Quantification, Fate and Hazard Assessment of HIV-ARVs in Water Resources

Volume 1
Analytical and Biological Aspects

Report to the
Water Research Commission

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WRC Report no. 2594/1/19
ISBN 978-0-6392-0122-1

March 2020

WATER RESEARCH COMMISSION

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This report forms part of a set of two reports. The other report is *Quantification, Fate and Hazard Assessment of HIV-ARVs in Water Resources, Volume 2: Social Aspects* (WRC Report No 2596/2/19).

EXECUTIVE SUMMARY

BACKGROUND

Since 1981, the world has struggled to cope with the extraordinary dimensions of a particular disease – HIV/AIDS. Specific, South Africa has faced some of the greatest challenges with regards to this. With an estimated 7.1 million people living with HIV/AIDS (PLWHA), South Africa has the largest and most high-profile HIV epidemic in the world (UNAIDS, 2018). In order to combat the HIV epidemic, South Africa has made great strides and currently possesses the largest HIV treatment programme in the world. In 2018, 4.4 million people were reported receiving treatment in South Africa, which equates to 61% of the PLWHA in the country (UNAIDS, 2018). South Africa's antiretroviral treatment (ART) programme has undertaken even more expansion, with the implementations of the World Health Organization's (WHO) 'test and treat' guidelines. The country has made big improvements in getting people to test for HIV and is on the verge of achieving the first of the 90-90-90 targets.

Irrespectively, South Africa still has one of the highest HIV incidence rates in the world, the largest treatment programme, and therefore the greatest consumption of antiretroviral drugs per capita (Wood et al., 2014), with prescribed amounts of up to several tons per year (Swanepoel et al., 2015). The national rollout of antiretrovirals began in 2005, with the objective of one service point in each of the 53 districts of South Africa. Since then, it has improved the quality of life and the historical pattern of mortality in South Africa. However, there may be subtle, yet unquantified effects and processes that need to be better understood. These include environmental concentrations of the drugs, secondary human exposures, effects on aquatic life, and social considerations. These interactions are the subject of this Water Research Commission report.

Certain antiretroviral compounds can be excreted largely unchanged after consumption (e.g. acyclovir didanosine and tenofovir) whereas other compounds undergo extensive biotransformation prior to elimination from the body (Galasso et al., 2002; Al-Rajab et al., 2010). Up to 90% of orally consumed pharmaceutical drugs reach wastewater in one form or another (Halling-Sørensen, 1998). Research also suggests that compounds such as emtricitabine, ganciclovir, and lamivudine are metabolized only to a small extent in the human body (10-30%), whereas abacavir and zidovudine are primarily metabolized to their glucuronide-adducts (Funke et al., 2016).

Swanepoel et al. (2015) documented the presence of various antiretrovirals (nevirapine, didanosine, stavudine, tenofovir, nelfinavir, nevirapine, and saquinavir) in drinking and groundwater samples. So also has the presence of antiretrovirals in South African waters been investigated by Schoeman et al. (2015), Wood et al. (2015) and Robson et al. (2017). It is therefore known that these pharmaceutical compounds are found in water resources, but knowledge of the implications and consequences thereof is exceptionally deficient.

The high prevalence and vast volume of antiviral drugs being consumed and excreted via various pathways into the natural environment poses an ever-increasing risk of pharmaceutical pollution. Many of the biological, economic, and social concerns of these highly bioactive compounds are unknown; endeavours to mount an effective solution are to date fragmented and vastly under-resourced. Subsequently, as a result of previous research and an increasing demand to fulfil the many gaps in knowledge about this particular subject, the North-West University project team set about to complete a thorough risk assessment, quantification, and fate of HIV-ARVs in the water resources of South Africa. The project team consists of specialists in environmental, social and health systems associated with this multidisciplinary topic, as well as the required partnerships and technology to conduct comprehensive research. Accordingly, the project contains many objectives and components as described below.

Towards an environmental perspective

A major objective of this study was to standardise a method for sampling, extraction, and analysis of antiretroviral and antifungal[1] compounds. It is already an achievement to identify these compounds in the environment at such low concentrations. However, these methods need to be accurate and trustworthy in order for universal standards and monitoring systems to be implemented in the future. This project includes the physical and chemical characteristics of the targeted compounds. All compounds have distinct physical and chemical properties that may undergo alterations as it is exposed to the various pathways leading to the receiving water resources. Therefore, the more properties identified for a compound, the more knowledge we have on the nature and fate of that substance. These properties can in turn help us to predict the fate of the substance and thus comprehend how it will react under various conditions and the risks that it may carry.

Pharmaceuticals may be discharged into the environment through various routes including domestic (solid) and sewage (liquid) wastes. Therefore, it is incumbent to investigate water quality up- and downstream from wastewater treatment plants (WWTPs), as well as other factors that may play a role in their release and changes. Due to the expected difference in concentrations, rivers from three different study areas were identified for water sampling purposes. Sites were selected based on their accessibility, safety, and their location in terms of the presence of WWTPs. Water was sampled up- and downstream from WWTPs in northern and southern Gauteng, at the Mooi River near Khutsong (North West province), and downstream of the Wonderfontein Spruit (Gauteng).

Analysis and identification of these pharmaceutical compounds in water resources then also leads to the question – how are these antiviral compounds affecting organisms and WWTPs? The continuous and cumulative usage of antiviral drugs has led to a heightened appreciation of possible viral resistance and toxicity. Antiviral drugs have toxic effects on certain microbes, algae, and fish (Al-Rajab et al., 2010; Kümmerer, 2008). It has also been confirmed that certain intermediates of antiviral agents can induce genotoxic effects on viral and microbial genomes. This is of significance for the reason that exposure to these drugs in the environment can lead to mutations, affecting genetic stability, viral transmissibility, and pathogenicity.

However, possible and probable non-specific antiviral effects of various antiviral substances against naturally occurring viruses remain undetermined. The present WRC study therefore investigates the potential of such non-specific effects on viruses in aquatic systems with the use of bacteriophages as surrogates. Bacteriophages help regulate population structure and prokaryotic composition in aquatic systems by influencing numerous ecological processes. According to Jain et al. (2013), antiviral substances and their intermediates are persistent in WWTPs where bacteriophages play a vital role in regulating microbial composition. By investigating this aspect, knowledge can be generated on the effectiveness of microbial organisms in the treatment of wastewater and further information can by gathered regarding the removal of pharmaceutical compounds in wastewater as well as the biological consequences associated with the presence of these pharmaceuticals.

The consumption of any type of exogenous drug by any organism in sufficient quantities may intervene with the regulation of metabolic systems and bring about adverse effects. The presence of antiretrovirals in water can be considered a hidden or latent risk – as mentioned before toxic effects have been linked between non-target organisms and antiviral compounds. Nonetheless, literature concerning ecotoxicological effects of pharmaceuticals rarely comprise of any chronic toxicity testing and predominantly entails acute toxicity in standardized tests. Once again, information, specifically regarding antiretroviral toxicity, is particularly lacking. Little to no information has been published on the ecotoxicological effects of the various antiretroviral drug classes; difference in toxicity experience by different phyla; the effects that antiretroviral metabolites have; the influence that environmental parameters have on pharmaceutical toxicity; the effect of combined antiretroviral drugs, nor the long-term indirect exposure of humans consuming contaminated water.

For the above reasons, this study investigated the biological effects of the active ingredients in selected antiretroviral formulations using a battery of cell lines capable of distinguishing between two mechanisms of action by which endocrine disruption can be mediated. Together with these cell lines, acute and chronic

[1] Antifungal compounds, specifically fluconazole, are often co-prescribed with antiretroviral compounds, due to co-infections.

exposure testing were investigated with freshwater snails. Snails were used because they can bioaccumulate various toxicants to such a degree that pollutants might reveal deleterious impacts at much lower environmental concentrations. Therefore, any toxic effects illustrated by these studies can help predict future environmental risks and can promote further toxicological investigation into long-term direct and indirect exposure. This information in conjunction with environmental factors can in turn be used to help inform the need for interventions and regulations.

In order to determine whether environmental exposure of antiretrovirals pose a threat to humans, a human health risk assessment was completed. The hazard assessment estimates the likelihood of adverse effects in a population at defined exposures to target compounds. The calculation of a threat based on the concentrations of the target compounds in the surface water can provide the probability of adverse health effects from a specific area. The risks posed by the exposure to ARVs are predicted by calculating the toxicity of these chemicals over a lifetime of exposure, exposure routes, and at concentrations determined at each site.

AIMS

The primary aim of this study was to offer an overview of the sources, travel pathways, behaviour, fate, and impact of antiretrovirals within the environment. Therefore, nine objectives were set to fulfil this aim. The first was to ascertain and document in a concise manner the present usage patterns and quantities of antiretrovirals, as well as to predict from historic data, potential impending scenarios. Next was to improve the sampling, extraction, and analytical practises, as well as to employ an independent QA/QC scheme. Other potential major metabolites were to be determined through co-analysis for the parent compounds. So also, was the spatial and temporal patterns of antiretrovirals in treated effluent, waste-, natural-, and drinking water to be determined. Possible biological impacts were to be investigated. This entailed using cell-based bioassays, and exposure testing with bacteriophages and freshwater snails. A hazard assessment was also to be conducted based on the data generated. This is followed by recommendations for actions and/or supplementary studies.

MATERIALS and METHODS

The material and methods of this study is separated into three main chapters (CHAPTER 2-4) and their specific components.

- Instrumental analysis;
- Biological effects associated with antiretroviral exposures;
- Hazard assessment for antiretrovirals in water.

The instrumental analysis component (CHAPTER 2:) contains a detailed description of analytical quality assurance and quality control measures. Quality control denotes the individual measures required in monitoring and utilising particular analytical operations to measure non-conforming method performance, whereas, quality assurance denotes the systems used to verify that the entire analytical process is operating within acceptable limits to ensure and regulate quality (Westgard, 2008). Antiretrovirals are present in the environment at very low concentrations. These compounds therefore needed to be concentrated during the extraction process to ensure that the analytical technique can detect the compounds. Water samples were extracted, cleaned-up, and analysed using a Liquid Chromatography/Mass Spectrometry-Quadrupole-Time-Of-Flight (Agilent) instrument. Compounds of interest were targeted, and the sample extracts were screened for other compounds.

In addition to the antiretroviral parent compounds, some antiretroviral metabolites were investigated. The data obtained from the parent antiretroviral analyses were utilised to screen for the antiretroviral metabolites. The scanning ability of the Agilent 6540 Accurate mass quadrupole time-of-flight mass spectrometer enables data gathering of many other potential compounds that might be present in the samples. However, the search was

limited to only antiretroviral metabolites. Fish tissue samples were also examined for the presence of antiretrovirals. These samples were subjected to the same Liquid Chromatography-Quadrupole-Time-Of-Flight method for antiretrovirals as for the water samples.

The biological effects component (CHAPTER 3:) is sub-divided into three aspects that tested for effects on *micro* and *macro* scale.

- The first aspect entails the potential impacts of antiretrovirals in the environment on bacteriophages. Here, six environmental comparable antiretroviral concentrations were evaluated for any effects on bacteriophages. A single colony of *Escherichia coli* WG5 bacteria was obtained and incubated with the various phage-host antiretroviral working solutions. Phages were analysed using Transmission Electron Microscopy and bacterial growth was investigated.
- The second aspect entails *in vitro* tissue culture assays where environmental extracts, active ingredients, and the formulations of the antiretrovirals were tested. By using receptor-mediated assays, the effects of antiretrovirals on non-target organisms can be inferred. Three reporter-gene cell lines capable of measuring (anti-)androgenic (MDA-kb2 cells), (anti-)oestrogenic (MVLN cells) effects, and aryl hydrocarbon receptor (AhR) (H4IIE-luc cells) activity were used.
- The third aspect contains the freshwater snail acute exposure testing. This aspect involves the investigation of the embryonic development of *Bulinus tropicus* (a freshwater snail indigenous to South Africa) after various antiretroviral exposures at different concentrations. Embryonic growth, hatching success, and mortality were investigated in the presences of four antiretrovirals (nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors).

In order to determine whether antiretrovirals in the environment pose a threat to human health, a hazard assessment was conducted (CHAPTER 4:). The hazard quotient for each antiretroviral was calculated using the maximum measured concentration of each antiretroviral across the sampling sites (described in CHAPTER 2: section 0). A hazard quotient was determined for the antiretrovirals and fluconazole, and the exposed individuals were considered safe if the hazard quotient was less than one. A hazard quotient exceeding unity (one) was considered indicative of whether (and by how much) an exposure concentration exceeded the reference concentration.

In addition, a complementary study was included (CHAPTER 5: Valuation of ecosystem services and disservices at health clinic gardens). This component is a qualitative-quantitative descriptive survey that include water aspects. Qualitative data was gathered in the form of interviews and observations, and quantitative data in the form of survey data. This study was added as a natural evolution of this project when additional complementary activities on this subject were discovered at the NWU.

RESULTS AND DISCUSSION

Instrumental analysis

The Olifantsfontein downstream 1 and 2, Olifantsfontein upstream, and Sunderland Ridge downstream 1 had the highest concentrations of antiretrovirals recorded from all the samples analysed. The three Olifantsfontein sites had high concentrations of lopinavir and efavirenz, with the maximum concentrations at 38 and 25 µg/L respectively. Sunderland Ridge downstream 1 only had a high concentration of lopinavir (34 µg/L). Almost all of the concentrations in drinking water samples were below limits of quantification, except for Sunderland downstream DW (efavirenz at 6.2 µg/L and fluconazole 0.173 µg/L), Waterval downstream 1 DW (nevirapine 0.038 µg/L) and Zeekoegat downstream 1 DW (fluconazole 0.4 µg/L, nevirapine 0.2 µg/L, lopinavir 9.03 µg/L, efavirenz 2.3 µg/L). The most frequently detected compound was fluconazole (28 detections from 72 samples), with concentrations ranging between 0.06-1.8 µg/L. Nevirapine and efavirenz were the second most detected compounds, both with 22 out of 72 samples. However, on average lopinavir and efavirenz had the highest concentrations of the compounds analysed. Didanosine and zidovudine were the least detected compounds (2 and 6 out of 72).

Of the 72 sites sampled, 69 sites have never before been investigated. The Rietvlei Dam (same as Hennops 1) and Zeekoegat downstream 1 and upstream 1 were sampled by Wood et al., (2015). The profile of antiretrovirals sampled and detected in South African water is different to international results. This can be ascribed to differences in the drugs and formulations prescribed, and because antiretroviral consumption in SA is much more prevalent than other countries. It is evident from the results obtained in these sampling events that the antiretrovirals (with the general exception of efavirenz) and fluconazole concentrate downstream of the WWTPs plants and are not efficiently removed.

In this study the concentration of efavirenz measured upstream of WWTPs, ranged from <LOD-20.4 µg/L, however, the site with the maximum concentration was at site Zeekoegat upstream 2, which was below the Baviaanspoort wastewater treatment plant. The next highest efavirenz concentration from an upstream site was at Olifantsfontein upstream (15.0 µg/L), the results correlate to those published by Schoeman et al. (2015 and 2017). The nevirapine concentrations determined in this study ranged from <LOD-1.3 µg/L at Zeekoegat upstream 1, which is about half the value that Schoeman et al. reported in 2015, but nearly double the concentration that Schoeman et al., reported in 2017. This is alarming and indicates the importance of determining the concentrations of pharmaceuticals in aquatic systems, and the potential effects that these compounds and mixtures of these compounds can pose to organisms encountering and consuming this water.

Fish samples

No antiretrovirals were detected in the fish tissue. These samples were collected in 2013 and 2014, therefore the accumulated antiretrovirals and/or fluconazole could have broken down by the time they were investigated. It is also possible that the fish metabolise the antiretrovirals, and metabolites might be present. Alternatively, the parent/metabolite may accumulate in an organ, like the liver, rather than muscle tissue.

Biological effects

In vitro tissue culture assays

Regarding the two reporter gene assays that were successfully performed, there was no activation of the respective receptors after exposure to environmentally relevant concentrations. However, there was evidence of inhibition of the androgen receptor by stavudine and lamivudine. Lopinavir also created slight inhibition at the highest concentration and the inhibition by efavirenz was dose-dependent; more inhibition was evident at the higher concentrations.

The data from the steroidogenesis assay provided evidence of a decreased production of both testosterone and oestradiol by the cells but this was not dose-dependent. Ritonavir also caused a decline in testosterone, but its influence on oestradiol was mixed (sometimes increasing its concentrations whilst decreasing other concentrations). Nonetheless, there was no relationship between the exposure concentration of the antiretroviral and oestradiol production. Lopinavir caused a lowering of both testosterone and oestradiol that was dose-dependent. Efavirenz seemed to have caused an increase in both the levels of testosterone and oestradiol at the lower exposure concentrations, but a decrease at the higher concentrations. Nevirapine mostly caused an abatement of testosterone levels but its influence on oestradiol response was not clear.

The human intestinal (Hutu) cells were exposed to concentrations equivalent to those in the formulations, therefore mimicking the levels the human intestine might experience after swallowing the tablets. The Hutu cells survived all the exposure concentrations, as well as at the three exposure concentrations. Efavirenz was the only active ingredient that caused a slight decrease in viability at the higher concentrations and at the 24 h and 36 h exposure periods.

Potential impacts on bacteriophages

There was no statistical significance in the optical density of bacteria between the control and other ARV exposure concentrations. However, there was a visible difference in the speed at which bacterial growth peaked in the control (after 70 min), compared to ARV exposures (100-120 min). Due to similar trends observed in the phage-bacteria mixture with and without ARV active compounds, it is possible that some were not affected, and standard activity took place. This could indicate either substrate binding saturation or

resistance to ARVs. Transmission Electron Microscopy results illustrated the potential effects on phage proteins due to the inhibition of targeted genetic sites of non-nucleoside reverse transcriptase inhibitor, nucleoside reverse transcriptase inhibitor, and protein inhibitor antiretrovirals. The heads (capsules) were separated from the tails and were assumed to have undergone morphological changes. Instead of the conventional icosahedral shape head, some appeared round while others had no specific shape. The active compounds of ARVs seem to have an impact on assemblage of viral proteins as exhibited by TEM images.

The mode of action of the ARV's could possibly have led to the disruption of certain bacteriophages and some may be resistant (Chauhan et al., 2019). The phages applied were direct isolates from aquatic bodies and may be a variety of bacteriophages thus as a result some may be susceptible, and some may carry resistant genes. It could be interesting to establish the individual and combined effect of ARVs on purified phage cultures.

WWTPs rely on the activity of bacteriophages to infect and kill a large portion of bacteria in the system. Phages rely on the metabolic activity of their bacterial hosts for their reproduction and survival in a system. It seems that these metabolic processes of bacteria are being impacted by the ARV compounds tested in this study, thereby causing a decrease in the total amount of phages in the system. A decrease in the number of phages in the system can lead to an increase in the number of bacteria in the WWTP effluent. Thus, ARV active compounds may have an effect on the productivity of a WWTP in removing bacteria from its water by impacting the phages in the system meant to kill and control these bacterial levels.

Disregarding the WWTP itself, the water bodies in receipt of effluent with ARV drug waste run the risk of bacterial blooms due to increased levels of bacteria being introduced. Such a decrease in microbial water quality produced by WWTP due to the activity of ARVs in the system could be harmful to the environment and cause an increased burden on human health, agriculture, and the environment, especially considering that pathogenic bacteria may be enhanced. Water quality may therefore be worse than the present tests imply due to the decreased metabolic activity of bacteria impacted by ARV active compounds.

Snail exposures

Comparisons of two different modes of antiretroviral action for their effects on the embryonic development of *B. tropicus*, demonstrated contrasting effects. Lamivudine and stavudine (NRTIs) generally had a stimulating effect on embryonic development, producing mean embryo lengths larger than that of the water controls. In turn, efavirenz and nevirapine (NNRTIs) demonstrated an overall inhibitory effect on embryonic growth. Such alterations in growth rate can greatly affect population densities and demography as well as interactions with other species and algae. The larger embryo length effect could be due to the variability in the extensive metabolism of efavirenz. The NNRTIs also illustrated the highest percentage of mortality, 35% for the 0.005 µg/L efavirenz exposure, and 34% for the 0.5 µg/L nevirapine exposure. Many of the embryos therefore, failed to make the necessary homeostatic adaptations to overcome the stress induced by these NNRTIs. Exposure of antiretrovirals towards *B. tropicus* affected embryonic length, hatching and rate of mortality. The influence of these antiretrovirals on the development and growth of the indicator organism therefore supports the necessity to conduct further investigations concerning ecological impact of these pharmaceutical compounds.

Hazard assessment of antiretrovirals

The study revealed that surface water sources from urbanised and industrial areas in South Africa were contaminated with high concentrations of antiretrovirals and fluconazole compared with other countries worldwide. An unacceptable risk to human health was identified when a hazard risk assessment was applied by using the minimum therapeutic dose approach. The hazard quotients calculated for the antiretrovirals and fluconazole were all greater than one and ranged from 1.7-130 with lopinavir having the highest hazard quotient. When considering the cumulative risk, the health index was also >1, indicating that the mixtures pose a significant risk to consumer's health. These results may be useful in setting water quality standards, and therefore operational targets for wastewater management and pollution prevention.

Valuation of ecosystem services and disservices at health clinic gardens

When considering supporting ecosystem services, the habitat service had an ecological value as it supports much different fauna and flora within different micro-gardens at the health clinics. The habitat provisioning for birds' ecosystem services had an ecological value, as habitat is created for different bird species. Even though there were some ecosystem disservices present in the health clinic gardens, the benefits of the gardens seem to outweigh the disadvantages. When considering the freshwater ecosystem service, very few stakeholders perceived the clinic gardens to be a source of fresh water, as only nine (out of 70) stakeholders perceived eight clinic gardens to be a source of fresh water. Most clinics receive their water from the municipality.

SUMMARISED RECOMMENDATIONS

Instrumental analysis

The following recommendations are presented for instrumental analysis of ARV compounds in water and tissues (CHAPTER 2:CHAPTER 2):

- It is recommended that the antiretrovirals metabolites are quantified by use of standards and a calibration curve, because the screening method did not reveal the presence of these compounds.
- The extraction method should be optimised for metabolites specifically.
- Further determination of antiretrovirals and metabolites should be investigated.
- It is advised to use the fish whole and analyses to be conducted in a timely manner after collection.
- If antiretrovirals and/or metabolites are detected in whole fish, it would be useful to determine in what tissue they accumulate, i.e. muscle, liver, brain, etc.
- It is advised to determine how other provinces in South Africa compare to the concentrations reported in Gauteng river systems.
- Lastly, future studies should take place during the months of March and/or September (when the highest concentrations of antiretrovirals were found in water sources).

Bacteriophages

The following recommendations are proposed for the potential impacts of antiretrovirals in the environment on bacteriophages (CHAPTER 3:):

- Biolog assays should be performed on bacterial hosts to establish which specific metabolic pathways are being affected by the presence of antiretroviral active compounds.
- It should be established what the minimum inhibitory concentration of antiretrovirals are for no effect on bacteria and bacteriophage metabolic/assembly.
- The effects on a single type of phage by several antiretroviral active compounds in a single mixture should be investigated.
- It is advised to investigate the effects on a phage-microcosm by several antiretroviral active compounds in a single mixture.
- Antiretrovirals using other mechanisms of inhibition should be tested, e.g. fusion inhibitors.
- The use of a purified single strain phage should be included in future studies (this could provide information on individual response mechanisms and effects of bacteriophages).
- Studies should be conducted to ascertain the effects of antiretrovirals on bacteria, fungi, and algae.
- It is recommended to establish whether individual or combined antiretroviral exposure affects purified phage cultures and microcosms differently.
- The viral ecology of fresh and marine waters is insufficiently understood. Investigations on the interactions between viruses and pollutants should be considered.
- The large scale, financial feasibility of implementing alternative faecal pollution indicators that do not rely on living/growing organisms should be investigated for example cholesterol.
- Establishing resistance patterns of bacteriophages and their hosts to ARVs is advised.
- Finally, to establish if there is a difference in effects on DNA phage and RNA phage by different ARVs respectively.

In vitro tissue culture assays

The following recommendations are proposed for the *in vitro* tissue culture assays (CHAPTER 3:):

- Steroidogenesis assays should be employed to test for the influence of the ARVs on the other steroid hormones expressed by the H295R cells (angiotensin-II-responsive steroid-producing adrenocortical cell line) used in the assay.
- It would be ideal if the analysis of the hormone levels could be substituted with instrumental analysis instead of the enzyme linked immune-sorbent assays.
- The instrumental analysis can determine the concentrations of multiple hormones in one run, whereas the enzyme linked immune-sorbent assays are developed for the single hormone. This makes the total analysis time consuming and expensive.
- It is also important to check for endocrine disruption via activation or inhibition of a working oestrogen reporter gene assay.

Snail exposures

The following recommendations are proposed for the snail exposures (CHAPTER 3:):

- It is imperative to conduct elaborate life-cycle studies on these and other potentially exposed organisms.
- In addition to supplementary acute exposure tests, future studies should investigate the effects of chronic exposure to antiretrovirals.
- Different modes of antiretrovirals action should be investigated, where investigations comprise of the active ingredient, precursors and metabolites.
- Future tests should not only include pure pharmaceutical compounds, but should also investigate the effects of pharmaceutical mixtures and combinations.
- All plausible roots of exposure should be considered in impending studies.
- It is recommended to experiment with other indicator species, for example fairy shrimp. (More comparative studies are required on different species).
- Micro- and mesocosm exposure tests are recommended to provide more environmentally relevant results.

Hazard assessment

The following recommendations are proposed for the human health risk assessment of antiretrovirals detected in water sources (CHAPTER 4:):

- More studies with different endpoints should be conducted by using the concentrations of antiretrovirals in African and South African surface water to obtain more toxicological information for further risk assessments.
- Tests should include standardised laboratory tests using *Daphnia*, zebrafish, or similar models.
- The effect of mixtures should also be investigated while individual drugs may exist within the predicted no effect concentration, mixture effects due to similar antiretrovirals with the same mechanism of action against the virus may lead to synergic or additive impacts against the virus.
- Another consideration is the analysis of antiretrovirals in different aquatic species, sediments, and soils to obtain more insights about their role and possible effects in the ecosystem.
- Targets in natural and drinking water should be set to protect human and environmental health. These targets may be guided by the results of this study.

Valuation of ecosystem services and disservices

The following recommendations are proposed for the valuation of ecosystem services and disservices at health clinic gardens in South Africa (CHAPTER 5:):

- Future studies should re-evaluate time management, so that there is enough time available to re-visit clinics to complete interviews with stakeholders, if needed.
- In future studies, a larger variety of illustrations for each concept should be used to indicate to the stakeholders the different scenarios in which the ecosystem services and ecosystem disservices could be present. Another option is not using any illustrations, but rather using many short questions to

obtain the perception of the stakeholders, instead of using three main questions for each ecosystem services (namely presence, value, and willingness to pay).
- It is advised to start with another ecosystem services (such as water) rather than food.
- Questionnaires should allow the option to discuss an opinion.
- Questionnaires should be altered to only include three values, namely ecological, economic and socio-cultural value as it was found that it is difficult to distinguish between social and cultural value.
- More comparative information is required from other health clinic gardens to provide an improved perspective of the spectrum of values offered by gardens (used to create management strategies for the health clinic gardens).
- Other aspects of health clinic gardens can be investigated such as including the abundance (e.g. percentage cover) of all the plant species (especially the food plant species) and not only the species richness.
- The inclusion of qualitative analysis in more ecological studies could also be implemented as this provides valuable information not obtained through quantitative studies.
- In some instances, water supply to clinics seems to be problematic. A study can be undertaken to investigate water supply and quality issues experienced by clinics, especially in rural areas.
- The ecosystem evaluation techniques may be applied to larger systems, and more focussed on water.

CONCLUSIONS

Humankind's relentless attempt to provide therapeutic benefits from chemicals is coupled to the inevitable discharge of pharmaceuticals into the natural and social environment. It is difficult to recognise the perils ahead, and even more difficult to provide an effective response. Nonetheless, if tipping points or thresholds are reached, it can lead to abrupt changes in the services provided by ecological systems, which, aside from triggering undesirable shifts in the natural balance, can have adverse social and economic consequences. Accordingly, this research has highlighted that the intersectionality of HIV expands into natural resources.

The research group has developed analytical methods with which the presence, concentrations, and spatial and temporal changes have been elucidated, improving sampling, extraction, and analytical procedures. Nearly all antiretrovirals were found in natural and some in drinking water, although none were found present in the fish tissue samples nor were antiretroviral metabolites found. However, the study provided evidence that antiretroviral exposure affected bacteriophages, embryonic development of freshwater snails, and of oestradiol and testosterone levels being influenced in the steroidogenesis (effects on steroid hormones).

Due to the need of millions of people to consume antiretrovirals every day, the relentless release of these compounds, based on our results, exhibit deleterious effects on non-target organisms in laboratory tests, and probably also in the environment. This may affect the ecology of natural aquatic ecosystems, and may alter the viral/bacterial interactions in WWTPs and receiving waters.

Humans are also exposed to these compounds via drinking water, and at concentrations exceeding calculated hazard quotients. Although not found in this study, humans and other organisms may potentially also ingest antiretrovirals and their breakdown products via aquatic organisms such as fish. As the antiretrovirals and fluconazole are consumed in combinations, mixtures of residues occur in the environment, together with other pharmaceuticals, adding to the risk of deleterious effects posed by them to humans, WWTP functioning, ecosystem functioning, and the organisms (including the natural viruses) associated with them.

Regardless of the environmental concerns about the high concentrations and mixtures of pharmaceuticals present in our drinking water, more research is needed to determine the risks to human health from chronic low-level exposures to single and mixtures of antiretrovirals and fluconazole. Further research on antiretroviral occurrence, behaviour, fate, and potential risks of parent compounds and metabolites in water sources are needed to establish guideline values for regulatory purposes and prioritisation of interventions. The concentrations of antiretrovirals in water sources of developing countries such as South Africa is expected to increase seeing as the countries with high antiretroviral treatment programs do not have treatment guidelines

regarding the presence of pharmaceuticals in wastewater treatment plant effluents. In addition, research to improve the drinking water treatment technology is required as the current water treatment processes are not able to remove effectively pharmaceuticals such as antiretrovirals and fluconazole.

It is inevitable that environmental considerations be accorded a lesser importance than poverty alleviation and disease prevention and management when dealing with emergencies. However, with this project we have now illustrated the links between the strategies employed to combat and manage the disease with a healthy, safe, and functioning ecosystem and her services. The protection of the environment, the management of the disease, and the moral obligations we have towards people living with HIV and AIDS, are irrevocably linked. Current and future health management should therefore mainstream the environment in strategy, planning, execution, and monitoring for optimal welfare for all.

FINAL RECOMMENDATIONS

1. The NWU research team have developed methods and knowledge on how the characterise the insidious problematics concerning HIV antiretrovirals on a regional scale for a specific disease. We have learned many lessons and gained tremendous experience. However, HIV antiretrovirals and the disease condition are not the only pharmaceutical and disease combinations that will result in environmental pollution. Although some of what we report is generic to the entire pharmaceutical spectrum, there are many situations that we do not know and understand, especially within a South African and African context. More studies are being published on the environmental side of pharmaceutical pollution in Africa. However, an integrated assessment as we have undertaken has nowhere been done. We therefore suggest that a much larger study, based on the experience and expertise developed by the current study would be appropriate for South Africa.
2. The science of pharmaceuticals in the environment is developing at a rapid pace. New findings on, *inter alia*, health and environmental impacts, risk assessments, analytical techniques, and mitigation measures are published almost daily. It is also likely that guideline values for concentrations of pharmaceuticals in water and food will be developed soon, and implemented or negotiated by authorities and international agencies. It would be incumbent for South Africa and the Water Research Commission to keep abreast of these developments and thereby provide guidance to local, regional, and national authorities, as well as water supply companies. This may be achieved by commissioning an annual summary report.

ACKNOWLEDGEMENTS

The authors would like to thank the Reference Group of the WRC Project K5/2594 for the assistance and the constructive discussions during the duration of the project. We would also like to thank the following people for their contributions to the project.

Reference Group	Affiliation
Dr E Ubomba-Jaswa	Water Research Commission (Chair)
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ACRONYMS and ABBREVIATIONS

3H-T	Radio-active testosterone
3TC	Lamivudine
ABC	Abacavir
ACN	Acetonitrile
ADD	Average daily dose
ADI	Acceptable daily intake
AhR	Aryl hydrocarbon receptor
AIDS	Acquired immune deficiency syndrome
AJS	Agilent Jet Stream
ANC	African National Congress
AR	Androgen receptor
ART	Antiretroviral therapy
ARV	Antiretroviral
ATV	Atazanavir
AZT	Zidovudine
BG	Baragwanath
cART	Combination antiretroviral therapy
CCMDD	Centralised chronic medicine dispensing and distribution
CCMT	Continuous care and monitoring of treatment
CCR5	Chemokine receptor antagonists
CD	Compact disc
CD_4	Cluster of differentiation 4
CE	Collision energies
cfu	Colony forming units
CHW	Community health workers
CI	Confidence interval
CPM	Counts-per-minute
d4T	2', 3' didehydro- 2', 3' dideoxythymine (Stavudine)
ddC	Zalcitabine
ddI	Didanosine
DNA	Deoxyribonucleic acid
DRE	Dioxin response element
DS	Downstream
E2	Oestradiol
EC	Effect concentration
EDL	Essential drug list
EDS	Ecosystem disservices
EFV	Efavirenz
ELISA	Enzyme linked immune-sorbent assays
EMB	Eosin Methylene Blue
ER	Oestrogen receptor

ES	Ecosystem services
ETR	Etravirine
FDC	Fixed dose combination
FIA	Flow injector analysis
FI	Fusion inhibitor
FRNA	F-specific RNA bacteriophages
FTC	Emtricitabine
GDP	Gross Domestic Product
GR	Glucocorticoid receptor
HAART	Highly active antiretroviral therapy
HLB	Hydrophilic-lipophilic balance
HLB-L	Hydrophilic-lipophilic balance low
HI	Health index
HIV	Human immunodeficiency virus
HIV-ARV	HIV-antiretroviral therapy
HHRA	Human health risk assessment
HPLC	Micro high-pressure liquid chromatograph
HREC	Health Research Ethics Committee
HQ	Hazard quotient
IS	Fluconazole-d4
ISO	International Organization for Standardization
IQR	Interquartile range
LAR	Luciferase assay reagent
LGBTI	Lesbian, gay, bisexual, transgender, and intersex
LC	Liquid chromatography
LC/MS	Liquid chromatography-mass spectrometry
LMICs	Low- and middle-income countries
LOD	Limit of detection
LOEC	Lowest-observed-effect-concentration
LOQ	Limit of quantification
LPV/r	Lopinavir/ritonavir
LSD	Lysergic acid diethylamine
MTT	2-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NFV	Nelfinavir
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NOEC	No-observed effect-concentration
NRTI	Nucleoside reverse transcriptase inhibitor
NSP	National strategic plan
NVP	Nevirapine
NWU	North-West University
OD	Optical Density
OR	Odds ratio
PBS	Phosphate buffered saline

PEG	Polyethylene glycol
PES	Payments for ecosystem services
PES	Polyethersulfone
pfu	Plaque forming unit
PI	Protein inhibitor
PPCPs	Pharmaceuticals and personal care products
PrEP	Pre-exposure prophylaxis
QC	Quality control
QTOF	Quadrupole time-of-fight
RAL	Raltegravir
RDD	Recommended daily dose
REP	Relative effects potency
RfD	Reference dose
RNA	Ribonucleic acid
RPV	Rilprivirine
RT-qPCR	Reverse transcription – real-time polymerase chain reaction
RTCA	Real-time cell analyser
RTV	Ritonavir
SC	Solvent control
SCG	Single-cell gel
SD	Standard deviation OR Single dose
SPE	Solid phase extraction
SQV	Lopinavir
SsMSA	Semi-solid Modified Scholtens' Agar
SsTYGA	Semi-solid Tryptone Yeast Glucose extract Agar
STG	Standard Treatment Guideline
T	Testosterone
TAF	Tenofovir alafenamide fumarate
TB	Tuberculosis
TCDD	2,3,7,8-tetrachlorodibenzo-*para*-dioxin
TDF	tenofovir
TEM	Transmission Electron Microscopy
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
TYGA	Tryptone-Yeast Glucose Extract Agar
UNAIDS	United Nations AIDS
US	Upstream
USA	United States of America
UTT	Universal test and treat
VL	Viral load
WHO	World Health Organisation
WV	Waterval
WWTP	Wastewater treatment plant

GLOSSARY

AIDS. A fatal disease caused by HIV in which there is a severe loss of the body's cellular immunity, greatly lowering the resistance to infection and malignancy.

Antiretrovirals. Antiretroviral drugs inhibit the reproduction of retroviruses (viruses composed of RNA rather than DNA) to aid in the treatment of HIV infections.

Ecosystem disservices. Refers to natural or anthropogenic-impacted ecosystem functions that negatively affect the well-being of humans. Some examples of ecosystem disservices include biological hazards, invasive species, floods, nutrient runoff, erosion, storms, heat waves and pests

Ecosystem services. The benefits people derive from the functions of ecosystems, e.g. social development and unity, improved health, access to fresh food, saving or financial profit, education, environmental equity, environmental sustainability, enhancing cultural heritage, life satisfaction, and increased biodiversity.

Geographic location. The geographical location in terms of this study is based on the geographic location of the prescriber practise and will be determined by the postal code of the practice of the prescriber of the ARV drugs. It will be determined at provincial- and district level. The postal code of the prescriber will be used as a proxy for the geographical location of the patient.

HAART. Refers to very potent ART regimen which almost invariably inhibits viral replication to undetectable levels in the blood. It comprises of a combination of antiretroviral drugs.

HIV. Human immunodeficiency virus is a sexually transmitted virus, which left untreated, can lead to the fatal disease AIDS. HIV infection leads to low levels of $CD4^+$ T cells through a number of mechanisms. When $CD4^+$ T cell numbers decline below a critical level, cell-mediated immunity is lost, and the body becomes progressively more susceptible to opportunistic infections.

Incidence. The HIV incidence is the number of new HIV infections in a population during a certain time period.

PPCP. Pharmaceuticals and personal care products refer to products used by individuals for personal health/well-being or for cosmetic purposes. Products targeted for use with pets or livestock, illegal drugs, or ubiquitous chemicals like caffeine also fall within the scope of PPCP. In terms of ingestible products, this would also include metabolites of the parent compound.

Prevalence. HIV prevalence is the percentage of people that are HIV+ in the population out of the total population at a given point in time.

qPCR. Is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR), also known as real-time PCR, which monitors the amplification of a targeted DNA molecule.

Titre. The strength of a solution or the concentration of a substance in solution as determined by titration. Titer testing employs serial dilution to obtain approximate quantitative information from an analytical procedure that inherently only evaluates as positive or negative. The titer corresponds to the highest dilution factor that still yields a positive reading.

Viramia. Medical condition in which viruses are present in the bloodstream. Viremia is analogous to bacteremia (the presence of bacteria in the blood) and parasitemia (the presence of a parasite in the blood). Bacteremia, parasitemia and viremia are all forms of sepsis (bloodstream infection).

Xenobiotics. A chemical compound (drug, pesticide, or carcinogen) that is foreign to a living organism.

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CHAPTER 1: INTRODUCTION

1.1 RATIONALE

The environmental fate and consequences of pharmaceuticals and personal care products (PPCPs) represent a crosscutting frontier in environmental chemistry and aquatic ecology (Rosi-Marshall et al., 2015). There is a global increase in the environmental burden from the parent compounds, pre-cursor compounds, and metabolites. These immanently bioactive compounds are often detected in low environmental concentrations. However, the continuous input of such compounds may lead to prolonged exposure and potential deleterious effects on non-target organisms and human health. One subject of concern is the release of contaminants into the environment, comprising of antiretrovirals (ARVs) in particular. This is of great environmental relevance, seeing that South Africa has one of the highest HIV incidence rates in the world, and subsequently the largest consumption of antiretroviral drugs per capita (Wood et al*.,* 2014), with prescribed amounts of up to several tons per year (Swanepoel et al*.,* 2015). Nonetheless, research to determine the ecotoxicological risks associated with these contaminants have only recently started to increase in magnitude and concern.

Resembling other pharmaceuticals, numerous antiviral drugs have been documented in aquatic environments including abacavir, acyclovir, lamivudine, nevirapine, and zidovudine (Prasse et al., 2010; Peng et al., 2014). In 2015, Swanepoel et al. revealed the presence of nevirapine (0.3-3.5 ng/L) and didanosine (0.4-3.3 ng/L) in drinking water samples. So was stavudine, tenofovir, nelfinavir, nevirapine and saquinavir quantified from seven of 18 groundwater samples, of which nevirapine (0.3-5.4 ng/L) was found in seven of the samples. Work carried out by Wood et al. (2015) also illustrated the simultaneous quantification of twelve antiretroviral compounds (abacavir, didanosine, efavirenz, indinavir, lamivudine, lopinavir, nevirapine, ritonavir, stavudine, tenofovir, zalcitabine, and zidovudine) in the surface water of South Africa. Schoeman et al. (2015) has also reported the presence of several ARVs in both treated and raw wastewater as well as in receiving waters with limits of quantification ranging between 0.2 and 10 ng/L for the following drugs: abacavir, acyclovir, lamivudine, nevirapine, oseltamivir, penciclovir, stavudine, and zidovudine. What's more, is that elevated concentrations of antiviral drugs have been identified in several surface waters with concentrations as high as 9 mg/L for zidovudine (K'Oreje et al., 2012; Wood et al., 2015), regardless of high removal rates during biological treatment in waste water treatment plants for example: abacavir >99% and acyclovir 98% (Prasse et al., 2010).

Another concern is that certain ARV compounds, can be excreted largely unchanged after consumption (e.g. acyclovir, didanosine and tenofovir), whereas other compounds can undergo extensive biotransformation prior to elimination from the body (Galasso et al., 2002; Al-Rajab et al., 2010). According to Funke et al. (2016), research suggest that compounds such as emtricitabine, ganciclovir, and lamivudine are metabolized only to a small extent in the human body (10-30%), whereas abacavir and zidovudine are primarily metabolized to their glucuronide-adducts. Research has also revealed that oseltamivir is not removed nor degraded during conventional wastewater treatment (Jain et al*.,* 2012). So also has the presence of the compound lamivudine in wastewater treatment plants (WWTPs) brought about the overall decrease in its efficiency (Vanková, as cited by Jain et al*.,* 2012). Although, the ARV efavirenz has been reported not to be quantifiable in a variety of environmental samples, Howard and Muir (2011) stated that the high production volume of this pharmaceutical is likely to become persistent and/or bio-accumulative in the environment. Accordingly, the environmental release of antiretroviral drugs are of immense concern due to prospective alterations in ecosystems as well as the problematic development of toxicological effects on humans and non-target organisms and even the cause of viral resistances (Prasse et al*.,* 2010) in the surface water of South Africa.

1.2 PROJECT AIMS

The main aim of this study was to provide an overview of the sources, travel pathways, behaviour, fate and impact of antiretrovirals within the environment. Which is achieved by means of the following objectives:

1. To determine and document in a concise manner, the current use patterns and amounts of HIV-ARVs, as well as predict, from historic data, possible future scenarios.
2. To improve the sampling, extraction and analytical procedures, as well as implement a QA/QC scheme that will be done independently.
3. To determine potential major metabolites that may need additional attention through co-analysis for the parent compounds.
4. To determine spatial and temporal patterns of ARVs in wastewater, treated effluent, natural water, and drinking water.
5. To determine potential impacts in the environment using cell-based bio-assays, exposing freshwater snails, and investigating potential impacts on bacteriophages.
6. To conduct a human health risk assessment, based on the data generated.
7. To propose ARV pathways from human consumption through the biopsychosocial determinants of health, discussed from a health perspective.
8. To describe the disruptive link between ARV consumption, challenged health systems and the environment.
9. To generate recommendations for action and/or supplementary studies.

Aims 7 and 8 are described and achieved in Quantification, fate and hazard assessment of HIV-ARVs in water resources: Volume 2 – Social aspects.

1.3 SCOPE AND LIMITATIONS

Table 1: Limitations associated with each research component.

RESEARCH COMPONENT	LIMITATIONS
Instrumental analysis	• The QTOF instrument used, is not as sensitive as QQQ. • Unable to fully simulate environmental water to act as matrix in a matrix matched calibration curve. • The use of deuterated-fluconazole as recovery standard is not desirable to simulate the properties of antiretrovirals.
Biological effects: Potential impacts of ARVs in the environment on bacteriophages	• Environmental concentrations are very low and the exposure studies using the microwell plate spectrophotometry only allows for 320µL total volume (this includes the bacteria and phage mixture). • Several large quantity dilution series of ARVs must first be made from the ARV stock to acquire appropriate concentration for investigating the effects of ARVs at environmental levels. • The spectrophotometry method does not allow for constant mixing of water as would be the case in the environment. • No other studies combining coliphages and ARV compounds have ever been performed before, thus there is a lack of literature to compare the experimental setup and results with.

RESEARCH COMPONENT	LIMITATIONS
In vitro tissue culture assays	• The effects of mixtures in environmental samples are difficult to determine. • Cannot determine which specific compound or mixture of compounds in the environmental sample causes oestrogenic activity.
Snail exposures	• Although *B. tropicus* is widely distributed throughout South Africa, it is not yet clear how similar phyla would react to comparable exposure testing. • Snail exposure testing is limited to the testing of a singular compound at a time, it does not reflect pharmaceutical mixture effects as found in the natural environment. • Exposure testing is limited by ideal laboratory conditions, toxicological effects thus do not reflect changing environmental conditions and factors. • Experimental system design is restricted to availability and access of pharmaceutically active ingredients as well as success of snail cultures. • There is no literature to compare results with.
Valuation of ecosystem services and disservices at health clinic gardens in South Africa	• Stakeholders hold opposing views of ESs and EDSs provided by health clinic gardens that differ from one stakeholder to the next and from one clinic to the next. • There are no associations between the actual values and the perceived values of the stakeholders. Only a relationship was tested between these values. It is possible stakeholders lack awareness of the benefits they could obtain from the garden or the value it has. • Stakeholders of the clinic gardens were not always present on the day of visitation. • The questions are mostly closed-ended and therefore more quantitative than qualitative.

1.4 SUMMARY OF WORK TO DATE

Table 2 and Table 3 summarise the work to date, revised due dates and deliverables submitted.

Table 2: Work completed to date.

No.	Task	Summary of work to date
1.	*Activity 6:* QA/AC	Training received as contracted from NMISA in instrumentation and theory of Agilent 1290 micro high-pressure liquid chromatograph (uHPLC) coupled to an Agilent quadrupole time-of-flight mass spectrometer (QTOF-MS). Sample sites were selected, and sampling was completed. Samples were analysed and the concentration of ARVs were determined in the different drinking and surface water samples.
2.	*Activity 11:* Bacteriophage investigation	Systematic literature review was completed. A single colony of *Escherichia coli* WG5 bacteria was obtained and incubated with the various phage-host antiretroviral working solutions. After which phages were analysed using Transmission Electron Microscopy and bacterial growth was investigated.
3.	*Activity 9:* Cellular bioassays	Active ingredients and formulations have been tested using *in vitro* cell-based assays. Three reporter gene cell lines capable of measuring (anti-)androgenic, (anti-)oestrogenic effects, and aryl hydrocarbon receptor (AhR) activity have been used to investigate the mode of action through which ARVs may affect non-target organisms. The (anti-)androgenic effects (MDA-kb2), activation of the AhR (H4IIE-luc), H295R steroidogenesis assay, and HuTu cytotoxicity assay have been completed.
4.	*Activity 10*: Snail exposures	*Bulinus tropicus* subjects have been exposed to active ingredients at various concentrations via water of which embryo toxicity, embryo development, hatching success and hatching growth has been investigated.
5.	Human risk assessment	An integrated literature review has been completed to provide a critical synthesis of HIV and AIDS from a health perspective into the realities were environmental- and health sciences can and should collaborate towards a transdisciplinary outcome. The four ARV pathways have been investigated and discussed.
6.	Valuation of ecosystem services and disservices	Literature study was completed. Research proposal was presented, and ethical clearance was obtained. Surveys have been conducted in the Kenneth Kaunda District Municipality of the North West province and the Phokwane Local Municipality, North Cape Province. A total of 32 health clinic gardens have been visited.

Table 3: Deliverable due dates and deliverables submitted to date.

No.	Deliverable	Status	Due date
1	Initial request.	Completed	30/05/2016
2	Detailed plan HIV-ARV use patterns, status quo regarding current use and predictions.	Completed	18/07/2016
3	Research progress report of components completed during past 12 months.	Completed	10/04/2017
4	Research progress report of the components completed during the past 24 months.	Completed	30/04/2018
5	Final report.	Completed	31/05/2019

BACKGROUND

1.5 THE PRESENCE OF PHARMACEUTICALS IN WATER

The intimate association between human wellbeing and the wellbeing of the environment makes ecocentric toxicity assessments indispensable. Disputably, this incentive resulted from the distinctive environmental transformations that have occurred on a scale unique to human history. Consequently, ecosystems have begun to lose their resilience and require greater protection from threats and pressures on their integrity (Cairins, 2004). One subject of concern is the release of contaminants into the environment comprising of PPCPs. Recent research has revealed that pharmaceuticals can transform and influence the structure of aquatic communities (Munoz et al., 2009; Rosi-Marshall et al., 2013) along with the behaviour of aquatic organisms (Brodin et al., 2013). However, in accordance with Daughton and Scuderi (2012), limited research on the presence and degradation of PPCPs in the surface waters of South Africa has been carried out even though it is widely comprehended that PPCPs are discharged into the environment (Ferrer and Thurman, 2012).

These pharmaceuticals may be discharged into the environment through various pathways including: domestic and sewage wastes, leakage of underground sewage lines, surface water runoff and the indirect excretion of un-metabolised parent compounds in the form of faeces or urine (Swati et al., 2011). In addition, the disposal of unwanted or expired medicinal compounds can also enter the environment via household waste that is confiscated to landfill sites that can leach to groundwater. The most common types of pharmaceuticals found in waste/natural receiving waters are anti-inflammatories, analgesics, anti-pyretyics, anti-epileptics, anti-depressants, beta-blockers, lipid-lowering medicines, antibiotics, etc. (Luo et al., 2014). The composition and concentrations in receiving matrixes are therefore likely to be determined by a combination of: the amounts utilised, the natural breakdown after ingestion, the method in which the residues are disposed of after excretion, the efficiency/lack of its removal from wastewater and soils, natural attenuation, dilution, and the environmental chemistry of the pharmaceuticals and their breakdown products. Moreover, the subsequent concentrations and associated risks may be further modified by the exploitation patterns in various regions, the urban and natural hydrological cycles in addition to the climate and geological/hydrological features of each region (Luo et al., 2014).

It is widely acknowledged that the ever-increasing introduction of biologically active materials may pose hazardous to humans and the natural environment (Kümmerer 2008; De Jongh et al., 2012). Pharmaceutically active ingredients (PAIs) are therefore thought to be a rather new class of environmental organic micropollutants (Carlsson et al., 2006; Christen et al., 2010). Due to their desired biological activity, pharmaceuticals are often susceptible to various breakdown mechanisms, can be transformed in countless potential, and confirmed breakdown products, many of which are also biologically active with non-desirable effects (Kümmerer 2008; Stuart et al. 2012; Saif Ur Rehman et al. 2013; Luo et al. 2014). These PAIs may thereby also induce unexpected effects in non-mammalian organisms, which might cause disturbance of the hormone and reproductive systems, neurobehavioral changes and even immune depression (Christen et al., 2010).

Most likely, the bulk of the risk associated with these compounds are mediated through water, considering that the majority of these pharmaceuticals eventually enter and end up in the fresh-, estuarine-, and marine water systems of South Africa. Madikizela et al. (2017), also proposes that the physiochemical properties of pharmaceuticals support their detection in water rather than in solid matrices such as sediments and aquatic plants. This suggests that the water cycle is of immense concern, as the pharmaceuticals are therapeutically designed to cause desired effects in an aqueous environment (such as the human body) at seemingly low concentrations. This insight adds further

concern about the role and effectiveness of WWTPs modulating the biological activity of the molecules to acceptable and innocuous levels for downstream users (Riviera-Utrilla et al. 2013; Luo et al. 2014) and the natural biological components (fish/snails, etc.) to the functioning of ecosystems, especially, since there are no worldwide accepted regulations pertaining to the removal of pharmaceuticals. Hence, there is a well-motivated need to monitor the occurrence and impacts of pharmaceuticals in the natural environment.

1.5.1 Pharmaceutical guidelines

Evaluating the potential health risks from exposure to pharmaceuticals in drinking water is complicated. Currently, there are no guidelines for pharmaceuticals in drinking water, no standardized prioritization of pharmaceuticals for further assessments, and no standardization of protocols for investigating and sampling pharmaceuticals which makes assessment and comparison of information difficult (Rodriguez-mozaz and Weinberg, 2010).

Comprehensive information on the fate and consequences of pharmaceutical compounds in the natural environment is exceedingly limited, especially to the public. Information on environmental effects are also greatly deficient since pharmacovigilance programs do not investigate short- and long-term effects of exposure to the environment. Although, some information is available on the effects of bioactive ingredients from clinical trials and mammalian studies. This information is largely endorsed by the pharmaceutical industry and unfortunately South Africa does not have large scale production of pharmaceuticals. Guidelines and regulations require set limits, adding further importance to investigating the complexity of emerging contaminants in South Africa as well,

By analysing mammalian and clinical trial data, in conjunction with methods of envisaging long-term consequences from low concentration exposure, scientists may be able to determine whether certain pharmaceutical compounds in the environment may constitute a threat to human and organism health (Seiler 2002; Huggett et al. 2003; Ankley et al. 2007; Berninger and Brooks 2010). This can in turn help to develop future guidelines and regulations.

Organisms exposed to PPCPs have revealed responses such as behavioural effects, histological alterations, biochemical reactions and even up- or down-regulation of genes (Ankley et al. 2007; Brooks et al. 2009; Corcoran et al. 2010). According to Boxall et al. (2012), these responses can take place at concentrations that are orders of magnitude lower than the concentrations at which effects are seen in regulatory tests, for example acute tests investigating the effects on invertebrate and fish mortality, or chronic tests investigating effects on growth and reproduction. The significance of these responses in relation to ecosystem functioning and population survival is inadequately understood. Yet, Boxall et al. (2012), argue that in order to fully comprehend the implications of nonstandard observations on ecosystem health and to establish the benefits of incorporating information from nonstandard ecotoxicology responses into retrospective and prospective risk assessment frameworks, these relationships need to be understood.

Given that our knowledge of the correlation between molecular level and whole-organisms level effects in humans are thoroughly developed, we can utilise this knowledge together with ecotoxicological responses to improve our understanding between molecular level and whole-organisms level endpoints for other organisms. This can in turn help improve ecologically important endpoints, such as growth, reproduction, and survival of species. Establishing indicator organisms, concentrations, endpoints, etc. is therefore vital in producing future guidelines.

1.5.2 Pharmaceutical mixtures and persistence

A singular PAI may pose hazardous to the environment, yet the environmental concerns are not limited to a singular drug. Concerns are much greater for the reason that hundreds of various classes of drugs are entering our water systems simultaneously. It can be expected that these chemical compounds are not just affected by environmental factors but are also acted upon one another.

Cleuvers (2003, 2004), has investigated the effects of pharmaceutical mixtures. The ecological prospective of anti-inflammatory drugs and of assorted acting pharmaceuticals in different sets of biotests were evaluated using various aquatic organisms. A mixture of NSAID (naproxen, acetylsalicylic acid, ibuprofen, diclofenac) was evaluated using acute *Daphnia magna* and *Desmodesmus subspicatus* (algae) tests. Interestingly, Cleuvers found the mixture toxicity at concentrations which the single compound indicated slight to no effects, followed the concept of concentration addition. Signifying that the concentrations of each compound acted in an additive manner.

To give further detail, acute toxicity tests were performed using *D. magna*, *D. subspicatus* and *Lemna minor* (macrophyte) in order to analyse the toxicity of nine drugs with dissimilar modes of action (carbamazepine, captopril, clofibric acid, diclofenac, ibuprofen, metformin, metoprolol, naproxen, propranolol). The combined effects of carbamazepine and clofibric acid illustrated the theory of concentration addition for *D. magna*. Yet, *L. minor* illustrated the theory of independent action. So also, where the results different for the combination test of NSAID, diclofenac and ibuprofen. *D. subspicatus* illustrated the theory of concentration addition, whereas *D. magna* illustrated that the combination effect was stronger (Cleuvers, 2003).

Pomati et al. (2008), also reported the mixture effect of 13 different pharmaceutical drugs at concentrations in the ng/L range and confirmed that the mixture effects could prevent the proliferation of human embryonic cells by influencing their morphology and physiology. Due to the concerning consequences posed by the mixture of contaminants, various other researchers have investigated the adverse effects of pharmaceutical mixtures at ng/L level (Zou et al., 2012; Vasquez et al., 2014; Wang et al., 2014).

The assessment of toxicity associated with chronic low-dose exposure to mixtures of pharmaceuticals requires chemical analysis or biomonitoring of drinking water for human hazard risk assessment. In order to measure low-dose chronic exposures, it is central that appropriate end points are studied. So also, could risk assessment gain from a prioritization listing of pharmaceuticals to assist in determining the risk based on specific factors such as therapeutic dose, mode of action, and environmental exposure (Lam, 2014). Though, pharmaceutical prioritization approaches are available in literature, no widely accepted prioritization list exists yet. In order to correct this, more information on the effects of individual as well as mixture of pharmaceuticals on human health is required.

1.5.3 Particular drug categories

The management of HIV/AIDS generally includes the use of multiple antiretroviral drugs in an endeavour to control and regulate the HIV infection. The various stages of the HIV life-cycle are acted upon by several classes of antiretroviral agents, subsequently ARV drugs are broadly classified by the phase of the retrovirus life-cycle that the drug inhibits. These classes comprise of nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), the protease inhibitors (PI), neuraminidase inhibitors and antiretroviral combinations, chemokine receptor antagonists (CCR5 antagonists), and fusion inhibitors (FIs) (Ruthban, 2017).

The various drug classes are used in combination, known as cART, whereas HAART refers to the use of multiple drugs that act on different viral targets. Its use began in 1997 and has since then contributed to the decreases in a patient's encumbrance of the HIV infection by maintaining function of the immune system and preventing opportunistic infections that often cause death. HAART has subsequently aided in the recent decline in AIDS hospitalization and morbidity. Therefore, it has become the standard of treatment (Moore and Chaisson, 1999).

According to Smyth et al. (2012), cART defends against resistance by suppressing HIV replication as far as possible, thereby reducing the potential pool of spontaneous resistance mutations. If one of the drugs being taken encounters a mutation that conveys resistance, the other drugs in combination will continue to suppress reproduction of that mutation. Customarily, combinations consist of three drugs from at least two different classes, commonly known as a 'triple cocktail' (Henkel, 1998). The number of useful combinations is limited by the fact that combinations of ARVs are subject to positive and negative synergies. Consequently, drug companies have worked together to combine complicated regimens into simpler formulas, termed fixed-dose combinations. For instance, there are now several options that combine three or four drugs into a single pill taken once daily (Bangalore et al., 2007). This greatly increases the ease with which medication can be taken, which in turn improves the consistency and adherence of the therapy.

1.5.3.1 Nucleoside reverse transcriptase inhibitors (NRTIs)

As mentioned before, NRTIs were some the first compounds accessible for the treatment of HIV infection. Although, less potent against HIV than NNRTIs, INSTIs, and PIs, the NRTIs have played a central role in ART exhibiting activity against HIV-1/HIV-2 and therefore continue to form part of the present-day standard of care (Shen et al., 2008). NRTIs disrupt the HIV replication cycle by means of competitive inhibition of HIV reverse transcriptase and termination of the DNA chain. Reverse transcriptase is an HIV-specific DNA polymerase which allows HIV RNA to be transcribed into single strand and ultimately double strand proviral DNA, which can then be incorporated into the host-cell genome (Weller and Williams, 2001). Proviral DNA chain elongation is obligatory before genome incorporation can occur, achieved by the addition of pyrimidine and purine nucleosides to the 3' end of the growing chain.

NRTIs comprise of closely associated structures, which are analogues of the nucleosides compulsory for DNA synthesis. The absence of a hydroxyl group at the 3' position of the ribose ring distinguishes NRTIs from natural nucleosides. These nucleosides analogues are prodrugs, which necessitate phosphorylation by intracellular kinase in order to be converted into a pharmacologically active tri-phosphorylated form (Karim and Karim, 2010). During the course of reverse transcription, the newly formed tri-phosphorylated nucleotide analogues attach to the viral reverse transcriptase enzyme, thereby contending with the naturally occurring nucleotides for integration into the DNA copy of HIV RNA. Once integrated into the proviral DNA chain, the absence of a 3'-hydroxyl group prevents the development of a new 3',5'-phosphodiesterase bond with the subsequent nucleotide, causing a chain termination (Figure 1).

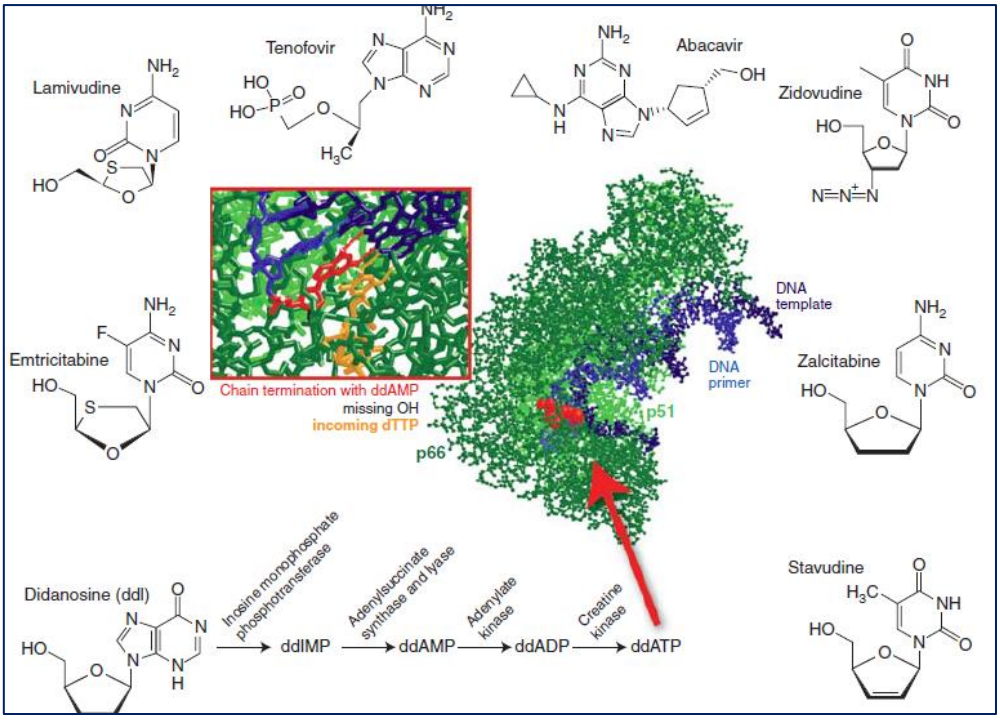


Figure 1: NRTIs and X-ray crystal structure of HIV-1 reverse transcriptase in complex with DNA primer/template chain terminated with ddAMP and with an incoming dTTP. The schematic illustration of crystal structure data was obtained, with permission, from Arts and Hazuda (2012).

According to Karim and Karim (2010), the activity of the ARVs is determined by the intracellular concentrations of tri-phosphorylated forms of the NRTIs, rather than the serum concentrations of the prodrugs. Resistance to NRTIs can result from several mechanisms; mutations in the *pol* gene, for instance, can alter the structure of reverse transcriptase that causes the preferential binding of natural deoxy-nucleotide tri-phosphates or the selective dismissal of the NRTIs. Chain termination can also be overturned by removing the 3' terminal nucleotide monophosphate by attaching to a free pyrophosphate or free nucleosides. NRTIs, such as AZT for example, require the precise acquisition of numerous mutations before the development of a noteworthy resistance (Esté and Cihlar, 2010). The association between NRTI-resistant mutations is relatively intricate with resultant alterations in the capacity of viral replication, cross resistance to other particular NRTIs, and even a reversal or delay of resistance to other constituents of this class. Analysis and interpretation of genotypic resistance patterns should be made in conjunction with the patient's past and present history of exposure to antiretrovirals (Azu, 2012). Interestingly, emtricitabine, lamivudine, and tenofovir exhibit activity against hepatitis B virus (HBV) in addition to HIV, and are therefore often incorporated into antiretroviral regimens for patients with HIV and HBV coinfection (Weller and Williams, 2001).

1.5.3.2 Protease inhibitors (PIs)

HIV protease inhibitors were first introduced in 1995 and form an integral part in the treatment of HIV infection. Though, PIs exhibit similar mechanisms of action, they have significant differences in their pharmacokinetics, efficacy, and adverse event profiles (Shen et al., 2008). PIs are structurally distinct molecules that do not necessitate any chemical alteration to become pharmacologically active. PIs are metabolised in the gut and liver by the P450 enzyme system and predominantly by the CYP3A4 isoenzyme, which results in several pharmacological interactions with other hepatically-metabolised drugs (Karim and Karim, 2010). The protease inhibitors' target is the HIV encoded protease enzyme, which is compulsory for the cleaving of an extensive HIV protein precursor transcript into gag, gag-pol

sub-units, prior to virion assemblage and the distribution of new infectious viruses from the initial infected cell.

They consist of small molecules that attach to the central, active cleavage site of the HIV protease heterodimer (Karim and Karim, 2010). The active binding site undergoes alterations due to resistance mutations clustering around the active site, with a consequential decrease in drug binding capability (Figure 2). PIs experience multiple coinciding patterns of resistance with specific single mutations, such as D30N, associated with joint resistance profiles for ritonavir and indinavir, with nelfinavir resistance, and distinctive patterns for amprenavir, saquinavir, and lopinavir (Bertrand et al., 2009).

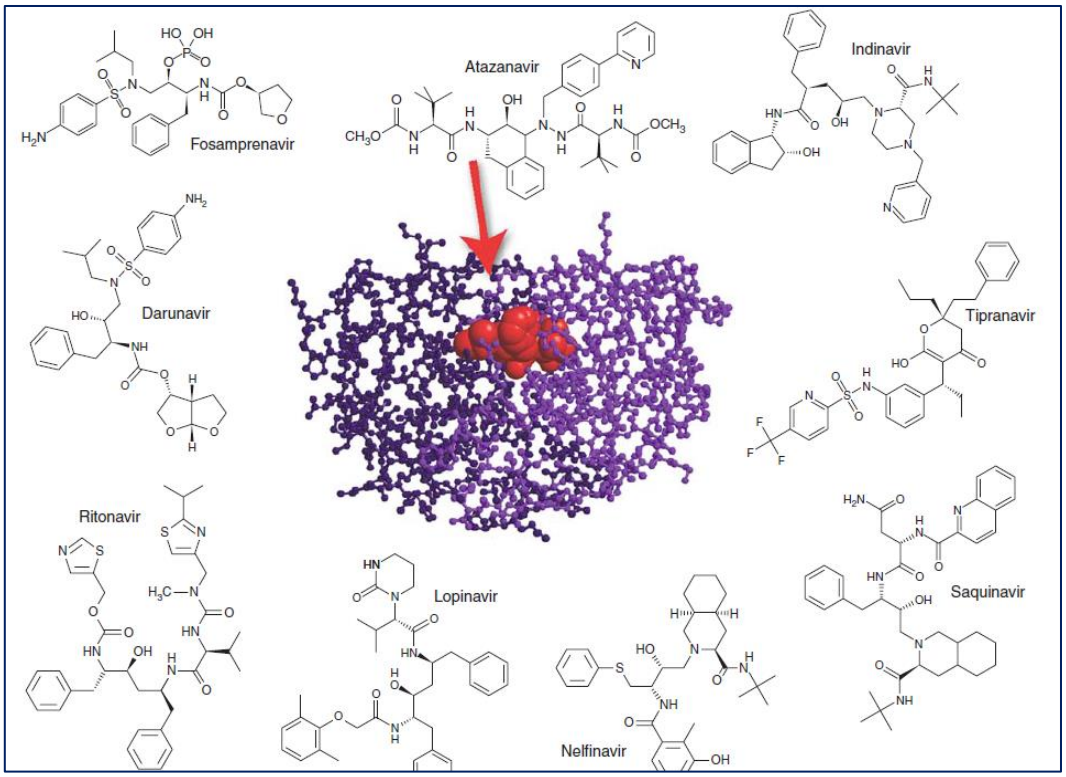


Figure 2: PIs and the crystal structure of HIV-1 protease complexed with atazanavir (as obtained, with permission, from Arts and Hazuda, 2012).

The capricious pathways to resistance affecting individual or assemblages of PIs allows for sequential use of antiretrovirals of this class following the incompetency of primary PI regimes (Azu, 2012). Class-related deleterious effects include acute gastrointestinal symptoms and long-term metabolic complications such as hyperlipidaemia, insulin resistance, and lipodystrophy (morphological changes in fat distribution). Both central fat and peripheral fat wasting accumulation have been documented after prolonged use of PI (Hagmann, 2003).

1.5.3.3 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

Non-nucleoside reverse transcriptase inhibitors were first introduced in the 1990s with the approval of nevirapine and have since then formed part of the preferred initial regimens that exhibited potent activity against HIV-1 infections. Efavirenz, in particular, confers the most significant inhibition of viral infectivity amongst NNRTIs (Shen et al., 2008). NNRTIs are reasonably free of long-term metabolic complexities related to the usage of PIs and have acquired acceptance and popularity as initial therapy when used in combination with two other nucleosides (Karim and Karim, 2010). Although, NNRTIs have no activity against HIV-2, they do not necessitate chemical alteration to become pharmacologically active.

The various members of NNRTIs have diverse chemical structures. They consist out of small molecules that function by non-competitive binding to an active receptor site adjacent to the polymerase domain of reverse transcriptase. Binding of NNRTIs to this catalytic receptor site causes alterations in the three-dimensional structure of reverse transcriptase, resulting in the impairment of polymerase activity (Esté and Cihlar, 2010). According to Rodgers et al. (1995), the NNRTI-binding pocket only subsists in the presence of NNRTIs and comprises of hydrophobic residues (F227, W229, Y181, Y188, and Y232) as well as hydrophilic residues (D192, E224, K101, K103, and S105) of the p66 subunit and E138 of the p51 subunit (Figure 3) (Arts and Hazuda, 2012).

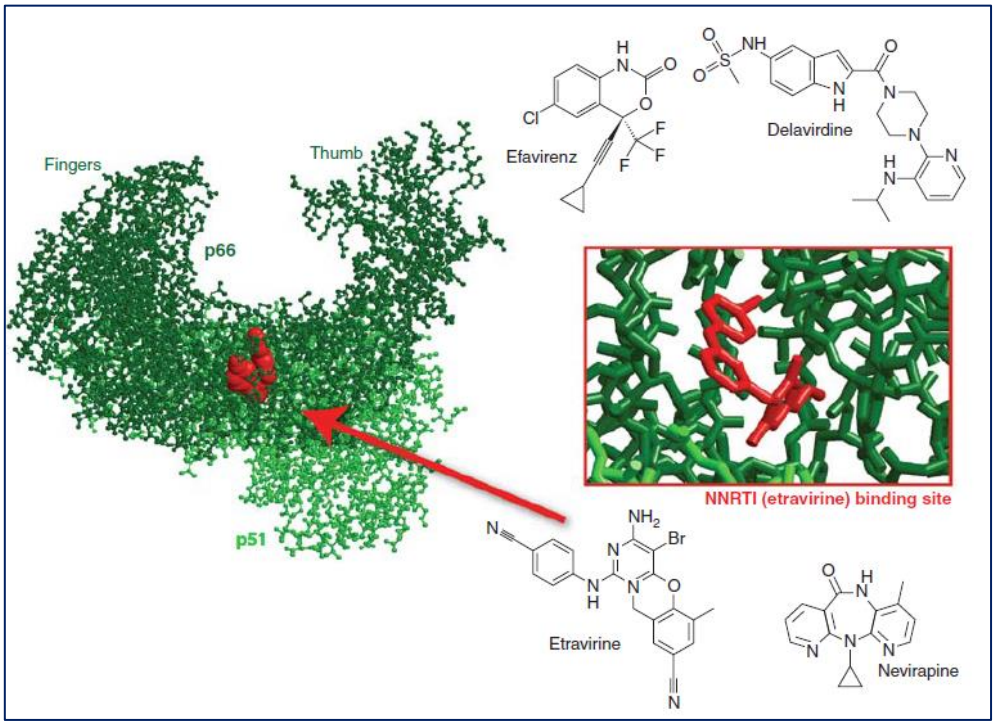


Figure 3: NNRTIs and the X-ray crystal structure of HIV-1 reverse transcriptase complexed with etravirine (as obtained from Arts and Hazuda, 2012).

Single point mutations modifying the NNRTI catalytic binding site, can cause the rapid development of high-level resistance if these agents are used as monotherapy. Other first generation NNRTIs also frequently experience cross-resistance. Class deleterious effects include acute skin rash and hepatotoxicity (Soriano et al., 2008; Kayode et al., 2011).

1.6 ENVIRONMENTAL CONCERNS

1.6.1 Latent risks of ARVs

The presence of HIV antiretrovirals (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 little historic concern, but which may have unexpected or unpredicted effects. Considering the number of patients on ART in South Africa, it is sensible to suspect that, despite some analyte loss due to their transformation and metabolism, ARV drugs contribute significantly to the complete pharmaceutical load in wastewater (Mosekiemang et al., 2019).

The existing data indicates that trace quantities of ARV residues are found in aquatic systems (Prasse et al. 2010; Peng et al. 2014; Russo et al., 2018). The persistence of some pharmaceuticals including ARVs, have been documented to be influenced by the aerobic physicochemical properties of water (Aminot et al., 2018). Half-lives of <5days under biochemical conditions in surface water and wastewater have been observed for abacavir, lamivudine, ritonavir and saquinavir.

Whereas, nevirapine and zidovudine was observed to be quite stable in surface water and wastewater (Aminot et al., 2018). According to the findings of Schoeman et al. (2017), the persistence of nevirapine was confirmed under most wastewater treatment practices (<22.5% removal). Ncube et al. (2018), argued that the removal efficiencies of the ARV drugs efavirenz (83-92%), lamivudine (24-59%), nevirapine (11-49%), and zidovudine (99%) as documented by K'oreje et al. (2016), suggest that certain ARV drugs are persistent enough to circumvent most wastewater treatment practices and persist in surface water.

ARVs are predominantly designed to interact with a specific receptor, enzyme or biological process to produce a desired therapeutic effect in humans. The introduction of ARVs into aquatic environments has the potential of affecting similar pathways in organisms comprising of identical target organs, tissues, cells or biomolecules, etc., however, it also has the potential for dissimilar modes of action to occur in those organisms lacking or with different receptors than those in humans.

Even though the precise environmental quantities are not yet defined, affects may vary considerably between species and even between individuals (Jones et al. 2004). ARVs in natural receiving waters may affect biota and ecosystems on many levels. Because physiology and behaviour of aquatic biota differ (e.g. ectothermic fish) compared to humans, there may be consequences and effects not yet known. Ectothermic animals mostly have slower physiological activities and consequently may metabolise xenobiotics at a much slower pace. ARVs, their modifying additives, and their breakdown products, may also illustrate side effects much different from their desired effects. It is not known for instance if they are toxic or endocrine disruptive at the measured or expected concentrations in the receiving or drinking waters, nor if there are any long-term effects due to exposure.

Information on the presence or absence of specific ARV targets in different taxa can help identify organisms and life stages most likely to be adversely affected in response to such exposure, even at low environmental concentrations (Seiler 2002; Huggett et al. 2003; Trudeau et al. 2005; Ankley et al. 2007; ECETOC 2008; Gunnarsson et al. 2008). Because of their intended biological activity, unintended side effects, and interactions with other compounds, ARVs in wastewater may affect the biological processes of the WWTPs, potentially even affect its own breakdown by acting on bacteriophages. Low-level exposure may also pose a risk to consumers of drinking water. Apart from side effects in healthy people, there is a possible latent risk of resistance development in people that are HIV-positive but that do not receive ART.

1.6.2 ARV research gaps

Numerous studies across the world have documented the presence of PPCPs in water sources, yet there is no comprehensibility how this will affect organism and human health. So also, should the absence of evidence not be confused with the perceived safety of continual drug waste exposure. Currently, literature about ecotoxicological effects of pharmaceuticals rarely comprise of any choric toxicity testing and predominantly entails acute toxicity in standardized tests. Once again, information regarding ARV toxicity is particularly lacking. Little information has been published on the ecotoxicological effects of the various antiretroviral drug classes; difference in toxicity experience by different phyla; the effects which ARV metabolites and by-products have; the influence which environmental parameters such as pH has on its toxicity, etc.; nor has the effect of combined

antiretroviral drugs been investigated. Only a hand full of authors have investigated the ecotoxicity of ARVs (Guo et al. 2015; Minguez et al. 2016; Ngumba et al. 2016a; Robson et al., 2017). Furthermore, not only are there uncertainties about long-term exposure to drug waste in water, there are uncertainties about the profound impact it will have on water quality and availability in conjunction with climate change and ecological disparities.

1.6.2.1 Metabolites and by-products

According to Mosekiemang et al. (2019), limited data on the environmental incidence of ARV drugs and their metabolites are likely contributed by the scarcity of commercially available standards for ARV drug metabolites in addition to intricate method development for target analytes of diverse physico-chemical properties.

Similar to other pharmaceuticals, NRTI and NNRTI ARV drugs are metabolised via phase I and II metabolic processes. This entails hydroxylation and excretion, or further glucuronidation of hydroxylated metabolites prior to excretion (Riska et al., 1999; Deng et al., 2015; Aouri et al., 2016). Zidovudine (NRTI) for example, is typically metabolised to zidovudine-glucuronide which has been documented in human urine together with the parent compound (Veal and Back, 1995). According to Riska et al. (1999) and Deng et al. (2015), efavirenz and nevirapine (NNRTIs), are excreted unchanged or metabolised into several hydroxylated metabolites, which may be further glucuronidated before excretion. Whereas the ARV class PI, are generally metabolised to produce simpler fragments of the parent compounds (Andrade et al., 2011). For instance, ritonavir is metabolised into various non-glucuronide derivatives, of which desthiazolylmethyloxycarbonyl ritonavir is the predominant metabolite (Denissen et al., 1997).

Russo et al. (2018), argues that in spite of the apparent efficiency of advanced oxidation processes in micropollutants removal, the potential for the formation of highly toxic by-products (Yuan et al., 2011; Rozas et al., 2016), demands for longer treatment times and for the further assessment of the ecotoxicity of treated water. A study performed by Wood et al. (2016), found that the *in vitro* toxicity and activity testing of nevirapine drug transformation products were none more toxic than the parent molecule. Nonetheless, the study concluded that antiviral activity is retained in some of the isolated fractions. Whether this can be accredited to a single molecule or through synergistic outcomes will only be explained once individual drug transformation products are isolated or synthesized (Wood et al., 2016).

Albeit the mixture effects of ARV drugs in aquatic systems have not yet been reported, these combinations of active substances are expected to demonstrate toxic consequences, as has been documented for other pharmaceutical classes of drugs (Pomati et al., 2008; Arnold et al., 2014). Based on the rational that evidence exists where the combination of various pharmaceutical drugs have different toxicity effects on different organisms and that ARV drugs in aquatic environments are known to be present, it is of great significance to conduct further investigation into the potential mixture effects of ARV drugs. Wood et al. (2016), also argues that it is essential to take into consideration that pharmaceuticals may be chemically altered and still retain biological activity.

A concerning statement made by Fent et al. (2006), stated that certain compounds analysed at concentrations lower than the individual non-observable effect concentration (NOEC) can still play a part in the total effect of the pharmaceutical mixture. Since ARVs are known to be a hazardous class pharmaceutical (Venhoff et al., 2007), various questions concerning the toxicological complexities of these drugs are brought to light. Do they exhibit similar combination effects? Do they bioaccumulate? Does their metabolites and breakdown products illustrate similar of even more toxic consequences?

What are the implications when antidepressants and/or antibiotics combine with ARVs in water resources? How do compounds from agricultural leakage react with ARVs, etc.? Supplementary research is therefore required to determine the impact of continued exposure to pharmaceutical mixtures.

1.7 TOXICOLOGICAL CONSEQUENCES

During a studied done by Sanderson et al. (2004), almost 3000 different compounds were analysed using (Q)SAR modelling of which antiviral drugs were predicted to be among the most hazardous therapeutic classes of drugs regarding their toxicity towards algae, daphnids and fish. Venhoff et al. (2007), also argues that ARV drugs can cause ecotoxicological effects especially NRTIs, which can cause deleterious effects in an organism's mitochondria as a result of their nucleosidic structure that can be integrated into DNA or RNA-strains. Ritonavir, a PI, has also been reported to display a high ecotoxicity potential (Escher et al., 2011). Zidovudine, which is excreted as a metabolite and parent compound via urine has been documented as the cause of hematological toxicity and carcinogenic effects in rodents (Vanková, 2010). There is a need to investigate the effects on different life stages and/or to examine the consequences on multigenerational life cycles in consideration of aquatic organisms which might be exposed to these compounds for their entire life expectancy.

As far as unmetabolised compounds are concerned, indinavir (PI) has been reported to be connected to certain side effects such as nephrolithiasis and crystalluria, urinary complications, renal atrophy, tubulointerstitial nephritis as well as hypertension (De Araujo and Seguro, 2002). Prolonged exposure to tenofovir has revealed to cause a reduction in bone mineral density (Fontana, 2009). As mentioned before, ARV drugs in wastewater may also affect the biological processes of the WWTPs, potentially even affect its own breakdown by acting on bacteriophages.

Guo et al. (2015), revealed in a study that the ecotoxicological exposure risk of ARVs towards algae due to human use is inconsequential. However, Minguez et al. (2016), documented that abacavir was harmful to green algae with an EC_{50} value of $57 mgL^{-1}$. So also did Ngumba et al. (2016a), document that nevirapine and zidovudine had potential ecotoxicological consequences on daphnia, fish and algae. Algae test subjects also reacted the most severe to ARV exposures. Similarly, Minguez et al. (2016) also concluded that abacavir was less toxic to diatom and crustacean test subjects and more toxic to algae.

1.7.1 Efavirenz case study

A study done by Robson et al. (2017), revealed that acute exposure of 10.3ng/L efavirenz to *Oreochromis mossambicus* caused severe damage to the liver and produced lower leukocyte levels. This study concluded that the introduction of efavirenz into the environmental has the risk of developing severe liver impairment in fish, such as hepatic steatosis, as a direct result from the exposure to this bioactive compound. Furthermore, Robson et al. (2017) found that regressive changes were the prevalent reaction pattern detected in the *O. mossambicus* study as a whole, indicating that the histopathological changes produced by acute efavirenz exposure may ultimately lead to functional organ loss and degenerated fish health. Yet Robson et al. (2017) were the first to describe the effects of environmentally pertinent concentrations of the drug efavirenz in South Africa waters on the wellbeing of *O. mossambicus.* Although, Swanepoel et al. (2015), also identified the presence of this antiretroviral drug compound (efavirenz) in the blood plasma of *Clarias gariepinus* from the Wasgoedspruit, North West Province, South Africa at a concentration of 135 ng/L.

According to Bastos et al. (2016), efavirenz is the third most utilised ARV in the world and a drug which is widely exploited in South Africa for the treatment of HIV-1 mutant strains. Unnervingly, efavirenz is considered hazardous in the environment due to its persistence and toxicity to aquatic life (Cayman Chemical, 2014; Stockholm County Council, 2014). As suggested before, environmental exposure to pharmaceutical compounds may illustrate similar effects on non-target organisms as in humans (Arnold et al., 2013; Kolpin et al., 2002). Robson et al. (2017), argues that this claim is seemingly true in relation to the liver damage observed for *O. mossambicus,* where comparable effects have been observed in patients receiving efavirenz treatment.

Rivero et al. (2007), stated that liver toxicity is the most prominent adverse effect of efavirenz treatment and frequently leads to the development of clinical hepatitis. So also has Macias et al. (2012), noted the association between treatment with efavirenz and the progression of hepatic steatosis. The duration of efavirenz treatment was also found to be correlated to the development of hepatic steatosis. In 2000, Sulkowski et al. (2000), reported that 18 out of 31 drugs producing hepatotoxicity in humans illustrated toxicity in the liver enzymes of animals.

Gatch et al. (2013), were some of the first scientists to document that efavirenz had a pharmacological profile consistent with psychoactivity. Even though efavirenz interacts with several molecular targets, Gatch et al. (2013) found that efavirenz predominate behavioural profile in rodents was comparable with lysergic acid diethylamine (LSD)-like properties mediated via the 5-HT2A receptor.

There are also other significant potential public health implications. Inaccurate dosing in HIV patients and in non-infected efavirenz users living in a high HIV prevalence environment, such as South Africa, produces near-optimal conditions for the emergence of HIV strains resistant to efavirenz and other ARV compounds. Regardless of whether efavirenz resistant strains of HIV develops in South Africa or somewhere else, it poses an eminent threat to health with a potential worldwide consequence. The studies performed by Gatch et al. (2013), at the molecular, cellular, and whole animal level propose that efavirenz has psychoactive properties equivalent to the hallucinogen LSD. Not only does this account for the cited abuse and misapplication potential, but also its dose-dependent side-effect profile comprising of adverse neuropsychiatric events.

1.7.2 Stavudine and zidovudine case study

Russo et al. (2018), highlighted that several studies have illustrated that there are differences between ARVs regarding genotoxic potency, chromosomal defacement and abnormalities induced *in vitro* and in perinatally to exposed infants and mice (Olivero et al., 2000; Olivero, 2007; Dutra et al., 2010). In light of investigating these discrepancies Russo et al. (2018), examined the ecotoxicity of treated and untreated solutions of stavudine and zidovudine using a series of ecologically pertinent testing species to evaluate the acute and chronic toxicities as well as the mutagenicity and genotoxicity.

The ecotoxicological test with *D. magna* and *Aliivibrio fischeri* as bioindicators did not evinced acute or chronic effects. *Raphidocelis subcapitata* illustrated a hormetic effect for stavudine and zidovudine treated with solutions at various UV_{245} doses after dilution from 1:10 to 1:100. However, specific tests with *Salmonella typhimurium* showed genotoxic and mutagenic activity of stavudine and zidovudine samples also at high dilution factors subjected to the type of photolytic treatment and substrate conversion.

The *Salmonella* mutagenicity assay results also indicated that mutagenic degradation intermediates could have been potentially formed at significant levels during the photolysis of zidovudine than stavudine. In addition, it was documented that the mutagenicity of the samples increased further at increasing UV_{254} doses. Residual mutagenic activity was also detected in the zidovudine samples (with concentrations of the same order of magnitude as those detected in African surface water) treated by UV_{254} photolysis for conversions higher than 90%, after a 1:1000 dilution (Russo et al., 2018).

It was also noted that in the presence of hydrogen peroxide, the UV_{245} photolysis reduces the ecotoxicological risk related to direct photolysis of the aqueous solutions containing the ARVs (Russo et al., 2018). This specific study also demonstrates the importance of selecting appropriate bioindicators depending on the biological and chemical characteristics of the selected xenobiotics detected in surface water and in WWTP effluents.

1.7.3 Alternative drugs

Despite the immense challenges ahead, certain opportunities have already presented itself. Fortunak (2014), for instances has highlighted some alternative solutions regarding ART, one being the extensive usage of rilpivirine (RPV) in first-line ART which has the potential to greatly reduce the costs and volume of API required to treat patients. According to Fortunak (2014), a mere 237 metric tons of rilpivirine would be required to treat 26 million patients each year, compared to 5,690 metric tons of efavirenz. Even at optimal pricing for efavirenz (120 USD/kg), using the alternative rilpivirine (400 USD/kg) has a potential costs reduction of 588 million USD/year due to lessened volume required to treat 26 million patients (Fortunak, 2014).

Zolopa et al. (2013) also argues that the use of alternative drugs can bring about a reduction in toxic effects. Substituting tenofovir disoproxil fumarate (TDF) with an alternative drug tenofovir alafenamide fumarate (TAF), has the prospective to enhance clinical benefits and decrease drug associated toxicity. TAF is dosed at 10-25mg a day versus 300mg of TDF a day. Hence, treating 26 million people with TDF will require 2,845 metric tons of API per year versus only 95-238 metric tons of TAF API a year. Once again treatment with an alternative drug is associated with a substantial reduction in costs (Zolopa et al., 2013).

These are but mere examples of a solution directly linked to the ability to produces non-toxic alternative drugs with low volume usage and is therefore dependant on technological advances in the health care industry. It is also not an all-encompassing resolution; however, it is a step in the right direction in lifting the environmental drug waste burden. Pressure should not only be placed on drug development and removal efficiencies of WWTPs, but also on ARV discharge in water and source of origin.

1.7.4 HIV-ARV compounds as used in South Africa

Presently, an arsenal of over 30 Food and Drug Administration (FDA) approved drugs are accessible for the treatment of HIV-1 infections (Arts and Hazuda, 2012), of which various combinations are accessible or approved in different countries. ART regimes are constantly changing due to new insights and product development. At the commencement of this project, various literature studies were considered in order to determine the most prevalent and utilized ARV compounds within South Africa on which the focus was to be drawn. The latest information available on which HIV-ARVs are generally used in South Africa, are contained in the Notice from the National Department of Health (appendix B), reproduced, and explained in a poster as found in appendix C. Even though, there are numerous bioactive compounds, this project set about to analyse the antiretrovirals as presented in Table 4.

This is supported by its vast consumption in South Africa, prescription quantities, availability of obtaining active ingredients as well as its occurrence and detection in the natural environment such as found in subsequent literature (refer to

Table 5).

Table 4: ARV classes and compounds analysed within this study.

Drug class	ARV generic name	Abbreviation	CAS no	FDA approval date
Nucleoside reverse transcriptase inhibitors	didanosine	ddI	69655-05-6	9 Oct 1991
	lamivudine	3TC	134978-17-4	17 Nov 1995
	stavudine	d4T	3056-17-5	24 Jun 1994
	zidovudine	ZDV/AZT	30516-87-1	19 Mar 1987
Non-nucleoside reverse transcriptase inhibitors	efavirenz	EFV	154598-52-4	17 Sep 1998
	nevirapine	NVP	129618-40-2	21 Jun 1996
Protein inhibitors	lopinavir	LVP	192725-17-0	15 Sep 2000
	ritonavir	RTV	155213-67-5	1 Mar 1987

According to Ncube et al. (2018), ARV concentrations in water systems are anticipated to increase in developing countries. This is based on the rational that countries with extensive ART programs do not have efficient treatment guidelines concerning the presence of pharmaceuticals in WWTP effluents. Insufficient sanitation systems in the majority of African countries also have the potential of augmenting ARV concentrations in surface water. The direct disposal of urine and faecal matter contaminated with ARV residues, may subsequently enter groundwater or accrue in rivers via runoff (Ncube et al., 2018).

Table 5: Antiretroviral drugs quantified in South African water bodies.

Compound	Sample	Maximum concentration (µg/L)	Reference
Atazanavir	Effluent	0.740	Abafe et al., 2018
Didanosine	Surface water	0.054	Wood et al., 2015
	Groundwater	0.003	Swanepoel et al., 2015
	Drinking water	0.003	Swanepoel et al., 2015
Darunavir	Effluent	17.000	Abafe et al., 2018
Efavirenz	Influent	17.4	Schoeman et al., 2015
	Effluent	7.1	Schoeman et al., 2015
	Influent	0.010	Robson et al., 2017
	Surface water	0.005	Robson et al., 2017
	Effluent	4.000	Schoeman et al., 2017
	Influent	34.000	Abafe et al., 2018
	Effluent	34.000	Abafe et al.., 2018
	Dam water	0.082	Rimayi et al., 2018
	River water	0.354	Rimayi et al., 2018
	Groundwater	0.005	Rimayi et al., 2018

Compound	Sample	Maximum concentration (µg/L)	Reference
Emtricitabine	River water	0.013	Rimayi et al., 2018
Indinavir	Effluent	0.042	Abafe et al., 2018
Lamivudine	Surface water	0.24	Wood et al., 2015
	Surface water	5.4	Schoeman et al., 2015
	Influent	2.200	Abafe et al., 2018
	Effluent	0.130	Abafe et al., 2018
Lopinavir	Surface water	0.31	Wood et al., 2015
	Surface water	0.001	Swanepoel et al., 2015
	Effluent	3.800	Abafe et al., 2018
Nevirapine	Surface water	1.48	Wood et al., 2015
	Surface water	4.9	Schoeman et al., 2015
	Influent	2.1	Schoeman et al., 2015
	Effluent	0.35	Schoeman et al., 2015
	Effluent	0.004	Swanepoel et al., 2015
	Drinking water	0.003	Swanepoel et al., 2015
	Groundwater	0.005	Swanepoel et al., 2015
	Effluent	0.473	Schoeman et al., 2017
	Influent	2.800	Abafe et al., 2018
	Effluent	1.900	Abafe et al., 2018
	Dam water	0.055	Rimayi et al., 2018
	River water	0.071	Rimayi et al., 2018
	Groundwater	0.013	Rimayi et al., 2018
Maraviroc	Effluent	0.039	Abafe et al., 2018
Raltegravir	Effluent	3.500	Abafe et al., 2018
Ritonavir	Effluent	1.500	Abafe et al., 2018
Rimantadine	Surface water	0.02	Wood et al., 2015
Stavudine	Surface water	0.78	Wood et al., 2015
	Drinking water	0.001	Swanepoel et al., 2015
Tenofovir	Surface water	0.24	Wood et al., 2015
	Effluent	0.002	Swanepoel et al., 2015
Zalcitabine	Surface water	0.07	Wood et al., 2015
Zidovudine	Surface water	0.97	Wood et al., 2015
	Effluent	0.003	Swanepoel et al., 2015
	Drinking water	0.002	Swanepoel et al., 2015
	Influent	53.000	Abafe et al., 2018
	Effluent	0.500	Abafe et al., 2018

Pharmacological studies reveal that the serum concentration of lamivudine and nevirapine increases when combined with fluconazole (Drugbank, 2017). So also does the metabolism of efavirenz, ritonavir, and zidovudine decrease when combined with fluconazole. Didanosine has been found to cause a decrease in the absorption of fluconazole ensuing a reduction in serum concentration and potential decrease in its efficacy (Drugbank, 2017). Due to the effects caused by fluconazole in association with the selected ARVs, the research team also investigated the ecotoxicological effects related to the release of fluconazole in the environment in conjunction with ARVs.

1.7.4.1 Fluconazole

Cryptococcosis is a conspicuous opportunistic infection amongst HIV-infected patients, predominantly in sub-Saharan Africa and Southeast Asia that is caused by the infection of *Cryptococcus* species. It is projected that cryptococcosis accounts for over 600 000 deaths each year globally (Park et al., 2009), of which cryptococcal meningitis is the most common clinical representative. Although, the worldwide prevalence of cryptococcosis has been substantially reduced by the widespread accessibility of ART, it is still a major obstacle in developing countries. Without pertinent treatment, cryptococcosis is fatal, therefore making early diagnosis and treatment key to treatment success. According to Srichatrapimuk and Sungkanuparph (2016), the treatment of cryptococcosis consists out of the optimum integration of three main aspects: intracranial pressure management for cryptococcal meningitis, antifungal therapy, and the restoration of immune function with ART.

It should be noted that the timing of ART in HIV-infected patients with cryptococcosis is extremely sensitive and should be executed with caution. Randomized trials have recently revealed that initiating ART up until five weeks after the commencement of antifungal therapy improved patient survival rates (Srichatrapimuk and Sungkanuparph, 2016). Treatment of cryptococcus has to do with its susceptibility to flucytosine, polyenes, and azoles. Consequently, fluconazole monotherapy is generally recommended for mild-to-moderate pulmonary disease, as well as during the consolidation and maintenance phases.

Fluconazole, a synthetic antifungal agent of the imidazole class, is available as a powder or for oral suspension. It is chemically designated as 2,4-difluoro-α,α1-bis(1H-1,2,4-triazol-1-ylmethyl)benzyl alcohol with a molecular formula of C13H12F2N6O and molecular mass of 306.3 (Drugbank). Fluconazole works by interacting with 14-α demethylase, a cytochrome P-450 enzyme required to convert ianosterol to ergosterol. As ergosterol is a vital component of the fungal cell membrane, inhibition of its synthesis marks an increase of cellular permeability producing leakage of cellular contents. Fluconazole may also inhibit endogenous respiration, inhibit the conversion of yeasts to mycelial forms, interact with membrane phospholipids, constrain purine uptake, and impair phospholipid and/or triglyceride biosynthesis (Dailymed, 2015).

Concern for the environmental release of this triazole antifungal agent relates to the fact that fluconazole is cleared predominantly by renal excretion, with approximately 80% of the administered dose emerging in urine as unchanged drug (Drugbank, 2017). Clinical studies have also revealed serious hepatic reactions during the treatment of fluconazole. According to the CLSI (2009), the spectrum of these hepatic effects has ranged from marginal transient elevations in transaminases to clinical hepatitis, cholestasis, and fulminant hepatic failure, as well as fatalities. Furthermore, is that cases of fatal hepatic reactions were noted to take place mainly in patients with serious underlying medical conditions such as AIDS and while taking multiple concomitant medications. Yet, limited information is available on the effect which fluconazole has on the environment and the potential risks related to it. Consequently, it necessitates further investigation.

CHAPTER 2: INSTRUMENTAL ANALYSIS

2.1 INTRODUCTION

The need for detection and quantification of ARVs is highlighted in previous chapters, hence, the aim of this chapter is to standardise a method for sampling, extraction, and analysis of antiretroviral compounds and the antifungal fluconazole. ARVs are present in the environment at low concentrations (

Table 5) and therefore the compounds need to be concentrated during the extraction process to ensure the analytical technique is capable of detecting the compounds. Water samples will be extracted, cleaned-up and analysed using a Liquid Chromatography/Mass Spectrometry-Quadrupole-Time-Of-Flight (LC/MS QTOF) (Agilent) instrument. Compounds of interest will be targeted, and the sample extracts will be screened for other compounds.

There have only been five studies published reporting on ARV concentrations in the environment in Africa and South Africa at the start of this study (Table 6). Various types of water sources were analysed.

Table 6: Overview of ARVs detected and quantified in environmental matrices.

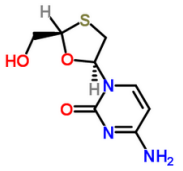
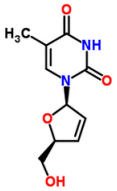
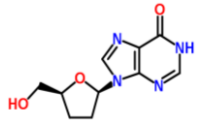
Sample site/type	Antiretrovirals	Concentration range	References
Wastewater treatment plants (WWTPs), receiving rivers, and wells in informal settlements.	nevirapine, lamivudine, zidovudine	1 000-9 000 ng/L	K'oreje et al., 2012
Surface water	lamivudine, zidovudine, nevirapine, efavirenz, amantadine, rimantadine	20-167 000 ng/L	K'oreje et al., 2016
Natural-, drinking-, bottled- and groundwater, fish plasma.	abacavir, efavirenz, didanosine, lamivudine, lopinavir, nelfinavir, nevirapine, ritonavir, stavudine, saquinavir, tenofovir, zidovudine	0.3-6.7 ng/L (water) 5.4-22 ng/L (plasma)	Swanepoel et al., 2014
Surface water sources downstream of WWTWs or urbanised environments.	zalcitabine, tenofovir, abacavir, efavirenz, lamivudine, didanosine, stavudine, zidovudine, nevirapine, indinavir, ritonavir, lopinavir	26.5-430 ng/L	Wood et al., 2015
Influent, effluent, chlorinated effluent from WWTP.	efavirenz, nevirapine	350-17 400 ng/L	Schoeman et al., 2015
Rivers flowing through informal settlements: upstream, midstream and downstream of informal settlements, and the WWTP effluent discharge point.	lamivudine, nevirapine, zidovudine	5 430-7 680 ng/L	Ngumba et al., 2016

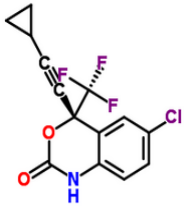
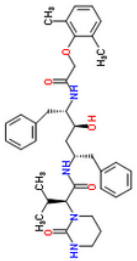
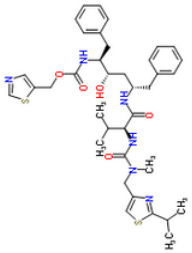
As mentioned before, didanosine, efavirenz, lamivudine, lopinavir, nevirapine, ritonavir, stavudine, and zidovudine have been chosen to be analysed based on their prevalence in the natural environment (

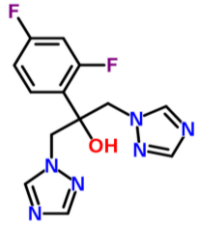
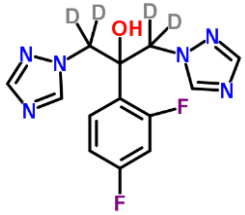
Table 5 and Table 6) and are extensively prescribed in South Africa. The antifungal, fluconazole will also be analysed because it is prescribed in conjunction with ARV medication (Meintjes et al., 2014).

The target compounds have been classified into different classes based in their mechanism of action. These compounds also have different chemical and physical characteristics (Table 7). In addition to the ARV parent compounds, some ARV metabolites were investigated. However, there is a lack of information on ARV metabolites in aquatic systems. Various metabolites are produced through different mechanisms such as human metabolism or microbial degradation. The metabolites include 8-hydroxyefavirenz, 2-hydroxy nevirapine and zidovudine-5'triphosphate (Andrade et al., 2011).

Table 7: Physical and chemical characteristics of the target compounds (As obtained from Chemspider, Drugbank and Pubchem).

	Compound	CAS#	Molecular formula	Monoisotopic mass	Structure	pKa		Water solubility
						Strongest acid	Strongest base	
ARVs	**Lamivudine**	134768-17-4	$C_8H_{11}N_3O_3S$	229.0521		14.29	-0.16	70 mg/mL
	Stavudine	3056-17-5	$C_{10}H_{12}N_2O_4$	224.0797		9.95	-3	83 mg/mL
	Didanosine	69655-05-6	$C_{10}H_{12}N_4O_3$	236.0909		6.94	2.75	15.8 mg/mL
	Nevirapine	129618-40-2	$C_{15}H_{14}N_4O$	266.1167		10.37	-5.06	0.705 mg/mL

Compound	CAS#	Molecular formula	Monoisotopic mass	Structure	pKa		Water solubility
					Strongest acid	Strongest base	
Zidovudine	30516-87-1	$C_{10}H_{13}N_5O_4$	267.0967		9.96	-3	20.1 mg/mL
Efavirenz	155213-67-5	$C_{14}H_9ClF_3NO_2$	315.0274		12.52	-1.5	0.093 mg/mL
Lopinavir	192725-17-0	$C_{37}H_{48}N_4O_5$	628.3624		6.26	-1.5	7.7×10^{-6} mg/mL
Ritonavir	155213-67-5	$C_{37}H_{48}N_6O_5S_3$	720.3127		13.68	2.84	1.26×10^{-3} mg/mL

	Compound	CAS#	Molecular formula	Monoisotopic mass	Structure	pKa		Water solubility
						Strongest acid	Strongest base	
Antifungal	**Fluconazole**	86386-73-4	$C_{13}H_{12}F_2N_6O$	306.1040		2.03	1.75	1.0 x 10^-3 mg/mL
Internal standard	**Fluconazole-d4**		$C_{13}H_8D_4F_2N_6O$	310.129		-	-	-

2.2 MATERIAL AND METHODS

2.2.1 Sampling

Sampling locations and regime

An initial sampling was conducted in rivers from three different study areas. These were chosen to focus on areas up- and downstream of WWTPs (Figure 4-Figure 7). Studies have documented that most WWTPs are unable to remove completely pharmaceuticals during the treatment process leading to contamination of water resources (Sun et al., 2014; Pereira et al., 2015). Exact sample locations were selected based on their accessibility and their location in terms of WWTPs (upstream and downstream). The sampling events took place in the summer, within the rainfall season in September-November 2017. Water was sampled up- and downstream from WWTPs in northern (Figure 5) and southern (Figure 6) Gauteng (Table 8), which consisted of Olifantsfontein, Sunderland Ridge, Vlakplaats, Waterval, Welgedacht, Zeekoegat, Flip Human, and Baragwanath. The third study area is the Mooi River, situated close to Khutsong (North West) (Table 9) and downstream of the Wonderfontein Spruit (Gauteng).

Table 8: Coordinates of Gauteng samples collected between October 2017 and October 2018.

Sample site	Coordinates	
Baragwanath DS	-26.259108	27.925885
Baragwanath US	-26.272759	27.950645
Flip Human DS	-26.185955	27.760417
Flip Human US	-26.177597	27.766307
Olifantsfontein DS1	-25.922349	28.227483
Olifantsfontein DS2	-25.910475	28.230592
Olifantsfontein US	-25.951971	28.207424
Sunderland DS	-25.822074	28.082248
Sunderland US	-25.840518	28.110386
Vlakplaats DS1	-26.376	28.170762
Vlakplaats DS2	-26.430108	28.160555
Vlakplaats US	-26.343632	28.170421
Waterval DS1	-26.453508	28.085552
Waterval DS2	-26.499893	28.070405
Waterval US	-26.421899	28.094211
Waterval US DW	-26.421171	28.098232
Welgedacht DS1	-26.199063	28.479604
Welgedacht DS2	-26.212292	28.48101
Welgedacht US	-26.191213	28.478078
Zeekoegat DS1	-25.642978	28.384984
Zeekoegat DS2	-25.60916	28.368198
Zeekoegat US1	-25.648713	28.328409
Zeekoegat US2	-25.662653	28.351033

Table 9: Coordinates of samples collected in the North West.

Sample site	Coordinates	
Khutsong 1	-26.31587	27.38189
Khutsong 2	-26.367696	27.270332
Khutsong 3	-26.253296	27.159809
Khutsong 4	-26.360294	27.13892
Khutsong 5	-26.514639	27.124767
Khutsong 6	-26.571739	27.103548
Khutsong 7	-26.684546	27.100692
Khutsong 8	-26.708126	27.106071
Khutsong 9	-26.724926	27.106132
Khutsong 10	-26.746121	27.098841

An additional sampling approach was conducted, whereby samples were collected from the Jukskei, Hennops, Klip, and Crocodile rivers (Table 10). These rivers were sampled in different months, namely March, April, May, August, and October 2018. The multiple sampling regime was adopted to determine fluxes in the concentrations over time, and seasonal effects. Some sites in this sampling event coincided with sample sites in the initial event, however they have different site labels, because during the second sampling event the sites were labelled based on the river systems, and not the WWTPs (as was the case in the first sampling event). These are Sunderland Ridge DS1 (same site as Hennops 8), Sunderland Ridge US (same as Hennops 7), Olifantsfontein US (same as Hennops 3) and Olifantsfontein DS1 (same as Hennops 4).

Table 10: Coordinates of samples collected in the Gauteng between March and October 2018.

Sample site	Coordinates	
Hennops 1	-25.878533	28.256195
Hennops 2	-25.888751	28.225992
Hennops 3	-25.951437	28.207384
Hennops 4	-25.922381	28.227477
Hennops 5	-25.88954	28.224148
Hennops 6	-25.829178	28.115976
Hennops 7	-25.840507	28.110298
Hennops 8	-25.822378	28.082272
Hennops 9	-25.793756	28.003707
Jukskei 1	-26.154441	28.130995
Jukskei 2	-26.084225	28.108553
Jukskei 3	-26.056121	28.118883
Jukskei 4	-26.032128	28.111185
Jukskei 5	-26.036721	28.055483
Jukskei 6	-26.009621	28.037822
Jukskei 7	-25.985702	28.002141
Jukskei 8	-25.955545	27.964965
Jukskei 9	-25.977999	27.962469
Jukskei 10	-25.955241	27.962657
Klip 1	-26.325093	27.987896
Klip 2	-26.328337	27.936846
Klip 3	-26.32011	27.941017
Klip 4	-26.306795	27.940254
Klip 5	-26.311805	27.918583
Klip 6	-26.336841	27.902994
Klip 7	-26.289923	27.885756
Klip 8	-26.239979	27.912726

Sample site	Coordinates	
Klip 9	-26.294204	27.836259
Crocodile 1	-25.895168	27.91374
Crocodile 2	-25.863174	27.93408
Crocodile 3	-25.797273	27.893952

These rivers were chosen based on a study done by Petersen et al., (2017). They identified these regions as potential sources to release emerging chemical pollutants based on their locality to medical centres, sewerage treatment works, high population density, and areas with an environmental vulnerability. We also used the results of the initial scouting sampling. Maps of the sampling sites are in Figure 4-Figure 7.

The Klip River flows through Soweto and the south of Johannesburg, and the Hennops River in the southern part of the city of Tshwane are the most vulnerable catchments to anthropogenic substances in Gauteng. This is due to: high population compared to the total population of Gauteng, many WWTPs, more than a third of Gauteng's medical facilities, and a critically threatened ecosystem status (Peterson et al., 2017). The Jukskei River is situated next to the Northern Johannesburg WWTP. This is the largest WWTP in the city of Johannesburg, receiving and treating approximately 450 million litres of sewage daily from 1.6 million people (DWAF, 2009; DWAF, 2011). There have been many reports about untreated sewage being released directly into the Jukskei River by this WWTP over the last decade.

The Crocodile River, and its main tributaries the Jukskei and Hennops rivers, flow though densely populated and heavily industrialised regions (Taylor et al., 2005). The river system drains an area between the City of Johannesburg and the Hartbeespoort Dam. This catchment area is severely water stressed, with the main users including mining, agriculture, industry, and urban. Approximately 1.25 million people reside in the Jukskei river catchment, with a large portion living in informal settlements (Neswiswi, 2014). A large part of the Jukskei River borders on urban townships, mainly the Alexandra and Diepsloot Townships, where free-standing (backyard) shacks are juxtaposed on formal settlements (Campbell, 1996; Fitchett, 2017). The increase in these backyard shacks is overloading the infrastructure, which includes the sewage system. The Alexandra township was originally designed for a population of 70 000, but it is estimated that it exceeds that number by at least four times (GJCM, 2000). The river is under immense pressure from the rapid urbanisation of the townships, with sewers frequently becoming blocked and overflowing into the nearby river (Jardine Da-Silva, 2016). Many of the settlements on the riverbanks are not connected to the sewerage system and rely on rudimentary services, such as portable toilets (GJCM, 2000).

Due to insufficient management of these services, it has resulted in sewage discharges into the Jukskei River. The river is prone to flash floods due to heavy rain, which affects the settlements on the riverbanks. This is also a contributing factor to the release of pollutants into the river, via storm water runoff. These untreated effluents are a major contributor of the pollution that the Jukskei River faces. The main river is joined by the Modderfontein, Braamfontein Spruit and Sandspruit, and ultimately flow into the Crocodile River. The Modderfontein runs through a nature reserve, and as a result, there is little influence from human activities (Moropa, 2015).

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 near the Hennops River. The Olifantsfontein WWTP has a treatment capacity of 105 ML/day and receives wastewater from mainly Tembisa and Ivory Park. The treated effluent is discharged into the Kaal Spruit, which confluences with the Olifants Spruit to become eventually the Hennops River. The Sunderland Ridge WWTP has a treatment capacity of 65 ML/day and treats wastewater generated in Centurion and Midrand. The treated effluent is discharged into the Hennops River (Roux et al., 2010). One of the most polluted

streams running into the Hennops River is the Kaal Spruit; it borders the highly populated Tembisa and Ivory Park informal settlements (Nawn, 2004). There is a build-up of municipal waste in the townships due to lack of funds and violence, which hinders the removal thereof (Hoffmann, 1995). The Hennops and Jukskei River leads into the Crocodile River, which ultimately ends up in the Hartbeespoort Dam.

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). There are several informal settlements located on the banks of the Klip River, as well as the Klipspruit and Harrington Spruit (which are major tributaries of the Klip River), which can contribute to the pollution of the river. Three WWTPs are located in the close vicinity of the Klip River, which consequently releases close to 500 ML/day flow discharge into the river (Vermaak, 2009).

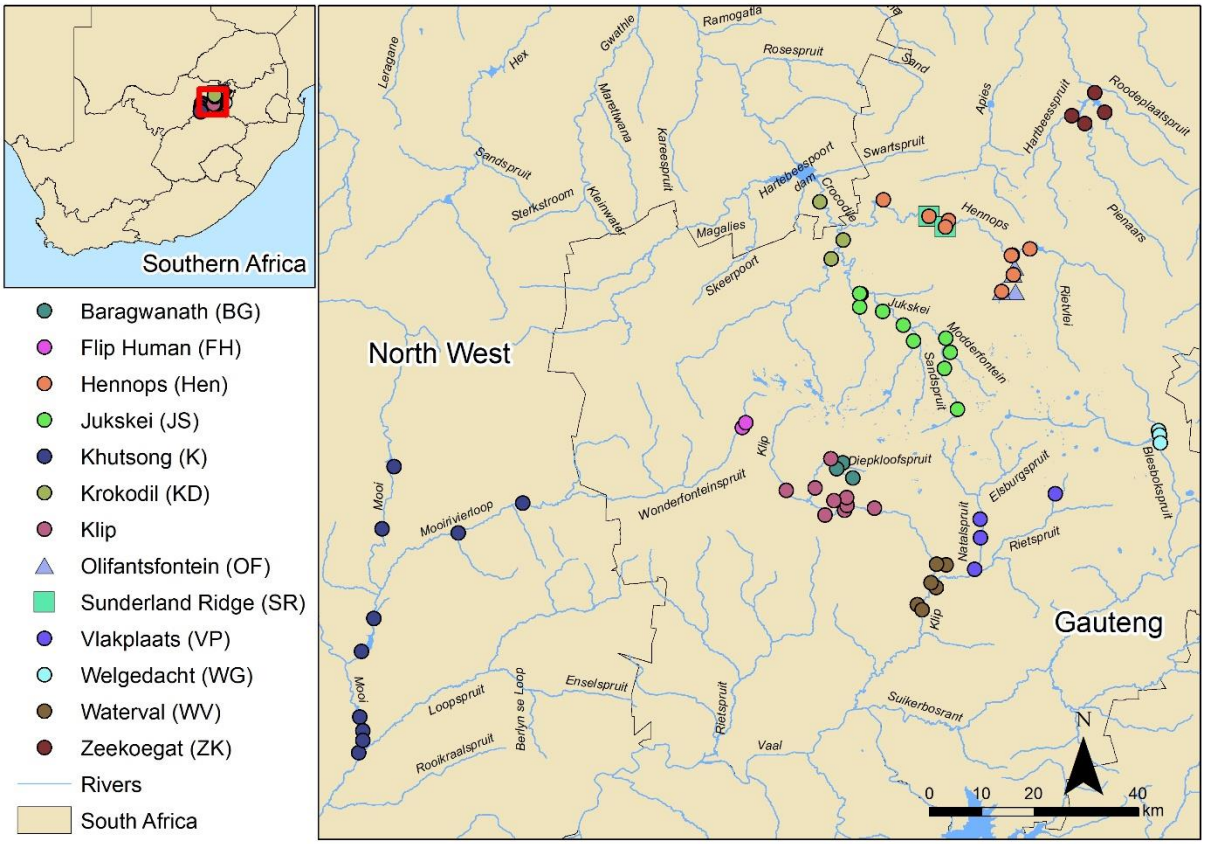


Figure 4: Map of the overall sampling area, across North West and Gauteng.

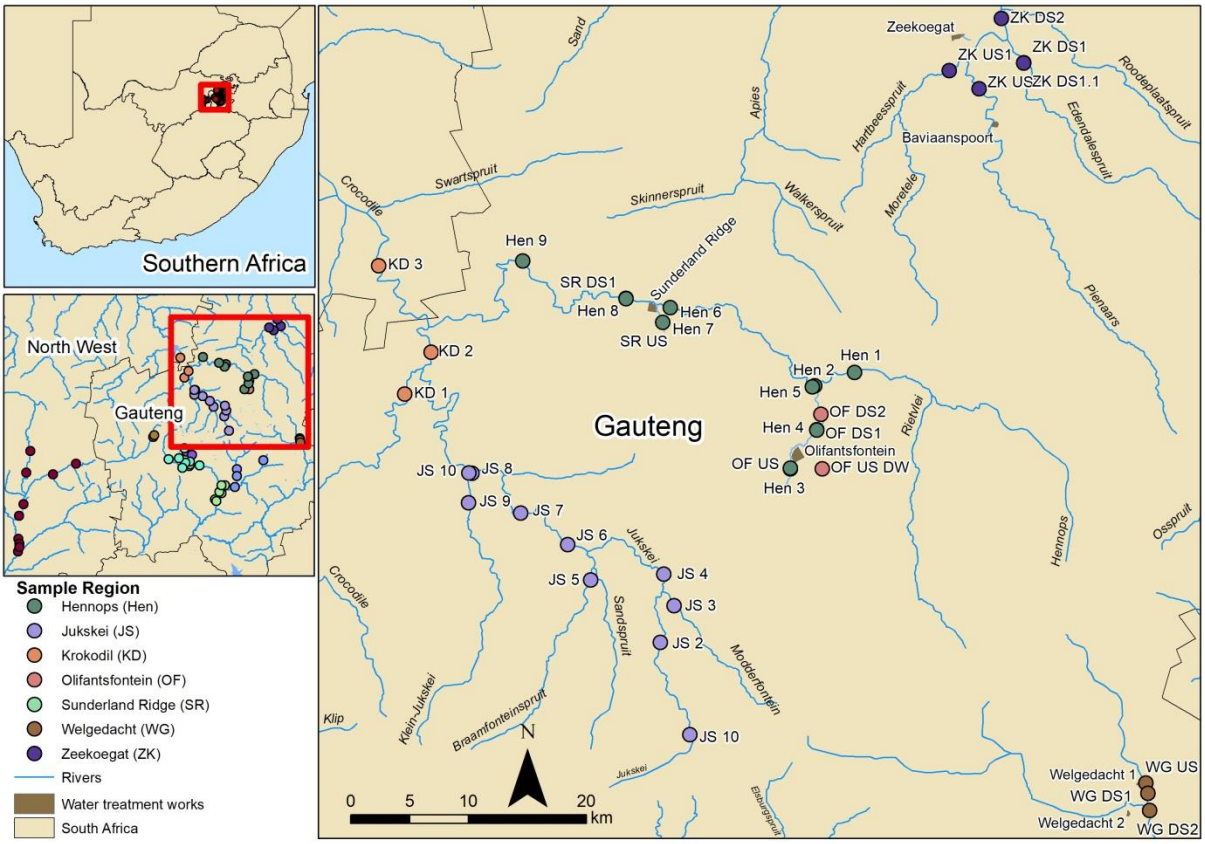


Figure 5: Map of the sampling sites situated in northern Gauteng.

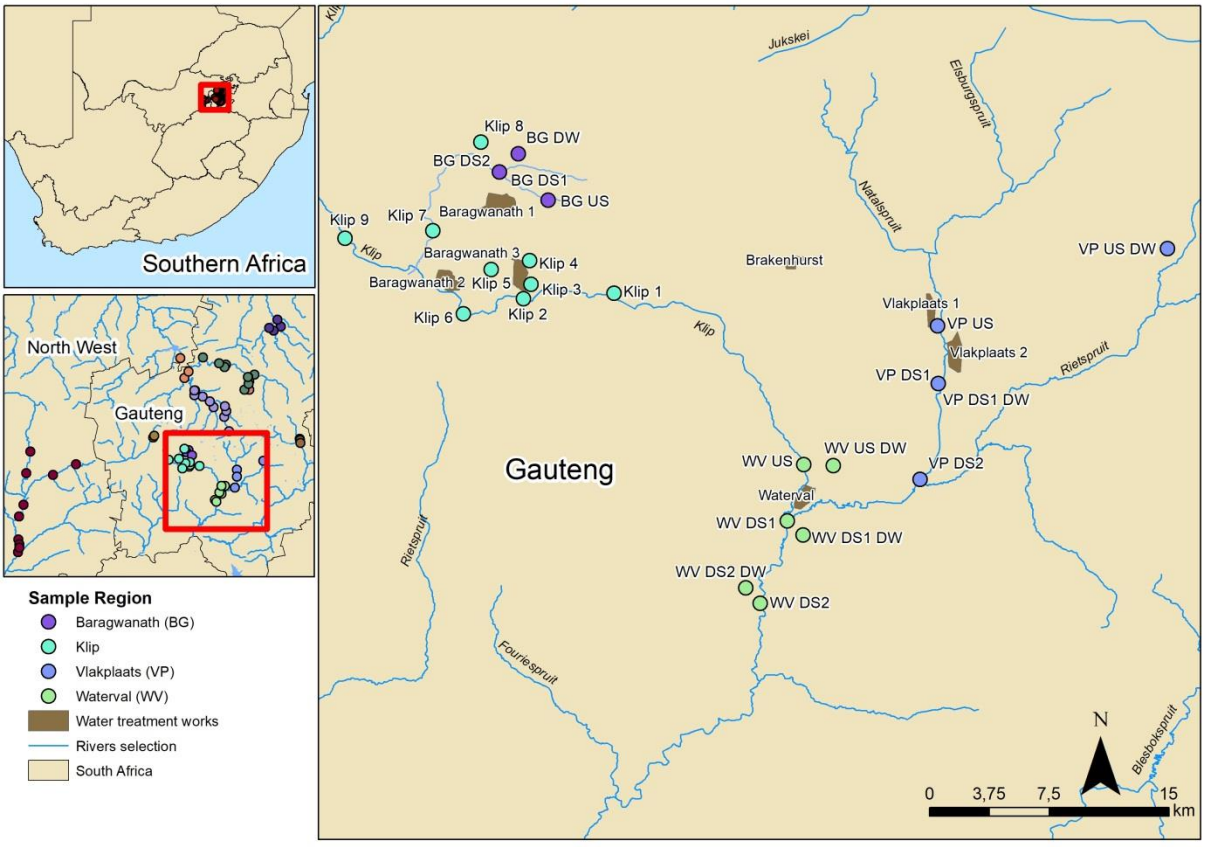


Figure 6: Map of the sampling sites in southern Gauteng area.

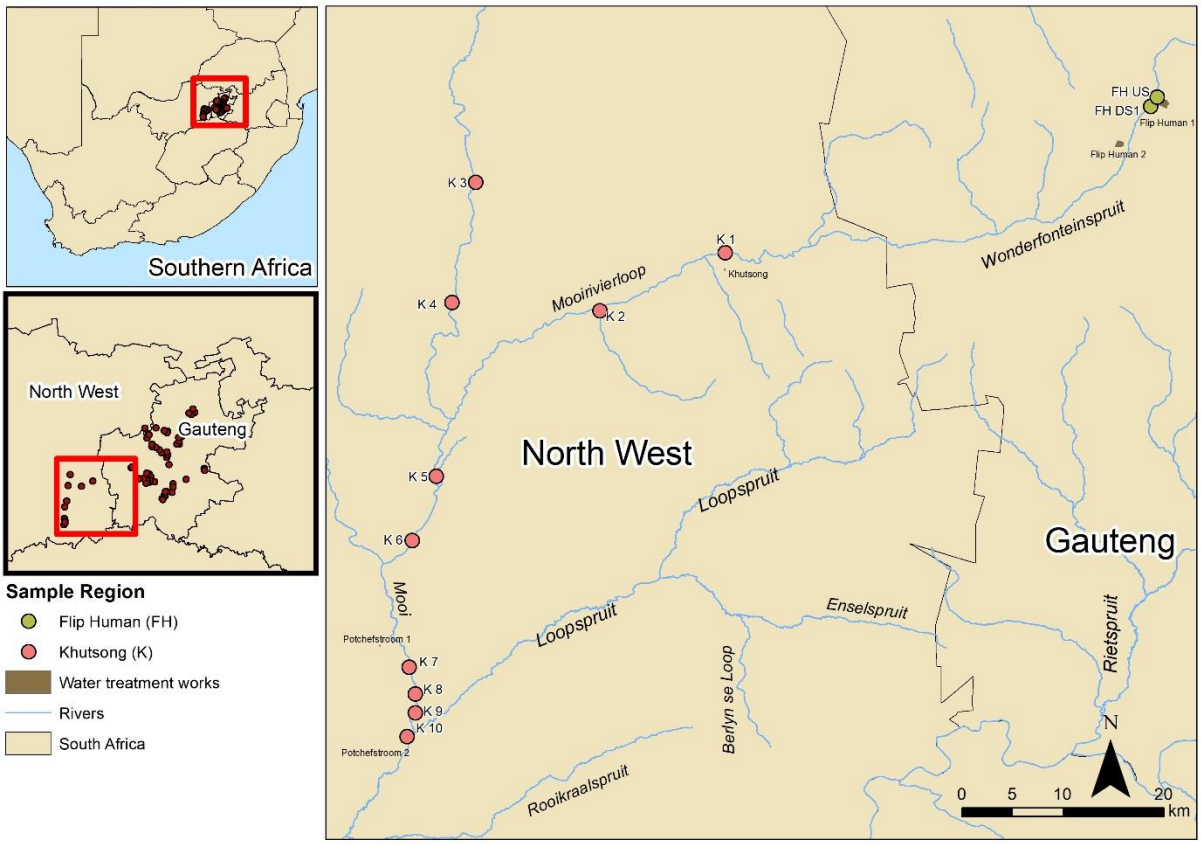


Figure 7: Map of sampling sites in the Mooi River (Khutsong, North West) and two Wonderfonteinspruit sites (Flip Human, Gauteng).

Additionally, drinking water samples (Table 11) were also collected near some river sites at petrol stations or shopping centres. This was conducted during both sampling regimes. Extraction and analysis were identical to that of the surface water samples.

Table 11: Coordinates of drinking water samples collected between September 2017 and October 2018.

Sample site	Coordinates	
Baragwanath DW	-26.259776	27.928923
Olifantsfontein US DW	-25.952014	28.231705
Sunderland US DW	-25.857579	28.109041
Vlakplaats DS DW	-26.376	28.17072
Vlakplaats US DW	-26.3	28.3
Waterval DS1 DW	-26.453508	28.085552
Waterval DS2 DW	-26.498918	28.066391
Waterval US DW	-26.421171	28.098232
Zeekoegat DS1 DW	-25.633561	28.394311
Jukskei DW 1	-26.168335	28.075730
Jukskei DW 2	-26.057332	28.102996
Jukskei DW 3	-26.036437	28.050118
Jukskei DW 4	-26.002509	28.012346
Klip DW 1	-26.262278	27.885836
Klip DW 2	-26.279450	27.934652
Klip DW 3	-26.339411	270928649
Hennops DW 1	-25.810506	28.096578
Hennops DW 2	-25.887581	28.106234

Sample site	Coordinates	
Hennops DW 3	-25.875911	28.223843
Croc DW 1	-26.899452	27.889038
Croc DW 2	-25.798498	27.891455

Sampling equipment

Glass containers were used for sampling (USEPA, 2007).

In order to remove contaminants from previously used glassware, they were cleaned as outlined below:

1. Acid wash (20% HCl) overnight
2. Rinse double distilled water (ddH_2O).
3. Sonicate 10 min 1:1 MeOH: ddH_2O
4. Rinse 3x ddH_2O
5. Rinse with MeOH
6. Dry
7. Screw on lid.

Sampling method

Samplers wore clean, non-powered nitrile gloves at all times during sampling to prevent the introduction of contaminants (Wood et al., 2015). To collect aqueous grab samples, the bottle was lowered to mid depth, rinsed three times with water before collecting the sample. Room was left in the bottle for mixing and thermal expansion (e.g. expansion during freezing). Three replicate samples at each site were collected.

Sample handling

Samples were protected from UV light (Prasse et al., 2010; Peng et al 2014). Each bottle was labelled with water resistant marker and covered with clear tape. The label included information such as: sampling area, sampling point number, date, initials of sampling person (Table 12).

During sampling, the water samples were kept on ice until being transported back to the laboratory. Upon arrival, the samples were placed in a fridge (4°C) until extraction, within 48 h (K'oreje et al., 2012).

Table 12: Sample identification system.

Site name	GPS coordinates	Site description	Matrix	Label

2.2.1.1 Fish sampling from same river system

Fish were sampled at four fish sites in the high flow season (October) in 2013 and 2014 at Lenasia, Fleurhof, Nancefield (Bushkoppies), and Orlando (East) (Figure 8). These samples were collected from sites during a previous studies' sampling event that overlapped with the Klip River and Baragwanath sites of the current study.

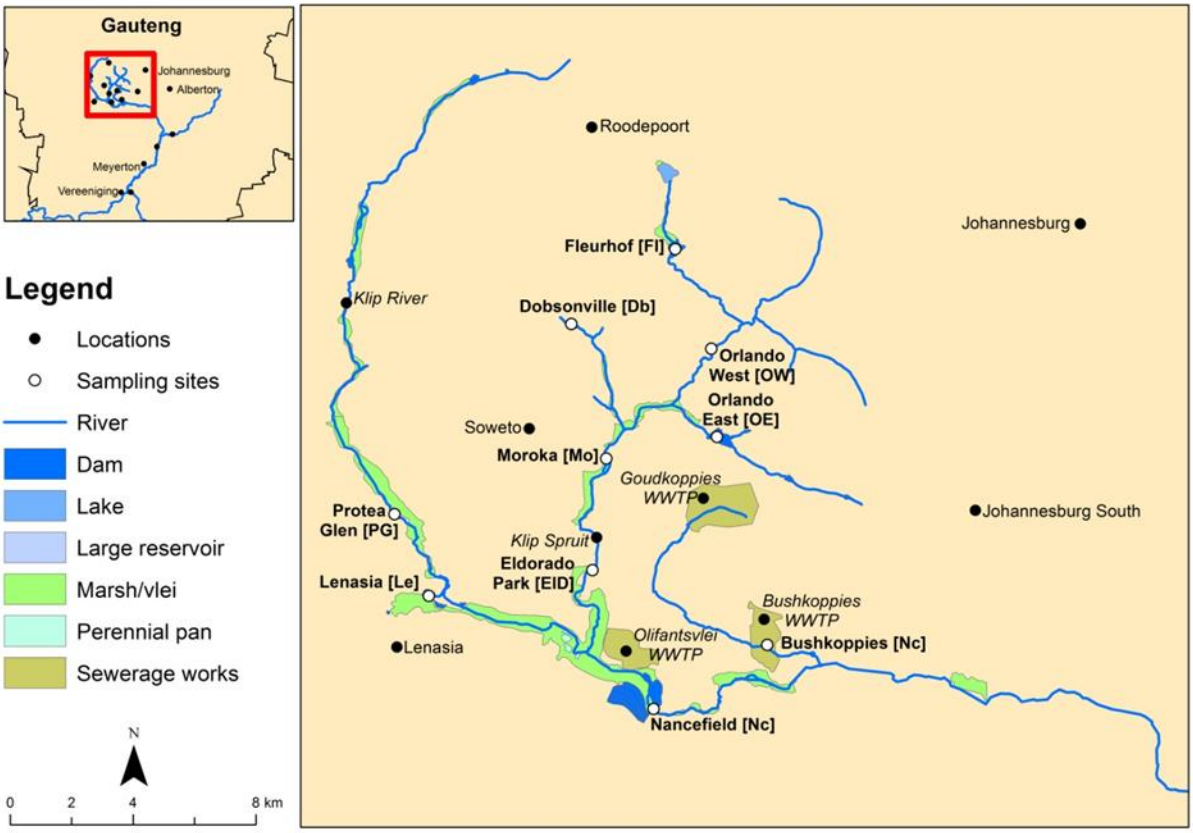


Figure 8: Sampling sites where fish were collected within the greater Soweto and Lenasia area.

2.2.2 Chemicals and Materials

Analytical reference standards (≥ 99% purity) obtained from the U.S. Pharmacopeial Convention, European Pharmacopoeia, British Pharmacopeia and World Health Organization were purchased from Stargate Scientific (Johannesburg, South Africa). Deuterated fluconazole (d-4) acted as internal standard and was obtained from Toronto Research Chemicals (Toronto, Canada). Honeywell Burdick and Jackson acetonitrile and methanol of spectrometry grade were purchased from Anatech, South Africa. Acetic and formic acid were obtained as 98% solutions for mass spectrometry from Fluka (Sigma, Germany). Ammonium acetate and ammonium formate were also obtained from Fluka (Sigma, Germany). Double distilled, nanopure, water was obtained from an ELGA water purification system. HLB-L (hydrophilic-lipophilic balance) (low) solid phase extraction (SPE) disks (47 mm) were obtained from Atlantic (Horizon Technology, Salem, NH, USA).

Individual stock solutions for each compound were prepared using a gravimetrical method by weighing 10 mg compound and dissolving it in 10 g (7.9 mL) of methanol resulting in 1 mg/mL. Stock solutions were prepared in amber glass vials with solid screw lids. These stock solutions were used to prepare standard mix solutions at 100, 10, 1 ng/mL. These mixes will be analysed to determine the stability of the compounds at -20°C, 4°C and 19°C. The internal standard, fluconazole d-4, was prepared to deliver a concentration of 5 µg/mL. During stock solution preparation an excel spreadsheet was used to determine amount of volume/g of solvent to be added to prepare desired concentration.

2.2.3 Extraction and clean-up of ARVs and fluconazole (antifungal)

2.2.3.1 Water samples

The extraction of target compounds from water was based on methods used by Ferrer et al. (2010), for pharmaceuticals. Water samples were spiked with 500 ng/mL internal standard (fluconazole-d4). Target compounds were concentrated 2 000 times by automated SPE using the SPE-DEX system (Horizon Technology, Salem, NH, USA). Oasis HLB disks were used because they are efficient at extracting analytes with various polarities and acid/base characteristics at different pHs (Pedrouzo et al., 2011). The disks are available in high (H), medium (M) and low (L) capacities. HLB-L was the best fit for our purpose because we have a low organic sample matrix. US EPA method 1694 for pharmaceuticals and personal care product analysis makes use of these disks.

The HLB extraction disks (47 mm, Horizon Technology) were conditioned with methanol twice, with 30-second soak times, and 15-second dry time, followed by an additional 10-second methanol conditioning with a two-second dry time. Further conditioning with water was done twice with 10-second soak time and a two-second dry time on the first cycle, and no dry time on the second cycle. The water sample was passed through the disk followed by a three-minute air dry. The target compounds were eluted with three methanol cycles. The first two cycles eluted for three minutes followed by a 20 second dry time, and the final cycle eluted for one minute followed by a one-minute dry time. The eluent was concentrated to near dryness using a gentle stream of nitrogen gas. The samples were reconstituted in methanol.

2.2.3.2 Fish samples

Fish muscle was accurately weighed to 0.5 g and was extracted using a Retsch (homogenizer) with 2 stainless steel beads and 1 mL extraction buffer consisting of acetonitrile and ethanol with 0.1% formic acid. Homogenate was centrifuged and frozen at -20°C overnight and centrifuged again the following day. The supernatant was collected and analysed. Blanks and calibration samples were subjected to the same treatment as the samples. All the samples and blanks were spiked with internal standard before extraction, but the matrix matched calibration samples were spiked with internal standard after extraction. These samples were analysed using the same LC-QTOF method for ARVs in the water samples.

2.2.4 Instrumentation

The UPLC system used consist of an Agilent 1290 Infinity Binary pump (G4220A); 1290 Infinity Autosampler (G4226A); and 1290 Infinity Thermostatted Column Compartment (G1316C) coupled to an Agilent 6540 Accurate mass Q-TOF/MS (G6540A) (Agilent Technologies, Santa Clara, CA, USA). The desolvation and ionisation of samples were achieved by positive and negative electrospray ionisation (ESI) enhanced with Agilent Jet Stream (AJS) technology. The QTOF was set to scan from 50 to 950 m/z and the instrument was set to extended dynamic range (2 GHz). Software used was MassHunter Data Acquisition (version B.05.00), MassHunter Qualitative Analysis (version B.05.00), and Quantitative Analysis for QTOF (version B.05.01). Mass axis calibration of QTOF was performed daily for positive and negative ionisation with tuning mixes (G1969-85000, Agilent). A reference solution with masses of 121.050873 [M+H] and 922.009798 [M+H] were constantly infused as accurate mass references.

2.2.4.1 Source optimisation

Several parameters of the AJS ESI source were optimised to improve the analytical sensitivity of each compound. These parameters are analytical instrumentation brand-specific and include drying gas

temperature (°C) and flow (L/min), nebuliser pressure (psi), sheath gas temperature (°C) and flow (L/min), VCap (V) and nozzle voltage (V). Additional parameters for the QTOF instrument are fragmentor (V), skimmer (V) and OCT RF Vpp (V). Optimisation was performed by means of a flow injector analysis (FIA) program. The FIA program consisted of segments in which one parameter varied four/five times. The default values were kept consistent throughout the optimisation process. In each segment, 1 uL of 100 ng/mL analytical standard was infused, and the QTOF was operated in scan mode. The mobile phase was isocratic at 50:50 H_2O:acetonitrile (ACN) at a flow rate of 0.3 mL/min. The complete FIA program is summarised in Table 9.

After the first 20 source parameter analysis, the results were inspected, and the optimal values were adjusted accordingly (Table 10). The FIA continued, still only changing one value at a time, but at optimal parameters for the specific compound. The FIA program was also used to optimise the nebuliser pressure, drying gas temperature, sheath gas temperature and flow, but with longer waiting periods to allow equilibration of the parameters set to change (Table 10). Nebuliser pressures of 15, 20, 30, 45 psi were investigated (Table 10). Drying gas temperatures tested were 250, 275, 300 and 350°C (Table 10). Sheath gas temperature (°C) and flow (L/min) were varied to 250:5, 300:6, 350:7, 400:10, 400:12 respectively (Table 10). Abundance obtained for all compounds after source optimisation (Table 11-Table 12) were used to set up a method consisting of all optimised parameters.

Table 13: FIA program used to optimise various source parameters (default values are in bold).

Segment	Injection time (min)	Parameter change (min)	Drying gas temp	Drying gas flow	Nebulizer pressure	Sheath gas temp	Sheath gas flow	Vcap	Nozzle voltage	Fragmentor	Skimmer	OCT RF Vpp	Collision energy
			°C	L/min	psi	°C	L/min	Vcap	V	V	V	V	V
1	0	0	300	6	20	400	12	3000	0	200	65	750	0
2	0.7	1	300	**8**	20	400	12	3000	0	200	65	750	0
3	1.4	2	300	10	20	400	12	3000	0	200	65	750	0
4	2.1	3	300	12	20	400	12	3000	0	200	65	750	0
5	2.8	4	300	10	20	400	12	3000	**0**	200	65	750	0
6	3.5	5	300	10	20	400	12	3000	100	200	65	750	0
7	4.2	6	300	10	20	400	12	3000	500	200	65	750	0
8	4.9	7	300	10	20	400	12	3000	1000	200	65	750	0
9	5.6	8	300	10	20	400	12	3000	0	130	65	750	0
10	6.3	9	300	10	20	400	12	3000	0	**175**	65	750	0
11	7	10	300	10	20	400	12	2750	0	200	65	750	0
12	7.7	11	300	10	20	400	12	2500	0	250	65	750	0
13	8.4	12	300	10	20	400	12	2250	0	200	45	750	0
14	9.1	13	300	10	20	400	12	3000	0	200	**55**	750	0
15	9.8	14	300	10	20	400	12	3000	0	200	65	750	0
16	10.5	15	300	10	20	400	12	3000	0	200	75	750	0
17	11.2	16	300	10	20	400	12	2500	0	200	65	750	0
18	11.9	17	300	10	20	400	12	**2750**	0	200	65	750	0
19	12.6	18	300	10	20	400	10	3000	0	200	65	750	0
20	13.3	19	300	10	20	400	8	4000	0	200	65	750	0

Table 14: FIA program (with longer waiting times) used to optimise more source parameters (default values are in bold).

Segment	Injection time (min)	Parameter change (min)	Drying gas temp	Drying gas flow	Nebulizer pressure	Sheath gas temp	Sheath gas flow	Vcap	Nozzle voltage	Fragmentor	Skimmer	OCT RF Vpp	Collision energy
1	0	0	300	10	15	400	12	3000	0	130	65	750	0
2	2.5	1	300	10	**20**	400	12	3000	0	130	65	750	0
3	5	3.6	300	10	30	400	12	3000	0	130	65	750	0
4	7.5	6.6	300	10	45	400	12	3000	0	130	65	750	0
1	0	0	250	10	20	400	12	3000	0	130	65	750	0
2	2.5	1	275	10	20	400	12	3000	0	130	65	750	0
3	5	3.6	**300**	10	20	400	12	3000	0	130	65	750	0
4	7.5	6.6	350	10	20	400	12	3000	0	130	65	750	0
1	0	0	300	10	20	250	5	3000	0	130	65	750	0
2	2.5	1	300	10	20	300	6	3000	0	130	65	750	0
3	5	3.6	300	10	20	350	7	3000	0	130	65	750	0
4	7.5	6.6	300	10	20	400	10	3000	0	130	65	750	0
5	10	9	300	10	20	**400**	**12**	3000	0	130	65	750	0

Table 15: Abundance data for source optimisation parameters.

		Positive ionisation						Negative ionisation			
		Fluconazole	**Fluconazole-d4**	**Ritonavir**	**Lamivudine**	**Lopinavir**	**Nevirapine**	**Efavirenz**	**Fluconazole**	**Fluconazole-d4**	**Zidovudine**
Drying gas flow (L/min)	6	0	47342	10590	0	8908	23555	31165	0	0	0
	8	890	34507	9201	0	8841	24228	30058	1517	8216	0
	10	507	27944	12489	68	7316	18910	32979	1602	8843	0
	12	1439	28960	9554	0	11218	21974	30562	1736	9070	0
Nozzle voltage (V)	**0**	821	26731	10206	95	10845	19303	30643	0	8221	0
	100	2090	25374	10764	0	8872	20224	31016	0	9021	0
	500	2257	57643	16724	76	16664	38454	50667	2982	14156	0
	1000	3443	85284	13978	55	17558	39293	63461	3758	18246	964
Fragmentor (V)	130	2836	102436	6711	2851	12652	19536	122994	106007	737779	1043
	175	176	65991	7041	0	10291	20306	83051	11049	97437	49356
	200	0	20297	8171	0	11995	19638	29618	1004	97437	1494
	250	156	0	2270	30	1083	3674	2439	0	7981	0
Skimmer (V)	45	379	6747	7507	13	7628	13361	16138	1189	0	0
	55	399	15464	8031	0	8494	18461	20684	1227	0	0
	65	258	25126	7520	0	10865	19877	29840	1457	7483	0
	75	1029	31726	8002	0	8833	10235	37091	1364	8963	736
Vcap (V)	2500	847	34882	8271	0	8913	26444	46557	0	0	0
	2750	847	26382	6667	0	9440	25967	33565	1501	0	0
	3000	681	24237	8533	75	9364	21916	33596	0	0	0
	4000	0	16610	6972	0	11792	13889	17367	1625	0	0

Table 16: Abundance data for source optimisation parameters.

		Positive ionisation						Negative ionisation			
		Fluconazole	**Fluconazole-d4**	**Ritonavir**	**Lamivudine**	**Lopinavir**	**Nevirapine**	**Efavirenz**	**Fluconazole**	**Fluconazole-d4**	**Zidovudine**
Nebulizer pressure (psi)	15	2713	41267	41267	3028	221710	155934	2930	38704	505775	78164
	20	1468	64275	64275	3867	291036	204342	4180	77012	845131	126747
	30	2572	121712	121712	12309	304889	238834	8406	162715	1595687	238485
	45	6561	200921	200921	14174	242562	232619	13716	243566	2225790	317778
Sheath gas temperature (°C)	250	1908	46926	115541	4572	79407	55362	55362	55362	55362	13892
	300	958	43401	82510	4275	64752	49266	49266	49266	49266	7349
	350	2170	51410	110506	3660	90377	71631	71631	71631	71631	8930
	400	3947	97595	183816	6610	171962	124060	124060	124060	124060	14817
	400	5498	176599	255393	11041	281855	215806	215806	215806	215806	8464
Drying gas temperature (°C)	250	4898	167975	182728	9063	222193	225871	372210	293640	2608394	18477
	275	5160	173262	197600	13598	247387	232077	383221	306848	2769208	14445
	300	5221	18I4343	207342	13278	234198	236970	388234	307006	2734004	22884
	350	5305	186613	193757	12592	228395	226100	377486	286754	2555909	15816

2.2.4.2 Column optimisation

The compounds to be analysed have different polarities (Table 7) which influences the separation technique to be used. We tested an Agilent ZORBAX C18 (stationary phase) and Poroshell 120 Bonus-RP columns. The best separation was achieved with the Bonus-RP column.

2.2.4.3 Mobile phase optimisation

The mobile phases used were chosen based on literature (Bezy et al., 2006; Avolio et al., 2008; Wood et al., 2015). Source optimisation indicated that responses from efavirenz, zidovudine, didanosine, fluconazole, and fluconazole-d4 (IS) are higher in negative ionisation. Nevirapine, ritonavir, lopinavir, and fluconazole-d4 (IS) were analysed in positive ionisation. Mobile phase optimisation (results not shown) revealed that for positive ionisation water (solvent A) and acetonitrile (solvent B) both containing 0.05% formic acid gives the best peak shapes. While for negative ionisation water (solvent A) and acetonitrile (solvent B) both with 0.1% acetic acid ensures the best peaks.

The extraction and analysis method were optimised and validated. Each sample was analysed in positive and negative ionisation according to the optimised parameters (Table 13). The compounds we were able to analyse include efavirenz, fluconazole, zidovudine, lopinavir, nevirapine, and ritonavir (Table 14). We were not able to create adequately a method that was sufficient for the analyses of stavudine, lamivudine, and didanosine.

Table 17: Optimised LC and MS parameters.

Parameters	**Positive ionisation**		**Negative ionisation**	
Injection volume	1 µL			
Column	Poroshell 120 Bonus-RP column (Agilent, 2.1 x 100 mm, 2.7 µm)			
Column temperature	25 ˚C			
Flow rate	0.6 mL/min		0.7 mL/min	
Mobile phase A	Water + 0.05% formic acid		Water + 0.1% acetic acid	
Mobile phase B	ACN + 0.05% formic acid		ACN + 0.1% acetic acid	
Gradient (min)	A (%)	B (%)	A (%)	B (%)
0/0	90	10	95	5
8.5/4.5	90	10	95	5
8.6/4.6	50	50	90	10
13/7	50	50	90	10
13.3/7.1	0	100	50	50
14.3/11	0	100	50	50
15/11.1	90	10	0	100
12.2	-	-	0	100
Post run-time	2 min		13.8 min	
Total run-time	17 min		15 min	
Drying gas temperature	275°C		275°C	
Drying gas flow	10 L/min		10 L/min	
Nebuliser pressure	45 psi		45 psi	
Sheath gas temperature	400°C		400°C	
Sheath gas flow	10 L /min		10 L /min	
VCap	3000 V		3000 V	
Nozzle voltage	0 V		300 V	
Fragmentor	130 V		130 V	
Skimmer	48 V		48 V	
OCT RF Vpp	750 V		750 V	

Table 18: Retention times and m/z values of ARVs and fluconazole to be analysed.

	Compounds	Retention time (min)	m/z
Negative ionisation	Efavirenz	10.0	314.0274
	Fluconazole	6.3	305.1040
	Zidovudine	3.6	266.092
	Didanosine	1.1	235.0849
	Fluconazole-d4 (IS)	6.3	309.1236
Positive ionisation	Fluconazole	3.8	307.1040
	Lopinavir	11.1	629.3729
	Nevirapine	7.5	267.1248
	Ritonavir	10.7	721.3185
	Fluconazole-d4 (IS)	3.8	311.1247

2.2.5 Screening of selected ARV metabolites

The data obtained from the parent ARV analyses were utilised to screen for the ARV metabolites. The scanning ability of the Agilent 6540 Accurate mass Q-TOF/MS enables data gathering of many other potential compounds that might be present in the samples. However, the search was limited to only ARV metabolites. Compound possibilities were generated based on molecular features and subjected to the MassHunter Personal Compound Database and Library (PCDL) software's Forensics and Toxicology library. The library can confirm the presence of compounds based on monoisotopic mass, isotope distribution, and retention time.

2.2.6 QC/QA

2.2.6.1 Method validation

The key element of method validation is error assessment (Westgard, 2008). Keeping track of random errors will improve an analytical method. There are many things to consider when validating a method. The following section highlights the quality control and quality assurance performed to complete the method validation.

2.2.6.2 Calibration

Due to possible matrix suppression and enhancement effects, an external matrix-matched calibration curve (that was spiked post-extraction) was used to account for matrix effects during quantification. To mimic natural occurring water as much as possible for the matrix-matched calibrations, double distilled water was supplemented with 0.7 mM $NaHCO_3$, 2 mM $CaCl_2{\cdot}2H_2O$, 0.5 mM $MgSO_4{\cdot}7H_2O$, and 75 µM KCl to create artificial freshwater (ISO, 2012). In an attempt to match the natural organic components of the sampled matrix, 25 mature freshwater snails (*Bulinus tropicus)* reared under clean laboratory conditions, were added to 1 L of the ISO prepared water for approximately 24 h. The matrix-matched water was prepared with 5% of this snail-infused water mixed into ISO water. Matrix-matched-calibrations were prepared by extracting 1 L of this matrix, and concentrated with nitrogen gas, followed by reconstituting in methanol and spiking with ARVs, fluconazole, and d4-fluconazole for an end volume of 0.5 mL.

The concentrations for the calibration curve were determined based on the expected levels of ARVs in the environment and the performance of the instrument. Samples are concentrated 2 000 times during extraction. This should be accounted for when choosing a calibration range. At first, the calibration levels differed for each sampling event to establish a range that will cover all the concentrations of ARVs in different samples. The calibration ranges for the four sampling events are specified in the results section. Dilutions were not serially prepared but originated from different stocks. These standards were analysed in triplicate to assess the

reportable range (Westgard, 2008). They were injected in order of increasing concentration, with blank injections in-between to prevent carry-over.

2.2.6.3 Limit of detection (LOD)/Limit of quantification (LOQ)

Sensitivity of an analytical method is defined as the increased response of the analyte linear to the analyte concentration (Whitmire et al., 2011). This is displayed with a calibration curve and the slope of the calibration curve. By using linear regression statistics, the uncertainties of the calibration curve can be used to calculate LOD and LOQ for the method from the external matrix matched calibration curve. By use of the y=mx+c model, LOD is calculated by 3*Sa/b and LOQ by 10*Sa/b; where Sa is the SD of the intercept (abundance) and b is the slope of the calibration curve (Schoeman et al., 2015). The LOD and LOQ of the method are summarised in Table 15-Table 17. These values differ for each sampling event, due to the different calibration curves that were prepared to compensate for the target compound concentration fluctuations between the three study areas.

2.2.6.4 Data analysis

Concentration of ARVs in samples will be determined by using this formula:

$$X_{ARV} = ((\text{native/stable isotope})-c) / m) \times \text{ISO conc} \quad [3]$$

where:

X_{ARV}	= calculated analyte concentration
Native	= native abundance
Stable isotope	= stable isotope abundance
c	= calibration curve y-intercept
m	= slope of calibration curve
ISO conc	= internal standard concentration

2.2.6.5 Precision

Repeatability of the method was determined by analysing 3 quality control (QC) samples spiked with a low, middle and high concentration. Precision was calculated using % RSD = (mean of SDEV of QCs/mean of QCs) x100. For great repeatability, the % RSD should be lower than 15% (Schoeman et al., 2015). Inter- and intralaboratory precision was determined by analysing the QC sample for three consecutive days in triplicate. Interlaboratory precision was acceptable (<15%) for all compounds except for didanosine with a %RSD between 7 and 29% for the different analysis sets (Table 15 and Table 17). Because of the poor RSD, it was decided to remove didanosine from the second set of analyses. The method will need to be adjusted to improve its results. Intralaboratory precision was acceptable for some compounds (fluconazole, ritonavir, didanosine, efavirenz, and zidovudine). However, there is room for improvement.

2.2.6.6 Accuracy

Accuracy was determined by the mean-recovery of spiked compounds at three different concentrations in the matrix to be analysed. The formula used was: % Accuracy = (mean value/true value). The accuracy was above 70% for fluconazole, nevirapine, and zidovudine. However, ritonavir, lopinavir, and didanosine were recovered between 55% and 63% (Table 15).

Table 19: QC/QA results for 1st sampling of Gauteng sites: BG, FH, OF, SR, VP, WV, WG and ZK.

	Linear range	Linearity	LOD µg/L	LOQ µg/L	Accuracy %	Interday precision %RSD	Intraday precision %RSD
Fluconazole	0, 0.4, 1.3, 4, 12, 36, 108, 326, 979, 2937	0.998	115	382	88	7.3	8.0
Nevirapine	0, 0.1, 0.3, 0.8, 2.5, 7.6, 22, 68, 204, 612	0.999	280	932	76	10.4	32.3
Lopinavir	0, 0.9, 2.7, 8.1, 24, 72, 217, 652, 1957, 5873	0.998	1161	3871	63	8.7	15.4
Ritonavir	0, 1.48, 4.4, 13.3, 40, 120, 360, 1080, 3242, 9728	0.997	482	1607	63	5.6	10.6
Didanosine	0, 0.1, 0.3, 1, 3, 9, 27, 81, 244, 734	0.998	28	93	55	7.1	9.9
Efavirenz	0, 0.9, 2.7, 8, 24, 72, 217, 652, 1957, 5873	0.982	1012	3375	196	9.7	12.9
Zidovudine	0, 7, 21, 64, 193, 580, 1740, 5220, 15662, 46987	0.999	1004	3346	80	5.4	8.3

Table 20: QC/QA for the Khutsong sites sampling event (K).

	Linear range	Linearity	LOD µg/L	LOQ µg/L
Fluconazole	0, 0.4, 1.3, 4, 12, 36, 108, 326, 979, 2937	0.998	115	382
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

Table 21: QC/QA results for sampling of the Hen, JK, Klip and KD rivers in Gauteng.

	Linear range (µg/L)	Linearity	LOD µg/L	LOQ µg/L	Interday precision %RSD	Intraday precision %RSD
Fluconazole	0, 5, 10, 20, 40, 80, 160, 330, 660, 1000	0.968	410	1368	12.9	5.7
Nevirapine	0, 5, 10, 20, 40, 80, 160, 330, 660, 1000	0.980	322	1074	4.1	5.5
Lopinavir	0, 5, 10, 20, 40, 80, 160, 330, 660, 1000	0.999	74	248	7.8	29.6
Ritonavir	0, 5, 10, 20, 40, 80, 160, 330, 660, 1000	0.938	578	1926	7.4	30.7
Didanosine	0, 5, 10, 20, 40, 80, 160, 330, 660, 1000	0.988	243	809	29.0	27.4
Efavirenz	0, 5, 10, 20, 40, 80, 160, 330, 660, 1000	0.972	386	1286	14.1	16.9
Zidovudine	0, 5, 10, 20, 40, 80, 160, 330, 660, 1000	0.979	333	1111	13.7	6.4

2.2.6.7 Linearity

Linearity of the calibration curve was assessed by determining the R^2 value of the calibration curve for each set of analysis (Table 15-Table 17). Good linearity is indicated with R^2 as close to one as possible (at least 0.9) (Miller and Miller, 2010). All the compounds had an R^2 of 0.9 or better for all the analyses.

2.3 RESULTS

There were no ARVs detected in the fish tissue. These samples were collected in 2013 and 2014, and if ARVs and fluconazole accumulated in the tissue, it might have broken down already. It is also possible that the fish metabolise the ARVs, and metabolites might be present. Alternatively, they may accumulate the parent/metabolite in an organ, like the liver, milt, or bile, rather than muscle tissue. It is recommended that fresh fish samples should be collected and extracted immediately. Whole fish homogenate should be used to determine uptake in the fish, not just in the muscle tissue. Metabolites should also be investigated.

Surface water was sampled during the summer season (wet and warm). In October 2017, the site around the WWTPs (Olifantsfontein, Sunderland Ridge, Vlakplaats, Waterval, Welgedacht, Zeekoegat, Flip Human, and Khutsong) and the medical facility (Baragwanath) were sample. Based on these results and a study done by Peterson et al. (2017), the Jukskei, Hennops, Crocodile, and Klip rivers were sampled over multiple months (March-October) to determine changes in the concentration over time and seasons. Peterson et al. (2017), identified that the Hennops and Klip rivers are sensitive to anthropogenic pollution. The Jukskei River receives possible pollution through the Northern Johannesburg WWTP. Some sites overlapped and are known under different site names: Sunderland Ridge DS1 (same site as Hennops 8), Sunderland Ridge US (same as Hennops 7), Olifantsfontein US (same as Hennops 3) and Olifantsfontein DS1 (same as Hennops 4). Additionally, drinking water (DW) samples were taken during both sampling regimes to determine if WWTPs are effectively removing the compounds from drinking water.

The results obtained between sampling events were grouped into tables according to the sampling regime-sampling event around WWTPs per system, river systems over time, and drinking water. The mean, standard deviation minimum, and maximum concentrations are indicated at the bottom of the results Table 22-Table 36.

The screening results of the ARV metabolites did not confirm the presence of any metabolites; however, this doesn't seem accurate after the high levels of parent ARV compounds were detected across the sampling sites. It may also be that the library did not contain an appropriate list of possible ARV metabolites. The only definite metabolite in the library was 8-hydroxyefavirenz, but this was still not detected in any of the samples.

2.3.1 Wastewater treatment plants

All targeted compounds, except for didanosine and zidovudine were quantified at the Olifantsfontein River sites (Table 22). Lopinavir and efavirenz had the highest mean concentrations at these sites, 26.37 and 13.96 µg/L respectively. The two Olifantsfontein DS sites had the highest concentrations of all target compounds. The Sunderland sites did not show clear trends regarding concentrations of the up- and downstream sites—some were higher in the upstream sites than the downstream.

In the Vlakplaats and Waterval system, the concentrations were relatively low, and ritonavir, didanosine, and zidovudine were not quantified in the samples (Table 23). The highest mean concentrations were for lopinavir and efavirenz, 1.58 and 1.53 µg/L respectively. Generally, the Vlakplaats DS1 had the highest concentrations but the DS2 and US sites were variable with regards to which one had higher concentrations—in some cases the US site had much higher concentrations than the DS. For the Waterval sites, the DS2 sites had higher concentrations than the other two sites, and there were no detections of any of the compounds of the target compounds.

The Welgedacht sites had measurable concentrations of most target compounds except for didanosine and zidovudine at the DS1 site (Table 24). By the time the water reached the DS2 site there were no quantifiable concentrations of any of the target compounds. The water upstream only had concentrations of nevirapine at relatively low concentrations (0.08 µg/L). Efavirenz and lopinavir had the highest mean concentrations, 2.43 and 1.78 µg/L respectively.

Concentrations of target compounds were greatest at the Zeekoegat US2 sites, except for nevirapine, which was greatest at the US1 site (Table 25). The downstream sites had lower concentrations, typically less than half that of the US sites. Lopinavir and efavirenz had the highest mean concentrations at 9.15 and 5.95 µg/L respectively. Didanosine was not quantified at these sites.

The Baragwanath sites had higher concentrations in the downstream site than the upstream site (Table 26). The upstream site only had low concentrations of fluconazole, and nevirapine, while the concentration of efavirenz was higher (1.25 µg/L). The downstream site also had quantifiable concentrations of lopinavir.

The two Flip Human sites had high concentrations, particularly the DS site. Lopinavir and efavirenz had the highest concentrations at these sites (Table 26). Further downstream from Flip Human are the Khutsong sites. Only Khutsong 1 had notable concentrations and the remaining sites had generally undetectable concentrations. Didanosine was quantifiable at two sites—Khutsong 3 and 5.

The most frequently detected compound was fluconazole with 28 detections from 72 samples with concentrations ranging 0.06-1.8 µg/L. Nevirapine and efavirenz were the second most detected compounds, both with 22 out of 72 samples. But on average lopinavir and efavirenz had the highest concentrations of the compounds analysed. Didanosine and zidovudine were the least detected compounds (2 and 6 out of 72).

Table 22: Concentrations (µg/L) of selected ARVs and fluconazole in surface water around the Sunderland Ridge and Olifantsfontein WWTPs from the initial sampling event (September 2017).

Sample site	Fluconazole		Nevirapine		Lopinavir		Ritonavir		Didanosine		Efavirenz		Zidovudine	
	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV
Olifantsfontein DS2	0.69	0.05	0.42	0.11	38.45	2.51	1.90	0.85	<LOD		19.96	9.57	<LOQ	
Olifantsfontein US	0.24	0.07	0.41	0.11	24.35	5.22	1.69	0.81	<LOD		15.05	6.62	<LOQ	
Olifantsfontein DS1	0.66	0.08	0.46	0.05	26.85	4.65	1.95	0.34	<LOD		24.60	6.44	<LOQ	
Sunderland US	0.63	0.06	0.09	0.02	8.55	0.50	0.64	0.05	<LOD		10.19	6.38	<LOD	
Sunderland DS	<LOD		0.32	0.05	33.64	2.32	1.77	1.11	<LOD		<LOD		<LOD	
Mean	0.44		0.34		26.37		1.59				13.96			
Standard deviation	0.31		0.15		11.41		0.54				9.48			
Minimum	<LOD		<LOD		<LOQ		<LOD		<LOD		<LOD		<LOD	
Maximum	0.69		0.46		38.45		1.95		<LOD		24.60		<LOQ	

Table 23: Concentrations (µg/L) of selected ARVs and fluconazole in surface water around the Vlakplaats and Waterval WWTPs from the initial sampling event (September 2017).

Sample site	Fluconazole		Nevirapine		Lopinavir		Ritonavir		Didanosine		Efavirenz		Zidovudine	
	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV
Vlakplaats DS1	0.19	0.01	0.23	0.03	5.9	0.52	<LOD		<LOD		4.61	1.79	<LOD	
Vlakplaats DS2	0.15	0.01	0.15	0.02	0.91	0.02	<LOD		<LOD		1.43	0.71	<LOD	
Vlakplaats US	0.11	0.02	0.22	0.01	1.85	0.03	<LOD		<LOD		1.98	1.3	<LOD	
Waterval DS1	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Waterval DS2	0.17	0.01	0.08	0.02	0.8	0.2	<LOD		<LOD		1.17	1.0	<LOD	
Waterval US	0.11	0.02	0		0		<LOD		<LOD		0		<LOD	
Mean	0.12		0.11		1.58						1.53			
Stdev	0.07		0.10		2.23						1.70			
Minimum	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Maximum	0.19		0.23		5.90		<LOD		<LOD		4.61		<LOD	

Table 24: Concentrations (µg/L) of selected ARVs and fluconazole in surface water around the Welgedacht WWTP from the initial sampling event (September 2017).

Sample site	Fluconazole		Nevirapine		Lopinavir		Ritonavir		Didanosine		Efavirenz		Zidovudine	
	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV
Welgedacht US	<LOQ		0.08	0.01	<LOD		<LOD		<LOD		<LOD		<LOD	
Welgedacht DS1	0.2	0.02	0.18	0.01	5.35	0.18	<LOQ		<LOD		7.28	3.83	<LOD	
Welgedacht DS2	<LOQ		<LOQ		<LOD		<LOD		<LOD		<LOD		<LOD	
Mean	0.07		0.09		1.78						2.43			
Stdev	0.11		0.09		3.09						4.20			
Minimum	<LOQ		<LOQ		<LOD		<LOD		<LOD		<LOD		<LOD	
Maximum	0.20		0.18		5.35		<LOQ		<LOQ		7.28			

Table 25: Concentrations (µg/L) of selected ARVs and fluconazole in surface water around the Zeekoegat WWTPs from the initial sampling event (September 2017).

Sample site	Fluconazole		Nevirapine		Lopinavir		Ritonavir		Didanosine		Efavirenz		Zidovudine	
	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV
Zeekoegat DS1	0.56	0.06	0.2	0.03	10.23	0.21	<LOQ		<LOD		1.46	0.8	<LOD	
Zeekoegat DS2	0.44	0.04	0.11	0.002	1.17	0.79	<LOD		<LOQ		0.85	0.43	<LOD	
Zeekoegat US2	1.0	0.01	0.3	0.03	20.11	3.83	1.36	0.34	<LOD		20.42	2.94	3.68	0.38
Zeekoegat US1	0.07	0.01	1.30	0.22	5.10	0.88	<LOQ		<LOD		1.05	0.60	<LOD	
Mean	0.52		0.48		9.15		0.34				5.95		0.92	
Stdev	0.38		0.56		8.19		0.68				9.65		1.84	
Minimum	0.07		0.11		1.17		<LOD		<LOD		0.85		<LOD	
Maximum	1.00		1.30		20.11		1.36		<LOQ		20.42		3.68	

Table 26: Concentrations (µg/L) of selected ARVs and fluconazole in surface water around the Baragwanath WWTPs and hospital from the initial sampling event (September 2017).

Sample site	Fluconazole		Nevirapine		Lopinavir		Ritonavir		Didanosine		Efavirenz		Zidovudine	
	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV
Baragwanath DS	0.21	0.01	0.04	0.004	2.04	0.12	<LOD		<LOD		1.53	1.47	<LOD	
Baragwanath US	0.10	0.004	0.06	0.01	<LOQ		<LOD		<LOD		1.25	0.97	<LOD	
Mean	0.16		0.05		1.02						1.39			
std dev	0.08		0.01		1.44						0.20			
min	0.10		0.04		<LOQ		<LOD		<LOD		1.25		<LOD	
max	0.21		0.06		2.04		<LOD		<LOD		1.53		<LOD	

Table 27: Concentrations (µg/L) of selected ARVs and fluconazole in surface water around the Khutsong and Flip Human WWTPs from the initial sampling event (October 2017).

	Fluconazole		Nevirapine		Lopinavir		Ritonavir		Didanosine		Efavirenz		Zidovudine	
Sample site	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV
Khutsong 1	0.43	0.014	<LOD		<LOD		<LOD		<LOD		5.7	0.32	<LOD	
Khutsong 2	<LOQ		<LOD		<LOD		<LOD		<LOD		<LOQ		<LOD	
Khutsong 3	<LOD		<LOD		<LOD		<LOD		0.029	0.02	<LOD		<LOD	
Khutsong 4	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Khutsong 5	<LOQ		<LOD		<LOD		<LOD		0.039	0.01	<LOD		<LOD	
Khutsong 6	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Khutsong 7	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Khutsong 8	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Khutsong 9	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Khutsong 10	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Flip Human DS	0.87	0.20	0.42	0.08	15.25	3.03	1.05	0.10	<LOD		20.295	8.43	<LOQ	
Flip Human US	0.17	0.03	0.10	0.01	5.5	1.01	<LOQ		<LOD		2.186	0.62	<LOD	
Mean	0.12		0.04		1.73		0.09		0.01		2.35			
Stdev	0.27		0.12		4.54		0.30		0.01		5.90			
Minimum	0.00		0.00		0.00		0.00		0.00		0.00		<LOD	
Maximum	0.87		0.42		15.25		1.05		0.04		20.30		<LOQ	

2.3.2 Systems monitored over time

2.3.2.1 Jukskei River

The concentrations of zidovudine in the Jukskei River were generally lowest in March and April, and commonly low in May (Figure 9a, Table 27). In August and October, the concentrations typically increase, and these months often had the highest concentrations across all sites. Jukskei 4 had the highest concentration recorded of zidovudine through all months and sites in October (2.74 µg/L), and there was no zidovudine detected at this site in any other month sampled. Concentrations in all months were generally lower at sites 5-10. Site 3 only had detectable concentrations in August, and this concentration was the lowest concentration recorded in August.

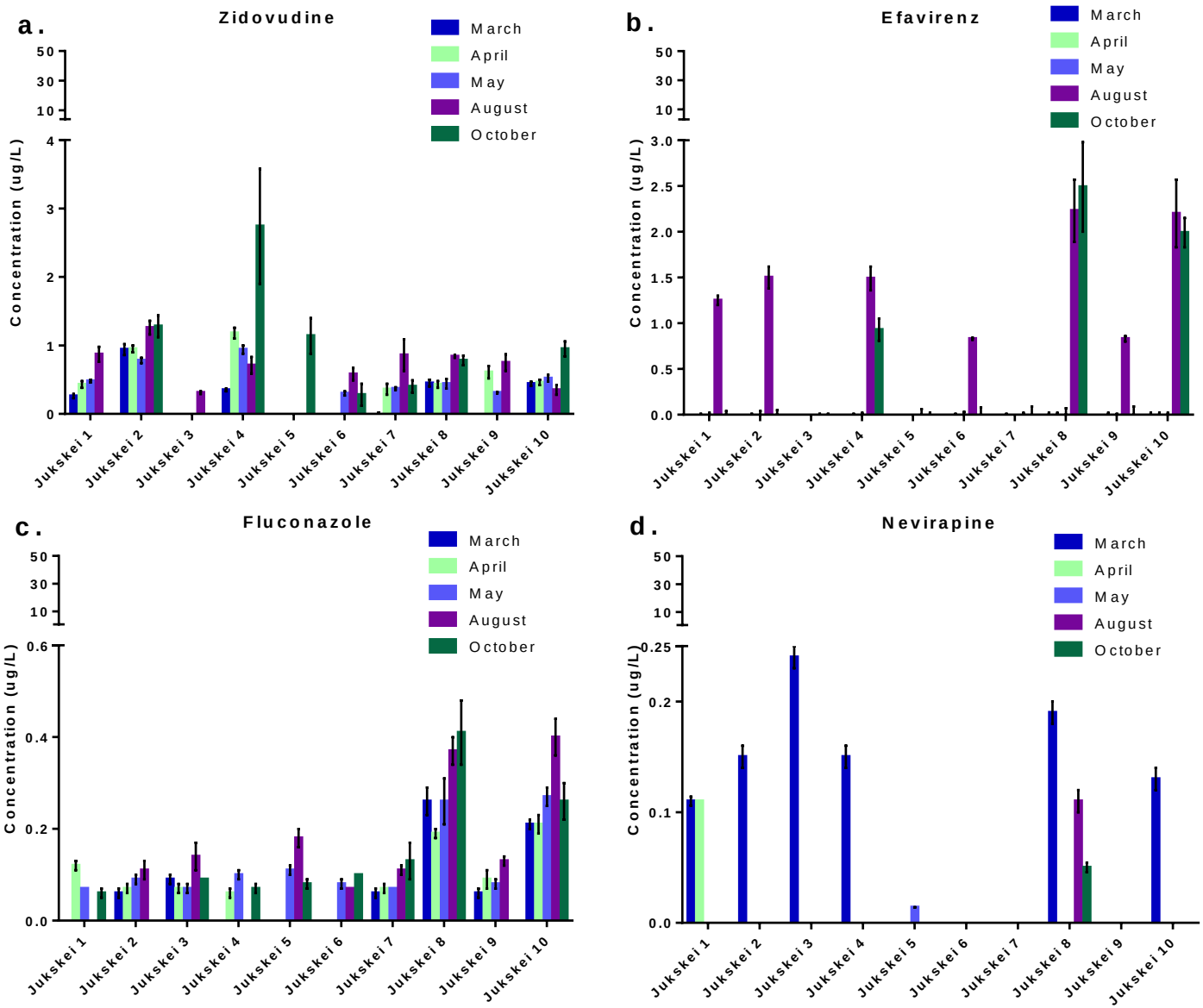
Efavirenz was seldom detected in water from the Jukskei River (Figure 9b, Table 27), and was mostly quantifiable in August (70% of the sites). Only three of the sites had quantifiable concentrations in October. The concentrations reported were greatest at site 8 and 10. Out of the three sites where efavirenz was detected in two months, the concentrations were greater in August than October twice. Site 8 was the exception, where the concentration in October was slightly higher.

Fluconazole was frequently quantified through the Jukskei River (Figure 9c, Table 27). The general trend was higher concentrations in August and October, and there were many instances where there were no detections in the month of March. Typically, the concentrations of fluconazole were greatest in August, with a few exceptions where it was greater in October. Jukskei 8 and 10 had the highest concentrations in all months compared to the other sites. The other sites tended to have similar concentrations across all months, with the exception of Jukskei 5, which had slightly higher concentrations in August compared to the other sites. Only sites 7, 8, and 10 had quantifiable concentrations of fluconazole in every sample month.

Nevirapine was seldom quantified in the Jukskei River, and at fairly low concentrations when it was quantifiable (Figure 9d, Table 27). It was most commonly quantified in March, and only one instance of quantification in the other months, distributed through the sites, revealing no clear trends. There was no nevirapine quantified at sites 6, 7, and 9 at any month. The highest concentration was at Jukskei 3.

Lopinavir had the highest concentrations of all compounds targeted in the Jukskei River. It was ubiquitously quantified across the sites. However, there were a few instances where it was not quantified in certain months (Figure 9e, Table 27). The concentrations were generally highest in March and August. Concentrations of lopinavir at sites 1, 2, and 4 were much higher in the month of March than the other months. At sites 8 and 10 the concentrations in March and August were far greater than the other months. Concentrations in April, May, and October were generally similar across all the sites.

Ritonavir was very infrequently quantified in the Jukskei River; there were only four instances where it could be quantified (Figure 9f, Table 27). These instances were at site 4 in March, site 8 in March and August, and site 10 in August. The concentrations in August were greater than in March.


a.
Zidovudine
March
April
May
August
October
Concentration (ug/L)
50
30
10
4
3
2
1
0
Jukskei 1
Jukskei 2
Jukskei 3
Jukskei 4
Jukskei 5
Jukskei 6
Jukskei 7
Jukskei 8
Jukskei 9
Jukskei 10
b.
Efavirenz
March
April
May
August
October
Concentration (ug/L)
50
30
10
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Jukskei 1
Jukskei 2
Jukskei 3
Jukskei 4
Jukskei 5
Jukskei 6
Jukskei 7
Jukskei 8
Jukskei 9
Jukskei 10
c.
Fluconazole
March
April
May
August
October
Concentration (ug/L)
50
30
10
0.6
0.4
0.2
0.0
Jukskei 1
Jukskei 2
Jukskei 3
Jukskei 4
Jukskei 5
Jukskei 6
Jukskei 7
Jukskei 8
Jukskei 9
Jukskei 10
d.
Nevirapine
March
April
May
August
October
Concentration (ug/L)
50
30
10
0.25
0.2
0.1
0.0
Jukskei 1
Jukskei 2
Jukskei 3
Jukskei 4
Jukskei 5
Jukskei 6
Jukskei 7
Jukskei 8
Jukskei 9
Jukskei 10

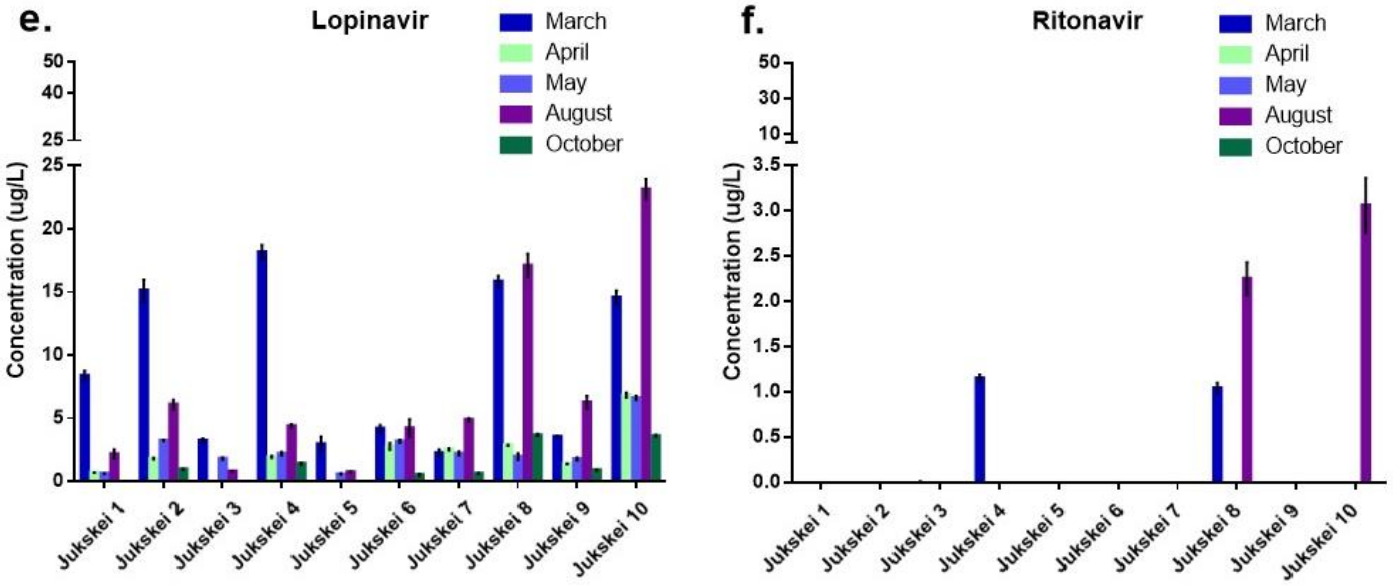


Figure 9: Concentrations of target compounds (a. zidovudine, b. efavirenz, c. fluconazole, d. nevirapine, e. lopinavir, f. ritonavir) from the Jukskei River over five months.

Table 28: Concentrations (µg/L) of selected ARVs and fluconazole in surface water from the Jukskei River, sampled over multiple months.

Month sampled	Sample Site	Zidovudine		Efavirenz		Fluconazole		Nevirapine		Lopinavir		Ritonavir	
		Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV
March	Jukskei 1	0.26	0.03	<LOD		<LOQ		0.11	0.00	8.38	0.36	<LOQ	
	Jukskei 2	0.94	0.08	<LOD		0.06	0.01	0.15	0.01	15.13	0.85	<LOQ	
	Jukskei 3	<LOD		<LOD		0.09	0.01	0.24	0.01	3.24	0.17	<LOD	
	Jukskei 4	0.35	0.02	<LOD		<LOQ		0.15	0.01	18.17	0.55	1.15	0.04
	Jukskei 5	<LOD		<LOD		<LOQ		<LOD		2.97	0.55	<LOQ	
	Jukskei 6	<LOQ		<LOD		<LOQ		<LOQ		4.19	0.28	<LOD	
	Jukskei 7	<LOQ		<LOD		0.06	0.01	<LOQ		2.26	0.26	<LOD	
	Jukskei 8	0.45	0.05	<LOD		0.26	0.03	0.19	0.01	15.83	0.44	1.04	0.06
	Jukskei 9	<LOQ		<LOD		0.06	0.01	<LOQ		3.54	0.06	<LOD	
	Jukskei 10	0.44	0.03	<LOD		0.21	0.01	0.13	0.01	14.56	0.53	<LOQ	
	Mean	0.24				0.07		0.10		8.83		0.22	
	std dev	0.31				0.09		0.09		6.39		0.46	
	min	<LOD		<LOD		<LOD		<LOD		2.26		<LOD	
	max	0.94				0.26		0.24		18.17		1.15	
April	Jukskei 1	0.43	0.05	<LOD		0.12	0.01	0.11	0.00	0.71	0.01	<LOD	
	Jukskei 2	0.95	0.05	<LOD		0.07	0.01	<LOQ		1.81	0.05	<LOD	
	Jukskei 3	<LOD		<LOD		0.07	0.01	<LOD		<LOQ		<LOD	
	Jukskei 4	1.18	0.08	<LOD		0.06	0.01	<LOQ		1.93	0.08	<LOD	
	Jukskei 5	<LOD		<LOD		<LOQ		<LOD		<LOD		<LOD	
	Jukskei 6	<LOQ		<LOD		<LOQ		<LOQ		2.78	0.26	<LOD	
	Jukskei 7	0.36	0.08	<LOD		0.07	0.01	<LOQ		2.53	0.08	<LOD	
	Jukskei 8	0.43	0.05	<LOQ		0.19	0.01	<LOQ		2.86	0.03	<LOD	
	Jukskei 9	0.61	0.09	<LOD		0.09	0.02	<LOD		1.38	0.01	<LOD	
	Jukskei 10	0.46	0.04	<LOQ		0.21	0.02	<LOQ		6.83	0.20	<LOQ	
	Mean	0.44				0.09		0.01		2.08			
	std dev	0.40				0.07		0.03		1.97			
	min	<LOD		<LOD		<LOQ		<LOD		<LOD		<LOD	
	max	1.18		<LOQ		0.21		0.11		6.83		<LOQ	
May	Jukskei 1	0.48	0.02	<LOD		0.07	0.00	<LOD		0.62	0.01	<LOD	
	Jukskei 2	0.78	0.04	<LOD		0.09	0.01	<LOQ		3.22	0.02	<LOD	

Month sampled	Sample Site	Zidovudine		Efavirenz		Fluconazole		Nevirapine		Lopinavir		Ritonavir	
		Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV
	Jukskei 3	<LOQ		<LOD		0.07	0.01	<LOQ		1.81	0.07	<LOD	
	Jukskei 4	0.94	0.06	<LOD		0.10	0.01	<LOQ		2.20	0.13	<LOD	
	Jukskei 5	<LOQ		<LOD		0.11	0.01	0.01	0.00	0.58	0.02	<LOD	
	Jukskei 6	0.30	0.03	<LOD		0.08	0.01	<LOQ		3.17	0.11	<LOD	
	Jukskei 7	0.37	0.02	<LOD		0.07	0.00	<LOQ		2.20	0.14	<LOD	
	Jukskei 8	0.44	0.07	<LOQ		0.26	0.05	<LOQ		1.98	0.24	<LOD	
	Jukskei 9	0.31	0.01	<LOD		0.08	0.01	<LOD		1.77	0.11	<LOD	
	Jukskei 10	0.52	0.05	<LOQ		0.27	0.02	<LOQ		6.61	0.15	<LOQ	
	Mean	0.42				0.12		0.00		2.42			
	std dev	0.30				0.08		0.00		1.72			
	min	<LOQ		<LOD		0.07		<LOD		0.58		<LOD	
	max	0.94		<LOQ		0.27		0.01		6.61		<LOQ	
August	Jukskei 1	0.87	0.11	1.25	0.05	<LOQ		<LOD		2.17	0.35	<LOQ	
	Jukskei 2	1.26	0.10	1.50	0.12	0.11	0.02	<LOD		6.09	0.36	<LOD	
	Jukskei 3	0.31	0.02	<LOQ		0.14	0.03	<LOD		0.80	0.01	<LOD	
	Jukskei 4	0.71	0.12	1.49	0.13	<LOQ		<LOQ		4.39	0.14	<LOD	
	Jukskei 5	<LOQ		<LOD		0.18	0.02	<LOD		0.74	0.02	<LOQ	
	Jukskei 6	0.58	0.09	0.83	0.01	0.07	0.00	<LOQ		4.23	0.69	<LOQ	
	Jukskei 7	0.86	0.23	<LOQ		0.11	0.01	<LOQ		4.88	0.12	<LOD	
	Jukskei 8	0.84	0.02	2.23	0.34	0.37	0.03	0.11	0.01	17.10	0.91	2.25	0.18
	Jukskei 9	0.75	0.12	0.83	0.03	0.13	0.01	<LOQ		6.26	0.50	<LOD	
	Jukskei 10	0.35	0.07	2.20	0.37	0.40	0.04	<LOQ		23.12	0.82	3.06	0.30
	Mean	0.65		1.03		0.15		0.01		6.98		0.53	
	std dev	0.36		0.85		0.14		0.03		7.33		1.14	
	min	<LOQ		<LOD		<LOQ		<LOD		0.74		<LOD	
	max	1.26		2.23		0.40		0.11		23.12		3.06	
October	Jukskei 1	<LOQ		<LOQ		0.06	0.01	<LOD		<LOQ		<LOD	
	Jukskei 2	1.28	0.16	<LOQ		<LOQ		<LOD		0.95	0.04	<LOD	
	Jukskei 3	<LOQ		<LOD		0.09	0.00	<LOD		<LOD		<LOD	
	Jukskei 4	2.74	0.84	0.93	0.12	0.07	0.01	<LOQ		1.40	0.06	<LOD	
	Jukskei 5	1.14	0.26	<LOD		0.08	0.01	<LOD		<LOD		<LOD	

Month sampled	Sample Site	Zidovudine		Efavirenz		Fluconazole		Nevirapine		Lopinavir		Ritonavir	
		Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV
	Jukskei 6	0.28	0.16	<LOQ		0.10	0.00	<LOQ		0.51	0.08	<LOD	
	Jukskei 7	0.40	0.09	<LOQ		0.13	0.04	<LOQ		0.62	0.02	<LOD	
	Jukskei 8	0.78	0.07	2.49	0.49	0.41	0.07	0.05	0.00	3.67	0.08	<LOQ	
	Jukskei 9	<LOQ		<LOQ		<LOQ		<LOQ		0.89	0.04	<LOD	
	Jukskei 10	0.95	0.11	1.99	0.16	0.26	0.04	<LOQ		3.60	0.06	<LOQ	
	Mean	0.76		0.54		0.12		0.01		1.16			
	std dev	0.85		0.95		0.13		0.02		1.38			
	min	<LOQ		<LOD		<LOQ		<LOD		<LOD		<LOD	
	max	2.74		2.49		0.41		0.05		3.67		<LOQ	

2.3.2.2 Klip River

Zidovudine was quantified at all sites in the Klip River (Figure 10a, Table 29). However, there were some months where it was not quantified at every site, and sites 3 and 8 where the only instances that it was quantifiable in every month. The highest concentration of zidovudine was recorded in April at site 3. Except for the aforementioned high concentration, the other concentrations remained relatively constant, with slight increases in May and August at site 7 and 8, concentrations in April and August are also slightly higher than the rest at site 4 and 5.

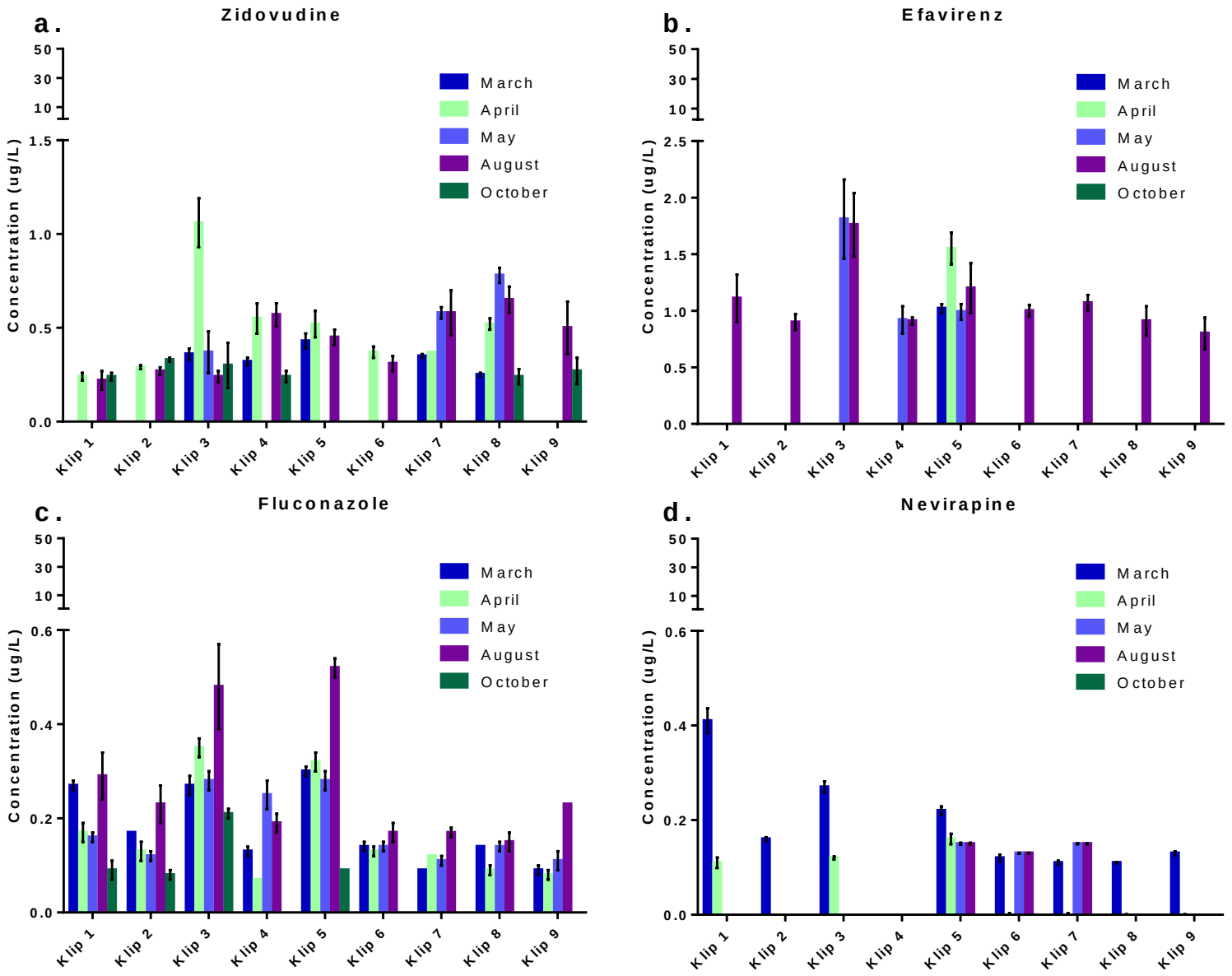
Efavirenz was consistently detected across all sites in the Klip, but only in August (Figure 10b, Table 29). It was seldom quantifiable in the other months—only at site 3, 4, and 5, was it quantified in other months. The highest concentration was at site 3, which had high concentrations in both May and August. The remaining sites had very similar concentrations. Klip 5, which had quantifiable concentrations in four months, had its highest concentration quantified in April, followed by August.

Fluconazole was detected at all sites, and all months barring October at sites 4, and 6-10 (Figure 10c, Table 29). The concentrations were typically greatest in August, with the exception of Klip 4, where May had a slightly higher concentration. Concentrations at site 3 and 5 show a similar trend through the months and similar concentrations. The remaining sites had similar concentrations, with site 1 and 2 being slighting higher than the rest.

Nevirapine was unfrequently quantified in the Klip River and was most frequently quantified in the samples collected in March. Only site 4 did not have any nevirapine quantified in any of the sampling months. The highest concentration recorded was at site 1 in March, followed by site 3 and 5. The remaining concentrations were fairly similar. Klip 5 had quantifiable concentrations of nevirapine in March, April, May, and August, and was the site with the most consistent quantification of nevirapine.

Lopinavir had the highest concentrations of any of the targeted compounds in the Klip River. With the highest concentrations quantified in August followed by March, except for site 5 where the concentration in March was greater than in August. The remaining months had much lower and similar concentrations, apart from Klip 5, where the concentration in April was greater than the aforementioned trend.

Ritonavir was quantified at all sites in the Klip, but typically only in March and August. Klip 5 was the only instance where it was quantified in another month—April. Concentrations in August were typically greatest in August, except for site 5, where it is greater in March. The concentration at Klip 4 in August has the highest concentration of ritonavir recorded in the Klip system.


a.
Zidovudine
Concentration (ug/L)
March
April
May
August
October
Klip 1
Klip 2
Klip 3
Klip 4
Klip 5
Klip 6
Klip 7
Klip 8
Klip 9
b.
Efavirenz
c.
Fluconazole
d.
Nevirapine

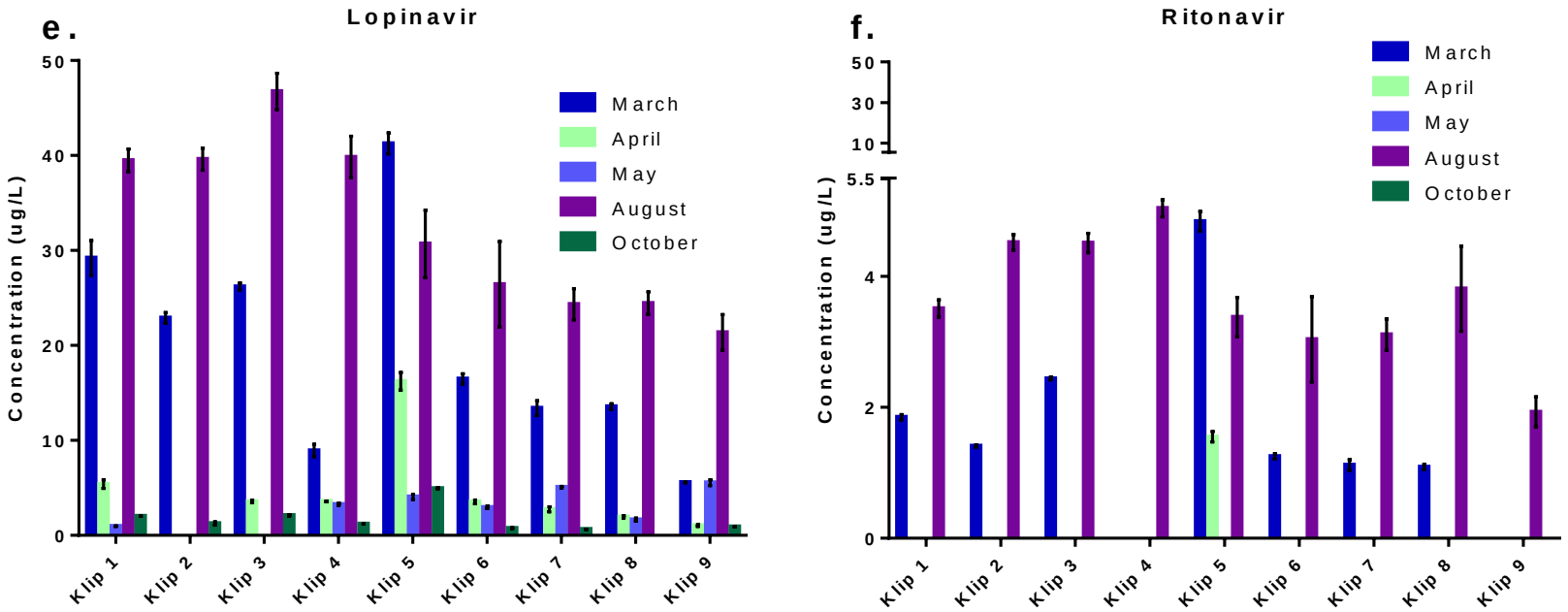


Figure 10: Concentrations of target compounds (a. zidovudine, b. efavirenz, c. fluconazole, d. nevirapine, e. lopinavir, f. ritonavir) from the Klip River over five months.

Table 29: Concentrations of selected ARVs and fluconazole in surface water from the Klip River, sampled over multiple months.

Month sampled	Site name	Zidovudine		Efavirenz		Fluconazole		Nevirapine		Lopinavir		Ritonavir	
		Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV
March	Klip 1	<LOQ		<LOQ		0.27	0.01	0.41	0.03	29.22	1.84	1.85	0.04
	Klip 2	<LOQ		<LOQ		0.17	0.00	0.16	0.00	22.91	0.56	1.41	0.02
	Klip 3	0.36	0.03	<LOQ		0.27	0.02	0.27	0.01	26.19	0.37	2.44	0.02
	Klip 4	0.32	0.02	<LOD		0.13	0.01	<LOQ		8.93	0.65	<LOQ	
	Klip 5	0.43	0.04	1.02	0.04	0.30	0.01	0.22	0.01	41.27	1.11	4.84	0.15
	Klip 6	<LOQ		<LOD		0.14	0.01	0.12	0.01	16.48	0.54	1.25	0.04
	Klip 7	0.35	0.01	<LOD		0.09	0.00	0.11	0.00	13.40	0.78	1.12	0.08
	Klip 8	0.25	0.01	<LOQ		0.14	0.00	0.11	0.00	13.57	0.30	1.09	0.04
	Klip 9	<LOD		<LOD		0.09	0.01	0.13	0.00	5.58	0.04	<LOD	
	Mean	0.19		0.11		0.18		0.17		19.73		1.56	
	std dev	0.19		0.34		0.08		0.12		11.24		1.46	
	min	<LOD		<LOD		0.09		<LOQ		5.58		<LOQ	
	max	0.43		1.02		0.30		0.41		41.27		4.84	
April	Klip 1	0.24	0.02	<LOQ		0.17	0.02	0.11	0.01	5.40	0.46	<LOQ	0.02
	Klip 2	0.29	0.01	<LOQ		0.13	0.02	<LOD		<LOD		<LOD	
	Klip 3	1.06	0.13	<LOQ		0.35	0.02	0.12	0.00	3.56	0.13	<LOQ	
	Klip 4	0.55	0.08	<LOQ		0.07	0.00	<LOQ		3.59	0.04	<LOQ	
	Klip 5	0.52	0.07	1.55	0.14	0.32	0.02	0.16	0.01	16.22	0.93	1.55	0.08
	Klip 6	0.37	0.03	<LOQ		0.13	0.01	<LOQ		3.54	0.20	<LOD	
	Klip 7	0.37	0.00	<LOQ		0.12	0.00	<LOQ		2.74	0.27	<LOD	
	Klip 8	0.52	0.03	<LOQ		0.09	0.01	<LOQ		1.96	0.11	<LOD	
	Klip 9	<LOQ		<LOQ		0.08	0.01	<LOQ		1.04	0.11	<LOD	
	Mean	0.44		0.17		0.16		0.04		4.23		0.17	
	std dev	0.29		0.52		0.10		0.07		4.77		0.52	
	min	<LOQ		<LOQ		0.07		<LOD		<LOD		<LOD	
	max	1.06		1.55		0.35		0.16		16.22		1.55	
May	Klip 1	<LOQ		<LOQ		0.16	0.01	<LOQ		0.96	0.03	<LOD	
	Klip 2	<LOQ		<LOQ		0.12	0.01	<LOD		<LOQ		<LOD	
	Klip 3	0.37	0.11	1.81	0.35	0.28	0.02	<LOD		<LOQ		<LOD	
	Klip 4	<LOQ		0.92	0.12	0.25	0.03	<LOQ		3.27	0.11	<LOD	

Month sampled	Site name	Zidovudine		Efavirenz		Fluconazole		Nevirapine		Lopinavir		Ritonavir	
		Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV
	Klip 5	<LOQ		0.99	0.07	0.28	0.02	0.15	0.00	4.06	0.27	<LOD	
	Klip 6	<LOQ		<LOQ		0.14	0.01	0.13	0.00	2.96	0.13	<LOD	
	Klip 7	0.58	0.03	<LOQ		0.11	0.01	0.15	0.00	5.07	0.09	<LOQ	
	Klip 8	0.78	0.04	<LOQ		0.14	0.01	<LOD		1.66	0.15	<LOD	
	Klip 9	<LOQ		<LOQ		0.11	0.02	<LOQ		5.55	0.31	<LOD	
	Mean	0.19		0.41		0.18		0.05		2.61			
	std dev	0.30		0.67		0.07		0.07		2.08			
	min	<LOQ		<LOQ		0.11		<LOD		<LOQ		<LOD	
	max	0.78		1.81		0.28		0.15		5.55		<LOD	
August	Klip 1	0.22	0.05	1.11	0.21	0.29	0.05	0.13	0.01	39.49	1.19	3.51	0.13
	Klip 2	0.27	0.02	0.90	0.07	0.23	0.04	<LOQ		39.62	1.16	4.52	0.12
	Klip 3	0.24	0.03	1.76	0.28	0.48	0.09	0.24	0.03	46.75	1.90	4.51	0.15
	Klip 4	0.57	0.06	0.91	0.03	0.19	0.02	0.12	0.00	39.84	2.20	5.04	0.13
	Klip 5	0.45	0.04	1.20	0.22	0.52	0.02	0.27	0.03	30.69	3.53	3.38	0.30
	Klip 6	0.31	0.04	1.00	0.05	0.17	0.02	0.27	0.02	26.44	4.50	3.04	0.65
	Klip 7	0.58	0.12	1.07	0.07	0.17	0.01	0.20	0.01	24.34	1.65	3.11	0.24
	Klip 8	0.65	0.07	0.91	0.13	0.15	0.02	0.21	0.03	24.45	1.18	3.81	0.65
	Klip 9	0.50	0.14	0.80	0.14	0.23	0.00	0.23	0.05	21.37	1.89	1.93	0.23
	Mean	0.42		1.07		0.27		0.18		32.55		3.65	
	std dev	0.16		0.29		0.14		0.09		9.03		0.95	
	min	0.22		0.80		0.15		<LOQ		21.37		1.93	
	max	0.65		1.76		0.52		0.27		46.75		5.04	
October	Klip 1	0.24	0.02	<LOD		0.09	0.02	<LOD		2.02	0.02	<LOD	
	Klip 2	0.33	0.01	<LOD		0.08	0.01	<LOD		1.25	0.18	<LOD	
	Klip 3	0.30	0.12	<LOQ		0.21	0.01	<LOD		2.12	0.08	<LOD	
	Klip 4	0.24	0.03	<LOD		<LOQ		<LOD		1.20	0.02	<LOD	
	Klip 5	<LOQ		<LOQ		0.09	0.00	<LOD		4.96	0.07	<LOQ	
	Klip 6	<LOQ		<LOD		<LOQ		<LOD		0.75	0.07	<LOD	
	Klip 7	<LOQ		<LOD		<LOQ		<LOD		0.64	0.01	<LOD	
	Klip 8	0.24	0.04	<LOD		<LOQ		<LOD		<LOQ	0.01	<LOD	
	Klip 9	0.27	0.07	<LOD		<LOQ		<LOD		0.92	0.03	<LOD	

Month sampled	Site name	Zidovudine		Efavirenz		Fluconazole		Nevirapine		Lopinavir		Ritonavir	
		Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV
	Mean	0.18				0.05				1.54			
	std dev	0.14				0.07				1.44			
	min	<LOQ		<LOD		<LOQ		<LOD		<LOQ		<LOD	
	max	0.33		<LOQ		0.21		<LOD		4.96		<LOQ	

2.3.2.3 Hennops River

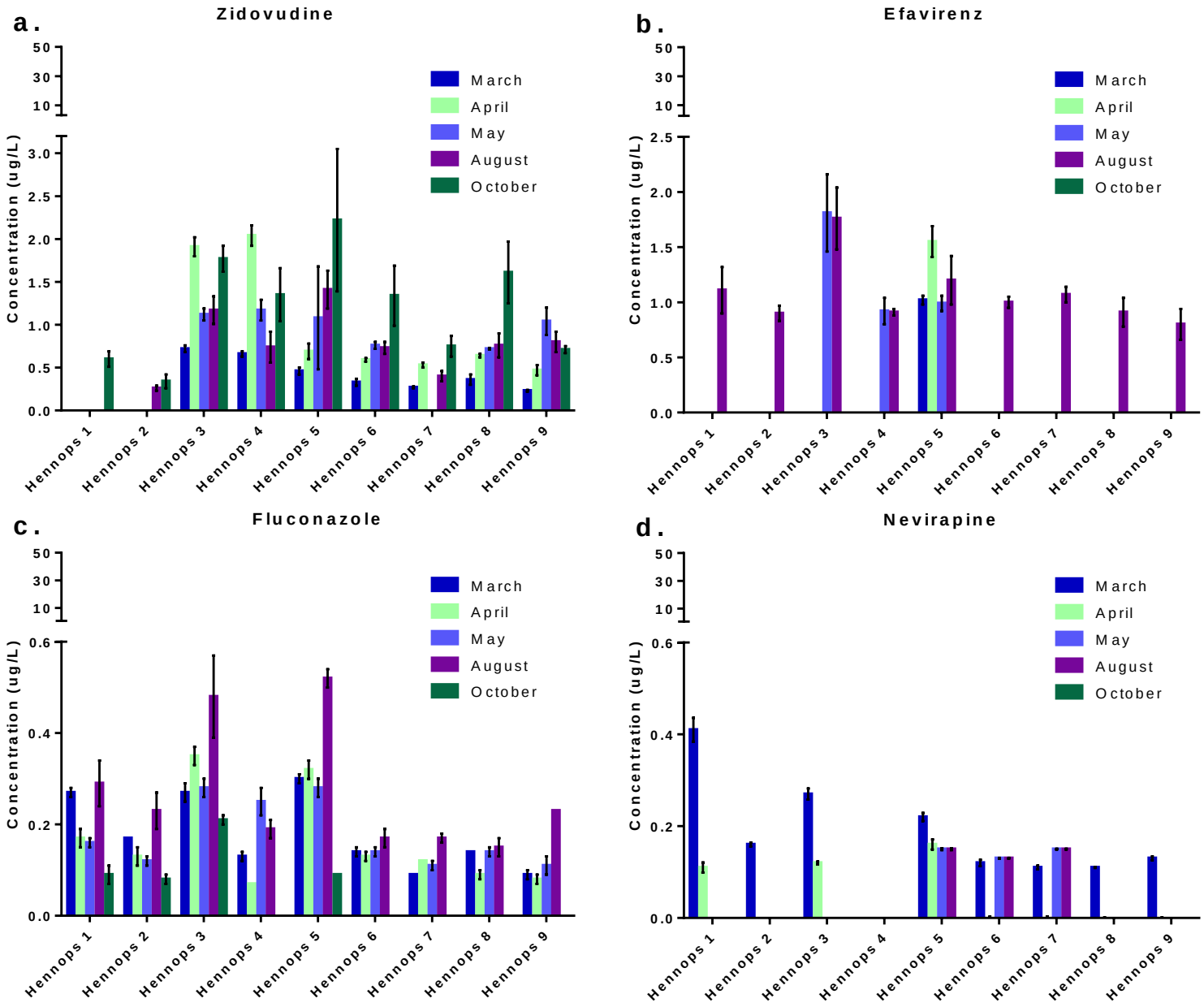
Zidovudine was detected in all months at all sites from Hennops River site 3-10, with the exception of May at site 7 (Table 30, Figure 11a). Zidovudine was only detected in October at Hennops site 1 and in August and October at Hennops site 2. Generally, the highest, or second highest concentration of zidovudine was quantified in October. In the instances where it was not the highest in October the highest concentration was recorded in April or May. The concentrations recorded in March were the lowest quantified in the Hennops River.

Efavirenz was only quantified at every site in the Hennops River during the August sampling event (Table 30, Figure 11b). It was also quantified at Hennops site 3 and 4 in May, and at site 5 in March-August. The concentration of efavirenz at site 3 had the greatest concentrations recorded through any of the quantified concentrations through the river in both May and August, with very similar concentrations.

Fluconazole was ubiquitous throughout the sample sites in the Hennops River, and was quantified in all months except October at sites 4, and 6-10 (Table 30, Figure 11c). The concentrations were greatest in August, with the exception of site 4, where the concentration was greatest in May. Site 3 and 5 had the highest recorded concentrations of fluconazole in every month compared to the other sites.

Lopinavir had the highest concentration of any of the targeted compounds in the Hennops River (Table 30, Figure 11e). Lopinavir was generally quantified at every site in all months sampled. The concentrations were consistently high in August, and concentrations in March were slightly lower. The concentrations in the other months were much lower than the aforementioned months, and these were all similar concentrations.

Ritonavir had a similar pattern to lopinavir—high concentrations in August and March, and a reversal at site 5 where the highest concentration was in March, not August (Table 30, Figure 11f). There was also a spike in the concentration in April at site 5 compared to the rest of the concentrations. Apart from these concentrations there were no other quantifiable concentrations in any of the other months at any of the other sites in the Hennops River.


a.
Zidovudine
Concentration (ug/L)
March
April
May
August
October
Hennops 1
Hennops 2
Hennops 3
Hennops 4
Hennops 5
Hennops 6
Hennops 7
Hennops 8
Hennops 9
b.
Efavirenz
c.
Fluconazole
d.
Nevirapine

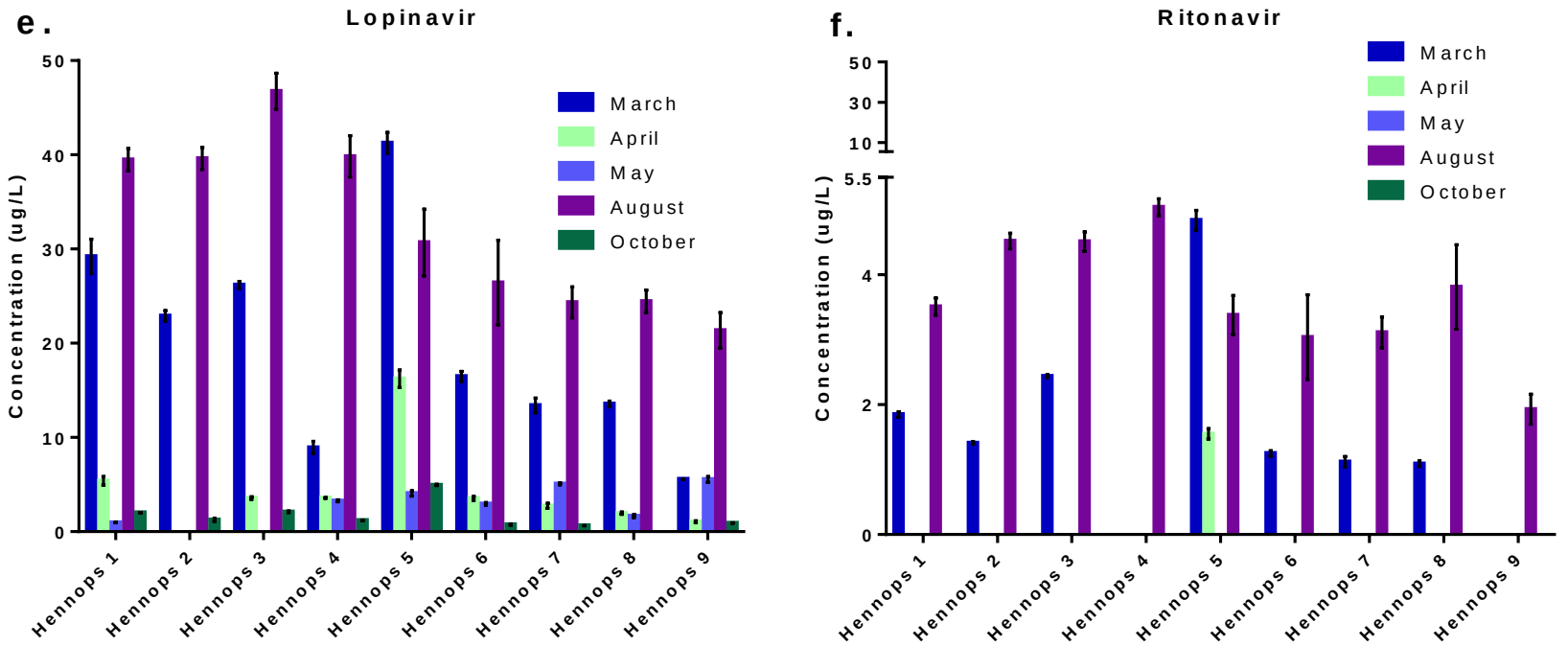


Figure 11: Concentrations of target compounds (a. zidovudine, b. efavirenz, c. fluconazole, d. nevirapine, e. lopinavir, f. ritonavir) from the Jukskei River over five months.

Table 30: Concentrations of selected ARVs and fluconazole in surface water from the Hennops River, sampled over multiple months.

Month sampled	Sample Site	Zidovudine		Efavirenz		Fluconazole		Nevirapine		Lopinavir		Ritonavir	
		Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV
March	Hennops 1	<LOD		<LOD		0.10	0.01	<LOQ		4.90	0.24	<LOD	
	Hennops 2	<LOD		<LOD		0.10	0.00	<LOQ		4.92	0.04	<LOD	
	Hennops 3	0.72	0.04	<LOQ		0.11	0.01	0.18	0.01	43.32	2.99	3.10	0.35
	Hennops 4	0.66	0.03	<LOQ		0.21	0.00	0.62	0.03	45.52	3.74	2.81	0.52
	Hennops 5	0.46	0.04	<LOD		0.21	0.02	0.48	0.02	34.12	1.33	2.48	0.12
	Hennops 6	0.33	0.04	<LOQ		0.19	0.03	0.55	0.01	63.69	1.56	5.14	0.09
	Hennops 7	0.27	0.01	<LOD		<LOQ		<LOQ		18.44	0.65	1.40	0.03
	Hennops 8	0.36	0.06	<LOD		0.21	0.03	0.34	0.02	53.15	1.53	4.30	0.10
	Hennops 9	0.23	0.01	<LOD		0.21	0.01	0.35	0.00	52.31	1.01	4.50	0.04
	Mean	0.34				0.15		0.28		35.60		2.64	
	std dev	0.25				0.08		0.25		21.55		1.88	
	min	<LOD		<LOD		<LOQ		<LOQ		4.90		<LOD	
	max	0.72		<LOQ		0.21		0.62		63.69		5.14	
April	Hennops 1	<LOD		<LOD		0.13	0.01	<LOQ		<LOQ		<LOD	
	Hennops 2	<LOD		<LOD		0.16	0.02	<LOQ		0.57	0.06	<LOD	
	Hennops 3	1.91	0.11	0.99	0.11	0.18	0.01	0.14	0.01	5.81	0.20	<LOQ	
	Hennops 4	2.04	0.12	<LOQ		0.24	0.02	0.22	0.01	3.17	0.03	<LOD	

Month sampled	Sample Site	Zidovudine		Efavirenz		Fluconazole		Nevirapine		Lopinavir		Ritonavir	
		Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV
	Hennops 5	0.69	0.09	<LOQ		0.15	0.01	0.13	0.00	3.65	0.19	<LOD	
	Hennops 6	0.59	0.02	<LOQ		0.13	0.01	<LOQ		2.62	0.16	<LOD	
	Hennops 7	0.53	0.03	<LOD		<LOQ	0.00	<LOD		1.21	0.10	<LOD	
	Hennops 8	0.64	0.02	<LOQ		0.15	0.01	<LOQ		3.44	0.08	<LOD	
	Hennops 9	0.47	0.06	<LOQ		0.16	0.01	<LOQ		3.85	0.04	<LOD	
	Mean	0.76		0.11		0.14		0.05		2.70		NA	
	std dev	0.73		0.33		0.06		0.08		1.83		NA	
	min	<LOD		<LOD		<LOQ		<LOD		<LOQ		NA	
	max	2.04		0.99		0.24		0.22		5.81		NA	
May	Hennops 1	<LOD		<LOD		0.09	0.01	<LOQ		4.11	0.17	<LOD	
	Hennops 2	<LOD		<LOD		0.10	0.00	<LOD		0.72	0.08	<LOD	
	Hennops 3	1.12	0.07	<LOQ		0.10	0.01	<LOD		0.98	0.02	<LOD	
	Hennops 4	1.17	0.12	1.14	0.01	0.32	0.03	<LOQ		<LOQ		<LOD	
	Hennops 5	1.08	0.60	<LOQ		0.16	0.02	<LOD		1.08	0.02	<LOD	
	Hennops 6	0.76	0.04	<LOQ		0.17	0.00	<LOD		<LOQ		<LOD	
	Hennops 7	<LOQ		<LOD		<LOQ		<LOD		0.69	0.02	<LOD	
	Hennops 8	0.72	0.01	<LOQ		0.17	0.01	<LOD		<LOQ		<LOD	

Month sampled	Sample Site	Zidovudine		Efavirenz		Fluconazole		Nevirapine		Lopinavir		Ritonavir	
		Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV
	Hennops 9	1.04	0.16	<LOQ		0.31	0.04	<LOQ		3.62	0.22	<LOD	
	Mean	0.65		0.13		0.16				1.25			
	std dev	0.51		0.38		0.10				1.55			
	min	<LOD		<LOD		<LOQ		<LOD		<LOQ		<LOD	
	max	1.17		1.14		0.32		<LOQ		4.11		<LOD	
August	Hennops 1	<LOQ		<LOD		0.29	0.02	<LOQ		2.32	0.25	<LOD	
	Hennops 2	0.26	0.03	<LOD		0.18	0.02	<LOQ		2.24	0.26	<LOD	
	Hennops 3	1.17	0.16	2.17	0.20	0.13	0.02	<LOD		18.01	2.61	2.23	0.56
	Hennops 4	0.74	0.18	4.18	0.17	0.43	0.02	<LOQ		35.35	2.42	4.34	0.10
	Hennops 5	1.41	0.22	3.62	0.18	0.37	0.03	0.13	0.00	31.31	0.49	3.40	0.15
	Hennops 6	0.73	0.07	2.74	0.33	0.35	0.03	0.64	0.07	32.03	1.47	3.48	0.42
	Hennops 7	0.40	0.06	<LOQ		0.15	0.01	0.53	0.03	7.77	0.38	<LOQ	
	Hennops 8	0.76	0.14	1.86	0.08	0.35	0.06	0.14	0.01	30.88	13.47	3.04	1.31
	Hennops 9	0.80	0.12	1.38	0.22	0.34	0.09	<LOQ		15.87	0.54	1.58	0.03
	Mean	0.70		1.77		0.29		0.16		19.53		2.01	
	std dev	0.44		1.58		0.11		0.25		13.34		1.69	
	min	<LOQ		<LOD		0.13		<LOD		2.24		<LOD	
	max	1.41		4.18		0.43		0.64		35.35		4.34	
October	Hennops 1	0.60	0.09	<LOD		0.21	0.02	<LOQ		0.86	0.04	<LOD	

Month sampled	Sample Site	Zidovudine		Efavirenz		Fluconazole		Nevirapine		Lopinavir		Ritonavir	
		Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV
	Hennops 2	0.34	0.08	<LOD		0.18	0.04	<LOQ		0.76	0.05	<LOD	
	Hennops 3	1.77	0.15	2.38	0.13	0.35	0.03	0.06	0.00	3.66	0.14	0.55	0.02
	Hennops 4	1.35	0.31	3.20	0.50	0.48	0.02	0.34	0.01	5.15	0.17	0.57	0.03
	Hennops 5	2.22	0.83	2.65	0.48	0.38	0.04	0.54	0.03	11.68	0.12	1.33	0.01
	Hennops 6	1.34	0.35	1.68	0.11	0.25	0.01	0.14	0.01	8.61	0.33	<LOQ	
	Hennops 7	0.75	0.12	<LOQ		0.06	0.00	<LOQ		2.40	0.08	<LOD	
	Hennops 8	1.61	0.36	1.40	0.22	0.23	0.06	0.13	0.01	7.72	0.24	<LOQ	
	Hennops 9	0.71	0.04	1.15	0.07	0.26	0.04	<LOQ	0.00	9.99	0.79	<LOD	
	Mean	1.19		1.38		0.27		0.13		5.65		0.27	
	std dev	0.62		1.21		0.12		0.19		4.03		0.47	
	min	0.34		<LOD		0.06		<LOQ		0.76		<LOD	
	max	2.22		3.20		0.48		0.54		11.68		1.33	

2.3.2.4 Crocodile River

Zidovudine was quantified at all the sampled sites in the Crocodile River. However, it was not quantified during every sampling event (Figure 12a, Table 31). The highest concentration recorded was during the April sampling at site Croc 2, and the next highest was from the same month at Croc 3.

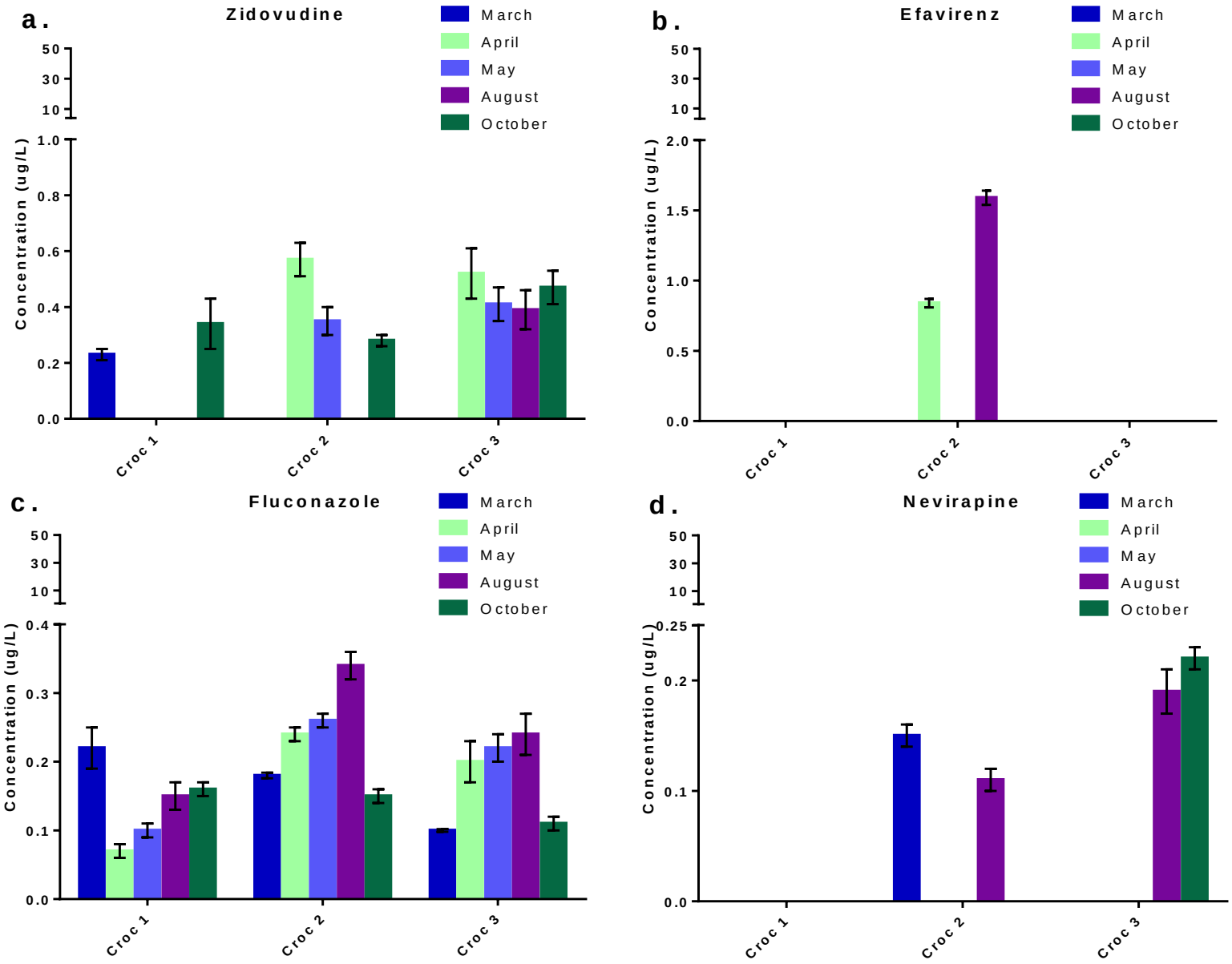
Efavirenz was only quantified twice, both at site Croc 2, in the April and August sampling event. The concentration in August was the highest (Figure 12b, Table 31).

Fluconazole was quantified at every site sampled in the Crocodile River, and during every sampling month (Table 31, Figure 12c). The concentrations tended to increase with each sampling event, with the concentration dipping again in October. Croc 1 was slightly different—the concentration in March was high, and the concentrations dropped for the April sampling, and steadily increased with each sampling event. The highest concentrations were at Croc 2, and the highest concentration was reported in the August sampling event.

Nevirapine was quantified on four occasions in the Crocodile River—at Croc 2 in March and August, and at Croc 3 in August and October (Figure 12d, Table 31). The highest concentrations were quantified at Croc 3 in October and August.

Lopinavir was quantified at all the Crocodile River samples in all the sample months. The highest concentration was at Croc 2 during August, and similarly high concentrations were reported at Croc 3 in August and October (Figure 12e, Table 31). Lopinavir had the highest concentrations of all the target compounds analysed in the Crocodile River.

Ritonavir was only quantified at three instances in the Crocodile River—in August at Croc 2, and August and October at Croc 3 (Table 31, Figure 12f). All of these concentrations were similar, but the highest was at Croc 3 in October.


a.
Zidovudine
March
April
May
August
October
Concentration (ug/L)
50
30
10
1.0
0.8
0.6
0.4
0.2
0.0
Croc 1
Croc 2
Croc 3
b.
Efavirenz
March
April
May
August
October
Concentration (ug/L)
50
30
10
2.0
1.5
1.0
0.5
0.0
Croc 1
Croc 2
Croc 3
c.
Fluconazole
March
April
May
August
October
Concentration (ug/L)
50
30
10
0.4
0.3
0.2
0.1
0.0
Croc 1
Croc 2
Croc 3
d.
Nevirapine
March
April
May
August
October
Concentration (ug/L)
50
30
10
0.25
0.2
0.1
0.0
Croc 1
Croc 2
Croc 3

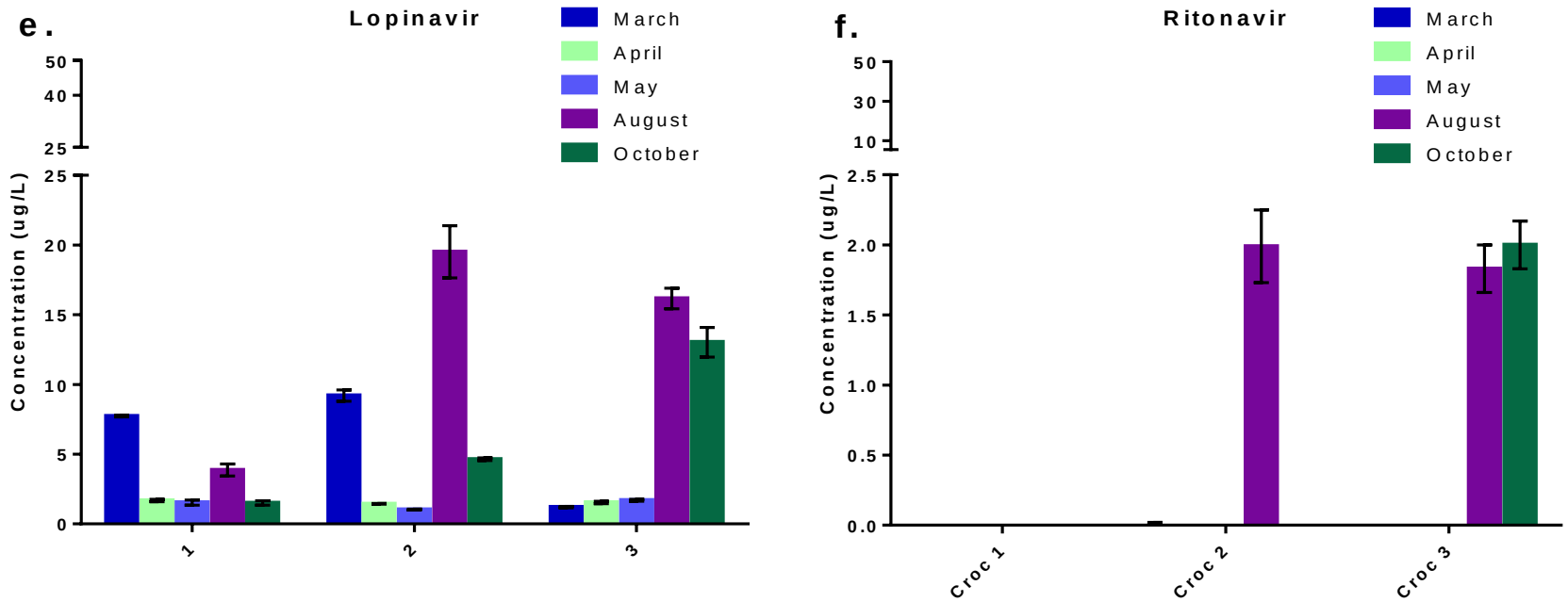


Figure 12: Concentrations of target compounds (a. zidovudine, b. efavirenz, c. fluconazole, d. nevirapine, e. lopinavir, f. ritonavir) from the Jukskei River over five months.

Table 31: Concentrations of selected ARVs and fluconazole in surface and drinking water from the Crocodile River, sampled over multiple months.

Sample month	Site name	Zidovudine		Efavirenz		Fluconazole neg		Nevirapine		Lopinavir		Ritonavir	
		Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV
March	Croc 1	0.23	0.02	<LOD		0.22	0.03	<LOQ		7.73	0.06	<LOQ	
	Croc 2	<LOQ		<LOD		0.18	0.00	0.15	0.01	9.20	0.40	<LOQ	
	Croc 3	<LOQ		<LOD		0.10	0.00	<LOQ		1.20	0.05	<LOD	
	Mean	0.08		NA		0.17		0.05		6.04			
	std dev	0.13		NA		0.07		0.09		4.26			
	min	<LOQ		NA		0.10		<LOQ		1.20		<LOD	
	max	0.23		NA		0.22		0.15		9.20		<LOQ	
April	Croc 1	<LOD		<LOD		0.07	0.01	<LOQ		1.68	0.09	<LOD	
	Croc 2	0.57	0.06	0.84	0.03	0.24	0.01	<LOQ		1.44	0.03	<LOD	
	Croc 3	0.52	0.09	<LOQ		0.20	0.03	<LOQ		1.54	0.09	<LOD	
	Mean	0.36		0.28		0.17		0.00		1.55			
	std dev	0.32		0.49		0.09		0.00		0.12			
	min	<LOD		<LOD		0.07		<LOQ		1.44		<LOD	
	max	0.57		0.84		0.24		<LOQ		1.68		<LOD	
May	Croc 1	<LOD		<LOD		0.10	0.01	<LOQ		1.53	0.19	<LOD	
	Croc 2	0.35	0.05	<LOQ		0.26	0.01	<LOQ		1.03	0.03	<LOD	
	Croc 3	0.41	0.06	<LOQ		0.22	0.02	<LOQ		1.69	0.07	<LOD	
	Mean	0.25				0.20				1.42			
	std dev	0.22				0.08				0.34			
	min	<LOD		<LOD		0.10		<LOQ		1.03		<LOD	
	max	0.41		<LOQ		0.26		<LOQ		1.69		<LOD	
August	Croc 1	<LOQ		<LOD		0.15	0.02	<LOD		3.86	0.43	<LOD	
	Croc 2	<LOQ		1.59	0.05	0.34	0.02	0.11	0.01	19.51	1.87	1.99	0.26
	Croc 3	0.39	0.07	<LOQ		0.24	0.03	0.19	0.02	16.17	0.74	1.83	0.17
	Mean	0.13		0.53		0.24		0.10		13.18		1.28	
	std dev	0.23		0.92		0.10		0.09		8.24		1.11	
	min	<LOQ		<LOD		0.15		<LOD		3.86		<LOD	
	max	0.39		1.59		0.34		0.19		19.51		1.99	
October	Croc 1	0.34	0.09	<LOD		0.16	0.01	<LOD		1.50	0.16	<LOD	
	Croc 2	0.28	0.02	<LOQ		0.15	0.01	<LOQ		4.64	0.09	<LOQ	

Sample month	Site name	Zidovudine		Efavirenz		Fluconazole neg		Nevirapine		Lopinavir		Ritonavir	
		Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV	Mean conc	STDEV
	Croc 3	0.47	0.06	<LOQ		0.11	0.01	0.22	0.01	13.03	1.06	2.00	0.17
	Mean	0.36				0.14		0.07		6.39		0.67	
	std dev	0.10				0.03		0.13		5.96		1.15	
	min	0.28		<LOD		0.11		<LOD		1.50		<LOD	
	max	0.47		<LOQ		0.16		0.22		13.03		2.00	

During the sampling around WWTPs, the drinking water samples generally did not have any target compounds detectable and/or quantifiable (Table 32). There were a few exceptions. Olifantsfontein DS1 had quantifiable concentrations of all target compounds except didanosine. The concentrations of lopinavir, ritonavir, and efavirenz were at concerning concentrations. Sunderland DS DW had concentrations of fluconazole and efavirenz, the concentration of the latter could pose a problem. Waterval DS1 had trace concentrations of nevirapine.

There were typically no quantifiable concentrations of target compounds in drinking water around the Jukskei sites in water collected in August and October (Table 33). Only zidovudine was quantified in both sampling months. The concentrations from the August event were higher than October, and these concentrations could be problematic.

There were no target compounds quantified in drinking water collected around the Klip River sample sites (Table 34).

Zidovudine was the only target compound quantified from the drinking water samples collected in the Hennops River region (Table 35). In both sampling events, Hennops DW3 was the only sample to contain zidovudine. Zidovudine was also the only target compound quantified in the Crocodile River region. It was only quantifiable in one instance—DW1 site in the August sampling event.

Table 32: Concentrations of selected ARVs and fluconazole in drinking water across the sampling area during February 2017.

Sample site	Fluconazole		Nevirapine		Lopinavir		Ritonavir		Didanosine		Efavirenz		Zidovudine	
	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV
Baragwanath DW	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Olifantsfontein DS1	0.66	0.08	0.46	0.05	26.85	4.65	1.95	0.34	<LOD		24.6	6.44	<LOQ	
Olifantsfontein US DW	<LOQ		<LOD		<LOQ		<LOD		<LOD		<LOD		<LOD	
Sunderland US DW	0.17	0.04	<LOD		<LOQ		<LOD		<LOD		6.24	2.38	<LOD	
Vlakplaats DS DW	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Vlakplaats US DW	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Waterval DS1 DW	<LOD		0.038	0.005	<LOD		<LOD		<LOD		<LOD		<LOD	
Waterval DS2 DW	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Waterval US DW	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Zeekoegat DS1 DW	0.42	0.12	0.21	0.04	9.04	2.45	<LOQ		<LOD		2.33	1.65	<LOD	

Table 33: Concentrations of selected ARVs and fluconazole in drinking water around the Jukskei region from August and October 2018.

	Sample site	Fluconazole		Nevirapine		Lopinavir		Ritonavir		Efavirenz		Zidovudine	
		Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV
August	Jukskei DW 1	<LOQ		<LOD		<LOD		<LOD		<LOD		0.27	0.13
	Jukskei DW 2	<LOQ		<LOD		<LOD		<LOD		<LOD		<LOQ	
	Jukskei DW 3	<LOQ		<LOD		<LOD		<LOD		<LOD		1.18	0.01
	Jukskei DW 3	<LOQ		<LOD		<LOD		<LOD		<LOD		0.52	0.18
	Mean											0.49	
	std dev											0.51	
	min	<LOD		<LOD		<LOD		<LOD		<LOD		<LOQ	
	max	<LOQ		<LOD		<LOD		<LOD		<LOQ		1.18	
October	Jukskei DW 1	<LOD		<LOD		<LOD		<LOD		<LOD		<LOQ	
	Jukskei DW 2	<LOD		<LOD		<LOD		<LOQ		<LOD		<LOQ	
	Jukskei DW 3	<LOQ		<LOD		<LOD		<LOD		<LOD		0.29	0.03
	Jukskei DW 3	<LOQ		<LOD		<LOD		<LOD		<LOD		<LOQ	
	Mean											0.07	
	std dev											0.15	
	min	<LOD		<LOD		<LOD		<LOD		<LOD		<LOQ	
	max	<LOQ		<LOD		<LOD		<LOQ		<LOD		0.29	

Table 34: Concentrations of selected ARVs and fluconazole in drinking water around the Klip region from August and October 2018.

	Sample site	Fluconazole		Nevirapine		Lopinavir		Ritonavir		Efavirenz		Zidovudine	
		Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV
August	Klip DW 1	<LOQ		<LOD		<LOQ		<LOD		<LOD		<LOQ	
	Klip DW 2	<LOD		<LOD		<LOD		<LOD		<LOD		<LOQ	
	Klip DW 3	<LOQ		<LOD		<LOQ		<LOD		<LOD		<LOD	
	Mean												
	std dev												
	min	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
	max	<LOQ		<LOD		<LOQ		<LOD		<LOD		<LOQ	
October	Klip DW 1	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
	Klip DW 2	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
	Klip DW 3	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
	Mean												
	std dev												
	min	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
	max	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	

Table 35: Concentrations of selected ARVs and fluconazole in drinking water around the Hennops region from August and October 2018.

	Sample site	Fluconazole		Nevirapine		Lopinavir		Ritonavir		Efavirenz		Zidovudine	
		Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV
August	Hennops DW 1	<LOQ		<LOD		<LOD		<LOD		<LOD		<LOQ	
	Hennops DW 2	<LOQ		<LOD		<LOD		<LOD		<LOD		<LOQ	
	Hennops DW 3	<LOD		<LOD		<LOD		<LOD		<LOD		0.45	0.34
	Mean											0.11	
	std dev											0.23	
	min	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
	max	<LOQ		<LOD		<LOD		<LOD		<LOD		0.45	
October	Hennops DW 1	<LOD		<LOD		<LOD		<LOD		<LOD		<LOQ	
	Hennops DW 2	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
	Hennops DW 3	<LOD		0.08		<LOD		<LOD		<LOD		0.35	0.06
	Mean			0.02								0.20	
	std dev			0.04								0.23	
	min	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
	max	<LOD		0.08		<LOD		<LOD		<LOD		0.35	

Table 36: Concentrations of selected ARVs and fluconazole in drinking water around the Crocodile region from August and October 2018.

	Sample site	Fluconazole Mean	Fluconazole STDEV	Nevirapine Mean	Nevirapine STDEV	Lopinavir Mean	Lopinavir STDEV	Ritonavir Mean	Ritonavir STDEV	Efavirenz Mean	Efavirenz STDEV	Zidovudine Mean	Zidovudine STDEV
August	Croc DW 1	<LOD		<LOD		<LOD		<LOD		<LOD		0.42	0.05
	Croc DW 2	<LOQ		<LOQ		<LOQ		<LOD		<LOD		<LOQ	
	Mean											0.30	
	std dev											0.21	
	min	<LOD		<LOD		<LOD		<LOD		<LOD		<LOQ	
	max	<LOQ		<LOQ		<LOQ		<LOD		<LOD		0.42	
October	Croc DW 1	<LOD		<LOD		<LOD		<LOQ		<LOD		<LOD	
	Croc DW 2	<LOD		<LOQ		<LOQ		<LOQ		<LOD		<LOD	
	Mean												
	std dev												
	min	<LOD		<LOD		<LOD		<LOQ		<LOD		<LOD	
	max	<LOD		<LOQ		<LOQ		<LOQ		<LOD		<LOD	

2.4 DISCUSSION

South Africa is the highest user of ARVs per capita in the world. These compounds enter the WWTPs that are not designed to remove pharmaceuticals (Wood et al., 2016). It has been shown that the levels of pharmaceuticals—like ARVs—and personal care products present in water samples are higher downstream of WWTPs than upstream because these compounds enter the WWTPs through sewage, are concentrated, and are not effectively removed (Schoeman et al., 2015).

2.4.1 Sampling around WWTPs

Generally, the samples collected downstream of the 11 sampled WWTPs had higher concentrations of target compounds than the upstream samples. There were, however, some exceptions. Zeekoegat, had high concentrations in the US 2 site in comparison with the other sites sampled in this locality, this can be attributed to being downstream of the Baviaanspoort WWTP (Figure 13). It was unfortunately only realised after sampling that this WWTP was located upstream of this site. The ZK US 1 site also had fairly high concentrations compared to other upstream sites. This could be explained by poor sanitation conditions and illegal sewage release in the area. South Africa is also a water scarce country which means that it is likely that concentration effects are taking place (Wood et al., 2016). Additionally, the time of the day could affect the concentrations, it has been documented that particular times of the day there is a high consumption of pharmaceuticals, leading to peaks of excretion (Amdany et al. 2015). These sites may not have been sampled in a close enough time frame, and it is possible that this “peak” occurred in the sample period.

Samples collected around the Olifantsfontein WWTP (Figure 14) had an increase in the two dominant compounds; lopinavir and efavirenz downstream of the WWTPs.

Sunderland Ridge sites had a different compound signature upstream than downstream. Efavirenz and lopinavir were dominant at the upstream site, while downstream lopinavir alone dominates, and is approximately three times as high as the upstream site, while the concentration of efavirenz was below the detection level (Figure 15). Schoeman et al. (2017), reported that concentrations of efavirenz in influent compared to effluent decreased from 27-71% by passing through the water treatment in WWTPs in Gauteng, which may be the reason for the decline in the concentration of efavirenz.

In the case of the Welgedacht sites, all the compounds were <LOD at the DS1 site, directly below the Welgedacht 1 water treatment plant (Figure 16). The DS2 site on the other hand, had high concentrations, which are attributed to the effluent coming from Welgedacht 2 water treatment plant (Figure 16).

The concentrations of compounds quantified at Vlakplaats (fluconazole, nevirapine, lopinavir, ritonavir and efavirenz) upstream of the Vlakplaats (VP) WWTP were lower than levels in the downstream samples. This is the first instance where efavirenz was not lower after going through the treatment plant (Figure 17). However, there is a dilution effect between DS1 and 2 which can be seen in the reduction of concentration of the compounds. In the case of the Waterval (WV) sites all of the compounds were below the detection limit, however further downstream (DS2) there were four quantifiable compounds. This might be due to external sources other than the WWTP (Figure 17).

At the Baragwanath sites, there was the expected increase in compound detections and concentrations, especially in the case of lopinavir (Figure 18). There is also a hospital between the two sites which could also be a source of the compounds.

Flip Human (FH) sites also show an increase in the concentrations of the target compound downstream compared with the upstream site. Efavirenz also shows this tendency, which was generally not observed at the other sites. These concentrations decrease, probably by dilution effects of tributaries flowing into the Wonderfonteinspruit, as seen at Khutsong 1 (K1). The concentrations at the Khutsong sites are generally low and showed no temporal patterns.

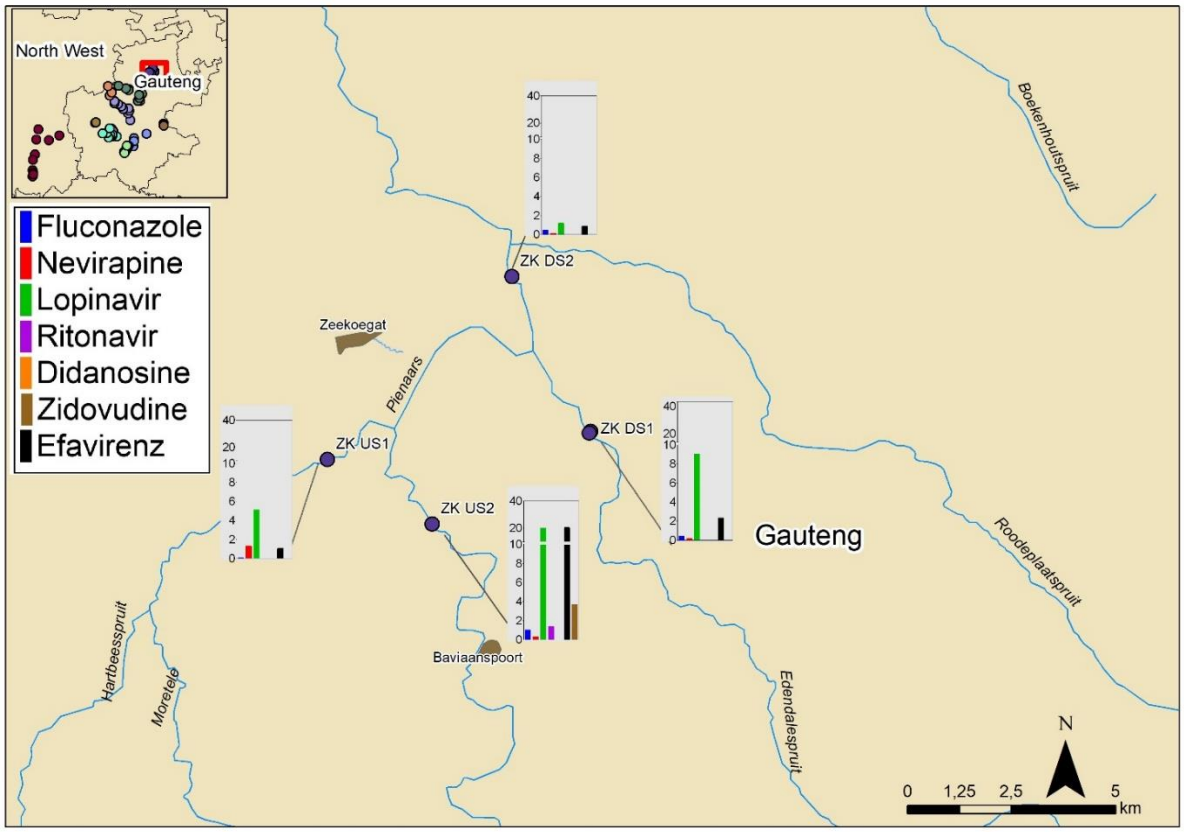


Figure 13: Map showing the concentrations of ARVs detected at the Zeekoegat sites, indicating the up- (US) and downstream (DS) sites of the Zeekoegat WWTP.

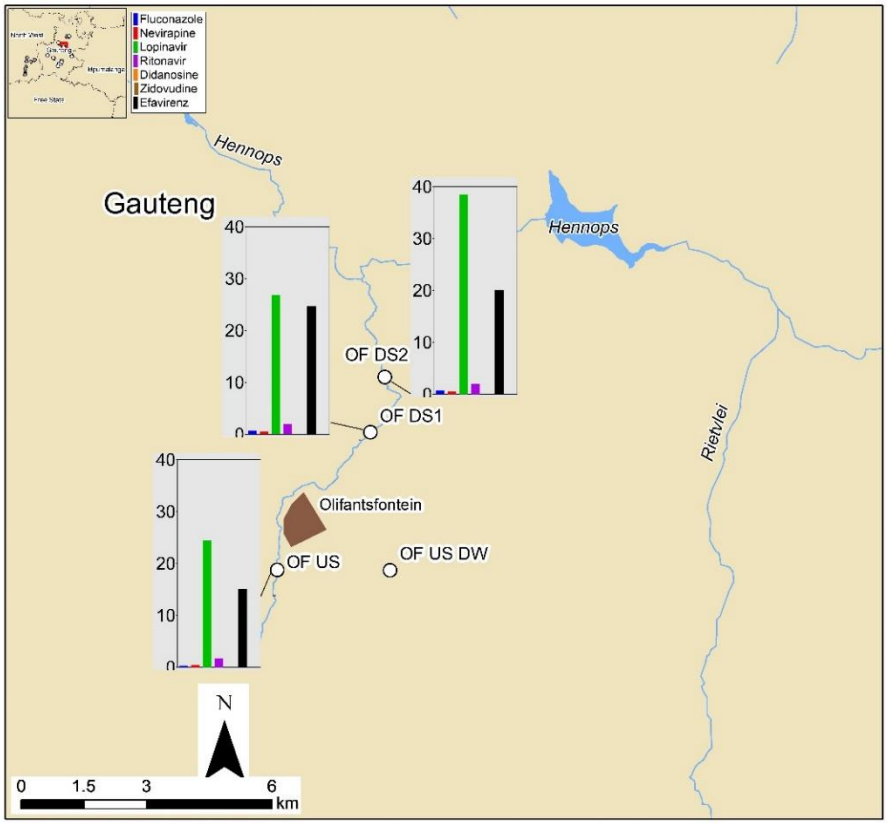


Figure 14: Map showing the concentrations of ARVs detected at the Olifantsfontein (OF) sites, indicating the up- (US) and downstream (DS) sites of the WWTPs.

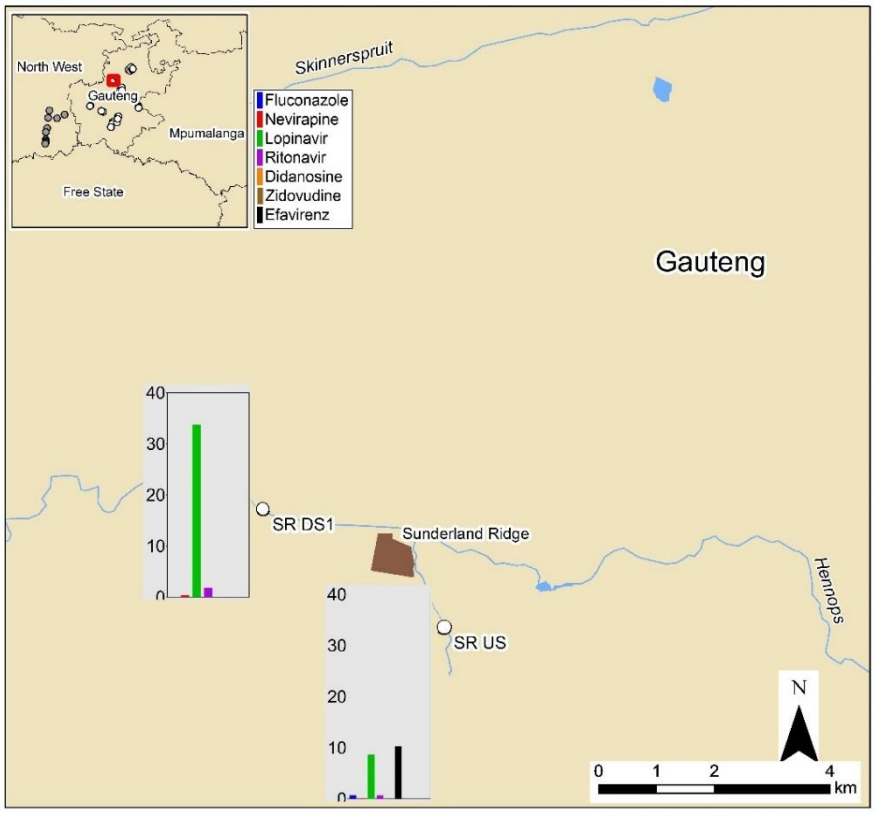


Figure 15: Map showing the concentrations of ARVs detected at the Sunderland Ridge sites, indicating the up- (US) and downstream (DS) sites of the WWTPs.

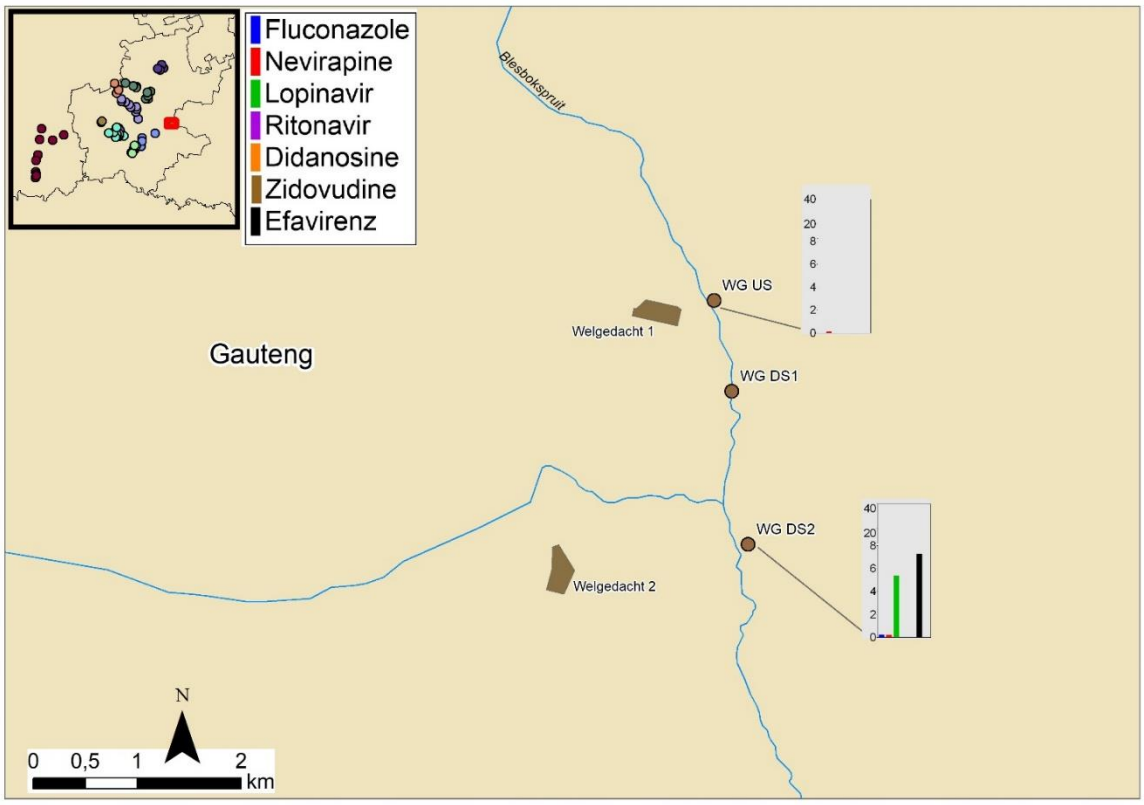


Figure 16: Map showing the concentrations of ARVs detected at the Welgedacht (WG) sites, indicating the up- (US) and downstream (DS) sites of the Welgedacht WWTPs.

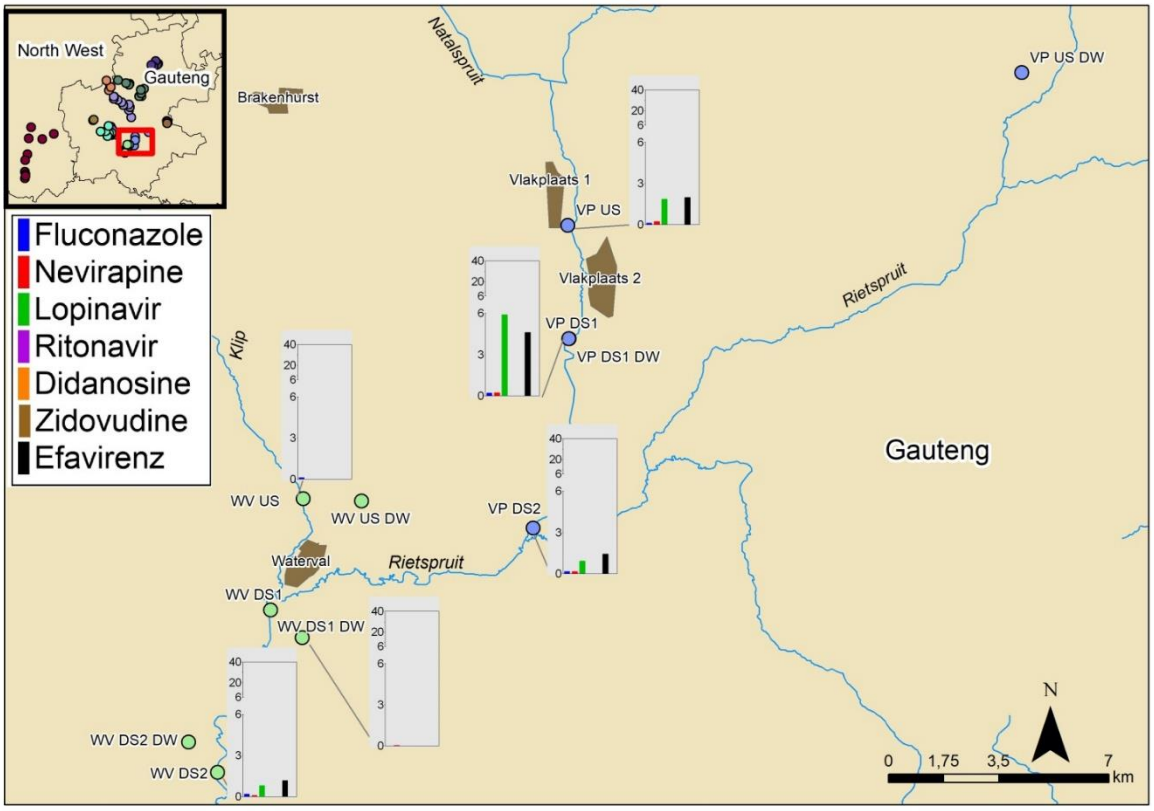


Figure 17: Map of the Vlakplaats (VP), and Waterval (WV) sites up- (US) and downstream (DS) of the two Vlakplaats, and the Waterval WWTPs.

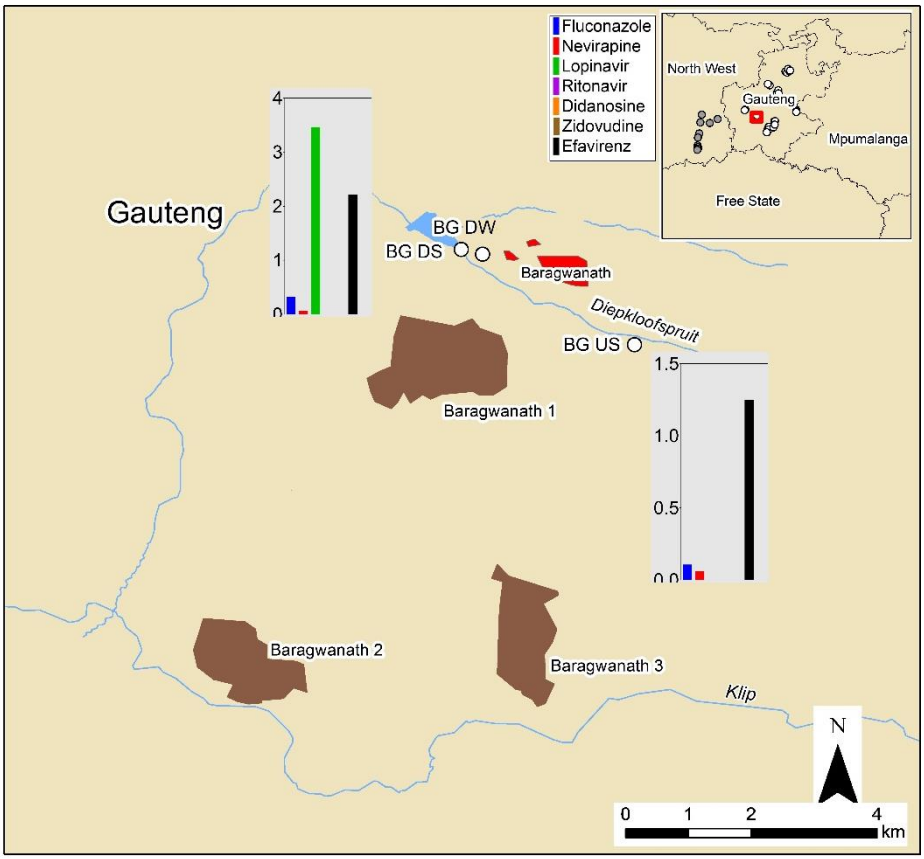


Figure 18: Map showing the concentrations of ARVs detected at the Baragwanath (BG) sites, indicating the up- (US) and downstream (DS) sites of the WWTPs. Also displayed are the Klip river sites (* indicates > calibration range).

Two sites from the Zeekoegat area (ZK US2 and DS2) were in the same locality as those in a study conducted by Wood et al. (2015). Didanosine was the only compound for which the results corroborated with concentrations reported by Wood et al. (2015)—below the detection limit. The concentrations of zidovudine, ritonavir, lopinavir, and efavirenz found at Zeekoegat US2 (Table 25) were all higher in the present study than results obtained by Wood et al. (2015), for the Pienaars River inflow site. However, the concentration of nevirapine was an order of magnitude lower in this study in comparison to that of Wood et al. (2015). The concentration of zidovudine and nevirapine at Zeekoegat DS2 (Table 25) were lower than reported by Wood et al. (2015), for the Roodeplaat Outflow site. However, this was not the case for lopinavir and efavirenz, which had higher concentrations than previously determined by Wood et al. (2015).

2.4.2 Spatial, temporal and compositional trends

Typically, the highest concentrations in the river systems monitored over multiple months indicated that the concentrations of the target compounds were greatest in the months of March and August. The high concentrations in March might be due to it being at the end of the rainy season, and target compounds accumulate in soils particularly in informal settlements (where there is inadequate sewerage and sanitation), and during a rain event these run off into the rivers (Segura, et al. 2015; Mzukisi, et al. 2017). The high concentrations in August could be explained by a concentration effect during the dry winter months, and a decline in October due to the sampling occurring after the first heavy rains, which causes an increase in water volumes and therefore a dilution effect. Lopinavir consistently had the highest concentrations of the target compounds in all of the river systems. Fluconazole and zidovudine were the most ubiquitous compounds present; this was different from the initial sampling event where zidovudine was seldom quantified.

The Jukskei River flows in a north western direction into the Crocodile River. The Northern Johannesburg WWTP is located upstream of Jukskei site 8 and 9, and this could explain the elevated concentrations in some of the months at site 8 and 10. The Jukskei River converges with the Crocodile River that flows in a northern direction towards the Hartbeespoort Dam, between Croc 1 and 2. Concentrations in all months tended to increase after the two rivers meet.

The Hennops River flows in a western direction and converges with the Crocodile River downstream of where the Jukskei confluence with the Crocodile River (just upstream of Croc 3). As the Hennops flows from the first sampling site (Hen 1 and 2) to the following site Hen 5, which is at the confluence of the Sesmylspruit and the Olifantspruit (which from there forth is known as the Hennops) the concentrations at Hennops 5 are typically highest, likely from the input from these aforementioned tributaries. The other site with elevated concentrations is Hen 3, which is the same as the OF US site (upstream of the Olifantsfontein WWTP). This latter site had similarly high concentrations of lopinavir and efavirenz. Hen 4 corresponds with the OF DS (downstream of the Olifantsfontein WWTP), however in the concentrations at Hen 4 were not as high as Hen 3, and often the target compounds were not quantified.

The Baragwanath (BG) and the Klip sites coincide. All the BG sites, and Klip 7 and 8 are located on a tributary of the Klip, Diepkloofspruit, initially flowing in a north west direction and then changing to flow in a southern direction into the Klip River. The Klip River flows from west to east. The Klip sites generally have peak concentrations at sites 3 and 5, these and site 4, are situated around Baragwanath 3 WWTP, which may be the source of these concentrations. While the Klip 8 and 7, which are downstream of the Baragwanath sites have more compounds detected than the BG sites. Of those that were detected at the BG sites (efavirenz and lopinavir), the Klip 8 and 7 have at least an order of magnitude greater concentrations of lopinavir and similar concentrations of efavirenz. The most western site on the Klip River (Klip 9) is situated close to the densely populated informal settlements (Protea Glen and Naledi) on the banks of the river and might account for the inputs in the region. Klip 6, is situated downstream of Baragwanath 2 WWTP. The Klip River flows southeast, to meet the Natalspruit, in the vicinity on the Waterval sites (Figure 17). The levels of the compounds at the Waterval US site are very low, which indicated a dilution effect. It should be kept in mind that the Klip River sites sampled around Baragwanath WWTP (BG) and the Waterval (WV) were taken in February 2017, and the Klip samples in March-October 2018.

Of the 72 sites sampled, 69 sites have never before been investigated. The Rietvlei Dam (same as Hennops 1) and Zeekoegat DS1 and US1 were sampled by Wood et al., (2015). The profile of ARVs sampled and detected in South African water is different to the international results. This can be ascribed to differences in the drugs and formulations that are prescribed, and because AIDS in SA is much more prevalent than other countries. It is evident from the results obtained in these sampling events that the ARVs (except for efavirenz, generally) and fluconazole concentrate in the WWTPs and are not efficiently removed.

Two studies conducted by Schoeman et al. (2015; 2017) published results on the concentrations of efavirenz and nevirapine from influent and effluent from undisclosed WWTPs in Gauteng. It was reported that the influent concentrations of efavirenz were 17.4 µg/L (Schoeman et al., 2015), and 5.5-14 µg/L (Schoeman et al., 2017). The concentrations in both studies were reduced by passing through the WWTP, with effluent concentrations of 7.1 µg/L (Schoeman et al., 2015), and 4.0 µg/L (Schoeman et al., 2017). The concentrations of nevirapine in influent was 2.1 µg/L (Schoeman et al., 2015), and 0.05-0.18 µg/L (Schoeman et al., 2015). In the first study the concentration of nevirapine decreased by passing through the WWTP to 0.35 µg/L (Schoeman et al., 2015), but in the subsequent study the concentration of nevirapine increased to 0.09-0.47 µg/L (Schoeman et al., 2017).

A study by Ngumba et al. (2016), reported concentrations of lamivudine (5.4 µg/L), zidovudine (7.7 µg/L), and nevirapine (4.9 µg/L) in surface water in Kenya. Another study in Kenya reported lamivudine, zidovudine, nevirapine, and efavirenz concentrations in surface water at 167, 17.4, 5.62, 0.65 µg/L respectively (K'oreje et al., 2016). In South African surface water lamivudine (0.24 µg/L), zidovudine (0.97 µg/L), nevirapine (1.48 µg/L), lopinavir (0.31 µg/L) concentrations were reported (Wood et al., 2015). In the current study the mean concentration of nevirapine in the rivers sampled were all below the concentrations recorded by these

studies. Most of the rivers sampled had mean concentrations of zidovudine that were less than the concentration reported by Wood et al. (2015); only the Zeekoegat sites had a similar mean concentration. However, there were individual sites that had concentrations higher than the study by Wood et al. (2015), but none of the sites had concentrations in excess of the other two studies. In most instances, the concentrations of efavirenz in the rivers sampled were above the concentrations reported in Kenya by K'oreje et al. (2016), and many samples had concentrations far exceeding them. The concentrations we report for lopinavir are much higher than those reported by Wood et al. (2015), lopinavir had the highest concentrations in the rivers sampled in this study.

In our study the concentration of efavirenz measured upstream of the WWTP, ranged from <LOD-20.4 µg/L (Table 22), however, the site with the maximum concentration was at site ZK UP2, which was actually below the Baviaanspoort WWTP, as mentioned before. The next highest efavirenz concentration from an upstream site was at Olifantsfontein US (15.0 µg/L), our results are in the same range as those published in these two Wood et al. (2015) articles. The nevirapine concentrations determined in this study ranged from <LOD-1.3 µg/L at Zeekoegat US1 (Table 25), which is about half the value that Schoeman et al. (2015) reported, but almost double the concentration that Schoeman et al. (2017) reported. Although, we did not sample influent directly, the concentrations we report are in the same range as the influent into these undisclosed WWTPs. This is alarming and indicates the importance of determining the concentrations of pharmaceuticals in aquatic systems, and the potential effects that these compounds, and mixtures of these compounds can pose to organisms encountering and consuming this water.

2.5 CONCLUSIONS

A variety of ARVs and a common antifungal agent (fluconazole) that is used in conjunction with ARVs have been quantified in various water bodies and drinking water sources across Gauteng and in the Mooi River in the North West. These compounds are pseudopersistent in the environment, and it is unknown what effects these might have on the biota that are exposed to these compounds. These may also accumulate in the tissues of organisms exposed. Humans can be exposed to these compounds if they consume organisms from these water sources. It is possible that these could cause detrimental effects, especially when other pharmaceuticals and their metabolites (resulting in a mixture effect) would also likely be present.

2.6 RECOMMENDATIONS

Summary of recommendations

The following recommendations are proposed for the instrumental analysis:

- It is recommended that the antiretroviral metabolites are quantified by use of standards and a calibration curve, because the screening method did not reveal the presence of these compounds.
- The extraction method should be optimised for metabolites specifically.
- Further determination of antiretrovirals and metabolites should be investigated.
- It is advised to use the fish whole and analyses to be conducted in a timely manner after collection.
- If antiretrovirals and/or metabolites are detected in whole fish, it would be useful to determine in what tissue they accumulate, i.e. muscle, liver, brain, etc.
- It is advised to determine how other provinces in South Africa compare to the concentrations reported in Gauteng river systems.
- Lastly, future studies should take place during the months of March and/or September (when the highest concentrations of antiretrovirals were found in water sources).

CHAPTER 3: BIOLOGICAL EFFECTS

3.1 INTRODUCTION

3.1.1 Potential impacts of ARVs in the environment on bacteriophages

3.1.1.1 Introduction

The development of antiviral agents over the last few decades has provided the clinical world with therapeutic possibilities previously unattainable (De Clercq and Li, 2016) However, the ever-increasing utilization of antiviral drugs has led to a heightened appreciation for the development of viral resistance and toxicity (Ncube et al. 2018). According to Al-Rajab et al. (2010), and Kummerer (2008), antiviral drugs have a toxic effect on certain microbes, algae, fish as well as *Daphnia magna*. Yet, non-specific antiviral effects of various antiviral substances against naturally occurring viruses remain undetermined. Prasse et al. (2010), demonstrated that intermediates of antiviral agents could have genotoxic effects on microbial and viral genomes. For instance, spontaneous mutations may arise during drug exposure, of which the biological consequences can include alterations in genetic stability, viral transmissibility, and pathogenicity. Of particular concern is the non-specific antiviral effects on naturally occurring aquatic viruses. Still, literature regarding this subject remains ominously inadequate and calls for further research. The danger of these toxins are that some may be expelled in the environment and treated water, in metabolized, conjugates and even active forms (Prasse et al. 2010). This alone, probes an investigation of the possible effect that ARVs may have against aquatic organisms, particularly aquatic bacteriophages.

Bacteriophages (phages) are viruses that infect bacteria (Douglas, 1975). They were first discovered in 1915 (Douglas, 1975). Bacteriophages are small, complex, protein-particles (Figure 19) and individuals can only be visualized by transmission electron microscopy (TEM) (Ackermann, 2012). Bacteriophages are host-specific, meaning each type of phage can only infect a certain range of bacterial hosts. Bacteriophages that infect *Escherichia coli*, or via *E. coli* structures are called coliphages. Coliphages that infect via receptors on *E. coli*'s cell wall are called somatic coliphages (Figure 20). For enumeration and isolation of somatic coliphages from aquatic environments ISO 10705-2 is employed (ISO, 2002). This method relies on the phages infecting a single *E. coli* in a solid mat of bacteria in a petri dish. After this initial infection, it multiplies exponentially continuing to infect and lyse new *E. coli* until a clear circle of lysed bacteria can be observed in the solid bacterial mat (Figure 21). This clear zone is termed a plaque and represents a single somatic coliphage in the original water sample (Nishihara, 2002).

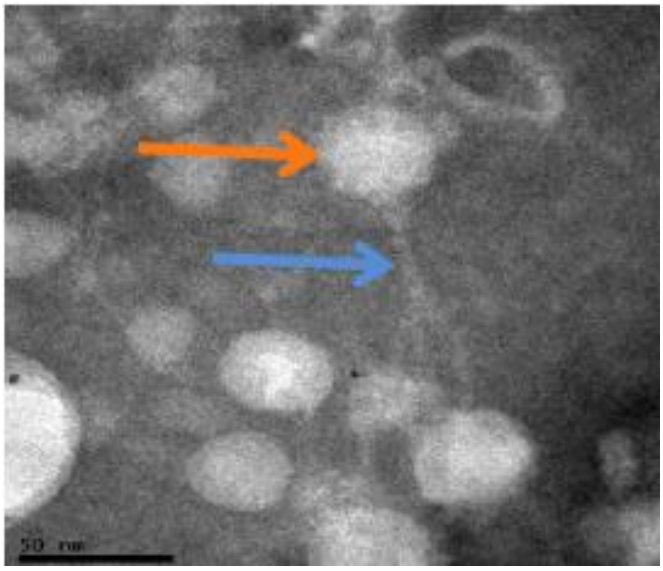


Figure 19: TEM image of a somatic coliphage. The orange arrow indicates a phage head. The blue arrow indicates a phage tail with three visible appendages.

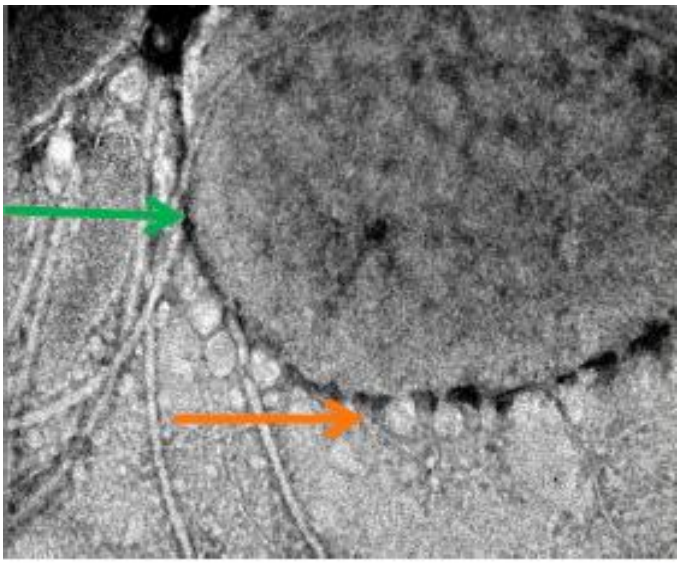


Figure 20: TEM image of phages absorbed to receptors on the cell wall of *E.coli* WG5. The green arrow indicates an *E. coli* cell. The orange arrow indicates a phage head.

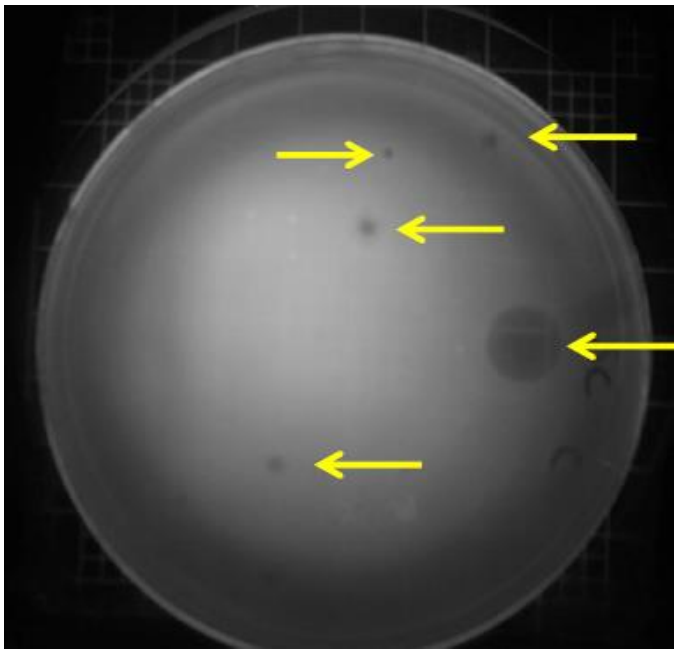


Figure 21: Photo of a Petri-dish containing one big clear, clean edge somatic phage plaque and four smaller clear, clean edge plaques.

Somatic coliphages are present in the gastro-intestinal tracts of all living animals and enter the aquatic ecosystem via the direct excretion of faecal matter into sewage or other water systems (Havelaar et al., 1993). Somatic coliphages have the ability to multiply spontaneously in the environment (Calender, 2005), and are therefore an important, naturally occurring, biological treatment mechanism in WWTPs. In water treatment plants somatic coliphages have been used as ideal indicators for water quality assessment (Skraber et al., 2009). They also play a central role in the regulation of prokaryotic composition and population structure in aquatic systems by affecting various ecological processes (Sandaa et al., 2009). It is known that antiviral substances and their intermediates are persistent in WWTPs (Jain et al., 2013) and that phages are important participants in regulating the microbial consortium composition in these aquatic systems (Sandaa, 2009). However, knowledge on the effects of antiviral substances on phages are unknown. The infectious cycle of somatic coliphage and *E. coli* is similar to that of HIV and CD4 T lymphocyte cells. It involves the absorption of the virus to the host cell, introduction of nucleic acid (RNA/DNA) into the host cell, transcription and translation of pro-virion RNA/DNA and ultimately the formation of new, mature virion-particles (Emmett et al., 2005). Since these mechanisms are similar the comparable virus model design for determination of ARV sensitivity on viruses is applicable and logic.

Bacteriophages are important in WWTP due to their ability to infect and kill persistent bacteria, thus these natural occurring viruses help improve the biological quality and safety of wastewater (Sandaa et al., 2009). We rely on bacteria to create more bacteriophages in the system in order for the viruses to keep the bacteria in check (Sandaa et al., 2009). The lack of activity by phages could cause larger biofilm formations, more bacteria in final effluent and bacterial blooms in water systems if left unchecked. Should aquatic phages, that form the basis of the food chain, be hampered by ARV pollution in the water, the food chain and efficiency of wastewater treatment could be hindered. Since a large portion of unchanged ARVs eventually end up in the WWTP, this system and its receiving water bodies could be viewed as hot spots to assess biological risks associated with ARVs in aquatic ecosystems (Madikizela et al., 2017). It must be emphasized that worldwide no regulations for wastewater effluents have been developed for ARVs and that WWTPs were not specifically designed to remove ARV compounds (Fernández-López et al., 2016). ARVs in aquatic systems could be considered a latent risk due to their biological activity, unintended secondary effects, and interactions with other compounds (Kummerer, 2008).

3.1.2 *In vitro* tissue culture assays

3.1.2.1 Introduction

In this study, biological effects of the active ingredients in selected ARV formulations were investigated using a battery of cell lines capable of distinguishing between two mechanisms of action by which endocrine disruption can be mediated. One mechanism of action specifically detects whether xenobiotics can mimic steroid hormones by binding to and activate, and/or inhibit their respective receptors. Hormonal effect can also be mediated by a second mechanism of action. This is achieved by disrupting the metabolism of the steroid hormones from cholesterol, resulting in changed levels of the various steroid hormones when compared to the unexposed levels.

Binding to the receptors were investigated by the so-called reporter gene assays. The general principle of the reporter gene assay is that ligand-bound nuclear receptors bind to a specific response element on the DNA and initiate transcription of the downstream gene. This downstream gene encodes a measurable feature such as luciferase (Poulsen et al., 2011). The measurement determines the potency of various ligands/chemicals to bind to the specific receptor and initiate or inhibit the transcriptional activity of the reporter.

Three cell lines testing the ability of a compound to bind to a receptor were used in the study: (1) (anti-)androgen activity via the androgen receptor (AR) by the MDA-kb2 cells (Wilson et al., 2002); (2) (anti-)oestrogen activity via the oestradiol receptor (ER) by the MVLN cells (Pons et al., 1990), and (3) binding to the aryl hydrocarbon receptor (AhR) by the H4IIE-luc cells (Aarts et al., 1993). The AhR regulates the expression of a wide range of genes, for example the CYP1A1 gene which codes for the xenobiotic-metabolizing enzyme cytochrome P450 (Denison and Nagy, 2003). And although the AhR *per se* is not a hormonal receptor, activation of it has been reported to exhibit anti-oestrogenic cross-talk with the ER (Chen et al., 2001), blocking it. This cross-talk mechanism between the AhR-ERα is complex, but involves the inhibition of oestradiol responsive genes by its dioxin response element (DRE) structures that bind to the AhR complex and so disrupting the oestrogen action through multiple mechanisms (Navas and Segner, 2000; Safe et al., 2000), which may lead to detrimental effects. All of the reporter gene cell lines used in this study have been genetically modified to express the firefly luciferase gene in addition to the usual expression of genes upon the successful binding of the respective receptors.

The stock of oestrogen cells (MVLN) that we have seemed to have aged to such an extent that they were unable to respond to even pure oestrogen. Unfortunately, this was realised only after all the other possible reasons for failing results were eliminated. A fresh batch of a similar, commercially available cell line, the T47Dkbluc cells, was acquired from the American Type Culture Collection and only arrived in January 2017. Because an honours student's research project depended on the successful running of the MVLN cells, their subsequent failure resulted in a new, initially unintended, project where the cytotoxic effects of the active ingredients of the selected ARVs were investigated on a human intestinal cell line. The HuTu-80 intestinal epithelial cells were used to act as a model for the human intestine to determine the time-and dose dependent cytotoxicity of various ARVs using the MTT-viability assay.

In order to determine if the respective ARV active ingredients can influence steroidogenesis (steroid hormone metabolism) the H295R adrenocortical carcinoma cells were used. Oestradiol (E2) and testosterone (T) are the most commonly measured hormones (OECD, 2011) for determining the effects of a compound on steroidogenesis. The concentrations of the hormones in the nutrient media of the cells can be quantified with analytical instrumentation or using ELISA where antibodies were raised against specific hormones. We report on the levels of E2 and T that we quantified using commercial ELISA kits.

3.1.3 Snail exposures

3.1.3.1 Introduction to Bulinus tropicus

The phylum Mollusca has the second most diverse and species-rich phyla known to scientists next to the phylum Arthropoda (Gruner, et al., 1993; Viljoen, 2010). Contributing considerably to the biomass of various trophic levels within an ecosystem, from primary consumers to top predators (Oehlmann et al., 2007). A general attribute of molluscs are their sensitivity to pollutants due to their restricted ability to metabolize exogenous organic chemicals together with a limited ability to eradicate pollutants via excretory organs (Lee, 1985; Legierse et al., 1998). It's believed that snails can bioaccumulate various toxicants to such a degree that pollutants might reveal deleterious impacts on snails at much lower environmental concentrations (Brown, 1981). Seeing that molluscs are generally sedentary, they can also be used to reflect previous and present environmental conditions at a specific site (Davies-Coleman and Palmer, 2004).

The functionality of these sentinel organisms in toxicological research has therefore been well recognized over years for demarcating anthropogenic activity and pollution in freshwater (Amiard-Triquet et al., 1987; Berthet et al., 1992; Traunspurger and Drews, 1996) marine (Kure and Depledge, 1994; G. Le Pennec and M. Le Pennec, 2001) as well as in terrestrial environments (Cortet et al., 1999; Scheifler et al., 2002). While most ecotoxicological studies emphasis soft tissue analysis, the calcified shell of molluscs can also be used as a pollution-concentrating organ (Osuna-Mascaro et al., 2015). Seeing that the shell forms an intricate part of the organism, they can also be preserved for analysis regarding past contamination events. A noteworthy example of perennial biomineralization with the potential of accurately recording environmental changes caused by pollution, in its chemical content, overall morphology as well as in both larval and adult stages (Walsh et al., 1995).

Bulinus is a large, extensive, and diverse genus. According to de Moor and Day (2007), its members are 'arduous' to identify, however they can be distinguished into four species-groups of which three occur in southern Africa. Of the three known species from southern Africa, *B. tropicus* is the most generic and extensive, with a wide distribution over the sub-continent (de Moor and Day, 2007). According to de Kock et al. (1986) *B. tropicus* is an ideal test subject since it has the shortest generation time of any of the other economically important freshwater snail species in South Africa. The rapid reproductive cycles of *B. tropicus* can therefore potentially be used as a bioindicator of a contaminated aquatic ecosystem, which in turn can provide useful information on the quality of the environment (Davies-Coleman and Palmer, 2004). Subsequently, this indigenous species can be acquired in large quantities, easily maintained/laboratory cultured and has renowned biological characteristics and prefaces.

3.2 MATERIAL AND METHODS

3.2.1 Potential impacts of ARVs in the environment on bacteriophages

This section contains the details of the methods and materials used to assess if the presence of ARVs have any possible effect on bacteriophages. Using concentrations comparable to those found present in the environment (CHAPTER 1:. The effects of ARV concentrations: 2 ng/L, 4 ng/L, 6 ng/L, 8 ng/L, 10 ng/L, 20 ng/L, 40 ng/L, 60 ng/L, and 80 ng/L; were assayed for all the ARVs mentioned hereafter. The effects of six ARVs of three different classes of ARVs were tested. The three classes were NNRTI, NRTI and PI. The six different ARVs were: efavirenz (NNRTI), nevirapine (NNRTI), stavudine (NRTI), lamivudine (NRTI), lopinavir (PI), and ritanovir (PI).

3.2.1.1 Sampling of environmental somatic coliphages

ISO 10705-2 was conducted on water sampled from a system having no ARVs present and known faecal pollution. This ensured a consortium of environmental somatic coliphages would be isolated without any prior known expression of resistance to ARV compounds. A 100 mL sterile glass Schott bottle was used for sampling. After sampling the bottles were placed in an insulated container and sealed shut until it reached the laboratory.

3.2.1.2 Cultivation and storage of bacterial-host stock

Known and pure, bacterial culture (*E. coli* WG 5) was streaked out on tryptone-soy agar (TSA) (Merck, Germany) plates and grown at 37°C overnight. After which the plates were sealed using para-film and stored at 4°C.

3.2.1.3 Cultivation and storage of somatic coliphage stock

Isolation of the bacteriophages from the aquatic medium mentioned in section 3.2.1.1 and its inhibitory constituents (like ARVs) was a multistep process. Firstly, 25 mL of glycine buffer (0.05 M glycine, 3% beef extract, pH 9.6) was added to 200 mL of sewage and mixed, to detach virions bound to organic material (Hjelmsø et al., 2017). The water samples were then filtered through 100 µm nylon filter paper by gravity to remove most unwanted organic solid materials. The water was then centrifuged at 4000xg for 60 minutes, and the collected supernatant was filtered through a 0.45 µm polyethersulfone (PES) membrane to remove bacterial and eukaryotic cells. Viruses were precipitated from the supernatant by incubation with Polyethylene glycol (PEG) 8000 (80 g/L) and NaCl (17.5 g/L) during agitation (100 rpm) overnight at 4°C, followed by centrifugation for 90 minutes at 13000xg. The resulting viral-containing pellet was eluted in 1 mL phosphate buffer saline (PBS) and stored at -80°C until further processing (Colombet et al., 2007).

Somatic coliphages were isolated from plaques obtained by ISO 10705-2 and stored in dH_20. In short, the method involves taking a single colony of WG5 obtained by methods mentioned in section 3.2.1.2 and transferring it aseptically into 50 mL tryptone soy broth (TSB) (Merck, Germany) and placed on a shaking incubator (100 rpm at 37°C) in an Erlenmeyer flask and grown to a concentration of 10^8 cfu/mL. 1.5 mL of this bacterial broth was added to 2.5 mL sample containing environmental phages (section 3.2.1.1). 6 mL of semi-solid tryptone yeast glucose extract agar (ssTYGA) was added to the respective mixture and poured out over a solid bottom layer of tryptone yeast glucose extract agar (TYGA). The ssTYGA was left to solidify in the petri-dishes, inverted and incubated overnight at 37°C. All plaques that formed in the ssTYGA were cut out and stored in dH_20 which served as somatic coliphage stock solution.

Concentration of this stock solution was determined by performing ISO 10705-2 (ISO, 2000) on a dilution series of the stock solution. When the final concentration of environmental phage stock was below 10^8 pfu/mL, cultivation of phages was necessary. This was done by mixing a 1 mL phage stock solution with 20 mL of TSB and 5 mL of overnight bacterial host culture. The mixture was placed into a 250 mL Erleyn-Meyer flask and agitated overnight at 100 rpm. The following day the mixture was centrifuged at 4000xg for 60 minutes, and the collected supernatant was filtered through a 0.45 µm PES membrane to remove bacterial and eukaryotic cells (Hjelmsø et al., 2017). Viruses were precipitated from the supernatant by incubation with PEG 8000 (80 g/L) and NaCl (17.5 g/L) during agitation (100 rpm) overnight at 4°C, followed by centrifugation for 90 minutes at 13000xg. The resulting viral-containing pellet was eluted in 1mL PBS and stored at -80°C until further processing. This was repeated until an ideal concentration of environmental phage was obtained.

3.2.1.4 Phage-host ARV exposure assay

A single colony of WG5 obtained by methods mentioned in section 3.2.1.2 was aseptically transferred to 50 mL tryptone soy broth (TSB) (Merck, Germany) and placed on a shaking incubator (100 rpm at 37°C) in an Erlenmeyer flask. They were grown to a concentration of 10^8 cfu/mL this was the bacterial working solution. Phages from the stock solution were suspended in sterilized TSB to a concentration of 10^4 pfu/mL this was the phage working solution. $MgCl_2$, and $CaCl_2$ was added to the phage working solution in volumes proportionate to those used in ISO 10705-2 to promote phage absorption.

An ARV, phage and bacterial host was added (in this order) into a single on the 96 well micro-plate in quantities to ensure desired ARV concentration in micro-well. The exact same mixture had 5 replicates for statistical purposes. Multi-well parameters were set at 37°C and to shake for two seconds just before the measurement taken. Measurements were taken every minute for 10 minutes, followed by a reading every 10 minutes for 50 minutes. Finally, measurements were taken every 30 minutes for a further 440 minutes. This was done for every ARV, at each of the nine (2, 4, 6, 8, 10, 20, 40, 60, 80 ng/mL) concentrations. A control with bacteria and sterilized TSB, as well as host and phage inoculated TSB, was measured alongside every concentration of every ARV. For control purposes, the concentration of bacterial host, on eosin methylene blue (EMB) agar spread plates, and phage by double agar-layer TYGA, used for each ARV at each concentration was established.

3.2.1.5 Transmission Electron Microscopy (TEM)

A single colony of WG5 obtained by methods mentioned in 3.2.1.2 was aseptically transferred to 50 mL tryptone soy broth (TSB) (Merk, Germany) and placed on a shaking incubator (100 rpm at 37°C) in an Erlenmeyer flask. After which the respective ARV and phage working solution was added to ensure the required ARV concentration. This mixture was then further incubated for 3 more hours on a shaking incubator (100 rpm at 37°C). After incubation-exposure of the phage-host mixture, in order to concentrate and purify phages enough for TEM analyses, samples were transferred to 50 mL falcon tubes and centrifuged at 11000 xg for 10 min. The supernatant was transferred to new sterile falcon tubes and 10% w/v PEG 8000 was added and left to incubate at 4°C overnight. The samples were then centrifuged for 10 min at 150 000xg and 4°C. After this ultra-centrifugation the supernatant was discarded and pellet was re-suspended in 1 mL ammonium acetate (Merck, Germany) (0.1M; pH7) and again centrifuged for 10 min at 150 000xg and 4°C. The aforementioned resuspension step was repeated twice. After the final discarding of ammonium acetate, 10 µL of MiliQ water was added to suspend phage particles in.

A drop of phage concentrated stock was applied to a 200-mesh copper-grid and left to air dry for two minutes, the excess liquid was drawn off with filter paper and the grid was allowed to dry completely. Afterwards a drop of 2%-phosphotungstic acid (aqueous), buffered to pH 6.5, was applied for 15 or 25 seconds to negative stain the background of the grid. Again, excess liquid was drawn off with filter paper and left to air dry completely. The grid was loaded into the TEM and phages were examined at 100 000-260 0009 (highest) magnification (Carey-Smith et al., 2006). Phages were visualized and images captured by A. Jordaan of the Electron Microscope Laboratory at the North-West University, South-Africa. A FEI Technai G12 High Resolution Transmission Electroscope was used at 120 kV, supported by Digital Micrograph software and a GATAN bottom mount camera.

3.2.2 *In vitro* tissue culture assays

The various cell lines were kept under their respective conditions and grown in their respective nutrient media (Table 37). Because all the cells are mammalian, they were kept at 37°C in humidified incubators supplemented with carbon dioxide, except for the androgen cell line (Table 37).

Table 37: The specific nutrient media and growth conditions for each cell line used.

Cell line	Nature of receptor	Nutrient media	Supplemented with 5% CO_2
MDA-kb2	androgen (AR)	Leibowitz-15 media, with phenol red and 10% foetal bovine serum (FBS)	No
H4IIE-*luc*	aryl hydrocarbon (AhR)	Dulbecco's Modified Eagle's Media (DMEM), with 10% FBS	Yes
HuTu-80	no receptor	DMEM, with 10% FBS	Yes
H295R	no receptor	DMEM-F12 HAM media, with 2.5% Nu-serum and 1% ITS Premix	Yes

Both the reporter gene-bioassays (MDA-kb2 and H4IIE-*luc*) were performed in white walled 96-well microtitre plates seeded with 120 000 cells/mL and 80 000 cells/mL respectively. The active ingredients lamivudine, stavudine, efavirenz, lopinavir, nevirapine and ritonavir were tested in triplicate. Six concentrations: 0.8, 8, 80, 800, 8 000, and 80 000 ng/L were tested for activating the receptor and in the case of the AR, also the inhibition of the ER. This concentration range was selected to cover the dose contained in each tablet to what has been reported in the aquatic environment of South Africa (Wood et al., 2015). Each plate also received, in triplicate, the vehicle control. The "vehicle" refers to the solvent into which the active ingredients were dissolved: methanol was the solvent for ritonavir, nevirapine, lopinavir and efavirenz, while water was the solvent for lamivudine and stavudine. Three wells acted as blank control, i.e. containing cells and their media only.

3.2.2.1 Reporter gene activation assays

When the ARVs were tested for activating the receptor, the plates also received the respective positive control: the androgen cells were dosed in triplicate with a serial dilution of testosterone (2.3 ng/mL, 0.46 ng/mL, 0.092 ng/mL, 18.4 fg/mL and 0.736 fg/mL) and the H4IIE-*luc* cells with 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD) at 480, 68.6, 9.8, 1.4, 0.2, and 0.03 µg/mL.

The MDA-kb2 cells have a second hormone receptor, the glucocorticoid receptor (GR) which might also be activated by the xenobiotic under investigation. To distinguish between AR and the GR, the activation assay must be repeated if there was activation during the first run. However, during the second run the AR receptor is blocked by a known inhibitor, flutamide. If activation is again observed, it is ascribed to successful binding to the GR and not the AR. Since there was no activation during initial, unblocked receptors, it was not necessary to repeat any activation assay and that part of the methods will not be discussed further.

The activation assays are five-day assays starting on the first day with seeding the plates and returning the plates to the incubator for 24 h (H4IIE-assay) and 48 h (androgen assay). The nutrient media of the androgen assay was supplemented with hormone-stripped FBS, to prevent false positive responses. After these incubation periods, the plates were dosed with the respective positive controls, vehicle controls, blank controls, and ARV active ingredients. The plates underwent a further incubation period—72 h for the H4IIE-assay and 48 h for the androgen assay, before the expressed luciferase enzyme was quantified.

After visual inspection of the plates, they were washed with phosphate buffered saline (PBS) supplemented with Mg^{2+} and Ca^{2+}. Lysis buffer was added to each well and the plates briefly frozen at -80°C to ensure that the cells' content is released. Upon thawing of the plates, they were placed inside a Berthold plate reader (Tristar LB941) to inject the luciferase assay reagent (LAR) and to record the luminescence. The LAR (20 mM of tricine, 1.07 mM $Mg(CO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4.7H_2O$, 0.1 mM EDTA-disodium salt, 33.3 mM dithiothreitol, 270 µM coenzyme A, 530 µM ATP and 470 µM beetle luciferin (Villeneuve et al., 1999)) was prepared in advance and stored in 50 mL aliquots at -80°C. The required volume is thawed on the day of the analysis.

Data analysis for the activation assays required that the response elicited by the tested ARV be expressed in terms of the positive control. To be able to do so, the responses by the positive are converted to a percentage of the maximum response. The responses elicited by the ARVs were also converted to a percentage of the maximum response elicited by the positive control and dose response curves are created for both control and ARV with log of the concentration on the x-axis and %control maximum on the y-axis. The straight-line equation of the dose-response curve for both control and ARV were determined in order to calculate the effects concentrations (ECs) at 80%, 50% and 20%. Relative effects potencies (REPs) were determined for 80%, 50% and 20% by dividing the EC of the positive control with the relevant EC of the ARV. By doing so the ARV's response is expressed in terms of the positive control-equivalent, enabling comparing the ARV potencies to that of the positive control, i.e. testosterone equivalents (androgen assay) or TCDD-equivalents (H4IIE-assay). All three, REP20-80 needed to be calculated because an ARV would not necessarily create the same response from the cells as the positive control, which would lead to dose-response curves with different slopes than that of the positive control. This would become evident because the REP20-80 values would be different. If the REP20-80 values are the same, it would indicate to the ARV having a similar potency as the positive control. This, however, is not often the case (Villeneuve et al., 2000).

3.2.2.2 Reporter gene inhibition assays

The inhibition assay for the androgen receptor was executed in a similar fashion as the activation assay with two exceptions: (i) the cells were seeded with a low concentration of testosterone (0.02 µg/mL) included in the nutrient medium and (ii) the negative control, flutamide, replaced the positive control of the activation assays. The flutamide concentration of 0.027 µg/mL was serially diluted four times to create another five concentrations. This negative control was not used in any quantification of inhibition, but rather to prove that the cells were responding well to a known inhibitor and that inhibition indications due to xenobiotic responses are valid.

The quantification of the inhibition effect differed from that explained for the excitation. Here, the mean of the luminescence recorded for the ARV exposure was compared with that from the solvent controlled cells. If the ratio $RLU_{ARV}/RLU_{solvent\ control}$ is smaller than 1 it indicated inhibition and the statistical significance was investigated with Mann-Whitney U test where $p < 0.05$ was regarded as significant. If there was no statistical significant difference determined, but there were repeatedly observed differences a test for practical significance was conducted (Ellis and Steyn, 2003).
This was done because statistical significance does not necessarily represent an important result in practice, however the practical significance (effect size) shows if there is a large enough effect in practice. To calculate the effect size the following formula was used (Ellis and Steyn, 2003):

$$r = \frac{z}{\sqrt{n}} \qquad [1]$$

where r = effect size; z = z-score provided by statistical software packages when a Mann-Whitney U-test is performed, and n = sample size. If r is less than or equal to 0.2 it is considered to have a small effects size. When $0.2 < r < 0.4$ it is considered to have a medium effect size and if $r > 0.4$ it has a large effect size. In this study, data with a large effect size was considered to have practical significance (Ellis and Steyn, 2003).

3.2.2.3 Viability assay for the reporter gene assays

A viability assay was run in parallel to the reporter gene assays to ensure that the dosed compounds had not interfered with the survival and growth of the cells. When noticing a decrease in light due to exposure, it could have been due to a decreased viability of the cells and not blocked AR receptors. The method is a colorometric assay based on the principle that viable cells metabolise yellow 2-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) solution to a dark blue formazan crystal (Mosmann, 1986).

A 96-well transparent micro titre plate was seeded and dosed in exactly the same manner as the reporter gene assays and underwent the same incubation periods. On the final day, the media were removed, the cells washed with PBS and treated to 0.5 mg/mL MTT in the nutrient media of the cells. After a 30 min incubation period at 37°C, the MTT solution was replaced with dimethyl sulphoxide to dissolve the blue formazan crystals. Absorbance was quantified at 560 nm in the plate reader and compared with that of control cells (unexposed cells). Viability was expressed as a percentage of the absorbance of exposed wells to the absorbance of the unexposed cells. Again, statistical significance of a difference was determined with the non-parametric Mann-Whitney U test.

3.2.2.4 Steroidogenesis assay

This assay was performed in transparent 24-well plates and each was seeded with 300 000 cells/mL (Hecker and Giesy, 2008). After a 24 h-incubation period the cells were dosed with controls and ARV active ingredients. Exposures were repeated in triplicate as for the reporter gene assays. A quality control (QC) plate was included in each experiment and served as an internal control for the hormone levels and the cell growth. The QC plate included six blank control wells, six vehicle control wells, triplicate wells of 1 µM and 10 µM forskolin, a known inducer of testosterone and oestradiol and three wells each of 0.3 µM and 3 of µM prochloraz, known inhibitors of the same two hormones (Nielsen et al., 2012).

After another 48 h incubation period, the media was removed from the wells and extracted for oestradiol and testosterone: The media (500 µL) was diluted with an equal volume of double distilled water containing 10 µL 0.14 Ci/mmol radio-active testosterone (3H-T) with which extraction efficiency was determined. This 1 mL aqueous medium was extracted twice with 5 mL anhydrous diethyl ether. The 10 mL ether was evaporated to near dryness under a gentle stream of nitrogen gas and reconstituted to 250 µL ELISA wash buffer. Extraction efficiency was determined by measuring the radio-active decay of the 3H-T in 10 µL of the reconstituted extract in 5 mL scintillation cocktail for two minutes recording the counts-per-minute (CPM). This was compared to the CPM of 10 µL 0.14 Ci/mmol 3H-T (stock) in 5 mL scintillation cocktail. Background CPM due to the cocktail was first subtracted before the percentage CPM was calculated (See equation):

$$\frac{(\text{CPM of sample - CPM of background}) \times \text{dilution factor}}{\text{CPM of stock - CPM of background}} \times 100 \qquad [2]$$

The dilution factor was 25.

The concentrations of the hormones produced by the cells were measured using 17β-oestradiol and testosterone specific competitive ELISA kits (IBL International: RE52041 and RE52151 respectively). Samples and hormone standards were added to the ELISA plate according to the manufacturer's instructions and absorbance measured at 450 nm in the plate reader. The concentrations of the hormones in the extracts were determined with the aid of the calibration curves and corrections were made for the extraction efficiencies. The concentrations of the hormones from the wells that received ARVs were compared to the respective concentrations produced by the unexposed cells and fold changes calculated. The significance of the fold changes was tested with Mann-Whitney U test ($p < 0.5$) as well as the effect size calculated.

3.2.2.5 Cytotoxicity of ARVs on human intestinal cells.

HuTu-80 cells were seeded at 80 000 cells/mL in transparent 96-well microtitre plates to investigate the toxicity of the active ARV ingredients. The exposure concentrations 1, 2, 4, 6, 8 and 10 mg/mL represented the prescribed concentrations and environmental levels of ARVs reported in previous studies (Thein et al., 2014). Three exposure periods were investigated: 12 h, 24 h, and 36 h. The different exposure periods were chosen to be comparable to other known toxicity tests. The MTT viability assay as previously described was used here.

3.2.3 Snail exposures

Fresh water snails of the *Bulinus tropicus* species were collected from the Potchefstroom district in the North West Province of South Africa (Figure 22). Certain species of the genus *Bulinus,* is often associated with parasitic infection and can act as the intermediate hosts for *Schistosoma* species. *B. tropicus* is also the most prominent intermediate host for *Calicophora microbothrium,* (de Kock et al*.,* 2002; de Moor and Day, 2007). In order to ensure that test subjects or exposures were not infected, they were laboratory cultured for several generations prior to exposure testing.

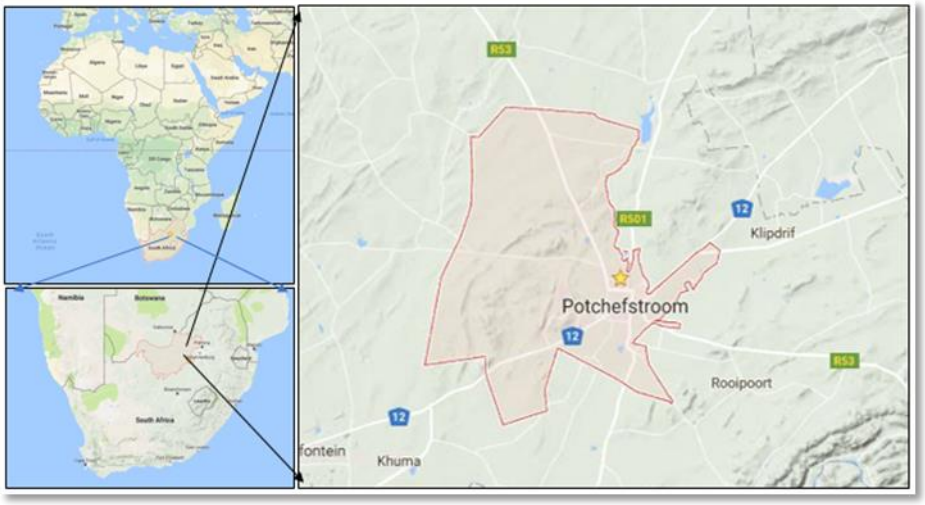


Figure 22: Map of Potchefstroom district, South Africa.

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Snails were cultured in synthetic water, as adopted from the International Organization of Standards (ISO, 2012), where known concentrations of calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$), magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), potassium chloride (KCl), and sodium hydrogen carbonate ($NaHCO_3$) where dissolved in ultra-pure distilled water (ELGA) to produce the ionic equivalent of environmental conditions (Figure 23). Each container was aerated with filtered air by means of glass pipettes. Snails were fed with commercial, Tetra Pro Algae fish food every third day and fluorescent light conditions were set on a 12h cycle. During the course of the various experiments, the water temperature was maintained at 25°C$\pm$1°C (de Kock et al*.,* 1986) and all test subjects were exposed to the corresponding laboratory conditions for the duration of exposures.

The method applied was adopted from (Minnaar, 2014) with minor alterations. Two sets of antiretroviral exposures were performed. One for the NRTI (lamivudine/stavudine) and one for the NNRTI (efavirenz/nevirapine). Roughly five reproductively active individuals were placed into separated Consol® glass containers under equivalent conditions for acute testing. These containers were monitored until two large egg clutches (comprising of ≥ 26 embryos per container) were laid. Egg clutches were only accepted when both were laid within the same time frame (few h). After which the reproductively active individuals were removed. Exposure concentrations series consisted out of: 0.005, 0.5, 50, and 5000 µg/L for lamivudine, stavudine, and nevirapine respectively. For the efavirenz exposures three different sets of concentrations were tested in replicated form, this was due to a limited amount of bioactive compound available for testing at the time. Exposure concentrations for efavirenz therefore consisted out of 0.005, 0.5, and 50 µg/L. These concentrations were all made up into 0.9 L of the ISO synthetic water.

Replicated water controls were also performed containing no bioactive antiretroviral compounds, as well as methanol controls to account for the methanol solubility of efavirenz (0.89 µg/L) and nevirapine (0.31 µg/L). Efavirenz exposures where first made up in a methanol stock solution and then diluted in ultra-pure distilled water. Each container was replaced with new water and specific exposures dose every third day. In accordance to literature the mean elimination half-life of efavirenz is 52-76h after a single dose and 45h for nevirapine (Li and Feng, 2012), although the mean elimination half-life for lamivudine is 5-7h and only 2.3h for

stavudine. Egg clutches were then monitored for four weeks by taking daily photos with a ProScope High Resolution microscope. Embryonic development was determined by monitoring embryo length up until the length at which the shell could be measure. According to Hickman et al. (2006), the length of a mollusc's shell will increase correspondingly with an increase in snail body mass. ImageJ software was used to measure from the edge of the opening to the apex of the shell. Calibration of each image was completed by means of ruler with 0.5 mm increments. Embryos were therefore measured according to each day's developmental progress. Those which displayed a clear lack of development (failure to increase in embryo length), failure to move when initiated, perceivable colour alteration as well as substantial algae overgrowth after successive days of monitoring, was noted as a sign of embryo mortality.

Figure 23: (a) Illustration of glass containers with various antiretroviral exposures (b) reproductively active adult snails of the *Bulinus tropicus* species (c)

3.2.3.1 Data analysis

Statistical interpretation of the data was performed by using Graphpad Prism version 5. Non-parametric tests were run for comparisons between the mean values of the various ARV exposures, water and methanol controls. The Kruskal-Wallis test was then used followed by Dunn's multiple comparison test. Statistical significance was measured at a $P<0.05$. Linear regressions were also performed for the various exposures, where no r^2 value lower than 0.7 was accepted.

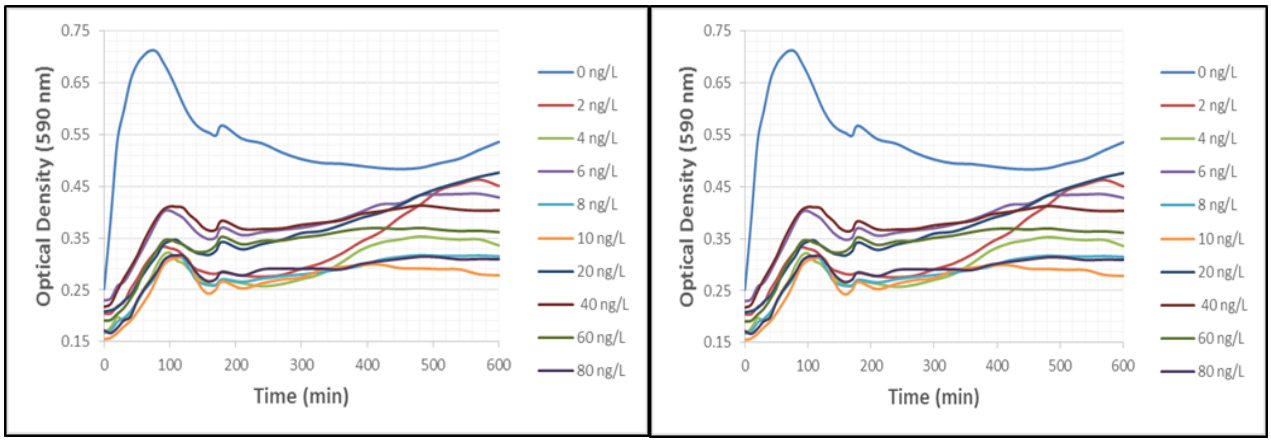
3.3 RESULTS

3.3.1 Potential impacts of ARVs on bacteriophages

This section contains quantitative and visual results of phage activity and survival after exposure to ARVs of classes NNRTI, NRTI, and PI.

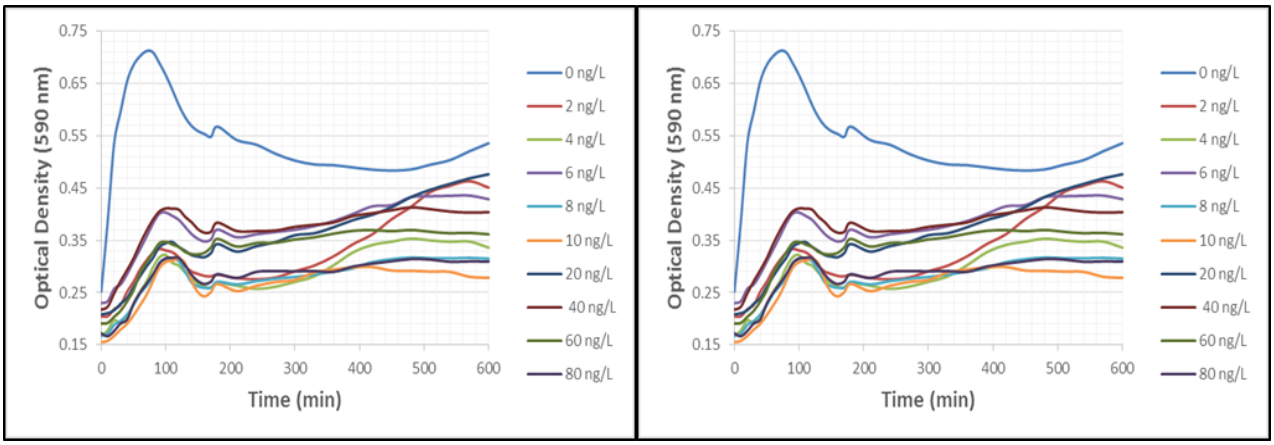
3.3.1.1 Phage-host ARV exposure study

This section contains quantitative results showing the increase/decrease of phage and host activity under exposure to various ARVs. An increase in optical density (OD) represents growth of bacteria WG5. A decrease in OD represents the lysis of WG5 due to phage activity.



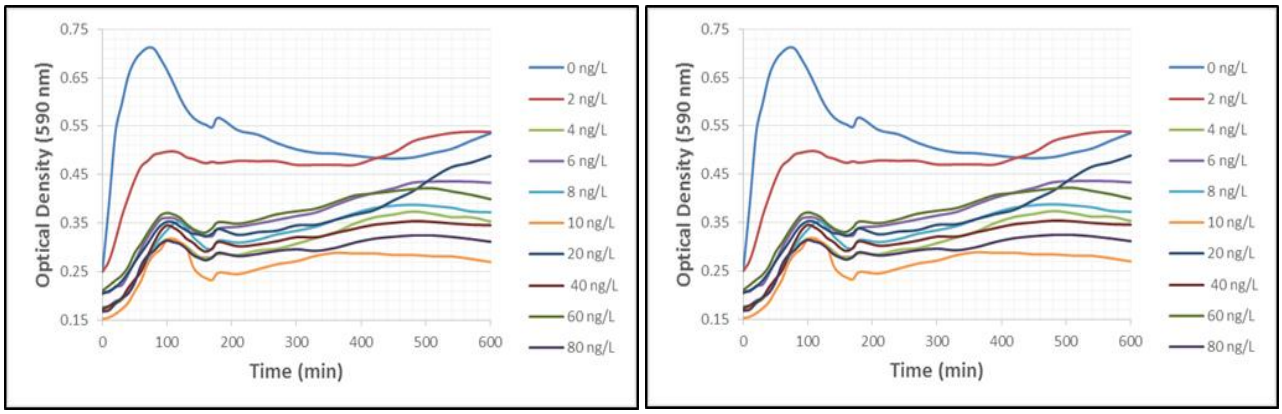
A B

Figure 24: OD results indicating death and growth of phage-host mixture while in the presence of NNRTI class ARVs of different concentrations. The effects of efavirenz exposure is represented in graph A and nevirapine in graph B.



C D

Figure 25: OD results indicating death and growth of phage-host mixture while in the presence of NRTI Class ARVs of different concentrations. The effects of stavudine exposure is represented in graph C and lamivudine in graph D.



E F

Figure 26: OD results indicating death and growth of phage-host mixture while in the presence of PI Class ARVs of different concentrations. The effects of lopinavir exposure is represented in graph E and ritonavir is represented in graph F.

There was no statistically significant or visible significant difference between the two different ARVs in each class (Figure 24 A and B) (Figure 25 C and D) (Figure 26 E and F). Neither was there a statistical significance between the control (0 ng/L) and the other ARV concentrations, however, there was a visible difference in the speed at which bacterial growth peaked in the control (70 min) and the rest (100-120 min). As well as the slope indicating lysis of bacteria due to phage activity is visibly steeper in the control as opposed to the other concentrations tested.

There is no statistically significant difference between the result of NNRTI, NRTI and PI. Visibly, OD results for the NNRTI (Figure 24) and NRTI (Figure 25) study showed more similarity than when visually compared to OD growth curves of PI (Figure 26) exposure study. The difference between the PI exposure study of 2 ng/L compared to the other PI concentrations and the 2 ng/L OD results of the NNRTI and NRTI assayed, was practically, yet, not statistically significant.

The control (0 ng/L) reached its peak 20 to 40 minutes before any of the samples containing ARV active compounds. Trends were similar to their controls indicating no direct effect on bacteriophages, but rather an effect on the growth/metabolic activities of the bacteria itself. There is no statistically significant difference between the increase of OD in the first 120 minutes (according to x^2 test), however there is an observable difference that should not be disregarded. Two lytic cycles can be observed, the first starting at 70 minutes for 0 ng/L ARV and in the presence of ARVs it starts only after 90-120 minutes, although this delay is also not statistically significant. There is a difference between the presence and absence of NNRTI ARVs. Bacterial growth starts to increase exponentially for 2 ng/L after 260 min and after 240 min for the 4 ng/L. The growth for the 2 ng/L stopped after 500 min and for the 4ng/L after 400 min. The other concentrations showed a linear increase in OD with the angle of 20 ng/L being the most substantial.

3.3.2 *In vitro* tissue culture assays

The (anti-)androgenic effects (MDA-kb2):

There was no evidence of activation in the MDA-kb2 cell line (Figure 27). The positive control, testosterone created a complete dose-response curve (left on Figure 27) showing that the AR was responding as it should. Also, none of the ARVs were cytotoxic to the MDA-kb2 cells at the investigated concentrations (MTT viability assay result not shown).

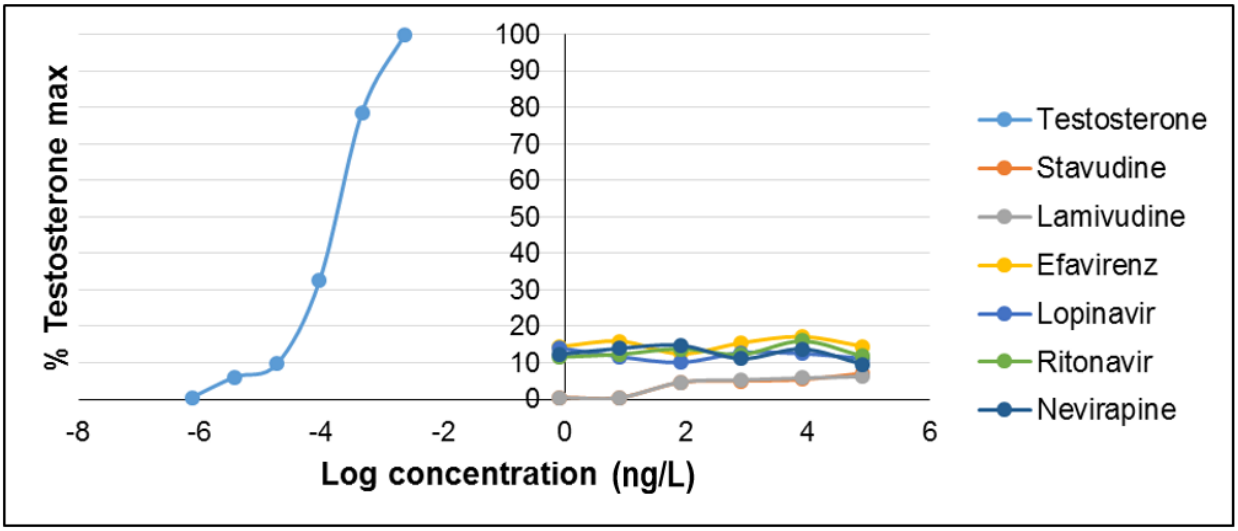


Figure 27: Comparison of AR activation from exposure to testosterone (positive control) and to the ARVs.

There was evidence of the ARV active ingredients inhibiting the AR receptor. None of the inhibitions were statistically significant, however, some where practically significant.

Figure 28 shows the inhibition of the two ARVs stavudine and lamivudine, with the same mechanism of action namely that of nucleoside and nucleotide reverse transcriptase inhibitors.

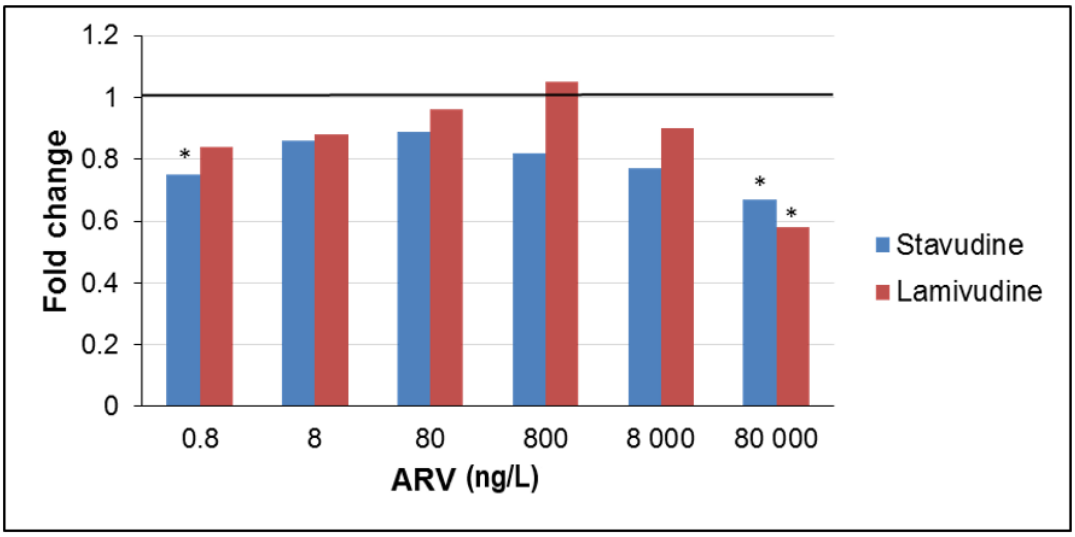


Figure 28: Fold change of stavudine and lamivudine at increasing concentrations. Asterisks indicate high effect size (r > 0.4) of the ARV exposed cells compared to the SC. The black line at 1 indicates the value of the SC.

Cells exposed to stavudine, at all concentrations tested, produced less light than that of the SC, meaning that exposure to all concentrations of stavudine inhibited the AR (only 0.8 and 80 000 ng/L had a large practical significance). Lamivudine showed inhibition at the 80 000 ng/L (the highest concentration tested) with a high practical significance. There was one instance of slight activation at 800 ng/L, but this was not supported by the activation assay and it was not practically significant.

Of the two protease inhibitors, lopinavir and ritonavir that were investigated it was ritonavir that did not show any inhibition (Figure 29). Lopinavir seemed to have had some activation evidence and even though it seemed to have been practically significant for the four lowest concentrations, this was not confirmed by the activation assays. The highest concentration lopinavir caused a practical significant inhibition.

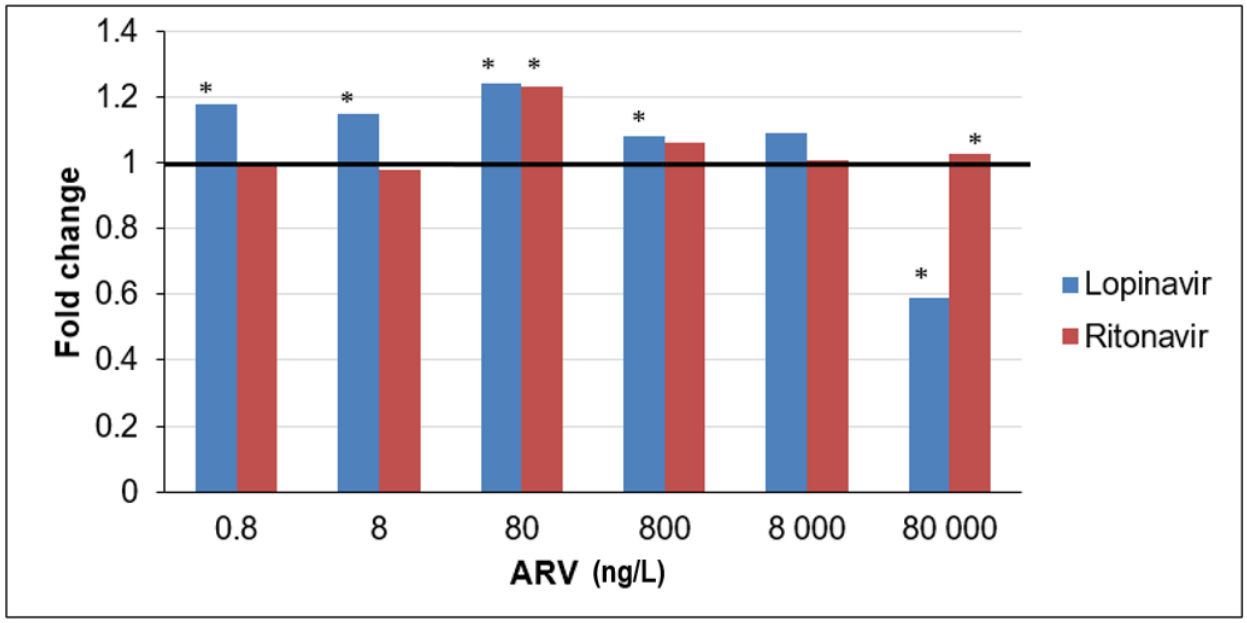


Figure 29: Fold change of lopinavir and ritonavir at increasing concentrations. Asterisks indicate high effect size (r > 0.4) of the ARV exposed cells compared to the SC. The black line at 1 indicates the value of the SC.

Efavirenz and nevirapine are non-nucleoside reverse transcriptase inhibitors and their inhibition is presented in Figure 30. Efavirenz showed dose-dependent inhibition with increasing inhibition as the concentration increased. This effect was practically significant at all the tested concentrations except for 80 ng/mL. Nevirapine showed inhibition at the lowest two concentrations that decreased as the concentrations increased and again experienced inhibition at the highest concentration (Figure 30).

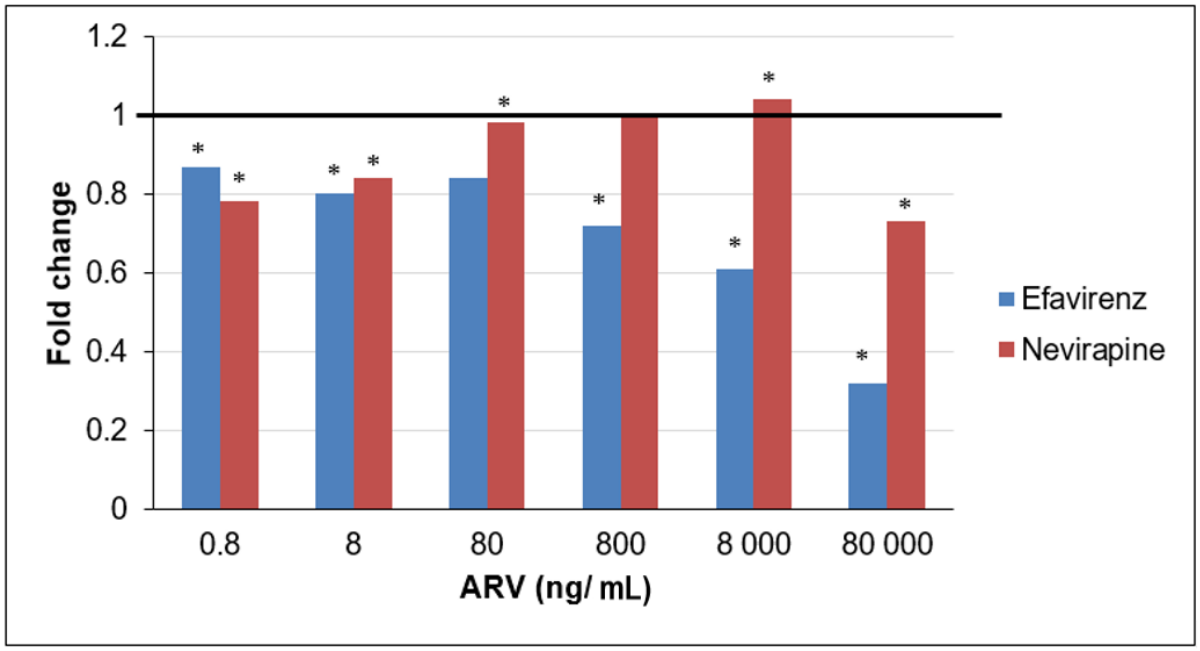


Figure 30: Fold change of efavirenz and nevirapine at increasing concentrations. Asterisks indicate high effect size (r > 0.4) of the ARV exposed cells compared to the SC. The black line at 1 indicates the value of the SC.

The above results presented AR inhibition evidence for five of the six ARV active ingredients at least for some of the tested concentrations. It was only ritonavir that did not have any significant inhibition effect at the targeted concentrations.

Activation of the AhR (H4IIE-luc):

The rat hepatoma cells used for this assay also were not negatively affected by the concentrations of the ARV active ingredients tested for binding to the AhR (data not shown).

Results show that the ARVs were inactive towards the AhR because no dose-response curves were obtained for any of the ARVs tested (i.e. lamivudine, stavudine, efavirenz, lopinavir, nevirapine, ritonavir) (Figure 31).

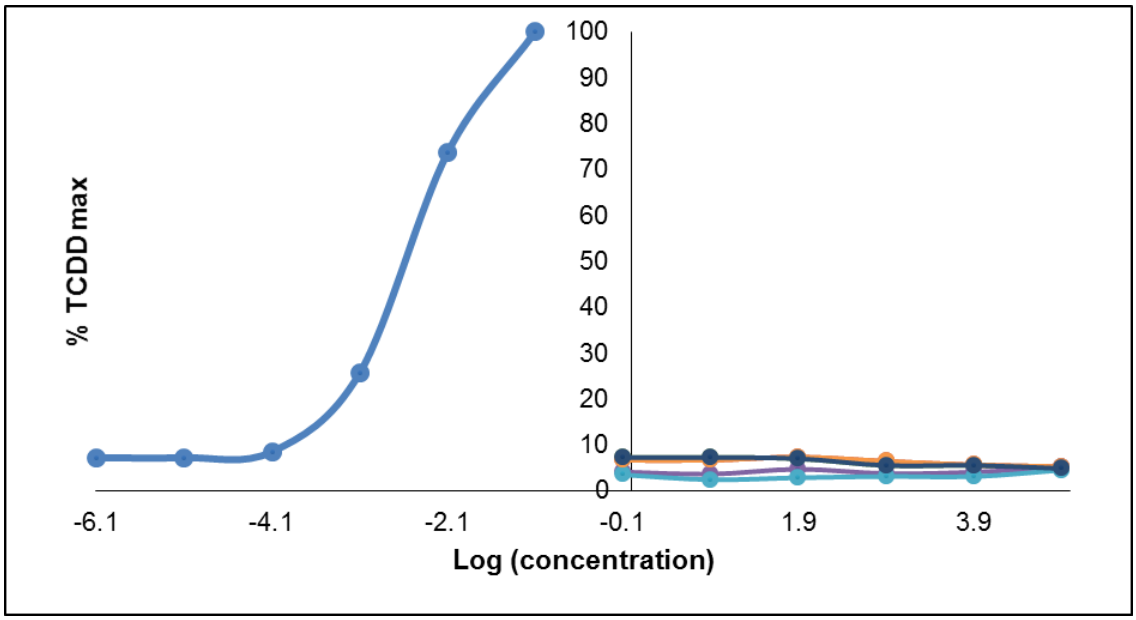


Figure 31: Responses of H4IIE-luc rat hepatoma cell assay to selected ARVs as TCDD. Doses expressed as a percentage of the maximum response observed for the TCDD standard (%-TCDD-max).

There was no significant AhR-mediated activity up to the highest concentration tested (lines on the right in Figure 31) whereas its well-known ligand, 2,3,7,8-TCDD created a full dose-response curve (on the left in Figure 31). It is clear from the results that the ARV active ingredients did not activate the AhR in the test cells, however it is known that some of them can cause the expression of other CYP enzymes, like CYP2B6, and CYP3A4 by both ritonavir and nevirapine (Terelius et al., 2016). The form of the CYP enzyme in these cells is CYP1A1, which is clearly not induced to be expressed by these xenobiotics.

3.3.2.1 H295R steroidogenesis assay:

3.3.2.1.1 *Lamivudine:*

After exposure to different concentrations of lamivudine, T production relative to the SC, showed a decline of T-levels at all concentrations, except the lowest (0.8 ng/L) which caused a practically significant increase in T (Figure 32). The five highest concentrations that caused inhibition were practically significant except for the 800 ng/L. Oestradiol levels were decreased at 8, 80, 8 000 and 80 000 ng/L lamivudine and increased at 0.8 and 800 ng/L (Figure 32). The 800 ng/L concentration did not fit the decline in the E2 levels of its neighbours (Figure 32), and probably is an artefact. This phenomenon warrants a repeated investigation. The 0.8 ng/L lamivudine—as for T—caused an increase, but it was not practically significant.

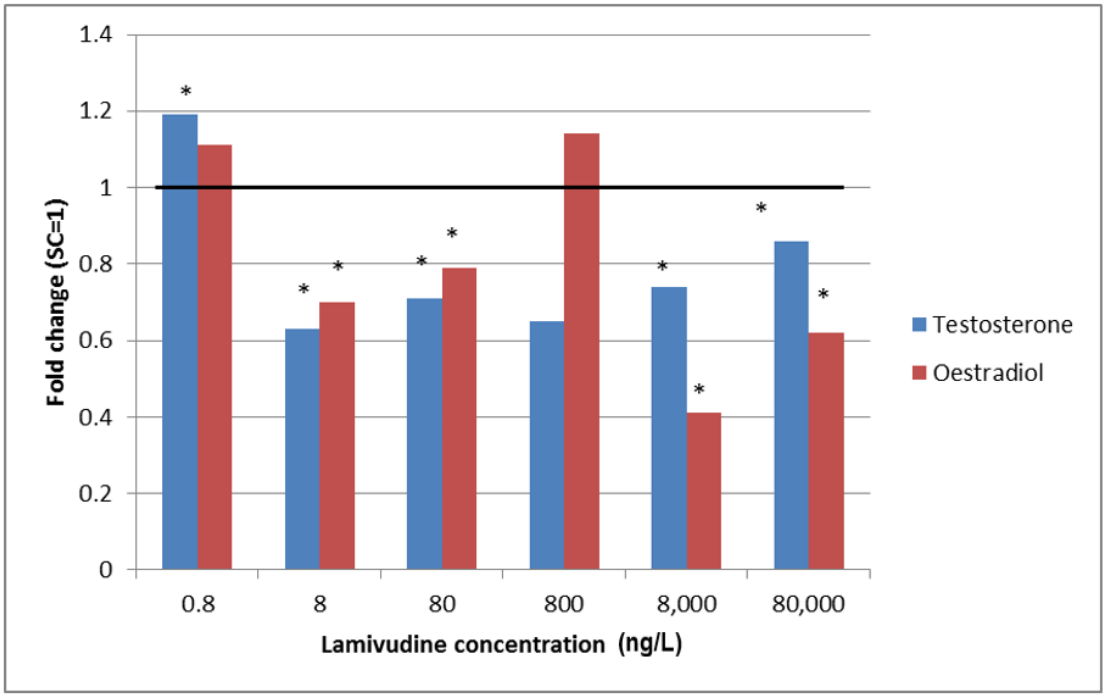


Figure 32: The effect of lamivudine on T and E2 production in exposed H295R cells. T and E2 is expressed as fold changes compared to solvent control (SC=1). Large practical significant changes (r > 0.4) are indicated by an asterisk (*). Fold change > 1 = induction, fold change < 1 = inhibition.

3.3.2.1.2 *Stavudine:*

Stavudine decreased T levels at all concentrations; and all, except 0.8 ng/L, caused a practically significant decrease that did not seem to be dose related (Figure 33).

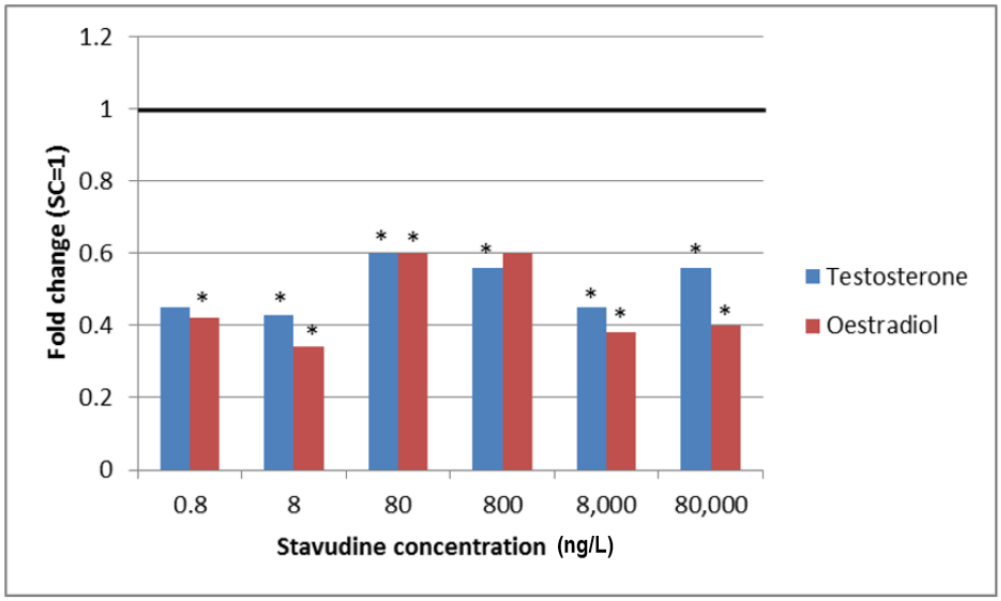


Figure 33: The effect of stavudine on T and E2 production in exposed H295R cells. T and E2 is expressed as fold changes compared to solvent control (SC = 1). Large practical significant changes (r > 0.4) are indicated by an asterisk (*). Fold change > 1 = induction, fold change < 1 = inhibition.

Oestradiol levels decreased practically significant at all but one concentration (800 ng/L) of stavudine (Figure 33).

3.3.2.1.3 *Ritonavir*

Ritonavir caused practically significant inhibition of T production at all concentrations tested; there were also no dose-dependent response (Figure 34). Oestradiol levels were decreased for some ritonavir concentrations and increased for others. However, there was no apparent pattern in this. Four concentrations ritonavir: 0.8, 80, 8 000 and 80 000 ng/L caused a practically significant decrease in E2 concentrations, where 8 and 800 ng/L apparently caused an increase in E2 (Figure 34). The latter might also be an artefact, and these exposures should be repeated.

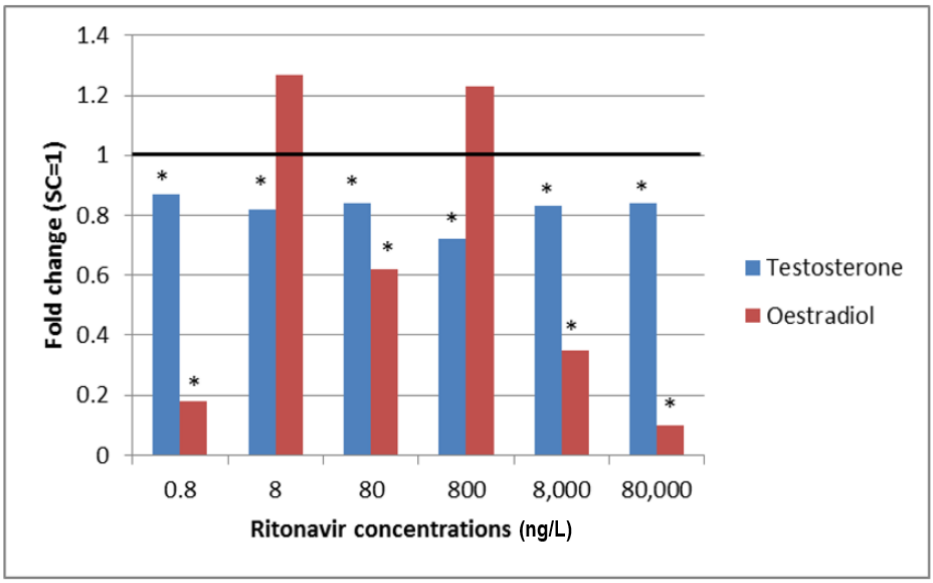


Figure 34: The effect of ritonavir on T and E2 production in exposed H295R cells. T and E2 is expressed as fold changes compared to solvent control (SC = 1). Large practical significant changes (r >0.4) are indicated by an asterisk (*). Fold change > 1 = induction, fold change < 1 = inhibition.

3.3.2.1.4 *Lopinavir:*

Lopinavir practically significantly abated T levels at all concentrations tested. Testosterone production showed no definite dose-dependent response (Figure 35). Oestradiol levels reduced dose-dependently with increased lopinavir concentrations response, with the highest concentrations showing more effect than the lower concentrations.

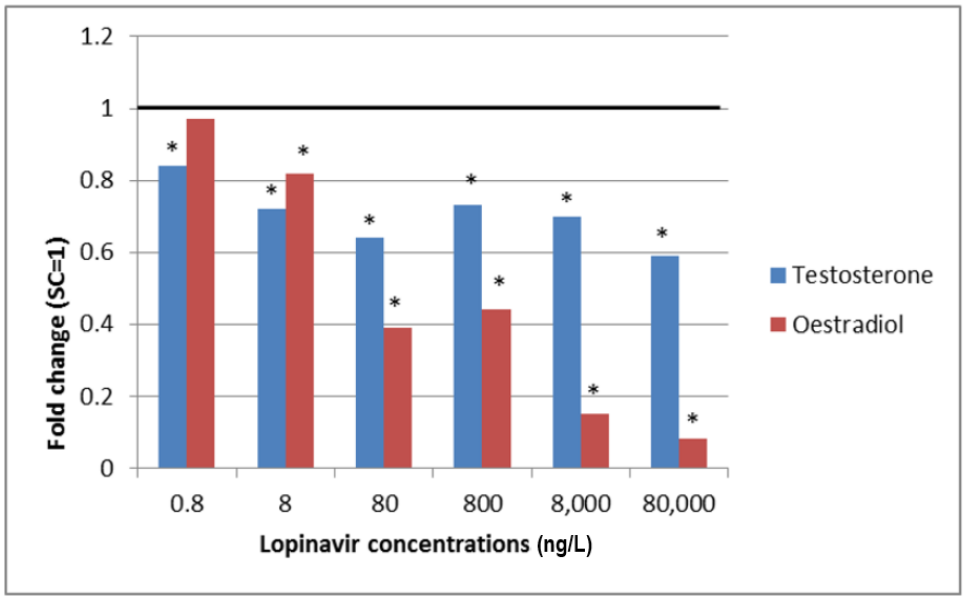


Figure 35: The effect of lopinavir on T and E2 production in exposed H295R cells. T and E2 is expressed as fold changes compared to solvent control (SC = 1). Large practical significant changes (r > 0.4) are indicated by an asterisk (*). Fold change > 1 = induction, fold change < 1 = inhibition.

3.3.2.1.5 *Efavirenz:*

Efavirenz induced a partial dose-dependent response for both testosterone and oestradiol. The fold change of testosterone had large practical significance at 0.8, 80, 8 000 and 80 000 ng/L, and medium practical significance at the remaining concentrations (Figure 36). The testosterone concentrations increased after exposure to the lowest three concentrations (Figure 36), was not changed by the 800 ng/L and was decreased at the two highest exposure concentrations. The E2 concentrations showed an approximation of the changes observed in the T levels. Oestradiol production showed a large practical significance at only two concentrations, 8 and 80 000 ng/L. Medium practical significance can be seen at 0.8 and 800 ng/L and small significance at 80 and 8 000 ng/L. The highest concentration (80 000 ng/L) caused large practical significance for both testosterone and oestradiol decrease (Figure 36).

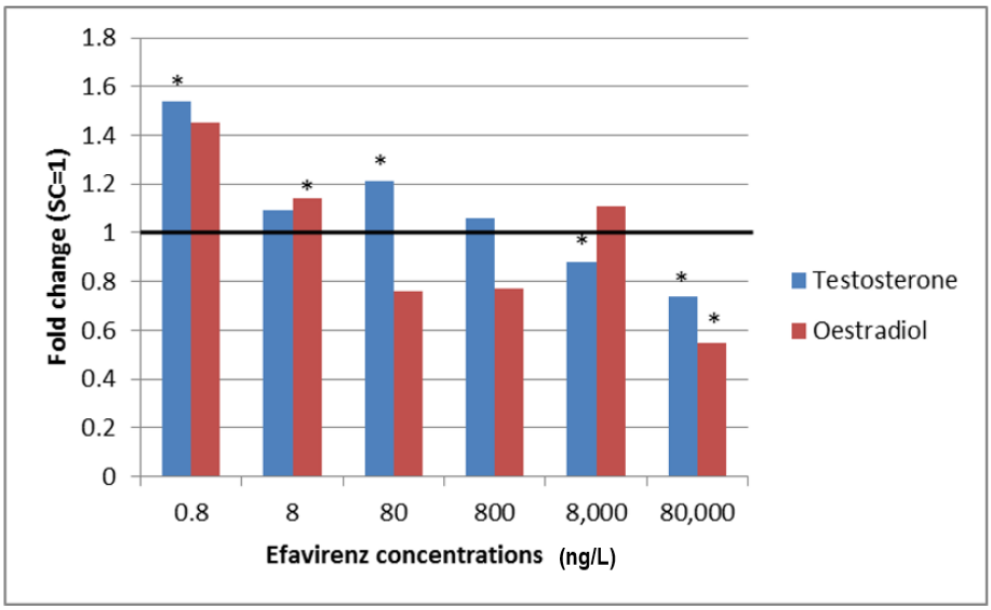


Figure 36: The effect of efavirenz on T and E2 production in exposed H295R cells. T and E2 is expressed as fold changes compared to solvent control (SC=1). Large practical significant changes (r>0.4) are indicated by an asterisk (*). Fold change > 1 = induction, fold change < 1 = inhibition.

3.3.2.1.6 *Nevirapine:*

Nevirapine exposure created a lowered T production at all the exposure concentrations except the highest 80 000 ng/L (Figure 37). The fold changed in the lowest five concentrations were practically significant. The E2 concentration changed in response to nevirapine exposure but the changes did not show a clear pattern (Figure 37).

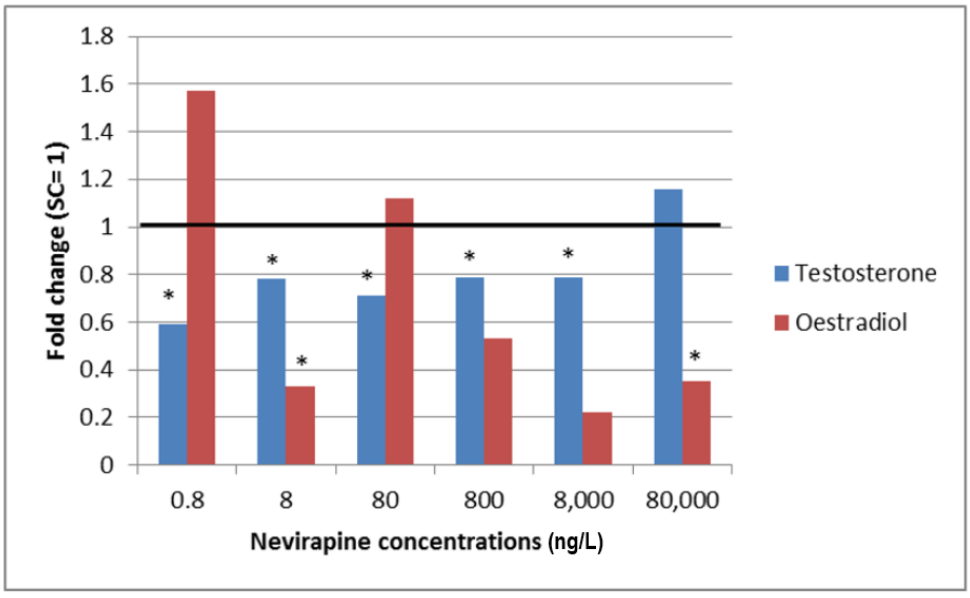


Figure 37: The effect of nevirapine on T and E2 production in exposed H295R cells. T and E2 is expressed as fold changes compared to solvent control (SC = 1). Large practical significant changes (r > 0.4) are indicated by an asterisk (*). Fold change > 1 = induction, fold change < 1 = inhibition.

3.3.2.2 HuTu cytotoxicity assay:

The results obtained for exposed HuTu-80 cells were grouped according to the ARV classes. This enabled us to investigate effects caused by ARVs with the same working mechanism.

3.3.2.2.1 *Lamivudine and stavudine*

These two compounds did not significantly change the viability of the human intestinal cells at any of the exposure concentrations and the exposure periods.

3.3.2.2.2 *Efavirenz and nevirapine*

Although here too there was no statistically significant changes to the viability of the cells at the different concentrations and periods, there was a slight tendency for efavirenz to decrease viability at increasing concentrations and at longer exposure periods (Figure 38).

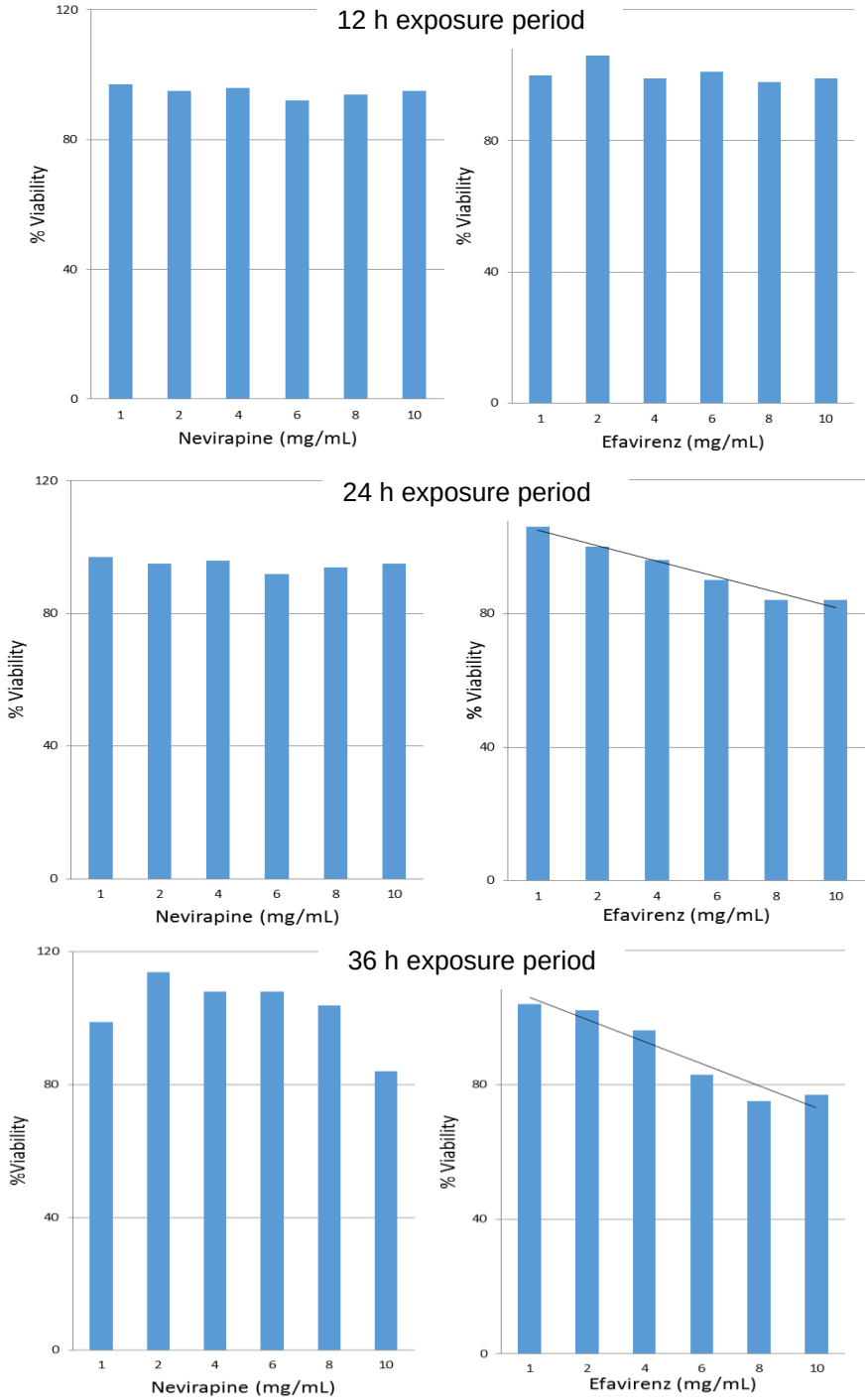


Figure 38: Percentage viability of the HuTu-80 cells after exposure to nevirapine and efavirenz (NNRTIs), after 12 h, 24 h, and 36 h.

3.3.2.2.3 *Lopinavir and ritonavir*

These two compounds did not significantly change the viability of the human intestinal cells at any of the exposure concentrations and the exposure periods.

3.3.3 Snail exposures

3.3.3.1 Efavirenz

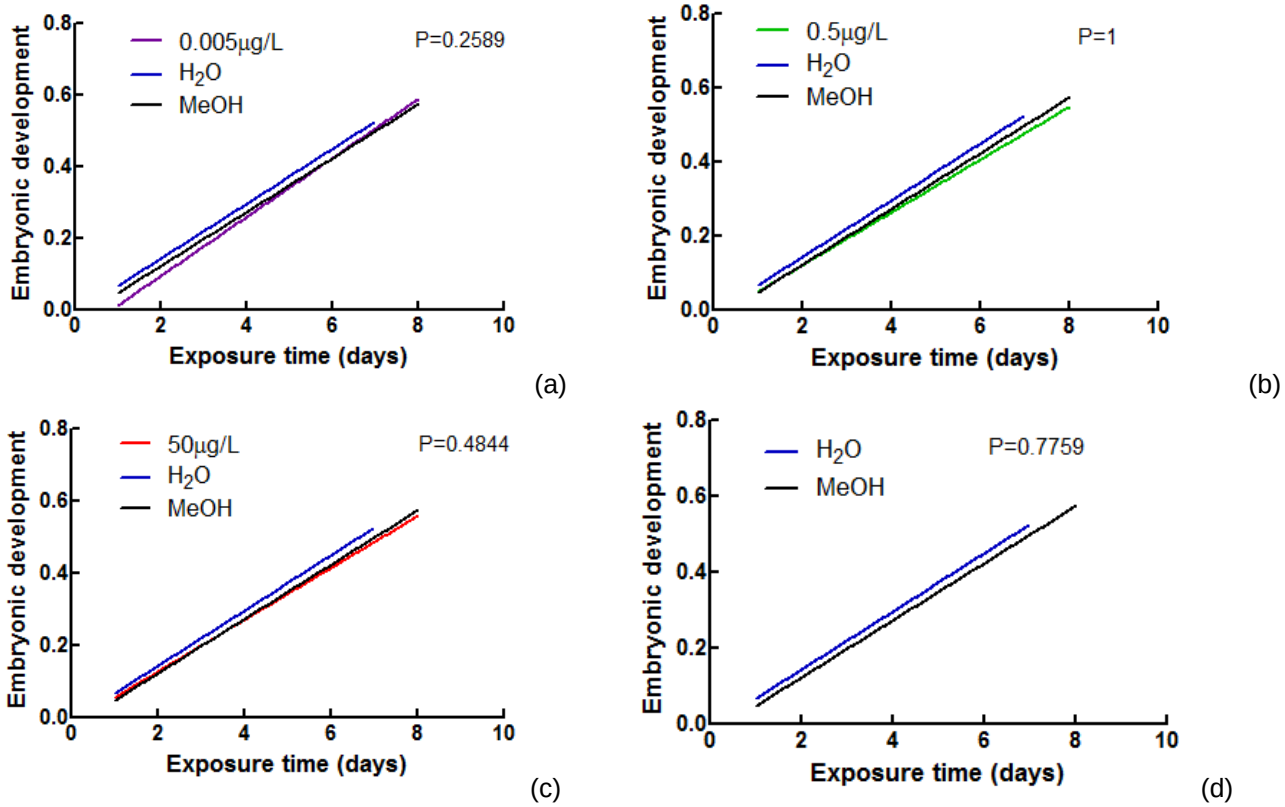


Figure 39: Linear regression were performed to determine whether exposure to efavirenz at various concentrations had an influence on the embryonic development of *B. tropicus* after several days. Exposure concentrations in comparison with controls are depicted as follow: (a) 0.005 µg/L, (b) 0.5 µg/L and (c) 50 µg/L. Graph also (d) depicts the comparison between the water and methanol controls.

All four graphs of the linear regressions of Figure 39 illustrated no statistically significant differences between the slopes when tested with a linear line F-test, as can be seen by the derived p-values on the various graphs. Individual F-tests for the various efavirenz exposures against the water and methanol control, respectively, were performed and revealed not to be statistically significant. These linear regressions portray the general growth trend of *B. tropicus* embryos, therefore where the regression lines stop, it is indicative of hatching and snails entering a new developmental phase. As can be seen in Figure 39 the water control displayed earlier hatching. The differences between elevations were also found to be statistically significant for (a) 0.005 µg/L (p=0.0092), (b) 0.5 µg/L (p=0.0001), and (c) 50 µg/L (p=0.0257) and (d) (p=0.0092). Figure 39 (a) and (b) revealed higher elevations for the water control than the 0.005 and 0.5 µg/L efavirenz exposures, while Figure 39 (c) revealed a higher elevation for the 50 µg/L efavirenz exposure than that of the controls.

3.3.3.2 *Nevirapine*

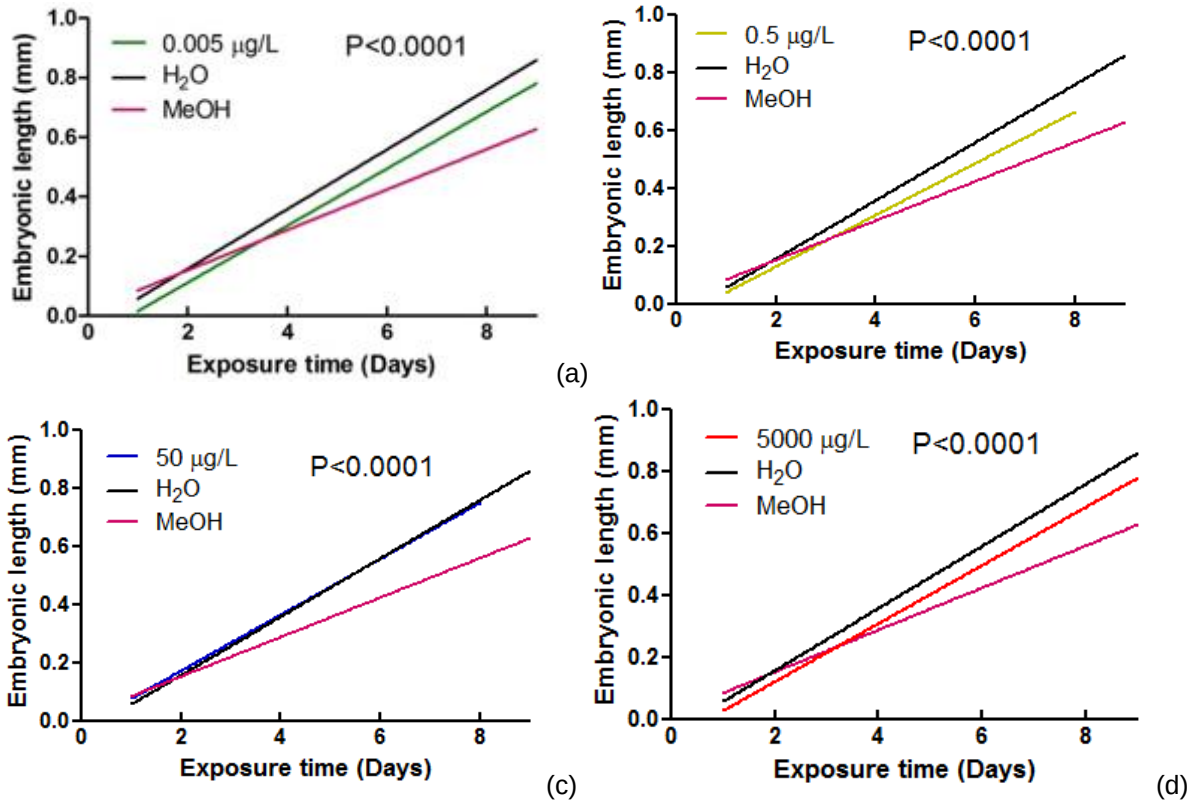


Figure 40: Linear regression of various concentrations of nevirapine exposure on embryonic development after several days. Exposure concentrations in comparison with controls are depicted as follow: (a) 0.005 µg/L, (b) 0.5 µg/L, (c) 50 µg/L and (d) 5000 µg/L.

All four graphs of Figure 40 illustrated statistically significant differences between the slopes when tested with a linear line F-test, as can be seen by the derived p-values on the various graphs. The 50 µg/L (c) and 0.5 µg/L (b) nevirapine exposures displayed earlier embryo hatching than that of either controls. The general trend of the nevirapine linear regressions are indicative of an inhibitory effect when compared to the water controls, although the methanol control seemed to exhibit a further deleterious effect on the embryonic development and growth of *B. tropicus,* with further inclined slopes.

3.3.3.3 *Lamivudine*

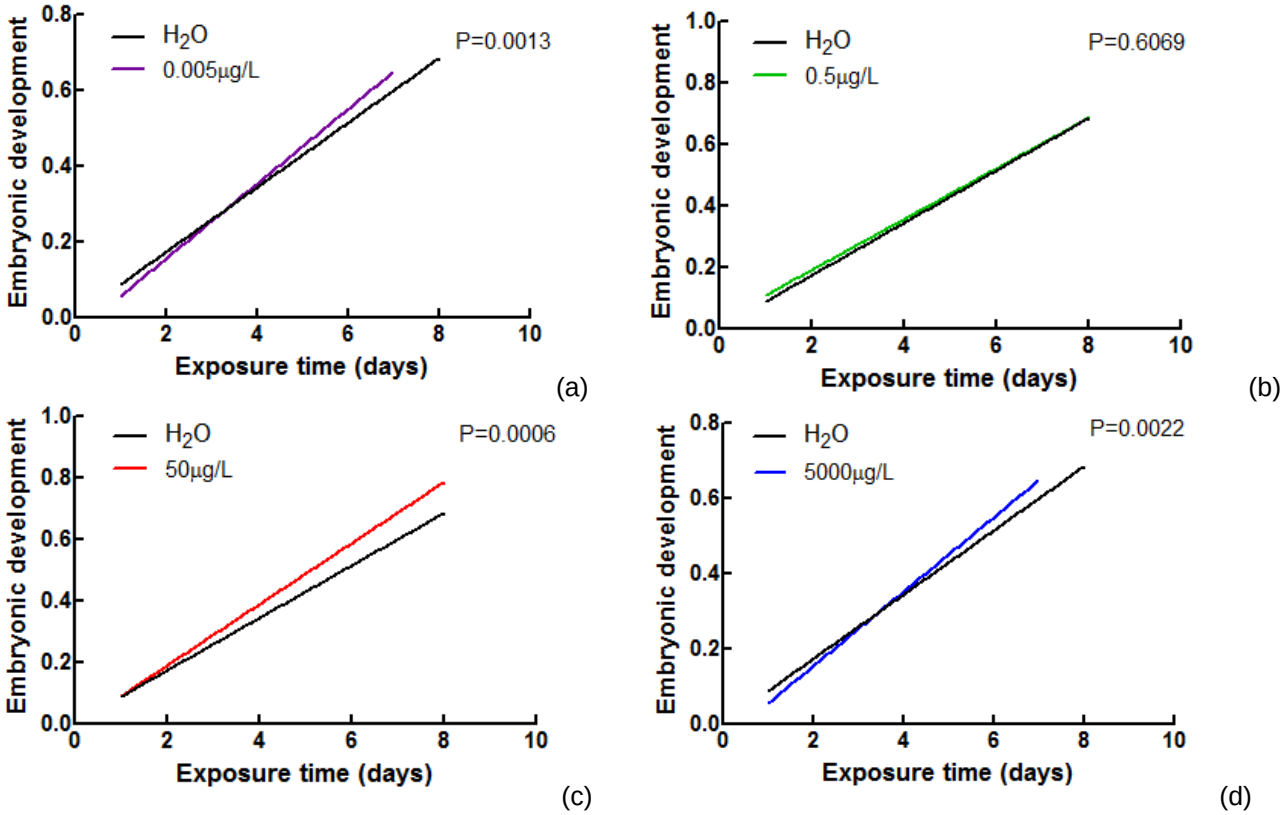


Figure 41: Linear regressions were conducted to determine whether embryonic development after several days was influenced by lamivudine exposures at either a concentration of: (a) 0.005 µg/L, (b) 0.5 µg/L, (c) 50 µg/L, or (d) 5000 µg/L in comparison with a water control.

The linear regressions of lamivudine (Figure 41) revealed that the differences in slopes for the 0.005, 50, and 5000 µg/L exposures were statistically very significant when tested with a linear line F-test. This can be seen by the p-values on the various graphs. However, the 0.5 µg/L exposure revealed not to be statistically significant regarding the difference in slopes. The lamivudine exposure concentrations for Figure 41 (a) and (d) therefore displayed earlier embryo hatching (24h). Both exposures start with elevations below that of the control but slopes cross (after about three days) and eventually represent slopes with steeper inclines than that of the water control. Figure 41 (c) revealed the greatest divergence in slopes. This 50 µg/L lamivudine exposure therefore represents an extremely statistically significant difference (p=0.0006) in slopes compared to that of the water control.

3.3.3.4 Stavudine

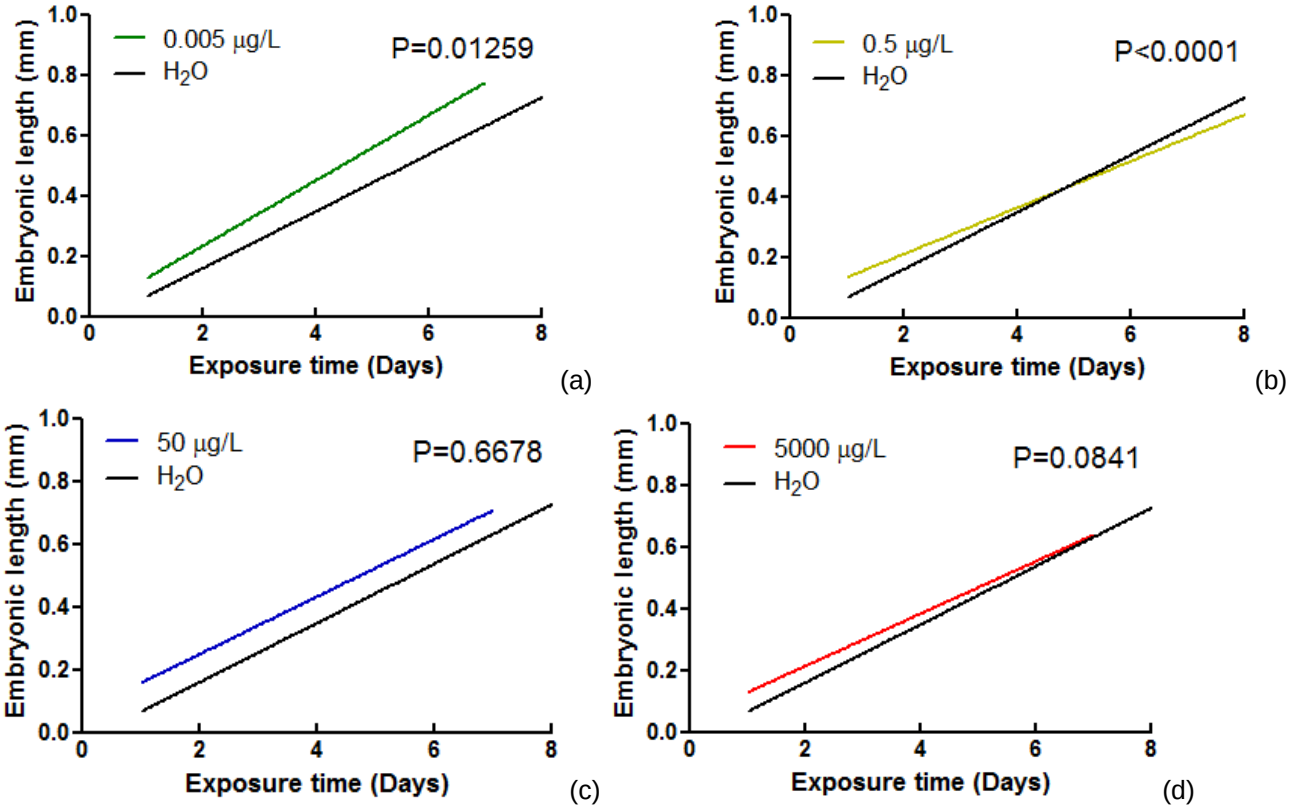


Figure 42: Linear regressions were conducted to determine whether embryonic development after several days was influenced by stavudine exposures at either a concentration of: (a) 0.005 µg/L, (b) 0.5 µg/L, (c) 50 µg/L, or (d) 5000 µg/L in comparison with a water control.

The linear regressions of stavudine (Figure 42) revealed that the differences in slopes for only the 0.005 µg/L (a) and the 0.05 µg/L (b) exposure was statistically very significant (P<0.0001) when tested with a linear line F-test. The elevations for the stavudine exposures where initially higher than that of the controls, with 50 µg/L (c) and 5000 µg/L (d) displaying earlier embryo hatching.

3.4 DISCUSSION

3.4.1 Potential impacts of ARVs on bacteriophages

Possible implications for the lower bacterial growth levels caused by ARVs in wastewater treatment plants may cause lower efficiency in nutrient removal in WWTPs (Sandaa et al., 2009). This new evidence should be investigated further by biolog assays and transcriptome studies to establish which specific pathways in the bacteria are being impacted. Furthermore, a decrease in production of new somatic coliphages in the system to kill *E. coli* implies that a higher burden of bacterial load further down the WWTP may exist (Sandaa et al., 2009).

In turn, this could cause substandard water quality in terms of microbial safety to exit the WWTP or the need for greater chlorine dosing to eliminate the bacteria that the coliphages failed to do. Greater chlorine dosing could have adverse effects on the receiving effluent water bodies and could cause interference with water quality tests that are culture dependant. Examples of this type of interference are issues in solid mat formation when ISO 10705 is used for testing phage levels in water (ISO, 2000), and other bacteriological cultivation dependent indicators (*E. coli*, faecal coliform, total coliform, and *Streptococci*) of water quality and faecal pollution. For instance, the Colilert method (Cooksey et al., 2019). It should be noted that the presence of ARVs in a WWTP should at this stage of research only be noted as a possible contributing factor to decreased WWTP efficiency and not a driving force.

The decrease in OD at minute 3-4 that was observed could possibly be due to the injection of DNA/RNA of the phages into the bacteria and start of phage protein degradation. It would be interesting to investigate if fusion inhibitor (FI) ARVs in wastewater treatment plants (such as: Enfuvirtide) (Greenberg and Cammack, 2004) can influence this occurrence/observation. The inability for phages to infect and ultimately kill bacteria in the WWTP would again cause interference with new total amount of phages being produced. Therefore, a decrease in the total number of coliphages present in the WWTP could decrease the biological control method established by phages in the system to decrease bacterial presence throughout the WWTP works. If in fact the FI does not impact the infection of bacteria by phages, it will support the claim that the H-protein in *E.coli*, proven to be similar than the human H protein is the affected site of NNRTI/NRTI in bacteria in the WWTP. This hypothesis can further be investigated by testing the effect of these ARVs on *Bacterioides fragilis* or other bacteria that have bacteriophages with tails (Tartera and Jofre, 1987; Jurczak-Kurek et al., 2016). The use of *Salmonella* WG49 and FRNA phages is not appropriate here since the absence of appendages of FRNA phages could cause variations in the results (Elbreki et al., 2014). Finally, the difference in reaction to the presence of ARVs in tests containing DNA phage and RNA phage respectively should also shed light on this aspect

Due to similar trends observed in the phage-bacteria mixture with and without ARV active compounds it is possible that some WG5 was not affected and normal activity was present. This could indicate either substrate binding saturation, resistance to ARV (Chauhan et al., 2019), or the presence of DNA phage that might not be affected in the same way RNA phage are. In the case of substrate binding saturation and if these results are to be reproduced it is of great importance to take note of the bacteria:phage ratio in this study. In this study the starting ratio for bacteria:phage in a single micro well was 2:1 to ensure initial growth curve first, whereas it is suspected by (Sandaa, 2009) to be 1:15 in aquatic environments. This is also an indication of a growing concern: the more ARVs in the water, the smaller this ratio could start to become.

The mode of action of the ARV's could possibly have led to the disruption of certain bacteriophages and some may be resistant (Chauhan et al., 2019). The phages applied were direct isolates from aquatic bodies and may be a variety of bacteriophages thus as a result some may be susceptible, and some may carry resistant genes. It could be interesting to establish the individual and combined effect of ARVs on purified phage cultures.

TEM results illustrated that phage proteins might possibly have been affected by the inhibition of the targeted genetic sites of NNRTI, NRTI, and PI ARVs. The heads (capsules) were separated from the tails as well as the head that seemed to have undergone morphological changes. Instead of the conventional icosahedral shape head, some seem round and others no specific shape.

WWTPs rely on the activity of bacteriophages to infect and kill a large portion of bacteria in the system. Phages rely on the metabolic activity of their bacterial hosts for their reproduction and survival in a system. It seems that these metabolic processes of bacteria are being impacted by the ARV active compounds tested in this study. Therefore, causing a decrease in the total amount of phages in the system. A decrease in the amount of phages in the system can lead to an increase in the number of bacteria in the WWTP effluent. Thus, ARV active compounds may have an effect on the productivity of a WWTP in removing bacteria from its water by impacting the phages in the system meant to kill and control these bacterial levels.

Furthermore, if one looks past the WWTP itself to the bodies of water that are receiving the effluent of a WWTPs impacted by ARVs, increased levels of bacteria being introduced into the environmental water could cause an increase in bacterial blooms in the receiving water bodies. Such a decrease in microbial water quality produced by WWTP due to the activity of ARVs in the system could be harmful to the environment and cause an increased burden of the health sector and agricultural sector. It could be harmful to the health sector, due to more potential pathogens being present in the water being used for household chores in rural areas. It could be harmful to the agricultural sector due to crops and livestock being irrigated and drinking water of poor microbial health and thus increasing the spread of disease.

Finally, due to the evidence seen in this study that ARVs influence bacterial metabolic processes it will be important to re-evaluate the use of culture dependant methods for testing microbial water quality at WWTPs and in the environment polluted with ARV active compounds. Water quality may be worse than those presented by tests imply due to the decreased metabolic activity of bacteria impacted by ARV active compounds.

3.4.2 Reporter gene assays

Regarding the two reporter gene assays that were successfully performed, there was not activation of the respective receptors after exposure to environmentally relevant concentrations. However, there was evidence of inhibition of the AR by stavudine and lamivudine. Lopinavir also created slight inhibition at the highest concentration and the inhibition by efavirenz was dose-dependent: more inhibition evident at the higher concentrations.

The data from the steroidogenesis assay gave evidence of a decreased production of both T and E2 by the cells but this was not dose-dependent. Ritonavir also caused a decline in T but its influence on E2 was mixed, sometimes increasing its concentrations and for other concentrations decreasing it levels, but with no relationship between exposure concentration of the ARV and E2 production. Lopinavir caused a lowering of both T and E2 and this was dose-dependent. Efavirenz seemed to have caused increase in both the levels of T and E2 at the lower exposure concentrations, but a decrease at the higher concentrations. Nevirapine mostly caused an abatement of T levels but its influence on E2 response was not clear.

The human intestinal cells were exposed to concentrations that might be equivalent to those in the formulations, therefore mimicking the levels the human intestine might experience after swallowing the tablets. The Hutu cells survived all the exposure concentrations as well as at the three exposure concentrations. Efavirenz was the only active ingredient that caused a slight decrease in viability at the higher concentrations and at the 24 h and 36 h exposure periods.

3.4.3 Snail exposures

Lowered fitness of organisms due to reproductive inadequacy is disputably among the most valuable sub-lethal effects measured in ecotoxicology. Studies completed by Wadji (1955), Najarian (1961) and Minnaar (2014), revealed that the laboratory isolation of *B. tropicus* did not diminish their egg-laying capacity and can therefore not be ruled as a factor influencing exposure results. When comparing the two different modes of ARV action for their effects on the embryonic development of *B. tropicus* (Figure 39-Figure 42), it is clear that the ARVs demonstrate contrasting effects.

Lamivudine and stavudine (NRTIs) generally had a stimulating effect on the embryonic development, producing mean embryo lengths larger than that of the water controls. This correlates to literature where Venhoff et al. (2007) argue that ARV drugs can cause ecotoxicological effects, especially NRTIs, which can cause effects in an organisms' mitochondria as a result of their nucleosidic structure that can be integrated into DNA or RNA-strains. Although, it is unclear what altered biological aspect brought about the positive fitness induced by the stressor of either lamivudine or stavudine. To the best of our knowledge, this is one of the only few documented examples of positive induced fitness produced by ARVs on freshwater organisms. However, this stimulating toxicant-induced fitness is also concerning because other organisms can be affected indirectly. Alterations in the abundance or fitness of susceptible species can lead to cascading indirect effects even on resistant species, at every trophic level within a community. Contaminant-induced increases in fitness in one species, can alter the competition and predation pressures on (and thus the behaviour of), other species within a community.

In turn, efavirenz and nevirapine (NNRTIs) demonstrated an overall inhibitory effect on embryonic growth. Disputably, due to the increasing energy demands in order to cope with metabolic or developmental disorders caused by the stressor of efavirenz or nevirapine. Such alterations in growth rate can greatly affect population densities and demography as well as other species interaction. According to Bae et al. (2011), uridine 5-diphosphate-glucuronosyltransferase (UGTs) is involved in the glucuronide formation of efavirenz and its three hydroxyl metabolites. UGTs have also been known to catalyse the glucuronide conjugation of numerous of endobiotic and xenobiotic compounds while contributing to their disposition and excretion. The larger embryo length variability could be due to the variability in the extensive metabolism of efavirenz.

The NNRTIs also illustrated the highest percentage of mortality with 35% for the 0.005 µg/L efavirenz exposure and 34% for the 0.5 µg/L nevirapine exposure. Many of the embryos therefore failed to make the necessary homeostatic adaptations to overcome the stress induced by these NNRTIs. It is; however, unclear which organs or response was affected. These results correspond with that in literature relating to an increase in sensitivity of juvenile organisms towards exposures with efavirenz (EMEA, 2005). What is clear is that exposure of ARVs towards *B. tropicus* affected embryonic length, hatching and rate of mortality. The influence of these ARVs on the development and growth of the indicator organism therefore supports the necessity to conduct further investigations with regards to ecological impact of these pharmaceutical compounds.

When coupled with differential metabolism, intra- and interspecies differences in behaviour, physiology and life history, produce substantial differences among species and individuals in responses and susceptibility to chemical contaminants. Regrettably, our knowledge of comparative mechanistic responses to contaminants remains inadequate, even for model laboratory organisms. There is also a phenomenal lack in information on the uptake through food webs, future studies should therefore place emphasis on comprehending the uptake routes of ARVs from a wide range of environments into a singular organism. These studies should also investigate the bioaccumulation in food webs covering different life cycle, size, and method of respiration traits. Substantiated on these studies, value can be added to models developed to estimate the uptake of these compounds in organisms and food webs.

3.5 CONCLUSIONS

3.5.1 Potential impacts of ARVs on bacteriophages

There was evidence that ARVs even at very low concentrations could affect the survival and reproduction of bacteriophages as well as their ability to infect their bacterial host. There was no statistically significant difference between the effects of different ARVs or their different concentrations on bacteriophage activity. It became evident that the effects of ARVs might not be limited to virus survival but also that a potential effect on bacterial metabolic processes and growth potential exists. Theoretically, the visible effects on virome assembly and growth of bacteria could contribute to the efficiency of WWTP substrate and pathogen removal.

3.5.2 *In vitro* tissue culture assays

There was no evidence of androgen activation and limited evidence of androgen inhibition at the concentrations that were tested. The AhR was not in any instance activated by any of the targeted ARVs. Unfortunately, there was no successful completion of an oestrogen assay and this is a pity because there is literature on ER activity by some of these ERs (Sikora et al., 2010). There was evidence of oestradiol and testosterone levels being influenced in the steroidogenesis assay showing the influence of the ARVs on the metabolism of these steroid hormones. There are more hormones that may be influenced by this mechanism of action, such as progesterone and cortisol and this should be investigated.

3.5.3 Snail exposures

Due to the rapid reproductive cycles, basic maintenance and availability it is feasible to state that *B. tropicus* can be used as a test subject for ARV ecotoxicity research, although further studies are required to conclude if it is a suitable biological indicator of ARV pollution. The results obtained adequately support the acceptance of the hypothesis that embryotic exposure to ARV compounds will have an effect on the developmental processes of *B. tropicus*. Both NNRTIs and NRTIs affected embryonic length, hatching and mortality in acute tests. The results are potentially indicative of an endocrine disruptive effect. For this study it was found that lamivudine and stavudine had a stimulatory effect whereas efavirenz and nevirapine had an inhibitory effect. We can therefore also conclude that the different mechanisms of ARV action had different effects on the early life stages of *B. tropicus.* With the potential to also have different effects on other aquatic organisms. In conclusion, although environmental concentrations are currently at low levels with little to no environmental effects found, ARVs pose potential deleterious effects on non-target organism, as the continuous input of such compounds into aquatic systems may lead to prolonged exposure. This study demonstrates that there is a requirement for advance knowledge on the facets of pharmaceutical pollutants, especially ARVs as well as the chemical and ecotoxicological interactions associated with these compounds in aquatic ecosystems

3.6 RECOMMENDATIONS

3.6.1 Potential impacts of ARVs on bacteriophages

Summary of recommendations

The following recommendations are proposed for the potential impacts of antiretrovirals in the environment on bacteriophages:

- Biolog assays should be performed on bacterial hosts to establish which specific metabolic pathways are being affected by the presence of antiretroviral active compounds.
- It should be established what the threshold concentration of antiretrovirals are for no effect on bacteria and bacteriophage metabolic/assembly.
- The effects on a single type of phage by several antiretroviral active compounds in a single mixture should be investigated.
- It is advised to investigate the effects on a phage-microcosm by several antiretroviral active compounds in a single mixture.
- Antiretrovirals using other mechanisms of inhibition should be tested, e.g. fusion inhibitors.
- The use of a purified single strain phage should be included in future studies (this could provide information on individual response mechanisms and effects of bacteriophages).
- Studies should be conducted to ascertain the effects of antiretrovirals on bacteria, fungi, and algae.
- It is recommended to establish whether individual or combined antiretroviral exposure affects purified phage cultures and microcosms differently.
- The viral ecology of fresh and marine waters is insufficiently understood. Investigations on the interactions between viruses and pollutants should be considered.
- The large scale, financial feasibility of implementing alternative faecal pollution indicators that do not rely on living/growing organisms should be investigated for example cholesterol.
- Establishing resistance patterns of bacteriophages and their hosts to ARVs.
- Finally, to establish if there is a difference in effects on DNA phage and RNA phage by different ARVs respectively.

3.6.2 *In vitro* tissue culture assays

Summary of recommendations

The following recommendations are proposed for the *in vitro* tissue culture assays (CHAPTER 3:):

- Steroidogenesis assays should be employed to test for the influence of the ARVs on the other steroid hormones expressed by the H295R cells (angiotensin-II-responsive steroid-producing adrenocortical cell line) used in the assay.
- It would be ideal if the analysis of the hormone levels could be substituted with instrumental analysis instead of the enzyme linked immune-sorbent assays.
- The instrumental analysis can determine the concentrations of multiple hormones in one run, whereas the enzyme linked immune-sorbent assays are developed for the single hormone. This makes the total analysis time consuming and expensive.
- It is also important to check for endocrine disruption via activation or inhibition of a working oestrogen reporter gene assay.

3.6.3 Snail exposures

Summary of recommendations

The following recommendations are proposed for the snail exposures:

- It is imperative to conduct elaborate life-cycle studies on these and other potentially exposed organisms.
- In addition to supplementary acute exposure tests, future studies should investigate the effects of chronic exposure to antiretrovirals.
- Different modes of antiretrovirals action should be investigated, where investigations comprise of the active ingredient, precursors and metabolites.
- Future tests should not only include pure pharmaceutical compounds, but should also investigate the effects of pharmaceutical mixtures and combinations.
- All plausible roots of exposure should be considered in impending studies.
- It is recommended to experiment with other indicator species, for example fairy shrimp. (More comparative studies are required on different species).
- Micro- and mesocosm exposure tests are recommended to provide more environmentally relevant results.

CHAPTER 4: HAZARD ASSESSMENT OF ANTIRETROVIRALS DETECTED IN WATER SOURCES

4.1 INTRODUCTION

The pharmacological active substances present in the water environment are mostly derived from prescription and over-the-counter drugs used for treatment of various illnesses by humans. Advances in analytical instruments and techniques made it possible to detect trace levels of various pharmaceuticals and their metabolites in sewage, river, lake, and groundwater all over the globe. One example of these compounds detected in water is ARV's that are used in the treatment of HIV/AIDS. The sources of ARVs are mostly municipal wastewater discharge, which includes both household and hospital wastewater (Kummerer, 2001).

ARVs have been detected in various water sources throughout South Africa and Africa (CHAPTER 1:). There is, however, not much evidence on the possible adverse effects that these compounds might have on non-target organisms (wildlife) and the environment. The fate of these compounds in the environment are also in question. It is of major public concern that these residual pharmaceuticals such as ARVs could unintentionally be consumed when drinking water contaminated by these compounds. Mainly because these substances could be pharmacologically and physiologically active and affect mechanisms in the human body at very low concentrations (Simazaki et al., 2015). This is specifically the case in countries that use surface waters as a source for drinking water, such as South Africa.

In order to determine whether ARVs pose a threat, a human health risk assessment should be conducted. There is however too few data available on ARVs to conduct a human health risk assessment and it was decided to rather do a hazard assessment (HA). This will estimate the likelihood of adverse effects in a population at defined exposures to target compounds. The calculation of a threat based on the concentrations of the target compounds in the surface water can provide the probability of health effects from a specific area. The risks posed by the exposure to ARVs is predicted by calculating the toxicity of these chemicals over a lifetime of exposure, exposure routes and at concentrations determined at each site.

The hazard assessment process includes 4 steps: 1) hazard identification; 2) hazard characterisation; 3) exposure assessment and 4) risk characterisation (WHO, 2010). The hazard identification step identifies the type and nature of adverse effects. Hazard characterisation includes a qualitative and quantitative description of the properties of an agent having the potential to cause adverse health effects. The exposure assessment evaluates the concentration of a particular agent that reaches a target population. The final step is risk characterisation that summarise the finding to use for advice in decision-making. The risks posed by chemically contaminated water were determined for physical contact while collecting water, fishing, swimming and bathing as well as oral ingestion for consumption of water. Hazard assessment of fish was omitted, as there were no quantifiable concentrations of ARVs detected in the fish tissue analysed from the same river system.

4.2 MATERIALS AND METHODS

4.2.1 Site selection

Water was sampled up- and downstream from WWTPs in northern (Figure 5) and southern (Figure 6) Gauteng (Table 8), which consisted of Olifantsfontein River, Sunderland Ridge, Vlakplaats, Waterval, Welgedacht, Zeekoegat, Flip Human and Baragwanath. The Mooi River was also sampled and is situated close to Khutsong (North West) (Table 9) and downstream of the Wonderfonteinspruit (Gauteng). More sampling sites included the Jukskei, Hennops, Klip, and Crocodile rivers (Table 10). Some sites in this sampling event coincide, however, they have different site labels: Sunderland Ridge DS1 (same site as Hennops 8), Sunderland Ridge

US (same as Hennops 7), Olifantsfontein US (same as Hennops 3) and Olifantsfontein DS1 (same as Hennops 4). The hazard assessment was done for the sampling sites of this project (detailed description in CHAPTER 2: section 0).

4.2.2 Chemicals and reagents

Analytical reference standards (≥ 99% purity) obtained from the U.S. Pharmacopeial Convention, European Pharmacopoeia, British Pharmacopeia and World Health Organization were purchased from Stargate Scientific (Johannesburg, South Africa). Deuterated fluconazole (d-4) acted as internal standard and was obtained from Toronto Research Chemicals (Toronto, Canada). Honeywell Burdick and Jackson acetonitrile and methanol of spectrometry grade were purchased from Anatech, South Africa. Acetic and formic acid were obtained as 98% solutions for mass spectrometry from Fluka (Sigma, Germany). De-ionised water was obtained from an ELGA water purification system. HLB-L (hydrophilic-lipophilic balance) (low) solid phase extraction (SPE) disks (47 mm) were obtained from Atlantic (Horizon Technology, Salem, NH, USA). Stock solutions of 1 mg/ml were prepared in methanol and stored at -20°C.

4.2.3 Extraction and LC-MS analysis

The extraction method used was based on Ferrer et al. (2010), for pharmaceuticals. Water samples were spiked with fluconazole-d4 (IS) and target compounds were extracted by Oasis HLB-L extraction disks using automated SPE (SPE-DEX system, Horizon Technology, Salem, NH, USA). The eluent was concentrated to near dryness using a gentle stream of nitrogen gas. The samples were reconstituted in methanol.

The UPLC system used consist of an Agilent 1290 Infinity Binary pump (G4220A); 1290 Infinity Autosampler (G4226A); and 1290 Infinity Thermostatted Column Compartment (G1316C) coupled to an Agilent 6540 Accurate mass Q-TOF/MS (G6540A) (Agilent Technologies, Santa Clara, CA, USA). The desolvation and ionisation of samples were achieved by positive and negative *e*lectrospray ionisation (ESI) enhanced with Agilent Jet Stream (AJS) technology. The QTOF was set to scan from 50 to 950 m/z and the instrument was set to extended dynamic range (2 GHz). Software used was MassHunter Data Acquisition (version B.05.00), MassHunter Qualitative Analysis (version B.05.00), and Quantitative Analysis for QTOF (version B.05.01). Mass axis calibration of QTOF was performed daily for positive and negative ionisation with tuning mixes (G1969-85000, Agilent). A reference solution with masses of 121.050873 [M+H] and 922.009798 [M+H] were constantly infused as accurate mass references.

The injection volume was 1 µl and the ARVs were separated using a Poroshell 120 Bonus-RP column (Agilent, 2.1 x 100 mm, 2.7 µm). Positive ionisation flow rate was 0.6 ml/min and negative ionisation flow was 0.7 ml/min. Positive ionisation mobile phases were water and acetonitrile both with 0.05% formic acid and negative ionisation mobile phases were water and acetonitrile with 0.1% acetic acid. For compound masses and retention times (Table 14).

4.2.4 Method validation

An external matrix matched calibration curve was used to account for matrix effects during quantification. The method was validated with satisfactory precision and accuracy. By use of the y=mx+c model, LOD is calculated by 3*Sa/b and LOQ by 10*Sa/b; where Sa is the SD of the intercept (abundance) and b is the slope of the calibration curve (Schoeman et al., 2015). Concentration of ARVs in samples will be determined by using this formula:

X_{ARV} = ((native/stable isotope-c) / m) x ISO conc

where: X_{ARV} = calculated analyte concentration; native = native abundance; stable isotope = stable isotope abundance; c = calibration curve y-intercept; m = slope of calibration curve and ISO conc = internal standard concentration. Refer to Chapter 2; section 0-0 for detailed information about the chemical analysis.

4.3 HAZARD ASSESSMENT

4.3.1 Hazard identification

ARVs and fluconazole are used in high volumes in South Africa. As highlighted throughout this project, ARVs have been detected in various water sources with the highest concentrations in Africa, and South Africa (CHAPTER 2). The compounds detected in this study include: ARVs (nevirapine, ritonavir, lopinavir, efavirenz, zidovudine) and the antifungal fluconazole. Although most pharmaceuticals are not persistent of nature, they are constantly introduced into the water sources. For information on chemical properties of ARVs and fluconazole refer to section 1.6.1-1.7.4. ARVs have not yet been classified to be hazardous to the environment or vertebrates that are indirectly exposed. For this specific hazard assessment, we estimated the worst-case scenario of the potential health risk to human by exposures to ARVs and fluconazole at concentrations detected in the current study. We calculated the hazard quotient (HQ) for each ARV detected across the sampling sites (Sharma et al., 2019).

4.3.2 Hazard characterisation

In the event of non-cancer effects, the benchmark for risks are expressed as exposure in milligrams per kilogram of body weight per day, known as the reference dose (RfD). These values are estimates based on exposure to humans that will result in no significant risk or adverse health effect if not exceeded (WHO, 2010). Usually toxicity data is incorporated in the RfD estimations, but in the absence of data, other sources or forms may be used (Newman, 2010).

There are no published RfDs available for ARVs. Therefor the RfD values for ARVs and fluconazole were calculated by using the therapeutic dose divided by uncertainty factors (composite uncertainty factors are 1 000 and used by default) (Table 38). These factors are built in to reduce the likelihood that actual risks to humans will be underestimated (WHO, 2010).

Table 38: Reference doses calculated for ARVs and fluconazole divided by 66.5 kg (average body mass for SA) and the therapeutic dose of each compound.

ARVs	Therapeutic dose Adults (mg/day)	Reference dose for non-carcinogenic effects mg/kg/d (safety factors included)
Efavirenz	600	0.009
Ritonavir	300	0.005
Lopinavir	200	0.003
Zidovudine	600	0.009
Nevirapine	200	0.003
Fluconazole	400	0.006

4.3.3 Exposure assessment

ARVs find their way into surface water bodies after excretion from the human body through release of untreated or improperly treated effluents from WWTPs, hospitals and production facilities. Another potential source of

ARVs into the environment is through improper sanitation and illegal disposal of both domestic and industrial waste into river sources.

The parameters used for exposure assessment is chosen to best represent the population in the sampling area. Exposure pathways for ARVs from this study are mostly related to water activities. These include drinking, food preparations, bathing and recreational activities. Other exposure pathways may be through food, if the water used for watering crops are contaminated with ARVs and fluconazole, and if they persist on the crops. This pathway has not been investigated yet and is not included in this risk assessment due to the lack of evidence on this assumption. The ARVs are mostly hydrophilic based on their chemical properties and they are therefore expected to only occur in the water surface. Previous studies investigated the likelihood of these compounds to attach to sediment or soil, but concluded that they do not bind to sediment or soil particles (Aminot et al., 2015).

4.3.4 Risk characterisation

Pharmaceuticals, such as ARVs and fluconazole, can enter the human body through ingestion and dermal contact. Oral intake is however the critical mode of entry, and therefore the risk assessment was done based on the average daily dose (ADD) and HQ via surface water that is consumed in areas where municipal treated drinking water is not always available. The ADD was calculated via the following formula:

ADD = C x IR x ED x EF/BM x AT

where C is the concentration of each ARV detected in surface water (ug/L), IR is the ingestion rate, ED refers to exposure duration, EF is the exposure frequency, BM is the average body mass of a South African and AT is the average lifetime.

Table 39: Parameters used to determine the average daily dose (ADD).

Formula abbreviation		Unit	Population variables Adults
BM	Body mass	kilogram	66.5
LE	Life expectancy	years	62.5
AT	Average lifetime	LE x 365 days	22 812.5
EF	Exposure frequency	days per year	365
ED	Exposure duration	years	30
IR water (IR)	Ingestion rate of water	L/day	1.41

A HQ was determined, and the exposed individuals were considered to be safe if the HQ < 1. A HQ exceeding 1 is not a statistical possibility of harm occurring, but instead, it's an indicator of whether (and by how much) an exposure concentration exceeds the reference concentration. The HQ was calculated by the following formula:

HQ = ADD/RfD

The risk effects of the mixture as a health index (HI) were determined by the sum of the HQ of all the ARVs quantified. A HI < 1 is an acceptable risk and HI > 1 means an unacceptable level of risk.

4.4 RESULTS AND DISCUSSION

The results of the hazard assessment are reflected in Table 40 to Table 43 and show that certain ARVs from different rivers, over 5 months of sampling, pose an unacceptable level of risk. The HQs calculated for the ARVs and fluconazole ranged from 0-216 with lopinavir having the highest HQ and also exceeding the HQ risk level of 1 in almost all of the samples. Fluconazole was the only compound that did not have a HQ great than

one in any of the samples. The majority of the sites located in the Hennops, Jukskei, Klip and Crocodile rivers, except for the drinking water sites, had a hazard index indicating cumulative risk, greater than 1 indicating that the mixtures pose a significant risk for consumer's health and that monitoring is needed.

Table 40: Hazard quotients and index calculated for ARVs and fluconazole detected in the Klip river.

	Zidovudine	Efavirenz	Fluconazole	Nevirapine	Lopinavir	Ritonavir	HI
Klip 1 (March)	0,00	0,00	0,45	**1,40**	**99,12**	**3,77**	**104,74**
Klip 2 (March)	0,00	0,00	0,28	0,53	**77,72**	**2,88**	**81,41**
Klip 3 (March)	0,40	0,00	0,46	0,93	**88,84**	**4,96**	**95,59**
Klip 4 (March)	0,37	0,00	0,23	0,00	**30,29**	0,00	**30,88**
Klip 5 (March)	0,48	**1,15**	0,51	0,74	**140,01**	**9,85**	**152,75**
Klip 6 (March)	0,00	0,00	0,24	0,41	**55,92**	**2,55**	**59,12**
Klip 7 (March)	0,40	0,00	0,15	0,37	**45,45**	**2,29**	**48,66**
Klip 8 (March)	0,28	0,00	0,24	0,38	**46,05**	**2,22**	**49,18**
Klip 9 (March)	0,00	0,00	0,15	0,46	**18,94**	0,00	**19,54**
Klip 1 (April)	0,27	0,00	0,30	0,37	**18,31**	0,00	**19,25**
Klip 2 (April)	0,32	0,00	0,23	0,00	0,00	0,00	0,55
Klip 3 (April)	**1,20**	0,00	0,59	0,41	**12,08**	0,00	**14,29**
Klip 4 (April)	0,63	0,00	0,11	0,00	**12,17**	0,00	**12,91**
Klip 5 (April)	0,59	**1,75**	0,54	0,56	**55,03**	**3,16**	**61,63**
Klip 6 (April)	0,42	0,00	0,22	0,00	**12,02**	0,00	**12,66**
Klip 7 (April)	0,41	0,00	0,20	0,00	**9,31**	0,00	**9,92**
Klip 8 (April)	0,59	0,00	0,16	0,00	**6,65**	0,00	**7,40**
Klip 9 (April)	0,00	0,00	0,14	0,00	**3,52**	0,00	**3,65**
Klip 1 (May)	0,00	0,00	0,28	0,00	**3,27**	0,00	**3,55**
Klip 2 (May)	0,00	0,00	0,20	0,00	0,00	0,00	0,20
Klip 3 (May)	0,42	**2,04**	0,48	0,00	0,00	0,00	**2,94**
Klip 4 (May)	0,00	**1,04**	0,43	0,00	**11,08**	0,00	**12,54**
Klip 5 (May)	0,00	**1,12**	0,47	0,51	**13,78**	0,00	**15,87**
Klip 6 (May)	0,00	0,00	0,23	0,43	**10,03**	0,00	**10,70**
Klip 7 (May)	0,65	0,00	0,18	0,51	**17,21**	0,00	**18,56**
Klip 8 (May)	0,88	0,00	0,24	0,00	**5,62**	0,00	**6,74**
Klip 9 (May)	0,00	0,00	0,19	0,00	**18,83**	0,00	**19,02**
Klip 1 (August)	0,25	**1,25**	0,48	0,00	**133,96**	**7,14**	**143,09**
Klip 2 (August)	0,30	**1,02**	0,39	0,00	**134,43**	**9,21**	**145,35**
Klip 3 (August)	0,27	**1,99**	0,82	0,00	**158,59**	**9,17**	**170,84**
Klip 4 (August)	0,65	**1,03**	0,33	0,00	**135,16**	**10,25**	**147,40**
Klip 5 (August)	0,51	**1,36**	0,88	0,51	**104,12**	**6,87**	**114,24**
Klip 6 (August)	0,35	**1,13**	0,29	0,43	**89,71**	**6,19**	**98,10**
Klip 7 (August)	0,66	**1,21**	0,28	0,51	**82,56**	**6,34**	**91,56**
Klip 8 (August)	0,73	**1,03**	0,25	0,00	**82,95**	**7,76**	**92,72**
Klip 9 (August)	0,56	0,90	0,38	0,00	**72,50**	**3,93**	**78,28**
Klip 1 (October)	0,27	0,00	0,16	0,00	**6,84**	0,00	**7,27**
Klip 2 (October)	0,37	0,00	0,14	0,00	**4,26**	0,00	**4,77**
Klip 3 (October)	0,33	0,00	0,35	0,00	**7,21**	0,00	**7,89**
Klip 4 (October)	0,27	0,00	0,00	0,00	**4,09**	0,00	**4,36**
Klip 5 (October)	0,00	0,00	0,16	0,00	**16,84**	0,00	**17,00**
Klip 6 (October)	0,00	0,00	0,00	0,00	**2,54**	0,00	**2,54**
Klip 7 (October)	0,00	0,00	0,00	0,00	**2,18**	0,00	**2,18**
Klip 8 (October)	0,27	0,00	0,00	0,00	0,00	0,00	0,27

	Zidovudine	Efavirenz	Fluconazole	Nevirapine	Lopinavir	Ritonavir	HI
Klip 9 (October)	0,30	0,00	0,00	0,00	**3,10**	0,00	**3,41**
Klip DW1 (August)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Klip DW2 (August)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Klip DW3 (August)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Klip DW1 (October)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Klip DW2 (October)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Klip DW3 (October)	0,00	0,00	0,00	0,00	0,00	0,00	0,00

HQ>1 indicated in bold; DW: drinking water; HI: hazard index (cumulative risk); HI>1 indicated in bold.

Table 41: Hazard quotients and index calculated for ARVs and fluconazole detected in the Jukskei river.

	Zidovudine	Efavirenz	Fluconazole	Nevirapine	Lopinavir	Ritonavir	HI
Jukskei 1 (March)	0,30	0,00	0,00	0,38	28,42	0,00	29,10
Jukskei 2 (March)	1,06	0,00	0,10	0,50	51,32	0,00	52,98
Jukskei 3 (March)	0,00	0,00	0,16	0,80	11,01	0,00	11,97
Jukskei 4 (March)	0,39	0,00	0,00	0,50	61,64	2,34	64,87
Jukskei 5 (March)	0,00	0,00	0,00	0,00	10,09	0,00	10,09
Jukskei 6 (March)	0,00	0,00	0,00	0,00	14,21	0,00	14,21
Jukskei 7 (March)	0,00	0,00	0,10	0,00	7,65	0,00	7,76
Jukskei 8 (March)	0,51	0,00	0,44	0,64	53,69	2,11	57,40
Jukskei 9 (March)	0,00	0,00	0,10	0,00	12,00	0,00	12,10
Jukskei 10 (March)	0,50	0,00	0,35	0,45	49,41	0,00	50,71
Jukskei 1 (April)	0,49	0,00	0,20	0,37	2,41	0,00	3,47
Jukskei 2 (April)	1,07	0,00	0,12	0,00	6,12	0,00	7,32
Jukskei 3 (April)	0,00	0,00	0,11	0,00	0,00	0,00	0,11
Jukskei 4 (April)	1,34	0,00	0,11	0,00	6,54	0,00	7,99
Jukskei 5 (April)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Jukskei 6 (April)	0,00	0,00	0,00	0,00	9,43	0,00	9,43
Jukskei 7 (April)	0,40	0,00	0,12	0,00	8,60	0,00	9,12
Jukskei 8 (April)	0,49	0,00	0,32	0,00	9,69	0,00	10,50
Jukskei 9 (April)	0,69	0,00	0,15	0,00	4,69	0,00	5,53
Jukskei 10 (April)	0,52	0,00	0,36	0,00	23,19	0,00	24,06
Jukskei 1 (May)	0,55	0,00	0,12	0,00	2,10	0,00	2,77
Jukskei 2 (May)	0,89	0,00	0,16	0,00	10,92	0,00	11,97
Jukskei 3 (May)	0,00	0,00	0,12	0,00	6,14	0,00	6,26
Jukskei 4 (May)	1,06	0,00	0,16	0,00	7,46	0,00	8,68
Jukskei 5 (May)	0,00	0,00	0,18	0,05	1,98	0,00	2,21
Jukskei 6 (May)	0,34	0,00	0,13	0,00	10,77	0,00	11,24
Jukskei 7 (May)	0,42	0,00	0,11	0,00	7,48	0,00	8,01
Jukskei 8 (May)	0,49	0,00	0,45	0,00	6,71	0,00	7,65
Jukskei 9 (May)	0,35	0,00	0,13	0,00	5,99	0,00	6,48
Jukskei 10 (May)	0,59	0,00	0,46	0,00	22,44	0,00	23,49
Jukskei 1 (August)	0,99	1,41	0,00	0,00	7,35	0,00	9,75
Jukskei 2 (August)	1,43	1,70	0,18	0,00	20,67	0,00	23,98
Jukskei 3 (August)	0,35	0,00	0,25	0,00	2,73	0,00	3,32
Jukskei 4 (August)	0,80	1,69	0,00	0,00	14,90	0,00	17,39
Jukskei 5 (August)	0,00	0,00	0,30	0,00	2,50	0,00	2,80
Jukskei 6 (August)	0,66	0,94	0,12	0,00	14,36	0,00	16,07
Jukskei 7 (August)	0,97	0,00	0,19	0,00	16,55	0,00	17,71

	Zidovudine	Efavirenz	Fluconazole	Nevirapine	Lopinavir	Ritonavir	HI
Jukskei 8 (August)	0,95	2,52	0,62	0,37	58,03	4,58	67,07
Jukskei 9 (August)	0,85	0,94	0,23	0,00	21,23	0,00	23,24
Jukskei 10 (August)	0,40	2,48	0,69	0,00	78,44	6,23	88,25
Jukskei 1 (October)	0,00	0,00	0,10	0,00	0,00	0,00	0,10
Jukskei 2 (October)	1,45	0,00	0,00	0,00	3,22	0,00	4,67
Jukskei 3 (October)	0,00	0,00	0,15	0,00	0,00	0,00	0,15
Jukskei 4 (October)	3,09	1,05	0,12	0,00	4,74	0,00	9,00
Jukskei 5 (October)	1,29	0,00	0,14	0,00	0,00	0,00	1,43
Jukskei 6 (October)	0,32	0,00	0,17	0,00	1,73	0,00	2,23
Jukskei 7 (October)	0,45	0,00	0,22	0,00	2,09	0,00	2,76
Jukskei 8 (October)	0,88	2,82	0,70	0,19	12,46	0,00	17,05
Jukskei 9 (October)	0,00	0,00	0,00	0,00	3,03	0,00	3,03
Jukskei 10 (October)	1,07	2,25	0,45	0,00	12,22	0,00	15,99
Jukskei DW1 (August)	0,31	0,00	0,00	0,00	0,00	0,00	0,31
Jukskei DW2 (August)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Jukskei DW3 (August)	1,34	0,00	0,00	0,00	0,00	0,00	1,34
Jukskei DW3 (August)	0,59	0,00	0,00	0,00	0,00	0,00	0,59
Jukskei DW1 (October)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Jukskei DW2 (October)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Jukskei DW3 (October)	0,33	0,00	0,00	0,00	0,00	0,00	0,33
Jukskei DW3 (October)	0,00	0,00	0,00	0,00	0,00	0,00	0,00

HQ>1 indicated in bold; DW: drinking water; HI: hazard index (cumulative risk); HI>1 indicated in bold.

Table 42: Hazard quotients and index calculated for ARVs and fluconazole detected in the Hennops river.

	Zidovudine	Efavirenz	Fluconazole	Nevirapine	Lopinavir	Ritonavir	HI
Hennops 1 (March)	0,00	0,00	0,16	0,00	**16,62**	0,00	**16,79**
Hennops 2 (March)	0,00	0,00	0,17	0,00	**16,70**	0,00	**16,87**
Hennops 3 (March)	0,82	0,00	0,19	0,60	**146,96**	**6,30**	**154,87**
Hennops 4 (March)	0,75	0,00	0,36	**2,11**	**154,43**	**5,71**	**163,36**
Hennops 5 (March)	0,52	0,00	0,36	**1,62**	**115,74**	**5,05**	**123,29**
Hennops 6 (March)	0,37	0,00	0,32	**1,87**	**216,09**	**10,47**	**229,12**
Hennops 7 (March)	0,30	0,00	0,00	0,00	**62,56**	**2,85**	**65,71**
Hennops 8 (March)	0,41	0,00	0,36	**1,14**	**180,31**	**8,75**	**190,96**
Hennops 9 (March)	0,26	0,00	0,35	**1,18**	**177,47**	**9,15**	**188,41**
Hennops 1 (April)	0,00	0,00	0,22	0,00	0,00	0,00	0,22
Hennops 2 (April)	0,00	0,00	0,27	0,00	**1,94**	0,00	**2,21**
Hennops 3 (April)	**2,16**	**1,12**	0,31	0,46	**19,70**	0,00	**23,75**
Hennops 4 (April)	**2,31**	0,00	0,41	0,74	**10,77**	0,00	**14,23**
Hennops 5 (April)	0,78	0,00	0,25	0,44	**12,37**	0,00	**13,85**
Hennops 6 (April)	0,66	0,00	0,23	0,00	**8,88**	0,00	**9,77**
Hennops 7 (April)	0,60	0,00	0,00	0,00	**4,12**	0,00	**4,72**
Hennops 8 (April)	0,73	0,00	0,25	0,00	**11,67**	0,00	**12,65**
Hennops 9 (April)	0,53	0,00	0,27	0,00	**13,06**	0,00	**13,86**
Hennops 1 (May)	0,00	0,00	0,15	0,00	**13,93**	0,00	**14,09**
Hennops 2 (May)	0,00	0,00	0,16	0,00	**2,44**	0,00	**2,60**
Hennops 3 (May)	**1,27**	0,00	0,17	0,00	**3,34**	0,00	**4,78**
Hennops 4 (May)	**1,32**	**1,29**	0,54	0,00	0,00	0,00	**3,15**
Hennops 5 (May)	**1,22**	0,00	0,27	0,00	**3,68**	0,00	**5,17**

	Zidovudine	Efavirenz	Fluconazole	Nevirapine	Lopinavir	Ritonavir	HI
Hennops 6 (May)	0,86	0,00	0,29	0,00	0,00	0,00	**1,15**
Hennops 7 (May)	0,00	0,00	0,00	0,00	**2,35**	0,00	**2,35**
Hennops 8 (May)	0,81	0,00	0,29	0,00	0,00	0,00	**1,11**
Hennops 9 (May)	**1,18**	0,00	0,53	0,00	**12,29**	0,00	**13,99**
Hennops 1 (August)	0,00	0,00	0,49	0,00	**7,88**	0,00	**8,37**
Hennops 2 (August)	0,29	0,00	0,30	0,00	**7,59**	0,00	**8,18**
Hennops 3 (August)	**1,32**	**2,45**	0,23	0,00	**61,10**	**4,54**	**69,64**
Hennops 4 (August)	0,84	**4,72**	0,73	0,00	**119,92**	**8,84**	**135,05**
Hennops 5 (August)	**1,60**	**4,09**	0,63	0,43	**106,23**	**6,93**	**119,91**
Hennops 6 (August)	0,83	**3,10**	0,60	**2,16**	**108,65**	**7,09**	**122,42**
Hennops 7 (August)	0,45	0,00	0,26	**1,80**	**26,35**	0,00	**28,86**
Hennops 8 (August)	0,86	**2,10**	0,59	0,47	**104,75**	**6,19**	**114,97**
Hennops 9 (August)	0,91	**1,56**	0,58	0,00	**53,84**	**3,23**	**60,11**
Hennops 1 (October)	0,68	0,00	0,36	0,00	**2,91**	0,00	**3,96**
Hennops 2 (October)	0,38	0,00	0,31	0,00	**2,57**	0,00	**3,26**
Hennops 3 (October)	**2,00**	**2,70**	0,59	0,22	**12,43**	**1,12**	**19,06**
Hennops 4 (October)	**1,53**	**3,62**	0,82	**1,14**	**17,49**	**1,16**	**25,75**
Hennops 5 (October)	**2,51**	**3,00**	0,65	**1,84**	**39,63**	**2,71**	**50,33**
Hennops 6 (October)	**1,51**	**1,90**	0,43	0,49	**29,20**	0,00	**33,52**
Hennops 7 (October)	0,84	0,00	0,10	0,00	**8,13**	0,00	**9,07**
Hennops 8 (October)	**1,82**	**1,58**	0,39	0,43	**26,20**	0,00	**30,43**
Hennops 9 (October)	0,80	**1,30**	0,44	0,00	**33,89**	0,00	**36,44**
Hennops DW1 (August)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Hennops DW2 (August)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Hennops DW3 (August)	0,51	0,00	0,00	0,00	0,00	0,00	0,51
Hennops DW1 (October)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Hennops DW2 (October)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Hennops DW3 (October)	0,40	0,00	0,00	0,27	0,00	0,00	0,66

HQ>1 indicated in bold; DW: drinking water; HI: hazard index (cumulative risk); HI>1 indicated in bold.

Table 43: Hazard quotients and index calculated for ARVs and fluconazole detected in the Crocodile river.

	Zidovudine	Efavirenz	Fluconazole	Nevirapine	Lopinavir	Ritonavir	HI
Crocodile 1 (March)	0,26	0,00	0,38	0,00	**26,23**	0,00	**26,87**
Crocodile 2 (March)	0,00	0,00	0,31	0,50	**31,20**	0,00	**32,02**
Crocodile 3 (March)	0,00	0,00	0,16	0,00	**4,08**	0,00	**4,25**
Crocodile 1 (April)	0,00	0,00	0,12	0,00	**5,70**	0,00	**5,82**
Crocodile 2 (April)	0,65	0,95	0,41	0,00	**4,90**	0,00	**6,91**
Crocodile 3 (April)	0,58	0,00	0,34	0,00	**5,21**	0,00	**6,14**
Crocodile 1 (May)	0,00	0,00	0,18	0,00	**5,20**	0,00	**5,38**
Crocodile 2 (May)	0,40	0,00	0,44	0,00	**3,51**	0,00	**4,35**
Crocodile 3 (May)	0,46	0,00	0,37	0,00	**5,74**	0,00	**6,57**
Crocodile 1 (August)	0,00	0,00	0,25	0,00	**13,09**	0,00	**13,34**
Crocodile 2 (August)	0,00	**1,79**	0,57	0,38	**66,18**	**4,06**	**72,99**
Crocodile 3 (August)	0,44	0,00	0,41	0,63	**54,86**	**3,73**	**60,08**
Crocodile DW 1 (August)	0,47	0,00	0,00	0,00	0,00	0,00	0,47
Crocodile DW 2 (August)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Crocodile 1 (October)	0,39	0,00	0,28	0,00	**5,10**	0,00	**5,76**
Crocodile 2 (October)	0,31	0,00	0,26	0,00	**15,74**	0,00	**16,31**
Crocodile 3 (October)	0,53	0,00	0,19	0,75	**44,21**	**4,07**	**49,74**

	Zidovudine	Efavirenz	Fluconazole	Nevirapine	Lopinavir	Ritonavir	HI
Crocodile DW 1 (October)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Crocodile DW 2 (October)	0,00	0,00	0,00	0,00	0,00	0,00	0,00

HQ>1 indicated in bold; DW: drinking water; HI: hazard index (cumulative risk); HI>1 indicated in bold.

In recent years ARVs have been under investigation in South Africa due to the high usage and load that end up in the surface water. The results of the present study revealed that the surface was contaminated with multiple ARVs up to concentrations of 38 ug/L. There are no existing guideline levels to which ARVs, and fluconazole can be compared to and it therefore warranted the need for a health risk assessment or hazard assessment which is second best.

The minimum therapeutic dose (MTD) was used to determine the reference dose in the current assessment, as the therapeutic doses of all ARVs are disclosed on the package inserts and are easily accessible. Traditionally, the acceptable daily intakes (ADIs) of target pharmaceuticals are used in risk assessments, because ADIs are determined by firm toxicological evidences obtained by controlled animal studies. These results are however hardly available and estimating the ADIs of the target pharmaceuticals and metabolites requires considerable effort, time and expertise in pharmacology. The therapeutic dose approach is more conservative because the therapeutic values are typically much smaller than those of ADIs (WHO, 2012). Application of the therapeutic dose in risk assessments has some limitations as it is determined by clinical trials that may include healthy volunteers, and thus a variety of the population that include sensitive or vulnerable individuals might not be considered. Nevertheless, the use of the MTD is a realistic method for determining human health risk (Simazaki et al. 2015). It should be kept in mind, that this hazard assessment was focused on the worst-case scenario of a long exposure duration through drinking water. This might not always be the case for all the humans exposed to these water sources. Various scenarios would play a role as the amount of water and exposure times may be less. Experimental data is needed to refine the hazard assessment applied in the current study.

4.5 CONCLUSION

This study revealed that surface water sources from an urbanised and industrial area were contaminated with high concentrations of ARVs and fluconazole compared to other countries worldwide. In this study, an unacceptable risk to human health was identified when a hazard assessment was applied by using the minimum therapeutic dose approach. To the best of our knowledge this is the first study to perform a hazard assessment on water sources in South Africa being contaminated by ARVs.

Despite the concerns about the high concentrations and mixtures of pharmaceuticals present in our drinking water, more research is needed to determine the risks to human health from chronic low-level exposures to single and mixtures of ARVs and fluconazole. Further research on ARV occurrence, behaviour, fate and potential risks of parent compounds and metabolites in water sources are needed to establish guideline values for regulatory purposes and prioritisation. The concentrations of ARVs in water sources of developing countries, such as South Africa, is expected to increase seeing as the countries with high ART programs do not have treatment guidelines regarding the presence of pharmaceuticals in WWTP effluents. In addition, research to improve the drinking water treatment technology is required as the current water treatment processes are not able to completely remove pharmaceuticals such as ARVs and fluconazole.

4.6 RECOMMENDATION

Summary of recommendations

The following recommendations are proposed for the hazard assessment of antiretrovirals detected in water sources:

- More studies with different endpoints should be conducted by using the concentrations of antiretrovirals in African and South African surface water to obtain more toxicological information for further risk assessments.
- Tests should include standardised laboratory tests using *Daphnia*, zebrafish, or similar models.
- The effect of mixtures should also be investigated while individual drugs may exist within the predicted no effect concentration, mixture effects due to similar antiretrovirals with the same mechanism of action against the virus may lead to synergic or additive impacts against the virus.
- Another consideration is the analysis of antiretrovirals in different aquatic species, sediments, and soils to obtain more insights about their role and possible effects in the ecosystem.
- Targets in natural and drinking water should be set to protect human and environmental health. These targets may be guided by the results of this study.

CHAPTER 5: VALUATION OF ECOSYSTEM SERVICES AND DISSERVICES AT HEALTH CLINIC GARDENS IN SOUTH AFRICA

5.1 INTRODUCTION

Home and community gardens are necessary components of urban and rural areas, as they provide ecosystem services to humans, and therefore play an essential role in food and health security (Cilliers et al., 2018). Values need to be determined for each of these ecosystem services to sensitise people about their importance in order to increase sustainable gardening practices (Cornelius, 2016). The North West Provincial Department of Health believed that gardens surrounding clinics could provide benefits to the society. As a result, most of the 300+ urban and rural community health clinics in the North West province have developed gardens on their premises (Cilliers et al., 2018). Health clinic gardens are government funded and managed gardens and were established to contribute to the nutritional needs of community members (Cilliers et al., 2018).

5.1.1 Clinic gardens and Tswana tshimo

According to Cornelius (2016), the health clinic gardens have a specific lay-out that is typically found in the home gardens of the Tswana people, namely the Tswana tshimo. The Tswana tshimo home garden lay-out could be considered to be a model of sustainable resource management in South Africa (Molebatsi et al., 2010). The Tswana tshimo consists of seven different micro-gardens, namely naga, lawn, vegetable garden, medicinal garden, ornamental garden, hedge and orchard (Molebatsi et al., 2010). The naga is natural areas where indigenous species grow, some species are harvested and used as food or medicine or the naga is used for grazing. The lawn contains regularly cut grass species and is more common in areas where water is available for irrigation. The vegetable garden is divided into sections in which different kinds of plants such as grains, leafy vegetables and fruit plants are cultivated. The medicinal garden contains different medicinal species planted and managed within a single area usually near the house. The ornamental garden consists of mainly flower beds that contain a large diversity of aesthetic species. Some plant species are shaped to form a hedge around the yard. The orchard consists of fruit trees which may also have medicinal value such as lemon trees. Molebatsi et al. (2010), also recognizes the lebala, which consists of the bare ground around the home that is kept open by sweeping and weeding as to keep snakes and other wild animals at a distance.

5.1.2 Community gardens

According to Kurtz (2001), "*community gardens are tangible arenas in which urban residents can establish and sustain relationships with one another, with elements of nature, and with their neighbourhood*". Guitart et al. (2012), found that community gardens have certain benefits, including social development and unity, improved health, access to fresh food, saving or financial profit, education, environmental equity, environmental sustainability, enhancing cultural heritage, life satisfaction, and increased biodiversity. These benefits are known as ecosystem services.

The disadvantages or challenges of community gardens include soil contamination, lack of water, safety issues, neighbourhood complaints, managing volunteers or volunteer drop off, theft of tools and vegetables/fruit, lack of funding, cultural difference matters, and lack of knowledge (Guitart et al., 2012). Some of these disadvantages or challenges are known as ecosystem disservices. According to Tzoulas et al. (2007), if green infrastructure and ecosystem health are improved it provides the environmental settings of public health. Public health includes physical, psychological, social and community health (Tzoulas et al., 2007).

Therefore, it could be said that a healthy environment, with a healthy supply of water, promotes the health of the people.

5.1.3 Ecosystem services

According to the Millennium Ecosystem Assessment (MEA) (2003), ecosystem services (ESs) refer to the benefits people derive from the functions of ecosystems. Ecosystems can provide many direct and indirect benefits in terms of providing provisioning, regulating, supporting, and cultural ESs (Cilliers et al., 2013; Gómez-Baggethun, et al. 2013). These benefits could also be provided by health clinic gardens. Provisioning services consist of material products such as food, genetic resources, fuel wood, medicinal resources, building material and fresh water. Regulating services includes regulation of ecosystem processes which humans benefit from, regulation of climate and water, noise reduction, biological control, pollination and soil quality. Supporting services maintain all other ecosystem services; it includes biomass production, nutrient and water cycling, provisioning of habitat, and maintenance of genetic diversity. Cultural services consist of spiritual enrichment, cognitive development, recreation and aesthetic experience that make up the non-material benefits humans obtain (TEEB, 2011; Cilliers et al., 2013; Gómez-Baggethun, et al. 2013). According to Davids et al. (2016), when ESs are adequately managed and protected human development was maintained.

5.1.4 Water as ecosystem service

Water is an essential resource that allows humans and other organisms to sustain healthy lives (Obi et al., 2006; Brouwer and Hassan, 2013). Water is also very important in the world's economy as it is essential in all human activities (Obi et al., 2006). Therefore, clean water is vital for socio-economic development (Obi et al., 2006). It has been found that rural areas, in comparison with urban areas, have lower levels of access to safe drinkable water (Ashton and Ramasar, 2002; Obi et al., 2006). Furthermore, South Africa is considered to be a "water stressed" country with 1000-1666 m^3/person/year (Ashton and Ramasar, 2002). Water stressed means the existence of "frequent seasonal water supply and quality problems, accentuated by occasional droughts" (Ashton and Ramasar, 2002).

When considering the environment, the Constitution of South Africa states that "everyone has the right to an environment that is not harmful to their health or well-being" (South Africa, 1996). This links to health care, food, water and social security which states that "everyone has the right to have access to sufficient food and water" (South Africa, 1996). Therefore, it is important to preserve ideal environments conditions to maintain human health. Knowledge about the value of water quality would be more significant to the public if modelled changes presented the risks associated with recreational opportunities and drinking water. These risks include the direct and indirect consequences of pharmaceutical exposures in surface and drinking water, which can in turn affect the value of water as an ecosystem service.

5.1.5 Ecosystem disservices

Ecosystem disservices (EDSs) refers to natural or anthropogenic-impacted ecosystem functions negatively affecting human well-being. Some examples of EDSs include biological hazards, invasive species, floods, nutrient runoff, erosion, storms, heat waves and pests (Lyytimäki and Sipilä, 2009; Van Döhren and Haase, 2015; Davids et al., 2016). According to Lyytimäki and Sipilä (2009), when one only considers ESs without considering the EDSs it may be counterproductive, for example invasive species can be seen to increase the biodiversity of an ecosystem (ESs) but, it could also lead to environmental problems such as competition with indigenous species (EDSs). Lyytimäki and Sipilä (2009), states that to manage urban environments successfully one must know what advantages and disadvantages are provided by the ecosystems.

5.1.6 Valuation of ecosystem services

Most people are not aware of the importance of ESs and how dependent people are on them, mainly since ESs are generally “not properly valued, priced or paid for” (Le Maitre et al., 2007). Most of the ESs, such as regulating services and cultural services, are basically ‘free’ and can therefore not be marketed, or are difficult to market (Bunse et al., 2015).

De Groot et al. (2002), divide the importance or value of ecosystems into three types: ecological, socio-cultural and economic value. The ecological value of a system is determined by the integrity of the regulation and habitat functions of the ecosystem and by ecosystem parameters such as complexity, diversity, and rarity (De Groot et al., 2002). Human perceptions play an important role in determining the socio-cultural value of an ecosystem. Social reasons play an important role in the identification of important environmental functions (De Groot et al., 2002). According to Sutherland et al. (2016), it is “important to trace the social and political trajectories of local practices and decision making” to better understand the changing value of ESs. The concept of ecological economics is used by Sutherland et al. (2016), which focuses on creating markets for ESs and payments for ecosystem services (PESs) programmes in order to value the environment. According to Tyrväinen (2001), monetary values are assigned to non-priced ecosystem services through the process of economic valuation. Limburg et al. (2002), further state that through economic valuation, one could determine the “difference something makes to well-being”.

According to Dallimer et al. (2014), it is apparent that the perceptions on the value of nature that humans have may be different from the derived economic value. This study will focus on a comparison between the values perceived by stakeholders and the economic value derived; therefore, it uses nonmonetary as well as monetary valuation techniques.

Economic valuation methods are divided into four basic types: (1) direct market valuation, (2) indirect market valuation, (3) contingent valuation, (4) group valuation, as seen in Table 44.

Table 44: A comparison of monetary valuation methods for ecosystem services adapted from De Groot et al. (2002), Farber et al. (2002), Brown et al. (2006), TEEB (2011), Miller and Lloyd-Smith (2012), and Czembrowski and Kronenberg (2016).

Methods		**Summary**	**Which services?**
Direct market valuation		Exchange value that ESs have in trade.	Provisioning services, such as food
Indirect market valuation	Avoided cost	How much spending was avoided because of ESs provided? ESs allow society to avoid costs that would have been incurred in the absence of those services.	Carbon sequestration Flood control Waste treatment by wetlands
	Replacement cost	ESs could be replaced with human-made systems. It is determined what the cost will be of replacing a lost good or service.	Regulating services, such as natural waste treatment
	Production function or Factor income	How much is the value-added by the ESs based on its input to production?	All provisioning services Moderation of extreme events Pollination Biological control
	Hedonic pricing	Service demand may be reflected in the prices people will pay for associated goods or properties. It can be described	Use values only, recreation and leisure

Methods		Summary	Which services?
		as the extra amount paid for higher environmental quality or more attributes of the properties.	
	Travel cost	Cost of visiting a site: travel costs (fares, car use) and also value of leisure expended. The travel costs can be seen as a reflection of the implied value of the service.	Use values only, recreation and leisure
Contingent valuation (Stated preferences)		How much is the survey respondent willing to pay to have more of a particular ESs. Social survey questionnaire is usually used to value a single service	All services
Group valuation	Participatory environmental valuation	Asking members of a community to determine the importance of a non-marketed ES relative to goods or services that are marketed.	All services

As seen in Table 44, it may be necessary to use more than one valuation method simultaneously for some ESs. Usually only one of the methods described above is used to determine individual values for all ESs. These individual values are then combined to obtain the value for all ESs of a specific area (Farber et al., 2002).

Contingent valuation is mainly used in this research project, as it can be applied to all ecosystem services. According to Christie et al. (2012), the contingent valuation method, which makes use of questionnaires to obtain people's willingness to pay (WTP), is the most widely applicable and used method.

5.1.7 Research aim and objectives

The research aim is to explore the actual and stakeholders' perceived presence and value of ecosystem services and disservices provided by health clinic gardens in the Dr Kenneth Kaunda District Municipality and Phokwane Local Municipality.
The following three objectives were identified for this study:

- Objective 1: To conduct an ecological survey to determine the social, cultural, economic and ecological value of a variety of ESs and EDSs (actual values of ESs).
- Objective 2: To develop and conduct a social survey to determine the social, cultural, economic and ecological value of a variety of ESs and EDSs as perceived by different stakeholders (perceived values of ESs).
- Objective 3: To compare/test relationships between the actual and perceived values of ESs.

5.2 MATERIALS AND METHODS

5.2.1 Research method

The design of the study is a qualitative-quantitative descriptive survey. Thus, this study comprises of a multiple method (or multimethod), i.e. quantitative and qualitative approaches were followed. Qualitative data in the form of interviews and observations, while quantitative data was in the form of survey data.

5.2.1.1 Study area

This study is part of a larger research project on health clinic gardens and community engagement in the North West Province and Northern Cape Province, South Africa. Within these two provinces, the following two municipalities were selected:

Dr Kenneth Kaunda District Municipality: This study forms part of an elaborated research initiative within this district, which was initially requested by the North West Provincial Department of Health. Within the Provincial Department of Health, a social franchising initiative was launched (Robinson, 2015) from where Prof Cilliers was requested to explore health clinic gardens as part of this initiative. This district municipality has 35 clinics with gardens.

Phokwane Local Municipality: As the Dr Kenneth Kaunda District Municipality is also one of the ten national pilot sites for the National Health Insurance (NHI) system, it can be argued that continuous research within one district doesn't provide a true reflection of health clinic gardens. To increase transferability, this research will also be extended to health clinics within the Phokwane Local Municipality. This particular municipality has been selected for two reasons, firstly because the AUTHeR has a long-standing relationship within this community, which enables an established network. In addition, the Phokwane Local Municipality represents the typical complexities of semi-urban communities with limited health resources. This local municipality only has five clinics with gardens.

5.2.1.2 Population and sampling

All voluntary stakeholders of the health clinic gardens in the North West and Northern Cape provinces have been interviewed, a total of 70 stakeholders. Stakeholders that have been interviewed include facility managers, groundskeepers, workers from the Community Works Programme (CWP), nurses, community health workers (CHW), clinic committee members, nutritionists and community members.

5.2.1.3 Data collection

5.2.1.3.1 *Interview schedule*

At each clinic, an interview schedule has been used to determine the perceived value the participants/interviewees/respondents/stakeholders (the term participant will be used) attach to ecosystem services and disservices found in the clinic garden. The interview schedule is in the form of an individual interview with structured and semi-structured questions. The interviews were digitally voice recorded and was transcribed. The interviews take approximately 20-40 minute's time to complete. Due to the language barrier an interpreter has been used to improve communication between the researcher and the participants.

It is important that the researcher differentiates between content and process during the interview (Greeff, 2011). The content of an interview is what the participants are saying, while the process is noticing how the participant talks and behaves during the interview (Greeff, 2011). The "content" has been obtained by asking open-ended questions throughout the interview to allow participants to express their perceptions as well as close-ended questions. In order to obtain the "process" the researcher has been making field notes and digitally

voice recording the interviews (Greeff, 2011). The field notes include what was heard, seen, experienced and thought about during the interview (Greeff, 2011; Schurink et al., 2011). These notes will help the researcher to remember what occurred during each interview (Greeff, 2011). The information obtained by using a digital voice recorder was transcribed soon after the data has been collected.

5.2.1.3.2 *Interview protocol*

Signed informed consent was obtained from each voluntary participant before the interview begins. Prior to starting, each participant was given a brief project description and an assurance of anonymity.

Following the introduction, the interview schedule begins with demographic questions. According to Cottrell (2003), literature indicates that socio-demographic variables, such as age, income, education, and political ideology, are often used as predictors of behaviour. Therefore, the participant's age, gender, home language, literacy level, occupational role, religion, health, political orientation, economic status and connectedness to nature has been obtained.

Each ESs concept has been explained to the participants until they seem to understand the concept. An illustration was made to represent each ESs. These illustrations help the participants to fully understand the different ESs, as it is a visual representation. The illustration was shown to the participants and questions asked about each ESs. The participants were asked whether he/she perceives the ESs to be present (don't know, no, average, or sufficient). The participant was then asked to indicate which values (economic, environment, social, and/or cultural – also shown with illustrations) he/she perceives the ESs to have, and to indicate the value's importance by organizing the values from most important to least important (the participant can place the illustrations in this order). Finally, the participant was asked if he/she is willing to pay for the ESs perceived to be present and if they are willing to pay, what is the amount they are willing to pay for each ESs.

The values economic, environmental, social and cultural are based on the five types of capital used in the sustainable livelihood approach, namely natural capital, human capital, economic or financial capital, physical capital and social capital (Morse and McNamara, 2013). Natural capital includes natural resource stocks such as soil, water, air and genetic resources as well as environmental services such as the hydrological cycle and pollution sinks (Morse and McNamara, 2013). Human capital concerns skills, knowledge and labour, while also including good health and physical capability (Morse and McNamara, 2013). Economic or financial capital is the capital base which includes cash, credit/debt, savings, and other economic assets (Morse and McNamara, 2013). Physical capital includes infrastructure such as buildings and roads, production equipment and technologies (Morse and McNamara, 2013). Social capital comprises social resources such as networks, social claims, social relations, affiliations and associations (Morse and McNamara, 2013). In the interview schedule four of these capitals are used, namely natural capital (environmental value), human capital (cultural value), economic or financial capital (economic value) and social capital (social value). The physical capital is not taken into consideration, since it comprises of human made assets and does not necessarily have a value when considering ESs or EDSs.

As previously mentioned, ESs are divided into four main categories, namely provisioning, regulating, supporting and cultural services. Provisioning services that were valuated through the interview schedule include: food, raw materials, medicinal plants and fresh water. Regulating services that were determined concerns the local climate and air quality regulation, carbon sequestration and storage, moderation of extreme events, wastewater treatment, erosion prevention and maintenance of soil fertility, pollination, and biological control. The provision of habitat for species and the maintenance of genetic diversity by means of supporting services will also be determined. Cultural services that were investigated include: recreation, health and well-being, spiritual experience and sense of place, aesthetical appreciation and inspiration for culture, art and design.

Questions on EDS was also asked, for example "do you think any of the plants in the garden can make you sick/ill/allergic" (Cornelius, 2016). Stakeholders working in the vegetable gardens have been asked about the amount of vegetables grown in the garden to determine the food supply per growth season, which is often

captured in a logbook. In addition to the social survey, an environmental survey will also be conducted to determine the species- and garden composition. Some tree measurements will also be made.

5.2.1.4 Ecological surveys

The ecological surveys were used to determine or calculate the actual value of some ESs. Therefore, some ESs and EDSs have been selected which are easier to determine the value off, as seen in Table 45 (ESs) and Table 46 (EDSs). The ecosystem disservices were chosen based on the paper by Shackleton et al. (2016), wherein ecosystem disservices are defined and characterised. Ecosystem services and disservices surveys have been conducted at the 27 health clinics in the DR Kenneth Kaunda District Municipality and the five health clinics in the Phokwane local municipality.

Table 45: A summary of the ES that were surveyed and the value type that was obtained from each ES.

Provisioning services	The market value of the vegetables and fruit produced in the health clinic gardens were determined.	Economic value
	It was determined for each plant species if they have any potential medicinal value or can be used as raw materials such as building material or firewood (literature).	Cultural value
Regulating services	The height, quantity and stem circumference at breast height/ground level, of all tree species in the clinic gardens were determined in order to calculate carbon sequestration (Van Rooyen et al., 2012).	Ecological and economic value
	Total tree cover was determined as a percentage of the clinic garden to determine possibility of climate regulation (heat island effect).	Ecological value
Supporting services	The layout of the garden (Tswana Tshimo) was determined as well as the area covered by each micro-garden (Ornamental, lawn, vegetable, naga, and lebala) and the total garden size in order to determine habitat diversity (Molebatsi et al., 2010).	Ecological value
	The plant diversity was calculated and determined for each micro-garden of each health clinic garden. The proportion of indigenous and native species and any red data species were determined.	
	There was observed whether there are any bird nesting sites in the trees that serves as habitat for bird species, bird species will then be identified if possible. Fuller et al. (2007), noted the bird species that were heard or seen in the green space boundary, which excluded overflying birds that were not actively feeding or hunting in the area.	
	It was determined what plant species have the potential to attract or house bird species (literature).	
	It was determined if there are any plant species that potentially attract butterflies and other insects (literature).	

Cultural services	It was determined if there are any plant species present in the gardens that could have spiritual or cultural value to the Tswana people (literature).	Cultural value
	Any sign that people use the garden for its aesthetical value was recorded by observing the presence, size and variety of ornamental micro-gardens.	Social and cultural value
	Recreation value – any sign that people use the garden benches and/or people sitting on the lawn was recorded.	Social and cultural value

Table 46: A summary of the EDSs that were surveyed.

Allergies	It was determined which of the plant species has the potential to cause allergies (literature).
Pests	It was observed if there is any pest damage on plants. There is no quantification of pests, only recognising if pests are present or not.
Accidents	It was determined if there are any branches that have the potential to fall and cause damage, for example branches dangerous bends of >45°.
Damage to buildings and other structures	It was observed if any plants cause damage to the buildings.
Blockage of view	It was observed if the plant species obstruct the view.

5.2.1.5 Data analysis

5.2.1.5.1 *Quantitative data*

The data was analysed by using PRIMER version 6 and STATISTICA version 13. Basic descriptive statistics and correlations such as ANOVA's and ordinations were applied to detect significant differences in species composition, and ecosystem provision. Regressions were also used to understand the influence of certain variables within groups. The demand for clinic gardens, and specific health and wellbeing benefits from them, were also determined through the above-mentioned analyses. Commonly available statistical guidance was used to select the right co-variance.

Contingent and direct market valuation techniques were used to determine the economic value of the ecosystem services in the clinic gardens. The stakeholders' perceptions were then compared to the calculated economic value of the ecosystem services. Quantitative and qualitative data sets were declared separately and only integrated in the discussion of the results.

5.2.1.5.2 *Qualitative data*

The qualitative data was analysed by using ATLAS.ti a software programme (Friese, 2014). ATLAS.ti is a tool used for supporting the process of qualitative data analysis (Friese, 2014).

5.2.1.6 Dissemination and publication

The results of the research were published in the form of a master's dissertation in fulfilment of the requirements for the degree *Magister Scientiae* in Environmental Sciences at the North-West University. The dissertation was published on completion of a MSc. study in the year 2019.

5.3 RESULTS

5.3.1 Presence and actual value of ecosystem services (ESs) and disservices (EDSs) provided by health clinic gardens

Clinic gardens were generally made up of a variety of different species influenced both by western and traditional practices (Molebatsi et al., 2010; Lubbe et al., 2011; Cornelius, 2016; Davoren et al., 2016). Health clinic gardens also had a variety of different micro-gardens (Molebatsi et al., 2010; Cornelius, 2016), that support a variety of different plant species contributing to the provisioning of ESs. These ESs had different values.

When considering the provisioning ESs, food had economic value as it has a monetary value. Raw materials had cultural value as it is used differently by different people and cultures (Van Wyk and Gericke, 2000). Medicinal resources are also used differently by different cultures (Van Wyk et al., 2009; Semenya and Potgieter, 2014), and therefore it could be said that it has cultural value. According to Van Wyk et al. (2009), each culture has developed solutions for various health aspects “that resonate in harmony with the worldview of that culture”. Semenya and Potgieter (2014), further stated that the practice of growing medicinal plant species in gardens may in the long term sustain the plant species and the indigenous knowledge about the species together with the cultural identity.

When considering regulating ESs, carbon had a monetary value, therefore, carbon sequestration and storage could be said to have economic value. Carbon sequestration also had ecological value as trees contribute to the removal of pollutants, such as ozone and nitrogen dioxide from the air which makes the atmosphere cleaner (Stewart et al., 2001). Stewart et al. (2001), also stated that trees have social, physical and health-related values as they are an integral part of an urban environment. Local climate regulation had ecological value as it plays a role in reducing the temperature of the environment at a local scale, which will only be confirmed when temperatures are measured in green and non-green urban areas and compared (Bowler et al., 2010).

When considering supporting ESs, the habitat service had an ecological value as it supports much different fauna and flora within different micro-gardens at the health clinics. The species diversity (maintenance of genetic diversity) ESs had an ecological value as the gardens support a variety of different species. Jansson and Polasky (2010), support this by stating that biodiversity supplies species with different traits that are needed to maintain functions for ESs to be generated. They further stated that this important function of biodiversity should not be taken for granted, as a decline in biodiversity can lead to a decline in the generation of ESs. The habitat provisioning for birds’ ESs had an ecological value, as habitat is created for different bird species.

When considering cultural ESs, the clinic gardens had social and cultural value due to recreational opportunities for patients and clinic personnel. The aesthetically pleasing nature of clinic gardens could also be said to have social and cultural value. The plants having spiritual or cultural significance to people is of cultural value, as different cultures view plants differently (Van Wyk and Gericke, 2000).

Even though there were some EDSs present in the health clinic gardens, the benefits of the gardens seem to outweigh the disadvantages. It is however important that these EDSs should be acknowledged and managed so that they do not become bigger challenges in the future.

5.3.2 Perceived presence and values of stakeholders on ecosystem services (ESs) and disservices (EDSs) provided by health clinic gardens

Most interviewed stakeholders were facility managers and groundskeepers, with an average age of 44.63 years. Most interviewed stakeholders were females, and most of them spoke Setswana as a home language. The highest level of education of most stakeholders was the completion of secondary education. Most of the stakeholders belong to a church which they attend once a week for Sunday services. Most stakeholders visit the clinic every few weeks or once a month for their health. Most stakeholders perceived the clinic to help the community with health care, while a majority feel that politics meant nothing or were not interested in politics. The income of most stakeholders was from the Department of health, and most interviewed stakeholders feel completely connected to nature.

ESs were stated by the stakeholders as either being absent, not sufficient (not enough) or sufficient (enough); there was also an option of “don’t know” if the stakeholders did not know anything about a specific ES. The average presence values given by all the interviewed stakeholders can be seen in Figure 43. The ESs perceived to be the most present was erosion prevention and the maintenance of soil fertility (2.26), as well as health and well-being (2.26). These were closely followed by the maintenance of genetic diversity (2.20) and carbon sequestration (2.14). The provisioning of fresh water was perceived to be the least present by the stakeholders.

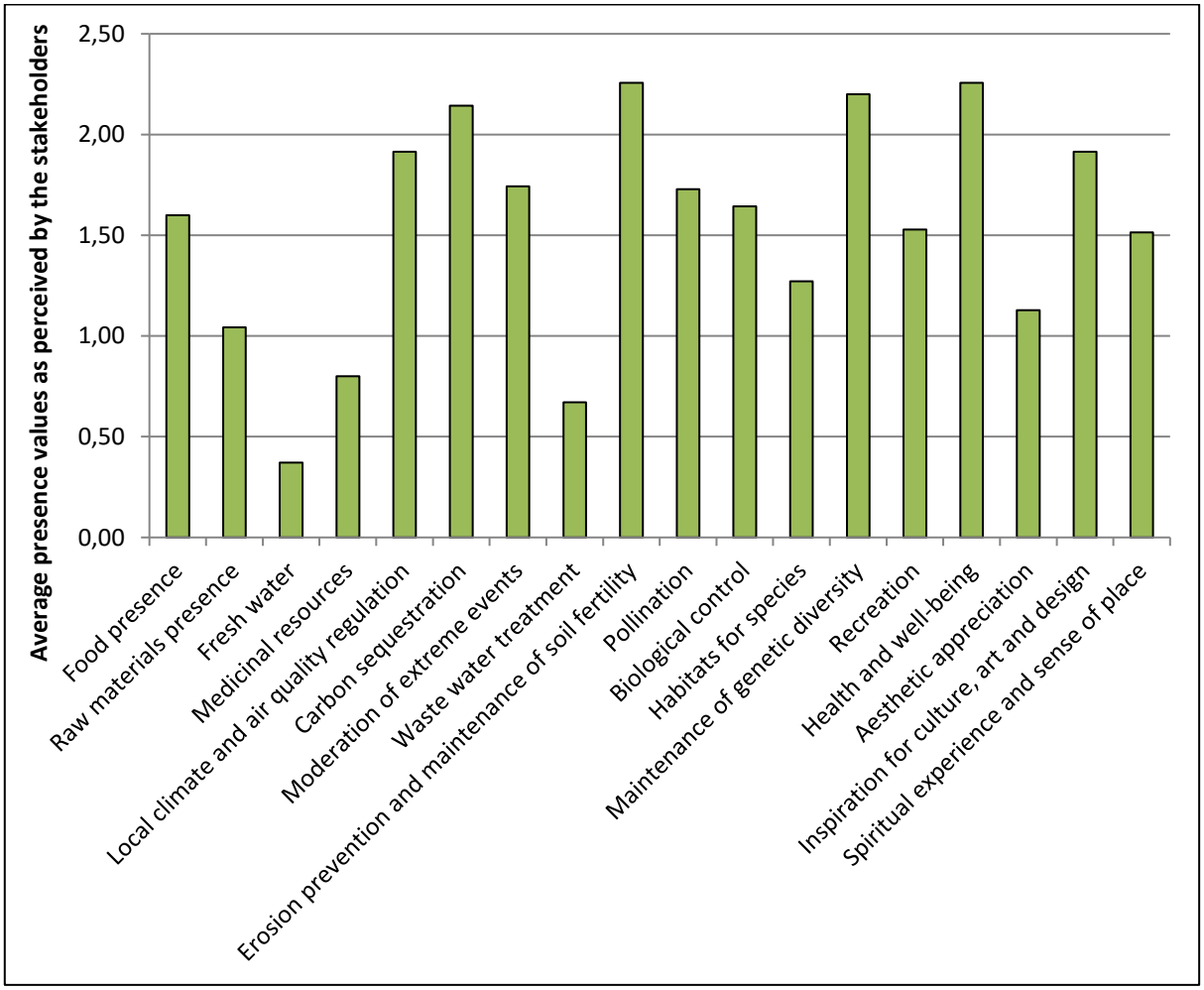


Figure 43: The average presence values for each ES as perceived by the stakeholders of all the health clinic gardens visited.

Stakeholders mostly stated that the ESs were not sufficient followed by not present (Figure 44). Most of the interviewed stakeholders did not perceive freshwater to be present in the health clinic gardens (Figure 44).

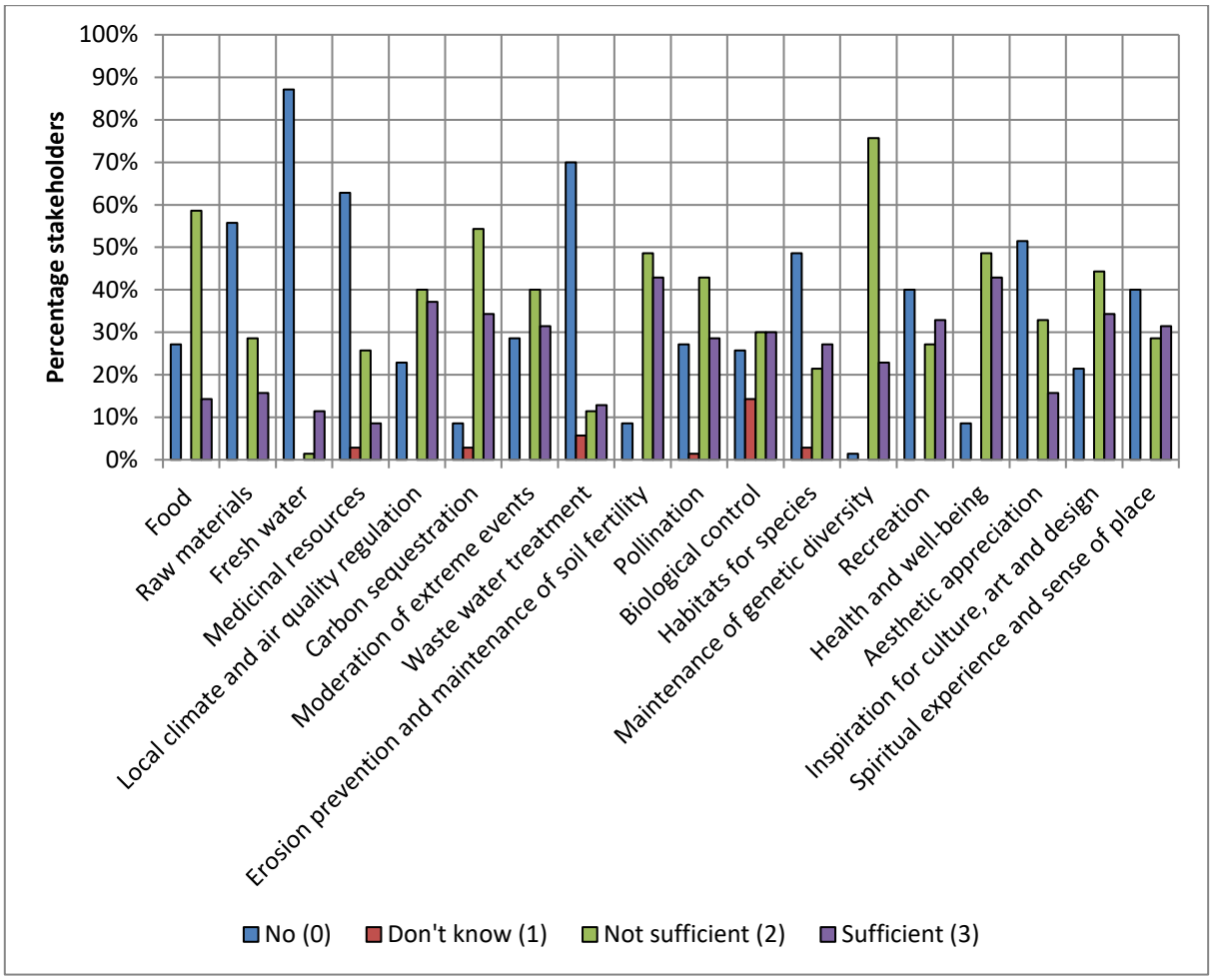


Figure 44: The percentage of stakeholders that perceived the ESs to be present at health clinic gardens.

Generally, there was no or very little difference in the perceptions of the different stakeholders. It would appear that greater contact with nature makes some stakeholders more aware of its benefits, for example, groundskeepers that were generally more actively involved in the clinic gardens may be more aware of the benefits nature provided.

It could be that there was more social cohesion in some sub-districts than others, in other words, some sub-districts could have more close-knit communities than others. It would appear that the Phokwane sub-district differs the most from all the other sub-districts, as it was the only sub-district included in the study from the Northern Cape Province.

Most stakeholders perceived the ESs to have ecological value (48.50%); this was followed by social value (45.64%), economic value (24.79%) and lastly cultural value (19.37%). These results were in contrast with the findings of Cornelius (2016), where cultural services were regarded as being the most important. In this study by Cornelius (2016), only the presence of ESs was recorded and not the real value. The value was assumed based on whether something was present in the clinic garden (Cornelius, 2016). Turpie et al. (2017), also found that cultural values were among the highest values associated with nature. Even though many ESs are related to different cultures, it seems that the stakeholders do not realise this or that it did not play a large role any more as people become more westernised.

Cornelius (2016), found that the ESs spiritual experience and sense of place was perceived to be the most important ESs, in this study this was not the case. An average of 32.14% of the interviewed stakeholders perceived this ESs to have any value, in contrast with the 98.61% that rated this service as valuable in the study of Cornelius (2016).

The provisioning of food was an important function as an average of 46.07% of the stakeholders perceived this service as being valuable. This result indicates the potential of health clinic gardens to be sources of food to the wider community, especially for more vulnerable patients.

People appear to enjoy being in the garden, as the clinic gardens provide many different benefits. The social value of the clinic gardens was also very important as it brings different people together. It is important that different stakeholders work together and share their knowledge in order to improve gardening practices. Costanza et al. (2017), stated that there is also a need to inform the wider community of the importance of ESs. The support of the public will determine the success of biodiversity conservation (Miller and Hobbs, 2002). In order to obtain broader public support, a wider, more diverse audience should be reached with a message that clearly states how important biodiversity is and how it influences human lives (Miller and Hobbs, 2002). People living in a city often perceive conservation as something that occurs somewhere else such as in a national park, wilderness area, or rainforest and is usually experienced second-hand on television or in a magazine (Miller and Hobbs, 2002). Thus, people attach importance to biodiversity in terms of entertainment value (Miller and Hobbs, 2002).

The protection of habitats may be promoted by compassionate and informed members of the local community, the so-called “champions” (Cornelius, 2016), because many land-use decisions are made by country officials, city administrators, and landowners (Miller and Hobbs, 2002). For example, in Australia community-based projects have been implemented to protect the ecological health of the landscapes so that flora and fauna are protected, and the farmers can make a living (Dilworth et al., 2000). A positive feedback loop is created by community-based efforts as they use local support, which in turn creates an interest in local conservation issues (Miller and Hobbs, 2002).

Champions are considered to be the individuals of a community that are inspiring and enthusiastic, who usually drive and motivate others in the community to act (Cornelius, 2016). Cornelius (2016), found that some health clinic gardens had examples of champions where one person was motivated to make a success of the clinic garden and to inspire other members of the community to participate. Cornelius (2016), stated that these champions should be appreciated and empowered so that they can play a larger role in the management of the community instead of “corrupt governmental institutions” having all the say. Tsegay et al. (2014), found that community champions include activists, community leaders, elders, traditional leaders, village heads, councillors and members of parliament. Some of the roles of champions include supporting community involvement and facilitating group discussions (Tsegay et al., 2014). These champions can, therefore, be vital in the survival of clinic gardens.

There were some negative associations made with the clinic gardens such as pests or allergies, even so, the percentage of stakeholders that perceived EDSs to be present was quite low, which indicates that the perception of the usefulness of the clinic gardens is overall more positive.

5.3.3 Qualitative data analysis, results and discussion

The qualitative data obtained from the interviews were analysed, by transcribing the relevant data and using ATLAS.ti software for analysis. A total of 18 transcripts were analysed which resulted in 87 codes which were grouped into 14 categories which were then grouped into three themes.

The themes contain codes relating to components, positive and negative, present in health clinic gardens which contribute to the success or failure of these gardens. The participants described the ecosystems of clinic gardens as providing raw materials, carbon sequestration and storage, erosion prevention and maintenance of soil fertility, wastewater treatment, moderation of extreme events, local climate regulation and pollination which were all seen as benefits provided by plants. The negative features or constraints by plants were described by the participants as causing damage to buildings and other structures, and allergies. Other negative features of plants were that they cannot survive wastewater chemicals, plants do not grow, plants being inaccessible and trees being too small. Animals were described by the participants as either being good

or bad. Good animals include birds and insects both responsible for pollination. Bad animals include ants, snakes, mice, rats, pigeons, sheep and spiders. It was also determined that the water source used in the clinic gardens was either from a borehole (“boorgat”) or from municipality accessible taps.

The understanding of the anthropogenic dimension of clinic gardens was that there were three main stakeholder groups, namely facility managers, groundskeepers and community members. These stakeholders had different perspectives on the role of the clinics, government, economy and politics play in the success of health clinic gardens. The perceived role of the clinics in comprehensive community health was assisting the community, being easily accessible, educating people, maintaining the trees in the garden, providing food to orphans and being a source of income. Many of these perceived roles were not actually the role of clinics, but become a burden to these clinics because they were not met by the government. The stakeholders perceive the role of the government to provide the clinics with seed and equipment, in order to make the clinic gardens successful. The provisioning of seed and equipment did not, however, occur at all the clinics. There seems to be a dissonance about the free versus the fee-for-vegetables. Some participants feel that vegetables and other ESs were free and cannot be paid for, while others perceive the ESs to have monetary value and were therefore important for the economy. Most participants acknowledge political views, but were not really interested. Therefore, political views did not seem to play a large role in the success or failure of health clinic gardens.

All of these components come together in the health clinic gardens creating interdependence between environmental and anthropogenic components of a clinic garden. In order for people to cultivate a garden both environmental and human elements were necessary, for example, plants (natural) and equipment (human). Emotional experiences as a valuable outcome of clinic gardens were achieved when a beautiful garden (nature) inspires feelings of happiness or love (human). Food production entails growing food such as fruit (nature) and giving it away to patients (human). Alternative medicines were obtained from the garden as plants such as aloes (nature) were used to treat ailments (human). Unfortunately, there were also reasons given by participants for why the vegetable gardens at the clinic were not successful. There were different reasons including a lack of plants and seeds (nature) and food being stolen (human).

Health clinic gardens were complex social-ecological systems dependent on many different components to be successful. There were adequate inputs and resources required to create, manage and maintain the clinic gardens.

5.3.4 Consolidation of results on “actual value” and “perceived value” of ecosystem services in health clinic gardens

It has been determined in South Africa that even economically deprived households with few resources still tend to plant, care for, and water trees (McHale et al., 2013). This care about nature could indicate that people are aware of how dependent they are on the resources provided by trees, which is supported by the fact that many communities meet their needs for food, cooking energy, medicine, construction material, and water from their surrounding environment (McHale et al., 2013). The same could be said about health clinic gardens that were sources of many different ESs.

When consideriing provisioning ESs, it was found that both Steve Tshwete clinic and Khuma clinic had among other clinics, the most potential food plants species, raw material plant species, and medicinal plant species. These two clinics could, therefore, serve as good examples of clinics that maximise their provisioning services output. Even so, the perceptions of the stakeholders indicate that they were not aware of this potential, as the stakeholders at the Steve Tshwete clinic perceived only food plant species to be present, and at Khuma clinic only one stakeholder perceived raw materials and medicinal resources to be present (both perceived food to be present). The clinics with the best potential to regulate the local climate and the carbon in the atmosphere were Jan Kempdorp CHC and Botshabelo clinic. The stakeholders seemed to acknowledge this value as all the stakeholders perceived these two ES to be present. Especially the stakeholders at the Jan Kempdorp CHC seemed to fully realise the potential as both stakeholders stated that these ES were sufficiently present. The

Botshabelo clinic had both many aesthetically pleasing plant species (ornamentals) and two potentially spiritual or cultural plant species, giving it a high cultural value. For the aesthetical appreciation, only one stakeholder perceived it to be insufficient, while for spiritual or cultural value all four stakeholders perceived this ES to be present, of which three stated it was sufficiently present.

The clinics that could serve as good examples of clinics where the economic value of ESs seemed to be realised were Mohadin, Botshabelo, Delekile Khoza, Bophelo and Nomimi Mothibi. The perceptions of the economic value were however different from the actual value, at only one of these clinics the actual and perceived value ratings were the same. Therefore, the perceptions of the stakeholders play an important role, as the clinics with the highest economic value ratings were Tswelelang 1 and Mohadin, while in the comparison the Botshabelo clinic was the clinic with the highest equal actual and perceived value ratings.

The Mohadin clinic could serve as a good example of a clinic where the ecological value of ESs seemed to be realised. However, the perceived value ratings are much higher than the actual value rating, possibly indicating that the stakeholders do not really realise the value of the ESs, or they might be maximising the use of the limited ESs.

Even though it may seem that the Jan Kempdorp CHC could serve as a good example of a clinic where the socio-cultural value of ESs was realised as this clinic also had the highest ecological value rating, the actual ecological value rating is higher than the perceived ecological rating. Therefore, there is still a need to inform the stakeholders of the actual ecological value of the ESs present in the clinic garden.

The values people place on ecosystem functions, structures and processes may not always express the full value, because humans are one of many species present in an ecosystem (Farber et al., 2002). According to Dallimer et al. (2014), the perceptions of the value of nature that humans have may be different from the economic value derived. Therefore, it is essential to consider both the actual value of ESs and the perceived value as stated by stakeholders. This has also indicated that the actual value of ESs must be communicated to the stakeholders so that they are aware of the actual benefits they derive from the health clinic gardens.

The importance of the qualitative results should also be expressed as this data contributed to the understanding of the perceptions of the stakeholders and in many cases provided reasons for emerging phenomena. It would, therefore, be recommended to anyone attempting to integrate social and ecological data to include a qualitative analysis.

5.3.5 Fresh water as ecosystem service

When considering the freshwater ecosystem service, very few stakeholders perceived the clinic gardens to be a source of fresh water, as only nine (of 70) stakeholders perceived eight clinic gardens to be a source of fresh water. Most clinics receive their water from the municipality. There were some stakeholders that said the clinic makes use of a borehole system, but there was often disagreement between stakeholders of the same clinic – indicating that in some cases the water just comes, and they do not know from where. Most stakeholders expressed their frustration of experiencing water shortages or water cuts mainly due to municipalities not paying their water bills. A few clinics said that they do not have vegetable gardens due to a shortage of water, as very little water can be given to the plants. There were however some clinics that had water tanks in which to store water.

5.4 DISCUSSION

In this study, two main aspects of health clinic gardens were studied, namely the actual value of selected provisioning, regulating, cultural and supporting ecosystem services (ESs) and the perceived value of provisioning, regulating, cultural and supporting ESs by different stakeholders, in the Dr Kenneth Kaunda District Municipality and Phokwane Local Municipality.

It was found that the health clinic gardens in the Dr Kenneth Kaunda District Municipality and Phokwane local municipality had a variety of micro-gardens that support a variety of plant species contributing to the provisioning of ESs. The ESs provided by the clinic gardens had different values. The vegetable and fruit plant species and carbon sequestration of plant species had economic value. Carbon sequestration, local climate regulation, habitat for species, and maintenance of genetic diversity had ecological value. Raw materials, medicinal plant species, recreational opportunities, aesthetically pleasing plant species, and plant species with spiritual or cultural significance had socio-cultural value. The health clinic gardens were also rated subjectively by using a scoring system where the health clinic gardens receive a value for the different ESs comparing the gardens with each other based on median values. These ratings indicate which clinics had economic, ecological, and/or socio-cultural value. These ratings can, therefore, be used to increase the different values at the other health clinic gardens. When considering only the ecological survey data, the clinics that had the highest rating for economic value were Tswelelang 1 and Mohadin, with Jan Kempdorp CHC achieving the highest rating for ecological and socio-cultural values.

Stakeholder perceptions of ESs and EDSs provided by health clinic gardens were determined, which differs from one stakeholder to the next and from one clinic to the next. This demonstrates the unique knowledge and perceptions each person has which together can lead to greater insight and knowledge on the subject. This exchange of knowledge between a diversity of knowledge systems could improve the capacity to react to conditions, change, responses, and in some cases fundamental relationships in the dynamics of social-ecological systems (Tengö et al., 2014). Health clinic gardens can thus be seen as pockets of social-ecological innovation (Cilliers et al., 2018), which could promote compassion in community members (Cornelius, 2016).

Generally, it seems that there were no associations between the actual values and the perceived values of the stakeholders. Only a relationship was tested between these values, but there could, however, be a lack of awareness of the benefits the stakeholders could obtain from the garden or the value it has. Because there were only three cases where the actual value and perceptions of stakeholders were statistically significant. The health clinic gardens were rated by using a subjective scoring system where the health clinic gardens received a value for the ecological data and social data based on median values. These ratings indicated which clinics had economic, ecological, and/or socio-cultural value. These ratings can, therefore, be used to increase the different values at the other health clinic gardens. There are a few clinics that could serve as a starting point for developing a management strategy that could be used to ensure that all health clinic gardens provide the maximum amount of ESs to benefit the community. There is however work needed to be done at all the health clinics concerning the ESs present in the gardens.

The health clinic gardens of the Dr Kenneth Kaunda District Municipality and the Phokwane sub-district were also compared to the health clinic gardens of the Bojanala District Municipality (Cornelius, 2016). It was found that the clinics had similar potential food plant species and potential medicinal plant species. Hedges were not common at any of the clinics. All the clinic gardens support many different species that seemed to be influenced by western and traditional practices (Tswana tshimo (Molebatsi et al., 2010)). Few stakeholders seemed to realise the potential of health clinic gardens to provide habitats for animals and plants, which may be due to a lack of understanding of this ES. In both studies EDSs were perceived to be a small problem, meaning that the benefits of the gardens are outweighing the disadvantages.

There were also some differences between the health clinic gardens of this study and of that conducted by Cornelius (2016). Cornelius (2016), found that the spiritual and sense of place ES was most abundant, while in this study erosion prevention and maintenance of soil fertility and health and well-being were most abundantly present. Overall the supporting ESs were perceived to be the least present in the Bojanala District Municipality (Cornelius, 2016), while in this study the provisioning ESs were perceived to be the least present and abundant. In the study by Cornelius (2016), only the presence of ESs were recorded and not the value of ESs as in this study. It, however, appeared that stakeholders valued cultural ESs most in the study of Cornelius (2016), as most stakeholders acknowledged it, while in this study most ESs were perceived to have ecological value and least ESs were perceived to have cultural value. Most of these differences could be ascribed to the fact that Cornelius (2016), did not have access to an interpreter throughout the study and did not make use of

illustrations for the ESs concepts. These additions to the methods used in this study seemed to make a big difference in the understanding of the concepts by the interviewed stakeholders.

The main aim of this study was to explore the actual and stakeholders' perceived presence and value of ESs and EDSs provided by health clinic gardens in the Dr Kenneth Kaunda District Municipality and Phokwane Local Municipality. Results showed that health clinic gardens provide a variety of ESs each with a value, which when communicated to the stakeholders of the health clinic gardens could contribute to more sustainable and resilient gardening practices that could be communicated to the larger community.

5.5 CONCLUSIONS

It is important to realise that the ESs provided by health clinic gardens have different values to different people. These values need to be recognised by the stakeholders so that when they communicate with the community, this knowledge can be shared. When people are more aware of the benefits provided by nature and the value thereof, more care might be taken in conserving these ESs. Some clinics could serve as good examples of gardens that have high economic, ecological, social and cultural value. It is, however, important to realise that to have a higher value does not necessarily mean that there should be more plant species planted; it is about managing the space available and making the best use of it.

Since the gardens studied were located at health clinics, it is a good place to increase opportunities to address various aspects such as nutritional deficiencies and stress reduction. Most people in the community visit the health clinics at some or other time and could be educated about the benefits and risks of gardens.

The information in this dissertation should be communicated to the Department of Health, all involved stakeholders, and the wider community. All relevant information and results should be communicated in a concise and clear manner. As it has been found, the services provided by a specific ecosystem can increase awareness and recognition in public policy (Costanza et al., 2017). A good option for communication is creating posters or pamphlets that could be distributed to the health clinics from where it is visible or obtainable for the community. The knowledge obtained from the posters or pamphlets could then be used by the stakeholders and the wider community to increase the deliverance of ESs in the health clinic gardens, as well as in their private home gardens.

5.6 RECOMMENDATIONS

There were certain limitations in this study that need to be addressed in future studies. At many health clinics, the stakeholders of the clinic gardens were not present on the day of visitation, mainly due to illness. Future studies should re-evaluate time management, so that there is enough time available to re-visit clinics to complete interviews with stakeholders, if needed.

Even though the illustrations of the ESs, values and EDSs were very helpful in explaining the different, often complex, concepts, they may have misled stakeholders and directed their answers. For example, the fact that an ES had an illustration may have influenced some of the stakeholders to feel that it, therefore, must be present in the clinic gardens. In future studies, a larger variety of illustrations for each concept could be created to indicate to the stakeholders the different scenarios in which the ESs and EDSs could be present. Another option is not using any illustrations, but rather using many short questions to obtain the perception of the stakeholders, instead of using three main questions for each ES (namely presence, value and willingness to pay).

In future studies, it would be a benefit to begin with another ES rather than food. Since food was the first ES asked about, the stakeholders seemed to think that the following ESs only concern the vegetable garden rather than the entire yard.

When considering the qualitative analysis of this data, the developed questionnaire has some shortcomings. The questions are mostly closed-ended and therefore more quantitative than qualitative. In order to broaden the understanding of the choice's stakeholders made during the interviews, more opportunity should be allowed to discuss their opinions.

The questionnaire should also be altered to only include three values, namely ecological, economic and socio-cultural value as it was found that it is difficult to distinguish between social and cultural value. There is a dissonance between the perceptions of different stakeholders. The one seemed to take the potential into account while the other only acknowledged the state the garden was in at the time of the interview. In future, it would add clarity if a time frame is given to the stakeholders so that such problems do not occur.

In order to broaden the scope of information available on health clinic gardens, data should be collected at other health clinic gardens throughout South Africa. Through comparing the results of a variety of health clinic gardens, a better perspective may be obtained of the spectrum of values offered by the gardens that may be used to create management strategies for the health clinic gardens.

Other aspects of health clinic gardens can be investigated such as including the abundance (e.g. percentage cover) of all the plant species (especially the food plant species) and not only the species richness. The inclusion of qualitative analysis in more ecological studies could also be implemented as this provides valuable information not obtained through quantitative studies.

Water plays an essential role, yet the water supply to clinics appears to be problematic. This aspect should be investigated further.

Summary of recommendations

The following recommendations are proposed for the valuation of ecosystem services and disservices at health clinic gardens in South Africa:

- Future studies should re-evaluate time management, so that there is enough time available to re-visit clinics to complete interviews with stakeholders, if needed.
- In future studies, a larger variety of illustrations for each concept should be used to indicate to the stakeholders the different scenarios in which the ecosystem services and ecosystem disservices could be present. Another option is not using any illustrations, but rather using many short questions to obtain the perception of the stakeholders, instead of using three main questions for each ecosystem services (namely presence, value, and willingness to pay).
- It is advised to start with another ecosystem services (such as water) rather than food.
- Questionnaires should allow the option to discuss an opinion.
- Questionnaires should be altered to only include three values, namely ecological, economic and socio-cultural value as it was found that it is difficult to distinguish between social and cultural value.
- More comparative information is required from other health clinic gardens to provide an improved perspective of the spectrum of values offered by gardens (used to create management strategies for the health clinic gardens).
- Other aspects of health clinic gardens can be investigated such as including the abundance (e.g. percentage cover) of all the plant species (especially the food plant species) and not only the species richness.
- The inclusion of qualitative analysis in more ecological studies could also be implemented as this provides valuable information not obtained through quantitative studies.
- In some instances, water supply to clinics seems to be problematic. A study can be undertaken to investigate water supply and quality issues experienced by clinics, especially in rural areas.
- The ecosystem evaluation techniques may be applied to larger systems, and more focussed on water.

CHAPTER 6: CONCLUSION

Humankind's relentless attempt to provide therapeutic benefits from chemicals is coupled to the inevitable discharge of pharmaceuticals into the natural and social environment. It is difficult to recognise the perils ahead, and even more difficult to provide an effective response. Nonetheless, if tipping points or thresholds are reached, it can lead to abrupt changes in the services provided by ecological systems, which, aside from triggering undesirable shifts in the natural balance, can have adverse social and economic consequences. Accordingly, this research has highlighted that the intersectionality of HIV expands into natural resources.

The research group has developed analytical methods with which the presence, concentrations, and spatial and temporal changes have been elucidated, improving sampling, extraction, and analytical procedures. Nearly all antiretrovirals were found in natural and some in drinking water, although none was found present in the fish tissue samples nor were antiretroviral metabolites found. However, the study provided evidence that antiretroviral exposure affected bacteriophages, embryonic development of freshwater snails, and of oestradiol and testosterone levels being influenced in the steroidogenesis (effects on steroid hormones).

Due to the need of millions of people to consume antiretrovirals every day, the relentless release of these compounds, based on our results, deleteriously effect non-target organisms in laboratory tests, and probably also in the environment. This may affect the ecology of natural aquatic ecosystems, and may alter the viral/bacterial interactions in WWTPs and receiving waters.

Humans are also exposed to these compounds via drinking water, and at concentrations exceeding calculated risk quotients. Although not found in this study, humans and other organisms may potentially also ingest antiretrovirals and their breakdown products via aquatic organisms such as fish. As the antiretrovirals and fluconazole are consumed in combinations, mixtures of residues occur in the environment, together with other pharmaceuticals, adding to the risk of deleterious effects posed by them to humans, WWTP functioning, ecosystem functioning, and the organisms (including the natural viruses) associated with them.

Regardless of the environmental concerns about the high concentrations and mixtures of pharmaceuticals present in our drinking water, more research is needed to determine the risks to human health from chronic low-level exposures to single and mixtures of antiretrovirals and fluconazole. Further research on antiretroviral occurrence, behaviour, fate, and potential risks of parent compounds and metabolites in water sources are needed to establish guideline values for regulatory purposes and prioritisation of interventions. The concentrations of antiretrovirals in water sources of developing countries such as South Africa is expected to increase seeing as the countries with high antiretroviral treatment programs do not have treatment guidelines regarding the presence of pharmaceuticals in wastewater treatment plant effluents. In addition, research to improve the drinking water treatment technology is required as the current water treatment processes are not able to remove effectively pharmaceuticals such as antiretrovirals and fluconazole.

However, solely relying on statements regarding end-of-pipe solutions that are often obvious and easy to hide behind are simplistic and not reflecting holistic reality. We demonstrated the critical intersections between the need to consume pharmaceuticals by PLWHA, the need to protect their dignity and confidentiality, the onus placed on PLWHA to adhere to prescriptions, the need to supply them adequately and timely with medication and health services, better understand the various social, psychological, psychiatric, and household constraints faced by PLWHA, reduce accumulation and the discard and waste, reduce off-label use, and to strengthen the understanding of ecosystem services at a scale wider than clinics.

An already stressed environment, inadequate waste management (solid and wastewater), social practices, and a strained health system, forced by the relentless need to distribute and adhere to antiretroviral therapy, all contribute towards environmental pollution, poverty and social tensions (including stigma, crime, and corruption). Consequently, the combination of these factors most likely burdens society, infrastructure, environment, and slows the alleviation of poverty.

It is a moral and historic obligation to the more than 39 million people who have died from this disease to relieve as far as possible the burdens placed on PLWHA (another 39 million), their families, and communities, and to reduce transmission to those not yet affected, from the hard lessons we have learned. South Africa have subscribed to the UNAIDS 90-90-90 strategy to improve HIV/AIDS management and quality of life. To reiterate;

- By 2020, 90% of all people living with HIV will know their HIV status.
- By 2020, 90% of all people with diagnosed HIV infection will receive sustained antiretroviral therapy.
- By 2020, 90% of all people receiving antiretroviral therapy will have viral suppression.

It is inevitable that environmental considerations be accorded a lesser importance than poverty alleviation and disease prevention and management when dealing with emergencies. However, with this project we have now illustrated the links between the strategies employed to combat and manage the disease with a healthy, safe, and functioning ecosystem and her services. The protection of the environment, the management of the disease, and the moral obligations we have towards people living with HIV and AIDS, are irrevocably linked. Current and future health management should therefore mainstream the environment in strategy, planning, execution, and monitoring for optimal welfare for all.

In the event of ARVs exhibiting an adverse environmental risk profile, alternative solutions need to be developed to abate or even remove the risk posed. This is a daunting task, one being reservations about the practically and effectiveness of many of the current posed solutions. These solutions include the following: improved waste water treatment options to lessen emissions to the environment; substitution of ARV drugs with more ecologically benign drugs; decreased number of expired drugs discarded; improved packaging and shelf life; modification in prescription practices; development of enhanced drug delivery systems to reduce the amount of compound required (Daughton 2003a, 2003b; START, 2008). Meticulous studies are therefore required to establish the different environmental and societal ramification associated with a particular mitigation strategy.

6.1 FINAL RECOMMENDATION

- The NWU research team have developed methods and knowledge on how to characterise the insidious problematics concerning HIV antiretrovirals on a regional scale for a specific disease. We have learned many lessons and gained tremendous experience. However, HIV antiretrovirals and the disease condition are not the only pharmaceutical and disease combinations that will result in environmental pollution. Although some of what we report is generic to the entire pharmaceutical spectrum, there are many situations that we do not know and understand, especially within a South African and African context. More studies are being published on the environmental side of pharmaceutical pollution in Africa. However, an integrated assessment as we have undertaken has nowhere been done. We therefore suggest that a much larger study, based on the experience and expertise developed by the current study would be appropriate for South Africa.

- The science of pharmaceuticals in the environment is developing at a rapid pace. New findings on, inter alia, health and environmental impacts, risk assessments, analytical techniques, and mitigation measures are published almost daily. It is also likely that guideline values for concentrations of pharmaceuticals in water and food will be developed soon, and implemented or negotiated by authorities and international agencies. It would be incumbent for South Africa and the Water Research Commission to keep abreast of these developments and thereby provide guidance to local, regional, and national authorities, as well as water supply companies. This may be achieved by commissioning an annual summary report.

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APPENDIX A: CLINICAL STAGES OF HIV AND AIDS

Table 47: Clinical stages of HIV/AIDS as defined by WHO (2007).

WHO Clinical Staging of HIV/AIDS for Adults and Adolescents	
Clinical Stage	Clinical conditions or symptoms
Primary HIV infection	Asymptomatic Acute retroviral syndrome
Clinical stage 1	Asymptomatic Persistent generalised lymphadenopathy
Clinical stage 2	Moderate unexplained weight loss (<10% of presumed or measured body weight) Recurrent respiratory infections (sinusitis, tonsillitis, otitis media, and pharyngitis) Herpes zoster Angular cheilitis Recurrent oral ulceration Pruritic Papular eruptions Seborrheic dermatitis Fungal nail infections
Clinical stage 3	Inexplicable severe weight loss (>10% of presumed or measured body weight) Unexplained chronic diarrhoea for >1 month Unexplained persistent fever for >1 month (>37.6°C, intermittent or constant) Persistent oral candidiasis (thrush) Oral hairy leukoplakia Pulmonary tuberculosis (current) Severe presumed bacterial infections (e.g. pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteremia) Acute necrotizing ulcerative stomatitis, gingivitis, or periodontitis Inexplicable anaemia (haemoglobin <8 g/dL) Neutropenia (neutrophils <500 cells/µL) Chronic thrombocytopenia (platelets <50,000 cells/µL)
Clinical stage 4	HIV wasting syndrome, as defined by the CDC (see Table 1, above) *Pneumocystis* pneumonia Recurrent severe bacterial pneumonia Chronic herpes simplex infection (orolabial, genital, or anorectal site for >1 month or visceral herpes at any site) Esophageal candidiasis (or candidiasis of trachea, bronchi, or lungs) Extra-pulmonary tuberculosis Kaposi sarcoma Cytomegalovirus infection (retinitis or infection of other organs) Central nervous system toxoplasmosis HIV encephalopathy Cryptococcosis, extra-pulmonary (including meningitis) Disseminated non-tuberculosis mycobacteria infection Progressive multifocal leukoencephalopathy Candida of the trachea, bronchi, or lungs Chronic cryptosporidiosis (with diarrhea) Chronic isosporiasis Disseminated mycosis (e.g. histoplasmosis, coccidioidomycosis, penicilliosis) Recurrent non-typhoidal *Salmonella* bacteremia

WHO Clinical Staging of HIV/AIDS for Adults and Adolescents
Lymphoma (cerebral or B-cell non-Hodgkin)
Invasive cervical carcinoma
Atypical disseminated leishmaniasis
Symptomatic HIV-associated nephropathy
Symptomatic HIV-associated cardiomyopathy
Reactivation of American trypanosomiasis (meningoencephalitis or myocarditis)

Source: World Health Organization. 2007. WHO Case Definitions of HIV for Surveillance and Revised Clinical Staging and Immunological Classification of HIV-Related Disease in Adults and Children.

APPENDIX B: ART regimes South Africa.

health
Department:
Health
REPUBLIC OF SOUTH AFRICA

Private Bag X828, **PRETORIA**, 0001, Civitas Building, Pretoria

TO:
HEADS OF DEPARTMENT
HEADS OF PHARMACEUTICAL SERVICES
CHIEF DIRECTORS: RESPONSIBLE FOR HIV

NOTICE: USE OF FIXED DOSE COMBINATIONS FOR FIRST AND SECOND LINE ANTIRETROVIRAL TREATMENT REGIMENS

There are four fixed dose combination (FDC) antiretroviral (ARV) products available on **HP13-2015ARV: Supply and Delivery of Antiretroviral Medicines April 2015 – March 2018**:

- Abacavir (ABC) 600mg + Lamividine (3TC) 300mg
- Tenofovir (TDF) 300mg + Emtricitabine (FTC) 200mg
- Tenofovir (TDF) 300mg + Emtricitabine (FTC) 200mg + Efavirenz (EFV) 600mg
- Zidovudine (AZT) 300mg + Lamivudine (3TC) 150mg

All clinically eligible patients on first and second line adult ARV regimens should be utilising the available FDCs as follows in accordance with National Antiretroviral Treatment (ART) guidelines:

First Line Regimens

Indication	Regimen	Fixed dose combination product/s to be used for this regimen	Addition of single ARVs to FDC combination
Preferred 1st line regimen	**TDF + 3TC or FTC + EFV**	TDF + FTC + EFV **FDC combination**	None
Contraindication to EFV	**TDF + 3TC or FTC + NVP**	TDF + FTC **FDC combination**	NVP
Contraindication to TDF	**AZT + 3TC + EFV**	AZT + 3TC **FDC combination**	EFV
Contraindication to TDF + EFV	**AZT + 3TC + NVP**	AZT + 3TC **FDC combination**	NVP
Contraindication to TDF + AZT	**ABC + 3TC + EFV**	ABC + 3TC **FDC combination**	EFV
Contraindication to TDF + AZT + EFV	**ABC + 3TC + NVP**	ABC + 3TC **FDC combination**	NVP

Note

- This notice does not apply to patients with clinical indications requiring doses not covered by the available fixed dose combinations.
- The use of Nevirapine in pregnancy should be avoided.

(a)

Second Line Regimens

Indication	Regimen	Fixed dose combination product/s to be used for this regimen	Addition of single ARVs to FDC combination
Failing a TDF-based first-line regimen	**AZT + 3TC + LPV/r**	AZT + 3TC **FDC combination**	LPV/r
Failing a TDF-based first-line regimen + if HBV co-infected	**AZT + TDF + 3TC + LPV/r**	AZT + 3TC **FDC combination**	TDF + LPV/r
		TDF + FTC **FDC combination**	AZT + LPV/r
Failing a D4T or AZT-based first-line regimen	**TDF + 3TC or FTC + LPV/r**	TDF + FTC **FDC combination**	TDF + 3TC + LPV/r
Patients with anaemia and renal failure	**ABC + 3TC + LPV/r**	ABC + 3TC **FDC combination**	LPV/r

TDF – Tenofovir, FTC – Emtricitabine, EFV – Efavirenz, NVP – Nevirapine, 3TC – Lamivudine, AZT – zidovudine, ABC – Abacavir, LPV/r – Lopinavir/Ritonavir, ATV/r – Atazanavir/Ritonavir, D4T – Stavudine

Note

- Patients unable to tolerate LPV/r should be switched to ATV/r:
  - ATV/r is part of a second line regimen not requiring specialist motivation;
  - ATV/r is not a combination product therefore both single agents of atazanavir and ritonavir are required.

Provinces and Health Care Facilities are requested to:

- Distribute and communicate this information in consultation with Pharmaceutical and Therapeutics Committees;
- Consult the National Antiretroviral Treatment Guidelines for comprehensive guidance on all regimens.

Comments and queries may be submitted to:

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Kind regards

DR T PILLAY
DEPUTY DIRECTOR: HEALTH REGULATION AND COMPLIANCE
DATE: 25/05/2015

DR Y PILLAY
DEPUTY DIRECTOR: HIV AND AIDS, TUBERCULOSIS, AND MATERNAL AND CHILD HEALTH
DATE 25/5/15

(b)

Figure 45: Notice from the National Department of Health regarding the use of fixed dose combinations for first- and second-line antiretroviral treatment regimes.

APPENDIX C: ART guidelines South Africa.

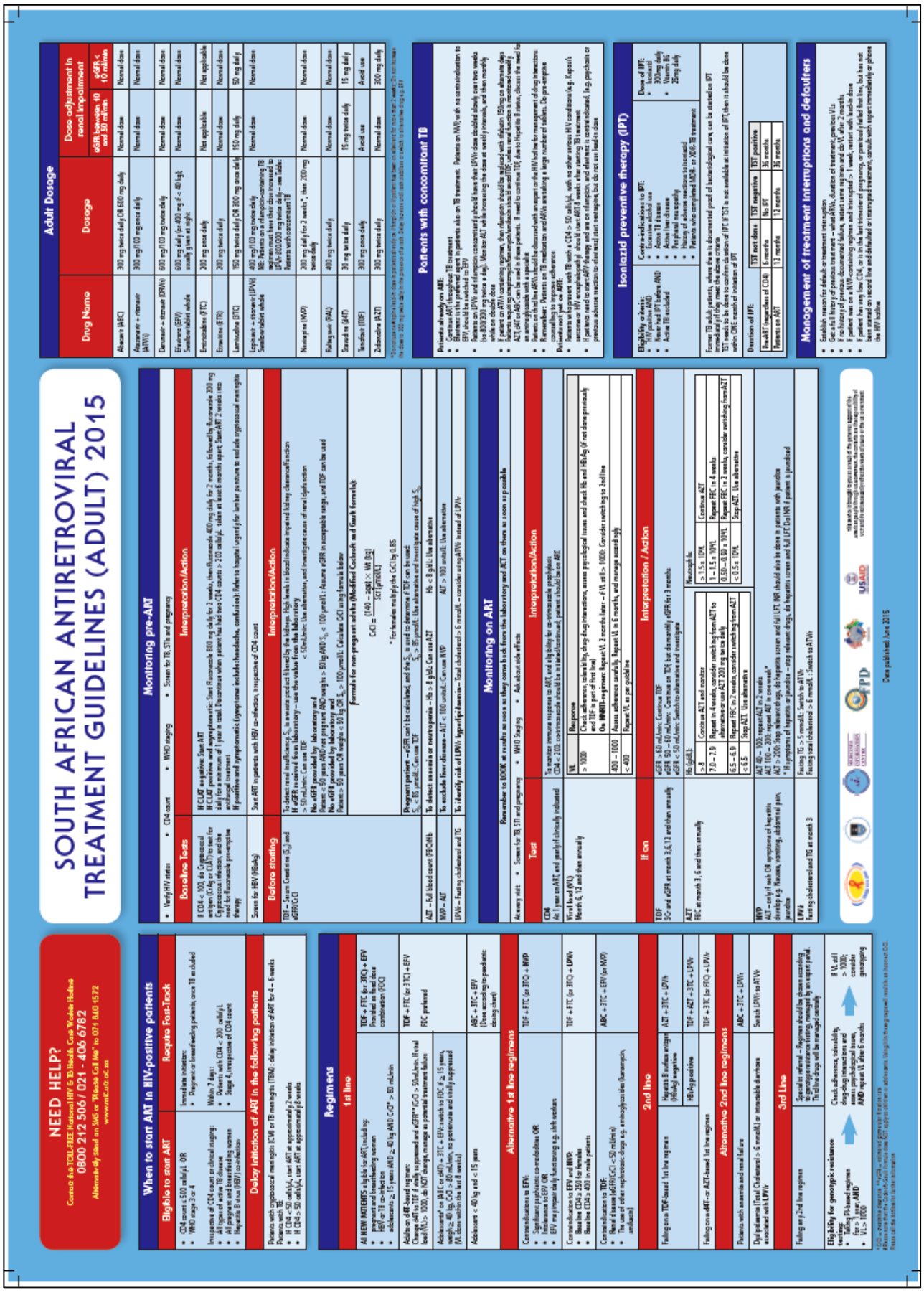

Figure 46: Antiretroviral treatment guidelines for adults in South Africa 2015.

APPENDIX D: SOUTH AFRICAN NATIONAL GUIDELINES FOR COMMENCING ANTIRETROVIRAL THERAPY.

Table 48: Eligibility criteria fir commencing ARVT in SA (Rossiter, 2014).

ELIGIBILITY CRITERIA
CD_4 count ≤350 cells/microliter regardless of stage or symptoms*
WHO stage 3 or 4 or other serious morbidity regardless of CD_4 count (Cryptococcal meningitis – defer ART for 4-6 weeks).
TB regardless of CD_4 count (TB meningitis – defer ART for 4-6 weeks).

*The WHO has moved the threshold for ART initiation to 500 cells/microliter.

APPENDIX E: CLASSIFICATION OF ANTIRETROVIRAL DRUGS.

Table 49: Classification and commentary of various ARV drug classes.

Antiretroviral drug class	Comments
Nucleoside/nucleotide reverse transcriptase inhibitors	No significant interaction. Triple NRTI combination may be considered as ART regimen in exceptional circumstances
Non-nucleoside reverse transcriptase inhibitors	Efavirenz is the preferred NNRTI for use with TB treatment. There is a moderate reduction in nevirapine concentrations. Leading-in dosing should be omitted. Monthly ALT monitoring is recommended.
Protease inhibitors	Dramatic reduction of concentrations of all PIs and none can be used without dose adjustment. In adults, limited evidence supports dose adjustments from the following agents (regular ALT monitoring essential: • Doubling the dose of lopinavir/ritonavir. Dose should be titrated up over 2 weeks to improve tolerability. The final dose is lopinavir 800 mg 12 hourly ritonavir 200 mg 12 hourly. • Saquinavir 400 mg 12 hourly + ritonavir 400 mg 12 hourly. In children: • Add ritonavir to match the lopinavir dose. Doubling the dose of lopinavir/ritonavir is not recommended as it results in sub therapeutic lopinavir concentrations

END VOLUME 1