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2020

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BEHAVIOUR OF SELECTED ANTI-RETROVIRALS' ACTIVE PHARMACEUTICAL INGREDIENTS AND THE ASSESSMENT OF THEIR BACTERIAL VIABILITY IN A SIMULATED WASTEWATER TREATMENT PLANT

PhD Research Thesis

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July 2020

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____

Lawrence Ikechukwu OBIDIKE

04 December 2019

ABSTRACT

The release of micropollutants into the environment via wastewater treatment plants (WWTP) is one of the issues which has long been considered a major source of environmental contamination due to the ineffectiveness of WWTPs in eliminating them. In these releases, a wide range of compounds is measured in trace amounts (ng/L to µg/L) such as pharmaceutical residues and hormones (PPHs), pesticides, phthalates, artificial sweeteners, chemical products, and personal care products. This study examined the effects of nevirapine, a non-nucleoside reverse transcriptase inhibitor (NNRTI) drug and with lamivudine, a nucleoside reverse transcriptase inhibitor (NRTI) drug in combination, on a simulated activated sludge process using actual wastewater from Bushkoppie Wastewater Treatment in Johannesburg, South Africa. Laboratory experiments were performed using duplicate samples of raw influent collected during June 2018 while the final laboratory experiments were performed using triplicate samples collected from raw influent during November 2018. 1, 15, and 25 mg/L concentrations of nevirapine were introduced into the wastewater and the concentrations of chemical oxygen demand (COD) and ammonia-nitrogen in the effluent of each experiment are recorded on a 2-hly basis until the 8th h when the experiment was terminated. Samples with 15 mg/L nevirapine inhibited the specific concentration variations of COD by 52.9% (standard deviation 27%) and the specific $N\text{-}NH_4^+$ concentration variation by 30% (standard deviation 21%). An increase in COD, as well as a decrease in total suspended solids (TSS), were observed in the wastewater with nevirapine. In order to assess nevirapine's time-kill activity, continuous experiments were conducted both in closed mode (batch equivalent) and imaging techniques combined with an L7007 LIVE/DEAD BacLight viability kit (Invitrogen, South Africa). The nevirapine toxicity in the wastewater was observed at lower concentration when exposure time increased. A 0.1 mg/L nevirapine concentration was toxic to heterotrophic bacteria on a closed mode, and inhibited nitrification. These findings agree with the microscopic studies, which showed a latency time before the lower nevirapine concentrations began to kill the bacteria. After 40 minutes there were 97 % (Standard Deviation 3.8) of living bacteria in control reactors, 76 % (Standard Deviation 3.1) in reactors that contained 0.1 mg/L nevirapine and 46 % (Standard Deviation 18.6) in the system that contained 10 mg/L nevirapine.

The influence of nevirapine and lamivudine on municipal sludge in batch reactors focuses on extracellular polymeric substances (EPS) as an indicator of bacteria sensitivity with respect to the above-mentioned drugs. The EPS were analyzed by FT-IR spectroscopies. It was found

that both drugs induced a significant increase of bound EPS in flocs. This may be attributed to a protection mechanism by the bacteria. However, only Nevirapine inhibited COD and nitrogen removal.

Key words: WWTP, Nevirapine, COD, Lamivudine, nitrification, sludge extracellular polymers substances, inhibition, emerging contaminants, HIV, ammonia-nitrogen

ACKNOWLEDGEMENT

I thank God for His love that led me through the challenges of this research programme and sustained me to complete it.

My profound gratitude goes to my supervisor, Prof Jean Mulopo who once again provided me with the insight, guidance and knowledge that saw me cross the finishing line.

This work would not have been done without the practical assistance of Thandeka Ngodela of Bushkoppie Wastewater Treatment, Johannesburg to whom I owe so much.

A special thanks to my loudest cheerleaders, my family - Patty, Stanley and Chinaka who have been my pillar of support and continues to be. Gratitude to my parents, Cyril and late Bessie (May her soul rest in peace) who persistently encouraged me to attain greater heights. My son, Chuka, has been my greatest motivation in all that I do, including this programme.

My profound appreciation to Dr Ezekiel Madigoe for his immense humanitarian support.

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LIST OF ABBREVIATIONS

3TC - Lamivudine

AE - Alkyl Ethoxylates

AES - Alkyl Ethoxy Sulphates

AMX - Amoxicillin

AMX-DKP - AMX diketopiperazine-2′,5′

AOB - Ammonia-oxidizing bacteria

APE - Alkylphenol ethoxylates

ART - Antiretroviral treatment

ARV - Antiretroviral

AS - Alkyl Sulphates

AS - Anionic Surfactants

BNR - Biological Nutrient Removal

BOD - Biochemical Oxygen Demand

CBOD - Carbonaceous Biochemical Oxygen Demand ()

CBZ - Carbamazepine

CLF - Clofibric

COD - Chemical Oxygen Demand

CS - Cationic Surfactants

CSTR - Continuous Stirred Tank Reactor

CYN - Cylindrospermopsin

DDT - Dichlorodiphenyltrichloroethane

DNA - Deoxyribonucleic acid

DOC - Dissolved Organic Carbon

DOM - Dissolved Organic Matter

EC - Emerging Contaminant

ENM - Engineered Nano Material

ERY – Erythromycin

ESI - Electron Spray Ionization

FD - Fluorescence Detector

FEN - Fenofibric

FID - Flame Ionization Detector

FNA - Free Nitrous Acid

GC - Gas Chromatography

HPLC - High Performance Liquid Chromatography

HRT - Hydraulic Retention Time

k_{biol} - Biodegradation Kinetics

LAS - Linear Alkylbenzene Sulphonate

LC - liquid chromatography

LCM - Lincomycin

MC - Microcystin

MCL - Maximum Contaminant Level

MLVSS – Mixed Liquor Volatile Suspended Solids

MPN - Most Probable Number

NIS - Non-ionic Surfactants

NNRTI - Non-Nucleoside Reverse Transcriptase Inhibitor

NOB - Nitrite-Oxidizing Bacteria

NP - Nanoparticles

NRTI - Nucleoside/Nucleotide Reverse Transcriptase Inhibitors

PPCP - Pharmaceuticals and Personal Care Products

PPH - Pharmaceutical Products and Hormone

PST – Primary Settling Tank

QAC - Quaternary Ammonium Compounds

RAS – Return Activated Sludge

RNA - Ribonucleic acid

SBR - Sequencing Batch Reactor

SCFA - Short-Chain Fatty Acid

SMX - Sulfamethoxazole

SRT	- Sludge Retention Time
SVI	- Sludge Volume Index
TC	- Tetracycline
TKN	- Total Kjeldahl Nitrogen
TP	- Transformation Product
VSS	- Volatile Suspended Solids
WMA	- Water Management Area
WS	- Waste Sludge
WWTP	- Wastewater Treatment Plant
WWTW	- Wastewater Treatment Work

RESEARCH OUTPUT

1. Obidike, L. and Mulopo, J. (2018). Effect of High Concentration of Nevirapine on the Growth of E. Coli in Wastewater Treatment. Proceedings of the World Congress on Engineering and Computer Science 2018 Vol II WCECS 2018, October 23-25, 2018, San Francisco, USA. http://www.iaeng.org/publication/WCECS2018/WCECS2018_pp532-537.pdf

2. The paper, “Evaluation of The Effects of Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) Drug (Nevirapine) on a Simulated Continuous Laboratory Scale Activated Sludge Wastewater Treatment Plant (WWTP)” has been submitted for publication in the academic journal, Environmental Science: Water Research & Technology with the manuscript ID, EW-ART-11-2019-001045.

3. The paper, “Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) Drug (Nevirapine) Time-Kill Activity at Low Concentration in A Simulated Closed Laboratory Scale Wastewater Treatment Plant (WWTP)” has been submitted for publication in the academic journal, Bulletin of Environmental Contamination and Toxicology with the tracking number, BECT-D-19-01219.

CONFERENCES ATTENDED

1. 3 – 4 September 2019: 10th Cross-Faculty Postgraduate Symposium: Showcasing Postgraduate Research at Wits

   Poster: Effect of Lamivudine on Nitrification during Biological Wastewater Treatment.

2. 2 September 2019: Wits School of Chemical and Metallurgical Engineering Research Day 2019

   Poster: Effect of High Concentration of Nevirapine on COD, Nitrate, and pH During Nitrification That Occurs in Biological Nutrient Removal of Municipal Wastewater.

3. 20 – 23 November 2018: 2018 Sustainable Sanitation Waste & Water Management Conference, Cape Town.

   Paper: Effect of Low Concentration of Nevirapine on the Growth of E. Coli in Wastewater Treatment.

4. 23 – 25 October 2018: World Congress on Engineering and Computer Science 2018, San Francisco, USA

   Paper Presented: Effect of High Concentration of Nevirapine on the Growth of E. Coli in Wastewater Treatment.

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CHAPTER 1: INTRODUCTION

1.1 BACKGROUND AND MOTIVATION

The harm that humans cause on the environment, either directly (pollution, hole in the ozone layer, acid rain, etc.) or indirectly (climatic disturbance), is an issue that has long been a concern for scientists. It is also expected that the increase in the world population, which could soon reach 9 billion, will exert more pressure on the environment. The large proportion of pollutants resulting from human activity, especially in urban areas, find their ways into water bodies, leading to significant water quality degradation. Thus, in many major cities a large panel of micropollutants are frequently found in surface water (Rehrl et al., 2020; Sousa et al., 2020; Gao et al., 2019). One of the major issues concerning micropollutants is their release into the environment through the wastewater treatment plants (WWTP), which have been long considered a major entry route for environmental contamination. In these releases, a wide range of compounds are measured in trace amounts (ng/L to μg/L) such as pharmaceutical products and hormones (PPHs), pesticides, phthalates, artificial sweeteners, chemical products, personal care (Choi et al, 2018; Chonova et al., 2018; Wang et al., 2019; Kemka et al., 2009).

These compounds are for the most part endocrine disruptors and/or toxic for humans and the environment. In this context, limiting the discharge of micropollutants into the environment has become a major preoccupation. Therefore, the contamination of WWTP effluents by micropollutants, as well as the efficiency of WWTPs to eliminate them has taken center stage particularly in today's context where the scarcity of water and the need for energy and food on global stage require that the feasibility of wastewater recycling and resource recovery be further explored (Meena et al., 2019; Lu et al., 2019). New pollutants management strategies and revised optimization strategies/pathways for WWTP processes are required in order to reduce the concentrations of new micropollutants discharges, and therefore in the environment.

Three main strategies have been broadly considered for dealing with WWTP effluents contamination namely, (i) reduction at the source, limiting/prohibiting the use or consumption of certain molecules, (ii) optimization of conventional treatment channels to improve the reductions in WWTP and (iii) the establishment of specific processes via a tertiary treatment of WWTP effluents. An additional challenge of micropollutants in sanitation is the potential export of micropollutants via sludge. Previous studies have shown that many micropollutants,

initially present in wastewater, may be adsorbed on primary and biological sludge (Guillossou et al., 2019; Coimbra et al., 2019; Kulandaivelu et al., 2020).

Emerging contaminants are substances characterized by a real or perceived threat to human health and the environment. The extent of the threat is uncertain mainly due to limited or no published scientific data elucidating their potential risk; there is no scientific basis for the development of the regulatory frameworks. These substances include, but not limited to, engineered nanomaterials (ENMs), pharmaceuticals, surfactants, pesticides, plasticizers, personal care products, endocrine disrupting compounds, and chemicals (including those in products and packaging) which eventually end up in water systems as the final sink. Most of these contaminants occur at trace levels (i.e. < 1 μg/L) and as a result, they create unique challenges for both removal processes at wastewater treatment plants and analytical detection. For instance, Kiser et al. (2009) measured 5-15 μg/L Ti (<0.7 μm) in the WWTPs effluents due to incomplete removal of ENMs (Limbach et al., 2008), and the absence of detection metrology is among the key hindrances in undertaking monitoring of ENMs in the actual environmental compartments (Maynard et al., 2006). Similar findings have been reported for other types of emerging contaminants in the aquatic environment. The increasing worldwide contamination of freshwater systems (including sources of drinking water) due to thousands of emerging industrial and natural chemical compounds is among the chief environmental problems facing humanity currently (Li et al., 2020). Although most emerging contaminants are present at low concentrations in the aquatic environments, many of them have raised considerable toxicological concerns (Nilsen et al., 2019; Bilal et al., 2020).

In the last two decades, the emission of the "emerging" or "new" unregulated contaminants has become a disproportional environmental problem, and there is widespread consensus that this form of contamination may merit urgent legislative intervention to avoid their potential long-term harm to human health and other ecological organisms (Gwenzi et al., 2018; Bwapwa and Jaiyeola, 2019; Gallo and Tosti, 2019; Brusseau and Artiola, 2019; Patel et al, 2020). The contaminants are mainly from products used in large quantities in everyday life, for example, human and veterinary pharmaceuticals, personal care products (e.g. cosmetics and sunscreens), surfactants, paints, and surfactant residues, plasticizers, and various industrial additives. Although these contaminants may be non-persistent in the environment (though for many of them, the studies are inconclusive), however, their high degree of potential transformation and removal rates are offset by their continuous introduction into the environment due to large usage volumes (Gil et al, 2019; Singh et al, 2019) and wide geographical loci of applications.

Globally and particularly in South Africa, owing to water scarcity, the partial or complete closure of water cycles is, and should be an integral part of the sustainable water-resource management. One option is by increasing the re-use of effluents for various purposes, especially within the industrial and agro/food production applications. However, because of the high cost of the end-of-pipe approach, indirect potable water re-use requires efficient treatment of wastewaters prior to their discharge. Nonetheless, it is without question, that the propensity for the contamination of freshwater will rise in the coming years because; (i) human population continues to grow, and/or (ii) patterns of natural surface water have become very low with the wastewater constituting the larger fraction of the flow. This is very true in the case of Gauteng province in South Africa, and other high notch economic hubs in South Africa. In view of these factors, among others, South Africa is particularly vulnerable as it is a semi-arid country with rapidly increasing annual population as well as industrial growth (Oberholster, 2018).

The situation is increasingly being exacerbated by additional factors like fluctuating natural seasonal flow as well as climate-change/prolonged drought that drastically alter the environmental concentrations of the emerging contaminants in water bodies due to no or low dilution. Therefore, the need for establishing the short-, medium-, and long-term effects of the emerging contaminants in the face of diminishing environmental absorption capability of the contaminants has become urgent, and of national importance. It is within this context that we seek to address this need through a scoping study and provide concise recommendations on how these challenges can be addressed via a systematic national research strategy specifically for emerging contaminants in drinking water.

Thus, the occurrence of trace organic contaminants in wastewaters, their behaviour during wastewater treatment and production of drinking water are key issues that require further investigation to highlight the need for a more comprehensive understanding of their environmental behaviour (Gil et al., 2019).

Of concern is the explosion of certain diseases in the last two decades in Africa that has introduced antiretroviral (ARV) drugs into the wastewater constituents, and in high concentrations (WHO, 2015). This is a consequence of its (ARV) use in the treatment and prophylaxis of various viral infections including influenza, hepatitis, herpes and HIV (De Clercq and Field, 2006; Kim et al., 2010; Olsen et al., 2006; Singer et al., 2006). The main pathway of ARVs into surface water bodies after excretion from the human body is through

the release of untreated or improperly treated effluents from WWTPs, hospitals and production facilities. From an African context, improper sanitation and illegal disposal of both domestic and industrial waste is another potential source of ARVs in the environment. A table of the ARVs that have been detected in environmental samples compiled by Ncube et al. (2018) indicates that the levels of ARVs in environmental water samples in Africa are higher than anywhere else. This might be related to the prevalence of the HIV/AIDS epidemic and the inherent large treatment programmes in the continent. The WHO Global Health Observatory data repository states that of the globally estimated 36.7 million people living with HIV/AIDS by end of 2016, 70% were from Africa, 10% in the South-East Asia, 9% in the Americas, 6% in Europe, 4% in the Western Pacific, and 1% in the Eastern Mediterranean (Porter et al., 2018). The estimated number of people receiving ARV treatment as of 2016 was 19.8 million. South Africa had the biggest antiretroviral treatment (ART) programme estimated at 3.9 million people receiving ARVs, about 24% of the world ART program. Even though South Africa, India and Kenya are the only countries with over a million of patients in their treatment programs, this only contributes 56, 49 and 64% of the infected individuals in those countries, respectively. In Nigeria, only 30% were on ART programmes despite having the second-highest number of people (3.2 million) living with HIV/AIDS in the world, behind South Africa. These huge numbers on ART programmes will lead to a high concentration of ARVs and their metabolites ending up in wastewaters. Worryingly, not much is known about the impact of the ARVs on the biological treatment process of wastewater and the activated sludge floc even though the success of pharmacokinetics of ARVs is recent with the first ARV drug, zidovudine only introduced in 1987 (Warnke et al., 2007). Release of untreated or less treated wastewater, as well as limited dilution of the effluents due to drought effects (low rainfall and high evapotranspiration), may be another factor. The situation is aggravated by the limited performance of available wastewater treatment processes against pharmaceuticals resulting in the release of contaminated effluents to surface water (Funke et al., 2016). The concentration of ARVs in surface water bodies of developing countries is expected to increase considering that the countries with high ART programmesdo not have treatment guidelines regarding the presence of pharmaceuticals in WWTP effluents. In addition, the lack of proper sanitation systems in certain parts of Africa could lead to the increase of ARVs in surface water due to the direct disposal of faecal matter and urine into the ground which could be carried into the rivers during the rainfall. Previous studies have also shown that zidovudine, lamivudine and nevirapine appear prominently in surface water (rivers and dams) with concentrations as high as 17 410, 167 000 and 5 620 ng L^{-1} respectively recorded in Kenya (K'oreje et al., 2016). In

WWTP effluents, these three ARVs have been recorded as high as 973, 31070 and 1357 ng L^{-1} respectively (K'oreje et al., 2016; Ngumba et al., 2016b; Wood et al., 2015). Lamivudine has the highest reported concentration in surface water (167 000 ng L^{-1}) recorded in Kenya (K'oreje et al., 2016). ARVs that have been found in drinking water are zidovudine (72.7 ng L^{-1}) in South Africa (Wood et al., 2015), darunavir (3.4 ng L^{-1}) in Poland (Giebułtowicz et al., 2018) and lamivudine (27.73 ng L^{-1}) in the USA (Furlong et al., 2017).

There has not been much research done nor written about the presence of ARVs, and those of their transformation products in surface water (Wood et al., 2015) even though some studies have shown that ARVs undergo a transformation during treatment of wastewater (Funke et al., 2016). Al-Rajab et al. (2010) showed that tenofovir is persistent in soils with no evidence of transformation products or microbial-based degradation. Prasse et al. (2015) have shown that ARVs under environmental conditions undergo both photo- and bio-transformation processes and provided evidence (Prasse et al., 2010) that conventional biological treatment followed by chemical phosphorus removal can substantially remove abacavir (>99%), lamivudine (>76%) and stavudine (>78%) from wastewater while nevirapine and zidovudine can by-pass these processes (Ncube, et al. (2018).

1.2 RESEARCH PROBLEM STATEMENT

One of the main problems of environmental sanitation is the release of micropollutants into the environment through the WWTP. These releases have long been considered an important source of environmental contamination. In these releases, a wide range of compounds is found in trace amounts, such as pharmaceutical products and hormones (PPH), pesticides, personal care products, etc. Therefore, limiting the release of these micropollutants into the environment is crucial because of the ineffectiveness of WWTP in eliminating them. The assessment of the ability of WWTP to eliminate micropollutants is essential to establish strategies and optimize treatment processes and pathways in order to reduce the concentration of the discharges into the environment. The presence of emerging contaminants in the environment raises numerous questions about risk to human health and other ecological organisms. This has led to some researchers attributing the adverse ecological effects to the presence of these compounds though there is no consensus on what forms of risks there are, if any to the human health. The lack of consensus is attributable to poor understanding of the mechanisms. For most contaminants, their mode of action in causing toxicological effects to the receptor organisms. In addition, it is only recently that the global scientific community initiated the process of

developing a better understanding of the occurrence, fate, toxicity, and transport of these emerging contaminants in the environment, including a better characterization of the human exposure via drinking water (Banik and Hossain, 2006). Presently in South Africa, there is very little or no information resource available that can provide scientific evidence to develop strategies that can address the management of these emerging contaminants both in surface and groundwater. In fact, this is yet to commence in the case of antiretroviral (ARV) drugs which are widely used in regions with high HIV infections.

In the context highlighted above, this PhD work focused on the impact and fate of two ARV emerging pollutants, namely nevirapine and lamivudine, in a selected conventional wastewater treatment system, and the potential contamination of the waste sludge (WS). This work investigated the extent to which the WWTP could eliminate nevirapine and lamivudine through an assessment of selected parameters i.e. nitrate in the wastewater effluent.

1.3 RESEARCH QUESTIONS

This research aims to address the question:

Does the influx of nevirapine and lamivudine into the sewage system derail the effectiveness of the WWTP? And what is their effect on the bacteria within the WWTP? Moreover, this work will consider the variation of selected bacterial communities i.e. e-coli in relation to the presence of nevirapine and lamivudine so that to elucidate the effects of these ARV drugs on the bacteria level, WWTP macrosystem level and on the sludge.

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CHAPTER 2: LITERATURE REVIEW

2.1 EMERGING CONTAMINANTS IN WASTEWATER

The major contaminants in wastewater are human faeces and urine, bathwater, food waste, personal and household maintenance products, and a wide range of other inorganic compounds in trace amounts (Metcalf and Eddy, 2014). Apart from the above-mentioned contaminants, for which the wastewater treatment plants were designed to remove, the presence of newly discovered contaminants, termed Emerging Contaminants (ECs), have compounded the task of treating wastewaters. ECs are compounds which have the potential to cause known or suspected adverse ecological or human health effects (Sorensen et al., 2015). They include newly synthesized substances as well as the ones that have long been present in the environment but whose presence and significance are only now being elucidated, especially their potential hazard on ecosystems and, or humans (Houtman, 2010; Daughton, 2004). This lag in the detection of ECs in the environment can be attributed to the slow development of commercial instruments that are capable of detecting very polar and highly water-soluble compounds like Pharmaceuticals and Personal Care Products (PPCPs) (Noguera-Oviedo and Aga, 2016). Their presence in various environmental compartments in water supplies have raised concern recently because they are likely to be a major threat to freshwater resources (Ellis, 2006; Prasse et al., 2010; Verlicchi et al., 2010; Watkinson et al., 2009). They are commonly derived from agricultural, industrial and municipal wastewater sources and pathways.

Researchers have attributed the presence of these organisms to the inadequacy of the wastewater treatment processes (Yu et al., 2012; Ferrer and Thurman, 2012; Luo et al., 2014; Wood et al., 2015) since the wastewater treatment plants (WWTP) are not specifically designed to remove them. Therefore, ECs are likely to be present at trace levels in effluents from WWTPs (Stackelberg et al., 2004). Barcelo (2003) gives a classification of emerging contaminants as listed in Table 2.1.

Table 2. 1 Classification of Emerging Contaminants (Barcelo, 2003).

Classes of EC	Examples
Pharmaceuticals	
Veterinary and human antibiotics	Trimethoprim, erythromycin, lincomycin, sulfamethoxazole
Analgesics and anti-inflammatory drugs	Codeine, ibuprofen, acetaminophen, acetylsalicylic acid, diclofenac, fenoprofen
Psychiatric drugs	Diazepam
Lipid regulators	Bezafibrate, clofibric acid, fenofibric acid
b-blockers	Metoprolol, propranolol, timolol
X-ray contrast media	Iopromide, iopamidol, diatrizoate
Steroids and hormones (contraceptives)	Estradiol, estrone, estriol, diethylstilbestrol
Personal care products	
Fragrances	Nitro, polycyclic and macrocyclic musks
Sunscreen agents	Benzophenone, Methyl benzylidene camphor
Insect repellents	N,N-diethyltoluamide (DEET)
Antiseptics	Triclosan, chloroprene
Surfactants and surfactant metabolites	Alkylphenol ethoxylates, alkylphenols (nonylphenol and octylphenol), alkylphenol carboxylates
Flame retardants	Polybrominated diphenyl ethers (PBDEs), Tetrabromo bisphenol A, Tris (2-chloroethyl) phosphate
Industrial additives and agents	Chelating agents (EDTA), aromatic sulfonates
Gasoline additives	Dialkyl ethers, Methyl-t-butyl ether (MTBE)
Disinfection by-products	Iodo-THMs, bromoacids, bromoacetonitriles, bromoaldehydes, cyanoformaldehyde, bromate, NDMA

2.1.1 Pharmaceuticals and Personal Care Products

The first large water quality survey conducted by the U.S. Geological Survey discovered that 80% of the 139 surface water sampled contain detectable levels of organic waste chemicals (Kolpin et al., 2002). In the qualitative survey of the drinking water undertaken by the WRC in

South Africa, they found the herbicides, atrazine and terbuthylazine, and the anticonvulsant, carbamazepine to be the major chemical determinants with the highest potential of having a negative health impact (Vosloo & Bouwman, 2005). A further extensive national survey that examined the concentration of these three chemicals in drinking water in several other metropolises showed that even the highest recorded levels of the three ECs never approached a possibly detrimental level on human health. The highest level of atrazine, terbuthylazine and carbamazepine were 163, 206, and 324 ng/ℓ, respectively. These values are well below the Maximum Contaminant Level (MCL) of 3 μg/L for atrazine and terbuthylazine, and 12 μg/L for carbamazepine (Vosloo & Bouwman, 2005). The MCL, set by the Environment Protection Agency of the USA, is a level where there is no known or expected risk to health. They had extracted the sediments through a technique called Accelerated Solvent Extraction (ASE) and the extract analyzed with high-resolution gas chromatography and mass spectrometry (GC/MS).

2.1.2 Hormones

Steroid hormones belong to the class of emerging contaminants called endocrine disruptors. They mimic or block the action of body hormones hence their presence in the environment is of great concern. Estradiol, estriol, ethinylestradiol and estrone are highly insoluble in water (Belfroid et al., 1999) which increases their persistence in water. The half-life of estrogenic steroids is estimated to be 2 – 6 days in water and sediments (Williams et al., 1999), but steroid hormones (testosterone, estradiol and progesterone) are both photo- and bio-degradable (Borch et al., 2008). Chang et al. (2011) results show that androgens and progestogens have high removal rates (91 - 100%) compared to estrogens (67-80%) with biodegradation being the major removal process in Wastewater Treatment Plants (WWTP). WWTPs are facilities in which a combination of various processes (e.g., physical, chemical and biological) are used to treat industrial wastewater and remove pollutants (Hreiz et al., 2015). When in contact with activated sludge, 17β-estradiol is oxidized to estrone whereas 17α-ethinylestradiol is persistent in aerobic conditions (Ternes et al., 1999).

Different hormones have been detected in different countries. In Western Cape, South Africa, estradiol, estriol and estrone were detected in 4 WWs with maximum concentrations of 4.7, 1.1 and 10.6 ng/l, respectively (Swart and Pool, 2007).

2.1.3 Surfactants

Surfactants comprise of a broad group of chemical compounds synthesized to exhibit tension-active properties that make them useful as a key ingredient of household and industrial detergents and in personal care products and pesticide formulations, among other applications. Surfactants consist mainly of three classes: cationic (CS), non-ionic (NIS) and anionic (AS). The commercial surfactants mostly used are linear alkylbenzene sulphonate (LAS), quaternary ammonium compounds (QAC), alkyl ethoxy sulphates (AES), alkyl sulphates (AS), Alkylphenol ethoxylates (APE) and alkyl ethoxylates (AE) (Liu et al., 2011). The presence of these surfactants in the environment has been a concern around the world, and especially in the developing countries as some of them, like APE, exhibit endocrine-disrupting properties (Kim, 2017).

APE has been detected in river waters and sediments in South Africa with concentration ranging from 0.25 to 93 ng/mL and 1.94 to 941 ng/gdw, respectively (Sibali et al., 2010). The fate of surfactants in source waters have been reported (García et al., 2001; Sun et al., 2003; Yoshida et al., 2009). Most surfactants are degradable although some may be persistent. Due to their chemical nature, some tend to adsorb onto the soil (sediments & sludge). Their sorption ability is in the order: cationic > non-ionic > anionic. The degradation of cationic surfactants (CS) is slow in river water and much slower in sediments (Ding et al., 1999; García et al., 2001). 30% was removed (through adsorption onto sediments) in a river running 3 km in 3 h (Sun et al., 2003). LAS degrade in sludge amended soil with a half-life of 7 – 33 days. LAS undergo aerobic biodegradation in both WWTP and river (3 days half-life) water and persist under anaerobic conditions. LAS was also removed by sorption onto the sludge & sediments. The higher the alkyl chain length of benzyl or methyl group, the lower the solubility of LAS and APE, hence their reduced biodegradability (García et al., 2001). APE in WWTP breaks down anaerobically into shorter chains which degrade aerobically. Nonylphenol (NP) has low solubility in water and high lipophilicity hence it tends to adsorb onto solids and bio-accumulate. Some NIS are easily degradable in both aerobic and anaerobic environment (Ying, 2006).

There is limited information on the toxicity of surfactants. Lewis (1991) found that chronic toxicity of ionic and non-ionic surfactants occurs at a concentration greater than 0.1 mg/L. In comparing the benzyl and methyl groups of CS, the methyl group was found to be more toxic than the benzyl group (García et al., 2001). Singh et al. (2002) report that CS is more toxic than

AS & NIS. EPA research has found that degradation by-products have higher toxicity than their parent compound (EPA, 1975).

Wide ranges of analytical techniques are available for the analysis of surfactants (Clara et al., 2007; Ding et al., 1999; Petrović and Barceló, 2004; Sibali et al., 2010). Gas Chromatography (GC) and liquid chromatography (LC) is the most commonly used methods for identification and quantification of surfactants in WWTP water, source water, sludge, soil and sediment. Gomez et al. (2011) analyzed non-ionic and anionic surfactant in WWTP, drinking water and seawater desalination plant water using Solid Phase Extraction (SPE) as a separating technique combined with LC Electron Spray Ionization (LC-ESI/MS). The method had a detection limit of 50 ng/L. Other methods used are Liquid-Liquid Extraction and Soxhlet Extraction for separation, and analyzed with (GC)/Flame Ionization Detector (FID) (Sibali et al., 2010) and ion-trap GC/MS (Ding et al., 1999). Clara et al. (2007) used different methods for different surfactants; LAS was analyzed by SPE-HPLC/Fluorescence Detector (FD) and nonylphenol, an APE by-product, by LC-ESI/MS/MS. Yoshida et al. (2008) used HPLC/FD.

2.1.4 Nanoparticles

Nanoparticles (NP) are usually described as particles with at least one dimension less than 100nm and are divided into natural and anthropogenic NP. Anthropogenic NP could either be synthesized (fullerenes, dendrimer, quantum dots, titanium dioxide, etc.) or incidental (sandblasting, diesel exhaust, etc.). Synthetic nanoparticles are increasingly used in everyday products, but little is known about their fate in the environment. WWTP is a major route of nanoparticles into an aquatic environment. Westerhoff et al. (2011) detected nanoparticles in both wastewater effluent and influent. Influent water TiO_2 concentration ranged from 181 to 1233 µg/L and <25 µg/L in effluent.

According to Fortner et al. (2005) and DiSalvo et al. (2008), the stability of nanoparticles in water depends on their chemical structure, water pH and temperature. Nanoparticles that are synthetically manufactured behave differently from similar but bigger particles. Some can be modified by attaching specific molecules to improve their properties. Limbach et al. (2008) and Jarvie et al. (2009) investigated the effect that surface functionality has on cerium oxide (CeO_2) and silicon dioxide (SiO_2) NP in WWTP. Both studies show that surface functionality plays an important role in removing particles from wastewater. In their investigation of the presence of silver sulphide (Ag_2S) NP in sewage sludge, Kim et al. (2010) reported that

wastewater treatment processes have roles to play in transforming nanoparticles. Ag_2S was formed from Ag particle (from production) and S (from WWTP) during the sedimentation process. Metal and metal oxides, carbon nanotubes and zeolites are some of the nanomaterials that could be used in water treatment.

2.1.5 Cyanobacteria

Cyanobacteria produce cyanotoxins which are classified into three groups:

1. Hepatotoxins: Microcystin (MC), Cylindrospermopsin (CYN) and Nodularin (N)
2. Neurotoxins: Anatoxin-a (AT), Anatoxin-a(s) (AS), Saxitoxin (ST) and Homoanatoxin-a
3. Dermatotoxins: Lipopolysaccharide, Lyngbyatoxin-a (LB) and Aplysiatoxin (AT).

MC is an important group of toxic compounds which have both acute and chronic hepatoxic effects on animals and humans (Shen et al., 2003). A provisional guideline value for drinking water of 1µg/l of Microcystin LR has been adopted by WHO and 20 µg/l for recreational activities. SA Department of Water Affairs recommends that MC concentration ranges from 0-0.8 µg/l in drinking water (London et al., 2005). Brazilian legislation for potable water recommends 3 and 15 µg/l for ST and CS, respectively (FUNASA, 2006).

The occurrence of cyanotoxins in freshwater have been reported (Brient et al., 2009; Everson et al., 2009; Makhera et al., 2010; Xu et al., 2011; Vogiazi et al., 2019; Bormans et al., 2019). Makhera et al. (2011) assessed the level of MC in Livuvhu river catchment in South Africa and found its concentration to range between 0.8 and 2 µg/l. In studies done in lakes in the North East of Germany, and in Cobaki Village Lake in New South Wales of Australia, CYN was detected (Fastner et al., 2007). In the Cobaki Village Lake, the maximum concentration of CYN detected was 38.2 µg/l (Everson et al., 2009).

In river water in France, MC and CYN were detected with concentrations of up to 0.72 µg/l and 1.55 - 1.95 µg/l, respectively (Brient et al., 2009). This concentration exceeded France maximum recommended concentration of CYN for drinking water (0.3 µg/l) (Brient et al., 2009). USGS scientists detected six types of cyanotoxins - AT, CYN, LB, MC, N and ST, in lake water with the following concentrations: MC (19000 µg/l), anatoxin-a (AT) (9.5 µg/l), CYN (0.14 µg/l), N (0.19 µg/l) and saxitoxins (ST) (0.19 µg/l) (Graham et al., 2010). Molica et al. (2005) investigated the presence of saxitoxins and anatoxin-a in Brazilian drinking waters and detected both. Hoeger et al. (2005) investigated the presence of MC in drinking water and

related water treatment plants in Germany and Switzerland and discovered that the toxin concentrations in samples from drinking water treatment plants ranged from 1.0 to >8.0 in raw water and <1.0 after treatment.

Song et al. (2007) studied the distribution and bioaccumulation of MC in water columns and toxin accumulation in 4 aquatic species, and the results show a high MC accumulation in these species. MC, CYN and N are photodegradable while MC and N biodegrade just as MC degrades in sediments (even at low conc.) but ST has shown to be persistent in the environment. Biodegradation of CYN in water bodies has been studied (Wormer et al., 2008) and no degradation of CYN could be observed during a 40-day period. Klitzke et al. (2009) studied the fate of CYN in sediments and concluded that different types of dissolved organic carbons affect CYN degradation. The presence of Dissolved Organic Carbon (DOC) released from lysed cells yield slow CYN degradation; 95% of CYN was degraded after 40 days. In Kenya, the phytoplankton in 3 reservoirs was studied. MC and endotoxin were the cyanotoxins produced by blooms during the dry season and their concentrations are reported to be well above recommended safe limits for drinking water (Mwaura et al., 2004).

MC & N have high toxicity with LD50 from 36 - 122 µg/kg in mice and rats (Dawson, 1998). (Badr et al., 2010) also illustrated the toxicity of MC daphnia and its occurrence in WWTP. CS is toxic to aquatics, damages liver, kidney, lungs, etc. In treatment, CYN can be converted to non-toxic products when controlled chlorination is applied (Falconer and Humpage, 2006).

2.1.6 Antiretroviral drugs

In general, ARV drugs are widely used in the treatment and prophylaxis of various viral infections including influenza, hepatitis, herpes and HIV (Kiso et al., 2004; De Clercq and Field, 2006; Olsen et al., 2006; Singer et al., 2006). In South Africa, they are used mostly in the treatment of HIV/AIDS. In fact, South Africa uses more antiretroviral compounds per capita than any other country. According to the World Health Organization (WHO), about 2 150 800 people received ARVs in 2012 in SA. This contrasts sharply with approximately 199 000 people on ARV therapy in Eastern Europe (WHO, 2013). In December 2014, South Africa had, with just over 3 million people on treatment, the world's largest antiretroviral therapy programme. The second largest programme was India's, with 830,707 people on treatment (WHO, 2015). With South Africa's high rate of HIV/AIDS, it is expected that a growing load of ARVs will be present in their river, having originated from the domestic sewage network

and thereafter evading the WWTP processes (Turton, 2008). Considering that the ARV drugs are relatively new in South Africa, and the WWTPs were not specifically designed to eliminate them from our wastewaters, it is hypothesized that their concentration will build up in our rivers. Since there has not been much research done nor written about their presence, and those of their metabolites in wastewater (Wood et al., 2015), there is adequate reason to become interested in this group of ECs.

For a country grappling with multi-varied spectra of social and economic challenges, it is of great concern that adequate resources might not be available to investigate and eliminate the ECs completely from our water. In their objective to develop a single LC-MS/MS method for the analysis of 12 commonly used anti-HIV compounds - Zalcitabine, Tenofovir, Abacavir, Efavirenz, Lamivudine, Didanosine, Stavudine, Zidovudine, Nevirapine, Indinavir, Ritonavir, and Lopinavir, Wood et al. (2015) took and analyzed samples from almost every major river and dam in SA. They found Nevirapine, Lopinavir and Zidovudine to be the most commonly occurring compounds. Stavudine, Nevirapine and Zidovudine had the highest averages, though their concentrations were in the low ng/L range.

2.1.6.1 Nevirapine

Nevirapine (NVP) is an antiretroviral drug in the form of white crystalline powder

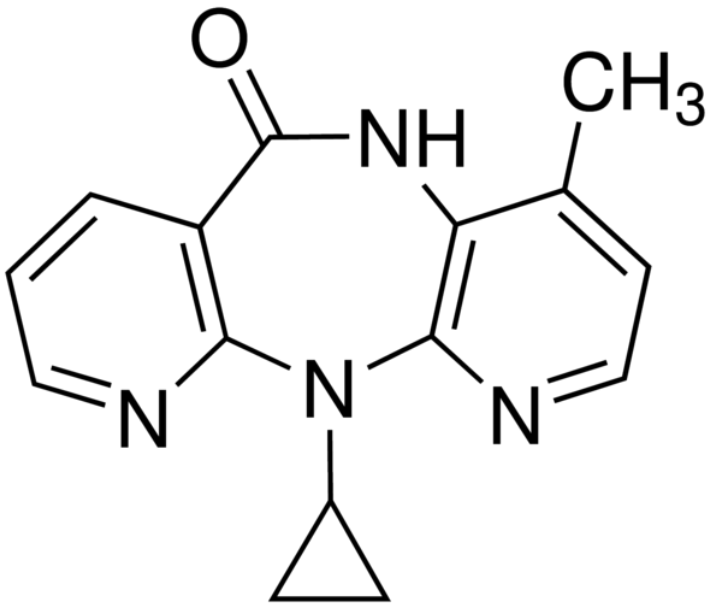


Figure 1. 1 Structure of Nevirapine (Source: Sigma Aldrich)

Having a molecular weight of 266.30 and a molecular formula, $C_{15}H_{14}N_4O$. The chemical name is 11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido [3,2-b:2', 3'-e][1,4] diazepin-6-one. It presents low aqueous solubility, which impacts its bioavailability. Structurally, nevirapine belongs to the dipyridodiazepinone chemical class. It is an oral medication used to treat and prevent retroviral infections primarily human immunodeficiency virus type 1 (HIV-1). HIV-1 is a virus that attacks mainly the CD4-T-cells responsible for the body's immune system. However, antiretroviral treatment against HIV-1 does not cure or kill the virus but rather prevents or slows down its multiplication (Deeks et al., 2013). It is generally recommended for use with other ARV medication (Saleem et al, 2019). Nevirapine is a non-nucleoside reverse transcriptase inhibitor (NNRTI) ARVD which is commonly given to pregnant women to inhibit the transfer of HIV to the unborn baby. The nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) act early in the replication viral cycle by preventing reverse transcription of the viral RNA of the HIV into its DNA, an essential step before the virus could integrate itself into the host cell. This is achieved by interfering with a viral enzyme responsible for this purpose called reverse transcriptase. Its excretion via urine is at 2.7% after ingestion (Schoeman et al., 2017; Swanopoel et al., 2015).

Log K_{ow}, the octanol/water partition coefficient is a parameter that lends insight into the behaviour of substances in solution. It is obtained when the test substance is added to N-octanol and water to determine its value. In general, compounds with log K_{ow} values lower than 3.0 are not expected to be significantly adsorbed to the particles thus exhibiting low removal efficiencies in the primary treatment (Behera et al., 2011). On the other hand, compounds with relatively high log K_{ow} values, and pKa values below the pH of the wastewater are expected to be dissociated in the aqueous phase and not bound to the particles as well (Thomas and Foster, 2005). Therefore, as Nevirapine's log K_{ow} value is 3.89 (Wood et al., 2015), which is less than 4, it indicates medium sorption potential and is less likely to bind to the primary sedimentary tank (PST) sludge. This is supported by the absence of nevirapine in the PST sludge (Evgenidou, 2015). It is thus expected that the presence of Nevirapine in wastewater will affect the nitrification process one way or the other hence will need to be eliminated completely by the biochemical process.

2.1.6.2 Lamivudine

Lamivudine (β-L-2′,3′-dideoxy-3′-thiacytidine, 3 TC), is one of the most investigated drugs in the fields of solid-state chemistry and crystal engineering with more than 47 solid forms

already discovered - 4 true polymorphs, 2 hydrates, salts, 7 cocrystals, 5 cocrystals of salts, 6 so-called duplexes and 1 solid solution (da Silva et al., 2019). It is a first-generation nucleoside reverse transcriptase inhibitor (NRTI) that was approved for the treatment of HIV-1 infection in 1995 and hepatitis B virus (HBV) infection in 1998 (EPIVIR, 2013; EPIVIR-HBV, 2016).

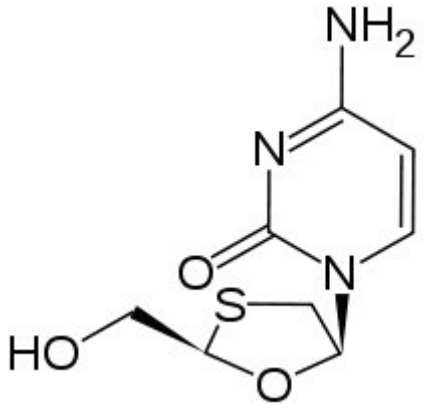


Figure 1. 2 Structure of Lamivudine

Since 2002, the World Health Organization (WHO) has been recommending treatment regimens for HIV infection, and both 3TC and emtricitabine (FTC) are the preferred components of nearly all fixed-dose combinations (Ford et al., 2017). However, Prasse et al. (2010) reported its concentration in water, 720 ng L^{-1} detected by HPLC/MS/MS (; An et al., 2011). Little is known concerning its behaviour in the environment and the fate in water environments, except that lamivudine is very stable to various forced decomposition conditions of hydrolysis (neutral), UV light and thermal stress as well as low concentration of H_2O_2 (Bedse et al., 2009; An et al., 2011). Thus, it may not easily be metabolized and can be excreted into the sewage by the human or animal metabolism though it has been reported to degrade to six products namely cytosine, uracil, lamivudine S-oxide (R-epimer), lamivudine S-oxide (S-epimer), 1-(2-(Hydroxymethyl)-1,3- oxathiolan-5-yl)pyrimidine- 2,4(1H,3H)-dione and 4-Amino-1-(2-methylene-1,3- oxathiolan-5-yl)pyrimidin-2(1H)- one (Bedse et al., 2009; Kurmi & Singh, 2017). No significant degradation was observed for the drug in neutral aqueous solutions (Kurmi & Singh, 2017).

2.2 EMERGING CONTAMINANTS IN SOUTH AFRICAN WASTEWATER

The South African Water Research Commission undertook a qualitative survey of the drinking water in two major cities in the country and found the herbicides, atrazine and terbutylazine,

and the anticonvulsant, carbamazepine to be the major chemical determinants with the highest potential of having a negative health impact (Vosloo & Bouwman, 2005). A further extensive national survey that examined the concentration of these three chemicals in drinking water in several other metropolia showed that even the highest recorded levels of the three ECs never approached a possibly detrimental level on human health. The highest level of atrazine, terbuthylazine and carbamazepine were 163, 206, and 324 ng/ℓ respectively. These values are well below the Maximum Contaminant Level (MCL) of 3 µg /ℓ for atrazine and terbuthylazine, and 12 µg/Lfor carbamazepine (Vosloo & Bouwman, 2005). The MCL, set by the Environment Protection Agency of the USA, is a level where there is no known or expected risk to health.

In South Africa, ARVs and their metabolites have joined the list of ECs (Obidike and Mulopo, 2018). Considering that there hasn't been much research done nor written about their presence, and those of their transformation products in surface water (Wood et al., 2015), there is enough reason to become interested in this group of EC. For starters, South Africa uses more antiretroviral compounds per capita than any other country. About 2 150 800 people received ARVs in 2012, which contrasts sharply with the approximate 199 000 people on ARV therapy in Eastern Europe (WHO, 2013.). Even though DDT, which is classified as a source of EC, is banned in 34 countries and severely restricted in 34 other countries, (http://www.popstoolkit.com/about/chemical/ddt.aspx), it is still being used in SA for malaria vector control.

These contaminants do not need to be persistent in the environment to be harmful to humans since their high transformation and removal rates can be offset by their continuous reintroduction into the environment (Daughton, 2004). They are commonly derived from agricultural, industrial and municipal wastewater sources and pathway. And since wastewater treatment plants (WWTP) are not specifically designed to remove these contaminants which are likely to be present in domestic and industrial wastewaters at trace levels, they are therefore likely to be present in effluents from WWTPs (Stackelberg et al., 2004).

2.2.1. Factors that exacerbate the danger of ECs in South African waters

The threat of ECs is a global problem (Kümmerer, 2009), and South Africa, like most developing countries, is in worse danger due to socio-economic constraints. Some of these factors are highlighted below.

2.2.1.1 Policies

The first large water quality survey, which reported the presence of pharmaceuticals and hormones in several U.S. streams was conducted by the U.S. Geological Survey in 2002 (Kolpin et al., 2002). Unfortunately, there hasn't been any comprehensive national survey undertaken on the presence of ECs in drinking water in South Africa (Vosloo & Bouwman, 2005). The only national survey was conducted by the Water Research Commission, a limited qualitative survey that focused only on polar, water-soluble compounds, in which they sampled drinking water in two major cities on multiple occasions (Patterton, 2013). Likewise, many of these organisms are not legislatively regulated in SA and their effects on health at different concentrations are unknown (WRC 2014). In fact, there is currently no legislation in SA to manage ECs in source and drinking water. Only recommended levels are advised on some (WRC 2014). Without a clear policy or guideline on regular monitoring of ECs, its danger is entrenched and hazardous.

2.2.1.2 Semi-arid Climate

The effects of seasonal changes on the concentration of contaminants, especially for pharmaceutical products have been highlighted by researchers (Vieno et al. 2005; Deblonde, et al. 2011). Since biodegradation and sorption have been accepted as major steps in the water treatment process, changes in temperature, precipitation rate and solar radiation have become critical criteria as they influence the number of molecules found in wastewater (Deblonde, et al. 2011). Increasing temperature causes a decrease in sorption rate but induces a more effective biodegradation process (Vieno et al., 2005; Loraine and Pettigrove, 2006). For many compounds, both will play major roles in the treatment of wastewater in South Africa where temperatures remain high for most of the year. Photodegradation rate would be high due to the high sunshine intensity in most parts of the country when solar radiation is high. Even in the normally short winter, which is usually between June and September, there is still a reasonable amount of sunshine.

With a mean annual rainfall of 450 mm which is well below the world average of 860 mm, South Africa is rightly considered a water-stressed country (Xulu et al., 2019). Like the rainfall, the country experiences high spatial variability of climate, ranging from desert in the west to sub-humid in the east. Since population density and economic activity is also spatially variable and does not follow patterns of rainfall, some water management areas (WMAs) experience

water scarcity, unlike others. Observations that over 90% of South Africa's incident precipitation remains untapped seem to indicate that the country has a surplus of available water (Backeberg and Sanewe, 2010).

Table 2.2 indicates the total rainfall in each of the country's nineteen (19) Water Management Areas (WMAs), the total yield, the user requirements and the deficit (surplus). The bulk of the country's economic activity is concentrated in the Johannesburg-Pretoria hub, Durban, and Cape Town which lie in the Crocodile (West) and Marico, Mvoti to Umzimkulu and the Berg WMAs, respectively. All these three WMAs cannot meet their requirements from within their boundaries and rely on inter-basin transfers, of which Johannesburg also relies on an international inter-basin transfer from the Lesotho Highlands Project. Although, the country currently has a surplus, it is estimated that this will be exhausted by the year, 2025. Table 2.2 shows that eleven (11) of the nineteen (19) WMAs experience physical water scarcity (WRC, 2012).

Table 2. 2 Rainfall Yields and user Requirements in the 19 WMAs (WRC, 2012)

Water Management Area	**Natural Mean Annual Rainfall (mm)**	**Available Yield (mm)**	**Total User Requirements (mm)**	**Deficit (mm)**
Limpopo	986	281	322	41
Luvuvhu/Letaba	1185	310	333	23
Crocodile West and Marico	855	716	1184	468
Olifants	2040	609	967	358
Inkomati	3539	897	844	(53)
Usuthu to Umhlatuze	4780	1110	717	(393)
Thukela	3799	737	334	(403)
Upper Vaal	2423	1130	1045	(85)
Middle Vaal	888	50	369	234
Lower Vaal	181	126	643	517
Mvoti to Umzimkulu	4798	523	798	275
Mzimvubu to Keiskamma	7241	854	374	480
Upper Orange	6981	4447	968	3479
Lower Orange	502	962	1028	(66)
Fish to Tsitsikamma	2154	418	898	(480)

Gouritz	1679	275	337	(62)
Olifants/Doring	1108	335	373	(38)
Breede	2472	866	633	233
Berg	1429	505	704	199
South Africa (Total)	**49040**	**13227**	**12871**	**(356)**

2.2.1.3 Wastewater Treatment

There is a great concern about the effectiveness of the wastewater treatment processes and plants in most cities in developing countries. Breakdowns are common and water quality suspect. This leads to a higher percentage of the wastewaters from most African cities getting returned to the streams without being treated, e.g. Nairobi and Dar-es-salaam (Table 2.3). A few cities, like Windhoek and Mbabane are the exception. Averagely, over 80% of the wastewater produced in large cities in sub-Saharan Africa are discharged in rivers or soil untreated (Nyenje et al., 2010). Table 2.3 below paints a clearer picture of the extent of the problem.

Table 2. 3 Estimated wastewater volumes in some mega-cities in sub-Saharan Africa (JMP, 1999; WHO, 2000; Nyenje, 2010)

Region	**City** (Country)	**Wastewater Production** ($10^6 m^3/y$)	**Treated** ($10^6 m^3/y$)	**Not Treated** ($10^6 m^3/y$)
East Africa	Nairobi (Kenya)	86.3	25.9	60.4
	Dar-es-salaam (Tanzania)	65.7	3.3	62.4
	Kampala (Uganda)	32.8	2.3	30.5
South Africa	Maputo (Mozambique)	31.0	7.7	23.2
	Windhoek (Namibia)	18.8	15.6	3.2
	Mbabane (Eswatini)	2.3	1.1	1.2
	Luanda (Angola)	17.5	3.0	14.5
	Lomé (Togo)	14.0	0.1	13.8

West Africa	Cotonou (Benin)	8.9	0.0	8.9
	Dakar (Senegal)	66.6	17.3	49.2

The most common immediate cause of poor water quality in the wastewater treatment plants is breakdowns of equipment and/or the delay in repairing them satisfactorily (CSIR and CIDB, 2007). Breakdowns are typically caused by an inappropriate plant, faulty operating procedures and lack of routine maintenance.

Researchers from Council for Scientific and Industrial Research (CSIR) visited water treatment works of three towns in the Northern Cape of South Africa and found that the treatment process in two of the three was not operating effectively (CSIR and CIDB, 2007). Raw water was flowing from the works into the towns' reticulation systems because, in one case, the chemical dosing system had broken down – a readily fixable problem, but beyond the ability of any of the municipal staff responsible. In the other case, poor design and construction, together probably with inappropriate operation, had led to the breakdown of the system, and the municipal staff responsible seemed not to have any idea on how to fix this problem. The third treatment works were currently delivering water to an acceptable standard, but the machinery showed signs of neglect. A year later, at least one of these works was still delivering "polluted water" (CSIR and CIDB, 2007). The processes have been adapted in some plants to accommodate the inadequacies of process units due to malfunctions hence they are not operating as designed on installation.

Table 2. 4 Specification of the quality of the wastewater to be discharged into Harrington Spruit from Bushkoppie WWTW (DWS, 2011).

Variable	**Limit**
pH	6.0 – 9.0
Electrical Conductivity	80 mS/m
Nitrates (as N)	4 mg/L
Ammonia (as N)	1.5 mg/L
Chemical Oxygen Demand	30 mg/L after removal
Typical (faecal) coli	0 (CFU/100mg/L)
Orthophosphate (as P)	0.5 mg/L
Suspended Solids	30 mg/L
Magnesium (Mg)	30
Chloride	75 (mg/L)
Fluoride	0.7

2.2.1.4 Population growth

Population growth is a major contributing factor towards the increase in the consumption of freshwater and consequent increase in wastewater turnover. Global population projections estimate that the world population of over 6 billion in 2000 will increase by 30% to 7.8 billion by 2025 (UNAID, 2015). This will put enormous strain on existing services, hence great effort will be put in securing freshwater and adequate sanitation. These increases will naturally cause an increase in pollution.

Urban-to-city drift, aggravated by the pull pressure of jobs demand due to the quest for a better standard of living, is growing daily. Immigrant population explosion from neighbouring poorer countries further compounds the population-growth problem (UNHCR, 2013). Faced with the reality of a total growth rate of 1.1% and an urban population growth rate of 1.6% for 2010 – 2015 (UNAID, 2015) being supported by dilapidating WWTPs, the danger posed by ECs in South Africa is clear.

2.2.1.5 Pesticides

As an agro-based economy, South Africa consumes huge amounts of pesticides which consequently, are expected to be found in their soil and surface waters. Long-term low-dose exposures to pesticides are increasingly thought to cause chronic health problems, including reproductive, immunological, respiratory, neurological and carcinogenic impacts (Kirkhorn and Schenker, 2002; Colosio et al., 2003; London et al., 2005). Strangely, the public health significance of pesticide pollution of water sources in South Africa has received relatively little attention from policy makers and regulators, unlike the microbiological quality of potable water. This oversight is reflected in the current drinking water guidelines in South Africa (DWAF, 1996a), which have detailed standards for inorganics and coliform content but only one standard for a pesticide, atrazine. Given that South Africa is the main market for pesticides in sub-Saharan Africa (Dinham, 1993; London et al., 2005), this is an important gap.

There are many factors that render the monitoring of pesticides in the water of poor countries difficult, and it is usually the poorest and most marginalized groups in those societies who bear the brunt of its environmental pollution (London and Rother, 1998). The high costs of analyses, and of the required analytical equipment (gas chromatography, high-pressure liquid chromatography, mass spectrometer, etc.) are crippling factors. Laboratory skills and institutional capacity available in South Africa for pesticide analyses are also in short supply (Rother and London, 1998), coupled with an absence of practical, feasible and cost-effective field monitoring protocols (Dalvie et al., 1999). The absence of a regulatory framework and water standards for and monitoring data on pesticides highlights the inadequacy of the country to address a potentially serious public health matter.

2.2.1.6 Metabolites

Parent chemicals are often excreted from the human body with many associated metabolites. As an example, ibuprofen is excreted as the unchanged drug (1%) and several metabolites: (þ)-2-40-(2-Hydroxy-2-methylpropyl)-phenylpropionic acid (25%), (þ)-2-40-(2-carboxypropyl)-phenylpropionic acid (37%) and conjugated ibuprofen (14%) (Kasprzyk-Hordern et al., 2008). However, only about 20% of ECs previously reported in UK waters were metabolites, which echoes a fact already stated in international studies (Gros et al., 2012; Hughes et al., 2012; Lopez-Serna et al., 2013). Admittedly, their determination has been restricted by a lack of available analytical reference standards (Petrie et al., 2015). The analysis of metabolites is

necessary since they can be found at concentrations much greater than the corresponding parent chemical and can also be pharmacologically active (Kasprzyk-Hordern et al., 2008). A major metabolite of carbamazepine (carbamazepine epoxide) has been found in influent wastewater at concentrations ranging from 880 to 4026 ng/l whereas the parent compound was found at <1.5 - 113 ng/l (Huerta-Fontela et al., 2010). Their release into the environment and the possibility of subsequent biotransformation and deconjugation makes their determination essential to better assess ecological risks (Petrie et al., 2015).

2.3 WASTEWATER TREATMENT UNITS

A conventional WWTP is made up of different treatment processes that ensures that dissolved and particulate pollution (colloids and suspended solids) are treated at different scales. The treatment process is generally divided into four main stages (Gallego-Schmid and Tarpani, 2019):

Pretreatment: this is a first physical treatment to remove easily separable contaminants from the water and may entail screening (coarse filtration), degreasing, and grit removal.

Primary treatment: This step allows for the elimination of particulate pollution composed of total suspended solids (TSS) and colloids. Often, this process is facilitated by the addition of coagulant and flocculant to form flocs of larger weights that settle much more easily and quickly.

Secondary or biological treatment: Primary treatment deals mostly with particulate pollution and not dissolved pollution such as carbon, nitrogen and phosphorus pollution. To remedy this, biological processes are implemented (activated sludge, biofiltration, membrane bioreactor etc.) with microbial cultures for the degradation of pollutant molecules, nitrification, and denitrification. In general, carbon is the polluting element present but in a smaller proportion compared with the needs of the bacteria. It is thus advisable to limit its consumption so that the other pollutants are also degraded. It is for that reason that the basin anoxic tank where denitrification is carried out is placed upstream in a ventilated basin where nitrification occurs. The disadvantage of this system is the need for recirculating the mixed liquor since the basin exits to the anoxic. Muds are separated from the water, purified by decantation and are recirculated at the head of the station in order to continue the treatment. Purified water can possibly undergo complementary treatments (tertiary treatments) before ejection into natural aquatic environments.

Sludge treatment: The various previous processes lead to the production of sludge, especially the primary and biological stages. If the generated wastes are not beneficiated via resources recovery or energy production, then specific treatment options must be developed.

It should be noted that there are several possible WWTP configurations (CSTR or plug flow hydrodynamics which provide different pollution abatement kinetics, the staging of used wastewater feeding to allow for better distribution of oxygen etc.). These different configurations may imply the different effect of pharmaceutical residues within WWTPs. For instance, in the case of high concentration of a residue in the WWTP feed stream, a plug flow arrangement is unlikely to reduce the effluent concentration below the specified threshold value. Some mainstream removal streams have evolved in South Africa over the years he commonly used configurations are listed below:

2.3.1 Wuhrmann Process

The Wuhrmann Process is a single sludge nitrification system followed by an anoxic zone for denitrification. The shortcoming of this system is its lack of a carbon source in the anoxic zone and the need for more alkalinity to maintain a steady pH in the aeration tank. Nitrogen gas in the clarifier inhibits settling.

2.3.2 The Ludzack-Effinger Process

The Ludzack-Effinger Process is like the Wuhrmann Process except that the positions of the anoxic and aeration tanks are interchanged. The anoxic zone is placed upstream of the nitrification reactor to take advantage of the carbon source in the influent. The nitrates formed are then introduced to the anoxic tank through the RAS. This system reduces the alkalinity demand and reduces the Carbonaceous Biochemical Oxygen Demand (CBOD) without the expense of adding air in the anoxic zone. The system is limited by the amount of nitrate returned in the RAS (Ferguson, 2019).

2.3.3 The Modified Ludzack-Effinger Process

This is the most common process used for biological nitrogen removal in municipal wastewater treatment. It relies on the nitrate formed in the aerobic zone being returned to the anoxic zone via the RAS thus increasing the nitrogen being removed. Because of this constraint, denitrification is very limited. This process has been improved through increased RAS recycle rates to prevent rising sludge in the secondary clarifiers due to denitrification (Metcalf and

Eddy, 2014) but it introduces dissolved oxygen in the anoxic zone which dilutes the substrate and reduces the performance (Gupta, 2018).

2.3.4 Four-Stage Bardenpho Process (Barnard, 1974)

The Four-stage Bardenpho process contains two anoxic zones to achieve a high level of total nitrogen removal. The Bardenpho process uses an anoxic basin followed by a standard BOD/ nitrification process, but no clarification. Mixed liquor is returned to the front of the process instead of RAS. Another extended anoxic basin is used to reduce the dissolved oxygen and create denitrification (Emaraa et al., 2014). The effluent passes to an aeration basin where the gas stripping process occurs and then to clarifiers. The RAS is returned to the head of the process. The main disadvantage of the Bardenpho process is the extended detention time in the two anoxic processes (Stensel et al., 2000).

2.3.5 Five-Stage Bardenpho Process

The Five-stage Bardenpho-process consist of the anaerobic tank in front, a pre-denitrification/ nitrification step with internal recirculation followed by an anoxic tank, and a final aeration step in order to avoid nitrate transfer to the anaerobic tank by the return sludge (Emara et al, 2014). It was developed to overcome incomplete denitrification of the MG/LE system and ensure nitrogen, phosphorus and carbon removal. The low nitrate concentration discharged from the aeration zone would be denitrified in a secondary anoxic zone placed after the aerobic zone. This delivers a relatively nitrate-free effluent. Prior to discharge to the clarifier, a flash aeration basin was introduced after the second anoxic zone to strip the nitrogen bubbles from the sludge flows to assist with the sedimentation of the sludge. The flash aeration also served to nitrify any ammonia released within the anoxic zone. This process has the theoretical propensity to remove all the nitrate, but in practice, this is not always possible. Instead, it achieves high P-elimination and is operated with very low loadings. Since the nitrates in the RAS are typically low (1-3 mg/L), they do not have the potential to significantly interfere with the phosphorus removal process (Emara et al., 2014). The main disadvantage of the Bardenpho process is the extended detention time in the two anoxic processes (Stensel et al., 2000). Numerous WWTP of this type operates in South Africa and North America.

2.3.6 Oxidation Ditches

The configuration of the Oxidation ditch is like Wuhrmann process and by changing the location of the aerators and mixers the oxidation ditch can operate like the Bardenpho process or the Modified Ludzack-Ettinger process. It can perform biological nitrogen removal by creating an anoxic zone in parts of the ditch. The oxidation ditch can create high recycle rates without the need for pumping the return.

2.3.7 University of Cape Town (UCT) Configuration (Rabinowitz and Marais, 1980)

The UCT-Process was developed at the University of Cape Town to avoid the remnant nitrate in the return sludge, as in the Bardenpho and Phoredox processes, impeding the release of phosphorus in the anaerobic tank. The main difference to the Bardenpho process is that the return sludge is fed to the anoxic pre-denitrification tank from where the denitrified activated sludge is returned to the anaerobic tank. Its major difference with the A^2O process is that it has three recycle streams, instead of two, but they share a similarity of the internal recycles feed, NO_x, being directed to the anoxic zone from the aerobic zone.

2.3.8 Modified University of Cape Town (UCT) Configuration (Rabinowitz and Marais, 1980)

The modified UCT process is like the original UCT process except that a second anoxic tank is introduced next to the first anoxic tank. This second anoxic tank receives the return of nitrate-rich MLSS from the end of the aerobic zone for denitrification.

2.3.9 Phoredox (Barnard, 1974)

The Phoredox-process is a simplification of the Bardenpho process but because of the low reduction rate in the second anoxic tank and the aeration tank, these steps were omitted. Nitrification does not occur to any appreciable extent in this process as it is designed with low aerobic SRT values, from 2 to 3 d at 20^{o}C and 4 to 5 d at 10^{o}C (Kroiss et al., 2011). At warmer climates, like in South Africa, avoidance of nitrification is difficult. The phosphorus removal is appreciable, but nitrification causes a decline in phosphorus removal.

2.3.10 Johannesburg (JHB) Process (Burke et al., 1986)

This process, which originated in Johannesburg, South Africa is a modification of the Phoredox process. Return sludge will be denitrified in a separate anoxic tank where it will have sufficient detention time to reduce the nitrate in the mixed liquor before it is fed to the anaerobic zone. Therefore, the input of the anaerobic tank is the inflow and nitrate-free return sludge.

2.3.11 Anaerobic/Oxic (A/O) Process

The A/O configuration is a modification of the Phoredox process with nitrification and denitrification and was developed in South Africa in 1974. Compared to the Phoredox process, the tanks are constructed as cascades and their order is identical. The detention time in the anoxic zone is 1 to 3 h, depending on the wastewater characteristics and the amount of nitrate to remove (Metcalf and Eddy, 2014). It is used where ammonia removal is not required so nitrates are not being returned to the anaerobic zone. Nitrate is recycled from the aerobic zone to the anoxic zone for denitrification. This process is operated in some plants in the US and Brazil.

2.3.12 Anaerobic/ Anoxic/ Oxic (A^2O) Process

This is a variant of the Anaerobic/ Oxic configuration but with the anoxic zone introduced between the anaerobic and aerobic zones. Nitrate rich MLSS is returned from the end of the aerobic zone to the anoxic zone for denitrification prior to RAS being returned to the anaerobic zone.

2.3.13 Biodenipho (Krüger, n.d.)

Two circular aeration tanks are operated with alternating nitrification and denitrification. Wastewater flow is added during the periods of denitrification where aeration is stopped. Thus, wastewater of the first activated sludge tank will be denitrified while the second activated sludge tank will be aerated and therefore nitrification will occur. As soon as all nitrate in the not aerated tank will be denitrified, the inflow and the aeration changes. Thus, the created nitrate of the aerated tank will be denitrified and the available ammonia in the aerobic tank will be oxidized. An upstream situated anaerobic tank enables a biological P-elimination.

In practice, the Bardenpho system for nitrogen removal is appropriate if the calculated effluent nitrate is less than 5 to 7 mg/L. But if greater than 5 to 7 mg/L (which is usually the case for

$^{TKN}/_{COD}$ ratios > 0.1 mg N/mg COD) then the MG/LE process is better suited for higher nitrogen removal efficiency.

2.3.14 Extended Anaerobic Sludge Contact (EASC) (Schoenberger, 1989)

This process was developed to implement biological P-elimination to existing WWTP. The primary sedimentation tank is used as an anaerobic reactor. Return sludge is fed to the sedimentation tank where anaerobic conditions enable sedimentation of primary sludge and RAS (Return activated sludge). Therefore, the residence time of the sludge extends and leads to an acidification of the raw wastewater. This acidification leads to improvement of the substrate quality for P-storing microorganisms. The runoff of the sedimentation tank and settled sludge will be fed to the anoxic tank together with the sludge recycled from the aerated nitrification tank.

2.3.15 ISAH (Austermann-Haun, 1998)

The ISAH is an approved process under unfavourable conditions (low temperature, dilution by external water or low substrate concentrations). RAS will be denitrified in a separate anoxic tank, which inhibits a possible disturbance of phosphate re-dissolution. Thus, the easily degradable wastewater inflow is fully available to phosphate-rich microorganisms.

2.3.16 Sequencing Batch Reactor (SBR) (ATV, 1997)

Sequencing Batch Reactor (SBR) perform all the necessary functions of nutrient removal in a single tank with variable water levels and timed aeration. This system requires a minimum of three tanks and advanced automation equipment to control the cycle times and phases. The control systems allow the operation to be configured to operate as almost any other suspended growth reactor by adjusting the cycle phases between fill, mix, aerated, settle and decant (Metcalf and Eddy, 2014). Compared to the continual flow processes, SBR works with only one tank in time sequences. One cycle passes the steps of filling (anoxic), filling and mixing (anaerobic), aeration (aerobic), sedimentation and removal of the treated effluent. Full-scale experience shows that Bio P-removal can be achieved with many aeration tank configurations where anaerobic zones or cascades occur and irrespective of whether the treatment efficiency is high i.e. with nitrification or only BOD-removal is achieved. Especially for treatment plants with low treatment efficiency (low sludge age), it must be considered that in secondary

clarifiers, anaerobic conditions can occur which will cause a release of stored phosphate to the effluent.

2.4 REMOVAL OF MICROPOLLUTANTS BY CONVENTIONAL WASTEWATER TREATMENT SYSTEM

The major objectives of biological treatment of domestic wastewater are to transform (oxidize) dissolved and particulate biodegradable constituents into acceptable end products, capture and incorporate suspended and non-settleable colloidal solids into a biological floc or biofilm, and to transform or remove nutrients, such as nitrogen and phosphorus. In some cases, specific trace organic constituents and compounds are removed (Metcalf and Eddy, 2014). For industrial wastewater, the removal or reduction of the concentration of the constituent organic and inorganic compounds is the main objective. Pretreatment is sometimes necessary since some of its constituents are toxic to microorganisms.

The nutrients that result in rapid eutrophication are introduced into waters through human activities. The concentration of these nutrients can be limited from point sources by biological trickling filters or activated sludge systems. Both systems utilize naturally occurring bacteria resident in the waters. The bacteria in these systems utilize the nutrients for growth and in this way, the nutrients pass from the liquid phase into the solid phase and are concentrated in the biological culture. Although both systems are effective in removing carbon, it is only the activated sludge works that can be designed to remove nitrogen and phosphorus from wastewater streams effectively. Biological Nutrient Removal (BNR) is the process of wastewater treatment by which carbon, nitrogen, and phosphorus are removed from the wastewater using microorganisms that are resident in the wastewater (Guimarães et al., 2020).

2.4.1 Carbon Removal

Carbon is the fourth most abundant element on earth and is very stable. Humans are 18 % carbon by volume. In wastewater, carbon occurs as organic and inorganic compounds and is measured using a test for BOD which represents the amount of oxygen consumed during the biochemical oxidation of the organic matter. The organic forms can be utilized by heterotrophs and inorganic compounds by autotrophs. Both forms of carbon are removed from the wastewater through a series of redox reactions, oxidizing the carbon source to carbon dioxide

and water. The carbon dioxide then escapes to the atmosphere, removing carbon from the waste stream.

Carbonaceous material in wastewater can be split into biodegradable and non-biodegradable forms. Each form has two fractions, namely soluble and particulate. The relative fractions of each constituent can vary considerably with different types of wastewater. The non-biodegradable material is not broken down within the treatment process. Generally, the non-biodegradable particulate material becomes enmeshed in the sludge, settles out in the sedimentation tanks and is removed from the system with the waste sludge. The non-biodegradable soluble fraction passes through the treatment process and is discharged with the effluent, sometimes giving rise to high effluent concentration. The biodegradable carbonaceous material is broken down in the treatment process by heterotrophic organisms under aerobic conditions (oxygen present) in an aeration basin. The soluble biodegradable material is rapidly used by the organisms (and is thus called readily biodegradable COD) whereas the particulate fraction is rapidly adsorbed but assimilated more slowly (and is thus called slowly biodegradable COD).

In the chemical reactions of the breakdown of carbonaceous material, electrons are transferred and accepted in redox reactions. The two main reaction paths within these reactions are termed the catabolic and anabolic pathways. In the catabolic pathway, a fraction of the organic molecules is taken up by the organism and oxidized to carbon dioxide and water. Associated with these reactions is the release of a large amount of energy. A small amount of this energy is captured by the organism and can be utilized e.g. for cell growth while the remaining energy is lost as heat.

The anabolic pathway is the pathway by which the organisms construct new cell mass i.e. grow. A small fraction of the organic molecules taken up in the catabolic pathway is modified to form part of the cell mass. These two cycles put together form the metabolism of the organism and in this manner, energy is removed from the wastewater. Under aerobic conditions, the amount of energy released in the anabolic and catabolic pathways is proportional to the mass of oxygen utilized for cell growth, which in mm can also be related to the number of electrons donated in the oxidation of organic compounds.

In order to determine the energy content of wastewater, and consequently the oxygen requirements for carbon removal, two main tests are used. These are the 5-day Biochemical Oxygen Demand (BOD) test and the Chemical Oxygen Demand (COD) test.

2.4.2 Nitrogen Removal

Nitrogen in wastewaters is one of the main nutrients of concern. It can be subdivided into two main forms: free and saline ammonia, and organically bound nitrogen. The organically bound nitrogen can be subdivided further into non-biodegradable and biodegradable, both forms having soluble and particulate fractions. Generally, wastewaters do not contain any nitrate or nitrite in the influent.

Nitrogen is characterized by the Total Kjeldahl Nitrogen (TKN) and the free and saline ammonia tests. Biodegradable organic nitrogen is broken down into free and saline ammonia within a sludge age of approximately 3 days. Non-biodegradable particulate nitrogen is generally settled out in sedimentation tanks and removed with the waste sludge stream. The non-biodegradable soluble nitrogen passes through the treatment system and is discharged with the effluent. The first step in the nitrogen removal process is called nitrification. In this process free and saline ammonia obtained from the breakdown of organic nitrogen is oxidized to nitrite (NO_2^-) and then nitrate (NO_3^-) in the presence of oxygen. The groups of organisms responsible for the oxidation are termed autotrophic organisms. These organisms behave differently compared to the carbon-removing organisms, the heterotrophs.

Two specific autotrophs perform the task of removing nitrogen from wastewater. *Nitrosomonas* converts free and saline ammonia to nitrite and *Nitrobacter* converts nitrite to nitrate. The oxygen requirement associated with this conversion amounts to 4.57 mg oxygen/mg N utilized. The rate of conversion of ammonia to nitrite by *Nitrosomonas* is much slower than the conversion *of* nitrite to nitrate by *Nitrobacter*. Therefore, the rate-limiting step is due to *Nitrosomonas.* The specific growth rate of these organisms is much slower than the growth rate *of* the heterotrophic organisms and consequently, the sludge age of a particular plant must be greater than the minimum time required for these organisms to multiply and survive within the system.

The nitrifying organisms are sensitive to the pH and alkalinity of the wastewaters. Their growth rate is severely inhibited outside the pH range of 7 to 8.5. During the conversion of ammonia to nitrate, hydrogen ions are released resulting in a decrease in the alkalinity of the wastewater.

Stoichiometrically, for every 1 mg of (NH_3-N) convened to nitrite or nitrate. 7.14 mg of alkalinity (as $CaCO_3$) is destroyed. If the alkalinity of wastewater drops below 40 mg/L (as $CaCO_3$), the pH becomes unstable resulting in a sharp decrease in nitrification efficiency due to the retarded growth rate of the autotrophs. Low pH values can also adversely affect the settling ability of the sludge and produce a corrosive effluent.

The disadvantages of nitrification discussed above can be partially overcome by the second step of the nitrogen removal process called denitrification. In this process, the nitrates from nitrification are reduced to nitrogen gas. This series of biological redox reactions take place in an anoxic zone and is the only zone in a treatment process in which substantial nitrogen removal is achieved. An anoxic zone implies that there are nitrite and nitrate present but no oxygen. Within this zone, the nitrite and nitrate formed in the aerobic zone are reduced to nitrogen gas which escapes to the atmosphere. The nitrite and nitrate serve the same function as oxygen within the aeration basin, i.e. as an electron/ hydrogen ion acceptor.

From stoichiometric relationships, when nitrate acts as an electron acceptor. 1 mg NO, as N is approximately equivalent to 2.86 mg O (as O) if oxygen was the terminal acceptor. If one thus combines the nitrification and denitrification processes, up to approximately 63% of the oxygen demand for nitrification can be recovered if complete denitrification is achieved. Even if denitrification is not complete, any amount *of* denitrification will result in some "oxygen recovery". Besides oxygen recovery, the effect of nitrification on pH and alkalinity can be reduced as during the denitrification step, for every mg of nitrate denitrified to nitrogen gas.

2.4.3 Phosphorus Removal

Phosphorus is an element in all living things even though it is not found in elemental form and is also very unstable. The typical municipal wastewater has a total phosphorus concentration of about 5 – 9 mg/L (Bashar et al., 2018). It consists mainly of two fractions - a soluble orthophosphate (PO_4^{3-}) and organically bound phosphorus which may be soluble or particulate in form. The orthophosphate ranges from 70 to 90 % of the total phosphorus. The organically bound phosphorus is converted to orthophosphate in the activated sludge process (Ekama and Marais, 1984). Phosphorus can be removed from wastewater by either biological means or by chemical precipitation. Oftentimes, chemical removal is used in conjunction with biological removal.

2.4.3.1 Biological removal

It is generally accepted that enhanced P removal occurs because of the ability of certain organisms, for example, *Acinetobacter* spp. to accumulate large quantities *of* polyphosphate (poly-p) within the cellular mass. The secret to designing and running an activated sludge plant successfully for P removal lies therefore in creating conditions in the plant which favour propagation and growth of these particular organisms (poly-P organisms) over organisms which do not have this propensity (non-poly- P organisms). In order to create conditions for the growth of poly-P and non-poly-P organisms, a plant must have three distinct zones: anaerobic, anoxic and aerobic zones.

Under aerobic conditions, the poly-P organisms are not able to compete with non-poly-P organisms for substrates (food sources) such as glucose or other saccharides. Under anaerobic conditions, and in the presence of short-chain fatty acids (SCFA), the poly-P organisms break down or hydrolyze stored polyphosphate. This process releases ortho-phosphate to the surrounding liquid. The bond energy released in hydrolyzing polyphosphate is utilized by the poly-P organisms to absorb, process and store the SCFA within dying organisms, thereby reserving substrate for their exclusive use when they enter an environment which contains external electron acceptors such as nitrate or oxygen. In this way, they do not have to compete with the non-poly-P organisms which are unable to utilize SCFA under the anaerobic conditions because of the lack of a suitable electron acceptor. When re-entering the aerobic environment, the poly-P organisms utilize the reserved SCFA both for growth and to replenish their poly-P pool by abstracting ortho-phosphate from the surrounding medium. This gives rise to the phenomenon known as excess P uptake which occurs in aerobic environments.

To promote the growth of these poly-P organisms, one needs:

(i) to create an anaerobic environment which receives or generates an adequate supply of SCFA: the mass of P released (and subsequent uptake in the aerobic zone) being proportional to the mass of SCFA obtained by the Poly-P organisms, followed by

(ii) an aerobic environment for P uptake by the Poly-P organisms,

Normally very little SCFA is present in the influent in South Africa. SCFA are generated in the anaerobic reactor by non-Poly-P acid fermenting organisms in the activated sludge mass acting on the influent sewage COD. However extensive experimental investigations have

shown that in the anaerobic reactor only the readily biodegradable fraction of the influent COD, the RBCOD fraction, is converted to SCFA. In South Africa, this fraction ranges around 20 per cent of the unsettled influent COD. It has been shown that the rate of generation of SCFA by the non-poly-P organisms within the anaerobic zone is a first-order reaction with respect to the RBCOD and the non-poly-P heterotroph active mass concentration and is therefore promoted if the anaerobic zone is subdivided into a series of two or more sub-zones. The rate of uptake (sequestration) of SCFA by the poly-P organisms usually is faster than the rate of generation of SCFA by the poly-P organisms so that usually no SCFA are measurable in the liquid phase in this zone.

In the activated sludge process, SCFA is very rapidly biodegraded by the non-poly-P organisms in the presence of an external electron acceptor such as oxygen or nitrate, at a rate much faster than the poly-P organisms can utilize SCFA.

Furthermore, in the presence of an external electron acceptor, the non-poly-P organisms will not generate and release SCFA from the RBCOD but will use the RBCOD. Therefore, in order to preserve and generate the SCFA in the anaerobic zone for the sole use of the poly-P organisms, great care has to be taken to minimize the introduction of oxygen and nitrate into that zone. If high concentrations of nitrate, for instance, are introduced into the anaerobic zone in the S-recycle, non-poly-P organisms will use the nitrate as an electron acceptor to metabolize the SCFA and RBCOD thereby reducing the supply of SCFA to the poly-P organisms and detrimentally affecting the biological P removal. It is thus critical to the process that as little oxygen and nitrate as possible is introduced into the anaerobic zone.

2.4.3.2 Chemical removal

As P can also be precipitated chemically in either a side-stream process (e.g. Phostrip) or in a main-stream process (e.g. in trickling filters) or in a process combining biological and chemical removal, it is necessary to discuss P removal mechanisms induced by chemical precipitation. The phosphates found in wastewater occur in three principal forms: orthophosphate, polyphosphate and organic phosphate. In the biological treatment process, most of the phosphates and converted to orthophosphate, which is the easiest form of P to precipitate chemically. The chemicals commonly used for phosphate precipitation are iron and aluminium salts and lime.

2.4.4 Advantages of Biological Nutrient Removal

Biological Nutrient Removal (BNR) of nitrogen and phosphorus has been widely used in wastewater treatment practice to control eutrophication in receiving water bodies because of its demonstrated economic and operational benefits. Their utilization is potentially more economic than conventional activated sludge treatment or physical/chemical processes. Its advantages are listed below:

a. Enhanced savings in chemical costs.
b. It does not add significantly to the salinity of receiving water.
c. The long sludge ages required by the process produce sludges which are not odorous.
d. It produces sludges suitable as soil conditioners if the wastewater does not contain excessive heavy metals.
e. Some of the alkalinity and oxygen used during nitrification is recovered in the nitrification process.

2.4.5 Disadvantages of Biological Nutrient Removal

Despite its numerous advantages and popularity, BNR has equally countering disadvantages, which are listed below:

a. The efficiency of the process is influenced by the characteristics of the wastewater.
b. The phosphorus-rich biological sludge wasted from the system will release phosphorus back into the liquid stream if it is not treated correctly.
c. The use of unaerated zones creates the need for relatively long solids retention times (sludge ages) in the biological reactors to ensure nitrification in winter, resulting in large biological reactors.
d. Anaerobic digestion of phosphorus-rich biological sludges in the presence of magnesium can result in struvite precipitation causing blockages in digester pipework. Unfortunately, the maximum concentration of magnesium to avoid precipitation is not known.
e. It requires greater skill from the operating staff.

2.5 WASTEWATER TREATMENT PLANTS & EMERGING CONTAMINANTS

Emerging pollutants are new products or chemicals without regulatory status and whose effects on the environment and human health are unknown. Many articles have reported their presence in wastewater and aquatic environments (Hirsch et al., 1999; Bendz et al., 2005; Castiglioni et al., 2006; Hummel et al., 2006; Verenitch et al., 2006; Huerta-Fontela et al., 2007; Terzić et al., 2008; Shao et al., 2009; Nelson et al., 2010; Evgenidou, 2015), and WWTPs are one of the pathways through which these compounds enter the environment (Evgenidou et al., 2015). Increasing evidence indicates possible adverse impact on the target organisms due to long-term and low-dose exposures to pharmaceuticals in the environment, including chronic toxicity, endocrine disruption, antibiotic resistance, as well as toxic effects on reproduction of terrestrial and aquatic organisms (Fent et al., 2006; Allen et al., 2010). Their removal at WWTPs is a complex process which may be only partial with many plausible mechanisms (Evgenidou, 2015).

Their removal efficiency can be effected either by the specific treatment processes employed (biological or chemical) in individual WWTPs or by the physicochemical characteristics of the pollutants treated, such as water solubility, tendency to volatilize, or to adsorb onto activated sludge, and degradation half-life by abiotic and biotic processes (Gulkowska et al., 2008; Behera et al., 2011; Luo et al., 2014). These physicochemical characteristics, which is their ability to interact with solid particles is a major factor because it facilitates their removal by physical-chemical (settling, flotation) or biological processes (biodegradation). Thus, compounds with low adsorption coefficients tend to remain in the aqueous phase, which favours their mobility through the WWTP and into the receiving waters (Ohlenbusch et al., 2000). Generally, compounds with the octanol/water partition coefficient (log K_{ow}) values lower than 3.0 are not expected to be adsorbed significantly to the particles thus exhibiting low removal efficiencies in the primary treatment (Behera et al., 2011) while compounds with relatively high log K_{ow} values and pK_a values below the pH of the wastewater are expected to be dissociated in the aqueous phase and not bound to the particles as well (Thomas and Foster, 2005).

The removal efficiency of PPCPs, IDs and their TPs is controlled by the operational and environmental conditions of the WWTPs. The most important of the operational conditions are: (a) the hydraulic retention time (HRT), (b) sludge retention time (SRT), and (c)

biodegradation kinetics (k_{biol}), while some of the major environmental conditions are (a) the temperature with lower efficiencies reported during winter periods and colder climates (Vieno et al., 2005; Gros et al., 2012; Verlicchi et al., 2012), (b) redox conditions (different removal efficiencies have been observed for anaerobic, and aerobic conditions), and (c) pH conditions effecting the degradation kinetics of the compounds (Suárez et al., 2008; Gros et al., 2012; Verlicchi et al., 2012).

In his investigation, Perez-Parada et al. (2011) identified four hydrolysis products of amoxicillin (AMX) antibiotic - two diastereomers of amoxilloic acid ((5S)-AMXO and (5R)-AMXO) and two diastereomers of AMX diketopiperazine-2′,5′ ((5R)- AMX-DKP and (5S)-AMX-DKP, respectively). All four TPs of AMX were detected in both influents and effluents of a WWTP in Spain thus indicating that complete removal of these target compounds after treatment in the WWTP was not possible.

A maximum value of 6000 ng/L for erythromycin-H_2O (ERY-H_2O) was detected by Hirsch et al. (1999) at WWTP effluents in Germany and their removal efficiency in WWTPs varies. According to McArdell et al. (2003), ERY-H_2O and macrolide antibiotics in general, cannot be eliminated completely in WWTPs, with removal rates of up to 19% and between 49 and 80%, respectively have been determined in WWTPs in Hong-Kong (Gulkowska et al., 2008) and Wisconsin, USA (Karthikeyan and Meyer, 2006). The secondary treatment with a longer hydraulic retention time of the sewage appeared more effective in reducing ERY-H_2O concentrations compared to the primary treatment (Evgenidou, 2015).

According to Gulkowska et al. (2008), concentrations of ERY-H_2O in some WWTPs in Hong-Kong were greater in the effluents than in the influents. Possible explanations for this aberration include (i) deconjugation of conjugated metabolites during the treatment process (Miao et al., 2002); (ii) an underestimation of the actual amount due to particulate matter with adsorbed ERY-H_2O being filtered out during sample preparation; and (iii) a change in the adsorption behaviour of the ERY-H_2O to particles during treatment processes, influencing the ratio between influent/ effluent water (Lindberg et al., 2005; Gulkowska et al., 2008). Xu et al. (2007) observed the same phenomenon and attributed it to the fact that particulate matter with sizes greater 0.45 mm was not included leading to an underestimation of the amount entering the WWTPs. Moreover, a signal suppression of the MS/MS detector in raw effluent samples due to high concentrations of organic matter could also be responsible for the lower concentrations in WWTP influents (Xu et al., 2007).

The hydroxylated derivative of the antibiotic metronidazole (METR-OH) was identified by Santos et al. (2013) in hospital effluents, and in WWTP influents and effluents in Portugal. In hospital effluents, its concentration was as high as 11 μg/L while it ranged at lower levels (n.d. –158 ng/L) in the influents in WWTP.

The lipid regulators, Clofibrate and etofibrate are hydrolysed within the body to yield the metabolite clofibric acid. Clofibric acid (CLF acid) was frequently detected in municipal, industrial and hospital wastewaters with variable concentrations. In Portugal, Salgado et al. (2011) detected concentrations that reached 41.4 μg/L. Discrepancies between countries were also observed depending on the prescription rates of the parent compounds. For example, CLF acid was not detected in the Norwegian samples since its parent compounds clofibrate and etofibrate are not prescribed at all in Norway (Weigel et al., 2004). It is also known to be present in rivers and ground or tap water (Lapworth et al., 2012; Leung et al., 2013). No definitive conclusion could be reached on the removal rates of CLF acid as the removal efficiencies depend on the specific treatment processes adopted in each WWTP and the operational regime of the concerned WWTPs. Ternes (1998) achieved a 15% removal rate using trickling filter treatment, 34% using activated sludge treatment and 51% in an activated sludge system using ferric chloride. However, Stamatis and Konstantinou (2013) achieved a 64% removal after the secondary treatment and 74% after tertiary treatment with chlorine and sand filtration. Behera et al. (2011) reported 76% removal after the secondary treatment in S. Korea while Salgado et al. (2012) exhibited very high removal (75%) after biological treatment in Portugal and very low removal by adsorption on activated sludge since only negligible amounts of CLF acid remained undegraded (after biological removal) and could be found adsorbed to the sludge. Roberts and Thomas (2006) achieved a 91% removal after tertiary treatment with chlorine disinfection in the UK.

Fenofibric acid (FEN acid), the TP of fenofibrate was detected in WWTPs in Spain with concentrations that ranged up to 349 ng/L by Rosal et al. (2010) and by Martínez Bueno et al. (2007). Rosal et al. (2010) also indicated that FEN acid was detected in every sample although its precursor was only detected in one sample and among other compounds had very low removal efficiency (1.3%) after the secondary treatment.

Acetylsalicylic acid is rapidly deacetylated in the body to form the active metabolite, Salicylic acid (SA) which was consistently detected in all samples - WWTP influents, effluents and in surface water samples collected near WWTP effluents (Verenitch et al., 2006). The influent's

concentration ranged up to 89.1 μg/L (Kosma et al., 2010) while the effluent's concentrations were up to 6825 ng/L (Kosma et al., 2014). However, Cruz-Morato et al. (2013) observed a 46% increase in the concentration of SA after treatment due to possible deconjugation of glucuronide metabolites during biological treatment. On the other hand, Lee et al. (2005) have found very low concentrations of SA in the effluents of the WWTPs although it was one of the most abundant acidic pharmaceuticals in the influents. They attributed this finding to the instability of the compound in sewage samples (Lee et al., 2003), thus presenting a removal of more than 90%. Stamatis and Konstantinou (2013) and Kosma et al. (2014) exhibited also high removal efficiencies of 85% and 70–100% respectively, after the secondary treatment, in agreement with Henschel et al. (1997) who demonstrated that SA presents high biodegradability with very high elimination rate after the secondary treatment. Moreover, Stamatis and Konstantinou (2013) demonstrated a total removal rate of 90% after tertiary treatment that was attributed mostly to chlorination since sand filtration proved inefficient due to its ionized group or to its low hydrophobicity.

Rosal et al., 2010 achieved a 17% efficiency for sulfamethoxazole (SMX) while Peng et al., (2006) achieved 98%. For tetracycline (TC), Spongberg and Witter (2008) recorded 12% while Karthikeyan and Meyer, 2006) got 80%. The removal of erythromycin-H_2O (ERY) by Rosal et al., (2010) improved from 4.3% to 72% while Miao et al., (2005) had less than 30% for carbamazepine (CBZ). Karthikeyan and Meyer, (2006) recorded 17% for lincomycin (LCM).

Meanwhile, pharmaceutical residues were frequently detected in biosolids at μg/kg to mg/kg levels (Gao et al., 2012). An average concentration of 68 μg/kg dry weight (dw) for SMX in activated sludge was reported by Göbel et al. (2005), and about 15 μg/kg dw for TC in biosolids was shown by Spongberg and Witter (2008). Moreover, SMX concentrations as high as 1020 μg/L was reported to present in surface water and groundwater (Lindsey et al., 2001).

2.6 EFFICIENCY OF CONVENTIONAL WASTEWATER TREATMENT PROCESSES IN REMOVING EMERGING CONTAMINANTS IN WWTP

Release of pharmaceuticals into the environment has received a lot of attention in recent years (Gao et al., 2012) and WWTPs is one of the pathways through which these compounds enter the environment (Evgenidou et al., 2015). A significant number of these trace level compounds

have been frequently detected both in aqueous (wastewater, drinking water, surface water, and groundwater) and solid (sludge, soil, and sediments) samples (Christian et al., 2003; Göbel et al., 2005; Kim and Carlson, 2007; Kümmerer, 2009). Increasing evidence indicates possible adverse impacts to the target organisms due to long-term and low-dose exposures to pharmaceuticals in the environment, including chronic toxicity, endocrine disruption, antibiotic resistance, as well as toxic effects on reproduction of terrestrial and aquatic organisms (Fent et al., 2006; Allen et al., 2010). Their removal at WWTPs is a complex process with many plausible mechanisms and may only be partial (Evgenidou, 2015).

2.6.1 Elimination by biodegradation

Because of measurement challenges and variability of the effluents and sludge, the effectiveness of WWTPs to remove emerging contaminants is still not well established. This is also compounded by the fact that the removal pathway varies depending on the molecules concerned. While most studies have shown that biological breakdown may be the major pathway for the removal of emerging contaminants (Bilal et al., 2019), others stipulate that emerging contaminants adsorption on sludge is likely the principal cause of the reduction in their concentration in water (Gulkowska et al., 2008; Behera et al., 2011). Therefore, the coefficient of sorption k_d in a particular WWTP system will determine the speed of emerging contaminant removal based on the concentration. That also implies that the configuration of the WWTP system is important, i.e. the plug flow configuration system may be more effective for removal via sludge adsorption than a perfectly agitated system.

Even though there are various groups of microorganisms in the activated sludge, it is unlikely that pharmaceuticals present in wastewater as microcontaminants can be effectively removed by biodegradation alone for three reasons (Wang, 2009). Firstly, compared with other pollutants in wastewater, pharmaceutical contaminants have relatively low concentration, which may be insufficient to induce enzymes that are capable of degrading pharmaceuticals. Secondly, many of the pharmaceutical contaminants are bioactive and this can inhibit the metabolism of microorganisms. As a result, it is impossible to use pharmaceutical contaminants as a favourable carbon or energy source by microorganisms. Thirdly, the nature of each compound and the operating condition of wastewater treatment plants will influence the performance of biodegradation.

Joss et al. (2006) used activated sludge to investigate the biodegradation of 25 pharmaceuticals, hormones and fragrances, including antibiotics, antiphlogistic, contrast agent, lipid regulator,

and nootropics, in batch experiments at typical concentration levels in municipal wastewater treatment. They indicated that only a few compounds, which were ibuprofen, paracetamol, 17β-estradiol, and estrone, could be degraded by more than 90%, while half of the target compounds were removed by less than 50%.

In general, WWTPs do not regulate the feed wastewater to the system and therefore are subjected to varying daily hydraulic shear forces and pollution loads. In WWTPs, the bacteria responsible for the decomposition of pollutants are in the form of flocs. Two types of bacteria are normally found in WWTPs namely the filamentous and nitrifying bacteria. The filamentous bacteria play a significant role in the structure of the flocs of which they form the "backbone" whereas the nitrifying bacteria, although present in minorities, facilitate the oxidation of ammoniacal nitrogen. Moreover, exopolymers secreted by these bacteria play also a role in the structure of the flocs, and their physicochemical properties.

2.6.2 Filamentous bacteria

Filamentous bacteria are normally present in the activated sludge in small amounts but proliferate under specific conditions (Kotay, 2011). Low phosphorus availability favours their growth in activated sludge treatment plants, which results in bad settling and the thickening of the sludge (Figueroa et al., 2015; Kroiss et al., 2011). In huge volume, they impede the treatment plant performance, causing formation and structuring of flocs which leads to sludge bulking or foaming (Yao et al., 2019; Ziegler et al., 1990) and end up degrading the soluble Biological Oxygen Demand (BOD). Impairment of the thickening properties of the sludge can be used as a sensitive indicator of phosphorus deficiency which strongly affects treatment efficiency. Excess dosing of phosphorus can result first in filling the P-storage capacity of the microorganisms and therefore will not immediately be detected by rising P-concentrations in the effluent (Nowak et al., 2000).

Biomass bulking in activated sludge processes is one of the main operational problems in activated sludge systems (Kotay, 2011). From wastewater treatment plants, several filamentous bacteria have been identified, isolated, and found responsible for biomass bulking viz. *Microthrix parvicella, Sphaerotilus natans, Eikelboom type 1702, Haliscomenobacter hydrossis, Nocardia, Thiothrix spp., Fungi, Acinetobacter, Beggiatoa, Chryseobacterium spp., Leucothrix mucor,* etc. (Figueroa et al., 2015; Metcalf and Eddy, 2014) Biomass bulking causes poor settling in the secondary clarifier and allows the unsettled biomass to escape with the effluent. Engineering manipulations are conventionally employed to solve the problem of

biomass bulking without understanding the relationship between the specific microorganisms and their role in filamentous bulking. In efforts toward controlling the biomass bulking, physicochemical methods like manipulation of flow rates of the return activated sludge, increased aeration, the addition of flocculants/coagulants and oxidants have been tried in the past (Xie et al., 2007). Surfactants and chlorine are also used to control the filamentous bulking in different attempts (Caravelli et al., 2007; Seka et al., 2003 and Xie et al., 2007). For various reasons, most of the above-mentioned attempts at mitigating filamentous bulking are not acknowledged as sustainable and/or cost-effective.

The sludge volume index (SVI) is often used to distinguish between good and bad biomass settling. SVI value of between 50 and 100 is considered good, but between 100 and 150 is filamentous growth and above 150 is bulking (Lee et al., 1983). In order to control the growth of these problematic bacteria, they need to be identified. However, identification based on morphotypes is known to be frequently inadequate for the reliable identification of many filamentous bacteria, since one morphotype can embrace several phylogenetically different organisms (Nielsen et al., 2009).

Haliscomenobacter hydrossis is a sheathed filamentous bacterium that has been detected worldwide in activated sludge samples because of its easily recognizable morphological appearance: rigid straight filament, length between 10 and 200 mm, diameter between 0.3 and 0.5 mm extending from the floc surface (thin needle shape appearance) and Gramnegative staining (Eikelboom, 2006). It has been suggested that they are surrounded by a translucent sheath which could protect them from antibiotics. When the bacteria leave their sheath, they can move thanks to their whips and can form another filamentous chain elsewhere.

2.7 NITRIFICATION AND NITRIFYING BACTERIA

The most common forms of nitrogen in wastewater are ammonia (NH_3), nitrogen gas (N_2), nitrite ion (NO_2^-), and nitrate ion (NO_3^-). Nitrite is very important in wastewater studies because it is extremely toxic to most fish and other aquatic species. Nitrite nitrogen is relatively unstable and is easily oxidized to the nitrate form. In wastewater, it seldom exceeds 1 mg/L and can be oxidized with chlorine. Nitrate nitrogen is the most oxidized form of nitrogen in wastewaters (Metcalf and Eddy, 2014). The South African Department of Water and Sanitation wastewater specification limits it to 4 mg/L (DWS, 2011). Nitrates concentration in wastewater may vary from 0 to 20 mg/L but if complete nitrification had taken place, its typical concentration in

effluents is 15 – 25 mg/L. Nitrous oxide (N_2O) is a greenhouse gas with an impact that is about 300 times that for CO_2 (Van Zwieten et al., 2015). NO_2 emanates mostly from agricultural activities but the combustion of fossil fuels and manufacturing of nylon also contributes greatly (Maier et al., 2009). It can be photolytically converted to nitric oxide. Internal combustion engines and power stations are major sources of nitrogen dioxide (NO_2).

Fresh wastewater nitrogen is primarily mixed with proteinaceous matter and urea but decomposition by bacteria readily changes this organic form of nitrogen to ammonia Jatana et al., 2020). Thus, the relative amount of ammonia present in wastewater gives an indication of the age of the wastewater. In the presence of abundant dissolved oxygen, bacteria can oxidize the ammonia nitrogen to nitrites and nitrates. The predominance of the nitrate indicates the stability of the wastewater with respect to oxygen demand (Tchinda et al., 2019). But then, nitrates can be used by plants and animals to form protein while death and decomposition of the plant and animal protein by bacteria will yield ammonia. The need, therefore, arises to reduce or remove the nitrogen in the wastewater in order to control these growths and possible eutrophication.

2.7.1 Nitrification

Ammonia is a very water-soluble colourless gas with a strong pungent odour that is easily liquefied and solidified. High concentrations of ammonia are toxic to humans and animals, hence the need to remove it from wastewaters. Untreated volumes of ammonia in wastewater can disperse in the air and harm those who have inhaled it, causing serious illnesses and skin disorders (Hazen and Sawyer, 2007).

Nitrification is a two-step biological process in which one type of autotrophic bacteria, *Nitrosomonas*, oxidize the ammonia in the water to nitrite and the second type of autotrophic bacteria, *Nitrobacter*, oxidize nitrite to nitrate (Carroll et al., 2005; Metcalf and Eddy, 2014) as shown in Equations 2 and 3, respectively. But the nitrates still acted as an aquatic fertilizer that created algae problems and results in a reduction of dissolved oxygen (DO) in the receiving waters (Emara et al., 2014). In a second step, nitrate is converted to gaseous nitrogen (Zhu et al., 2008) and is removed from the wastewater.

$$2NH_4^- + 3O_2 \xrightarrow{\text{Nitrosomonas}} 2NO_2^- + 4H_2O + 4H^+ + \text{NEW CELLS} \quad (2)$$

$$2NO_2^- + O_2 \xrightarrow{\text{Nitrobacters}} 2NO_3^- + \text{NEW CELLS} \quad (3)$$

Nitrite is an intermediate in both nitrification and denitrification. The partial nitrification of ammonia to nitrite has been named nitritation, and the subsequent direct reduction of nitrite to N2 gas, denitritation (Jenicek et al., 2004). The application of nitritation–denitritation could lead to a considerable saving in aeration costs and external carbon sources as compared to the complete nitrification-denitrification (Zhu et al., 2008; Fux et al., 2006). For the treatment of domestic wastewater, nitritation and denitritation are particularly significant because the organic carbon source in it is typically limiting (Zeng et al., 2003).

Denitrification is known to proceed as conversion of nitrates to nitrites and subsequent conversion of nitrites to nitric oxide, nitrous oxide and nitrogen gas (Sincero and Sincero, 2002). It is impacted by the amount of biodegradable organic compound present (Metcalf and Eddy, 2014) and it is generally taken to be an anaerobic process as some of the reductase reactions are inhibited in intact cells by the presence of oxygen. However, there are indications that certain organisms may escape this control by oxygen under some conditions (Ferguson, 2019). The reaction rates are slow, being limited by the slow growth rates of the bacteria involved (Metcalf and Eddy, 2014). They are also not very efficient as yields are typically low.

2.7.2 Nitrifying bacteria

The diversity of specific bacterial groups in activated sludge is crucial in maintaining the stability of the wastewater treatment system as it influences the functioning of the reactor (Daims et al., 2001). Ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) are aerobic chemoautotrophs because they use CO_2 for their carbon source and require dissolved oxygen to oxidize any inorganic compound (NH_4^-N or NO_2^-N) to obtain cell energy (Metcalf and Eddy, 2014). Since ammonia oxidation to nitrites is the limiting step in nitrogen removal from wastewater, ammonia-oxidizing bacteria (AOB) are of universal importance in activated sludge technologies (Zielinska et al., 2012). The growth rate of AOB is slow and the activities of their population are strongly affected by operational conditions.

Many researchers have shown that Nitrosomonas was common in activated sludge systems (Enfrin et al., 2019) while Geets et al. (2006) showed that the Nitrosospira-related strain dominates the AOB population (Metcalf and Eddy, 2014). Park and Noguera (2004) showed that at a low DO (0.12–0.24 mg/L), the AOB community in activated sludge was dominated by members of the *Nitrosomonas europaea* lineage, whereas at high DO (up to 8.5 mg/L), members of the *Nitrosomonas oligotropha* lineage were prevalent. However, with the passage

of time the AOB community in the high-DO reactor shifted from the *Nitrosomonas oligotropha* lineage to the *Nitrosomonas europaea* lineage without loss of nitrification efficiency. These considerations suggest that AOB lineages include species showing different affinities for oxygen.

The rate of conversion of ammonia to nitrite, by the *Nitrosomonas,* is much slower than that of nitrite to nitrate, by the *Nitrobacter* (Ekama and Marais, 1984). Often, any nitrite that is formed is converted virtually immediately to nitrate. Consequently, very little nitrite is observed in the effluent from a plant operating on an influent that does not contain substances that inhibit the *Nitrobacter.* Thus, the limiting rate in the nitrification sequence is that due to the *Nitrosomonas*. Because the nitrite produced is virtually immediately converted to nitrate in terms of the growth rate of the *Nitrosomonas,* it can be assumed that the conversion is from ammonia to nitrate directly. On this basis, the kinetics of nitrification reduce to the kinetics of the *Nitrosomonas* (Ekama & Marais, 1984).

All bacteria need oxygen for respiration. While aerobic bacteria get their oxygen from dissolved oxygen in the water, anaerobic and facultative bacteria get oxygen by stripping oxygen from sugars, starches, and sulfates when no DO is present, and subsequently releasing CO_2, CH_4, and H_2S. Denitrifying bacteria are facultative and can use oxygen from nitrates. Denitrification, which follows the nitrification process utilizes denitrifying bacteria to remove the oxygen from the nitrate compounds and convert them into nitrogen gas (N_2) which effectively removes the nitrogen from the effluent by aeration (Benisch et al., 2007; Emara et al., 2014), as shown in Equation (4) below.

$$NO_3 + BOD \longrightarrow N_2 + CO_2 + H_2O + OH^- + CELLS \quad (4)$$

But for them to use this chemically bound oxygen, an anoxic condition must be created - the DO must be less than 0.1 mg/L (Benisch, 2007).

2.7.3 Inhibition of the autotrophic bacteria and the nitrifying bacteria

Recent researches have shown that the risk for WWTPs for not meeting the effluent standards in relation to the variations of the composition of the influents is larger when considered from nitrogen than DCO perspectives (Zeng, 2010). Nitrification can thus be regarded as a major bottleneck in activated sludge WWTP processes. It has been shown that the difference between the speed of growth and the speed of mortality is weaker for the nitrifying bacteria than for the

heterotrophic bacteria (Pomeroy et al., 2001.). Thus, for the same mortality between nitrifying bacteria and heterotrophic bacteria caused by poison at a time t, nitrification would be affected longer (Zielinska, et al., 2012).

Nitrite formation occurs in both nitrification and denitrification. The partial nitrification of ammonia to nitrite has been named nitritation, and the subsequent direct reduction of nitrite to N_2 gas, denitritation (Jenicek et al., 2004). For the treatment of domestic wastewater, nitritation and denitritation are particularly significant because the organic carbon source in it is typically limiting (Zeng et al., 2003).

In order to achieve nitritation–denitritation, the oxidation of ammonia to nitrite needs to be controlled instead of the oxidation of ammonia to nitrate, and the build-up of nitrite. Therefore, the nitrite-oxidizing bacteria (NOB) have to be inhibited or eliminated while ammonia-oxidizing bacteria (AOB) have to become the dominant nitrifying bacteria (Hellinga et al., 1998). The factors affecting nitrite build-up and the community structure such as higher temperature (Hellinga et al., 1998; Yoo et al., 1999), higher free ammonia (FA) and free nitrous acid (FNA) concentration (Vadivelu et al., 2007; Peng et al., 2008; Park and Bae, 2009), inhibitor (Grunditz et al., 1998), low dissolved oxygen (DO) concentration (Ruiz et al., 2006; Blackburne et al., 2008; Guo et al., 2009), control of hydraulic retention time (HRT) or aeration time (Peng et al., 2007; Gao et al., 2009; Zeng et al., 2009) have been investigated. Natural temperatures of domestic wastewater vary in the range of 10–25℃ with the seasons. In this temperature range, high nitrite build-up is difficult to maintain because the specific growth rate of NOB is higher than that of AOB (Hellinga et al., 1998). The FA and FNA concentrations in domestic wastewater cannot reach levels that are inhibitory to NOB. Consequently, control of both DO concentration and HRT seems to be the only available method to establish nitritation. AOB has a stronger affinity to oxygen than NOB at low DO concentrations, which causes nitrite build-up as well as savings in aeration cost (Garrido et al., 1997; Pollice et al., 2002).

2.7.4 Exopolymers

Exopolymers (EPS) is a self-produced, biopolymeric network that provides the three-dimensional (3-D) architecture. (De Beer and Stoodley, 2013; Flemming, 2011). They are high molecular weight secretions from the micro-organisms to which the residues of cellular lysis and hydrolysis of macromolecule are added. The major components in EPS are carbohydrates and the proteins though organic matter can also adsorb itself on it. The main role of biofilm EPS is to provide cell-to-cell scaffolding, while securing irreversible attachment of the entire

consortium to the surface as “bio-anchorage” (De Beer and Stoodley, 2013; Flemming, 2011; Flemming and Wingender, 2010; Nadell et al, 2009). The term EPS is frequently ascribed to sessile microbial biofilms. (De Beer and Stoodley, 2013; Flemming and Wingender, 2010; Stoodley et al., 2002; Nadell et al, 2009).

It is very broad and refers to any extracellular dissolved organic matter (DOM) released by prokaryotes or eukaryotes (Bhaskar and Bhosle, 2005; De Beer and Stoodley, 2013; Flemming, 2011). The released material usually includes a mixture of polysaccharides, proteins, lipids, and nucleic acids. (Bhaskar and Bhosle, 2005; Flemming, 2011; Decho, 2010; Herzberg, 2009; Flemming and Wingender, 2010). Divalent cations (mainly Ca^{2+}) initiate multiple cross-links between the dissolved polysaccharide chains to form the semi-stable gelatinous network characteristic of EPS (Flemming, 2011; Decho, 2010; de Kerchove, and Elimelech, 2008). Hydrophobic interactions, which drive the spontaneous association of apolar hydrophobic solutes in water may also be involved in the formation of these gels (Verdugo et al., 2004; Ding et al., 2007).

2.8 FACTORS THAT INHIBIT ACTIVATED SLUDGE IN WWTPs

The inhibition of the activated sludge WWTPs by emerging contaminants have also been the subject of recent researches. In most of the studies, synthetic wastewaters were used. Successful wastewater treatment operations depend on the knowledge and control of the parameters that affect its performance. Inefficient systems increase the cost of operation and might render the process uneconomical (Sharma & Ahlert, 1977). The rates of nitrification reaction are highly dependent on some environmental factors. These include the substrate and oxygen concentrations, temperature, pH, and the presence of toxic or inhibiting substances. Parameters affecting nitrification are summarized next.

2.8.1 Substrate concentration

In most cases, the rate of the reaction is limited by the first step, the oxidation of the ammonia. At steady state, the amount of NO_2^- present is usually small and can be ignored (Ekama and Marais, 1984).

2.8.2 Temperature

The effect of temperature changes in wastewater is critical as it is multifaceted. Although it greatly affects the rate of nitrification, it is a parameter that is most difficult to control from an

operational point of view (Sharma & Ahlert, 1977). Nitrification reactions follow the van't Hoff-Arrhenius law up to 30℃ (Metcalf & Eddy, 1973). Thus, nitrification proceeds better in warmer seasons or climates because nitrifying bacteria are temperature sensitive. Wastewater is usually warmer than the local water supplies due to warmer waters emanating from households and industries. With an increase in the rate of reaction due to the increase in temperature and considering that oxygen is less soluble in warmer water, depletion of dissolved oxygen in the water endangers aquatic life. It also deters some species of fish that prefer colder receiving waters.

The growth constants of nitrifying bacteria are affected greatly by temperature (Sharma & Ahlert, 1977), though rapid changes in temperature do not produce rapid changes in growth rates. A slow adaptation period, with a lower than the expected growth rate, follows such changes. The overall optimum temperature for the growth of nitrifying bacteria appears to be in the range 28-36℃, although optimum temperatures up to 42℃ have been reported for *Nitrobacter* (Painter, 1970; Sharma & Ahlert, 1977). Knowles, Downing & Barrett (1965) have estimated that the temperature coefficient (increase in maximum specific growth rate constant) for *Nitrosomonas* was 9.5% per ℃. The temperature coefficient for *Nitrobacter* was found to be about 5.9% per ℃. However, literature values for a relative change in nitrifier growth rate or activity for a given change in temperature vary considerably (Focht & Chang, 1975; EPA, 1975; Wong-Chong & Loehr, 1975). In measuring the effect of rapid temperature changes on nitrification reactions via batch studies on a marine nitrifying filter system, Srna & Baggaley (1975) revealed that a 4℃ increase in temperature caused about a 50% increase in the rate of nitritification; a I℃ drop caused a 30% decrease when compared with calculated values at 21.3℃. A similar increase in temperature was reported to result in a 12% increase in the rate of nitratification, while a 1.5℃ decrease lowered the rate by 8%. Barritt (1933) found that the thermal death point of a pure culture of *Nitrosomonas* was between 54 and 58℃. Little or no growth of nitrifying bacteria is expected below 4℃ (Buswell *et al.*, 1954; Painter, 1970; Sharma & Ahlert, 1977).

2.8.3 Dissolved Oxygen Concentration

Nitrifying bacteria are particularly sensitive to low oxygen concentrations since they utilize oxygen during the oxidation reactions (Sharma & Ahlert, 1977). Therefore, providing adequate oxygen in wastewater treatment plants is critical for increased efficiency of the process. Nitrification rates increase up to DO concentrations of 3 to 4 mg/L (Metcalf & Eddy, 2014).

However, high DO concentrations, up to 33 mg/L do not appear to affect nitrification rates significantly. Low oxygen concentrations, however, do reduce the nitrification rate (Ekama & Marais, 1984). According to the equations of the oxidizing reactions below,

$$NH_4^+ + 1.5O_2 \longrightarrow 2H^+ + H_2O + NO_2^- + 58\text{-}84 \text{ kcal} \qquad 2.8.1$$

$$NO_2^- + 0.5O_2 \longrightarrow NO_3^- + 15.4\text{-}20.9 \text{ kcal} \qquad 2.8.2$$

3.43 mg of oxygen is required for the nitrification of 1 mg NH_3-N (Sharma, 1977).

Stenstrom and Poduska (1980) have suggested a formula to relate DO to the growth rate as follows:

$$\mu no = \mu nmo \frac{O}{Ko + O}$$

where

O - oxygen concentration in bulk liquid (mgO/l)

K_o - half saturation constant (mgO/l)
μ_{nmo} - maximum specific growth rate (/d)
μ_{no} = specific growth rate at mgO/L oxygen(/d).

The value attributed to K_o has ranged from 0.3 to 2 mg O/l, i.e. at DO values below K_o, the growth rate will decline to less than half the rate where oxygen is present in adequate concentrations. The wide range of K_o probably has arisen because the concentration of DO in the bulk liquid is not necessarily the same as inside the biological floc where the oxygen consumption takes place. Consequently, the value will depend on the size of floc, mixing intensity and oxygen diffusion rate into the floc. Furthermore, in a full-scale reactor, the DO will vary over the reactor volume due to the discrete points of oxygen input (with mechanical aeration) and the impossibility of achieving instantaneous and complete mixing. For these reasons, it is not exactly possible to establish a generally applicable minimum oxygen value - each reactor will have a value specific to the conditions prevailing in it. In nitrifying reactors, with bubble aeration a popular DO lower limit, to ensure unimpeded nitrification, is 2 mg O/l at the surface of the liquid.

Under cyclic flow and load conditions, difficulties of ensuring an oxygen supply matching the oxygen demand and a lower limit for the DO arises. Equalization of the lead probably presents

the best practical means to facilitate control of the oxygen level in the reactor. In the absence of equalization, mitigation of the adverse effects of low oxygen concentration during peak oxygen demand periods is by having sludge ages significantly longer than the minimum necessary for nitrification.

2.8.4 pH

pH is a measure of the hydrogen-ion concentration in a solution. The specific growth rate of the nitrifiers, μ_n is extremely sensitive to the pH of the culture medium (Ekama and Marais, 1984). Hence, pH has a strong effect on nitrification rates. The reactions occur fastest in a pH range of 7.5 - 8, but ammonia oxidation rate declines significantly at values below 7 (Metcalf and Eddy, 2014). It seems that the activities of both the hydrogen ion (H^+) and hydroxyl ion (OH^-) act inhibitorily outside this range. This happens when the pH increases above 8.5 [increasing (OH^-)] or decreases below 7 [increasing (H^+)]. If the bacteria exist in flocs or films, the pH at the cell surface will be lower than the bulk pH due to the production of H^+ ions. The exact mechanisms by which pH affects the reaction rates are not fully understood, although Anthonisen (1974) believed that inhibitions, particularly from the neutral NH_3 and HNO_2 species become important. Anthonisen postulated that when the intracellular pH of a nitrifying organism is lower than the pH of the extracellular environment, free ammonia (FA) will penetrate the cell membrane. Ionized ammonia (NH_4^+) is postulated to remain in the extracellular environment. Similarly, when intracellular pH is higher than that of the extracellular environment, free nitrous acid (FNA) permeates the cell, not nitrite ion. Anthonisen proposes that the ability of FA and FNA to penetrate the nitrifying organism makes them more inhibitory than ammonium and nitrite ions (Sharma & Ahlert, 1977).

Suzuki et al. (1974) examined the effect of pH on the oxidation of ammonia by *Nitrosomonas cells as* well as extracts and found that FA, rather than ionized ammonia, is the substrate for *Nitrosomonas.* Similarly, O'Kelley et al. (1970) presented data suggesting FNA as the substrate for the nitrite oxidase system of *Nitrobacter.*

2.8.5 Inhibiting Substances

Many substances can potentially inhibit nitrification reactions, especially metals. Skinner and Walker (1961) have shown complete inhibition of ammonia oxidation at 0.25 mg/L nickel, 0.25 mg/L chromium, and 0.10 mg/L copper. When exposed to more than one inhibitor, the extent of inhibition increases greatly.

According to Anthonisen (1974), the degree of inhibition of nitrification in substrate and product inhibition depends upon the ammonia-ammonium and the nitrite-nitrous acid equilibria. Other researchers (Prakasam & Loehr, 1972; Boon & Laudelout, 1962) support the suggestion that this aspect of inhibition is due to free ammonia and undissociated nitrous acid. Normal ammonia and nitrite ion concentrations in domestic wastewaters (Schwinn & Dickson, 1972) are not in the inhibiting ranges. However, substrate and product inhibition are of significance in the treatment of industrial, poultry and agricultural wastes (Sharma & Ahlert, 1977).

2.8.6 Flow symmetry and loading

It is proven that cyclic flow and load conditions of the wastewater decreases the efficiency of nitrification. From simulation studies, even though the nitrifiers are operating at their maximum rate during high flow, it is extremely difficult to oxidize all the ammonia available, hence an increased ammonia concentration is discharged in the effluent. This reduces the concentration of nitrifiers formed during the process. This adverse effect due to cyclicity becomes more emphasized as the amplitude of the flow and load cycles increase. It is amended as the aerobic sludge age increases. Simulation studies of the cyclic effect have indicated that an approximate relationship can be established between the effluent ammonia concentration under steady-state and that under cyclic conditions (van Haandel et al., 1981).

2.8.7 Effect of surfaces and turbulence

It is not clear whether the presence of surfaces, surface type, or the level of turbulence play a significant role in nitrification. DeMarco *et al.* (1967) concluded that high turbulence causes an increase in the initial rate of ammonia oxidation as compared to low turbulence though no measurements of dissolved oxygen concentration, pH, or the *degree* of turbulence were included, and did not determine whether the ammonia removed was recovered as nitrite or nitrate. Some effects of surfaces and/or turbulence are quite possible in nature, though. Finstein & Matulewich (1974) observed that interface muds contained about 450 times as many ammonia oxidizers and 256 times as many nitrite oxidizers as did surface waters on a volumetric basis. Tuffey (1973), in an intensive field study of nitrification in streams and estuaries, found that shallow streams had luxuriant surface colonies. Collins (1969) recommended surface muds of stratified lakes as a good source of nitrifiers. It is generally agreed that in the sea, also, nitrifying bacteria are found in sediments (Vargues & Brisou, 1963, cited in Painter, 1970). Sims & Little (1972) reported that the number of nitrifiers on the zeolite

particles used in an aeration unit was ten times greater than when zeolite was not used. Curtis et al. (1975), as part of a study on nitrification in the Trent Basin of England, estimated that at least 80% of the ammonia oxidation occurred in the sediments. However, as observed by Finstein & Matulewich (1974) in their study, it is difficult to say whether nitrifiers, enumerated by the most-probable-number (MPN) method in the laboratory, were actually active while in the sediments.

2.8.8 Concentration of nitrifiers

The number (or mass) of nitrifying organisms influences the rate of nitrification observed (Downing, 1968; Downing & Knowles, 1970; Eckenfelder, 1970). There is a paucity of quantitative information on this point, perhaps due to difficulties in enumerating nitrifiers (Finstein, 1968). The concentration range of sewage samples, as estimated by the most-probable-number technique, is 1,000-10,000 per ml (Strom et *al.*, 1976). Much of the available data, with some exceptions, is on the basis of volatile suspended solids (VSS) (Sharma & Ahlert, 1977).

DeMarco *et al.* (1967) reported that the initial rate of ammonia oxidation increased with increasing initial seed concentration in VSS. The time required for a given degree of ammonia removal was inversely proportional to the concentration of nitrifiers present. Similarly, Metcalf & Eddy (2014) found that the time to completely nitrify a given amount of ammonia nitrogen per gram of mixed-liquor VSS (MLVSS) was constant, given the same environmental conditions (MLVSS studied range from 800-6000 mg/L).

The rate of nitrification depends on the ratio of ammonia-to-nitrite oxidizers and probably does not remain first-order with respect to nitrifier concentration. Typical values for the rates of nitritification-nitratification, expressed as mg N oxidized/g cells/h at 20°-30°C, are in the range of 110 - 250 for pure cultures and 0.5 - 6 for activated sludge-type systems (EPA, 1975), although rates up to 20-25 have been observed in one case (EPA, 1975). Reports by Srinath *et al.* (1974) and Wong-Chong & Loehr (1975) bear the first point out. With a reactor treating mineral salts media with mixed nitrifying populations, Wong-Chong and Loehr ascertained the ammonia oxidation rate (in mg N/I/h) to have a Monod-type relationship, with the nitrifier concentration (in VSS mg/l). The data of Srinath *et al.* for pure cultures, seem to follow a similar behaviour. The influence of nitrifier concentration on nitrification has an added significance in the case of the biochemical oxygen demand (BOD) test (Olenik, 1974).

Table 2. 5 Concentrations of some substances which inhibit the activity of nitrifying bacteria in activated sludge (Tomlinson et al., 1966)

Molar concentration inhibiting oxidation by 75 %		
Substance	**Ammonia**	**Nitrite**
Hydrazine sulphate	2×10^{-3}	1.5×10^{-3}
Sodium azide	3.6×10^{-4}	2.2×10^{-4}
Sodium arsenite	1.3×10^{-2}	5×10^{-2}
Sodium cyanide	3.5×10^{-3}	5.7×10^{-5}
Sodium cyanate	2.5×10^{-3}	2.5×10^{-3}
2-4 dinitrophenol	2.5×10^{-3}	2.2×10^{-3}
Dithio-oxamide	1.5×10^{-5}	3.5×10^{-4}
Methylamine	2.3×10^{-2}	5×10^{-2}
Trimethylamine	2×10^{-3}	4.3×10^{-3}
Potassium chromate	3.5×10^{-3}	2.8×10^{-2}
Potassium chlorate	2×10^{-2}	2×10^{-3}
Nickel sulphate	4×10^{-4}	5×10^{-3}

In the table above, sodium cyanate inhibited ammonia by 40% while sodium arsenite and methylamine inhibited nitrite by 65% and 50%, respectively.

2.8.9 Effect of light

There has been some suggestion in the literature and backed by positive results of investigations, that light might inhibit the activity of nitrifying bacteria. Warington (1878) found that nitrification proceeded more rapidly in cultures placed in a dark cupboard than on an open bench (Meiklejohn, 1954). Ulken (1963) determined that the ratio of oxygen uptake in the dark to that in light (4000 lux) at 25°C was 1.22 for *Nitrosomonas* and 1.5 for *Nitrobacter* (Painter, 1970), while Hooper & Terry (1973) observed complete inhibition of *Nitrosomonas* activity by a 200-watt bulb (420 lux).

2.8.10 Sludge age, organic loading and detention time

In the operation of suspended-growth treatment plants, several operational parameters indirectly reflect the effect of nitrifier concentration on nitrification. Among these are the sludge age, detention time, and organic loading required to achieve a given degree of

nitrification. With other parameters (temperature, pH, dissolved oxygen, etc.) held constant, the degree of nitrification decreases significantly with an increase in organic loading (Prakasam & Loehr, 1972). Similarly, a sludge age of approximately three to four days appears to be required in most suspended-growth systems to achieve a high degree of nitrification (Poduska, 1973; Poduska & Andrews, 1974, 1975).

Detention time is not an inclusive parameter as its value depends upon a number of system parameters. High detention times may be needed if either of the concentration of nitrifying bacteria, sludge age or system temperature is low.

2.9 VIABILITY STUDY

The characterization of bacterial viability is crucial for assessing the impact of pharmaceutical residues on WWTP efficiencies. Conventionally, bacterial viability is determined by a series of dilutions of the test sample followed by culture in Petri dishes and counting of the number of bacteria formed. This implies a delay generally between 24h and 5 days. Another flaw in this technique is that it cannot provide information on the spatial distribution of the location of dead and living bacteria. Finally, this technique can only be used when the bacteria studied are cultivable in synthetic culture media. This is not the case for the vast majority of bacteria in activated sludge treatment plants. In fact, only a small fraction of activated sludge bacteria is cultivable (Su et al., 2018).

Cell viability is defined as the ratio of initial cell number minus dead cell number to the initial cell number (Cole and Lupták, 2019). The parameters that define cell viability in a particular experiment can be as diverse as the redox potential of the cell population, the integrity of cell membranes, or the activity of cellular enzymes. Each assay provides a different snapshot of cell health and can individually or together form the basis of an assay for cell viability, cytotoxicity, or drug efficacy with several integrated components.

Specially designed cell viability indicators have been developed for sensing the different characteristics and providing a visual readout of cell health using a fluorescence microscope, microplate reader, or flow cytometer. All indicators have positive and negative attributes; however, their sensitivity, reliability, and compatibility with relevant cell lines are important factors in determining utility.

The bacteria viability kit is used in this study. The commercially available LIVE/DEAD BacLight kit is popular among researchers in various fields (Boulos et al., 1999). The kit consists of two stains, propidium iodide (PI) and SYTO9, which both stain nucleic acids. Green fluorescing SYTO9 can enter all cells and is used for assessing total cell counts, whereas red fluorescing PI enters only cells with damaged cytoplasmic membranes. The emission properties of the stain mixture bound to DNA change due to the displacement of one stain by the other and quenching by fluorescence resonance energy transfer (Stocks, 2004). LIVE/DEAD staining was shown to work not only with (eu) bacteria (Boulos et al., 1999) but also with archaea (Leuko et al., 2004) or eukaryotic cells, such as yeast (*Saccharomyces cerevisiae*) (Zhang and Fang, 2004). Although this kit enables differentiation only between bacteria with intact and damaged cytoplasmic membranes, it is often used to differentiate between active and dead cells (Gasol et al., 1999; Sachidanandham et al., 2005). While it seems accurate to assume that membrane-compromised bacterial cells can be considered dead (Berney et al., 2006; Nebe-von-Caron et al., 2000), the reverse (that intact cells are active cells) is not necessarily true (Joux and Lebaron, 2000). Microscopic assessment of LIVE/DEAD-stained bacterial cells is usually simplified to either "green"-labelled (live) or "red"-labelled (dead) cells. However, experience with this dye combination and flow cytometry during the last few years has shown that the staining of bacterial cells with SYTO9 and PI does not always produce distinct "live" and "dead" populations; intermediate states are also observed (Barbesti et al., 2000; Berney et al., 2006; Christiansen et al., 2003; Hoefel et al., 2003; Joux and Lebaron, 2000). This intermediate state is referred to as "unknown" and can lead to difficulties in the interpretation of results and can be critical when, for example, decisions must be made about the effectiveness of disinfection methods or the number of viable bacteria in water distribution systems. Nevertheless, to our knowledge, the nature of these intermediate states has not been fully clarified.

The death of a bacterial cell has long been defined as the inability of a cell to grow to a visible colony on bacteriological media. However, with traditional culture methods, one can observe bacterial death only in retrospect (Postgate, 1989). Intermediate states like cell injury are difficult to detect with the plating method (Berney et al., 2007). Nevertheless, there are several viability indicators that can be assessed at the single-cell level without culturing cells. These indicators are based mostly on fluorescent molecules, which can be detected with epifluorescence microscopy, solid-state cytometry, or flow cytometry. Each indicator is based on criteria that reflect different levels of cellular integrity or functionality. Over the last 20

years, multiparameter flow cytometry has become a powerful tool in microbiology, particularly in biotechnological processing, food preservation, and chemical disinfection processes (Berney et al., 2006; Hewitt et al., 1999; Joux and Lebaron, 2000; Nebe-von-Caron et al., 2000; Porter et al., 1997), because it is fast and allows single-cell analysis.

2.10 CONCLUSION

The pharmaceutical and emerging contaminants residues pose several major problems. Several lines of evidence suggest that their presence in the environment, even in low concentrations, is likely to have an impact on WWTPs and could cause disturbances in the operation. Though it would be desirable for WWTPs to efficiently remove most of these contaminants, however, modern wastewater treatment plants (WWTP) were not designed to deal with complex chemical compounds as found in pharmaceutical residues and/or most emerging contaminants. In general, primary treatment by physicochemical methods such as coagulation and flocculation, and chlorination have also not been shown to be effective in removing many of pharmaceuticals. The effectiveness of WWTPs in removing micropollutants depends on the treatment process used, especially at the tertiary stage, and operational configuration of the wastewater treatment facility among other variables. In general, WWTPs mostly reduce solids and bacteria by oxidizing the water. For instance, the removal of clofibric acid (CLF) through various methods showed that better results are obtained with tertiary treatment. Ternes (1998) achieved a removal rate of 15% (trickling filter treatment), 34% (activated sludge treatment), and 51% (activated sludge system using ferric chloride) while Stamatis and Konstantinou (2013) improved it with 64% (secondary treatment) and 74% (tertiary treatment with chlorine and sand filtration).

Advanced wastewater treatment processes including ozonation, activated carbon and membrane nanofiltration and reverse osmosis, and advanced oxidation technologies have shown more promise because removal rates above 99% for targeted compounds have been achieved through these processes. Adsorption by activated carbon and ozone and UV advanced oxidation have been effective in removing some EDCs and PPCPs. However, this can be dangerous in the rare cases where oxidation changes previously innocuous compounds into more harmful by-products. Also, the need to regenerate the carbon used in this process arises as much if it ends up in a landfill and the process uses a lot of energy and may lead to greater environmental risks. Ultraviolet photolysis has been considered but was only able to remove

50 - 80% of target compounds even when the dose was over a hundred times of a typical disinfection dose.

An informed risk assessment using combined analyses and models of Predicted No-Effect Concentration, PNECs is crucial and needs to be developed before significant resources are spent in upgrading infrastructure. Even the most advanced wastewater treatment technology will not always be able to completely remove all pharmaceuticals. It is therefore important to consider the costs of these often very expensive technologies relative to the potential toxicity of the compounds they are intended to remove.

However, the need for the reduction or prohibition of micropollutants at the source is one of the major strategies being considered for dealing with WWTP effluents contamination which will curtail the cost of treatment. It is exemplified by the discrepancies in concentrations being observed and which depends on the prescription rates of the parent compounds between countries. CLF concentrations that reached 41.4 μg/L were detected in Portugal but was not detected in Norway since its parent compounds, clofibrate and etofibrate are not prescribed in Norway.

The rise in the concentration of compounds after treatment is an oddity whose cause has been explained as the deconjugation of the composite molecules during biological treatment. Salicylic acid (SA), an active metabolite of Acetylsalicylic acid increased in concentration by 46% after biological treatment due to possible deconjugation of glucuronide metabolites. However, very low concentrations of SA were found in the effluents of the WWTPs although it was one of the most abundant acidic pharmaceuticals in the influents. This was attributed to the instability of the compound in sewage samples.

Despite the vast body of knowledge that exists on the concentrations of PPCPs in the aquatic environment, published reports on their environmental concentrations in both sewage and sludge are few. Much less is known of the occurrence and fate of their TPs. Some compounds that appear to have relatively high removal rates may be absorbed into sludge, meaning that if they are used as fertilizer later, they may end up in groundwater or enter the surface waters through runoff. In this sense, measuring concentrations in influent and effluent of treatment plants may not be a true indicator of environmental impact from certain compounds. The sewage sludge and biosolids can serve as the main reservoir of TP residues underling the importance of sludge management strategies. This will improve the removal rate of PPCPs and

their TPs and curtail this vicious circle of their treatment and reintroduction into surface waters through the environment.

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CHAPTER 3: MATERIALS AND METHODS

3.1 SAMPLES COLLECTION & MATERIALS

Samples of wastewater were collected after screening (Figure 3.1) while the sludge was taken from the recirculation loop from Bushkoppie Wastewater Treatment (BWT) (Johannesburg South Africa: 26°18'40"S 27°56'6"E). The samples were collected during the morning h (before 9 am) and repeated ten times from June 2017 to October in 2018. Triplicates of grab samples (1 L) were collected from the inflow stages. Primary sewage (wastewater) and activated sludge are collected after the primary screening with the removal of grit. All samples (n = 10) were transported to the laboratory on ice and processed within 4 h of collection to reduce bacterial activity.



Figure 3. 1 Collecting wastewater in BWT

All the tanks, as shown in Figure 3.2 were constructed using the transparent material, Perspex of 6 mm thickness except for the Primary settling tank which was constructed with High-Density Polyethylene. Transparent flexible pipes are used to connect the tanks and the pumps in the simulation.

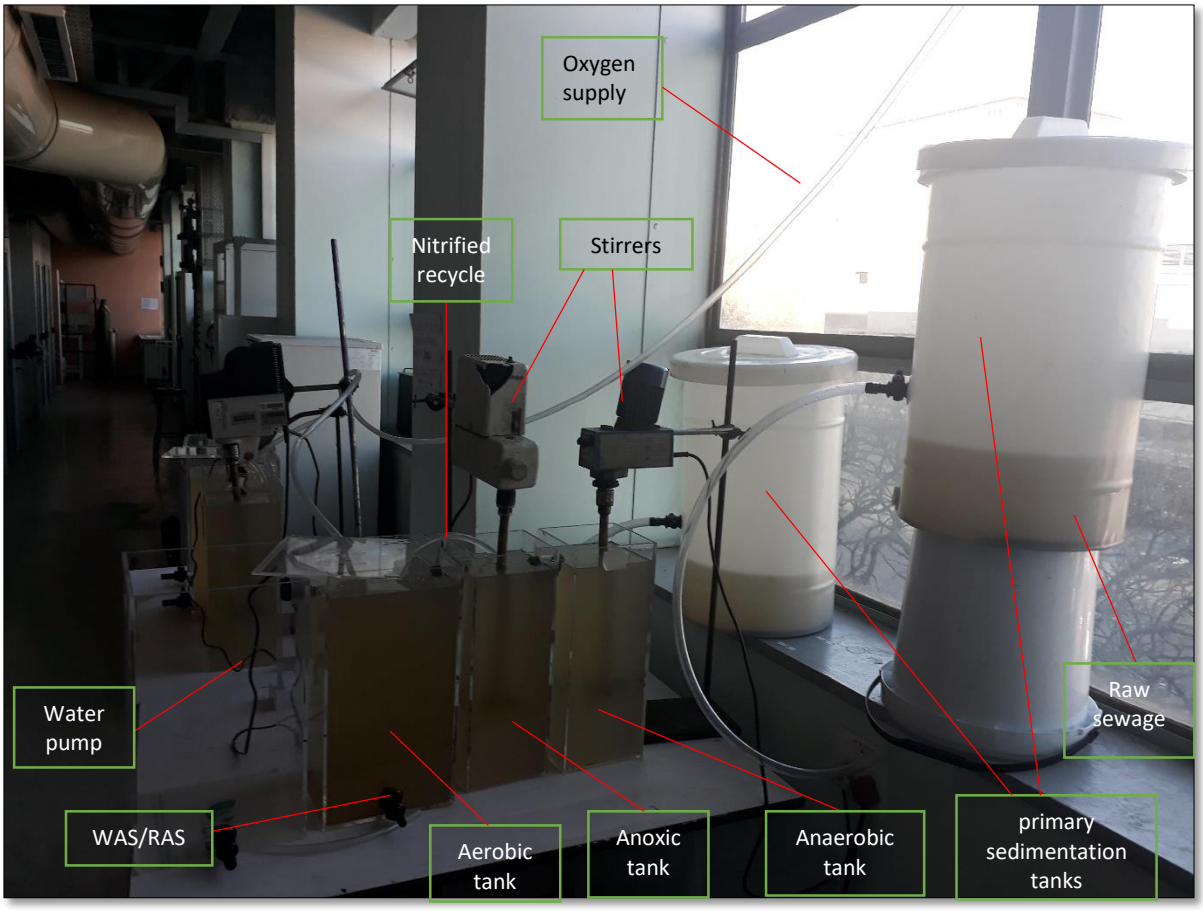


Figure 3. 2 The wastewater treatment in progress in the lab

The BWT plant has a capacity of 200 million litres per day (Ml/d) of wastewater whose properties fall within the ranges shown in Table 3.1, below.

Table 3. 1 Inlet Wastewater daily average parameters for the BWT

Parameter	**Units**	**Value**
pH	-	6.87 ± 0.33
Conductivity	µS/cm	726 ± 52
Salinity	Psu	0.42 ± 0.09
Total dissolved solids (TDS)	mg/L	353 ± 43
Dissolved oxygen (DO)	mg/L	3.55 ± 0.38
Chemical oxygen demand (COD)	mg/L	643 ± 162
Dissolved organic carbon (DOC)	Mg/L . C	17 ± 6
Total suspended solids (TSS)	mg/L	688 ± 37

3.2 METHODS

3.4.1 Laboratory-scale WWTP preparation

Two locally constructed laboratory-scale wastewater treatment plants were constructed in accordance with the OECD design guidelines (OECD, 2007) and used in the simulation experiments, each containing the following: A model wastewater treatment plant was constructed in accordance with the OECD design guidelines. The model comprised of an aerated stirred tank and a clarifier simulating biological treatment using activated sludge system (Figure 4.1). All the tanks in the pilot-scale modules were constructed using the transparent material, Perspex of 6 mm thickness except for the initial storage tank which was constructed with High-Density Polyethylene. Transparent flexible pipes are used to connect the tanks and the pumps. Two model units, a test unit and a control unit, were run concurrently for parallel exposure experiments for the ARVs. The wastewater and

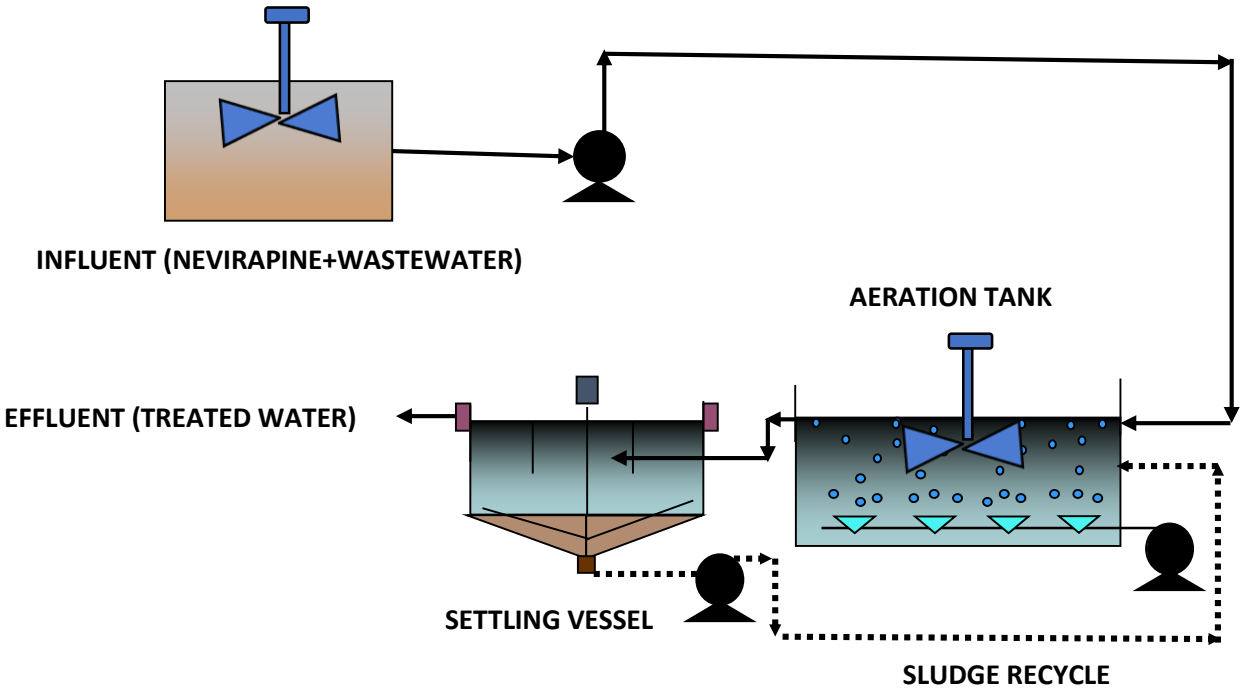


Figure 4. 1 A schematic representation of the simulated activated sludge wastewater treatment plant.

the activated sludge were mixed at a 1:1 ratio and fed into the WWTP using Watson-Marlow 120S/DV pumps (Falmouth, Cornwall, UK) into the test and control units at 10 ml/min continuously for at least 240 h and allowed to reach steady state. Steady-state was established

through monitoring the chemical oxygen demand (COD) removal. COD is a measurement of the oxygen required to oxidize soluble and particulate organic matter in water.

At the beginning of the experiment, the storage tank was filled with activated sludge. The system which was exclusively used as a control had raw wastewater containing no ARV added to the sludge in this reactor. The other system contained raw wastewater spiked with ARV (nevirapine/lamivudine) (Aspen Pharmacare, South Africa) of between 0.1 and 30 mg/L concentrations.

Upon reaching steady-state conditions, wastewaters containing ARV and no ARV were then fed continuously using Watson-Marlow 120S/DV pumps (Falmouth, Cornwall, UK) into the pre-equilibrated test and control units. The aerated tank reactor was aerated at 100 L/h using compressed air introduced through a distributor at the bottom. A Watson-Marlow SCI Q 323S pump was used daily for 10 min to pump out sludge. The model unit design allowed for a hydraulic residence time (HRT) of 10 h in the completely stirred tank reactor (aeration time). Treated effluent and activated sludge samples (200 ml each) were collected daily from the clarifier of each unit. The sampling interval was based on the combined residence time of 12 h in both vessels of each unit. As per the standard practice, part of the sludge collected from the clarifier was introduced as return activated sludge (RAS) to the completely stirred tank reactor using a Watson-Marlow SCI Q 323S pump for both the test and control unit. This was also meant to prevent wash-out of the sludge. The activated sludge collected was split into two equal parts, the waste activated sludge (WAS) and the return activated sludge (RAS). The RAS was re-introduced into the aeration chambers daily to replenish the biomass. The WAS samples were used for analysis. Primary sewage (wastewater) was collected from the BWT plant commissioned to cater for the inhabitants of the southern suburbs of Johannesburg, Soweto East and from the industries to the south of Johannesburg including Lenasia. It has a capacity of 200 million litres per day of wastewater. The wastewater was collected just after the primary screening bar upon the removal of grit. The activated sludge was collected in the recycle line from the BWT plant. In this plant, the daily average characteristics of the wastewater are shown in Table 4.2.

Table 4. 1 Average characteristics of raw domestic wastewater used in the simulated wastewater treatment plant study

Parameter	**Units**	**Value**

pH	-	6.87 ± 0.33
Conductivity	µS/cm	726 ± 52
Salinity	Psu	0.42 ± 0.09
Total dissolved solids (TDS)	mg/L	353 ± 43
Dissolved oxygen (DO)	mg/L	3.55 ± 0.38
Chemical oxygen demand (COD)	mg/L	643 ± 162
Dissolved organic carbon (DOC)	Mg/L. C	17 ± 6
Total suspended solids (TSS)	mg/L	688 ± 37

Wastewater was collected once per week and stored in a cold room at 4°C to slow down bacterial activity before it was fed into a simulated WWTP. The wastewater and the activated sludge are fed into the WWTP at a ratio of 1:1. We focused mainly on the 15 mg/L nevirapine concentration in order to study variability caused by changes in the biomass characteristics of the WWTPs. Furthermore, we did not adhere strictly to the ISO 9509:2006 recommendations for inhibition tests on nitrifying bacteria because real wastewater was used to make the experiment as realistic as possible (ISO 9509:2006) hence it was not necessary to run inhibition tests prior to the experiments. Both experimental setups were run concurrently and under the same experimental conditions.

3.4.2 Sample and wastewater measurements

Both systems were monitored, and samples (effluent from the aerobic tank) were regularly taken from the reactors, with each reactor being sampled every 2 h until the 8th h. After filtration (paper filter pore size ≈ 1.5 µm), ammonia levels were determined using the Hach Nessler Method 8038 on a Hach DR/2400 spectrophotometer (Hach) (error ± 0.6 N-NH_4+ mg/L). Nitrite and nitrate were measured by ion chromatography (error ± 1.5 mg N-NO_3-/L). Suspended solids were obtained after filtration (pore size = 0.5 µm) of 10 mL of mixed liquor and by drying at 105°C for 24 h. High-resolution transmission electron microscope (TEM) images of sludge were obtained using a JEOL JEM 2100F with a LaB6-cathode operated at a voltage of 200 kV (Tokyo, Japan), and high-resolution SEM measurements were made on FEI NovaNanoSEM230 operated at 2−5 keV (Tokyo, Japan). The dissolved organic carbon (DOC), dissolved oxygen (DO), chemical oxygen demand (COD) were measured using the fusion

UV/Persulfate TOC Analyser (TELEDYNE TEKMAR, USA), oxygen meter (HANNA Instruments, Portugal), and photometer (HACH, DR 3900, USA), respectively.

3.4.3 Spectro-photocolorimetry (COD and ammonium)

We measured COD and ammonium with a Hach® DR / 2400 spectrophotometer. The wavelength of this device ranges from 400 to 800 nm (± 1 nm). The selection of the wavelength can be done manually in single signal mode or automatic in programme mode. We used the automatic mode. The results can be read in transmission, absorption or concentration mode but we used the absorption mode.

The determination of Chemical Oxygen Demand (COD) is widely used in municipal and industrial laboratories to measure the overall level of organic contamination in wastewater. The contamination level is determined by measuring the equivalent amount of oxygen required to oxidize organic matter in the sample. Chemetrics offers two dichromate reactor digestion methods for fast, easy, safe determinations of low-, mid-, and high-range COD levels in wastewater: the USEPA-approved Method*, and a mercury-free method. The products using the USEPA-approved method contain mercuric sulfate in the reagent to eliminate chloride interferences. The more readily disposable mercury-free product line is applicable when chloride interference is not a concern and USEPA reporting is not required. Chemetrics leakproof reagent vials contain premeasured solutions of sulfuric acid and potassium dichromate. To perform the COD determination, the analyst simply removes the Teflon-lined screw cap from the vial, adds a sample to the vial, and replaces the cap. The vial is then heated for two h at 150 ℃ in a standard digestor block. Once digestion is completed, results are obtained using any photometer that accepts 16-mm diameter cells. Chemetrics COD vials can be directly used in our V-2000 multi-analyte photometer, Chemetrics single analyte COD photometers, as well as in Hach1 spectrophotometers. Built-in Hach COD methods and calibrations can be used without the need for a new calibration. A generic calibration table is included within the Chemetrics kit for use with other spectrophotometers. COD is a global measure of chemically oxidizable pollution. It is also a global measure of pollution chemically oxidizable by a sulfochromic mixture. This is the amount of oxygen equivalent to the amount of dichromate consumed by the pollution during its hot oxidation. After the reaction, the value is measured by absorbance spectrophotometry. We used the range 0 -750 nm. The protocol detailed has four steps:

1. 2 mL of sample is placed in a Hach® tube and mixed with an acidic solution (3.5 mL). This digestion solution is a mixture of potassium dichromate (a strong oxidizer) and mercury sulfate (to eliminate interference with chloride ions). The acidic solution is composed of concentrated sulfuric acid (acidic medium) and silver sulphate (catalyst).

2. The sample is heated under reflux for 2 h at 147 °C in a CSB / DCO Aqualytic Reactor AL31 furnace and allowed to cool in the open air for 20 minutes.

3. The outside of the tubes is cleaned with ethanol-soaked paper to remove any traces that could interfere with the absorbance measurement. Absorbance is measured at 620 nm. The average of four absorbance measurements on the same sample makes it possible to have a more precise value. A blank is each time made from a sample of demineralized water that has been treated as a sample to be assayed.

4. A calibration line gives the correspondence between the absorbance of the sample and its COD.

3.4.4 Ammoniacal nitrogen

The Hach® method 380 over a range of 0 to 2.5 mg N/L based on Nessler's reagent has been adapted for the sampling of 10 mL. Samples (filtered) were diluted to the 20^{th} power using a Hamilton® automatic diluter and demineralized water. The reagent of Nessler (alkaline potassium iodo-mercurate) is decomposed in the presence of ammonium ions with the formation of dimercuriammonium iodide producing a yellow colour. Its concentration is measured by absorbance at 425 nm. The assay error is evaluated to ± 0.5 mg $N\text{-}NH_4$/L.

3.4.5 Ionic Chromatography: Nitrites and Nitrates

The analysis by ion chromatography is carried out using a DIONEX® apparatus using the ion exchange column IonPac AS18. The ions or polar compounds are driven by the mobile phase and separated by the effect of their interactions with the ionic sites of the stationary phase. The ion exchange layer of the stationary phase of column AS18 consists of quaternary ammoniums. The eluent used (mobile phase) is Potassium Hydroxide (KOH). The higher the charge density of an anion, the more it will be retained by the stationary phase and the longer the residence time in the column will be important. Thus, one can differentiate the ions by their residence

time in the column. The detection is based on the measurement of the conductivity of the mobile phase at the exit of the column.

3.4.6 Baclight^{TM} viability marking

Sludges collected from both the test and control units after 7 days were used for bacterial viability assessments. The bacterial viability in each sample was analyzed using L7007 LIVE/DEAD BacLight viability kit (Invitrogen, South Africa). The kit consists of two stains, namely: propidium iodide (PI) and SYTO®9. Both PI and SYTO®9 stain nucleic acids were used to differentiate between cells that were intact (live organisms – stained in green) and damaged cells (dead organisms – stained in red), respectively. Ten µL aliquots of each stain were mixed together and 3 µL of the mixture was used for analyzing 1 mL volume of bacterial suspension. The dye and bacterial suspensions were mixed thoroughly and incubated at room temperature (~ 250℃) in the dark for 15 minutes. Thereafter, 5 µL of the stained bacterial suspension was placed on a microscope slide and covered with a cover slip. The slides were examined under fluorescence microscopy using a BX51 microscope (Olympus, South Africa) fitted with an UPlanFl 100x/1.3 oil immersion objective. The excitation/emission maxima for the dyes were approximately 480/500 nm for SYTO®9 stain and 490/635 nm for propidium iodide. The images were captured and analyzed using a CC12 soft imaging system.

3.4.7 Escherichia coli enumeration

The membrane filter technique was used to isolate Escherichia coli from the wastewater. This method was easy to perform with large volume of samples and the results could be obtained rapidly, compared to other methods. The target organisms are fecal indicators of water quality and have the potential to develop nevirapine/lamivudine resistance. Nutrient Agar with MUG Medium: MUG (4-methyl umbrelliferyl β -D gluconoride) media (Fisher Scientific, Hampton, NH) was used for the detection and enumeration of Escherichia coli in wastewater sample. Approximately, 23.1 grams of nutrient agar in 1 L distilled water was boiled to dissolve completely. The agar solution was sterilized at 121 - 124℃ for 15 minutes and allowed to cool to 50℃. Precise amounts of nevirapine/lamivudine were added to the nutrient agar and the solution was stirred for a few seconds to ensure that they were completely distributed in the solution. 10 ml of nutrient agar was transferred into each pre-sterilized Petri dish. The dishes could dry for 24 h and stored at 4℃. Serial dilution techniques were used. Samples collected from the WWTP were diluted in Benzer dilution fluid (BDF). The BDF solution consisted of

7 grams of NaCl and 1 gram of Trypticase soy agar mixed in 1 L of distilled water. BDF solution was sterilized in the autoclave and used for dilution of wastewater samples.

Five pre-sterilized test tubes were filled with 9 mg/L of BDF solution. Sample bottles were shaken in order to evenly distribute the bacteria present in the wastewater. One-milliliter aliquot of the sample was transferred into the first tube and mixed to produce a 1/10 dilution. One mg/L of diluted sample from this first tube was transferred to the second tube and mixed to produce a 1/100 dilution and the process was repeated until the 1/1000, 1/104, 1/105 dilutions had been prepared in the five tubes. Aliquots were transferred using sterilized pipettes. The plates were labeled with sampling location, date and sample number. The samples were filtered using a 0.45 µm, 47 mm, diameter, cellulosic white grid filter (Fishers scientific, Hampton, NH) placed on fa 3-prong filter holder. Approximately, 25ml of distilled water was first added to wet the filter paper. Precise volumes of diluted samples were then transferred on to the filter. A vacuum pump system was used to expedite the passage of water through the filter while bacteria were collected on the membranes. Filters were carefully removed and transferred with the help of sterilized forceps on to the selective media plates. All inoculated agar plates were incubated in a temperature-controlled shaker (Environ Shaker 3597, Lab-Line Instruments Inc IL) at temperature (37℃) and time (2-4 h). Colonies were randomly picked from the agar plates and washed with phosphate saline solution three times and finally stored in cryovials containing TSB with 50% glycerol at −80℃ for further analysis. The species of these isolates were further confirmed by real time polymerase chain reaction. Briefly, cultures grown overnight were lysed in a bead mill for 60 s at 5000 rpm and the debris was removed by centrifugation. The DNA concentration at the end of the crude extraction was measured using a UV-spectrophotometer (NanoDrop™ 2000, Thermo Scientific, Wilmington, DE, USA). Each DNA extract was analyzed in duplicate by the EC23S857 assay for E. coli. The reaction mixture contained 12.5 µl of Environmental Master Mix 2.0, 2.5 µL of 2 mg/L bovine serum albumin, 1 µM of each primer, 2 µL of DNA-free water and 5 µL of the DNA extracts for a total reaction volume of 25 µL; and the thermal cycling protocols were 10 min at 95℃, followed by 40 cycles of 15 s at 95℃ and 60 s at 56℃. A positive control (E. coli) and a no template control were also run during the analysis for quality.

3.4.8 Nevirapine tablets

The nevirapine drug (manufactured by Aspen, South Africa) came in tablets of 200 mg (Figure 3.3) was bought from a local pharmacy in Johannesburg, South Africa. It was ground in a

mortar and pestle and varying concentrations of the powdery form were introduced into the wastewater treatment reactor.



Figure 3. 3 The nevirapine drug used for the experiments

3.4.9 Lamivudine tablets

The lamivudine drug (manufactured by Aspen, South Africa) came in tablets of 150 mg was bought from a local pharmacy in Johannesburg, South Africa. It was ground in a mortar and pestle and varying concentrations of the powdery form were introduced into the wastewater treatment reactor.



Figure 3. 4 The lamivudine drug used for the experiments

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CHAPTER 4: EVALUATION OF THE EFFECTS OF NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR (NNRTI) DRUG (NEVIRAPINE) ON A SIMULATED CONTINUOUS LABORATORY SCALE ACTIVATED SLUDGE WASTEWATER TREATMENT PLANT (WWTP)

Abstract

One of the main issues related to sanitation is the release of micropollutants into the environment via wastewater treatment plants (WWTP) which have been long considered as a major entry point for environmental contamination. In these releases, a wide range of compounds is measured in trace amounts (ng/L to μg/L) such as pharmaceutical residues and hormones (PPHs), pesticides, phthalates, artificial sweeteners, chemical products, and personal care products. These compounds are for the most part endocrine disruptors and/or toxic for humans and the environment. In this context, limiting the discharge of micropollutants to the environment has become a major preoccupation of scientists. Understandably, the contamination of WWTP effluents by micropollutants, as well as the efficiency of WWTPs to eliminate the micropollutants has taken centre stage particularly since the scarcity of water and the need for energy and food on the global stage require that the feasibility of wastewater recycling and resource recovery be further explored. This paper examines the effects of nevirapine on a simulated activated sludge process using actual wastewater from an urban WWTP.

Nevirapine belongs to a class of drugs known as non-nucleoside reverse transcriptase inhibitors (NNRTIs) and it is used as an inhibitor of human immunodeficiency virus type 1 (HIV-1) in South Africa. Laboratory experiments were performed using duplicate samples of raw influent collected during June 2018 while final experiments were performed using triplicate samples collected from raw influent during November 2018. 1, 15, and 25 mg/L concentrations of nevirapine are introduced into the wastewater and the concentrations of chemical oxygen demand (COD) and ammonia-nitrogen in the effluent of each experiment are recorded on a 2-hourly basis until the 8th h when the experiment ended. Wastewater samples with 15 mg /L nevirapine inhibited the specific concentration variations of COD by 52.9% (standard deviation

27%) and the specific $N\text{-}NH4^{+}$ concentration variation by 30% (standard deviation 21%). An increase in COD, as well as a decrease in total suspended solids, were observed in the wastewater with nevirapine.

Keywords: WWTP, nevirapine, COD, nitrification, inhibition, emerging contaminants, HIV, ammonia-nitrogen

4.1 INTRODUCTION

Globally, and particularly in South Africa, owing to water scarcity, the partial or complete closure of water cycles should be an integral part of the sustainable water resource management (Pollard and D. Du Toit, 2008). One option is by increasing the re-use of effluents for various purposes especially within the industrial and agro/food production applications (Zajda and Aleksander-Kwaterczak, 2019). However, because of the high cost of the end-of-pipe approach, indirect potable water re-use requires efficient treatment of wastewaters prior to their discharge (Zajda and Aleksander-Kwaterczak, 2019). Nonetheless, it is without question that the propensity of freshwater contamination will rise in the coming years because (i) human population continues to grow, and/or (ii) patterns of natural surface water have become very low with the wastewater constituting the larger fraction of the flow (Chapman, 2018). This is very true in the case of the Gauteng region in South Africa, and other high notch economic hubs in South Africa. In view of these factors, among others, South Africa is particularly vulnerable as it is a semi-arid country with rapidly increasing annual population as well as industrial growth (Oberholster, 2018).

The situation is increasingly being exacerbated by factors like fluctuating seasonal flow as well as prolonged drought due to climate-change that drastically alter the environmental concentrations of the emerging contaminants in water bodies emanating from low or no dilution (Chen et al., 2018). Therefore, the need for establishing the short-, medium-, and long-term effects of the emerging contaminants in the face of diminishing environmental absorption capability of the contaminants has become urgent, and of national importance (Costa et al., 2018). Thus, the occurrence of trace emerging contaminants in wastewaters, their behaviour during wastewater treatment and production of drinking water are key issues that require further investigation to highlight the need for a more comprehensive understanding of their environmental behaviour (Tran et al., 2018).

On the other hand, the explosion of certain new diseases in the last 3-4 decades in Africa has introduced antiretroviral (ARV) drugs into the wastewater constituents, and in high concentrations. This is a consequence of ARV use in the treatment and prophylaxis of various viral infections including influenza, hepatitis, herpes and HIV (De Clercq and Field, 2006; Kim et al., 2010; Kiso et al., 2004; Olsen et al., 2006; Singer et al., 2006). The main pathway of ARVs into surface water bodies after excretion from the human body is through the release of untreated or improperly treated effluents from WWTPs, hospitals and production facilities (Ncube et al., 2018). From an African context, improper sanitation and illegal disposal of both domestic and industrial waste may be another potential source of ARVs in the environment (Ncube et al., 2018). A table of the ARVs that have been detected in environmental samples compiled by Ncube et al. (2018) indicates that the levels of ARVs in environmental water samples in Africa are higher than anywhere else. This might be related to the prevalence of the HIV/AIDS epidemic and the inherent large treatment programme in South Africa. The WHO Observatory data repository states that of the globally estimated 36.7 million people living with HIV/AIDS by end of 2016, 70% were from Africa, 10% in the South-East Asia, 9% in the Americas, 6% in Europe, 4% in the Western Pacific, and 1% in the Eastern Mediterranean (Porter et al., 2018). The estimated number of people receiving ARV treatment as of 2016 was 19.8 million. South Africa had the biggest antiretroviral treatment (ART) programme estimated at 3.9 million people receiving ARVs, about 24% of the world ART program. Nevirapine (NVP) is an oral medication used to treat and prevent retroviral infections primarily human immunodeficiency virus type 1 (HIV-1). HIV-1 is a virus that attacks mainly the CD4-T-cells responsible for the body's immune system. However, antiretroviral treatment against HIV-1 does not cure or kill the virus but rather prevents or slows down its multiplication (Deeks et al., 2013). It is generally recommended for use with other ARV medication. Nevirapine is a non-nucleoside reverse transcriptase inhibitor (NNRTI) ARVD which is commonly given to pregnant women to inhibit the transfer of HIV to the unborn baby. The nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) act early in the replication viral cycle by preventing reverse transcription of the viral RNA of the HIV into its DNA, an essential step before the virus could integrate itself into the host cell. This is achieved by interfering with a viral enzyme responsible for this purpose called reverse transcriptase. Its excretion via urine is at 2.7% after ingestion (Schoemnan et al., 2017; Swanepoel et al., 2015). Examples of ARV drugs concentrations in wastewater and river water are presented in Table 4.1.

Table 4. 2 Examples of ARV drugs concentration

	ARV drug	Type	Influent Conc. (pg/L)			Effluent Conc. (pg/L)			Reference
			Median	Min	Max	Median	Min	Max	
Raw urban wastewater		Macrolide	0.34	0.07	12				Karthikeyan & Meyer, 2006
	OSC-CAR		42.7	n.d.	42.7	17.3	n.d.	-	Prasse et al., 2010
	OSC-CAR		29.4	n.d.	29.4	12.2	n.d.	-	Prasse et al., 2010
	Lamivudine		720			n.d.			Prasse et al., 2010
	Zidovudine		380			n.d.			Prasse et al., 2010
	Abacavir		220			n.d.			Prasse et al., 2010
	Acyclovir		1800			n.d.			Prasse et al., 2010
	Penciclovir		<50			n.d.			Prasse et al., 2010
	Stavudine		<50			n.d.			Prasse et al., 2010
	Nevirapine		2200			400			Schoeman et al., 2015
	Nevirapine		1200			1000			Schoeman et al., 2015
	Efavirenz		17300			7000			Schoeman et al., 2015
	Efavirenz		3600			3000			Schoeman et al., 2015
	Efavirenz		9200			5100			Schoeman et al., 2015
	Zidovudine		30			14.1			Funke et al., 2016
	Abacavir		21			n.d.			Funke et al., 2016
	Emtricitabine		980			n.d.			Funke et al., 2016
	ABV-COOH		960			n.d.			Funke et al., 2016
River water									
	OSC		15						Prasse et al., 2010
	OSC-CAR		24						Prasse et al., 2010
	Zidovudine		170						Prasse et al., 2010
	Acyclovir		190						Prasse et al., 2010
	EMT-COOH		152.50	25	280	80			Funke et al., 2016
	ACV-COOH		404	58	750	41			Funke et al., 2016
	LMV-COOH		123	16	230	84			Funke et al., 2016

OSC: Oseltavir; OSC-CAR: Oseltavir Carboxylate; EMT-COOH: Emtricitabine Carboxylate; ACV-COOH: Acyclovir Carboxylate; LMV-COOH: Lamivudine Carboxylate

Nitrification and denitrification are performed by bacteria in activated sludge, and like all living organisms, they have different requirements on environmental, physical and chemical conditions. Many chemical substances and mixtures are toxic to bacteria if they are present in too high concentrations and can cause a decrease of the biological process as in the case of a WWTP. As the nitrifying bacteria are most sensitive to toxic chemicals, they are often used as test organisms to detect chemicals in toxic concentrations. The toxicity to nitrifying bacteria can be measured by inhibition of nitrification. WWTPs are expected to partially eliminate

micropollutants as ARV drugs even though they are for the most part designed to reduce nitrogen and chemical oxygen demand (COD) and not micropollutants such as ARV drugs that can be toxic to their biomass. This toxicity could reduce the chemical oxygen demand (COD) removal rate and the nitrification rate, which are key components of WWTPs treatment efficiency. There is still a lack of data describing the effects of non-nucleoside reverse transcriptase inhibitor drug such as nevirapine on wastewater treatment processes, especially on the COD removal rate inhibition and nitrification rate

4.2 MATERIALS AND METHODS

The materials and methods for carrying out the experiments are discussed in Chapter 3 using 1, 15, and 25 mg/L concentrations of nevirapine.

4.2.1 Removal rate and inhibition calculations

Nitrification is a two-step biological process in which one type of autotrophic bacteria, *Nitrosomonas*, oxidize ammonia to nitrite (NO_2-N) (Equation 4.1) and the second type of autotrophic bacteria, *Nitrobacter* oxidize nitrite to nitrate (NO_3-N) (Equation 4.2) (Carroll et al., 2005). The rate of conversion of ammonia to nitrite, by the Nitrosomonas, is much slower than that of nitrite to nitrate, by the *Nitrobacter* (Ekama and Marais, 1984). Often, any nitrite that is formed is converted virtually immediately to nitrate. Consequently, very little nitrite is observed in the effluent from a plant operating on an influent that does not contain substances that inhibit the *Nitrobacter*. Thus, the limiting rate in the nitrification sequence is that due to the *Nitrosomonas* and it can be assumed that the conversion is directly from ammonia to nitrate. On this basis, the kinetics of nitrification reduces to the kinetics of the *Nitrosomonas* (Ekama and Marais, 1984).

$$NH_4^+ + 1{,}5O_2 \rightarrow NO_2^- \quad (4.1)$$

$$NO_2^- + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \quad (4.2)$$

The specific concentration variations of the COD and nitrogen to ammonium (N-NH_4+) as well as the specific nitrification rates were calculated during the first 4 h of the experiment as follows:

Specific N-NH_4^+ concentration variation= d ([N-NH_4^+]) / dt / suspended solid,

where t = time;

Specific COD concentration variation = d ([COD]) / dt / suspended solid; and

Specific nitrification rate = d ([N-NO_2^-] + [N-NO_3^-]) / dt / suspended solid, with

nitrite (N- NO_2^-) in mg N/L and nitrate (N-NO_3^-) in mg N/L.

The degree of inhibition was calculated as:

Percent inhibition = [(rate control – rate nevirapine reactor) / rate control] × 100.

% **inhibition** = [1 - (X - MIN)/ (MAX - MIN)] x 100

4.3 RESULTS AND DISCUSSION

4.3.1 Removal rates and inhibition

The nevirapine concentrations used in the current work were between 1 and 30 mg/L). The average specific COD concentration variation in the control system was -21 mgO_2/(gSS·h). The average specific COD evolution rate in the system containing wastewater spiked with 15 mg/L nevirapine was -6.7 mgO_2/(gSS·h), and the average inhibition was 52.9 % (standard deviation 27 %) (Figures 4.1 and 4.3). The inhibition of the COD evolution rate may be caused by the death of bacteria and the release of biomass by-products in the wastewater. However, the results show a temporary increase in COD in the system with 15 mg/L nevirapine at shorter times (< 15 minutes) (Figure 4.6).

In the system containing wastewater spiked with nevirapine, measurement of suspended solids shows biomass loss due to a possible mass transfer mechanism. Deterioration of activated sludge was detected via image analysis as also shown in Figure 4.10.

On the other hand, the average N-NH_4^+ specific concentration variation in the control system was -1.23 mgN/(gSS·h). This rate depends on initial N-NH_4^+ concentrations in the wastewater consistent with Monod kinetics. The average N-NH_4+ specific evolution rate in reactors with 15 mg/L of nevirapine was -0.79 mgN/(gSS·h) (Figure 4.2)

The results further show that the specific nitrification rates were inhibited in nevirapine-spiked sludge from the Bushkoppie WWTP. Other researchers also reported inhibition of nitrification in WWTP (Ellis, 2010; Jain et al., 2013). A summary of N-NH_4^+ and COD concentration variation as a function of nevirapine concentration is shown in Table 4.2. While variability

with respect to the sampling date was evident, no differences in the behaviour of the sludge between summer experiments and winter experiments could be established for the triplicates.

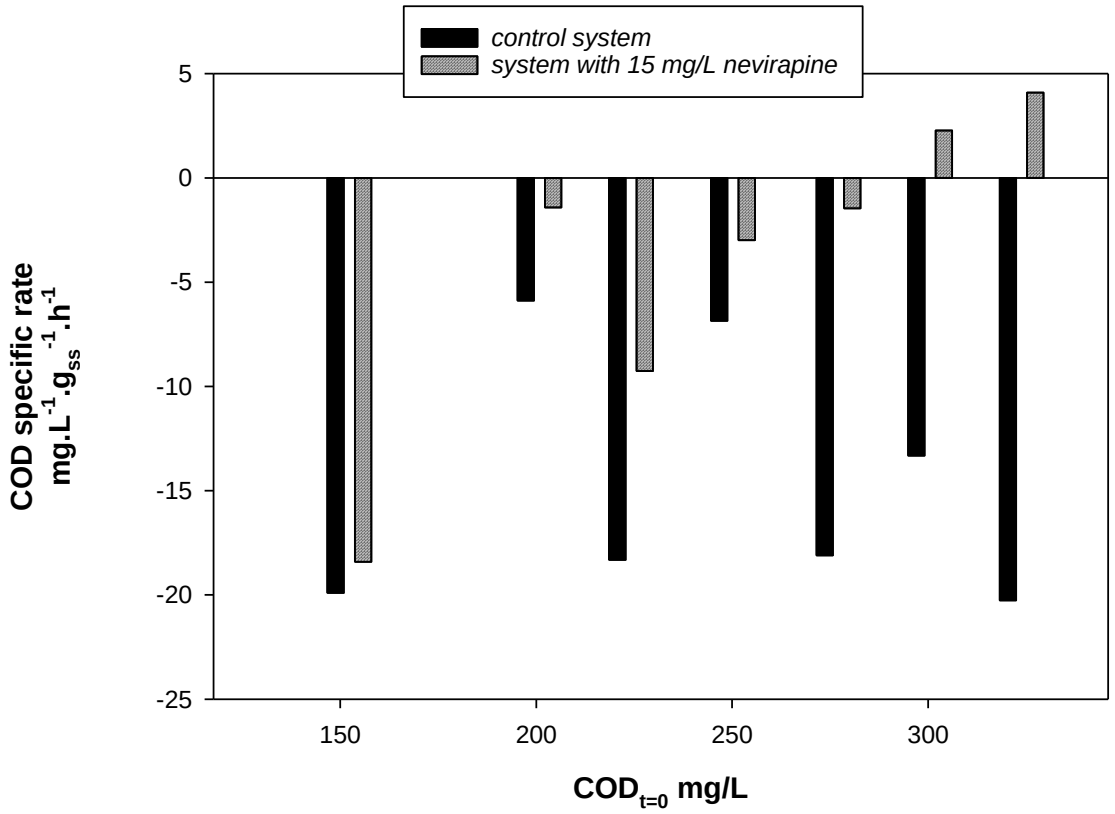


Figure 4. 1 Specific $N\text{-}NH_4^+$ concentration variation as a function of the initial $N\text{-}NH_4^+$ concentration in control system and system containing wastewater spiked with 15 mg/L nevirapine.

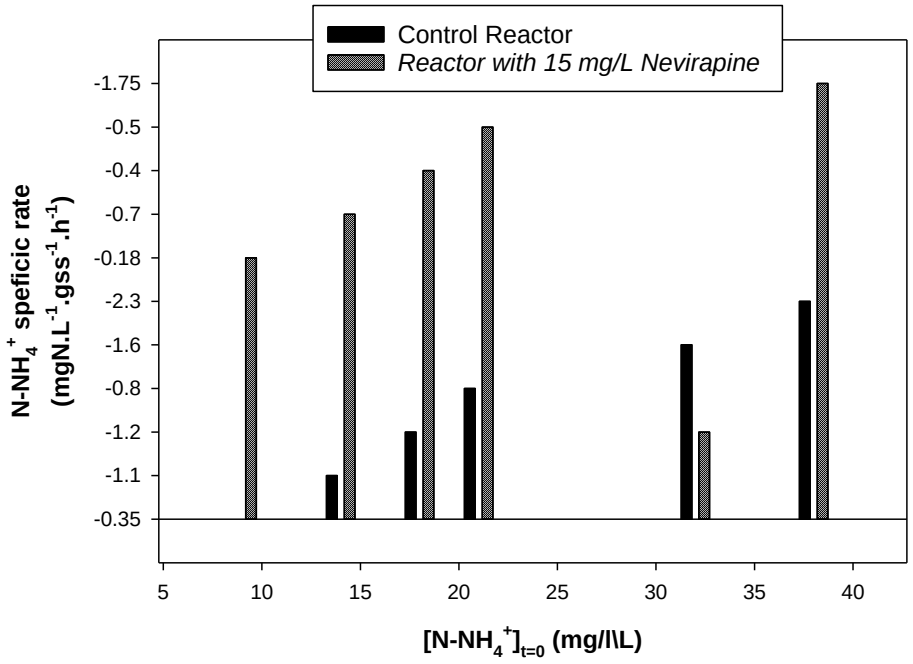


Figure 4. 2 Specific COD concentration variation as a function of the initial COD in the control system and system containing wastewater spiked with 15 mg/L nevirapine.

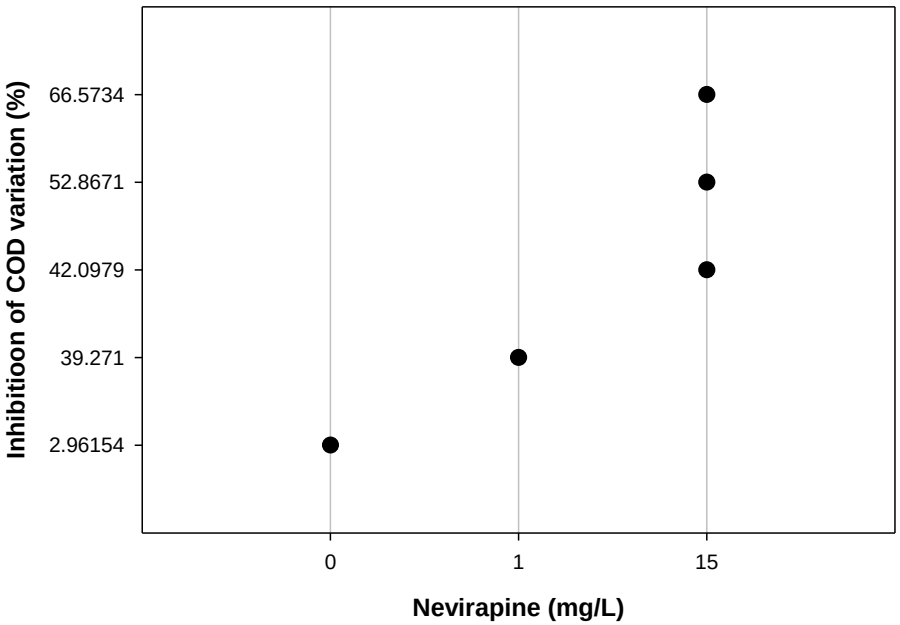


Figure 4. 3 Inhibition of the specific COD evolution rate as a function of Nevirapine concentration.

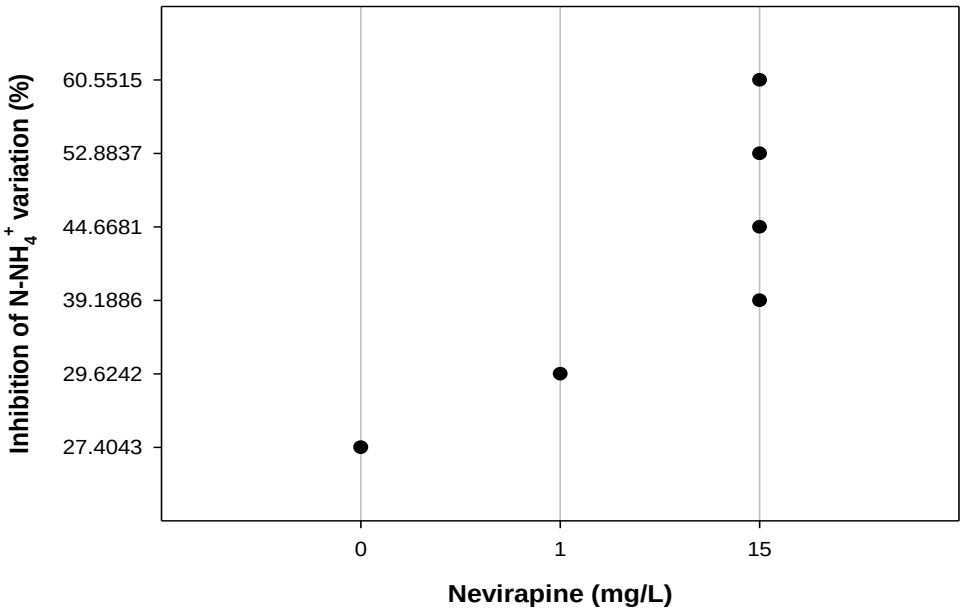


Figure 4. 4 Inhibition of the specific N-NH_4+ evolution rate as a function of Nevirapine concentration

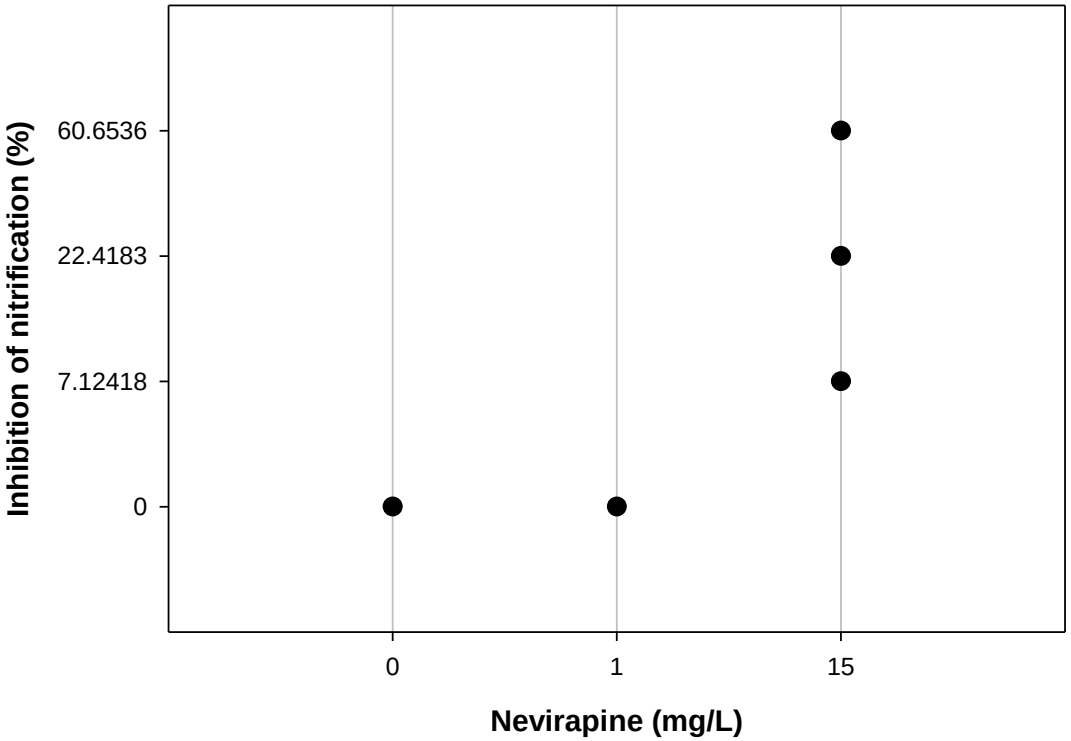


Figure 4. 5 Inhibition of the specific nitrification rate as a function of Nevirapine concentration

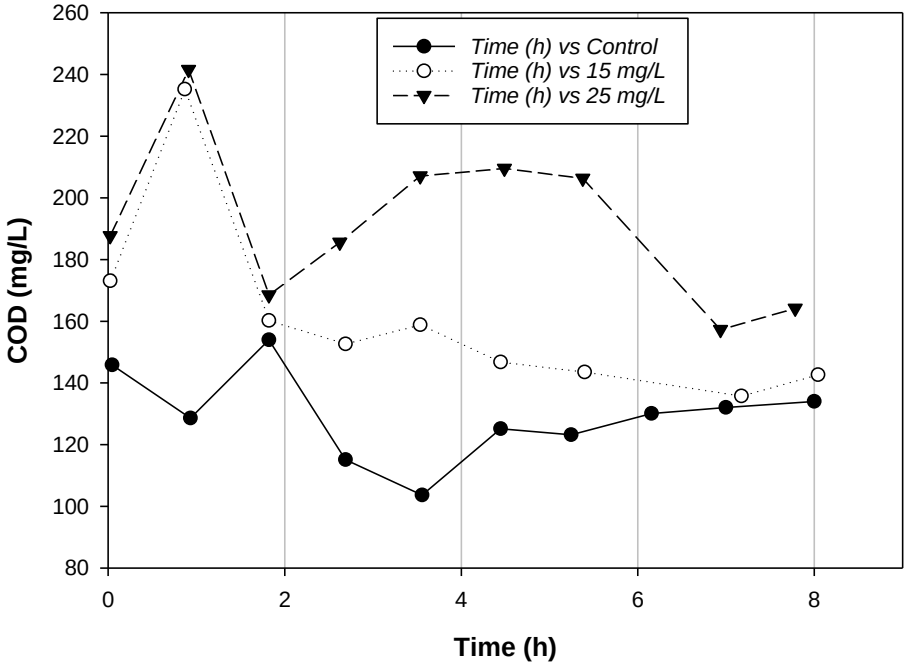


Figure 4. 6 Example of the evolution of COD versus time as a function of nevirapine concentration (control, 15 mg/L, 25 mg/L).

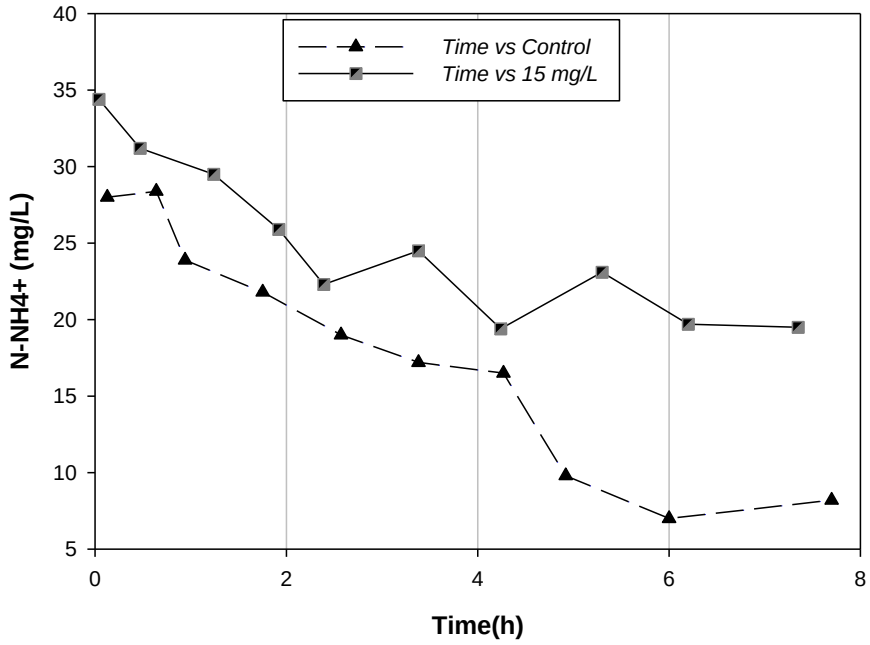


Figure 4. 7 Example of $N\text{-}NH_4^+$, $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ concentration over time as a function of Nevirapine concentration (control, 15 mg/L) for the BWT

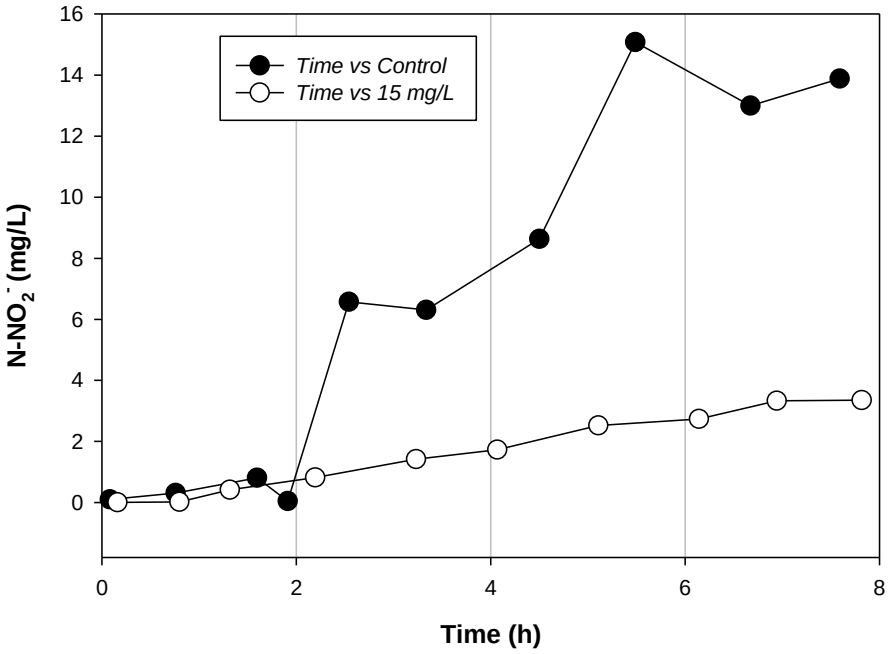


Figure 4. 8 Example of N-NH_4+, N-NO_2- and N-NO_3- concentration over time as a function of Nevirapine concentration (control, 15 mg/L) for the BWT sludge

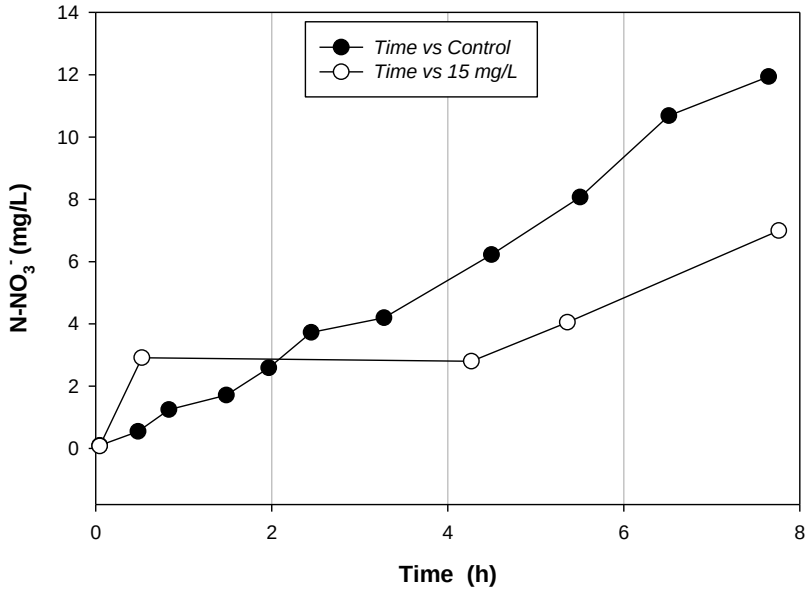


Figure 4. 9 Example of N-NH_4^+, N-NO_2^- and N-NO_3^- concentration over time as a function of Nevirapine concentration (control, 15 mg/L) for the BWT sludge.

Table 4. 3 Summary of $N\text{-}NH_4^+$ and COD concentration variation as a function of Nevirapine concentration

Nevirapine	$N\text{-}NH_4^+$ specific rate	COD specific rate	Number of experiments
mg/L	mg N/ (gSS.h)	mgO_2/ (gSS.h)	
1	-0.04	-22	(n=2)
15	-0.03	-13	(n=3)
25	-0.4	-18	(n=2)

The inhibition of nitrification was high and varied for different samples with 15 mg/L nevirapine, probably indicating an even discharge of the inhibitory substance. Another possible explanation could be that the samples were taken as random samples and not daily samples, resulting in fluctuations in the chemical composition caused by a batch-wise release of different process streams. But overall, the experiments with 15 mg/L nevirapine showed consistently higher inhibition of nitrification than those without nevirapine. To some extent, the inhibition of nitrification has been shown to be related to the toxicity of substance (s) in the wastewater (Klein and Tenno, 2019). However, inhibition of nitrification varies depending on the sludge type at WWTPs, because sludge bacteria that are exposed to certain toxicants under a period might adapt to these and work up a steady growth under the chemical exposure (Jönsson, 2001). It is therefore important to take note that results from the inhibition of nitrification may differ greatly depending on the bacterial consortium involved (Jönsson, 2001; Kelly et al., 2004).

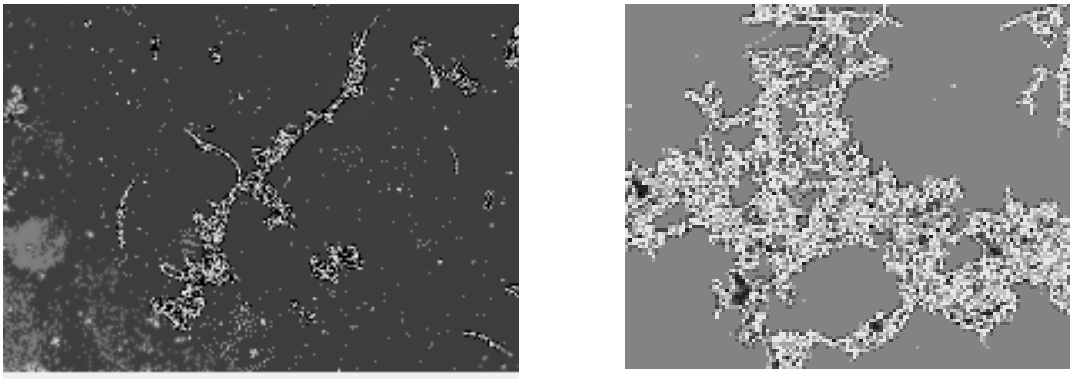


Figure 4. 20 Activated sludge floc images (from BWT sludge) at the beginning of the experiment (left) and after 240 min (right) in a system containing wastewater spiked with 15 mg/L Nevirapine.

4.4 CONCLUSION

The non-nucleoside reverse transcriptase inhibitors (NNRTIs) drug (Nevirapine) was found to be toxic to the activated sludge from BWT. Though the global performances can be evaluated conventionally (in %), in this study the quantity of pollutant removed per unit of total suspended solids (TSS) removed was used as a normalization parameter. Nevirapine reduced the specific COD and $N\text{-}NH_4^+$ concentration variation rates. Although the limit values for nitrification should be measured from consistent daily samples, the random samples showed a clear indication of nevirapine introducing disturbances in the WWTP. However, no definitive conclusion about the inhibition of nitrification in the actual WWTPS cannot be drawn yet, as the true concentrations of nevirapine or similar non-nucleoside reverse transcriptase inhibitors (NNRTIs) drug in the existing WWTPs are not known.

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CHAPTER 5: NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR (NNRTI) DRUG (NEVIRAPINE) TIME-KILL ACTIVITY AT LOW CONCENTRATION IN A SIMULATED CLOSED LABORATORY SCALE WASTEWATER TREATMENT PLANT (WWTP)

Abstract

The fate of micropollutants in the environment has become an increasing issue in the last two decades, especially in heavily urbanized areas. In parallel with traditional pollutants, a large number of molecules such as pesticides, pharmaceuticals and personal care products (PPCPs), flame retardants, etc. which pose a potential threat to the environment are detected in it. Furthermore, the fate of pollutants within the WWTPs is well studied today and WWTP effluents are generally considered an important source of contamination for a long time, especially in concentrated urban areas. This demands an imperative for a better understanding of micropollutants' behaviour within wastewater treatment processes. This paper investigates nevirapine toxicity toward activated sludge as a function of exposure time at low concentration. Experiments were conducted both in closed mode (batch equivalent) and imaging techniques combined with an L7007 LIVE/DEAD BacLight viability kit (Invitrogen, South Africa), allowed to assess nevirapine time-kill activity. The nevirapine toxicity was observed at low concentration when exposure time increased. A 0.1 mg/L nevirapine concentration was toxic to heterotrophic bacteria on a closed mode and inhibited nitrification. These findings are in agreement with the microscopic studies, which showed a latency time before the lower nevirapine concentrations began to kill the bacteria. After 40 minutes, there were 97 % (SD 3.8) of living bacteria in control reactors, 76 % (SD 3.1) in reactors that contained 0.1 mg/L nevirapine and 46 % (SD 18.6) in the system that contained 10 mg/L nevirapine.

5.1 INTRODUCTION

The impact of humans on their environment either directly (pollution, hole in the ozone layer, acid rain, etc.) or indirectly (climatic disturbance) is an issue that has long been a concern for scientists (Evgenidou 2015; Gao et al. 2012; Deblonde et al. 2011). It is also expected that the increase of the world population, which could soon reach 9 billion, will add more pressure to the environment (Nagarajan et al. 2019; Azizi et al. 2013). The large proportion of pollutants resulting from human activity, especially in urban areas, find their ways into water bodies, resulting in significant water quality degradation. Thus, in many major cities, a large panel of micropollutants is frequently found in surface water (Cronin et al. 2003; Wakida and Lerner 2005; Kemka et al. 2006). The estimated number of people receiving ARV treatment as of 2016 was 19.8 million. South Africa had the biggest antiretroviral treatment (ART) programme estimated at 3.9 million people receiving ARVs, about 24% of the world ART program. Nevirapine (NVP) is an oral medication used to treat and prevent retroviral infections primarily human immunodeficiency virus type 1 (HIV-1). HIV-1 is a virus that attacks mainly the CD4-T-cells responsible for the body's immune system. However, antiretroviral treatment against HIV-1 does not cure or kill the virus but rather prevents or slows down its multiplication (Deeks et al. 2013). It is generally recommended for use with other ARV medication. Nevirapine is a non-nucleoside reverse transcriptase inhibitor (NNRTI) ARVD which is commonly given to pregnant women to inhibit the transfer of HIV to the unborn baby. The nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) act early in the replication viral cycle by preventing reverse transcription of the viral RNA of the HIV into its DNA, an essential step before the virus could integrate itself into the host cell. This is achieved by interfering with a viral enzyme responsible for this purpose called reverse transcriptase. Its excretion via urine is at 2.7% after ingestion (Schoeman et al. 2017; Swanepoel et al. 2015). In the last two decades the emission of the “emerging” or “new” unregulated contaminants has become disproportional environmental problem, and there is widespread consensus that this form of contamination may merit urgent legislative intervention to avoid their potential long-term harm to human health and other ecological organisms (Stumpf et al. 1999; Kummerer 2001; Heberer et al. 2002; Carballa et al. 2004; Ternes et al. 2004; Breggin and Pendergrass 2006; Cunningham 2006; Powell et al. 2008). The contaminants mainly are from products used in large quantities in everyday life, for example, human and veterinary pharmaceuticals, personal care products (e.g. cosmetics and sunscreens), surfactants, paints, and surfactant residues, plasticizers, and various industrial additives. Although these contaminants may be non-persistent in the environment

(though for many of them the studies are inconclusive), however, their high degree of potential transformation and removal rates are offset by their continuous introduction into the environment due to large usage volumes (Snyder et al. 2003) and wide geographical loci of applications. Many chemical substances and mixtures are toxic to bacteria if they are present in very high concentrations and can cause a decrease of the biological process as in the case of a WWTP. The discharge of emerging drugs and their residues can disturb aquatic ecosystems [Sacca et al. 2009; Singh et al. 2008; Accinelli et al. 2007] and may increase bacterial resistance in water environments [Bezuidenhout et al. 2016; Christen et al. 2010; Fent et al. 2006; Santos et al. 2010; Baquero et al. 2008]. Whether the resistance develops in the WWTP is currently under discussion (Li et al. 2010; Storteboom et al. 2010; Gao et al. 2012). However, most WWTPs focus on bacterial activity and are not designed to cope with emerging drugs that can be toxic to their biomass. This toxicity can reduce chemical oxygen demand (COD) removal and nitrification, which are key processes in WWTPs. Besides influent and effluent characteristics, the biomass activity should be monitored, since the bacteria are responsible for pollutant degradation.

There is still a lack of data describing the effects of emerging drugs on wastewater treatment processes, especially when it comes to studying their long-term effects on living organisms. Nevertheless, a few studies have already highlighted the importance of these effects [Petrovic et al. 2003] and have reported that chronic toxicity of low concentrations of emerging drugs can decrease the WWTP efficiency [Tran et al., 2018]. As most WWTPs rely on the bacterial consortium to treat pollution (Emara et al. 2014; Evgenidou 2015), it seems reasonable within the South African context to investigate the potential effect of a widely used antiretroviral drug such as nevirapine, on activated sludge. Consequently, this study uses short- and long-term toxicity tests in closed operating systems, and imaging techniques to assess nevirapine toxicity on activated sludge processes.

5.2 MATERIALS AND METHODS

The materials and methods for carrying out the experiments are discussed in Chapter 3. 0.1, and 10 mg/L concentrations of nevirapine were used for each batch of the wastewater treatment.

5.3 RESULTS AND DISCUSSION

5.3.1 24-H Closed Tests

24-h experiments showed the acute toxicity of nevirapine. Inhibition levels seem to increase with the increase in nevirapine concentration. When using the highest nevirapine concentrations (10 mg/L), nitrification inhibition was noticed from the beginning of the experiment. Despite a low N-NO_3- production, there were an N-NH_4^+ accumulation and no N-NO_2^- in the system spiked with 10 mg/L nevirapine (Figure 5.1). In contrast, in the system spiked with 0.1 mg/L nevirapine, the N-NO_2^- and N-NO_3^- production was not inhibited during the first h of the experiments but was inhibited during the last 4 h period (Figure 5.1 and 5.2). However, for the same 10 mg/L concentration, there was an N-NH_4^+ accumulation during the first period and again during the last 4h period. This N-NH_4^+ overproduction is probably the results of the ammonification of biomass by-products coming from the destroyed biomass. The release of biomass by-products increased the soluble COD as shown in Figure 5.3. The decrease in nitrification rate was not observed for a 0.1 mg/L nevirapine concentration. However, COD measurement showed a release of by-products from the biomass that increased the soluble COD even for the 0.1 mg/L nevirapine concentration.

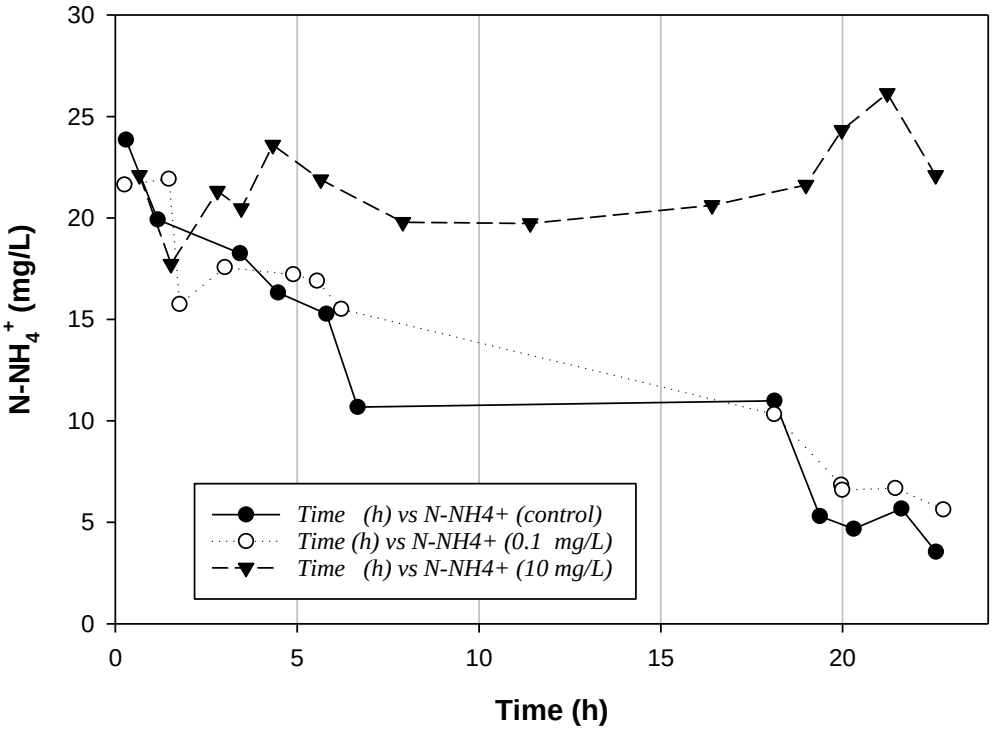


Figure 5. 1 Specific $N\text{-}NH_4^+$ concentration variation versus time as a function of nevirapine concentration

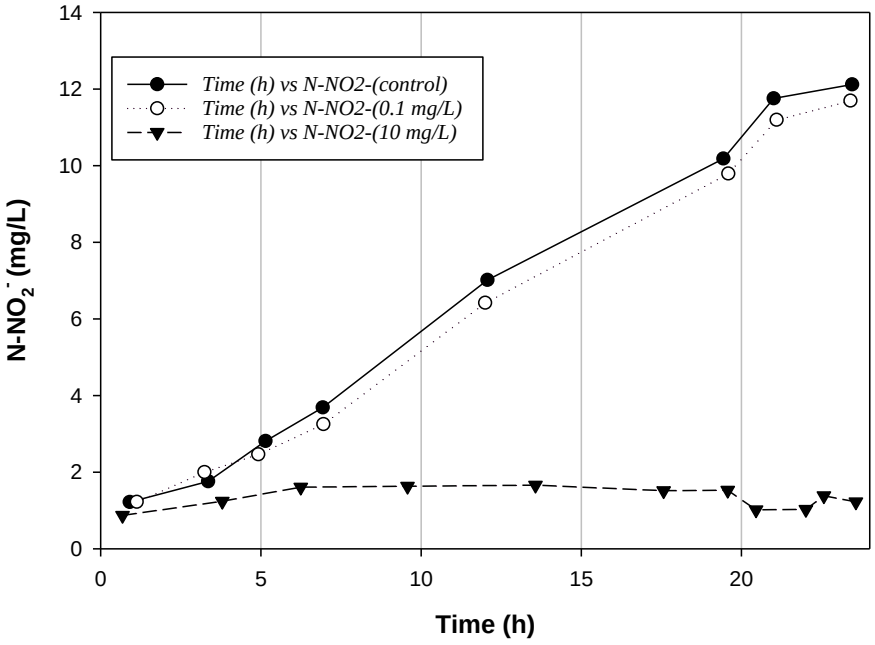


Figure 5. 2 Specific $N\text{-}NO_2^-$ concentration variation versus time as a function of nevirapine concentration

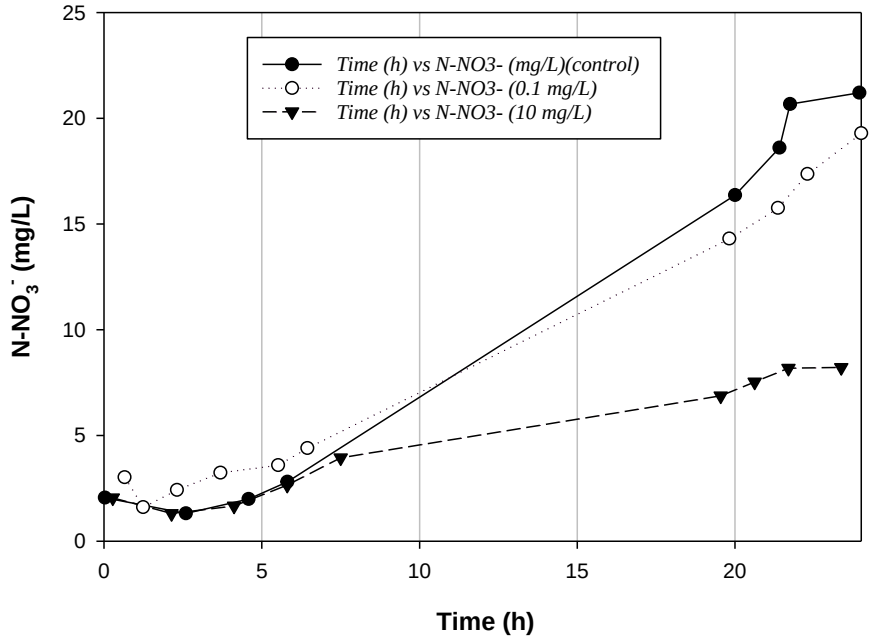


Figure 5. 3 Specific $N\text{-}NO_3^-$ concentration variation versus time as a function of nevirapine concentration

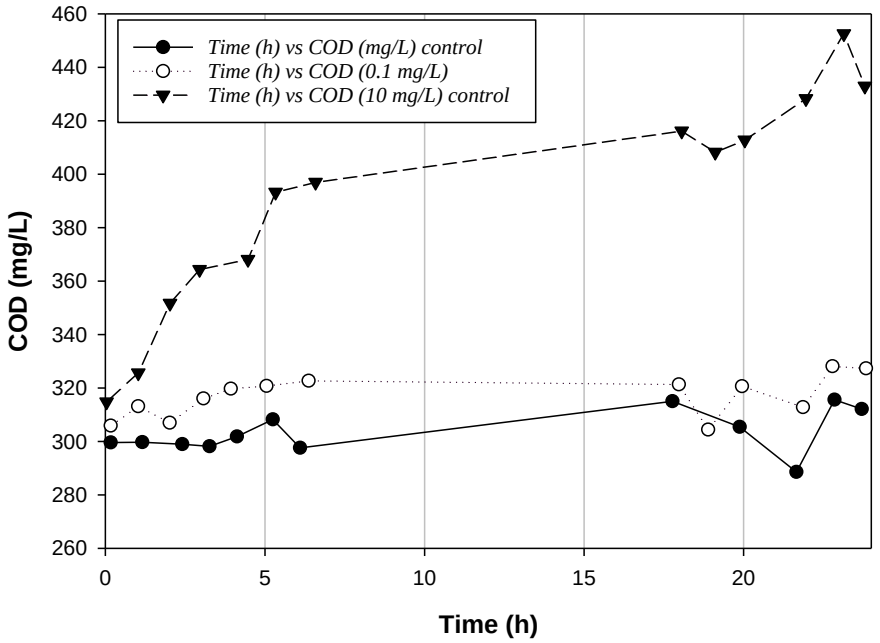


Figure 5. 4 Soluble COD concentration variation versus time as a function of Nevirapine concentration

5.3.2 7-Days Closed Tests

Nevirapine toxicity was observed at lower concentrations during a prolonged test period of 7 days on both nitrifiers and heterotrophic bacteria as 0.1 mg/L nevirapine concentration inhibited the nitrification (Figure 5.5).

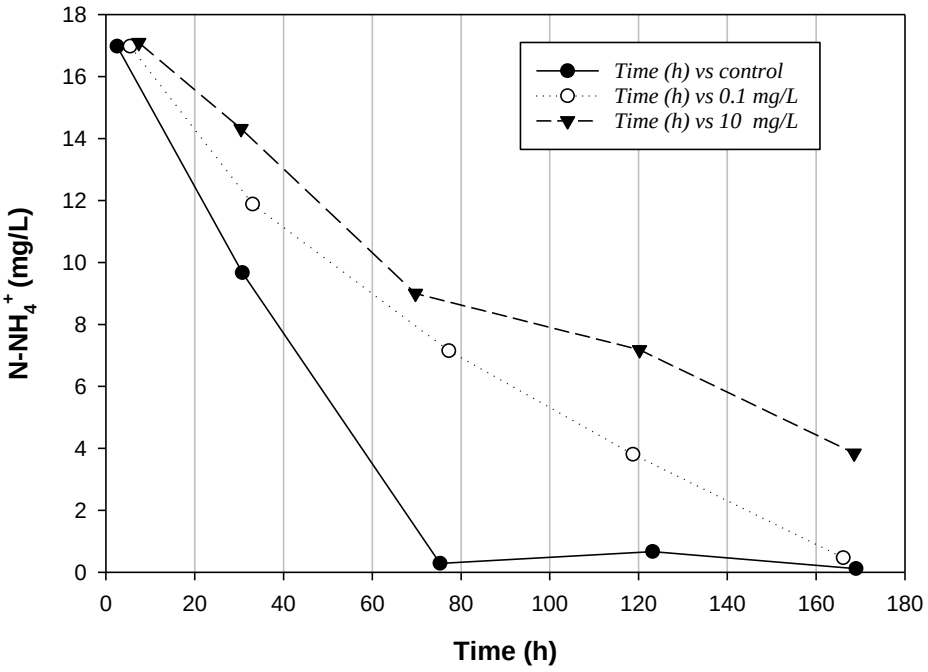


Figure 5. 5 $N\text{-}NH_4^+$ concentration variation versus time as a function of the nevirapine concentration

However, only a small difference was observed between the control reactors and reactors with 0.1 mg/L nevirapine during the first 20 h (Figure 5.5). A possible explanation could be that the nevirapine toxicity on activated sludge depends not only on the nevirapine concentration but also on the exposure time. Therefore, one would expect a reduced nevirapine transfer rate into the exopolysaccharide barrier and the cells membranes at nevirapine concentration are reduced. This suggests that the nevirapine even at low concentrations as one would expect in urban WWTP influents could still be toxic to activated sludge. A 7 days period closed test with lower biomass concentration was found to be a valuable experimental set up to study low nevirapine concentrations effects on activated sludge.

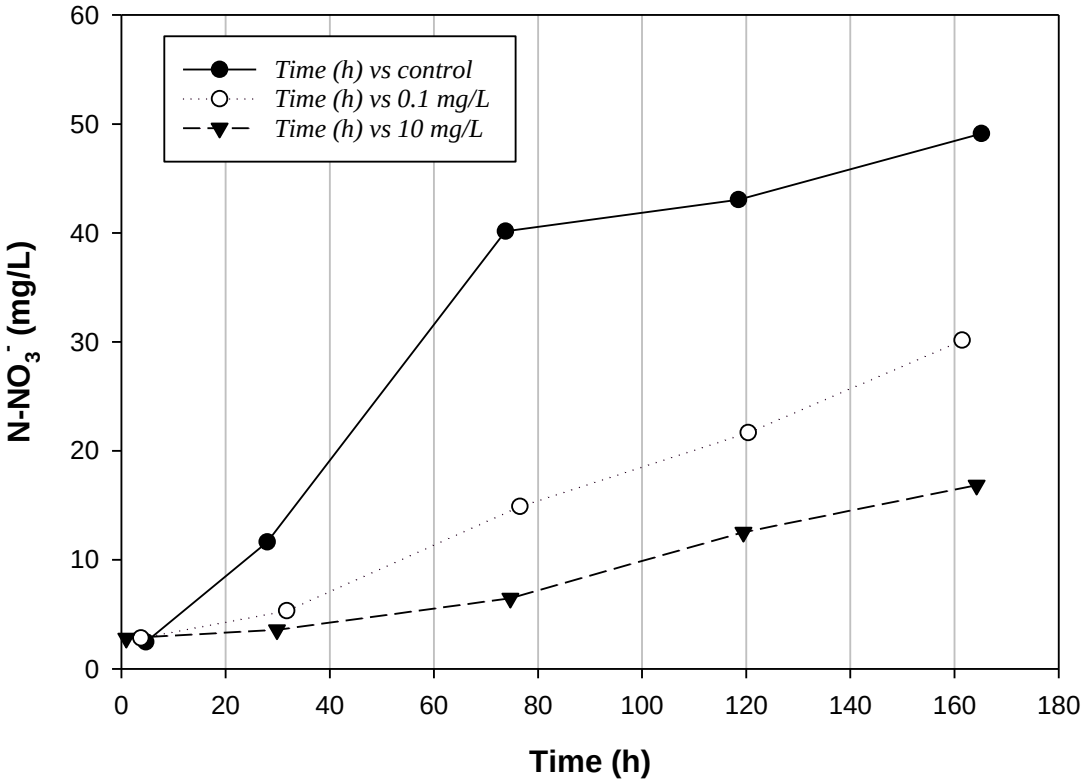


Figure 5. 6 N-NO_3- concentration variation versus time as a function of the nevirapine

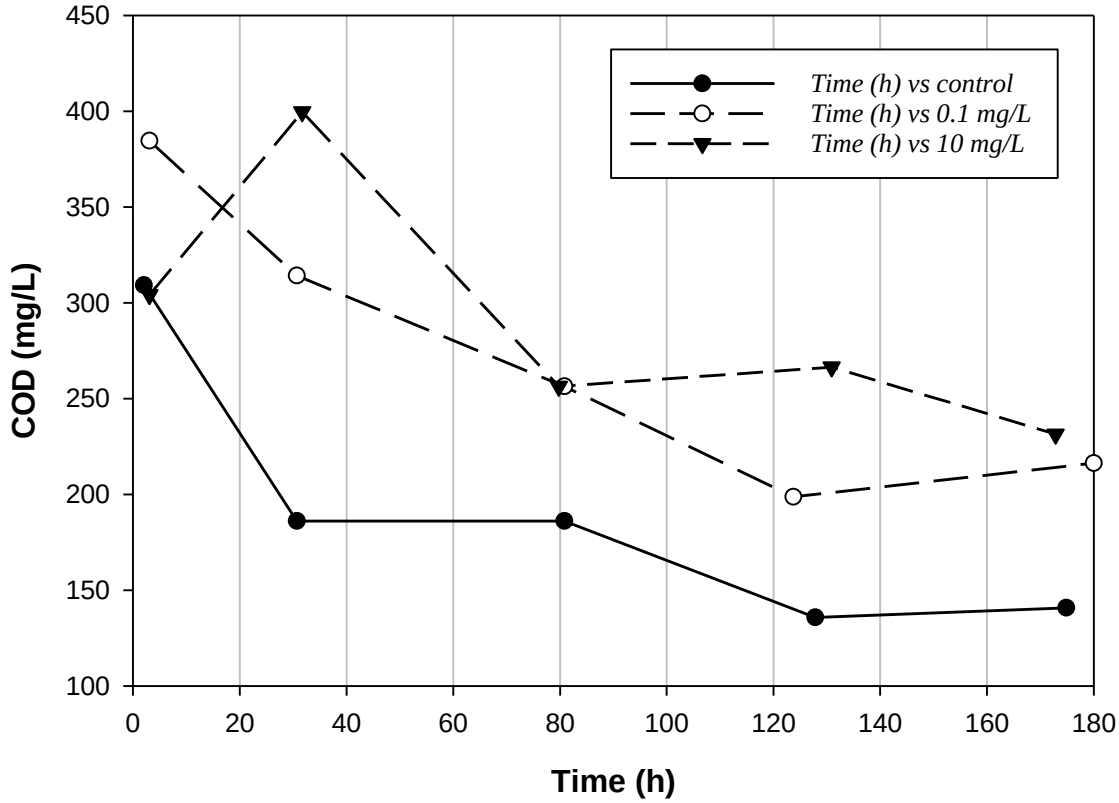


Figure 5. 7 Soluble COD concentration variation versus time as a function of Nevirapine concentration

5.3.3 Activated Sludge Morphology & Toxicity Assessment

The deterioration of activated sludge morphology was monitored by image analysis for different nevirapine concentrations. The LIVE/DEAD® BacLight bacterial viability kit technique was used to examine bacterial viability because of its distinctive advantage to visually discriminate between live and dead cells for a broad range of bacterial species found in wastewater. The results of the control and test units showed sparse agglomerates of cells as evidence of floc disintegration (Figure 5.9). Results showed also a latency period before the bacteria began to die. Although a contingent of fluorescent red cells in test units spiked with nevirapine (Figures 5.10b and c) may indicate some bacterial cell membrane damage compared with the negative controls (Figure 5.10a), a large contingent of fluorescent green cells is still evident in these systems. Moreover, some cells with compromised membranes were also observed in the control units due to natural mortality. The ability of the activated sludge to remove COD and degrade organic material was slightly hampered in the test units (0.1 mg/L and 10 mg/l nevirapine). Figure 5.10 shows the bacterial viability in the control and test unit

activated sludge flocs viewed by fluorescence microscopy, with live (green fluorescent) and dead (red fluorescent) bacterial cells. The wastewater system showed some resilience upon short term exposure to nevirapine as shown in Figure 5.8. This latency time was shorter with high nevirapine concentrations. After 40 minutes there were 97 % (SD 3.8) of living bacteria in control reactors, 76 % (SD 3.1) in reactors that contained 0.1 mg/L nevirapine and 46 % (SD 18.6) in the system that contained 10 mg/L nevirapine (Figure 5.8). As a result of biomass destruction, a floc breakage was observed as shown in Figure 5.7 for the wastewater spiked with 10 mg/L nevirapine. It is possible that this biomass deflocculation could promote the environmental release of bacteria and nevirapine-resistant genes possibly acquired by bacterial species in the WWTP. The latency period before the bacteria began to die confirmed the importance of both concentration and exposure time. The implication of these results is that both the nevirapine concentration variations in the wastewater influent and the WWTP hydrodynamics could impact nevirapine toxicity on activated sludge. At the scale of flocs, the toxicity should also depend on the hydrodynamics, as it was found that bacteria in the outer surface of flocs were the first ones to die.

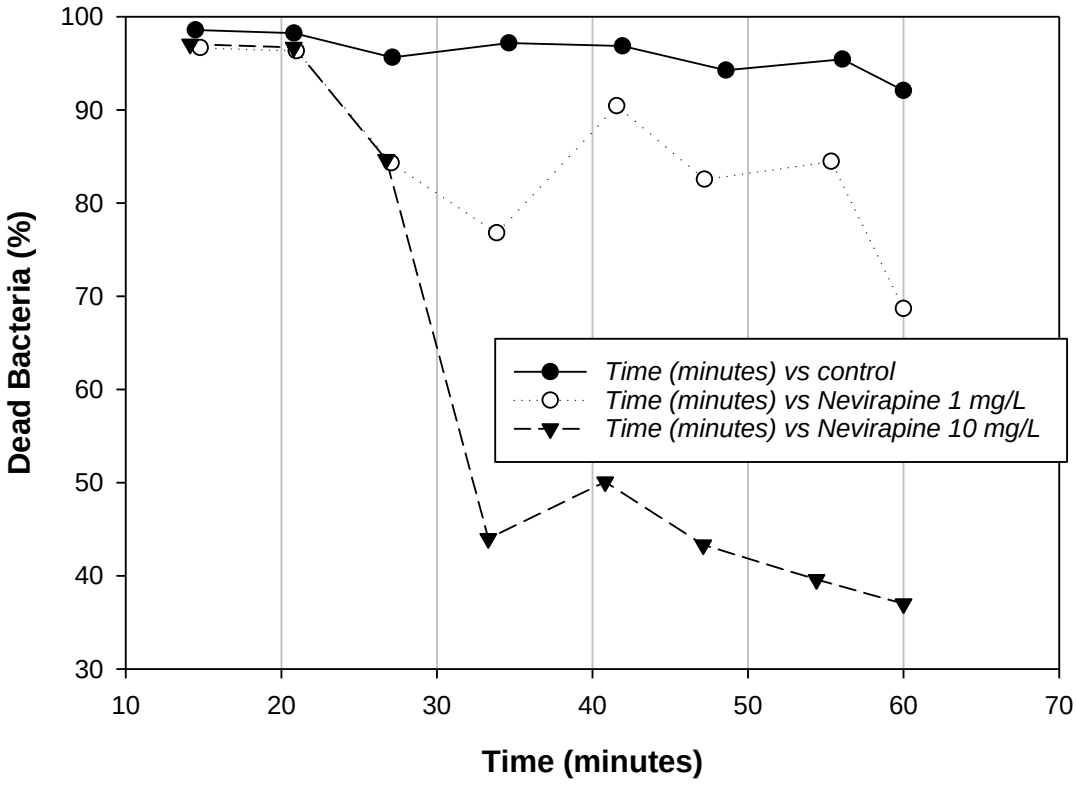


Figure 5. 8 Per cent live bacteria evolution versus time in microscopic slide wells in the function of nevirapine concentration

However, further studies examining quantitative resilience limits of specific bacteria and protozoa to nevirapine exposure may be needed, particularly over an extended period of time to investigate possible bacterial adaptation or potential of being comprised after a certain time. Moreover, many older and smaller WWTPs employ fixed-film biological reactors (e.g. trickling filters) rather than the suspended biomass systems reported in this paper. On that basis, further research on the removal of nevirapine by attached microbial communities is therefore needed. Additional research would also be needed to understand the possible release of nevirapine or similar non-nucleoside reverse transcriptase inhibitor (NNRTI) drug from sludge incineration as well as their impact to the soil where wastewater sludge is used for agricultural purposes.

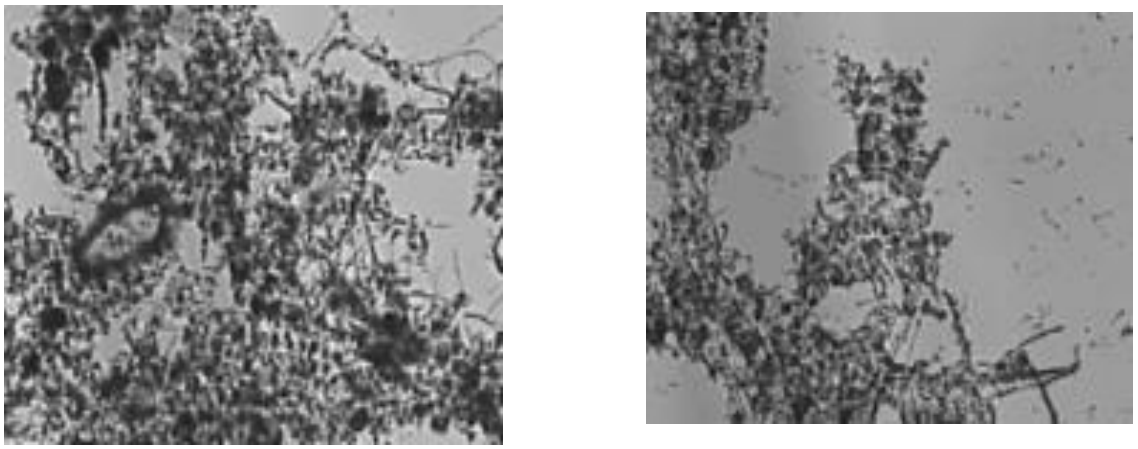
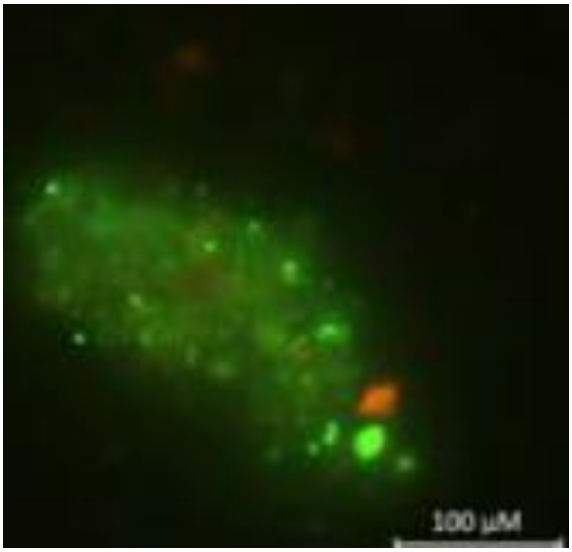
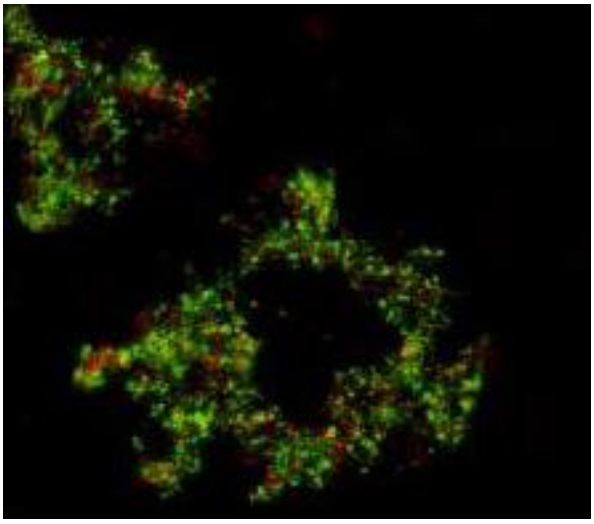


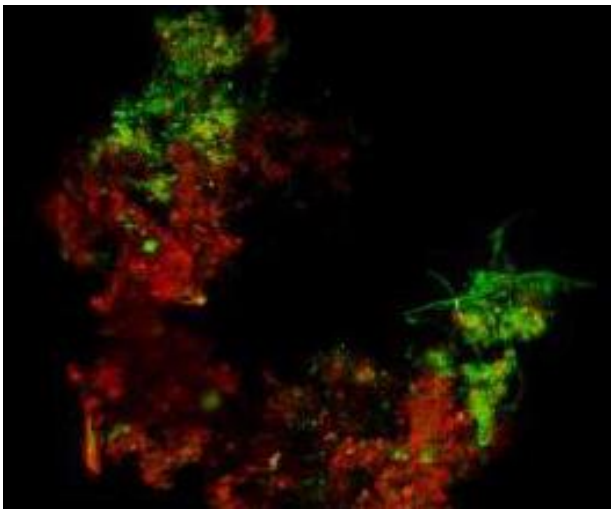
Figure 5. 9 Floc morphology evolution versus time in the system with 10 mg/L nevirapine concentration after 20 minutes (left), 4 h (right)

(a)





(b)



(c)

Figure 5. 1 Bacterial viability in control activated sludge (a) and bacterial viability in activated sludge exposed to (b) 0.1 mg/L nevirapine (c) 10 mg/L over 168 hr viewed by fluorescent microscopy

5.4 CONCLUSION

The non-nucleoside reverse transcriptase inhibitors (NNRTIs) drug (Nevirapine) was found to be toxic to the activated sludge Bushkoppie WWTP. The results showed that both the concentration and exposure time should be taken into account when studying nevirapine toxicity on activated sludge bacteria. The effect of low concentrations was observed after the four-h' time period usually used in toxicity tests. Fluorescence microscopy was useful to assess nevirapine time-kill activity. However, further studies may be required to examine the

quantitative resilience limits of specific bacteria and protozoa to nevirapine exposure, particularly over an extended period of time to investigate possible bacterial adaptation or potential of being compromised after a certain period time. Moreover, many older and smaller WWTPs employ fixed-film biological reactors (e.g. trickling filters) rather than the suspended biomass systems reported in this paper. On that basis, further research on the removal of nevirapine by attached microbial communities is therefore needed.

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CHAPTER 6: ACTIVATED SLUDGE BEHAVIOUR IN BATCH MODE IN THE PRESENCE OF BOTH NON-NUCLEOSIDE AND NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR DRUGS (NEVIRAPINE & LAMIVUDINE): EFFECTS ON EXTRACELLULAR POLYMERIC SUBSTANCES (EPS)

Abstract

Limiting the discharge of micropollutants to the environment has become a major preoccupation. Consequently, the ineffectiveness of WWTPs to eliminate them as well as the contamination of WWTP effluents by micropollutants has taken centre stage particularly. The scarcity of water and the need for energy and food on the global stage require that the feasibility of wastewater recycling and resource recovery be further explored. This paper examines the influence of Nevirapine and Lamivudine on municipal sludge in batch reactors. The study focused on extracellular polymeric substances (EPS) as an indicator of bacteria sensitivity to the mentioned drugs. The EPS were analyzed by FT-IR spectroscopies. It was found that both Nevirapine and Lamivudine induced a significant increase of bound EPS in flocs. This may be attributed to a protection mechanism by the bacteria. However, only Nevirapine inhibited COD and nitrogen removal.

6.1 INTRODUCTION

Globally and particularly in South Africa, owing to water scarcity, the partial or complete closure of water cycles should be an integral part of the sustainable water resource management (Pollard and Du Toit, 2008). One option is by increasing the re-use of effluents for various purposes especially within the industrial and agro/food production applications (Zajda and Aleksander-Kwaterczak, 2019). However, because of the high cost of the end-of-pipe approach, indirect potable water re-use requires efficient treatment of wastewaters prior to their discharge (Zajda and Aleksander-Kwaterczak, 2019; Petrovic et al., 2003). Nonetheless, it is without question that the propensity of freshwater contamination will rise in the coming years because (i) human population continues to grow, and/or (ii) patterns of natural surface water have become very low with the wastewater constituting the larger fraction of the flow

(Chapman, 2018). This is very true in the case of Gauteng province and other high notch economic hubs in South Africa. In view of these factors, among others, South Africa is particularly vulnerable as it is a semi-arid country with rapidly increasing annual population and steady industrial growth (Mkonda and He, 2018; Oberholster, 2018).

The situation is increasingly being exacerbated by additional factors like fluctuating natural seasonal flow as well as climate-change/prolonged drought that drastically alter the environmental concentrations of the emerging contaminants in water bodies due to no or low dilution (Chen et al., 2018). Therefore, the need for establishing the short-, medium-, and long-term effects of the emerging contaminants in the face of diminishing environmental absorption capability of the contaminants has become urgent, and of national importance (Costa et al., 2018). Thus, the occurrence of trace emerging contaminants in wastewaters, their behaviour during wastewater treatment and production of drinking water are key issues that require further investigation to highlight the need for a more comprehensive understanding of their environmental behaviour (Xing et al., 2018; Tran et al., 2018).

South Africa has the biggest antiretroviral treatment (ART) programme estimated at 3.9 million people receiving ARVs, about 24% of the global ART program. Nevirapine (NVP) is an oral medication used to treat and prevent retroviral infections primarily human immunodeficiency virus type 1 (HIV-1). HIV-1 is a virus that attacks mainly the CD4-T-cells responsible for the body's immune system. However, antiretroviral treatment against HIV-1 does not cure or kill the virus but rather prevents or slows down its multiplication (Deeks et al. 2013). It is generally recommended for use with other ARV medication.

Nevirapine is a non-nucleoside reverse transcriptase inhibitor (NNRTI). It was the first NNRTI to be approved by the Federal Drug Administration (FDA) for use in combination therapy of HIV-1 infection in 1996. It has been approved for use in children of 2 months or older and has been widely used as single-dose prophylaxis for prevention of mother-to-child HIV transmission (MTCT) in resource-poor settings (Wood, 2005). NNRTIs are non-competitive inhibitors of HIV-1 RT because they are mainly metabolized by the CYP3A subfamily of cytochrome P450 enzymes. Nevirapine (NVP) can induce its own metabolism mediated by CYP450 with the production of several hydroxylated metabolites, with subsequent glucuronidation. Other NNRTIs are delavirdine and efavirenz.

Lamivudine is a nucleoside reverse transcriptase inhibitor (NRTI) whose behaviour and fate in water environments is quite unknown, except that it is very stable to various forced

decomposition conditions of hydrolysis (neutral), UV light and thermal stress as well as low concentration of H_2O_2 (Bedse et al., 2009; An et al., 2011). They are considered the backbone of HIV treatment because they act early in the replication viral cycle by preventing reverse transcription of the viral RNA into its DNA, an essential step before the virus could integrate itself into the host cell. This is achieved by interfering with a viral enzyme responsible for this purpose called reverse transcriptase (Ncube et al., 2018). Its excretion via urine is at 2.7% after ingestion (Schoeman et al. 2017; Swanepoel et al. 2015). Other NRTIs are zidovudine, didanosine, zalcitabine, stavudine, abacavir, and emtricitabine.

The reverse transcriptase (RT) of HIV is actually the target for three classes of inhibitors: nucleoside RT inhibitors (NRTIs), nucleotide RT inhibitors (NtRTIs) and non-nucleoside RT inhibitors (NNRTIs). The NRTIs and NtRTIs interact with the catalytic site (that is the substrate-binding site) of the enzyme, whereas the NNRTIs interact with an allosteric site located a short distance from the catalytic site. For the NRTIs and NtRTIs to interact with the substrate-binding site, they need to be phosphorylated to the triphosphate and diphosphate forms, respectively. As competitive inhibitor of the normal substrate, the ddNTP will inhibit incorporation of this substrate into the growing DNA chain; as alternate substrate, it will be incorporated into this chain (as ddNMP), thereby acting as a chain terminator (since the ddNMP is missing the 3’-hydroxyl group required for further chain elongation) (Andrande, 2011; De Clercq, 2007).

Very little attention has been paid in South Africa to the ARV drugs effects on bacterial behaviour, especially on extracellular polymeric substances (EPS) from the metabolism of bacteria. EPS production is a general property of microorganisms and is a response to stress situations (Lotti et al., 2019), so they can be regarded as an indicator of bacteria's "well-being." Microorganisms liable for pollutant degradation in wastewater treatment grow and develop in aggregated forms as biofilm or flocs wherein the main aspect are EPS (from 50 % up to 90 % of total natural count). The EPS are key constituents of the floc and biofilms and ultimately determine their physicochemical and biological properties (Shao et al., 2019).

The impact of Nevirapine and Lamivudine on activated sludge in batch reactors was investigated. The responses of real sludge issued from a municipal WWTP to shock load of ARV drugs was examined. Hence, no acclimation period was planned, and the experiments were carried out in batch reactors with relatively high drugs concentration (25 mg/L). Both the EPS in flocs and the soluble part in the bulk water phase, the total protein and total

polysaccharide contents were examined by UV-Vis spectroscopy. The removal efficiency of the pollutants, COD, and nitrogen was also followed.

6.2 MATERIALS AND METHODS

The materials and methods for conducting the experiments are discussed in Chapter 3.

6.3 RESULTS AND DISCUSSION

The mixed liquor suspended solids (MLSS) were measured at the beginning (t = 0 h) and at the end of the experiment (t = 24 h) (Figure 6.1). A decrease of suspended solids appeared in the first series of experiments in the presence of Nevirapine and Lamivudine (Figure 6.1). This was probably due to the release of organic particulate matter from flocs. In the second series of experiments (Figure 6.2) no significant variation of MLSS was observed and the biomass seemed to be less sensitive to ML than in the case shown in Figure 6.1. The sludge was sampled five weeks later in the municipal WWTP when the microbial consortium could have been exposed to different conditions. The total protein and total polysaccharide concentrations which were determined by classical ultraviolet-visible spectrophotometry are presented in Figure 6.3. It can be seen that their distribution is different in soluble and in bound phases. Soluble phases contain higher polysaccharide content than that of protein. In Figures 6.3 and 6.4, an increase of protein and polysaccharide concentrations was seen mainly in bound EPS in the presence of Nevirapine and to a lesser extent in the presence of Lamivudine.

COD and nitrogen removal are also assessed in all the experiments. Nevirapine and Lamivudine did not alter the bacteria capacity to degrade the pollutants. The COD and nitrogen removal performance were very close in the control reactor and in the reactors with Lamivudine. However, Nevirapine inhibited the biological removal of pollutants. In the reactor with Nevirapine (Figures 6.5 and 6.6), the COD removal was inhibited and the NH_4^+

concentration was reduced only slightly (13%).

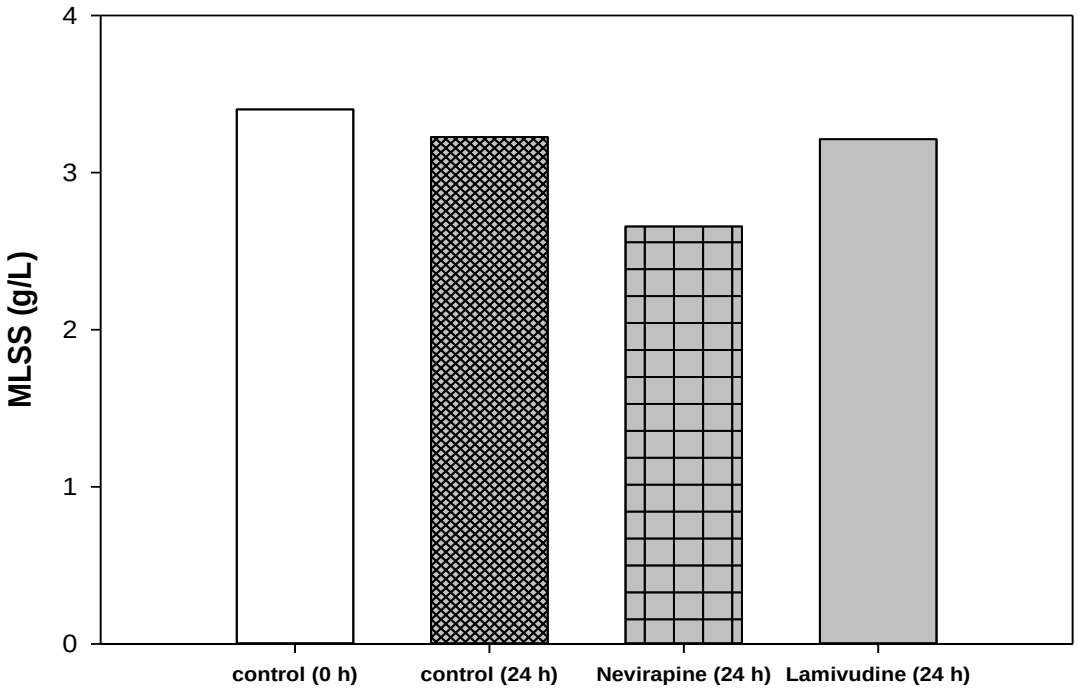


Figure 6. 1 Mixed liquor suspended solids in the control reactor at the beginning of the experiment and after 24 h in reactors with different drugs.

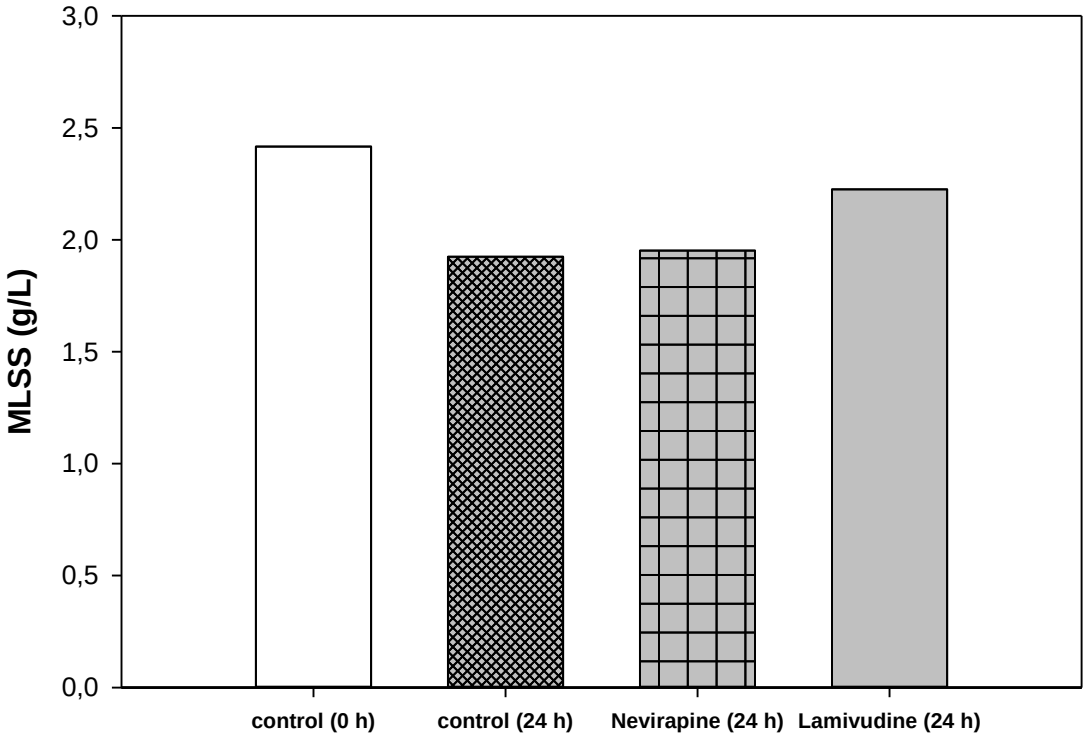


Figure 6. 2 Mixed liquor suspended solids in the control reactor at the beginning of the experiment and after 24 h in reactors with different drugs (repeat experiment)

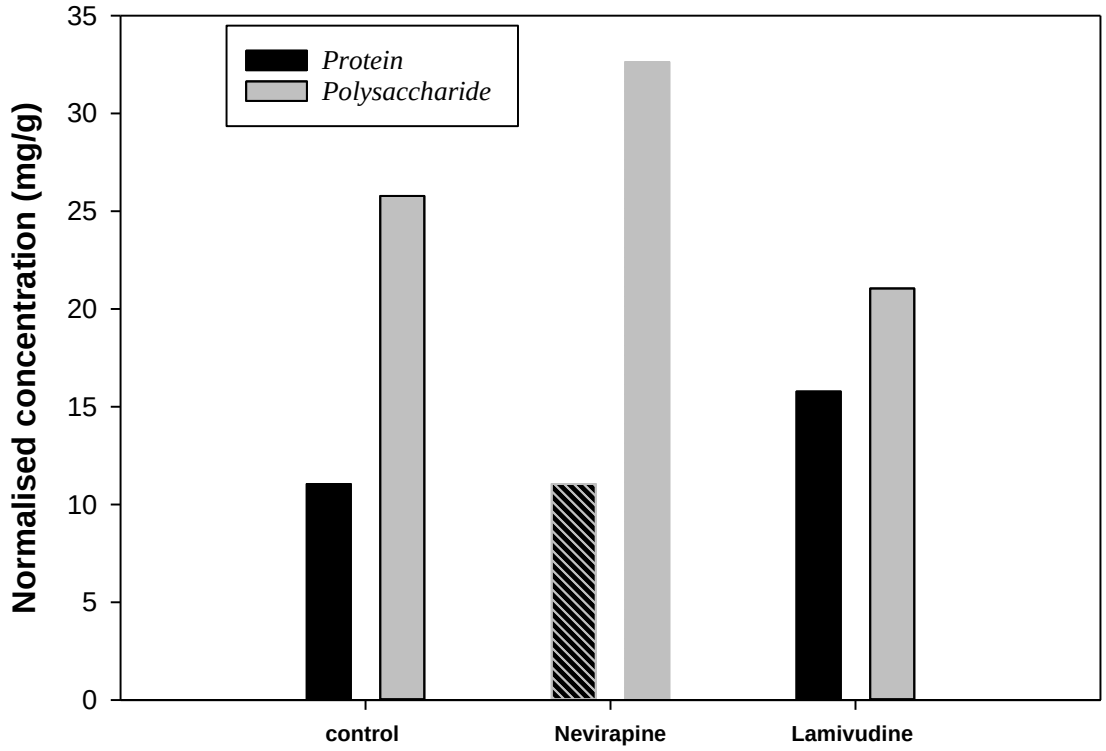


Figure 6. 3 Normalized concentrations (per gram of MLSS) of protein and polysaccharide present in supernatant for control reactor and for reactors with drugs using Ultraviolet–visible spectrophotometry

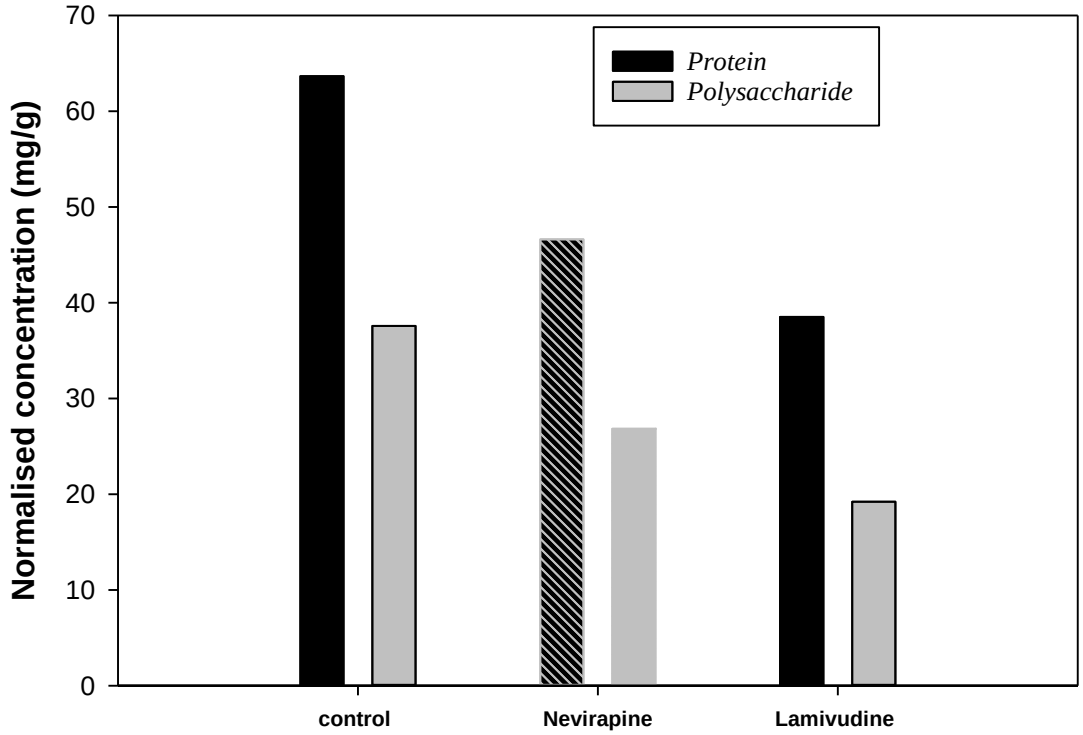


Figure 6. 4 Normalized concentrations (per gram of MLSS) of protein and polysaccharide present in bound EPS for control reactor and for reactors with drugs using Ultraviolet–visible spectrophotometry.

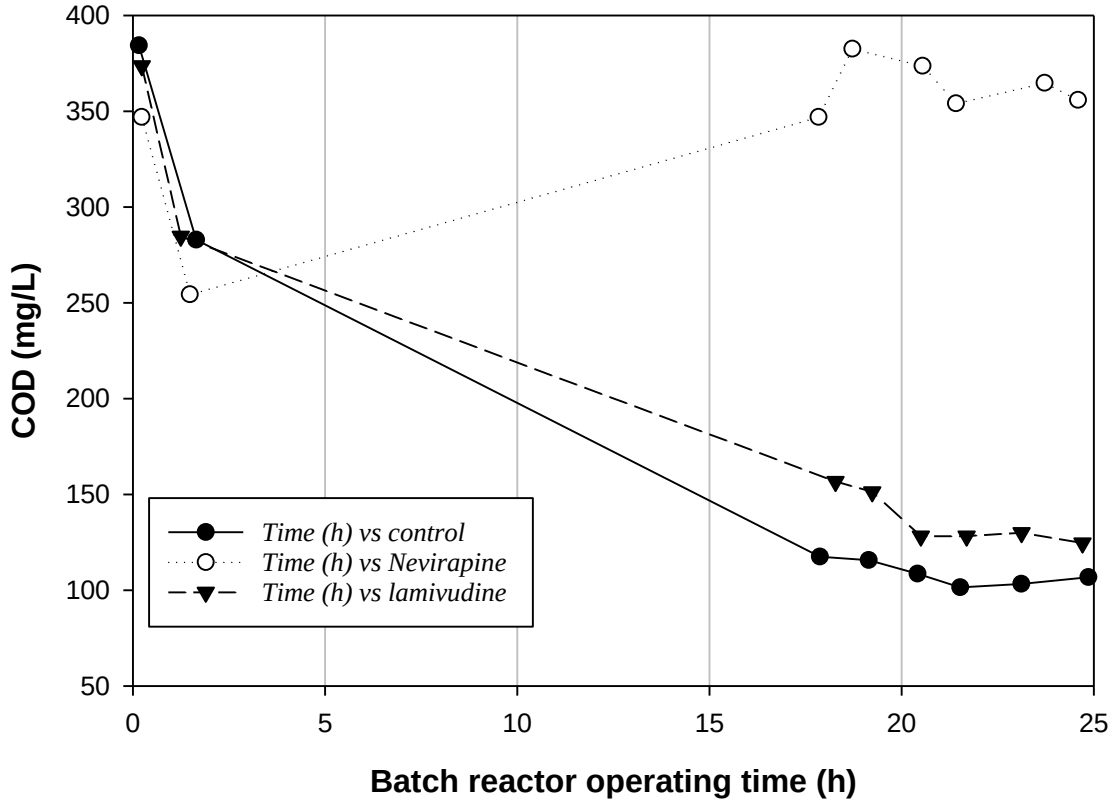


Figure 6. 5 COD over the operation time: in control reactor and in reactors with drugs

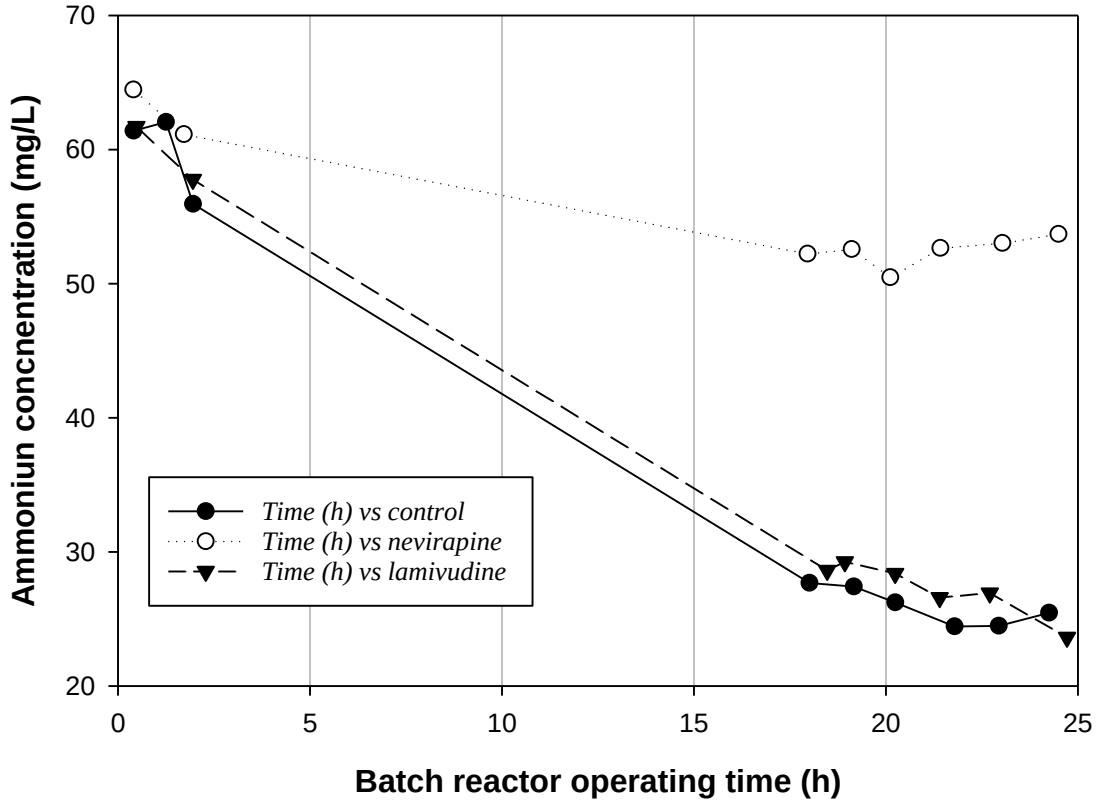


Figure 6. 6 Nitrogen removal over the reactor's operation time: in control reactor and in reactors with drugs

Ultraviolet–visible spectrophotometry and FT-IR analysis, showed that Nevirapine and Lamivudine not only induced an increase of the bound EPS concentration in flocs but also were responsible for a partial disintegration of flocs (observed by the decrease of MLSS) which may be the cause of the release of floc organic matter into the bulk wastewater. The enhanced EPS production in flocs may be a bacteria response to the stress situation to compensate for the loss of protective barrier. Moreover, Nevirapine inhibited the pollutants removal, suggesting that it has a stronger toxic effect on the biomass than Lamivudine.

6.4 CONCLUSION

The influence of Nevirapine (a non-nucleoside reverse transcriptase inhibitor drug) and Lamivudine (a nucleoside reverse transcriptase inhibitor drug) on the biological properties of the activated sludge was studied in lab-scale batch reactors. The study of EPS (bound and soluble fraction) allowed to assess the bacteria sensitivity to these ARV drugs. Nevirapine and Lamivudine induced an increase of bound EPS in flocs. The release of flocs organic matter to bulk wastewater was also observed. The toxic effect of Nevirapine on the biomass was more impactful than that of Lamivudine. The presence of Nevirapine inhibited the removal of the COD and nitrogen. However, it is noteworthy that biomass in WWTP can vary depending on the treatment process, pollutants or climatic conditions. Thus, it may be sensitive to the two drugs mentioned above.

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CHAPTER 7: GENERAL CONCLUSION

One of the main problems of environmental sanitation is the release of micropollutants into the environment through the WWTP. These releases have long been considered an important source of environmental contamination. This study has looked at both the concentration and exposure time with respect to the toxicity of two selected ARV drugs, Nevirapine and Lamivudine on activated sludge bacteria. Nevirapine destroyed flocs and reduced the specific Chemical Oxygen Demand evolution rate and the nitrification rate even though the total nitrogen cycle should be monitored. SEM Microscopy and combined with the BacLightTM were used to assess the Nevirapine time-kill activity. The results showed that both the concentration and exposure time should be taken into account when studying nevirapine toxicity on activated sludge bacteria. The effect of low concentrations was observed after the four-h time period usually used in toxicity tests. Nevirapine reduced the specific COD and N-NH_4^+ concentration variation rates. Although the limit values for nitrification should be measured from consistent daily samples, the random samples showed a clear indication of nevirapine disrupting the WWTP process. However, definitive conclusion about the inhibition of nitrification in the actual WWTPS cannot be drawn yet, as the true concentrations of nevirapine or similar non-nucleoside reverse transcriptase inhibitors (NNRTIs) drug in the existing WWTPs are not known.

However, further studies may be required to examine the quantitative resilience limits of specific bacteria and protozoa to nevirapine exposure, particularly over an extended period of time to investigate possible bacterial adaptation or potential of being compromised after a certain period time. On that basis, further research on the removal of nevirapine by attached microbial communities is therefore needed. Examination of other WWTP sludge is needed to further elucidate the underlying reasons for some of the variability in the behaviour of biomass containing Nevirapine.

The influence of Nevirapine and Lamivudine on the biological properties of the activated sludge and the study of EPS (bound and soluble fraction) showed that both ARV drugs induced an increase of bound EPS in flocs and the release of flocs of organic matter into bulk wastewater. The toxic effect of Nevirapine on the biomass was more pronounced than that of Lamivudine, inhibiting the removal of the COD and nitrogen.

ACKNOWLEDGEMENTS

This work is supported by the Innovation and Doctoral scholarship (Grant number 102501) from the National Research Foundation (NRF) of the Republic of South Africa.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.