Assessment of raw and treated sewage using *in vitro* assays

by

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Sewage treatment

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Total coliforms

Escherichia coli

Steroids

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Progestins

Humoral immunity

ABSTRACT

Water scarcity is becoming an increasingly relevant problem for urban centres, especially in Southern Africa. However, water availability is not the only concern for consumers, because water quality is just as relevant. Many studies have revealed adverse health effects in organisms exposed to polluted waters, and the main source of that water pollution was traced back to sewage treatment works (STWs). Physiological systems that are affected include the endocrine system (as well as the reproductive system) and the immune system.

Recently, the Stellenbosch STW started upgrading its facility, but this procedure would also affect the STW's operations.

Stellenbosch STW uses an activated sludge treatment, but also employs trickling filters (biofilters). After screening and grit removal, wastewater enters trickling filters, and then undergoes activated sludge treatment (aerobic basin). After activated sludge treatment (and settling) some water is chlorinated before entering a maturation pond. The other water goes directly to a larger maturation pond (for a longer period), instead. The final effluent then gets discharged into the Veldwagters River.

Since STW operations is an important factor in STW effluent quality, this study aimed to investigate the water quality (at Stellenbosch STW) during the upgrade. Specifically, the bacterial quality, the steroidal quality (testosterone, progesterone, estrone: E1, 17 β-estradiol: E2 and 17 α-ethinyl estradiol: EE2) and the potential immunotoxic quality of waters were assessed. Water samples were collected after the grit removal (influent),

after the trickling filters (biofilter effluent), while it was leaving the aerobic basin (activated sludge effluent) and as it was leaving the maturation ponds (final effluent).

To determine bacterial quality a semi-quantitative ReadyCult® assay was performed on raw water samples (detects total coliforms and *Escherichia coli*). Bacterial levels were high for all influent samples, water from the biofilter, water from the aerobic digester (activated sludge) and the final effluent (most days). The first collection date, however, showed less than 1cfu/mL of both *E. coli* and total coliforms for the final effluent.

Raw water also underwent solid phase extraction, before the steroid concentrations were determined by enzyme-linked immunosorbent assays (ELISAs). Steroid levels were very high in the influent. Each treatment progressively reduced the steroid concentration. However, progesterone concentration increased during the biofilter treatment. The increase in progesterone was probably due to bacterial de-conjugation of hydrophilic-progesterone-conjugates. Nonetheless effluent steroid levels were significantly lower than the influent. Steroid reduction through the Stellenbosch STW was 96%, 95%, 55%, 78% and 87% for testosterone, progesterone, estrone, estradiol and ethinyl estradiol respectively. Much variability in steroid concentrations was noted between sampling dates. The activated sludge treatment was the best at reducing steroid concentration. Nonetheless, the STW still discharged steroids into the environment.

Finally, the humoral immune effects of Stellenbosch STW influent and effluent was determined by using hybridoma cells and assessing affects on antibody production. Antibody levels were then detected by ELISA. No adverse effects to antibody synthesis/secretion were noted as a result of exposure to either influent or effluent. This

implies that the waters tested were not immunotoxic. However, the hybridoma cells could have been less sensitive to toxicants than other assays. Furthermore, more tests (*in vitro* and *in vivo*) are necessary to confirm the apparent non-immunotoxic effects of the Stellenbosch STW samples.

In conclusion, during the initial phases of the Stellenbosch STW upgrade, the influent showed high levels of faecal bacteria and steroids. Therefore, the influent quality was poor. The effluent also showed high bacterial levels and still contained steroids. Furthermore, no immunotoxic effects of either influent or effluent was noted.

Declaration

I, **Robin Alvin Booysen**, declare that ***Assessment of raw and treated sewage using in vitro assays*** is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Signed November 2014

Robin Alvin Booysen

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"...Blessed be the name of God forever and ever, for wisdom and might are His.
And He changes the times and the seasons; He removes kings and raises up kings; He gives wisdom to the wise and knowledge to those who have understanding.
He reveals deep and secret things; He knows what *is* in the darkness, and light dwells with Him.

"I thank You and praise You, O God of my fathers; You have given me wisdom and might..""
Daniel 2: 20-23 (The Holy Bible: New King James Version)

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17 α-ethinyl estradiol (also known as ethinyl estradiol)	EE2
17 β-estradiol (also known as estradiol)	E2
Activator protein-1	AP-1
Alkylphenol ethoxylate metabolite	APEM
Anaerobic sludge blanket	UASB
Androgen receptor	AR
Antibody-dependent cell-mediated cytotoxicity	ADCC
Antigen presenting cell	APC
Antigen-binding fragment	Fab
B cell receptor	BCR
Bisphenol A	BPA
Cluster of differentiation	CD
Colony forming unit	CFU
Cytochrome P450 cholesterol side-chain cleavage enzyme	P450scc/ CYP11A1
Cytolytic/cytotoxic T cell	T_C cell
Dehydroepiandrosterone	DHEA
Deoxyribonucleic acid	DNA
Dichlorodiphenyltrichloroethane	DDT
Dimethyl sulfoxide	DMSO
Emerging organic contaminant	EOC
Endocrine disrupting compound/chemical	EDC
Endoplasmic reticulum	ER
Enzyme-linked immunosorbent assay	ELISA

Escherichia coli	*E. coli*
Estriol	E3
Estrogen receptor	ER
Estrogenic EDC	e-EDC
Estrone	E1
Follicle stimulating hormone	FSH
Fragment crystallizable	Fc
Germinal centre	GC
Gonadotropin releasing hormone	GnRH
Heavy chain	HC
Helper T cells	T_H
Hypothalamic-pituitary-gonad axis	HPG axis
Immunoglobulin (also known as antibody)	Ig
Immunoreceptor tyrosine-based activation motif	ITAM
Interleukin	IL
Light chain	LC
Lipopolysaccharides	LPS
Lipoprotein lipase	LPL
Litre	L
Luteinizing hormone	LH
Major histocompatibility complex	MHC
Mega (million) litre	ML
Membrane attack complex	MAC
Microgram	µg
Microlitre	µL

Micrometer	μm
Millilitre	mL
Molar	M
Multixenobiotic resistance	MXR
Municipal solid waste	MSW
Nanogram	ng
Nanometer	nm
Natural killer cell	NK cell
Nonylphenol	NP
Nuclear factor-κB	NF-κB
Octylphenol	OP
Olive mill wastewaters	OMW
Organochlorine pesticide	OCP
Pathogen associated molecular pattern	PAMP
Persistent organic pollutant	POP
Pharmaceuticals and personal care products	PPCPs
Phosphate buffered saline	PBS
Phosphoinositide 3-kinase	PI3K
Phospholipase C-γ2	PLCγ2
Picogram	pg
Plasma cell	PC
Polychlorinated biphenyl	PCB
Polycyclic aromatic hydrocarbon	PAH
Progesterone receptor	PR
Progesterone	P4

Reverse-osmosis	RO
Revolutions per minute	rpm
Sewage treatment works	STW
T cell receptor	TCR
Total coliforms	TC
Ultraviolet	UV
Wastewater treatment works	WWTW
Whole blood culture	WBC

CHAPTER 1: SCOPE OF PROJECT AND SITE DESCRIPTION

1.1 Water Scarcity (and importance)

Water is one of the world's most important resources. All living organisms on Earth need water, since water is the medium in which biological reactions (metabolism) occur and is consequently known as the medium of life (Stikker, 1998; Pandey, 2006). In terrestrial environments, the main sources of freshwater are rivers and dams/lakes, but humans also use groundwater. Environmental waters are used for many functions. Environmental water: serves as a drinking water supply (not only for humans, but for other animals and as water for plants as well), is used for aesthetic purposes, is used in industry (including generation of electricity), is used for recreational purposes, is used for transportation and is even used for wastewater disposal (Barber *et al*, 2011; Shinn *et al*, 2009; UNGEMS/Water Programme, 2008). In addition, environmental waters also serve as the habitat for many freshwater aquatic fauna and flora (Stikker, 1998). The additional uses of environmental water simply emphasize the great reliance the biosphere has on water. The functions of water also reveal how essential it is for us to protect our environmental waters.

The Earth has a finite volume of freshwater that is continually reused (hydrological cycle), so the quantity of freshwater is relatively unchanged (Stikker, 1998). However, the number of water users (specifically people) is increasing, thus making water scarcer (less water available for each individual). UN (United Nations) predictions suggest that the global population will be 7.4-10.6 billion by the year 2050 (UN, 2004). The UN's extrapolations also show that

by the year 2300, the world population could reach as high as 36.4 billion individuals, or could drop to as low as 2.3 billion individuals. The African population was predicted to grow from 795.7 million individuals in 2000, to 1,803.3 million individuals in the year 2050 (UN, 2004). However, Southern Africa's population is not expected to change much between the years 2000 and 2300. Nonetheless, most people live in urban areas with high population densities making cities require larger water supplies.

According to Showers (2002), the proportion of Africans living in urban areas, as compared to rural areas, increased from the 1970s to the 1990s. Urban growth has created a greater demand for water in urban areas. Consequently, many African cities started using more water bodies, from more distant locations to meet their water needs. For example in the 1970s, the city Cape Town used the Eerste River, Berg River and Steenbras River to supply water demands, and in the 1990s Cape Town's water sources increased to include the Riviersonderend River and Palmiet River (Showers, 2002). Furthermore, based on population growth predictions, annual rainfall and climate change; le Blanc and Perez (2008) showed that by 2050, more than 80% of the South African population will live in areas classed as water tense despite the fact that they occupy a small portion of the South African land area (urban centres). These two studies show how water demand of cities have increased, and likely will continue to increase over time, not even addressing agricultural water use.

Currently agriculture is responsible for 85% of global water consumption (Shiklomanov and Rodda, 2003). Therefore, Pfister *et al* (2011) endeavoured to determine how water demands will change, regionally, in order to meet the food

needs of the predicted global population of the year 2050. Pfister *et al* (2011) assumed that the world could adopt one of four strategies to meet global food demands: increasing agricultural intensity, agricultural expansion to pastures only, agricultural expansion to pastures and natural ecosystems, or both increased agricultural intensity and expansion to only pastures. A water stress index was used to evaluate different global regions. Based on the used options, it appears that South Africa will remain a water stressed country in 2050, with a larger portion of her land area being classified as such than is currently the case (Pfister *et al*, 2011). All sources, thus far point to the same conclusion: unless something changes for the better, South Africa is likely to face a water crisis in the near future.

Furthermore, water scarcity is not as simple as comparing water demand to water supply, as one should also consider water quality. Water quality is often ignored in water scarcity studies (Rijsberman, 2006), giving misleading information on the actual water availability status of a region. Individuals who effectively do not have access to sufficient clean water to meet their needs are called water insecure (Rijsberman, 2006). A region is classified as water scarce when a community living there is water insecure (Rijsberman, 2006). According to Rijsberman's (2006) definition, polluted water should not be considered as part of the available water in a region. Instead, according to Rijsberman (2006), water scarcity assessments should use a specific water quality and consider how much water possessing that quality level is accessible, where it is or when it can be obtainable.

1.2 Environmental water quality

1.2.1 Occurrence of water pollution

It is unfortunate that water (and sediment) pollution appears to be a common occurrence around the world, especially where there are large human settlements (Sakan *et al*, 2011; Hoai *et al*, 2011; Kosjek *et al*, 2012; Xu *et al*, 2011; Gómez *et al*, 2011; Chase *et al*, 2012). Pollution places extra stress on water users as many of the pollutants are present at biologically relevant concentrations (Calisto and Esteves, 2009). Environmental water bodies tend to be contaminated with complex mixtures of pollutants that may produce unknown adverse health effects to organisms exposed to them (Hoai *et al*, 2011; Kosjek *et al*, 2012; Eggen *et al*, 2010; Xu *et al*, 2011; Musolff *et al*, 2009). Some common contaminants include endocrine disrupting compounds/chemicals (EDCs), heavy metals and pathogens.

The prevalence of water pollution can be clearly illustrated by studies such as the one conducted by Sakan *et al* (2011), which presented the presence of metals in the sediments of the major Serbian rivers and their tributaries (15 rivers in total). These rivers were shown to contain cadmium, zinc, nickel, copper, lead, chromium, cobalt, iron, manganese, beryllium and/or vanadium. Each metal ranged from not being detected in some rivers, to as high as 476mg/L (mg: milligrams; L: litre) (Sakan *et al*, 2011). Although Sakan *et al* (2011) revealed pollution in Serbian rivers, the researchers have limited their investigation to metals only. There may have been many more pollutants in those waters, including organic contaminants. Nonetheless, Sakan *et al* (2011) still gives an indication of how common environmental water pollution is.

A study, by Xu *et al* (2011), found that surface waters in Singapore were polluted with an array of chemicals including pharmaceuticals, EDCs and alkylphenol ethoxylates. The most abundant pollutants were the alkylphenol ethoxylate metabolites (APEMs), ranging from 980–7720ng/L (ng: nanograms) (Xu *et al*, 2011). The steroids estrone (E1) and estriol (E3) were also detected at <1–304ng/L and <3–451ng/L respectively (Xu *et al*, 2011). Xu *et al* (2011) was more focused on searching for emerging organic contaminants (EOCs) (13 EOCs were found); however, the researchers found many more other organic pollutants as well. The combination of pollutants discovered, at the concentrations present, could have many unknown detrimental effects on organisms exposed to it.

Other investigations reflect the Xu *et al* (2011) study, revealing the ubiquity of organic contaminants in environmental water. Reported organic contaminants include polychlorinated biphenyls (PCBs) (Ho *et al*, 2003), polycyclic aromatic hydrocarbons (PAHs) (Ho *et al*, 2003), bisphenol A (BPA) (Quednow and Püttmann, 2008; Musolff *et al*, 2009), nonylphenol (NP) (Choi *et al*, 2011; Quednow and Püttmann, 2008; Musolff *et al*, 2009), 4-*tert*-OP (4-*tert*-Octylphenol) (Quednow and Püttmann, 2008), caffeine (Musolff *et al*, 2009), pharmaceuticals (Kosjek *et al*, 2012; Camacho-Muñoz *et al*, 2010; Calisto and Esteves, 2009; Musolff *et al*, 2009), synthetic musks (Chase *et al*, 2012; Musolff *et al*, 2009), perfluorinated compounds (Bossi *et al*, 2008; Gómez *et al*, 2011), pesticides (Hoai *et al*, 2011), steroids (Barel-Cohen *et al*, 2006; Camacho-Muñoz *et al*, 2010), pathogens (Ho *et al*, 2003), and even antibiotic resistance genes (Thevenon *et al*, 2012). Organic contaminants are commonly found at concentrations of ng/L, and are consequently also known as micropollutants.

1.3 Sewage treatment works and water cleansing

The purpose of sewage treatment works (STWs) is to convert wastewater into clean water that is safe to discharge into the environment (Drinan and Whiting, 2001). This poses a great challenge to municipal STWs, because they often deal with combined wastewaters from various industries as well as domestic sources.

Studies have shown that conventional STWs typically reduce pollutants in wastewater, but do not remove all pollutants (Yu *et al*, 2006). Studies have also shown that different STWs can produce effluents of vastly different quality, despite using similar (or even the same) treatment processes (Samie et al, 2009). Various factors contribute to the effluent quality of a STW such as the influent quality, weather conditions at the time of sampling and operational differences (e.g. different hydraulic retention times) between STWs. Thus, maintenance work on STWs that causes specific reactors to be unused for a period could reduce the quality of that STWs effluent for that period.

Wastewater treatment involves a series of treatment steps that each contributes to improving the quality of the water being treated. The primary treatments employ physical processes (Drinan and Whiting, 2001). Secondary treatment uses physical and biological processes and different tertiary treatments use various more advanced (non-conventional) options (Drinan and Whiting, 2001). The Stellenbosch STW employed conventional methods of sewage treatment during the sampling period of this study. In 2012, The Stellenbosch STW initiated and upgrade procedure that may also have temporarily reduced its treatment capacity from 20.4ML/day (M: million) to 12ML/day. Therefore, water samples were collected on different dates during the initial phase of the upgrade to assess its

quality. The following section will give an overview of the Stellenbosch STW layout before the upgrade and the proposed changes that the upgrade would bring.

1.4 Stellenbosch sewage treatment works description

Prior to the upgrade process, the Stellenbosch STW's maximum dry weather flow capacity was approximately 20.4ML/day. Stellenbosch STW employed two biological treatments, namely tricking filters (bio-filters) and activated sludge (refer to **Figure 1.1**).

1.4.1 Pre-treatment and Primary Treatment

Raw sewage passes through bar screens before entering a vortex de-gritter. Thereafter, the wastewater travelled through one of four primary settling tanks. Some sludge is removed at the primary settling tank and travels to a pumping station.

1.4.2 Secondary Treatment

After the primary treatment, wastewater enters one of three operational trickling filters, after which it enters one of two aerobic basins for the activated sludge treatment. Effluent from the first aerobic basin is split and sent to two secondary settling tanks before being chlorinated and then entering a maturation pond. Effluent from the second aerobic basin is split and sent to four secondary settling tanks before travelling directly to the second (larger) maturation pond. Sludge is collected from the secondary settling tanks and sent to pump stations. The final effluent from Stellenbosch STW would be discharged into the Veldwagters River.

1.4.3 Sludge Treatment

From the sludge pump stations, most sludge enters one of four up-flow anaerobic sludge blanket digesters (UASB); the remainder is sent directly for dewatering. After anaerobic digestion, sludge is thickened before dewatering. Some thickened sludge is returned to the UASB. After dewatering, the sludge is composted together with wood chips before being sold to the public.

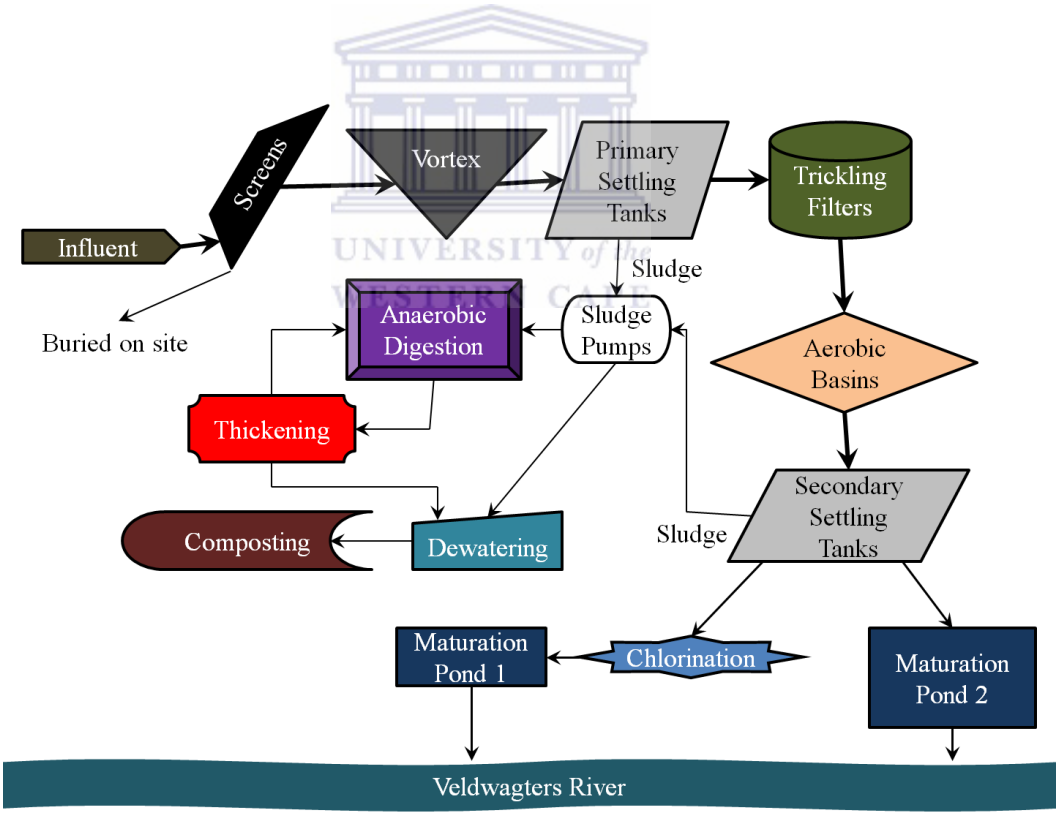

Figure 1.1. Layout of the Stellenbosch STW before the upgrade.

1.4.4 Upgrade

The proposed upgrade of Stellenbosch STW involves the conversion of one of the two aerobic basins into an anaerobic basin. A new aerobic basin will also be constructed together with secondary settling tanks. The aim of the upgrade procedure is to improve effluent quality and to increase the STW's capacity. Therefore, additional upgrades are also proposed to permit the STW to handle larger water volumes during the other treatments as well.

1.5 Aims of study

The broad purpose of this investigation is to assess the effectiveness of the Stellenbosch sewage treatment works at removing physiologically relevant pollutants during a STW upgrading procedure. Portions of the STW were temporarily shut down to allow for that facility's upgrading, but the quality of the final effluent after a sub-optimal treatment needed evaluation. Wastewater samples were thus collected at different points throughout the sewage treatment process on different days, and were used in number of bioassays.

The first objective was to assess the bacterial quality of the waters from Stellenbosch STW. The determination of bacterial loads of water is a standard used around the world. For an easily interpreted result, scientists search for specific indicator organisms, such as the coliforms, which are considered as indicators of faecal contamination. A chromogenic medium was used to provide a semi-quantitative assay for determining *Escherichia coli* and total coliforms in the water.

Since steroids form an important class of endocrine disrupting compound, their levels were also assessed in this study. The second objective of the study was to determine the steroid levels in the water using commercially available enzyme-linked immunosorbent assays. Five steroid hormones (estrone, 17 β-estradiol, 17 α-ethinyl estradiol, progesterone and testosterone) were quantified in the water samples.

The third objective of this study was to determine whether the wastewaters from the Stellenbosch STW showed any immunotoxic activity. For the immunotoxicity screen, a hybridoma cell line was employed. The hybridoma cells produce monoclonal antibodies against lipoprotein lipase. Wastewater samples were tested for effects on antibody production by the hybridoma cells. Antibody synthesis was quantitated by an enzyme-linked immunosorbent assay (ELISA).

1.6 References

Barber LB, Brown GK, Nettesheim TG, Murphy EW, Bartell SE, Schoenfuss HL. 2011. Effects of biologically-active chemical mixtures on fish in a wastewater-impacted urban stream. Science of the Total Environment. 409, 4720–4728.

Barel-Cohen K, Shore LS, Shemesh M, Wenzel A, Mueller J, Kronfeld-Schor N. 2006. Monitoring of natural and synthetic hormones in a polluted river. Journal of Environmental Management. 78, 16–23.

Bossi R, Strand J, Sortkjær O, Larsen MM. 2008. Perfluoroalkyl compounds in Danish wastewater treatment plants and aquatic environments. Environment International. 34, 443–450.

Calisto V, Esteves VI. 2009. Psychiatric pharmaceuticals in the environment. Chemosphere. 77, 1257–1274.

Camacho-Muñoz MD, Santos JL, Aparicio I, Alonso E. 2010. Presence of pharmaceutically active compounds in Doñana Park (Spain) main watersheds. Journal of Hazardous Materials. 177, 1159–1162.

Chase DA, Karnjanapiboonwong A, Fang Y, Cobb GP, Morse AN, Anderson TA. 2012. Occurrence of synthetic musk fragrances in effluent and non-effluent impacted environments. Science of the Total Environment. 416, 253–260.

Choi M, Furlong ET, Moon H, Yu J, Choi H. 2011. Contamination of nonylphenolic compounds in creek water, wastewater treatment plant effluents, and sediments from Lake Shihwa and vicinity, Korea: comparison with fecal pollution. Chemosphere. 85, 1406–1413.

Drinan JE, Whiting NE. 2001. Water & wastewater treatment, a guide for the nonengineering professional. CRC Press. Boca Raton. 316pp.

Eggen T, Moeder M, Arukwe A. 2010. Municipal landfill leachates: a significant source for new and emerging pollutants. Science of the Total Environment. 408, 5147–5157.

Gómez C, Vicente J, Echavarri-Erasun B, Porte C, Lacorte S. 2011. Occurrence of perfluorinated compounds in water, sediment and mussels from the Cantabrian Sea (North Spain). Marine Pollution Bulletin. 62, 948–955.

Ho KC, Chow YL, Yau JTS. 2003. Chemical and microbiological qualities of the East River (Dongjiang) water, with particular reference to drinking water supply in Hong Kong. Chemosphere. 52, 1441–1450.

Hoai PM, Sebesvari Z, Minh TB, Viet PH, Renaud FG. 2011, Pesticide pollution in agricultural areas of Northern Vietnam: case study in Hoang Liet and Minh Dai communes. Environmental Pollution. 159, 3344-3350.

Kosjek T, Perko S, Zupanc M, Zanoški Hren M, Landeka Dragičević T, Žigon D, Kompare B, Heath E. 2012. Environmental occurrence, fate and transformation of benzodiazepines in water treatment. Water Research. 46, 355-368.

le Blanc D, Perez R. 2008. The relationship between rainfall and human density and its implications for future water stress in Sub-Saharan Africa. Ecological Economics. 66, 319–336.

Musolff A, Leschik S, Möder M, Strauch G, Reinstorf F, Schirmer M. 2009. Temporal and spatial patterns of micropollutants in urban receiving waters. Environmental Pollution. 157, 3069–3077.

Pandey S. 2006. Water pollution and health. Kathmandu University Medical Journal. 4, 128-134.

Pfister S, Bayer P, Koehler A, Hellweg S. 2011. Projected water consumption in future global agriculture: scenarios and related impacts. Science of the Total Environment. 409, 4206–4216.

Quednow K, Püttmann W. 2008. Endocrine disruptors in freshwater streams of Hesse, Germany: changes in concentration levels in the time span from 2003 to 2005. Environmental Pollution. 152, 476-483.

Rijsberman FR. 2006. Water scarcity: fact or fiction? Agricultural Water Management. 80, 5–22.

Sakan S, Đorđević D, Dević G, Relić D, Anđelković I, Đuričić J. 2011. A study of trace element contamination in river sediments in Serbia using microwave-assisted *aqua regia* digestion and multivariate statistical analysis. Microchemical Journal. 99, 492–502.

Samie A, Obi CL, Igumbor JO, Momba MNB. 2009. Focus on 14 sewage treatment plants in the Mpumalanga Province, South Africa in order to gauge the efficiency of wastewater treatment. African Journal of Biotechnology. 8, 3276-3285.

Shiklomanov IA, Rodda JC. 2003. World water resources at the beginning of the 21st century. Cambridge: Cambridge University Press. 435pp.

Shinn C, Dauba F, Grenouillet G, Guenard G, Lek S. 2009. Temporal variation of heavy metal contamination in fish of the River Lot in Southern France. Ecotoxicology and Environmental Safety. 72, 1957–1965.

Showers KB. 2002. Water scarcity and urban Africa: an overview of urban–rural water linkages. World Development. 30, 621–648.

Stikker A. 1998. Water today and tomorrow. Futures. 30, 43–62.

Thevenon F, Adatte T, Wildi W, Poté J. 2012. Antibiotic resistant bacteria/genes dissemination in lacustrine sediments highly increased following cultural eutrophication of Lake Geneva (Switzerland). Chemosphere. 86, 468–476.

United Nations Environment Programme Global Environment Monitoring System (GEMS)/Water Programme. 2008. Water quality for ecosystem and human health, 2nd edition. Ontario: UNEP GEMS/Water Programme. 120pp.

United Nations. 2004. World Population to 2300. New York: UN. 240pp.

Xu Y, Luo F, Pal A, Yew-Hoong Gin K, Reinhard M. 2011. Occurrence of emerging organic contaminants in a tropical urban catchment in Singapore. Chemosphere. 83, 963–969.

Yu JT, Bouwer EJ, Coelhan M. 2006. Occurrence and biodegradability studies of selected pharmaceuticals and personal care products in sewage effluent. Agricultural Water Management. 86, 72–80.

CHAPTER 2: LITERATURE REVIEW

Due to the complex chemical makeup of water bodies, this review will focus on a number of general traits noted in these waters around the world. It will mention some sources of pollution, a number of commonly found pollutants, and studies on the biological activity of individual pollutants vs. mixtures of pollutants. Lastly, this review will specifically look at steroidogenesis and the actions of a few steroids (as well as some endocrine disrupting chemicals/compounds: EDCs).

2.1 Pollutant classification

The classification of environmental pollutants is quite complicated, as different researchers classify the same pollutants in different ways. Some researchers classify pollutants based on chemical properties (inorganic and organic pollutants), while other researchers do so based on environmental abundance (macropollutants and micropollutants). There are also researchers who classify pollutants based on the chemicals' origins (industrial pollutants, agricultural pollutants, municipal wastes, etc) or the chemicals' commercial uses (pesticides, pharmaceuticals, flame retardants, etc).

When researchers consider the organic pollutants, there are even more categories that are used. There are harmful (organic) pollutants which are fairly common in the environment, due to them having long half-lives. These contaminants are classified as persistent organic pollutants (POPs). Other pollutants were discovered relatively recently to be in the environment and are termed emerging organic contaminants (EOCs). Furthermore many of the organic pollutants were

shown to modulate the endocrine systems of vertebrates, and were consequently named endocrine disrupting chemicals/compounds (EDCs).

When examining the sources of pollutants, we find that the sources of water pollution are categorized as either point-sources or nonpoint-sources. Nonpoint sources of pollution are also known as diffuse sources. This is because nonpoint-sources are not well defined and the precise origin of pollutants is not easy to trace. Nonpoint sources of water pollution tend to come from large geographic regions, and include runoff from storms, geological weathering and atmospheric deposition. Nonpoint sources of pollution usually contribute minimally to the levels of pollution found in our environmental waters (Lapworth *et al*, 2012).

Point-sources of pollution are distinct locations from whence pollutants are discharged into the environment, and are easier to indentify as pollution sources (Lapworth *et al*, 2012). Therefore, point sources tend to have higher pollutant loads and are easier to trace. Typical point-sources of pollution include industrial effluents, municipal solid waste (MSW) leachate (Eggen *et al*, 2010), and sewage treatment works (STW) effluent.

2.2 Sewage as a source of pollution

Of all the water pollution sources the single largest polluter is sewage, both raw sewage and treated effluent from STWs. According to Camacho-Muñoz *et al* (2010) surface waters and wastewater treatment works (WWTW) effluents in Doñana watersheds (Spain), contained at least 16 pharmaceutically active compounds. The steroids 17 α-ethinyl estradiol (EE2), 17 β-estradiol (E2, also known as estradiol), estriol (E3) and estrone (E1) were detected in wastewaters at

<0.01 µg/L (µg: micrograms; L: litre), <0.02-0.24 µg/L, <0.003-0.16 µg/L and <0.16 µg/L respectively (Camacho-Muñoz *et al*, 2010).

Musolff *et al* (2009), revealed that urban waters from Leipzig (Germany) contained the micropollutants bisphenol A (BPA), nonylphenol (NP), caffeine, carbamazepine, galaxolide and tonalide; and noted seasonal differences in surface water pollutant loads. Reports from Musolff *et al* (2009) show variable pollutant distributions, with wastewater influent usually possessing highest pollutant loads and groundwater usually possessing lowest loads. BPA and NP were the pollutants with highest mean concentrations in both surface- and groundwater. Musolff *et al* (2009) indicates that treated wastewater was the source of micropollutants for the other water compartments.

The studies by Camacho-Muñoz *et al* (2010) and Musolff *et al* (2009) are supported by several other investigations that showed similar results (Esperanza *et al*, 2007; Choi *et al*, 2011; Díaz-Cruz *et al*, 2009; Yu *et al*, 2006; Xu *et al*, 2011; Gómez *et al*, 2011; Loos *et al*, 2010). Treated wastewater as a source of pollution is somewhat ironic, since the purpose of STWs is to remove harmful contaminants from wastewaters (Drinan and Whiting, 2001). Often, municipal STWs have to treat complex mixtures of pollutants in influents, because municipal STWs receive both domestic and industrial wastewaters. Many of the water pollutants are new, having only been produced recently (e.g new drugs), and the conventional STW was not designed to remove the new chemicals (Hendricks, 2011). Consequently new chemicals are not properly eliminated from the wastewaters.

Unfortunately, the incomplete removal of pollutants form wastewater poses a potential threat to wildlife and human populations in the area. An important route for human exposure to wastewater pollutants is via ingestion. According to Rabitto *et al* (2011), fish living in the Samuel Reservoir of the Amazon were contaminated by mercury and dichlorodiphenyltrichloroethane (DDT). They found mercury and DDT in the muscle tissue of the fish investigated, thus the investigators concluded fish to be a potential rout for human exposure to mercury and DDT in the Amazon (Rabitto *et al*, 2011). Another study, conducted in northern Vietnam, showed that both fish and plant (tea) tissues were contaminated with the pesticides used by farmers (Hoai *et al*, 2011). In a different study, Blocksom *et al* (2010) investigated the extent of pollution in the Mississippi River (USA). According to Blocksom *et al* (2010), fish tissues showed unexpectedly high levels of organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs). This discovery was surprising as OCPs and PCBs were banned for at least twenty years by the sampling date (Blocksom *et al*, 2010). Blocksom *et al* (2010) point out that the chemicals in question are highly lipid soluble and can bioaccumulate in the environment; hence their persistence after decades.

STWs remove some pollutants from influents. The influent and effluent of the Back River WWTW in Baltimore (United States of America: USA) was examined for the presence of pharmaceuticals and personal care products (PPCPs) (Yu *et al*, 2006). This analysis showed PPCPs in both influent and effluent. The effluent PPCP levels were usually significantly lower than the influent levels. The mechanisms by which STWs remove these contaminants are by adsorption,

biodegradation (biotransformation) and photocatalysis (Hamid and Eskicioglu, 2012).

Another dimension to be considered when evaluating water pollution is 'self-purification' of the water body. Self-purification is the process whereby a water body (usually a river) appears to clean itself of pollutants discharged into it. Self-purification is accomplished by the aquatic biota, which all play a role in removing pollutants from the water phase, and/or degrading/transforming the pollutants (Ostroumov, 2010). Although self-purification is encouraging, it does have limits, simply because self-purification relies largely on living organisms (Ostroumov, 2010). Pollutants that are toxic could eliminate certain species from a river, leaving a role in self-purification vacant. Alternately, the concentration, or mixture of pollutants present could be of such a nature that the pollutants inhibit self-purification. Without active self-purification, many pollutants would remain in the environment, which could ultimately affect the wildlife and human populations (UNGEMS/Water Programme, 2008).

2.3 Water pollution and biology

Environmental water pollution poses a notable threat to exposed living organisms. A number of studies have revealed how different chemicals can harm different organisms. Pollutants affect both plants and animals, which can cause changes in the biodiversity of the studied ecosystems.

2.3.1 Plants and animals

According to Karaouzas *et al* (2011), olive mill wastewaters (OMW) caused a decrease in biodiversity of the Evrotas River Basin (Greece) during olive mill operation seasons. Karaouzas *et al* (2011) found that, even when OMW was highly diluted, it had a great impact on the aquatic fauna exposed to it. Suquía River (Argentina) was also shown to have reduced biodiversity, specifically of fish (Merlo *et al*, 2011). However, the pollutants in Suquía River came from industry, municipal wastewater, agriculture and other nonpoint sources. Fish biodiversity was also noted as impaired at Brush creek (USA) (Porter and Janz, 2003), likely due to municipal sewage discharges.

One possible explanation for impaired fish diversity is that water pollutants may alter fish movements; as was the case in an investigation conducted by Durrant *et al* (2011). Durrant *et al* (2011) demonstrated that fish, that are unaccustomed to the polluted waters, would avoid entering polluted water. Thus the pollution produced a change in the investigated fish species' movements (Durrant *et al*, 2011).

Plants are also affected by pollution of their environment. In experiments by Perron and Juneau (2011), the investigators demonstrated how common environmental pollutants affect plant photosynthesis. It was also shown that plants could even retain pesticides in their tissues to possibly pass them on to humans via ingestion (Hoai *et al*, 2011). Another study, by Sun *et al* (2010), revealed how certain heavy metals and PAHs (Polycyclic aromatic hydrocarbons), from industries, accumulate in pine needles over time (Sun *et al*, 2010). In a review by Patra *et al* (2004) it is clear that heavy metals are also genotoxic to plants. For

example lead caused mutations in plants during mitosis, other mitotic interferences, increased micronucleus formation, diminished DNA (deoxyribonucleic acid) synthesis and inhibited cell growth, which lead to cell death (Patra *et al*, 2004).

In addition to decreasing biodiversity and harming plant metabolism, the same pollutants have been reported to affect many animal organ systems. The animal organ systems affected include, the immune system, the male reproductive system, the renal system, nervous system, and more. Many of the investigations on vertebrate physiology were done using fish species native to the studied region or by exposing test organisms to the pollutants (Stoker *et al*, 2003; Kamei *et al*, 2008; Jobling *et al*, 2009; Bellingham *et al*, 2012; Müller *et al*, 2009; Maceda-Veiga *et al*, 2010; Jeffries *et al*, 2011; Kumar *et al*, 2008; Porter and Janz, 2003; Folmar *et al*, 1996; Prado *et al*, 2011; Shinn *et al*, 2009; Luvizutto *et al*, 2010; Garcia-Reyero *et al*, 2011; Liu *et al*, 2011; Culioli *et al*, 2009; Vicente-Martorell *et al*, 2009; Silva *et al*, 1999; Cazenave *et al*, 2009; Albertsson *et al*, 2007).

2.3.2 Studies on individual pollutants

A number of common environmental water pollutants, such as BPA, endosulfan, fluoxetine, etc, have been studied individually for potentially harmful effects on organisms. These studies often expose organisms to environmentally relevant concentrations of the pollutant. The primary objectives of these studies are to determine whether the tested substance is physiologically active at specific concentrations, as well as determining the pollutant's mode of action.

The reproductive and endocrine systems are probably the systems that are most often studied as targets for pollutants, mainly EDCs. BPA caused sex reversals (in what was supposed to be male *Caiman latirostris* fish) at a much lower dose (20-100 times lower) than *in vitro* studies suggested (Stoker *et al*, 2003). OP induced/stimulated testosterone production by adult rat Leydig cells *in vitro* (Murono and Derk, 2002). An investigation by Kamei *et al* (2008) revealed that OP reduced female rat femur width and growth, when exposed perinatally and postnatally. Other chemicals affecting animal reproduction include 4-OP (Bendsen *et al*, 2001), fluoxetine (Mennigen *et al*, 2010) and dieldrin (Fowler *et al*, 2007).

Some other reported effects of individual pollutants include altered gene expression (Elango *et al*, 2006), altered enzyme activity (Estey *et al*, 2008; Smith *et al*, 2012), decreased hepatic activity (Estey *et al*, 2008), altered embryo histology (Osterauer *et al*, 2010), bioaccumulation (Ballesteros *et al*, 2011) and altered nervous system development (Pillon *et al*, 2012).

2.3.3 Chemical mixtures react different to their individual components

Although the findings from exposures to single chemicals are concerning, many studies point to mixtures of pollutants as being more harmful than the individual chemicals are. Synergism is mentioned as a mechanism for chemical interactions to produce a more pronounced effect than would be expected. A study by Jobling *et al* (2009) demonstrated that the male reproductive abnormalities discovered in fish, from UK (United Kingdom) rivers, caused by exposure to treated wastewater are likely to come from the effects of chemical mixtures, rather than solely steroid

estrogens. According to Bellingham *et al* (2012), exposure to a cocktail of EDCs during gonad development could cause permanent changes in gametes of adult animals, in both sexes.

An investigation by Khalaf *et al* (2009) revealed that an environmental mixture of the pharmaceuticals reduced both activator protein-1 (AP-1) activity (AP-1 is induced by mitogen activated protein kinase pathway), and Nuclear factor-κB (NF-κB) activity (NF-κB initiates inflammatory response). Furthermore, according to Khalaf *et al* (2009), mixtures of pharmaceuticals produced more pronounced effects on inflammatory markers than the individual drugs did.

According to Bellingham *et al* (2012), environmental exposure to EDCs results in low tissue levels of almost any given pollutant and their distribution in the organism depends on multiple factors. However, exposure to multiple EDCs could likely affect multiple systems within the organism (Bellingham *et al*, 2012). A review by Smital *et al* (2004) indicates that many common environmental pollutants possess the ability to inhibit multixenobiotic resistance (MXR) in aquatic organisms; and therefore sensitize exposed organisms to bioaccumulate pollutants more easily. Smital *et al* (2004) repeated that mixtures of certain chemicals appear to be more potent MXR inhibitors than the pure individual chemicals (Smital *et al*, 2004).

Thus, the precise effects of even known mixtures of chemicals on living organisms are unknown. This makes studies on physiological activity of STW effluent more important to better understand the potential dangers that exposure to those waters pose.

2.3.4 Studies on chemical mixtures (environmental water samples)

As is the case with individual pollutants, mixtures of pollutants produce various harmful effects in organisms exposed to them. Refer to **Table 2.1** for an overview of some health effects noted in organisms due to exposure to pollutant mixtures.

As can be seen from the literature, there have been many studies on the effects of pollutants on living organisms. It is also clear that although the individual chemicals found in the environment are harmful to living organisms, the mixtures found in rivers (primarily originating from sewage) produce more pronounced harmful effects in exposed organisms. Toxic effects of treated sewage indicate that current wastewater treatment technologies are not sufficient to preserve the environment; and that we need to better monitor the state of our environment. Furthermore, we also need to develop new technologies that are better able to remove all pollutants throughout the year without being so expensive that it is unfeasible to be used by all municipalities.

Table 2.1. Some studies on health effects of water pollution exposure in living organisms (or tissue).

Sample source	Region, Country	Effects noted	Species studied	Reference
Polluted river(s)	Near Gothenburg, Sweden	Altered protein expression	Fish: *Oncorhynchus mykiss*	Albertsson *et al*, 2007
Polluted estuary	Huelva, Spain	Reduced ability to detoxify essential elements	Fish: *Sparus aurata* and *Solea senegalensis*	Vicente-Martorell *et al*, 2009
STW influent and effluent	Western Cape, South Africa	Cytotoxicity	Tissue culture: MCF-7 cells	Swart *et al*, 2011
STW influent and effluent	Beijing, China	Cytotoxicity	*Vibrio fischeri*	Wang *et al*, 2003
MSW lechate	Lucknow, India	Toxic effects	*Drosophila melanogaster*	Bhargav *et al*, 2008
STW effluent	Northern India	Toxic effects	Albino rats (*Rattus norvegicus)*	Kumar *et al*, 2008
STW sludge	São Paulo State, Brazil	Centrilobular hepatocyte hyperplasia	Wistar rat	Luvizutto *et al*, 2010
Polluted stream	North-Eastern Spain	Micronuclei in their erythrocytes	Fish: *Barbus meridionalis*	Maceda-Veiga *et al*, 2010
STW effluent	Canada	Spleen tissue damage	Fish: *Oncorhynchus mykiss*	Müller *et al*, 2009
Polluted river(s)	São Paulo State, Brazil	Induced tissue lesions	Wistar rats	Silva *et al*, 1999
Polluted river(s)	Jiangsu Province, China	Depressed testis cell viability	Sprague-Dawley rats (tissue culture): Spermatogenic, Sertoli and Leydig cells	Wu *et al*, 2011

Polluted river(s)	Minas Gerais, Brazil	Possible delayed gonad maturation	Fish: *Astyanax fasciatus*	Prado *et al*, 2011
Polluted river(s)	Minas Gerais, Brazil	High incidence of intersex gonads	Fish: *Astyanax fasciatus*	Prado *et al*, 2011
Polluted river(s)	Jiangsu Province, China	Inhibited testosterone production	Sprague-Dawley rats (tissue culture): Spermatogenic, Sertoli and Leydig cells	Wu *et al*, 2011
Polluted river(s)	Minnesota, USA	Reduced circulating testosterone	Fish: *Cyprinus carpio*	Folmar *et al*, 1996
Polluted river(s)	Minnesota, USA	Vitellogenin induction in males (estrogenic effects)	Fish: *Cyprinus carpio*	Folmar *et al*, 1996
STW effluent	Minnesota, USA	Estrogenic effects	Fish: fathead minnows	Garcia-Reyero *et al*, 2011
Polluted river(s)	Minnesota, USA	Elevated estrogen/androgen ratio	Fish: *Cyprinus carpio*	Folmar *et al*, 1996
STW effluent	Northern India	Androgenic effects	Albino rats (*Rattus norvegicus)*	Kumar *et al*, 2008
STW effluent	Oklahoma, USA	Elevated serum testosterone levels	Fish: *Lepomis megalotis*	Porter and Janz, 2003
Polluted watersheds	Nebraska, USA	De-feminization of females	Fish: fathead minnows	Jeffries *et al*, 2011
STW effluent	Minnesota, USA	Altered sexual behaviour	Fish: fathead minnows	Garcia-Reyero *et al*, 2011
MSW lechate	Lucknow, India	Stress response induction	*Drosophila melanogaster*	Bhargav *et al*, 2008
MSW lechate	Lucknow, India	HSP 70 induction	*Drosophila melanogaster*	Bhargav *et al*, 2008
MSW lechate	Lucknow, India	Lipid peroxidation	*Drosophila melanogaster*	Bhargav *et al*, 2008

Polluted river(s)	São Paulo State, Brazil	Reduced antioxidant defence system	Wistar rats	Silva *et al*, 1999
MSW lechate	Lucknow, India	Antioxitant enzyme induction	*Drosophila melanogaster*	Bhargav *et al*, 2008
Polluted river(s)	Santa Fe, Argentina	Induction of antioxidant system	Fish: *Prochilodus lineatus*	Cazenave *et al*, 2009
STW sludge	São Paulo State, Brazil	Slightly increased aspartate aminotransferease activity	Wistar rat	Luvizutto *et al*, 2010
Polluted river(s)	São Paulo State, Brazil	Increased serum amylase activity	Wistar rats	Silva *et al*, 1999
Polluted river(s)	São Paulo State, Brazil	Increased creatinine levels	Wistar rats	Silva *et al*, 1999
Polluted river(s)	Santa Fe, Argentina	Slight reduction in haemoglobin concentration	Fish: *Prochilodus lineatus*	Cazenave *et al*, 2009
STW effluent	Canada	Altered blood cellular makeup	Fish: *Oncorhynchus mykiss*	Müller *et al*, 2009
Polluted river(s)	Santa Fe, Argentina	Altered leukocyte levels	Fish: *Prochilodus lineatus*	Cazenave *et al*, 2009
Polluted river(s)	Santa Fe, Argentina	Increased neutrophil population	Fish: *Prochilodus lineatus*	Cazenave *et al*, 2009
Polluted river(s)	Santa Fe, Argentina	Reduced lymphocyte population	Fish: *Prochilodus lineatus*	Cazenave *et al*, 2009
Polluted stream	North-Eastern Spain	Increased phagocytosis	Fish: *Barbus meridionalis*	Maceda-Veiga *et al*, 2010

STW effluent	Canada	Reduced T and B lymphocyte proliferation	Fish: *Oncorhynchus mykiss*	Müller *et al*, 2009
STW effluent	Canada	Increased NK cell activity	Fish: *Oncorhynchus mykiss*	Müller *et al*, 2009
STW effluent	Canada	Increased surface Ig expression	Fish: *Oncorhynchus mykiss*	Müller *et al*, 2009
STW effluent	Canada	Increased MHC II activation	Fish: *Oncorhynchus mykiss*	Müller *et al*, 2009
Polluted river(s)	Ilorin, Nigeria	High microbial loads in tissues	Fish: *Clarias gariepinus*	Kolawole *et al*, 2011
Polluted river(s)	Santa Fe, Argentina	Reduced liver somatic index	Fish: *Prochilodus lineatus*	Cazenave *et al*, 2009
STW effluent	Northern India	Reduced liver and kidney weight gain	Albino rats (*Rattus norvegicus)*	Kumar *et al*, 2008
STW effluent	Oklahoma, USA	Liver hypertrophy	Fish: *Lepomis megalotis*	Porter and Janz, 2003
Polluted river(s)	Minas Gerais, Brazil	Reduced body size	Fish: *Astyanax fasciatus*	Prado *et al*, 2011
Polluted river(s)	Corsica, France	Bioaccumulation of pollutants	Fish: *Salmo trutta*	Culioli *et al*, 2009
Polluted river(s)	South-Western France	Bioaccumulation of pollutants	Fish: *Abramis brama*	Shinn *et al*, 2009
Polluted estuary	Huelva, Spain	Bioaccumulation of pollutants	Fish: *Sparus aurata* and *Solea senegalensis*	Vicente-Martorell *et al*, 2009

2.4 The importance of steroids

Although many pollutants have been identified in the environment, recently organic contaminants have received a lot of attention, especially EDCs. One of the most important classes, if not the most important class, of EDC is the estrogenic EDCs (e-EDCs). E-EDCs are important because many STW effluents are described as estrogenic, and their feminising effects have been noted for years. Furthermore, many noted adverse health effects of EDCs were attributed to e-EDCs (Swart, 2008). Synthetic and natural steroids are an important class of e-EDC. The natural steroids (and the synthetic steroid EE2) are released into the sewage systems via urine and faeces, which end up at STWs before discharge into the environment. The following section will review steroid biosynthesis and will mention how EDCs may interfere with endocrine function.

2.4.1 Hypothalamic-pituitary-gonad axis

Steroidogenesis is regulated by the hypothalamic-pituitary-gonad (HPG) axis. The hypothalamus secretes gonadotropin releasing hormone (GnRH), which acts on the pituitary gland. The pituitary gland then releases luteinizing hormone (LH) and follicle stimulating hormone (FSH) which travel to the gonads via the general circulation. LH can trigger steroidogenesis in mammalian gonads. FSH stimulates the release of inhibin, from either the ovaries or the testes; which provides negative feedback by inhibiting GnRH release from the hypothalamus. In addition, FSH also plays a role in female steroidogenesis.

2.4.2 Steroidogenesis

Cholesterol is the precursor used for steroidogenesis and is transported to the inner mitochondrial membrane (Ghayee and Auchus, 2007; Miller and Auchus, 2011). Inside the mitochondrion, cholesterol becomes cleaved by CYP11A1 (also known as cytochrome P450 cholesterol side-chain cleavage enzyme: P450scc), to produce pregnenolone (Ghayee and Auchus, 2007; Miller and Auchus, 2011). CYP11A1 is the protein that distinguishes steroidogenic tissues from non-steroidogenic tissues. **Figure 2.1** shows an overview of the steroidogenic pathway.

2.4.3 Steroidogenic tissues

There are a number of different organs that play roles in vertebrate steroidogenesis. The steroidogenic organs include the adrenal glands, the testes in males and the ovaries in females. Although the steroidogenic organs may produce any of the steroids, the reality is that each tissue is more likely to produce and secrete specific steroids and less likely to produce and secrete the other steroids (Miller and Auchus, 2011).

In the adrenal glands, the zona glomerulosa and zona fasciculata possess tissue specific differences that result in each zone producing different steroids. Aldosterone is synthesized by zona glomerulosa, whereas cortisol and corticosterone are synthesized at the zona fasciculata (Miller and Auchus, 2011). The adrenals also produce small amounts of testosterone (Miller and Auchus, 2011).

The testes are the primary organs where androgen biosynthesis occurs in male vertebrates. The steroidogenic tissue of the testes is comprised of Leydig cells. The main steroid produced by the Leydig cells is testosterone. They readily convert dehydroepiandrosterone (DHEA) to androstenedione and then to testosterone.

The ovaries of vertebrates use a two cell system to produce mainly estrogenic steroids (however, under specific conditions, this organ will produce mainly progestins). LH induces the production of progesterone and pregnenolone by the granulosa cells (Miller and Auchus, 2011). This then diffuses into the theca cells, where they are converted to androgens (Miller and Auchus, 2011; Jamnongjit and Hammes, 2006). The androgens (primarily androstenedione) return to the FSH-stimulates granulosa cells to be converted to estrogens (Miller and Auchus, 2011; Jamnongjit and Hammes, 2006). However, this description only applies to the follicular phase of the female menstrual cycle. Granulosa cells can become 'luteinized' to produce progesterone and pregnenolone, by gonadotropin exposure (Jamnongjit and Hammes, 2006).

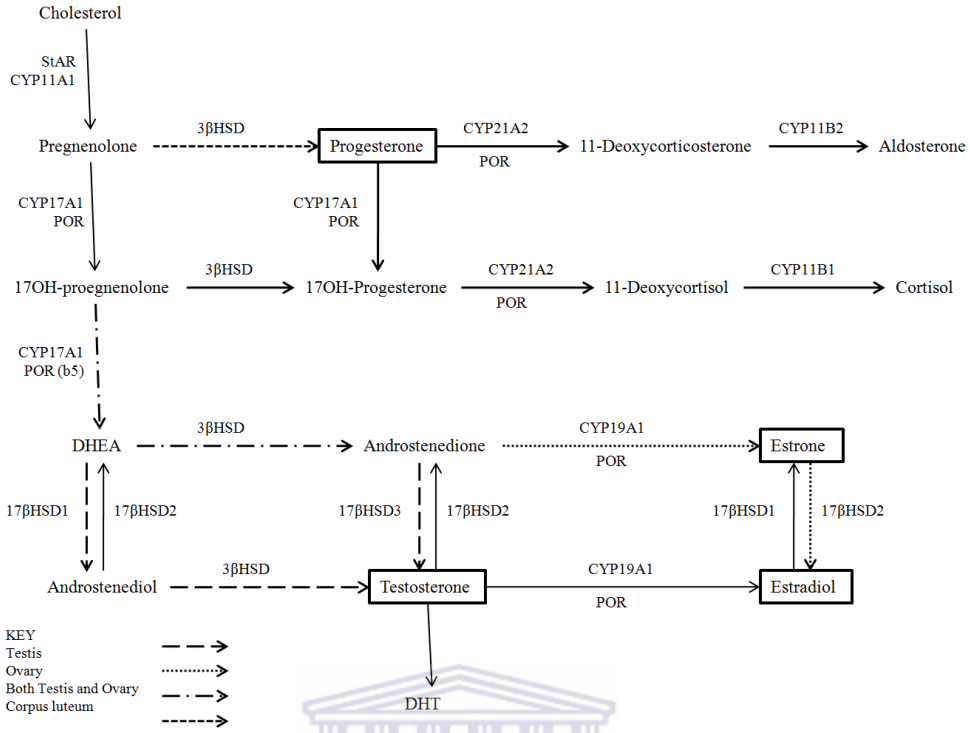

Figure 2.1. An overview of the steroidogenic pathway (Based on Ghayee and Auchus, 2007; Miller and Auchus, 2011). Abbreviations used: steroidogenic acute regulatory protein (StAR); β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (βHSD, eg 3βHSD); 17α-hydroxypregnenolone (17OH-pregnenolone); dehydroepiandrosterone (DHEA); dihydrotestosterone (DHT).

2.4.4 Actions of sex steroids

A number of studies over the years have revealed that the sex steroids (androgens, estrogens and progestins) play many important roles in the male (mainly androgens) and female (mainly estrogens and progestins) reproductive systems. Each of these steroid classes could act via 'genomic' or 'nongenomic' routes. Steroids are transported through the general circulation while bound to sex hormone-binding globulin, and dissociate before interacting with its target tissue.

The genomic route involves the steroid diffusing across the cell plasma membranes to be transported to the nucleus. At the nucleus, the steroid binds a specific nuclear steroid receptor, which eventually allows for expression of specific genes. This process takes some time before the response is fully evident. A quicker response for steroidal action may involve specific membrane steroid receptors, which forms the nongenomic route.

2.4.4.1 Androgens

The primary active androgen in the human is testosterone. Androgens act by binding to androgen receptors (ARs) to stimulate various physiological responses. Testosterone plays a role in nervous system development and neuroprotection (Białek *et al*, 2004). Androgens produce male secondary sexual characteristics, including increased muscle mass, strength and power as well as the growth of facial and body hair (Białek *et al*, 2004; Bhasin *et al*, 2001). In addition, testosterone plays roles in lipid and bone metabolism, as well as vascular behaviour (Białek *et al*, 2004; Bhasin *et al*, 2001; Campelo *et al*, 2012). Testosterone also promotes the development of spermatozoa in males, amongst other functions (Białek *et al*, 2004; Campelo *et al*, 2012; Bhasin *et al*, 2001).

2.4.4.2 Estrogens

The most potent and important estrogen in vertebrates is E2; however E1 and E3 may also be present. Estrogens act via estrogen receptors (ERs), of which, there are at least two nuclear subtypes; ERα and ERβ. Estradiol is essential for female reproduction, by stimulating endometrial growth and indirectly promoting follicle

maturation, amongst others (Rosenfeld *et al*, 2001; Young and Lessey, 2010; Halasz and Szekeres-Bartho, 2013). Estradiol also appears to aid male reproduction (Sharpe, 1998; Hess *et al*, 2001). Estrogens maintain skeletal integrity, by inhibiting osteoclast recruitment, thus preventing bone resorption (Punyadeera *et al*, 2003; Sharpe, 1998). Estrogens also allow for closing of the epiphyses (Sharpe, 1998). Estrogens appear to play a role in muscle metabolism of lipids (Oosthuyse and Bosch, 2012), enhancing central nervous system activities (Punyadeera *et al*, 2003), and improving cardiovascular profiles, amongst others (Punyadeera *et al*, 2003; Sharpe, 1998).

2.4.4.3 Progestins

The principal progestin in vertebrates is progesterone (P4). P4 acts via the progesterone receptors (PRs) of which there are two nuclear subtypes (PR-A and PR-B) as well as membrane PRs (Young and Lessey, 2010; Halasz and Szekeres-Bartho, 2013; Ismail *et al*, 2003; Singh and Su, 2013; Thomas and Pang, 2013; Punyadeera *et al*, 2003). P4 downregulates endometrial ERs, and thus antagonizes estrogen-induced endometrial growth (Young and Lessey, 2010; Halasz and Szekeres-Bartho, 2013). P4 also causes uterine receptivity that permits the successful implantation of an embryo (Young and Lessey, 2010; Halasz and Szekeres-Bartho, 2013). Progestin hormones suppress the maternal immune response to the embryo (Halasz and Szekeres-Bartho, 2013); and play a role in mammary gland development in preparation for lactation (Ismail *et al*, 2003). In females that are not pregnant, P4 prepares the endometrium for menstruation (Young and Lessey, 2010). In addition, P4 appears to display neuroprotective

effects (Singh and Su, 2013), as well as a number of beneficial effects on the cardiovascular system (Thomas and Pang, 2013).

2.4.5 Some endocrine disrupting chemical/compound mechanisms

The basic definition of an endocrine disrupting compound/chemical is an agent which has the ability to interact/interfere with the endocrine system functions of living organisms. Thus, EDCs' actions often affect the functional molecules of endocrine systems, which are hormones. However, hormones do not act alone; all hormones use receptors to signal a desired action (UNEP and WHO, 2013). Hormone receptors are located on cell membrane surfaces or within the cytosol of specific cells, and are highly specific for the hormone which activates it. The highly specific hormone-receptor interaction in part, allows a low dose of hormone to cause the desired affect (UNEP and WHO, 2013). Furthermore cells can become either more or less sensitive to hormones by respectively, increasing or decreasing the number of specific receptors they possess (UNEP and WHO, 2013).

There are a number of levels at which EDCs could interfere with normal endocrine functioning. EDCs may act like hormones by binding to specific hormone receptors. The binding action could result in stimulation of that response, or its inhibition through the blocking action of the EDC (Wuttke *et al*, 2010). EDCs might also alter the timing of hormone delivery (UNEP and WHO, 2013). EDCs could antagonize hormones; alter a hormone's structure, hormone synthesis, hormone receptor levels or hormone elimination (Swart, 2008). EDCs

could also possibly affect the epigenetics of an EDC-exposed individual, which may be passed on to following generations (Wuttke *et al*, 2010).

Thus we find that e-EDCs may cause feminization of males. Many e-EDCs are also anti-androgens, which accentuates male feminization. Since there is substantial homogeny amongst vertebrate endocrine physiology; e-EDC exposure could result in decreased fertility of many exposed animal populations, which may contribute to the extinction of many threatened species. Ultimately EDCs are likely to affect the human population as well. In fact EDCs may already be harming humanity, as human fertility has gradually decreased over the past few decades, such that infertility already affects one in every six to ten couples globally; that is approximately 80 million individuals (Cousineau and Domar, 2007; Inhorn, 2003; Shefi and Turek 2006). Statistics such as these should prompt us to find better ways to eliminate EDCs from our environment, or at least to reduce their presence and usage. For the former, we should take a closer look at our primary source of environmental water pollution, which are STWs.

2.5 Overview

There are many different types of pollutants found in environmental waters and EDCs are a particularly important class of pollutant. These pollutants have various sources, but the effluents from sewage treatment works seem to be the major water polluter around the world. A number of studies were conducted that show many adverse effects of water pollution on living organisms. People have investigated the effects of known environmental pollutants on different models and shown some of the harmful effects they produce. However, it was also found

that mixtures of pollutants (as can be found in the environment) appear more toxic to organisms than the individual pollutants do. Some of the physiological systems affected by exposure to polluted water include the reproductive system (via effects on the endocrine system) and the immune system amongst others.

Often STW effluents are described as estrogenic, making e-EDCs one of the most relevant classes of pollutant in the environment. The most potent e-EDCs are natural steroids (estrogens), but other natural steroids are also important.

Steroidogenesis is extremely important to mammals, as steroids perform or permit a number of actions within the host that are essential to reproduction. The synthesis of steroids from cholesterol, in the steroidogenic tissues are under the control of the HPG-axis. Each steroidogenic tissue synthesizes a specific set of steroids, but has the ability to produce the other steroids as well.

EDCs can potentially interfere with steroidogenesis, hormone delivery, hormone-receptor binding, it can alter hormone receptor abundance, and can harm the host in many more ways.

2.6 List of references:

Albertsson E, Kling P, Gunnarsson L, Larsson DGJ, Förlin L. 2007. Proteomic analyses indicate induction of hepatic carbonyl reductase/20β-hydroxysteroid dehydrogenase B in rainbow trout exposed to sewage effluent. Ecotoxicology and Environmental Safety. 68, 33–39.

Ballesteros ML, Gonzalez M, Wunderlin DA, Bistoni MA, Miglioranza KSB. 2011. Uptake, tissue distribution and metabolism of the insecticide endosulfan in *Jenynsia multidentata* (Anablepidae, Cyprinodontiformes). Environmental Pollution. 159, 1709-1714.

Bellingham M, Fiandanese N, Byers A, Cotinot C, Evans NP, Pocar P, Amezaga MR, Lea RG, Sinclair KD, Rhind SM, Fowler PA. 2012. Effects of exposure to environmental chemicals during pregnancy on the development of the male and female reproductive axes. Reproduction in Domestic Animals. 47, 15–22.

Bendsen E, Laursen SB, Olesen C, Westergaard LG, Andersen CY, Byskov AG. 2001. Effects of 4-octylphenol on germ cell number in cultured human fetal gonads. Human Reproduction. 16, 236-243.

Bhargav D, Singh MP, Murthy RC, Mathur N, Misra D, Saxena DK, Chowdhuri DK. 2008. Toxic potential of municipal solid waste leachates in transgenic *Drosophila melanogaster* (*hsp70-lacZ*): *hsp70* as a marker of cellular damage. Ecotoxicology and Environmental Safety. 69, 233–245.

Bhasin S, Woodhouse L, Casaburi R, Singh AB, Bhasin D, Berman N, Chen X, Yarasheski KE, Magliano L, Dzekov C, Dzekov J, Bross R, Phillips J, Sinha-Hikim I, Shen R, Storer TW. 2001. Testosterone dose-response

relationships in healthy young men. American Journal of Physiology - Endocrinology and Metabolism. 281, E1172–E1181.

Białek M, Zaremba P, Borowicz KK, Czuczwar SJ. 2004. Neuroprotective role of testosterone in the nervous system. Polish Journal of Pharmacology. 56, 509-518.

Blocksom KA, Walters DM, Jicha TM, Lazorchak JM, Angradi TR, Bolgrien DW. 2010. Persistent organic pollutants in fish tissue in the mid-continental great rivers of the United States. Science of the Total Environment. 408, 1180–1189.

Camacho-Muñoz MD, Santos JL, Aparicio I, Alonso E. 2010. Presence of pharmaceutically active compounds in Doñana Park (Spain) main watersheds. Journal of Hazardous Materials. 177, 1159–1162.

Campelo AE, Cutini PH, Massheimer VL. 2012. Cellular actions of testosterone in vascular cells: mechanism independent of aromatization to estradiol. Steroids. 77, 1033–1040.

Cazenave J, Bacchetta C, Parma M J, Scarabotti PA, Wunderlin DA. 2009. Multiple biomarkers responses in *Prochilodus lineatus* allowed assessing changes in the water quality of Salado River basin (Santa Fe, Argentina). Environmental Pollution. 157, 3025–3033.

Choi M, Furlong ET, Moon H, Yu J, Choi H. 2011. Contamination of nonylphenolic compounds in creek water, wastewater treatment plant effluents, and sediments from Lake Shihwa and vicinity, Korea: comparison with fecal pollution. Chemosphere. 85, 1406–1413.

Cousineau TM, Domar AD. 2007. Psychological impact of infertility. Best Practice & Research Clinical Obstetrics and Gynaecology. 21, 293-308.

Culioli J, Calendini S, Mori C, Orsini A. 2009. Arsenic accumulation in a freshwater fish living in a contaminated river of Corsica, France. Ecotoxicology and Environmental Safety. 72, 1440–1445.

Díaz-Cruz MS, García-Galán ML, Guerra P, Jelic A, Postigo C, Eljarrat E, Farré M, López de Alda MJ, Petrovic M, Barcelo D. 2009. Analysis of selected emerging contaminants in sewage sludge. Trends in Analytical Chemistry. 28, 1263-1275.

Drinan JE, Whiting NE. 2001. Water & wastewater treatment, a guide for the nonengineering professional. CRC Press. Boca Raton. 316pp.

Durrant CJ, Stevens JR, Hogstrand C, Bury NR. 2011. The effect of metal pollution on the population genetic structure of brown trout (*Salmo trutta* L.) residing in the River Hayle, Cornwall, UK. Environmental Pollution. 159, 3595-3603.

Eggen T, Moeder M, Arukwe A. 2010. Municipal landfill leachates: a significant source for new and emerging pollutants. Science of the Total Environment. 408, 5147–5157.

Elango A, Shepherd B, Chen TT. 2006. Effects of endocrine disrupters on the expression of growth hormone and prolactin mRNA in the rainbow trout pituitary. General and Comparative Endocrinology. 145, 116–127.

Esperanza M, Suidan MT, Marfil-Vega R, Gonzalez C, Sorial GA, McCauley P, Brenner R. 2007. Fate of sex hormones in two pilot-scale municipal

wastewater treatment plants: conventional treatment. Chemosphere. 66, 1535–1544.

Estey C, Chen X, Moon TW. 2008. 3-Hydroxy-3-methylglutaryl coenzyme A reductase in rainbow trout: effects of fasting and statin drugs on activities and mRNA transcripts. Comparative Biochemistry and Physiology, Part C. 147, 386–398.

Folmar LC, Denslow ND, Rao V, Chow M, Crain DA, Enblom J, Marcino J, Guillette Jr LJ. 1996. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environmental Health Perspectives. 104, 1096-1101.

Fowler PA, Abramovich DR, Haites NE, Cash P, Groome NP, Al-Qahtani A, Murray TJ, Lea RG. 2007. Human fetal testis Leydig cell disruption by exposure to the pesticide dieldrin at low concentrations. Human Reproduction. 22, 2919–2927.

Garcia-Reyero N, Lavelle CM, Escalon BL, Martinović D, Kroll KJ, Sorensen PW, Denslow ND. 2011. Behavioral and genomic impacts of a wastewater effluent on the fathead minnow. Aquatic Toxicology. 101, 38–48.

Ghayee HK, Auchus RJ. 2007. Basic concepts and recent developments in human steroid hormone biosynthesis. Reviews in Endocrine and Metabolic Disorders. 8, 289–300.

Gómez C, Vicente J, Echavarri-Erasun B, Porte C, Lacorte S. 2011. Occurrence of perfluorinated compounds in water, sediment and mussels from the Cantabrian Sea (North Spain). Marine Pollution Bulletin. 62, 948–955.

Halasz M, Szekeres-Bartho J. 2013. The role of progesterone in implantation and trophoblast invasion. Journal of Reproductive Immunology. 97, 43– 50.

Hamid H, Eskicioglu C. 2012. Fate of estrogenic hormones in wastewater and sludge treatment: a review of properties and analytical detection techniques in sludge matrix. Water Research. 30, 1-21.

Hendricks R. 2011. Assessment of the biological quality of raw and treated effluents from three sewage treatment plants in the Western Cape, South Africa. Doctoral Thesis, University of the Western Cape. 240pp.

Hess RA, Bunick D, Bahr J. 2001. Oestrogen, its receptors and function in the male reproductive tract — a review. Molecular and Cellular Endocrinology. 178, 29–38.

Hoai PM, Sebesvari Z, Minh TB, Viet PH, Renaud FG. 2011, Pesticide pollution in agricultural areas of Northern Vietnam: case study in Hoang Liet and Minh Dai communes. Environmental Pollution. 159, 3344-3350.

Inhorn MC. 2003. Global infertility and the globalization of new reproductive technologies: illustrations from Egypt. Social Science & Medicine. 56, 1837–1851.

Ismail PM, Amato P, Soyal SM, DeMayo FJ, Conneely OM, O'Malley BW, Lydon JP. 2003. Progesterone involvement in breast development and tumorigenesis—as revealed by progesterone receptor "knockout" and "knockin" mouse models. Steroids. 68, 779–787.

Jamnongjit M, Hammes SR. 2006. Ovarian steroids: the good, the bad, and the signals that raise them. Cell Cycle. 5, 1178–1183.

Jeffries MKS, Conoan NH, Cox MB, Sangster JL, Balsiger HA, Bridges AA, Cowman T, Knight LA, Bartelt-Hunt SL, Kolok AS. 2011. The anti-estrogenic activity of sediments from agriculturally intense watersheds: assessment using *in vivo* and *in vitro* assays. Aquatic Toxicology. 105, 189–198.

Jobling S, Burn RW, Thorpe K, Williams R, Tyler C. 2009. Statistical modeling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in English rivers. Environmental Health Perspectives. 117, 797-802.

Kamei S, Miyawaki J, Sakayama K, Yamamoto H, Masuno H. 2008. Perinatal and postnatal exposure to 4-*tert*-octylphenol inhibits cortical bone growth in width at the diaphysis in female mice. Toxicology. 252, 99–104.

Karaouzas I, Skoulikidis NT, Giannakou U, Albanis TA. 2011. Spatial and temporal effects of olive mill wastewaters to stream macroinvertebrates and aquatic ecosystems status. Water Research. 45, 6334-6346.

Khalaf H, Salste L, Karlsson P, Ivarsson P, Jass J, Olsson PE. 2009. *In vitro* analysis of inflammatory responses following environmental exposure to pharmaceuticals and inland waters. Science of the Total Environment. 407, 1452–1460.

Kolawole OM, Ajayi KT, Olayemi AB, Okoh AI. 2011. Assessment of water quality in Asa River (Nigeria) and its indigenous *Clarias gariepinus* fish. International Journal of Environmental Research and Public Health. 8, 4332-4352.

Kumar V, Chakraborty A, Viswanath G, Roy P. 2008. Androgenic endocrine disruptors in wastewater treatment plant effluents in India: their influence on reproductive processes and systemic toxicity in male rats. Toxicology and Applied Pharmacology. 226, 60–73.

Lapworth DJ, Baran N, Stuart ME, Ward RS. 2012. Emerging organic contaminants in groundwater: a review of sources, fate and occurrence. Environmental Pollution. 163, 287-303.

Liu J, Wang R, Huang B, Lin C, Wang Y, Pan X. 2011. Distribution and bioaccumulation of steroidal and phenolic endocrine disrupting chemicals in wild fish species from Dianchi Lake, China. Environmental Pollution. 159, 2815-2822.

Loos R, Locoro G, Contini S. 2010. Occurrence of polar organic contaminants in the dissolved water phase of the Danube River and its major tributaries using SPE-LC-MS2 analysis. Water Research. 44, 2325–2335.

Luvizutto JFL, Solano MLM, Passareli D, Franchi CAS, Umbuzeiro GA, de Camargo JLV. 2010. Subchronic toxicity evaluation of a treated urban sewage sludge. Journal of Toxicology and Environmental Health, Part A. 73, 916–925.

Maceda-Veiga A, Monroy M, Viscor G, De Sostoa A. 2010. Changes in non-specific biomarkers in the Mediterranean barbell (*Barbus meridionalis*) exposed to sewage effluents in a Mediterranean stream (Catalonia, NE Spain). Aquatic Toxicology. 100, 229–237.

Mennigen JA, Lado WE, Zamora JM, Duarte-Guterman P, Langlois VS, Metcalfe CD, Chang JP, Moon TW, Trudeau VL. 2010. Waterborne

fluoxetine disrupts the reproductive axis in sexually mature male goldfish, *Carassius auratus*. Aquatic Toxicology. 100, 354–364.

Merlo C, Abril A, Amé MV, Argüello GA, Carreras HA, Chiappero MS, Hued AC, Wannaz E, Galanti LN, Monferrán MV, González CM, Solís VM. 2011. Integral assessment of pollution in the Suquía River (Córdoba, Argentina) as a contribution to lotic ecosystem restoration programs. Science of the Total Environment. 409, 5034–5045.

Miller WL, Auchus RJ. 2011. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocrine Reviews. 32, 81-151.

Müller C, Ruby S, Brousseau P, Cyr D, Fournier M, Gagné F. 2009. Immunotoxicological effects of an activated-sludge-treated effluent on rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology, Part C. 150, 390–394.

Murono EP, Derk RC. 2002. Exposure to octylphenol increases basal testosterone formation by cultured adult rat Leydig cells. Journal of Steroid Biochemistry & Molecular Biology. 81, 181–189.

Musolff A, Leschik S, Möder M, Strauch G, Reinstorf F, Schirmer M. 2009. Temporal and spatial patterns of micropollutants in urban receiving waters. Environmental Pollution. 157, 3069–3077.

Oosthuyse T, Bosch AN. 2012. Oestrogen's regulation of fat metabolism during exercise and gender specific effects. Current Opinion in Pharmacology. 12, 363–371.

Osterauer R, Kohler HR, Triebskorn R. 2010. Histopathological alterations and induction of hsp70 in ramshorn snail (*Marisa cornuarietis*) and zebrafish (*Danio rerio*) embryos after exposure to $PtCl_2$. Aquatic Toxicology. 99, 100–107.

Ostroumov SA. 2010. Biocontrol of water quality: multifunctional role of biota in water self-purification. Russian Journal of General Chemistry. 80, 2754–2761.

Patra M, Bhowmik N, Bandopadhyay B, Sharma A. 2004. Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance. Environmental and Experimental Botany. 52, 199–223.

Perron MC, Juneau P. 2011. Effect of endocrine disrupters on photosystem II energy fluxes of green algae and cyanobacteria. Environmental Research. 111, 520–529.

Pillon D, Cadiou V, Angulo L, Duittoz AH. 2012. Maternal exposure to 17-alpha-ethinylestradiol alters embryonic development of GnRH-1 neurons in mouse. Brain Research. 1433, 29–37.

Porter CM, Janz DM. 2003. Treated municipal sewage discharge affects multiple levels of biological organization in fish. Ecotoxicology and Environmental Safety. 54, 199–206.

Prado PS, Souza CC, Bazzoli N, Rizzo E. 2011. Reproductive disruption in lambari *Astyanax fasciatus* from a Southeastern Brazilian reservoir. Ecotoxicology and Environmental Safety. 74, 1879–1887.

Punyadeera C, Verbost P, Groothuis P. 2003. Oestrogen and progestin responses in human endometrium. Journal of Steroid Biochemistry & Molecular Biology. 84, 393–410.

Rabitto IS, Bastos WR, Almeida R, Anjos A, de Holanda IBB, Galvão RCF, Neto FF, de Meneze ML, dos Santos CAM, Ribeiro CAO. 2011. Mercury and DDT exposure risk to fish-eating human populations in Amazon. Environment International. 37, 56–65.

Rosenfeld CS, Wagner JS, Roberts RM, Lubahn DB. 2001. Intraovarian actions of oestrogen. Reproduction. 122, 215–226.

Sharpe RM. 1998. The roles of oestrogen in the male. Trends in Endocrinology & Metabolism. 9, 371-377.

Shefi S, Turek PJ. 2006. Definition and current evaluation of subfertile men: review article. International Brazilian Journal of Urology. 32, 385-397.

Shinn C, Dauba F, Grenouillet G, Guenard G, Lek S. 2009. Temporal variation of heavy metal contamination in fish of the River Lot in Southern France. Ecotoxicology and Environmental Safety. 72, 1957–1965.

Silva AMM, Novelli ELB, Fascineli ML, Almeida JA. 1999. Impact of an environmentally realistic intake of water contaminants and superoxide formation on tissues of rats. Environmental Pollution. 105, 243-249.

Singh M, Su C. 2013. Progesterone and neuroprotection. Hormones and Behavior. 63, 284–290.

Smital T, Luckenbach T, Sauerborn R, Hamdoun AM, Vega RL, Epel D. 2004. Emerging contaminants—pesticides, PPCPs, microbial degradation

products and natural substances as inhibitors of multixenobiotic defense in aquatic organisms. Mutation Research. 552, 101–117.

Smith EM, Iftikar FI, Higgins S, Irshad A, Jandoc R, Lee M, Wilson JY. 2012. *In vitro* inhibition of cytochrome P450-mediated reactions by gemfibrozil, erythromycin, ciprofloxacin and fluoxetine in fish liver microsomes. Aquatic Toxicology. 109, 259–266.

Stoker C, Rey F, Rodriguez H, Ramos JG, Sirosky P, Larriera A, Luque EH, Muñoz-de-Toro M. 2003. Sex reversal effects on *Caiman latirostris* exposed to environmentally relevant doses of the xenoestrogen bisphenol A. General and Comparative Endocrinology. 133, 287–296.

Sun F, Wen D, Kuang Y, Li J, Li J, Zuo W. 2010. Concentrations of heavy metals and polycyclic aromatic hydrocarbons in needles of Masson pine (*Pinus massoniana* L.) growing nearby different industrial sources. Journal of Environmental Sciences. 22, 1006–1013.

Swart JC, Pool EJ, van Wyk JH. 2011. The implementation of a battery of *in vivo* and *in vitro* bioassays to assess river water for estrogenic endocrine disrupting chemicals. Ecotoxicology and Environmental Safety. 74, 138–143.

Swart JC. 2008. The development and implementation of biomarker assays for estrogenic endocrine disruptors. Doctoral Thesis, University of the Western Cape. 194pp.

Thomas P, Pang Y. 2013. Protective actions of progesterone in the cardiovascular system: potential role of membrane progesterone receptors (mPRs) in mediating rapid effects. Steroids. 78, 583–588.

United Nations Environment Programme Global Environment Monitoring System (GEMS)/Water Programme. 2008. Water quality for ecosystem and human health, 2nd edition. Ontario: UNEP GEMS/Water Programme. 120pp.

United Nations Environment Programme, World Health Organization. 2013. State of the science of endocrine disrupting chemicals–2012. Geneva: UNEP, WHO. 260pp.

Vicente-Martorell JJ, Galindo-Riaño MD, García-Vargas M, Granado-Castro MD. 2009. Bioavailability of heavy metals monitoring water, sediments and fish species from a polluted estuary. Journal of Hazardous Materials. 162, 823–836.

Wang C, Wang Y, Kiefer F, Yediler A, Wang Z, Kettrup A. 2003. Ecotoxicological and chemical characterization of selected treatment process effluents of municipal sewage treatment plant. Ecotoxicology and Environmental Safety. 56, 211–217.

Wu J, Wang X, Ying F, Hu G, Wang X, Li D, Yu H, Han X. 2011. *In vitro* assessment of reproductive toxicity on rats induced by organic contaminants of source water. Ecotoxicology and Environmental Safety. 74, 1756–1764.

Wuttke W, Jarry H, Seidlovα-Wuttke D. 2010. Definition, classification and mechanism of action of endocrine disrupting chemicals. Hormones. 9, 9-15.

Xu Y, Luo F, Pal A, Yew-Hoong Gin K, Reinhard M. 2011. Occurrence of emerging organic contaminants in a tropical urban catchment in Singapore. Chemosphere. 83, 963–969.

Young SL, Lessey BA. 2010. Progesterone function in human endometrium: clinical perspectives. Seminars in Reproductive Medicine. 28, 5-16.

Yu JT, Bouwer EJ, Coelhan M. 2006. Occurrence and biodegradability studies of selected pharmaceuticals and personal care products in sewage effluent. Agricultural Water Management. 86, 72–80.

CHAPTER 3: THE MAMMALIAN IMMUNE SYSTEM

Research has shown that the immune system of mammals is affected by water pollution. This chapter will briefly review the mammalian immune system with some extra attention being placed on humoral immunity.

The mammalian immune system has two main arms that work in concert to protect the host from pathogenic invasions, namely the innate immune system (immunity present at birth) and the acquired immune system (also known as adaptive immunity) (Tosi, 2005). The innate immune system is fully functional at birth in healthy individuals, whereas the acquired immune system requires prior exposure to an antigenic agent before it can react and produce long lasting immunity to that agent. In general, the innate immune system is the first line of defence against infection, followed by the acquired immune system, which only becomes fully active after exposure to pathogens to provide long-term protection. Both systems work together and complement each other in protecting the host.

3.1 Innate Immunity

3.1.1 Barrier system

The first line of defence is the barrier system. The barrier system is composed of the epithelium, as well as many extra-epithelial structures. The barrier system employs both physical and chemical means of preventing and controlling the development of infections. Some physical defences are: the impermeable nature of the epithelium, wash action of sweat and urine flow, removal of dead epithelium, and the cillial movements (Chaplin, 2006; Chaplin, 2010). Epithelial cells also

possess the ability to secrete antimicrobial agents, such as the defensins, lysozyme and cathelicidin (which also acts as a chemotactic agent to white blood cells) (Tosi, 2005). In addition, epithelial cells also secrete cytokines to attract and/or activate immune cells (Tosi, 2005). With the barrier system intact, it is highly unlikely that a mammal would become ill due to a pathogenic cause.

The purpose of the immune system is to protect the host from all pathogens, and to ideally do so without harming the host. In order to tolerate host tissues, the immune system must have a means of distinguishing 'self' antigens from 'non-self' antigens. The way the immune system does this is by using receptors that recognize 'non-self' antigens. In addition, the healthy immune system generally cannot recognize 'self' antigens (natural killer cells are an exception).

3.1.2 Phagocytosis

It is almost inevitable that any living and moving creature would, at some stage in its life, be subject to some force that causes an open wound injury. When this occurs, the pathogen may encounter a type of white blood cell; white blood cells are the effectors of the immune system once the barrier is compromised. Due to their abundance in the body, the first white blood cell that a pathogen encounters is likely to be a phagocyte, like a neutrophil or macrophage (Tosi, 2005).

Many phagocytes possess pathogen associated molecular pattern (PAMP) receptors (eg. the Toll-like receptors in humans: TLR), which recognise specific common pathogenic molecules, such as the lipopolysaccharides (LPS) found on Gram negative bacterial walls (Chaplin, 2006; Chaplin, 2010; Tosi, 2005). In addition, phagocytes also possess receptors that interact with the Fc (fragment

crystallizable) region of antigen-bound antibodies (also known as immunoglobulins: Igs), thus antibodies can act as opsonins (Chaplin, 2006). Once a foreign antigen interacts with a PAMP receptor on a phagocyte (or with an Fc receptor, via an appropriate antibody), receptor-mediated endocytosis follows (Chaplin, 2006; Tosi, 2005). The foreign agent is then surrounded by a phagocytic vesicle (the phagosome), which fuses with a lysosome to form a phagolysosome (Tosi, 2005).

Ideally, the lysosomal enzymes (together with other toxic agents, such as nitric oxide, superoxide, hypochlorite, and chloramines) should kill and degrade the foreign agent yielding pathogen peptides which will be processed further to activate acquired immunity (Chaplin, 2006; Chaplin, 2010; Tosi, 2005). In addition, the reacted phagocyte also releases inflammatory mediators and other cytokines. During the process of killing the foreign agent, the phagocyte often dies, with the dead phagocytes forming pus at the site of infection.

3.1.3 Natural killer cells

A special class of T lymphocyte, known as natural killer (NK) T cells, plays an important role in innate immunity (Chaplin, 2006; Chaplin, 2010; LaRosa and Orange, 2008; Riley, 2008). NK cells do not possess special antigen receptors like other lymphocytes (Chaplin, 2006; Chaplin, 2010; LaRosa and Orange, 2008; Riley, 2008). However, NK cells interact with MHC I (major histocompatibility complex class I) molecules on somatic cells to produce an inhibitory signal in the NK cell (Chaplin, 2006; Chaplin, 2010; LaRosa and Orange, 2008; Riley, 2008). Thus, cells that do not express enough MHC I-peptide complexes will be killed by

the NK cells. This prevents any cells infected with intracellular pathogens from being unnoticed by the immune system (Riley, 2008). NK cells kill their targets by use of perforin and inducing apoptosis, and are thus potent anti-cancer agents in the body (Tosi, 2005; LaRosa and Orange, 2008; Riley, 2008). NK cells also possess activating receptors, though; and once activated, NK cells also secrete cytokines to aid the immune response (Tosi, 2005; Riley, 2008).

3.2 Acquired Immunity

3.2.1 Effectors of Acquired Immunity

The two primary cell types that are the effectors of acquired immunity are the T cells/lymphocytes and the B cells/lymphocytes and their derivatives. Both T and B cells are derived from the bone marrow in adults (or foetal liver) (Bonilla and Oettgen, 2010). Thereafter T cells develop in the thymus, hence the name T cell, and B cells develop in the bursa equivalent (which is the bone marrow in humans), hence the name B cell (Bonilla and Oettgen, 2010).

Although recent studies have revealed more sub-classes, T cells (possessing antigen receptors) have two main subsets: those expressing the cluster of differentiation (CD) 4 molecule ($CD4^+$ cells) and $CD8^+$ cells (Chaplin, 2006; Chaplin, 2010). $CD8^+$ cells kill somatic cells that are infected with intracellular pathogens (cell-mediated immunity); they are thus called cytolytic/cytotoxic T (T_C) cells (Chaplin, 2010). The $CD4^+$ cells provide signals that help other lymphocytes to execute their functions (Chaplin, 2010). They are consequently called helper T (T_H) cells (Chaplin, 2010). Helper T cells are further divided into two groups that perform mostly mutually exclusive functions. T_H 1 cells stimulate

T_C cell effector function, and T_H 2 cells stimulate B cell effector functions (Chaplin, 2006; Chaplin, 2010). B cells mature into plasma cells (PCs) which secrete high levels of specific antibody (humoral immunity). In addition to their effector functions, both T cells and B cells can be stored as memory lymphocytes to provide long-term immunity against the relevant antigen.

3.2.2 Major histocompatibility complex -peptide complexes

In order to interact with virtually any foreign antigen the host comes in contact with, the acquired immune system needs a more sophisticated means of detecting foreign antigens than simply using PAMP receptors. For this reason, acquired immunity employs the use of major histocompatibility complex (MHC) molecules (Chaplin, 2006; Chaplin, 2010).

Once pathogen peptides are obtained via phagocytosis and/or proteasome activity, it must be presented to other immune cells to activate the acquired immune system. Antigen presentation is mainly done using one of two different types of MHC molecules (Chaplin, 2006; Chaplin, 2010). MHC class I (MHC I) is expressed in all somatic cells, whereas MHC II is only expressed in a group of cells (which includes macrophages, B cells and dendritic cells) which are called antigen presenting cells (APCs) (Chaplin, 2006; Chaplin, 2010).

3.2.2.1 Major histocompatibility complex class I

Inside a healthy somatic cell, a proteasome degrades some of the proteins synthesized by the cell to give ‘self’ peptides (Chaplin, 2010). If the somatic cell was infected with an intracellular pathogen, like a virus, some of the pathogenic

proteins would also have been degraded by the proteasome to give pathogen peptides (Chaplin, 2010).

The peptides (either pathogen peptides or 'self' peptides) are then transported to the endoplasmic reticulum (ER), where they are attached to MHC class I molecules (Chaplin, 2010). Thereafter, the MHC I-peptide complexes pass through the Golgi apparatus into exocytic vesicles so that it can translocate to the extracellular surface of the plasma membrane (Chaplin, 2010). MCH I-foreign peptide complexes activate Tc cells possessing complimentary T cell receptors (TCRs) to kill the infected cell (Chaplin, 2010).

3.2.2.2 Major histocompatibility complex class II

The synthesis of MCH class II molecules begins with an APC, which samples some of the extracellular medium, either by endocytosis of potential pathogens or pinocytosis (Chaplin, 2006; Chaplin, 2010). In this case pathogen peptides are generated either by the killing of the microbe (as was described), or by the proteasome's activity.

Foreign peptides are processed onto MHC class II molecules and transported to the APC's external surface via the ER and Golgi, just like with MHC class I (Chaplin, 2010). MCH II-foreign peptide complexes activate (or contribute to the activation of) naive B cells that possess complimentary B cell receptors (BCRs). The activated B cells can then produce antibodies against the activating antigen.

3.2.3 Lymphoid tissue and germinal centre formation

Unfortunately, there will be very few B cells and T cells present which can interact with the required antigen, bound to MHC on an APC (or infected somatic cell). The B (or T_C) cell may need T_H cell signals to be fully activated and so, must come into contact with a rare T_H cell which can also interact with that same antigen. To increase the likelihood for this to occur, the lymphoid tissues have special zones where B cells and T cells accumulate after their antigen-independent development. The secondary lymphoid tissues are highly vascularised, which permits circulating B and T cells to enter the tissue and form germinal centres (GC). These tissues also are connected to the lymphatics, permitting the exchange of APCs and activated lymphocytes. Once activated, B cells either remain here to become short-lived plasma cells (for T cell independent antigens) that secrete high levels of Ig; or they migrate to primary B cell follicles to form a GC with T_H2 cell interaction (Kalia *et al*, 2006; LaRosa and Orange, 2008; Maddaly *et al*, 2010; Schroeder and Cavacini, 2010).

3.2.4 Immunoglobulin structure

The B lymphocyte antigen receptor (BCR) is composed of Ig components. Essentially, the BCR main structure is an immobilised antibody located on the outer surface of the B cell (or B cell derivative). The BCR can only interact with one specific antigen, the same antigen that the given B cell produces antibodies against (Chaplin, 2010; Schroeder and Cavacini, 2010). Thus, the genetic alterations that determines the BCR, also determines the antibody that the given B cell secretes.

Immunoglobulins are composed of two identical light chains (LC) and two identical heavy chains (HC) (Chaplin, 2010; Schroeder and Cavacini, 2010), see **Figure 3.1**. There are two types of light chain (κ and λ), and nine heavy chain types (IgA1, IgA2, IgD, IgE, IgG1-4 and IgM) (Bonilla and Oettgen, 2010). Immunoglobulins possess an antigen-binding fragment (Fab) and an effector (Fc: fragment crystallizable) region (Schroeder and Cavacini, 2010). The heavy and light chains are held together by disulfide bridges (Schroeder and Cavacini, 2010). The five Ig isotypes each have different half-lives and are present at different concentrations within a healthy host (Schroeder and Cavacini, 2010). In addition, IgA and IgM often form dimeric and pentameric associations, respectively; which allows them to be present in body secretions (Schroeder and Cavacini, 2010). For more on Ig structure and isotypes, see Schroeder and Cavacini (2010).

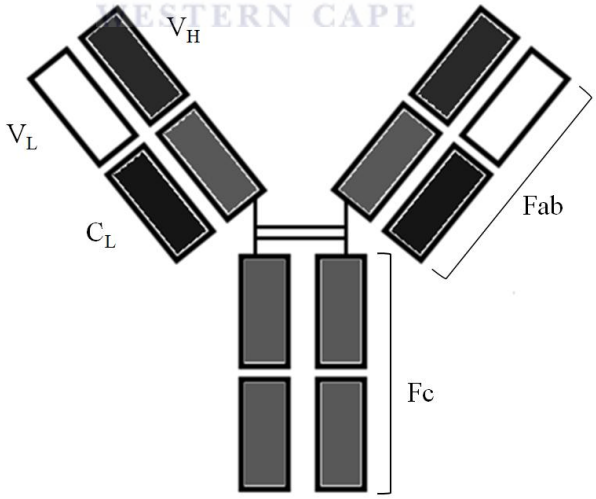

Figure 3.1. The structure of the IgG molecule (based on Schroeder and Cavacini, 2010). V: variable segment, C: constant segment, L: light chain, H: heavy chain, Fab: antigen binding fragment, Fc: fragment crystallizable.

3.2.5 Antibody actions

Once antibodies are produced, it engages the antigen and initiates different means of eliminating the threat. Immunoglobulins may be used to initiate Fc-mediated phagocytosis (described earlier). They could also be used for antibody-dependent cell-mediated cytotoxicity (ADCC), where specific cells (eg. NK cells) kill their targets via the release of perforin and granzymes. Finally, an antigen-bound antibody could also activate a complement pathway, which produces the membrane attack complex (MAC) to kill the foreign agent. Although the complement pathways are one means of attacking potential pathogens, an antibody is not necessary, and so the complement forms part of both innate and adaptive immunity.

3.3 Summary

Clearly, the mammalian immune system is complex, using a number of different mechanisms to protect the host from infections. Firstly, innate immunity provides protection against a range of potential pathogens by preventing an invasion (barriers) and actively eliminating potential pathogens (phagocytosis and NK cell activity). Once the barriers are compromised, phagocytosis yields pathogen peptides which are then processed onto MCH molecules to be presented to cells of the acquired immune system (B and T lymphocytes). B and T cells each possess unique antigen receptors that can only interact with specific antigens. Once the lymphocyte engages its antigen, it becomes activated (likely with the aid of T_H cells). B cells and T_H2 cells form germinal centres in lymphoid tissues to permit

their activation. B cells undergo mitosis to produce either plasma cells or memory B cells. Plasma cells secrete antibodies and memory cells are generally inactive and wait for reactivation by its antigen. Activated T cells also divide to produce effector cells and memory cells. Once antibodies are released, they can initiate the elimination of a foreign agent by any one of a number of physiological processes, like a compliment pathway.

3.4 List of references:

Bonilla FA, Oettgen HC. 2010. Adaptive immunity. The Journal of Allergy and Clinical Immunology. 125, S33-S4.

Chaplin DD. 2006. Overview of the human immune response. The Journal of Allergy and Clinical Immunology. 117, S430-S435.

Chaplin DD. 2010. Overview of the immune response. The Journal of Allergy and Clinical Immunology. 125, S3-S23.

Kalia V, Sarkar S, Gourley TS, Rouse BT, Ahmed R. 2006. Differentiation of memory B and T cells. Current Opinion in Immunology. 18, 255–264.

LaRosa DF, Orange JS. 2008. Lymphocytes. The Journal of Allergy and Clinical Immunology. 121, S364-S369.

Maddaly R, Pai G, Balaji S, Sivaramakrishnan P, Srinivasan L, Sunder SS, Paul SFD. 2010. Receptors and signaling mechanisms for B-lymphocyte activation, proliferation and differentiation – Insights from both in vivo and in vitro approaches. FEBS Letters. 584, 4883–4894.

Riley J. 2008. NK cells and their receptors. Reproductive BioMedicine Online. 16, 173-191.

Schroeder HW, Cavacini L. 2010. Structure and function of immunoglobulins. The Journal of Allergy and Clinical Immunology. 125, S41-S52.

Tosi MF. 2005. Innate immune responses to infection. The Journal of Allergy and Clinical Immunology. 116, 241-249.

CHAPTER 4: ANALYSIS OF GRAB SAMPLES AT VARIOUS STAGES OF SEWAGE TREATMENT FOR STEROID AND MICROBE CONTENT AND EFFECTS ON HUMORAL IMMUNITY DURING A SEWAGE TREATMENT PLANT UPGRADE

4.1 Abstract

Environmental water quality assessment has become an important process in protecting human and environmental health. This is because sewage treatment works (STWs) discharge pollutants like EDCs (endocrine disrupting chemicals/compounds) into the environment due to their incomplete removal. Natural steroids are an important class of EDC due to their potency, and are excreted by humans in both a free and a conjugated form. Faecal bacteria may deconjugate steroid-conjugates to produce more free steroids. Presence of total coliforms and *Escherichia coli* are standard bioindicators for human faecal contamination, and are often used in water quality assessment. A newer approach to water quality assessment is the determination of net physiological effects (e.g humoral immune effects) of water. Therefore, this investigation aimed to determine the quality of wastewater throughout the Stellenbosch STW during an upgrading procedure. Concentrations of five steroids (testosterone, progesterone, estrone, estradiol and ethinyl estradiol) were assayed, as well as the presence of total coliforms and *E. coli* and the effects of wastewater on humoral immunity.

Grab samples were collected after primary treatment, after biofilter treatment, after activated sludge treatment and just prior to environmental discharge. Total coliforms and *E. coli* presence was assessed using a chromogenic medium. Water

also underwent solid phase extraction before ELISAs (enzyme-linked immunosorbent assays) were performed for each of the steroids assayed. Humoral immune effects were determined using hybridoma cells and assessing their antibody secretion by ELISA.

Influent steroid concentrations varied greatly between sampling days, and were higher than in other STWs. Influent concentrations were 199.7, 209.8, 166.1, 143.3, and 63.9 pg/mL (pg: pictograms; L: litre) for testosterone, progesterone, estrone, 17 β-estradiol, 17 α-ethinyl estradiol, respectively. The STW significantly reduced the concentration of each steroid, and the activated sludge treatment was the most effective at removing all five steroids. The Stellenbosch STW reduced testosterone, progesterone, E1, E2 and EE2 concentrations by 96.2%, 95.1%, 55.1%, 78.5% and 87.4%, respectively. Nonetheless, steroids were still being discharged into the environment.

The bacterial quality of all influent and most samples exceeded 1000CFU/mL (CFU: colony forming unit; mL: millilitre) for both total coliforms and *E. coli*, which is of poor quality. On the first collection date the effluent quality was better though (<1CFU/mL for both total coliforms and *E. coli*).

Furthermore, neither the influent nor effluent samples showed any significant effects on humoral activity. Cytotoxicity was assessed in another study and also shows no cytotoxicity for either influent or effluent. Studies on the effects of these samples on other immune pathways are recommended, as well as *in vivo* studies, to provide more conclusive information on immunotoxicity.

4.2 Introduction

The quality of environmental water is one of the major concerns of the 21st century because many water bodies are contaminated with arrays of pollutants (Gómez *et al*, 2011; Hoai *et al*, 2011; Sakan *et al*, 2011; Xu *et al*, 2011; Chase *et al*, 2012; Kosjek *et al*, 2012). Various physiological systems, including the immune system, were studied using different species around the world, and revealed adverse health effects of exposure to water pollution (Khalaf *et al*, 2009). Pollutants often enter the environment though sewage treatment works (STWs), due to inefficient treatment of their vastly complex influents (Drinan and Whiting, 2001; Yu *et al*, 2006; Díaz-Cruz *et al*, 2009; Xu *et al*, 2011). Therefore, effluent quality monitoring is becoming an increasingly important procedure in assuring the health of communities.

One of the important classes of pollutants is the endocrine disrupting compounds/chemicals (EDCs), which have the ability to interact with the endocrine systems of vertebrates (Eertmans *et al*, 2003). Natural steroids are some of the more prominent types of EDCs, since they tend to be more potent than synthetic EDCs (Eertmans *et al*, 2003; Schwarzenbach *et al*, 2010). The steroids enter the environment via mammalian excrement. Due to their hydrophobicity, steroids undergo Phase II metabolism to produce steroid conjugates that are easier to excrete (Daughton and Ternes, 1999). These conjugated steroids may well be an important source of free steroids in the environment, since bacteria such as those found in wastewater and STWs, can deconjugate them; thus, making these steroids active EDCs (Esperanza *et al*, 2007).

An important parameter in water quality assessment is the evaluation of pathogenic microorganisms in water. Due to challenges in quantifying individual pathogens in water, scientists instead use indicator organisms, such as total coliforms (TC) and *Escherichia coli* to monitor water quality (Drinan and Whiting, 2001). *Escherichia coli* and TC are bacteria that signify water contamination by human faeces. Testing for indicator organisms has become a standard for water quality assessment, and many governments have set water quality standards for these parameters (Drinan and Whiting, 2001; UNGEMS/Water Programme, 2008).

More recently, scientists have also begun assessing the net physiological effects of water samples. This is because research has shown that mixtures of pollutants sometimes interact synergistically with one another (Jobling *et al*, 2009; Khalaf *et al*, 2009; Bellingham *et al*, 2012). This means that we presently cannot predict the exact response polluted water would cause in a living organism. Biological assays are currently the only means of assessing the net physiological effects of water pollution. These assays have proven very useful as they produce data that is more relevant to human health than pollutant quantification studies do.

This investigation, aims to determine the quality of the Stellenbosch STW during an upgrade procedure. Water samples were collected on four different dates. On each sampling day, water was collected after the primary treatment, after the trickling filter treatment, after the activated sludge treatments and just prior to discharge in the environment. The water quality parameters assessed were presence of total coliforms and *E. coli*, the concentrations of five steroids

(testosterone, progesterone, estrone, 17 β-estradiol and 17 α-ethinyl estradiol) and effects on humoral immunity.

4.3 Materials and Methods

4.3.1 Overview of Stellenbosch's sewage treatment works layout

Wastewater entering the STW is passed through a bar screen to remove large debris that may damage the machinery within the STW. Thereafter the water enters a vortex, followed by a primary settling tank. The secondary treatment follows, with water being passed through a trickling filter (biofilter), followed by an aerobic basin and a secondary settling tank (activated sludge). Once the secondary treatment is complete, the wastewater is split up. Some of the water is chlorinated before entering a pond (Pond 1), the rest of the water enters a different (larger) pond, without chlorination (Pond 2). Pond 1 has a retention time of 16 hours, and Pond 2's retention time is 25 hours. Thereafter, the water is joined once more and discharged into the Veldwagters River.

4.3.2 Water Collection

Water samples were collected in two litre pre-cleaned glass bottles. The bottles were first cleaned with sodium hypochlorite, and then soap. The bottles were then thoroughly rinsed out with tap water, followed by a distilled water rinse. Thereafter the bottles were rinsed out with ethanol, inverted, and allowed to dry overnight. After this procedure, each of the bottles were sterilised by autoclaving.

Grab samples were collected from different locations within the sewage treatment works. These samples came from the primary settling tank, the trickling filter, the aerobic basin and the final effluent (just before being discharged into the Veldwagters River). The water samples were immediately transported to the

laboratory for the solid phase extraction and bacterial assays; the remaining water was stored at -80°C (°C: degrees Celsius) until needed.

4.3.3 Bacteriology Assay

A one millilitre (mL) aliquot from each water sample was used to perform an *E. coli* and total coliforms test. A serial dilution of each sample was prepared in pre-made ReadyCult® Coliforms medium (Merck, Germany). This was allowed to incubate overnight at 37°C. After incubation, the colour of the medium was recorded, both under white and UV (ultraviolet) light. A colour change to blue-green, under white light, indicated the presence of at least one colony forming unit (CFU) of total coliforms in the sample. A blue fluorescence of the sample, under UV light, indicated the presence of at least one *E. coli* CFU in the sample.

4.3.4 Solid Phase Extraction

SP-18 columns were used to prepare hydrophobic extracts of the water samples collected, as previously described (Hendricks, 2011). In brief, the columns were prepared by passing two millilitres of methanol through each column, followed by 2mL phase B, 2mL of ethanol and another 2mL of ethanol. Care was taken not to let the columns dry out during this entire procedure. Phase B consists of 40% Hexane, 45% Methanol and 15% Propanol, by volume. Thereafter, each tube was filled to the top (approximately 7mL) with distilled water, and allowed to run through completely.

All water samples were centrifuged for five minutes at 2473Xg, to remove all large debris from the samples. The supernatant was passed through the prepared

SP-18 columns to commence the hydrophobic extraction. After the water sample had passed through the SP-18 column, the column was dried under suction (-50kPa) for 60 minutes. At this point, the columns could be stored at room temperature. Just prior to elution, the columns were again dried using a vacuum pump at -50kPa.

For elution of hydrophobic substances, 2X one millilitre of Phase B was passed through each column and collected in labelled glass vials. The eluates were then dried under a gentle air current. The dried eluate was reconstituted in dimethyl sulfoxide (DMSO) to produce a 1000X concentrated extract of the water samples. These hydrophobic extracts were stored at 5°C until the steroid analysis was performed.

4.3.5 Hormone Analysis

Hormone analysis was performed on the hydrophobic water extracts by use of commercially available enzyme-linked immunosorbent assays (ELISAs) for each of the steroids tested (testosterone, progesterone, estrone, estradiol and ethinyl-estradiol) (DRG Instruments GmbH, Germany).

All hydrophobic extracts were allowed to thaw and were vortexed before being diluted in 1X wash buffer. The remainder of the ELISA procedures were done according to the manufacturer's instructions.

Briefly, the control, standards and diluted sample extracts were dispensed into the given microtiter wells. The enzyme conjugate was then added and this was incubated. Thereafter, the plates were washed and dried before the substrate solution was added. This was incubated and then the enzymatic reaction was

stopped (adding stop solution). The absorbance was read in a spectrophotometer at 450nm (nm: nanometres). The standards were used to construct a standard curve and the concentration of steroid hormone in the sample was calculated using the standard curve.

4.3.6 Humoral Immunity Assessment

A hybridoma cell line was used to assess the effect of Stellenbosch STW water samples, collected during an upgrade procedure, on humoral immunity. The hybridoma cells produce monoclonal antibodies against lipoprotein lipase. Wastewater samples were tested for their effects on hybridoma antibody synthesis, which was quantified by ELISA.

4.3.6.1 Tissue Culture

The hybridoma cell line ($4C_2E_3H_{11}G_5$) secreting antibodies against lipoprotein lipase (LPL) was maintained in a 10% conditioned Full Medium. By volume, conditioned Full Medium consisted of 0.25% sodium pyruvate (Sigma, United Kingdom: UK), 0.5% gentamycin (Sigma, United States of America: USA), 1% antibiotic-antimycotic (Sigma, Germany), 1% glutamax (Gibco, UK), 10% conditioned medium and 87.25% Ex-Cell Medium (Sigma-Aldrich, USA). The conditioned full medium was sterile filtered before use. The hybridoma cells were also maintained at 37°C under 5% carbon dioxide (CO_2) until needed, or split.

Under sterile conditions, the tissue culture flask containing the hybridoma cells was tapped to dislodge cells. The bottom surface of the flask was also rinsed a few times (with the medium already in the flask) after the tapping, to further aid cell

dislodging. The cell suspension was centrifuged at 155Xg for five minutes and the supernatant was decanted. The cell pellet was resuspended in a small volume of fresh 10% conditioned Full Medium and counted. The cell suspension concentration was adjusted as required to allow a final concentration of 1000 cells per well in the 96 well, flat bottomed, tissue culture plate (Falcon USA).

Before adding the water samples to the 96 well plate, each water sample was thawed and sterile filtered using a 0.45µm (µm: micrometer) filter (once sterile filtered, the water samples were stored at -20°C). By volume each well received 10% sterile-filtered water sample and 90% hybridoma cell suspension in Full Medium (except the dilution curve wells). The tissue culture plate was incubated overnight at 37°C and under 5% CO_2. After the incubation period, 50-100µL (µL: microlitre) of the supernatant from each well was removed, under sterile conditions, and placed in a sterile storage plate. The storage plate was stored at -20°C until needed. To assess the effects of the water samples on the cell line's ability to produce/secrete antibodies, an ELISA for LPL was conducted on the culture supernatants.

4.3.6.2 Enzyme-linked immunosorbent assay for lipoprotein lipase

A clean 96 well Maxisorp® ELISA plate (Nunc, Denmark) was first coated with 50µL of 1µg/mL (µg: microgram; mL: millilitre) LPL (Southern Biotech, USA) in phosphate buffered saline (PBS) (Lonza, Belgium). This was incubated at room temperature for two hours on a plate shaker at 450 revolutions per minute (rpm). Thereafter the plate was decanted and tapped dry onto absorbent paper. Once coated like this, the plate could be stored at -20°C until needed. If the coated plate

was stored, it would first be thawed for a minute or two before proceeding to the next step.

After coating the plate, 200µL of a block solution was added to each well. The block solution was made up of 1% human serum albumin (Western Cape Regional Blood Bank, South Africa) in PBS. The plates were then incubated at room temperature for one hour on a plate shaker at 450rpm. While the plate was being blocked, the supernatants to be assayed were prepared. The supernatants from the hybridoma experiment were diluted to 1/100 in PBS containing human serum albumin and 0.01% Tween 20.

After the 60 minute blocking of the ELISA plate, the plate was decanted, washed thrice in wash buffer (composed of 0.01% Tween 20 in PBS), and tapped dried onto absorbent paper. Next, 50µL of the 1/100 diluted hybridoma culture supernatants was added to their pre-assigned wells. This was incubated for one hour at room temperature on a plate shaker at 450rpm.

After that the plate was again decanted, washed thrice in wash buffer and tapped dried onto absorbent paper. Thereafter 50µL of an enzyme-conjugate solution was added to each well. The enzyme-conjugate used was a rabbit anti-mouse IgG-HRP (Southern Biotech, USA), and was diluted 1/5000 in the diluted block solution as was used earlier to dilute the cell line supernatants. The plates were then incubated at room temperature for one hour on a plate shaker at 450rpm.

The plate was then decanted and washed seven times as before. Then 100µL of pre-warmed (to 37°C) substrate solution was placed into each well. The substrate used was TMB microwell peroxidise substrate (Sure Blue) (KPL, USA). The plates were placed in the dark at room temperature for 15 minutes, before adding

50µL of 0.5M (M: Molar) H_2SO_4 (sulphuric acid) as the stop solution. Finally the plate absorbance was read at 450nm.

4.3.7 Statistical Analysis

Statistical Analysis was performed using the SigmaPlot program (Version 12.0). One way analysis of variance (ANOVA) was carried out on data and a $P<0.05$ (P: Probability) was considered significant.

4.4 Results and Discussion

During 2012, the Stellenbosch Sewage Treatment Works (STW) initiated an upgrade that caused the STW management to temporarily shut down portions of the plant. The change in STW operations were likely to affect the quality of effluent that was released into the environment. Due to the potential for adverse effects on ecosystems in the area, the quality of the STW waters during the upgrade process was determined.

The effluents of different sewage treatment processes from the Stellenbosch STW were analysed for estrone (E1), 17 β-estradiol (E2), 17 α-ethinyl estradiol (EE2), progesterone and testosterone concentrations on four different occasions (18 May, 20 June, 26 June and 10 July, 2012). This was achieved by employing an enzyme-linked immunosorbent assay (ELISA) for each steroid. The STW effluents that were assessed came from the grit chamber (labelled as influent), the trickling filter (biofilter), the aerobic basin (activated sludge) and the final effluent (from the ponds and chlorination).

4.4.1 Validation of steroid assays

To assess water quality, one can either assay the presence of known pollutants or determine affects of the water on a living tissue and/or organism. ELISAs are fast and easy to perform quantitative assays and are also fairly sensitive. ELISAs were successfully used in other studies to determine steroid levels in sewage effluents from the Western Cape, South Africa (Swart, 2008; Hendricks, 2011). Therefore, ELISAs were chosen as the quantization assay for this research project.

To determine pollution of wastewaters, this investigation evaluated the concentrations of five steroids, namely testosterone, progesterone, estrone, estradiol and ethinyl-estradiol. Steroids were assessed, because they are an important class of EDC known to be discharged by STWs (Camacho-Muñoz *et al*, 2010). The following section validates the steroid ELISAs

4.4.1.1 Testosterone enzyme-linked immunosorbent assay

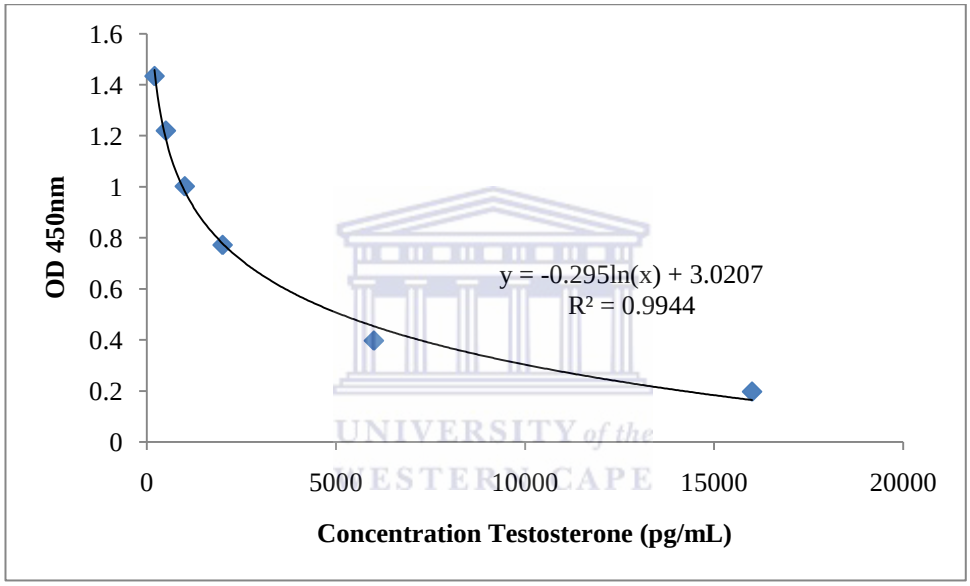

Figure 4.1. A Standard Curve for quantization of testosterone in Stellenbosch STW samples.

Water samples from the Stellenbosch STW were assayed for testosterone using an ELISA. The water samples were first subject to SP-18 solid phase extraction and resuspended in DMSO. Thus the concentrated hydrophobic extracts were diluted in wash buffer, before performing the ELISA according to the manufacturer's instructions. There is a strong correlation between the optical density and testosterone concentration (R^2=0.9944). Recovery of a sample with known

testosterone concentration using the current ELISA is 101±1%. Therefore, this ELISA appears to be reliable for determining testosterone concentration in the sewage samples used.

4.4.1.2 Progesterone enzyme-linked immunosorbent assay

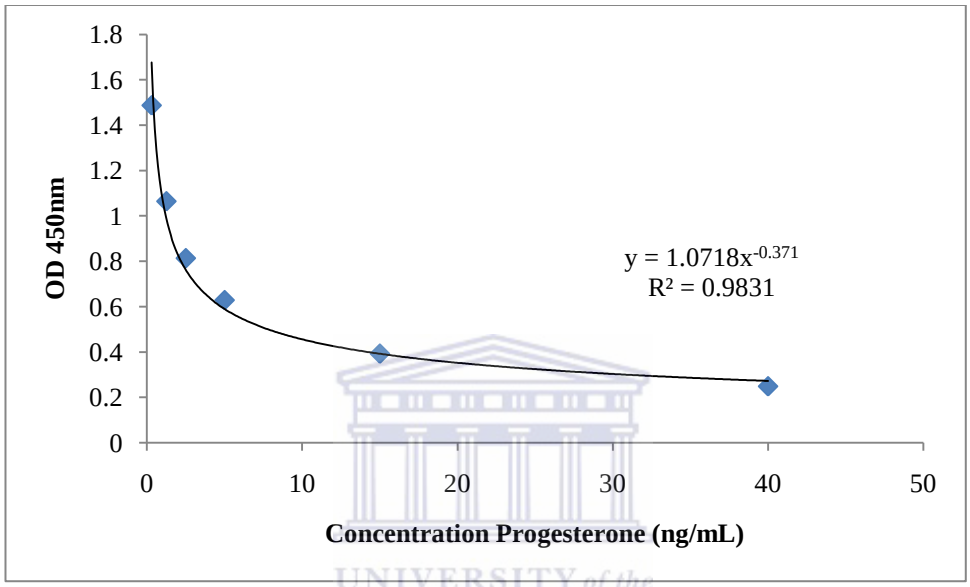

Figure 4.2. Standard Curve for quantization of progesterone in Stellenbosch STW samples.

Water samples from the Stellenbosch STW were assayed for progesterone concentration via an ELISA. The water samples were first subject to SP-18 solid phase extraction and resuspended in DMSO. Thus the concentrated hydrophobic extracts were diluted in wash buffer, before performing the ELISA according to the manufacturer's instructions. There is a strong correlation between the optical density and progesterone concentration (R^2=0.9831). Recovery of a sample with known progesterone concentration using the current ELISA is 107±15%.

Therefore, this ELISA appears to be reliable for determining progesterone concentration in the sewage samples used.

4.4.1.3 Estrone enzyme-linked immunosorbent assay

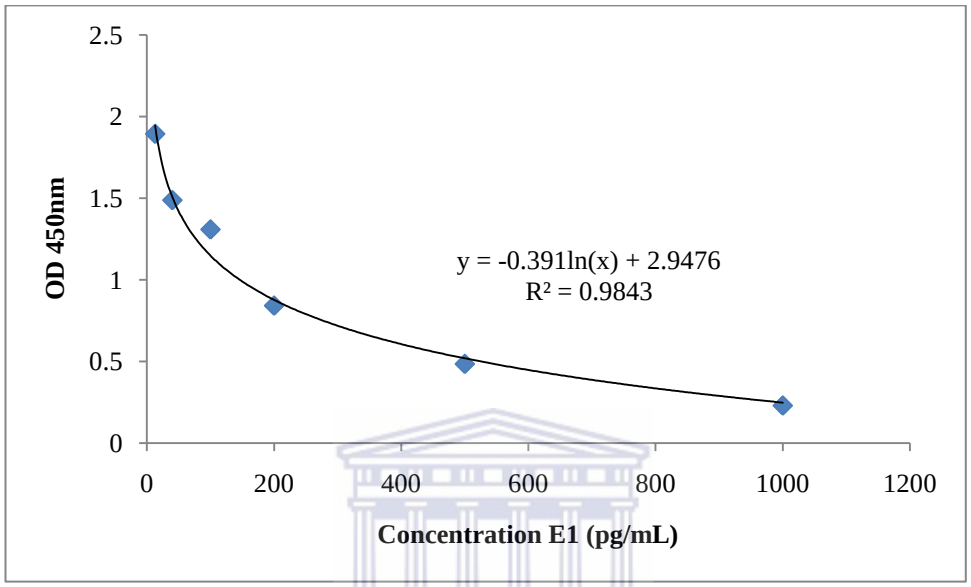

Figure 4.3. A Standard Curve for quantization of estrone in Stellenbosch STW samples.

Water samples from the Stellenbosch STW were assayed for estrone concentration via an ELISA. The water samples were first subject to SP-18 solid phase extraction and resuspended in DMSO. Thus the concentrated hydrophobic extracts were diluted in wash buffer, before performing the ELISA according to the manufacturer's instructions. There is a strong correlation between the optical density and estrone concentration (R^2=0.9843). Recovery of a sample with known estrone concentration using the current ELISA is 109±9%. Therefore, this ELISA appears to be reliable for determining estrone concentration in the sewage samples used.

4.4.1.4 17 β-estradiol enzyme-linked immunosorbent assay

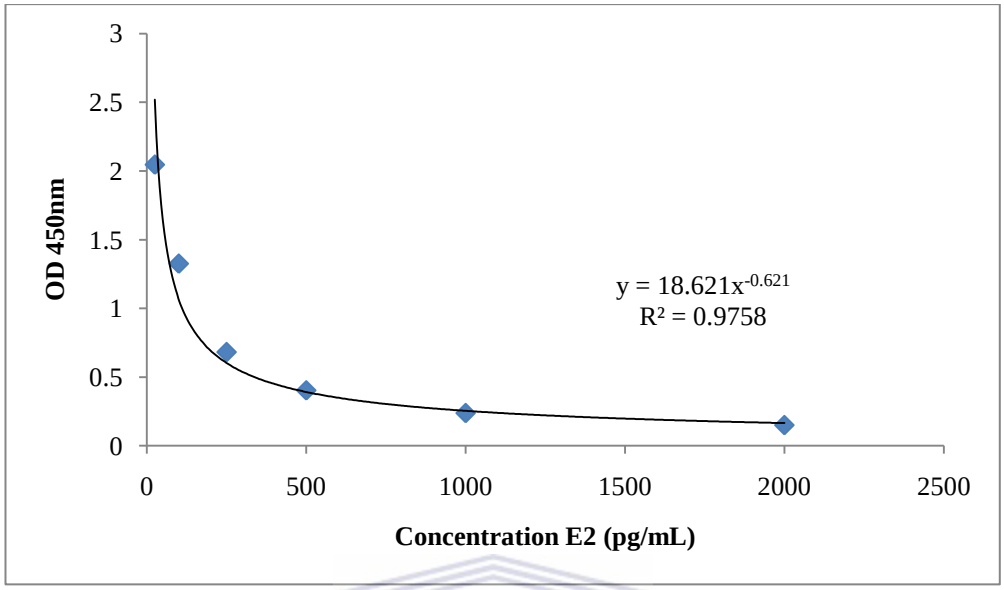

Figure 4.4. Standard Curve for quantization of 17 β-estradiol in Stellenbosch STW samples.

Water samples from the Stellenbosch STW were assayed for 17 β-estradiol concentration via an ELISA. The water samples were first subject to SP-18 solid phase extraction and resuspended in DMSO. Thus the concentrated hydrophobic extracts were diluted in wash buffer, before performing the ELISA according to the manufacturer's instructions. There is a strong correlation between the optical density and 17 β-estradiol concentration (R^2=0.9758). Recovery of a sample with known estradiol concentration using the current ELISA is 95±0.4%. Therefore, this ELISA appears to be reliable for determining 17 β-estradiol concentration in the sewage samples used.

4.4.1.5 17 α-ethinyl estradiol enzyme-linked immunosorbent assay

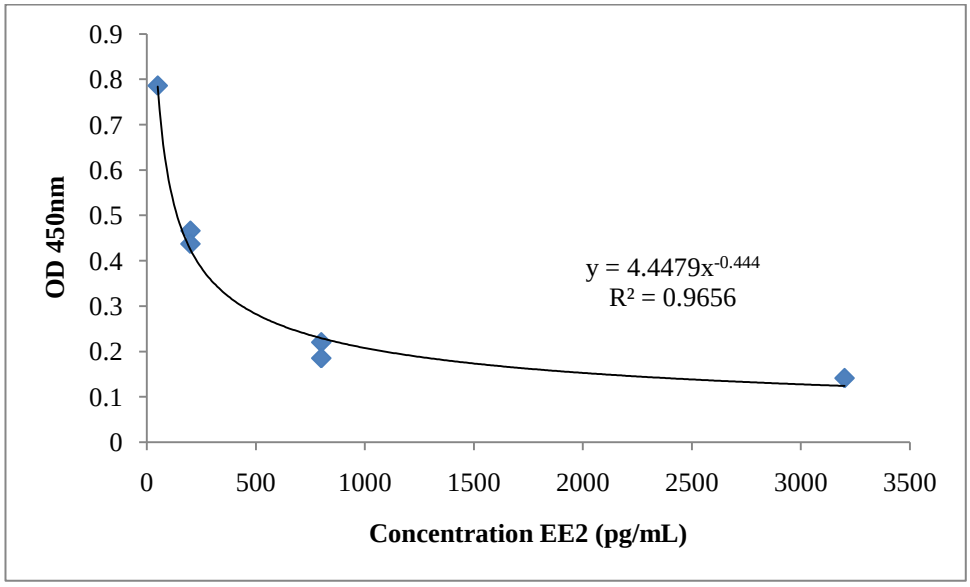

Figure 4.5. Standard Curve for quantization of 17 α-ethinyl estradiol in Stellenbosch STW samples.

Water samples from the Stellenbosch STW were assayed for 17 α-ethinyl estradiol concentration via an ELISA. The water samples were first subject to SP-18 solid phase extraction and resuspended in DMSO. Thus the concentrated hydrophobic extracts were diluted in wash buffer, before performing the ELISA according to the manufacturer's instructions. There is a relatively strong correlation between the optical density and 17 α-ethinyl estradiol concentration (R^2=0.9656). Recovery of a sample with known ethinyl estradiol concentration using the current ELISA is 90±18%. Therefore, this ELISA appears to be reliable for determining 17 α-ethinyl estradiol concentration in the sewage samples used.

4.4.1.6 Conclusion of Steroid Assay Validation

In order to assess environmental water pollution, it is important that the main sources of pollution be monitored, which are STWs (Musolff *et al*, 2009). When an STW operates at sub-optimal operations (such as during an upgrade), it becomes increasingly important to determine the quality of its treated waters. STW effluents have been known to possess EDCs for many years (Folmar *et al*, 1996). Therefore quantifying steroids in STW effluent is an important step in evaluating the human impact on the biosphere.

The data obtained from standard curves for each assay shows that these assays can effectively reveal steroid levels in sewage samples.

4.4.2 Testosterone

The influent testosterone concentrations ranged from 123.3 pg/mL (pg: picograms) to 317.1 pg/mL; with a mean of 199.7 pg/mL across the sampling period (refer to **Figure 4.6** and **Table 4.1**). These levels were similar to wastewater from three other Western Cape STWs (Hendricks, 2011), but were considerably higher than at Chinese STWs (Chang *et al*, 2011; Liu *et al*, 2011). The Chinese STWs' influent testosterone concentrations were below 100ng/L (Chang *et al*, 2011; Liu *et al*, 2011). The Stellenbosch mean influent testosterone concentration was also significantly higher than the negative control ($P<0.05$). However, influent testosterone concentration at Stellenbosch was lower than at the Darvill wastewater treatment works (WWTW) (KwaZulu-Natal, South Africa), which showed mean levels of 343ng/L (ng: nanograms; L: litre) across their sampling period (Manickum and John, 2014).

After the biofilter treatment, the testosterone levels dropped significantly ($P<0.05$) to a mean of 114.78 pg/mL (42.52% removal). The effluent from the aerobic basin (activated sludge) showed an average testosterone concentration of 7.16 pg/mL; thus reducing testosterone concentration by 93.76%. The activated sludge treatment was, therefore, the best treatment at reducing testosterone concentration. The final effluent showed no significant difference to the activated sludge effluent, with testosterone levels of 6.87-10.69 pg/mL. The large reduction in testosterone concentration was consistent with that of Chinese STWs (Chang *et al*, 2011), and other South African STWs (Hendricks, 2011; Manickum and John, 2014) which also report approximately 96% reduction in testosterone concentration. Testosterone removal was efficient enough to produce no significant difference between either the activated sludge effluent or the final effluent and distilled water. Nonetheless, low levels of testosterone were still being released into the environment due to an incomplete testosterone removal. According to Chang *et al* (2011), the primary process for testosterone and progesterone removal appears to be biodegradation. However, studies on testosterone removal and fate are scarce, making this study an important contribution to the occurrence of androgens in the environment.

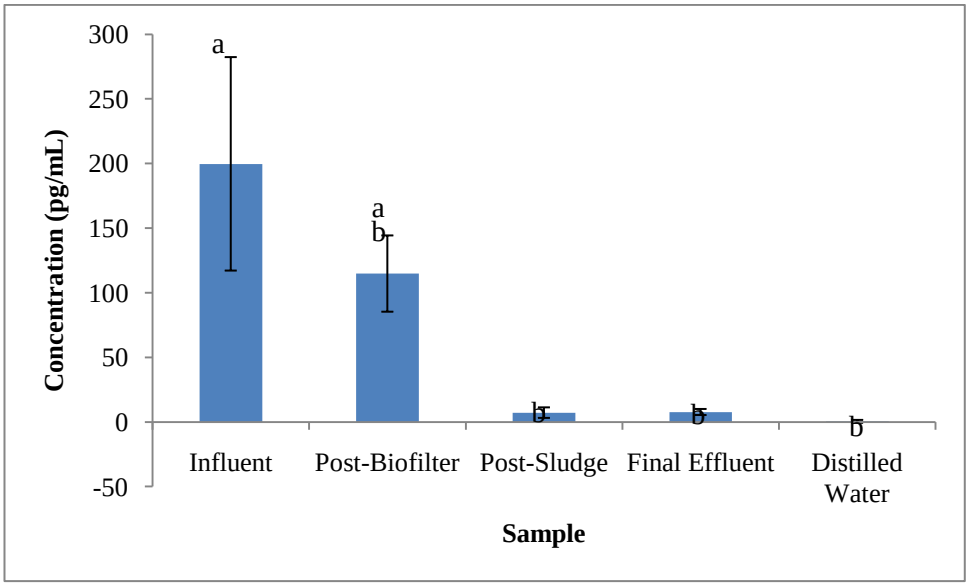

Figure 4.6. Mean concentrations of Testosterone throughout Stellenbosch STW for the four collection dates. a: significant difference compared to the Distilled water sample (P<0.05); b: significant difference compared to the Influent sample (P<0.05).

4.4.3 Progesterone

The mean influent progesterone concentration was 209.8 pg/mL (refer to **Figure 4.7** and **Table 4.1**); which was higher than those of Chinese STWs, where Liu *et al* (2011) found 6.1ng/L and Chang *et al* (2011) found 35-108ng/L progesterone in their influents. However, Stellenbosch influent progesterone concentration was lower than at Darvill WWTW, were it ranged from 163-904ng/L across their sampling period (Manickum and John, 2014). Nonetheless, progesterone concentration in the Stellenbosch influent was significantly higher (P<0.05) than the negative control.

After the biofilter treatment, the progesterone concentration showed a significant increase (P<0.05) to 414.67 pg/mL; almost a 100% increase. Much variation in

the biofilter effluent's progesterone concentration was noted between sampling days. This was likely due to weather conditions amongst other factors. The increase in progesterone concentration may have resulted from bacterial deconjugation of progesterone glucuronide and sulphate conjugates from phase II metabolism. Phase II metabolism of progesterone is virtually the same as for other natural steroids (Greaves *et al*, 2014); and according to D'Ascenzo *et al* (2003), glucuronide-estrogen conjugates are readily deconjugated by sewage bacteria (*E. coli*). Glucuronide-progesterone conjugates also appear to be the predominant form of the steroid in human urine (Meng *et al*, 1997); therefore the bacteria that deconjugate glucuronide-estrogen conjugates are likely to produce the same effect for progesterone-conjugates.

Nonetheless, the activated sludge treatment produced a significant decrease ($P<0.05$) in the progesterone levels to an average of 9.23 pg/mL, which was statistically no different to the final effluent. These results were slightly higher than that of Chang *et al* (2011), but were similar to the Darvill WWTW (Manickum and John, 2014). The Stellenbosch STW produced a progesterone removal of 95.11%, likely by biodegradation (Chang *et al*, 2011). The final effluent progesterone level was not significantly different to distilled water, but still contained progesterone. Like testosterone, studies on progesterone removal and fate are scarce, making this a valuable contribution to our understanding of steroids in the environment.

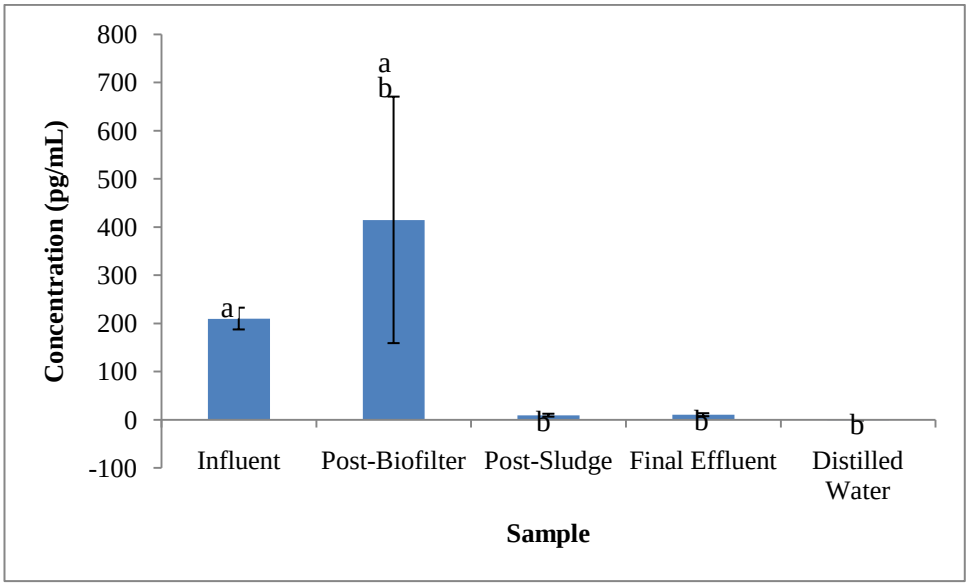

Figure 4.7. Mean concentrations of Progesterone throughout Stellenbosch STW for the four collection dates. a: significant difference compared to the Distilled water sample (P<0.05); b: significant difference compared to the Influent sample (P<0.05).

4.4.4 Estrone

Estrone concentrations showed a lot of variation between sampling days (refer to **Figure 4.8** and **Table 4.1**). The range of the influent estrone levels was 76.56 pg/mL to 362 pg/mL (mean of 166.77pg/mL), which was higher than in other STWs around the world (D'Ascenzo *et al*, 2003; Chang *et al*, 2011; Kumar *et al*, 2011; Limpiyakorn *et al*, 2011; Liu *et al*, 2011; Atkinson *et al*, 2012; Zhou *et al*, 2012a; Manickum and John, 2014). A review of estrogens in STWs found a mean E1 concentration of approximately 47ng/L, which is considerably lower than the Stellenbosch samples (Limpiyakorn *et al*, 2011). According to D'Ascenzo *et al* (2003), human estrogen excretion levels vary with sex, age and gestational status. Therefore differences in population makeup and practices could contribute to the

high E1 levels detected at Stellenbosch compared to other STWs. This is partially confirmed by Hendricks (2011), where three other Western Cape STWs were analysed for steroids, and revealed similar influent E1 levels. The exact origin of wastewaters for the Stellenbosch STW influent at the sampling period is unknown, though, so the specific reason for high E1 concentration cannot be confirmed.

The mean estrone levels after the biofilter treatment was 144.41 pg/mL. Although there was an 11.8% reduction in mean estrone levels at this step, no significant difference was noted compared to the influent. Another reduction in estrone concentration was noted after the activated sludge treatment. However, this showed no statistically significant difference compared to the biofilter effluent, despite showing a 51.2% mean reduction in E1 levels. The range for E1 concentration throughout the STW was substantial across the sampling period (eg. E1 range after activated sludge treatment was: 14.88pg/mL-114.87pg/mL), which likely caused the lack of statistical support to the E1 reduction. Nonetheless, the estrone concentration of the activated sludge effluent was significantly lower ($P<0.05$) than that of the influent. The final effluent possessed an average estrone concentration of 73.33 pg/mL. Although this was high compared to some other studies (D'Ascenzo *et al*, 2003; Swart, 2008; Chang *et al*, 2011; Hendricks, 2011; Kumar *et al*, 2011; Liu *et al*, 2011; Zhou *et al*, 2012b; Manickum and John, 2014), Zhou *et al* (2012a) detected up to 74.2ng/L of estrone in their effluent sample. Another Western Cape STW showed even higher average E1 effluent concentration (149pg/mL) than Stellenbosch did in this study (Hendricks, 2011).

The entire STW produced a 55.22% reduction in the estrone levels, which was the lowest reduction for all five steroids assayed. According to Limpiyakorn *et al* (2011), it is not uncommon for E1 removal at STWs to be lower than other natural estrogens. However, the Stellenbosch STW produced a reduction in E1 concentration that was almost 14 percentiles below average (Limpiyakorn *et al*, 2011). The final effluent estrone concentration was significantly greater ($P<0.05$) than that of distilled water, which confirms the incomplete removal of E1.

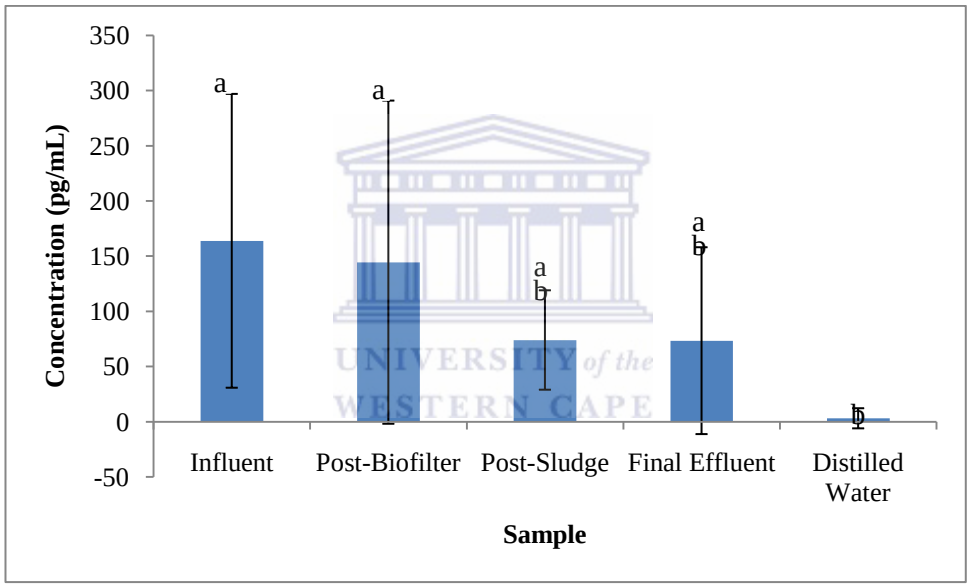

Figure 4.8. Mean concentrations of Estrone throughout Stellenbosch STW for the four collection dates. a: significant difference compared to the Distilled water sample ($P<0.05$); b: significant difference compared to the Influent sample ($P<0.05$).

4.4.5 Estradiol

The estradiol levels in the influent averaged at 143.27 pg/mL (refer to **Figure 4.9** and **Table 4.1**), which was significantly higher than the negative control ($P<0.05$).

The influent E2 concentration was also fairly high compared STWs in other countries (D'Ascenzo *et al*, 2003; Chang *et al*, 2011; Kumar *et al*, 2011; Limpiyakorn *et al*, 2011; Liu *et al*, 2011; Atkinson *et al*, 2012; Zhou *et al*, 2012a); however, it was similar to other South African STWs (Hendricks, 2011; Manickum and John, 2014). Estradiol concentration was significantly ($P<0.05$) decreased (35.69% reduction) to a concentration of 92.15 pg/mL, by the biofilter treatment. The greatest decrease in estradiol levels was noted after the activated sludge treatment; which lowered estradiol levels to 39.89 pg/mL, a significant reduction ($P<0.05$) of 72.16% of the influent levels. Detected estradiol concentrations in effluent (13.07pg/mL-54.94pg/mL) fall within the range reported in China (Zhou *et al*, 2012b) and South Africa (Hendricks, 2011; Manickum and John, 2014). The final effluent was significantly lower ($P<0.05$) than the influent, which indicates that the STW reduced E2 levels. The final effluent was not significantly different to the negative control. However, the entire STW removed only 78.46% of estradiol from the raw wastewater, which was lower than at other STWs (D'Ascenzo *et al*, 2003; Chang *et al*, 2011; Hendricks, 2011; Zhou *et al*, 2012a). The lack of statistical support to the incomplete removal of E2 could be attributed to differences between sampling dates (range).

According to Limpiyakorn *et al* (2011), E2 is primarily removed from wastewater by biodegradation, but sorption onto sludge is also an important mechanism for E2 removal. Estradiol removal at the Stellenbosch STW was similar to that of three other South African activated sludge STWs (Hendricks, 2011; Manickum and John, 2014), as well as other studies (Limpiyakorn *et al*, 2011). Nonetheless,

the Stellenbosch STW did not remove all E2 from wastewater, and may affect biota in receiving waters.

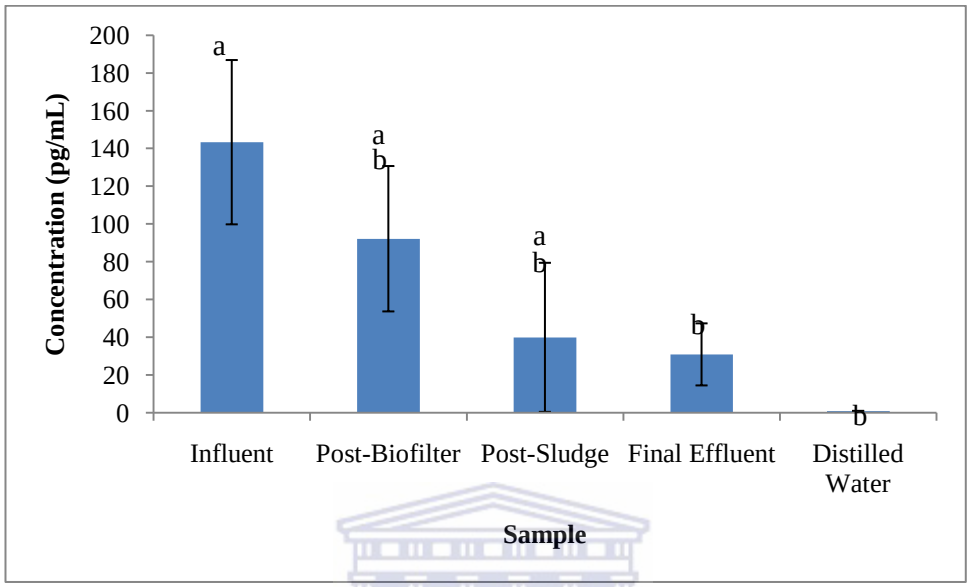

Figure 4.9. Mean concentrations of Estradiol throughout Stellenbosch STW for the four collection dates. a: significant difference compared to the Distilled water sample (P<0.05); b: significant difference compared to the Influent sample (P<0.05).

4.4.6 Ethinyl-estradiol

Across the sampling period, the EE2 influent levels fluctuated; ranging from 38.28 pg/mL to 103.78 pg/mL (refer to **Figure 4.10** and **Table 4.1**). This range was similar to that of Zhou *et al* (2012a), as well as Manickum and John (2014). Nonetheless, these EE2 levels remain higher than reports from other investigations (Kumar *et al*, 2011; Atkinson *et al*, 2012); and was significantly higher than the negative control (P<0.05). The biofilter caused a significant EE2 reduction to 53.16% of the influent levels (46.84% reduction). The activated

sludge reactor further reduced EE2 concentration to 14.2% of the influent concentration (9.07pg/mL). Therefore, the activated sludge treatment was the most efficient at reducing EE2 concentration. The final effluent (retaining 12.62% of the influent EE2 concentration) showed no significant difference compared to the activated sludge effluent. The removal of EE2 from wastewaters at Stellenbosch STW was similar to a Chinese STW (Zhou *et al*, 2012a), UK STWs (Kumar *et al*, 2011) and to another South African STW (Manickum and John, 2014). According to Limpiyakorn *et al* (2011), EE2 reduction at the Stellenbosch was above average for STWs in general, which is approximately 58%. The main mechanisms for EE2 removal at STWs are biodegradation and sorption, as with natural steroids (Zhou *et al*, 2012a). The activated sludge effluent and final effluent concentrations of EE2, were statistically no different to that of distilled water, likely due to high variability between sampling dates. Nonetheless, EE2 was still being discharged into the environment via effluents from the Stellenbosch STW.

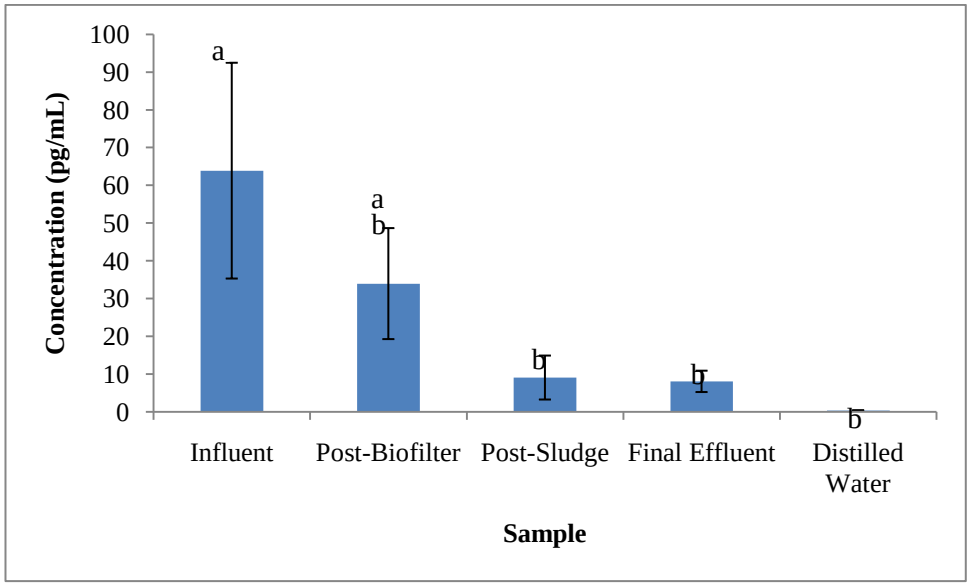

Figure 4.10. Mean concentrations of Ethinyl-estradiol throughout Stellenbosch STW for the four collection dates. a: significant difference compared to the Distilled water sample ($P<0.05$); b: significant difference compared to the Influent sample ($P<0.05$).

Table 4.1. Influent vs. effluent steroid concentrations for the four sampling days. E1: estrone; E2: 17 β-estradiol; EE2: 17 α-ethinyl estradiol; SD: standard deviation; pg: pictograms; mL: millilitre.

Steroid		Influent concentration	Effluent concentration	Percentage steroid remaining
E1	Mean	163.77pg/mL	73.33pg/mL	44.78%
	SD	133.07	84.62	21.36
E2	Mean	143.27 pg/mL	30.87 pg/mL	21.54%
	SD	43.52	16.44	14.86
EE2	Mean	63.87 pg/mL	8.06 pg/mL	12.62%
	SD	28.58	2.83	6.68
Progesterone	Mean	209.83 pg/mL	10.25 pg/mL	4.89%
	SD	22.62	3.13	1.65
Testosterone	Mean	199.70 pg/mL	7.67 pg/mL	3.84%
	SD	82.6	2.34	2.25

4.4.7 Bacteriology

Raw water samples were diluted (1/10, 1/100, 1/1000 and 1/10000) in the ReadyCult® coliforms medium and incubated overnight, to determine the presence of *Escherichia coli* and total coliforms in the water samples. A blue-green colour change in the medium under white light signalled the presence of total coliforms, whilst fluorescence under UV (ultraviolet) light signalled the presence of *E. coli*. According to the manufacturer, the detection limit of ReadyCult is one total coliforms or *E. coli* CFU/100mL water sample. This, however, was the detection limit before the dilution was made. The manufacturer's instructions required 100mL of water sample to be mixed with medium powder. In this experiment, only 1mL of the water sample was used, and it was diluted in sterile pre-made liquid medium. This means that a positive result for the 1/10 dilution now represents at least 1 CFU/mL water sample, a positive for the 1/100 dilution represents at least 10 CFU/mL, etc.

The influent and biofilter water samples tested positive for total coliforms and *E. coli* at all dilutions used and on all collection dates (refer to **Tables 4.2** and **4.3**). This means that the total coliforms and *E. coli* concentration in these samples were at least 1000 CFU/mL each. On 18 May, 20 June and 26 June the activated sludge water samples also had at least 1000 CFU/mL of total coliforms and *E. coli* (positive for both at all dilutions used); as well as the final effluents of 20 June, 26 June and 10 July. The final effluent of 18 May showed less than 1 CFU/mL *E. coli* and total coliforms. The activated sludge sample of 10 July showed a total coliforms concentration of 100-999 CFU/mL (positive at all dilutions, except the last). The *E. coli* analysis for this sample (10 June sludge) revealed a

concentration of 1-9 CFU/mL. A sterile distilled water sample was also tested on both 26 June and 10 July. Both of these samples showed less than one CFU of both total coliforms and *E. coli*.

According to the South African Department of Water Affairs and Forestry (1996), water with total coliform levels of 0-5 CFU/100mL poses negligible health risk to humans who occasionally consume it. Therefore most STW samples were all unacceptable for human consumption. The effluent from 18 May 2012 may have been satisfactory, since humans are unlikely to ever consume raw STW effluent. However, the *E. coli* concentration of 18 May was inconclusive with regards to human health risk. The South African Department of Water Affairs and Forestry (1996) states that water *E. coli* levels above 20 CFU/100mL poses a risk to human health. Thus all samples assayed (expect for the 18 May effluent) posed a risk to human health.

Table 4.2. Concentration of total coliforms at the different levels in Stellenbosch STW on each of the collection dates. cfu: colony forming units; mL: millilitre.

Total Coliforms	18-May	20-Jun	26-Jun	10-Jul
Influent	> 1000 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL
Post-Biofilter	> 1000 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL
Post-Sludge	> 1000 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL	100-999 cfu/mL
Effluent	< 1 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL
Distilled Water			< 1 cfu/mL	< 1 cfu/mL

Table 4.3. Concentration of *E. coli* at the different levels in Stellenbosch STW on each of the collection dates. cfu: colony forming units; mL: millilitre.

E. coli	18-May	20-Jun	26-Jun	10-Jul
Influent	> 1000 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL
Post-Biofilter	> 1000 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL
Post-Sludge	> 1000 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL	1-9 cfu/mL
Effluent	< 1 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL	> 1000 cfu/mL
Distilled Water			< 1 cfu/mL	< 1 cfu/mL

4.4.8 Humoral Immunity Assay

Sterile filtered influent and effluent samples from the Stellenbosch STW were assessed for its effects on humoral immunity. A reverse-osmosis (RO) water sample was also assayed as the negative control. An easy and quick humoral immunity assay was provided by a hybridoma cell line. The levels of antibody synthesized/secreted by the hybridoma cells, were measured to determine if the water samples shows any effects on humoral immunity. Antibody levels were determined by ELISA, and compared to the RO water sample (RO water was assumed not to affect antibody synthesis/secretion, and thus represented 100% antibody secretion).

4.4.8.1 Validation of Humoral Immunity Assay

When determining the effects of a sample on physiology, one can either use an *in vitro* assay or an *in vivo* assay. *In vitro* assays are very useful in studying physiology due to their similarities to the *in vivo* condition, low costs, speed of processing, and reduced ethical concerns (Taju *et al*, 2012). However, *in vitro* assays lack the (potential) interactions of multiple physiological systems that are necessary to draw convincing conclusions on physiological activity of a sample. Thus, *in vitro* studies are usually an initial investigation that must be confirmed *in vivo*. An *in vitro* assay would then be followed by an *in vivo* assay only if the results obtained are acceptable.

Studies have revealed that polluted water can affect the physiology of organisms exposed to it, including the immune system (Khalaf *et al*, 2009). Therefore, this investigation included an assessment on the effects of Stellenbosch STW samples

on humoral immunity, by determining antibody synthesis of hybridoma cells. The following section validates the humoral immunity assay used here.

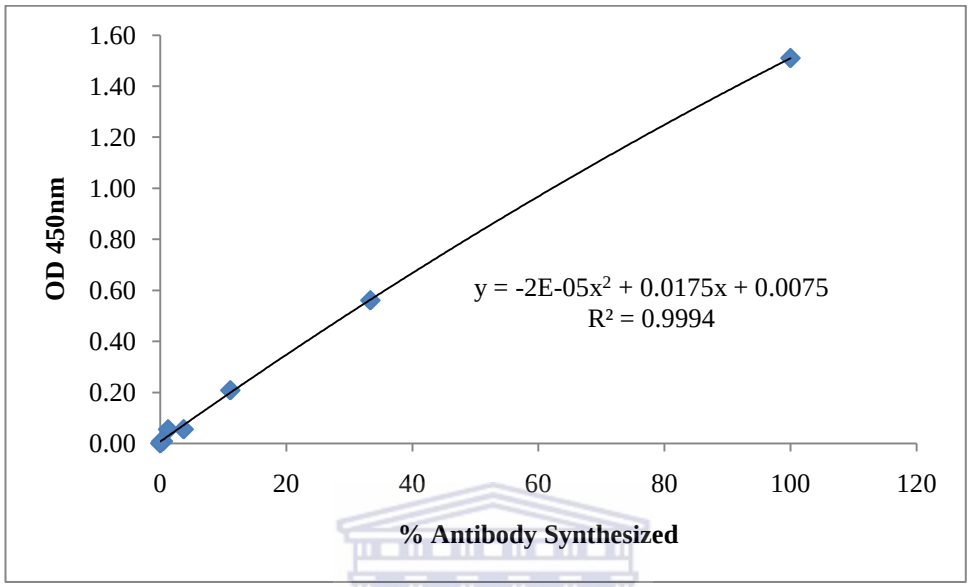

Figure 4.11. Standard Curve for assessment of antibody synthesis by hybridoma cells.

Supernatants from the water sample-exposed hybridoma cells were assayed for antibodies via an ELISA. The reverse osmosis water-treated cells were assumed to produce antibodies at 100%. A standard curve was generated by performing a serial dilution of the 100% antibody sample. A very good correlation (R^2=0.9994) was noted between the optical density achieved and the dilution factor. Recovery of a sample with known antibody concentration (% antibody synthesized) using the current ELISA is 99%. Therefore, the ELISA appears to be reliable for determining antibody concentration by the hybridoma cells used.

4.4.8.2 Humoral Immune Effects

No significant difference in antibody synthesis was noted between either the mean influent or effluent samples and the RO water sample (refer to **Figure 4.12**).

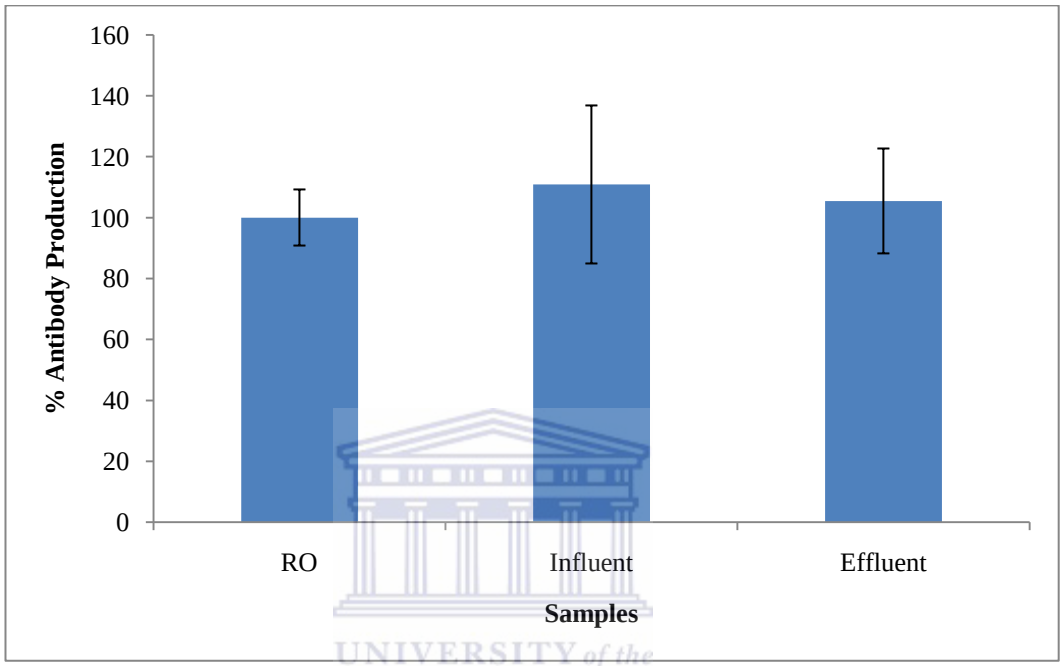

Figure 4.12. Mean (for all collection dates) percentage antibody produced by hybridoma cells during exposure to the water samples. RO: reverse osmosis water; Influent: primary treatment effluent (after sedimentation); Effluent: final effluent (juts prior to discharge). a: significant difference compared to RO water.

Due to the noted variability in the steroid levels from the treatments on different collection days, the influent and effluent samples of each collection date was also compared to one another (**Figure 4.13**). Once more, most samples showed no significantly different antibody concentrations compared to the RO water. The only exception was the Influent sample collected on 26 June 2012, which was

significantly higher (P<0.05). Therefore, the 26 June 2012 influent caused a stimulation of antibody production.

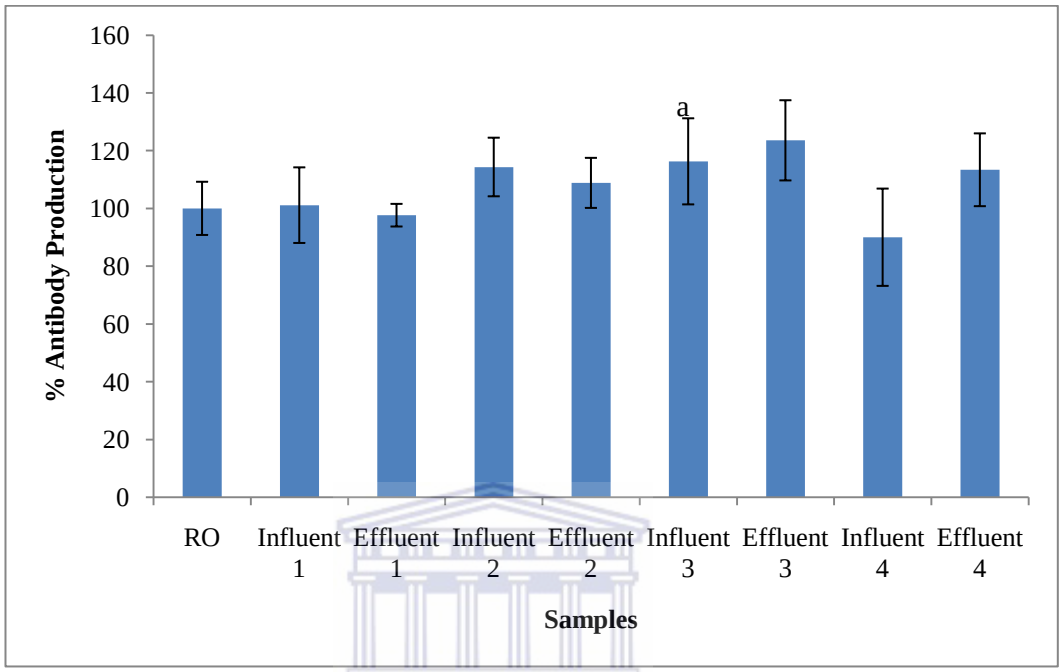

Figure 4.13. Percentage of antibody produced by water sample-exposed cells for each collection day. RO: reverse osmosis water; Tap: tap water; Influent: primary treatment effluent (after sedimentation); Effluent: final effluent (juts prior to discharge); Sample1: 18 May 2012; Sample 2: 20 June 2012; Sample 3: 26 June 2012; Sample 4: 10 July 2012. a: significant difference compared to RO water.

Taken together, the Stellenbosch STW influent and effluent samples appear not to suppress humoral immunity. In fact, the influent and effluent samples appear to stimulate antibody synthesis, although not significantly. According to Hendricks (2011), three other Western Cape STW influents and effluents also produced stimulation of the humoral immune system. Influents also showed higher stimulation than effluents and both were higher than the negative control

(Hendricks, 2011). In that study, though, the differences in stimulatory effects of the samples were statistically significant. This implies that the Stellenbosch STW samples were less likely to affect humoral immunity than the STWs evaluated by Hendricks (2011). However, Hendricks (2011) used a whole blood culture (WBC) instead of hybridoma cells, and that assay may have been more sensitive. During WBC, human whole blood is co-incubated with a (water) sample. Thereafter, the levels of specific cytokines are measured to determine which immune pathways are affected. According to Pool *et al* (2000), a whole blood culture can simulate an animal model when it comes to inflammation. In addition, Pool *et al* (2000) have also mentioned that the WBC is very sensitive to inflammatory agents (more sensitive than *in vivo* assays). Therefore, it is also likely that the WBC is more sensitive to humoral activity than a monoclonal hybridoma cell line. Hybridoma cells can produce antibodies without stimulation, unlike cytokine secretion by whole blood, which needs stimulation. Thus, the differences between stimulated and unstimulated cultures may be more pronounced in whole blood than hybridoma cells, making WBC more sensitive. Antibody synthesis/secretion might also be a slower process than cytokine secretion, which would require the hybridoma cells to have longer incubation times. Furthermore, the hybridoma cells were cultured in a conditioned medium, which may have already provided some stimulation to the hybridoma cells. Perhaps an unconditioned medium should be used instead.

In order to confirm that the water samples were immunotoxic and not simply cytotoxic, a cytotoxicity assay is usually included in immune modulation studies.

A cytotoxicity assay was not included in this study, but was performed using the same samples in another investigation (Lategan, 2014, unpublished). According to Lategan (2014, unpublished), the influent and effluent water samples were statistically no different to either one another or the negative control (distilled water). This was very similar to the antibody secretion results presented here. Therefore, the water samples assayed showed no effects on the humoral immune system, and they were not cytotoxic either.

Unfortunately, this study cannot conclusively state that the water samples assayed were not immunotoxic. According to the US Congress Office of Technology Assessment (1991), immunotoxicity is any undesirable change in immune function resulting from exposure to some foreign agent. Therefore, more assays will need to be carried out in order to assess other pathways of the immune system. A study conducted by Pool and Magcwebeba (2009) to assess immunotoxicity of river water, included tests to determine the effects of the water on cell-mediated immunity and inflammation. These would be good parameters to consider for future studies on wastewater. *In vivo* studies would also be needed to confirm the non-immunotoxic effects observed *in vitro*.

4.5 Conclusion

In conclusion, it is clear that the Stellenbosch STW reduced the levels of steroids from raw wastewater. All influent steroid levels were significantly higher than the negative control, and generally were also higher than at other STWs. Steroid concentration decreased after each treatment process, with the exception of progesterone after the biofilter. Increase in progesterone concentration was likely due to deconjugation of glucuronide conjugates in the wastewater. Variability in steroid levels between sampling days suggests that the STW steroid removal was good (efffuent was similar to distilled water). However, steroids were still detected in effluents. Testosterone was the best removed steroid assayed and estrone was the worst at approximately 96% removal and 55% removal respectively. The treatment process that contributed the most to steroid reduction was activated sludge (for all steroids assayed).

Bacterial quality of water from Stellenbosch STW was poor, as most samples showed at least 1000 CFU/mL (maximum limit of the assay). However, the effluent from the first collection date may have been good. This could not be confirmed due to the detection limits of the assay employed.

Lastly, influent and effluent waters were not shown to be either cytotoxic or immunotoxic. In fact the waters appear to slightly stimulate antibody synthesis/secretion. However, the hybridoma assay used here may be less sensitive than other assays. Although this study suggests that water from Stellenbosch STW may not be immunotoxic, it is inconclusive as only one pathway of the immune system was assessed (humoral immunity). It is recommended that additional assays be included to determine cell mediated

immunity and inflammatory effects. A comparison test (between the hybridoma assay used here and other assays for humoral immunity) should also be carried out to establish their sensitivities; as well as *in vivo* studies to confirm our findings.

4.6 References

Atkinson, SK. Marlatt, VL. Kimpe, LE. Lean, DRS. Trudeau, VL. Blais, JM. 2012. The occurrence of steroidal estrogens in south-eastern Ontario wastewater treatment plants, Science of the Total Environment, 430, 119-125.

Bellingham M, Fiandanese N, Byers A, Cotinot C, Evans NP, Pocar P, Amezaga MR, Lea RG, Sinclair KD, Rhind SM, Fowler PA. 2012. Effects of exposure to environmental chemicals during pregnancy on the development of the male and female reproductive axes. Reproduction in Domestic Animals. 47, 15–22.

Camacho-Muñoz MD, Santos JL, Aparicio I, Alonso E. 2010. Presence of pharmaceutically active compounds in Doñana Park (Spain) main watersheds. Journal of Hazardous Materials. 177, 1159–1162.

Chang, H. Wan, Y. Wu, S. Fan, Z. Hu, J. 2011. Occurrence of androgens and progestogens in wastewater treatment plants and receiving river waters: Comparison to estrogens , Water Research , 4 5, 7 3 2-7 4 0.

Chase DA, Karnjanapiboonwong A, Fang Y, Cobb GP, Morse AN, Anderson TA. 2012. Occurrence of synthetic musk fragrances in effluent and non-effluent impacted environments. Science of the Total Environment. 416, 253–260.

D`Ascenzo, G. Di Corcia, A. Gentili, A. Mancini, R. Mastropasque, R. Nazzari, M. Samperi, R. 2003. Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities, The Science of the Total Environment, 302, 199-209.

Daughton CG, Ternes TA. 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environmental Health Perspectives. 107, 907-938.

Department of Water Affairs and Forestry (RSA). 1996. South African Water Quality Guidelines (Second Edition), Volume 1: Domestic Use.

Díaz-Cruz MS, García-Galán ML, Guerra P, Jelic A, Postigo C, Eljarrat E, Farré M, López de Alda MJ, Petrovic M, Barcelo D. 2009. Analysis of selected emerging contaminants in sewage sludge. Trends in Analytical Chemistry. 28, 1263-1275.

Drinan JE, Whiting NE. 2001. Water & wastewater treatment, a guide for the nonengineering professional. CRC Press. Boca Raton. 316pp.

Eertmans F, Dhooge W, Stuyvaert S, Comhaire F. 2003. Endocrine disruptors: effects on male fertility and screening tools for their assessment. Toxicology in Vitro. 17, 515–524.

Esperanza M, Suidan MT, Marfil-Vega R, Gonzalez C, Sorial GA, McCauley P, Brenner R. 2007. Fate of sex hormones in two pilot-scale municipal wastewater treatment plants: conventional treatment. Chemosphere. 66, 1535–1544.

Folmar LC, Denslow ND, Rao V, Chow M, Crain DA, Enblom J, Marcino J, Guillette Jr LJ. 1996. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environmental Health Perspectives. 104, 1096-1101.

Gómez C, Vicente J, Echavarri-Erasun B, Porte C, Lacorte S. 2011. Occurrence of perfluorinated compounds in water, sediment and mussels from the Cantabrian Sea (North Spain). Marine Pollution Bulletin. 62, 948–955.

Greaves, RF. Jevalikar, G. Hewitt, JK. Zacharin, MR. 2014. A guide to understanding the steroid pathway: Newinsights and diagnostic implications, Clinical Biochemistry, http://dx.doi.org/10.1016/j.clinbiochem.2014.07.017

Hendricks R. 2011. Assessment of the biological quality of raw and treated effluents from three sewage treatment plants in the Western Cape, South Africa. Doctoral Thesis, University of the Western Cape. 240pp.

Hoai PM, Sebesvari Z, Minh TB, Viet PH, Renaud FG. 2011, Pesticide pollution in agricultural areas of Northern Vietnam: case study in Hoang Liet and Minh Dai communes. Environmental Pollution. 159, 3344-3350.

Jobling S, Burn RW, Thorpe K, Williams R, Tyler C. 2009. Statistical modeling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in English rivers. Environmental Health Perspectives. 117, 797-802.

Khalaf H, Salste L, Karlsson P, Ivarsson P, Jass J, Olsson PE. 2009. In vitro analysis of inflammatory responses following environmental exposure to pharmaceuticals and inland waters. Science of the Total Environment. 407, 1452–1460.

Kosjek T, Perko S, Zupanc M, Zanoški Hren M, Landeka Dragičević T, Žigon D, Kompare B, Heath E. 2012. Environmental occurrence, fate and transformation of benzodiazepines in water treatment. Water Research. 46, 355-368.

Kumar, V. Nakada, N. Yasojima, M. Yamashita, N. Johnson, AC. Tanaka, H. 2011. The arrival and discharge of conjugated estrogens from a range of different sewage treatment plants in the UK, Chemosphere, 82, 1124–1128.

Lategan, K. 2014. The validation of *Saccharomyces cerevisiae* as a rappid tool to assess toxicity, unpublished manuscript.

Limpiyakorn, T. Homklin, S. Ong, SK. 2011. Fate of Estrogens and Estrogenic Potentials in Sewerage Systems, Critical Reviews in Environmental Science and Technology, 41, 1231–1270.

Liu, S. Ying, G. Zhao, J. Chen, F. Yang, B. Zhou, L. Lai, H. 2011. Trace analysis of 28 steroids in surface water, wastewater and sludge samples by rapid resolution liquid chromatography–electrospray ionization tandem mass Spectrometry, Journal of Chromatography A, 1218, 1367–1378.

Manickum, T. John, W. 2014. Occurrence, fate and environmental risk assessment of endocrine disrupting compounds at the wastewater treatment works in Pietermaritzburg (South Africa), Science of the Total Environment, 468–469, 584–597.

Meng, L. Reyes, H. Palma, J. Hernandez, I. Ribalta, J. Sjovall, J. 1997. Profiles of bile acids and progesterone metabolites in the urine and serum of women with intrahepatic cholestasis of pregnancy, Journal of Hepatology, 21, 346-357.

Musolff A, Leschik S, Möder M, Strauch G, Reinstorf F, Schirmer M. 2009. Temporal and spatial patterns of micropollutants in urban receiving waters. Environmental Pollution. 157, 3069–3077.

Pool, EJ. Magcwebeba, TU. 2009. The Screening of River Water for Immunotoxicity Using an In Vitro Whole Blood Culture Assay, Water Air Soil Pollution, 200, 25–31.

Pool, EL. Van Wyk, JH. Leslie, AK. 2000. Inflammatory activity as an indicator of water quality: the use of human whole blood cultures, Journal of Immunoassay, 2(14), 387-399.

Sakan S, Đorđević D, Dević G, Relić D, Anđelković I, Đuričić J. 2011. A study of trace element contamination in river sediments in Serbia using microwave-assisted aqua regia digestion and multivariate statistical analysis. Microchemical Journal. 99, 492–502.

Schwarzenbach RP, Egli T, Hofstetter TB, von Gunten U, Wehrli B. 2010. Global water pollution and human health. Annual Review of Environment and Resources. 35, 109-136.

Swart JC. 2008. The development and implementation of biomarker assays for estrogenic endocrine disruptors. Doctoral Thesis, University of the Western Cape. 194pp.

Taju G, Abdul Majeed S, Nambi K.S.N, Sarath Babu V, Vimal S, Kamatchiammal S, Sahul Hameed A.S. 2012. Comparison of *in vitro* and *in vivo* acute toxicity assays in *Etroplus suratensis* (Bloch, 1790) and its three cell lines in relation to tannery effluent. Chemosphere. 87, 55–61.

United Nations Environment Programme Global Environment Monitoring System (GEMS)/Water Programme. 2008. Water quality for ecosystem and human health, 2nd edition. Ontario: UNEP GEMS/Water Programme. 120pp.

US Congress, Office of Technology Assessment. 1991. Identifying and Controlling Immunotoxic Substances-Background Paper, OTA-BP-BA-75, Washington DC, USA.

Xu Y, Luo F, Pal A, Yew-Hoong Gin K, Reinhard M. 2011. Occurrence of emerging organic contaminants in a tropical urban catchment in Singapore. Chemosphere. 83, 963–969.

Yu JT, Bouwer EJ, Coelhan M. 2006. Occurrence and biodegradability studies of selected pharmaceuticals and personal care products in sewage effluent. Agricultural Water Management. 86, 72–80.

Zhou, YQ. Zha, JM. Wang, ZJ. 2012a. Occurrence and fate of steroid estrogens in the largest wastewater treatment plant in Beijing, China, Environmental Monitoring and Assessment, 184, 6799-6813.

Zhou, YQ. Zha, JM. Xu, YP. Lei, BL. Wang, ZJ. 2012b. Occurences of six steroid estrogens from different effluents in Beijing, China, Environmental Monitoring and Assessment, 184, 1719-1729.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 General conclusions

Water quality assessment is important for us to ensure the health of human communities. This is because many known water pollutants have been shown to affect mammalian physiology. Steroids are potent endocrine disrupting chemicals/compounds (EDCs) that are known to be released by sewage treatment works (STWs), and other contaminants have been shown to affect the immune system. The Stellenbosch STW recently initiated an upgrade procedure that would affect the STW's operations, and likely their effluent quality as well. Therefore, the aim of this investigation was to assess the quality of Stellenbosch STW's treated and untreated waters during their upgrade procedure.

The first objective of this study was to determine the presence of total coliforms and *Escherichia coli* throughout the Stellenbosch STW. The concentration of total coliforms and *E. coli* was at least 1000CFU/mL (CFU: colony forming units; mL millilitre) in the influent, on all collection dates. The effluent also showed total coliforms and *E. coli* concentrations of at least 1000CFU/mL for most collection dates. However, the first collection date showed total coliforms and *E. coli* levels of below 1CFU/mL. Therefore the influent and effluent bacterial loads were generally high.

Our second objective was to determine the concentrations of five steroids throughout the Stellenbosch STW. Influent concentrations of steroids were high compared to other STWs around the world. The activated sludge treatment was the most effective at reducing steroid levels. The final effluent concentration of

steroids was not significantly higher than the negative control. However, much variation in steroid levels was noted between collection dates. This gave a high range for most steroids and likely reduced statistical significance of our observations. The removal of steroids was as follows: 96%, 95%, 55%, 78% and 87% for testosterone, progesterone, E1 (estrone), E2 (17 β-estradiol) and EE2 (17 α-ethinyl estradiol) respectively. Thus testosterone was the best removed and E1 the worst, which is similar to the performance of other STWs. This investigation has thus, shown that the Stellenbosch STW has discharged steroids into the environment during our sampling period.

The third, and final, objective of this investigation was to determine the effects of the influent and effluent on humoral immunity. No significant difference was noted between the effects of the STW samples and the negative control on antibody synthesis/secretion.

5.2 Recommendations

This study found that neither STW influent nor effluent affect antibody production by hybridoma cells. Contrary to this, previous work done by our research group showed that STW influents, and to a lesser extent effluents, modulate immune pathway biomarkers of whole blood cultures. This could be due to the fact that hybridoma cells are less sensitive than a human whole blood culture. Therefore, tests are recommended to directly compare the sensitivity of the hybridoma cells used in this experiment with the human whole blood culture. *In vivo* studies, as well as studies on different immune pathways, would also be necessary to confirm the apparent non-immunotoxic effects of the Stellenbosch

STW water observed here. In addition, it would be good to also assess the effects of this water on other physiological systems as well as assessing generational effects to get a fuller understanding of any dangers this water may pose.

The ELISAs done for screening selected steroids revealed the presence of high levels of the steroids estrone, 17 β-estradiol, 17 α-ethinyl estradiol, progesterone and testosterone in the STW influent. The STW effluent showed significantly lower concentrations of each of the assayed steroids. A good idea for further investigations would be to confirm the data of this study using an alternative method such as high performance liquid chromatography, tandem mass spectrometry. These methods could also be used to help determine the fate of the pollutants tested here.

Lastly, a re-evaluation of the Stellenbosch STW water, after the completion of the upgrade, is also recommended. In this study, water samples were collected during late autumn to mid-winter. Samples collected during mid-summer (perhaps also mid-autumn and mid-spring) should be included in future studies. It would also be good to compare Stellenbosch STW to other STWs in the Western Cape sampled during the same seasons. This could be especially valuable if the STWs selected would include both conventional and modern STWs.