Detection and Evaluation of the Fate of Estrogen Endocrine Disrupting Chemicals in Wastewater Treatment

Submitted in fulfilment of the requirements of the degree of Doctorate of Technology: Biotechnology in the Faculty of Applied Sciences at the Durban University of Technology

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APPROVAL

I hereby approve the final submission of the following dissertation.

Detection and Evaluation of the Fate of Estrogen Endocrine Disrupting Chemicals in Wastewater Treatment

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This _____ day of _____ , 2014, at the Durban University of Technology.

ABSTRACT

All over the world concerns have been raised over the possible adverse effects that may occur when exposed to chemicals that have the potential to interfere and affect the endocrine system. The concern is directed at both humans and wildlife. There is still a lack of public awareness regarding Endocrine Disrupting Chemicals (EDCs) and the harmful effects on humans and wildlife. It has only been within the last decade that South Africa began the actual task for proper management and control for water and wastewater quality. There are many ways to detect these EDCs all of which are very laborious and most of the cases these EDCs are either in the pico or nano gram per litre range, too minute for many methods to detect effectively; so therefore the research project aimed to use rapid and sensitive techniques to determine the quickest means to detect the very low concentrations of theses EDCs. Two techniques were researched, i.e., Enzyme Linked immunoassays (ELISAs) and Radio-immunoassays (RIAs). The research study thus assessed the solid phase extraction (SPE) technique for total recovery of hormones; the ELISA and RIA techniques for rapid detection of natural (estrone (E1), estradiol (E2) and estriol (E3) and synthetic ethinylestradiol (EE2) by validating the precision and reproducibility . These techniques were then applied to determine hormone EDC removal first at laboratory scale investigations and then applied to full scale wastewater treatment plants (WWTP) with different configurations in order to deduce removal efficiency of each type of plant. The next phase assessed the toxicity of individual and combined estrogen standards as well as the toxicity in the WWTPs and classify and to determine if there was a correlation between hormone concentration and toxicity in final effluents. The assessment of the SPE and the immunoassay procedures (ELISA AND RIA) using standards and controls found that both these assays can be utilised to quantify hormone estrogens in wastewater. The small sample volume required reduced the labour time and application of the procedure made it cost effective and reliable techniques. The intra-assay and inter-assay validation procedures as well as the standard recoveries confirmed reproducibility and precision of the immunoassays. The % CV were <10% for both the intra-assay and inter-assay validations. The laboratory scale investigations included the operation of a modified Ludzak-Ettinger (MLE) process which enabled control and manipulation over the operational parameters in order to establish how certain parameters influenced the removal of hormone EDCs. One such parameter that was manipulated was the

sludge retention time (SRT). The MLE tests showed that the SRTs definitely have an effect on the removal of hormones from the influent as well as the overall performance of sewage treatment. The 10 day SRT proved that longer SRTs will definitely aid in the removal of hormones and possibly other EDCs in raw sewage. During the 10 day SRT the influent hormone concentrations (E1: 59.11 ng/L, E2: 61.40 ng/L) were almost double than the influent hormone concentrations (E1: 26.46 ng/L, E2: 27.60 ng/L) during the 5 day SRT, which impacted on the removal efficiency. The 5 day SRT had an overall average E2 and E1 removal of 78.11% and 81.71% respectively while the 10 day SRT had average E2 and E1 removal of 91.24 % and 80.56% respectively. The 24 hour batch test provided evidence of the reversible metabolism of the E2 hormone. This was seen by the rapid decrease of E2 and the rapid increase of E1 in less than 3 hours, which proved that E2 can be metabolized in to E1. An average reduction of 94.44% of E2 was seen after 5 hours and after 10 hours was no longer detected. After 13 hours E1 could no longer be detected. This finding also provided clarity as to the lower percentage removal of E1 during the 10 day SRT of the MLE process. The *Vibrio fischeri* biotox method implemented was the most economic and easiest way to conduct the toxicity tests. The validation of the test used a 52.9 mg/L $K_2Cr_2O_7$ standard which provided a Cr (VI) concentration of 18.7 mg/L in the final test suspension which is the theoretical effective concentration causing 50% inhibition (EC_{50}). This specific concentration of the Cr (VI) exhibited an EC_{50} at 20.08 mg/L. The toxicity investigations of the individual and mixed hormone standards revealed that at the 10 ng/L concentration the individual E2 standard had the highest percentage inhibition (%INH) of 45.99% after the 30 minute contact time (T_{30}), and when this standard was further diluted to 5 and 1 ng/L also showed higher % INH (26.04 and 23.66 %INH, respectively) than the individual EE2 standard (21.92 %INH) at 10 ng/L. . According to the toxicity classification system and after interpretation of the data, all the hormone standards were classified as Class II as they all exhibited slight acute toxicity. The 10 ng/L E2 standard had Toxicity Units (TU) of 0.8 which was close to the Class III level; however when it was in a mixture with E1 and E3, the TU was much lower (0.6 TU). The synthetic EE2 hormone also showed slight acute toxicity and had the lowest TU of 0.4. The application of the above mentioned techniques to full scale WWTPs with different configurations showed different removal efficiencies. The WWTPs ranged from the most primary consisting of just oxidation ponds to biological trickling filters, to biological nutrient removal (BNR) to conventional activated sludge (AS) plants. Removal rates ranged from 29% to 96% for E2, 0% to 89% for E1 and 0% to 100% for EE2.

The overall ranking of the WWTPs from the most efficient to least efficient in terms of hormone removal were as follows: Plant E (91%) = Plant D (before UF) (91%) > Plant B (east side) (88%) > Plant B (west side) (77%) > Plant C (east side) (71%) > Plant D (after UF) (57%) > Plant A (56%) > Plant C (west side) (12%). Using the *Vibrio fischeri* method to evaluate the reduction of toxicity in WWTPs C, D and E proved effective. It was seen immediately after secondary biological treatment in the clarifier effluent the toxicity was reduced. Plants C, D and E had reduced the toxicities by 100, 80 and 97 % immediately after secondary biological treatment, while after the addition of the Chlorine disinfectant in the final stage of treatment the toxicity increased having %INH of 99.9, 15.7 and 99.9 respectively. In conclusion the SPE can be used as an extraction procedure for hormones in wastewater and the immunoassays can be used as rapid techniques for quantification of hormone EDCs in wastewater. The ELISA technique proved to be the slightly superior to the RIA in terms of facilities required. The laboratory scale procedures proved that some hormones can be oxidised to other hormones and therefore longer sludge retention times may be required to improve the removal. The study of the different WWTPs configuration showed that plant configuration and operational parameters impact the removal of hormone EDCs. The composition of the influent received by the plant also has an effect on the removal, i.e., whether it's industrial, domestic or a mixture of both. Results concluded that plants which have either mixing and/or aeration with activated sludge and longer SRTs of more than 10 days have a higher rate of hormone removal than those plants with shorter SRTs and that the activated sludge processes were capable of reducing the toxicity of the influent. Overall results indicated that hormone EDCs are indeed being discharged with the effluents from WWTPs in South Africa. However whether the concentrations left in the final effluents will still have an adverse effect on the aquatic life is a question that still remains unanswered. The aquatic ecosystems are inevitably being polluted with these EDCs and their breakdown products.

DECLARATION

Detection and Evaluation of the Fate of Estrogen Endocrine Disrupting Chemicals in Wastewater Treatment

SWASTIKA SURUJLAL-NAICKER

I hereby declare that the dissertation represents my own work. It has not been submitted before for any diploma/degree or examination at any other Technikon/University.

_____ _____

SWASTIKA SURUJLAL-NAICKER **Date**

2014

Reference Declaration in Respect of a Doctorate's Dissertation

I, **Swastika Surujlal-Naicker** (full name of student) and, **Professor Faizal Bux** (full name of supervisor) do hereby declare that in respect of the following dissertation:

Detection and Evaluation of the Fate of Estrogen Endocrine Disrupting Chemicals in Wastewater Treatment

- as far as we know and can ascertain:
- no other similar dissertation exists;
- the only similar dissertation(s) that exist(s) is/are referenced in my dissertation as follows:

_____

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- All references as detailed in the dissertation are complete in terms of all personal communications engaged in and published works consulted.

_____ _____

Signature of Student **Date**

_____ _____

Signature of Supervisor **Date**

PREFACE

Some of the material in this thesis has/will been/be published and presented elsewhere:

Publications:

- Surujlal-Naicker, S. and Bux, F. Application of Radio-immunoassays to assess the fate of estrogen EDCs in full scale wastewater treatment plants. Journal of Environmental Science and Health, Part A (2013) 48, 1–11.Copyright C _ Taylor & Francis Group, LLC. ISSN: 1093-4529 (Online) DOI: 10.1080/10934529.2012.707832
- Surujlal-Naicker, S., Gupta, S.K. and Bux, F. Evaluating the acute toxicity estrogen hormones and wastewater effluents using *Vibrio fischeri*. [In Print: Journal of Human and Ecological Risk Assessment] (ID: 955767 DOI:10.1080/10807039.2014.955767)
- Surujlal-Naicker, S., Gupta, S.K. and Bux, F. Using ELISAs to investigate removal of hormone EDCs in WWTPs with different configurations in South Africa. [Submitted]

Conferences:

- International Water Association (IWA) World Water Congress: Sustainable Water Management Practices, Beijing, China, 10-14 September 2006. Short Platform Presentation and Poster: Detection of Hormone Endocrine Disrupting Chemicals in Final Effluents from South African Wastewater Treatments Plants using the application of Radio-immunoassays.

- International Water Association (IWA) and Groundwater Resources Association (GRA) of California. Micropol and Ecohazard. 6th IWA/GRA Specialised conference on assessment and control of micropollutants/hazardous substances in water. June 8 – 10 2009. San Francisco, California. Poster Presentation: Determining the Removal Capacity of Hormone Endocrine Disrupting Chemicals in South African Wastewater Treatment Plants.

DEDICATION

This thesis is dedicated to those who care about saving our water resources and creating a better environment for future generations!

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To God Almighty for providing me with the strength and courage to achieve this goal!

To my parents, it is through your love, guidance and encouragement I have managed to make it this far in life. Thank you! I am eternally grateful!

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ABBREVIATIONS

ASP - Africa Stockpiles Programme

BNR - Biological Nutrient Removal

BOD - Biological Oxygen Demand

CNS - Central Nervous System

COD - Chemical Oxygen Demand

Cr (VI) - Hexavalent Chromium

CV - Coefficient of Variation

DDE - Dichlorodiphenylethylene

DDT - Dichlorodiphenyltrichloroethane

DEHP - Di(2-ethylhexy)-Phthalate

DOC - Dissolved Organic Carbon

DWA - Department of Water Affairs

E1 - Estrone

E2 - Estradiol

E3 - Estriol

EC_{50} - Effective Concentration causing 50 % inhibition

EDCs - Endocrine Disrupting Chemicals

EDs - Endocrine Disruptors

EE2 - Ethinylestradiol

EEC - Estimated Environmental Concentration

EEQ - Estradiol Equivalents

ELISA - Enzyme Linked Immunoassay

EPA - Environmental Protection Agency

ER - Estrogen Receptor

EU - European Union

$FeCl_3$ - Ferric Chloride

GCMS - Gas Chromatography Mass Spectrophotometry

GR - Glucocorticoid Receptor

HCHs - Hexchlorocyclohexanes

HDCs - Hormone Disrupting Chemicals

HRT	-	Hydraulic Retention Time
% INH	-	Percentage Inhibition
$K_2Cr_2O_7$	-	Potassium Dichromate
KF	-	Correction Factor
Kg	-	Kilogram
L	-	Litre
LAS	-	Linear Alkylbenzene sulphonates
LCMS/MS	-	Liquid Chromatography Mass Spectrophotometry/ Mass Spectrophotometry
LOEC	-	Lowest Observed Effect Concentration
MATC	-	Maximum Acceptable Toxicant Concentration
MCL	-	Maximum Concentration Limits
mL	-	Millilitre
Ml	-	Mega litre
MLE	-	Modified-Ludzak Ettinger
MLSS	-	Mixed Liquor Suspended Solids
MLVSS	-	Mixed Liquor Volatile Suspended Solids
N	-	Nitrogen
NAPLs	-	Non Aqueous Phase Liquids
ND	-	Not Detected
ng	-	Nanogram
NGOs	-	Non-governmental Organizations
NOEC	-	No Observed Effect Concentration
NP	-	Nonylphenol
NSB	-	Non-specific Binding
NSCM	-	Night Soil Composting Microorganisms
OUR	-	Oxygen Utilisation Rate
P	-	Phosphorus
PAH	-	Polyaromatic Hydrocarbons
PBDEs	-	Polybrominated Diphenyl Ethers
PCBs	-	Polychlorinated Biphenyls
PCDD	-	Polychlorinated Dibenzene Diphenyls
PCDF	-	Polychlorinated dibenzo Furans
PE	-	Percentage Effect

pg	-	Pico gram
PGMs	-	Platinum Group Metals
PNEC	-	Predicted No Effect Concentration
POPs	-	Persistent Organic Pollutants
ppt	-	Parts per trillion
PST	-	Primary Settling Tank
RIA	-	Radio Immunoassay
RLU	-	Relative Light Units
SANS	-	South African National Standards
SPE	-	Solid Phase Extraction
SRT	-	Solids Retention Time
STW	-	Sewage Treatment Works
TU	-	Toxicity Units
UF	-	Ultra filtration
UK	-	United Kingdom
UNEP	-	United Nations Environment Programme
USEPA	-	United States Environmental Protection Agency
UWWT	-	Urban Wastewater Treatment Plants
VTG	-	Vitellogenin
WHO	-	World Health Organisation
WWF	-	World Wildlife Federation
WWTP	-	Wastewater Treatment Plant

CHAPTER 1
INTRODUCTION

1.1 BACKGROUND

All over the world concerns have been raised over the possible adverse effects that may occur when exposed to chemicals that have the potential to interfere and affect the endocrine system. Many studies have been done to identify a number of these chemicals (Scheringer *et al*., 2012; Stewart *et al*., 2014) as well as the adverse effects they may have (Payan-Renteria *et al.*, 2012). These chemicals are known as endocrine disrupting chemicals (EDCs) (Nasu *et al.*, 2001). The concern is directed at both humans and wildlife (Jiang *et al.*, 2012). In developing countries like South Africa, the public is not aware of these effects; there is also a lack of knowledge among Health Workers, Regulators and Industry, with regards to the potential effect of EDC's on public health (Awofolu and Fatoki, 2003).

Many of the chemicals listed as endocrine disrupting chemicals by developed and developing countries are still being used in South Africa today, which includes Dichlorodiphenyltrichloroethane (DDT) to control the malaria epidemic (Awofolu and Fatoki, 2003). South Africa is still assessing methods that must be employed to detect/screen for EDCs that have an impact on animals and human endocrine systems. The National Water Act (no 36 of 1998) excludes low dose exposures and concerns only toxicological variables. Most of the effluents that reach natural water resources include effluents from sewage plants, effluents which have pesticide contamination and run-off in agricultural areas as well as industrial effluents. The endocrine disruption in wildlife, which includes developmental abnormalities, infertility, bisexuality, sex reversals, immune, neural and metabolic processes is based on unconfirmed cases and an epidemiological data linking human health to EDC's is still to be made.

1.2 WHAT IS AN EDC?

An EDC has collectively been defined as "*an exogenous substance that can cause adverse health effects in an intact organism or its progeny, by interfering with the synthesis, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of development processes"* (Lopez and Barcelo, 2001; Menditto and Baldassarri, 1999; Zhou *et al*., 2010).

1.3 THE IMPORTANCE OF THE ENDOCRINE SYSTEM

In order for the body to function properly, a constant internal environment needs to be maintained so that organs can communicate with each other. When changes in the internal and external environments occur, it is essential that an organism responds to these changes which are done by communication between various parts of the body (Sturmhofel and Bartke, 1998).

There are 3 major systems in the human body with the endocrine system being one of them. The endocrine system works like an enabler who helps the body to communicate with the external environment (Gore and Dickerson, 2012). There are two systems that help to ensure communication, i.e., the nervous and the hormonal (neuroendocrine) system. The nervous system provides transmission of information between different body regions to occur rapidly. Contrary to the nervous system, the hormonal communication system depends on the production, release and transport of hormones in the bloodstream, which is adequate for instances that need extensive and longer lasting regulatory actions. As such, these two communication systems work hand in hand with each other as stimuli from one system to the other can induce the release of certain hormones and vice versa (Gore and Dickerson, 2012).

1.4 WHAT ARE HORMONES?

Endocrine glands produce hormones. The various hormones that are produced are peptides, proteins, steroids and catecholamines. The various glands include those of the hypothalamus, pituary gland, adrenal glands, gonads (i.e. testes and ovaries), thyroid gland, parathyroid glands and pancreas (Gore and Dickerson, 2012).

The term “endocrine” means obtaining a response to a signal produced by the glands (i.e., hormones) which are released into the bloodstream. The hormones are then transported to their specified target cells. There are hormones which are hydrophilic and hydrophobic. The hydrophilic hormones cannot permeate across the target cell wall and therefore bind to the receptors which are located on the surface of the cell wall. The hydrophobic hormones are capable of diffusing across the target cell wall and bind to receptors inside the cell. The hormone-receptor can thus start a series of biochemical reactions in the cell which can modify the cells activity. Steroid hormones are hydrophobic signalling molecules (Chinoy, 2010).

1.5 TYPES AND SOURCES OF EDC’S

Agonists and antagonists are key agents in the chemistry of the human and animal bodies. An agonist produces an action where an antagonist is the opposite and can block a reaction. An agonist works in such a way that it binds to a receptor site and stimulates a response, which often imitates the natural body reaction and when the antagonist binds to the receptor, the receptor becomes inactive and cannot be recognised (Rich and Myszka, 2002). The concern about endocrine disruptors has focused on compounds that are estrogen receptor (ER) agonists. The many names of compounds like these have been referred to as “estrogenic”, “estrogen-like”, “environmental estrogens”, or “xenoestrogens”. These ER agonists and antagonists mimic or block the functions of endogenous estrogens (Li *et al*., 2006; Snyder *et al.*, 2001).

People in their everyday life come into contact with chemicals with estrogenic effects, as these chemicals are found in over a thousand products. EDCs commonly found in people include DDT, Polychlorinated Biphenyls (PCBs), Bisphenol A, Polybrominated Diphenyl Ethers (PBDEs) and a variety of Phthalates (Fourth National Report on Human Exposure to Environmental Chemicals, 2009).

There are many other chemicals suspected of being EDCs such as polychlorinated dibenzo-dioxins (PCDDs) and furans (PCDFs), polycyclic aromatic hydrocarbons (PAHs), their phenol derivatives and many pesticides, the contraceptive 17-alphaethinylestradiol, as well as

naturally occurring phytoestrogens (Falconer *et al*., 2006). There are studies that have reported these chemicals to have estrogenic activity (Falconer *et al*., 2006, Jiang *et al.*, 2012).

According to Phillips and Harrison (1999) the main sources of human exposure to endocrine disruptors (ED's) are from food, pharmaceuticals, occupational exposure, air and drinking water (from contamination of ground water). Jiang *et al.* (2012) found the main compounds responsible for estrogenic activity in source drinking waters in China, ranging from 39% to 97% to be E2, EE2 and 4-Nonylphenol.

Environmental estrogens which are made up of synthetic chemicals and natural plant compounds have been found to exhibit estrogens in both animals and humans. These have been found to cause adverse effects in both male and female reproductive systems (Sikka *et al*., 2004). The problem is that it is difficult to predict which natural synthetic chemicals will behave in this way in living organisms as they cannot be identified by the structure alone. The two most studied groups of phytoestrogens are the isoflavones, found in soybeans and other plants and ligands present in foodstuffs, including flaxseed and cereals (Tapiero *et al.*, 2002).

The potential sources of estrogen in sewage are the female steroid sex hormones, i.e., the 3 natural estrogens (estrone, estradiol and estriol) (Ying *et al*., 2002). These are produced by both female and male vertebrates. The amount produced and excreted varies between the two sexes throughout life.

Estradiol is metabolized both reversibly and irreversibly. In the reversible metabolism estradiol is oxidised to estrone, while in the irreversible metabolism, estradiol is converted to catechol estrogen or estriol. The sulphate and glucuronide conjugates are products of metabolism and excreted in the urine and therefore become more water soluble (Hamid and Eskicioglu, 2012). These are significant sources of natural estrogens into the sewage system. A small amount is excreted via faeces as unconjugated metabolites (Christiansen *et al.*, 2002; Ying *et al.*, 2002). The highest amount of estrogen is excreted during pregnancy. The hormone contraceptive contains ethinylestradiol which is excreted as an un-metabolised but conjugate form (Christiansen *et al.*, 2002).

The debate amongst the scientific research community regarding the claim that these chemicals have been disrupting the endocrine system is still ongoing. However, there are many that believe there is evidence that these chemicals possess some risk to human health. (Colborn, 2004; Sharpe and Irvine, 2004; Solomon and Schettler, 2000).

1.6 TOXICITY OF EDC'S

In assessing the potential toxicity of a chemical in the environment, the bioavailability and bioaccumulation are important parameters that need to be determined (Geyer *et al*., 2000). Bioavailable compounds are generally free or water extractable, while those that are attached to dissolved organic matter or solids are less available. The bioavailability of a chemical is dependent on the routes of exposure. For example, the actual uptake can be greatly influenced by living in or ingesting contaminated sediments (Lai *et al.*, 2002).

Bioconcentration is when an organism takes up a chemical directly from its surrounding phase and biomagnification is the indirect uptake of food. An important factor that determines the extent of bioaccumulation is bioconcentration (Geyer *et al*., 2000). The use of the bioaccumulation factor is important when conducting risk assessments as the increase in concentration in the tissue may cause adverse effects in organisms and this can also increase the exposure to predators by way of dietary consumption in the food chain (Lai *et al.*, 2002).

1.7 THE WASTEWATER TREATMENT PLANT (WWTP) AND ITS ENVIRONMENT

There are various types of WWTPs, the most basic being waste stabilization ponds, followed by the activated sludge process and the most advanced being the biological nutrient removal (BNR) activated sludge plant. These are the 3 types of plants chosen for this study, providing the different degrees of treatment for wastewater.

1.8 TYPES OF WASTEWATER TREATMENT PROCESSES

1.8.1 Waste Stabilization Ponds

These types of ponds are the simplest configuration of wastewater treatment. These types of ponds are large, shallow excavations, where the raw sewage is treated naturally by algae and bacteria. The usual arrangement of these ponds is in series where each successive pond receives the water from the previous pond, hence there is a continuous flow of water. The efficiency of treatment depends on the number of ponds in series as well as the retention times in each pond. Due to the ponds relying on sunlight for energy, the treatment is slow which results in long hydraulic retention times. This is a disadvantage of these ponds as they also need large land space (Sperling, 2007). The advantage is that even though the main purpose of the waste stabilization ponds is the removal of pathogenic microorganisms, they still have the capability of producing an effluent with a low biological oxygen demand (BOD) and nutrient concentration. They also have a high resistance to hydraulic and organic shock loads and can tolerate influent with heavy metal concentrations of up to 30 mg/L (Mara, 1996; Horan, 1990). Lastly they are not dependant on an electricity supply or mechanical maintenance giving good reliability.

The types of waste stabilization ponds are facultative, anaerobic followed by facultative, facultative aerated lagoons, complete mixed aerated lagoons with sedimentation ponds and maturation ponds (Sperling, 2007).

a) Facultative Ponds

These types of ponds are dark green in colour due to the large amounts of micro-algae that grow naturally. The major role of facultative ponds is for BOD removal. Both aerobic and anaerobic metabolism and environments occur in this pond. The algae provide the dissolved oxygen which is a product of photosynthesis and some of the oxygen is from the atmosphere through the ponds surface. The oxygen is then utilized by the heterotrophic bacteria which remove the BOD and in doing so release carbon dioxide which is in turn utilized by the algae, resulting in symbiosis between the algae and the bacteria. As the pond depth increases the

amount of dissolved oxygen decreases creating anaerobic metabolism towards the bottom of the pond (Mara, 1996; Horan, 1990).

b) Anaerobic Ponds followed by facultative ponds

This type of pond lacks dissolved oxygen as the name implies. The organic material is therefore degraded by fermentative pathways, which is firstly enzyme hydrolysis of the polymers (protein, fats) in to monomers (amino acids, fatty acids), followed by volatile fatty acid formation (alcohols, propionate, butyrate) and then methanogenesis, where methane and carbon dioxide is produced. These ponds are also capable of removing 40 to 50% BOD (Mara, 1996; Horan, 1990). The remainder of the BOD is removed in the facultative ponds (Sperling, 2007).

c) Facultative aerated lagoons

The mechanism is similar to facultative ponds, however mechanical stirrers is used to introduce oxygen (Sperling, 2007).

d) Complete mixed aerated lagoons with sedimentation ponds

These are lagoons with high energies and have a higher biomass concentration which removes a higher percentage of BOD. However this result in the effluent having a high load of suspended solids which is normally removed in the sedimentation ponds before being discharged (Sperling, 2007).

e) Maturation Ponds

The main purpose of these types of ponds is the removal of pathogenic organisms such as bacteria, viruses and helminths. By having long retention times, the removal of these organisms is achieved. The algae also play a role in removal in nitrogen and phosphorus (Mara, 1996; Horan, 1990).

1.8.2 The Activated Sludge Process

The activated-sludge process comprises of a biological treatment process which contains a diverse community of microorganisms in an aerobic environment. These organisms use carbonaceous organic matter found in wastewater to obtain energy in order to generate new cells while at the same time releasing energy by converting organic matter with lower energy. Some organisms gain their energy by converting ammonia nitrogen to nitrate nitrogen (Awad, 2011).

1.8.3 The Biological Nutrient Removal Activated Sludge Process

In the conventional activated sludge processes, plant nutrients such as nitrogen (N) and phosphorus (P) are only removed in limited quantities. The substantial amounts that remains are still present in the effluent and can cause eutrophication in the receiving water body. Thus a biological nutrient removal (BNR) process was developed to remove these unwanted nutrients.

A process called nitrification occurs in the anoxic zone of a BNR plant where nitrogen is oxidised by nitrifying bacteria into nitrates. The chemical reaction can be seen in equation 1.

$$NH_4^+ + 2O_2 \longrightarrow NO_3^- + 2H^+ + H_2O \qquad (1)$$

In the aerobic zones of the BNR plant the process of denitrification takes place where by the dissolved oxygen is the preferred source for the bacteria. The remaining nitrogen will escape as nitrogen gas to the atmosphere. The chemical reaction is shown in equation 2.

$$NO_3^- + H^+ \longrightarrow 1{,}25O_2 + 0{,}5N\uparrow + 0{,}5H_2O \qquad (2)$$

The anaerobic zone in the BNR process aids with phosphorus removal. In this zone where no oxygen or nitrates are present the normal aerobic bacteria cannot function, however a group of organisms called the poli-phosphate (poly-P) organisms proliferate as they can obtain energy by releasing phosphate complexes and when conditions become aerobic, they take up more phosphates than is needed for their normal metabolic requirements. Since they are slow growers they are not present in the conventional activated sludge reactors (Horan, 1990; Lilley *et al.*, 1997).

In addition to these, BNR plants removing nutrients, there is also a need to reduce the suspended solids and organic materials as well as pathogenic microorganisms. However no single unit can best achieve this, and thus a combination of unit processes are usually used. Thus there are various stages that the influent sewage go through to produce an effluent which has little or no ecological effect on the environment once discharged.

1.8.4 Complimentary Unit Processes:

a) Preliminary Treatment:

This includes screening of the raw sewage where floating materials like paper, rags, wood etc., are removed and grit removal where inorganic particles that have a diameter of >0.2 mm is removed (Horan, 1990; Mara, 1996).

b) Primary Sedimentation:

In this unit all particles with a size range between 0.5 - 10 mm are removed, these are known as settleable solids. It is not essential to have a primary sedimentation tank and some biological treatment plants are designed to operate without them. However a sedimentation tank can remove about 40% of the BOD load. The raw settled solids, known as raw sludge are then sent to a primary digester for sludge treatment and the effluent, or settled sewage is then sent to the next stage of treatment (Horan, 1990; Mara, 1996).

c) Secondary Treatment

The most common secondary treatment is the activated sludge unit. Many plants have only an aerated system; however in BNR treatment there are 3 different zones, the anaerobic, anoxic and aerobic. This is the basic setup for removal of nitrogen and phosphorus (Horan, 1990; Mara, 1996).

d) Secondary Sedimentation

The secondary sedimentation tank receives effluent from secondary treatment. This is where clarification takes place to separate the biomass from the supernatant which produces an effluent with very low or zero solids concentration (Horan, 1990; Mara, 1996).

e) Tertiary Treatment/Advanced Wastewater Treatment

There are many types of tertiary treatment processes, the most commonly applied one is the use of chlorine after secondary sedimentation. The various types of treatment can include one or a combination of chlorine, micro screening, filtration, precipitation and coagulation, reverse osmosis, ion exchange, ozonation etc. (Horan, 1990; Mara, 1996). Rosal *et al.*, (2010) found that for majority of the pollutants tested for, less than 20% was removed by the sewage treatment works (STW) and with the addition of ozone doses of <90 µM allowed the removal of majority of the pollutants.

1.9 FATE OF ESTROGENS IN WWTPs

According to many researchers, WWTPs are the major sources of releasing estrogens in to receiving water bodies, such as rivers etc. According to Baronti *et al.* (2000) the effluents emanating from WWTPs primarily with domestic inputs are greatly suspected to be a significant source of natural and synthetic estrogens. Women, men and female animals all naturally excrete the 17β-estradiol and estrone hormones (Gower, 1975 as cited by Ying *et al.*, 2002). Estradiol, for example, has a Low Observed Effect Concentration (LOEC) for some effects of 10 pM. The total daily excretion of natural estrogens by a million people is about 200 g. While this is minute compared with environmental releases of many synthetic chemicals; the high potency of estrogen makes it a significant release.

Research on effects of different plant sewage effluents on fish have shown that the estrogenic nature of sewage effluents, estrogens or estrogen chemicals are causing hormone disruption (Barber *et al*., 2012). Christiansen *et al*. (2002) and Filby *et al*. (2007) reported that the sign of the disruption was feminisation in male fish, where vitellogenin, (the female yolk protein) was produced as a response to an estrogen exposure. A few males also had early stages of egg

cells in the testis and some also developed the female duct and other adverse effects were noted (Christiansen *et al.*, 2002; Filby *et al*., 2007). Studies with early life roach, *Rutilis rutilis*, found that after 300 days of exposure to treated wastewater effluent lead to feminisation of male roach (Liney *et al.*, 2006).

For this reason, during the year 2002, the United Kingdom Environment Agency launched its strategy on endocrine disrupting substances in the environment. Development of environmental quality targets for estrogen steroids (such as estrone, estriol, 17β-estradiol and 17α-ethinylestradiol) was one of the actions in the strategy, as well as to highlight high risk areas which then allowed monitoring and development of Predicted-No-Effect-Concentrations (PNEC) to protect aquatic life.

Johnson *et al.* (2000) showed that males and females (i.e., women who were menstruating, menopausal, pregnant or those just on the pill) excreted estrogenic steroids. He estimated the daily values excreted which can be seen in Table 1.1. The levels of estrogen expected to be found in rivers are in ng/L, taking into consideration the dilution factor and previous measurements. With this in mind researchers have still yet to find suitable and rapid methods of detection for measurement of these minute concentrations. The most widely used methods, amongst others, thus far have been Gas Chromatography Mass Spectrophotometry (GCMS) and Liquid Chromatography. However these methods are laborious and time consuming. The more recent approach has been the use of RIAs and ELISAs. The RIAs have been used for the detection of estrogens in faecal wastes and in soil. This has not yet been applied to wastewater for detection of natural and synthetic estrogen.

Table 1.1: Daily Excretion (µg) of estrogenic steroids in humans (Johnson *et al.*, 2000)

Category	E2	E1	E3	EE2
Males	1.6	3.9	1.5	-
Menstruating females	3.5	8	4.8	-
Menopausal females	2.3	4	1	-
Pregnant women	259	600	6000	-
Women	-	-	-	35

E1: Estrone; E2: Estradiol; E3: Estriol; EE2: Ethinylestradiol

1.9.1 The Test Principle of Radioimmunoassay's

The principle is based on the competition between radioactive and non-radioactive antigens for a fixed number of antibody sites. The amount of the [I-125] radiolabelled analyte which is bound to the antibody is inversely proportional to the concentration of the unlabelled analyte. By using a double antibody system, the separation of free and bound antigen is attained (Source from RIDASCREEN: RIA kit insert).

1.9.2 The Test Principle of Enzyme Linked Immunoassays

The principle is also based on the antigen-antibody reaction. The capture antibodies which are directed against the anti-hormone antibodies are covered in the microtiter wells. When the sample or standard is added and the hormone enzyme conjugate and anti-hormone antibodies are added, they compete for the antibody binding sites, hence the term, competitive enzyme immunoassays (Source from RIDASCREEN: ELISA kit insert).

1.10 ACUTE TOXICITY MONITORING USING *Vibrio Fischeri*

Toxicity evaluation of environmental substances such as wastewater, contaminated water bodies has become an important part of environmental pollution monitoring and toxicity measurements based on microorganisms has grown steadily (Jennings *et al.*, 2001; Parvez *et al.*, 2006,).

The toxicity test is commercially known as Microtox and has been used to evaluate the toxicity in environmental samples. The toxicity test utilises the naturally luminescent bacterium *Vibrio fischeri* (Boluda *et al.*, 2002). The sensitivity of the bioluminescent test is similar to that of acute lethality tests using fish. Comparison of the bioluminescent test with other bacterial assays such as nitrification inhibition, ATP luminescence, respirometry and enzyme inhibition proved to be most sensitive (Parvez *et al*., 2006).

The test principle of using luminescent bacteria to determine toxicity of a substance is based on the light intensity that the bacterium exhibits. The toxicity is determined after measuring the decrease in light intensity after 5, 15 and 30 minute contact times and is calculated as the percentage inhibition (%INH) (ISO 11348-3, 2007).

By determining the acute toxicity of organic and inorganic substances in waters entering and leaving the WWTP, one can prevent plant and environmental aquatic damages from occurring (Gutierrez *et al.*, 2002).

1.11 AREAS OF UNCERTAINTY

In any research study, there are always those grey areas of uncertainty. When examining the biochemical pathway of estrogen, there are many pathways that the three forms of estrogen can take. For example, the estradiol molecule can be oxidized to estrone and the estrone can also revert back to estradiol. Certain compounds can be derived from the hydroxylation of estrone (Fig. 1.1). These compounds are the most prevalent metabolites of estradiol and estrone. They are also known as catechol estrogens, which can be further metabolized by the enzyme catechol aminotransferase.

The conjugation of estrogens occurs in the human body as they have to be transformed into more soluble forms so they can be excreted via the kidneys. When conjugation occurs the estrogens lose their activity. This process of conjugation is enzymatically mediated and it generates sulphate and glucuronic acid esters of the hydroxyl groups in the 3- and 7- position

of the basic structure. Both the E2 and EE2 have two hydroxyl groups, and can therefore possibly form eight different conjugates, while for E1 there are only 2. The conjugated form is only found in the human urine and can be transformed back into parent substances by microorganisms and enzymes in the sewage (Kjolholt *et al*., 2004).

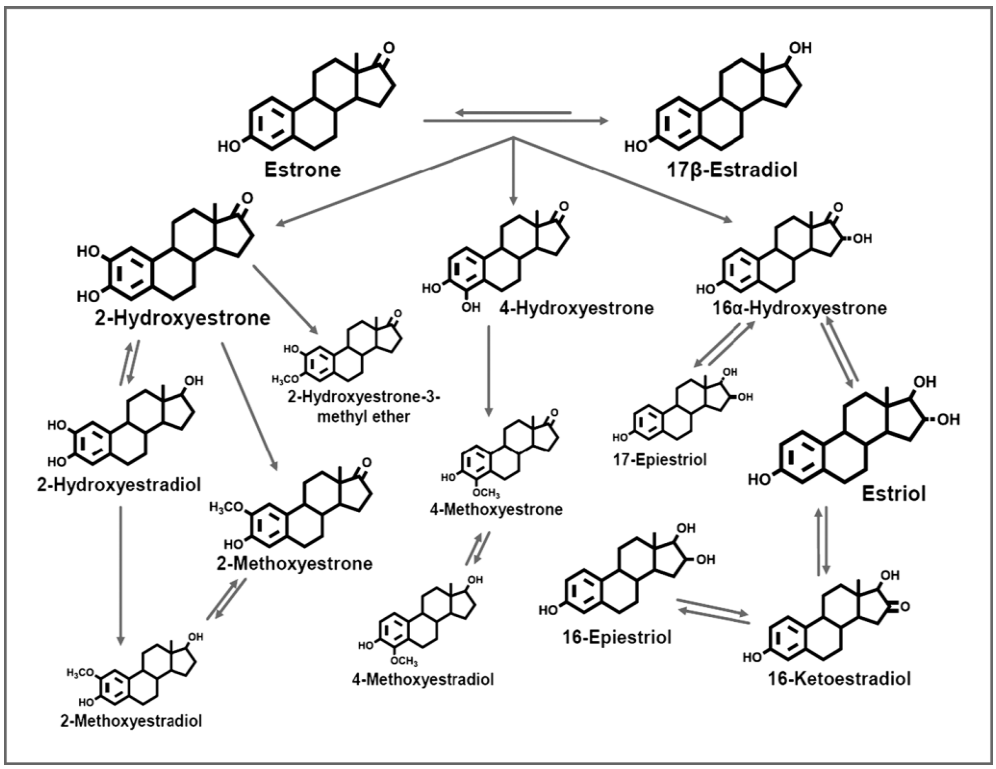


Figure 1.1: Diagram showing the metabolites of Estrogens (Fuhrman *et al*., 2012)

1.12 AIMS AND OBJECTIVES

The focus on this research study was to first assess the solid phase extraction technique and determine the precision, reproducibility of the ELISA and RIAs as rapid and quantitative techniques, as a suitable alternative to conventional methods. The next phase was to select wastewater treatment plants with different configurations.

Therefore the aims of this research study were to assess techniques for rapid detection of natural (estradiol, estrone, and estriol) and synthetic (ethinylestradiol) estrogens in the different wastewater treatment plants and to investigate the acute toxicity of the hormones as well as the final effluents of each plant.

The objectives of the study were:

1. To assess Radio-immunoassays (RIAs) and Enzyme Linked Immunoassays (ELISA) techniques for detection of steroid hormones in wastewater.
2. To conduct laboratory scale investigations to determine the fate of these compounds under controlled conditions
3. To select wastewater treatment plants (WWTP) that represents the various configurations of treatment processes and to determine their removal capabilities of steroid hormones.
4. To assess the acute toxicity of the estrogen standards as well as the toxicity of the final effluents using the bioluminescent marine bacterium *Vibrio fischeri*.

1.13 NOVELTY OF THE RESEARCH

The novelty of this research resides in adapting RIAs to determine estrogenic compounds in wastewater; however there has been some progress in the use of ELISAs to detect hormone EDCs in addition the assessment of determining the acute toxicity using *Vibrio fischeri* of estrogen standards is novel. No evidence has been found in determining the toxicity of estrogen standards and there is very little research in determining the toxicity of wastewater effluents.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

During the autumn of 1995, the Danish Environmental Protection Agency (EPA) published the report "Environmental Project no. 290: Male Reproductive Health and Environmental Chemicals with Estrogenic Effects". Soon after the report the Danish government started phasing out the use of pesticides. Based on information available to the Danish EPA on the composition of individual pesticides, and on information from importers and manufacturers, the Danish EPA had prepared a list (appendix 1) which contained the trade names of just under 70 pesticides containing auxiliary matters which - in view of the present state of knowledge - the Danish EPA considers to have estrogenic effects or to be capable of decomposing into compounds with estrogenic effects.

During this time the "endocrine disruptor hypothesis" was formulated as the concern over certain chemicals biological impact on wildlife arose (Colborn and Clement, 1992). Theo Colborn observed changes in the wildlife in and around the Great Lakes of the United States and theorised that there were chemical compounds which were mimicking the sex hormone estrogen and accumulated in animals. The accumulation caused problems such as behavioural, morphological and reproductive in a number of resident species, especially those that were on top of the food chain (Colborn *et al*., 1996). Since then, studies have shown that chemicals discarded into the environment have caused adverse effects in the development and functionality of the endocrine system of both wildlife and humans (Filby *et al.*, 2007; Lutz and Kloas, 1999).

These "endocrine disrupting chemicals" (EDCs) or the newly emerging term "endocrine active chemicals", are capable of altering the control of gene expressions and cause interferences with homeostatic feedback loops at both the developmental and functional level (Ferry, 2011). It was further hypothesized that if any process is facilitated by chemical signals then it can become disrupted indicating the advancement of biological signalling mechanisms in ecosystems (McLachlan, 2001). Interference in the signalling process especially during

development can cause effects which can range from abnormalities at birth to subtle changes which only become apparent long after exposure (WHO, 2002).

When high doses of EDCs occur, it can result in acute toxicity like immediate death of the cell however at very low levels; the expressions of genes can be altered during development which can result in permanent endocrine system changes (Welshons, *et. al.*, 2003). It thus becomes difficult to statistically detect changes when there are different EDCs interfering with different signalling pathways and mechanisms especially when combined with other chemical compounds in mixtures (Weiss, 2002).

Initially the main area of focus was on estrogenic chemicals as estrogenic effects were first to be seen, however other hormones, like the androgens, anti-estrogens etc. also exist, amongst others (National Academy of Sciences, 2003a).

Additional chemical products intended for consumers, include pharmaceuticals, which were designed to be pharmacologically active, and personal care products, many of which contain EDCs, are found in the world's waterways. It was also stated that the amount of pharmaceuticals and personal care products which enter the environment annually is comparable to the amounts of pesticides used annually and that many of these EDCs have been found in humans at much higher levels where endocrine disrupting effects are observed in animals (Daughton, 2001).

Endocrine disruptors have now been found to be the new source of evolutionary variation similar to environmental stochasticity as they enter the biological signals from outside the organism can enter the cell and change the gene expression. Estrogens are believed to be the first signalling molecule as this receptor is found in vertebrates and invertebrates (McLachlan, 2001). Also predicting the outcomes of the gene alteration can become difficult since many different organisms may also use the same signalling molecule for different functions (Daughton and Ternes, 1999).

The volatile and persistent nature of some chemicals, such as DDT and PCBs, has enabled them to travel long distances through atmospheric distribution (Byrne, 2009). However even through the banning of such chemicals, the World Health Organisation (WHO) has reported that the levels of DDT and PCBs have decreased but the by-products such as PBDEs have increased (WHO, 2002).

Similar results were noted by the World Wildlife Foundation (WWF) during their United Kingdom (UK)-wide blood survey. The survey showed if strict control over the use of these chemicals is applied, the contamination levels to humans can be reduced. For example, two types of pesticides which have been banned in the UK did not show up in the survey and that PCB levels were gradually decreasing from levels found in the UK more than 10 years ago (WWF, 2003). One thus needs to re-evaluate the significance these chemical compounds have on the environment with regards to the changes it can have on the biodiversity and extinction of species (Johnson, 2004).

2.2 THE REALITY OF THE SITUATION REGARDING HUMAN EXPOSURE TO CHEMICALS

As regards the ubiquitous nature of these compounds Dr. J. P. Myers, one of the authors of "Our Stolen Future" had this to say in a speech at the Rio + 5 Forum Five years after the Earth Summit, UN Conference on Environment and Development, 14 March 1997, makes these points from the book:

Let me challenge you with two simple facts.

1. *First, every one of you sitting here today is carrying at least 500 measurable chemicals in your body that were not part of human chemistry before the 1920's. We are walking experiments, differing from all previous generations of human ancestry in this regard.*
2. *And second, there is now incontrovertible scientific proof that a mother shares some of these man-made chemicals with her baby while it is in her womb. No baby has been born on the planet for at least two decades without some exposure to novel chemicals in the womb. Some with little exposure. Some with a lot. But none with none.*

He goes on to say:

> *In all likelihood, some, perhaps many of these compounds will turn out to be benign, with no impact. But some we know already cause problems...*

It is this sentence, hidden away in Dr. Myers speech that defines the technical problem. Chemical compounds, new to the world or new to a particular environment, are not part of our environment, they are our environment. We cannot return to a pre-industrial condition and few would advocate that we do that. Our task is thus to find those compounds, among Dr. Myers' 500, that are not benign (as quoted in Jost, 1998).

Such compounds are suspected to be a part of the cause to different types of cancers such as the breast cancer, testicular cancer, hyperspadism, as well as a progressive fall in sperm count. Many synthetic and natural chemical compounds can behave as estrogens, anti-estrogens, anti-androgens, and thyro-active agents and cause disruption, but most instances have been the 'feminization' occurrence which is through estrogenic effects (Colborn *et al*., 1993). The possible negative and long-lasting effects of these chemicals have now grown to include other neurodevelopment issues. For example, Colborn, (2004) explored the possibility that some chemicals can also add to the problems associated with neurodevelopment and behavioural problems.

Until recently, the knowledge base on environmental-estrogens, had in many cases, fuelled speculation and arguments, and yielded few definitive answers. For example, in 1997 the USEPA prepared a document that provided an overview of the scientific research of environmental endocrine disruption. This document served as an "*interim assessment and analysis of the environmental endocrine disruption hypothesis"* until a more detailed investigation of environmental endocrine disruption could be done (USEPA, 1997). Further, as stated by Miyamoto and Klein, (1998) there are many specific cases of well known or suspected endocrine mediated effects in the environment, however these effects cannot be considered of general importance due to the wide difference in ecology and species. These differences may take place due to differences in the mechanisms of sex differentiation, in structure of receptor and function and in metabolism. In some cases the natural hormones may

be responsible for the apparent adverse effects. Additional studies are needed to determine the action of natural and synthetic chemicals ability to cause endocrine disruption in the environment and the need to develop screening tools for early detection (Miyamoto and Klein, 1998).

The situation in Africa could perceivably be multiplied as, unlike; many other countries which are actively phasing out many of these compounds, huge stockpiles of obsolete suspected EDCs are found in Africa. For example, it is well known that almost every country in Africa has stockpiles of these compounds and that the continent has a total of more than 50,000 metric tons of these wastes. It is, also, well known that these stocks pose a significant threat to human health and the environment through direct exposure, contamination of soil and groundwater, and the reuse of contaminated products. Therefore, the Africa Stockpiles Program (ASP), which is partially funded by the World Bank, will confront this threat through clean-up and disposal of the stockpiles in three or four phases over a period of 10 to 15 years. The ASP will also work to build capacity and put in place measures to help prevent the re-accumulation of such stocks. The program is being undertaken by a partnership that includes African regional organizations, non-governmental organizations (NGOs), the private sector, and international organisations, in particular, the Food and Agriculture Organization of the United Nations (The World Bank, 2003).

2.3 TYPES AND FUNCTIONS OF EDCs

While numerous compounds, mainly xenobiotics, can be considered potential endocrine disruptors, attention has been focussed, for the most part, on persistent environmental pollutants with weak estrogenic or antiandrogenic properties (See table 2.1 for classification). A comprehensive description of all environmentally relevant hormonally active chemicals, estrogens, androgens or gestagens, would be exceedingly difficult to fulfil, due to the high number and wide variations in structure of these compounds. Therefore the following only concentrates on some of the major EDCs analysed in nature.

For this chapter, the term "endocrine disruptor (ED)" will be used). Of significance here is the idea that endocrine disruptors include not only the environmental estrogens and but also

includes any chemical/agent that can cause disruption to the endocrine system. Endocrine disrupters, under some circumstances are hypertrophic (stimulatory) agents and tumour promoters. When assessing the adverse effects of an ED it is important to consider the dose, body burden, timing and duration of exposure at critical periods of life. The effects may not be expressed immediately, or it could be reversible or irreversible (USEPA, 1997).

Table 2.1: Classification of the Sources of EDCs (Christiansen *et al.*, 2002)

CLASSIFICATION	EDC
Herbicides	Dichlorophenoxyacetic acid, atrazine, methoxychlor, methylphenol
Pesticides	Chlordecane, DDT Endosulfan, Heptachlor, Kepone Malathion, Mirex
Organochlorinated Pesticides	Aldrin, Benzophenone, Dieldrin, Lindane
Polychlorinated Biphenyl (PCBs)	Arochlor 1254, 1260
Plasticizers	Butyl benzyl phthalate (BBP), Bisphenol A, Pthalates: n-butyl phthalate, Diphenyl
Industrial Materials	Nitro toluene, ‘n-Butylbenzene, p-Cresol, Toluene
Phenols	Alkylphenolic compounds: 4-octylphenol, 4nonylphenol, 4nonylphenol-di-ethoxylates, Dichlorophenol, Nonylphenol, octylphenol
Pharmaceutical products	O’p’DDD, Adiol, caffeine: Synthetic estrogens: DES, EE2, Mestranol, Estradiol benzoate
Endogenous estrogens	Enterodiol, Estrone, Estradiol
Plant substances containing estrogen – like compounds	Flavonols, Isoflavones, Phytoestrogens, coumestrol, genistein, salsolidine, salsoline, salsolinol
Naturally occurring substances that contain estrogen- like compounds	Boron, Broccoli, Cholesterol, Immunoglobin G (IgG), Milk from pregnant cows, Mycotoxins, Phytohormones, Xenobiotics.

The abovementioned compounds have marked differences such as the molecular size, volume, structure, thereby highlighting the diversity of the ligands that can bind to the estrogen receptor (ER). This diversity has compelled different agencies worldwide to prioritise, standardise and classify the various compounds and their effects in an ordered and logical fashion. Therefore, Groshart and Okkerman, 2000, (BKH Consulting Engineers, Delft, the Netherlands), were commissioned by the European Commission to carry out a study on endocrine disruption which focused on man-made chemicals following the establishing of a priority list of substances for further investigation into their roles in ED. This list comprised of 564 substances which had some evidence of ED and then further reduced to 146 substances after further investigation. These 146 substances were further categorised by a panel of experts in the field and 3 categories were formed. Following the expert meeting 66 substances were placed in Category 1, 51 in to Category 2 and 29 in Category 3. Further grouping of Category 1 was substances having high, medium and low exposures. The list can be changed at any time and chemicals can be added or removed (Groshart and Okkerman, 2000).

The main compounds of concern and research, have in all likelihood, been the environmental estrogens and related hormonal compounds. The reason is that these hormonal compounds are effective at very low concentrations. For this reason during the year 2002 the United Kingdom Environmental Agency started its strategy on EDCs in the environment. One of the actions aimed for in this strategy was to develop environmental quality targets for the estrogen hormones in order to highlight high risk areas and to then conduct monitoring in to order to determine the Predicted-No-Effect-Concentrations (PNEC) to protect the aquatic ecosystem.

As part of this strategic development, monitoring studies showed that concentrations of steroid estrogens can vary considerably in sewage effluents, with representative values given in Table 2.2 (United Kingdom Environmental Agency, 2002). While table 2.3, indicates the Maximum Concentration Limits (MCL) allowed in drinking water.

Table 2.2: Minimum, Maximum and Typical Concentrations of Estrogen Compounds Monitored in the UK (UK Environment Agency, 2002).

Compound	Minimum - Maximum Concentrations (ng/L)	Typical Concentrations (ng/L)	PNECs (ng/L)
Estrone	0.35 – 220	5 – 20	-*
17β-Estradiol	2.7 – 88	1 – 10	1.0
17α-Ethinylestradiol	0.13 – 62	0.1 – 10	0.1

The toxicity studies available for estrone and estriol are currently insufficient to propose PNECs for the protection of freshwater or saltwater life, although a suggested possible target value range is for estrone.

Table 2.3: A Few Representative Maximum Concentration Limits (MCL) Allowed in Drinking Water According to the EU, WHO, and USEPA (UK Environment Agency, 2002)

COMPOUND	EU (µg/L)	WHO (µg/L)	USEPA (µg/L)
DEHP	10	8	6
PCBs	1.0	-	0.5
PAHs	0.2	0.7	0.2
PCDD/PCDF	-	-	0.00003

There are many synthetic hormones found in contraceptive agents such as ethinylestradiol, diethylstilbestrol and mestranol. It is very difficult to get the quantities of these synthetic hormones even though they have potency similar to the natural hormones. Synthetic hormones have been detected in samples from various environments, although, here again, no definitive trends can be compiled. For example, in a previous UK study, neither ethinylestradiol nor mestranol was frequently found in treated sewage effluent. However, in a similar study conducted in Germany, all samples analysed had ethinylestradiol (median concentration 17

ng/L) and some rivers had concentrations of 1–4 ng/L. These concentrations are significantly higher than those recorded in studies of rivers in the Netherlands which were found to have 0.3 ng/L of ethinylestradiol. Further, in the German study, final effluents from 12 WWTPs had mestranol at a median concentration of 4 ng/L. These results serve to indicate that there is relevant exposure in the aquatic environment (Miyamoto and Klein, 1998).

Apart from the synthetic and/or xenobiotic compounds already mentioned, various naturally occurring compounds have also shown different degrees of endocrine disruption such as the phyto-estrogens. The estrogenic isoflavone content of most soy proteins is mainly represented by daidzein and genistein. The latter are transformed by the intestinal bacterial flora into equol, which has a more potent nature than either genistein or daidzein, but has a limited capacity for inducing cornification of the vaginal epithelium in post-menopausal women (19% as opposed to 8% among controls) (Preziosi, 1998).

2.4 PERSISTENT ORGANIC POLLUTANTS (POPs)

Persistent Organic Pollutants (POPs) are synthetic and toxic chemicals which have negative effects on humans, wildlife and the environment. These substances include a variety of organic compounds which includes pesticides, dioxins, furans, many being by products generated as a result of human and natural activity. Many of these POPs exhibit endocrine disrupting effects. POPs have found to "survive" for long periods as they take many years to degrade and can be transported by wind and water thus accumulating in the food chain with highest levels being in the marine mammals (Hagen and Walls, 2005;Scheringer *et al*., 2012; WHO, 2002).

POPs and other EDCs have important common characteristics (The World Bank, 2001) identified as follows:

- Acute toxic effects have been found causing birth defects, damages to reproductive systems, as well as some carcinogenic effects. It was found that most women and infants appear to be vulnerable to certain POPs.
- They are found to be extremely persistent in the environment as they resist the natural biodegradation processes.

- Many POPs and EDCs are soluble in fatty tissue, which causes exponential bioaccumulation. Bioconcentration also occurs whereby the animal absorbs high amount of POPs directly from the environment, as opposed to eating other animals. For example, some POPs present in water have bioaccumulated in the fatty tissue of fish by factors going up to 70,000 times as the same POP present in the water column (National Academy Sciences, 2003b).
- Many of these compounds are semi-volatile and therefore can travel great distances via cycles of evaporation and atmospheric cycling and deposition also known as the "grasshopper effect". Other natural carriers are wind and water. POPs can reach their highest concentrations in cooler regions in the arctic regions and around the world as they condense at cooler temperatures and are volatile at warm temperatures. POPs have thus been found on all continents, and in all climatic zones, which includes the open ocean and deserts, and in all wildlife species and human beings. (Brun, *et. al*, 2004; Byrne, 2009; The World Bank, 2001).

There is no question that many chemicals, especially at elevated concentrations, cause human disease. Most people are exposed to a mixture of chemical compounds at both high and low concentrations, but majority at lower concentrations. It has been the norm to study the effects that chemicals have on humans, wildlife and the environment either singly or at higher concentrations as it is easier to study a single compound to determine the dose-response information. Studies by Payan-Rentira *et al*., (2012) found that farm workers exposed to pesticide had acute poisoning as well asvarious numerous alterations of their digestive, neurological, respiratory, circulatory, dermatological, renal, and reproductive systems as opposed to the control group of workers who were not exposed.

The present use of fossil fuels has increased the concentrations and exposed humans to a greater range of hydrocarbons and their by-products. However, by far the greatest challenge to the environment must, surely, be as a result of the increasing chemical, plastics and pharmaceutical industries. Out of the 80 000 chemicals being used today, about 10% have shown to be carcinogenic and many of them have not been properly tested for human toxicity (Carpenter, 2002)

It could be pointed out that, although, all living organisms are being challenged, daily, by mixtures of compounds most, if not all, would be at very low concentrations which would imply a low hazard rating. However, it has been shown that low doses over a period of time could have significant effects (National Academy Sciences, 2003a).

This ubiquitous nature of many organochlorine compounds was very elegantly demonstrated by a study that looked at their concentrations in tree bark. It was found that from over 90 worldwide sites of analysing tree barks, it still had concentrations ranging from 1000 to 10 000 ng/g of DDE (National Academy Sciences, 2003a).

The combination of all of the abovementioned complicating factors in the ubiquitous nature of these compounds and their drastic effects has spurned many international bodies to take action. For example, as of March 18, 2002, there have been 122 countries which signed the at the Stockholm convention, South Africa included to reduce the use of POPs (The World Bank, 2001).

Since then South Africa has put in place a National Implementation Plan (NIP) for Stockholm convention which is implemented by the South African Governments Department of Environmental Affairs (DEA) to phase out the POPs listed. The NIP has the following expected outcomes:

- *"to protect South Africans' health from the effect of POPs;*
- *to promote a cleaner South African environment;*
- *to improve South Africa's capacity to manage POPs;*
- *to reduce South Africa's contribution to global pollutant loading; and*
- *to contribute to meeting South Africa's commitments under the Stockholm Convention"* (National Implementation Plan, 2011).

From all of the above it is clear that there are a variety of different types of EDCs which all have different modes of action, sources, effects and physico-chemical characteristics. Further, the amount and types of EDCs in any given environment will be a product of numerous

interacting variables which include the relative biodegradability, and thus, the half-life, of the compound, the hydrophilic versus hydrophobic nature, the partition coefficients between air, water and soil/solids and have been extensively described by other authors (Alexander, 1999; Atlas and Bartha, 1993). However, there are, in general, certain classes of EDCs which will tend to concentrate in a specific media. For example, highly volatile compounds will tend to volatilise into the atmosphere while strongly hydrophilic compounds will tend to be dissolved in the aqueous phase. A major determining factor is the partitioning between aqueous and solid phase, including biota. Total organic carbon and hydrophobicity of the EDCs are crucial in the prediction of partitioning of for example, estrogens to sediment and both salinity and the presence of other hydrophobic compounds are likely to influence this process. Additionally, microbial activity will determine the rate of biotransformation of the compound, while the physico-chemical characteristics will influence the compound's ability for bioconcentration, bioavailability, and bioaccumulation. The interaction between these variables and their relative effects on the degradability of any compound is exceedingly complex and has been described by other researchers (Alexander, 1999; Atlas and Bartha, 1993).

2.5 TRANSFER TO AND EXPOSURE FROM WATER, WASTEWATER AND SLUDGE

Worldwide numerous governmental bodies are trying to implement water policies which aim to promote sustainable water use and they all have as one of their major objectives the long-term reduction of contaminant discharges to aquatic environments. Although important, it must be pointed out that the input of EDCs into the aquatic environment is only one of a series of challenges that face the sustainable conservation of water. For example, other challenges include salination of water bodies, increases in algal growth and toxins, ingress of agricultural nutrients, pathogenic organisms, as well as heavy metals from mining. The two main routes of entry of EDCs into an aquatic system are by point- and non-point sources. Point sources consist mainly of industrial discharges and wastewater treatment plant effluents. There are also many potentially toxic contaminants that can transfer to sewer sludge (which is a product of wastewater treatment) (European Commission, 2001).

2.6 BIOTIC FATE

The relative disappearance of any compound, and, especially, steroidal hormones, is due to many interacting and complex processes, of which, biodegradation is just one. For example, on entering the aqueous environment the kinds of interactions that can occur for 17β-estradiol, in receiving waters, is dilution and sorption and, within limits, biodegradation. Estradiol has been found in wastewater effluents in both the UK and Germany at levels of 2.7–4.8 ng/L and >1 ng/L respectively. This contributes to a significant level of environmental exposure (Miyamoto and Klein, 1998). It has also been suggested that 17α-ethinylestradiol is more persistent than the natural estrogens in surface waters, and has a higher potential to sorb to sediment(United Kingdom Environmental Agency, 2002).

There have been reports that the concentrations in the domestic effluent have shown to stimulate vitellogenin (VTG) in some fish (Liney, 2006). Studies on intersex in roach in British rivers are not clear whether this is attributed to steroid estrogens or by other chemicals in the effluent. However, there is strong evidence that steroid estrogens in wastewater treatment plant effluents are responsible for inducing VTG in fish: a process known to be estrogen reliant. A factor influencing the impact of EDCs in effluents is the dilution factor as in Europe and the UK effluents can be a large portion of flow in summer months (Johnson *et al.*, 2000).

This has necessitated that governments all over the world have had to re-evaluate their discharge limits and to re-assess the risk posed by these compounds. The United Kingdom has, following critical evaluation of ecotoxicological studies, found it possible to propose a Predicted-No-Effect-Concentrations (PNECs) value for 17a-ethynylestradiol of 0.1 ng/L and a 'tentative' PNEC value for 17β-estradiol of 1 ng/L. The toxicity research available for estrone and estriol are currently insufficient to impose PNECs for the protection of freshwater or saltwater life (United Kingdom Environmental Agency, 2002).

Despite these risk assessments and determinations of concentrations which should not result in negative impacts on the river ecosystem, and especially, fish populations, it has been shown that very low concentrations of compounds, not necessarily dissolved in the aqueous phase, can have significant effects. The study by Peck *et al.*, (2003) found high levels of estrogenic

activity in the sediment both upstream and downstream of WWTP, which ranged between 21.3 and 29.9 ng Estradiol equivalents (EEQ)/kg as opposed to levels <3 ng/L in the river and surface waters. Analysis of the steroid estrogens of the sediments found high estrone activity with lower amounts of 17β-estradiol which imply that sediments are a major source of persistent of estrogenic contaminants (Peck, *et al.*, 2003).

2.7 TOXICITY MONITORING

Toxicity monitoring is rapidly becoming an integral part of environmental monitoring programmes of water boards, local authorities for most waters. This is mainly due to the high costs and laborious procedures of chemical analysis to determine extent of polluted waters. Most toxicity testing makes use of bioluminescent bacteria. Luminescent bacteria are an abundant group of light-emitting bacteria found in fresh water, marine waters and terrestrial environments. These bacteria are gram negative mobile rods, which also have the capacity of functioning as facultative anaerobes (Girotti *et al*., 2002).

A marine bacterium that has become a widespread assay in toxicity monitoring of wastewaters, industrial effluents as well as contaminated waters is *Vibrio fischeri* as it is said to be the most sensitive out of all the bioluminescent bacteria (Logar and Vodovnik, 2007). *Vibrio fischeri* has been proven to be rapid, simple and cost effective and can quickly measure acute toxicity of individual or complex mixtures (Kaiser, 1998).

The application of using toxicity monitoring has extended to screening of wastewaters entering WWTPs, as well as to predict the toxicity of a range of chemicals as well as assessment of hazardous waste sites (Gutierrez *et al*., 2002;Parvez *et al*., 2006;USEPA, 1991).

2.8 ENVIRONMENTAL LIMITATIONS

In the past the degradation of organic and inorganic pollutants has been mainly investigated at higher relative concentration (ppm and/or ppb) ranges. The main reason for this is that microorganisms grow far better at higher nutrient concentrations and it was much easier to monitor microbial growth and/or substrate disappearance at these higher concentrations.

However, as interest grew in the possible degradation of, mainly recalcitrant organics, low concentration contaminants other unforeseen issues become apparent. One such issue was the existence of a concentration of a nutrient source which is too low for microorganisms to inhabit and grow (Aboul-Kassim and Simoneit, 2001).

An additional complication with certain non-aqueous phase liquids (NAPLs) which are characterised by very low partitioning coefficients is that the final concentration in the water phase - in which the microbes function - may be very low and even lower than the minimum level for biodegradation and, thus, below the level of transformation.

To uphold its viability, every organism must utilise energy. In animals and humans, the energy used is reflected in basal metabolism while in microorganisms, the amount of energy to permit the organism to remain alive is designated as the maintenance energy. Heterotrophic organisms derive their energy from the oxidation of organic substances. Due to the presence of fairly constant levels of dissolved organic carbons (DOC) in the sea, the threshold was formulated. The low carbon concentrations were not enough to support microbial proliferation and mineralisation of carbon. The level of DOC is approximately 1 mg/L (ppm) in marine waters and <5 mg/L in oligotrophic fresh waters (Alexander, 1999). Although this theory does not take into account many other factors the phenomenon of threshold values has been recorded often in nature. For example, in a plume of contaminated water derived from secondary sewage effluent subjected to rapid infiltration, a number of compounds were found to have persisted at low concentrations in the aquifer for more than 30 years; the average concentrations in the groundwater were 20 - 70 ng/L of 2, 3-dimethyl - 2 - butanol, 2-methyl-2-hexanol, ethylbenzene and propylbenzene isomers, all compounds which are metabolized at higher concentrations (Alexander, 1999).

2.9 CONCLUSION

Human nature is such that we believe that when a mess is made it can be washed away with no repercussions. Managers in the field of water and wastewater understand what society needs to realise. We need to understand that every pollutant that leaves our home, cities must end up somewhere and most of the time it ends up in rivers, bays and oceans. With the increase of pollution especially emanating from metropolitan centers, highly concentrated

waste streams are being produced, the results of which lead to direct effects on the health and prosperity of our civilisation.

There are many ways to detect these EDCs all of which are very laborious and most of the cases these EDCs are either in the pico or nano gram per litre range, too minute for many methods to detect effectively; therefore the research project aimed to also use rapid and sensitive techniques to determine the quickest means to detect the very low concentrations of theses EDCs. Two techniques were researched, i.e., Enzyme Linked immunoassays and Radio-immunoassays. The following chapters assessed the use of these techniques, followed by laboratory scale investigations and then the fate and toxicity of these EDCs indifferent wastewater treatment processes.

CHAPTER 3
ASSESSMENT OF THE IMMUNOASSAY PROCEDURES

3.1 INTRODUCTION

Techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry/mass spectrometry (LCMS/MS) have generally been employed for the quantitative analysis of EDCs. Even though these can be reliable methods there are certain potential drawbacks, including the large volumes of sample required, the extensive purification process, expensive instrumentation, required expertise in operation and the long period for the analysis. These shortcomings can be cost and time prohibitive should a large number of samples need to be analysed. Due to the increasing awareness of the drastic effects especially hormone disruptors have ((Fourth National Report on Human Exposure to Environmental Chemicals, 2009; Larsson *et al.*, 1999; Lai *et al.*, 2002; Kashiwada *et al.*, 2002; Phillips and Harrison, 1999; Sharpe and Irvine, 2004; Tabata *et al.*, 2001; Willingham and Crews, 1999), there is a great need, one could also perhaps add urgent need for fast, simple, reliable and cost effective methods for quantifying hormone EDCs. Two such methods investigated were the ELISAs and RIAs. There is little research done using RIAs for detecting hormone EDCs (Lopez de Alda and Barcelo, 2001; Snyder, 1999). ELISAs have many applications as measuring PCBs, insecticides, herbicides, polynuclear aromatic hydrocarbons and heavy metals (Goda *et al.*, 2000).

Upon initiation of this project the types of hormones to be tested were depended on the availability of the test kits as well as the standards. Upon researching the available kits for the ELISA, it was found that at the time only 1 of the 3 natural estrogens and one of the synthetic hormone kits were available to purchase i.e., a 17ß-Estradiol (E2) and 17 ß –Ethinylestradiol (EE2).

For the RIA kits all three of the natural hormones were available, except for the synthetic estrogen, ethinylestradiol. The kits that were available were the Estradiol (E2) DSL-4400, the Estrone (E1) DSL-8700 and the Estriol (E3) DSL-3700. All kits were purchased from Biopharm, Amersham.

This chapter focused on assessing using immunoassay procedures using standards and controls and applied them to the kits mentioned above to determine the recovery of the standards.

The aim was to assess, adapt and if need be optimise the ELISA and RIA technique for detection of natural and synthetic estrogens by using standards and controls.

3.2 MATERIALS AND METHOD

A Solid Phase Extraction (SPE) using Carbon 18 Cartridges was used. C18 cartridges (Art No.R2002) were purchased from Microsep and the following procedure applied to standards, controls and unknown samples. All glasswares was washed and prepared according to appendix 2.

3.2.1 Conditioning of the C18 Cartridge

The procedure for conditioning one of the C18 cartridges took approximately 5 – 10 minutes after preparation of the solutions. The C18 cartridge was rinsed with 3 mL of 100% methanol and then equilibrated with 2 mL 20 mM Tris Buffer pH 8.5 / 20% methanol (appendix 3). A volume of 10 mL of standards/controls and 100 mL of unknown samples were applied to the cartridge. The cartridge was rinsed with 2 mL 20 mM Tris Buffer pH 8.5 in 20% methanol and then rinsed with 3 mL 40% methanol (appendix 4). The fluid residues removed by positive pressure or vacuum and the cartridge dried for 2 min by floating it with nitrogen gas.

3.2.2 Elution from Cartridges

Standards were eluted with 80% methanol (appendix 5) using a flow rate of 15 drops per minute and collected in a new vial. The eluent was collected by applying positive pressure. The eluate was then dried using nitrogen gas and reconstituted to 1mL with 80% methanol and stored in a freezer until application to the ELISA or RIA kits.

The equipment used for the ELISAs was a DAS Microplate reader with tungsten lamps and 2 filters (450 nm and 630 nm). All standards and samples were read at 450nm. The RIDASCREEN 17ß-Estradiol, (Art. No.: R2301) was a competitive enzyme immunoassay for the quantitative analysis of 17ß-Estradiol in bovine plasma. The RIDASCREEN Ethinylestradiol, (Art. No.: R2501) was a competitive enzyme immunoassay for the quantitative analysis of Ethinylestradiol in urine and bovine plasma.

All reagents needed for the enzyme immunoassay – which also included standards were contained in the test kit. Each micro-titre plate was sufficient for 96 determinations. Each of the 96 wells was coated with antibodies directed against anti-17β-Estradiol or anti-17β-Ethinylestradiol. The following reagents were also provided with each kit:

- *17B-Estradiol / Ethinylestradiol enzyme conjugate*

The 17 β -Estradiol and the Ethinylestradiol enzyme conjugate were provided as a concentrate. Only the amount needed was reconstituted due to the diluted enzyme conjugate having a limited stability. The conjugate concentrate was diluted 1:11 in buffer (e.g. 200 µL conjugate concentrate + 2 mL buffer, ready to use was sufficient for 4 microtiter strips.)

- *Anti-17β-Estradiol / Anti-ethinylestradiol antibody*

The anti-17 β -Estradiol / anti- Ethinylestradiol antibody was prepared as previously reported for the 17B-Estradiol / Ethinylestradiol enzyme conjugate.

3.2.3 Procedure for Estradiol (E2) and Ethinylestradiol (EE2) ELISA

The standards were put through the SPE as outlined in 3.2.1 and 3.2.2 above and then applied to the relevant ELISA kits. The basis of the method is the antigen-antibody reaction. In brief; microtiter wells covered with capture antibodies which were directed against anti-estradiol or anti-ethinyestradiol antibodies were inserted in the microwell holder. All standards were prepared in duplicate. Refer to appendix 6 for details on the full procedure. The standard curve was drawn and then compared to the typical standard curve data supplied with the test

kit. The absorption was inversely proportional to the estradiol and ethinylestradiol concentration in the sample/standard.

The time required for sample and reagent preparation was approximately 1 hour and the test implementation was approximately 2.5 hours. Each standard was applied in duplicate and the average absorbance readings at 450 nm were then used to calculate the percentage absorbance. Standards 1 to 6 were provided in a 40% methanol/water solution each having a concentration of 0, 50 ng/L, 200 ng/L, 800 ng/L, 3 200 ng/L and 12 800 ng/L respectively. The mean lower detection limit was established by the supplier.

The ELISA standards were compared by calculating the % absorbed. This was calculated by dividing the mean absorbance values of a standard by standard 1 (0 mg/L) and multiplied by 100 shown in Equation 1 below. The standard 1 therefore equalled 100% and the absorbance values were quoted in percentages.

Equation 1

$$\% \text{ Absorbance} = \frac{\text{Absorbance standard (or sample)}}{\text{Absorbance zero standard}} \times 100 \quad (1)$$

The values that were calculated using Equation 1 for the standards were entered in a system of coordinates against both the E2 and EE2 concentration in [ng/L]. The calibration curve was almost linear in the 50 – 3200 ng/L range. Thus using the calibration curve the hormone concentration in ng/L (ppt) corresponding to the absorbance was determined in unknown samples.

3.2.4 Procedure for the Radioimmunoassay RIA)

A Captus 600 Multichannel analyser was used to read the tubes for the RIA analysis. This equipment was calibrated and then optimised before doing the count. RIA kits supplied materials/reagents for the quantitative measurement of estrogens in serum or plasma. The procedures followed the basic principle of RIAs where competition occurred between

radioactive and a non-radioactive antigen for a fixed number of antibody binding sites. The amount of [I-125] labelled estradiol bound to the antibody was inversely proportional to the concentration of the unlabelled estradiol present. A double antibody system was used to achieve separation of bound and unbound antigen for the E2 and E1 test kits. For the E3 test kit the separation was achieved by emptying out the antibody-coated tubes.

All standards and controls for each test were subjected to the SPE procedure outlined in 3.2.1 and 3.2.2.

3.2.4.1 Test procedure for the E2 RIA kits (DSL-4400)

Estradiol standards were provided in serum with sodium azide as a preservative and each having a concentration of 0 pg/mL, 20 pg/mL, 50 pg/mL, 250 pg/mL, 750 pg/mL, 2000 pg/mL and 6000 pg/mL. There were also two controls which were at 242.7 pg/mL and 1003.9 pg/mL. Refer to appendix 7 for details on full procedure.

3.2.4.2 Test procedure for E1 RIA kits (DSL-8700)

Estrone standards were provided in serum in a protein-based buffer with sodium azide as a preservative and each having a concentration of 0pg/mL, 15 pg/mL, 35 pg/mL, 100 pg/mL, 300 pg/mL, 900 pg/mL and 2000 pg/mL. There were also two controls which were at 40.1 pg/mL and 287.4 pg/mL. Refer to appendix 8 for details on full procedure.

3.2.4.3 Test procedure for E3 RIA kits (DSL-3700)

Estriol standards were provided in serum with a non-mercury preservative and each having a concentration of 0ng/mL, 0.1 ng/mL, 0.3 ng/mL, 1.0 ng/mL, 3.0 ng/mL, 10 ng/mL and 30 ng/mL. There were also two controls which were at 0.53 ng/mL and 2.84 ng/mL. Refer to appendix 9 for details on full procedure.

The standard curve for each kit was drawn and then compared to the typical standard curve data. The Multichannel Captus Counter was optimised to get as close as possible to the typical standard curve data.

The RIA standards were compared by using the counts per minute of bound antibody. The sample preparation was approximately 1hour and the test implementation was ~2hours 15 minutes for E1, ~2 hours for E2 and ~1 hour 30 minutes for E3.

For the E1 and E2 RIA kits the percentage mean sample counts were calculated using Equation 2 below. Where B = the mean sample counts and Bo = Mean counts of the 0 pg/mL standard, NSB = Non Specific Binding.

Equation 2

$$\% \, B/Bo = \frac{\text{Mean sample counts} - \text{NSB counts}}{\text{Mean counts of 0pg/mL std} - \text{NSB counts}} \times 100 \qquad (2)$$

The E3 RIA kit was calculated slightly differently as shown in Equation 3. The reason for the difference was that the E3 kits have test tubes which contain the rabbit anti-estriol polyclonal antibody immobilized on the inside and therefore there was no non-specific binding to the test tube.

Equation 3

$$\% \, B/Bo = \frac{\text{Mean sample counts}}{\text{Mean counts of 0pg/mL std}} \times 100 \qquad (3)$$

The values that were calculated for the standards were entered in a system of coordinates against the E1, E2 or E3 concentrations. Thus using the calibration curve the hormone concentration corresponding to the percentage counts can be determined in unknown samples. However when assessing the test kit performance, actual counts per minute of bound antibody was used and not the percentage counts.

3.2.5 Validation of the ELISA and RIA Procedures

In order to determine the precision and reproducibility of the ELISA and RIA kits it was necessary to estimate the precision quantitatively. The calculations applied were from the study by Rodbard (1974). The intra-assay and inter-assay were performed using the following calculations:

1) The mean of the replicate (r) counts observations were calculated
2) If there were two values (x) the differences between the two values were calculated, if there were more than two values the range was used.
3) The sample standard deviations (s) for the replicate measurements were calculated using the following Equation 4:

$$s = \frac{(x_1 - x_2)}{\sqrt{r}} \qquad (4)$$

If r = 2 then the standard has only one degree of freedom (df = r - 1).

Next the estimate was then expressed as a percentage coefficient of variation (CV) by dividing the standard deviation (s) by the mean and multiplying by 100 as show by Equation 5:

$$\% CV = 100 \frac{s}{x} \qquad (5)$$

3.2.5.1 Recovery of E2 standard using ELISA

A 17β-Estradiol standard was purchased from Sigma Aldrich and 3 working solutions of 2 µg/L, 5 µg/L and 10 µg/L were made up from a stock concentration of 1 g/L and applied to the ELISA kit in order to determine percentage recoveries and kit performance. The working solutions were also subjected to the C18 cartridges.

3.2.5.2 Recovery of E1, E2 and E3 controls using the RIA

The controls that were provided in each RIA kit were used to determine percentage recoveries as well as kit performance. There were two controls at different concentrations for E1, E2 and E3 that were provided in each RIA kit. The controls were also subjected to the C18 cartridges.

3.3 RESULTS

3.3.1 Comparison of the ELISA standard curves

The application of the E2 ELISA kit was user friendly and was simple and easy to follow. Figure 3.1 displayed the typical standard curve data and two test runs and the in a logarithmic scale. The correlation coefficients were 0.9741, 0.9978 and 0.9939 respectively. The % recovery for test one ranged from 79 to 104% with an average recovery of 92.27% and test two ranged from 80 to 123% with an average recovery of 101.50 %. The average coefficients of variation were 5.18 and 5.46 for test one and two respectively. Statistical evaluation was performed on the average absorbance, percentage absorbance as well as the calculated standards concentrations between each test and the typical standard data using the *t*-test, with Tails = 2 and Type = 1. The Pearson correlation was also performed on the calculated standard concentration.

The average absorbance showed no significant difference with P = 7.18% and 60.71% for the first and second test respectively. However the percentage absorbance showed a significant difference for the second test with P = 1.06 % and no significant difference for the first test with P = 42.75%. Even though there was this difference in the % absorbance the calculated standard concentrations showed no significant difference for the second test having P = 29.1% and the first test having P = 35.51%. The Pearson's correlation also showed perfect correlation for the calculated standard concentrations for both tests when compared to the typical standard concentrations.

The application of EE2 ELISA kit procedure was also user friendly. Two test runs were also performed for the assessment and the standards were done in duplicate. Figure 3.2 shows the typical standard curve data and the standard curve data of the two test runs in a logarithmic scale. The correlation co-efficients were 0.97415, 0.9971 and 0.998. The percentage recovery for test one ranged from 85 to 114 % with an average recovery of 101.11% and the range for test two were from 89 to 115% with an average recovery of 100.51%. The coefficients of variation were 3.51 and 2.71 for test one and two respectively. Statistical evaluation of the average absorbance readings found P= 82.55 % and P= 49.40 %, while the

percentage absorbance showed a significant difference for both the first test and the second tests with P = 2.72 % and 0.67 % respectively. However the calculated standard concentrations found a perfect correlation with the Pearsons correlation and no significant differences with P = 28.3 % and 62.49 % for test one and two respectively.

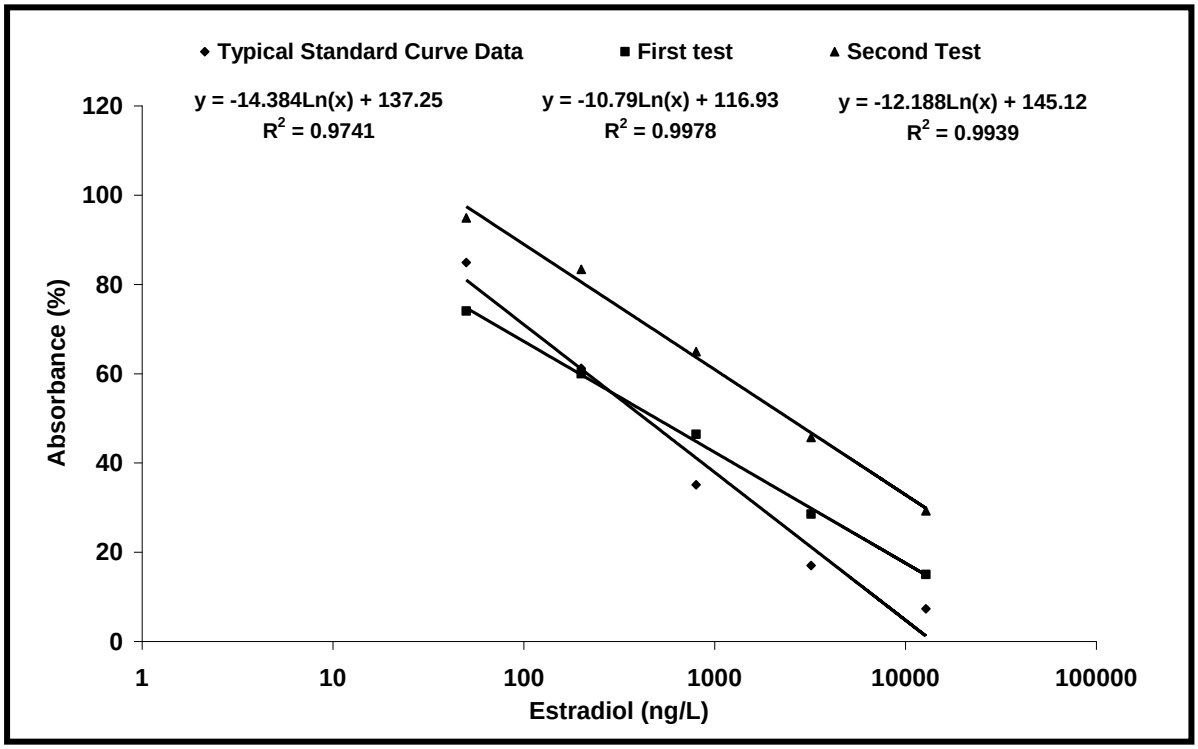


Figure 3.1 Typical, first and second test standard curves of E2 ELISA

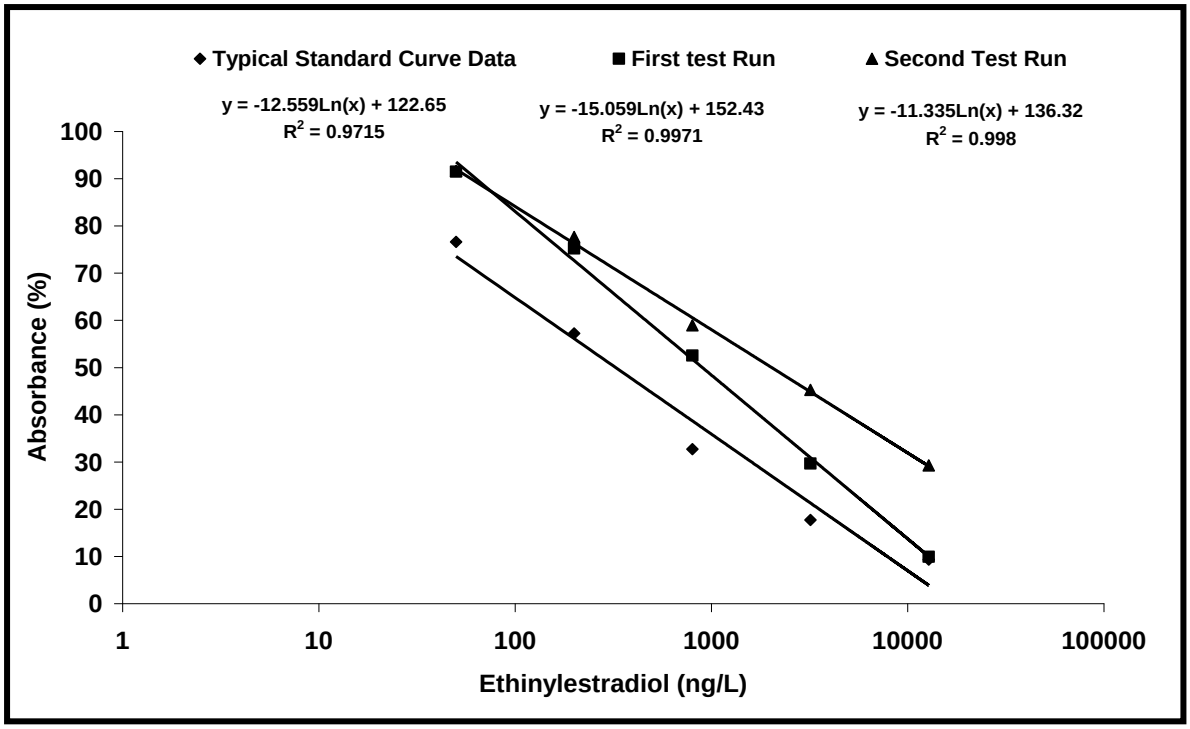


Figure 3.2 Typical, first and second tests standard curves of EE2 ELISA

3.3.2 Comparison of the RIA standard curves

The first trial run for the RIAs application yielded low total counts when compared to the Typical Standard Data Counts for the test kits. It was found that the Energy scale influenced the counts. The test procedures for the RIA kits did not indicate under which energy scale the tubes should be read in, therefore it was necessary to then establish which energy scale to work with, in order for a maximum reading to be obtained. The total counts which consisted of only the zero standard and no other solution was used to determine the energy scale which gave a value close to the Typical Standard Counts.

Table 3.1 shows the total counts at the different energy scales for E2, E1 and E3. The trial run was at 84 KeV. It was found that at 100 KeV yielded counts close to the Typical Standard Counts. The other configuration parameters of the Gamma counter were Peak counts = 100, counting time of 60 seconds and Counting Time Base = Real. Each standard was applied in duplicate and the average Gamma counts after 60 seconds were taken.

Table 3.1 Total Gamma counts at different energy scales for E2, E1 and E3

	Typical Standard Total counts (cpm)	Total Count at 84 KeV	Total Count at 100 KeV
E2	**59287**	19966	42570
E1	**30271**	20801	28641
E3	**72187**	25091	68125

Figure 3.3 displays the typical, 84 KeV and 100 KeV standard curve data of E2, in a logarithmic scale with correlation coefficients of 0.979, 0.9513 and 0.9766 respectively. It was noted that even though the counts for the 84 KeV yielded lower total count values, the % recoveries were not affected. The overall % recoveries for the 84 KeV and 100 KeV were 108% and 104% respectively. The statistical evaluation show significant differences for the 84 KeV counts and the percentage counts with P = 2.97 and 2.16% respectively. The 100 KeV show no significant difference for the counts with P =56.59%. The calculated standard concentrations for both the 84 and 100 KeV show no significant differences when compared to the typical standard concentrations with P = 23.64 and 59.12% respectively. The calculated standard concentrations also yielded perfect correlation in comparison to the typical standard concentration.

The E1 Standard Curve data is shown in figure 3.4 and has correlation coefficients of 0.998, 0.9915 and 0.9984 for the Typical Standards, at 84 KeV and 100 KeV respectively. The overall % recoveries of E1 standards were 100.91 and 100.14 for the 84 KeV and 100 KeV energy scales. A similar trend of the E1 counts and E1 % counts were also noted. The 84 KeV total counts and % counts showed significant differences where P <5% but the calculated standard concentrations showed no significant differences to the typical standard concentrations where P = 38.13 and 76.33% for the 84 KeV and 100 KeV respectively. The calculated concentrations also yielded perfect correlations of 1.

The E3 standard curve is shown in Figure 3.5 and has correlation coefficients of 0.9875, 0.9978 and 0.9989 for the Typical, 84 KeV and 100 KeV respectively, and both energy scales

yielded recoveries of 100%. The same trend was noted for E3 where there was a significant difference in the total counts and % counts where P < 5 % while the calculated standard concentrations showed no significant differences for the 84 KeV. The 100 KeV showed no significant differences to the typical standard data. Perfect correlation was also noted for the calculated standard concentrations at the 84 and 100 KeV.

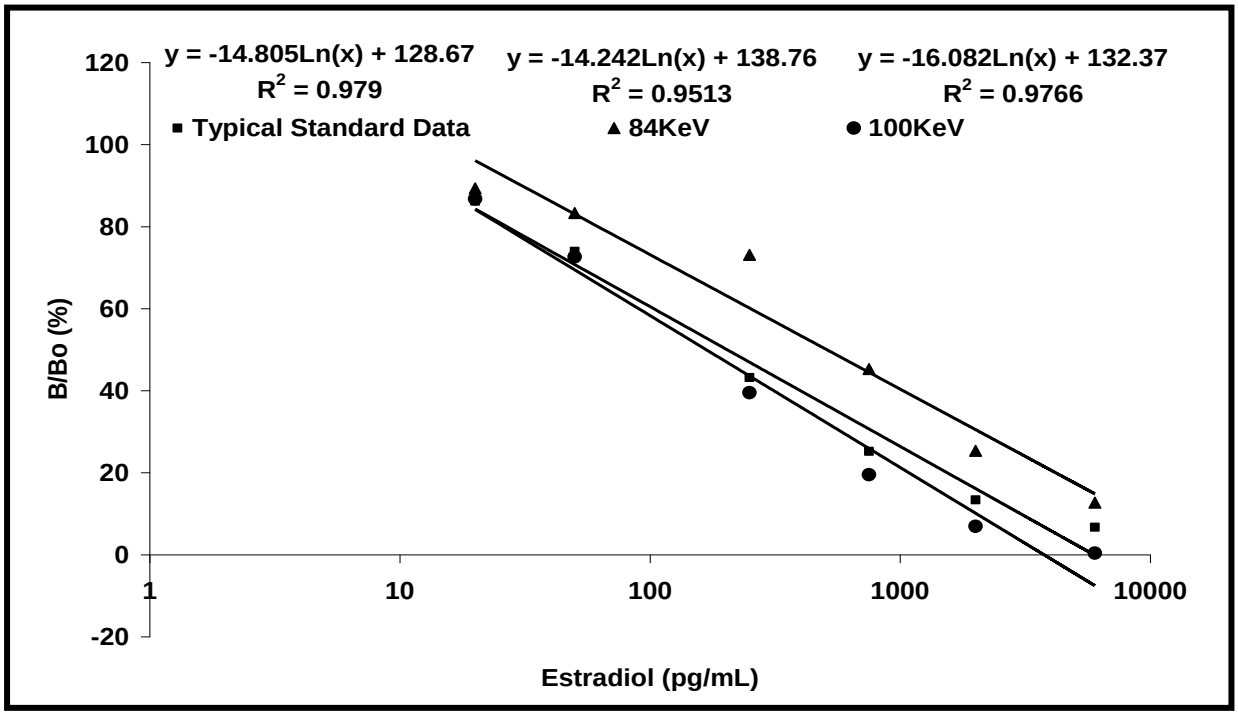


Figure 3.3 Typical, 84 KeV and 100 KeV standard curves of E2 RIA

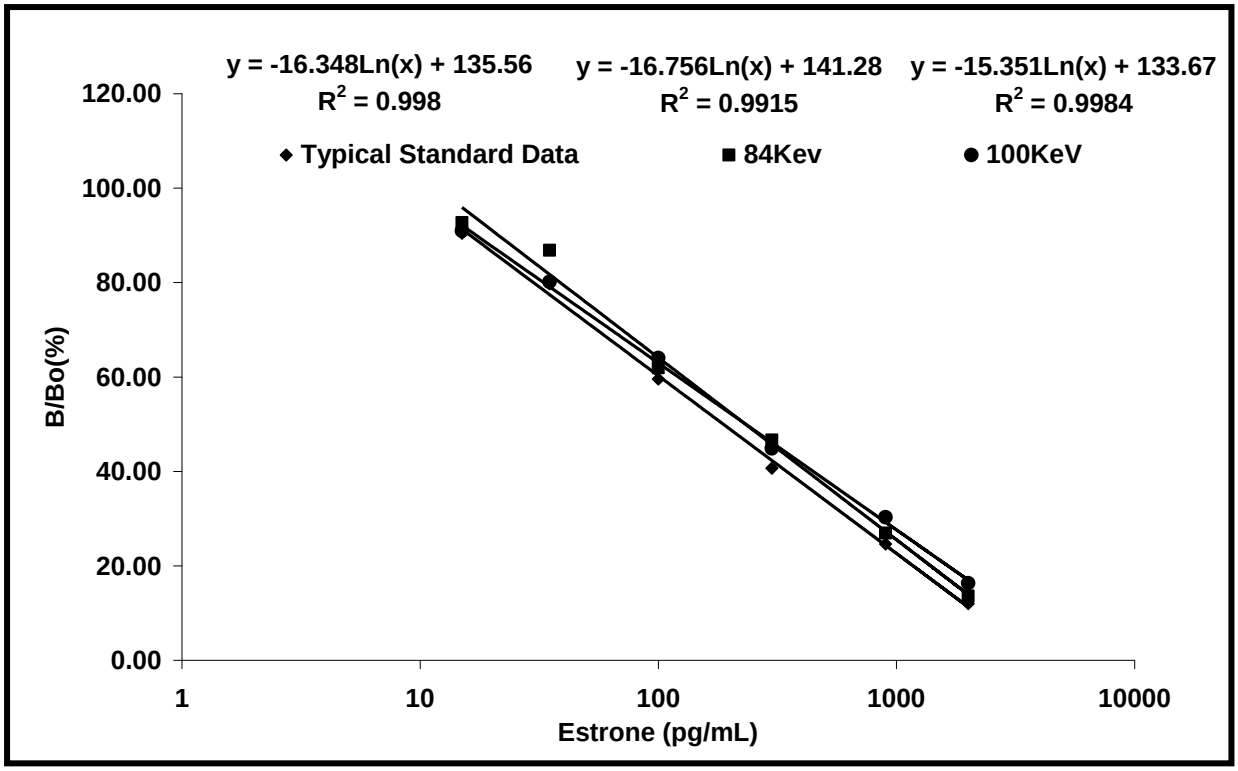


Figure 3.4 Typical, 84 KeV and 100 KeV standard curves of E1 RIA

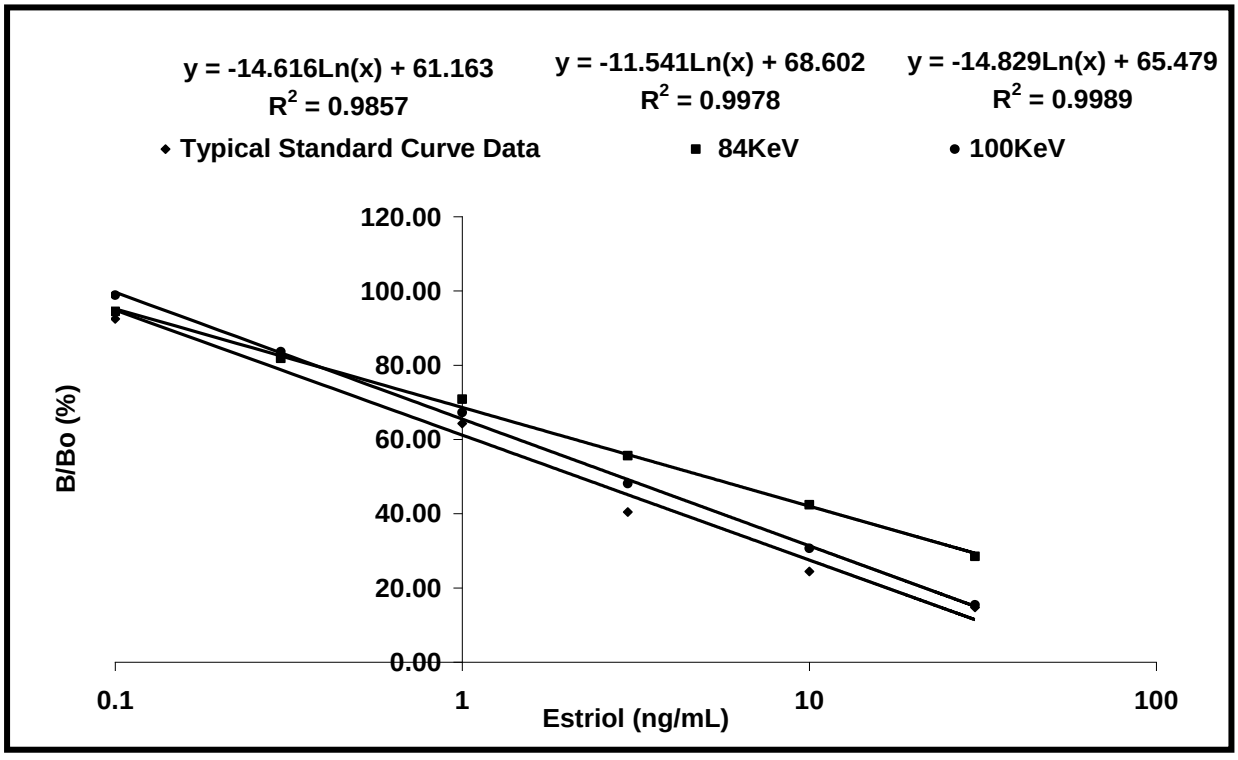


Figure 3.5 Typical, 84 KeV and 100 KeV standard curves of E3 RIA

3.3.3 Intra-assay and inter-assay Validations

The ELISA intra-assay precision was calculated from the means of 4 replicate of each standard, conducted on the same assay, shown in Table 3.2 and the ELISA inter assay (Table 3.3) was analysed using the mean of average duplicates for two separate runs. The RIA intra-assay precision was calculated from the means of 3 replicate counts of each standard, shown in Table 3.4 for E2, E1 and E3. The inter-assay precision is shown in Table 3.5 for E2, E1 and E3 and was analysed from the mean of average duplicates for two separate runs.

The intra-assay variation for E2 ELISA was <10% for all standards and the inter-assay variation for E2 ELISA was <7% for all standards. The EE2 ELISA showed intra-assay variations <6% for all standards and inter-assay variations <7% for all standards.

The intra-assay variation for E2 RIA was <8% for standards 1 to 6. The E2 RIA inter-assay variation was <11% for standards 1 to 6. The E1 RIA had less than 8% for both intra-assay and inter-assay variations. E3 RIA also showed variations below 8% for both the intra and inter-assay variations for all standards.

Statistical evaluation of the intra and inter-assays were conducted using the ANOVA function. The results indicated that there was no significant differences with P >5 % for all sets of data.

When evaluating the precision regarding reproducibility of the different methods, the mean RIAs intra and inter-assay variations were used. The mean intra assay variation E2, E1 and E3 RIA were 3.7%, 4.5%, and 3.0%, respectively. The mean inter-assay variation for E2, E1 and E3 RIA were 4.6%, 3.9% and 1.7%, respectively. These results are in line with the study done by Swart and Pool, 2007 where ELISAs were used to determine E2, E1 and E3 concentrations and the validation of the ELISA test kits had intra and inter-assay variations less than 5.6%, 8.2% and 4.5% for E3, E1 and E2, respectively. The correlation coefficients (R^2) for the intra assay ranged from 0.0.9897 to 0.998 and the R^2 for the inter assay ranged from 0.9876 to 0.9959.

Table 3.2 Coefficient of Variation (%) of the ELISA Intra-Assay Validation

Standard	n	E2	EE2
2	**4**	3.5	2.7
3	**4**	6.1	4.5
4	**4**	9.5	4.2
5	**4**	7.5	5.3
6	**4**	0.8	2.1

Table 3.3 Coefficient of Variation (%) the ELISA Inter-Assay Validation

Standard	n	E2	EE2
2	**2**	5.7	6.4
3	**2**	7.1	4.2
4	**2**	3.5	5.7
5	**2**	4.9	2.8
6	**2**	0.7	6.4

Table 3.4: Coefficient of Variations (%) for the RIA Intra-Assay Validation

Standard	Number	E2	E1	E3
1	**3**	4.7	2.6	0.9
2	**3**	7.5	6.2	2.6
3	**3**	2.5	1.0	3.1
4	**3**	3.0	7.6	7.0
5	**3**	3.2	4.2	1.0
6	**3**	1.5	5.2	3.0

Table 3.5: Coefficient of Variation (%) for the RIA Inter-Assay Validation

Standard	Number	E2	E1	E3
1	**2**	2.1	4.2	2.3
2	**2**	0.7	0.7	0.3
3	**2**	8.5	0.0	1.3
4	**2**	0.7	6.5	3.4
5	**2**	10.6	4.3	2.8
6	**2**	4.9	7.8	0.0

3.3.4 Recovery of E2 standard using the ELISA

Due to the ELISA kit not containing controls, an external E2 standard was purchased from Sigma Aldrich and different concentrations made up and applied to the ELISA. Figure 3.6 shows the standard curve used to determine the concentrations of the E2 standards. The correlation coefficient was 0.9828. Three working solutions were made up to theoretical concentrations of 2, 5 and 10 μg E2/L from a stock solution of 1 g/L. Table 3.6 shows the actual concentrations obtained as well as the % recovery. Standards 2, 5 and 10 μg E2/L had % recoveries of 109, 113 and 109 respectively.

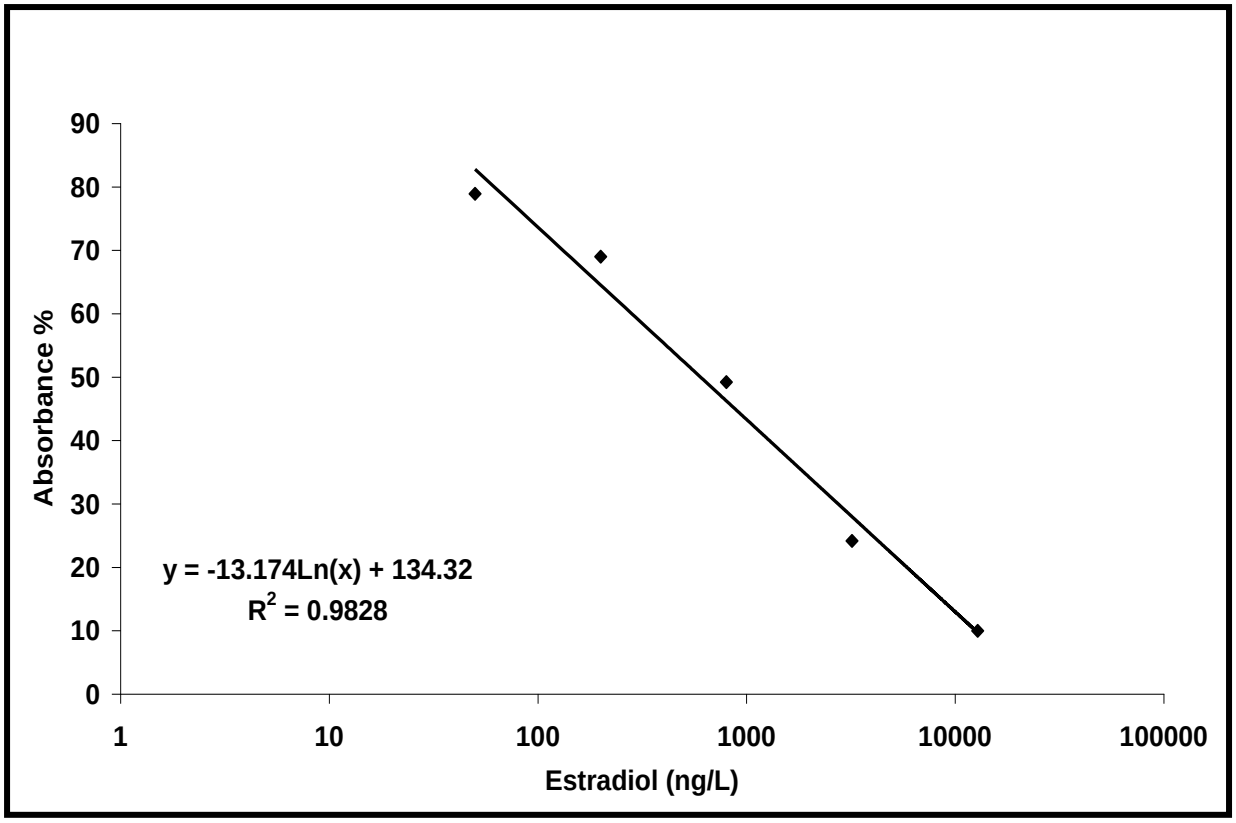


Figure 3.6 Standard curve obtained for the E2 standard concentrations.

Table 3.6 Average E2 ELISA concentrations (± 95 % CI) of working standard solutions

E2 Working Solutions (µg/L)	Actual Concentration µg/L	Standard Deviations	% Recovery
2	2.17 ± 5.3	0.38	108.53
5	5.65 ± 2.2	0.16	113.09
10	10.94 ± 6.4	0.46	109.43

3.3.5 Recovery of E1, E2 and E3 using the RIA

The E1 control I and II had recoveries of 97.88% and 105.03% at the 84 KeV respectively and 101.87 % and 97.93 % at the 100 KeV energy scales. The E2 control I and II had recoveries of 98.83 and 104.95% respectively at the 84 KeV and recoveries of 104.94% and 101.85% at 100 KeV energy scale proving again that even though the counts were low at the 84 KeV it did not interfere with the recovery. The % recovery for E3 control I was 98.11 and 103.77 for

the 84 KeV and 100 KeV energy scales respectively and at both energy levels the recovery was the same for control II at 97.54%. Table 3.7 shows the concentrations as well as the % recovery for the controls.

Table 3.7 Average RIA Control Concentrations (± SD) (%Recovery) for E1, E2 and E3

	Typical Control Concentration	Concentration at 84KeV (cpm) (% Recovery)	Concentration at 100KeV (cpm) (% Recovery)
E1 (pg/mL)	**I: 40.1**	39.25 ± 0.6 (97.88)	40.85 ± 0.53 (101.87)
	II: 287.4	301.87 ± 10.23 (105.02)	281.46 ± 4.2 (97.93)
E2 (pg/mL)	**I: 242.7**	239.9 ± 2.0 (98.83)	254.7 ± 8.5(104.94)
	II: 1003.9	1053.6 ± 35.1 (104.95)	1022.5 ± 13.1 (101.85)
E3 (ng/mL)	**I: 0.53**	0.52 ± 0.01 (98.11)	0.55 ± 0.01 (103.77
	II: 2.84	2.77 ± 0.05 (97.54)	2.77 ± 0.05 (97.54)

3.4. DISCUSSION

It is extremely important to determine a proper extraction and clean-up procedure especially when dealing with a matrix that is complex such as wastewater. According to Gomes *et al.* (2004) the more intense the extraction and clean up procedure is the greater the chance for analyte losses which can result in lower recoveries. Many researchers have used the C18 cartridges for solid phase extractions (SPE) for use in either GCMS or LCMS (D'Ascenzo *et al.*, 2003; Gibson *et al.*, 2005; Johnson *et al.*, 2000; Onda *et al.*, 2003) and a few for ELISA (Desbrow *et al.*, 1998; Drewes *et al.*, 2005; Gibson *et al.*, 2005; Ternes *et al.*, 1999).

Lopez de Alda and Barcelo (2001) in the review of analytical methods stated that the advantage of using the cartridges for SPE is that it is open to system automation as there are specific apparatuses which are available for unattended washing, conditioning, sample loading, washing, drying and elution of a large number of samples. The Lopez de Alda and Barcelo (2001) review also reported that subsequent drying of the cartridges with either air or nitrogen gas does not lead to losses of analyte.

The approach of using RIAs for detecting estrogens in wastewater can be regarded as a novel approach. This technique had to be optimised for this application and therefore there is a limited amount of literature with regards to comparative evaluation of these findings. There are two studies that we are aware of who applied RIAs (Shore *et al.*, 1993 as cited by Lopez de Alda and Barcelo, 2001; Snyder *et al.*, 1999). However the controls that were provided for the RIA kits served as a good indication of the recovery of the standards and unknown samples from the C18 cartridges and the RIA technique. If the controls did not show values close to the theoretical values then one could establish if the application was correct or not.

The intra and inter-assay validations in this study yielded coefficient of variations <8%. The study by Snyder *et al.*, 1999, found that the coefficient of variations for the RIA analysis was <12% which was in line with this study. Rodbard (1974) stated that it is essential that a quality control system be developed which can provide information on the reproducibility. Rodbard (1974) also found that making use of duplicates or triplicates within each of several assays is usually most efficient, especially if the interest lies in estimating the intra assay and inter assay variations.

The volumes of samples required for an ELISA and RIA procedure is much less as compared to the volumes needed for other techniques. A review of analytical methods done by Lopez de Alda and Barcelo (2001) showed that the volume of sample analysed was depended on the sensitivity of the procedure used for the final analysis. The Lopez de Alda and Barcelo (2001) study showed that the RIAs only required 50 mL for extraction as compared to the 20L and 80L required by liquid-liquid partition and analysed by gas-liquid chromatography.

The results yielded in this study showed good recoveries after the extractions and application to ELISAs and RIAs (Tables 3.6 and 3.7). The use of 80% methanol eluted majority or all of the estrogens from the C18 cartridge. A study by Desbrow *et al.*, 1998 also illustrated that majority of estrogenic activity was eluted from the C18 columns between 80% and 85% methanol. However there were some instances whereby the value of the standard were higher than the theoretical value. Onda *et al.*, (2003) also confirmed that when using methanol eluate of SPE and a dichloromethane eluate SPE, the estrogenic activity for methanol yielded higher theoretical values while the dichloromethane eluate nearly matched the theoretical values.

These results show the existence of unknown estrogenic substances contained in the methanol eluate. Unfortunately for these tests no comparison was done using dichloromethane as the eluate. A study by Pool (2008) found that after subjecting the sample to C18 SPE, the sample was dissolved in different percentages of analytical grade ethanol (10 and 20%). The author found that there were no major differences between percentage recoveries for the E1, E2 and E3 at the different ethanol concentrations.

Lopez de Alda and Barcelo, (2001) also reported that the evaporation and concentration of the eluted extracts in analyte recovery is also important and if no precautions taken can result in losses of the volatile compounds. However if precautions are taken such as controlling the flow rate and temperature especially when nitrogen gas is used for evaporation and by protecting the sample from light and ensuring the extract is not kept dry for prolonged periods can minimize and reduce possible losses.

Both the ELISA and RIA have shown good recoveries of standards and controls proving the accuracy and precision of the immunoassay procedures (Tables 3.2 to 3.5). Goda *et al*. (2000) mentioned that ELISAs offers an advantage over the conventional methods (GC-MS, LC-MS/MS) in that no special skills is necessary, ease of handling, small sample volume, relatively fast measurement, high sample turnover, low detection limits and acceptable costs. ELISAs can also be utilised in the routine monitoring of EDCs existence in the environment. The study by Snyder *et al.* (1999) found that the RIA also allowed extremely sensitive detection of E2 and EE2 without an intense clean up procedure but is also sensitive to compounds which have a similar structure to the analyte. Kfir and Genthe (1993) found in the comparisons of ELISA to RIA, the principle of ELISA is similar to RIA; however the difference is the labeling of the antibody or antigen which is achieved by using an enzyme instead of a radioisotope. The sensitivity of ELISAs to RIAs was also found to be equal.

A study done by Pool (2008) evaluated different bio-assays i.e., Estradiol ELISA, Estrone ELISA, the yeast screen and the Xenopus liver assay. This study found that the ELISA test kits had a much shorter total time assay (2 days) as compared to the other bio-assays which

took more than 7 days and another finding was that it was much cheaper to conduct the ELISA bioassays. The cost factor worked out to be R270 per sample done in optimum batch for the ELISA, R1350 for the Yeast screen and R675 for the Xenopus liver assay. If a similar factor was applied to the RIA test kits, then the cost per sample would be the same as the ELISA test kits as the labour and non- labour times would be close to the ELISA test kits.

Research by Huang and Sedlak, (2001) revealed that the total time taken to conduct the ELISA analysis was less than 5 hours which is similar to the turnaround times for conducting the assay in this study.

Ingerslev and Halling-Sorensen (2003) found in their comparative study of the different techniques, that *"immunochemical techniques have the lowest cost and are 35 times less expensive than the most advanced LC-MS-MS. GC-MS-MS is less expensive than LC-MS-MS while single GC-MS is half the price of single LC-MS*".

The study by Pool (2008) also recommends that support be given to build capacity in ELISA technology especially in South Africa

3.5 CONCLUSIONS

It has been shown that having the right extraction and clean up procedure prior to immunoassay application plays an important role in analyte recovery. The use of SPE with C18 cartridges was used by many researchers before application to the respective analytical procedure for hormone detection. ELISA and RIA immunoassays can be employed for use as rapid techniques for hormone EDC detection. The small sample volume required shortens the labour times and application of the procedure makes it a cost effective and reliable technique. The intra-assay and inter-assay validation procedures confirmed reproducibility and the results of the controls and standards during the investigation showed good recoveries.

However, although the RIA procedure used was easy to follow, did have a few setbacks. Firstly there was a need for facilities that houses a radioactive laboratory which is not commonly accessible. Special precautions had to be taken during application, like having absorbent paper in case of spillage of radioactive material, working behind a lead brick to avoid radio-activity contact and special containers were required for proper disposal of the tubes. There was a storage facility whereby the time taken for the radio activity to "die-off" had to be used and once the time had elapsed the tubes could then be discarded. Comparatively RIAs are more intensive with regards to equipment required. The Gamma counter for the RIAs had to be calibrated each time before use.

The RIA method was also a rapid method which can definitely be employed for determining estrogen hormones in unknown samples. However the method can only be employed if the right facility is available.

It is easier to employ an ELISA method for determining estrogen hormones as there is no need for special facilities or expensive equipment than what is needed for RIAs. This can be seen by the larger number of researchers who employ ELISAs rather than RIAs and the high recommendations of the use of ELISAs over the conventional GC-MS, LC-MS techniques. ELISA has also been recommended for use in routine monitoring of hormone disrupting chemicals in the environment due to its acceptable costs, relatively fast measurements, ease of handling and high sample turnover.

On adapting the techniques, the following chapter focuses on testing the techniques of ELISA and RIA in determining the fate of these estrogens wastewater using laboratory scale investigations.

CHAPTER 4
DETERMINING THE FATE OF ESTROGENS IN LABORATORY SCALE INVESTIGATIONS

4.1 INTRODUCTION

A laboratory scale Modified Ludzak Ettinger (MLE) system was used to determine the fate and degradation potential of these natural and synthetic estrogens under controlled conditions. In the 1960's Ludzak and Ettinger were the first to propose a single sludge process which utilizes the biodegradable material in the influent as an energy source. Barnard later in the 1970's improved on this process by separating the anoxic and aerobic reactors, recycling the underflow from the settler to the anoxic reactor, and introducing an additional recycle from the aerobic to the anoxic reactor. The influent is fed directly into the anoxic zone. This zone is practically free of oxygen but does contain nitrite and nitrate. The aerobic zone is kept aerated by introducing air or oxygen. In this zone the utilization of the biodegradable organic matter is completed. A recycle (A-cycle) from the aerobic zone recycles the mixed liquor back to the anoxic zone. The underflow recycle (S-recycle) from the clarifiers also recycles mixed liquor to the anoxic basin. The total flow from the aerobic reactor is discharged directly with the effluent and is not recycled back to the anoxic zone (Lilley *et al.*, 1997). By operating laboratory scale processes conditions could be manipulated to see the effect of hormone removal under controlled conditions. Laboratory scale investigations are generally aimed at making a realistic prediction of the behaviour of substances in the environment.

Toxicity evaluation of environmental substances such as wastewater, contaminated water bodies has become an important part of environmental pollution monitoring and has grown steadily (Girotti *et al*., 2002; Girotti *et al*., 2008; Kovats *et al*., 2012; Ma *et al*., 2014; Parvez *et al*., 2006; Serafim *et al*., 2013; Smital *et al*., 2011). Recent trends in ecotoxicological research has focused substantially on the development of several types of rapid, robust, convenient, and cost-effective batteries of bioassays for the qualitative and quantitative toxicity characterization of various environmental mutagens (Burga-Perez *et al*., 2013; Celebi and Sponza, 2012). The luminescent bacteria toxicity test commercially known as Microtox,

utilises the naturally luminescent bacterium *Vibrio fischeri*, and is being used for ecotoxicological risk assessment (Boluda *et al., 2002;* Jalova *et al., 2013*). The toxicity characterization by Microtox tests are being used for the evaluations of dispersant toxicity and are done by intimate contact between toxicants and luminescent bacterium for a very short period (1 hours or less). The exposure to toxicants inhibits the luciferase enzymes responsible for the light emission of luminescent bacterial cells thus the luminescence intensity. The inhibition is directly proportional to the toxicity of the compounds being tested and is therefore used to quantify the toxicity in comparison to the controls (Ma *et al*., 2014).

The approach by Hewitt and Marvin (2005) whose review is based on the environmental effects-directed analysis of effluents which allows "*biological end-point to direct chemical manipulations of a mixture to separate active components from inactive ones"* thereby allowing analytical efforts to focus on the most relevant compounds which are not necessarily known has sparked this investigation of determining the toxicity of the hormone standards. This ultimately confirms toxicants and eliminates those not associated with the effect of concern.

Thus this decision to include toxicity monitoring was to determine the level of toxicity that an individual hormone could exhibit and following in the next chapter if there is a correlation between the toxicity of the hormone itself and hormones in the final effluents in wastewater treatment plants.

This chapter thus investigated the fate and removal of hormones using laboratory-scale processes under controlled conditions in order to obtain information to assess behaviour in full scale WWTPs and the assessment of the Biotox kit using the marine bacterium, *Vibrio fischeri* to determine the toxicity of the estrogen hormone standards.

4.2 MATERIALS AND METHOD

4.2.1 The laboratory scale MLE process

The feed of the MLE process was taken from the influent of wastewater treatment plant B which consisted of a split treatment facility viz, simple activated sludge process on the west side and a biological nutrient removal process (BNR) on the east side (further description and schematic diagram of this plant is given in Chapter 5). The MLE process contained the seed sludge (Mixed Liquor Suspended Solids (MLSS) of 2500 mg/L from the WWTP B and the influent was collected after the coarse and fine screens in 25 L containers and stored at 4ºC. The influent was collected on a weekly basis.

The influent was also passed through a sieve with pore size of approximately 1mm diameter to get rid of suspended matter and this served as a feed for the process and was maintained at 24 L/d. This laboratory scale process consisted of a 6 L anoxic zone, followed by an 8L aerobic zone, and a 2.5 L clarifier to separate the mixed liquor from the effluent (Fig 4.1). The COD was maintained at approximately 500 mg/L. The anoxic zone was created by having only a stirrer with gentle mixing, while the aerobic zone was created by diffusing air via an air stone connected to an oxygen utilisation meter, which maintained the DO at 2 – 5 mg/L/hr.

The influent and effluent Chemical Oxygen Demand (COD) and Mixed Liquor Volatile Suspended Solids (MLVSS) were monitored in order to determine the systems performance. The standard method for determination of the COD (appendix 10) and MLSS (appendix 11) were used.

The MLE system was also manipulated to control the sludge retention time (SRT). For the first four months a 5 day SRT was applied and then for the latter time period a 10 day SRT was run. During the entire duration of both the SRTs, the fate and degradation potential of the natural and synthetic estrogens were monitored for the influent and effluent samples as well as samples after each zone in the process after the process reached steady state. The

ELISA and RIA were employed to detect estrogens. The laboratory scale process was given a one month acclimatisation period. The process was operated for a total of 10 months but actual plant monitoring and analysis began in the second month.

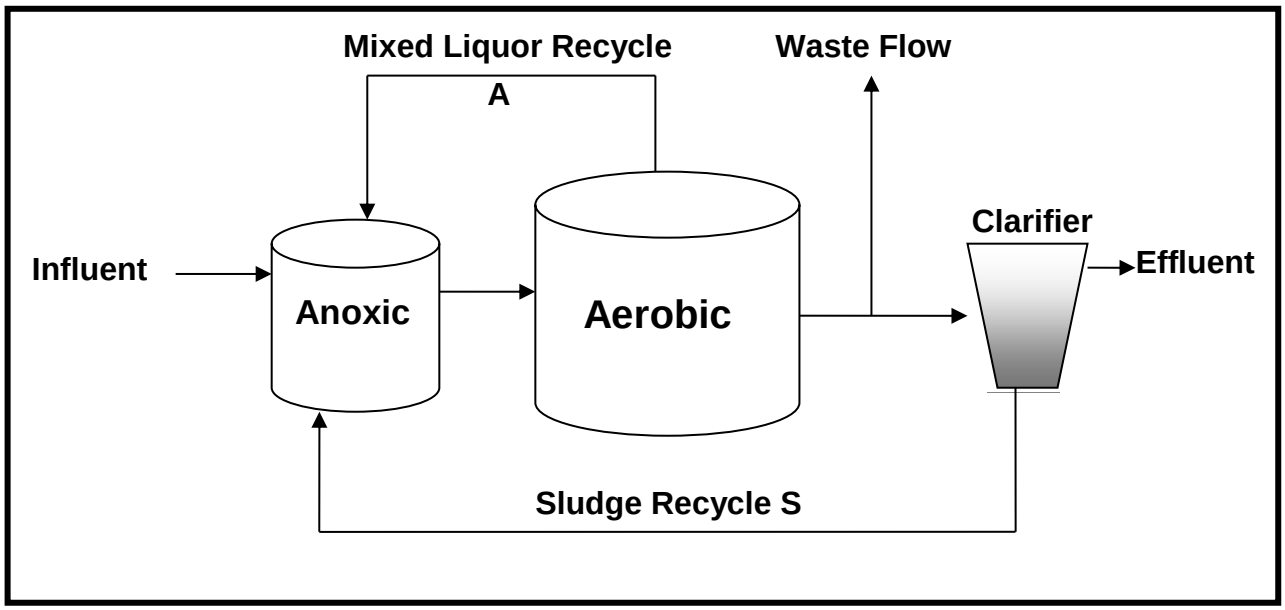


Figure 4.1 Schematic diagram of the laboratory scale MLE Process

4.2.2 The use of a 24 hour batch test in assessing 17β-E2 degradation

Pure E2 standard was purchased as a powder from Sigma. Through the use of a batch test, the behaviour of 17β-E2 in an activated sludge process was studied. Activated sludge (MLSS 1000 mg/L) from the MLE system was used. For this process a 10 L reactor was used. Influent from a domestic wastewater plant was spiked with the E2 standard to obtain a concentration of 1ng/mL. The batch consisted of 500mL of sludge and 4.5 L of influent. The DO was set between 4 to 6 mg/L/hr. The batch test was run for 24 hours. At appropriate time intervals, 100mL of the suspension was taken and centrifuged and supernatants analysed for E1 and E2.

4.2.2.1 Preparation of Samples

All glassware was cleaned according to the method described in appendix 2. The cuvettes used were discarded after a single use.

Samples with sludge were centrifuged at 3000 rpm for 6 minutes and the supernatant was filtered through a glass fibre filter paper with 0.3-0.6 μm pore size. The filtrate was kept in amber glass bottles and preserved with 1% formaldehyde (appendix 12) if it was not used immediately. Samples were stored in the fridge at approximately 4°C. Samples were then subjected to SPE procedures and applied to the respective immunoassays.

4.2.3 Toxicity of E2, EE2 and a mixture of E1, E2 and E3 standards

The E2, EE2 and mixture of E1, E2 and E3 hormone standards from ELISA kits were procured from Tox Solutions, South Africa. The 1 μg/LE2, 3 μg/L EE2 and mixture of E1, E2 and E3 stock solutions were diluted using ultra-pure water to a concentration of 10 ng/L and the 10 ng/L E2 was further diluted to 1, 5 ng/L and the toxicity determined using the *Vibrio fischeri* Biotox procedure purchased from Tox Solutions, South Africa. The *Vibrio fischeri* is a marine bacterium therefore all tests had to be performed at a salinity of 2%. A Kikkoman luminometer was used to read the relative light unit (RLU) for each sample. The assay is based on inhibition of luminescence of the combined diluted test sample and the luminescent bacteria. After contact times of 15 (T_{15})and/or 30 minutes (T_{30}), the decrease of light intensity is measured. The inhibitory effect of diluted samples was calculated using the BioTox software by comparison to a toxin free control which provided the percentage inhibition (% INH). The % INH was then plotted against the dilution factor and the curve produced was used to calculate the Effective concentration (EC) giving 50% inhibition (EC_{50}) of light output of the sample.

The test procedure in brief: All reagents, samples and the room temperature were kept at 15ºC. Prior to the toxicity testing the 9 g Biotox sodium chloride (NaCl) tablet was left overnight to dissolve in 45 mL deionised water to obtain a 20% NaCl solution. The following day the lyophilised *Vibrio fischeri* was reconstituted with the reagent diluent. This reconstituted reagent was allowed to stabilise for 30 minutes in the fridge and then 30 minutes at 15ºC before pipetting in to the cuvettes. See appendix 13 for full procedure.

A screening test was first performed on the respective standards. A control was prepared by diluting the 20% NaCl to 2%. The control of 2% was also used in the definitive test.

A 10 mL sample with 1 mL of the 20% NaCl was used for the screen test. Once the *Vibrio fischeri* was stabilised a 500 µL aliquot was added to the test tube and read at time zero. The control and the sample aliquot also of 500 µL were added to the *Vibrio fischeri* and the luminescence read. The next reading was after 15 minutes and then 30 minutes for each standard. All controls and samples were done in duplicate.

If a sample proved to have a toxic value of more than 50% then the definitive test was performed by adding a 2 mL aliquot of the 2% NaCl to cuvettes to provide five dilutions for each standard. Then 2 mL of sample was added to the first cuvette and four dilutions were made (Refer to appendix 13 for full details on the procedure).

If the BioTox software is not used then Equation 1 can be used to determine the correction factor (KF) which can then be applied in equation 2 to determine the INH % (an example of 15 minutes contact time is used):

Equation 1:

$$KF = \frac{IC_{15}}{IC_0} \qquad (1)$$

Equation 2:

$$INH\% = 100 - \frac{IT_{15}}{KF \times IT_0} \times 100 \qquad (2)$$

Where:

- KF = Correction factor.
- IC_{15} = Luminescence intensity of control after contact time (15 min) in RLU.
- IC_0 = Initial luminescence intensity of control sample in RLU.
- IT_{15} = Luminescence intensity of test sample after contact time (15 min) in RLU.
- IT_0 = Initial luminescence intensity of the test sample in RLU.

This study used the BioTox software to calculate the all the %INH.

The toxicity classification system is created using a scoring system that can be applied to any kind of liquid waste either with or without treatment that is discharged into any natural water source. This also includes leachates from waste dump sites or from contaminated soils. This systems scoring is based on two values the first comprising of the acute toxicity ranking and the second is the weight score for each toxicity class (Persoone *et al.*, 2003). The acute toxicity classes are calculated from the results of the INH % which are transformed into Toxic Units (TU) (Equation 3).

Equation 3:

$$TU = \frac{1}{L(EC_{50})} \times 100 \qquad (3)$$

The samples are thus classified into one of five categories based on the number of TU in a test. The five classes are as follows:

***"Class I – no acute toxicity**: none of the tests showed a toxic effect (i.e., an effect value higher than that in the controls)*

***Class II – slight acute toxicity**: the percentage effect (PE) observed when at least one toxicity test was significantly higher than that of the control but is below 50 % (< 1 TU). (NB. The scoring system for natural waters, the 20 % effect level can be used as the lowest PE considered having a significant toxic impact. The 20 % corresponds to 0.4 TU (because the 50 % effect = 1 TU, 20 % effect is equivalent to 0.4 TU, 30 % effect = 0.6 TU, and 40 % effect = 0.8 TU).*

***Class III – acute toxicity**: the $L(E)C_{50}$ reached or exceeded in at least one test, but in the 10-fold dilution of the sample the effect was less than 50 % (=1 – 10 TU)*

***Class IV – high acute toxicity**: the $L(E)C_{50}$ reached in the 10-fold dilution for at least one test but not in the 100-fold dilution (=10 – 100 TU).*

***Class V – very high acute toxicity:** the $L(E)C_{50}$ reached in the 100-fold dilution for at least one test (≥ 100 TU)"* (Persoone *et al.*, 2003).

4.3 RESULTS

4.3.1 The laboratory scale MLE Process

The laboratory scale MLE system was set up to mimic a wastewater treatment plant. The major purpose was to determine the effect of different sludge retention times on hormone removal. Prior to determining hormone concentrations under controlled conditions, it was important to first establish that the laboratory scale process was operating efficiently which was achieved by monitoring the COD removal rates and the MLVSS concentrations until process reached steady state. Operation of the MLE process enabled us to control and change operational parameters in order to establish how certain parameters influenced the removal of hormone EDCs. One such parameter that was manipulated and controlled was the SRT.

The process monitoring and analysis was started in mid-autumn April 2008 and continued for a period of 9 months. The system performance was determined by the % COD removal. Figure 4.2 shows the average COD removal rates (± SD) and MLVSS concentrations (± SD) for the MLE process during the monitoring period.

During the 5 day SRT the MLVSS concentrations were below 2000 mg/L and averaged at 1642 mg/L, while the average COD removal was 82% (fig. 4.2). In the 5^{th} month the SRT was changed to 10 days. A steady increase in the MLVSS was noted reaching approximately 2500 mg/L in month 6 showing that the MLE system was working at its optimum level. However, unfortunate power failures caused a disturbance in the system which resulted in a decrease in the removal efficiency and a fall in biomass concentration but the system maintained an average COD removal of 90.73% and 2165 mg/L MLVSS until the process was shut down.

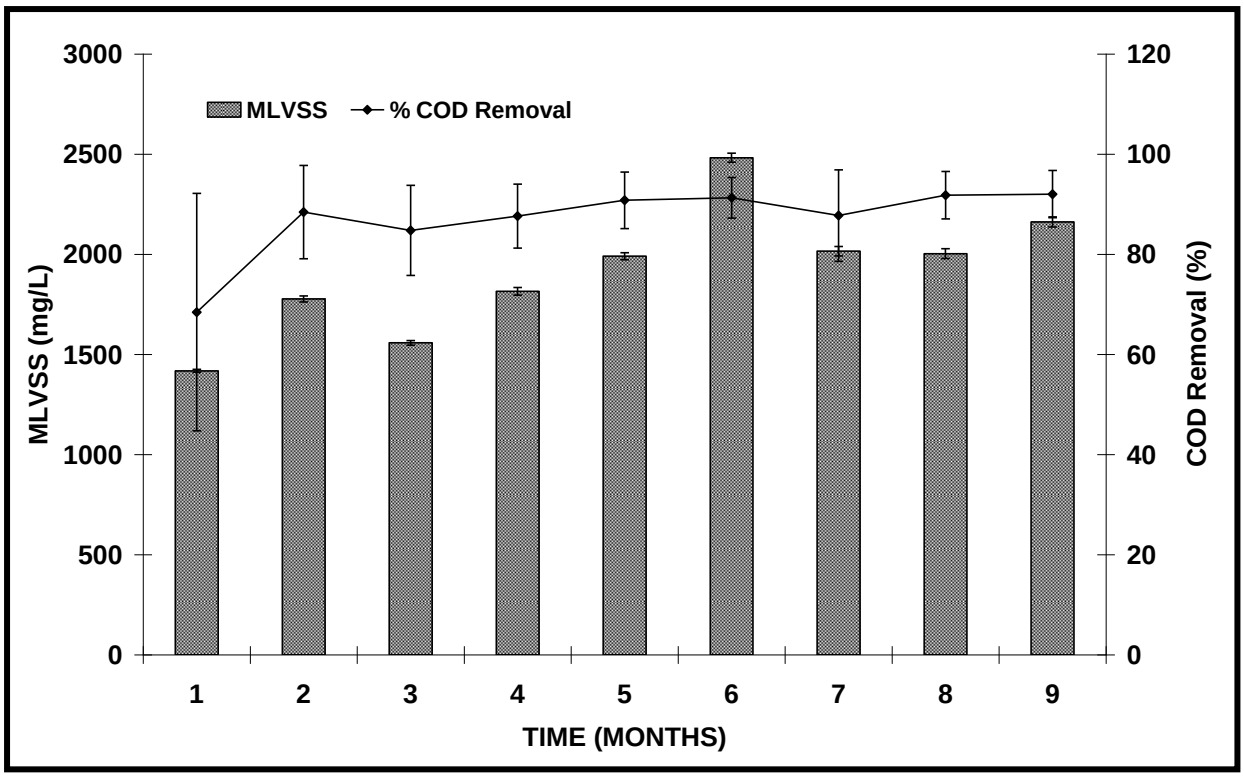
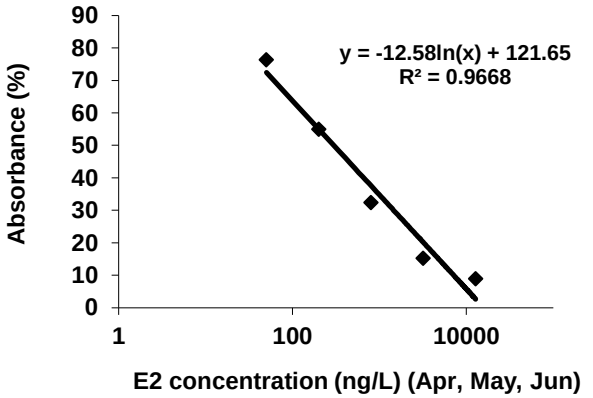
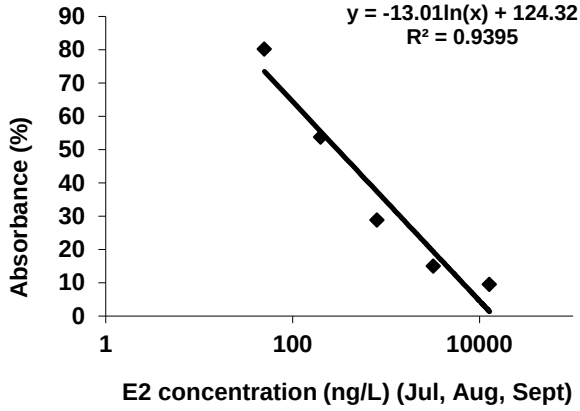


Figure 4.2 Percentage COD removal and mean MLVSS concentrations for the laboratory scale MLE Process

4.3.1.1 Fate of estrogens in the laboratory scale MLE system

Samples were taken from the different components of the MLE system i.e., influent, anoxic, aerobic and effluent zones. Samples were taken twice a month and analysed for E2 and EE2 using ELISA and E1 and E2 using RIAs. The standard curves for the E2 and EE2 ELISA are shown in figures 4.3 and 4.4 respectively, and the E1 and E2 RIA standard curves are shown in figures 4.5 and 4.6 respectively. The correlation coefficients (R^2) for all the standard curves were between 0.9395 and 0.9925.





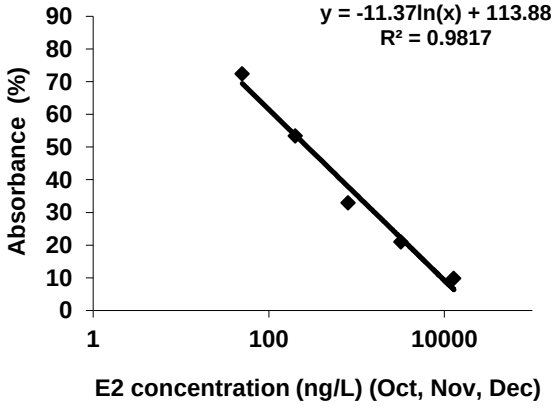
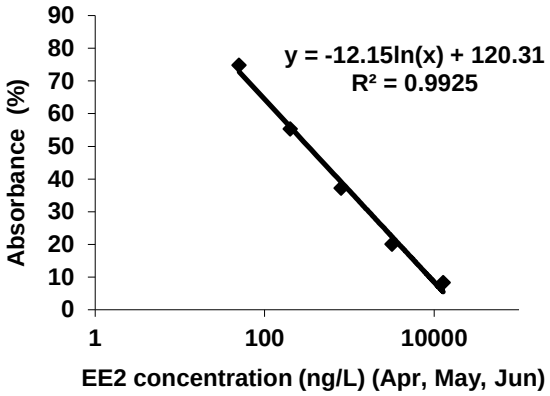
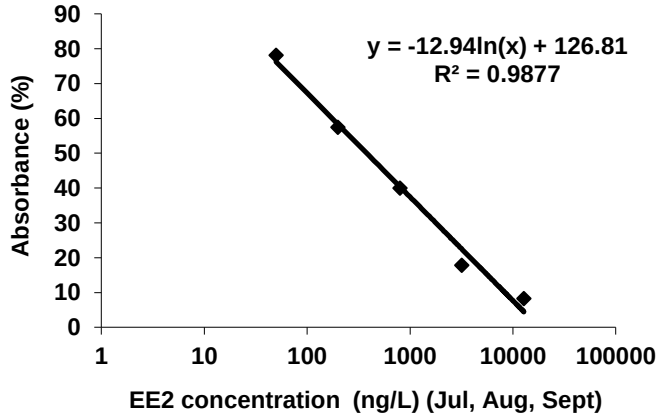


Figure 4.3 Standard curves used to calculate the E2 ELISA concentrations during the MLE process from April to December 2008.





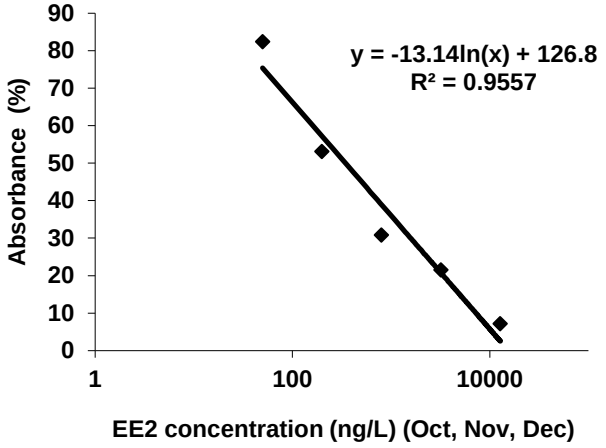
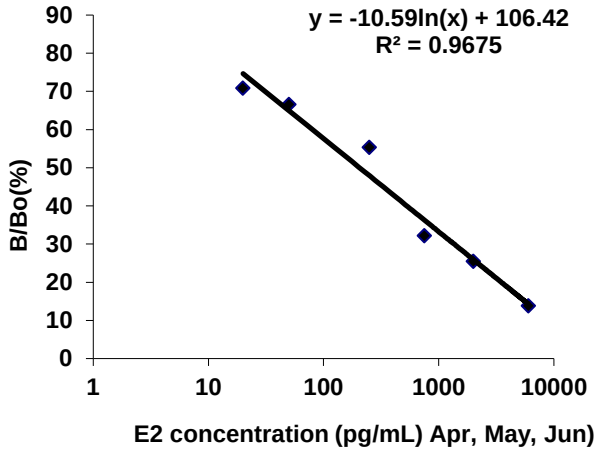
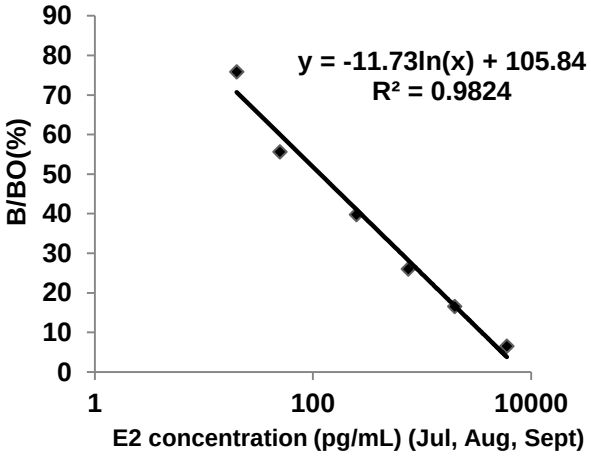


Figure 4.4 Standard curves used to calculate the EE2 ELISA concentrations for the MLE process from April to December 2008





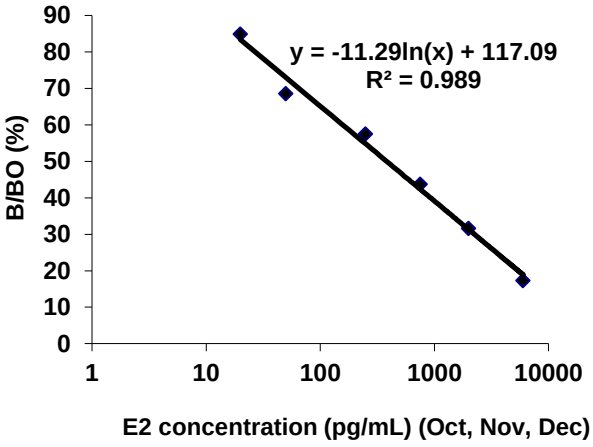
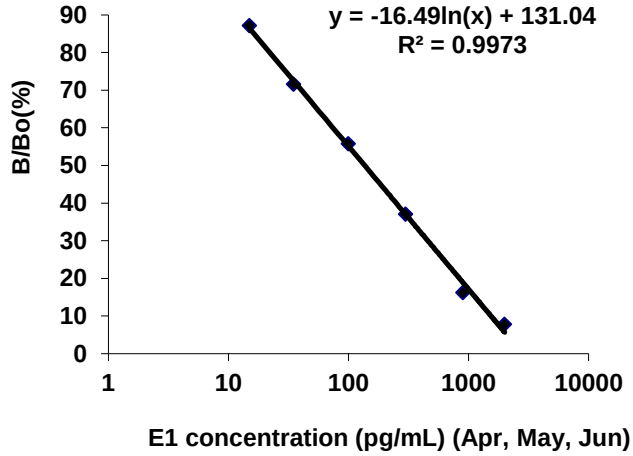
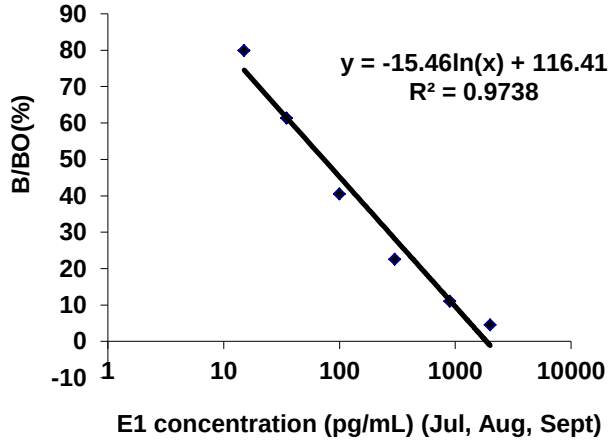


Figure 4.5 Standard curves used to calculate the E2 RIA concentration for the MLE process from April to December 2008





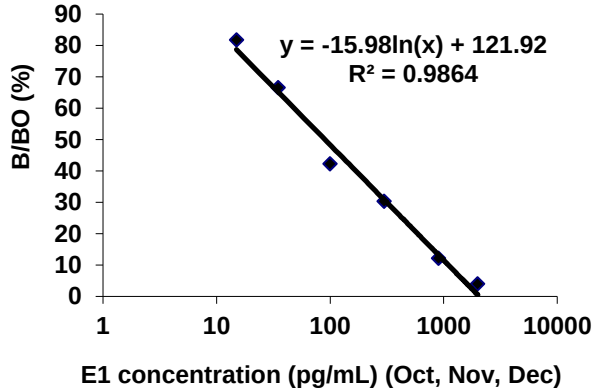


Figure 4.6 Standard curves used to calculate the E1 RIA concentrations for the MLE process from April to December 2008

(a) Assessment of Estradiol (E2) removal

During the first six months of operation the concentration of E2 in the influent sample ranged between 23. 57 ± 1.36 ng/L to 33.61 ± 0.24 ng/L for the ELISA and 23.00 ± 0.48 pg/mL to 31 .06 ± 1.97 pg/mL for the RIA (Fig. 4.7 (ELISA) and 4.8 (RIA)). From the 7th month, an increase of the influent E2 concentrations were noted and ranged from 65.06 ± 3.19 ng/L to 97.06 ± 4.75 ng/L for the ELISA and 63.46 ±2.69 pg/mL to 90.96 ± 3.86 pg/mL for the RIA.

During the period of the 5 day SRT the average percentage removal of E2 was 84% (fig. 4.7) an 4.8). The longer SRT of 10 days showed improved results in months 5 and 6 with a removal of 100 %. In months 7 to 9 due to the increase in the E2 levels in the influent the removal efficiency was higher. Months 7 to 9 had an average removal of 97% E2. Effluent E2 concentrations ranged from Not Detected (ND) to 6.76 ± 0.13 ng/L.

When assessing the overall E2 removal for each component of the MLE system the following was found:

- A removal of 67% took place in the anoxic zone.
- A further 65% was removed in the aerobic zone,
- And a further decrease of 39% in the final effluent was noted.

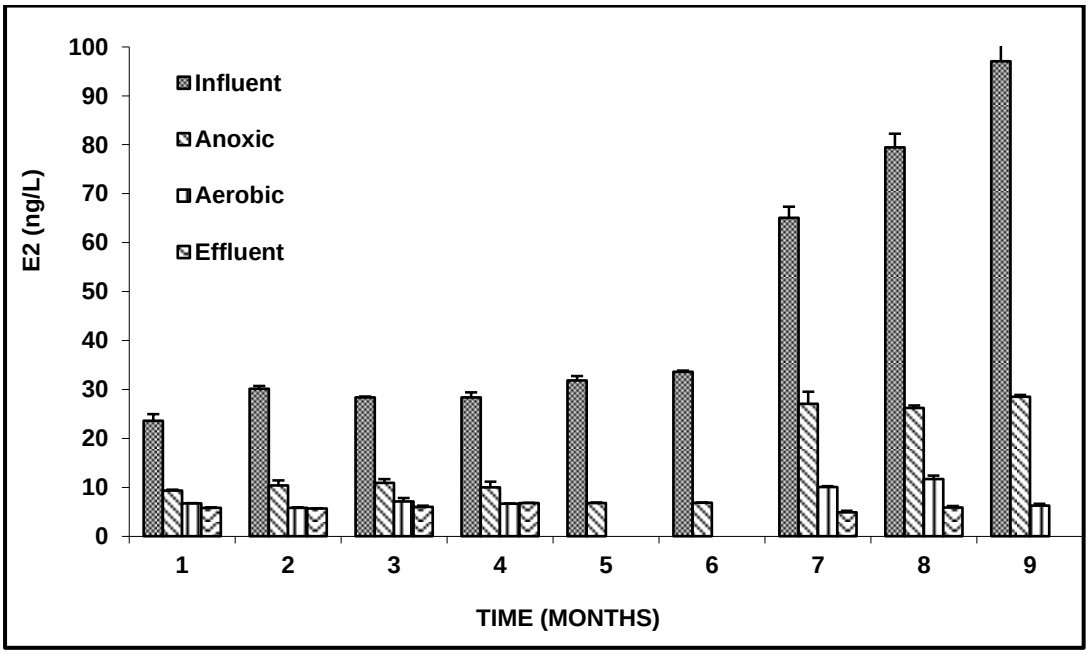


Figure 4.7 E2 concentrations in the different samples during the laboratory scale MLE process as determined by ELISA

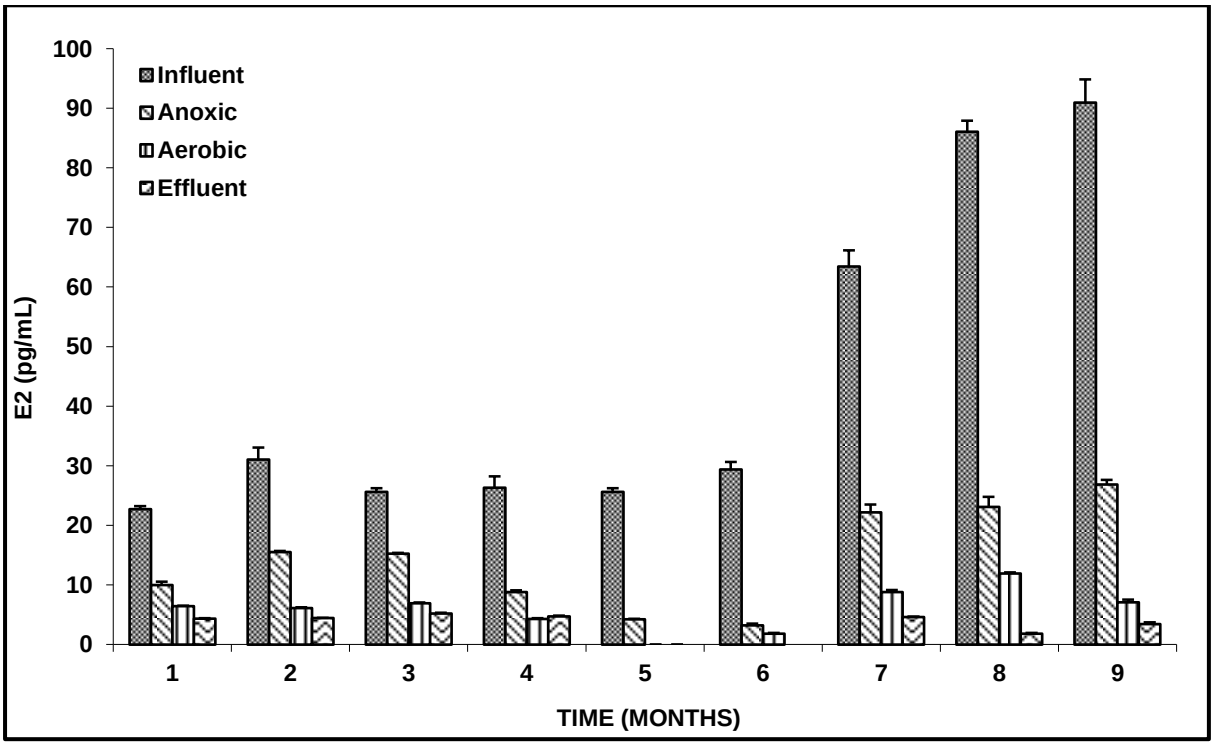


Figure 4.8 E2 concentrations in the different samples during the laboratory scale MLE process as determined by RIA

(b) Assessment of Ethinylestradiol (EE2) removal

The EE2 concentrations also showed similar trends to the E2 with regards to the increase in the last 3 months (Fig. 4.9). The influent concentrations of EE2 ranged from ND to 12 ± 0.46 ng/L. Where EE2 could not be detected a 100% removal value was assigned to that sample, therefore 100% removal efficiency was noted throughout the entire process even after the increase in the influent in the latter 3 months and irrespective of whether the SRT was 5 or 10 days.

Assessment of the EE2 removal in each component of the MLE system showed a removal of 77% in the anoxic zone when compared to influent concentration and was not detected in any of the samples from the aerobic zone or effluent.

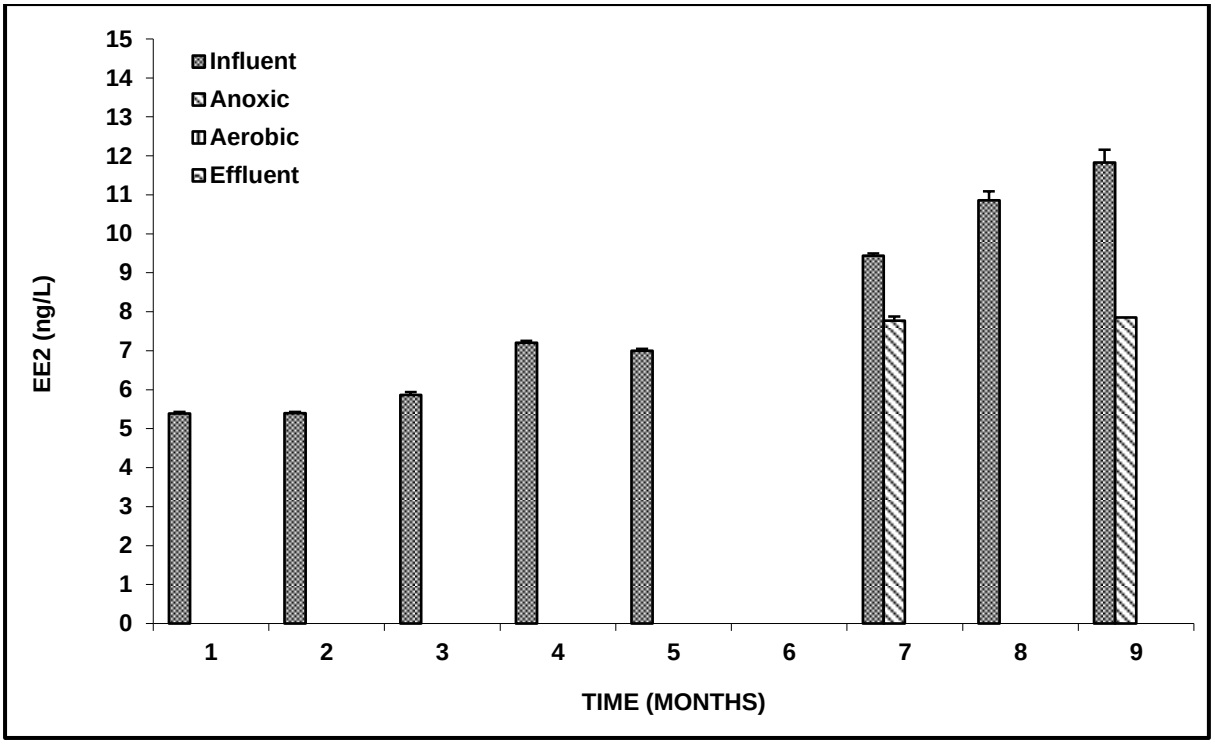


Figure 4.9 EE2 concentrations in different samples during the laboratory scale MLE process as determined by ELISA

(c) Assessment of Estrone (E1) removal

The influent E1 concentrations shown in figure 4.10 ranged from 43 ± 1.29 to 50 ± 4.88 pg/mL in the first six months and the increase in the influent concentration in the last 3 months was also experienced. The E1 concentrations in the latter 3 months ranged from 97 ± 4.75 to 115 ± 6.79 pg/mL.

When analysing E1 removal in each zone of the MLE system, there were definitive decreases in E1 concentrations. The percentage removal from the anoxic zone when compared to the influent concentrations was 47.49%, while removal from the anoxic to aerobic zones was 57.81% and from the aerobic to the final effluent there was a further decrease of 13.86%.

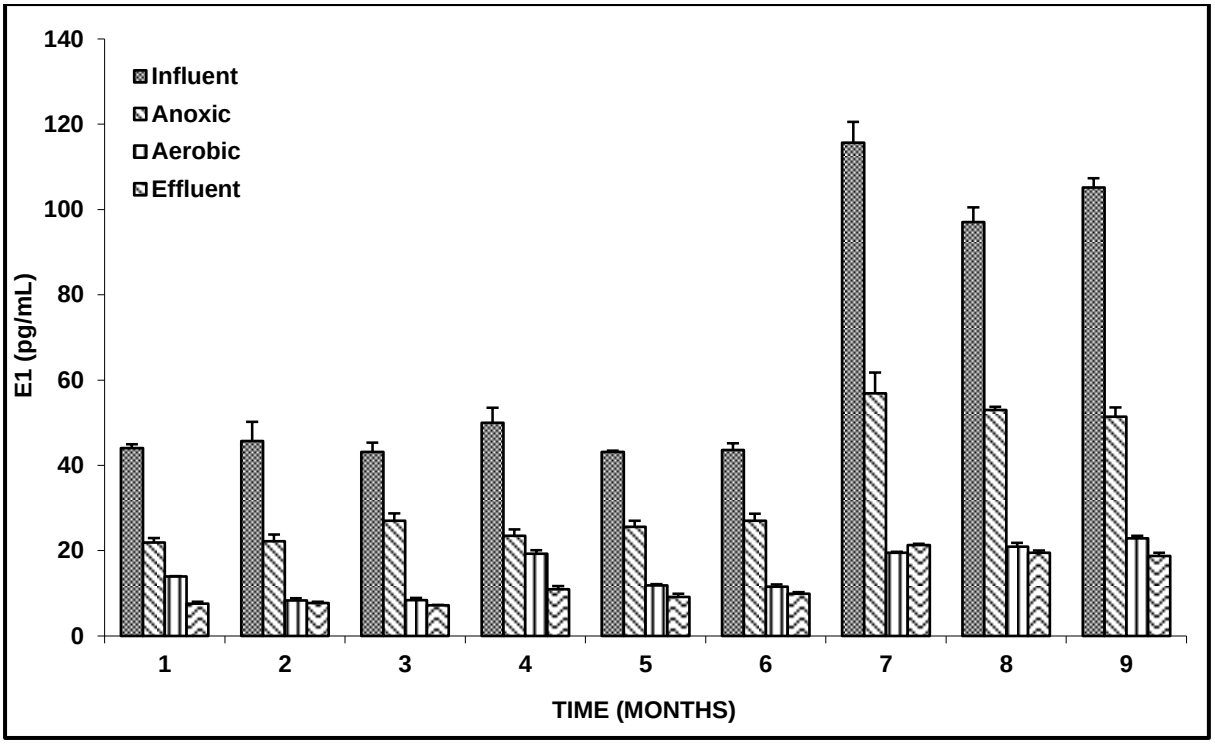


Figure 4.10 E1 concentrations in different samples during the laboratory scale MLE process as determined by RIA

(d) Overall Hormone Removal in MLE process

Table 4.1 summarises the hormone concentrations over the 5 and 10 day SRT and the overall percentage removal. It can be seen that the 5 day SRT had an overall average E2 and E1 removal of 78.11% and 81.71% respectively while the 10 day SRT had average E2 and E1 removal of 91.24% and 80.56% respectively.

Table 4.1 Mean E2 and E1 concentrations (± 95 % CI) in each zone of the laboratory scale MLE process during the 5 and 10 day SRT and percentage removal.

	5 DAY SRT				10 DAY SRT			
	E2 (ng/L)	**Std Dev**	**E1 (pg/mL)**	**Std Dev**	**E2 (ng/L)**	**Std Dev**	**E1 (pg/mL)**	**Std Dev**
Influent	27.60 ± 1.11	0.8	45.73 ± 3.85	2.78	61.40 ± 2.68	1.94	80.92 ± 3.44	2.48
Anoxic	10.14 ± 1.15	0.83	23.65 ± 2.03	1.46	19.07 ± 1.00	0.72	42.78 ± 3.03	2.18
Aerobic	6.56 ± 0.34	0.25	12.48 ± 0.69	0.49	9.33 ± 0.6	0.43	17.34 ± 0.73	0.53
Effluent	6.04 ± 0.19	0.14	8.36 ± 0.53	0.38	5.38 ± 0.45	0.32	15.73 ± 0.75	0.54
% Removal	**78.11**		**81.71**		**91.24**		**80.56**	

4.3.2 Fate of E2 standard in the 24 hour batch test

The operation of the batch test proved very useful to assess the behaviour of the E2 and E1 hormones during activated sludge treatment. The standard curves for E1 and E2 are shown in figure 4.11. The correlation coefficients were 0.956 and 0.964 respectively for E1 and E2. During the 24 hour batch experiments (fig. 4.12) a sharp decrease in the E2 concentration within the first 3 hours was noted and E1 increased by 98 %, with regard to the initial concentration of 0.013 ng/mL. This finding proved that the reversible metabolism of E2 does exist, however the irreversible metabolism of E2 to E3 was not investigated. The E1 concentration gradually decreased but still remained higher than the E2 concentration and at 13 hours the E1 concentration was 0.019 ng/mL and thereafter no longer detected. An average reduction of 94.44% of E2 was seen after 5 hours and after 10 hours was no longer detected. The correlation coefficients were 0.956 to 0.9916.

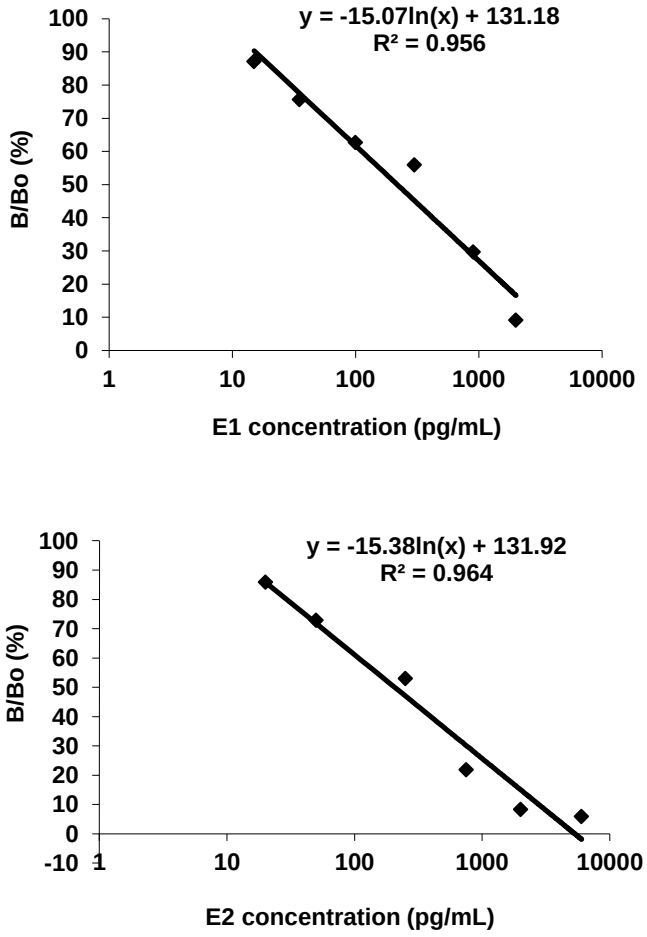


Figure 4.11 Standard curves used to determine E1 and E2 standard concentrations

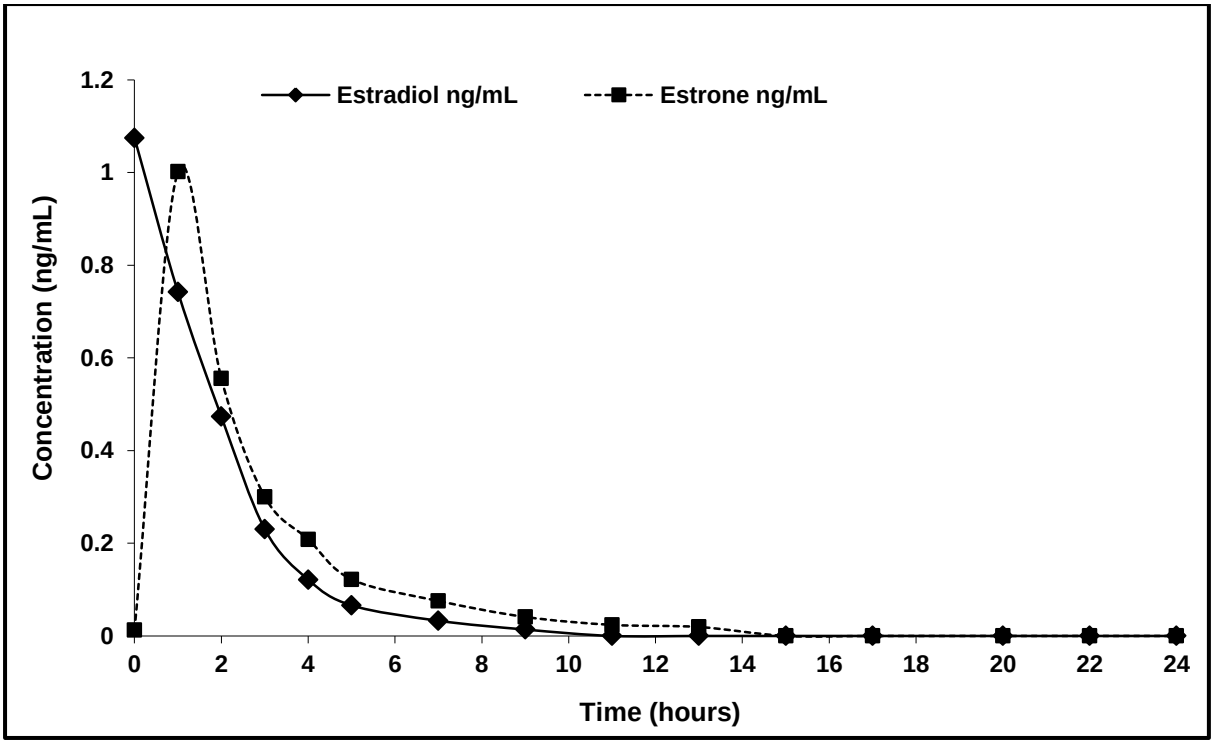


Figure 4.12 E2 and E1 RIA concentrations in the 24 hour batch test.

4.3.3 Toxicity of the Hormone Standards

For the toxicity investigation a comparison of the E2, EE2 and a mixture of E1, E2 and E3 was conducted at a concentration of 10 ng/L and a further dilution of E2 at 1 and 5 ng/L. The reason that motivated toxicity monitoring of the estrogens hormones was to determine how toxic the low concentrations of hormones really are and also establish where in the hazard classification system it fell. Therefore the microbiotest *Vibrio fischeri* method was used to conduct the toxicity tests.

In all bioassays it is always ideal to perform a validity test to determine precision. For the *Vibrio fischeri* biotox assay the validity was determined by using a reference solution. There are three reference solutions that could be used whereby each of these solutions must cause a 50 % inhibition after 30 min contact time at their respective concentrations in the final test suspension. Anyone of the following solutions may be used:

a) 3.4 mg/L 3,5-dichlorophenol
b) 2.2 mg/L Zn (II), equivalent to 9.67 mg/L Zinc sulphate heptahydrate

c) 18.7 mg/L Cr(VI), equivalent to 52.9 mg/L Potassium dichromate.

For the validity, a definitive test (appendix 13) was performed in order to determine the EC_{20} and EC_{50} values. In this study, Potassium Dichromate ($K_2Cr_2O_7$) was selected as a reference solution. Figure 4.13 shows the EC_{20} and EC_{50} values obtained for the 52.9 mg/L $K_2Cr_2O_7$ solution after a 30 minute contact time. The theoretical value to obtain an EC_{50} of Chrome VI is 18.7 mg/L after 30 minutes. The EC_{20} and EC_{50} value obtained after 30 minutes in this study was 3.87 ± 5 mg/L and 20.08 ± 5.7 respectively. The correction factor (KF) was 0.246.

Table 4.2 indicates the results of the different hormones of the same concentration at the 15 and 30 minute contact times. The results at the 10 ng/L revealed that after the 15 minute contact time the E2 standard showed the highest % inhibition at 39.97%, following was the mixture of E1, E2, E3 standards with 29.81% and the EE2 having the lowest % inhibition at 21.92%. After the 30 minute contact time the results showed a further increase in toxicity for the E2 standard reaching 45.99%, the EE2 had a slight decrease in the % INH to 19.54% while the mixture of hormones increased slightly to 31.54%. The lower concentrations of 1 and 5 ng/L E2, revealed that after the 15 minute contact time % INH was 23.7 and 26 respectively and after the 30 minute contact time % INH was 23.6 and 24.9 ng/L respectively. Since all final percentages were not above 50% there was no need to continue with the definitive test to determine the $L(E)C_{50}$. A definitive test would have only been performed if the final outcome of the screening test showed an inhibition above 50%. The results indicated that the E2 hormone on its own exhibits higher toxicity than in combination with other hormones.

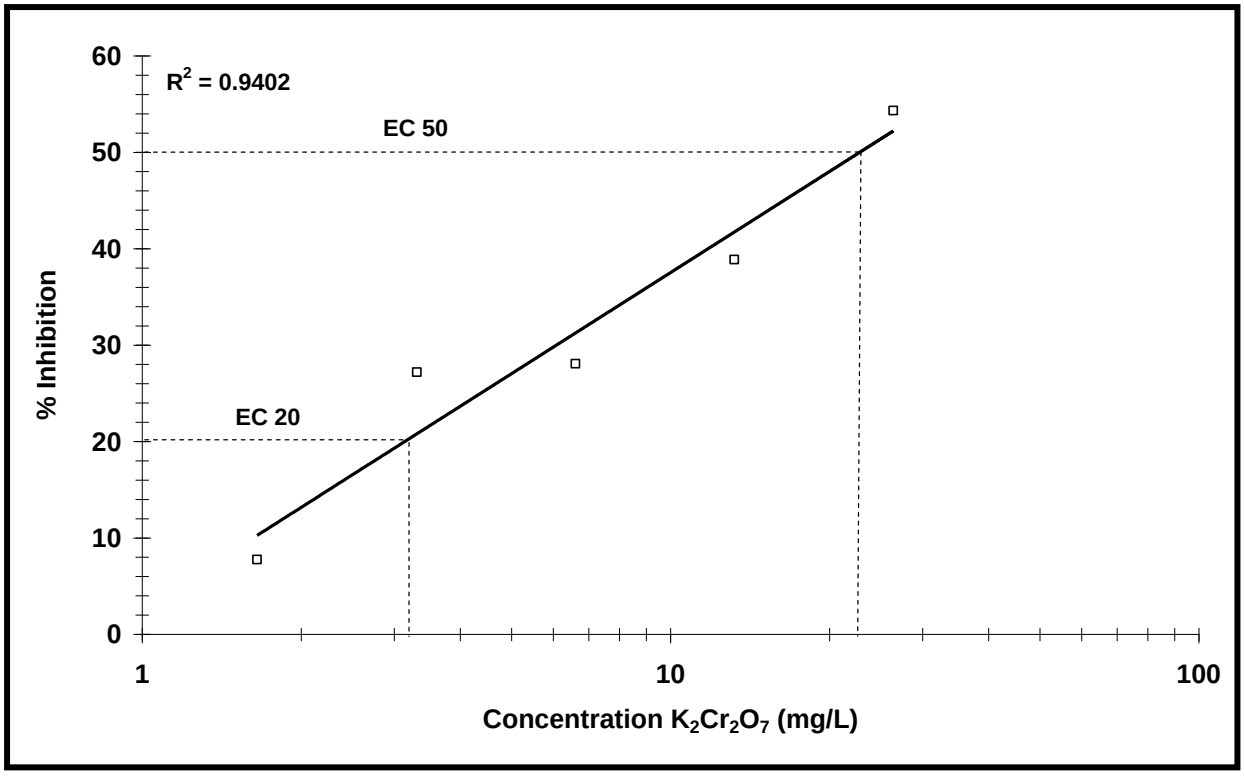


Figure 4.13 EC_{20} and EC_{50} of the $K_2Cr_2O_7$ reference solution

Table 4.2 % Inhibition (± SD) of the E2, EE2 and mixture of E1, E2, E3 Standards

	T15	**T30**
E2 (1 ng/L)	23.66 ± 2.43	23.60 ± 5.24
E2 (5 ng/L)	26.04 ± 5.46	24.85 ± 0.69
E2 (10 ng/L)	39.97 ± 0.27	45.99 ± 0.84
EE2 (10 ng/L)	21.92 ± 4.52	19.54 ± 0.70
E1,2,3 (10 ng/L)	29.81 ± 3.58	31.54 ± 6.78
Correction factor	0.637	0.609

Hence Table 4.3 shows the table that was compiled with symbols by Persoone *et al.* (2003) to establish a hazard classification table to easily classify substances.

Table 4.3 Hazard classification system for wastes discharged into an aquatic environment (Persoone *et al.*, 2003).

TU	Classification	Toxicity	Symbol
<0.4	Class I	No acute toxicity	
0.4 < TU < 1	Class II	Slight acute toxicity	
1 < TU < 10	Class III	Acute toxicity	
10 < TU < 100	Class IV	High acute toxicity	
TU> 100	Class V	Very high acute toxicity	

Therefore by extrapolating the information from the % INH (Table 4.2) and the toxicity classification system (Table 4.3) and applying this to the results of the toxicity monitoring of the standards; then the different hormones at the specified concentrations can thus be classified according to their TU. Table 4.4 shows the results of the toxicity classification system for the different standards. The % inhibition after 30 minutes was used to thus calculate the TU and then classify each of the different hormones. All the analysed hormones fell in the Class II toxicity classification system which exhibited slight acute toxicity. The E2 hormone was quite close to the Class III level which exhibits acute toxicity.

Table 4.4 The toxicity classification of E2, EE2 and mixture of E1, E2, E3 standards after the 30 minutes contact time

	TU	Class	Symbol
E2 (10 ng/L)	0.8	II	☹
E2 (1 and 5 ng/L)	0.5	II	☹
EE2 (10 ng/L)	0.4	II	☹
E1, E2, E3 (10 ng/L)	0.6	II	☹

4.4 DISCUSSION

4.4.1 The Laboratory Scale MLE System

Finding other research to compare removal of hormones within each component of the MLE laboratory scale processes was a challenge. Literature found was that of research done on influent and effluents of full scale wastewater treatment works which is discussed in the next chapter.

The manipulation of the SRT in the MLE system showed that the SRT can to some extent play a significant role in the removal of estrogens as indicated in Table 4.1. The increase of MLVSS and higher COD removal shown in Figure 4.2 during the 10 day SRT proves that the longer SRT can improve the system performance especially for those hormones which are more persistent like the E1 which was due to the conversion of E2. With regards to hormone removal the longer 10 day SRT also removed a higher percentage of E2 than the 5 day SRT (Table 4.1). Lopez-Fernandez *et al*. (2013) who compared a laboratory scale conventional activated sludge process and a membrane bioreactor over a 10 and 20 day SRT found that the removal of E2 was >90% for both the systems. Our findings also confirmed previous research by Lopez-Fernandez et al., (2013) that in the 10 day SRT of the activated sludge MLE system, the removal of E2 was also >90%.

However for the E1 removal both the 5 and 10 day SRTs were almost on par with the 5 day SRT being a percentage higher in reducing the E1 concentrations. The reason for the 10 day SRT not having a better removal could be attributed to the increase in hormone concentration that occurred in the influent. The period from October to December was summer months and the area that the WWTP serves where the samples were taken was a popular vacation area. It is postulated that during the summer months the population increased in that area which caused an increase of the hormone concentrations in the influent. The increase was almost double than what was found during the period of the 5 day SRT. The reason for the lower removal of E1 was due the increase of hormone concentrations in the influent which attributed to a higher percentage of E2 being oxidised to E1 thereby increasing the overall E1 concentration and thus reducing the removal efficiency. Hence the increase of E1 due to these

reasons made it difficult for the system to remove it more efficiently. Perhaps due to this factor of E2 converting to E1, a longer SRT period might be required to enhance E1 removal from the system. A study by Johnson *et al*. (2005) also found that with an increased HRT and SRT the removal of E1 increased. A 100 % removal of EE2 occurred for both the SRT periods. Kreuzinger *et al.* (2004) also found that with an increased SRT and hydraulic retention time (HRT) the removal efficiencies can be improved. Johnson *et al.* (2000) mentions that the longer the water remains in the works (i.e., increasing the HRT) the greater the time available for degradation. Increasing the SRT also has a great impact not only on the biota but also on the floc particles. By having proper floc formation in the sludge influences the hydrophilic and hydrophobic properties which can have an effect on the sorbent properties for estrogen compounds, (Johnson *et al.*, 2000).

4.4.2 The 24 hour Batch Process

The results from the 24 hour batch test showed a similar trend as found by the Ternes *et al.* (1999) study, where a sharp decrease of E2 was noted between 1 – 3 hours and a rapid increase of E1 during those times were noted (fig. 4.12). . The E2 was almost quantitatively oxidised to E1. The Ternes *et al.* (1999) study also used a 1 ng/mL spiking level and found that the removal of E2 and formation and elimination of E1 was accelerated and that after 5 hours both the E1 and E2 were not detected. In this study E2 was not detected after 9 hours and E1 after 15 hours.

Research by Shi *et al.* (2004) who used nitrifying activated sludge (NAS) to degrade hormones in batch tests, found that 98% of E2 was degraded within 2 hours and that E1 was also generated during the E2 degradation. This study by Shi *et al.*, (2004) also looked at degradation of hormones by an ammonia oxidising bacteria, *N. europaea* which found that during E2 degradation, E1 was not generated, which indicated that NAS and *N. europaea* exhibited different degradation pathways for E2. It was also concluded that the generation of E1 from E2 occurred in both NAS and conventional activated sludge, but the E2 degradation via E1 is caused by other heterotrophic bacteria and not by the nitrifying bacterium. Onda *et al.* (2003) also showed similar trends where the pilot scale AS process removed 94.7% of E2, while in the batch test with AS rapid removal of E2 and conversion of E2 to E1 was noted.

The Onda *et al*. (2003) study also recommended that operational parameters for removal be based on E1 concentrations as it had lower percentage removal rates in the AS process of 69.2%.

4.4.3 The toxicity of the E2, EE2 and mixture of E1, E2 and E3 Standards

To our knowledge, there are no reports which exist in the literature which have determined the toxicity of individual hormone standards. There are reports of using *Vibrio fischeri* in determining toxicity and estrogenic activity in wastewater plants (Schiliro *et al.*, 2004), chemicals in industrial effluents (Barbusinski, 2005; Wang *et al.*, 2002), and using bioluminescent bacteria to determine toxicity of other toxic chemicals other than hormones (Girotti *et al.*, 2002).

Persoone *et al.* (2003) performed a battery of microbiotests and established two classification systems with the first being a hazard classification system for natural waters and the second a toxicity classification system for wastes discharged in to the aquatic system. The latter will be discussed as the toxicity effect of the different hormone standards falls in to this category (See section 4.2.3 for the calculation of the classification system).

According to the toxicity classification system and after interpretation of the data, all the hormone standards fell in the Class II category and all exhibited slight acute toxicity. The E2 hormone at 10 ng/L had a TU close to the Class III level (Table 4.2); however when it was in a mixture with E1 and E3, the toxicity was much lower than on its own. It can thus be postulated that the toxic effects of the E1 and E3 are not as great as E2 and thus when in mixtures, the E2 toxicity level is reduced. The synthetic EE2 hormone also showed slight acute toxicity but the % TU was lower as compared to the natural hormones. When further diluted E2 standards at 1 and 5 ng/L were analysed, results revealed that at both these lower concentrations the E2 hormone still had % INH higher than EE2.

Hence if one can relate these findings to the other chemicals found in wastes which are either with or without treatment one can thus establish the toxic effect that these chemicals can have on the aquatic environment.

Study by Dalzell *et al.* (2002) where five rapid direct toxicity assessment methods were compared found that out of the five tests the most sensitive bioassay was the *Vibrio fischeri*. Kaiser (1998) has also concluded that the use of *Vibrio* bacterium can provide a simple, rapid and cost effective means of measuring the acute toxic effect of either individual substances or complex mixtures.

It is however imperative that when assessing a potential toxicity of chemicals in the environment, important parameters that must be taken in to consideration are the bioavailability and the bioaccumulation. Bioavailable compounds are generally free or water extractable, while those that are less available are those that are bound to dissolved organic matter or solids. The bioavailability of a chemical is dependent on exposure routes, for example, organisms/animals living in or ingesting sediments that are contaminated may drastically influence the actual uptake. The two significant factors that determine the degree of bioaccumulation are bioconcentration, which is the uptake via the surrounding phase and biomagnification which is the uptake via food. The bioaccumulation factor is important for risk assessments as the increase in tissue concentration may cause undesirable effects in organisms and also increase the exposure of predators by way of dietary consumption in the food chain (Lai *et al.*, 2002).

4.5 CONCLUSION

The laboratory scale investigations aided in determining the fate of the hormone EDCs under controlled conditions. One was thus able to prove theoretical outcomes without having outside interferences. Operation of the MLE process enabled us to control and change operational parameters in order to establish how certain parameters influenced the removal of hormone EDCs. The MLE tests showed that the sludge retention times definitely have an

effect on the removal of hormones from the influent as well as the overall performance of sewage treatment. The 10 day SRT proved that longer SRTs will definitely aid in the removal of hormones and possibly other EDCs in raw sewage.

The batch test provided an insight into the fate of the E2 hormone. This 24 hour batch test has proven that within an activated sludge process there was definite removal of E2 and that part of the removal was definitely due to conversion to E1. E1 has also proven to be more persistent due to the oxidation of E2 to E1 and also possibly due to the de-conjugation of the glucorinides and sulphates of E1 conjugates. This was shown by the longer time taken for complete removal of E1 to occur.

The determination of toxicity of the different standards was to establish how great the toxic effect was for individual and mixed hormones were and to also try and establish at what concentration an EC_{50} could be obtained. However, for the concentrations that were investigated the EC_{50} could not be established as all the standards had % inhibitions below 50%, but this does not mean that the standards do not have a toxic effect.

Toxicity monitoring can be applied to any type of substance, be it a chemical, waste discharge, soil etc. By first conducting the screening toxicity test one can establish if further analysis needs to be conducted. If the sample shows no toxic effect then no further investigation needs to be conducted. If the sample shows that it has a toxic effect, then a detailed chemical fractionation can be done whereby different compounds are separated according to their chemical groups. Thereafter the EC_{50} and TU can be determined and consequentially the toxicity of sample can be attributed to a specific chemical group. The screening of samples using toxicity can thus reduce the number of samples to be analysed for chemical fractionation, thus saving time and the cost of doing unnecessary tests if the samples has no toxic effect.

CHAPTER 5
INVESTIGATING THE FATE OF ESTROGENS IN FULL SCALE WASTEWATER TREATMENT PLANTS

5.1 INTRODUCTION

The ubiquitous occurrence of EDCs in sewage effluents and aquatic systems has become a major concern worldwide as its lower concentrations may impair the gene expression resulting in deleterious and permanent changes in endocrine systems whereas the higher concentration are lethal to the aquatic animals (Welshons *et al*., 2003). The main compounds of concern and research, have in all likelihood, been the environmental estrogens and related hormonal compounds. The sewage effluents contain wide range of natural and synthetic estrogens as well as numerous synthetic compounds and pharmaceuticals having varying estrogenic effects. (Baronti *et al*., 2000). The major sources of estrogens (E1, E2 and E3) in the sewage are the female steroid sex hormones as well as the synthetic estrogen known as hormone contraceptives (birth control pills), which contain ethinylestradiol (EE2) (Christiansen *et al*., 2002; Desbrow *et al.*, 1998; Routledge *et al*., 1998). The estradiol (E2) and its metabolites such as estrone (E1) and estriol (E3), due to presence of hydroxyl group, these estrogens easily conjugate with sulphate and glucorinides present in the domestic sewage and wastewater (Christiansen *et al*., 2002).

According to Baronti *et al.* (2000) the effluents emanating from the sewage treatment plants primarily with domestic inputs are strongly suspected to be a significant source of natural and synthetic estrogens. The hormones 17β-E2 and E1 are naturally excreted by men and women as well as in female animals (Ying *et al*., 2002). The levels of estrogen expected to be found in rivers are in trace amounts (ng/L), taking into consideration the dilution factor and previous measurements.

Estrogens being released with the final effluents WWTPs have shown to be detrimental to aquatic life and have been documented in various studies (Christiansen *et al*., 2002;Desbrow *et al*., 1998; Folmar *et al.*, 1996;Tabata *et al*., 2001). For example, in a gonad hepatotoxicity

study, sexual differences such as female like ducts were formed in male fish exposed to 80% of sewage effluents containing E1 concentrations between 6.5 to 8.6 ng/L, E2 0.7 – 3.6 ng/L(Liney *et al*., 2006).

South Africa, in global terms, has been categorised as a water stressed country. The National government has found it difficult to control pollution as the management of water and wastewater is a complex business and needs proper institutional arrangements (Muller *et al*., 2012). In South Africa, for all wastewater treatment plants it is imperative to operate at the optimum in order to meet the General Authorisation wastewater disposal standards, stipulated in Government Gazette No. 32820, (2013). Table 5.1 shows the standards applicable to those plants that discharge up to 2000 m^3/day which includes physical, chemical and bacterial parameters.

Many WWTP find it difficult to comply with standards listed in Table 5.1 and thus the ability of these plants to remove EDCs could also be comprised. Even though most of these substances are not being readily degradable, they are removed from the sewage with different efficiencies depending on the layout of the wastewater treatment plant and their adsorption behaviour to activated sludge. The analysis of these hormone EDCs in waste water is a crucial analytical challenge, as firstly, the wastewater matrix can be extremely complex, which normally contains an array of compounds that can cause interferences in the analysis of the target analytes, and secondly, because these compounds have very low limits of detection as they have been reported to affect living organisms endocrine systems at extremely low concentrations.

Table 5.1 Wastewater limit values applicable to discharge of up to 2000 m^3/day wastewater into a water resource (Government Gazette No. 36820, 2013)

Substance/Parameter	General Limit	Special Limit
Faecal Coliforms (per 100 ml)	1 000	0
Chemical Oxygen Demand (mg/l)	75	30
pH	5,5-9,5	5,5-7,5
Ammonia (ionised and un-ionised) as Nitrogen (mg/l)	6	2
Nitrate/Nitrite as Nitrogen (mg/l)	15	1,5
Chlorine as Free Chlorine (mg/l)	0,25	0
Suspended Solids (mg/l)	25	10
Electrical Conductivity (milli Siemens/metre)(mS/m)	70 mS/m above intake to a maximum of 150 mS/m	50 mS/m above background receiving water, to a maximum of 100 mS/m
Ortho-Phosphate as phosphorous (mg/l)	10	1 (median) and 2,5 (maximum)
Fluoride (mg/l)	1	1
Soap, oil or grease (mg/l)	2,5	0
Dissolved Arsenic (mg/l)	0,02	0,01
Dissolved Cadmium(mg/l)	0,005	0,001
Dissolved Chromium (VI) (mg/l)	0,05	0,02
Dissolved Copper (mg/l)	0,01	0,002
Dissolved Cyanide (mg/l)	0,02	0,01
Dissolved Iron (mg/l)	0,3	0,3
Dissolved Lead (mg/l)	0,01	0,006
Dissolved Manganese (mg/l)	0,1	0,1
Mercury and its compounds (mg/l)	0,005	0,001
Dissolved Selenium (mg/l)	0,02	0,02
Dissolved Zinc (mg/l)	0,1	0,04
Boron (mg/l)	1	0,5

In this chapter, WWTPs with different types of configurations were studied and the hormone EDCs analysed to determine removal capacity and the toxicity determined using the Biotox method.

5.2 MATERIALS AND METHOD

5.2.1. Selection of wastewater treatment plants

The WWTPs were selected to provide a range of the different configurations, from the most basic to the more advanced. WWTPs A and B were from the Kwa-Zulu Natal region and these plants were analysed using both the ELISA and RIA techniques. At later stage in the project, Plants C, D and E from the Western Cape region were included and were analysed only using the ELISA technique and toxicity.

5.2.1.1 Wastewater treatment plant A

Plant A had a design capacity of 1 Ml/day. Plant configuration consisted of a coarse grid to remove rags and plastics and 4 oxidation ponds in series having only a domestic influent (Fig. 5.1).

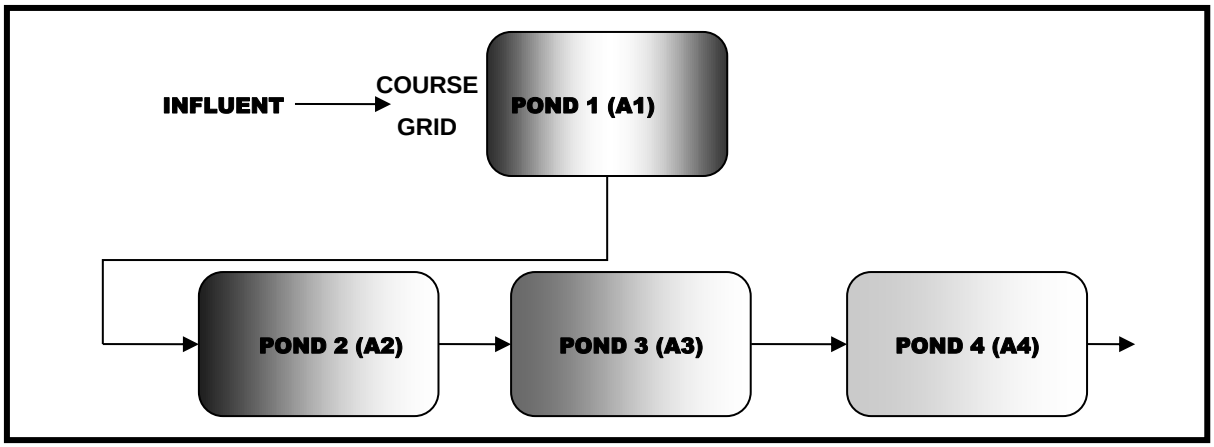


Figure 5.1 Schematic Diagram of WWTP A with sampling points (A1 - A4)

5.2.1.2 Wastewater treatment plant B

This plant has an influent consisting of approximately 5% industrial effluent and 95% domestic influent. The plant configuration has a split treatment facility viz, simple activated sludge (AS) process on the west side and a biological nutrient removal process (BNR) on the east side (Fig. 5.2). There is also a course grid before the influent splits. This plant has a design capacity of 7.2Ml/d.

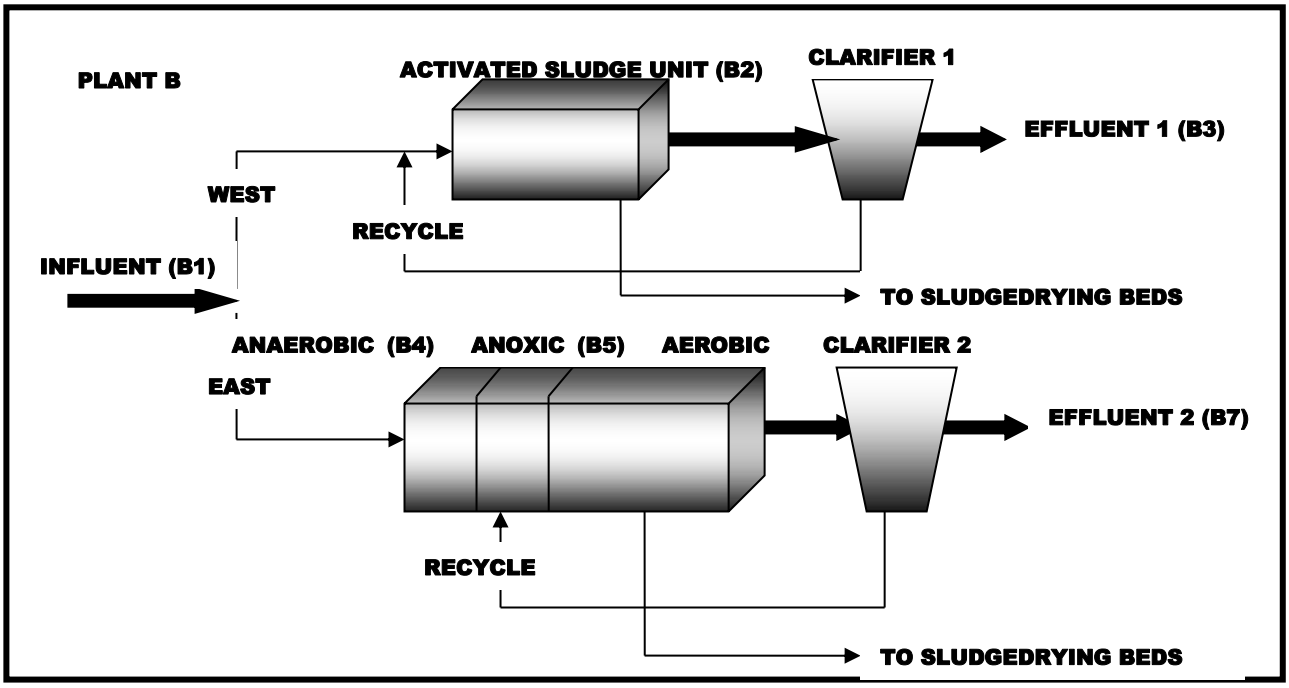


Figure 5.2 Schematic diagram of WWTP B with sampling points. B1: Influent after screens and grits, B2: Activated Sludge Unit, B3: Effluent 1, B4: Anaerobic, B5: Anoxic, B6: Aerobic, B7: Effluent 2

5.2.1.3 Wastewater treatment plant C

Plant C initially consisted of primary sedimentation tanks, biological trickling filters and humus tanks and four secondary sludge dams. Thereafter a new plant was built on the east side where two of the four secondary dams were converted into a biological nutrient removal plant. The one dam was

converted in to the anaerobic and anoxic zones and the second dam was converted into the aerobic tank. Two new clarifiers were also built. Plant C was also upgraded as there were many other parameters that were not meeting the standards as set out in Table 5.1This plant receives f a mixed influent of 80% domestic and 20% industrial stream with a design capacity of 11ML/day. When initial sampling began the plant configuration consisted of only the west side (Fig. 5.3). Thereafter a BNR plant was built on the east side, having a design capacity of 7.3ML/d. The west side configuration was mothballed and therefore samples could only be taken from the east side. The influent passed through a course grid and a drum screen before going to the respective plants.

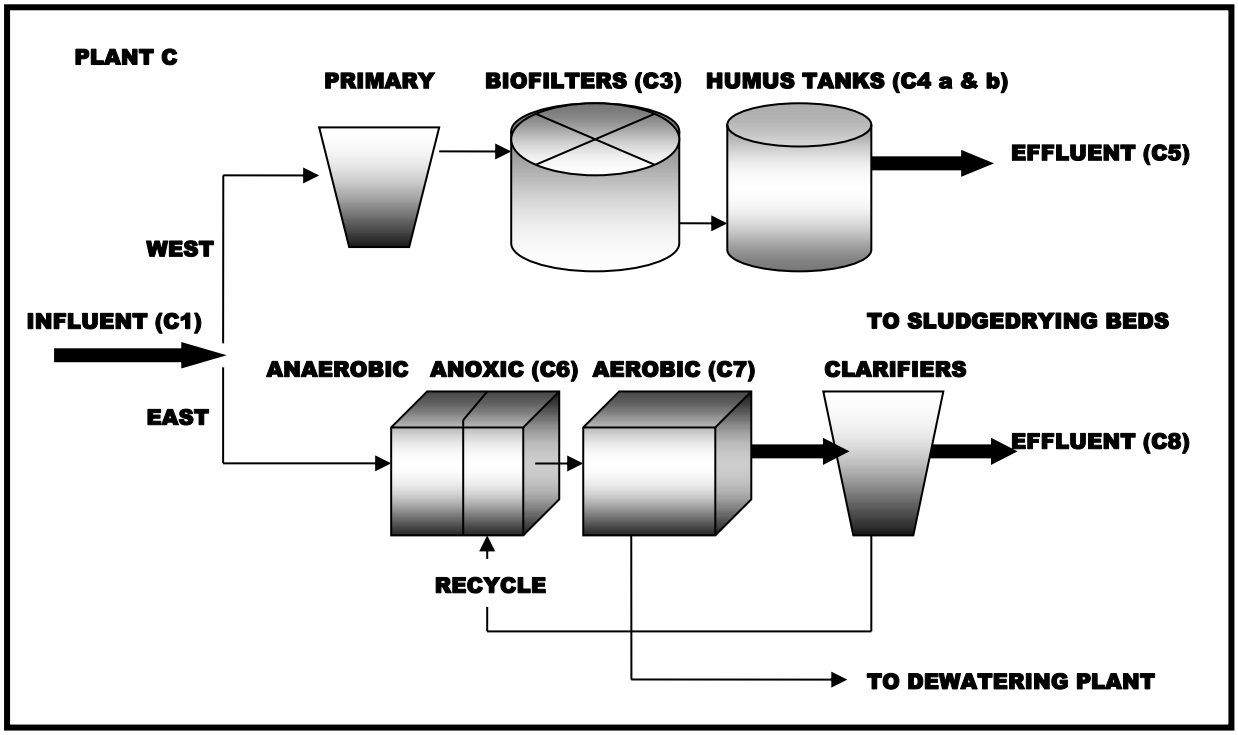


Figure 5.3 Schematic diagram of WWTP C and sampling points. C1:Influent after screens and grits, C2: Primary Sedimentation Tanks (PST), C3: Biofilters, C4a: Humus tank 1 and C4b: Humus tank 2, C5: Effluent from the West, C6: Anoxic, C7: Aerobic, C8: Effluent.

5.2.1.4 Wastewater treatment plant D

Plant D consisted of a simple activated sludge process with extended aeration and an ultra-filtration (UF) plant which supplies water to the areas water catchment dam. . This plant had a mixed influent of 60% domestic and 40% industrial water. This plant has a design capacity of 15 ML/day (Fig. 5.4). The influent also passed through a course grid, drum screens and also has sand trap before going through the AS process. This plant also has an Ultrafiltration (UF) plant which receives its feed from the clarifier effluent. The UF plant was built as an emergency project due to the severe droughts experienced in this area. The UF plant consists of 3 skids each holding 100 membranes of pore size of 0.1μm

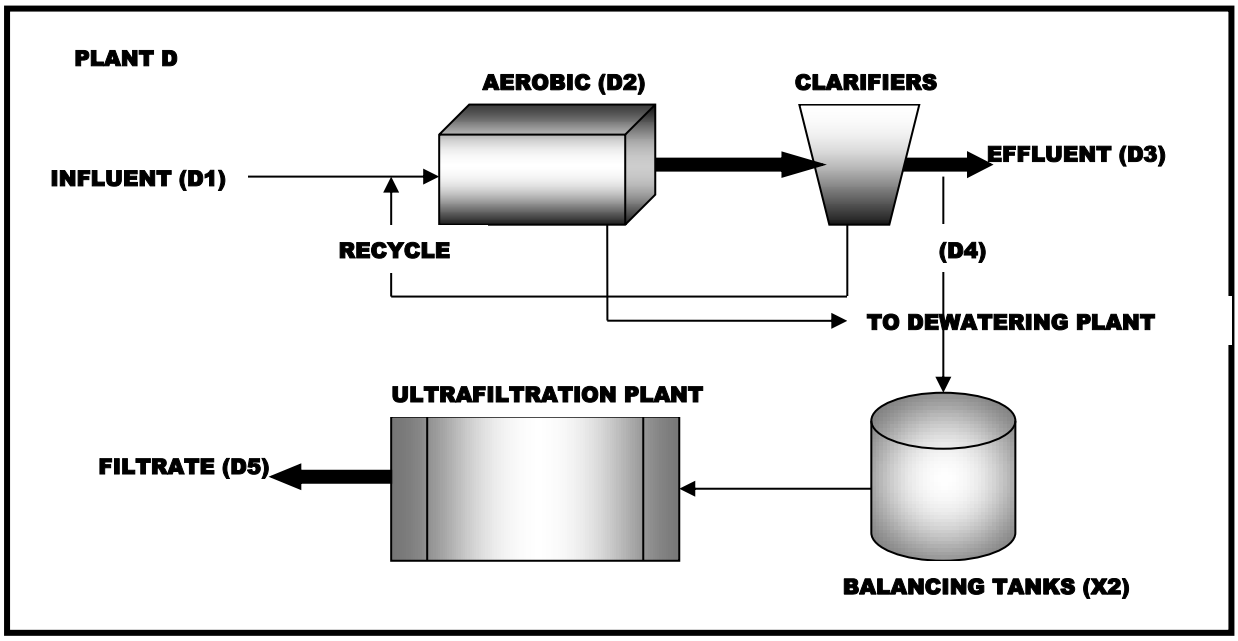


Figure 5.4 Schematic diagram of WWTP D and sampling points.D1:Influent after screens and grits, D2: Aerobic, D3: Effluent (after chlorination), D4: Clarifier effluent, D5: Filtrate

5.2.1.5 Wastewater treatment plant E

Plant E consisted of a simple activated sludge plant and receives only a domestic influent feed. The design capacity is 1.2ML/day (Fig. 5.5). The influent first also passes through a coarse grid.

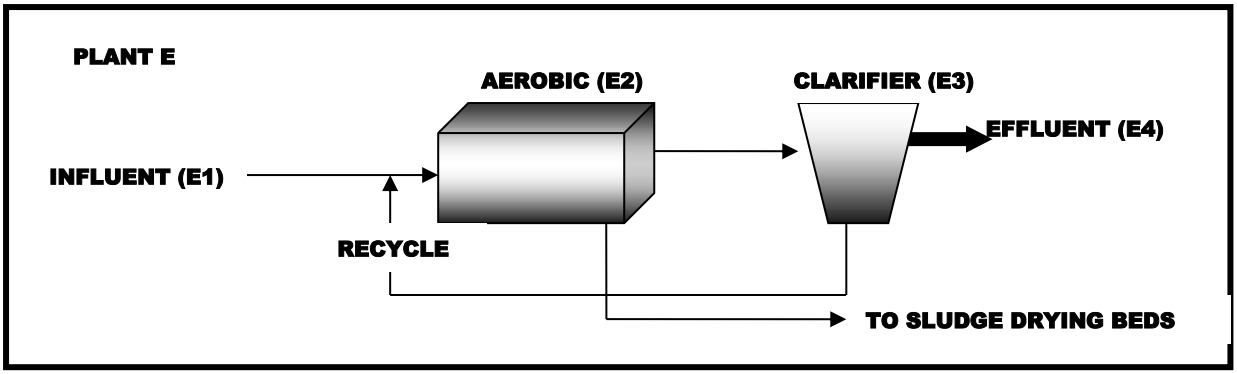


Figure 5.5 Schematic diagram of WWTP E and sampling points. E1: Influent after screens and grits, E2: Aerobic, E3: Clarifier effluent, E4: Effluent after chlorination

5.2.2 Sample Collection and Preparation of Wastewater Samples for ELISA and RIA

For the wastewater treatment plant, the retention times of the influent were determined in each treatment process. Twenty four hour composite 1L samples of the influent and 1L grab samples at other points were collected and stored in clean and dry amber colour glass bottles. All glassware was cleaned according to the method described in appendix 2.

The samples were prepared in the following manner:

1. Samples with sludge were centrifuged at 3000rpm for 6 minutes and the supernatant was filtered through a glass fibre filter paper with 0.3 – 0.6μm pore size.
2. The influent and effluent samples were only filtered through pre-treated glass fibre filters, pore size 0.3 – 0.6μm.
3. The filtrate was kept in amber glass bottles and preserved with 1 % formaldehyde (appendix 12) if it was not used immediately. Samples were stored in the fridge at approximately 4°C.

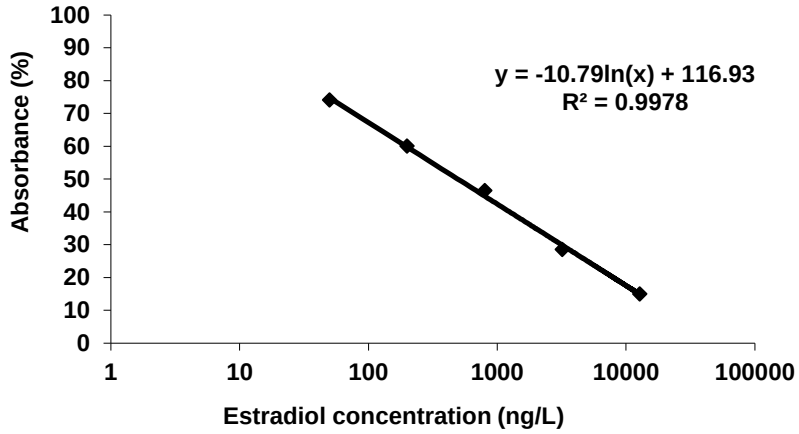
Samples were then subjected to SPE and applied to the respective immunoassays. The SPE and immunoassays procedures are described in the previous chapters.

5.2.3 Toxicity of Wastewater Samples from Plants C, D and E

For the toxicity determination of wastewater samples from the influent, clarifier effluent and final effluent after chlorine disinfection were collected in 100mL amber glass borosilicate bottles. The bottles were cleaned according to the procedure described in appendix 2.
The samples were analysed immediately after being brought to the laboratory and there was no filtering of samples or pH adjustments. Samples were analysed without any prior treatment and adjustments to determine the toxicity. The Biotox, *Vibrio fischeri* procedure was followed as described in Chapter 4 and appendix 13. Other parameters determined on the influent and effluent samples were the COD, Ammonia as Nitrogen (appendix 14) and pH (appendix 15). The free and total Chlorine (appendix 16 and 17 respectively) were also determined in final effluent samples.

5.3 RESULTS

The standard curves used for all the WWTPs for the determination of the E2 and EE2 ELISA is shown in figure 5.6 and the standard curve used for all the WWTPs for the determination of E1 and E3 RIA are shown in figure 5.7. The correlation coefficients (R^2) for all the standard curves were ranged from 0.9712 to 0.9999.



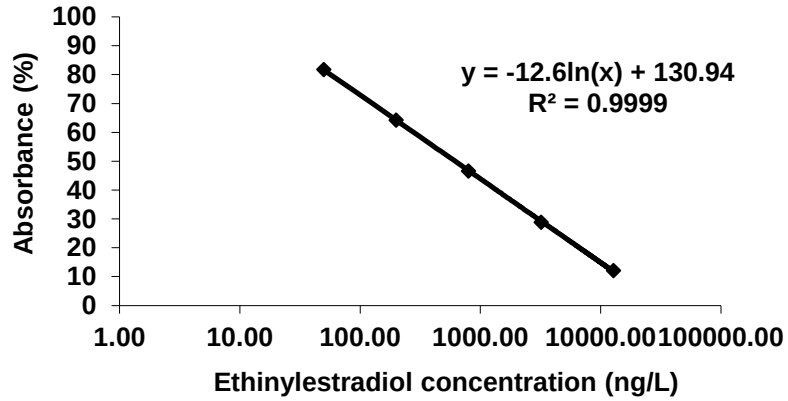
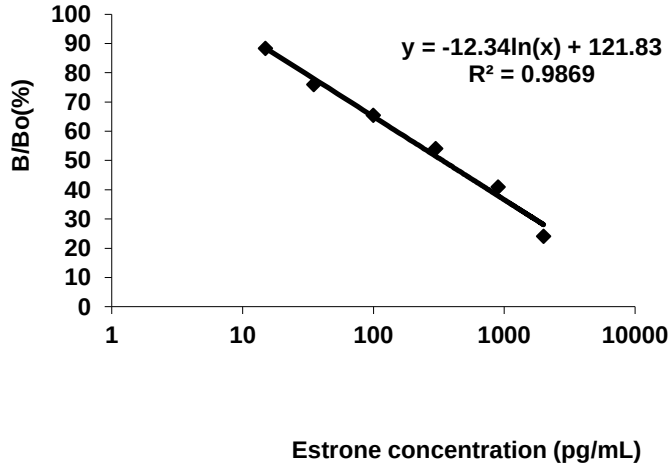


Figure 5.6 Standard curves obtained for E2 and EE2 ELISA



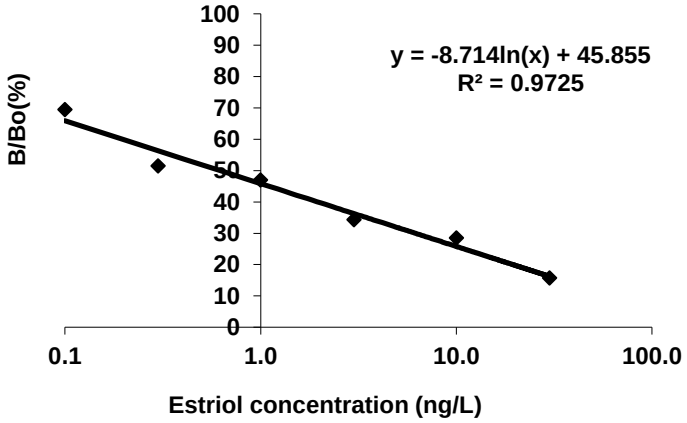


Figure 5.7 Standard curves obtained for E1 and E3 RIA

5.3.1 Fate of Estrogens in WWTPs A and B

Plants A and B are WWTP situated in the Kwa-Zulu Natal area. Plant A consists of four oxidation ponds and Plant B has one influent stream which splits and goes to the west and east sides of the plant (refer to Table 5.2 for plant design and operating parameters).

When analysing the removal capacity of the different plant configurations, it was found that the concentrations of E1, E2 and EE2 were reduced by each of the different processes in plants A and B; however E3 concentrations were not reduced at all by plant A (Table 5.3 and 5.4). In fact an increase 0.006ng/mL of E3 was noted in the effluent as compared to the influent in Plant A. Plant A had an average removal efficiency of 70.10% E2, 55.05% E1 and 0% E3. The average removal of estrogens in Plant B were 73.95% E2, 56.18% E1 and 76.92% E3 in the simple activated sludge process while the BNR process in Plant B removed an average of 84.65% E2, 80.40% E1 and 86.66% E3. The EE2 concentrations for both plants were only detected in the influents samples and in the anaerobic zone of the BNR process. There was no detection (ND) of estrogens in the anoxic, aerobic and final effluents in Plant B. Thus a value of 100% removal was assigned to those samples.

Table 5.2 Design and Operating Parameters of WWTPs A and B

	Plant Design Capacity (ML/d)	Actual Operating Capacity (ML/d)	COD Influent (mg/L)	COD Effluent (mg/L)	Sludge Retention Time (SRT) (Days)
Plant A	1.0	0.3	500	<75	-
Plant B: East side **Plant B: West side**	7.2 total	4.69 total	800	<75	15

The influent hormone concentrations for Plants A and B, ranged from 43 –99ng/LE2, and 13 –16ng/L EE2 (Table 5.3) and 47 – 104 pg/mL E1 and11 – 52 ng/mL E3(Table 5.4) and the effluent hormone concentrations ranged from 6 – 30ng/L E2 (Table 5.3), 9 – 47pg/mL E1, 0.007 – 0.017ng/mL E3(Table 5.4) and EE2 was not detected in any of the effluent samples. In conjunction with determining the hormone concentrations in the wastewater samples, 2 controls for each hormone were run simultaneously as shown in Table 5.4 for the RIAs. The % coefficients of variations were all below 10. The standard deviation was calculated using the theoretical concentrations for each control and the actual concentration after analysis.

The effluent E1 levels were also higher than the E2 levels for all systems; however the E1concentrations did not increase more than the influent E1 concentrations. This displayed similar trends to the 24hour batch test.

Table 5.3 Average E2 and EE2 ELISA concentrations (±SD) in WWTPs A and B

		E2(ng/L)	EE2 (ng/L)
Plant A	**Pond 1**	98.73 ± 2.39	12.75 ± 0.38
	Pond 2	65.11 ± 5.73	0.00
	Pond 3	42.53 ± 1.67	0.00
	Pond 4	29.55 ± 6.34	0.00
	% Removal	**70.10**	**100.00**
Plant B	**Influent**	42.96 ± 1.7	15.41 ± 0.45
(West)	**Aeration**	28.55 ± 3.35	0.00
	Clarifier 1	10.33 ± 0.51	0.00
	Effluent 1	11.19 ± 0.55	0.00
	% Removal	**73.95**	**100.00**
Plant B	**Influent**	42.96 ± 1.70	15.41 ± 0.45
(East)	**Anaerobic**	44.71 ± 0.87	13.99 ± 2.05
	Anoxic	22.88 ± 0.89	0.00
	Aerobic	16.22 ± 1.74	0.00
	Clarifier 2	9.21± 0.35	0.00
	Effluent 2	6.60 ± 0.72	0.00
	% Removal	**84.65**	**100.00**

Table 5.4 Average E1 and E3 RIA Concentrations (± SD) in WWTPs A and B

		E1(pg/mL)	E3(ng/mL)
Plant A	**Pond 1**	103.56 ± 4.06	0.011 ± 0.0
	Pond 2	72.42 ± 9.92	0.008 ± 0.0
	Pond 3	89.23 ± 8.74	0.007 ± 0.001
	Pond 4	46.55 ± 2.73	0.017 ± 0.001
	% Removal	**55.05**	**0**
Plant B West Side	**Influent**	47.47 ± 1.86	0.052 ± 0.001
	Aerobic	20.91 ± 1.23	0.007 ± 0.001
	Effluent 1	20.80 ± 0.22	0.012 ± 0.001
	% Removal	**56.18**	**76.43**
Plant B East Side	**Influent**	47.47 ± 1.86	0.052 ± 0.001
	Anaerobic	30.29 ± 2.38	0.009 ± 0.001
	Anoxic	76.34 ± 2.24	0.014 ± 0.001
	Aerobic	26.98 ± 0.26	0.008 ± 0.001
	Effluent 2	9.30 ± 0.36	0.007 ± 0.00
	% Removal	**80.40**	**86.66**
Controls	**I**	46.52 ± 6.29	0.51 ± 0.02
	II	280.06 ± 7.19	2.63 ± 0.21

5.3.2 Fate of Estrogens in WWTPs C, D & E

Plants C, D, E are situated in the Western Cape area having different types of configurations. Table 5.5 shows each plants design capacity and process parameters. Both plants C and D receive a mixed influent of domestic and industrial feeds.

Table 5.5 Design parameters of WWTPs C, D and E.

	Plant Design Capacity (ML/d)	Actual Operating Capacity (ML/d)	COD Influent (mg/L)	COD Effluent (mg/L)	Sludge Retention Times (SRT) (Days)
Plant C West Side	11	11	650	72	-
Plant C East Side	7.3	6	950	<75	20 - 25
Plant D: Activated Sludge Process with Extended Aeration	15	12	1000	<75	20
Plant D: Ultrafiltration	10	9	-	-	-
Plant E	1.5	1.2	800	<75	10

Table 5.6 shows the average E2, E1 and EE2 concentrations for these 3 plants. When analysing the removal of hormones on the west side of plant C; results revealed there was only a 24.084% reduction of E2 and 13.17 % reduction of E1. This plants capability of removing E1 and E2 was very inefficient and in fact released major portions of these hormones into the aquatic ecosystem. Unfortunately EE2 was not analysed for at the time. However when analysing the removal of the BNR process (East), the results showed a 90.57% reduction for E2 and 88.89% for E1 was achieved. The levels of EE2 were very low at 3ng/L and 2 ng/L for the influent and effluent; respectively.

Table 5.6 Average E2, E1 and EE2 ELISA concentrations (± SD) in WWTPs C, D and E

		E2 (ng/L)	E1 (ng/L)	EE2 (ng/L)
Plant C	**Influent**	62.27 ± 0.48	63 ± 1.6	-
West Side	**PST 1**	60 ± 0.01	-	-
	Biofilter2	81 ± 0.01	-	-
	Humus 1	36 ± 0.01	-	-
	Humus 2	49 ± 0.02	-	-
	Effluent	47.28 ± 0.21	54.7 ± 1.8	-
	% Removal	**24.08**	**13.17**	
East Side	**Influent**	53 ± 1.0	81 ± 3.0	3.00 ± 1.0
	Anoxic	9 ± 1.0	9 ± 1.0	0
	Aerobic	6 ± 1.0	12 ± 0.2	3.00 ± 1.0
	Effluent	5 ± 0.0	9 ± 1.0	2.00 ± 0.02
	% Removal	**90.57**	**88.89**	**33.33**
Plant D	**Influent**	55.21 ± 0.04	77.55 ± 1.2	4 ± 0.0
	Aerobic with extended aeration	22.00 ± 0.50	9 ± 0.5	2 ± 0.0
	Effluent	2.00 ± 0.25	18.4 ± 1.6	0
	% Removal	**96.38**	**76.27**	**100**
UF Plant	**Clarifier Effluent**	8.10 ± 0.10	13.9 ± 0.5	10 ± 5.0
	Combined Filtrate	6.40 ± 1.10	14 ± 0.55	13 ± 1.0
	% Removal	**20.99**	**0**	**0**
Plant E	**Influent**	56.57 ± 0.44	74.3 ± 2.65	7 ± 1.0
	Aerobic	11.47 ± 0.0	10 ± 0.1	0
	Effluent	2.20 ± 0.17	17 ± 0.9	0
	% Removal	**96.11**	**77.12**	**100**

When analysing plant D's removal efficiency, the simple activated sludge process had a reduction of 96.38% E2 and 76.27% E1 before ultrafiltration treatment. Comparison of the removal efficiency of the UF plant was calculated using the clarifier effluent which was the feed to the UF plant and the combined filtrate concentrations. After the UF treatment the E2 concentration in the clarifier effluent was further reduced from 8.1 ng/L to 6.4 ng/L. and no further removal of E1 and EE2 were noted. In fact there was also a slight increase of 3 ng/L in EE2 concentration after the UF process when compared to the clarifier effluent. Figure 5.8 shows the different types of membrane filtrations with the substances that can be retained or pass through. From the results of the samples after the ultrafiltration process, estrogens are still able to pass through and are not retained by this type of membrane.

Plant E showed a reduction in all the hormone concentrations with % removals of 96.11, 77.12 and 100 % for E2, E1 and EE2 respectively.

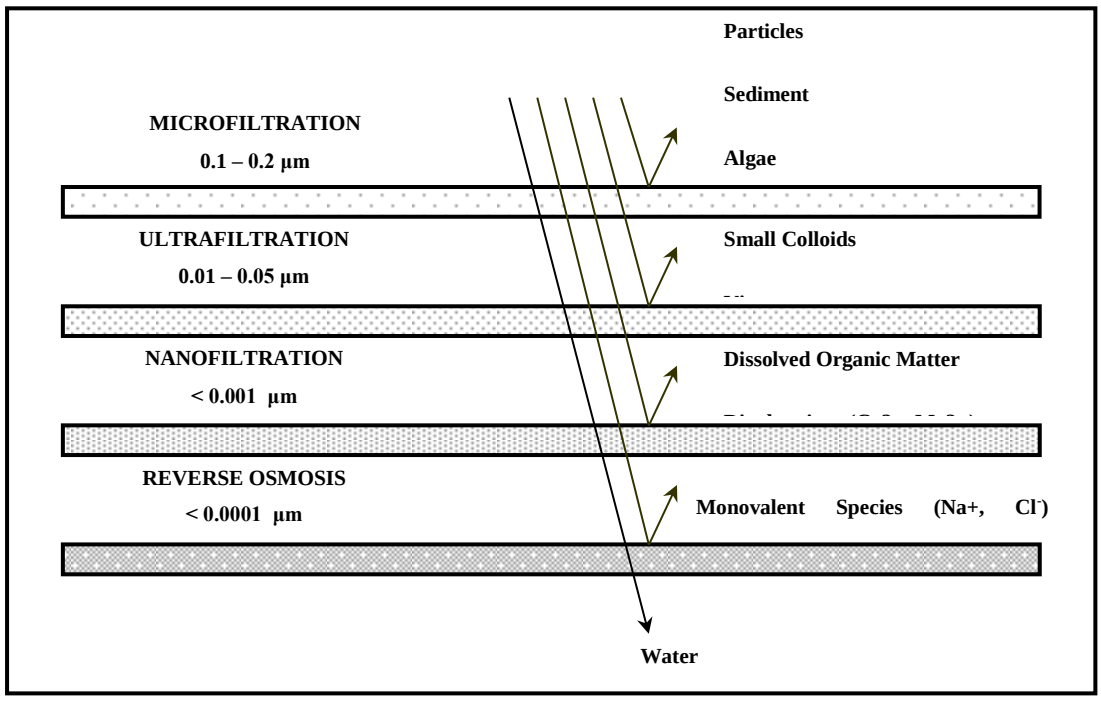


Figure 5.8 Hierarchy showing the different types of pressure-driven membrane filtrations (adapted from Pirnie, 2005)

5.3.3 Overall Plant Performance for Hormone Removal

In this study it was found that a mixed wastewater influent stream of domestic and industrial origin also has an effect on the removal of the estrogens. This was shown when Plant E had the best overall percentage removal and this plant receives only domestic influent. When comparing plants with a mixed influent of domestic and industrial water, Plant D (activated sludge with extended aeration) before the UF proved to have the highest removal capabilities. Table 5.7 shows the ranking of the plants selected from highest to lowest estrogen removal. The ranking of plants was based on results of samples taken at the time and was not indicative of comparative removal efficiency, as process configuration, operating conditions, influent quality at the time can also influence outcome of results and change rank positions. The WWTP ranged from the most primary consisting of just oxidation ponds to the BNR process. Removal rates ranged from 29% to 96% for E2, 0% to 89% for E1 and 0% to 100% for EE2.

Table 5.7 Ranking of WWTPs in order of highest to lowest estrogen removal

Rank	Plant	Configuration	Type of Influent	Overall % Removal
1	E	Activated Sludge	Only domestic	91.07
2	D (before UF)	Activated Sludge with Extended Aeration	Mixed Domestic and Industrial	90.88
3	B (east side)	BNR	Domestic with 5 % Industrial	87.93
4	B (west side	Activated sludge with extended aeration	Domestic with 5 % Industrial	76.64
5	C (east side)	BNR	Mixed Domestic and Industrial	70.93
6	D (After UF)	Ultra filtration	Mixed Domestic and Industrial	56.80
7	A	Oxidation Ponds	Only Domestic	56.29
8	C (west side)	Biological Trickling filters	Mixed Domestic and Industrial	12.04

5.3.4 Acute Toxicity in WWTPs C, D and E

The toxicity of influent, clarifier effluent and final effluents after chlorination disinfection was measured for the plants in the Western Cape area. Table 5.8 shows the toxicity as well as the conventional parameters for plants C, D and E. The influents of all 3 plants showed high acute toxicity values and was classified as a Class IV waste. The clarifier effluent of all 3 plants showed no acute toxicity and was classified as Class I. However the final effluent of Plant C and E also showed high acute toxicity and was classified as a Class IV waste. The high toxicity was attributed to the chlorine disinfectant together with other chemicals. However if one had to assess the performance of the wastewater works to remove toxicity then the toxicity of the clarifier effluent must be used and not the final effluent after chlorine disinfection. The results have shown that those plants with biological treatment can reduce the toxicity of the influent coming in to the plant. The reason that the toxicity was analysed on samples with and without chlorine was to establish if the plants are capable of reducing the toxicity or not and establish that the high final effluent toxicity was due to the chlorine disinfectant and not the actual effluent itself. If one had to only take the influent and final effluent and determine the toxicity the results will have reflected as though the plants were not capable of reducing the toxicity. The percentage toxicity reduction of plants C, D and E calculated from the influent and clarifier effluent were 100, 80 and 97%, respectively (Table 5.8). The COD and ammonia levels (Table 5.8) also indicated the operational status of the plant and was used to determine if the toxicity was due to poor plant performance or not. The COD and ammonia were well within the standards indicated in Table 5.1, however the free chlorine limit indicated in Table 5.1 is 0.25 mg/L and only Plant D was within this limit having a free chlorine concentration of 0.13 mg/L (Table 5.8). Plant C and E had free chlorine of 0.57 and 0.59 mg/L (Table 5.8) respectively which was more than twice the applicable discharge standard. Therefore the major component of the final effluent toxicity can be attributed by the chlorine disinfectant and not from the operational performance of the plants.

Table 5.8 Toxicity and conventional parameters of samples in WWTPs C, D and E

	SAMPLE	% INH (30 min) ± SD	%CV	TU	CLASS	COD (mg/L)	AMMONIA (mg/L)	PH	FREE /TOTAL CHLORINE (mg/L)
PLANT C (BNR PROCESS)	Influent	75.2 ± 0.07	0.1	10 < TU < 100	IV	360	26	6.94	-
	Clarifier Effluent	-56.1 ± 2.53	3.5	<0.4	I	22	0.01	6.8	-
	% Toxicity Reduction	100 %							
	Effluent with Chlorine	99.9 ± 0.01	0.02	10 < TU < 100	IV	53	4	6.96	0.57/0.61
PLANT D	Influent	69.7 ± 1.61	2.2	10 < TU < 100	IV	285	33	7.57	-
	Clarifier Effluent	14.1 ± 0.99	1.4	<0.4	I	72	0.2	7.18	-
	% Toxicity Reduction	80 %							
	Effluent with Chlorine	15.7 ± 6.93	9.6	<0.4	I	66	0.3	7.25	0.13/0.20
PLANT E	Influent	89.7 ± 2.03	2.8	10 < TU < 100	IV	580	63	6.89	-
	Clarifier Effluent	3.0 ± 10.83	15.0	<0.4	I	23	2	6.72	-
	% Toxicity Reduction	97 %							
	Effluent with Chlorine	99.9 ± 0.00	0.0	10 < TU < 100	IV	27	2	6.75	0.59/1.53

INH: Inhibition, SD: Standard Deviation, CV: Coefficient of Variation, TU: Toxicity Units, COD: Chemical Oxygen Demand

5.4 DISCUSSION

Upon collection of the samples for the immunoassay analysis, the sludge samples were centrifuged and the other samples filtered. The reason for centrifuging/filtering was that wastewaters usually have a high loading of organic and inorganic material as well as suspended particles, therefore filtration becomes essential when subsequent extraction of the sample is through SPE. The suspended solids can cause blockages on the adsorbent bed when the analysis is performed by immunochemical assay, and this can cause unwanted adsorption on to the antibodies. Most studies used glass fibre filters with a pore size between 0.22 and 1.2μm, (Lopez de Alda and Barcelo, 2001). It is also suggested that estrogens are mostly in the colloidal or dissolved fraction as Braga *et al.* (2005) also found that there was little difference between pre-filtered and filtered samples. A 1% formaldehyde solution was used to preserve samples. According to a study by Lopez de Alda and Barcelo (2001) when water was stored in bottles that were preserved by 1% formaldehyde, no huge losses were encountered, but found significant losses when no sample preservation took place. In this study all samples for the immunoassays were processed within 2 to 3 days with preservation and samples for toxicity was analysed immediately.

The reason for using ELISAs for E2 and EE2 and RIAs for E1 and E3 was the availability of the test kits at the time. The E1 and E3 were not available in the ELISA kits and E2 and EE2 were not available for the RIAs, hence two methods had to be employed to get the concentrations for the required hormone EDCs.

5.4.1 Fate of Estrogens in WWTPs

Can et al. (2014) found in their comparative study of 3 wastewater treatment plants with different configurations, that the percentage removal of steroid hormones was higher in the plant with the advanced treatment than the plant with only the primary treatment. Findings of our study also suggest that the configurations of WWTPs definitely played an important role as far as removal of estrogen EDCs was concerned. When comparing the hormone removal capacity of final effluents of WWTPs A and B; Plant B east side (BNR process) had the best removal capacity for all 3 estrogens having an overall removal of 84.3%, while Plant B west

side (simple activated sludge process) had a 69.3% removal and Plant As, oxidations ponds had the poorest removal capabilities of 42.8% (Table 5.7). The biological trickling filters of plant C; had a 24.08% reduction of E2 and no reduction of E1. Svenson *et al.* (2003) found in the study of estrogenic removal in Swedish municipal WWTPs, the activated sludge treatment was more effective than trickling filters in removing estrogenicity and where chemical precipitation was used without biological treatment had little effect in the removal. The Marti and Batista, (2014) study also found that only a 24 % removal of estrogens occurred in the aqueous phase of the trickling filter and more than 90 % estrogen removal in the aqueous phase of the BNR and the non-BNR plants. The Marti and Batista, (2014) study also used ELISAs for quantification of estrogens.

It was also noted throughout all processes in laboratory scale and at the WWTPs investigation; the removal of E1 was not that efficient and was more persistent. When estrogens are released from the body in urine they are released in a conjugated form, bound either as glucorinides of sulphates. These conjugates are biologically inactive until cleaved somewhere between houses and the WWTP. Once cleaved these hormones become active. It is these hormones that can contribute to the increase of estrogens. Estrone concentrations become elevated due to the unconjugated forms of E1 as well as the further oxidation of E2 to E1(Belfroid *et al.*,1999;D'Ascenzo *et al.*, 2003; Desbrow *et al.*, 1998; Ternes *et al.*, 1999).

According to Johnson *et al*. (2005) the poorest removal capabilities were also found in the final effluent of the WWTP that only used primary treatment and had values of 13 ng/L E2 and 35ng/L of E1. In plant A the E3 hormone levels in final effluent was higher than the influent The latter could be due to plant A only being a primary treatment facility. As indicated in figure 1.1, the increase of E3 in the final pond could be attributed to the breakdown of E2 and other metabolites in the system. It is very difficult to compare E3 concentrations with other findings as Ying *et al.*, (2002), also found that E3 was rarely reported as it was not detected in most influents studied, as out of the 8 WWTP investigated E3 was only found in one WWTP at concentrations of 0.43 – 18 ng/L.

The current operational WWTWs in the Western Cape area had overall average removal efficiencies of 94.35% for E2 and 80.76 % for E1 (Table 5.7). Nakada *et al.* (2006) showed

similar figures in studies from WWTWs that use primary and secondary treatment with activated sludge in Tokyo where the overall average removal of E2 was 90% and E1 was 86%.

When analysing the fate of EE2 in the different wastewater plants, EE2 was not found in the effluents of plants receiving purely domestic wastewater. However in the plants that had a mixture of domestic and industrial feeds, EE2 concentrations were still prevalent. Whether the mixed influent does have an impact on the EE2 removal has not been determined. Christiansen *et al.* (2002) have also found that biodegradation rates of estrogens with activated sludge showed a higher rate of removal in sludges from domestic sewage treatment works than in sludge from industrial sewage works. Ternes *et al.* (1999) also found that EE2 was persistent under aerobic conditions in an activated sludge treatment process. In this study the levels of EE2 in samples analysed was extremely low when compared to other studies (Table 5.3 and 5.6). Wang *et al.* (2010) found that EE2 levels in influents ranged from 133.1 –403.2 ng/L and effluents ranged from 35.3 – 269.1ng/L and also found EE2 was persistent along with Bisphenol A in the effluents of the WWTP studied.

The study by Braga *et al.* (2005) also compared two types of treatment plants. The first was an advanced sewage treatment plant (STP) consisting of activated sludge process having anoxic and aerobic basins with tertiary treatment having microfiltration, reverse osmosis and chlorination/de-chlorination. The second plant was an enhanced primary STP with $FeCl_3$ addition. The removal of E1 and E2 from the advanced STP secondary effluent was 85% and 96% respectively; slightly further reduction was noted after microfiltration and was not detected after reverse osmosis and later chlorination. Therefore a WWTP especially with advanced treatment technology can decrease the levels of EDCs in effluents. This was also seen in studies by Jin *et al.* (2010) where a combination of nanofiltration and reverse osmosis with a chemical treatment greatly enhanced the removal of the E1 hormone from the WWTP effluent.A study by Weber *et al.* (2004) which investigated the use of nanofiltration at different pressures for elimination of steroids showed that at a pressure of 10 bars there was more than 99% retention for E2 and E1 and 82% retention for EE2. At higher pressures the retention levels decreased. Unfortunately the UF operating parameters were not changed to determine the removal of estrogens at different pressures

When assessing overall plant performance depicted in Table 5.7, the plants with some form of aeration with sludge had removal efficiencies above 80%. Servos *et al.* (2005) in their study of hormone removal in 18 WWTPs found that more than 75% and up 98% E2 removal was achieved in systems with secondary treatment and if was noted that those plants with high SRTs were also effective in reducing hormone levels. Such findings were also noted by Carballa *et al.*, (2004) and Stanford and Weinberg, (2010), where the aerobic treatment showed excellent removal of steroid estrogens.

Plant C which incorporated a BNR process had the best overall plant performance and removed majority of E1 and E2 (Table 5.7) and also had the longest SRT of between 20 – 25 days. Plant D which was second best also had an SRT of 20 days which was close to Plant Cs BNR process. Plant B BNR process had an SRT of 15 days, unfortunately the SRT for Plant A was not provided and Plant E had an SRT of 10 days. This could have been one of the factors that played a role in the higher removal of estrogens in the WWTPs with retention times greater than 10 days. Kreuzinger *et al*. (2004) compared the removal efficiency of activated sludge plants related to the SRTs. This Kreuzinger *et al*. (2004) study looked at plants who's SRTs ranged from 0.3 – 275 days. The plants ranged from conventional AS, SBR and membrane technology. It was found that those factors that play a role in efficient removal of micro pollutants is the sludge age and hydraulic retention times (SRT, HRT), the food to micro-organism ratio, as well as the dilution factor which also needs to be taken into consideration. Kreuzinger *et al*. (2004) study also found that high loaded AS plants with an SRT of 1 day showed no removal of estrogens and concluded that with increasing SRT, the biological degradation of micro pollutants also increases. Johnson *et al*. (2005) also found that an increased HRT and SRT increased the amount of E1 removal. Removal or disappearance of the hormones during the WWTP process does not necessarily mean that the compound of interest is degraded; it could also be adsorped to sludge. Research by Ivashechkin *et al.*, (2004), showed that Bisphenol A along with the estrogens possessed a high affinity to anaerobically digested sludge and can therefore be discharged with the sludge into the environment. However if the sludge is conditioned then desorption can occur. Further studies need to take the excess sludge into consideration to determine if adsorption to sludge occurs and if so what the concentrations of these EDCs in the sludge are.

There were many factors that played a role for the difference in removal capacities. A major contributing factor for the higher removal efficiency occurred in those plants which had some form of aeration with activated sludge. Studies carried out with activated sludge showed that the mechanism for removal of most estrogens from the aqueous phase is aerobic biodegradation (Baronti *et al.*, 2000; Onda *et al.*, 2003;Ternes *et al.*, 1999). Other studies indicate the main mechanism of removal is sorption to particles and not biotransformation (Huang and Sedlak, 2001 as cited in Kuster *et al.*, 2004). Those plants which didn't have any form of aeration had extremely poor removal efficiencies like the biological trickling filter process in plant C. The plant which had the Ultrafiltration (UF) after the conventional sludge showed no further decreases in E2, E1 and EE2 concentrations. The same effect was seen in the study by Clara *et al.*, (2004), which found that the UF membranes in the membrane bioreactor (MBR) plant also did not lead to further retention of the observed compounds. Figure 5.8 which showed the different types of membranes, indicated that the UF membranes pore size was still too large for the hormones to pass through, perhaps the flocculation process prior to the UF process needed to be further optimised to get larger flocs in order to retain the hormones on the membrane. The idea behind many of the UF plants constructed in SA was to recycle wastewater to enhance the water catchments' areas in times of severe drought. In some areas where the recycled water was used directly for drinking water purposes reverse osmosis (RO) membranes were used. Unfortunately no samples were available at the time to determine the removal efficiency of the RO membranes. However results from an advanced water recycling demonstration plant in Australia proved that from ozonation, microfiltration, nanofiltration and RO; treatment by RO was the most successful in removing pharmaceutically active residuals and hormones from sewage effluent (Khan *et al.*, 2004). Studies which researched removal of E1 from treated sewage effluent using nanofiltration and RO found that E1 removal was enhanced by the presence of hydrophobic acid (HpoA) in the feed solution and that if the of calcium ions were removed via the pre-treatment also helped to improve the E1 removal (Jin *et al.*, 2010). Studies by Margot *et al.* (2013) have also found that the tertiary treatment after the conventional activated sludge treatment using ozonation and Powdered Activated Carbon combined with Ultrafiltration (PAC-UF) removed a higher percentage of hormone EDCs.

It was also hypothesized that due to the mixture of domestic and industrial feeds coming into these plants, perhaps the complex mixture of industrial effluents can interfere with the removal of EDCs, the theory above was hypothesized after seeing the removal efficiency of Plant E which is solely a domestic influent plant with an activated sludge process and had higher reductions of estrogens than the other plants

Even though most wastewater treatment plants with activated sludge have the ability to reduce the hormones in final effluents, many effluents still have estrogenic activity which can be attributed to other chemicals which can stem from industrial effluents. In a study by Mahomed *et al.* (2008) where estrogenic activity was analysed in waters from industries in Pretoria, it was found that the estrogenic activity was detected in all of the samples tested. There are a lot of other factors that need to be considered such as the biomass present and the design and operation of the specific WWTP (Mastrup *et al.*, 2005).

Estrogens being released with the final effluents have shown to be detrimental to aquatic life and have been documented (Christiansen *et al.*, 2002; Desbrow *et al.*, 1998; Folmar *et al.*, 1996; Kashiwada *et al.*, 2002; Larson *et al.*, 1999; Routledge *et al.*, 1998; Tabata *et al.*, 2001). In studies by Metacalf (2001) (as cited by Christiansen *et al.*, 2002) found that there were reproductive interferences in fish populations that were exposed to effluents from WWTPs. A concentration of 5ng/L of 17ß-Estradiol induced the production of yolk protein in male fish and at 10ng/L induced intersex. It was found that EE2 was even more potent than the natural estrogens which caused an induction in yolk protein and intersex at 0.1ng/L.

The study by Liney *et al.* (2006) exposed fish to different concentrations of sewage effluent of 20%, 40% and 80%. The chemical analysis of the effluent showed E1 concentrations between 6.5 – 8.6 ng/L, E2 0.7 – 3.6 ng/L and EE2 was not detected in any of the sampling points. The health effects of the fish exposed to the effluents revealed that at 80% of effluent treatment the gonad histopathology all the male fish had sexual differences where female-like ducts formed. Vitellogenin (VTG) was also permeated in many tissues and was also detected in the liver, kidney and gonads of the fish exposed to 80% effluent. Genetic damage was also very prominent which found breaks in the single strand DNA. The DNA damage was more significant in the fish exposed to the 40 and 80 % effluents (Liney *et al.*, 2006).

5.4.2 Toxicity Removal from WWTPs using *Vibrio fischeri*

Using the *Vibrio fischeri* method to evaluate the reduction of toxicity in WWTPs proved effective. It was seen immediately after secondary biological treatment in the clarifier effluent for all plants the toxicity was reduced (Table 5.8). Research by Farre *et al.* (2002) also observed high toxicity levels in the influents of the two WWTPs investigated and that only the after the biological stage most of the toxic compounds analysed for were removed. The acute toxicity testing of the influents, clarifier effluent and final effluents of the Plants C, D, and E have shown no correlation with regards to the hormone concentrations in the clarifier effluent and final effluents being discharged (Table 5.8). If the chlorine was removed from the final effluent there will be no toxic effect even though there are hormone estrogens in the effluent. Studies by Pignata *et al*. (2012) who used 3 types of toxicity testing with *Vibrio fischeri* being one of the test methods, found low toxicity in effluents without sodium hypochlorite disinfectant and very high TU in effluents after the use of the disinfectant.

A study by Bicchi *et al.* (2009) whose study looked at estrogenic activity in effluent samples from a municipal wastewater works and assayed the same untreated samples for acute toxicity using *Vibrio fischeri* also found no correlation could be established between estrogenic activity and toxicity. Studies by Schiliro *et al.* (2004) also revealed no correlation between toxicity and estrogenic behaviours. The Bicchi *et al*. (2009) study also found the effluent samples to be weakly toxic or toxic ($0.4 > TU < 7.6$), but did not indicate if toxicity was related to the disinfectant or not.

Even though results indicate that there are estrogens in the final effluents and no toxicity studies have found that the estrogens in the aquatic environment can cause effects when working together in mixtures (Christiansen *et al*, 2002; Desbrow *et al.*, 1998; Folmar *et al.*, 1996; Kashiwada *et al.*, 2002; Larson *et al.*, 1999; Routledge *et al.*, 1998; Tabata *et al.*, 2001). One such study by Gibson *et al*. (2005) where rainbow trout was exposed to a mixture of estrogen contaminants in effluents from two WWTPs; consisting of E1, E2 and EE2. The effluent before the exposure period had concentrations of 195 ng/L, 38.9 ng/L and 7.9 ng/L of E1, E2 and EE2 respectively and after the exposure period the concentrations were 10.3 ng/L,

0.8 ng/L and 1.1 ng/L of E1, E2 and EE2 respectively. Upon quantification of the estrogenic contamination that accumulated in the bile of the fish revealed that the major components were E2 and E1 which accounted for 55 – 60% and 32 – 37% respectively which shows that steroidal estrogens can bioaccumulate in fish and are major contaminants.

It has been reported by a few researchers that bioluminescent bacterium and ecotoxicity tests are already being used for water, wastewater and industrial management in many countries (Hewitt and Marvin, 2005; Jennings, *et al.*, 2001;Mendonca *et al.*, 2011). Research by Mendonca *et al.* (2011) where comparisons of a number of ecotoxicological tests for acute toxicity on influent and effluent samples from 4 different WWTPs and found that the acute toxicity was dependant on the treatment level, and that after secondary treatment and tertiary treatment there was either slight toxicity or no toxicity. The Mendonca *et al.* (2011) study also found that the bacterium *Vibrio fischeri* proved to be the most sensitive species in the wastewater ecotoxicological evaluation.

5.5 CONCLUSION

The results from the different types of WWTWs show that the removal of estrogens did occur from the influents when compared to the concentrations in the final effluents. The different configurations definitely play an extremely important role in efficient removal of estrogen EDCs. This was seen from the poor removal of all estrogens in plant A which only consisted of oxidation ponds and no other forms of secondary or tertiary treatment. Even though this plant only had a domestic feed it still performed poorly. Plant B which had two different types of treatment processes on one plant, proved that the BNR plant performed more efficiently in reducing the estrogen concentrations, while the simple conventional activated sludge process had a better efficiency than plant A but less efficient than the BNR process.

Plant C with the biological filter configuration did not perform well at all in removing any of the estrogens, and in fact had higher concentrations in the effluent than the influent. After this plant was upgraded to a BNR plant the capacity in removing the estrogens improved

tremendously especially for E2 and E1. However the EE2 concentrations were very low in the influent and effluent but only managed a 33% removal in the upgraded plant.

In Plant D the conventional activated sludge process was also quite efficient in removing the estrogens concentrations from the influent. However the inclusion of the ultrafiltration process did not help in reducing the concentrations further. Being the first plant of this kind in the country perhaps one needs to still optimise the operating parameters to enhance removal of these EDCs.

The toxicity evaluation using the bioluminescent bacteria *Vibrio fischeri* showed that the different WWTPs with biological treatment can reduce the toxicity of the influent coming into the plant; however the addition of the chlorine disinfectant proved to increase the toxicity of the final effluent. No correlation could be made with the hormone concentration and the Chlorine toxicity of the final effluents.

Hence it can be concluded that estrogens and to an extent toxicity can be reduced in certain WWTP. However this would depend on the plant configuration, the type of feed and the operating parameters which could all play a role either individually or in harmony in reducing these estrogens. An area of concern that requires further investigation is the effect of residual low concentration estrogens that are still present in the final effluents and how the latter impact on the receiving aquatic ecosystems.

CHAPTER 6
GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 GENERAL DISCUSSION

In South Africa there is still a lack of public awareness regarding EDCs and their harmful effects on humans and wildlife. Ecotoxicological and health risk assessment lag behind most of the developed countries in the world today. It has only been within the last decade that South Africa began the actual task for proper management and control of water and wastewater quality. This has been promulgated by the Department of Water Affairs (DWA) who has launched an incentive based regulation for stringent control of water and wastewater quality. This regulation has been enforced since 2008 for all local authorities to obtain a Blue Drop or Green Drop status or both. The Blue drop regulates the drinking water quality while the Green drop regulates the wastewater effluent quality. By putting together such a regulation has forced all local authorities to prioritise the management of both the water and wastewater treatment plants. Each of the regulations has a set of criteria with points attached to each criterion. The local authorities need to adhere to and achieve more than 95% in order to qualify for a Blue Drop status and greater than 90% to achieve a Green Drop Status. Within the set of criteria one of the major components is the water quality monitoring compliance. It is within this set of criteria that the water and wastewater must comply with the South African National Standards (SANS).

Even though the SANS has quite a strict list of standards to abide by, the list still does not include hormone such as EDCs. South Africa is still a country that uses and probably abuses most of the chemicals listed in most developed countries as potential EDCs, which also include DDT to combat malaria (Dalvie *et al.*, 2004; IWA 2nd World Water Congress, 2002).

There are few research groups in South Africa which use biomarkers to assess the effects of EDCs on wildlife species. The research conducted to determine estrogenic activity are in-vitro tests such as the E-screen (MCF-7 cells), recombinant yeast screen bioassay and catfish-vitellogenin (cf-Vtg) coupled with ELISAs as well as toxicity testing (Aneck-Hahn *et al.*, 2005; Aneck-Hahn *et al.*, 2006; Burger, 2008). Within these research groups there is very little information on the concentrations of estrogen hormones being discharged with wastewaters into aquatic ecosystems.

This research project focussed on the use and application of rapid immunoassay techniques (ELISAs and RIAs) to quantify estrogens concentrations in wastewater effluents collected from different parts of South Africa, i.e., Kwa-Zulu Natal and Western Cape Provinces as well as to determine the capability the different plant configurations had on the removal of hormone EDCs.

The assessments of the immunoassay kits were done to determine the performance in terms of precision, accuracy and reproducibility of the procedures and the recovery from SPE procedures. The performance evaluations of the selected procedures were done through the analysis of various replicates of controls and standards before subjecting unknown samples to SPE and immunoassay procedures.

The assessment results of the ELISA technique showed that very good recoveries were achieved during the intra-assay and inter-assay validation tests. Both the E2 and EE2 procedures were found to be precise and reproducible. The same was found during the assessment of the RIA technique. The E1, E2 and E3 test kits were also found to be precise and reproducible. Both techniques had recoveries above 95%. An added advantage of the RIA test kits were the provision of controls that were with the kit. The controls were used to determine if there was good recovery from the SPE and if the RIA procedure was followed correctly. The controls were good indicators of overall precision and accuracy. If the concentration of the controls after application to the SPE and ELISA were not close to theoretical value then one would need to retrace steps and determine where the error was made and where to optimise the procedure to improve the recovery.

For the ELISA, standards were purchased and made up to provide different concentrations as controls. These were used to determine the SPE recoveries and procedure performance. The recoveries were also >95% for the ELISAs. The volumes of samples required for an immunoassay procedure is much less as compared to the volumes needed for other techniques. A review of analytical methods done by Lopez de Alda and Barcelo (2001) showed that the amount of sample processed varied with the sensitivity of the technique used. The study showed that the Immunoassays only required 50 mL for extraction as compared to the 20 L and 80 L required by liquid-liquid partition and analysed by gas-liquid chromatography. This study used 100 mL samples.

The fate of these hormones under various controlled conditions was determined in the second phase of this research project. This was done by conducting laboratory-scale experiments by optimizing various conditions to achieve maximum removal of estrogens. One such process was the operation of a laboratory scale MLE process which was operated for 9 months. This process mimics most wastewater treatment plants (WWTP) that uses an activated sludge process. The continuous operation of the MLE system was maintained by consistent feeding of influent from WWTP B at the rate of 24 L/day. It was found that the SRTs played an important role in the removal of the estrogen EDCs from the MLE system. The process was run first at a 5 day SRT and then at a 10 day SRT. The 10 day SRT showed definitive increases in removal efficiencies not only for the estrogen but also for other physico-chemical parameters such as the COD. Depending on the plant configuration, plant design capacities and perhaps longer SRTs can further enhance the removal of EDCs. Johnson *et al.*, (2000) and Kreuzinger *et al*. (2004) also found that SRTSs and the HRTs played an important role in EDC removal. It was also found by Holbrook *et al.* (2002), that sorbent characteristics of the MLSS may fluctuate with SRT and that the SRT is a significant operational parameter to take into account when removal of estrogenic contaminants from WWTP are concerned. Furthermore by adjusting the SRT, process controllers have the ability to influence the sorption potential of the biosolids.

The second laboratory experiment which used batch tests to determine the fate of E1 and E2 in activated sludge slurry over time found that the concentration of E1 increased drastically within the first 3 hours to above the E2 concentrations and then decreased with time. It was also found that the E1 took longer to degrade than the E2. A 94% reduction of E2 was seen after 5 hours and was no longer detected after 10 hours. After 13 hours E1 could no longer be detected. According to Ternes *et al.* (1999) E1 is formed from the breakdown of E2 and also from the deconjugation of E1-glucuronides and E1-sulfates, therefore the increase of E1 could also be possibly due to the net change of E1 concentration between the influent and effluent.

The other possibility of the decrease of the concentrations of E1 and E2 could also be attributed to the adsorption onto the sludge. A study by (Shore *et al.*, 1993) as cited by Lai *et al.* (2002) observed that 45% of removal of hormones was due to partitioning to the activated sludge. Therefore the decrease of estrogens, especially E2, observed could also be due to the same effect; however biodegradation through binding to sediment was not investigated in this study.

The toxicological evaluation of the EDCs was done in the third phase of this research project through microbiological toxicity tests. The luminous marine bacterium *Vibrio fischeri* toxicity test was simple, rapid, cost effective and easy to conduct; therefore the use of intact luminous bacteria for assessment of toxicity has some advantages over other tests using fish or other aquatic invertebrate such as daphnids, rotifers and ostrocods etc. (Dalzell, 2002; Kaiser, 1998). The use of a luminometer enabled the detection of the luminescence emitted by the bacteria at 490 nm.

The test was based on the principle that the physical, chemical or biological stressors affect cell respiration, electron transport systems, ATP generation which then alters the level of luminescence (Jennings *et al.*, 2001). Therefore various toxic substances such as heavy metals, PCBs, PAHs or steroid hormones EDCs etc., even though having different characteristics, may exert an effect on metabolic responses of bacterial cells thus affecting

the bacteria's luminescence. Thus measuring the effect of a very low concentration of a wide range of toxicants is possible through comparison of the luminescence level of *Vibrio fischeri* with a clean water control after short incubation periods.

The basis of selecting different hormone standards was due to the fact that earlier reports have shown that very low concentrations of hormone EDCs have adverse effects on aquatic life (Liney *et al.*, 2006). Therefore by determining the toxicity and applying the toxicity classification system on individual hormone standards one can now classify these substances and determine no effect concentrations to provide information for environmental limits.

Many researchers from different countries showed that wastewater treatment plants are major contributors of hormone EDCs into the aquatic ecosystem (Baronti *et al.*, 2000; Belfroid *et al.*, 1999; Braga *et al.*, 2005; Christiansen *et al.*, 2002; Desbrow *et al.*, 1998; Liney *et al.*, 2006; Ternes *et al*., 1999). Due to scarcity of water resources, many countries have started recycling wastewater for drinking water purposes, therefore concerns have been raised regarding the health risk of EDCs through drinking water (Falconer, 2006; Touraud, *et al*., 2011). The South African WWTP effluents are also not different from the rest of the world. The different plant configurations in this study showed different removal efficiencies (Table 5.6 and 5.7)

As seen from the 24 hour batch experiment whether the decrease in concentrations was attributed to degradation or to sorption is yet to be determined (fig 4.12). According to Ying *et al.* (2002) due to the low solubility of the estrogens (E1, E2, E3 and EE2); these hormones are more hydrophobic compounds thereby causing sorption to soil/sediment or sludge which can account for the reduction in the aqueous phase. In a case study Gomes *et al.* (2004) found that the sludge from the activated sludge (AS) process had a higher % recovery of E1, E2, E3 and EE2 as compared to the sludge from the sequencing batch reactor (SBR). However it was not clear whether the higher recovery for the AS was attributed to process efficiency or the sludge matrix for analysis.

When determining the decomposition of EDCs in sludge, the study by Minamiyama *et al.* (2008) which determined the fate of nonylphenols and E2 in composted sewage sludge after land application, found that there were high concentrations in the leachate and in the soil. However, there was rapid degradation under 300 days. It was also found that an acclimation of micro-organisms were necessary before the decomposition of E2 began. It can thus be assumed if a majority of estrogens can be removed via sorption to sludge in a WWTP and if the sludge is disposed at a landfill site, the estrogens may be degraded within a year.

Different environmental processes have a great influence on the fate of estrogens, especially in soil. For example, it has been shown by Yu *et al*. (2003) that the total organic carbon (TOC) content, concentration and hydrophobicity of the estrogens are determining factors when predicting the partitioning of estrogens to sediment, further, both salinity and the presence of other hydrophobic compounds also influence this process. Yu *et al.* (2003) showed the completion of the sorption equilibrium needed approximately 2 d when aqueous estrogen concentrations (C_t s) were 25 to 50% of their solubility limits (S_W s), but required longer periods of up to 10 to 14 days when the C_t was 20 times lower than the S_W. Further, the measured sorption isotherms were all nonlinear, with n ranging from 0.475 – 0.893. Therefore, the Yu *et al*. (2003), study suggested that these estrogenic chemicals at sub-micrograms per litre levels may exhibit even slower movement rates and increased capacities of sorption by soils and sediments.

Despite relatively limited amount of adsorption of selected estrogen molecules in the soil, it has been shown that as a result of different attenuating factors active in soil, the movement of these compounds is limited (Yu *et al.*, 2003). It must be pointed out that the different physico-chemical characteristics within the same type and between different types of EDCs will have a significant impact on the relative rates of movement through soils and the final fate of the different compounds (Mansell and Drewes, 2004).

Ying *et al.* (2004) on the other hand showed that E2 showed fast biodegradation properties with a half-life value (time for 50% loss) of ~2 days under aerobic conditions while the degradation rates were much lower under anaerobic conditions, but EE2 was found to resist biodegradation and remained for up to 70 days under both aerobic and anaerobic conditions (Ying, *et al.*, 2004).

There are not many reports which researched the isolation and culture of the acclimatised micro-organisms and/or their enzymes that aided in the decomposition of estrogens (Fujji *et al.*, 2002; Suzuki *et al.*, 2003). Shi *et al.* (2004) investigated the microbial degradation of E1, E2, E3 and EE2 by activated sludge from Korea (ASK) and night soil composting microorganisms (NSCM). The results showed that both ASK and NSCM degraded close to 100% of the natural estrogens but not the synthetic estrogen. The estrogenic activity was also determined after degradation reached below detection limits and after 14 days of incubation the natural estrogens were degraded and their estrogenic activities were also removed.

Even though the WWTP are contributing EDCs into the aquatic system; they do reduce the amounts being discharged when comparing the influents to the effluents (Table 5.7). Tanaka *et al*. (2001) have also indicated that there was a significant reduction in estrogenic activity from the influent after activated sludge treatment. Therefore WWTP definitely play a significant role in the removal of these EDCs. The design of a plant is crucial in determining the overall discharge effluent quality. A plant with activated sludge definitely contributes to a higher removal efficiency of EDCs. Other important factors are the sludge and hydraulic retention times. As Kreuzinger *et al.* (2004) published higher SRTs allow the enhancement of slow growing bacteria and as a result the establishment of a more diverse bacterial range with more extensive physiological capabilities compared to sewage treatment plants with low SRTs.

Other studies have also shown that activated sludge treatment processes tended to be more effective than other treatment processes in removal of estrogens (Christiansen *et al.*, 2002; Kjolholt *et al.*, 2004; Svenson *et al.*, 2003). Stanford and Weinberg, (2010) have shown an increase in steroid estrogens removal when an advanced pre-treatment such as the aerobic filtration was included in the process.

The use of immunoassays and toxicity tests are becoming more economically viable in today's world due to the ease of use, lower costs and the rapid time frames taken to obtain quantitative results. When purchasing large expensive instrumentation one has to also make provision for housing the instruments as well as the gases that are required for the operation whereas the purchasing of instrumentation that caters for these immunoassay and toxicity techniques is far more cost effective as far less space and no gases are required for the instruments. It thus makes more economic sense to pursue and develop more immunoassays that targets a wider variety of compounds. These rapid tests are fast becoming readily available and have proved to be very sensitive, robust and cost effective techniques for quantitative analysis.

6.2 CONCLUSIONS

The findings from this study have found that ELISAs and RIAs can be used as a simple and rapid tool for the monitoring of hormone EDCs in wastewater. Both immunoassays have shown good precision and accuracy to determine these micropollutants. The ease of use, rapid turnaround times and low costs make these test kits a better alternative than the conventional methods such as GC-MS etc. However the use of the ELISA as a rapid and cost effective analytical tool for monitoring micropollutants is becoming more popular than RIAs and is growing rapidly in South Africa and worldwide. Even though RIAs also have a simple procedure to follow, the presence of the radioactive material in the test kit reagents requires a more stringent control/procedure for discarding the reagents after use.

The laboratory scale MLE investigations showed that the longer SRTs of 10 days or more do play a vital role in the removal of hormone EDCs and overall plant efficiency. Whether the removal was based on biodegradation or sorption to sludge has to still be investigated. The batch test revealed that the increase and persistence of E1 in final effluents was definitely due to the oxidation of E2 to E1.

The toxicity tests revealed that the E2, EE2 and mixture of E1, E2, E3 standards all showed slight acute toxicity falling into Class II of the toxicity classification system. The E2 standard showed higher toxicity levels as a single standard as compared to when it was in a mixture with E1 and E3. The E2 standard at 10 ng/L also had the highest toxicity out of all the standards and was close to the Class III level for acute toxicity after T_{30}. The EE2 hormone had the lowest toxicity after T_{30}.

The study of the different WWTPs configuration show that plant configuration and operational parameters has a huge impact on the removal of hormone EDCs. The composition of the receiving influent into the plant also has an effect on the removal, i.e., whether it's industrial, domestic or a mixture of both. Results concluded that plants which have aeration or activated sludge have a higher rate of hormone removal than those plants without.

The toxicity tests have shown that the WWTP procedures definitely have capabilities of reducing the toxicity of the influent.

Overall results indicate that hormone EDCs are indeed being discharged with the effluents from WWTPs in South Africa. It can thus be concluded that in order to remove the hormone concentrations from effluents, a plant having some form of mixing and/or aeration with bio solids is imperative. However, whether the concentrations left in the final effluents will still have an adverse effect on the aquatic life is a question that still remains unanswered. The aquatic ecosystems are inevitably being polluted with these EDCs and their breakdown products which are often used as a drinking or food source.

6.3 RECOMMENDATIONS

Further studies in this field could include investigations into the irreversible metabolism of E2 and E3 as well as the adsorption capabilities of hormone EDCs to the waste sludge. The reason is that there are different pathways that the hormones can metabolise into. It will be interesting to note what the final breakdown product is and to also determine if it holds any estrogenic activity. It will also be of interest to find out how much of these hormones is adsorped to the waste sludge and if desorption can occur.

A more detailed programme for biomonitoring should also be included to determine exactly what the health effects of these low concentrations of estrogens are not only on the aquatic life but also human health downstream of the South African WWTPs. There is still a debate regarding the effect of EDCs on human health, as the effects, if any, can only be seen many years after exposure has occurred.

There also needs to be an increase in awareness made to the public regarding the use of water downstream from WWTPs. Many people, who live along river banks and downstream from a WWTP, use the water for bathing, food preparation and also for drinking. They need to be made aware of the potential health risks of using this water.

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APPENDICES

APPENDIX 1: LIST OF PESTICIDES PREPARED BY THE DANISH EPA (Danish EPA, 1995)

NR	CASNR	Name	HPV/pers.	ECO	HUM	Total	Concern
11	12789-03-6	Chlordane	Highly Pers	2	1	1	High
12	57-74-9	Chlordane (cis- and trans-)	Highly Pers	2	1	1	High
20	143-50-0	Kepone = Chlordecone	Highly Pers	2	1	1	High
21	2385-85-5	Mirex	Highly Pers	2	1	1	High
24	8001-35-2	Toxaphene = Camphechlor	Highly Pers	2	1	1	High
42	50-29-3	DDT (technical) = clofenotane	HPV	1	1	1	High
56	50-29-3	p,p'-DDT = clofenotane	HPV	1	1	1	High
57	3563-45-9	Tetrachloro DDT = 1,1,1,2-Tetrachloro-2,2-bis(4-chlorophenyl)ethane	Highly Pers	1	2	1	High
63	50471-44-8	Vinclozolin	HPV	3	1	1	High
69	12427-38-2	Maneb	HPV	3	1	1	High
70	137-42-8	Metam Natrium	HPV	3	1	1	High
73	137-26-8	Thiram	HPV	3	1	1	High
74	12122-67-7	Zineb	HPV	3	1	1	High
78	58-89-9	Gamma-HCH = Lindane	HPV	2	1	1	High
87	330-55-2	Linuron (Lorox)	HPV	3	1	1	High
142	1912-24-9	Atrazine	HPV	2	1	1	High
163	34256-82-1	Acetochlor	HPV	3	1	1	High
164	15972-60-8	Alachlor	HPV	2	1	1	High
191	100-42-5	Styrene	HPV	3	1	1	High
198	118-74-1	Hexachlorobenzene = HCB	HPV	3	1	1	High
270	85-68-7	Butylbenzylphthalate (BBP)	HPV	3	1	1	High
279	117-81-7	Di-(2-ethylhexyl)phthalate (DEHP) = Dioctylphthalate (DOP)	HPV	3	1	1	High
286	84-74-2	Di-n-butylphthalate (DBP)	HPV	3	1	1	High
326	80-05-7	2,2-Bis(4-hydroxyphenyl)propan = 4,4'-isopropylidenediphenol = Bisphenol A	HPV	1	1	1	High
396	1336-36-3	PCB	Pers.		1	1	High
408	35065-27-1	PCB153	Pers.		1	1	High
410	32774-16-6	PCB169	Pers.		1	1	High
417	2437-79-8	PCB47	Pers.		1	1	High
422	32598-13-3	PCB77	Pers.		1	1	High
427	53469-21-9	Aroclor 1242	Highly Pers		1	1	High
428	12672-29-6	Aroclor 1248	Pers.		1	1	High
429	11097-69-1	Aroclor 1254	Highly Pers		1	1	High
430	11096-82-5	Aroclor 1260	Pers.		1	1	High
438	59536-65-1	PBBs = Brominated Biphenyls (mixed group of 209 Congeners)	Pers.		1	1	High
467	40321-76-4	1,2,3,7,8 Pentachlorodibenzodioxin	Pers.		1	1	High
472	No CAS 140	2,3,7,8 Tetrachlorodibenzo-p-dioxin (TCDD)	Pers.		1	1	High
487	57117-31-4	2,3,4,7,8 Pentachlorodibenzofuran	Pers.		1	1	High
525	688-73-3	Tributyltin	Metal	1	2	1	High
526	No CAS 050	Tributyltin compounds	Metal	1	2	1	High
527	56-35-9	Tributyltin oxide = bis(tributyltin) oxide	HPV/ Metal	1	2	1	High
504	26354-18-7	2-propenoic acid, 2-methyl-, methyl ester = Stannane, tributylmeacrylate	Metal	1	2	1	High
512	No CAS100	Methoxyetylacrylate tinbutyltin, copolymer	Metal	1	2	1	High
514	4342-30-7	Phenol, 2-[[(tributylstannyl)oxy]carbony	Metal	1	2	1	High
515	4342-36-3	Stannane, (benzoyloxy)tributyl-	Metal	1	2	1	High
516	4782-29-0	Stannane, [1,2-phenylenebis(carbonyloxy)	Metal	1	2	1	High
517	36631-23-9	Stannane, tributyl = Tributyltin naphtalate	Metal	1	2	1	High

APPENDIX 1: CONTINUED….

NR	CASNR	Name	HPV/pers.	ECO	HUM	Total	Concern
518	85409-17-2	Stannane, tributyl-, mono(naphthenoyloxy	Metal	1	2	1	High
519	24124-25-2	Stannane, tributyl[(1-oxo-9,12-octadecad	Metal	1	2	1	High
520	3090-35-5	Stannane, tributyl[(1-oxo-9-octadecenyl)	Metal	1	2	1	High
521	26239-64-5	Stannane, tributyl[[[1,2,3,4,4a,4b,5,6,1	Metal	1	2	1	High
522	1983-10-4	Stannane, tributylfluoro-	Metal	1	2	1	High
524	2155-70-6	Tributyl[(2-methyl-1-oxo-2-propenyl)oxy]stannane	Metal	1	2	1	High
528	No CAS 099	Tributyltincarboxylate	Metal	1	2	1	High
529	26636-32-8	Tributyltinnaphthalate	Metal	1	2	1	High
530	No CAS 101	Tributyltinpolyethoxylate	Metal	1	2	1	High
531	2279-76-7	Tri-n-propyltin (TPrT)	Metal	1	3	1	High
532	No CAS 051	Triphenyltin	Metal	1	3	1	High
509	900-95-8	Fentin acetate	Metal	1	3	1	High
536	95-76-1	3,4-Dichloroaniline	HPV	1	2	1	High
560	108-46-3	Resorcinol	HPV	3	1	1	High
141	61-82-5	Amitrol = Aminotriazol	HPV	3	1	1	Medium
182	1836-75-5	Nitrofen	HPV	3	1	1	Medium
216	140-66-9	4-tert-Octylphenol=1,1,3,3-Tetramethyl-4-butylphenol	HPV	1	1	1	Medium
254	25154-52-3	Phenol, nonyl-	HPV	1	1	1	Medium
523	1461-25-2	Tetrabutyltin (TTBT)	HPV/ Metal	1	2	1	Low
538	99-99-0	4-Nitrotoluene	HPV	3	1	1	Low

APPENDIX 2: PROCEDURE FOR CLEANING OF BOTTLES

1. 500 mL amber glass bottles were washed with detergent and rinsed thoroughly under running tap water
2. Bottles were then rinse 4 times with distilled water
3. The last rinse was with high quality ethanol (approximately 25 ml per 100 ml bottle).
4. Bottles were then allowed to dry upside down on rack.

APPENDIX 3: 20 mM TRIS BUFFER (pH 8.5) + 20% METHANOL

0.242 g Tris was dissolved in 80 mL distilled water and 20 mL methanol (100%) in a 100 mL volumetric flask.

APPENDIX 4: 40% METHANOL

A 40 mL aliquot of Methanol (100%) was diluted with distilled water and made up to the mark in a 100 mL volumetric flask.

APPENDIX 5: 80% METHANOL

An 80 mL aliquot of Methanol (100%) was diluted in 20 mL distilled water in a 100 mL volumetric flask.

APPENDIX 6: TEST PROCEDURE FOR THE ESTRADIOL AND ETHINYLESTRADIOL ELISA KIT

All reagents were allowed to reach room temperature (~ 25°C) and mixed thoroughly by gentle inversion before use.

1. A sufficient number of wells were inserted in to the microwell holder for all standards and samples to be run in duplicate. Positions of standards and sample were recorded.
2. A 20 µL aliquot of each standard or prepared sample was applied to separate duplicate wells.
3. A 50 µL aliquot of diluted enzyme conjugate was added to the bottom of each well.
4. A 50 µL aliquot of diluted anti-17B-Estradiol / Ethinylestradiol solution, respectively, was added to the bottom of each well. Plates were mixed gently by rocking the plate manually and incubated for 2 h at room temperature (20 - 25°C) in the dark.
5. After incubation the liquid was poured out of the wells and tapped upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Wells were filled with 250 µL of distilled water and the liquid decanted again. This was repeated twice more.
6. A 50 µL aliquot of substrate and 50 µL of chromogen were added to each well, mixed gently by rocking the plate manually and incubated for 30 min at room temperature (20 - 25°C) in the dark.
7. The final step was the addition of 100 µL of the stop solution to each well, followed by gentle rocking of the plate manually to mix and measured at 450 nm against an air blank. Wells were read within 60 minutes after addition of stop solution.

APPENDIX 7: TEST PROCEDURE FOR THE DSL ESTRADIOL- 4400 RIA KIT

All reagents were allowed to reach room temperature (~ 25°C) and mixed thoroughly by gentle inversion before use.

1. Tubes were labelled and arranged in duplicate for total counts, non-specific binding (NSB), standards, controls and unknowns using 12 x 75 mm test tubes.
2. A 100 µL of the Estradiol standards, controls and unknowns were pipetted to the bottom of the appropriate tubes. To the NSB a 200 µL aliquot of the 0 pg/mL Estradiol Standard (A) was added.
3. A100 µL aliquot of the Estradiol [I^{-125}] reagent was added to each tube.
4. A100 µL aliquot of the Estradiol antiserum was added to all tubes except NSB and total count tubes.
5. Tubes were vortexed gently for 1-2 seconds.
6. Tubes were covered and incubated at room temperature for 30 minutes.
7. A 1 mL aliquot of the precipitating reagent was added to all tubes, except the total count tubes and immediately vortexed. This reagent needed to be mixed thoroughly before use.
8. Tubes were incubated at room temperature for 15 - 20 minutes.
9. All tubes except the total count tubes were centrifuge for 15 - 20 minutes at 1500 x g.
10. All tubes, except the total count tubes, were decanted by simultaneous inversion with a sponge rack into a radioactive waste receptacle. Tubes were allowed to drain on absorbent material for 15 - 30 seconds and gently blotted to remove any droplets adhering the rim before returning them to the upright position. Failure to blot tubes adequately may result in poor replication and spurious values.
11. All tubes were counted in a gamma counter for one minute.

APPENDIX 8: TEST PROCEDURE FOR THE DSL ESTRONE 8700 RIA KIT

All reagents were allowed to reach room temperature (~ 25°C) and mixed thoroughly by gentle inversion before use.

1. Tubes were labelled and arranged in duplicate for total counts, non-specific binding (NSB), standards, controls and unknowns using 12 x 75 mm test tubes.
2. A 50 µL aliquot of the Estrone standards, controls and unknowns were pipetted to the bottom of the appropriate tubes. To NSB a 150 µL aliquot of the 0 pg/mL Estrone standard was added.
3. A 100 µL aliquot of the Estrone [I^{-125}] reagent was added to each tube.
4. A 100 µL aliquot of the Estrone Antiserum was added to all tubes except NSB and total count tubes.
5. Tubes were vortexed gently for 1-2 seconds.
6. Tubes were covered and incubated at room temperature for one hour.
7. After incubation a 1 mL aliquot of the precipitating reagent was added to all tubes, except the total count tubes and immediately vortexed. This reagent needed to be mixed thoroughly before use.
8. Tubes were incubated at room temperature for 15 minutes.
9. All tubes were centrifuged except the total count tubes for 20 - 30 minutes at 1500 x g.
10. All tubes were decanted, except the total count tubes, by simultaneous inversion with a sponge rack into a radioactive waste receptacle, allowed to drain on absorbent material for 15 - 30 seconds and gently blotted to remove any droplets adhering to the rim before returning them to the upright position. Failure to blot tubes adequately may result in poor replication and spurious values.
11. All tubes were counted in a gamma counter for one minute.

APPENDIX 9: TEST PROCEDURE FOR THE DSL ESTRIOL- 3700 RIA KIT

All reagents were allowed to reach room temperature (~ 25°C) and mixed thoroughly by gentle inversion before use.

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1. Two plain (Uncoated) tubes for total counts were labelled. The unconjugated Estriol antibody-coated tubes were labelled and arranged in duplicate for standards, controls and unknowns.
2. A 50 µL aliquot of the Unconjugated Estriol standards, controls or unknowns were pipetted to the bottom of the appropriate tubes.
3. A 500 µL aliquot of the Unconjugated Estriol (I^{-125}) Reagent was added to all tubes and vortexed.
4. All tubes were covered and incubated at 37°C for 60 minutes. .
5. All tubes except the total count tubes were, by simultaneous inversion with a sponge rack into a radioactive waste receptacle. Tubes were struck sharply on absorbent material to facilitate complete drainage and then allowed to drain on absorbent material for a minimum of 2 minutes. The tubes were blotted to remove any droplets adhering to the rim before returning them to the upright position. Failure to blot tubes adequately may result in poor replication and spurious values.
6. All tubes were counted in a gamma counter for one minute..

APPENDIX 10: STANDARD METHOD 5220B FOR OPEN REFLUX COD METHOD DETERMINATION (Clesceri *et al*., 1998)

Apparatus

a. Reflux apparatus, consisting of 250 mL or 500 mL Erlenmeyer flasks with ground-glass 24/40 neck and 300 mm jacket Liebig, West, or equivalent condenser with 24/40 ground-glass joint, and a hot plate having sufficient power to produce at least 1.4 W/cm^2 of heating surface, or equivalent.
b. Blender.
c. Pipets, Class A and wide-bore
d. 25 mL Burette

Reagents

a. Standard potassium dichromate solution, 0.04167M: 12.259 g $K_2Cr_2O_7$, primary standard grade, previously dried at 150°C for 2 h, was dissolved in distilled water and diluted to 1000 mL. This reagent undergoes a six-electron reduction reaction; the equivalent concentration is 6 x 0.04167 M or 0.2500 N.

b. Sulfuric acid reagent: Ag_2SO_4, reagent or technical grade, crystals or powder, was added to concentrated H_2SO_4 at the rate of 5.5 g Ag_2SO_4 /kg H_2SO_4. It was left to stand for 1 to 2 d to dissolve, thereafter it was mixed.

c. Ferroin indicator solution: 1.485 g 1,10-phenanthroline monohydrate and 695 mg $FeSO_4 \cdot 7H_2O$ was dissolved in distilled water and diluted to 100 mL. This indicator solution may be purchased already prepared.*

d. Standard ferrous ammonium sulfate (FAS) titrant, approximately 0.25 M: 98 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was dissolved in distilled water. 20 mL concentrated H_2SO_4, cool, and was added and diluted to 1000 mL. This solution was standardize daily against standard $K_2Cr_2O_7$ solution as follows:

25.00 mL standard $K_2Cr_2O_7$ was diluted to about 100 mL. 30 mL concentrated H_2SO_4 was added and cool. The solution was titrated with FAS titrant using 0.10 to 0.15 mL (2 to 3 drops) ferroin indicator.

APPENDIX 10: Contin…

Molarity of FAS solution was calculated by the following formula:

$$= \frac{\text{Volume 0.04167 M } K_2Cr_2O \text{ solution titrated, mL}}{\text{Volume FAS used in titration, mL}} \times 0.2500$$

e. Mercuric sulfate, $HgSO_4$, crystals or powder.

f. Potassium hydrogen phthalate (KHP) standard, $HOOCC_6H_4COOK$: The KHP was lightly crushed and then dried to constant weight at 110°C. A 425 mg portion was diluted in distilled water and diluted to 1000 mL. KHP has a theoretical COD of 1.176 mg O_2/mg and this solution has a theoretical COD of 500 mg O_2/ mL. This solution was prepared on a weekly basis and kept in a fridge.

Procedure

a. Treatment of samples with COD of > 50 mg O_2/L: Samples were blended if necessary and 50 mL pipetted into a 500 mL refluxing flask. For samples with a COD of > 900 mg O_2/L, a smaller portion was diluted to 50.00 mL. A 1 g $HgSO_4$ aliquot was added, with several glass beads, and very slowly 5.0 mL sulfuric acid reagent was added, with mixing to dissolve $HgSO_4$. The mixture was cooled while mixing to avoid possible loss of volatile materials. A 25.00 mL aliquot of 0.04167 M $K_2Cr_2O_7$ solution was added and mixed. The flask was attached to the condenser and the cooling water turned on. The remaining sulfuric acid reagent (70 mL) was added through the open end of condenser with continued swirling and mixing.

The open end of condenser was covered with a small beaker to prevent foreign material from entering the refluxing mixture and refluxed for 2 h. The condenser was cooled and washed down with distilled water and disconnected. The mixture was diluted to about twice its volume with distilled water and cooled to room temperature. The excess $K_2Cr_2O_7$ was titrated with FAS, using 0.10 to 0.15 mL (2 to 3 drops) ferroin indicator. The end point was taken when the first sharp color change from blue-green to reddish brown that persisted for 1 min or longer. Duplicate determinations should were agreed within 5 % of their average.

APPENDIX 10: Contin…

b. Alternate procedure for low COD samples: The above procedure was followed but the sample volume was adjusted by adding all reagents to a sample larger than 50 mL and reduced total volume to 150 mL by boiling in the refluxing flask open to the atmosphere without the condenser attached.

Calculation

$$\text{COD as mg } O_2/L = \frac{(A - B) \times M \times 8000}{\text{mL sample}}$$

where:

A = mL FAS used for blank,

B = mL FAS used for sample,

M = molarity of FAS, and

8000 = milliequivalent weight of oxygen X 1000 mL/L.

APPENDIX 11: PROCEDURE FOR MLSS MLVSS DETERMINATION

Principle

a. MLSS is the total amount of organic and mineral suspended solids contained in the mixed liquor of the activated sludge reactor. This value offers the system operator a crude measure of the biomass contained within the process.

b. MLVSS, or Mixed Liquor Volatile Suspended Solids, determines the amount of volatile suspended solids found in a sample of mixed liquor. Volatile solids are those solids which are burnt up when a sample is heated to 550°C in a furnace.

Apparatus

- Desiccator
- Centrifuge tubes (50 mL)
- Centrifuge capable of 3000 rpm
- Drying oven, for operation at 103 to 105°C
- Analytical balance, capable of weighing to 0.1 mg
- Magnetic stirrer with TFE stirring bar
- Wide-bore pipets
- Graduated cylinder
- Low-form beaker
- Stop watch
- Muffle furnace for operation at 550°C

Procedure

A Sample volume of 50 mL was added in to a centrifuge tube. Tubes were then centrifuged at 300 rpm for 6 minutes. The supernatant was discarded and the sludge pellet was quantitatively scooped into a pre-weighed crucible. The crucible was placed in the drying oven and left overnight to dry after 24 hours it was removed from the oven and placed in the desiccator. The cooled crucible was then re-weighed. The determination of the volatile solids is determined

APPENDIX 11: Contin…

thereafter by putting the reweighed crucible in to the furnace for 1 hour. The crucible is then removed and placed in a desiccator and weighed until a constant reading is achieved.

Calculation

$$\text{MLSS, mg/L} = \frac{(A - B) \times 1000}{\text{(Sample volume, mL)}}$$

where:

A = weight of crucible + dried residue, mg, and

B = weight of crucible, mg.

The volatile solids calculation is as follows:

$$\text{Volatile solids, mg/L} = \frac{C - D \times 1000}{\text{(Sample volume, mL)}}$$

Where:

C = Sample and crucible weight from MLSS test, mg

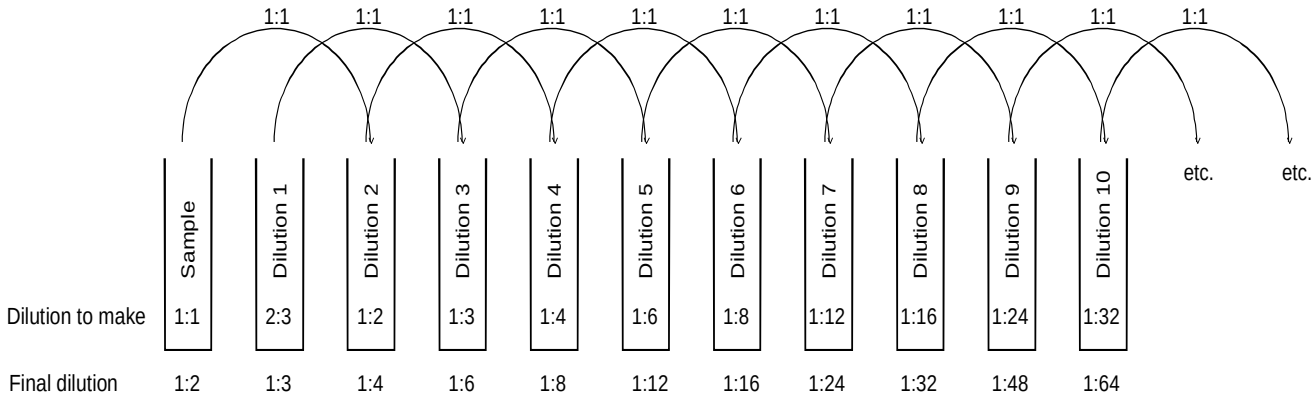
D = Sample and crucible weight after ignition in muffle furnace, mg

APPENDIX 12: 1% FORMALDEHYDE

A 2.5 mL aliquot of Formaldehyde (40%) was diluted in 97.5 mL distilled water in a 100 mL volumetric flask. (Used to preserve samples if not used immediately).

APPENDIX 13: THE *Vibrio fischeri* TOXICITY PROCEDURE

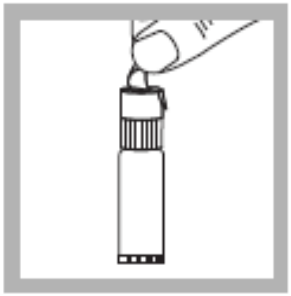
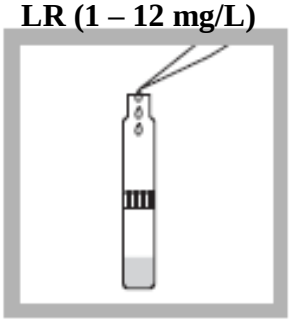
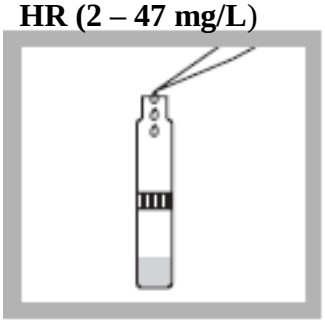
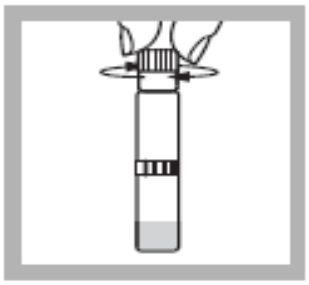
1. The salinity of the sample was adjusted to be equivalent to 2% NaCl solution. With freshwater or low salinity samples add either solid NaCl to a final concentration of 2% w/v or 1/10 of the final volume of 1243-552 Sample Diluent.
2. The *Vibrio fischeri* reagent was reconstituted by adding the contents of one vial of ±4 °C 1243-551 Reagent Diluent. The reconstituted reagent was equilibrated at ±4°C for 30 minutes and then stabilised at ±15 °C for at least 30 min before pipetting into the cuvettes.
3. A volume of 0.5 ml of the final bacterial suspension was pipetted in the cuvettes needed for performing the tests. All dilutions and controls were done as duplicate samples. The bacteria were allowed to stabilise in the cuvettes for at least 15 minutes at ±15 °C.
4. A sufficient volume of 1243-552 Sample Diluent was diluted 1:10 with distilled water (for example 20 ml Sample Diluent and 180 ml distilled water).
5. The principle of the dilution procedure is shown in the picture below for the definitive test:



6. The luminescence intensity (I_0) from the first cuvette (no. 1) containing bacterial suspension was measured and immediately 0.5 ml of sample was added to the cuvette. Equal time intervals between each sample was used
7. Sample dilutions at ±15 °C were incubated for the chosen contact time (15 and 30 minutes). The luminescence intensity (I_t) from the first sample was determined (cuvette number 1). Equal time intervals between each sample were used.

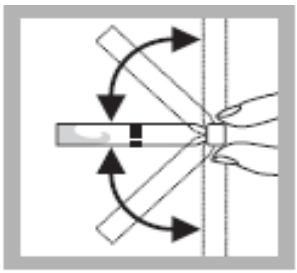
8. The EC_{50}-value was determined by using standard linear regression analysis. If the range of value pairs cannot be linearized, the EC_{50}-value can be determined graphically using a double logarithmic co-ordinate system. The % INH was plotted on the y-axis and the concentration (in mg/l, mol/l or % of original sample) on the x-axis.

APPENDIX 14: NITROGEN AMMONIA (SALICYLATE METHOD) HACH DR 5000 TEST N TUBE 831 (LOW RANGE) AND 832 (HIGH RANGE) PROCEDURE (DR5000 procedure manual)

	LR (1 – 12 mg/L)	HR (2 – 47 mg/L)
		
1. Carefully remove the protective foil lid from the DosiCap™ ***Zip***. Unscrew the cap from the vial.	**2.** Carefully pipet 0.5 mL (500 µL) of sample into the vial. Immediately proceed to step 3.	**2.** Carefully pipet 0.2 mL (200 µL) of sample into the vial. Immediately proceed to step 3.
		
3. Flip the DosiCap ***Zip*** over so that the reagent side faces the vial. Screw the cap tightly onto the vial.	**4.** Shake the capped vial 2–3 times to dissolve the reagent in the cap. Verify that the reagent has dissolved by looking down through the open end of the DosiCap ***Zip***.	

APPENDIX 14: Continued…

5. Wait 15 minutes.



6. After 15 minutes, invert the sample an additional 2–3 times to mix.

The color remains constant for an additional 15 minutes after the timer expires.

7. Thoroughly clean the outside of the vial.

8. Insert the prepared vial into the cell holder. Close the lid.

The instrument reads the barcode, then selects and performs the correct test. Results are in mg/L NH_3–N.

No instrument Zero is required.

APPENDIX 15: pH USING THE CRISON MM41 MULTYMETER

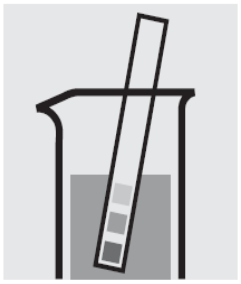
Apparatus

a. Crison pH/EC meter with magnetic stirrer

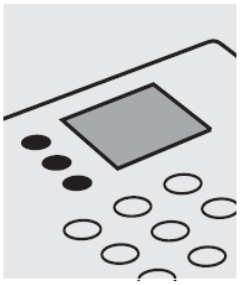
Procedure:

a. *Instrumentation Calibration*: The pH probe was calibrated using 4.00, 7.01 and 9.10 calibration buffers.
b. *Measurement of pH*: A 50 mL sample was poured in glass beakers and pH probe submersed in sample. Stirrer was turned on and the pH reading was recorded once the reading stabilised.

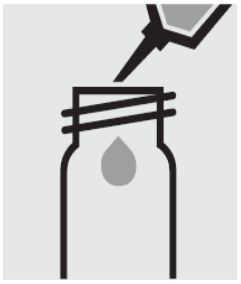
APPENDIX 16: FREE CHLORINE USING THE MERCK PICCO METER (Picco meter procedure manual)



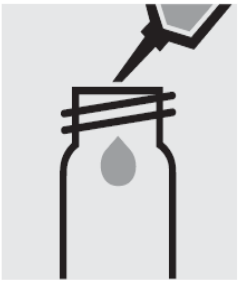
Check the pH of the sample, specified range: pH 4 – 8.
If required, add dilute sodium hydroxide solution or sulfuric acid drop by drop to adjust the pH.



Select method (1)(3)(0), select subitem >>free.

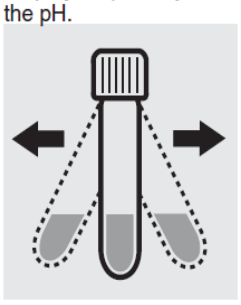


Fill approx. 10 ml of distilled water into an empty 16-mm cell (**do not add any reagents!**), close with the screw cap.
(Blank cell)



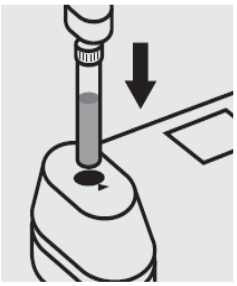
Pipette 5.0 ml of the sample into a round cell.

Add 1 level blue micro-spoon of **Cl_2-1**, close with the screw cap.

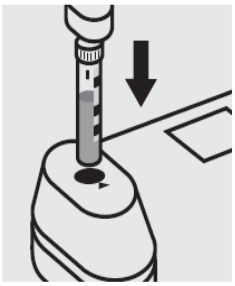


Shake the cell vigorously to dissolve the solid substance.

Reaction time:
3 minutes
Press (Enter) to start the countdown.

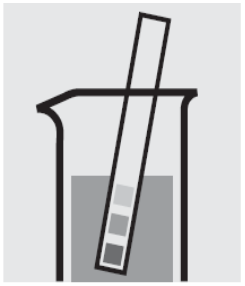


Insert the blank cell into the cell compartment.
Press (Zero).

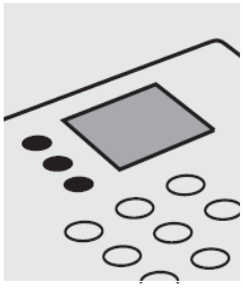


Insert the cell containing the sample into the cell compartment. Align the mark on the cell with that on the photometer.
Press (Test).

APPENDIX 17: TOTAL CHLORINE USING THE MERCK PICCO METER (Picco meter procedure manual)

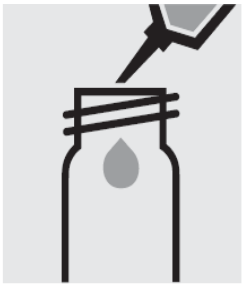


Check the pH of the sample, specified range: pH 4 – 8.
If required, add dilute sodium hydroxide solution or sulfuric acid drop by drop to adjust the pH.



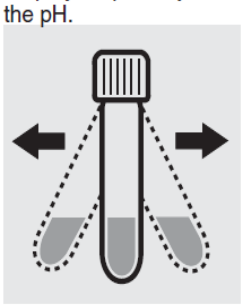
Select method (1)(3)(0), select subitem >>total.

Fill approx. 10 ml of distilled water into an empty 16-mm cell (**do not add any reagents!**), close with the screw cap.
(Blank cell)

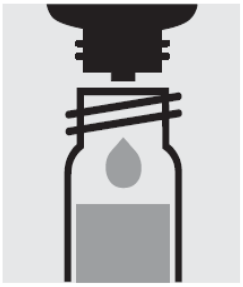


Pipette 5.0 ml of the sample into a round cell.

Add 1 level blue micro-spoon of **Cl_2-1**, close with the screw cap.

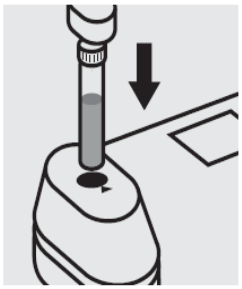


Shake the cell vigorously to dissolve the solid substance.

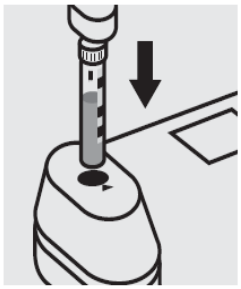


Add 2 drops of **Cl_2-2**, close with the screw cap, and mix.

Reaction time:
3 minutes
Press (Enter) to start the countdown.



Insert the blank cell into the cell compartment.
Press (Zero).



Insert the cell containing the sample into the cell compartment. Align the mark on the cell with that on the photometer.
Press (Test).

APPENDIX 18: PUBLISHED MANUSCRIPTS

1. Application of radio-immunoassays to assess the fate of estrogen EDCs in full scale wastewater treatment plants
2. Evaluating the Acute Toxicity of Estrogen Hormones and Wastewater Effluents Using Vibrio fischeri