Investigating The Efficacy Of A Moving Bed Biofilm Reactor For The Removal Of The Antiretrovirals Tenofovir, Emtricitabine, Nevirapine, Ritonavir And Efavirenz From Synthetic Wastewater.

BY

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DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in any candidature for any degree.

Signed…… Date……..

STATEMENT 1

This thesis is being submitted in partial fulfilment for the requirements for the Doctor of Philosophy (PhD): Biotechnology.

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STATEMENT 2

This thesis is the result of my own independent investigation, except where otherwise stated. Other sources are acknowledged by giving explicit references. A bibliography is appended.

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STATEMENT 3

I hereby give consent for my dissertation, if accepted, to be available for photocopying and for inter-library loan, and for title summary to be made available to outside organisation.

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LIST OF ABBREVIATIONS

ACE	Abundance-based coverage estimators
AIDS	Acquired Immunodeficiency Syndrome
AOPs	Advanced oxidation processes
API	Active pharmaceutical ingredients
ART	Antiretroviral therapy
ARVs	Antiretroviral drugs
AS	Activated sludge
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
E2	17β-estradiol
EC_{50}	Half maximal effective concentration
EC	Emerging contaminants
EDCs	Endocrine disrupting chemicals
EE2	17-α-Ethinylestradiol
EFV	Efavirenz
ErC_{50}	Concentration which results in a 50 percent reduction in growth rate
FDC	Fixed-dose combination
FTC	Emtricitabine
HIV	Human Immune Deficiency Virus
HPLC	High Performance Liquid Chromatography
HPLC/MS/MS	High Performance Liquid Chromatography tandem mass spectrometry
HRT	Hydraulic retention time

LC/MS/MS	Liquid Chromatography with tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limits of detection
LOQ	Limits of quantification
MBBR	Moving Bed Biofilm Reactor
MOA	Mode of action
N	Nitrogen
NH_4-N	Ammonium-Nitrogen
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NO_2	Nitrite
NO_3	Nitrate
NSAIDs	Non-steroidal anti-inflammatory drugs
NtRTIs	Nucleotide analogue reverse transcriptase inhibitors
O_3	Ozone
OECD	Organization for Economic Co-operation and Development
OLR	Organic loading rate
OM	Organic matter
OUT	Operational taxonomic unit
P	Phosphorus
PAOs	Phosphorus accumulating organisms
PCoA	Principal Coordinates Analysis
PCPs	Personal Care Products
PhACs	Pharmaceuticals
PIs	Protein inhibiters
PO_4-P	Orthophosphate as phosphorus
QTOF	Quadrupole time of flight
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
rRNA	Ribosomal RNA

SALR	Surface area loading rate
SND	Simultaneous nitrification and denitrification
SOP	Standard operating procedure
SPE	Solid phase extraction
SR -	Synergistic ratios
STP	Sewage treatment plant
TDF	Tenofovir
TN	Total Nitrogen
UHPLC	Ultra High Pressure Liquid Chromatography
UHPLC-QTOF-MS/MS	Ultra High Pressure Liquid Chromatography coupled to quadrupole time of flight mass spectrometry
UV	Ultraviolet
VAS	Volatile attached solids (Average attached biomass)
VSS	Volatile suspended solids (Average suspended biomass)

ABSTRACT

South Africa utilises more antiretroviral (ARV) compounds per capita than any other nation in the fight against Human Immune Deficiency Virus (HIV) or acquired immunodeficiency syndrome (AIDS). Considering the main entrance pathways of antiviral drugs into the urban water cycle, excretions via urine or faeces from treated individuals play a dominant role. Due to the limited efficiency of conventional biological treatment (activated sludge), ARVs were detected in South African wastewater treatment plant effluents and surface waters. This poses a threat to aquatic environments due to the toxicity of ARVs and can be a potential contributor to ARV resistance due to persistent low level ARV exposure in the general population. This study investigated the efficacy of a moving bed biofilm reactor (MBBR) for ctybtri8nthe elimination of five ARV compounds i.e., tenofovir, emtricitabine, nevirapine, ritonavir and efavirenz from synthetic wastewater. Furthermore, the study also looked at the shift in microbial community compositions of biofilms in the MBBR due to exposure to the ARV compounds. Lastly, the ecotoxicity of the MBBR's influent and effluent along with the actual ARV compounds were examined.

The capacity of ARV degradation by the MBBR was investigated by spiking synthetic wastewater influent with 10 µg/L of five ARV compounds. Actual removal during treatment was assessed by sampling the inlets and outlets of the reactor. A targeted solid phase extraction method with Ultra High Pressure Liquid Chromatography coupled to quadrupole time of flight mass spectrometry (LC-MS/MS) was used to quantify the five ARV compounds. Microbial diversity (alpha-diversity) of seeded sludge from a full-scale municipal WWTP and biofilm samples from a laboratory scale MBBR system during pre- and post-introduction of ARV compounds was investigated by Illumina sequencing of the 16S rRNA gene. Ecological toxicity of the MBBR's influent and effluent along with the five ARV compounds was determined using the *Vibrio fischeri*, *Daphnia magna* and *Selenastrum capricornutum* toxicity test kits and measured as EC_{50}.

After MBBR treatment; Nevirapine, Tenofovir, Efavirenz, Ritonavir and Emtricitabine all showed marked reduction in concentration between the influent and effluent of the MBBR. On average, the percentage removed for Nevirapine, Tenofovir, Efavirenz, Ritonavir and

Emtricitabine was 62.31%, 74.18%, 93.62%, 94.18% and 94.87% respectively. Microbial diversity results demonstrated that the introduction of antiretroviral drugs affects the bacterial community composition and diversity considerably. For instance, *Nitrosomonas, Nitrospira* and *Alicycliphilus* were found to be higher in post introduction of ARV compounds biofilm samples than in biofilm samples before the introduction of ARV compounds. The EC_{50} for Tenofovir, Emtricitabine, Nevirapine, Ritonavir and Efavirenz were 82.5, 41.7, 39.3, 60.3 and 0.21 mg/L respectively for *S. capricornutum*; 81.3, 50.7, 49, 87.1 and 0.43 mg/L respectively for *D. magna*; and 73.5, 55.1, 41.3, 83.6 and 0.55 mg/L respectively for *V. fischeri*. The EC_{50} of the influent and effluent were found to be above 100% concentration, therefore they could not be specifically determined. The ecotoxicity results show that ARV compounds are potentially toxic to the environment, with efavirenz being more toxic than the other four ARV compounds tested. Since there were no toxic effects observed from the effluent, it can be assumed that mineralisation has occurred, or the transformation products are of less or equal toxicity to the influent (because the influent did not show any toxic effects to the model organisms tested).

CHAPTER ONE

1. General introduction

1.1 Occurrence of pharmaceuticals in the urban water cycle and the environment

The shortage of fresh water is an increasing concern to many nations, with studies showing that human water consumption exceeds sustainable levels (Postel, 2000; Kummu et al., 2016). In many areas, extracting more fresh water for use in cities, agriculture, and industry puts additional pressure on aquatic ecosystems. Water recycling and reuse can provide tremendous benefits to the environment and to society, by decreasing freshwater uptake through the use of reclaimed water for landscape irrigation, to recharge groundwater aquifers or for drinking. However, several contaminants of emerging concern such as micropollutants can compromise the reliability of reclaimed water, by contaminating vital aquatic compartments such as surface water, groundwater and eventually drinking water (Barbosa et al., 2016). The occurrence of these micropollutants in the aquatic environment has become an increasing environmental concern because of their potential negative effects on human and ecological health.

Micropollutants, also known as emerging contaminants (mostly organic), are usually of anthropogenic origin and are commonly present in waters at trace concentrations ranging from a few ng/L to several µg/L (Kasprzyk-Hordern et al., 2007; Baker and Kasprzyk-Hordern, 2013). The compounds in question are derived from three broad categories, namely Pharmaceuticals (PhACs), Personal Care Products (PCPs) and Endocrine Disrupting Compounds (EDCs) (Ferrari et al., 2003; Diamanti-Kandarakis et al., 2009; Al Aukidy et al., 2012; Verlicchi and Zambello, 2015). Most of these chemicals reach natural waters mainly through municipal (domestic) and industrial wastewater streams (Edokpayi et al. (2017). To date, many research papers have indicated the widespread occurrence of micropollutants in the environment (Kolpin et al., 2004; Zhou et al., 2010; Gomez et al., 2012; Blair et al., 2016; Biswas and Vellanki, 2021). The most thought-provoking class of these emerging contaminants are pharmaceuticals. Pharmaceuticals are compounds that are designed to prevent, cure and treat diseases and improve health (Jelic et al., 2011). However, many pharmaceutical drugs are not completely degraded in the human body and are excreted after

slight transformation or in an unchanged form (Debska et al., 2004). Excreted drugs are transported into wastewater treatment plants (WWTPs) through sewage pipes. It was initially thought that most pharmaceutical products underwent complete mineralization, i.e., a conversion to CO_2 and H_2O, during the wastewater treatment process. However, it has been scientifically demonstrated that most WWTPs are unable to remove pharmaceutical drugs completely during the sewage treatment process, mostly due to their low concentrations and diversity (Luo et al., 2014; Ngqwala, & Muchesa 2020; Ternes et al. 2015). As a result, many of these compounds end up in the aquatic environment, posing a threat to wildlife and humans alike.

1.2 Risks associated with the occurrence of pharmaceuticals in the environment

Active pharmaceutical ingredients (API) of human medicinal products have been detected in different environmental compartments, such as surface water, groundwater, soil and in wastewater at low concentrations ranging from sub-ng/L levels to µg/L (Hirsch et al., 1999; Kolpin et al., 2004; Archer et al., 2017). Of particular concern is the exposure of non-target organisms to pharmaceuticals, as these compounds are designed to be biologically active and low exposure can elicit physiological change (Fent et al., 2006; Pruden et al., 2006). Synthetic estrogens used as contraceptives such as 17-α-Ethinylestradiol (EE2), have been shown to disrupt the endocrine system by altering sex determination, delaying sexual maturity, and decreasing the secondary sexual characteristics of exposed organisms at environmentally relevant concentrations in the low ng/L-range by mimicking its natural analogue, 17β-estradiol (E2) (Aris et al., 2009; Saaristo et al., 2010; Saaristo et al., 2019). The review by Brausch et al. (2012) gives a comprehensive overview on the toxic effects of human pharmaceuticals in aquatic organisms, endpoints (Effective concentration and Lethal concentration), mode of action (MOA) and toxicity of pharmaceutical classes. Acute toxicities of 150 pharmaceutical substances, comprised of 35 pharmaceutical classes, are presented. In the same study, chronic toxicities are given for 65 pharmaceutical substances from 20 pharmaceutical classes. The review describes standard and advanced ecotoxicological test systems that have been applied to study the MOA in aquatic organisms. Toxic effects are proved from molecular (e.g. inhibition of cyclooxygenase) to population levels i.e. behavioural changes and effects on reproduction (Brausch et al., 2012). An even more dramatic effect has

been reported by Oaks et al. (2004) who showed that the decrease of vulture populations in Pakistan is directly linked to the concentrations of diclofenac in these birds (Oaks et al., 2004). While the potential environmental effects that most pharmaceutical drugs pose in surface waters remain largely unknown, several studies have predicted antiviral drugs as one of the most hazardous therapeutic classes in relevance to their toxicity towards daphnids, fish and algae (Almeida et al. 2021; Sanderson et al. 2004).

The application of living organisms or bioassay as biological indicator for measuring toxicity levels of substances such as pharmaceuticals has long been demonstrated. The basis of bioassays is that chemical exposure at a sufficient concentration imparts negative impacts on the physiology and behaviour of an exposed species or population. When conducting bioassays, it is recommended to perform toxicity tests representing at least three of the four trophic levels (bacteria, algae, invertebrate and vertebrate) (Jooste and Herbst 2004). The toxicity of toxins is described in terms of the dose/concentration that causes a particular effect/response in a specified population (measurement endpoints). Typically, these measurement endpoints are expressed as: the effective concentration (EC) or the lethal concentration (LC) that elicits a response (e.g., immobility, lethality, etc.) from 50% of the exposed population (Shayne, 2005). Toxicity bioassays may evaluate either acute or chronic end points. Acute toxicity tests are short-term tests that measure the effects of exposure to relatively high concentrations of chemicals. The measurement endpoint generally reflects the extent of lethality. Chronic toxicity tests, on the other hand, generally are longer-term tests that measure the effects of exposure to relatively lower, less toxic concentrations. For a chronic toxicity test, the measurement endpoint concerns a sublethal effect (e.g., reproduction, growth) or both lethality and sublethal effect.

Even though numerous studies have shown the occurrence of pharmaceuticals in water sources and their adverse effects towards standardized aquatic toxicity models, there is still no clear evidence or knowledge of their effects on human health. The absence of evidence though does not mean that humans are safe from any adverse effects of continual exposure to drug residues in water. For instance, after reporting a number of antiretroviral drugs (ARVs) in different water matrices, including wastewater effluents, surface water and drinking water (Wood et al., 2015; Schoeman at al., 2015; Wood et al., 2016; Abafe et al., 2018), Swanepoel et al. (2015) have suggested that low concentrations of antiretroviral drugs (ARVs) may be

consumed via drinking water, maintaining low concentrations of ARVs in consumers that are infected with human immunodeficiency virus (HIV) but are not receiving Antiretroviral therapy (ART), suggesting the possibility of resistance development by HIV.

1.3 ARVs and HIV/AIDS in South Africa

The discovery of HIV as the causative organism of acquired immunodeficiency syndrome (AIDS) together with an ever-increasing understanding of the virus replication cycle have been instrumental in providing researchers with the necessary knowledge and tools to develop specific anti-HIV drugs (Arts & Hazuda, 2012). So far, there are 25 compounds which have been formally approved by the US Food and Drug Administration (FDA) for the treatment of HIV-1 infections (Clercq, 2010). These drugs are classified into six distinct categories based on their point of intervention within the HIV replicative cycle (refer to Figure 1.1):

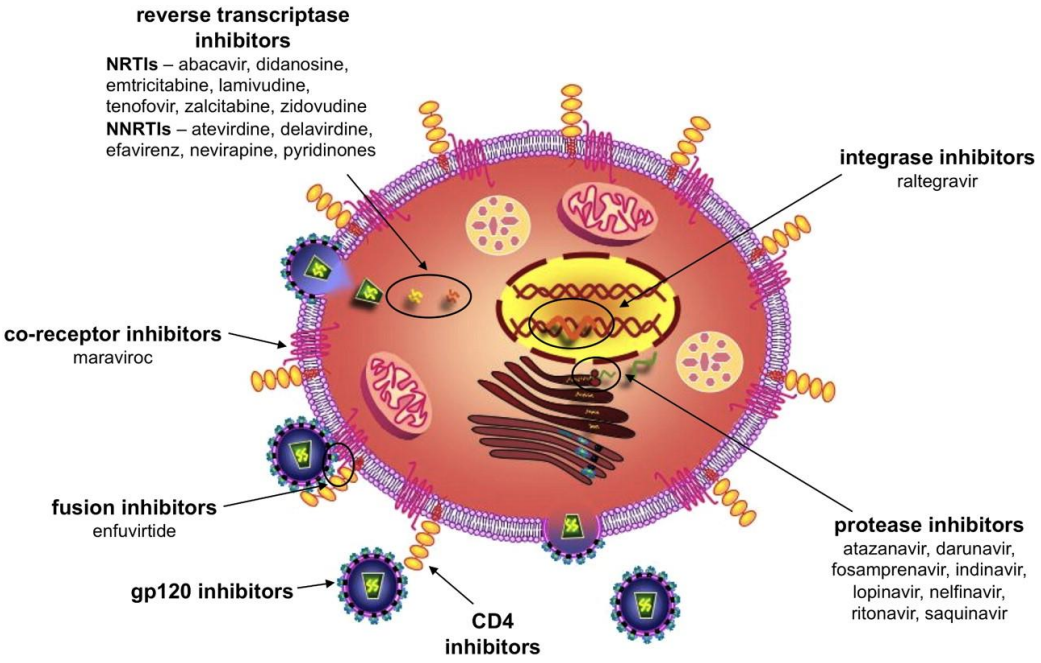


Figure 1. 1: Illustration of the inhibition of HIV-1 replication at different steps in the viral life cycle (Pirrone et al., 2011).

1. Nucleotide analogue reverse transcriptase inhibitors (NtRTIs).
    - Emtricitabine, Tenofovir disoproxil fumarate, Zidovudine, Didanosine, Zalcitabine, Stavudine, Lamivudine and Abacavir.
2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

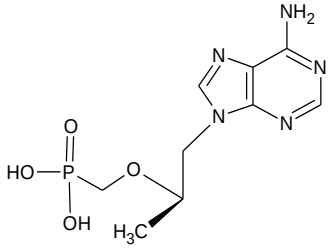
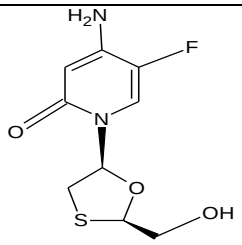
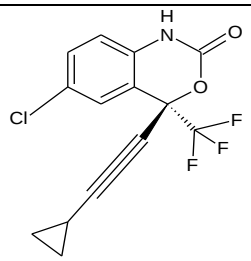
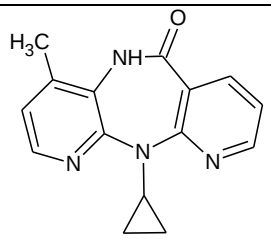
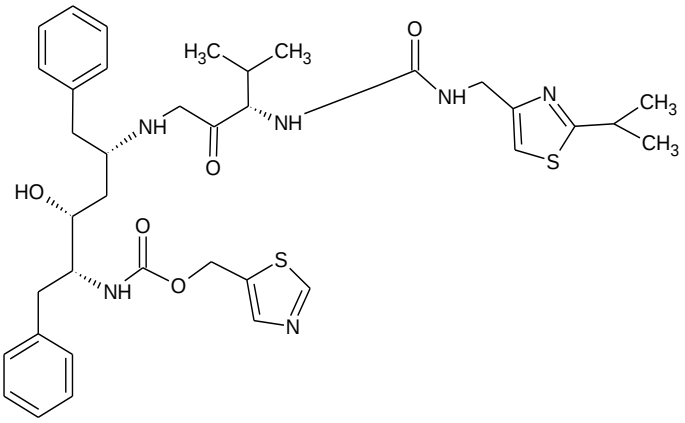
- Nevirapine, Delavirdine, Efavirenz and Etravirine.

3. Integrase inhibitors
   - Raltegravir.
4. Protease inhibitors (PIs)
   - Saquinavir, Ritonavir, Indinavir, Nelfinavir, Amprenavir, Lopinavir, Atazanavir, Fosamprenavir, Tipranavir and Darunavir.
5. Fusion inhibitors
   - Enfuvirtide.
6. Co-receptor antagonists.
   - Maraviroc.

Early in the epidemic, specific HIV antiviral drugs were given as monotherapy. However, monotherapies frequently led to treatment failures because the virus quickly developed resistance to the single drug. Following the advent of highly active antiretroviral therapy (HAART), which involves a combination of at least two or more antiretroviral compounds from different classes, dramatic improvements in HIV-1-infected patient health and survival were realized as more refined combination therapies resulted in reductions in viral loads and increases in CD4 T-cell counts (Pirrone et al., 2011).

South Africa has the world's largest antiretroviral therapy (ART) programme, with approximately 7.2 million of its population estimated to be living with HIV and about 4.5 million people receiving antiretroviral therapy by the end of 2017 (WHO, 2018). South Africa has made impressive progress in the rollout of ART since the start of the public sector ART programme in 2004. In 2012, the South African National Department of Health (NDoH), announced the award of a new antiretroviral (ARV) tender worth R5.9 billion that, for the first time since the start of the ARV programme, includes a triple fixed-dose combination (FDC) tablet. The FDC tablet contains 300 mg Tenofovir, 200 mg Emtricitabine and 600 mg Efavirenz (Clinicians Society, 2013). In some cases, nevirapine is given to pregnant patients to prevent mother to child transmissions of HIV during pregnancy and breastfeeding. If Efavirenz and Nevirapine are not compatible for someone, they are replaced with Ritonavir as a first-line of defence (Meintjes et al., 2017).

Table 1. 1: Properties of Tenofovir, Emtricitabine, Efavirenz, Nevirapine and Ritonavir.

Name	**Structure**	**Molecular Mass, g/mol**
Tenofovir		287.21
Emtricitabine		247.25
Efavirenz		315.68
Nevirapine		266.89
Ritonavir		720,94

Emtricitabine and Tenofovir belong to a class of antiretroviral drugs known as NtRTIs, which block reverse transcriptase, an enzyme crucial to viral production in HIV infected people. Therapeutically, the prodrug tenofovir disoproxil fumarate and emtricitabine are orally administered. They both have enhanced oral absorption and are rapidly converted intracellularly to the active form tenofovir diphosphate and emtricitabine 5'-triphosphate, respectively. When tenofovir is administered, 70-80% of the dose is recovered in the urine as unchanged drug within 72 hours of administration, while 86% and 14% of the dose of emtricitabine was recovered unchanged in urine and faecal samples, respectively (National Centre for Biotechnology Information, 2016).

Efavirenz is a synthetic purine derivative that is a non-nucleoside reverse transcriptase inhibitor (NNRTI). It binds directly to the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase, an RNA-dependent DNA polymerase, blocking its function in viral DNA replication (Muchiri et al., 2013). In combination with other antiretroviral drugs, this agent has been shown to significantly reduce HIV viral load, preventing damage to the immune system and reduce the risk of developing AIDS (Borand et al., 2014). Efavirenz is excreted principally in the faeces, both as an unchanged drug and metabolites. Excretion of efavirenz has been evaluated in individuals receiving 400 mg daily for 1 month. Following oral administration of 400 mg of radio-labelled efavirenz on day 8, 14-34% of the dose was excreted in urine (less than 1% as unchanged drug), and 16-61% was excreted in faeces (predominantly as unchanged drug) (Rakhmanina & Anker 2010).

Nevirapine, a NNRTI is excreted from the body primarily in urine and a small amount of the drug is excreted in faeces, both as unchanged drug (5%) and metabolites (2-Hydroxynevirapine glucuronide (18.6%), 3-hydroxynevirapine glucuronide (25.7%), and 12-hydroxynevirapine glucuronide (23.7%) were the major metabolites recovered in urine) following oral administration of 200 mg daily (Fan-Havard et al., 2013).

Ritonavir is an HIV protease inhibitor that interferes with the reproductive cycle of HIV. Ritonavir taken as a 100 mg tablet is eliminated from the body predominantly in faeces as an unchanged drug and metabolites (86% of unchanged drug and metabolites), with minor urinary elimination (11%, mostly metabolites) (Hardman et al., 2001).

The excretion of unmetabolized ARV compounds from the body, suggests that a large fraction of the administered ARV drugs have the potential to find their way to sewage treatment facilities. Swanepoel et al. (2015) estimated that a daily dose of combination ARV therapy (mean of 991 mg/day/person, range 590-1996) equates to a total of 529 000 kg of ARV compounds ingested per year (assuming 1.5 million people on ART). Using the same formula, given that the number of people receiving ARV therapy has increased to an estimated 4.5 million to date the amount of ARV compounds ingested per year equates to 1 587 000 kg. A number of research studies conducted have identified ARV drugs in South African waters i.e. wastewater effluents, ground water, surface waters and even drinking water (Swanepoel et al., 2015; Wood et al., 2016; Schoeman at al., 2017; Abafe et al., 2018), highlighting the limitation of WWTPs to completely remove ARV drugs from the water systems. With the expanding ARV load and the current limitation of wastewater treatment plants to completely remove these emerging contaminants, it is envisaged that an increasing concentration of ARV compounds will soon be entering the environment through WWTP discharges (Horn et al. (2021; Kümmerer, 2008).

1.4 Removal of pharmaceuticals from water

The occurrence of a large spectrum of pharmaceuticals in the environment clearly shows that conventional WWTPs are not capable of fully eliminating these compounds. Poor removal efficiencies of pharmaceuticals during the wastewater treatment process have been reported in literature (Gaffney et al., 2017; Kodom et al., 2021; Tambose et al., 2010). The removal efficiencies reported for antiviral drugs lamivudine and nevirapine in Kenya were found to be 24-59% and 11-49%, respectively (K'oreje et al., 2016). Prasse et al. (2010) also recorded poor removal efficiency for nevirapine, zidovudine and oseltamivir in Germany. Kruglova et al. (2014) showed no biodegradation of the anti-epileptic drug Carbamazepine. Antibiotics, one of the most ubiquitously used class of pharmaceuticals, are also known for their recalcitrance to biological wastewater treatment processes (Brown 2006; Minh et al., 2009; Polesel et al., 2016). This is not surprising as today's WWTPs have primarily been designed to minimize effluent nutrient loads, in particular nitrogen and phosphorous, to prevent eutrophication of receiving waters. Therefore, new strategies are needed to minimize the release of pharmaceuticals and their transformation products into the aquatic environment. Currently

there is no specific treatment available to ensure the complete removal of various micropollutants due to their diverse properties. Reliable processes that are able to eliminate both bulk substances as well as micropollutants are yet to be developed. However, several options have been proposed for the removal of pharmaceuticals, such as activated carbon adsorption, ozonation, advanced oxidation processes (AOPs), membrane bioreactors and attached growth treatment processes (Bolong et al., 2008; Derco et al. (2021). Depending on the pollutant concentration in the effluent and the cost of the process, different methods can be chosen.

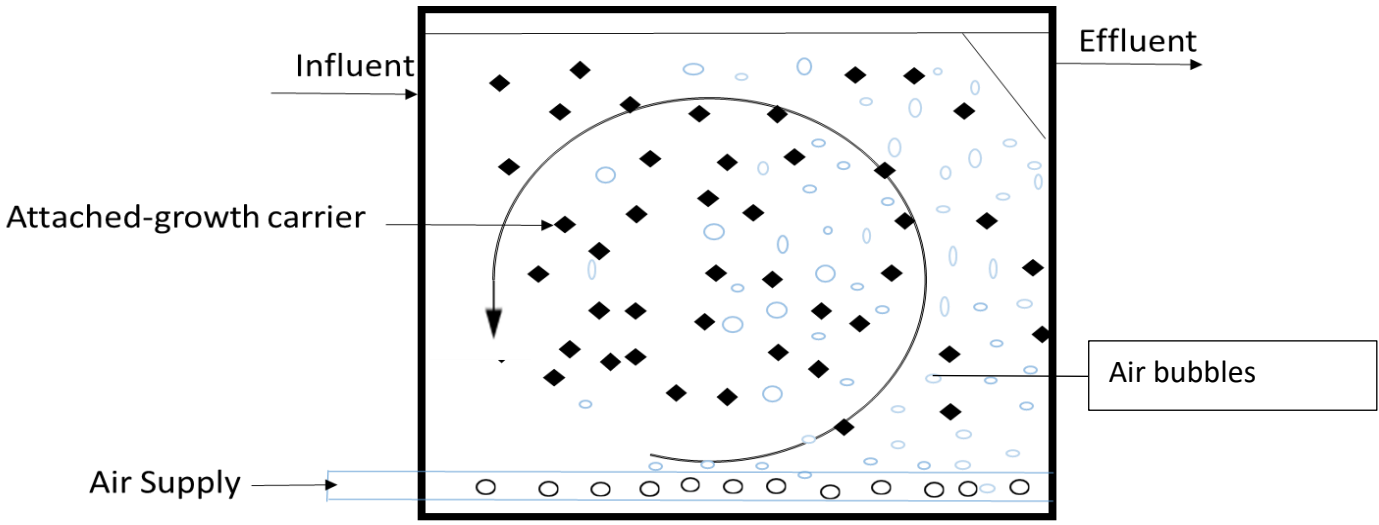
The efficiency of ozone (O_3) in removing pharmaceuticals from wastewater has been tested in both laboratory and pilot scale experiments (Ternes et al., 2003; Huber et al., 2005; Bahr et al., 2007; Benner and Ternes, 2009; Hollender et al., 2009; Hansen et al., 2010; Zimmermann et al., 2011). During ozonation, organic compounds are degraded by means of chemical oxidation either directly or indirectly. During direct oxidation, ozone reacts specifically with electron-rich chemical moieties such as double bonds, activated aromatic systems and deprotonated amines (von Gunten, 2003) since it only reacts selectively with nucleophilic molecules (Stockinger et al., 1995). Therefore, pharmaceuticals such as estrogens, beta blockers or macrolide and sulfonamide antibiotics are readily removed from treated waters by ozonation, whereas X-ray contrast media such as iopromide are insufficiently eliminated (Huber et al., 2005; Dodd et al., 2006).

During indirect oxidation, ozone reacts with water constituents, thereby decomposing primarily to form free hydroxyl radicals which belong to the strongest oxidants in water (also known as AOP). Hydroxyl free radicals are strong oxidants with an oxidation potential of 2.80 V compared to ozone (2.07 V) and chlorine (1.36 V) and can oxidize organics including those resistant to ozone such as some pharmaceuticals, pesticides, and volatile organic compounds (Wang and Xu, 2012). The main drawback of ozonation is that it does not lead to a complete mineralization of organic compounds but to the formation of oxidation products (OPs), which might be potentially toxic as they contain aldehyde, ketone, or nitroso moieties (von Gunten, et al., 2001; von Gunten, 2003; Li et al., 2008; Dantas et al., 2008; Teixeira et al., 2011). Therefore, one of the main challenges of the application of ozonation in water treatment is the possibility of toxic by-product formation.

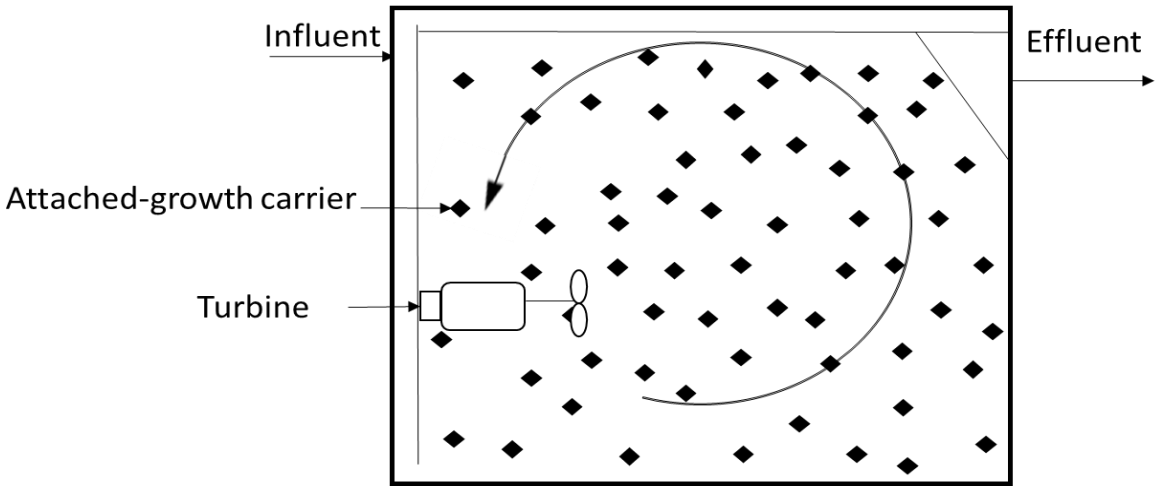
Activated carbon has very high adsorption capacities attributable to its microscopic structure and its enormous specific surface area which enables the removal of a large number of pharmaceuticals (Reungoat et al., 2010; Chang et al., 2015). Although virgin activated carbon often has high adsorption capacities, the presence of other constituents in water, such as natural organic matter (NOM), could significantly reduce the activated carbon adsorption capacity for pharmaceuticals (Fukuhara et al., 2006). As this technique is based on the adsorptive removal of organic compounds, the activated carbon has to be reactivated or disposed of (by incineration) after use.

Membrane processes do not enable degradation of the contaminant, but only its transference to a new phase (the membrane), where it is present in a more concentrated form. Reverse osmosis, nano and ultrafiltration are sensitive processes to temperature, organic material occurring naturally in the water matrices and the concentration of dissolved salts. The occurrence of high concentrations of non-target compounds can cause membrane structure deterioration or fouling (Homem and Santos, 2011). Therefore, these techniques are most often used in combination with other methodologies (as advanced treatment processes).

Attached growth technology is a promising alternative to activated sludge processes for wastewater treatment which involves attached growth on inert carriers either fixed or mobilized in suspension within the reactor (Luo et al., 2014). The moving bed biofilm technology has demonstrated high full-scale applicability for the biodegradation of pharmaceuticals in hospital wastewater (Falas et al., 2012; Casas et al., 2015; Mazioti et al., 2015; Tang et al., 2017; Ooi et al., 2017; Ooi et al., 2018). A suspended porous polymeric substance is used as a carrier which moves continuously in the aeration tank and the active biomass grows as a biofilm on the surface of the carrier (Loukidou and Zouboulis, 2001). In this context, more than 90% of the biomass is attached to the media rather than suspended in the liquid (Schmidt and Schaechter, 2011). The circulation of carriers (Figure 1.2) in the reactor is caused by aeration in an aerobic reactor or by mechanical stirring when anoxic or anaerobic treatment is used (Jahren and Ødegaard 2000).



a) Aerobic MBBR treatment.



b) Anoxic/Anaerobic MBBR treatment

Figure 1. 2: Diagrams of aerobic and anaerobic MBBR systems

The major strength of the moving bed biofilm techniques is that they combine the advantages of different biological treatment technologies (i.e. activated sludge and biofilm systems). Demonstrated advantages of employing moving bed biofilm reactors (MBBRs) include operation at higher biomass concentration, less sensitivity to toxic compounds, lack of long sludge settling period (Loukidou and Zouboulis, 2001), less prone to the process upsets from poorly settling biomass (Schmidt and Schaechter, 2011), increased solid retention favouring slow growing organisms such as nitrifiers (Guo et al., 2010; Shore et al., 2012), removal of slowly-degradable compounds such as micropollutants (Falas et al., 2012) and cost effectiveness (Fang, 2011). The MBBR has a relatively small footprint, requiring one fifth to

one third of that needed for traditional activated sludge treatment. The effect of temperature on biological nitrification is also less of a concern due to the stability of the biofilm (Salvetti et al., 2006).

The performance of MBBR systems depends on several factors, including hydraulic retention time (HRT), surface area loading rate (SALR), dissolved oxygen (DO), carrier size and shape, and carrier percent fill. Several styles of carriers with varying shapes and sizes have been developed to suit different applications (Figure 1.3). Odegaard et al. (2000) have highlighted that the filling degree may be chosen depending on the type of application. The MBBR organic loading rates per carrier area (i.e. g COD/m².d) play a crucial role in the treatment efficiency of MBBR reactors. Higher area has shown to considerably improve the performance of those systems, whereas the influence of the carrier area shows no distinction in removal rates between carriers with different shapes but the same surface area (Ødegaard et al., 2000).

Figure 1. 3: Examples of conventionally used MBBR carriers with inner protected surface area (clockwise from bottom left corner): Chip P, K3, Chip M and K1 (Veolia Water Technologies, AnoxKaldnes).

As highlighted by Andreottola et al. (2003), COD removal in MBBR systems is influenced by hydraulic retention time (HRT) with an HRT higher than 5 hrs considered most efficient. A higher HRT provides enough contact time for the biodegradation of organic matter (OM) and therefore, an extended contact time between support media and effluent enhances the waste material removal efficiency. Aygun et al. (2008) evaluated the impact of organic loading

rates on COD removal in a moving bed biofilm reactor using the biofilm carrier (K1) at 50% filling ratio and organic loading rates at 6, 12, 24, 48 and 96 g COD/m².d. The study showed that an increase in organic loading rate was followed by a decline in organic removal (at 95.1%, 94.9%, 89.3%, 68.7% and 45.2%, respectively). The percentage of carrier media in an MBBR is governed by the volume of the reactor and can be limited to 70% (Ødegaard et al., 2000). However, the percentage of media required is based on wastewater characteristics and specific treatment goals. Optimization studies carried out by Azizi et al. (2013) at fill ratios ranging from 20% to 30%, 40%, 50%, and 60% with an HRT of 6 h, noted that a fill ratio of 40% was optimal for effective treatment, while an increasing fill ratio showed no additional COD removal.

The effect of dissolved oxygen (DO) concentration on the rate of biological nitrification in wastewater treatment processes has been investigated by numerous researchers. Optimization studies have shown that DO concentration in an MBBR is required to be maintained higher than 2 mg/L for optimal COD removal (Stenstrom and Poduska 1980; Weon et al., 2004; Wang et al. 2005; Odegaard 2006; McQuarrie and Boltz 2011). Wang et al. (2005) reported that by decreasing the DO from 2 mg/L to 1 mg/L, the COD removal efficiency declined by 13%, highlighting the fact that DO is a limiting factor on MBBR efficiency. On the other hand, increasing the DO from a level of 2 to 6 mg/L raised the COD removal efficiency slightly by 5.8%. Furthermore, their results also showed that concurrently, simultaneous nitrification and denitrification (SND) may well be achieved within a single MBBR reactor with an HRT of six hours due to the limitation of oxygen diffusion into the biofilm. The best N-removal rate (89.1% on average) was obtained once the DO was maintained at 2 mg/L. At lower DO concentrations, anoxic conditions occurred and ammonia conversion to nitrite (NO_2) or nitrate (NO_3) was restricted and at higher DO concentrations anoxic denitrification within the deeper layers of the biofilm did not occur.

1.5 Analytical methods for the determination of pharmaceuticals in water bodies

The growing concern in the scientific community regarding the occurrence of pharmaceuticals in different water matrices and the potential risks towards aquatic and human health from exposure to traces of pharmaceuticals, arise from the fact that they are not considered under the legislation which regulates water quality. However, they may be introduced in future

legislation because many organizations, such as the World Health Organization, have elaborated the need for more systematic studies on the transport, occurrence, and fate of pharmaceuticals in drinking water sources. One important limitation of such studies is the availability of sufficiently sensitive and reliable analytical techniques for these investigations (Caban et al., 2016). Based on these concerns there is a need for a sensitive and reliable detection technique.

Considerable progress has been made in developing analytical equipment and methodologies to enable the determination of pharmaceuticals at lower and lower concentration levels in different environmental matrices. A survey of the literature reveals various techniques available for the determination of antiviral drugs such as High Performance Liquid Chromatography (HPLC) (Woolf et al., 2002; Jullien et al., 2003; Sentenac et al., 2003; Kandagal et al., 2008), Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS) (Clark et al., 2004; Koal et al., 2005; Clark et al., 2006; King et al., 2006; Gomes et al., 2008) or Ultra High Performance Liquid Chromatography/Tandem Mass Spectrometry (UHPLC-MS/MS), Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF-MS) (Ferrer and Thurman, 2012), UV Spectrophotometry (Dube and Vyas, 2009). However, most spectrophotometric techniques suffer from low sensitivity, high detection limits, tedious experimental conditions, and complex procedures for the preparation of samples or standard solutions. In general, HPLC and LC-MS based techniques are expensive, but rapid analysis and a high degree of resolution make them the first choice for most researchers.

LC-MS has been applied progressively as an accurate, sensitive, and specific analytical technique for the analysis of drugs and biochemical compounds (Jamal, 2000). Unlike the classical HPLC, chromatographic detection of a drug using MS relies on monitoring the mass of the molecular or fragment ion of the drug rather than monitoring its UV absorbing properties. In this respect, determination of weak UV absorbing compounds with conventional UV detectors is always a problem, however these compounds are simply determined using mass spectrometric analysis. Moreover, due to higher specificity of LC-MS, a compound can be easily recognized by its molecular ion and any other interference from co-existing compounds can be identified, even if they co-elute (Mohammed, 2000). According to the literature, the most employed LC-MS system for quantitative analysis of target compounds is the LC-MS/MS (Zwiener and Frimmel, 2004.). LC-MS/MS combines the

separating power of liquid chromatography (LC) with the highly sensitive and selective mass analysis capability of triple quadrupole mass spectrometry (QqQ-MS). A sample solution containing analytes of interest are pumped through a stationary phase (LC column) by a mobile phase flowing through at high pressure. Chemical interaction between the components of the sample, the stationary phase and the mobile phase affects different migration rates through the LC column affecting a separation. The wide variety of stationary phase and mobile phase combinations allow for customizing a separation to suit many complex solutions (Jamal, 2000).

After elution from the LC column, the sample is directed to the mass spectrometer (MS). The MS for an LC/MS/MS system has an ionization source where the LC column emission is nebulized, desolvated and ionized creating charged particles. These charged particles then migrate under high vacuum through a series of mass analysers (quadrupole) by applying electromagnetic fields. A specific mass/charge precursor ion (or parent ion) is targeted to pass through the first quadrupole, excluding all other mass/charge ratio particles. In the collision cell, the selected mass/charge ions are then fragmented into product ions (or daughter ions) by collision with an inert gas. The third quadrupole is used to target specific product ion fragments. The resulting isolated product ions are then quantified with an electron multiplier. This transition of ions from the precursor to product ion is highly specific to the structure of the compound of interest and therefore provides a high degree of selectivity (Jamal, 2000). The strength of this technique lies in the separation power of LC for a wide range of compounds combined with the capability of the MS to quantify compounds with a high degree of sensitivity and selectivity based on the unique mass/charge (m/z) transitions of each compound of interest.

Direct analysis of samples using LC-MS is possible, but it is usually the case that samples will need to be cleaned up to remove the worst interferences and to concentrate the sample if the analyte is only present at very low concentrations. For the extraction of target analytes, most techniques involve liquid-liquid extraction (LLE) and/or solid phase extraction (SPE) (Söderström et al., 2006; Lindegardh et al., 2007). SPE is the preferred technique since it presents the advantages of simplicity, reproducibility, and applicability. For trace analysis, SPE is generally used to selectively isolate and concentrate a known target analyte within the sample. For instance, SPE is a good way to pre-concentrate water samples prior to the final

determination to decrease detection limits (Buchberger, 2011). Typical SPE protocols for trace analysis involve the following steps (Dean, 1998; Bulletin 910: Guide to Solid Phase Extraction, 1998):

1. Wetting and conditioning – to solvate and activate the sorbent to retain analytes of interest.

2. Sample loading/retention – application of the sample to the cartridge (sorbent) where analytes of interest are retained.

3. Wash/rinse – any lesser retained interferences are washed off the sorbent.

4. Elution – removal of analytes of interest into a collection tube using a relatively small volume of suitable solvent.

A detailed literature survey on antiretroviral drugs reveals that most techniques for determination of antiretroviral drugs individually and in combination have been from pharmaceutical formulations, drug substance, and biological matrices (Venkatesan and Kannappan, 2014). Recently few studies have shown the detection of these compounds from environmental matrices i.e., soil, surface water, ground water, influent, and effluent samples (Swanepoel et al., 2015; Wood et al., 2015; Abafe et al., 2018; Ngumba et al., 2020). In this study an LC-MS/MS was used to determine the efficacy of an MBBR in the removal of five antiretroviral drugs from a spiked synthetic wastewater.

1.6 Problem statement

The current conventional wastewater treatment plant facilities in South Africa are not equipped for the complete removal of emerging contaminants which may result in a discharge of low quality (or untreated) municipal effluents into the environment (Schoeman et al., 2015). Of particular concern is the prevalence of ARVs due to the large number of people currently receiving ARV treatment in South Africa. A number of antiretroviral medicines have been detected in soil, surface water, ground water and wastewater treatment plant effluent samples (Swanepoel, 2015; Wood, 2015; Abafe, 2018). Studies show that South Africa has a growing antiretroviral load (Clinicians Society 2013; Bessong et al., 2021; van Schalkwyk et al., 2021); this means that over time more and more of these ARV compounds may enter the human population because of the inadequate treatment of sewage water. Therefore, it would

be expedient to establish a system for the complete removal of these recalcitrant antiretroviral compounds.

1.7 Rationale/Motivation

Antiretroviral drugs are not completely metabolized in patients and are excreted in large amounts from the body through faeces or urine as unchanged compounds (Galasso et al., 2002; Prasse et al., 2010). Therefore, they can enter the environment by means of wastewater treatment plant (WWTP) discharges since most pharmaceuticals are only partly removed during wastewater treatment (Prasse et al., 2010). The environmental release of antiretroviral drugs is of considerable concern due to potential ecosystem alterations and the development of viral resistance as a result of persistent low level ARV exposure in the general population. Studies have shown that antiviral drugs are among the predicted most hazardous therapeutic classes with regard to their toxicity toward algae, daphnids and fish (Sanderson et al., 2004; Nannou, et al., 2020). To date, very few studies have been published on the impact of antiretroviral compounds used in South Africa.

1.8 Research aim

The aim of this study was to investigate the efficacy of a moving bed biofilm reactor for the removal of the antiretrovirals tenofovir, emtricitabine, nevirapine, ritonavir and efavirenz from synthetic wastewater.

1.9 Objectives

1. To construct, operate and optimise the performance of a laboratory scale moving bed biofilm reactor (MBBR).

2. To investigate the efficacy of the MBBR in the removal of tenofovir, emtricitabine, efavirenz, ritonavir and nevirapine from spiked synthetic wastewater using LC-MS/MS

3. To assess the shift in the microbial biofilm consortium after the introduction of ARV compounds in the MBBR system.

4. To examine the toxicity of the bioreactor effluent and five antiretroviral compounds (tenofovir, emtricitabine, efavirenz, ritonavir and nevirapine) towards *Vibrio fischeri*, *Selenastrum capricornutum* and *Daphnia magna*.

1.10 Thesis outline

Construction and optimization of a moving bed biofilm reactor.

Chapter 2 Provides a detailed description on the construction, operation, and optimization of a lab scale MBBR.

Investigation into the efficacy of an MBBR for the removal of ARV compounds.

Chapter 3 This chapter describes the quantification, by liquid chromatography tandem mass spectrometry (LC-MS/MS), of five ARV compounds in samples generated from the MBBR. Samples were extracted by Solid Phase Extraction (SPE) cartridges and injected onto the LC-MS/MS for analysis. Quantification was performed against an external calibration curve.

Understanding the dynamics of microbial consortium prior and post introduction of ARV compounds in an MBBR system.

Chapter 4 Describes the shift in microbial communities as a result of the introduction of ARV compounds in the MBBR system. The microbial consortium was determined by extracting DNA from the biofilm on carriers. The 16S rDNA gene target was amplified using primers E517F (5'-GCCAGCAGCCGCGGTAA-3') and E969-984 (5'-GTAAGGTTCYTCGCGT-3'). The pooled libraries underwent sequencing using the MiSeq system (SY-410-1003, Illumina Inc.) with MiSeq Reagent kit v3 (600 cycle) (Illumina, MS-102-3003). The Mothur pipeline was used for the entire sequence data processing according to the Mothur SOP.

Toxicity evaluation of specific antiretroviral drugs used in South Africa.

Chapter 5 Reports the toxicity of antiretroviral drugs used in South Africa. The toxicity was assessed using *Vibrio fischeri*, *Selenastrum capricornutum* and *Daphnia magna*.

Final conclusions

Chapter 6 Discusses the main outcomes of the studies mentioned above, draws final conclusions, and outlines potential future research opportunities.

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CHAPTER TWO

2. Construction and optimization of a moving bed biofilm reactor.

2.1. Introduction

Wastewater treatment plants based on conventional activated sludge (CAS) are originally designed for removing nitrogen, phosphate, and organic matter, most of which consist of naturally occurring biodegradable organic pollutants (Grady et al., 1998). As a result, a number of non-biodegradable pharmaceuticals have been detected in WWTP effluents and surface waters (Snyder, 2008). Due to the limited efficiency of conventional biological treatment, innovative solutions are being explored to improve the removal of trace contaminants & residues in wastewater. Activated carbon has been demonstrated to have high capacity to adsorb pharmaceuticals when used in post-treatment/polishing steps for CAS treatment (Rivera-Utrilla et al., 2009; Simazaki et al., 2008). Ozonation is currently the typical process to remove organic micropollutants from wastewater (Hollender et al., 2009). However, compared with biological treatment processes, both activated carbon and ozone increase energy consumption and maintenance cost related to wastewater treatment. Falås et al. (2012) found that there were distinct differences in removal efficiencies of pharmaceuticals by activated sludge and suspended biofilm carriers. Higher degradation rates per unit of biomass were achieved with the biofilm reactor compared to activated sludge. The moving bed biofilm reactor (MBBR) technology was developed in the late 1980s in Norway for enhanced BOD and nitrogen management (nitrification and denitrification) in compact reactors (Hem et al., 1994). An MBBR is an aerated attached-growth biological wastewater treatment system that consists of free-floating plastic media. The plastic media or carriers are made of a material with a density close to the density of water (1 g/cm^3), such as polyethylene. In MBBR, the type of biofilm carrier, its shape, surface area and filling ratio are the key parameters determining the efficiency and performance of treating wastewater (Garcia et al. 2008). More carriers can provide more sites for the attachment and growth of microorganisms. However, Pham et al. (2008) demonstrated that increasing the filling ratio of carriers decreased oxygen transfer efficiency, promoting an increase in the aeration rate to fluidize carriers, which could enhance the shear stress on the biofilm. Moreover, a high aeration rate increases the operational cost (Anderson et al., 2013). Therefore, the carrier

filling ratio and aeration rate of MBBR must be optimized to meet the pollutant removal requirements. The amount of volume occupied by the carriers within the reactor is usually between 25-70% of the total reactor volume (Odegaard et al., 2000). In this study, a continuous-flow laboratory scale system was designed, built, installed, and operated under different filling ratios, organic loading rates (OLR) and hydraulic retention times (HRT) in order to optimize its performance. The systems were monitored over time for the elimination of conventional wastewater parameters i.e., Biological Oxygen Demand, Chemical Oxygen Demand, and nutrients (i.e., Ammonium ion, Total Nitrogen and Phosphate).

2.2 Experimental

2.2.1 Materials

2.2.1.1 Synthetic wastewater

A synthetic wastewater (simulating medium strength municipal wastewater primary influent) spiked with ARV compounds was used in this study. A concentrated stock solution was prepared according to Table 2.1 (Lee et al., 2003). The stock solution was stored in a refrigerator at 4°C. The synthetic wastewater (SW) was produced by diluting the stock solution with demineralised water and was fed continuously and evenly to the treatment system to avoid any fluctuation in the feed concentration and provide a sufficient source of biodegradable organic pollutants such as glucose, ammonium sulphate and potassium dihydrogen orthophosphate. The synthetic wastewater contains chemical oxygen demand (COD) of 320–360 mg/L, total organic carbon (BOC) of 100–120 mg/L, NH_4-N of 13–16 mg/L, NO_2-N of 0.01–0.02 mg/L, NO_3-N of 0.4–1.1 mg/L and PO_4-P of 3.0–3.5 mg/L. $NaHCO_3$ or H_2SO4 was used to adjust the pH in the MBBR to a constant value of 7. Depending on the experimental condition, the medium composition was varied in order to obtain the desired influent COD concentrations.

Table 2. 1: Composition and concentrations of synthetic wastewater.

Composition	**Molecular weight (g/mol)**	**Concentration (mg/L)**
Organics and nutrients		
Glucose ($C_6H_{12}O_6$)	180.0	280
Ammonium sulfate ($(NH_4)_3SO_4$)	132.1	72
Potassium phosphate (KH_2PO_4)	136.1	13.2
Trace nutrients		
Calcium chloride ($CaCl_2.2H_2O$)	147.0	68
Magnesium sulfate ($MgSO_4.7H_2O$)	246.5	5.07
Manganese chloride ($MnCl_2.4H_2O$)	197.9	0.275
Zinc sulfate ($ZnSO_4.7H_2O$)	287.5	0.44
Ferric chloride anhydrous ($FeCl_3$)	162.2	1.45
Cupric sulfate ($CuSO_4.5H_2O$)	249.7	0.391
Cobalt chloride ($CoCl_2.6H_2O$)	237.9	0.42
Sodium molybdate dehydrate ($Na_2MoO_4.2H_2O$)	242.0	1.26
Yeast extract		30

2.2.1.2 Selected ARV compounds

A set of five ARV compounds widely used in South Africa were selected, namely Tenofovir, Emtricitabine, Ritonavir, Efavirenz and Nevirapine. Concentrated stock solutions containing 1 mg/ml of each ARV compound was prepared in pure DMSO and kept in a freezer. The stock solution was added to the artificial wastewater to attain a concentration of 10 µg/L of each ARV.

2.2.1.3 MBBR Carrier Media

The biofilm media used in this study was ECOBM-100 Biofilter provided by Ecotao Enterprice, South Africa. This media resembles AnoxKaldnes K1, a polyethylene carrier with a density of

about 125 kg/m^3. The carrier is designed to provide interspaces for suspended microorganisms, offer a high specific surface area of up to 1200m^2/m^3 and has 4 rooms/chambers (Figure 2.1) with a diameter of 10 mm and a height of 10 mm. During the initial stages of the MBBR operation, the carriers were acclimatized for a period of 30 days using activated sludge collected from Khutsong WWTP fed with synthetic wastewater without the addition of the ARV micropollutants.

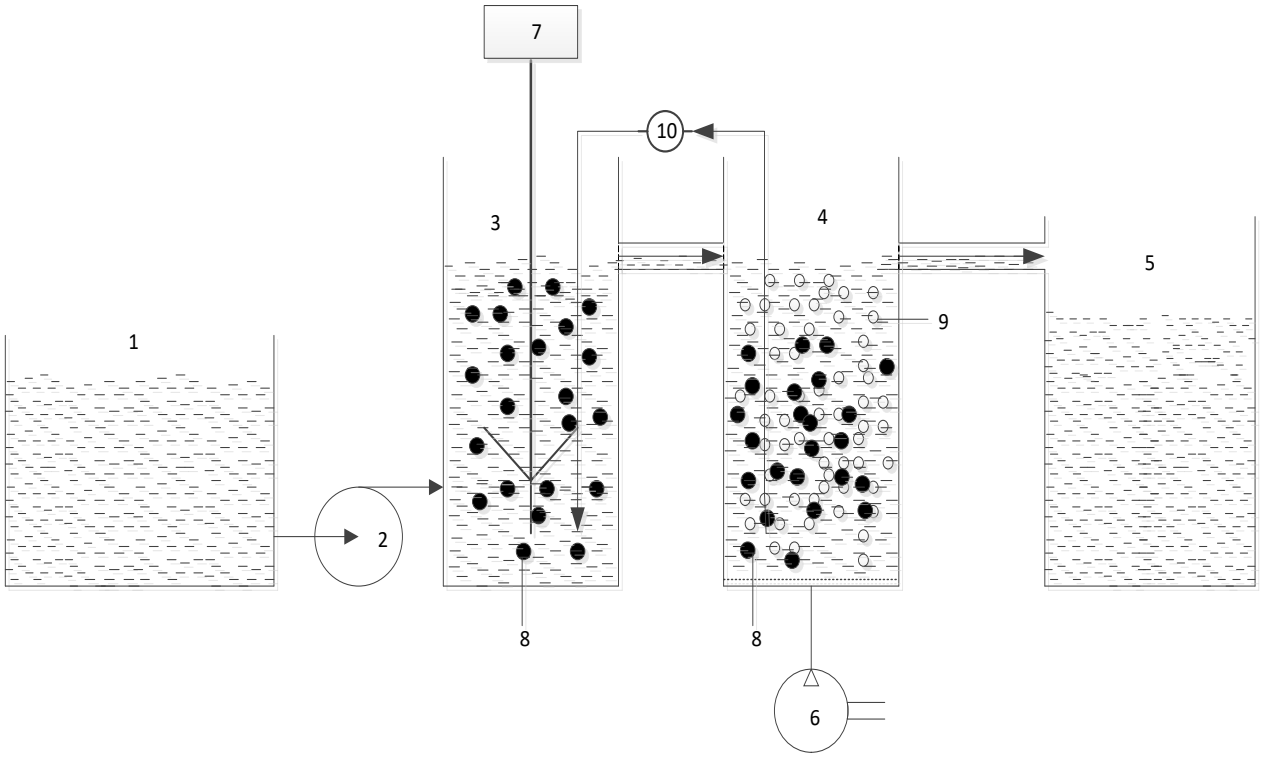
Figure 2. 1: The attached-growth carriers (ECOBM-100 Biofilter) used in this study.

2.2.2 Experimental setup and operation protocol

2.2.2.1 MBBR system optimization

A bench-scale MBBR system with a working volume of 30 L was used in the study (Figure 2.2). The system consisted of two reactors, anoxic and aerobic, connected in series. The SW was first passed through an anoxic zone where denitrification occurred, followed by an aerated aerobic zone with nitrification. Before the optimization experiments were carried out, the carriers were acclimatized in the reactor tanks filled with synthetic wastewater and activated sludge collected from a wastewater treatment plant located in Potchefstroom, North West Province, South Africa. The acclimatization of carriers lasted for 30 days. The reactor was operated at a flowrate of 20 ml/min with an internal recycling flowrate at 60 ml/min from the

aerated aerobic zone to the anoxic zone. To avoid excessive detachment of the biosolids on the carriers, the aeration of the aerobic MBBR was adjusted to around 4 L/min to achieve gentle circulation of the carriers. The DO concentration of the MBBR was in the range between 4 - 4.5 mg/L.



1. Feed
2. Peristaltic pumps
3. Anoxic reactor
4. Aerobic (Oxic) reactor
5. Secondary sedimentation tank
6. Aeration pump
7. Overhead stirrer
8. Attached growth carriers
9. Bubbles
10. Inline pump

Figure 2. 2 Schematic flow diagram of the MBBR system used in the study.

The optimization was divided into three stages. Synthetic wastewater was prepared in the laboratory and used in all stages of the experiment with constant composition. However, the concentration of synthetic wastewater in the influent was varied depending upon the organic loading rate (OLR) conditions (using Eq. 2.1). In the first stage of the study, the effect of carrier filling rate on the performance of the MBBR in terms of COD, BOD and nutrient removal was examined using 10%, 20%, 30%, 40 and 50% of carriers at HRT of 24 h and organic loading

rate (OLR) of 0.32 - 0.36 kg COD/m^3. d^{-1} In the second stage, the effect of OLR on the performance of the MBBR in terms of COD, BOD and nutrient removal was examined. Four different OLRs of 0.15, 0.3, 0.6, 0.9 and 1.2 kg COD/m^3. d^{-1} were tested under the optimized carrier filling rate and HRT of 24 h. The third stage of this study was to investigate the effect of three different hydraulic retention times (HRT) on the performance of the MBBR in terms of COD, BOD, and nutrient removal. HRTs of 6, 12 and 18 h were examined at the optimized filling rate and optimized OLR and the optimum HRT was achieved. During the HRT optimization, the flowrate varied depending upon the HRT conditions. However, the internal recycling flowrate was always three times the flowrate of the system. Subsequently, optimized operating conditions for the MBBR performance were obtained within this experiment in terms of COD, BOD, and nutrient removal.

$$OLR = \frac{\mathrm{ICOD} * \mathrm{F}}{\mathrm{V}} \qquad \text{(Eq. 2.1)}$$

Where: OLR is organic loading rate (kg/m^3. d^{-1})

ICOD is the COD concentration of synthetic wastewater influent (mg/L)

F is the flow rate of synthetic wastewater (mL/min)

V is the volume of the reactor (L)

$$HRT = \frac{\mathrm{V}}{\mathrm{F}} \qquad \text{(Eq. 2.2)}$$

Where: HRT is hydraulic retention time (h)

F is the flow rate of synthetic wastewater (mL/min)

V is the volume of reactor (L)

2.2.2.2 MBBR system experimental procedure

The reactor was run at a flow rate of 20 ml/min and a COD loading of 0.32–0.36 kg/ m^3.d^{-1}. To avoid excessive detachment of the biosolids on the carriers, the aeration of the aerobic MBBR was adjusted to around 4 L/min to achieve gentle circulation of the carriers. The DO concentration of the MBBR was in the range between 4 - 4.5 mg/L. After the optimisation stage, the MBBR system was acclimatised to the synthetic wastewater spiked with ARV

compounds. The wastewater with ARV compounds was continuously introduced to the MBBR for a period of 30 days before the investigation of ARV compounds removal was carried out. After the acclimatisation period, grab samples were then collected continuously over a period of 10 days to assess the removal efficiency. BOD, COD, PO_4, NH_4 and total nitrogen (TN) were also monitored daily during the course of the experiment.

Removal efficiency (percentage) was calculated by comparing concentrations between influent wastewater (spiked synthetic wastewater) and the effluent of the reactor using Equation 2.3:

$$Removal\ (\%) = \left(1 - \frac{Concentration\ of\ final\ effluent}{Concentration\ of\ influent}\right) \text{x}\ 100\% \qquad \text{(Eq. 2.3)}$$

2.2.3 Analytical methods

2.2.3.1 Nutrients, pH, BOD, COD and DO

Samples for analysing nutrients, pH, BOD, COD and DO were collected daily (from Day 1 to Day 30) in brown bottles from the MBBR effluent and analysed immediately.

2.2.3.1.1 Nutrients, pH, BOD, COD and DO Analytical methods.

Biological oxygen demand (BOD) of the influent and effluent was measured using a BOD analyzer (OxiTop IS 12, from labotec Midrand, South Africa). COD, NH_4-N, TN-N and PO_4-P were measured by spectrophotometric methods using a NANOCOLOR® 500 D (MACHEREY-NAGEL, from Separations scientific Roodepoort, South Africa) kit. The pH and DO of the reactor were measured everyday using a pH meter (BANTE instruments Labotec Midrand, South Africa, Multi Meter 900) and DO meter (model no. HI98198, from Hanna instruments Johannesburg, South Africa), respectively. All parameters were analysed in triplicate.

2.4.1 Statistical analysis.

The data obtained in this research were analysed by Microsoft Excel software. Statistical analysis was used to indicate significant differences ($P < 0.05$). The difference is detected as statistically significant if P-value is lower than 0.05 and non-significant if the P-value is greater than 0.05. Microsoft Excel software was also used to carry out descriptive statistics.

2.3. Results and Discussion

2.3.1 MBBR optimization.

After inoculation of the reactors with activated sludge from a municipal wastewater treatment plant, 30 days was necessary to achieve constant attached biomass concentration. This period is referred to as the start-up or acclimatisation phase, during which the plastic carriers were being colonized by the microorganisms at the initially applied organic loading rate (0.32 - 0.36 kg COD/m^3. d^{-1}).

2.*3.1.1 Effect of carrier filling rate on the performance of moving bed biofilm reactor in terms of organic matter removal.*

The main principle of MBBR is based on the use of freely moving plastic carrier elements with density a little lighter than that of water in which microorganisms form biofilms (Odegaard et al., 2000). This particular feature is especially beneficial for the establishment and development of slow growing organisms which frequently suffer from washout as a result of hydraulic and organic shock loadings (Hu et al., 2011) and/or insufficient solids retention time (Head and Oleszkiewicz, 2005). In this regard, ECOBM-100 carriers can provide an appropriate support area for rapid and stable attachment of microorganisms. Since collision and attrition of media in the reactor causes biofilm detachment from the outer surface of media (Majeed et al., 2012), ECOBM-100 carriers provide interspaces for suspended microorganisms, with 4 rooms/chambers inside a cylindrical shaped carrier element that also has outer fins to protect biofilm loss and promote growth of biofilm. An active layer is formed inside and outside of the carriers and leads to removal of organic matter (OM) and nutrients from wastewater. The high specific area of the carrier media, which allows very high biofilm concentrations in a small reactor volume, controls the system performance. It was reported that typical biofilm concentrations range from 3000 to 4000 g TSS m^{-3} (Odegaard et al. 1994), which is similar to values obtained in activated sludge processes with high sludge ages.

2.3.1.1.1 Organic removal

BOD and COD removal efficiency at HRT 24 h, OLR of 0.36 kg/m^3.d^{-1}, flow rate of 20 mL/min, aeration rate of 4.5 L/min and different ECOBM-100 carrier filling rates are shown in Figures 2.3 and 2.4, respectively.

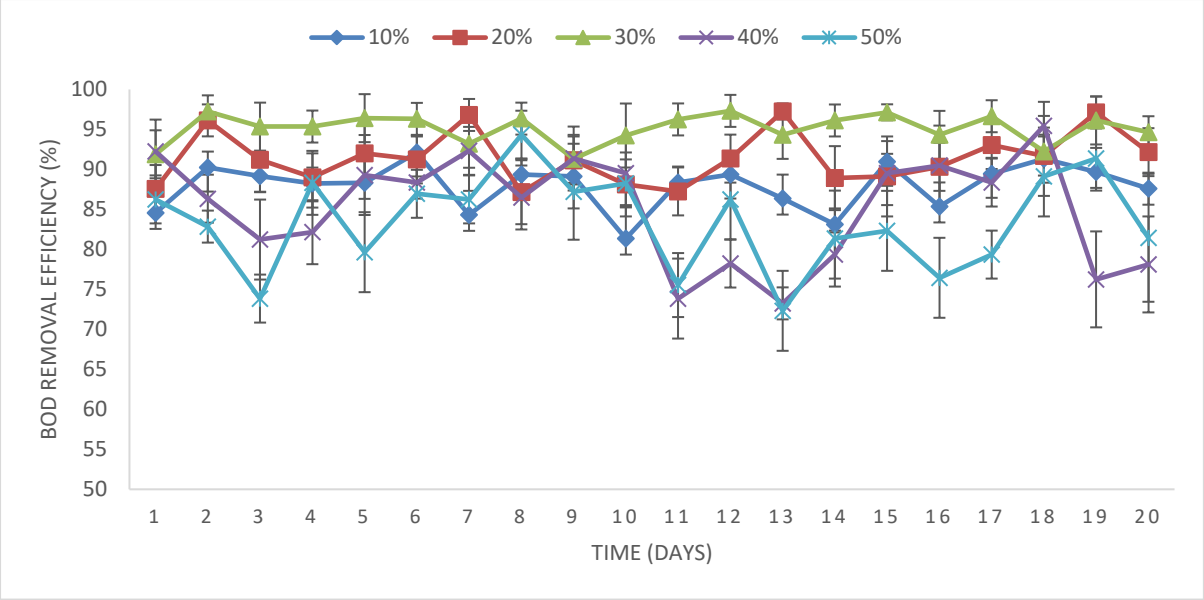


Figure 2. 3 BOD removal efficiency different carrier filling rates.

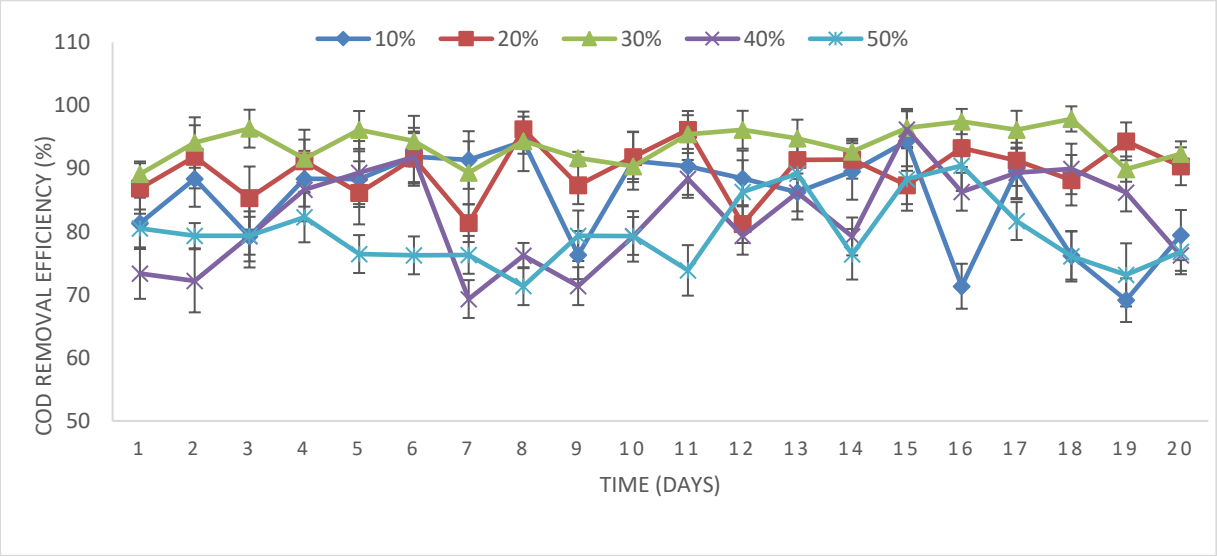


Figure 2. 4: COD removal efficiency at different carrier filling rates.

The results indicated that the average BOD removal efficiency at 10, 20, 30, 40 and 50% carrier filling rates were 87.92, 91.43, 94.09, 89.11 and 83.47%, respectively; with a p-value below 0.05, suggesting that the mean values of the five carrier filling rates were significantly different. Furthermore, the fluctuations in the trend of BOD removal efficiency at 10, 20, 40 and 50% carrier filling rates might have resulted from the unstable growth conditions for

microorganisms caused by hydraulic shearing effects. In comparison, average removal efficiency of 30% carrier filling rate was found to be slightly higher and steadier which may be as a result of an adequate amount, evenly distributed and smooth biofilm layer present on the carrier's surface to enable transport of substrate and oxygen to the biofilm.

In the same study, Figure 2.4 shows that the average COD removal efficiency was significantly affected by different filling rates. 85.27, 89.72, 93.97, 82.31 and 79.64% COD removal efficiency were obtained at 10, 20, 30, 40 and 50% carrier filling rates respectively, with a p-value less than 0.05. It can be seen that COD removal efficiency increased up to 8.7% with increasing carrier filling rate from 10% to 30%. However, adding a higher amount of carriers (from 40 to 50%) in the reactor led to a sharp decrease in COD removal efficiency (i.e. 14.33%) which may have been due to slower movement of carriers in the reactor resulting in accumulation of biomass on carrier surfaces. Adequate turbulence is ideal for efficient system performance. Sufficient turbulence sloughs off excess biomass and maintains adequate thickness of the biofilm (Bolton et al., 2006). In this regard, thick and fluffy biofilms are not desired for this system. Trapani et al. (2008) also observed that a fill fraction of 35% had higher COD removal efficiency than a 66% fill-fraction.

2.3.1.1.2 Nutrient removal

PO_4-P, NH_4-N and TN-N removal efficiencies at HRT of 25 h, OLR of 0.36 kg/m^3.d^{-1}, flow rate of 8 mL/min, aeration rate of 4.5 L/min and different PE carrier filling rates are shown in Figures 2.5, 2.6 and 2.7, respectively.

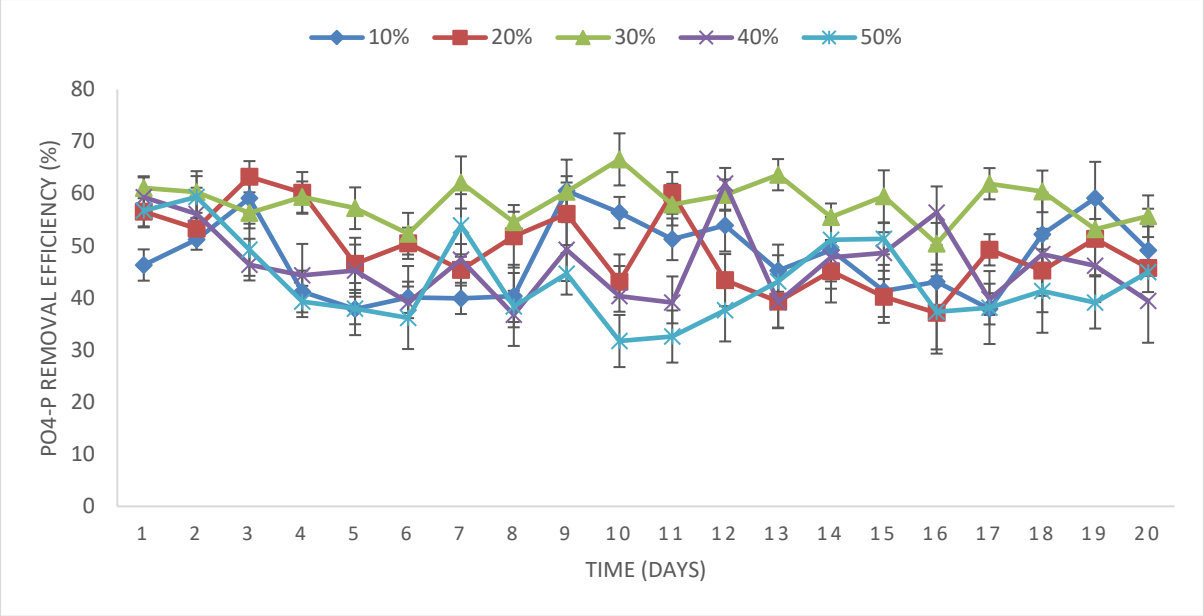


Figure 2. 5 PO_4-P removal efficiency at different carrier filling rates.

A biological phosphorus removal process utilizes bacterial consortiums for their ability to take up phosphorus as they grow in the system. This process is termed enhanced biological phosphorus removal (EBPR) (Helness and Odegaard, 1999). The bacteria responsible for phosphorus removal are phosphorus accumulating organisms (PAOs) which play a significant role in phosphorus removal (Okunuki et al., 2004). The average PO_4-P removal efficiency at five carrier filling rates of 10, 20, 30, 40 and 50% were 47.77, 49.18, 58.41, 46.55 and 43.21% respectively (Figure 2.5). With the mean values showing significant difference at $p < 0.05$. As with the BOD and COD, PO_4-P removal efficiency was affected by different carrier filling rates. There was a 10.64% improvement in PO_4-P removal efficiency from 10% to 30% filling ratio, this may be as a result of the low amount of active biofilm at a low filling rate (10 and 20%), Odegaard et al. (2000) proved that the treatment performance of MBBR is proportional to the installed biofilm surface area, so treatment upgrades can be performed by simply adding additional carriers to the same tank. On the other hand, there was a decrease in phosphate consumption above 30% carrier filling rates (i.e. up to 15.2% difference from 30% to 50% filling ratios). This may be due to the competition that occurs between the fast growing non-PAO heterotrophs and PAOs which prefer to use more energy in aerobic conditions to maintain glycogen and polyphosphate cycles than bacterial growth (Saito et al., 2004).

Nevertheless, Figure 2.5 indicates that the MBBR system had the highest and steadiest PO_4-P removal efficiency at a 30% carrier filling rate.

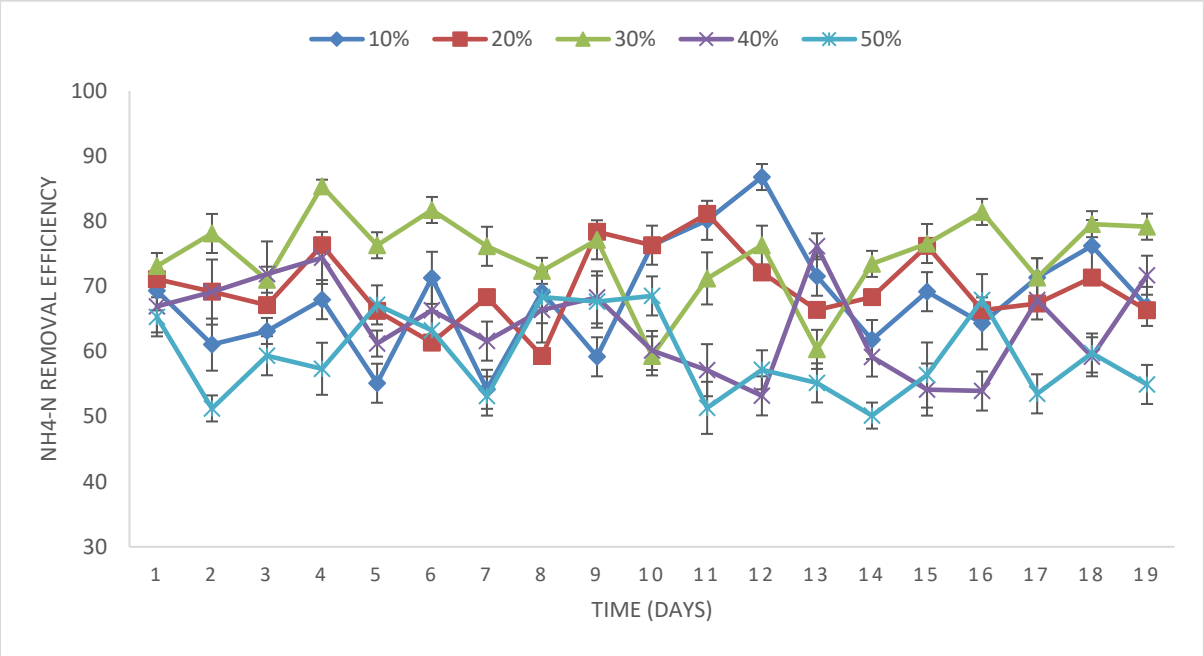


Figure 2. 6: NH_4-N removal efficiency at different carrier filling rates.

The removal of NH_4-N in the system was also monitored during the operation of MBBR at different carrier filling rates (Figure 2.6). NH_4-N can be removed from wastewater by assimilation into biomass or biological nitrification and denitrification processed under aerobic and anoxic conditions (Al-Rekabi, 2015). In nitrification-denitrification nitrogen removal takes place by two steps. In the first step (nitrification) ammonium nitrogen is converted into nitrite by autotrophic microorganisms called *Nitrosomonas* and further oxidized into nitrate by *Nitrobacter*. The second step (denitrification) nitrate is first converted to nitrite (NO^{2-}) and then to nitrous oxide or laughing gas (N_2O), nitric oxide (NO), and finally to nitrogen gas (N_2) (Wang, 2006). Figure 2.4 shows that NH_4-N average removal efficiency was 68.16, 69.96, 74.74, 64.14 and 59.35 at 10, 20, 30 40 and 50% carrier filling rates, respectively: at $p < 0.05$. The results also demonstrated that biofilm density on carriers in MBBR plays a key role in system performance and 30% filling rate can provide a sufficient amount of biomass on carriers for PO_4-P and NH_4-N removals. There was a steady increase in the efficiency of NH_4-N removal from 10% - 30% filling rate; however, a sharp decrease in NH_4-N removal was observed at filling rates above 30%. The nitrification rate is strongly

dependant on the oxygen concentration, as more and more biomass accumulates (due to sluggish movement of carriers at higher than 30% filling rates) the fast growing heterotrophs outcompete the slow growing nitrifiers for oxygen and space, hence the decrease in the efficiency of NH_4-N removal (Hem et al., 1994; Sun et al., 2016).

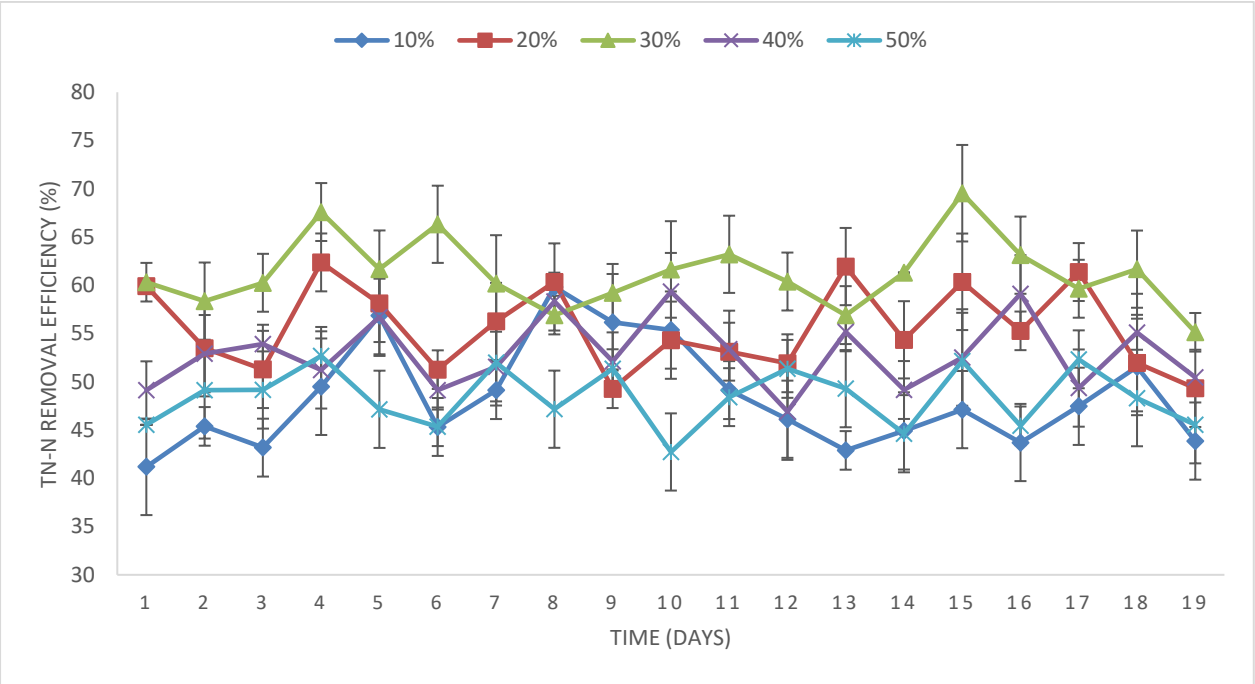


Figure 2. 7: TN-N removal efficiency at different carrier filling rates.

TN-N average removal efficiency was 48.35, 55.59, 61.23, 52.92 and 48.40% at 10, 20, 30 40 and 50% carrier filling rates, respectively. Comparable with the other parameters above, it was noted that a 30% filling rate is optimal for effective treatment. As with the NH_4-N removal efficiency, an increase in media fill ratio brings about a steady decrease in TN-N removal efficiency. Decreasing the filling rate below 30%; however, results in significant reduction in the TN-N removal efficiency due to availability of less surface area for microbial biofilm formation.

2.3.1.1.3 Microbial growth

Biomass plays a key role in MBBR systems, and it is one of the major factors which controls the system's performance. Thus, attachment of biomass to the carrier's surface and the activity of attached microorganisms were considered in many studies (Jianlong et al., 2000; Hosseiny and Borghei, 2002; Nguyen et al., 2010; Ahmed, 2011; Hajipour et al., 2011). MBBR

is capable of retaining a considerable quantity of attached biomass which provides successful performance and achieves appreciable organic and nutrient removal.

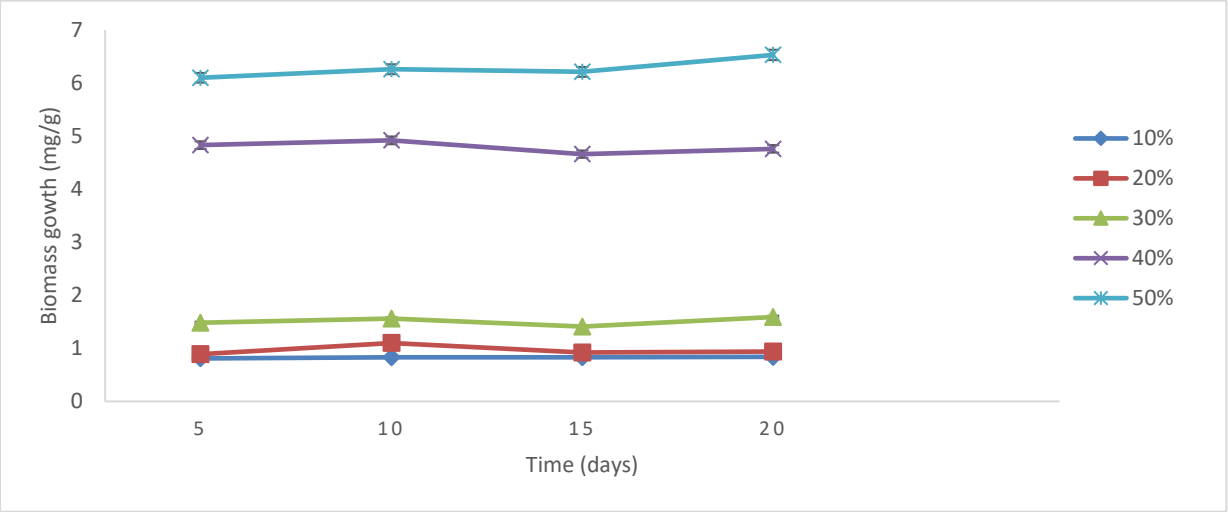


Figure 2. 8: Microbial growth on ECOBM-100 carriers at different carrier filling rates in the aerobic (oxic) reactor.

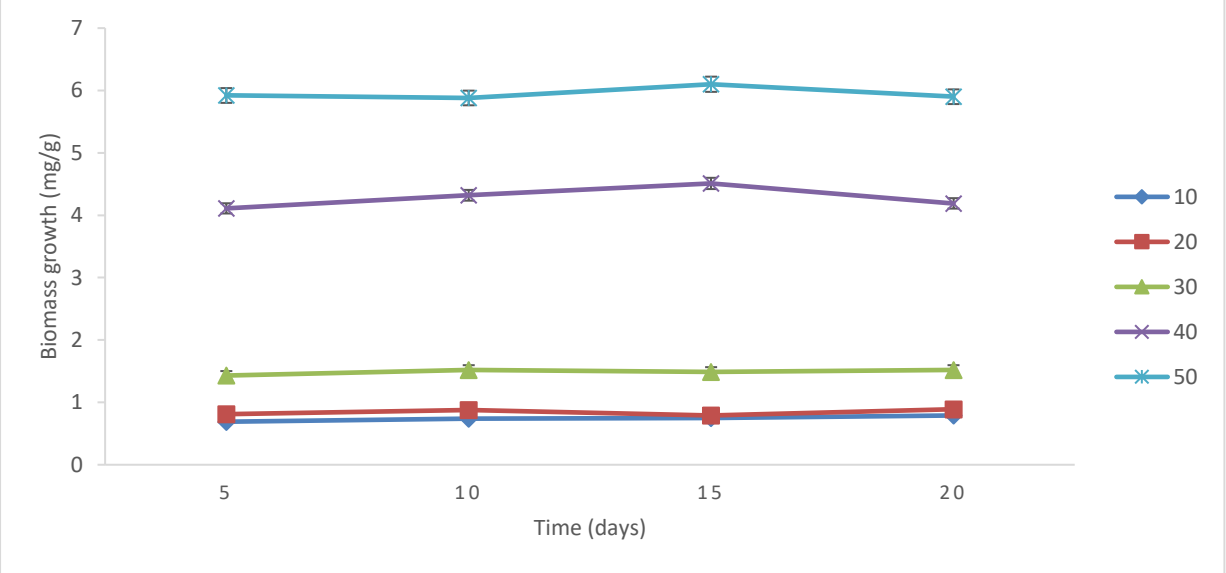


Figure 2. 9: Microbial growth on ECOBM-100 carriers at different carrier filling rates in the anoxic reactor.

As can be seen in Figure 2.8, at 10, 20, 30, 40 and 50% carrier filling rates and aeration rate of 4.5 mg/L, average biomass growth was 0.83, 0.96, 1.51, 4.79 and 6.28 mg/g, respectively. At 10% and 20% carrier filling rates, due to the quick movement of carriers in comparison to other filling rates, attachment of suspended microorganisms to the carrier was obstructed. In

addition, food consumption by attached microorganisms was hindered. This fact resulted in lower biomass growth and consequently, lower organic matter (OM) and nutrient removal compared to other carrier filling rates. At 30% filling rate, carriers moved uniformly and slowly which was the favourable condition for the attached microorganisms to obtain nutrients from the wastewater. Meanwhile, the suspended microorganisms had a better chance to attach to the carrier's surface and colonize when 30% of the reactor volume was filled with carriers.

Moreover, in Figure 2.9, the same trend was observed for the anoxic reactor at 10, 20, 30, 40 and 50% carrier filling rates and turbine rotational speed of 50 rev/min, average biomass growth was 0.74, 0.84, 1.49, 4.28 and 5.95 mg/g respectively. Similarly, at lower carrier filling rates (10 and 20%) attachment of suspended microorganisms to the carriers was hindered due to the quick movement of carriers. At 30% filling rate microorganisms had a reasonable chance of attaching to the carrier's surface and could form an adequate biofilm layer.

In both the aerobic and anoxic reactors, accumulation of microorganisms on carriers was observed at 40 and 50% carrier filling rates due to the slower movement of carriers inside the reactor. This fact resulted in the formation of a thick and unsatisfactory biofilm layer. While swift and slow movements were the main concerns of 10/20 and 40/50% carrier filling rates, respectively; uniform movement was observed at 30% filling rate which allowed for a thin, evenly distributed, and smooth biofilm to form on the carrier's surface. Consequently, higher OM and nutrient removal were obtained at this condition which was due to the better transportation of substrate and oxygen diffusion in the biofilm layer.

Alves et al. (2002) reported that when the carrier concentration was very high, the fluidization of carriers was difficult, and more aeration flux or higher turbine rotational speed was needed to suspend the carriers. As a result, the operational cost of the biofilm process increased. In this regard, thick and fluffy biofilms, which formed at 40% and 50% carrier filling rates are not desired for this system. The same results were reported by Chen et al. (2008) where pesticide wastewater was used from a pesticide factory in Hebei Province, North of China and they found the same trend for contaminants removal and microbial growth on carriers by augmentation of carrier filling rate.

2.3.1.2 Effect of organic loading rate on the performance of MBBR in terms of organic matter removal.

Organic loading rate can be calculated on the basis of either variation in the influent COD or reduction in the HRT. During this stage of the experiment, the initial OLR was set at 0.15 kg COD/m^3.d with influent COD concentration of 156.25 mg/L, HRT of 24h, carrier filling rate of 30%, flow rate of 20 mL/min and aeration rate of 4.5 L/min. The OLR was then increased gradually to 0.3, 0.6, 0.9 and then to 1.2 kg COD/m^3.d by increasing the influent COD concentration to 312.5, 625, 937.5, and then to 1250 mg/L, respectively.

2.3.1.2.1 Organic removal.

The removal efficiency of BOD at different OLRs of 0.15, 0.3, 0.6, 0.9 and 1.2 kg COD/m^3.d are shown in Figure 2.10. The results showed that BOD removal efficiency increased proportionally with increasing OLR from 0.15 to 1.2 kg COD/m^3.d. The average BOD removal efficiency at OLR of 0.15, 0.3, 0.6, 0.9 and 1.2 kg COD/m^3.d were 83.16, 93.55, 93.85, 94.25 and 95.18%, respectively. Low BOD removal efficiency at OLR of 0.15kg COD/m^3.d could be related to the low concentration of dissolved organic matter in the influent which resulted in low biofilm biomass formation. When Tijhuis et al. (1994) studied the formation and growth of heterotrophic aerobic biofilms on small suspended particles in airlift reactors, they observed that the substrate surface loading rate has an effect on the amount of biomass on the biofilm particle i.e. a higher surface load leads to a thicker biofilm.

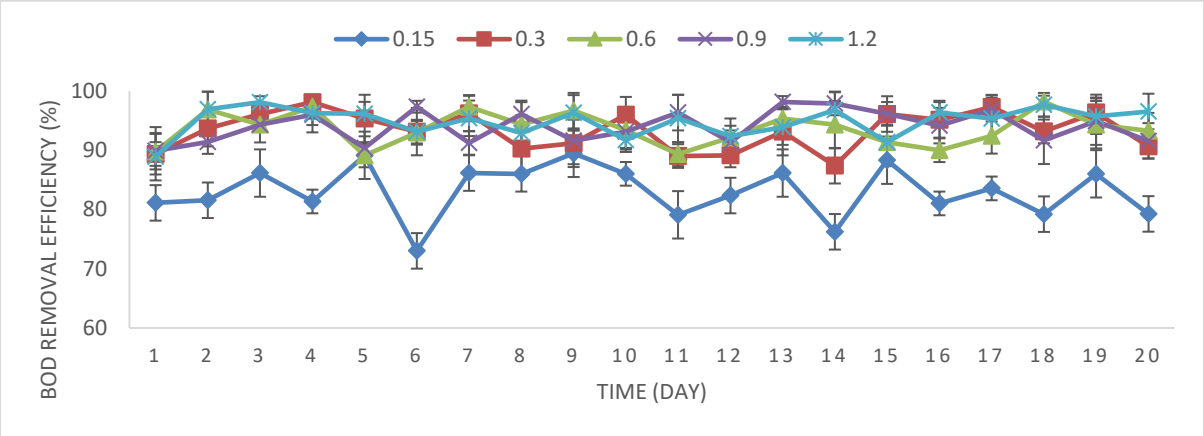


Figure 2. 10: BOD removal efficiency at different carrier filling rates.

It can be seen from Figure 2.11 that the highest BOD removal efficiencies were achieved at OLRs of higher than 0.3 kg COD/m3.d. This could be explained as a result of enhancement of microbial growth and augmentation of their attachment on carriers when the OLR was increased.

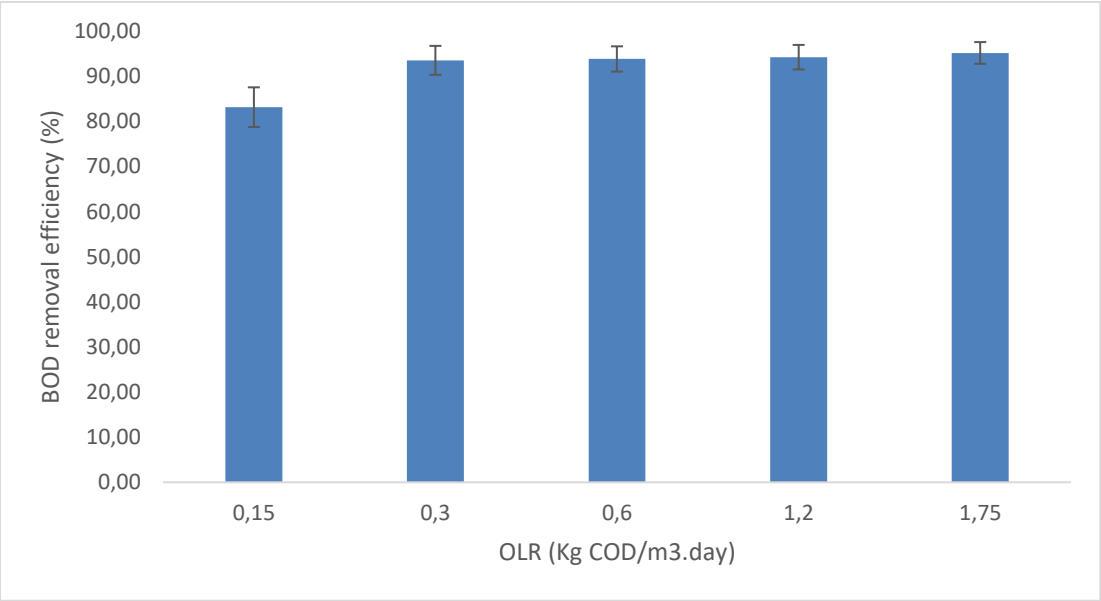


Figure 2. 11: Performance of MBBR at different OLRs in terms of BOD removal.

Carbon and nitrogen sources are essential nutrients for cell growth and protein biosynthesis in all organisms. Microorganisms cause the degradation of carbon sources and nutrients when enough oxygen is provided. As a result, an increasing carbon source results in faster growth of microorganisms and enhancement of removal efficiency. This condition is ideal for increasing the performance of MBBR. Hence, microbial concentration was enhanced with increasing OLR in this experiment and this resulted in high BOD and COD removal efficiency.

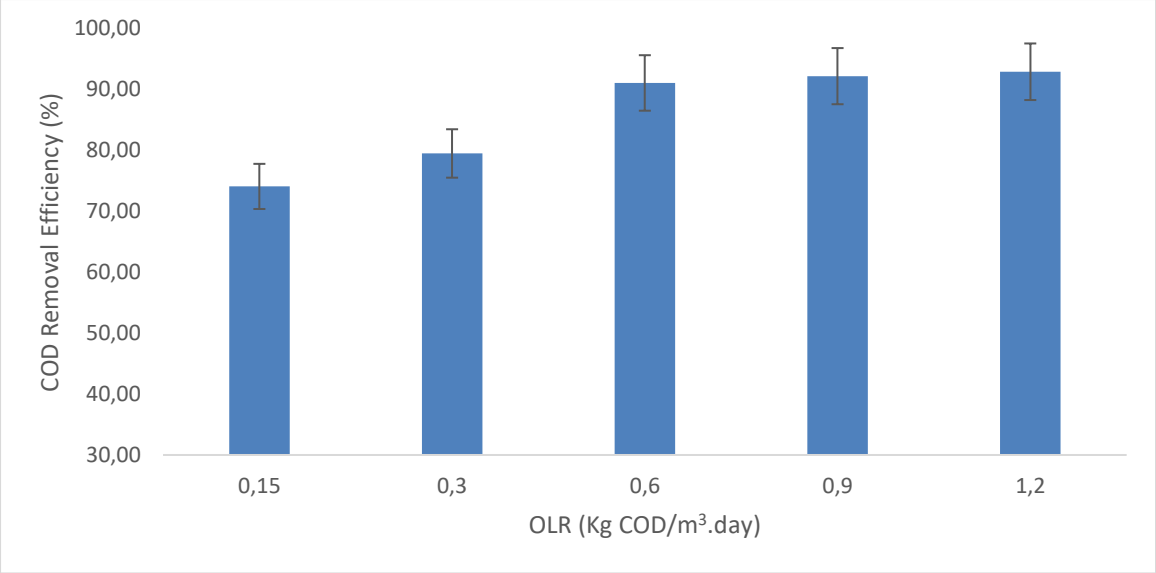


Figure 2. 12: Performance of MBBR at different OLRs in terms of COD removal.

The results for COD removal with MBBR showed a similar trend as that of BOD removal at different OLRs. Average COD removal efficiency increased from 74.07 to 92.89% with increasing OLR from 0.15 to 1.2 kg COD/m3.d^{-1}, respectively (Figure 2.12).

When Hajipour et al. (2011) studied aerobic thermophilic treatment of landfill leachate in a moving-bed biofilm bioreactor, they found the same trend in COD removal efficiency at different OLRs. In their experiment it was found that the COD removal efficiency increased primarily with increase in OLR. However, after it reached a constant value of about 6 kg COD/m3.d, COD removal efficiency started to decrease significantly. The loading rate at which the removal rate does not increase with OLR is termed maximum loading capacity of a bioreactor. Previous studies proved that increasing OLR results in an increase in COD removal efficiency until it reaches a plateau (Hajipour et al., 2011; Nguyen et al., 2011). However, this maximum appears to differ depending on the system configuration. For instance, while Hajipour et al. (2011) achieved maximum loading capacity at constant OLR of 6 kg COD/m^3.d^{-1}, Nguyen et al. (2011) reached a lower OLR of 1.2 kg COD/m3.d^{-1}. Chen et al. (2008) used a MBBR system with an anaerobic–aerobic arrangement. The contribution of the anaerobic MBBR to total COD removal efficiency reached 91% at an OLR of 4.08 kg COD/m^3.d^{-1} at HRT of 4 days, and then gradually decreased to 86% when feed OLR increased to 15.70 kg COD/m^3.d at HRT of 0.5 days. The total COD removal efficiency of the system had a slight

decrease from 94% to 92% even though the feed OLR was increased from 4.08 to 15.70 kg COD/m^3.d.

2.3.1.2.2 Nutrient removal.

PO_4-P, NH_4-N and TN-N removal efficiencies at HRT of 24 h (1 day), carrier filling rate of 30%, flow rate of 8 mL/min, aeration rate of 4.5 L/min and different OLRs are shown in Figures 2.13, 2.14 and 2.15, respectively.

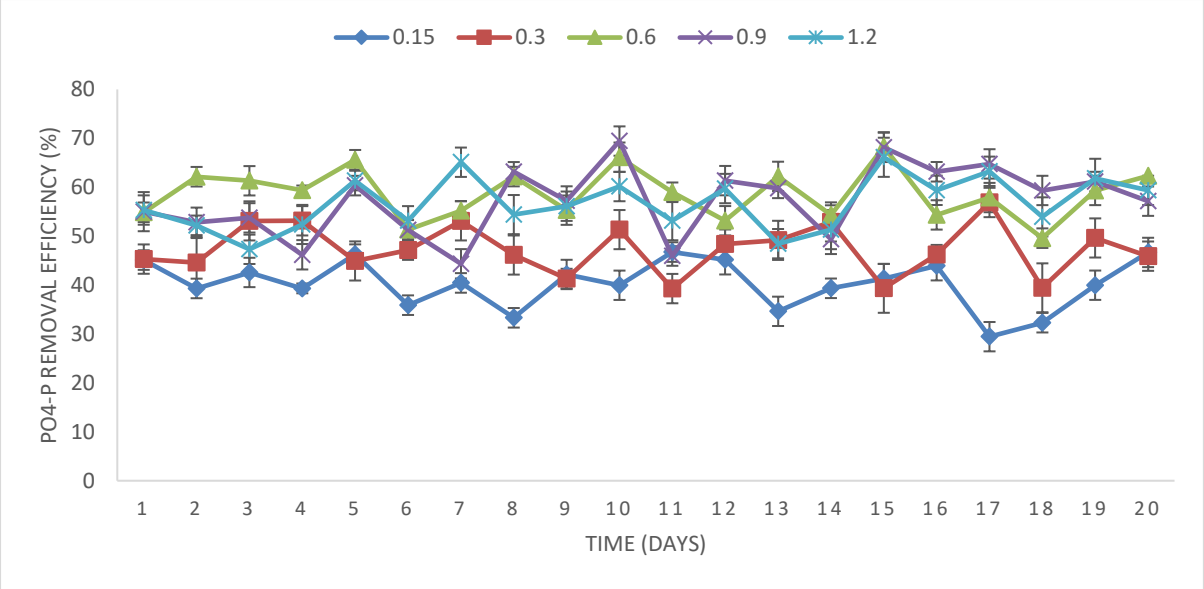


Figure 2. 13: PO_4-P removal efficiency at different OLRs.

As stated above, biological phosphorus removal is achieved mostly by PAOs, these microorganisms have the ability to accumulate phosphate over and above what is required for growth. Since EBPR is achieved by incorporation of phosphorus in the biomass, a high concentration of phosphorus accumulating biomass in the process is an advantage. Due to the low amount of biofilm concentration at OLRs of 0.15 and 0.30 kg COD/m^3.d^{-1}, the phosphate removal efficiency was 40.20 and 47.36%, respectively. However, by increasing OLR to 0.6 kg COD/m^3.d^{-1} the phosphate removal efficiency increased to 58.69%, while further increment to 1.2 kg COD/m^3.d^{-1} showed a steady decrease in phosphorus removal efficiency to 56.70%. Previous studies also demonstrated that attached growth systems could not achieve high removal efficiency as compared to activated sludge system (Wang et al., 2006; Nguyen et al., 2011). This is because phosphorus is removed from the process by withdrawal of excess sludge. In a biofilm process the concentration of suspended solids in the influent to

the sludge separation will be much lower than in an activated sludge process (Helness and Ødegaard, 1999) resulting in lower phosphorus removal.

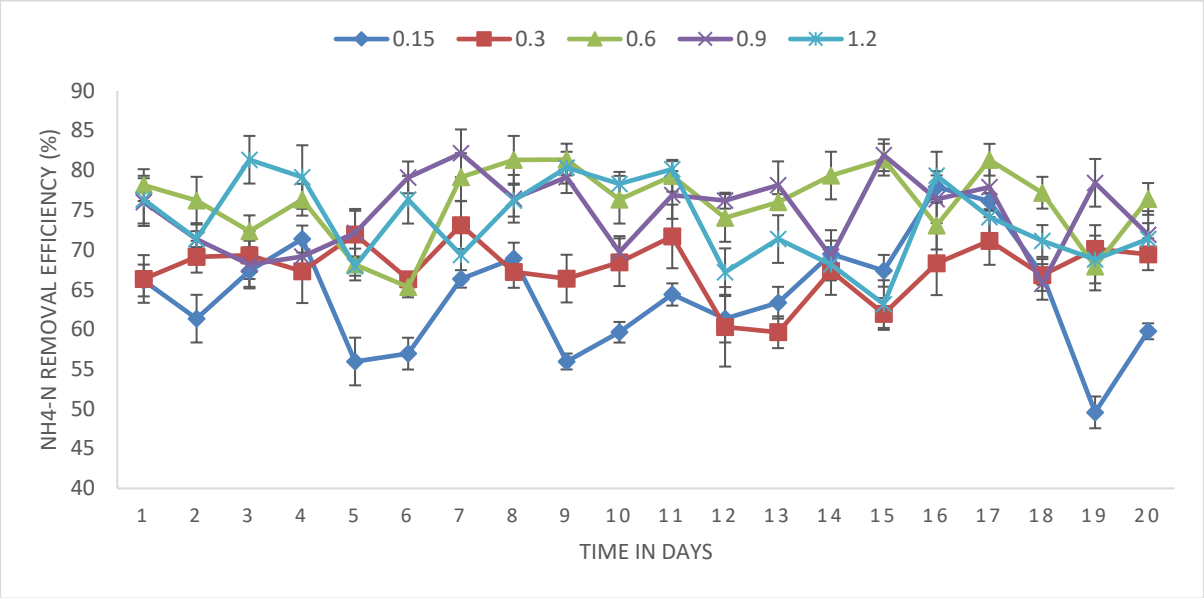


Figure 2. 14: NH_4-N removal efficiency at different OLRs.

The outcomes of NH_4-N removal showed an increase in removal efficiency rate from 64.31 to 76.03% when the OLR enhanced from 0.15 to 0.6 kg $COD/m^3.d^{-1}$, respectively. Although Li et al. (2011) reported that higher COD removal efficiency could be achieved at higher OLR in the aerobic reactor. In contrast Zielinska and Wojnowska-Baryla (2000) showed that OLR may negatively affect nitrification rate at higher OLR. High COD loads favour the development of fast-growing heterotrophs (Rittmann and Manem, 1992; Rusten et al. 1995). As autotrophic nitrifiers compete poorly for oxygen and nutrients, especially in high loaded attached growth processes, they tend to be overgrown by heterotrophic organisms (Figueroa and Silverstein, 1992; Ohashi et al., 1995). In MBBR there is a limited amount of growth area available, nitrifiers are potentially forced deeper into the biofilm, where a greater mass transport resistance is experienced (Rittmann and Manem, 1992). Hence, nitrification in the biofilm was

negatively impacted resulting in a steady decrease in NH_4-N above 0.6 kg COD/m^3.d^{-1} OLR because of the heterotrophic growth.

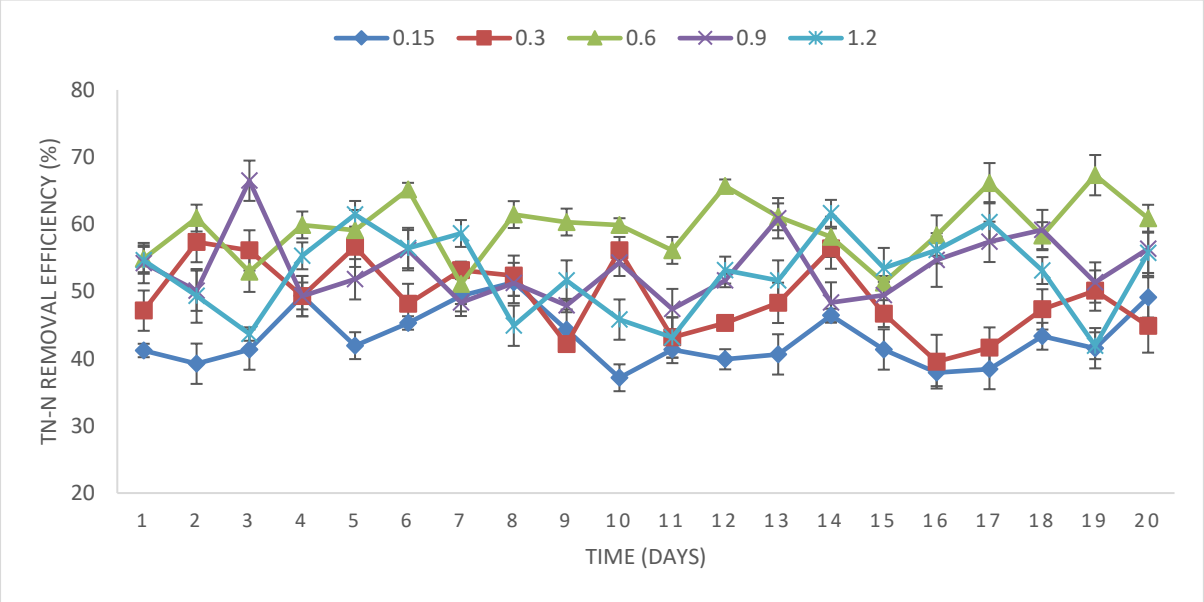


Figure 2. 15:TN-N removal efficiency at different OLRs.

Figure 2.15 shows that TN-N average removal efficiency was 43.04, 49.10, 59.46, 53.33 and 52.62 at 0.15, 0.3, 0.6, 0.9 and 1.2 kg COD/m^3.d^{-1} OLRs, respectively. Demonstrating the same trend as the NH_4-N removal efficiency, it was noted that 0.6 kg COD/m^3.d^{-1} OLR was optimum for effective removal of TN-N. As with the NH_4-N removal efficiency, an increase in OLR above 0.6 kg COD/m3.d^{-1}, brought about a steady decrease in TN-N removal efficiency. While lower OLRs, resulted in significant reduction in the TN-N removal efficiency due to availability of less sustenance for microbial biofilm formation.

2.3.1.2.3 Microbial growth

The growth trends of attached biomass at different OLRs is shown in Figure 2.16 and Figure 2.17. The higher organic loading rates applied in this experiment, promoted the growth of bacteria. The results showed that the amount of attached biomass developed in the MBBR increased as the organic loading rate was increased. In the MBBR reactor used in this study, biofilms reached an average concentration of 0.88, 1.64, 3.29, 7.22 and 15.37 mg/g in the aerobic reactor and 0.86, 1.45, 3.27, 6.71 and 14.37 in the anoxic reactor, both at 0.15, 0.3,

0.6, 0.9 and 1.2 kg COD/m^3.d^{-1} OLRs, respectively. Bassin et al. (2016) reported the same occurrence when studying the effect of organic loading rate on the performance of MBBRs filled with different carriers. As the chemical oxygen demand input was gradually increased, the biofilm became thicker on the carrier media and the surface detachment rates were enhanced. Consequently, the amount of suspended solids increased considerably to levels commonly found in hybrid bioreactors.

The dynamics of the volatile suspended and attached biomass over the five different OLRs is displayed in Figure 2.18 and Figure 2.19. For comparison with the amount of biomass present in suspension, the sludge adhered to the carriers was also expressed in mg/L.

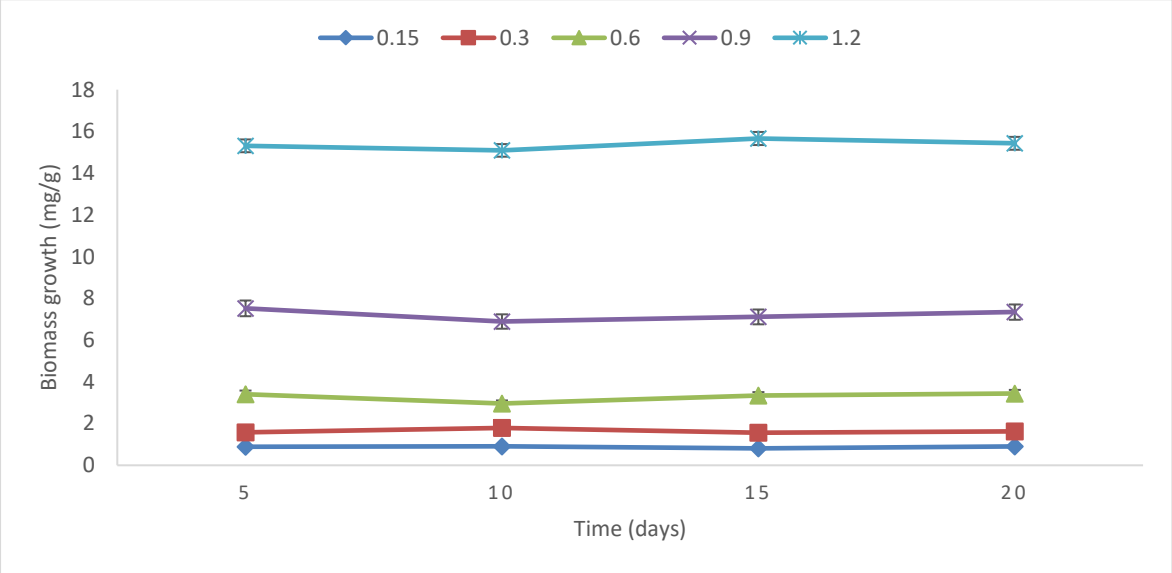


Figure 2. 16: Microbial growth on ECOBM-100 carriers at different organic loading rate in the aerobic (oxic) reactor.

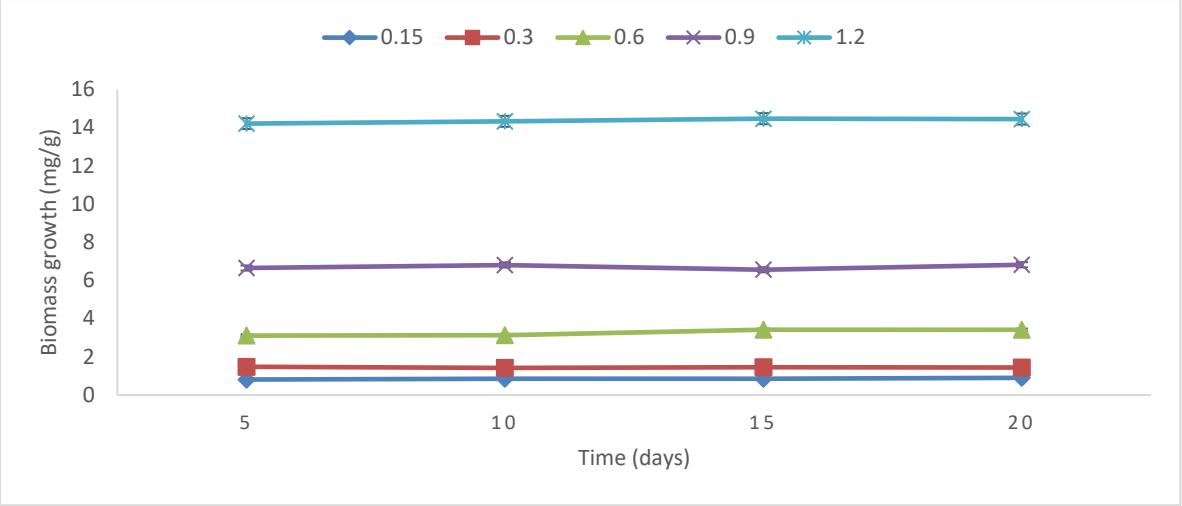


Figure 2. 17: Microbial growth on ECOBM-100 carriers at different organic loading rate in the anoxic reactor.

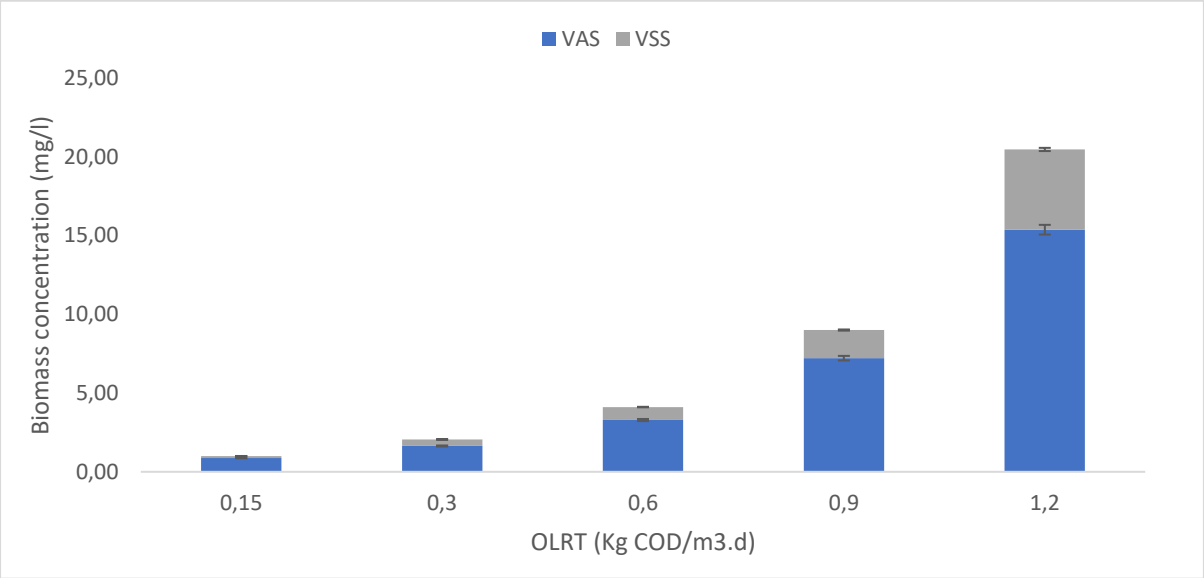


Figure 2. 18; Average attached (VAS) and suspended (VSS) biomass concentrations obtained in different OLRs in the aerobic (oxic) reactor.

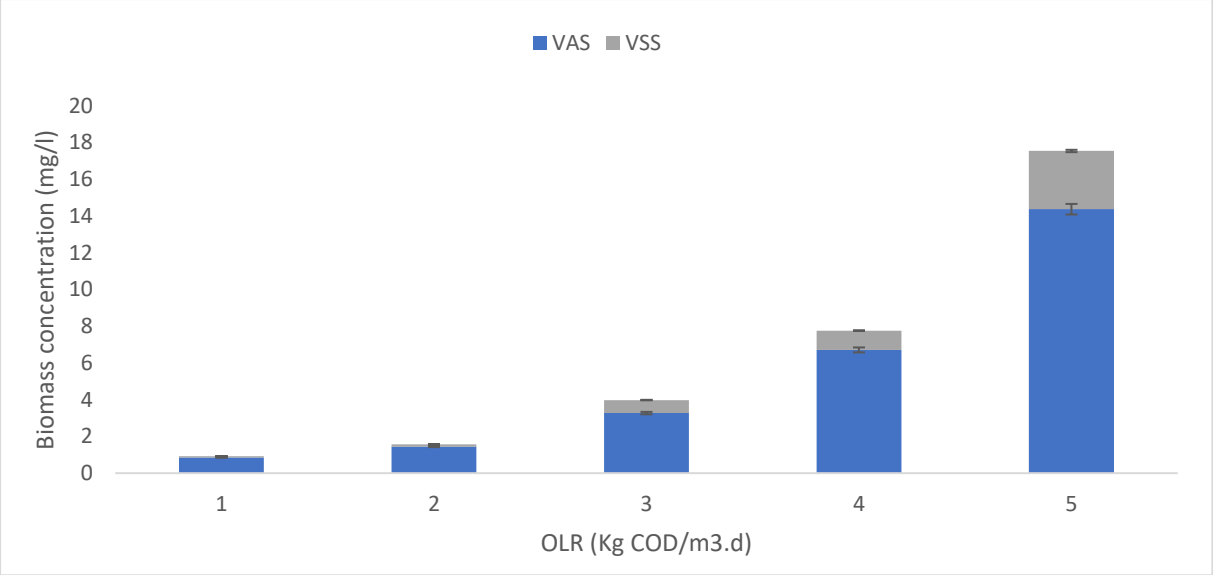


Figure 2. 19: Average attached (VAS) and suspended (VSS) biomass concentrations obtained in different OLRs in the anoxic reactor.

With regards to the volatile suspended solids, it represented only a fraction of 9% of the total volatile solids suspended biomass + attached biomass) in the aerobic reactor, whereas in the anoxic reactor it was only 8% at the lowest organic load (0.15 kg COD/m^3.d^{-1}). However, further increase in the COD loading rate from 0.15 - 1.2 kg COD/m^3.d^{-1} was accompanied by a considerable increase in amount of suspended biomass. At 1.2 kg COD/m^3.d^{-1} OLR, VSS accounted for 25% of the total amount of biomass in the aerobic reactor and 22% in the anoxic reactor. At this particular condition, the plastic carriers in the reactor exhibited the thickest biofilm and were completely saturated with biomass. With the enhanced heterotrophic growth resulting from the gradual increase of the influent organic load the COD removal efficiency increased, while the ammonium and total nitrogen removal efficiency gradually decreased at OLRs above 0.6 kg COD/m^3.d^{-1}. This shows that although there was an augmentation of VSS with an increase in OLR, it could be inferred that the contribution of the suspended biomass to the total nitrifying activity was very faint. This is due to washouts of the slow growing nitrifiers that occur in continuous operational systems.

2.3.1.3 Effect of hydraulic retention time (HRT) on the performance of MBBR in terms of organic matter removal

Hydraulic retention time is an important operational variable which can be easily controlled (Elefsiniotis and Oldham, 2006). It can be defined as the average length of time a molecule of liquid remains in the reactor and can also be defined as the volume of the reactor divided by

the average influent flow rate. Selecting suitable HRT is necessary as it directly affects the removal effect in biofilm processes (Smitha and Udayashankara 2017).

2.3.1.3.1 Organic Removal

To understand the effect of hydraulic retention time (HRT) on MBBR performance, the HRT was changed by varying the flow rate in this phase of the experiment. In this regard, this stage of the experiment was conducted at the optimal conditions and maintained a constant OLR of 0.6 kg COD/m^3.d^{-1} (Table 2.3). In order to decrease the HRT while the OLR is constant, the influent COD concentration decreased from 450 to 300 and then to 150 mg/L by changing the HRT from 18 to 12 and then to 6h, respectively (Table 2.3). The results for BOD and COD removal efficiency at different HRTs are discussed as follows.

Table 2. 2: Moving bed biofilm reactor operating condition at the third stage of the experiment.

HRT (h)	**18**	**12**	**6**
OLR (kg COD/m^3.d^{-1})	0.6	0.6	0.6
Influent COD concentration (mg/L)	450	300	150
Flow rate (mL/min)	27.8	41.7	83.3

At the initial stage of this phase, the HRT of 24h was selected, which required a flow rate of 20 mL/min. Long contact time between carriers and influent were provided. This was to enhance the rate of microbial attachment and growth on carriers. As soon as completing this period of the cycle, the HRT variation was started (at 18, 12 and 6h) with the aim of understanding the effect of HRT on MBBR performance in this study. Figure 2.20 shows BOD and COD removal efficiency at different HRTs. The results showed no significant difference at all three HRTs on the removal efficiency of BOD and COD (with $p > 0.05$). The system achieved more than 95 % of BOD and COD removal efficiency at 18, 12 and 6h. Due to the fact that the wastewater consisted mainly of biodegradable organic pollutants, 6h was enough time for the fast growing heterotrophic microorganisms to multiply and biodegrade the organic pollutants.

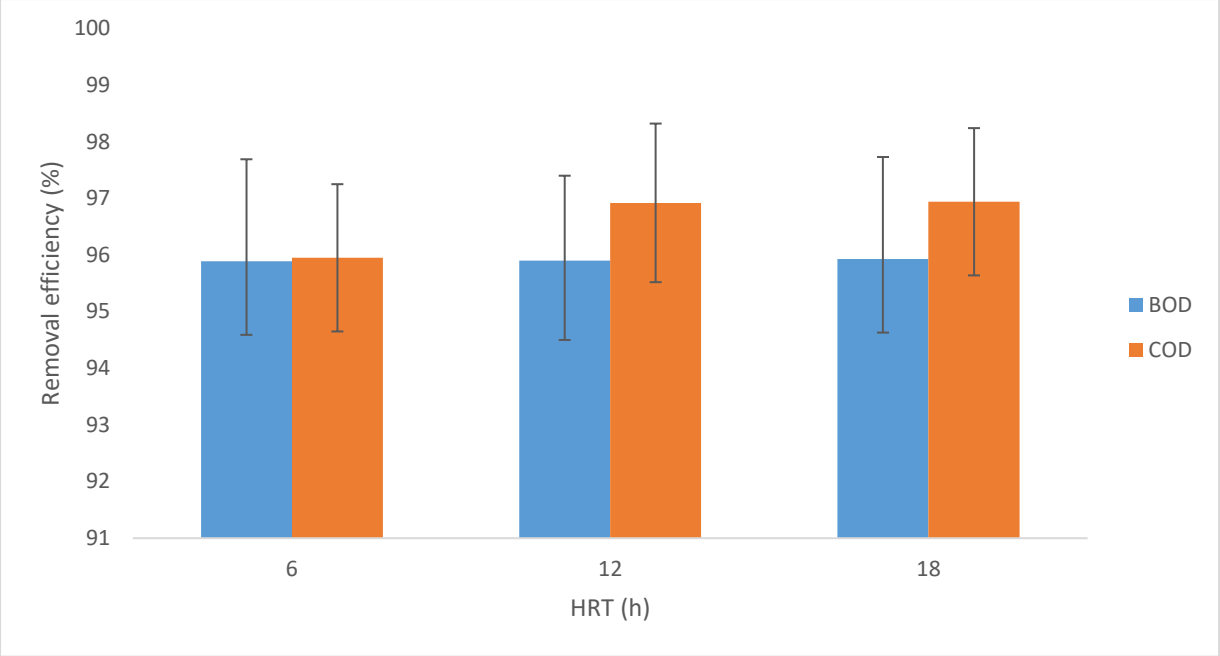


Figure 2. 20: BOD and COD removal in the MBBR unit at HRTs of 18 h, 12 h, and 6 h.

2.3.1.3.2 Nutrient removal

Figures 2.21, 2.22 and 2.23 show the effect of HRT on PO_4-P, NH_4-N and TN-N removal, respectively. Figure 2.21 shows a notable increase in PO_4-P removal efficiency by gradually increasing the HRT. The average PO_4-P removal increased significantly from 52.15 to 63.04% by increasing HRT from 6h to 18h. While in the previous optimization (OLR) study when HRT was at 25h and OLR of 0.6 Kg/COD $m^3.d^{-1}$ the PO_4-P removal efficiency reached 58.40%, indicating that HRT above 18h may not be suitable for PO_4-P removal. Jiang et al. (2018) reported the same trend when studying the effect of hydraulic retention time on the performance of a hybrid moving bed biofilm reactor-membrane bioreactor system for micropollutants removal from municipal wastewater. Within the shortest contact time of 6h between mixed liquor and biomass, phosphate was consumed by microorganisms for microbial growth (Jiang et al., 2018). In addition, Figure 2.21 shows that as the HRT increased to 12 and 18h respectively, larger amounts of phosphate were consumed and accumulated by PAOs.

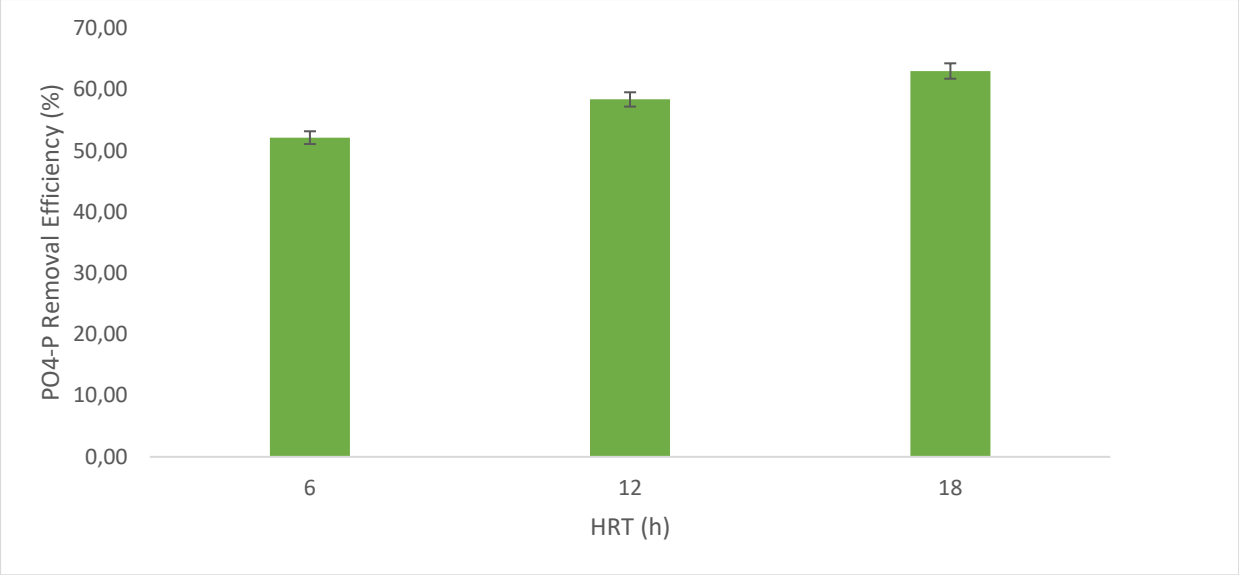


Figure 2. 21: PO_4-P removal in the MBBR unit at HRTs of 18 h, 12 h, and 6 h.

Figure 2.22 demonstrates that the NH_4-N removal efficiency was higher at longer HRT. In this regard, the average NH_4-N removal efficiencies obtained were 56.78, 65.30 and 74.80% at HRT of 6, 12 and 18 h, respectively. This significant increase in the trend of NH_4-N removal from HRT of 6 to 18 h could be attributed to the active biofilm layer presented on the carrier's surface at the longest HRT which resulted in augmentation of the nitrification rate. The same trend for NH_4-N removal was reported by Jiang et al. (2018). Since nitrifiers are slow growing microorganisms (Liu et al., 2004), they needed a longer HRT for their development on the biofilm layer.

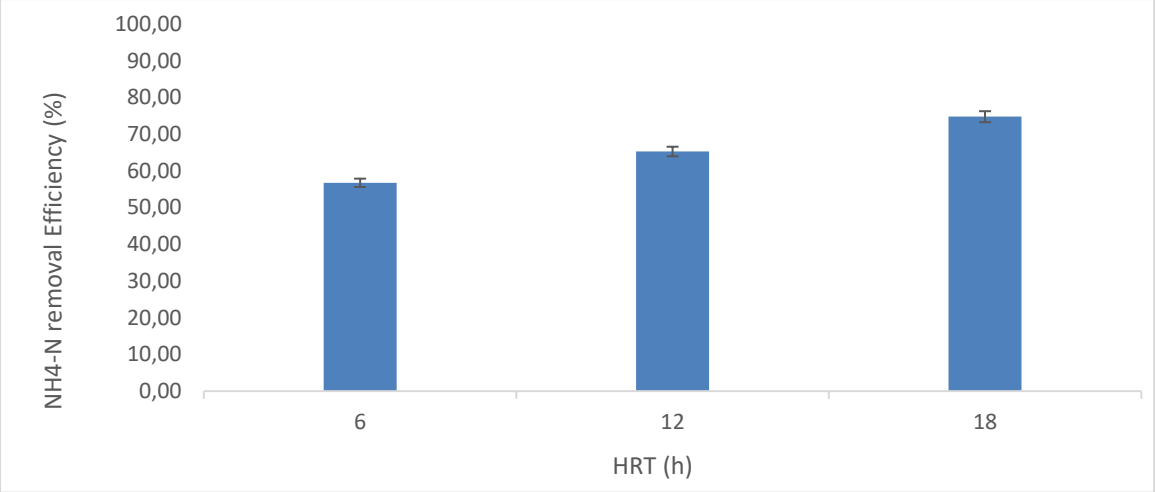


Figure 2. 22: NH_4-N removal in the MBBR unit at HRTs of 18 h, 12 h, and 6 h.

The same trend as NH_4-N was observed for T-N removal. The results revealed that when the HRT of the MBBR increased from 6 to 12 and then to 18 h, T-N removal increased from 47.46 to 51.52 and then to 62.62%, respectively. The results suggested that nitrification was enhanced by increasing HRT.

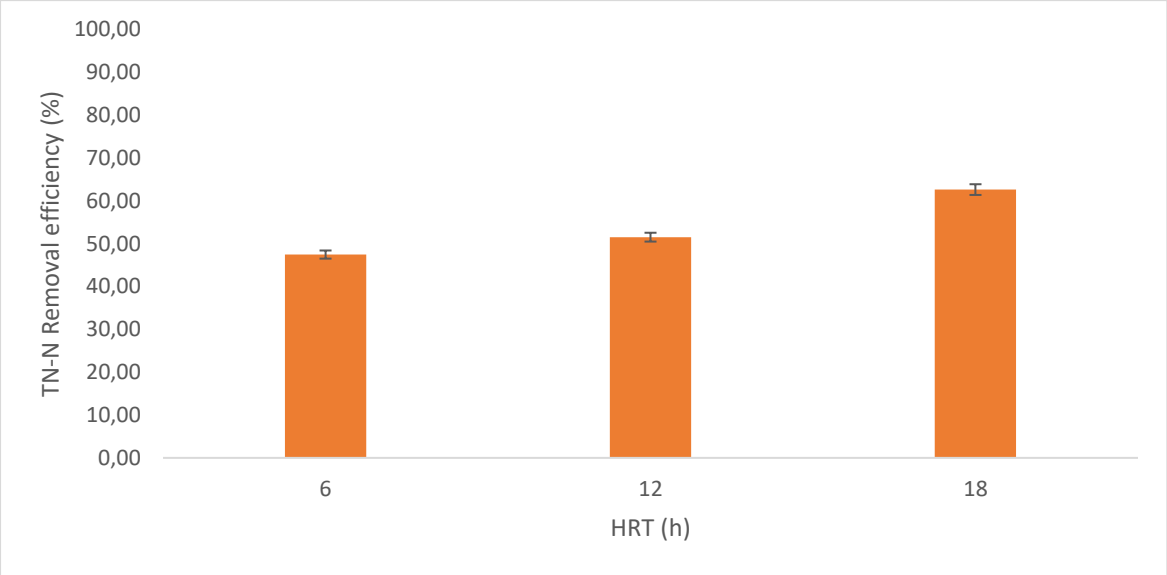


Figure 2. 23: NH_4-N removal in the MBBR unit at HRTs of 6 h, 12 h, and 18 h.

2.3.1.3.3 Microbial growth

The results of the 20-day experiment of the effect of HRT on microbial growth (VAS) in the MBBR is shown in Figures 2.24 and 2.25.

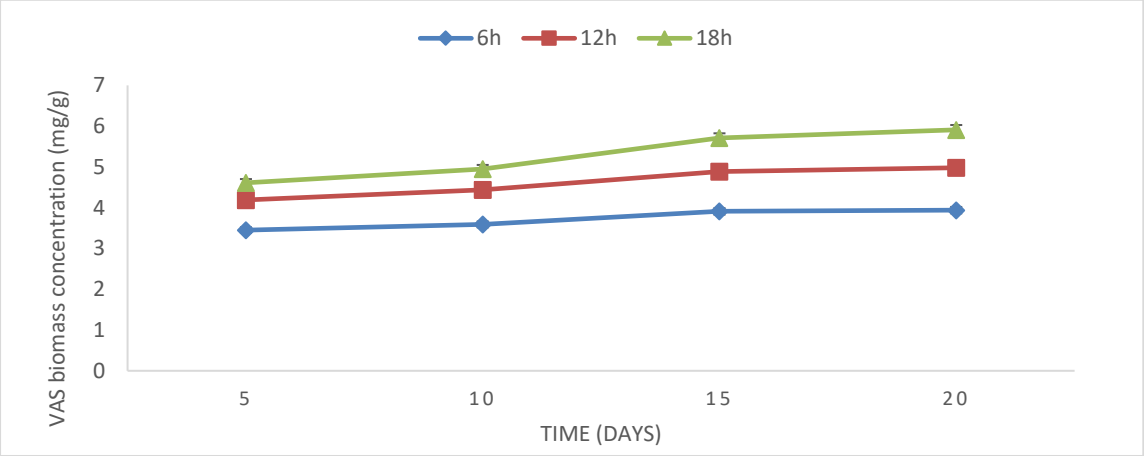


Figure 2. 24: Microbial growth on ECOBM-100 carriers at different HRTs in the aerobic reactor.

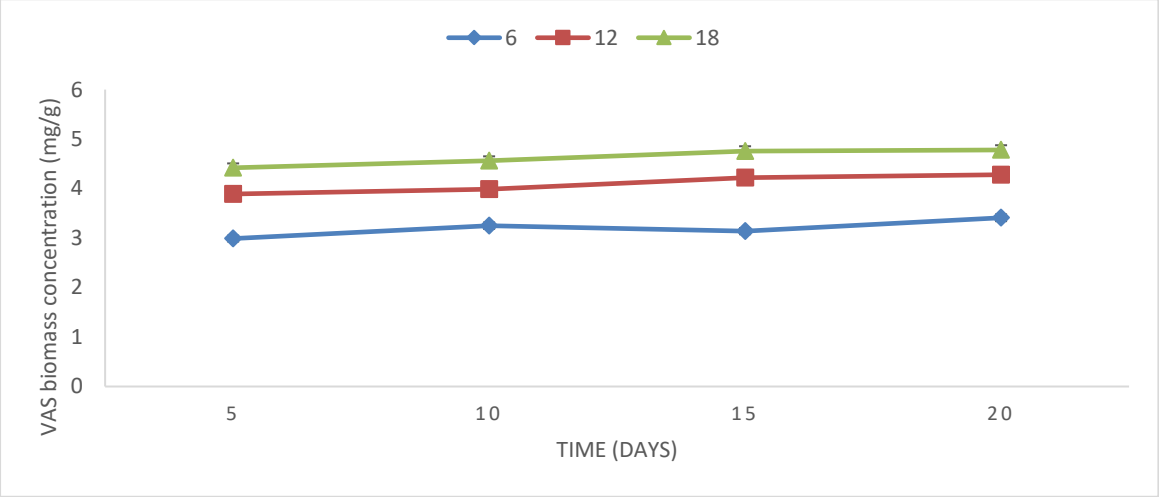


Figure 2. 25: Microbial growth on ECOBM-100 carriers at different HRTs in the anoxic reactor.

Higher biomass concentration was observed when HRT increased successively from 6 to 18 h in both aerobic and anoxic reactors. In this regard, with the increment of HRT from 6, 12 and then to 18 h, attached biomass concentration increased from 3.72, 4.63 and 5.30 mg/g, respectively in the aerobic reactor and 3.20, 4.10 and 4.63 mg/g respectively in the anoxic reactor. This improvement in microbial growth resulted in subsequent enhancement in

nutrient removal. The same observation was reported by González-Martínez et al. (2013) when studying the effect of hydraulic retention time on ammonium removal. They indicated that the use of longer HRT (of 0.5 day) determines the formation of highly specialized biofilms (mainly by *Nitrosomonas* sp.), which are effective in the biotransformation of ammonium into nitrite. On the contrary, the use of shorter HRT (of 0.4 day), determines the formation of more heterogeneous biofilms. Thus higher HRTs are more desirable in MBBR systems.

2.4 Conclusion

The MBBR was shown to effectively remove BOD, COD, and nutrients (phosphates and nitrates). The optimum operating conditions for the MBBR were obtained at a carrier filling rate of 30%, organic loading rate of 0.6 kg COD/m^3.d^{-1} OLR, hydraulic retention time of 18h and flow rate of 27.8 mL/min. These running conditions were then maintained for ARV removal efficiency studies in chapter three.

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3. Quantification of the ARV removal efficiency of a moving bed biofilm reactor.

3.1 Introduction

Scientific literature demonstrates that anthropogenic organic compounds such as pharmaceuticals, biocides and industrial chemicals are today ubiquitously present in the aquatic environment (Boyd et al., 2003; Snyder et al., 2003; Chen et al., 2006; Kim et al., 2007; Kumar et al., 2010; Capdeville, 2011). Several other studies have revealed that micropollutants may have adverse effects on aquatic life even at very low concentrations (Walters et al., 2005; Peck and Hornbuckle, 2006; Kümmerer, 2009; Santos et al., 2010;Mehinto et al., 2010; Hallmann et al., 2014; Jonsson et al., 2014). In surface and ground waters, a broad range of inorganic and organic contaminants are controlled by legislation outlined by the Department of Water and Sanitation's water quality management (Department of Water and sanitation, 2016). However, these have traditionally been industrial or agricultural chemicals. Following the recent proposal of the pharmaceuticals E2, EE2 and diclofenac as priority hazardous substances (European Commission, 2008), there is a need for legislation to include a greater number of municipal derived chemicals described as emerging contaminants (ECs). The groups of pharmaceuticals that are being detected in aqueous samples worldwide include non-steroidal anti-inflammatory drugs (NSAIDs), β-blockers, antibiotics, anti-epileptics, antipsychotics, steroid hormones and ARVs (Madikizela et al., 2017). The occurrence and fate of ARVs in particular has not been well documented; however, there has recently been some progress in the analysis of ARV drugs in aquatic environments (Ncube et al., 2018). This could emanate from the steady increase in the number of people that receive ARV treatment. To date, in South Africa alone, there is an estimated 4.5 million people receiving ARV therapy compared to about 616 000 in 2009 (WHO, 2018). This places South Africa as the country with the largest ART programme in the world. As a result, there are an increasing number of reports on the occurrence of ARVs in South African waters (Wood et al., 2015; Schoeman at al., 2015; Swanepoel et al., 2015; Wood et al., 2016; Abafe et al., 2018). As with most (human) pharmaceuticals, ARVs are partly metabolised in treated individuals, while large fractions are excreted unchanged via urine or faeces (Daughton and Ternes, 1999; Galasso et al., 2002) and thus find their way into WWTPs.

Given that the current WWTPs are not designed to eliminate the ECs and their metabolites, municipal wastewater treatment facilities are viewed as one of the principal discharge sources for the release of emerging contaminants into the environment (Ternes et al., 2004; Petrie et al., 2015). However, considerable progress has been made in the efforts to eliminate the release of pharmaceuticals from WWTPs to the environment. A number of investigations have shown that biological treatment can accomplish more, if optimised for enhanced removal of micropollutants. MBBR systems (a biological wastewater treatment process) have been used both in pilot plant studies and in full scale plants for the treatment of different types of wastewaters (Barwal and Chaudhary, 2014). The basic principle of the MBBR is the use of plastic carriers on which microorganisms can grow in biofilms, where different bacterial groups compete and co-exist in different niches. With microorganisms growing in biofilms rather than in suspended flocs, it is possible to fit more active biomass into the treatment plant, hence creating very compact treatment solutions. When comparing activated sludge (AS) to MBBR on the biodegradation of benzotriazoles and hydroxybenzothiazole, Mazioti et al. (2015) reported that the biomass developed in the MBBR system had greater capacity for removal than the AS, especially when operated under low organic loading. Accinelli et al. (2012) examined the removal of bisphenol-A, atrazine and oseltamivir with bioplastic carriers inoculated with specific bacterial strains. The results from the study showed that when wastewater samples were incubated with freely moving carriers, greater removal of the three chemicals was observed. This provides a potential solution to the removal of ARVs from wastewater. The main objective of this study was to examine the ability of an MBBR to remove ARVs from municipal/domestic wastewater. A continuous-flow laboratory scale system was designed, built, installed, and operated under different organic loading rates (OLR), hydraulic retention times (HRT), and filling rates in order to optimize its performance. The systems were monitored over time for the elimination of conventional wastewater parameters i.e., Biological Oxygen Demand, Chemical Oxygen Demand, and nutrients.

3.2 Experimental

3.2.1 Selected ARV compounds

A set of five ARV compounds widely used in South Africa were selected, namely Tenofovir, Emtricitabine, Ritonavir, Efavirenz and Nevirapine. Concentrated stock solutions containing 1 mg/ml of each ARV compound was prepared in pure dimethyl sulfoxide DMSO and kept in a

freezer. The stock solution was added to the artificial wastewater to attain an initial concentration of 10 µg/L of each ARV.

3.2.2 MBBR system experimental procedure

After the optimisation stage (Chapter 2), the MBBR system was acclimatised to the synthetic wastewater spiked with ARV compounds (10 µg/L of each ARV). The wastewater with ARV compounds was continuously introduced to the MBBR for a period of 30 days before the investigation of ARV compounds removal was carried out. After the acclimatisation period, grab samples were then collected continuously over a period of 10 days to assess the removal efficiency. BOD, COD, Phosphate, Ammonium and total nitrogen (TN) were also monitored daily during the course of the experiment.

Removal efficiency (percentage) was calculated by comparing concentrations between influent wastewater (spiked synthetic wastewater) and the effluent of the reactor using Equation 3.1:

$$Removal\ (\%) = \left(1 - \frac{Concentration\ of\ final\ efluent}{Concentration\ of\ influent}\right) \text{x } 100\% \qquad \text{(Eq. 3.1)}$$

3.2.3 Analytical methods

3.2.3.1 Nutrients, pH, BOD, COD and DO

Samples for analysing nutrients, pH, BOD, COD and DO were collected daily (from day 1 to day 30) in brown bottles from the MBBR effluent and analysed immediately.

3.2.3.1.1 Nutrients, pH, BOD, COD and DO Analytical methods.

Biological oxygen demand (BOD) of the influent and effluent was measured using a BOD analyzer (OxiTop IS 12, from labotec Midrand, South Africa). COD, NH_4-N, TN-N and PO_4-P were measured by spectrophotometric methods using a NANOCOLOR® 500 D (MACHEREY-NAGEL, from Separations scientific Roodepoort, South Africa) kit. The pH and DO of the reactor were measured everyday using a pH meter (BANTE instruments Labotec Midrand, South Africa, Multi Meter 900) and DO meter (model no. HI98198, from Hanna instruments Johannesburg, South Africa), respectively. All parameters were analysed in triplicate.

3.2.3.2 ARV compounds

ARV compounds in the samples generated from the MBBR were quantified by using liquid chromatography tandem mass spectrometry (LC-MS/MS). Samples were pre-concentrated and extracted by Solid Phase Extraction (SPE) cartridges and injected onto the LC-MS/MS for analysis. Quantification was performed against an external calibration curve.

3.2.3.3 ARV Analytical Standards

Efavirenz, Nevirapine and Ritonavir were purchased from Industrial Analytical (Pty) Ltd (Khayalame, South Africa). Tenofovir and Emtricitabine were purchased from Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). 10 mg of the analytical standards (Tenofivir, Emtracitabine, Nevirapine, Ritonavir and Efiverenz) were dissolved in 10 ml Dimethyl Sulfoxide (DMSO), resulting in a 1000 mg/L solution. This solution was then diluted 10 times to produce a 100 mg/L working stock solution. Appropriate dilutions were then made using the synthetic wastewater as a diluent to produce to 0.5, 1, 3, 5, 8, 10 and 12 µg/L standards. The 0.5, 1, 3, 5, 8, 10 and 12 µg/L standards were then injected to produce a calibration graph for all standards.

3.2.3.4 Sample Preparation

Samples were vacuum filtered through 0.45 µm (Pall, USA) filters on an Agilent Vacuum Manifold (Agilent, Santa Clara, California) prior to processing. Solid Phase Extraction (SPE) was carried out as described previously by Wood et al. (2017). The Smart Prep automated SPE system (Horizon, USA) was used to extract the samples. Oasis HLB (Waters, USA) SPE cartridges (6 cc, 500 mg) were conditioned with 4 mL of methanol (Labscan, Poland), followed by 6 mL of HPLC-Grade water (Burdick and Jackson, USA) and loaded with 500 mL of filtered sample. Flow rates were 10 mL/min for each step. The cartridges were then dried under nitrogen and eluted with 5 mL of methanol into 500 µL of dimethyl sulfoxide (Sigma Aldrich, Germany). Eluted samples were then evaporated under a stream of nitrogen at room temperature to 500 µL and stored at -20 ^{o}C until analysis.

3.2.3.5 LC-MS/MS Analysis

LC analysis was performed on an Agilent 6460 triple Quad LC/MS system with water (A) and acetonitrile (B), both with 0.1% formic acid as mobile phase (All chemicals from Burdick and

Jackson (Honeywell)). Separation was on an Agilent Poroshell HPH-C8, 2.7µm, 3.0 x 50 mm column at a flow rate of 0.5 mL/min with a 10 µL injection volume. Elution started at 20% B (organic) for 0-5mins, 5-15mins to 90% B and 15-20mins down to 20% (B). Column eluent passed into an Agilent 6460 triple quad LC/MS with a jet stream electrospray ionisation source operated in a positive mode. Source and acquisition parameters are presented in Table 3.1 below.

Table 3. 1: Agilent 6460 triple Quadrupole LC/MS parameters for the analysis of antiretroviral compounds with positive electrospray ionisation mode.

Parameter	**Value**
Gas Temperature	300°C
Drying Gas	5.1 L/min
Nebuliser	45 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	11 L/min
Nozzle Voltage	300V
Fragmentor	135 V
Acquisition range	50- 1500 m/z
Acquisition rate	0.33 cycles/s

Reactor samples that had been spiked with 10 µg/L of the five ARV drugs were analysed quantitatively against external calibration curves. These values are compared to the spiked unreacted influent water. Each sampling time was analysed in triplicate (Table 2.5). Qualitative and quantitative data analysis were performed using Agilent MassHunter Qualitative and Quantitative software respectively.

3.2.3.6 Calibration curve

External calibration curves were generated by injecting triplicates (10 µL) of serially diluted standards (Nevirapine, Emtricitabine, Ritonavir, Efavirenz, and Tenofovir) onto LC-MS/MS.

Peak areas were measured, and a plot of area against concentration was used to prepare the seven-point calibration curves for each ARV compound (0.5 - 12µg/L).

3.2.3.7 Method validation

Method validation is an important part of analytical chemistry to confirm that the method employed for a specific test is suitable for its intended use (Green, 1996). The method was validated for all the five analytes according to the ICH guidelines, and the following parameters were tested for the validation:

i. linearity,

ii. repeatability,

iii. % recovery,

iv. matrix match effect, and

v. limit of detection (LOD) and limit of quantitation (LOQ).

i. Linearity

The linearity of the calibration was determined from the correlation coefficient (R^2) of the calibration curve recorded when seven different concentrations between 0.5 – 12 µg/L were injected in triplicate onto the LC-MS/MS system.

ii. Repeatability

The instrumental intra-day and inter-day repeatability of the instrument was assessed by three different concentrations (5 µg/L, 10 µg/L and 15 µg/L) of mixed standards. The samples were injected six times (*n = 6*) at a different time of the day for intra-day repeatability and in three consecutive days for inter-day repeatability. The percentage relative standard deviation (RSD) of the responses were then determined.

iii. Percentage recovery

The SPE recovery was evaluated with three different concentrations of 5 µg/L, 10 µg/L and 15 µg/L respectively. For each concentration, triplicate samples of 1000 mL Milli-Q water were divided into two 500 mL portions A and B, respectively. Triplicate samples of portion A for

each concentration was spiked with the stock mixed standard solution to the concentrations of 5 µg/L, 10 µg/L and 15 µg/L, respectively. The second part (B) was not spiked. Both A and B were passed through the SPE process. B was later spiked with the stock solution to 5 µg/L, 10 µg/L and 15 µg/L, respectively, after the SPE process. The concentrates of both A and B were reconstituted into 1mL with acetonitrile/water (20:80 v/v) in 2mL chromatographic bottle and injected onto the LC-MS/MS.

$$\% \text{ recovery} = \frac{A}{B} \times 100 \qquad \text{(Eq 3.2)}$$

Where:

A = peak area of A (peak area of analyte added before the SPE process);

B= peak area of B (peak area of analyte added after the SPE process).

iv. Matrix effect (ME).

Matrix effects are often caused by the alteration of ionization efficiency of target analytes in the presence of co-eluting compounds in the same matrix. Matrix effects can be observed either as a loss in response (ion suppression) or as an increase in response (ion enhancement). In this study synthetic wastewater (SW) without the ARV drugs was used for matrix match blank sample. The SW was used to evaluate the matrix effect as follows. Triplicate samples of both Milli-Q water and SW samples were spiked with a standard solution to 10 µg/L before and after the SPE process, respectively. Then, the peak areas of the samples were measured in the LC-MS/MS, and the ME was evaluated using Equation 3.3.

$$\text{ME (\%)} = \frac{X}{Y} \times 100 \qquad \text{(Eq. 3.3)}$$

Where:

X = the peak area of the ARV standard recorded for the Milli-Q water.

Y = the peak area of the ARV standard recorded for the extracted synthetic wastewater sample spiked with ARV standard after SPE.

v. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined by injecting 1 mg/L of standard ten times onto the LC-MS/MS. The LOD and LOQ for each analyte were defined as the lowest concentration producing a

signal-to-noise ratio (S/N) of 3 and 10, respectively. The LOD and LOQ were determined by analysing spiked Synthetic wastewater.

3.3.2 ARV drug quantification

3.3.2.1 Calibration curve

Standard calibration curves for the five analytes were prepared as described in section 3.2.3.6. Triplicate measurements of peak areas of seven different concentrations (0.5 – 12 µg/L) for Nevirapine, Emtricitabine, Ritonavir, Efavirenz, and Tenofovir were determined using the LC-MS/MS. The plot of area against concentration was used to prepare the 7-points calibration curves for each pharmaceutical compound as shown in Appendix 1 to Appendix 5 (see supplementary information section). Typical chromatograms for the calibration standard are presented from Figure 6 to Figure 11 (see supplementary information section).

3.3.2.2 Method validation

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose (Rao, 2018). Methods need to be validated or revalidated before their introduction into routine use (Agalloco, 1995). The analytical validation performed in this research included linearity, percentage recovery, repeatability, matrix effect, the limit of detection and limit of quantification.

i. Linearity

The result of the linearity of the analytical method is presented in Table 3.2 and it was determined as described in section 3.2.3.7 (i). The response area of the three analytes were linear to the measured concentrations of the standards. The R^2 values for the three compounds were 0.979, 0.998, 0.988, 0.989, and 0.983 for Nevirapine, Emtricitabine, Ritonavir, Efavirenz and Tenofovir, respectively. The result showed that a good correlation was obtained between the peak areas and concentrations.

ii. Repeatability

The results of intra-day and inter-day evaluated are presented in Table 3.2. These were estimated based on relative standard deviation (RSD) at the same experimental conditions as described in the experimental procedure contained in section 3.2.3.7 (ii). The values for the three concentrations examined ranged between 1 – 1.82 and 1.30 – 3.28 for intra-day and

inter-day, respectively. The RSDs for the compounds are within the acceptable limit (less than 20%) for repeatability test for the HPLC method (United Nations Office on Drugs and Crime, 2009). This shows that the method was repeatable and reliable.

iii. Percentage recovered.

Percentage recoveries for the five compounds are presented in Table 3.2, it was determined by the experimental procedure described in section 3.2.3.7 (iii). Among the five compounds, Tenofovir, Emtricitabine and Efavirenz had the least recovery compared to the other compounds. This could be attributed to their high solubility in aqueous media that limited its retention on SPE (WHO, 2010; European Medicines Agency, 2017). On the other hand, Ritonavir and Nevirapine maintained the highest recoveries at the three levels of determinations, and this can be attributed to their low polarity and low solubility in water (DeGoey, 2009; WHO, 2009).

iv. Matrix effect.

The results for the matrix effects are as shown in Table 3.2. Experimental procedure for matrix effect was described in section 3.2.3.7 (iv). All five compounds were prone to matrix effects. The matrix effects ranged between 71 and 79% which increased in the following order: Ritonavir > Nevirapine > Emtricitabine > Efavirenz > Tenofovir. Although, LC–MS is one of the most sensitive and selective analytical techniques, it often suffers from matrix effects, especially when using ESI for analysing extracts of complicated matrices (Matuszewski et al., 2017). Matrix effects are often caused by the alteration of ionization efficiency of target analytes in the presence of co-eluting compounds in the same matrix. This may be suppression or enhancement of signal or response from the target analyte thus affecting the accuracy of analytical methods (Cimetiere et al., 2013). Analytical inaccuracy could result from different sources which include sample composition, compounds released during sample pre-treatment or extraction, mobile phase additives, sample to matrix ratios, matrix type and extraction methodology (Wood et al., 2015). To avoid the effect, samples may either be analysed by external calibration using matrix-matched standards or by standard addition (Cimetiere et al., 2013; Wood et al., 2015).

v. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) of an analytical technique is the lowest concentration of an analyte that can be detected or distinguished (though not quantified) from the noise of an analytical procedure or of an instrument. It is usually stated as a concentration at a signal to noise ratio 3:1 (Bhardwaj et al., 2015; Vidushi et al., 2017). The limit of quantitation (LOQ) on the other hand is the lowest concentration of an analyte that can be successfully quantified in a sample with satisfactory precision and accuracy under specified working conditions (Bhardwaj et al., 2015). It is usually calculated as a signal: noise ratio 10:1 as recommended by ICH (Bhardwaj et al., 2015; Vidushi et al., 2017). Both LOD and LOQ were determined as described in section 2.2.3.7 (v). Table 3.2 presents the LOD for the five pharmaceutical formulations and it varied from 0.12 – 0.16 ng/ml. LOQ ranged between 0.40 – 0.53 ng/ml.

Table 3. 2: Calibration data for five ARV drugs, diluted serially and analysed by LC-MS-MS in a positive ionisation mode.

Drug	**LOD**	**LOQ**	**Linearity**	**% Recovery (SD)**			**%**	**Intra-day repeatability (RSD)**			**Inter-day repeatability (RSD)**		
	(ng/mL)	(ng/mL)	(R^2)	5ng/mL	10ng/mL	15ng/mL	**Metrix effect (SD)**	5ng/mL	10ng/mL	15ng/mL	5ng/mL	10ng/mL	15ng/mL
Nevirapine	0.13	0.44	0.979	94.24 (±3.71)	90.51 (±3.55)	90.33 (±4.16)	76 (±2.41)	1.22	1.26	1.26	1.34	1.56	1.56
Emtricitabine	0.14	0.47	0.998	83.21 (±5.31)	83.81 (±4.54)	83.67 (±4.32)	74 (±3.16)	1.41	1.50	1.53	1.56	1.59	2.01
Ritonavir	0.12	0.40	0.988	95.26 (±4.93)	90.87 (±3.91)	91.11 (±5.19)	79 (±3.34)	1.21	1.29	1.33	1.38	1.41	1.41
Efavirenz	0.16	0.53	0.989	88.37 (±3.12)	84.26 (±5.11)	85.12 (±5.23)	73 (±2.55)	1.33	1.41	1.43	1.55	1.57	1.56
Tenofovir	0.14	0.47	0.983	85.23 (±2.94)	82.52 (±3.82)	82.85 (±4.66)	71 (±2.72)	1.34	1.56	1.58	1.66	1.62	1.67

3.3.2.3 Analysis of Reactor Samples

Figure 3.1 to Figure 3.5 illustrates the efficacy of the MBBR on the removal of five selected ARV drugs. Nevirapine, Tenofovir, Efavirenz, Ritonavir and Emtricitabine all showed marked reduction in concentration between the influent and effluent of the MBBR. On average, the percentage removed for Nevirapine, Tenofovir, Efavirenz, Ritonavir and Emtricitabine are 62%, 74%, 93%, 94% and 94% respectively, with a P-value of 0.015.

The low level of removal results of Nevirapine (Figure 3.1) could be attributed to its persistent nature (Wood et al., 2015). An in-vitro investigation of the removal rate of Nevirapine carried out by Vankova and co-workers showed that Nevirapine has low biodegradability (up to 3%) in a closed bottle system (Vanková et al., 2010). However, K'Oreje and co-workers have reported a much higher average removal percentage of 37% from a conventional aerobic wastewater treatment plant in Kenya (K'Oreje et al., 2016). Comparable results were also reported in South Africa by Abafe et al., (2018) who reported an average removal percentage of 32% from a municipal WWTP in KwaZulu-Natal province. In contrary, K'Oreje also reported an increase in nevirapine concentrations at the outlet from another WWTP in Kenya (850 ng/L at the inlet and 1000 ng/L at the outlet) and attributed this increase in concentration to the de-conjugation of the hydroxylated metabolites of nevirapine in the WWTP. Tenofovir was the second least biodegraded drug from the MBBR with 74% removal efficiency (Figure 3.2). Like Nevirapine, Tenofovir has been reported to be persistent in the environment. A study by Al-Rajab et al., (2010) in London has shown that Tenofovir is relatively persistent in soils with no evidence of transformation products. After introducing the drug to varying temperature treated soils (range 4 ^{0}C to 30 ^{0}C and autoclaved) mineralization in the soil increased with temperature, and did not occur in autoclaved soil, suggesting a microbial based degradation (Al-Rajab et al., 2010). Although this hypothetical finding addresses the compound's biodegradability by microorganisms, several studies have reported the detection of Tenofovir in WWTP effluents and surface waters confirming the refractory nature of this drug (Mlunguza et al., 2019; Schoeman et al., 2015; Wood et al., 2015). Despite the fact that Nevirapine and Tenofovir were the least biodegraded antiretroviral drugs investigated in this study, the MBBR presents a promising alternative when compared to findings reported for conventional wastewater treatment plants (Table 3.3).

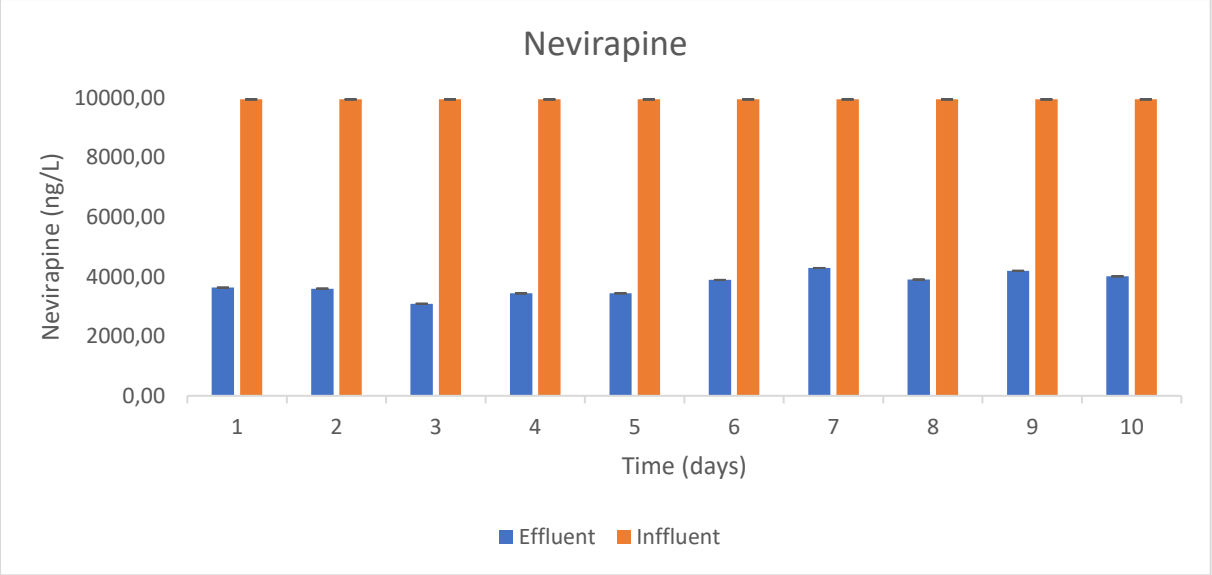

Figure 3. 1: The concentration of Nevirapine over time in a bioreactor effluent as compared to the bioreactor influent, with an average removal efficiency of 62%.

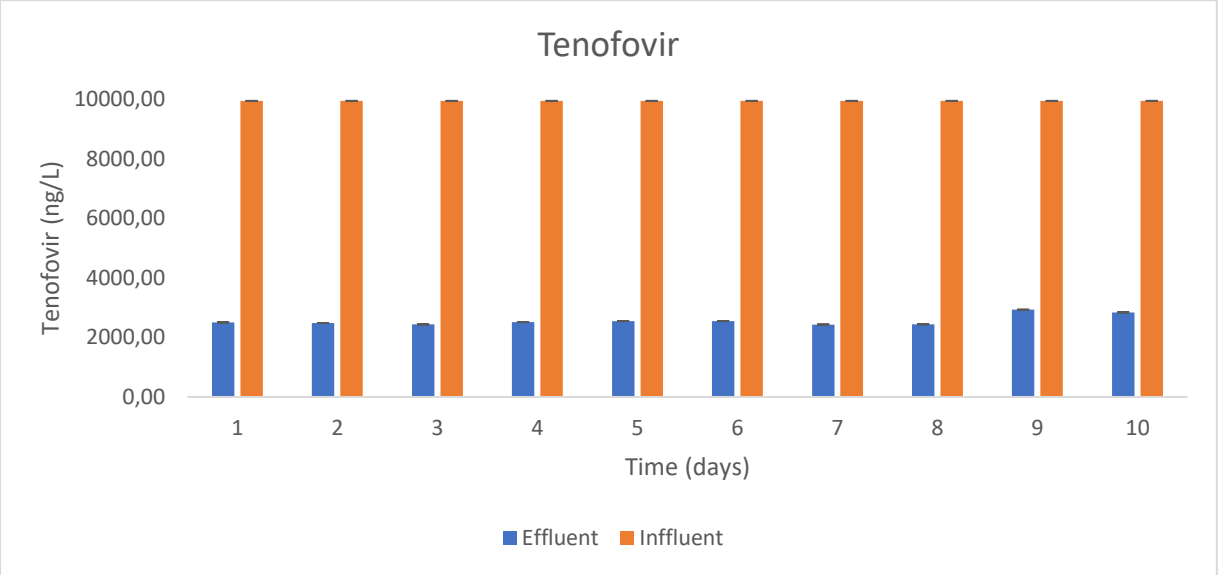

Figure 3. 2: The concentration of Tenofovir over time in a bioreactor effluent as compared to the untreated bioreactor influent, with an average removal of 74%.

Even though literature has reported the five ARV drugs investigated in this study to be recalcitrant (Al-Rajab et al., 2010; Prasse et al., 2010; Jain et al., 2013; Wood et al., 2015), Efavirenz, Emtricitabine and Ritonavir were extensively biodegraded in the MBBR at an average removal rate of 93.62%, 94.18% and 94.87% respectively (Figure 3.3 - Figure 3.5). In this regard, the MBBR showed much better removal percentage of the three ARV drugs when

compared to reported removal percentage data on conventional WWTPs. In a previous study in South Africa Schoeman et al., (2015) investigated the ability of a municipal WWTP to remove Efavirenz. The compound concentrations entering the WWTP ranged between 5500 to almost 14 000 ng/L and the removal percentage ranged between 27 and 71%. Abafe et al. (2018) have reported varying removal percentages of Ritonavir from three different municipal WWTPs in South Africa. 53% removal percentage was reported for decentralized wastewater treatment (DEWATS), 43% for Northern WWTP and 71% for Phoenix WWTP. There were no removal efficiency studies reported on Emtricitabine by the time of this study, however the MBBR has shown to remove Emtricitabine almost completely at 95% (Figure 3.5). Like many other emerging contaminants, ARV drugs have been reported in the ng/L range in influents of conventional WWTPs (Table 3.3). These low concentrations may be the result in poor adaptation or development of activated sludge bacteria to degrade these ARV drugs. Unlike the activated sludge, MBBR has an increased solid retention time favouring slow growing organisms such as nitrifiers (Guo et al., 2010; Shore et al., 2012) and/or conceivably organisms that have developed the necessary genetics to degrade these antiviral drugs.

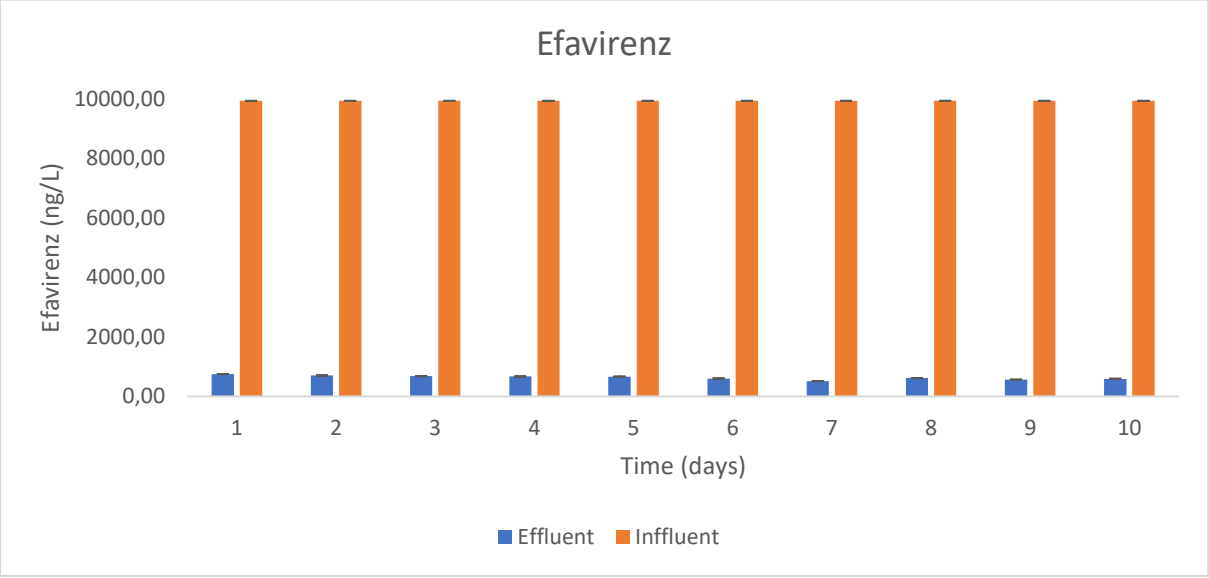

Figure 3. 3: The concentration of Efavirenz over time in a bioreactor effluent as compared to the untreated bioreactor influent, with an average removal of 94%.

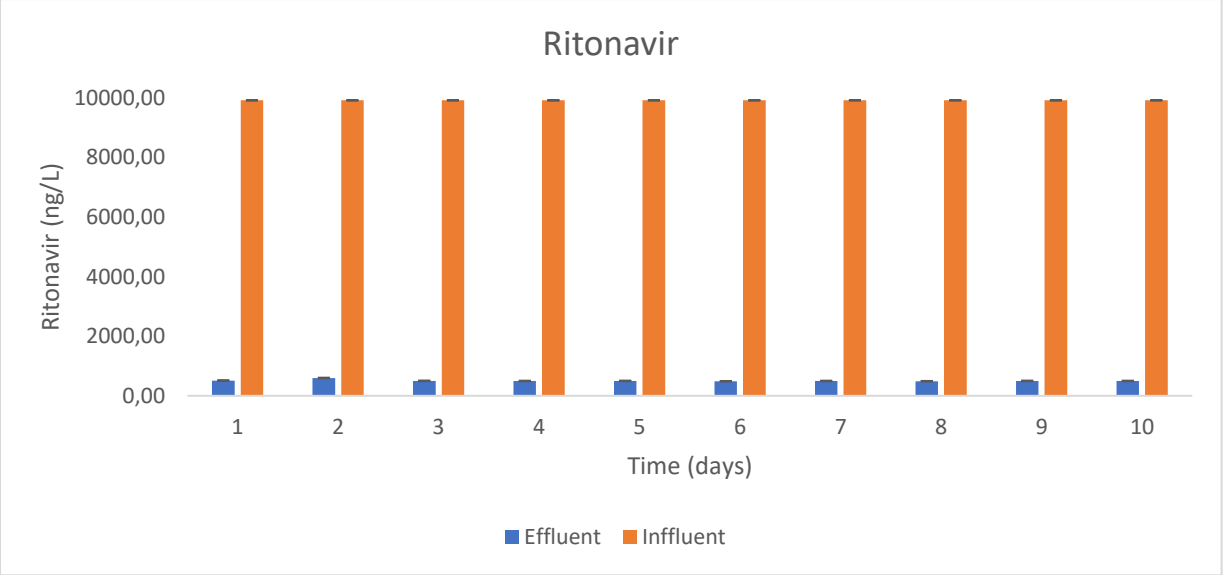

Figure 3. 4: The concentration of Ritonavir over time in a bioreactor effluent as compared to the untreated bioreactor influent, with an average removal of 94%.

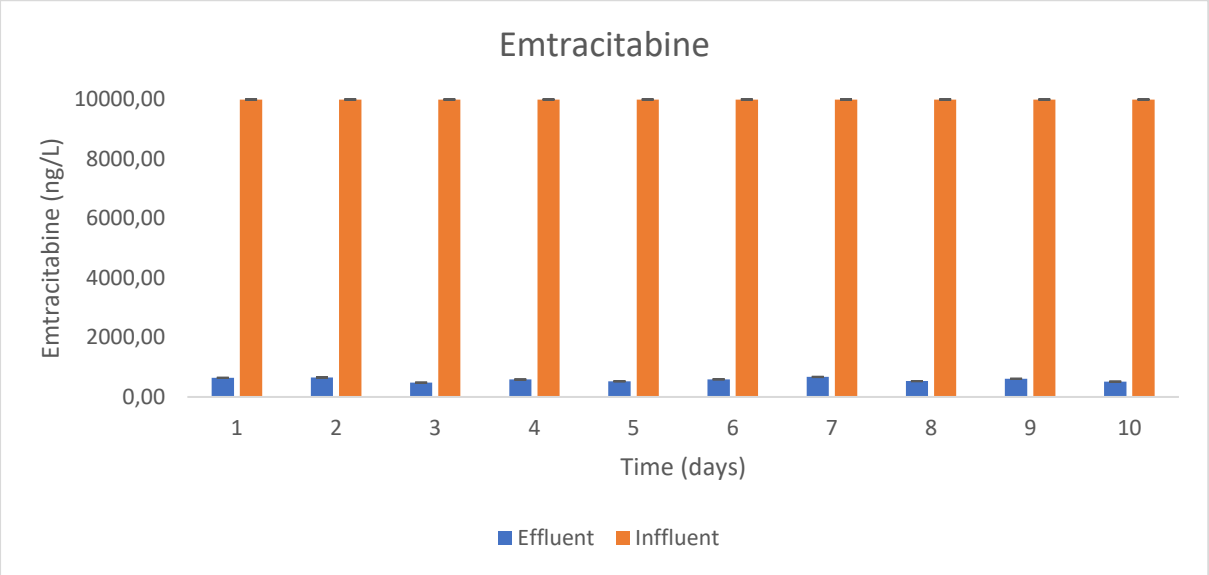

Figure 3. 5: The concentration of Emtricitabine over time in a bioreactor effluent as compared to the untreated bioreactor influent, with an average removal of 95%.

Table 3. 3: Antiretroviral drug removal efficiency of different wastewater treatment plants

ARV	Country	Removal Percentage (%)	References
Nevirapine	South Africa	15	Schoeman 2015
	South Africa (Northern WWTP)	19	Abafe et al. 2018
	South Africa (Decentralized)	9	Abafe et al. 2018
	South Africa (Phoenix)	32	Abafe et al. 2018
	Kenya (Nyalenda)	37	K'Oreje et al. 2016
	Kenya (Kisat)	2	K'Oreje et al. 2016
	Kenya (Dandora)	-76	K'Oreje et al. 2016
	MBBR	62	This study
Efavirenz	South Africa	27 - 71	Schoeman 2015
	South Africa (Decentratilze)	0	Abafe et al. 2018
	South Africa (Nothern)	-37	Abafe et al. 2018
	South Africa (Phoenix)	41	Abafe et al. 2018
	Kenya (Nyalenda)	67	K'Oreje et al. 2016
	Kenya (Kisat)	89	K'Oreje et al. 2016
	Kenya (Dandora)	87	K'Oreje et al. 2016
	MBBR	94	This study
Tenofovir	MBBR	74	This study
Ritonavir	South Africa (Decentralized)	53	Abafe et al. 2018
	South Africa (Northern WWTP)	43	Abafe et al. 2018
	South Africa (Phoenix WWTP)	71	Abafe et al. 2018
	MBBR	95	This study
Emtricitabine	MBBR	95	This study

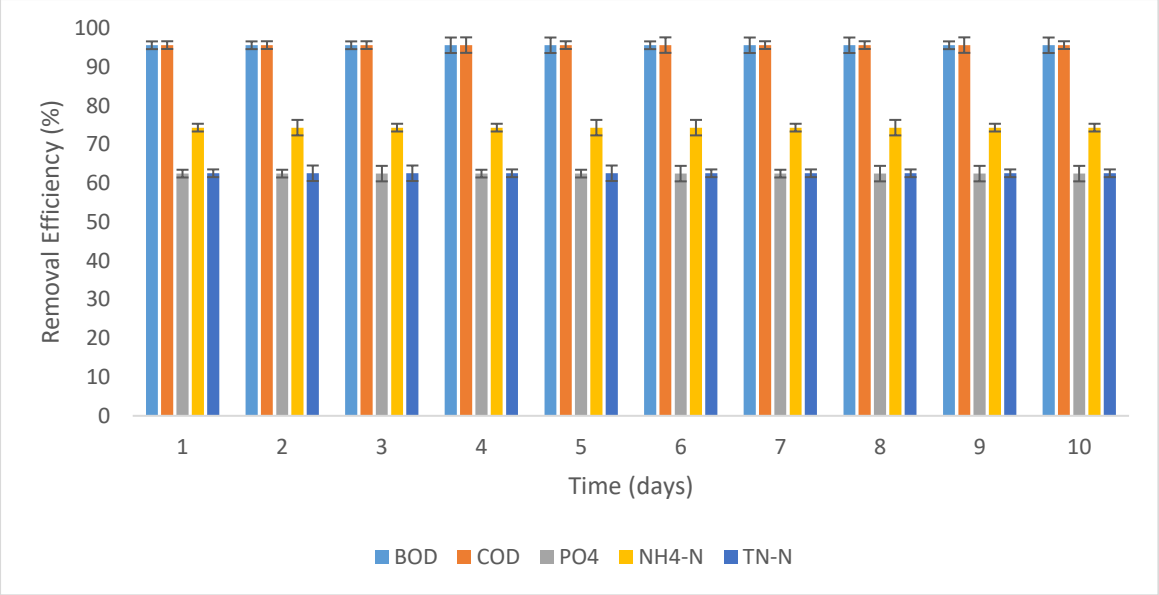

Figure 3. 6: Average removal efficiency of BOD, COD, PO_4, NH_4 and TN after the introduction of ARV compounds, with an average removal of 95.65, 95.69, 62.52, 74.39 and 62.61%, respectively.

Figure 3.6 shows the average removal efficiency of BOD, COD, PO_4, NH_4 and TN after the introduction of ARV compounds. The results indicated that the average BOD, COD, PO4, NH4 and TN removal efficiency were 95.65, 95.69, 62.52, 74.39 and 62.61%, respectively. These results are comparable to the ones before the introduction of the ARV compounds, suggesting that the introduction of ARV compounds did not have any significant effect on the performance of the MBBR in terms of the BOD, COD and nutrient removal. This may have been as a result of the advantage that MBBR has compared to activated sludge i.e., the ability to develop and retain more diverse microbial biofilms on carriers. Moreover, MBBR systems have previously been reported to be stable against toxic shock loads (Hosseini and Borghei 2005).

3.4 Conclusion

The literature review revealed that current WWTPs are not able to provide an absolute barrier for the elimination of ECs. Therefore, this study investigated the efficacy of an MBBR to remove antiretroviral drugs from synthetic municipal wastewater. The MBBR demonstrated the capacity to degrade pharmaceuticals that have thus far been considered as recalcitrant as they are poorly degraded by activated sludge in WWTPs, while at the same time maintaining its effectiveness to remove organic matter (BOD and COD) and nutrients (NH_4, TN and PO_4). The reductions of BOD and COD were consistently high (>90%) during pre and post introduction of antiretroviral drugs. NH_4, TN and PO_4 were also significantly eliminated (>70, >60 and >60%, respectively) in both cases. The MBBR was found to efficiently remove Nevirapine, Tenofovir, Efavirenz, Ritonavir and Emtricitabine from the synthetic influent sample with an average removal of 62%, 74%, 94%, 94% and 95% respectively after 10 days of operation.

3.5 References

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CHAPTER FOUR

4 Understanding the dynamics of a microbial consortium prior to and post the introduction of ARV compounds in an MBBR system.

4.1. Introduction.

The principal objective of biological wastewater treatment is to employ biocenosis of microorganisms to completely oxidise all of the organic components of the wastewater into carbon and water. The activated sludge (AS) process is a widely used technology for wastewater treatment because of its low operational cost and high efficiency (Wagner and Loy 2002; Yildiz 2014; Saunders et al., 2016). The microbial community structures in biological treatment systems are extremely diverse in composition and are largely dependent on the influent characteristics such as chemical composition (Ju and Zhang, 2015; Lee et al., 2015). AS systems are constantly exposed to pharmaceuticals from a variety of sources (Slater et al., 2010). However, the effective toxicity of pharmaceuticals towards AS remains unclear, especially their impact on nutrient removal performance. There is recent evidence that bacterial neuraminidases are important in biofilm formation (Soong et al., 2006; Parker et al., 2009; Wren et al., 2017). Consequently, antiviral neuraminidase inhibitors themselves may inhibit bacterial neuraminidases (Glanz et al., 2018), which could prove detrimental to the structure of the suspended biofilms that make up AS. A moving bed biofilm reactor (MBBR) has been applied to WWTPs for enhanced BOD and nitrogen management (Biswas et al., 2014; Oliveira, 2014) and has provided various advantages, such as stability against hydraulic and toxic shock loads, avoidance of sludge recirculation, and flexibility of shape and operating load. The use of this approach for removal of micropollutants has recently been stated in several publications (Chen et al., 2008; Falås et al., 2012, 2016; Luo et al., 2015; Tanget al., 2017). Falås et al. (2012) found that there were distinct differences in removal efficiencies of pharmaceuticals by activated sludge and suspended biofilm carriers, the higher degradation rates per unit of biomass were achieved with the biofilm reactor compared to activated sludge. Additionally, diclofenac was clearly but slowly degraded by the biofilm, while activated sludge was unable to degrade this compound. MBBRs have been demonstrated to have considerably higher removal rates for selected pharmaceuticals than the activated sludge process and membrane bioreactors (Vieno and Sillanpää, 2014; Zupanc et al., 2013).

Various removal mechanisms (e.g. sorption, biodegradation and abiotic degradation) may contribute to the total elimination of organic contaminants. However, for polar acidic pharmaceuticals, microbial degradation is believed to be the most important removal process in biological wastewater treatment (Quintana et al., 2005). A broad bacterial consortium is required to achieve the desired biological conversions and the performance of wastewater treatment largely depends on the bacterial diversity present (Saikaly et al., 2005). Therefore, a fundamental understanding of the microbial community structure and stability as well as its response to different chemicals entering the wastewater, are desirable for stable and efficient WWTP operation. The aim of this study was to evaluate the influence of commonly used ARV compounds in South Africa on the structure of MBBR bacterial communities in a laboratory scale wastewater treatment bioreactor.

4.2. Materials and methods

4.2.1. Seed sludge and synthetic wastewater composition

Activated sludge was obtained from a WWTP located in Potchefstroom, North West Province, South Africa. The synthetic wastewater contained chemical oxygen demand (COD) of 320–360 mg/L, total organic carbon (BOC) of 100–120 mg/L, NH_4-N of 13–16 mg/L, NO_2-N of 0–0.02 mg/L, NO_3-N of 0.4–1.1 mg/L and PO4-P of 3.0–3.5 mg/L. $NaHCO_3$ or H_2SO4 was used to adjust the pH in the MBBR to a constant value of 7 (Lee et al., 2003).

4.2.2. Selected ARV compounds

A set of five ARV compounds widely used in South Africa were selected, namely Tenofovir, Emtricitabine, Ritonavir, Efavirenz and Nevirapine. Concentrated stock solutions containing 1 mg/ml of each ARV compound was prepared in pure DMSO and kept in a freezer. The stock solution was added to 30L of the synthetic growth medium to attain a final spiked concentration of 10 µg/L for each compound during the course of the experiments.

4.2.3. Bioreactor setup and operation conditions

A bench-scale MBBR system with a working volume of 30 L was used in the study. The system consisted of two reactors connected in series i.e. an anoxic reactor and an aerobic reactor. The water was first lead into an anoxic zone where denitrification occurred, followed by an aerated aerobic zone with nitrification. Before the optimization experiments were carried out, the carriers were acclimatized in the reactor tanks filled with synthetic wastewater and

activated sludge from a wastewater treatment plant. The acclimatization of carriers lasted for 30 days. The reactor was operated at a flowrate of 20ml/min with an internal recycling flowrate at 60ml/min from the aerated aerobic zone to the anoxic zone.

4.2.4. DNA extraction, PCR, and sequencing

Biofilms for microbial community determination were obtained at day 0 (original seed sludge before incubation in MBBR, termed H1); after optimisation of the MBBR but before introduction of ARVs from attached biomass in the anoxic reactor (termed H5) and aerobic reactor (H3); and again after the introduction of ARV compounds in the anoxic reactor (H9) and aerobic reactor (H7). At each stage, five randomly selected bio-carriers were removed from the reactor. The biofilms were detached from the carriers, and filtered through 0.45 µm filter paper. The filter was immersed in 50% (v/v) ethanol, and then stored at -20°C for further DNA extraction. Total genomic DNA was extracted from the biofilms by using a Quick-DNA™ Fecal/Soil Microbe Microprep kit (ZYMO Research, USA) according to the manufacturer's protocol. The DNA concentrations were determined with a NanoDrop ND-1000 spectrophotometer. The DNA was diluted to a final concentration of 1 ng/µL by using sterile water and stored at −80 °C for subsequent analysis.

The 16S rDNA gene target was amplified using primers E517F (5'-GCCAGCAGCCGCGGTAA-3') and E969-984 (5'-GTAAGGTTCYTCGCGT-3') (Wang & Qian 2009) specific for the V4 and V5 hypervariable region of the 16S ribosomal subunit coding gene (16S rRNA) found in bacteria. PCR reactions were set up with a final concentration of: 0.7 U of AccuPOL High Fidelity DNA polymerase Taq (Ampliqon, A211102) , 1 X buffer containing $MgCl_2$, 0.2 mM of dNTP's, 0.3 µM per primer and diluted with molecular grade water to a final volume of 50 µl. Thermocycling consisted of an initial denaturing step for 5 minutes at 98˚C; then five cycles of 98˚C (45 seconds), 45˚C (30 seconds) and 72˚C (45 seconds); followed by a further 20 cycles of 98 ˚C (45 seconds), 50˚C (30 seconds) and 72˚C (45 seconds); and completed with a final elongation at 72˚C for 5 minutes. The amplified PCR products were purified from the gel using the FavorPrep™ GEL/PCR Purification kit (FAGCk001-1, Favorgen Biotech Corp.). Dual indices and Illumina sequencing adaptors were added by PCR as per the manufacturer specifications using the Nextera XT Index kit (Illumina FC-131-1001). The resultant amplicon libraries were purified using AMPure XP beads (Agencourt, A63881), quantified using the Qbit 4 flourometer (Qubit dsDNA HS Assay, ThermoFisher Scientific, Q32854) and the pooled libraries validated

using the Bioanalyzer 2100 (Agilent, G2939BA) and DNA High Sensitivity DNA chips (Agilent, 5067-4627). The pooled libraries were sequenced using the MiSeq system (SY-410-1003, Illumina Inc.) with MiSeq Reagent kit v3 (600 cycle) (Illumina, MS-102-3003).

4.2.5. Sequence Processing and Analysis

The Mothur pipeline was used for the entire sequence data processing according to the Mothur SOP. Briefly, error removals were performed through screening sequences that did not align to the Silva database (Quast, 2013), and pre-clustering to merge rare sequences into larger sequences if the difference was within one or two base pairs. Chimeric sequences were removed by using UCHIME (Edgar, 2011). Taxonomic classification was done based on Ribosomal Database Project (RDP) classifier (Cole et al., 2009) training set version 9, followed by non-bacterial sequence removal. Operational taxonomic units (OTUs) were calculated at distance 0.03 using the Mothur subroutine cluster.split. Microbial community dissimilarity was analysed based on the Yue and Clayton theta coefficient calculated by the tree.shared Mothur subroutine.

4.3. Results and Discussion

4.3.1 Diversity of microbial community and composition

As shown in Table 4.1, Mothur analysis revealed a total of 8909 OTUs across all samples at a cut off level of 0.03. The microbial diversity index is listed in Table 4.1, comprising of community richness (Ace, Chao) and community diversity (Shannon, Simpson). Each sample was more than 99% of the coverage, indicating that the depth of the sequence was sufficient. The patterns of Chao1, ACE, Shannon and Simpson values were very similar to the OTU numbers. According to the OTU number, sample H5 had the richest microbial diversity, followed closely by H3, whereas sample H9 displayed considerably less microbial richness. According to Table 4.1, the values of Ace, Chao, Shannon and Simpson indices demonstrate that H5 had the highest microbial diversity, while H9 had the lowest microbial diversity. As shown in Figure 4.1, the rarefaction curves of these samples are approaching plateaus, this suggested that the number of OTUs was sufficient to reveal the authentic bacterial communities within each sample.

Table 4. 1: Richness and diversity indices of microbial communities for sludge and biofilm samples (OTUs at 0.03 cut off).

Samples	OTUs	Chao1	ACE	Shannon	Simpson
H1	1661	1187	1033	6.20	0.993
H3	1984	1331	1124	6.26	0.994
H5	2092	1432	1271	6.52	0.996
H7	1591	1106	1033	6.0	0.991
H9	1581	1110	908	5.89	0.988

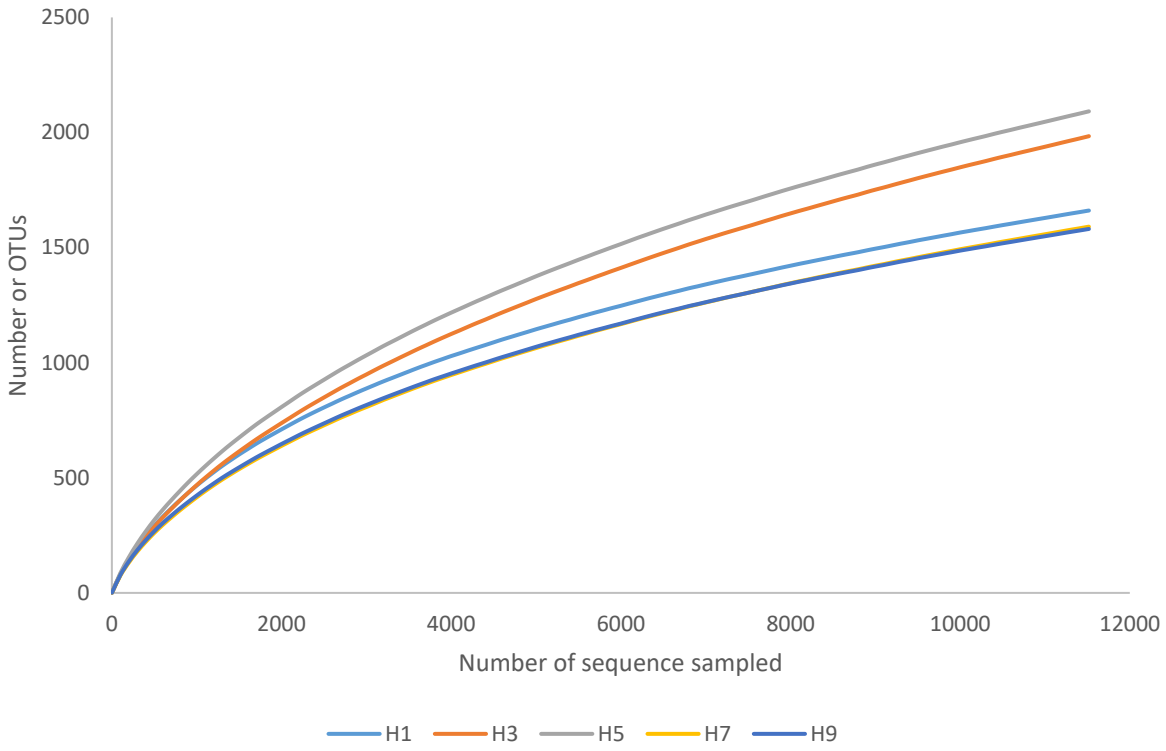

Figure 4. 1: Rarefaction curve. The number of OTUs at 0.03 (species) detected vs the number of sequences sampled.

Various aerobic microorganisms were identified in the anoxic units. A possible explanation for this observation was that the lab scale MBBR used in this study was an open structure (this was to mimic practical wastewater treatment systems), and oxygen could have dissolved into the wastewater. Again, recirculation of aerobic effluent into the anoxic unit to ensure denitrification was providing another source of dissolved oxygen. Therefore, strict anoxic operating conditions were not observed in the current study. Similar conclusions were also reported by several other studies (Xu et al., 2017; Zhang et al., 2018a; Zhang et al., 2018b). Zhou et al. (2015) have also observed that the microbial richness in the system operated alternatively between anoxic and aerobic conditions was superior to that in exclusive anaerobic or aerobic units.

4.3.2. Taxonomic profiles of the microbial community composition

The RDP Classifier was used to assign the effective bacterial sequences to different phylogenetic taxa. In total, 38 identified phyla were observed. As shown in Figure 4.2,

Proteobacteria was the most dominant phylum in most of the samples, except for sample H3 where it was the second most dominant phylum at a relative abundance of 21.19%. *Proteobacteria* constitute the largest and phenotypically most diverse division among prokaryotes (Gupta 2000). As reported in other studies, this widespread phylum was dominant in many environments, notably soil and aquatic environments (Spain et al., 2009; Wang et al., 2012; Sun et al. 2015; Huang et al., 2019). Other phyla such as *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Firmicutes* and *Acidobacteria* were frequently detected in activated sludge (Juretschko et al., 2002; Kragelund et al., 2007; Seviour et al., 2008; Kragelund et al., 2008; Zhang et al., 2012; Xu et al., 2018). In this study, however, it was notable that the microbial community compositions of biofilm samples from post introduction of ARV compounds differed from other biofilm samples before the introduction of the ARV compounds. For example, the relative abundance of *Bacteroidetes* and *Chloroflexi* in H7 (21.95 and 21.28%) and H9 (20.73 and 19.87%) was much higher than that in other biofilm samples i.e. H3 (10.29 and 6.48%) and H5 (12.73 and 12.85%). Whereas phyla of *Actinobacteria*, *Verrucomicrobia*, and *Patescibacteria* in H7 and H9 exhibited less abundance. Also, on class level, *Alphaproteobacteria* in H7 and H9 (32.55 and 25.12%) was much more abundant than that in other biofilm samples before the exposure to ARV compounds (13.07 and 13.21%).

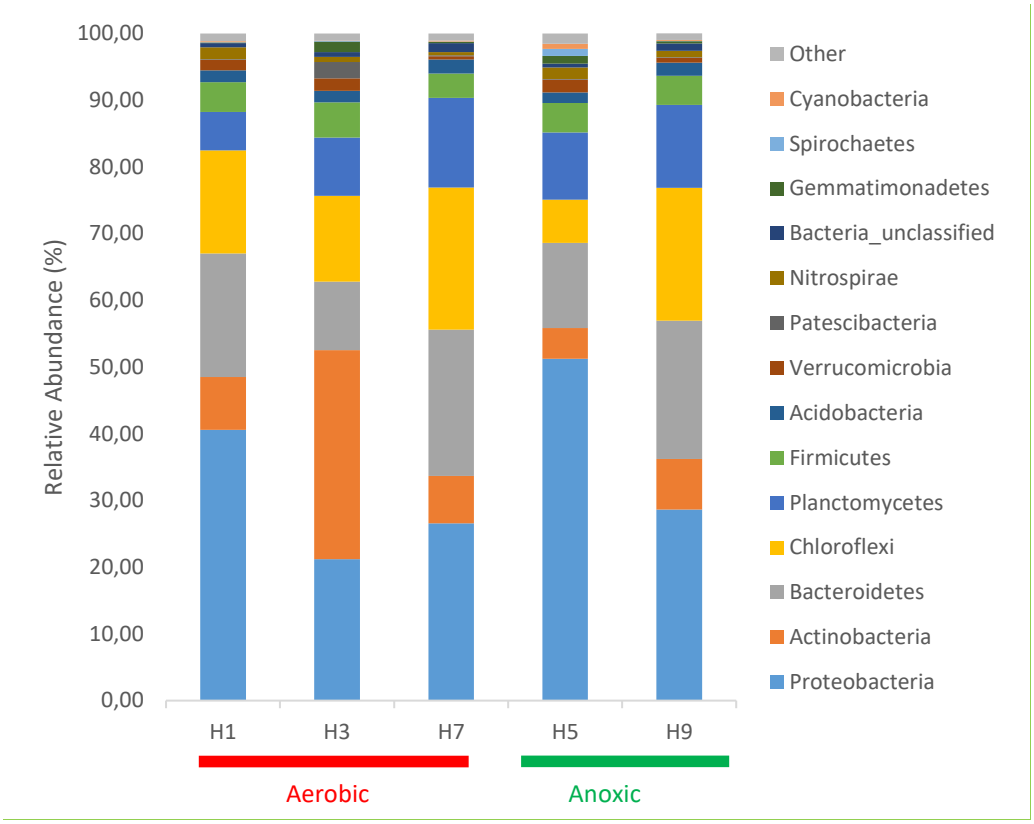

Figure 4. 2: Relative abundance of different phyla in one sludge and four biofilm samples.

Within Proteobacteria, Gammaproteobacteria was the most dominant group (54.9-77.55%). Alphaproteobacteria, accounting for 13.21 to 32.55%, was the second dominant class, followed by Deltaproteobacteria (7.78–13.59%). The rest of unclassified Proteobacteria occurred at the lowest level in the range from 0.34 to 1.54%.

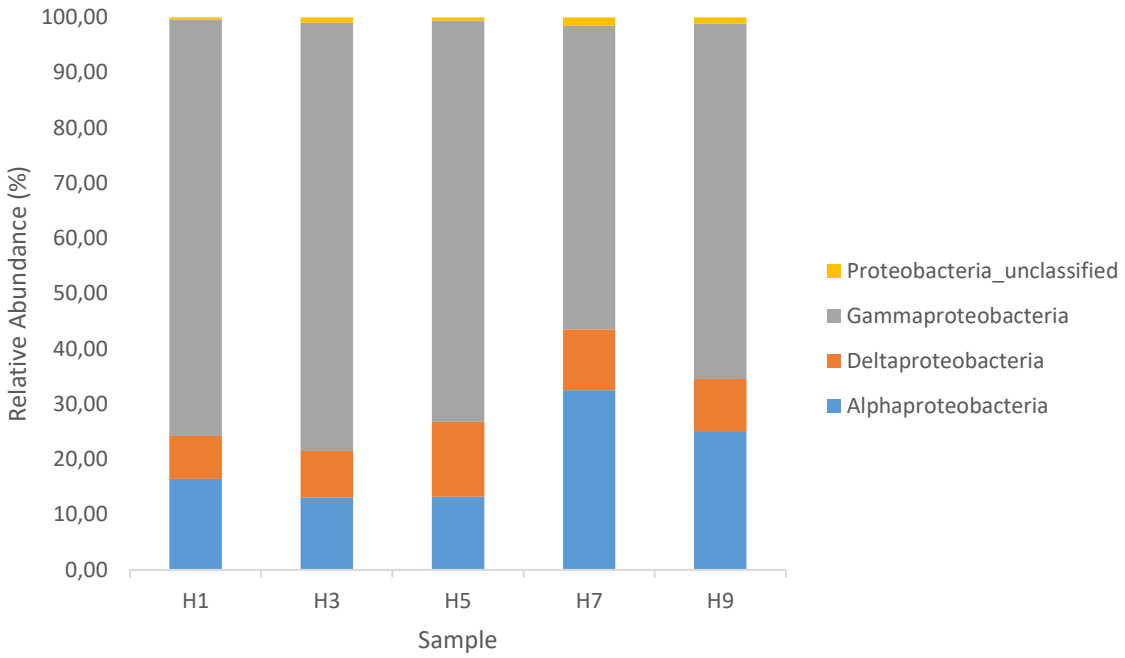

Figure 4. 3: Relative abundance of different classes within Proteobacteria.

Within the *Gammaproteobacteria*, 39 orders were identified. *Betaproteobacteriales* was the most abundant order between 50.61 and 74.56% of all samples, followed by *Xanthomonadales* (3.07–16.1%) and *Competibacterales* (1.01-13.69%). In contrast, the other 36 taxonomic groups including *Steroidobacterales*, *Gammaproteobacteria_unclassified*, *Cellvibrionales*, *Pseudomonadales*, *Legionellales*, *Arenicellales*, *Methylococcales*, *Run-SP154*, *Chromatiales*, *Diplorickettsiales*, *Gammaproteobacteria_Incertae_Sedis*, *R7C24*, *Acidithiobacillales* and others had lower abundances. However, the distribution of these orders generally demonstrated location-specific trends. For example, *Run-SP154* accounted for 1.9 - 3.7% in H1, H3 and H5 (samples before exposure to ARV compounds) and *Diplorickettsiales* accounted for 1.2-2.2% in the same samples, whereas their presence in other samples (H7 and H9 i.e. samples after exposure to ARV compounds) were not detected. Other organisms such as *R7C24* and *Acidithiobacillales* were only detected in samples after exposure to ARV compounds.

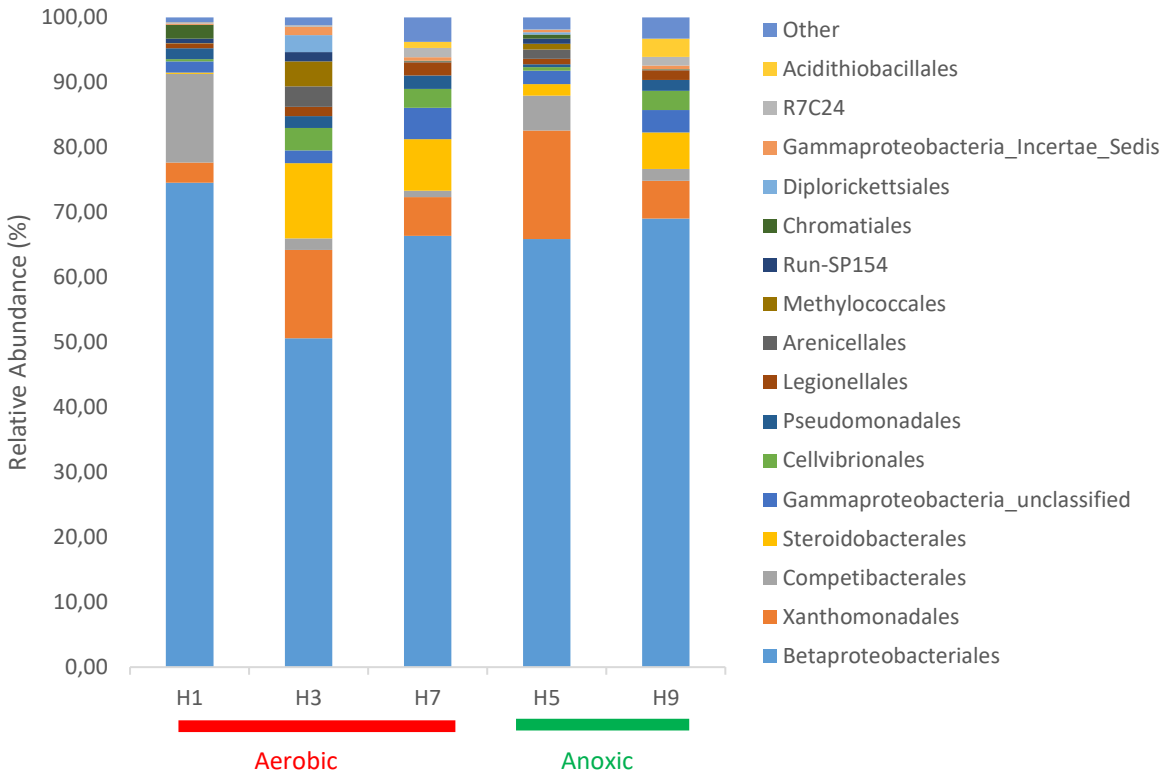

Figure 4. 4: Relative abundance of different orders within Gammaproteobacteria in one sludge and four biofilm samples.

A total of 254 orders were obtained using RDP Classifier. Among them, 117 orders were commonly shared by all the samples. Nine dominant (>1% relative abundance in any sample) orders accounted for 66.97 - 76.98% of the classified sequences, including *Betaproteobacteriales*, *Ardenticatenales*, *Chitinophagales*, *Microtrichales*, *Clostridiales*, *Solirubrobacterales*, *Pirellulales*, *Rhizobiales*, *Propionibacteriales*, *Sphingobacteriales*, *Gemmatales*, *Xanthomonadales*, *Caldilineales*, *Corynebacteriales*, *Flavobacteriales*, *Planctomycetales*, *Myxococcales*, *Cytophagales*, *Competibacterales*, and *Micrococcales*, in which *Betaproteobacteriales* and *Ardenticatenales* were two of the most abundant orders, accounting for 8.32–24.52% and 4.12–16.04% of the classified sequences, respectively. Several rare orders were detected in only one sludge or biofilm sample, such as *Gracilibacteria* and *RCP2-54* in H1; *Sneathiellales*, *Ignavibacteria_unclassified* and *ADurb.Bin180* in H3; *Elsterales*, *Cyanobacteria_unclassified*, *Candidatus_Abawacabacteria*, *Candidatus_Kaiserbacteria*, *RCP2-54*, and *Brevinematales* in H5; *Pasteurellales*, *Salinisphaerales*, *Lineage_IV* and *Lineage_IIc* in H7; and *Aegiribacteria*, *Caldisericales*,

Gastranaerophilales, and *Elusimicrobia_unclassified* in H9, which constituted <0.1 % relative abundance of the classified sequences in each sample.

At the family level, a total of 468 families were obtained. Among them, 121 families including *Rhodocyclaceae*, *Burkholderiaceae*, *Pirellulaceae*, *Saprospiraceae*, *Gemmataceae*, *Competibacteraceae*, *Caldilineaceae*, *Chitinophagaceae* etc. were commonly shared by all sludge samples. However, only five families were the most commonly abundant (>1% relative abundance in any sample), including *Rhodocyclaceae*, *Burkholderiaceae*, *Pirellulaceae*, *Saprospiraceae*, *Gemmataceae*, in which *Rhodocyclaceae* accounted for the most in the range from 10.2 to 16.23% followed by *Burkholderiaceae* (5.02-8.7%). H7 and H9 had much more abundance of *Pirellulaceae* (7.2 and 4.98% respectively), *Saprospiraceae* (4.1 and 5.81%, respectively), *Nitrosomonadaceae* (2.1 and 3.9% respectively), and *Gemmataceae* (4.75 and 6.17%, respectively) as compared with other samples with abundance ranges of 0.35-0.86%, 0.31-0.93%, 0.65-0.88% respectively.

In total, 800 genera were detected in all seven sludge samples. Among them, a total of 215 genera were commonly shared, accounting for 96.66 to 97.28% of the classified sequences. However, there were a total of 178 rare genera that appeared in only one sample and accounted for <0.3% of the classified sequences, indicating a wide diversity of bacterial taxa in activated sludge samples. The top 12 genera accounting for the most reads (a total of 30 genera for the sludge and all the four biofilm samples) were used to generate the heat map (Figure 4.5).

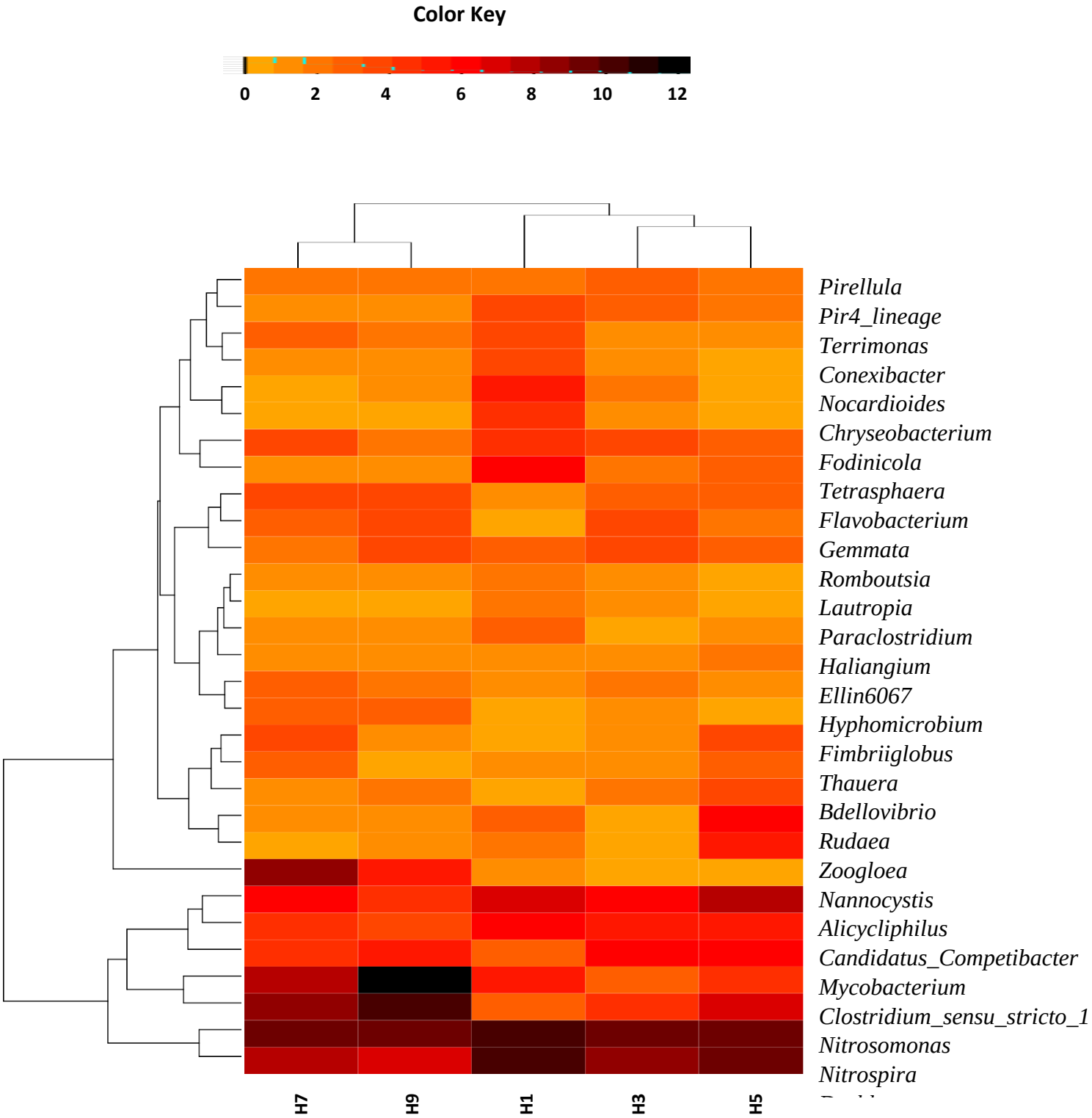

Figure 4. 5: Heat map of the top ten dominant genera in each sample. The selected genera in a total of 30 genera for all the samples were compared with their relative abundances. The colour intensity (percentage) in each panel represents the percentage of a genus in a sample, referring to the colour key at the bottom.

Genus level analysis (Figure 4.5) can provide further information on microbial adaptation in response to exogenous perturbation. Among the commonly abundant genera (>1% relative abundance), many have been identified in activated sludge samples. For instance, *Dechloromonas* was a genus abundant (>1% relative abundance) in all these five samples, which was found to significantly correlate with the carbon source utilization of activated sludge communities (Yang et al., 2011). Also, this genus has been reported as a denitrifier to remove nitrate (Ginige et al., 2004) and a phosphate-accumulating microorganism to enhance phosphorus removal (Liu et al., 2005). In the current study, *Nitrosomonas* spp. was found to be more abundant in samples after the introduction of ARV compounds (12.3% for H9 and 8% for H7) than in samples before the introduction of ARV compounds (2.5–4.4 %). *Nitrosomonas* is a genus of ammonia-oxidizing *Proteobacteria* that oxidise ammonia to nitrite (mostly *Nitrosomonas europaea*) (Pynaert et al., 2004; Sun et al., 2010). *Nitrospira* has also been reported as one of the major ammonia- and nitrite-oxidizing bacteria (e.g., *Nitrospira defluvii*, etc.) in aerobic/anoxic wastewater treatment systems (Limpiyakorn et al., 2011; Yang et al., 2011; Kim et al., 2013). In this work, *Nitrospira* in biofilm samples from post introduction of ARV compounds (9.1% for H9 and 7.9% for H7) was higher than that in other biofilm samples before the introduction of ARV compounds (4.2% for H3 and 5.7% for H5). The presence and enrichment of *Nitrosomonas* and *Nitrospira* post introduction of ARV compounds in the MBBR suggest the potential of these nitrifiers to degrade these pharmaceuticals. The biofilm carriers in the reactor have played a significant role in retaining these essential yet slow-growing organisms (which suffer wash outs in conventional activated sludge systems), since in the sludge sample *Nitrosomonas* and *Nitrospira* showed low abundance at 4.6 and 2.8%, respectively. *Alicycliphilus* has already been reported to be a promising candidate for participating in the development of novel xenobiotics bioremediation processes. Mechichi et al. (2003) isolated a *Alicycliphilus* strain from a municipal sewage plant which under denitrifying conditions used cyclohexanol as a sole carbon source and nitrate as an electron acceptor. In this study *Alicycliphilus* was found to be relatively abundant in H7 and H9 (5.3 and 8.9% respectively) as compared to other samples ranging from 0.1 to 1.1%. Meanwhile, members of *Candidatus*, such as *Candidatus accumulibacter* and *Candidatus competibacter* detected in this study have previously been reported in wastewater treatment (McIlroy at al., 2013). These organisms aid in simultaneous phosphate and nitrate removal in aerobic/anoxic-anaerobic wastewater treatment systems (Rubio-Rincón et al., 2017). The abundance of the

Candidatus spp. was relatively similar in the sludge and biofilm samples before and after the introduction of ARV compounds. The presence and enrichment of these bacteria with various physiological traits indicated the metabolic versatility of the collected sludge and biofilm samples.

4.3.3. Similarity comparison of sludge samples and biofilm samples

Principal Coordinates Analysis (PCoA) was used to visualize and compare the dissimilarity matrix of the relative abundance of OTUs among different biofilm samples and sludge sample (Figure. 4.6). The two first ordination axes explained 78.7% of the variability found in the microbial community compositions.

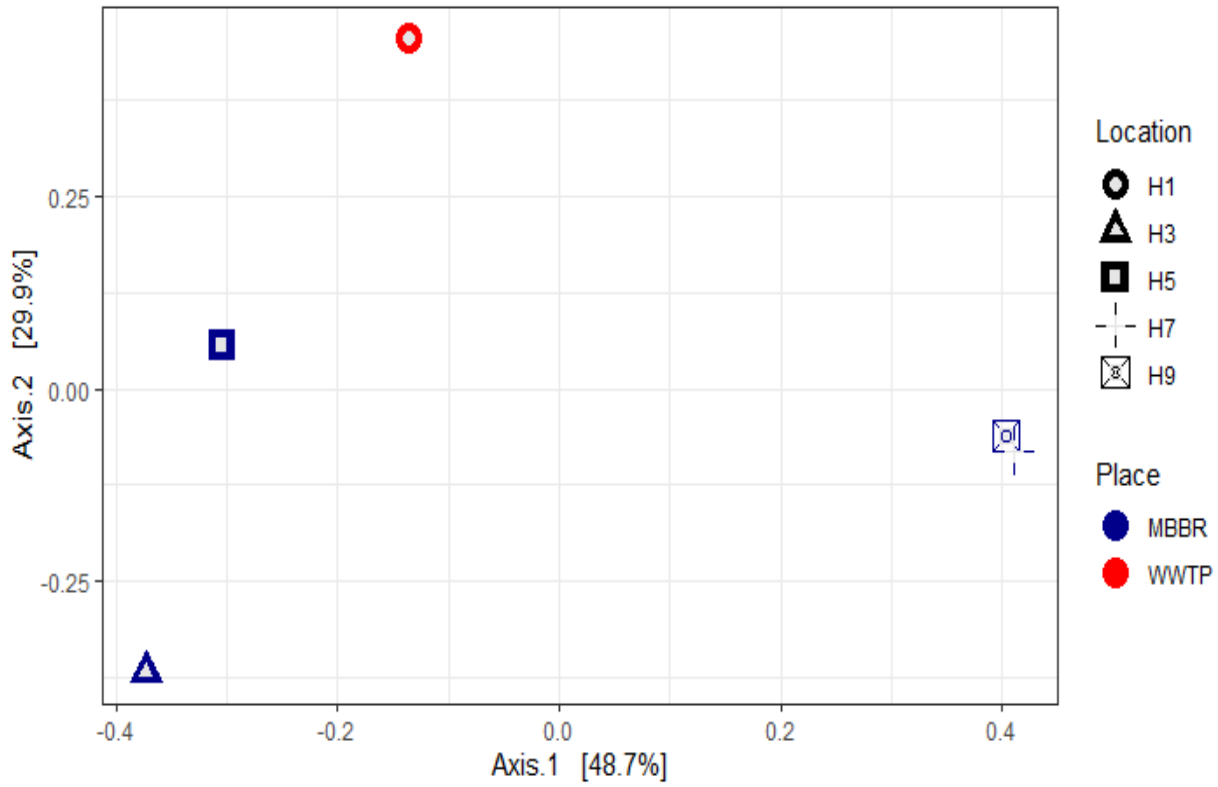

Figure 4. 6: PCoA plot analysis based on the sequencing data. The axes are the percentage of variation explained by the components.

As indicated from PCoA (Figure 4.6), distinct microbial communities developed except those in H7 and H9, which were clustered together, indicating that distinct microbial communities might evolve from different treatment facilities and different treatment zones. The marked shift in microbial communities from the seeded sludge through the start-up of the MBBR to post introduction of ARV compounds in the reactor, might have been caused by a variety of

factors including influent characteristics, environmental variables, and operating modes. The sludge (H1) collected from a municipal WWTP had the most distantly related microbial community as compared to the MBBR biofilm samples. This was due to the different operating variables of the WWTP and the MBBR, such as influent organic loading, wastewater composition, pH, temperature, DO etc. A number of studies have also reported a shift in microbial communities as a result of different operational parameters (Yadav at al., 2014; Ju et al., 2014; Ye et al., 2016). In addition, biofilm carriers in the MBBR allowed for the retention of slow-growing microorganisms by forming aggregates of cells (biofilm) on carriers. In this study the species of *Flavobacterium* were relatively more abundant (>1%) in the biofilm samples (H3, H5, H7 and H9) than in the sludge sample (H1). Jo et al. (2016) have also reported that species of *Flavobacterium* were more abundant in full-scale membrane bioreactors than in AS. These results suggest that this genus probably prefers biofilm environments and may contribute to biofilm formation. Introduction of ARV compounds in the MBBR have also affected a shift in microbial communities in the biofilm samples from pre- and post-introduction of the ARVs. This can be seen by the distance between the microbial communities from H3 (pre-exposure to ARVs) and H7 (post-exposure to ARVs) aerobic samples; and also H5 (pre-exposure to ARVs) and H9 (post-exposure to ARVs) anoxic samples (Figure 3.6). Moreover, the introduction of ARVs has shown to force microbial communities in the aerobic and anoxic zones to become more closely related. This can be seen by clustering together of sample H7 and H9. As shown on the heatmap in Figure 4.5, there were a number of genera that became more abundant in samples after the introduction of ARVs, such as *Nitrosomonas, Nitrospira* and *Alicycliphilus*.

4.5. Conclusion

A thorough knowledge of microbial community and diversity in WWTP is essential and needed for controlling the operating processes and improving the treatment performance such as removal of organic matter, nutrients and recalcitrant organic compounds. As a result, high-throughput sequencing was employed in this study to investigate the microbial community compositions of sludge from a full-scale municipal WWTP and biofilm samples from a laboratory scale MBBR system during pre- and post-introduction of antiretroviral drugs. Similar to the previous studies, it was found that Proteobacteria were the most dominant phylum, within which the Gammaproteobacteria were the most abundant class. PCoA was performed between bacterial community compositions, and the results demonstrated that a set of physicochemical and operating parameters were the important factors affecting the distribution of microorganisms and influencing the overall microbial communities. Moreover, the biofilm carriers played a significant role in retaining essential slow-growing organisms, since in the sludge sample *Nitrosomonas*, *Nitrospira* and *Alicycliphilus* showed a lower abundance than in the biofilm samples. When comparing the biofilm samples, the introduction of antiretroviral drugs was found to affect the bacterial community composition and diversity considerably. For instance, *Nitrosomonas*, *Nitrospira* and *Alicycliphilus* were found to be higher in post introduction of ARV compounds biofilm samples than in biofilm samples before the introduction of ARV compounds. The presence and enrichment of *Nitrosomonas*, *Nitrospira* and *Alicycliphilus* in post introduction of ARV compounds in the MBBR suggest the potential of these organisms to degrade the ARV compounds. Advanced molecular techniques, such as metagenomics and metaproteomics, can be used in future to analyse the metabolic potentials of the biofilms in wastewater treatment systems.

4.6. References

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CHAPTER FIVE

5. Toxicity evaluation of specific antiretroviral drugs used in South Africa.

5.1 Introduction

Antiretroviral drugs (ARVs) are medications used in the treatment of retroviral infections primarily human immunodeficiency virus (HIV). Since the country's first case of HIV in 1982, South Africa has come a long way to reduce HIV/AIDS-related rates of infection, morbidity, and mortality. During the early 1990s the first HIV-1 specific antiviral drugs were given as monotherapy. In recent years the standard of HIV-1 care evolved to include the administration of a cocktail of antiretroviral compounds (Arts and Hazuda 2012). Combination antiretroviral therapy dramatically suppresses viral replication and the plasma HIV-1 viral load to below the limits of detection of the most sensitive clinical assays (Autran et al., 1997; Komanduri et al., 1998; Lederman et al., 1998). Thus, they are prescribed in several tons per year in developing countries (Nattrass, 2008). In order to make the treatment simpler, more effective and cheaper, in 2015 a fixed dose combination (FDC) of antiretroviral drugs was introduced in South Africa as the first-line treatment for HIV positive patients in addition to other commonly used ARVs. FDC involves a single pill that contains a combination of two or more drugs. The single pill contains a combination of tenofovir (300 mg), emtricitabine (200 mg) and efavirenz (600 mg) (NDoH, 2015). Like many other pharmaceuticals, some antiretroviral drugs are not completely metabolized in patients and are excreted in large amounts from the body through faeces or urine as unchanged compounds (Galasso et al., 2002; Prasse et al., 2010). Therefore, they can enter the environment by means of wastewater treatment plant (WWTP) discharge since most pharmaceuticals are only partly removed during wastewater treatment (Prasse et al., 2010). A number of ARVs were detected in South African WWTP effluents and surface waters (Wood et al., 2015; Abafe et al., 2018). The environmental release of antiretroviral drugs is of considerable concern due to potential ecosystem alterations and the development of viral resistance. In June 2006, a new “Guideline on the Environmental Risk Assessment of Medicinal Products for Human Use” (EMEA, 2006) was released in the European Union, which requires an environmental risk assessment for all marketing authorization applications. However, since efavirenz, emtricitabine and tenofovir were already present on the European market with the originator's product for several years with recognized efficacy and an acceptable level of safety, the introduction of the generic

product onto the market was deemed unlikely to result in any significant increase in the environmental exposure and would thus not be expected to have an adverse effect upon the environment. With this in mind, and under the guidance of the Committee for Medicinal Products for Human Use (CHMP) Guideline on the Environmental Risk Assessment of Medicinal Products for Human Use (EMEA/CHMP/SWP/4447/00), a formal environmental risk assessment for efavirenz, emtricitabine and tenofovir was not considered necessary. However, it is also of interest to investigate whether compounds that are already on the market pose a hazard to the environment, particularly those with growing market shares in South Africa such as antiretroviral drugs. Studies have shown that antiviral drugs are among the predicted most hazardous therapeutic classes with regards to their toxicity toward algae, daphnids and fish (Sanderson et al., 2004). In this study, the acute toxicity of a selected group of antiretroviral drugs used in South Africa has been determined for *Vibrio fischeri*, *Daphnia magna* and *Selenastrum capricornutum*. The ARVs studied are tenofovir, emtricitabine, efavirenz and ritonavir. In addition, nevirapine used as part of antiretroviral therapy to prevent mother to child transmission of HIV during pregnancy and breastfeeding has also been included in this study. In order to obtain ecologically relevant data on their potential ecological risks, the toxicity of these five chemicals alone and in combination was tested in order to mimic environmentally realistic mixtures. With regards to the constitution of the mixtures, it was hypothesized that the combined effects may lead to synergistic interactions between the compounds, i.e., the toxic effects caused by the mixture might exceed the sum of effects of the separate constituents.

5.2 Experimental

5.2.1 Chemicals

Efavirenz, Nevirapine and Ritonavir were purchased from Industrial Analytical (Pty) Ltd (Kyalami, South Africa). Tenofovir and Emtricitabine were purchased from Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). Stock solutions of ARVs were prepared in 3% (v/v) dimethyl sulfoxide (DMSO, from Sigma-aldrich) at a concentration of 100 mg/L. To determine EC_{50} of the target compounds, two-fold serial dilutions (50 mg/L, 25 mg/L, 12,5 mg/L, 6.25 mg/L, 3.125 mg/L, 1.56 mg/L, 0.78 mg/L, 0.39 mg/L, 0.19 mg/L, 0.097 mg/L, 0.05 mg/L, 0.025 mg/L, 0.012 mg/L) were done in deionized water for the *V. fischeri*

bioluminescence assay and the osmolality was adjusted to 2% NaCl for the optimal performance of this bioassay. For *D. magna* and *S. capricornutum*, serial dilutions were done using their respective growth media as described in their toxicity test kits. Based on the EC_{50} values of the molecule alone, mixtures were realised by using the lowest toxic compounds in order to test the combined toxic effects between the compounds. Binary mixtures of Tenofovir, Emtricitabine, Ritonavir and Nevirapine were prepared by mixing together two antiviral drugs with the ratio of 1:1(v/v). Ternary mixture of Tenofovir, Emtricitabine and Ritonavir was also prepared by mixing the three compounds together with the ratio of 1:1:1 (v/v). The ecotoxicity of the influent (where the 100% influent contained 10µg/l of each ARV compound) and effluent of the MBBR was also investigated. The effluent contained 515 ng/L Emtricitabine, 592 ng/L Efavirenz, 4013 ng/L Nevirapine, 499 ng/L of Ritonavir, 2838 ng/L Tenofovir. Efavirenz was left out of the mixture toxicity assessments because of its toxic effects at very low concentrations.

5.5.5 V. fischeri bioluminescence assay.

Toxicity was evaluated using the bioassay based on the inhibition of the luminescence emitted by the bacteria *V. fischeri*. Light emitted from bacteria is a result of the interaction of the enzyme luciferase, reduced flavin, and a long-chain aldehyde in the presence of oxygen. The metabolic energy generated in this pathway is converted to chemical energy, through the electron transport system, into visible light. This metabolic pathway is intrinsically linked to cellular respiration, so disruption of normal cellular metabolism causes a decrease in light production. Bacteria *V. fischer*i was supplied as freeze-dried reagent (1243-500 BioTox™ Kit), supplied by ToxSolutions Kits & Services, Johannesburg, South Africa. The reagent was stored at -20˚C and rehydrated prior to testing. The toxicity bioassay was carried out according to ISO 11348-3 guidelines (International Organization for Standardization, 1998). The acute toxicity endpoint was determined for 15 and 30 min as the effective concentration (EC_{50}) of a chemical that causes a 50% reduction in the bioluminescence of the bacteria. Light production from luminescent bacteria was measured with a luminometer (Kikkoman® Lumitester C-110), samples were incubated at 15˚C in a chilling block (Echothem™ Chilling/Heating Plate) between measurements. The EC_{50} value was determined using the BIOTOX program.

5.2.3 D. magna toxicity bioassay.

The microbiotest is a 24 – 48 h assay based on immobility or mortality of the test organisms, with calculation of the EC_{50} or LC_{50}. *Daphnia magna* was supplied as dormant eggs (ephippia) (DAPHTOXKIT F^{TM} magna), supplied by ToxSolutions Kits & Services, Johannesburg, South Africa. Before the assay was conducted, ephippia were hatched to supply the live biota. This was done by transferring the ephippia into a hatching petri dish with Standard Freshwater and incubated for three days, at 20-22°C, under continuous illumination of min. 6000 lux. Acute toxicity tests were conducted according to the Organization for Economic Co-operation and Development (OECD) standard procedures with neonates (less than 24 h-old) of *D. magna* (OECD, 2004). Five neonates were placed in 10 ml test solution for toxicity analysis. In each toxicity test, five or more dilutions of a sample and a control with four replicates were prepared in six wells of tissue plates. Toxicity tests were conducted at 20 ± 2˚C with a 16 h light: 8 h dark photoperiod for 48 hours. Test species were not fed during the test period. The acute toxicity endpoint was determined for 24 and 48 hours as the EC_{50}. Immobilization of the test species was used to calculate the EC_{50} values using the Probit analysis via Microsoft excel.

4.2.4 S. capricornutum toxicity bioassay.

The toxicity tests with microalgae were conducted according to the OECD standard procedures for growth inhibition of *Selenastrum capricornutum* (OECD 201, 2006). *S. capricornutum* was supplied immobilized as algal beads (ALGALTOXKIT F^{TM}) by ToxSolutions Kits & Services, South Africa. The 72-h growth inhibition test was performed in long cell test vials, with *S. capricornutum* de-immobilized from algal beads. After preparing the toxicant dilution series, the flasks were inoculated with the concentrated algal suspension in order to obtain an initial algal concentration of 1.10^4/mL in each test solution. After thorough shaking, 25 mL of the algae-toxicant dilutions from each flask was transferred into a corresponding long cell. The cells were then incubated randomly for 72 h in a temperature controlled room with constant uniform illumination (min. 4000 lux bottom illumination), at 21-25°C. Optical density (OD) was determined at 670 nm every 24 hours, over a 72 h period. The 72 h fifty percent reduction in growth rate (ErC50) was then calculated using the mean daily OD values for the 3 replicate cells according to OECD Guideline 201.

5.2.5 Toxicity of mixtures

Joint-action toxicity data were analysed based on the Synergistic Ratio (SR) model described by Hewlett and Plackett (1959):

$$\text{SR index} = \frac{\text{EC50 of chemical acting alone}}{\text{Mixture (EC50 of chemical + additive)}} \quad \text{(Eq.5.1)}$$

Where SR = 1 describes that the joint action is additive.

SR < 1 describes antagonism.

SR > 1 describes synergism.

5.2.6 Toxicity category criteria

The toxicity categories based on the EC_{50} values were determined as, ''*very toxic to aquatic organisms*'' (EC_{50} ≤ 1 mg/ l), ''*toxic*'' (EC_{50} in the range of 1–10 mg/1), and ''*harmful*'' (EC_{50} in the range of 10–100 mg/l), which are established in legislation (Directive 93/67/EEC) by the Commission of the European Communities (1996), are applied in this study to classify the target compounds.

5.3 Results and Discussion

The occurrence of pharmaceuticals in the aquatic environment may affect the growth of aquatic organisms including cyanobacterial mats which have important ecological position and essential roles in nutrient cycling and oxygen production (Breitholtz et al., 2006; Yasser and Adli 2015).

5.3.1 Toxicity of single compounds

The results showed a considerable variability of toxicity among the different ARV compounds tested. Similar trends were observed with the different model organisms used (Table 5.1). The EC_{50} rankings of relative toxicity towards *S. capricornutum*, *D. magna* and *V. fischeri*, were determined as 82.5, 81.3 and 73.5 mg/L, respectively for tenofovir; 41.7, 50.7 and 55.1 mg/L respectively for emtricitabine; 0.21, 0.43 and 0.55 mg/L, respectively for efavirenz; 60.3, 87.1

and 83.6 mg/L respectively for ritonavir; and 39.3, 49 and 41.3 mg/L, respectively for nevirapine. The three model organisms showed more sensitivity to efavirenz than the other ARV compounds tested, with the lowest value of EC_{50} of 0.21, 0.43 and 0.55 mg/L. While tenofovir showed the least toxicity towards the three model organisms.

Table 5. 1: Half maximal effective concentration (EC_{50} in mg/L) of the five ARV compounds towards *S. capricornutum*, *D. magna* and *V. fischeri*.

ARV	EC_{50}		
	S. capricornutum	*D. magna*	*V. fischeri*
Efavirenz	0.21	0.43	0.55
Tenofovir	82.5	81.3	73.5
Emtricitabine	41.7	50.7	55.1
Nevirapine	39.3	49	41.3
Ritonavir	60.3	87.1	83.6

The literature review demonstrates a lack of ecotoxicological data for pharmaceuticals and particularly for antiretroviral drugs. However, recently Omotola et al., (2021) have shown the potential environmental toxicity of lamivudine. In the study Daphnia magna toxicity test revealed a mortality rate of 85% on exposure to 100 µg/L lamivudine in freshwater, which increased to 100% at 48-h exposure. At lower concentrations of 10 µg/L lamivudine, 90% and 55% survival rates were observed at 24 h and 48 h, respectively. Currently there is no data available for efavirenz, ritonavir, tenofovir, nevirapine, and emtricitabine toxicity in aquatic organisms. In this study, the levels of exposure to which the test organisms were acutely exposed were above those already reported in the municipal water effluent and surface water. However, the extensive production, prescription and release of antiretroviral drugs will continue to grow in the future, and consequently their loadings to the environment can result in potential long-term ecological risks to aquatic biota. According to the classification of the Commission of the European Communities (1996), the antiretroviral drug efavirenz was very toxic to all the species studied since the acute toxicity data were determined from concentrations below 1 mg/L. Tenofovir, emtricitabine, nevirapine and ritonavir were

considered harmful to all the three model organisms studied (EC_{50} in the range of 10– 100 mg/l). These results were consistent with the findings of Sanderson et al. (2004) who proposed that antiviral drugs are among the predicted most hazardous therapeutic classes with regard to their toxicity toward algae, daphnids and fish. In addition, Escher et al. (2010) have also indicated the toxicity of the compound oseltamivir ethylester (Tamiflu® an antiviral agent for the treatment of influenza A and B) towards *V. fischeri* and green algae *Desmodesmus subspicatus*.

5.3.2 Toxicity of mixtures

Since aquatic organisms are continuously exposed to complex mixtures of emerging contaminants, there is a need to evaluate mixture toxicity. When two or more chemicals are applied simultaneously to living organisms, the combined effect may result in the addition of toxic effect of one chemical to the other (non-interactive or additive action), or the toxic effects caused by the mixture being significantly less than the sum of the toxic effects of the separate constituents (antagonism model), or the toxic effects caused by the mixture may significantly exceed the sum of effects of the separate components (synergism model) (Stenersen, 2004).

Table 5. 2: Half maximal effective concentration (EC_{50} in mg/L) of binary and ternary mixture of ARV compounds towards *S. capricornutum*, *D. magna* and *V. fischeri*.

ARV Mixture	EC_{50}		
	S. capricornutum	*D. magna*	*V. fischeri*
Emtricitabine + Tenofovir = B1	40.22	45.5	50.3
Emtricitabine + Ritonavir = B2	41.1	49.5	55.12
Emtricitabine + Nevirapine = B3	30.2	41.1	35.8
Ritonavir + Tenofovir = B4	60.5	76.8	75.3
Ritonavir + Nevirapine = B5	40.1	50.7	40.5
Nevirapine + Tenofovir = B6	38.4	44.2	39.4
Tenofovir + Emtricitabine + Ritonavir = T1	39.25	41.7	34.2

The toxicity of mixtures varied from 30.2 to 81.3 mg/L (EC_{50} values) in the three model organisms tested (Table 5.2). On the basis of EC_{50}, all the mixtures were found to be slightly

more toxic to the three species than the chemicals applied alone with a p-value of less than 0.05. There was a low variability in the SR ratios ranging from 0.97 to 2.44 mg/L (Table 5.3). In the *S. capricornutum*, most SR ratios calculated with reference to emtricitabine, tenofovir, ritonavir and nevirapine agreed with the model of synergism action (SR > 1); by contrast, a model of additive and antagonistic action was suggested in the case of ritonavir in the binary mixture B4 (SR = 1) and nevirapine in the binary mixture B5 (SR<1). In *D. magna*, the joint-action toxicity of the ritonavir + nevirapine mixture with reference to nevirapine agreed with antagonistic models (SR <1), whereas the SR for the rest of the mixtures with reference to emtricitabine, tenofovir and ritonavir were consistent with a synergistic model (SR > 1). In *V. fischeri* bioluminescence assay, most mixtures fitted with a synergistic model (SR < 1). While the SR for the ritonavir + tenofovir mixture conformed to the antagonistic model.

Table 5. 3: Synergistic ratios (SR) of binary and ternary mixtures calculated according to the synergistic ratio model (Hewlett and Plackett 1959) where SR = 1 describes that the joint action is additive, SR < 1 describes antagonism, and SR > 1 describes synergism

Test species		T1	B1	B2	B3	B4	B5	B6
S. capricornutum	Emtricitabine	1.06	1.04	1.01	1.38	-	-	-
	Tenofovir	2.1	2.05	-	-	1.36	-	2.15
	Ritonavir	1.54	-	1.47	-	1.00	1.50	-
	Nevirapine	-	-	-	1.30	-	0.98	1.02
D. magna	Emtricitabine	1.22	1.11	1.02	1.23	-	-	-
	Tenofovir	1.95	1.79	-	-	1.06	-	1.84
	Ritonavir	2.09	-	1.76	-	1.13	1.72	-
	Nevirapine	-	-	-	1.19	-	0.97	1.11
V. fischeri	Emtricitabine	1.61	1.10	1.00	1.54	-	-	-
	Tenofovir	2.15	1.46	-	-	0.98	-	1.87
	Ritonavir	2.44	-	1.52	-	1.11	2.06	-
	Nevirapine	-	-	-	1.15	-	1.02	1.05

Emtricitabine + Tenofovir = B1; Emtricitabine + Ritonavir = B2; Emtricitabine + Nevirapine = B3; Ritonavir + Tenofovir = B4; Ritonavir + Nevirapine = B5; Nevirapine + Tenofovir = B6; and Tenofovir + Emtricitabine + Ritonavir = T1

Pharmaceutical compounds of a heterogeneous mixture i.e. from different therapeutic drug classes often have different modes of action and toxic sites such that the model of addition or synergism may not be expected (Broderius et al., 2005). Interestingly, Yang et al. (2008) revealed that binary mixtures of several antibacterial agents (e.g., triclosan, tylosin, triclocarban, and tetracyclines), that were expected to have the same mode of action, showed additive, synergistic, and even antagonistic effects in *S. capricornutum*. It has been reported that the types of interactions exhibited by the components of mixtures largely depend on the proportion of the occurrence in the mixtures (Otitoloju, 2005). However, as observed in this study, it was found that most of the mixtures studied, acted additively or together in a synergistic manner.

5.3.3. Toxicity of influent and effluent from the MBBR

To assess if the MBBR degrades and mitigate the toxicity of the ARVs or make toxic the effluent by producing metabolites (transformation products), since degradation does not always equate to detoxification (Celiz et al., 2009), it was necessary to investigate the toxicity of the influent and the effluent. At the concentrations at which the study was conducted, the EC_{50} of the influent and effluent were found to be above 100% of the influent and effluent (where the 100% influent contained 10µg/l of each ARV compound).

Table 5. 4: Half maximal effective concentration (EC_{50} in %) of the influent and effluent towards *S. capricornutum*, *D. magna* and *V. fischeri*.

Toxicant (%)	EC_{50}		
	S. capricornutum	*D. magna*	*V. fischeri*
Influent	>100	>100	>100
Effluent	>100	>100	>100

5.4 Conclusion

In their natural habitat organisms are exposed not only to a single chemical but simultaneously or sequentially to multiple mixtures of compounds. This study investigated single and joint effects of binary and ternary mixtures of antiretroviral drugs. In summary, the results showed diverse toxicity effects of the tested molecules with the antiretroviral drug

efavirenz being particularly toxic to freshwater and marine organisms. Based on the EC_{50} values, acute effects seemed to be unlikely since the median toxicity concentrations were at minimum 10-fold higher than actual environmental levels. However, significant combination effects of substances can occur even if the toxicity of the single substances is low (Feron et al. 1998). In this study, the toxicity of binary and ternary mixtures was not different, but the mixtures were much more toxic than the chemicals individually and eventually led to either synergistic or additive interactions. Though, in certain instances transformation products resulting from biodegradation of pharmaceuticals can be equally or more toxic than the parent compound (Walgren et al., 2005; Celiz et al., 2009; Radjenovic et al., 2009). Toxicity results of the effluent showed no toxic effects on the model organisms tested, assuming complete mineralisation or production of equally (since the influent also showed no toxic effects) or less toxic transformation products. The data presented in this study are preliminary, and repeated testing and careful chemical analyses are necessary to confirm that these interactive effects occur consistently at these exposure concentrations. However, the realization that an increase in toxicity could result when pollutants occur in mixtures emphasises the need for an awareness of cocktail effects for better ecological risk assessment.

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CHAPTER SIX

6. Final Conclusion and Prospects for Future Research

6.1 Final Conclusion

The work presented in this study was aimed at elucidating the potential of biofilm systems, specifically MBBR, as a biological treatment technology for the removal of antiretroviral compounds (i.e. Efavirenz, Tenofovir, Emtricitabine, Nevirapine and Ritonavir) in municipal WWTP. This was accomplished by means of experiments performed in laboratory-scale reactors. The MBBR system consisted of two reactors, anoxic and aerobic, connected in series. The influent was first passed through an anoxic zone where denitrification occurred, followed by an aerated aerobic zone for nitrification, with an internal recycling from the aerated aerobic zone to the anoxic zone. The results obtained from this study clearly demonstrate that the MBBR system has the potential to be used as an efficient biological treatment technology for the treatment of municipal wastewater to eliminate antiretroviral compounds while at the same time maintaining the efficacy to also remove overall organic matter and nutrients. The MBBR was found to efficiently remove Nevirapine, Tenofovir, Efavirenz, Ritonavir and Emtricitabine from the synthetic influent sample with an average of 62.31%, 74.18%, 93.62%, 94.18% and 94.87% of the ARV drugs removed respectively after 10 days of operation.

In addition, the dynamics of the microbial consortium prior and post introduction of ARV compounds in the MBBR system was also investigated. This was done to understand the shift in microbial communities due to the introduction of the ARVs. Microbial diversity (alpha-diversity) of seeded sludge from a full-scale municipal WWTP and biofilm samples from a laboratory scale MBBR system during pre- and post-introduction of ARV compounds were investigated by Illumina sequencing of the 16S rRNA gene. The results indicated that a wide diversity of microorganisms inhabited the reactor prior to and post-introduction of the ARV compounds. When PCoA was performed between bacterial community compositions, the results demonstrated that the biofilm carriers played a significant role in retaining essential slow-growing organisms, since in the sludge sample *Nitrosomonas*, *Nitrospira* and *Alicycliphilus* showed a lower abundance than in the biofilm samples. In addition, the introduction of antiretroviral drugs was found to affect the bacterial community composition

and diversity considerably i.e. there was a shift in microbial community compositions post-introduction of ARV compounds. For instance, *Nitrosomonas*, *Nitrospira* and *Alicycliphilus* were found to be higher in post introduction of ARV compounds biofilm samples than in samples before the introduction of ARV compounds. The presence and enrichment of these organisms in post introduction of ARV compounds in the MBBR suggests their potential to degrade the ARV compounds. Understanding shifts in microbial communities due to the introduction of specific ARV compounds can help improve the operations of the MBBR to increase the likelihood of enhancing the microbial community of the taxa performing specific functionality which likely catalyse the biotransformation of the micropollutants.

This study also investigated the toxicity of antiretroviral drugs towards the environment by using *S. capricornutum, D. magna* and *V. fischeri* as model organisms. The ecotoxicity results demonstrated that efavirenz was >100-fold more toxic than the other four antiretroviral drugs studied, at EC_{50} values of 0.21, 0.43 and 0.55 mg/L towards *S. capricornutum, D. magna* and *V. fischeri*, respectively. The toxicity of binary and ternary mixtures were also evaluated. It was observed that the mixtures were more toxic than the chemicals individually and eventually led to either synergistic or additive interactions. These results substantiate the need to develop more efficient biodegradation techniques to prevent environmental contamination, given the inadequacy of conventional activated sludge systems to remove these emerging contaminants. Toxicity results of the effluent showed no toxic effects on the model organisms tested, assuming complete mineralisation or production of equally (since the influent also showed no toxic effects) or less toxic transformation products. This was expected as the degradation/removal of the ARVs by the MBBR should theoretically result in an effluent that is less environmentally toxic than the influent. In future an influent with higher toxic concentrations of the ARVs could be used to determine if the MBBR does in fact reduce toxicity and in what ways it is affected by higher concentrations of the ARVS.

6.2 Prospects for future research

In this PhD thesis, it was shown that the MBBR system has the potential to be used as an efficient biological treatment technology for the treatment of municipal wastewater to eliminate antiretroviral compounds by efficiently removing Nevirapine, Tenofovir, Efavirenz, Ritonavir and Emtricitabine. Although it was shown that the MBBR efficiently removed the five ARVs tested, there is increasing evidence in the literature that most pollutants are not

completely removed in wastewater treatment (i.e. mineralised) but are often only slightly modified (Quintana et al., 2005; Schulz et all., 2008; Kormos et al., 2010), resulting in transformation products with a comparable or increased toxicity compared to parent compounds (Walgren et al., 2005; Celiz et al., 2009; Radjenovic et al., 2009). Future research should also include transformation products when studying the fate and ecotoxicity of these ARV compounds after wastewater treatment.

In chapter three, it was noted that the structure and function of microbial communities can be affected by influent characteristics and environmental variables. This was shown by the marked shift in microbial communities from the seeded sludge through the start-up of the MBBR to post introduction of ARV compounds in the reactor. In order to improve the efficiency of the MBBR for the removal of ARV compounds, there is a need to thoroughly study the microbial community and diversity of the MBBR biofilm post-introduction of the ARV compounds, by using advanced molecular techniques such as metagenomics and metaproteomics in order to identify and isolate specific organisms that can biodegrade ARV compounds and possibly other recalcitrant micropollutants. This would offer opportunities for the targeted inoculation of WWTPs in combination with moving bed biofilms as a potential method for the improved removal of recalcitrant micropollutants.

The toxicity of ARV compounds against the algae *S. capricornutum*, the bacterium *V. fischeri* and the crustacean *D. magna* was measured. However, it would be relevant to investigate the toxicity of these compounds towards non-target viruses as viruses such as bacteriophages play a significant role in aquatic ecosystems (Sime-Ngando, 2014). The review by Suttle (2007) presented that viruses kill approximately 20% of the oceanic microbial biomass daily, which has a significant impact on nutrient and energy cycles.

6.3 References

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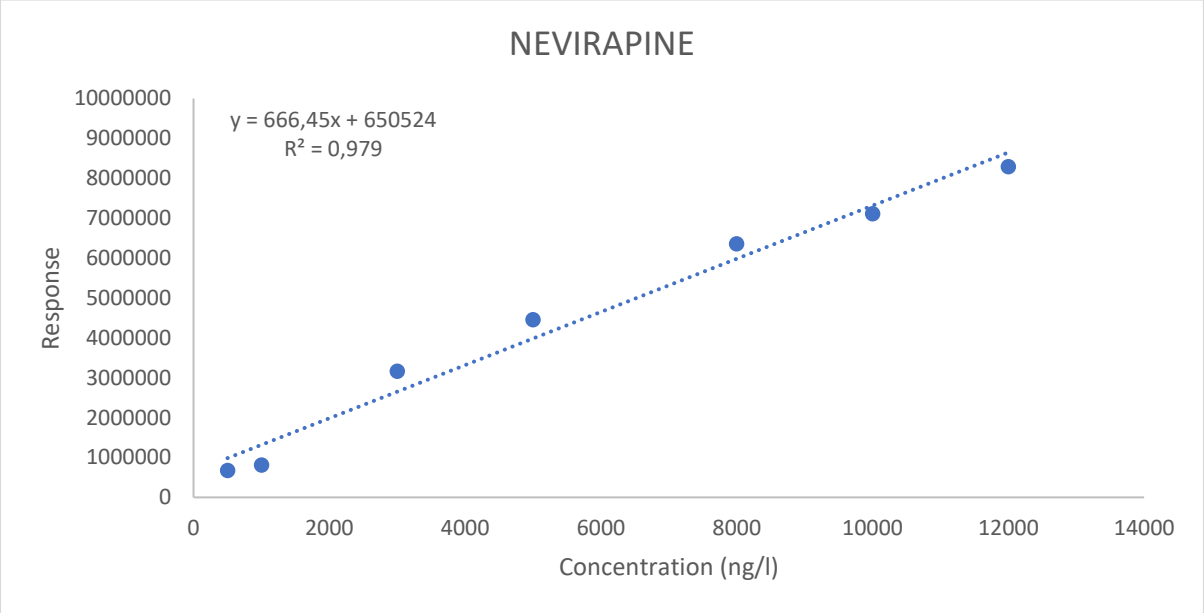
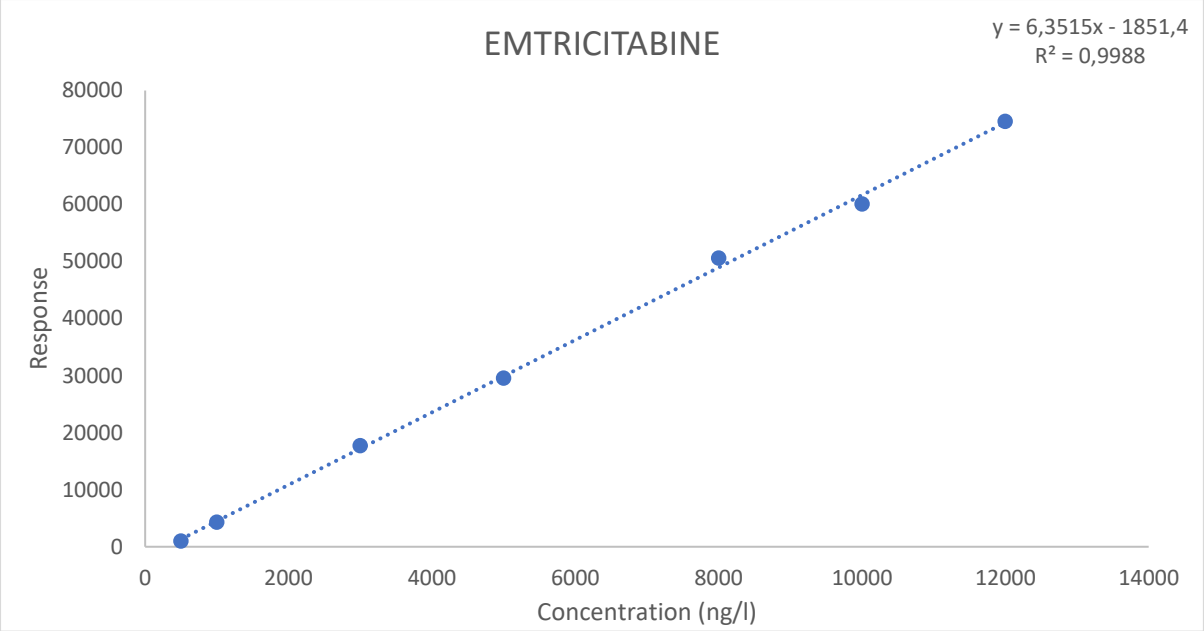
Supplementary Information

Calibration and Linearity

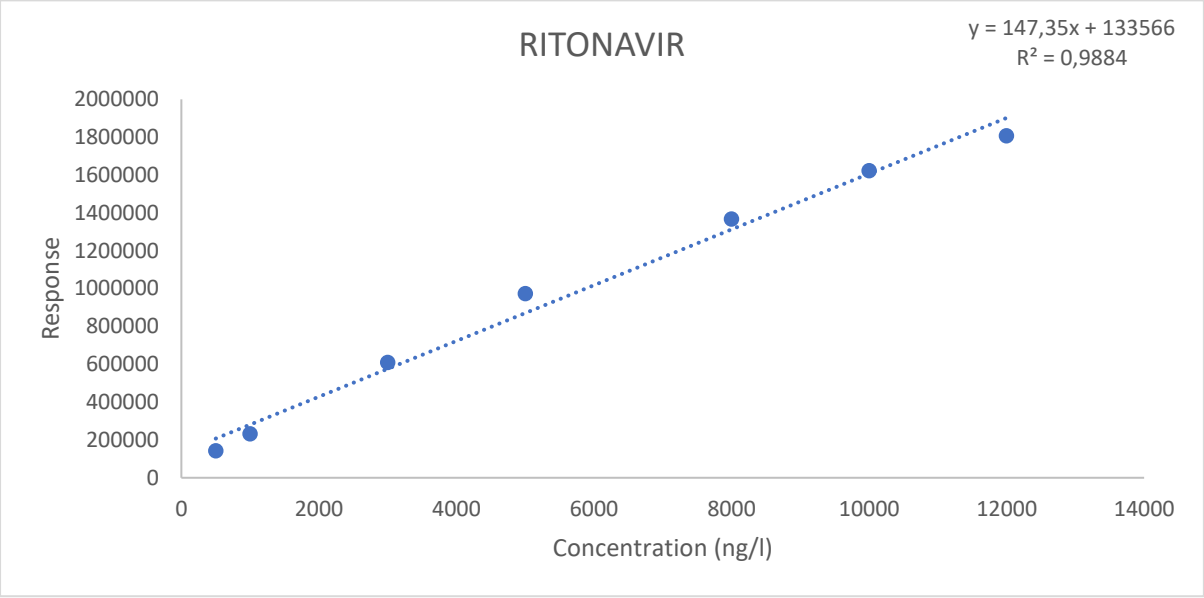
External calibration curves were generated by injecting 10µL of serially diluted mixture of standards onto LC-MS/MS (1000, 3000, 5000, 8000, 10 000 and 12 000 ng/L). A seven-point calibration was performed.



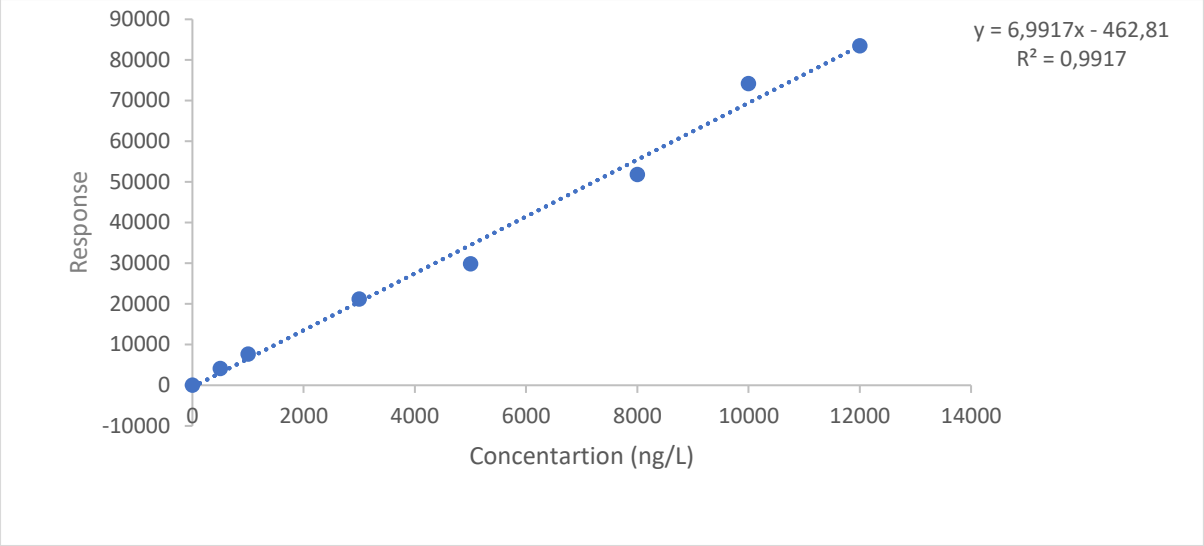
Appendix A: Calibration curve obtained fron the analysis of nevirapine (1000, 3000, 5000, 8000, 10000 and 12000 ng/L) by postive electrospray LC-MS/MS.



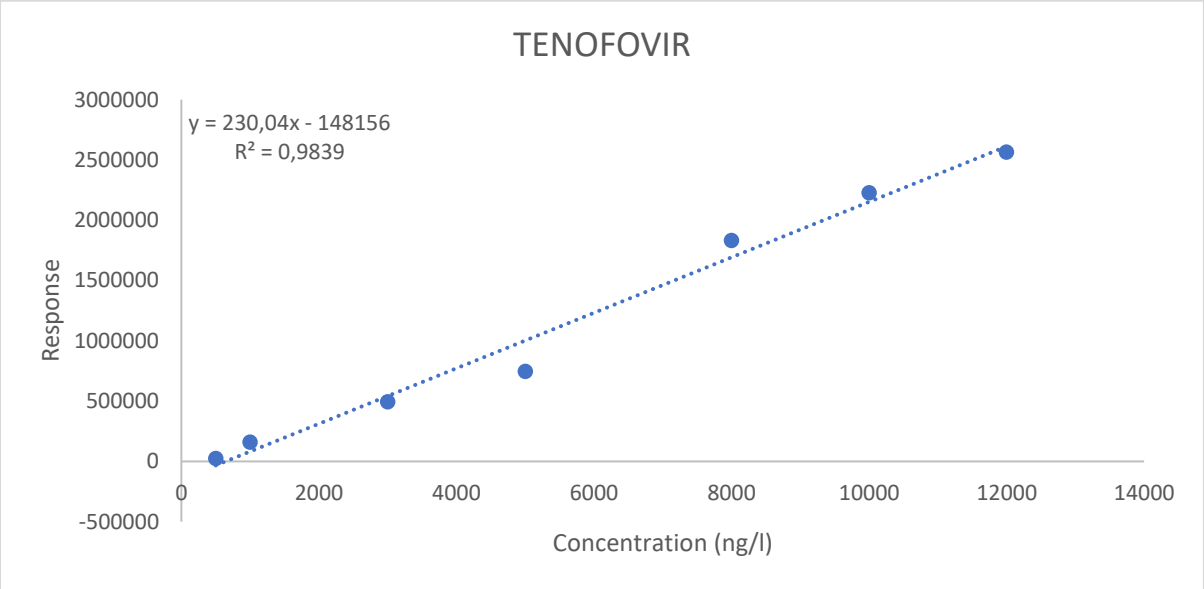
Appendix B: Calibration curve obtained fron the analysis of emtricitabine (1000, 3000, 5000, 8000, 10 000 and 12 000 ng/L) by postive electrospray LC-MS/MS.



Appendix C: Calibration curve obtained from the analysis of Ritonavir (1000, 3000, 5000, 8000, 10 000and 12 000 ng/L) by positive electrospray LC-MS/MS.

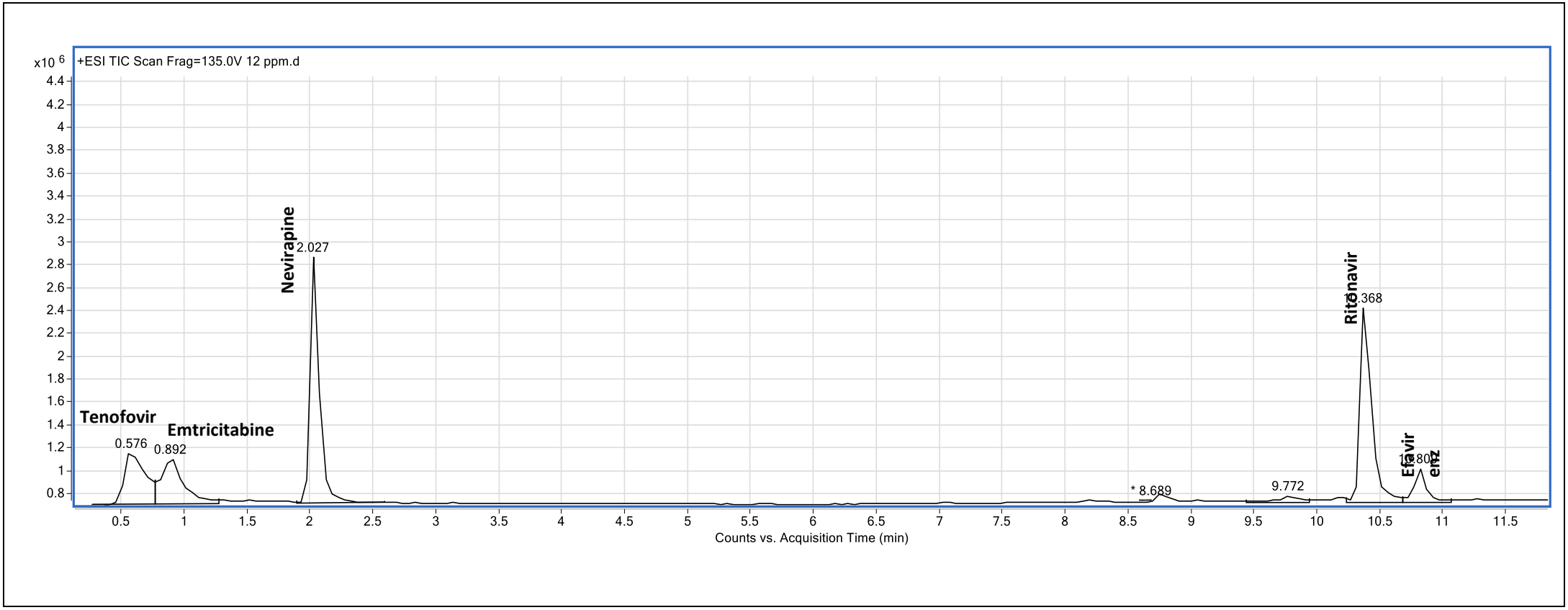


Appendix D: Calibration curve obtained from the analysis of Efavirenz (1000, 3000, 5000, 8000, 10 000 and 12 000 ng/L) by positive electrospray LC-MS/MS.

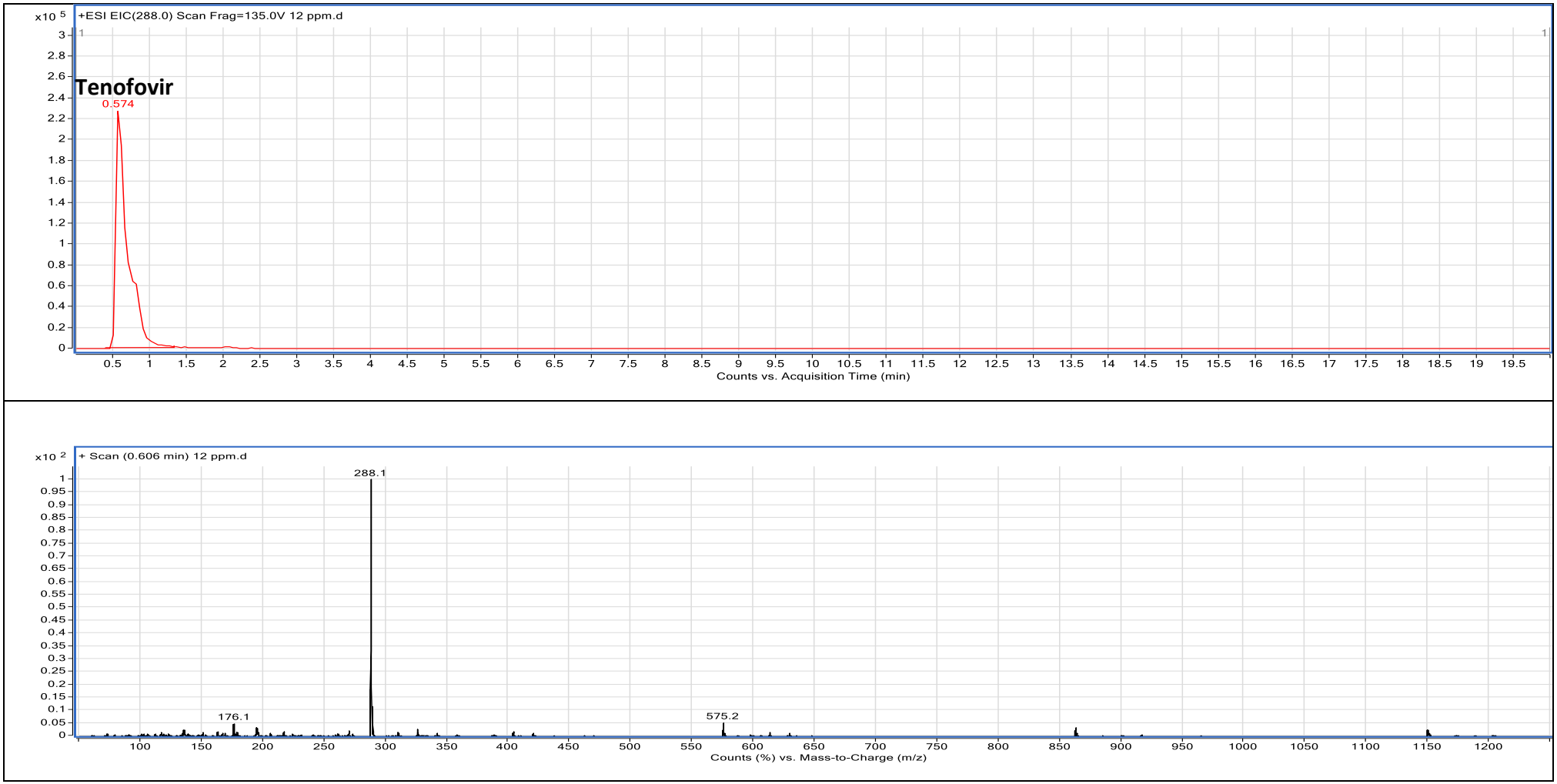


Appendix E: Calibration curve obtained fron the analysis of Tenofovir (1000, 3000, 5000, 8000, 10000 and 12000 ng/ml) by positive electrospray LC-MS/MS.

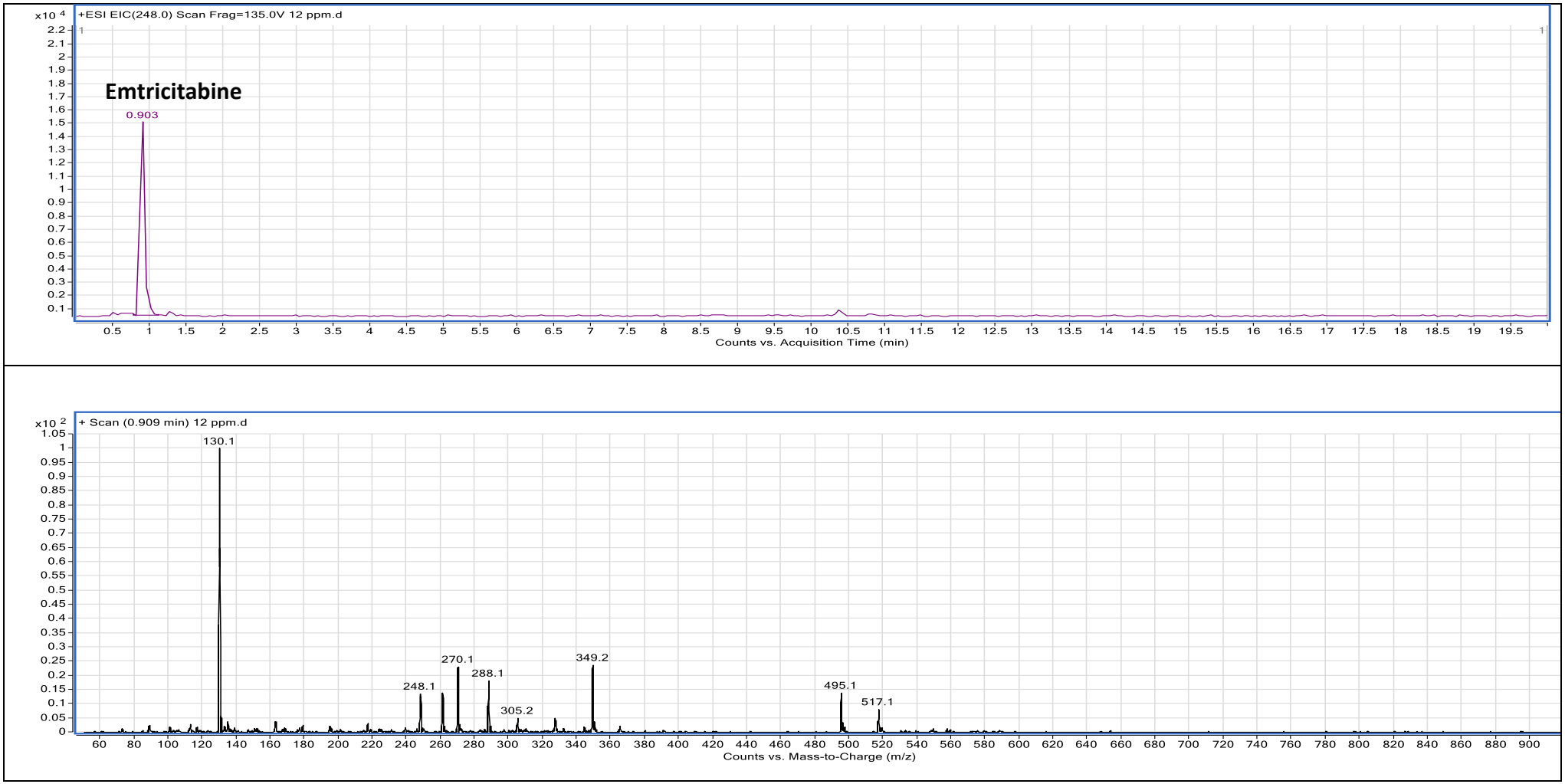
A full scan chromatogram of a mixture of all the compounds is presented in Appendix F. Model extracted ion chromatograms and mass spectra for each of the targeted compounds are presented in Appendix G to Appendix K.



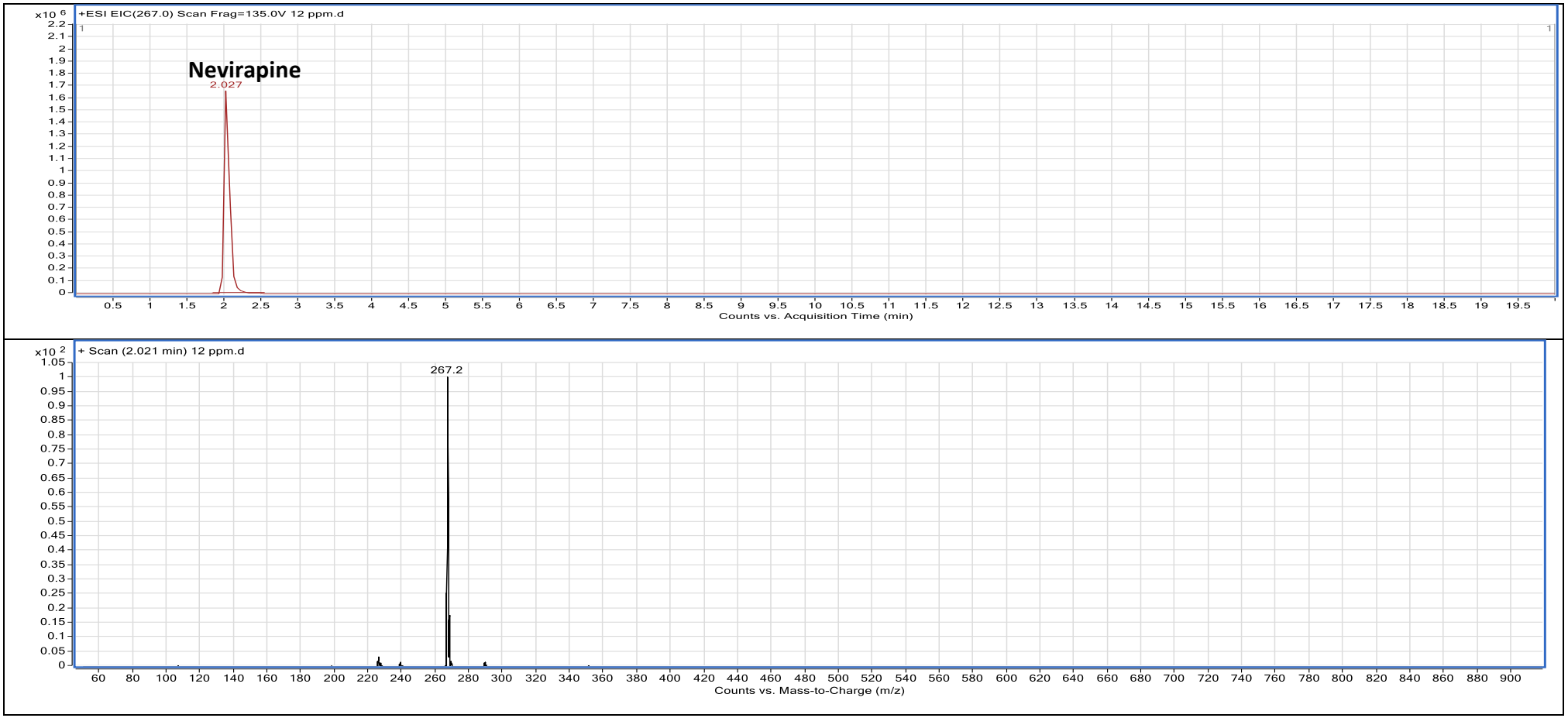
Appendix F: Full Chromatogram of a mixture of the Five Antiretroviral Drugs by LC-MS/MS



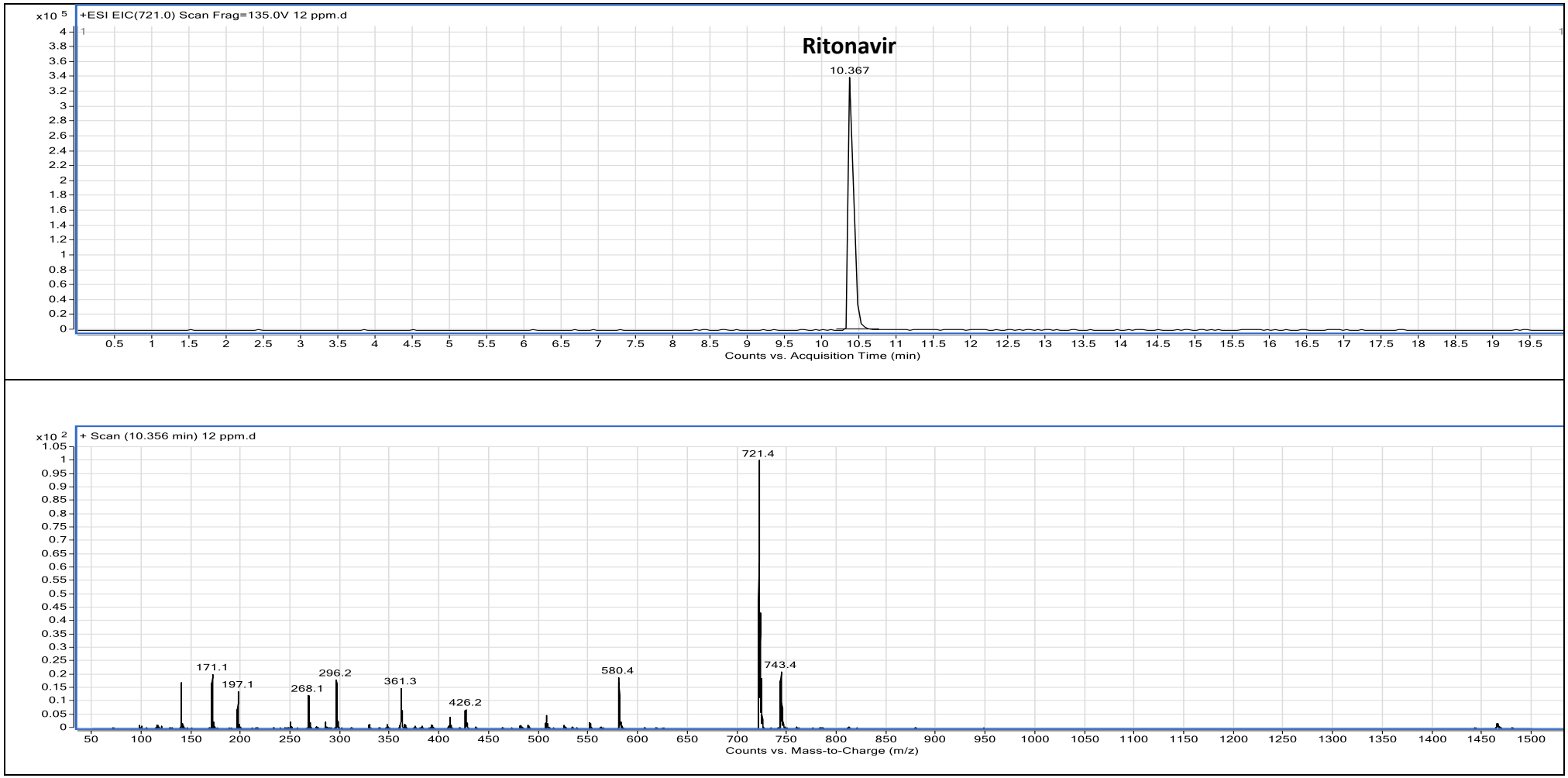
Appendix G: EIC and mass spectrum of Tenofovir by LC-MS/MS



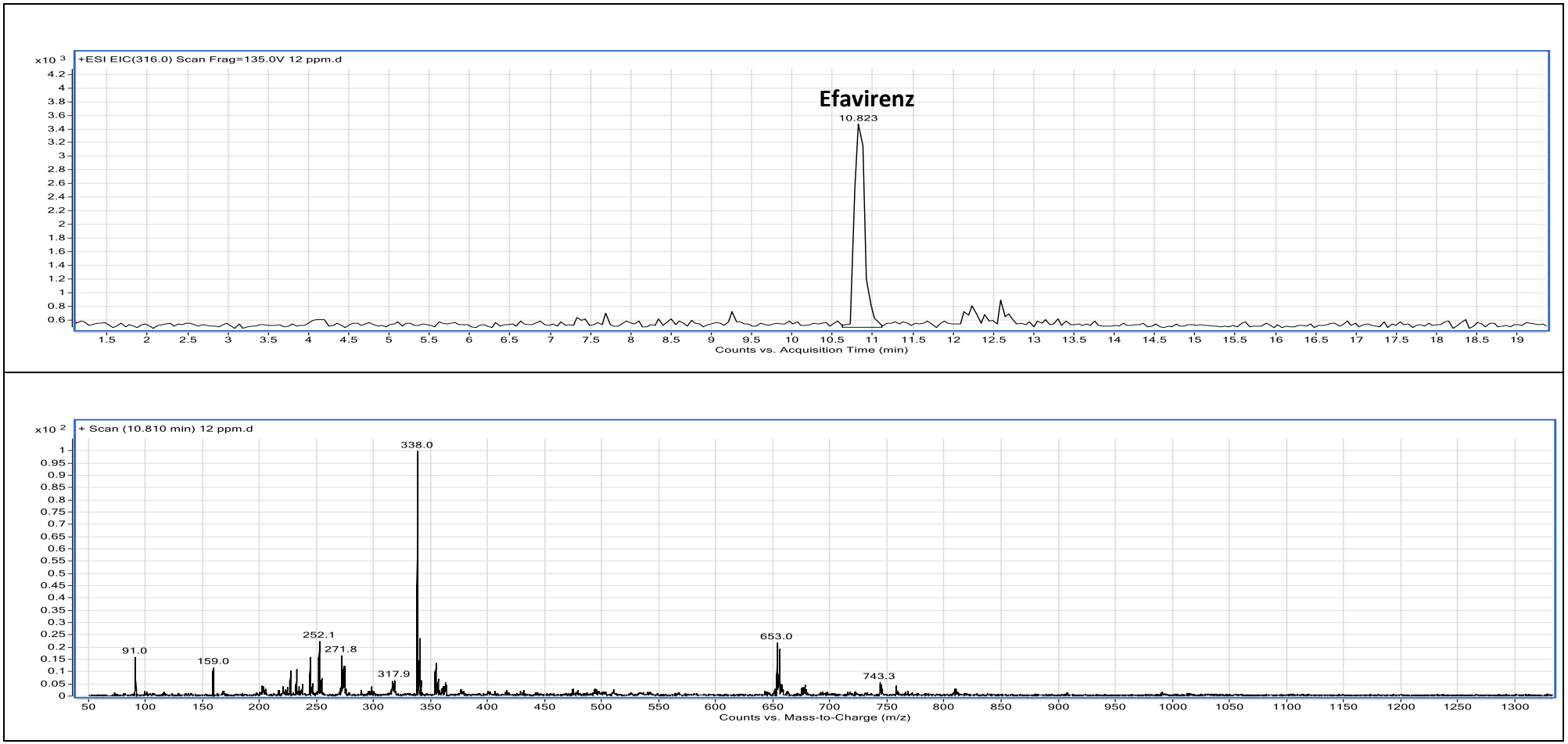
Appendix H: EIC and mass spectrum of Emtricitabine by LC-MS/MS



Appendix I: EIC and mass spectrum of Nevirapine by LC-MS/MS



Appendix J: EIC and mass spectrum of Ritonavir by LC-MS/MS



Appendix K: EIC and mass spectrum of Efavirenz by LC-MS/MS