The occurrence and wastewater chlorination fate of selected pharmaceuticals in South African surface water

By

Timothy Paul Wood

Submitted in partial fulfilment of the requirements for the degree

PHILOSOPHIAE DOCTOR

Chemistry

Supervisor: Professor Egmont Rohwer

In the Faculty of Natural and Agricultural Sciences

Department of Chemistry

University of Pretoria

July 2017

I declare that the thesis, which I hereby submit for the degree *Philosophiae Doctor* (Chemistry) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

_____ _____

T P Wood Date

Dedication:

To Olga.

Summary

The world's water is a rapidly deteriorating and declining resource, which stands to negatively impact every nation on earth. Recently, it has been noted that pharmaceuticals and their disinfection transformation products are present in water supplies. These compounds as well as endocrine disruptors and personal care products are collectively known as micropollutants. Water quality is of special interest to the defence community and as a result the South African National Defence Force initiated this work, to be carried out at Protechnik Laboratories, a division of ARMSCOR SOC Ltd.

The major aims of this research are concerned with the detection and quantification of micropollutants, specifically pharmaceuticals relevant to South African society, in surface water on a national scale; and how these compounds change as a result of wastewater chlorination. The differences between the original parent compound and the disinfection transformation products' *in vitro* toxicity and activity are also addressed. The thesis is divided into separate publications, each with its own topic, yet they are all bound in that they serve to describe particular themes: how mass spectrometry has evolved to answer environmental chemistry questions and how the interface between chemistry and biology, as well as an understanding of each effectively serves to answer some of the most difficult scientific questions.

Very little work regarding the detection and quantification of antiretroviral compounds (ARVs) has been described in the literature; most likely due to the regional prevalence of human immunodeficiency virus (HIV). A novel method for the simultaneous quantification of 12 ARVs using the standard addition method was developed. South African surface water, from across the country was concentrated by solid phase extraction (SPE) and analysed by liquid chromatography coupled to a triple quadrupole tandem mass spectrometer (LC-MS/MS). Matrix effect was found to substantially affect sample quantitation with an average corrected method detection limit of 90.4 ng/L. This is the first report of a

countrywide survey of South African surface water for the quantification ARVs with average concentrations ranging between 26.5 and 430 ng/L.

Using a broader approach, liquid chromatography coupled to quadrupole time of flight mass spectrometry (LC-QTOF) was utilised to quantify 99 pharmaceuticals in SPE extracts of South African surface water on a national level. In addition to this, a non-targeted approach was developed in which three commercially available mass spectral databases, combined into a single searchable entity and parallelized by cluster computing were utilised to screen water samples. This broad approach was combined with automatically triggered tandem mass spectrometry to yield fragmentation data for unknown compounds; for database comparison. Limits of quantification were in the low ng/L range for the majority of the compounds and it was found that nationally both Lamotrigine and Nevirapine occurred most often. Prednisolone and Ritonavir were present at the highest average concentration; 623 and 489 ng/L respectively. It is noteworthy that more than 50% of the targets chosen for analysis are not detectable in any of the samples, which highlights the utility of untargeted, database driven screening; prior to the use of costly analytical standards. The untargeted approach, which was stringent, detected 45 % of the compounds found in targeted mode, and furthermore tentatively identified a total of 4273 unique compounds across the samples. Automatic tandem mass spectrometry yielded 92 unique hits with greater than 95 % confidence. From these results it is clear that untargeted screening should precede the targeted approach as a matter of economy and to guide the selection of targets for quantification. There is still room for improvement in the field as the computational analysis of the large data sets created in this research represents a substantial bottleneck in the analytical workflow.

The wastewater disinfection process, in addition to releasing pharmaceuticals into the environment, has been found to lead to the transformation of these compounds; resulting in the formation of a variety of undescribed disinfection transformation products (DTPs). The biological activity and toxicity of these DTPs are largely unknown. We investigated the laboratory scale chlorination of the

commonly used anti-HIV drug Nevirapine, which occurs ubiquitously in surface water, as shown by our earlier research. The chlorination kinetics of the compound was determined under realistic wastewater treatment concentrations and by scaling up the chlorination reactions the various DTPs were characterised by LC-QTOF. DTPs were produced, isolated by preparative chromatography and subjected to *in vitro* toxicity and antiviral activity analyses.

Nevirapine was found to be highly resistant to chlorination degradation, which may serve to explain in part its wide spread prevalence in South African aquatic systems. A large variety of DTPs were formed during simulated chlorination, the mass spectral characteristics of which were incorporated into a searchable database that was used to screen surface water samples. It was found that a variety of these DTPs are detectable in the environment as they are released from wastewater treatment works albeit at very low levels. Interestingly, a number of the purified chlorination reaction fractions retained antiviral activity and were found to be less toxic than intact Nevirapine.

Similarly, the chlorination profile of the ARV Zidovudine, which is also prolific in South African surface water, was investigated at realistic wastewater treatment conditions. Chlorination reaction products of Zidovudine, at various concentrations, were characterised by LC-QTOF and a DTP database was constructed. When chlorine was at a 25 times molar excess, Zidovudine was found to degrade slowly which partially accounts for its environmental release. Interestingly, after an initial decline in Zidovudine concentration the drug increased in solution to a stable point (20 % of the initial concentration). It was found that Zidovudine would dimerise with itself as well as its DTPs. These would then degrade over time and release Zidovudine back into solution. It is theorised that dimerization could afford Zidovudine protection from chlorine degradation and in part account for its environmental prevalence. It is more likely however that Zidovudine, by merit of its low concentration in the environment, would associate with dissolved organic compounds in wastewater.

The DTPs characterised in the laboratory were however not detectable in the environment. DTPs were isolated by preparative chromatography and subjected to *in vitro* toxicity and antiviral activity screening. The total chlorination reaction mixture and the resulting DTP fractions were found to be less toxic than Zidovudine and a variety of the fractions retained antiviral activity.

The work presented in this thesis represents the first large scale quantitative analysis of South African surface water for pharmaceuticals. New technology, especially high resolution mass spectrometry coupled to liquid chromatography, has radically changed the approach towards monitoring organic water pollutants. The degradation and transformation of two compounds relevant to South African society was also studied. This research may serve as a useful point of departure to consider: instituting national regulations regarding the discharge of pharmaceuticals in the environment, as well as the toxicology of DTPs in South African ecosystems.

Preface

Working as a “biologist” I found that without knowledge of chemistry and its related techniques my research could lack depth of understanding. One also risks neglecting possible research opportunities by staying focused on a particular subset of science. For this, and a number of other reasons, the world is moving towards appreciating the “chemistry-biology interface”.

I personally believe that beauty is to be found at frontier conditions; where one extreme may be compared to another, and in doing so appreciate each for what it is. It is at this interface between chemistry and biology that I found interest, and indeed beauty.

This project was born from military research which was aimed at developing methods for the detection of trace amounts of chemical and biological warfare agents in the environment. The subject matter however, although not related to these agents, provides the opportunity for a “real-life” scenario which could be utilised to developed analytical methodologies. In doing so however, we have developed a national water baseline which may be utilised in future if South African water is ever threatened.

Acknowledgements

First and foremost, I would like to thank my supervisor Professor Egmont Rohwer, for taking a chance on a lost biologist who found his way into analytical chemistry and loved it immediately.

To Dr Philip Coleman, thank you for encouraging me to pursue a PhD and planting the seed in my head with his phrase: “We’ll make a chemist of you yet, Tim!” To Dr Manelisi Shumane, the Senior Manager of Protechnik Laboratories, thank you for encouraging me to continue with the work. Thank you to Mr Jan Venter the ARMSCOR project manager for your understanding and encouragement.

Thank you to Colonel Ben Steyn for initially securing the funding for the work and Colonel Ngqukavana for ensuring its continuation. Without their help this work would never have been realised.

To the scientific staff of Protechnik Laboratories, thank you all for your support and help over the time it took to complete this research. I believe I frustrated a number of you with oddball questions over the years (Taki and Ferdie). Thank you for your patience and help.

The support staff of Protechnik need special thanks and acknowledgement since without them, no science could be performed at the institute. Thank you to all of you for ensuring the continuous stream of coffee required for motivation to do the work and making sure the infrastructure, chemicals and consumables are available to do the actual work.

To my collaborators: Cornelia, Thembi, Adri, Adriaan and Chris; thank you all for your hard work and support.

My sincere thanks to the South African National Defence Force and the South African Military Health Services for funding this work.

Finally to my family, without your support this would not have been possible. Olga, Vera, Agatha, Maryna and Justin; thank you.

Publications and Presentations

The thesis is based on the following manuscripts:

Published Papers

Chapter 2 - Wood, T.P., Duvenage, C.S., Rohwer, E., 2015. The occurrence of anti-retroviral compounds used for HIV treatment in South African surface water. Environmental Pollution 199, 235–243.

Chapter 3 - Wood, T.P., du Preez, C., Steenkamp, A., Duvenage, C.S., Rohwer, E., 2017. Database-Driven Screening of South African Surface Water and the Targeted Detection of Pharmaceuticals Using Liquid Chromatography - High Resolution Mass Spectrometry. Environmental Pollution, 230, 453-462.

Chapter 4 - Wood, T.P., Basson, A.E., Duvenage, C., Rohwer, E.R., 2016. The chlorination behaviour and environmental fate of the antiretroviral drug Nevirapine in South African surface water. Water Research 104, 349-360.

Papers in Press/Preparation

Chapter 5 - Wood, T.P., Ntlemeza, T., Basson, A.E., Duvenage, C., Rohwer, E.R., 2016. Transformation of the Anti-HIV Drug Zidovudine During Simulated Wastewater Chlorination and the Subsequent Formation of Biologically Active Zidovudine Heterodimers. *In Preparation*.

Oral Presentations:

- South African Military Health Services Surgeon General's Show and Tell 2013 – Wood, T.P. (presenter), Duvenage, C.S., Rohwer, E., Research

Highlights and the Environmental Prevalence of Pharmaceuticals in Surface Water.

- South African Water Institute 2014 - Wood, T.P., Duvenage, C.S., Rohwer, E. (presenter), Group Research Highlights: The Detection and Quantification of Selected Pharmaceuticals in South African Surface Water.
- South African Military Health Services Academic Day 2014 - Wood, T.P. (presenter), Duvenage, C.S., Rohwer, E., The Quantification of Anti-retroviral drugs in South African Surface Water.
- South African Military Health Services Command Council 2015 – Wood, T.P. (presenter), Research History.

Table of Contents

Abbreviations

AIDS	Acquired immunodeficiency syndrome
APCI	Atmospheric pressure chemical ionisation
ARMSCOR	Armaments corporation of South Africa
ARV	Antiretroviral
AZT	Zidovudine
BPC	Base peak chromatogram
CAS	Chemical abstract services
CE	Collision energy
CID	Collision induced dissociation
CPU	Central processing unit
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOS	Disk operating system
DPD	Dipropyl-p-phenylenediamine
DTP	Disinfection transformation product
DWAF	Department of water affairs
EI	Electron impact
EIC	Extracted ion chromatogram

EMV	Electron multiplier voltage
EPA	Environmental protection agency
ESI	Electrospray ionisation
FAC	Free available chlorine
FBF	Find by formula
FDA	Food and drug administration
FTICR	Fourier transform ion cyclotron resonance
FTMS	Fourier transform mass spectrometry
GB	Gigabyte
GC	Gas chromatography
GPS	Global positioning system
GUI	Graphic user interface
HAART	Highly active antiretroviral therapy
HDD	Hard drive
HIV	Human immunodeficiency virus
HLB	Hydrophilic lipophilic balanced
HPLC	High performance/pressure liquid chromatography
HPTLC	High performance thin layer chromatography
HRMS	High resolution mass spectrometry
IC_{50}	50% inhibitory concentration
LC	Liquid chromatography

LOD	Limit of detection
LOQ	Limit of quantification
MDL	Method detection limit
MFE	Molecular feature extraction
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NHLS	National health laboratory services
NICD	National institute for communicable diseases
NIH	National institutes of health
NMR	Nuclear magnetic resonance
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
OS	Operating system
PCDL	Personal compound database library
PCP	Personal care product
POCIS	Polar organic chemical integrative sampler
PPCP	Pharmaceuticals and personal care products
PTFE	Polytetrafluoroethylene
QSAR	Quantitative structure activity relationship

QTOF	Quadrupole time of flight
RAM	Random access memory
RNA	Ribonucleic acid
RPM	Revolutions per minute
RRHD	Rapid resolution high definition
RRHT	Rapid resolution high throughput
RSD	Relative standard deviation
RT	Retention time
SADC	Southern African developing community
SAMHS	South African military health services
SANDF	South African national defence force
SOC	State owned company
SPE	Solid phase extraction
TB	Tuberculosis
TIC	Total ion chromatogram
TOF	Time of flight
TOFMS	Time of flight mass spectrometry
UHPLC	Ultra-high pressure liquid chromatography
UPLC	Ultra-pressure liquid chromatography
URL	Uniform Resource Locator
US	United States

USA	United States of America
USEPA	United States environmental protection agency
UV	Ultraviolet
WHO	World health organisation
WWTP	Wastewater treatment plant
WWTW	Wastewater treatment works
WWW	World Wide Web
ZID	Zidovudine

List of Tables

List of Figures

Chapter 1: Introduction

Water is arguably our most precious natural resource. Unfortunately it is under constant threat from amongst others drought, misuse and pollution. The latter is a multifaceted problem that affects plant, animal and human health. Traditionally, water pollution is thought of as the introduction of refuse or harmful factory-discharged chemicals into water courses; but in recent years the micropollutant concept has arisen. Micropollutants in water systems describes: pharmaceuticals, pesticides, endocrine disruptors, personal care products (PCPs) as well as all of the aforementioned compounds' transformation products (Daughton & Scuderi 2012). Although these compounds are diverse in their chemistry and origin, the binding characteristics that group them as micropollutants are their low environmental concentrations (ng-µg/L range), their human origin and their potential to negatively affect plant, animal and human wellbeing.

South Africa is a water scarce region; therefore any exogenous pollutants released into aquatic systems tend to have higher concentrations than countries with higher rainfall (Azzouz & Ballesteros 2013). Water scarcity in conjunction with decreasing quality can therefore have a wide ranging impact on amongst others: food security, hygiene, urbanisation, electricity generation, civil unrest and national security (Duse et al. 2003; Thopil & Pouris 2016; Schreiner & Baleta 2015; Gulati et al. 2013).

Since water is such a vital resource it has importance in the realm of national defence as well as the operational aspects of military forces. The South African Military Health Services (SAMHS), a branch of the South African National Defence Force (SANDF), initiated the work described in this thesis. The South African Defence Review (South African Defence Review, 2014), which is a document that roadmaps the development of the SANDF for the next 30 years, predicts that

globally there will be an increase in competition for mineral resources, land and water. It is therefore important to have scientific methods to describe the quality and safety of water used by South African soldiers at home and abroad.

The work for this thesis was carried out at Protechnik Laboratories, a division of ARMSCOR SOC Ltd; an institute that is concerned with research and development in the field of chemical and biological warfare and terrorism. Water security is a key factor in national defence and counter terrorism, and Protechnik has developed various methods to detect chemical and biological warfare agents in water. Research into micropollutants provides Protechnik with the opportunity to test and develop techniques utilised in trace chemistry using a “real world” scenario, as opposed to simulated chemical weapons incidents. Doctrine can therefore be practiced and refined using lessons learned in this work. In addition to this, as will be described later, the research has led to the development of a national water chemistry baseline. This is in the form of a searchable mass spectrometric data set created from samples taken from every major river and dam in South Africa. This resource allows for a comparative baseline that can be used in the event of a threat to South African water resources.

The research is concerned with manifold overlapping fields: public defence/counter terrorism, public health, environmental health, trace analytical chemistry, microbiology and toxicology. The separation and detection of pharmaceuticals using various liquid chromatographic and mass spectral techniques in South African surface water is described. The transformation as a result of wastewater chlorination of selected compounds is described on a laboratory scale and in the environment. Following this the *in vitro* toxicity and activity of the purified disinfection transformation products (DTPs) is described.

This work is however a first step, in this country, to determine the extent of pharmaceutical pollution. As Yin (2017) points out in a recent article, environmental science focuses primarily on the presence of pharmaceuticals, as opposed to their metabolites, in surface water. The metabolites, DTPs and metabolite

transformation products are neglected in the field as a whole. Their detection would represent one of the next steps upon completion of this current research project.

1.1 Liquid Chromatography Mass Spectrometry, Sample Collection and Processing in Trace Analyses

Before exploring the thesis of this research or specific methods utilised in environmental water research, a discussion of the various technologies utilised in the field is merited. There are many ways to analyse small molecules in complex matrices, yet most inevitably consist of two parts: separation of the sample into simpler components, followed by detection of the aforementioned components. In the majority of cases, in this particular field, separation is achieved by liquid chromatography and detection by any of a number of mass spectral techniques. It is important however to note that, regardless of the instrumental sensitivity, the success of most analyses is largely affected by the pre-analytical sample collection and preparation.

1.1.1 Sample Collection and Processing

Sample collection and processing/preparation are arguably two of the most important steps in the analytical workflow. Bias introduced at this point often times cannot be detected or remediated regardless of the advanced nature of the analytical technique used for final characterisation of pollutants.

A number of regulatory bodies, such as the United Sates Environmental Protection Agency (EPA) prescribe protocols for the extraction of micropollutants from environmental samples (USEPA 2008). Sample collection for the detection of micropollutants usually involves grab sampling (Petrie et al. 2014) or passive cumulative sampling (Soulier et al. 2016; Li et al. 2016).

The type of sample extraction methodology chosen is not only determined by the sample type but also the analytical procedure. The sample must be prepared in such a way as to consider the type of separation by LC, the ionisation method and the mass spectral method chosen (Henion et al. 1998). In an ideal world, the sample would be introduced directly into the liquid chromatography mass spectrometer (LC-MS) but this is not always possible due to amongst others: the presence of interfering compounds that require removal by sample preparation, the low concentration of the target analyte (that requires concentration) or the presence of compounds (e.g. particulate matter) that could damage the instrument.

Liquid-liquid extraction is one of the simplest extraction modalities in which a water sample is combined with an immiscible organic solvent. The method relies on the analytes partitioning into the organic phase and being collected by centrifugation. The organic solvent is then evaporated and the dried targets are reconstituted in the appropriate mobile phase. The selectivity of the extraction is dependent on analyte pKa and solubility as well as solution pH and ionic strength. The methodology is however cost and waste intensive and tends not to be amenable to automation (Henion et al. 1998)

Solid phase extraction (SPE) is the most popular sample preparation technique in environmental chemistry (Prasse et al. 2015). It relies on partitioning between a solid phase (SPE sorbent material) and a liquid phase (sample). By altering the sorbent material one is able to selectively capture and concentrate analytes of interest. This is achieved by passing a sample through a pre-conditioned sorbent bed, washing (to remove unwanted molecules) and elution of the target analytes. These may then be further concentrated by evaporation. One of the main advantages of this method is its ability to concentrate low abundance analytes from a large sample volume (Nováková & Vlcková 2009).

It is incredibly important to consider the efficacy of a particular sampling technique and what conclusions may be drawn with regards to the prevalence of the target molecule in a particular system. It has been found that often researchers do not consider internationally accepted guidelines concerning water sampling. Short term

variations in target concentrations is often not measured and conclusions drawn regarding long term concentration changes may be as a result of sampling artefacts (Ort et al. 2010).

1.1.2 Liquid Chromatography

Chromatography can be defined as the separation process in which a sample mixture is distributed between two phases, namely the stationary and mobile phases. The stationary phase may be solid, porous or surface active material coated onto particles, or, a thin layer of liquid applied to the inside of a column or solid support. The mobile phase may be gas or liquid, which defines the name of the particular technique, i.e. gas or liquid chromatography (Meyer 2013). Liquid chromatography (LC) has become extremely popular, more so than gas chromatography (GC), in the separation and analysis of pharmaceutical compounds because these can include polar and temperature labile chemicals that do not elute well in GC. Focus will therefore be placed on the various aspects of this technique.

The LC system most commonly consists of two pumps (binary or quaternary), a solvent selection valve, a switching valve, an injector, a column heater and a column. With improvements in technology, systems and columns are able to produce and withstand higher pressures, thereby increasing analytical throughput (McMaster 2005). Similarly column technology has improved (narrower column bores, improved particle packing/homogeneity/coating and reduced particle size, pH range characteristics etc.) to yield improved theoretical plate values (a measure of column efficiency) and in turn better chromatographic separations in less time (MacNair et al. 1997). Reduced particle size and the associated higher pressures required to drive the mobile phase through the length of the column at an increased optimum linear flow rate distinguishes modern Ultrahigh Performance Liquid Chromatography (UHPLC) from the older high performance/pressure liquid chromatography (HPLC). With the advent of UHPLC, analysis times, resolution and

sensitivity have all improved. This has allowed for further development in the field enabling scientists to pursue multi-dimensional liquid chromatographic separations (two dimensional LC) with exceptionally short 2nd dimension run times and better overall resolution (François et al. 2009).

The chemistry (molecular interaction between the target analyte and chromatographic system) and therefore the separation are largely determined by the mobile phase selection and the stationary phase composition. Liquid chromatography can be separated into, amongst others: reversed phase, normal phase, ion exchange, size exclusion, ion-pair and affinity chromatography (Meyer 2013). Due to the nature of the target analytes, reverse phase chromatography is used most often in the analysis of pharmaceuticals in water supplies (Ferrer et al. 2003).

Reverse phase chromatography describes a form of partition chromatography in which the analyte moves between the non-polar stationary phase and the polar mobile phase. Elution from the stationary phase is achieved by decreasing the polarity of the mobile phase by introducing (incrementally) a less polar organic solvent. Very polar or ionic species tend not to be retained on non-polar columns and non-polar species elute as the polarity of the mobile phase mixture is reduced. With improvements in bonded phase technology and types, chromatographers are able to select the polarity of analytical columns (e.g. from C18 to CN, in increasing polarity); thereby optimising the separation of target molecules (Willoughby et al. 2002).

1.1.3 Sample Ionisation

After analytes elute from the end of an analytical column they have to be vaporised (to remove mobile phase) and ionised in order to be analysed by mass spectrometry; a technique that operates in the gaseous phase and in a vacuum. There are a variety of ionisation techniques available to LC-MS and the selection of a particular type is dependent on the chemistry of the target analyte as well as the

desired flexibility of the analysis. LC-MS ionisation, in most cases, tends to be “softer” than gas chromatography mass spectrometry (GC-MS) ionisation, often yielding intact ionised molecular ions only, as opposed to electron impact (EI) ionisation that yields some molecular ions but mostly fragments following a “hard” impact by a high energy electron in vacuum. As there are a number of LC-MS ionisation techniques available, for the sake of brevity, only the two most commonly used methods in the field of environmental analysis will be described.

One of the more popular LC-MS ionisation techniques is electrospray ionisation (ESI); a technique that is able to positively or negatively charge ions prior to analysis. LC column eluent is sprayed through a hollow needle, with high electrical potential, at atmospheric pressure, yielding charged droplets providing effective evaporation by the “Coulomb explosion” model. In most cases the spray is dried and focused using heated nitrogen. The heat is only required to prevent evaporative cooling and freezing during evaporation, providing essentially a cool ionization process that prevents thermal degradation of labile compounds. Evaporation of the droplets results in the spontaneous formation of ions (positive or negative, according to the high voltage selected on the needle and therefore on the droplets) which can be sampled by the mass spectrometer by passing into a high vacuum inlet, the design of which varies between manufacturers (Willoughby et al. 2002; Smith et al. 1990). ESI is considered revolutionary in the field, as almost any ionisable molecule (from low molecular weight compounds to high molecular weight proteins and nucleic acids) is made amenable to mass spectrometry (Ho et al. 2003).

Atmospheric pressure chemical ionisation (APCI) interfaces similarly to ESI, in that column eluent is sprayed from an aperture and evaporated under atmospheric pressure conditions, however, without the high electric potential facilitating the electrospray and corresponding spontaneous ionisation. APCI is similarly a soft ionisation technique that provides mostly pseudomolecular ions and few fragments. The distinction between the two techniques is found in the mechanism of ionisation. APCI ionisation occurs using a source of electrons that is introduced on

axis with the heated spray. The analyte ions are therefore produced by (i) electron ionisation of mobile phase and source gases (usually nitrogen or air) under atmospheric pressure conditions, followed by the formation of secondary reagent ions through ion molecule reactions and (ii) the chemical ionisation of analytes by these reagent ions during collisions through typically proton transfer. Under positive APCI conditions the reagent ions like hydronium ion-water clusters ($H_3O^+(H_2O)_n$) can transfer a proton during a collision with analyte molecules. The gas phase analytes will gain and retain the proton (and its charge) as long as their proton affinity is greater than that of water. That is, they must have some molecular features like a lone pair of electrons that can act as a base to accept the proton. In negative ion APCI, negative reagent ions can abstract a proton from a weakly acidic analyte, to result in the quasi-molecular (M-1) negative ion that lost a proton. Depending on the chemical nature of the analyte, either positive or negative APCI may thus be the more appropriate technique selected (McMaster 2005; Willoughby et al. 2002).

Important to note is that, unlike “collisionless” EI ionisation, ESI and APCI are not universal ionisation techniques and will e.g. not detect alkanes as these do not have features to retain any charge even after undergoing collisions with the reagent ions. The multitude of ion molecule collisions in ESI and APCI have another drawback in that the chemical ionisation of the analyte is affected by the momentary composition of the reagent ion species at a given chromatographic retention time. Unlike in GC-(EI) MS, ionisation efficiency therefore becomes matrix dependent, complicating quantitative analysis when using external calibration with pure analyte solutions.

1.1.4 Mass Spectrometric Analysers

A variety of mass analysers are found in commercial instrumentation such as ion cyclotron resonance (or Fourier Transform, FT), magnetic sector, double sector, Time of Flight (TOF), quadrupole, ion trap and Orbitrap mass spectrometers. All

analysers determine the mass of an ion by measuring an ion's mass-to-charge ratio (*m/z)* under vacuum conditions. The analysers are distinguished by: resolution, scan speed, mass accuracy, mass range, sensitivity and dynamic range (Willoughby et al. 2002). As TOFs, quadrupoles and ion traps are most commonly used in environmental analysis, focus will be placed on these instruments. A brief comparison of the instruments is provided in Table 1-1.

Quadrupole analysers consist of parallel rods (four, six or eight), or "quads", arranged around a central axis. Ions are introduced along this axis and may be focused or selected by applying voltages to opposing sets of rods. This creates a "mass filter", allowing only one particular *m/z* ion through at any given time. By ramping the voltages across the poles one is able to convey the complete (nearly) set of ions to the detector. The triple quadrupole instrument, as the name suggests, consists of three sequential quads. This allows researchers to select an ion of interest in the first quad, fragment it in the second quad (using a collision gas) and filter one or more or all of the product ions in the third quad, in order to perform MS-MS. The nature of this instrument is therefore particularly suited to targeted analyses in that known precursor ions are screened in a sample and their identity is validated with the detection of known product ions and their relative intensity. This is commonly known as "reaction monitoring", which may involve single or multiple pairs of precursor/fragment ions resulting in exceptional selectivity in target compound detection (Willoughby et al. 2002). These instruments are perfectly suited to quantitative analyses, as targeted ion abundances may be related to known target concentrations using a calibration curve; easily achieving detection limits in the parts per trillion range (Kang 2012).

Ion trap analysers are variations of the quadrupole in that they electrodynamically focus ions in a three dimensional volume, or "trap" (as opposed to along a quad axis). The ion trap consists of two end caps and a ring; and the ions are detected by selectively placing them in unstable orbits which force them from the trap onto a detector. Specific ions may be selected by the trap and using collision induced dissociation MS/MS as well as MS^n can be carried out (Willoughby et al. 2002).

Both quadrupoles and ion traps, although providing high levels of sensitivity, display limited mass resolution and yield nominal mass (integer mass) information only as in the classical bar graph mass spectra. High resolution (reflectron) TOF and QTOF instruments however are able to accurately measure the mass and isotopic distribution of an ion which provides information regarding its atomic composition. The TOF analyser works on the simple principle that ion velocity is mass dependent when ions are accelerated in the ion source with a fixed electrical potential difference. The TOF pulses a packet of ions (generated by the source and selected by the quadrupole in the case of a QTOF) down a flight tube. The ions which received the same amount of energy at the pulser separate in the flight tube based on their mass (and thus speed). Ions are then reflected back, by a reflectron that corrects for small energy differences of identical mass ions in the initial ion packet. The ions of different mass then arrive at the detector at different times. The time taken to traverse the flight tube is then related to the mass of the ion. With the addition of a quadrupole to TOF technology, MS/MS experimentation is possible (precursor selection by quadrupole at nominal mass only), which enables structural elucidation in addition to the molecular formula data gathered from the accurate mass measurement of the quasi-molecular ion in full scan mode. TOFs have improved in sensitivity over the past number of years to rival that of quadrupole instruments in the single ion mode, and the fact that they gather full scan data enables researchers to adopt a non-targeted screening approach; which is not possible with quadrupoles or ion traps at the same low concentration levels. In addition to these considerations, TOFs have an extremely high upper mass limit, which makes them particularly suited to the analysis of complex molecules such as proteins (Willoughby et al. 2002; Ferrer & Thurman 2009).

Table 1-1: Comparison of the main advantages and disadvantages between quadrupole time of flight (QTOF), triple quadrupole and quadrupole ion trap instrumentation. Adapted from (Thurman & Ferrer 2003).

Instrument	Unique Features	Advantages	Disadvantages
QTOF (MS/MS)	Generates accurate mass of precursor and product ions	Very sensitive when performing precursor ion scans and provides accurate mass for product ion identification. The instrument constantly gathers full scan information.	Very expensive technology. Quasi-selected reaction monitoring and neutral loss experimentation is not possible.
Triple quadrupole (MS/MS)	Neutral loss MS-MS mode	Very sensitive during multiple reaction monitoring.	Not sensitive in full scan mode. Provides nominal mass values.
Quadrupole ion trap (MS/MS)	MS^n	Sensitive in scanning mode. Least expensive. Fragmentation pathway can be deduced easily.	Less reliable for quantitation than triple quad MRM. Neutral loss scanning not possible.

As alluded to earlier, with the ability to accurately measure an ion's mass its chemical formula may be determined. This information allows for the determination of the molecular formulae of unknown compounds as well as to elucidate their structures (based on MS/MS data). It is however not quite that simple, as demonstrated by Kind & Fiehn (2006). The authors showed that monoisotopic mass alone could not yield a definitive chemical identity, in that there are a large number of possible atomic configurations that could lead to near identical masses (excluding isotopes) even at mass accuracy levels approaching 1 ppm. The

authors show that a theoretical instrument with a mass error of 3 ppm and a 2 % isotopic abundance error, could outperform an instrument with a 0.1 ppm mass error that does not provide isotopic information. Isotopic abundance is therefore essential in assigning correct chemical formulae to ions. High resolution analysers allow the more reliable determination of isotope patterns as they identify each isotopic mass via accurate mass measurement and prevent undue overlap with co-eluting compounds containing isobaric (same nominal mass) peaks.

Kind & Fiehn (2007) went on to propose seven golden rules for filtering molecular formulae, which involve: (1) the number of possible elements should be restricted, (2) LEWIS and SENIOR rules, (3) isotopic patterns, (4) carbon-hydrogen ratios, (5) the elemental ratio between nitrogen, oxygen, phosphorus, and sulphur compared to carbon, (6) “element ratio probabilities” and (7) the presence of trimethyl-silylated chemicals. With these considerations in mind one is able to approach the identification of unknown compounds in logical and conservative way (Figure 1-1).

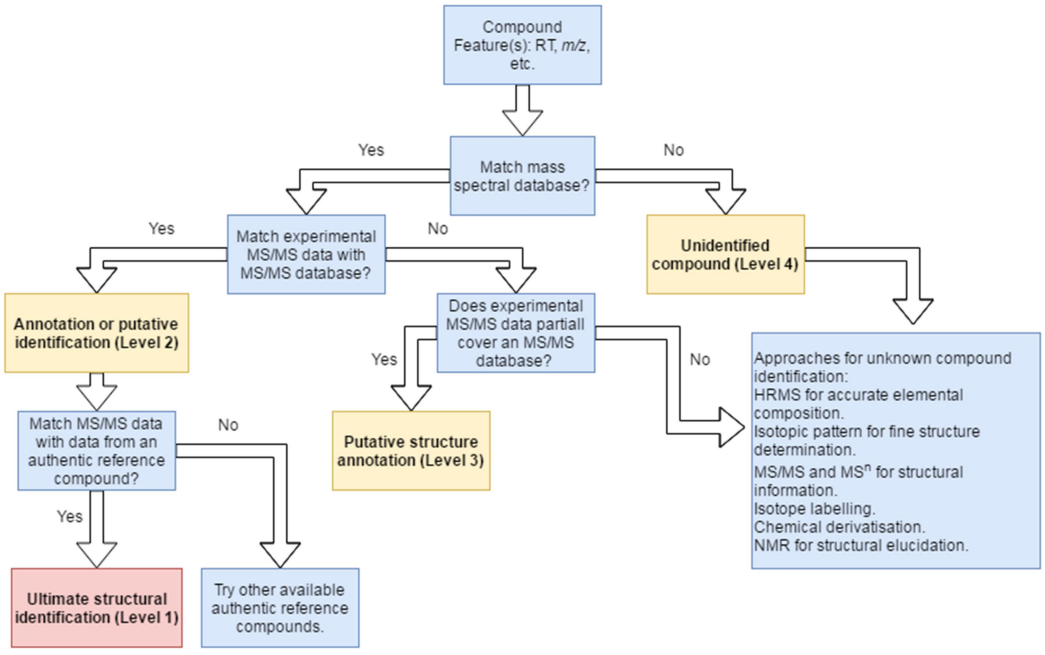


Figure 1-1: An unknown compound analysis decision tree adapted from Rathahao-Paris et al. 2016.

1.1.5 LC-MS Limitations: The Matrix Effect

LC-MS has proven itself to be an exceptionally useful technique, but it is in no way infallible. One of its major drawbacks, when considering quantitative analysis, is matrix effect. The concept of matrix effect in mass spectrometry describes the influence that the sample matrix may have on the quantitative accuracy of the analytical process. As explained in section 1.1.3 earlier, components of the matrix may induce either ion suppression or enhancement as they influence the chemical species transferring the charge during collision with the analyte, leading to inaccurate quantitative data. Many authors address this problem in their work, each taking an approach most suitable to their available resources (Dams et al. 2003; Ito & Tsukada 2002; Patel 2011).

Various steps may be taken to account for this phenomenon, each with advantages and shortfalls. The addition of internal labelled isotopes to each sample is the most effective way in combating matrix effect (Gosetti et al. 2010), yet unfortunately these compounds are not readily available for all targets (e.g. the antiretroviral (ARV) class of drugs). Another approach is the utilisation of matrix matched standards (Gosetti et al. 2010), which are environmental water samples with identical matrix to the target samples; yet do not contain the target chemicals. Calibration curves are then generated by diluting the target chemicals into these matrices. Unfortunately due to the diverse range of water samples processed in the research described in this work it would be virtually impossible to find a universal matrix to be used across all samples. In addition to this, since the environmental prevalence of ARVs is so high, one may not find a truly blank matrix.

The standard addition method is utilised in the literature (Zwiener & Frimmel 2004; Hibbert & Gooding 2006; Ye et al. 2007; Ort et al. 2010; Padhye et al. 2014), albeit to a lesser extent. This is most likely due to the labour intensive nature of the method as a calibration curve must be created for each sample that is analysed. The sample is divided and increasing amounts of target analyte is spiked into each aliquot. The concentration of the targets in the un-spiked aliquot is then determined

by linear regression. This is an aspect addressed in the quantitative approach taken in Chapter 4 (Wood et al. 2015).

1.2 The Presence of Pharmaceuticals in Water Supplies

For the past number of years it has been shown that various pharmaceuticals and personal care products are polluting surface water supplies across the world (Kümmerer 2009a; Kümmerer 2009b; Daughton & Scuderi 2012; Jain et al. 2013). These micropollutants find their way into water systems as a direct result of human activities such as: discharge from wastewater treatment works following human consumption and excretion (Wang & Wang 2016), through leaching of landfilled pharmaceuticals (Lu et al. 2016; Cook et al. 2012; Peng, Ou, et al. 2014), the use of pit latrines in resource limited areas (Graham & Polizzotto 2013) and illegal sewage dumping from septic tank trucks (personal observation).

As there are many ways of pharmaceuticals entering the environment, they are present in a diverse array of ecosystems and parts of the water cycle. Pharmaceuticals have been detected in rivers (Agunbiade & Moodley 2014), lakes and dams (Zhang et al. 2015), groundwater (K'oreje et al. 2016), soil (Dodgen & Zheng 2016) and in the ocean (Alygizakis et al. 2016). The ubiquitous distribution of these compounds could imply that a large variety of ecosystems are under threat as a result of the compounds' toxicity or biological activity.

Although this is a worldwide phenomenon, very little data exists regarding the prevalence of pharmaceuticals in South African surface water (Hendricks & Pool 2012; Agunbiade & Moodley 2014; Agunbiade & Moodley 2015; Schoeman et al. 2015; Wood et al. 2015). In addition to this, the South African picture would differ from trends found in developed countries as many of the SA citizens do not have access to proper sanitation and their pharmaceutical usage profile differs greatly. This is due to the large burden placed on South African society by Human Immunodeficiency Virus (HIV) and tuberculosis (TB) prevalence in the country.

South Africa utilises more antiretroviral (ARV) compounds to treat HIV per capita than any other nation on earth (WHO 2013). It is therefore reasonable to expect, and as will be shown later in this thesis, that these compounds would be more prevalent in South African surface water than in any other country. For this reason, the approach to the detection and quantification of pharmaceuticals and their DTPs in surface water should be uniquely tailored to consider the South African environment.

1.2.1 Targeted detection

In the early years of this field of research, scientists tended to utilise a targeted approach to the detection of these compounds in water. This is largely due to the type of mass spectral equipment that was available at the time. The first report describing the presence of pharmaceuticals entering the environment via wastewater treatment works (WWTWs) was by Keith (1981) and co-workers of the United States Environmental Protection Agency (EPA). The researchers used liquid-liquid extraction (with dichloromethane) followed by derivatisation and GC-MS to detect clofibric acid and salicylic acid. European scientists (Richardson & Bowron 1985; Watts et al. 1983) similarly used GC-MS to detect a handful of pharmaceuticals in the environment in the low µg/L range.

The research at this point in time was limited to the chemical characteristics of the target molecules. GC-MS analysis requires that the analytes have a high enough vapour pressure to be separated in the gas phase at reasonable temperatures or are amenable to derivatisation to increase their vapour pressure. This severely limited the range of molecules that could be analysed as most pharmaceuticals are polar compounds. For this reason the field shifted to the use of liquid chromatography coupled to mass spectrometry (LC-MS), with “soft” electrospray ionisation (ESI) for these analyses.

Researchers favoured triple quadrupole mass spectrometers which provided the highest possible levels of selectivity and sensitivity. As an example Ternes et al.

(2001) described the detection of nine neutral pharmaceuticals using solid phase extraction (SPE) and liquid chromatography tandem mass spectrometry (LC-MS/MS) detection. The analysis required pre-concentration of the sample (by a factor of 1000 times) prior to analysis to achieve low ng/L detection levels. As technology has improved in the preceding decade this pre-concentration step has become less necessary with researchers showing that direct sample injection without prior manipulation is possible to achieve similar limits of detection (Boix et al. 2015; Lucini et al. 2015).

Vanderford et al. (2003) developed a method to analyze trace levels of 27 compounds, 16 originating from pharmaceuticals (e.g. Carbamazepine, Diazepam, Ibuprofen), five originating from personal care products such as caffeine and the common ingredient found in sunscreen, oxybenzone, five different steroids including estrogen, progesterone and testosterone, and one pesticide, Atrazine. Their method employed SPE and LC-MS/MS with ESI in both positive and negative modes as well as atmospheric pressure chemical ionization (APCI) and yielded detection limits of ~1.0 ng/L. The methodology highlights the efficiency of LC-MS to separate and analyse chemically diverse (in polarity, solubility etc.) compounds in a single analysis; a feature not easily reproduced by GC-MS, as described earlier.

Stackelberg et al. (2004) analyzed 106 wastewater related compounds using a combination of LC-MS and GC-MS in a water purification plant before and after treatment. In their study they analyzed 25 different antibiotics using LC-MS (in selected ion monitoring mode), 22 prescription and non-prescription drugs such as warfarin, codeine, and ibuprofen by high performance liquid chromatography (HPLC)-ESI-MS as well as several wastewater related compounds such as caffeine by GC/MS. The study revealed the presence of prescription and non-prescription drugs and their respective metabolites in surface water as well as finished drinking water. This type of study shows the circular nature of pollution in that exogenous chemicals released by mankind often find their way back to the resources (i.e. drinking water) man needs for survival.

As the importance of micropollutants in water supplies has gained attention, laboratories across the world have developed targeted methodologies to detect these compounds. The number of compounds detected simultaneously has also increased over recent years. Papageorgiou et al. (2016) tracked the seasonal occurrence of 55 pharmaceuticals using LC-MS/MS in a municipal WWTW in central Greece. Spanish surface and treated water was evaluated by Gros et al. (2012) to detect 81 pharmaceuticals, using LC coupled to a linear ion trap, a less popular technique compared to triple quadrupole tandem mass spectrometric analysis.

The above mentioned articles all refer to the identification and quantification of pharmaceuticals other than antiretroviral drugs (ARVs). Very few articles have been published on the detection and quantification of antiretroviral drugs in biological matrices such as urine, plasma, serum, saliva, cerebrospinal fluid and semen; let alone in wastewater. The reason for this is most likely due to the regional prevalence of HIV (i.e. predominantly in developing countries).

Methods for clinical sample analysis can thankfully be evaluated and adapted for application to the detection of these compounds in surface water matrices. Anbazhagan et al. (2005) developed a method for the simultaneous quantification of the ARVs Stavudine, Lamivudine and Nevirapine in tablets by using UV spectroscopy, reverse phase LC and high performance thin layer chromatography (HPTLC) whereas Chi et al. (2002) simultaneously detected five protease inhibitors in human plasma by LC/MS/MS. An overall review on the HPLC analysis methods used to detect and quantify protease inhibitors in various biological matrices has been published by Aarnoutse et al. (2001).

The studies mentioned here, do not describe the detection of ARVs in wastewater and only recently, the first publication of this kind was published by Prasse et al. (2010) where they describe the analysis of nine ARV compounds (Acyclovir, Abacavir, Lamivudine, Nevirapine, Oseltamivir, Penciclovir, Ribavirin, Stavudine and Zidovudine) and one active metabolite (Oseltamivir carboxylate) in raw and treated wastewater using SPE and LC-ESI-MS, with detection limits as low as 0.2

ng/L. All but one (Ribavirin) were detected in wastewater influent and it was found that wastewater treatment was unable to remove all the compounds leading to their release and subsequent detection in the Hessian Ried river system in Germany. At the onset of our research we hypothesized that if these compounds are present in German water systems they should be present in South African surface water, at much higher levels, based on national usage profiles (and this was indeed found (Wood et al. 2015) to be the case).

In the five years subsequent to Prasse's research, ARVs have been detected in geographically diverse areas from China (Peng, Wang, et al. 2014) to Kenya (K'oreje et al. 2016). The importance and prevalence of the antiviral class of compound, including those used for the treatment of common conditions such as flu virus (Tamiflu) is garnering attention in the research community (Jain et al. 2013). Prasse's group have since continued and shown that a number of traditionally used ARVs are resistant to wastewater treatment by both chemical and biological treatment processes; partially explaining their prevalence in the environment (Postigo & Richardson 2014; Prasse et al. 2015; Bollmann et al. 2016; Funke et al. 2016).

1.2.2 Non-targeted screening and discovery

As the field of micropollutant detection has matured, researchers realised that an ever increasing number of targets needed to be included in their analyses. Targeted detection was becoming cumbersome and the constant threat of missing relevant compounds, or those for which standards are not available, continued due to the nature of targeted analyses. In addition to this, targeted analyses could not be mined retrospectively or altered after the fact. Thankfully, high resolution mass spectrometry (HRMS) instrumentation has improved over the years leading to increased selectivity and sensitivity (Xian et al. 2012). Researchers are now able to screen for a large number of targets in a "full scan" fashion and simultaneously gather data relating to unknown compounds which can be interpreted at a later

date. The data also allows for the tentative identification, and in some cases structural elucidation, of completely unknown compounds (Bader et al. 2016).

High resolution mass spectrometry is a feature of instrumentation such as Time of Flight (TOF), Quadrupole Time of Flight (QTOF), Orbitrap and Fourier transform ion cyclotron resonance (FTICR) mass spectrometers (Xian et al. 2012). These instruments accurately measure ion masses and are able to resolve isotopic patterns even in the presence of co-eluting isobaric peaks. From this data researchers can determine molecular formulae for unknown compounds as well as searching data against public and commercially available databases. This high mass resolving power also makes inter-laboratory comparison of data possible, a feature not easily possible with LC-MS in the past (Rivier 2003).

In a seminal paper Ferrer & Thurman (2012) described the detection of 100 pharmaceuticals and their degradation products in surface water using UHPLC-QTOF. The authors used a generic sample preparation method in order to capture the broadest possible range of compounds. Using a combination of chromatographic retention time data and accurate mass values they were able to demonstrate how isobars and isomers (compounds with the same nominal and accurate mass respectively) could be distinguished from each other. It is also important to note that they achieved limits of detection ranging between one and 1000 ng/L, which is comparable to targeted screening methods using triple quadrupole instruments.

The increased analytical capacity was demonstrated by Cotton et al. (2016) in a study in which they simultaneously detected (semi-quantitatively) 536 pharmaceutical and pesticide residues in water in as little as 36 minutes; using an Orbitrap mass spectrometer. The researchers used accurate mass, retention time and collision induced dissociation (CID) ion information as confirmation for the presence of target analytes.

In order to deal with temporal fluctuations of micropollutants in groundwater samples Soulier et al. (2016) utilised a passive Polar Organic Chemical Integrative

Sampler (POCIS) system. This methodology provides a cumulative picture as compared to grab sampling, which inherently may miss transient pollutants. By using targeted, suspect (based on a database) and unknown screening (based on molecular formula determination) the researchers were able to demonstrate the utility of the method in detecting compounds not commonly identified during grab sampling or targeted screening.

Using HRMS a large number of compounds may be tracked simultaneously. Parry & Young (2016) compared targeted and non-targeted screening to assess the efficiency of a pilot scale water treatment reactor. When evaluating the reactor they found that 100% of the 12 target pharmaceuticals were removed by more than 50% whereas only 74% of the compounds tracked by non-targeted methods were similarly removed. This demonstrates the two very different conclusions that would be drawn when using targeted *versus* non-targeted analyses.

Boix et al. (2016) analysed WWTW samples from 10 Italian cities to detect intact Omeprazole and Venlafaxine as well as 23 Omeprazole and four Venlafaxine metabolites. Omeprazole was not detected in any of the samples, yet a number of its metabolites were detectable. This highlights the utility of HRMS in comparison to targeted analysis, in that the wrong conclusion would have been drawn (i.e. absence of Omeprazole) if targeted analysis was utilised.

Besides environmental detection, the same research group utilised HRMS to study pharmaceutical biotransformation as a precursor to detection research (Boix, Ibáñez, Sancho, et al. 2016). Five commonly used pharmaceuticals were investigated in laboratory scale transformation experiments (with surface water and activated sludge) and using HRMS, 22 transformation products were tentatively identified. By retrospectively mining environmental sample data, the researchers found up to 14 of these transformation products were present in the environment.

With advances in detection and characterisation technology, the field of “sewage epidemiology” has arisen. Researchers are able to detect illicit drugs (narcotics) in WWTW influent and in turn infer population usage profiles within a particular

suburb or city (Kasprzyk-Hordern et al. 2008; Bartelt-Hunt et al. 2009; Hernández et al. 2014; Baz-Lomba et al. 2016). This allows researchers to advise law enforcement officials and policy makers on the efficacy of a particular prevention strategy or the adoption of new regulatory policies (Asimakopoulos & Kannan 2016). As an example Baz-Lomba et al. (2016) used UHPLC-QTOF with offline solid phase extraction for the detection and quantification of 51 psychoactive substances in sewage. Sensitivities in the low ng/L range were achieved and the methodology was designed in such a way as to be applied to surface water and pooled urine samples as well.

With the breadth of data that is available as a result of QTOF analyses, researchers are now able to harmonise and combine various other data sources in order to make accurate toxicological predictions and provide more accurate feedback as to regulatory/safety steps that need to be taken. Rager et al. (2016) combined HRMS data with *in silico* toxicity predictions (US EPA's Expocast) and *in vitro* toxicity data (Tox 21 Consortium). By analysing dust samples from homes the researchers were able to identify thousands of molecular features, which were subsequently narrowed down to a handful of toxic "chemicals of interest", many of whose household prevalence had not been described in the literature before. The researchers rightly conclude that HRMS should be utilised prior to targeted analysis when performing toxicological research.

1.3 Disinfection Transformation Products of Pharmaceuticals

It has been found that although pharmaceuticals may not be detected in water supplies, certain by-products of these compounds are created during the disinfection process. These disinfection transformation products (DTPs) have been shown to be toxic (Bedner, 2006) and their effect on humans and microbes alike are largely unknown. Bedner and Maccrehan (2006) simulated the disinfection process in the laboratory and tracked the transformation of acetaminophen (paracetamol) over time. The authors found that the toxicants 1,4-benzoquinone

and *N*-acetyl-*p*-benzoquinone imine were formed as a result of acetaminophen chlorination; the latter being the toxic compound associated with acetaminophen overdose.

It is proposed that pharmaceuticals chosen for investigation in screening studies should also be evaluated to determine if these compounds would form DTPs. The structure, nature and behaviour of these compounds should be formalised to determine their possible toxicity or their ability to promote drug resistance in micro-organisms. From an academic standpoint, the chlorination fate of many of these pharmaceuticals (specifically TB and HIV drugs) has yet to be determined. From a review of the literature it was found that this research, to the best of our knowledge, had not been conducted in South Africa before.

Rodil et al. (2012) studied the chlorination behavior of phenazone-type drugs in order to determine their environmental fate. The researchers found that the main pathways for these drugs consisted of halogenation, hydroxylation and dealkylation. The chlorination half-lives of the compounds were found to be very short (up to 173 seconds) and the various degradation products were identified using LC-QTOF. Interestingly, some of the degradation products remained stable after a number of days of chlorination. In addition to this the researchers also found that chlorination of these compounds occurs in real water matrices (e.g. tap water) and not only under laboratory conditions.

Rodriguez-Cabo and co-workers studied the reactivity of fungicides in water containing free chlorine with LC-QTOF technology. Three of the six compounds studied proved to be unstable and showed reaction variability based on pH, bromine trace levels and water matrix type. The researchers found that the compounds generated a number of reaction products when combined with chlorine-spiked surface water or even tap water. Using accurate mass measurements and mass spectral analysis the authors were able to suggest reaction pathways for the less stable fungicides (Rodriiguez-Cabo et al. 2013).

Padhye et al. (2014) performed a year-long evaluation of drinking water treatment plant efficiency for the removal of personal care products from water. The researchers found that a number of pharmaceuticals persisted in the finished treated water and that ozonation was the most effective method for their removal. This type of research serves to highlight that chlorination and the associated treatment processes for water purification may not completely remove pharmaceuticals and other sterilization methods should be sought as an adjunct

Knowledge of the second order rate constants of the reactions between pharmaceuticals and chlorine are essential to predict whether or not they will be removed during wastewater treatment. Acero et al. (2013) studied the chlorination behavior of five commonly used pharmaceuticals and found that their chlorination rate is a function of pH. Interestingly the results for bromination of these compounds were identical to their chlorination reaction rate behavior. In addition to this, the authors found that the presence of bromine in the solution slightly enhanced the degradation of these compounds by chlorination.

N-nitrosamines, which have recently been found to occur in treated water as DTPs have strong carcinogenic and mutagenic properties. Secondary and tertiary amines are the dominant precursors of N-nitrosamine formation during wastewater disinfection. Wu et al. (2015) developed an ultrafast LC-MS method for the detection of seven N-nitrosamine precursors, dimethylamine, ethylmethylamine, diethylamine, dipropylamine, trimethylamine, 3-(dimethylaminomethyl)indole and 4-dimethylaminoantipyrine in a drinking water system. The method required no sample preparation, save filtration, and could be completed in 11 minutes. The majority of sampling sites in the mid-western United States showed N-nitrosamine precursor levels below the method limits of detection, yet at selected sites dimethylamine was detected at concentrations of up to 25 µg/L.

Wang et al. (2015) studied the effect of chlorine, ozone and chlorine dioxide on four pharmaceuticals (ranitidine, nizatidine, doxylamine, and carbinoxamine). Additionally, besides removal of the parent amine based compound, the formation of N-Nitrosodimethylamine was evaluated. N-Nitrosodimethylamine can be formed

from tertiary amine moieties such as those present in these pharmaceuticals. It was found that all three disinfectants effectively removed the parent compounds but only ozonation prevented N-Nitrosodimethylamine formation.

The chlorination of methyl-amide and amide containing pharmaceuticals was also studied by Šakić and colleagues in Croatia. The researchers posit that very little is known regarding the reaction mechanisms of this class of drugs. Using a computational chemistry approach they propose that an iminol intermediate is formed which immediately reacts with HOCl. This serves to provide a mechanistic explanation for amide-hypochlorite reactivity (Šakić et al. 2014).

Bulloch et al. (2015) studied the effluent from 10 WWTWs in southern California (USA) to determine the concentrations of various pharmaceuticals, personal care products and 21 of their halogenated DTPs. The authors found that the pharmaceutical concentrations ranged between >10 and 3830 ng/L and the chloro/bromo DTPs between >4 and 370 ng/L. DTP concentration, they found, depended on amongst others: the concentration of the parent compound in wastewater influent, hydraulic retention time and chlorine contact times. Salicylic acid was found to halogenate readily whereas bisphenol A DTPs were not detectable.

As mentioned earlier, narcotic studies during “sewer epidemiology” may provide useful information to law enforcement officials. Similarly data gleaned from the environmental fate of these compounds could also be beneficial. The environmental and chlorination fate of cocaine was studied using UHPLC-QTOF. The researchers subjected cocaine spiked surface water to photodegradation, chlorination and hydrolysis. Up to 16 degradation products were tentatively identified, with 8 of these as a result of chlorination (Bijlsma et al. 2013).

Besides chlorination, one should also bear in mind the effects of chloramination on target compounds. Monochloramine is added to finished drinking water as a preservative (as chlorine degrades too rapidly within the water network). Bi et al. (2013) studied the degradation behavior of Oxytetracycline. The authors identified

six degradation products and once again the reaction rate was found to be dependent on pH.

It may be concluded from a review of the literature that the study of the chlorination behavior of pharmaceuticals is a rapidly expanding field (Noguera-Oviedo & Aga 2016; Postigo et al. 2016). With that said though, very little work has been performed to address the biological questions that arise as a result of this research (e.g. what are the toxicological effects of these chlorinated compounds?).

1.4 Toxicology of Pharmaceutical Disinfection Transformation Products and Environmental Drug Resistance

The high burden of disease created by HIV/AIDS and tuberculosis in South Africa, and the extensive use of antiretroviral and antibiotic compounds leads one to ask: to what extent are these compounds found in the South African water supply? This paradigm leads to a number of further questions: (1) How do these pharmaceuticals affect the promotion of bacterial/viral drug resistance? (2) What is the sub-therapeutic human toxicity of these compounds? (3) How do these compounds affect aquatic fauna/flora?

The full impact these compounds have on the environment has yet to be shown, but when considering antimicrobial agents, their presence in the environment could potentially lead to the promotion of antibiotic or antiviral drug resistance in the target organism. As an example, Soderstrom (2009) has suggested that the overuse of Tamiflu (Osaltamivir) and its ineffective removal from wastewater, could promote drug resistance in the flu virus through constantly exposing the natural reservoir (ducks) to low levels of the compound in nature. The virus in the ducks would be constantly exposed to low levels of the antiviral compound thereby developing resistance. Once the strain reaches the human population Osaltamivir would therefore be ineffective.

Matsushita et al. (2015) described the effects of chlorination on the mutagenicity of five commonly used iodinated X-ray contrast media. Iopamidol, iohexol, iopromide, iomeprol, and diatrizoate chlorination reaction mutagenicity and toxicity were evaluated by the Ames and bioluminescence inhibition assays respectively. The researchers found that of the five compounds tested only iopamidol was degraded by chlorine and the reaction was shown to increase mutagenicity and toxicity, proportional to chlorine concentration.

Wastewater is often discharged into water catchment areas (e.g. manmade dams) and it has been found that a variety of the effluents components can negatively affect an ecosystem. More than three decades ago Jones & Lee (1984) showed that the increased nutrient load in wastewater effluent promoted eutrophication in the Roodeplaat dam system. It has also been demonstrated that the sentinel fish species in the dam are affected by water pollution. Fish in the dam display histological features associated with liver toxicity (Van Dyk et al. 2012). It is evident that human activity negatively affects fauna and flora, yet since the cause of these are found in water, a resource also utilised by man, it should be a topic of concern. The pervasiveness of micropollutants from the source of discharge, into the environment and back to man has yet to be studied thoroughly.

Besides direct exposure to waterborne pathogens that have acquired resistance through sub-therapeutic exposure to antimicrobial compounds, man is also at risk from other avenues. The use of treated wastewater for crop irrigation is a very viable agricultural solution in water scarce regions, but, there are a number of risks associated with this practice. The soil microbiome (i.e. the total soil bacterial compliment) would be exposed to low levels of antimicrobial agents, which may promote drug resistance; although there hasn't been documented proof of this phenomenon. Besides exposure to antibiotics inducing resistance, microbes are able to transfer resistance to each other through a process known as "lateral gene transfer". Microbes present in WWTWs that have acquired resistance may therefore transfer resistance gene elements to human pathogens (Gatica & Cytryn 2013).

Very little research has been performed to determine the toxicity or mechanism of action of DTPs. Yet it may be assumed, as will be demonstrated successfully later in this thesis, that if the DTP structure remains similar enough to the parent compound, then the quantitative structure activity relationship (QSAR) may be the same; i.e. the DTP would have a similar biological activity to the parent compound. The reason for this lack in research could be found in the fact that the majority of environmental detection and characterisation research is carried out by chemists and published in chemistry related journals. This serves to highlight one on the themes of the thesis: how chemistry and biology, as well as an understanding of each, can benefit a particular research field.

1.5 Aims

Four interlinked aims are associated with this work:

1. To determine the extent of pharmaceutical pollution in South African water supplies;
2. To elucidate the chlorination behaviour of selected pharmaceuticals compounds relevant to South Africa;
3. To determine the extent of the presence of these chlorinated compounds in South African water supplies;
4. To determine the comparative *in vitro* toxicity and biological activity between selected pharmaceuticals and their disinfection transformation products.

These aims may be further divided into a number of objectives and activities:

1. The development a single analytical technique (LC-MS) for the detection of selected target chemicals;
2. Characterisation of the ESI-MS properties of each target compound;

3. Characterisation of the ESI-MS/MS fragmentation of each target compound;
4. Determination of pharmaceutical compound and DTP pseudomolecular and product ion accurate masses;
5. The development of a universal extraction technique for target chemicals from water samples;
6. The quantification of target chemicals in environmental water samples;
7. The *in silico* prediction of pharmaceutical modification as a result of wastewater treatment (chlorination);
8. The determination of the environmental fate of selected compounds, as affected by the South African water purification strategy (wastewater disinfection by chlorination);
9. Laboratory scale creation, purification and mass spectral characterisation of pharmaceutical disinfection transformation products;
10. Determination of the antimicrobial properties of DTPs;
11. Estimation of the human toxicity of selected DTPs *in vitro*.

1.6 Themes of the Thesis

As this thesis is a compilation of publications, i.e. a set of free standing entities, it is not always possible to convey the thematic message of the work as a whole. It may therefore be useful to state the themes:

1. How advances in separation science and mass spectrometry have aided environmental analysis;
2. The concept of the holistic picture of ecotoxicology in that micropollutants have a diverse effect, ranging from immediate toxicity, endocrine disruption and the development of drug resistance;

3. The utility of the research from a defence perspective and the development of a national water chemistry baseline in South Africa;

4. How chemistry and biology, as well as an understanding of each, and the interface between them, can benefit a particular research field.

1.7 Significance of the Work

Through a review of the literature, it has been found that this type of work has not been done in South Africa before. In addition to this, international focus has not been on HIV or TB drugs, due to the fact that they are not as commonly used in Europe and America as they are in sub-Saharan Africa.

Through a personal communication with Dr Christian Daughton (US Environmental Protection Agency) and a review of the "US EPA Bibliographic Database of Publications Relevant to Pharmaceuticals and Personal Care Products", which contains over 13 000 citations (Daughton & Scuderi 2012), only one citation was found regarding the detection of pharmaceuticals in South African water; at the time of initiating this research project.

Drug resistance is a major problem in South Africa, specifically in the case of *Mycobacterium tuberculosis*; this work would aid in explaining certain epidemiological aspects of the promotion of drug resistance in this organism. Although humans are the natural reservoir of *M. tuberculosis, M. bovis* and *M. avium* should also be considered as both are known zoonotic organisms and may be affected by the presence of TB drugs in the environment.

Water security (supply and quality) is fast becoming a worldwide concern. The introduction of micropollutants into an already strained system could exacerbate the problem. The work therefore seeks, as a first point of departure, to determine the extent of this pollution in the South African context. This point furthers the aims of Green Chemistry (water treatment), in that chemistry will be utilised to identify a

problem which can hopefully be solved in the future through modification of behaviour and/or chemical-engineering solutions.

1.8 Structure of the Thesis

The four research chapters, each of which is an independent publication, are not presented in chronological order; rather by theme. For this reason the style and formatting (which is journal-dependent) may not be consistent between the chapters. As each chapter is expected to stand alone, there would also be a certain level of repetition in the technical aspects of the thesis that the reader should bear with.

The first two publications deal with the detection and quantification of pharmaceuticals in South African surface water on a national level. The two papers differ in the number of targets addressed as well as the mass spectrometric methods used. This difference addresses one of the themes of the thesis and shows how improved technology has enabled us to increase the number of targets detected simultaneously (when using a triple quadrupole in the first article for 12 ARV targets versus a QTOF in the second chapter for 96 pharmaceutical targets of various classes).

The second two publications explore the chlorination behaviour, environmental fate, toxicity and biological activity of two antiretroviral compounds. Chapter Four deals with Nevirapine chlorination whereas Chapter Five addresses the disinfection transformation, by chlorine, of the antiretroviral drug Zidovudine. The DTPs for both compounds were produced on a laboratory scale, purified and tested for *in vitro* activity and toxicity.

Finally, concluding remarks addressing individual aspects and the research project as a whole are presented in Chapter Six.

1.9 References

1. Aarnoutse, R.E. et al., 2001. High-performance liquid chromatography of HIV protease inhibitors in human biological matrices. *Journal of Chromatography. B, Biomedical Sciences and Applications*, 764(1-2), 363–84.

2. Acero, J.L. et al., 2013. Chlorination and bromination kinetics of emerging contaminants in aqueous systems. *Chemical Engineering Journal*, 219, 43–50.

3. Agunbiade, F.O. & Moodley, B., 2015. Occurrence and distribution pattern of acidic pharmaceuticals in surface water, wastewater and sediment of the Msunduzi River, Kwazulu-Natal, South Africa. *Environmental Toxicology and Chemistry*, 35(1), 36-46.

4. Agunbiade, F.O. & Moodley, B., 2014. Pharmaceuticals as emerging organic contaminants in Umgeni River water system, KwaZulu-Natal, South Africa. *Environmental Monitoring and Assessment*, 186(11), 7273–7291.

5. Alygizakis, N.A. et al., 2016. Occurrence and spatial distribution of 158 pharmaceuticals, drugs of abuse and related metabolites in offshore seawater. *Science of the Total Environment*, 541, 1097–1105.

6. Anbazhagan, S. et al., 2005. Simultaneous quantification of stavudine, lamivudine and nevirapine by UV spectroscopy, reverse phase HPLC and HPTLC in tablets. *Journal of Pharmaceutical and Biomedical Analysis*, 39(3-4), 801–4.

7. Asimakopoulos, A.G. & Kannan, K., 2016. Neuropsychiatric pharmaceuticals and illicit drugs in wastewater treatment plants: a review. *Environmental Chemistry*, 13, 541–576

8. Azzouz, A. & Ballesteros, E., 2013. Influence of seasonal climate differences on the pharmaceutical, hormone and personal care product

removal efficiency of a drinking water treatment plant. *Chemosphere*, 93(9), 2046–2054.

9. Bader, T. et al., 2016. General strategies to increase the repeatability in non-target screening by liquid chromatography-high resolution mass spectrometry. *Analytica Chimica Acta*.

10. Bartelt-Hunt, S.L. et al., 2009. The occurrence of illicit and therapeutic pharmaceuticals in wastewater effluent and surface waters in Nebraska. *Environmental Pollution*, 157, 786–791.

11. Baz-Lomba, J., Reid, M.J. & Thomas, K.V., 2016. Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF. *Analytica Chimica Acta*, 914, 81–90.

12. Bi, X. et al., 2013. Monochloramination of oxytetracycline: kinetics, mechanisms, pathways and Disinfection By-products formation. *CLEAN-Soil, Air, Water*, 10, 969-975.

13. Bijlsma, L. et al., 2013. Investigation of degradation products of cocaine and benzoylecgonine in the aquatic environment. *Science of The Total Environment*, 443, 200–208.

14. Boix, C., Ibáñez, M., Sancho, J.V., et al., 2016. Biotransformation of pharmaceuticals in surface water and during waste water treatment: Identification and occurrence of transformation products. *Journal of Hazardous Materials*, 302, 175–187.

15. Boix, C. et al., 2015. Fast determination of 40 drugs in water using large volume direct injection liquid chromatography-tandem mass spectrometry. *Talanta*, 131, 719–727.

16. Boix, C., Ibáñez, M., Bagnati, R., et al., 2016. High resolution mass spectrometry to investigate omeprazole and venlafaxine metabolites in wastewater. *Journal of Hazardous Materials*, 302, 332–340.

17. Bollmann, A.F. et al., 2016. Occurrence and fate of amisulpride, sulpiride, and lamotrigine in municipal wastewater treatment plants with biological treatment and ozonation. *Journal of Hazardous Materials*, 15, 204-215.

18. Bulloch, D.N. et al., 2015. Occurrence of Halogenated Transformation Products of Selected Pharmaceuticals and Personal Care Products in Secondary and Tertiary Treated Wastewaters from Southern California. *Environmental Science & Technology*, 49(4), 2044-51.

19. Chi, J. et al., 2002. Simultaneous determination of five HIV protease inhibitors nelfinavir, indinavir, ritonavir, saquinavir and amprenavir in human plasma by LC/MS/MS. *Journal of Pharmaceutical and Biomedical Analysis*, 30(3), 675–684.

20. Cook, S.M. et al., 2012. Life cycle comparison of environmental emissions from three disposal options for unused pharmaceuticals. *Environmental Science & Technology*, 46(10), 5535–5541.

21. Cotton, J. et al., 2016. Development and validation of a multiresidue method for the analysis of more than 500 pesticides and drugs in water based on on-line and liquid chromatography coupled to high resolution mass spectrometry. *Water Research*, 104, 20–27.

22. Dams, R. et al., 2003. Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid. *Journal of the American Society for Mass Spectrometry*, 14(11), 1290–1294.

23. Daughton, C. & Scuderi, M., 2012. Pharmaceuticals and Personal Care Products (PPCPs): Relevant Literature. *US Environmental Protection Agency, Las Vegas, NV*.

24. Dodgen, L. & Zheng, W., 2016. Effects of reclaimed water matrix on fate of pharmaceuticals and personal care products in soil. *Chemosphere*, 156, 286–293.

25. Duse, A.G., da Silva, M.P. & Zietsman, I., 2003. Coping with hygiene in South Africa, a water scarce country. *International journal of environmental health research*, 13 Suppl 1, S95–105.

26. Ferrer, I. & Thurman, E.M., 2012. Analysis of 100 pharmaceuticals and their degradates in water samples by liquid chromatography quadrupole time-of-flight mass spectrometry. *Journal of Chromatography A*, 1259, 148–157.

27. Ferrer, I. & Thurman, E.M., 2009. *Liquid chromatography time-of-flight mass spectrometry*, John Wiley & Sons.

28. Ferrer, I., Thurman, E.M. & others, 2003. *Liquid chromatography/mass spectrometry, MS/MS and time of flight MS*, American Chemical Society; Distributed by Oxford University Press.

29. François, I., Sandra, K. & Sandra, P., 2009. Comprehensive liquid chromatography: fundamental aspects and practical considerations—a review. *Analytica Chimica Acta*, 641(1), 14–31.

30. Funke, J., Prasse, C. & Ternes, T.A., 2016. Identification of transformation products of antiviral drugs formed during biological wastewater treatment and their occurrence in the urban water cycle. *Water Research*, 98, 75–83.

31. Gatica, J. & Cytryn, E., 2013. Impact of treated wastewater irrigation on antibiotic resistance in the soil microbiome. *Environmental Science and Pollution Research*, 20(6), 3529–3538.

32. Gosetti, F. et al., 2010. Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A*, 1217(25), 3929–3937.

33. Graham, J.P. & Polizzotto, M.L., 2013. Pit latrines and their impacts on groundwater quality: a systematic review. *Environmental Health Perspectives*, 121(5), 521–530.

34. Gros, M., Rodriguez-Mozaz, S. & Barceló, D., 2012. Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. *Journal of Chromatography A*, 1248, 104-21.

35. Gulati, M. et al., 2013. The Water-energy-food Security Nexus: Challenges and opportunities for food security in South Africa. *Aquatic Procedia*, 1, 150–164.

36. Hendricks, R. & Pool, E.J., 2012. The effectiveness of sewage treatment processes to remove faecal pathogens and antibiotic residues. *Journal of Environmental Science and Health, Part A*, 47(2), 289–297.

37. Henion, J., Brewer, E. & Rule, G., 1998. Peer Reviewed: Sample Preparation for LC/MS/MS: Analyzing Biological and Environmental Samples. *Analytical Chemistry*, 70(19), 650A–656A.

38. Hernández, F. et al., 2014. Investigation of pharmaceuticals and illicit drugs in waters by liquid chromatography-high-resolution mass spectrometry. *TrAC Trends in Analytical Chemistry*, 63, 140–157.

39. Hibbert, D.B. & Gooding, J.J., 2006. *Data analysis for chemistry*, Oxford University Press New York.

40. Ho, C. et al., 2003. Electrospray ionisation mass spectrometry: principles and clinical applications. *Clinical Biochemist Reviews*, 24(1), 3–12.

41. Ito, S. & Tsukada, K., 2002. Matrix effect and correction by standard addition in quantitative liquid chromatographic-mass spectrometric analysis of diarrhetic shellfish poisoning toxins. *Journal of Chromatography A*, 943(1), 39–46.

42. Jain, S. et al., 2013. Occurrence and Removal of Antiviral Drugs in Environment: A Review. *Water, Air, & Soil Pollution*, 224(2), 1–19.

43. Jones, R.A. & Lee, G.F., 1984. Impact of phosphorus load reductions on eutrophication-related water quality of Roodeplaat Dam(Reservoir), Republic of South Africa. *Water S. A.*, 10(3), 115–120.

44. K'oreje, K. et al., 2016. Occurrence patterns of pharmaceutical residues in wastewater, surface water and groundwater of Nairobi and Kisumu city, Kenya. *Chemosphere*, 149, 238–244.

45. Kang, J.-S., 2012. Principles and applications of LC-MS/MS for the quantitative bioanalysis of analytes in various biological samples. In *Tandem Mass Spectrometry-Applications and Principles*. Intechopen, 441–492.

46. Kasprzyk-Hordern, B., Dinsdale, R.M. & Guwy, A.J., 2008. The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. *Water Research*, 42(13), 3498–3518.

47. Keith, L.H., 1981. Identification and analysis of organic pollutants in water. In Ann Arbor Science, Ann Arbor, MI.

48. Kind, T. & Fiehn, O., 2006. Metabolomic database annotations via query of elemental compositions: mass accuracy is insufficient even at less than 1 ppm. *BMC Bioinformatics*, 7(1), 1.

49. Kind, T. & Fiehn, O., 2007. Seven golden rules for heuristic filtering of molecular formulas obtained by accurate mass spectrometry. *BMC Bioinformatics*, 8(1), 1.

50. Kümmerer, K., 2009a. Antibiotics in the aquatic environment-a review-part I. *Chemosphere*, 75(4), 417–434.

51. Kümmerer, K., 2009b. Antibiotics in the aquatic environment-a review-part II. *Chemosphere*, 75(4), 435–441.

52. Li, Z., Sobek, A. & Radke, M., 2016. Fate of Pharmaceuticals and their Transformation Products in four small European Rivers receiving treated Wastewater. *Environmental Science & Technology,* 50, 5614−5621.

53. Lu, M.C. et al., 2016. Occurrence and treatment efficiency of pharmaceuticals in landfill leachates. *Waste Management*, 55, 257-264.

54. Lucini, L. et al., 2015. QqQ and Q-TOF liquid chromatography mass spectrometry direct aqueous analysis of herbicides and their metabolites in water. *International Journal of Mass Spectrometry*, 392, 16–22.

55. MacNair, J.E., Lewis, K.C. & Jorgenson, J.W., 1997. Ultrahigh-pressure reversed-phase liquid chromatography in packed capillary columns. *Analytical Chemistry*, 69(6), 983–989.

56. Matsushita, T. et al., 2015. Changes in mutagenicity and acute toxicity of solutions of iodinated X-ray contrast media during chlorination. *Chemosphere*, 135, 101–107.

57. McMaster, M.C., 2005. *LC/MS: a practical user's guide*, John Wiley & Sons.

58. Meyer, V.R., 2013. *Practical high-performance liquid chromatography*, John Wiley & Sons.

59. Noguera-Oviedo, K. & Aga, D.S., 2016. Lessons learned from more than two decades of research on emerging contaminants in the environment. *Journal of Hazardous Materials*, 316, 242–251.

60. Nováková, L. & Vlcková, H., 2009. A review of current trends and advances in modern bio-analytical methods: chromatography and sample preparation. *Analytica Chimica Acta*, 656(1), 8–35.

61. Ort, C. et al., 2010. Sampling for pharmaceuticals and personal care products (PPCPs) and illicit drugs in wastewater systems: Are your conclusions valid? A critical review. *Environmental Science & Technology*, 44(16), 6024–6035.

62. Padhye, L.P. et al., 2014. Year-long evaluation on the occurrence and fate of pharmaceuticals, personal care products, and endocrine disrupting chemicals in an urban drinking water treatment plant. *Water Research*, 51, 266–276.

63. Papageorgiou, M., Kosma, C. & Lambropoulou, D., 2016. Seasonal occurrence, removal, mass loading and environmental risk assessment of 55 pharmaceuticals and personal care products in a municipal wastewater treatment plant in Central Greece. *Science of The Total Environment*, 543, 547–569.

64. Parry, E. & Young, T.M., 2016. Comparing targeted and non-targeted high-resolution mass spectrometric approaches for assessing advanced oxidation reactor performance. *Water Research*, 104, 72-81.

65. Patel, D., 2011. Matrix Effect in a View of LC-MS/MS: An Overview. *International Journal of Pharma & Bio Sciences*, 2(1), 559-564.

66. Peng, X., Ou, W., et al., 2014. Occurrence and ecological potential of pharmaceuticals and personal care products in groundwater and reservoirs in the vicinity of municipal landfills in China. *Science of The Total Environment*, 490, 889–898.

67. Peng, X., Wang, C., et al., 2014. Profile and behavior of antiviral drugs in aquatic environments of the Pearl River Delta, China. *Science of The Total Environment*, 466, 755–761.

68. Petrie, B., Barden, R. & Kasprzyk-Hordern, B., 2014. A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring. *Water Research*, 72, 3-27.

69. Postigo, C. et al., 2016. Characterization of iodinated disinfection by-products in chlorinated and chloraminated waters using Orbitrap based gas

chromatography-mass spectrometry. *Analytical and Bioanalytical Chemistry*, 408(13), 3401–3411.

70. Postigo, C. & Richardson, S.D., 2014. Transformation of pharmaceuticals during oxidation/disinfection processes in drinking water treatment. *Journal of Hazardous Materials*, 279, 461–475.

71. Prasse, C. et al., 2010. Antiviral drugs in wastewater and surface waters: a new pharmaceutical class of environmental relevance? *Environmental Science & Technology*, 44(5), 1728–1735.

72. Prasse, C. et al., 2015. Spoilt for choice: A critical review on the chemical and biological assessment of current wastewater treatment technologies. *Water Research*, 87, 237–270.

73. Rager, J.E. et al., 2016. Linking high resolution mass spectrometry data with exposure and toxicity forecasts to advance high-throughput environmental monitoring. *Environment International*, 88, 269–280.

74. Rathahao-Paris, E. et al., 2016. High resolution mass spectrometry for structural identification of metabolites in metabolomics. *Metabolomics*, 12(1), 1–15.

75. Richardson, M.L. & Bowron, J.M., 1985. The fate of pharmaceutical chemicals in the aquatic environment. *Journal of Pharmacy and Pharmacology*, 37(1), 1–12.

76. Rivier, L., 2003. Criteria for the identification of compounds by liquid chromatography-mass spectrometry and liquid chromatography-multiple mass spectrometry in forensic toxicology and doping analysis. *Analytica Chimica Acta*, 492(1), 69–82.

77. Rodil, R., Quintana, J.B. & Cela, R., 2012. Transformation of phenazone-type drugs during chlorination. *Water Research*, 46(7), 2457–2468.

78. Rodriiguez-Cabo, T. et al., 2013. Liquid chromatography time-of-flight mass spectrometry evaluation of fungicides reactivity in free chlorine containing water samples. *Journal of Mass Spectrometry*, 48(2), 216–226.Šakić, D. et al., 2014. Chlorination of N-methylacetamide and amide-containing pharmaceuticals. Quantum-chemical study of the reaction mechanism. *The Journal of Physical Chemistry A*, 118(12), 2367–2376.

79. Schoeman, C. et al., 2015. Quantification of Selected Antiretroviral Drugs in a Wastewater Treatment Works in South Africa Using GC-TOFMS. *Journal of Chromatography & Separation Techniques*, 6, No. 4.

80. Schreiner, B. & Baleta, H., 2015. Broadening the Lens: A Regional Perspective on Water, Food and Energy Integration in SADC. *Aquatic Procedia*, 5, 90–103.

81. Smith, R.D. et al., 1990. New developments in biochemical mass spectrometry: electrospray ionization. *Analytical Chemistry*, 62(9), 882–899.

82. Soulier, C., Coureau, C. & Togola, A., 2016. Environmental forensics in groundwater coupling passive sampling and high resolution mass spectrometry for screening. *Science of the Total Environment*, 563, 845–854.

83. South African Defence Review Committee, 2014. South African defence review. *Pretoria: Department of Defence and Military Veterans*, 2, p.28.

84. Stackelberg, P.E. et al., 2004. Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water-treatment plant. *Science of the Total Environment*, 329(1), 99–113.

85. Ternes, T., Bonerz, M. & Schmidt, T., 2001. Determination of neutral pharmaceuticals in wastewater and rivers by liquid chromatography-electrospray tandem mass spectrometry. *Journal of Chromatography A*, 938(1), 175–185.

86. Thopil, G.A. & Pouris, A., 2016. A 20 year forecast of water usage in electricity generation for South Africa amidst water scarce conditions. *Renewable and Sustainable Energy Reviews*, 62, 1106–1121.

87. Thurman, E. & Ferrer, I., 2003. Comparison of quadrupole time-of-flight, triple quadrupole, and ion-trap mass spectrometry/mass spectrometry for the analysis of emerging contaminants. In *ACS Symposium Series*. 14–31.

88. USEPA, 2008. Sampling Guidance for Unknown Contaminants in Drinking Water.

89. Vanderford, B.J. et al., 2003. Analysis of endocrine disruptors, pharmaceuticals, and personal care products in water using liquid chromatography/tandem mass spectrometry. *Analytical Chemistry*, 75(22), 6265–6274.

90. Van Dyk, J., Cochrane, M. & Wagenaar, G., 2012. Liver histopathology of the sharptooth catfish Clarias gariepinus as a biomarker of aquatic pollution. *Chemosphere*, 87(4), 301–311.

91. Wang, J. & Wang, S., 2016. Removal of pharmaceuticals and personal care products (PPCPs) from wastewater: A review. *Journal of Environmental Management*, 182, 620–640.

92. Wang, X. et al., 2015. Effect of oxidation on amine-based pharmaceutical degradation and N-Nitrosodimethylamine formation. *Water Research*, 87, 403–411.

93. Watts, C. et al., 1983. Analysis of organic micropollutants in water. *Reidel, Dordrecht*, 120–131.

94. WHO, 2013. Global update on HIV treatment 2013: results, impact and opportunities.

95. Willoughby, R.C., Sheehan, E. & Mitrovich, S., 2002. *A global view of LC/MS: how to solve your most challenging analytical problems 2nd Ed*, Global View Publ.

96. Wood, T.P., Duvenage, C.S. & Rohwer, E., 2015. The occurrence of anti-retroviral compounds used for HIV treatment in South African surface water. *Environmental Pollution*, 199, 235–243.

97. Wu, Q. et al., 2015. Determination of secondary and tertiary amines as N-nitrosamine precursors in drinking water system using ultra-fast liquid chromatography-tandem mass spectrometry. *Talanta*, 131, 736–741.

98. Xian, F., Hendrickson, C.L. & Marshall, A.G., 2012. High resolution mass spectrometry. *Analytical Chemistry*, 84(2), 708–719.

99. Ye, Z., Weinberg, H.S. & Meyer, M.T., 2007. Trace analysis of trimethoprim and sulfonamide, macrolide, quinolone, and tetracycline antibiotics in chlorinated drinking water using liquid chromatography electrospray tandem mass spectrometry. *Analytical Chemistry*, 79(3), 1135–1144.

100. Yin, L., et al., 2017. Pay special attention to the transformation products of PPCPs in environment. *Emerging Contaminants*, In Press. , http://dx.doi.org/10.1016/j.emcon.2017.04.001.

101. Zhang, Q.-Q. et al., 2015. Comprehensive evaluation of antibiotics emission and fate in the river basins of China: source analysis, multimedia modeling, and linkage to bacterial resistance. *Environmental Science & Technology*, 49(11), 6772–6782.

102. Zwiener, C. & Frimmel, F.H., 2004. LC-MS analysis in the aquatic environment and in water treatment-a critical review. *Analytical and Bioanalytical Chemistry*, 378(4), 851–861.

Chapter 2: The Occurrence of Anti-Retroviral Compounds used for HIV Treatment in South African Surface Water

This chapter was published in Environmental Pollution. The format reflects the style set by the journal.

Wood, T.P., Duvenage, C.S., Rohwer, E., 2015. The occurrence of anti-retroviral compounds used for HIV treatment in South African surface water. Environmental Pollution 199, 235–243.

The Occurrence of Anti-Retroviral Compounds used for HIV Treatment in South African Surface Water

Timothy Paul Wood[a,c] *(Corresponding Author), Cornelia S.J. Duvenage*[b]*, Egmont Rohwer*[c]

[a] Protechnik Laboratories, a division of ARMSCOR SOC Ltd., 103 Combretum Crescent, Centurion, Pretoria, 0001, South Africa. Tel: +27 12 665 9444, Fax: +27 12 665 0240, timw@protechnik.co.za

[b]Department of Internal Medicine, 1 Military Hospital, South African Military Health Services, Voortrekker Street, Pretoria, 0001, South Africa. Tel: +2783 273 3382, corneliaduv@mtnloaded.co.za

[c] Department of Chemistry, University of Pretoria, Lynwood Road, Pretoria, 0001, South Africa. Tel: +27 12 420 3772 email: egmont.rohwer@up.ac.za

Keywords: antiretroviral, HIV, surface water, LC-MS, solid phase extraction, personal care products

Abstract

The study and quantification of personal care products, such as pharmaceuticals, in surface water has become popular in recent years; yet very little description of these compounds' presence in South African surface water exists in the literature. Antiretrovirals (ARVs), used to treat human immunodeficiency virus (HIV) are rarely considered within this field. A new method for the simultaneous quantification of 12 antiretroviral compounds in surface water using the standard addition method is described. Water samples were concentrated by a generic automated solid phase extraction method and analysed by ultra-high pressure liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). Substantial matrix effect was encountered in the samples with an average method detection limit of 90.4 ng/L. This is the first reported countrywide survey of South African surface water for the quantification of these compounds with average concentrations ranging between 26.5 and 430 ng/L.

Capsule:

This work represents the first quantitative description of antiretrovirals, as a group, in surface water using a modified standard addition method and UHPLC-MS/MS.

Highlights:

- An LC-MS/MS method for the detection of 12 antiretroviral drugs was developed
- The compounds were detected in South African surface water for the first time
- Targets occurred in the low to mid ng/L range
- Nevirapine occurred ubiquitously across all the samples tested
- Matrix effect was corrected for using a modified standard addition method

2.1 Introduction

Concerns regarding the presence of personal care products (PCPs), such as pharmaceuticals, in water supplies have arisen recently with various researchers showing that a wide variety of pharmaceuticals are discharged into the environment as a result of inadequate wastewater treatment (Ferrer and Thurman, 2012; Yu et al., 2012; Luo et al., 2014). This appears to be a global phenomenon (Kümmerer, 2009), and in addition to discharge from waste-water treatment works (WWTWs) one should also consider alternative sources of contamination such as improper disposal of expired pharmaceutical stocks e.g. leachate from pharmaceutical landfilling (Peng, Ou, et al., 2014), or, pit latrines (Graham and Polizzotto, 2013) in developing countries. There is a marked gap in the literature, regarding this global phenomenon, describing the situation in Africa.

Very little research has been carried out in South Africa to determine the presence of pharmaceuticals and their degradation products in surface water using mass spectrometry; as determined by searching the curated "US EPA Bibliographic Database of Publications Relevant to Pharmaceuticals and Personal Care Products" (Daughton and Scuderi, 2012). Also, South Africa utilises more anti-retroviral compounds per capita than any other nation in the fight against HIV/AIDS, with approximately 2 150 880 people receiving ARVs in 2012 as contrasted to the approximate 199 000 people on ARV therapy in Eastern Europe (WHO, 2013). This presents a novel problem with regards to the presence and transformation of these compounds in the environment. Since South Africa uses more of these compounds than any other nation it has been theorized that these compounds should be present in the environment to a much greater extent. This phenomenon should also be exacerbated by the overall low rainfall and water scarcity in sub-Saharan Africa; which would lead to lower environmental dilution of the target compounds.

Anti-HIV compounds such as nucleoside and non-nucleoside reverse transcriptase inhibitors, protease inhibitors, fusion inhibitors, entry inhibitors and integrase strand transfer inhibitors, are used to treat HIV ("FDA Antiretroviral drugs used in the

treatment of HIV infection," 2014) and to prevent mother-to-child transmission (Mofenson, 2010). The breadth of the compound class therefore poses an interesting analytical challenge, and to our knowledge no other research addressing their simultaneous detection, in any environmental matrix, has been carried out.

Prasse and colleagues (2010) studied the presence of five anti-HIV compounds in addition to other anti-virals in the Hessian Ried river systems and found their presence as a result of WWTW discharge. Peng and co-workers (2014) utilised a similar methodology to detect antiviral drugs, including Stavudine and Zidovudine, in the Pearl River Delta in China; but could not detect these compounds in surface water. Given the global usage of these pharmaceuticals and since the compounds have been detected in European surface water and WWTW influent and effluent, it is predicted that higher concentrations should be present in South African water supplies due to higher usage in the population. These compounds can be seen as additional candidates for consideration as emerging pollutants.

The main objective of this work was to develop a single LC-MS/MS method for the analysis of 12 commonly used anti-HIV compounds, concentrated by generic solid phase extraction (SPE), to quantitatively determine their prevalence in South African surface water. This work also represents the first step in a nationwide survey for the detection of pharmaceuticals in surface water in South Africa.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

Analytical reference standards obtained from the US, British and European Pharmacopoeia as well as Toronto Research Chemicals (Toronto, Canada) were purchased from Anatech (Johannesburg, South Africa). All compounds were of 97% purity or better as indicated by the vendor. Zalcitabine, Tenofovir, Abacavir, Efavirenz, Lamivudine, Didanosine, Stavudine, Zidovudine, Nevirapine, Indinavir, Ritonavir, Lopinavir and caffeine stock solutions (1 mg/mL) were prepared in

methanol and stored at -20 °C until use. 13C3-trimethyl caffeine, 100 µg/mL in methanol, was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA) and diluted to 20 µg/mL in methanol before use. Standards were prepared and handled in a separate room from samples in order to prevent cross contamination. LC-MS grade acetonitrile, methanol and water were purchased from Lab-Scan (Gliwice, Poland) and formic acid and ammonium formate from Merck (Johannesburg, South Africa). No South African-origin water was used as a reagent in the course of this research.

2.2.2 Environmental Sample Collection and Extraction

Grab samples were collected from various surface water sources in South Africa, detailed in Table S2-3 (Supporting Information). Sampling locations were selected based on their proximity to WWTWs and the distance from major bodies of water. None of the samples were part of a "pristine" water course and all were either downstream of WWTWs or urbanised environments. Samples were collected in "virgin" borosilicate Schott bottles while wearing nitrile gloves (to prevent the introduction of contaminants) and transported, protected from light, to the laboratory at room temperature. Samples were stored at -20°C until extraction, after which extracts were stored at -20°C until analysis.

Five hundred millilitres of each sample was filtered using a 1 µm glass-fibre syringe-driven filter (Pall, USA) and extracted using the Smart Prep Extraction System (Horizon, USA), which is an automated offline solid phase extraction instrument. The extraction procedure was modified from a method developed by Ferrer and Thurman in order to maintain a level of universality (Ferrer and Thurman, 2012). Briefly, 6 cc Oasis HLB, 500 mg SPE cartridges (Waters, Milford, MA, USA) were conditioned with 4 mL of methanol followed by 6 mL of HPLC-grade water. Five hundred millilitres of sample was introduced at a flow rate of 10 mL/min after which cartridges were dried under nitrogen for three minutes. Cartridges were eluted twice with 5 mL of methanol at 10 mL/min and the eluate

dried under a gentle stream of nitrogen to 500 µL. All extractions were performed at 18 °C (+- 0.5 °C) in a dedicated area.

The standard addition method was used to quantify all target analytes, with modification from the traditional approach (Conley et al., 2008). Each extracted sample (190 µL) was combined with 10 µL 13C3-caffeine standard and divided into four aliquots of 45 µL each. To these, 5 µL of either 10 000 ng/mL, 1000 ng/mL, 100 ng/mL or 0 ng/mL standard mixture in methanol was added. The samples were analysed in triplicate in order of increasing concentration, with blank injections between each in order to prevent and evaluate carry-over. Standard addition data was analysed using Mass Hunter Quant (Agilent, Santa Clara, USA). The data obtained from these analyses were compared to an external calibration curve, generated by injecting a mixture of standards in methanol at 1, 10, 100 and 1000 ng/mL, in order to assess the effects of the matrix. Retention time reproducibility was checked periodically by injecting a standard mixture after every batch analysis.

2.2.3 LC-MS/MS Analysis

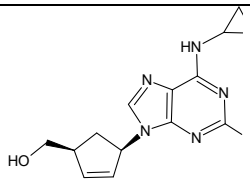
An array of similar chromatographic columns were tested to optimise separation. The XDB-C8 1.8 µm 3.0x50 mm, Eclipse Plus RRHD C18 1.8 µm 2.1x50 mm (Agilent), Kinetex PFP 1.7 µm 50x2.1 mm, Kinetex XB-C18 1.7 µm 2.1x50 mm, Kinetex C8 2.6 µm 50x4.6mm and Kinetex C18 1.7 µm 50x2.1 mm (Phenomenex) were evaluated. Combinations of mobile phases such as water and either acetonitrile or methanol with either 0.1% formic acid or 5 mM ammonium formate were tested, to lead to the optimized method. Extensive method validation was carried out and may be found described further in the Supplementary information.

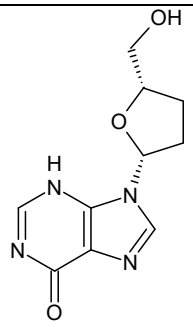
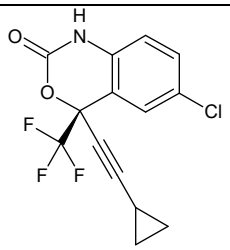
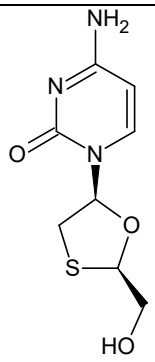
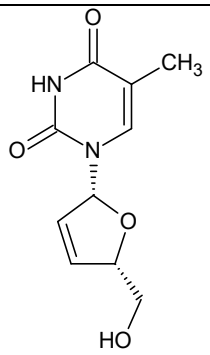
SPE extracts were analysed by LC-ESI-MS/MS. Target compounds were separated using an Agilent 1290 series UHPLC and mobile phases consisted of water (A) and acetonitrile (B) both with 0.1% formic acid. Following a 15 µL injection onto a Zorbax Eclipse C8 XDB, 3.0x50mm, 1.8 µm column, the chromatographic gradient was as follows: 0% B, 3 min; 100% B 20 min; 100% B 25

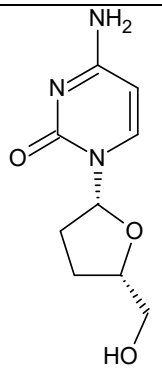
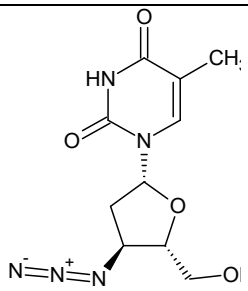
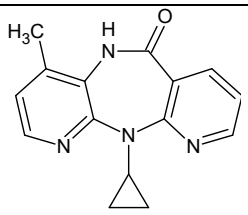
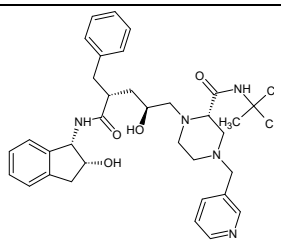
min; 0% B 30 min; 0% B 40 min. A flow rate of 0.4 mL/min was used, and the column was maintained at 22 °C with no column effluent splitting. The UHPLC was coupled to an Agilent 6460 triple quadrupole, equipped with a Jet Stream electrospray ionization (ESI) source. All analyses were performed in positive ion mode.

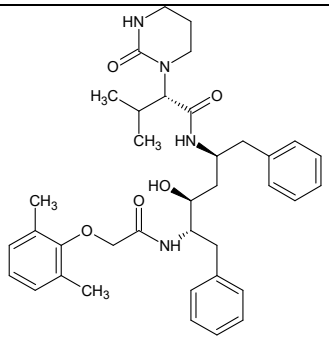
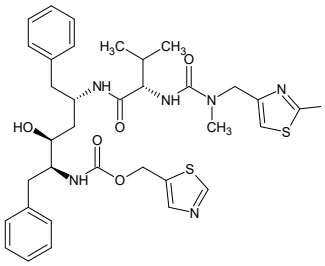
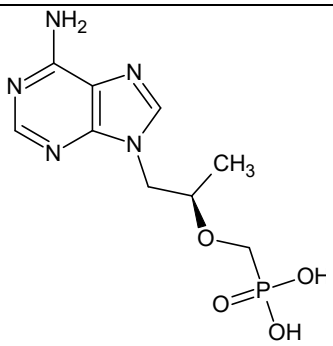
MS/MS optimisation was performed automatically using the Agilent Optimizer software package (Table 2-1) and verified manually. These settings were then combined into a single dynamic MRM method with the following ESI Jet Steam source conditions: Delta EMV 400 V, gas temperature 250 ºC, gas flow 8 L/min, nebuliser pressure 35 psi, sheath gas temperature 300 ºC, sheath gas flow 10 L/min and capillary voltage 3000 V.

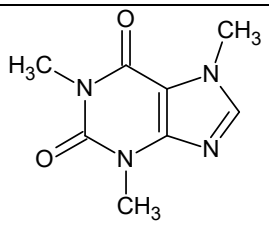
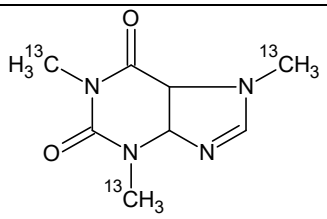
Table 2-1: Compound names, CAS numbers, precursor ions, product ions, collision energies, fragmentor voltages, structures and predicted Log Kow and pKa values of target pharmaceutical analytes.

Name (CAS No.)	RT (Min)	*m/z* Precursor Ion	m/z Product Ion 1 (Collision Energy eV)	m/z Product Ion 2 (Collision Energy eV)	Fragmentor Voltage (V)	Structure	Log Kow **	pKa Strongest Acid/Base ***
Nucleoside Reverse Transcriptase Inhibitors								
Abacavir (136470-78-5)	6.0	87.2	191 (17)	150 (29)	108		1.54	15.41/5.77

Name (CAS No.)	RT (Min)	*m/z* Precursor Ion	m/z Product Ion 1 (Collision Energy eV)	m/z Product Ion 2 (Collision Energy eV)	Fragmentor Voltage (V)	Structure	Log Kow **	pKa Strongest Acid/Base ***
Didanosine (69655-05-6)	5.3	237.1	137 (6)	76 (40)	80/76*		-3.14	6.94/2.75
Efavirenz (154598-52-4)	13.9	316	299.1 (0)	237 (8)	80/84*		4.15	12.52/-1.5
Lamivudine (134678-17-4)	2.9	230.1	112 (8)	95 (42)	144		-2.62	14.29/-0.16
Stavudine (3056-17-5)	5.5	225.1	127 (10)	99 (0)	10		-0.79	9.95/-3

Name (CAS No.)	RT (Min)	*m/z* Precursor Ion	m/z Product Ion 1 (Collision Energy eV)	m/z Product Ion 2 (Collision Energy eV)	Fragmentor Voltage (V)	Structure	Log Kow **	pKa Strongest Acid/Base ***
Zalcitabine (7481-89-2)	0.6	212.1	112 (6)	95 (38)	40		-1.72	14.67/0.18
Zidovudine (30516-87-1)	6.7	268.1	127 (8)	110 (32)	76		-7.05	9.96/-3
Non-nucleoside reverse transcriptase inhibitor (nNRTI)								
Nevirapine (129618-40-2)	8.2	267.1	226 (24)	80 (44)	120		3.89	10.37/5.06
Protease inhibitors								
Indinavir (150378-17-9)	8.8	614.4	421.3 (32)	97.1 (58)	164/160*		2.66	13.19/7.37

Name (CAS No.)	RT (Min)	*m/z* Precursor Ion	m/z Product Ion 1 (Collision Energy eV)	m/z Product Ion 2 (Collision Energy eV)	Fragmentor Voltage (V)	Structure	Log Kow **	pKa Strongest Acid/Base ***
Lopinavir (192725-17-0)	13.5	629	183 (20)	155 (40)	50		6.26	13.39/-1.5
Ritonavir (155213-67-5)	13.2	721.3	296 (14)	140 (58)	144		5.28	13.68/2.84
nucleotide analogue reverse transcriptase inhibitors								
Tenofovir (147127-20-6)	1.9	288.1	176.1 (24)	159 (32)	50		-1.57	1.35/5.12

Name (CAS No.)	RT (Min)	*m/z* Precursor Ion	m/z Product Ion 1 (Collision Energy eV)	m/z Product Ion 2 (Collision Energy eV)	Fragmentor Voltage (V)	Structure	Log Kow **	pKa Strongest Acid/Base ***
Xanthines								
Caffeine (58-08-2)	6.2	195	138 (16)	110 (20)	100		0.16	-/-0.92
Caffeine (Trimethyl 13C3) (78072-66-9)	6.2	198.2	140.2 (16)	112.1 (20)	100		0.16	-

* Product ion 1 Fragmentor Voltage / Product Ion 2 Fragmentor Voltage

**Log Kow prediction from www.Chemspider.com (EPI suite prediction)

***pKa from www.drugbank.ca

2.3 Results and Discussion

2.3.1 Chromatography and Mass Spectrometry

A variety of chromatographic programs, columns and solvents were tested; yet it was decided to sacrifice optimal conditions for the sake of universality. A number of the compounds are polar and were not effectively retained on C18 columns. A short C8 column (50 mm) was therefore chosen in order to retain more polar compounds and reduce elution times of non-polar compounds. A shorter column maximises the effect of column flushing at the end of each run since it was found that narrow bore columns rapidly became blocked, displaying retention time and peak shape aberrations, regardless of sample filtration steps. A chromatographic programme, with longer holds and flushing times, was chosen to maximise column

lifetime and to ensure chromatographic reproducibility across analyses since the use of short gradients could promote the co-elution of interfering compounds that may enhance matrix effect, thereby eliminating the inherent purification properties of chromatography.

The retention time for each of the compounds on six different 50 mm columns from two major manufacturers; calculated from averaged triplicate injections of a 1 µg/mL mixture of the targets were utilised to compare column suitability (Figure S2-1, Supporting Information). The columns are highly comparable yet it was found that the Agilent XDB-C8 column yielded the best peak shape. When comparing compounds with the least retention time difference, the Agilent XDB-C8 column showed the highest resolution (0.19 min). Stavudine and Tenofovir display poor peak shapes on the majority of columns; with Stavudine eluting in the dead-volume on the Kinetix XB-C18 column (Phenomenex). The XDB-C8 column was also chosen because it is amenable to the addition of a greater variety of target compounds at a later stage.

These compounds are rarely analysed in a single run by mass spectrometry. Due to their divergent nature, as illustrated by their varying Kow values (Table 2-1), average source settings were chosen to yield the most optimal conditions for the group, which in most cases is to the detriment of individual compounds' levels of sensitivity. The Agilent Mass Hunter Optimiser was used to automatically adjust fragmentor voltages and collision energies for each compound, which yielded a variety of product ions (Table S2-3, Supporting Information). Fragmentor and collision energy settings, resulting in optimal quantifier and qualifier ions (based on abundance and size), were chosen for each target compound.

2.3.2 Environmental sample analysis

The extraction and detection method was validated using a variety of techniques which would describe the effect of the matrix upon the analysis as well as the analytical limitations of quantitating the target compounds in a complex water

matrix. Results of these studies are provided in the Supplementary Information (Figure S2-2).

Samples were collected during varying weather conditions and different times of day from dams and rivers across South Africa. Where logistically possible a body of water was sampled at multiple locations, with each sample representing a snapshot at any given time (Figure S2-3, Supporting Information). Inferences regarding the continual contamination of a particular water system can only be made if it is sampled continuously using passive samplers (Bartelt-Hunt et al., 2009) or over a long period of time (Kasprzyk-Hordern et al., 2008). The initial aim of this research however was only to determine the presence of ARVs in South African surface water (Table 2-2). This aim was largely achieved as a sample was taken and analysed from almost every major river and dam in the country.

Table 2-2: Pharmaceutical concentrations (ng/L) at various locations across South Africa as determined by automated solid phase extraction, standard addition and analysis by UHPLC-ESI-MS/MS. Standard deviation of triplicate analysis presented in brackets.

		Drug Concentration (ng/L)												
	GPS Co-Ordinates	**Zalcitabine**	**Tenofovir**	**Lamivudine**	**Didanosine**	**Stavudine**	**Abacavir**	**Caffeine**	**Zidovudine**	**Nevirapine**	**Indinavir**	**Ritonavir**	**Lopinavir**	**Efavirenz**
Roodeplaat Dam System														
Pienaars River Inflow	-25.678677, 28.357116	n.d	243 (0.5)	242 (2.9)	n.d	778 (26.1)	n.q	149 (1.4)	339 (3.0)	1480 (13)	n.d	n.q	n.q	n.d
Zeekoegat WWTW Outflow	-25.624620, 28.341890	n.d	n.d	n.d	n.d	n.d	n.d	n.q	973 (3.5)	n.q	n.d	n.q	n.q	n.q
Angling Area	-25.626404, 28.345692	n.d	n.d	n.d	n.d	102 (11.3)	n.q	243 (2.9)	627 (4.0)	236 (5.3)	n.q	n.q	n.q	n.d
S.E Bank	-25.637763, 28.344150	n.d	n.d	184 (0.4)	n.q	n.d	n.q	397 (1.9)	298 (0.9)	337 (4.0)	n.d	n.q	n.q	n.q
Motorboat Launch	-25.618238, 28.358642	n.d	n.d	n.d	n.q	n.q	n.q	366 (4.8)	293 (3.1)	316 (5.2)	n.d	n.q	n.q	n.q
Rowing Club	-25.623345, 28.349842	n.d	n.d	151 (0.1)	n.q	n.d	n.q	295 (2.9)	306 (2.5)	294 (4.1)	n.d	n.q	n.q	n.d
Roodeplaat Outflow	-25.608244, 28.367231	n.d	n.d	n.d	n.q	413 (7.7)	n.q	303 (2.8)	224 (2.0)	347 (6.0)	n.q	n.q	n.q	n.q
Rietvlei Dam														
Southern Bank	-25.881576, 28.268585	n.d	n.d	132 (0.2)	n.d	n.d	n.d	181 (1.0)	156 (0.9)	n.q	n.d	n.d	n.q	n.d
Northern Bank	-25.876767, 28.279846	n.d	n.d	94.5 (0.1)	n.d	n.d	n.d	249 (1.5)	188 (0.6)	177 (1.6)	n.d	n.d	n.q	n.d
Orange River System														
Orange River (Bethulie)	-30.534670, 26.022975	71.3 (0.0)	189 (4.4)	n.d	n.d	n.d	n.q	53.9 (0.1)	n.q	n.q	n.q	n.q	n.d	n.d

		Drug Concentration (ng/L)												
	GPS Co-Ordinates	**Zalcitabine**	**Tenofovir**	**Lamivudine**	**Didanosine**	**Stavudine**	**Abacavir**	**Caffeine**	**Zidovudine**	**Nevirapine**	**Indinavir**	**Ritonavir**	**Lopinavir**	**Efavirenz**
Gariep Dam Oviston	-30.692147, 25.761238	n.d	n.d	n.d	54.0 (0.0)	n.d	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.d
Gariep Dam (N.E)	-30.603858, 25.503609	n.d	145 (0.03)	n.d	n.d	n.d	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.d
Vaal confluence	-29.070882, 23.637209	n.d	n.d	n.d	n.d	n.d	n.d	n.q	n.d	n.q	n.d	n.d	n.d	n.d
Orange confluence	-29.072898, 23.638936	n.d	n.d	n.d	n.d	n.d	n.d	n.q	n.d	n.q	n.d	n.d	n.d	n.d
Confluence	-29.071810, 23.635868	n.d	n.d	n.d	n.d	n.d	n.d	n.q	n.d	n.q	n.d	n.d	n.d	n.d
Cape Region														
Eerste Rivier	-33.941603, 18.857078	n.d	n.d	n.d	n.d	n.d	n.d	n.q	n.d	n.q	n.d	n.d	n.d	n.d
Theewaterskloof Dam	-34.027283, 19.208261	n.d	n.d	n.d	n.d	n.d	n.d	n.q	n.d	n.q	n.d	n.d	n.d	n.d
Vaal Dam														
Dam wall	-26.883278, 28.116047	n.d	n.d	n.d	n.d	n.d	n.d	78.1 (1.0)	n.q	n.q	n.d	n.q	n.q	n.d
Oranjeville	-26.999155, 28.214893	n.d	n.d	n.d	n.d	n.d	n.d	n.q	n.q	n.q	n.d	n.d	n.q	n.d
Vaal Dam Inflow	-27.020575, 28.608589	n.d	n.d	n.d	n.d	n.d	n.d	80.7 (1.1)	51.7 (1.1)	n.q	n.d	n.q	n.q	n.q
Vaal Dam Out Flow	-26.874950, 28.115583	n.d	n.d	n.d	n.d	n.d	n.d	158 (2.8)	n.q	n.q	n.d	n.d	n.q	n.q

		Drug Concentration (ng/L)												
	GPS Co-Ordinates	**Zalcitabine**	**Tenofovir**	**Lamivudine**	**Didanosine**	**Stavudine**	**Abacavir**	**Caffeine**	**Zidovudine**	**Nevirapine**	**Indinavir**	**Ritonavir**	**Lopinavir**	**Efavirenz**
Single system samples														
Hartebeesfontein WWTW Outflow	-26.030715, 28.291084	n.d	n.d	n.d	n.d	n.d	n.d	333 (3.1)	452 (3.4)	n.q	n.q	n.q	130 (2.1)	n.q
Ditholo	-25.320242, 28.340728	n.d	n.d	n.q	n.d	n.q	n.d	n.q	n.d	143 (0.4)	n.d	n.d	n.q	n.d
Hartbeespoort Dam, Meerhof (2011)	-25.760775, 27.891871	n.d	n.d	n.d	n.d	n.d	n.d	927 (5.7)	350 (0.8)	130 (0.8)	n.q	n.q	283 (5.7)	n.d
Hartbeespoort Dam, Meerhof (2014)	-25.760775, 27.891871	28.2 (1.7)	n.d	n.q	54.1	n.d	n.q	402 (1.5)	139 (2.5)	137 (5.2)	n.q	n.q	305 (37.9)	n.q
Hartbeespoort Dam, Tap Water Sample	-25.745594, 27.911238	8.4 (0.03)	n.d	n.d	n.d	n.d	n.d	263 (0.7)	72.7	n.q	n.d	n.q	n.q	n.q
Renosterkop	-25.108639, 28.887359	n.d	n.d	n.d	n.q	n.d	n.d	441 (5.9)	n.q	n.q	n.d	n.d	n.d	n.d
Inanda Dam	-29.673792, 30.854874	n.d	n.d	n.d	n.q	n.d	n.d	232 (0.1)	n.q	n.q	n.d	n.d	n.q	n.d
Inanda Dam offshore	-29.674016, 30.860239	n.d	n.d	n.d	n.q	n.d	n.d	40.7 (0.1)	n.q	n.q	n.d	n.d	n.q	n.d
Total no. of occurrences across all locations *		3	3	7	9	5	10	29	23	29	8	16	22	9
Average concentrations quantified (ng/L)**		36.0	192	160	54.1	431	n.q	273	319	360	n.q	n.q	239	n.q

* Quantified and unquantified occurrences
** Average values determined from only samples in which targets compounds were quantified.
n.d – Not detected; values below instrumental LOD.
n.q – Not quantified; values above instrumental LOD but below MDL.

Nevirapine, Lopinavir and Zidovudine were found most frequently throughout the survey. The drug concentrations are in the low ng/L range with Stavudine, Nevirapine and Zidovudine showing the highest averages.

The larger systems, such as the Vaal River was tracked from an urbanised environment to where it terminates into the Orange River at a large confluence. Similarly, the Orange River was followed from the Gariep Dam, its largest impoundment, near an urban area to the confluence. Compounds detected near urban areas become undetectable as the water course progresses. This may be due to either dilution or biodegradation.

Figure S2-4 shows the chromatographic profile of one of the environmental samples spiked with the highest calibration level. Good peak shape is obtained for the majority of compounds although the relative response between the compounds is quite large. This is due to the generic nature of the analytical method.

Greater attention was given to the Roodeplaat Dam system due to its proximity to the laboratory as well as the fact that two WWTWs fed into the dam (Zeekoegat and Baviaanspoort). Multiple samples were collected in the dam with a maximum distance between the sampling locations of no more than 1.3 Km. The Roodeplaat Dam is a hypertrophic man-made impoundment of the Pienaars, Hartebees and Edendale rivers. The dam wall was built in 1959 and provides water for both domestic and irrigation use. The dam is located in the most densely populated province of South Africa and along with the Hartbeespoort dam is subject to high levels of eutrophication. Roodeplaat has a 668 Km^2 catchment area with a 397 ha surface area and a mean depth of 10.3 m (van Ginkel and Silberbauer, 2007).

The presence of pharmaceuticals in this body of water has not been described in the literature yet there are studies that describe the effects of pollution on eutrophication (Jones and Lee, 1984; van Ginkel and Silberbauer, 2007). Researchers have found that the ecosystem has changed over the past two decades with changes in the dominant phytoplankton species in the system (van Ginkel and Silberbauer, 2007). Physiological changes in indicator fish species have

also been attributed to human activities (Marchand, 2009; Van Dyk et al., 2012). The impact and significance of the presence of personal care products (e.g. pharmaceuticals) has had on this aquatic system has yet to be determined.

The concentrations of the compounds were found to vary between the two WWTW (that feed into the Roodeplaat Dam) but a sample could not be taken upstream of the Baviaanspoort plant therefore the background contribution made by the Pienaars River (which flows through an urbanized area) could not be made. The majority of the target compounds were found in the Roodeplaat system at varying concentrations. This highlights the importance of collecting multiple samples from different points in a system before significant inferences can be made. This is further substantiated by the differences in concentration detected at the Orange-Vaal River confluence. Samples from the Vaal River, Orange River and from the confluence (all taken within 100 meters of each other) were found to differ significantly.

In the Roodeplaat system, as in most cases, the drug concentration is lower at the outflow than any other point sampled within the dam. There are points of higher concentration within the dam e.g. at the recreational angling area. This may be due to the depth and mixing of the water in the area. The shallow angling area is approximately 100 m from the Zeekoegat WWTW outflow, which did not contribute all of the compounds detected in the angling area. The data generated from this body of water are potentially significant because the dam is used for recreation (fishing and water sport) as well as a source for potable water.

The Roodeplaat Dam is a warm monomitic body of water, and as a result, mixing occurs once annually in winter when ambient temperatures cool. As samples were taken in late winter, one could presume that the concentrations detected are a combination of recent additions (from the WWTW) and persistent compounds that rose from the bottom of the dam. Since temperature stratification occurs in many lakes, surface sampling may only be relevant at certain times of year.

Nevirapine was detected in all of the surface water samples but only reliably quantified in nine out of the 24 sampling locations. The compound is a non-nucleoside reverse transcriptase inhibitor that is widely used for the treatment of HIV as well as the prevention of mother-to-child transmission (Coovadia et al., 2012). The prevalence of this compound can most likely be attributed not only to its frequent therapeutic use, but also the compound's persistence in the environment. The compound has been found to be non-biodegradable in a "closed bottle" *in vitro* system (Vanková and others, 2010). This type of *"in vitro"* research shows that it is most likely persistent in the environment, yet it does not address the compounds' behaviour in WWTWs. This may only be determined by measuring WWTW outflows. Two such outflows, from the Zeekoegat and Hartebeesfontein WWTW were sampled at the point where they discharge into the environment and many of the target compounds were detected. It must be noted that this is not a measure of WWTW efficacy; which may only be achieved by comparing target levels in the raw sewage as compared to the discharge.

It has been shown in Europe though that compounds such as Nevirapine and Zidovudine are not removed by WWTW whereas compounds such as lamivudine, Stavudine and Abacavir are removed with greater than 80% efficacy. The compounds have been detected in the low ng/L range in German surface water (Prasse et al., 2010) and as anticipated are present at much higher levels in South Africa.

Caffeine is commonly used an anthropogenic marker for surface water contamination by wastewater (Buerge et al., 2003) and it appears ubiquitously throughout the sampled areas. However no relationships were observed between caffeine levels and target compound levels as determined by the variability in the ratio between target and caffeine concentration. Targets were detected in samples where caffeine could not be quantified. It can be speculated that caffeine may have a shorter environmental half-life than the targets or the compounds are not removed with equal efficiency by WWTW. Potentially, the WWTW in a specific area is effective or that the population is not consuming as many ARVs as other more

urbanised areas, which is evident in the samples taken from rural areas where population density is lower. A large, impounded body of water may provide a cumulative picture of human activity. Even if target compound influx is not detectable, due to the persistent nature of these compounds, concentrations may increase over time.

Additional considerations must be made in sub-Saharan Africa that may not be relevant to similar European studies. Inadequate sanitation in certain parts of the country, the use of pit latrines and malfunctioning WWTWs indicates that untreated human waste is quite often discharged into water systems. The presence of these compounds in the environment cannot be solely attributed to functional WWTW discharge.

The standard addition method was utilized since a specific quantitative answer was required for this research. The method is highly labour and cost intensive as a single sample analysis consists of a minimum of 17 injections. For screening purposes, it is suggested that an external calibration curve consisting of spiked matrix in conjunction with isotopically labelled standards should be used. The standard addition method does not lend itself to routine screening but has proven to be vital when quantifying novel contaminants for the first time in a particular water system. It is proposed that once the linearity of a standard addition method calibration is established in a particular matrix, the number of calibrators used for routine work may be reduced, thereby reducing costs and analytical time.

One of the major pitfalls of LC-ESI analyses is the effect of the matrix on analytical accuracy. Co-eluting compounds either suppress or enhance target signals leading to inaccurate quantitation. Analytical inaccuracy is a result of the synergistic effect of: sample components, compounds released during pre-treatment or extraction as well as mobile phase additives. Sample to matrix ratios, matrix type, extraction methodology, chromatography and mass spectrometer type all influence the extent of matrix effect (Gosetti et al., 2010). Samples may either be analysed by external calibration using matrix matched standards (Chen et al., 2010) or by standard addition (Ito and Tsukada, 2002; Conley et al., 2008; Cimetiere et al., 2013).

Matrix matched calibration has been found to be inaccurate in cases with high matrix load (Stüber and Reemtsma, 2004). Since matrix blanks are often unavailable and a single sample locality is chosen to provide matrix calibration for multiple locations; the incorrect assumption that all matrices are equivalent is made. Matrix effect can be accounted for by using isotopically labelled standards followed by correction. However, unless a standard for each target is used, an incorrect assumption is made that all of the target compounds have identical chemical properties (Gosetti et al., 2010).

One important benefit of the standard addition method is its inherent quality control. Instrumental variation that occurs over time is accounted for since each sample is injected with its calibrators every time. This, is as opposed to batch calibration which may show error over time with large sample numbers.

For these reasons a modified standard addition method (Conley et al., 2008) was applied to environmental analyses to account for matrix interference. The environmental relevance of the presence of antiretrovirals in water supplies is not immediately clear, as is the case with antibiotics, in that they may promote drug resistance. HIV does not have a non-human host and does not occur in the environment, as is the case with other viruses or bacteria. Also, the model of transfer of resistance genes between species cannot be applied when considering the virus. The influence of these compounds on environmental retroviruses has not been established in the literature, and although the presence of these compounds in the environment may not affect the target virus, but they may still promote the development of drug resistance in other pathogens.

2.4 Conclusion

The LC-MS method presented efficiently separates and analyses the major members of the ARV drug class. The standard addition method accurately quantifies these compounds in complex matrices and is a more viable alternative to the use of isotopically labelled standards, many of which are not commercially

available for this group of compounds. The extraction and analytical method was developed to remain generic and amenable to the addition of more compounds to the analytical paradigm. This work represents a nationwide survey of surface water and presents, for the first time, qualitative data for many of these pharmaceuticals in the environment. Compounds such as Nevirapine have been detected in European surface waters, yet the majority of the compounds targeted in this work have not been previously described in the literature. The ecotoxicity of many emerging pollutants and their degradation products has not been established and further research may aid in determining the consequences of discharging these compounds into the environment.

A variety of studies describe the modification of pharmaceuticals as a result of simulated waste water treatment (Acero et al., 2013; Huerta-Fontela et al., 2012; Shen and Andrews, 2011; Soufan et al., 2012) and it has been found that potentially innocuous compounds such as acetaminophen (Bedner and MacCrehan, 2006) may be converted into toxic by-products as a result of chlorination. As this is the first description of many of these ARVs in the environment further research describing their biotransformation is needed.

Acknowledgements:

We gratefully acknowledge the South African Military Health Services for funding this research.

Supporting Information:

The supporting information describes chromatographic optimisation, sampling locations, a list of product ions for each of the compounds obtained by mass spectrometry, method validation studies, a map of sampling locations and an example of a chromatogram obtained during standard addition analysis.

2.5 References

1. Acero, J.L., Benitez, F.J., Real, F.J., Roldan, G., Rodriguez, E., 2013. Chlorination and bromination kinetics of emerging contaminants in aqueous systems. Chemical Engineering Journal 219, 43–50.
2. Bartelt-Hunt, S.L., Snow, D.D., Damon, T., Shockley, J., Hoagland, K., 2009. The occurrence of illicit and therapeutic pharmaceuticals in wastewater effluent and surface waters in Nebraska. Environmental Pollution 157, 786–791.
3. Bedner, M., MacCrehan, W.A., 2006. Transformation of acetaminophen by chlorination produces the toxicants 1, 4-benzoquinone and N-acetyl-p-benzoquinone imine. Environmental Science & Technology 40, 516–522.
4. Bijlsma, L., Boix, C., Niessen, W., Ibáñez, M., Sancho, J.V., Hernández, F., 2013. Investigation of degradation products of cocaine and benzoylecgonine in the aquatic environment. Science of the Total Environment 443, 200–208.
5. Buerge, I.J., Poiger, T., Müller, M.D., Buser, H.-R., 2003. Caffeine, an anthropogenic marker for wastewater contamination of surface waters. Environmental Science & Technology 37, 691–700.
6. Chen, F., Ying, G.-G., Yang, J.-F., Zhao, J.-L., Wang, L., 2010. Rapid resolution liquid chromatography-tandem mass spectrometry method for the determination of endocrine disrupting chemicals (EDCs), pharmaceuticals and personal care products (PPCPs) in wastewater irrigated soils. Journal of Environmental Science and Health Part B 45, 682–693.
7. Cimetiere, N., Soutrel, I., Lemasle, M., Laplanche, A., Crocq, A., 2013. Standard addition method for the determination of pharmaceutical residues in drinking water by SPE-LC-MS/MS. Environmental Technology 34, 3031–3041.
8. Conley, J.M., Symes, S.J., Kindelberger, S.A., Richards, S.M., 2008. Rapid liquid chromatography-tandem mass spectrometry method for the determination of a broad mixture of pharmaceuticals in surface water. Journal of Chromatography A 1185, 206–215.

9. Coovadia, H.M., Brown, E.R., Fowler, M.G., Chipato, T., Moodley, D., Manji, K., Musoke, P., Stranix-Chibanda, L., Chetty, V., Fawzi, W., others, 2012. Efficacy and safety of an extended nevirapine regimen in infant children of breastfeeding mothers with HIV-1 infection for prevention of postnatal HIV-1 transmission (HPTN 046): a randomised, double-blind, placebo-controlled trial. The Lancet 379, 221–228.
10. Daughton, C., Scuderi, M., 2012. Pharmaceuticals and Personal Care Products (PPCPs): Relevant Literature. US Environmental Protection Agency, Las Vegas, NV.
11. Englert, B., 2007. Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS. US Environmental Protection Agency (EPA) 1–72.
12. FDA Antiretroviral drugs used in the treatment of HIV infection [WWW Document], n.d. URL http://www.fda.gov/ForConsumers/byAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm (accessed 5.26.14).
13. Ferrer, I., Thurman, E.M., 2012. Analysis of 100 pharmaceuticals and their degradates in water samples by liquid chromatography quadrupole time-of-flight mass spectrometry. Journal of Chromatography A 1259, 148–157.
14. Gosetti, F., Mazzucco, E., Zampieri, D., Gennaro, M.C., 2010. Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. Journal of Chromatography A 1217, 3929–3937.
15. Graham, J.P., Polizzotto, M.L., 2013. Pit latrines and their impacts on groundwater quality: a systematic review. Environmental Health Perspectives 121, 521–530.
16. He, Y., Chen, W., Zheng, X., Wang, X., Huang, X., 2013. Fate and removal of typical pharmaceuticals and personal care products by three different treatment processes. Science of the Total Environment 447, 248–254.

17. Huerta-Fontela, M., Pineda, O., Ventura, F., Galceran, M.T., 2012. New Chlorinated Amphetamine Type Stimulants Disinfection-By-Products Formed During Drinking Water Treatment. Water Research 3304–3314.
18. ICH, 2008. Validation of Analytical Procedures: Text and Methodology, Q2 (R1), Current Step 4 Version, Parent Guidelines on Methodology Dated November 6 1996, Incorporated in November 2005, in: International Conference on Harmonisation, Geneva, Switzerland, www.ich.org.
19. Ito, S., Tsukada, K., 2002. Matrix effect and correction by standard addition in quantitative liquid chromatographic-mass spectrometric analysis of diarrhetic shellfish poisoning toxins. Journal of Chromatography A 943, 39–46.
20. Jones, R.A., Lee, G.F., 1984. Impact of phosphorus load reductions on eutrophication-related water quality of Roodeplaat Dam(Reservoir), Republic of South Africa. Water S. A. 10, 115–120.
21. Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2008. The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. Water Research 42, 3498–3518.
22. Kümmerer, K., 2009. Antibiotics in the aquatic environment-a review-part I. Chemosphere 75, 417–434.
23. Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S., Wang, X.C., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Science of The Total Environment 473, 619–641.
24. Marchand, M.J., 2009. A histology-based fish health assessment to determine the health status and edibility of two indicator fish species from the Roodeplaat Dam. MSc Thesis, University of Johannesburg.
25. Mofenson, L.M., 2010. Prevention in neglected subpopulations: prevention of mother-to-child transmission of HIV infection. Clinical Infectious Diseases 50, S130–S148.
26. Peng, X., Ou, W., Wang, C., Wang, Z., Huang, Q., Jin, J., Tan, J., 2014a. Occurrence and ecological potential of pharmaceuticals and personal care

products in groundwater and reservoirs in the vicinity of municipal landfills in China. Science of The Total Environment 490, 889–898.

27. Peng, X., Wang, C., Zhang, K., Wang, Z., Huang, Q., Yu, Y., Ou, W., 2014b. Profile and behavior of antiviral drugs in aquatic environments of the Pearl River Delta, China. Science of the Total Environment 466, 755–761.
28. Prasse, C., Schlüsener, M.P., Schulz, R., Ternes, T.A., 2010. Antiviral drugs in wastewater and surface waters: a new pharmaceutical class of environmental relevance? Environmental Science & Technology 44, 1728–1735.
29. Shen, R., Andrews, S.A., 2011. Demonstration of 20 pharmaceuticals and personal care products (PPCPs) as nitrosamine precursors during chloramine disinfection. Water Research 45, 944–952.
30. Soufan, M., Deborde, M., Legube, B., 2012. Aqueous chlorination of diclofenac: Kinetic study and transformation products identification. Water Research 46(10), 3377-86.
31. Stüber, M., Reemtsma, T., 2004. Evaluation of three calibration methods to compensate matrix effects in environmental analysis with LC-ESI-MS. Analytical and Bioanalytical Chemistry 378, 910–916.
32. Van Dyk, J., Cochrane, M., Wagenaar, G., 2012. Liver histopathology of the sharptooth catfish Clarias gariepinus as a biomarker of aquatic pollution. Chemosphere 87, 301–311.
33. Van Ginkel, C.E., Silberbauer, M.J., 2007. Temporal trends in total phosphorus, temperature, oxygen, chlorophyll a and phytoplankton populations in Hartbeespoort Dam and Roodeplaat Dam, South Africa, between 1980 and 2000. African Journal of Aquatic Science 32, 63–70.
34. Vanková, M., *et al.*, 2010. Biodegradability analysis of pharmaceuticals used in developing countries; screening with OxiTop C-110. Thesis. Tampere University of Technology.
35. WHO, 2013. Global update on HIV treatment 2013: results, impact and opportunities.

36. Yu, K., Li, B., Zhang, T., 2012. Direct rapid analysis of multiple PPCPs in municipal wastewater using ultrahigh performance liquid chromatography-tandem mass spectrometry without SPE pre-concentration. Analytica Chimica Acta 738, 59-68.

2.6 Supporting Information

The Occurrence of Anti-Retroviral Compounds used for HIV Treatment in South African Surface Water

Timothy Paul Wood[a,c] (Corresponding Author), Cornelia S.J. Duvenage[b], Egmont Rohwer[c]

[a] Protechnik Laboratories, a division of ARMSCOR SOC Ltd., 103 Combretum Crescent, Centurion, Pretoria, 0001, South Africa. Tel: +27 12 665 9444, Fax: +27 12 665 0240, timw@protechnik.co.za

[b]Department of Internal Medicine, 1 Military Hospital, South African Military Health Services, Voortrekker Street, Pretoria, 0001, South Africa. Tel: +2783 273 3382, corneliaduv@mtnloaded.co.za

[c] Department of Chemistry, University of Pretoria, Lynwood Road, Pretoria, 0001, South Africa. Tel: +27 12 420 3772 email: egmont.rohwer@up.ac.za

2.6.1 Materials and Methods

2.6.1.1 Environmental Sample Collection and Extraction

Table S2-3: Grab sample collection log, detailing: locations, dates, weather conditions, time of day, season, sampling strategy and observations.

Sample Name	GPS Co-Ordinates	Date Collected	Weather Conditions and Time of Day	Season	Comments on Sampling Strategy and Visual Description
Roodeplaat Dam System*					
Pienaars River Inflow	-25.678677, 28.357116	12/08/2013	Clear, warm day. Early afternoon.	Winter	Clear sample, taken by hand 1 m

Sample Name	GPS Co-Ordinates	Date Collected	Weather Conditions and Time of Day	Season	Comments on Sampling Strategy and Visual Description
					into the river.
Zeekoegat WWTW Outflow	-25.624620, 28.341890	22/07/2014	Clear and mild. Mid-morning.	Winter	Rapidly flowing. Sample opaque and foul smelling.
Angling Area	-25.626404, 28.345692	22/07/2014	Clear and mild. Mid-morning.	Winter	Collected 100 m from the Zeekoegat inlet at the angling area. +- 2 m into the dam. Sample clear with algae and silt.
S.E Bank	-25.637763, 28.344150	22/07/2014	Clear and mild. Mid-morning.	Winter	+- 2 m into the dam. Sample clear.
Motorboat Launch	-25.618238, 28.358642	22/07/2014	Clear and mild. Mid-day.	Winter	Collected +- 1 m into the dam. Sample clear with arthropods.
Rowing Club	-25.623345, 28.349842	22/07/2014	Clear and mild. Mid-day.	Winter	Collected from a jetty +- 5 m into the dam. Sample

Sample Name	GPS Co-Ordinates	Date Collected	Weather Conditions and Time of Day	Season	Comments on Sampling Strategy and Visual Description
					clear with algae.
Roodeplaat Outflow	-25.608244, 28.367231	22/08/2014	Clear and mild. Mid-day.	Winter	Collected from the top of a +- 10 m bridge. Very clear water.
Rietvlei Dam*					
Southern Bank	-25.881576, 28.268585	17/07/2014	Clear and warm. Mid-day.	Winter	Collected from a bird-hide +- 15 m into the dam. Water clear with arthropods.
Northern Bank	-25.876767, 28.279846	17/07/2014	Clear and warm. Mid-day.	Winter	Collected from the bank. Water clear with algae.
Orange River System					
Orange River (Bethulie)	-30.534670, 26.022975	27/02/2014	Clear, hot day. Early afternoon.	Summer	Collected from a +- 30 m bridge over the Orange river. Fast flowing from earlier rains. . Water brown, completely opaque and

Sample Name	GPS Co-Ordinates	Date Collected	Weather Conditions and Time of Day	Season	Comments on Sampling Strategy and Visual Description
					silt laden.
Gariep Dam Oviston	-30.692147, 25.761238	27/02/2014	Clear, hot day. Midday.	Summer	Collected from a jetty +- 3 m into the dam. Water brown, completely opaque and silt laden.
Gariep Dam (N.E)	-30.603858, 25.503609	27/02/2014	Clear, hot day. Early afternoon.	Summer	Collected +- 2 m from the dam bank inside a natural cove. Water clear.
Vaal confluence	-29.070882, 23.637209	19/02/2014	Overcast and hot. Late morning.	Summer	10 m from the bank and +- 100 m from the confluence. Slow flowing river. Water opaque.
Orange confluence	-29.072898, 23.638936	19/02/2014	Overcast and hot. Late morning.	Summer	10 m from the bank and +- 100 m from the confluence. Slow flowing river. Water opaque.
Confluence	-29.071810, 23.635868	19/02/2014	Overcast and hot. Late	Summer	10 m from the bank. Slow flowing

Sample Name	GPS Co-Ordinates	Date Collected	Weather Conditions and Time of Day	Season	Comments on Sampling Strategy and Visual Description
			morning.		river. Water opaque.
Cape Region					
Eerste Rivier*	-33.941603, 18.857078	09/02/2014	Mid-Morning. Clear and warm.	Summer	Collected from a +-2 m bridge over the river. Water clear.
Theewaterskloof Dam	-34.027283, 19.208261	09/02/2014	Early morning. Clear and cool.	Summer	Collected from a +-15 m bridge over the dam. Water clear.
Vaal Dam					
Dam wall	-26.883278, 28.116047	25/02/2011	Clear, mid-day, afternoon.	Summer	Collected by boat +- 30 m from the dam wall. Water slightly silty.
Oranjeville	-26.999155, 28.214893	21/02/2014	Overcast and mild. Morning.	Summer	Collected from a bridge bisecting part of the dam. Water beige-opaque.
Vaal Dam Inflow	-27.020575, 28.608589	21/02/2014	Overcast and mild. Morning.	Summer	Collected from a +- 15 m bridge over the Vaal River.

Sample Name	GPS Co-Ordinates	Date Collected	Weather Conditions and Time of Day	Season	Comments on Sampling Strategy and Visual Description
					Water clear.
Vaal Dam Out Flow	-26.874950, 28.115583	21/02/2014	Raining. Late afternoon.	Summer	Collected from a bridge below the dam wall. Rapidly flowing water. Water clear.
Single system samples					
Hartebeesfontein WWTW Outflow*	-26.030715, 28.291084	24/02/2011	Clear and hot. Midday.	Summer	Collected +- 10 m from WWTW discharge. Water clear.
Ditholo	-25.320242, 28.340728	10/01/2014	Clear and hot. Midday.	Summer	Collected from a natural lake. Dark brown water.
Hartbeespoort Dam, Meerhof (2011)*	-25.760775, 27.891871	02/02/2011	Clear and hot. Midday.	Summer	Collected +- 2 m from the bank. Water green and opaque.
Hartbeespoort Dam, Meerhof (2014)*	-25.760775, 27.891871	25/02/2014	Clear and hot. Midday.	Summer	Collected +- 2 m from the bank. Water green and opaque.
Hartbeespoort Dam, Tap Water Sample*	-25.745594, 27.911238	25/02/2014	Clear and hot. Midday.	Summer	Collected from a filling station faucet.

Sample Name	GPS Co-Ordinates	Date Collected	Weather Conditions and Time of Day	Season	Comments on Sampling Strategy and Visual Description
Renosterkop	-25.108639, 28.887359	21/07/2013	Clear and mild. Midday.	Winter	Sample is slightly opaque and light brown.
Inanda Dam	-29.673792, 30.854874	01/03/2014	Clear and hot. Midday.	Summer	Collected +- 3 m from the bank. Water clear with algae.
Inanda Dam offshore	-29.674016, 30.860239	01/03/2014	Clear and hot. Midday.	Summer	Collected by boat at the approximate centre of the dam. Water clear.

* Indicates that the sample was taken in or near an urban environment.

2.6.1.2 Method Validation

To estimate the limit of detection and limit of quantification as well as the potential carry-over of the system, a mixture of the target compounds was serially diluted in 10 fold increments in Milli-Q water (Millipore, Billerica, MA, USA) in a range between 0.1 ng/L and 10 µg/L. A volume of 500 mL of each spike sample was extracted in triplicate with appropriate blank extractions between each. Residual target concentrations carried over into the blank samples were determined and extractions were performed from the highest to the lowest concentration in order to determine the maximal amount of expected carry-over.

Instrumental limits of detection and quantification (LOD and LOQ) were determined by repeat injection of a low concentration mixture of the targets diluted in methanol. The LOD and LOQ were defined as concentrations yielding a signal to noise ratio

of 3 and 10 respectively (ICH, 2008). Chromatographic variability was determined by calculating the relative standard deviation percentage (%RSD) for retention times of each compound.

Method detection limits (MDL) and recoveries were determined by processing 24 environmental water samples from the Hennops River. Half of these were spiked at 100 ng/L and quantified by standard addition. The average concentration of each of the analytes from the unspiked samples was subtracted from these values and the MDL for each compound was calculated using the following equation (Conley et al., 2008):

$$MDL = T_{(n-1,\alpha=0.01)} \times S$$

where T = 2.718 for 11 degrees of freedom with α = 0.01 and S is the standard deviation of the averaged triplicate (injection) concentrations of the 12 blank-subtracted spiked samples. In addition to this, LOQ was calculated as described previously. Recovery was calculated as a percentage of the average measured concentration compared to the known spiked concentration.

2.6.2 Results and Discussion

2.6.2.1 Chromatography and Mass Spectrometry

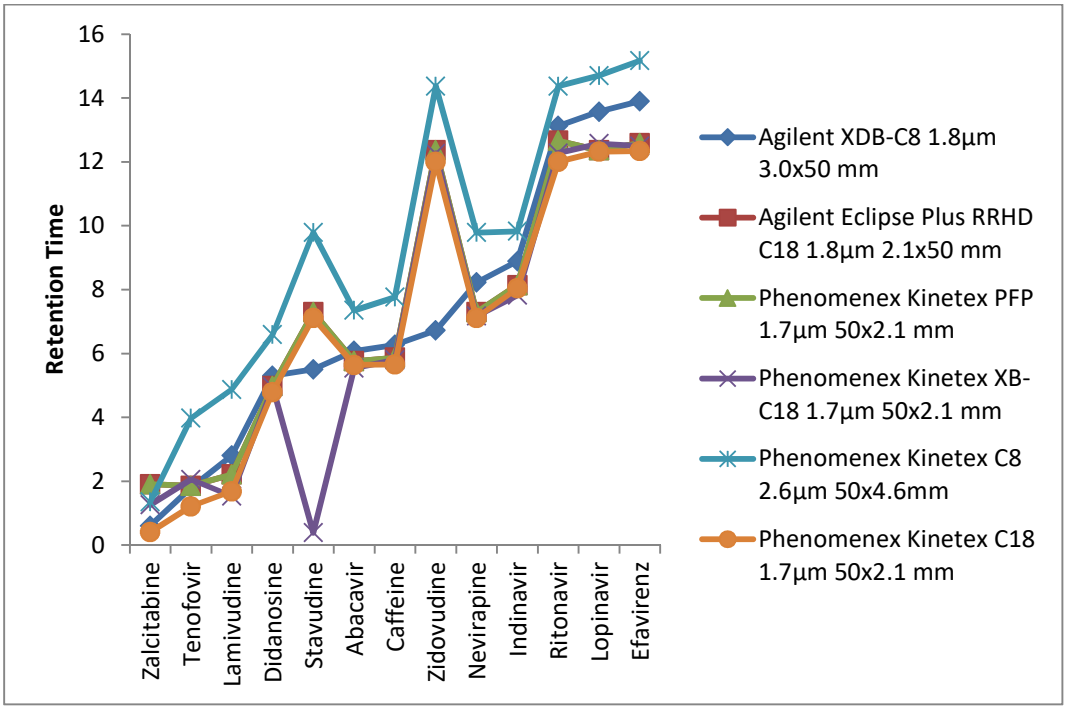


Figure S2-1: Comparison of the Agilent XDB-C8 1.8 µm 3.0x50 mm, Agilent Eclipse Plus RRHD C18 1.8 µm 2.1x50 mm, Phenomenex Kinetex PFP 1.7 µm 50x2.1 mm, Phenomenex Kinetex XB-C18 1.7 µm 50x2.1 mm, Phenomenex Kinetex C8 2.6 µm 50x4.6mm and Phenomenex Kinetex C18 1.7 µm 50x2.1 mm columns for the analysis of 12 ARVs and caffeine by LC-MS.

2.6.2.2 Method Validation

Extraction of spiked MilliQ water was compared to spiked surface water to determine differences in extraction efficiency as well as the potential signal enhancement or suppression caused by the matrix (described in Supplementary Information).

Oasis HLB cartridges are described in the EPA method for the analysis of water for PCPs (Englert, 2007) and a number of researchers have adopted these to extract a variety of compounds with variations in cartridge sorbent mass, conditioning, load volumes and elution (Bijlsma et al., 2013; Cimetiere et al., 2013; He et al., 2013). A

more universal approach developed by Ferrer and co-workers (Ferrer and Thurman, 2012) was automated and validated for ARV compounds (Table S2-4).

Instrumental limits of detection and quantification were based on the calculation of concentrations that would yield signal to noise ratios of 3 and 10, respectively. This was achieved through replicate (six) injections of calibration standards across the predicted detection range (0.1-1000 ng/L). Linearity for a five point curve proved to be acceptable with a lowest R^2 value of 0.994. The chromatographic retention time reproducibility was high for all compounds except Tenofovir (% RSD=2.07), which exhibited peak broadening during analysis.

Spiked MilliQ water was extracted in triplicate, and similarly LOD and LOQ was determined. These data showed that the “best case” extraction scenario without matrix proved to be effective for all of the target compounds. The Horizon Smart Prep carryover was found to be lower than 0.5 % for the majority of compounds.

Samples collected from the Hennops River were spiked with the target compounds at 100 ng/L, extracted 12 times, and processed using the standard addition method. This river was chosen because it flows through an urban environment, may present a “worst case” of matrix interference, and because of its proximity to the laboratory. The spike concentration was chosen as a value near the concentration of the compound with the highest instrumental LOQ (Stavudine). Linearity for the four point calibration was greater than R^2=0.996, and the method detection limit proved to be in the low ng/L range for the majority of compounds. The effect of the matrix on recovery was determined and these values were utilized to adjust the final environmental quantitation data (Table S2-4).

Table S2-4: Instrumental and method limitations with method efficacy parameters for the analysis of 12 ARVs and caffeine by solid phase extraction and LC-ESI-MS/MS.*

	Instrumental				Spiked MilliQ			Matrix Spike		
Drug Name	**R^2**	**LOD (ng/mL)**	**LOQ (ng/mL)**	**RT %RSD**	**LOD (ng/L)**	**LOQ (ng/L)**	**Carry-over (%)****	**LOQ (ng/L)**	**MDL (ng/L)**	**% Recovery**
Zalcitabine	0.998	0.01	0.04	0.00	7.0	23.3	0.1	0.1	21.9	16
Tenofovir	0.999	0.2	0.5	2.07	14.4	48.0	n.d	6.4	25.1	9
Lamivudine	0.996	3.1	10.4	0.99	0.5	1.7	0.03	1.3	13.4	20
Didanosine	0.999	0.1	0.3	0.07	0.06	0.2	0.2	0.04	40.4	66
Stavudine	0.998	8.1	26.9	0.19	5.4	18.1	n.d	18.2	36.6	49
Abacavir	0.994	0.02	0.06	0.03	0.002	0.01	0.1	0.04	43.1	89
Caffeine	0.995	0.1	0.4	0.06	0.3	1.07	n.d	0.07	35.3	62
Zidovudine	0.995	0.1	0.5	0.05	0.4	1.2	0.1	0.2	38.6	57
Nevirapine	0.995	0.03	0.1	0.04	0.01	0.02	0.1	10	92.7	74
Indinavir	0.991	0.2	0.6	0.34	1.4	4.5	4.0	0.04	35.3	44
Ritonavir	0.999	0.06	0.2	0.05	0.04	0.15	0.4	0.1	156.6	125
Lopinavir	0.999	0.09	0.3	0.03	0.15	0.5	0.3	0.05	117.2	88
Efavirenz	0.999	3.6	12.1	0.03	1.40	4.7	0.2	3	519.0	102

*Limits of Detection (LOD) and Quantification (LOQ) determined by calculation of concentrations that would yield signal to noise ratios of 3 and 10 respectively. Method Detection Limit (MDL) was calculated from the standard deviation of 12 spiked matrix samples.

** The percentage carry-over is calculated as a function of the amount of analyte detected in the blank sample that follows directly after the highest concentration spiked sample. No detection is reported as (n.d).

The accepted standard addition practice is to dilute the sample with increasing volumes of standard, yet the method used here relies on the addition of equal volumes of standards with increasing concentrations. Isotopically labelled caffeine

was introduced after extraction in order to correct for errors in pipetting small volumes, evaporation in the LC autosampler and variability in liquid chromatography.

Compound extraction efficiency and detection sensitivity varied within the group and may be attributed to the divergent nature of the compounds. Higher method limits of detection, when compared to instrumental limits, were most likely due to the variability introduced by variations in the SPE sorbent and the multiple pipetting steps of small volumes in the method. Also, a major source of variability is the final concentration step, in which volumetric estimation was performed by visual inspection. It is noteworthy that by using signal to noise to calculate the LOQ of the standard addition method, the sensitivity is much higher than when accounting for standard deviation between samples.

The spiked environmental samples were quantified using an external calibration curve (targets in HPLC-grade water), and these data were compared to concentrations derived from standard addition analysis (Figure S2-2, Supporting Information). Six of the 12 targets exhibited marked signal enhancement (i.e. over estimation of concentration by external calibration) as a result of the matrix. This over estimation is not due to the co-elution of a false positive compound as the unspiked samples showed no presence of signals similar to the target molecules. In addition to this, targets occurring in the un-spiked blanks were quantified and subtracted from the spiked samples. Tenofovir, Ritonavir and Efavirenz displayed substantial signal suppression with the chromatographic peak shape (spreading) of Tenofovir affected by the matrix. Nevirapine, the most frequently occurring target, appeared to be unaffected by the matrix.

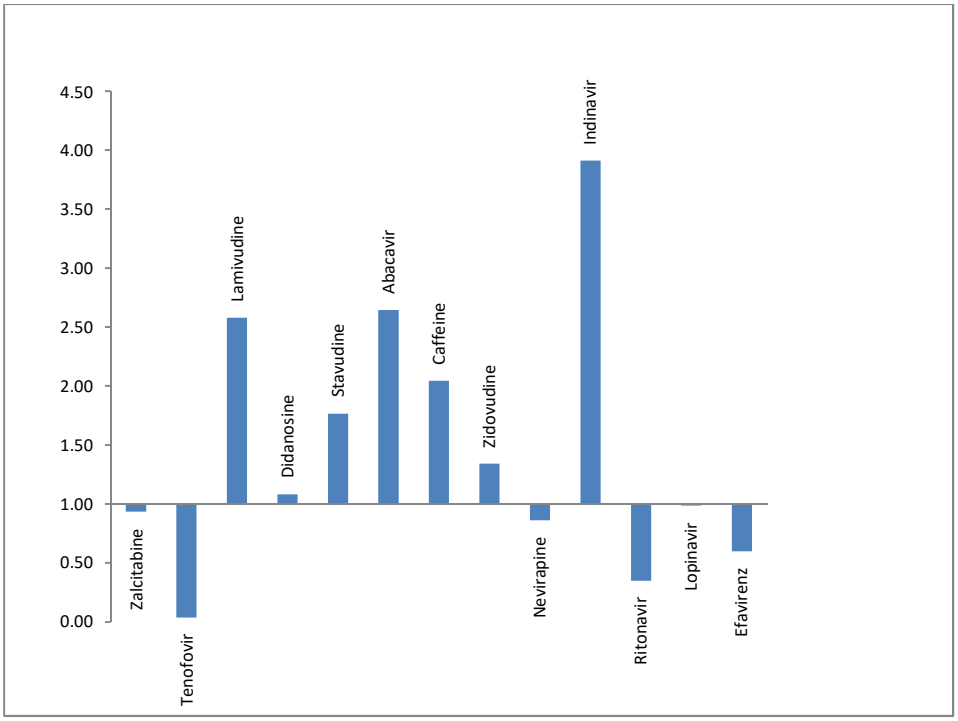


Figure S2-2: Comparison of spiked matrix quantified by external calibration or using the standard addition method. Concentrations for each target are presented as a ratio (external over spiked) with numbers greater than 1 indicating signal enhancement and less than 1 signal suppression as a result of matrix interference.

2.6.2.3 Environmental sample analysis

Figure S2-3: A Google Maps Engine log of environmental sample collection points across South Africa with a zoomed insert depicting the Roodeplaat Dam system.

Table S2-5: Product ions, listed in order of abundance, generated by LC-ESI-MS/MS at various collision energies and fragmentor voltages for the analysis antiretroviral compounds used for the treatment of HIV.

Compound Name	Fragmentor Range	CE Range	Product ions*
Abacavir	102-108	17-46	191, 150, 79.1, 134, 174
Caffeine	100	16-28	138, 110, 69.1, 83.1
Didanosine	50-80	0-50	137, 119.1, 55.1, 110, 121, 178.9, 147.9
Efavirenz	80-128	4-49	53.1, 237, 299.1, 149, 243.9, 193, 167, 102.1, 281.1, 187.9, 123.1
Indinavir	140-180	4-49	421.3, 97.1, 421, 465.3, 364.3, 133, 415.2, 346, 341.2
Lamivudine	76-144	4-54	112, 95.1, 69.1, 45.1, 68, 172.1
Lopinavir	1-120	8-40	155.1 , 183.1, 120.1, 447.2
Nevirapine	120-128	24-44	226, 80, 107, 198, 197, 183.6
Ritonavir	138-144	14-58	140, 296, 268, 171, 197.1
Stavudine	72-128	4-36	105, 77, 127, 208.9, 192.9, 146.9, 149, 144.2, 155.3
Tenofovir	1-50	16-32	176.1, 159, 270, 136
Zalcitabine	60-132	0-36	112, 95, 195, 55.1, 57.1, 69.1, 89.1, 177.1, 133.1
Zidovudine	76-100	0-50	127, 110, 54.1, 195, 136, 233, 96.1

* Listed in order of decreasing abundance.

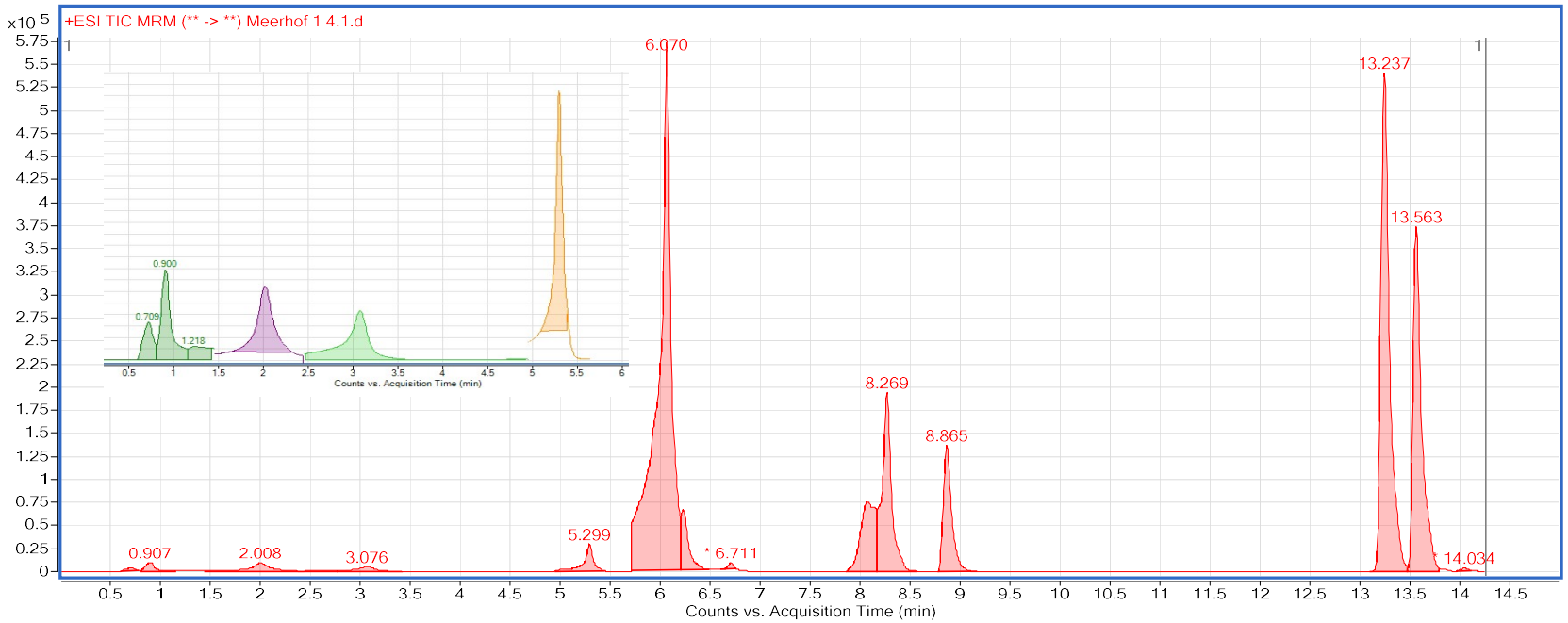


Figure S2-4: LC-MS/MS total ion chromatogram of the highest calibrator (1000 ng/ml) of a grab sample from the Hartbeespoort Dam extracted by SPE. Inset: extracted ion chromatogram for the most abundant transitions for lower intensity targets.

2.6.3 References in SI

1. Bijlsma, L., Boix, C., Niessen, W., Ibáñez, M., Sancho, J.V., Hernández, F., 2013. Investigation of degradation products of cocaine and benzoylecgonine in the aquatic environment. Science of The Total Environment 443, 200–208.

2. Cimetiere, N., Soutrel, I., Lemasle, M., Laplanche, A., Crocq, A., 2013. Standard addition method for the determination of pharmaceutical residues in drinking water by SPE-LC-MS/MS. Environmental Technology 34, 3031–3041.

3. Conley, J.M., Symes, S.J., Kindelberger, S.A., Richards, S.M., 2008. Rapid liquid chromatography-tandem mass spectrometry method for the determination of a broad mixture of pharmaceuticals in surface water. Journal of Chromatography A 1185, 206–215.

4. Englert, B., 2007. Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS. US Environmental Protection Agency (EPA) 1–72.

5. Ferrer, I., Thurman, E.M., 2012. Analysis of 100 pharmaceuticals and their degradates in water samples by liquid chromatography quadrupole time-of-flight mass spectrometry. Journal of Chromatography A 1259, 148–157.

6. He, Y., Chen, W., Zheng, X., Wang, X., Huang, X., 2013. Fate and removal of typical pharmaceuticals and personal care products by three different treatment processes. Science of The Total Environment 447, 248–254.

7. ICH, 2008. Validation of Analytical Procedures: Text and Methodology, Q2 (R1), Current Step 4 Version, Parent Guidelines on Methodology Dated November 6 1996, Incorporated in November 2005, in: International Conference on Harmonisation, Geneva, Switzerland, www.ich.org.

Chapter 3: Database-Driven Screening of South African Surface Water and the Targeted Detection of Pharmaceuticals Using Liquid Chromatography - High Resolution Mass Spectrometry

This chapter was pulished in Environmental Pollution. The format reflects the style set by the journal.

Wood, T.P., du Preez, C., Steenkamp, A., Duvenage, C.S., Rohwer, E., 2017. Database-Driven Screening of South African Surface Water and the Targeted Detection of Pharmaceuticals Using Liquid Chromatography - High Resolution Mass Spectrometry. Environmental Pollution, 230, 453-462.

Database-Driven Screening of South African Surface Water and the Targeted Detection of Pharmaceuticals Using Liquid Chromatography - High Resolution Mass Spectrometry

Timothy Paul Wood[a,d] (Corresponding Author)

Christiaan Du Preez[b]

Adriaan Steenkamp[b]

Cornelia Duvenage[c]

Egmont R Rohwer[d]

[a] Protechnik Laboratories, a Division of ARMSCOR SOC Ltd, 103 Combretum Crescent, Centurion, Pretoria, 0001, South Africa. Tel: +27 12 665 9444, timw@protechnik.co.zamailto:timw@protechnik.co.za

[b] Flamengro, a Division of ARMSCOR SOC Ltd, Cnr Delmas road and Nossob street, Pretoria, 0001, South Africa. Tel: +27 12 428 1911 Chris@flamengro.co.za

[c]Department of Internal Medicine, 1 Military Hospital, South African Military Health Services, Voortrekker Street, Pretoria, 0001, South Africa.
Tel: +27832733382, corneliaduv@mtnloaded.co.za

[d] Department of Chemistry, University of Pretoria, Lynwood Road, Pretoria, 0001, South Africa. Tel: +27 12 420 3772 email: egmont.rohwer@up.ac.zamailto:egmont.rohwer@up.ac.za

Highlights

- A method for the quantification of 96 pharmaceuticals by UHPLC-QTOF was developed and applied to South African surface water
- Lamotrigine and Nevirapine occurred most often
- Prednisolone and Ritonavir were present at the highest average concentration in surface water samples
- An analytical procedure to screen samples prior to costly targeted analysis is presented
- Cluster computation was used to parallelize standard analytical software to reduce total processing times

Abstract

Pharmaceuticals and personal care products are released into aquatic environments, largely as a result of ineffectual removal during wastewater treatment. Here we present a screening strategy based on the use of three commercially available mass spectral databases, combined into a single searchable entity and parallelized by cluster computing. In addition to this, a targeted solid phase extraction method with Ultra High Pressure Liquid Chromatography coupled to quadrupole time of flight mass spectrometry (UHPLC-QTOF) was used to quantify 99 pharmaceuticals in South African surface water on a national level. Limits of quantification were in the low ng/L range for the majority of the compounds and it was found that nationally both Lamotrigine and Nevirapine occurred most often. Prednisolone and Ritonavir were present at the highest average concentration; 623 and 489 ng/L respectively. It is however shown that more than 50% of the targets chosen for analysis are not detectable in any of the samples, which highlights the utility of untargeted, database driven screening; prior to the use of costly analytical standards. Untargeted screening detected 45 % of the compounds detected in targeted mode, and furthermore tentatively identified a

total of 4273 unique compounds across the samples. Automatically triggered MS/MS analyses yielded 92 unique hits with greater than 95 % confidence. It is therefore suggested that untargeted screening should precede the targeted approach as a matter of economy and to guide the selection of targets for quantification. There is however great room for improvement in current commercial database search methodologies as a large bottleneck exists due to processing time.

3.1 Introduction

In recent years concerns have arisen regarding the presence of pharmaceuticals in surface and drinking water (Boorman, 1999; Rodriguez-Mozaz et al., 2015; Wode et al., 2015; Zhang et al., 2015; Papageorgiou et al., 2016). These compounds are present in the environment largely due to excretion by humans and livestock that have ingested them. Other routes of surface water contamination that should be considered are pit latrines (Graham and Polizzotto, 2013), improper pharmaceutical destruction (Peng et al., 2014), malfunctioning sewage treatment plants and illegal sewage disposal. Many of these factors are unique to the African continent and other “developing regions” and are not sufficiently addressed in the literature.

The presence of pharmaceuticals in the environment has been proven worldwide, yet little work has been done in South Africa and Africa as a whole on this topic. The “African picture”, which is unique in that many regions are water scarce and people often utilize unpurified water for drinking, has been largely neglected. This has been established through searching the curated USEPA database (Daughton and Scuderi, 2012) on this field. It is heartening though that missing pieces of the picture are now being added regularly by researchers across the African continent (Agunbiade and Moodley, 2014, 2015; Schoeman et al., 2015; K’oreje et al., 2016).

Since South Africa faces a number of unique challenges such as: water scarcity, malfunctioning waste water treatment, HIV burden, a high Tuberculosis (TB)

prevalence, TB drug resistance and reduced access to fresh water; this topic is extremely relevant to the population as a whole. The behavior and fate of these compounds in the environment has yet to be established completely in the literature (Westerhoff et al., 2005; Farré et al., 2008; Miège et al., 2009; Padhye et al., 2014) and it is believed that their presence in the environment could contribute to drug resistance (Khetan et al., 2007; Kümmerer, 2009; Gatica et al., 2013; Jain et al., 2013). This is especially important given the TB challenges faced in South Africa.

Ferrer and Thurman (2012) utilized liquid chromatography coupled to high resolution mass spectrometry to detect 100 pharmaceuticals in surface water. The researchers highlighted the utility of accurate mass analysis in this field. Isomers do however exist for chemicals of interest necessitating the use of chemical standards, which could hopefully be distinguished chromatographically or by mass spectral fragmentation. High resolution mass spectrometry is rapidly gaining popularity in the field of micropollutant detection and researchers are able to screen for an ever increasing number of targets (Alygizakis et al., 2016; Bletsou et al., 2016; Cotton et al., 2016; González-Mariño et al., 2016; Soulier et al., 2016).

Similarly high resolution mass spectrometry is being used to determine the environmental fate of pharmaceuticals and their transformation as a result of wastewater treatment. The technology allows researchers to detect and characterize novel disinfection transformation products with a high level of sensitivity (Boix et al., 2016; Ibáñez et al., 2016; Rager et al., 2016; Wood et al., 2016). This means that if even though the original parent molecule is not detectable in a sample, the detection of its transformation products allows researchers to draw inferences relating to its prevalence in wastewater treatment works (WWTW) systems.

High resolution mass spectrometric instrumentation is improving rapidly and what once was used only for characterization now allows one to perform sensitive quantification studies. Since the majority of pharmaceutical compounds are ionized

and measured, the technology yields a broader picture of a particular sample (Glauser et al., 2016).

Having had success with the detection of antiretrovirals (ARVs) (Wood et al., 2015) in surface water, the research is followed by this work to include a broader variety of compounds. Samples have been taken from across South Africa from almost all the major rivers and dams (man-made) in the country. This has led to the development of an important data resource which is the establishment of a national baseline for these types of compounds. By screening this type of sample using high resolution mass spectrometry research is not limited by the pharmaceutical standards that are available to use for targeted comparisons; the data may be mined in various ways to identify targets for validation at a later stage.

Some of the drawbacks of LC-MS analysis are difficulties of inter-laboratory reproducibility (Rivier, 2003) as well as a lack of a comprehensive cross-platform database. With high resolution instruments producing accurate mass values for unknowns, data can now be compared more easily between laboratories. In addition to this a number of commercial databases have become available for a variety of compounds. The size of the high resolution mass spectral data files and the current algorithms used in comparing them against even small databases (+-80 000 compounds) is at this time proving cumbersome, with a single LC-MS data file search routine taking up to 12 hours. For this reason we investigate the utility of massively parallel cluster computing to alleviate the computational bottleneck. The advantage of this approach is that the user interface remains the same, only that a single user is able to process multiple iterations of a software package simultaneously from a single interface. This allows a user that is only familiar with the mass spectral software to process samples without necessitating further training on aspects of cluster computation.

In this work not only the prevalence of these compounds but also the analytical procedure, which involves three distinct injections of the same sample, each with its own mass spectral acquisition mode is described. Targeted screening in Full Scan mode without fragmentation, which provides unfragmented pseudomolecular

ion information, is followed by untargeted screening in which high abundance ions are automatically selected for fragmentation (Auto MS/MS), subjected to compound dependent collision energy, yielding tandem mass spectra. In the third method, low abundance ions, that are missed by Auto MS/MS selection are fragmented by applying a fixed collision energy to all the ions detected by the instrument (All Ions mode, no precursor ions selected). This yields fragmentation information relatable to the complementary data generated in the Full Scan approach via the accurate masses (and elemental composition of ions) available from both runs at identical retention times. With this methodology, the maximum amount of information can be gleaned from the data set. The data set also has a certain amount of longevity in that it can be re-mined as new targets and scientific questions arise.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Analytical reference standards were obtained from the US, British and European Pharmacopoeia, Toronto Research Chemicals (Toronto, Canada) and Sigma Aldrich (Johannesburg, South Africa). All compounds were of 97% purity or better as indicated by the vendor. Compounds were prepared in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/mL and stored at -20°C until use. Standards were grouped into five sets according to molecular mass to ensure no isobars were present in the same set and diluted to 10 µg/mL in DMSO. The sets were stored at -20°C until use and combined to produce the appropriate working solutions. ^{13}C3-trimethyl caffeine, 100 µg/mL in methanol, was obtained from Cambridge isotope Laboratories (Tewksbury, MA, USA) and diluted to 10 µg/mL in methanol before use. Standards were prepared and handled in a separate area from samples in order to prevent cross contamination. LC-MS grade acetonitrile, methanol, DMSO and water were purchased from Lab-Scan (Gliwice, Poland) and premixed acetonitrile and water each with 0.1% formic acid were obtained from Burdick and

Jackson (Milwaukee, MI, USA). No South African-origin water was used as a reagent in the course of this research.

3.2.2 Method Optimization

Thirty six of the target compounds were randomly selected from the 5 pharmaceutical groups and diluted in DMSO to 1 µg/mL and injected in triplicate (1 ng on column) to compare the chromatographic characteristics of six columns, namely the: Zorbax Eclipse XDB C8 RRHD 4.6 x 150 mm, 1.8 µm; Zorbax Eclipse XDB C8 RRHT 2.1 x 100 mm, 1.8 µm; Poroshell 120 SB-C8 2.1 x 100 mm, 2.7 µm; Poroshell 120 EC-C8 2.1 x 100 mm, 2.7 µm; Poroshell 120 SB-C18 2.1 x 150 mm, 2.7 µm and the Poroshell HPH C-8 2.1 x 100 mm, 2.7 µm.

Compounds were separated using acetonitrile and water, both with 0.1% formic acid, at a flow rate of 0.5 mL/min, at 30 °C on an Agilent 1290 series UHPLC using the following program: 2 % organic for 3 min to 100%, 22 min; 100%, 25 min to 2%, 27 min and 2% at 30 min. Eluted compounds were detected using an Agilent 6550 QTOF in Full Scan mode. The flow rate was optimized using peak shape and average height as indicators of efficacy.

Various mass spectral parameters were evaluated to result in average optimum conditions for the majority of compounds. Three different ion source conditions were utilized (Table S3-5) and compared by injecting 1 ng on column of each of the five groups and quantifying the results against an external calibration curve generated with one of the source settings methods using the Agilent software package “MassHunter Quant”. Following the identification of optimal source conditions, the effect of pre-collision cell (“Fragmentor”) voltage on target sensitivity was evaluated. This was achieved through repeat injections of pharmaceutical mixtures with Fragmentor voltage set at 0, 50, 100, 200, 300 and 365. The optimal source conditions were then applied to the Full Scan, Auto MS/MS and All Ions approaches utilized in the research.

Instrument sensitivity was determined by triplicate injection of target mixtures near the predicted limit of detection (LOD) followed by regression to extrapolate the

LOD and limit of quantification (LOQ) for each compound, as well as the standard deviation and linearity of the curve. This was performed in order to predict the instrument's dynamic range for a particular compound, with LOD and LOQ defined as the injected amounts presenting a signal to noise ratio of 3 and 10 respectively.

3.2.3 Use of Dimethyl Sulfoxide (DMSO) as an Injection Solvent

In order to evaluate the effect of DMSO as an injection solvent, *versus* methanol which is a popular injection solvent in the literature, the five pharmaceutical groups were made up to 1 ng/mL in either DMSO or methanol. Peak shape and retention time were compared and the effect of injection volume was evaluated. The Poroshell HPH 2.1 x 100 mm, 2.7 µm as well as the Zorbax Eclipse Plus XDB C8, 2.1 x 50 mm, 1.8 µm columns were tested using both long (30 min) as well as short gradients (10 min).

3.2.4 Sample Extraction

Environmental samples were collected as part of a multiyear project between 2013 and 2016. Grab samples were collected from various surface water sources in South Africa. Sampling locations were selected based on their proximity to WWTWs and the distance from major bodies of water. Samples were collected in "virgin" 2 L borosilicate Schott bottles while wearing nitrile gloves (to prevent the introduction of contaminants) and transported, protected from light, to the laboratory at room temperature. Samples were stored at -20°C until extraction after which extracts were stored at -20°C until analysis.

500 mL of each sample was filtered using a 1 µm glass-fibre syringe-driven filter (Pall, USA) and extracted using the Smart Prep Extraction System (Horizon, USA); an automated offline solid phase extraction instrument. The extraction procedure was modified from a method developed by Ferrer and Thurman in order to maintain a level of universality (Ferrer and Thurman, 2012). Briefly, 6 cc Oasis HLB, 500 mg SPE cartridges (Waters, Milford, MA, USA) were conditioned with 4

mL of methanol followed by 6 mL of HPLC-grade water. Five hundred millilitres of sample was introduced at a flow rate of 10 mL/min after which cartridges were dried under nitrogen for three minutes. Cartridges were eluted twice with 5 mL of methanol into 500 µL DMSO containing ^{13}C3 Caffeine (1 µg/mL). Eluates were then dried under a gentle stream of nitrogen to 500 µL. Samples that had been extracted during earlier research and stored in methanol (at -20°C) were combined with equal volumes of DMSO (containing ^{13}C3 Caffeine, 1 µg/mL) and dried under nitrogen at room temperature to remove the methanol. All extractions were performed at 18°C (± 0.5°C) in a dedicated area.

The extraction method was validated using surface water from the Roodeplaat dam system (Rowing Club site) which was collected in a 25 L polypropylene carbouy and spiked with varying concentrations of the 5 pharmaceutical groups (0, 1, 10 and 100 ng/L). The large sampling volume was utilized to promote homogeneity. These were processed by the solid phase extraction method described previously and analysed in triplicate using a UHPLC-QTOF (Agilent, USA). Data was then exported to MassHunter Quant and compared to an external calibration curve. LOD and LOQ were defined as the concentration yielding a signal to noise of 3 and 10 respectively (Taylor, 1987; Hanke et al., 2007; Sanagi et al., 2009).

3.2.5 Environmental Sample Analysis

Samples were analysed by UHPLC-QTOF with 10 µL of each sample (in DMSO) separated on a Poroshell HPH C8, 2.1 x 100 mm, 2.7 µm column with a gradient of acetonitrile and water (both with 0.1% formic acid) at 30°C described earlier.

Separated compounds were detected on an Agilent 6550 QTOF by positive electrospray ionization with a “JetStream” source in Full Scan mode, Auto MS/MS as well as in All Ions Mode (i.e. three injections per sample). Optimised source conditions were: gas temp. 200 ºC; gas flow 15 L/min; nebulizer pressure 40 psig; sheath gas temp. 400ºC; capillary voltage 3500 V; nozzle voltage 500 V and fragmentor voltage 350 eV.

An Auto MS/MS method was developed to increase the breadth of the data generated. Collision energy was determined using a formula which adjusts collision voltage according to the target's mass with a slope of 6 and an offset of 4 eV; where collision energy = (slope x *m/z*)/100 + offset. Nitrogen was used as a collision gas throughout. A scan rate of 3 and 5 spectra per second was used for MS and MS/MS data acquisition respectively. Data was analysed by MassHunter Qual in which the algorithm filters the full data set to extract only the spectra generated by fragmentation of the ions that were automatically selected by the instrument during acquisition ("Find by Auto MS/MS"). This was run followed by elemental formula generation for the automatically selected intact masses, comparison against an accurate mass library as well as against a spectral database for collision fragment comparison (fragment accurate masses and relative ratios).

All Ions mode was utilized to create fragmentation data for those species that may have been missed by screening or Auto MS/MS. The method is identical to the Full Scan approach, except that during half of the acquisition time a collision energy of 40 eV is applied to all the ions exiting the quadrupole in the total ion transmission mode (i.e. fragmentation from unspecified precursor ions). This therefore leads to the fragmentation of all the ions transmitted to the collision cell that are able to dissociate at a given energy.

3.2.6 Database-Driven Screening

Three commercially available databases, namely the Agilent: Metlin, Veterinary Drugs and Forensics/Toxicology databases; were combined into a single searchable entity. This was achieved using the Agilent Personal Compound Database Library (PCDL) Manager software package. A subset database was created from the Metlin database and the remaining two data sets were imported manually. Where possible duplicates were identified by manual sorting and removed.

The MassHunter Qual software was deployed in a VirtualBox (Oracle®). Each virtual box may be set up to utilize only a user defined amount of the Central Processing Unit (CPU) resources and the Random Access Memory (RAM) resources. The virtual boxes are then spawned on a single IBM BladeCenter to a maximum of 10 in all. In addition, permission was obtained from Agilent to deploy many instances of the licensed query database against which the sample is compared. This was necessary in order to allow each virtual box to access the database in parallel. If the licensing model only allows queries from a single Virtual Box, no speed-up will be possible since a database query from one Virtual Box will effectively disallow queries from other Virtual Boxes during the same period. Finally, scripting was used to automate the entire workflow so that sample queries may be queued and that the results and reports are written to the hard disc drive (HDD) automatically in a .csv format file for each sample. The .csv files were then processed using a Microsoft Excel macro which automatically sorted and filtered the data to present results within the criteria specified by the user.

The hardware used was an IBM H523 Blade Server. Each Virtual Box was assigned 4 of the CPU resources, 16 GB of the RAM resources and was loaded on a 100 GB partition. Results were written to an IBM H523 storage Blade. The base operating system was Linux Red Hat enterprise with Windows 7 loaded in each virtual environment. The Virtual Boxes were created using Oracle VirtualBox 5.0.10. Scripting was performed using DOS batch files and the backup software used was NovaBackup DataCenter 5.3.

In the Agilent Qual software, Full Scan data molecular features were extracted with the following criteria: target type - small molecules with peaks greater than 100 counts; positive ion species included H, Na, K and NH_4 adducts. The isotope model followed common organic molecules, with no limits on atom numbers and with charge state limited to 2. No filters were placed on relative or absolute height of the compounds and a compound quality score of 80% was applied. This score considers: signal to noise ratio, retention time, peak shape and width, ion retention time, ion species mass differences and isotope pattern. No mass or mass defect

filters were applied. Mass defect describes the difference between an ion's exact mass and its integer mass value. Filtering by mass defect requires an *a priori* knowledge or preference, thereby limiting a search to a particular set of compounds. This data analysis step was similarly applied to the All Ions data set.

The "Find Compounds by MS/MS" utility in the Agilent MassHunter Qual software was utilized to determine the complexity of the Auto MS/MS data set. The retention time window was set to 0.1 min with a positive TIC threshold of 1000 counts and an *m/z* tolerance of 0.05. Persistent background ions were excluded if their precursor *m/z* occurred more than 5 times in a given time period, unless the intensity was greater than 100000 counts. No masses, besides these were actively excluded in the analysis.

The "compounds" identified by Molecular Feature in the three datasets (from the separate injections in the Full Scan MS, Auto MS/MS and All Ions modes) were then screened against the combined database. A Molecular Feature describes the selection of related ions by the software (e.g. un-adducted and adducted ion species are identified as having the same origin) with exclusion of background ions. Spectral searching was applied to the Auto MS/MS data. In addition to this, the datasets were screened using the "Find by Formula" functionality and compared. The Find by Formula algorithm screens the data set for the masses and isotopic patterns of molecular formulae contained in the database. This methodology is unfortunately by far the most time and computational resource intensive. A database match was only included with a 95% score and flagged for quality if it was lower than 98%.

Unfortunately, the inclusion of manufacturer-specific jargon has been unavoidable in this manuscript. A short glossary of terms is available at the end of the supplementary information.

3.3 Results and Discussion

3.3.1 Method Optimisation

Compounds were grouped in different standard vials in order to avoid the presence of isobars in the same sample to prevent confusion in peak assignment during method development. As an example, Doxycylcine hyclate and Tetracycline have identical masses (444.456). Similarly, Oseltamivir and Praziquantel have a 0.0016 amu mass difference. Without MS/MS analyses these compounds could not be distinguished from another without *a priori* knowledge of their retention times.

Since such a diverse array of compounds was being analysed, a column amenable to their effective separation was required. To this end, 6 reverse-phase columns were compared, using identical UHPLC-QTOF conditions. As expected, no optimum conditions could be found simultaneously applicable to all of the compounds, only for a best average of results. Figure 3-1 shows the overlaid data for Nevirapine (1 ng on column). Three of the six columns showed signs of overloading (peak fronting and spreading), which was found to be compound specific; i.e. peak shapes were good for the majority of compounds on all of the columns. This means that the capacity of these columns is relatively low for a particular compound and they are less suitable for use with a real world sample that has a large target dynamic range. Following this (peak shape-based exclusion) the remaining columns were evaluated based on height and resolution. The Agilent Poroshell HPH C8, 2.1 x 100 mm, 2.7 µm, provided the best resolution between the target compounds with a value of 0.19 min for the closest eluting compounds and was chosen for further use.

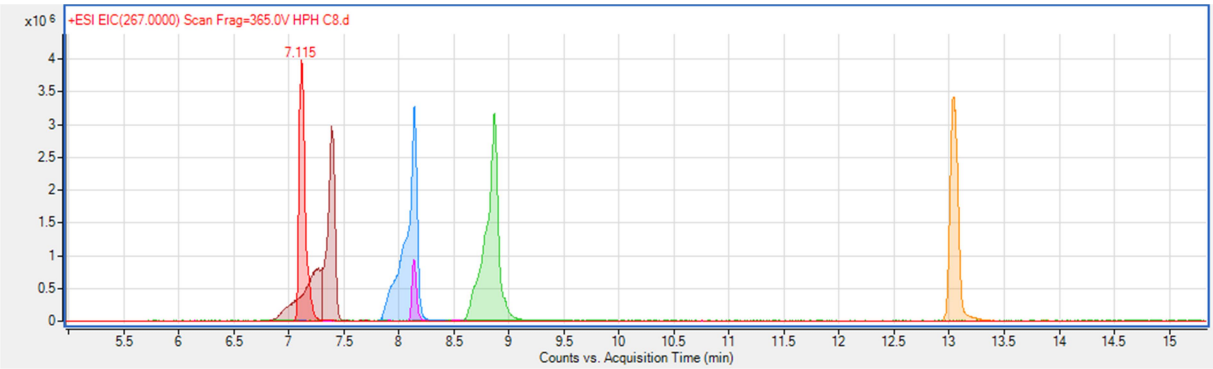


Figure 3-1: Overlaid EIC m/z 267 (+-0.5) for all six chromatography columns depicting Nevirapine at 1 ng on column. Traces: Poroshell HPH C8, 2.1 x 100 mm, 2.7 µm (red), Poroshell 120 EC-C8 2.1 x 100 mm, 2.7 µm (brown), Poroshell 120 SB-C8 2.1 x 100 mm, 2.7 µm (blue), Zorbax Eclipse XDB C8 RRHT 2.1 x 100 mm (pink), Poroshell 120 SB-C18 2.1 x 150 mm, 2.7 µm (green) and Poroshell 120 SB-C18 2.1 x 150 mm (orange).

Mass spectrometric optimization was achieved quantitatively by comparing the signal generated for each compound, for each of the three methods, against an external calibration curve created on one acquisition method. The calibration curve was run using Method 3, and if the target showed a signal higher than what was spiked one could assume that the method used is better than Method 3 for that particular compound. Using this strategy, the five compound groups were run and averages were drawn from the members in order to choose the most effective method (Table S3-6 to Table S3-10). In Group 1 (Table S3-6), Method 3 provided the best results; yet there were notable outliers such as Lamivudine (which shows greater signal in Method 1 and 2). This highlights the fact that optimal conditions for a single target are sometimes sacrificed to provide the optimal conditions for the group. Following source condition optimization, Fragmentor Voltage optimization was carried out. This was achieved through multiple injections of the targets at different concentrations and the optimal setting was found to be 365 eV for most of the targets (Figure S3-11).

3.3.2 Use of DMSO as an Injection Solvent

The aprotic solvent DMSO (MacGregor, 1967) is widely considered a "universal solvent" and was chosen for inclusion in the method because if its solvation power as well as the fact that it does not evaporate readily. In previous research it was found that one of the major sources of variability is the use of methanol as an extraction eluent and injection solvent. The final step of methanol evaporation under nitrogen, after solid phase extraction, is determined by visual inspection and micropipette. This leads to high level of variability between samples. In addition to this, when dealing with small sample volumes, it was found that methanol evaporates on the lab bench as well as in the UHPLC autosampler. Again, this creates unwanted variability in the analyses. Due to DMSO's high boiling point, these factors can be eliminated entirely. DMSO may be used as a "keeper" by adding it to the final methanol elution prior to evaporation (Whelan et al., 2010; Wagner and Oehlmann, 2011).

DMSO as an injection solvent was evaluated on two columns, the Poroshell HPH 2.1 x 100 mm, 2.7 µm as well as the Zorbax Eclipse Plus XDB C8, 2.1 x 50 mm, 1.8 µm. It was found that although DMSO produced a much more intense solvent front, it did not negatively affect peak shape or retention times for the target compounds (Figure S3-12). Compound signal (i.e. peak height) on average is similar when using DMSO as opposed to methanol as an injection solvent. It was noted though that the later eluting compounds showed higher peak signals with DMSO (Figure S3-13).

It was found, however, that DMSO markedly increases column backpressure with high injection volumes (40 µL) and if a short gradient is used on the Poroshell HPH column the system rapidly reaches the column's maximum pressure. To overcome this, it was found that longer gradients on this column are more amenable to large volume injections of DMSO and the system stays within allowable pressure limits.

DMSO is relatively persistent in the chromatographic system, which in most cases is unwanted; yet when using high resolution mass spectrometry this could be an unexpected boon in that the protonated monomer and dimer of DMSO may be

used as an additional internal accurate mass calibrant. Internal calibration is one of the strong points of this instrument type, as a reference mass is continuously infused during the analysis. This strengthens the ability for inter-laboratory comparison and the utility of accurate mass databases. Using DMSO as an injection solvent is advantageous because the pseudomolecular ion as well various DMSO ion species (Table 3-1) are relatively pervasive throughout the analysis. These species may then be used as backup mass calibrants if ion suppression of the infused calibration mixture occurs.

Table 3-1: Possible DMSO ion species found in positive electrospray ionisation experiments.

Calculated Monoisotopic *m/z*	**Species (DMSO = M)**
78.0139	M
79.0212	$(M+H)^+$
101.0032	$(M+Na)^+$
120.0478	$(M+CH_3CN)^+$
137.0749	$(M+CH_3CN+NH_4)^+$
157.0351	$(2M+H)^+$
179.0171	$(2M+Na)^+$
235.0491	$(3M+H)^+$

3.3.3 Method Validation

The method was first validated by determining the “best case” of detection in which the instrument sensitivity was determined (Table S3-11). This shows the instrument’s capability without accounting for interfering compounds in the matrix. LOD and LOQ for the majority of compounds were found to be acceptable. Method

validation of the solid phase extraction was carried out and concentrations that yield a signal to noise ratio of 3 and 10 were used for the LOD and LOQ respectively. This was performed for each compound (Table S3-11) and it was found that the majority of compounds were extracted effectively (96 out of 99). In addition to this, the LOQ for most of the compounds is in the low ng/L range, but, as expected due to their divergent nature and hence ionization efficiencies, there is a large spread of LOD across the calibration range. Forty eight percent of the compounds LOQ was lower than 100 ng/L; 12% between 100 and 500 ng/L; 21% between 500 and 1000 ng/L, 16% >1000 ng/L and 3% undetectable. The undetectable compounds were Leflunomide, Chloroquine and Zalcitabine. Their absence can be accounted for by SPE material binding efficiency, in the case of Leflunomide and Chloroquine, and MS settings for Zalcitabine. There is also the possibility of adduct formation, leading to missed identification.

3.3.4 Environmental Sample Analysis

Samples taken from across South Africa, from every major river and dam system, were concentrated by SPE and analysed by UHPLC-QTOF. The analysis consisted of the three methodologies described earlier: Full Scan, Auto MS/MS and All Ions.

These three strategies therefore provide an incredibly rich data set which can be utilized to quantify targets or identify targets to be investigated at a later stage. Table 3-2 illustrates the average numbers of molecular features identified using the three analytical strategies across the various sampling points. Although the Molecular Feature algorithm is imperfect, in that it at times identifies fragment ions (from collision induced dissociation) as intact species, the functionality does provide insight into sample complexity; and in this case the extent of pollution. Sampling sites near WWTW outflows (Zeekoegat and Baviaanspoort) as well as those in urban areas were found to contain more “molecular features” than samples taken in rural areas.

In this work, focus was given to the Roodeplaat Dam system due to its proximity to the laboratory as well as the fact that it impounds the outflow of two WWTWs (Zeekoegat and Baviaanspoort) and is a source of potable water.

Table 3-2: Average "Molecular Feature" descriptors for Full Scan, Auto MS/MS and All Ions of environmental water samples concentrated by SPE and analysed by positive electrospray UHPLC-QTOF.

Sample Name	GPS Co-Ordinates	No. of Full Scan Molecular Features	No. of Auto MS/MS Molecular Features	No. All Ions Molecular Features
Roodeplaat Dam System*				
Pienaars River Inflow	-25.678677, 28.357116	7604	968	11835
Zeekoegat WWTW Outflow	-25.624620, 28.341890	8438	885	14244
Angling Area	-25.626404, 28.345692	7879	894	11771
S.E Bank	-25.637763, 28.344150	6532	1369	10040
Motorboat Launch	-25.618238, 28.358642	6669	993	10526
Rowing Club	-25.623345, 28.349842	6123	873	9475
Roodeplaat Outflow	-25.608244, 28.367231	5678	1041	9235
Rietvlei Dam*				

Sample Name	GPS Co-Ordinates	No. of Full Scan Molecular Features	No. of Auto MS/MS Molecular Features	No. All Ions Molecular Features
Southern Bank	-25.881576, 28.268585	5760	1538	9619
Northern Bank	-25.876767, 28.279846	5415	1487	8391
Orange River System				
Orange River (Bethulie)	-30.534670, 26.022975	4295	676	7566
Gariep Dam Oviston	-30.692147, 25.761238	2095	398	3568
Gariep Dam (N.E)	-30.603858, 25.503609	3841	576	7396
Vaal River side of confluence	-29.070882, 23.637209	4040	546	7890
Orange River side of confluence	-29.072898, 23.638936	3906	530	7462
Confluence	-29.071810, 23.635868	3622	482	7379
Cape Region				
Eerste Rivier*	-33.941603, 18.857078	3378	583	6696
Theewaterskloof Dam	-34.027283, 19.208261	3496	533	6317
Stellenbosch Tap Water Sample	-33.940642,	1844	324	3455

Sample Name	GPS Co-Ordinates	No. of Full Scan Molecular Features	No. of Auto MS/MS Molecular Features	No. All Ions Molecular Features
	18.827443			
Vaal Dam				
Dam wall	-26.883278, 28.116047	4800	727	5857
Oranjeville	-26.999155, 28.214893	3776	686	7099
Vaal Dam Inflow	-27.020575, 28.608589	3555	685	6312
Vaal Dam Out Flow	-26.874950, 28.115583	4012	745	7023
Vaal River	-29.04938, 23.76872	3258	565	5980
Single system samples				
Hartebeesfontein WWTW Outflow*	-26.030715, 28.291084	6582	886	8999
Ditholo	-25.320242, 28.340728	6280	867	7703
Hartbeespoort Dam, Meerhof	-25.760775, 27.891871	7900	1024	14108
Hartbeespoort Dam, Tap Water Sample*	-25.745594, 27.911238	8783	1188	15171
Hartbeespoort Inflow, Crocodile River	-25.775818, 27.901601	4249	785	7978
Renosterkop	-25.108639,	4839	731	7356

Sample Name	GPS Co-Ordinates	No. of Full Scan Molecular Features	No. of Auto MS/MS Molecular Features	No. All Ions Molecular Features
	28.887359			
Inanda Dam	-29.673792, 30.854874	4385	578	5073
Inanda Dam offshore	-29.674016, 30.860239	4223	514	4717

The highest number of molecular features of Full Scan data was found in the Hartbeespoort potable water samples, followed by Zeekoegat WWTW outflow samples. The highest in Auto MS/MS and All Ions features were found in the Rietvlei and Meerhof (Hartbeespoort Dam) samples respectively. The lowest number of molecular features for all three analytical methods was in the Stellenbosch potable water sample and the Oviston (Orange River system) samples.

By comparing the complexity of the two potable water samples taken from geographically distinct areas, one is able to possibly judge the efficacy of the water treatment process used to create a specific potable water sample. The source of these samples (Hartbeespoort and Theewaterskloof Dams respectively) also differ greatly in sample complexity and Theewaterskloof does not receive WWTW outflow. As expected if the source water contains a higher level of pharmaceuticals the probability of the finished drinking water containing these compounds increases.

Water samples were screened in a targeted fashion, and quantified against external calibration curves, for 96 of the initial 99 target compounds and it was

found that the concentration ranges of each differed significantly for the various sampling sites (Table 3-3).

Table 3-3: Quantitation range and prevalence of 99 pharmaceuticals is South African surface water by positive electrospray UHPLC-QTOF in Full Scan mode of 72 samples.

COMPOUND NAME	COMPOUND CLASS	AVERAGE (ng/L)	HIGHEST (ng/L)	LOWEST (ng/L)	NUMBER OF OCCURRENCES
Abacavir	ARV	n.q	n.q	n.q	4
Acetaminophen	Analgesic	127	233	8	13
Acyclovir	Antiviral	0	0	0	0
Amikacin	Aminoglycoside Antibiotic	0	0	0	0
Amoxicillin	B-Lactam Antibiotics	70	207	9	7
Aspartame	Sweetener	0	0	0	0
Atorvastatin	Statin	0	0	0	0
Azathioprine	Immunosuppressive Antimetabolite Pro-Drug	0	0	0	0
Azithromycin	Macrolide Antibiotic	n.q	n.q	n.q	4
Benzylpenicillin	B-Lactam Antibiotics	0	0	0	0
Caffeine	Stimulant	141	358	27	47
Captopril	Angiotensin-Converting Enzyme (ACE) Inhibitor	0	0	0	0
Carbamazepine	Anticonvulsant	n.q	n.q	n.q	30
Carvedilol	Beta Blocker	0	0	0	0
Cefaclor	Cephalosporin Antibiotic	0	0	0	0
Cefotaxime	Cephalosporin Antibiotic	0	0	0	0
Ceftriaxone	Cephalosporin Antibiotic	0	0	0	0
Chloramphenicol	Amphenicol Antibiotic	0	0	0	0

COMPOUND NAME	COMPOUND CLASS	AVERAGE (ng/L)	HIGHEST (ng/L)	LOWEST (ng/L)	NUMBER OF OCCURRENCES
Chlorhexidine	Anti-Septic	5	5	5	2
Chloroquine Phosphate	Antimalarial Agent	0	0	0	0
Chlorothiazide	Thiazide Diuretic	251	468	90	8
Chlorpheniramine Maleate	Histamine H1 Antagonist	n.q	n.q	n.q	11
Chlorpropamide	Antihyperglycemic	0	0	0	0
Cholecalciferol	Vitamin	0	0	0	0
Cimetidine	Histamine Congener,	39	52	30	12
Clarithromycin	Macrolide Antibiotic	0	0	0	0
Clindamycin	Lincosamide Antibiotic	0	0	0	0
Clotrimazole	Antimycotic	n.q	n.q	n.q	4
Cloxacillin	B-Lactam Antibiotics	0	0	0	0
Cyclosporin A	Immunosupressant	0	0	0	0
Dextromethorphan Hydrobromide Monohydrate	Codeine Analog	0	0	0	0
Diclofenac Sodium Salt	Non-Steroidal Anti-Inflammatory Agent (NSAID)	81	125	40	11
Didanosine	ARV	0	0	0	0
Diphenhydramine Hydrochloride	Histamine H1 Antagonist	47	54	39	3
Doxycycline Hyclate	Tetracycline Antibiotic	n.q	n.q	n.q	3
Efavirenz	ARV	174	696	3	21
Emtricitabine	ARV	361	361		5
Enalapril Maleate	Angiotensin-Converting Enzyme (ACE) Inhibitor	11	16	3	9
Erythromycin	Macrolide Antibiotic	n.q	n.q	n.q	2

COMPOUND NAME	COMPOUND CLASS	AVERAGE (ng/L)	HIGHEST (ng/L)	LOWEST (ng/L)	NUMBER OF OCCURRENCES
Ethambutol	Antimycobacterial	n.q	n.q	n.q	10
Ethionamide	Antimycobacterial	0	0	0	0
Famotidine	Histamine H2-Receptor Antagonist	0	0	0	0
Flucloxacillin	B-Lactam Antibiotics	0	0	0	0
Fluconazole	Triazole Antifungal	41	130	8	37
Fluoxetine Hydrochloride	Selective Serotonin-Reuptake Inhibitors (SSRIs)	42	42	0	1
Furosemide	Benzoic-Sulfonamide-Furan	0	0	0	0
Gabapentin	Analgesic	181	206	157	2
Gemfibrozil	Anticholesterolemic	0	0	0	0
Gentamicin	Aminoglycoside Antibiotic	0	0	0	0
Guaifenesin	Expectorant	n.q	n.q	n.q	2
Hydrochlorothiazide	Thiazide Diuretic	0	0	0	0
Hydrocortisone	Corticosteroid	13	25	1	3
Ibuprofen	Anti-Inflammatory	0	0	0	0
Indinavir	ARV	0	0	0	0
Indomethacin	Non-Steroidal Anti-Inflammatory Agent (NSAID)	n.q	n.q	n.q	3
Isoniazid	Antimycobacterial	n.q	n.q	n.q	17
Ketoconazole	Imidazole Antifungal	0	0	0	0
Ketoprofen	Non-Steroidal Anti-Inflammatory Agent (NSAID)	0	0	0	0
Labetalol Hydrochloride	Alpha/Beta Adrenergic Antagonist	135	370	10	44

COMPOUND NAME	COMPOUND CLASS	AVERAGE (ng/L)	HIGHEST (ng/L)	LOWEST (ng/L)	NUMBER OF OCCURRENCES
Lamivudine	ARV	21	21	-	1
Lamotrigine	Anticonvulsant	190	586	0	46
Lansoprazole	Proton-Pump Inhibitor	n.q	n.q	n.q	1
Leflunomide	Antirheumatic	410	644	120	44
Lidocaine	Anesthetic	n.q	n.q	n.q	9
Loperamide	Antidiarrheals	n.q	n.q	n.q	3
Lopinavir	ARV	204	859	1	36
Loratadine	Histamine H1 Receptor Antagonist	n.q	n.q	n.q	4
Lovastatin	Statin	201	839	23	
Mesalamine	Anti-Inflammatory	0	0	0	0
Metformin Hydrochloride	Antihyperglycemic	91	179	4	2
Methocarbamol	Muscle Relaxant	0	0	0	0
Methotrexate	Antineoplastic Antimetabolite	0	0	0	0
Methylparaben (Methyl Parahydroxybenzoate)	Anti-Fungal	305	494	93	8
Metoclopramide Hydrochloride	Antiemetic	0	0	0	0
Metoprolol Tartrate	B1-Adrenergic Blocking Agent	0	0	0	0
Metronidazole	Nitroimidazole	51	60	43	1
Naproxen	Anti-Inflammatory	0	0	0	0
Nevirapine	ARV	123	379	n.q	46
Ofloxacin	B-Lactam Antibiotics	0	0	0	0
Omeprazole	Antisecretory	0	0	0	0
Oseltamivir	Antiviral	0	0	0	0

COMPOUND NAME	COMPOUND CLASS	AVERAGE (ng/L)	HIGHEST (ng/L)	LOWEST (ng/L)	NUMBER OF OCCURRENCES
Praziquantel	Anthelmintic	90	167	21	7
Prednisolone	Glucocorticoid	623	1083	257	18
Prednisone	Anti-Inflammatory Glucocorticoid	355	355	n.q	4
Pyrazinamide	Antimycobacterial	445	528	270	7
Ranitidine Hydrochloride	Non-Imidazole Blocker	0	0	0	0
Rifampicin	Antimycobacterial	0	0	0	0
Ritonavir	ARV	489	1130	59	8
Stavudine	ARV	0	0	0	0
Sulfamethoxazole	Sulfonamide Antibiotic	94	252	13	44
Taurine	Bile Component	0	0	0	0
Tenofovir	ARV	0	0	0	0
Tetracycline Hydrochloride	Polyketide Antibiotic	0	0	0	0
Theophylline	Phosphodiesterase Inhibitor	200	0	0	34
Tobramycin	Aminoglycoside Antibiotic	0	0	0	0
Trimethoprim	Pyrimidine Inhibitor Antibiotic	0	0	0	0
Valsartan	Angiotensin-Receptor Blocker (ARB)	126	425	8	22
Zalcitabine	ARV	0	0	0	0
Zidovudine	ARV	0	0	0	0

*n.d. - Not Detected (S/N<3); n.q. - Not Quantified (S/N<10).

Caffeine was detected most frequently, in 66% of the samples, followed by Lamotrigine and Nevirapine (64% of the samples). Nevirapine use in South Africa

is exceptionally high due to the high HIV burden suffered in the country (Wood et al., 2015). In addition to this, the molecule is relatively persistent in the environment (Wood et al., 2016). This means that besides considering the amount of compound discharged into the environment (by merit of patient consumption data) one should also consider the compound's behavior during waste water treatment and its biodegradability when studying its environmental prevalence.

Forty eight out of the 99 target compounds were detected in the environmental samples. This highlights one of the inherent flaws of targeted screening as a strategy to begin with, in that a 48% hit rate was achieved. This is considering the cost of the remaining 51 analytical reference standards, and the time taken to prepare them, may not have been required for the analysis. It may therefore be more economical to screen samples from undescribed water sources against a database (using accurate mass and MS/MS data) and then confirm targets based on the acquired data.

Due to the large amount of data generated for each environmental sample, it is convenient to present one sample as an example of the analytical procedure. Figure 3-2 shows the total ion chromatogram (TIC) of a sample taken on the Pienaars River before it reaches the Roodeplaat dam. The sample is downstream of the Baviaanspoort WWTW and as expected, contains a number of the target compounds as well as a plethora of other unidentified contaminants. The first large peak is the solvent front (of DMSO) and obscures a number of the earlier eluting (polar) compounds.

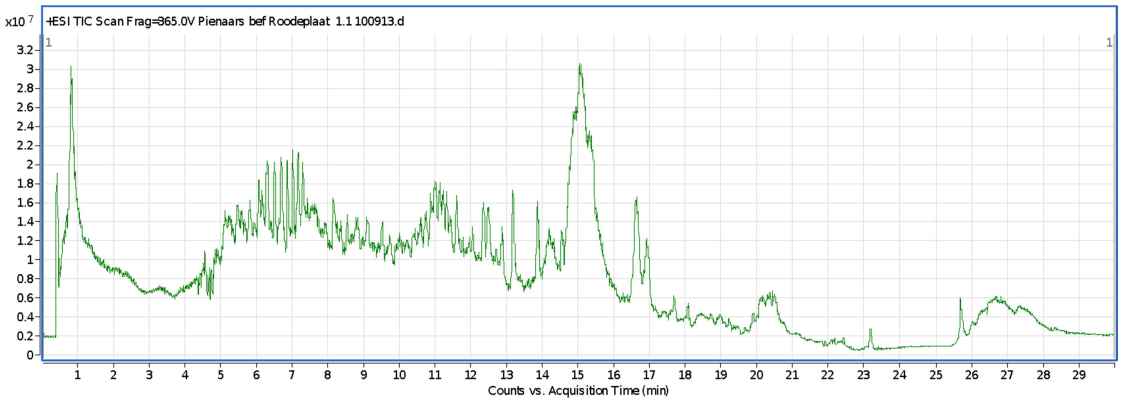


Figure 3-2: Total ion chromatogram of a Pienaars River water sample concentrated by SPE and analysed by UHPLC-QTOF in Full Scan mode.

The sample complexity becomes clearer in Figure 3-3 where only the base peaks of the data are presented as a chromatogram. From this broad view, a target is identified via non-targeted screening, i.e. through accurate mass screening against a database. One such target's (Valsartan) characteristic ions (mass window +- 0.5 *m/z*) are extracted in Figure 3-4. The target molecule appears at the expected retention time (+- 0.2 min), as determined by comparison to an analytical standard, and shows a clear peak.

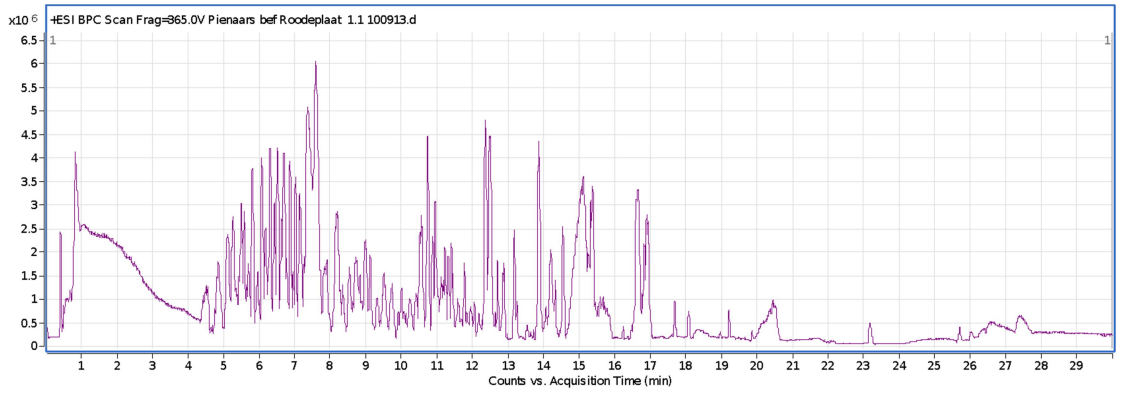


Figure 3-3: Base peak chromatogram of a Pienaars River water sample concentrated by SPE and analysed by UHPLC-QTOF in Full Scan mode.

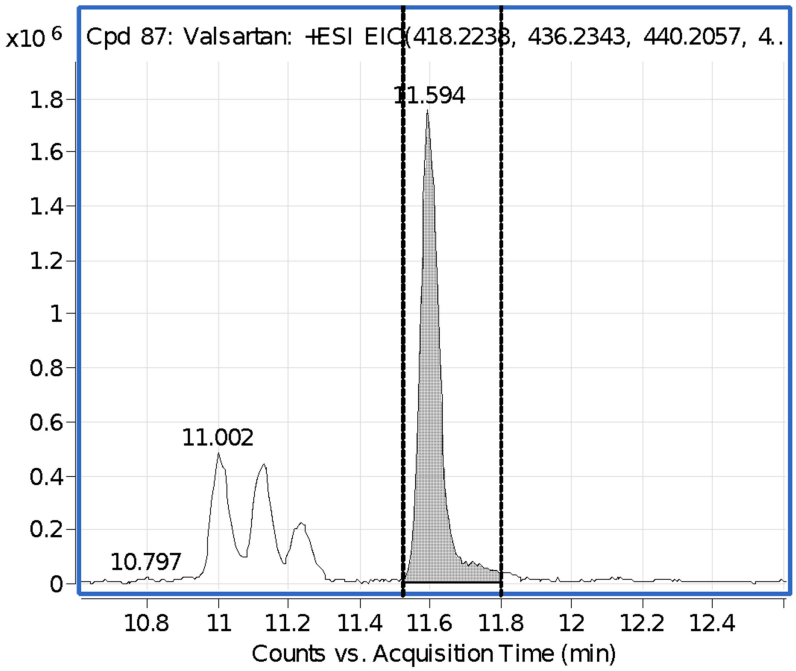


Figure 3-4: Extracted ion chromatogram (EIC) of Valsartan pseudomolecular ion (theoretical M+H = 436.2343) and characteristic adducts and losses detected in a Pienaars River water sample concentrated by SPE and analysed by UHPLC-QTOF in Full Scan mode.

The software is then able to compare the expected mass spectra (isotopic ratio) to the data that was generated for the target (Figure 3-5). The Find by Formula algorithm in MassHunter Quant (Agilent) generates a theoretical isotopic ratio for the target and then overlays this with the acquired data. In this way and accuracy of match score may be generated and presented to the user. The “Score (Tgt)” result of the Find by Formula algorithm is a measure of how closely a detected compound matches the mass, isotopic pattern and retention time of database entry compound. This value gives an indication of the certainty by which the presence of a target may be reported. An additional level of confidence is gained in the automatic identification of adducts as well as dimers and dimer adducts (e.g. the [2M+Na]+).

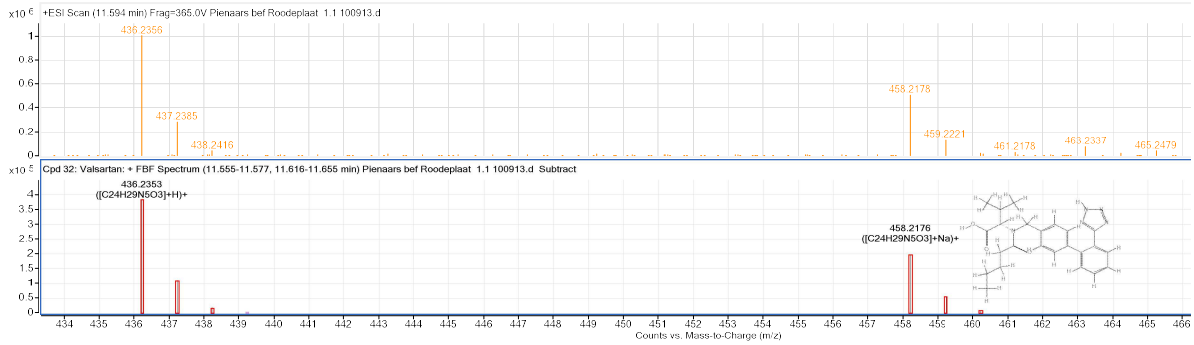


Figure 3-5: Total ESI mass spectrum (top) and Find by Formula (FBF) spectrum (bottom) of Valsartan detected in a Pienaars River water sample concentrated by SPE and analysed by UHPLC-QTOF in Full Scan mode. Red boxes overlaying spectrum peaks in the FBF spectrum represent the theoretical isotopic distribution of Valsartan (theoretical M+H = 436.2343).

The data set is extremely useful in that it allows the user to discriminate between true targets and potential false positives. The retention time (RT) of the hit as well as the difference between the hit and the database is presented. A RT difference of no more than 0.2 minutes is accepted by most laboratories. The second level of certainty is provided by the mass error. This is the difference between the measured and theoretical mass of the target compound. The acceptable level of mass error between a measured ion and library compound can be determined by comparing the measured mass difference between the infused calibrant and its theoretical mass, in the same analysis.

In order to maximize the amount of data generated from a sample, non-targeted approaches were investigated. By using Auto MS/MS the instrument selects prominent ions and then fragments them by MS/MS. In this case collision energy was determined by a formula which uses the target ion's mass to determine the amount of energy used for fragmentation. The "Find by Auto MS/MS" algorithm was utilised to extract target ions (Figure 3-6) from the Pienaars River sample. Auto MS/MS data may be analysed using a number of approaches: "Molecular Formula Generator" can be utilised to propose formulae for the unknown masses that were selected and searched against a library or an online database, "Molecular Features" can be extracted and similarly searched, or the resulting

collision spectra of the compounds selected for Auto MS/MS can be compared against a database; such as the composite database generated during this study (> 80 000 compounds).

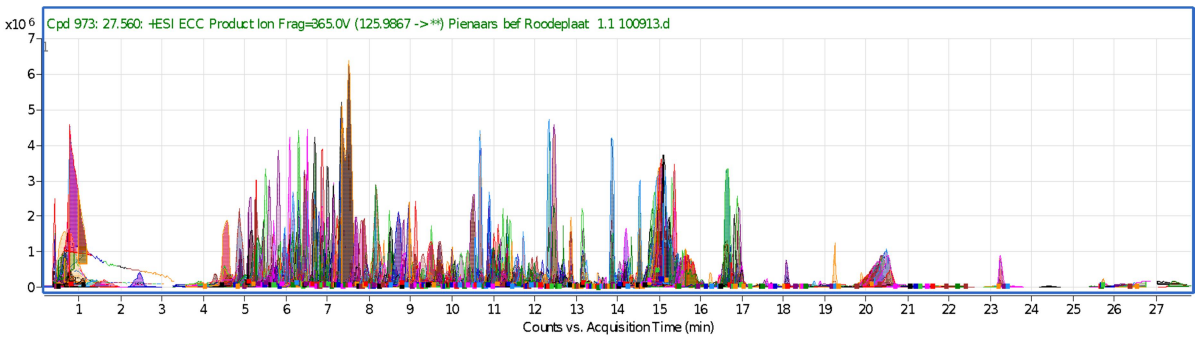


Figure 3-6: Overlaid EICs of compounds found by Auto MS/MS of a Pienaars River water sample concentrated by SPE and analysed by UHPLC-QTOF in Auto MS/MS mode.

In the case of Valsartan, the positive pseudomolecular ion (*m/z* 436.2817) was selected automatically for MS/MS fragmentation (at 30.2 eV). The target mass spectrum (Figure 3-7) may then be identified by comparing it to a spectral database (Figure 3-8).

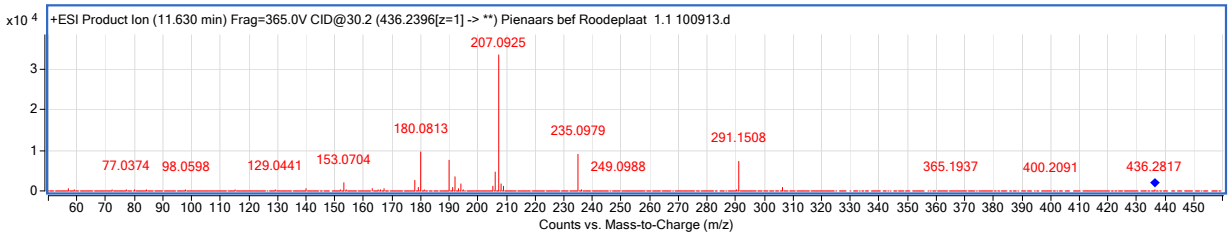


Figure 3-7: MS/MS spectrum (CID 30.2 eV) of an SPE extract of the Pienaars River before the Roodeplaat dam analysed by UHPLC QTOF in Auto MS/MS mode. Precursor ion is the Valsartan pseudomolecular ion (m/z 436.2817).

From these figures it is clear that the suspected target was present in the sample since chromatographic retention times matched closely, the pseudomolecular

mass matched closely with the theoretical mass and a number of the collision induced dissociation ion products matched with a spectral database library.

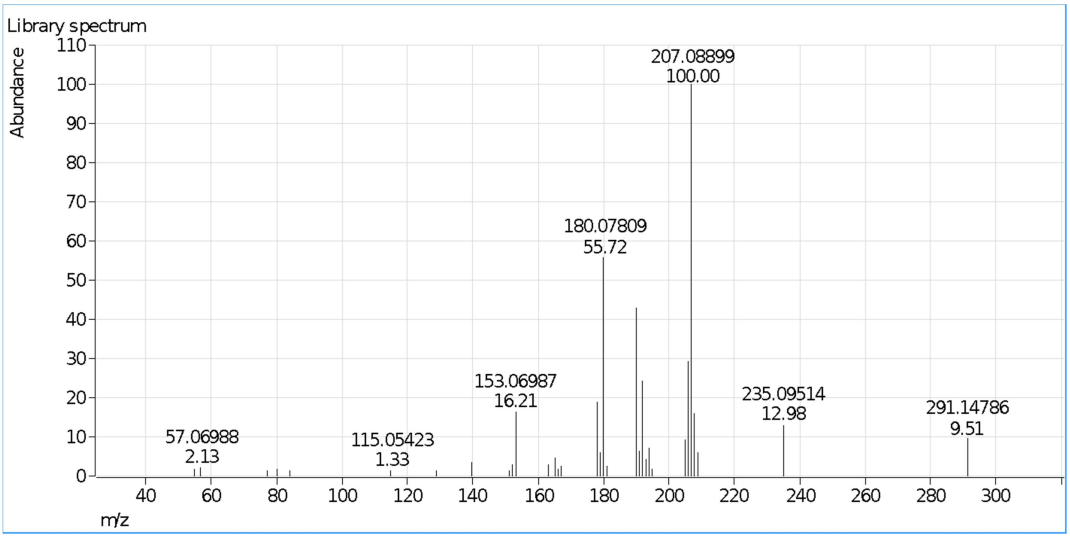


Figure 3-8: Library MS/MS spectrum of Valsartan CE = 40 in positive ESI.

The last analytical approach utilized for the analysis of environmental samples is All Ions analysis. This is a Full Scan methodology which consists of multiple "Experiments" within a single run. The first experiment scans across the given range at three spectra per second and is directly followed by another experiment in which collision energy is applied to the collision cell. This means that all ions passing to the collision cell (i.e. the quadrupole is in total transmission mode) are fragmented at the desired constant voltage, leading to a non-discriminatory highly fragmented mass spectrum alternating the with the quasi-simultaneously generated unfragmented electrospray mass spectrum.

The advantage of this approach is that the unfragmented Full Scan data as well as the CID data is available for near identical time points throughout the analysis. This means that low intensity ions that may have been missed by Auto MS/MS could still be fragmented and extracted from the data. The All Ions TIC of the same Pienaars River sample that was analysed previously in Full Scan mode is identical

to the Full Scan MS experiment. Figure 3-9 shows the TIC of "Experiment 2" in which collision energy (40 eV) is applied to all the ions in the experiment. This TIC is nearly identical to Experiment 1 albeit with a markedly reduced intensity. This is expected, as intense ions are fragmented into groups of less intense product ions.

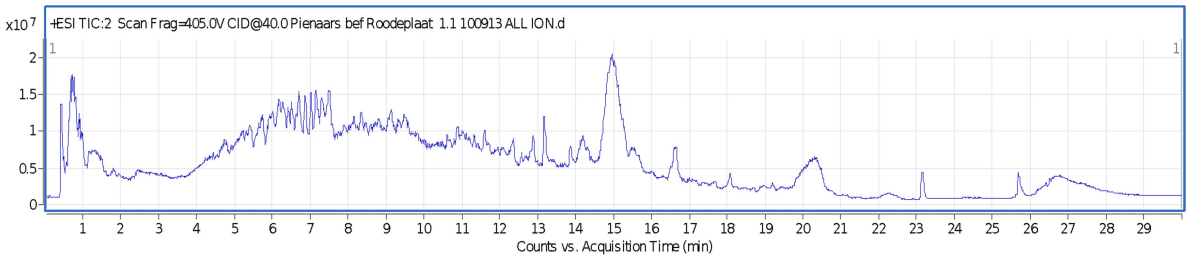


Figure 3-9: TIC of experiment 2 (CE = 40 eV) in an All Ions analysis of a Pienaars River water sample concentrated by SPE and analysed by UHPLC-QTOF.

Once again, to provide continuity, Valsartan will be used as an example. In this data set the compound was automatically identified using the "Find by Formula" algorithm and then may be further characterized by its CID spectrum (Figure 3-10). Assuming that Valsartan was not chosen for Auto MS/MS analysis (due to exclusion because of its precursor ion intensity) but its pseudomolecular ion was detected with high confidence in Full Scan analysis; the All Ions approach would provide a means of manual identification by identifying the compound's MS/MS product ions that are produced during the non-discriminatory CID component of the analysis.. When comparing the acquired spectrum to the database all the characteristic ions of Valsartan are present in addition to a number of others. These are from co-eluting compounds as well as the fragmentation of background ions that would not normally be present in a (CID) mass spectrum. Fragment confirmation may also be automated during the Find by Formula algorithm, in which the number of desired fragments may be specified.

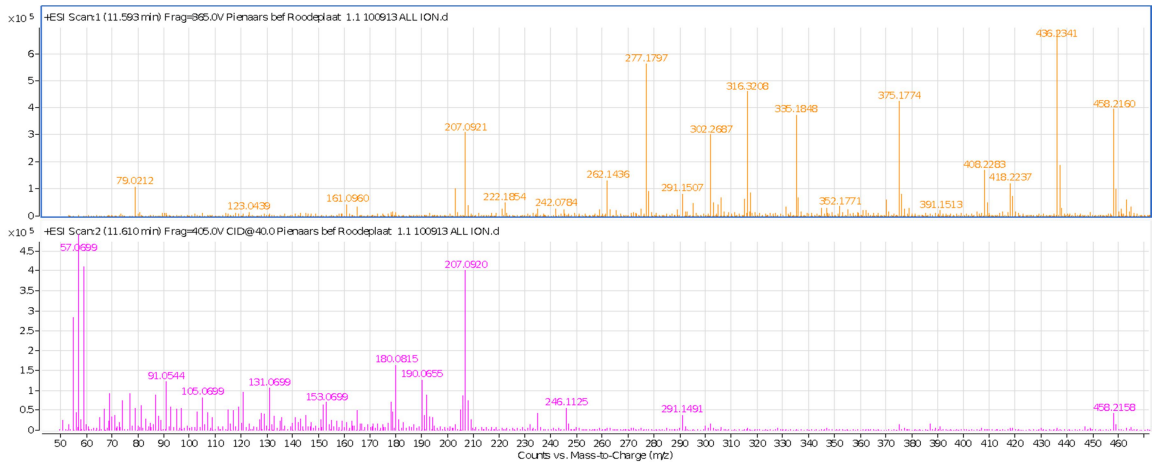


Figure 3-10: Full Scan mass spectrum at CE = 0 eV of a peak identified as Valsartan (top) and subsequent CID of all the ions in the peak at CE = 40 eV (bottom).

Interestingly, more compounds are identified for this experiment (on the same sample) than with the Full Scan experiment when using the Find by Formula approach with the Forensic Toxicology database. Two hundred and eighty three compounds were detected with greater than 90% confidence as compared to the 190 in the earlier experiment. It is, however, believed that a number of these should be false positives as a fragment ion may have been misidentified as an intact ion in a number of cases. This is because the algorithm may assign a mass match from the database to a fragment ion, believing that it is an intact pseudomolecular ion. It is therefore important to utilize confirmation ions during these experiments.

By utilizing this three pronged high resolution mass spectrometry approach information may be mined from data at any stage after the analysis since the sample has been analysed so fully. This means that as new scientific questions arise, analysts may return to the data and re-analyse it from a fresh perspective. This is important from the standpoint of novel micropollutants in that new compounds are discovered regularly and having a historical pollution profile baseline of a water system may retrospectively provide insight by tracking the appearance in time of the novel chemical species. It is therefore also important to

repeat this type of national screening campaign on a regular basis in order to not only track known micropollutants but also to identify new emerging water contaminants as and when they appear.

3.3.5 Database-Driven Screening

Fortunately a number of databases are becoming commercially available, which will do much to aid in the inter-laboratory comparison of LC-MS data. In this work, three such databases were combined into a single searchable entity (Table 3-4).

Table 3-4: The number of compounds and spectra in the Agilent Forensics and Toxicology, Metlin and Veterinary Drugs Personal Compound Database Libraries (PCDLs).

Database Name	No. Compounds	No. Spectra*
Agilent Forensics and Toxicology PCDL	9008	8934
Agilent Metlin AM PCDL	64092	25880
Agilent Veterinary Drugs PCDL	1029	1796

* The number of spectra in a PCDL may exceed the number of compounds due to multiple spectral entries for a single compound

Unfortunately, when using high resolution mass spectrometry, the data file size is quite large, which leads to an increase in the time it takes to compare it to a database. A single query may take up to 12 hours per data file, which represents a severe bottleneck in the analytical strategy. The methodology adopted is designed to increase the speed with which samples are identified against the database.

The architecture of the MassHunter software does not allow parallelization and subsequent computational speed-up of the query algorithm and therefore, in order to achieve the stated aim, many samples (e.g. 10 samples) will be analysed at the same time where each identification task will take 12 hours. This will effectively

reduce the query time to 1.2 hours (since 10 queries are processed at the same time). It is expected that the speed-up will be slightly more than 1.2 hours since each Virtual Box must use the same communication hardware on the CPU and between the CPU, the RAM and the hard disk drive (HDD). It was however found that a single parallel instance completes the query in slightly less time (9%) than a serial instance.

The results from this approach were very encouraging indicating that an effective sample identification time of 1.2 hours can be achieved with the present setup compared to the sample identification time of 12 hours for the individual search instance. MassHunter running natively in a Windows 7 OS takes approximately 12 hours to complete an analysis, the same file running in a single Virtual Box takes 13 hours to complete, while running in a Virtual Box in parallel on the same hardware takes approximately 13-14 hours to complete. At least 30 instances of MassHunter may be spawned and executed at any one time with the possibility of completing two such cycles in a 24 hour period. This will allow a user to identify 60 samples per day compared to the 2 samples per day throughput that is currently possible. This represents a 3000 % increase in throughput.

The Virtual Box environment allows an operator that is only familiar with Windows-based commands to effectively operate the MassHunter Software on the cluster. During the course of this work it was found that the MassHunter process could be launched from the DOS command line. This is not described in any previous training and to the best of our knowledge is not a common practice. This is since most analysts operate from a graphic user interface (GUI) as opposed to the command line. This strategy allows a batch script to be created in a simple text file that could be launched to execute serially the analysis of multiple data files.

As mentioned earlier, up to three instances of MassHunter can be run simultaneously on a single virtual box. This was found to not be possible on a personal computer of similar hardware allocation. It is most likely due to the fact that no other resources are utilised, save those for MassHunter, on the virtual box. It is evident that the CPU capacity is the limiting factor during the analysis, with

almost all of the CPU capabilities permanently engaged. The RAM allocation on the other hand is a small fraction of the total RAM available (6 GB used out of 16 GB in most instances).

When all the environmental samples were screened using this strategy, a vast data set was reduced into a more manageable number of potential leads. On average, by Full Scan analysis, 195 compounds were identified per sample by database matching. This is out of 4273 potential compounds flagged by molecular feature across all the samples, many of which occur in multiple samples. As mentioned earlier, 48 out of the 99 targets chosen for analysis could be detected in the environment. Of these, 22 were found automatically in Full Scan analysis. The reason that not all 48 were detected was the level stringency applied to the search. Lower concentration targets, i.e. those below the peak height threshold, were excluded. The top 10 most frequently occurring compounds, identified by Full Scan analysis (Table S3-12) were not, however, pharmaceuticals. The data may be visualized by compound class (Table S3-13) and it was found that pesticides occurred most frequently. With regards to compounds relevant to this study, stimulants and analgesics occurred most frequently across the whole sample set.

Through an automated MS/MS analysis of the sample set, 92 unique compounds, occurring multiple times across the data set were identified. The reduction of compound numbers identified using this approach can be related to a combination of the stringency at which the ions were selected for MS/MS and the composition of the database (i.e. compounds selected for MS/MS were not present in the database). Of the 10 most commonly occurring compounds (Table S3-14) four were pharmaceuticals. When arranged by compound class (Table S3-15) it was found that seven of the 10 most frequently occurring compound classes were of a pharmaceutical nature. Thirty of the 92 compounds identified by Auto MS/MS are also identified by Full Scan analysis. The reasons why not all 92 are identified also relate to analytical stringency as well as the fact that Full Scan analysis often presents a number of isomers as results.

It must be borne in mind that there are various areas in the analytical workflow where bias can be introduced. This could range from ionization efficiency, resulting in the preferential detection of certain compounds, to the type of database used (e.g. pharmaceutical, pesticide, toxicology etc.), leading to an inaccurate perception of which compound types occur most/least frequently (e.g. if a greater number of a particular compound type is represented in the database, compared to others).

There is therefore a need to expand databases, commercial and public, in order to obtain a clearer picture of the chemical constituents of surface water. In addition to this, it is suggested that multiple analytical paradigms be adopted, integrated and compared with each other; and utilized to screen surface water routinely. Routine analysis previously only allowed a narrower view inherent to targeted screening. With advances in high resolution mass spectrometry targeted quantification as well as untargeted screening is now possible on a single instrument. The amount of information obtainable will soon prove to outweigh the cost of such sophisticated infrastructure.

3.4 Conclusions

In this work the utility of high resolution mass spectrometry with targeted and untargeted screening as a strategy to analyse surface water for the presence of a diverse array of pharmaceuticals is demonstrated. For the first time the diversity of pharmaceutical pollutants in South African surface water, on a national scale, has been shown. A searchable data set that may serve as a national pollution baseline for future use has been created. To accomplish this, a SPE extraction and UHPLC-QTOF method was developed that achieved limits of quantification in the low to mid ng/L range for the majority of compounds. It is however important to note that LC-MS is subject to matrix effect, which may not always be controlled when performing quantification studies using external calibration. This has been shown in earlier work (Wood, 2015) and methodologies such as the standard addition method merit further investigation within the paradigm presented in this

manuscript. Initially 99 compounds were selected and 96 of these were screened for in South African surface water. A wide spread of concentration was noted for the targets, with Lamotrigine and Nevirapine jointly being the most prevalent. Prednisilone and Ritonavir had the highest average concentrations at 623 and 489 ng/L respectively. It is evident that the antiretroviral (anti-HIV) class of drug occurs frequently in South African surface water, which may be attributed to the high usage of these compounds in the country; due to the extremely high national HIV burden.

Mass spectrometry provides comprehensive data enabling the detection and identification of trace amounts of a wide variety of analytes, also through retrospective data mining. The sheer amount of data from the analysis poses challenges relating to fast interpretation of the data in order to support decision making. Cluster computing may be used to parallelize mass spectrometric analyses. Although no reduction in time per analysis is achieved, the overall throughput of the process is increased by at least 3000%.

High resolution mass spectrometry continues to prove its utility in environmental analyses, yet much work is still to be done on the data processing aspect of this analytical strategy. Once data analyses become automated and faster, the technology would become more appealing for routine screening purposes, potentially replacing the generally used targeted screening approach.

3.5 References

1. Agunbiade, F.O., Moodley, B., 2014. Pharmaceuticals as emerging organic contaminants in Umgeni River water system, KwaZulu-Natal, South Africa. Environmental Monitoring and Assessment 186 (11), 7273–7291.
2. Agunbiade, F.O., Moodley, B., 2015. Occurrence and distribution pattern of acidic pharmaceuticals in surface water, wastewater and sediment of the Msunduzi River, Kwazulu-Natal, South Africa. Environmental Toxicology and Chemistry 35(1), 36-46.

3. Alygizakis, N.A., Gago-Ferrero, P., Borova, V.L., Pavlidou, A., Hatzianestis, I., Thomaidis, N.S., 2016. Occurrence and spatial distribution of 158 pharmaceuticals, drugs of abuse and related metabolites in offshore seawater. Science of the Total Environment 541, 1097–1105.
4. Boix, C., Ibáñez, M., Sancho, J.V., Parsons, J.R., de Voogt, P., Hernández, F., 2016. Biotransformation of pharmaceuticals in surface water and during waste water treatment: Identification and occurrence of transformation products. Journal of Hazardous Materials 302, 175–187.
5. Boorman, G.A., 1999. Drinking water disinfection byproducts: review and approach to toxicity evaluation. Environmental Health Perspectives 107 (Suppl 1), 207.
6. Cotton, J., Leroux, F., Broudin, S., Poirel, M., Corman, B., Junot, C., Ducruix, C., 2016. Development and validation of a multiresidue method for the analysis of more than 500 pesticides and drugs in water based on on-line and liquid chromatography coupled to high resolution mass spectrometry. Water Research 104, 20–27.
7. Daughton, C., Scuderi, M., 2012. Pharmaceuticals and Personal Care Products (PPCPs): Relevant Literature. US Environmental Protection Agency, Las Vegas, NV.
8. Farré, M. la, Pérez, S., Kantiani, L., Barceló, D., 2008. Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. TrAC Trends in Analytical Chemistry 27 (11), 991–1007.
9. Ferrer, I., Thurman, E.M., 2012. Analysis of 100 pharmaceuticals and their degradates in water samples by liquid chromatography quadrupole time-of-flight mass spectrometry. Journal of Chromatography A 1259, 148–157.
10. Gatica, J., Cytryn, E., others, 2013. Impact of treated wastewater irrigation on antibiotic resistance in the soil microbiome. Environmental Science and Pollution Research International 20, 3529-3538.
11. Glauser, G., Grund, B., Gassner, A.-L., Menin, L., Henry, H., Bromirski, M., Schutz, F., McMullen, J., Rochat, B., 2016. Validation of the mass-

extraction-window for quantitative methods using liquid chromatography high resolution mass spectrometry. Analytical Chemistry 88 (6), 3264–3271.

12. González-Mariño, I., Gracia-Lor, E., Bagnati, R., Martins, C.P., Zuccato, E., Castiglioni, S., 2016. Screening new psychoactive substances in urban wastewater using high resolution mass spectrometry. Analytical and Bioanalytical Chemistry, 1–13.
13. Graham, J.P., Polizzotto, M.L., 2013. Pit latrines and their impacts on groundwater quality: a systematic review. Environmental Health Perspectives 121 (5), 521–530.
14. Ibáñez, M., Borova, V., Boix, C., Aalizadeh, R., Bade, R., Thomaidis, N., Hernández, F., 2016. UHPLC-QTOF MS screening of pharmaceuticals and their metabolites in treated wastewater samples from Athens. Journal of hazardous materials http://dx.doi.org/10.1016/j.jhazmat.2016.03.078.
15. K'oreje, K., Vergeynst, L., Ombaka, D., De Wispelaere, P., Okoth, M., Van Langenhove, H., Demeestere, K., 2016. Occurrence patterns of pharmaceutical residues in wastewater, surface water and groundwater of Nairobi and Kisumu city, Kenya. Chemosphere 149, 238–244.
16. Hanke, G., Wollgast, J., Loos, R., Jiménez, J.C., Umlauf, G., Mariani, G., Müller, A., Huber, T., Christoph, E.H., Locoro, G., others, 2007. Comparison of Monitoring Approaches for Selected Priority Pollutants in Surface Water. JRC Scientific and Technical Reports.
17. Jain, S., Kumar, P., Vyas, R.K., Pandit, P., Dalai, A.K., 2013. Occurrence and Removal of Antiviral Drugs in Environment: A Review. Water, Air, & Soil Pollution 224 (2), 1–19.
18. Khetan, S.K., Collins, T.J., others, 2007. Human pharmaceuticals in the aquatic environment: a challenge to green chemistry. Chemical Reviews-Columbus 107 (6), 2319–2364.
19. Kümmerer, K., 2009. Antibiotics in the aquatic environment-a review-part II. Chemosphere 75 (4), 435–441.
20. MacGregor, W.S., 1967. The chemical and physical properties of DMSO. Annals of the New York Academy of Sciences 141 (1), 3–12.

21. Miège, C., Choubert, J., Ribeiro, L., Eusèbe, M., Coquery, M., 2009. Fate of pharmaceuticals and personal care products in wastewater treatment plants-Conception of a database and first results. Environmental Pollution 157 (5), 1721–1726.
22. Padhye, L.P., Yao, H., Kung'u, F.T., Huang, C.-H., 2014. Year-long evaluation on the occurrence and fate of pharmaceuticals, personal care products, and endocrine disrupting chemicals in an urban drinking water treatment plant. Water Research 51, 266-276.
23. Papageorgiou, M., Kosma, C., Lambropoulou, D., 2016. Seasonal occurrence, removal, mass loading and environmental risk assessment of 55 pharmaceuticals and personal care products in a municipal wastewater treatment plant in Central Greece. Science of The Total Environment 543, 547–569.
24. Peng, X., Ou, W., Wang, C., Wang, Z., Huang, Q., Jin, J., Tan, J., 2014. Occurrence and ecological potential of pharmaceuticals and personal care products in groundwater and reservoirs in the vicinity of municipal landfills in China. Science of The Total Environment 490, 889–898.
25. Rager, J.E., Strynar, M.J., Liang, S., McMahen, R.L., Richard, A.M., Grulke, C.M., Wambaugh, J.F., Isaacs, K.K., Judson, R., Williams, A.J., others, 2016. Linking high resolution mass spectrometry data with exposure and toxicity forecasts to advance high-throughput environmental monitoring. Environment International 88, 269–280.
26. Rivier, L., 2003. Criteria for the identification of compounds by liquid chromatography-mass spectrometry and liquid chromatography-multiple mass spectrometry in forensic toxicology and doping analysis. Analytica Chimica Acta 492 (1), 69–82.
27. Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Research 69, 234–242.

28. Sanagi, M.M., Ling, S.L., Nasir, Z., Hermawan, D., Wan Ibrahim, W.A., Naim, A.A., 2009. Comparison of signal-to-noise, blank determination, and linear regression methods for the estimation of detection and quantification limits for volatile organic compounds by gas chromatography. Journal of AOAC International 92 (6), 1833–1838.
29. Schoeman, C., Mashiane, M., Dlamini, M., Okonkwo, O., 2015. Quantification of Selected Antiretroviral Drugs in a Wastewater Treatment Works in South Africa Using GC-TOFMS. Journal of Chromatography & Separation Techniques, 6, No. 4.
30. Soulier, C., Coureau, C., Togola, A., 2016. Environmental forensics in groundwater coupling passive sampling and high resolution mass spectrometry for screening. Science of the Total Environment 563, 845–854.
31. Taylor, J.K., 1987. Quality assurance of chemical measurements. CRC Press.
32. Wagner, M., Oehlmann, J., 2011. Endocrine disruptors in bottled mineral water: Estrogenic activity in the E-Screen. The Journal of Steroid Biochemistry and Molecular Biology 127 (1), 128–135.
33. Westerhoff, P., Yoon, Y., Snyder, S., Wert, E., 2005. Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. Environmental Science & Technology 39 (17), 6649–6663.
34. Whelan, M., Kinsella, B., Furey, A., Moloney, M., Cantwell, H., Lehotay, S.J., Danaher, M., 2010. Determination of anthelmintic drug residues in milk using ultra high performance liquid chromatography-tandem mass spectrometry with rapid polarity switching. Journal of Chromatography A 1217 (27), 4612–4622.
35. Wode, F., van Baar, P., Dünnbier, U., Hecht, F., Taute, T., Jekel, M., Reemtsma, T., 2015. Search for over 2000 current and legacy micropollutants on a wastewater infiltration site with a UPLC-high resolution MS target screening method. Water Research 69, 274–283.

36. Wood, T.P., Duvenage, C.S., Rohwer, E., 2015. The occurrence of anti-retroviral compounds used for HIV treatment in South African surface water. Environmental Pollution 199, 235–243.
37. Wood, T.P., Basson, A.E., Duvenage, C., Rohwer, E.R., 2016. The chlorination behaviour and environmental fate of the antiretroviral drug nevirapine in South African surface water. Water Research 104, 349-360.
38. Zhang, Q.-Q., Ying, G.-G., Pan, C.-G., Liu, Y.-S., Zhao, J.-L., 2015. Comprehensive evaluation of antibiotics emission and fate in the river basins of China: source analysis, multimedia modeling, and linkage to bacterial resistance. Environmental Science & Technology 49 (11), 6772–6782.

3.6 Supporting Information

Database-Driven Screening of South African Surface Water and the Targeted Detection of Pharmaceuticals Using Liquid Chromatography - High Resolution Mass Spectrometry

Timothy Paul Wood[a,d] (Corresponding Author)

Chris Du Preez[b]

Adriaan Steenkamp[b]

Cornelia Duvenage[c]

Egmont R Rohwer[d]

[a] Protechnik Laboratories, a Division of ARMSCOR SOC Ltd, 103 Combretum Crescent, Centurion, Pretoria, 0001, South Africa. Tel: +27 12 665 9444, timw@protechnik.co.zamailto:timw@protechnik.co.za

[b] Flamengro, a Division of ARMSCOR SOC Ltd, Cnr Delmas road and Nossob street, Pretoria, 0001, South Africa. Tel: +27 12 428 1911 Chris@flamengro.co.za

[c]Department of Internal Medicine, 1 Military Hospital, South African Military Health Services, Voortrekker Street, Pretoria, 0001, South Africa.
Tel: +27832733382, corneliaduv@mtnloaded.co.za

[d] Department of Chemistry, University of Pretoria, Lynwood Road, Pretoria, 0001, South Africa. Tel: +27 12 420 3772 email: egmont.rohwer@up.ac.zamailto:egmont.rohwer@up.ac.za

3.6.1 Method Optimisation

Table S3-5: Three mass spectrometric source conditions used for the analysis of pharmaceutical compounds in surface water.

	Method		
Parameter	**1**	**2**	**3**
Gas Temp. (°C)	150	290	200
Gas Flow (L/min)	17	8	15
Nebuliser (psig)	40	27	40
Sheath Gas Temp (°C)	350	380	400
Sheath Gas Flow (L/min)	12	11	12
Capillary Voltage (V)	3500	3500	3500
Nozzle voltage (V)	500	500	500
Fragmentor	175	120	365
Skimmer 1	65	65	65

Table S3-6: Group 1 pharmaceuticals analysed using three different methods and quantified against a standard curve produced using Method 3.

Compound	Method No.		
	1	2	3
Lamivudine	1658.1	1121.9	998.9
Pyrazinamide	1043.5	435.8	999.6
Amoxicillin	1100.7	789.0	1000.2
Theophylline	819.0	512.4	1002.5
Chloroquinephosphate	751.2	873.0	995.9
Chlorothiazide	909.0	734.1	1000.0
Methotrexate	931.8	898.8	1000.9
Azathioprine	738.0	607.5	996.4
MetoprololTartrate	982.0	920.1	997.2
Methocarbamol	755.6	606.0	993.1
Erythromycin	1076.8	1342.1	999.4
Methylparaben(Methylparahydroxybenzoate)	537.2	202.6	1000.1
Prednisone	908.8	982.2	1000.4
FluoxetineHydrochloride	1099.0	934.9	997.9
Benzylpenicillin	881.9	747.0	1000.5
Ketoprofen	891.6	708.9	1000.4
Valsartan	984.4	1144.9	1000.8
Cholecalciferol	7626.2	493.4	980.1
Averages	900.7	780.8	998.0

For Group 2 it appears at first that Method 1 is the most optimal based on the average values; yet upon closer inspection it is found that there are outliers (Mesalamine and Zalcitabine) that have extremely good responses that are skewing the data. When these are removed Method 1 performs the worst out of the three methods for the remaining targets.

Therefore it is also important to describe the deviation from the mean in order to interpret these data.

Table S3-7: Group 2 pharmaceuticals analysed using three different methods and quantified against a standard curve produced using Method 3.

Compound	Method No.		
	1	2	3
Taurine	1026.1	649.4	1001.1
Zalcitabine	2717.3	758.3	998.8
Mesalamine	3384.9	172.3	1000.0
Emtricitabine	563.2	802.9	991.7
Famotidine	903.1	598.8	997.7
Abacavir	908.5	729.8	995.6
Caffeine	791.3	470.1	1001.0
cefotaxime	921.6	836.3	1001.0
Cefaclor	897.1	903.9	1000.0
Oseltamivir	1020.3	822.1	1000.6
Chlorhexidine	586.4	1248.9	996.9
Diphenhydramine Hydrochloride	1375.6	916.6	996.7
Chloramphenicol	928.2	352.8	1000.5
Prednisolone	1082.5	953.0	1000.4
Clarithromycin	1189.3	1530.5	998.1
cloxacillin	1014.7	887.8	1000.5
Naproxen	515.7	223.8	1000.3
Leflunomide	767.5	381.6	1002.0
Diclofenac sodium salt	834.1	762.6	1000.6
Lovastatin	814.6	980.4	992.6
Average	1112.1	749.1	998.8

Table S3-8: Group 3 pharmaceuticals analysed using three different methods and quantified against a standard curve produced using Method 3.

Compound	Method No.		
	1	2	3
Metformin Hydrochloride	897.5	440.1	992.2
Tobramycin	952.8	793.1	1001.7
Tenofovir	878.9	408.8	1000.9
Ethionamide	1805.4	3288.8	829.8
Hydrochlorothiazide	1055.6	881.3	996.7
Lidocaine	924.4	800.3	1000.5
Doxycycline hyclate	886.2	621.5	997.4
Lamotrigine	866.3	1694.4	996.9
Azithromycin	866.3	1694.4	996.9
Captopril	877.5	612.6	1004.5
Guaifenesin	842.6	425.9	992.8
Labetalol Hydrochloride	916.4	791.7	998.9
Indinavir	908.2	1334.0	1001.3
Dextromethorphan hydrobromide monohydrate	1070.7	1089.9	994.7
Lansoprazole	1072.8	871.9	999.7
Ketoconazole	862.5	952.4	1003.2
Clotrimazole	3366.9	610.0	1001.7
Praziquantel	878.9	990.4	1000.8
Gemfibrozil	1872.1	368.1	1012.6
Average	1147.5	982.6	990.7

Table S3-9: Group 4 pharmaceuticals analysed using three different methods and quantified against a standard curve produced using Method 3.

Compound	Method No.		
	1	2	3
Isoniazid	1215.2	718.9	999.3
metronidazole	914.4	460.2	999.8
Didanosine	683.7	638.9	983.4
Ranitidine Hydrochloride	926.6	697.9	994.0
Cimetidine	946.6	583.3	996.9
Stavudine	1121.7	399.6	1001.1
Trimethoprim	965.2	825.5	995.1
Ofloxacin	977.7	733.2	999.8
ceftriaxone	954.8	1126.5	1000.0
Metoclopramide Hydrochloride	958.6	845.8	997.4
Tetracycline Hydrochloride	944.6	837.4	1002.2
Chlorpheniramine Maleate	922.1	724.3	994.8
Omeprazole	1020.7	775.6	1000.1
Nevirapine	945.4	607.4	999.8
Enalapril Maleate	960.4	815.3	1000.7
Carvedilol	937.5	789.4	998.6
Loperamide	1128.1	1274.1	996.2
Rifampicin	890.6	1068.7	1002.7
Lopinavir	665.9	1329.0	1000.6
Average	951.6	802.7	998.0

Table S3-10: Group 5 pharmaceuticals analysed using three different methods and quantified against a standard curve produced using Method 3.

Compound	Method No.		
	1	2	3
Gentamicin	0.0	0.0	0.0
Ethambutol	812.0	565.7	996.8
Acyclovir	1614.0	783.0	999.1
Acetaminophen	825.4	558.3	1000.1
Gabapentin	904.7	478.2	1002.6
Aspartame	915.2	772.2	1008.1
Zidovudine	1692.0	515.0	1024.5
Fluconazole	987.7	729.0	1000.2
Clindamycin	961.2	950.4	1000.4
Sulfamethoxazole	875.6	569.2	1000.6
Hydrocortisone	887.2	1056.2	1000.3
Carbamazepine	720.9	719.0	994.1
Loratadine	965.0	849.3	999.9
Chlorpropamide	903.1	798.0	1000.6
Flucloxacillin	1047.4	811.2	999.3
Atorvastatin	801.5	1108.5	1000.6
Indomethacin	950.0	1008.6	1000.1
Ritonavir	522.9	1814.1	1001.3
Efavirenz	965.2	843.9	1000.1
Cyclosporin A	497.8	2566.9	1001.0
Average	892.4	874.8	951.5

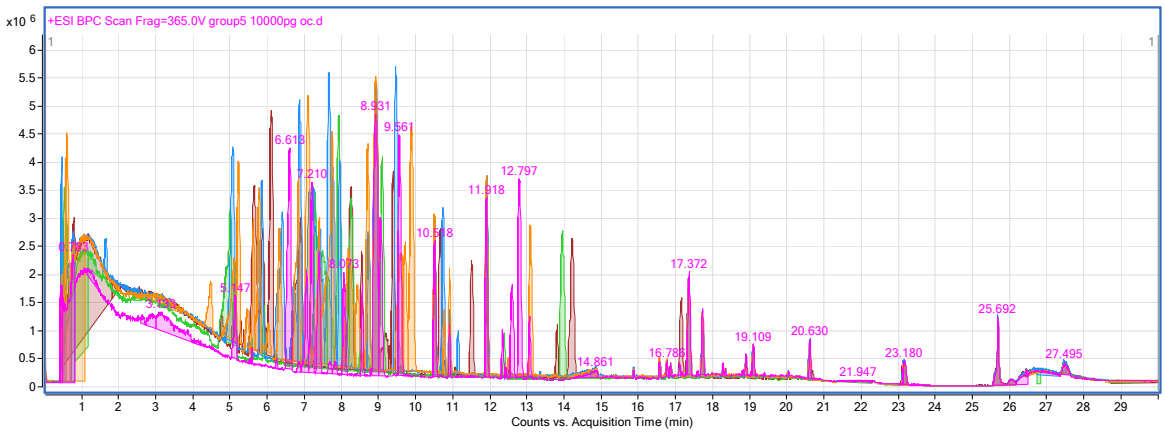


Figure S3-11: Base Peak Chromatogram overlay of five pharmaceutical groups, 10 ng on column, analysed by UHPLC positive electrospray QTOF under optimized conditions.

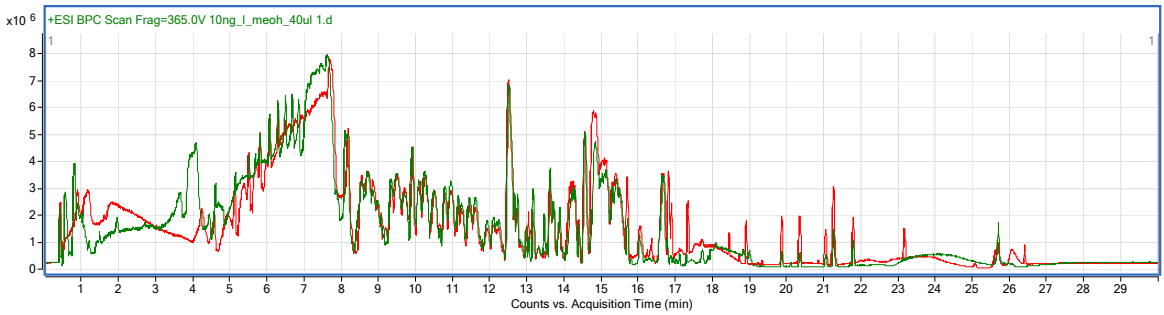


Figure S3-12: BPC overlay of targets spiked into surface water at 10 ng/L with either methanol (green trace) or DMSO (red trace) as the injection solvent (40 µL).

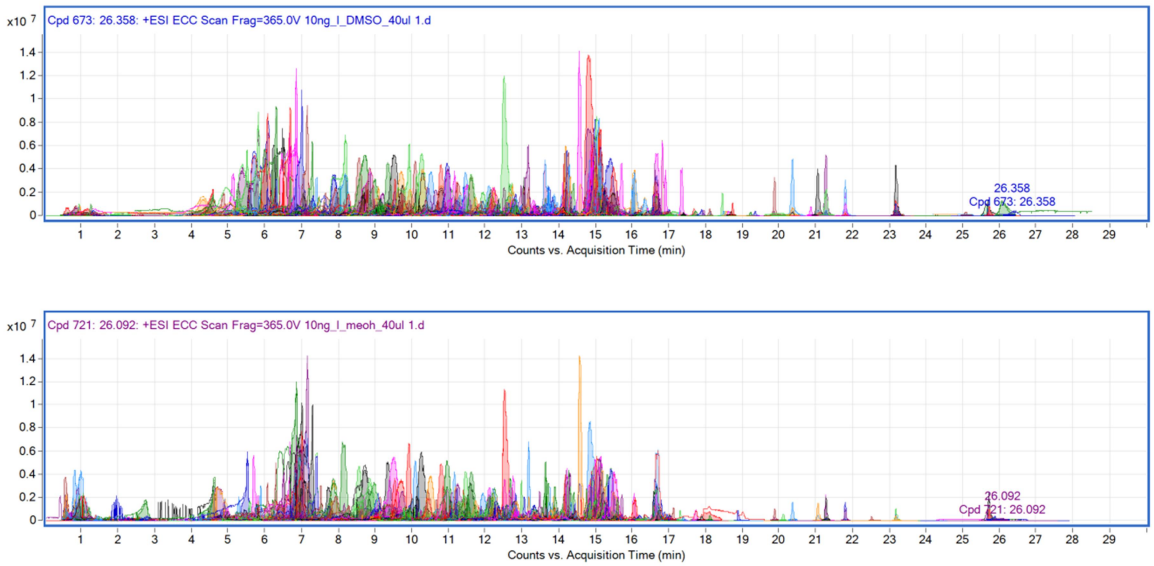


Figure S3-13: Overlay of Extracted compound chromatograms (ECC) from a molecular feature extraction of MFE extract of targets spiked into surface water at 10 ng/L with either DMSO (top) methanol (bottom) as the injection solvent (40 µL).

Table S3-11: Instrument sensitivity (LOD and LOQ) based on injection of standard mixtures in DMSO. LOD and LOQ are presented as pg on column. Method limits of detection based on spiked surface water processed by SPE.

	Instrumental Limits					Method Limits (ng/L)	
Compound	LOD (pg)	STDEV	LOQ (pg)	STDEV	LINEARITY	LOD	LOQ
Abacavir	0.19	0.02	0.62	0.07	0.994	4.3	14.5
Acetaminophen	18.81	0.7	62.7	2.35	0.9977	229	763.4
Acyclovir	13.41	3.5	44.7	11.65	0.9941	755.8	>1000
Amikacin	n.d	-	n.d	-	-	269.3	897.6
Amoxicillin	23.9	2.92	79.66	9.75	0.999	298.4	994.6
Aspartame	9.2	1.64	30.67	5.47	0.9913	655.9	>1000
Atorvastatin	1.43	0.33	4.77	1.1	0.999	13.6	45.2
Azathioprine	0.86	0.02	2.87	0.07	0.999	16.3	54.2
Azithromycin	0.44	0.25	1.46	0.83	0.999	3.6	11.9
Benzylpenicillin	32.98	4.8	109.92	16	0.999	292.4	974.8
Caffeine	5.27	0.35	17.57	1.18	0.999	2.2	7.4
Captopril	356.91	55.47	1189.7	184.91	0.9952	487.3	>1000
Carbamazepine	0.2	0.02	0.68	0.07	0.9901	4.5	15.1
Carvedilol	0.62	0.08	2.06	0.26	0.999	15	49.9
Cefaclor	902.31	199.37	3007.69	664.56	0.991	171.2	570.7
Cefotaxime	0.82	0.32	2.72	1.06	0.999	19.8	66
Ceftriaxone	n.d	-	n.d	-	-	336.8	>1000
Chloramphenicol	98.16	1.21	327.19	4.03	0.999	174.7	582.3
Chlorhexidine	1.85	0.33	6.16	1.1	0.999	796.2	>1000
Chloroquine	0.81	0.14	2.71	0.46	0.999	n.d.	n.q.
Chlorothiazide	73.53	2.02	245.1	6.73	0.999	58.6	195.3
Chlorpheniramine Maleate	0.34	0.02	1.13	0.07	0.9953	4.5	15
Chlorpropamide	14.39	2.29	47.98	7.64	0.9982	54.6	182.1
Cholecalciferol	292.19	18.78	973.97	62.61	0.991	255.4	851.2
Cimetidine	2.29	0.26	7.65	0.88	0.999	4.2	14.1
Clarithromycin	0.25	0.03	0.84	0.09	0.9971	2.5	8.3
Clindamycin	0.55	0.06	1.83	0.21	0.999	19	63.4
Clotrimazole	217.85	26.05	726.16	86.82	0.998	209.3	697.7
Cloxacillin	27.16	4.29	90.52	14.31	0.999	401.5	>1000
Cyclosporin A	196.84	16.01	656.14	53.37	0.9931	38.2	127.5
Dextromethorphan Hydrobromide Monohydrate	0.08	0.01	0.27	0.28	0.9863	3.3	11.1
Diclofenac Sodium Salt	10.74	2.16	35.8	7.21	0.999	15.2	50.7
Didanosine	13.08	2.05	43.59	6.85	0.9963	283.7	945.5
Diphenhydramine Hydrochloride	0.54	0.02	1.81	0.06	0.9963	12.6	41.9
Doxycycline Hyclate	4.43	0.18	14.77	0.62	0.999	619.8	>1000
Efavirenz	5.1	3.08	17.01	10.27	0.999	8.7	28.9
Emtricitabine	26.76	4.3	89.2	14.32	0.999	9.8	32.7

	Instrumental Limits					Method Limits (ng/L)	
Compound	**LOD (pg)**	**STDEV**	**LOQ (pg)**	**STDEV**	**LINEARITY**	**LOD**	**LOQ**
Enalapril Maleate	3.1	0.14	10.33	0.48	0.999	73.8	246.1
Erythromycin	0.57	0.07	1.91	0.25	0.999	4.3	14.4
Ethambutol	0.13	0.05	0.44	0.17	0.9951	468.4	>1000
Ethionamide	4.69	0.45	15.65	1.51	0.999	420.3	>1000
Famotidine	3.95	0.52	13.18	1.74	0.999	4.9	16.2
Flucloxacillin	82	16.59	273.33	55.29	0.9957	193.2	644
Fluconazole	0.69	0.06	2.31	0.19	0.999	16.2	53.9
Fluoxetine	0.28	0.03	0.94	0.1	0.9985	75.6	251.9
Furosemide	463.79	88.58	1545.97	295.26	0.997	133.1	443.5
Gabapentin	72.09	4.65	240.3	15.48	0.9935	297.9	993.1
Gemfibrozil	n.d	-	n.d	-	-	182.2	607.2
Gentamicin	204.13	28.31	680.43	94.38	0.9895	273.6	912
Guaifenesin	0.13	0	0.42	0.02	0.9821	459.4	>1000
Hydrochlorothiazide	393.14	19.26	1310.48	64.19	0.9984	257.1	856.9
Hydrocortisone	9.96	0.36	33.21	1.18	0.968	134.2	447.3
Ibuprofen	n.d	-	n.d	-	-	122.7	409.1
Indinavir	0.58	0.13	1.92	0.45	0.999	13.9	46.2
Indomethacin	13.51	2.19	45.02	7.29	0.999	62.6	208.7
Isoniazid	1.87	0.13	6.23	0.45	0.999	3	9.9
Ketoconazole	0.51	0.05	1.71	0.18	0.999	7.5	25
Ketoprofen	5.48	0.41	18.26	1.36	0.999	200.8	669.5
Labetalol Hydrochloride	0.66	0.03	2.19	0.1	0.9987	17.1	56.9
Lamivudine	9.43	0.36	31.45	1.19	0.9985	74.9	249.7
Lamotrigine	0.31	0.07	1.05	0.22	0.9978	0.4	1.4
Lansoprazole	0.47	0.22	1.57	0.74	0.999	2.7	9
Leflunomide	17.01	2	56.69	6.68	0.9925	n.d	n.q
Lidocaine	0.25	0.02	0.83	0.08	0.9924	3.1	10.2
Loperamide	0.18	0.02	0.61	0.07	0.993	6.2	20.7
Lopinavir	2.9	0.29	9.68	0.97	0.999	6.1	20.4
Loratadine	0.75	0.04	2.5	0.13	0.9953	23	76.7
Lovastatin	0.18	0.01	0.6	0.03	0.98	8.5	28.4
Mesalamine	n.d	-	n.d	-	-	171.7	572.5
Metformin Hydrochloride	0.11	0.01	0.36	0.04	0.9594	5.9	19.6
Methocarbamol	0.13	0	0.43	0.01	0.9793	7.8	25.8
Methotrexate	7.11	1.01	23.71	3.35	0.999	135.4	451.3
Methylparaben	51.23	0.85	170.78	2.85	0.999	465.1	>1000
Metoclopramide Hydrochloride	0.22	0.04	0.72	0.12	0.9966	1.5	4.9
Metoprolol	0.18	0.01	0.58	0.03	0.993	8.6	28.8
Metronidazole	2.61	0.38	8.69	1.25	0.999	10.3	34.2

	Instrumental Limits					Method Limits (ng/L)	
Compound	**LOD (pg)**	**STDEV**	**LOQ (pg)**	**STDEV**	**LINEARITY**	**LOD**	**LOQ**
Naproxen	67.21	7.21	224.02	24.04	0.999	368.6	>1000
Nevirapine	0.52	0.06	1.73	0.2	0.999	3.2	10.6
Ofloxacin	0.41	0.07	1.37	0.24	0.999	6.8	22.7
Omeprazole	0.39	0.23	1.32	0.77	0.999	17.7	58.9
Oseltamivir	0.9	0.08	2.99	0.25	0.999	15.8	44.1
Praziquantel	5.05	0.33	16.83	1.1	0.999	11.1	37.1
Prednisolone	11.02	1.74	36.72	5.8	0.999	395.9	>1000
Prednisone	6.71	0.82	22.35	2.72	0.999	948.2	>1000
Pyrazinamide	77.52	5.92	258.4	19.73	0.999	240.7	802.2
Ranitidine Hydrochloride	1.95	0.14	6.5	0.46	0.999	4	13.3
Rifampicin	38.37	4.11	127.89	13.69	0.998	305.5	>1000
Ritonavir	5.64	1.04	18.81	3.45	0.999	25.1	83.7
Stavudine	16.8	1.44	56	4.81	0.999	164.1	547.1
Sulfamethoxazole	3.86	0.38	12.88	1.28	0.999	1.8	6.1
Taurine	43.53	4.21	145.09	14.02	0.999	250.6	835.5
Tenofovir	3.17	1.57	10.56	5.22	0.9967	367.6	>1000
Tetracycline Hydrochloride	6.66	0.19	22.2	0.64	0.999	273.9	913.1
Theophylline	8.81	0.61	29.38	2.04	0.9986	9.8	32.6
Tobramycin	734.22	148.09	2447.41	493.63	0.9967	274.8	916
Trimethoprim	0.15	0.03	0.48	0.11	0.9902	2.5	8.2
Valsartan	4.98	0.41	16.59	1.36	0.999	40.6	135.2
Zalcitabine	563.71	11.45	1879.03	38.18	0.9987	n.d	n.q
Zidovudine	445.86	8	1486.2	26.66	0.993	17.8	59.2

Table S3-12: The top 10 most frequently occurring compounds tentatively identified by full scan analysis of samples analysed by UHPLC-QTOF, screened against a combination of three commercially available databases.

Compounds Identified by Full Scan Analysis	No. of Occurrences	Compound Description
Pentadecanal	50	long-chain fatty aldehyde
C16 Sphinganine	48	Lipid
Xestoaminol C	47	Amino alcohol
C17 Sphinganine	45	Sphingolipid
(22E)-3alpha,12alpha-Dihydroxy-5beta-chol-22-en-24-oic Acid	44	Lipid
Tris(butoxyethyl)phosphate	44	Flame retardant
(4OH,8Z,t18:1) sphingosine	42	Sphingolipid
2S-hydroxylauric acid	42	Fatty acid
[6]-Shogaol	40	Plant metabolite
Capsiamide	39	Plant metabolite

Table S3-13: The top 10 most frequently occurring compound classes tentatively identified by full scan analysis of samples analysed by UHPLC-QTOF, screened against a combination of three commercially available databases.

Compound Class	No. of Occurrences
Pesticide	185
Stimulant	89
Synthetic	85
Analgesic	72
Antimycotic	58
Biomolecule	57
Antiphlogistic	49
Dermatic	46
Bronchodilator	44
Anticholesteremic	38

Table S3-14: The top 10 most frequently occurring compounds tentatively identified by AutoMS analysis of samples analysed by UHPLC-QTOF, screened against a combination of three commercially available databases.

Compound Name	No. Occurrences	Compound Description
9-Octadecenamide (Oleamide)	47	Fatty acid
Phthalic acid Mono-2-ethylhexyl Ester	34	Plasticizer
Aldimorph	28	Fungicide
Carbamazepine	27	Anticonvulsant / Analgesic
Stearidonic Acid	23	Fatty acid
Tramadol	17	Analgesic
Nevirapine	16	Anti-retroviral
Val Phe	15	Dipeptide
Lamotrigine	14	Anticonvulsant
O-DT / O-Desmethyltramadol	14	Tramadol metabolite

Table S3-15: The top 10 most frequently occurring compound classes tentatively identified by AutoMS analysis of samples analysed by UHPLC-QTOF, screened against a combination of three commercially available databases.

Compound type	No Occurrences
Designer drug; Fatty acid	44
Equine drug; Anticonvulsant	41
Pesticide; Fungicide	28
synthetic	21
virustatic	19
Equine drug; Potent analgesic	17
Designer drug	14
antiepileptic metabolite	12
Designer drug; Equine drug; Hypnotic	11
Veterinary drug; Antibiotic	11

3.6.2 Glossary of Manufacturer-Specific Terms

1. All Ions – Unselective fragmentation of all the ions transmitted to the collision cell at a fixed collision energy.
2. Auto MS/MS – Automatic precursor selection based on intensity followed by MS/MS fragmentation at an automatically adjustable collision energy determined by the mass of the precursor.
3. Experiment - A time section of a spectral acquisition program. User defined timing.
4. Full Scan – Unselective and unfragmented accurate mass scanning of all the ions transmitted through the quadrupole.
5. Find by Auto MS/MS – MassHunter Qual algorithm to filter data based on compounds selected automatically for MS/MS fragmentation.
6. Find by Formula - MassHunter Qual algorithm that isolates targets by comparison to an accurate mass database.
7. Find by Molecular Feature - MassHunter Qual algorithm that identifies a compound based on its ion species' resemblance to an intact pseudomolecular ion and its associated ions (e.g. isotopic pattern, salt adducts, dimers etc.).
8. Molecular Feature - Describes the selection of related ions by the software (e.g. un-adducted and adducted ion species are identified as having the same origin) and exclusion of background ions.

Chapter 4: The Chlorination Behaviour and Environmental Fate of the Antiretroviral Drug Nevirapine in South African Surface Water

This chapter was published in *Water Research*. The format reflects the style set by the journal.

Wood, T.P., Basson, A.E., Duvenage, C., Rohwer, E.R., 2016. The chlorination behaviour and environmental fate of the antiretroviral drug nevirapine in South African surface water. Water Research 104, 349-360.

The Chlorination Behaviour and Environmental Fate of the Antiretroviral Drug Nevirapine in South African Surface Water

T P Wood[a,d]

A E Basson[b]

C Duvenage[c]

E R Rohwer[d]

[a]Protechnik Laboratories, a Division of ARMSCOR SOC Ltd, Pretoria, South Africa.
timw@protechnik.co.za

[b]Centre for HIV and STI: HIV Virology Section (Morris Laboratory), National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS), Johannesburg, South Africa

[c] Department of Internal Medicine, 1 Military Hospital, South African Military Health Services, Pretoria, South Africa

[d] Department of Chemistry, University of Pretoria, Pretoria, South Africa.

Abstract

The wastewater treatment process, besides discharging pharmaceuticals into the environment, has been found to result in the formation of a variety of undescribed compounds. Here we investigate the laboratory scale chlorination of the commonly used anti-HIV drug Nevirapine, characterise its disinfection transformation products (DTPs), and using liquid chromatography with high resolution mass spectrometry, screen environmental surface water for these DTPs. Chlorination of Nevirapine was scaled up, fractioned by preparative chromatography and the fractions were tested *in vitro* for toxicity and anti-HIV activity. Nevirapine was found to be resistant to degradation at relevant chlorination levels, which may partially explain its ubiquitous presence in South African surface water. During simulated chlorination, a variety of DTPs with varying properties were formed, some of which were detected in the environment, close to wastewater treatment plants. Interestingly, some of these compounds, although not as toxic as Nevirapine, retained antiviral activity. Further purification and synthesis is required to fully characterise these novel molecules.

Keywords

Nevirapine, chlorination, wastewater, LC-MS, surface water, LC-QTOF

Highlights

- Nevirapine is resistant to chlorination; may explain its environmental prevalence
- Nevirapine forms a variety of chlorination disinfection transformation products *in vitro*
- These compounds were detected in the environment by UHPLC-QTOF
- The disinfection transformation products were non-toxic *in vitro*, with slight antiviral activity
- Potentially biologically active modified pharmaceuticals are released into the environment

4.1 Introduction

Over the past two decades researchers have shown that pharmaceuticals and personal care products (PPCP) are released into water courses as a result of human use (Ternes et al., 2001; Peng et al., 2014). The effect these compounds, at low concentrations, have on human health or aquatic fauna and flora have yet to be determined fully (Petrie et al., 2014; Roden et al., 2015). Furthermore, the development and promotion of drug resistance in bacterial populations has been postulated (Kümmerer, 2009).

Besides releasing pharmaceuticals into the environment, these compounds have also been found to be modified as a result of wastewater treatment. The resulting disinfection transformation products (DTPs), many of which are undescribed, are then released into the environment, which further complicates the impact of PPCPs on the environment. The mechanism and type of transformation product formation is dependent on the type of disinfection utilised. It has been found that pharmaceuticals may be modified by ozonation (Zimmermann et al., 2011), chloramination (Zhai et al., 2014) and chlorination (He et al., 2013; Bulloch et al., 2015). Chlorination is one of the more popular methods used to disinfect wastewater and has therefore received the most attention in the literature. In South Africa water disinfection is commonly achieved using chlorine gas. The Department of Water Affairs and Forestry (DWAF) requires that discharged wastewater should contain zero faecal coliforms per 100 mL with the caveat that residual chlorine may not be higher than 0.25 mg/mL (Leopold and Freese, 2009).

Chlorination, as a mechanism to treat wastewater and drinking water, has been the method of choice for a long time since it is a cost effective and broad spectrum method of disinfection. In addition to this, chloramination of treated water ensures a longer duration of disinfection as chloramines have a longer half-life than free residual chlorine (Leopold and Freese, 2009).

Unfortunately chlorination has a number of drawbacks, such as the formation of disinfection by-products during the disinfection process. A large number of toxic

compounds may be formed through the interaction between chlorine and dissolved organic matter. Compounds such as the trihalomethanes and the haloacetic acids have been identified in previous decades and are now strictly regulated (Richardson et al., 2007).

Very little is known about the chemical characteristics of pharmaceutical DTPs and their toxicity profiles cannot always be based on those of the parent compound. This was shown in the case of the chlorination of acetaminophen, which resulted in the production of the toxic compounds 1,4-benzoquinone and N-acetyl-p-benzoquinone imine (Bedner and MacCrehan, 2006).

Besides adding complexity to the potential toxicity profile, the biological activities of many of the degradation products are not known. The degradation products of antivirals or antibiotics may retain antimicrobial properties or even gain additional activities. Inroads into understanding the transformation of antibiotics are being made by various researchers and it has been found that while most antibiotics lose their activity during water disinfection, a few do form biologically active transformation products (Dodd et al., 2009; Escher and Fenner, 2011; Mestankova et al., 2012; Keen and Linden, 2013). In addition to understanding the chemistry behind their transformation, various technologies are in development to effectively remove pharmaceuticals and their disinfection transformation products from wastewater (Prasse et al., 2015). These technologies are however in their infancy and have yet to be adopted widely in “first world countries”, let alone in developing countries such as South Africa.

Recent research on the prevalence of HIV-1 antiretroviral compounds (ARVs) in South African surface water has shown that Nevirapine occurs ubiquitously in the environment (Wood et al., 2015). The drug is a non-nucleoside reverse transcriptase inhibitor (NNRTI) that is commonly used to prevent mother to child transmission of HIV (Mofenson, 2010; Coovadia et al., 2012). Prasse and colleagues have shown that the compound also occurs in European surface water and its presence is attributable to inefficient removal during wastewater treatment (Prasse et al., 2010).

Vankova and co-workers showed that Nevirapine has low biodegradability in a closed bottle system (Vanková *et al.*, 2010). Although this theoretical finding addresses the compound's ubiquitous presence in South African surface water (Wood et al., 2015), it does not describe how the compound reacts during wastewater treatment, if at all.

The antiretroviral class of compound has not been studied extensively in surface water across the world. This is most likely due to the regional prevalence of HIV. In addition to this, no research, to our knowledge, concerning the transformation behaviour of these drugs during the disinfection process has been described.

South Africa utilises more ARVs per capita than any other country in the world (WHO, 2013) which indicates that high amounts of these compounds would enter wastewater treatment works (WWTWs) that were not designed to remove pharmaceuticals. In addition to ineffective WWTWs, improper sanitation and illegal sewage release should also be considered. These factors, as well as the reduced expected dilution, in a water scarce region such as South Africa, led to the hypothesis that ARVs and their degradation products should be prevalent in the environment.

Here the reactivity of the antiretroviral drug Nevirapine to chlorine, in the form of sodium hypochlorite, is qualitatively studied. The degradation products that are formed as a result of chlorination are described and related to environmental water samples collected in South Africa. It is then shown that although these disinfection transformation products of Nevirapine are not toxic, they may have the same or similar biological activity as the parent molecule. The environmental impact of releasing active, undescribed molecules from WWTWs has yet to be determined.

4.2 Materials and Methods

4.2.1 Chemical reagents

Nevirapine was purchased from the United States Pharmacopeia, through Industrial Analytical (Johannesburg, South Africa) and stock solutions (1 mg/mL) were made up in methanol and stored at -20°C until use. LC-MS grade water, methanol and dimethyl sulfoxide (DMSO) were purchased from Lab-Scan (Gliwice, Poland). Sodium hypochlorite from Merck (Johannesburg, South Africa), 10-14%, was diluted in water to 0.4 M and the concentration was found to be stable over time by iodometric titration. Monobasic and dibasic potassium phosphate (Merck) were used for buffering Nevirapine and NaOCl solutions to a final concentration of 10 mM. Ammonium Chloride, sodium thiosulphate and ascorbic acid were purchased from Radchem (Johannesburg, South Africa), formic acid from Sigma-Aldrich (Johannesburg, South Africa) and 20 mL borosilicate amber vials with PTFE caps from Macherey-Nagel (Düren, Germany). Pharmaceutical Nevirapine was obtained from Aspen (Johannesburg, South Africa) and utilized for large scale experimentation to reduce costs. Water and acetonitrile, each with 0.1% formic acid were obtained from Burdick & Jackson (Muskegon, USA). All buffers and reagents were formulated using LC-MS grade water (non-South African origin).

4.2.2 Chlorination reactions

Nevirapine (20 µg/mL) diluted in either LC-MS grade water, 10 mM phosphate buffer pH 5.8 or 10 mM phosphate buffer pH 8 was combined in equal volumes with NaOCl diluted in either of the aforementioned solvents (to yield 50, 100, 200 or 500 µM NaOCl) and stirred at room temperature (20 °C +-1 °C). Aliquots were taken from the reaction at 1, 5, 10, 20, 30, 60 and 120 min and then again at 24 hours. Aliquots were analysed by HPLC-UV, UHPLC-QqQ and UHPLC-QTOF.

To identify an effective quenching agent, sample fractions (1 mL) for each time course were added to either sodium thiosulphate, ascorbic acid or ammonium chloride to yield a twofold molar excess (compared to NaOCl concentration),

analysed by LC-MS plug injection and compared to unquenched data. In order to generate the most accurate data for a particular time point, unquenched reactions were incubated in the LC autosampler. Plug injections were performed using an Agilent 1290 series UHPLC coupled to an Agilent 6460 triple quadrupole (Agilent). Mobile phases consisted of water (A) and acetonitrile (B) both with 0.1% formic acid, held at 50% B at a flow rate of 0.4 mL/min. Sequential 15 µL plug injections (no column) of a sample incubated on the LC-MS autosampler, held at (20°C +-1°C), were analysed by mass spectrometry in MS2 scan mode by positive electrospray ionisation. Source conditions: gas temperature, 250ºC; gas flow, 8 L/min; nebulizer, 35 psi; sheath gas temperature, 300 ºC; sheath gas flow, 10 L/min; capillary voltage, 3000 V and nozzle voltage, 0 V.

For kinetics studies Nevirapine (4 µM) diluted in either LC-MS grade water, 10 mM phosphate buffer pH 5.8, 10 mM phosphate buffer pH 8 or WWTW effluent from the Zeekoegat plant was combined in equal volumes with NaOCl diluted in either of the aforementioned solvents (to yield 2 µM Nevirapine and 20 µM NaOCl) and stirred at room temperature (20 °C +-1 °C). Aliquots were taken from the reaction at 10 second intervals and combined with NH_4Cl to give a two-fold molar excess with respect to NaOCl. Samples were analysed by UHPLC-QTOF immediately after the last time course was sampled. An external 6 point calibration of Nevirapine in the matching reaction solvent was utilised for quantitative purposes and analysed in MassHunter Quant (Agilent).

4.2.3 LC-UV analysis of Chlorination Reactions

The LC-UV system consisted of an Agilent 1100 series binary pump LC coupled to an Agilent diode array detector (254 nm with 400 nm reference). A 1.8 µm Zorbax Eclipse Plus C18, 2.1x50mm, column was used to separate a 15 µL injection of the reaction products at a flow rate of 0.2 mL/min at room temperature. Mobile phase A consisted of water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The gradient was as follows: 0 min, 5% B; 20 min, 95%B; 22 min 95% B; 30 min, 5% B; 40 min, 5%B.

4.2.4 Large Scale Preparation of Nevirapine Chlorination Reaction Products

Pharmaceutical Nevirapine (Aspen) tablets (1.8 g) were crushed and resuspended in 25 % HCl. The solution was clarified by centrifugation (1811 x g for 30 min) and the supernatant containing approximately 1 g of Nevirapine was collected. The yield of this acid extraction was determined by UHPLC-QTOF analysis as compared to an external calibration curve.

Acid extracted Nevirapine was diluted in either 100 mM phosphate buffer (pH 8) or 10 mM Phosphate buffer (pH 5.8) to 1.5 mg/mL, 0.4 M NaOCl was added daily for a period of four days and the reactions were monitored by UHPLC-QTOF analysis. Upon reaching the maximum diversity of reaction products, the mixtures were dried by rotary evaporation (Buchi, Switzerland) at 40ºC under vacuum. The total basic and acidic reactions were diluted in dimethyl sulphoxide to 1 mg/mL for toxicity studies.

The dried and crushed acidic reaction mixture was dissolved in water and fractioned by centrifugation into aqueous and non-aqueous fractions. These were dried, as before, and diluted in dimethyl sulphoxide to 1 mg/mL for toxicity studies.

Both the acidic and basic reactions were separated using preparative chromatography. Each reaction in water (100 mg of 100 mg/mL) was loaded onto a Biotage SNAP Ultra C18 (12 g) column and separated on a gradient of acetonitrile and water (both with 0.1 % formic acid) over 30 min at a flow rate of 10 mL/min. Fractions were collected and dried by vacuum centrifugation (Martin Christ RVC 2-33IR) at 2 mBar, 40 ºC, 900 rpm for 12 hours. Dried fractions were resuspended in DMSO (1 mg/mL) and subjected to toxicity and activity screening.

4.2.5 Environmental Sample Collection and Extraction

Grab samples were collected from all the major rivers and lakes (man-made) in South Africa as part of a multi-year water quality study. Samples were collected in “virgin” borosilicate Schott bottles and transported to the laboratory at room temperature. Sampling locations were chosen based on proximities to wastewater

treatment works (WWTW) and human settlements. To provide a comprehensive picture of a specific body of water, multiple samples were taken from different locations in a sampling site. Samples were stored at -20°C until extraction after which extracts were stored at -20°C until analysis.

Each environmental sample (500 mL) was filtered using a 1 µm glass-fibre syringe driven filter (Pall) and extracted using the Smart Prep Extraction (Horizon, Salem, USA); an automated offline solid phase extraction instrument. Briefly 6 cc Oasis HLB, 500 mg (Waters) cartridges were conditioned with 4 mL methanol followed by 6 mL of HPLC Grade water. The sample (500 mL) was then introduced at a flow rate of 10 mL/min after which cartridges were dried under nitrogen for three minutes. Cartridges were then eluted twice with 5 mL of methanol and dried under a gentle stream of nitrogen to 500 µL. All extractions were performed at 18 °C (+-2 °C).

4.2.6 UHPLC QTOF Analysis

Environmental concentrations of Nevirapine were reported previously and national samples were analysed by UHPLC-QTOF in full scan, “Auto MS/MS” and “all ions” mode. Similarly, chlorination reactions of Nevirapine at varying concentrations of NaOCl and pH (after 1 min) were analysed in order to characterise the resulting reaction products. In addition to these, the large scale chlorination reactions were monitored by UHPLC-QTOF.

Each sample (1 µL) was separated on an Agilent 1290 UHPLC using an HPH 2.1 x 100 mm Poroshell column (Agilent) with water and acetonitrile (both with 0.1% formic acid) as mobile phases at a flow rate of 0.5 mL/min. The gradient was as follows: 3 min, 2% B (organic); 22 min 100%; 25 min, 100%; 27 min, 2% and 30 min, 2%.

Eluting compounds were analysed by positive electrospray QTOF fitted with an iFunnel source. Source conditions: gas temperature, 200ºC; gas flow, 15 L/min;

nebulizer, 40 psi; sheath gas temperature, 400 ºC; sheath gas flow, 12 L/min; capillary voltage, 3500 V, nozzle voltage, 500 V and fragmentor, 365 eV.

Data obtained from full scan and auto-MS analysis of Nevirapine reactions were manually inspected using MassHunter Qual (Agilent) and exported to Mass Profiler (Agilent) for analysis. Features present in the control reactions (Nevirapine without NaOCl and NaOCl without Nevirapine) and blank injections were subtracted from the reaction data sets to determine unique reaction products. These features were inspected manually and exported to MassHunter PCDL Manager (Agilent) to create a database containing information on each reaction product's accurate mass, retention time and MS/MS behaviour. This database was then applied to environmental samples to determine the presence of reaction products in the environment.

Molecular Structure Correlator (Agilent) was used to calculate precursor and fragment formulas and correlate actual MS/MS spectra with theoretical fragments of proposed structures. The structures were proposed (manually) for each of the major reaction products.

4.2.7 In Vitro Toxicity and Antiviral Activity

The CellTiter 96 AQueous One Solution Cell Proliferation Assay System (Promega, Madison, USA) was used to determine the toxicity (Barltrop et al., 1991) of Nevirapine chlorination reaction product fractions in 293T cells (Graham et al., 1977). This was compared to the toxicity of pharmaceutical grade as well as analytical grade Nevirapine. Sample toxicity, as reflected by cell viability, was measured by the bioreduction of a MTS tetrazolium compound (MTS) to a coloured formazan product in the culture medium. The formazan product was spectrophotometrically quantified with a VesraMax microplate reader (Molecular Devices, Sunnyvale, USA). The degree of sample toxicity was related to the MTS-to-formazan conversion by the cells.

Sample titration into complete Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Johannesburg, South Africa) supplemented with 10 % foetal bovine serum (Hyclone, GE, Little Chalfont, UK), 1 M HEPES (Life Technologies, Johannesburg, South Africa) and 10 mg/mL Gentamicin (Sigma-Aldrich, Johannesburg, South Africa) provided a range of concentrations for toxicity testing. Unexposed cells were used as a negative control. Incubation commenced for 2 days at 37^{o}C under 5% CO_2 in a humidified atmosphere. After the addition of the MTS reagent, plates were incubated for 1.5 hours after which spectrophometric absorbances were read at 490 nm, with 690 nm as reference wavelength. A viability of 0% indicates total cell death while a viability of 100% indicates full cell viability. Cytotoxic concentration-50 (CC_{50}) values were calculated and indicate the sample concentration at which 50% of the cells are viable.

Anti-HIV activity was determined over non-toxic sample concentrations. Dilutions of each of the reaction product fractions were titrated in 96-well culture plates as described above. After the addition of cells and virus, the plates were incubated for 48 hours at 37^{o}C under 5% CO_2 in a humidified atmosphere. A standard HIV-1 subtype C isolate, MJ4, was used to screen for antiviral activity of the samples. A medium control (cells, virus and growth medium) was included. After incubation the Bright Glo™ Reagent (Promega) was used to assay for the expression of firefly luciferase in the plate wells (De Wet et al., 1987). Bioluminescence was quantified on a Victor-3 1420 Multi Label Counter (Perkin Elmer). The medium control was used as the uninhibited control. The percent viral activity was calculated as the factor of the bioluminescence of the test sample and that of the virus control.

A viral activity of 0% indicates complete viral inhibition while a viral activity of 100% indicates no inhibition (full viral activity). Inhibitory concentration-50 (IC_{50}) values were calculated and indicated the concentration of sample at which 50% of the viruses were inhibited. During the activity screening, a decrease in luciferase activity can be observed over toxic concentrations as a result of compromised cell viability, and not as a result of inhibition of the virus. An untreated cell control (media only) is used as a reference for 100% cell viability (no toxicity).

4.3 Results and Discussion

4.3.1 Chlorination Reactions

Various chromatographic systems were utilised throughout this research and although they differ by instrument type (UV detection, triple quadrupole and qTOF) a high level of correlation was found between the data. Three concentrations of Nevirapine were used in this research in order to represent environmental concentrations (low, 2 µM) as well as having concentrations high enough to detect (medium, 37 µM) transformation products and purify them (high, 5620 µM).

The efficacy of ammonium chloride, sodium thiosulfate and citric acid as quenching agents was evaluated. Researchers (Bedner and MacCrehan, 2006; Soufan et al., 2012) have warned of the potential of sodium thiosulphate to reverse chlorination reactions, and this was found to be the case for Nevirapine. This was found by comparing quenched and unquenched LC-MS plug injection spectra in which the ion intensity of the Nevirapine pseudomolecular ion was compared. This highlights the fact that Nevirapine oxidation by NaOCl may be reversed (with the addition of a reducing agent such as sodium thiosulphate). Similarly, ascorbic acid was found to reverse Nevirapine chlorination reactions. Fortunately NH_4Cl was found to halt the reaction while not inducing additional reactions, as shown by the un-quenched control. Autosampler reactions (unquenched) and quenched stirred reactions were found to be comparable when analysed by plug injection, which indicates that the reaction is not adversely influenced by an increase in volume. Thus, large scale (10 mL) stirred reactions were utilised throughout in order to analyse identical samples multiple times across a number of analytical platforms.

UV analysis of chlorination reactions (37 µM Nevirapine to 50, 100, 200 and 500 µM NaOCl) showed that the compound reacts almost immediately (the earliest time course was one minute) and then remains relatively constant over the time course up to 24 hours. In Figure 4-1 the 1 minute reaction of Nevirapine with NaOCl is overlaid with the 24 hour reaction (at pH 8). The peak area of Nevirapine remains constant, whereas the reaction products show variability over time. The dynamic

range of Nevirapine detection was verified by injection of a calibration curve and was found to be linear across the concentration range tested.

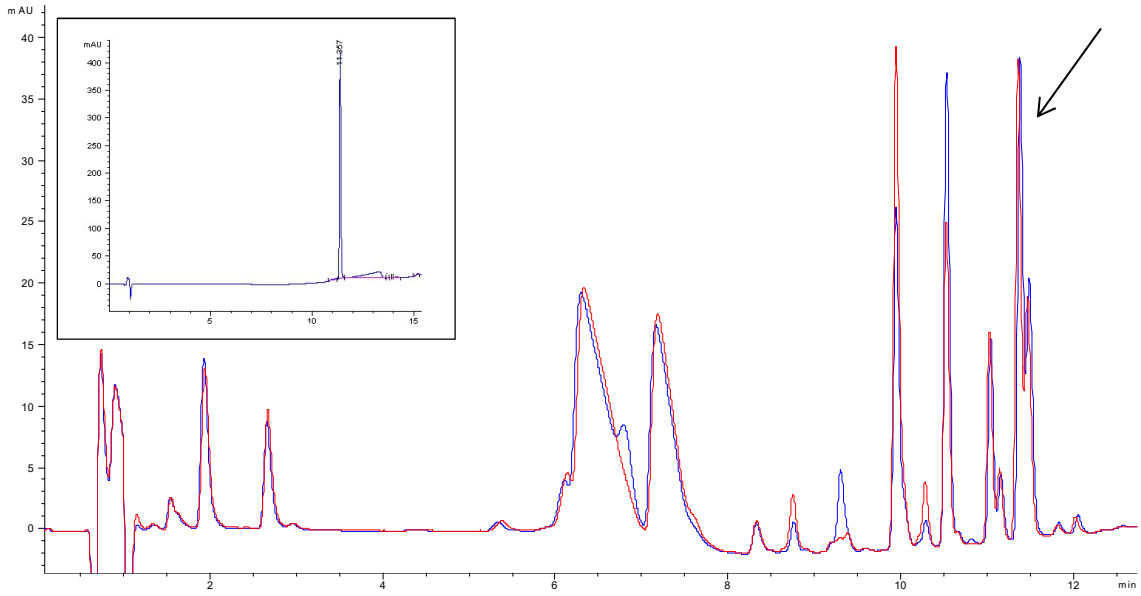


Figure 4-1: Overlaid LC-UV trace (254 nm) of 10 µg/mL Nevirapine (37 µM), indicated by an arrow, reacted with 100 µM NaOCl (pH 8) after one minute (blue trace) and 24 hours (red trace). The figure inset shows the LC-UV trace of unreacted Nevirapine (10 µg/mL in phosphate buffer).

The reactivity of Nevirapine in the LC-UV data was validated by monitoring the intensity of the extracted *m/z* 267 ion (Nevirapine pseudomolecular ion) in LC-MS experiments and data were found to be comparable to LC-UV results. The extent of reaction was found to be dependent on NaOCl concentration as well as pH. The 37 µM Nevirapine was exhausted in as little as one minute when treated with 500 µM NaOCl in the buffered as well as unbuffered reactions. In the basic reaction (pH 8) with 200 µM NaOCl the Nevirapine is undetectable at 1 minute whereas it remains at a low level in the pH 5.8 and unbuffered reactions (Figure S4-5).

Although the reaction was found to be stable over time, it was found that pH greatly affected the reaction's speed well as the nature of the reaction products. LC-UV analysis (Figure S4-6) showed that a variety of distinct products were formed under basic and acidic conditions. In the mid-range concentration reactions (37 µM)

Nevirapine was found to be resistant to degradation at the levels of chlorine typically used in WWTWs with up to 50% of the Nevirapine remaining intact after 24 hours when treated with a 5 fold molar excess of NaOCl. The concentration of Nevirapine in the samples were however much higher than what could be expected to be found in the environment. The reactions were found to occur fastest at pH 8, although all reactions occurred rapidly and stabilised after one minute. It is also important to note that these experiments were carried out in a buffer with no other organic components.

As is the case with the chlorination of many pharmaceuticals, the kinetics of the Nevirapine reaction are first order with respect to each reactant, with second order overall (Deborde and von Gunten, 2008). Low Nevirapine (2 µM) concentrations treated with 10 fold free available chlorine (FAC) were utilised to simulate realistic environmental conditions and determine reaction kinetics. The reactions started within 10 seconds (the earliest measurement) and proceeded to completion with the assumption that free available chlorine (FAC) was in excess. The reaction of Nevirapine (NVP) with NaOCl as FAC can be described by the following equations,

$$\frac{\mathrm{d[NVP]}}{\mathrm{d}t} = -\kappa[\mathrm{FAC}][\mathrm{NVP}] \qquad (1)$$

$$\frac{\mathrm{d[NVP]}}{\mathrm{d}t} = -\kappa_{obs}.[\mathrm{NVP}] \qquad (2)$$

$$\ln\left(\frac{[\mathrm{NVP}]_t}{[\mathrm{NVP}]_0}\right) = -\kappa_{obs}.t \qquad (3)$$

where [NVP] is the total concentration of Nevirapine and [FAC] is the concentration of free available chlorine (in excess). κ is the second-order rate constant and the observed pseudo first-order rate constant is κ_{obs}, which was calculated based the slope of the linear component of the graph of equation (3); with $\kappa_{obs} = \kappa$ [FAC] and [FAC] = $[\mathrm{FAC}]_0$. The rates at various pH conditions at NaOCl concentrations similar to WWTW are shown in Table 4-1 as lower limit second order rate constants, as the reactions occurred too rapidly to measure in a batch format.

Table 4-1: Pseudo first order κ_{obs} and lower limit second-order Rate Constants, κ (M-1.s-1), for nevirapine (2 µM) reacted with 20 µM free available chlorine from NaOCl with phosphate buffer (pH 5.8 or 8) and without buffer.

Sample	Pseudo first order κ_{obs}	Second-order Rate Constants, κ ($M^{-1}.s^{-1}$), 20 µM FAC
pH 5.8*	0.0008	4.0×10^{1}
pH 8	0.0204	1.02×10^{3}
Unbuffered	0.1175	5.88×10^{3}

* The Nevirapine is not consumed entirely in this reaction.

The reaction occurred most rapidly in the unbuffered reactions, followed by reactions at basic pH, in acidic buffer and in WWTW effluent respectively. In the basic reaction the Nevirapine was completely consumed after 90 s, whereas in the acidic reaction the Nevirapine consumption ceases at 110 s, with up to 80 % Nevirapine remaining (Figure S4-7). The unbuffered reaction proceeds most rapidly, with total Nevirapine consumption. This is most likely due to the fact that the continued reactivity of the transformation products is unencumbered by the buffer and its resulting effect on compound speciation.

The wastewater matrix composition can greatly affect the efficacy of pharmaceutical transformation due to the chlorine demand exerted by dissolved organic molecules, nitrites and ammonia (Lee and von Gunten, 2010). During wastewater treatment, Nevirapine degradation would most likely be even less effective due to the increased chlorine demand caused by these molecules in the wastewater. Where wastewater was used as a reaction matrix for kinetics studies, it was found that 95 % of the Nevirapine remained intact when treated with a 10 fold molar excess of chlorine. A second order rate constant could not be determined as the chlorine did not remain in excess due to the demand placed on it by dissolved organic matter.

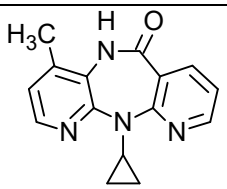
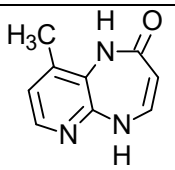
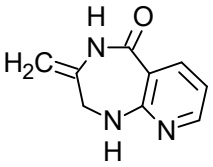
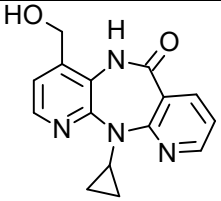
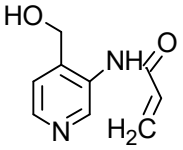
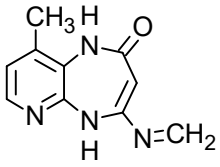
pH was found to significantly affect the extent of Nevirapine degradation in all three reaction formats tested in this work, with greater reactivity seen at a high pH than in acidic reactions. The OCl^- species dominates at a basic pH and is a weaker oxidizer than HOCl. The increased reactivity of Nevirapine when OCl^- is predominant may therefore be as a result of the speciation (i.e. anionic, cationic or neutral) of Nevirapine.

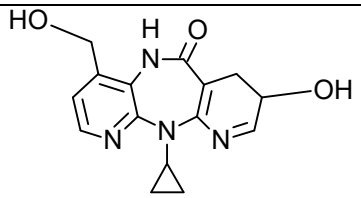
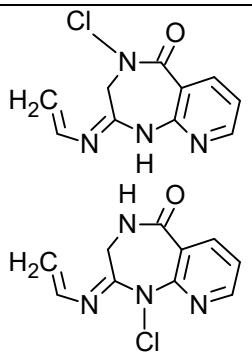
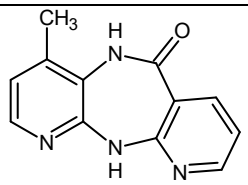
During wastewater treatment, chlorine is utilised as a disinfectant; and optimal disinfection is achieved when the HOCl species dominates (at low pH). As we have demonstrated these are the exact opposite conditions required for effective Nevirapine removal. The concept of reactivity related to pH is further confounded, as it was found that some compounds are effectively removed at low pH (e.g. sulfamethoxazole) while others (e.g. ciprofloxacin) are better removed at a high pH where the reaction is dependent on reagent speciation (Li and Zhang, 2012). These data provide credence to earlier research in that Nevirapine is one of the most ubiquitously occurring ARVs in the environment (Wood et al., 2015).

4.3.2 LC-qTOF Analysis

Accurate masses generated from analyses of various chlorination reactions were used to generate formulae for each mass. Formulae were then used to propose theoretical structures for the most prominent reaction products using the Nevirapine skeleton as a starting point (Table 4-2).

Table 4-2: Accurate mass measurements and proposed structures for the most prominent chlorination reaction products of Nevirapine. Theoretical structures were compared to MS/MS data using Agilent Molecular structure Correlator.

Accurate Mass Measurement of the Pseudomolecular Ion and Proposed Formula	Proposed Structures
Nevirapine (267.1254) $C_{15}H_{14}N_4O$	
176.0808 $C_9H_{10}N_3O$	 
283.1185 $C_{15}H_{14}N_4O_2$	
179.0807 $C_9H_{10}N_2O_2$	
203.0921 $C_{10}H_{10}N_4O$	

Accurate Mass Measurement of the Pseudomolecular Ion and Proposed Formula	**Proposed Structures**
301.1284 $C_{15}H_{16}N_4O_3$	
237.0538 $C_{10}H_9ClN_4O$	
226.0834 $C_{12}H_{10}N_4O$	

MS/MS spectra for each of these structures were then compared to theoretical mass spectra generated using Molecular Structure Correlator (MSC). When multiple proposed structures existed for a compound, MSC scores were utilised to discriminate between candidates. Only structures with scores greater than 98% (intact) and 90 % (for fragments) were accepted for further consideration.

Data from the large scale as well as the 1 minute chlorination reactions were analysed to identify abundant and unique chemical entities (not present in the system or controls). The compounds' masses, retention times, MS/MS spectra and proposed formulae were collated into a searchable database containing 42 putative compounds (Table S4-6), using PCDL Manager (Agilent). It was found that several

molecules yielding highly similar accurate mass measurements existed that were only distinguishable by their retention times. These are most likely stereoisomers as it was found that a variety of potential nevirapine reaction products share the same mass.

Although the chlorination of Nevirapine at chlorine levels similar to those used in WWTWs yielded a wide variety of major and minor reaction products, certain well described functional groups within the molecule may be used to predict transformation. Nevirapine contains a tertiary amine, a moiety which reacts with chlorine as described in various studies (Prütz, 1998; Mitch and Schreiber, 2008; Shah et al., 2011; Selbes et al., 2012). Using these models the loss of cyclopropane as seen in a number of the DTPs may be explained (Figure 4-2). Unfortunately though, this may not be applied to all such compounds, as highlighted by Deborde and von Gunten (2008) in which Ciprofloxacin and Enrofloxacin (both contain a similar cyclopropane moiety) react preferentially with chlorine at another amine.

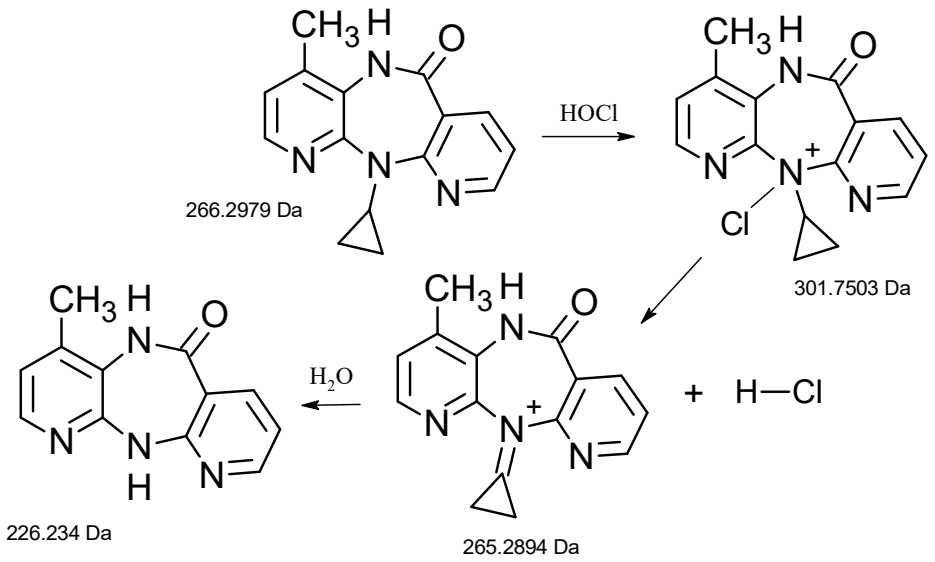


Figure 4-2: Proposed scheme for the reaction of the Nevirapine tertiary amine during chlorination.

The molecule *m/z* 226 was detected a number of times in the analyses (of the chlorination reactions) but since it is one of the major MS/MS and CID

fragmentation products of Nevirapine and its transformation products, its presence as a standalone molecule could not be confirmed with confidence in environmental samples, i.e. it may be a mass spectrometric fragmentation product and not an intact molecule.

4.3.3 Detection of Nevirapine and its chlorination products in the environment

The SPE technique utilised here was chosen for its universality. Unfortunately limits of detection and quantitation for the method could not be performed as reaction mixtures consisting of multiple components were used as standards. However, this work is largely qualitative with a lower limit of detection defined as an amount providing a signal to noise ratio of 3.

Samples from every major river and water body in South Africa (Table S4-7) were screened against a database of the most ubiquitous Nevirapine chlorination reaction products. From these data (Table S4-8), positive identification in the environment was only accepted if the mass of the pseudomolecular ion, the retention time and MS/MS spectra matched to the compound present in the *in vitro* chlorination reactions. As anticipated, the compounds were only detected in samples taken close to WWTWs in dense human settlements.

Full scan mass spectra provided a wealth of information regarding a particular sample and intense ions were automatically fragmented, in a separate injection, to provide MS/MS data. Fragmentation energy was set automatically based on the precursor's mass. Many of the prominent chlorination products were detected in the environment. As an example, at the Zeekoegat sampling site, the compound with the *m/z* 203.0938 was detected (Figure 4-3).

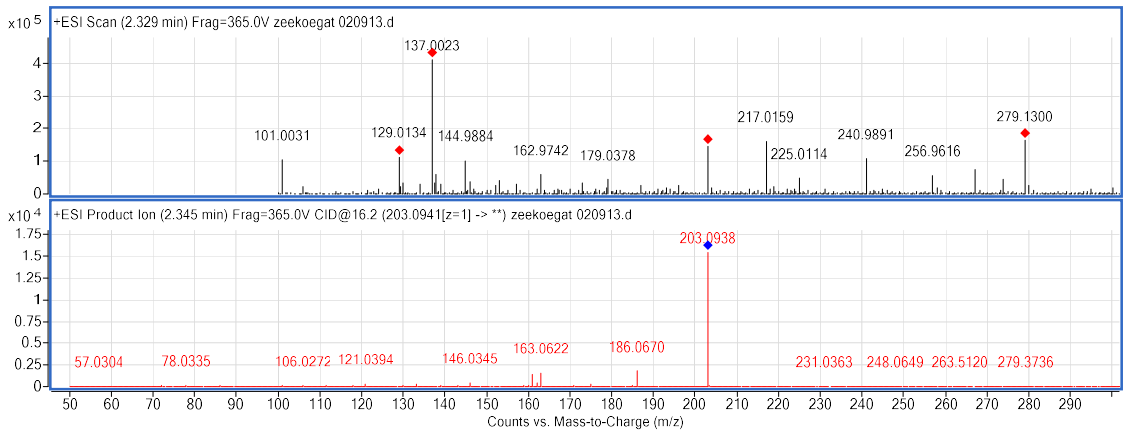
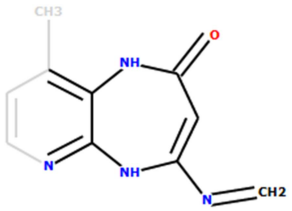
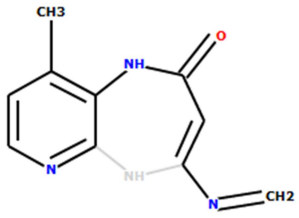
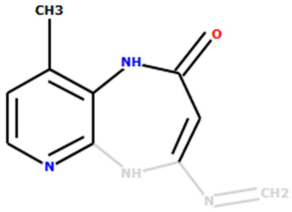


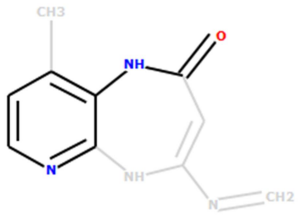
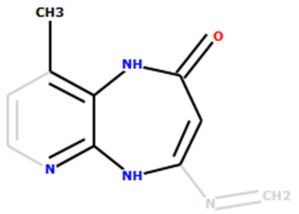
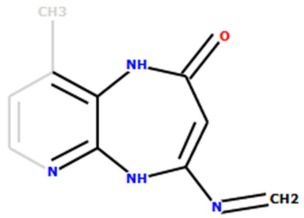
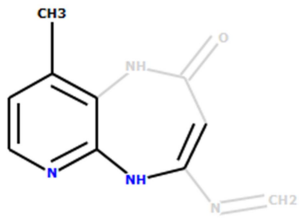
Figure 4-3: Full scan ESI spectrum (top) and ESI auto-MS/MS spectrum of *m/z* 203.0941+- 0.01 (bottom) of an SPE extract of water taken from the Zeekoegat WWTW discharge, analysed by UHPLC-QTOF (retention time: 2.3 minutes). Red squares (top) indicate ions automatically chosen for MS/MS and the blue square (bottom) indicates the precursor ion, fragmented at 16.2 eV.

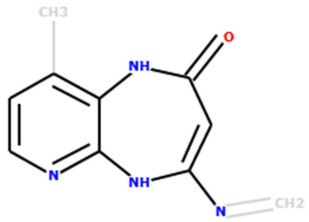
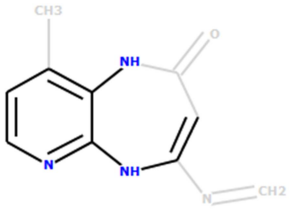
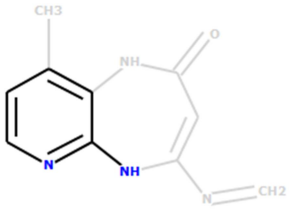
The QTOF operates in “Auto MS/MS” mode in which intense ions are selected during full scan mode for MS/MS fragmentation. The instrument selects the collision energy to use on a case by case basis depending on the particular ion’s mass. Using this approach, accurate mass measurements of the intact species as well as the resulting fragments (and their ratios) may be utilised to compare samples to analytical standards; or even generate structures by interpretation.

The MS/MS spectrum for this compound was predicted by the Molecular Structure Correlator (Agilent) software (Table 4-3).

Table 4-3: Measured mass spectrum compared to theoretical fragmentation generated for the MS/MS of *m/z* 203.0941 by Molecular Structure Correlator (Agilent) with the difference between measured and proposed masses and structures (grey text indicates fragmentation); for the 10 most intense ions.

Measured *m/z*	Nominal Intensity	Proposed Formula	Mass difference (ppm)	Proposed Structure
163.0615	100	$C_7H_7N_4O$	-0.4	
186.0664	83.11	$C_{10}H_8N_3O$	-1.1	
161.071	83.09	$C_9H_9N_2O$	-0.4	

Measured *m/z*	**Nominal Intensity**	**Proposed Formula**	**Mass difference (ppm)**	**Proposed Structure**
121.0393	28.88	$C_6H_5N_2O$	2.8	
146.0353	22.62	$C_7H_4N_3O$	-2.8	
162.0536	16.33	$C_7H_6N_4O$	0.1	
133.0756	11.41	$C_8H_9N_2$	3.2	

Measured *m/z*	**Nominal Intensity**	**Proposed Formula**	**Mass difference (ppm)**	**Proposed Structure**
175.0609	7.87	$C_8H_7N_4O$	3.1	
130.0397	7.53	$C_7H_4N_3$	2.1	
93.0441	5.26	$C_5H_5N_2$	6.7	

Although the software assumes that the pseudomolecular ion is fragmented fully, when proposing the ions' nominal intensities, the ratio between the fragment ions agrees with the measured standard (Figure 4-4). Similarly, for this compound, and many like it, the ion ratios measured in the environment, match the "standards" generated in the laboratory.

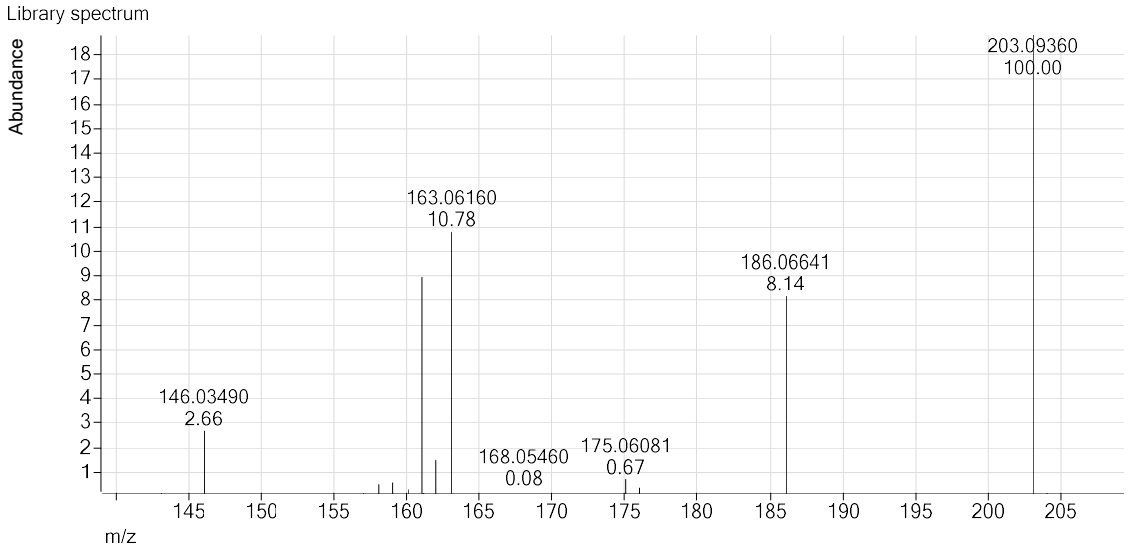


Figure 4-4: ESI auto-MS/MS spectrum of *m/z* 203.0941+-0.01, from a 1 minute chlorination reaction of Nevirapine, pH 8, 200 µM NaOCl; analysed by UHPLC-QTOF. The "standard" spectrum was exported from PCDL Manager (Agilent).

It was found, and it is self-evident, that the DTPs are more prevalent in highly populated areas that are near WWTWs. The prevalence of these compounds could also be related to Nevirapine concentration, in that samples that contained lower levels of Nevirapine were found to not contain detectable amounts of the DTPs. The majority of the DTPs were detected in the Roodeplaat system (Table 4-4), which is influenced by two of the three WWTWs that were sampled in this research.

Table 4-4: An excerpt of Nevirapine chlorination products detected by positive ESI UHPLC-QTOF analysis of SPE extracts of surface water samples from the Roodeplaat Dam.

Sampling Location (GPS Coordinates)	*m/z*	RT	RT Diff (Tgt)*	Diff (DB, mDa)**	Mass	Proposed Formula***
Roodeplaat Dam Outflow (-25.608244, 28.367231)	160.0854	3.222	0.006	0.45	159.0783	$C_9 H_9 N_3$
Roodeplaat Dam (-25.623345, 28.349842)	160.087	3.18	-0.036	-0.84	159.0795	$C_9 H_9 N_3$
Roodeplaat Dam (-25.626404, 28.345692)	160.0855	3.238	0.022	0.9	159.0778	$C_9 H_9 N_3$
Roodeplaat Dam Outflow (-25.608244, 28.367231)	160.0858	3.225	0.009	1.4	159.0773	$C_9 H_9 N_3$
Pienaars River (-25.678677, 28.357116)	188.0825	4.662	-0.061	-0.49	187.0756	$C_{10} H_9 N_3 O$
Roodeplaat Dam (-25.618238, 28.358642)	188.0822	4.673	-0.05	-1.69	187.0768	$C_{10} H_9 N_3 O$
Roodeplaat Dam (-25.618238, 28.358642)	188.0823	4.656	-0.067	-1.65	187.0767	$C_{10} H_9 N_3 O$
Pienaars River (-25.678677, 28.357116)	317.0813	7.234	-0.017	-1.13	316.0742	$C_{15} H_{13} Cl N_4 O_2$
Roodeplaat Dam (-25.618238, 28.358642)	203.0922	2.288	-0.007	1.15	202.084	$C_{10} H_{10} N_4 O$
Roodeplaat Dam (-25.626404, 28.345692)	351.1577	5.312	-0.019	1.62	175.0737	$C_9 H_9 N_3 O$
Zeekoegat WWTW Outflow (-25.624620, 28.341890)	319.1144	9.616	-0.026	2.42	296.1254	$C_{16} H_{16} N_4 O_2$
Roodeplaat Dam (-25.626404, 28.345692)	319.1142	9.632	-0.009	2.63	296.1252	$C_{16} H_{16} N_4 O_2$

* RT Diff (Tgt) - The difference between the measured retention time and that of the standard (minutes)

** Diff (DB, mDa) The difference between the measured mass and that of the true mass of the "standard"

*** Proposed formula, generated by MassHunter Qual (Agilent)

The environmental prevalence (Table S4-8), is therefore relatively low, compared to the ubiquitous distribution of the parent molecule (30 locations across South Africa were sampled and it was found that Nevirapine is detectable at the majority of the sites).

The concept of the minimal criteria required for identification of a compound by mass spectrometry is a widely discussed topic, as inter-laboratory LC-MS comparison is not always fully possible. This is because variability in instrumentation type and conditions would lead to variability of data generated for identical compounds (Rivier, 2003). Therefore in this work, even though DTP "standards" were not isolated and characterised in pure form and only analysed as a mixture, a positive identification of a DTP in the environment was only accepted if: retention time matched within 0.1 min, MS/MS spectra matched, the accurate mass difference was no greater than 1 ppm and that the overall MassHunter match factor was greater than 80 % (a value that encompasses all of the aforementioned factors).

It is exceptionally important to bear in mind that the lower molecular weight species described during the chlorination reactions and subsequently found in surface water, may not necessarily originate from the degradation of Nevirapine. This is because the probability of a shared feature between Nevirapine and another molecule increases as the fragment size decreases.

4.3.4 Large Scale Preparation and Separation of Chlorination Reaction Products

The small-scale chlorination studies could not be scaled-up to maintain the molar ratio between nevirapine and NaOCl, as it would have led to very high volume reactions; and it was for this reason that the products of the reactions differed significantly. Compounds identified in the small scale reactions were not present in the scaled-up versions and visa-versa. Similarly, a plethora of novel compounds were found in the scaled-up reactions that were not present in the small scale reactions. This phenomenon could be attributed to not only the transience of some of the reaction products, but also the low concentrations of the small scale reactions. This is important since many researchers utilise this small scale *in vitro* approach to simulate an industrial process (wastewater treatment); and it may not provide a true reflection of the myriad of potential reactions that pharmaceuticals undergo in this situation.

A total of 45 and 29 fractions were prepared for the basic and acidic reactions, respectively. From UHPLC-QTOF analysis it was found that each fraction contained multiple compounds. This is due to the high levels of similarity between them, as they all arose from the same parent molecule. Chromatographic separation of these highly similar compounds would therefore prove to be challenging. Nevirapine was also present in a number of fractions and its presence was borne in mind when attributing antiviral activity to a particular fraction.

Nevirapine, and subsequently the total chlorination reaction were found to be highly insoluble in water and the majority of commonly used laboratory solvents. The varying levels of solubility of the reaction components further confounds chromatographic purification.

4.3.5 In Vitro Toxicity and Activity

The total and preparative chromatography samples of both acidic and basic chlorination reactions of Nevirapine were subjected to toxicity and activity studies, *in vitro*. The concentration at which 50% of the cells' growth is inhibited (IC_{50}), is inversely proportional to the level of toxicity of a test compound. Thus, a low IC_{50} value is indicative of a high level of toxicity. This is determined by comparing the spectrophotometric absorbance, generated through the measurement of MTS, of the test sample to an untreated control. The ratio is then presented as a percentage to indicate cell survival. The total reactions were much less toxic than Nevirapine (Table 4-5) and none of the preparative fractions were found to be more toxic than the parent molecule in 293T cells.

Table 4-5: Inhibitory concentrations (µg/mL) at which 50% of 293T cells *in vitro* are killed, as determined by MTS assay. The total chlorination reactions of Nevirapine (in basic or acidic phosphate buffer; pH 8 or 5.8 respectively) as compared to a Nevirapine control. Standard deviation presented in brackets.

	Average IC_{50} (µg/mL)
Nevirapine basic reaction	73.7 (0.4)
Nevirapine acid reaction	34.1 (2.1)
Nevirapine control	0.03 (0.01)

That is not to say however that the compounds would not produce toxicity in another fashion (e.g. hepatotoxicity, carcinogenicity etc.) or exhibit novel aspects of environmental toxicity. This should be determined by further *in vitro* studies. But, this provides a heartening indication that the chlorination products do not represent yet another anthropogenic source of toxicity that is being discharged into the environment.

The inhibition of viral replication was determined in a single cycle of infection. Virus-like particles were used that contain HIV-1 subtype C reverse transcriptase, integrase and protease, as well as the RNA transcript of the firefly luciferase protein. Once the virus infects the cell, the firefly RNA is reverse transcribed by the HIV-1 reverse transcriptase to a complementary DNA (cDNA) and integrated into the host cell's chromosomal DNA by HIV-1 integrase. Upon integration, the firefly luciferase gene is expressed to produce active firefly luciferase that can quantified by measuring its bioluminescence. In the absence of inhibitors, this signal is directly proportional to the number of infectious virus particles present in the initial inoculum. Since inhibitors (e.g. DTPs) decrease the number of firefly luciferase gene copies that are integrated into the host's genome, a decrease in the amount of bioluminescence will also be observed. The bioluminescence ratio between an exposed sample and an unexposed control is presented as a percentage to indicate the effect of a test compound on viral activity.

During antiviral activity studies a number of the preparative fractions (Table S4-9 and Table S4-10) showed antiviral activity. The majority of these however can be attributed to the presence of intact Nevirapine. This is because many of the novel compounds share structural similarity with Nevirapine and because of Nevirapine's insolubility, they could not be separated effectively by preparative chromatography. This could be circumvented by comparing the ratio of the UHPLC-QTOF extracted ion (*m/z* 267.1) peak area to the antiviral activity intensity (Figure S4-8) between the fractions. The fractions that had antiviral properties not attributed to Nevirapine could then be identified as active derivatives (i.e. an increase in antiviral activity not associated with an equivalent increase in Nevirapine concentration).

Since many of these compounds either share the Nevirapine "backbone" or are only slightly modified, it is reasonable to speculate that they would also share Nevirapine's structure activity relationship. Nevirapine displayed an IC_{50} value of 0.03 µg/mL, which is substantially lower than the levels at which the total chlorination reaction showed antiviral properties. The various purified fractions however had wide ranging IC_{50} values (0.02 to 20 µg/mL). Higher values may be due to reduced activity properties in the particular molecules or due to the fact that the compounds were not tested in pure form; thereby providing aberrant compound mass-to-activity results.

Once these compounds are fully characterised by mass spectrometry (e.g. MS^n studies) and Nuclear Magnetic Resonance (NMR), they may be synthesised in their pure form. This will lead to a deeper understanding of their mechanism of action.

4.4 Conclusion

From kinetics studies, where chlorine was in excess, it was found that Nevirapine would not be degraded effectively during wastewater treatment because of: increased chlorine demand by dissolved organic matter and reduced degradation at the acidic pH used in wastewater treatment. The latter is most likely due to the

speciation of the Nevirapine molecule. This serves to explain the ubiquitous environmental prevalence in South African surface water, as shown in earlier research (Wood et al., 2015). Although the molecule is relatively persistent, in this work it was shown that Nevirapine is still subject to modification by chlorination, producing a number of DTPs, and degrades entirely at a basic pH.

Through a UHPLC-QTOF analysis of South African surface water it was shown that the DTPs described from *in vitro* analysis are present in the environment. But, the national prevalence of these compounds is exceptionally low since the parent molecule is only found in trace amounts. This is because only a small proportion of an already low concentration of Nevirapine reacts to form these compounds and the distribution is further limited due to the dilution of WWTW discharge.

The chlorination reaction of Nevirapine was scaled up in order to isolate the DTPs identified in nature and in the small-scale stirred reactions; yet it was found that the scale of the reaction affects the nature of the reaction products. This serves to highlight that laboratory scale chlorination may not always be truly representative of industrial wastewater treatment, in that compounds identified in laboratory-scale reactions may not be present in wastewater purification scale reactions and vice versa. This is largely due to concentration differences between the two as well as the addition of a number of uncontrollable variables (e.g. reactive chemicals) in wastewater. With that said though, selected compounds that were identified in the small scale reactions (lower total concentration of both Nevirapine and NaOCl) were incorporated into a database and subsequently detected in WWTW effluent.

Through *in vitro* toxicity and activity testing it was found that none of the nevirapine DTPs are more toxic than the parent molecule. It was however also found that antiviral activity is retained in some of the isolated fractions. Whether this can be attributed to a single molecule or through synergistic effects will only be clarified once individual DTPs are isolated or synthesized.

It is important to consider the fact that pharmaceuticals may be modified and still retain biological activity. It is therefore clear that there is a need to consider the

total impact of not only discharging pharmaceuticals but also their reaction products into the environment. The wastewater treatment process should be scrutinized to not only remove pharmaceuticals through chemical degradation but also their resulting byproducts. Various authors have noted that pharmaceuticals may form biologically active transformation products during wastewater purification (Dodd et al., 2009; Escher and Fenner, 2011; Mestankova et al., 2012; Keen and Linden, 2013). Further investigation into the purification and characterization of the reaction products identified in this work is required in order to obtain a clear picture of how the disinfection of these types of compounds affect the environment.

Acknowledgements:

Christoph Mueller of Agilent Technologies for his kind help on the UHPLC-QTOF method development.

The 293T cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: 293 from Dr. Andrew Rice.

Funding Source:

We would like to gratefully acknowledge the South African Military Health Services for funding this research. The funding agency did not play a role in the study design or data interpretation.

4.5 References

1. Barltrop, J.A., Owen, T.C., Cory, A.H., Cory, J.G., 1991. 5-(3-carboxymethoxyphenyl)-2-(4, 5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium, inner salt (MTS) and related analogs of 3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) reducing to purple water-soluble formazans as cell-viability indicators. Bioorganic & Medicinal Chemistry Letters 1 (11), 611–614.

2. Bedner, M., MacCrehan, W.A., 2006. Transformation of acetaminophen by chlorination produces the toxicants 1, 4-benzoquinone and N-acetyl-p-benzoquinone imine. Environmental Science & Technology 40 (2), 516–522.
3. Bulloch, D.N., Nelson, E.D., Carr, S.A., Wissman, C.R., Armstrong, J.L., Schlenk, D., Larive, C.K., 2015. Occurrence of Halogenated Transformation Products of Selected Pharmaceuticals and Personal Care Products in Secondary and Tertiary Treated Wastewaters from Southern California. Environmental Science & Technology 49, 2044-2051.
4. Coovadia, H.M., Brown, E.R., Fowler, M.G., Chipato, T., Moodley, D., Manji, K., Musoke, P., Stranix-Chibanda, L., Chetty, V., Fawzi, W., others, 2012. Efficacy and safety of an extended nevirapine regimen in infant children of breastfeeding mothers with HIV-1 infection for prevention of postnatal HIV-1 transmission (HPTN 046): a randomised, double-blind, placebo-controlled trial. The Lancet 379 (9812), 221–228.
5. Deborde, M., von Gunten, U., 2008. Reactions of chlorine with inorganic and organic compounds during water treatment—Kinetics and mechanisms: A critical review. Water Research 42 (1), 13–51.
6. Dodd, M.C., Kohler, H.-P.E., Von Gunten, U., 2009. Oxidation of antibacterial compounds by ozone and hydroxyl radical: elimination of biological activity during aqueous ozonation processes. Environmental Science & Technology 43 (7), 2498–2504.
7. Escher, B.I., Fenner, K., 2011. Recent advances in environmental risk assessment of transformation products. Environmental Science & Technology 45 (9), 3835–3847.
8. Graham, F., Smiley, J., Russell, W., Nairn, R., 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. Journal of General Virology 36 (1), 59–72.
9. He, Y., Chen, W., Zheng, X., Wang, X., Huang, X., 2013. Fate and removal of typical pharmaceuticals and personal care products by three different treatment processes. Science of The Total Environment 447, 248–254.

10. Keen, O.S., Linden, K.G., 2013. Degradation of antibiotic activity during UV/H2O2 advanced oxidation and photolysis in wastewater effluent. Environmental science & technology 47 (22), 13020–13030.
11. Kümmerer, K., 2009. Antibiotics in the aquatic environment-a review-part II. Chemosphere 75 (4), 435–441.
12. Lee, Y., von Gunten, U., 2010. Oxidative transformation of micropollutants during municipal wastewater treatment: comparison of kinetic aspects of selective (chlorine, chlorine dioxide, ferrate VI, and ozone) and non-selective oxidants (hydroxyl radical). Water Research 44 (2), 555–566.
13. Leopold, P., Freese, S.D., 2009. A Simple Guide to the Chemistry, Selection And Use Of Chemicals For Water And Wastewater Treatment (TT 405/09).
14. Li, B., Zhang, T., 2012. pH significantly affects removal of trace antibiotics in chlorination of municipal wastewater. Water Research 46 (11), 3703–13.
15. Mestankova, H., Schirmer, K., Escher, B.I., von Gunten, U., Canonica, S., 2012. Removal of the antiviral agent oseltamivir and its biological activity by oxidative processes. Environmental Pollution 161, 30–35.
16. Mitch, W.A., Schreiber, I.M., 2008. Degradation of tertiary alkylamines during chlorination/chloramination: implications for formation of aldehydes, nitriles, halonitroalkanes, and nitrosamines. Environmental Science & Technology 42 (13), 4811–4817.
17. Mofenson, L.M., 2010. Prevention in neglected subpopulations: prevention of mother-to-child transmission of HIV infection. Clinical Infectious Diseases 50 (Supplement 3), S130–S148.
18. Peng, X., Ou, W., Wang, C., Wang, Z., Huang, Q., Jin, J., Tan, J., 2014. Occurrence and ecological potential of pharmaceuticals and personal care products in groundwater and reservoirs in the vicinity of municipal landfills in China. Science of the Total Environment 490, 889–898.
19. Petrie, B., Barden, R., Kasprzyk-Hordern, B., 2014. A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring. Water Research 72, 3-27.

20. Prasse, C., Schlüsener, M.P., Schulz, R., Ternes, T.A., 2010. Antiviral drugs in wastewater and surface waters: a new pharmaceutical class of environmental relevance? Environmental Science & Technology 44 (5), 1728–1735.
21. Prasse, C., Stalter, D., Schulte-Oehlmann, U., Oehlmann, J., Ternes, T.A., 2015. Spoilt for choice: A critical review on the chemical and biological assessment of current wastewater treatment technologies. Water Research 87, 237–270.
22. Prütz, W., 1998. Reactions of hypochlorous acid with biological substrates are activated catalytically by tertiary amines. Archives of Biochemistry and Biophysics 357 (2), 265–273.
23. Richardson, S.D., Plewa, M.J., Wagner, E.D., Schoeny, R., DeMarini, D.M., 2007. Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. Mutation Research/Reviews in Mutation Research 636 (1), 178–242.
24. Rivier, L., 2003. Criteria for the identification of compounds by liquid chromatography-mass spectrometry and liquid chromatography-multiple mass spectrometry in forensic toxicology and doping analysis. Analytica Chimica Acta 492 (1), 69–82.
25. Roden, N.M., Sargent, E.V., DiFerdinando Jr, G.T., Hong, J.-Y., Robson, M.G., 2015. The Cumulative Risk to Human Health of Pharmaceuticals in New Jersey Surface Water. Human and Ecological Risk Assessment: An International Journal 21 (1), 280–295.
26. Selbes, M., Kim, D., Ates, N., Karanfil, T., 2012. The roles of tertiary amine structure, background organic matter and chloramine species on NDMA formation. Water Research 47, 945-953.
27. Shah, A.D., Kim, J.-H., Huang, C.-H., 2011. Tertiary amines enhance reactions of organic contaminants with aqueous chlorine. Water Research 45 (18), 6087–96.

28. Soufan, M., Deborde, M., Legube, B., 2012. Aqueous chlorination of diclofenac: Kinetic study and transformation products identification. Water Research 46, 3377-3386.
29. Ternes, T., Bonerz, M., Schmidt, T., 2001. Determination of neutral pharmaceuticals in wastewater and rivers by liquid chromatography-electrospray tandem mass spectrometry. Journal of Chromatography A 938 (1), 175–185.
30. Vanková, M., others, 2010. Biodegradability analysis of pharmaceuticals used in developing countries; screening with OxiTop C-110. Thesis. Tampere University of Technology.
31. De Wet, J.R., Wood, K., DeLuca, M., Helinski, D.R., Subramani, S., 1987. Firefly luciferase gene: structure and expression in mammalian cells. Molecular and Cellular Biology 7 (2), 725–737.
32. WHO, 2013. Global update on HIV treatment 2013: results, impact and opportunities.
33. Wood, T.P., Duvenage, C.S., Rohwer, E., 2015. The occurrence of anti-retroviral compounds used for HIV treatment in South African surface water. Environmental Pollution 199, 235–243.
34. Zhai, H., Zhang, X., Zhu, X., Liu, J., Ji, M., 2014. Formation of brominated disinfection byproducts during chloramination of drinking water: New polar species and overall kinetics. Environmental Science & Technology 48 (5), 2579–2588.
35. Zimmermann, S.G., Wittenwiler, M., Hollender, J., Krauss, M., Ort, C., Siegrist, H., von Gunten, U., 2011. Kinetic assessment and modeling of an ozonation step for full-scale municipal wastewater treatment: Micropollutant oxidation, by-product formation and disinfection. Water Research 45 (2), 605–617.

4.6 Supporting Information

The Chlorination Behaviour and Environmental Fate of the Antiretroviral Drug Nevirapine in South African Surface Water

Timothy Paul Wood[a,d] (Corresponding Author)

Adriaan Erasmus Basson[b]

Cornelia Duvenage[c]

Egmont Richard Rohwer[d]

[a] Protechnik Laboratories, a Division of ARMSCOR SOC Ltd, 103 Combretum Crescent, Centurion, Pretoria, 0001, South Africa. Tel: +27 12 665 9444, timw@protechnik.co.za

[b]Centre for HIV and STI: HIV Virology Section (Morris Laboratory), National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS). 1 Modderfontein Road, Sandringham, 2131, South Africa. Tel: +27 11 386 6541, adriaanb@nicd.ac.za

[c]Department of Internal Medicine, 1 Military Hospital, South African Military Health Services, Voortrekker Street, Pretoria, 0001, South Africa. Tel: +27832733382, corneliaduv@mtnloaded.co.za

[d] Department of Chemistry, University of Pretoria, Lynwood Road, Pretoria, 0001, South Africa. Tel: +27 12 420 3772 email: egmont.rohwer@up.ac.za

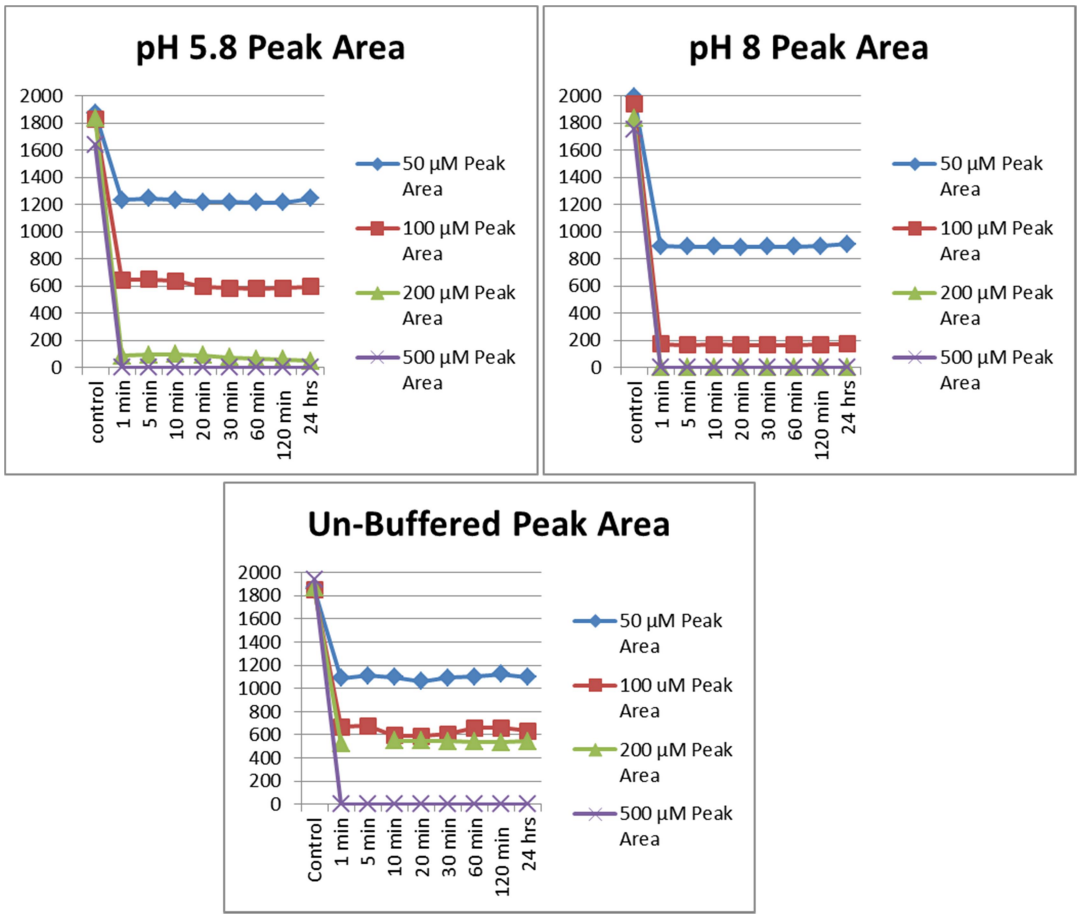


Figure S4-5: The pseudomolecular ion (m/z 267) peak area of Nevirapine (37 µM) reacted with varying NaOCl over time at pH 5.8 or pH 8 in 10 mM phosphate buffer or un-buffered, analysed by LC-QqQ.

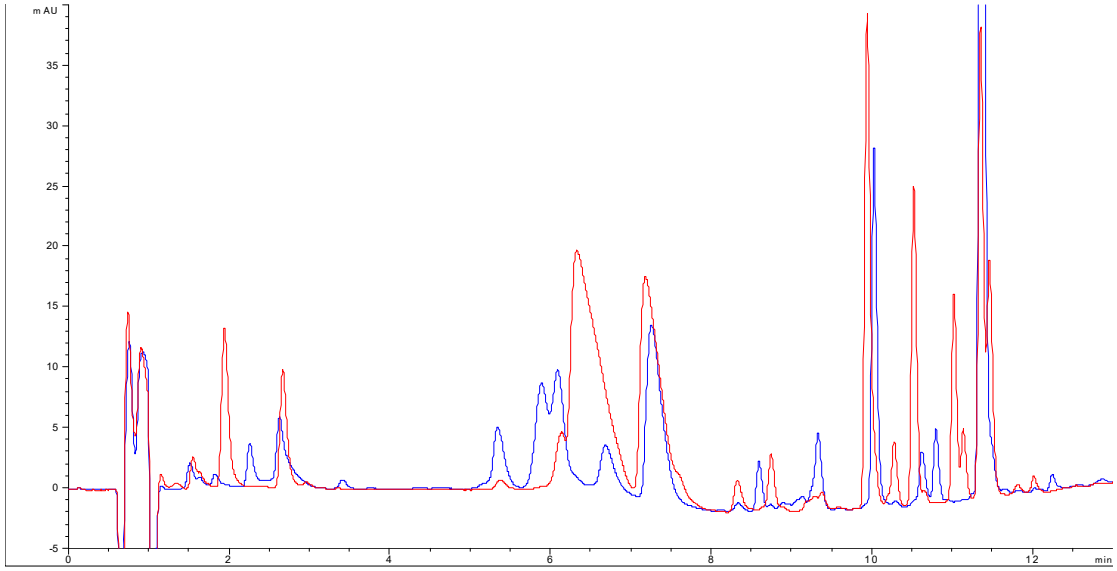


Figure S4-6: Overlaid LC-UV trace (254 nm) of Nevirapine reacted with 100 µM NaOCl at pH 8 (blue trace) and pH 5.8 (red trace) in 10 mM phosphate buffer after 24 hours.

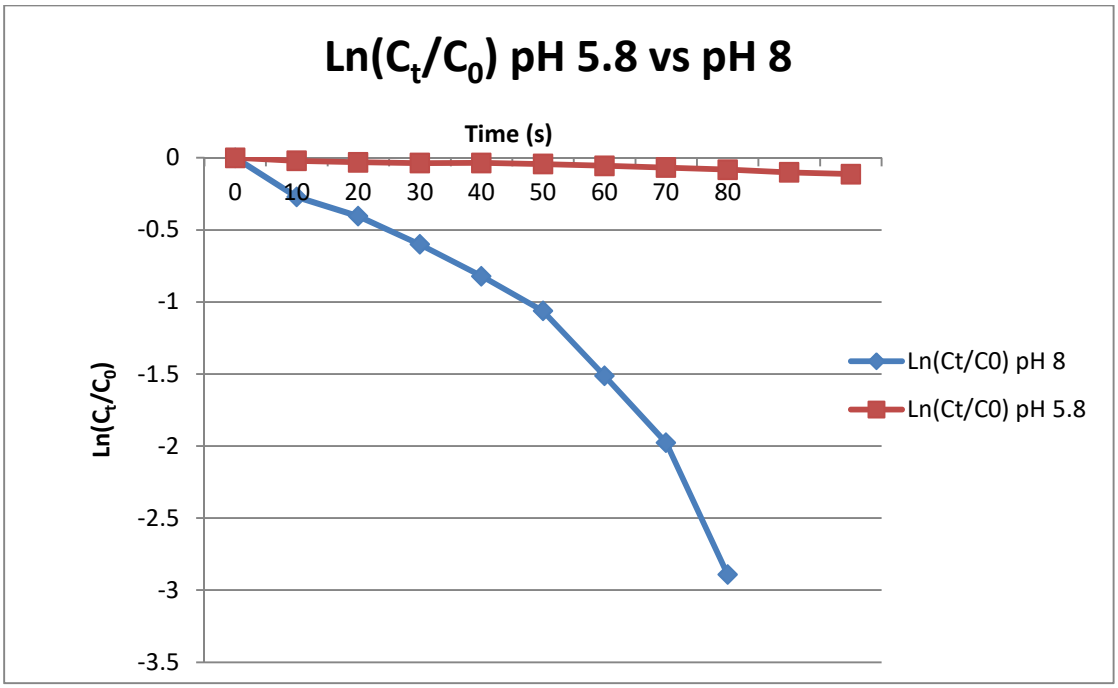


Figure S4-7: Ln(Ct/C0) over time for kinetic measurements of Nevirapine (2 µM) degradation at pH 5.8 or 8 when chlorine is at a 10 fold excess (20 µM).

Table S4-6: The most abundant unique entities identified from the Nevirapine chlorination reactions with proposed formulae, accurate mass, retention time (RT) and MS/MS fragment ions collated into a searcheable database with Agilent PCDL Manager.

Compound Name	Proposed Formula*	Measured Mass	RT (min)	MS/MS Fragment Ions
1	-	121.9176	0.442	
2	$C_9H_9N_3$	159.0787	3.216	120.0547, 97.9662, 56.9407
3	$C_9H_8N_2O$	160.0665	0.663	133.0762, 78.0338, 118.0638, 121.0403
4	$C_9H_9N_3O$	175.0753	5.331	136.0503, 135.0425, 148.0509, 116.9766, 120.0565
5	$C_9H_{10}N_2O_2$	178.0742	1.211	136.9316, 118.9201, 56.9641
6	$C_{10}H_9N_3O$	187.0751	4.723	148.0505, 171.0556, 133.0751, 93.0435
7	$C_{11}H_8N_2O_2$	200.0593	5.588	80.0491, 122.023, 183.0555
8	$C_{11}H_8N_2O_2$	200.0596	6.304	122.0236, 183.0550, 186.0431, 173.0721
9	$C_{10}H_{10}N_4O$	202.0851	2.295	163.0616, 186.0664, 161.0711, 121.0391
10	$C_{10}H_9N_3O_2$	203.0704	5.698	161.0708, 204.0760, 133.0755, 78.0330, 118.06490
11	-	205.8791	0.442	-
12	-	207.8773	0.44	-
13	-	211.8456	0.468	-
14	$C_{10}H_{10}N_4O_2$	218.0828	0.631	161.0468, 219.0881, 201.0775
Nevirapine	$C_{15}H_{14}N_4O$	266.1168	7.178	226.0858, 107.0605, 161.0712, 198.0906
15	$C_{14}H_{13}N_3O_3$	271.0964	6.226	254.0923, 214.0608, 161.0707, 272.1019, 186.0657
16	$C_{14}H_{13}N_3O_3$	271.0965	6.351	254.0929, 214.0610, 161.0708, 186.0656, 272.1019, 133.0763, 118.0654

Compound Name	Proposed Formula*	Measured Mass	RT (min)	MS/MS Fragment Ions
17	$C_{15}H_{14}N_4O_2$	282.1117	5.409	-
18	$C_{15}H_{14}N_4O_2$	282.1117	6.206	161.0710, 242.0793, 214.0845, 123.05520
19	$C_{15}H_{14}N_4O_2$	282.1118	6.853	255.1243, 243.0877, 242.0798, 213.0777, 215.0938, 161.0701
20	$C_{16}H_{16}N_4O_2$	296.1278	9.642	282.1106, 297.1339, 267.0869, 137.0705, 256.0960
21	$C_{15}H_{13}N_3O_4$	299.0911	6.498	122.0233, 96.0439, 217.0618, 187.0499, 189.0659
22	$C_{15}H_{16}N_4O_3$	300.1222	5.37	161.0712, 133.0764, 78.0338, 106.0303
23	$C_{15}H_{15}N_3O_4$	301.1058	5.588	122.0235, 94.0280, 98.0601, 96.0446
24	$C_{15}H_{15}N_3O_4$	301.1061	6.303	-
25	$C_{15}H_{15}N_3O_4$	301.1061	6.305	-
26	-	309.8221	0.523	212.8518, 174.8963, 94.9287
27	$C_{15}H_{14}N_4O_4$	314.1014	6.033	269.1039, 315.1080, 297.0994, 241.1089, 203.0943
28	$C_{15}H_{14}N_4O_4$	314.1018	5.099	161.0710, 133.0756, 118.0660, 78.0326, 105.0456
29	$C_{15}H_{14}N_4O_4$	314.102	6.43	269.1038, 315.1081, 229.0721, 297.0972, 241.1080, 242.0794
30	$C_{15}H_{13}ClN_4O_2$	316.0731	7.251	161.0708, 276.0408, 158.0240, 248.0462, 157.0166
31	$C_{15}H_{15}N_3O_5$	317.1013	6.498	-
32	$C_{19}H_{12}O_5$	320.0676	6.853	208.0979, 252.3384, 321.1343, 92.9652

Compound Name	Proposed Formula*	Measured Mass	RT (min)	MS/MS Fragment Ions
33	$C_{16}H_{17}N_3O_5$	331.1167	7.565	-
34	$C_{16}H_{12}N_8O$	332.1136	1.501	227.0923, 333.1186, 287.1137, 245.1028, 251.0925, 186.0565
35	-	347.7782	0.49	-
36	$C_{21}H_{14}O_6$	362.0781	5.47	283.1199, 345.0749, 161.0715, 242.0800
37	$C_{21}H_{14}O_6$	362.0785	5.409	-
38	$C_{21}H_{14}O_7$	378.0731	5.134	243.0877, 188.0815, 161.0699, 379.0804, 253.1089
39	-	379.7677	0.452	-
40	-	379.7678	0.454	-
41	$C_{18}H_{12}N_2O_8$	384.0605	5.419	283.1188, 305.0983, 161.0695, 265.1084
42	-	483.7118	0.504	-

* - denotes an entity for which a formula could not be proposed with greater than 90 % certainty and/or cases for which automatic MS/MS did not record spectra for a particular compound.

Table S4-7: GPS co-ordinates of sampling areas.

Sample Name	GPS Co-Ordinates
Roodeplaat Dam System*	
Pienaars River Inflow	-25.678677, 28.357116
Zeekoegat WWTW Outflow	-25.624620, 28.341890
Angling Area	-25.626404, 28.345692
S.E Bank	-25.637763, 28.344150
Motorboat Launch	-25.618238, 28.358642

Sample Name	GPS Co-Ordinates
Rowing Club	-25.623345, 28.349842
Roodeplaat Outflow	-25.608244, 28.367231
Rietvlei Dam*	
Southern Bank	-25.881576, 28.268585
Northern Bank	-25.876767, 28.279846
Orange River System	
Orange River (Bethulie)	-30.534670, 26.022975
Gariep Dam Oviston	-30.692147, 25.761238
Gariep Dam (N.E)	-30.603858, 25.503609
Vaal confluence	-29.070882, 23.637209
Orange confluence	-29.072898, 23.638936
Confluence	-29.071810, 23.635868
Cape Region	
Eerste Rivier*	-33.941603, 18.857078
Theewaterskloof Dam	-34.027283, 19.208261
Vaal Dam	
Dam wall	-26.883278, 28.116047
Oranjeville	-26.999155, 28.214893
Vaal Dam Inflow	-27.020575, 28.608589
Vaal Dam Out Flow	-26.874950, 28.115583
Single system samples	
Hartebeesfontein WWTW Outflow*	-26.030715, 28.291084
Ditholo	-25.320242, 28.340728
Hartbeespoort Dam, Meerhof (2011)*	-25.760775, 27.891871
Hartbeespoort Dam, Meerhof (2014)*	-25.760775, 27.891871
Hartbeespoort Dam, Tap Water Sample*	-25.745594, 27.911238
Hartbeespoort Inflow, Crocodile River	-25.775818, 27.901601
Renosterkop	-25.108639, 28.887359

Sample Name	GPS Co-Ordinates
Inanda Dam	-29.673792, 30.854874
Inanda Dam offshore	-29.674016, 30.860239

* Indicates that the sample was taken in or near an urban environment.

Table S4-8: Nevirapine and its chlorination products detected by positive ESI UHPLC-QTOF analysis of SPE extracts of South African surface water samples.

GPS	Description	m/z	RT*	RT Diff (DB)**	Diff (DB, mDa)**	Mass	Peak Height	Proposed Formula
Roodeplaat Dam System								
-25.678677, 28.357116	Pienaars River before Roodeplaat Dam	170.0722	4.792	-0.069	-0.32	187.0754	51552	$C_{10}H_9N_3O$
-25.678677, 28.357116	Pienaars River before Roodeplaat Dam	188.0824	4.685	0.038	-0.16	187.0753	582284	$C_{10}H_9N_3O$
-25.678677, 28.357116	Pienaars River before Roodeplaat Dam	267.1248	7.142	0.036	-0.76	266.1175	3247866	$C_{15}H_{14}N_4O$ (Nevirapine)
-25.678677, 28.357116	Pienaars River before Roodeplaat Dam	283.1192	6.159	0.047	-0.37	282.1121	1473596	$C_{15}H_{14}N_4O_2$
-25.678677, 28.357116	Pienaars River before Roodeplaat Dam	317.0807	7.231	0.02	-0.67	316.0738	57884	$C_{15}H_{13}ClN_4O_2$
-25.626404, 28.345692	Roodeplaat Angling Area	267.1243	7.132	0.046	-0.22	266.117	1200003	$C_{15}H_{14}N_4O$ (Nevirapine)
-25.626404, 28.345692	Roodeplaat Angling Area	188.0824	4.683	0.04	-0.23	187.0753	95604	$C_{10}H_9N_3O$
-25.626404, 28.345692	Roodeplaat Angling Area	375.1588	4.828	-0.105	-2.38	187.0775	81153	$C_{10}H_9N_3O$
-25.624620, 28.341890	Zeekoegat WWTW Outflow	188.0823	4.655	0.068	0.01	187.0751	321806	$C_{10}H_9N_3O$

GPS	Description	m/z	RT*	RT Diff (DB)**	Diff (DB, mDa)**	Mass	Peak Height	Proposed Formula
-25.637763, 28.344150	Roodeplaat S.E. Bank	188.0829	4.626	0.097	-0.74	187.0758	99686	$C_{10}H_9N_3O$
-25.637763, 28.344150	Roodeplaat S.E. Bank	267.1252	7.153	0.025	-0.39	266.1172	1668882	$C_{15}H_{14}N_4O$ (Nevirapine)
-25.618238, 28.358642	Roodeplaat Motor Boat Launch	188.0824	4.662	0.061	-0.22	187.0753	126361	$C_{10}H_9N_3O$
-25.618238, 28.358643	Roodeplaat Motor Boat Launch	267.1251	7.16	0.018	-1.16	266.1179	1742427	$C_{15}H_{14}N_4O$ (Nevirapine)
-25.623345, 28.349842	Roodeplaat Rowing Club	188.0825	4.652	0.071	-0.33	187.0754	125822	$C_{10}H_9N_3O$
-25.608244, 28.367231	Roodeplaat Dam Outflow	267.125	7.15	0.028	-1.03	266.1178	1492262	$C_{15}H_{14}N_4O$ (Nevirapine)
Orange-Vaal System								
-30.534670, 26.022975	Orange River (Bethuli)	267.1236	7.133	0.045	0.33	266.1164	21351	$C_{15}H_{14}N_4O$ (Nevirapine)
-29.071810, 23.635868	Orange-Vaal River Confluence	267.124	7.133	0.045	-0.07	266.1168	46847	$C_{15}H_{14}N_4O$ (Nevirapine)
-29.070882, 23.637209	Vaal River Confluence	267.1242	7.133	0.045	-0.24	266.117	44894	$C_{15}H_{14}N_4O$ (Nevirapine)
-30.603858, 25.503609	Gariep Dam N.E Bank	267.1241	7.137	0.041	-0.06	266.1168	50227	$C_{15}H_{14}N_4O$ (Nevirapine)
-26.999155,	Oranjeville Bridge	283.1197	6.399	-0.193	-1.15	282.1128	47585	$C_{15}H_{14}N_4O_2$

GPS	Description	m/z	RT*	RT Diff (DB)**	Diff (DB, mDa)**	Mass	Peak Height	Proposed Formula
28.214893								
-26.874950, 28.115583	Vaal Dam Outflow	267.1253	7.14	0.038	-1.25	266.118	305071	$C_{15}H_{14}N_4O$ (Nevirapine)
Hartbeespoort/Crocodile River System								
-25.760775, 27.891871	Hartbeespoort Dam, Meerhof	267.1251	7.134	0.044	-1.24	266.118	790461	$C_{15}H_{14}N_4O$ (Nevirapine)
-25.775818, 27.901601	Hartbeespoort Inflow, Crocodile River	267.1251	7.087	0.091	-1.13	266.1179	429248	$C_{15}H_{14}N_4O$ (Nevirapine)
-25.775818, 27.901601	Hartbeespoort Inflow, Crocodile River	283.1196	6.398	-0.192	-0.98	282.1127	560137	$C_{15}H_{14}N_4O_2$
-25.775818, 27.901601	Hartbeespoort Inflow, Crocodile River	188.0822	4.613	0.11	-0.02	187.0751	81918	$C_{10}\ H_9\ N_3\ O$
Single Samples								
-26.030715, 28.291084	Hartbeesfontein WWTW	267.124	7.139	0.039	-0.08	266.1168	799749	$C_{15}H_{14}N_4O$ (Nevirapine)
-26.030715, 28.291084	Hartbeesfontein WWTW	283.1187	6.901	-0.048	-0.35	282.1122	36425	$C_{15}H_{14}N_4O_2$
-26.030715, 28.291084	Hartbeesfontein WWTW	158.0723	5.175	0.156	0.03	175.0753	2041054	$C_9H_9N_3O$
-25.881576, 28.268585	Rietvlei Dam	267.1248	7.128	0.05	-0.77	266.1175	744658	$C_{15}H_{14}N_4O$ (Nevirapine)

GPS	Description	m/z	RT*	RT Diff (DB)**	Diff (DB, mDa)**	Mass	Peak Height	Proposed Formula
-25.881576, 28.268585	Rietvlei Dam	158.0715	5.166	0.165	0.75	175.0745	1145107	$C_9H_9N_3O$
-29.673792, 30.854874	Inanda Dam Bank	267.1233	7.14	0.038	0.59	266.1162	247851	$C_{15}H_{14}N_4O$
-29.674016, 30.860239	Inanda Dam Centre	267.1233	7.125	0.053	0.65	266.1161	319778	$C_{15}H_{14}N_4O$

Table S4-9: The *in vitro* viral activity of the fractions of the basic Nevirapine chlorination reaction. A value of 100% or more indicates that a fraction has no antiviral properties. The concentration at which 50 % of the virus is inhibited (IC_{50}) was estimated from the average viral activity measured after exposure to each fraction at 10 µg/mL.

	Percent virus activity				Estimated IC_{50} (µg/mL)*
NVP Basic Fractions:	**1**	**2**	**Average**	**Standev**	
Nev. Rxn. FC C1	103.6%	102.4%	103.0%	0.8%	-
Nev. Rxn. FC C2	97.7%	98.4%	98.1%	0.5%	19.6
Nev. Rxn. FC C3	60.8%	61.4%	61.1%	0.5%	12.2
Nev. Rxn. FC C4	4.7%	3.9%	4.3%	0.6%	0.9
Nev. Rxn. FC C5	4.9%	4.3%	4.6%	0.4%	0.9
Nev. Rxn. FC C6	8.7%	7.7%	8.2%	0.7%	1.6
Nev. Rxn. FC C7	14.0%	12.4%	13.2%	1.1%	2.6
Nev. Rxn. FC C8	18.4%	16.6%	17.5%	1.2%	3.5
Nev. Rxn. FC C9	8.5%	8.1%	8.3%	0.3%	1.7
Nev. Rxn. FC C10	26.2%	26.0%	26.1%	0.2%	5.2
Nev. Rxn. FC C11	29.4%	29.8%	29.6%	0.2%	5.9
Nev. Rxn. FC C12	36.6%	37.7%	37.1%	0.8%	7.4
Nev. Rxn. FC C13	12.4%	11.6%	12.0%	0.6%	2.4
Nev. Rxn. FC C14	2.5%	1.7%	2.1%	0.6%	0.4
Nev. Rxn. FC C15	4.5%	4.4%	4.4%	0.0%	0.9
Nev. Rxn. FC C16	21.1%	17.9%	19.5%	2.2%	3.9
Nev. Rxn. FC C17	38.5%	36.0%	37.2%	1.7%	7.4
Nev. Rxn. FC C18	45.6%	42.7%	44.2%	2.1%	8.8

	Percent virus activity				Estimated IC_{50} (µg/mL)*
NVP Basic Fractions:	**1**	**2**	**Average**	**Standev**	
Nev. Rxn. FC C19	42.7%	39.0%	40.9%	2.6%	8.2
Nev. Rxn. FC C20	29.2%	26.1%	27.7%	2.2%	5.5
Nev. Rxn. FC C21	29.8%	29.9%	29.9%	0.1%	6.0
Nev. Rxn. FC C22	50.8%	52.0%	51.4%	0.9%	10.3
Nev. Rxn. FC C23	59.1%	58.7%	58.9%	0.3%	11.8
Nev. Rxn. FC C24	50.7%	53.1%	51.9%	1.7%	10.4
Nev. Rxn. FC C25	71.2%	73.9%	72.5%	1.9%	14.5
Nev. Rxn. FC C26	89.6%	88.9%	89.3%	0.5%	17.9
Nev. Rxn. FC C27	89.1%	94.7%	91.9%	4.0%	18.4
Nev. Rxn. FC C30	83.4%	92.9%	88.1%	6.7%	17.6
Nev. Rxn. FC C31	89.6%	92.2%	90.9%	1.9%	18.2
Nev. Rxn. FC C32	91.4%	94.2%	92.8%	2.0%	18.6
Nev. Rxn. FC C33	100.6%	101.1%	100.9%	0.4%	-
Nev. Rxn. FC C34	92.2%	93.4%	92.8%	0.9%	18.6
Nev. Rxn. FC C35	94.9%	99.5%	97.2%	3.2%	19.4
Nev. Rxn. FC C36	91.3%	98.2%	94.7%	4.9%	18.9
Nev. Rxn. FC C37	94.3%	94.1%	94.2%	0.1%	18.8
Nev. Rxn. FC C38	95.5%	95.1%	95.3%	0.2%	19.1
Nev. Rxn. FC C39	101.4%	95.6%	98.5%	4.1%	19.7
Nev. Rxn. FC C40	90.2%	91.6%	90.9%	1.0%	18.2
Nev. Rxn. FC C41	94.4%	96.6%	95.5%	1.6%	19.1

	Percent virus activity				Estimated IC_{50} (µg/mL)*
NVP Basic Fractions:	**1**	**2**	**Average**	**Standev**	
Nev. Rxn. FC C42	95.8%	100.1%	97.9%	3.0%	19.6
Nev. Rxn. FC C50	95.2%	88.6%	91.9%	4.7%	18.4
Nev. Rxn. FC C51	99.1%	89.7%	94.4%	6.7%	18.9
Nev. Rxn. FC C52	99.5%	88.1%	93.8%	8.0%	18.8
Nev. Rxn. FC C53	92.4%	88.6%	90.5%	2.7%	18.1
Nev. Rxn. FC C54	114.8%	113.4%	114.1%	1.0%	-

* The estimation is based on the assumption that the relationship between antiviral activity and test compound concentration is linear; - where viral activity is ≥ 100 % at 10 µg/mL, the IC_{50} could not be calculated.

Table S4-10: The in vitro viral activity of the fractions of the acidic Nevirapine chlorination reaction. A value of 100% or more indicates that a fraction has no antiviral properties. The concentration at which 50 % of the virus is inhibited (IC_{50}) was calculated from the average viral activity measured after exposure to each fraction at 10 µg/mL.

	Percent virus activity				Calculated IC_{50}
NVP Acidic Fractions:	**1**	**2**	**Average**	**Standev**	**(µg/mL)***
Nev. Acid Rxn. FC D1	87.5%	84.7%	86.1%	1.9%	17.2
Nev. Acid Rxn. FC D2	101.1%	98.3%	99.7%	2.0%	19.9
Nev. Acid Rxn. FC D3	102.9%	105.6%	104.2%	1.9%	-
Nev. Acid Rxn. FC D4	101.8%	107.1%	104.5%	3.8%	-
Nev. Acid Rxn. FC D5	100.8%	97.5%	99.1%	2.4%	19.8
Nev. Acid Rxn. FC D6	97.5%	97.6%	97.5%	0.1%	19.5
Nev. Acid Rxn. FC D7	96.3%	96.8%	96.5%	0.4%	19.3
Nev. Acid Rxn. FC D8	100.5%	99.7%	100.1%	0.5%	-
Nev. Acid Rxn. FC D9	101.8%	98.4%	100.1%	2.4%	-
Nev. Acid Rxn. FC D11	105.4%	103.1%	104.2%	1.6%	-
Nev. Acid Rxn. FC D12	96.1%	94.5%	95.3%	1.1%	19.1
Nev. Acid Rxn. FC D14	102.1%	97.8%	99.9%	3.0%	20.0
Nev. Acid Rxn. FC D15	103.4%	95.2%	99.3%	5.8%	19.9
Nev. Acid Rxn. FC D16	100.9%	98.6%	99.7%	1.6%	19.9
Nev. Acid Rxn. FC D17	104.2%	100.6%	102.4%	2.5%	-
Nev. Acid Rxn. FC D18	110.7%	104.4%	107.5%	4.4%	-
Nev. Acid Rxn. FC D19	95.8%	92.9%	94.4%	2.1%	18.9
Nev. Acid Rxn. FC D20	50.8%	44.9%	47.8%	4.2%	9.6

	Percent virus activity				Calculated IC_{50}
NVP Acidic Fractions:	**1**	**2**	**Average**	**Standev**	**(µg/mL)***
Nev. Acid Rxn. FC D21	57.6%	54.3%	56.0%	2.4%	11.2
Nev. Acid Rxn. FC D22	26.2%	21.6%	23.9%	3.3%	4.8
Nev. Acid Rxn. FC D23	1.2%	0.9%	1.1%	0.2%	0.2
Nev. Acid Rxn. FC D24	0.0%	0.1%	0.1%	0.0%	0.02
Nev. Acid Rxn. FC D25	0.1%	0.1%	0.1%	0.0%	0.02
Nev. Acid Rxn. FC D26	16.6%	14.8%	15.7%	1.3%	3.1
Nev. Acid Rxn. FC D27	72.2%	65.1%	68.7%	5.0%	13.7
Nev. Acid Rxn. FC D28	99.9%	93.0%	96.5%	4.9%	19.3
Nev. Acid Rxn. FC D29	101.7%	89.6%	95.7%	8.6%	19.1
Nev. Acid Rxn. FC D30	107.4%	85.6%	96.5%	15.4%	19.3
Nev. Acid Rxn. FC D34	104.1%	91.1%	97.6%	9.2%	19.5

* The calculation is based on the assumption that the relationship between antiviral activity and test compound concentration is linear; - where viral activity is ≥ 100 % at 10 µg/mL, the IC_{50} could not be calculated.

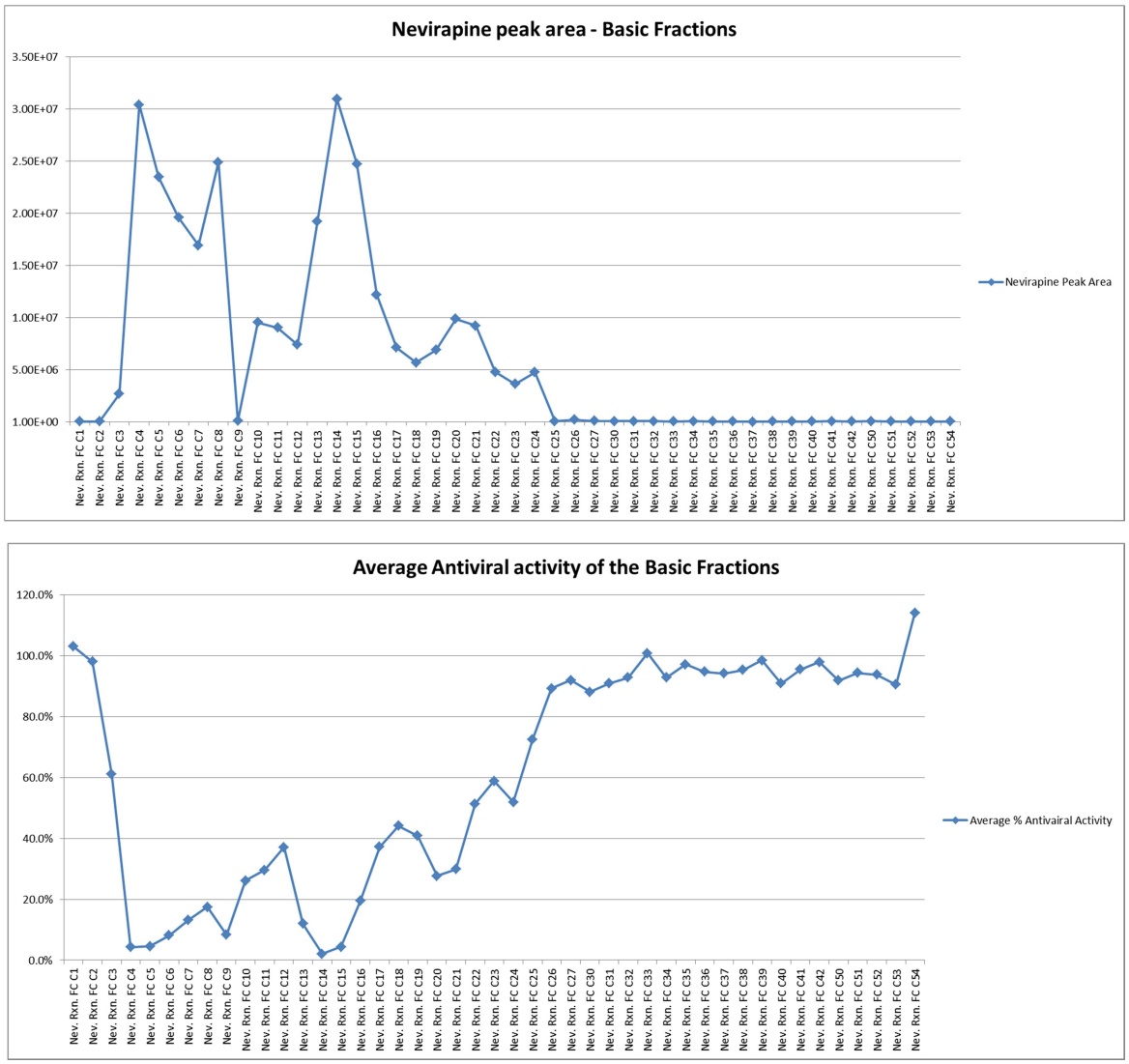


Figure S4-8: The peak area of the extracted Nevirapine ion analysed by UHPLC-QTOF (top) and the average antiviral activity (bottom) of each of the chlorination reaction preparative chromatography fractions.

Chapter 5: Transformation of the Anti-HIV Drug Zidovudine During Simulated Wastewater Chlorination and the Subsequent Formation of Biologically Active Zidovudine Heterodimers

This chapter is in preparation for publication. The format reflects the style set by the intended journal.

Transformation of the Anti-HIV Drug Zidovudine During Simulated Wastewater Chlorination and the Subsequent Formation of Biologically Active Zidovudine Heterodimers

Timothy Paul Wood[a,d] (Corresponding Author)

Thembela Ntlameza[a]

Adriaan Erasmus Basson[b]

Cornelia Duvenage[c]

Egmont Richard Rohwer[d]

[a] Protechnik Laboratories, a Division of ARMSCOR SOC Ltd, 103 Combretum Crescent, Centurion, Pretoria, 0001, South Africa. Tel: +27 12 665 9444, timw@protechnik.co.za

[b]Centre for HIV and STI: HIV Virology Section (Morris Laboratory), National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS). 1 Modderfontein Road, Sandringham, 2131, South Africa. Tel: +27 11 386 6541, adriaanb@nicd.ac.za

[c]Department of Internal Medicine, 1 Military Hospital, South African Military Health Services, Voortrekker Street, Pretoria, 0001, South Africa.
Tel: +27832733382, corneliaduv@mtnloaded.co.za

[d] Department of Chemistry, University of Pretoria, Lynwood Road, Pretoria, 0001, South Africa. Tel: +27 12 420 3772 email: egmont.rohwer@up.ac.za

Highlights

- The anti-HIV drug Zidovudine is resistant to degradation by chlorination during simulated wastewater treatment conditions
- The nature of the disinfection by-products formed during chlorination is dependent on Zidovudine concentration
- Zidovudine forms oxidised heterodimers which may afford protection from degradation
- The disinfection transformation products are not more toxic than Zidovudine in an *in vitro* cell culture system and some retain antiviral activity
- Transformation products are not detectable in wastewater effluent

Keywords

UHPLC-QTOF, disinfection transformation product, wastewater, Zidovudine, HIV

Abstract

Pharmaceutical compounds, besides being released into the environment due to ineffectual waste water treatment, undergo modifications as a result of the disinfection process. Zidovudine, an antiretroviral (ARV) compound used to treat human immunodeficiency virus (HIV) infection, has been detected in South African surface water. Waste water treatment chlorination was simulated and Zidovudine reactivity was determined. Transformation products were characterised using Ultra High Pressure Liquid Chromatography coupled to Time of Flight Quadrupole mass spectrometry (UHPLC-QTOF). During chlorination, at 25 times excess of chlorine to drug, Zidovudine was found to degrade slowly (4.6, 5.3 and 5.0 $M^{-1}.s^{-1}$ in unbuffered water, at pH 8 and at pH 5.8), which partially accounts for its environmental release. After an initial decline, Zidovudine concentration increased in solution to a stable point (20 % of the initial concentration). With increased Zidovudine concentrations it was found that the molecule would form heterodimers following chlorination, which could degrade over time and release Zidovudine back into solution. If dimerisation could afford protection from chlorine disinfection, it

would explain in part the compound's environmental prevalence. Disinfection transformation products were isolated and found to be no more toxic than Zidovudine *in vitro,* while retaining antiviral properties. The compounds were compiled into a database and used to screen effluent samples from four wastewater treatment works (WWTWs), all of which discharged Zidovudine but not the disinfection transformation products (DTPs). Further study into the environmental impact of releasing biologically active pharmaceutical transformation products is merited.

5.1 Introduction

It has been widely established in the literature that pharmaceuticals are present in surface water (Kümmerer, 2009a, 2009b; Daughton and Scuderi, 2012; Luo et al., 2014; Petrie et al., 2014). This may be as a result of ineffectual removal during wastewater treatment (Rodriguez-Mozaz et al., 2015; Wode et al., 2015; Papageorgiou et al., 2016), factory discharge (Cardoso et al., 2014), landfilling of pharmaceuticals (Peng et al., 2014) or the use of pit latrines in developing nations (Graham and Polizzotto, 2013). The latter point highlights the unique factors that should be considered in this type of research when studying resource-limited areas. Very few studies have been conducted in this field on the African continent (Agunbiade and Moodley, 2014, 2015; Schoeman et al., 2015; Wood et al., 2015) and this is important since the pharmaceutical usage profiles in developing nations would be characterized by a higher consumption of antimycobacterial, antimalarial and antiretroviral drugs per capita when compared to other countries.

South Africa has the highest HIV burden worldwide and utilises more antiretroviral drugs than any other nation (WHO, 2013). It was shown in earlier research that these compounds are ubiquitous in South African surface water (Wood et al., 2015). The most likely avenue of entry of these drugs into aquatic systems in urban environments is through ineffectual removal at WWTWs (Schoeman et al., 2015). Anti-HIV drugs and other similar antiviral compounds have been detected in surface water world-wide (Jain et al., 2013). This is especially relevant since many

viruses have non-human hosts e.g. adenoviruses in water fowl that may be exposed to antiviral agents, thereby inducing the development of drug resistant viral strains (Järhult, 2012). Although HIV has no environmental component to its lifecycle, the ecotoxicological effect of these compounds in surface water is still unknown.

Besides being present in surface water, it has been found that pharmaceuticals are also modified during wastewater treatment and the preparation of potable water. The disinfection processes of chlorination (Matsushita et al., 2015), chloramination (Li and Zhang, 2013), ozonation (Wang et al., 2015) and bromination (Acero et al., 2013; Zhai et al., 2014) have all been found to lead to the modification of a variety of pharmaceutical compounds.

Bedner and co-workers showed that seemingly innocuous compounds such as acetaminophen may be transformed into toxic disinfection transformation products (DTPs) as a result of chlorination (Bedner and MacCrehan, 2006). Similarly the toxicity and mutagenicity of X-ray contrast agents were found to increase as a result of chlorination (Matsushita et al., 2015).

The effects of disinfection on antiretroviral compounds used for the treatment of HIV are largely unknown. Prasse et al. (2015) found that Abacavir, Acyclovir, Lamivudine, Emtricitabine and Zidovudine all underwent photo and biotransformation in a simulated system. The products of oxidation/halogenation as a result of chlorination during wastewater treatment conditions has however not been studied extensively in the literature for many of these drugs.

A variety of compounds are utilised for the treatment of HIV infection. Among them is the Nucleoside-analogue Reverse Transcriptase Inhibitor, Zidovudine, the first drug approved for the treatment of HIV infection (Adediran et al., 2016). The compound is generally used as part of a Highly Active Antiretroviral Therapy (HAART) regimen, and may have toxic side effects such as the induction of anaemia in patients (Mulenga et al., 2016; Rougemont et al., 2016).

In this research the transformation of the antiretroviral drug Zidovudine during simulated wastewater treatment was studied. The degradation kinetics are studied and putative structures for the resulting DTPs are proposed. The chlorination reaction for the drug was scaled up and a number of the DTPs were isolated and screened for toxicity and antiviral activity. A database of these compounds was compiled and the effluent of four WWTWs screened against it to determine their environmental prevalence.

5.2 Materials and Methods

5.2.1 Chemicals

Potassium iodate, potassium permanganate, sodium sulphate pentahydrate, sodium carbonate, potassium iodide, the Spectroquant dipropyl-p-phenylenediamine (DPD) Chlorine test kit, sodium hypochlorite (10-14%), monobasic and dibasic potassium phosphate were obtained from Merck Chemicals (Johannesburg, South Africa). Sulphuric acid (made up to 10%), analytical grade Zidovudine (Fluka), ammonium chloride and sodium thiosulphate were purchased from Sigma Aldrich (Johannesburg, South Africa). Zidovudine utilised for comparative toxicity and antiviral activity assays was obtained from the US National Institute of Health (NIH AIDS Reagent Program, Germantown, MD, USA). Glacial acetic acid and ascorbic acid were purchased from Glassworld (Johannesburg, South Africa). A domestic starch powder was used to produce a 2% starch indicator solution. Premixed mobile phases, acetonitrile and water; each with 0.1% formic acid, and pure water were purchased from Burdick & Jackson (Muskegon, USA).

Diluted sodium hypochlorite (10-14 %), in MilliQ water, was utilised as a stock solution and was quantified by DPD colorimetry at 515 nm in Nunc Maxisorp microtitre plates (Nunc, Denmark) on a Multiskan GO Spectrophotometer (Thermo, Helsinki, Finland). In addition to this, standard iodometric titration was utilised to

ensure the stability of the chlorine concentration. No South African origin water was used during the analytical component of this research.

5.2.2 Autosampler Reactions

Zidovudine was diluted in either water, 10 mM phosphate buffer (pH 5.8 or 8) or WWTW effluent (from the Zeekoegat WWTW, Pretoria) to 8 µM. Similarly, chlorine in the form of NaOCl was diluted to 200, 400 and 1000 µM. The Zidovudine and each of the respective hypochlorite samples were combined in equal volumes (40 µL of each) in inert low volume vial inserts (Agilent, Santa Clara, USA). Following addition of the Zidovudine to the hypochlorite, the solutions were mixed (40 µL, with rapid expulsion), incubated in the autosampler at 20 ºC and 3 µL was injected onto the UHPLC. Subsequent injections were programmed to yield first a 30 second then further 1 hour intervals of incubation time for each sample. The time between combining the drug and chlorine with mixing followed by injection was determined to be 30 seconds. Negative controls (Zidovudine without chlorine and *vice versa*) were included at the end of each analysis. Quantification of Zidovudine was achieved by external calibration *via* MassHunter Quant (Agilent, Santa Clara, USA) using either the pseudomolecular ion *m/z* 268 or the major in-source fragmentation ion *m/z* 127 for lower concentration samples. These data were utilised to determine the reaction kinetics of Zidovudine chlorination.

Compounds were separated on a Zorbax Eclipse Plus C8 RRHD column (2.1 mm x 50 mm, 1.8 µm) with an Agilent 1290 liquid chromatograph. Mobile phase A consisted of premixed HPLC grade water with 0.1% formic acid while mobile phase B was acetonitrile with 0.1 % formic acid (Burdick & Jackson, Muskegon, USA). The column was maintained at 30 ºC with a flow rate of 0.4 mL/min. Each sample (3 µL) was separated according to the gradient: 2% B for 0.3 min, to 100% B at 3 min, hold 1 min, to 2% B at 4.3 min and equilibrate to 5 min.

5.2.3 Stirred Reactions

Stirred 10 mL reactions, with 10 µg/mL Zidovudine (37 µM) to 100 µM NaOCl, were utilised to characterise the Zidovudine disinfection transformation products, as the autosampler reactions (at environmentally realistic Zidovudine concentrations) were too dilute for these purposes. The reactions were sampled at 0, 1, 2, 3, 4, 5, 24 and 48 hours and analysed by UHPLC-QTOF in full scan and automatically triggered MS/MS (Auto MS/MS) mode. Compounds were separated on a Zorbax Eclipse Plus C8 RRHD column (2.1 mm x 50 mm, 1.8 µm) with mobile phases as described previously. The gradient consisted of: 2% B for 1 min to 100% B over 5 min, 100 % B for 1 min and 2 % B for 1 min.

Quenching agents (sodium thiosulphate, ascorbic acid and ammonium chloride) were investigated at two-fold molar excess (to NaOCl) at equivalent times to determine their utility in halting the reactions. The concentration of Zidovudine and its transformation products were compared between quenched and unquenched samples.

5.2.4 Large Scale Reactions and Purification

One gram of Zidovudine was diluted in 100 mL of 100 mM phosphate buffer (pH 8) and 0.04 M NaOCl was added daily, for seven days. The reaction was monitored daily, prior to each further addition of NaOCl, by UHPLC-QTOF. The end point was determined by the diversity of compounds present in the reaction as well as the amount of Zidovudine remaining. The final product was dried overnight by vacuum concentration in a rotary evaporator (Buchi, Switzerland) at 40ºC.

Reverse phase flash chromatography was used to separate the large scale reaction mixtures. This was carried out on a Buchi Sepacore binary pump system equipped with a UV detector (254 nm). Mobile phases A and B were water and acetonitrile, each with 0.1% formic acid, respectively. The product (400 mg) was dissolved in 1 ml of 0.1% formic acid in HPLC grade water and injected onto a

Maechery Nagel C18 (4 g) column and separated on a gradient (2 % B for 5 min, to 100% B over 30 min) at a flow rate of 15 ml/min.

The fractions were collected every 30 seconds and subsequently dried using a centrifugal vacuum evaporator (Martin Christ), at 30 ºC for 6 hours at a rotation of 600 RPM and a pressure of 5 mBar. Each fraction was diluted to 1 mg/ml in DMSO and all fractions showed complete solubility in DMSO. Each of these was diluted further and analysed by UHPLC-QTOF in full scan and Auto MS/MS mode prior to *in vitro* toxicity and activity studies.

5.2.5 UHPLC-QTOF Analysis

The column eluent from the two different UHPLC separations were analysed by positive electrospray mass spectrometry using an Agilent 6550 QTOF with a dual spray Jet Stream source. Source parameters were: Gas Temperature 200 ºC, Gas Flow 15 L/min, Nebuliser 35 psig, Sheath Gas Temperature 400 ºC and Sheath Gas Flow 12 L/min. Auto MS/MS conditions consisted of acquisition at 3 spectra/s in MS mode and 5 spectra/s in MS/MS. Collision energy was selected based on the precursor mass with a slope of 6 and offset of 4 eV. A maximum of 4 precursors per cycle were selected based on an abundance threshold of 3500 units. Accurate mass correction was achieved by the continuous infusion of a low mass calibration solution (Agilent). Data was evaluated qualitatively using the MassHunter Qual package (Agilent) and where necessary Zidovudine was quantified by external standard calibration using the Agilent Quant software package.

5.2.6 In Vitro Toxicity and Antiviral Activity

The toxicity of Zidovudine chlorination reaction product fractions was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay System (Promega, Madison, USA) in 293T cells. These data were compared to the toxicity of analytical grade Zidovudine and each fraction's toxicity, determined by cell

viability, as measured by the bioreduction of a MTS tetrazolium compound (MTS) to a coloured formazan product in the culture medium (Barltrop et al., 1991). This product was spectrophotometrically quantified with a VersaMax microplate reader (Molecular Devices, Sunnyvale, USA). The level of sample toxicity was related to the MTS-to-formazan conversion by the cells in the samples *versus* the unexposed controls.

A range of concentrations for toxicity testing was achieved through sample titration into complete Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Johannesburg, South Africa) supplemented with 10% fetal bovine serum (Hyclone, GE, Little Chalfont, UK), 25 mM HEPES (Life Technologies, Johannesburg, South Africa) and 0.05 mg/mL Gentamicin (Sigma-Aldrich, Johannesburg, South Africa). 293T cells were combined with the dilution and incubated for 2 days at 37^{o}C with 5% CO_2 in a humidified atmosphere. After adding the MTS reagent, plates were incubated for 1.5 hours after which spectrophometric absorbances were read at 490 nm, with a 690 nm reference wavelength. Cell viability was assessed by reference to the media (no drug) control: 0% viability indicates total cell death while a viability of 100% indicates complete cell survival. Cytotoxic Concentration-50 (CC_{50}) values were determined and indicate the sample concentration at which 50% of the cells are alive and metabolizing after treatment.

Non-toxic sample concentrations were used to determine the antiviral activity of the various fractions. A wild-type HIV-1 subtype C pseudovirus (p8.9MJ4) (Gupta et al., 2010) was used to screen for antiviral activity of the Zidovudine DTPs *in vitro*. Dilutions of each preparative chromatography fraction were titrated in 96-well culture plates and after the addition of 293T cells and pseudovirus, the plates were incubated for 48 hours at 37 ^{o}C with 5% CO_2 in a humidified atmosphere. Following incubation the Bright Glo™ Reagent (Promega) was used to determine the expression of firefly luciferase in infected cells. A Victor-3 1420 Multi Label Counter (Perkin Elmer) was used to quantify bioluminescence and the medium control was used as the untreated control. An unexposed (negative) control (cells, virus and growth medium) was included in the analysis. The viral activity was calculated as

the factor of the bioluminescence of the test sample compared to that of the unexposed control and expressed as a percentage. A viral activity of 0% indicates complete viral inhibition by the test substance, while 100% indicates full viral activity and no inhibition as a result of exposure to the compound(s) tested. Inhibitory concentration-50 (IC_{50}), is the concentration of sample at which 50% of the viruses were inhibited by a particular test compound. It should be noted that a decrease in luciferase activity can be observed over toxic concentrations as a result of compromised cell viability, and not as a result of inhibition of the virus; therefore an untreated cell control (100% cell viability, without virus) is used.

5.2.7 WWTW Effluent Analysis

Grab samples were collected from the environmental effluent discharge points of four WWTWs (three in Gauteng and one in Kwa-Zulu Natal province) in unused borosilicate Schott bottles (Glassworld, Johannesburg, South Africa). Samples were stored at -20ºC prior to extraction and again at -20ºC until analysis. Samples were filtered with a 1 µm glass-fibre syringe driven filter (Pall) and extracted with the Smart Prep Extraction System (Horizon, Salem, USA). Oasis HLB (Waters, Milford, USA) solid phase extraction (SPE) cartridges (6 cc, 500 mg) were conditioned with methanol and HPLC-grade water (4 and 6 mL respectively) and loaded with 500 mL of sample at a flow rate of 10 mL/min. Cartridges were dried under nitrogen for three minutes, eluted with 5 mL of methanol into 500 µL DMSO and dried under nitrogen to 500 µL. Extractions were performed at 18 ºC (+-2ºC) in a dedicated area.

Samples were analysed by UHPLC-QTOF in full scan and Auto MS/MS modes using source conditions as described previously. Each environmental sample (15 µL) was separated on an HPH C8 2.1 x 100 mm Poroshell column (Agilent) with water and acetonitrile (both with 0.1% formic acid) at 0.5 mL/min. The gradient was: 3 min, 2% B (organic); 22 min 100%; 25 min, 100%; 27 min, 2% and 30 min, 2%.

A representative group of the chlorination reactions and the various preparative chromatography fractions were analysed similarly and a searchable database was created using the Agilent PCDL Manager Software. Full scan and Auto MS/MS WWTW samples were screened against this database with a 0.2 minute retention time allowance and an 80% match factor cut-off criterion, which included the mass match factor of 1 ppm and isotopic distribution match.

5.3 Results and Discussion

The antiretroviral drug Zidovudine has been found to undergo a number of interesting changes in the literature (Table S5-3). Aparna et al. (2010) found that Zidovudine underwent photocatalytic reactions in pill form, resulting in a number of impurities. Amongst these is a dimer in which the azide group of one Zidovudine molecule is displaced while binding to the thymine component of another Zidovudine molecule. Similarly, the azide group may be displaced and form a double bond in the ring structure, resulting in the creation of Stavudine (another ARV drug). The transformation of Zidovudine by biological and photochemical processes in the open water wetland treatment process was described by Prasse et al. (2015). The authors found that the azide group was highly reactive during photochemical degradation, while the hydroxyl groups were mainly involved during biological transformation; resulting in carboxyl group formation (Table S5-3).

The reaction products arising as a result of Zidovudine chlorination have to the best of our knowledge not been described in the literature. This drug is subject to speciation by merit of its structure, which could mean that its reactivity profile would be highly dependent on pH.

5.3.1 Zidovudine Reaction Kinetics

Reaction kinetics were determined for Zidovudine using reactions incubated in the LC autosampler, this provides the minimum possible time from when the sample is reacted to when it is analysed. The only way to reduce this time would be to infuse

a reaction directly into the mass spectrometer without chromatography. Direct infusion was attempted in this research as described by Vanderford et al. (2008) yet it was found that the reaction buffer induced ion suppression of the targets and it was therefore abandoned. The autosampler-based approach was adopted since it was found that none of the commonly used quenching agents could be utilised to halt the reactions. Each measured time point occurred in a separate reaction vial when incubation times were less than the chromatographic time; otherwise the chromatographic runs were used to space the sampling time points.

Zidovudine (4 µM) in either water or 10 mM phosphate buffer (pH 5.8 or 8) was reacted with an excess of chlorine (100, 200 or 500 µM) in the equivalent buffer and analysed every hour for 24 hours. The Zidovudine was found to decrease rapidly and then increase over time to a stable point (Figure S5-5). No significant difference was noted between the reactions with 100, 200 or 500 µM NaOCl, as the reagent was in excess and did not affect the kinetics.

The reaction kinetics of Zidovudine, much like many pharmaceuticals (Deborde and von Gunten, 2008), are expected to be first order for each reactant with second order overall. Realistic environmental conditions were simulated by treating a low concentration of Zidovudine with an excess of free available chlorine (FAC) (25, 50 and 125 times molar excess) in order to determine reaction kinetics. A concentration of 4 µM Zidovudine was chosen since lower concentrations would necessitate sample preparation/concentration which could introduce bias. The reactions started within 30 seconds which was the earliest possible measurement as determined by measuring the time taken between addition of the NaOCl to the Zidovudine in the autosampler and the UHPLC injection. The reaction proceeded with the assumption that FAC was in excess as seen by the fact that there is no significant difference between the 100, 200 and 500 µM NaOCl reactions in each buffer. The reaction of Zidovudine (Zid) with FAC (NaOCl) can be described as follows (Soufan et al. 2012):

$$\frac{\mathrm{d[Zid]}}{\mathrm{d}t} = -\kappa[\mathrm{FAC}][\mathrm{Zid}] \qquad (1)$$

$$\frac{\mathrm{d[Zid]}}{\mathrm{d}t} = -\kappa_{obs}.\,[\mathrm{Zid}] \qquad (2)$$

where [Zid] is the total concentration of Zidovudine and [FAC] is the concentration of free available chlorine (in excess). κ is the second-order rate constant and the observed pseudo first-order rate constant is κ_{obs}; with $\kappa_{obs} = \kappa$ [FAC] and [FAC] = $[FAC]_0$. The κ values for Zidovudine chlorination (4 µM to 100 µM FAC), determined from the initial linear component of the natural logarithm of the reaction (Figure S5-6), were 4.6, 5.3 and 5.0 $M^{-1}.s^{-1}$ in water, at pH 8 and at pH 5.8 respectively. To place this in perspective, the antibiotic Levofloxacin is removed at a rate of 4400 $M^{-1}.s^{-1}$ under similar conditions (Najjar et al. 2013).

These values show that Zidovudine would not be removed effectively under realistic wastewater treatment conditions as has been shown in earlier research, with the prevalence of the drug in South African surface water (Wood et al., 2015). Even at chlorine levels 125 times that of Zidovudine, the drug persisted in the reactions. The realistic range of pH used during wastewater treatment was not seen to affect the degradation rate of Zidovudine appreciably. Kumar et al. (2014) found that Zidovudine could only be removed effectively at pH 3, a condition not likely to occur during real-world waste water treatment. This can most likely be attributed to the speciation of the molecule and that the HOCl species, which is a stronger oxidiser than OCl^-, would dominate at acidic pH levels.

It has been shown that wastewater matrix composition has a large impact on the efficacy of pharmaceutical transformation. This is due to the chlorine demand exerted by dissolved organic molecules, nitrites and ammonia (Lee and von Gunten, 2010). As a result of this, during wastewater treatment, it could be expected that Zidovudine transformation/degradation by chlorine would be even less efficient. Furthermore, an accurate second order rate constant could then not be determined as chlorine would not be in excess (at realistic levels) due to the demand placed on it by dissolved organic materials.

It is interesting to note that in the chlorination reactions the Zidovudine concentration decreases between 0 and 1 hour and then steadily increases to a

stable point over a number of hours (Figure S5-5). Overall, 20 % (+- 1.3 %) of the Zidovudine remains in all of the reactions at various pH and NaOCl concentrations.

As will be shown in the chlorination reactions utilising higher Zidovudine concentrations (stirred and large scale reactions); the molecule forms unstable conjugates with itself, its components and chlorinated components of itself. These molecules then react further, releasing intact Zidovudine back into the reaction. These conjugate compounds are unstable and are therefore most likely not present in the environment but could afford protective properties (from chlorination) to the intact Zidovudine which would be released into the environment once these conjugates/dimers degrade. In addition to this, the propensity of Zidovudine to form covalent interactions (Aparna et al. 2010) with itself may mean that it is as likely to interact with any one of a vast number of dissolved organic compounds during wastewater treatment; that could similarly provide protection against chlorination.

5.3.2 Zidovudine Disinfection Transformation Products

In order to identify and characterize the DTPs formed during Zidovudine chlorination, larger scale stirred reactions were utilized. This is because these molecules' concentrations were too low to detect in the autosampler reactions (under realistic WWTW concentration conditions). Reactions occurred at the same buffer and pH conditions as the autosampler reactions.

A number of authors utilize quenching agents to halt the chlorination process (Cai et al. 2013; Najjar et al. 2013). Yet it has also been found in the literature that these chemicals may reverse the reaction by reducing the chlorination products (Bedner and MacCrehan, 2006). For this reason, the most commonly used quenching agents, ascorbic acid, ammonium chloride and sodium thiosulfate were tested in single vial reactions as described previously. A 100 µM chlorination of Zidovudine was quenched with a twofold molar excess of each agent and analysed by UHPLC-QTOF (Figure S5-7). It was found that none of the quenching agents could be used to halt the chlorination reactions without adversely affecting the reaction

products. For all three quenching agents, the Zidovudine concentration was comparable to an untreated Zidovudine standard. This shows that the majority of the early reaction products should be relatively simple and readily reducible back to the original parent compound.

Analysis of the accurate mass and isotopic patterns of the reaction products revealed that the major mechanism of product formation is oxidation. Aliquots of the 100 µM chlorination reaction (pH 8 and pH 5.8 in 10 mM phosphate buffer) of Zidovudine were removed and analysed at various time points while the original reaction was allowed to continue. Zidovudine was quantified by external calibration against a curve generated by MassHunter Quant, which showed very good linearity ($R^2 > 0.999$) over a large dynamic range.

Both reactions (at pH 5.8 and 8) rapidly consume Zidovudine (30 % remaining), yet over time the Zidovudine concentration increases to a stable point (70-80 % remaining; Figure S5-8 and Figure S5-9). This, as with the quenching agent results, highlights the reversible nature of the DTPs produced during chlorination.

The most abundant product (*m/z* 161) in the stirred reactions was found to be a chlorinated thymine molecule (Figure 5-1). Thymine is the most abundant peak in the Zidovudine mass spectrum (*m/z* 127) and arises as a result of collision induced disassociation (CID) in the ion source. The DTP (*m/z* 161) clearly displays an isotopic pattern indicative of the presence of chlorine.

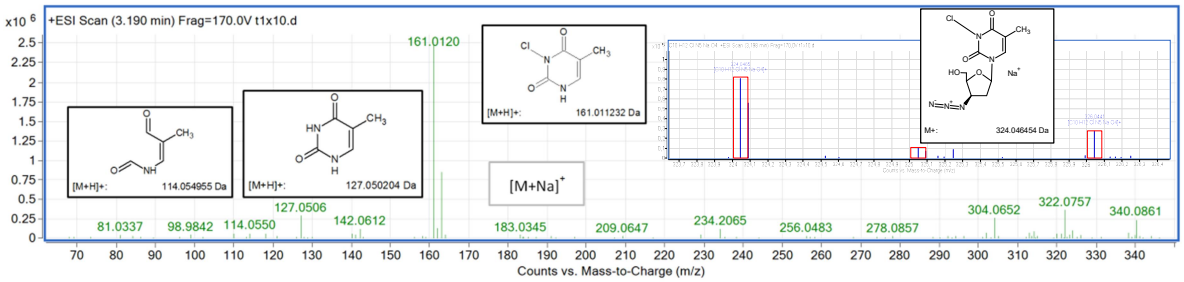


Figure 5-1: Positive ESI mass spectrum of the major disinfection transformation product resulting from the chlorination Zidovudine. Putative structures and fragments are presented as insets as well as the zoomed mass spectrum of the sodiated intact molecule. Theoretical isotopic fit (97 %) is presented as red bars over spectrum peaks with putative formulae.

There appears to be two pathways to the formation of the thymine-chloride molecule (Figure 5-2), resulting in two isomeric forms depending on which of thymine's two nitrogens are chlorinated. As these are isomers they are indistinguishable during full scan analysis. It is possible that the molecules' retention times may differ (there is a peak shoulder in the extracted chromatogram of *m/z* 161), yet their identity can only be fully resolved by comparison to an analytical standard.

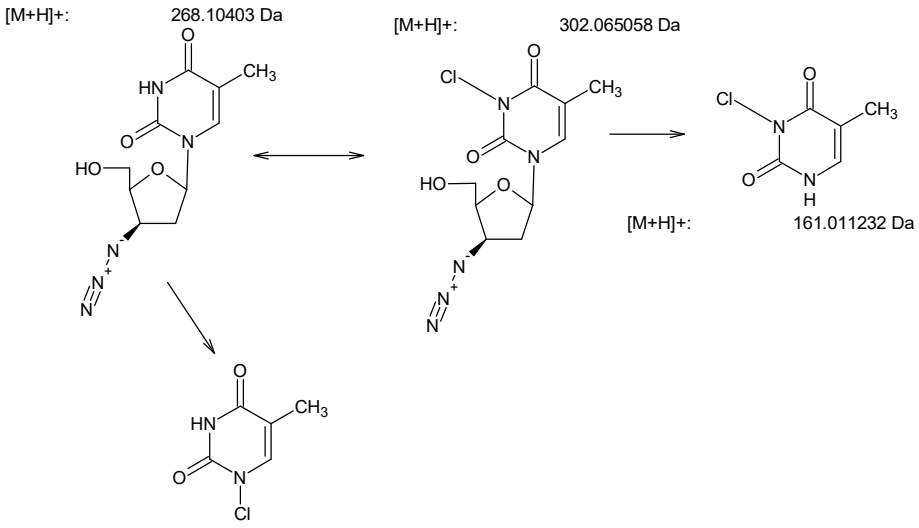


Figure 5-2: The proposed chlorination reaction pathway of the thymine component of Zidovudine leading to the formation of two thymine-chloride isomers (*m/z* 161).

The rationale behind proposing the second pathway is due to the presence of higher molecular mass species in the spectrum. Once the neutral loss of the other half of the Zidovudine molecule was added, the calculated pseudomolecular ion (161 + 141 = *m/z* 302.065) was found in the spectrum with high mass accuracy, albeit at low intensity. The sodiated pseudomolecular ion (spectrum presented as an inset in Figure 5-1) however was present at a higher intensity, with characteristic chlorine isotopes present (theoretical isotopic fit 97 %). There are a number of unidentified high mass species in this region of the spectrum, which co-elute and may be closely related on a structural level.

The *m/z* 161 ion was monitored over the time course (Figure S5-10) and it is clear that the compound is not stable. The *m/z* 302 compound, of which *m/z* 161 is a part, followed an inverted concentration trend when compared to Zidovudine degradation. The compound's reduction may account for the reintroduction of intact Zidovudine back into the reaction mixture.

Zidovudine's azide group has been described in the literature to undergo elimination and rearrangement of hydrogen, resulting in a loss of either N_2 or HN_3

during mass spectrometric fragmentation (Xiao et al., 2003). This phenomenon was not encountered with the mass spectrometric conditions used in this work for the analysis of a pure Zidovudine standard. The hydrogen elimination was however noted as a result of chlorination of Zidovudine and this compound (*m/z* 210) and its hydroxylated form was detected as a peak shoulder of the Zidovudine remaining in solution after chlorination (Figure 5-3).

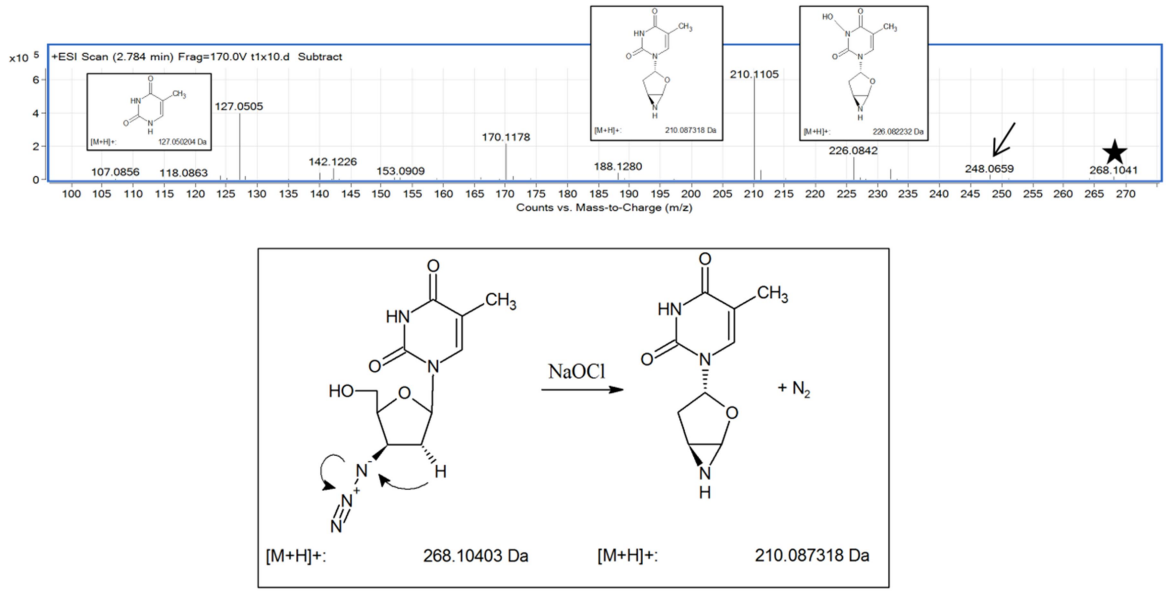


Figure 5-3: Positive ESI mass spectrum of a Zidovudine disinfection transformation product following treatment with NaOCl. Insets describe protonated mass spectral fragments; the sodiated pseudomolecular ion is indicated by an arrow and a peak impurity is indicated (Zidovudine) with a star as the compound of interest co-eluted with Zidovudine. The proposed reaction pathway consists of a rearrangement involving the azide group (Xiao et al., 2003).

5.3.3 Large Scale Reactions

The Zidovudine reaction was scaled up in order to isolate the chlorination DTPs for characterization, toxicity testing and antiviral studies. A solid light brown product was obtained from the up-scaled stirred chlorination reaction. The reaction mixture is highly soluble in water (up to 600 mg/ml) yet it was found to be insoluble/partially soluble in most of the solvents commonly used in preparative chromatography such as: hexane, ethyl acetate, dichloromethane and methanol. The reaction

products are therefore most likely highly polar. Compounds were separated using the same mobile phases as selected for the analytical chromatography.

It was found that the large majority of compounds co-eluted in a single peak, early in the chromatographic run. Each fraction was analysed by UHPLC-QTOF and although all of the fractions contained at least two compounds, a number of unique fractions were obtained. Unfortunately, the most polar compounds bled into all of the fractions due to over-loading of the column. The column was intentionally overloaded in order to obtain enough of the lower concentration products.

Further proof of the Zidovudine adduct formation concept is found in the mass spectral analysis of the large scale Zidovudine chlorination reaction and the subsequent preparative chromatography fractions. Analysis of these reveals the ubiquitous presence of the Thymine component of Zidovudine in a number of the DTPs (Figure S5-11). These are most likely heteromers composed of intact and partial fragments of the Zidovudine molecule.

The major reaction product in the large scale reaction was found to be a Zidovudine heterodimer. The structure proposed is a molecule composed of Zidovudine associated by non-covalent interaction with another oxidized Zidovudine molecule. Mass spectrometric analysis of these compounds (Figure 5-4) showed the prominent *m/z* 127 peak; which is the main collision dissociation product of the original Zidovudine molecule.

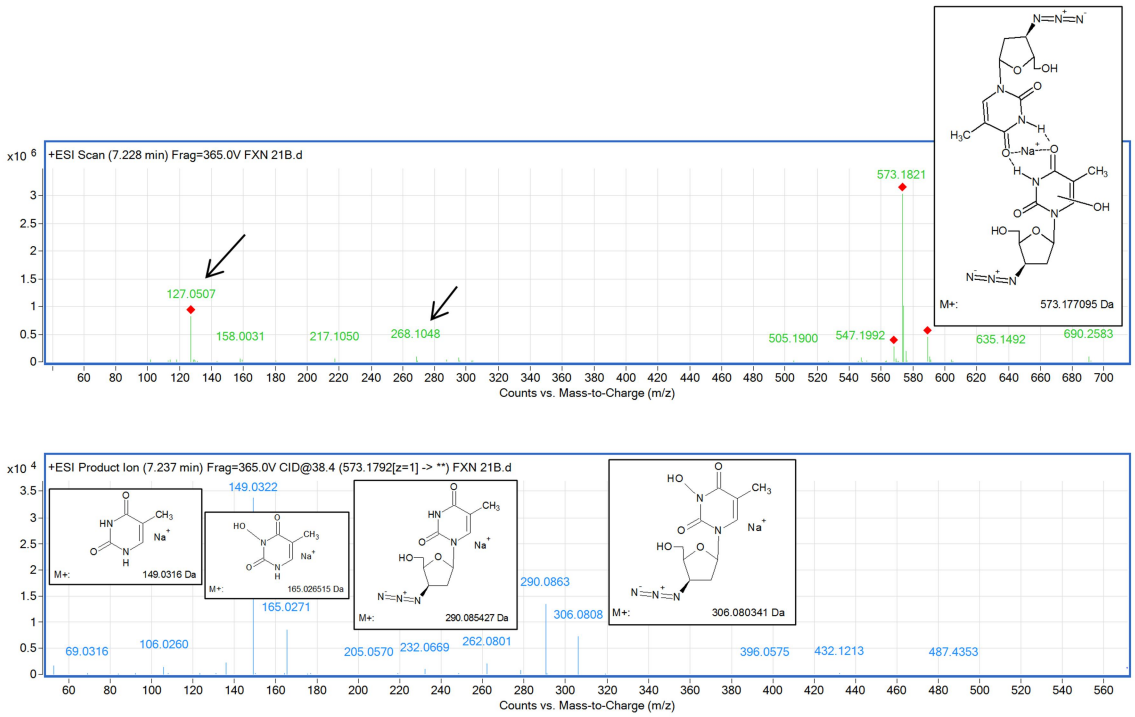


Figure 5-4: Positive ESI full scan (top) and Auto MS/MS (CID 38.4 eV) mass spectra of the major large scale Zidovudine disinfection transformation product ($[M+Na]^+$ *m/z* 573). This is a sodiated oxidized heterodimer of Zidovudine formed through hydrogen bonding as proposed by Pan (2008). In full scan analysis the Zidovudine pseudomolecular ion (*m/z* 268) as well as its major in-source fragmentation product (*m/z* 127) appear as CID products (arrows). Ions selected for Auto MS/MS are indicated with red diamonds. MS/MS of *m/z* 573 shows a neutral loss of Zidovudine resulting in the (sodiated) intact second member of the heterodimer (*m/z* 306) as well as the equivalent loss of the heterodimer to produce the sodiated Zidovudine. Correspondingly both the sodiated thymine and oxidized thymine adducts (m/z 149 and 165) are found. The ready loss of Zidovudine in the gas phase ionic fragmentation could be a reflection of the equivalent ready decomposition of the aqueous heterodimer to revert back to free Zidovudine.

In addition to this, the pseudomolecular Zidovudine ion is also present in the spectrum. This shows that Zidovudine is liberated during in-source fragmentation. It is proposed that the sodium molecule is “trapped” in the hydrogen bonded dimer, as similarly proposed by Pan (2008), who showed that a stable eight member ring with a hydrogen bridge may be formed with similar chemical moieties during electrospray ionisation. It is proposed that a similar phenomenon occurs for Zidovudine hetrodimerisation in solution prior to MS analysis. The reaction product

that contains Zidovudine cannot be confused with the Zidovudine remaining in the mixture since their chromatographic retention times differ substantially (> 1 minute). The molecule could also not arise as an artifact of mass spectral interaction between the oxidised Zidovudine and intact Zidovudine, for the same reason (i.e. differing retention times). The heterodimer therefore forms in solution and becomes sodiated during MS analysis.

Auto MS/MS analysis selected the *m/z* 573 ion for fragmentation, which lead to a neutral loss of intact Zidovudine (*m/z 573* → 306). The *m/z* 306 ion represents the intact sodiated-oxidised Zidovudine molecule, which is the second member of the heterodimer. Through mass spectral interpretation it could be determined that it is the thymine component of this molecule that is oxidized and sodiated (through the presence of the *m/z* 165 and 149 ions which are sodiated thymine with and without oxygen respectively). There appears to be a relatively strong association between sodium and this molecule as it remains bound to the molecule through the various stages of mass spectral fragmentation.

The phenomenon of non-covalent interaction of pharmaceuticals with themselves in solution and their subsequent release as the chlorination reaction proceeds may serve to explain their presence in the environment. The interaction could afford the molecule protection from degradation during disinfection, to be again discharged into the environment via a reversible reaction. This phenomenon is more prevalent, *in vitro*, when studying higher concentrations of Zidovudine, and can be explained by the proximity of the molecules to each other in solution.

In the 11 fractions that showed anti-HIV activity (Figure S5-12) up to 12 unique compounds could be found. Some of these are unique to each fraction while others are present in all the active fractions. The activity may therefore be attributed to a single compound or even as a result of synergy between multiple compounds. The true nature and activity of these compounds may only, however, be fully understood after further purification and structural elucidation studies.

5.3.4 In Vitro Toxicity and Antiviral Activity

Neither the total Zidovudine chlorination reaction product (Table 5-1) nor the flash chromatography fractions (Table 5-2) thereof showed any signs of toxicity *in vitro* as compared to the two control Zidovudine standards from Fluka or the NIH. Although Zidovudine shows little toxicity *in vitro* (up to 100 µg/mL), the compound does induce anaemia in patients (Adediran et al., 2016), which highlights the need to test compounds in multiple cell systems and in patients before unequivocal statements regarding toxicity can be made. From an ecotoxicological perspective it is heartening however that the disinfection transformation products of Zidovudine are not overtly toxic or at least not more so than the parent molecule. Again it must be borne in mind that only one cell line was used for screening. Specific aspects such as hepatotoxicity or neurotoxicity cannot be inferred from this data.

Table 5-1: Total acidic Zidovudine chlorination product toxicity and antiviral activity as compared to two Zidovudine controls (Fluka and NICD). Average cell viability (293T cells) determined as a percentage compared to an unexposed control with standard deviation presented in brackets. Viral activity is expressed as a percentage where 100% indicates that the compound had no effect on the virus and 0 % indicates full viral inhibition; standard deviation in brackets (n = 3).

	Toxicity			**Antiviral Activity**		
Concentration	**% Cell Viability (Std Deviation)**			**% Viral Activity (Std Deviation)**		
µg/mL	Total Zidovudine Chlorination Reaction Product	Zidovudine Control (Fluka)	Zidovudine Control (NICD)	Total Zidovudine Chlorination Reaction Product	Zidovudine Control (Fluka)	Zidovudine Control (NICD)
100.0	100 (1.9)	99.1 (1.2)	100 (1.4)	7.2 (1.2)	0.0 (0.0)	0.0 (0.0)
33.3	100 (13.8)	100 (1.7)	96.8 (1.5)	17.30 (0.3)	0.1 (0.0)	0.0 (0.0)
11.1	97.6 (1.2)	100 (2.1)	97.8 (0.6)	29.8 (2.8)	0.1 (0.0)	0.1 (0.0)
3.7	100 (1.0)	100 (0.7)	100 (1.0)	47.5 (2.8)	0.5 (0.0)	0.2 (0.0)
1.2	97.5 (3.2)	96.7 (0.5)	100 (4.6)	65.1 (1.3)	2.2 (0.3)	2.1 (0.1)
0.4	97.9 (2.9)	99.8 (2.6)	100.1 (1.9)	74.6 (3.1)	6.5 (0.1)	6.5 (0.6)
0.1	96.5 (4.0)	99.3 (3.4)	104.3 (7.6)	87.3 (4.2)	14.8 (0.4)	15.2 (0.7)
0.05	99.0 (2.4)	98.8 (1.2)	99.4 (2.0)	90.5 (1.4)	28.1 (0.9)	28.7 (4.2)
0.02	100 (0.1)	101.6 (0.5)	99.3 (1.4)	94.6 (3.8)	42.4 (1.4)	43.3 (4.9)
0.005	99.1 (3.1)	100 (0.5)	98.2 (2.6)	96.9 (2.9)	54.6 (1.2)	60.3 (4.0)
0.002	100 (2.1)	100 (0.1)	100 (1.8)	100 (0.3)	61.1 (0.9)	65.7 (16.4)

Table 5-2: Zidovudine chlorination reaction flash chromatography fraction toxicity screening data. Average cell viability (293T cells) of each fraction at 10 µg/mL determined as a percentage compared to an unexposed control with standard deviation presented in brackets. Viral activity is expressed as a percentage where 100% indicates that the compound had no effect on the virus and 0 % indicates full viral inhibition after exposure to each fraction at 10 µg/mL; standard deviation in brackets (n = 3).

	Toxicity	**Antiviral Activity**
Zidovudine Chlorination Reaction Fraction	**% Cell Viability (Std Deviation)**	**% Antiviral Activity (Std Deviation)**
Zid. Rxn. FC B1	97.9 (0.9)	107.9 (8.6)
Zid. Rxn. FC B2	99.0 (0.6)	97.8 (2.7)
Zid. Rxn. FC B3	100 (4.9)	100 (4.3)
Zid. Rxn. FC B4	100 (0.6)	91.1 (5.4)
Zid. Rxn. FC B5	100 (0.8)	97.6 (3.6)
Zid. Rxn. FC B6	100 (1.5)	96.4 (4.0)
Zid. Rxn. FC B7	100 (2.7)	97.5 (4.2)
Zid. Rxn. FC B8	100 (0.6)	86.9 (1.6)
Zid. Rxn. FC B9	100 (1.3)	93.0 (1.8)
Zid. Rxn. FC B10	100 (1.7)	92.5 (1.3)
Zid. Rxn. FC B11	100 (2.0)	95.6 (3.7)
Zid. Rxn. FC B12	100 (1.5)	96.5 (1.6)†
Zid. Rxn. FC B13	100(1.4)	100(4.3)†
Zid. Rxn. FC B14	100(1.4)	90.1 (2.3)*†
Zid. Rxn. FC B15	100(0.5)	76.8 (2.0)*†
Zid. Rxn. FC B16	100 (6.4)	73.2 (1.2)†
Zid. Rxn. FC B17	98.5 (1.3)	77.4 (3.7)†
Zid. Rxn. FC B18	100 (0.3)	76.4 (3.2)
Zid. Rxn. FC B19	97.9 (1.2)	69.4 (4.1)**††
Zid. Rxn. FC B20	97.6 (4.9)	3.2 (0.1)**††
Zid. Rxn. FC B21	100 (2.6)	0.7 (0.1)**††

	Toxicity	Antiviral Activity
Zidovudine Chlorination Reaction Fraction	**% Cell Viability (Std Deviation)**	**% Antiviral Activity (Std Deviation)**
Zid. Rxn. FC B22	100 (1.1)	3.2 (0.6)††
Zid. Rxn. FC B23	100 (3.0)	22.3 (0.3)
Zid. Rxn. FC B24	100 (2.6)	32.6 (1.9)
Zid. Rxn. FC B25	100 (1.0)	34.4 (4.9)
Zid. Rxn. FC B42	100 (1.2)	82.7 (1.9)
Zid. Rxn. FC B44	100 (1.2)	94.2 (4.5)
Zid. Rxn. FC B50	100 (0.9)	95.0 (0.3)
Zid. Rxn. FC B51	100 (6.9)	92.8 (5.1)
Zid. Rxn. FC B54	99.9 (0.8)	95.4 (2.3)
Zid. Rxn. FC B57	100 (1.1)	103.9 (2.9)

* Contains the Zidovudine pseudomolecular ion (*m/z* 268) at the same retention time as a Zidovudine standard.

** Contains the Zidovudine pseudomolecular ion (*m/z* 268) at a different retention time to a Zidovudine standard, arising as a result of dimerization.

† Contains the Zidovudine thymine CID ion (*m/z* 127) at the same retention time as a Zidovudine standard.

†† Contains the Zidovudine thymine CID ion (*m/z* 127) at a different retention time to a Zidovudine standard.

The antiviral activity assay investigates the inhibition of viral replication in a single cycle of infection. Virus-like particles that contain HIV-1 subtype C reverse transcriptase, integrase and protease were used. The virus-like particle also contains the RNA transcript of the firefly luciferase protein. During successful infection, complementary DNA (cDNA) is produced by HIV-1 reverse transcriptase from the firefly RNA template and integrated into the host cell's DNA by HIV-1 integrase. Once integrated, the firefly luciferase gene is expressed to produce active firefly luciferase that can then be assayed via a bioluminescence reaction and quantified. There is a directly proportional relationship between the bioluminescent signal and the number of infectious virus particles present in the initial inoculum. Treatment with pharmaceuticals (e.g. ARVs) or DTPs inhibits viral

activity and decreases the number of firefly luciferase gene copies that are integrated into the host's genome resulting in a concomitant decrease in the amount of bio-luminescence. The ratio of bioluminescence between an exposed sample and an unexposed control is presented as a percentage. This would give an indication of the effect of a test compound on viral activity; i.e. its utility as a therapeutic compound.

Control Zidovudine obtained from Fluka and the NIH both showed anti-HIV activity *in vitro.* The total Zidovudine chlorination reaction product (Table 5-1) was less potent, only showing anti-HIV activity at higher concentrations than the parent molecule (Figure S5-13). The chlorinated Zidovudine fractions show activity in the mid-range of the chromatographic separation (Table 5-2, fractions B15 to B42). Once analysed by UHPLC-MS, it was found that Zidovudine is present in some of the active fractions (B20, B21 and B22), but not all of them. The concentration of Zidovudine in these fractions is however not high enough to account for the fractions' levels of antiviral activity. A number of the (impure) fractions showed marked anti-HIV activity, and further purification must be carried out before final assessments regarding activity of each reaction product can be made.

Twelve of a total of 31 preparative chromatography fractions showed viral inhibition higher than 10 %. In 6 of the 31 fractions, protonated thymine ions were detectable at Zidovudine's retention time (+- 0.1 min) and in two of these the intact Zidovudine pseudomolecular ion (*m/z* 268) could be detected (Figure S5-14). Of these six, four fractions displayed viral inhibition >10%. In four of the fractions, protonated thymine ions could be detected at a retention time different to Zidovudine (> 1 min) and all displayed a viral inhibition > 10% (Figure S5-15). Five fractions displayed diminished viral activity (with the lowest at 22.3% activity) in which neither the Zidovudine pseudomolecular ion nor the thymine ion could be detected. Fraction B21, which had the highest concentration of the Zidovudine heterodimer (described in Figure 5-4) showed almost complete viral inhibition (0.7 % remaining) comparable to Zidovudine at a similar concentration. It is postulated that once the

heterodimer enters the culture medium it degrades and reverts to the original Zidovudine molecule, thereby inducing an identical mechanism of action.

5.3.5 WWTW Effluent Analysis

A database containing the retention time, measured pseudomolecular mass, predicted mass and where possible MS/MS spectra was created for the DTPs generated in the laboratory at the various scales of reaction and for the isolated preparative chromatography fractions. The effluent from four WWTWs were screened against this database and none of the DTP compounds were detectable in samples. Zidovudine was however detected (S/N > 10) in all of the samples at the expected retention time with a mass accuracy of less than 1 ppm. Quantification was not carried out as this had been performed in earlier research (Wood et al., 2015).

The reasons for the absence of the DTP compounds in the environment may be threefold: they are not formed during industrial wastewater disinfection, they are present below the limits of detection or they are so short-lived that they do not enter the water cycle in the dimer form. Alternatively, with the discovery of Zidovudine's ability to dimerise in solution, the molecule may form associations with dissolved organic molecules during wastewater treatment, thereby affording it protection from chlorination. It is more likely that the Zidovudine molecule would form conjugates with dissolved organic compounds other than itself purely as a result of proximity (due to the low concentration of Zidovudine in solution which reduces the probability of self-association). The nature and identity of the Zidovudine DTP compounds require further investigation.

5.4 Conclusion

The antiretroviral drug Zidovudine has been found to be resistant to chlorination under conditions (pH and chlorine to drug ratios) typically used during waste water treatment. The drug degrades slowly, compared to other pharmaceuticals during

disinfection, and up to 20 % of the compound may remain in solution even when free available chlorine is in excess. These findings may serve to explain the discharge of Zidovudine from WWTW and its subsequent presence in surface water.

As Zidovudine concentration increases, relative to chlorine, an increased propensity for the formation of Zidovudine conjugates is found. The dimer formation, although removing free Zidovudine from solution, may contribute to the drug's environmental presence by affording a level of protection to the chlorination reaction. It may be speculated that the dimer could then enter the environment, degrade and release intact Zidovudine. In wastewater streams with more realistic Zidovudine concentrations, temporary association with dissolved organic species could outweigh the above reaction pathway.

In this work, the *in vitro* behavior of Zidovudine chlorination products is reported for the first time. None of these new compounds were found to be more toxic than the original parent compound. This is a positive eventuality from an environmental perspective, yet before inferences can be made, full toxicity studies should be performed.

We have for the first time shown that Zidovudine may react to form novel active anti-HIV compounds when chlorinated. This was established by isolating active fractions by preparative chromatography. Although HIV does not have an environmental host or an environmental component to its "lifecycle", the release of biologically active molecules (intact parent drugs or DTPs) may have a negative effect on the environment justifying further research into the antiretroviral degradation products.

Acknowledgements

Our sincere thanks to Dr Taki Netshiozwi for his advice and support.

Funding Source

We gratefully acknowledge the South African Military Health Services for funding this research. The funding agency did not play a role in the study design or data interpretation. The *in vitro* toxicity and activity screening was supported by the National Health Laboratory Services Research Trust.

5.5 References

1. Acero, J.L. et al., 2013. Chlorination and bromination kinetics of emerging contaminants in aqueous systems. *Chemical Engineering Journal*, 219, 43–50.
2. Adediran, A. et al., 2016. Impact of HIV Infection and Zidovudine Therapy on RBC Parameters and Urine Methylmalonic Acid Levels. *Interdisciplinary Perspectives on Infectious Diseases*, ID 5210963.
3. Agunbiade, F.O. & Moodley, B., 2015. Occurrence and distribution pattern of acidic pharmaceuticals in surface water, wastewater and sediment of the Msunduzi River, Kwazulu-Natal, South Africa. *Environmental Toxicology and Chemistry,* 35(1), 36-46.
4. Agunbiade, F.O. & Moodley, B., 2014. Pharmaceuticals as emerging organic contaminants in Umgeni River water system, KwaZulu-Natal, South Africa. *Environmental Monitoring and Assessment*, 186(11), 7273–7291.
5. Aparna, P. et al., 2010. Identification, isolation, and characterization of potential degradation products in a triple combination lamivudine, zidovudine, and nevirapine tablet for oral suspension. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, 65(5), 331–335.
6. Barltrop, J.A. et al., 1991. 5-(3-carboxymethoxyphenyl)-2-(4, 5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium, inner salt (MTS) and related analogs of 3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) reducing to purple water-soluble formazans as cell-viability indicators. *Bioorganic & Medicinal Chemistry Letters*, 1(11), 611–614.

7. Bedner, M. & MacCrehan, W.A., 2006. Transformation of acetaminophen by chlorination produces the toxicants 1, 4-benzoquinone and N-acetyl-p-benzoquinone imine. *Environmental Science & Technology*, 40(2), 516–522.
8. Cai, M. et al., 2013. Influencing factors and degradation products of antipyrine. *Environmental Science & Technology*, 40(2), 516–522.
9. Cardoso, O., Porcher, J.-M. & Sanchez, W., 2014. Factory-discharged pharmaceuticals could be a relevant source of aquatic environment contamination: Review of evidence and need for knowledge. *Chemosphere*, 115, 20-30.
10. Daughton, C. & Scuderi, M., 2012. Pharmaceuticals and Personal Care Products (PPCPs): Relevant Literature. *US Environmental Protection Agency, Las Vegas, NV*.
11. Deborde, M. & von Gunten, U., 2008. Reactions of chlorine with inorganic and organic compounds during water treatment—Kinetics and mechanisms: A critical review. *Water Research*, 42(1), 13–51.
12. Graham, J.P. & Polizzotto, M.L., 2013. Pit latrines and their impacts on groundwater quality: a systematic review. *Environmental Health Perspectives*, 121(5), 521–530.
13. Gupta, R.K. et al., 2010. Full length HIV-1 gag determines protease inhibitor susceptibility within in vitro assays. *AIDS (London, England)*, 24(11), p.1651.
14. Jain, S. et al., 2013. Occurrence and Removal of Antiviral Drugs in Environment: A Review. *Water, Air, & Soil Pollution*, 224(2), 1–19.
15. Järhult, J.D., 2012. Oseltamivir (Tamiflu) in the environment, resistance development in influenza A viruses of dabbling ducks and the risk of transmission of an oseltamivir-resistant virus to humans—a review. *Infection Ecology & Epidemiology*, 2.
16. Kumar, P., Kumar, P.S. & Kulkarni, R., 2014. Fate of Zidovudine through water treatment with chlorine: a kinetic study. *International Research Journal of Environment Sciences*, 3(9), 50–55.

17. Kümmerer, K., 2009a. Antibiotics in the aquatic environment-a review-part I. *Chemosphere*, 75(4), 417–434.
18. Kümmerer, K., 2009b. Antibiotics in the aquatic environment-a review-part II. *Chemosphere*, 75(4), 435–441.
19. Lee, Y. & von Gunten, U., 2010. Oxidative transformation of micropollutants during municipal wastewater treatment: comparison of kinetic aspects of selective (chlorine, chlorine dioxide, ferrate VI, and ozone) and non-selective oxidants (hydroxyl radical). *Water Research*, 44(2), 555–566.
20. Li, B. & Zhang, T., 2013. Different removal behaviours of multiple trace antibiotics in municipal wastewater chlorination. *Water Research*, 47(9), 2970–82.
21. Luo, Y. et al., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Science of The Total Environment*, 473, 619–641.
22. Matsushita, T. et al., 2015. Changes in mutagenicity and acute toxicity of solutions of iodinated X-ray contrast media during chlorination. *Chemosphere*, 135, 101–107.
23. Mulenga, V. et al., 2016. Abacavir, zidovudine, or stavudine as paediatric tablets for African HIV-infected children (CHAPAS-3): an open-label, parallel-group, randomised controlled trial. *The Lancet Infectious Diseases*, 16(2), 169–179.
24. Najjar, E. et al., 2013. Aqueous chlorination of levofloxacin: Kinetic and mechanistic study, transformation product identification and toxicity. *Water Research*. 47, 121-129.
25. Pan, H., 2008. A non-covalent dimer formed in electrospray ionisation mass spectrometry behaving as a precursor for fragmentations. *Rapid Communications in Mass Spectrometry*, 22(22), 3555–3560.
26. Papageorgiou, M., Kosma, C. & Lambropoulou, D., 2016. Seasonal occurrence, removal, mass loading and environmental risk assessment of 55 pharmaceuticals and personal care products in a municipal wastewater

treatment plant in Central Greece. *Science of The Total Environment*, 543, 547–569.

27. Peng, X. et al., 2014. Occurrence and ecological potential of pharmaceuticals and personal care products in groundwater and reservoirs in the vicinity of municipal landfills in China. *Science of The Total Environment*, 490, 889–898.
28. Petrie, B., Barden, R. & Kasprzyk-Hordern, B., 2014. A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring. *Water Research*, 72, 3-27.
29. Prasse, C. et al., 2015. Co-occurrence of Photochemical and Microbiological Transformation Processes in Open-Water Unit Process Wetlands. *Environmental Science & Technology*, 49(24), 14136–14145.
30. Rodriguez-Mozaz, S. et al., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Research*, 69, 234–242.
31. Rougemont, M. et al., 2016. Safety of zidovudine dose reduction in treatment-naïve HIV infected patients. A randomized controlled study (MiniZID). *HIV Medicine*, 17(3), 206–215.
32. Schoeman, C. et al., 2015. Quantification of Selected Antiretroviral Drugs in a Wastewater Treatment Works in South Africa Using GC-TOFMS. *Journal of Chromatography & Separation Techniques*, 6, No. 4..
36. Soufan, M., Deborde, M. & Legube, B., 2012. Aqueous chlorination of diclofenac: Kinetic study and transformation products identification. *Water Research*, 46, 3377-3386.
33. Vanderford, B.J. et al., 2008. Real-time detection and identification of aqueous chlorine transformation products using QTOF MS. *Analytical Chemistry*, 80(11), 4193–4199.
34. Wang, X. et al., 2015. Effect of oxidation on amine-based pharmaceutical degradation and N-Nitrosodimethylamine formation. *Water Research*, 87, 403–411.

35. WHO, 2013. Global update on HIV treatment 2013: results, impact and opportunities.
36. Wode, F. et al., 2015. Search for over 2000 current and legacy micropollutants on a wastewater infiltration site with a UPLC-high resolution MS target screening method. *Water Research*, 69, 274–283.
37. Wood, T.P., Duvenage, C.S. & Rohwer, E., 2015. The occurrence of anti-retroviral compounds used for HIV treatment in South African surface water. *Environmental Pollution*, 199, 235–243.
38. Xiao, Q. et al., 2003. Electrospray ionization mass spectrometry of AZT H-phosphonates conjugated with steroids. *Rapid Communications in Mass Spectrometry*, 17(13), 1405–1410.
39. Zhai, H. et al., 2014. Formation of brominated disinfection byproducts during chloramination of drinking water: New polar species and overall kinetics. *Environmental Science & Technology*, 48(5), 2579–2588.

5.6 Supporting Information

Transformation of the Anti-HIV Drug Zidovudine During Simulated Wastewater Chlorination and the Subsequent Formation of Biologically Active Zidovudine Heterodimers

Timothy Paul Wood[a,d] (Corresponding Author)

Thembela Ntlemeza[a]

Adriaan Erasmus Basson[b]

Cornelia Duvenage[c]

Egmont Richard Rohwer[d]

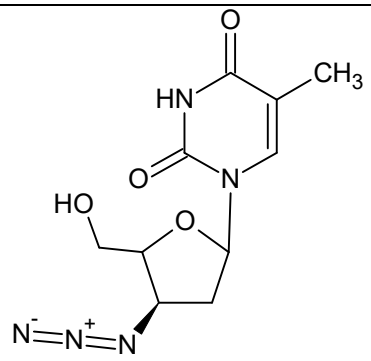
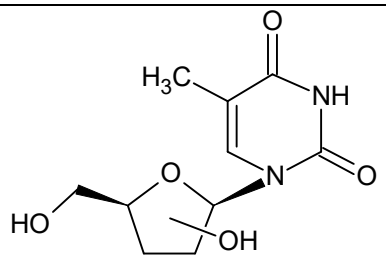
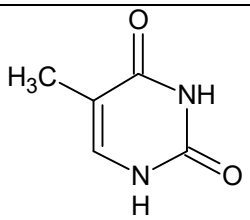
[a] Protechnik Laboratories, a Division of ARMSCOR SOC Ltd, 103 Combretum Crescent, Centurion, Pretoria, 0001, South Africa. Tel: +27 12 665 9444, timw@protechnik.co.za

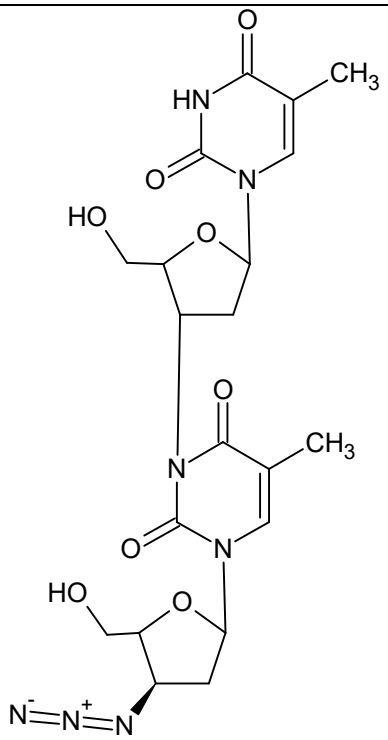
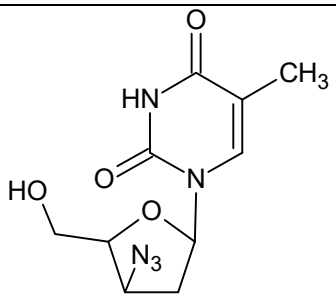
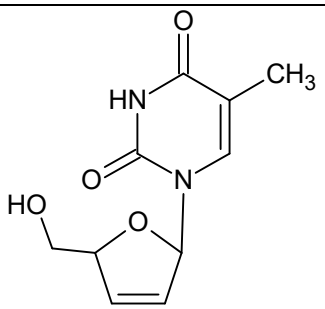
[b]Centre for HIV and STI: HIV Virology Section (Morris Laboratory), National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS). 1 Modderfontein Road, Sandringham, 2131, South Africa. Tel: +27 11 386 6541, adriaanb@nicd.ac.za

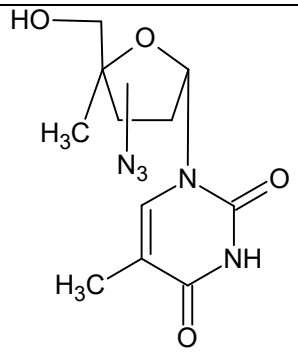
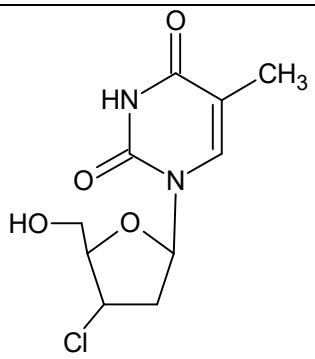
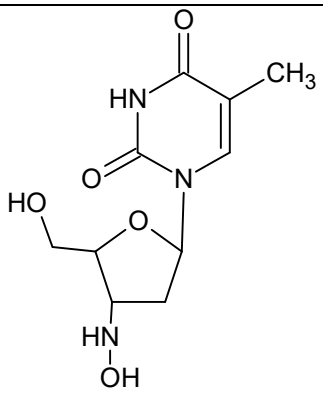
[c]Department of Internal Medicine, 1 Military Hospital, South African Military Health Services, Voortrekker Street, Pretoria, 0001, South Africa. Tel: +27832733382, corneliaduv@mtnloaded.co.za

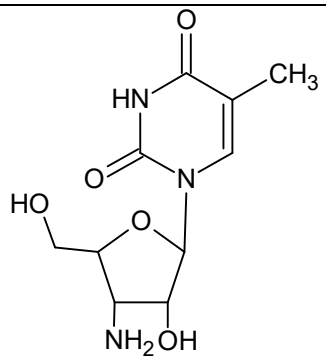
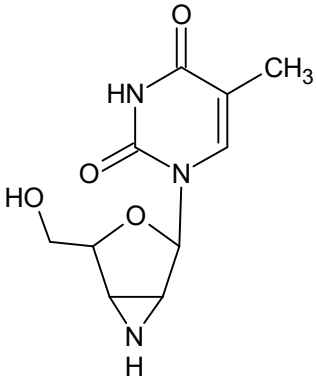
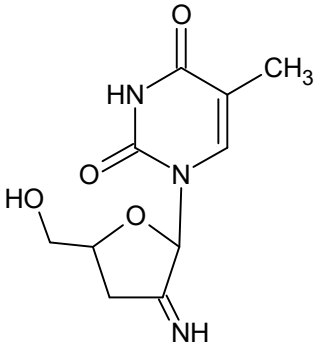
[d] Department of Chemistry, University of Pretoria, Lynwood Road, Pretoria, 0001, South Africa. Tel: +27 12 420 3772 email: egmont.rohwer@up.ac.za

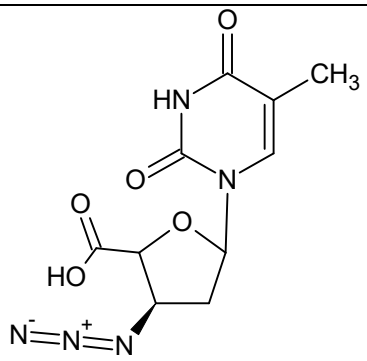
Table S5-3: Selected structures of Zidovudine transformation and degradation products from the literature.

Name	Structure
Zidovudine	 [M+H]+: 268.10403 Da
Pharmaceutical Zidovudine Tablet Degradation Products and Impurities (Aparna et al., 2010)	
Beta-thymidine	 [M+H]+: 243.097548 Da
Thymine	 [M+H]+: 127.050204 Da

Name	Structure
Dimer	 [M+H]+: 492.183737 Da
β-Azido Zidovudine	 [M+H]+: 268.10403 Da
Stavudine	 [M+H]+: 225.086983 Da

Name	Structure
α-Thymidine Analog	 [M+H]+: 282.11968 Da
Chloro impurity	 [M+H]+: 261.063661 Da
Photo- and Biodegradation Products of Zidovudine (Prasse et al., 2015)	
Zidovudine TP257	 [M+H]+: 258.108447 Da

Name	Structure
Zidovudine TP126	 [M+H]+: 258.108447 Da
Zidovudine TP239	 [M+H]+: 240.097882 Da
Zidovudine TP239	 [M+H]+: 240.097882 Da

Name	Structure
Zidovudine Carboxylate	 [M+H]+: 282.083295 Da

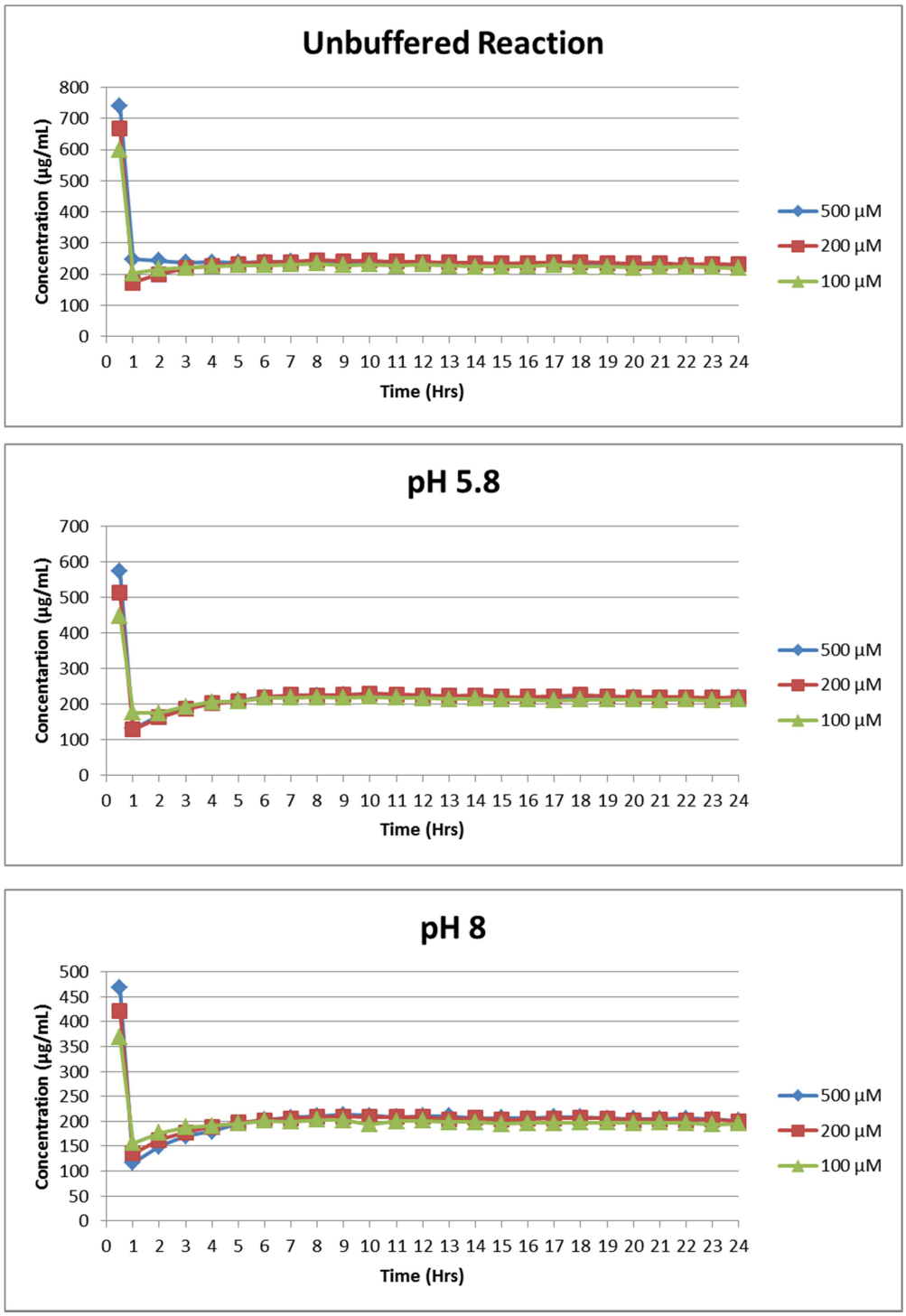


Figure S5-5: The concentration of Zidovudine (4 µM) over time after chlorination by NaOCl (100, 200 and 500 µM) in 10 mM Phosphate buffer (pH 5.8 and 8) or without buffer. Analysed by UHPLC-QTOF with external calibration. t0 = 30 seconds.

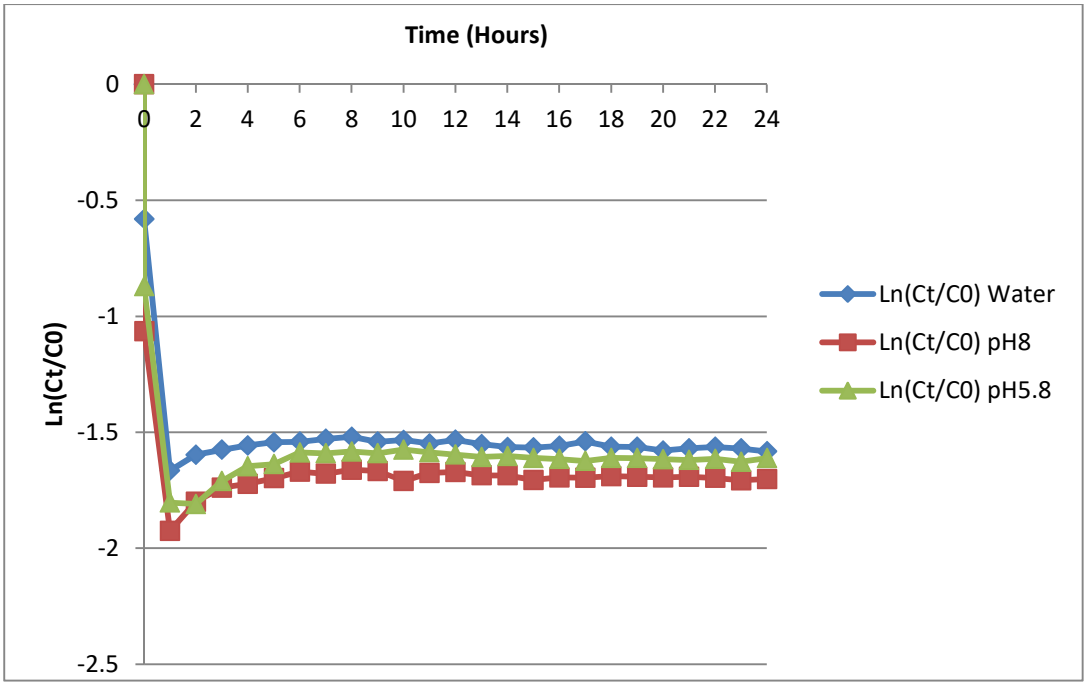


Figure S5-6: Ln(Ct/C0) over time for the chlorination (100 µM) of Zidovudine in solution (water, pH 8 and pH 5.8).

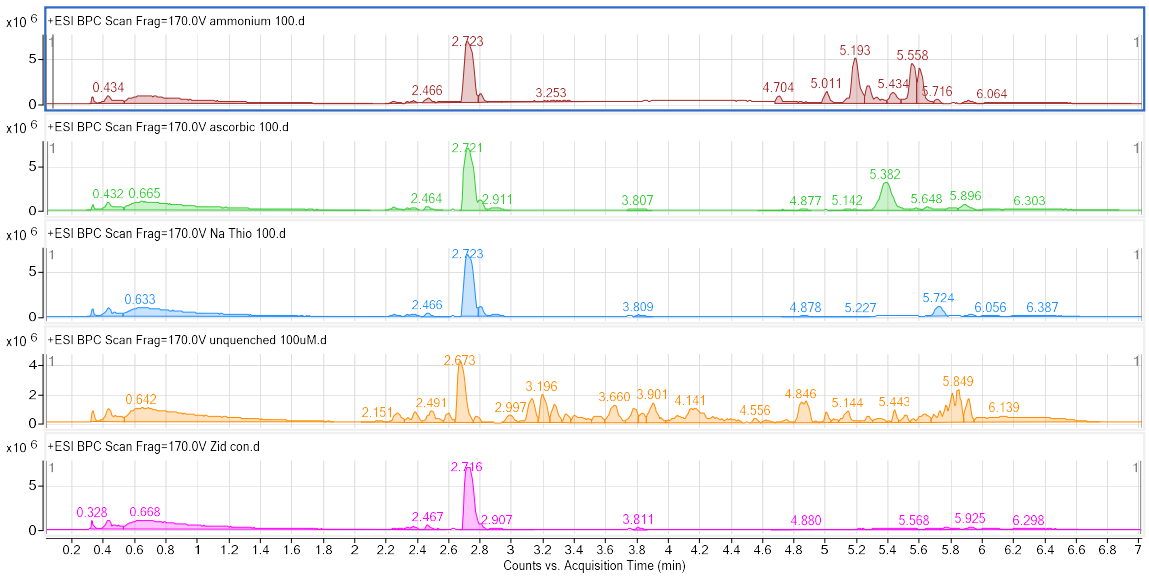


Figure S5-7: Base peak chromatograms of (from top) - 100 µM chlorination reactions quenched by ammonium chloride, ascorbic acid or sodium thiosulphate, an unquenched reaction and a Zidovudine standard (10 µg/ml).

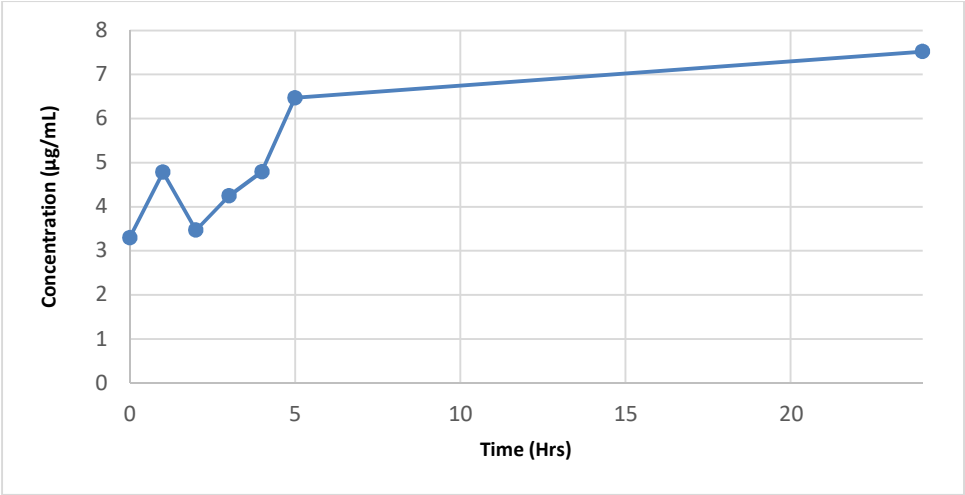


Figure S5-8: Concentration of Zidovudine over time in a stirred chlorination reaction. 10 µg/ml of Zidovudine treated with 100 µM NaOCl at pH 8 in 10 mM phosphate buffer, analysed by UHPLC-QTOF at 0, 1, 2, 3, 4, 5 and 24 hours.

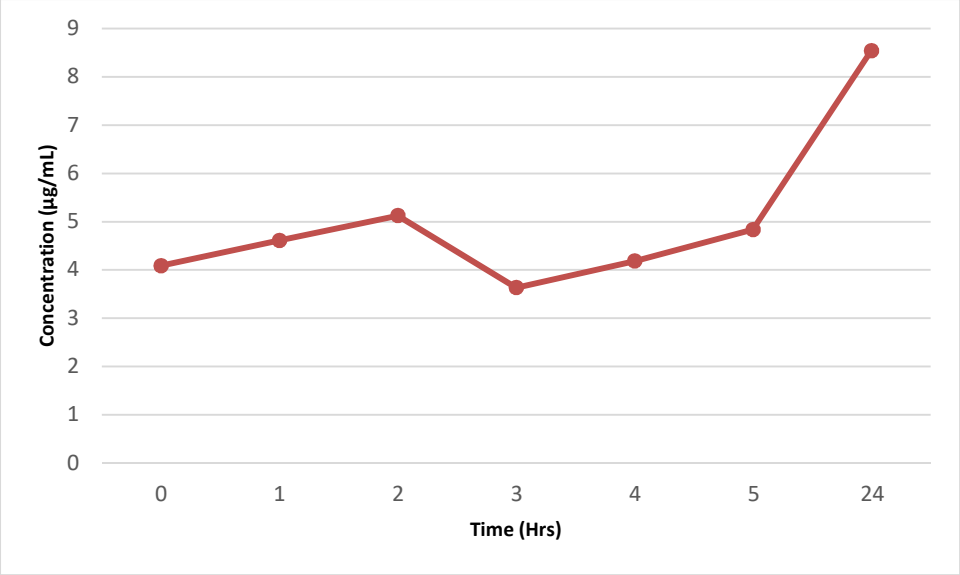


Figure S5-9: Concentration of Zidovudine over time in a stirred chlorination reaction. 10 µg/ml of Zidovudine treated with 100 µM NaOCl at pH 5.8 in 10 mM phosphate buffer, analysed by UHPLC-QTOF at 0, 1, 2, 3, 4, 5 and 24 hours.

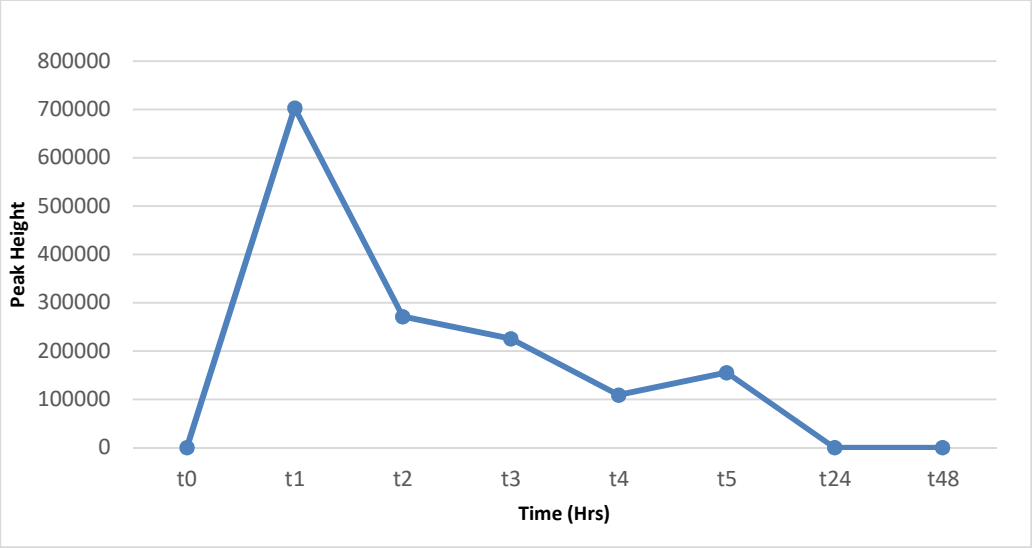


Figure S5-10: Peak height of the extracted ion chromatogram of *m/z* 161 from UHPLC-QTOF analysis. These are plotted against time for the chlorination of 10 µg/ml Zidovudine treated with 100 µM NaOCl at pH 8 in 10 mM phosphate buffer.

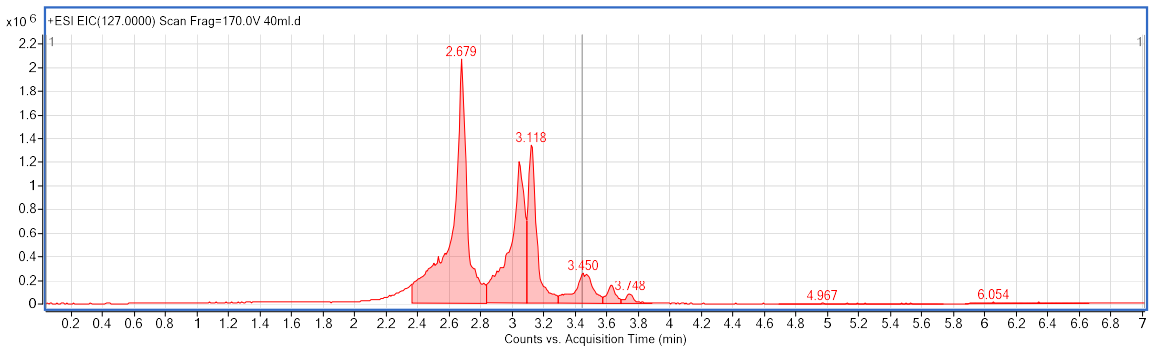


Figure S5-11: Extracted ion chromatogram (*m/z* 127; thymine) of positive ESI analysis of the total large scale chlorination reaction of Zidovudine.

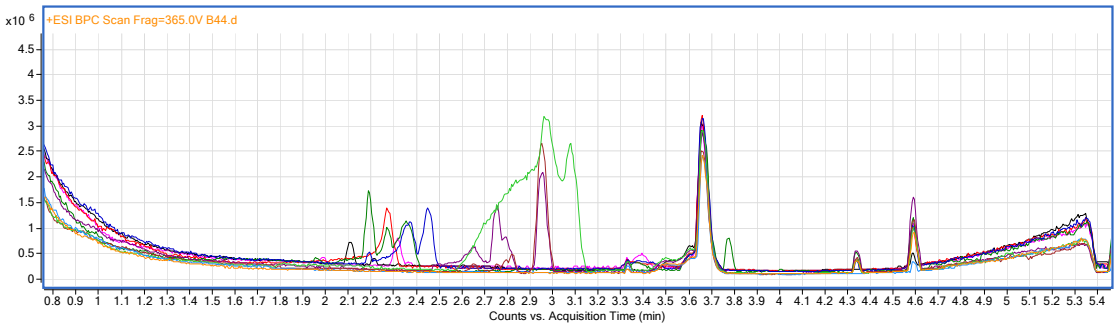


Figure S5-12: Overlaid zoomed base peak chromatograms (BPCs) of the Zidovudine chlorination reaction fractions that did not contain zidovudine but showed anti-HIV activity. Analysis performed by positive ESI UHPLC-QTOF.

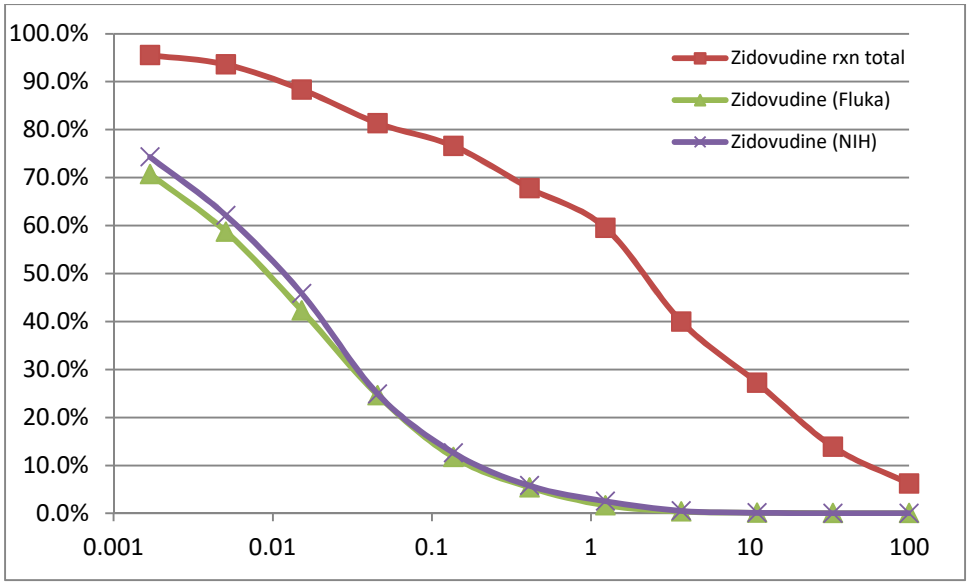


Figure S5-13: The anti-HIV activity of the total Zidovudine chlorination reaction product compared to control Zidovudine from two sources (Fluka and NICD). Viral activity (%) is plotted against concentration (µg/mL).

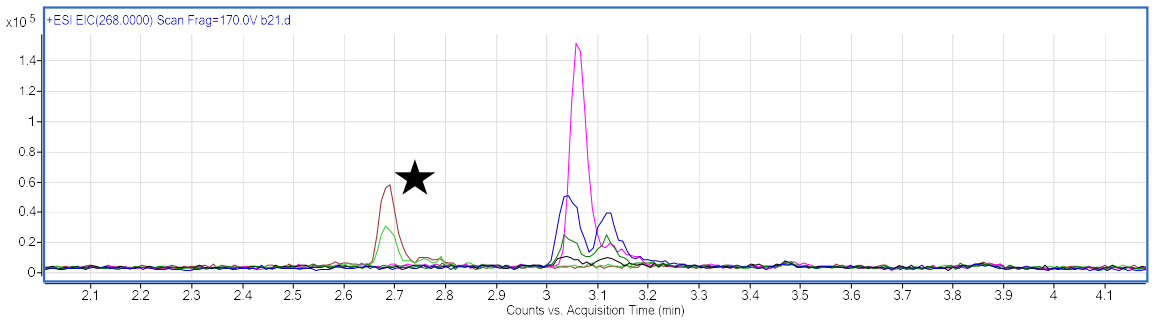


Figure S5-14: Overlaid EIC (*m/z* 268) of six large scale chlorination fractions that contain the Zidovudine pseudomolecular ion. Two of the six contain Zidovudine (star).

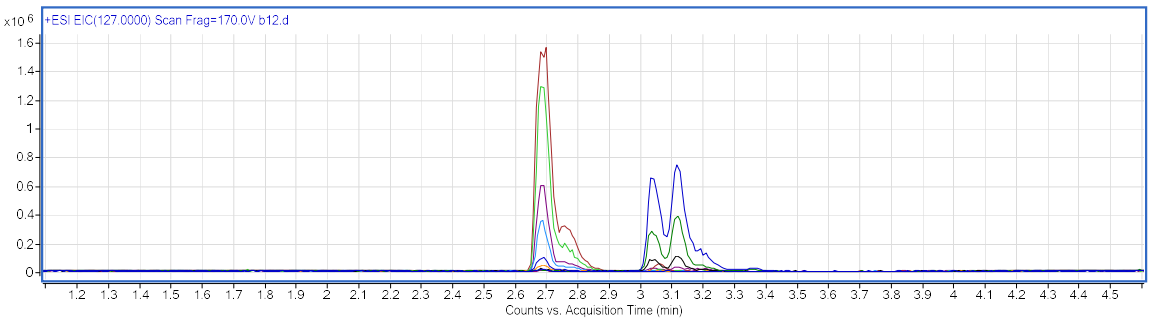


Figure S5-15: Overlaid EIC (*m/z* 127) of 10 large scale chlorination fractions that contain the protonated thymine ion.

Chapter 6: Conclusion

The work presented in this thesis is divided into four stand-alone articles, each of which addresses one or more of the original aims and themes of the research. With the first aim we sought to determine the nationwide prevalence of pharmaceuticals in South African surface water; research that had until this time not been performed before on this scale. Initially, national surface water was screened for 12 antiretroviral using a triple quadrupole LC-MS. The method efficiently separated and analysed the major members of the ARV drug class. It was however limited in flexibility and the number of targets included in the analysis.

In order to overcome matrix effect and accurately quantify these compounds (accuracy was necessary as it was a “first report” of these compounds’ prevalence in the literature) a standard addition method was developed. The method accurately quantified these compounds in complex matrices and is a more viable alternative to the use of isotopically labelled standards, many of which are not commercially available for this group of pharmaceuticals. The extraction and analytical technique was developed in such a way as to remain generic and amenable to the addition of more compounds to the analytical paradigm, as was shown in research that was to follow.

The work represents a nationwide survey of surface water and presents, for the first time, quantitative data for many of these pharmaceuticals in the environment. Compounds such as Nevirapine have been detected in European surface waters, yet the majority of the compounds targeted in this work have not been previously described in the literature.

In the second chapter we expanded upon the first aim to increase the number of pharmaceutical targets from 12 to 96 compounds. Here we demonstrated the utility of high resolution mass spectrometry with targeted and untargeted screening as a strategy to analyse surface water for the presence of a diverse array of

pharmaceuticals. This serves to address one of the themes of the thesis in that we are able to show how advances in mass spectrometry, from a triple quadrupole to a QTOF, has yielded improvements in the field of environmental chemical analysis.

We have for the first time shown the diversity of pharmaceutical pollutants in South African surface water on a national scale and have created a searchable data base that may serve as a national pollution baseline for future use. This outcome fulfills the third theme of the thesis, which is to utilize the products of this work for national defence purposes.

To perform these analyses, a SPE extraction and UHPLC-QTOF method was developed that achieved limits of quantification in the low to mid ng/L range for the majority of compounds. A broad concentration range was noted for the targets, with Lamotrigine and Nevirapine jointly occurring most frequently. Prednisilone and Ritonavir had the highest average concentrations in surface water at 623 and 489 ng/L respectively. It was found that the antiretroviral (anti-HIV) class of drug occurs frequently in South African surface water, which may be attributed to the high usage of these compounds in the country; due to the extremely high national HIV burden.

This outcome highlights the fact that these studies should be performed in each country as there is variability in national pharmaceutical usage, sewage management system availability and efficiency as well as rainfall. Each of these factors would in turn affect the prevalence and concentration of a particular pharmaceutical in national surface waters.

In this research it was shown that high resolution mass spectrometry provides an extremely large and comprehensive data set. This enables researchers to detect and identify a wide variety of analytes. The richness of the data set does however lead to analytical challenges relating to rapid data analysis in order to support decision making. As shown in Chapter Three, cluster computing may be used to parallelize mass spectrometric analyses and although no reduction in time per analysis was achieved, the overall throughput was increased by 3000%.

The second, third and fourth aims of the research were to: elucidate the chlorination behavior of selected pharmaceuticals compounds relevant to South Africa; determine the extent of the presence of the chlorinated compounds in South African water supplies; and determine the comparative *in vitro* toxicity and biological activity between selected pharmaceuticals and their disinfection transformation products. These are all addressed in Chapter Four and Five for the degradation, fate, toxicity and biological activity of Nevirapine and Zidovudine, following chlorination, respectively.

The ubiquitous prevalence of Nevirapine in surface water can partially be explained by its resistance to degradation by chlorine as demonstrated by kinetics studies, where chlorine was added in excess to environmentally plausible Nevirapine concentrations in the laboratory. From this work it is proposed that Nevirapine would not be degraded completely during wastewater treatment due to: increased chlorine demand by dissolved organic matter and reduced degradation at the mildly acidic pH used in wastewater treatment.

It is further hypothesised that chlorination efficacy is related to the speciation of the Nevirapine molecule, which is pH dependent. The molecule degrades entirely at a highly basic pH, which is the exact opposite condition to that found during wastewater chlorination. A slightly acidic pH is preferred during wastewater treatment in order to favour the formation of HOCl, which is a stronger disinfectant. At basic pH, the OCl^- species dominate, which may react more favourably with Nevirapine in its basic speciation state.

Although at environmentally relevant concentrations, the molecule is relatively persistent, it was shown that Nevirapine is still subject to modification by chlorination, producing a number of DTPs when reactions were scaled up. Through a UHPLC-QTOF analysis of South African surface water it was shown that the DTPs described from *in vitro* analysis are present in the environment. This was achieved through the creation of a searchable mass spectrometric database of Nevirapine DTPs (and later Zidovudine DTPs).

The national prevalence of these compounds is exceptionally low since the parent molecule is only found in trace amounts. This is most likely because only a small fraction of an already dilute substrate is converted to form DTPs. These are then further diluted as they enter the water course, rendering them undetectable (i.e. beneath the current method limits of detection). For these reasons, and as expected, the Nevirapine DTPs are only detectable at WWTW outlets. These findings satisfy the third aim of the research in that the national prevalence of the Nevirapine DTPs was determined, and found to be very low.

In order to isolate the Nevirapine DTPs identified in nature and in the small-scale stirred reactions; the reaction scale had to be increased. It was however found that as the relative molarity between the substrates changed the nature of the reaction products also changed. This shows that laboratory scale chlorination studies of pharmaceuticals may not accurately reflect real-world occurrences during industrial wastewater treatment; as compounds identified in laboratory-scale reactions may not be present in wastewater purification scale reactions and *vice versa*. This phenomenon is most likely due to the differences in concentration between laboratory and industrial scale chlorination as well as the presence of a plethora of reactive organic molecules in wastewater. These represent an uncontrolled variable in the process which not only places a demand on chlorine but may also react with pharmaceuticals. Be that as it may, a variety of the DTPs characterised during the small scale reactions were detectable in WWTW effluent.

The final aim of this research was to determine the *in vitro* toxicity and biological activity of the isolated DTPs and compare these findings to that of the parent drug. Through *in vitro* toxicity screening in human cells it was found that none of the DTP fractions from the Nevirapine chlorination reaction were more toxic than the Nevirapine itself. Interestingly, however, it was found that a number of the DTPs retained antiviral properties. This may be explained by the close quantitative structure activity relationship between the parent and DTPs. The activity may be as a result of the action of a single DTP, or synergy between a number of similar

compounds. This may only be clarified once the individual molecules are synthesised in pure form.

Using a similar methodology, in order to expand upon the three previously mentioned aims, it was found that the ARV Zidovudine is also resistant to chlorination under conditions (pH and chlorine to drug ratios) typically used during waste water treatment. The compound degrades slowly compared to other pharmaceuticals during disinfection and as much as 20 % remains in solution; even when there is an excess of free available chlorine. The resistance of Zidovudine to chlorine degradation may in part explain its environmental prevalence.

By increasing Zidovudine concentration in the laboratory scale reaction, relative to chlorine, there was a noted increase in the formation of Zidovudine conjugates. These conjugates were found to be dimers as well as heterodimers, in that they were composed of Zidovudine DTPs that formed associations with the Zidovudine molecule as well as with each other. The ability of Zidovudine to associate with itself, or with its DTPs, although initially removing it from solution could contribute to the drug's environmental prevalence. The association may afford the molecule a level of protection against chlorine attack and subsequent degradation. It is then possible that the dimer could enter the water course and by reverse reaction, release intact Zidovudine and potentially a Zidovudine DTP into the water system. When considering the environmental concentration of Zidovudine, it is more likely that Zidovudine will associate with dissolved organic molecules in wastewater than with itself. This phenomenon, although not studied in this work may present a novel mechanism in which this and potentially other compounds escape degradation during chlorine disinfection.

In this research we report for the first time the *in* vitro behaviour of Zidovudine DTPs. None of the compounds arising from the Zidovudine chlorination reactions and those subsequently purified into fractions were found to be more toxic than the original parent compound. This is heartening from an environmental impact and

toxicology perspective but further toxicity studies are necessary before final conclusions regarding environmental effects can be made.

In addition to toxicity studies, the *in vitro* anti-HIV effects of the novel DTP fractions were studied and it was shown, for the first time, that Zidovudine DTPs retain biological activity. The active fractions were isolated by preparative chromatography and shown to inhibit HIV replication *in vitro*. This serves to display that the structure activity relationship is retained to an extent when Zidovudine is transformed during chlorination.

HIV does not have an environmental component to its lifecycle as many viruses and bacteria do; therefore the presence of ARVs or ARV DTPs in the environment would not affect the virus directly. With that said, though, we have shown that these DTP compounds are biologically active and their effects on other microbes, fauna and flora cannot be ignored. It is important to bear in mind that pharmaceuticals, besides being discharged into the environment, form biologically active DTPs. Many of these are completely uncharacterised and may gain novel mechanisms of action in addition to or instead of those of the parent molecule.

Drug resistance is fast becoming a major problem in South Africa and around the world. Many organisms are found in the environment e.g. *Mycobacterium bovis* which is closely related to *Mycobacterium tuberculosis*. Through constant sub-therapeutic exposure of these organisms to wastewater discharged pharmaceuticals and their active DTPs we may be inducing a novel mechanism of drug resistance development. Thereby preparing the microbes for the treatment they would be exposed to if they were to infect animals or humans, and in doing so render the treatment ineffective.

In light of this new information, the wastewater process in South Africa should be re-evaluated in order to not only remove pharmaceuticals during wastewater processing but also to remove their DTPs. Ideally wastewater disinfection should be re-worked to not produce DTPs at all, while still removing pharmaceutical residues.

The objectives outlined in the introductory chapter of the thesis were all achieved. This can be stated with confidence as all the aims of the research were met. This work represents the first steps in a field that has received very little attention in South Africa and just begins to describe what is most likely a larger problem than initially anticipated.

By drawing the themes as well as the aims of this work together we are able to show a holistic picture of ecotoxicology. Micropollutants have a diverse effect, ranging from immediate toxicity, endocrine disruption and the development of drug resistance. In order to demonstrate this, we have shown that chemistry and biology, as well as an understanding of both, and the interface between them, can benefit a particular research field.

A large amount of work is still required in the field as a whole and in South Africa in particular. The toxicity and mechanisms of action for most of the newly described DTPs have yet to be determined. The biological effects of these compounds on the target organism as well as non-target organisms and the environment must be determined. There may also not be immediate toxicity, but the induction of long term toxic effects cannot be ignored.

In addition to pharmaceutical DTPs, the extent of metabolite and metabolite DTP pollution should be determined. In many cases a higher concentration of metabolite is excreted when compared to the intact drug. Therefore more of these compounds would enter the environment. In addition to this, if the parent compound concentration is higher than the metabolites in the environment, it may provide an indication of pharmaceuticals entering the water cycle directly (e.g. dumping, landfilling, etc.). This may therefore prompt formal investigations regarding the treatment of expired medication in South Africa.

The large body of work dedicated to the detection of pharmaceuticals and DTPs in surface water should now be refocused to address environmental relevance as well as being used as a starting point to institute regulatory and legislative

processes. These could then serve to encourage or enforce the reduction of the introduction of micropollutants into the environment.

In the end it can be seen that the tools that are currently available to the analytical chemist, and indeed also to the biologist, may serve to save quality water; which is arguably our most important natural resource.