Assessment of the biological quality of raw and treated effluents from three sewage treatment plants in the Western Cape, South Africa

by

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Declaration

I, Rahzia Hendricks declare that the thesis entitled óAssessment of the biological quality of raw and treated effluents from three sewage treatment plants in the Western Capeô is my work and has not been submitted for any degree or examination at any other university and that all sources of my information have been quoted as indicated in the text and/or list of reference.

Rahzia Hendricks

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May 2011

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1 Liter	1 L
17Ŭethinylestradiol	EE2
17ɓ-Estradiol	E2
4 Nitro Quinoline Oxide	4NQO
5,5`-Dithio-bis-2-nitrobenzoic acid	DTNB
5-thio-2-nitro-benzoate	TNB
Acetylcholinesterase	AChE
Acetylthiocholine iodide	ATI
acid dissociation	pKa
activated charcoal cloths	ACC
activated charcoal fibers	ACF
adenylate cyclase adenosine 3, 5-cyclic monophosphate	cAMP
alcohol ethoxylates	AE
alkyl polyglycoside	APG
alkylphenol polyethoxylates	APE
alkylphenoxy acetic acid	APEC1
alkylphenoxy ethoxy acetic acid	APEC2
analysis of variance	ANOVA
biochemical oxygen demand	BOD
bisphenol A	BPA
chemical oxygen demand	COD
correlation coefficients	R^2
colony forming units per milliliter	CFU/ml
complement-reactive protein	CRP
Deoxyribonucleic acid	DNA
dimethyl sulfoxide	DMSO
endocrine disrupting chemicals	EDCs
Enzyme Linked Immunosorbent Assays	ELISAs
Escherichia coli	E.coli
estriol	E3
Estrogen receptor alpha	ERŬ
Estrogen receptor beta	ERɓ
Estrone	E1
follicle-stimulating hormone	FSH
granular activated charcoal	GAC
Interferon gamma	IFN-ɔ
Interleukin-10	IL-10
Interleukin-6	IL-6

International Programme on Chemical Safety	IPCS
kilograms	Kg
Lactate dehydrogenase	LDH
linear alkylbenzene sulphonates	LAS
lipopolysaccharide	LPS
luteinizing hormone	LH
Membrane bioreactor	MBR
membrane filter technique	MFT
mestranol	MeEE2
methyl ester ethoxylate	MEE
microgram per day	µg/day
microgram per liter range	µg/L
milligram per liter	mg/L
Micrometers	µm
multiple-tube fermentation	MTF
nanograms per litre	ng/L
natural killer	NK
Nicotinamide adenine dinucleotide	NAD+
nonylphenol ethoxylates	NPE
nonylphenol polyethoxylates	NPEO
nonylphenols	NP
octophenols	OP
octylphenol ethoxylates	OPE
optical density	OD
P450 cholesterol side chain cleavage enzyme	P450scc/CYP11A1
perfluorinated compounds	PFCs
personal care products	PCP
phytohemagglutinin	PHA
polyethylene glycols	PEG
powdered activated charcoal	PAC
Roswell Park Memorial Institute 1640	RPMI-1640
solid phase extractions	SPE
solid retention times	SRT
Standard deviation	SD
Standard Error of the mean	SEM
sulfophenyl acids	SPC
ultraviolet light	U.V.
whole blood cultures	WBC
Xiphophorus maculatus	*X.maculatus*
ɓ-D-glucoronidase activity	GLUase activity

ABSTRACT

Wastewater contains contaminants such as bacteria, heavy metals, industrial chemicals, pesticides, pharmaceuticals, personal care products, steroid hormones and surfactants. Pollutants enter receiving waters via agricultural run-off, wash-off from roadways, industrial wastewaters and domestic sewage. Pollutants can incur adverse effects to the environment, human and animal health. The aim of this study was to compare the water quality of raw wastewater and treated sewage effluents from three different sewage treatment plants in the Western Cape, South Africa. The treatment plants investigated are on the same river system. Sewage treatment plant 1 and 2 use older technologies, while sewage treatment plant 3 has been upgraded and new technologies (membrane bioreactor) were incorporated in the treatment processes.

The first objective was to determine the occurrence of total coliforms, *Escherichia coli* (*E. coli*) and fluoroquinolone and sulfamethoxazole antibiotic residues in raw wastewater and treated sewage effluents. Bacteria in treated sewage effluents can result in diseases such as dysentery, gastroenteritis, and typhoid upon exposure. A chromogenic test was used to screen for coliforms and *E. coli.* Enzyme linked Immunosorbent Assays (ELISA) were used to quantitate antibiotic residues (fluoroquinolones and sulfamethoxazole) in raw wastewater and treated sewage effluents. This study showed that bacteria are present in raw wastewater and residual bacteria are released with treated sewage effluents from sewage treatment plant 1. Fluoroquinolones and sulfamethoxazole are present in raw wastewater entering all sewage treatment plants. Fluoroquinolones were not eliminated by the sewage treatment processes and detectable levels were released with treated sewage effluents. Sulfamethoxazole was eliminated by the treatment processes used by sewage treatment plant 2 and 3 only.

The second objective of this study was to compare the occurrence of the steroid hormones estradiol, estrone and testosterone in raw wastewater and treated sewage effluents. Steroids in water bodies are associated with endocrine disruption. ELISAs specific for the steroid hormones were used to assess the samples collected from the sewage treatment plants. Estradiol, estrone and testosterone were detected in raw wastewater entering all sewage treatment plants. Sewage treatment plant 3 displayed low efficiencies for estradiol removal. Treatment plant processes at sewage treatment plant 1 and 2 removed estrone effectively. Testosterone was removed efficiently by the treatment processes.

The third objective of the study was to determine the occurrence of the surfactants alkylphenol ethoxylates (APE) and alcohol ethoxylates (AE) in raw wastewater and treated sewage effluents. Surfactants in water bodies are associated with adverse effects in aquatic organisms. ELISAs specific for the selected surfactants were used to assay the sewage samples. APE and AE surfactants were detected in significant concentrations in raw wastewater entering all investigated sewage treatment plants. Results of this study showed that APE was not removed effectively by treatment plant 1. However, APE was removed by the treatment processes used in sewage treatment plant 2 and 3. In addition, this study showed that AE levels in treated sewage effluents for all sewage treatment plants were reduced, irrespective of treatment technology used.

Biomarkers for toxicity are useful to determine potential adverse effects to humans and animals. Lactate dehydrogenase (LDH) release from cells is used as a biomarker to determine cellular cytotoxicity. Acetylcholinesterase (AChE) inhibition is used as a biomarker to determine neurotoxic contaminants in the aquatic environment. The SOS chromotest is often used to determine genotoxicity of samples. The fourth objective of this study was to validate and use these screening tests to determine the toxicity of raw

wastewater and treated sewage effluents. Raw wastewater and treated sewage effluents were screened for cytotoxicity using LDH release as a biomarker. This study also focused on validating the AChE inhibition assay to screen raw wastewater and treated sewage effluents for potential AChE inhibitors. Raw wastewater and treated sewage effluents were also tested for genotoxicity using the SOS chromotest. The results of this study showed that raw wastewater and treated sewage effluents from all sewage treatment plants were not cytotoxic. Results of this study also showed that raw wastewater entering sewage treatment plants contain AChE inhibitors. The sewage treatment processes are ineffective in eliminating these inhibitors from treated sewage effluents. In addition, raw wastewater samples tested positive for genotoxicity. Treated sewage effluents from all sewage treatment plants displayed no genotoxicity. This indicates effective removal of genotoxins by all three sewage treatment plants investigated. This study makes use of only screening assays to determine toxicity therefore care should be taken into interpreting results. Results of this study could reflect unique characteristics of the analyzed samples and therefore not a true representation of raw wastewater and treated sewage effluents over an extended period of time.

The fifth objective of this study was to screen raw wastewater and treated sewage effluents from three different sewage treatment plants for its toxic effects on specific immune pathways using an *in vitro* whole blood culture assay and cytokine monitoring. Mammals possess immune systems that are particularly vulnerable or sensitive to exposure to pollutants. Therefore, the immune system can be used to monitor pollutant exposure. Sewage effluents consist of a mixture of chemicals, pollutants, microorganisms, debris, heavy metals, pesticides and pharmaceuticals. These sewage effluents or environmental pollutants may have effects on the immune system of humans. Interleukin-6 (IL-6) was used as a biomarker for inflammation. Interleukin-10 (IL-10) was used as a biomarker for humoral

immunity. ELISAs specific for these two cytokines were used to assay the samples. Results of this study showed that raw wastewater and treated sewage effluent samples produced an immunotoxic effect on the IL-6 and IL-10 immune pathways. Despite different technologies used by the sewage treatment plants in this study, contaminants in the effluents still resulted in immunotoxic effects.

The final objective of this study was to determine the efficiency of activated charcoal for the removal of steroids and surfactants from treated sewage effluents from a sewage treatment plant. Several concentrations of activated charcoal were added to treated sewage effluents from the sewage treatment plant and allowed to incubate for 2 hours. Treated sewage effluents and activated charcoal adsorbed treated sewage effluents were assessed for the occurrence and removal of estradiol, estrone, testosterone and APE. Specific ELISAs were used to monitor estradiol, estrone, testosterone and APE. Results showed that activated charcoal is effective in removing the steroids and surfactants. Adding activated charcoal as a final sewage treatment step could potentially provide a method that could be employed by sewage treatment plants to reduce residual contaminants in treated sewage effluents.

Chapter 1: Literature Review

1.1. Introduction to emerging contaminants found in sewage

The human population is growing exponentially and this has resulted in an increase in the demand, production and use of new chemicals and resources. It has also resulted in an increase in the amount of pollutants that are released into the environment. These emerging contaminants have impacted our freshwater resources and have become one of the most worrying environmental issues of the 21st century (Kolpin et al., 2002). These inorganic and organic contaminants are discharged to sewers and wastewater treatment plants (Bolong et al., 2009). Inorganic contaminants include chemicals such as heavy metals, asbestos, and nitrates (Lee et al., 2005) (see Figure 1.1). Typically, organic compounds are mixtures of carbon, hydrogen, oxygen and nitrogen (Boari et al., 1997) (see Figure 1.2). The organic contaminants include chemicals such as steroid sex hormones, pharmaceuticals, personal care products (PCP), illegal drugs (unlawful drugs), flame retardants and perfluorinated compounds (PFCs) (Diaz-Cruz et al., 2009). Other contaminants frequently found in sewage or wastewater include surfactants (Schröder et al., 1999), and pesticides (Fernández-Alba et al., 2001). The occurrence of these substances in the environment has been studied extensively (Ternes et al., 1999a; Ternes et al., 1999b; Desbrow et al., 1998). However, only a few studies on the occurrence of these contaminants in South African waters have been done (Gordon et al., 2009; Samie et al., 2009; Sibali et al., 2010; Kinge et al., 2010; Dungeni and Momba, 2010). Bolong et al. (2009) discussed three key issues to highlight challenges associated with these contaminants in the environment. The first issue concerns the lack of limiting regulations for the release of these chemicals into the environment. Secondly,

endocrine disruptors are on the increase and consist of several diverse chemicals. Finally, the difficulty in analyzing these compounds poses a challenge, since each compound has a different mechanism of action (Bolong et al., 2009). This review provides an overview of various contaminants found in sewage as well as the different treatment processes used by sewage treatment plants.

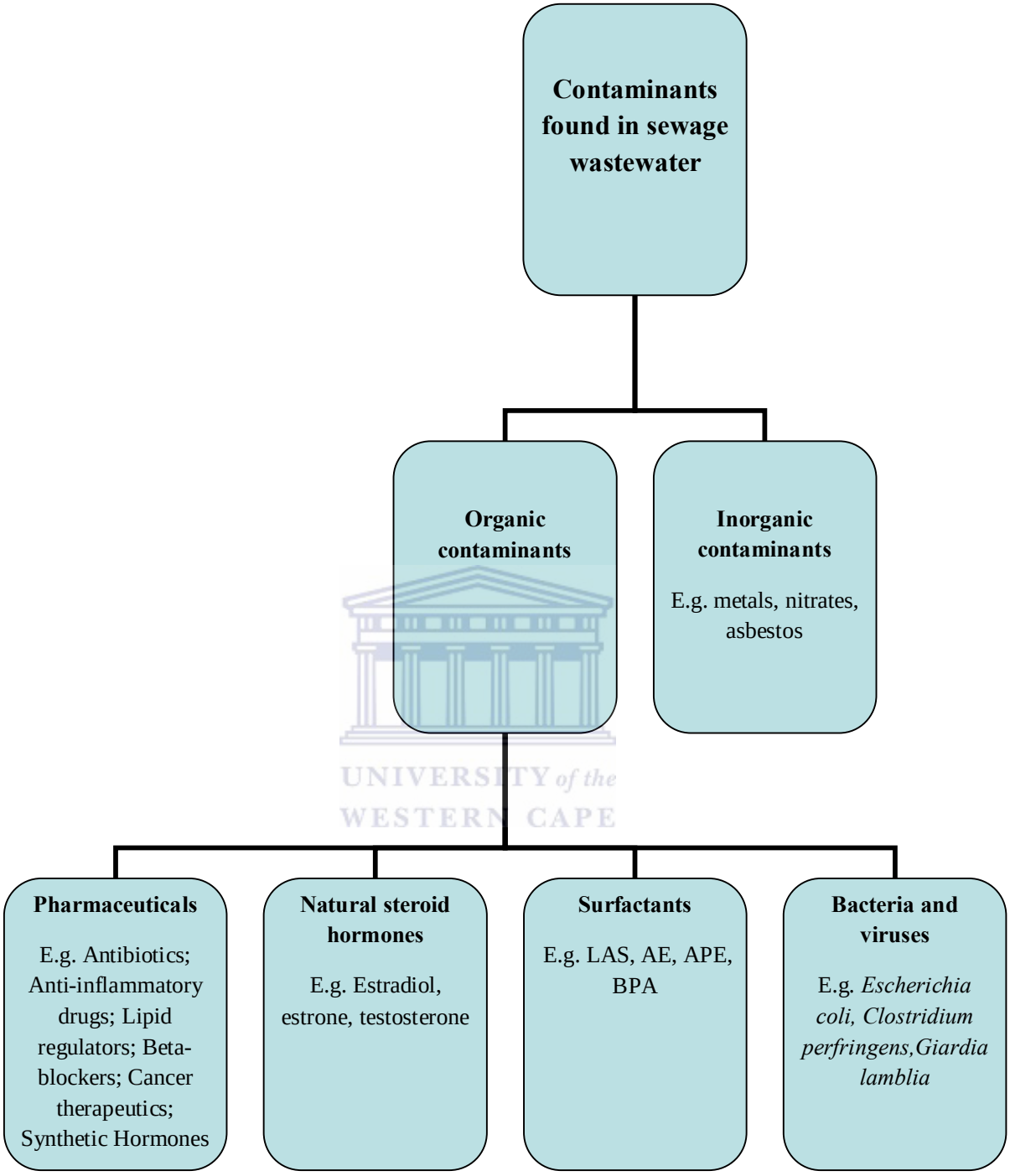


Figure 1.1. Inorganic and organic contaminants found in sewage.

1.2. Pathogens in wastewater

Raw wastewater from industries, residential areas and hospitals collectively enter the sewer (Samie et al., 2009). In turn, the raw wastewater enters municipal sewage treatment plants to be treated before entry into receiving waters. Bacteria are found in the intestines of humans and animals and these are released into the sewage system. Consequently, raw wastewater is contaminated with high loads of faecal bacteria and pathogens.

Pathogens found in raw wastewater include viruses (Prado et al., 2011), bacteria (Wéry et al., 2008) and protozoa (Bertrand and Schwartzbrod, 2007). The DNA viruses, adenoviruses, rotavirus, and hepatitis A are often found in urban sewage and may cause adverse effects to humans and animals (Girones et al., 2010). Viral infections can result in respiratory diseases, gastroenteritis and acute hepatitis (Girones et al., 2010). On the other hand, common pathogenic bacteria isolated from water may result in bacterial gastroenteritis. These bacteria include the *Salmonella* and *Campylobacter* species. Other waterborne bacterial pathogens such as *Shigella*, *Yersinia* and *Vibrio cholera* are associated with contaminated water and can result in outbreaks of disease (Gaffga et al., 2007). Protozoa such as *Cryptosporidium*, *Giardia*, and *Entamoeba* can be found in contaminated water. These parasites are common waterborne and food-borne pathogens that result in diarrhoea in their hosts (Ho and Tam, 1998).

A commonly used bioindicator for faecal contamination of water is faecal coliforms. Yields of total coliforms and faecal coliforms in raw sewage are typically 10^7-10^9 and 10^6-10^8 colony forming units per milliliter (CFU/ml) respectively (Rose et al., 1996). Inadequately treated sewage effluents with residual pathogens pose a risk to humans and animals. Moreover, the direct discharge of untreated sewage into receiving waters could result in

adverse effects to humans. Indeed, high levels of *Giardia lamblia* have been found in river sites where treated sewage effluents have been discharged (Ho and Tam, 1998). In addition, the contamination of drinking water by sewage has resulted in outbreaks of cholera (Gaffga et al., 2007).

Sewage treatment plants use different methods to eliminate pathogens from raw wastewater. Methods of treatment include sedimentation, mesophilic or themophilic anaerobic digestion or composting (Sahlstrom et al., 2004). The ability of sewage treatment plants to eliminate pathogens are variable. A study performed by George et al. (2002) using plate counts and enzymatic methods, investigated twelve different wastewater treatment plants for their efficiency to removal faecal coliforms. Of the twelve wastewater treatment plants, only one had an ultraviolet (U.V.) disinfection treatment step. Wastewater treatment plants with high retention times were the most efficient at removing culturable faecal coliforms. On the other hand results for *Escherichia coli* (*E. coli)* ɓ-D-glucoronidase activity (GLUase activity) showed the same removal pattern as the plate counts, however with the tertiary U.V. light disinfection no reduction of GLUase was measured. Taken together, these results indicate that removal efficiencies of faecal coliforms and pathogens are dependent on the type of treatment.

In a similar study, Samie et al. (2009) investigated the efficiency of fourteen different sewage treatment plants in the Mpumalanga Province, South Africa using microbiological and physicochemical parameters. Results of the study showed that only two sewage treatment plants were able to produce zero faecal coliforms in treated effluents. In addition, pathogenic bacteria such as *Shigella* and *Salmonella* were still isolated from some of the treated sewage effluents.

The increase in the population worldwide and the scarcity and growing need for potable water throughout the world has put added pressure on water quality criteria. In the future, reclaimed water will be used for irrigation of parks and crops (George et al., 2002). Today, the Windhoek Goreangab water reclamation plant is the worldôs first potable water plant, with 35 % of drinking water being reclaimed (du Pisani, 2006). In order to reduce the adverse effects associated with these contaminated sewage effluents a need to improve sewage treatment processes arises. Moreover, regular monitoring of treatment processes will prevent release of untreated effluents due to malfunctioning sewage treatment plants (Samie et al., 2009).

1.3. Pharmaceuticals

Pharmaceuticals are drugs or chemicals that are used in diagnosis, treatment and prevention of disease (Daughton and Ternes, 1999). Pharmaceuticals are a group of emerging contaminants in the environment. Pharmaceuticals are used in both human and veterinary medicine. A significant amount of pharmaceuticals are used throughout the world. In particular, the European Union uses 3000 different drugs in human medicine (Fent et al., 2006). Various types of pharmaceuticals used in human medicine include antibiotics, analgesics, anti-inflammatory drugs, contraceptives, beta-blockers, lipid regulators and neuroactive compounds (Daughton and Ternes, 1999). In addition, antibiotics and anti-inflammatory drugs are used in veterinary medicine.

After the ingestion of pharmaceuticals, they are metabolized in the body in several phases. Phase I metabolic reactions consist of oxidation, reduction and hydrolysis of the parent structure. The compounds formed after this metabolic reaction are often more toxic and reactive than the original compound. Thereafter, Phase II reactions occur which results in conjugation of the drug. This stage produces inactive products or compounds. Phase I and Phase II metabolic reactions result in alteration of the chemical behaviour of the compounds since metabolic reactions always results in more hydrophilic substances being produced compared to the original compound (Halling-Sørensen et al., 1998).

Pharmaceuticals are not digested completely by humans and animals, and therefore enter the environment via human urine or faeces. These pharmaceuticals are discharged into municipal sewage. Other sources of environmental pharmaceuticals are hospital wastewater (Langford and Thomas, 2009), discharge of unused and unwanted drugs (Glassmeyer et al., 2009), illegal drug discharge (Kasprzyk-Hordern et al., 2009), landfill leachates (Nikolaou et

al., 2007) and wastewater from pharmaceutical manufacturers (Larsson et al., 2007; Sim et al., 2011). Pharmaceuticals either adsorb to sewage sludge (Golet et al., 2003) or undergo biodegradation in sewage treatment plants (Tixier et al., 2003). Most sewage treatment processes are inefficient in removing pharmaceuticals from sewage effluents and pharmaceutical residues are released into the environment (Ternes, 1998; Koutsouba et al., 2003). Pharmaceuticals found in sewage effluents include antibiotics (Hirsch et al., 1999), blood lipid regulators (Pedrouzo et al., 2010), synthetic steroid hormones (Ternes, 1998) and non steroidal inflammatory drugs (Kosjek et al., 2005). These pharmaceuticals often enter rivers, lakes, surface waters and sometimes drinking water (Pedrouza et al., 2010) (Figure 1.2). In addition, animal manure applied as fertilizer to agricultural soil provides a source of veterinary medicine to surface waters (Jørgensen and Halling-Sørensen, 2000). Taken together, the occurrence of these pharmaceuticals in the environment can impact aquatic systems.

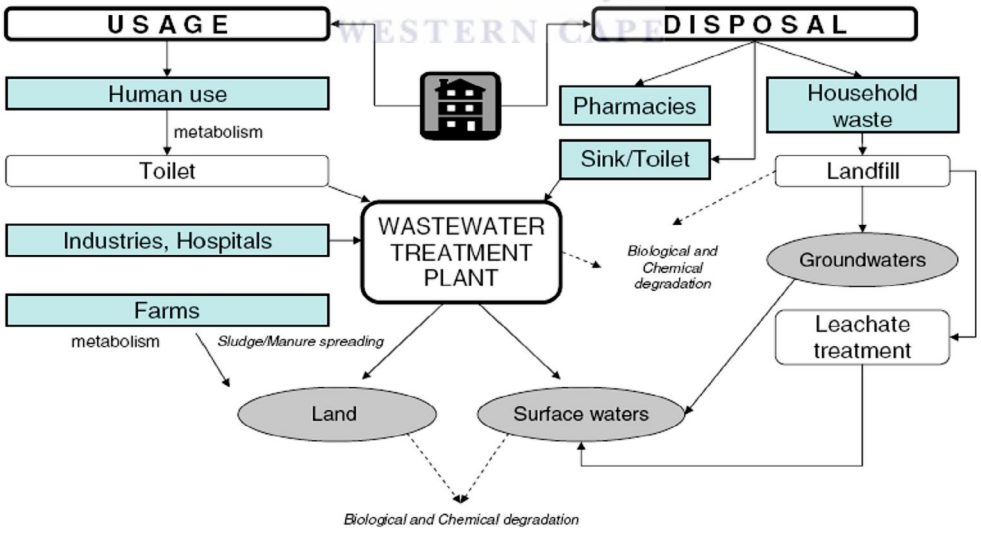


Figure 1.2. The origin and distribution of pharmaceuticals in the environment (Nikolaou et al., 2007).

1.3.1. Antibiotics

Antibiotics are used to treat infections in both humans and animals. The main groups of antibiotics used are tetracyclines, sulfonamides, chloramphenicol and fluoroquinolones. Hirsh et al. (1999) studied the occurrence of 18 antibiotics in German sewage treatment plant effluents and river waters. According to the results of the study only dehydrated erythromycin, roxithromycin, clarithromycin, sulfamethoxazole and trimethoprim were detected in sewage treatment plant effluents and surface waters. These antibiotics were detected in the microgram per liter range (µg/L). Tetracycline and penicillin were not detected in effluents due to either hydrolysis or binding to sediment (Hirsh et al., 1999).

The older conventional activated sludge processes are 85 % effective in removal of antibiotics from sewage effluents (Peng et al., 2006). Despite treatment of raw wastewater, some antibiotics remain persistent in treated sewage effluents (Karthikeyan and Meyer, 2006). A decrease in antibiotic concentration in receiving waters is not frequently observed. Antibiotics such as sulfamethoxazole, ciprofloxacin and clindamycin were detected 100 meters downstream from its discharge point (Batt et al., 2006). These antibiotics in receiving waters could possibly present a threat to aquatic and human life.

Environmental antibiotics are thought to result in bacterial strain resistance (Hirsh et al., 1999). However, contrasting data suggest that antibiotics have no effect on resistance in bacterial strains (Armisen et al., 2010). Further investigations in South African wastewaters indicate high resistance of *E. coli* to antibiotics such as chloramphenicol, tetracycline, ampicillin and erythromycin (Kinge et al., 2010). These results indicate arbitrary use of these antibiotics by humans and also poor quality treated sewage effluents produced by treatment plants.

Furthermore, the toxic effects of the antibiotics lincomycin, sulfamethoxazole and ofloxacin were studied on aquatic organisms (Isidori et al., 2005). Results of the study showed that ofloxacin was genotoxic and that sulfamethoxazole and lincomycin were mutagenic (Isidori et al., 2005). Moreover, fish downstream of sewage treatment plants may potentially be carriers of bacteria that are resistant to antibiotics and therefore could pose a health risk to consumers of contaminated fish (Miranda and Zemelman, 2001).

1.3.2. Synthetic steroid hormones

Synthetic steroids such as 17Ŭ ethinylestradiol (EE2) and mestranol (MeEE2) are often prescribed to individuals as oral contraceptives (Figure 1.3) (Heberer, 2002). Approximately 50 kilograms (kg) of EE2 is prescribed in Germany annually (Ternes et al., 1999b). The amount of EE2 excreted by an individual is estimated to be 32 microgram per day (µg/day) (Johnson et al., 2000). Several studies done in Europe and North America showed that EE2 levels released by sewage treatment plants are between 1 ï 9 nanogram per liter (ng/L) (Ternes et al., 1999b; Baronti et al., 2000; Johnson et al., 2005). EE2 is also found in surface waters such as rivers and estuaries. However, concentrations detected in these water bodies were below the quantification limit of < 0.1 ng/L (Belfroid et al., 1999).

Removal of EE2 from sewage treatment plants is either by biodegradation or adsorption on sludge (Andersen et al., 2005; Czajka and Londry, 2006). Bacterial degradation of EE2 has been studied by Czajka and Londry (2006) under methanogenic, sulfate-, iron-, and nitrate-reducing conditions. Cultures from lake and water sediments were used to determine anaerobic degradation of EE2. Under long incubation periods, anaerobic degradation was not observed. However, Sarmah and Northcott (2008), observed rapid degradation of EE2 in

river water sediments and groundwater aquifer material under aerobic conditions. Therefore EE2 biodegradation efficiency is considerably higher under aerobic conditions (Cajthaml et al., 2009). Moreover, Andersen et al. (2005) postulated that the sorption of EE2 during sewage treatment processes is insignificant compared to biodegradation.

Very high elimination rates for EE2 were found in sewage treatment plants in Brazil (78%) (Ternes et al., 1999b). This is in accordance with Baronti et al. (2000) in which EE2 was effectively removed by activated sludge treatment (85 %). In contrast, Ternes et al. (1999a) showed that EE2 levels was not reduced after treatment in a German sewage treatment plant, with EE2 still being detected in sewage treatment discharges. These results are consistent with Ternes et al. (1999b) who did not observe reduced EE2 levels in aerobic batch experiments containing diluted activated sludge from a sewage treatment plant in Frankfurt, Germany.

Very low concentrations of EE2 are detected in sewage effluents and contribute to the estrogenicity of these effluents (Desbrow et al., 1998). EE2 induces biological effects such as vitellogenin synthesis in rainbow trout (Verslycke et al., 2002).

1.4. Natural steroid hormones

Naturally produced steroid hormones are synthesized from cholesterol and have a cyclopentan-o-per hydrophenanthrene ring (Ying et al., 2002). These steroid hormones are synthesized by several organs in the body particularly the adrenal cortex, testis and ovary (Ying et al., 2002). Natural steroid hormones have the potential to alter the endocrine system and have therefore been the focus of several studies.

1.4.1. 17β-Estradiol (E2) and Estrone (E1)

Estrogens are C-18 steroids (Hall and Phillips, 2005). These steroids have an aromatized ring and a phenolic hydroxyl group at C-3, with either a hydroxyl group (estradiol) or a ketone group (estrone). Gonadal steroids are all derived from cholesterol (C-26). Cholesterol is converted to androstenedione, which in turn is converted to estradiol (C-18) by the removal of carbon side chains. Estrogen is mainly produced by the ovaries. The enzyme aromatase converts androstenedione to estradiol, and is also responsible for extra glandular conversion of androgens to estrogens (Figure 1.3). Estradiol is synthesized in the ovary, whereas estrone is a product of peripheral conversion. Estriol (E3) is produced through metabolism of both estradiol and estrone (Hall and Phillips, 2005).

Estrogen production is regulated by the hypothalamic pituitary axis. Gonadotropin-releasing hormone secreted by the hypothalamus activates the pituitary gland. In turn, the pituitary gland secretes luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH stimulates the theca cells in the ovary to produce androstenedione. On the other hand, FSH allows the granulose cells to convert androstenedione to 17ß-estradiol. A negative feedback

system on the pituitary gland occurs if excess 17ß-estradiol is produced (Hall and Phillips, 2005).

Estrogen functions in sexual differentiation and reproductive functions (Tsuchiya et al., 2005; Acconcia and Kumar, 2006). It also plays a role on the cardiovascular system by inducing vasodilation (White, 2002). The action of estrogen is exerted by two receptors, namely Estrogen receptor alpha (ERŬ) and Estrogen receptor beta (ERß). Metabolism of estrogen is predominantly in the liver and catalysed by liver enzymes (Tsuchiya et al., 2005). In the liver estrogens are oxidized, hydroxylated and methylated before conjugation with either glucuronic acid or sulphate (Ying et al., 2002).

Several studies have shown that estrogen and estrone is excreted in urine and faeces (DôAscenzo et al., 2003; Johnson et al., 2000; Johnson et al., 2005). Menstruating females excrete approximately 3.5 µg/day of E2, 8 µg/day E1 and 1.5 µg/day E3 in urine. However, menopausal females excrete lower levels of estrogens (2.3 µg/day E2, 4 µg/day E1 and 1 µg/day of E3). Daily excretion rates of estrogen from males are comparable to excretion rates by menopausal females (1.6 µg/day E2, 3.9 µg/day E1 and 1.5 µg/day E3). Pregnant females excrete the highest 17ß-estradiol (259 µg/day), estrone (600 µg/day) and estriol (6000 µg/day) (Johnson et al., 2000).

The steroids in urine and faeces enter sewers and are released into sewage treatment plants. Baronti et al. (2000) investigated the occurrence of natural and synthetic steroid hormones in raw wastewater and treated effluents from several activated sludge sewage treatment plants. Results of the study showed that raw wastewater concentrations of estradiol and estrone were 12 ng/L and 52 ng/L respectively. The efficiencies of estradiol and estrone removal were 87 % and 61 % respectively. Residual concentrations of estradiol and estrone were also detected

in water downstream from the sewage treatment plants (Ternes et al., 1999b; Baronti et al., 2000).

Estrogenic potency of treated sewage effluents is a major concern. The estrogenic compounds present in treated sewage effluents potentially elicit biological responses in animals. Studies have shown that sewage treatment plants successfully remove steroids from raw wastewater, with negligible release in treated sewage effluents (Körner et al., 2000). In turn, the decrease in steroid concentration results in a decrease in estrogenic activity of the treated sewage effluents (Körner et al., 2000). In spite of decreased estrogenicity of effluents, biological activity is still observed in organisms. Panter et al. (1998) reported that the levels of 17ϐ-estradiol and estrone found in sewage effluents result in altered reproductive function in fathead minnows. Several other studies have assessed the estrogenicity of treated effluents on fish species (McArdle et al., 2000; Solé et al., 2002; Solé et al., 2003; Carbella et al., 2004; Diniz et al., 2005). Diniz et al. (2005) found that male crucian carp (*Carassius carassius*) exposed to municipal sewage effluents showed an increase in vitellogenin production. Moreover, results of this study showed that the testes of the fish were severely altered. In addition, the occurrence of oocytes in the gonads was observed in 20 % of male fish exposed to 100 % sewage effluents. Furthermore, exposure of fish to estrogenic sewage effluents may also result in induction of the enzyme CYP1A, a biomarker enzyme used to determine pollutant exposure (McArdle et al., 2000).

1.4.2. Testosterone

The function of the testis is to produce sperm and steroid hormones for sexual function and reproduction (Andersen and Tufik, 2006). The steroid sex hormone, testosterone, is produced

by Leydig cells in the testis upon stimulation of the pituitary gland gonadotropin, LH (Wang and Stocco, 2005; Midzak et al., 2009). LH binds to cell plasma membrane receptors in the Leydig cells. Thereafter, adenylate cyclase is activated to produce adenosine 3, 5-cyclic monophosphate (cAMP) (Ascoli et al., 2002). In turn, cAMP stimulates the transport of cholesterol to the inner mitochondrial membrane (Midzak et al., 2009). The P450 cholesterol side chain cleavage enzyme (P450scc/CYP11A1) results in the metabolism of the cholesterol molecule to form the intermediate molecule pregnenolone. Thereafter, pregnenolone is converted to testosterone by enzymes of the smooth endoplasmic reticulum (Figure 1.4). The chemical structure of testosterone is shown in Figure 1.5.

Plasma testosterone production by adult males are on average 300 - 1000 µg/L while adult females produce 20 - 75 µg/L. These levels are much higher than estrogen levels produced by both female and males (Leusch et al., 2006).

It is important to determine the presence of androgens such as testosterone in the environment to reduce adverse effects to human and animal life. Chronic exposure of *Daphnia magna* to the androgen, 4-hydroxyandrostenedione has resulted in mortality to neonates (Barbosa et al., 2008). Other effects of androgenic effluent exposure have resulted in the masculinization of fish (Howell et al., 1980; Bortone et al., 1989; Cody and Bortone, 1997; Larsson et al., 2000). This however was disputed by a study that showed that masculinization of fish and *in vitro* steroid production are unrelated biological endpoints (Bandelj et al., 2006). Only limited data for the occurrence of testosterone in sewage treatment effluents are available and therefore there is a need to lessen this knowledge gap. Vulliet et al. (2007) reported testosterone concentrations of 1 ï 30 ng/L in wastewater from France. In a similar study twelve municipal sewage treatment plant effluents were analyzed using gas chromatography/tandem mass spectrometry for the occurrence of steroid hormones. Results of the study showed that

the androgens, testosterone and androstenedione were detected at concentrations of 6.1 ng/L and 4.5 ng/L respectively (Kolodziej et al., 2003). More recent data indicate that androgens contribute to 96 % of total hormone concentration in wastewater treatment effluents in Beijing, China (Chang et al., 2010). The removal efficiency of androgens in wastewater treatment effluents in Beijing, China are between 91 - 100 % (Chang et al., 2010). Additionally, Leusch et al. (2006) demonstrated androgenic effects by municipal raw sewage from Australia and New Zealand using rainbow trout androgen receptor assays. The androgenic activity was observed to be 50 ï 100 fold higher than estrogenic activity. This was attributed to higher levels of testosterone excretion by humans compared to estrogen excretion. Activated sludge treatment of the raw sewage resulted in high removal efficiencies of the androgens (Leusch et al., 2006).

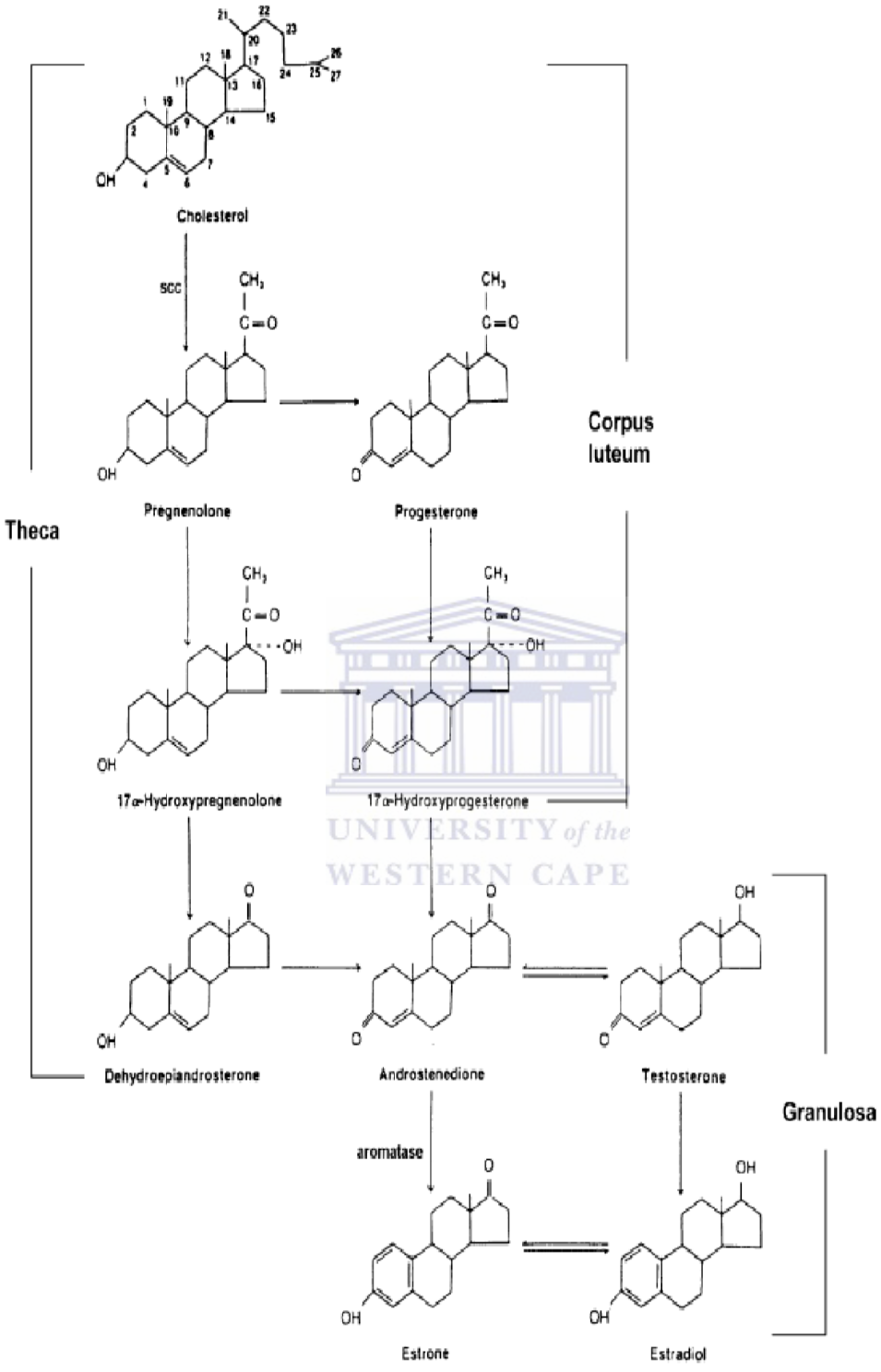


Figure 1.3. Estrogen biosynthesis in the ovary (Hall and Phillips, 2005).

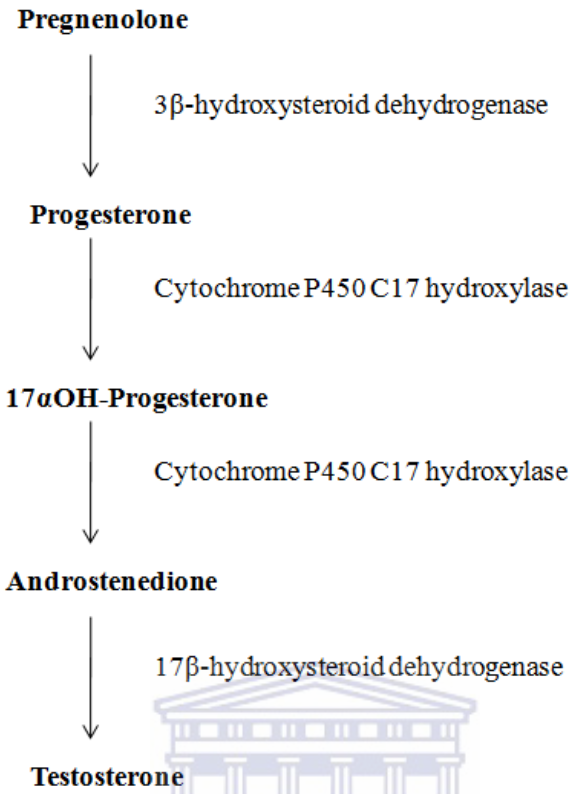


Figure 1.4. Testosterone synthesis pathway from pregnenolone (Midzak et al., 2009).

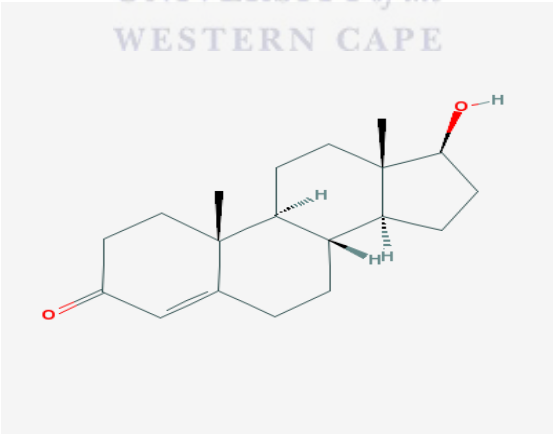


Figure 1.5. Chemical structure for testosterone (Kolodziej et al., 2003).

1.5. Surfactants

Synthetic surface active substances, also known as surfactants, are used in household chemicals as well as in industries. Surfactants are important components in detergents, fabric softeners, emulsifiers, paints, adhesives and biocides (Clara et al., 2005). Surfactants can be classed into four groups namely anionic, non-ionic, cationic and zwitterionics (Yangxin et al., 2008). Currently used detergents contain a mixture of all classes of surfactants to enhance performance (Yangxin et al., 2008). The focus of this review will be on surfactants that are discharged into the environment where they may potentially cause adverse effects to aquatic and human life. Surfactants enter the environment in various ways. In particular, sewage sludge that is contaminated with surfactants are used as fertilizer and consequently enter soil and surface waters. Domestic use results in surfactants release via sewage. These chemicals enter sewage treatment plants and due to inefficient removal of surfactants by sewage treatment processes, low levels of the contaminants are still discharged to receiving waters (Scott and Jones, 2000). Another source of environmental surfactants is direct discharge of untreated domestic sewage into freshwater and marine sites (Eichhorn et al., 2002).

1.5.1. Anionic surfactants

Anionic surfactants are predominantly used by manufacturers since they are most cost effective and because of their ease of use (Yangxin et al., 2008). Anionic surfactants are amphipatic and have hydrophobic and hydrophilic chains of various lengths (Cserháti et al., 2002). The most popular anionic surfactant used by manufacturers are linear alkylbenzene sulphonates (LAS). The estimated worldwide use of LAS was 2.8 million tons in 1998 (Verge et al., 2000).

In nature and sewage treatment plants biodegradation of surfactants entails the transformation of these surfactants by microbial activity (Dhouib et al., 2003). In sewage treatment plants the process of biodegradation of surfactants is a valuable component to prevent its discharge into the environment. Biodegradation of surfactants in the environment can be affected by various factors such as chemical structure and physical properties of the geographical area (Ying, 2006).

The degradation of LAS by various aerobic microorganisms has been shown. LAS are degraded into mono- and dicarboxylic sulfophenyl acids (SPC). These intermediates are formed by ι -oxidation of the alkyl chain terminal carbon and ϐ-oxidation (Di Corcia and Samperi, 1994). Anaerobic degradation of LAS is not favoured (De Wolf and Feijtel, 1998). LAS and its intermediates have been found in river water and more than 99 % of LAS is degraded by natural microbial flora (Perales et al., 1999).

Chronic exposure of 0.2 milligram per liter (mg/L) LAS to rainbow trout fry results in hypertrophy of the lamellar gill epithelia and a reduction of swimming capacity (Hofer et al., 1995). LAS has the ability to affect the immune system of fish (Bakirel et al., 2005). Lower phagocytic activity of leukocytes and a decrease body weight of rainbow trout fry chronically exposed to 0.4 mg/L LAS have been observed (Bakirel et al., 2005).

1.5.2. Non-ionic surfactants

Non-ionic surfactants are used in detergents, emulsifiers, wetting agents and industrial dispersing chemicals. Examples of non-ionic surfactants are alkylphenol ethoxylates (APE) (Morales et al., 2009) and alcohol ethoxylates (Yangxin et al., 2008).

Biodegradation of APE by bacteria in sewage treatment plants begins with a shortening of the ethoxylate chain. This results in production of shorter chain APE. Intermediates of APE are the alkylphenols (e.g. nonylphenol and octophenols), short chain alkylphenol ethoxylates (1 - 4 ethoxylate units) and carboxylates (alkylphenoxy acetic acid [APEC1] and alkylphenoxy ethoxy acetic acid [APEC2]) (Ying, 2006).

APE breakdown products are often detected in sewage treatment plant effluents. Johnson et al. (2005) surveyed 14 sewage treatment plants for nonylphenol concentrations in sewage effluents. Nonylphenol was detected in all 14 sewage treatment plants with a median of 0.31 µg/L and values ranging from 0.05 to 1.31 µg/L. These results were similar to those found in other sewage treatment plants in Germany (median value 0.56 µg/L, values ranging from 0.25 - 2.3 µg/L) and Italy (values ranging from 0.7 - 4 µg/L) (Spengler et al., 2001; Di Corcia and Samperi, 1994). The measured removal rates of nonylphenol ethoxylates (NPE) by sewage treatment plants varied from 37 to 90 % in Spain (Céspedes et al., 2008), from 78 to 91 % in Beijing (Lian et al., 2009) and from 60 to 75 % in China (Yu et al., 2009) indicating only partial degradation of APE (Ying, 2006).

Bioconcentration of 4-nonylphenol in adult medaka (*Oryzias latipes*) results in adverse alterations to fecundity and fertility (Ishibashi et al., 2006). In addition, as a result of maternal transfer, accumulation of 4-nonylphenol occurs in medaka embryos. This results in changes in hatchability and time to hatching of the embryos (Ishibashi et al., 2006). Other studies confirm that nonylphenol acts as a xenoestrogen resulting in mixed secondary sex characteristics and gonadal intersex in exposed fish (Balch and Metcalfe, 2006).

Fatty alcohol ethoxylates (AE) has a higher detergency than LAS and is grouped as non-ionic surfactants (Yangxin et al., 2008). These surfactants were introduced as an alternative to APE. AE is also used in laundry detergents. AE undergoes degradation using two

mechanisms (Steber and Wierich, 1987). Central cleavage of AE to polyethylene glycols (PEG) and the respective alcohol and the ι and ϐ-oxidation of the alkyl chain occur during biodegradation. Linear AE is known to be readily biodegraded. Moreover, the elimination of AE from sewage treatment plants was predicted to be dependent on the structure of AE. However, removal of AE is mostly due to biodegradation and sorption to primary sludge (Kiewiet et al., 1997; Mezzanotte et al., 2002). Morral et al. (2006) reported that more than 99 % of AE is removed by sewage treatment plants in the U.S. Moreover, Belanger et al. (2006) reported that AE posed low risks to the aquatic environments of Europe and North America.

1.6. Sewage treatment technologies

The scarcity of clean water throughout the world indicates that there is a need for appropriate management of available water resources (Aiyuk et al., 2006). Environmental protection agencies aim to re-use treated wastewater and by-products produced by sewage treatment plants (Lettinga et al., 2001). Sewage treatment plants are equipped with various treatment processes. Treatment processes employed by different sewage plants can be broken up into different stages namely, primary, secondary and tertiary stages. These treatment processes are implemented to treat raw wastewater to make it suitable for entry into receiving waters. Furthermore, these stages can be divided into physical, chemical and biological unit operations to remove contaminants (ESCWA, 2003). Figure 1.6. provides a description of the processes within each class. To implement management practices or pollution control of the environment, and to determine the occurrence of contaminants in receiving waters, it is necessary to briefly review different sewage treatment processes used today.

1.6.1. Wastewater treatment stages

The above mentioned methods of wastewater treatment are combined and often used in sequence as a pre-treatment, primary treatment, secondary treatment and tertiary treatment in sewage treatment plants.

1.6.1.1. Pre-treatment

This is a preliminary treatment of the raw wastewater that eliminates all unfavourable objects that might harm the operation of the subsequent treatment processes. The pre-treatment

processes include screening, comminution and grit removal (Sutherland, 2007; ESCWA, 2003).

1.6.1.2. Primary treatment of wastewater

During this process aeration and mechanical flocculation occurs. This stage functions as an initial process before entering the secondary treatment stage. This stage also produces sludge that needs to be removed and disposed of appropriately (Sutherland, 2007; ESCWA, 2003).

1.6.1.3. Secondary treatment of wastewater

Secondary treatment functions to remove soluble and colloidal organics that have eluded the primary treatment stage (ESCWA, 2003; Sutherland, 2007). The processes are carried out by the activated sludge system.

1.6.1.4. Tertiary treatment

This process is often the last process in the sewage treatment plant. Tertiary treatment removes significant amounts of nitrogen, phosphorus, heavy metals, bacteria and viruses. In addition, unit operations such as chemical coagulation, flocculation and sedimentation are employed by sewage treatment plants. Advanced treatments such as activated carbon, membrane bioreactor technology and reverse osmosis may be used (ESCWA, 2003; Sutherland, 2007).

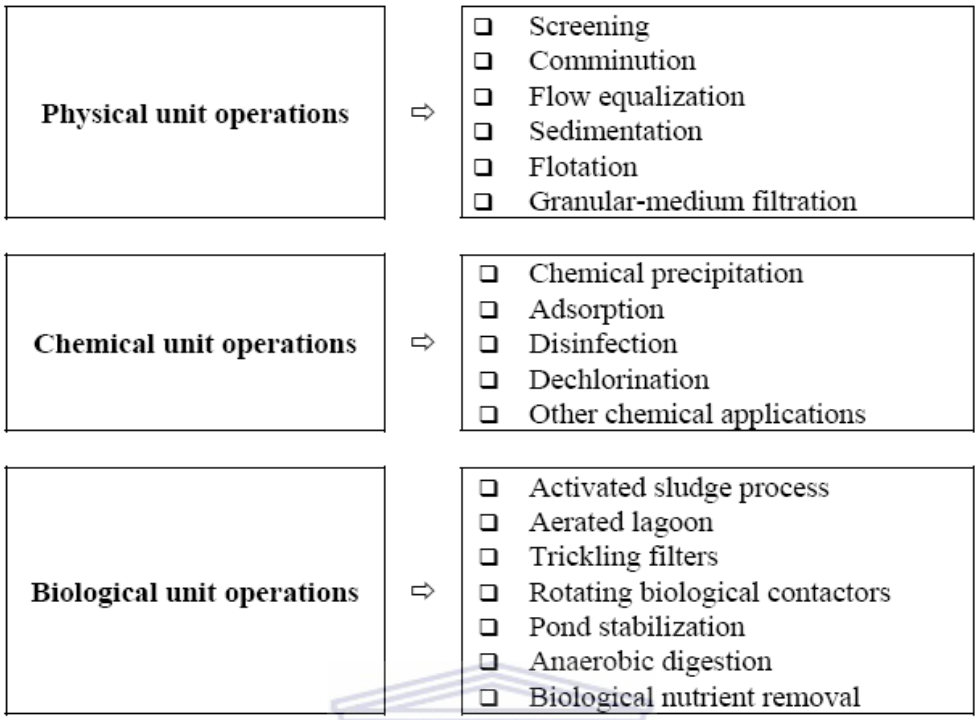


Figure 1.6. Overview of physical, chemical and biological categories and unit operations employed in sewage treatment plants (ESCWA, 2003).

1.6.2. Physical methods to process wastewater

This primary stage uses physical forces to remove contaminants from sewage. The physical methods used in sewage treatment plants include screening techniques, comminutors, flow equalization and sedimentation (Pokhrel and Viraraghavan, 2004).

1.6.2.1. Screening

Screening filters out large particles and solid objects from sewage. Screening of wastewater protects equipment downstream from the sewage flow and prevents objects from floating in

the primary settling tank (ESCWA, 2003). There are different types of screens used by sewage treatment plants. These screening technologies include coarse screens, fine screens and rods or wires (EPA, 1999; ESCWA, 2003). Solid objects such as rags, plastics and metals are either incinerated or buried.

Coarse screens consist of mechanical cleaned bar screens and trash racks. Bar screens are vertical or inclined steel bars that allow for wastewater flow. On the other hand trash racks are parallel rectangular or round steel bars with clear openings (ESCWA, 2003). These bar screens and trash racks were designed to prevent logs and large solids entering the treatment processes (EPA, 1999).

Fine screens include perforated plates, wire mesh, woven wire cloth and wedge-shaped wire. These screens have size openings of 1.5 ï 6 mm and therefore they need to be continuously cleaned with jets of water or steam (ESCWA, 2003; Mittal, 2006).

1.6.2.2. Comminutors and Grit removal

Comminutors are installed in wastewater treatment plants to reduce the size of large floating objects. Comminutors consist of blades that shred the material into smaller sizes. These comminutors help to prevent odour, flies and unsightliness. Wastewater from the comminutors usually enters grit removal chambers. These grit removal chambers remove sand, gravel or cinder (ESCWA, 2003).

1.6.2.3. Flow equalization

To enhance the process of secondary and advance treatment of wastewater, sewage treatment plants use flow equalization. This procedure entails correcting operating parameters according to the sewage treatment plant. Parameters that are adjusted include flow, pollutant levels and temperature of the wastewater (ESCWA, 2003).

1.6.2.4. Sedimentation

This technique is used globally in several sewage treatment plants. This process allows the suspended material that is heavier than water to settle (Matko et al., 1996). Settling of suspended material usually occurs as a result of gravitational force. A clarifier or also known as a sedimentation tank is used to allow the settling process to occur (Bansal et al., 2003). This process also produces a concentrated sludge (ESCWA, 2003; Lee et al., 2006).

1.6.3. Chemical methods to process wastewater

Chemical reactions are used to treat wastewater. These technologies are used in combination with physical parameters of treatment. Chemical reactions carried out by wastewater treatment plants include, chemical precipitation (Kornboonraksa et al., 2009), adsorption, disinfection and dechlorination (ESCWA, 2003).

1.6.3.1. Chemical precipitation

Chemical precipitation or chemical coagulation of wastewater encourages flocculation of solids so that they form settled flocculants. This process promotes the removal of suspended solids and phosphorus, and a better biochemical oxygen demand (BOD) (Wang et al., 2006). The quality of the wastewater produced is dependent on the amount of chemicals used and the management of the process (ESCWA, 2003).

1.6.3.2. Disinfection

Disinfection or sterilization of wastewater is defined as the removal and destruction of pathogens (Hassen et al., 2000). This is an important process to prevent outbreaks of diseases to humans. Different disinfection technologies are employed by sewage treatment plants. These disinfection processes use heat, light, radiation and or chemical reagents. The most commonly used chemical reagent is chlorine and its various derivatives (Verlicchi et al., 2009). Chlorine is effective for the treatment of wastewater because of its high oxidizing potential and it does not produce residual chlorine throughout the treatment processes (Sadiq and Rodriguez, 2004). The process of dechlorination follows the treatment of wastewater with chlorine. This process entails the removal of free and total combined chlorine residue by using sulfite (MacCrehan et al., 2005). Chlorination of wastewater leads to the formation of chlorination by-products, with more than 300 discovered (Becher, 1999). However, dechlorination does not remove these toxic by-products that are formed.

1.6.4. Biological methods to process wastewater

Biological methods of treating wastewater focus on the removal of the contaminant by using the metabolic activity of living organisms, particularly bacteria (Schultz, 2005). These processes separate the dissolved organic matter into flocculent settleable organic and inorganic solids. Colloidal and carbonaceous organic matter are products of the microorganisms activity. The organic matter either escape as gases or is removed by sedimentation tanks. These processes are also used in combination with physical and chemical treatments. Specific chemical parameters assessed during these stages are biological oxygen demand (BOD), chemical oxygen demand (COD) and nitrogen and phosphorus contents. Biological processes implemented by sewage treatment plants are grouped into five categories. These include aerobic processes, anoxic processes, anaerobic processes, combined processes and pond processes (ESCWA, 2003).

1.6.4.1. Activated sludge process

The activated sludge process (Figure 1.7) is used globally as treatment processes in municipal sewage treatment plants (Liu, 2003). This technology uses the bacterial biomass to remove contaminants from wastewater (Gernaey et al., 2004). Various Gram negative bacteria and protozoa are involved in the activated sludge process. The bacteria in the aeration basin degrade organic matter to carbon dioxide and water (ESCWA, 2003; Liu, 2003; Gernaey et al., 2004). The bacterial cells are partitioned from the purified wastewater into a concentrated form and is known as sludge (Liu, 2003). The sludge produced is a secondary solid waste and therefore needs to be disposed of appropriately (Liu, 2003). The aeration in the basin is sustained by either diffused or mechanical aeration. These techniques aid in

mixing of the wastewater to ensure efficient aeration. After retaining the wastewater for a specific time, it is passed to a secondary clarifier. In the secondary clarifier, the sludge produced is allowed to settle and a purified effluent is released. Some of the settled sludge is recycled back to the aeration basin. In this way, the activated sludge concentration is maintained (Rigopoulos and Linke, 2002).

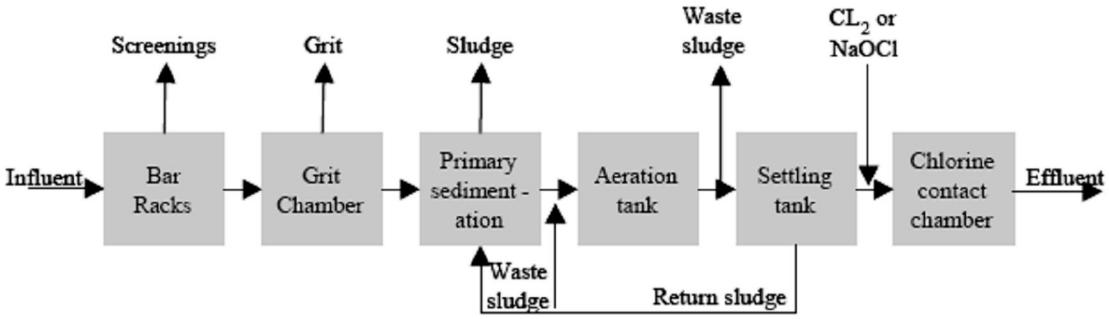


Figure 1.7. Overview of a typical activated sludge process (ESCWA, 2003).

1.6.4.2. Biological nutrient removal

The activated sludge process can be complicated further depending on the operational goals of the process. Biological nutrient removal involves removing nitrogen and phosphorus from wastewater (Kim et al., 2009). It is important to remove these nutrients from wastewater to prevent adverse impacts to the aquatic environment as it may result in a decrease of dissolved oxygen in receiving waters (ESCWA, 2003).

1.6.4.3. Nitrogen removal

Nutrient removal techniques are used to prevent eutrophication of water bodies. Two processes are used in waste water treatment plants, namely nitrification and denitrification. Nitrification transforms ammonia to nitrite or nitrate. Nitrification in wastewater is achieved

by two genera of bacteria, namely the *Nitrosomonas* and *Nitrobacter* (Andrade do Canto et al., 2008). The *Nitrosomonas* species oxidize ammonia to nitrite. On the other hand, *Nitrobacter* species converts nitrite to nitrate. Denitrification entails the removal of nitrogen by nitrate to nitrogen gas (Wicht, 1996). The nitrification and denitrification processes use both aeration and anoxic zones in the activated sludge tank (ESCWA, 2003).

1.6.5. Advanced treatment methods

Classic methods of sewage or wastewater treatments were mainly intended to remove human waste of natural origin (Daughton and Ternes, 1999). These older technologies (conventional activated sludge system) used in sewage treatment plants today are thus unable to remove the emerging contaminants such as pharmaceuticals, surfactants, heavy metals and paints. These contaminants are discharged with treated sewage effluents into rivers and surface waters and may result in adverse effects to humans and aquatic life. Several new treatment technologies have been introduced to sewage treatment plants with the hope of producing better quality effluents. Several investigations into using advanced technologies such as membrane bioreactor technology (Wintgens et al., 2002; Weiss and Reemtsma, 2008) and activated charcoal (Dash et al., 2009) to treat wastewater has been reported.

1.6.5.1. Membrane bioreactor technology

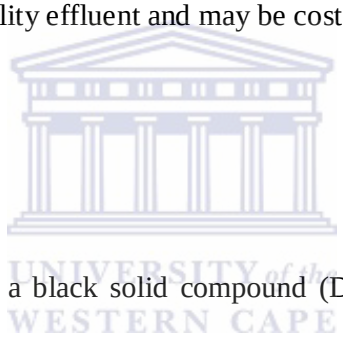
Membrane bioreactor (MBR) technology is a fairly new treatment technology implemented by sewage treatment plants. This MBR technology is used in conjunction with the activated sludge process (Le-Clech et al., 2006; González et al., 2008). The MBR technology consists of filtration membranes, namely ultra or micro filtration membranes, which are submerged

into the activated sludge mixture (Weiss and Reemtsma, 2008). These membranes are used to separate the treated wastewater from solid wastewater (Fenu et al., 2010). Pore sizes of membranes range between 0.05 to 0.4 micrometers (µm), and therefore, it is efficient in separating the sewage mixtures (Fenu et al., 2010). Several advantages of membrane bioreactor technology compared to activated sludge technology have been recognized. High sludge retention times for MBR technology compared to the activated sludge system is associated with high performance of the treatment in terms of COD removal (Cirja et al., 2008). For an activated sludge system typical solid retention times (SRT) are from 8 - 25 days, however for MBR higher SRT are from 25 ï 80 days (Winnen et al., 1996). High SRT promotes the diversity of specialized microorganisms which can potentially result in the degradation of persistent compounds (Rosenberger et al., 2002). However, in activated sludge systems these microorganisms can be washed away (Visvanathan et al., 2000). Other advantages of using MBR technology is the high removal of pathogens without chemical disinfection (Liu et al., 2009).

MBR technology produces a higher quality effluent compared to the activated sludge system alone (Sipma et al., 2010). A study by González et al. (2008) on the elimination and degradation of the surfactants APE, LAS and their degradation products in a membrane bioreactor and conventional activated sludge sewage treatment plants showed that the elimination of ethoxy chains of nonylphenol polyethoxylates (NPEO) averaged at 50 % in the activated sludge plant, whereas MBR technology eliminated 94% NPEO. On the other hand, LAS showed similar elimination in both sewage treatment plant systems. In addition, in the MBR system, ammonia, COD and total suspended solids quality was always superior compared to the activated sludge system. MBR technology also shows better removal of pharmaceuticals (Quintana et al., 2005). Though care should be taken when interpreting

results since removal efficiencies are based on differences in influent and effluent pharmaceutical levels which may not result in a true reflection of the effectiveness of sewage treatment (Sipma et al., 2010).

The use of MBR technology in wastewater treatment plants are on the increase. However, implementation of this technology is not that forthcoming because of its perceived high operating costs. Owen et al. (1995) reviewed the economics of membrane processes for water and waste water applications. It was concluded that the implementation of membranes and the costs associated with it, can be equivalent or less than using conventional systems depending on the application. Consequently, adding this technology to sewage treatment plants could provide a better quality effluent and may be cost effective.



1.6.5.2. Activated Charcoal

Activated charcoal or carbon is a black solid compound (Dwivedi et al., 2008). It can be found in a granular or a powdered form (Dias et al., 2007). This substance is produced from coconut shells; lignin, wood and bone char (Pollard et al., 1992). Factors such as surface area, micro-porous structure and surface reactivity provide the adsorptive characteristics of activated charcoal (Mohan and Pitmann Jr., 2006).

Activated carbon is used as an effective adsorbent for several pollutants such as surfactants (Xiao et al., 2005), synthetic chemicals (Zytner, 1992), and pesticides (Pelekani and Snoekyink, 2000). The removal of these potential endocrine disruptors is mainly studied in terms of their removal efficiency in various water systems and the contribution of physicochemical properties of the endocrine disruptors (Liu et al., 2009).

A study by Snyder et al. (2007) showed activated charcoal removed more than 90 % of contaminants in wastewater. However, powdered activated charcoal (PAC) was more efficient in eliminating emerging contaminants in wastewater than granular activated charcoal (GAC). PAC in wastewater plants is advantageous since it is freshly added to treatment processes as a chemical feed and is not recycled. Therefore, this adsorbent may be added seasonally to treatment processes, when risk of contaminants in sewage effluents is high (Snyder et al., 2007). On the other hand, GAC use, although effective, may allow more hydrophilic contaminants to breakthrough compared to hydrophobic contaminants. Indeed, both PAC and GAC have great potential to remove trace contaminants in sewage effluents (Snyder et al., 2007).

1.7. Objectives of the current study

Emerging contaminants or pollutants in sewage are on the increase. These contaminants include natural steroid hormones, synthetic steroid hormones, surfactants, antibiotics, illegal drugs and flame retardants. These contaminants have been known to cause harmful effects to animals and humans. Sewage treatments plants were mainly intended to operate as plants to remove human waste of natural origin. Consequently, low concentrations of emerging pollutants may still enter the environment with sewage effluents. Since older technologies (conventional activated sludge system) are not effective in removing these contaminants from wastewater, new advanced methods are needed. Many countries monitor the presence of emerging contaminants in sewage effluents. However, research and information still remain limited about the extent and impact of sewage effluent pollutants in South Africa. This study aims to compare the water quality of raw wastewater and treated sewage effluents from three different sewage treatment plants in the Western Cape, South Africa. The treatment plants investigated are on the same river system. Sewage treatment plant 1 and 2 use the older technologies (conventional activated sludge system) to treat wastewater. Sewage treatment 3 has been upgraded and new technologies (membrane bioreactor) were incorporated in the treatment processes. Sewage treatment plant 2 and 3 receive domestic effluents only. However, sewage treatment plant 1 receives both domestic and industrial raw wastewater. Raw wastewater and treated sewage effluents will be monitored for the occurrence of total coliforms, *E. coli*, antibiotics, surfactants and natural steroid hormones. In addition, the raw wastewater and treated sewage effluents will be assessed for immunotoxicity and cytotoxicity. Finally, the efficiency of activated charcoal in removing steroid hormones and surfactants from treated sewage effluents will be evaluated as a potential final step in cleaning treated sewage.

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Chapter 2: Problem Identification and Site description

Pollutants enter waters via agricultural run-off, wash-off from roadways, industrial wastewaters, municipal sewage, and domestic sewage (Bolong et al., 2009). These pollutants are treated by sewage treatment plants. However, some pollutants are not removed effectively by sewage treatment plants, resulting in their release into the environment. Furthermore, these pollutants can also enter surface water. Surface water contaminated with sewage effluents are often recycled for drinking water, recreational activities and agricultural purposes (Weinberg et al., 2004). Consequently, it is important to eliminate these pollutants to prevent potential adverse effects on human and animal health.

Classic methods of sewage or wastewater treatments were mainly intended to operate as plants to remove human waste of natural origin (Daughton and Ternes, 1999). Since older technologies (conventional activated sludge system) are not effective in removing some of the contaminants from wastewater, new advanced methods are needed. New methods of sewage treatment such as activated charcoal filtration or ozonation are being used to eradicate micropollutants (Lündstrom et al., 2010). Studies have shown that more advanced processes of sewage treatment result in reduced release of pollutants in effluents (Andreozzi et al., 2008; Teske & Arnold, 2008). Consequently, advanced treatment technologies could become a vital additional step in the treatment of sewage effluents. However, other studies demonstrated that regardless of treatment processes, the effluent produced still released the same amount of pollutants. Consequently, the effluents produced the same adverse biological effects on animals (Lundström et al., 2010).

Alarming data exist for pollutants in sewage effluents for some countries. Also, the effects of pollutants on humans and animals have been well researched (Jobling et al., 1998). In South

Africa, screening of sewage effluents for organic pollutants is however not that well established. Information still remains limited about the extent and impact of sewage effluent pollutants in South Africa. More studies are needed to provide a comprehensive picture of pollutants in South Africa.

Sewage effluent quality can be assessed by monitoring pollutants in raw wastewater and treated sewage effluents from both older plants and plants using new technologies (membrane bioreactor) for pollutant removal. The impact of these sewage effluents on the environment and health can be assessed by determining the toxicity. Therefore, the aim of this study was to compare the water quality of raw wastewater and treated sewage effluents from three different sewage treatment plants in the Western Cape, South Africa.

2.1. Hypothesis

H0- Upgraded plants do not decrease pollutants in sewage effluents compared to older plants.
H1- Upgraded plants decrease pollutants in sewage effluents compared to older plants.

2.2. Site Description

Raw wastewater and treated sewage effluents were collected from three different sewage treatment plants in the Western Cape, South Africa. The treatment plants investigated are on the same river system. Sewage treatment plant 1 and 2 use older technologies (conventional activated sludge system) to treat wastewater. Sewage treatment 3 has been upgraded and new technologies (membrane bioreactor) were incorporated in the treatment processes. Sewage treatment plant 2 and 3 receive domestic effluents only. However, sewage treatment plant 1 receives both domestic (85 % flow intake) and industrial raw wastewater (15 % flow intake).

A detailed description of sewage treatment technologies for the different sewage treatment plants are illustrated below (Figure 2.1; 2.2). The older technologies (conventional activated sludge system) used at the sewage treatment plants are divided into three processes, namely:

(i) Primary treatment which includes pre-treatment of raw wastewater by coarse and fine screens for grit removal. This process includes sedimentation tanks to allow the heavier organic particles to settle.

(ii) Secondary treatment of raw wastewater using activated sludge. This process involves using aerated biological digestion by bacteria to remove remaining suspended and dissolved material. In addition, nitrification and de-nitrification of wastewater is also used as treatment processes within the sewage treatment plants. Thereafter, the wastewater enters the secondary sedimentation tank to allow separation of the liquid and solid phase. After secondary sedimentation the wastewater enters maturation ponds for further pathogen removal.

(iii) Tertiary treatment is the final step in the conventional activated sludge system used by sewage treatment plant 1 and 2. Ultraviolet light (used only at sewage treatment plant 1) or chlorine (used only at sewage treatment plant 2) are the disinfection processes used, before the treated sewage effluent enter the receiving waters.

Sewage treatment plant 3 uses an additional treatment technology (membrane bioreactor) concurrently with conventional or older treatment technologies as seen in Figure 2.2. The membrane bioreactor technology consists of microporous membranes. These micro-filtration and ultra-filtration membranes separate liquid and solids.

Water collected from the Eerste River in Jonkershoek, Stellenbosch, South Africa (33º55ô51ôôS, 18º51ô16ôôE) was used as a negative control site. This site is situated in the Stellenbosch mountain and there is no human activity upstream from this area.

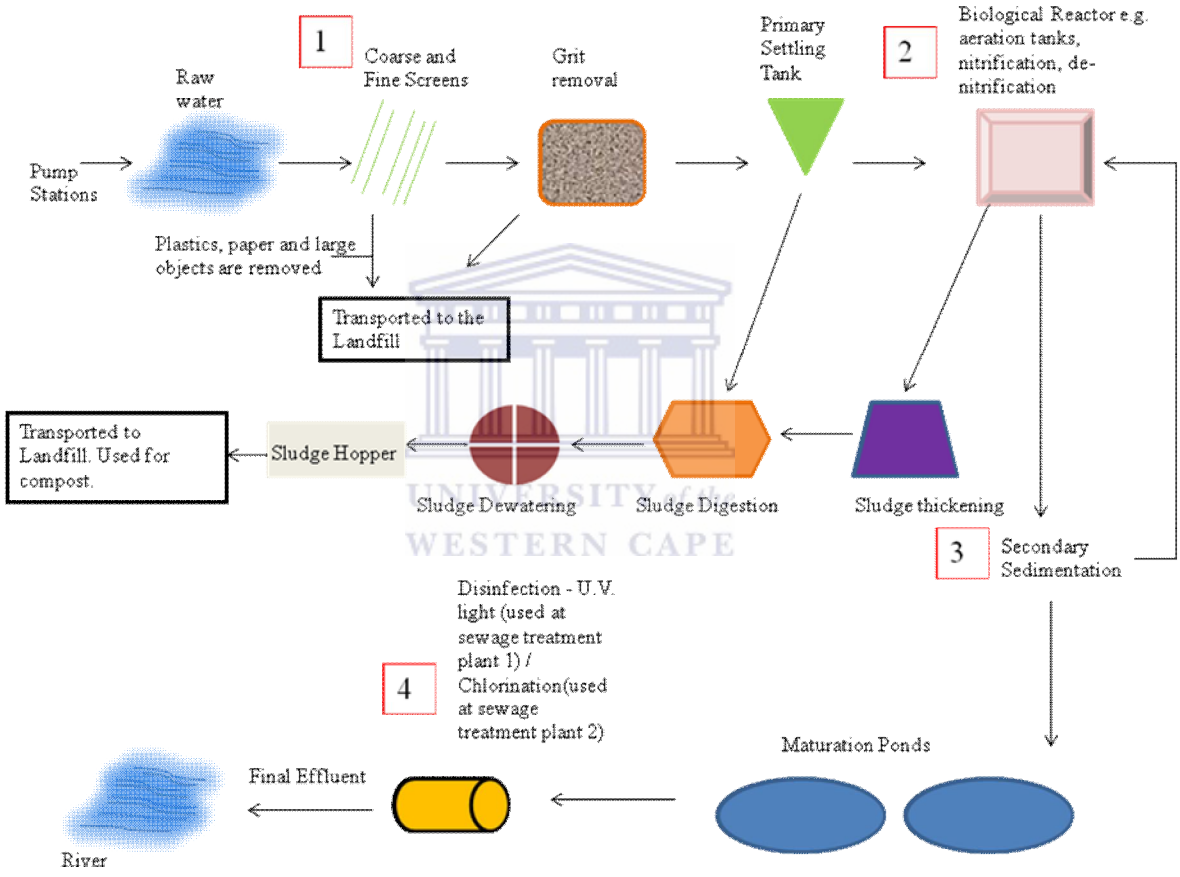

Figure 2.1. Older sewage treatment plant technologies (conventional activated sludge system) used at sewage treatment plant 1 and 2.

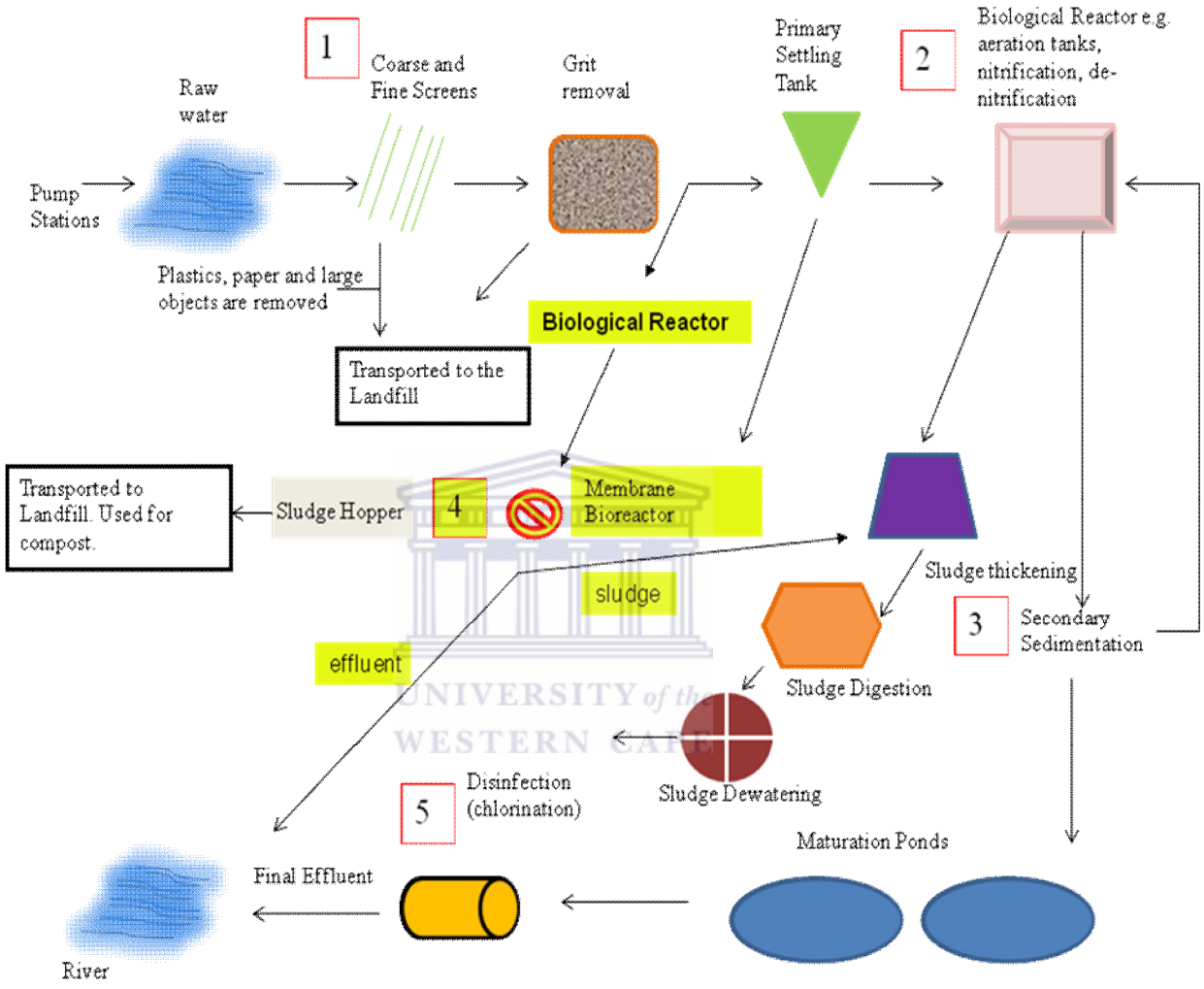

Figure 2.2. Sewage treatment plant 3 uses an additional treatment technology (membrane bioreactor, 4) concurrently with conventional or older treatment technologies.

2.3. References

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Chapter 3: The effectiveness of sewage treatment processes to remove faecal pathogens and antibiotic residues

3.1. Abstract

Pathogens and antibiotics in the aquatic environment are increasing due to increased population. These contaminants enter the aquatic environment via sewage effluents and may pose a health risk to wild life and humans. Monitoring the occurrence and removal of these contaminants in sewage effluents are vital in protecting the environment. The aim of this study was to determine the levels of faecal bacteria, and selected antibiotic residues in raw wastewater and treated sewage effluents from three different sewage treatment plants in the Western Cape, South Africa. The treatment plants investigated are on the same river system. Sewage treatment plant 1 and 2 use older technologies, while sewage treatment plant 3 has been upgraded and membrane technologies were incorporated in the treatment processes. Coliforms and *E. coli* were used as bioindicators for faecal bacteria. A chromogenic test was used to screen for coliforms and *E. coli*. ELISAs were used to quantitate antibiotic residues in raw and treated sewage. Raw intake water at all the sewage treatment plants contained total coliforms and *E. coli.* High removal of *E. coli* by sewage treatment processes was evident for sewage treatment plant 2 and 3. Sewage treatment technologies at sewage treatment plant 1 were ineffective in removing total coliforms and *E. coli*. Fluoroquinolones and sulfamethoxazole are commonly used antibiotics and were selected to monitor the efficiency of sewage treatment processes for antibiotic removal. Fluoroquinolones and sulfamethoxazole were detected in raw wastewater from all sewage treatment plants. Sewage treatment plant processes at sewage treatment plant 1 did not reduce the fluoroquinolone

concentration in treated sewage effluents. Sewage treatment plant processes at sewage treatment plant 2 and 3 reduced the fluoroquinolone concentration by 21 % and 31 % respectively. The reduction of fluoroquinolone by the sewage treatment plants was not statistically significant and residual concentrations were released with treated sewage effluents. Sewage treatment processes at sewage treatment plant 1 did not reduce the sulfamethoxazole concentration in treated sewage effluents. Sewage treatment processes at sewage treatment plant 2 and 3 reduced sulfamethoxazole by 34 % and 56 %, respectively. The reduction of sulfamethoxazole was not statistically significant and residual concentrations were released with treated sewage effluents. This study showed that bacteria and antibiotic residues are still discharged into the environment. The release of these contaminants may pose a threat to aquatic and human life. Further research needs to be undertaken to improve sewage treatment technologies, thereby producing a better quality treated sewage effluent.

3.2. Introduction

Faecal contaminants enter environmental water via various routes. Non-human faecal contamination can occur by domestic animals such as dogs and cats (Whitlock et al., 2002). Other significant sources of faecal contamination to environmental water are via rats, beavers, gulls, waterfowl and pigeons (Siewick et al., 2007).

Humans and other warm-blooded animals have coliforms as intestinal flora. These coliforms are excreted and are discharged to be treated by municipal sewage treatment plants. However, if the wastewater remains untreated, bacterial pathogens present in the sewage effluents can result in diseases such as dysentery, typhoid, and gastroenteritis upon exposure to the contaminated water (Rose et al., 1996). Inefficient treatment processes result in microorganisms being released with treated effluents in the aquatic environment (George et al., 2002). The effluents then enter aquatic ecosystems and become a major source of faecal contamination. Faecal contaminants pose a health risk to humans and animals upon exposure to contaminated water (George et al., 2002). Monitoring faecal contamination of sewage could provide valuable information on urban land uses and potential routes of faecal contamination (Young and Thackston, 1999; Whitlock et al., 2002). Indicator organisms to monitor bacteriological quality of water include *Escherichia coli* (*E. coli*) and coliforms (Rompé et al., 2002).

Various methods can be employed to examine faecal contamination of water sources. The classical methods used to screen faecal contaminants include the multiple-tube fermentation (MTF) technique and the membrane filter technique (MFT). Briefly, the MTF technique is carried out using different dilutions of the water sample in test tubes. After 48 hours of incubation, gas production, acid formation and growth of organisms can then be determined.

A confirmatory test for the target organisms then follows a presumptive positive reaction (Rompé et.al 2002). On the other hand, the MFT consists of filtering a water sample using a sterile filter (0.45µm). This filtering technique traps bacteria on the filter. The filter can then be cultured on selective media and enumeration can be done (Rompé et al., 2002).

The classical methods used have several advantageous and disadvantageous characteristics. For instance, the MTF method allows for semi-quantitative enumeration of coliforms but is labour intensive. The MTF method is also time consuming and a subculture stage for confirmation is needed (Rompé et al., 2002).

Chromogenic tests to monitor total coliforms and *E. coli* are commercially available. Chromogenic tests are effective and are able to detect total coliforms and *E. coli* in different water sources. In addition, these tests take advantage of enzymatic properties of coliforms. These tests are specific and only total coliforms and *E. coli* that feed on defined substrate nutrients in the medium can release a chromogen or fluorochrome. Chromogen or fluorochrome production indicates the presence of the microbes (Rompé et al., 2002). These tests are easy and rapid to use and can save on costs (Rompé et al., 2002).

Modern disease management strategies have resulted in increased pharmaceutical use, particularly the use of antibiotics. Additionally, antibiotics are also used in veterinary medicine (Hirsch et al., 1999). In humans and animals, antibiotics exit via urine or faeces. Antibiotics are not always metabolized and a large amount of biologically active ingredients are discharged with urine and faeces. These unchanged or partially metabolized antibiotics then enter sewage where it may either be eradicated by sewage treatment processes or released with sewage effluents into the aquatic environment (Hirsch et al., 1999). Antibiotic residues in the environment could elicit potential adverse consequences such as bacterial resistance. Moreover, antibiotics and their metabolites could display synergism or additional

unintended effects and pose a health risk to aquatic species and consumers of the contaminated water (Gulkowska et al., 2008).

4-Quinolones and synthetic pharmaceuticals such as fluoroquinolones, quinolones and quinolone carboxylic acids are used extensively as antibiotics in human and veterinary medicine (Martinez et al., 2006). Fluoroquinolones have a broad spectrum of activity and enhanced pharmokinetic properties (Picó and Andreu, 2007). Some of the flouroquinolone antibiotics include ciprofloxacin, ofloxacin, levofloxacin and norfloxacin (Picó and Andreu, 2007). Fluoroquinolones have been found in raw and treated sewage effluents (Golet et al., 2003; Vieno et al., 2007). The release of fluoroquinolones into the environment can have adverse effects on aquatic microorganisms (Lindberg et al., 2007).

The sulphonamides are components of sulfanilamide (Zhang and Wang, 2009). One of the sulphonamide antibiotic residues include sulfamethoxazole. Sulfamethoxazole is used extensively as an antimicrobial in animals and humans (Peng et al., 2006). Sulfamethoxole can be discharged into the environment, via sewage effluents, where it remains persistent (Hong et al., 2008).

Many countries are monitoring the presence, removal and fate of contaminants in raw wastewater and treated sewage effluents (Ternes, 1998; Ternes et al., 1999). In South Africa several studies have focussed on the presence of bacteria in sewage effluents (Samie et al., 2009; Dungeni and Momba, 2010; Omar and Barnard, 2010). Little is known about other contaminants in wastewater and treated effluents from sewage treatment plants in South Africa. The National Water Act of South Africa (Act no. 36 of 1998) consists of several chapters. In particular Chapter 3, Part 4, deals with pollution prevention. Certain requirements need to be implemented by the owner of the properties where activities or processes occur that can result in pollution of a water source. Measures include containing

and preventing the release of pollutants into the environment, eliminating any sources of pollutants and to remedy the effects of the pollution.

The South African constitution also has several acts that pertain to environmental rights. Section 24 (a) states that: ñ Every human has the right to an environment that is not harmful to human health or well-beingò. The constitution further states in Section 24 (b) that: ñ Everyone has the right to have the environment protectedò. Water is a scarce commodity and needs to be protected to ensure sustainable usage. The aim of this study was to determine the occurrence of faecal bacteria, and antibiotic residues in raw and treated sewage effluents from three different sewage treatment plants in the Western Cape, South Africa. Total coliforms and *E. coli* were used as bioindicators to evaluate the effectiveness of the treatment plant disinfection processes. Fluoroquinolone and sulfamethoxazole are commonly used antibiotics and were used to monitor the efficiency of sewage plants to remove antibiotics.

3.3. Materials and Methods

3.3.1. Site Description and water collection

Raw wastewater and treated sewage effluents were collected from three different sewage treatment plants in the Western Cape, South Africa. The treatment plants investigated are on the same river system. Sewage treatment plant 1 and 2 use older technologies (conventional activated sludge system) to treat wastewater. Sewage treatment 3 has been upgraded and new technologies (membrane bioreactor) were incorporated in the treatment processes. Sewage treatment plant 2 and 3 receives domestic effluents only. However, sewage treatment plant 1 receives both domestic (85 % flow intake) and industrial raw wastewater (15 % flow intake).

A detailed description of sewage treatment technologies for the different sewage treatment plants are as follows. The older technologies (conventional activated sludge system) used at the sewage treatment plants can be divided into three processes, namely:

(i) Primary treatment which includes pre-treatment of raw waste water by coarse and fine screens for grit removal. This process includes sedimentation tanks to allow the heavier organic particles to settle.

(ii) Secondary treatment of raw water using activated sludge. This process involves aerated biological digestion by bacteria to remove remaining suspended and dissolved material. In addition, nitrification and de-nitrification of wastewater is also used as treatment processes within the sewage treatment plants. Thereafter, the wastewater enters the secondary sedimentation tank to allow separation of the liquid and solid phase. After secondary sedimentation the wastewater enters maturation ponds for further pathogen removal.

(iii) Tertiary treatment is the final step in the conventional activated sludge system used by sewage treatment plant 1 and 2. Ultraviolet light (used only at sewage treatment plant 1) or chlorine (used only at sewage treatment plant 2) are the disinfection processes used, before the treated sewage effluent are released from plants.

Sewage treatment plant 3 uses an additional treatment technology (membrane bioreactor) concurrently with conventional or older treatment technologies. The membrane bioreactor technology consists of microporous membranes. These micro-filtration and ultra-filtration membranes separate liquid and solids.

Water collected from the Eerste River in Jonkershoek, Stellenbosch, South Africa (33º55ô51ôôS, 18º51ô16ôôE) was used as a negative control. This site is situated in the Stellenbosch mountain and there is no human activity upstream from this area.

Samples were collected in pre-cleaned 1 Liter (1 L) plastic bottles and transported to the laboratory in a cooler.

3.3.2. Monitoring of total coliforms and *E. coli* in wastewater samples

Raw wastewater and treated sewage effluents from all sewage treatment plants were collected over a four week sampling period, during winter (21 June 2010 ï 12 July 2010). Total coliforms and *E. coli* in wastewater samples were monitored by using the Readycult Coliforms 100 test (Merck, Germany). The test was performed according to the manufacturerôs instructions. The Readycult Coliforms 100 is a chromogenic test that simultaneously detects total coliforms and *E. coli*. Tests for total coliforms and *E. coli* were done using 10 ml, 1 ml or 0.1 ml of water samples. Raw wastewater and treated sewage

effluent samples were incubated overnight at 37 °C, before analysis. Coliforms are indicated by a yellow to blue-green colour change of medium, while fluorescence under U.V. light is indicative of *E. coli* in the sample. To confirm the presence of *E. coli*, Kovacôs reagent was used.

3.3.3. Solid Phase Extraction of water samples

Samples were filtered with filter paper (Munktell, 15 µm, 240 mm) (Lasec, SA) before extraction. Water samples were then extracted using C-18 columns (Sigma Aldrich, South Africa). Columns were conditioned with 2 ml of Phase B mixture (45 % methanol, 40 % hexane and 15 % propanol), then 2 ml ethanol and lastly 4 ml distilled water. After the washing step, 100 ml of water sample was passed through the column. The columns were then dried using a vacuum pump (PALL vacuum pump, LifeSciences, 60 Hz, 1.92 Amperes, 220-240 Volts). The hydrophobic analytes attached to the resin were eluted with 2 ml of Phase B mixture. The eluates were dried under a stream of air. The dried eluate was reconstituted with dimethyl sulfoxide (DMSO) to make a 1000 times concentrated sample stock solution. Extracts were diluted in 10 % methanol at a ratio of 1:100 for the fluoroquinolone ELISA.

3.3.4. Fluoroquinolone analysis of raw wastewater and treated sewage effluent extracts

Fluoroquinolone ELISA kits were purchased from Abraxis, Warminister, PA. This ELISA cross-reacts with the fluoroquinolones Enroflaxacin (100 %) and Danofloxacin (100 %). Samples were analyzed according to the instructions included in the kit. All reagents required were supplied in the kit. The ELISA plate was precoated with antibodies specific to

a unique antigenic site on the fluoroquinolone molecule. Samples or standards and fluoroquinolone enzyme conjugate were pre-mixed in an uncoated microplate (100 εl of each solution). Thereafter, 100 εl of the pre-mixture was transferred per well of the coated plate. The plate was then incubated for 1 hour at room temperature. Thereafter, the wells were washed five times with wash solution and tapped dry. After washing, 100 εl of substrate was added to all wells and incubated for 30 minutes at room temperature. The enzyme reaction was stopped by adding 100 εl of stop solution to all wells. The optical density was read at 450 nm with a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). The 0 µg/L standard results in maximum binding of the enzyme conjugate. All data was expressed as a percentage of 0 µg/L standard. A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

3.3.5. Sulfamethoxazole analysis of raw wastewater and treated sewage effluents

Raw wastewater and treated sewage effluents were sterilized with 0.45 µm sterile filters (Lasec, SA) prior to use in the sulfamethoxazole ELISA. Sulfamethoxazole ELISA kits were purchased from Abraxis, Warminister, PA. This ELISA displays 100 % cross-reactivity with sulfamethoxazole. Samples were analyzed according to the instructions included in the kit. All reagents required were supplied in the kit. The ELISA plate was precoated with antibodies specific to a unique antigenic site on the sulfamethoxazole molecule. Samples or standards were added to the precoated microplate (75 εl/well). Thereafter, 50 εl/well of the anti-sulfamethoxazole antibody solution were added to the microplate. The contents of the wells were then mixed for 20-30 seconds. After mixing, the plate was incubated at room temperature for 20 minutes. After the incubation period, 50 εl/well of the sulfamethoxazole

enzyme conjugate solution was added to each well of the microplate. After mixing as before, the plates were then incubated for 40 minutes at room temperature. Thereafter, the wells were washed four times with wash solution and tapped dry. After washing, 150 εl of substrate solution was added to all wells and incubated for 30 minutes at room temperature. The enzyme reaction was stopped by adding 100 εl of stop solution to all wells. The optical density was then read at 450 nm with a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). The 0 µg/L standard results in maximum binding of the enzyme conjugate. All data was expressed as a percentage of 0 µg/L standard. A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

3.3.6. Statistical analysis

One way analysis of variance (ANOVA) was used to compare results for the antibiotic assays, with $P<0.050$ considered as significant. Statistical analysis was done using SigmaPlot Version 11.

3.4. Results

3.4.1. The detection of total coliforms and *E. coli*

The Readycult Coliforms 100 is a chromogenic test that simultaneously detects total coliforms and *E. coli*. A yellow to a green-blue colour change of the culture broth indicates the presence of total coliforms. Fluorescence of the broth under ultraviolet light indicates the presence of *E. coli*. Confirmation of the presence of *E. coli* was further done by addition of Kovac`s reagent (Merck, Germany) to the broth. Table 3.1 shows the detection of total coliforms and *E. coli* in raw wastewater and treated sewage effluents from all sewage treatment plants over the four week sampling period.

For the Jonkershoek negative control sample 1-10 CFU/100ml of total coliforms was detected at each of the collection times. However, *E. coli* was not found in the Jonkershoek negative control samples. All the raw wastewater samples tested positive with more than 1000 CFU/100ml total coliforms and *E. coli* detected.

Total coliforms and *E. coli* were detected at levels more than 1000 CFU/100ml in treated sewage effluent for sewage treatment plant 1. The total coliforms and *E. coli* levels in treated sewage effluents from sewage treatment plant 2 were less than 1 CFU/100ml. Total coliforms were detected at 1-10 CFU/100ml in treated sewage effluents from sewage treatment plant 3. This is similar to the levels found in the Jonkershoek negative control water sample. The *E. coli* levels in treated sewage effluents produced by sewage treatment plant 3 were less than 1 CFU/100ml.

3.4.2. Detection of fluoroquinolones in raw wastewater and treated sewage effluents from the three sewage treatment plants

Raw wastewater and treated sewage effluents from all sewage treatment plants were analysed for the presence of fluoroquinolones. The standard curve for the fluoroquinolone ELISA is shown in Figure 3.1. There is a good inverse correlation (R^2 = 0.9871) between the percentage of the maximum binding and the log of fluoroquinolone concentration. Results for the detection of fluoroquinolones in raw wastewater and treated sewage effluents from all sewage treatment plants are illustrated in Tables 3.2, 3.3, and 3.4, respectively. Concentrations of fluoroquinolones are represented as Mean ± Standard deviation (SD). The percentage reduction of fluoroquinolones from raw wastewater to treated sewage effluents are also given in the tables. Very low levels of fluoroquinolones were detected in the Jonkershoek negative control (below (<) Limit of detection, LOD = 0.016 ng/ml).

Fluoroquinolones detected in both domestic and industrial raw wastewater from sewage treatment plant 1 were 90 ± 24 ng/L and 89 ± 28 ng/L. The combined concentration of fluoroquinolone for the mixture of domestic and industrial raw wastewater from sewage treatment plant 1 was 90 ± 19 ng/L. Fluoroquinolone concentration in domestic and industrial raw wastewater and the combined mixture concentration was higher when compared to the Jonkershoek negative control ($P<0.050$). Fluoroquinolone concentration in treated sewage effluents from sewage treatment plant 1 was 92 ± 29 ng/L. There was no difference in the fluoroquinolone concentration of the domestic raw wastewater, industrial raw wastewater, the combined mixture and treated sewage effluents from sewage treatment plant 1. Fluoroquinolone concentration in the treated sewage effluents was significantly higher compared to the Jonkershoek negative control ($P<0.050$). The conventional activated

sludge process at sewage treatment plant 1 reduced the fluoroquinolone concentration by 2 %.

Fluoroquinolone concentration detected in raw wastewater from sewage treatment plant 2 was 92 ± 11 ng/L. Fluoroquinolone concentration in the raw wastewater were higher when compared to the Jonkershoek negative control ($P<0.050$). Fluoroquinolone concentration in treated sewage effluents from sewage treatment plant 2 was 72 ± 34 ng/L. There was no difference in the fluoroquinolone concentration of the raw wastewater and treated sewage effluents from sewage treatment plant 2. Fluoroquinolone concentration in the treated sewage effluents was significantly higher compared to the Jonkershoek negative control ($P<0.050$). The conventional activated sludge process at sewage treatment plant 2 reduced the fluoroquinolone concentration by 21 %.

Fluoroquinolone concentration detected in raw wastewater from sewage treatment plant 3 was 99 ± 11 ng/L. Fluoroquinolone concentration in the raw wastewater was higher when compared to the Jonkershoek negative control ($P<0.050$). Fluoroquinolone concentration in treated sewage effluents was 68 ± 33 ng/L. There was no difference in the fluoroquinolone concentration of raw wastewater and treated sewage effluents from sewage treatment plant 3. Fluoroquinolone concentration in the treated sewage effluents was significantly higher compared to the Jonkershoek negative control ($P<0.050$). The membrane bioreactor process at sewage treatment plant 3 reduced the fluoroquinolone concentration by 31 %.

3.4.3. Detection of sulfamethoxazole in raw wastewater and treated sewage effluents from the three sewage treatment plants

Raw wastewater and treated sewage effluents from all sewage treatment plants were analysed for the presence of the antibiotic sulfamethoxazole. The standard curve for the sulfamethoxazole ELISA is shown in Figure 3.2. There is a good inverse correlation (R^2 = 0.9775) between the percentage of the maximum binding and the log of sulfamethoxazole concentration. Results for the detection of sulfamethoxazole in raw wastewater and treated sewage effluents in all sewage treatment plants are illustrated in Tables 3.2, 3.3, and 3.4, respectively. Concentrations of sulfamethoxazole are represented as Mean ± Standard deviation (SD). The percentage reduction of sulfamethoxazole from raw wastewater to treated sewage effluents are also given in the tables. Very low levels of sulfamethoxazole were detected in the Jonkershoek negative control (< LOD =0.0015 ng/L).

The sulfamethoxazole concentration detected in domestic and industrial raw wastewater from sewage treatment plant 1 were 111 ± 4 ng/L and 156 ± 12 ng/L, respectively. The combined concentration of sulfamethoxazole for the mixture of domestic and industrial raw wastewater from sewage treatment plant 1 was 118 ± 3 ng/L. Sulfamethoxazole concentration in domestic and industrial raw wastewater, and the combined mixture was higher when compared to the Jonkershoek negative control ($P<0.050$). Sulfamethoxazole concentration in treated sewage effluents from sewage treatment plant 1 was 121 ± 28 ng/L. There was no difference in the sulfamethoxazole concentration of the domestic raw wastewater, industrial raw wastewater and treated sewage effluents of sewage treatment plant 1. Sulfamethaxole concentration in the treated sewage effluents was significantly higher compared to the

Jonkershoek negative control (P<0.050). The conventional activated sludge process at sewage treatment plant 1 reduced the sulfamethoxazole concentration by 4 %.

Sulfamethoxazole concentration detected in raw wastewater from sewage treatment plant 2 was 153 ± 7 ng/L. The sulfamethoxazole concentration in the raw wastewater was higher when compared to the Jonkershoek negative control (P<0.050). Sulfamethoxazole concentration in treated sewage effluents from sewage treatment plant 2 was 101 ± 44 ng/L. Sulfamethoxazole concentration in raw wastewater was significantly higher than levels found in the treated sewage effluents for sewage treatment plant 2 (P<0.050). Sulfamethaxole concentration in the treated sewage effluents was significantly higher compared to the Jonkershoek negative control (P<0.050). The conventional activated sludge process at sewage treatment plant 2 reduced the sulfamethoxazole concentration by 34 %.

The concentration of sulfamethoxazole detected in raw wastewater from sewage treatment plant 3 was 170 ± 4 ng/L. Sulfamethoxazole concentration in the raw wastewater was higher when compared to the Jonkershoek negative control (P<0.050). Sulfamethoxazole concentration in the treated sewage effluents from sewage treatment plant 3 was 76 ± 23 ng/L. Sulfamethoxazole concentration in raw wastewater was significantly higher than levels found in the treated sewage effluents for sewage treatment plant 3 (P<0.050). Sulfamethaxole concentration in the treated sewage effluents was significantly higher compared to the Jonkershoek negative control (P<0.050). The membrane bioreactor process at sewage treatment plant 3 reduced the sulfamethoxazole concentration by 56 %.

Table 3.1. Detection of total coliforms and E. coli in raw wastewater and treated sewage effluents from three sewage treatment plants in the Western Cape, South Africa. Y = the detection of coliforms or E. coli, N= no detection of coliforms or E. coli.

		Week 1		Week 2		Week 3		Week 4	
Sample name	**Sample vol. (ml)**	**Coliforms**	***E. coli***	**Coliforms**	***E. coli***	**Coliforms**	***E. coli***	**Coliforms**	***E. coli***
Jonkershoek (control site)	10	Y	N	N	N	Y	N	Y	N
	1	N	N	N	N	N	N	N	N
	0.1	N	N	N	N	N	N	N	N
STP 1 Domestic Raw water	10	Y	Y	Y	Y	Y	Y	Y	Y
	1	Y	Y	Y	Y	Y	Y	Y	Y
	0.1	Y	Y	Y	Y	Y	Y	Y	Y
STP 1 Industrial Raw water	10	Y	Y	Y	Y	Y	Y	Y	Y
	1	Y	Y	Y	Y	Y	Y	Y	Y
	0.1	Y	Y	Y	Y	Y	Y	Y	Y
STP 1 Final Effluent	10	Y	Y	Y	Y	Y	Y	Y	Y
	1	Y	Y	Y	Y	Y	Y	Y	Y
	0.1	Y	Y	Y	Y	Y	Y	Y	Y
STP 2 Raw water	10	Y	Y	Y	Y	Y	Y	Y	Y
	1	Y	Y	Y	Y	Y	Y	Y	Y
	0.1	Y	Y	Y	Y	Y	Y	Y	Y
STP 2 Final Effluent	10	N	N	N	N	N	N	N	N
	1	N	N	N	N	N	N	N	N
	0.1	N	N	N	N	N	N	N	N
STP 3 Raw water	10	Y	Y	Y	Y	Y	Y	Y	Y
	1	Y	Y	Y	Y	Y	Y	Y	Y
	0.1	Y	Y	Y	Y	Y	Y	Y	Y
STP 3 Final Effluent	10	Y	Y	Y	N	Y	N	Y	N
	1	N	N	N	N	N	N	N	N
	0.1	N	N	N	N	N	N	N	N

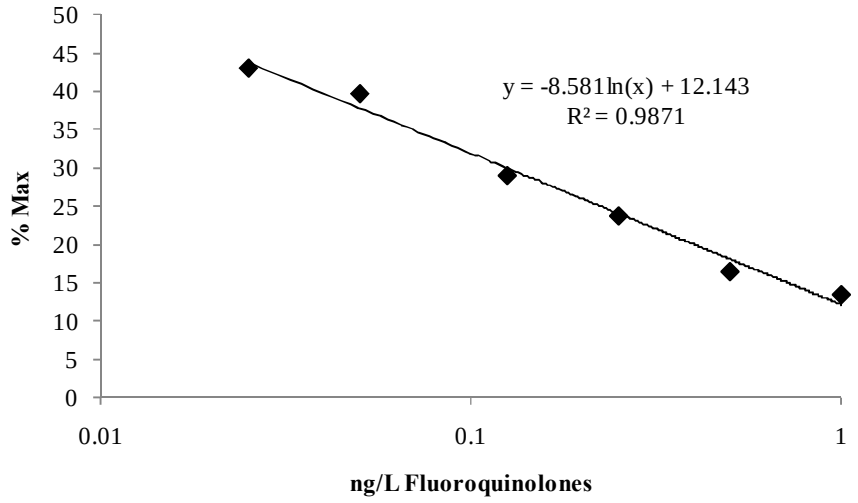

Figure 3.1. Standard curve obtained for the fluoroquinolone ELISA.

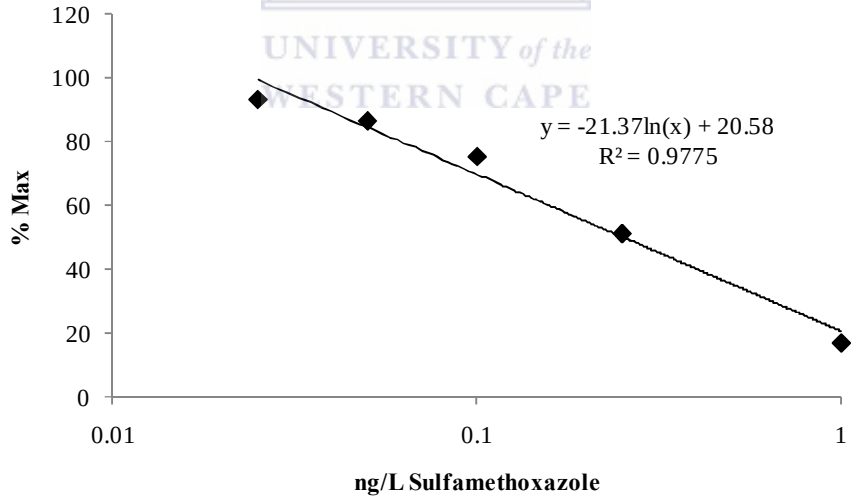

Figure 3.2. Standard curve obtained for the sulfamethoxazole ELISA.

Table 3.2. Mean concentration (ng/L ± SD) of selected antibiotics found in domestic and industrial raw wastewater and treated sewage effluents for sewage treatment plant 1 (n=8). Sewage treatment plant 1 uses the conventional activated sludge system as wastewater treatment processes. Water collected at Jonkershoek was used as a negative control sample. This site is not impacted by human activity.

Selected Antibiotics	Jonkershoek Negative Control	SEWAGE TREATMENT PLANT 1				
		Domestic Raw Wastewater	Industrial Raw Wastewater	Calculated value of mixture	Treated Sewage Effluents	Percentage reduction (%)
Fluoroquinolones (ng/L)	2 ± 2	90 ± 24 [a]	89 ± 28 [a]	90 ± 19 [a]	92 ± 29 [a]	2 ± 10
Sulfamethoxazole (ng/L)	0 ± 0	111 ± 4 [a]	156 ± 12 [a]	118 ± 3 [a]	121 ± 28 [a]	4 ± 1

[a] Statistically different to negative control (P<0.050).

Table 3.3. Mean concentration (ng/L ± SD) of selected antibiotics found in raw wastewater and treated sewage effluents for sewage treatment plant 2 (n=8). Sewage treatment plant 2 uses the conventional activated sludge system as wastewater treatment processes. Water collected at Jonkershoek was used as a negative control sample. This site is not impacted by human activity.

Selected Antibiotics	Jonkershoek Negative Control	SEWAGE TREATMENT PLANT 2		
		Raw Wastewater	Treated Sewage Effluents	Percentage reduction (%)
Fluoroquinolones (ng/L)	2 ± 2	92 ± 11 [a]	72 ± 34 [a]	21 ± 5
Sulfamethoxazole (ng/L)	0 ± 0	153 ± 7 [ab]	101 ± 44 [a]	34 ± 9

[a] Statistically different to negative control (P<0.050).

[b] Statistically different to treated sewage effluents (P<0.050).

Table 3.4. Mean concentration (ng/L ± SD) of selected antibiotics found in raw wastewater and treated sewage effluents for sewage treatment plant 3 (n=8). Sewage treatment plant 3 uses the newer membrane technology as an additional wastewater treatment process. Water collected at Jonkershoek was used as a negative control sample. This site is not impacted by human activity.

Selected Antibiotics	Jonkershoek Negative Control	SEWAGE TREATMENT PLANT 3		
		Raw Wastewater	Treated Sewage Effluents	Percentage reduction (%)
Fluoroquinolones (ng/L)	2 ± 2	99 ± 11 [a]	68 ± 33 [a]	31 ± 3
Sulfamethoxazole (ng/L)	0 ± 0	170 ± 4 [ab]	76 ± 23 [a]	56 ± 9

[a] Statistically different to negative control (P<0.050).

[b] Statistically different to treated sewage effluents (P<0.050).

3.5. Discussion

Treated sewage effluents containing residual pollutants are often discharged into surface water. These effluents can contribute to the pathogens in the environment (Ottoson et al., 2006). A group of bacteria, known as the coliforms are used to monitor the microbiological quality of water (Wutor et al., 2009). The occurrence of non pathogenic faecal coliforms in water can indicate the occurrence of pathogenic microorganisms that are of faecal origin (Wutor et al., 2009). One of the main bacterial indicators of faecal contamination is *E. coli* (Molleda et al., 2008). Studies have shown that gastrointestinal and respiratory diseases are linked to polluted waters that have increased numbers of indicator bacteria (Thurston et al., 2001). Monitoring the bacteriological quality of water is an important parameter to limit these diseases.

In this study, total coliforms and *E. coli* were detected in raw wastewater from all sewage treatment plants. Since wastewater from homes, hospitals and commercial buildings collects in sewers and flows to sewage treatment plants, high faecal bacteria counts were expected in the raw sewage (Samie et al., 2009).

High loads of total coliforms and *E. coli* present in treated sewage effluents from sewage treatment plant 1, show that the treatment processes and disinfection by the U.V. light at this plant are ineffective in removing faecal bacteria. The maximum for no risk is 0 CFU/100 ml for faecal coliforms and 10 CFU/100 ml for total coliforms (DWAF, 1998). Consequently these guidelines set out by the Department of Water Affairs and Forestry of South Africa, DWAF (1998) imply that the treated sewage effluents from sewage treatment plant 1 is of poor microbiological quality. The results of this study confirm data obtained in previous

studies that have shown that U.V. light disinfection of sewage effluent does not reduce microbial populations as effectively as disinfection by chlorine (Guo et al., 2009).

Treatment technologies employed by sewage treatment plant 2 are similar to that of sewage treatment plant 1, except at the tertiary treatment where chlorination is used instead of U.V. disinfection. The treated sewage effluents produced by sewage treatment plant 2 was of acceptable microbiological quality with both total coliforms and *E. coli* below the recommended levels. However, studies have shown that other properties play a role in treatment of wastewater. Conductivity, pH, dissolved oxygen, nitrogen and phosphate content may have an effect on bacterial communities present in sewage (Moura et al., 2007; Samie et al., 2009). Sewage effluent treatment with chlorine may have adverse effects on aquatic life. Chlorination results in the formation of some toxic by-product formation and these can have adverse effects on the aquatic life (Brungs, 1973). The disinfection by-product 2,2,4-trichloro-5-methoxycyclopenta-4-ene-1,3-dione (TCMCD) was found to induce mortality in zebrafish embryos and may potentially be mutagenic to humans (Shen et al., 2010).

E. coli was not detected in treated sewage effluents from sewage treatment plant 3. This therefore implies that the membrane bioreactor technology employed by the plant was effective in removing *E. coli* from sewage. These results are consistant with studies that showed high removal rates of *E. coli* from sewage upon membrane bioreactor treatment (Bolzonella et al., 2010).

The global consumption of antimicrobials is estimated to be between 100 000 and 200 000 tons per year (Senta et al., 2008). The occurrence of pharmaceuticals in raw wastewater is dependant on different factors (Vieno et al., 2007). For instance, the total consumption of antibiotics by different populations and countries may vary.

Fluoroquinolones are the most widely prescribed antibiotics (Picó and Andreu, 2007). Fluoroquinolones were detected in all raw wastewater samples. The levels detected ranged from 89 ng/L to 92 ng/L. Seasonal variations in antibiotic levels in sewage can occur. The samples in this study was collected during winter. Moreover, during winter months people are more likely to become sick and therefore increased levels of antibiotics are prescribed. Castiglioni et al. (2004) has shown that antibiotic use in winter is considerably more than in summer. However, the levels of antibiotics can differ between sewage treatment plants at different time periods (Tixier et al., 2003).

This study shows that fluoroquinolones were not effectively eliminated by the treatment processes at the three sewage treatment plants investigated (Table 3.2, 3.3, 3.4). Treated sewage effluents contained significantly higher fluoroquinolones than the Jonkershoek negative control site ($P < 0.050$). No significant difference in fluoroquinolone concentration between raw wastewater and treated sewage effluents were found, indicating that sewage treatment processes used by the three plants are inefficient at removing this antibiotic from sewage. The results show that high loads of fluoroquinolones are discharged into the environment. The results of this study are consistent with previous studies that showed high levels of fluoroquinolones in effluents from sewage treatment plants (Nakata et al., 2005). Fluoroquinolones have been measured in sewage treatment plant effluents in European countries such as France (300 - 500 ng/L); Italy (300 - 500 ng/L); Greece (500 ng/L) and Switzerland (30 - 1100 ng/L) (Nakata et al., 2005).

The type of treatment technology used may aid in the removal of antibiotics from wastewater (Xu et al., 2007). In this study sewage treatment plant 1 and 2 use the conventional activated sludge process only for treatment. In addition to the conventional activated sludge process, sewage treatment plant 3 also use membrane bioreactor technology for sewage treatment.

The percentage reduction of fluoroquinolones differed according to the sewage treatment processes used. A 2 % and 21 % reduction of fluoroquinoloes for the conventional activated sludge processes at sewage treatment plant 1 and 2 were calculated. For the membrane bioreactor technology at sewage treatment plant 3, a calculated value of 31 % was found. These results indicate that despite the different treatment technologies used, elimination of the fluoroquinolones from treated sewage effluents are minimal.

The nature of the drug also plays a role in its removal from wastewater. The fluoroquinolone antibiotics are very hydrophilic compounds (Vieno et al., 2007). Elimination of fluoroquinolones are mainly via sorption to sludge (Golet et al., 2003). In contrast, other studies have shown higher removal rates of fluoroquinolones from wastewater (Gulkowska et al., 2008) with approximately 78 % removed . However, this was not evident in this study.

Several other factors need to be taken into consideration. Studies have suggested that the dilution of raw wastewater by heavy rain can result in the reduction of pharmaceutical removal by sewage treatment plants (Ternes, 1998). Other factors such as temperature of the wastewater, the hydraulic and solid rentention time, age of the activated sludge, environmental conditions and characteristics of the raw influent may all play a role in the elimination of pharmaceuticals in wastewater (Zuccato et al., 2006; Vieno et al., 2007).

Moreover it is important to prevent the discharge of these pharmaceutical compounds to receiving waters since it may result in adverse effects to fish species and an eventual health risk to consumers of fish caught in contaminated water bodies (Miranda and Zemelman, 2001; Pathak and Gopal, 2005).

Sulfamethoxazole is an antibiotic used widely in human and veterinary medicine (Zhang and Wang, 2009). Sulfamethoxazole is resistant to breakdown and has been found in

environmental ecosystems (Holm et al., 1995; Kummerer, 2001). Sulfamethoxazole was detected in all raw wastewater samples analysed in this study (Tables 3.2, 3.3, 3.4). The sulfamethoxazole concentration of the treated sewage effluents were significantly higher than the Jonkershoek negative control ($P<0.050$), indicating incomplete removal during sewage treatment processes. Sewage treatment plant 1 did not reduce the sulfamethoxazole concentration and the antibiotic was released at very high levels in the treated sewage effluents. These results are similar to those published by Zuccato et al. (2010). A significant decrease of sulfamethoxazole concentration in treated sewage effluents compared to raw wastewater can be seen for sewage treatment plant 2 and 3 ($P<0.050$). The percentage reduction of sulfamethaxazole for sewage treatment plant 2 and 3 was 34 % and 56 %, respectively. Watkinson et al. (2007) has shown that the mean removal rate of sulfamethoxazole in conventional activated sludge plants was 92 %, however, concentrations in the ng/L range are still present in treated sewage effluents. In contrast, removal rates of sulfamethoxazole in membrane bioreactor plants are higher (Radejenoviĺ et al., 2009).

3.6. Conclusion

The present study indicated the occurrence of faecal bacteria in raw wastewater and treated sewage effluents from certain sewage treatment plants. U.V light disinfection showed inefficient removal of faecal bacteria compared to chlorination. Newer technologies such as the membrane bioreactor technology in sewage treatment plant 3 reduced the faecal bacteria in treated sewage effluents. However, other factors such as pH and conductivity of wastewater may play a role in bacterial communities that survive.

The results of this study also show that due to inefficient removal by treatment processes, antibiotic residues are still present in treated sewage effluents. Therefore, wastewater with high raw influent concentrations of antibiotics will require some form of additional treatment to reduce their concentration in treated sewage effluents. This study also showed that membrane bioreactor technology could potentially be helpful in reducing the amount of contaminants released into the environment.

The National Water Act of SA (Act no. 36 of 1998) needs to be strictly enforced by government in order to ensure the conservation of our water sources. Further research needs to be undertaken to improve sewage treatment technologies, thereby producing a better quality treated sewage effluent.

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Chapter 4: The effectiveness of sewage treatment processes to remove selected steroid hormones

4.1. Abstract

Natural steroid hormones regulate several physiological systems. The presence of steroid hormones in the environment may result in endocrine disruption. Major steroid hormone groups found in the environment are the estrogens, estradiol and estrone. Limited data is available on the occurrence and effects of testosterone in the environment. The aim of this study was to compare the levels of estradiol, estrone and testosterone in raw intake wastewater and treated sewage effluents from three different sewage treatment plants in the Western Cape, South Africa. Sewage treatment plant 1 and 2 use older technologies, while sewage treatment plant 3 has been upgraded and new technologies (membrane bioreactor) were incorporated in the treatment processes. ELISAs specific for the steroid hormones were used to assess steroids in the samples collected from the sewage plants. Estradiol, estrone and testosterone were detected in raw wastewater from all sewage treatment plants. Estradiol levels ranged from 87 - 115 pg/ml in raw wastewater and, 14 - 76 pg/ml in treated sewage effluents. Treatment plants processes at sewage treatment plant 3 displayed low efficiencies for estradiol removal. Estrone levels ranged from 87 ï 227 pg/ml in raw wastewater and 20 ï 149 pg/ml in treated sewage effluents. Only treatment plant processes at sewage treatment plant 1 and 2 remove estrone effectively. Testosterone levels ranged from 121 ï 212 pg/ml in raw wastewater and, 9 - 21 pg/ml in treated sewage effluents. Testosterone was removed effectively by the treatment processes. Although new technologies (membrane bioreactor) have been incorporated to improve sewage treatment processes, high levels of steroid

hormones are still released into the environment with the treated sewage effluents. These discharged sewage effluents may have adverse effects on the aquatic environment. Further studies are needed to improve sewage treatment processes and to determine the biological activity of these sewage effluents.

4.2. Introduction

Endocrine disrupting chemicals (EDCs) are a group of environmental contaminants that are a major concern. Kavlock (1996) defines an EDC as: ñAn exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processesò. The International Programme on Chemical Safety (IPCS) describes EDCs as: ñAn exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub) populationsò (IPCS, 2002).

EDCs include several chemical classes such as natural and synthetic hormones, plant components, pesticides, substances used in the plastic industry, as well as in consumer products. Other EDCs may be found in industrial by-products and pollutants (IPCS, 2002).

EDCs can act in several ways to potentially result in harmful effects. EDCs can bind to receptors at the cell surface, cytoplasm or nucleus which then result in alterations in gene expression (Birnbaum, 1994). These alterations in gene expression can disrupt normal biological functions such as cell proliferation and differentiation, and normal development (IPCS, 2002). EDCs can also result in inhibition of hormone synthesis, transport and metabolism, and activation of receptors through receptor phosphorylation (IPCS, 2002).

Natural hormones such as the steroid hormones influence the hormonal system and consequently may result in endocrine disruption if released into the environment (Ternes, 1999a). Animals and humans excrete steroid hormones daily (Ying et al., 2002). Steroid hormones include progestogens, glucocorticoids, mineralcorticoids, androgens and estrogens. These steroid hormones are secreted by the adrenal cortex, testis, ovary and placenta. Most

estrogenic steroid hormones such as estradiol, estrone and estriol are excreted by females. These hormones function *in vivo* to protect and support the reproductive tissues, breasts, skin and brain. Females naturally excrete approximately 5 µg/day of estrone and estradiol (Shore and Shemesh, 2003).

Water resources can become contaminated with EDCs as a result of municipal sewage, industrial wastewaters, agricultural run-off and underground contamination (Bolong et al., 2009). In French sewage treatment plants, the concentrations of 17ϐ-estradiol and estrone ranged from 3 - 18 pg/ml to 1 - 3 pg/ml respectively. Estrone and 17ϐ-estradiol concentrations have also been detected in sewage effluent in Rome (52, 12 pg/ml); Britain (1-50, 80 pg/ml); Germany (27, 15 pg/ml) and Brazil (40, 21 pg/ml) respectively (Baronti et al., 2000; Desbrow et al., 1998; Ternes et al., 1999b). Removal rates of estrogens by treatment in sewage treatment plants differ. Sorption and biodegradation are the main estrogen removal mechanisms during sewage treatment (Anderson et al., 2005). Data suggest that the average removal rates of 17ϐ-estradiol and estrone during sewage treatment are approximately 80 % (Johnson and Williams, 2004; Anderson et al., 2005). Steroid hormones can result in adverse effects to the aquatic life at very low concentrations (Jobling et al., 1998). In addition, exposure to estrogens in the environment may potentially impact plant species. Contaminated sewage effluent used for irrigation has resulted in increased levels of phytoestrogens in Alfafa (Shore et al., 1995).

Environmental steroids have been studied extensively (Ternes et al., 1999b; Johnson and Sumpter, 2001; Johnson et al., 2005). Less literature is available on the occurrence of androgens in the environment. The natural androgens such as testosterone and dihydroepiandrosterone are predominantly male steroid hormones. Males excrete approximately 10 mg of androgen per day (Shore and Shemesh, 2003). Androgens are

discharged into surface waters by sewage effluents (Kolpin et al., 2002). In addition, testosterone has been found in soil and paper and pulp industrial effluents (Finley-Moore et al., 2000; Bandelj et al., 2006). The concentrations of testosterone in the environment vary. Kolpin et al. (2002) reported testosterone levels of up to 214 pg/ml in streams. The median level for testosterone reported in this study was 116 pg/ml. Testosterone levels reported for sewage treatment plant effluents vary between 1 and 50 pg/ml (Trenholm et al., 2006; Kim et al., 2007; Vulliet et al., 2007). In addition, androgenic substances present in wastewater effluents can result in biological responses in animals (Bandelj et al., 2006).

Several countries monitor steroid hormones in sewage effluents (Ternes et al., 1999a; Ternes et al., 1999b). Only limited studies have been done in South Africa that focused on the presence of steroid hormones in treated sewage effluents (Swart and Pool, 2007). The aim of this study was to monitor the levels of steroid hormones in raw wastewater and treated sewage effluents from three different sewage treatment plants in the Western Cape, South Africa. ELISAs were used to assess the levels of specific steroid hormones in the sewage samples collected (Swart and Pool, 2007).

4.3. Materials and Methods

4.3.1. Site description and collection of water samples

Raw wastewater and treated sewage effluents were collected from three different sewage treatment plants in the Western Cape, South Africa. The treatment plants investigated are on the same river system. Sewage treatment plant 1 and 2 use older technologies (conventional activated sludge system) to treat wastewater. Sewage treatment 3 has been upgraded and new technologies (membrane bioreactor) were incorporated in the treatment processes. Sewage treatment plant 2 and 3 receive domestic effluents only. However, sewage treatment plant 1 receives both domestic (85 % flow intake) and industrial raw wastewater (15 % flow intake).

A detailed description of sewage treatment technologies for the different sewage treatment plants are as follows. The older technologies (conventional activated sludge system) used at the sewage treatment plants can be divided into three processes, namely:

(i) Primary treatment which includes pre-treatment of raw water intake by coarse and fine screens for grit removal. This process includes sedimentation tanks to allow the heavier organic particles to settle.

(ii) Secondary treatment of raw water using activated sludge. This process involves aerated biological digestion by bacteria to remove remaining suspended and dissolved material. In addition, nitrification and de-nitrification of wastewater is also used as treatment processes within the sewage treatment plants. Thereafter, the wastewater enters the secondary sedimentation tank to allow separation of the liquid and solid phase. After secondary sedimentation the wastewater enters maturation ponds for further pathogen removal.

(iii) Tertiary treatment is the final step in the conventional activated sludge system used by sewage treatment plant 1 and 2. Ultraviolet light (used only at sewage treatment plant 1) or chlorine (used only at sewage treatment plant 2) are the disinfection processes used, before the treated sewage effluent are released from plants.

Sewage treatment plant 3 uses an additional treatment technology (membrane bioreactor) concurrently with conventional or older treatment technologies. The membrane bioreactor technology consists of microporous membranes. These micro-filtration and ultra-filtration membranes separate liquid and solids.

Water collected from the Eerste River in Jonkershoek, Stellenbosch, South Africa (33º55ô51ôôS, 18º51ô16ôôE) was used as a negative control. This site is situated in the Stellenbosch mountain and there is no human activity upstream from this area.

Samples were collected in pre-cleaned 1 Liter (1 L) plastic bottles and transported to the laboratory in a cooler.

4.3.2. Solid Phase Extraction of raw wastewater and treated sewage effluents for assays

Samples were filtered with filter paper (Munktell, 15 µm, 240 mm) (Lasec, SA) before extraction. Water samples were then subjected to solid phase extractions (SPE) using C-18 columns (Sigma, Aldrich). The SPE columns were conditioned with 2 ml of Phase B mixture (45 % methanol, 40 % hexane and 15 % propanol), then 2 ml ethanol and lastly 4 ml distilled water. After the washing step, 100 ml of water sample was allowed to run through the columns, respectively. The columns were then dried using a vacuum pump (PALL vacuum pump, LifeSciences, 60 Hz, 1.92 Amperes, 220-240 Volts). The hydrophobic molecules

attached to the resin were eluted with 2 ml of Phase B mixture. The eluates were dried under a stream of air. The dried eluate was reconstituted with DMSO to make a 1000 times concentrated sample stock solution. Extracts were diluted with 0.1% BSA in saline at a ratio of 1:100 for the estradiol, estrone and testosterone ELISAs.

4.3.3. Estradiol (E2) analysis of water extracts

E2 kits were purchased from DRG Instruments GmbH, Germany. All the reagents required were supplied in the kit. The wells of a microtiter plate were pre-coated with antibody directed towards a unique antigenic site on the E2 molecule. This ELISA has a 100 % cross-reactivity with E2. Samples and standards were applied at 25 εl/well to the anti-estradiol coated plate. Thereafter, 100 εl of enzyme conjugate (Estradiol horseradish peroxidase) was added to all wells. The mixture was incubated for 2 hours at room temperature on a plate shaker (Stuart, Microtiter Plate Shaker, SSMS). After incubation, the wells were washed five times with wash solution and tapped dry. Thereafter, 100 εl of substrate was added to all wells and incubated for 30 minute at room temperature. The reaction was stopped by adding 50 εl of stop solution to all wells. The absorbances were then read at 450 nm with a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

4.3.4. Estrone (E1) analysis of water extracts

E1 kits were purchased from DRG Instruments GmbH, Germany. All the reagents required were supplied in the kit. The wells of a microtiter plate were pre-coated with antibody

directed towards a unique antigenic site on the E1 molecule. This ELISA has 100 % cross reactivity with E1. Samples and standards were applied at 50 εl/well to the anti-estrone coated plate. Thereafter, 100 εl of enzyme conjugate (Estrone horseradish peroxidase) was added to all wells. The mixture was incubated for 1 hour at room temperature on a plate shaker (Stuart, Microtiter Plate Shaker, SSMS). After incubation, the wells were washed five times with wash solution and tapped dry. Thereafter, 150 εl of substrate was added to all wells and incubated for 30 minutes at room temperature. The reaction was stopped by adding 50 εl of stop solution to all wells. The absorbances were then read at 450 nm with a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

4.3.5. Testosterone analysis of wastewater extracts

Testosterone kits were purchased from DRG Instruments GmbH, Germany. All the reagents required were supplied in the kit. The wells of a microtiter plate were pre-coated with antibody directed towards a unique antigenic site on the testosterone molecule. This ELISA has 100 % cross-reactivity with testosterone. Samples and standards were applied at 50 εl/well to the anti-testosterone coated plate. Thereafter, 100 εl of enzyme conjugate (Testosterone horseradish peroxidase) was added to all wells. The mixture was incubated for 1 hour at room temperature on a plate shaker (Stuart, Microtiter Plate Shaker, SSMS). After incubation, the wells were washed five times with wash solution and tapped dry. Thereafter, 150 εl of substrate was added to all wells and incubated for 30 minutes at room temperature. The reaction was stopped by adding 50 εl of stop solution to all wells. The absorbances were then read at 450 nm with a microtiter plate reader (Thermo Electron Corporation, Original

Multiskan Ex). A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

4.3.6. Statistical analyses

One way analysis of variance (ANOVA) was used to compare results for the steroid hormone assays, with $P<0.050$ considered as significant. Statistical analysis was done using SigmaPlot Version 11.

4.4. Results

4.4.1. Detection of selected steroids in raw wastewater and treated sewage effluents of three different sewage treatment plants

The standard curves for the estradiol, estrone and testosterone ELISAs are shown in Figure 4.1. The correlation coefficients (R^2) for all the standard curves are between 0.9546 and 0.9908. These standard curves show good inverse correlations between the optical density and the steroid concentration. Estradiol, estrone and testosterone concentrations detected in raw wastewater and treated sewage effluents for the three different sewage treatment plants are shown in Table 4.1; 4.2 and 4.3, respectively. Concentrations of the selected steroids are represented as Mean ± Standard Error of the mean (SEM). Very low levels of the selected steroids were detected in the Jonkershoek negative control (< LOD of E2 = 9.714 pg/ml; < LOD = 6.3 pg/ml E1, < LOD = 0.0083 pg/ml testosterone).

4.4.2. Steroid levels in sewage treatment plant 1 raw wastewater and treated sewage effluents

Estradiol concentrations detected in domestic and industrial raw wastewater were 87 ± 8 pg/ml and 94 ± 10 pg/ml, respectively. The combined concentration of estradiol for the mixture of domestic and industrial raw wastewater from sewage treatment plant 1 was 88 ± 9 pg/ml. Estradiol concentrations for domestic and industrial raw wastewater, and the combined mixture was higher when compared with the Jonkershoek negative control

(P<0.050). There was no difference in the estradiol concentration of domestic and industrial raw wastewater for sewage treatment plant 1.

Estradiol concentrations detected in treated sewage effluent for sewage treatment plant 1 was 14 ± 5 pg/ml. There was no difference in the estradiol concentrations of the treated sewage effluent for sewage treatment plant 1 and the Jonkershoek negative control. Estradiol concentrations for domestic and industrial raw wastewater, and the combined mixture was higher than treated sewage effluent concentrations (P <0.050). The conventional activated sludge process at sewage treatment plant 1 reduced the estradiol concentration by 84 %.

Estrone concentrations detected in domestic and industrial raw wastewater for sewage treatment plant 1 was 87 ± 56 pg/ml and 109 ± 49 pg/ml, respectively. The combined concentration of estrone for the mixture of domestic and industrial raw wastewater from sewage treatment plant 1 was 91 ± 52 pg/ml. Estrone concentrations for domestic and industrial wastewater, and the combined mixture was higher when compared with the Jonkershoek negative control (P<0.050). There was no difference in the estrone concentration of the domestic and industrial raw wastewater for sewage treatment plant 1.

Mean estrone concentrations detected in treated sewage effluent for sewage treatment plant 1 was 20 ± 7 pg/ml. There was no difference in the estrone concentrations of the treated sewage effluent for sewage treatment plant 1 and the Jonkershoek negative control. Estrone concentrations for domestic and industrial raw wastewater, and the combined mixture was higher than treated sewage effluent concentrations (P <0.050). The conventional activated sludge process at sewage treatment plant 1 reduced the estrone concentration by 78 %.

Testosterone concentrations detected in domestic and industrial raw wastewater for sewage treatment plant 1 was 121 ± 51 pg/ml and 150 ± 59 pg/ml, respectively. The combined concentration of testosterone for the mixture of domestic and industrial raw wastewater from

sewage treatment plant 1 was 111 ± 55 pg/ml. Mean testosterone concentrations for domestic and industrial wastewater, and the combined mixture was higher compared with the Jonkershoek negative control ($P<0.050$). There was no difference in the testosterone concentration of domestic and industrial raw wastewater for sewage treatment plant 1.

Testosterone concentrations detected in treated sewage effluent for sewage treatment plant 1 was 19 ± 5 pg/ml. There was no difference in the testosterone concentration of treated sewage effluent for sewage treatment plant 1 and the Jonkershoek negative control. Mean testosterone concentrations detected in domestic and industrial wastewater, and the combined mixture was higher compared to the treated sewage effluent for sewage treatment plant 1 ($P<0.050$). The conventional activated sludge process at sewage treatment plant 1 reduced the testosterone concentration by 86 %.

4.4.3. Steroid levels in sewage treatment plant 2 raw wastewater and treated sewage effluents

Estradiol concentrations detected in raw wastewater were 97 ± 7 pg/ml. Estradiol concentration for raw wastewater was higher when compared with the Jonkershoek negative control ($P<0.050$).

Estradiol concentrations detected in treated sewage effluent for sewage treatment plant 2 were 22 ± 17 pg/ml. There was no difference in the estradiol concentrations of the treated sewage effluent for sewage treatment plant 2 and the Jonkershoek negative control. Estradiol concentrations detected in raw wastewater were higher compared to the treated sewage effluent ($P<0.050$). The conventional activated sludge process at sewage treatment plant 2 reduced the estradiol concentration by 78 %.

Estrone concentrations detected in raw wastewater were 178 ± 59 pg/ml. Estrone concentration for raw wastewater was higher when compared with the Jonkershoek negative control ($P<0.050$).

Estrone concentrations detected in treated sewage effluent for sewage treatment plant 2 were 40 ± 39 pg/ml. There was no difference in the estrone concentration of the treated sewage effluent for sewage treatment plant 2 and the Jonkershoek negative control. Estrone concentrations detected in raw wastewater were higher compared to the treated sewage effluent ($P<0.050$). The conventional activated sludge process at sewage treatment plant 2 reduced the estrone concentration by 77 %.

Testosterone concentrations detected in raw wastewater were 211 ± 57 pg/ml. Testosterone concentration for raw wastewater was higher compared with the Jonkershoek negative control ($P<0.050$).

Testosterone concentrations detected in treated sewage effluent for sewage treatment plant 2 were 21 ± 4 pg/ml. There was no difference in the testosterone concentration of the treated sewage effluent for sewage treatment plant 2 and the Jonkershoek negative control. Testosterone concentrations detected in raw wastewater were higher compared to the treated sewage effluent ($P<0.050$). The conventional activated sludge process at sewage treatment plant 2 reduced the testosterone concentration by 90 %.

4.4.4. Steroid levels in sewage treatment plant 3 raw wastewater and treated sewage effluents

Estradiol concentrations detected in raw wastewater were 115 ± 4 pg/ml. Estradiol concentration in raw wastewater was higher when compared with the Jonkershoek negative control ($P<0.050$).

Estradiol concentrations detected in treated sewage effluent for sewage treatment plant 3 were 76 ± 6 pg/ml. Estradiol concentrations in the treated sewage effluent were significantly higher compared to the Jonkershoek negative control ($P<0.050$). Estradiol concentrations for raw wastewater were higher compared with the treated sewage effluent ($P<0.050$). The membrane bioreactor process at sewage treatment plant 3 reduced the estradiol concentration by 34 %.

Estrone concentrations detected in raw wastewater were 227 ± 24 pg/ml. Estrone concentration in raw wastewater were higher when compared with the Jonkershoek negative control ($P<0.050$).

Estrone concentrations detected in treated sewage effluent for sewage treatment plant 3 were 149 ± 38 pg/ml. Estrone concentrations in the treated sewage effluent were higher compared to the Jonkershoek negative control ($P<0.050$). There was no difference in the estrone concentration of the raw wastewater and treated sewage effluent for sewage treatment plant 3. The membrane bioreactor process at sewage treatment plant 3 reduced the estrone concentration by 34 %.

Testosterone concentrations detected in raw wastewater were 212 ± 62 pg/ml. Testosterone concentration for raw wastewater were higher compared with the Jonkershoek negative control ($P<0.050$).

Testosterone concentrations detected in treated sewage effluent for sewage treatment plant 3 were 9 ± 5 pg/ml. There was no difference in the testosterone concentration of the treated sewage effluent for sewage treatment plant 3 and the Jonkershoek negative control. Testosterone concentration for raw wastewater were higher compared with the treated sewage effluent ($P<0.050$). The membrane bioreactor process at sewage treatment plant 3 reduced the testosterone concentration by 96 %.

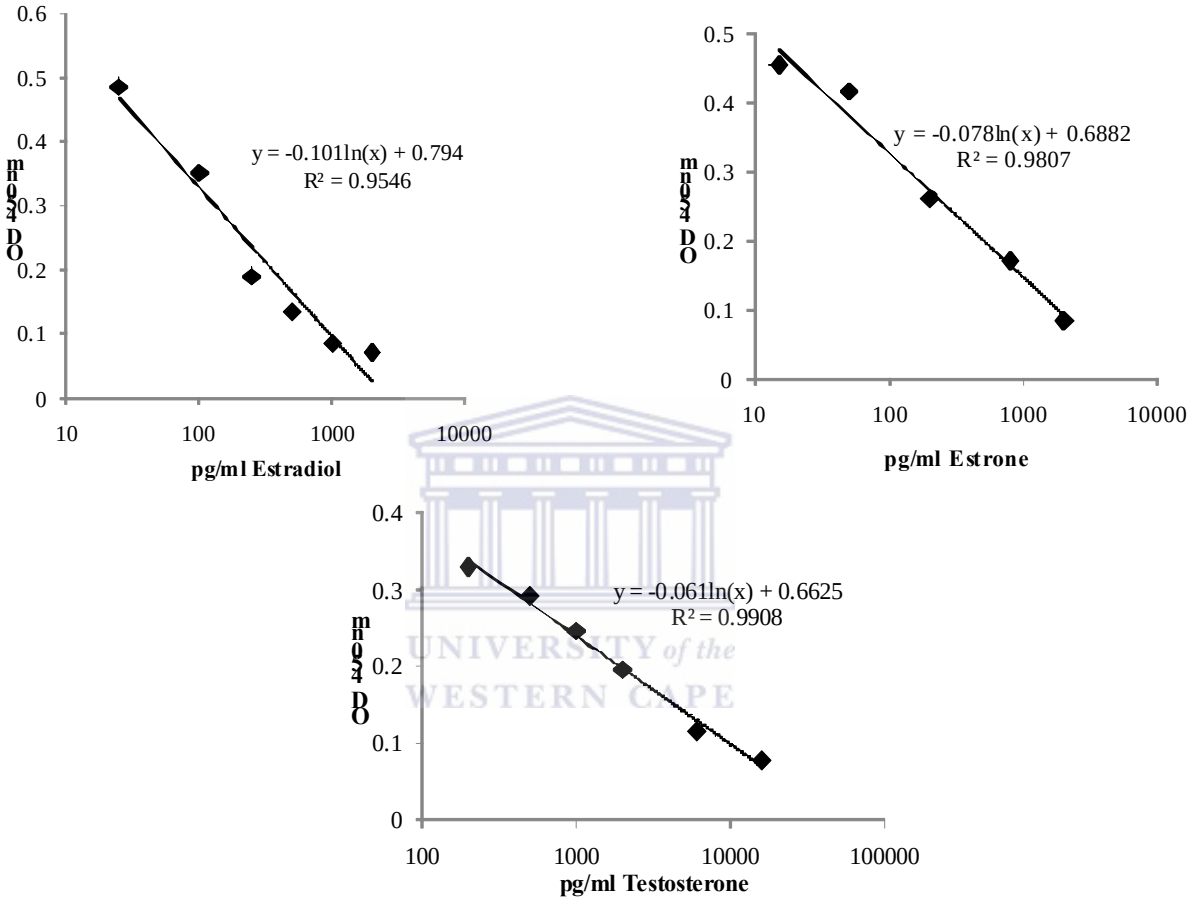

Figure 4.1. Standard curves obtained for the estradiol, estrone and testosterone ELISAs.

Table 4.1. Mean concentration (pg/ml ± SEM) of selected steroids found in raw wastewater and treated sewage effluents for sewage treatment plant 1 (n=8). Sewage treatment plant 1 uses the conventional activated sludge system as wastewater treatment processes. Water collected at Jonkershoek was used as a negative control sample. This site is not impacted by human activity.

Selected Steroid Hormones	Jonkershoek Negative Control	SEWAGE TREATMENT PLANT 1				
		Domestic Raw Water	Industrial Raw Water	Calculated value of mixture	Treated Sewage Effluents	Percentage Reduction (%)
Estradiol (pg/ml)	0 ± 0	87 ± 8 [ab]	94 ± 10 [ab]	88 ± 9 [ab]	14 ± 5	84 ± 3
Estrone (pg/ml)	2 ± 1	87 ± 56 [a]	109 ± 49 [a]	91 ± 52 [ab]	20 ± 7	78 ±52
Testosterone (pg/ml)	21 ± 8	121 ± 51 [ab]	150 ± 59 [a]	111 ± 55 [ab]	19 ± 5	86 ± 6

[a] Statistically different to negative control (P<0.050).

[b] Statistically different to treated sewage effluent (P<0.050).

Table 4.2. Mean concentration (pg/ml ± SEM) of selected steroids found in raw wastewater and treated sewage effluents for sewage treatment plant 2 (n=8). Sewage treatment plant 2 uses the conventional activated sludge system as wastewater treatment processes. Water collected at Jonkershoek was used as a negative control sample. This site is not impacted by human activity.

Selected Steroid Hormones	Jonkershoek Negative Control	SEWAGE TREATMENT PLANT 2		
		Raw Water	Treated Sewage Effluents	Percentage reduction (%)
Estradiol (pg/ml)	0 ± 0	97 ± 7 [ab]	22 ± 17	78 ± 2
Estrone (pg/ml)	2 ± 1	178 ± 59 [ab]	40 ± 39	77 ± 49
Testosterone (pg/ml)	21 ± 8	211 ± 57 [ab]	21 ± 4	90 ± 38

[a] Statistically different to negative control (P<0.050).

[b] Statistically different to treated sewage effluent (P<0.050).

Table 4.3. Mean concentration (pg/ml ± SEM) of selected steroids found in raw wastewater and treated sewage effluents for sewage treatment plant 3 (n=8). Sewage treatment plant 3 uses the newer membrane technology as an additional wastewater treatment process. Water collected at Jonkershoek was used as a negative control sample. This site is not impacted by human activity.

Selected Steroid Hormones	Jonkershoek Negative Control	SEWAGE TREATMENT PLANT 3		
		Raw Water	Treated Sewage Effluents	Percentage reduction (%)
Estradiol (pg/ml)	0 ± 0	115 ± 4 [ab]	76 ± 6 [a]	34 ± 2
Estrone (pg/ml)	2 ± 1	227 ± 24 [a]	149 ± 38 [a]	34 ± 10
Testosterone (pg/ml)	21 ± 8	212 ± 62 [ab]	9 ± 5	96 ± 40

[a] Statistically different to negative control (P<0.050).

[b] Statistically different to treated sewage effluent (P<0.050).

4.5. Discussion

Raw wastewater and treated sewage effluents were collected from the three different sewage treatment plants in the Western Cape, South Africa. The two older sewage treatment plants were of the activated sludge type. Sewage treatment plant 1 and 2 differ however in the tertiary treatment, with the latter using chlorination and the former using U.V. light disinfection. Sewage treatment plant 3 consists of both the activated sludge process with additional membrane bioreactor technology for treatment of raw wastewater. The wastewater samples collected were analysed for the occurrence of the steroid hormones estradiol, estrone and testosterone.

In humans, natural steroids are excreted in urine as biologically inactive glucuronide or sulphated conjugates (D`Ascenzo et al., 2003). However, elimination of steroids in the faeces is predominantly as unconjugated forms. This is as a direct result of high levels of *E. coli* present in the gut (Desbrow et al., 1998). In addition, raw wastewater contains a high population of *E. coli* which, are actively producing ɓ-glucuronidase (Panter et al., 1998). ɓ-glucuronidase is the enzyme that results in deconjugation of steroid hormones to its unconjugated forms. This study showed that estradiol concentrations in raw wastewater from all three sewage treatment plants were significantly higher compared to the Jonkershoek control site ($P<0.050$), (Table 4.1, 4.2, 4.3). Very low or no detection of estradiol were found in the Jonkershoek negative control sample. No human activity occurs at the control site therefore levels of *E. coli* in the water sample is minimal. Consequently, it may be possible that the *E. coli* present in the raw wastewater from all sewage treatment plants resulted in the unconjugated and conjugated form of estradiol, which was detected in this study. Furthermore, estradiol concentrations in raw wastewater from all sewage treatment plants

were higher compared to the treated sewage effluent concentrations ($P<0.050$). This therefore implies that the different treatment technologies used by the sewage treatment plants, were able to decrease the estradiol concentrations in treated sewage effluents. However, the estradiol level in effluents from sewage treatment plant 3 were higher than estradiol levels in the Jonkershoek negative control sample ($P<0.050$), indicating incomplete removal of estradiol by the treatment processes, with only a 34 % reduction. Treatment processes at sewage treatment plant 1 and 2 was more successful in eliminating estradiol concentrations in treated sewage effluents compared to the other plants, with a reduction of 84 % and 78 %, respectively. However, treatment plants do not remove all the estradiol from wastewater and low levels of estradiol are released into the environment with treated sewage effluents (Baronti et al., 2000). Membrane bioreactor technology proved to be effective for removal of hormones, with approximately 99 % removed (Kim et al., 2007). However, this was not evident in treated sewage effluents from sewage treatment plant 3, which employ membrane bioreactor technology as a treatment process. Studies suggest that estradiol may not only be transformed prior to entering the sewage treatment plant, but also during the biological treatment resulting in higher yields of free estrogens in the sewage effluents (Johnson and Sumpter, 2001). In addition, estradiol is mainly biodegraded and has a low affinity for adsorption to particulate matter (Fürhacker et al., 1999). Consequently, the persistence of estradiol in treated sewage effluents could be explained by its dissociation from large flocculate particles during the clarification stage (Johnson and Sumpter, 2001).

High concentrations of estrone were detected in raw wastewater from all the sewage treatment plants compared to the Jonkershoek negative control ($P<0.050$), (Table 4.1, 4.2, 4.3). Estrone is eliminated by humans as a sulfonide conjugate (Johnson and Sumpter, 2001). Estrone is seen as more persistent than estradiol since the arylsulfatase enzyme is not

abundant in raw wastewater to allow for cleavage (Johnson and Sumpter, 2001). Scientists proposed that biodegradation of estradiol in raw wastewater may result in the formation of its by-product, estrone. Alternatively, estrone in raw wastewater can be explained by deconjugation of its sulfonide (Johnson and Sumpter, 2001; Baronti et al., 2000).

In this study, high estrone levels are observed in treated sewage effluents from sewage treatment plant 3 compared to the Jonkershoek negative control ($P<0.050$). The conventional activated sludge processes at sewage treatment plant 1 and 2 were more successful in eliminating estrone concentrations in treated sewage effluents, with a reduction of 78 % and 77 %, respectively. Consequently, despite the different sewage treatment technologies used by the sewage treatment plants, estrone concentrations in treated sewage effluents were not effectively eliminated. These results are in accordance with studies that have shown that estrone is more resistant to treatment and thus sewage treatment plants are ineffective at eliminating the steroid from sewage effluents (Baronti et al., 2000; Ternes et al., 1999b).

The differences observed between the conventional activated sludge process and the membrane bioreactor process at the sewage treatment plants could be attributed to various factors. Hydrophobicity, chemical structure and temperature of the wastewater all play a role in removal of micropollutants from sewage effluents (Cirja et al., 2008). Moreover, the low removal of estrone from sewage effluents may be associated to breakdown of estradiol during sewage treatment (Baronti et al., 2000).

Estrone has half the potency of estradiol, but higher levels of estrone have been found in sewage effluents (Ternes et al., 1999b; Baronti et al., 2000; Johnson and Sumpter, 2001). Complete elimination of estrone by improved sewage treatment processes will decrease the estrogenicity of the treated sewage effluents (Johnson and Sumpter, 2001). In terms of concentration, estrone should be considered the most important endocrine disruptor (Johnson

and Sumpter, 2001). This is an important issue and extensive studies have shown that treated sewage effluents result in endocrine disruption in fish (Diniz et al., 2005; Ma et al., 2005).

In this study, higher testosterone concentrations were detected in raw wastewater from all three sewage treatment plants compared to the control ($P<0.050$), (Table 4.1, 4.2, 4.3). These results are consistent with reports that high amounts of androgens are excreted by humans daily (Shore and Shemesh, 2003). Testosterone production by males is approximately 6500 µg/day, compared to female production of approximately 240 µg/day (Shore and Shemesh, 2003). Limited data is available on testosterone levels in sewage treatment plants.

In this study, domestic and industrial raw wastewater from sewage treatment plant 1 contained a higher mean testosterone concentration compared to the treated sewage effluent ($P<0.050$). This was also evident for raw wastewater from sewage treatment plant 2 and 3. Furthermore, no significant difference of mean testosterone concentration in treated sewage effluents from all sewage treatment plants compared with the control was observed. The conventional activated sludge process at sewage treatment plant 1 and 2 produced a 86 % and 90 % reduction of testosterone, respectively. The membrane bioreactor process at sewage treatment plant 3 produced a 96 % reduction of testosterone. Furthermore, the data suggest that high removal of testosterone occurs during treatment processes at all sewage treatment plants. This is in accordance with Chang et al. (2010) who observed high removal efficiencies of androgens from sewage effluents. It is postulated that the removal of testosterone from the aqueous phase is by means of sorption to activated sludge (Esperanza et al., 2004). However, there is limited data on the biodegradation and fate of testosterone in the environment. Studies that investigate the biodegradation and distribution of testosterone in the environment are needed in order to lessen the gap in knowledge. The impact of androgens to the aquatic life has been demonstrated. Ellis et al. (2003) has shown that

exposure of mosquitofish to androgenic substances in paper and pulp effluents has resulted in its masculinization. Furthermore, the impact of androgenic substances in wastewater has also been investigated in terrestrial animals. Kumar et al. (2008) demonstrated that androgenic substances in wastewater influents and effluents can result in endocrine disruption in rats.

4.6. Conclusion

This study showed the occurrence of significant concentrations of estradiol, estrone and testosterone in raw wastewater for the investigated sewage treatment plants. The natural steroid estradiol was detected in sewage effluents from sewage treatment plant 3, due to its low removal during treatment. Furthermore, this study showed that the conventional activated sludge processes at sewage treatment plant 1 and 2 successfully removed estrone from sewage effluents. Older and additional technologies employed showed high removal of testosterone in treated sewage effluents from all sewage treatment plants. Sewage effluents represent a large source of steroid hormones to the environment. The steroid hormones released into the environment with the sewage effluents may potentially result in adverse effects to aquatic life. Further studies need to be done in order to determine the biological activity of these sewage effluents. Investigations to improve and enhance existing sewage treatment processes should be carried out.

4.7. References

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Chapter 5: The effectiveness of sewage treatment processes to remove selected surfactants

5.1. Abstract

Surfactants are made up of a polar head group that is easily soluble in water and a non-polar hydrocarbon tail, which does not easily dissolve in water. Surfactants have been found at different concentrations in surface waters, sediments and sludge-amended soils. The aim of this study was to determine the occurrence of the surfactants alkylphenol ethoxylates (APE) and alcohol ethoxylates (AE) in raw wastewater and treated sewage effluents from three sewage treatment plants in the Western Cape, South Africa. Raw wastewater and treated sewage effluents were collected from the three different sewage treatment plants. Sewage treatment plant 1 and 2 employs older technologies. Sewage treatment plant 3 employs newer technologies for treatment. ELISAs specific for the selected surfactants were used to assay the samples. APE and AE were detected in raw wastewater entering all sewage treatment plants investigated. Results of this study showed that APE was not effectively removed by sewage treatment plant 1. However, APE was removed by the treatment processes used in sewage treatment plant 2 and 3. In addition, this study showed that AE levels in treated sewage effluents for all sewage treatment plants were reduced, irrespective of treatment technology used. Additional studies should be implemented to determine the fate and biological effects of the surfactants in treated sewage effluents from sewage treatment plants.

5.2. Introduction

Surface active agents, also known as surfactants, are components of laundry and household products (Yangxin et al., 2008). Detergent formulations today consist of a mixture of surfactants to enhance the cleaning capability. Surfactants are made up of a polar head group that is easily soluble in water and a nonpolar hydrocarbon tail, which does not easily dissolve in water (Ying, 2006). These hydrophobic and hydrophilic molecules are extensively used in cleaning detergents, personal care products, pesticide formulations, paints, textiles, pharmaceuticals, pulp and paper industries (Ying, 2006). Surfactants have been found in surface waters, sediments and sludge-amended soils (Ying, 2006). Alkylphenol ethoxylates (APE) and alcohol ethoxylates (AE) are the most widely studied surfactants.

Alkylphenol ethoxylates (APE) can be subdivided into nonylphenol ethoxylates (NPE) and octylphenol ethoxylates (OPE) (Ying, 2006). APE has been shown to have a better detergent capability than AE (Yangxin et al., 2008). APE are primarily used as detergents, emulsifiers, solubilizers, wetting agents and dispersants (David et al., 2009). In 1997, it was estimated that the global use of APE was 500 000 tons per annum and that 80 % of this was NPE, while OPE accounted for 20 % (Ying, 2006). NPE and OPE are readily biodegraded during sewage treatment processes and in the environment. These molecules then lose their ethoxy groups and form nonylphenols (NP) and octophenols (OP) including other mono-, di- and tri-ethoxylates (David et al., 2009). NP mimics estrogen (17ß-estradiol) by binding to estrogen receptors. Nonylphenols are therefore known as xenoestrogens (Giesy et al., 2000).

Nonylphenol can adversely affect aquatic organisms. NP concentrations higher than 1 mg/L resulted in mortality of zebra mussel (*Dreissena polymorpha*) after 50 day exposure (Quinn et al., 2006). NP also alters the sex ratios of Japanese medaka, *Oryzias latipes* (Tabata et al.,

2001). NP acts as a xenoestrogen by increasing vitellogenin synthesis in male *Xiphophorus maculatus* (*X.maculatus*) fish (Kinnberg et al., 2000). NP has also been found to have an effect on testicular structure and may have an effect on the fertility of male fish (Kinnberg et al., 2000).

Non-ionic surfactants such as alcohol ethoxylates (AE) have been widely used in combination with anionic surfactants in laundry and personal-care agents (Yangxin et al., 2008). Other non-ionic surfactants include alkylphenol ethoxylate, methyl ester ethoxylate (MEE), ethoxylated amine and alkyl polyglycoside (APG).

AE surfactants are often included in detergents and are formed by the reaction of a fatty alcohol and ethylene oxide. Alcohol ethoxylates are widely used and concern of environmental contamination is high (Belanger et al., 2006). These surfactants end up being discharged into the environment and entering wastewater. AE are highly biodegradable, however due to the large volumes produced they pose an environmental risk (Wong et al., 2004). Monitoring of municipal wastewater treatment plants for AE has been done in Europe, Canada, and North America (Eadsworth et al., 2006; Morrall et al., 2006). Studies have shown that AE pollution of the environment poses a health risk to aquatic life (Cardellini and Ometto, 2001; Mann and Bidwell, 2001). *Xenopus laevis* embryos and tadpoles displayed both teratogenic and toxic effects after 72 hour exposure of AE (Cardellini and Omette, 2001).

South African river waters contain surfactants (Gordon et al., 2009; Sibali et al., 2010). However, very little or no information has been reported on these surfactants in sewage effluents. Many surfactants enter receiving waters and can adversely affect aquatic species. More specifically frogs displayed nonspecific narcosis following exposure to nonylphenol ethoxylate and alcohol alkoxylate (Man and Bidwell, 2001). Monitoring the occurrence of

these surfactants in raw wastewater and treated sewage effluents can provide data on the effectiveness of sewage treatment processes to prevent release of these pollutants into the environment where they may pose adverse effects on aquatic animals.

The aim of this study was to determine the occurrence of the surfactants APE and AE in raw wastewater and treated sewage effluents from three different sewage treatment plants in the Western Cape, South Africa. ELISAs specific for surfactants were used to assay sewage samples. APE and AE are commonly used surfactants in industries and were used to monitor the efficiency of sewage plants to remove surfactants.

5.3. Materials and Methods

5.3.1. Site description and collection of water samples

Raw wastewater and treated sewage effluents were collected from three different sewage treatment plants in the Western Cape, South Africa. The treatment plants investigated are on the same river system. Sewage treatment plant 1 and 2 use older technologies (conventional activated sludge system) to treat wastewater. Sewage treatment 3 has been upgraded and new technologies (membrane bioreactor) were incorporated in the treatment processes. Sewage treatment plant 2 and 3 receive domestic effluents only. However, sewage treatment plant 1 receives both domestic (85 % flow intake) and industrial raw wastewater (15 % flow intake).

A detailed description of sewage treatment technologies for the different sewage treatment plants are as follows. The older technologies (conventional activated sludge system) used at the sewage treatment plants can be into three processes, namely:

(i) Primary treatment which includes pre-treatment of raw waste water by coarse and fine screens for grit removal. This process includes sedimentation tanks to allow the heavier organic particles to settle.

(ii) Secondary treatment of raw water using activated sludge. This process involves aerated biological digestion by bacteria to remove remaining suspended and dissolved material. In addition, nitrification and de-nitrification of wastewater is also used as treatment processes within the sewage treatment plants. Thereafter, the wastewater enters the secondary sedimentation tank to allow separation of the liquid and solid phase. After secondary sedimentation the wastewater enters maturation ponds for further pathogen removal.

(iii) Tertiary treatment is the final step in the conventional activated sludge system used by sewage treatment plant 1 and 2. Ultraviolet light (used only at sewage treatment plant 1) or chlorine (used only at sewage treatment plant 2) are the disinfection processes used, before the treated sewage effluents are released from plants.

Sewage treatment plant 3 uses an additional treatment technology (membrane bioreactor) concurrently with conventional or older treatment technologies. The membrane bioreactor technology consists of microporous membranes. These micro-filtration and ultra-filtration membranes separate liquid and solids.

Water collected from the Eerste River in Jonkershoek, Stellenbosch, South Africa (33º55ô51ôôS, 18º51ô16ôôE) was used as a negative control. This control site is situated in the Stellenbosch mountain and there is no human activity upstream from this area.

Samples were collected in pre-cleaned 1 Liter (1 L) plastic bottles and transported to the laboratory in a cooler.

5.3.2. Solid Phase Extraction of raw wastewater and treated sewage effluents water for surfactant assays

Samples were filtered with filter paper (Munktell, 15 µm, 240 mm) (Lasec, SA) before extraction. Water samples were then subjected to solid phase extractions (SPE) using C-18 columns (Sigma, Aldrich). The SPE columns were conditioned with 2 ml of Phase B mixture (45 % methanol, 40 % hexane and 15 % propanol), then 2 ml ethanol and lastly 4 ml distilled water. After the washing step, 100 ml of water sample was allowed to run through the columns, respectively. The columns were then dried using a vacuum pump (PALL vacuum

pump, LifeSciences, 60 Hz, 1.92 Amperes, 220-240 Volts). The hydrophobic molecules attached to the resin were eluted with 2 ml of Phase B mixture. The eluates were dried under a stream of air. The dried eluate was reconstituted with DMSO to make a 1000 times concentrated sample stock solution. Extracts were diluted in 10 % methanol at a ratio of 1:100 for the APE ELISA. For the AE ELISA extracts were diluted at a ratio of 1:100 in 30 % methanol.

5.3.3. APE analysis of raw wastewater and treated sewage effluent extracts

APE ELISA kits were purchased from Ecologiena, Tokiwa Chemical Industries Co. Ltd, Japan. Samples were analyzed according to the instructions included in the kit. All reagents required were supplied in the kit. The monoclonal antibody has a high specificity to APE with various polyethoxylic chain length (n=1-22) and doesnôt cross-react with other surfactants or compounds of similar structure. The ELISA plate was precoated with antibodies specific to a unique antigenic site on the APE molecule. Samples or standards and enzyme conjugate were pre-mixed in an uncoated microplate (100 εl of each solution). Thereafter, 100 εl of the pre-mixture were transferred per well of the coated plate. The plate was then incubated for 1 hour at room temperature. Thereafter, the wells were washed five times with wash solution and tapped dry. After washing, 100 εl of substrate was added to all wells and incubated for 30 minutes at room temperature. The enzyme reaction was stopped by adding 100 εl of stop solution to all wells. The optical density was then read at 450 nm with a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

5.3.4. AE analysis of raw wastewater and treated sewage effluent extracts

AE ELISA kits were purchased from Ecologiena, Tokiwa Chemical Industries Co. Ltd, Japan. Samples were analyzed according to the instructions included in the kit. All reagents required were supplied in the kit. The monoclonal antibody has a high specificity to AE (C10-12) with various chain length (n=1-25) and doesnôt cross-react with other surfactants or compounds of similar structure. The ELISA plate was precoated with antibodies specific to a unique antigenic site on the AE molecule. Briefly, 70 εl of enzyme conjugate was dispensed into the wells of the coated plate. Thereafter, 30 εl of the samples or standards were added to the wells. The plate was then incubated for 1 hour at room temperature. Thereafter, the wells were washed five times with wash solution and tapped dry. After washing, 100 εl of substrate was added to all wells and incubated for 30 minutes at room temperature. The enzyme reaction was stopped by adding 100 εl of stop solution to all wells. The optical density was then read at 450 nm with a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

5.3.5. Statistical analysis

One way analysis of variance (ANOVA) was used to compare results for the surfactant assays, with $P<0.050$ considered as significant. Statistical analysis was done using SigmaPlot Version 11.

5.4. Results

5.4.1. Detection of surfactants in raw wastewater and treated sewage effluents in the sewage treatment plants

Extracts of raw and treated sewage effluent samples from all sewage treatment plants were analysed for the surfactants APE and AE. The standard curves for the APE and AE ELISAs are shown in Figure 5.1 and 5.2, respectively. The correlation coefficients (R^2) for the standard curves were 0.9555 and 0.9968 for APE and AE, respectively. These standard curves show good inverse correlations between the optical density and the surfactants concentration.

The mean concentrations of APE and AE detected in raw wastewater and treated sewage effluents for all sewage treatment plants are shown in Table 5.1, 5.2 and 5.3. Concentrations of the selected surfactants are represented as Mean ± Standard Error of the mean (SEM). Very low levels of the selected surfactants were found in the Jonkershoek negative control.

5.4.2. APE levels in sewage treatment plant 1 raw wastewater and treated sewage effluents

APE concentrations detected in domestic and industrial raw wastewater were 96 ± 2 µg/L and 100 ± 1 µg/L, respectively. The combined concentration of APE for the mixture of domestic and industrial raw wastewater from sewage treatment plant 1 was 97 ± 1 µg/L. APE concentrations for domestic and industrial raw wastewater were higher when compared with

the Jonkershoek negative control (P<0.050). There was no difference in the APE concentration of the domestic and industrial raw wastewater for sewage treatment plant 1.

APE concentration detected in treated sewage effluent for sewage treatment plant 1 was 53 ± 14 µg/L. APE concentration in the treated sewage effluent was significantly higher compared to the Jonkershoek negative control (P<0.050). APE concentrations for domestic and industrial raw wastewater were higher than treated sewage effluent concentrations (P<0.050). The conventional activated sludge process at sewage treatment plant 1 reduced the APE concentration by 45 %.

5.4.3. APE levels in sewage treatment plant 2 raw wastewater and treated sewage effluents

APE concentration detected in raw wastewater from sewage treatment plant 2 was 96 ± 1 µg/L. APE concentration detected in treated sewage effluent from sewage treatment plant 2 was 20 ± 2 µg/L. APE concentration for raw wastewater was higher when compared with the Jonkershoek negative control and the treated sewage effluent (P<0.050). There was no difference in the APE concentration of the treated sewage effluents for sewage treatment plant 2 and the Jonkershoek negative control. The conventional activated sludge process at sewage treatment plant 2 reduced the APE concentration by 79 %.

5.4.4. APE levels in sewage treatment plant 3 raw wastewater and treated sewage effluents

APE concentration detected in raw wastewater from sewage treatment plant 3 were 90 ± 3 µg/L. APE concentration detected in treated sewage effluent from sewage treatment plant 3 was 17 ± 3 µg/L. APE concentration for raw wastewater were higher when compared with the Jonkershoek negative control and the treated sewage effluent ($P<0.050$). There was no difference in the APE concentration of the treated sewage effluents for sewage treatment plant 3 and the Jonkershoek negative control. The membrane bioreactor process at sewage treatment plant 3 reduced the APE concentration by 81 %.

5.4.5. AE levels in sewage treatment plant 1 raw wastewater and treated sewage effluents

AE concentrations detected in domestic and industrial raw wastewater were 51 ± 2 µg/L and 63 ± 0 µg/L, respectively. The combined concentration of AE for the mixture of domestic and industrial raw wastewater from sewage treatment plant 1 was 52 ± 1 µg/L. AE concentrations for domestic and industrial raw wastewater were higher when compared with the Jonkershoek negative control ($P<0.050$). There was no difference in the AE concentration of the domestic and industrial raw wastewater for sewage treatment plant 1. AE concentration detected in treated sewage effluent for sewage treatment plant 1 was 17 ± 11 µg/L. There was no difference in the AE concentration of the treated sewage effluents for sewage treatment plant 1 and the Jonkershoek negative control. AE concentrations for domestic and industrial raw wastewater were higher than treated sewage

effluent concentrations (P <0.050). The conventional activated sludge process at sewage treatment plant 1 reduced the AE concentration by 73 %.

5.4.6. AE levels in sewage treatment plant 2 raw wastewater and treated sewage effluents

AE concentration detected in raw wastewater from sewage treatment plant 2 was 64 ± 0 µg/L. AE concentrations detected in treated sewage effluent from sewage treatment plant 2 was 9 ± 3 µg/L. AE concentration for raw wastewater was higher when compared with the Jonkershoek negative control and the treated sewage effluent (P<0.050). There was no difference in the AE concentration of the treated sewage effluents for sewage treatment plant 2 and the Jonkershoek negative control. The conventional activated sludge process at sewage treatment plant 2 reduced the AE concentrations by 83 %.

5.4.7. AE levels in sewage treatment plant 3 raw wastewater and treated sewage effluents

AE concentration detected in raw wastewater from sewage treatment plant 3 was 64 ± 0 µg/L. AE concentration detected in treated sewage effluent from sewage treatment plant 3 was 16 ± 4 µg/L. AE concentration for raw wastewater was higher when compared with the Jonkershoek negative control and the treated sewage effluent (P<0.050). There was no difference in the AE concentration of the treated sewage effluents for sewage treatment plant 3 and the Jonkershoek negative control. The membrane bioreactor process at sewage treatment plant 3 reduced the AE concentration by 70 %.

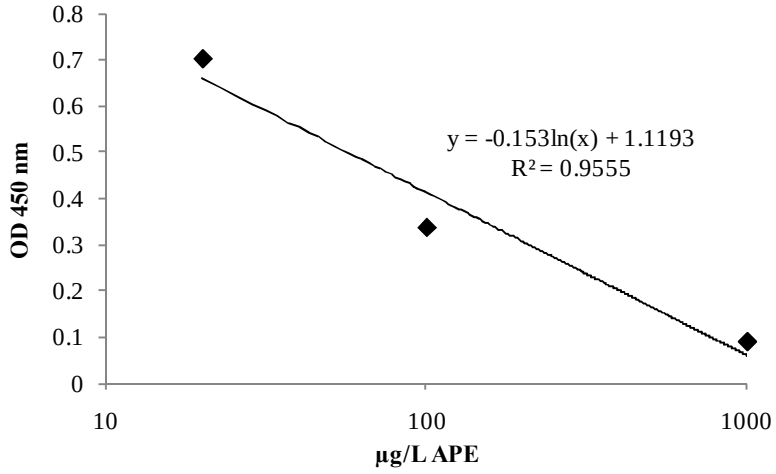

Figure 5.1. Standard curve obtained for alkylphenol ethoxylate ELISA.

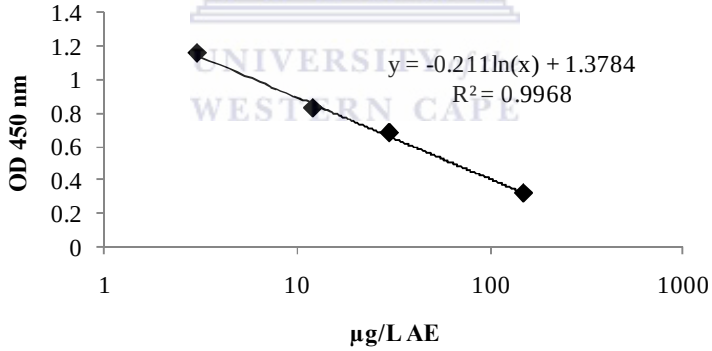

Figure 5.2. Standard curve obtained for alcohol ethoxylate ELISA.

Table 5.1. Mean concentration (µg/L ± SEM) of selected surfactants found in raw wastewater and treated sewage effluents for sewage treatment plant 1 (n=8). Sewage treatment plant 1 uses the conventional activated sludge system as wastewater treatment processes. Water collected at Jonkershoek was used as a negative control sample. This site is not impacted by human activity.

Selected Surfactants	Jonkershoek Negative Control	SEWAGE TREATMENT PLANT 1				
		Domestic Raw Water	Industrial Raw water	Calculated value of mixture	Treated Sewage Effluent	Reduction percentage (%)
APE(µg/L)	1 ± 0	96 ± 2 [ab]	100 ± 1 [ab]	97 ± 1 [ab]	53 ± 14 [a]	45 ± 1
AE(µg/L)	4 ± 3	51 ± 2 [ab]	63 ± 0 [ab]	52 ± 1 [ab]	17 ± 11	73 ± 1

[a] Statistically different to negative control (P<0.050).

[b] Statistically different to treated sewage effluent (P<0.050).

Table 5.2. Mean concentration (µg/L ± SEM) of selected surfactants found in raw wastewater and treated sewage effluents for sewage treatment plant 2 (n=8). Sewage treatment plant 2 uses the conventional activated sludge system as wastewater treatment processes. Water collected at Jonkershoek was used as a negative control sample. This site is not impacted by human activity.

Selected Surfactants	Jonkershoek Negative Control	SEWAGE TREATMENT PLANT 2		
		Raw Water	Treated Sewage Effluent	Reduction percentage (%)
APE(µg/L)	1 ± 0	96 ± 1 [ab]	20 ± 2	79 ± 2
AE(µg/L)	4 ± 3	64 ± 0 [ab]	9 ± 3	83 ± 3

[a] Statistically different to negative control (P<0.050).

[b] Statistically different to treated sewage effluent (P<0.050).

Table 5.3. Mean concentration (µg/L ± SEM) of selected surfactants found in raw wastewater and treated sewage effluents for sewage treatment plant 3 (n=8). Sewage treatment plant 3 uses the newer membrane technology as an additional wastewater treatment process. Water collected at Jonkershoek was used as a negative control sample. This site is not impacted by human activity.

Selected Surfactants	Jonkershoek Negative Control	SEWAGE TREATMENT 3		
		Raw water	Treated Sewage Effluent	Reduction percentage (%)
APE(µg/L)	1 ± 0	90 ± 3 [ab]	17 ± 3	81 ± 3
AE(µg/L)	4 ± 3	64 ± 0 [ab]	16 ± 4	70 ± 1

[a] Statistically different to negative control (P<0.050).

[b] Statistically different to treated sewage effluent (P<0.050).

5.5. Discussion

Raw wastewater and treated sewage effluents were collected from three different sewage treatment plants in the Western Cape, South Africa. Sewage treatment plant 1 and 2 uses older technologies (conventional activated sludge system) for treatment of wastewater. Sewage treatment plant 3 uses an additional treatment technology (membrane bioreactor) concurrently with conventional or older treatment technologies. The samples collected were analyzed for the occurrence of the surfactants APE and AE. ELISAs specific for the surfactants were used to assay the samples.

APE are surfactants that are widely used in detergents, paints, pesticides, textile and petroleum recovery chemicals and personal products (Scott and Jones, 2000). APE surfactants can be subdivided into both NPE and OPE (Loyo-Rosales et al., 2007). NPE and OPE are predominantly used in industry today (Loyo-Rosales et al., 2007). With degradation of APE, toxic metabolites are produced such as the alkylphenols (Loyo- Rosales et al., 2007; Ying, 2006). The alkylphenols include nonylphenol and octylphenol (Ying, 2006). These breakdown products are capable of causing detrimental effects to animals (Schüürmann, 1991). Consequently, monitoring APE levels play an important role in preventing adverse effects in the environment.

In this study, very low detection of APE were found in the Jonkershoek negative control sample. No human activity occurs at the control site therefore levels of APE in the water sample is minimal. Among the samples studied, high levels of APE were detected in raw wastewater from all sewage treatment plants compared to the Jonkershoek negative control ($P<0.050$). This is therefore an indication of its discharge from industries and domestic areas. Moreover, APE

concentrations in raw wastewater from all sewage treatment plants were significantly higher than treated sewage effluent concentrations ($P<0.050$). This therefore is an indication that the different treatment technologies employed by the sewage treatment plants were able to eliminate APE from treated sewage effluents. However, the APE level in treated sewage effluents from sewage treatment plant 1 were higher than APE levels in the Jonkershoek negative control sample ($P<0.050$), indicating incomplete removal of APE by the treatment processes, with only a 45 % reduction. This may be explained by differences in loading levels of the sewage treatment plants, since sewage treatment plant 1 receives raw wastewater from both industries and domestic areas. Furthermore, APE undergoes degradation in the presence of oxygen, however with the formation of breakdown products. These breakdown products are not always as accessible to microorganisms for degradation (Scott and Jones, 2000). Consequently, incomplete degradation of APE may have resulted in higher levels seen in treated sewage effluents from sewage treatment plant 1. Sewage treatment processes at sewage treatment plant 2 and 3 reduced the APE concentration by 79 % and 81 %, respectively. The high percentage reductions indicate effective removal of the surfactant APE, by both the conventional activated sludge process at sewage treatment plant 2 and the membrane bioreactor process at sewage treatment plant 3. Studies have shown higher elimination efficiencies of APE in membrane bioreactor units compared to conventional treatments (González et al., 2007). Additionally, González et al. (2007) showed that degradation of APE in membrane bioreactor units produced fewer breakdown products.

AE are surfactants that are composed of a hydrophobic alkyl chain and hydrophilic ethoxylate units (Ren, 2008). Ionization of AE surfactants do not occur in aqueous solution therefore they are known as non-ionic surfactants. This type of surfactant was created as an alternative to APE

to decrease the environmental loading and is used in household detergents today (Scott and Jones, 2000). Therefore it is not surprising that high AE concentrations were found in raw wastewater for all sewage treatment plants compared to the Jonkershoek negative control ($P<0.050$). Moreover, mean AE concentrations in raw wastewater from all sewage treatment plants was significantly higher than treated sewage effluent concentrations ($P<0.050$). High percentage reductions of AE by both the conventional activated sludge processes at sewage treatment plant 1 (73 %) and 2 (83 %) and the membrane bioreactor process at sewage treatment plant 3 were calculated (70 %). Consequently, despite the different treatment technologies used by the sewage treatment plants, AE concentrations in treated sewage effluents were all effectively eliminated. Several studies have been done where AE is effectively removed through the treatment process of aerobic biodegradation (Mezzanotte et al., 2003).

5.6. Conclusion

In conclusion, APE and AE surfactants were detected in significant concentrations in raw wastewater from all investigated sewage treatment plants. Results of this study showed that APE was not efficiently eliminated by sewage treatment plant 1. In addition, this study showed that AE concentration in treated sewage effluents for all sewage treatment plants were similar irrespective of treatment technology used. Using newer treatment technologies, such as membrane bioreactors can be advantageous in eliminating and reducing surfactant release into the environment. Additional studies should be implemented to determine the fate and biological effects of the surfactants in treated sewage effluents from sewage treatment plants.

5.7. References

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Chapter 6: Rapid *in vitro* tests to determine the toxicity of raw wastewater and treated sewage effluents

6.1. Abstract

Wastewater consists of a complex mixture of substances. During wastewater treatment these harmful substances can be eliminated or broken down. However, persistent compounds released with the treated sewage effluents, enter the environment and pose a risk to animal and human life. To determine the potential risks involved, screening tests are needed to monitor wastewater for potential toxic contaminants. The aim of this study was to validate and use screening tests to determine the toxicity of raw wastewater and treated sewage effluents from three sewage treatment plants in the Western Cape, South Africa. Raw wastewater and treated sewage effluents were screened for cytotoxicity using Lactate Dehydrogenase (LDH) release from cells as biomarker, neurotoxicity using acetylcholinesterase (AChE) inhibition and genotoxicity using the SOS test. Results showed no cytotoxicity for both raw wastewater and treated sewage effluents from all sewage treatment plants. Additionally, raw wastewater from all sewage treatment plants contained AChE inhibitors. The sewage treatment processes were not effective at eliminating these AChE inhibitors. This study also showed that raw wastewater from all sewage treatment plants tested positive for genotoxicity. Treated sewage effluents from all sewage treatment plants displayed no genotoxicity. This indicates effective removal of genotoxins by all three sewage treatment plants investigated.

6.2. Introduction

Pollutants and untreated industrial effluents can enter the environment. These contaminants can pose a major risk to the environment and aquatic life. Many of these pollutants are persistent in the environment and are not readily biodegraded (Wepener et al., 2001; Yadav et al., 2009).

Lactate dehydrogenase (LDH) release from cells is extensively used as a biomarker for necrosis or oncotic cell death (Valentovic and Ball, 1998; Kendig and Tarloff, 2007). Upon toxic injury to cells, the membrane integrity is impaired. LDH that normally occurs intracellularly, then leach into the incubation medium and can be monitored as a biomarker of cell damage (Kendig and Tarloff, 2007). LDH catalyses the oxidation of L-lactate to pyruvate in the presence of nicotinamide adenine dinucleotide (NAD^+) (Sepp et al., 1996). This reaction can then be measured colorimetrically using a spectrophotometer (Sepp et al., 1996). As a rule, the amount of LDH released is directly related to the amount of cytotoxicity caused by the chemical or toxicant (Sepp et al., 1996). Several studies have used LDH release as a method to determine cytotoxicity of chlorinated drinking water and treated sewage effluents. Human cell lines and aquatic organisms are often used as bioindicators of cytotoxicity (Yuan et al., 2005; Chourpagar and Kulkarni, 2009).

Acetylcholinesterase (AChE) has been used as a biomarker to determine neurotoxic contaminants in the aquatic environment (Yadav et al., 2009). AChE is the enzyme that catalyses the hydrolysis of the neurotransmitter acetylcholine, to form choline and acetic acid (Sakar et al., 2006). AChE has several molecular forms and is usually found in the membranes of erythrocytes (Sakar et al., 2006). Many natural toxins and man-made poisons play a part in neurotoxicity by inhibiting the enzyme AChE (Yadav et al., 2009). These

pollutants include organophosphates, heavy metals and carbamate insecticides. AChE is also the target of drugs that are used for neuromuscular disorders and diseases such as myasthenia gravis, glaucoma and Alzheimer`s disease (Silman and Sussman, 2005). The inhibition of AChE has been extensively used to determine exposure to anticholinesterase agents (Menezes et al., 2009). The inhibition of AChE can be measured by using the Ellman method (1961).

Organophosphate insecticides can contaminate surface waters and lead to inhibition of AChE. This inhibition of AChE can be used as a diagnostic tool to monitor organophosphates. Inhibition of AChE activity can potentially be used as a warning sign of adverse or harmful sublethal toxic effects on aquatic life, populations and communities (Day and Scott, 1990). AChE activity has been assessed in various aquatic organisms exposed to fertilizer industry effluents and secondary treated industrial effluents (Yadav et al., 2009; Ghedira et al., 2009; Wepener et al., 2005).

Contaminants in wastewater can potentially be genotoxic. Genotoxic micropollutants can present undesirable effects to humans and animals. Genotoxic substances induce deoxyribonucleic acid (DNA) damage and mutations. In humans, genotoxic substances can potentially result in cancer development (Çegura et al., 2009). Commonly used tests to determine genotoxicity includes the Comet assay, the Ames *Salmonella* mutagenicity assay and the micronucleus test (Kim and Margolin, 1999; Sugihara et al., 2000; Pellacani et al., 2006). Other common tests include a set of responses from a group of genes known as the SOS genes. The SOS chromotest is based on the detection of DNA damaging agents. It involves incubation of a specially developed *Escherichia coli* (*E. coli*) strain (PQ37) with the test substance of concern. The *lacZ* operon and *sfiA* gene in the *E. coli* strain are fused. The *lacZ* operon is the structural gene for ɓ-galactosidase, which is then under the control of the

sfiA gene (Quillardet and Hofnung, 1985). If a SOS response occurs, *lacZ* is then expressed and is measured photometrically by measuring ß-galactosidase (Sundermann et al., 1996). The level of ß-galactosidase secreted is an indication of the genotoxicity of the test sample. The SOS chromotest has been used to determine the genotoxicity of a variety of chemicals, metal compounds, hospital effluents, and complex environmental extracts (Mersch-Sundermann et al., 1996; Lantzsch and Gebel, 1997; Jolibois et al., 2003; White et al., 1996).

Wastewater consists of a complex mixture of substances. Sewage treatment plant processes are inefficient at eliminating all contaminants from treated effluents. These contaminants then enter the environment and can pose a risk to animal and human life. To determine the potential risks involved, screening tests are needed to monitor wastewater for potential toxic contaminants. Particularly, they have to be easy to use and not require highly skilled staff. The tests have to be reproducible and not expensive. Also, tests should be able to examine large numbers of samples with the use of minimum reagents (Fuerhacker et al., 2005). The aim of this study was to validate and use screening tests to determine the toxicity of raw wastewater and treated sewage effluents from three different sewage treatment plants in the Western Cape, South Africa. Toxicity was investigated using LDH inhibition as biomarker for cytotoxicity, AChE inhibition as biomarker for neurotoxicity, and ß-galactosidase as biomarker for genotoxicity.

6.3. Materials and Methods

6.3.1. Site description and water collection

Raw wastewater and treated sewage effluents were collected from three different sewage treatment plants in the Western Cape, South Africa. The treatment plants investigated are on the same river system. Sewage treatment plant 1 and 2 use older technologies (conventional activated sludge system) to treat wastewater. Sewage treatment 3 has been upgraded and new technologies (membrane bioreactor) were incorporated in the treatment processes. Sewage treatment plant 2 and 3 receive domestic effluents only. However, sewage treatment plant 1 receives both domestic (85 % flow intake) and industrial raw wastewater (15 % flow intake).

A detailed description of sewage treatment technologies for the different sewage treatment plants are as follows. The older technologies (conventional activated sludge system) used at the sewage treatment plants can be divided into three processes, namely:

(i) Primary treatment which includes pre-treatment of raw wastewater by coarse and fine screens for grit removal. This process includes sedimentation tanks to allow the heavier organic particles to settle.

(ii) Secondary treatment of raw wastewater using activated sludge. This process involves using aerated biological digestion by bacteria to remove remaining suspended and dissolved material. In addition, nitrification and de-nitrification of wastewater is also used as treatment processes within the sewage treatment plants. Thereafter, the wastewater enters the secondary sedimentation tank to allow separation of the liquid and solid phase. After secondary sedimentation the wastewater enters maturation ponds for further pathogen removal.

(iii) Tertiary treatment is the final step in the conventional activated sludge system used by sewage treatment plant 1 and 2. Ultraviolet light (used only at sewage treatment plant 1) or chlorine (used only at sewage treatment plant 2) are the disinfection processes used, before the treated sewage effluent enters the receiving waters.

Sewage treatment plant 3 uses an additional treatment technology (membrane bioreactor) concurrently with conventional or older treatment technologies. The membrane bioreactor technology consists of microporous membranes. These micro-filtration and ultra-filtration membranes separate liquid and solids.

Water collected from the Eerste River in Jonkershoek, Stellenbosch, South Africa (33º55ô51ôôS, 18º51ô16ôôE) was used as a negative control. This site is situated in the Stellenbosch mountain and there is no human activity upstream from this area.

Samples were collected in pre-cleaned plastic bottles (1 L) and transported to the laboratory in a cooler at 4 °C.

6.3.2. Collection of blood for LDH and AChE assays

Blood was collected from consenting healthy male subjects (20-26 years of age). Criteria for blood collection were that donors were not on medication for the month prior to blood collection. Blood was collected by venipuncture using endotoxin-free evacuated blood collection tubes (Greiner Bio One GmBH) containing sodium citrate (3.2%).

6.3.3. Solid phase extraction of raw wastewater and treated sewage effluents for assays

Samples were filtered with filter paper (Munktell, 15 µm, 240 mm) (Lasec, SA) before extraction. Water samples were then subjected to solid phase extractions (SPE) using C-18 columns (Sigma, Aldrich). The SPE columns were conditioned with 2 ml of Phase B mixture (45 % methanol, 40 % hexane and 15 % propanol), then 2 ml ethanol and lastly 4 ml distilled water. After the washing step, 100 ml of water sample was allowed to run through the columns, respectively. The columns were then dried using a vacuum pump (PALL vacuum pump, LifeSciences, 60 Hz, 1.92 Amperes, 220-240 Volts). The hydrophobic molecules attached to the resin were eluted with 2 ml of Phase B mixture and dried under a stream of air. The dried eluate was reconstituted with dimethyl sulfoxide (DMSO) to make a 1000 times concentrated sample stock solution.

6.3.4. Lactate dehydrogenase assay to determine cellular cytotoxicity of raw wastewater and treated sewage effluents

All experiments were performed under sterile conditions in a laminar flow cabinet. For the assay, aliquots of raw wastewater and treated sewage effluents were sterilized using a 0.45 µM sterile filter (Lasec, S.A.). Samples and controls were added to eppendorfs (100 µl/eppendorf). Blood was diluted 1:9 with Roswell Park Memorial Institute 1640 (RPMI-1640) medium. The diluted blood was added to samples (900 µl/eppendorf). Samples were incubated at 37 °C for 24 hours.

LDH from blood cells released into culture medium was used as a biomarker for cellular toxicity. LDH was measured using a commercially available kit (Biovision, USA). The

assay was performed according to the manufacturer`s instructions. Briefly, cell free culture supernatants (10 µl) were transferred into ninety six well microtiter plates (Nunc, Apogent, Denmark). For the 100 % cytotoxicity standard, a control blood sample cell was lysed with 2 µl of TritonX-100 detergent. Addition of the detergent results in immediate lysis of the blood cells. The sample was mixed and an aliquot of the lysate was diluted with 0.9 % saline at a ratio of 1:9. This lysate was used as the 10 % cytotoxicity control. A standard curve was constructed using dilutions of this sample. Thereafter, LDH reaction mixture was prepared and 100 µl added to all cell free supernatants and standards. The mixture was incubated for 1 hour. Optical densities were read at 492 nm at time-zero and after 1 hour, using a microtiter plate reader (Thermo Electron, Original Multiskan Ex). Optical densities for the standards were used to construct a standard curve. The cytotoxicity of the samples was read off this curve. Cytotoxicity is expressed as % LDH released ± Standard error of the mean (% LDH ± SEM).

6.3.5. Optimization of the AChE inhibition assay

AChE was extracted from human blood. Assays were conducted in ninety six-well microtiter plates (Nalge Nunc International, Thermo Fisher Scientific, NY, U.S.A.). Freshly collected blood were diluted with distilled water (1:3) to lyse blood. Thereafter, doubling dilutions of the lysed blood was performed in assay buffer (0.1 M sodium phosphate buffer) and added to all wells of the microplate (50 µl/well). This was followed by the addition of substrate mixture to all wells (50 µl/well). The substrate mixture contained 0.075 M Acetylthiocholine iodide (ATI) and 0.01 M 5,5`-Dithio-bis-2-nitrobenzoic acid (DTNB) in assay buffer. The plate was then incubated away from light for 1 hour. Optical densities were measured at 405 nm at 5 min intervals during the 1 hour incubation period using a microplate reader (Thermo

Electron, Original Multiskan Ex). A curve was drawn from the optical densities obtained and the optimal dilution factor of blood to be used in the AChE assay was read off this curve.

6.3.6. Optimization of positive control (chlorpyrifos) for use in the AChE inhibition assay

Chlorpyrifos (Efekto, reg. no. L5676) is an organophosphate insecticide that inhibits acetylcholinesterase and was therefore used as a positive control. For the assay, ninety six-well microtiter plates (Nalge Nunc International, Thermo Fisher Scientific, NY, U.S.A.) were used. The initial concentration of the chlorpyrifos used in the assay was 960 µg/ml. A dilution series of this concentration of chlorpyrifos was prepared in distilled water and then applied to all the wells (50 µl/well). After this 25 µl of lysed blood, diluted 1 in 40 in assay buffer, was added to all wells. This was followed by addition of the substrate mixture to all wells (50 µl/well). Optical densities were measured at 405 nm at 5 min intervals during the 1 hour incubation period using a microplate reader (Thermo Electron, Original Multiskan Ex). A curve was drawn from the optical densities obtained and the concentration of chlorpyrifos to be used in the AChE assay was read off this curve.

6.3.7. Screening of raw wastewater and treated sewage effluents for AChE inhibitors using the validated AChE assay

All assays were performed in ninety six-well microtiter plates (Nalge Nunc International, Thermo Fisher Scientific, NY, U.S.A.). As a negative control DMSO was diluted 1: 9 (v/v) in assay buffer. As a positive control chlorpyrifos stock (60 µg/ml in DMSO) was diluted 1:7 (v/v) with assay buffer. For the assay, 25 µl/well of the negative and positive controls were

added to the microtiter plate, respectively. Water extracts were diluted 1:9 (v/v) in assay buffer and added to the wells (25 µl/well). Subsequently, 25 µl of a 1:40 dilution of blood in assay buffer was added to all wells. The plate was then incubated for 15 minutes. This was followed by the addition of 50 µl of substrate mixture to all wells. Optical densities were measured at 405 nm at 5 min intervals during the 1 hour incubation period using a microplate reader (Thermo Electron, Original Multiskan Ex). The inhibition of AChE was calculated as a percentage in terms of the negative control. Data is expressed as percentage AChE inhibition ± Standard error of the mean (% Ache inhibition ± SEM).

6.3.8. SOS chromotest to determine genotoxicity of raw wastewater and treated sewage effluent samples

The SOS chromotest was purchased from Environmental Bio Detection Products Incorporated (EBPI), Ontario, Canada. The assay was performed according to the manufacturer`s instructions. All reagents were supplied in the kit. Briefly, growth medium was added to the lyophilized bacteria (*E. coli* PQ37 strain) and incubated for 4 - 5 hours at 37 °C. Thereafter, the bacteria grown were tested for turbidity at 600 nm and the bacterial suspension was diluted to give an optical density of 0.05 nm. Raw wastewater and treated sewage effluent extracts were diluted 1/100 in DMSO. Two-fold serial dilutions of the positive control, 4 Nitro Quinoline Oxide (4NQO, 100 ng/ml) in DMSO was prepared. Thereafter, 10 µl of each sample and control was added to a 96 well microtiter plate. Thereafter, 100 µl of the bacterial suspension was added to all the wells of the microtiter plate. The plate was then incubated for two hours at 37 °C, followed by the addition of 100 µl of the substrate solution (ɓ-galactosidase) to all the wells for 1 hour. The colour reaction was then stopped by adding 50 µl of stop solution. Optical densities were then measured at

620 nm and 405 nm using a microplate reader (Thermo Electron, Original Multiskan Ex). Genotoxicity of the samples and standards were calculated by a conversion factor. The conversion factor was calculated by dividing the optical densities of 620 nm and 405 nm. This conversion factor was then used to correct the optical densities of the samples and standards. A standard curve was then constructed using the concentration and toxicity equivalents of the positive control per millilitre. The genotoxicity of raw wastewater and treated sewage effluents were then read off this standard curve. Data is expressed as equivalents of the positive control.

6.3.9. Statistical analysis

One way analysis of variance (ANOVA) was used to compare results for the different assays, with $P<0.050$ considered as significant. Statistical analysis was done using SigmaPlot Version 11.

6.4. Results

6.4.1. Cytotoxicity assessment of raw wastewater and treated sewage effluents from the three sewage treatment plants using whole blood cultures

The LDH standard curve is shown in Figure 6.1. The correlation coefficient (R^2) for the standard curve is 0.9821. This standard curve shows a good correlation between the percentage of LDH released and the optical density. The percentage LDH released by the whole blood cultures after incubation with the raw wastewater and treated sewage effluents were extrapolated using the standard curve (Table 6.1).

The percentage LDH released by raw wastewater and treated sewage effluents for all sewage treatment plants were significantly lower compared to the positive control ($P<0.050$). The percentage LDH released by raw wastewater, treated sewage and water from the Jonkershoek control site are similar indicating no cytotoxicity for any of the samples investigated.

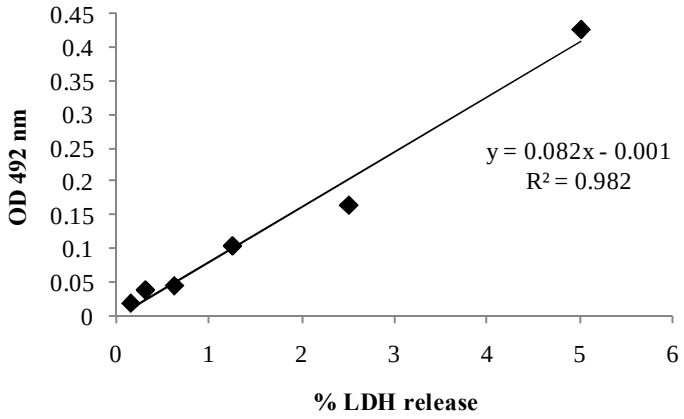

Figure 6.1. Standard curve for the LDH assay. The standard curve obtained shows a good correlation ($R^2 = 0.9821$) between the optical density (OD) and percentage (%) LDH released.

Table 6.1. Mean percentage LDH release (% LDH ± SEM) by raw wastewater and treated sewage effluents from all sewage treatment plants in the Western Cape, South Africa (n= 8).

			SEWAGE TREATMENT PLANT 1			SEWAGE TREATMENT PLANT 2		SEWAGE TREATMENT PLANT 3	
	Positive Control	**Jonkershoek negative control**	**Domestic Raw Wastewater**	**Industrial Raw Wastewater**	**Treated Sewage Effluent**	**Raw Wastewater**	**Treated Sewage Effluent**	**Raw Wastewater Raw water**	**Treated Sewage Effluent**
LDH	11 ± 0	1 ± 1[a]	5 ± 1[a]	5 ± 1[a]	5 ± 1[a]	5 ± 1[a]	5 ± 1[a]	7 ± 2[a]	5 ± 1[a]

[a] Statistically different to positive control (P<0.050)

6.4.2. Optimization of blood and chlorpyrifos concentration for AChE assay

To establish the optimum dilution of blood to use in the AChE assay, a doubling dilution of blood in assay buffer was performed. The optimization curve shows that there is a good correlation (R^2= 0.9938) between the absorbance and dilution factor for blood (Figure 6.2). The dilution factor for the blood selected for future assays are 1/40, since this dilution gives optical densities in the linear region of the assay curve.

To establish a dilution factor or concentration of the positive control to use in the AChE assay, a doubling dilution of the positive control in assay buffer was performed (Figure 6.3). The dilution of the positive control selected was 60 µg/ml, since this dilution gives optical densities in the linear region of the inhibition curve.

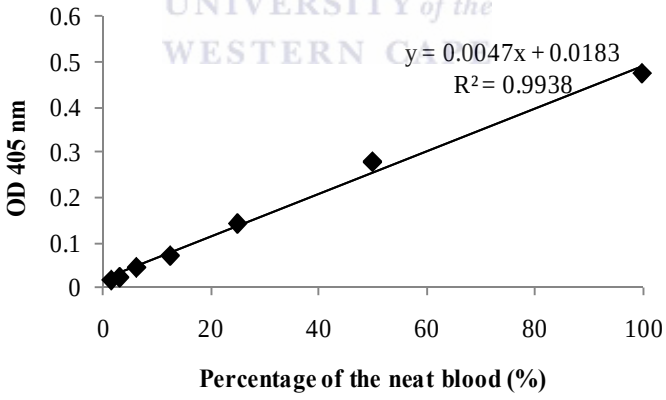

Figure 6.2. Optimization curve of the blood to be used in AChE assay.

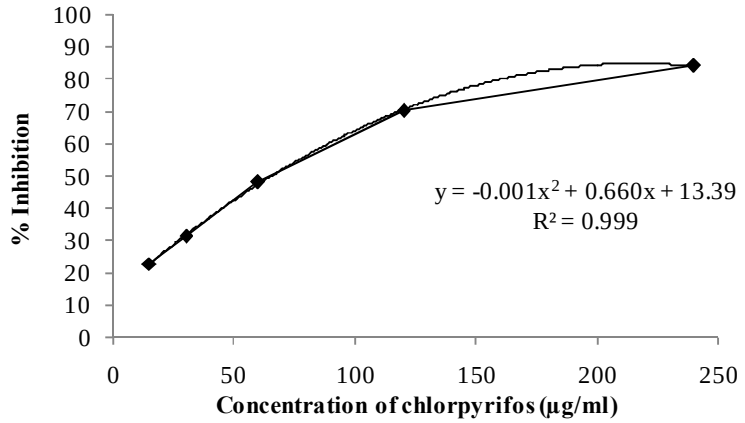

Figure 6.3. Optimization curve for the positive control.

6.4.3. Inhibition of AChE by raw wastewater and treated sewage effluents for the three sewage treatment plants

The inhibition of AChE of raw wastewater and treated sewage effluents for the three sewage treatment plants are shown in Table 6.2. The AChE inhibition for the domestic, industrial raw wastewater and treated sewage effluents were significantly higher compared to the Jonkershoek negative control ($P<0.050$). There was no difference in the AChE inhibition of domestic, industrial raw wastewater and treated sewage effluents.

The AChE inhibition of raw wastewater from sewage treatment plant 2 was significantly higher compared to the Jonkershoek negative control ($P<0.050$). The AChE inhibition of raw wastewater from sewage treatment plant 2 was significantly higher compared to the treated sewage effluents ($P<0.050$).

The AChE inhibition of raw wastewater from sewage treatment plant 3 was significantly higher compared to the Jonkershoek negative control ($P<0.050$). The AChE inhibition of raw

wastewater from sewage treatment plant 3 was significantly higher compared to the treated sewage effluents ($P<0.050$).

Table 6.2. Inhibition of AChE (% AChE inhibition ± SEM) by raw wastewater and treated sewage effluents from the three sewage treatment plants in the Western Cape, South Africa (n=8).

	Positive Control	Jonkershoek negative control	SEWAGE TREATMENT PLANT 1			SEWAGE TREATMENT PLANT 2		SEWAGE TREATMENT PLANT 3	
			Domestic Raw Wastewater	Industrial Raw Wastewater	Treated Sewage Effluent	Raw Wastewater	Treated Sewage Effluent	Raw Wastewater	Treated Sewage Effluent
AChE	33 ± 0	-1 ± 0	19 ± 4[a]	29 ± 5[a]	24 ± 15[a]	28 ± 13[ab]	3 ± 13[a]	9 ± 13[ab]	-14 ± 10[a]

[a] Statistically different to negative control (P<0.050).

[b] Statistically different to treated sewage effluent for the same sewage treatment plant (P<0.050).

6.4.4. Genotoxicity of raw wastewater and treated sewage effluents for the three sewage treatment plants

The standard curve for the SOS chromotest is shown in Figure 6.4. The correlation coefficient (R2) for the standard curve is 0.9943. This standard curve shows a good correlation between the toxicity and the equivalents of 4NQO. The SOS chromotest results for genotoxicity of raw wastewater and treated sewage effluents are shown in Table 6.3. The genotoxicity of the raw wastewater and treated sewage effluents are expressed in ng/ml 4 NQO equivalents.

The results of the test show that the Jonkershoek negative control sample is not genotoxic (0 ± 0). Genotoxicity equivalents of raw wastewater from all sewage treatment plants were significantly higher than the Jonkershoek negative control sample ($P<0.050$). The results of the test show that treated sewage effluents from all sewage treatment plants are non-genotoxic (0 ± 0).

Both the domestic raw and industrial raw wastewater from sewage treatment plant 1 tested positive for genotoxicity (116 ± 37 ng/ml; 112 ± 63 ng/ml respectively). The genotoxicity equivalents of the domestic and industrial raw wastewater were significantly higher compared to the treated sewage effluents from sewage treatment plant 1 ($P<0.050$).

Raw wastewater from sewage treatment plant 2 also tested positive for genotoxicity (194 ± 56 ng/ml). The genotoxicity equivalents of raw wastewater was higher compared to the treated sewage effluents from sewage treatment plant 2 ($P<0.050$).

Raw wastewater from sewage treatment plant 3 tested positive for genotoxicity (736 ± 412 ng/ml). The genotoxicity equivalents of raw wastewater was higher compared to treated sewage effluents from sewage treatment plant 3 ($P<0.050$).

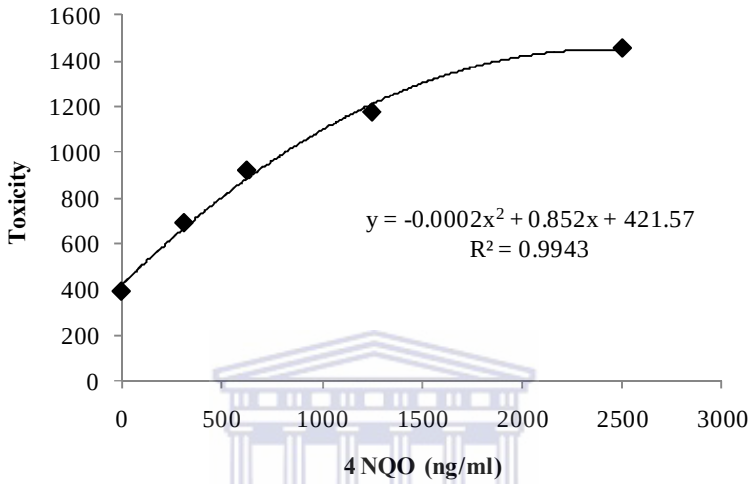

Figure 6.4. Standard curve for the SOS genotoxicity assay. The standard curve obtained shows a good correlation ($R^2 = 0.9948$) between the toxicity and equivalents of 4NQO (ng/ml).

Table 6.3. Genotoxicity of raw wastewater and treated sewage effluents expressed as ng/ml 4 NQO equivalents (n = 8).

		SEWAGE TREATMENT PLANT 1			SEWAGE TREATMENT PLANT 2		SEWAGE TREATMENT PLANT 3	
	Jonkershoek Negative Control	**Domestic Raw Wastewater**	**Industrial Raw Wastewater**	**Treated Sewage Effluent**	**Raw Wastewater**	**Treated Sewage Effluent**	**Raw Wastewater**	**Treated Sewage Effluent**
SOS Chromotest	0 ± 0	116 ± 37 [a]	112 ± 63 [a]	0 ± 0	194 ± 56 [a]	0 ± 0	736 ± 412 [a]	0 ± 0

[a] Statistically different to the treated sewage effluent from the same sewage treatment plant ($P<0.005$).

6.5. Discussion

The aim of this study was to validate and use screening tests to determine the toxicity of raw wastewater and treated sewage effluents from three different sewage treatment plants in the Western Cape, South Africa. Raw wastewater and treated sewage effluents were collected from the three different sewage treatment plants and analyzed. The raw wastewater and treated sewage effluents were tested for potential cytotoxicity, neurotoxicity and genotoxicity.

LDH release from whole blood cultures was used as a biomarker for cell cytotoxicity. In this study a higher percentage of LDH released from the positive control compared to the raw wastewater and treated sewage effluent samples from all sewage treatment plants was observed ($P<0.050$). The raw wastewater and treated sewage effluent samples from all sewage treatment plants assayed at a 10 % concentration, resulted in no observable cytotoxicity. The presence of toxic contaminants in raw wastewater and treated sewage effluents can result in the loss of cell membrane integrity and therefore the loss of viable cells. However, this was not evident in whole blood cells in this study. Conversely, other studies have shown cytotoxicity of chlorinated drinking water produced from polluted raw wastewater (Yuan et al., 2005).

Acetylcholinesterase has been used as a biomarker to determine potential neurotoxic contaminants in the aquatic environment (Yadav et al., 2009). This study focused on the validation and implementation of the Ellman method (1961) to screen raw wastewater and treated sewage effluents for potential AChE inhibitors. The Ellman method (1961) has been used in several studies to determine acetylcholinesterase activity of tissue extracts, homogenates and neurotoxic compounds (Pfeifer et al., 2005). The method is based on

colorimetric measurement of enzyme activity. ATI is converted to thiocholine by the enzyme AChE. The released thiocholine then reacts with 5,5`-Dithio-bis-2-nitrobenzoic acid (DTNB). This reaction then results in the production of a yellow anion, 5-thio-2-nitro-benzoate (TNB) (Ellman et al., 1961). This colorimetric reaction can then be measured using a spectrophotometer. This study shows that lysed blood contains high levels of AChE. The AChE in lysed blood is very sensitive to chlorpyrifos inhibition and can thus be used for AChE inhibition assays. Chlorpyrifos at 54 µg/ml results in half maximal inhibition of AChE. AChE activity is an important biomarker to determine pollutant exposure to aquatic and terrestrial animals. Exposure of the freshwater teleost, *Channa striatus* (Bloch), to fertilizer industry effluents resulted in significant decrease of AChE activity (Yadav et al., 2009). In addition, contaminants present in United Kingdom estuaries resulted in decreases in AChE activity in muscle tissue of flounder fish (*Platichthys flesus*) (Kirby et al., 2000).

To test the applicability of the validated AChE inhibition assay, raw wastewater and treated sewage effluents were screened. The results obtained show that the AChE inhibition by raw wastewater from all sewage treatment plants were significantly higher than the Jonkershoek negative control ($P<0.050$). No difference of AChE inhibition between raw wastewater and treated sewage effluents for sewage treatment plant 1 were found. However, AChE inhibition by raw wastewater samples from sewage treatment plants 2 and 3 was lower than inhibition by treated sewage effluents. Taken together, these results show that the sewage treatment processes at all sewage treatment plants did not effectively eliminate potential AChE inhibitors. Also, a higher inhibition of AChE by treated sewage effluents from sewage treatment plant 2 and 3 could be a result of additional substances added during the treatment processes. AChE inhibitors may not necessarily be organophosphates or carbamates but may include other low level contaminants such as heavy metals or detergents, present in urban

rivers, estuaries and paper mill effluents (Payne et al., 1996). The AChE inhibitors present in treated sewage effluents could harm animals and humans (Kirby et al., 2000).

The SOS Chromotest has previously been used to determine genotoxicity of hospital and surface drinking waters (Jolibois et al., 2003; Guzzella et al., 2004). Jolibois et al. (2003) attributes the genotoxicity of hospital wastewater effluents to compounds such as anticancer drugs and antibiotics such as ciprofloxacin. The SOS Chromotest indicates potential DNA damaging agents present in the samples. In this study, the SOS Chromotest was used to assay raw wastewater and treated sewage effluents from three sewage treatment plants for potential genotoxicity. Results of this study showed that water from the control site has no genotoxicity. This result is expected since the control site is not impacted by human activity. All the raw wastewater samples assayed, tested positive for genotoxicity. The genotoxicity equivalent of sewage treatment plant 3 was higher than the genotoxicity equivalents of both sewage treatment plant 1 and 2. These results indicate that raw wastewater samples contain contaminants that could result in potential genotoxicity. In addition, differences in genotoxicity equivalents between sewage treatment plants could be attributed to differences in loading levels by each sewage treatment plant and the degree of different products used by the surrounding population. Treated sewage effluents from all sewage treatment plants displayed no genotoxicity. This indicates effective removal of genotoxins by all three sewage treatment plants investigated.

6.6. Conclusion

No cytotoxicity of whole blood cultures was observed for both raw wastewater and treated sewage effluents from all sewage treatment plants. In addition, this study showed that raw wastewater from all sewage treatment plants has potential AChE inhibitors. The sewage treatment processes are not effective at eliminating these AChE inhibitors. This study also showed that raw wastewater tested positive for genotoxicity. Sewage processes were effective in removing genotoxic substances. Taken together, these results suggest that the absence of cytotoxicity in raw wastewater and treated sewage effluents does not necessarily indicate the absence of other toxicities in these samples. The cytotoxicity method used in this study does not exclude effects on other specific cellular pathways (Ganey et al. 1993). This study showed that although treated effluents tested negative for cytotoxicity and genotoxicity, AChE inhibitors were still present after treatment processes.

Using these rapid tests to screen samples for toxicity is easy and does not require specialized skills. In addition, large numbers of samples can be screened at the same time. This study makes use of only screening assays to determine toxicity therefore care should be taken into interpreting results. Results of this study could reflect unique characteristics of the analyzed samples and therefore not a true representation of raw wastewater and treated sewage effluents over an extended period of time. Consequently, additional studies should be performed to determine *in vivo* effects of raw wastewater and treated sewage effluents. These tests could include a comparative toxicity assessment using a battery of *in vivo* tests. The mudsnail, *Potamopyrgus antipodarum* and the annelid *Lumbriculus variegates* often used for bioaccumulation studies could be ideal test species to determine biological effects of raw and treated effluents in whole organisms.

6.7. References

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Chapter 7: The Immunotoxic effects of raw wastewater and treated sewage effluents from three different sewage treatment plants in the Western Cape, South Africa

7.1. Abstract

The function of the immune system is to eliminate pathogens or chemicals from the host. The immune system can be subdivided into the innate immunity and adaptive immunity pathways. Mammals possess immune systems that are particularly vulnerable or sensitive to exposure to pollutants. Therefore, the immune system can be used to monitor pollutant exposure. Sewage effluents consist of a mixture of chemicals, microorganisms, debris, heavy metals, pesticides and pharmaceuticals. These sewage effluents or environmental pollutants may have an effect on the immune system of humans. However, very limited information is available on this subject. Moreover, studies that investigate the effects of wastewater on the immune system are imperative to lessen the knowledge gap. The aim of this study was to screen raw wastewater and treated sewage effluents from three different sewage treatment plants for its immunotoxic effects, using an *in vitro* whole blood culture assay and cytokine monitoring. Specific cytokines of the immune system were used as biomarkers. IL-6 was used as a biomarker for inflammation. IL-10 was used as a biomarker for humoral immunity. ELISAs specific for these two cytokines were used to assay the samples. Results of this study showed that raw wastewater and treated sewage effluents produced an immunotoxic effect on the IL-6 and IL-10 immune pathways. Despite differing technologies used by the sewage treatment plants in this study, contaminants were still able to result in an immunotoxic effect. Taken together, this study shows that sewage effluents may contain

contaminants that can cause adverse effects to the immune system of humans. Further *in vivo* studies are needed to clarify the mechanisms by which these immunotoxic effects occur.

7.2. Introduction

The function of the immune system is to eliminate pathogens or chemicals from the host (Schultz and Grieder, 1987). The immune system can be subdivided into the innate immunity and adaptive immunity pathways (Pruett, 2003). The first line of defense against microbes is the physiological and anatomical barriers. These barriers include the undamaged skin, mucous membranes, the bacteriolytic enzyme and lysozyme in tears and saliva (Turvey and Broide, 2010).

Innate immunity is considered as the host`s natural immunity and is inborn (Wolowczuk et al., 2008). Innate immunity is executed by both hematopoietic and nonhematopoietic cells (Turvey and Broide, 2010). Macrophages, dendritic cells, mast cells, neutrophils, eosinophils, natural killer (NK) and NK T cells all form part of the hematopoietic cells involved in innate immunity (Turvey et al., 2010). To enhance the innate immunity other mechanisms come into play such as complement protein, Lipopolysaccharide (LPS) binding protein, complement-reactive protein (CRP) and defensins (Turvey and Broide, 2010). Moreover, the molecular mediators, cytokines play a role in inflammation (Pruett, 2003).

Acquired or adaptive immunity elicits a memory response upon successive attacks of pathogens or microbes (Pruett, 2003). Several cell types such as B-cells, T-cells, T-cytotoxic cells, and antigen presenting cells play a role in adaptive immunity (Pruett, 2003). Adaptive immunity generates both cellular and humoral immunity (Perdigon et al., 1995). Cellular mediated responses are brought about by the T-helper 1 cells or T-cytotoxic cells. The T-helper 1 cells produce cytokines, which activate macrophages and allow for the destruction of microbes. On the other hand, T-cytotoxic cells release cytokines in order to directly kill off cells that are virus-infected or other intracellular parasites (Pruett, 2003). Humoral immunity

is characterized by antibody production by B-lymphocytes (Perdigon et al., 1995; Pruett, 2003; Weng, 2008). B-cell secretion of antibodies allows for pathogen destruction (Weng, 2008).

Cytokines are protein molecules that modulate the innate and adaptive immune system (Hansson et al., 2002). Cytokines function to repair tissues during infections and tissue trauma (Hopkins, 2003). Interleukins is a term used for cytokines that are produced by leukocytes (Parkin and Cohen, 2001). Several interleukins play a role in immunity.

Interleukin-6 (IL-6) is produced by various cell types. Cell types include T-cells, B-cells, fibroblasts, monocytes and endothelial cells. This interleukin is pleiotropic and displays both an anti-inflammatory and a pro-inflammatory effect (Gadient and Otten, 1997). IL-6 also functions in hematopoeisis, induction of acute phase proteins and maintenance of tissue function (Gadient and Otten, 1997; Fonseca et al., 2009). IL-6 also plays a role in the chronic phase of inflammation by directing monocytes to the site of injury (Fonseca et al., 2009). Consequently, this interleukin has the ability to result in autoimmunity (Fonseca et al., 2009).

Interleukin-10 (IL-10) is a cytokine that may be found in several cells. Cellular sources of IL-10 include mast cells, macrophages, B-cells and T-cells (Rennick et al., 1992). IL-10 possesses both stimulatory and suppressive properties. Its stimulatory role aids in humoral immunity. Humoral immunity is brought about by B-cell stimulation and the secretion of immunoglobulins. IL-10 secretion also results in the development of cytotoxic T-cells. On the other hand, IL-10 may suppress cytokine production by macrophages and Interferon gamma (IFN-ɔ) production in T- helper 1 cells (Rennick et al., 1992; Fiorentino et al., 1989).

Mammals possess immune systems that are particularly vulnerable or sensitive to exposure to pollutants (Secombe et al., 1992). Therefore, the immune system can be used to monitor

pollutant exposure. Sewage effluents consist of a mixture of chemicals, pollutants, microorganisms, debris, heavy metals, pesticides and pharmaceuticals (Salo et al., 2007). Environmental pollutants may have an effect on the immune system of humans (Luster and Rosenthal, 1993). IL-6 production by whole blood cultures (WBC) has been used as a biomarker for water quality (Pool et al., 2000). Furthermore, cytokine production from mouse splenocytes has been used as immunological biomarkers to evaluate treatment efficiency of reclaimed wastewaters (Kontana et al., 2008). Therefore, studies that investigate the effects of wastewater on the immune system are imperative to lessen the knowledge gap. The aim of this study was to screen raw wastewater and treated sewage effluents from three different sewage treatment plants for its immunotoxic effects, on specific immune pathways using an *in vitro* whole blood culture assay and cytokine monitoring.

7.3. Materials and Methods

7.3.1. Site description and collection of water samples

Raw wastewater and treated sewage effluents were collected from three different sewage treatment plants in the Western Cape, South Africa. The treatment plants investigated are on the same river system. Sewage treatment plant 1 and 2 use older technologies (conventional activated sludge system) to treat wastewater. Sewage treatment 3 has been upgraded and new technologies (membrane bioreactor) were incorporated in the treatment processes. Sewage treatment plant 2 and 3 receives domestic effluents only. However, sewage treatment plant 1 receives both domestic (85 % flow intake) and industrial raw wastewater (15 % flow intake).

A detailed description of sewage treatment technologies for the different sewage treatment plants are as follows. The older technologies (conventional activated sludge system) used at the sewage treatment plants can be divided into three processes, namely:

(i) Primary treatment which includes pre-treatment of raw waste water by coarse and fine screens for grit removal. This process includes sedimentation tanks to allow the heavier organic particles to settle.

(ii) Secondary treatment of raw water using activated sludge. This process involves aerated biological digestion by bacteria to remove remaining suspended and dissolved material. In addition, nitrification and de-nitrification of wastewater is also used as treatment processes within the sewage treatment plants. Thereafter, the wastewater enters the secondary sedimentation tank to allow separation of the liquid and solid phase. After secondary sedimentation the wastewater enters maturation ponds for further pathogen removal.

(iii) Tertiary treatment is the final step in the conventional activated sludge system used by sewage treatment plant 1 and 2. Ultraviolet light (used only at sewage treatment plant 1) or chlorine (used only at sewage treatment plant 2) are the disinfection processes used, before the treated sewage effluents are released from plants.

Sewage treatment plant 3 uses an additional treatment technology (membrane bioreactor) concurrently with conventional or older treatment technologies. The membrane bioreactor technology consists of microporous membranes. These micro-filtration and ultra-filtration membranes separate liquid and solids.

Water collected from the Eerste River in Jonkershoek, Stellenbosch, South Africa (33º55ô51ôôS, 18º51ô16ôôE) was used as a negative control. This site is situated in the Stellenbosch mountain and there is no human activity upstream from this area.

Samples were collected in pre-cleaned plastic bottles (1 L) and transported to the laboratory in a cooler.

7.3.2. Collection of blood

Informed consent documents were signed prior to collecting blood from donors. Blood was drawn from healthy male subjects (20-26 years of age). Criteria for blood collection were that donors were not on medication for the past month. Blood was collected via venipuncture using endotoxin-free evacuated blood collection tubes (Greiner Bio One GmBH) containing sodium citrate (3.2 %). All experiments were performed under sterile conditions. Blood was diluted 1:9 with Roswell Park Memorial Institute 1640 (RPMI-1640) medium.

7.3.3. Determination of inflammatory activity of water samples using WBC

Raw wastewater and treated sewage effluent samples were filtered through a 0.45 µM sterile filter (Lasec, S.A.). The samples were then added to eppendorfs (100 µl/eppendorf). For the positive control, 100 µl of a 10 µg/ml Lipopolysaccharide (LPS) (Sigma ï Aldrich, U.S.A.) solution was added to an eppendorf. For the negative control, 100 µl of distilled water was added to an eppendorf. Thereafter, 900 µl/eppendorf of diluted blood was added to the samples and controls. The WBC were incubated for 24 hours at 37 °C. After the incubation period, culture supernatants were collected and assayed for IL-6. IL-6 was used as a biomarker for inflammatory activity.

7.3.4. Determination of humoral activity of water samples using WBC

Raw wastewater and treated sewage effluent samples were filtered through a 0.45 µM sterile filter (Lasec, S.A.). The samples were then added to eppendorfs (100 µl/eppendorf). For the positive control, 100 µl of a 1.6 mg/ml phytohemagglutinin (PHA) (Sigma ï Aldrich, U.S.A.) solution was added to an eppendorf. For the negative control, 100 µl of distilled water was added to an eppendorf. Thereafter, 900 µl/eppendorf of diluted blood were added to the samples and controls. The WBC were incubated for 48 hours at 37 °C. After the incubation period, culture supernatants were collected and assayed for IL-10. IL-10 was used as a biomarker for humoral activity.

7.3.5. Lactate Dehydrogenase (LDH) assay

LDH released from culture supernatants was used as a biomarker for cellular cytotoxicity. LDH was measured on all culture supernatants using a commercially available kit (Biovision, USA). The assay was performed according to the manufacturerôs instructions.

7.3.6. IL-6 and IL-10 ELISA

Human ELISA Ready-Set-Go cytokine kits were purchased from eBioscience, USA. The ELISA kits were used according to the recommendations of the manufacturer. Briefly, ninety six well microtiter plates (Nunc, Apogent, Denmark) were coated with capturing antibody (purified anti-human IL-6 or IL-10 respectively) at a final dilution of 1: 250 in coating buffer (50 µl/well). The microtiter plates were allowed to incubate at 4 °C overnight. All successive incubations were performed on a plate shaker (Stuart, Microtiter Plate Shaker, SSMS). Subsequently, the binding sites in the wells were blocked with 200 µl/well blocking solution at room temperature for 1 hour. The plates were then washed 5 times with wash buffer. Standards (recombinant IL-6 or IL-10; 1 µg/ml) diluted in assay diluent were prepared. Standards or blood culture supernatants were added to the plates (50 µl/well) and incubated at room temperature for 2 hours. After 5 sequential washes with wash buffer, 50 µl of biotinylated detection antibody prepared at a final dilution of 1: 250 in assay diluent were added to all wells. The plate was then incubated for 1 hour at room temperature. After washing as before, avidin horseradish peroxidase (HRP) prepared at a final dilution of 1: 250 in assay diluent were added to all wells and incubated for 30 minutes at room temperature. After washing as before, 50 µl of 3.3ô, 5.5ô-tetramethylbenzidine (TMB) soluble substrate was added to all wells and the plate was incubated in the dark for 20 minutes. The enzymatic

reaction was then stopped by addition of 50 µl of 2 M sulphuric acid (H_2SO_4) to all wells. The optical densities were measured at 450 nm with a microtiter plate reader (Thermo Electron, Original Multiskan Ex). IL-6 and IL-10 production were calculated as a percentage of the positive control.

7.3.7. Statistical analysis

One way analysis of variance (ANOVA) was used to compare results, with $P<0.050$ considered as significant. Statistical analysis was done using SigmaPlot Version 11.

7.4. Results

7.4.1. Effects of raw wastewater and treated sewage effluents on cytotoxicity

Raw wastewater and treated sewage effluent samples were tested for cellular cytotoxicty using an LDH assay. Results showed that all samples were non-cytotoxic (data not shown).

7.4.2. Effects of raw wastewater and treated sewage effluents on inflammatory activity

IL-6 was used as a biomarker to determine the inflammatory response induced by raw wastewater and treated sewage effluent samples. The standard curve for the IL-6 ELISA is shown in Figure 7.1. The standard curve was used to calculate the concentrations of IL-6 in samples. The standard curve shows that there is a good correlation ($R^2 = 0.991$) between the absorbance and IL-6 concentration. Results are expressed as a percentage of the positive control (LPS). Results for IL-6 production by unstimulated WBC exposed to raw wastewater and treated sewage effluent samples for sewage treatment plant 1, 2, and 3 are shown in Table 7.1; 7.2 and 7.3 respectively. There was no difference in the percentage IL-6 produced between the Jonkershoek negative control site and the negative control (distilled water).

The percentage of IL-6 produced by domestic and industrial raw wastewater from sewage treatment plant 1 is significantly higher compared to the percentages produced by the Jonkershoek negative control site ($P<0.050$). There was no difference in the percentage IL-6 produced between the domestic and industrial raw wastewater for sewage treatment plant 1 compared to the LPS positive control. There was no difference in the percentage IL-6 produced by the domestic and industrial raw wastewater compared to the treated sewage

effluents for sewage treatment plant 1. A higher percentage of IL-6 was produced by treated sewage effluents compared to the Jonkershoek negative control ($P<0.050$).

The percentage IL-6 produced by raw wastewater and treated sewage effluents from sewage treatment plant 2 is significantly higher compared to the percentage produced by the Jonkershoek negative control site ($P<0.050$). There was no difference in the percentage IL-6 produced between the raw wastewater and the treated sewage effluents from sewage treatment plant 2 and the LPS positive control. There was no difference in the percentage IL-6 produced between the raw wastewater and treated sewage effluents for sewage treatment plant 2.

The percentage IL-6 produced by raw wastewater and treated sewage effluents from sewage treatment plant 3 is significantly higher compared to percentage produced by the Jonkershoek negative control site ($P<0.050$). There was no difference in the percentage IL-6 produced between the raw wastewater for sewage treatment plant 3 and the LPS positive control. The percentage IL-6 produced by the raw wastewater was significantly lower compared to the treated sewage effluents ($P<0.050$). The percentage of IL-6 produced by treated sewage effluents of sewage treatment plant 3 was significantly higher compared to the LPS positive control ($P<0.050$).

7.4.3. Effects of raw wastewater and treated sewage effluents on humoral activity

IL-10 was used as a biomarker for humoral immunity. The standard curve obtained for the IL-10 ELISA is shown in Figure 7.2. The standard curve showed a good correlation (R^2 = 0.999) between the absorbance and IL-10 concentration. Standard curves were used to calculate the concentrations of IL-10. Results were expressed as a percentage of the positive

control (PHA). The results for IL-10 production by unstimulated WBC exposed to raw wastewater and treated sewage effluent samples are shown in Table 7.1; 7.2 and 7.3 respectively. No significant difference of the percentage IL-10 produced by the Jonkershoek negative control site and the negative control (distilled water) was found.

The percentage IL-10 produced by domestic and industrial raw wastewater from sewage treatment plant 1 were significantly higher compared to the percentages produced by the Jonkershoek negative control site and the PHA positive control ($P<0.050$). Also, a higher percentage of IL-10 produced by the domestic and industrial raw wastewater compared to the treated sewage effluent was found ($P<0.050$). There was no difference in the percentage IL-10 produced between the treated sewage effluents of sewage treatment plant 1 and the PHA positive control. A higher percentage of IL-10 was produced by treated sewage effluents compared to the negative control site ($P<0.050$).

The percentage IL-10 produced by raw wastewater and treated sewage effluents from sewage treatment plant 2 were significantly higher compared to the percentages produced by the Jonkershoek negative control site ($P<0.050$). The percentage IL-10 produced by the raw wastewater was significantly higher than the positive control and the treated sewage effluents ($P<0.050$). There was no difference in the percentage IL-10 produced between the treated sewage effluents of sewage treatment plant 2 and the PHA positive control.

The percentage IL-10 produced by raw wastewater and treated sewage effluents from sewage treatment plant 3 were significantly higher compared to the percentages produced by the Jonkershoek negative control site ($P<0.050$). The percentage IL-10 produced by the raw wastewater was significantly higher than the positive control and the treated sewage effluents ($P<0.050$). There was no difference of percentage IL-10 produced between the treated sewage effluents of sewage treatment plant 3 and the PHA positive control.

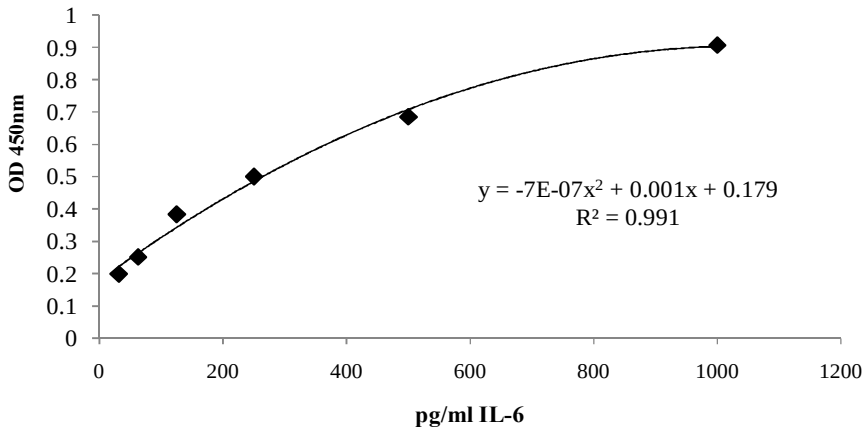

Figure 7.1. Standard curve for the IL-6 ELISA. The standard curve obtained shows a good correlation ($R^2 = 0.991$) between the optical density (OD) and IL-6 concentration.

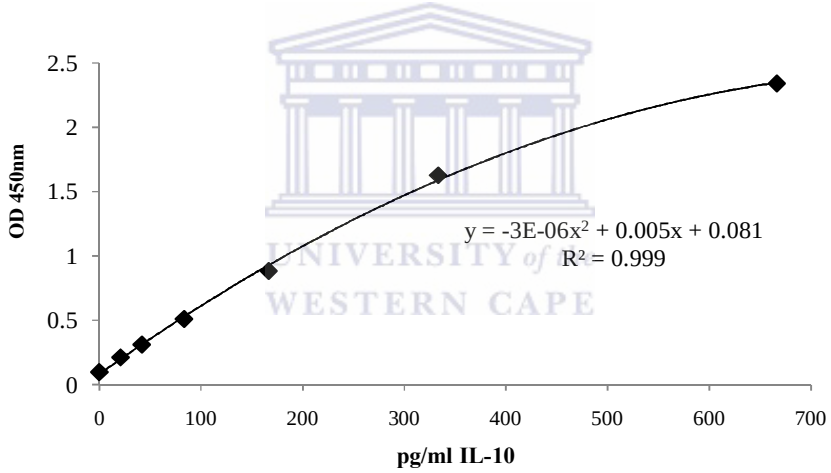

Figure 7.2. Standard curve for the IL-10 ELISA. The standard curve obtained shows that there is a good correlation ($R^2 = 0.999$) between the optical density (OD) and IL-10 concentration.

Table 7.1. IL-6 and IL-10 production (% ± SD) by WBC exposed to raw wastewater and treated sewage effluents from sewage treatment plant 1. Results are expressed as a percentage of the positive control. Sewage treatment plant 1 uses the conventional activated sludge system as wastewater treatment processes. Water collected at Jonkershoek was used as a negative control sample. This site is not impacted by human activity (n=32).

	Positive Control	Jonkershoek Negative Control Site	SEWAGE TREATMENT PLANT 1		
			Domestic Raw Water	Industrial Raw Water	Treated Sewage Effluent
IL-6 (% of positive control)	97 ± 1	0 ± 0 [b]	92 ± 46 [a]	89 ± 48 [a]	80 ± 48 [a]
IL-10 (% of positive control)	100 ± 8	0 ± 0 [b]	139 ± 58 [abc]	168 ± 78 [abc]	105 ± 33 [a]

[a] Statistically different to negative control site (P<0.050).

[b] Statistically different to positive control (P<0.050).

[c] Statistically different to treated sewage effluent (P<0.050).

Table 7.2. IL-6 and IL-10 production (% ± SD) by WBC exposed to raw wastewater and treated sewage effluents from sewage treatment plant 2. Results are expressed as a percentage of the positive control. Sewage treatment plant 2 uses the conventional activated sludge system as wastewater treatment processes. Water collected at Jonkershoek was used as a negative control sample. This site is not impacted by human activity (n=32).

	Positive Control	Jonkershoek Negative Control Site	SEWAGE TREATMENT PLANT 2	
			Raw Water	Treated Sewage Effluent
IL-6 (% of positive control)	97 ± 1	0 ± 0 [b]	125 ± 79 [a]	89 ± 46 [a]
IL-10 (% of positive control)	100 ± 8	0 ± 0 [b]	179 ± 53 [abc]	87 ± 24 [a]

[a] Statistically different to negative control site (P<0.050).

[b] Statistically different to positive control (P<0.050).

[c] Statistically different to treated sewage effluent (P<0.050).

Table 7.3. IL-6 and IL-10 production (% ± SD) by WBC exposed to raw wastewater and treated sewage effluents from sewage treatment plant 3. Results are expressed as a percentage of the positive control. Sewage treatment plant 3 uses the newer membrane technology as an additional wastewater treatment process. Water collected at Jonkershoek was used as a negative control sample. This site is not impacted by human activity (n=32).

	Positive Control	Jonkershoek Negative Control Site	SEWAGE TREATMENT PLANT 3	
			Raw Water	Treated Sewage Effluent
IL-6 (% of positive control)	97 ± 1	0 ± 0 [b]	96 ± 57 [ac]	151 ± 113 [ab]
IL-10 (% of positive control)	100 ± 8	0 ± 0 [b]	145 ± 65 [abc]	77 ± 29 [a]

[a] Statistically different to negative control site (P<0.050).

[b] Statistically different to positive control (P<0.050).

[c] Statistically different to treated sewage effluent (P<0.050).

7.5. Discussion

Wastewater contains a mixture of contaminants that may enter the sewage treatment plant. These contaminants include pharmaceuticals, personal care products (PCP), surfactants and industrial chemicals, bacteria and viruses (Bolong et al., 2009). Hospitals, septic tanks and livestock activities are all sources of contaminants (Focazio et al., 2008). Contaminants can exit via sewage effluents into the environment. Contaminants in the environment can pose a threat to animals and humans (Adams et al., 2008; Sturve et al., 2008; Reyero et al., 2008). The immune systems of humans are sensitive to exposure to pollutants (Secombe et al., 1992). Therefore, the immune system can be used to monitor pollutant exposure. The aim of this study was to screen raw wastewater and treated sewage effluents from three different sewage treatment plants for its immunotoxic effects using an *in vitro* whole blood culture assay. The inflammatory cytokine IL-6 and the humoral cytokine IL-10 were assessed respectively.

IL-6 was used as a biomarker for inflammatory actitivity and used to determine wastewater quality (Pool et al., 2000). The results of this study showed that WBC exposed to raw wastewater and treated sewage effluents from all sewage treatment plants produced an inflammatory response compared to the Jonkershoek negative control site ($P<0.050$) (Table 7.1; 7.2 and 7.3). The negative control site is not impacted on by human activity. Therefore no stimulation or suppression of the immune cytokines occurs when this sample water is exposed to WBC. On the other hand, raw wastewater contains bacteria, viruses and various other contaminants that can potentially activate T-cells, monocytes and macrophages. In turn the activation of the various cells then result in IL-6 synthesis. IL-6 production plays a role in inflammation which can be beneficial to the host. However, over-production of IL-6 can impact

the host negatively by resulting in chronic inflammation and autoimmunity. Furthermore, sewage treatment processes were not effective in eliminating contaminants that result in the inflammatory response since no significant difference of IL-6 between raw wastewater and treated sewage effluents from sewage treatment plant 1 and 2 were found. A higher percentage of IL-6 produced by treated sewage effluents from sewage treatment plant 3 compared to the raw wastewater and positive control occurred ($P<0.050$). This could be attributed to low removal rates of the contaminants in the sewage effluents or breakage of pathogens that may release endotoxins during sewage processes. Therefore, despite the different treatment technologies employed by the three sewage treatment plants, an induction of IL-6 can still be produced. These effects may still be seen downstream of the sewage treatment plant (Reyero et al., 2008; Khalaf et al., 2009).

IL-10 was used as a biomarker for humoral immunity and water quality (Pool and Magcwebeba, 2009). The results of this study show that WBC exposed to raw wastewater and treated sewage effluents from all sewage treatment plants has an immunotoxic effect on the humoral response compared to the Jonkershoek negative control site ($P<0.050$) (Table 7.1; 7.2 and 7.3). IL-10 is a cytokine that results in the activation and differentiation of B-cells. B-cells then synthesize antibodies known as immunoglobulins. These antibodies or immunoglobulins aid in protecting the host from intestinal parasites, bacteria and fungi (Twigg, 2005). Induction of IL-10 by raw wastewater could thus be an indication of the presence of bacteria, fungi and intestinal parasites that have been discharged into sewage. Several studies have shown an increase in IL-10 production upon exposure to bacteria, fungi and protozoa (Gazzinelli et al., 1992; Carvalho et al., 2002; Oderda et al., 2007; Jeurink et al., 2008).

In addition, results of this study show that WBC exposed to raw wastewater from all sewage treatment plants produces a significantly higher IL-10 compared to the PHA positive control ($P<0.050$). This therefore implies that raw wastewater from all sewage treatment plants results in hyperstimulation of the humoral response. Furthermore, raw wastewater from all sewage treatment plants results in a higher IL-10 production compared to the treated sewage effluents ($P<0.050$). However, treated sewage effluents from all sewage treatment plants produced higher IL-10 levels compared to the Jonkershoek negative control ($P<0.050$). These results therefore imply that the sewage treatment processes were efficient in lowering the IL-10 stimulant levels. However, treated sewage effluents still produce an induction on the humoral response. Incomplete removal of contaminants in sewage effluents can result in alteration of the immune system of animals (Hoeger et al., 2005; Salo et al., 2007).

Sewage effluents are often recycled for drinking water, recreational activities and agricultural purposes (Weinberg et al., 2004). Therefore, monitoring sewage effluents for its immunotoxic effects become an important water quality parameter. This study showed that raw wastewater and treated sewage effluents can have immunomodulatory effects. In addition, sewage treatment technologies used by sewage plants are ineffective in removing the contaminants that produce the immune effects.

7.6. Conclusion

This study only examines the *in vitro* effects of raw wastewater and treated sewage effluents on the immune system. To get a better understanding of the immunotoxic effects of sewage, *in vivo* studies need to be done. Furthermore, the results of this study are based on the sum of

contaminants present in sewage, and do not take into consideration the individual effects. Further work to identify individual contaminants that produce immunotoxic effects need to be done.

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Chapter 8: The use of activated charcoal to remove selected steroid hormones and surfactants from treated sewage effluents

8.1. Abstract

Wastewaters contain many contaminants which may enter receiving waters via agricultural run-off, wash-off from roadways, industrial wastewaters, municipal sewage, and domestic sewage. These contaminants have many adverse effects on human and animal health. Therefore, removal of these toxic compounds from sewage effluents is imperative to prevent adverse effects. A potential solution could be the absorption of pollutants by powdered activated charcoal. Previous studies have shown that sewage treatment processes are not effective in removing steroid hormones and surfactants from wastewaters. The aim of this study was to determine the efficiency of activated charcoal for the removal of steroids and surfactant residues from treated sewage effluents from a sewage treatment plant. Varying concentrations of activated charcoal (0; 25 mg/L; 50 mg/L; 100 mg/L) were added to treated sewage effluents and allowed to incubate for 2 hours. After the incubation the charcoal treated effluents were assayed for estradiol, estrone, testosterone and alkylphenol ethoxylate (APE) using Enzyme Linked Immunosorbent Assays (ELISAs). Results showed that all assayed concentrations of activated charcoal are effective in removing the steroids and surfactant APE from treated sewage effluents. The use of 100 mg/L of activated charcoal to ensure complete removal of steroids and surfactants is recommended. This study provides a potential method that could be employed by

sewage treatment plants as a final step to reduce steroids and surfactants in treated sewage effluents.

8.2. Introduction

Municipal wastewaters contain many pollutants such as pharmaceuticals, personal care products (Carballa et al., 2004), steroid hormones (Svenson et al., 2003) and surfactants (Mezzanotte et al., 2003). These pollutants enter receiving waters via agricultural run-off, wash-off from roadways, industrial wastewaters, municipal sewage, and domestic sewage (Bolong et al., 2009). Pollutants have many deleterious or negative impacts on the environment, human and animal health. For instance, survival ratios of mature male medaka exposed to varying concentrations of 17ϐ-estradiol (E2), nonylphenol (NP) and bisphenol A (BPA) declined. Moreover, E2, NP and BPA induce estrogenic effects in the male medaka (Tabata et al., 2001). Therefore, it is imperative that pollutants be removed from treated sewage effluents prior to entry into receiving waters.

Many sewage treatment plants are not equipped with technology to effectively remove all pollutants from wastewater (Petroviĺ et al., 2003). Low concentrations and difficulty in analyzing these pollutants mean that sewage treatment processes are not being monitored effectively for minimal adverse effects (Bolong et al., 2009). Therefore, low concentrations of pollutants may still enter receiving waters. However, concentration levels of compounds present in wastewater can vary according to differences in loading level of the sewage treatment plant, plant size and population background (Bolong et al., 2009). Differences of influent and effluent concentrations of pollutants can be used to determine pollutant removal efficiency of a plant (Zhang and Zhou, 2008). Different treatment processes such as physicochemical, biological and other more advanced treatments are used in sewage treatment plants to eliminate contaminants. Pollutants have various chemical properties that may impact on their removal efficiency (Bolong

et al., 2009). Certain pollutants may be water soluble, non-reactive to particles and stable in water. Therefore, pollutants may persist in the dissolved phase and thus be more bioavailable. If pollutants are hydrophobic, they will react with particles and be adsorbed. The bioavailability of the hydrophobic pollutants will thus decrease (Bowman et al., 2002). Furthermore an understanding of the various chemical properties of pollutants will allow for better removal efficiencies at sewage treatment plants. Also improved technologies to remove contaminants are vital in preventing adverse effects to aquatic life.

The use of adsorption systems to remove organic contaminants in wastewater is becoming prominent in sewage treatment plants (Stalter et al., 2010). Inorganic and organic contaminants present in the wastewater are removed by binding to an adsorbent. A frequently used adsorbent is the extremely porous activated carbon, also known as activated charcoal.

Activated charcoal is a black solid compound (Dwivedi et al., 2008). There are several forms of activated charcoal namely, granular activated charcoal (GAC), powdered activated charcoal (PAC), activated charcoal fibers (ACF) and activated charcoal cloths (ACC) (Dias et al., 2007). These forms have different properties. GAC is made up of bigger particle sizes than the PAC (Dwivedi et al., 2008). GAC can be produced by hard substances such as coconut shells. GAC is used specifically as a column filler and for gas and liquid treatments. On the other hand, raw material for PAC includes using wood sawdust. PAC mixes with liquid substances and is discharged of thereafter. Adsorption using PAC is very successful, however, its small particle size means that it tends to settle and remove slower than GAC and thus is more difficult to remove from treated effluent (Dias et al., 2007).

Interactions between the activated charcoal and the adsorbate are brought about by electrostatic and non-electrostatic forces (Dias et al., 2007). Characteristics of the adsorbate that play a role in the adsorption processes include the molecular size, solubility and acid dissociation (p*K*a) (Dias et al., 2007).

The use of activated charcoal is extremely expensive and costs of this adsorbent can be reduced by using cheaper raw material for its production (Lafi, 2001). The use of waste materials to produce activated charcoal has become an increasingly attractive prospect (Dias et al., 2007). Moreover, studies on agricultural waste material have also been investigated as potential ingredients to produce activated charcoal (González and Montoya, 2007).

Activated charcoal is known to be an excellent adsorbent for micropollutants and contaminants in wastewater and gas treatments (Chen et al., 1996; Dwivedi et al., 2008). It is also used extensively in drinking water plants as part of the purification processes (Zytner, 1992). Several studies have been undertaken to determine the effectiveness of activated charcoal for removing contaminants (Ayotamuno et al., 2006; Dash et al., 2009). GAC and PAC were 96 % and 99.9 % effective respectively in removing petroleum-hydrocarbon from ground water (Ayotamuno et al., 2006).

In South Africa, activated charcoal is mainly used as an adsorbent in drinking water treatment plants and is not normally incorporated in sewage treatment plants. Adding this additional step to the treatment processes of wastewater could reduce contaminant concentrations in effluents and produce purer treated sewage effluents entering the environment. This will improve sustainability of the natural resource. The aim of this study was to determine the efficiency of activated charcoal for the removal of steroids and surfactants from treated sewage plant effluents.

8.3. Materials and Methods

8.3.1. Site Description

Treated sewage effluents were collected from a sewage treatment plant in the Western Cape, South Africa. This sewage treatment plant has been upgraded and new technologies (membrane bioreactor) were incorporated in the treatment processes. The design capacity of the sewage treatment plant is 80 Mega Liters Per Day (ML/d). The sewage treatment plant uses an additional treatment technology (membrane bioreactor) concurrently with the conventional activated sludge process as seen in Figure 8.1. The membrane bioreactor technology consists of microporous membranes. These micro-filtration and ultra-filtration membranes separate liquid and solids.

8.3.2. Sample collection

Samples were collected in pre-cleaned 1 Litre (1 L) plastic bottles and transported to the laboratory in a cooler. Samples were filtered with filter paper (Munktell, 15 µm, 240 mm) (Lasec, SA) before adsorption tests and extraction.

8.3.3. Adsorption tests with activated charcoal

Different concentrations of powdered activated charcoal were added to 1 L of treated sewage effluents in 1 L bottles. Concentrations of activated charcoal added to treated sewage effluents

were from 25 mg/L, 50 mg/L, and 100 mg/L. As a control, 1 L of treated sewage effluent was left untreated (no activated charcoal added). All samples were put on a plate shaker for 2 hours. Thereafter, activated charcoal was allowed to settle out of the liquid phase by incubation at 4 °C overnight.

8.3.4. Solid phase extraction

Water samples were subjected to solid phase extractions (SPE) using C-18 columns (Sigma, Aldrich). The SPE columns were conditioned with 2 ml of Phase B mixture (45 % methanol, 40 % hexane and 15 % propanol), then 2 ml ethanol and lastly 4 ml distilled water. After the washing step, 100 ml of water sample was allowed to run through the columns, respectively. The columns were then dried using a vacuum pump (PALL vacuum pump, LifeSciences, 60 Hz, 1.92 Amperes, 220-240 Volts). The hydrophobic molecules attached to the resin were eluted with 2 ml of Phase B mixture and dried under a stream of air. The dried eluate was reconstituted with DMSO to make a 1000 times concentrated sample stock solution. Extracts were diluted with 0.1% BSA in saline at a ratio of 1:100 for the Estradiol and Estrone ELISAs and 1:10 for the testosterone ELISA. For the alkylphenol ethoxylate (APE) ELISA, extracts were diluted in 10 % methanol at a ratio of 1:100.

8.3.5. Estradiol (E2) analysis of final sewage effluent and activated charcoal treated sewage effluents extracts

E2 kits were purchased from DRG Instruments GmbH, Germany. All the reagents required were supplied in the kit. The wells of a microtiter plate were pre-coated with antibody directed towards a unique antigenic site on the E2 molecule. Samples and standards were applied at 25 εl/well to the anti-estradiol coated plate. Thereafter, 100 εl of enzyme conjugate (Estradiol horseradish peroxidase) were added to all wells. The mixture was incubated for 2 hours at room temperature on a plate shaker (Stuart, Microtiter Plate Shaker, SSMS). After incubation, the wells were washed five times with wash solution and tapped dry. Thereafter, 100 εl of substrate were added to all wells and incubated for 30 minutes at room temperature. The reaction was stopped by adding 50 εl of stop solution to all wells. The absorbances were then read at 450 nm with a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

8.3.6. Estrone (E1) analysis of treated sewage effluents and activated charcoal treated sewage effluents extracts

E1 kits were purchased from DRG Instruments GmbH, Germany. All the reagents required were supplied in the kit. The wells of a microtiter plate were pre-coated with antibody directed towards a unique antigenic site on the E1 molecule. Samples and standards were applied at 50 εl/well to the anti-estrone coated plate. Thereafter, 100 εl of enzyme conjugate (Estrone

horseradish peroxidase) were added to all wells. The mixture was incubated for 1 hour at room temperature on a plate shaker (Stuart, Microtiter Plate Shaker, SSMS). After incubation, the wells were washed five times with wash solution and tapped dry. Thereafter, 150 εl of substrate were added to all wells and incubated for 30 minutes at room temperature. The reaction was stopped by adding 50 εl of stop solution to all wells. The absorbances were then read at 450 nm with a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

8.3.7. Testosterone analysis of treated sewage effluents and activated charcoal treated sewage effluents extracts

Testosterone kits were purchased from DRG Instruments GmbH, Germany. All the reagents required were supplied in the kit. The wells of a microtiter plate were pre-coated with antibody directed towards a unique antigenic site on the testosterone molecule. Samples and standards were applied at 50 εl/well to the anti-testosterone coated plate. Thereafter, 100 εl of enzyme conjugate (Testosterone horseradish peroxidase) were added to all wells. The mixture was incubated for 1 hour at room temperature on a plate shaker (Stuart, Microtiter Plate Shaker, SSMS). After incubation, the wells were washed five times with wash solution and tapped dry. Thereafter, 150 εl of substrate were added to all wells and incubated for 30 minutes at room temperature. The reaction was stopped by adding 50 εl of stop solution to all wells. The absorbances were then read at 450 nm with a microtiter plate reader (Thermo Electron

Corporation, Original Multiskan Ex). A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

8.3.8. APE analysis of treated sewage effluents and activated charcoal treated sewage effluents water extracts

APE ELISA kits were purchased from Ecologiena, Tokiwa Chemical Industries Co. Ltd, Japan. Samples were analyzed according to the instructions included in the kit. All reagents required were supplied in the kit. The ELISA plate was precoated with antibodies specific to a unique antigenic site on the APE molecule. Samples or standards and antigen enzyme conjugate were pre-mixed in an uncoated microplate (100 ε1 of each solution). Thereafter, 100 ε1 of the pre-mixture was transferred per well of the coated plate. The plate was then incubated for 1 hour at room temperature. Thereafter, the wells were washed five times with wash solution and tapped dry. After washing, 100 ε1 of substrate were added to all wells and incubated for 30 minutes at room temperature. The enzyme reaction was stopped by adding 100 ε1 of stop solution to all wells. The absorbances were then read at 450 nm with a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

8.3.9. Statistical analysis

One way analysis of variance (ANOVA) was used to compare results for the steroid hormone and surfactant assays, with $P<0.050$ considered as significant. Statistical analyses were done using SigmaPlot Version 11.

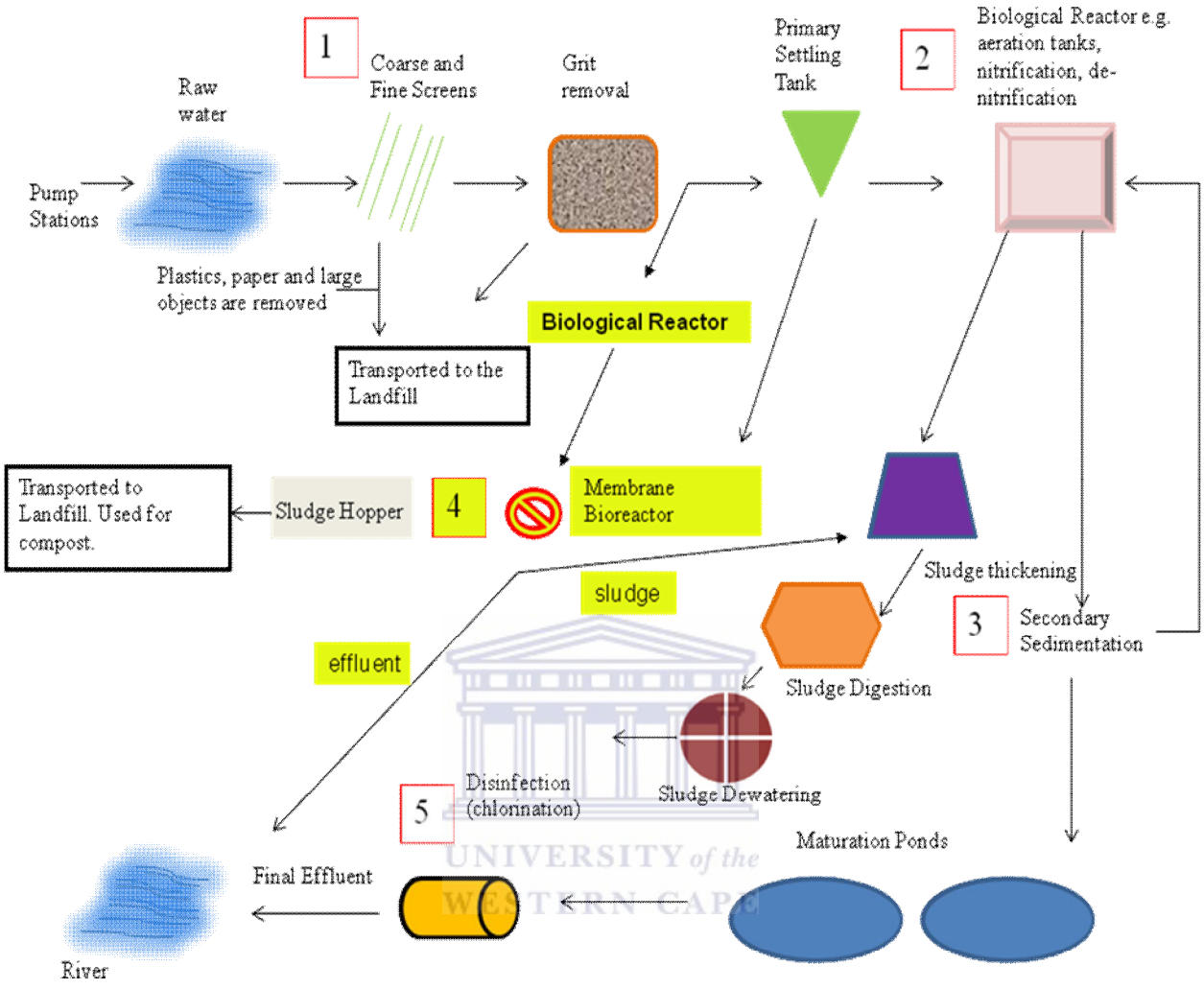

Figure 8.1. An additional membrane bioreactor technology (4) concurrently with older or conventional treatment processes are used to treat wastewater at the sewage treatment plant.

8.4. Results

8.4.1. Estradiol levels in treated sewage effluents and activated charcoal treated sewage effluents

The activated charcoal treatment had a major decrease on residual estradiol levels in sewage effluents (Figure 8.2). Estradiol concentrations are represented as the Mean ± Standard Error of the mean (SEM). The highest estradiol concentration observed was for the treated sewage effluents (4 ± 0 pg/ml). Lower estradiol concentrations for activated charcoal treated sewage effluents were found. These lower concentrations were significantly different compared with the treated sewage effluents (P<0.050).

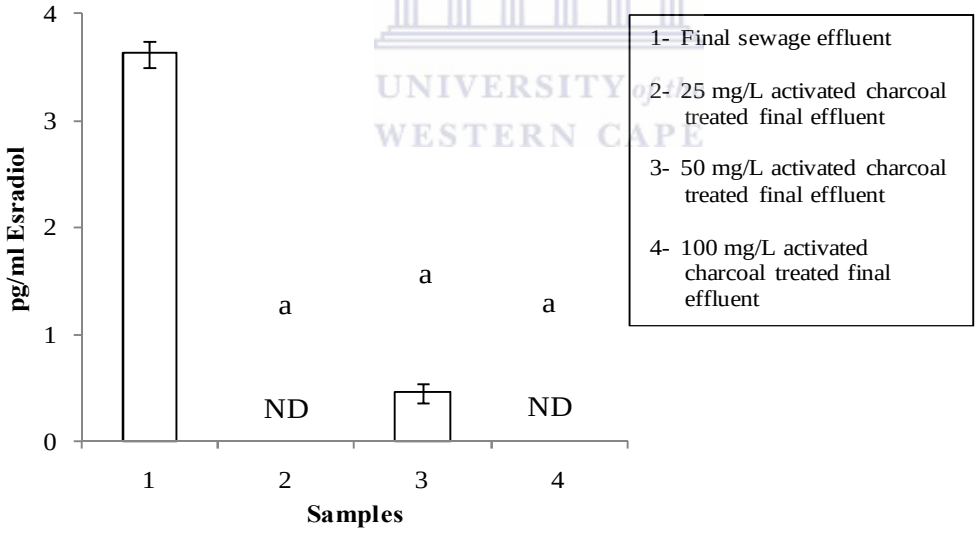

Figure 8.2. Mean estradiol concentrations (pg/ml) for treated sewage effluents and activated charcoal treated sewage effluents. [a] Statistically different compared to the final sewage effluent (P<0.050). Bars = standard error of the mean. ND = not detected.

8.4.2. Estrone levels in treated sewage effluents and activated charcoal treated sewage effluents

The activated charcoal treatment had a major decrease on residual estrone levels in sewage effluents (Figure 8.3). Estrone concentrations are represented as the Mean ± Standard Error of the mean (SEM). The highest estrone concentration observed was for the treated sewage effluents (12 ± 0 pg/ml). Lower estrone concentrations for the activated charcoal treated sewage effluents were found. These lower concentrations were significantly different compared with the treated sewage effluents ($P<0.050$).

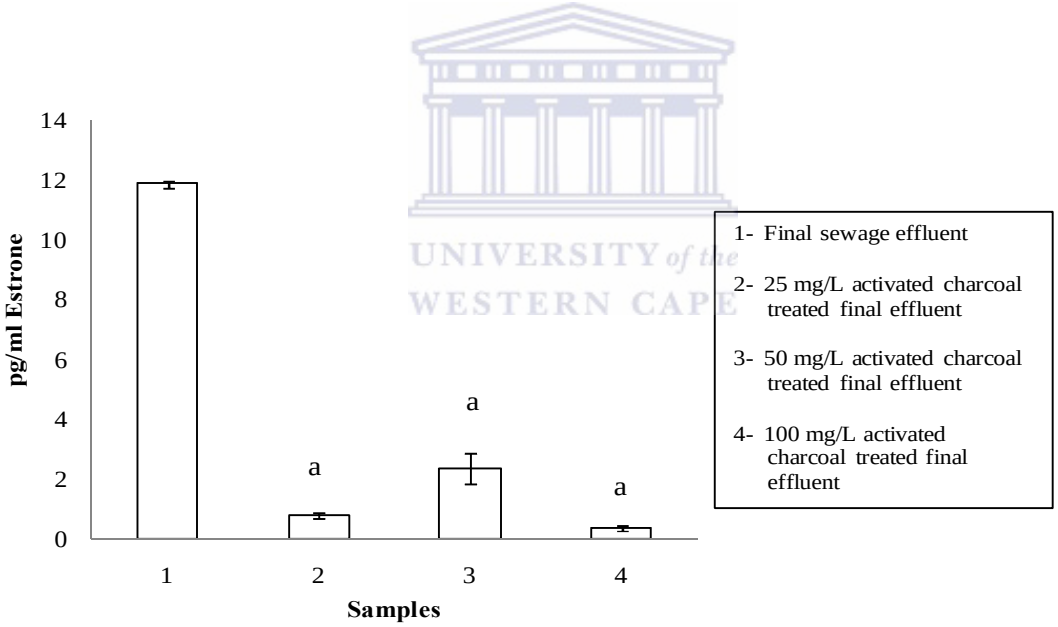

Figure 8.3. Mean estrone concentrations (pg/ml) for treated sewage effluents and activated charcoal treated sewage effluents. [a] Statistically different compared to the final sewage effluent ($P<0.050$). Bars = standard error of the mean.

8.4.3. Testosterone levels in treated sewage effluents and activated charcoal treated sewage effluents

The activated charcoal treatment had a major decrease on residual testosterone levels in sewage effluents (Figure 8.4). Testosterone concentrations are represented as the Mean ± Standard Error of the mean (SEM). The highest testosterone concentration observed was for the treated sewage effluents (100 ± 10 pg/ml). Lower testosterone concentrations for activated charcoal treated sewage effluents were found. These lower concentrations were significantly different compared with the treated sewage effluents ($P<0.050$).

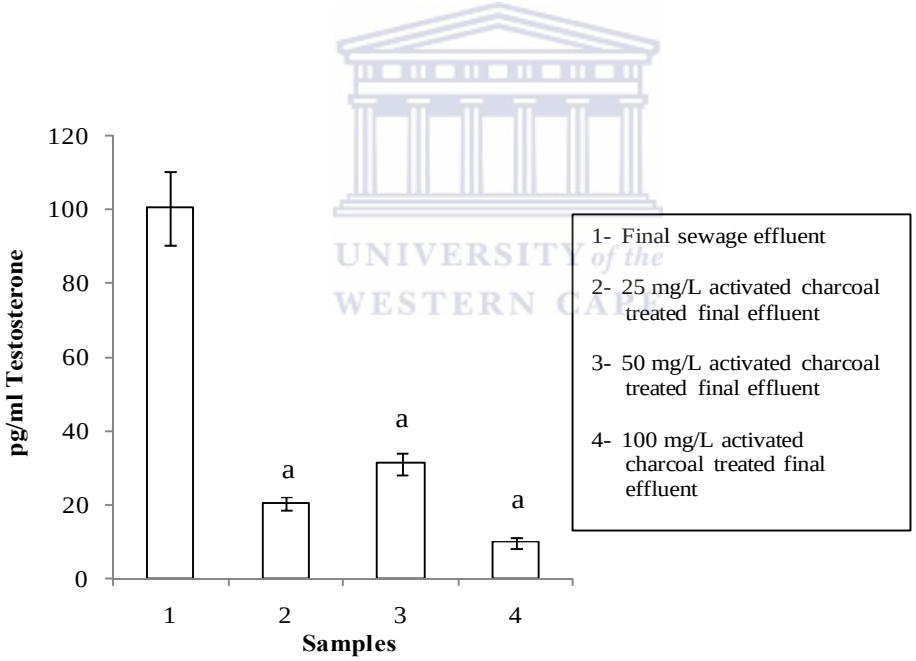

Figure 8.4. Mean testosterone concentrations (pg/ml) for treated sewage effluents and activated charcoal treated sewage effluents. [a] Statistically different compared to the final sewage effluent ($P<0.050$). Bars = standard error of the mean.

8.4.4. APE levels in treated sewage effluents and activated charcoal treated sewage effluents

The activated charcoal treatment had a major impact on residual APE levels in treated sewage effluents (Figure 8.5). APE concentrations are represented as the Mean ± Standard Error of the mean (SEM). The highest APE concentration observed was for the treated sewage effluents (7 ± 1 µg/L). Lower APE concentrations for effluents treated with 50 mg/L and 100 mg/L activated charcoal compared to the original sewage effluents were found ($P<0.050$). Treatment with 25 mg/L activated charcoal did not result in a reduction of APE in the sewage effluent.

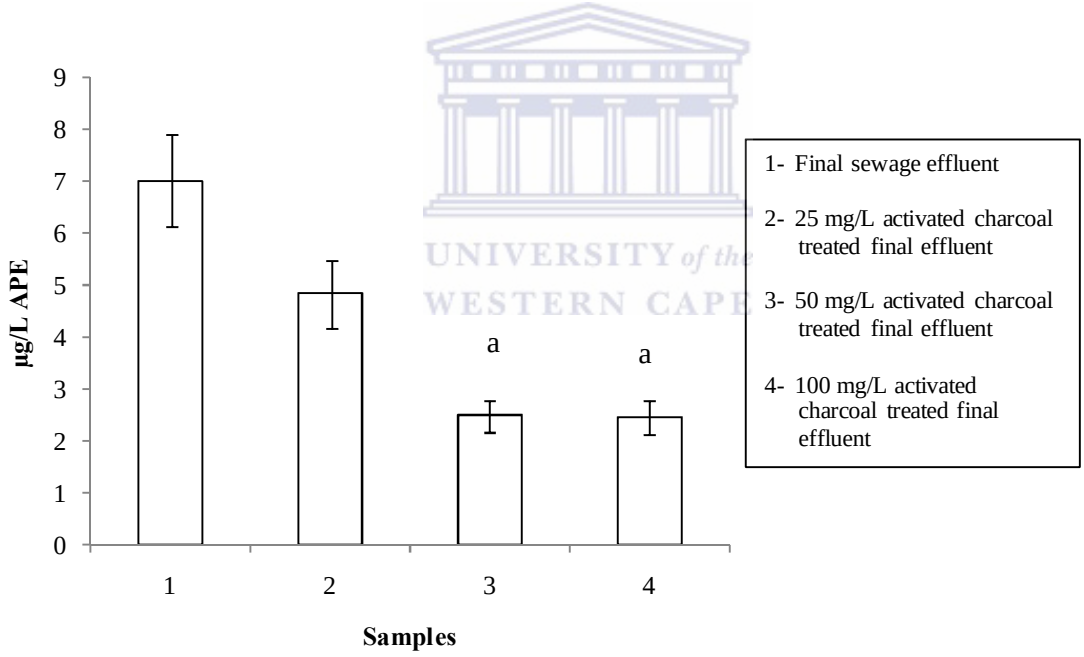

Figure 8.5. Mean APE concentrations (µg/L) for treated sewage effluents and activated charcoal treated sewage effluents. [a] Statistically different compared to the treated sewage effluents ($P<0.050$). Bars = standard error of the mean.

8.5. Discussion

Treated sewage effluents were collected from a sewage treatment plant in the Western Cape, South Africa. The treated sewage effluents are known to have residual concentrations of steroid hormones still present, despite treatment. Improved technologies are needed to completely remove pollutants from treated sewage effluents. This will limit environmental pollution and prevent adverse effects to humans and animals. The aim of this study was to evaluate if PAC is effective in removing steroid hormones and surfactants from treated sewage effluents.

Low concentrations of pollutants or contaminants from sewage treatment plants are released into receiving waters due to inefficient treatment processes. Many adverse effects have been shown in animals exposed to treated sewage effluents still contaminated with micropollutants (Fenlon et al., 2010; Ma et al., 2005). Japanese medaka exposed to sewage effluents experienced impairment of liver functioning and reproduction (Ma et al., 2005). Moreover, modulation of the sex ratio of daphnids occurred upon exposure to sewage effluents (Baer et al., 2009). In addition, vitellogenin synthesis and the presence of oocytes in male fish upon sewage effluent exposure indicate that estrogenic substances are still present in these treated effluents (Diniz et al., 2005). Technologies are needed to improve removal of contaminants from sewage treatment effluents. A popular method implemented by drinking water plants is the use of activated charcoal (Chen et al., 1996). Activated charcoal is either used as a primary treatment or as the final step in treatment of effluents (Dias et al., 2007).

This study showed that activated charcoal treatment of sewage effluents reduced estradiol, estrone and testosterone levels ($P<0.050$). This also implies that the activated charcoal treatment

of treated sewage effluents was effective, since lower or no concentrations of steroids were found compared to the treated sewage effluents. This data supports Zhang and Zhou (2005) and Snyder et al. (2007) which showed effective removal of estrone and estradiol by activated charcoal. The results of this study show that very low or no detection of steroid hormones were found in activated charcoal treated sewage effluents. Therefore, this implies that using activated charcoal to remove steroids from treated sewage effluents could potentially reduce adverse effects to aquatic life. Moreover, the extent of removal of a particular contaminant is dependent on the molecular characteristics of the contaminant in question (Snyder et al., 2007). Another factor that plays a role in adsorption of contaminants includes the physical properties of the wastewater (Fuerhacker et al., 2001).

The surfactant APE is widely used in industry today as components of detergents, paints, pesticides, textiles and personal care products (Scott and Jones, 2000). Residual surfactants released with sewage effluents could potentially result in a hormonal response in certain fish species (Solé et al., 2000). It is important to remove these harmful pollutants to prevent adverse effects to the environment. This study showed that high levels of APE are still released with treated sewage effluents. This is an indication of inefficient removal of APE by the sewage treatment processes. Sewage effluents treated with 50 mg/L and 100 mg/L activated charcoal resulted in a significant decrease in APE levels (Figure 8.5).

The use of 100 mg/L activated charcoal as a final treatment step in the treatment process is recommended to remove steroid hormones and surfactants from sewage effluents.

8.6. Conclusion

Advantages of using powdered activated charcoal seem warranted to remove contaminants from wastewater. However, because of its high cost and the inefficient disposal of contaminated PAC, it may induce further harm to the environment. Therefore, the benefits and adverse impacts must be weighed to determine overall efficiency of using this technology. Nonetheless, activated charcoal use provides an exciting prospect to remove adverse contaminants from wastewater. It could prove valuable to improve effluent quality when the effluent is used for reclamation, irrigation and recreation. Consequently, treated wastewater effluents recycled for industrial and agricultural purposes could be of a higher quality and therefore pose less adverse effects to humans.

This study showed that activated charcoal is efficient in removing steroid hormones and surfactants from sewage treatment plant effluents. A minimum concentration of 100 mg/L of activated charcoal is advised. This study provides a potential method that could be employed by sewage treatment plants to improve sewage effluent quality.

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Chapter 9: General conclusions and recommendations

9.1. General conclusions

The occurrence of contaminants found in sewage treatment effluents have been investigated by researchers globally. The technologies employed by sewage treatment plants are inefficient to remove all contaminants and these are discharged into receiving waters. Several reports have linked adverse health effects in humans and animals to contaminated sewage effluents. In addition, these contaminants can be transferred to soil and ground water.

This study compared the water quality of raw wastewater and treated sewage effluents from three different sewage treatment plants in the Western Cape, South Africa. The treatment plants investigated are on the same river system. Sewage treatment plant 1 and 2 use older technologies (conventional activated sludge system) to treat wastewater. Sewage treatment plant 3 has been upgraded and new technologies (membrane bioreactor) were incorporated in the treatment processes. Sewage treatment plant 2 and 3 receives domestic effluents only. However, sewage treatment plant 1 receives both domestic (85 %) and industrial (15 %) raw wastewater. In order to achieve the aims of this study, several objectives had to be reached.

The first objective of this study was to determine the occurrence of total coliforms, *E. coli* and selected antibiotics (fluoroquinolones and sulfamethoxazole) in raw wastewater and treated sewage effluents. A chromogenic test was used to screen for coliforms and *E. coli.* ELISAs were used to quantitate antibiotic residues in raw wastewater and treated sewage effluents. Raw

wastewater from all sewage treatment plants contained total coliforms and *E. coli.* Total coliforms and *E. coli* were removed effectively by sewage treatment plants 2 and 3. Sewage treatment plant 1 released total coliforms and *E. coli* above the recommended levels. Fluoroquinolones and sulfamethoxazole are commonly used antibiotics and were selected to monitor the efficiency of sewage treatment processes for antibiotic removal. Fluoroquinolones and sulfamethoxazole were detected in raw wastewater from all sewage treatment plants. Sewage treatment plant processes at sewage treatment plant 1 did not reduce the fluoroquinolone concentration in treated sewage effluents. Sewage treatment plant processes at sewage treatment plant 2 and 3 reduced the fluoroquinolone concentration by 21 % and 31 % respectively. The reduction of fluoroquinolone by the sewage treatment plants was not statistically significant and antibiotic residues were released with treated sewage effluents. Sewage treatment processes at sewage treatment plant 1 did not reduce the sulfamethoxazole concentration in treated sewage effluents. Sewage treatment processes at sewage treatment plant 2 and 3 reduced sulfamethoxazole by 34 % and 56 %, respectively. The reduction of sulfamethoxazole was not statistically significant and residues of this antibiotic were released with treated sewage effluents. This study successfully showed that treatment technologies used by some sewage treatment plants are inefficient in eliminating bacteria and antibiotics from treated sewage effluents. Indeed, this study shows that sewage treatment technologies need to be improved. Furthermore, examining the incidence of outbreaks of disease in humans downstream of sewage treatment plants could provide valuable insight into impacts of poor quality treated effluents on populations. Additional research could be undertaken to determine the toxic effects of these discharged antibiotics to aquatic life by examining resistant bacterial strains in aquatic species.

The second objective of this study was to compare the occurrence of the steroid hormones estradiol, estrone and testosterone in raw wastewater and treated sewage effluents. ELISAs were used to assess these steroids in the samples collected from the sewage plants. High levels of estradiol, estrone and testosterone were detected in raw wastewater from all sewage treatment plants. Measured concentrations of estradiol ranged from 87 - 115 pg/ml in raw wastewater and 14 - 76 pg/ml in treated sewage effluents. Treatment plants processes at sewage treatment plant 3 displayed low efficiencies for estradiol removal. Estrone levels ranged from 87 ï 227 pg/ml in raw wastewater and 20 ï 149 pg/ml in treated sewage effluents. Only treatment plants processes at sewage treatment plant 1 and 2 remove estrone effectively. Testosterone levels ranged from 121 ï 212 pg/ml in raw wastewater and 9 - 21 pg/ml in treated sewage effluents. Testosterone was removed effectively by all the treatment plants. Although new technologies (membrane bioreactor) have been incorporated to improve sewage treatment processes, high levels of steroid hormones are still released into the environment with the treated sewage effluents. These discharged sewage effluents may have adverse effects on the aquatic environment. This study successfully showed that despite the different technologies employed, ineffective removal of steroids from raw wastewater occurs. Residual steroid hormones are released into the environment with treated sewage effluents. Further studies are needed to improve sewage treatment processes and to determine the biological activity of these treated sewage effluents.

The third objective of this study was to determine the occurrence of the surfactants APE and AE in raw wastewater and treated sewage effluents. ELISAs specific for the selected surfactants were used to assay the sewage treatment samples. Alkylphenol ethoxylates (APE) and alcohol ethoxylates (AE) are the most widely studied surfactants. APE and AE surfactants were detected in significant concentrations in raw wastewater from all investigated sewage treatment plants.

This study showed that sewage treatment processes did not reduce the surfactant levels. Additional studies should be implemented to determine the fate and biological effects of the surfactants in receiving waters.

The fourth objective of this study was to validate and use screening tests to determine the toxicity of raw wastewater and treated sewage effluents. Biomarkers were used to determine toxicity. LDH release from cells was used as a biomarker to determine cellular cytotoxicity. Acetylcholinesterase (AChE) inhibition was used as a biomarker to determine neurotoxic contaminants in the sewage samples. The SOS chromotest was used to determine genotoxicity of the samples. The results of this study showed none of the raw wastewater and treated sewage effluents investigated were cytotoxic. Raw wastewater from all sewage treatment plants contains AChE inhibitors. The conventional activated sludge system and membrane bioreactor technology were unable to eliminate the AChE inhibitors. Raw wastewater samples from all sewage treatment plants tested positive for genotoxicity. Treatment processes effectively removed genotoxicity. The rapid tests were successfully employed to assess raw wastewater and treated sewage effluents for toxicity. This study provides valuable information on the potential toxic effects of wastewater to human and aquatic life. Further research into improving sewage treatment processes to completely eliminate all toxic contaminants will prove valuable. Future studies should examine the toxic effects of raw wastewater and treated sewage effluents *in vivo*.

The fifth objective of the study was to screen raw wastewater and treated sewage effluents from the three different sewage treatment plants for its immunotoxic effects, on the specific immune pathways using an *in vitro* whole blood culture assay and cytokine monitoring. IL-6 was used as a biomarker for inflammation. IL-10 was used as a biomarker for humoral immunity. ELISAs

specific for these two cytokines were used to assay the samples. Results of this study showed that raw wastewater and treated sewage effluent samples produced an immunotoxic effect on the IL-6 and IL-10 immune pathways. Despite differing technologies used by the sewage treatment plants in this study, contaminants were still able to result in an immunotoxic effect. Taken together, this study shows that sewage effluents contain contaminants that can potentially have adverse effects on the immune systems of humans. Future studies should look at the impacts of these contaminants on immune defense mechanisms *in vivo*.

The final objective of this study was to determine the efficiency of activated charcoal for the removal of steroids and surfactants from treated sewage effluents from a sewage treatment plant in the Western Cape, South Africa. Results showed that activated charcoal is effective in removing steroid hormones and the surfactant APE from treated sewage effluents. The use of 100 mg/L of activated charcoal to ensure complete removal of steroids and surfactants is recommended. This study successfully showed that activated charcoal could be used as a final treatment process to remove residual steroids and surfactants from wastewater. This will produce a better quality of sewage effluents entering the environment and improve sustainability of resources.

Due to inefficient treatment processes in sewage treatment plants, pollutants are discharged into the environment and may have adverse effects on humans and aquatic life. Monitoring raw wastewater and treated sewage effluents for various pollutants will provide much needed information on the efficacy of treatment processes. In this study, the occurrence of total coliforms, *E. coli*, antibiotics, surfactants and natural steroid hormones were assessed. This study also investigated the potential toxic and immunotoxic effects of raw wastewater and

treated sewage effluents from the sewage treatment plants. Adding activated charcoal treatment as an additional treatment step in sewage treatment plants could be valuable to reduce concentrations of contaminants entering the environment with the treated sewage effluents.

Several countries monitor the occurrence of contaminants in sewage effluents. In South Africa, screening of sewage effluents for organic pollutants is not well established. This study provides insight into the origin of some pollutants in South Africa. Moreover, monitoring the pollutants in sewage effluents will highlight problems that exist and will hopefully result in the research and implementation of methods that will reduce pollution of the environment. A summary table of the results obtained for treatment plant efficiencies to remove pollutants are given in Table 9.1.

Table 9.1. Summary of the microbial and chemical pollutant removal efficiencies of the three sewage treatment plant investigated.

Pollutant monitored	Sewage treatment plant 1	Sewage treatment plant 2	Sewage treatment plant 3
Total coliforms (TC)	U.V. light disinfection ineffective at removing TC in sewage effluents	Chlorine disinfection effective at removing TC in sewage effluents	Membrane bioreactor, chlorine disinfection is effective at removing TC in sewage effluents
E. coli	U.V. light disinfection ineffective at removing *E. coli* in sewage effluents	Chlorine disinfection effective at removing *E. coli* in sewage effluents	Membrane bioreactor effective at removing *E. coli* in sewage effluents
Fluoroquinolones	92 ng/L (2% reduction)	72 ng/L (21% reduction)	68 ng/L (31% reduction)
Sulfamethoxazole	121 ng/L (4% reduction)	101 ng/L (34% reduction)	56 ng/L (56% reduction)
Estradiol	14 pg/ml (84% reduction)	22 pg/ml (78% reduction)	76 pg/ml (34% reduction)
Estrone	20 pg/ml (78% reduction)	40 pg/ml (77% reduction)	149 pg/ml (34 % reduction)
Testosterone	19 pg/ml (86% reduction)	21 pg/ml (90 % reduction)	9 pg/ml (96 % reduction)
Alkylphenol ethoxylate	53 µg/L (45% reduction)	20 µg/L (79% reduction)	17 µg/L (81% reduction)
Alcohol ethoxylate	17 µg/L (73% reduction)	83 µg/L (83% reduction)	16 µg/L (70% reduction)

Table 9.1. shows that chlorine disinfection processes at sewage treatment plant 2 and 3 are effective at eliminating total coliforms and *E. coli* in sewage effluents. The membrane bioreactor processes at sewage treatment plant 3 are more effective at reducing fluoroquinolones and sulfamethoxazole antibiotics in sewage effluents compared to the conventional processes. The membrane bioreactor processes at sewage treatment plant 3 did not reduce the levels of the steroid hormones estradiol and estrone effectively. Treatment processes at all three sewage

plants investigated removed testosterone effectively. Similar reduction percentages were obtained for the surfactants alkylphenol ethoxylate and alcohol ethoxylate in sewage effluents from sewage treatment plant 2 and 3.

9.2. Recommendations

Various ELISAs are used in this study to determine the concentration and presence of organic pollutants in question. These ELISAs are commercially available and test for organic pollutants such as steroid hormones and surfactants. Using this testing method a monitoring programme to determine the quantity of organic pollutants entering and exiting sewage treatment plants can be implemented. Future studies could include determining the source of particular contaminants. If a problem exists, source control strategies need to be put in place. These strategies may include substituting or using alternative industrial chemicals in order to lessen the footprint to organisms downstream of sewage treatment plants. Furthermore, using ELISAs, the transport of the target compounds can be assessed by investigating quantities of these compounds during each stage of the sewage treatment process. If a perceived problem then arises implementation of additional advanced technologies as a final treatment step is recommended. The use of activated charcoal or a multi-barrier approach using membrane bioreactor technology followed by reverse osmosis or nanofiltration may be ideal treatment technologies to prevent residual organic pollutants entering receiving waters. In addition, the fate of organic compounds could be assessed downstream of the sewage treatment plants. Lastly, effects of residual organic pollutants to wildlife such as frogs could also be assessed.