

**DETECTION OF *AEROMONAS* SPECIES IN RELATION TO THE OCCURRENCE  
OF ESTROGENS AND TESTOSTERONE IN VARIOUS WATER SOURCES IN  
LIMPOPO PROVINCE, SOUTH AFRICA AND LUSAKA, ZAMBIA**

BY

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of Science (MSc) in Microbiology

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## DECLARATION

I Manavhela Murendeni, student no: 11606607 hereby declare that this dissertation submitted for the Degree MSc in Microbiology at the University of Venda, is my original work and has not been submitted to this or any other institution of higher education. I further declare that all sources cited or quoted are indicated and acknowledged by means of a compiled list of references.

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## Dedication

I dedicate this dissertation to my son.

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## List of abbreviations

<b>AVP</b>	Arginine Vasopressin
<b>AK</b>	Amikacin
<b>β</b>	Beta
<b>Bp</b>	Base pair
<b>Bla</b>	Beta-lactamase
<b>°C</b>	Degree Celsius
<b>CAZ</b>	Ceftazidime
<b>CDC</b>	Center for Disease Control and prevention
<b>CFU</b>	Colony forming units
<b>CM</b>	Centimeter
<b>CRO</b>	Cetriazone
<b>CSLI</b>	Clinical and Laboratory Standards Institute
<b>CXM</b>	Cefuroxime
<b>DNA</b>	Deoxyribonucleic acid
<b>E</b>	Erythromycin
<b>ESBL</b>	Extended spectrum beta-lactamases
<b>E1</b>	Estrone
<b>E2</b>	Estradiol
<b>E3</b>	Estriol
<b>EE2</b>	Ethinylestradiol
<b>EF</b>	Free estriol
<b>ET</b>	Total estriol
<b>ELISA</b>	Enzyme-linked immunosorbent assay

<b>EDCs</b>	Endocrine disrupting chemicals
<b>g</b>	Gram
<b>GN</b>	Gentamicin
<b>hrs</b>	Hours
<b>KZ</b>	Cefazolin
<b>MIC</b>	Minimal inhibitory concentration
<b>ml</b>	Milliliter
<b>NOR</b>	Norofloxacin
<b>NA</b>	Nalidix Acid
<b>PK</b>	Proteinase k
<b>SPSS</b>	Statistical Package for the Social Science
<b>TMP</b>	Trimethoprim
<b>TE</b>	Tetracyclines
<b>TOB</b>	Tobramycin
<b>TMP-SMZ</b>	Trimethoprim-Sulfamethoxazole
<b>μl</b>	Microliter
<b>UK</b>	United Kingdom
<b>WHO</b>	World Health Organization
<b>WRC</b>	Water Research Council
<b>WWTPs</b>	Wastewater treatment plants

## Research output emanating from the project

From the present study, five manuscripts have been developed. Of these one has been submitted to a peer reviewed Journal for consideration for publication while the last four are being finalized.

1. Manavhela M, Sichilima A, Samie A “Occurrence of 17 $\beta$ -Estradiol (E2) and potential effect on *Aeromonas* diversity in wastewater and fresh water fish samples”. Submitted to international journal of environmental science and technology.
2. Manavhela M and Samie A “Occurrence and distribution of steroid hormones in wastewater, surface water and fish samples from South Africa and Zambia”.
3. Manavhela M and Samie A “Prevalence of *Aeromonas* species from various sources of water and fish samples”.
4. Manavhela M and Samie A “Antibiotic resistance and detection of beta-lactamase genes of *Aeromonas* species isolated from aquatic sources and antimicrobial activity of medicinal plants”.
5. Manavhela M and Samie A “Influence of steroid hormones on the diversity of *Aeromonas* species isolated from aquatic sources”.

## SUMMARY

**Background:** The occurrence of microorganisms and endocrine disrupting chemicals (EDCs) in water poses a serious concern due to their effects on humans, animals and environment. In recent years, EDCs have been increasingly reported in rivers that receive large amounts of wastewater effluents. Of all the EDCs, natural and synthetic hormones are among those that are recognized for their potential to mimic or interfere with normal hormonal functions of humans and animals. The present study aimed at assessing the occurrence of these hormones in relation to the molecular diversity of *Aeromonas* and evaluating the resistance of *Aeromonas* to antibiotics as well as to assess anti-bacterial activity of two selected traditional medicinal plants.

**Methods:** Wastewater, water and fish samples were collected from various sources (rivers, wastewater treatment plants, taps, and dams) for the detection of hormones and isolation of *Aeromonas* species. The analysis of hormones from various organs of the fish and from water samples was conducted, after extraction using enzyme-linked immunosorbent assays (ELISA). Different types of hormones including Estriol, Estradiol, Ethinylesradiol and Testosterone were detected, and their concentrations determined. *Aeromonas* spp were isolated from the samples using microbiological methods and Conventional PCR was used for genotyping as well as for detection of the beta-lactamase genes. Kirby-bauer method was used to determine the susceptibility profiles of *Aeromonas* to different antibiotics. Microdilution assay was used to determine the Anti-bacterial activity of the plant (*Annoniceae* and *Zornia milneana*) extracts against *Aeromonas* species.

**Results:** A total of 144 samples were collected from 23 different locations in two countries: South Africa and Zambia. These included wastewater and treated wastewater, River water, fish and tap water. 17 $\alpha$ -ethinylestradiol (EE2) was detected

in most of the samples (92.7%) with concentrations varying from 0.59 ng/ml to 65 ng/ml. The hormones were also detected from drinking water, with testosterone detected at high concentrations of up to 140 ng/ml in tap water. Most sewage treatment plants were not able to remove the EE2 from the wastewater as the concentration of this hormone in the final effluent was almost always higher than that in the influent. These hormones were also detected in drinking water at high concentrations of up to 53.49 ng/ml in the tap water for EE2 and 1777 ng/ml for E2. The overall detection of *Aeromonas* species in the samples was 84.5%. *A. caviae* was the most prevalent species accounting for 73.6%, followed by *A. veronii* with 64.6%. The bacteria were completely resistant to cefuroxime accounting for 100% resistance. *Aeromonas* isolates also showed high resistance to trimethoprim (88.7% for *A. hydrophila*), cefazolin (highest 97.8% for *A. caviae*), and ceftazidime (83.9% for *A. sobria*). TEM was the most prevalent beta-lactamase gene with detection rate of 87%. All isolates lacked the presence of the CTX-M3 gene. Also, wastewater had the highest prevalence of *A. veronii* and *A. caviae* accounting for 87.5% and 82.5% respectively. Multiple antibiotic resistance was also observed with the *Aeromonas* isolates being resistant to up to 11 antibiotics. High prevalence of 77.1% of *Aeromonas hydrophila* was observed in the presence of ethinylestradiol (EE2). *Aeromonas veronii* and *Aeromonas caviae* were the most predominant species in the presence of total estriol, *A. veronii* had a prevalence of 57.1% and *A. caviae* had a prevalence of 52.8%. *Aeromonas hydrophila* and *Aeromonas caviae* had the lower prevalence in the presence of hormones with the percentages of 26.1% and 27.8% respectively. The methanol extracts of both *Zornia milneana* and *Annona* species showed good activity against the *Aeromonas* spp with the lowest MIC of 0.078 mg/ml. Ethyl acetate extracts were the least effective.

**Conclusion:** This study has shown high occurrence of steroid hormones in all types of environmental samples tested. These included tap water, river water, wastewater and fish both in Zambia and South Africa. Therefore, steroid hormones constitute an important health problem in the Southern African Sub-Region. The incapacity of the wastewater treatment plants to remove EE2 is an important problem that needs to be tackled immediately. The prevalence of *Aeromonas* species is very high in our environmental water as well as in drinking water, with the highest prevalence observed in fish and wastewater. It was also revealed that there is a relationship between steroid hormones and *Aeromonas* species, with the hormones supporting the growth of *Aeromonas* species. The presence of beta-lactamase genes which causes *Aeromonas* to be resistant to antibiotics was also noted. Methanol extracts of *Zornia milneana* and *Annona spp* were the most effective against *Aeromonas spp* and could serve as primary sources for the isolation of lead compounds.

**Keywords:** *Aeromonas*, Wastewater, ELISA, Hormones, Estrogenic activity, Limpopo, Southern Africa, beta-lactamase genes, Antibiotic resistance

## CHAPTER ONE: INTRODUCTION

### 1.1 Introduction

Estrogens are ubiquitous, small steroid compounds that function as hormones and are key regulators of physiological changes associated with reproduction in males and females. Estrogens regulate many other important physiological processes, including immune functions and mineral homeostasis (Pinto et al., 2014). The levels of estrogens in water have become a global concern for more than a decade now, due to the risk it poses to human and animal life (Naldi et al., 2016). For example, the exposure to natural and synthetic hormones may interfere with reproduction and development in humans and animals (Faul et al., 2014; Mohagheghian et al., 2014). The most significant entry route for these compounds into the environments is their release from waste water treatment plants, and this is supported by their common detection at high levels in wastewater effluents (Singh et al., 2010). In rural areas toxic/estrogenic compounds that originates from agriculture, industrial and households waste, end up contaminating water and in some cases could go undetected (Apia, 2018). Thus, there is an elevated risk of human exposure to environmental contamination (Aneck-Hahn et al., 2009). Steroid hormones (estrogens, androgens bisphenol A and others) are among those toxic compounds which contaminate the environment.

Estrogenic steroids such as estrone (E1), estradiol (E2), estriol (E3), and ethinylestradiol (EE2) are among the most potent endocrine disrupting compounds (Mohagheghian et al., 2014). Many studies done in the UK, Japan and North America, have shown that consistent exposure of fish to high concentrations of estrogens have

caused reproductive abnormalities (Manickum et al., 2011). Globally, estrogens are the primary pollutants contributing to the estrogenic activity (which is defined as the compounds/chemicals that have the ability to mimic the normal functions of naturally occurring estrogens) in water bodies (Snyder et al., 1999; Garriz et al., 2015). Among the steroidal hormones, estrogens have been receiving a lot of attention; but there are also other types of steroid hormones that have been detected in the aquatic environment such as androgens. Like estrogens, androgens have been detected in surface water as well as sewage effluents (Örna, 2016). In 2002, Kolpin and colleagues detected 40% of reproductive hormones in streams in 30 different states (Kolpin et al., 2002). In Chinese streams, surface water was found to have 54.5% of steroid hormones. Estradiol was found to be the main contributor of estrogenicity with a ratio of 82.8% (Yao et al., 2018).

Water is an essential element of life on earth and people rely on water for drinking and for domestic purposes. However, water also contains microorganisms and other aquatic organisms as well as chemicals (steroid hormones) that pose threat to human and animal health (Ying et al., 2011). Water is also considered as a vehicle for the spread of human opportunistic and pathogenic bacteria. *Aeromonads* for example; are known to cause infections in humans and fish. They are found in habitats such as fresh and brackish water, as well as in soil (Catagay et al., 2014). The Occurrence of *Aeromonas* pathogens in water could be used as indicators of fecal contamination and its presence may have severe health implications on consumers especially those who are immunocompromised (Mulamattathil et al., 2014).

Members of the genus *Aeromonas* are known to cause a wide spectrum of diseases in man and animals (Ghenghesh et al., 2008). Some studies have shown that some motile *Aeromonas* species are becoming food and waterborne pathogens of

increasing importance (Araujo et al., 2002; Ansari et al., 2011). They have been associated with several food-borne outbreaks and are progressively being isolated from patients with traveller's diarrhea (Gravenitz et al., 2007).

Presently, as a presumed emerging enteric pathogen, *Aeromonas* species can grow in water distribution systems, especially in biofilms, where they may be resistant to chlorination (Chauri et al., 2001). For example, *Aeromonas hydrophila* is listed in the contaminant candidate list, and Environmental protection agency method 1605 has validated its detection and enumeration in drinking water systems (Igbinosa et al., 2012). Elsewhere, the use of antibiotics in treating bacterial infections in humans and aquaculture has increased. Generally, in aquaculture they are incorporated in the feed or by directly putting them in water for the prevention of the spread of diseases (Defoirdt et al., 2011). The extensive use of the antibiotics has in turn led to microorganisms developing resistance against them. *Aeromonas* species are among bacteria that are becoming resistant to antibiotics, this is mainly due to the wastewater discharges that contains different chemicals including antibiotics as well (Odeyemi and Ahmad, 2017). Antibiotic resistance of *Aeromonas* species to a variety of antibiotics is a human health concern since the resistant bacteria can be acquired by humans from aquatic environments through food chains (Scarano et al., 2018). CDC has reported that in the US 2 million people are infected with antibiotic-resistant bacteria, and a minimum of at least 23,000 people die yearly. Many microorganisms are known to produce beta lactamases, with *Escherichia coli* and *Klebsiella* being among the most documented in literature. The emergence of Extended Spectrum Beta Lactamases (ESBLs) is an important concern for human health especially in countries where second line of treatment is not available (Guiral et al., 2018). The emergence of drug resistance has drawn the attention of researchers to explore medicinal plants in order

to develop new medicines that are effective against resistant microorganisms (Romha et al., 2018).

Traditionally, herbal medicine has been used to treat inflammation, pain, and inflammatory-mediated pain. Medicinal plants are known to contain a variety of nutritional values and bioactive compounds that have activity against inflammation disorders and different microorganisms (Abu Bakar et al., 2018). Medicinal plants are an integral part of the development of modern medicine (Akram et al., 2014). In developing countries many people still rely on the use of traditional medicine for therapeutic purposes (Hayta et al., 2014). In this study, we evaluated the antibacterial activity of *Zornia milneana* and *Annoniciae* against *Aeromonas* species.

## 1.2 Study rationale

In recent years the use of contraceptive pills among women has increased, and these pills contain estrogens at high levels particularly the synthetic  $17\alpha$  ethynilestradiol. Their disposal in the sewage influent increases their levels in the water environment. People end up drinking water containing those high levels of hormones which may cause health complications. They interfere with the normal functioning of animals and human body systems (WRC., 2011). High levels of synthetic and normal estrogens in the human body are known to cause diseases such as breast cancer, testicular cancer, testicular dysgenesis syndrome (low sperm count). In a study done by Stachefeld in 2008, it was observed that estradiol in young women lowers the osmotic threshold for arginine vasopressin (AVP) and the stimulation during saline infusion and dehydration. In postmenopausal women, estradiol reduced the osmotic threshold for

AVP release during hypertonicity. Therefore, since AVP plays a role in water homeostasis if its release is reduced, it could lead to dehydration because the volume of water lost in the body will not be replaced (Sharman and Low., 2008).

In the aquatic organisms with special reference to fish, exposure to steroid hormones is known to cause negative effects such as sex reversals, intersex, alteration in mating, affects gonad maturation and they induce vitelogenin and finally they also cause feminisation (Carvalho et al., 2014). These hormonal compounds are thought to serve as nutrients to bacterial communities because they are degraded metabolically. Therefore, their presence in environmental waters may cause overflow of pathogenic bacteria which are harmful to both man and aquatic organisms, and some studies have reported on the increase of cytotoxicity to aquatic organisms with the increase of estrogenic activity (Bornman et al., 2007). Therefore, this study focused on *Aeromonas* species since they are known pathogens to man and fish. They cause a wide spectrum of diseases. For example, in man they cause septicaemia and gastroenteritis and in fish they cause furunculosis, and *Aeromonas* motile septicaemia. *Aeromonas* have been reported to be responsible for food borne and water-borne outbreaks; due to consistent isolation of these organisms from patients with traveller's diarrhea and also on the various organs of fish (Igbinosa et al., 2012). It is vital that a comprehensive study be conducted with intention to evaluate the occurrence of *Aeromonas* in various water sources including drinking water as well as in the various organs of fish. Although, other studies have reported on the occurrence of *Aeromonas* species in tissues of fish and in various water sources, there is dearth of information on the occurrence of *Aeromonas* in various water sources in Limpopo. In this province there are a lot of rural areas where people still use river water, natural springs for drinking purposes, and for washing their clothes (this was observed during sample

collection). Those who live in urban areas of the Province rely on ground water, recycled water and bottled water for drinking; and most plastic bottles contains compounds such as Bisphenol A which have estrogenic activity. People are not aware of estrogenic contaminants in these sources of water, and how prolonged exposure to these contaminants can cause human health problems

Of major concern is the toxicity in drinking water which causes human health problems which needs to be attended to; therefore, it is important to assess the toxicity in drinking water and sewage effluents for the sake of wildlife (Guang-Guo et al., 2011). There is one study that has assessed estrogenic activity in Limpopo province, South Africa (Aneck-hahn et al., 2009). However, the data collected was from one District of the province: the Waterberg District. Our study provided a chemical assessment of the presence and distribution of selected hormones and their potential effects on *Aeromonas* growth in the water environment. Currently, as far as we know there is only limited data available on the estrogenic contamination in various sources of water in South Africa as a whole. Furthermore, there is insufficient information on the occurrence of *Aeromonas* in relation to the concentration of hormones in wastewater treatment plants influents and effluents, drinking water, dams and rivers found in Limpopo province of South Africa. Few studies showed that estrogens have a significant impact on the growth and diversity of microorganisms in soil (Durham and Perry, 1956; Zhang et al., 2014). However, in the aquatic environments we are also facing the problem of other wide variety of chemicals including antibiotics compounds. Their persistence in aquatic environments and microorganisms being exposed to them for a long time, leading to microorganisms developing antibiotic resistance (WHO 2014).

Bacterial resistance to antibiotics is a rising global health problem. This calls for more research to develop new drugs to try and control infections (Carvalho and Santos, 2016). As far as we know, no study has been conducted to assess the effects of estrogens/steroid hormones on microorganisms in water in the Vhembe district or anywhere in South Africa. Therefore, this study will provide information on the occurrence of steroid hormones and assess their influence on microbial growth with special reference to *Aeromonas* species and their antimicrobial resistance profile.

### **1.3 Objectives of the study**

#### **1.3.1 Main objective**

The main objective of the present study is to assess the occurrence and molecular characteristics of *Aeromonas* species and to determine concentrations of steroid hormones in various water sources (wastewater, drinking water, surface water) in Limpopo Province, South Africa and in the Lusaka area, in Zambia.

#### **1.3.2 Specific objectives**

The specific objectives are:

- To isolate and identify *Aeromonas* species from various water sources (wastewater and drinking water and surface water) in the study area.
- To determine the occurrence and concentration of hormones (Estriol, 17  $\beta$  Estradiol, 17 $\alpha$  ethynilestradiol and testosterone) in various water sources.
- To assess the correlation between the occurrence of hormones and that of the *Aeromonas* isolates and their diversity in various water sources.

- To determine the antibiotic susceptibility profiles as well as to determine the presence of antibiotic resistance genes among the *Aeromonas* isolates.
- To determine the potential antimicrobial activity of two selected medicinal plants against the *Aeromonas* isolates.

## 1.4 Hypotheses

In the present study we hypothesise that:

- The increase in the concentration of hormones leads to the increase of *Aeromonas* concentration and diversity.
- Antibiotic resistance is common among *Aeromonas* species isolated from water sources around the Limpopo Province.
- Medicinal plants can be used in order to control the occurrence of *Aeromonas* isolates.

## 1.5 Reference

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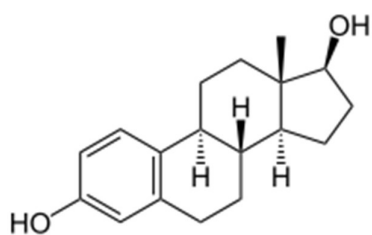
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## CHAPTER 2: Literature review

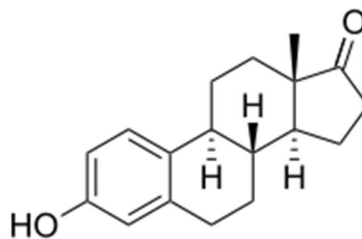
### 2.1 Steroid hormones

#### 2.1.1 What are hormones?

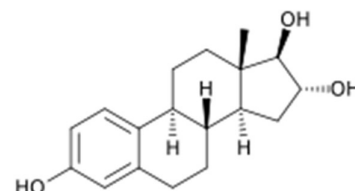
Hormones are chemical messengers, or regulatory substances produced by specialised tissues called ductless glands and are transported in blood stream to stimulate specific tissues into function (Pinto et al., 2014). These hormones affect important functions such as metabolism, growth and sexual maturation, reproduction and adaptation to the environment. Hormones are a group of biologically active compounds that are synthesized from cholesterol and have in common a cyclopentan-o-perhydrophenanthrene ring (Petrivic et al., 2013). Natural hormones are secreted by the adrenal cortex, testis, ovary and placenta in human and animal, and include progestogens, glucocorticoids, mineralocorticoids, androgens and estrogens. In addition, there are some synthetic hormones such as ethynylestradiol (EE2) and mestranol (MeEE2) used as contraceptives (Ying et al., 2002). Different structure of steroid hormones are shown in figure 2.1 below.



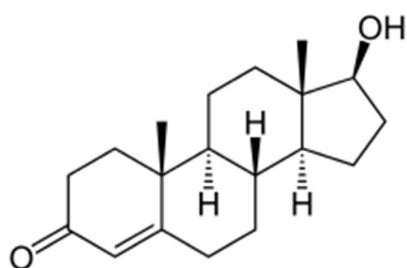
Estradiol



Estrone



Estriol



Testosterone

Figure 2.1 Chemical structures of different steroid hormones (Barron., 2011)

## 2.1.2 Classification of hormones

Hormones are classified by various criteria; based on the proximity of their site of synthesis to their site of action, chemical structure and the degree of their solubility in aqueous medium (Losel and Wehling, 2003).

Classification by proximity of their site of synthesis to site of action include 3 classes of hormones, namely: 1. Autocrine hormones: hormones that act on the same cell that synthesised them. 2. Paracrine hormones: hormones that are synthesised close to their site of action. 3. Endocrine hormones: hormones that are synthesised by endocrine glands and transported in the blood to target cells that contain the appropriate receptors (Melmed, 2016).

Classification based on chemical structure includes 4 classes of hormones: steroidal hormones (Estradiol, Testosterone, Cortisol, and Aldosterone), protein hormones (Insulin), fatty acids derivatives (Eicosanoids) as well as amino acid derivatives (Thyroid hormones, Adrenaline, Catecholamines) (Farquhar and Palade, 1964,).

Figure 2.2 shows a simple tree diagram that shows how hormones are classified.

# Hormone Classification

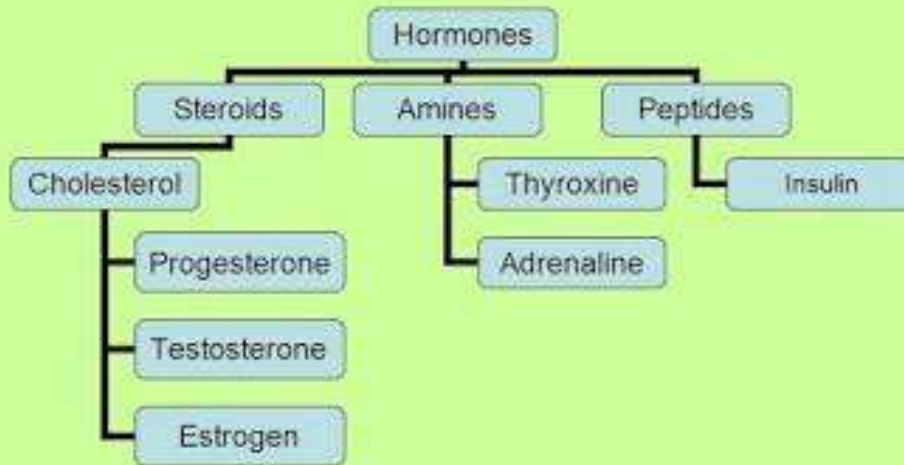


Figure 2.2 Classification of steroid hormones (<https://slideplayer.com/slide/8383541/>)

### 2.1.3 Physical and chemical properties of hormones

The physical and chemical properties of estrogens and testosterone are summarized in a table 2.1 below.  $17\beta$  estradiol has a molecular weight (Mw) of 272.39  $\mu\text{g/mol}$ , and the octanol/water partition coefficient ( $k_{ow}$ ) of 13. There is no data available for the PKa and the EFF for testosterone.

Table 2.1: Physical and chemical properties of estrogens and testosterone considered in this study.

Steroid hormone	Molecular weight [g/mol]	Water solubility 20 C [mg l <sup>-1</sup> ]	Log Kow at pH 7	pKa	EEF
$17\beta$ -Estradiol	272.39	3.94	13	10.6	1
Estriol	288.39	2.81	13	10.05	$5.9 \times 10^{-3}$
$17\alpha$ -Ethinyl estradiol	296.41	4.15	4.8	10.4	1.25
Testosterone	288.4	18.0-25.0	3.2	-	-

Ma and Yates, 2018;

## 2.1.4 Daily excretions of steroid hormones

Males secrete around 6500  $\mu\text{g/day}$  of androgens, particularly testosterone. Cycling women also excrete large amounts of estrogens with estimated 82-695  $\mu\text{g/day}$  of Estradiol (E2), and they in urine they excrete 3-65  $\mu\text{g/day}$ . Males also secrete estrogenlike Estrone (6.5  $\mu\text{g/day}$ ) and Estradiol (35  $\mu\text{g/day}$ ), see table 2.2 below

Table 2.2: Human production and excretion of estrogens.

Sex steroid	Amount excreted in urine ( $\mu\text{g/day}$ )	Amount produced ( $\mu\text{g/day}$ )	Sex
17 $\beta$ -Estradiol		82–695	Female (cycling)
17 $\beta$ -Estradiol	0.3-5	13	Female (pre-pubertal)
17 $\beta$ -Estradiol	-	48	Male
17 $\beta$ -Estradiol	1.5	6.5	Male (pre-pubertal)
Estriol	-	–	Female (pregnant)
Estrone	3-65	110–497	Female (cycling)
Estrone		41	Female (pre-pubertal)
Estrone	2-20	88	Male
Estrone	-	35	Male (pre-pubertal)
Androgens	3	6500 (testosterone)	Male
Androgens	-	240 (testosterone)	Female

Shore and Shemesh, 2003

### **2.1.5 Natural roles of steroid hormones**

Estrogens are female hormones, and they mainly maintain the health of the reproductive tissues, breasts, skin and brain (Manickum and John, 2011; Cui et al., 2013). Progestogens can be thought of as a hormonal balancer, particularly of estrogens. Androgens play a key role in tissue regeneration, especially the skin, bones and muscles. According to integrative wellness and research center inc., 2016; Glucocorticoids are produced by the adrenal glands in response to stressors such as emotional upheaval, exercise, surgery, illness or starvation. All the hormones exert their actions by passing through the plasma membrane and binding to intracellular receptors (Guang-guo et al., 2002). In 2015, Rochira and colleagues described that estrogens accelerate bone elongation at puberty, epiphyseal closure, achievement of peak bone mass and it maintains the bone mass in males (Rochira et al., 2015). In females estrogens are mainly responsible for endosteal bone formation.

### **2.1.6 Water quality and sources of hormonal contamination**

Water is essential for life and humans use it for their different need including drinking and cleaning. For water to be considered drinkable (potable) it has to fulfil a certain number of criteria including microbial, aesthetic and chemical qualities. If one of these water qualities is compromised, then the quality of water is considered poor. Poor water quality, be it drinking water or environmental water is affected by many things including runoffs, erosion, increased sedimentation, chemicals, pesticides and others. All these factors contribute to reducing the quality of water and they are all from different sources (Chapman and WHO, 1996).

There are different sources of contamination of water sources by steroid hormones. These may include sewage effluents (containing human waste), industrial waste, agricultural runoffs, animal wastes as well as landfills. From our homes, industries and different institutions, sewage treatment plants receive large amounts of steroid hormones that we excrete through urine, which during treatment they are not completely removed and therefore they end up in surface water such as rivers, dams, streams, and ponds (Li et al., 2016). People in different communities both in urban as well as rural areas dispose off some of the waste products in landfills and during runoffs they end up in environmental waters. Everyday animals excrete quite large amounts of hormones also (especially cows because they are fed estrogens in order to improve milk production) which end up in surface waters where aquatic organisms are exposed and some people use surface water for domestic purposes (Shore and Shemesh, 2003). Table 2.3 shows estrogen concentration produced by animals daily and figure 2.3 below shows the different sources of hormone contamination. Table 2.4 shown the occurrence of the hormones in different water and wastewater sources.

Table 2.3: Summary of estrogen concentrations (ng/l) in the environment. (NDA=no data available).

Sample type	E1	17 $\alpha$ -E2	17 $\beta$ -E2	E3	EE2
Slurry in swine pit	5900–150,000	4000–84,000	1800–49,000	NDA	NDA
Swine farm effluent	5200–5400	650–680	1000–1500	2200-3000	NDA
Slurry in dairy pit	2500–80,000	2000–5000	800–27,000	NDA	NDA
Treated cattle feedlots	720	1100	1250	NDA	NDA
Dairy farm wastewater	370–2356	1750–3270	351–957	NDA	NDA
Lagoon pond	650	NDA	NDA	NDA	NDA
Biogas digestate	593	50	24	NDA	NDA
Sow urine	416-490	NDA	85-97	127-193	NDA
Grazing land water	78	31	18	NDA	NDA
Swine manure	70	175	15	NDA	NDA
Swine manure leachate	68.1	2.5	NDA	NDA	NDA
1m deep fround water	68.1	NDA	2.5	NDA	NDA
STP/effluent	12-196	6.4–12.6	6.2-42.2	NDA	0.59-5.6
Sea water	NDA	NDA	0.83	NDA	4.67

(Adeel et al., 2017)

Table 2.4: Literature on estrogenic activity on various sources of water

Country	Water source	Oestrogenic activity	Bioassay used	Reference
Japan	Sewage treatment effluents	5-15 ng/l	YES	Matsui et al., 2000
	Lake water	1 ng/l	YES	Matsui et al., 2000
Korea	River water	0.5 pg/l - 7.4 ng/l	E-screen	Oh et al., 2000
Korea	River water	0.005-1.92 ng/l	E-screen	Oh et al., 2006
China, Lake Taihu	Lake water	4.8-12.1 ng/l 1	4.8-12.1 ng/l	Shen et al., 2001
China, Lake Taihu	Lake water	2.2-8.3 ng/l	HGELN*	Shen et al., 2001
Belgium	Rivers	10 ng/l	YES	Witters et al., 2001
United Kingdom	Streams	0- 26.5 ng/l	YES	Matthiessen et al., 2006
South Africa	Surface, ground and river water	0.31-2.1 ng/l	YES	Aneck-Hahn et al., 2008
South Africa	Small industry effluents	0.001-5.8 ng/l	YES	Mahomed et al., 2008
South Africa	Surface, ground and river water	0.16- 1.92 ng/l	YES	Bornman et al., 2007
South Africa	Surface, ground and river water	0.32-16.0 ng/l	ER-Calux®	Bornman et al.
South africa	Surface and ground water		RBCA	Aneck-Hahn et al., 2009

Aneck-hahn et al., 2009

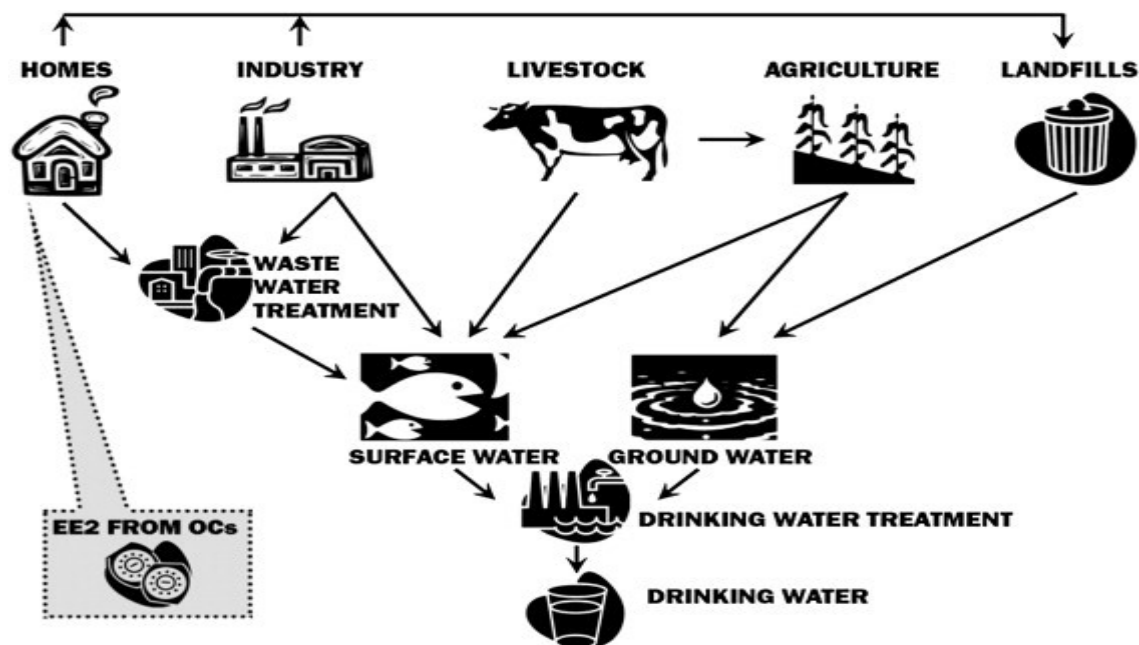


Figure 2.3. Sources of steroid hormones in the environment (wise et al.,2010)

### 2.1.7 Effects of steroid hormones in humans and animals.

In humans, steroid hormones are known to have health implications as well as in animals. These compounds could affect wildlife and human health by disrupting their normal endocrine systems. These steroids have been detected in effluents of sewage treatment plants and surface water (Ying et al., 2002). The presence of the steroids in the environment poses a significant potential problem of interference with normal function of the endocrine systems of wildlife and can thus affect reproduction and development in wildlife (Manickum and John, 2014).

In humans, steroid hormones cause cancers such as testicular and breast cancer in men and women and they also cause a condition known as testicular dysgenesis (low sperm count) which leads to infertility problems (Lubber et al., 1992). In fish they cause

intersex, sex reversals, induction of vitelogenin and changes in sexual behaviour of several other aquatic organisms such as frogs, algae and benthic organisms (Pal et al., 2010; Esteban et al., 2014). During developmental stages, previous studies have reported that elevated concentrations of estradiol in pre-pubertal children may result in excessive rapid growth and early onset of puberty in females and late onset of puberty in males. (Heffron et al., 2016) in post pubertal development estrogens can induce testicular and ovarian cancer and can stimulate endometriosis, heart diseases, osteoporosis and Alzheimer's diseases (Wright-Walters et al., 2007; Heffron et al., 2016), as well as promoting the development of thyroid cancer especially in women because they are more exposed to high levels of estrogens during menstruation cycle, lactation and pregnancy (Zhang et al., 2017).

#### **2.1.8 Effects of steroid hormones on microorganisms**

The effect of hormones on microorganisms varies. In some cases, it might be beneficial while in some cases it might be inhibitory for the growth of microorganisms. Steroid hormones have been reported to serve as nutrients for some bacterial organisms (Purdom et al., 1994). A study done by Zhang and colleagues in soil showed that estrogens enhanced the growth of soil bacterial community (Zhang et al., 2014). This suggests that synthetic hormones do have an effect on microorganisms in soil. In a study which investigated the influence of the synthetic hormone trenbolone on sediment microbial enzyme activity, trenbolone was reported to have an inhibitory effect on sediment N-acetyl-glucosaminidase activity (Radl et al., 2005; Hotchkiss et al., 2007).

Other than modulation of the immune system, endogenous steroid hormones have been reported to have a direct effect on the metabolism, growth and expression of virulence factors of bacteria. For example, it is said that when a woman is pregnant the proportion of certain bacterial species associated with plaque microbiota is altered with significant increase in the ratio of anaerobic and facultative bacteria (Garcia-Gomez et al., 2012). Bacteria such as *Prevotella intermedius* is reported to be among the anaerobic and it's said to uptake estradiol and progesterone which enhances its growth. It has also been demonstrated that progesterone inhibits the growth of *Neisseria gonorrhoea* and *N. meningitis* (Shah et al., 1990). However, very few studies have been conducted in water.

### **2.1.9 Degradation of steroid hormone**

There are different methods used in the degradation of hormones, which occurs in two forms: aerobic and anaerobic degradation.

#### **Aerobic degradation**

There are several microorganisms that have been identified as utilizers of estrogens that were isolated from media such as soil, sandy aquifers, compost and seas. It has been reported that these isolates belong to the  $\alpha$ ,  $\beta$ , and  $\gamma$  proteobacteria. There are two known marine microbial isolates that were observed to degrade testosterone and  $\beta$ -estradiol (Yu et al., 2013).

#### **Anaerobic degradation**

It has been reported that some intestinal microbes are able to degrade estrogens aerobically or in anaerobic conditions. In activated sludge it was observed that E2 was

degraded to E1 anaerobically when amended with acetone. In 2006 Czajka and Londry noted that there was a reversible conversion between E2 and E1 anaerobically. They also observed that E1 and E2 can be converted to EE2 under anaerobic conditions (Czajka and Londry, 2006; yu et al., 2013). Figure 2.4 shows the transformation of steroid hormones by microorganisms.

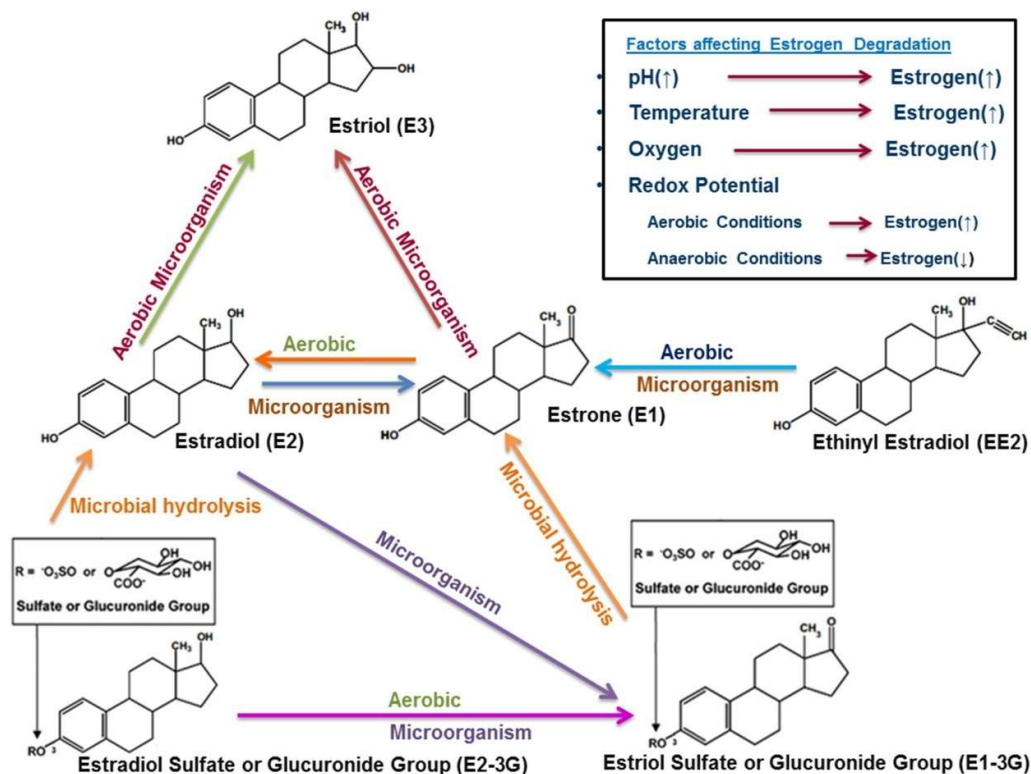


Figure 2.4: Biodegradation of steroid hormones by microorganisms in the environment.

(Adeel et al., 2017)

## 2.1.10 Methods for the removal of hormones from water

Current methods used for the Removal of estrogens from the water involves physical and biological processes.

### 2.1.10.1 *The physical processes include sorption and membrane filtration*

#### **a) Sorption**

The removal of estrogenic contaminants by sorption depends on the estrogen uptake from the aqueous phase onto the solid phase which a solid phase is the sorbent. This includes sorption onto activated sludge and sorption onto adsorbent materials (Ren et al., 2007).

#### **b) Membrane filtration**

This is a process that involves a membrane which acts as a separation wall which allow some substances to pass through and retains some. Nowadays many water and waste water treatment plants use membrane filtration. There are variety of membrane filtration processes including microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). Previous studies reported that that these processes were the promising option for the removal of EDCs, including steroid hormones from water. (Bolong et al., 2009; Yoon et al., 2006). The elimination of EDCs by membranes includes combination of processes, adsorption, size exclusion and charge repulsion. The energy consumption and pressusure requirements for these processes follows the following order  $RO > NF > UF > MF$ , this shows that reverse osmosis will give the best results but because it consumes high energy it wouldnt be convenient. Few studies reported that reverse osmosis and nanofiltration membranes have rejection efficiency that strongly depend on the physicochemical properties of the EDCs (e.g.

molecular weight, 1-octanol-water partition coefficient ( $K_{ow}$ ), Water solubility ( $S_w$ ) and electrostatic properties, membrane operating conditions (flux and water feed quality), membrane properties (permeability, pore size, surface charge and hydrophilicity or hydrophobicity) as well as membrane parameters such as pH, temperature and salinity. All these factors determine the efficiency of the membranes (Suzuki and Maruyama, 2006).

#### *2.1.10.2 The biological processes include bacteria from activated sludge and microalgae*

##### **i. Activated sludge**

Activated sludge is a type of biological treatment that is applied in many wastewater treatment plants and is performed by a mixture of different microorganisms (aerobic and anaerobic microorganisms). This culture of microorganisms is mostly comprised of bacteria than fungi and protozoa. These types of bacteria gain energy during this process from organic matter that contains carbon (Li et al., 2005). Even bacteria that benefit from conversion of ammonia nitrogen to nitrate nitrogen. This process involves the adsorption of hormone compounds onto the activated sludge and then decomposition by microorganisms takes place within few hours, but for ethinylestradiol (EE2) takes up to 6 days for it to be degraded by a nitrifying activated sludge (Suzuki and Murayama, 2005).

##### **ii. Microalgae**

Shi et al., in 2010 investigated biodegradation and adsorption of estrone, estradiol and ethinylestradiol in ponds that have duckweed and algae. They employed lemma species as the duckweed and the algae was a mixture of cultures of about six algae

genera. They found that the presence of algae and duckweed speed up the removal of the hormones and after that the sorbed hormones were then biodegraded by the duckweed, microorganisms and algae.

## 2.2 AEROMONAS

### 2.2.1 What are *Aeromonas*?

*Aeromonas* are described as aerobic and anaerobic Gram-negative rod-shaped bacilli that are indigenous in aquatic environment and are opportunistic pathogens to aquatic organisms and humans belonging to the family *Aeromonadaceae* (Aravena-Roman et al., 2013; Abbot et al., 2003; Martinez-Murcia, 2016). They are oxidase and catalase positive, and they are facultative anaerobic rods which are all motile except for one (*Aeromonas salmonicida*) which is non-motile. *Aeromonas* are chemoorganotrophic showing oxidative and fermentative metabolism of glucose. They produce variety of exoenzymes such as Arylamidases, Amylase, Dnase, Chitinase, Hemolysins, Esterases, Peptidases, Proteases, and Chondroitinase (Horneman and Ali, 2011).

### 2.2.2 Classification of *Aeromonads*

The current classification of the genus *Aeromonas* is based on DNA-DNA hybridization and 16S ribosomal DNA relatedness. The genera of the family *Aeromonadaceae* now include *Oceanimonas*, *Aeromonas*, *Tolumonas (incertaesedis)*, and *Oceanisphaera* (Carriero et al., 2016). *Aeromonas* species are classified as mesophilic species

because all of them except *Aeromonas salmonicida* which does not grow at 37°C but instead grows at cold temperatures (Janda and Abbot, 2010).

The formal description of the genus was made by Stainer in 1943, and in the 1970s *Aeromonas* species were classified into 2 groups based on their growth temperature, motility and pigment production (Janda and Abbot, 2010). One group comprised mesophilic strains able to grow at 37°C, motile and non-pigmented, mainly associated with human clinical infections and represented by *A. hydrophila*. The other group comprised psychrophilic strains (optimum growth at 22-28°C), non-motile and pigmented, which were mainly fish pathogens represented by *A. salmonicida*. In the 1980s, DNA-DNA hybridization assays allowed the differentiation of various genospecies or hybridization groups (Hidalgo and Figueras, 2012).

### **2.2.3 Biology/Morphology of *Aeromonas* species**

The genus *Aeromonas* is made up of straight, coccobacillary-to-bacillary Gram-negative bacteria with surrounding ends measuring 0.3–1.0 × 1.0–3.5µm (Roman et al., 2013). Most motile strains produce a single polar flagellum, while peritrichous or lateral flagella may be formed on solid media in some species. *Aeromonas* spp. grow readily on basic laboratory media such as heart infusion agar (Janda and Abbot, 2010). *Aeromonas hydrophila* is Gram-negative, non-spore-forming and rod-shaped to coccoid cell with rounded ends. They are oxidase and catalase positive, produce nitrate to nitrite and ferment D-glucose (Sarkar et al., 2012).

#### **2.2.4 Pathogenesis of *Aeromonads***

Most of the *Aeromonas* species are opportunistic pathogens. The most common ones being *A. veronii*, *A. hydrophila*, *A. caviae*, *A. sobria* well as *A. salmonicida* (Sun et al., 2016). The pathogenesis of *Aeromonas* infections results from the multiple virulence related factors such as biologically active substances, adhesion to organs and extracellular factors such as enzymes and toxins (Von Gravenitz, 2007; Janda and Abbot, 2010; Sun et al., 2016).

*Aeromonas* species produce a broad range of extracellular enzymes (such as beta-lactamases, proteases, lipases, amylases), some of which are thought to contribute to pathogenesis. Although the virulence of *Aeromonads* is known to depend on several factors, it is still incompletely understood despite decades of intense research (Ghenghesh et al., 2008). *Aeromonas* is said to be pathogenic because it possesses all the requirements of pathogenic bacteria. It attaches and enters into host cells through production of flagella, pili and adhesin. Multiplication in host tissue is assisted by the production of siderophores and outer membrane proteins, while production of capsule, Slayer, lipopolysaccharide, and porins contributes to their resistance to host defence mechanisms. Enterotoxins, proteases, phospholipases, and hemolysins cause damage to host cells leading to cell death (Isoken et al., 2012).

#### **2.2.5 Laboratory diagnosis of *Aeromonas* species**

In the laboratory there are different methods that are used to diagnose infections caused by *Aeromonas* species. Samples collected for laboratory diagnosis are cultured using different media, mainly used include *Aeromonas* selective agar and

blood agar (Chen et al., 2012). Samples mostly are enriched in alkaline peptone water for overnight in most cases and then the following day they are cultured on agar and incubated overnight at 37°C, followed by isolation of single colonies to enrich them in nutrient broth for extraction of DNA. Samples collected for laboratory diagnosis includes stool samples, blood samples and blood from wound infections.

Polymerase chain reaction (PCR) technique is a molecular method used to identify *Aeromonas* species that may be causing different infections in man and fish.

### **2.2.6 Treatment of infections caused by *Aeromonas* infections**

*Aeromonas* species are susceptible to some antibiotics. However, they are also resistant to a number of antibiotics. But for treating some *Aeromonas* infections in humans, drugs of choice includes fluoroquinolones such as ciprofloxacin and Norfloxacin; third generation cephalosporins: ceftriaxone and cefuroxime; trimethoprim—sulfamethoxazole (TMP-SMZ) and lastly but not the least the tetracyclines (TE) (Chen et al., 2012).

### **2.2.7 Antibiotic resistance of *Aeromonads***

*Aeromonas* species are becoming resistant to a number of antibiotics. This may be due to the fact that water bodies receive large amounts of animals and human wastes which contain a lot of antimicrobial agents that are making the normal microflora in water to become resistant (Goni-Urriza, 2000; Odeyemi and Ahmad, 2015). *Aeromonas* are believed to harbor some resistant genes which is also increasing their

resistance to antimicrobials (Schmidt et al., 2001; Kore et al., 2014). The resistance to antibiotics has been observed in *Aeromonas* isolates isolated from heavily polluted waters (Huddleson et al., 2006; Aravena-Roman et al., 2012; Odeyemi and Ahmad, 2015). In general, the emergence and spread of antibiotic resistance among the pathogenic bacteria has been a growing public health concern (Frieri et al., 2017). Bacterial resistance mostly stems from treatment failure. These days antibiotic resistance results from misusing antibiotics as growth promoters in animal husbandary. Due to the consumption of these animal products by humans, it's been reported that resistant strains may spread from animals to humans. In environments such as sewage that contains rich nutrients horizontal gene transfer processes are possible and studies have reported detection of antimicrobial resistance in wastewater and drinking water (Cisar et al., 2014). Rawal et al., 2016 found *Aeromonas* species to have antibiotic resistance that ranges from 0 -100%.

Several studies have reported on high antibiotic resistance. In a study done by Scarano et al in 2018 they found that *Aeromonas* isolates were resistant to more than one antibiotic and some to even 11 antibiotics. They also reported the resistance to be as high as 100%. Their results suggested that isolates were exposed to high risk sources of contamination with broad use of antibiotics Scarano et al 2018. Other studies also indicated high antibiotic resistance of *Aeromonas* species. (Dumontet et al., 2000; Nguyen et al., 2014). The antibiotics most frequently associated with multiple resistance were amoxicillin, ampicillin, cephalothin, erythromycin, streptomycin, sulfadiazine and trimethoprim. In a study conducted on antibiotic resistance of gram-negative bacteria 91% was found to be resistant to ampicillin, cefalotin, cefuroxime, ceftriaxone, cefepime each accounted 16 (70%). Another study showed very high

resistance to cefazolin, amoxicillin–clavulanic acid and cefuroxime; 8 (80%), 7 (70%), 7 (70%) respectively (Belachew et al., 2018).

## **2.3 Medicinal plants used for the control of microorganisms**

Plants and their products have always been used for medicinal purposes in the past and their use is still applied today by many people as a primary care. The knowledge of the use of herbal medicine have been transferred from generations to generations (Heinrich, 2010). Majority of people particularly in rural areas in developing countries consider medicinal plants very valuable to them as they still rely on their use for treating different infections and diseases and as a resource for food. Millions of people in the world rely on medicinal plants for livelihood improvement and income generation, nourishment as well as primary healthcare (WHO, 2002; Polat et al., 2013). Plant species that are known to be used for medicinal purposes either traditionally or as modern medicine belong to between 50 000 and 70 000 species (Schippmann et al., 2006, Hayta et al., 2014).

The use of medicinal plants in treating infectious diseases is because they are believed to have lower side effects and toxicity and they are easy to access and much cheaper compared to conventional medicines. Because medicinal plants constituents have structural and biological diversity, many modern pharmaceuticals have been discovered. Medicinal plants are also used to treat animal diseases (Ren et al., 2016; Romba et al., 2015; Romba et al., 2016). Different parts of plants such as leaves, twigs, and rhizomes are used in medicinal preparations traditionally. Medicinal plants are known to treat viral, bacterial, parasitical and fungal infections as well as ailments such as diarrhoea, kidney problems, eczema, sinusitis, fevers, arthritis and other

infections and disorders. The medicinal plants used in the present study are those that are commonly used by traditional healer for the treatment of diarrheal diseases.

### 2.3.1 *Zornia milneana*

*Zornia milneana* is one of the species belonging to the family of Fabaceae and it is named after a British botanist, Mr Edgar Milne-Redhead. Its common name in Tshivenda is Lukandulula. It is native in Zimbabwe; however; it is distributed in Angola, Zambia, Namibia, Botswana, and some parts of South Africa as well. Ecologically, *Zizeeria Knysna* (butterfly) also known as sooty blue are known to feed on the plant (Chapano and Mugarisanwa, 2003).

*Zornia* is described as a perennial herb that has decumbent or prostrate stems, measuring from 8cm to 75 cm long. Its leaves are of 4-foliolate, with linear leaflets or narrow elliptic, pubescent and gland-dotted beneath. *Zornia milneana* flowers are yellow with red veining on the standard, and each flower is clasped between large green leaf-like bracts that have hairy margins. The plant's pods are in constricted segments, covered in spiny bristles. It grows in altitude range of between 900 – 1540m, and are habitats of sandy soils of various grassland, woodland and sometimes in old cultivation areas and flowering time is between November and April (Kirby, 2013). According to traditional healers in the Vhavenda region, *Zornia milneana* is used for the treatment of childhood diarrhea (Samie et al., 2009) as well as flue and headaches.

### 2.3.2 *Annona* species

*Annona spp* is a member of the *Annonaceae* family. In the *Annona* genus there are six species (*A. cherimola*, *A. squamosa*, *A. atemoya*, *A. reticulata*, *A. muricata* and *A. diversifolia*) that are known to produce eatable fruits (Escribano et al., 2007; Anuragi et al., 2016). Most of the *Annona* species originated from South America, but *A. muricata* is said to have been a native to Africa (Pinto et al., 2005). The *Annona* pulp is vitamins and minerals rich (Gyamfi et al., 2011). The fatty acids, the carbohydrates, certain minerals and edible fibres among others contribute to the high nutritional value of *A. cherimola*. Different parts of the *Annona* species such as the roots, leaves, barks, fruits as well as the seeds (basically, all parts of *Annonas*) are sources of compounds of medicinal importance (Anuragi et al., 2016). They have been described to be the source of natural antioxidants for several diseases. Traditionally to treat diseases like dysentery, cardiac problems, worm infestation, ulcers, constipation, fever, as well as fainting *A. squamosa* is used. Also *A. muricata* is orally used for treatment of fevers, diarrhoea and also used as an enhancer of the production of breast milk (Baskar et al., 2006).

Some *Annona* species such as *A. coriacea* have been reported to have antifungal activity. The methanol extract of this plant was found to have activity against *Cryptococcus neoformans* (which causes *cryptococcal meningitis*) and *Cryptococcus gattii* (Almeida-Apolonio et al., 2018). It is reported that the major chemical constituents detected in the family *Annonaceae* are the Alkaloids, in which large quantities of isoquinoline alkaloids are found and are thought to have some antibacterial and anti-tumour effects (Rabelo et al., 2014; Rinaldi et al., 2017). Anti-malaria and anti-*Mycobacterium tuberculosis* activities have been reported to be the

result of bioactive compounds such as glycoside flavonoids (quercetin and luteolin) in some of the *Annona* species (Araujo et al., 2014; Pimenta et al., 2014). Methanol extract of *A. mucosa* has been observed to inhibit the growth of some gram-positive bacteria. Members of the *Annonaceae* genus are also reported to have activity against norfloxacin resistant strains of *Staphylococcus aureus* (deBarboza et al., 2015).

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## **CHAPTER 3: Occurrence and distribution of steroid hormones in wastewater, surface water and fish samples from South Africa and Zambia**

### **3.1 Abstract**

**Background:** Natural estrogens and androgens are hormones produced in humans and animals and they often end up in environmental water through sewage discharge and run-offs on landfill as well as through the disposal of animal waste. Synthetic ethinylestradiol (EE2) is the most potent estrogen associated with interference with the endocrine system and altering the reproduction of humans and animals. These hormones have been reported to occur in surface waters. However, data on their distribution in surface water and wastewater remains scanty in the Southern African region.

**Method:** Samples were collected from Rivers, Wastewater treatment plants, Dams, taps as well as fish samples collected from these water sources in the Northern region of South Africa and the region around Lusaka in Zambia. Hormones were extracted from these samples using ethanol concentration procedure and commercial ELISA kits were used to measure their concentrations in the sample types.

**Outcome:** A total of 144 samples were collected.  $17\alpha$ -ethinylestradiol was detected in high concentration of up to 65  $\mu\text{g/ml}$ . Drinking water was also contaminated with hormones, with testosterone detected at high concentration of 140  $\text{ng/ml}$  in tap water. Mean concentrations ranged from 2.36 – 68.2  $\text{ng/ml}$ . Rivers accounted for 43% prevalence of the steroid hormones, 28% was detected from sewage samples and 9% was detected in tap water.

**Conclusion:** Action by the municipality is needed in removing the hormones from effluents, and quality drinking water that is free from steroid hormone contaminants needs to be provided to avoid pressing health issues.

### 3.2 Introduction

Natural estrogens are produced in humans and animals by the testes, ovary, adrenal cortex and the placenta (Hamid and Eskicioglu, 2012; Adeel et al., 2017). These hormones end up in environmental water through sewage discharge and run-offs on landfill as well as through the disposal of animal waste. Steroid hormones act as endocrine disrupting chemicals interfering with normal functions of the endocrine system of human and animals (Yan-you Gou, 2016). Among the estrogens; synthetic ethinylestradiol (EE2) is the most potent estrogen associated with interference with the endocrine system and altering the reproduction of humans and animals (king et al., 2016). EE2 is used in contraceptives, owing their detection in the environment in high concentrations. Steroidal hormones are also being detected from treated wastewater released from sewage treatment plants; in many cases; into the rivers where they end up affecting aquatic organisms. However, there is very little literature on their occurrence in the Limpopo Province and in Zambia. Furthermore, EE2 has been known for having morphological and physiological effects on fish, altering the sex and causing vitellogenin in males (Lange et al., 2001; Fenske, 2005; Henriksen et al., 2016).

One of the major factors that is contributing to the abundance of steroid hormones contamination in aquatic ecosystems is the use of animal manure that is used in enhancing the soil fertility, and these manures adds to the risk of having these

pollutants that end up disrupting the endocrine systems of aquatic organisms (Hensen et al., 1998; Prater et al., 2015). Estrogens concentrations ranging from as low as 1ng/ml and 0.1 ng/ml of synthetic ethinylestrogens have been described to cause serious negative effects on aquatic organisms (Young et al., 2002; Vymazal et al., 2015). Pregnant women are reported to excrete 30mg of estrogens per day, and other women secrete between 10 and 100  $\mu\text{g}$  of E1, E2, E3 and synthetic EE2. Estrogens are said to go under some transformation in mammals and humans such as methylation, conjugation, hydroxylation and oxidation. It has been observed that in activated sludge natural 17 $\beta$ -estradiol is oxidised to estrone in just few hours and is further slowly converted to estriol which is the most excreted. But the degradation of EE2 is reported to occur very slowly taking days to months to occur (Kostich et al., 2013; De mes et al., 2005; Jurgenes et al., 2002; Vymazal, 2015).

Knowing the distribution of steroidal hormones in the environment is important so that correct measures can be taken in trying to eradicate these contaminants from our waters to ensure safety of the population. Many studies worldwide have focused on the effects of these steroid hormones on aquatic organisms and report less on their distribution (Armstrong et al., 2016). It is of significance to investigate the occurrence of these hormones in different water sources. Such information will be critical to further understand if the problems that humans are experiencing such as infertility and cancer may also be due to water that we use and that are contaminated by these pollutants (steroid hormones). The present chapter describes the occurrence and distribution of steroid hormones in water sources and wastewater in the Limpopo Province of South Africa and some fish samples from Lusaka, Zambia.

### **3.3 Materials and methods**

#### **3.3.1 Ethical clearance**

Authorization to collect samples from sewage treatment plants was obtained from the municipalities. Ethical clearance was obtained from the University of Venda ethics and health committee.

#### **3.3.2 Study area**

The study was conducted in all the 5 Districts of the Limpopo Province. The Limpopo Province is the northernmost province of South Africa which comprises of 5 districts namely: Waterberg, Sekhukhune, Vhembe, Mopani and Capricorn district. It is named after the Limpopo River that flows on the northern borders of the Province. The Province has a population of about 5 million according to census 2015. This Province covers approximately one tenth of the total surface area of South Africa. It comprises an area of 116 824 km<sup>2</sup>, only 14% of which is arable land (Meintjies et al., 1995; Aneck-Hahn et al., 2009). A map of the Limpopo Province is shown in Figure 3.1.

A small number of samples included in this study were collected from Lusaka and Chilanga, in Zambia. Chilanga is located 20km south of Lusaka city which is the capital city of Zambia. Kafue River is the longest river within Zambia at about 1600 km long.

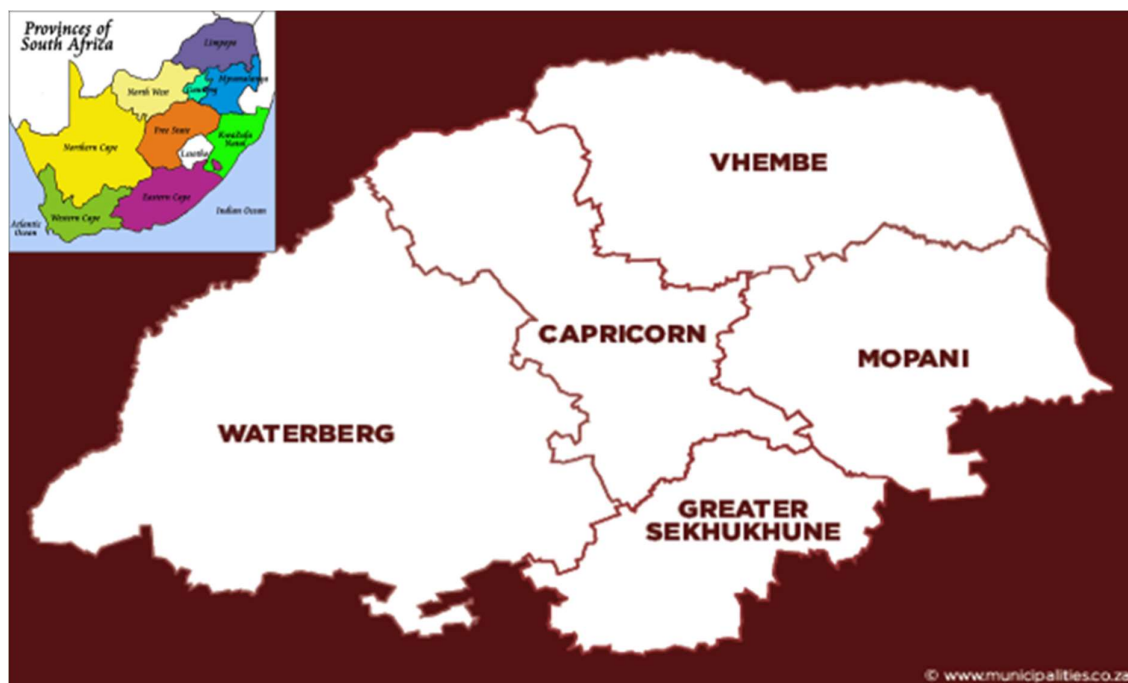


Figure 3.1: A map showing the different districts in the Limpopo province of South Africa where samples were collected.

### 3.3.3 Sample collection

Wastewater, surface water, as well as tap water samples were collected using sterile 1l plastic bottles. Prior to sample collection bottles were rinsed with ethanol to rid any contaminants. Fish were collected from the fish farm and crabs from Kafue River in Lusaka, Zambia. All samples were kept at 4°C for transportation to the University of Venda.

### **3.3.4 Extraction of hormones**

#### ***3.3.4.1 Extraction of hormones from water samples***

The hormones were extracted by mixing 100 ml of the water sample with 200 ml of absolute ethanol. The mixture was dried at 55 degrees Celsius using a rotary evaporator until the volume was reduced to 1ml. Then 20 ml of absolute methanol was added, and evaporation was continued till 5ml of the solution remained. One hundred ml absolute ethanol was further added, and then anhydrous sodium sulphate was added to remove water traces then moved to another flask. The volume of the extract was reduced to 5ml using a rotary evaporator at 55 degrees Celsius.

#### ***3.3.4.2. Extraction of hormones from fish samples***

The estrogens from fish samples (gills, intestines and flesh) were extracted using the following method (modified from the method used for hormones extraction from water samples). About 5g of gills, intestine and meat were grinded using pastel and mortar and was mixed with sterile water, after which 14ml of the mixture was mixed with 30ml of absolute ethanol. The mixture was dried at 55 degrees Celsius using a rotary evaporator until the volume was reduced to 1ml then 20ml of absolute methanol was added and evaporation was continued till 5ml of the ethanol remained. One hundred mills (100 ml) of absolute ethanol was added and then anhydrous sodium sulphate was added to dry the extract then moved to another flask. The volume of the extract was reduced to 5ml using a rotary evaporator at 55 degrees Celsius.

### **3.3.4.3 Enzyme-linked immunosorbent assay (ELISA)**

ELISA was used as a tool to detect and measure the concentrations of the hormones from fish and water samples. The assays were performed according to the manufacturer's instructions (MP Biomedicals, Solon, Ohio). The principle of the ELISA used is based on competitive binding. ELISA was conducted for testosterone, estriol, estradiol (E2) as well as ethinylestradiol (EE2).

### **3.3.5 Statistical analysis**

All results were recorded in an excel spreadsheet and the concentrations of all hormones were calculated using Microsoft excel. The standard graphs were drawn using Microsoft excel as well. Distribution was analysed using IBM SPSS version 25, a  $p \leq 0.05$  was considered significant.

## **3.4 Results**

### **3.4.1 Frequency of occurrence of hormones in various water sources**

A total of 144 samples were collected from Zambia and South Africa. Of these, 28 were from fish samples, 14 were drinking water, 40 were sewage samples from the wastewater treatment plants, and 62 were River samples. Four hormones were tested including 17  $\alpha$  Ethinylestradiol (EE2), 17  $\beta$  Estradiol (E2), Estriol (Total estriol and free estriol), and Testosterone. The concentrations of the hormones varied from 0 to up to

5702.83 ng/ml for testosterone while for EE2 it varied from 0 to 65 ng/ml (Table 3.1). The highest frequency of occurrence was observed for EE2 with a percentage of 92.7%, followed by 64.0% for free estriol with testosterone having the lowest frequency (Table 3.2). The variation in the total number tested was based on the availability of the kits and the number of replicates to be tested.

Table 3.1. Concentration of the hormones tested in samples collected from South Africa and Zambia.

Hormones tested	Number tested	Mean	Standard Deviation	Min	Max
Testosterone	137	68.22379	506.8726	0	5702.833
17 $\beta$ Estradiol	99	92.28606	275.4426	3.844	1777.936
17 $\alpha$ ethynil estradiol	137	19.43945	16.75804	0	65.061
Total estriol	50	37.2218	164.4463	0	1154.374
Free estriol	86	2.355663	3.82345	0	15.028

Table 3.2. Overall positivity of steroid hormones in environmental samples from South Africa and Zambia.

Hormones	Frequency of positive samples	Total tested	Percent
Testosterone	61	138	44.2
Ethinylestradiol	127	137	92.7
Free estriol	55	86	64.0
Total estriol	27	50	54.0
Estradiol	99	99	100.0

### 3.4.2 Occurrence of hormones in the districts of Limpopo and Zambia

In all the five districts of the Limpopo province and Zambia; 3 districts (Waterberg, vhembe and capricorn) as well as Zambia had high prevalence of steroid hormones. With 22.2%, 23.6% and 27.1% respectively. The results are shown in table 3.3.

Table 3.3: Frequency of all hormones based on the different districts of the Limpopo province and from Zambia

Districts	Percentage %
Waterberg	22.20
Mopani	7.60
Capricorn	13.90
Vhembe	23.60
Sekhukhune	5.60
Zambia	27.10
Total	100.0

### 3.4.3 Frequency of occurrence of hormones based on the location

High frequency of occurrence was noted in locations such as Nzhelele, Polokwane and Chilanga with 16.0%, 9.0% and 8.3% respectively. Low frequency among others was noted in mokgopong with a percentage of 0.7%.

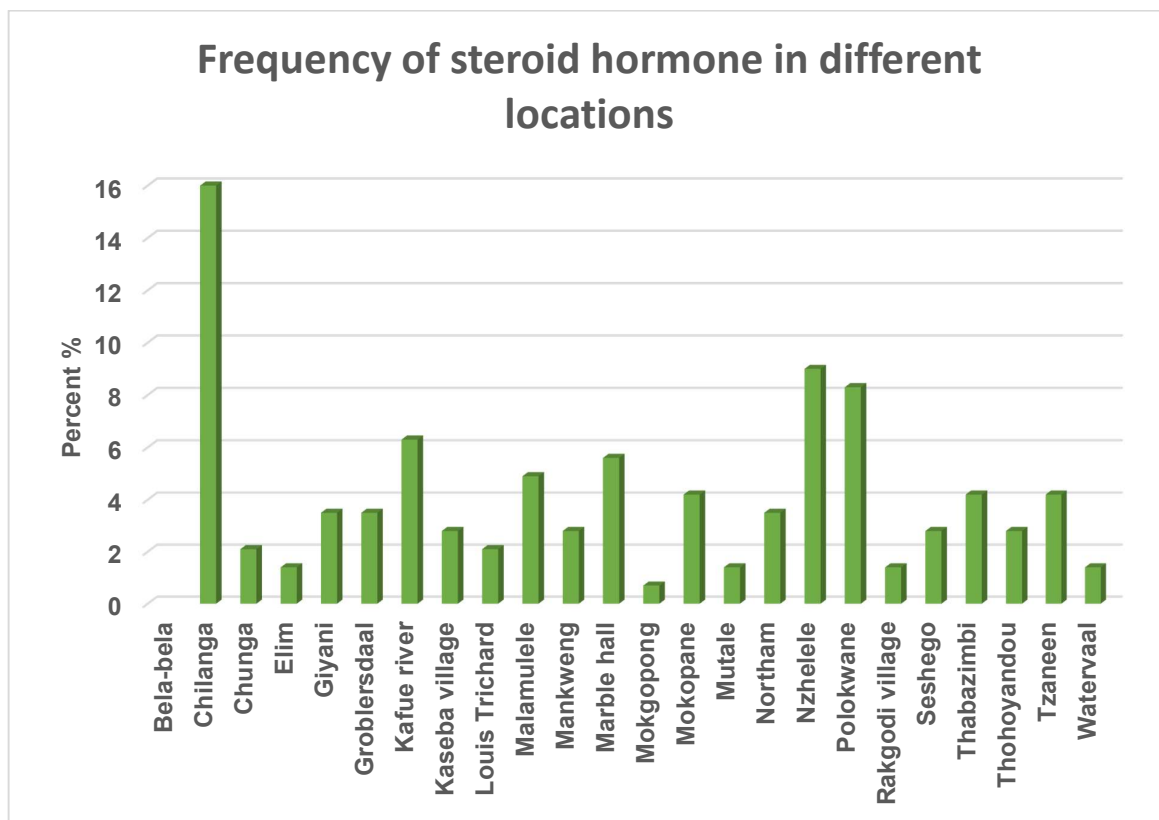


Figure 3.2: Frequency of steroid hormone in different locations of the Limpopo province and Zambia

### 3.4.5: Frequency of occurrence of hormone levels in water and fish samples

Highest observed frequency of occurrence was with testosterone with a percentage of 75 (54.7%). All different levels Estradiol (E2) was widely distributed ranging from 14.1%- 39.4%. Ethinylestradiol (EE2) also had high frequency occurrence from level 1 to level 4 with varying percentages of 11.7% to 27%. Total and free estriol fell under three categories (level 0 to level 2), of these categories, high occurrence was observed in all ranging from 14.0% to 50.0% (Table 3.4).

Table 3.4: Overall Frequency of occurrence of hormone levels in water and fish samples.

	levels	Frequency	Percent %
<b>Testosterone</b>	0	1	0.7
	0-5 ng/ml	75	54.7
	5-20 ng/ml	40	29.2
	21-100 ng/ml	15	10.9
	>100 ng/ml	6	4.4
	Total	137	100.0
<b>Estradiol</b>	0-5 pg/ml	31	31.3
	5-20 pg/ml	39	39.4
	21-100 pg/ml	15	15.2
	>100 pg/ml	14	14.1
	Total	99	100.0
<b>Ethinylestradiol</b>	0	16	11.7
	0-5 ug/ml	29	21.2
	5-20 ug/ml	38	27.7
	21-100 ug/ml	27	19.7
	>100 ug/ml	27	19.7
	Total	137	100.0
<b>Total estriol</b>	0	23	46.0
	0-5 ng/ml	14	28.0
	5-20 ng/ml	13	26.0
	Total	50	100.0
<b>Free estriol</b>	0	31	36.0
	0-5 ng/ml	43	50.0
	5-20 ng/ml	12	14.0
	Total	86	100.0

### 3.4.6 Distribution of Ethinylestradiol, Estriol, Testosterone and Estradiol according to the country where the sample was collected

All hormones were highly distributed in both countries with high percentages except for free estriol for which only one sample was positive in Zambia. The highest occurrence was observed with Ethinylestradiol (EE2) in both countries, with South Africa having 69.5% and Zambia 90.6%. Total and free estriol was also prevalent in South Africa, and Zambia with both countries having a high prevalence. Estradiol also had high prevalence in Zambia and South Africa. Results are shown in table 3.5 below.

Table 3.5: Occurrence of hormones in South Africa and Zambia

Hormones	South Africa	Zambia	Total	Statistics (p value)
Ethinylestradiol	73(69.5%)	29(90.6%)	102(74.5%)	0.017
Free estriol	54(64.3%)	1(50.0%)	55(64.0%)	0.678
Total estriol	19(86.4%)	8(28.6%)	27(54.0%)	0.000
Estradiol	26(28.9%)	3(33.3%)	29(29.3%)	0.780
Testosterone	7(6.7%)	2(6.1%)	9(6.5%)	0.166

### 3.4.7: Distribution of steroid hormones in the different districts of the Limpopo province

Total estriol was not prevalent in Capricorn, Sekhukhune and Waterberg, but free estriol was prevalent in all districts. High percentages of ethinylestradiol and free estriol as well as Estradiol was observed in all districts and Zambia, with the highest percentages in Vhembe, Sekhukhune and Mopani. Testosterone had low prevalence in all the districts ranging from 0-25.0%. Results are shown in table 3.6 below

Table 3.6: Distribution of steroid hormones based on the different districts

Districts	EE2	EF	ET	E2	Testosterone
Vhembe	28 (82.4%)	15(78.9%)	14(87.5%)	7(28.0%)	2(5.9%)
Mopani	9 (81.8%)	5(83.3%)	5(83.3%)	1(16.7%)	1(9.1%)
Capricorn	11 (55.0%)	13(68.4%)	0	6(31.6%)	0
Sekhukhune	8 (87.5%)	6(75.0%)	0	6(75.0%)	2(25.0%)
Waterberg	18 (56.3%)	15(46.9%)	0	6(18.8%)	2(6.3%)
Zambia	29 (90.6%)	1(50.0%)	8(28.6%)	3(33.3%)	2(6.1%)
Total	102(74.5%)	55(64.0%)	27(54.0%)	29(29.3%)	9(6.5%)
Statistics	P=0.007 $\chi^2=16.099$	P=0.177 $\chi^2= 7.636$	P=0.000 $\chi^2=16.596$	P=0.065 $\chi^2=10.387$	P=0.350 $\chi^2=11.091$

### 3.4.8 Distribution of steroid hormones in different type of samples

Fish, crayfish and tap water had the highest prevalence of ethinylestradiol EE2 and total estriol. All these types of samples mentioned above except for tap water were negative for free estriol. Estradiol was also absent from fish, groundwater and ponds. Tap water did not include total Estradiol as well. The occurrence of EE2 was statistically significant with a p value of 0.014. However, the occurrence of free estriol and Estradiol E2 was not statistically significant. Results are tabulated below (table 3.7)

Table 3.7: Occurrence of steroid hormones in different types of samples

Type of sample	Ethinyl-estradiol	Free estriol	Total estriol	Estradiol	Testosterone
Crayfish	6(100.0%)	0	1(16.7%)	0	0
Dam	4(100.0%)	0	3(75.0%)	2(50.0%)	0
Fish	14(93.3%)	0	4(26.7%)	0	0
Ground water	1(100.0%)	1(100.0%)	0	0	0
Pond water	3(60.0%)	2(66.7%)	0	0	0
River	38(71.7%)	27(61.4%)	7(77.8%)	11(25.0%)	1(1.6%)
Sewage	25(62.5%)	18(69.2%)	12(85.7%)	13(39.4%)	6(15.0%)
Tap water	11(84.6%)	7(58.3%)	0	3(25.0%)	2(15.4%)
Total	102(74.5%)	55(64.0%)	27(54.0%)	29(29.3%)	9(6.5%)
statistics	0.0136	0.881	0.005	0.365	0.069

### 3.4.9 Occurrence of steroid hormones in sewage and non-sewage samples

In order to observe the impact of wastewater as the origine of these hormones, we compared the occurrence in wastewater to other sample types. Surprisingly EE2 was more common in all the other types of samples including drinking water compared to wastewater. Wastewater samples had the highest prevalence of steroid hormones with total estriol having a percentage of 85.7%. Non-waste water samples also had high occurrence of the steroid hormones. The difference between the percent occurrence in wastewater and other types of samples was statistically significant for EE2 and both free and total estriol however was not for Estradiol and testosterone. The results are presented in Table 3.8.

Table 3.8: Distribution of steroid hormones in wastewater and non-wastewater

<b>Steroid hormones</b>	<b>Non sewage samples</b>	<b>Sewage samples</b>	<b>Total</b>	<b>Statistics</b>
Ethinylestradiol	77(79.4%)	25(62.5%)	102(74.5%)	0.039
Free estriol	37(61.7%)	18(69.2%)	55(64.0%)	0.036
Total estriol	15(41.7%)	12(85.7%)	27(54.0%)	0.005
Estradiol	16(24.2%)	13(39.4%)	29(29.3%)	0.118
Testosterone	3(3.1%)	6(15.0%)	7(6.5%)	0.259

### 3.4.10 Occurrence of steroid hormones according to sample type

Free estriol and Estradiol were absent from fish and total estriol was not prevalent in tap water as well. Steroid hormones are highly distributed across all these sample types with highest prevalences observed with EE2 in fish with a percentage of 95.2% and total estriol in sewage with a high percentage of 85.7%. The difference for the occurrence of estradiol was not statistically significant with a p value of 0.295, however the p value for the occurrence of total estriol was significant with  $p = 0.001$ . Table 3.9 shows the results.

Table 3.9: Distribution of steroid hormones according to sample types

Sample type	Ethinyl-estradiol	Free estriol	Total estriol	Estradiol	Testosterone
River	46(73.0%)	30(62.5%)	10(71.4%)	13(24.1%)	1(1.6%)
Sewage	25(62.5%)	18(69.2%)	12(85.7%)	13(39.4%)	6(15.0%)
Tap water	11(84.6%)	7(58.3%)	0	3(25.0%)	2(15.4%)
Fish	20(95.2%)	0	5(23.8%)	0	0
Total	102(74.5%)	55(64.0%)	27(54.0%)	29(29.3%)	9(6.5%)
Statistics	0.036	0.770	0.001	0.295	0.024

### **3.4.11 Distribution of steroid hormones in different levels in South Africa and Zambia**

The hormones and bacteria were categorised in different levels (based on the concentrations). Both South Africa and Zambia had the highest prevalence of testosterone in level 1 and 2 (Lower concentrations). In South Africa Estradiol was prevalent in all levels, but in Zambian samples estradiol was not present in level 3. The prevalence of total estriol was high in all the different levels, with highest occurrence in level 0 with a percentage of 71.4% in Zambia. Free estriol was prevalent in all levels in South Africa but in Zambia level free estriol was not present in level 0. All levels had high prevalence of ethinylestradiol EE2 in South Africa with highest prevalence observed in level 2 in both countries, with South Africa having 26.7% and Zambia 31.3%. Results recorded in a table 3.10 below.

Table 3.10: Occurrence of hormones in South Africa and Zambia in varying categories

Hormones	level	South Africa	Zambia	Total
<b>Testosterone</b>	0	1(1.0%)	0(0.0%)	1(0.7%)
	0-5 ng/ml	53(50.5%)	22(68.8%)	75(54.7%)
	5-20 ng/ml	33(31.4%)	7(21.9%)	40(29.2%)
	21-100 ng/ml	13(12.4%)	2(6.3%)	15(10.9%)
	>100 ng/ml	5(4.8%)	1(3.1%)	6(4.4%)
<b>Estradiol</b>	0-5 pg/ml	28(31.1%)	3(33.3%)	31(31.3%)
	5-20 pg/ml	36(40.0%)	3(33.3%)	39(39.4%)
	21-100 pg/ml	15(16.7%)	0(0.0%)	15(15.2%)
	>100 pg/ml	11(12.2%)	3(33.3%)	14(14.1%)
<b>Total estriol</b>	0	3(13.6%)	20(71.4%)	23(46.0%)
	0-5 ng/ml	11(50.0%)	3(10.7%)	14(28.0%)
	5-20 ng/ml	8(36.4%)	5(17.9%)	13(26.0%)
<b>Free estriol</b>	0	30(35.7%)	1(50.0%)	31(36.0%)
	0-5 ng/ml	42(50.0%)	1(50.0%)	43(50.0%)
	5-20 ng/ml	12(14.3%)	0(0.0%)	12(14.0%)
<b>Ethinylestradiol</b>	0	16(15.2%)	0(0.0%)	16(11.7%)
	0-5 ug/ml	25(23.8%)	4(12.5%)	29(21.2%)
	5-20 ug/ml	28(26.7%)	10(31.3%)	38(27.7%)
	21-100 ug/ml	20(19.0%)	7(21.9%)	27(19.7%)
	>100 ug/ml	16(15.2%)	11(34.4%)	27(19.7%)

### **3.4.12 Occurrence of different levels of steroid hormones in sewage and non-sewage samples**

Estradiol E2 was prevalent in all the different levels in both the wastewater and the non-wastewater samples with the highest prevalence (42.4% in non-sewage and 33.3% in sewage samples) observed in level 2. The prevalence of testosterone was low in level 0 of both non-sewage and sewage samples and in level 4 of non-sewage samples. Ethinylestradiol EE2 showed varied prevalence in sewage and non-sewage samples with the highest prevalence of 30.9% observed in level 2 from non-sewage samples. The difference for the occurrence of EE2 was not statistically significant. Total and free estriol were also prevalent in both types of the sample. High prevalence was observed in level 0 (58.3%) in non-sewage as well as in total estriol. Level 1 showed high prevalence of free estriol (61.5%) in sewage samples. The occurrence of total estriol was statistically significant with P value of 0.016, however the difference for free estriol was not statistically significant with a pvalue of 0.312. Results are presented in the table 3.11 below.

Table 3.11: Distribution of hormones in wastewater and non-waste water in varying levels.

Hormones	levels	Non sewage samples	Sewage samples	Total	Statistics
<b>Estradiol</b>	0-5 pg/ml	22(33.3%)	9(27.3%)	31(31.3%)	0.011
	5-20 pg/ml	28(42.4%)	11(33.3%)	39(39.4%)	
	21-100 pg/ml	12(18.2%)	3(9.1%)	15(15.2%)	
	>100 pg/ml	4(6.1%)	10(30.3%)	14(14.1%)	
<b>Testosterone</b>	0	0(0.0%)	1(2.5%)	1(0.7%)	0.011
	0-5 ng/ml	57(58.8%)	18(45.0%)	75(54.7%)	
	5-20 ng/ml	30(30.9%)	10(25.0%)	40(29.2%)	
	21-100 ng/ml	9(9.3%)	6(15.0%)	15(10.9%)	
	>100 ng/ml	1(1.0%)	5(12.5%)	6(4.4%)	
<b>EE2</b>	0	10(10.3%)	6(15.0%)	16(11.7%)	0.276
	0-5 ug/ml	18(18.6%)	11(27.5%)	29(21.2%)	
	5-20 ug/ml	30(30.9%)	8(20.0%)	38(27.7%)	
	21-100 ug/ml	17(17.5%)	10(25.0%)	27(19.7%)	
	>100 ug/ml	22(22.7%)	5(12.5%)	27(19.7%)	
<b>Total estriol</b>	0	21(58.3%)	2(14.3%)	23(46.0%)	0.016
	0-5 ng/ml	7(19.4%)	7(50.0%)	14(28.0%)	
	5-20 ng/ml	8(22.2%)	5(35.7%)	13(26.0%)	
<b>Free estriol</b>	0	23(38.3%)	8(30.8%)	31(36.0%)	0.312
	0-5 ng/ml	27(45.0%)	16(61.5%)	43(50.0%)	
	5-20 ng/ml	10(16.7%)	2(7.7%)	12(14.0%)	

### 3.4.13 Distribution of steroid hormones in different sample types

Testosterone showed to be widespread in all sample type in level 1 and 2, however lowest prevalence was observed in level 0 of all the sample type ranging from 0.0% - 2.5% although the difference was not statistically significant with  $p= 0.052$ . Ethinylestradiol was widespread with highest prevalence percentage of 47.6% in fish samples of level 4, the difference was significant with a p value of 0.010. Total estriol is also widespread, however, low distribution to non distribution was observed in fish samples, and the occurrence was statistically significant with a value of 0.010. Free estriol was not prevalent in fish samples and the difference was no statistically significant. Table 3.12 shows the results.

Table 3.12: Occurrence of steroid hormones in different levels in sample type

Hormones	Level	River	Sewage	Tap water	Fish	Total	Statistics
<b>Testosterone</b>	0	0(0.0%)	1(2.5%)	0(0.0%)	0(0.0%)	1(0.7%)	0.052
	0-5 ng/ml	36(57.1%)	18(45.0%)	5(38.5%)	16(76.2%)	75(54.7%)	
	5-20 ng/ml	22(34.9%)	10(25.0%)	4(30.8%)	4(19.0%)	40(29.2%)	
	21-100 ng/ml	5(7.9%)	6(15.0%)	3(23.1%)	1(4.8%)	15(10.9%)	
	>100 ng/ml	0(0.0%)	5(12.5%)	1(7.7%)	0(0.0%)	6(4.4%)	
<b>EE2</b>	0	9(14.3%)	6(15.0%)	1(7.7%)	0(0.0%)	16(11.7%)	0.083
	0-5 ug/ml	12(19.0%)	11(27.5%)	4(30.8%)	2(9.5%)	29(21.2%)	
	5-20 ug/ml	21(33.3%)	8(20.0%)	3(23.1%)	6(28.6%)	38(27.7%)	
	21-100 ug/ml	12(19.0%)	10(25.0%)	2(15.4%)	3(14.3%)	27(19.7%)	
	>100 ug/ml	9(14.3%)	5(12.5%)	3(23.1%)	10(47.6%)	27(19.7%)	
<b>Estradiol</b>	0-5 pg/ml	19(35.2%)	9(27.3%)	3(25.0%)	31(31.3%)		0.33
	5-20 pg/ml	22(40.7%)	11(33.3%)	6(50.0%)	39(39.4%)		
	21-100 pg/ml	11(20.4%)	3(9.1%)	1(8.3%)	15(15.2%)		
	>100 pg/ml	2(3.7%)	10(30.3%)	2(16.7%)	14(14.1%)		
<b>Total estriol</b>	0	4(28.6%)	2(14.3%)	1(100.0%)	16(76.2%)	23(46.0%)	0.010
	0-5 ng/ml	5(35.7%)	7(50.0%)	0(0.0%)	2(9.5%)	14(28.0%)	
	5-20 ng/ml	5(35.7%)	5(35.7%)	0(0.0%)	3(14.3%)	13(26.0%)	
<b>Free estriol</b>	0	18(37.5%)	8(30.8%)	5(41.7%)		31(36.0%)	0.661
	0-5 ng/ml	22(45.8%)	16(61.5%)	5(41.7%)		43(50.0%)	
	5-20 ng/ml	8(16.7%)	2(7.7%)	2(16.7%)		12(14.0%)	

### 3.4.4 Variation of Ethinylestradiol (EE2) concentrations in different samples

Figure 3.3 shows the concentration of ethinylestradiol in different samples. Some of the samples had high concentration with one fish sample having 65.1  $\mu\text{g/ml}$ . the concentrations ranged from 0.027  $\mu\text{g/ml}$ - 65.061  $\mu\text{g/ml}$ .

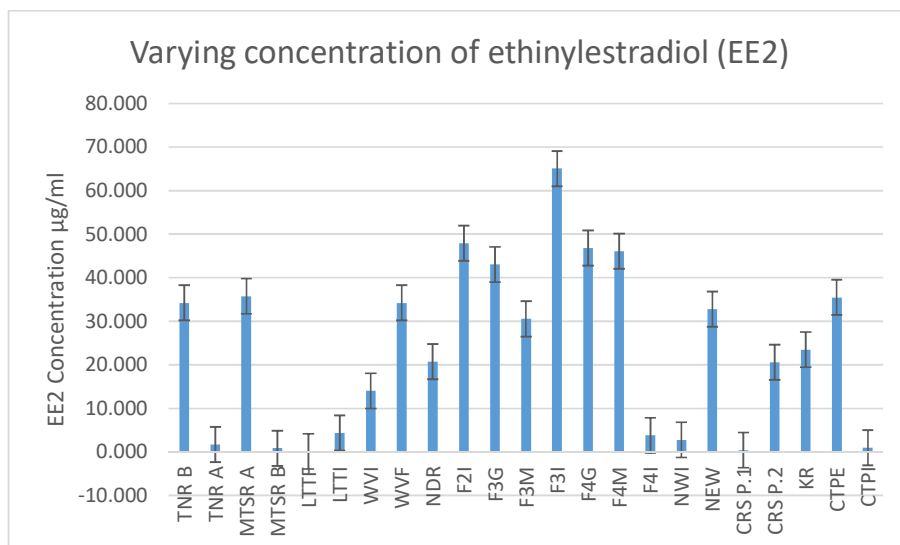


Figure 3.3: A bar graph showing ethinylestradiol (EE2) concentration in wastewater, fish and river samples

### 3.4.5 Testosterone concentration in wastewater, river and fish samples

Testosterone concentration varied from 0.030 ng/ml – 140 ng/ml; with the highest amount being from a tap water sample from mokopane in a village called Mahwelereng in the Waterberg district, Limpopo South Africa. The concentrations are shown in figure 3.4

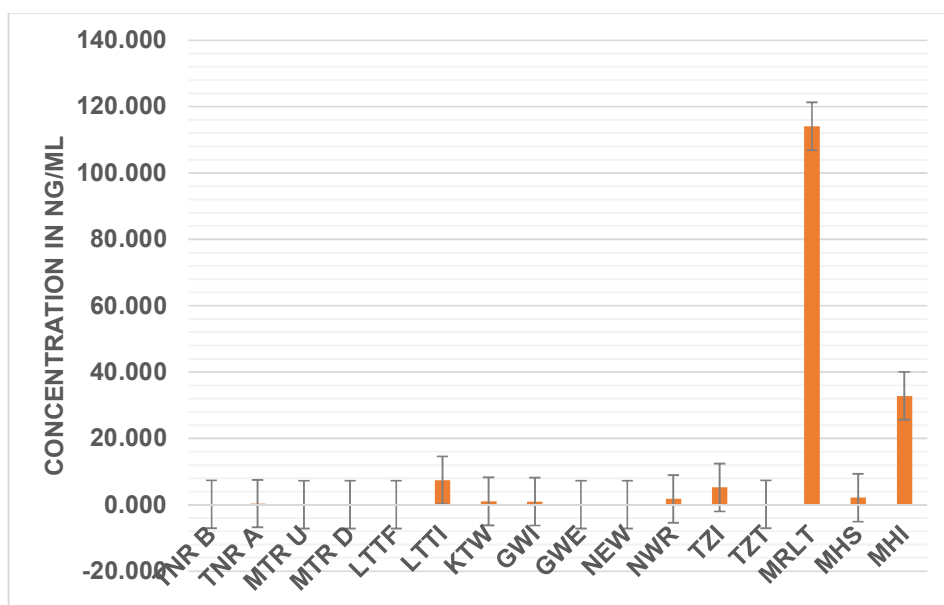


Figure 3.4: A bar graph showing the concentration of Testosterone in the water and fish samples

### 3.4.6 Estradiol concentration in selected wastewater, river and tap water samples

Estradiol concentration was high in most samples varying from 3.850 pg/ml – 1777.936 pg/ml. The highest concentration was observed on tap water sample.

Results are shown in figure 3.5 below.

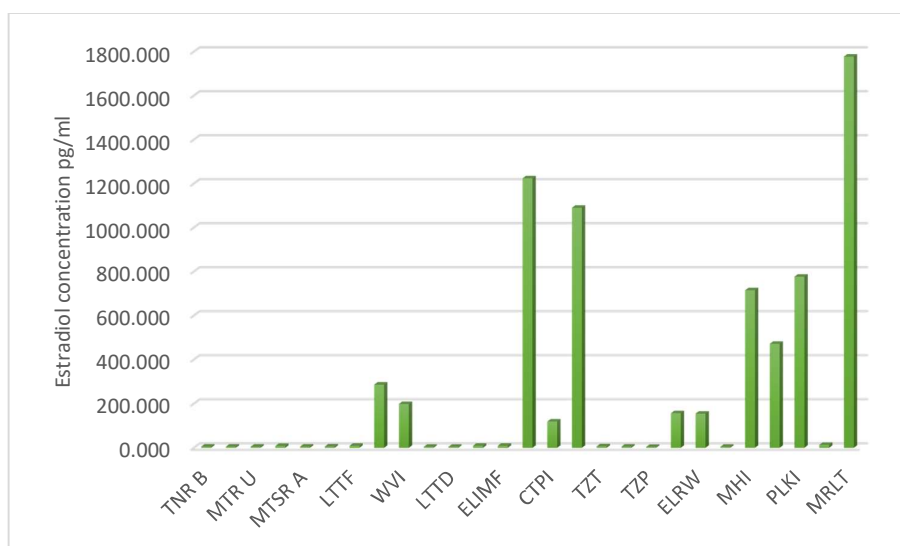


Figure 3.5: A bar graph showing Estradiol concentration in wastewater and water samples from South Africa and Zambia

### 3.4.7 Total and free estriol concentrations from different samples

Samples were chosen randomly to test for free and total estriol. The total estriol had much higher concentrations compared to those of free estriol which is normal. Total estriol varied between 0 – 1154.374 ng/ml while free estriol varied from 0 – 14.995 ng/ml. Figure 3.6 and 3.7 shows the results.

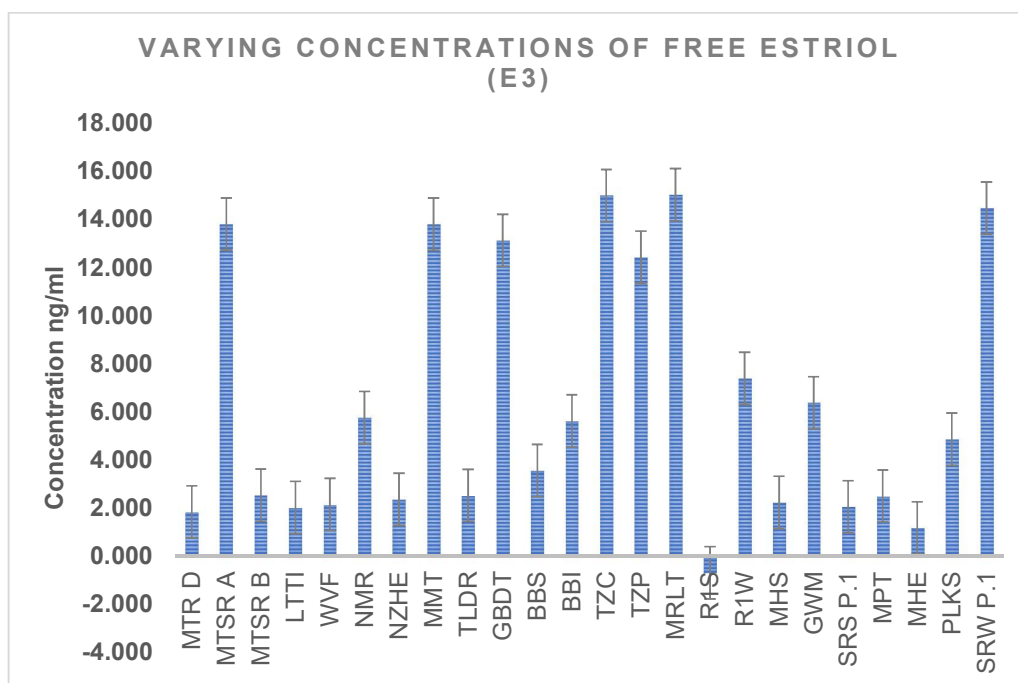


Figure 3.6: A bar graph showing free estradiol (E3) concentrations

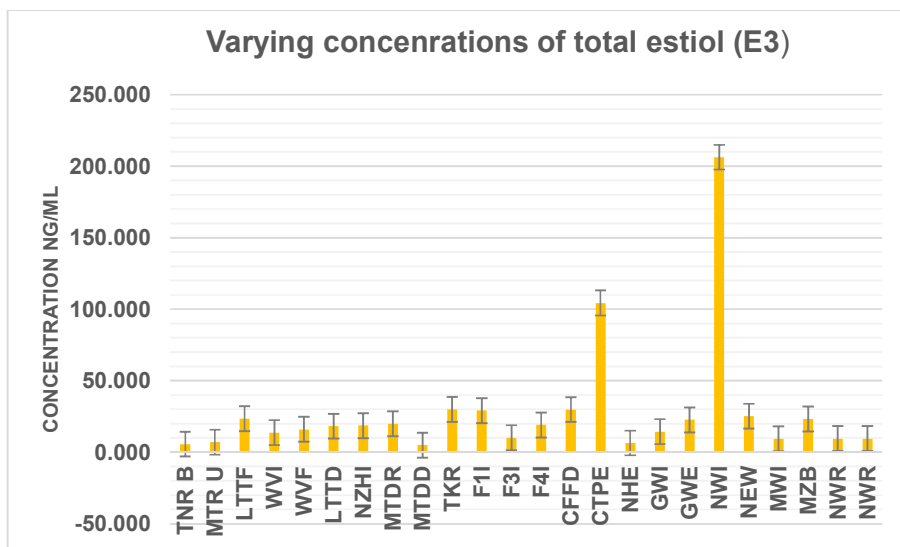


Figure 3.7: A bar graph presenting varying concentrations of total estriol (E3)

### 3.5 Discussion

Large quantities of hormones being produced and released in the environment everyday by humans and animals have been a growing cause of concern for more than two decades (Combalbert and Hernandez-Raquet, 2010). Steroid hormones are potent chemicals known to alter normal development of fish and human (Rao et al., 2012). Our study focused on steroidal estrogens and testosterone, type of steroid hormones that are produced by humans and are commonly detected in wastewater effluents. The results indicate that these hormones are quite common in our environment and even the effluents contain wide range of estrogens as well as the androgen testosterone.

Man-made ethinylestradiol was detected in high amounts from wastewater effluents to rivers as well as fish tissue. This strongly suggests that our WWTPs are not effective or are not equipped enough to remove the hormonal contaminants. The common detection of these estrogens and androgens in effluents (Racz and Goel, 2009) and rivers as well as drinking water is of very serious public health concern that needs to be addressed before we reach a point of no return. Already these contaminants have been implicated in chronic diseases such as cancer and pressing health issues like sterilisation (Massawe et al., 2017).

These results are in line with studies done in other countries such as Korea, China, UK, Japan and Belgium just to mention a few (Matsui et al., 2000; Oh et al., 2006; Shen et al., 2001; Witters et al., 2001 and Matthuessen et al., 2006). Also, few studies have been done in South Africa that reported high concentrations of estrogens exceeding 0.1 ng/L which is the lowest concentration that has been described to elicit health problems in human beings resulting from a prolonged exposure (Manickum and

John et al., 2014). Aneck-Hahn et al., in 2011 observed oestrogenic activity in drinking water in one district of the Limpopo province, their findings are also similar to the findings of this study because we also found tap water samples do have steroid hormone concentrations of considerable concern. There was one sample in particular from an area next to where they conducted their study which had a very high amount of testosterone, implying that the water provided in that area are highly contaminated with hormone in the Waterberg district.

Ethinylestradiol was also detected in very high concentration of up to 65 µg/ml yet a concentration as low as 0.1 ng/ml can cause adverse effects to aquatic organisms and human alike. King and colleagues also detected high concentration of ethinylestradiol (EE2), predicting that in the Australian wastewater, a mass of 0.02 µg/ person enters the wastewater treatment plants, and in the United Kingdom, Johnson and Williams suggested that each person contributes 0.08 µg to 0.19 µg of EE2 that enters the wastewater treatment plants (King et al., 2016; Johnson and William., 2004).

Some studies indicated that Estradiol can be efficiently removed during wastewater treatment process. For instance, in a study done in Namibia by Faul et al., in 2013, their results indicated that Estradiol was successfully removed during the treatment process, but because estradiol can be oxidised to estrone, they detected estrone in high concentrations (Faul et al., 2013; Carballa et al., 2008). Testosterone levels were detected at very low concentration in our study, much lower than that of Estradiol with concentrations ranging from 0- 140 ng/ml. But in the case of those samples with high concentration of testosterone, the results are supported by studies published by Shore and Shemesh, (2003) which suggested that the reason for high concentration of testosterone is because human and animals excrete large amounts of natural steroid hormones; of course, with testosterone being one of them. The results are also similar

to those obtained in other studies that detected higher concentration of testosterone in environmental waters (Leush et al., 2006). But in china, Chang et al., (2011) detected lower concentrations of testosterone. Presently, there is still lack of information in South African waters on the occurrence and implication of hormonal contaminants in combination with other pollutants.

### **3.6 Conclusion**

The present study showed a high occurrence of steroid hormones particularly the synthetic hormone  $17\alpha$  ethinyl estradiol. From these findings it is very clear that actions need to be taken from the municipality side in coming up with strategies in trying to remove these contaminants from WWTPs effluents and from drinking water, because if consumption of these contaminants is prolonged for years, it can then lead to very serious health concern. Therefore, quality drinking water needs to be provided to avoid pressing health issues.

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## Chapter 4: Prevalence of *Aeromonas* species from various water sources, wastewater and fish samples from South Africa and Zambia

### 4.1 Abstract

**Background:** *Aeromonas* species are responsible for diseases among animals and Humans. *Aeromonads* are divided into two groups, one being motile mesophilic *Aeromonads* (including *A. hydrophila*) which cause diseases mainly in humans the other group being psychrophilic non-motile *Aeromonads* comprising of species such as *Aeromonas salmonicida* responsible for causing diseases among fish. The goal of the study was to report on the distribution of *Aeromonas* species from various sources in South Africa and Zambia.

**Method:** *Aeromonas* were isolated using the *Aeromonas* selective agar enriched with ampicillin from water and fish samples. Genomic DNA was extracted using a bioflux genomic DNA extraction kit. The Extracted DNA was subjected to conventional PCR for *Aeromonas* detection and identification. Then PCR products were gel electrophoresed using agarose and the gels were viewed using a Bio-rad gel doc machine under 3% of ethidium bromide.

**Results:** Overall detection of *Aeromonas* species in the samples was 84.5%. *A. caviae* was the most prevalent species accounting for 73.6%, followed by *A. veronii* with 64.6%. Also, wastewater had the highest prevalence of *A. veronni* and *A. caviae* accounting for 87.5% and 82.5% respectively. River water had high prevalence of *Aeromonas caviae* with 95.1% compared to 80.0% of *A. veronii*. Tap water was negative for three *Aeromonas* species except for *Aeromonas caviae* which was

positive (7.7%). Removal efficiency of *Aeromonas* by the wastewater treatment plants ranged from 71% to 100%.

**Conclusion:** Species of *Aeromonas* are well distributed in water sources in the region, with rivers and wastewater having abundance of *Aeromonas* species. *Aeromonas veronii* and *Aeromonas caviae* were the most prevalent in many areas.

**Key words:** *Aeromonas spp*, water, fish, DNA extraction, and gel electrophoresis, epidemiology.

## 4.2 Introduction

*Aeromonads* are divided into two groups, one being motile mesophilic *Aeromonads* including *A. hydrophila*, which cause diseases such as wound infections, septicaemia and gastrointestinal infections, mainly in humans (Parker and Shaw, 2011; Hoel et al., 2017). The other group being psychrophilic non-motile *Aeromonads* comprising of species such as *Aeromonas salmonicida* responsible for causing diseases such as septicemia, furunculosis and ulcer formation in fish (Stratev and Odeyemi, 2016). Members of the genus *Aeromonacidae* are ubiquitous in aquatic environments and are opportunistic pathogens of humans and animals. Species belonging to the genus *Aeromonas* are ubiquitous, found in river, bottled water, chlorinated and unchlorinated water, and drinking water as well as other sources (Abilhamd, 2010; Odeyemi, 2012; Odeyemi, 2017). Members of this family are gram-negative, oxidase positive, catalase positive.

*Aeromonas* species have been receiving increased attention as primary and opportunistic pathogens in humans. Four species of *Aeromonas* (*i.e* *Aeromonas caviae*, *Aeromonas veronii biovar sobria*, *Aeromonas dhakensis* as well as *Aeromonas*

*hydrophila* are mostly implicated in human infections (Teunis and Figueras, 2016). There are several virulence factors that have been described in *Aeromonas* species including enterotoxins, cytotoxins, surface structures, secretory systems as well as haemolysins (Chopra et al., 2007; Aravena-Roman et al., 2014).

Infections caused by *Aeromonas* species have been observed to also occur in patients who are suffering from some different diseases such as diabetes, hepatitis and malignant tumours (Janda, 1998; Ko, 1995; Miyagi 2016). *Aeromonas* infections are also reported to occur through trauma (CDC, 1990; Voss, 1992; Miyagi, 2016). In August 2005 when hurricane Katrina occurred in New Orleans, *Aeromonas spp* were detected in flood water samples in approximately 107CFU/ml (Presley, 2006). In Southern Thailand during December 2004, in patients with skin and soft-tissue infections *Aeromonas* species were found in 145 of 641 isolates (Hiranthusikul, 2005, Miyagi, 2006). One of the *Aeromonas* species; *A. dhakensis* has been described to occur in the environment and has also been identified as a causative agent of diarrhoea in humans but can also cause infection in animals as well (Chen et al., 2016)

It is of importance to study the distribution of *Aeromonas* species in sources of water as they are the most common causative agents of diseases in aquatic organisms mainly fish, which humans use as a source of food; because consuming contaminated fish may lead to infections in humans as well. Furthermore, these sources may serve as transmission mechanisms to humans. Therefore, the present study determined the distribution of *Aeromonas* species in environmental water sources and wastewater as well as fish organisms.

## 4.3 Materials and methods

### 4.3.1 Isolation of bacteria

#### 4.3.1.1 Isolation of bacteria from fish

*Aeromonas* selective agar was used for the isolation of *Aeromonas* from fish. Sterile swabs were used to collect sample from surface, intestines, gills and flesh of the fishes. The bacteria were isolated by inoculating on *Aeromonas* selective agar (Oxoid, England) and incubating for 24hrs in 37 °C. After incubation, colony forming units were counted per plate and recorded. From these, 3-4 colonies from each plate were taken and inoculated in nutrient broth and incubated overnight. After incubation DNA was purified from each sample and was stored at -20 °C.

#### 4.3.1.2 Isolation of bacteria from water samples

*Aeromonas* selective agar enriched with ampicillin was used for the primary isolation of *Aeromonas* from water samples. Briefly, 50 µl Aliquots of each water sample (or the dilution of the sample for the wastewater) were inoculated on *Aeromonas* selective agar and incubated at 37 °C for 24 hours. Then after incubation colony forming units were counted per each plate and recorded; then 3-4 colonies from each plate were inoculated in a nutrient broth and incubated for 24 hrs. Then after incubation DNA was extracted from each sample and stored in -20 °C until further use.

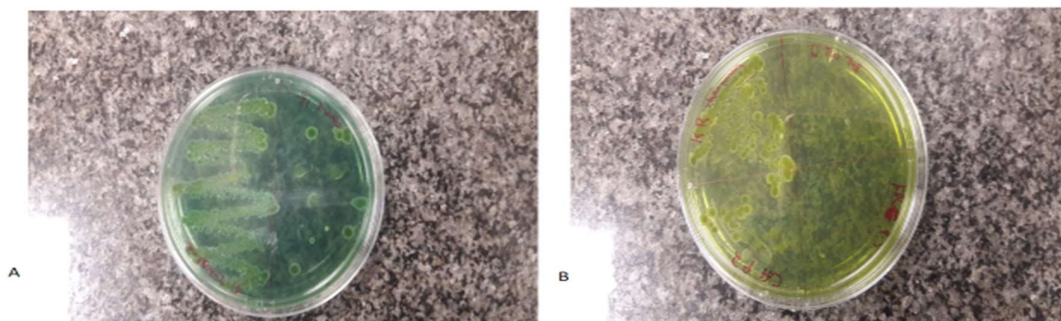


Figure 4.1: (A; B ) petri-dish plates showing colonies of *Aeromonas* after culturing

### 4.3.2 DNA isolation

*Aeromonas* DNA from water and fish samples was isolated using a bioflux bacterial genomic DNA extraction kit according to the manufacturer's instructions (Bioer Technology Co., Ltd.). The protocol was as follows: Harvested 1 mL of bacterial suspension from culture and swabs of field tissue was transferred to 2 mL centrifuge tubes and centrifuged at  $16\ 000 \times g$  for 1 min. Supernatant was discarded and the pellet was dissolved in 100  $\mu\text{L}$  EL buffer and mixed thoroughly and then incubated at  $37^\circ\text{C}$  for 15 minutes. After incubation 100 $\mu\text{L}$  of RS buffer was added with 10  $\mu\text{L}$  of PK solution and the mixture was mixed thoroughly and incubated at  $59^\circ\text{C}$  for 20 minutes to lyse the cells and 200  $\mu\text{L}$  GA buffer was added and the mixture was mixed. Then centrifuged at  $13\ 000 \times g$  for 1 min and the supernatant was transferred to a new 1.5ml tube and 400 $\mu\text{L}$  BA buffer was added. The mixture was transferred to a spin column and centrifuged at  $13\ 000 \times g$  for 1 minute, the flow through was discarded, after which 500 $\mu\text{L}$  of the G binding buffer was added to the spin column and centrifuged at  $10\ 000 \times g$  for 1 minute and the flow through was discarded. 500  $\mu\text{L}$  of washing buffer was added to the spin column twice and centrifuged at  $10\ 000 \times g$  for 1 minute and each time the flow through was discarded. After centrifuging for the second time the spin columns were centrifuged for an additional 1 minute at  $10\ 000 \times g$ , then the spin

columns were transferred into new sterile micro-centrifuged tubes after which 100µL of elution buffer was added and the mixture was incubated at room temperature for 1 minute and then centrifuged at 10 000 × g for 1 minute. The spin columns were removed, and the DNA was in the buffer inside the micro-centrifuged.

#### 4.3.3 Genotyping of *Aeromonas* by PCR

Extracted genomic DNA was genotyped for *Aeromonas spp* using singleplex conventional PCR as previously described by Lee et al., (2002); Wang et al., (2003), and Khan and Cerniglia, (1996). Refer to table 4.1 and table 4.2 for Primers and cycling conditions used.

Table 4.1. List of primers that were used for the identification of *Aeromonas* species

Primer name	Sequence 5'-3'	Size	Reference
ASA1F ASA1R	5'-TAAAGGGAAATAATGACGGCG-3' 5-'GGCTGTAGGTATCGGTTTTTCG-3'	249	Wang et al, 2003
Aer.hyF Aer.verF Reverse	5'-GAAAGGTTGATG CCTAATACGTA-3' 5'-GAGGAAAGGTTGGTAGCTAATAA-3 5'-CGTGCTGGCAACAAAGGACAG-3'	685 658	Dorsch et al, 1994
AER8 AER9	5'-CTGCTGGCTGTGACGTTACTCGCAG-3' 5'-TTCGCCACCGGTATTCCTCCAGATC-3'	260	Khan and Cerniglia, 1996
PAAS1 PAAS2	5'-CGTTGGATATGGCTCTTCCT-3' 5'-CTCAAACGGCTGCGTACCA-3'	243	Byers et al, 2003
AER1 AER2	5'-ACGCAGCAGATATTAGCTTACG-3' 5'-ACGCAGCAGATATTAGCTTACG-3'	316	Khan and Cerniglia, 2003

Table 4.2: Cycling conditions for primers used to detect *Aeromonas* species.

Primer	Cycling conditions
ASA1F ASA1R	initial denaturation at 95 °C for 5mins, at 95 °C for 5 min, 50 cycles of denaturation at 95 °C for 30s, annealing at 59 °C for 30s and extension at 72 °C for 30s, and 72 °C for 7 min
Aer.hyF Aer.verF Reverse	initial denaturation at 98 °C for 150s, 5 cycles of denaturation at 93 °C for 30s, annealing 64c for 30s, extension at 72 °C for 30s and 28 cycles of denaturation at 93 °C for 30s, annealing at 60 °C for 30s, extension at 72 °C for 45s and final extension at 72 °C for 5mins.
AER1 AER2	initial denaturation at 95 °C for 2 mins, 35 cycles of denaturation at 94 °C for 1 min, annealing at 68 °C for 1min, extension at 72 °C for 1min and final extension at 72 °C for 3mins

## 4.4 Results

### 4.4.1 Molecular detection of *Aeromonas* species

Four species of *Aeromonas* (*Aeromonas caviae*, *Aeromonas hydrophila*, *Aeromonas veronii*, *Aeromonas sobria*) were detected using conventional PCR. The PCR products were those expected for each species as shown in figure 4.2 and figure 4.3 respectively.

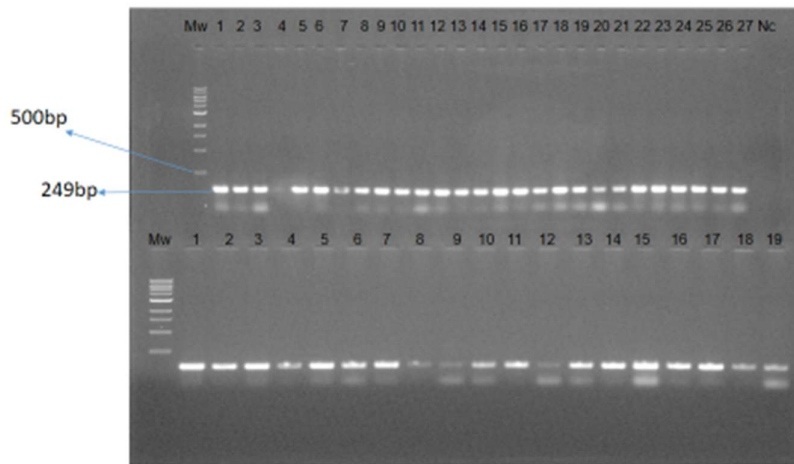


Figure 4.2. PCR amplification products for *A. sobria* from various water sources samples. Representative of 1% agarose gel photograph stained with 3% ethidium bromide. Row 1: Mw= molecular marker, lanes 1-27= wastewater, surface water, dams and fish samples and lane 28 is the negative control. Row 2: Mw= molecular marker, lanes 1-19= wastewater, surface water, dams and fish samples

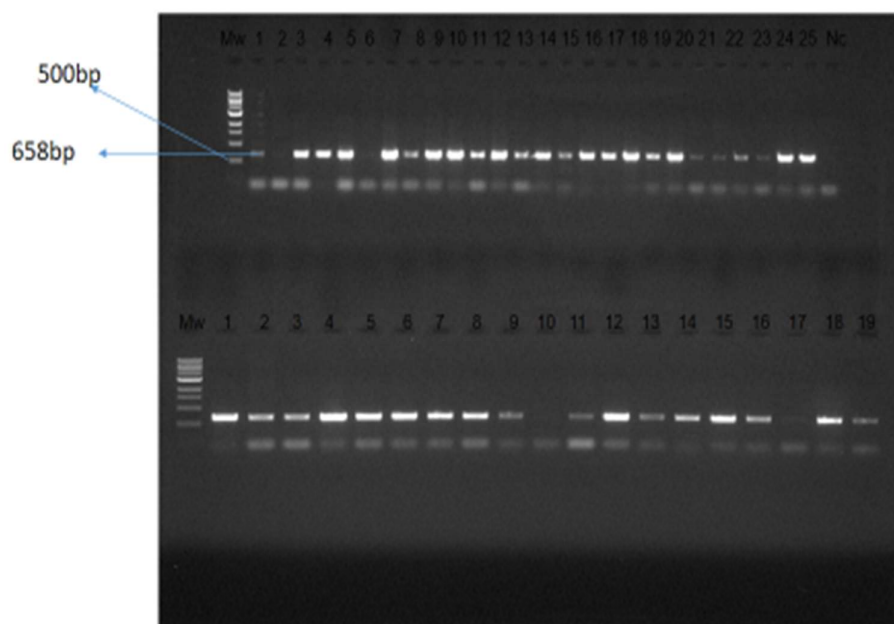


Figure 4.3 PCR amplification products for *A. veronii* from samples of different water sources. Representative of 1% agarose gel photograph stained with 3% ethidium bromide. Row 1: Mw= molecular marker, lanes 1-25= wastewater, surface water, dams and fish samples and lane Nc is the negative control. Row 2: Mw= molecular marker, lanes 1-19= wastewater, surface water, dams and fish samples

#### 4.4.2 Frequency of occurrence of *Aeromonas* species in different sources of water and fish as well as the occurrence of *Aeromonas* in different levels.

Four species of *Aeromonas* were identified using PCR. The overall detection rate of *Aeromonas* species was 84.25%. *A. caviae* was the most prevalent species in the samples with 73.6 %, followed by *A. veronii* with 64.6% prevalence. All the tested *Aeromonas* species had high prevalence with the least percentage being that of *A. sobria* with 43.8%. The prevalence results are shown in table 4.3.

Table 4.3: Overall occurrence of *Aeromonas* species in different sources from South Africa and Zambia

Organisms	Frequency	Percent %
<i>A. hydrophila</i>	75	52.4
<i>A. veronii</i>	93	64.6
<i>A. sobria</i>	63	43.8
<i>A. caviae</i>	106	73.6

#### 4.4.3 Frequency of occurrence of the bacteria

The occurrence of *Aeromonas* in different levels was high in level (from 0-10) with the least being in level level 3 with a percentage of 22.2%. Results are tabulated below (table 4.4)

Table 4.4: Frequency of occurrence of the different bacteria levels

Bacteria level (CFU/ml)	Frequency	Percent %
0	33	22.9
0-10	36	25.0
10-100	43	29.9
>100	32	22.2
Total	144	100.0

#### 4.4.4 Distribution of *Aeromonas* species divided into levels in sewage and non-sewage samples

All the samples were obtained from 10 sources that were either wastewater or non-wastewater namely: ground water, dams, canals, tap water, ponds, fish, crayfish, and river. Low prevalence of 7.5% of *Aeromonas* was observed in sewage samples in level 1 (0-10). The occurrence of *Aeromonas* in different levels in both sewage and non-sewage samples was statistically significant with  $p=0.00$ . It can be seen from Table 4.5 that the sewage samples were the most contaminated by *Aeromonas* spp with higher percentage of samples containing more than 100 CFU/ml.

Table 4.5: Comparison between the occurrence of *Aeromonas* in sewage samples and all the other type of samples.

Bacteria level (CFU/ml)	Non-sewage samples	Sewage samples	Total	Statistics
0	27(26.0%)	6(15.0%)	33(22.9%)	0.000
0-10	33(31.7%)	3(7.5%)	36(25.0%)	
10-100	30(28.8%)	13(32.5%)	43(29.9%)	
>100	14(13.5%)	18(45.0%)	32(22.2%)	

#### 4.4.5 Occurrence of *Aeromonas* species in sample types in different levels

Table 4.6 below shows the prevalence of different of *Aeromonas* in various sample types. Most tap water (92.3%) did not have *Aeromonas*. This is an indication that the microbial quality of tap water was generally good. High prevalence of *Aeromonas* in level 1 (0-10) and 2 (10-100) was also demonstrated in samples from a river source. The difference was statistically significant.

Table 4.6: Occurrence of *Aeromonas* species in sample types in different levels

Bacteria level (CFU/ml)	River	Sewage	Tap water	Fish	Total
0	6(9.5%)	6(15.0%)	12(92.3%)	9(32.1%)	33(22.9%)
0-10	31(49.2%)	3(7.5%)	1(7.7%)	1(3.6%)	36(25.0%)
10-100	23(36.5%)	13(32.5%)	0(0.0%)	7(25.0%)	43(29.9%)
>100	3(4.8%)	18(45.0%)	0(0.0%)	11(39.3%)	32(22.2%)

#### 4.4.6 Distribution of *Aeromonas* based on country

Table 4.7 shows prevalence of *Aeromonas* species in the two different countries where samples were obtained. The occurrence of *Aeromonas* species was predominant in Zambia more than South Africa with only *Aeromonas sobria* that was more prevalent in South Africa with 46.7%. The difference for the occurrence of *A. hydrophila* was statistically significant with a p value of 0.014, however, the occurrence of other species were not statistically significant with p values > 0.05.

Table 4.7: Distribution of *Aeromonas* species based on which country they come from

Organisms	Country		Total	Statistics
	South Africa	Zambia		
<i>A. hydrophila</i>	48(46.2%)	27(69.2%)	75(52.4%)	0.014
<i>A. veronii</i>	66(62.9%)	27(69.2%)	93(64.6%)	0.477
<i>A. sobria</i>	49(46.7%)	14(35.9%)	63(43.8%)	0.247
<i>A. caviae</i>	77(73.3%)	29(74.4%)	106(73.6%)	0.901

#### 4.4.7 Distribution of *Aeromonas* in different districts in South Africa and Zambia

Table 4.8 shows the prevalence of *Aeromonas* species in different districts and Zambia, *A. hydrophila* and *A. caviae* had high prevalence in all the districts. Between the two, there was no statistical significance in their prevalence with p values that were more than 0.05. The Mopani districts had low prevalence compared to other districts.

Table 4.8: Distribution of *Aeromonas* in South Africa and Zambia

Districts	<i>A. hydrophila</i>	<i>A. veronii</i>	<i>A. sobria</i>	<i>A. caviae</i>
Vhembe	17(50.0%)	18(52.9%)	21(61.8%)	26(76.5%)
Mopani	3(27.3%)	7(63.6%)	3(27.3%)	7(63.6%)
Capricon	10(50.0%)	15(75.0%)	7(35.0%)	16(80.0%)
Sekhukhune	4(50.0%)	4(50.0%)	3(37.5%)	4(50.0%)
Waterberg	14(45.2%)	22(68.8%)	15(46.9%)	24(75.0%)
Zambia	27(69.2%)	27(69.2%)	14(35.9%)	29(74.4%)
Total	75(52.4)	93(64.6%)	63(43.8%)	106(73.6%)
Statistics	0.156	0.504	0.183	0.629

#### 4.4.8 Occurrence of *Aeromonas* species wastewater and non-wastewater samples

The results in table 4.9 show the prevalence of *Aeromonas* species isolated from samples comparing sewage and non-sewage samples. Detection of *Aeromonas* species was at higher rates in sewage samples. The results were statistically significant for the prevalence of *Aeromonas veronii* and *Aeromonas caviae* ( $p < 0.05$ ).

Table 4.9: Occurrence of *Aeromonas* species wastewater in and non-wastewater samples

Organisms	Non-sewage samples	Sewage samples	Total	Statistics
<i>A. hydrophila</i>	54(52.4%)	21(52.5%)	75(52.4)	0.994
<i>A. veronii</i>	60(57.7%)	33(82.5%)	93(64.6%)	0.005
<i>A. sobria</i>	42(40.4%)	21(52.5%)	63(43.8%)	0.189
<i>A. Caviae</i>	71(68.3%)	35(87.5%)	106(73.6%)	0.019

#### 4.4.9 Occurrence of *Aeromonas* based on sample type

*Aeromonas veronii* and *Aeromonas caviae* were predominant in sewage samples. Tap water had low prevalence of *Aeromonas* species with *Aeromonas caviae* accounting for only 7.7%. The prevalence of *Aeromonas* species in different sample types is statistically significant with  $p=0.00 - 0.001$ . The results are presented in table 4.10

Table 4.10: Occurrence of *Aeromonas* based on sample type

Organisms	River	Sewage	Tap water	Fish	Total	Statistics
<i>A. hydrophila</i>	33(52.4%)	21(52.5%)	0(0.0%)	21(75.0%)	75(52.4)	0.000
<i>A. veronii</i>	39(61.9%)	33(82.5%)	0(0.0%)	21(75.0%)	93(64.6%)	0.000
<i>A. sobria</i>	34(54.0%)	21(52.5%)	0(0.0%)	8(28.6%)	63(43.8%)	0.001
<i>A. caviae</i>	48(76.2%)	35(87.5%)	1(7.7%)	22(78.6%)	106(73.6%)	0.000

#### 4.4.10. The efficiency of wastewater treatment plants in removing *Aeromonas* cells during treatment

The efficiency of many wastewater treatment plants in removing the bacteria was above 70%. There are four wastewater treatment plants from Giyani, Tzaneen, Elim and Kaseba village (Zambia) that managed to remove bacteria by 100%. The removal efficiency ranged from 71% - 100%. Figure 4.4 shows the results.

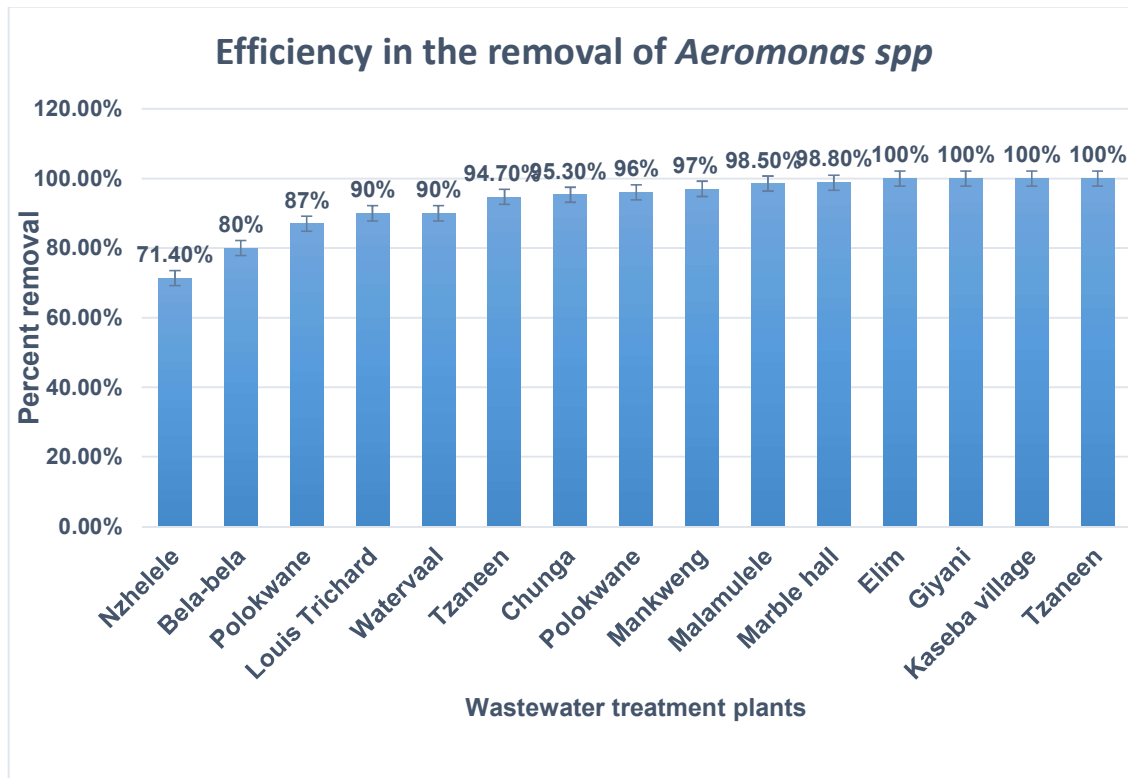


Figure 4.4: A bar graph showing the efficiency of wastewater treatment plants in the removal of *Aeromonas* cells during treatment.

## 4.5 Discussion

*Aeromonas* are microorganisms often found in aquatic environments (Miyagi et al., 2016). In our study *Aeromonas* species was found to be abundant in surface water, wastewater, and Ground water. The study found that *Aeromonas caviae* was the most predominant species. Similar results were also obtained by Bousaic et al., in 1991. Most of the wastewater samples including the effluents had high number of *Aeromonas* cells implying that *Aeromonas* species are not totally removed after the treatment. Similar results have also been described by Martone-Roch et al., 1991 and Popovic et al., 2015.

*Aeromonas caviae* had low prevalence in fish tissue, although *Aeromonas hydrophila* was abundant in fish tissue. The risk of microorganisms; in particular bacteria; to penetrate fish's tissue is when the total count exceeds over  $10^4$  CFU/ml (Popovic et al., 2015). *A. hydrophila* is also common in environmental samples. Igbinosa and colleagues in 2012 described *Aeromonas hydrophila* to be the most common cause of traveler's diarrhea, which suggests its abundance in environmental waters; showing that water is a common route for transmission. However, in our study *A. hydrophila* was the second most prevalent species following the predominant *Aeromonas caviae*.

In a study done by Didugu et al., 2015, *Aeromonas* species were prevalent in various water sources including drinking water, wastewater, bottled water and well water (Didugu et al., 2015). In a study conducted in Japan by Miyagi et al., (2016), *Aeromonas* spp was found to have high prevalence in environmental water samples and was reported to occur at over 1000 CFU/100ml. Our results are also in agreement with theirs, because they also found *Aeromonas veronii* to be the most predominant

species (Miyagi et al., 2016). Pathogenic *Aeromonas* strains detection represent potential risk for human health in that they would cause serious infections. Uche and colleague revealed that *Aeromonas* were implicated in diarrhea in children more than in adults (Uche and Kennedy, 2014). Constant practice of good hygiene and monitoring of reduction of pathogenic bacteria from drinking water should be encouraged.

*Aeromonas* was found to be more prevalent (75%) in water samples compared to 54.5% in fish samples by Latif-eugenin et al., (2016). Our results were also similar to those obtained by Kivanc et al., (2011) who found high prevalence of *Aeromonas* species in river water. However, our findings differ with theirs because we also detected *Aeromonas caviae* in tap water, whereas from their findings drinking water was free from any species of *Aeromonas*. Our results were also supported by Ghenghesh et al., (2015) who also concluded that *Aeromonas* are found in different sources of water including treated water. Although the removal efficiency of the wastewater treatment plants was very high, we still found high prevalence of *Aeromonas* species in the effluents

## 4.6 Conclusion

The study revealed that *Aeromonads* are widely distributed in our environmental waters, calling for an action in reducing their occurrence from our water sources as well as the wastewater effluents. The abundance of *Aeromonas* species in the environmental waters, especially in drinking water will continue to pose threat to public human health. This is because consumption of water and food contaminated by

*Aeromonas* will always lead to diseases such as diarrhoea, wound infection and others.

## 4.7 References

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## Chapter 5: Antibiotic resistance and detection of beta-lactamase genes of *Aeromonas* species isolated from aquatic sources

### 5.1 Abstract

**Background:** *Aeromonas* is an important pathogen of fish and humans, responsible for infections such as septicaemia, wound infection, ulcer and many others. *Aeromonas* resistance to antibiotics has been observed in environmental samples however, there is no regular reporting system for resistance profiles as well as genes that might be responsible for antibiotic resistance of *Aeromonas* from the northern part of South Africa.

**Methods:** Pure colonies of *Aeromonas* were obtained by sub-culturing preserved *Aeromonas* Isolates on *Aeromonas* selective agar. Mueller-Hinton broth and agar were used to evaluate the antibiotic susceptibility of *Aeromonas* isolates against twelve selected antibiotics. PCR was used to detect beta-lactamase genes responsible for antibiotic resistance in *Aeromonas* species.

**Results:** The bacteria was completely resistant to cefuroxime accounting for 100% resistance. *Aeromonas* isolates showed high resistance to Trimethoprim (88.7% for *A. hydrophila*), cefazolin (highest 97.8 for *A. caviae*), and ceftazidime (83.9% for *A. sobria*). TEM was the most prevalent beta-lactamase gene with detection rate of 87% followed by OXA-1 with 26.4%. All isolates lacked the presence of CTX-M3.

**Conclusion:** Many *Aeromonas* species carry the TEM beta-lactamase gene, which account for their resistance to penicillins and more particularly ampicillin. *Aeromonas* resistance to antibiotics poses great danger to aquatic and human health.

## 5.2 Introduction

*Aeromonas* are important pathogens of fish and humans, responsible for infections such as septicaemia and furunculosis (Brifias et al., 2003; Samie et al., 2005). Increase in antibiotic resistance of *Aeromonas* species has been reported by various studies, however, there is limited literature on antibiotic resistance of these organisms in South Africa in general and in the Northern region in particular. Antibiotic resistance in *Aeromonads* owes to the abundance of antimicrobial agents in sewage effluents and water bodies that receives large amounts of these effluents (Pitrowska et al., 2017). Antibiotic resistance of microorganisms in general is a public health concern since the resistance will result in difficulties to control infections caused by these microorganisms. *Aeromonas* are being targeted in aquatic environments as indicator bacteria in monitoring the antimicrobial resistance because of their ability to acquire and exchange antibiotic resistance genes (Patil et al., 2016).

*Aeromonas* resistance to antibiotics has been observed in environmental samples (Igbinosa et al, 2012; Huddleston et al, 2006, Aravena-roman et al, 2012; Odeyemi and Ahmad, 2017). Beta-lactamase genes are the ones that are most responsible for antibiotic resistance in *Aeromonas*. In aquaculture it is believed that the use of prophylactic antibiotics reduces mortality and contributes to growth and increases production and revenue as well (Pridgeon and Klesius, 2013). Such compounds are used for treatment and prevention of diseases in fish caused by *Aeromonas spp* such as haemorrhagic septicaemia, red-sore disease, *Aeromonas* septicaemia and ulcer

disease. It is a common practice to administer these compounds together with fish feed (Swan and White, 1991; Patil, 2016). Though antibiotics are enhancing production in aquaculture there is a concern that the use of these compounds selects for antibiotic resistant bacteria and antibiotic resistance genes, mostly in cases of over-doses or less doses of antibiotics administered. But this is increasing antibiotic resistance at high level through water discharge into environmental waters and fish consumption (Kolar, 2001).

The Extended Spectrum Beta-lactamases (ESBL) are the most common responsible for antibiotic resistance. These are the derivatives of well-known  $\beta$ -lactamases (SHV and TEM  $\beta$ -lactamases) that went through substitution of one or more amino acids near the active site of the enzyme (Osman et al., 2017). The increased challenge of tackling bacterial infections is due to the emergence of these  $\beta$ -lactamases (Sana et al., 2011). ESBL producing bacteria are resistant to penicillins, and cephalosporins (Adenaike et al., 2013; She et al., 2015). More than 200  $\beta$ -lactamases have been described, and according to their structural and functional characteristics they are divided into 4 major groups and 8 subgroups. TEM, OXA-type, SHV are the most common ones usually detected from species belonging to Enterobacteriaceae family (Bush et al., 1995; bush and Jacoby, 1997; Bradford, 2001; Delmani et al., 2017).

The resistance of *Aeromonas* to  $\beta$ -lactam antimicrobial agents is mediated by  $\beta$ -lactamases that inactivate the antibiotics by hydrolysing the beta-lactam ring (Livermore, 1995; Delmani et al., 2017). Most studies have focused on antibiotic resistance from clinical samples mostly with limited report on antibiotic resistance from environmental samples. The present study focuses on the evaluation of antibiotic resistance from environmental samples and also detect the extended spectrum beta

lactamases by PCR, including blaTEM, blaCTX-M3, blaCTX-M9, blaCMY-type, blaOXA.

## **5.3 Materials and methods**

### **5.3.1 Antimicrobial testing**

Antimicrobial susceptibility test of *Aeromonas* isolates was performed on Mueller-Hinton agar plate using the Kirby-Bauer disk diffusion method following the CLSI recommendations. A total of 54 isolates were selected so as to be representative of all types of samples to be evaluated for their susceptibility against 12 antimicrobial agents including; amikacin, ceftazidime, ceftriaxone, cefuroxime, gentamicin, nalidixic acid, tobramycin and trimethoprim-sulfamethoxazole, trimethoprim, norfloxacin, cefazolin and erythromycin. Growth of the isolates was enriched in Mueller-Hinton broth before culturing on the Mueller-Hinton agar by selecting 3 or more colonies from the *Aeromonas* selective agar plate and inoculate in the broth and incubate the suspension for 2 hours at 37 °C. Then 10µl of the suspension was inoculated on two Mueller-Hinton agar plates using a sterile cotton swab by streaking the swab over the entire sterile agar surface 3 times. Then onto each plate, 6 antimicrobial disks were placed at the recommended distance from each other. The petri plates were aerobically incubated at 37 °C for 24 hours before the size of the zone of inhibition were recorded.

### **5.3.2 DNA extraction**

The protocol was described in the previous chapter (chapter 4)

### 5.3.3 Detection of beta-lactamase genes by PCR

Polymerase chain reaction was used to detect the presence of different beta-lactamase genes (TEM, OXA, CMY-type, CTX-M3, and CTX-M9) in 95 samples that were randomly selected to represent the different sample types. Polymerase chain reaction was performed using T100 thermal cycler (Bio-Rad) using the primers as shown in Table 5.1 and Table 5.2. Amplified DNA products were subjected to gel electrophoresis through 2% agarose gel containing ethidium bromide and the gels were viewed using a gel-doc machine from Bio-Rad Company. Each 25 $\mu$ l contained 12.5  $\mu$ l dreamtag, 2.5  $\mu$ l IQ solution, 0.6  $\mu$ l primers (forward and reverse primers), and 3.8  $\mu$ l nuclease free water to bring the total volume to 25  $\mu$ l.

Table 5.1: Primers used for detection of  $\beta$ -lactamase genes

Primer	Sequence	Product size	Reference
TEM	F: TTC TTG AAG ACG AAA GGG R: ACG CTC AGT GGA ACG AAA AC	1207 bp	Ho et al., 2005
CTX-M3	F: CCG TTT CCG CTA TTA CAA AC R: GTT ACA ATG TGT GAG AAG CAG	891 bp	
CTX-M9	F: GTG ACA AAG AGA GTG CAA CGG R: ATG ATT CTC GCC GCT GAA GCC	857 bp	
CMY-type	F: GAT TCC TTG GAC TCT TCA R: TAA AAC CAG GTT CCC AGA TAG	1807 bp	
OXA-1	F: ACA CAA TAC ATA TCA ACT TCG R: AGT GTG TTT AGA ATG GTG ATC	936 bp	

SHV	F: CAC TCA AGG ATG TAT TGT G R: TTA GCG TTG CCA GTG CTC G	885 bp	Brinas et al., 2003
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Table 5.2: Cycling conditions for primers used in amplification of beta-lactamase genes.

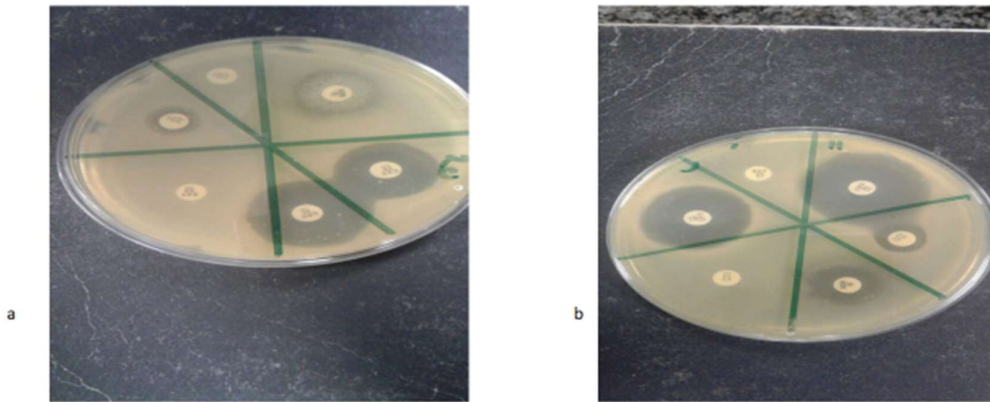
Primer	Cycling conditions
SHV, TEM, CMY-type, OXA-1	Initial denaturation at 94 °c for 5min, 35 cycles of denaturation at 94 °c for 30s, annealing at 60 for 30s, extension at 72 °c for 30s and final extension at 68 °c for 10 min.
CTX-M-3	Initial denaturation at 94 °c for 10min, 30 cycles of denaturation at 94 °c for 60s, annealing at 55 °c for 60s, extension at 72 °c for 2min and final extension at 72 °c for 5min min.
CTX-M-9	Initial denaturation at 94 °c for 10min, 25 cycles of denaturation at 94 °c for 30s, annealing at 55 °c for 30s, extension at 72 °c for 60s and final extension at 72 °c for 10 min.

Ho et al., 2005, Kaye et al., 2004, Brinas et al., 2003

## 5.4 Results

### 5.4.1 Antibiotic susceptibility of *Aeromonas* to selected antibiotics

A total of 54 isolates were tested against 12 antibiotics. The diameters of the zone of inhibition (Figure 5.1) were measured recorded and analysed. All isolates were resistant to cefuroxime and strong resistance was also observed against ceftriaxone (51.9%).



**Figure 5.1: (a, b) Petri-plates showing anti-bacterial activity of some of the tested antibiotics**

### 5.4.2 Overall frequency of antibiotic resistance of *Aeromonas spp*

Figure 5.2 shows the overall frequency of antibiotic resistance of *Aeromonas spp* to 12 antibiotics. No resistance was observed to cefuroxime (CXM). High resistance to cefazolin (KZ) was observed in 98.1% followed by resistance to trimethoprim (TMP) encountered in 87.0%. Overall, high resistance to antibiotics was observed.

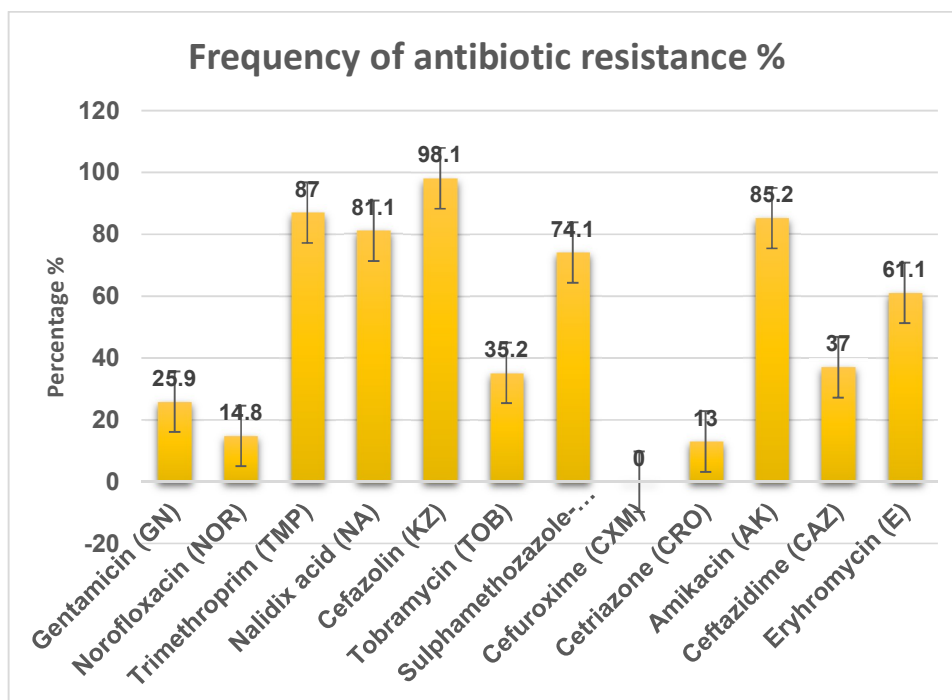


Figure 5.2: Overall frequency of antibiotics in *Aeromonas* isolates

### 5.4.3 Antimicrobial resistance of *Aeromonas hydrophila*

The table 5.3 below presents the resistance profile of *Aeromonas hydrophila* to different antibiotics. Most of the isolates (96.9%) were resistant to Cefazolin (KZ), after which high resistance was also observed in 75.0% against Nalidix acid (NA). A lower resistance was noted against Norfloxacin (NOR) with a percentage of 12.6%. There was no statistical significance on the resistance of *Aeromonas* species to all these different antibiotics. *Aeromonas veronii* was mostly resistant to Cefazolin (KZ) accounting for 97.8%. This high resistance was followed by a notably high resistance to Trimethoprim (TMP) accounting for 88.9%. Sulphamethozazole-Trimethoprim (SXT) accounted for high resistance of 77.8%. The resistance of *Aeromonas veronii* to antibiotics was not statistically significant with  $p > 0.05$ . Antibiotic resistance profile of *Aeromonas sobria* showed high resistance to Cefazolin (KZ), Trimethoprim (TMP), Nalidix Acid (NA) and Sulphamethozazole-Trimethoprim (SXT) was observed for *Aeromonas sobria* with 96.8%, 80.6%, 70.0% and 67.7% respectively. There was statistical significance on the resistance of *Aeromonas sobria* to Nalidixic Acid (NA) with  $p = 0.018$  and Amikacin (AK) with  $p = 0.022$ . However, the difference for the resistance of *A. sobria* to other antibiotics was not significant. The observed resistance of *Aeromonas caviae* to all antibiotics was very high. Cefazolin (KZ) and Trimethoprim (TMP) accounted for the highest prevalence with 97.8% and 87.0% respectively. The difference for less resistance to Erythromycin was statistically significant with a  $p$  value of 0.050.

Table 5.3: Antimicrobial resistance profile of *Aeromonas* species

Antibiotics	<i>A. hydrophila</i>	<i>A. veronii</i>	<i>A. sobria</i>	<i>A. caviae</i>
Gentamicin (GN)	9(28.1%)	12(26.7%)	10(32.3%)	13(28.3%)
Norofloxacin (NOR)	4(12.55)	8(17.8)	7(22.6%)	8(17.45)
Trimethoprim (TMP)	27(8.4%)	40(88.9%)	25(80.6%)	40(87.0%)
Nalidix acid (NA)	24(75.0%)	35(79.5%)	<b>21(70.0%)</b>	38(84.4%)
Cefazolin (KZ)	31(96.9%)	44(97.8%)	30(96.8%)	45(97.8%)
Tobramycin (TOB)	9(28.1%)	16(35.6%)	13(41.9%)	15(32.6%)
Sulphamethozazole-Trimethoprim (SXT)	22(68.8%)	35(77.8%)	21(67.7%)	34(73.9%)
Cefuroxime (CXM)	32(100.0%)	45(100.0%)	31(100.0%)	46(100.0%)
Cetrazone (CRO)	22(68.8%)	16(35.6%)	10(32.3%)	17(37.0%)
Amikacin (AK)	21(65.6%)	28(62.2%)	<b>23(74.2%)</b>	29(63.0%)
Ceftazidime (CAZ)	28(87.5%)	39(86.7%)	26(83.9%)	39(84.8%)
Erythromycin (E)	6(18.8%)	7(15.6%)	<b>8(25.8%)</b>	<b>5(10.9%)</b>

### 5.4.7 Multiple Antibiotic Resistance of *Aeromonas* species

Of the 54 isolates tested for antibiotic resistance, many were resistant. Some isolates were resistant to more than one antibiotic of which 18.5% were resistant to 2 antibiotics and 5.6 % were resistant to 4 antibiotics. The results are shown in the table 5.4.

Table 5.4: Frequency of occurrence of multiple antibiotic resistance among *Aeromonas* isolates

Number of antibiotics	Frequency	Percent (%)
4	3	5.6
5	7	13.0
6	9	16.7
7	13	24.1
8	12	22.2
9	5	9.3
10	4	7.4
11	1	1.9
Total	54	100.0

### 5.4.7 Frequency of the beta-lactamase genes

A total of 95 samples were tested for the presence of different beta-lactamase genes. TEM was the most frequent gene occurring in 78 (87.6%) samples, followed by OXA-1 23 (25.3%) and SHV positive in 23 (25.3%) samples respectively. All isolates were negative for the CTX-M-3. Some isolates had two or more genes. The results are shown in table 5.5.

Table 5.5. Over all frequency of occurrence of the beta lactamase genes in all the samples

<b>β-lactamase genes</b>	<b>Frequency</b>	<b>Percent %</b>
<b>TEM</b>	78	87.6
<b>CTX M-3</b>	0	0
<b>CTXM-9</b>	3	3.4
<b>CMY-type</b>	7	7.8
<b>OXA-1</b>	23	25.3
<b>SHV</b>	23	25.3

#### 5.4.8 Distribution of beta-lactamase genes from *Aeromonas* isolates in South Africa and Zambia

Table 5.6 below presents prevalence of beta-lactamase genes in South Africa and Zambia. South Africa presented high prevalence of some beta-lactamase genes. TEM was the most predominant accounting for 91.7% in Zambia and 87% in South Africa. This high prevalence of TEM was followed by OXA-1. CTXM-9 had the lowest prevalence found in 3.9% of samples in South Africa, but it was not common in samples collected from Zambia. CTXM-3 was not common in samples from both countries.

Table 5.6: Distribution of beta-lactamase genes from *Aeromonas* isolates in South Africa and Zambia

Beta lactamase	South Africa	Zambia	Total	Statistics
TEM	67(87.0%)	11(91.7)	78(87.6%)	X <sup>2</sup> = 0.208 P= 0.649
CTXM3	0	0	0	
CTXM9	3(3.9%)	0	3(3.4%)	
CMY-TYPE	6(7.8%)	1(7.7%)	7(7.8%)	
OXA-1	21(27.6%)	2(18.2)	23(26.4%)	
SHV	21(26.6%)	2(16.7%)	23(25.3%)	

#### 5.4.9 Distribution of beta-lactamases per the different districts

The results in table 5.7 shows the distribution of the beta lactamase genes in various districts. Sekhukhune district lacked CTXM3, CTXM9, CMY-type and OXA-1 and had low prevalence other b-lactam genes. Vhembe district also lacked the presence of CXM3, CTXM9 as well as CMY-type. Waterberg, however showed consistent prevalence of the b-lactam genes with TEM detected in 90.9% and OXA-1 was detected in 33.3%. Mopani and capricorn also presented various prevalence of the b-lactam genes with TEM dominating.

Table 5.7: Distribution of beta-lactamases per the different districts

Districts	TEM	CTXM3	CTXM9	CMY-TYPE	OXA-1	SHV
Vhembe	22(78.6%)	0	0	0	6(21.4%)	2(7.1%)
Mopani	6(100.0%)	0	0	2(33.3%)	2(33.3%)	2(28.6%)
Capricorn	15(88.2%)	0	2(12.5%)	1(6.3%)	6(35.3%)	7(43.8%)
Sekhukhune	4(100.0%)	0	0	0	0	1(25.0%)
Waterberg	20(90.9%)	0	1(4.3%)	3(13.0%)	7(33.3%)	9(37.5%)
Zambia	11(91.7%)	0	0	1(7.7%)	2(18.2%)	2(16.7%)
Total	78(87. %)	0	3(3.4%)	7(7.8%)	23(26.4%)	23(25.3%)

#### 5.4.10 Occurrence of beta-lactamase genes in *Aeromonas* isolates based on type of samples

Like in previous results, CTXM3 was not detected in any of the types of samples. Beta-lactamase genes were detected mostly in sewage samples followed by pond water. In all these types of samples TEM was the predominant gene with highest prevalence except in tap water. In tap water only OXA-1 was observed in 50.0%. TEM was also the only gene to be detected in ground water sample. Results are tabulated below.

Table 5.8: Occurrence of beta-lactamase genes in *Aeromonas* isolates based on type of samples

Type of sample	TEM	CTXM3	CTXM9	CMY-TYPE	OXA-1	SHV
Crayfish	2(100.0%)	0	0	0	0	1(50.0%)
Dam	4(100.0%)	0	0	0	2(50.0%)	0
Fish	3(100.0%)	0	0	0	0	0
Ground water	1(100.0%)	0	0	0	0	0
Pond water	5(100.0%)	0	0	1(20%)	1(25.0%)	1(20.0%)
River	39(90.7%)	0	0	0	8(19.0%)	8(18.3%)
Sewage	24(80.0%)	0	3(10.0%)	6	11(39.3%)	13(41.9%)
Tap water	0(0.0%)	0	0	0	1(50.0%)	0
Total	78(87.6%)	0	3(3.4%)	7(7.8%)	23(26.4%)	23(25.3%)
Statistics	0.130	constant	0.527	0.103	0.352	0.219

#### 5.4.11 Occurrence of beta lactamase genes in *Aeromonas* isolates from wastewater and non wastewater sources

TEM was observed in 54(91.5%) of non-sewage samples, followed by 20.3% prevalence of OXA-1 in non-sewage samples. Sewage samples had high prevalences for TEM (80.0%) followed by SHV with 41.9%, with lower prevalence observed in CTXM9 with 10.0%. results are shown in table 5.9 below.

Table 5.9: Occurrence of beta lactamase genes in *Aeromonas* isolates from wastewater and non wastewater sources

Beta-lactamase	Non-sewage	Sewage	Total
TEM	54(91.5%)	24(80.0%)	78(87.6%)
CTXM3	0	0	0
CTXM9	0	3(10.0%)	3(3.4%)
CMY-TYPE	1(1.7%)	6(20.0%)	7(7.8%)
OXA-1	12(20.3%)	11(39.3%)	23(26.4%)
SHV	10(16.7%)	13(41.9%)	23(25.3%)

#### 5.4.12 Occurrence of beta lactamases genes according to sample type

Samples from fish had the lowest prevalence of beta-lactamase genes with the detection of TEM and SHV only. Samples from the sewage had the high prevalence of beta-lactamase genes harbouring all types of beta-lactamase except for the CTXM3 that was absent in all the samples. CTXM9 was observed only in samples obtained from sewage with the percentage of Oxa-1 and SHV were the most predominant in

sewage samples. TEM was highly prevalent in sample from fish (100.0%) followed by samples from the river (92.5%). Results are recorded in the table below.

Table 5.10: Prevalence of beta-lactamase genes in various sample types

Sample type	TEM	CTXM3	CTXM9	CMY-TYPE	OXA-1	SHV
River	49(92.5%)	0	0	1(1.9%)	11(21.6%)	9(16.7%)
Sewage	24(80.0%)	0	3(10.0%)	6(20.0%)	11(39.3%)	13(41.9%)
Tap water	0	0	0	0	1(50.0%)	0
Fish	5(100.0%)	0	0	0	0	1(20.0%)
Total	78(87.6%)	0	3(3.4%)	7(7.8%)	23(26.4%)	23(25.3%)

#### 5.4.13 Occurrence of beta-lactamases in isolates that were positive for *Aeromonas hydrophila*

Table 5.11 below shows the prevalence of beta-lactamase genes in samples positive for *Aeromonas hydrophila*. TEM was observed in 50 (90.9%) of *A. hydrophila* positives. This was followed by the detection of OXA-1 in 24.5%. And the lowest detection of beta-lactamase was observed in CTXM9 with the percentage of 5.5%. There was no statistical significance on the occurrence of beta-lactamase in relation to occurrence of *Aeromonas hydrophila* with p values that were greater than 0.05.

High prevalence of TEM was observed in 88.7% of samples positive for *Aeromonas veronii*, followed by the detection of SHV in 28.8%. Low prevalence was also observed in CTXM9 and CMY-TYPE with 4.2% and 8.5% respectively. However, the difference

was not significant with  $p > 0.05$ . CTXM9 showed the lowest prevalence with detection in only 1.9%. High prevalence of TEM was observed in 87.0% followed by the prevalence of OXA-1 which was detected in 32.7%. The difference for the occurrence of beta lactamase genes was not statistically significant with  $p > 0.05$ .

Table 5.16 below shows prevalence of beta-lactamase in the samples that were positive for *Aeromonas caviae*. Likewise, TEM was the most predominant detected in 86.6%, followed by detection of Oxa-1 in 25.6% of the samples positive for *A. caviae*. SHV was observed in 23.8%. Lowest prevalence was observed in CTXM9 and CMY-type with 3.7% and 8.5% respectively. The difference for occurrence of beta-lactamase in *A. caviae* positive samples was not significant.

Table 5.11: Occurrence of beta lactamases in isolates that were positive for *Aeromonas species*

Beta-lactamase	<i>A. hydrophila</i>	<i>A. veronii</i>	<i>A. sobria</i>	<i>A. caviae</i>
TEM	50(90.9%)	63(88.7%)	47(87.0%)	71(86.6%)
CTXM3	0	0	0	0
Ctxm9	3(5.5%)	3(4.2%)	1(1.9%)	3(3.7%)
CMY type	4(7.3%)	6(8.5%)	4(7.4%)	7(8.5%)
OXA-1	13(24.5%)	17(25.4%)	17(32.7%)	20(25.6%)
SHV	13(23.6%)	21(28.8%)	12(21.8%)	20(23.8%)

#### 5.4.17 Frequency of multiple beta-lactamase genes in *Aeromonas* isolates

All isolates that were tested for beta-lactamase genes were negative for the CTX-M3 gene. Of those that were positive, 11 isolates had no beta-lactamase genes at all, and 46(48.9%) isolates had one gene and 25(26.6%) had the presence of two genes. Two isolates (2.1%) harboured all the other genes (TEM, SHV, CTX-M9 and CMY-type). Results are shown in Table 5.12.

Table 5.12: Table showing multi-resistance beta-lactamase genes of *Aeromonas* isolates from aquatic sources.

Multi- $\beta$ -lactamase genes	Frequency	Percent %
0	11	11.7
1	46	48.9
2	25	26.6
3	10	10.6
4	2	2.1
Total	94	100.0

#### 5.4.18 Multiple antibiotic resistance of *Aeromonas* isolates from South Africa and Zambia

Table 5.13 below shows the resistance of *Aeromonas* species to multiple antibiotics based on the country where the samples were collected. High resistance to multiple antibiotics was observed in South Africa more than Zambia. 24.0% was resistant to 7 and 8 antibiotics in South Africa yet in Zambia 1(25.0% was) resistant to 7 antibiotics. 3(75.0%) was observed to be resistant to 5 antibiotics in Zambia. Only 2% was resistant to 11 antibiotics in South Africa. No resistance was observed for 2-3 and 12 antibiotics in both countries. The difference was statistically significant with  $p=0.030$

Table 5.13: Multiple resistance of *Aeromonas* isolates from South Africa and Zambia

Number of antibiotics	Country		Total	Statistics
	South Africa	Zambia		
4	3(6.0%)	0(0.0%)	3(5.6%)	0.030
5	4(8.0%)	3(75.0%)	7(13.0%)	
6	9(18.0%)	0(0.0%)	9(16.7%)	
7	12(24.0%)	1(25.0%)	13(24.1%)	
8	12(24.0%)	0(0.0%)	12(22.2%)	
9	5(10.0%)	0(0.0%)	5(9.3%)	
10	4(8.0%)	0(0.0%)	4(7.4%)	
11	1(2.0%)	0(0.0%)	1(1.9%)	

### 5.19 Resistance of *Aeromonas* species to multiple antibiotics based on the different districts

The table 5.49 below represents the resistance of *Aeromonas* species to multiple antibiotics according to the different districts where samples were obtained. The Vhembe districts showed prevalence of multi-antibiotic from 4 to 7 antibiotics with the highest resistance in 4(50.0%) to 6 antibiotics. Capricorn district also showed high resistance to 8 antibiotics which was observed in 46.7%, for Capricorn district, no resistance was noted for 6 and 11 antibiotics. Sekhukhune and Waterberg also demonstrated quite high prevalence of *Aeromonas* resistance to multiple antibiotics with 40.0% being resistant to 5 antibiotics in Sekhukhune. In Waterberg, 36.4% was resistant to 7 antibiotics followed by 18.2% which was resistant to 6 and 8 antibiotics

Table 5.14: Resistance of *Aeromonas* species to multiple antibiotics based on the different districts

MAR	Vhembe	Capricorn	Sekhukhune	Waterberg	Zambia	Total
4	2(25.0%)	1(6.7%)	0(0.0%)	0	0	3(5.6%)
5	1(12.5%)	1(6.7%)	2(40.0%)	0(0.0%)	3(75.0%)	7(13.0%)
6	4(50.0%)	0	1(20.0%)	4(18.2%)	0	9(16.7%)
7	1(12.5%)	2(13.3%)	1(20.0%)	8(36.4%)	1(25.0%)	13(24.1%)
8	0	7(46.7%)	1(20.0%)	4(18.2%)	0	12(22.2%)
9	0	2(13.3%)	0	3(13.6%)	0	5(9.3%)
10	0	2(13.3%)	0	2(9.1%)	0	4(7.4%)
11	0	0	0	1(4.5%)	0	1(1.9%)

#### 5.4.20 Multiple antibiotic resistance of *Aeromonas* from various sources

High resistance of *Aeromonas* species to multiple antibiotics among the different districts was observed in sewage samples with sewage samples being resistant to different antibiotics from 4-11. One dam sample and ground water samples were resistant to 5(100.0%) antibiotics only. Also, one tap water sample was observed to be resistant to 9 antibiotics. 28.0% of river samples was observed to be resistant to 6, 7 and 8 antibiotics. In sewage samples highest resistance was observed in 22.7% to 7 antibiotics, followed by 18.2% to 5 and 8 antibiotics. Table 5.15 shows the results

Table 5.15: Multiple antibiotic resistance of *Aeromonas* from various sources

Number of antibiotics	Dam	Ground water	Pond water	River	Sewage	Tap water	Total
4	0	0	0	2(8.0%)	1(4.5%)	0	3(5.6%)
5	1(100%)	1(100%)	1(25.0%)	0	4(18.2%)	0	7(13.0%)
6	0	0	0	7(28.0%)	2(9.1%)	0	9(16.7%)
7	0	0	1(25.0%)	7(28.0%)	5(22.7%)	0	13(24.1%)
8	0	0	1(25.0%)	7(28.0%)	4(18.2%)	0	12(22.2%)
9	0	0	0	1(4.0%)	3(13.6%)	1(100%)	5(9.3%)
10	0	0	1(25.0%)	1(4.0%)	2(9.1%)	0	4(7.4%)
11	0	0	0	0	1(4.5%)	0	1(1.9%)

#### 5.4.21 Resistance of *Aeromonas* to various number of antibiotics in isolates from wastewater and non-wastewater sources

Between sewage and non-sewage samples, there was a varied resistance in both types of samples to multiple antibiotics. In non-sewage samples resistance to 7 and 8 antibiotics was observed in 8(25.0%). No resistance was observed against 11 antibiotics. In sewage samples, high resistance was observed in 22.7% to 7 antibiotics. The resistance of *Aeromonas* species from sewage and non-sewage samples to multiple antibiotics was not statistically significant with a p value of 0.685.

Table 5.16: Resistance of *Aeromonas* to various number of antibiotics in isolates from wastewater and non-wastewater sources

Number of antibiotics	Sewage samples		Total
	Non-sewage samples	Sewage samples	
4	2(6.3%)	1(4.5%)	3(5.6%)
5	3(9.4%)	4(18.2%)	7(13.0%)
6	7(21.9%)	2(9.1%)	9(16.7%)
7	8(25.0%)	5(22.7%)	13(24.1%)
8	8(25.0%)	4(18.2%)	12(22.2%)
9	2(6.3%)	3(13.6%)	5(9.3%)
10	2(6.3%)	2(9.1%)	4(7.4%)
11	0	1(4.5%)	1(1.9%)

#### 5.4.22 Multi-antibiotic resistance of *Aeromonas* based on sample type

Samples from obtained from tap water had low prevalence of multiple resistance to a number of antibiotics, with resistance observed in 1(100.0%) to 9 antibiotics. High resistance was observed in sample from the river source with 25.8% being resistant to 7 and 8 antibiotics, followed by 22.7% resistant to 6 antibiotics. Sample obtained from sewage showed high resistance to 7 antibiotics and to 8 antibiotics with a lower resistance observed in 4 (4.5%) and 11 (4.5%) antibiotics. The difference was not significant with  $p= 0.336$ , results are shown in table 5.17.

Table 5.17: Multi-antibiotic resistance of *Aeromonas* based on sample type

MAR	Sample type			Total
	River	Sewage	Tap water	
4	2(6.5%)	1(4.5%)	0	3(5.6%)
5	3(5.6%)	4(18.2%)	0	7(13.0%)
6	7(22.6%)	2(9.1%)	0	9(16.7%)
7	8(25.8%)	5(22.7%)	0	13(24.1%)
8	8(25.8%)	4(18.2%)	0	12(22.2%)
9	1(3.2%)	3(13.6%)	1(100.0%)	5(9.3%)
10	2(6.5%)	2(9.1%)	0	4(7.4%)
11	0	1(4.5%)	0	1(1.9%)

## 5.5 Discussion

Antibiotic resistance of microorganisms in general is a global problem, because these microorganisms are responsible for causing different infections and their resistance to antibiotics is making it difficult to treat those infections. Therefore, it is important to investigate microorganisms and their resistance patterns to commercial antibiotics (Odeyemi and Ahmad, 2015). Two modes of antibiotic resistance was described by Kummerer and colleagues in a review published in 2009. First, there is what is termed vertical resistance which is the indigenous resistance that exist naturally in microorganisms and is spread across progeny by cell-division during growth. Secondly, is what is named the horizontal resistance transfer which refers to acquired antibiotic resistance, resulting from the contact between antibiotics and microorganisms in the environment through therapeutic use of antibiotics or naturally (Kummerer, 2009). *Aeromonas* species are becoming more resistant to antibiotics and they harbour extended spectrum beta-lactamase genes which codes for the resistance. Some studies have found that ESBL producing microorganisms are resistant to cephalosporins (ceftriaxone and cefuroxime) which supports our finding of complete resistance to cefuroxime (Oli et al., 2017).

The resistance of *Aeromonas* species in the environment can also be attributed to their exposure to commercial antibiotics in the aquatic environment through sewage contamination. Aquatic organisms, mainly fish may then acquire these resistant bacteria since they are abundant in their living space (Scarano et al., 2018). Then humans and animals may also get the resistant bacteria by coming into contact with the water such as seawater, drinking water and wastewater as well as consuming food that have the bacteria, like fish, sea cucumber, shrimps and others. Our study revealed

that *Aeromonas* isolates were resistant to cefuroxime, trimethoprim, ceftazidime, cefazolin and nalidix acid. Antibiotic resistance of *Aeromonas* in the aquatic environment poses a great danger to our fresh water food but also to sea food, because it will increase the chances of having infections that cannot be treated.

Many gram-negative bacteria are known to produce ESBL, which are the derivatives of the TEM and SHV genes by mutation that alters the amino sequence on the active site of these beta-lactamases (Chandra and Goswami, 2014). Many studies have been conducted concerning ESBL producing microorganisms, mainly focusing on *E. coli*, *Klebsiella pneumoniae* and others (Yi et al., 2014). The present study showed that the CTX-M3 gene was not common in the *Aeromonas* isolates tested. The results confirmed high prevalence of the TEM gene (87.6%), followed by SHV and OXA-1. While the prevalence of other genes was very low. These results supported our expectation in detection of high prevalence of TEM gene because it is responsible for ampicillin resistance in *Aeromonas*. This is similar to results obtained by Yi et al., in 2014 who found high prevalence of TEM gene among *Aeromonas* isolates. In our study the prevalence of the CTX-M9 was very low (3.4%), while the CTX-M-3 was not found at all. These results are supported by a study done in 2013 by Pual and colleagues who found no blaCTX-M genes in *Aeromonas* (Pual et al., 2013), although many studies have also observed low prevalence of these blaCTX-M gene, a study that was conducted in South Korea on *Aeromonas* isolated from fish, they found high prevalence of the blaCTX-M genes (Yi et al., 2015). However, there are other studies that found low prevalence of blaTEM among *Aeromonas* species. For example a study done by Igbiosa et al., (2017) found low prevalence (8.3%) of blaTEM among *Aeromonas* isolates. Some studies have observed blaCTX M-15 previously in *Aeromonas spp* and also the detection of CTX-M genes are said to be increasing in

gram-negative ESBL-producing organisms globally (Osman et al., 2017). Some isolates carried more than one beta-lactamase gene which increases the resistance profiles of these organisms to different antibiotics.

## 5.6 Conclusion

In this study, *Aeromonas* spp isolated from different aquatic sources were shown to carry the TEM beta-lactamase gene, which attributes to their resistance to Beta-lactam antibiotics. Multi-resistance was also observed among the *Aeromonas* isolates. Such high resistance poses great danger to aquatic and human health in the region. Regular monitoring of the aquatic environments for antibiotic resistant bacteria is recommended in order to control and minimises chances of infection with untreatable bacteria.

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## Chapter 6: Influence of steroid hormones on the diversity of *Aeromonas* species isolated from aquatic sources

### 6.1 Abstract

**Background:** Different contaminants, mainly chemical compounds including steroid hormones (estrogens and androgens) are released from manufacturing industries and through wastewater treatment effluents that end up in our environmental waters which contributes to the state of poor water quality. Water is a home to a variety of aquatic organisms as well as a wide range of microorganisms such as *Aeromonas* spp. The interactions between the contaminants and the microorganisms found in the water environment has not been thoroughly studied.

**Method:** Samples were collected from Rivers, Taps and WWTPs around Limpopo province and in Lusaka, Zambia. *Aeromonas* was isolated and identified to species level by specific PCR protocols. ELISA was used for measuring concentrations of different steroid hormones. The potential effects of steroid hormones on *Aeromonas* isolation was studied through the student t test as well and the multivariate analysis test and the difference was considered significant if the p value was less than 0.05.

**Results:** High prevalence of 77.1% of *Aeromonas hydrophila* was observed in the presence of ethinylestradiol (EE2). *Aeromonas veronii* and *Aeromonas caviae* were the most predominant species in the presence of total estriol, *A. veronii* had a prevalence of 57.1% and *A. caviae* had a prevalence percentage of 52.8%. *Aeromonas hydrophila* and *Aeromonas caviae* had the lowest prevalence with the percentages of 26.1% and 27.8% respectively

**Conclusion:** The presence of steroid hormones in the water samples influenced the diversity of *Aeromonas* spp. This is a possible indication that these hormones can be metabolised by the *Aeromonas* species that can then be used for the removal of these hormones in the water environment.

## 6.2 Introduction

As it is well-known that *Aeromonas* species are opportunistic pathogens; meaning that generally they do not cause infections but if it happens that they get an opportunity to do so they cause infections. *Aeromonas* mainly in aquatic environments cause infections to aquatic organisms due to stress conditions such as poor water quality, overcrowding, or rough handling (Noga, 2010; Beaz-Hildago and Figueras, 2013; Dar et al., 2016). Pollution is responsible for the poor quality of environmental waters. Water can be polluted by different contaminants mainly chemical compounds that are released from manufacturing industries and through wastewater treatment effluents that end up in our environmental waters. Of these compounds are steroid hormones which have been described to occur more and more often in environmental waters and soil. Furthermore, it has been suggested that soil microbial community may use these hormones as nutrients which is good in keeping the soil bacteria abundant because they play a role in ecological processes. However, in aquatic environment it would cause a problem in that it will encourage the growth of many opportunistic pathogenic bacteria which in turn would end up causing infections as they become abundant in aquatic environments (Alexander, 1977; Bonilla et al., 2012; Zhang et al., 2015). Of the *Aeromonas* species, *A. caviae* has been identified and described as a

potential indicator in sewage pollution because it is the most dominant species in sewage contaminated waters (Ramteke et al., 1993; Popovic et al., 2015).

The most dominant steroid hormone pollutant in environmental waters is ethinylestradiol (EE2), which is a synthetic hormone (a derivative of natural hormone Estradiol (E2)). It is associated with negative effects in aquatic organisms as well as humans, causing disorders such as feminisation in male fish, intersex in fish, and is implicated in cancer in humans (Luzio et al., 2016). EE2 and other chemicals cause environmental waters to be of poor quality. Although EE2 also alters the endocrine system of the aquatic organisms, it has been suggested to serve as nutrient to microorganism leading to their overflow (Pauwels et al., 2008). Degradation of steroid hormones is thought to be mediated by microorganisms with transformation being oxidation of E2 and EE2 into E3 (Fernandes et al., 2003; Verlicchi et al., 2012).

There is dearth of information on the effects of steroid hormones on the diversity of microorganisms. The present study sought to determine the potential effect of steroid hormones on *Aeromonas species*.

## **6.3 Materials and methods**

### **6.3.1 Sample collection**

Wastewater, surface water, as well as tap water samples were collected using 1l plastic bottles. Prior to sample collection bottles were rinsed with ethanol to rid any contaminants. Fish were collected from the fish farm and crabs from Kafue River in Zambia, Lusaka. All samples were kept at 4 °C during transportation to the University of Venda.

### **6.3.2 Extraction of hormones and isolation and identification of *Aeromonas* spp**

The samples tested for the present study were collected and *Aeromonas* spp were isolated and identified as described earlier in chapter 3 and 4.

### **6.3.3. Statistical analysis**

The potential effects of steroid hormones on *Aeromonas* isolation was studied through the student t test as well and the multivariate analysis test and the difference was considered significant if the p value was less than 0.05. and to obtain the efficiency of the wastewater treatment removal of hormones we used the formula [ concentration of influent – concentration of effluent].

## **6.4 Results**

### **6.5.1 Occurrence of *Aeromonas* species in relation to ethinylestradiol (EE2)**

The prevalence of *Aeromonas* species in the presence of ethinylestradiol (EE2) was very high, with the highest prevalence observed in *Aeromonas hydrophila* with percentage of 77.1%. This was followed by the predominance of *Aeromonas sobria* with 74.2%, *Aeromonas veronii* with 71.35, and *Aeromonas caviae* with 71.0%. There was no statistical difference in the occurrence of *Aeromonas* in the presence of ethinylestradiol (EE2). The results are shown below in table 6.1.

Table 6.1: Distribution of *Aeromonas* species in the presence of ethinylestradiol (EE2)

Organisms	EE2		Statistics
	Negative	Positive	
			P=0.429
<i>A. hydrophila</i> negative	19 (28.8%)	47(71.2%)	
<i>A. hydrophila</i> positive	16 (22.9%)	54 (77.1%)	
Total	35(25.7%)	101 (74.3%)	
<i>A. veronii</i> negative	10 (20.0%)	40 (80.0%)	p=0.259
<i>A. veronii</i> positive	25 (28.7%)	62 ( <b>71.3%</b> )	
Total	35 (25.5%)	102 (74.5%)	
<i>A. sobria</i> negative	19(25.3%)	56(74.7%)	P= 0.950
<i>A. sobria</i> positive	16(25.8%)	46(74.2%)	
Total	35(25.5%)	102(74.5%)	
<i>A. caviae</i> negative	6(16.2%)	31(83.8%)	P=0.128
<i>A. caviae</i> positive	29(29.0%)	71( <b>71.0%</b> )	
Total	35(25.5%)	102(74.5%)	

#### 6.4.2 Occurrence of *Aeromonas* species in relation to the presence Total Estriol (E3)

Table 6.2 shows the prevalence of *Aeromonas* species in the presence of total Estriol. The most predominant species observed in the presence of total estriol is *Aeromonas sobria*. Lowest prevalence was observed for *Aeromonas hydrophila* with 42.9%.

*Aeromonas veronii* had a prevalence of 57.1% and *Aeromonas caviae* had a prevalence percentage of 52.8%. The occurrence of these species was not significant statistically with  $p > 0.05$ .

Table 6.2: Prevalence of *Aeromonas* species in the presence of total estriol (E3)

Organisms	Total estriol		Statistics
	Negative	Positive	
<i>A. hydrophila</i> negative	7(31.8%)	15(68.2%)	P= 0.075
<i>A. hydrophila</i> positive	16(57.1%)	12( <b>42.9%</b> )	
Total	23(46.0%)	27(54.0%)	
<i>A. veronii</i> negative	11(50.0%)	11(50.0%)	P=0.615
<i>A. veronii</i> positive	12(42.9%)	16(57.1%)	
Total	23(46.0%)	27(54.0%)	
<i>A. sobria</i> negative	14(53.8%)	12(42.2%)	P= 0.247
<i>A. sobria</i> positive	9(37.5%)	15(62.5%)	
Total	23(46.0%)	27(54.0%)	
<i>A. caviae</i> negative	6(42.9%)	8(57.1%)	P=0.781
<i>A. caviae</i> positive	17(47.2%)	19( <b>52.8%</b> )	
Total	23(46.0%)	27(54.0%)	

### 6.4.3 Prevalence of *Aeromonas* species in the presence of free estriol

The table 6.3 below shows the prevalence of *Aeromonas* species in the presence of free estriol. High prevalence was observed with *Aeromonas caviae* 63.5%. Followed by *Aeromonas hydrophila* with the prevalence of 63.4%. *Aeromonas veronii* and *Aeromonas sobria* were also dominant with prevalence percentages of 62.1% for *A. veronii* and 61.1% for *A. sobria*. The difference was not significant for the occurrence of these *Aeromonas* species in relation to free estriol with a p value that was greater than 0.05. Results are shown in table 6.3.

Table 6.3: Occurrence of *Aeromonas* species in the presence of free estriol (E3)

Organisms	Free estriol		Statistics
	Negative	Positive	
<i>A. hydrophila</i> negative	16(36.4%)	28(63.6%)	P=0.983
<i>A. hydrophila</i> positive	15(36.6%)	26(63.4%)	
Total	31(36.5%)	54(63.5%)	
<i>A. veronii</i> negative	9(32.1%)	19(67.9%)	P=0.600
<i>A. veronii</i> positive	22(37.9%)	36( <b>62.1%</b> )	
Total	31(36.0%)	55(64.0%)	
<i>A. sobria</i> negative	17(34.0%)	33(66.0%)	P= 0.641
<i>A. sobria</i> positive	14(38.9%)	22( <b>61.1%</b> )	
Total	31(36.0%)	55(64.0%)	
<i>A. caviae</i> negative	8(34.8%)	15(65.2%)	P=0.883
<i>A. caviae</i> positive	23(36.5%)	40(63.5%)	
Total	31(36.0%)	55(64.0%)	

#### 6.7.4 Occurrence of *Aeromonas* species in the presence of Estradiol (E2)

*Aeromonas veronii* was the most predominant species in the presence of estradiol E2 with a percentage of 32.3%. The occurrence of *Aeromonas veronii* was not statistically significant. This was followed by *Aeromonas sobria* which was the second predominant species with a prevalence of 29.8%. The difference was not significant with a p value of 0.918. *Aeromonas hydrophila* and *Aeromonas caviae* had the lower prevalence with the percentages of 26.1% and 27.8% respectively. Their occurrence was not statistically significant. Results are shown in table 6.4.

Table 6.4: Prevalence of *Aeromonas* species in the presence of estradiol (E2)

Organisms	Estradiol		Statistics
	Negative	Positive	
<i>A. hydrophila</i> negative	36(69.2%)	16(30.8%)	P=0.609
<i>A. hydrophila</i> positive	34(73.9%)	<b>12(26.1%)</b>	
Total	70(71.4%)	28(28.6%)	
<i>A. veronii</i> negative	28(75.7%)	9(24.3%)	P=0.401
<i>A. veronii</i> positive	42(67.7%)	20(32.3%)	
Total	70(70.7%)	29(29.3%)	
<i>A. sobria</i> negative	37(71.2%)	15(28.8%)	P= 0.918
<i>A. sobria</i> positive	33(70.2%)	14(29.8%)	
Total	70(70.7%)	29(29.3%)	
<i>A. caviae</i> negative	18(66.7%)	9(33.3%)	P=0.589
<i>A. caviae</i> positive	52(72.2%)	<b>20(27.8%)</b>	
Total	70(70.7%)	29(29.3%)	

## 6.4.5 Prevalence of *Aeromonas* species in relation to testosterone In the presence of testosterone

Low prevalence of *Aeromonas* species was observed with *A. hydrophila* having the lowest prevalence of 4.2%, this was followed by *A. veronii* with .7%, *A. sobria* had the prevalence of 56.5%, and *A. caviae* had a low prevalence of .9%%. However, the difference for occurrence of these organisms in relation to testosterone was not statistically significant with  $p > 0.05$ . Results are recorded in table 6.5.

Table 6.5: Prevalence of *Aeromonas* species in the presence of Testosterone

Organisms	Testosterone		Statistics
	Negative	Positive	
<i>A. hydrophila</i> negative	37(56.1%)	5(7.6%)	P= 0.690
<i>A. hydrophila</i> positive	40(56.3%)	<b>3(4.2%)</b>	
Total	77(56.2(%)	8(5.8%)	
<i>A. veronii</i> negative	28(56.0%)	4(8.0%)	p=0.851
<i>A. veronii</i> positive	49(55.7%)	<b>5(5.7%)</b>	
Total	77(55.8%)	9(6.5%)	
<i>A. sobria</i> negative	43(56.6%)	5(6.6%)	P= 0.975
<i>A. sobria</i> positive	34(54.8%)	4(6.5%)	
Total	77(55.8%)	9(6.5%)	
<i>A. caviae</i> negative	22(59.5%)	2(5.4%)	P=0.859
<i>A. caviae</i> positive	55(54.5%)	7(6.9%)	
Total	77(55.8%)	9(6.5%)	

#### **6.4.6 Efficiency of removal of steroid hormones by the wastewater treatment plants**

Four wastewater treatment plant that are all in South Africa showed to be very efficient in removing hormones with 100.0% removal efficiency. Three of them (from Bela-bela, Elim and Polokwane) were efficient in removing free estriol and testosterone (from Mankweng). Testosterone and free estriol were removed even though the percentage for some treatment plants was low. Although some treatment plants seemed to remove EE2, many of them failed because there is a high number of EE2 increase with a high increase concentration of of up to 6176%. From Zambia, one wastewater treatment plant from Chunga shows high efficiency in removing testosterone, E2 and EE2 by over 90%. And the one from Kaseba village is failing to removing these steroid hormones where there is even an increase on the concentration of testosterone by 198%.

Table 6.6: Efficiency of removal of steroid hormones by the wastewater treatment plants.

Country	Location	Testos Removal	EE2 Removal	Ef Removal	Et Removal	E2 Removal
South Africa	Bela-bela	-5.24%	99.90%	100.00%	-	36.26%
South Africa	Elim	78.50%	-357.27%	100.00%	-	1.01%
South Africa	Giyani	96.20%	-2021.80%	-	-57.84%	-
South Africa	Louis Trichard	99.00%	97.46%	-	-	96.79%
South Africa	Malamulele	29.00%	-29.70%	-	-	-1259.87%
South Africa	Mankweng	100.00%	21.74%	-274.85%	-	20.38%
South Africa	Marble hall	99.60%	-147.87%	0.00%	-	98.83%
South Africa	Mokopane	-	-	1.15%	-	-
South Africa	Nzhelele	28.16%	-6176.00%	-	-	-
South Africa	Polokwane	-177.68%	-53.19%	-	-	5.09%
South Africa	Polokwane	83.50%	97.80%	100.00%	-	98.28%
South Africa	Tzaneen	-9.12%	1.44%	-	-	6.50%
South Africa	Tzaneen	97.45%	-1099.78%	-229.20%	87.78%	-
South Africa	Watervaal	67.38%	-144.08%	-	-16.61%	97.84%
Zambia	Chunga	99.00%	97.20%	-	-	90.26%
Zambia	Kaseba village	-198.60%	-	-	-20.00%	36.32%

Note: Negative sign means accumulation.

### 6.4.6 Association between Estradiol (E2) and multiple infections

The scatter graph (figure 6.1) below shows the correlation between Estradiol (E2) with multiple infection. As the concentration of Estradiol increases it seems the number of multi-infections increases as well.

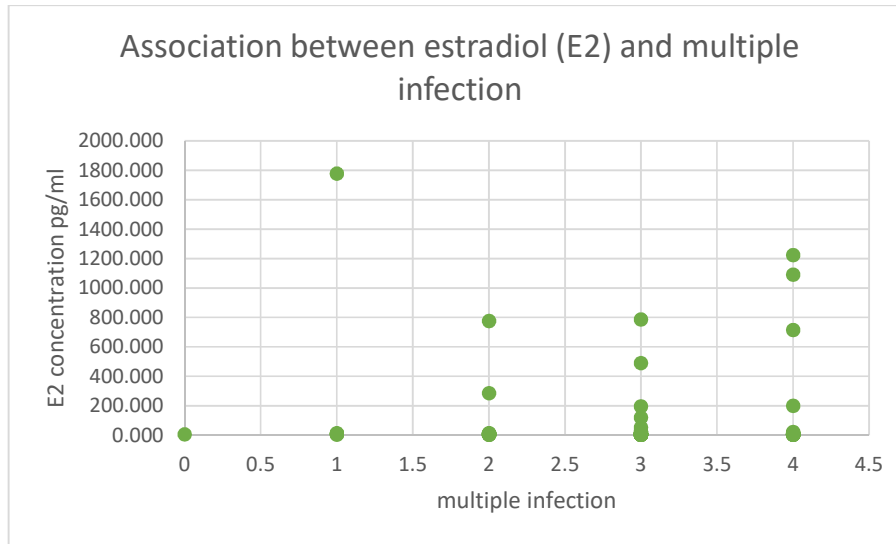


Figure 6.1: A scatter graph showing the potential association between Estradiol and number of multiple infections.

### 6.4.7 The correlation between testosterone and number of infections

Testosterone did not seem to favour the growth of many *Aeromonas* isolates, but with few that were detected in the presence of testosterone showed a tendency of many infections at a lower concentration. Results are plotted below on a scatter graph (figure 6.2)

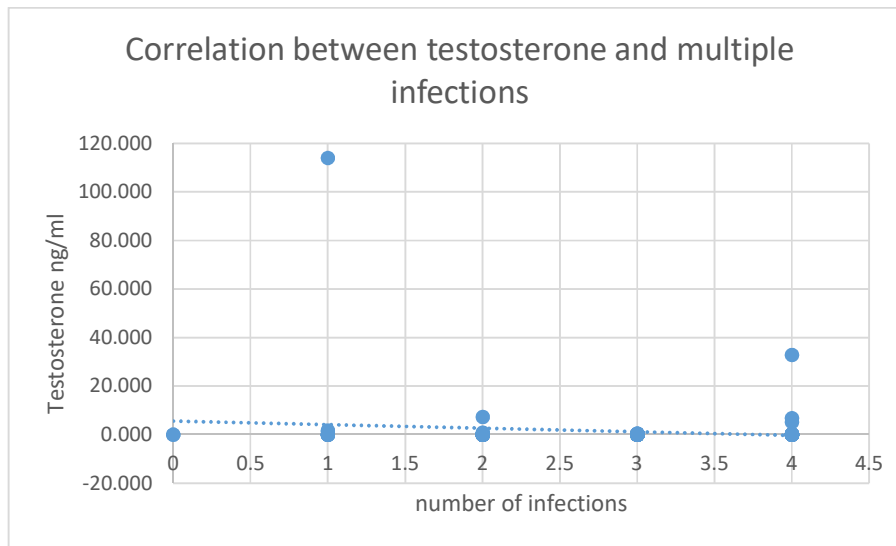


Figure 6.2: A scatter plot showing the correlation between testosterone and multiple infections.

### 6.7.8 The relationship between ethinylestradiol (EE2) and multiple infection

High concentration of Ethinylestradiol had high number of *Aeromonas* species, because as the concentration of EE2 increased the number of *Aeromonas* species increased by a large number. Results are shown in figure 6.3 below.

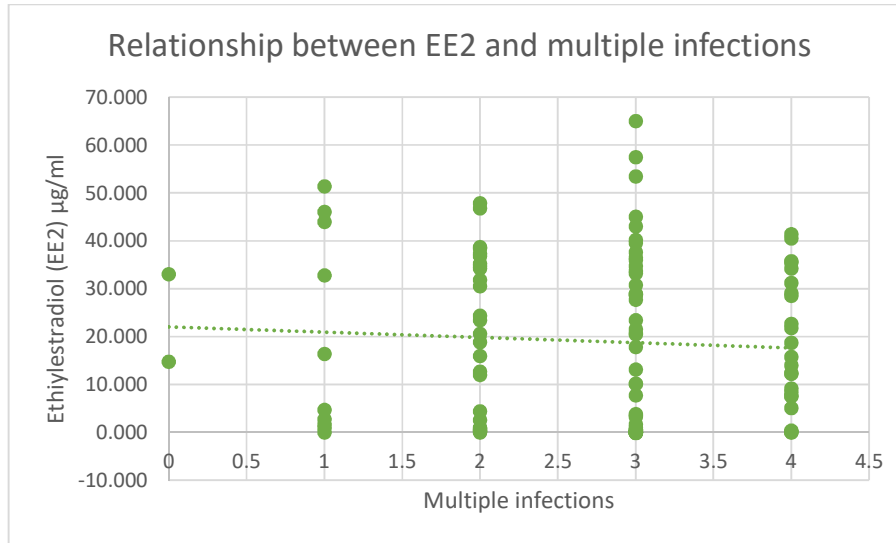


Figure 6.3: A scatter graph showing the relationship between EE2 and number of infections

### 6.7.9 The correlation between total estriol (E3) and multiple Infection

Total estriol supported the growth of *Aeromonas* species at a lower level, high concentration of total estriol did not show to favour the *Aeromonas* diversity (Figure 6.4).

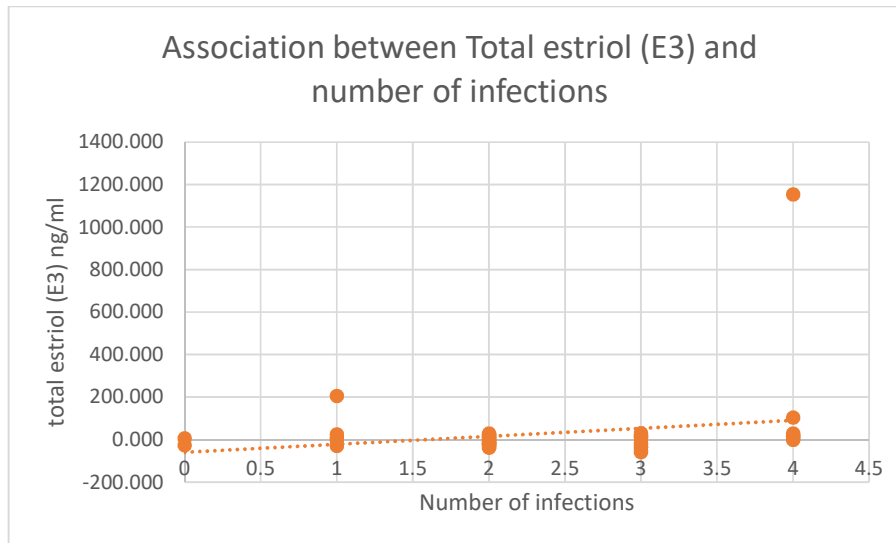


Figure 6.4: A scatter plot showing correlation between total estriol (E3) and multiple infections by *Aeromonas* species.

### 6.4.10 Relationship between free estriol (E3) and number of multiple infection

*Aeromonas* species strived very well at low concentration, though some species occurred at higher concentration. Results are shown in figure 6.5 below

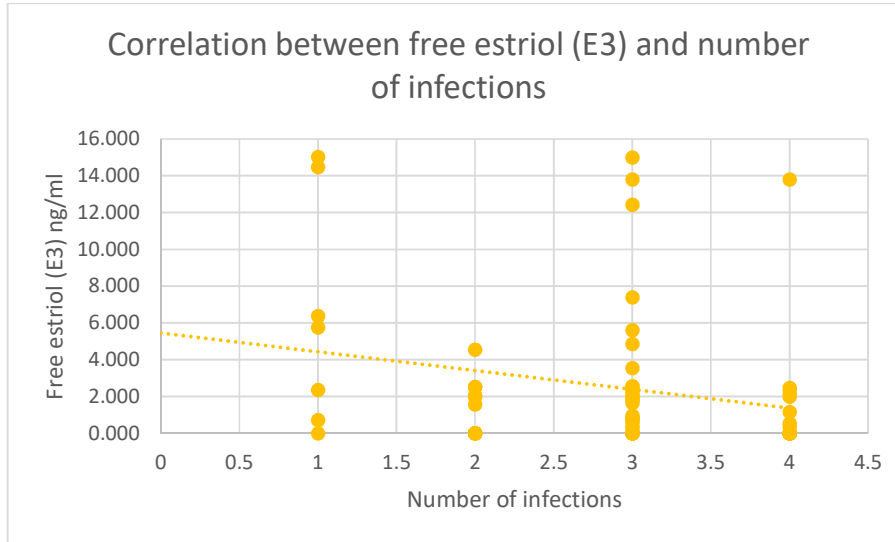


Figure 6.5: A scatter plot presenting the correlation between free estriol (E3) and number of *Aeromonas* infection

## 6.5 Discussion

The aim of this study was to seek knowledge on the effects of steroidal hormones (estrogens and testosterone) on *Aeromonas* species diversity. Estrogens exist as normal and as synthetic compounds that are considered detrimental to aquatic organisms (Purdom et al., 1994) as well as humans by their interaction with the estrogen receptors (they bind to estrogen receptors and mimic the estrogenic activity, hence disrupting the endocrine system). They exist either as halogenated, unconjugated or conjugated estrogens. These hormones are excreted from the body naturally as conjugated forms which is attached to glucuronide and sulphate groups (Griffiths, 2013).

In our study, *Aeromonas* species were used as an indicator of steroid hormone pollution so that we can determine the potential influence of steroid hormones in microorganisms found in the aquatic environment. The results from this study showed that *Aeromonas* species thrive very well in the presence of many steroid hormones with the highest prevalence observed in the presence of synthetic ethinylestradiol (EE2) from 71.0 to 77.1 %. This suggest that *Aeromonas* could be useful in trying to remove this synthetic hormone from our environments since its degradation is very slow compared to other steroid hormones making it resistant in the environment (Mosechet and Hollender, 2009) leading to its accumulation and therefore making it the most dangerous. In 2009, (Mosechet and Hollender, 2009) suggested that EE2 is thought to be co-metabolically removed by an enzyme of a denitrifying bacteria. In a study done by Song et al., (2017) it was discovered that degradation of hormones was possible by nitrifying and ammonia -oxidising bacteria (AOB), and degradation of EE2

was effective when it was co-metabolically removed by heterotrophic microorganisms and nitrifying bacteria.

*Aeromonas hydrophila* did not show high prevalence in the presence of testosterone but had high prevalence in the presence of free estriol and EE2. And *A. veronii* had low prevalence in the presence of estradiol. This could imply that as *A. hydrophila* is able to metabolically degrade free estriol, although it might use less of the estradiol to gain energy as such. *A. veronii* also did not seem to utilise more of the free estriol. This implies that unconjugated and conjugated forms of steroid hormones are not used the same way by microorganisms. These are similar to results obtained by Zhang et al., (2015) who found that steroid hormones in soil served as nutrient sources for soil bacteria to enhance reproduction of new strains as well as to improve soil microbial activity. So, in this case, our results show that estrogens are used as nutrient sources by *Aeromonas* species.

Our results also indicated low prevalence of *Aeromonas* species in the presence of testosterone, suggesting that testosterone does not have much influence on the growth of *Aeromonas* compared to other hormones tested in our study. This is not very similar to a study done by Moschet and Hollender, (2009) who described that testosterone is well degraded metabolically.

Apart from low detection of *Aeromonas* species in the presence of testosterone, the results suggested that estradiol which is the normal estrogen produced by human and animals through urine and faeces did not seem to support the growth of all the *Aeromonas* species tested in this study as their prevalence in relation to estradiol was lower than in other hormones. From this, it's not conclusive to say that estradiol serves as nutrients for *Aeromonas* species and also the microbial community in the aquatic

environments. Although some organisms such as *Pseudomonas* spp have been identified as decomposers of testosterone (Fernandes et al., 2003), no study has investigated the degradation of these compounds by *Aeromonas*.

The results showed that *Aeromonas hydrophila* and *Aeromonas caviae* as well as *Aeromonas veronii* were able to reduce the steroid hormones. With *Aeromonas hydrophila* and *Aeromonas caviae* showing significant reduction of total estriol. Ethinylestradiol (EE2) was also significantly reduced by *Aeromonas veronii* and *Aeromonas caviae*. In fact, *Aeromonads* have been involved in the degradation of a number of chemicals from water (Ramteke et al., 1993), although they have not yet been identified as metabolisers of these steroids before. The removal efficiency of steroid hormones varied, but many wastewater treatments plants has shown to remove testosterone and Estradiol by more than 70%. This supports the low detection of testosterone in the samples tested. Although the results indicates reduction of EE2 by *A.veronii* and *A. caviae*, many wastewater treatment plants did not show any efficiency in the removal of ethinylestradiol EE2. Other organisms such as *Nitrosomonas europaea* and *Nitrospira multiformis* have been involved in the biodegradation of EE2 (Gauke et al., 2008). In our study, the concentration of EE2 increased by over 100% in many cases showing that ethinylestradiol accumulates over time instead of being reduced. Similar results have been described in other studies where it was observed that the concentration of this compounds actually increases in the sewage treatment plants (Pauwels et al., 2008).

## 6.6 Conclusion

Our results indicated differences in the prevalence of *Aeromonas* species in the presence of different steroid hormones. More research needs to be conducted to investigate the influence of EE2 using variety of microorganisms, as well as investigate differences on the influence of free and total (conjugated and unconjugated) estrogens as our study revealed that the prevalence of *Aeromonas* species varied in the presence of free and total estriol.

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## Chapter 7: Anti-microbial activity of two selected medicinal plants against *Aeromonas* species

### 7.1 Abstract

**Background:** Medicinal plants provide natural solution at minimal cost for treating different infections. Many people in developing countries use medicinal plants in treating infections and diseases more than they use western medicine, mainly because the plants are cost effective. The aim of this study was to assess the antimicrobial activity of *Zornia milneana* and *Annona* spp against *Aeromonas* spp isolated from water and wastewater samples.

**Method:** The plants were extracted with methanol, ethyl acetate and Acetone respectively. All the extracts were assessed for antimicrobial activity against *Aeromonas* species using the disc diffusion method and the MICs were determined using the microdilution method. The extracts were tested against 05 isolates of *Aeromonas*.

**Results:** The plant extracts did not show activity against *Aeromonas* species on disc diffusion assay. Methanol extracts of both *Zornia milneana* and *Annona* species showed good activity at lowest concentration of 0.078 mg/ml. Ethyl acetate extracts were the least effective.

**Conclusion:** The plants used could serve as source of compounds that can be used to control infections by *Aeromonas* species.

Key words: *Zornia milneana*, *Annona*, Antimicrobial activity and *Aeromonas*

## 7.2 Introduction

Medicinal plants as it is known, provide natural solution at minimal cost for treating different infections (Obi et al., 2007). Medicinal plants have pharmacological and biological activities as they are a very good secondary metabolites sources; and are also used as complementary medicines in managing cancer (Khan et al., 2018). One species of the *Annonaceae* family was found to have inhibitory activity against fungal species.

Several studies conducted previously have reported antimicrobial activity in different *Annona* species. For example, the leaves of *Annona vepretorum* showed antimicrobial activity against *Staphylococcus aureus*, *E. coli* and *Klebsiella pneumoniae* (Almeida et al., 2014). Methanol, dichloromethane and ethyl acetate extracts from *Annona hypoglauca* stems inhibited the growth of some bacteria such as *Staphylococcus aureus* and *Enterococcus faecalis* (Rinaldi et al., 2016). So many medicinal plants have compounds that have antimicrobial activities such as flavonoids and others, these compounds have been described in the *Annona* species as well including *Annona glabra* (Zhang et al., 2004; Bao et al., 2015; Galvao et al., 2016).

Some *Zornia* species have been reported to have pharmacological effects; *Zornia diphylla* for example has been reported to have spasmolytic activity and is mainly used by the Queretaro Otomi Indians in Mexico (Rojas et al., 2012). In some places in India the plant is used for treating cancer and fungal diseases, dysentery, venereal diseases and is also used to induce sleep in children (Arunkumar et al., 2012, Khare, 2007, Govindarajan et al., 2016). Geetha and colleagues reported methanol extract of this plant to have anticonvulsant activity, and in French Guiana they make use of *Zornia latifolia* juice to calm inflamed intestines (Geetha et al., 2012, DeFilips et al., 2004; Cornara et al., 2018). The present study sought to determine the antimicrobial activity

of *Zornia milneana* and one of the *Annona species* because there is very less to almost no information on the antimicrobial activity of these two species. Methanol, acetone and ethyl acetate extracts of these two plants were tested on selected *Aeromonas* isolates.

## **7.3 Materials and methods**

### **7.3.1 Collection of plant material**

Whole plant of *Zornia milneana* was collected at Ha-Mulima, in Vhembe district. The plant was identified by botanists at the University of Venda from the department of botany to confirm the plant. The *Annona* plant was collected from Fouban, western region of Cameroon.

### **7.3.2 Processing of the plant material**

The plants were washed and dried over a period of two weeks. Dried samples were milled into fine powder by pounding manually with a clean and sterile mortar and stored in a cool dry place until further use.

### **7.3.3 Extraction of plant material**

Dried ground material (5 g) was extracted in 50ml of methanol, ethyl acetate and acetone. The plant materials were soaked in these solvents for 48hrs with constant shaking. Then the mixtures were filtered using Whattman no.1 filter paper to remove the solid particles. The filtered products were evaporated at 40 °C using the rotary evaporator to concentrate the extracted products.

#### **7.3.4 Antimicrobial activity by agar diffusion method**

After the plants products were dried, 10 mg of each plant product was weighed and dissolved in 1ml of the original solvents to obtain a concentration of 10 mg/ml. Fifty microliter of each of the 25 samples of *Aeromonas* species that were randomly selected were cultured on Mueller-Hinton agar and 8 wells per plate were dug using by placing large hole of a pipette tip on the agar and press it down to cut a hole in the agar; after which 10 $\mu$ l of each plant product were put into the wells respectively and the plates were incubated at 37 °C for 24hrs.

#### **7.3.5 Antimicrobial activity by disc diffusion method**

After the plant's products were dried, 10 mg of each plant product was weighed and dissolved in 1ml of the original solvents to obtain a concentration of 10mg/ml then 200 small paper discs were impregnated with the plant extracts, 25 discs per plant extract for 25 *Aeromonas* samples. Fifty microliters of each of the 25 samples that were randomly selected were cultured on Mueller-Hinton agar and 8 paper discs that were impregnated by different plant extracts were placed on each plate and the plates were incubated at 37 °C for 24hrs

#### **7.3.6 Broth dilution method**

Micro dilution method was used for determination of minimal inhibitory concentration (MIC). Briefly, a 96 well plate was used for the antimicrobial activity. In the first row 184  $\mu$ l of Mueller-Hinton broth was added, and then 100  $\mu$ l Mueller-Hinton broth of was added in the remaining wells. In the first raw, 16 $\mu$ l of a positive (ciproflaxicin) and a

negative control (distilled water) were added in the first and second column respectively, then the plant extract in the remaining wells of the first row. A series of dilutions of the plant extracts with reduced concentrations was carried out to cover all the wells and the controls by taking 100µl in each column from the first row and mixing with the second row to give a two-fold dilution in the last row. Then 100 µl was of the test culture was added in each well and the plates were incubated overnight at 35 °C. The next day 40 µl of 0.2 mg/ml INT (iodonitrotetrazolium) was added in each well and incubated for 10 mins. After 10 mins the results were read by observing the colour change determining the MIC. Wells that changed colour indicated bacterial growth. And results were also read using a plate reader at 450nm.

## 7.4 Results

### 7.4.1 Antibacterial activity through agar diffusion method

All plant extracts of different solvents showed no activity against some *Aeromonas* isolates using the agar diffusion method. But in other plates the activity seemed to be there, but the plant extracts run below the agar (Figure 7.1).

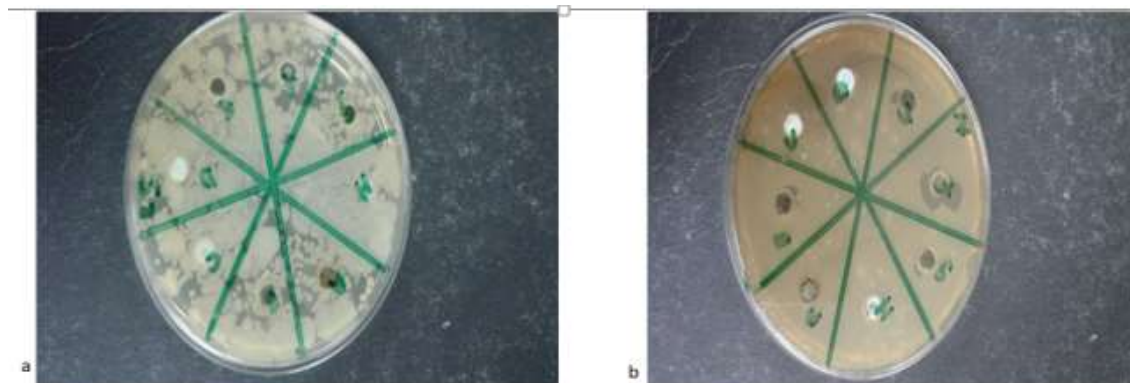


Figure 7.1: (a, b) Petri-plates showing the results of plant extracts

#### **7.4.2 Minimum Inhibitory Concentration assay: Antimicrobial activity of the plant extracts against *Aeromonas* isolates from different sources of water**

MIC were run in duplicates on 5 isolates of *Aeromonas* species, bacterial growth was observed in all wells by colour change. Ciprofloxacin was used as positive control and distilled water was used as negative control, the results were recorded as mean MIC. The highest concentration of plants extract tested was 10mg/ml and the lowest was 0.078mg/ml.

#### **7.4.3 Antimicrobial activity of the plant extracts against *Aeromonas* isolates from a river**

Ethyl acetate extracts of all the plants were least effective against *Aeromonas* isolate 1 with an MIC value of 10mg/ml, and the methanol extract of *Zornia milneana* twigs showed good activity with an MIC value of 0.078mg/ml. also the methanol extract as well as the acetone extract of *Zornia milneana* leaves showed good activity with an MIC value of 1.25 mg/ml. Refer to table 7.1 below.

Table 7.1: Mean MIC values of all the plants extracts against *Aeromonas* species from a river source

Plants	Methanol Extract	Acetone extract	Ethyl acetate extract
<i>Annona</i> species	10mg/ml	10mg/ml	10mg/ml
<i>Zornia milneana</i> leaves	1.25 mg/ml	1.25mg/ml	10mg/ml
<i>Zornia milneana</i> twiggs	0.078mg/ml	-	10mg/ml

#### 7.4.4 Antimicrobial activity of the plant extracts against *Aeromonas* isolates from a dam

The methanol extract of the *Annona* species showed good activity with an MIC value of 0.078 mg/ml. And the acetone and ethyl acetate extracts were less effective with an MIC value of 10mg/ml. methanol extracts of *Zornia milneana* were less effective as well at 10 mg/ml (Table 7.2). None of the plant extracts were bactericidal.

Table 7.2: Mean MIC values of all the plants extracts against *Aeromonas* species isolated from a dam

Plants	Methanol Extract	Acetone extract	Ethyl acetate extract
<i>Annona</i> species	0.078mg/ml	10mg/ml	10mg/ml
<i>Zornia milneana</i> leaves	10 mg/ml	10mg/ml	10mg/ml
<i>Zornia milneana</i> twiggs	10mg/ml	-	10mg/ml

#### 7.4.4 Antimicrobial activity of the plant extracts against *Aeromonas* isolates from wastewater treatment plant

*Annona* species ethyl acetate extract showed very good activity against *Aeromonas* with an MIC value of 0.125 mg/ml. *Zornia milneana* twigs methanol extract was also effective with an MIC value of 5 mg/ml. However, the methanol extract and the acetone extract of *Annona* and *Zornia milneana* were very much less effective with an MIC of 10mg/ml.

Table 7.3: Mean MIC values of all the plants extracts against *Aeromonas* species isolated from wastewater

Plants	Methanol Extract	Acetone extract	Ethyl acetate extract
<i>Annona</i> species	10mg/ml	10mg/ml	0.125mg/ml
<i>Zornia milneana</i> leaves	10 mg/ml	10mg/ml	10mg/ml
<i>Zornia milneana</i> twiggs	5mg/ml	-	10mg/ml

#### 7.4.5 Antimicrobial activity of the plant extracts against *Aeromonas* isolates from a river

All the plant extracts showed good activity against *Aeromonas* isolated from a river sample except for ethyl acetate plant extract of *Zornia milneana* twigs. With *Annona* species showing the best activity with an MIC value of 0.078 mg/ml and methanol extract of *Zornia milneana* twigs and acetone extract of *Annona* species being effective with an MIC value of 0.625 mg/ml. Methanol extract of *Zornia milneana* and leaves and ethyl acetate were active with an MIC value of 2,5 mg/ml. Ethyl acetate extract of *Zornia milneana* leaves also showed very good activity with an MIC value of 0.312 mg/ml. results are shown in table 7.4 below.

Table 7.4: Mean MIC values of all the plants extracts against *Aeromonas* species from river sample

Plants	Methanol Extract	Acetone extract	Ethyl acetate extract
<i>Annona</i> species	0.078mg/ml	0.625mg/ml	2.5mg/ml
<i>Zornia milneana</i> leaves	2.5 mg/ml	5mg/ml	0.312mg/ml
<i>Zornia milneana</i> twiggs	0.625mg/ml	-	10mg/ml

#### 7.4.6 Antimicrobial activity of the plant extracts against *Aeromonas* isolates from different a river

All the methanol, acetone and ethyl acetate plant extracts of *Annona* species, *Zornia milneana* leaves as well as the twigs were all very less effective with an MIC value of 10mg/ml. results are tabulated below (Table 7.5)

Table 7.5: Mean MIC values of all the plants extracts against *Aeromonas* species from river

<b>Plants</b>	<b>Methanol extract</b>	<b>Acetone extract</b>	<b>Ethyl acetate extract</b>
Annona species	10mg/ml	10mg/ml	10mg/ml
<i>Zornia milneana</i> leaves	10 mg/ml	10 mg/ml	10mg/ml
<i>Zornia milneana</i> twiggs	10mg/ml	-	10mg/ml

## 7.5 Discussion

The aim of this study was to investigate if *Annona* species and *Zornia milneana* have antimicrobial activity against *Aeromonas*. *Aeromonas* species cause infections in a wide range of aquatic organisms as well as humans and antibiotic resistance in this bacterium has been reported to have increased (Ramena et al., 2018). This has opened a gap for more research in trying to develop new drugs that bacteria and other microorganisms are susceptible to. In this study we tested two plants that are known to be used traditionally as an alternative to treat different infections.

Many studies have reported on the antibacterial activity of medicinal plant extracts of different solvents. These reports have aided in coming up with new effective medicines in treating diseases. Our study also demonstrated varying results of different solvents extracts of *Zornia milneana* and that of the *Annona* species. Several reports are available on the antimicrobial activity of different *Annona* species, including *A. squamosa*, *A. senegalsis* and many more (de Souza Barboza et al., 2015; Rinaldi et al., 2017). The present study demonstrated that many of the *Annona* extracts were not effective, however some extracts of this plant showed good activity against some *Aeromonas* isolates. The extract that showed activity with the lowest concentration was a methanol extract of the *Annona* plant with an MIC value of 0.078mg/ml. However, all the extracts including methanol, acetone, and ethyl acetate showed good activity with an MIC value of 0.078 mg/ml, 0.625 mg/ml and 2.5 mg/ml respectively against one isolate. This suggests that the extracts of *Annona* can be used in developing good drugs that can act against *Aeromonas* in treating infections caused by *Aeromonas* species. Ethyl acetate extract of *Annona* species did not yield very good results, the extract was effective against only one isolate of *Aeromonas*. The

results from our study are not similar to those obtained by de Barboza et al., (2015) who found that methanolic extracts of *Annona mucosa* had antibacterial activity in inhibiting the growth of a gram-positive *Bacillus subtilis*. Pathak et al., (2010) observed that methanol extracts of *Annona muricata* was also effective in inhibiting the growth of *Streptococcus pyogenes*. Their extracts showed no growth inhibition against all for the gram-negative bacteria.

*Zornia milneana* is traditionally used by the Vhavenda people to treat headaches and flue as well as diarrhea. The plant is very common amongst the Vhavenda people and is sometimes used in combination with other medicinal plants. As far as our knowledge is concerned this plant has been understudied. In the present study we set out to determine antimicrobial activity of different extracts *Zornia milneana* against *Aeromonas* species. Our results revealed that methanol extracts of *Zornia milneana* were the most effective than those of acetone and ethyl acetate, the highest activity was observed with an MIC value of 0.078 mg/ml. Acetone extracts of this plant's leaves were effective on two isolates; one with an MIC value of 1.25 mg/ml and the other with an MIC value of 5mg/ml. Ethyl acetate extracts of *Zornia milneana* leaves showed good activity with an MIC value of 0.312 mg/ml. This shows that methanol extracts of *Zornia milneana* are very good in inhibiting bacterial growth with the least being ethyl acetate extracts, although it can be because ethanol itself is vey effective in inhibiting bacterial growth.

When these extracts with the same concentrations were tested using agar diffusion and disc diffusion method no positive results were obtained. We suggest that further studies be conducted using different concentrations on different microorganisms (including fungi, gram-positive and gram-negative bacteria).

## 7.6 Conclusion

This study shows that methanol extracts of both plants have good anti-bacterial activity compared to extracts of other solvents. Based on this study it can be concluded that methanol extracts of *Annona* spp and *Zornia milneana* are good in suppressing bacterial growth. More research needs to be conducted especially on the toxicity of these plants and also to identify the active compounds.

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## Chapter 8: Overall discussion, conclusion and recommendations

### 8.1 Overall discussion and conclusion

Occurrence of detrimental contaminants described as endocrine disrupting chemicals in water sources and wastewater has always been a growing concern for human populations as well as wild life. These endocrine disrupting chemicals comprise of pharmaceuticals, surfactants, personal care products, steroid hormones and various other industrial additives. Their occurrence in water sources should not be ignored for the adverse effects they have on aquatic life and human health and they should be constantly monitored. Steroid hormones are one of the leading contaminants of major concern, considering synthetic estrogens that are resistant to degradation causing them to be found in excess in our environmental waters as well as drinking water. Although the microbial quality of drinking water was good, we found high concentrations of the hormones in drinking water which is an indication that these compounds are not been removed by traditional water treatment procedures. These results though alarming are not out of the ordinary since other studies have found similar results in drinking water (Torres et al., 2015).

Previous research has indicated that these endocrine disrupting chemicals; steroid hormones in particular; cause negative effects to aquatic organisms (mainly fish) such as induction of vitellogenin, feminisation of male fish and intersex. To humans they have been reported to have implications on chronic diseases such as cancer and serious conditions including sterility (Lubbert et al., 1992). It has also been reported that concentrations as low as a pico-molar could be detrimental to aquatic organisms (Griffiths, 2009).

The present study demonstrated that steroid hormones are widely distributed across the Limpopo province with effluents from different WWTPs having high concentrations, which are released in nearby rivers in most cases; which are homes to variety of organisms as well as microorganisms. On top of that people in some areas, particularly rural areas are dependent on rivers for drinking purposes and other things like bathing and washing (Bolong et al., 2009). Some of these people, however, treat the water while some do not, leading to them consuming such contaminants. This study went as far as detecting high concentration of testosterone (140ng/ml) in drinking water, bringing us or to the conclusion that our water treatment are not efficient as well in removing hormonal contaminants.

Infections such as septicaemia in both human and fish as well as diarrhoea and wound infections and others are caused by *Aeromonas species* which in this study we used as an indicator of how microorganisms behave in the presence of steroid hormones. Our results indicated that these microorganisms are also fairly distributed across our environmental waters, and minimally in drinking water. The results showed that some of the *Aeromonas* grow very well in the presence of steroid hormones but with differences in free or total estrogens. Further research should be carried out to determine the effects of conjugated and unconjugated steroid hormones on microorganisms.

The present study also indicated that *Aeromonas species* are resistant to a wide variety of antibiotics with highest observed resistance to cefuroxime, other studies also observed the same results (Oli et al., 2017). The resistance of *Aeromonas species* or even other bacteria in general is often attributed to the presence of chemical compounds in the environment; with reference to water; where these microorganisms are found. The chemical compounds are reported to end in the environmental water

through failure of the wastewater treatment plants to remove those (Lim et al., 2017). The resistance of *Aeromonas* to antibiotics was complimented by detection of the beta-lactamase genes which are responsible for their resistance. This study indicated high prevalence of the TEM gene as well as the SHV and OXA-1. The resistance to antibiotics is a problem for treating infections caused by resistant microorganisms.

Since microorganisms that are causing infections are becoming more and more resistant to antibiotics, researchers are busy trying to find new drugs. We also decided to test two different plants (*Annona* spp and *Zornia milneana*) for their anti-bacterial activity against *Aeromonas* because this bacteria is also resistant to most antibiotics. Of the five isolates of *Aeromonas* tested from various sources (Wastewater, dam and river) our study revealed that most of them were susceptible to methanol extracts of both plants. The lowest concentration that was able to inhibit growth was 0.078mg/ml. We recommend that more studies should be done on these plants, and the active compounds of these plants should be isolated and tested as to which are effective in inhibiting bacterial growth. From the microdilution assay, no concentration was bactericidal.

This study demonstrated that Limpopo province is one of the most contaminated places with steroid hormones. The wastewater effluents contained high concentrations of these hormones, and drinking water is also not safe from contamination by the steroid hormones. The prevalence of *Aeromonas* species is very high in our environmental water as well as in drinking water, with the highest prevalence observed in fish and wastewater. It was also revealed that there is a relationship between steroid hormones and *Aeromonas* species, with the hormones supporting growth of

*Aeromonas* species. The presence of beta-lactamase genes which cause *Aeromonas* to be resistant to antibiotics were also detected. It was demonstrated that methanol extracts of *Zornia milneana* and *Annona spp* were the most effective compared to that of ethyl acetate and acetone.

## 8.2 Recommendations

In future, it is highly recommended that the government should upgrade wastewater treatments to try and eradicate these contaminants from our waters to reduce their accumulation in recreational waters as well as drinking water sources. The use of filtration technics is of importance because it's said to be efficient in removing EDCs (Bolong et al., 2008). Zhang et al 2014 suggested that it will be very useful if it can be determined as to which part of the wastewater treatment plant is efficient in removing the hormones. More research need to be conducted to try and identify which micro-organisms are more efficient in metabolizing the steroid hormones to improve their removal from the environment.

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