PRESENCE, CONCENTRATIONS AND POTENTIAL IMPLICATIONS OF HIV-ANTI-RETROVIRALS IN SELECTED WATER RESOURCES IN SOUTH AFRICA

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND

In 2013 an estimated 6.3 million people in South Africa were living with HIV and AIDS, more than in any other country. In 2013, 200 000 were estimated to have died from this disease. South Africa as a country currently has the greatest number of people that use HIV anti-retrovirals (HIV-ARVs) as anti-retroviral therapy (ART) in the world. Some HIV-ARVs used in South Africa include Zidovudine, Satvudine, Lamivudine, Nevaripine, Indinavir and Ritonavir. HIV-ARVs are used in combination therapies, called Highly Active Antiretroviral Therapy (HAART). A daily dose of combination therapy of HIV-ARVs (mean of 991 mg/day/person, range 590-1996) equates to a total of 529 000 kg of HIVARV compounds ingested per year (assuming 1.5 million people on ART). Excretion of HIV-ARVs varies depending on compound, but some such as Tipranivir, are excreted at 80%, and Nevaripine at 2.7% via urine. Assuming a mean 30% excretion to sewage via urine and faeces, we estimate that about 159 000 kg of HIV-ARV could reach the aquatic systems of South Africa every year, but this is based on assumptions. From the above it is obvious that PPCPs can be expected in natural waters in South Africa. Fish and other aquatic biota are therefore exposed to and may even accumulate HIV-ARVs directly from water via their gills, through food, or both. It is not known what effects these exposures and/or accumulation may have. Fish in our project will serve as a distant surrogate for secondary human exposure, acknowledging that fish might be exposed to much higher concentrations in natural waters than humans would through drinking water.

AIMS

The following were the aims of the project:

1. To conduct a literature survey on HIV-ARV presence in the environment.
2. To determine the major HIV-ARV compounds used in SA.
3. Develop extraction and analytical procedures for selected HIV-ARVs from water and fish.
4. Collect treated waste water from four different waste water treatment works, at least three rivers, and at least three impoundments.
5. Collect fish from the same sites as for the natural waters.
6. Analyse the water and fish as per developed methodology.
7. Analyse and interpret the data, generate conclusions and recommendations, and report via various means.

METHODS

An analytical method was developed to extract and quantify determination of 11 antiretroviral drugs (Abacavir, Efavirenz, Didanosine, Lamivudine, Lopinavir, Nelfinavir, Nevirapine, Ritonavir, Stavudine, Saquinavir, Tenofovir, and Zidovudine) in water and fish. The Horizon Technology's SPE-DEX 4790 automated extraction system, the Horizon Technology DryVap® Concentrator System, and the HPLC/QTOF-MS (Agilent Technologies, Germany) consisting of a HPLC (High pressure liquid chromatograph) (series 1100) and 6540 Accurate Mass QTOF-MS (Quadrupole time-of-fight – Mass spectrometer) were used for extraction, evaporation and analysis of samples, respectively. From a list drawn up of presently used HIV-ARVs in SA, the following were selected for this study: Stavudine, Lamivudine, Zidovudine, Abacavir sulfate, Efavirenz, Nevirapine, Saquinavir, Mesylate, Ritonavir, Nelfinavir, Didanosine, Lopinavir and Tenofovir. Quantification was done using standards. The LOQ (Limit of Quantification) was defined as the lowest calibration point in the linear regression (determined by MassHunter), with a signal to noise ratio of 10. Calibration curves showed excellent linearity over the calibration range, with correlation coefficients greater than 0.90 for all analytes. LOQs ranged between 0.02-1.0 ng/l, and recoveries from spiked water ranged between 88-108%.

RESULTS AND DISCUSSION

More than 100 samples were collected, composed of natural water, drinking water (including bottled water), fish plasma, and later also groundwater. Sample collection was successfully completed and fish blood samples were collected from five different dams and rivers. Extraction and analytical methods were developed. Efavirenz and Saquinavir were not detected in any of the 47 natural water samples. All other compounds were detected at least once, with Nevirapine found in six samples. The lowest concentration was 0.3 ng/l (Zidovudine) and the highest was Nevirapine at 6.7 ng/l. Of the four WWTP treated effluent, only Nevirapine (0.4 ng/l), Zidovudine (0.3 ng/l) and Tenofovir were quantified in two of the WWTPs. Of the 31 drinking water samples, Lamivudine, Tenofovir, Efavirenz, Lopinavir, and Saquinavir were not quantifiable. Nevirapine occurred in five samples (0.3-3.5 ng/l), and Didanosine in three (0.4-3.3 ng/l). Stavudine, Nevirapine, Tenofovir, Nelfinavir and Saquinavir were quantified from seven of 18 ground water samples. Nevirapine was found in eight (0.3-5.4 ng/l). In 33 fish plasma samples from four sites, nine samples had quantifiable amounts of HIV-ARVs: Stavudine (n=2), Didanosine (n = 3), Abacavir (n = 1), Efavirenz (n = 1), Nelfinavir (n = 1), and Saquinavir (n = 2). Plasma concentrations ranged from 5.4-22 ng/l. All sites, including a supposed ‘clean site’, upstream of Upington, had one or more quantifiable HIV-ARV residues. No HIV-ARVs were detected in any of the 25 bottled water samples. Due to the scattered nature of sample locations, and the variable presence and concentrations in samples, no meaningful statistics could be done.

CONCLUSIONS

The sampling, extraction, and analytical procedures used, successfully provided quantifiable concentration data for HIV-ARVs in environmental samples. All eleven HIV-ARVs were quantifiable in at least one matrix. Two of the four WWTPs had quantifiable amounts of HIV-ARVs in their effluent, indicating that effective removal (below LOQ) is possible. The likely (unconfirmed) presence of many other compounds in the waters that we analysed also indicates that other PPCPs, pesticides, and industrial pollutants, may be present. If mainly unstable HIV-ARVs are found in the samples that were analysed, it is therefore likely that similar unstable compounds would be present, and indicates that more stable compounds, whether PPCPs or other, will also survive intact for variable periods in waters. The unconfirmed likely presence of many compounds in our waters needs urgent attention to inform the need for more protection of the sources, as well as the ecology and consumers.

RECOMMENDATIONS

The following recommendations can be made:

- The amounts of HIV-ARVs being used in South Africa should be monitored on a continuous basis, and, if possible, better quantified, as well as their concentrations in the environment.
- The existing extraction and analytical procedures can be further refined.
- The potentially larger concentrations of stable breakdown products should be investigated.
- The inflowing and outflowing concentrations over time on a range of different WWTPs with different efficiencies (based on Green Drop data) needs to be determined.
- Studies on the effects of HIV-ARVs on the viral ecology of WWTPs should be investigated.
- Risk assessment studies on potential human dietary exposure via fish should be investigated.
- In parallel with many of the investigations listed above, additional PPCPs should be investigated. A likely target list should be constructed with input from the pharmaceutical community, standards obtained, and presence and concentrations confirmed. Further actions can then be based on the results.

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ACRONYMS & ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
AJS	Agilent jet steam
ART	Antiretroviral therapy
ARV	Antiretroviral
DEET	N,N-diethyl-meta-tulamide
EDTA	Ethylene-diamine-tetra-acetic acid
ESI	Electron spray ionization
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HIV-ARVs	Human immunodeficiency virus-antiretrovirals
HPLC	High pressure liquid chromatography
HSRC	Human science research centre
LOQ	Limit of quantitation
MS	Mass spectrometer
NWU	North West university
PPCPs	Pharmaceuticals and personal care products
QTOF/MS	Quadrupole time-of-flight mass spectrometer
SPE	Solid phase extraction
TB	Tuberculosis
TOF	Time-of-flight
WHO	World Health Organization
WRC	Water Research Commission
WWTP	Waste water treatment plant

CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Antiretroviral therapy (ART) is the main treatment for HIV/AIDS in South Africa. The aim of ARV (anti-retroviral) drugs is to keep the amount of HIV viruses in the body at a low level, in order to stop the weakening of the immune system. It also helps the immune system to recover somewhat if already compromised by HIV.

HIV can become resistant to one drug quickly. Thus, combination therapy is a growing trend in countries with high HIV concurrency (currently 29% in SA). This entails of a patient taking two or three antiretroviral drugs at the same time. In developing countries such as South Africa, ARVs are also given alongside TB (Tuberculosis) therapy.

There are currently more than 20 approved antiretroviral drugs, but not all are available in every country (AVERT, 2014). The most common ARV drugs used in South Africa are: Lamivudine, Stavudine, Efavirenz, Nevirapine, Didanosine, Zidovudine, Lopinavir; Ritonavir, Abacavir, Tenofovir, Saquinavir, Nelfinavir (all generic names). These drugs are then used in a variety of combinations for first-line defence (the combination of drugs a person is given at the beginning of treatment) or second line defence (a new combination of drugs given to a person when HIV has become resistant to the drugs first given).

The estimation of the number of people that need ART treatment globally is 14 000 000 (UNAIDS, 2013). In 2013, it was estimated that about 2 500 000 people need treatment in South Africa, as an estimated 29% of the population is HIV positive (UNAIDS, 2013). The reported number of people actually receiving ART globally is 9.7 million (WHO, 2013). Presently, 1.9 million people in South Africa are taking triple antiretroviral therapy on a daily basis, which makes the programme the largest in the world (Motsoaledi, 2012). The latest update on the Treatment Action Campaign website states that 1 500 000 people in South Africa are now on ART (TAC, 2008). In 2010, the President of the Republic of South Africa, President Zuma, signed a contract that focused on four key issues:

- increasing life expectancy;
- decreasing maternal and child mortality;
- decreasing the burden of disease related to HIV and TB; and
- strengthening the effectiveness of the health system (Motsoaledi, 2012).

A daily dose of combination therapy of HIV-ARVs (mean of 991 mg/day/person, range 590-1996) equates to a total of 529 000 kg of HIV-ARV compounds ingested per year (assuming 1.5 million people on ART). The calculation is probably an over-estimation as not all people have access to municipal sewage disposal systems. It is also not known how much of the compounds are destroyed by sewage treatment works and

through natural attenuation in the environment. However, it does indicate a potentially serious load of bioactive compounds to South African waters. This amount is likely to increase as more people receive ART. The issue of HIV-ARVs in water can be considered a hidden or latent risk. These risks have not been recognised before, or have existed for some time, but are only now becoming recognised or discovered. Latent risk chemicals are assumed to be in the environment, or are un-assessed chemicals of historic little concern, but which may have unexpected or unpredicted effects. Presently, we see two major issues and one lesser issue associated with HIV-ARVs in waters, although there may be others:

1) Low concentrations of HIV-ARVs may be consumed via drinking water, implying the possibility of resistance development by HIV. Maximally-suppressive AR therapies or HAART, which reduce the likelihood of viral mutations, are the best tool to minimize the occurrence of resistance. Sub-optimal regimens, interrupted regimens, and poor adherence to regimens are major factors determining the development of resistance, as the therapeutic concentrations in the body drops, but may still exert selective pressure. Our interpretation is that it may be possible (but hopefully unlikely) that resistance may be promoted by low concentrations of HIV-ARVs in drinking water, maintaining low concentrations of HIV-ARVs in consumers that are HIV positive but are not receiving ART. The development of resistance is not a straightforward matter though, and needs careful assessment. Although the possibility of resistance development from residues in drinking water may be small (the dilution and attenuation from discharge to eventual uptake may be large enough), it needs urgent investigation to discard this possibility, as the amount used in SA is higher than anywhere else in the world. Pollution might also be concentrated through a small number of STPs into finite receiving natural waters. From here, it may end up again in drinking water, through either treated water or direct consumption. At present, we do not know what residue concentrations may result in resistance but that will be part of this project through literature searches and consultations with role players.
2) HIV-ARVs may affect the natural virus component of municipal wastewater treatment works and the ecological function of viruses in receiving natural waters. Aquatic viruses may play a very important but yet poorly understood role in the ecosystem. Viruses are highly abundant in water and have been termed virioplankton. It has major influences on aquatic bacteria and algae and may have a regulatory role in phosphorous cycling and primary production.

Excretion of HIV-ARVs varies depending on compound, but some such as Tipranivir, are excreted at 80%, and Nevirapine at 2.7% via urine. Assuming a mean 30% excretion to sewage via urine and faeces, we estimate that about 159 000 kg of HIV-ARV could reach the aquatic systems of South Africa every year, but this is based on assumptions. An additional issue with getting more people on ARV is that the prevalence rate for the country will increase as more people survive due to treatment, while new infections add to the proportion infected.

ARV treatment in South Africa is essential, and with 1.9 million people on treatment it begs the question; if these ARV drugs find their way into our environment and waterways, what could the effects on the ecosystem, or perhaps even human health be? Literature indicates that the presence of ARV drugs may create problems for aquatic organisms, raising suspicions and urgency for their removal from water and wastewater. Because of this, ARV drugs are considered as emerging pollutants, belonging to the class of Pharmaceutical and Personal Care Products (PPCPs). Some antiviral agents go through biotransformation when consumed, while other are excreted from the body unchanged (Galasso *et al*., 2002). Therefore, there exists an immediate need to remove ARV drugs from water as some have non-biodegradable metabolites (Mascolo *et al*., 2010 and Prasse *et al.,* 2010). They are also deemed an extremely hazardous therapeutic class due to their toxicity (Sanderson *et al.,* 2004).

ARV drugs enter the environment through the two main different pathways:

1. Through direct disposal of medicine into environment (landfill sites that leach into ground and groundwater, and toilets (Bound and Voulvoulis, 2005).

2. Through domestic and sewage wastes (septic tanks and surface water runoff) (Bound and Voulvoulis, 2005). The un-metabolised ARV drugs are excreted via faeces or urine, and are thus most likely present in waste water (Accinelli *et al*., 2007 and Prasse *et al.,* 2010).

Many drugs are only partially removed during water purification (Prasse *et al.,* 2010), and thus enter waterways through WWTP (waste water treatment plant) discharge, some ARVs enter the WWTP in an inactive form, but due to water treatment process, may enter the environment in a biological active form (Kummerer, 2008).

Due to South Africa's high HIV prevalence and the large-scale production, prescription, administration, and use of ARV drugs, HIV-ARV drugs have likely found their way into the aquatic ecosystem. Because of a global lack of knowledge that exists for the efficient detection and removal of ARVs from wastewater, as well as the non-assessment of eco-toxicological risks associated with ARV drugs in the environment, an urgent need exists to investigate presence, concentrations, removal as well as detection methods of HIV-ARVs in water, and to identify drug sources, amounts released into waste water and their potential effects on human and animal health.

1.2 PROJECT AIMS

This project aims to establish the presence and contamination levels, as well as potential implications of HIV-ARs in discharged wastewater effluent, natural receiving waters, treated drinking water, and fish from natural surface water reservoirs in South Africa. Since the presence and concentrations are not known, it is premature to determine if there would be any effects on the viral biology of natural waters, or if the concentrations in drinking water (treated drinking water or drinking water from groundwater sources) may cause resistance. Experimental determinations and risk assessment can be motivated in follow-up studies if warranted by the results of this study.

Objectives

1. To conduct a literature survey on HIV-ARV presence in the environment. A first screening showed no reports whatsoever. The literature review therefore had to be expanded to generic pharmaceuticals as well as other ARVs.
2. To determine the major HIV-ARV compounds used in SA. This was done in collaboration with role players such as the pharmaceutical industry and Departments of Health. There were too many compounds of concern, so a cross section was selected based on modes of action.
3. To develop extraction and analytical procedures for selected HIV-ARVs from water and fish. An HPLC-QTOF/MS was used for this purpose, as well as other extraction equipment used in other projects looking at POPs.

4. To collect and analyse water samples from final wastewater effluent from four different wastewater treatment works, at least three rivers, and at least three impoundments. The localities were determined after the literature survey and in consultation with the project reference group.
5. To collect and analyse fish samples from the same sites as for the natural waters in the previous objective were collected, where possible.
6. To interpret the data, generate conclusions, as well as recommendations.

1.3 SCOPE AND LIMITATIONS

The scope of this study entails:

- Establishing the presence and concentrations of HIV-ARV drugs and their metabolites in treated water, drinking water and natural water;
- Establishing the presence and concentrations of HIV-ARVs accumulated in fish in natural waters exposed to sewage treated effluent and storm runoff;
- Establishing the effects of HIV-ARV drugs on the environment, and possible health effects as a result of these ARV drug concentrations in water, or their accumulation in fish;
- Generating motivations for follow-up studies, if warranted by the results of this study.

The limitations of this study may include:

- The sampling area. To sample all of the nine provinces of SA would be too costly, also adding to the time requirements. Therefore, only selected provinces were considered, and not for all matrixes.
- Predicting effects on viral ecology and human resistance knowing the concentrations of the compounds of interest in the water was beyond the scope of this study.
- Claims that all anti-viral medication detected in the environment is due to HIV treatment only when the medication may also be used in the treatment of other infections (e.g. Hepatitis B), which may also lead to resistance in humans and super infections in other infections was not tested for;
- The stabilities of the ARV drugs found in the environment are unknown.

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

Ecologists and toxicologists started expressing concern about the potential adverse effects of pharmaceuticals in the water supply in the mid-1960s, but it was not until the 1970s that the presence of pharmaceuticals was documented (Snyder et al., 2003). Since the 1990s, water contamination with pharmaceuticals has been a major environmental concern (Doeer-MacEwen and Haight, 2006). Until this time, pharmaceuticals in water systems were largely ignored because of their relative solubility and containment in waterways compared to other conventional pollutants such as industrial chemicals, agrochemicals, and industrial wastes and by-products (Water Encyclopaedia, 2009). Since then, a great deal of attention and research has been devoted to the ecological and physiological risks of associated with pharmaceuticals in water. In the early 2000s, most of this research focused on steroid hormones (for their endocrine disrupting capabilities) and antibiotics (Daughton, 2008).

To detect pharmaceuticals in the environment, powerful analytical methods are needed. Because of the high polarity of some pharmaceuticals, liquid chromatography (LC) is the preferred analytical method to separate the raw water sample or the pre-cleaned and concentrated extract. There exists a need, however, for modern advanced mass spectrometry (MS) to distinguish between multiple pharmaceuticals and interfering matrix compounds and to obtain sufficient sensitivity. Now, tandem MS/MS making use of multiple reaction monitoring (MRM) with quadrupole instruments is at the top of the list when it comes to pharmaceutical quantification technology (DeMeestere *et al*., 2010). However due to limitations of the MS/MS it is only suitable for specific target analysis.

There is an increased interest in the development of methods that permit multi-residue screening in full-scan or non-targeted way. In this regard, accurate mass-based high-resolution mass spectrometry (HRMS) is receiving increased attention. HRMS is a technique that is much more flexible and will lead the field of multi-residue analysis in the near future (Kaufmann *et al.,* 2010). Among the available HRMS technologies are the QTOF mass analysers.

2.2 PHARMACEUTICALS AND PERSONAL CARE PRODUCTS (PPCPs)

2.2.1 Introduction

Pharmaceuticals and personal care products (PPCPs) are a diverse group of thousands of chemicals used as painkillers, antibiotics, contraceptives, beta-blockers, lipid regulators, impotence drugs, dental care products, soaps, sunscreen agents, and hair care products. The WHO estimated in 2001 that antibiotics were often the largest single group of drugs purchased in developing countries. Intact and metabolized pharmaceuticals are excreted by humans and animals and discharged into the aquatic environment via the release of municipal wastewater and direct discharge, while personal care products will also enter the aquatic environment through showering and bathing in natural waters. In general, municipal waste water treatment plants (WWTPs) and personal product manufacturing plant effluents are the major sources of these chemicals. In some instances, up to a 100 ng/l of individual PPCPs have been measured in treated water. Concerning antibiotics, veterinary medicine/livestock husbandry practices, aquaculture, and human medicinal treatment constitute the major sources.

The fate of PPCPs in WWTPs is dependent on the plant's process and efficiency. In some cases, the substances pass through the plant more or less unchanged. In other cases, the substance is metabolized, inactivated, or destroyed. Environmental concentrations of various PPCPs have been reported in many countries including Japan, Denmark Germany, UK, USA, and Brazil. Synthetic steroids, antibiotics, cytostatics, drugs for lowering blood lipid levels, anticonvulsants, beta-blockers, x-ray contrast agents, and fluoxetine formulations are of environmental concern based on recent studies. A recent study from Germany found that anti-inflammatory drugs degraded and were almost completely removed from sewage during biological treatment. Musk fragrances were only partially removed as they absorb onto suspended solids. The partial removal of these substances by STPs leads to their presence in the environment. The authors concluded that existing WWTPs should be upgraded in order to reduce releases. The removal efficiencies of different PPCPs in WWTPs vary considerably due to the low concentrations of individual PPCPs and their properties.

Because of their low volatility, PPCP distribution through the environment will mainly occur through aqueous transport and food chain dispersal (Richardson et al., 2005). PPCPs, such as di(ethylhexyl)phthalate, benzophenone, ibuprofen, and triclosan have been detected in drinking water from California, in concentrations between 10.0 and 0.16 µg/l. Treated water from a well-run water treatment plant in Finland showed that although most pharmaceuticals were removed by treatment, ciprofloxacin was not removed. In China N,N-diethyl-meta-toluamide (DEET) and bezafibrate was found to survive water treatment. Compounds such as meprobamate, atenolol, carmemazepine and fluoxetine were detectable in US drinking water. In most cases, mention was made of potential metabolites forming during treatment processes, which, potentially, are also toxic.

2.2.2 Routes of PPCPs into the environment

Pharmaceutical residues may find their way in the environment via various routes. A substantial fraction of the global production of pharmaceuticals takes place in low-cost production countries. Recent reports demonstrate that such production sites may emit very large quantities of e.g. antibiotics to local surface waters. The major route of pharmaceutical substances to reach the aquatic environment is excretion from humans. Since many pharmaceutical substances are not metabolized in the body, they are excreted in biologically active form via the urine. Many pharmaceutical substances are not fully taken up from the intestine into their blood stream; these will be excreted via the faeces. Hence, both urine and faeces from treated patients contain pharmaceutical residues. An additional source to environmental pollution with pharmaceuticals is improper disposal of unused or expired drugs. Some pharmaceutical residues may bind strongly to soil particles, with little tendency to leak out to ground water or to local surface waters, while other more water-soluble residues can leach with rain and reach both groundwater and surface waters. Wastewater treatment plants (WWTPs) are not designed to manage pharmaceutical product and remnants deposited into the water systems and environment from human consumption and excretion, and improper disposal of these products. Once in the water they can have adverse effects on organisms and the aquatic ecology of a habitat (Daughton, 2008; Jerker, 2009). The effects of these pharmaceuticals on human health have however not been determined.

2.2.3 Fate of Pharmaceuticals in the environment

The fate of incoming pharmaceutical residues in the WWTP is quite unpredictable. Some substances seem to be more or less completely eliminated, while others pass the different steps in the WWTP unaffected. There is no systematic knowledge at hand to predict how and why this occurs. Pharmaceutical residues that have been conjugated (bound to a bile acid) before being excreted from the patients may undergo de-conjugation in the WWTP, yielding higher concentrations of free pharmaceutical substance in the outlet from

the WWTP than in its incoming water. Some pharmaceuticals with large sales volumes have not been detected in the incoming water to the WWTP, indicating that complete metabolism and degradation must have occurred already in the patient or during the transport of sewage from the household to the WWTP.

2.2.4 Health effects of PPCPs on humans

The scope of human exposure to pharmaceuticals and personal care products from the environment is a complex function of many factors. These factors include the concentrations, types, and distribution of pharmaceuticals in the environment; the pharmacokinetics of each drug; the structural transformation of the chemical compounds either through metabolism or natural degradation processes; and the potential bioaccumulation of the drugs (Daughton, 2008). More research is needed to determine the effects on humans of chronic exposure to low concentrations of pharmaceuticals. The full effects of mixtures of low concentrations of different pharmaceuticals are also unknown (American Water Works, 2009).

Although research has shown that PPCPs are present in water bodies throughout the world, no studies have shown a direct impact on human health. However, even with the absence of empirical data, the possibility of adverse outcomes due to interactions or long-term exposures to these substances cannot be ruled out.

2.2.5 Environmental effects

While the full effects of most PPCPs on the environment are not understood, there is concern about the potential they have for harm because they may act unpredictably when mixed with other chemicals from the environment or concentrate in the food web. Additionally, some pharmaceuticals are active at very low concentrations, and are often released continuously in large or widespread quantities.

Because of the high solubility of most pharmaceuticals, aquatic organisms are especially vulnerable to their effects. The increased presence of estrogen and other synthetic hormones in wastewater due to birth control and hormonal therapies has been linked to increased feminization of exposed fish and other aquatic organisms (Washington State University, 2009). The chemicals within these pharmaceuticals products could either affect the feminization or masculinization of different fishes, therefore affecting their reproductive rates (Siegrist et al., 2004). Studies also show that pharmaceuticals tend to accumulate in fish (Jerker, 2009), making fish particularly vulnerable.

2.3 ARVs AND HIV/AIDS IN SOUTH AFRICA

The Human Sciences Research Council (HSRC) (2011), a South African institution, estimates 10.9% of all South Africans have HIV/AIDS. Additionally, the Central Intelligence Agency (2007) estimates that 310 000 individuals died in South Africa from HIV/AIDS in the year 2009. The rising prevalence rate has increased from 10.6% in 2008 to 12.2% in 2012. In 2012 alone, the HSRC reported 470 000 new diagnoses – or nearly 1 100 new infections every day. That is 100 000 more than was seen just one year earlier in 2011. There are currently 1.9 million people in South Africa on therapy, and this makes up 30% of the 8 million people worldwide on ARVs, according to SA's Minister of Health, Dr Aaron Motsoaledi (Erasmus, 2014). Some of the other facts he mentioned included:

- Forty-nine percent of women who die in childbirth and pregnancy are HIV-positive.
- Thirty-five percent of children who die under the age of five are HIV-positive.
- In 2004, there were 400 000 people on ARVs in SA and now there are 2.4 million.
- The high numbers of people receiving their ARV medicines once a month are contributing to the lengthy waiting times at government clinics.

- It is hoped to have 4.6 million South Africans on ARVs by the year 2016.
- The battle against TB will not be won unless the battle against HIV/AIDS is won, as TB kills 90% of HIV-positive people.
- Twenty million people in SA have been tested for HIV.

The HIV-ARVs chosen for this study are listed in Table 2.1. Selection criteria were:

(i) used in South Africa
(ii) consumption or prescription quantities,
(iii) occurrence and frequency of the detection of these compounds in the environment as conveyed in literature (Prasse *et al.,* 2009), and
(iv) availability of analytical standards.

Table 2.1: HIV ARVs used for HIV treatment in South Africa

CLASS	NAME	MOL MASS (g/mol)	(M+H) *m/z*	SOLUBLE IN	CAS
NRTIs	Zidovudine	267.242	268.104	Water	30516-87-1
	Stavudine	224.213	225.087	Water	3056-17-5
	Didanosine	236.227	237.227	Water	69655-05-6
	Tenofovir	287.0256	288.0856	Methanol	147127-20-6
	Lamivudine	229.2598	230.0594	Water	134678-17-4
	Abacavir	286.332	287.1615	Water	216699-07-9
NNRTIs	Efavirenz	432.9734	433.9734	Methanol	154598-52-4
	Nevirapine	266.888	568.3204	Methanol	129618-40-2
PIs	Lopinavir	628.3697	629.3697	Methanol	192725-17-0
	Ritonavir	720.32	721.32	Methanol	155213-67-5
	Saquinavir	766.95	767.95	Methanol	149845-06-7
	Nelfinavir	663.9	267.124	Methanol	159989-65-8

Table 2.1 shows the 11 ARVs that were used in the development of the method for analysing ARV in water. Their molecular mass as well as their mass to charge ratio is shown. The mass to charge ratio is used to identify the compound once it has been analysed with the HPLC-QTOF/MS. Some of the ARV standards are water soluble, while others are methanol soluble. The solubility of the ARV is important to know with regard to protocols used when extracting the ARV from water.

CHAPTER 3: MATERIALS AND METHODS

3.1 INTRODUCTION

An analytical method was developed for the determination of 11 antiretroviral drugs (Abacavir, Efavirenz, Didanosine, Lamivudine, Lopinavir, Nelfinavir, Nevirapine, Ritonavir, Stavudine, Saquinavir, Tenofovir, and Zidovudine) in various waters and in fish plasma. Compain et al. (2005) used solid phase extraction (SPE) for the extraction of Lamivudine, Stavudine and Zidovudine from plasma samples. SPE is also the method used for the analysis of antiviral drugs in natural waters and has been used in the detection of Osetamivir Carboxylate in surface and wastewaters (Soderstrom et al., 2009 and Ghosh et al., 2009). The simultaneous analysis of a broad spectrum of antiviral drugs is made more challenging due to different chemical properties and different pKa-values. Because of their high polarity, and use of larger sample volumes (sample volumes of plasma is about 300 µL, where sample volumes of water is ranges from 200 mL to 1000 mL) antiviral drugs are probably not effectively retained on common SPE sorbent materials. The same is true for reverse-phase columns used in liquid chromatography, which may have consequences with regard to chromatographic resolution (Hemstrom and Irgum, 2006). However, one of the main objectives of this study was the development of a method for the simultaneous analysis of 11 antiretroviral HIV drugs in environmental water and fish using SPE and HPLC-QTOF/MS.

3.2 WATER SAMPLING

Water samples were collected at various sites across South Africa. Water samples collected were natural waters (surface water), drinking water (from taps), final effluent from WWTPs, and groundwater (used for irrigation and domestic purposes, including drinking).

It became clear that water samples had to be collected as close as possible in time to analyses, and had to be analysed within a matter of days. Water samples were collected in 500 mL Shott bottles that had been rinsed with a mixture of hexane and methanol. Water was collected from bridges by means of a rope tied to the bottle. Samples from dams were collected by grab sampling from approximately 30 cm below the water surface. This was done by hand in shallow areas. Drinking water from taps and stored water from groundwater sources was also collected as above. The water was allowed to flow for about 30 seconds from taps, before a drinking water sample was taken. After sampling, water samples were transported to the lab on ice in cooler boxes. Table 3.1-3.4 indicate the sites where samples were collected, along with the GPS coordinates. Samples were collected in selected provinces in South Africa, including Free State (FS), Gauteng (GAU), KwaZulu-Natal (KZN), Northern Cape (NC), North West (NW), Eastern Cape (EC), Western Cape (WC) and Mpumalanga (MP).

Table 3.1: Drinking water sampling sites and their GPS coordinates

	Site	*Site code*	*Province*	*GPS coordinates*	
1	Bloemfontein	DW 1 FS	FS	29.04	-26.10
2	Parys	DW 2 FS	FS	26.54	-27.26
3	Viljoenskroon	DW 3 FS	FS	27.12	-26.57
4	Kutsong	DW 1 GAU	GAU	26.21	-27.18
5	Welverdiend	DW 2 GAU	GAU	26.23	-27.16
6	Lenasia	DW 3 GAU	GAU	26.17	-27.49
7	The cradle	DW 4 GAU	GAU	26.02	-27.44
8	Carletonville	DW 5 GAU	GAU	26.20	-27.22
9	Fochville	DW 6 GAU	GAU	26.28	-27.29
10	Fochville 2	DW 7 GAU	GAU	26.29	-27.29
11	Gold reef city	DW 8 GAU	GAU	26.14	-28.00
12	Rivonia	DW 9 GAU	GAU	26.03	-28.03
13	Central PTA	DW 10 GAU	GAU	*25.44*	-28.11
14	Garsfontein Rd	DW 11 GAU	GAU	24.47	-28.16
15	John Voster Dr	DW 12 GAU	GAU	25.52	-28.11
16	Krugersdorp	DW 13 GAU	GAU	26.05	-27.45
17	Sunninghill	DW 14 GAU	GAU	26.02	-28.03
18	Durban	DW 1 KZN	KZN	29.52	-31.02
19	Pongola	DW 2 KZN	KZN	27.22	-31.36
20	Colesberg	DW 1 NC	NC	30.42	-25.07
21	Hopetown	DW 2 NC	NC	29.59	-24.10
22	Potchefstroom	DW 1 NW	NW	26.41	-27.05
23	Klerksdorp	DW 2 NW	NW	26.53	-26.39
24	Orkney	DW 3 NW	NW	26.58	-26.40
25	Vaal reefs	DW 4 NW	NW	26.56	-26.43
26	Vredefort	DW 5 NW	NW	36.53	-27.15
27	Coligny	DW 6 NW	NW	26.20	-26.18
28	Biesiesvlei	DW 7 NW	NW	26.22	-27.05
29	Sannieshof	DW 8 NW	NW	26.31	-25.54
30	Muiskraal	DW 9 NW	NW	26.15	-27.09
31	Nelspruit	DW 1 MP	MP	25.28	-30.58

Table 3.2: Wastewater treatment plant effluent sampling sites and their GPS coordinates

	Site	*Site code*	*Province*	*GPS coordinates*	
1	Boskoppies	WWTP 1 GAU	GAU	26.19	-27.56
2	Kutsong	WWTP 2 GAU	GAU	26.21	-27.18
3	Potchefstroom	WWTP 1 NW	NW	26.45	-27.05
4	Atlantis	WWTP 2 WC	WC	33.36	-18.28

Table 3.3: Surface water sampling sites and their GPS coordinates

	Site	Site code	Province	*GPS coordinates*	
1	Sundays River – Port Elizabeth	NW 1 EC	EC	33.41	-25.49
2	Central Port Elizabeth	NW 2 EC	EC	33.57	-25.35
3	Orange River – Aliwal Noord	NW 3 EC	EC	30.85	-26.70
4	Stormberg spruit	NW 4 EC	EC	30.85	-26.50
5	Vaal River – Viljoenskroon	NW 1 FS	FS	27.11	-26.41
6	Orange River – Gariep Dam	NW 2 FS	FS	30.62	-25.50
7	Caledon River – Wepener	NW 3 FS	FS	27.72	-26.96
8	Caledon River – Maseru	NW 4 FS	FS	29.29	-27.45
9	Caledon River – Fiksburg	NW 5 FS	FS	28.88	-27.88
10	Loch logan – Bloemfontein	NW 6 FS	FS	29.11	-26.20
11	Modder river	NW 7 FS	FS	28.96	-26.35
12	Groot vet River	NW 8 FS	FS	28.63	26.90
13	Klein vet River	NW 9 FS	FS	28.63	-26.95
14	Sand River (Senekal)	NW 10 FS	FS	28.32	-27.61
15	Wonderfontein – Carletonville	NW 1 GAU	GAU	26.21	-27.18
16	Suid van Kutsong	NW 2 GAU	GAU	26.21	-27.18
17	Loopspriut Fochville	NW 3 GAU	GAU	26.29	-27.30
18	Wonderfontein bo-loop	NW 4 GAU	GAU	26.18	-27.22
19	Lenasia Wetland	NW 5 GAU	GAU	26.72	-27.53
20	Pongola River – Pongola	NW 1 KZN	KZN	27.23	-31.40
21	Msunduzi river – Pietermaritzburg	NW 2 KZN	KZN	29.36	-30.24
22	Inanda dam	NW 3 KZM	KZN	29.40	-30.51
23	Schoemanskloof spruit	NW 1 MP	MP	25.23	-30.30
24	Vaal River – Rob Ferreira	NW 2 MP	MP	27.88	-25.19
25	Botshabelo stream	NW 3 MP	MP	29.20	-28.70
26	Vaal river – Barkley west	NW 1 NC	NC	28.33	-24.31
27	Orange River – Vanderkloof dam	NW 2 NC	NC	28.59	-24.44
28	Harts River – Spritzkop dam	NW 3 NC	NC	28.12	-24.49
29	Vaal River – Barkley west	NW 4 NC	NC	28.54	-24.55
30	Orange River – Douglas	NW 5 NC	NC	29.16	-23.69
31	Orange River – Vanderkloof dam	NW 6 NC	NC	29.59	-24.43
32	Vaal River – Bloemhof dam	NW 1 NW	NW	27.28	-25.40
33	Mooi River – Potchefstroom	NW 2 NW	NW	26.42	-27.06
34	Loopspriut – Potchefstroom	NW 3 NW	NW	26.43	-27.08
35	Mooi River – Boskop dam	NW 4 NW	NW	26.30	-27.07
36	Klipdrift Dam	NW 5 NW	NW	26.36	-27.53
37	Modder dam	NW 6 NW	NW	26.42	-27.09
38	Vaal River – upstream from Orkney	NW 7 NW	NW	26.56	-26.51
39	Vaal River – downstream from Orkney	NW 8 NW	NW	27.06	-26.31
40	Vaal River – Potchefstroom	NW 9 NW	NW	26.56	-27.03
41	Mooi River – upstream from Potchefstroom	NW 10 NW	NW	26.35	-27.05
42	Mooi River – Muiskraal	NW 11 NW	NW	26.26	-27.07
43	Harts river – Sannieshof	NW 12 NW	NW	26.31	-25.49

44	Harts River – Baberspan	NW 13 NW	NW	26.35	-25.34
45	Vaal River – Bloemhof dam inlet	NW 14 NW	NW	27.63	-25.68
46	Vaal River – Bloemhof Dam outlet	NW 15 NW	NW	27.66	-25.61
47	Zeekoevlei	NW 1 WC	WC	34.33	-18.30

Table 3.4: Groundwater sampling sites and their GPS coordinates

	Site	**Site code**	**Province**	***GPS coordinates***	
1	Wildfontein 1	GW 1 GAU	GAU	26.17	-27.23
2	Wonderfontein	GW 2 GAU	GAU	26.24	-27.14
3	Vereeniging	GW 3 GAU	GAU	26.38	-27.38
4	Wildfontein 2	GW 4 GAU	GAU	26.17	-27.23
5	Schoemanskloof	GW 1 MP	MP	25.23	-30.30
6	Botshabelo	GW 2 MP	MP	29.23	-26.68
7	Potchefstroom – Bult	GW 1 NW	NW	26.41	-27.05
8	Potchefstroom – Haaskraal	GW 2 NW	NW	26.47	-27.04
9	Potchefstroom – Vyfhoek 831	GW 3 NW	NW	26.41	-27.07
10	Potchefstroom – Vyfhoek 826	GW 4 NW	NW	26.40	-27.09
11	Potchefstroom – Wilgeboom	GW 5 NW	NW	26.46	-27.03
12	Potchefstroom – Miederpark	GW 6 NW	NW	26.44	-27.05
13	Potchefstroom – Skubarts	GW 7 NW	NW	26.41	-27.06
14	Boskop – Muiskraal	GW 8 NW	NW	26.26	-27.02
15	Potchefstroom – Wilgeboom 2	GW 9 NW	NW	26.45	-27.05
16	Boskop Garage	GW 10 NW	NW	26.34	-27.07
17	Renosterbult	GW 11 NW	NW	26.21	-27.08
18	Baberspan	GW 12 NW	NW	26.57	-25.58
19	Potchefstroom – excelsior	GW 13 NW	NW	26.42	-27.05

3.3 FISH SAMPLING

3.3.1 Fish samples – The African Catfish

The African catfish (*Clarias gariepinus*) is a dominant freshwater fish found in most natural water in South Africa. It can grow to 2 m long and can weigh up to 59 kg. Its body colouration varies from olive green, to brown and black. The ventral side of its body are pale olive to white. It is a heavy boned, flat-headed fish. The head has four pairs of long trailing sensory organs known as ‘barbells’ around its mouth giving it a similar appearance to a cat, hence the name catfish. It has a high number of gill folds varying from 24 to 110, the number increasing with the size of the fish. The body is elongated with long, low dorsal and anal fins and a smoothly rounded tail fin. The skin is leathery and has no scales. It has a small but powerful pectoral fin set immediately in front of the anal fin that has a serrated spine. The eyes are small and set far forward in a flat and bony head. At the back of the head, there is a subsidiary breathing organ above the gills that enables this animal to breathe air directly.

Figure 3.1: The African catfish (Species: *Clarias gariepinus*).

Table 3.5: Sample locations of fish and number of fish samples taken

No.	***Place***		***Sampling date***	***Province***	***Samples analysed***
001	Vaal River	Vanderbijlpark	28.01.2014	GAU	2 samples
002	Vaal River	Schoemansdrift	30.01.2014	NW	14 Samples
003	Bloemhof Dam	Bloemhof	10.02.2014	NW	10 samples
004	Lenasia-west dam	Lenasia	04.03.2014	GAU	7 samples
005	Wonderfontein	Carletonville	20.02.2014	GAU	15 samples
006	Vaal River	Upington	04.02.2014	NC	10 samples

3.3.2 Fish sampling protocols

The field crew was informed in detail about the equipment to be employed for the project, based on the existing information and expectations of the project. The field crew understood the general physical and biological principles that affect fish aggregation and the biology and habitat preferences of the selected species. Sampling was under permit and with NWU ethical clearance. Before sampling, all vials were labelled with a unique code for the fish. The fish was killed by cutting the spine.

3.3.3 Blood sampling

Blood was taken from the ventral aspect of the tail (caudal vein) with an EDTA vacutainer vial and syringe; about 4-6 ml of blood was taken per fish. All blood samples collected were put on ice immediately after sample collection. Once one fish was finished, the process was repeated using the next fish.

3.4 MATERIALS AND METHODS

Materials and ARV standards consisted of Stavudine, Lamivudine, Zidovudine, Abacavir Sulfate, Efavirenz, Nevirapine, Saquinavir Mesylate, Ritonavir, and Nelfinavir. The standards were acquired from Sigma-Aldrich (Johannesburg; South Africa). Didanosine, Lopinavir and Tenofovir were acquired from Industrial Analytical (Johannesburg; South Africa). Formic acid was supplied by Sigma-Aldrich (Johannesburg; S.A.) All solvents were supplied by Anatech Technologies. All solvents used were of HPLC grade and at >99% purity. Cleanert SPE cartridges were used for the extraction of ARVs from fish, and were supplied by Stargate Scientific. Extraction of ARV drugs from water was done on a SPE-DEX 4790 Automated extractor (Horizon Technology, Salem, NH) using 1 µm Walters© filters and C18 filters from Horizon Technologies.

3.4.1 Sample extraction

3.4.1.1 *Water samples*

Extraction of ARV drugs from water samples was done using Horizon Technology's SPE-DEX 4790 automated extraction system. This system is a fully automated SPE system designed to process rapidly environmental water samples using SPE for trace concentration analysis of organic chemical contaminants. Sample volumes ranging between 40 ml up to 8 l are accommodated, although the sample volume used in this study was 500 ml of water. SPE-DEX 4790 is the only extractor system available to handle both clean and particulate-laden environmental matrixes such as; ground water, waste water, drinking water, WWTP water (influent and effluent), and natural water. It is for this reason that it was used in the extraction of ARV drugs from water samples. The programmed method used is shown in Table 3.6. C18 filters and 1 µm filters were used during extraction (Horizon Technology, Inc.).

Table 3.6: Programmed method for SPE of ARV drug from water

Cycle	Reagent	Soak time	Air dry time
Pre-conditioning of filters	Methanol with 0.1% formic acid	1.5 min	15 seconds
Sample loading	N/A	N/A	N/A
Wash cycle	none	none	none
Air dry cycle	N/A	N/A	5 min
Elution	Reagent water	4 min	1 min
	Methanol	4 min	1.5 min

* N/A – not applicable

3.4.1.2 *Blood samples*

Blood samples were collected from fish, as described in Section 3.3.3. Blood samples were centrifuged for 2 min at 6000 rpm to generate plasma from blood samples. Plasma samples (300 µl) were extracted with C18 Cleanert 500 mg/6 ml SPE cartridges (Stargate Scientific, Johannesburg). SPE cartridges were conditioned with 1 ml methanol and 0.1% formic acid, before the plasma sample was loaded onto the cartridge. The sample was washed with 4 ml hexane, and eluted with 4 ml of 50:50 (v/v) methanol/water. It was then dried completely under nitrogen flow using the DryVap (Horizon Technologies), and eluted with 750 µl of mobile phase A (water with 0.1% Formic acid) and 750 µl of mobile phase B (acetonitrile and 0.1% formic acid) into sample vials, and injected into the HPLC-TOF/MS for analysis.

3.4.2 Sample analysis

3.4.2.1 *Sample preparation*

Water samples were prepared for analysis by filtering 2.5 ml water through a 1 µm glass fibre filter (Sigma Aldrich, South Africa). The sample was placed into Sample vials and analysed on HPLC- QTOF/MS. Because the QTOF is an accurate mass-based spectrometer, it can be run on both full-scan and targeted scan modes, allowing the analyses to be done without pre-concentration.

Water and fish samples were evaporated to dryness (under nitrogen flow) using the Horizon Technology DryVap® Concentrator System. This system is designed to automate the drying, evaporation, and

concentration of organic extracts prior to chromatographic analysis, combining what were once manual steps into one automated process. After evaporation, the samples were washed into auto sampler sample vials with 500 µl of mobile phase A, and 500 µl of mobile phase B, and then analysed by the HPLC-QTOF/MS.

3.4.2.2 *Sample analysis*

Water and fish plasma extracts were analysed on the HPLC-QTOF/MS (Agilent Technologies, Germany) consisting of a HPLC (high-pressure liquid chromatograph) (series 1100) and 6540 Accurate Mass QTOF-MS (Quadrupole time-of-fight – Mass spectrometer) (Agilent Technologies, Waldbronn, Germany) (Figure 3.2). This HPLC-QTOF/MS features Agilent jet stream Thermal Focusing Technology for improved sensitivity, as well as the enhanced MassHunter Workstation software for superior data mining and analysis capabilities. These features allow the Agilent HPLC-QTOF/MS to deliver exceptional sensitivity, excellent mass accuracy, fast data acquisition, and streamlined qualitative and quantitative analysis.

Figure 3.2: The HPLC-QTOF/MS used for the detection of HIV-ARVs in water and fish plasma samples. All calculations were done using MassHunter (Version 5.0) Software (Agilent; Germany).

3.4.2.3 *Instrument Conditions and Parameters*

Samples were injected using 0.2 µl into the HPLC, and ran with polar mobile phase A (water with 0.1% formic acid) and organic mobile phase B (acetonitrile with 0.1% formic acid). The flow rate was 0.200 ml/min, isocratically (solvents ran on 50% mobile phase A and 50% mobile phase B). An Agilent C18 column was used in the analysis. Stop-time was set at 8 min, with no post time, and pressure limits of 1-1000bar.

The source used for the QTOF/MS was a Dual AJS ESI, with the source conditions being: Gas temperature, 300°C; Drying gas, 8l/min; Nebulizer at 45 psig; Sheath gas temperature at 300°C, and the sheath gas flow was 8l/min. The VCap was run at 4000 V and the nozzle voltage at 0V (default values). All standard stock solutions were run on positive ionization, and on MS (segmented) mode. The acquisition mass minimum and maximum ranges were 100-1000 *m/z* and included the reference masses of 121 *m/z* and 922 *m/z.*
The MS-TOF conditions were ran on default at: fragmentor voltage 200V, skimmer at 65V, and with collision energy of 0 V. In the infusion method, no fragmenting is needed.

MassHunter software was used to identify the specific ARV in the water samples analysed, by searching for the compounds via their chemical formulas, with a match made if their chemical formula, retention time, and mass to charge ratio of the compounds correspond.

3.5 QUANTIFICATION AND METHOD VALIDATION

Calibration curves were prepared by spiking 500 ml of surface dam water with limited contamination (determined with prior analyses for no interfering or competing compounds). This gives a best representation of the matrixes that were analysed. Water was spiked with the analytes at concentrations of 0.1, 0.2, 0.4, 1, 2, 5, 10, 12, 40, and 100 ng/l. A concentration of 10 ng/l of internal standard (Ritonavir) was added to all of these aliquots. Samples were then subjected to the SPE extraction as described in Section 3.4. Linear regression was applied to the calibration curves with a weighting factor of 1/x for water, and 5/x for fish plasma.

The sensitivity of the method was assessed by determining the LOQ for analytes in the calibration standards using 500 mL of surface water spiked with different concentrations of the various analytes. The sample was then extracted by SPE. The LOQ was defined as the lowest calibration point in the linear regression (determined by MassHunter), with a signal to noise of 10. The accuracy of the method was determined by spiking known amounts of analytes (100 ng) into limited contaminated surface water (three replicates of each). Relative recoveries were calculated by dividing the measured amount of analytes, with the spiked quantities. Blanks were subjected to the same preparation, extraction, and analysis process.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 INTRODUCTION

This section presents the quantitative data for all ARV drugs found at the various water sampling sites (natural water, drinking water, effluent from WWTP and groundwater) and in fish plasma, as well as a general discussion of the results. The concentrations of the identified ARV drugs are given in ng/l, and were determined and calculated using Agilent MassHunter software.

4.2 VALIDATION OF THE ANALYTICAL METHOD

The analytical method was evaluated for sensitivity and accuracy. Relative recoveries were done by dividing the measured concentrations with the original spiked concentrations. Calibration curves showed excellent linearity over the calibration range (Table 4.1), with correlation coefficients greater than 0.98 for all analytes. Percentage relative recoveries varied between 88 ± 13 to 108 ± 3. The LOQ of the analytical method ranged between 0.02 and 1 ng/l. The mobile phases used in this method (water with 0.1% formic acid as mobile phase A and Acetonitrile with 0.1% formic acid) showed good results, although literature states that formic acid has a negative effect on the ionization of analytes (Prasse et al., 2009). The lack of a buffer can however result in peak broadening, and retention time instability. It is because of this reason that 0.1 % formic acid continued to be used in the mobile phases. Calibration curves showed a very good linearity over the calibration range (0.98-0.99) and percentage relative recoveries ranging from 88 ± 13 to 108 ± 3 (Table 4.1). Different matrix effects can account for the varied differences in the extraction efficiencies of ARV drugs.

Table 4.1: Linearity calibration curves, LOQ, and recoveries of ARV drugs in water.

						Surface water
	Analyte	**CAS**	**Purity**	**Linearity**	**LOQ (ng/l)**	**Recoveries (%)**
1	**Stavudine**	3056-17-5	99.9 %	0.9990	0.1	95 +/- 5
2	**Lamivudine**	134678-17-4	99.9 %	0.9994	1	100 +/- 10
3	**Didanosine**	69655-05-6	99.9 %	0.9994	0.1	104 +/- 4
4	**Nevirapine**	129618-40-2	99.9 %	0.9892	0.1	106 +/- 4
5	**Zidovudine**	30516-87-1	99.9 %	0.9999	0.1	100 +/- 3
6	**Abacavir**	216699-07-9	99.9 %	0.9940	0.02	108 +/- 3
7	**Tenofovir**	147127-20-6	99.9 %	0.9996	0.4	101 +/- 4
8	**Efavirenz**	154598-52-4	99.9 %	0.9809	0.1	92 +/- 6
9	**Nelfinavir**	159989-65-8	99.9 %	0.9987	0.02	88 +/- 13
10	**Lopinavir**	192725-17-0	99.9 %	0.9988	0.02	101 +/- 6
11	**Saquinavir**	149845-06-7	99.9 %	0.9991	0.1	97 +/- 5
12	**Ritonavir**	155213-67-5	99.9 %	Internal standard		

4.3 POLLUTANT SCREENING IN WATER AND FISH SAMPLES

During analysis of ARV drugs from water and fish, tox screens (toxicity tests) were also done on the samples. These tox screens revealed the presence of other pharmaceuticals, industrial chemicals, and biological compounds in the water. Some classes of chemical compounds found in the water were; acardicides, adhesives, anabolics, analgesics, androgens, anaesthetics, antihelmintics, antiamebics, antiarrhythmics, antibacterials, antibiotics, anticholesteremics, anticoagulants, anticonvulsants, anticulcervatives, antidepressants, antidiabetics, antidots, antiestrogens, antifungals, antihistamines, antihypertensives, antimalarials, antimigraines, antimycotics, antineoplastics, antioxidants, antiparkinsonians, antiphlogistics, antirheumatics, antiseptics, antispasmotics, antitussives, antivirals, artificial sweeteners, beta-blockers, bronchodilators, cardiotonics, chemotherapeutics, choleretics, cytistatics, dermatics, designer drugs, disinfectants, diuretics, DNA cross-linking agents, doping agents, dyes, enzyme inhibitors, estrogens, explosives, fungicides, gyrase inhibitors, h2-blockers, hallucinogens, illicit drugs, hemostatics, herbicides, hypnotics, illegal drugs, immune modulators and suppressants, insecticides, laxatives, local anaesthetics, mollusicicides, markers of taxus poisoning, muscle relaxants, neuroleptics, nootropics, parasymatholytics, pesticides, progestins, psychedelics, radiation protectants, rodenticides, scabicides, sedatives, spasmolytics, stimulants (egg. caffeine), stomachics, tranquilizers, tuberculostatics, uriosurics, vasodilators, virucides, virustatics, and x-ray contrasts or contrasting agents, amongst others. Many pharmaceuticals found in the water samples such as antifungals, tuberculostatics, and antimalarials are also given to HIV positive patients, together with ART, for the prevention of certain illnesses (WHO, 2014).

4.4 OCCURRENCE OF HIV ANTIRETROVIRAL DRUGS IN WATER SAMPLES

The results are presented in tabular form (Tables 4.2-4.6) and graphically on maps (Figures 4.1-4.5).

Table 4.2 shows the concentrations of HIV_ARVs in natural water from across South Africa. Most of the 47 samples had no detectable residues. Only 13 samples had detectable residues, of which two had more than one. Stavudine, Lamivudine, Neverapine, Zidovidine, Abacavir, Tenofovir, Nelvinavir and Lopinavir were detected. Figure 4.1 shows the geographic distribution of these data. The highest concentrations were in the Gauteng and North West provinces.

Table 4.3 presents the results of the HIV_ARVs in WWTP effluent from four plants. Two of the plants had quantifiable residues, both from Gauteng. Figure 4.2 shows the data represented geographically.

Table 4.4 contains the data from municipal drinking water. Ten of the 31 samples had quantifiable concentrations of HIV-ARVs. One sample had two HIV-ARVs (DW 1 GAU). The same data are presented geographically in Figure 4.3.

Groundwater also had quantifiable residues (Table 4.5). Seven of the 18 samples had quantifiable residues, and the data are presented geographically in Figure 4.4. One sample (GW 3 NW) had three residues that could be quantified.

Table 4.6 shows the results of the fish plasma analyses. Nine of the 33 samples had quantifiable residues. One sample had two different HIV-ARVs at quantifiable concentrations. The data are presented in a geographic format in Figure 4.5. No quantifiable residues were found in 25 different bottled drinking water samples.

Table 4.2: The results and concentrations (ng/l) of ARVs in natural waters from various sites across South Africa.

	Site	Stavudine	Lamivudine	Didanosine	Nevirapine	Zidovudine	Abacavir	Tenofovir	Efavirenz	Nelfinavir	Lopinavir	Saquinavir
1	NW 1 EC	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2	NW 2 EC	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**1.6**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
3	NW 3 EC	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
4	NW 4 EC	**1.1**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5	NW 1 FS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6	NW 2 FS	<LOQ	**0.89**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
7	NW 3 FS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
8	NW 4 FS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
9	NW 5 FS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
10	NW 6 FS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
11	NW 7 FS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
12	NW 8 FS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
13	NW 9 FS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
14	NW 10 FS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
15	NW 1 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
16	NW 2 GAU	**0.73**	<LOQ	<LOQ	**4.0**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**1.3**	<LOQ
17	NW 3 GAU	<LOQ	<LOQ	<LOQ	**2.4**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
18	NW 4 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
19	NW 5 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
20	NW 1 KZN	<LOQ	<LOQ	<LOQ	**0.84**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
21	NW 2 KZN	<LOQ	<LOQ	<LOQ	<LOQ	**0.6**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
22	NW 3 KZM	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
23	NW 1 MP	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

24	NW 2 MP	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
25	NW 3 MP	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
26	NW 1 NC	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
27	NW 2 NC	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
28	NW 3 NC	<LOQ	<LOQ	<LOQ	<LOQ	**0.87**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
29	NW 4 NC	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**1.2**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
30	NW 5 NC	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
31	NW 6 NC	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
32	NW 1 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
33	NW 2 NW	<LOQ	<LOQ	<LOQ	<LOQ	**0.31**	<LOQ	<LOQ	<LOQ	**0.82**	<LOQ	<LOQ
34	NW 3 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**0.56**	<LOQ	<LOQ	<LOQ	<LOQ
35	NW 4 NW	<LOQ	<LOQ	<LOQ	**0.52**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
36	NW 5 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
37	NW 6 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
38	NW 7 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
39	NW 8 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
40	NW 9 NW	<LOQ	<LOQ	<LOQ	**0.7**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
41	NW 10 NW	<LOQ	<LOQ	<LOQ	**6.8**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
42	NW 11 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
43	NW 12 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
44	NW 13 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
45	NW 14 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
46	NW 15 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
47	NW 1 WC	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

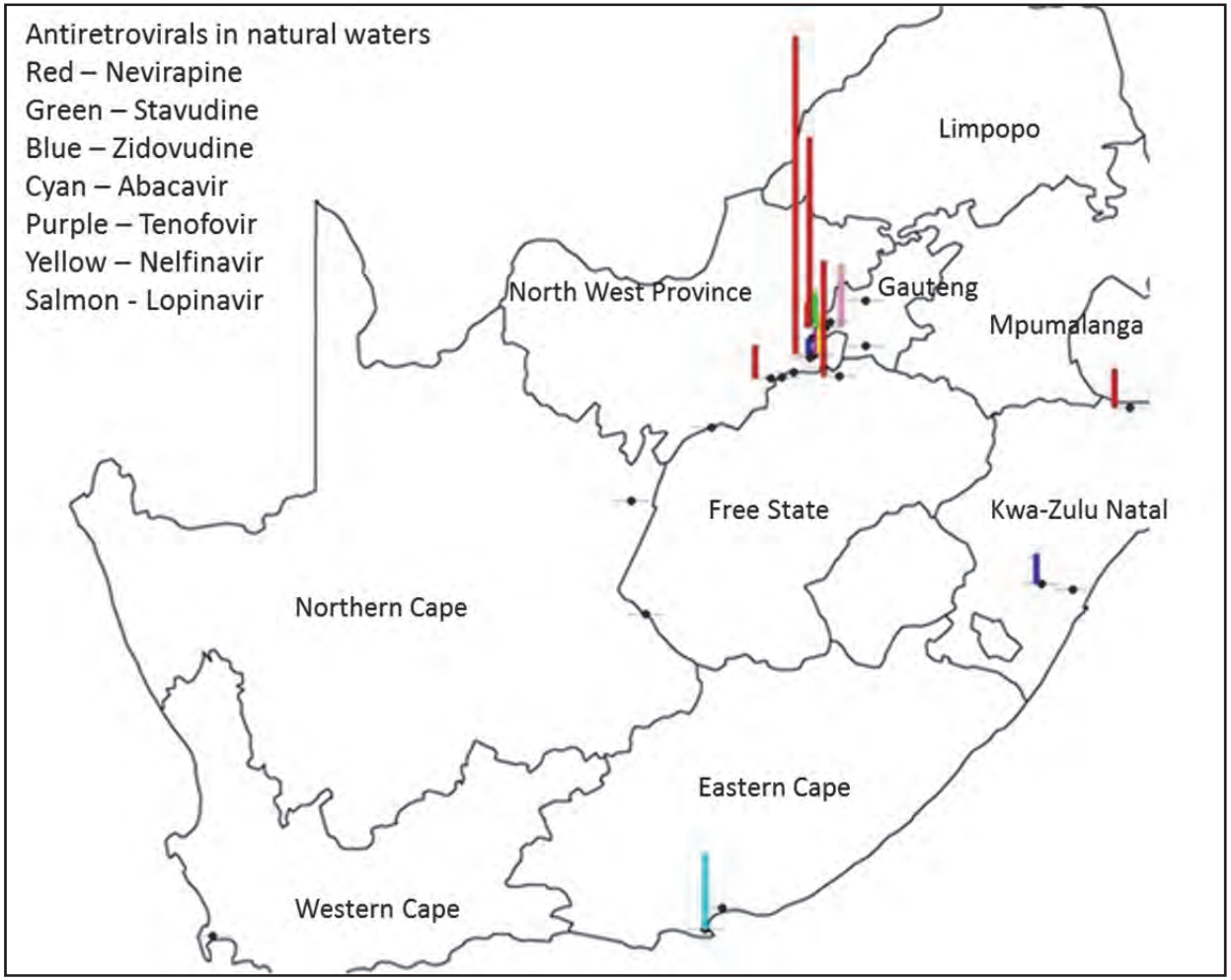

Figure 4.1: A geographical representation of the ARV drugs found in natural waters at different sampling sites across South Africa. Sites with no quantifiable residues are indicated with a black dot. (Max concentration is 6.8 ng/l)

Table 4.3: The results and concentrations (ng/l) of HIV-ARVs in WWTP effluent at various sampling points in South Africa.

		HIV antiretroviral (ng/l)										
	Site	**Stavudine**	**Lamivudine**	**Didanosine**	**Nevirapine**	**Zidovudine**	**Abacavir**	**Tenofovir**	**Efavirenz**	**Nelfinavir**	**Lopinavir**	**Saquinavir**
1	WWTP 1 GAU	<LOQ	<LOQ	<LOQ	**0.43**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2	WWTP 2 GAU	<LOQ	<LOQ	<LOQ	<LOQ	**0.29**	<LOQ	**1.6**	<LOQ	<LOQ	<LOQ	<LOQ
3	WWTP 1 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
4	WWTP 1 WC	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

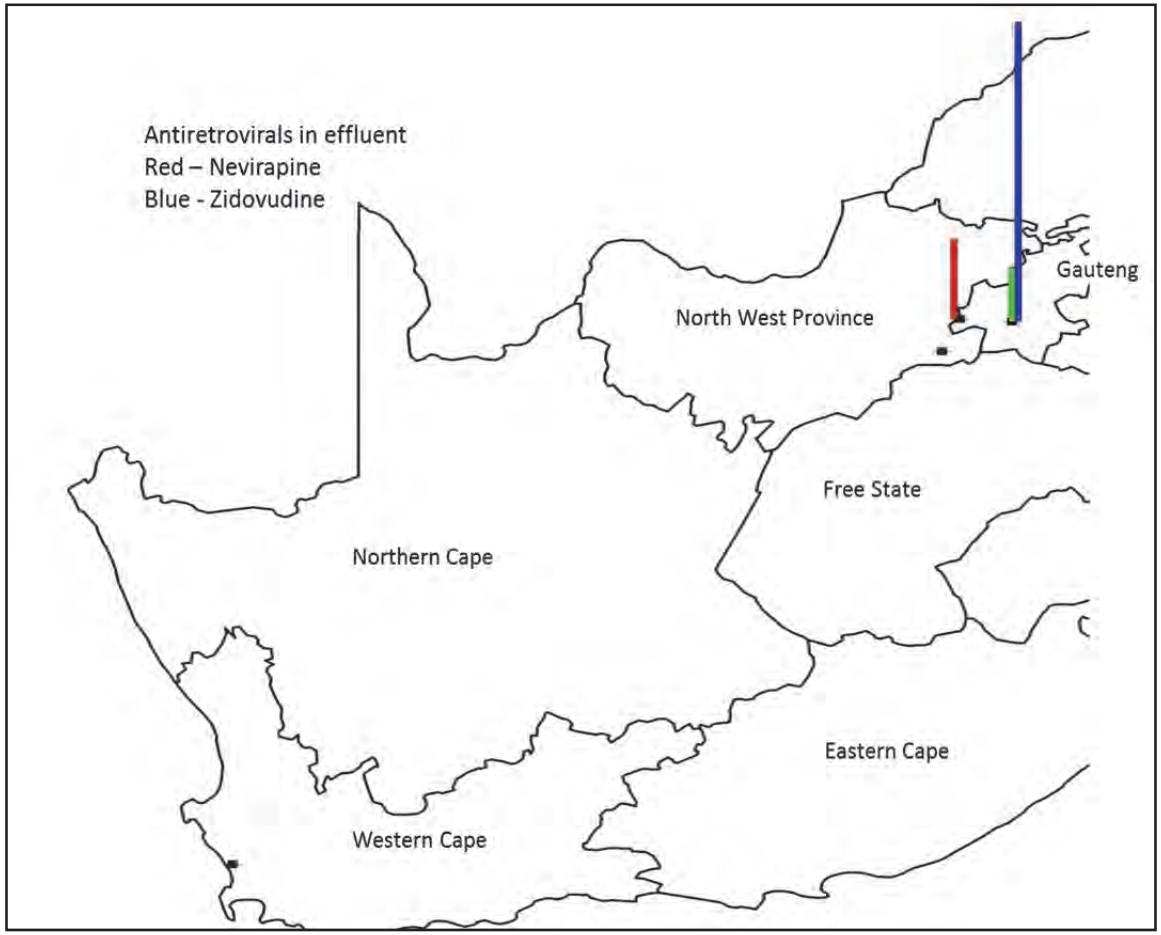

Figure 4.2: HIV-ARV concentrations in WWTP effluent water samples from different sampling sites. (Max concentration is 1.6 ng/l)

Table 4.4: The results and concentrations (ng/l) of HIV-ARVs in municipal tap water from sites across South Africa.

		HIV antiretroviral (ng/l)										
	Site	Stavudine	Lamivudine	Didanosine	Nevirapine	Zidovudine	Abacavir	Tenofovir	Efavirenz	Nelfinavir	Lopinavir	Saquinavir
1	DW 1 FS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2	DW 2 FS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
3	DW 3 FS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
4	DW 1 GAU	<LOQ	<LOQ	<LOQ	**3.5**	**1.9.**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5	DW 2 GAU	<LOQ	<LOQ	<LOQ	<LOQ	**0.4**	**0.5**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6	DW 3 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
7	DW 4 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
8	DW 5 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
9	DW 6 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
10	DW 7 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
11	DW 8 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
12	DW 9 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
13	DW 10 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
14	DW 11 GAU	<LOQ	<LOQ	**0.61**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
15	DW 12 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
16	DW 13 GAU	<LOQ	<LOQ	<LOQ	**0.5**	<LOQ	<LOQ	<LOQ	<LOQ	**1.1**	<LOQ	<LOQ
17	DW 14 GAU	<LOQ	<LOQ	<LOQ	**0.7**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
18	DW 1 KZN	**0.9**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
19	DW 2 KZN	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
20	DW 1 NC	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
21	DW 2 NC	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
22	DW 1 NW	<LOQ	<LOQ	**0.4**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

23	DW 2 NW	<LOQ	<LOQ	<LOQ	**0.3**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
24	DW 3 NW	<LOQ	<LOQ	**3.3**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
25	DW 4 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
26	DW 5 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
27	DW 6 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
28	DW 7 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
29	DW 8 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
30	DW 9 NW	<LOQ	<LOQ	<LOQ	**1.0**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
31	DW 1 MP	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

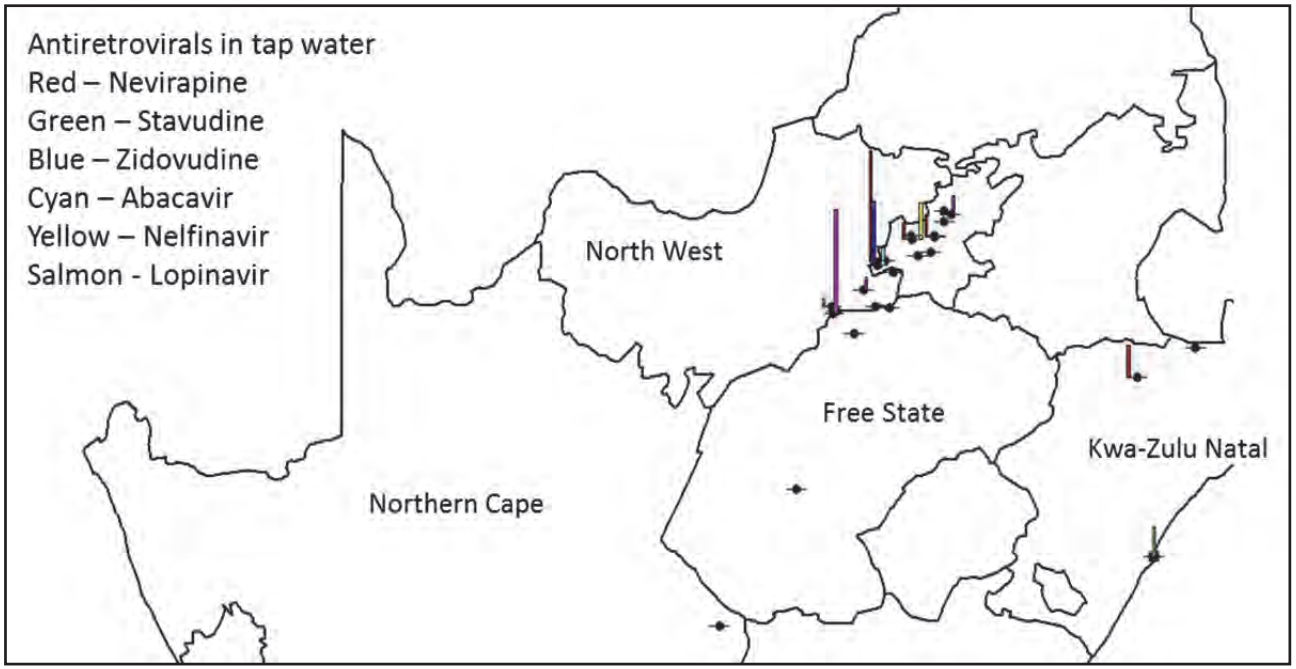

Figure 4.3: Concentrations of HIV-ARVs in tap water samples from various sampling sites in South Africa. (Max concentration is 3.5 ng/l).

Table 4.5: The results and concentrations (ng/l) of HIV-ARVs in groundwater from various sites across South Africa.

	Site	Stavudine	Lamivudine	Didanosine	Nevirapine	Zidovudine	Abacavir	Tenofovir	Efavirenz	Nelfinavir	Lopinavir	Saquinavir
1	GW 1 GAU	<LOQ	<LOQ	<LOQ	**2.5**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2	GW 2 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
3	GW 3 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
4	GW 4 GAU	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5	GW 1 MP	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6	GW 2 MP	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
7	GW 1 NW	**0.9**	<LOQ	<LOQ	**0.3**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
8	GW 2 NW	<LOQ	<LOQ	<LOQ	**0.6**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
9	GW 3 NW	**0.3**	<LOQ	<LOQ	**5.3**	<LOQ	<LOQ	<LOQ	<LOQ	**0.9**	<LOQ	<LOQ
10	GW 4 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
11	GW 5 NW	<LOQ	<LOQ	<LOQ	**0.5**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**1.3**
12	GW 6 NW	<LOQ	<LOQ	<LOQ	**0.6**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
13	GW 7 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
14	GW 8 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
15	GW 10 NW	<LOQ	<LOQ	<LOQ	**4.9**	<LOQ	<LOQ	**2.4**	<LOQ	<LOQ	<LOQ	<LOQ
16	GW 11 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
17	GW 12 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
18	GW 13 NW	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

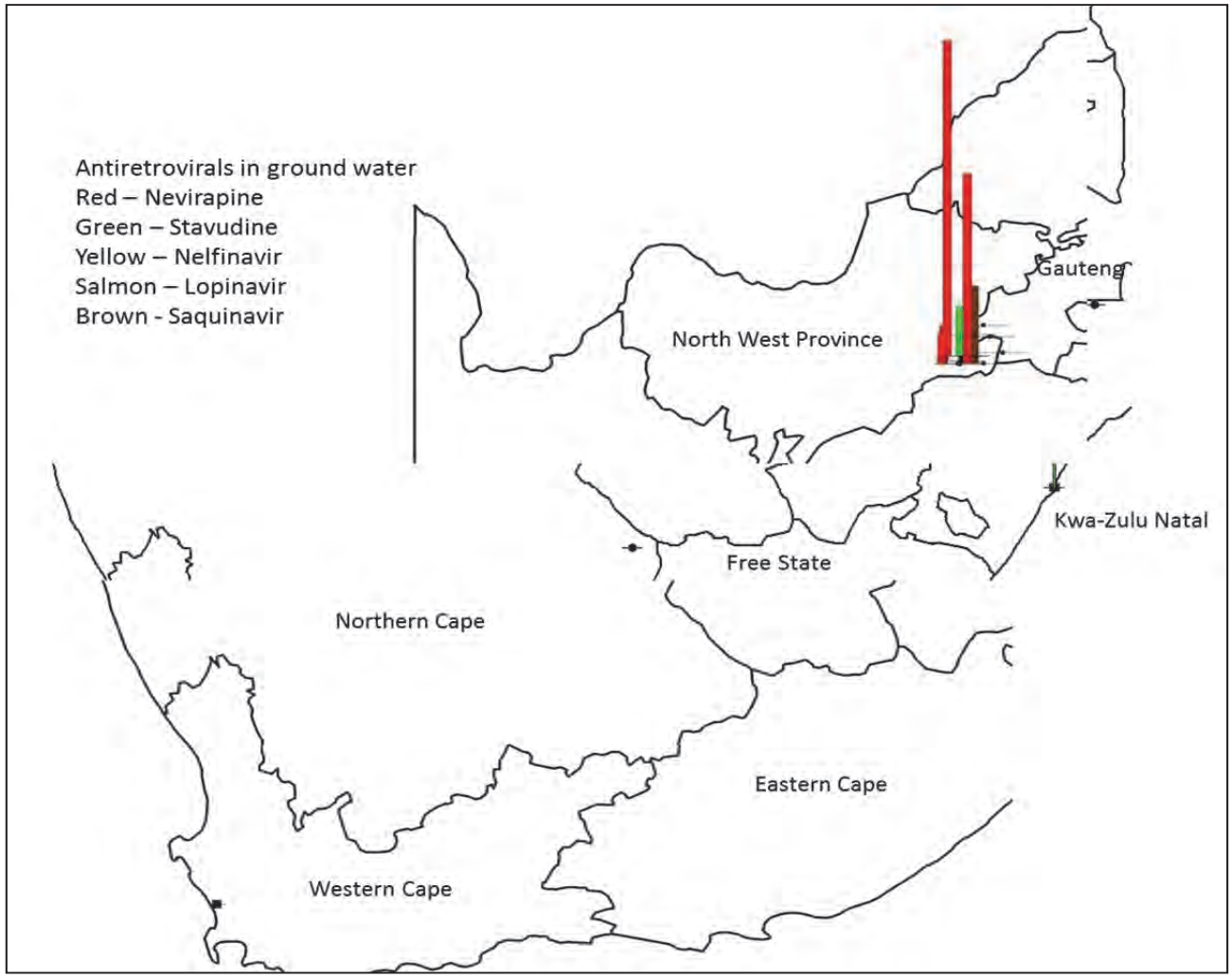

Figure 4.4: Concentrations of HIV-ARVs in ground water from various sites in the North West and Gauteng Provinces. (Max concentration is 4.9 ng/l).

4.5 OCCURRENCE OF ANTIRETROVIRAL DRUGS IN FISH PLASMA

Table 4.6: The results and concentrations (ng/l) of HIV-ARVs in fish plasma samples, caught at various sites across South Africa.

Site	Stavudine	Lamivudine	Didanosine	Nevirapine	Zidovudine	Abacavir	Tenofovir	Efavirenz	Nelfinavir	Lopinavir	Saquinavir	Ritonavir
Bloemhof dam												
BH 1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BH 2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BH 3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BH 4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BH 5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BH 6	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**36**	<LOQ	<LOQ	<LOQ	<LOQ	**47**	<LOQ
BH 7	**95**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BH 8	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BH 9	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BH 10	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**50**	<LOQ
Upington												
UP 1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
UP 2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
UP 3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
UP 4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
UP 5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
UP 6	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
UP 7	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
UP 8	**110**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Wasgoedspruit												
WS 1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
WS 2	<LOQ	<LOQ	**55**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
WS 3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
WS 4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**135**	<LOQ	<LOQ	<LOQ	<LOQ
WS 5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
WS 6	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
WS 7	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
WS 8	<LOQ	<LOQ	**46**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
WS 9	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
WS10	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
WS 11	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
WS 12	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
WS 13	<LOQ	<LOQ	**39.5**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Vanderbijlpark												
VP 1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**90**	<LOQ	<LOQ	<LOQ
VP 2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

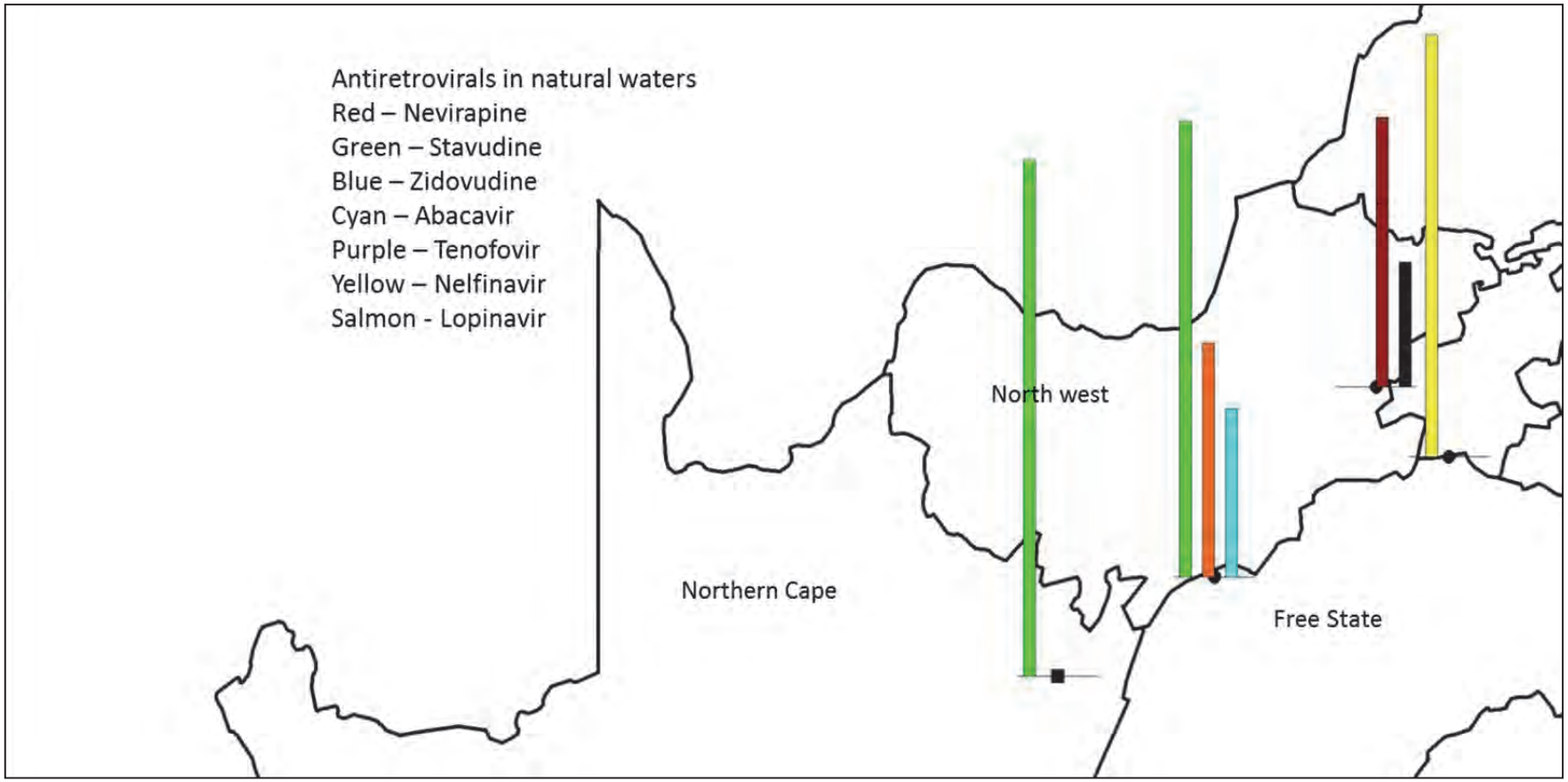

Figure 4.5: ARVs found in fish samples from various sampling sites in the North West, Gauteng and Northern Cape Provinces. (Max concentration of 135 ng/l).

4.6 GENERAL DISCUSSION

4.6.1 Presence of HIV/ARV drugs in water

HIV-ARV drugs have been found at variable concentrations in different water sources in Germany (Prasse *et al.,* 2009 and K'oreje *et al.*, 2012). In this study, the majority of samples had no quantifiable concentrations. In natural waters, quantifiable concentrations of Stavudine, Lamivudine, Nevirapine, Zidovudine, Abacavir, Tenofovir, and Nelfinavir were found. Nevirapine was detected in six samples. The lowest concentration was 0.3 ng/l (Zidovudine) and the highest was Nevirapine at 6.8 ng/l. Efavirenz and Saquinavir were not detected in any of the 47 surface samples.

Of the four WWTP treated effluent samples, only Nevirapine (0.4 ng/l), Zidovudine (0.3 ng/l) and Tenofovir (1.6 ng/l) were quantified in two of the WWTPs. In previous studies, Prasse *et al*. (2009) reported that 87-99% of Lamivudine, Abacavir and Stavudine are removed during the WWTP processes. Elevated concentrations of Zidovudine may occur after WWTP processes; this may be because of the cleavage of glucuronide conjugates, known to be excreted by humans by up to 70% (Veal and Black, 1995). Two sites yielded no quantifiable ARV concentrations even though the drugs, Zidovudine and Nevirapine, are not removed from the water during normal WWTP processes (Prasse *et al.,* 2009).

Of the 31 drinking water samples, Lamivudine, Tenofovir, Efavirenz, Lopinavir, and Saquinavir concentrations were not quantifiable. Nevirapine occurred in five samples (0.3-3.5 ng/l), and Didanosine in three (0.4-3.3 ng/l). Abacavir and Stavudine were identified each in a sample, and Zidovudine was identified in two drinking water samples.

Stavudine, Nevirapine, Tenofovir, Nelfinavir and Saquinavir were quantified from seven of 18 ground water samples, while Nevirapine was found in eight samples (0.3-5.4 ng/l).

Very little literature exists that informs about antiretroviral drugs in drinking water and ground water. As far as we know, this is the first report of such data in South Africa. Due to the scattered nature of sample locations, and the variable presence and concentrations in samples, no meaningful statistics could be done. Because of the high prevalence of HIV in South Africa (12.2%) (Shisana *et al.,* 2014), consumption of antiretroviral drugs is high. Most of the people that are infected with HIV have to be on ART throughout their lives. Considering that, ARV drugs have been reported in all types of water elsewhere (Prasse *et al.,* 2009), there is now enough cause to further consider the implications of potentially contaminated drinking water for human consumption.

4.6.2 Presence of HIV/ARV drugs in fish

In 33 fish plasma samples from four sites, nine samples had quantifiable amounts of HIV-ARVs: Stavudine (n=2), Didanosine (n=3), Abacavir (n=1), Efavirenz (n=1), Nelfinavir (n=1), and Saquinavir (n=2). The concentrations of ARVs in fish blood plasma ranged from 27-135 ng/l. All sites, including a supposedly pristine ('clean') water sample, upstream of Upington, had one or more quantifiable HIV-ARV residues. To our knowledge, this is the first report of HIV antiretroviral drug occurrence in fish. However, since South Africa has a large poor population, many people depend on fish that are caught from river and dams and used as food on a daily basis (CGIAR, 2011). These concentrations indicate that the HIV-ARVs are bio-available, and could possibly bioaccumulate as well. This raises issues such as potential bioconcentration through food webs. This is an issue that may need further investigation.

4.7 SUMMARY

Tox screens (toxicity test) revealed the presence of other pharmaceuticals, industrial chemicals, and biological compounds in the water. Some classes of chemical compounds found in the water were; acardicides, adhesives, anabolics, analgesics, androgens, anesthetics, antihelmintics, antiamebics, antiarrhythmics, antibacterials, antibiotics, anticholesteremics, anticoagulants, anticonvulsants, anticulcervatives, antidepressants, antidiabetics, antidots, antiestrogens, antifungals, antihistamines, antihypertensives, antimalarials, antimigraines, antimycotics, antineoplastics, antioxidants, antiparkinsonians, antiphlogistics, antirheumatics, antiseptics, antispasmotics, antitussives, antivirals, artificial sweeteners, beta-blockers, bronchodilators, cardiotonics, chemotherapeutics, choleretics, cytistatics, dermatics, designer drugs, disinfectants, diuretics, DNA cross-linking agents, doping agents, dyes, enzyme inhibitors, estrogens, explosives, fungicides, gyrase inhibitors, h2-blockers, hallucinogens, illicit drugs, hemostatics, herbicides, hypnotics, illegal drugs, immune modulators and suppressants, insecticides, laxatives, local anaesthetics, mollusicicides, markers of taxus poisoning, muscle relaxants, neuroleptics, nootropics, parasymatholytics, pesticides, progestins, psychedelics, radiation protectants, rodenticides, scabicides, sedatives, spasmolytics, stimulants (egg. caffeine), stomachics, tranquilizers, tuberculostatics, uriosurics, vasodilators, virucides, virustatics, and x-ray contrasts or contrasting agents, amongst others. Many pharmaceuticals found in the water samples such as antifungals, tuberculostatics, and antimalarials are also given to HIV positive patients, together with ART, for the prevention of certain illnesses (WHO, 2014).

In natural waters, quantifiable concentrations of Stavudine, Lamivudine, Nevirapine, Zidovudine, Abacavir, Tenofovir, and Nelfinavir were found. Nevirapine was detected in six samples. The lowest concentration was 0.3 ng/l (Zidovudine) and the highest was Nevirapine at 6.8 ng/l. Efavirenz and Saquinavir were not detected in any of the 47 surface samples. Of the four WWTP treated effluent samples, only Nevirapine (0.4 ng/l), Zidovudine (0.3 ng/l) and Tenofovir (1.6 ng/l) were quantified in two of the WWTPs. Two sites yielded no quantifiable ARV concentrations even though the drugs. Of the 31 drinking water samples, Lamivudine, Tenofovir, Efavirenz, Lopinavir, and Saquinavir concentrations were not quantifiable. Nevirapine occurred in five samples (0.3-3.5 ng/l), and Didanosine in three (0.4-3.3 ng/l). Abacavir and Stavudine were identified each in a sample, and Zidovudine was identified in two drinking water samples. Stavudine, Nevirapine, Tenofovir, Nelfinavir and Saquinavir were quantified from seven of 18 ground water samples, while Nevirapine was found in eight samples (0.3-5.4 ng/l). With regards to fish samples, of the 33 fish plasma samples collected from four sites, nine samples had quantifiable amounts of HIV-ARVs: Stavudine (n=2), Didanosine (n=3), Abacavir (n=1), Efavirenz (n=1), Nelfinavir (n=1), and Saquinavir (n=2). The concentrations of ARVs in fish blood plasma ranged from 27-135 ng/l.

These findings suggest that HIV-ARVs are more prevalent in groundwater at slightly higher concentrations. This may be due to lower microbial action and less sunlight that would otherwise enhance the breakdown of the compounds. The HIV-ARVs in groundwater samples were associated with urban areas and areas closely located to some of the WWTPs, including one that was clearly dysfunctional. In many areas, groundwater may be contaminated by non-treated sewage. Since untreated groundwater is also used in many areas for drinking water, this route of exposure seems the most likely to contribute towards human exposure. This should be one area of urgent investigation to get a better understanding of actual exposure and risk.

Drinking water (from taps) had variable amounts of quantifiable HIV-ARVs in some samples. This indicates that processes to prepare tap water seem not to be able to clean intake water completely, and this potentially exposes the general human population to low concentrations of HIV-ARVs. It would be advisable in the future to do time-based analyses of intake and final water samples to get a better understanding of human

exposures. Natural water samples from the same systems had variable presence and amounts of HIV-ARVs. With the present sampling system, it was not possible to determine whether HIV-ARVs were carried downstream, but there are some such indications from our results. It is therefore possible that dysfunctional WWTPs could contaminate drinking water sources of downstream communities. This would be another area of further investigation, as the results would indicate that WWTPs should be urgently upgraded to protect downstream communities from exposure.

The fish plasma data, although with very scattered presence and generally at higher concentrations of HIV-ARVs than in water, does indicate the potential of chronic human uptake from low concentrations in drinking water, and the possibility that humans may also accumulate low concentrations of HIV-ARVs from this source. The highly variable data indicates that the compounds are likely to be unstable in fish, and therefore also in humans. It should be noted though, that human exposures are likely to be chronic. Even if the molecules themselves break down quickly, it the chronic presence that is of concern. Whether the concentrations could affect HIV resistance is not known, but this should be investigated. The relatively high concentrations in fish plasma are indicative of bio-accumulation and bio-magnification. This raises the possibility that higher trophic level organisms, such as piscivorous birds, may have even higher concentrations of HIV-ARVs. This aspect needs further investigation. Since there are no data on the effects of HIV-ARVs on natural viruses, further investigations are required. If the presence and concentrations of HIV-ARVs in the environment can increase with time and due to more people on ART, then the possible impact of HIV-ARTs on viruses should be investigated, as bacterial composition and activity is regulated to some extent by viral phages. Especially, the microbial action upon which WWTPs depend may be affected, although it should be noted that different types of viruses might be affected differently by compounds aimed specifically at retroviruses.

Very little literature exists that informs about antiretroviral drugs in water and fish. As far as we know, this is the first report of such data in South Africa. Due to the scattered nature of sample locations, and the variable presence and concentrations in samples, no meaningful statistics could be done. However, since South Africa has a large poor population, many people depend on fish that are caught from river and dams and used as food on a daily basis (CGIAR, 2011). These concentrations indicate that the HIV-ARVs are bio-available, and could possibly bioaccumulate as well. This raises issues such as potential bioconcentration through food webs. This is an issue that may need further investigation.

CHAPTER 5: CONCLUSIONS & RECOMMENDATIONS

The aim of the project was to establish the presence, concentrations, and potential implications of HIV-ARVs in final treated sewage effluent, natural receiving waters, tap water, and fish. During the project, bottled water and borehole water was added. The following are conclusions and recommendations from this study.

Presence and sources of HIV-ARVs in water

HIV-ARVs have been found in quantifiable concentrations in all water sources, except in bottled water. The South African Department of Health and the Human Sciences Research literature indicated that the different HIV-ARVs are used at different intensities. The most utilised drugs include NRTIs such as Zidovudine, Stavudine, Didanosine, Tenofovir, Lamivudine and Abacavir, NNRTIs such as Efavirenz, Nevirapine and PIs such as Lopinavir, Ritonavir, Saquinavir and Nelfinavir. Based on these findings, the following recommendations are made:

- The amounts of HIV-ARVs being used in South Africa should be monitored on a continuous basis, and, if possible better quantified.
- To keep track of HIV-ARVs that are taken out of circulation and new compounds introduced (especially in the public sector), to anticipate changes in amounts used and released. E
- It may eventually be possible to predict increases and risks for specific compounds, based on their physical and chemical characteristics, use patterns, and their behaviour in the environment. This monitoring will also allow the testing the adequacy of existing extraction and analytical techniques in anticipation of new compounds before being used.

Development of extraction and analytical procedures for selected HIV-ARVs

Extraction and quantification procedures were developed by this project using the HPLC-QTOF/MS, and good linearity and recoveries were obtained for all selected compounds at below the ng/l range. For future studies, the following is recommended:

- The existing extraction and analytical procedures can be further refined.
- Attention should also be given to confirm and quantify the many other compounds that were detected in water sources. This will assist in eventual relative risk determinations, as well as inform on improved waste water treatments, drinking water preparation, groundwater protection, and conservation.
- The risk of external contamination through the sampling procedures used is exceedingly low, but any possible doubt should be addressed.
- Attention should be given to better describe each source and sample and accurately describe the water sampling procedures.
- Attention should be given to better describe the fish plasma sampling procedure. The risk of contamination through the sampling procedures used is exceedingly low, but any possible doubt should be addressed.

Presence and concentrations of HIV-ARVs in water sources

Analyses and quantification was successful with the methods developed. Many of the water samples had no quantifiable residues. All 11 HIV-ARVs were however quantified at least once, but very few samples had more than compound. The concentrations in the samples degraded markedly over a period of days. This quick disappearance of the parent compound raises concerns of higher concentrations of stable breakdown products (from both biological and physical decomposition). HIV-ARVs were determined in different water samples and biota. Fish plasma proved the best matrix for quantifying ARVs. Based on these findings, it is recommended that potentially larger concentrations of stable breakdown products be also investigated. This

could be done by looking at the clinical literature where the pharmacodynamics and pharmacokinetics for specific compounds are probably better known. The stable metabolites can then be selected for targeted analyses from water and fish. The availability of analytical standards however, may limit the scope of this investigation.

Interpretations of the data and conclusions.
Two of the four final treated sewage effluents had quantifiable concentrations of HIV-ARVs. This seems to correlate with the apparent efficiencies of the WWTPs. It also indicates that WWTPs that operate well can eliminate HIV-ARVs to below LOQ. The presence of HIV-ARVs in final treated sewage water logically indicates that it will also be present in the inflow at higher concentrations. What the effects of these compounds could be on the viral-bacterial ecology of the various treatment processes are unknown, but the potential consequences are of concern. Based on this, the following can be recommended:

- Determining inflow and outflowing concentrations over time on a range of different WWTPs with different efficiencies (based on Green Drop data).
- It should be attempted to correlate the inflow concentrations with the catchment characteristics (relating to ART) of the WWTPs, including hospitals and clinics.
- Conduct studies on the effects of HIV-ARVs on the viral ecology of WWTPs.

Natural waters
Receiving waters had variable concentrations of quantifiable compounds. With the present sampling system, it was not possible to determine whether HIV-ARVs were carried downstream, but there are such indications. It is therefore possible that suboptimal functioning WWTPs could contaminate drinking water sources of downstream communities. People directly consuming surface water may also be exposed. The effects of the HIV-ARVs on the natural aquatic viral component of the natural ecology of streams are unknown. Based on these findings, the following recommendations can be made:

- Conduct intense and time-based studies on the concentrations of HIV-ARV concentrations in the receiving waters of various WWTPs.
- Conduct studies to determine the half-lives of HIV-ARVs in natural waters.
- Conduct analyses of waters downstream of WWTPs and determine if HIV-ARVs are carried far enough to potentially contaminate the drinking water treatment plants of downstream communities.
- Consideration should be given to what may happen in future scenarios when more people are likely to receive ART.
- Conduct studies on the possible effect of HIV-ARVs on the virioplankton of natural waters and in WWTPs.

Groundwater
Groundwater, as determined from the quantifiable presence of HIV-ARVs, seems to have slightly higher concentrations than surface water. These higher concentrations might be due to less exposure to sunlight and microbial action compared to surface water. There are also some indications that groundwater close to WWTP are contaminated by WWTPs. The detectable HIV-ARVs in groundwater also suggest that people that use ground water as drinking water may consume water containing HIV-ARVs. Bottled water had no detectable HIV-ARVs, although some other compounds were detected but not confirmed. For future studies, the following recommendations can be made:

- Conduct a study to investigate the sources of HIV-ARVs to groundwater. Plume analyses of the compounds may indicate likely sources.
- Determine the speed of horizontal and vertical movement of the compounds in groundwater, as well as their half-lives and factors governing half-lives.
- Conduct studies of potential human uptake and exposure at communities depending on bore-hole water.
- If human exposure should be investigated, and if confirmed, risk assessments must be conducted.

Tap water

The data from tap water from different communities had variable presence of detectable and quantifiable HIV-ARVs. This suggests that the treatment processes to derive drinking water may not be able to reduce the concentrations in raw intake water to below LOQ. It also suggests that people consuming tap water may be exposed. The patterns that were detected are not clear however, and in-depth studies are needed. For future studies, it is recommended that:

- Intensive time-based studies on the concentrations of HIV-ARVs in raw water used to derive drinking water through treatment, as well as the product, are conducted.
- Intensive time-based studies on the eventual water reaching consumers are conducted.
- If such exposures are confirmed, risk assessments will have to be conducted.

Fish

The presence of concentrations of HIV-ARVs in some fish plasma samples from various localities indicates potential bio-accumulation, either from water, or food, or both. Since fish was used as a distant indicator for human exposure, it suggests that similar processes may be involved with higher trophic level organisms and humans. The effects of these concentrations on humans or aquatic animals are unknown. Recommendations are as follows:

- Conduct in-depth studies on potential bio-accumulation by and effects on aquatic biota.
- Conduct a risk assessment on potential human dietary exposure via fish.

Other compounds

This study detected many other anthropogenic compounds, especially PPCPs. Their actual presence cannot be confirmed without analytical standards, but it is likely that at least some of these compounds are present. It is therefore recommended that, in parallel with many of the investigations listed above, additional compounds should be investigated. A likely target list should be constructed with input from the pharmaceutical community, standards obtained, and presence and concentrations confirmed. Further actions can then be based on the results.

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