

Endocrine disrupting effects of HIV antiretrovirals in the South African aquatic environment

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Abstract

The presence of endocrine disrupting chemicals (EDCs) in the environment is a growing concern as they pose potential risks to human and environmental health. These compounds interfere with natural homeostasis in living organisms, and have been reported to influence reproductive, endocrine and nervous systems of wildlife and humans. Due to the high prevalence of people living with the human immunodeficiency virus (HIV) in South Africa, as well as the subsequent high usage of antiretrovirals (ARVs) to treat this disease, these compounds are likely to end up in the environment. Wastewater treatment plants (WWTPs) are a major contributor of pharmaceuticals into surface water, and it has been found previously that ARVs are present in effluent intended to be released into receiving waters. The effects of these compounds on non-target organisms, such as aquatic species that spend a large portion of their life in direct contact with contaminated water, are largely unknown. These ARVs can have possible endocrine disrupting effects, but as of yet there are few studies done to determine this. The aryl hydrocarbon receptor (AhR) is responsible for metabolising xenobiotics from the environment and can bind to a wide variety of structurally diverse compounds from high to low affinity. Activation of the AhR can also cause possible endocrine disruption as it is involved in cross-talk with the oestrogen receptor (ER), acting as an antagonist. The aim of this study was to determine the concentrations of ARVs (efavirenz, lopinavir, ritonavir, nevirapine, zidovudine and didanosine) in surface water receiving WWTP effluent through instrumental analysis and to determine their possible biological response by using the H4IIE-*luc* reporter gene bioassay. The H4IIE-*luc* cells have been genetically modified to express the firefly luciferase enzyme in response to AhR activation as a result of *CYP1A1* transcription. Seven WWTPs were chosen in the Gauteng province, and samples were collected upstream and downstream of the effluent release point. ARVs were quantified at 17 of the 20 surface water samples, varying in concentrations and detection frequencies. Efavirenz and lopinavir were found at the highest concentrations across the study. These two compounds were also the highest concentration found in South Africa for surface water, when compared with other studies. The WWTP are a point source for ARVs (and pharmaceuticals as a whole) in receiving waters. The water samples had no AhR-mediated activity, despite the high levels of ARVs present at some sites. The ARVs are thus unlikely to bind to the AhR and influence the ER through receptor cross-talk. These compounds will not have any endocrine disrupting effects through this pathway.

Keywords: H4IIE-*luc*, aryl hydrocarbon receptor, wastewater treatment plants, Gauteng rivers, instrumental analysis

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List of acronyms and abbreviations

#

3TC Lamivudine

A

AhR Aryl hydrocarbon receptor
AMP Adenosine monophosphate
AR Androgen receptor
ARNT Aryl-hydrocarbon nuclear translocator
ART Antiretroviral therapy
ARV Antiretroviral
ATP Adenosine triphosphate
AZT Zidovudine

B

BC Blank control
BEQ Biological toxicity equivalent
bHLH-PAS Basic helix-loop-helix PER-ARNT-SIM

C

CaCl₂·2H₂O Calcium chloride dehydrate
CV Coefficient of variation
CYP Cytochrome P450

D

d4T Stavudine
ddH₂O Double distilled water (18 MΩ distilled water)
ddl Didanosine
DDT Dichlorodiphenyltrichloroethane
DMEM Dulbecco's Modified Eagle's Medium
DMSO Dimethyl sulfoxide
DRE Dioxin responsive element
DS Downstream
DW Drinking water
DWA Department of water affairs
DWS Department of water and sanitation

E

EC Effective concentration

EDC	Endocrine disrupting chemical
EFV	Efavirenz
EPA	Environmental Protection Agency
ER	Oestrogen receptor
ERWAT	East Rand Water Care Association
ESI	Electrospray ionisation
EtOH	Ethanol
<u>F</u>	
FBS	Foetal bovine serum
FH	Flip Human
Flu-d4	Deuterated fluconazole
<u>G</u>	
GC	Gas chromatography
<u>H</u>	
HAART	Highly active antiretroviral therapy
HAH	Halogenated aromatic hydrocarbons
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
HPLC/MS/MS	High performance liquid chromatography with tandem mass spectrometry
HSP	Heat shock proteins
<u>I</u>	
INSTI	Integrase nuclear strand transfer inhibitors
ISO	International Organization for Standardisation
<u>K</u>	
KCl	Potassium chloride
<u>L</u>	
LAR	Luciferase activating reagent
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
LPV/r	Lopinavir/ritonavir
Luc	Luciferase

M

MeOH	Methanol
$\text{Mg}(\text{CO}_3)_4\text{Mg}(\text{OH})_2$	Magnesium carbonate hydroxide
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	Magnesium sulphate heptahydrate
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

N

NaHCO_3	Sodium bicarbonate
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NR	Nuclear receptor
NRTI	Nucleoside reverse transcriptase inhibitors
NVP	Nevirapine

O

OD	Optical density
OF	Olifantsfontein

P

PAH	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyls
PI	Protease inhibitors
PPCP	Pharmaceuticals and personal care products
PPI	Inorganic pyrophosphate

Q

Q-TOF/MS	Quadrupole time-of-flight mass spectrometry
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R

RE	Removal efficiencies
REACH	Registration, evaluation, authorisation and restriction of chemicals
REP	Relative potency values
RLU	Relative light units

S

S/N	Signal-to-noise
SBSE	Stir bar sorptive extraction
SC	Solvent control
SPE	Solid phase extraction
SR	Sunderland Ridge

StatsSA	Statistics South Africa
<u>I</u>	
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TDF	Tenofovir
<u>U</u>	
UPLC	Ultra-high performance liquid chromatography
US	Upstream
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
<u>V</u>	
VP	Vlakplaats
<u>W</u>	
WG	Welgedacht
WHO	World Health Organisation
WV	Waterval
WWTP	Wastewater treatment plants
<u>Z</u>	
ZG	Zeekoegat

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Chapter 1: Introduction

There is a growing concern regarding pharmaceuticals and personal care products (PPCPs) found in the environment of South Africa originating from industries, agriculture and household use (Aneck-Hahn et al., 2005). The presence of pharmaceuticals, such as antibiotics, antiretrovirals (ARVs), and steroids, are well documented in the aquatic environment (Archer et al., 2017a) and have been found in surface water, ground water and even drinking water all over the world (Santos et al., 2010). Pharmaceuticals find their way to wastewater treatment plants (WWTPs) via the excretion of the active compounds and their metabolites, as well as through the environmentally unsafe disposal of unused medication. These WWTPs are more often than not inadequate to completely remove the compounds and their metabolites (Petrovic et al., 2009), which results in them entering the surface water. Pharmaceuticals are continuously being used and thus have a constant presence in the environment (Ebele et al., 2017). Due to the continuous release into the aquatic environment, they might then exhibit the same exposure potential as true persistent pollutants, such as polychlorinated biphenyls (PCBs) (an industrial chemical) and dichlorodiphenyltrichloroethane (DDT) (an organochlorine pesticide) (Daughton and Ternes, 1999). The low volatility of pharmaceuticals means that they will mainly occur in aqueous phases, making them easily transported through the aquatic environment (Daughton and Ternes, 1999). Unpolluted natural water in South Africa is essential as it is already a water-scarce country (DWA, 2013) and it has a rapid population growth (StatsSA, 2018), increasing the demand for clean water. Pharmaceuticals can have a potentially major impact on the deterioration of water quality of South Africa.

Numerous natural and anthropogenic compounds are suspected to act as endocrine disruptors. These compounds can mimic or modulate endogenous hormones and are known as 'endocrine-disrupting chemicals' (EDCs) (Snyder et al., 2001). Pharmaceuticals can act as EDCs, but their endocrine disrupting abilities are largely unknown (Crawford et al., 2017).

The effects of EDCs are mainly determined by their ability to interfere with the transcriptional activity of nuclear receptors (NRs), such as the oestrogen receptor (ER) (Klett et al., 2010). Other pathways can also be affected such as mediation of the aryl hydrocarbon receptor (AhR) (Swedenborg et al., 2009). The AhR is involved in the metabolism of xenobiotics (Swedenborg et al., 2009), mainly through the cytochrome P450 (CYP) 1A1 enzyme (Hukkanen, 2000). *CYP1A1* can metabolise xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs), into more toxic or mutagenic metabolites (Delescluse et al., 2000).

Activation of the AhR is known to antagonise the ER and interfere with its transcriptional activity (Ramadoss et al., 2005). Disruption of the normal hormone signalling of the ER pathway could result in adverse effects such as the disruption of hormone homeostasis (Ramadoss et al., 2005). Ligands that are capable of activating the AhR can thus not only cause toxic and/or carcinogenic effects, but can also cause endocrine disruption, through interaction with the ER.

The human immunodeficiency virus (HIV) is a considerable problem in South Africa, as a large portion of the population is affected by this disease. It is the fifth leading cause of death after tuberculosis, diabetes and various heart diseases (StatsSA, 2016). To combat this problem, South Africa rolled out the antiretroviral therapy (ART) programme, which is based on the use of antiretroviral (ARV) drugs to treat the virus. Due to the high prevalence of the disease, the country also has the largest ART programme in the world (Avert, 2018), with an estimated of 2.2 million people receiving ARVs in 2012 (WHO, 2013). It is expected that due to the large amount of ARVs being used in South Africa, they will be present in the aquatic environment. ARVs have been found in the freshwaters of South Africa in previous studies (Schoeman et al., 2015; Swanepoel et al., 2015; Wood et al., 2015). They have also been found in freshwaters in Kenya (K'oreje et al., 2016; Ngumba et al., 2016a) and Germany (Prasse et al., 2010). Nothing is known about the possible endocrine disrupting abilities of ARVs on aquatic animals and/or humans.

The following questions can therefore be raised: In what quantities are the ARVs present in the freshwater environment of South Africa, and can these compounds present in water samples cause possible endocrine disrupting effects through modulating the AhR?

Because ARVs are found in low concentrations in water, large volumes of water must be extracted and the target compounds concentrated. The target compounds can be quantified using an ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometer (UPLC-Q-TOF/MS). *In vitro* reporter gene assays can be used to determine the modulation of the AhR by the ARVs found in the water samples. The H4IIE-*luc* cells are rat hepatoma cells stably transfected with a dioxin responsive element (DRE) promoter-luciferase reporter gene construct (Sanderson et al., 1996). The increased expression of *CYP1A1* is then used as an indicator of AhR activation (Xiao et al., 2015).

The *in vitro* assays are commonly done alongside instrumental analysis, to screen for possible indicators of endocrine disrupting activity and to give a more comprehensive characterisation of the potential of a sample to mediate the AhR, which can result in possible toxic effects, or indirect modulation of the ER (Snyder et al., 2001).

1.1. Aims and objectives

Hypothesis: ARVs present in environmental freshwater samples of South Africa have the potential to activate the aryl hydrocarbon receptor (AhR).

The aims and associated objectives of this study were:

1. to quantify the six ARVs commonly used in South Africa for HIV treatment (ritonavir, lopinavir, efavirenz, nevirapine, zidovudine and didanosine) in environmental samples upstream and downstream of WWTP.

Objectives:

- Selecting an appropriate extraction method: e.g. solid phase extraction (SPE).
 - Using UPLC-Q-TOF/MS.
2. to determine if the ARVs in aquatic samples activates the AhR using the H4IIE-*luc* cell line.

Objectives:

- The ability of the water extracts that are quantified (see no. 1) to activate the AhR will be determined using the H4IIE-*luc* cells. The compounds in the water samples will be extracted using SPE and concentrated if the ARV concentrations are too low to be detected by the *in vitro* assay.
- The responses of the environmental samples will be compared with that of known concentrations of the active ingredients of the ARV treatment.
- The cytotoxicity of the environmental samples will also be assessed using the MTT viability assay.

Chapter 2: Literature review

2.1. Water as a natural resource

Water is a vital natural resource and essential for all elements of life, from everyday function, such as drinking and food production, to providing a habitat for aquatic organisms and a reproduction medium for some terrestrial organisms. Despite its importance, water is poorly managed and facing a decline in water quality due to water pollution. South Africa is a water-scarce country, with unevenly distributed rainfall tied to seasonal changes, as well as high evaporation rates leading to reduced levels of water run-off and less availability of surface water (DWA, 2013). The average annual rainfall for South Africa is 490 mm, with 21% of the country receiving less than 200 mm. This is considerably less than the 814 mm average annual rainfall of the world (WWF, 2016).

South Africa has had a rapid increase in population growth, from 49.9 million in 2010 to an estimated current population of 57.73 million people (StatsSA, 2018). With the increase in the number of people living in South Africa, there is also an increase in the demand for proper water and sanitation services.

The availability of clean freshwater is one of the major problems facing the world today. Drinking water is obtained from surface water (rivers, lakes, impoundments and wetlands) and groundwater sources. Surface water sources are sinks for waste, both natural and anthropogenic, as many receive sewage effluent, and as a result these aquatic environments are often polluted (Fawell and Nieuwenhuijsen, 2003). There has been improvement in the provision of clean drinking water to rural communities in the past years, but some still rely on untreated or inadequately treated water resources for daily use (Archer et al., 2017b).

2.1.1. Sources of pollution

The sources of pollution of surface waters can be classified into two categories: point and non-point sources. Point sources are defined as discharge directly into a receiving environment from a definitive point of entry. Wastewater treatment plants are the most common contributor to point source pollution, with industrial wastewaters also contributing. Non-point sources are harder to identify as it does not have a single point of entry into the waterway, and are typically from run-off from agricultural land and urban areas (Li, 2014).

Point source pollution:

Point sources are continuous and can easily be monitored by measuring the discharge at a single point over time. They can thus also be easily controlled through the treatment of the source (Carpenter et al., 1998). WWTP effluents are major contributors to this pollution problem. This is because of improper infrastructure as well as inadequate management. An estimated 85% of South Africa's WWTPs are rundown: they are either dysfunctional or non-functional, with millions of litres of sewage being released into rivers daily (Bateman, 2010).

Return flows (which include treated effluent of WWTPs) at 14% make up a substantial portion of the total available water of South Africa (DWA, 2009). It is thus important that the treated effluent released into receiving water must be of best, technically achievable, quality. For most WWTPs that is not the case, because they are not equipped to completely remove all the compounds that enter them, some of which are ultimately discharged into the aquatic environment (Petrovic et al., 2009). In 2008 the national Department of Water and Sanitation (DWS) of South Africa launched a Green Drop programme to properly evaluate the performance of the WWTPs in the country. The Green Drop status of municipal, private and department of public works wastewater systems were determined from 2009 until 2013: 39% of the municipal WWTPs did not comply with the DWS standards and therefore did not receive a Green Drop status (DWS, 2014). These standards apply to the quality of the WWTP effluent and/or the management of the plant.

Non-point source pollution:

Non-point sources are linked to irregular events such as heavy rainfall and agricultural activity (Carpenter et al., 1998). Urban run-off is a result of impervious surfaces such as roads, housing and construction sites, which are typical elements of urbanisation. During heavy rainfall, this run-off washes pollutants from these surfaces into the storm sewers that carry the run-off directly into the surface water, completely avoiding WWTPs.

2.2. Pharmaceuticals as endocrine disrupting chemicals

Endocrine disrupting effects has been shown for pharmaceuticals, such as steroid drugs which may cause an increase of oestrogenic responses and alteration in development and reproduction (Hernando et al., 2006). The risk to non-target organisms of most pharmaceuticals in the environment is largely unknown (Crawford et al., 2017).

EDCs are a special group of compounds that have come under attention of international health organisations, like the World Health Organisation (WHO) and the REACH (registration, evaluation, authorisation and restriction of chemicals) program in Europe, and national organisations such as the United States Environmental Protection Agency (USEPA), because of the adverse health effects that they can have on humans and wildlife. The USEPA defines an endocrine disruptor as follows: “... exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for maintenance of homeostasis, reproduction, development, and/or behaviour” (Kavlock et al., 1996). Living organisms all rely on the endocrine system, which produces hormones that act as chemical messengers to control basic functions such as growth, maintenance and reproduction (Birkett and Lester, 2002). Numerous natural and anthropogenic chemicals are known or suspected to act as endocrine disruptors. This group of chemicals are comprised of different sub-categories and differ in their source, chemical properties and fate in the environment (Bergman et al., 2013; Van Wyk, 2012).

A variety of pollutants, such as pesticides, pharmaceuticals and personal care products (PPCPs), plasticisers, and industrial by-products have been shown to have endocrine activity (Archer et al., 2017b; Conley et al., 2017). When these pollutants enter surface waters, they may pose a risk to aquatic organisms and humans directly utilising the water.

Endocrine disruption can occur via: 1) agonists/antagonists of hormonal receptors, 2) selective modulators in coactivator/corepressor recruitment or 3) cross-talk between different receptor types (Yeung et al., 2011). EDCs can interfere with the hormones naturally produced in the body: from altering the amount of hormone synthesised and transported by the circulatory system, to the amount that reaches the target organs and how potently the hormone can activate its receptor (Gore et al., 2014). EDCs thus have the ability to interfere with the fundamentals of hormone signalling.

2.2.1. Pharmaceuticals

Pharmaceuticals are natural or synthetic chemicals found in over-the-counter and prescription human medicine and veterinary drugs (WHO, 2012), used in treatment and prevention of human or animal diseases (Boxall et al., 2012). Personal care products are those used in the improvement of daily life such as moisturisers, shampoos and deodorants (Boxall et al., 2012). These groups of chemicals are collectively known as PPCPs.

In recent decades, there has been an increasing focus on PPCPs as pollutants in the aquatic environment. The presence of pharmaceuticals (like anti-inflammatories, antibiotics, antiseptics, antihypertensive medicine, antiepileptic, ARVs, beta-blockers, blood lipid regulators, oestrogens, steroids as well as a long list of other substances) are well-documented in the aquatic environment (Archer et al., 2017a; Bottoni et al., 2010). Pharmaceuticals have been detected in WWTP influent and effluent, surface water, marine water, groundwater and drinking water (Santos et al., 2010). The types of pharmaceuticals found are dependent on social, technological, cultural and agricultural factors and can differ between different geographical areas (Matongo et al., 2015).

PPCPs are designed to exert a biological effect in the intended consumer, and are thus of concern when found in surface water, because it can have unknown unintentional effects on non-target organisms directly exposed to the compounds (Azuma et al., 2016). Aquatic organisms are especially of concern because they can be subjected to multigenerational exposure due to their habitat (Halling-Sørensen et al., 1998). Humans and terrestrial animals can be exposed to pharmaceutical contaminants through consumption of contaminated aquatic organisms and drinking water (Bottoni et al., 2010).

- Sources of pharmaceuticals in aquatic environment

The potential exposure routes of pharmaceuticals into the environment are depicted in Figure 2.1. The most common source of pharmaceuticals in the environment is sewage, mainly from urban areas, which ultimately find its way to WWTPs (Archer et al., 2017b). PPCPs cannot be completely removed during conventional WWTP processes (Jelic et al., 2011) and are detectable in reclaimed water (Ebele et al., 2017) at low concentrations ranging from ng/L to µg/L (Yu and Wu, 2011). After pharmaceuticals are ingested by the target consumer, they undergo metabolic processes in the human and animal bodies. Large fractions of the compounds are excreted with urine and faeces, as unchanged parent compounds, residues or metabolites of the drug (Dębska et al., 2004), depending on the nature of the compound (Jjemba, 2006), and are therefore present in municipal sewage. The WWTPs produce treated waste in two forms: aqueous effluent, that is discharged into the freshwater environment, and sewage sludge (Ebele et al., 2017; Jones-Lepp and Stevens, 2007).

Pharmaceutical-tainted treated aqueous effluent can be discharged into surface waters or reclaimed as irrigation water for agriculture, where it can enter the surface- and groundwater through run-off and leaching (Deo, 2014) (Figure 2.1). Untreated sewage can also be discharged into surface water during flood overflow events or leaking sewage pipes (Daughton and Ternes, 1999).

Sorption of pharmaceuticals to sludge during the wastewater treatment process plays a vital role in the removal of these compounds from the aqueous phase (Subedi et al., 2013). After treatment, this sludge (biosolids) is often applied as fertilisers in agriculture (Ebele et al., 2017) (Figure 2.1) to improve the soil properties because it is rich in nutrient and organic matter (Wu et al., 2010). Despite the sludge being beneficial, it can also contain pollutants, amongst which are pharmaceuticals (Wu et al., 2010). Following the application of biosolids on soil, the PPCPs can enter ground- and surface water through leaching and run-off, and subsequently contaminate drinking water (Ebele et al., 2017; Heberer, 2002; Wu et al., 2010).

The disposal of unwanted or out-of-date medicine is also a notable source of pharmaceutical pollution in the environment (Bound and Voulvoulis, 2005). The unused drugs are disposed of via the (a) toilet/sink, where they end up in the WWTPs, or (b) household waste that is ultimately taken to landfills, where they can leach into groundwater (Bound and Voulvoulis, 2005; Daughton and Ternes, 1999) (Figure 2.1).

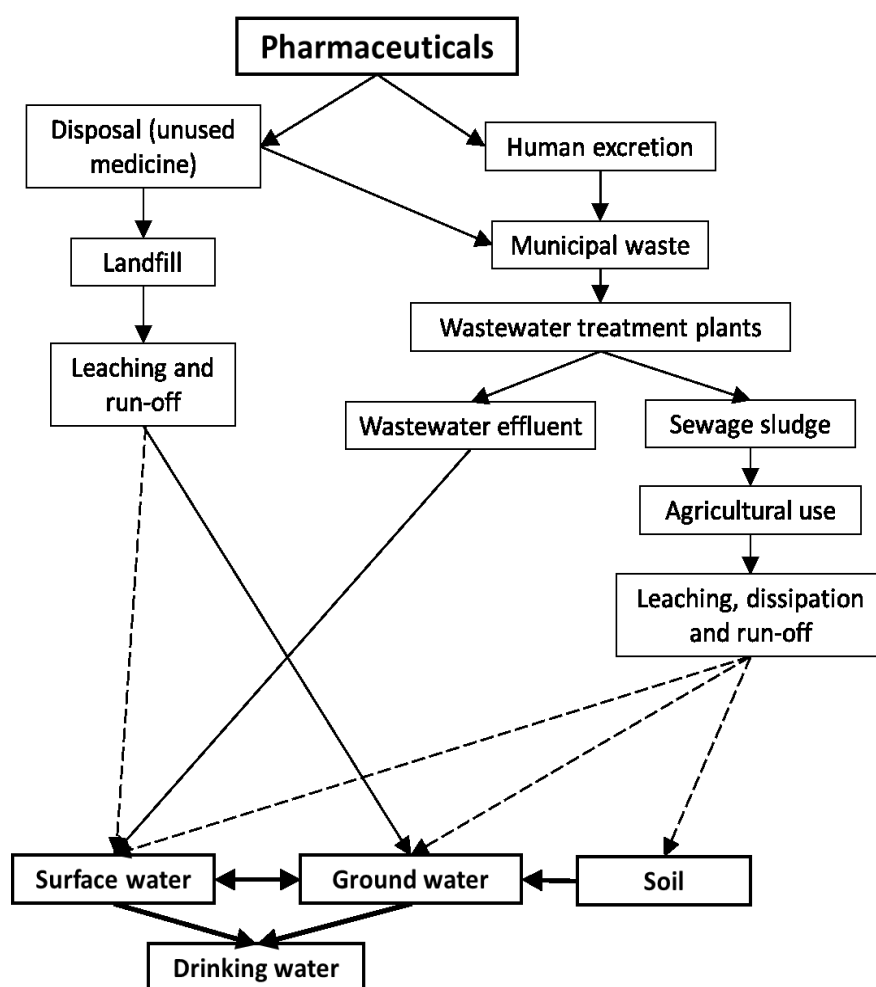


Figure 2.1: Potential exposure routes of pharmaceuticals into environment (adapted from Heberer, 2002).

Most PPCPs are not persistent, but due to their continuous use and release into the aquatic environment, they can be classified as being pseudo-persistent. These compounds are designed to degrade easily after excretion, but have a constant presence in the environment due to their continuous release (Aminot et al., 2015; Ebele et al., 2017). This can result in chronic effects in non-target organisms due to the long term exposure (Arnold et al., 2014).

Pharmaceuticals are designed with a specific mode of action and to exert biological effects, at low concentrations, on the target consumer (Cleuvers, 2003). However, some non-target organisms can be more susceptible to PPCPs because they do not have the same detoxification and metabolic systems as the intended consumer (Arnold et al., 2014).

2.3. HIV & ARVs

The human immunodeficiency virus (HIV) is a prominent health problem in South Africa, with an estimated 13.1% of the population infected (StatsSA, 2018). HIV is a virus that spreads through bodily fluids and attacks the body's immune system. The virus destroys CD4+ T-helper cells, also known as CD4 cells (CD4 is a glycoprotein found on the surface of T helper cells), which are white blood cells that help the immune system fend off infections and diseases. Left untreated, the HIV will reduce the number of CD4 cells in the body, making the person more susceptible to opportunistic infections (tuberculosis) and diseases (cancers) (Avert, 2018). There is no cure for HIV, but the use of ARVs as part of the antiretroviral therapy (ART) can control the virus levels in the patient, allowing them to live longer. Due to South Africa's high prevalence of HIV, we also have the largest antiretroviral therapy (ART) programme in the world, with an estimated 2.2 million people receiving ARVs in 2012 (WHO, 2013). According to Osunmakinde et al. (2013), ARV drugs are the third most prescribed type of drugs in the public health sector of South Africa, after hypertensives and analgesics.

There are five ARV classes currently available in South Africa, targeting different stages of HIV replication and inhibiting key enzymes—reverse transcriptase, protease and integrase—needed by HIV to replicate or acting on a different stage of the HIV infection (Azu, 2012; Meintjes et al., 2014).

- 1. Nucleoside reverse transcriptase inhibitors (NRTIs) and 2. non-nucleoside reverse transcriptase inhibitors (NNRTIs):** The HIV gene is carried in RNA, thus a DNA copy (proviral DNA) needs to be made from the RNA of the virus, and the reverse transcriptase enzyme is responsible for this. NRTIs and NNRTIs block the action of this enzyme (Engelman and Cherepanov, 2012). NRTIs require phosphorylation by cellular kinase in order to exert their activity, whereas NNRTIs require no intracellular metabolism (Esposito et al., 2012).

The most commonly used NRTIs are didanosine (ddl), lamivudine (3TC), stavudine (d4T), tenofovir (TDF) and zidovudine (AZT). The chemical structures of only ddl and AZT are given in Figure 2.2 because they were used in this study. The common NNRTIs (Figure 2.3) in South Africa are efavirenz (EFV) and nevirapine (NVP) (Meintjes et al., 2014).

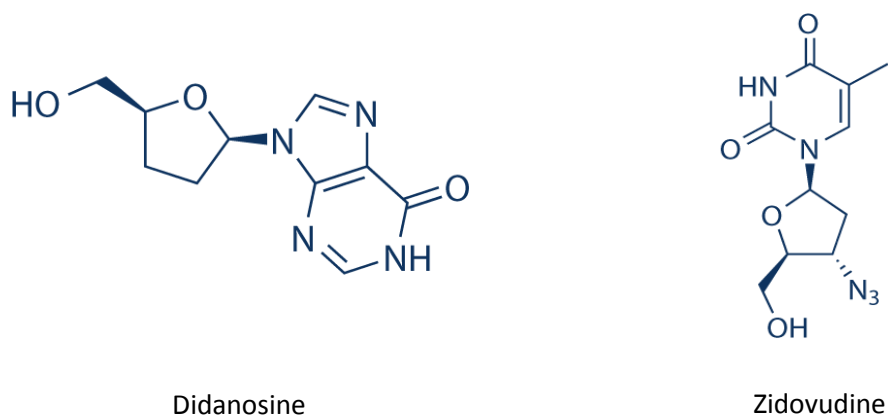


Figure 2.2: The chemical structure of the commonly used NRTIs, didanosine and zidovudine.

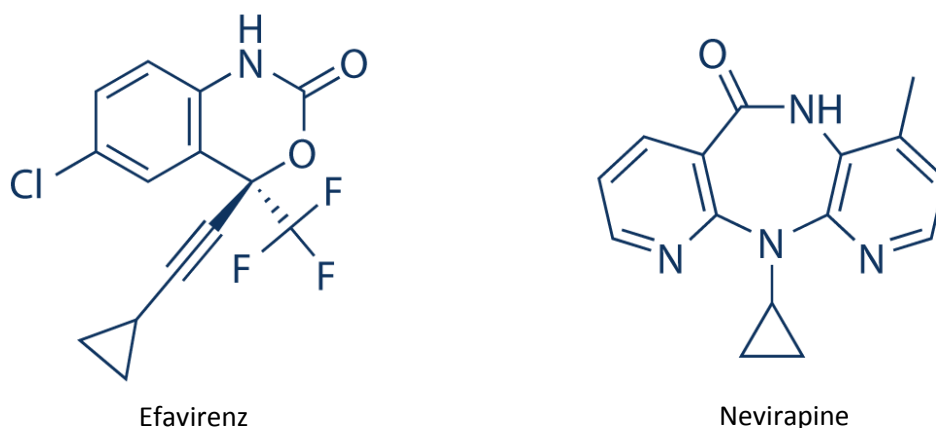


Figure 2.3: The chemical structure of the commonly used NNRTIs, efavirenz and nevirapine.

3. **Protease inhibitors (PIs):** The protease enzyme is necessary for the maturation of the viral particles budding from infected cells. The PIs block the activity of the enzyme and result in the formation of non-infective viral particles (Engelman and Cherepanov, 2012). Protease inhibitors do not require chemical alterations to become pharmaceutically active. Lopinavir boosted with ritonavir (LPV/r) (Figure 2.4) is the most commonly used PI in South Africa (Meintjes et al., 2014).

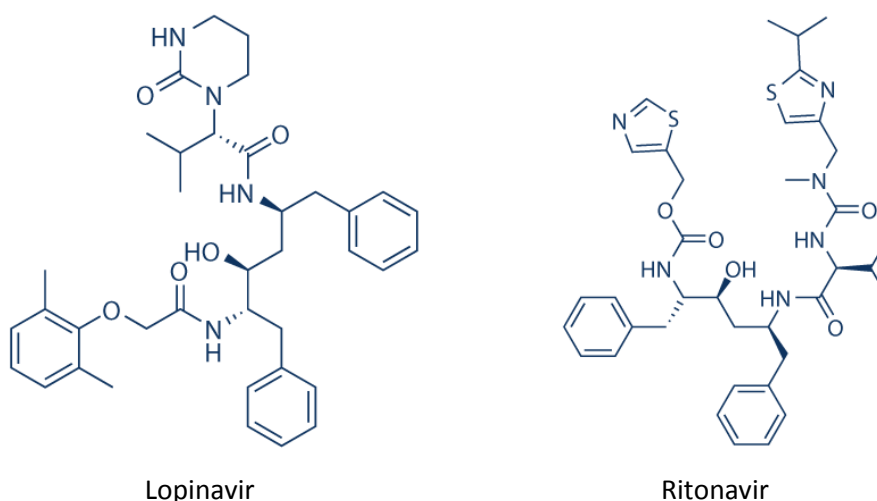


Figure 2.4: The chemical structure of the commonly used PIs, lopinavir and ritonavir.

4. **Integrase nuclear strand transfer inhibitors (INSTIs):** The integrase enzyme is responsible for the integration of the proviral DNA into the host DNA (Engelman and Cherepanov, 2012) and INSTIs prevent this from happening. Raltegravir is a INSTIs commonly prescribed in South Africa (Meintjes et al., 2014).
5. **Entry inhibitors:** HIV targets CD4 receptors on the outside of T-cells, and fuses to the outside of the cell. Entry inhibitors prohibit the binding to the host surface proteins, making entry into the host cells more difficult (Haqqani and Tilton, 2013). The entry inhibitor used in South Africa is maraviric (Meintjes et al., 2014).

The highly active antiretroviral therapy (HAART) programme is the use of a combination of ARV drug classes, in order to minimise the development of resistant strains (Mallipeddi and Rohan, 2010). First-line HAART treatment is based on the combination of two NRTIs and one NNRTIs (Meintjes et al., 2014). According to the WHO guideline for the treatment and prevention of HIV (WHO, 2016), the recommended first-line regimen for adults would include TDF + 3TC + EFV. If contraindication occurs, AZT should replace TDF as NRTI, and/or NVP should replace EFV (AZT + 3TC + EFV (or NVP)). Second-line HAART for adults should include two NRTIs and one ritonavir boosted PI, such as LPV/r. The recommended regimen consists of AZT + 3TC + LPV/r. According to Osunmakinde et al. (2013) EFV, 3TC and d4T is the most prescribed in the public health sector of South Africa.

2.3.1. ARVs in the aquatic environment

These ARVs, similar to other pharmaceuticals, are excreted and end up in the wastewater system that are often not designed to specifically remove these compounds and they ultimately end up in the environment (Deziel, 2014).

Another source of ARVs into the environment is through the illegal street drug named Nyaope (or Whoonga). Nyaope is a highly addictive drug that can be found in the impoverished townships of South Africa, and is a concoction of heroin, morphine, marijuana, methamphetamine and may also contain the ARV drugs efavirenz or ritonavir (Thomas and Velaphi, 2014). The ARVs are stolen from HIV positive patients in order to sell and/or use in Nyaope (Rough et al., 2014). This can lead to resistance to these ARV drugs, leading to the use of other ARVs, increasing the diversity of the compounds being used. This can result in more people consuming ARVs than only people living with HIV, increasing the risk of it entering the surface waters.

Metabolites of the ARVs may also be excreted alongside the parent compounds. As mentioned in Section 2.3 above, only the NRTIs require metabolism in order to exert a pharmacological action. The metabolites produced during this step can thus exert more detrimental effects on non-target organisms (Archer et al., 2017a). The NNRTIs and PIs are metabolised into HIV inactive forms.

Some ARVs have been found globally in WWTP influent and effluent and the freshwater environment. Examples include Kenya (K'oreje et al., 2016; Ngumba et al., 2016a), Germany (Prasse et al., 2010), Finland (Ngumba et al., 2016b) and Belgium (Vergeynst et al., 2015). Studies done by Wood et al. (2015), Schoeman et al. (2015), Wooding et al. (2017) and Afafe et al. (2018) have also found some ARVs in the aquatic environment of South Africa.

The Gauteng Province is the smallest province in South Africa, yet it has the highest population density and is highly industrialised (StatsSA, 2011g). With 25.3% of the country's population living in Gauteng, they have the largest share of the population (StatsSA, 2017). Gauteng has the fifth highest prevalence of HIV by province, with 12.4% (Shisana et al., 2014).

The Ekurhuleni Metro in Gauteng and eThekweni in KwaZulu-Natal had the highest prevalence of HIV in South Africa by city, with the City of Tshwane and City of Johannesburg being only slightly lower than the national average (Shisana et al., 2014). The HIV prevalence in informal settlements (25.8%) is also higher than formal settlement (13.9%) (Shisana et al., 2005). Informal settlements are often located near a water source, such as a river (Gangoo, 2003), which can contribute to surface water pollution through improper disposal of waste and inadequate sanitation.

2.3.2. Side effects of ARVs

The consumption of ARVs results in short-term toxicities in the liver, kidney and bone marrow, and cutaneous reactions like hypersensitivity. Long-term side effects can include morphological complications and metabolic abnormalities. Lipoaccumulation, breast enlargement (especially in men) and lipomas are examples of morphological complications, where metabolic abnormalities include insulin resistance and abnormalities of glucose metabolism (Marfatia and Makrandi, 2005). Different adverse side effects are attributed to the different ARV classes (Kwara et al., 2005) (Table 2.1).

Table 2.1: Adverse side effects attributed to different antiretroviral (ARV) classes (Kwara et al., 2005).

Class of antiretroviral	Adverse side effects
NRTIs	Hepatic steatosis (fatty liver)
	Lactic acidosis
	Lipodystrophy (redistribution of body fat)
	Mitochondrial toxicity
NNRTIs	Hepatotoxicity (liver damage) & elevated liver enzymes
	Skin rash
PIs	Hyperglycaemia
	Lipodystrophy & dyslipidaemia
	Hepatotoxicity

Despite the long list of known side effects of ARVs, the unintentional endocrine disrupting effects is largely unknown. This study aims to shed some light on some of these possible unknown effects.

2.4. Determining effects of EDCs

The studies on the effect of EDCs on the endocrine system have mainly focused on their direct effect on reproductive processes mediated by the family of NRs. The NRs are ligand-regulated transcription factors and are responsible for reproduction, homeostasis and metabolism, as well as responding to xenobiotics (Swedenborg et al., 2009). Members of the NR family include the ER and the androgen receptor (AR). Generally, chemicals with oestrogenic-, androgenic- and thyroid-like activity are mediated through the respective nuclear receptors, but other modes of actions are also possible such as the interaction with the steroidogenesis pathway (Van Wyk, 2012) or the AhR (Swedenborg et al., 2009).

The AhR is a member of the basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) protein family and is involved in the metabolism of xenobiotics (Swedenborg et al., 2009) by inducing drug-metabolising enzymes such as the cytochrome P450 (CYP) enzymes (Hukkanen, 2000). The CYP1A1 enzyme is the most studied xenobiotic-metabolising enzyme, as it is the most consistently and commonly expressed isoform (Denison and Nagy, 2003; Shanle and Xu, 2010).

The AhR is bound to heat shock proteins (HSPs) in the absence of ligands in the cytoplasm. When bound to a ligand, it translocates into the nucleus, and the HSPs dissociate from the ligand-bound AhR. The bound AhR forms a heterodimer by binding with the aryl-hydrocarbon nuclear translocator (ARNT) protein. This dimer-complex binds to the specific DNA recognition site for the AhR, the dioxin-response element (DRE). Binding to the DRE leads to the transcription of adjacent responsive genes (Figure 2.5), (Frye et al., 2012; Giesy et al., 2002) such as the *CYP1A1* gene (Mackowiak and Wang, 2016). The enhanced expression of the *CYP1A1* in turn increases the biotransformation of xenobiotics (Mackowiak and Wang, 2016).

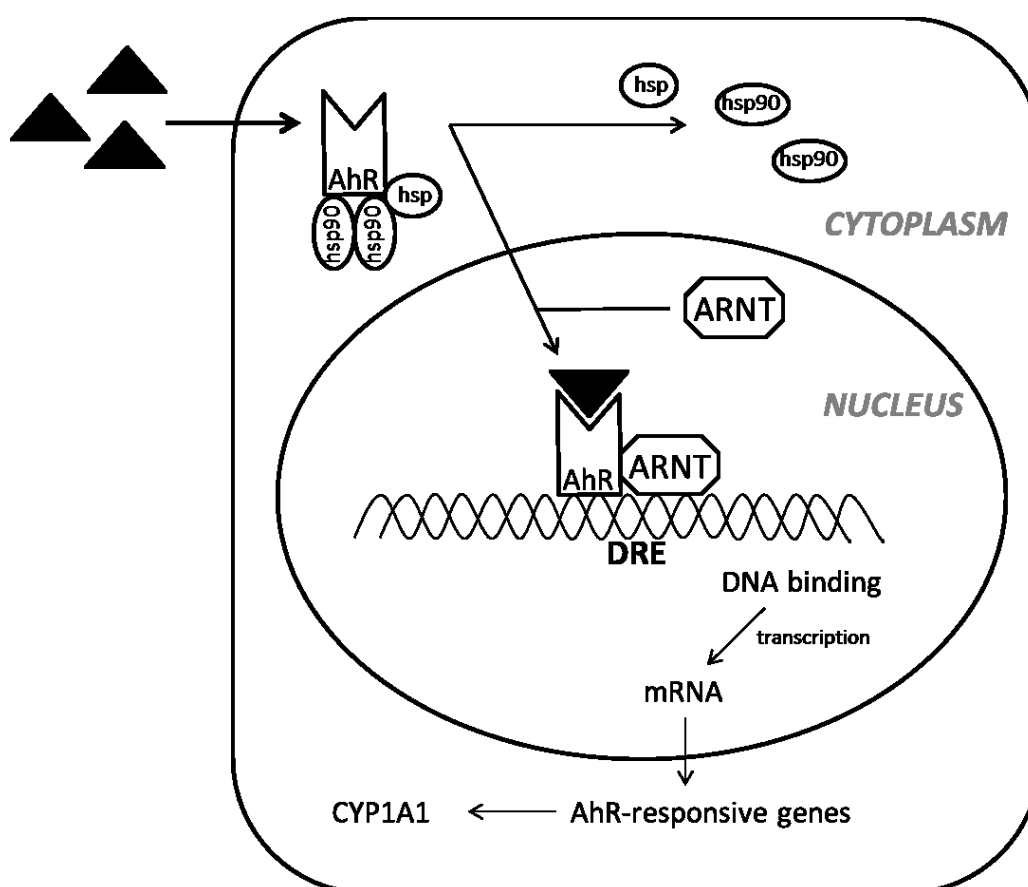


Figure 2.5: Mechanism of action of the aryl hydrocarbon receptor (AhR) in response to ligand binding (adapted from Giesy et al., 2002) (ARNT = aryl-hydrocarbon nuclear translocator; DRE = dioxin response element; hsp = heat shock protein).

Examples of well-known high-affinity AhR-ligands are halogenated aromatic hydrocarbons (HAHs) (like polychlorinated biphenyls), and polycyclic aromatic hydrocarbons (PAHs), as well as some structurally related compounds (Denison and Nagy, 2003; Giesy et al., 2002). The most potent inducer of the *CYP1A1* gene is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). It is also a known human carcinogen (Connor and Aylward, 2006). The AhR facilitates the biological response to environmental contaminants (Mackowiak and Wang, 2016; Swedenborg et al., 2009), as it is either involved in detoxification or bio-activation of these xenobiotics (De Coster and Van Larebeke, 2012).

Some xenobiotics (like PAHs) can cause toxicity in humans and wildlife by being metabolised into cytotoxic or genotoxic intermediates (Madak-Erdogan and Katzenellenbogen, 2011). The AhR also binds with low to moderate affinity to compounds with different structures and physiochemical properties (Denison and Nagy, 2003), such as structurally diverse pharmaceuticals (Jin et al., 2014).

Beside the activation of CYP enzymes, the AhR can also modulate specific cellular signalling pathways, such as the ER (Janošek et al., 2006). Disruption of the signalling pathways of receptors by AhR-mediated events can result in numerous effects such as, endocrine disruption, immunotoxicity, carcinogenesis, and developmental and reproductive toxicity (Janošek et al., 2006; Safe, 2001).

2.4.1. Endocrine disrupting effects by activation of AhR

Cross-talk can occur between the AhR and the signalling pathways of NRs such as the ER (Janošek et al., 2006). Activation of the AhR has been shown to exert ER independent anti-oestrogenic effects through the interference with ER signalling pathways (Janošek et al., 2006), resulting in reduced levels of oestrogens (Göttel et al., 2014). According to Mortensen and Arukwe (2007) and Shanle and Xu (2010), there are four possible mechanisms for the anti-oestrogenic effect of the AhR:

- Increased metabolism rate of oestradiol
- Decreased ER isoforms
- Suppression of oestradiol induced transcription
- Competition for transcriptional cofactors by ER and AhR

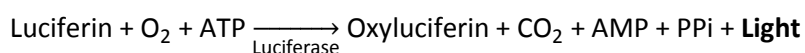
AhR ligands thus have the ability to not only cause toxicity and carcinogenesis, but also disruption in hormone systems, through interaction with the ER.

2.4.2. Relevance of bioassays

In vitro bioassays are a bioanalytical technique commonly used to characterise the biological effects of compounds/samples as it integrates the effects of unidentified compounds as well as potential mixtures (Wagner and Oehlmann, 2011). These *in vitro* techniques are helpful as a first-line screening of hormonally active compounds with specific mechanisms of action in environmental samples (Štěpánková et al., 2011; Svobodová and Cajthaml, 2010). They can be used to detect the biological effects of environmentally relevant concentrations, which are often below detection limits of chemical analysis (Chapman and Leusch, 2014).

The genetic composition of eukaryotic cells can be manipulated to produce a specific gene product in response to a stimulus (New et al., 2003). A reporter gene, whose expression can be easily monitored, is added downstream of the promoter in the plasmid (Liu et al., 2009) and is under control by the same signals as the target gene (Bauer, 2011). This reporter gene product contains enzymatic activity that can be monitored easily e.g. luciferase and thus 'reporting' on the stimulus (New et al., 2003). The use of these cell lines to determine the endocrine disruptive effects of compounds are called reporter gene assays. These assays are sensitive, rapid, reproducible and easy (Schenborn and Groskreutz, 1999).

The luciferase (*luc*) gene is a bioluminescent gene found naturally in several species of firefly, that encodes for the luciferase enzyme (New et al., 2003). These genes have been cloned and transfected into mammalian cells. Beetle luciferin (also called D-luciferin) is a photon-emitting substrate (Fan and Wood, 2007) and is added externally and is catalysed by luciferase in the presence of oxygen and adenosine triphosphate (ATP) to produce light (560 nm) (Köhler et al., 2000). The amount of light emitted is directly proportional to the enzymatic activity (New et al., 2003). The reaction can be summarised as follows:



The advantages of using the luciferase reporter gene are that it has a high specific activity, a broad linear range and no endogenous activity. The disadvantages include the requirement of luciferin to be added and the presence of ATP and O₂, as well as the need for cell lysis prior to the addition of the substrate (Naylor, 1999).

Other disadvantages include the natural organic material, such as dissolved organic carbon, present in surface waters which may affect the *in vitro* bioassays by working directly on the pathway of interest (either activation or inhibition), which may cause variation in the results of the study (Rosenmai et al., 2018). Cytotoxic compounds that might be present in especially extracts from the environment might cause the cells to die, and no responses would be recorded.

H4IIE-luc reporter gene assay

The H4IIE-luc bioassay is the best characterised reporter gene assay model for determining AhR-mediated responses to ligands (Janošek et al., 2006). The H4IIE-luc cells are rat hepatoma cells that had been stably transfected with a luciferase reporter gene under the control of the DRE (Sanderson et al., 1996; Vrzal et al., 2005). This liver-derived cell line re-expresses the biotransformation enzyme CYP1A1 (Jacobs et al., 2008). The response, when a ligand binds to the AhR leading to the expression of luciferase, is shown in Figure 2.66. When luciferin is then added externally it will produce light as a measurable response.

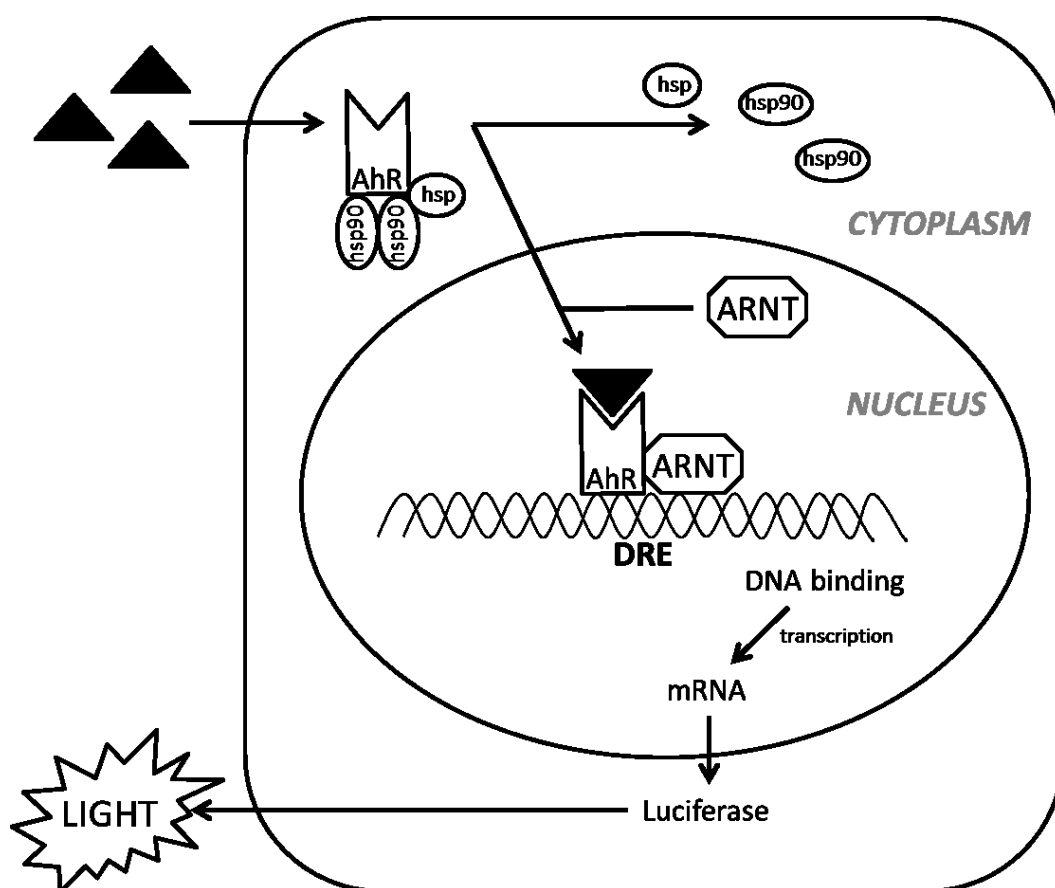


Figure 2.6: Mechanism of action of the luciferase reporter gene response of the aryl hydrocarbon receptor (AhR) in response to ligand binding (adapted from Giesy et al., 2002) (ARNT = aryl-hydrocarbon nuclear translocator; DRE = dioxin response element; hsp = heat shock protein).

2.4.3. Hormonal effects of ARVs

Stavudine, nevirapine and zidovudine were determined to have slight luciferase activity by binding to the AhR in the H4IIE-*luc* bioassay (Hu et al., 2007), thus inducing the expression of the CYP1A1 enzyme.

Some ARVs can act as EDCs, through interaction with the ER. Studies done by Sikora et al. (2010) and Svärd et al. (2014) found that the NNRTI efavirenz binds to the ER and increases transcriptional activity. Xiang et al. (2014) found that the PI ritonavir acts as antagonist of the ER. These studies were not done using environmentally relevant concentrations, thus the effects may differ when environmentally relevant concentrations are used. The unintentional exposure of wildlife and humans to ARVs in the environment can present any of these side-effects and many more.

Relatively little is known about the biological effect of environmentally relevant concentrations of ARVs. Robson et al. (2017) exposed *Oreochromis mossambicus* to environmentally relevant concentrations of efavirenz (10.3 ng/L) for 96 hours, and found that after this acute exposure, efavirenz can cause severe liver damage (steatosis). The overall health of the fish was determined with a histology-based fish health assessment index. At a higher concentration (20.6 ng/L) efavirenz was shown not only to impact the liver, but also the overall health of the fish.

Ngumba et al. (2016a) determined the possible ecotoxicological risk of zidovudine (0.77 µg/L) and nevirapine (0.49 µg/L) on algae, daphnia and fish using risk quotients. They found that zidovudine had potential high risk effects on algae, while the risk to daphnia and fish are low, and nevirapine had a high risk of causing effects in all three groups. High risk to algae, such as decreased growth, is concerning as they are primary producers in the aquatic ecosystem and many organisms rely on this as their main sources of food (Ncube et al., 2018).

The current knowledge on the hormonal effects of ARV drugs is very limited. Further studies are needed to determine if these drugs and their metabolites have any biological activity, and if so to what extent. This needs to be done with a combination of different end-point assays at different levels of biological organisation, such as *in vitro* assays (using the AhR to determine xenobiotic activity or the ER and AR for hormonal activity, and biomarkers) or *in vivo* assays (daphnia reproduction).

2.5. Instrumental analysis

The detection of pharmaceuticals in the aquatic environment can be challenging due to them being present at trace levels as well as the complexity of the matrix (Fatta-Kassinos et al., 2011). The matrix can influence the ability to recover a compound. Sensitive analytical methods are needed to determine pharmaceutical residues in different matrices as low as ng/L (Reddersen and Heberer, 2003). Pharmaceuticals in environmental samples have been reported at µg/L and ng/L levels, thus sensitive detectors are needed.

Mass spectrometry (MS) is a valuable detection method due to its high sensitivity and selectivity and is often coupled with either liquid chromatography (LC), for non-volatile compounds, or gas chromatography (GC), for volatile compounds (Yan et al., 2008). GC-MS was the preferred method for analysing pharmaceuticals in the environment because it is less impacted by matrix effects. However, LC-MS has recently been considered as the better choice, because most pharmaceuticals lack the suitable volatility for GC and thus need derivatisation. High performance liquid chromatography (HPLC) is the go-to analytical technique for the analysis of endocrine disruptors (Sosa-Ferrera et al., 2013).

Sample preparation in environmental samples is an important step because it enables the removal of major matrix interferences (sample clean-up) and pre-concentration, or the so-called enrichment step, of the analyte. For pharmaceutical analysis, solid phase extraction (SPE) is the preferred method for sample preparation (Madikizela et al., 2017; Petrović et al., 2005).

Instrumental analysis of a water sample provides quantitative and qualitative information about contaminants in the aquatic environment, while bioassays measure the collective effect of compounds that exert effects through the same mode of action (Lv et al., 2016). The downside is that screening of compounds using instrumental analysis does not give any information of their possible toxic properties, and bioassays on the other hand cannot identify the compounds that elicits a response (Kolkman et al., 2013). These limitations can be overcome by using a combination of the chemical screening and bioassays; this provides a powerful method for determining the biological activity of a sample (Gong et al., 2014).

2.6. Quantification of ARVs in the environment and their effects on the AhR

Surface water resources around the world—in first and third world countries—have been shown to be contaminated by ARVs. Some ARVs have indicated possible endocrine disrupting abilities through the activation of the AhR, as well as interfering with the transcriptional activity of the ER. The ARVs, as part of pharmaceuticals, are likely to have low to moderate affinity to the AhR.

Due to the continuous release of these compounds into surface waters and the high levels in which they are found, their ability for binding and activating the AhR can differ from the expected.

South Africa has a very high percentage of the population living with HIV, thus the use of ARVs are also high, and these compounds will likely be found at high concentrations in surface water. The endocrine disruption that these compounds may elicit in mixtures and at environmentally relevant concentrations is largely unknown.

The AhR mediated response of ARVs can be determined using the H4IIE-*luc* bioassay, which would indicate their detrimental effects, while instrumental analysis can determine the presence of ARVs, and their concentration. This can be used to explain the responses observed from the bioassay.

Chapter 3: Methods and materials

3.1. Sampling sites

The study area consisted of five river systems in Gauteng, that each receives the final discharge from WWTPs within their catchment. These include (Table 3.1):

- The Klip River, which receives the final effluents from both the Waterval and Vlakplaats WWTPs.
- The Blesbok Spruit near the town of Springs (east of Johannesburg), receives the treated effluent of the Welgedacht plant.
- The Hennops River that receives discharges from both Sunderland Ridge and Olifantsfontein plants which are situated near the towns of Centurion and Midrand, respectively.
- The Pienaars River receives treated effluent from the Zeekoegat plant that is located on the banks of the Roodeplaat Dam.
- The Wonderfontein Spruit receives the discharge from the Flip Human plant.

Table 3.1: The treatment capacity, water service authority, location and river into which the WWTP drains.

Wastewater treatment plants	Capacity (ML/day)	Water service authority	Coordinates	River
Waterval	155	Ekurhuleni metropolitan municipality	-26.437277 28.100972	Klip River
Vlakplaats	83	Ekurhuleni metropolitan municipality	-26.346666 28.181972	Klip River
Welgedacht	35	Ekurhuleni metropolitan municipality	-26.191472 28.474138	Blesbok Spruit
Olifantsfontein	105	Ekurhuleni metropolitan municipality	-26.940222 28.216111	Hennops River
Sunderland Ridge	65	City of Tshwane metropolitan municipality	-25.829056 28.104079	Hennops River
Zeekoegat	85	City of Tshwane metropolitan municipality	25.622513 28.333131	Roodeplaat Dam/Pienaars River
Flip Human	30	Mogale City local municipality	-26.184746 27.773073	Wonderfontein Spruit

Sampling sites in river systems were selected based on their accessibility and location in terms of WWTPs (e.g. upstream and downstream). The sampling areas were chosen to determine the impact of treated effluent from WWTPs on the concentrations of ARVs in receiving waters. The location of the discharge point into the rivers was used as a reference point to subdivide the study area into river areas upstream, as well as downstream, from the discharge point. In Table 3.2, the chosen sampling points with regards to the sampling area is shown.

Figure 3.1 indicates the position of all the sampling areas within the Gauteng province. Rivers from different areas in Gauteng were selected. According to the Green Drop status of Gauteng, South Africa, four of the chosen WWTPs received high risk status. These include the Welgedacht, Sunderland ridge, Zeekoegat and Flip Human WWTPs, which had a Green Drop status of between 69.4–71.2% (the provincial status being 78.8%) (DWA, 2011). These plants are at risk of discharging untreated (or only partially treated) effluent into the surface water, which can become polluted if the sewage is not correctly treated. It is thus necessary to determine the impact of these WWTPs on the concentrations of ARVs. This was done by sampling upstream of the effluent discharge as well as downstream (at two locations if possible).

Informal settlements situated on the edges of rivers were also taken into account. Many of these townships have inadequate access to basic water and sanitation services and as a result many of them are located close to a water source, particularly rivers (Gangoo, 2003). The lack of sanitation forces these communities to make use of pit latrines or river banks, the residue of which can enter the rivers through surface run-off. This could potentially be a major source of contamination of surface waters.

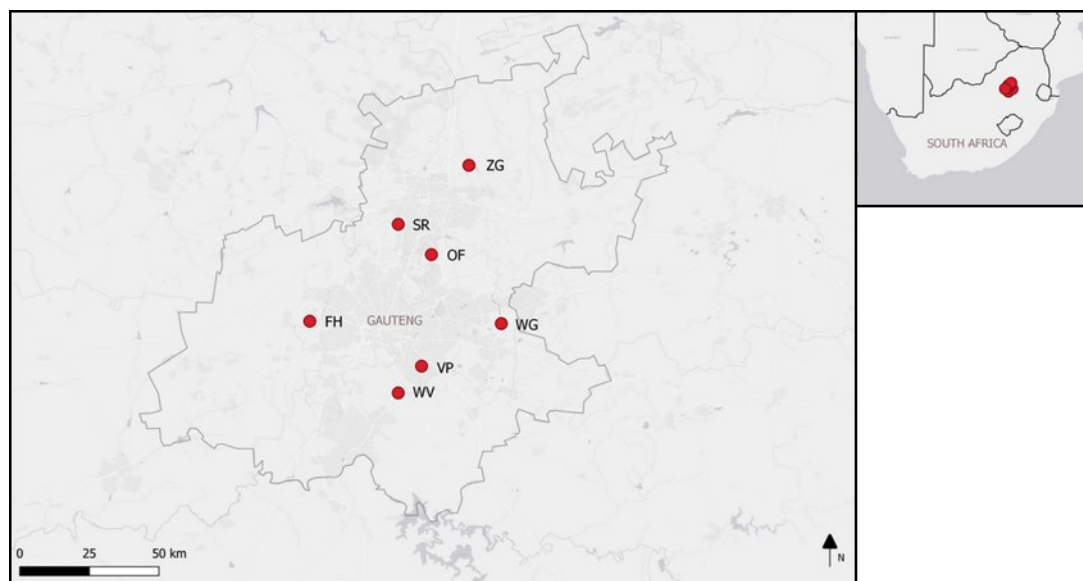


Figure 3.1: Area map of the wastewater treatment plants sampled in Gauteng, South Africa (WV = Waterval; VP = Vlakplaats; WG = Welgedacht; OF = Olifantsfontein; SR = Sunderland Ridge; FH = Flip Human; ZG = Zeekoegat).

Table 3.2: Sampling coordinates for Waterval, Vlakplaats, Welgedacht, Olifantsfontein, Sunderland Ridge, Zeekoegat and Flip Human and their approximate distance in terms of the sampling area. (US = upstream; DS = downstream; WWTP = wastewater treatment plant).

Sampling area name	Location in terms of sampling area		Coordinates	
Waterval (WV)	US	1 km upstream of WWTP	-26.421899	28.094211
	DS1	1 km downstream of WWTP	-26.453508	28.085552
	DS2	6 km downstream of WWTP	-26.499893	28.070404
Vlakplaats (VP)	US	1 km upstream of WWTP	-26.343632	28.170421
	DS1	1 km downstream of WWTP	-26.376000	28.170762
	DS2	6 km downstream of WWTP	-26.430108	28.160555
Welgedacht (WG)	US	0.5 km upstream of WWTP	-26.191213	28.478078
	DS1	1 km downstream of WWTP	-26.199063	28.479604
	DS2	2 km downstream of WWTP	-26.212292	28.481010
Olifantsfontein (OF)	US	1 km upstream of WWTP	-25.951971	28.207242
	DS1	2 km downstream of WWTP	-25.922349	28.227483
	DS2	3 km downstream of WWTP	-25.910475	28.230592
Sunderland Ridge (SR)	US	1 km upstream of WWTP	-25.840518	28.110386
	DS1	2 km downstream of WWTP	-25.822074	28.082248
Zeekoegat (ZG)	US1	3 km upstream of WWTP	-25.648713	28.328409
	US2	6 km upstream of WWTP	-25.662653	28.351033
	US3	8 km upstream of WWTP	-25.642978	28.384984
	DS1	4 km downstream of WWTP	-25.609160	28.368198
Flip Human (FH)	US	1 km upstream of WWTP	-26.177597	27.766307
	DS	1 km downstream of WWTP	-26.185955	27.760417

3.1.1. Klip River and the Waterval and Vlakplaats WWTPs

The Klip River is situated in the southern part of Johannesburg (Vermaak, 2009) and feeds into the Vaal River, which is a key source of drinking water for the city of Johannesburg (Mothetha, 2016), thus reaching a larger population group. There are several informal settlements located on the banks of the Klip River that can contribute to the pollution of the river. The Klip River also receives the effluent from the Waterval WWTP that is situated on the banks of the southern part of the River.

The Vlakplaats plant is located between the Kathlehong and Vosloorus townships. Kathlehong has a population of 407 294 (StatsSA, 2011c) and Vosloorus has 163 216 (StatsSA, 2011e). The WWTP treats sewage from mainly Vosloorus and Boksburg, and has a capacity to treat 83 ML/day (Table 3.1). The Dekema plant is a smaller plant capable of treating only 36 ML/day, and bypasses some of its screened sewage to the Vlakplaats plant (ERWAT, 2018a). Dekema receives sewage from Kathlehong and Alberton; the Vlakplaats plant thus also receives sewage from these areas.

Both plants discharge their treated effluent into the Natal Spruit, which ultimately leads in to the Klip River below the Waterval plant (Figure 3.2). The US site is located downstream of the Dekema WWTP and the two DS sites are located below the Vlakplaats plant, a few kilometres apart (Table 3.2).

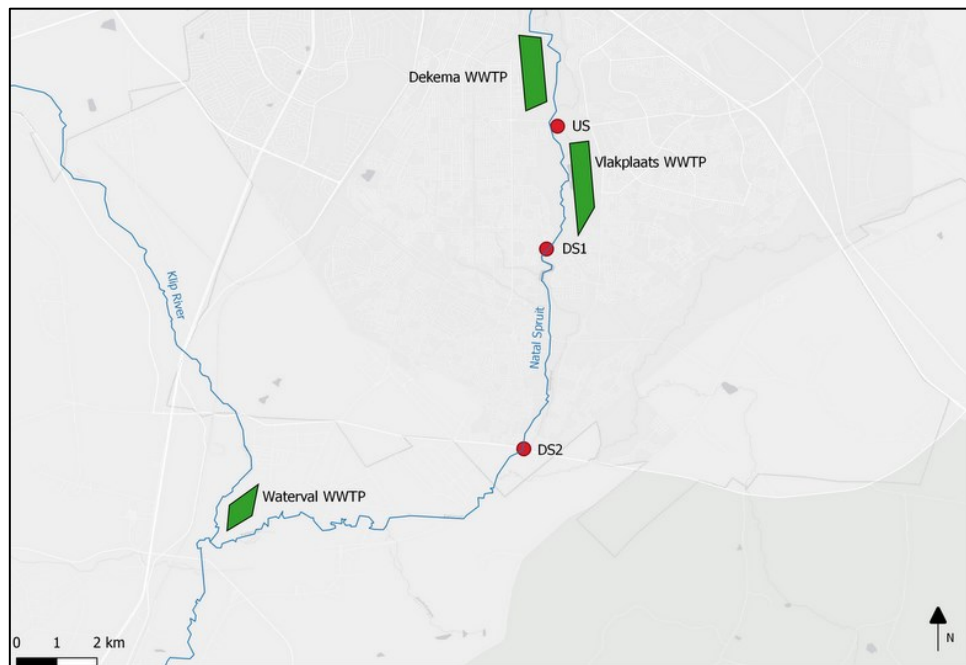


Figure 3.2: Sampling locations around the Vlakplaats WWTP (as well as the Dekema and Waterval WWTPs which is located upstream and downstream of VP) (US = upstream; DS = downstream; WWTP = wastewater treatment plant).

The upstream site of the Waterval plant is located on the Klip River after it runs through mainly agricultural or undeveloped land for approximately 30 km from Soweto, which is the largest township in the Gauteng province. Despite its proximity, it is not likely that this upstream site of WV is strongly being influenced by Soweto. The WWTP receives effluent from Alberton, Kathlehong, Vosloorus and Boksburg (ERWAT, 2018b) and has a treatment capacity of 155 ML/day (Table 3.1). The two downstream sites are also located on the Klip River, the first just after the effluent of the WWTP entered the river and the second a few kilometres further downstream after the river ran through agricultural lands (Table 3.2). The Natal Spruit (that receives effluent from the Vlakplaats WWTP) also runs into the Klip River upstream of DS2.

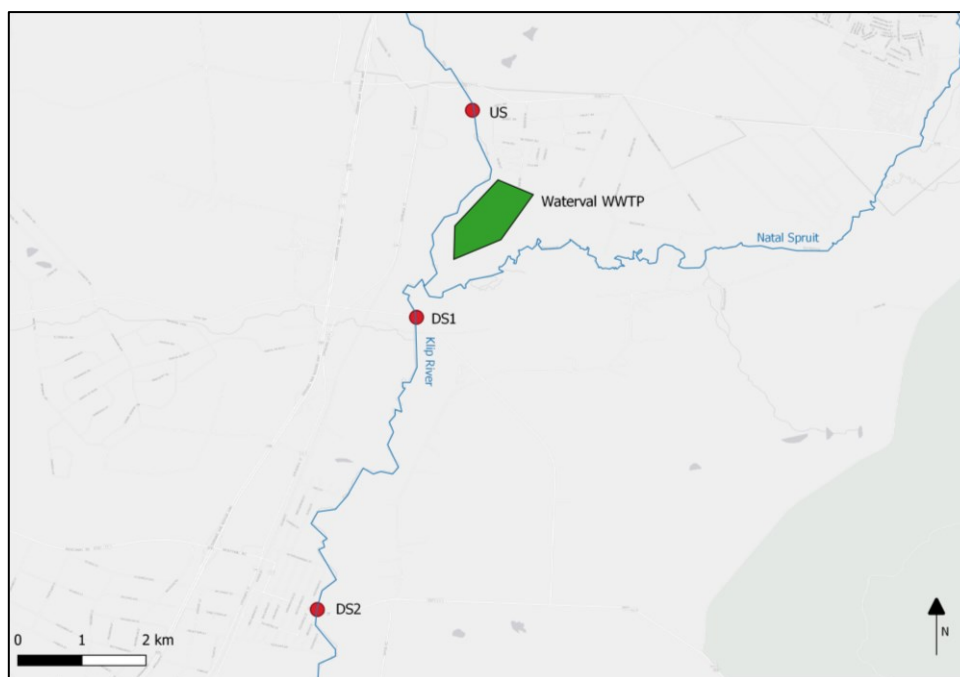


Figure 3.3: Sampling locations around the Waterval WWTP (US = upstream; DS = downstream; WWTP = wastewater treatment plant).

3.1.2. Blesbok Spruit and the Welgedacht WWTP

The Blesbok Spruit located in the eastern side of Gauteng flows southwards before it joins the Suikerbosrand River. This Spruit forms one of the largest wetlands in South Africa and the wetland is classified as a Ramsar wetland, because of its significant value to the country (Ambani and Annegarn, 2015). Since it has been shown that constructed wetlands improve the removal of some pharmaceuticals when used as a part of the wastewater treatment process (Matamoros and Salvadó, 2012), it is assumed that natural wetlands should also be able to aid in the removal of these types of compounds. The Welgedacht WWTP is located next to the Blesbok Spruit near Springs and has a capacity to treat 35 ML sewage/day (Table 3.1) (making it one of the smaller plants in this study) (Figure 3.4). The Daveyton Township upstream of the US site and has a population of 127 967 people (StatsSA, 2011b). The Cowles Dam drains into the Blesbok Spruit upstream of the DS2 site (Figure 3.4).

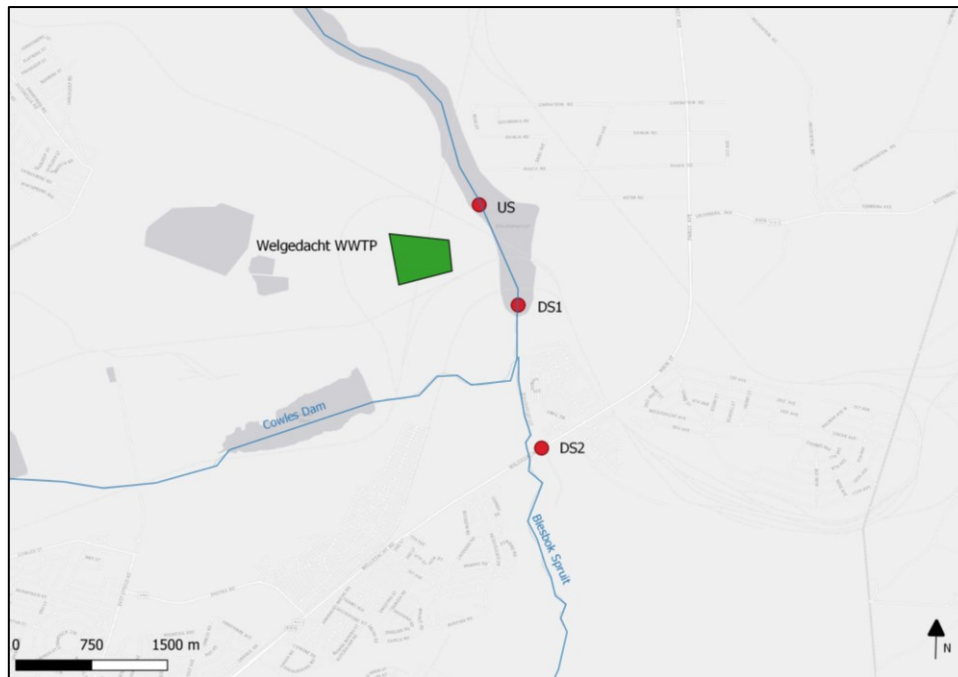


Figure 3.4: Sampling locations around the Welgedacht WWTP (US = upstream; DS = downstream; WWTP = wastewater treatment plant).

3.1.3. Hennops River and the Olifantsfontein and Sunderland Ridge WWTPs

The Hennops River flows from Kempton Park, through Centurion and finally into the Crocodile River. This river flows through informal and formal residential-, business-, recreational- and industrial areas. The Olifantsfontein WWTP and Sunderland Ridge WWTP are located in the Hennops River catchment (Figure 3.5 and Figure 3.6), with the Olifantsfontein WWTP upstream of the Sunderland Ridge WWTP. Both plants have been reported to release untreated/partially treated sewage into the Hennops River (Bega, 2018; Meijer, 2015; Milford, 2017).

The Sunderland Ridge WWTP has a treatment capacity of 65 ML/day and treats wastewater generated in Centurion and Midrand (Table 3.1). The treated effluent is discharged into the Hennops River (Roux et al., 2010). The upstream site is located on the Riet Spruit that runs through mainly industrial and formal urban areas. The Riet Spruit flows past the Sunderland Ridge WWTP before it flows into the Hennops River. The downstream site on the Hennops River is situated after the effluent discharge point of the plant (Figure 3.5).

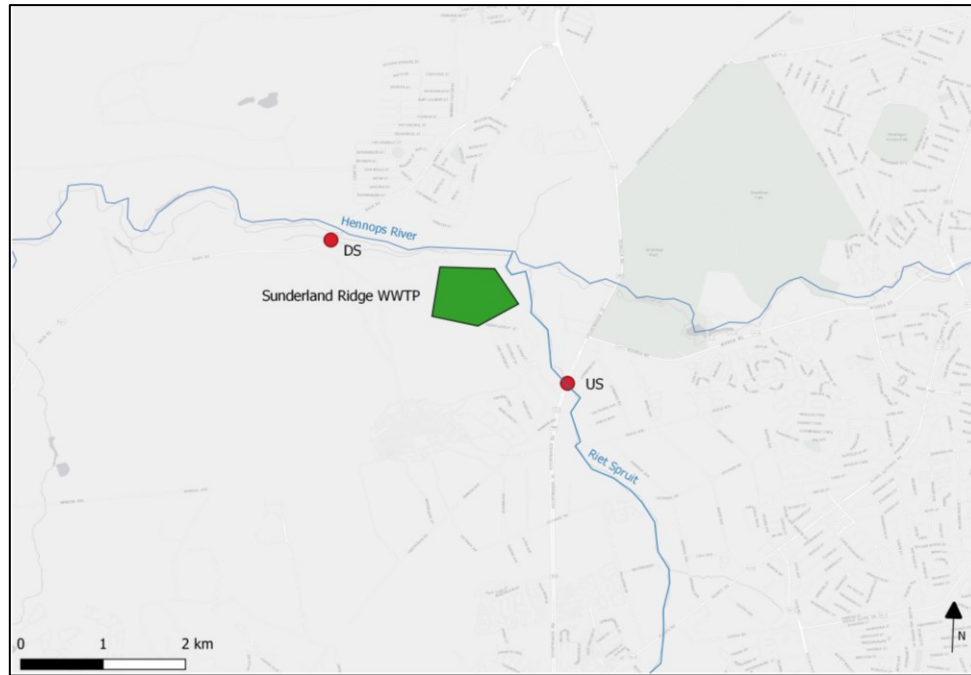


Figure 3.5: Sampling locations around the Sunderland Ridge WWTP (US = upstream; DS = downstream; WWTP = wastewater treatment plant).

The Olifantsfontein WWTP has a treatment capacity of 105 ML/day and receives wastewater from mainly Tembisa and Ivory Park (Table 3.1). The treated effluent is discharged into the Kaal Spruit, which runs into the Olifant Spruit. The US site is located on the Kaal Spruit, and the two downstream sites on the Olifant Spruit (Figure 3.6). The Olifant Spruit confluences with the Sesmyl Spruit (which flows from the Rietvlei Dam) to eventually become the Hennops River (Roux et al., 2010).

Kaal Spruit is one of the most polluted streams running into the Hennops River; it borders the highly populated Tembisa and Ivory Park informal settlements (Nawn, 2004). There is a build-up of municipal waste in the townships which is not regularly removed due to violence and lack of funds (Hoffmann, 1995). Tembisa is the second largest township in Gauteng, after Soweto, with a population of 463 109 people and a population density of 10 820 people/km² (StatsSA, 2011d). This township mainly consists of formal housing (Hoffmann, 1995) and 84.7% of the community has access to toilet connected to sewage systems. Ivory Park borders Tembisa and has a smaller population of 184 383 people (StatsSA, 2011a), but it has a much higher population density (20 020 people/km²), where people mainly live in informal housing (squatter camps) (Hoffmann, 1995). About 88% of this township is connected to a sewage system, but in Ivory Park the system is in a very poor state due to lack of management and untreated sewage are released directly into the Kaal Spruit (Hoffmann, 1995). The upstream site is located just after the border of these townships, thus receives all the possible pollutants from them. The downstream sites are located after the treated effluent is released into the Kaal Spruit, before it becomes the Hennops River (Figure 3.6).

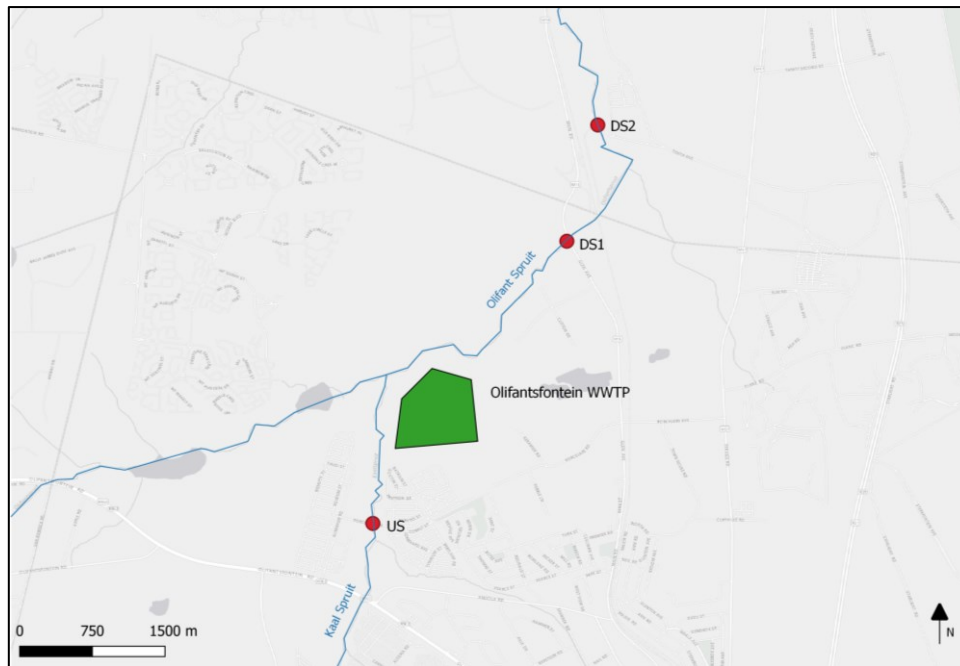


Figure 3.6: Sampling locations around the Olifantsfontein WWTP (US = upstream; DS = downstream; WWTP = wastewater treatment plant).

3.1.4. Pienaars River/Roodeplaat Dam and the Zeekoegat WWTP

This sampling area is unconventional, as it is not the direct impact of the Zeekoegat WWTP that was determined in this study, but rather the influence of the inflow of the rivers into the Roodeplaat Dam. The dam receives waters from the Pienaars River (US2) (43% of inflow), Hartbees Spruit (US1) (27% of the inflow), Edendale Spruit (US3) (8% of inflow) and the Zeekoegat WWTP (22% of the inflow) (Silberbauer and Esterhuyse, 2014) (Figure 3.7). The Zeekoegat plant is located on the banks of the Roodeplaat Dam (Figure 3.7), which is situated on the Pienaars River. It is a medium sized sewage treatment plant and has a capacity to treat 85 ML sewage per day (Table 3.1).

The Pienaars River flows through the Mamelodi Township before entering the Roodeplaat Dam. This township has a population of 334 577 people, with 70.4% of the household that has access to toilets connected to a sewage system. The Pienaars River also receives treated effluent from the Baviaanspoort WWTP seven kilometres before it enters the Roodeplaat Dam. The Pienaars River then continues north after the dam (this part of the river is the only downstream site). The Edendale Spruit also flows through the outer edges Mamelodi, but mainly borders agricultural lands. The Hartbees Spruit flows through an urban area of Pretoria before it reaches the dam. The three upstream sites were located near the point where the Hartbees Spruit (US1), Pienaars River (US2) and Edendale Spruit (US3) enter the Roodeplaat Dam (Figure 3.7). This sampling area only has one downstream site (DS1) that is situated on the outflow of the Pienaars River from the Dam. The flow of this part of the river is controlled by a constructed dam wall.

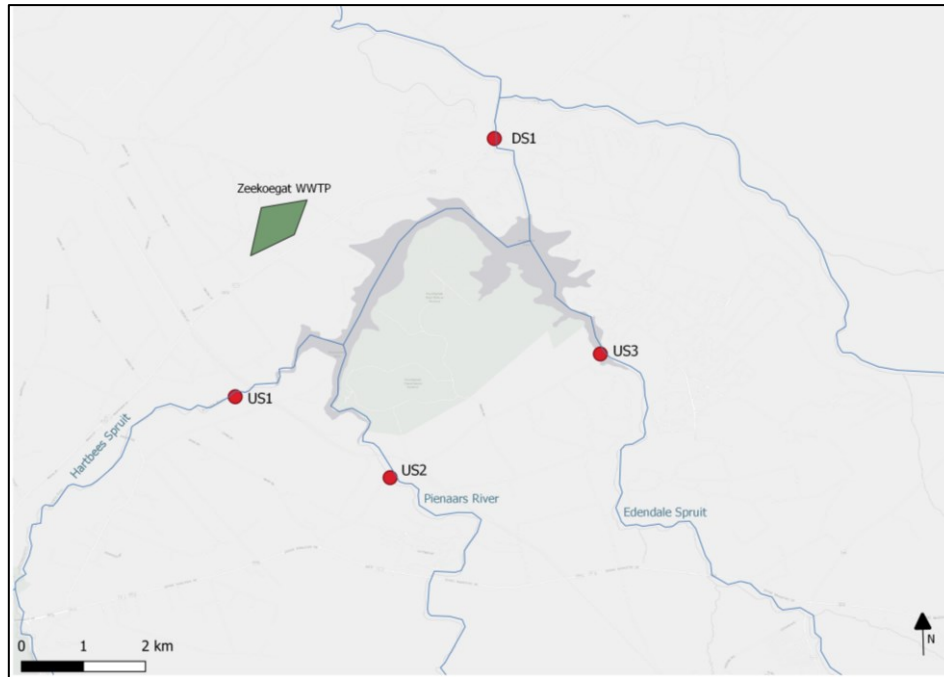


Figure 3.7: Sampling locations around the Zeekoegat WWTP and the Roodeplaat Dam (US = upstream; DS = downstream; WWTP = wastewater treatment plant).

3.1.5. Wonderfontein Spruit and the Flip Human WWTP

The Wonderfontein Spruit starts in Krugersdorp in Gauteng and flows towards Potchefstroom in the North-West province. The Flip Human plant is located near the town of Randfontein and discharges its effluent into the Wonderfontein Spruit (Figure 3.8). It has a sewage treatment capacity of 30 ML/day (Table 3.1).

Kagiso is a township in Randfontein with a population of 115 802 people living in a density of 8 173 people/km². It is located along the Wonderfontein Spruit upstream of the WWTP (above the upstream site). About 90% of the community has access to sanitation services (flush toilets connected to sewage systems), but some areas are still relying on pit toilets and buckets (StatsSA, 2011f).

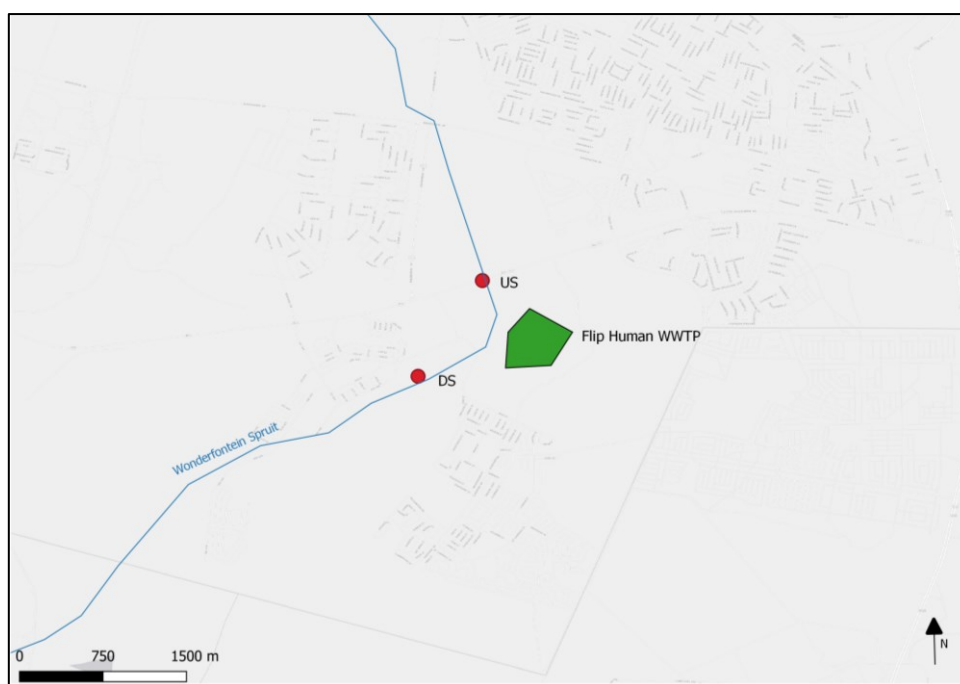


Figure 3.8: Sampling locations around the Flip Human WWTP (US = upstream; DS = downstream; WWTP = wastewater treatment plant).

3.1.6. Drinking water samples

A few drinking water (DW) samples were taken to assess the presence of ARVs within them. Although this was not an initial aim of the study, I made use of the opportunity to sample water from taps in the areas close to the WWTPs. The presence of pharmaceuticals in surface water is well-known, but in drinking water (tap water) it is largely unknown as there is no routine monitoring programme for these compounds (WHO, 2012). Seven tap water samples were taken at different areas from Gauteng (Table 3.3). Most of the drinking water for Gauteng is supplied by Rand Water, which mainly use the Vaal River as a water source.

Table 3.3: Coordinates for drinking water sampling (DW = drinking water).

Sampling site	Coordinates	
Olifantsfontein DW	-25.952014	28.109041
Zeekoegat DW	-25.633561	28.394311
Waterval DW1	-26.421171	28.098232
Waterval DW2	-26.453508	28.085552
Waterval DW3	-26.498918	28.066391
Vlakplaats DW1	-26.340354	28.180533
Vlakplaats DW2	-26.376000	28.170720

3.2. Sampling

The sites were sampled once in the month of October 2017. By only doing one sampling round, seasonal variation would not have been observed. This once-off sampling thus only gives an indication of the ARVs levels for that specific time period. Clear glass Schott bottles (1 L) were used for sampling. These bottles were washed in a 10% v/v hydrochloric acid (HCl) bath for 24 hours, and then washed three times with 18 MΩ distilled water (ddH₂O). The cleaning method was validated by analysing blanks (ddH₂O) after glassware was cleaned. Results showed that no ARVs were present after the cleaning procedure. Powder free nitrile gloves were worn at all times during sampling to prevent contamination. Grab samples were taken at all sites. The clean bottle was lowered and rinsed three times at the water sources before collecting the sample. Three subsamples were taken at each site, one for instrumental analysis, one for the biological assay, and one as a back-up.

The target compounds were protected from break down by ultraviolet (UV) radiation (Prasse et al., 2010) and microbial activity by transporting them at 4°C in the laboratory. No preservatives were added to the sample to prevent contamination (Ferrer and Thurman, 2012). Extraction was done within 48 hours of sampling (K'oreje et al., 2016). Each bottle was labelled with information such as: the sample area, sampling point number, date and the initials of the sampling persons.

3.3. Clean-up and extraction of water samples

The methods used to extract the target compounds from the water samples were based on methods by Ferrer and Thurman (2012).

Only the water samples (1 L) used for chemical analysis were spiked with 50 ng of the surrogate standard, deuterated (d-4) fluconazole (flu-d4) (Toronto Research Chemicals, Canada), to determine the extraction efficiency. As the extraction method was designed to target for pharmaceutical compounds, the flu-d4 was used as it ionises in positive and negative ionisation. The d-4 standard was used to combat the interference from the environmental matrices, where typical standards would not be sufficient. Samples were extracted with an automated solid phase extraction (SPE) process using the SPE-DEX 4790 system (Horizon Technology, Salem, NH, USA). This system uses time (and not volume) to standardise the volumes used for washing, and elution. The Atlantic HLB (hydrophilic-lipophilic balance) extraction disks (47 mm, Horizon Technology) were used because of their efficiency to extract analytes with various polarities and pH values (Yu and Wu, 2011). The HLB disks are available in low, medium, and high capacities. The low capacity was chosen as it is used in the EPA 1694 method for determining pharmaceuticals and personal care products in water, soil, sediment and biosolids by high performance liquid chromatography with tandem mass spectrometry (HPLC/MS/MS) (Englert, 2007). All solvents were of analytical grade.

The disks are created with 30 µm particle size sorbents. This allows most of the particles to be trapped on the disk. Smaller particles are likely to be trapped between the sorbent particles allowing for solutes trapped on them to be eluted.

The disks were conditioned twice with methanol (MeOH) with 30 second soak times, and 15 second dry time, followed by an additional 10 second methanol conditioning with a two second dry time. It was further conditioned twice with 18 mΩ H₂O with the first cycle having a 10 second soak time and a two second dry time, and the second cycle having a 10 second soak time and no dry time. The sample passed through the disk followed by a 15 minute air dry period. The target compound on the extraction disks were eluted three times with MeOH after a predetermined soak time. The target compounds were all soluble in MeOH (to different degrees) (Xu and Madden, 2011), and the analytes adsorbed to the fine particles in the sample would thus be eluted with the MeOH.

It was soaked twice with MeOH for three minutes followed by a dry time of 20 seconds, and with the third cycle soaked for 1 minute followed by a 1 minute air drying time in air. The eluent was concentrated to dryness under a gentle stream of nitrogen gas while heated at 35°C. The samples were then reconstituted in 500 µL MeOH. This meant the target compound was concentrated 2 000 times.

For biological analysis, the samples were extracted in the same manner as previously explained, without the addition of the internal standard, flu-d4. These samples were reconstituted in 500 µL ethanol (EtOH).

3.4. Instrumental analysis

An UPLC system coupled to a Q-TOF/MS was used for instrumental analysis. The instrument consisted of an Agilent 1290 liquid chromatograph coupled with an Agilent 6540 Accurate Mass Q-TOF/MS (Agilent Technologies, Santa Clara, CA, USA).

Liquid chromatography separation was performed at 25°C using a Poroshell 120 Bonus-RP column (Agilent, 2.1 x 100 mm, 2.7 µm). The flow rate of the mobile phase was 0.6 mL/min, with an injection volume of 1 µL and column temperature of 25°C. Electrospray ionisation (ESI) was used for mass spectrometric measurements. Due to the different polarities of each compound, each sample was analysed in positive and negative ionisation according to the optimal mobile phase gradient parameters.

Positive ionisation:

Nevirapine, ritonavir, lopinavir and flu-d4 were analysed in positive ionisation. The following gradient was applied using water and 0.05% formic acid (A) and acetonitrile and 0.05% formic acid (B) as solvents (Table 3.4).

Table 3.4: Liquid chromatography method parameters for positive ionisation.

Gradient (min)	A (%)	B (%)
0	90	10
8.5	90	10
8.6	50	50
13	50	50
13.3	0	100
14.3	0	100
15	90	10

Negative ionisation:

Efavirenz, zidovudine, didanosine and flu-d4 were analysed in negative ionisation. The following gradient was applied using water and 0.1% acetic acid (A) and acetonitrile and 0.1% acetic acid (B) as solvents (Table 3.5).

Table 3.5: Liquid chromatography method parameters for negative ionisation.

Gradient (min)	A (%)	B (%)
0	95	5
4.5	95	5
4.6	90	10
7	90	10
7.1	50	50
11	50	50
11.1	0	100
12.2	0	100

Agilent MassHunter Quantitative software was used for data processing.

Quality assurance and quality control

The sensitivity of the method was determined using the calibration curve. Linear regression statistics were used to calculate the LOD and LOQ. The LOD was set at a signal-to-noise (S/N) ratio of 3, and the LOQ was set at 10 (Schoeman et al., 2015). The LOD and LOQ is presented in Table 3.6 was the concentration detected by the instrument, before the concentration factor was added to the value.

A matrix matched calibration curve (spiked post extraction) was used to account for possible matrix effects during quantification. Artificial freshwater was created to attempt to mimic natural occurring water as closely as possible, 18 mΩ H₂O was supplemented with 0.7 mM sodium bicarbonate (NaHCO₃), 2 mM calcium chloride dehydrate (CaCl₂·2H₂O), 0.5 mM magnesium sulphate heptahydrate (MgSO₄·7H₂O) and 0.75 mM potassium chloride (KCl) (ISO, 2012). To match the organic content of the sampled matrix, 25 mature freshwater snails (*Bulinus tropicus*) were kept in 1 L of the prepared water for a minimum of 24 hours. The matrix matched water was made up of 5% snail-housed water and 95% prepared water. The calibrations were prepared by extracting 1 L of this matrix water, undergoing the same procedure as for the sampled water, evaporated with nitrogen gas and reconstituted in methanol, as well as spiked with the ARVs and flu-d4, for an end volume of 1 mL.

The calibration curve concentrations were based on expected levels of ARVs in the environment and the performance of the instrument (Table 3.6). When the calibration range was chosen, it was taken into account that the samples were concentrated 2 000 times during extraction.

The linearity of the calibration curve was determined by the R² value of the curve (Table 3.6). Linearity with R² as close to one as possible is desired. All compounds had linearity above 0.9.

Table 3.6: The linear range, linearity, limit of detection (LOD) and limit of quantification (LOQ) for each ARV compound detected (µg/L).

Compound	Linear range	Linearity	LOD (µg/L)	LOQ (µg/L)
Nevirapine	0, 0.1, 0.3, 0.8, 2.5, 7.6, 22, 68, 204, 612	0.999	280	932
Lopinavir	0, 0.9, 2.7, 8.1, 24, 72, 217, 652, 1957, 5873	0.998	1161	3871
Ritonavir	0, 1.48, 4.4, 13.3, 40, 120, 360, 1080, 3242, 9728	0.997	482	1607
Didanosine	0, 0.1, 0.3, 1, 3, 9, 27, 81, 244, 734	0.998	28	93
Efavirenz	0, 0.9, 2.7, 8, 24, 72, 217, 652, 1957, 5873	0.982	1012	3375
Zidovudine	0, 7, 21, 64, 193, 580, 1740, 5220, 15662, 46987	0.999	1004	3346

3.4.1. Statistical analysis

To determine if WWTPs significantly contribute to the increase of the respective ARVs downstream in the river, the levels of those ARVs of which levels could be quantified were compared between up- and downstream sites. These ARVs turned out to be efavirenz, nevirapine, lopinavir and ritonavir. The data was investigated for normality and when it was discovered that the data was not normally distributed, the Mann-Whitney U test was performed. A 95% confidence interval was used as statistical significance ($p < 0.05$).

Effect sizes were also calculated because it is an objective measure of the likelihood of a difference having a practical significance. Because the data was not normally distributed, effect sizes were calculated using the following formula: $r = |z|/\sqrt{n}$ where z is the z-statistic obtained from the non-parametric test Mann-Whitney test and n is the number of data points of both groups. The resulting effect size (r) can be interpreted as follows: $r = 0.1$ has a small likelihood of having a practical significance; $r = 0.3$ has a medium likelihood; and $r = 0.5$ has a large likelihood.

3.5. Biological analysis

The H4IIE-*luc* cells were donated by Professor John Giesy, currently at the University of Saskatoon, Canada.

All cultures were maintained in a sterile environment, with all work surfaces regularly cleaned with 70% ethanol. The H4IIE-*luc* cells were maintained in a humidified environment at 37°C under atmospheric conditions containing 5% CO₂ and cultured in Dulbecco's Modified Eagle's Medium (DMEM), containing L-glutamine, without phenol red (Sigma-Aldrich), containing 10% foetal bovine serum (FBS) (Biowest), and 0.04 M sodium bicarbonate (Sigma-Aldrich). The cells were grown until they were 90% confluent, by adding fresh media every 2–3 days. Once they were confluent, they were passaged. The cells were removed from the tissue culture dish using trypsin-EDTA (0.25% trypsin) (Biowest).

3.5.1. Bioassay procedure

The bioassay procedure was modified from Tillitt et al. (1991).

Day 1:

The reporter gene assays were performed in 96-well white walled micro-titer plates (Greiner bio-one) with flat transparent bottoms. The inner 60 wells were seeded with 250 µL cells per well at a density of 80 000 cells/mL (20 000 cells/well) (Figure 3.9).

The cell suspension used during seeding was prepared in hormone-free media, because the hormones could influence the response of the cells. The source of the hormones, the FBS, was treated with dextran coated charcoal to absorb any free hormones (Lindquist, 1987). The exterior wells received phosphate-buffered saline (PBS) (Sigma-Aldrich) to create a homogenous environment for cell growth across all cell containing wells. The plates were incubated for 24 h at 37°C in 5% CO₂.

Day 2:

Two different bioassays were performed, one for the extracted water samples and one for the single active compound of ARV treatment.

1. Extracted water samples

Six dilutions (three time dilution) were dosed as 100%, 33%, 11%, 3.7%, 1.2% and 0.4% of the raw extract. The cells were dosed in triplicate with 2.5 µL of the sample dilution series. Each plate also received the solvent control (SC) (to determine possible effects from the solvent, ethanol, used) in triplicate. A blank control (BC) was also added to each plate in triplicate and these contained only cells and media. The reference control standard 2,3,7,8-tetrachloro dibenzo-*p*-dioxin (TCDD) dissolved in ethanol was added to every second plate at 120, 30, 7.5, 1.9, 0.5, 0.1 pg TCDD/well (Figure 3.9). The plates were then incubated for a further 72 h at 37°C in 5% CO₂.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		TCDD 1	TCDD 1	TCDD 1	Blank Control	Sample A 100%	Sample A 100%	Sample A 100%	Sample B 100%	Sample B 100%	Sample B 100%	
C		TCDD 2	TCDD 2	TCDD 2	Blank Control	Sample A 33%	Sample A 33%	Sample A 33%	Sample B 33%	Sample B 33%	Sample B 33%	
D		TCDD 3	TCDD 3	TCDD 3	Blank Control	Sample A 11%	Sample A 11%	Sample A 11%	Sample B 11%	Sample B 11%	Sample B 11%	
E		TCDD 4	TCDD 4	TCDD 4	Ethanol	Sample A 3.7%	Sample A 3.7%	Sample A 3.7%	Sample B 3.7%	Sample B 3.7%	Sample B 3.7%	
F		TCDD 5	TCDD 5	TCDD 5	Ethanol	Sample A 1.2%	Sample A 1.2%	Sample A 1.2%	Sample B 1.2%	Sample B 1.2%	Sample B 1.2%	
G		TCDD 6	TCDD 6	TCDD 6	Ethanol	Sample A 0.4%	Sample A 0.4%	Sample A 0.4%	Sample B 0.4%	Sample B 0.4%	Sample B 0.4%	
H												

Figure 3.9: Typical plate layout of a 96 well plate for the bioassay. (TCDD = positive control, Ethanol = solvent control).

2. Active compounds of ARV treatment

The procedure was the same for the extracted water samples. The active ingredients efavirenz, lopinavir, nevirapine and ritonavir were tested for the activation of the AhR. Six concentrations: 0.8, 8, 80, 800, 8 000 and 80 000 ng/L were dosed in triplicate (Figure 3.10). The ARVs and the reference compound were dissolved in MeOH (SC).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		TCDD 1	TCDD 1	TCDD 1	Blank Control	Efavirenz A	Efavirenz A	Efavirenz A	Lopinavir A	Lopinavir A	Lopinavir A	
C		TCDD 2	TCDD 2	TCDD 2	Blank Control	Efavirenz B	Efavirenz B	Efavirenz B	Lopinavir B	Lopinavir B	Lopinavir B	
D		TCDD 3	TCDD 3	TCDD 3	Blank Control	Efavirenz C	Efavirenz C	Efavirenz C	Lopinavir C	Lopinavir C	Lopinavir C	
E		TCDD 4	TCDD 4	TCDD 4	Methanol	Efavirenz D	Efavirenz D	Efavirenz D	Lopinavir D	Lopinavir D	Lopinavir D	
F		TCDD 5	TCDD 5	TCDD 5	Methanol	Efavirenz E	Efavirenz E	Efavirenz E	Lopinavir E	Lopinavir E	Lopinavir E	
G		TCDD 6	TCDD 6	TCDD 6	Methanol	Efavirenz F	Efavirenz F	Efavirenz F	Lopinavir F	Lopinavir F	Lopinavir F	
H												

Figure 3.10: Typical plate layout of a 96 well plate for the ARV active compound bioassay. (TCDD = positive control, Methanol = solvent control).

Day 5:

The media was removed from the inner 60 wells and the cells were washed thrice with PBS containing Ca^{2+} and Mg^{2+} . This ensures that there is an excess of these ions, which are the limiting factors during the light production reaction of the luciferase assay. After all liquid was discarded, 25 μL of cell lysis buffer (Promega) was added to each well. The plates were stored at -80°C for a minimum of 30 mins to ensure complete lysis of the cell membranes. After which the cells were thawed and placed in a luminometer (Berthold, LB941) to read luminescence. Luciferase activating reagent (LAR) was added automatically to each well at a volume of 100 μL . The LAR consists of: 20 mM tricine (Sigma-Aldrich), 1.07 mM $\text{Mg}(\text{CO}_3)_4\text{Mg}(\text{OH})_2$ (Sigma-Aldrich), 2.67 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma-Aldrich), 0.1 mM EDTA-disodium salt (Sigma-Aldrich), 33.3 mM dithiothreitol (DTT) (Melford), 0.27 mM coenzyme A (Melford), 0.53 mM ATP (Melford), and 0.47 mM beetle luciferin (Promega) (Villeneuve et al., 1999). The bioluminescence was quantified as relative light units (RLU).

Data collection and calculation:

The RLU values were imported to Microsoft Excel (2010) for further calculations. The mean, standard deviation and coefficient of variation (CV) were calculated. The CV had to be less than 20% for quality assurance.

The RLUs of the samples were expressed in terms of the maximum response caused by the positive control and was referred to as %TCDD-max. Dose-response curves were created for the samples as well as the positive control by expressing the logarithm of the TCDD concentration (or volume of the sample per well) on the x-axis and the %TCDD-max on the y-axis.

The effective concentrations (EC) were calculated for the reference curve and the samples, by determining the concentrations (or volumes) responsible for 20%, 50% or 80% (EC₂₀, EC₅₀ and EC₈₀) of luminescence elicited by the cells using the straight line section of the graph. A minimum of three data points above the 15% response were required to create a valid straight line. The ECs were used to determine the relative potency values (REP) of each sample. This was assessed as the ratio of the EC₂₀-80 of the reference over the EC₂₀-80 of the sample. Due to the samples having a small efficacy (most only reached EC₂₀), the EC₂₀ was used to determine the biological activity equivalents (BEQs).

$$REP_{20} = \frac{EC_{20} \text{ TCDD (pg/}\mu\text{L)}}{EC_{20} \text{ SAMPLE (}\mu\text{L)}}$$

The LOD value was calculated using the mean of the EC₀ values (where no response was elicited) of all TCDD dose response curves. The 95% confidence interval was added to the average to obtain a ngTCDD/L LOD value (Nieuwoudt et al., 2009).

Viability assay

The viability assay was run in parallel to the luminescence assay and exposed to the same concentrations of samples and standards. Cytotoxicity was determined using a MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) viability assay. This assay is based on the conversion of yellow tetrazolium salt into an insoluble blue/purple formazan product by metabolically active cells (Mosmann, 1983; Van Meerloo et al., 2011). This assay was performed to prevent false negative results and to ensure that the cells were viable during exposures.

For the MTT viability assay, cells were seeded in clear 96-well plates (TPP) and followed the same procedure as the luminescence assay. On the fifth 100 μ L MeOH, was added to the negative controls, after which all wells received 100 μ L MTT solution at a concentration of 0.5 mg/mL (Žegura et al., 2009). The plates were then incubated for at 37°C, 5% CO₂ for 30 mins. The MTT solution was discarded and the plate washed thrice with PBS without magnesium and calcium salts, after which 200 μ L dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. After a further 30 mins (where the plates were kept at room temperature), the optical density (OD) was measured at 560 nm (Berthold, TriStar LB941). The OD was directly proportional to the amount of living cells in the wells (Carmichael et al., 1988).

Chapter 4: Results

The results of the instrumental analysis for surface water and drinking water samples, as well as the AhR activity of the surface water extracts are presented below.

4.1. Instrumental analysis

The concentrations of different ARVs for surface water are summarised in Table 4.1. The most frequently detected compound was nevirapine with 19 detections out of 20 sites. Lopinavir and efavirenz also had high detection frequencies with 17 and 16 detections respectively. Ritonavir was detected at 12 sites. Zidovudine and didanosine were the least detected compounds with 5 and 1 out of 20 respectively (Table 4.1).

Despite its high prevalence at the sampling sites, nevirapine concentrations ranged from 0.04 ± 0.004 (BG DS) to 1.30 ± 0.22 $\mu\text{g/L}$ (ZG US1), which were very low concentrations in comparison with the other compounds detected. Lopinavir concentrations measured ranged from 0.80 ± 0.19 (WV DS2) to 38.45 ± 2.51 $\mu\text{g/L}$ (OF DS2). Ritonavir ranged from 0.64 ± 0.05 (SR US) to 1.95 ± 0.34 $\mu\text{g/L}$ (OF DS1). Efavirenz concentrations ranged from 0.85 ± 0.43 (ZG DS1) to 24.60 ± 6.44 $\mu\text{g/L}$ (OF DS1). Zidovudine was only quantified at Zeekoegat US2 (3.68 ± 0.38 $\mu\text{g/L}$) (Figure 4.1A-E). Didanosine could not be quantified at any site, and was only found below LOQ at Zeekoegat DS1 (Table 4.1).

Olifantsfontein US, -DS1 and -DS2, Sunderland Ridge US and -DS, Flip Human DS and Zeekoegat US2 had the highest concentrations of ARVs of all the surface water samples. Olifantsfontein DS1 had the highest concentrations of ritonavir and efavirenz (Figure 4.1C and Figure 4.1D), and Olifantsfontein DS2 had the highest levels of lopinavir found in this study (Figure 4.1B). Efavirenz and lopinavir were usually the ARVs with the highest concentrations of all those analysed at each site (Figure 4.1).

Table 4.1: Concentration (µg/L) for six antiretrovirals at various surface water locations across Gauteng. (LOD = limit of detection; LOQ = limit of quantification; US = upstream; DS = downstream). Standard deviation is also presented. The highest concentration for each compound is indicated in bold. Results were corrected in term of recovery of the sample.

Sample site	Recovery (%)	Drug concentration (µg/L)						Total ARV concentration
		Nevirapine	Lopinavir	Ritonavir	Efavirenz	Zidovudine	Didanosine	
OF US	22	0.41 ± 0.11	24.35 ± 5.22	1.69 ± 0.81	15.04 ± 6.62	<LOQ	<LOD	41.5
OF DS1	31	0.46 ± 0.05	26.84 ± 4.65	1.95 ± 0.34	24.59 ± 6.44	<LOQ	<LOD	53.8
OF DS2	20	0.42 ± 0.11	38.45 ± 2.51	1.90 ± 0.85	19.96 ± 9.57	<LOQ	<LOD	60.7
SR US	19	0.09 ± 0.02	8.55 ± 0.50	0.64 ± 0.05	10.18 ± 6.38	<LOD	<LOD	19.5
SR DS	44	0.32 ± 0.05	33.63 ± 2.32	1.77 ± 1.11	<LOD	<LOD	<LOD	35.7
VP US	85	0.22 ± 0.01	1.85 ± 0.03	<LOD	1.98 ± 1.30	<LOD	<LOD	4.1
VP DS1	73	0.23 ± 0.03	5.90 ± 0.52	<LOQ	4.61 ± 1.79	<LOD	<LOD	10.8
VP DS2	81	0.15 ± 0.02	0.91 ± 0.02	<LOD	1.43 ± 0.71	<LOD	<LOD	2.5
WV US	88	<LOQ	<LOQ	<LOD	<LOQ	<LOD	<LOD	-
WV DS1	81	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	-
WV DS2	90	0.08 ± 0.02	0.80 ± 0.20	<LOD	1.17 ± 1.00	<LOD	<LOD	2.1
WG US	85	0.08 ± 0.01	<LOD	<LOD	<LOD	<LOD	<LOD	0.1
WG DS1	83	0.18 ± 0.01	5.35 ± 0.18	<LOQ	7.28 ± 3.38	<LOD	<LOD	12.8
WG DS2	63	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	-
ZG US1	30	1.30 ± 0.22	5.10 ± 0.88	<LOQ	1.05 ± 0.60	<LOD	<LOD	7.5
ZG US2	11	0.30 ± 0.03	20.11 ± 3.83	1.36 ± 0.34	20.41 ± 2.94	3.68 ± 0.38	<LOD	42.2
ZG US3	25	0.20 ± 0.03	10.23 ± 0.21	<LOQ	1.46 ± 0.80	<LOD	<LOD	11.9
ZG DS1	38	0.11 ± 0.00	1.17 ± 0.79	<LOD	0.85 ± 0.43	<LOD	<LOQ	2.1
FH US	28	0.10 ± 0.01	5.50 ± 1.01	<LOQ	2.19 ± 0.62	<LOD	<LOD	7.8
FH DS	26	0.42 ± 0.08	15.25 ± 3.03	1.05 ± 0.10	20.29 ± 8.43	<LOQ	<LOD	37.0
Average		0.30	12.75	1.48	8.83	3.68	<LOQ	
Detection frequency (%) (n=20)		95	85	60	80	25	5	

Comparing upstream concentrations of efavirenz, nevirapine, lopinavir and ritonavir to the downstream levels resulted in no significant differences between up- and downstream ($p > 0.05$), and the practical significant tests also resulted in small likelihoods (Table 4.2) that the differences could be meaningful. However, the type II errors for these data sets were large (Table 4.2) which means that no conclusions regarding the role of WWTPs in contributing to higher downstream levels of ARVs can be done at this stage. More data points are necessary.

Table 4.2: Summary of up- and downstream concentrations of ARVs that occurred at quantifiable levels and the outcome of the statistical comparison.

	Efavirenz		Nevirapine		Lopinavir		Ritonavir	
	Upstream (ug/L)	Downstream (ug/L)	Upstream (ug/L)	Downstream (ug/L)	Upstream (ug/L)	Downstream (ug/L)	Upstream (ug/L)	Downstream (ug/L)
	15.04	24.59	0.41	0.46	24.35	26.84	1.69	1.95
	10.18	0	0.09	0.32	8.55	33.63	0.64	1.77
	1.98	4.61	0.22	0.23	1.85	5.9	0	0
	0	0	0	0	0	0	0	0
	0	7.28	0.08	0.18	0	5.35	0	0
	2.19	20.29	1.1	0.42	5.5	15.23	0	1.05
	1.05	19.96	1.3	0.42	5.1	38.45	0	1.9
	20.41	1.43	0.3	0.15	20.11	0.91	1.36	0
	1.46	1.17	0.02	0.08	10.23	0.8	0	0
		0		0		0		0
		0.85		0.11		1.17		0
n	9	11	9	11	9	11	9	11
Mann-Whitney (p)		>0.05		>0.05		>0.05		>0.05
Mann-Whitney (z)		-0.38		-0.152		-0.114		-0.49
Effect size r		0.08		0.03		0.03		0.11
β^*		0.935		0.81		0.914		0.918

*Beta is the probability of making a type II error, i.e. assuming that there is no difference between the levels of the up- and downstream sites

Seven DW samples were analysed for the presence of ARVs (Table 4.3). Most compounds in the drinking water (DW) samples were below LOD, except for Zeekoegat DW (nevirapine 0.2 µg/L; lopinavir 9.0 µg/L; efavirenz 2.3 µg/L; ritonavir <LOQ), Welgedacht DW2 (nevirapine 0.04 µg/L) and Olifantsfontein DW (lopinavir <LOQ) (Table 4.3). Out of 25 samples in this entire study lopinavir and efavirenz had the 9th highest concentration in ZG DW (Figure 4.1B and Figure 4.1D), and nevirapine had the 10th highest concentration at ZG DW (Figure 4.1A), which was quite high (especially when drinking water was expected to be a clean sample). Levels of ARVs in drinking water were generally low, with ZG DW being the exception.

Table 4.3: Concentration (µg/L) for six antiretrovirals at various drinking water (tap water) locations across Gauteng. (LOD = limit of detection; LOQ = limit of quantification; DW = drinking water). Standard deviation is also presented. The highest concentration for each compound is indicated in bold. Results were corrected in term of recovery of the sample.

Sample site	Recovery (%)	Drug concentration (µg/L)						Total ARV concentration
		Nevirapine	Lopinavir	Ritonavir	Efavirenz	Zidovudine	Didanosine	
OF DW	44	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	-
VP DW1	78	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	-
VP DW2	92	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	-
WV DW1	93	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	-
WV DW2	79	0.04 ± 0.05	<LOD	<LOD	<LOD	<LOD	<LOD	0.1
WV DW3	92	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	-
ZG DW	45	0.21 ± 0.04	9.04 ± 2.45	<LOQ	2.33 ± 1.65	<LOD	<LOD	11.6
Average		0.13	9.04	-	2.33	-	-	
Detection frequency (%) (n=7)		29	29	14	14	0	0	

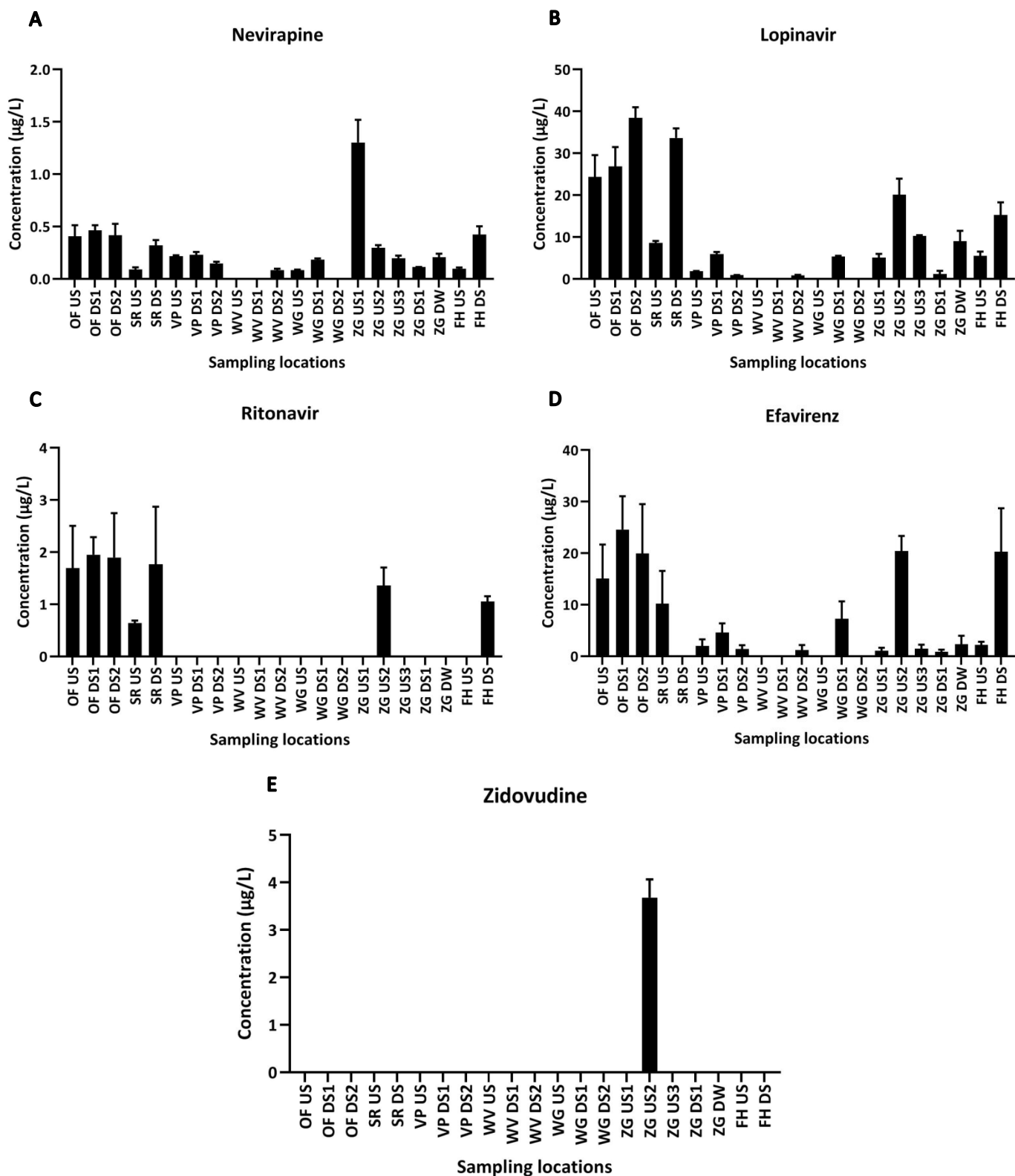


Figure 4.1: Concentrations (µg/L) of nevirapine (A), lopinavir (B), ritonavir (C), efavirenz (D) and zidovudine (E) in terms of the sampling locations. (Only the Zeekoegat drinking water sample was included as it was the only DW sample with high ARV levels) (OF = Olifantsfontein; SR = Sunderland Ridge; VP = Vlakplaats; WV = Waterval; WG = Welgedacht; ZG = Zeekoegat; FH = Flip Human; US = upstream; DS = downstream; DW = drinking water).

4.2. Luciferase bioassay

4.2.1. Cell viability

The cytotoxic effects of the extracted water samples on the H4IIE-*luc* cells were evaluated by the MTT assay. This assay determined the viability of cells in terms of the ability to convert tetrazolium salt into water soluble formazan product. The viability of the majority of the samples was above 80%, viability below 80% was an indication of cytotoxicity, i.e. the sample extract hampered the viability of the cells (López-García et al., 2014). Cells exposed to SR US, OF US, OF DS2 and WG DS1 had exceptionally low viability which ranged from 0% to 5.7%, WV DS2 and OF DS1 also had a low viability of 30.6% and 69.5% (Figure 4.2). All raw extracts from Olifantsfontein showed cytotoxicity.

These low viabilities can be attributed to the mixtures of compounds present in the concentrated sample. Lower cell viability can lead to reduced luciferase response that was not due to the compounds in the extract inhibiting the AhR but rather its toxicity. Lowered cell viability would also have decreased the amount of luminescence in the event of AhR agonists together with moderate cytotoxicity. The cell viabilities reported in Figure 4.2 are for the highest exposure concentration.

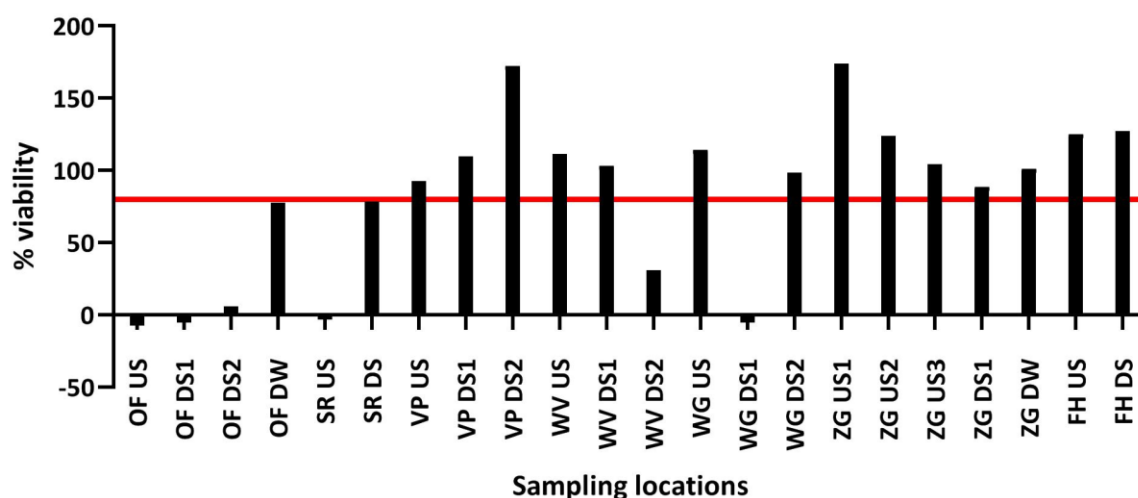


Figure 4.2: Cell viability results of highest exposure concentration of the water extracts for the H4IIE assay. The red line indicates 80% viability (OF = Olifantsfontein; SR = Sunderland Ridge; VP = Vlakplaats; WV = Waterval; WG = Welgedacht; ZG = Zeekoegat; FH = Flip Human; US = upstream; DS = downstream).

Some water samples exceeded 100% viability, with ZG US1 and VP DS2 having the highest (174% and 171%, respectively) (Figure 4.2). The viability of the solvent control, ethanol in this case, represents 100% viability. These high viability values were an indication of compounds in the extracts to which the cells responded to and which caused increased cell growth, i.e. possibly enhancing cancer growth.

4.2.2. Reporter gene bioassay

The ability of the environmental extracts to act as agonists of the AhR was determined using the H4IIE bioassay. The response was quantified as %TCDDmax, the percentage relative to the maximum TCDD (positive control) response. Bioassay derived toxic equivalents (TCDD-eq) were determined based on the REP calculated with the dose response of both the standard (TCDD) and the sample. The REP values were back calculated to the volume of water initially extracted.

The raw extracted samples varied in response depending on the sampling location, ranging from 6 to 38 %TCDDmax. All sites of Olifantsfontein (US, DS1 and DS2) had a %TCDDmax response above 20, with 22%, 33% and 21%, respectively (Table 4.4). These raw extracts were also extremely cytotoxic to the cells, which may have caused a lowered luminescence response. Sunderland US had a response of 20%, whereas SR DS were <LOD. Waterval DS2 barely reached the 20% response mark and this was the lowest of the samples that showed any agonistic AhR response. Flip Human DS had the highest response of 38%, and also had a high cell viability. Unfortunately, despite this clear evidence of AhR activation, the dose-response curve was such that an EC20 could not be quantified (see section Data collection and calculation in section 3.5.1). Flip Human DS had a definitive response to the raw extract, but an EC20 could not be determined and therefore their responses were indicated as <LOQ (Figure 4.3). The TCDD-eq can only be quantified when the linear part of the dose response curve has three points above 15% TCDDmax.

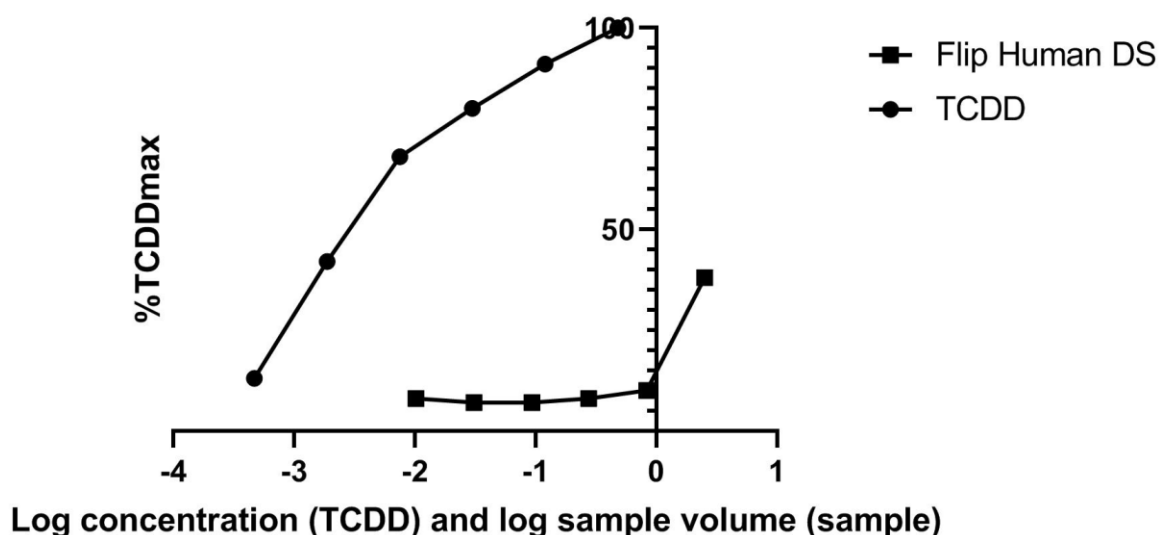


Figure 4.3: The dose-response curve of TCDD compared with the dose-response curve of Flip Human DS.

The single active compounds of the ARVs (efavirenz, lopinavir, ritonavir and nevirapine) had no AhR-mediated activity up to the highest tested concentration (80 000 ng/L) (data not shown). TCDD created a dose-response curve.

Table 4.4: The %TCDDmax response of each extracted sample, their toxic equivalents (pg TCDD-eq/μL) with the back calculated value (per L) and the cell viability (%). (LOD = limit of detection; LOQ = limit of quantification; US = upstream; DS = downstream; DW = drinking water).

Samples		%TCDDmax	REP20 (pg TCDD-eq/μL)	% viability
Olifantsfontein	US	22	<LOQ	72
	DS1	33	<LOQ	24
	DS2	21	<LOQ	6
	DW	11	<LOD	78
Sunderland Ridge	US	11	<LOQ	95
	DS	15	<LOD	78
Vlakplaats	US	7	<LOD	93
	DS1	7	<LOD	110
	DS2	8	<LOD	171
Waterval	US	12	<LOD	111
	DS1	14	<LOD	102
	DS2	19	<LOQ	31
Welgedacht	US	9	<LOD	113
	DS1	8	<LOD	87
	DS2	9	<LOD	99
Zeekoegat	US1	8	<LOD	174
	US2	7	<LOD	124
	US3	7	<LOD	104
	DS1	10	<LOD	87
	DW	9	<LOD	100
Flip Human	US	8	<LOD	124
	DS	38	<LOQ	127

A majority of the samples were reported as being below LOD; this indicates that there was no detectible decrease/increase in RLUs. The %TCDDmax of these samples was all below 15% (Table 4.4). Vlakplaats, Welgedacht and Zeekoegat samples were all <LOD. The response from the extracted samples was not high enough to elicit an EC50 or EC80 value, thus the EC20 value was only used when available.

The high levels of ARVs found in the surface water samples through instrumental analysis (Table 4.1), were expected to elicit some biological response, but some samples had no responses in the biological assay, while some had only a slight response (<LOQ) (Table 4.4).

Chapter 5: Discussion

This chapter first features the individual wastewater treatment plants and this is followed by a comparison of the results of this study to South African as well as global levels for ARVs in the environment. The biological responses and the meaning thereof are discussed at the end.

5.1. Wastewater treatment plants (instrumental and biological analysis)

A majority of the compounds had higher concentrations downstream of the wastewater treatment plants (WWTP) when compared with upstream sites. The most frequently detected compounds nevirapine, lopinavir and efavirenz had higher concentrations at the downstream sites of all WWTPs, except for the Sunderland Ridge WWTP where efavirenz had a higher concentration upstream (Figure 4.1). Ritonavir had higher levels downstream for all the sites where it was detected (Olifantsfontein, Sunderland Ridge, Welgedacht and Flip Human). However, none of the differences between up- and downstream levels were statistically significant for any of the compounds at any of the WWTPs. Didanosine was not detected at any sites.

In most of the sampling locations in this study, lopinavir was found at the highest levels in this study, followed by efavirenz (Figure 5.1). Nevirapine was the most frequently detected but at very low concentrations compared with lopinavir and efavirenz (Figure 5.1). WV US, WV DS1 and WG DS2 sample had no quantifiable ARV concentrations (Table 4.1 and Figure 5.1). The Kaalspruit and Olifantspruit into which the Olifantsfontein WWTP discharges its effluent (Figure 3.6) and the Pienaars River that flows into the Roodeplaat Dam was the most polluted river areas in this study. This was shown in Figure 5.1, where OF US, -DS1 and -DS2, and ZG US2 had the highest total ARV concentrations. The Olifantsfontein, Sunderland Ridge and Flip Human WWTP contributed the most to the levels of ARVs into the receiving water (the downstream total ARV concentration was higher than upstream) (Figure 5.1).

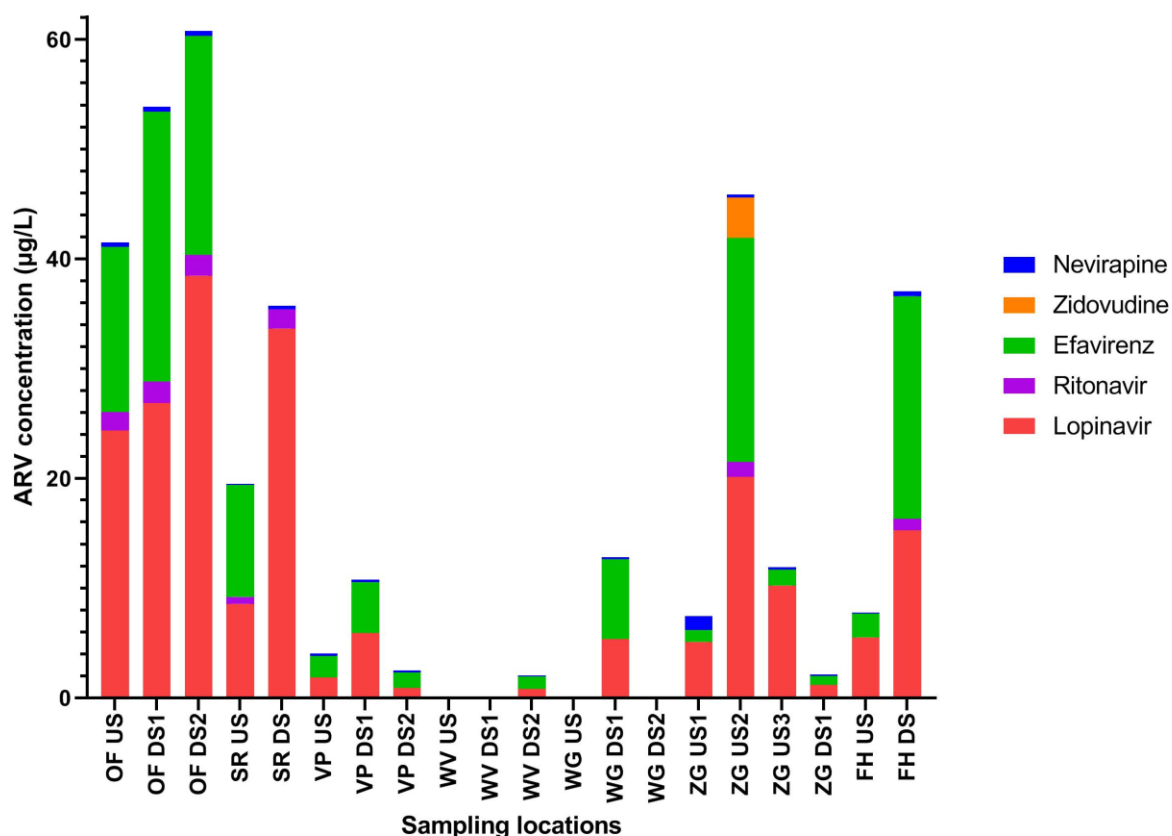


Figure 5.1: Total ARV concentrations grouped for each different sampling site (only the Zeekoegat drinking water sample was included as it was the only DW sample with high ARV levels) (OF = Olifantsfontein; SR = Sunderland Ridge; VP = Vlakplaats; WV = Waterval; WG = Welgedacht; ZG = Zeekoegat; FH = Flip Human; ZG = US = upstream; DS = downstream).

To determine the possible impact of WWTPs on the levels of ARVs found in the river, the upstream and downstream sites were compared with each other. When the downstream levels are higher than those found in the upstream sites, it shows that the WWTP acts as a point source of ARVs in the receiving river. Some of the WWTPs are at high risk to discharge untreated sewage into the receiving waters and these plants can be responsible for releasing a larger amount of pollutants as a whole into the rivers, contributing to the high levels found for efavirenz, lopinavir and ritonavir (particularly downstream of the plant) in this study. Due to the large number of informal settlements located along the banks of the studied rivers, their possible influence as a non-point source of ARVs should also be noted because wastewater often runs unhindered from them directly into the river. The prevalence of HIV in informal townships are generally higher than in formal areas (Shisana et al., 2005). This may result in a higher usage of ARVs in these areas. As these communities often lack proper sanitation services, sewage (containing ARVs) can overflow into the streets and enter the rivers through surface run-off. The WWTPs that service these areas are also under much pressure due to the large sudden increase in population.

5.1.1. Olifantsfontein

The Olifantsfontein WWTP had a no-risk Green Drop status (DWA, 2011), implying that this plant was not expected to release a large quantity of pollutants into receiving waters. The effluent from this plant is released into the Kaal Spruit that eventually runs into the Hennops River.

The concentrations of nevirapine, lopinavir, ritonavir and efavirenz were higher at downstream sites, than in the upstream sites (Figure 5.2). This indicates that the Olifantsfontein WWTP probably contributed to the presence of these compounds in the Kaal Spruit into which the effluent is released. All compounds detected, except for lopinavir, showed a decrease in levels from DS1 to DS2, indicating that these compounds likely do not have any additional sources releasing it into the river between the two sites. Lopinavir had a steady increase in concentrations from US to DS2, and this could indicate a possible source of this compound into the river before the DS2 site (Figure 5.2). Something similar happened downstream of the Waterval WWTP, but not at Vlakplaats and Welgedacht WWTPs, all of which had more than one downstream site (Table 4.1).

Upstream concentrations of lopinavir and efavirenz were also very high, this can likely be contributed to the densely populated Tembisa and Ivory Park informal settlements that are located near the river (Figure 3.6). The population of Tembisa is much greater than that of Ivory Park, but the latter has twice the population density (StatsSA, 2011a; StatsSA, 2011d). The infrastructure for Ivory Parks sanitation system is in a poor state and is under increasing pressure to serve the large community (Hoffmann, 1995). Lack of adequate infrastructure can lead to sewage system overflows, which would result in the release of untreated sewage into the rivers through surface run-off. The concentrations of ARVs can be expected to be higher at the upstream site than normally expected.

The high overall concentrations of ARVs can possibly be attributed to the high prevalence of HIV in the Ekurhuleni Metropolitan area. According to Shisana et al. (2014) this is one of the cities, together with eThekweni in KwaZulu-Natal, that has the highest HIV infected population. The WWTP is operated by the Ekurhuleni Metropolitan Municipality (Table 3.1). The Olifantsfontein plant is under pressure to treat the high volumes of sewage from the informal settlements mentioned above. This can lead to an increase in the overall ARV levels in the WWTP effluent that was released into the Kaal Spruit. Figure 5.1 shows that the Kaal- and Olifant Spruit has high total levels of ARVs compared with others in this study, indicating that these two river sections is heavily polluted.

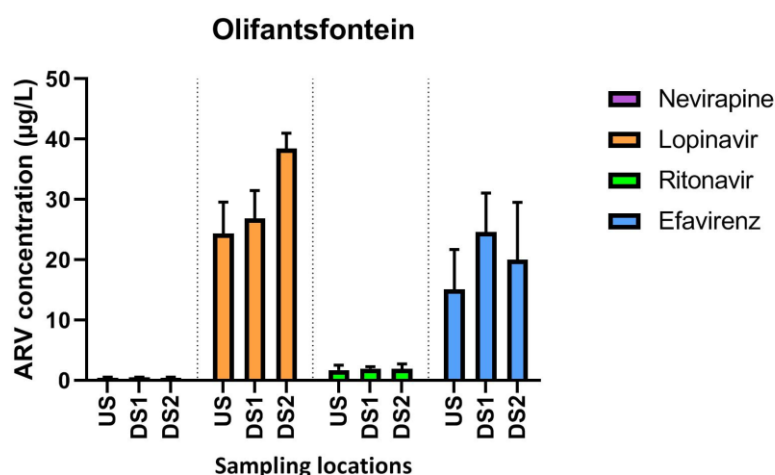


Figure 5.2: Grouped concentrations of ARVs based on location within Olifantsfontein sampling area. Bars indicate standard deviation (US = upstream; DS = downstream).

Extreme cytotoxicity can be seen for the raw extracts of US and DS1 (0% viability) (Table 4.4), indicating that if these water sample (1 L) was concentrated 2 000 times, there were cytotoxic effects. The cytotoxicity influenced the %TCDDmax response of the two samples; if it were not cytotoxic there could have been an even higher %TCDDmax (Figure 5.3). The DS2 sample was not as cytotoxic as the other samples, but still had a very low viability (6%). It was evident that this sample could generate a response to the raw extract, but the response was possibly altered by the low cell viability. Overall, the US and DS1 samples had a higher response than the DS2, but raw extract response was influenced by their influence on cell viability (Figure 5.3).

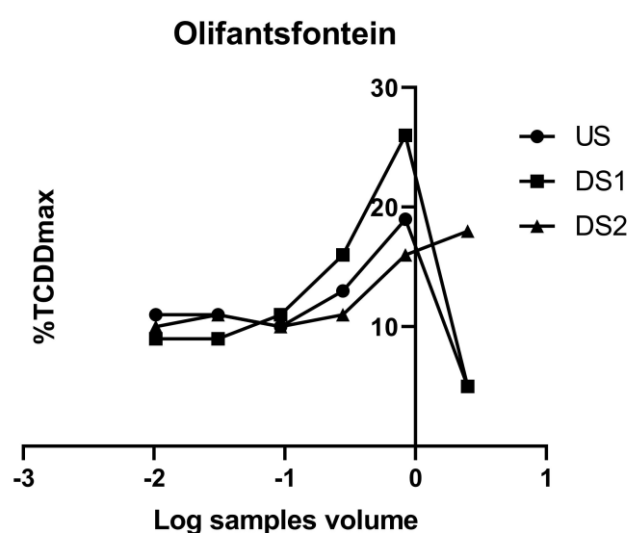


Figure 5.3: Dose response curves of Olifantsfontein sampling area expressed as %TCDDmax (US = upstream; DS = downstream).

The total concentrations of ARVs in the Olifantsfontein samples were: for the US $\Sigma 41.5 \mu\text{g/L}$, for DS1 $\Sigma 53.8 \mu\text{g/L}$ and for DS2 $\Sigma 60.7 \mu\text{g/L}$ (Table 4.1). Except for Zeekoegat US2 ($\Sigma 42.2 \mu\text{g/L}$), these three sites had the highest total concentrations of ARVs for the entire study. This can mean that the levels of possible polar AhR ligands were also higher in these samples, which would explain the high biological responses of the diluted extracts of US and DS1 (Figure 5.3). The extremely low cell viability of the cells exposed to these samples at US and DS1 can also be explained by these high concentrations.

5.1.2. Sunderland Ridge

The Sunderland Ridge plant is designated to be at risk, due to its low Green Drop status (DWA, 2011). It was expected that this plant would contribute to the levels of ARVs in the water. The downstream sample had higher levels of nevirapine, lopinavir and ritonavir when compared with the US site (Figure 5.4) (nevirapine with $0.32 \mu\text{g/L}$ and $0.09 \mu\text{g/L}$; lopinavir with $33.63 \mu\text{g/L}$ and $8.55 \mu\text{g/L}$; ritonavir with $1.77 \mu\text{g/L}$ and $0.64 \mu\text{g/L}$) (Figure 5.1). There was a notable increase of lopinavir between the US and DS sites. Contradictory to lopinavir, efavirenz was detected at the upstream site only, indicating that the WWTP was not contributing to, at least, efavirenz levels already in the receiving waters (Figure 5.4). Robson et al. (2017) also found a higher efavirenz level upstream of a WWTP ($0.01 \mu\text{g/L}$) than downstream ($<\text{LOQ}$).

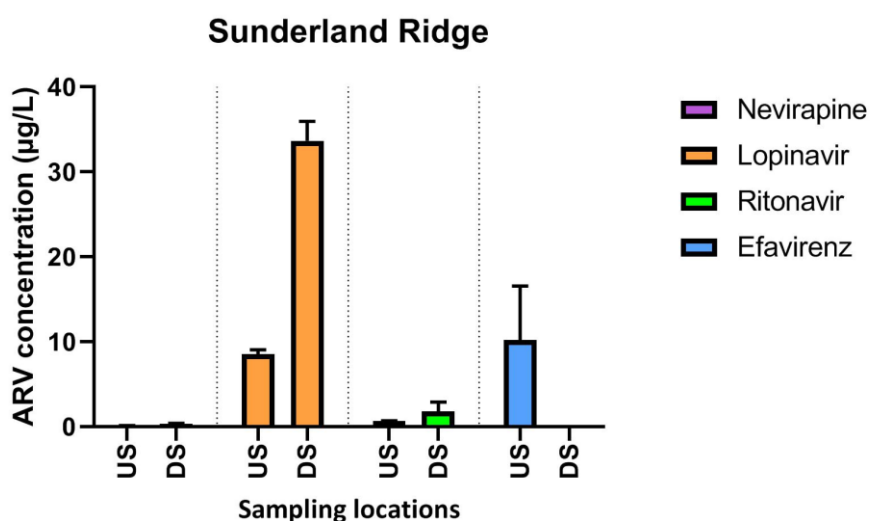


Figure 5.4: Grouped concentrations of ARVs based on location within Sunderland Ridge sampling area. Bars indicate standard deviation (US = upstream; DS = downstream).

The cell viability of SR US and DS differed considerably, with an exceptionally low viability with the US sample (0%) and a moderate viability at DS (78%) (Table 4.4). The US sample response were $<\text{LOQ}$, where the DS sample was $<\text{LOD}$. The US sample had a higher response than the DS (Figure 5.5).

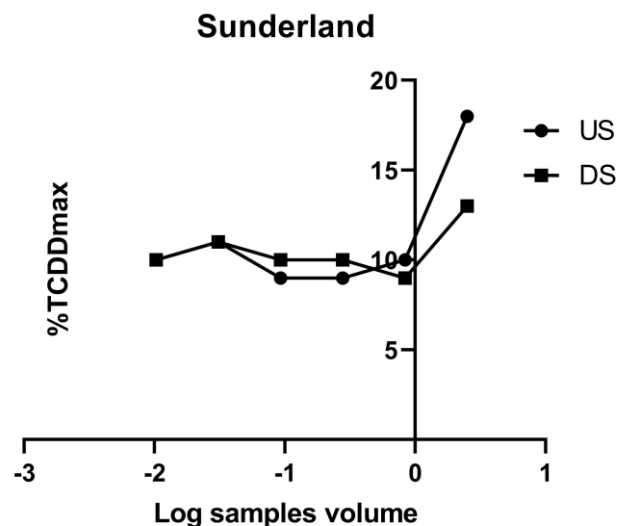


Figure 5.5: Dose response curves of Sunderland Ridge sampling area expressed as %TCDDmax (US = upstream; DS = downstream).

The US site had a $\Sigma 19.5 \mu\text{g/L}$ compared with the DS with $\Sigma 35.7 \mu\text{g/L}$ (Table 4.1), indicating that there was a higher level of ARVs (and possibly other unknown compounds) in the DS site. Because of this situation the biological activity was expected to be higher for the DS, but this was not the case (Figure 5.5).

5.1.3. Vlakplaats

The Vlakplaats WWTP was expected to have some input of ARVs into the receiving water, due to the large capacity of the plant as well as the informal settlements whose sewage it treats. The plant does however have an adequate Green Drop status (DWA, 2011), classifying it at no-risk.

The first downstream (DS1) site had higher concentrations of nevirapine, lopinavir and efavirenz than US and DS2. The upstream site (US) had higher concentrations than DS2 (but still lower than DS1) for all compounds detected (Figure 5.6).

The Dekema WWTP is situated just upstream of the Vlakplaats WWTP and discharges the effluent into the Natal Spruit above the US site of the Vlakplaats plant (Figure 3.2). This situation might explain the levels of nevirapine, lopinavir and efavirenz already present at the upstream site of VP. The second downstream (DS2) site is located six km from the WWTP. The river runs along the edges of Kathlehong and Vosloorus, and the former has a big population, but it seems that these townships do not have a large contribution to the levels of ARVs into the surface waters. The Vlakplaats WWTP contributed to the higher overall ARV concentration at the downstream site (Figure 5.1).

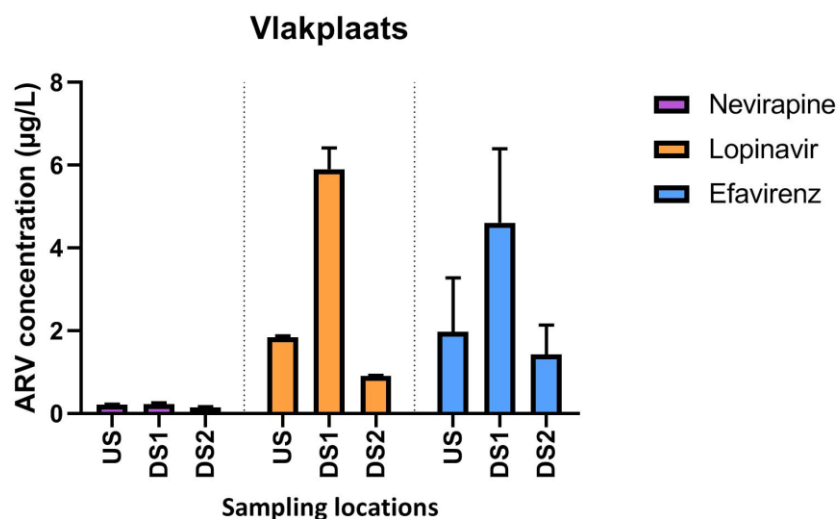


Figure 5.6: Grouped concentrations of ARVs based on location within Vlakplaats sampling area. Bars indicate standard deviation (US = upstream; DS = downstream).

There was no AhR response to any of the samples and the cells had a high viability (>80%) (Table 4.4), indicating that the lack of light (AhR activation) was due to the absence of AhR ligands and not cytotoxicity (Figure 5.7).

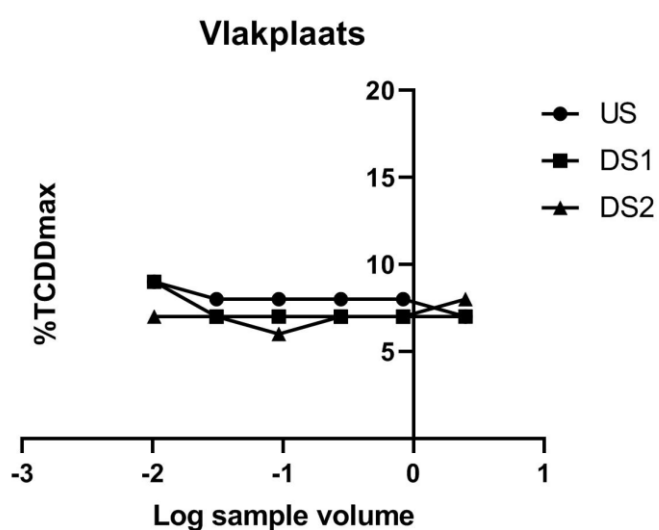


Figure 5.7: Dose response curves of Vlakplaats sampling area expressed as %TCDDmax (US = upstream; DS = downstream).

Vlakplaats had generally low total ARVs, with US at $\Sigma 4.1 \mu\text{g/L}$, DS1 at $\Sigma 10.8 \mu\text{g/L}$ and DS2 at $\Sigma 2.5 \mu\text{g/L}$ (Table 4.1).

5.1.4. Waterval

The Waterval plant has a no-risk Green Drop status (DWA, 2011), the impact of this plant to the receiving waters was expected to be minimal.

There were no compounds quantified in the US or DS1 sites. All concentrations at DS1 were below LOD, while nevirapine, lopinavir and efavirenz were <LOQ at the US site (Table 4.1). DS2 had the highest concentration for nevirapine, lopinavir and efavirenz for the sampling area (Figure 5.8). The higher concentrations at DS2 could possibly be due to the use of sludge as fertiliser for agricultural purposes upstream of the site (Envirolution Consulting, 2014). Sludge can still contain traces of pharmaceuticals, that can enter surface water through leaching and surface run-off (Ebele et al., 2017). Some pharmaceuticals have been detected in dewatered sludge (used for agricultural purposes) by Dong et al. (2016) and Yu et al. (2011) in China and Magnér et al. (2016) in Sweden. ARVs (as part of pharmaceuticals) can potentially also enter the aquatic environment through this manner. Another possible source of the quantified ARVs at DS2 can be from the Natal Spruit that joins the Klip River upstream from DS2, and the Natal Spruit carries effluent from the Vlakplaats WWTP. Vlakplaats DS2 had slightly higher levels of nevirapine, lopinavir and efavirenz when compared with WV DS2 (Table 4.1).

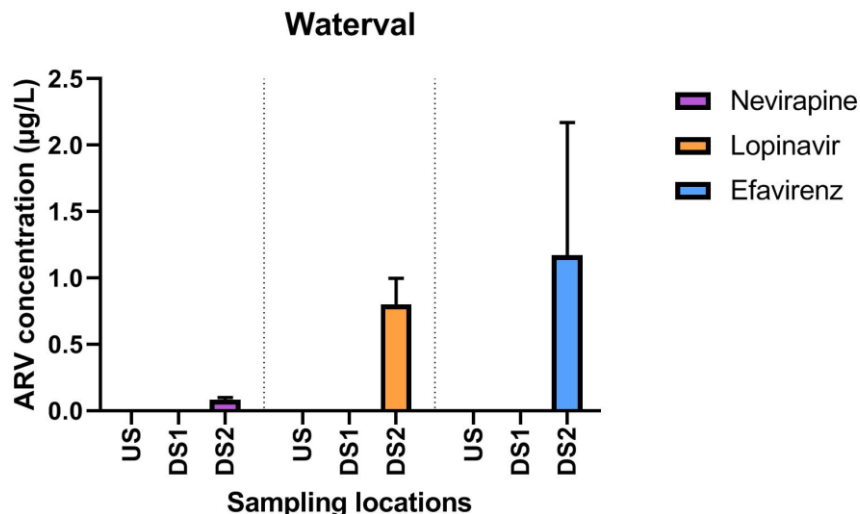


Figure 5.8: Grouped concentrations of ARVs based on location within Waterval sampling area. Bars indicate standard deviation (US = upstream; DS = downstream).

Waterval DS2 was the only sample that had a response with a 19% TCDDmax, but the viability of this sample was also lower (31%), when compared with US and DS1 (>100%) (Table 4.4). The response of DS2 could potentially be higher, if not for the low cell viability. DS1 and DS2 were <LOD, whereas US was <LOQ (Figure 5.9).

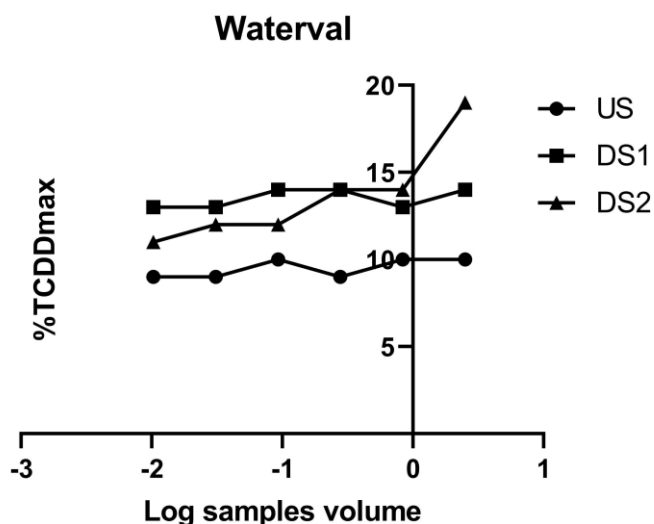


Figure 5.9: Dose response curves of Waterval sampling area expressed as %TCDDmax (US = upstream; DS = downstream).

Waterval DS2 was the only sample that had quantifiable levels of ARVs which might explain the biological activity, indicating slight activation of the AhR.

The total ARVs for US and DS1 could not be determined as nothing could be quantified. DS2 had a total ARV concentration of $\Sigma 2.1 \mu\text{g/L}$ (Table 4.1), which was very low, indicating that another unknown compound might be present in the environment, which caused the higher biological response.

5.1.5. Welgedacht

The concentrations of ARVs in this sampling area were expected to be lower than at any of the other sampling sites due to the natural wetland formed by the Blesbok Spruit (Figure 3.4) that can filter out pollutants from the water. Because the Welgedacht WWTP is also one of the smaller plants in this study, the input of ARVs into the river was expected to be lower compared with the others (Table 3.1).

Of the two downstream sites, nevirapine, lopinavir and efavirenz were quantified at only DS1 (Table 4.1). Nevirapine was the only compound also quantified the US site (Figure 5.10). The drastic decrease at DS2 in levels of the compounds could be explained by the inflow of the Cowles Dam, which drains into the Blesbok Spruit upstream of DS2 (Figure 3.4).

Although this is one of the smaller WWTPs in this study, the contribution of overall ARV levels downstream of the plant was higher than for example the Vlakplaats plant, which has more than double the treatment capacity (Table 3.1 and Figure 5.1). This can be due to the low Green Drop status of the Welgedacht plant (DWA, 2011).

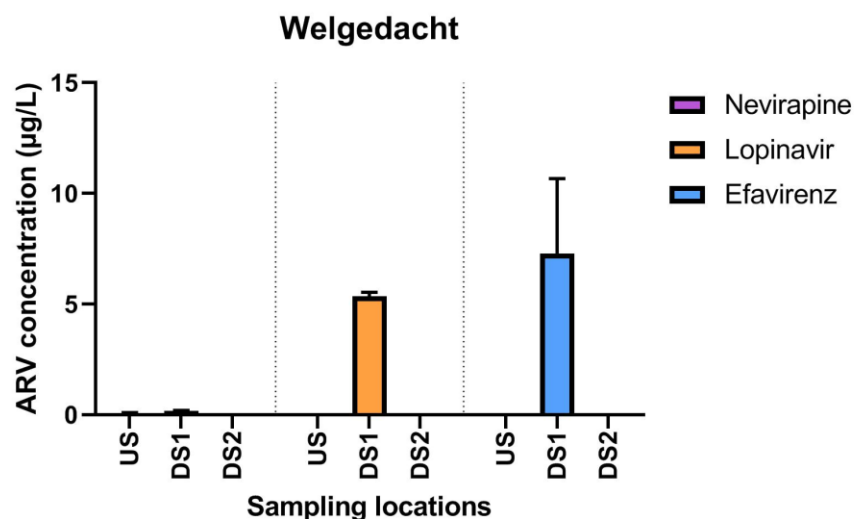


Figure 5.10: Grouped concentrations of ARVs based on location within Welgedacht sampling area. Bars indicate standard deviation (US = upstream; DS = downstream).

There were no biological responses with any of the samples (Figure 5.11). Samples from US and DS2 did not hamper cell viability which indicated that there were no AhR ligands in the extracts. However, the raw extract of DS1 was cytotoxic (Table 4.4) and a lack of biological response was attributed to the fact.

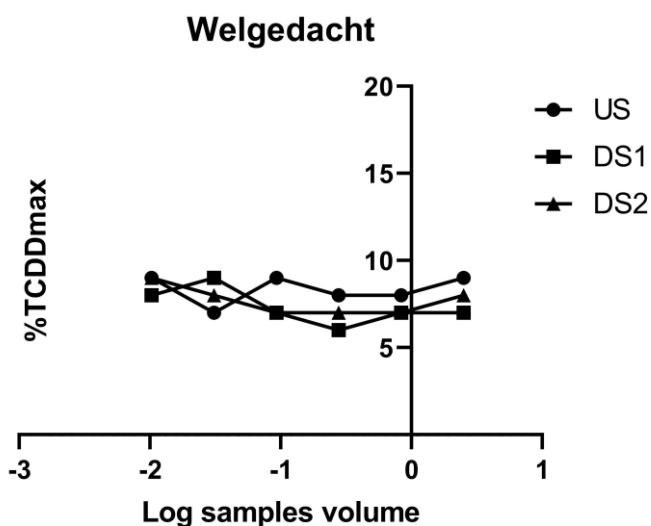


Figure 5.11: Dose response curves of Welgedacht sampling area expressed as %TCDDmax (US = upstream; DS = downstream).

US had a total ARV concentration of $\sum 0.1 \mu\text{g/L}$ and DS1 had $\sum 12.8 \mu\text{g/L}$ (Table 4.1). The total for DS2 could not be determined as no compound was quantified. Together with sampling sites around Waterval WWTP, Welgedacht WWTP sampling sites had the lowest occurrence of target compounds between the sites (Figure 5.1).

5.1.6. Zeekoegat

Zeekoegat (Figure 3.7) US2 is the only site in the entire study that had quantifiable levels of five of the six ARV compounds tested for in this study (Table 4.1). This sampling area was different from the others, as a single river system around the WWTP was not used. This WWTP is situated on the bank of the Roodeplaat Dam with all of its US sites upstream of the Dam, all in different streams (Figure 3.7). Zidovudine and ritonavir were both quantified at the US2 site (Pienaars River) only, and this was also the only site in this study where zidovudine was found. The highest levels of lopinavir and efavirenz for this sampling area were found at US2 at 20.11 µg/L and 20.42 µg/L, respectively (Table 4.1). The Pienaars River (US2) flows through the densely populated Mamelodi Township, which can contribute non-point source pollutants into the river. The Baviaanspoort WWTP, which has a higher treatment capacity than the Zeekoegat WWTP, discharges its effluent into the Pienaars River upstream of US2. As this river contributes 43% of the inflow into the Roodeplaat Dam, it can be a main source of pollutants into the Dam. The high levels of lopinavir, efavirenz and zidovudine in this sample were thus to be expected.

US3 (Edendale Spruit) also had a high concentration of lopinavir at 10.23 µg/L (Figure 5.12). The Edendale Spruit also flows through parts of Mamelodi, and can also receive some run-off pollution, possibly explaining the high levels of lopinavir in this sample (Figure 3.7). The Hartbees Spruit (US1) had the highest nevirapine level in the entire study (Table 4.1), but low concentrations of lopinavir and efavirenz when compared with those of US2 and US3 (Figure 5.12). DS1 had very low nevirapine, lopinavir and efavirenz levels compared with the three US sites (especially US2 and US3) (Table 4.1). The outflow of the Roodeplaat Dam (DS1) is controlled by a constructed dam wall. It seems as if the target compounds were trapped in the dam, resulting in low levels of ARVs at the DS1 site.

The inflow streams (US1, -2 and -3) were shown to possibly have a bigger impact on the ARV levels in the Roodeplaat Dam, than the WWTP itself, as the inflow sites had a larger total ARV concentration than the outflow (DS1) site (Figure 5.1). This needs to be verified by sampling the WWTP effluent of the Zeekoegat plant itself.

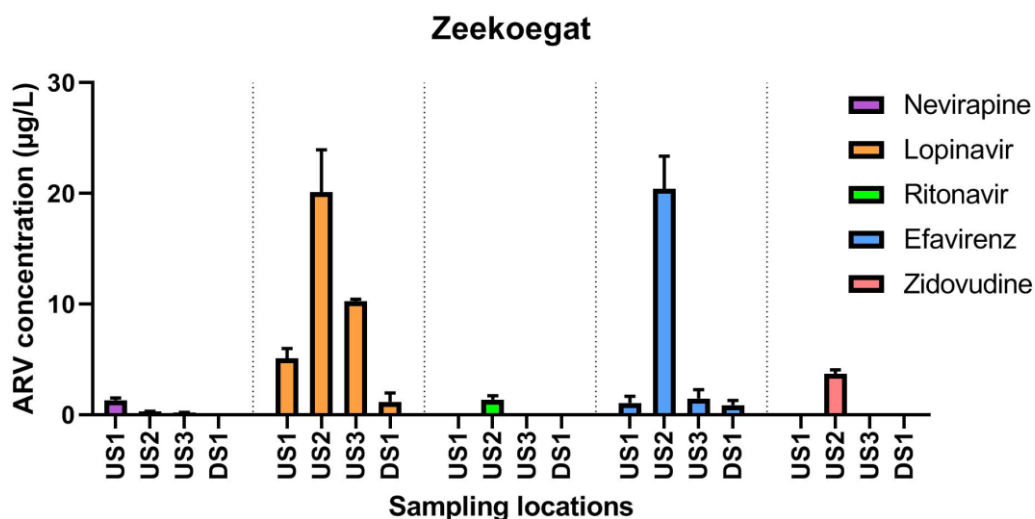


Figure 5.12: Grouped concentrations of ARVs based on location within Zeekoegat sampling area. Bars indicate standard deviation (US = upstream; DS = downstream).

None of the samples had any response or were cytotoxic to the cells (>80% viability) (Table 4.4), indicating that nothing in the sample were AhR agonists (Figure 5.13).

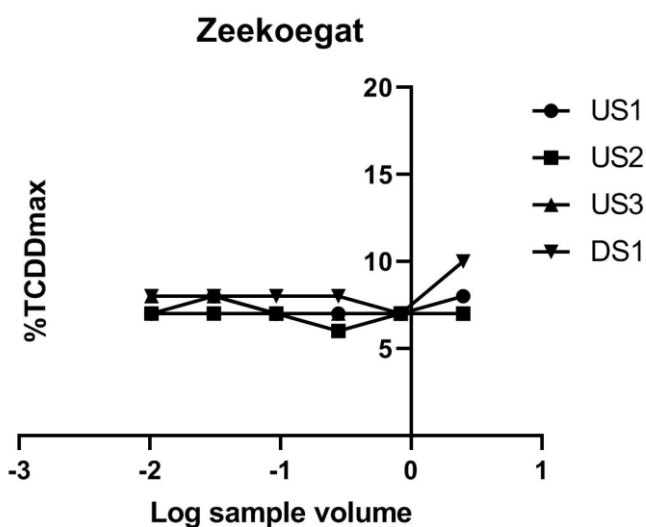


Figure 5.13: Dose response curves of Zeekoegat sampling area expressed as %TCDDmax (US = upstream; DS = downstream).

The fact that there was no biological response to any of the samples was surprising, as there were high concentrations of ARVs present, especially in US2 and US3. The Σ ARV concentration for US1 was 7.5 µg/L, for US2 was 42.2 µg/L, for US3 was 11.9 µg/L and for DS1 was 2.1 µg/L (Table 4.4). The high total concentration for US2 was exceptionally high (third highest Σ ARV concentration of the current study), giving an indication that some biological response was to be expected.

5.1.7. Flip Human

The downstream site of Flip Human had higher concentrations for nevirapine, lopinavir, ritonavir and efavirenz than upstream of the WWTP, as was expected (Figure 5.1 and Table 4.1). For lopinavir and efavirenz there was a large difference between the sites. Lopinavir increased three fold (5.50 µg/L and 15.25 µg/L), and efavirenz increased 10 fold (2.19 µg/L and 20.30 µg/L), indicating that the WWTP contributed to the abundance of these two compounds (Figure 5.14). This increase was not observed at any of the other WWTPs. The location of the Kagiso Township next to the Wonderfontein Spruit upstream of US can also possibly contribute to these levels (Figure 3.8).

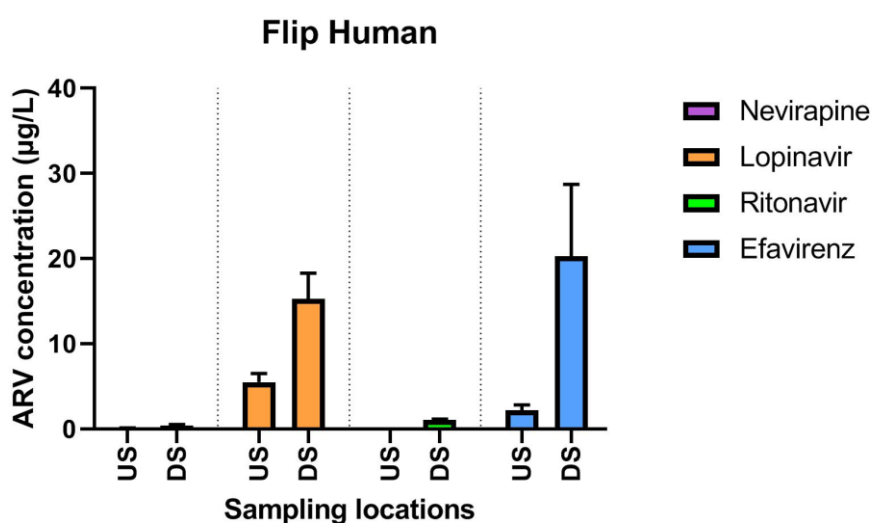


Figure 5.14: Grouped concentrations of ARVs based on location within Flip Human sampling area. Bars indicate standard deviation (US = upstream; DS = downstream).

The US sample had no biological response, whereas DS had the second highest %TCDDmax response (31%) (Figure 5.15). There was no cytotoxicity for the highest concentration and this response was thus not influenced by the viability of the cells (Table 4.4).

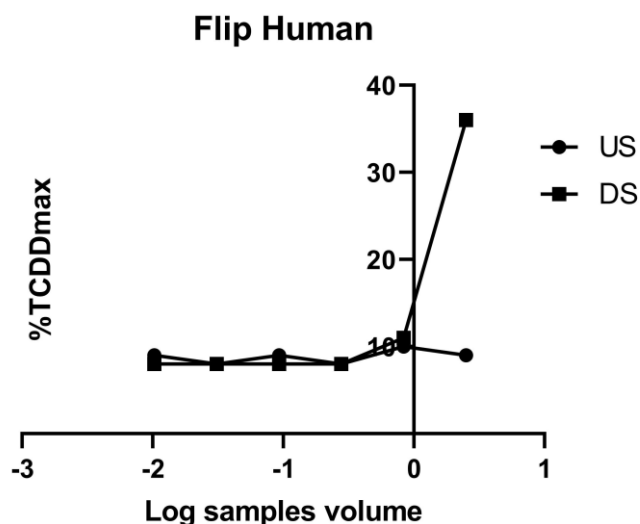


Figure 5.15: Dose response curves of Flip Human sampling area expressed as %TCDDmax (US = upstream; DS = downstream).

The Σ ARV concentration for DS was higher than US, with 37.0 $\mu\text{g/L}$ at DS and 7.8 $\mu\text{g/L}$ at US (Table 4.4). This indicated that the ARVs present in the DS sample (as well as possible other compounds) could have contributed to the high biological response in Figure 5.15.

5.2. Drinking water

Seven drinking water samples were analysed to determine the presence of ARVs in the sample. Zeekoegat DW had very high levels of efavirenz and nevirapine (Table 4.3), even higher than some surface waters samples (Table 4.1). Welgedacht DW2 had low levels of nevirapine (Table 4.3). Out of the seven DW samples taken only 2 had quantifiable levels of ARVs. Nevirapine and lopinavir were detected the most frequent in DW samples with 29% each, while ritonavir and efavirenz were detected at 14% of the sites. Zidovudine and didanosine were not detected at any of the DW sites.

Only two were used to determine possible biological responses, Zeekoegat DW and Olifantsfontein DW (Table 4.4). These samples showed no AhR mediated activity. This is good news, as clean drinking water is an essential resource for humans. Although ZG DW had high levels of ARVs in the water (Table 4.1), it had no AhR activity.

5.3. Overview of instrumental analysis

Arranging the compounds with the highest concentrations to the lowest for this study were lopinavir > efavirenz > zidovudine > ritonavir > nevirapine > didanosine.

Katende-Kyenda et al. (2011) analysed the South African national medicine claims database for three years from 2005 to 2007 and reported that lopinavir/ritonavir were prescribed at higher doses than recommended, at 1066/264 mg (the recommended daily dose is 800/200 mg). If this issue has not been resolved in the meantime, this can possibly explain the high concentrations found for lopinavir in some surface water samples. When rifampicin (an antibiotic) was administered to treat tuberculosis, which is a common co-infection in HIV positive patients, it causes a reduction in lopinavir levels in the patient by inducing the CYP enzymes (Sunpath et al., 2014).

It was recommended that the dose of lopinavir/ritonavir be doubled to 1600/400 mg daily, as an interim solution to this problem (Sunpath et al., 2014). This would explain the high levels of lopinavir found in the environment. Although lopinavir and ritonavir are always prescribed in combination, ritonavir was detected less frequently than lopinavir in my study (60% vs 85%) (Table 4.1). Aminot et al. (2015) found that ritonavir would rather absorb to the suspended solid particles than in the water at 5–40% of the total ARV concentration. However, in their study, they collected 20 L, whereas we collected only 1 L, and they pre-filtered the sample to remove the suspended solids for separate analysis. The water samples in the current study were not pre-filtered to remove the suspended solids before extraction, so in theory the compounds absorbed to the suspended solids should also be present in the sample extract.

The high levels of efavirenz can be attributed to the use of this compound as part of the recommended first-line regimen for HIV patients. It is also one of the most prescribed ARV in the public health sector of South Africa (Osunmakinde et al., 2013). Efavirenz is also believed to be a component of the South African street drug, Nyaope (Thomas and Velaphi, 2014). Due to the belief that this ARV is used to make this drug, it is often stolen from HIV positive patients, pharmacies and clinics (Rough et al., 2014). This means that more efavirenz may be present in communities and the levels in the environment is likely to increase as well.

Nevirapine is prescribed to prevent mother-to-child transmission of HIV in South Africa (Burton et al., 2015). This prevents the transmission of HIV from the mother to the child. The South African government was ordered by the constitutional court in 2002 to provide nevirapine to all pregnant women who are HIV positive, and is now available free of charge to qualifying patients (Njoroge, 2002). Aminot et al. (2018) found that nevirapine were relatively stable in biotic conditions of WWTPs, which can lead to an increase in levels and occurrence of this compound released from WWTPs into receiving waters. This explains the high detection frequency of nevirapine found in this study (Table 4.1).

Zidovudine undergoes photo-transformation when exposed to sunlight (Prasse et al., 2015), indicating that if the WWTP effluent containing zidovudine was released into the receiving waters, it will biodegrade. This can indicate why zidovudine was only quantified at only one sample (ZG US2) in this entire study (Table 4.1).

The ecotoxicological risks of ARVs in the water are not adequately known. The only study done to determine the risk to aquatic organisms was done by Ngumba et al. (2016a) using risk quotient values. They determined the acute toxicity at three trophic levels in the aquatic ecosystem (algae, daphnia, and fish). They found that nevirapine (at 0.49 µg/L) in the environment will have possible high risk to algae, daphnia and fish, while zidovudine (at 0.77 µg/L) will have high risk to only algae. Both of the compounds will possibly interfere with the algae (primary producers) in the ecosystem by affecting their growth, and this will affect organisms in higher trophic levels as well. For nevirapine in the current study, only one site had a concentration higher than 0.49 µg/L that was predicted to cause acute toxicity in all three trophic levels by Ngumba et al. (2016a) at Zeekoegat US1 (1.3 µg/L) (Table 4.1). Zidovudine was only quantified at one site in my study at Zeekoegat US2 (3.68 µg/L) (Table 4.1) and was higher than the predicted acute risk concentration of 0.77 µg/L for algae (Ngumba et al., 2016a). The Roodeplaat Dam system is at risk of acute toxicity to invertebrates and freshwater fish due to high levels of nevirapine and lopinavir at the inflow sites (ZG US1 and US2) of the Dam. The levels could be diluted when the river feeds into the Dam, and the effects to the organisms living in centre of the Dam could be lowered. The levels of ARVs in the Dam should also be monitored in future studies.

Comparing concentrations with other studies

The levels of ARVs found in this study were compared with those reported for surface water in other studies across the globe (Table 5.1). This should be done with caution because most papers report on grab samples, as my study as well. However, ARVs are prescribed to be taken at very specific times of the day that would likely influence the period at which the highest levels are eventually found in the environment. If sampling does not coincide with effluent release, the levels would differ much when compared with sampling just before effluent release (Ncube et al., 2018). The results from the current study could differ from those of other studies as different seasons were sampled with different sample sizes. Other studies generally used the same analytical procedure as in this study (LC-MS/MS). The concentrations of efavirenz, lopinavir and ritonavir in surface water in the present study exceeded all others reported in literature.

Wood et al. (2015) sampled at various locations across South Africa in 2013 and 2014, some locations were the same as in the present study, such as in the Roodeplaat Dam and the Rietvlei Dam. ZG US2 and ZG DS1 were sampled in the same river section in the Pienaars River. Wood et al. (2015) found zidovudine and nevirapine at the Pienaars River inflow (ZG US2) at 0.4 and 1.5 µg/L, while in the present study, zidovudine was found at much higher concentrations of 3.7 µg/L (Table 4.1), while nevirapine was found at a slightly lower level at 0.3 µg/L (Table 4.1).

Zidovudine and nevirapine were also found at the Roodeplaat Dam outflow (ZG DS1) by Wood et al. (2015) at 0.2 and 0.4 µg/L, respectively. An irrigation canal is responsible for the outflow of water from the Dam, and does not represent the quality of the surface water of the Dam (Sartory, 1980). In the present study, both these compounds were lower, with nevirapine at 0.1 µg/L and zidovudine <LOD (Table 4.1). Lopinavir, ritonavir and efavirenz were quantified at ZG US2 and DS1 in the present study, but not by Wood et al. (2015).

Wood et al. (2015) also sampled at the Rietvlei Dam at two locations, at the northern banks of the Dam and in the Sesmyl Spruit that flows from the Rietvlei Dam. The Sesmyl Spruit eventually merges with the Olifant Spruit to become the Hennops River. They found zidovudine and nevirapine at low concentrations in the Rietvlei Dam area, both at 0.2 µg/L. The Olifant Spruit receives effluent from the OF WWTP and had high concentrations of lopinavir, efavirenz and ritonavir in this study, but Wood et al. (2015) (Table 4.1) reported <LOD for efavirenz and ritonavir. They did not quantify lopinavir. Nevirapine levels were slightly higher (highest with 0.46 µg/L at OF DS1) than those found by Wood et al. (2015) and zidovudine was not detected in this study (Table 4.1). The Rietvlei Dam was also sampled in 2015 by Wooding et al. (2017) at six locations, for efavirenz and nevirapine. They found efavirenz (highest of 0.15 µg/L) at the closest level to that of the highest concentrations found in the present study of 24.6 µg/L for efavirenz (OF DS1) (Table 5.1).

Wood et al. (2015) also sampled surface water from the Gariep Dam in the Free State province, the Vaal Dam (situated in the Free State, Gauteng and Mpumalanga), the Hartbeespoort Dam in Gauteng, and the Inanda Dam in KwaZulu-Natal. Robson et al. (2017) sampled in the Vhembe district of the Limpopo province. They sampled upstream and downstream of a WWTP (Muledeni), and found higher levels of efavirenz (0.01 µg/L) upstream of the WWTP, than downstream (<LOQ) (as was found in this study at Sunderland Ridge in Figure 5.4). The Hartbeespoort Dam and various locations along the Jukskei River in Gauteng was sampled by Rimayi et al. (2018).

Efavirenz concentrations reported for the present study were higher than those found in other South African studies and in Kenya (Table 5.1). The efavirenz level that was the closest to the highest level of 24.6 µg/L downstream of the Olifantsfontein WWTP in the Olifant Spruit of the current study was 0.15 µg/L reported by Wooding et al. (2017) for the Sesmyl Spruit upstream of the Rietvlei Dam sampled in 2015 (Table 5.1). This difference can be due to the difference in sampling years and different extraction methods. Wooding et al. (2017) used stir bar sorptive extraction (SBSE), whereas SPE was used in this study. The levels in this study were comparable to those found in WWTP influent by Schoeman et al. (2015) in Gauteng and Afafe et al. (2018) in Durban, KwaZulu-Natal (Table 5.1).

Lopinavir was found at 38.5 µg/L at Olifantsfontein DS2 (Table 4.1), which was considerably higher than the 0.31 µg/L found by Wood et al. (2015) in 2014 in the Hartbeespoort Dam (surface water), the Olifant Spruit (OF DS2) runs into the Hennops River, which eventually reaches the Hartbeespoort Dam. This difference in lopinavir levels can be due to the degradation of the compound from OF DS2 to the Hartbeespoort Dam. Lopinavir levels were also higher than in WWTP influent and effluent of Durban (Afafe et al., 2018) and in WWTP effluent of the Hartbeesfontein WWTP (located upstream of the Rietvlei Dam) (Wood et al., 2015) (Table 5.1).

The highest level of ritonavir for this study was found at 1.95 µg/L in the Olifant Spruit (OF DS1) (Table 4.1), but although it was detected by Wood et al. (2015), they could not quantify it (LOQ < 0.2 µg/L) at Hartbeespoort Dam in 2014 (Table 5.1). This difference could be explained by the same principle as for lopinavir mentioned above. Ritonavir levels in this study was within the same range Afafe et al. (2018) found in wastewater influent and effluent in Durban WWTPs (Table 5.1).

The levels of nevirapine for the present study was in the same range Wood et al. (2015) found in the Roodeplaat Dam system and the Hartbeespoort Dam in South Africa (Table 5.1), but lower than those found in Kenya (K'oreje et al., 2016), who reported a maximum concentration of 5.62 µg/L against the 1.3 µg/L found in the present study (Table 5.1) in the Hartbees Spruit which flows into the Roodeplaat Dam (ZG US1) (Figure 3.7).

Zidovudine had the highest surface water concentration in South Africa in this study at the Edendale Spruit that flows into the Roodeplaat Dam (ZG US3) at 3.68 µg/L (Table 4.1). The highest zidovudine concentration found by Wood et al. (2015) was in the Roodeplaat Dam system at 0.63 µg/L (Table 5.1). Surface water levels of zidovudine in Kenya were higher than those found in the present study, as well as by Wood et al. (2015), with a maximum concentration of 17.41 µg/L (K'oreje et al., 2016) (Table 5.1).

Didanosine was detected in this study at 5% of the sampling sites (Table 4.1), but was not quantified making comparison with other studies difficult. Lamivudine and stavudine were not included in the current study but they have been found in South African surface water previously (Table 5.1).

Table 5.1: Minimum and maximum concentrations (µg/L) of ARV drugs found in different matrixes globally. (LOQ = Limit of quantification; LOD = limit of detection; WWTP = wastewater treatment plant).

Compound name	Sample matrix	Country	Minimum and maximum concentration reported (µg/L)	Reference
Didanosine	Surface water	South Africa	0.054–0.0541	Wood et al. (2015)
Efavirenz	Surface water	South Africa	<12.1 (LOQ)	Wood et al. (2015)
		South Africa	0.0013–0.010	Robson et al. (2017)
		South Africa	0.0003–0.15	Wooding et al. (2017)
		South Africa	0.002–0.35	Rimayi et al. (2018)
		South Africa	0.85–24.6	This study
		Kenya	0.02–0.56	K'oreje et al. (2016)
	Drinking water	South Africa	<12.1 (LOQ)	Wood et al. (2015)
	WWTP influent	South Africa	17.4	Schoeman et al. (2015)
	WWTP effluent		7.1	
	WWTP influent	Kenya	1.02	K'oreje et al. (2016)
	WWTP effluent		0.11	
	WWTP influent	South Africa	24–34	Abafe et al. (2018)
	WWTP effluent		20–34	
Lamivudine	Surface water	South Africa	0.095–0.24	Wood et al. (2015)
		Kenya	5.43	Ngumba et al. (2016a)
		Kenya	6.51–167.1	K'oreje et al. (2016)
		Finland	0.012	Ngumba et al. (2016b)
	WWTP effluent	Kenya	3.99	Ngumba et al. (2016a)
	WWTP influent	Finland	0.037–0.055	Ngumba et al. (2016b)
	WWTP effluent		0.02–0.022	
	WWTP influent	Kenya	60.68	K'oreje et al. (2016)
	WWTP effluent		31.07	
	WWTP influent	Germany	0.21	Prasse et al. (2010)
	WWTP effluent		<0.05 (LOQ)	

	WWTP influent WWTP effluent	Belgium	0.51 <LOD	Vergeynst et al. (2015)
	WWTP influent WWTP effluent	South Africa	0.84–2.2 <0.02 (LOD)–0.13	Abafe et al. (2018)
Lopinavir	Surface water	South Africa South Africa	0.28–0.31 0.8–38.45	Wood et al. (2015) This study
	Drinking water	South Africa	<0.3 (LOQ)	Wood et al. (2015)
	WWTP effluent	South Africa	0.13	Wood et al. (2015)
	WWTP influent WWTP effluent	South Africa	1.2–2.5 1.9–3.8	Abafe et al. (2018)
Nevirapine		South Africa	0.13–1.48	Wood et al. (2015)
		South Africa	0.04–0.23	Wooding et al. (2017)
		South Africa	0.006–0.071	Rimayi et al. (2018)
	Surface water	South Africa	0.036–1.30	This study
		Kenya	4.86	Ngumba et al. (2016a)
		Kenya	0.03–5.62	K'oreje et al. (2016)
		Germany	0.005–0.017	Prasse et al. (2010)
	Drinking water	South Africa	<0.1 (LOQ)	Wood et al. (2015)
	Ground water	Kenya	0.02–1.6	K'oreje et al. (2016)
	WWTP effluent	South Africa	0.35	Schoeman et al. (2015)
	WWTP effluent	Kenya	1.36	Ngumba et al. (2016a)
	WWTP influent WWTP effluent	Finland	0.013–0.019 0.008–0.01	Ngumba et al. (2016b)
	WWTP influent WWTP effluent	Kenya	3.3 2.1	K'oreje et al. (2016)
	WWTP influent WWTP effluent	Germany	0.0048 0.0072	Prasse et al. (2010)
	WWTP influent WWTP effluent	South Africa	0.67–2.1 0.54–1.9	Abafe et al. (2018)
Ritonavir	Surface water		<0.2 (LOQ) 0.64–1.95	Wood et al. (2015) This study
	Drinking water	South Africa	<0.2 (LOQ)	Wood et al. (2015)

	WWTP influent		1.6–3.2	
	WWTP effluent		0.46–1.5	Abafe et al. (2018)
Stavudine	Surface water	South Africa	0.102–0.78	Wood et al. (2015)
		Germany	0.002–0.0029	Prasse et al. (2010)
	WWTP influent		0.012	
	WWTP effluent	Germany	<0.02 (LOQ)	Prasse et al. (2010)
Zidovudine		South Africa	0.052–0.63	Wood et al. (2015)
		South Africa	3.68	This study
	Surface water	Kenya	7.68	Ngumba et al. (2016a)
		Kenya	0.05–17.41	K'oreje et al. (2016)
		Germany	0.005–0.17	Prasse et al. (2010)
	Drinking water	South Africa	0.073	Wood et al. (2015)
	Ground water	Kenya	0.02–0.03	K'oreje et al. (2016)
	WWTP effluent	South Africa	0.45–0.97	Wood et al. (2015)
	WWTP effluent	Kenya	0.51	Ngumba et al. (2016a)
	WWTP influent		0.046–0.06	
	WWTP effluent	Finland	0.02–0.037	Ngumba et al. (2016b)
	WWTP influent		20.13	
	WWTP effluent	Kenya	0.11	K'oreje et al. (2016)
	WWTP influent		0.31	
	WWTP effluent	Germany	0.098	Prasse et al. (2010)
	WWTP influent		6.9–53	
	WWTP effluent	South Africa	0.087–0.5	Abafe et al. (2018)

- Detection frequency

Due to the high detection frequencies of nevirapine (95%), lopinavir (85%), efavirenz (80%) and ritonavir (60%) in the surface water, these compounds have a widespread occurrence in the river systems of Gauteng (Table 4.1). Wood et al. (2015) also found that nevirapine (100%) and zidovudine (75%) were most frequently detected in surface waters. They found these compounds mainly in the Roodeplaat Dam system, Rietvlei Dam and Hartbeespoort Dam. Ngumba et al. (2016a) and K'oreje et al. (2016) also found high detection frequencies for nevirapine (>90%) in Kenya.

Nevirapine and lopinavir were also detected the most in this study, whereas zidovudine was only detected at five sites (25%) unlike in other studies that had detection frequencies >80% (K'oreje et al., 2016; Wood et al., 2015). Recently, the use of zidovudine in the ART regimen is being discouraged due to its toxicity (aggravating renal failure and anaemia) (Meintjes et al., 2017).

As Wood et al. (2015) determined the zidovudine levels in 2014, and this study was done in 2017, this could possibly explain the decrease in detection frequencies of zidovudine.

- Drinking water:

ARVs were not commonly found in drinking water samples. Wood et al. (2015) found zidovudine at 0.07 µg/L, whereas efavirenz, nevirapine, lopinavir and ritonavir had levels below LOQ. In this study, zidovudine was not quantified in drinking water samples, but nevirapine (0.2 µg/L), lopinavir (9.04 µg/L) and efavirenz (2.33 µg/L) were quantified at Zeekoegat DW. These concentrations were higher than some of the surface water samples, which is concerning as it poses a direct threat to human health through consumption of contaminated water. This site is located near the Roodeplaat Dam, but the source of the tap water is unknown. Since Rand Water (which mainly use the Vaal River as a water source) supplies the central area of Gauteng with drinking water (Rand Water, 2018) and this area is part of its extended supply area, it may be assumed that the water originated there. The City of Tshwane also has municipal water treatment works that may also supply this area with drinking water. The possibility that the property owners use groundwater as source also needs to be accounted for. Overall ARVs were found in very low concentrations or not detected at all in drinking water (Table 4.1).

- WWTP influents and effluents:

This study was done to determine if the WWTPs can be a significant point source contributor to the levels of ARVs in the receiving surface waters. WWTP influents mainly have higher concentrations of ARVs than effluents (Table 5.1), indicating that the WWTP was partially successful of removing these compounds, and thus have high removal efficiencies (REs). The REs differ between the compound as well as the WWTPs. This can be due to the different techniques that each WWTP use to remove potentially harmful compounds.

Efavirenz showed good REs in studies by Schoeman et al. (2015) and K'oreje et al. (2016), but Abafe et al. (2018) found very low to none removal of efavirenz from the WWTP (Table 5.1). Lamivudine showed higher concentrations in influent than in effluent in all studies (Table 5.1), although the REs ranged from low (24–59%) (K'oreje et al., 2016) to high (>76%) (Prasse et al., 2010).

Lopinavir was only studied by Abafe et al. (2018) and had a negative RE. Nevirapine had low (11–49%) (K'oreje et al., 2016) to negative REs (Abafe et al., 2018; Prasse et al., 2010). Nevirapine concentrations increased during the removal process in WWTPs, but this is possibly explained by the de-conjugation of hydroxylated metabolites of the compound (Schoeman et al., 2017). Stavudine and zidovudine had high REs in all studies, with the lowest being 60% (Ngumba et al., 2016b).

Low REs indicates that the WWTPs were unsuccessful in removing the ARVs, and ARVs were released into the receiving waters. Efavirenz, lopinavir, and nevirapine had low REs, and it can be expected that these compounds will be found more frequently and at higher concentrations. This was supported by the present study, as these three compounds have the highest detection frequency of all the compounds, and efavirenz and lopinavir were present at high concentrations (Table 4.1). Zidovudine was reported to have high REs, indicating less probability of detection compared with the other compounds. As zidovudine had a detection frequency of only 25% and was quantified at only one site, the low detection can be attributed to the high REs of this compound (Table 4.1).

The Green Drop status of a WWTP can have an impact on the extent to which the ARVs are present in receiving waters, but this is not always the case. As Welgedacht, Sunderland Ridge, Flip Human and Zeekoegat WWTPs had low Green Drop statuses (DWA, 2011), indicating that they might be at higher risk of releasing these compounds into the surface water. All these plants (except for Zeekoegat) showed that they are a source of ARVs into the receiving waters (Figure 5.1). Olifantsfontein and Vlakplaats had adequate Green Drop status, but they were still responsible for an increase in ARV levels (Figure 5.1).

- Groundwater:

Nevirapine and zidovudine were found in groundwater as by K'oreje et al. (2016) at levels of 0.02–1.6 µg/L and 0.02–0.03 µg/L, respectively. The presence of ARVs in groundwater can be a direct threat to human health because groundwater is often used as drinking water without any pre-treatment (K'oreje et al., 2016).

5.4. Biological analysis

Compounds that are typically associated with the binding to the AhR, such as PAH, are generally planar, hydrophobic compound, and are likely to accumulate in sediment (Denison and Nagy, 2003). These 'typical' ligands bind with high affinity to the AhR, but there are some 'non-typical' compounds that may be low affinity ligands that are weak inducers of the production of CYP1A1 (Denison and Nagy, 2003).

Pharmaceuticals are generally characterised as hydrophilic and polar compounds and will predominately be found in aqueous phases (Schoeman et al., 2017), which does not conform to the typical structure of AhR ligands. Pharmaceuticals are reported as being selective AhR modulators (Jin et al., 2012), the ligands are tissue-specific AhR agonists (Mulero-Navarro and Fernandez-Salguero, 2016). Several pharmaceuticals (such as anti-inflammatory, anti-allergic, anti-androgen and anti-arrhythmic drugs) have been proven to induce CYP1A1 in rodent systems (Jin et al., 2012) and can be used as anti-cancer agents (Safe et al., 2017).

Some ARV drugs are indeed metabolised by the cytochrome P450 (CYP) enzymes. The CYP3A4 enzyme is involved in the metabolism of all NNRTIs (efavirenz, nevirapine) and PIs (lopinavir, ritonavir). The two NNRTIs is also metabolised by CYP2B6, and the PI ritonavir by CYP2D6 (Makinson et al., 2010). Ritonavir is also the most potent inhibitor of CYP3A4 and also induces CYP1A2 (Walubo, 2007). NRTIs are not metabolised in the liver and does not have an effect on the CYP enzymes (Makinson et al., 2010).

The luciferase activity of nevirapine and zidovudine (along with 145 other pharmaceutical compounds) were determined by Hu et al. (2007) using the H4IIE cell line. This is based on the ability to activate the CYP1A1 enzyme. It was found that these two ARVs caused slight activation of the AhR, with a response of 10.7 ± 8.9 %TCDDmax (nevirapine), and 1.6 ± 10.0 %TCDDmax (zidovudine), respectively. The concentrations tested were 10 μ M (approximately 2 600 μ g/L). The concentrations of nevirapine and zidovudine found in this study were much lower (maximum 1.3 μ g/L and 3.68 μ g/L, respectively), this indicated that the concentrations these compounds needs to be extremely higher to contribute to the luciferase activity.

A majority of the extracted samples were reported as <LOD, indicating that there was no activation of the AhR. Some samples did have a slight %TCDDmax response, such as Olifantsfontein US (22%), -DS1 (33%) and -DS2 (21%), Sunderland Ridge US (20%), Waterval DS2 (19%) and Flip Human (38%) (Table 4.4), but were all <LOQ, as an EC20 value could not be determined. Toxic equivalents could thus also not be determined for the samples (Table 4.4).

The ability of single ARV compounds to activate the AhR was also tested in this study. There was no AhR-mediated activity for any of the compounds up to 80 μ g/L. This concentration tested was higher than levels of all compounds found with instrumental analysis (Table 4.1).

Efavirenz had a maximum of 24.6 µg/L (OF DS1), nevirapine had 1.3 µg/L (ZG US1), lopinavir had 38.5 µg/L (OF DS2) and ritonavir had 1.9 µg/L (OF DS1) (Table 4.1). As Hu et al. (2007) found that nevirapine and zidovudine caused very low activation of the AhR at much higher concentrations than those found, it can be said that ARVs can cause very low AhR-mediated responses.

These single ARVs compounds can thus not explain the slight biological response (Table 4.4). It is possible that mixtures of these compounds, as well as other unknown compounds in the water extract, could have caused this effect. The low biological activity of the samples observed can be due to the possibility that the ARVs present in the sample did not fit the typical structure of AhR ligands. Another possibility is that because the ARV drugs are not metabolised by CYP1A1 specifically, it will not be able to activate the AhR.

The activation of the AhR for FH DS (Table 4.4) are most likely attributed to polar compounds other than ARVs present in the sample, but their identity is unknown. The induction of CYP1A1 by other compounds in the sample, may ultimately alter the bioavailability of the ARV drugs (Kumar et al., 2015).

5.4.1. Cell viability assay

The low viabilities of the samples can be attributed to the mixtures of compounds present in the concentrated sample, as well as any other particles still present from the environmental sample. Lower cell viability can lead to reduced luciferase response that is not due to the compounds in the extract but rather its toxicity.

The use of ARVs as part of the antiretroviral therapy program has been linked to liver toxicity (Kalyesubula et al., 2011). The extreme cytotoxicity of some of the samples can thus possibly be linked to the impact of the ARV compounds on the H4IIE cells, as they are derived from rat liver cells. The Mozambique tilapia (*Oreochromis mossambicus*) fish exposed to efavirenz were shown to cause severe liver damage at 10.3 ng/L, and could impact the overall health of the fish at higher concentrations (Robson et al., 2017). Efavirenz had high concentrations in all samples that were cytotoxic to the H4IIE cells: OF US, OF DS1, OF DS2, SR US and WG DS1 had extremely low cell viabilities (Table 4.4). As efavirenz were found at the highest concentration of 24.60 µg/L (or 24 600 ng/L) at OF DS1 (Table 4.1), this can indicate that the cytotoxicity in these samples were due to the high levels of this compound. Efavirenz, at 8 µg/L, caused a low cell viability of 53%, and decreased to 51% at 80 µg/L (data not shown). This compound was responsible for the lowest cell viability of all compounds tested, and provides some evidence that it was the efavirenz that could have contributed to the low cell viability.

Antiretrovirals, especially NRTIs, are known to induce mitochondrial toxicity (Xue et al., 2013). Efavirenz, an example of a NNRTI, also impacts hepatic cells by inhibiting mitochondrial function in patients (Blas-García et al., 2010). A decrease in mitochondrial function by these drugs can result in an increase of cytotoxicity. This can be seen as samples with high levels of efavirenz, were cytotoxic to the cells.

High viability of the cells (Table 4.4) at Vlakplaats DS2 and Zeekoegat US1 could be from the compounds present in the water which increase cell proliferation (Dai et al., 2015), and as the H4IIE cells used in the MTT assay are essentially cancer cells, it can be deduced that these compounds (or the mixtures thereof) can possible increase cancer cell growth.

Chapter 6: Conclusion and recommendations

Overall, this study has shown that WWTPs contribute to ARV concentrations in receiving waters. At the Olifantsfontein, Sunderland Ridge, Vlakplaats, Welgedacht and Flip Human WWTPs (five of seven) the levels of ARVs downstream of the WWTPs were higher than at the corresponding upstream sites (not statistically significant). The level of efavirenz and lopinavir in surface water found in this study were higher than in any other South African study. Efavirenz levels were also higher than those found in Kenya. The compounds and the extent to which they were present in surface water differed from plant to plant.

The service area of the WWTPs also plays a role in the amount of ARVs present in the surface waters into which the effluent is discharged. For example, Olifantsfontein WWTP is located near the Tembisa and Ivory Park Townships that are largely informal settlements that has a large population and density. Olifantsfontein had the overall highest total ARV concentrations compared with all other sites. This indicates that the areas from which the WWTP receives its effluent, plays an important role in the contribution to the load of pharmaceuticals as a whole present in surface waters.

The AhR-mediated reporter gene assay was used to determine if the extracted samples had possible AhR agonists. None of the samples had any quantifiable biological response, even though there were high levels of ARVs present. The slight response seen for some sample sites (such as Flip Human DS) can be attributed to other compounds that act as AhR agonists, but the identities of these are unknown. The single active compounds were also tested using the same biological assay and there was no response at even the highest dose (80 µg/L). None of the compounds were found at such a high concentration in the surface water samples. It is unlikely that the levels of ARVs found in surface water will result in AhR-mediated responses by inducing the CYP1A1 gene.

These ARVs can thus not display endocrine disrupting effects via the cross-talk with the oestrogen receptor, as the AhR needs to be activated to act as antagonist to the ER. Although the ARVs were not able to cause toxicity through mediation of the AhR (and possible endocrine disruption), they still have several life threatening adverse effects though long-term use. These compounds were proven to be present in various surface water locations across Gauteng and even at high concentrations in drinking water. When non-target humans and wildlife ingest these compounds unintentionally through contaminated water, it can lead to any of the possible side effects of the drugs.

As HIV positive patients also consume these ARVs through the water as well as their daily regimen of ARV drugs, it can lead to resistance of the virus. This hinders the treatment of the patient and another combination of drugs needs to be prescribed, increasing the amount of different ARV compounds being used in South Africa.

The presence of these compounds in the surface water is thus of major concern as there is little known about the ecotoxicological impact of ARVs on non-target organisms.

Recommendations

The use of target analysis to determine specific compounds in an environmental sample is not ideal as it does not give an indication of other compounds present or to what extent. Broad screening of surface water in South Africa is needed to monitor routinely the presence of pharmaceuticals and to what extent they are present in the environment.

The presence of metabolites of ARVs in surface waters and their biological effects should be determined in future studies.

Collection and analysis of water should be routinely (at the same time daily for a few weeks, or every few hours at the same site) done to determine a trend in for when the highest level will occur per day. This will give an indication of the variation of the ARV drugs in the receiving waters. A grab sample only gives a snapshot of the compounds present within that certain period, which can change constantly. The magnitude of the WWTP contributions should be determined based on the mass-balance as well as the flow of receiving surface waters.

As the extracted water samples were not able to produce an AhR-mediated response in the H4IIE cells, other cell lines should be used, such as the T47D-KB*luc* cells that can determine (anti-) oestrogenic activity of the water extract through the ER. This can give a clear indication of the possibility of the ARVs to have endocrine disrupting effects, not just through possible cross-talk of the AhR with the ER. The MDA-kb2 bioassay can also be used to determine possible (anti-) androgenic effects. To determine if the ARVs can have effects through a different mode of action, the H295R steroidogenesis assay can be used.

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