrine disrupter activity in fish, namely: 1) gross morphology (i.e., secondary sexual characteristics), 2) vitellogenin (VTG) levels (Emmersen et al., 1979; Jobling and Sumpter, 1993; Purdom et al., 1994; White et al., 1994; Folmar et al., 1996, 2000; Hyllner et al., 1991; Oppen-Bernsten et al., 1992; Larsson et al., 1994; Arukwe et al., (1997, 1998); Denslow et al., 1997; Murata et al., 1997; Celius and Walther (1998a,b); Knudsen et al., 1998; Hemmer et al., 2001), and 3) gonadal histology (Gimeno et al., 1996; Jobling et al., 1996; Gray and Metcalfe, 1997; Panter et al., 1998; Metcalfe et al., (2000, 2001)).

Several fish based biomarker assays, measuring the expression of estrogen-regulated gene products such as VTG have been validated. Synthesis of one such product, vitellogenin (the egg yolk precursor phospholipoglycoprotein), is generally associated with oviparous reproduction and forms the basis of many screening programs for estrogenic compounds in aquatic systems (Sumpter and Jobling, 1995; Hiramatsu et al., 2005; Porte et al., 2006). VTG is normally only present in the plasma of female fish. Male fish do have the VTG gene and when exposed to estrogens, VTG can be expressed and accumulate in the plasma of male fish (Copeland et al., 1986; Purdom et al., 1994). A very useful feature of VTG as a biomarker for

estrogenicity is its magnitude of induction when an oviparous animal such as fish is exposed to estrogens. Reports have indicated that VTG plasma levels can change up to a million fold from nanogram per millilitre to milligram per millilitre when exposed to estrogens (Purdom et al., 1994; Tyler et al., 1996). This biomarker can be used for both *in vitro* (e.g., hepatocyte cultures) and *in vivo* (animal whole body) studies (Tyler et al., 1999). VTG as a biomarker can be equally as valuable in females for screening anti-estrogens by both directly (i.e., estrogen receptor agonists; Panter et al., 2000a) and indirectly (e.g., inhibitor of aromatase, Ankley et al., 2002) as well as for some types of androgens (Ankley et al., 2003).

In many fish species VTG protein induction is extremely sensitive to estrogen exposure (Sumpter and Jobling 1995; Porte et al., 2006; Hiramatsu et al., 2005). However, it seems that sensitivity differs between species and is also dependent on the specific stage of sexual development. The most sensitive species (minimum exposure period to lowest estrogen concentration that significantly induce VTG) found until now is sexually mature male trout (OECD, 2004). These fish synthesized significantly higher VTG than control fish upon exposure to 0.3 ng/l EE2 for 28 days (Sheahan et al., 1994). Juvenile fathead minnow exposed from 24 hours post fertilization to 30 days post hatch showed a lowest observed effective concentration (LOEC) for VTG induction at 50 ng/l 17β-estradiol (E2) (Tyler et al., 1999). In comparison, mature fathead minnow males that were exposed for 21 days showed significant VTG induction compared to the controls with a LOEC of 320 ng/l for E2 (Panter et al., 1998). In another study conducted by Panter et al. (2000), mature male fathead minnow exposed to E2, showed a LOEC of 30 ng/l for VTG induction. Mature male Medaka exposed for 4 days to E2 showed a LOEC for VTG induction of 20 µg/l (Foran et al., 2000). Zebrafish juveniles exposed for 21 days to E2 showed a LOEC for VTG induction at 100 ng/l, whereas adults also exposed for 21 days showed a LOEC at 25 ng/l (Brion et al., 2004).

The use of small teleosts fish species is preferred for the investigation of endocrine disrupting chemicals (Environmental Agency, 2000). Fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*) and zebrafish (*Danio rerio*) has been proposed by the OECD as the most likely species to be used as bio indicators for endocrine disruption. The fathead minnow has a strong regulatory history in the United States, whereas the zebrafish has been similarly used in Europe and the medaka in Japan (OECD, 2004). These species tolerate a wide range of water-quality and water-temperature conditions, require small culture space, overall easy to culture, produce the required number of embryos

needed for testing and have a small size at maturity, which reduces maintenance costs. The tilapiine species, *Orechromis mossambicus,* (Mozambique Tilapia), is extensively used in aquaculture (Trewavas, 1983) around the world. Tilapia, similar to fathead minnow, medaka and zebrafish, is easy to culture, tolerate a wide range of water-quality and importantly, is native to Southern Africa. Tilapia juveniles and their ability to induce VTG upon estrogen exposure, have not yet been investigated as an *in* vivo screen for estrogenicity. In order for tilapia juveniles to be used in an *in vivo* screen for estrogenicity, it is necessary to investigate natural as well as E2 inducible VTG protein levels during early development.

The aim of this study was to evaluate an un-sexed population of tilapia juveniles as a bio-indicator for estrogenicity using VTG as a biomarker. One of the objectives of this study was to determine the endogenous VTG levels of tilapia hermaphroditic juveniles to give us a better understanding of the natural biomarker background. This objective complies with OECD recommendations which states: it is important to have a better understanding for the "noise of the VTG biomarker" to clarify the range of endogenous VTG levels in both juvenile and adult fish (OECD, 2006). The second objective of the study was to determine the critical stage where juveniles are most sensitive for estrogen exposure and VTG induction. If exposure to estrogenic endocrine disruptors during early development is sufficient to be manifested by VTG induction, this may considerably lower the costs as well as make the maintenance of fish easier when testing environmental samples for estrogenic effects. The third objective of this study was to determine the lowest concentration of the synthetic estrogen, diethylstilbestrol (DES) that induces VTG expression in juvenile tilapias (less than 70 days post fertilization). Although the OECD recommends the use of ethinylestradiol as a positive control, we used DES as an alternative because of difficulty obtaining ethinylestradiol in South Africa.

7.3 Methods and Materials

7.3.1. Test Fish

Fourteen days post fertilization (dpf) swim-up fry stage *Oreochromis mossambicus* juveniles were obtained from the Welgevallen experimental farm, University of Stellenbosch, South Africa. The juveniles used for each experiment were derived from a single breeding hatch of a single adult breeding pair (one female and male) tilapias for each experiment. Fish were kept in 10 litre stock tanks (30 x 22 x 16 cm) filled with tap water. The water was constantly aerated and filtered through activated charcoal. Fish were maintained in water of similar quality for all exposure experiments, but without activated charcoal filters. Fish were maintained at a temperature of 27 °C (± 1 °C) with a light regime of 14:10 (light:dark) throughout the duration of all experiments. Fish were fed daily with crushed commercial tilapia pellets (AquaNutro, South Africa). Water was replaced every 5 days.

7.3.2. Test chemicals and exposure design

All chemicals in this study were from either Sigma Chemical Company (St Louis, MO, USA) or Roche (South Africa) unless otherwise stated. All solvents and chemicals used were of analytical grade. Diethylstilbestrol (DES) was used for all estrogen exposures and was supplied by Sigma Chemical Company (St Louis, MO, USA). DES stock solutions were prepared to a concentration of 1mg/ml using either analytical grade ethanol or dimethyl sulfoxide (DMSO) as a solvent. The final solvent concentration never exceeded 0.01 % (v/v) according to OECD recommendations (OECD, 2004). Estrogen exposures were performed under semi static conditions. Exposures were performed in 1 litre glass bottles filled with 500 ml of water. Half the volume of the test solution was exchanged every five days. Prior to experiments, all glass containers were washed with Neptune Liquid salt (Bell Products, www.bellchemicals.com). Before all experiments, fish tanks and exposure systems were washed with Hibitane (5 % antiseptic) washing agent soap, followed by a thorough rinse with water that has been charcoal filtered. Tanks were then stripped with 100 % methanol followed by a rinse with charcoal filtered water. Tanks were filled with the required amount of water and aerated at 27 °C for 48 hours prior to putting fish into it.

Juvenile tilapias were transferred to the 1 liter exposure tanks. DES or vehicle control was added to the water and the fish were then exposed for 5 days. At the end of the exposure period the fishes were anaesthetized with MS-222 (100 mg/l water containing 200 mg $NaHCO_3$). The fish were then used for further analytical procedures. The exposures were

done with groups of fish at various days post fertilization. The age of fish used for this study ranged between 15 to 70 days post fertilization.

7.3.3. Fish weight and length measurement

Fish were photographed using a Leica EZ4D microscope and measurements taken (to the nearest µm) using Leica Version 2.4.0 image analysis software (Leica Microsystems (Switzerland) Limited). The mass of juvenile fish was determined with a bench top precision scale (Ohaus Precision Scale) to nearest 0.01g.

7.3.4. Protein extraction and quantification

All the protein extraction procedures were carried out on ice. Protein extraction buffer (saline (0.9 % (w/v) NaCl in distilled water) and 0.01 % (v/v) PMSF) was added to the juvenile fish at a weight to volume ratio of 1g per 10 ml extraction buffer. Sample were then sonicated (Omni-Ruptor 400; Omni International INC.) at 40 % power. Samples were sonicated for 15 seconds in total, 5 second bursts at a time, followed by 1 min incubation on ice. Cell rests were removed by centrifugation at 12000 x g for 10 minutes at 4 ˚C. Cell pellets were discarded and the supernatants were aliquoted and stored at -80 ˚C until further use. Protein concentrations of the samples were measured according to the method of Bradford, (1976) using biovine serum albumin (BSA) as a standard protein (Sigma).

7.3.5. Vitellogenin quantification

The VTG content of the whole body homogenate preparations was determined using a competition ELISA. An ELISA system similar to the one described in chapter 6 was used to quantify VTG in juvenile fish. In this ELISA an in-house VTG antibody was used to detect VTG. Results and specifications regarding this ELISA system have been submitted elsewhere for publication (Journal of Immuno Assay and Immunochemistry). Nunc-Immuno Maxisorp® plates (Nalge Nunc, Denmark) were used for all ELISA assays. Plates were coated overnight at 4°C with 50 µl per well of 1/2000 diluted anti-VTG antiserum in saline. At the end of the incubation period the antiserum was decanted and the plate was washed four times with saline. Following the wash procedure, the remaining adsorption sites were blocked by dispensing 0.2 ml of block solution (2 % v/v human serum albumin [HSA] in saline) per well. The plate was then incubated for one hour at room temperature. The plate was washed as before where after samples or purified VTG standards (50 µl) as well as 50 µl biotinylated-VTG were added to each well. Plates were then incubated for three hours at room

temperature. The plate was washed using the same procedure as earlier described. Avidin horse radish peroxidase (AV–HRP) was diluted 1/2000 with saline containing 1 % (w/v) HSA and dispensed at 50 µl per well. Plates were incubated for one hour at room temperature where after it was decanted and washed eight times with saline. BM Blue POD soluble substrate were heated to 37°C and dispensed at 50 µl per well. Plates were incubated at room temperature for twenty minutes followed by addition of 50 µl per well of stop solution (0.5 M H_2SO_4). The optic density was lastly determined at 450 nm. A standard curve was drawn from the VTG standards. The VTG concentration of juvenile tilapia homogenates were calculated using the standard curve.

7.3.6. 17β-estradiol quantification

Estradiol was quantified using a commercial ELISA kit (catalogue number RE52041, IBL, Germany) using the manufacturer's instruction manual. In brief: microtitre plate strips precoated with rabbit anti-estradiol was removed from the strip holder and fixed firmly in the ELISA plate. All assays were done in duplicate. Samples and standards were transferred to the wells (25 µl/well). Estradiol – horseradish peroxidase conjugate was added to all the wells (200 µl/well). The solutions were mixed by gently tapping the plate, after which it was incubated for 120 minutes at room temperature. At the end of the incubation period the solutions in the wells were decanted. The wells were then washed three times with 300 µl/well of wash solution. Substrate was then dispensed at 100 µl per well after which the plate was incubated for 15 minutes at room temperature. The reaction was stropped by addition of stop solution (100 µl/well). The absorbance was determined at 450 nm using a plate reader. A standard curve was drawn using the reading obtained for the standards and the concentration of the samples was read off this curve.

7.3.7. Statistical analysis

Variation in body mass, length, total protein concentration, VTG concentration during development, as well as fold induction of VTG following exposure to DES and 17β-estradiol was assed by using single factor ANOVAs and pairwise multiple comparison procedure (Student-Newman-Keuls Method). A Pairwise multiple comparison procedure (Tukey's HSD) was used to indicate significant different groups ($P < 0.05$).

7.4. Results

7.4.1. Growth of juvenile Mozambique tilapia

The incremental average body length of juvenile tilapia fish between 15 and 70 dpf is displayed in figure 7.1. Two way analysis of variance of the average length between the control group and exposed group showed no statistical ($P > 0.05$).

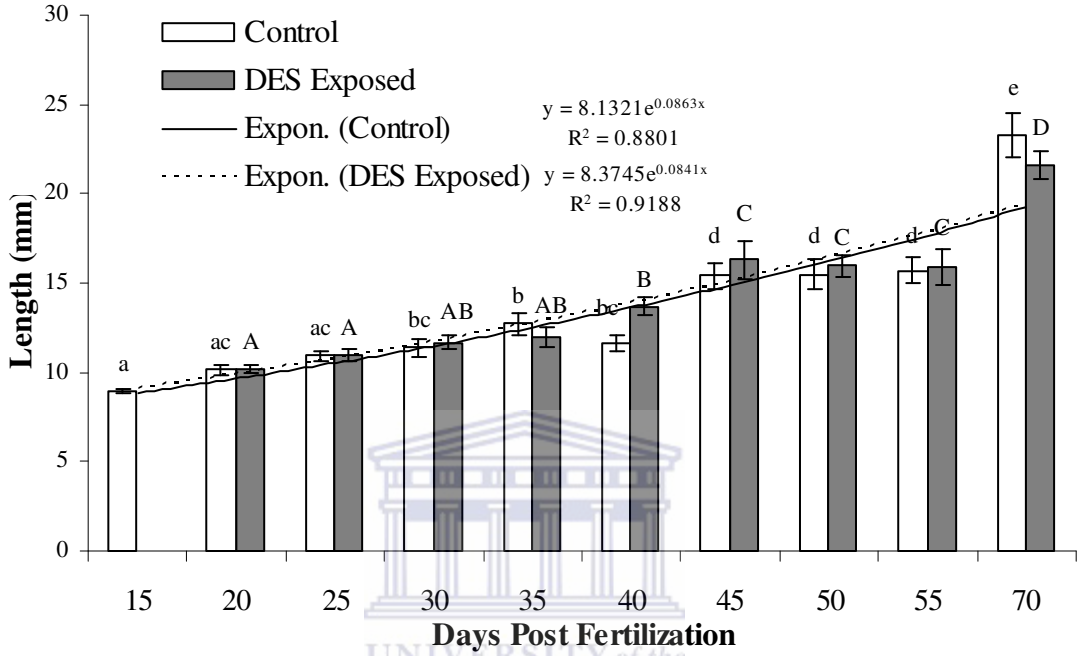

Figure 7.1. Length of juvenile tilapias determined at 5 day intervals. Tilapia juveniles were exposed to 100 µg/l DES and to the same volume of vehicle control (DMSO). At the end of the exposure period fish were sacrificed and measured. Vertical error bars represent the standard error of the mean. Each data bar is the average length obtained for 10 fish. Small letters above the data bars represent statistical comparison among the control group, whereas capitalized letters represents statistical comparison among DES exposed fish.

The body length of both the control and exposed group increased exponentially between 15 and 70 dpf. Data obtained for the body lengths of the juveniles fit exponential plots with an R^2 value of 0.89 and 0.92 for the control and exposed group respectively. The average body length of the control group of juveniles at 15 dpf was 9.0 mm (± 0.2). These fish showed no significant difference ($P > 0.05$) in body length compared to fish measured at 20 and 25 dpf. Fish measured at 30 dpf was 11.4 mm (± 0.5) long and showed no significant difference with fish measured at 35 and 40 dpf. Fish measured at 45 dpf was 14.5 mm (± 0.7) and showed no significant difference ($P > 0.05$) in length with fish measured at 50 and 55 dpf. Fish measured at 70 dpf was 23.3 (± 1.2) in length and was significant higher than the previous group measured. It can be observed from this graph that juvenile tilapia fish between 15 and 70 dpf in a laboratory environment takes on average 10 days to significantly increase in length.

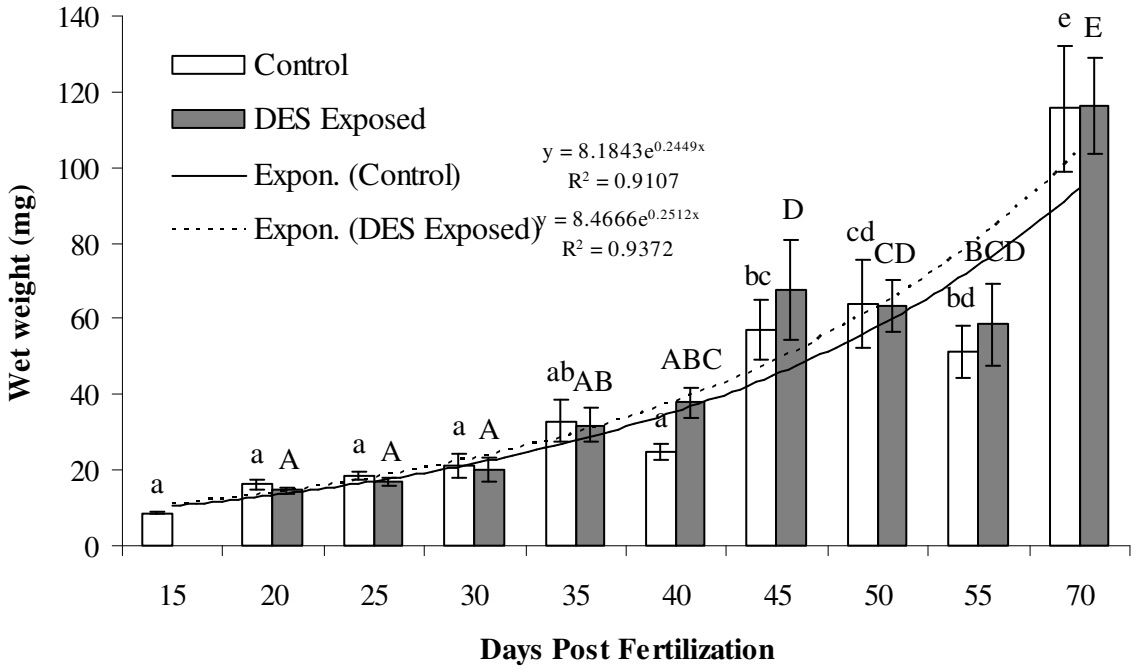

Figure 7.2. Wet weight of juvenile tilapias weighed at 5 day intervals. Tilapia juveniles were exposed to 100 µg/l DES and to the same volume of vehicle control (DMSO). At the end of the exposure period fish were sacrificed and measured. Vertical error bars represent the standard error of the mean. Each data bar is the average length obtained for 10 fish. Small letters above the data bars represent statistical comparison among the control group, whereas capitalized letters represents statistical comparison among DES exposed fish.

7.4.2. Wet weight of juvenile tilapias

The average body mass of juvenile tilapia fish weighed between 15 dpf and 70 dpf is displayed in figure 7.2. Two way analysis of variance of the average wet weight between the control group and exposed group showed no statistical ($P > 0.05$). At 15 dpf juveniles had a body mass of 8.5 mg ± 0.3 which increased to 115.6 mg ± 16.6 at 70 dpf. The body mass of juvenile tilapias between 15 and 70 dpf, weighed at 5 day intervals increased exponentially. Data obtained for the fresh weight of both the control and exposed groups fit exponential plots with R^2 values of 0.91 and 0.94 respectively. Statistical analyses (ANOVA) of the data show that tilapia weight between 15 and 40 dpf in both the control and exposed group show no significant differences. Tilapia weight between 45 and 55 dpf again showed no significant differences but were significantly higher than fish weighed between 15 and 40 dpf. Fish weight at 70 dpf was statically the heaviest.

7.4.3. Total protein yield of juvenile tilapias

At the end of each 5 day exposure period fish were sacrificed. Total protein was extracted and this was quantified using Bradford assays (Fig. 7.3).

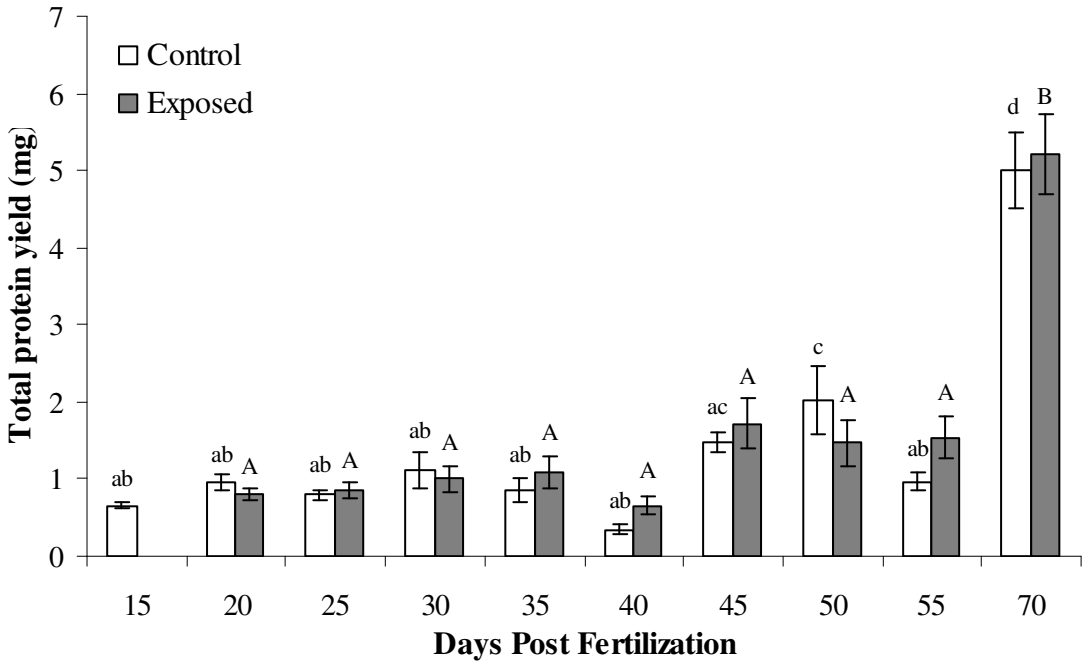

Figure 7.3. Total protein yield of juvenile tilapias determined at 5 day intervals. Tilapia juveniles were exposed to 100 µg/l DES and to the same volume of vehicle control (DMSO). At the end of the exposure period fish were sacrificed total protein yield determined. Vertical error bars represent the standard error of the mean. Each data bar is the average total protein yield obtained for 10 fish. Small letters above the data bars represent statistical comparison among the control group, whereas capitalized letters represents statistical comparison among DES exposed fish.

Pair wise analysis (Tukey's HSD) of control and DES exposed fish at each dpf data point showed no statistical variation in total protein yield. Moreover, the total protein yield of juveniles between 15 and 55 dpf also showed no statistical differences (ANOVA, $P > 0.05$). However, control fish at 50 dpf showed a protein yield significantly higher (ANOVA, $P < 0.05$) than the younger fish. The total protein yield from 70 dpf fish was significantly higher (ANOVA, $P < 0.05$) than all the other sampling points. At this dpf the total amount of extracted protein were approximately 5 times higher than the average total protein extracted from 50 dpf sampling fish.

7.4.4. Vitellogenin in juvenile tilapias

Fish that received the DMSO vehicle control showed no significant differences ($P > 0.05$) in VTG protein levels compared to fish that were maintained in water without DMSO and therefore represent natural VTG levels in juvenile tilapias (Results not shown). Natural VTG levels showed little/small variation (1.3 - 10.7 µg VTG/mg total protein) between 15 dpf through 45 dpf.

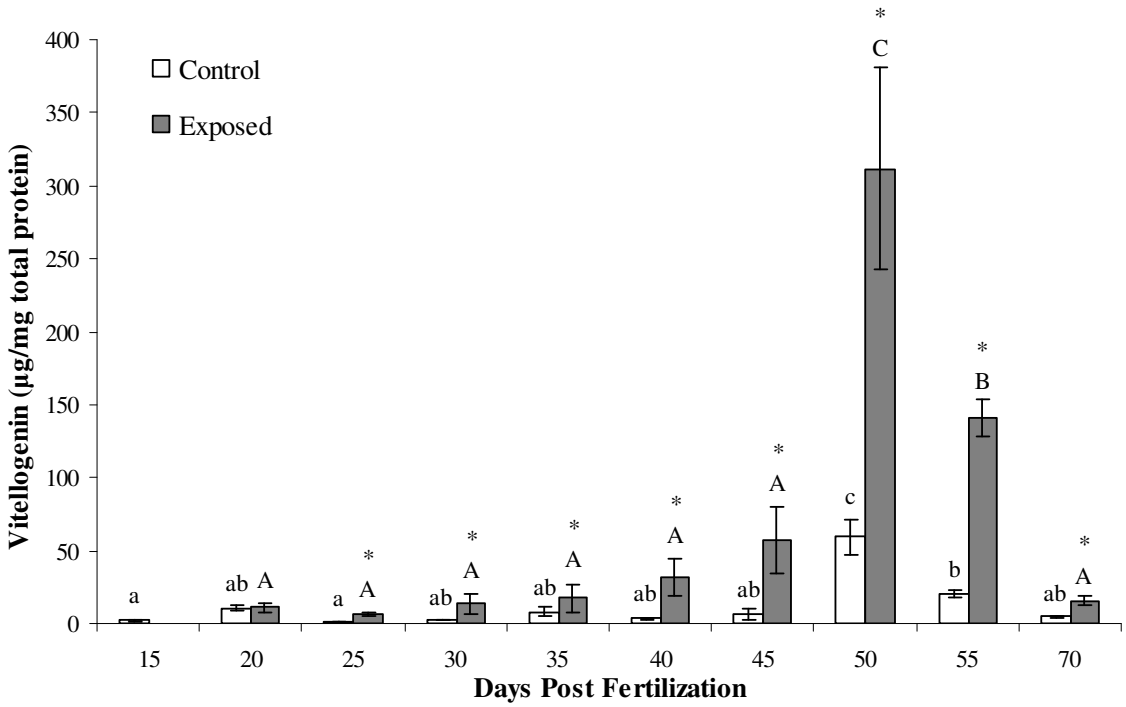

Figure 7.4. VTG protein levels in juvenile tilapias. Tilapia juveniles were exposed to 100 µg/l DES and to the same volume of vehicle control (DMSO) for 5 days per exposure. At the end of the exposure period fish were sacrificed and VTG protein levels quantified. Vertical error bars represent the standard error of the mean for 10 individual fish. Small letters above the data bars represent statistical comparison among the control group, whereas capitalized letters represents statistical comparison among DES exposed fish. Significant VTG levels of the exposed fish in comparison with the control group at a specific dpf is indicated by a (*) above the appropriate data bar.

Statistical analyses (ANOVA) of the VTG protein levels showed that this variation was non-significant ($P > 0.05$) among these treatment groups. At 50 dpf there was a 5 fold increase in natural VTG protein levels that were significantly (ANOVA, $P < 0.001$) higher than the other dpf analyzed. However, VTG levels obtain from 55 dpf were again reduced and showed no significant differences ($P > 0.05$) compared to VTG levels observed in fish from 30 – 45 dpf and 70 dpf (see figure 7.4).

VTG levels in fish exposed to DES were significantly higher compared to their respective control groups at all the dpf analyzed, except for 20 dpf. Similar to the results obtained for the natural VTG levels, DES induced VTG levels varied between 10.9 and 57.4 µg VTG/mg total protein between 20 and 45 dpf. Statistical analysis showed no significant differences between VTG levels at the different time points (ANOVA, $P > 0.05$). Again a 14 fold increase in VTG levels in DES exposed fish was observed at 50 dpf that were significantly ($P < 0.001$) higher than VTG levels between 20 and 45 dpf. In contrast with the natural VTG levels a similar

stimulatory effect was observed for 55 dpf fish (6 fold induction). DES induced VTG levels decreased and returned to levels similar to the pre – 50 dpf levels at 70 dpf.

7.4.5. Fold VTG induction

The ratio of DES induced VTG over the natural VTG (fold VTG induction) was calculated for each time point as explained in the Methods and Materials section (Fig. 7.5). The fold VTG induction was determined in order to assess at which stage of tilapia juvenile development fish are most sensitive for estrogen dependent VTG induction. The fold VTG induction of fish between 20 and 35 dpf revealed no significant differences, except for fish at 25 dpf that were significantly higher. However, the fold VTG induction of fish exposed to DES from 40 to 55 dpf were significantly higher than fish between 20 and 35 dpf. The fold VTG induction of fish from 40 to 55 dpf ranged between 5.3 to 8.0 fold, whereas 20, 30 and 35 dpf fish showed VTG induction levels of 1.0, 2.2 and 1.1 respectively.

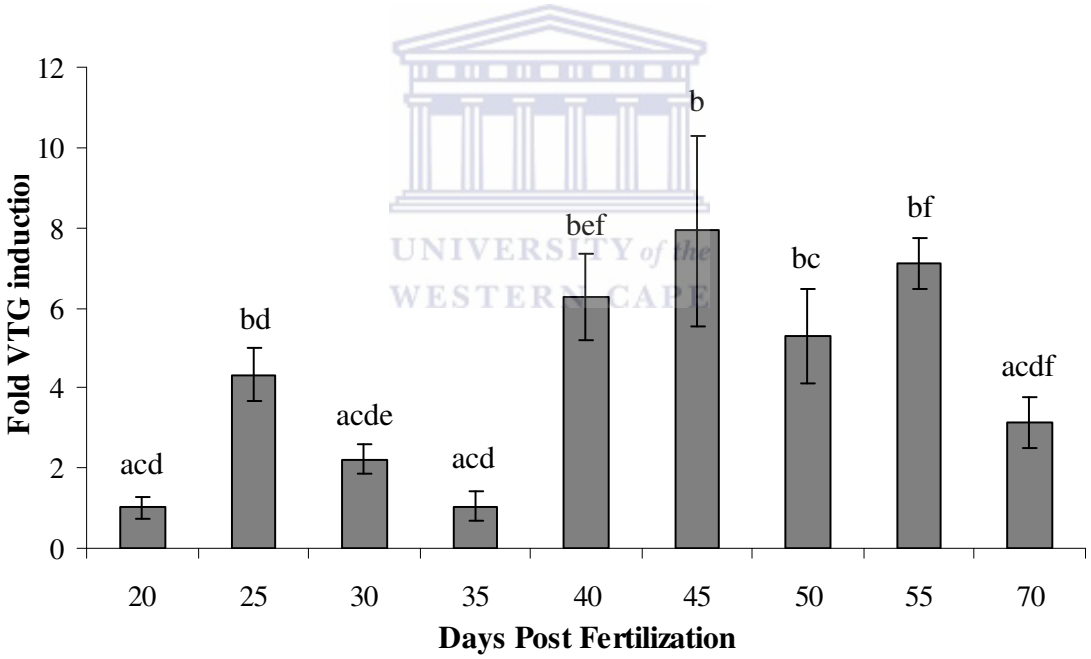

Figure 7.5. Fold VTG induction of juveniles during development. Tilapia juveniles were exposed to either 100 µg/l DES, or to the same volume of vehicle control (DMSO) for 5 days per exposure. At the end of the exposure period fish were sacrificed and VTG protein levels quantified. The fold VTG induction was calculated as the DES induced VTG protein concentration of 10 individual fish divided by the average natural VTG concentration of 10 fish from that respective dpf. Vertical error bars represent the standard error of the mean for 10 individual fish. Letters above the data bars represent significant differences in VTG levels among different dpf compared.

7.4.6. Albumin levels in juvenile tilapias

Albumin (Alb) levels were determined in the same whole body homogenates that were used for total protein preparations and VTG quantifications and are displayed in figure 7.6. Alb concentrations were expressed as mg Alb/mg total protein. No significant differences in the Alb levels were observed when DES exposed fish and control fish were compared at all of the dpf analyzed (ANOVA, $P > 0.05$). Alb concentrations significantly increased ($P < 0.05$) from 0.4 – 0.9 mg Alb/mg total protein from 15 – 25 dpf in control fish. No significant differences ($P > 0.05$) in Alb levels however, could be observed between 25 and 55 dpf, although Alb concentrations varied between 0.65 and 0.95 mg Alb/mg protein in the control fish. At 70 dpf Alb levels decreased again and showed no significant differences ($P > 0.05$) in comparison to Alb concentrations of 15 and 20 dpf fish. Similar trends in Alb concentrations can be observed for fish exposed to DES compared to the control group. Alb levels steadily increase from 15 to 25 dpf reaches a plato and then decrease from around 50 – 55 dpf to 70 dpf in our study.

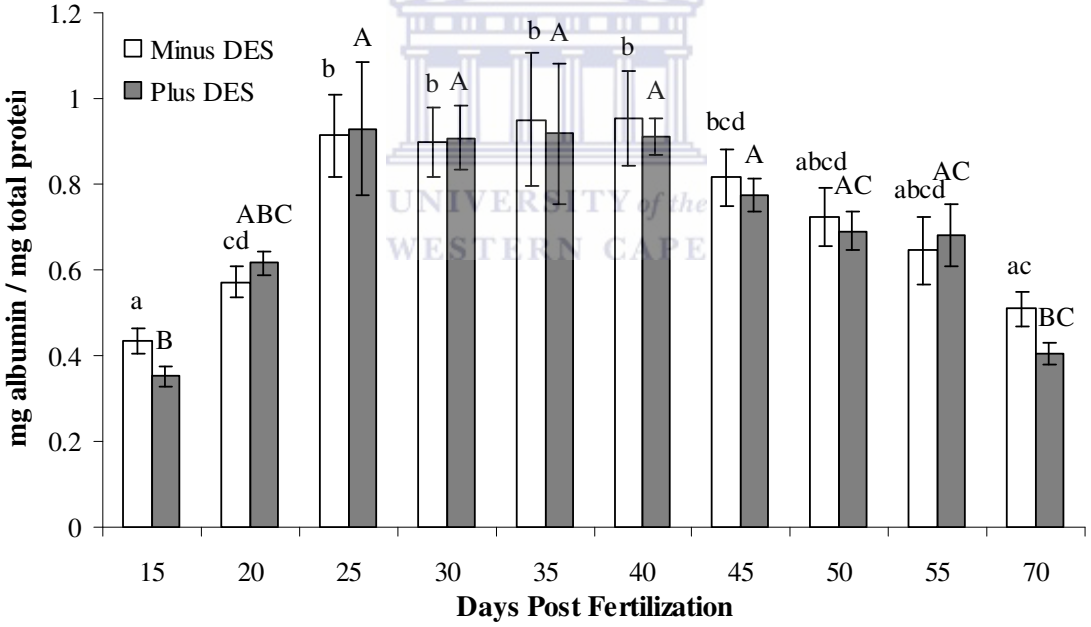

Figure 7.6. Albumin protein levels in juvenile tilapia. Tilapia juveniles were exposed to 100 µg/l DES and to the same volume of vehicle control (DMSO) for 5 days per exposure. At the end of the exposure period fish were sacrificed and Alb protein levels were determined. Vertical error bars represent the standard error of the mean for 10 individual fish. Small letters above the data bars represent statistical comparison among the control group, whereas capitalized letters represents statistical comparison among DES exposed fish.

7.4.7. 17β-estradiol (E2) levels in juvenile tilapia

E2 concentrations were determined in tilapia whole body homogenate preparations that ranged between 45 - 70 dpf (Fig. 7.7). E2 levels were 10.5 ± 1.1, 22.4 ± 2.5, 9.7 ± 3.5 and 4.3

± 0.4 pg E2/mg total protein at 45, 50, 55 and 70 dpf respectively. No significant differences ($P > 0.05$) in E2 could be detected in fish at 45, 55 and 70 dpf. A significant ($P < 0.001$) two fold increase in E2 was observed at 50 dpf fish compared to the other time points investigated.

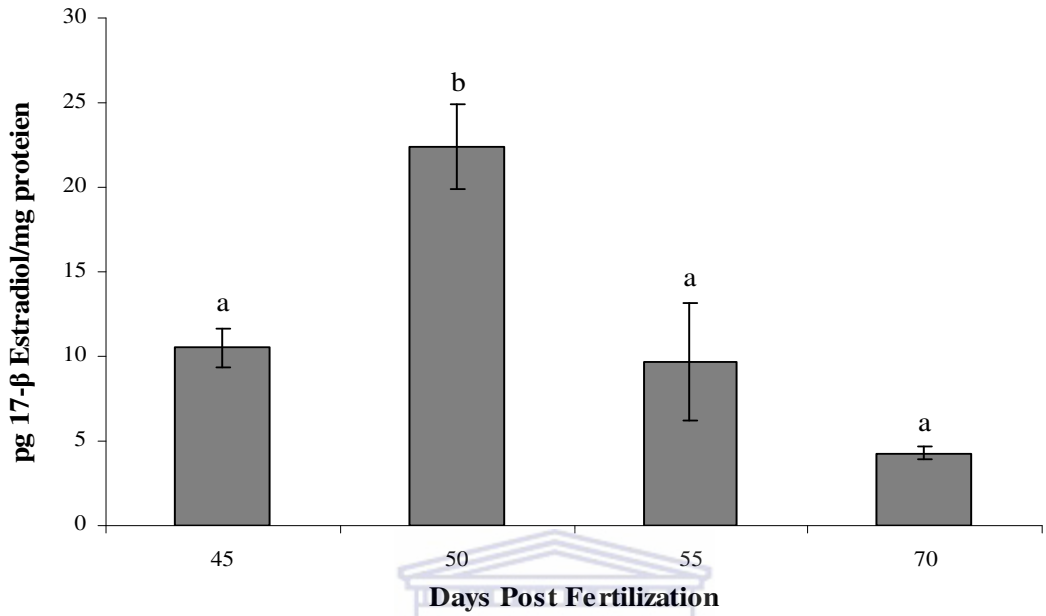

Figure 7.7. 17β-estradiol levels in juvenile fish. Estradiol levels were determined in the same control fish that were used for VTG quantification. Vertical error bars represent the standard error of the mean for 10 individual fish. Letters above the data bars represent statistical comparison of estradiol levels between the different dpf.

7.4.8. The effect of DES dose on VTG induction by tilapia juveniles

In this experiment tilapia juveniles were exposed for a period of 5 and 10 days to a series of different DES concentrations (Fig. 7.8). Exposure of the juveniles for 5 days to a series of different DES concentrations showed that only fish exposed to 100 µg/l DES were able to induce significantly higher ($P < 0.001$) VTG compared with the DMSO control. A three fold induction of VTG was observed after 5 days exposure to 100 µg/l DES. An exposure period of 10 days shows that the minimum DES concentrations that could induce significantly higher ($P < 0.001$) VTG compared to the control was 0.2 µg/l.

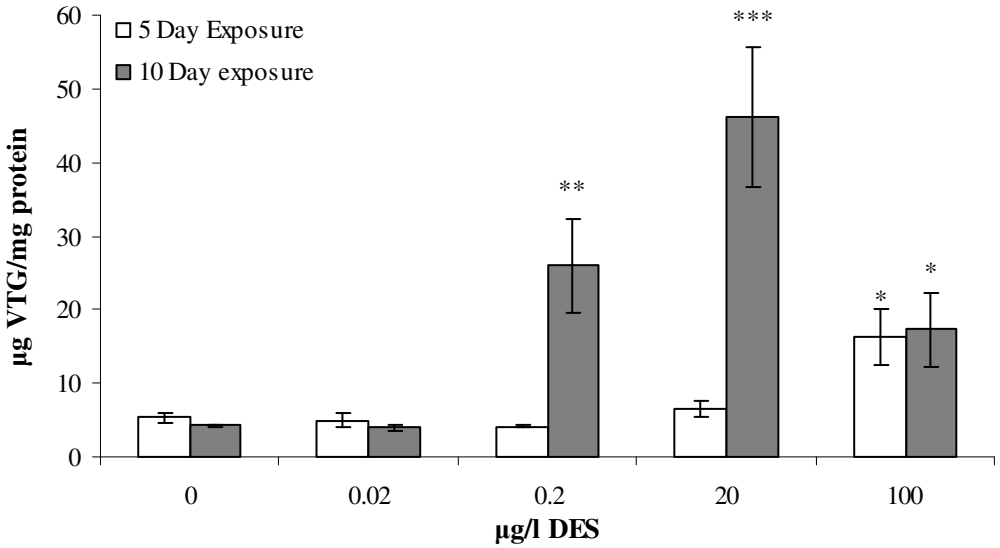

Figure 7.8. DES dose responsive induction of VTG in tilapia juveniles. Tilapia juveniles (35 dpf) were exposed for 5 and 10 days to a series of different DES concentrations. At the end of the exposure period, WBH protein preparations were prepared, followed by the quantification of VTG. Vertical error bars represents the standard error of the mean of 10 individual fish. Significant differences ($P < 0.001$) in VTG levels at a certain DES concentration in comparison with the DMSO control is indicated by (*).

A 6-,10- and 4-fold induction of VTG protein levels compared to the control was observed after exposing fish for 10 days to 0.2, 20 and 100 µg/l DES respectively. Exposure of juveniles for 10 days to a series of DES concentrations ranging from 0.02 – 100 µg/l DES show that 20 µg/l DES induced the highest levels of VTG protein (46.0 ± 9.5 µg VTG/mg protein), whereas 100 µg/l DES induced the lowest levels of VTG protein (17.3 ± 5.0 µg VTG/mg protein) compared to the control group (4.2 ± 0.3 µg VTG/mg protein).

7.5. Discussion

The administration of E2 to male tilapia, *Oreochromis mossambicus* has previously been reported to induce VTG synthesis in the liver with a subsequent increase in plasma VTG (Takemura and Kim, 2001). The aim of this study was to investigate VTG induction in juvenile tilapias as a potential *in vivo* screening assay for environmental estrogens. Tilapia is indigenous to Southern Africa (Matthews and Brandt, 2004). This species is also indigenous to other parts of Africa as well as the Middle East and is used for mainly aquaculture purposes. Tilapias are hardy fishes, with a wide environmental tolerance and they reach sexual maturity at a relatively young age, which allows for rapid population growth ideal for aquaculture (Trewavas, 1983; Matthews and Brandt, 2004).

Exposure of juveniles to DES had no significant effect on somatic growth (wet weight, length and total protein yield) (Fig. 7.1, 7.2, 7.3) between 15 and 70 dpf. Although we have used a static exposure system in our study, previous studies using flow through systems have also found that DES at these relevant concentrations have no significant effect on somatic growth (Lye, 1998; Panter et al., 2002). However, longer exposure periods to xenoestrogens may influence somatic growth. For instance a study conducted by Länge et al. (2001) on fathead minnows showed that the potent synthetic estrogen 17α-ethynylestradiol significantly inhibited somatic growth over an exposure period of 305 days at 4 ng/l. In our juvenile tilapia study, the longest period that fish were exposed to DES was 10 days. Therefore, it is unlikely that DES exposure will influence somatic growth over such a short exposure period.

In order to validate a biomarker for endocrine disruption using whole animals, it is necessary to know the endogenous level of the biomarker during the specific developmental stage being characterized. Natural endogenous VTG levels of fish were determined every 5 days from 15 - 70 dpf. VTG concentrations were statistically similar ($P > 0.05$) between 15 and 45 dpf and varied between 1.3 - 10.7 µg VTG/mg total protein (1.3 - 10.7 µg/ml VTG) (Fig. 7.4). Inter study comparisons of VTG levels are always difficult when VTG protein concentrations are expressed as the amount of protein/ml whole body homogenate as the ratio of fish wet weight to protein extraction buffer differs between studies. The natural baseline VTG levels for juvenile tilapia fish in our study correspond to values reported in previous studies. For example, Panter et al. (2002) reported plasma VTG concentrations around 1 µg/ml, in fathead minnow (between 45 and 100 days post hatch), Brion et al. (2004) reported 0.1 µg/ml at 21 dpf and 1 µg/ml VTG at 42 dpf in zebrafish. However, the five fold increase in natural VTG

levels at 50 dpf has not yet been reported before. These elevated VTG levels, however, returned to the baseline VTG concentration at 70 dpf. Similar to the peak in VTG levels found at 50 dpf in control fish, this peak was also observed in juveniles exposed to 100 µg/l DES at 50 dpf (Fig. 7.4).

Further investigations showed that this peak in VTG at 50 dpf may have been a result of natural endogenous E2 levels. Tilapia juvenile whole body homogenates from 45, 50, 55 and 70 dpf were used to determine endogenous E2 concentrations. The increment of VTG protein levels at 50 dpf coincide with a two fold increase of endogenous 17β-estradiol at this specific dpf. In order to rule out the possibility that there may have been a discrepancy with the protein extraction or determination assay at 50 dpf (both VTG and E2 concentrations are expressed per mg total protein), albumin levels were determined in WBH (Fig. 7.6). In figure 7.6 it is clear that there was no significant difference ($P > 0.05$) in albumin levels between 45 – 55 dpf. Moreover, the total protein yield of 50 dpf juveniles was significantly higher than that of 45 and 55 dpf. VTG and E2 data, although significantly higher, may thus appear even lower than their actual levels because protein concentrations are also elevated at 50 dpf. The increase in both plasma E2 and VTG concentrations at 50 dpf may mark the onset of female sex differentiation. In support hereof, histological gonadal differentiation in juvenile tilapia has been reported to occur around 55 dpf (Esterhuyse et al., 2008). Moreover, the latter study reports an increase in aromatase gene expression around 25 dpf thus illustrating the possible onset of steroidogenesis preceding vitellogenesis observed at 50 dpf in the present study. The timing of sexual differentiation differs widely among species. In the Japanese medaka, sex differentiation of the female occurs before hatching while, in the male, differentiation of the testis takes place around 13 days post hatch (Yamamoto, 1953, 1975). In carp sexual differentiation in the females normally occurs between 50 and 60 dph while the male gonad remained undifferentiated until 90 dph (Komen et al., 1995). In zebrafish sex differentiation began between 21 and 28 dph and was completed at approximately 40 dph (Takahashi, 1977; Uchida et al., 2004). This phenomenon however needs to be further investigated in tilapias.

Although the peak in endogenous VTG expression was observed at 50 dpf, a significant VTG induction upon estrogen exposure was observed at 25 dpf (Fig. 7.4). Legler et al. (2000) demonstrated in transgenic zebrafish that the estrogen receptor subtypes α and β mRNA is detectable in all developmental stages as early as 1 dpf. This study showed that zebrafish ER α mRNA expression reached a peak at 35 dpf, whereas ER β mRNA were the highest from 28

– 35 dpf during development. Esterhuyse et al. (2008) showed that ER gene expression is detectable from 20 dpf in tilapia juveniles. Expression of the ER subtypes is a physiological prerequisite for VTG expression. In fathead minnow VTG protein synthesis was shown to occur in fish exposed to E2 from 24 h post fertilization to 30 d post hatch in a dose-dependent manner (Tyler et al., 1999). One of the major objectives of the present study was to identify the developmental stage of juvenile tilapias, between 15 and 70 dpf, where fish can be used as an *in vivo* screen for estrogens. The ability of juveniles to produce the maximum VTG concentrations in comparison with control fish was used as a measure to identify this developmental stage. Fish were exposed to the maximum sub-lethal level of DES (OECD, 2004) for the minimum time period and the fold VTG induction compared to natural VTG levels were calculated (Fig. 7.5). DES exposure of juveniles for a 5 – 10 day period ending between 40 and 55 dpf show the highest VTG induction compared to non-exposed controls (5 – 8 fold inductions). Although younger fish (less than 40 dpf) revealed some VTG induction potential, the DES induction response was much lower (1 - 2 fold) compared to the controls. A possible explanation may be that endogenous VTG during this stage is relatively high on account of various physiological needs (Hiramatsu et al., 2005)

Juvenile tilapias (35 dpf) were exposed to a series of DES concentrations in order to determine what the lowest concentration of DES is that can induce significant VTG levels (Fig. 7.8). Our results support previous findings that the concentration and time of estrogen exposure to induce VTG are inversely correlated. For example, the lower the concentration of estrogen the longer the exposure period required to significantly induce VTG (OECD, 2004). Exposure of juveniles for five days revealed that the lowest level of DES that can induce significantly higher levels of VTG compared to the control is 100 µg/l, whereas 200 ng/l DES was the minimum concentration of DES that could induce significantly higher VTG levels after 10 days of exposure. It was observed from figure 7.8 that 10 day exposure of juveniles to 100 ug/l DES suppressed VTG induction compared to 20 µg/l DES. In previous studies the minimum concentration of E2 that could induce significant VTG levels in juveniles or adults (rainbow trout (*Oncorhynchus mykiss*) ranged between 10 ng/l (Thorpe et al., 2001) to between 27 and 100 ng/l in other cyprinid fish (fathead minnow; Kramer et al., 1998; Panter et al., 1998; roach, *Rutilus rutilus*; Routledge et al., 1998). In cyprinodont fish (sheepshead minnow, *Cyprinodon variegates*), the least effective concentration for plasma vitellogenin induction was 200 ng/l (Folmar et al., 2000).

In summary this study shows that exposure of juvenile tilapias to DES between 15 and 70 dpf does not influence somatic growth. Tilapia juveniles at this very early stage of development have the ability to express significant amounts of VTG when exposed to DES for a period of 5 days. Analysis of the natural VTG levels of a mixed sex population of juveniles between 15 and 70 dpf shows a peak in VTG expression at 50 dpf. This peak in VTG expression coincides with a peak in endogenous E2 levels and may mark the start of sexual differentiation and puberty. In order to use tilapia juveniles as an *in vivo* screen for estrogenicity it is important to identify the developmental stage where animals are most sensitive for estrogen and VTG induction. We showed that tilapia juveniles used for *in vivo* estrogenicity assays should be between 35 – 45 dpf for maximum sensitivity. This is a very short window period of 10 days where juvenile tilapias shows the maximum ability to induce VTG upon E2 exposure. Dose response experiments revealed that the lowest concentration of DES that induces VTG is 200 ng/l after an exposure period of 10 days. In this study we showed that juvenile tilapias can be successfully employed as a screen for estrogenicity. We identified the age at which juveniles are most sensitive for estrogen exposure to induce VTG. We also established the baseline VTG levels of a mixed sex population of juveniles. In future studies this *in vivo* screen for estrogenicity should be validated for other identified synthetic estrogens, anti-estrogens, as well as environmental water extracts.

We propose further studies on the development of *Oreochromis mossambicus* as indigenous Africa indicator species for environmental estrogens.

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Chapter 8

Assessment of water collected from the Eerste River for estrogenicity

Key words: e-EDCs, bio-assay, battery assays, ELISA, ERα, MCF-7, VTG

8.1. Abstract

To evaluate the estrogenic activity of environmental water samples a panel of *in vitro* and *in vivo* bio-assays, which are based on different action mechanisms, are required. In the current study a test battery containing four assays were used to analyse water from the Eerste River, South Africa for estrogenicity. Three sites were used for analysis, namely Jonkershoek (control site situated in the mountains at the origin of the Eerste River), sewage effluent from Stellenbosch sewage treatment works and Spier (sampling site on the Eerste River downstream from Stellenbosch). Estrogenicity was determined using the estrone ELISA, estrogen induced proliferation of MCF-7 cells (E-screen), estrogen induced suppression of ERα in MCF-7 cells (ERα assay), and by monitoring estrogen induced vitellogenin synthesis in juvenile *Oreochromis mossambicus* (VTG assay). Low levels of estrone were detected in samples collected from Jonkershoek. Water from this site shows no estrogenic activity when the E-screen, the ERα assay, nor the VTG assay was used for monitoring. The estrone levels in the sewage effluent extracts, as well as Spier extracts, ranged between 14.76 and 19.42 ng/l. The assays using ERα induction by the MCF-7 cell line, MCF-7 proliferation and *in vivo* VTG synthesis by juvenile tilapia showed that these samples are estrogenic. Results obtained for the assays in the battery are comparable.

8.2. Introduction

The occurrence of estrogenic compounds in the environment is a well studied phenomenon (Gellert 1978; Hammond et al., 1979; Kupfer and Bulger, 1987; Krishnan et al., 1993; White et al., 1994; Newbold 1995; Katzenellenbogen 1995; Nimrod and Benson, 1996). Most environmental estrogens are synthetic compounds produced as a result of industrial, domestic and agricultural activities (Kuiper et al., 1998). However, not all estrogenic compounds are man-made. Some environmental estrogens originate from natural sources (Kuiper et al., 1998; Giesy et al., 2002). Examples of these are the phyto- and the myco-estrogens. Several studies, including the previous chapters of this thesis, have focused on the development and implementation of assays to monitor environmental estrogens. Pollutants can act at several points on the endocrine/reproductive system to give estrogenic or anti-estrogenic effects (Reviewed by Baker, 2001). Some pollutants mediate their action by directly binding to the estrogen receptor, while other pollutants act by for instance modulating the estrogen biosynthesis pathways resulting in either increased or decreased circulatory estrogen levels (Sharpe and Irvine, 2004). Moreover, several pollutants demonstrate effects that are species, tissue- and cell-specific, as well as being influenced by metabolism (WHO/IPCS, 2002). Due to the varied biological actions of estrogenic pollutants, current strategies for monitoring estrogens favour the use of a battery of tests, representative of several potential points on the estrogenic response pathways, for monitoring (Reel et al., 1996; Shelby et al., 1996; Baker et al., 1999a,b, 2000).

South Africa, like most countries in the world, faces ecological challenges due to EDCs found in the aquatic environment. The aim of the current study is to implement a newly developed bio-monitoring battery of tests as a first tier screen for estrogenicity of a river. This battery consists of three *in vitro* and one *in vivo* screen for estrogenicity. Water samples were firstly analysed for the specific female hormone, estrone. Total estrogenicity of the water samples were determined using two more *in vitro screens*, namely a modified MCF-7 proliferation assay (E-Screen) and a MCF-7 ERα determination ELISA. Vitellogenin induction in juvenile tilapias was used as a short term exposure *in vivo* assay. The river that was selected for this study is the Eerste River, Stellenbosch, South Africa. Several studies have investigated this river system. A study conducted by Reinecke et al., (2003) found significant concentrations of Mn, Cu, Pb, Cd and Zn in both the water (ranging between 0.006 – 0.222 mg/l) and sediment (ranging between 0.43 – 184.7 mg/kg). The same study reported total coliforms concentrations of 1.2 x 10^5 / 100ml as well as faecal coliforms concentrations of 1.5 x 10^4 /

100 ml from water that passed through Stellenbosch town centre. In another study, all the water samples collected from the Eerste River showed inflammatory potential using interleukin 6 syntheses by whole blood cultures as biomarker (Pool et al., 2000). Figure 8.1 is a map of the river indicating the sites that were used for this study. Sites were selected to include water not affected by human activity (Jonkershoek) as well as samples from sites severely affected by agricultural, industrial and domestic pollution.

8.3. Methods and Materials

8.3.1. Study Area

The origin of the Eerste River is in the Jonkershoek Valley of the Western Cape in South Africa. It flows through the Jonkershoek Forest Reserve, the Assegaaibosch Nature Reserve and several vineyards before reaching the town of Stellenbosch (33˚56'10"S; 18˚51'34"E). For the rest of its course the river is bordered by agricultural land, industrial areas, formal and informal housing before reaching the sea on the False Bay coast (Fig. 8.1.). Eerste River water samples were collected at Jonkershoek, Stellenbosch sewage treatment works (Stellenbosch STW) and near Spier wine estate.

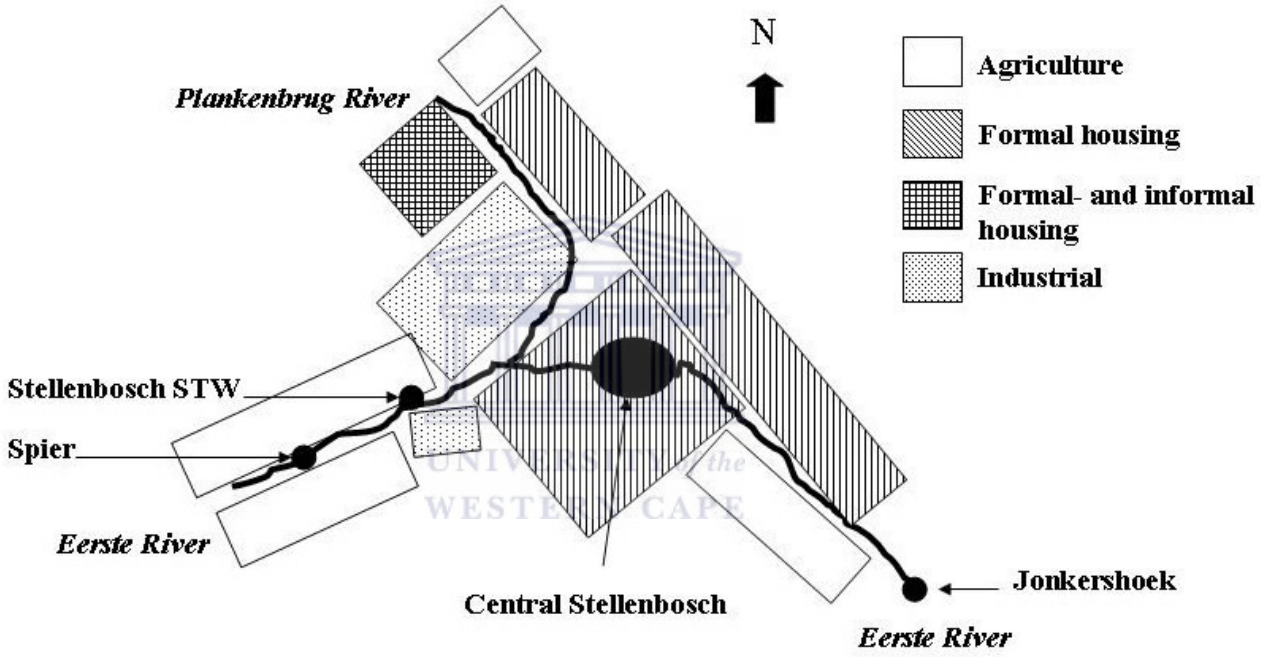

Figure 8.1. Map of the Eerste River in Stellenbosch, South Africa. Water samples were collected at Jonkershoek, Stellenbosch STW and Spier. Water samples were collected in January during high summer as well as April after the first winter rains. Water samples were collected in 2007 and 2008 for analysis.

8.3.2. Water collections

Water samples were collected in clean glass bottles. The bottles were cleaned by washing with soap, followed by rinsing with tap water. The bottles were then rinsed with HPLC grade water and finally it was rinsed with HPLC grade ethanol (Merck, Germany). The bottles were then allowed to dry up-side down in a dry rack. Samples were collected in January, at the peak of the dry summer season and at the beginning of the rain season in April. Water samples were taken to the laboratory and were processed immediately.

8.3.3. Water hydrophobic content extractions

Extracts were prepared as previously described by Swart and Pool, (2007). The dried water extracts were reconstituted in dimethyl sulfoxide (DMSO) to give a final volume 1/1000 times that of the original sample volume.

8.3.4. Estrone ELISA

The estrone levels of the water extracts were quantified as previously described by Swart et al., (2007).

8.3.5. MCF-7 assays

The MCF-7 total cellular protein, total cellular LDH and ERα levels were determined as described in chapters 4 and 5 of this thesis.

8.3.6. Juvenile tilapia *in vivo* bio-assay for estrogenicity

The induction of vitellogenin by juvenile tilapias was used as an *in vivo* bio-assay for environmental estrogenicity and toxicity. The tilapias were exposed to estrogen controls or samples as described previously (Chapter 7). At the end of the exposure period, juveniles were sacrificed and protein was extracted from whole body homogenates. In short, fish were weighed, placed in protein extraction buffer (Saline containing 0.01 % (w/v) Phenylmethylsulphonylfluoride [PMSF]) pre-cooled to 4 ˚C, at 10 ml buffer per gram body mass). Samples were then sonicated (Omni-Ruptor 400; Omni International INC.) at 40 % power. Samples were sonicated for 15 seconds at 5 second bursts followed by 1 min incubation on ice. Cells rests were removed by centrifugation at 12000 x g for 10 minutes at 4 ˚C. Cell pellets were discarded and the supernatants were aliquoted and stored at -80 ˚C until further use. Protein was measured according to the method of Bradford (1976) using bovine serum albumine (BSA) as a standard protein (Sigma).

8.3.7. Tilapia VTG ELISA

The VTG concentrations of the whole body homogenates were determined using a competition ELISA. An ELISA system similar to the one described in chapter 6 was used to quantify VTG in juvenile fish. In this ELISA, an in-house VTG antibody was used to detect VTG. Results and specifications regarding this ELISA system have been submitted elsewhere for publication (Journal of Immuno Assay and Immunochemistry). Nunc-Immuno Maxisorp® plates (Nalge Nunc, Denmark) were used for all ELISA assays. Plates were coated overnight

at 4°C with 50 µl per well of 1/2000 diluted anti-VTG antiserum in Saline. At the end of the incubation period wells were decanted and washed four times with Saline. Following the wash procedure, remaining adsorption sites were blocked by dispensing 0.2 ml of block solution (2 % v/v human serum albumin [HSA] in Saline) per well. The plate was then incubated for one hour at room temperature. The plate was washed as before where after samples or purified VTG standards (50 µl) as well as 50 µl biotinylated-VTG were added to each well. Plates were then incubated for three hours at room temperature. The plate was washed using the same procedure as earlier described. Avidin horse radish peroxidase (AV–HRP) was diluted 1/2000 with Saline containing 1 % (w/v) HSA and dispensed at 50 µl per well. Plates were incubated for one hour at room temperature where after it was decanted and washed eight times with Saline. BM Blue POD soluble substrate were heated to 37°C and dispensed at 50 µl per well. Plates were incubated at room temperature for twenty minutes followed by addition of 50 µl per well of stop solution (0.5 M H_2SO_4). The optical density was lastly determined at 450 nm. A standard curve was drawn from the VTG standards. The VTG concentration of juvenile tilapia homogenates were calculated using the standard curve.

8.3.8. Statistical analysis

The results were analysed by using single factor ANOVAs and pairwise multiple comparison procedure (Student-Newman-Keuls Method). A Pairwise multiple comparison procedure (Tukey's HSD) was used to indicate significant different groups ($P < 0.001$).

8.4. Results

In the present study, water from three sites of the Eerste River namely: Jonkershoek, Stellenbosch STW and Spier were analysed for estrogenicity using a battery of biological assays as described in the introduction section. Water samples were collected in 2006 and 2007 during January (Jan: high summer) and April (Apr: after first winter rains). Table 8.1 displays the total rainfall and average temperature data obtained from the South African weather office (www.weathersa.co.za) for the area at the sample collection times. No rain was reported for both Jan 2006 and 2007 whereas 41 mm and 88 mm of rain were reported for Apr 2006 and 2007 respectively. The average maximum temperature was 32˚C and the average minimum temperature 18˚C for Jan, during both 2006 and 2007. The average maximum temperature was 26˚C and the average minimum temperature was 13˚C for Apr, during both 2006 and 2007.

Table. 8.1. Rainfall and temperature recorded. Total rainfall and temperature recorded for Boland area (Stellenbosch) during Jan and Apr for both 2006 and 2007

	2006		**2007**	
	Jan	**Apr**	**Jan**	**Apr**
Total Rainfall (mm)	0	41	0	88
Average Max temperature (˚C)	32.08	26.18	32.88	26.04
Average Min temperature (˚C)	18.09	13.25	18.22	12.9

8.4.1. Jonkershoek analysed for estrogenicity

Table 8.2 displays the results obtained for assessing water samples collected from Jonkershoek for estrogenicity using the battery of assays. The concentration of estrone in the control site was very low, near the detection limit of the assay and was statistically similar ($P > 0.05$) to the assay negative control. Similar to the results obtained for estrone, none of the samples collected at Jonkershoek were estrogenic when analysed with the MCF-7 total LDH assay or the MCF-7 ERα assay because test assay values obtained with the latter two assay were statistically similar ($P > 0.05$) to their respective assay negative controls. Exposure of juvenile tilapias to water extracts collected during Apr 2006 and Apr 2007 resulted in statistically similar ($P > 0.05$) VTG concentrations compared to the control fish. However, exposure of juveniles to water extracts collected during Jan 2006 and Jan 2007 showed significantly ($P < 0.001$) higher VTG levels in comparison with the assay negative control.

Table 8.2. Jonkershoek analysed for estrogenicity using the battery of assays. Water samples were collected from Jonkershoek in Jan (high summer) as well as Apr (after first rains) during 2006 and 2007. Water samples were analysed for estrone, estrogen dependent proliferation of MCF-7 cells using LDH as biomarker, estrogen dependent reduction of ERα expression by MCF-7 cells as well as estrogen dependent induction of VTG in Tilapia juveniles. Samples significantly higher ($P < 0.001$) than the –ve control are indicated with **.

Jonkershoek					
		2006		**2007**	
Assay	**-ve**	**Jan**	**Apr**	**Jan**	**Apr**
Estrone (n = 4) (ng/l)	1.823 ± 0.320	1.430. ± 0.492	2.234 ± 0.433	1.659 ± 0.398	1.809 ± 0.457
MCF-7 total LDH (n = 8) (% of –ve)	100.000 ± 2.879	101.021 ± 6.065	96.086 ± 5.892	106.109 ± 7.3123	106.049 ± 8.275
MCF-7 Erα (n = 8) (OD/mg protein, % of –ve)	100.000 ± 7.385	98.636 ± 14.265	97.39 ± 6.204	94.814 ± 12.604	91.727 ± 11.535
Tilapia VTG (n = 8) (µg/mg total protein)	6.786 ± 1.447	11.506 ± 1.649 **	9.811 ± 1.825	13.066 ± 1.195 **	9.114 ± 1.092

8.4.2. Stellenbosch STWs analysed for estrogenicity

Estrone concentrations were significantly higher ($P < 0.001$) compared to the assay negative control ranging between 14.756 ± 0.519 ng/l and 19.421 ± 0.066 ng/l for water samples collected during Jan and Apr of 2006 and 2007 at Stellenbosch STWs (Table 8.3). The estrone concentration was significantly higher ($P < 0.001$) in water samples collected during Jan 2006 compared to Apr 2006. Water samples collected in Jan 2007 contained significantly higher ($P < 0.001$) estrone concentrations compared to Apr 2007.

Stellenbosch STWs extract collected Apr 2006 was the only sample that tested positive with the MCF-7 proliferation assay. MCF-7 cells exposed to water extracts from this sampling date at Stellenbosch STWs resulted in a 1.3 fold increase ($P < 0.001$) in total LDH compared to the assay negative control. No significant differences ($P > 0.05$) in the total LDH levels could be detected comparing MCF-7 cells exposed to water extracts collected during Jan 2006 and Jan 2007. The total LDH levels of MCF-7 cells exposed to water extracts collected in Apr 2007 were significantly ($P < 0.001$) lower compared to that of the assay control. MCF-7 cells exposed to water extracts from Apr 2006 resulted in significantly higher ($P < 0.001$) total LDH levels compared to Jan 2006.

Stellenbosch STW water extract collected in Jan 2006 was the only sample that resulted in a statistically similar ($P > 0.05$) ERα expression value compared to the assay negative control. STW water extracts collected in Apr 2006, Jan 2007 and Apr 2007 all resulted in significantly lower ($P < 0.001$) ERα expression values compared to the assay negative control. ERα expression values were significantly lower ($P < 0.001$) for MCF-7 cells exposed to water extracts collected during Apr 2006 compared to Jan 2006, whereas no significant differences ($P > 0.05$) could be detected comparing ERα expression values of MCF-7 cells exposed to water extracts collected during Jan and Apr 2007.

Tilapia juveniles exposed to Stellenbosch STWs water extracts collected in Jan 2006 synthesized statistically similar ($P > 0.05$) VTG concentrations compared to control fish. Exposure of tilapias to Stellenbosch STW water extracts from all the other sampling dates resulted in significantly higher ($P < 0.001$) VTG concentrations. Juveniles exposed to water extracts collected during Apr 2007 resulted in significantly higher ($P < 0.001$) VTG concentrations compared to Jan 2006.

Table 8.3. Stellenbosch STWs analysed for estrogenicity using the battery of assays. Water samples were collected from Jonkershoek in Jan (high summer) as well as Apr (after first rains) during 2006 and 2007. Water samples were analysed for estrone, estrogen dependent proliferation of MCF-7 cells using LDH as biomarker, estrogen dependent reduction of ERα expression by MCF-7 cells as well as estrogen dependent induction of VTG in Tilapia juveniles. Samples significantly higher ($P < 0.001$) than the –ve control are indicated with **. Assay values printed in bold indicates significant higher values ($P < 0.001$) of a specific assay compared between the Jan and Apr samples analysed for a specific year.

Stellenbosch STW					
		2006		**2007**	
Assay	**-ve**	**Jan**	**Apr**	**Jan**	**Apr**
Estrone (ng/l)	1.823 ± 0.320	**17.814 ± 0.215** **	16.513 ± 0.431 **	**19.421 ± 0.066** **	14.756 ± 0.519 **
MCF-7 total LDH (n = 8) (% of –ve)	100.000 ± 2.879	108.794 ± 2.439	**130.163 ± 1.800** **	**103.016 ± 6.399**	69.875 ± 5.984 **
MCF-7 ERα (n = 8) (OD/mg protein, % of –ve)	100.000 ± 7.385	87.447 ± 6.630	**61.946 ± 3.756** **	33.300 ± 1.576 **	28.265 ± 1.283 **
Tilapia VTG (n = 8) (µg/mg total protein)	6.786 ± 1.447	7.752 ± 1.753	**12.894 ± 1.720 ****	**17.167 ± 2.108 ****	10.636 ± 1.299 **

8.4.3. Spier analysed for estrogenicity

Table 8.4 displays the results obtained for assessing water samples collected at Spier for estrogenicity using the battery of assays. The results obtained among the test assays were similar for all the assays when water samples collected at Spier was analysed. All the test assays showed significant different ($P < 0.001$) values for each of the sampling dates being analysed compared to their respective assay negative controls. Estrone concentrations ranged between 10.316 ± 0.570 ng/l and 22.989 ± 0.078 ng/l for water samples collected in Jan and April during both 2006 and 2007. Water samples collected in Jan 2006 contained significant higher ($P < 0.001$) estrone concentrations compared to April 2006, whereas water samples collected in April 2007 contained significant higher estrone concentrations compared to Jan 2007.

MCF-7 cells exposed to water extracts from Spier resulted in an significant increase in total LDH ranging between 15.196 % and 29.851 % compared to the assay negative control for water samples collected in both 2006 and 2007. No significant differences could be observed ($P > 0.05$) comparing total LDH levels of MCF-7 cells exposed to water extracts of Jan and Apr 2006. The latter is also true comparing total LDH levels of MCF-7 cells exposed to water extracts collected during Jan and Apr 2007.

Exposure of MCF-7 cells to water extracts from Spier also resulted in a significant ($P < 0.001$) decrease in ERα expression levels ranging between 51.504 ± 3.078 % and 11.564 ± 4.721 % compared to the assay negative control. MCF-7 cells exposed to water extracts from Spier, April 2006 resulted in significantly ($P < 0.001$) lower ERα expression levels compared to Jan 2006. No significant differences ($P > 0.05$) in ERα expression levels could be observed for the Jan 2007 and Apr 2007 water extracts.

Tilapia juveniles exposed to water extracts from Spier collected during Jan and Apr 2006 and 2007 synthesized significantly higher ($P < 0.001$) VTG compared to control fish. VTG concentrations ranged between 12.988 ± 2.014 and 13.958 ± 1.563 µg/mg. Fish exposed to water extracts collected during Apr 2006 induced significantly ($P < 0.001$) higher VTG concentrations compared to water collected during Jan 2006. No significant differences in VTG concentrations were observed for tilapia juveniles exposed to water extracts collected during Jan and Apr 2007.

Table 8.4. Spier analysed for estrogenicity using the battery of assays. Water samples were collected from Jonkershoek in Jan (high summer) as well as Apr (after first rains) during 2006 and 2007. Water samples were analysed for estrone, estrogen dependent proliferation of MCF-7 cells using LDH as biomarker, estrogen dependent reduction of ERα expression by MCF-7 cells as well as estrogen dependent induction of VTG in Tilapia juveniles. Samples significantly higher ($P < 0.001$) than the –ve control are indicated with **. Assay values printed in bold indicates significant higher values ($P < 0.001$) of a specific assay compared between the Jan and Apr samples analysed for a specific year.

Spier					
		2006		**2007**	
Assay	**-ve**	**Jan**	**Apr**	**Jan**	**Apr**
Estrone (ng/l)	1.823 ± 0.320	**16.050 ± 0.280** **	10.316 ± 0.570 **	12.077 ± 0.065 **	**22.989 ± 0.078** **
MCF-7 total LDH (% of –ve)	100.000 ± 2.879	115.196 ± 8.377 **	115.840 ± 3.214 **	122.970 ± 4.366 **	129.851 ± 5.418 **
MCF-7 Erα (OD/mg protein, % of –ve)	100.000 ± 7.385	51.504 ± 3.078 **	**11.564 ± 4.721** **	28.394 ± 3.217 **	35.701 ± 5.844 **
Tilapia VTG (µg/mg total protein)	6.786 ± 1.447	10.119 ± 2.014 **	**12.988 ± 1.894 ****	13.076 ± 1.884 **	13.958 ± 1.563 **

8.5. Discussion

Earlier studies have indicated that seasonal variation in river water quality and EDCs concentration do occur (Ouyang et al., 2006; Eun-Joung et al., 2007). Climatic data from the Stellenbosch area for 2006 and 2007 (Table 8.1) show that there are major difference in the total rainfall and average temperature between Jan and Apr. The run rates as well as quality of the river water differ between Jan and Apr (Ngwenya F., 2006). During Jan the river contains very little or no water and high concentrations of sewage and industrial effluents. During the winter the concentration of sewage and industrial effluents are highly diluted because of the high rainfall. Due to this, water was collected during high summer (Jan) and after the first winter rains (Apr) to analyse for estrogenicity using a battery of assays. The Eerste River was chosen to implement this battery of assays, because several previous studies have investigated contaminants in this river (Pool et al., 2000; Reinecke et al., 2003). The Eerste River also provides an ideal study area because of various degrees of domestic, agricultural and industrial activities along the river. Water samples were firstly analysed for the specific female hormone, estrone. Total estrogenicity of the water samples were determined using two more *in vitro screens* namely a modified MCF-7 proliferation assay (E-Screen) and a MCF-7 ERα protein determination ELISA. Vitellogenin induction in juvenile tilapias was used as a short term exposure *in vivo* assay.

Analysis of the water samples collected at Jonkershoek was negative for the estrone ELISA, MCF-7 LDH assay as well as the MCF-7 ERα assay. Each of the above mentioned assays produced assay results statistically similar ($P > 0.05$) compared to the respective assay negative control. The VTG ELISA suggests that samples collected from Jonkershoek Jan 2006 and Jan 2007 was estrogenic. However, none of the other test assay could confirm this result. Moreover, significant VTG induction was only observed for fish exposed to water samples collected during Jan which is high summer when water flow is restricted and fungal growth was observed in the water. No human, agricultural or industrial activity occurs at Jonkershoek. Therefore, a possible explanation for the induction of VTG in juvenile tilapias exposed to water samples collected during Jan 2006 and 2007 may be that the water was contaminated with estrogenic mycotoxins produced by fungi or even phytoestrogens. Estrogenic mycotoxins produced by *Fusarium* species have previously been detected in river water and sediment samples (Wu et al., 1990). It is also well known from previous studies that phytoestrogens do have the ability to disrupt endocrine disruption (Kuiper et al., 1998; Giesy et al., 2002). However, the sensitivity of juvenile tilapias towards phytoestrogens

exposure and estrogenic mycotoxins in comparison with other *in vitro* assays for estrogenicity for instance the E-Screen assay has not been investigated before.

Analysis of the sewage treatment works' effluent entering the Eerste River for estrogenicity resulted in very consistent data using the battery of assays. All the assays except for the estrone ELISA suggested that water collected during Jan 2006 are not estrogenic. In contrast, all the assays suggested that water samples collected from Stellenbosch STWs during April 2006 are estrogenic. Similar, all the assays except for the MCF-7 LDH assay also suggests that water samples collected during Jan 2007 and Apr 2007 are estrogenic. The estrone concentrations detected in water samples collected from Stellenbosch STWs are similar to the lower range of estrone concentrations detected in sewage effluent from Britain (Desbrow et al., 1998), Italy (Baronti et al., 2000), Germany (Ternes et al., 1999a), Canada and Netherlands (Belfroid et al., 1999). No apparent trend could be detected, comparing the estrogenicity of water samples collected during Jan and April for 2006 and 2007 respectively.

The Spier collection site is on the Eerste River, downstream from Stellenbosch and water at this point in the Eerste River contains pollutants from formal and informal housing development, agricultural and industrial activities (Fig 8.1.). All of the assays used to assess the estrogenicity of the water samples collected at all four sampling dates during 2006 and 2007 suggest that the water was estrogenic. Water samples analysed with the estrone ELISA, MCF-7 LDH assay, MCF-7 ERα assay and the juvenile tilapia VTG assay resulted in significantly different ($P < 0.001$) assay results compared to the respective assay negative control, suggesting that the samples are estrogenic. The estrone concentrations detected in water samples collected from Spier are similar to the estrone concentrations detected in river water samples from previous studies. A study conducted by Xiao et al., (2001) on the river Thames in England reported estrone levels ranging from 0.2 – 17 ng/l. A study on two lakes and three streams in Denmark that receive no or little sewage effluent reported estrone levels ranging from 0.2 – 3 ng/l (Christiansen and Plesner, 2001). Similar to the analysis of Stellenbosch STWs, no apparent trend could be noticed when comparing the estrogenicity of samples collected during Jan and April for 2006 and 2007 respectively.

It is evident from this study that a single test assay is insufficient to prove if a water sample is estrogenic or not. The use of a combination of assays dependent on different modes of action are required to confidently analyse water samples as a first tier screen for estrogenicity. It is

evident from this study that the different assays that were used can be successfully employed as a battery of assays to screen environmental water samples for estrogenicity. Results obtained from this battery of assays should be interpreted as a first tier screen for estrogenicity and not monitoring of the Eerste River for estrogenicity. Samples that tested positive should be further investigated using second and third tier screens with routine sampling in order to monitor the Eerste River for estrogenicity.

To our knowledge this is the first study that has evaluated the estrogenicity of a river in South Africa and more specifically Stellenbosch, using a battery of assays consisting of both *in vitro* and *in vivo* screens. Inefficient sewage treatment works, industrial as well as agricultural activities are polluting our natural environment. The development and implementation of both *in vitro* and *in vivo* screens for estrogenicity, which are applicable to the Southern African context, i.e. rapid assays that are affordable and uses indigenous species is of cardinal importance to monitor our environment.

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Chapter 9

9.1. General Conclusion

The hypothesis regarding the concerns that chemicals found in the environment may display estrogenic effects as well as the ability to disrupt normal endocrine function, is not new at all (Allen and Doisy, 1923. Burlington and Lindeman, 1950). Various reports have linked adverse health effects in humans and wildlife to estrogenic endocrine disrupting chemicals (e-EDCs) (Kavlock et al., 1996). One of the major concerns is that these e-EDCs have been found in wastewater, surface waters, sediments, groundwater and even drinking water (Benfenati et al., 2003; Petrović et al., 2003; Snyder et al., 2003; Peterović et al., 2004). Consequently, organizations such as the US Environmental Protection Agency (EPA) and Organization for Economic Cooperation and Development (OECD) have taken great interest in the development of test assays as well as guidelines for monitoring EDCs. As a result, a large number of *in vitro* and *in vivo* tests have been developed for the screening of environmental EDCs (ECETOC, 1996; Holmes et al., 1998; Kavock et al., 1996). The continued development and inter laboratory evaluation of assays to detect and routinely monitor e-EDC are some of the major goals of the EPA and OECD.

For this reason, the aim of our study was the development and validation of a battery of screens encompassing both *in vitro* and *in vivo* assays to monitor e-EDCs. The secondary aim of this study was to implement this battery of assays to evaluate the Eerste River, South Africa for environmental e-EDCs. In order to achieve the aims of this study, several objectives had to be reached. The first objective of this study was the validation of commercially available ELISA kits to quantify natural estrogenic hormones in complex environmental mixtures. These ELISA kits were originally designed by the manufacturer to quantify natural hormones in human plasma. The ELISA kits were validated to quantify estrone, estriol and 17β-estradiol in hydrophobic extracts of water. These ELISA assays proved to be highly repeatable with inter- and intra assay variation of less than 5.6 %, 8.2 % and 4.5 % for the quantification of estriol, estrone and 17β-estradiol respectively. The applicability of these ELISA kits to determine steroid hormone levels in sewage effluent were further supported by good parallelism between dilution curves of the kit standards and sewage effluent samples. Analysis of sewage effluent entering the Kuils River water catchment area revealed similar estriol, estrone and 17β-estradiol concentrations to those obtained for sewage effluents from Britain (Desbrow et al., 1998), Italy (Baronti et al., 2000), Germany (Ternes et al., 1999a), Canada and Netherlands (Belfroid et al., 1999).

The second objective of this study was the development of a multi biomarker estrogen receptor (ER) dependent whole cell bio-assay to assess total environmental estrogenicity. The human breast cancer cell line, MCF-7 was used to introduce some modification to the well known E-screen assay described by Soto et al., (1992). The culture of MCF-7 cells in a 96-well format using chemically defined serum replacement factors instead of FBS was validated. Several endpoints of the E-Screen assay as biomarkers for estrogenicity and cytotoxicity were also evaluated. Total LDH activity, total protein yield as well as XTT activity were evaluated as proliferation endpoints and biomarkers for estrogenicity. Total LDH activity of MCF-7 cells was the most sensitive biomarker of these three endpoints and had a detection range for E2 between 100 pM E2 and 1000 nM E2. The third objective of this study involved the setting up and validation of an ELISA to quantify ERα levels as a biomarker for estrogenicity. This is a novel amendment to the E-Screen assay for this ELISA is performed on cultured MCF-7 cells (exposed to estrogen standards or environmental water extracts) that have been fixed to the 96-well culture plates. The sensitivity of this screen for environmental estrogens was assessed by determining ERα levels of MCF-7 cells that were cultured at different E2 concentrations. The linear range for the ERα direct ELISA was between 1 – 1000 nM E2. This bio-assay was successfully implemented to determine the total estrogenicity of a pristine site as well as sewage effluent from Stellenbosch sewage treatment works.

The fourth objective of this study was to setup and validate a competition ELISA to quantify vitellogenin (VTG) from *Oreochromis mossambicus* (tilapia). VTG, which is the precursor to several egg yolk proteins in oviparous vertebrates, has been identified as a valuable biomarker to detect e-EDCs in several fish species (Purdom et al. 1994; Jobling et al. 1995; Sumpter, 1995). However, few quantitative VTG ELISAs for tilapia have been validated using commercially available anti-VTG that shows cross reactivity with tilapia VTG. The newly developed ELISA uses commercially available seabream anti-VTG that shows cross reactivity with several fish species. A competition ELISA was developed to quantitatively assess tilapia serum and liver culture supernatants for VTG. This tilapia VTG competition ELISA has a broad detection range between 80 ng/ml – 5.4 µg/ml VTG. The ELISA is highly repeatable with intra- and interassay variations less than 3.4 %. We also showed that this ELISA can be used to quantify VTG that has been produced *in vitro* (sliced liver cultures) and *in vivo* (juvenile whole body homogenates).

The fifth objective of the study entailed using this quantitative VTG ELISA to validate the use of tilapia juveniles (less than 70 dpf) as a short term *in vivo* screen for estrogenicity. It is evident from this study that juvenile tilapias have the ability to produce VTG upon estrogen exposure from a very early stage of development (25 dpf). This observation is in agreement with studies conducted in zebrafish (Legler et al., 2000) and fathead minnow (Tyler et al., 1999). Our study shows that juvenile tilapias aged between 35 – 45 dpf synthesized the highest concentration of VTG upon estrogen exposure. At this age 200 ng/l of DES was the lowest concentration of estrogen that could induce significant VTG levels after 10 days of exposure. The detection limit of this short term *in vivo* assay for estrogenicity is in the same range that was found for other cyprinodont fish species (Folmar et al., 2000). Analysis of the natural VTG levels of a mixed sex population of tilapia juveniles from 15 to 70 days post fertilization (dpf) juveniles revealed interesting results. A sudden significant 5 fold increase in natural VTG levels was observed in fish that are 50 dpf of age. This sudden increase in VTG protein levels coincided with a significant 2 fold increase in natural 17β-estradiol levels. This observation may possibly mark the onset of sexual differentiation of hermaphroditic juvenile tilapias.

Our study was concluded by implementing this battery of assays to screen the Eerste River, South Africa for e-EDCs. In this study, three sites were used for analysis namely Jonkershoek (control site situated in the mountains), sewage effluent from Stellenbosch sewage treatment works and Spier (sampling site on the Eerste River downstream from Stellenbosch). These three sites were analysed for the specific steroid hormone estrone. The MCF-7 breast cancer cell line was used to assess the ability of water extracts from these tree sites to induce cellular proliferation as well as reduce ERα protein levels, which are both biomarkers for estrogenicity. Juvenile tilapias were also exposed to water extracts from these sites and were analysed for VTG protein levels (short term *in vivo* assay). Estrone in the control site ranged between 0.7 and 1.8 ng/l whereas Stellenbosch STWs contained estrone levels ranging between 14.76 and 19.42 ng/l. Water samples analysed from Spier contained estrone levels ranging between 12.08 and 16.05 ng/l. Results obtained from the other three assays i.e., the quantification of ERα protein levels expressed by MCF-7 cells, the MCF-7 proliferation assay as well as the tilapia juvenile short term *in vivo assay* gave similar results. It was concluded that water samples collected from Stellenbosch STWs and Spier were estrogenic based on the analyses using this battery of assays.

Endocrine disrupting chemicals can act via a wide range of mechanisms, which includes receptor dependent and independent process. EDCs may display species, tissue- and cell-specific effects, as well as being influenced by metabolism. EDCs may also display synergistic or antagonistic effects when present in complex environmental mixtures. The use of a battery of assays that encompasses both *in vitro* and *in vivo* tests is therefore essential to comprehensively assess the estrogenic potential of complex environmental mixtures. In this study we developed, validated and also implemented a battery of assays encompassing both *in vitro* and *in vivo* assays, based on different biological mechanisms, to detect estrogenic EDCs. To our knowledge, this is the first study that has used a battery of bio-assays to specifically assess the estrogenicity of South Africa rivers. Studies like these are necessary to develop and evaluate test assays using species which are applicable to the country of origin. In order for us to preserve our natural environment and also ourselves, it is necessary for the continued development of test assays to detect EDCs.

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