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Quantification of some ARVs' removal efficiency from wastewater using a moving bed biofilm reactor

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ABSTRACT

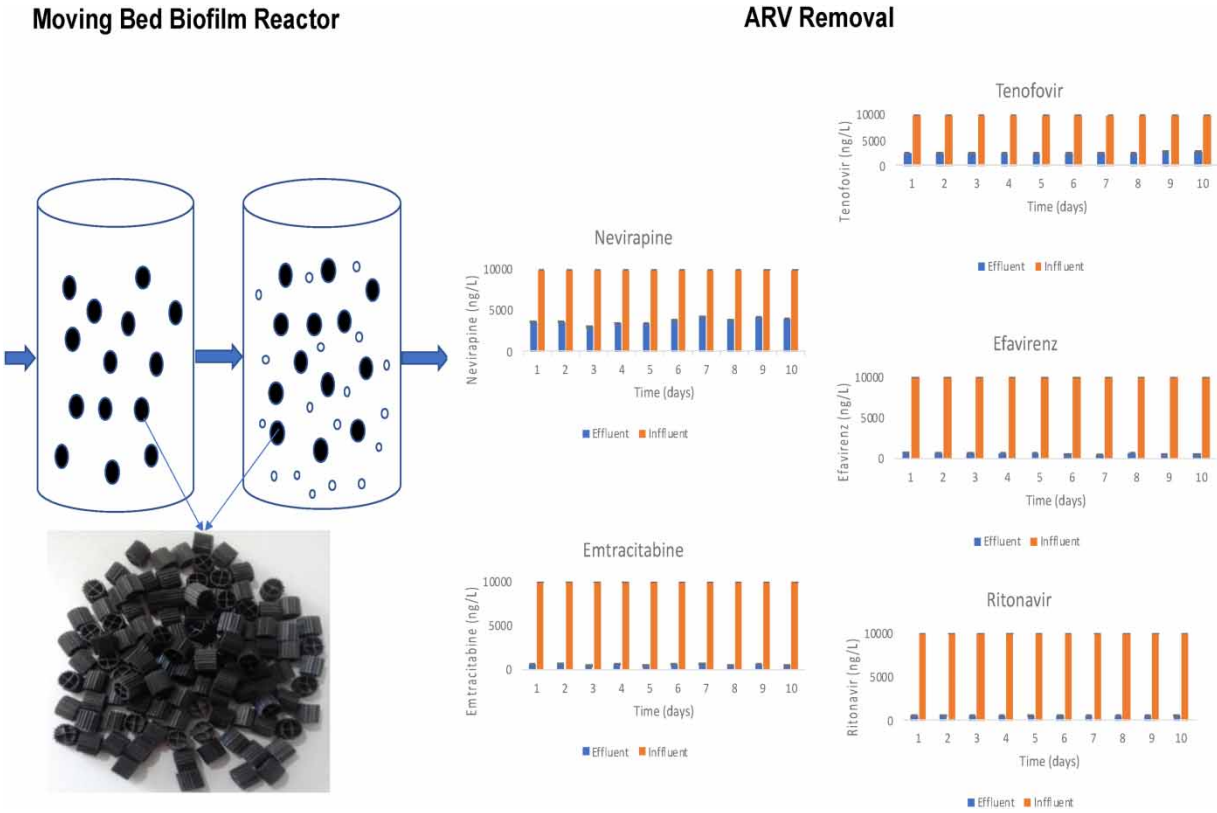
To date, in South Africa alone, there are an estimated 4.5 million people receiving antiretroviral (ARV) therapy. This places South Africa as the country with the largest ARV therapy programme in the world. As a result, there are an increasing number of reports on the occurrence of ARVs in South African waters. Achieving efficient and bio-friendly methods for the removal of these pollutants is considered as a concern for environmental researchers. This study aims at studying the efficiency of a moving bed biofilm reactor (MBBR) system for removing ARVs from wastewater. A continuous-flow laboratory scale system was designed, built, installed, and operated at a carrier filling rate of 30%, an organic loading rate of 0.6 kg COD/m_3.d_{-1} OLR, a hydraulic retention time of 18h, and a 27.8 mL/min flow rate. The systems were monitored over time for the elimination of conventional wastewater parameters i.e., Biological Oxygen Demand, Chemical Oxygen Demand, and nutrients. The results showed that the MBBR system as a bio-friendly method has high efficiency in removing 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.

Key words: antiretrovirals, moving bed biofilm reactor, quantification, removal efficiency, wastewater

HIGHLIGHTS

- A moving bed biofilm reactor (MBBR) system for removing ARVs from wastewater.
- MBBR system as a bio-friendly method has high efficiency in removing Nevirapine, Tenofovir, Efavirenz, Ritonavir and Emtricitabine from wastewater.
- A continuous-flow laboratory scale system was designed, built, and operated under different organic loading rates, hydraulic retention times, and filling rates to optimize its performance.

GRAPHICAL ABSTRACT



INTRODUCTION

Micropollutants, also known as emerging contaminants (primarily organic), are usually of anthropogenic origin and are commonly present in waters at trace concentrations ranging from ng/L to several μg/L (Kasprzyk-Hordern *et al.* 2007; Baker & Kasprzyk-Hordern 2013). The compounds in question are derived from three broad categories: Pharmaceuticals, Personal Care Products, and Endocrine Disrupting Compounds (Ferrari *et al.* 2003; Diamanti-Kandarakis *et al.* 2009; Al Aukidy *et al.* 2012; Verlicchi & Zambello 2015). These chemicals reach natural waters mainly through municipal (domestic) and industrial wastewater streams (Edokpayi *et al.* 2017).

The most thought-provoking class of these emerging contaminants is pharmaceuticals. Given that 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., conversion to CO_2 and H_2O, during the biological wastewater treatment process. However, it has been scientifically demonstrated that most WWTPs cannot remove pharmaceutical drugs during sewage treatment (Kolpin *et al.* 2004; Zhou *et al.* 2010; Cimetiere *et al.* 2013; Capdeville & Budzinski 2011; Gómez *et al.* 2012; Blair *et al.* 2016), mainly because wastewater treatment plants based on conventional activated sludge (CAS) were initially 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, several pharmaceuticals have been detected in WWTP effluents and surface waters (Snyder 2008).

To date, in South Africa alone, there are 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 world's most extensive ARV therapy program. As a result, there is an increasing number of reports on the occurrence of ARVs in South African waters (Schoeman *et al.* 2015; Swanepoel *et al.* 2015; Wood *et al.* 2015, 2016; Abafe *et al.* 2018). As with most (human) pharmaceuticals, ARVs are partly metabolized in treated individuals, while large fractions are excreted unchanged via urine or feces (Daughton & Ternes 1999; Galasso *et al.* 2002) and thus find their way into WWTPs. After reporting several ARV drugs in different water matrices,

including wastewater effluents, surface water, and drinking water (Schoeman *et al.* 2015; Wood *et al.* 2015, 2016; Abafe *et al.* 2018), Swanepoel *et al.* (2015) have suggested that low concentrations of ARV drugs 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 ARV therapy, suggesting the possibility of resistance development by HIV. Furthermore, studies have shown that antiviral drugs are among the predicted most hazardous therapeutic classes concerning their toxicity toward *algae*, *daphnids*, and *fish* (Sanderson *et al.* 2004; Nannou *et al.* 2020).

Due to the limited efficiency of conventional biological treatment, it would be necessary to explore innovative solutions to improve the removal of trace contaminants and residues in wastewater. Activated carbon has been demonstrated to have a high capacity to adsorb pharmaceuticals when used in post-treatment/polishing steps for CAS treatment (Simazaki *et al.* 2008; Rivera-Utrilla *et al.* 2009). 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. In addition, a number of investigations have shown that the biofilm reactors such as membrane biofilm reactors, fixed film bed bioreactors, and moving bed biofilm reactors (MBBRs) can accomplish more if optimized for enhanced removal of pharmaceutical contaminants (Dolar *et al.* 2012; Sengupta *et al.* 2021). Among these biofilm bioreactors, MBBR is the most preferred method because of its low operational cost and its simplicity of operation. Currently, MBBR systems have been used both in pilot plant studies and in full-scale plants for the treatment of wastewater (Barwal & 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.

Moreover, the primary 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 benefits 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 & Zouboulis 2001), less prone to the process upsets from poorly settling biomass (Schmidt & Schaechter 2011), increased solid retention favoring slow-growing organisms such as nitrifiers (Guo *et al.* 2010; Shore *et al.* 2012), and cost-effectiveness (Fang 2011). The MBBR has relatively tiny footprint operational requirements, 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). Moreover, when comparing activated sludge to MBBR on the removal of benzotriazoles and hydroxybenzothiazole, Mazioti *et al.* (2015) reported that the biomass developed in the MBBR system had a greater capacity for removal than the activated sludge, especially when operated under low organic loading. Accinelli *et al.* (2012) also 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. From this perspective, these previous studies provide a potential solution to the removal of ARVs from wastewater. However, the research work on the removal of ARV pollutants such as Tenofovir, Emtricitabine, Nevirapine, Ritonavir, and Efavirenz from wastewater using MBBR has not been investigated.

Hence, the main objective of this study was to examine the ability of an MBBR to remove Tenofovir, Emtricitabine, Nevirapine, Ritonavir, and Efavirenz from synthetic municipal wastewater. A continuous-flow laboratory-scale system was designed, built, installed, and optimized. The optimum operating conditions for the MBBR were obtained at a carrier filling rate of 30% and an organic loading rate of 0.6 kg COD/$m^3 \cdot d^{-1}$ OLR, a hydraulic retention time of 18 h, and a 27.8 mL/min flow rate. The operational conditions were maintained and used to eliminate ARV pollutants of interest. The system was also monitored over time to eliminate conventional wastewater parameters, i.e., Biological Oxygen Demand (BOD), Chemical Oxygen Demand (BOD), and nutrients, and the results obtained were discussed.

EXPERIMENTAL METHODS

Chemicals and reagents

A set of five ARV compounds widely used in South Africa were selected, namely Tenofovir, Emtricitabine, Ritonavir, Efavirenz, and Nevirapine. 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). Ten milligrams of the analytical standards (Tenofovir, Emtricitabine, Nevirapine, Ritonavir, and Efiverenz) was dissolved in 10 ml Dimethyl Sulfoxide (DMSO), resulting in a 1,000 mg/L solution. This solution was diluted 10 times to produce a 100 mg/L working stock solution. Appropriate dilutions were then made using the synthetic wastewater to produce 0.5, 1, 3, 5, 8, 10, and 12 μg/L standards. The 0.5, 1, 3, 5, 8, 1,0, and 12 μg/L standards were then injected to produce a calibration graph for all standards.

The biofilm media used in this study was ECOBM-100 Biofilter provided by Ecotao Enterprise, South Africa. The carrier media (Figure 1) are made of polyethylene and are shaped like a small cylinder (with a normal diameter of 10 mm and a normal length of 10 mm), with a cross inside the cylinder and longitudinal fins on the outside, and a density of about 125 kg/m^3. The carrier is designed to provide interspaces for suspended microorganisms and offers a high specific surface area of up to 1,200 m^2/m^3.

Sample prepaaration

Concentrated stock solutions containing 1 mg/mL of each ARV compound were 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 drug.

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 °C until analysis.

MBBR system experimental procedure

After the optimization stage (see Supplementary information for MBBR system optimization), the MBBR system was acclimatized to the synthetic wastewater spiked with ARV compounds (10 μg/L of each ARV). All the experiments were conducted at pH 7 ± 0.4 by using a 50 mM phosphate buffer solution. The wastewater with ARV compounds was continuously introduced to the MBBR for a period of 30 days before the investigation of ARV compound removal was carried out. After the acclimatization period, grab samples were then collected continuously over a period of 10 days to assess the removal efficiency. Reactor samples that had been spiked with 10 μg/L of the five ARV drugs were analyzed quantitatively against external calibration curves. These values are compared to the spiked unreacted influent water. Each sampling time was

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

analyzed in triplicate. Qualitative and quantitative data analysis was performed using Agilent MassHunter Qualitative and Quantitative software respectively. 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 SPE cartridges and injected into the LC-MS/MS for analysis. Quantification was performed against an external calibration curve.

Removal efficiency (percentage) was calculated by comparing concentrations between influent wastewater (spiked synthetic wastewater) and the effluent of the reactor using Equation (1):

$$Removal\ (\%) = \left(1 - \frac{Concentration\ of\ final\ efluent}{Concentration\ of\ influent}\right) \times 100\% \quad (1)$$

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, 2.7 μm, 3.0 × 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 0–5 mins, 5–15 mins to 90% B and 15–20 mins down to 20% (B). Column eluent passed into an Agilent 6460 triple quad LC/MS with a jet stream electrospray ionization source operated in a positive mode. Source and acquisition parameters are presented in Table 1.

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 five analytes according to the International Council for Harmonisation (ICH) guidelines, and the following parameters were tested for the validation: linearity, repeatability, % recovery, matrix match effect, limit of detection (LOD), and limit of quantitation (LOQ).

Accuracy, precision, calibration, and limit of quantification

The instrumental intra-day and inter-day repeatability tests of the instrument were assessed at 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 different times of the day for intra-day repeatability and on three consecutive days for inter-day repeatability. The percentage relative standard deviation (RSD) of the responses was then determined. 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 and 12 μg/L were injected in triplicate onto the LC-MS/MS system. 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 the area against concentration was used to prepare the seven-point calibration curves for each ARV compound (0.5–12 μg/L). LOD and LOQ were determined by injecting 1 mg/L of standard ten times onto the LC-MS/MS. The LOD

Table 1 | Agilent 6460 triple Quadrupole LC/MS parameters for the analysis of antiretroviral compounds with positive electrospray ionization 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	300 V
Fragmentor	135 V
Acquisition range	50–1500 m/z
Acquisition rate	0.33 cycles/s

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 analyzing spiked Synthetic wastewater.

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 1,000 mL Milli-Q water were divided into two 500 mL portions, A and B, respectively. Triplicate samples of portion A for each concentration were 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 1 mL with acetonitrile/water (20:80 v/v) in a 2 mL chromatographic bottle and injected onto the LC-MS/MS.

$$\%\text{recovery} = \frac{A}{B} \times 100 \tag{2}$$

where A = peak area of A (peak area of analyte added before the SPE process); and B = peak area of B (peak area of analyte added after the SPE process).

Matrix effect (ME)

Matrix effects are often caused by the alteration of the 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 ARV drugs was used for the 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).

$$\text{ME } (\%) = \frac{X}{Y} \times 100 \tag{3}$$

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 the ARV standard after SPE.

Chemical analytical methods

Samples for analyzing nutrients, pH, BOD, COD, and dissolved oxygen (DO) were collected daily (from day 1 to day 30) in brown bottles from the MBBR effluent and analyzed immediately. BOD, COD, phosphate, ammonium, and total nitrogen (TN) were also monitored daily during the course of the experiment.

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 every day 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 analyzed in triplicate.

Statistical analysis

The data obtained in this research were analyzed by Microsoft Excel software. Statistical analysis by t-test was used to indicate significant differences ($P < 0.05$) between the influent and the effluent of each ARV compound in the reactor. The difference is detected as statistically significant if the 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.

RESULTS AND DISCUSSION

The designed reactor was inoculated with activated sludge from a municipal wastewater treatment plant. Thirty days was necessary to achieve constant attached biomass concentration. This period is referred to as the start-up or acclimatization phase, during which the plastic carriers were being colonized by the microorganisms at the initially applied OLR (0.32–0.36 kg COD/m^3·d^{-1}). Additionally, all the experiments were conducted at pH 7 ($\pm$0.4). The assessment of the removal efficiency of ARV pollutants was achieved by collecting grab samples continuously over a period of 10 days. It is important to note that the results reported and discussed in this study focus on the results obtained after the optimization of the system, meaning the 10 days assessment period.

Nutrients, pH, BOD, COD, and DO analytical methods

Figure 2 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, PO_4, NH_4, and TN removal efficiencies 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 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 & Borghei 2005).

Analysis of reactor samples

Figures 3–7 illustrate the efficacy of the MBBR on the removal of five selected ARV drugs. Nevirapine, Tenofovir, Efavirenz, Ritonavir, and Emtricitabine all showed a marked reduction in concentration between the influent and effluent of the MBBR. On average, the percentage removed for Nevirapine, Tenofovir, Efavirenz, Ritonavir, and Emtricitabine is 62, 74, 93, 94, and 94%, respectively, with *P*-values of 0.019, 0.018, 0.002, 0.001, and 0.003 respectively. The *P*-value confirms that there was a statistically significant difference between the average means of the influent and effluent for each of the ARV compounds in the MBBR system.

The low removal results of Nevirapine (Figure 3) 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 showed that Nevirapine has low biodegradability (up to 3%) in a closed bottle system (Vankova 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 the KwaZulu-Natal province. On the contrary, K'Oreje *et al.* (2016) also reported an increase in

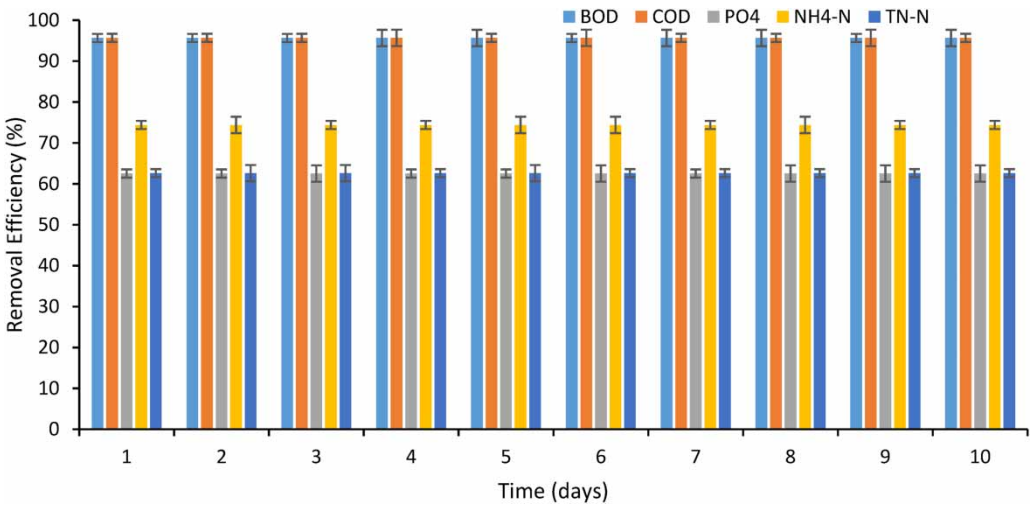


Figure 2 | Average removal efficiency of BOD, COD, PO_4, NH_4, and TN after the introduction of ARV compounds, with an average removal of 95.65, 95.69, 62.52, 74.39, and 62.61%, respectively.

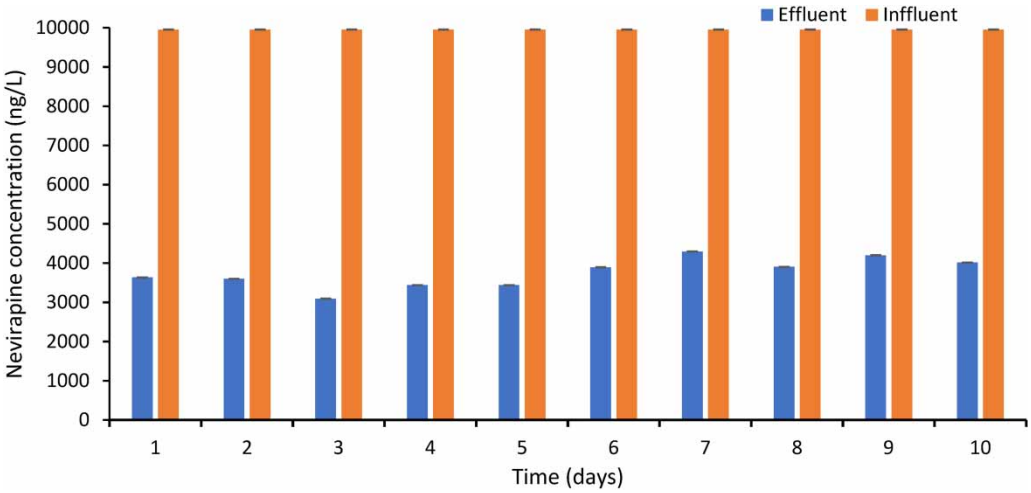


Figure 3 | The concentration of Nevirapine over time in a bioreactor effluent as compared to the bioreactor influent, with an average removal efficiency of 62%.

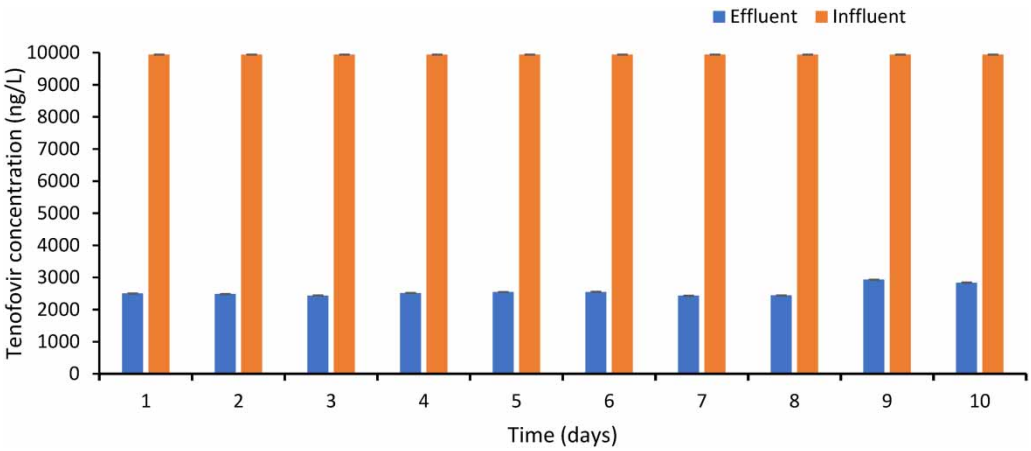


Figure 4 | The concentration of Tenofovir over time in a bioreactor effluent as compared to the untreated bioreactor influent, with an average removal of 74%.

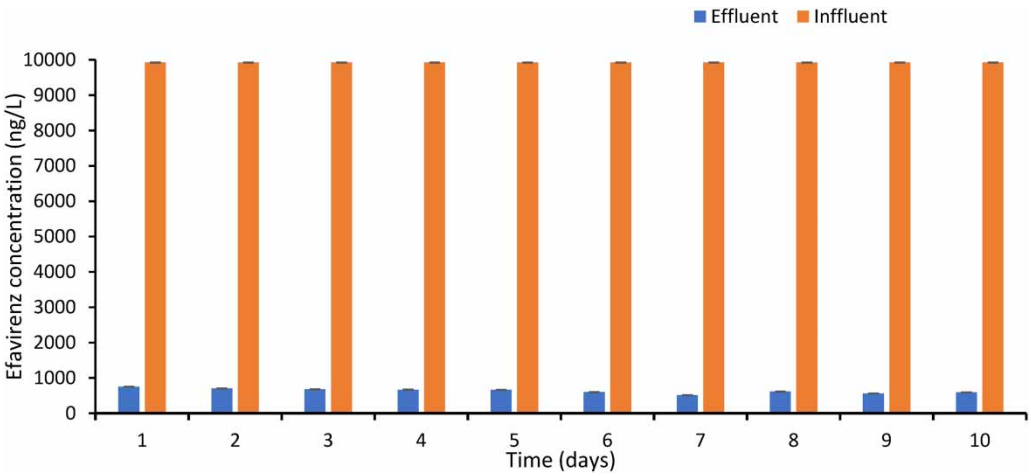


Figure 5 | The concentration of Efavirenz over time in a bioreactor effluent as compared to the untreated bioreactor influent, with an average removal of 94%.

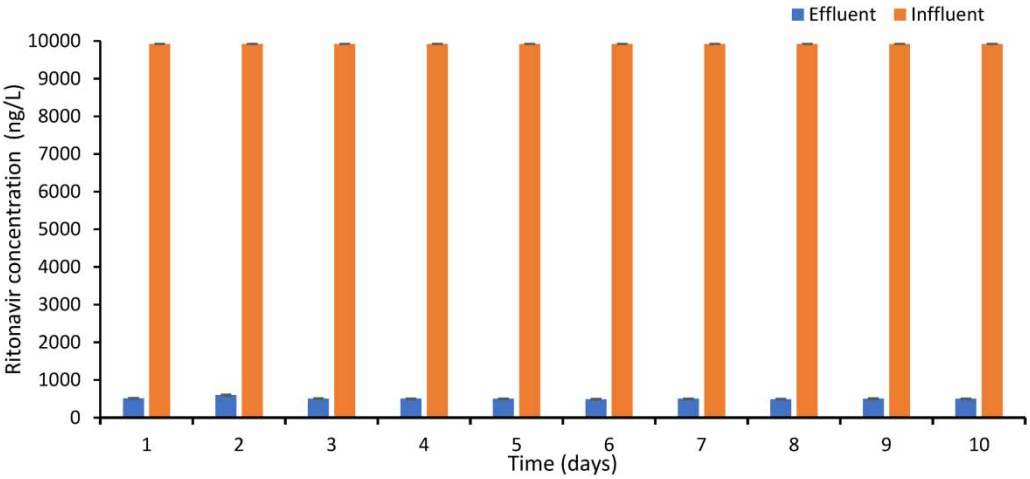


Figure 6 | The concentration of Ritonavir over time in a bioreactor effluent as compared to the untreated bioreactor influent, with an average removal of 94%.

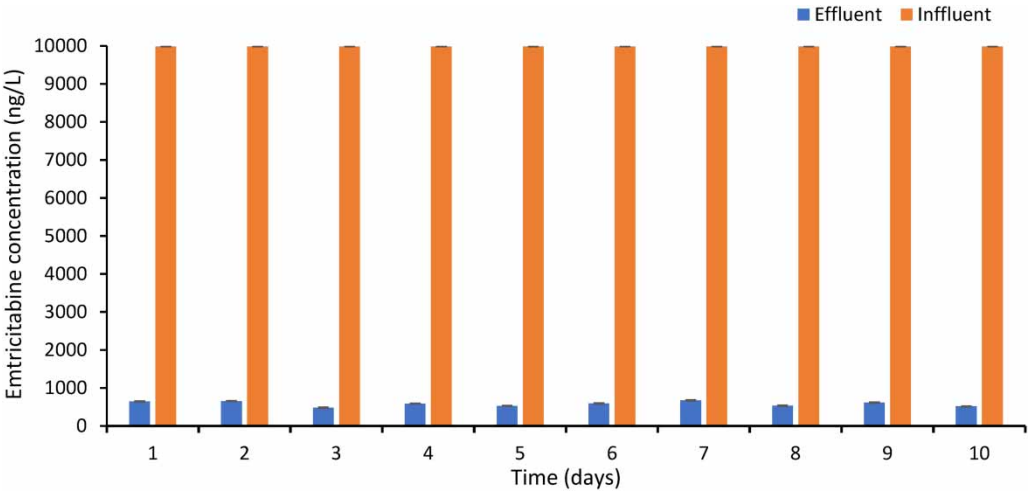


Figure 7 | The concentration of Emtricitabine over time in a bioreactor effluent as compared to the untreated bioreactor influent, with an average removal of 95%.

nevirapine concentrations at the outlet from another WWTP in Kenya (850 ng/L at the inlet and 1,000 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 4). Like Nevirapine, Tenofovir has been reported to be persistent in the environment. Al-Rajab *et al.* (2010) in London have shown that Tenofovir is relatively persistent in soils with no evidence of transformation products. After introducing the drug to varying temperatures in treated soils (range 4 °C to 30 °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 (Schoeman *et al.* 2015; Wood *et al.* 2015; Mlunguza *et al.* 2019). Even though Nevirapine and Tenofovir were the least biodegradable antiretroviral drugs investigated in this study, the MBBR presents a promising alternative when compared to findings reported for conventional wastewater treatment plants (Table 2).

Table 2 | Antiretroviral drug removal efficiency of different wastewater treatment plants

ARV	Country	Removal Percentage (%)	References
Nevirapine	South Africa	15	Schoeman *et al.* (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 *et al.* (2015)
	South Africa (Decentralized)	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

Comparison of antiretroviral removal efficiency

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 removed in the MBBR at an average removal rate of 93.62%, 94.18%, and 94.87% respectively (Figures 5–7). In this regard, the MBBR showed a 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 5,500 and 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. A removal percentage of 53% was reported for decentralized wastewater treatment (DEWATS), 43% for Northern WWTP, and 71% for Phoenix WWTP. No removal efficiency studies were reported on Emtricitabine by the time of this study. However, the MBBR has been shown to remove Emtricitabine almost entirely at 95% (Figure 7). Like many other emerging contaminants, ARV drugs have been reported in the ng/L range in the influents of conventional WWTPs. These relatively low concentrations may result in poor adaptation or development of activated sludge bacteria to degrade these ARV drugs, since slow-growing microorganisms suffer flush-outs in conventional activated sludge systems due to the low solid retention time.

Method validation

Accuracy, precision, calibration, and limit of quantification

The objective of the 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 the limit of quantification. The result of the linearity of the analytical method is presented in Table 3, and it was determined as described in the validation and linearity section (see Supplementary Information). The response area of the three analytes was 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. The limit of detection

Table 3 | Calibration data for five ARV drugs, diluted serially and analyzed by LC-MS-MS in a positive ionization mode

				% Recovery (SD)				Intra-day repeatability (RSD)			Inter-day repeatability (RSD)		
Drug	LOD (ng/mL)	LOQ (ng/mL)	Linearity (R^2)	5 ng/mL	10 ng/mL	15 ng/mL	% Matrix effect (SD)	5 ng/mL	10 ng/mL	15 ng/mL	5 ng/mL	10 ng/mL	15 ng/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

(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 of 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 of 10:1 as recommended by ICH (Bhardwaj *et al.* 2015; Vidushi *et al.* 2017). Table 3 presents the LOD for the five pharmaceutical formulations and it varied from 0.12 to 0.16 ng/ml. LOQ ranged between 0.40 and 0.53 ng/ml. 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 the area against concentration was used to prepare the seven-point calibration curves for each pharmaceutical compound as shown in Appendix A to Appendix E (see Supplementary information). Typical chromatograms for the calibration standard are presented in Appendix F to Appendix K (see Supplementary information).

The results of intra-day and inter-day evaluations are presented in Table 3. These were estimated based on relative standard deviation (RSD) at the same experimental conditions. The values for the three concentrations examined ranged between 1 and 1.82 and between 1.30 and 3.28 for intra-day and inter-day, respectively. The RSDs for the compounds are within the acceptable limit (less than 20%) for the repeatability test for the high performance liquid chromatography (HPLC) method (United Nations 2009). This shows that the method was repeatable and reliable.

Percentage recovered

Percentage recoveries for the five compounds are presented in Table 3. From the results obtained, Tenofovir, Emtricitabine, and Efavirenz had the lowest recovery compared to the other compounds. This could be attributed to their high solubility in aqueous media, which limited their 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 *et al.* 2009; WHO 2009).

Matrix effect

The results for the matrix effects are shown in Table 3; it can be noticed that 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 liquid chromatography-mass spectrometry (LC-MS) is one of the most sensitive and selective analytical techniques, it often suffers from matrix effects, especially when using electrospray ionization (ESI) for analyzing extracts of complicated matrices (Matuszewski *et al.* 1998). Matrix effects are often caused by the alteration of the 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 be analyzed either by external calibration using matrix-matched standards or by standard addition (Cimetiere *et al.* 2013; Wood *et al.* 2015; Panuwet *et al.* 2016).

CONCLUSION

The literature review revealed that current WWTPs are not able to provide an absolute barrier to the elimination of emerging contaminants. 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, total nitrogen (TN), and PO_4). The optimum operating conditions for the MBBR were obtained at a carrier filling rate of 30% and an organic loading rate of 0.6 kg COD/m^3·d^{-1} OLR, a hydraulic retention time of 18 h, and a 27.8 mL/min flow rate. At these operating conditions, the MBBR achieved the highest removal rate of BOD, COD, 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.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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