SUBSTANCES OF EMERGING CONCERN IN SOUTH AFRICAN AQUATIC ECOSYSTEMS

Volume 2:
Evaluation of antibiotic resistance bacteria and chemical contaminants' removal efficiency using various treatment technologies

Report

to the Water Research Commission

by

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The publication of this report emanates from Water Research Commission (WRC) project K5/2733, titled “Micropollutants and endocrine-disrupting contaminants (EDCs) in wastewater treatment systems: Approach towards effective water reclamation, new treatment technologies, and development of an adverse outcome pathway network for risk assessment”.

Volume 1: Substances of emerging concern in South African aquatic ecosystems: Fate, environmental health risk characterisation and substance use epidemiology in surrounding communities (**WRC Report no. 2733/1/20**)

Volume 2: Substances of emerging concern in South African aquatic ecosystems: Evaluation of antibiotic resistance bacteria and chemical contaminants’ removal efficiency using various treatment technologies (this report)

EXECUTIVE SUMMARY

BACKGROUND

The presence of contaminants of emerging concern (CECs) and associated endocrine-disrupting contaminants (EDCs) in environmental waters is mostly attributed to the discharge of wastewater effluents from wastewater treatment works (WWTW). This is because conventional WWTW are not necessarily designed to remove the vast number of pollutants that are regularly used by households, industry and/or agriculture. As a result, a vast range of CECs is readily shown to persist in the final treated effluent within the low ng/ℓ to high µg/ℓ range, highlighting the need for investigations into additional or alternative treatment technologies that may improve CEC degradation to mitigate against the exposure of humans and ecosystems to health risks. Much advancement has been made to promote both suspended and attached microbial growth during wastewater treatment to improve overall nutrient removal. This is achieved through either fixed bed bioreactors (FBBs) or moving bed bioreactors (MBB). Although the focus of such treatment technologies is mostly on improving sludge maturity for nutrient removal, the optimisation of these technologies, with the focus on CEC removal, will gain much focus in the future.

Moreover, although much advancement has been shown to promote an increased state of biodegradation of CECs within secondary WWTW processes, it is still highlighted that tertiary treatment through advanced oxidation pathway (AOP) processes may be needed to completely mineralise the CEC rather than creating metabolically active transformation products or simply retaining them within the treatment system. Apart from the reported recalcitrance of CECs in existing WWTW technologies, there is a renewed global focus on the potential role of CECs as trigger factors for the development of antibiotic resistance (AR) in water environments. In recent literature, WWTWs have been reported as “hotspots” for the proliferation of AR-related genes and virulence factors. With AR becoming a well-known global crisis, many investigations into factors that promote its emergence have occurred. For example, some studies have shown the potential of recalcitrant antibiotics at WWTWs to catalyse the development of antimicrobial resistance (AMR) genes.

AIMS OF THE STUDY

The current study was aimed at investigating two main aspects:

- Through the known knowledge of the presence and fate of CECs in South African WWTWs, to investigate the potential of alternative treatment technologies to improve on CEC and EDC degradation. In this regard, two advanced water treatment systems were evaluated: the HYBrid ACtivated Sludge (HYBACS®) and the Carbon-based ElectroChemical Oxidation (CabECO) treatment systems

- To investigate the presence and fate of antibiotic-resistant bacteria (ARB) and AMR-related genes at two WWTWs that utilise activated sludge (AS) treatment processes, but through two different treatment setups (complete-mix and plug-flow systems)

EFFICIENCY OF THE HYBACS® TREATMENT SYSTEM IN REMOVING PRIORITY MICROPOLLUTANTS IN WASTEWATER

In the first case study, the performance of a HYBACS® treatment system that includes advanced secondary treatment through the incorporation of Shaft Mounted Advanced Reactor Technology (SMART™) modules prior to conventional activated sludge (CAS) treatment steps was investigated for the removal of 13 selected CECs. Performance of the treatment system was evaluated by measuring the levels of these CECs and the estradiol-equivalent (EEQ) concentrations before and after treatment.

Results obtained showed an increase in EEQ levels during primary treatment and at the onset of the advanced secondary treatment. Moreover, a high level of cytotoxicity was recorded in the recombinant yeast bioassay during the primary treatment steps. However, cytotoxicity was significantly reduced in the liquor after the advanced secondary treatment. This may imply that inert cytotoxic components in the primary treatment modules may interfere with the biochemical pathways in the bioassay, suppressing total estrogenicity responses. Although estrogenicity was significantly reduced after the anoxic stage of the AS treatment step, the calculated EEQ levels were again shown to increase at the overflow of the secondary clarification and effluent after chlorination. The removal analysis for 13 CECs during the treatment process showed six CECs having high mass balance reduction, five showing moderate mass balance reduction and two metabolic by-products of carbamazepine showing negative mass balances, indicating an accumulation during the treatment process or recirculation of these metabolic by-products during sludge maturity in the secondary treatment steps.

It was concluded that, although the treatment technology was adequately monitored during the study, the high level of cytotoxicity during primary treatment steps and some operational issues during the period of sample acquisition may have skewed the final results on the performance of the upgraded treatment works. Nevertheless, the study still confirmed that the WWTW discharge may even contribute to improved water quality of the recipient river system, as high loads of CECs, cytotoxicity and endocrine-disrupting activity were recorded in the river system located upstream of the WWTWs, with a marked reduction in the concentrations of some pollutants further downstream of the works.

EVALUATION OF THE CABECO TREATMENT SYSTEM FOR THE REMOVAL OF PRIORITY MICROPOLLUTANTS IN WASTEWATER

A CabECO treatment process was also investigated for its potential to degrade priority CECs and its estrogenic endocrine-disrupting activities for the purpose of including such a treatment process as a polishing step for water reclamation. The optimisation and implementation of the treatment technology form the focus of a project that was granted by the European Union Horizon 2020 Research and Innovation Programme – SafeWater Africa (SWA) (Project No. 689925). The work presented here explores the forays into the degradation of priority CECs and estrogen receptor-mediated endocrine disruption outcomes.

The results showed that the CabECO technology was effective for the degradation of two CECs (carbamazepine and sulfamethoxazole) from environmental water samples, as well as the reduction of anti-estrogenic responses of micropollutants that were analysed by a recombinant yeast estrogen screen (YES) using ozonation technology. When environmental samples were treated with CabECO, some compounds, such as diclofenac, benzotriazole, caffeine and atrazine, still proved to be recalcitrant. The treatment technology is still under evaluation and optimisation and shows promise for a decentralised treatment option in rural and/or urban areas that are burdened with insufficient sanitary water provision.

PRESENCE AND FATE OF ANTIBIOTIC RESISTANT BACTERIA AND RESISTANT GENES IN WASTEWATER

It was observed that ARB were found throughout the investigated WWTWs, regardless of the stage of the treatment process or the season of sample acquisition. Although high numbers of ARB were present in post-chlorination effluent, the numbers were reduced compared to the influent. *Morganella morganii* was the most dominant species of the isolated bacteria. However, selection bias may have occurred as this species has intrinsic resistance to certain antibiotics and hence will outcompete other biota during culturing. As the identified species were obtained from the effluent, it can be expected that these organisms are transferred to surface waters, which may have serious implications if water is reclaimed for food production, recreation or other forms of exposure to the water.

Target resistance genes were found to be better removed in WWTWs that make use of a plug-flow system and maturation tank prior to chlorination of the treated effluent, thus showing that extended residence time provides better removal efficiency for both resistant bacteria and plasmid-mediated resistance genes. Determining the effect of antibiotics on biofilm metabolism needs further investigation, including the ability for a biofilm to recover after exposure to high antibiotic concentrations.

CONCLUSION AND RECOMMENDATIONS

The recombinant yeast estrogen screen (YES) assay served as a sufficient first-tier scoping assay to evaluate a biochemical response of all chemical substances within the wastewater and surface water sample matrices that may interfere with a specific endocrine system pathway. Follow-up research should thus include a tiered approach to evaluate interference with specific endocrine system pathways using a combination of effect-based monitoring (EBM) tools for more refined intervention. This holds value to evaluate the quality of surface water systems that are affiliated with both WWTW discharge and areas that are being highly polluted by additional anthropogenic practices. Moreover, combining a variation of EBM assays with non-targeted chemical analyses to identify priority CECs and substances that are of high concern to exert adverse health effects will allow for more defined mitigation strategies to reduce the burden of pollution into freshwater ecosystems. The CEC removal calculations for the WWTW study included the estimation of daily loads (g/day) of the target analytes for more refined mass balance estimations, but did not consider the hydraulic residence time (HRT) of the treatment system and should thus be considered in such studies. Both the YES and chemical analysis results for the environmental surface waters highlighted the extent of pollution that may originate from alternative sources that are not associated with WWTW discharge – a common observation that is reported for many other areas in the South Africa where sanitation services does not meet the demand of rising population growth.

The CabECO technology shows potential for application in a decentralised drinking water system for rural Southern African regions. The ozone generated by the electrochemical process is sufficient for the disinfection of various environmental samples, as well as the abatement of several micropollutants. Retention time is extremely important to include in the final design of this system. For the application of this system in a real-world scenario, it is important to consider that ozone concentration is reduced considerably, with a substantial portion of the ozone being depleted by various sources of organic matter other than the target compounds and microbes. Thus, CabECO appears better suited as a disinfectant or “polishing step” after water treatment. The standard procedures for generating potable water (flocculation, sedimentation, filtration) always demand the addition of a disinfectant post-treatment. Currently, chlorine is the go-to disinfectant, but has the disadvantage of being hazardous to human health and having a negative environmental impact. CabECO is an attractive alternative for the disinfection step in water treatment, or for polishing water with a micropollutant footprint. For disinfection, it has the disadvantage of lacking residuals, due to the instability of ozone. Thus, retreatment before use, after storage, would be recommended.

For the identification of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) during wastewater treatment, it was found that bacterial isolates from the waster matrices showed much higher minimum inhibitory concentrations (MICs) for the antibiotics colistin, amoxicillin and sulfamethoxazole compared to their reported CLSI or EUCAST MIC resistance breakpoint criteria for pure laboratory strains of the same organism, which highlights that environmental pathogens have a much stronger tolerance to the selected antibiotics. However, comparing MICs from isolates obtained from the wastewater matrices that were either enriched with- or without the addition of an antibiotic substance in the growth media showed inconsistent results over the larger range of identified organisms and highlight the complexity of drawing definite conclusions whether pre-exposure of environmental microbiota with environmentally-relevant concentrations of antibiotics does indeed cause an increase in ABR profiles

that may be driven by various biotic- and/or abiotic factors in the environment. Future recommendations to follow up on these methodologies will be to consider pre-exposure and/or enrichment using a combination of antibiotics that are similar to pharmaceutical prescriptions, as environmental strains is shown to be less susceptible to a single antibiotic treatment.

The identification of target antibiotic-resistant genes (ARGs) within the wastewater systems showed a predominant abundance of ARGs associated with sulfamethoxazole resistance (*sul1* and *sul2*). Moreover, the identification of a colistin resistant gene (*mcr3*) was also identified and raised concern that inert environmental resistance is present in wastewater systems, as colistin is considered as a last-resort antibiotic drug for Gram-negative bacterial infections and should receive focus during routine surveillance initiatives.

Lastly, the study included an initial evaluation of sulfamethoxazole resistance profiles in a laboratory-scale study using a carbon dioxide evolution measurement system (CEMS) for real-time evaluation of the effect of environmentally-relevant concentrations of antibiotic exposure on established surface-attached microbial consortiums (biofilms). Although initial results showed limited effects of sulfamethoxazole exposure on the metabolism of a pure-culture biofilm over a 210-hour period, it was recorded that the biofilm of the pure culture recovered from a five-fold higher concentration of sulfamethoxazole than its determined MIC, which raised the issue of biofilms that will have a much larger resistance profile than planktonic cells in similar batch- or conventional MIC experimentation. On-going studies using this experimental setup is being done at the research facility, where follow-up studies will include the administration of antibiotic combinations similar to prescribed medications and/or co-exposure with inorganic substances for more defined ABR profiling.

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ACRONYMS AND ABBREVIATIONS

AMR	Antimicrobial Resistance
AMX	Amoxicillin
AOP	Advanced Oxidation Pathway
AR	Antibiotic Resistance
ARB	Antibiotic-resistant Bacteria
ARG	Antibiotic-resistance Gene
AS	Activated Sludge
ATZ	Atrazine
AWaRe	Access, Watch and Reserve
BEG	Benzoylecgonine
BNR	Biological Nutrient Removal
BZT	Benzotriazole
CabECO	Carbon-based ElectroChemical Oxidation
CA	Corrected Absorbance
CAFF	Caffeine
CAS	Conventional Activated Sludge
CBZ	Carbamazepine
CBZ-diol	10,11-dihydro-10,11-trans-dihydroxycarbamazepine
CBZ-ep	Carbamazepine-10,11-epoxide
CDC	Centres for Disease Control
CDDEP	Centre for Disease Dynamics, Economics and Policy
CEC	Contaminants of Emerging Concern
CEMS	Carbon Dioxide Evolution Measurement System
CFU	Colony-forming Unit
CLSI	Clinical Laboratory Standards Institute
COC	Cocaine
COD	Codeine
CPRG	Chlorophenol red §-D-galactopyranoside
CSIR	Council for Scientific and Industrial Research
CST	Colistin
DCF	Diclofenac
dh-hCBZ	10,11-dihydro-10-hydroxy carbamazepine
DHPS	Dihydropteroate Synthase
DWS	Department of Water and Sanitation
E_2	Estradiol
EC_{50}	Concentration which shows an effect in 50% of the experimental population
EDC	Endocrine-disrupting Contaminant

EEQ	Estradiol Equivalent
ERWAT	East Rand Water Care Company
ESBL	Extended Spectrum β-lactamase
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FBB	Fixed Bed Bioreactor
GD	Green Drop
GES	GES-β-lactamase
GM	Gentamicin
h-dhCBZ	10-hydroxy-10,11-dihydro carbamazepine
hER	Human Estrogen Receptor
HGT	Horizontal Gene Transfer
HLB	Hydrophilic-lipophilic-balanced
HPLC	High-performance Liquid Chromatography
HYBACS®	HYBrid ACtivated Sludge
IC_{50}	Inhibitory concentration which shows an effect in 50% of the experimental population
KPC	Klebsiella pneumoniae carbapenemase
LC-MS	Liquid Chromatography-Mass Spectrometry
LMIC	Low- to Middle-Income Countries
LPS	Lipopolysaccharide
MAC	MacConkey
MBB	Moving Bed Bioreactor
MDL	Method Detection Limit
MDR	Multi-drug Resistant
MeOH	Methanol
METH	Methamphetamine
METHA	Methaqualone
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MRM	Multiple-reaction Monitoring Mode
MS/MS	Triple-Quadrupole Mass Spectrometer
NCBI	Nucleotide Sequence Database Collaboration
NCID	National Institute for Communicable Diseases
NDH	National Department of Health
NDM	New Delhi Metallo-β-lactamase
NHLS	National Health Laboratory Service
NIC	Nominal Inhibitory Concentration

NICD	National Institute for Communicable Diseases
NSAID	Non-steroidal Anti Inflammatory
OD	Optical Density
OH^-	Hydroxyl Radical
OXA	OXA-β-lactamase
PABA	P-aminobenzoic Acid
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PPCP	Pharmaceuticals and Personal Care Products
PST	Primary Sediment
qPCR	Real-time Polymerase Chain Reaction
RAS	Return Activated Sludge
RO	Reverse Osmosis
SMART™	Shaft Mounted Advanced Reactor Technology
SMX	Sulfamethoxazole
SNP	Single Nucleotide Polymorphism
SPE	Solid-phase Extraction
SPM	Solid Particulate Matter
SST	Secondary Settling Tank
SWA	Safe Water Africa
TB	Tuberculosis
TSB	Tryptic Soy Broth
UPLC	Ultra-performance Liquid Chromatograph
VBNC	Viable but Non-culturable
WAO	Wet Air Oxidation
WHO	World Health Organisation
WWTW	Wastewater Treatment Works
YAES	Yeast Anti-estrogen Screen
YES	Yeast Estrogen Screen

CHAPTER 1: BACKGROUND

1.1 EMERGING SUBSTANCES OF CONCERN IN WATER

1.1.1 Introduction

Providing safe drinking water is a global challenge. The demand and access to safe potable water resources are impacted on by rapid population growth and urbanisation on a global scale. Furthermore, changes in annual weather patterns, especially for arid countries such as South Africa, which has experienced low rainfall in most parts, emphasise the need for the improved management of existing water resources, including more efficient reuse. The reallocation of freshwater resources will become the norm in years to come, with treated wastewater already being reused for agricultural purposes. In many developing countries, including South Africa, many sewerage and wastewater treatment facilities are malfunctioning, overloaded or not compliant with national standards, which limits the ability of these countries to practise water reclamation.

For this reason, the Department of Water and Sanitation (DWS) implemented the Green Drop Report in South Africa to audit municipal and private WWTWs. Assessment criteria taken into account when compiling the report includes the design capacity of the treatment plant, capacity exceedance, effluent quality (referring to microbial, physical and chemical compliance) and technical skills (management and operation) (Department of Water Affairs, 2013). Despite its implementation, the non-compliance of WWTWs was evident and the Green Drop system was discontinued, with the most recent available data being from 2013, shown in Figure 1-1 (Department of Water Affairs, 2013). These statistics show the potential of WWTWs to serve as pollution “hotspots” for emerging substances of concern, such as recalcitrant chemical micropollutants (including antibiotics), as well as pathogenic and AMR microorganisms within environmental surface waters, which create several health concerns for both humans and aquatic ecosystems.

1.1.2 Contaminants of emerging concern

The presence of emerging organic micropollutants in freshwater ecosystems has gained increasing interest regarding their health risks for freshwater ecosystems and humans. Of these CECs, the focus has been placed on the discharge of pharmaceuticals and personal care products (PPCPs) from either direct or diffuse point sources. The presence of CECs in environmental waters is attributed to the discharge from WWTWs , which are not necessarily designed to remove the vast number of CECs that are regularly used by households, industry and/or agriculture (Petrie et al., 2014). Such incomplete removal of CECs is dependent on numerous factors, such as the type of wastewater treatment and the operational challenges they face, the source of the wastewater (industrial or domestic), spatial and temporal fluctuations in the use of certain CECs (for example, antibiotics during colder months and anti-histamines during spring), and the chemo-physical properties of the CECs (partitioning, volatilisation, thermal stability, water solubility, etc.). Due to so many factors affecting the fate of CECs in wastewater and receiving waters, a vast range of CECs is readily shown to persist in WWTW effluent, mainly within the low ng/ℓ to high µg/ℓ range (Petrie et al., 2014; Archer et al., 2017a; Archer et al., 2017b; Baalbaki et al., 2016; Baalbaki et al., 2017). Establishing the security and resilience of freshwater resources has become an increasing global concern, especially for drought-stricken countries (including South Africa), which also face exponential population increases and urbanisation. For this reason, improving the quality of reclaimed wastewater holds value to serve as an additional resource for both potable and non-potable reuse. Although compliance criteria are set for such treatment systems in most parts of the world, they do not include the assessment of CECs, which may not only have deleterious health effects to the environment and humans over the long term, but also impact on the safeguarding of existing freshwater ecosystems that are used for human consumption.

For this reason, understanding the fate of priority CECs during currently implemented wastewater treatment technologies is key to assessing the potential and need for improved reclamation technologies that may drive sustainable development.

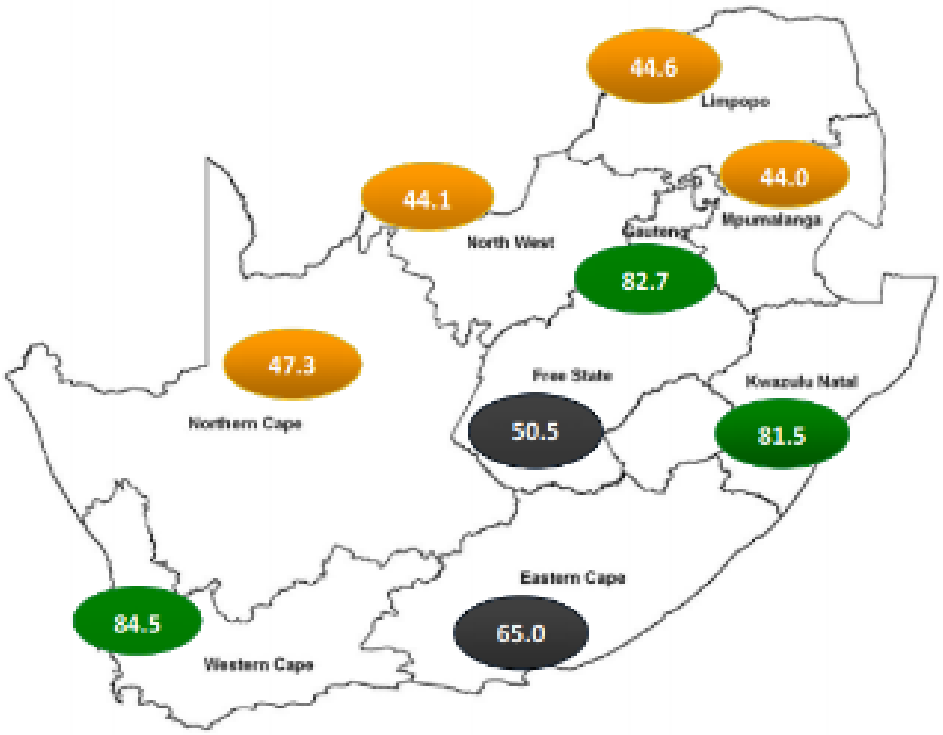

Figure 1-1: Green Drop Report scores (as a percentage) for provinces in South Africa as indicated in the Green Drop report of the Department of Affairs, 2013

1.1.3 Antibiotics and antibiotic resistance

Antibiotics have cured and prevented many life-threatening diseases, and their use has been a fundamental part of public health since the mid-1900s (Ling et al., 2015). However, the threat of AMR, which leads to a post-antibiotic era, has long been of scientific interest. The dangers of multi-drug resistant (MDR) bacteria or co-called “superbugs” have been highlighted to the public in various news platforms to create awareness of this issue (Ismail, 2016; Pillay, 2017), such as the example of a pan-resistant *Klebsiella pneumoniae* isolate that has shown resistance to all 26 antibiotics available in the United States (Branswell, 2017). Similar cases have been reported globally, including in South Africa (National Department of Health, 2016). Furthermore, AMR infection statistics suggest that Europe and the USA see 50,000 deaths annually, while 700,000 deaths are observed globally each year. (Audi et al., 2016; Branswell, 2017; Karlamangla, 2017; O’Neill, 2014). This figure is predicted to reach 10 million annual deaths on a global scale by 2050, with 4.15 million of these deaths predicted to be attributed to Africa alone (Figure 1-2). The increase in global travel in the late 1900s and subsequently the 21st century has contributed to the rapid spread of resistant bacteria across country borders (Cantas et al., 2013). While the discovery of penicillin in 1928 was a revolutionary event for the treatment of infectious disease, its use in a clinical environment was not established until 1945.

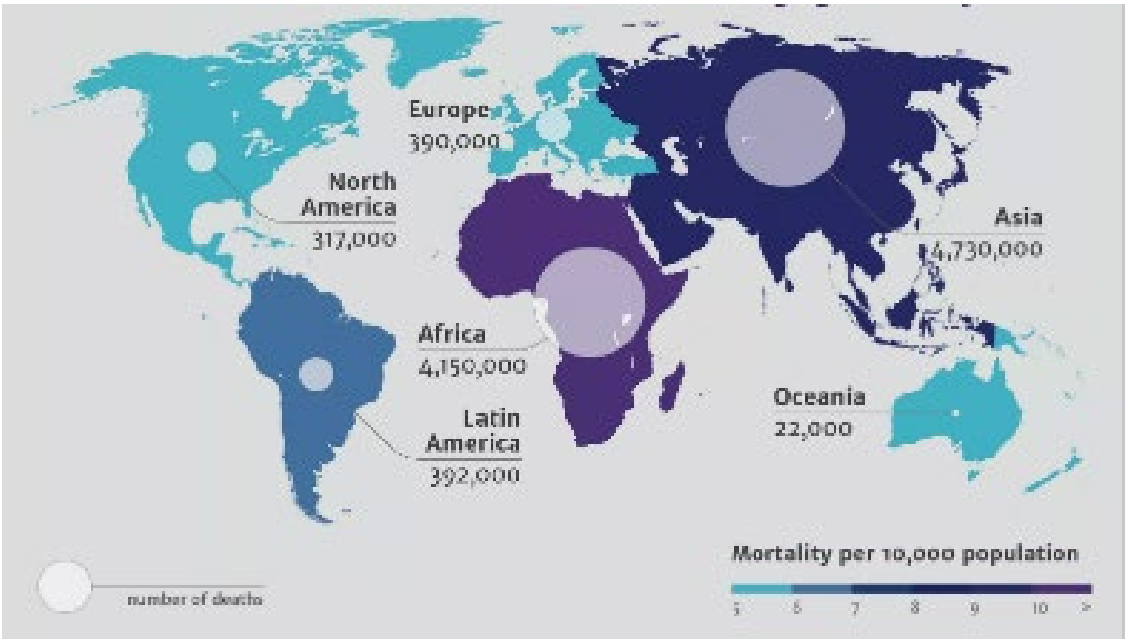

Figure 1-2: Deaths attributable to AMR each year by 2050 (O'Neill, 2014)

Despite this, resistance of *Staphylococcus pyogenes* to penicillin was recorded as early as 1945 (Barber and Rozwadowska-Dowzenko, 1948). It has been found that, in most cases, resistance to an antibiotic develops rapidly after its clinical introduction. Figure 1-3 represents a timeline showing the development of resistance in relation to the discovery of the respective antibiotic (Alcock, 2018). Cephalosporins are an isolated case where antibiotic resistance predates the clinical use of the drug (Figure 1-3). Since many of the active ingredients of antibiotics are found in the environment, bacteria are often exposed to antibiotic-selective pressures before the antibiotic is discovered and commercialised. As a result, the development of resistance to antimicrobial compounds is inevitable (Barber and Rozwadowska-Dowzenko, 1948; Laxminarayan et al., 2013).

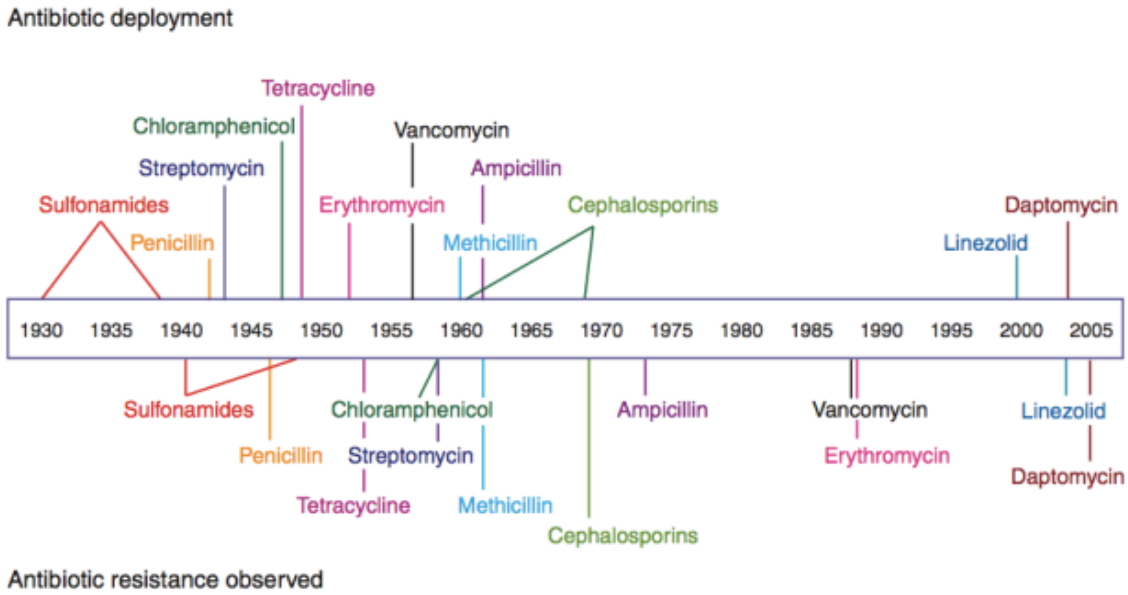

Figure 1-3: Timeline of the discovery of various antibiotics and the subsequent emergence of resistance to the respective classes (Alcock, 2018)

The relationship between the use of antibiotics and the emergence of resistant bacteria is well known (Gelband et al., 2015). Moreover, the over-prescription and misuse of antibiotics have contributed to the global antibiotic resistance crisis. For example, the South African National Department of Health (NDH), as well as the US Centres for Disease Control (CDC), has estimated that 50% of all prescribed antibiotics for human consumption are unnecessary as a result of incorrect diagnoses (Centres for Disease Control and Prevention, 2013; National Department of Health, 2016). In addition to antibiotic use for human health, an overwhelming use of antibiotics in animals and livestock has been observed (Van Hoek et al., 2011; Centres for Disease Control and Prevention, 2013; Ventola, 2015). A study by Van Boeckel et al. (2015) reported that 63,000 tons of antibiotics were used worldwide in animal husbandry. This is a substantially larger amount of antibiotic usage compared to human usage, and is anticipated to reach 105,000 tons in the next decade (Van Boeckel et al., 2015).

In South Africa, 80% of available antibiotics are used for agricultural, livestock and domestic animal purposes (National Department of Health, 2016), not only to treat infection, but also as preventative measures to facilitate growth promotion (Laxminarayan et al., 2013). This emphasises the impact of veterinary antibiotic use on the development of AMR, which, in turn, has a negative impact on human health. The connection between the use of antibiotics in animals and the development of AMR was recorded in the Swann Report in 1969. In this report, the UK government suggested limiting the use of human antibiotics for prophylactic purposes in animals to try and prevent the emergence of resistance (Swann et al., 1969). As a result of this report, many countries in the European Union (EU) banned the use of antibiotics for growth promotion. However, it was not until 2006 that antibiotic use in livestock was banned completely in some areas (Laxminarayan et al., 2013).

Clearly, multiple factors may influence AMR development on a global scale. With the vast differences between anthropogenic practices among continents and countries (such as agriculture, socio-economic differences and pharmaceutical use), it is vital for each location or country to establish the priority areas that need to be addressed for the control and mitigation of AMR. To understand the impact and current situation of antibiotic resistance specific to a South African environmental context, the following factors need to be addressed, among others:

- Priority AMR genes that are found in the environment, including “AMR hotspots”, where the development of these genes is increased
- Mechanisms of resistance on gene transfer to evaluate whether non-pathogenic microorganisms will afford environmental pathogens with resistance profiles
- Biotic and abiotic factors (including anthropogenic influences) that affect the expression of the resistance genes in microbial cohorts

While it is known that other contaminants, including heavy metals, also induce AMR in the environment (Yazdankhah et al., 2018), this study focuses on sub-inhibitory concentrations of antibiotics.

1.2 MICROPOLLUTANTS IN WASTEWATER TREATMENT WORKS

1.2.1 Micropollutant sources and entry into wastewater treatment works

Organic micropollutants, such as pesticides, PPCPs and industrial waste products, can enter surface waters from anthropogenic activities through various point and non-point sources,. These chemicals are mostly destined for WWTWs when excreted after consumption and/or wrongful disposal. These contaminants are then either further metabolised in the sewage pipelines or will be transported to WWTWs in their parent forms at concentrations ranging from low (ng/ℓ) to high ($\mu g \cdot \ell^{-1}$) levels. Micropollutants can enter WWTWs through multiple points, as shown in Figure 1-4.

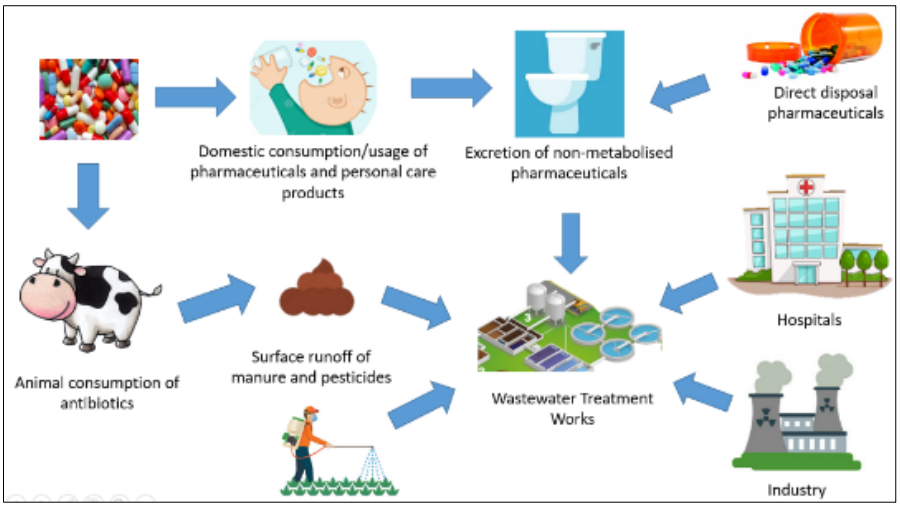

Figure 1-4: Sources of micropollutant entry into WWTWs

Briefly, human excretion from both domestic and commercial sectors, as well as waste products from manufacturing and other industrial processes, enter the sewerage system (Samadi et al., 2015; Wellington et al., 2013). While WWTWs are designed to produce effluents that comply with standards set by the Department of Water and Sanitation (DWS), they are not required to screen for micropollutants and pathogenic bacteria, which are often able to persist in these environments, despite the treatment process. Many of these compounds have been found to have detrimental effects on wildlife, as well as human health (Archer et al., 2017a).

1.2.2 Removal of micropollutants in wastewater treatment works

As safe, potable water is a necessity for all life forms, the occurrence of micropollutants in wastewater, and their potential distribution in the environment and through the food chain, is therefore of concern. Most WWTWs rely on primary treatment, followed by secondary and tertiary treatment. Primary treatment involves the screening and degritting of coarse particulate matter in raw wastewater, followed by the sedimentation of solid waste through settled sludge. Various studies have shown the benefit of primary treatment to significantly lower the loads of certain CECs, such as the biocide triclosan (Baalbaki et al., 2016). However, such removal at primary sedimentation mostly relies on the physio-chemical properties of the analytes, such as their high tendency to partition into solids, rather than their biodegradation (Jones et al., 2005). Therefore, most CECs are still found to persist after primary treatment. Secondary treatment of most WWTWs during recent years consists of a series of reactors that are mostly driven by biological processes under variable redox conditions. This generates a diversity of microbiota, which maintains an AS treatment process under suspended growth.

A WWTW monitoring study by Baalbaki et al. (2016) included a comprehensive analysis of the levels of CECs at various treatment processes. The authors reported on the removal of 26 CECs and showed that a high proportion of removal is driven by secondary treatment. The removal of micropollutants during AS treatment relies on the bio-degradation, sorption, hydrolysis, photolysis, and/or volatilisation of organic matter to mainly lower the high nutrient load of contaminated water resources before discharging it into the environment or for further treatment by means of secondary sedimentation and tertiary treatment steps (Min et al., 2018). Such CAS treatment processes are widely adopted at WWTWs and have been shown to generate an added benefit by lowering the loads of CECs that are not necessarily monitored or required for compliance by the WWTW operator. However, as research continues to identify an increasing load of CECs, which pose either a lethal or a non-lethal health risk to ecosystems, the need for the reliant monitoring of priority CECs is needed.

Another factor to consider is the formation of metabolite or breakdown products of CECs that are driven by biodegradation processes within WWTWs. This is of importance, as it is unknown whether such transformation products may exert a more deleterious environmental impact. Furthermore, several studies have reported on the occurrence of negative mass balances of CECs during CAS wastewater treatment, implying a build-up of target analytes at the treated effluent compared to untreated influent, and may be ascribed to the deconjugation or back-transformation of CECs and their breakdown products under various biotic and abiotic conditions within WWTWs. Taking all of this into consideration, it is evident that the development of more than enough treatment processes, through modifications of existing AS systems and/or tertiary treatment processes, is needed to combat an ever-increasing list of CECs that may enter natural freshwater resources.

An example of modifying CAS treatment systems includes the incorporation of both suspended growth and surface-attached growth to promote increased biological degradation. The CAS processes are sufficient for seeding such attached growth, raising various advantages such as securing a more stable microbial community within treatment processes, which are more resilient to operational and climatic changes within the treatment plant, and promoting a higher yield of biomass for improved nutrient removal, especially for nitrifying communities with slow growth rates (Serrano et al., 2011; Di Trapani et al., 2013; Luo et al., 2014). As an added benefit, CECs that show high or moderate affinity to be removed through biodegradation will have increased exposure to a variety of biotic conditions by serving as labile nutrient sources. However, many CECs that do not show high affinity to be associated with particulate matter and/or biodegradation during wastewater treatment are still problematic. For example, the anti-epileptic carbamazepine and the antibiotic sulfamethoxazole regularly serve as model analytes to assess whether moderate to highly persistent compounds can be removed from conventional WWTW systems. For such compounds, negative mass balances are regularly reported (Baalbaki et al., 2017), and highlight the need to investigate both the transformation pathways of such recalcitrant contaminants and improved tertiary treatment for their removal.

1.2.3 Association of micropollutants with suspended and attached microbial growth during wastewater treatment

Much advancement has been made to promote both suspended and attached growth during wastewater treatment to improve overall nutrient removal. This is achieved through FBB and MBB, respectively. Such treatment strategies can also assist with the improved removal capabilities of WWTWs to eradicate persistent CECs through increased residence times and contact with microbial communities, which may drive their biodegradation. In a bench-scale study to investigate the removal of micropollutants using a hybrid MBB process, it was shown that the elimination of recalcitrant pollutants, such as the anti-inflammatories diclofenac and mefenamic acid and the herbicide clofibric acid, were more rapidly removed than in CAS processes (Falås et al., 2013).

A similar bench-scale study, using a sponge-based MBB reactor, also showed improved removal of the pharmaceuticals ibuprofen, metronidazole, naproxen, primidone and even triclosan, compared to CAS treatment systems (Luo et al., 2014). A pilot-scale study treating hospital effluent using a three-stage MBB reactor showed good removal of the pharmaceuticals atenolol, citalopram, clindamycin, trimethroprim and metroprolol within the first bioreactor (Casas et al., 2015). However, the authors noted that pharmaceuticals such as sulfamethoxazole, tramadol and erythromycin still showed relative persistence through the treatment process. This highlights the fact that treatment technologies that are reliant on biodegradation and dependent on prolonged residence time within the system are still facing constraints, especially for recalcitrant CECs such as carbamazepine and sulfamethoxazole, which do not regularly degrade under CAS treatment. Regardless, the addition of an added FBB or MBB step to CAS treatment systems has shown great promise to lower a substantial proportion of CECs that show moderate persistence within WWTWs.

1.3 ANTIBIOTICS IN WASTEWATER

1.3.1 Antibiotics of interest

Countless antibiotics are considered a major concern for public health, and are being placed on the list of "critical antimicrobials" by the World Health Organisation (WHO) (World Health Organisation, 2011). This list is extensive, which reinforces the severity of the AR crisis. From this list, aminoglycosides, carbapenems, quinolones, β-lactams, macrolides and multiple drugs used for tuberculosis (TB) treatment are among the antimicrobials that should receive high priority (World Health Organisation, 2011). In particular, the polymyxin antibiotic colistin, the penicillin-like compound amoxicillin, and the aminoglycoside gentamicin, form part of the critically important antimicrobials, while the sulphonamide antibiotic sulfamethoxazole has been listed as highly important (World Health Organisation, 2011). These antimicrobials were selected for the current study based on their regular prescription and use in the Western Cape, along with their known resistance profiles in concerning pathogens, as discussed below.

1.3.1.1 Amoxicillin

Amoxicillin, a bactericidal β-lactam that inhibits cell wall synthesis, is a semi-synthetic antibiotic that is effective against gram-negative and gram-positive infections, as well as many anaerobic bacteria (Huang et al., 2011; Kaur et al, 2011). Amoxicillin is listed in the WHO's Access, Watch and Reserve (AWaRe) Index under the "access" group of antibiotics that are first- or second-choice empirical treatments for common or severe clinical syndromes and should always be available. However, because of the increased prevalence of extended spectrum β-lactamase (ESBL)-producing bacteria, the β-lactamase inhibitor clavulanic acid has regularly been co-administered with amoxicillin since the 1980s (Kaur et al., 2011). Such co-administration shows effectiveness as a first-line, broad-spectrum antibiotic for various common infections, such as respiratory and dermal infections, tonsillitis and ear infections (McIntosh, 2017). However, as stipulated in the Antimicrobial Resistance National Strategy Framework for 2018-2024, issued by the South African Department of Health and the Department of Agriculture, Forestry and Fisheries, one of the key drivers of AMR that needs to be addressed is the reliance of broad-spectrum antibiotics that will select for a wide range of resistant bacterial populations in comparison to more defined narrow-spectrum antibiotics. Moreover, other resistance profiles, such as altered penicillin-binding protein structures, reduced porin expression or increased efflux pump expression, can also lead to amoxicillin resistance, even with co-administration to combat specific AMR mechanisms (Drawz and Bonomo. 2010).

1.3.1.2 Colistin

Colistin falls into the polymyxin class of drugs, which are cationic cyclic polypeptides derived from *Paenibacillus polymyxa* strains. It is listed in the WHO-AWaRe Index under the "reserve" group of antibiotics that need to be considered as a last-resort treatment and used as key targets for antimicrobial stewardship programmes. Its use in humans was also previously avoided due to its renal and neural toxicity effects (Cai et al., 2012). It functions by targeting the negatively charged Lipid A component of the lipopolysaccharide (LPS) found on the outer membrane of gram-negative bacteria. This binding facilitates the diffusion of the polymyxin across the periplasmic space, intercalating into the inner membrane of the cell, resulting in pore formation and cellular components leaking out of the cell, resulting in cell death (Gao et al., 2016). Although its use in humans has been limited, colistin has previously been used extensively in livestock feed, especially in swine and poultry farms, as well as in veterinary medicine. This has been identified as the root of the emergence of colistin resistance, which is beginning to be observed in a clinical setting (Gales et al., 2011; Newton-Foot et al., 2017; Rhouma et al., 2016). As a result, its use in livestock has been banned by the South African Veterinary Council (Gouws, 2016).

The use of colistin for human treatment is slowly increasing as a result of the increase of bacteria that are resistant to commonly prescribed antimicrobials (Cai et al., 2012; Liu et al., 2016). As a result, the South African Department of Health lists colistin as an antibiotic that requires ongoing surveillance against pathogenic organisms such as *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* (National Department of Health, 2016). This classification of colistin reinforces the urgent need to prevent the emergence of full-blown resistance to this last-resort antimicrobial and limit the spread of resistance genes to other pathogens. The *mcr-1* gene was the first plasmid-mediated colistin-resistance gene identified in China (Liu et al., 2016). This gene has so far been identified in several gram-negative organisms, including *E. coli, Salmonella* spp. and *K. pneumoniae* (El Garch et al., 2017; Gao et al., 2016). The *mcr-1* protein has been shown to aid resistance to colistin by the addition of a phosphatidylethanolamine molecule to the Lipid A component of the LPS at the 4' phosphate group, thus reducing the affinity of Lipid A to colistin (Liu et al., 2016). Since its discovery, numerous *mcr* genes have been identified, the most recent being *mcr-9,* isolated from *Salmonella typhimurium.* The emergence of these genes is concerning and puts the reality of the post-antibiotic era into perspective.

1.3.1.3 Gentamicin

Gentamicin, an aminoglycoside antibiotic obtained from *Micromonospora purpura* in the 1960s, is a broad-spectrum protein synthesis inhibitor. Despite this, it is mostly used to treat infections caused by gram-negative bacteria (Samadi et al., 2015; Van Hoek et al., 2011). Gentamicin exhibits a large percentage of resistance to a number of bacterial species in South Africa (National Institute for Communicable Disease, 2015). The main mechanisms of resistance are aminoglycoside-modifying enzymes such as acetyltransferase, aminoglycoside phosphotransferase and aminoglycoside nucleotidyl transferase. These enzymes are encoded by *aac, aph* and *ant* plasmid-mediated gene variations, respectively, and alter the antibiotic through the adenylation, phosphorylation and acetylation of amine and hydroxyl groups (Duran et al., 2012; Samadi et al., 2015; Van Hoek et al., 2011). In addition, intrinsic mechanisms of resistance to aminoglycosides include efflux pumps, decreased permeability and alteration of ribosomes (Van Hoek et al., 2011).

1.3.1.4 Sulfamethoxazole

As a member of the sulphonamide class of antibiotics, sulfamethoxazole acts by interfering with the biosynthetic metabolic pathway that produces folate in bacterial cells. P-aminobenzoic acid (PABA) plays a vital role in this metabolic pathway as dihydropteroate synthase (DHPS) acts on it to produce folate. However, sulphonamides are structural analogues of PABA, thus compete with it and inhibit the enzyme activity of DHPS (Van Hoek et al., 2011). Resistance to these antibiotics occurs through genetic mutations in the gene encoding DHPS (Suzuki et al., 2015). To combat this mechanism, sulphonamide is prescribed, together with trimethoprim. This combination is used to treat a large variety of infections such as urinary tract infections, *Nocardia* spp., *Toxoplasma* spp. and methicillin-resistant *Staphylococcus aureus.* In addition, HIV-positive patients are treated with low doses of sulphonamide-trimethoprim to prevent opportunistic infections (Benson et al., 2009). This makes sulphonamide relevant in a South African context.

1.3.2 Levels of antibiotics in wastewater

A number of studies have shown that many antibiotics, as well as antibiotic-resistance genes (ARGs), are not effectively removed during the wastewater treatment process and there is significant variation in concentrations of the same compound in WWTWs (Archer et al., 2017a; Petrie et al., 2015). This occurs because of the different sectors that feed into WWTWs. For example, a WWTW that receives influent from hospitals and domestic sectors will have a different micropollutant profile than those that receive influent from industrial or agricultural sectors.

In addition, different treatment facilities utilise different treatment methods, which can lead to differences in the efficiency of micropollutant removal. Xu et al. (2015), showed that only 11.6% of sulphonamides were removed from sewerage treatment plants and were found in concentrations ranging from 648.1 ng/ℓ to 2,518 ng/ℓ between different treatment plants. In this group of antibiotics, sulphonamides are often the most commonly detected in WWTWs (Zhang and Li, 2011).

One of the few studies indicating antibiotic prevalence in South African wastewater focused on sulphonamide concentrations in KwaZulu-Natal. These were found to be 48.2 ng/ℓ in rural surface waters, 2,561 ng/ℓ in urban surface waters and 3,612 ng/ℓ in sewerage treatment plant effluent (Suzuki et al., 2015). When compared to Gauteng, sulphonamides were found at an average of 1,344.8 ng/ℓ in wastewater effluent with a removal efficiency below 25% (Archer et al., 2017a). These high concentrations, especially in an African setting, make sense due to the high use of this antibiotic for the treatment of bacterial infections in patients from countries where immunocompromising diseases such as HIV are rife (Suzuki et al., 2015). The median concentrations of sulphonamides were also found to be among the highest of a number of antibiotics detected in hospital effluent (wastewater influents (250 ng/ℓ), effluents (50 ng/ℓ) and environmental water (8 ng/ℓ)) in a study performed in Australia (Watkinson et al., 2009).

The highest concentration of sulphonamides detected in WWTW influent was 5,597 ng/ℓ (Peng et al., 2008), while that of the effluent was 6,000 ng/ℓ (Batt et al., 2006). Trimethoprim, a drug used in combination with sulphonamides in the ratio 1:5, was also shown to persist in wastewater effluent at concentrations of 10 ng/ℓ (Watkinson et al., 2009). The highest concentration of trimethoprim in WWTW effluent (3,052 ng/ℓ) was detected in the United Kingdom (Kasprzyk-Hordern et al., 2009). In addition, trimethoprim was detected in South African wastewater effluent at a concentration of 1,446 ng/ℓ (Archer et al., 2017a). These results show that there are substantial concentrations of this antibiotic in the WWTW effluent globally, which poses a large environmental concern for AMR development.

Of the β-lactam class of antibiotics, oxacillin, cloxacillin, ampicillin, amoxicillin, penicillin G and penicillin V are the most frequently detected in WWTWs (Zhang and Li, 2011). Of these, penicillin V was found in the highest concentration in WWTW influent (13,800 ng/ℓ) and effluent (2,000 ng/ℓ), while 1,400 ng amoxicillin was detected per litre influent in the same study (Watkinson et al., 2009). Quinolones are also frequently detected in high concentrations in WWTWs. The most prominent antibiotics from this group are ofloxicin, ciprofloxacin and norfloxacin. These antibiotics are widely used in numerous countries, and as a result are detected in WWTWs worldwide (Zhang and Li, 2011).

Quinolone concentrations in a Chinese WWTW ranged from 728.8 ng/ℓ to 3,866 ng/ℓ (Xu et al., 2015), while the highest concentration of quinolones in a Hong Kong study was 7,870 ng/ℓ (Minh et al., 2009). Of the macrolides, another of the antimicrobial groups of concern mentioned previously is a metabolite of erythromycin, erythromycin-H_2O, which was detected in the highest concentrations (10,025 ng/ℓ and 4,330 ng/ℓ) in influent and effluent, respectively (Kasprzyk-Hordern et al., 2009).

Few studies reported on the presence of colistin in environmental waters, which is due to the poor metabolism of this drug, with patients treated with this drug excreting 80% of the colistin prodrug, colistin methane sulfonate, which is primarily destined for WWTWs (Labuschagne et al., 2016). Recently, a study by Hembach et al. (2017) showed the occurrence of the *mcr-1* gene within bacterial isolates from a German WWTW effluent. These results support earlier contentions that complex mixtures of antibiotics, biocides, disinfectants and pharmaceuticals may provide selective pressures to promote horizontal gene transfer within the WWTW system.

It is evident that concentrations of the antibiotics in wastewater have a large range and differ within and between treatment plants, as well as countries. Multiple environmental and technical factors may result in such variability. For instance, the timing of sampling, the sampling method and the downstream processing of samples may all lead to the preferential detection of certain compounds.

The physio-chemical properties of different antibiotics can also affect the way they interact in the environment. Some may attach to other compounds and thus the chemical structure changes, making them undetectable, while others may break down into various metabolites. In addition, inflow from various sources or rain events can dilute the water and alter the concentrations in the treatment plant. It was also noted that more commonly used antibiotics were detected in WWTWs in higher concentrations (Zhang and Li, 2011). The presence of these antimicrobials in WWTWs and other environmental waters can play a major role in the development and dissemination of AMR.

1.4 ANTIBIOTIC RESISTANCE PROFILING IN WASTEWATER TREATMENT PLANTS

1.4.1 Microbiology of WWTWs

The aim of biological treatment is to remove unwanted organic compounds from wastewater. These include chemicals from industry, agriculture and domestic sectors. The microbial communities in AS form mobile biofilms that are suspended in the mixed liquor. These aggregations are known as flocs. Microorganisms present in aeration tanks utilise compounds such as carbon, nitrogen and phosphorous as nutrients to multiply in wastewater. By doing this, microbes remove the organic compounds from the wastewater. Although compounds are not completely removed, these microbial communities still play a major role in the wastewater treatment process (Miralles-Cuevas et al., 2017; Nagwekar, 2014).

As microorganisms are ubiquitous, they also enter the WWTW through a variety of avenues seen for micropollutants in Figure 1-6.. These include normal gut microbiota that are excreted and enter WWTWs through the sewage network, as well as those excreted by patients with bacterial infections, immune responses or those taking medication. Microbes used in industrial processes such as the mass production of enzymes or wine production, and environmental microorganisms or those present within agriculture (crops, livestock and in soils) can also be flushed into the WWTW by rain or irrigation. Previously, to determine the prevalence of certain bacterial species or the bacterial composition of a sample, culturing techniques were used. While this provides an indication of the culturable microbes in an environment, it typically accounts for less than 2% of the bacteria that are present in that environment (Wade, 2002). The remaining 98% is known as viable but non-culturable (VBNC) bacteria. Metagenomics is a powerful tool that enables the description of bacterial composition, including the VBNC bacteria of a sample. As a result, the use of this tool for environmental microbiology is increasing, however it is still limited due to the high cost involved.

Proteobacteria have been found to be highly prevalent throughout the WWTW process, particularly in activated sludge, as shown in studies conducted in China and Finland (Guo et al., 2017; Hultman et al., 2018). This phylum includes Enterobacteriaceae, *Salmonella*, *Vibro* and *Helicobacter* genera, among others. Many of these species are pathogens that are threats to public health and are listed as “critically important” by the WHO (World Health Organisation, 2017). Bacteroidetes and Firmicutes are phyla that are also observed in relatively high quantities (Guo et al., 2017; Hultman et al., 2018). Many authors have indicated that WWTWs are reservoirs for horizontal gene transfer (HGT) between microorganisms, which could lead to an increase in the spread of resistance genes between species and promote the emergence of a resistant bacterial community (Lin et al., 2016; Madsen et al., 2012; Pruden et al., 2013; Rizzo et al., 2013; Wellington et al., 2013). This community would add to the microbes that entered the WWTW already containing resistance genes. Organisms receiving ARGs can be both pathogenic and non-pathogenic. While the non-pathogenic ARB may seem less threatening, they may still have the potential to transfer resistance genes to pathogens (Samadi et al., 2015).

Due to the fact that biofilms (flocs in the case of activated sludge) are closely associated communities of bacteria and are in close proximity to each other, the likelihood of HGT occurring is likely (Van Hoek et al., 2011).

The relatively high concentrations of some antibiotics in WWTWs, in addition to the low removal efficiency of others, allows for the application of a constant selective pressure to the microbiological communities present in the WWTW (Xu et al., 2015). It has also been shown that sub-inhibitory concentrations of antibiotics can select for resistant organisms. For example, Gullberg et al*.,* (2011) exposed a susceptible strain to an antibiotic concentration 1/30 of the minimum inhibitory concentration (MIC). This strain grew 15% slower compared to when the strain was not exposed to antibiotics. In contrast, the growth of a mutant strain, containing resistant genes, was not affected by exposure to sub-MICs. This suggests that resistant strains can outcompete susceptible strains when exposed to sub-MICs, resulting in the emergence of resistant bacterial populations.

Furthermore, the study showed that exposing susceptible organisms to a quarter of the MIC of an antibiotic may result in the development of resistance. Within 200 to 400 generations, mutants were observed with MICs between two and 16 times higher than the MIC of the starting strain. After 500 to 600 generations, MICs increased to 24 to 32 times higher than the starting MIC (Gullberg et al., 2011). Some microorganisms, such as *Pseudomonas* spp., utilise a biofilm as a defence mechanism when exposed to antibiotics. Biofilm formation limits the penetration of antibiotics to bacterial cells at the centre or base of the biofilm, enhancing their ability to persist in the environment (Balcázar et al., 2015). In addition to selective pressure, HGT may arise due to other environmental factors. While the wastewater treatment process is designed to remove microbial content in the effluent, lapses in the functioning and maintenance of these processes have the potential to allow AMR organisms to persist downstream from WWTW effluents. In addition, the microbial composition has been found to differ between influent and effluent, with a larger diversity of bacteria being present in the effluent opposed to the influent (Tang et al., 2016). A South African study indicated that pathogenic strains of *E. coli* present in wastewater effluent were resistant to multiple antibiotics, especially tetracycline (60.1%) and ampicillin (55.6%) (Adefisoye and Okoh, 2016).

In addition to WWTWs harbouring and transmitting AMR to effluent-receiving waters, rural and peri-urban settlements are mostly impacted on by poor health and sanitation services and practices. For example, many informal settlements are not connected to municipal sewage or greywater drainage systems or do not have access to communal toilets or potable water distribution systems (Greenberg et al., 2007). As a result, the discharge of sewage and wastewater from daily use normally ends up in the proximity of households or communal areas, and eventually also in natural freshwater systems. As water safety and security are vital for the establishment and maintenance of public health, the presence of both high-risk pathogens and antimicrobials of concern in these environments needs to be established. In addition, the effect that antimicrobials and high-risk pathogens may have on the development and dissemination of AMR needs to be investigated in environmental waters (Friedrich et al., 2009).

1.4.2 High-risk bacterial pathogens

The Infectious Diseases Society of America has prioritised the most concerning pathogens in terms of AR. These six organisms are collectively known as the ESKAPE organisms and include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp. (Rice, 2008). These organisms are responsible for many infections and often contain genes encoding multiple mechanisms of resistance, which not only make the infection more difficult to treat, but also prolong the duration of illness for the patient, giving resistant bacteria more time to be transmitted to other individuals (Du et al., 2016; Laxminarayan et al., 2013).

The South African National Institute for Communicable Diseases (NCID) utilises resistance maps to indicate the percentage resistance of individual species to an antibiotic on a provincial level. Many of the ESKAPE organisms are already highly resistant to numerous antibiotics in South Africa. For example, the percentage of *A. baumannii* isolates that have resistance to gentamicin in public hospitals across South Africa is shown in Figure 1-5a, while that of *E. faecium* to ampicillin/amoxicillin is shown in Figure 1-5b.

In addition, the WHO released a priority pathogens list at the beginning of 2017. This list was established by a number of experts in a variety of fields and is split into three risk categories: critical, high and medium (World Health Organisation, 2017).

Carbapenemase producers such as *A. baumannii, P. aeruginosa* and Enterobacteriaceae, which include organisms such as *E. coli* and *K. pneumoniae,* fall into the "critical" category. This is due to the fact that these organisms cause infections that are difficult to treat, and are – in some cases – untreatable (Du et al., 2016; Laxminarayan et al., 2013; World Health Organisation, 2017). The "high-risk" category of the WHO's priority pathogens list includes vancomycin-resistant *E. faecium* and *S. aureus*, methicillin-resistant *S. aureus*, clarithromycin-resistant *Helicobacter pylori*, fluoroquinolone-resistant *Campylobacter*, fluoroquinolone-resistant *Salmonella* spp., and third-generation cephalosporin- and fluoroquinolone-resistant *Neisseria gonorrhoeae*. Finally, the medium-risk priority organisms include penicillin-non-susceptible *Streptococcus pneumoniae*, ampicillin-resistant *Haemophilus influenza* and fluoroquinolone-resistant *Shigella* spp. (World Health Organisation, 2017).

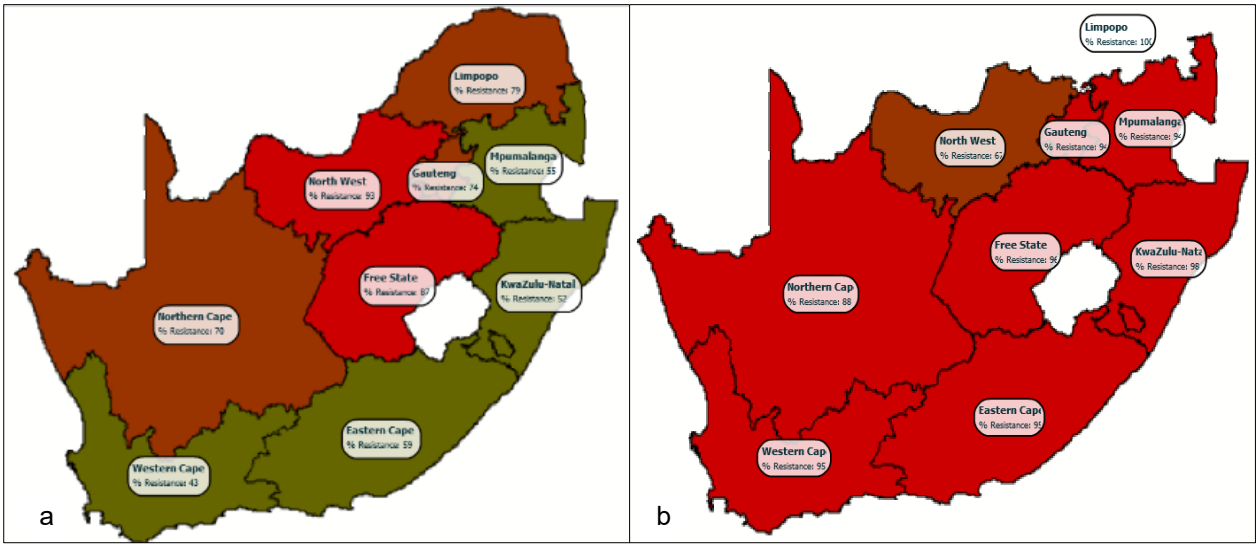

Figure 1-5: Percentage resistance of clinical isolates of (a) *A. baumannii* to gentamicin and (b) *E. faecium* to ampicillin/amoxicillin in NHLS provincial hospitals in South Africa (adapted from NICD, 2015)

The NICD indicates the ratio of susceptibility of *P. aeruginosa* in 2014/15 to various antibiotics as seen in Figure 1-6. Some 56% of the antibiotics shown had a decreased susceptibility ratio in 2015 as opposed to 2014, indicating that *P. aeruginosa* resistance to these antibiotics (amikacin, piperacillin-tazobactam, cefazolin/cephalexin, cefoxitin, cefotaxime/ceftriaxone, ceftazidime, imipenem, meropenem and ciprofloxacin) has increased (NICD, 2015). While the susceptibility ratio is expected to decrease over time, the percentage decrease over one year for some antibiotics is alarming (a 13% decrease for cefazolin and a 3% for amikacin). Amoxicillin and trimethoprim-sulfamethoxazole have very low susceptibility ratios (15% and 34%, respectively), which reinforces the concern around AMR as these are regularly prescribed antimicrobials. In the USA, Enterobacteriaceae resistance to carbapenems has increased by 1.4% in a decade (Laxminarayan et al., 2013). Some 71% of *Klebsiella* spp. isolates and 50% of *E. coli* isolates obtained from neonates in developing countries were found to be resistant to gentamicin, while 60-70% of *E. coli* isolates and close to 100% of *Klebsiella* spp. isolates are resistant to ampicillin (Zaidi et al., 2005). Iweriebor et al. (2015) showed that 67-100% of *Enterococcus* isolates from WWTWs and hospital effluents in the Eastern Cape were resistant to a number of different antibiotics.

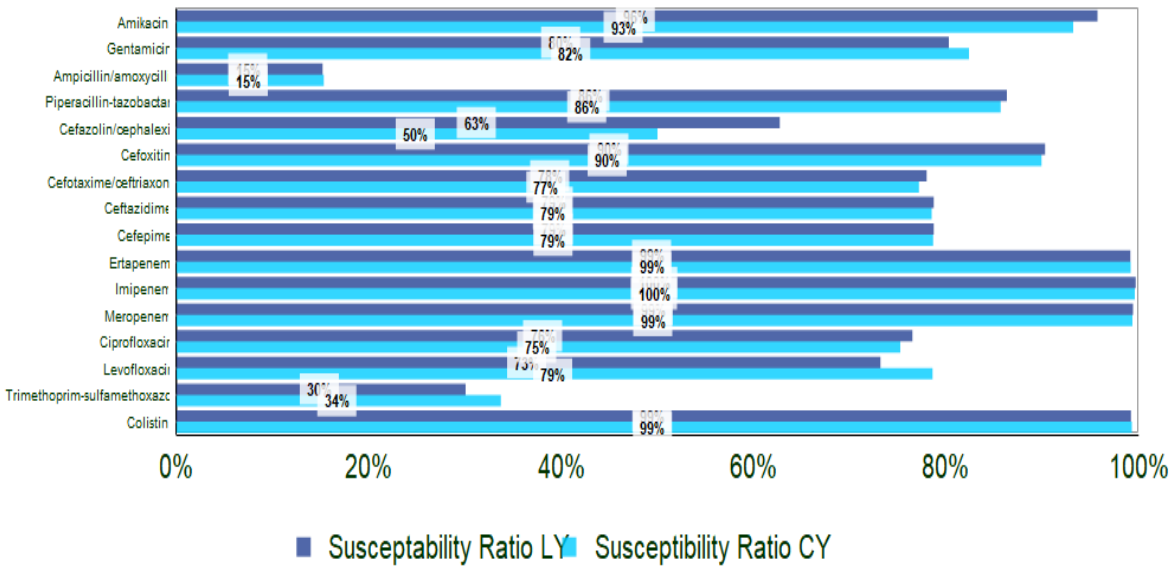

Figure 1-6: Susceptibility ratios of *P. aeruginosa* to various antimicrobials in 2014 (dark blue) and 2015 (light blue)

Duran et al. (2012), examining Staphylococcal resistance profiles against 10 different antibiotics, showed that 87.9% of the clinical isolates were resistant to penicillin, followed by 52.3% to ciprofloxacin 32% to trimethoprim-sulfamethoxazole, 28.9% to amoxicillin-clavulanic acid and 31.5% to gentamicin. From this, a high level of resistance to commonly used antibiotics is seen for staphylococcal isolates, reinforcing the WHO's listing of this genus of pathogen as a high-priority organism (Duran et al., 2012; World Health Organisation, 2017). ESBL producers, which result in resistance to penicillin antibiotics, have also given rise to concern (Wellington et al., 2013). In the study of Duran et al. (2012), it was found that 93.5% of the isolates tested contained the *blaZ* gene, encoding resistance to penicillin (Duran et al., 2012). The number of isolates that contain these genes is higher than the number of isolates that exhibit phenotypic penicillin resistance. This indicates that even though a resistance gene is present, it may or may not be expressed and confer resistance to a particular antibiotic (Duran et al., 2012).

1.4.3 Resistance genes in wastewater effluents

1.4.3.1 Prevalence of antibiotic resistant genes in wastewater

As the wastewater treatment process was not specifically designed to remove resistance genes, they often persist in effluent, which may have detrimental consequences for human, plant and animal life. New emerging ARGs that confer resistance to aminoglycosides, β-lactams and fluoroquinolones in clinical settings have been identified in wastewater effluent, indicating that genetic material from clinical microorganisms that enter the WWTW can be transferred to environmental bacteria (Szczepanowski et al., 2009). The most reported ARGs found in wastewater are those that encode resistance to amino-glycosides, tetracyclines, sulphonamides and β-lactams.

A study by Hultman *et al.* (2018) showed that the quantity of four resistance genes found within bacterial orders in the influent of a WWTW in Finland was higher than that found in the effluent. It was observed in some cases that resistance genes that persist in the effluent were present in different bacterial orders than in the influent, showing that HGT indeed occurs in WWTWs (Hultman et al., 2018). Similar results were obtained in a study conducted in Michigan, USA, where it was shown that a maximum of 2.33 x 10^4 ARG copies per mℓ of wastewater effluent were present, while 4.32 x 10^9 ARG copies·g^{-1} of bio solids were detected (Munir et al., 2011).

Similarly, another study by Yang et al. (2016) showed that copy numbers of carbapenem resistance genes ranged from 1.54×10^3 copies·mℓ$^{-1}$ to 2.14×10^5 copies·mℓ$^{-1}$ in WWTW effluents as opposed to bio-solids where the gene copy number was substantially higher and ranged from 6.51×10^9 copies·g^{-1} to 6.18×10^{10} copies·g^{-1} (Yang et al., 2016). In a third study, roughly 94% of β-lactam resistance genes were detected in activated sludge, while 85% of these genes were detected in WWTW effluent (Szczepanowski et al., 2009). The high ARG copy number in bio-solids could influence the dissemination of ARG and ARB if the bio-solids are repurposed for farming instead of being incinerated or taken to a landfill.

Despite there being a consensus that ARG quantities decrease from influent to effluent, other studies suggest that there is very little or no change in certain ARG copy numbers after the wastewater treatment process (Munir et al., 2011; Xu et al., 2015). After chlorination, blaKPC$_{-2}$, bla$_{GES-1}$ and bla$_{IMP-1}$ were detected, predominantly in *A. baumannii* and *E. coli,* in WWTW effluent (Yang et al., 2016). In addition, the study by Munir et al. (2011) showed that there was no significant decrease in either ARG or ARB before chlorination, compared to after chlorination. Other research has suggested that chlorination selects for ARB and induces the emergence of ARG. However, the underlying mechanisms are unknown (Murray et al., 1984).

With the rise of carbapenem-resistant bacteria, particularly *K. pneumoniae* and other Entero-bacteriaceae, the β-lactam class of antibiotics has become a major concern with a little under 1,000 different β-lactamases being identified in the past two decades (Laxminarayan et al., 2013). Of these, the New Delhi Metallo-β-lactamase (NDM), OXA-β-lactamase (OXA), GES-β-lactamase (GES) and Klebsiella pneumoniae carbapenemase (KPC) are of most concern. As a result, the WHO has listed carbapenem antibiotics as a critically important antimicrobial (World Health Organisation, 2011). The *AmpC*, *bla*$_{TEM}$ *bla*$_{SHV}$ are common ARGs encountered for amoxicillin resistance (Féria et al., 2002). Sulphonamide-resistance genes are among the most frequently encountered in WWTWs.

Among the plasmid-mediated sulphonamide-resistance genes are *sul1, sul2* and *sul3,* which were first identified in the 1980s (Van Hoek et al., 2011). In areas that have not been recently exposed to sulphonamide antibiotics, it has been found that *sul* genes, in addition to tetracycline-resistance genes (*tet*), persist in sediment for long periods of time (Muziasari et al., 2014). The *sul1* and *sul2* are commonly detected in high frequencies in WWTW effluent. However, *sul3,* appears to be less prominent (Czekalski et al., 2014; Hultman et al., 2018; Suzuki et al., 2015; Xu et al., 2015). Multiple studies have shown that *sul3* is present in microorganisms found in animals, but not those found in humans (Guerra et al., 2003; Suzuki et al., 2015; Wu et al., 2010).

Information regarding antibiotics and their respective resistance genes in South African wastewaters is limited, with the study performed by Suzuki et al. (2015) in KwaZulu-Natal being among the first of its kind. That study found that the copy numbers of *sul1* and *sul2* were similar, being 10^{-2} -10^{-1} per 16S copy number (Suzuki et al., 2015). A study performed by Suzuki et al. (2013) showed that *sul3* was not detected in bacteria that had been cultured, indicating that this gene is more likely to occur in VBNC organisms. Until recently, gene-coding mechanisms of resistance to colistin were only chromosomal. However, as mentioned previously, Liu et al. (2016) identified the first plasmid-mediated colistin-resistance gene, *mcr-1*. Since its first discovery, this gene has been identified globally in *E. coli* isolated from food, animals or clinical samples (Gao et al., 2016). There have been very few studies that isolated colistin-resistance genes from bacteria in WWTWs.

1.4.3.2 Mechanisms of action and their transfer to other organisms

Antibiotic resistant genes can encode several mechanisms of resistance to either single or multiple antibiotics. Multiple genes can be responsible for resistance to a particular antibiotic and it is possible for more than one resistance gene to be expressed simultaneously in a single cell (Duran et al., 2012).

One of the best-researched resistance mechanisms is the production of the modification enzyme, β-lactamase. This enzyme renders penicillin antibiotics inactive by hydrolysing the β-lactam ring present in the antibiotic (Duran et al., 2012). Other mechanisms of resistance include altering cell wall permeability, preventing the antibiotic from entering the target site and the presence of efflux pumps, which can actively remove the antibiotic from the microbial cell before it is able to exhibit its function. In addition, alternative metabolic pathways can be acquired by the cell, which bypass a crucial metabolic step that an antibiotic would normally inhibit, and enzymes usually targeted by an antimicrobial compound can be over-produced (Van Hoek et al., 2011; Lin et al., 2015).

1.5 PROJECT AIMS

This report addresses two main topics:

- Through the known knowledge of the presence and fate of CECs in South African WWTWs, to investigate the potential of alternative treatment technologies to improve on CEC and EDC degradation
- To investigate the presence and fate of ARB- and AMR-related genes in WWTWs.

These topics correspond with aims 4, 6 and 7 of the original aims of the project, which are as follows:

- Report on the presence and fate of EDCs and priority micropollutants at a WWTW that utilises the HYBACS® treatment system (Aim 6)
- Report on the presence and fate of EDCs and priority micropollutants during the treatment of wastewater by the CabECO treatment system (Aim 7)
- Investigate the development of AMR caused by environmental concentrations of persistent antimicrobial pollutants (Aim 4)

CHAPTER 2: EFFICIENCY OF VARIOUS TECHNOLOGIES FOR THE REMOVAL OF PRIORITY MICROPOLLUTANTS IN WASTEWATER

2.1 INTRODUCTION

The focus of most secondary treatment technologies is on improving sludge maturity for nutrient removal. However, the optimisation of these technologies for the removal of CECs will gain much focus in the future. Although there has been much advancement to promote an increased state of biodegradation of CECs within conventional WWTW treatment processes, it is still evident that tertiary treatment processes, such as AOPs, may be needed to completely mineralise the CECs, rather than create metabolically active transformation products or simply retain them within the treatment system (Beltrán and Rey, 2018). Such tertiary treatment systems are based on the formation of highly reactive species such as hydroxyl radicals (OH^{-}), which drive the mineralisation of CECs (Klavarioti et al., 2009). Examples of AOPs include photolysis (ultraviolet) combined with peroxide, Fenton oxidation, electrochemical oxidation, ultrasound irradiation, subcritical wet air oxidation, heterogenous photocatalysis and ozonation, of which the latter two have received the most attention for their potential for CEC degradation (Klavarioti et al., 2009).

In contrast to advanced secondary treatment, tertiary treatment technologies require improved infrastructure of the WWTW and are also associated with high costs for their operation and maintenance. However, the rapid response of such treatment steps to eradicate CECs compared to lengthy exposure or residence time needed for secondary treatment favours AOPs to serve as both centralised and decentralised treatment technologies. The implementation of such tertiary treatment technologies usually requires optimisation in its performance for the area or treatment system, where it is destined for use. Where deployed, such treatment technologies have been shown to be useful to lower CECs, which show persistence in WWTWs and environmental waters. The following case studies are aimed at investigating the potential of an advanced secondary treatment process and a tertiary treatment process using photochemical oxidation to remove CECs that show variable persistence in conventional WWTW systems.

2.2 CASE STUDY 1: FATE OF EMERGING CONTAMINANTS IN A WWTW USING A HYBRID ACTIVATED SLUDGE (HYBACS®) TREATMENT SYSTEM

2.2.1 Background

The current case study reports on the assessment of the presence and fate of priority micropollutants and endocrine-disrupting ligands within the treatment modules of the WWTW after the new treatment module has been implemented. The initial treatment processes of the plant allowed for an average capacity of 10.8 mℓ per day, serving an estimated 35,397 population equivalents. The plant deals with a high domestic load of sewage and grey water from the district. With increasing urbanisation within the area, the wastewater load and capacity of the plant needed to be adjusted. This led to the installation of a patented HYBACS® by Bluewater Bio (London, UK) to primarily increase plant operation (nutrient removal and biomass formation) and treatment capacity.

2.2.2 The HYBACS® system

The HYBACS® system at the site consists of 12 attached-growth SMART™ units. This reactor system is based on a rotating structure that allows for the formation of biomass onto mesh plates that are rotated within the wastewater liquor and thus allow for attached biomass growth through aerobic, anaerobic and anoxic conditions (Figure 2-1).

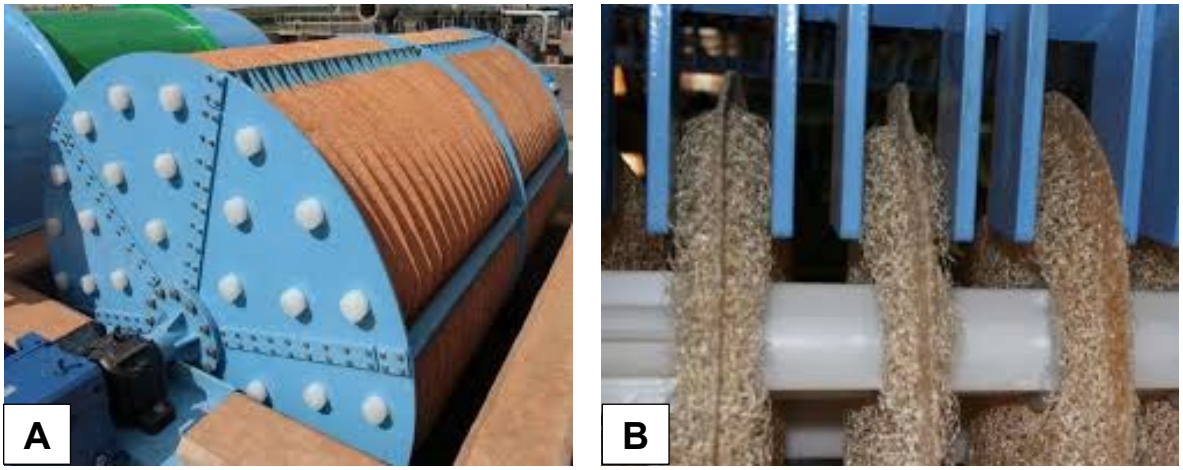

Figure 2-1: Image A is from http://www.degremont-technologies.com/cms_medias/pdf/cs_Infilco_HYBACS-Botleng.pdf, and image B is from https://www.mi-wea.org/docs/McGovern-Hybrid_Activated_Sludge_Process.pdf.

The SMART™ treatment system is then coupled with a CAS system that provides suspended biomass contact. The addition of this reactor allows for improved contact time of wastewater with both surface-attached and suspended sludge for nutrient removal, as well as aeration prior to further AS treatment. The upgrade to the plant allows for an increased wastewater treatment capacity of more than 20 mℓ per day, equating to a population estimate of 75,000 people. Apart from the improvement of hydrological parameters and capacity through the SMART™ reactor, this system also shows promise to assist with the metabolism and attenuation of persistent organic pollutants, such as pesticides and PPCPs. However, such a study has not yet been done on this system.

2.2.3 Study site

The study was done at a WWTW south of Tsakane (Mashona) in the Ekurhuleni Metropolitan, Gauteng (Figure 2-2). The most recent census for the township estimated a population of 135,994 (Stats SA 2011). The WWTW receives domestic wastewater, a fraction of which is from a recently built shopping centre. Initially, the plant processes included a central inlet works containing screening and grit removal, followed by primary sediment (PST) and a CAS process that contains a three-stage biological nutrient removal (BNR) basin (anaerobic, anoxic and aerobic) and a secondary settling tank (SST) as a final clarification basin. The overflow of the SST is then chlorinated before being discharged into a tributary leading towards the Blesbokspruit River (Figure 2-2). The river itself flows through a densely populated area upstream of the WWTW, with smaller populations and more agricultural land situated downstream from the point of wastewater discharge.

Figure 2-2: Map layout of the Tsakane WWTW. Images derived from Google Maps. The white arrow shows the flow direction of the tributary of the Blesbokspruit River.

2.2.4 Sampling and sample processing

Sampling of the aqueous matrix within the WWTW was done at the raw inlet after screening and degritting the outflow of the final SMART™ module at the overflow of the PST, anaerobic, anoxic and aerobic bioreactors, the overflow of the clarifier module, and the treated wastewater effluent after chlorination (Figure 2-3). Grab samples (500 mℓ) were taken from the modules within the plant, and time-proportional composite samples (500 mℓ) were taken from the raw inlet and treated effluent. Grab samples from river water located 50 m upstream and 50 m downstream from the WWTW point of discharge were also taken on a single day during the sampling period. Raw and treated effluent samples were collected over a seven-day period from 14 to 20 June 2018, and samples between treatments were collected on 18 June 2018.

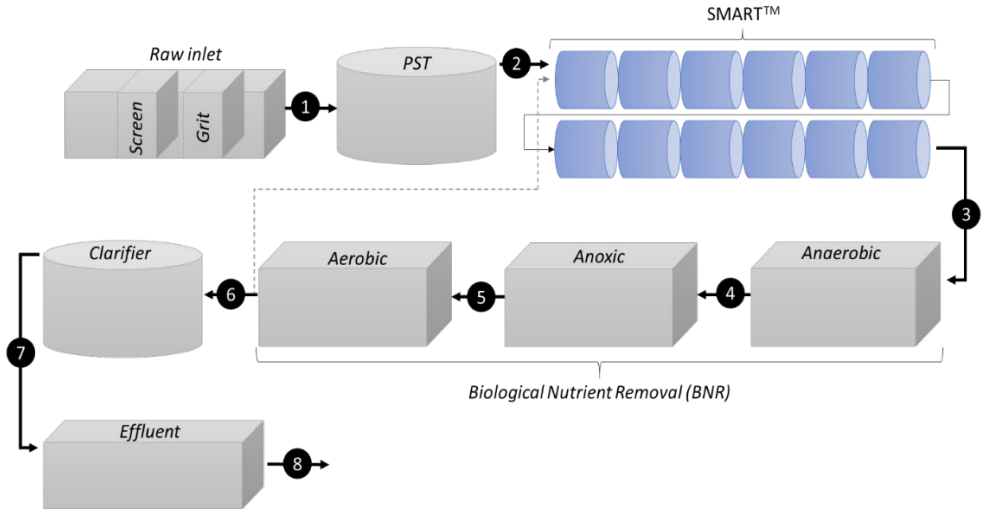

Figure 2-3: Schematic and sampling points (1-8) of the Tsakane WWTW after the installation of the HYBACS® treatment system

The collected aqueous samples from the various treatment modules and river water were split into duplicate water samples for liquid chromatography-mass spectrometry (LC-MS) and a 300 mℓ water sample for the YES assay. All samples were pre-filtered using 1.2 um glass fibre filters and a vacuum filtering device prior to solid-phase extraction (SPE). For raw wastewater and AS samples from the SMART™ anaerobic, anoxic and aerobic modules, the water samples were centrifuged (5,000 rpm, 15 minutes, 4 °C) to separate the solid particulate matter (SPM) from the aqueous matrix prior to pre-filtration. For the chemical analyses, duplicate aqueous samples from each WWTW process (50 mℓ) and river water (100 mℓ) were spiked with a labelled internal standard mixture at a concentration of 1 µg/ℓ prior to SPE. Aqueous samples for the YES assay of each WWTW treatment step and river water (300 mℓ) did not include any labelled internal standard spike and were extracted in singlet.

Extraction of target analytes was done using Oasis hydrophilic-lipophilic-balanced (HLB) cartridges (3 cc, 60 mg; Waters) for LC-MS, and Oasis HLB cartridges (6 cc, 200 mg; Waters) for the YES assay. The 3 cc cartridges for LC-MS were pre-conditioned with 2 mℓ high-performance liquid chromatography (HPLC)-grade methanol followed by 2 mℓ deionised water (MilliQ). The 6 cc cartridges for the YES assay were pre-conditioned with 4 mℓ HPLC-grade methanol (MeOH), followed by 4 mℓ deionised water (MilliQ). All cartridge pre-conditioning was performed under gravity. The aqueous samples were then loaded through the cartridges using a 12-channel SPE vacuum manifold (SUPELCO, VISIPREP™, Sigma-Aldrich) at a flow rate of 5 mℓ per minute and dried for 30 minutes under vacuum. Elution of the samples was done using 4 mℓ HPLC-grade MeOH silanised glass vials and evaporated under nitrogen and a heating mantle (35 °C). The dried samples were then reconstituted in either 1 mℓ MeOH for the LC-MS samples or 300 µℓ MeOH for the YES assay samples, giving a 50x concentration for the WWTW samples (for LC-MS), a 100x concentration for the river water samples (LC-MS), and a 1,000x concentration for the YES assay samples.

2.2.4.1 The Yeast Estrogen Screen

The yeast-based estrogen receptor binding screen (YES) followed the protocol described by Sohoni and Sumpter, 1998. Upon the 72-hour incubation of the yeast cells with the SPE-extracted water samples, the assay plates were measured for colour change using a spectrophotometer. The absorbance was measured at 570 nm for colour change of chlorophenol red §-D-galactopyranoside (CPRG) caused by steroid hormone-mediated β-galactosidase production, and 620 nm for turbidity change and cytotoxicity. The threshold for cytotoxicity in the samples was determined using Equation 2-1:

$$\text{Cytotoxicity} = \text{Median Blank}_{620nm} - (3*\text{stdev}_{blank620nm}) \quad \text{[Eq. 2-1]}$$

To correct for turbidity that may provide false positive responses in the assay, a corrected absorbance (CA) was calculated for each sample in the assay using Equation 2-2:

$$\text{Corrected absorbance (CA)} = (OD_{570nm} - [OD_{620nm} - \text{blank}_{620nm}]) \quad \text{[Eq. 2-2]}$$

where OD_{570nm} and OD_{620nm} refer to the optical density of the sample measured at 570 and 620 nm, respectively, and Blank_{620nm} refers to the median optical density measured for the blank wells in each assay plate at 620 nm.

Water samples were only considered for further analysis if the corrected absorbance was above a detection threshold using Equation 2-3:

$$\text{Detection} = \text{Median blank}_{CA} + (3*\text{stdev}_{\text{blankCA}}) \quad \text{[Eq. 2-3]}$$

The CA of water samples that showed no cytotoxicity and was above the detection threshold of the assay was then compared with the responses of the estradiol (E_2) standard curve in the assay to calculate an estimated EEQ value for each sample.

2.2.4.2 Liquid chromatography-mass spectrometry for targeted CEC analysis

Acquisition and quantification of the target CEC analyses were achieved using an ultra-performance liquid chromatograph (UPLC) coupled with a triple-quadrupole mass spectrometer (MS/MS; Waters) and a multiple-reaction monitoring mode (MRM) method as previously described in Volume 1 of this project report (WRC Report K5/2733). The target CECs that were investigated included the non-steroidal anti-inflammatory (NSAID) diclofenac, the anti-convulsant carbamazepine, carbamazepine-10,11-epoxide and 10,11-dihydro-11-hydroxycarbamazepine, the opioid codeine, the corrosion inhibitor benzotriazole, the lifestyle chemical caffeine, the herbicide atrazine, the antibiotic sulfamethoxazole and the illicit drugs methamphetamine, methaqualone, cocaine and benzoylecgonine.

2.2.5 Results and discussion

2.2.5.1 Yeast estrogen screen

Estimated EEQ values from the various treatment steps showed a gradual decrease in estrogenicity during the sampling campaign that was done before the installation of the HYBACS® upgrade, with an estimated removal of 78.6% of total estrogenicity at the final treated effluent (Figure 2-4). Although a high rate of removal was recorded, the EEQ value in the final discharge was still within the 5 ng/ℓ level shown to potentially trigger estrogenic outcomes in aquatic organisms (Caldwell et al., 2012). These concentrations also surpassed risk-based trigger values, as mentioned previously in the current report (Part 1 in WRC Report K5/2733).

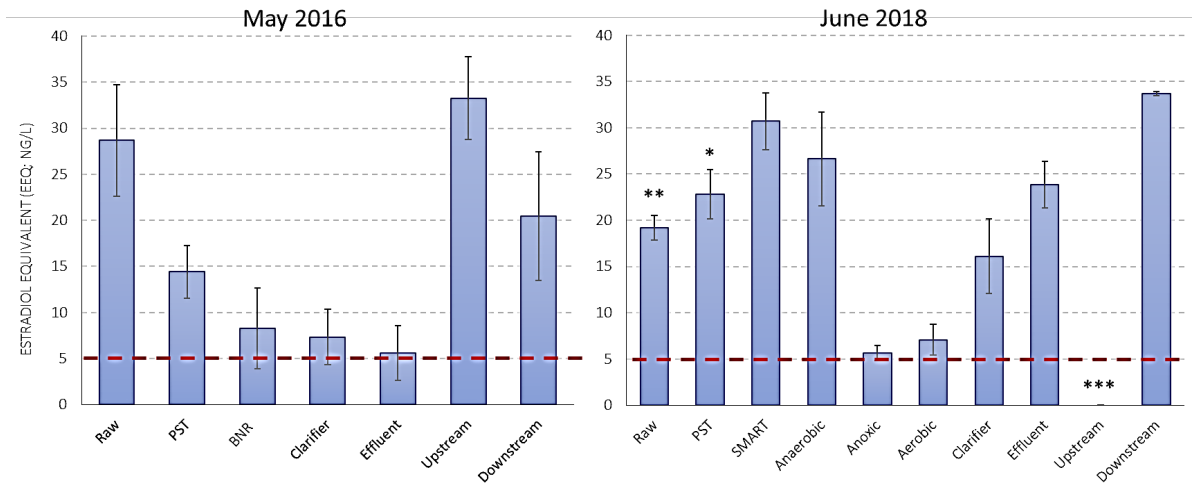

Figure 2-4: **Yeast estrogen screen performed between treatment steps in May 2016 and June 2018 before and after the installation of the HYBACS® treatment system, respectively. Values expressed as EEQ (ng/ℓ) as generated by a 17β-estradiol standard curve. Abbreviations: PST - Primary sedimentation tank; BNR - Biological nutrient removal through AS treatment; SMART™ - Shaft Mounted Advanced Reactor Technology. The asterisk indicates cytotoxicity observed within the assay (*: Low; **: Medium; ***: High).**

On the other hand, the average EEQ values for the water samples within the river system located upstream of the plant showed an even higher average concentration than in raw sewage, indicating extreme pollution from alternative sources within this river system. Consequently, the estimated EEQ concentrations were lower in downstream river samples than upstream of the plant (Figure 2-4), showing that the discharge of the WWTW still contributes to lowering the total estrogenicity in the river system.

For the assessment of total estrogenicity in the various treatment modules after the implementation of the HYBACS® system, calculated EEQ values increased in the overflow of the PST and in the liquor after the SMART™ treatment (Figure 2-4). Although it seems that the EEQ concentration increased, it should be noted that some cytotoxicity was observed in the assay for the raw wastewater and PST overflow, with no cytotoxicity in the liquor after the SMART™ treatment (Figure 2-4). This raises concern that some inert cytotoxic components in these treatment modules may have interfered with the biochemical pathways in the YES (altered receptor binding, altered transcription and translation of β-galactosidase and/or the metabolism of CPRG in the assay media), ultimately suppressing the total estrogenicity outcomes of the assay. Regardless, estrogenicity was then significantly reduced after the anoxic treatment step (Bonferroni's multiple comparisons test, $P < 0.001$) (Figure 2-4).

However, calculated EEQ values were again shown to increase at the overflow of the secondary clarification and effluent after chlorination. The cause for this increase is still unknown. However, it was observed that the WWTW experienced operational challenges during the sampling period, in which a high load of bulking was observed on the surface of the secondary clarifiers, along with moderate turbidity in the final effluent. Whether these conditions may have resulted in a build-up of estrogenic analytes and/or desorption of recalcitrant analytes from sludge during the AS process is still unclear.

Like the observed cytotoxic effects, which could have influenced the estrogenic response within the WWTW treatment steps, a high load of estrogenicity was observed in surface waters located downstream from the WWTW compared to the upstream sample, in which the upstream sample was completely cytotoxic in all of the concentrated ranges of the sample in the assay.

The high estrogenic load could have originated from analytes in upstream surface waters, from the high estrogenic load in the final effluent or from a combination of the two. No cytotoxicity was observed for the downstream water samples in the YES assay, which at least confirms that the discharge of the WWTW still contributes to lowering the cytotoxic load in the water body. Even so, the measured EEQ concentration in both the downstream and treated effluent samples is shown to be much higher than the EEQ concentration that is shown to exert endocrine-disrupting effects in freshwater biota, and which thus require further intervention (Figure 2-4). Although natural and synthetic steroid hormones are shown to be the most potent contributors to the total estrogenicity in aqueous matrices (Holbrook et al., 2002, Snyder et al., 2001), the detection of high estrogenicity in river water located upstream from the WWTW discharge may also be derived from a mixture of estrogen-mimicking contaminants, such as preservatives (e.g. methylparaben) and plasticisers (e.g. bisphenol-A), both of which have been shown to mimic the natural binding of a steroid hormone to a hormone receptor, although at much lower potencies (Sun et al., 2016; Lazúrová and Lazúrová, 2013) and may easily be associated with pollution products along water bodies. Moreover, unintentional or intentional discharge of sewage (human waste products) may also explain the high load of estrogenicity in the water body, as many peri-urban communities are either not connected to or have poor sanitation infrastructure. Regardless of the source, it is still difficult to correlate effect-based results (such as estrogenicity determined by the YES) with targeted analytical chemistry, as the complexity of physical, chemical and microbial mixture interactions may complicate the drawing of definite conclusions. Regardless of whether the targeted CECs in the study may have contributed to the estrogenic responses in the YES for the same samples, it is worth recording this to evaluate whether the different CECs showed the same fate during the WWTW process as observed for effect-based monitoring.

2.2.5.2 Target CECs during the treatment process

The acquired concentrations of the priority CECs during the WWTW process are shown in Table 2-1, along with the average removal of the target analytes throughout the seven-day sampling period. A removal value below 25% was considered as *poor* removal, between 25 and 75% as *moderate*, and above 75% as *high* removal. Six of the 13 CECs tested showed high average removal and included the illicit drugs methamphetamine, cocaine and benzoylecgonine, the analgesic codeine, the anti-corrosive benzotriazole and the lifestyle chemical caffeine. Five of the 13 CECs showed moderate removal, including the antibiotic sulfamethoxazole, the herbicide atrazine, the illicit drug methaqualone, the anti-epileptic carbamazepine and the NSAID diclofenac. Two metabolic by-products of carbamazepine – carbamazepine-10,11-epoxide and 10,11-dihydro-10-hydroxy carbamazepine – showed an average negative mass balance throughout the sampling period (Table 2-1).

It was clear that a large variation in removal profiles over the seven-day sampling period was observed for sulfamethoxazole, atrazine, methaqualone, carbamazepine and its metabolites (Table 2-1). Due to the differences in their physio-chemical properties, no direct conclusion could be drawn for this observation of removal variance between days. However, it should be noted that the current removal calculation did not consider the hydraulic residence of the treatment system, and therefore merely compared the mass loads (grams per day) of the CECs at the incoming wastewater with the treated effluent on the same day. Again, as some CECs will have a tendency to be retained in either the aqueous or solid phase of treated wastewater, these compounds may persist over long periods of time within the WWTW, especially considering the fact that a proportion of AS from the aerobic module is returned to the start of the SMART^{TM} treatment system to promote sludge maturity for the HYBACS® treatment system.

Some discussion on the most significant findings during the CEC analyses follows. The concentration of the anti-inflammatory diclofenac showed an average decrease of 41.5% (± 18.7) throughout the current study period (Table 2-1). This correlates with various other studies showing that diclofenac reduction during AS treatment rarely exceeds 50% removal (Radjenovic et al., 2009; Falås et al., 2013; Petrie et al., 2013; Larsson et al., 2013). The most prominent reduction was observed after primary sedimentation and can be attributed to the Log K_{ow} of diclofenac, which is shown to range between 4.02 and 4.51 (EPI Suite, Version 4.1, KOWWIN).

Table 2-1: Concentrations (ng/ℓ, ± standard deviation) of the target analytes throughout the WWTW processes on 18 June 2018 and daily load reduction (percentage, ± standard deviation) estimated using the calculated mass loads (g/day) at the raw and final effluent wastewater samples during the seven-day sampling period

CEC	WWTW process concentration (ng/ℓ ± standard deviation)								Removal (%)	Upstream	Downstream
	Raw	PST	SMART™	Anaerobic	Anoxic	Aerobic	Clarifier	Effluent			
DCF	967.0 ±1.9	536.8 ±10.2	519.5 ±27.4	510.8 ±16.5	454.3 ±13.2	390.5 ±3.0	426.1 ±55.0	310.2 ±43.9	41.1 ±18.7	208.7 ±16.3	383.8 ±40.9
CBZ	448.1 ±3.7	312.9 ±15.8	595.8 ±10.0	648.4 ±1.7	641.8 ±5.6	764.4 ±2.8	815.1 ±12.8	885.1 ±10.3	31.6 ±53.6	250.0 ±11.0	440.9 ±21.3
CBZ-ep	861.7 ±6.8	199.3 ±23.3	318.6 ±39.0	309.6 ±31.7	316.7 ±17.9	337.1 ±17.4	378.6 ±24.1	331.5 ±41.5	-13.4 ±69.5	318.1 ±2.4	319.6 ±10.0
h-dhCBZ	621.5 ±14.9	337.8 ±22.0	1095.2 ±102.3	1134.3 ±45.4	976.2 ±4.3	993.4 ±7.0	1240.7 ±60.2	1293.6 ±142.0	-4.2 ±60.1	830.4 ±37.4	767.2 ±61.2
BZT	848.3 ±0.2	120.9 ±3.0	40.1 ±5.2	32.0 ±11.8	37.0 ±1.1	0.8 ±1.1	< MQL	< MQL	93.4 ±10.0	< MDL	< MDL
COD	123.8 ±22.2	54.2 ±5.9	76.6 ±9.6	55.3 ±7.8	31.4 ±7.2	4.9 ±4.4	12.3 ±1.0	5.2 ±1.1	85.6 ±13.0	24.2 ±10.5	1.4 ±2.0
METH	40.4 ±4.8	24.4 ±5.1	8.8 ±1.9	1.6 ±2.3	2.3 ±2.3	< MQL	< MQL	0.1 ±0.2	91.1 ±14.2	2.5 ±3.1	< MQL
METHA	413.8 ±16.8	391.5 ±20.7	568.3 ±22.2	613.2 ±13.7	612.9 ±23.1	322.4 ±37.5	637.0 ±37.0	996.3 ±9.4	25.3 ±68.9	686.5 ±13.9	298.4 ±27.2
COC	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	100.0*	14.4 ±6.7	< MDL
BEG	13.8 ±1.7	42.9 ±10.1	14.2 ±1.1	17.2 ±1.5	7.1 ±7.9	< MQL	8.7 ±3.2	13.5 ±4.4	87.7 ±11.2	20.9 ±1.8	4.9 ±6.9
ATZ	98.3 ±32.0	92.8 ±15.6	94.5 ±4.7	90.2 ±14.7	114.5 ±21.3	92.7 ±17.6	94.9 ±15.6	86.5 ±3.6	34.0 ±30.7	220.4 ±39.8	99.8 ±14.2
SMX	3547.5 ±341.5	1630.0 ±93.1	3807.4 ±215.5	3084.6 ±31.0	1876.7 ±58.4	314.7 ±12.4	837.5 ±72.2	1129.8 ±56.6	45.7 ±40.0	1797.5 ±7.5	805.1 ±40.6
CAFF	22201.3 ±601.9	10668.4 ±260.0	4158.8 ±101.5	3621.3 ±375.1	989.3 ±38.0	513.0 ±49.7	n.d	755.9 ±43.5	89.7 ±10.6	7342.4 ±89.7	1220.7 ±5.4

* Effluent concentrations were below the method detection limit (MDL) and were therefore considered to be lowered significantly during wastewater treatment. Raw influent concentrations were only below the MDL on the sampling day when all the treatment processes were analysed and ranged between 0.03 and 0.16 g/day during the rest of the sampling period.

DCF: Diclofenac; CBZ: Carbamazepine; CBZ-ep: Carbamazepine-10,11-epoxide; h-dhCBZ: 10-hydroxy-10,11-dihydro carbamazepine; BZT: Benzotriazole; COD: Codeine; METH: Methamphetamine; METHA: Methaqualone; COC: Cocaine; BEG: Benzoylecgonine; ATZ: Atrazine; SMX: Sulfamethoxazole; CAFF: Caffeine.

This value suggests that diclofenac may associate with the solid matrix in wastewater, including settled sludge and suspended sludge. However, Topuz et al. (2014) concluded from sludge recovery studies that diclofenac does not show high adsorption to WWTW sludge under aerobic, anoxic or anaerobic conditions within AS treatment modules, and that the lower concentrations measured in final effluent are due to partial biodegradation rather than its retention in solid matrices.

It is further shown that low removal efficiencies of diclofenac are observed during anaerobic digestion, which is mediated by high oxidation rates (Ávila et al., 2013). This was also shown during the current study, where the concentrations lowered slightly in the anoxic and anaerobic reactor overflows, compared to anaerobic digestion (Table 2-1). On the other hand, a study by Fan et al. (2014), showing the biodegradation profile of diclofenac under sterilised and AS reactions, concluded that the sterilised conditions were more favourable for their overall removal or partitioning over a biologically AS condition. The authors mentioned that the amine, halogen and carboxylic functional groups of diclofenac are mostly ascribed to its resistance against biodegradation.

For this reason, the partial biodegradation and partitioning to the solid matrix may drive the moderate reduction of diclofenac during WWTW processes. This is partially shown in the current study, where diclofenac concentrations did not necessarily reduce much throughout the AS treatment processes. Therefore, diclofenac will not necessarily be returned from the aerobic treatment to the SMART™ treatment system during AS maturation and its decrease will be determined by hydraulic residence within the secondary and tertiary treatment steps.

The degradation products of diclofenac should also be considered if the removal of the parent compound is under investigation. Kosjek et al. (2009) identified the degradation product 1-(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one, which forms during thermal decomposition and/or free OH^-. The metabolite 4-hydroxy-diclofenac has also been shown as intermediate of diclofenac biodegradation during AS treatment, although its presence is much lower than the parent compound (Ávila et al., 2013). The presence of 4-hydroxydiclofenac within the wastewater samples could not be determined due to difficulties with its quantification using existing LC-MS methods, and warrants the need for further optimisation to include this analyte in future studies. Although moderate removal for diclofenac was observed during the current study, the final effluent concentrations ranged from 261.9 to 363.5 ng/ℓ.

A study by Efosa et al. (2017) showed that an exposure as low as 29.6 ng/ℓ may induce an up-regulation of hepatic vitellogenin, and a down-regulation of gonadal *Srd5a2* gene expression in male African clawed frogs (*Xenopus laevis*), with a concentration of 2,961.4 ng/ℓ causing a further up-regulation of gonadal aromatase gene expression and lower ratios of plasma testosterone over E_2 concentrations in the same test species. This confirms that diclofenac may exert endocrine-disrupting effects at environmentally relevant concentrations by mediating an anti-androgenic response. For the assessment of the efficacy of the HYBACS® treatment system to improve diclofenac degradation, it does not seem that the addition of attached growth SMART™ modules in the current study have any significant improvement on the removal of diclofenac, but requires further investigation. This will include assessing the partitioning of diclofenac and its major metabolic by-product, 4-hydroxy-diclofenac, within the solid matrix of the AS modules, along with its partitioning within the SMART™ attached biomass.

The anti-epileptic carbamazepine is shown in numerous monitoring studies to be extremely recalcitrant and rarely biodegradable in WWTWs using AS treatment (Petrie et al., 2014; Archer et al., 2017a; Baalbaki et al., 2017). Its Log K_{ow} is calculated between 2.25 and 2.45 (EPI Suite, Version 4.1, KOWWIN), indicating a low tendency to be associated with solid matrices, and is retained within the aqueous component of surface waters. An estimated 25 to 30% of the orally administered dose of carbamazepine is excreted unchanged from the human body, while absorbed carbamazepine goes through excessive metabolism (Zhang et al., 2008).

Carbamazepine-10,11-epoxide is a therapeutically active metabolite, which is excreted from the body at 2% of the orally administered dose. It is therefore detected in reclaimed wastewater in a concentration range of 50-120 ng/ℓ (Bahlmann et al., 2014). The 10,11-dihydro-10,11-trans-dihydroxycarbamazepine (CBZ-diol), which is not therapeutically active, is the most common carbamazepine metabolite found in urine (30% of the oral dosage). The CBZ-diol is the predominant metabolite detected in reclaimed wastewater in concentrations equal to or higher than the parent compound (Leclercq et al., 2009).

During the current study, concentrations of carbamazepine increased during the plant treatment processes, but not during the entire sampling period (Table 2-1). A variety of possibilities for such an increased concentration during wastewater treatment has been proposed: First, carbamazepine reaches the plant in a conjugated form, which may then undergo deconjugation under microbial metabolic conditions, therefore rendering a higher parent compound concentration as treatment commences. Secondly, as only a fraction of administered carbamazepine is excreted in its parent form (± 5%), its primary and secondary metabolites rather reach the plant and may be back-transformed to the parent compound rather than further biodegradation, or thirdly, carbamazepine persists within return activated sludge (RAS) and will reside within the secondary treatment steps for long periods.

Alternatively, all three the abovementioned hypotheses may be true to explain the negative mass balance of carbamazepine during wastewater treatment. The fact that metabolic by-products of carbamazepine are shown to be present at higher concentrations within wastewater, however, implicates that the second hypothesis may well contribute to a large extent to the higher loads of carbamazepine in treated effluent. As mentioned before, the concentrations of the primary metabolite of carbamazepine, CBZ-ep, was higher in raw wastewater compared to carbamazepine and decreased after the primary sedimentation step, followed by a slight increase at the end of the SMART™ process. It then remained constant until treated effluent (Table 2-1).

A similar trend was found for a secondary metabolite of carbamazepine, 10,11-dihydro-10-hydroxy carbamazepine (dh-hCBZ), although a higher concentration increase was observed from the SMART™ process onwards (Table 2-1). Both CBZ-ep and dh-hCBZ have a much lesser tendency to be associated with solid matrices, with estimated Log K_{ow} values of 0.95 and 0.93, respectively (EPI Suite, Version 4.1, KOWWIN). This depicts the possibility of both metabolites being retained within the aqueous matrix of WWTW treatment processes, which can be highly persistent when AS is returned to the start of the treatment process. The CBZ-ep readily undergoes further metabolism to CBZ-diol, but this analyte was unfortunately not included for the current study. Nevertheless, the average removal of both CBZ-ep and dh-hCBZ showed negative mass balances over the sampling period (Table 2-1).

This increase of both carbamazepine and its metabolic by-products may well consist of a variation of factors, as mentioned before, including deconjugation, metabolic back-transformation and hydraulic retention without biotic or abiotic degradation. However, the most prominent increase in the levels of carbamazepine and dh-hCBZ was estimated between the SMART™ reactor and PST overflow. As matured activated sludge, or return activated sludge (RAS), is returned from the aerobic bioreactor to the SMART™ system, it can be assumed that these compounds are retained in the plant for extended periods of time throughout the plant treatment processes without necessarily being degraded. A small-scale batch experiment study using a similar approach to the HYBACS® system has shown that exposure to carbamazepine showed no change within either carrier (attached growth) or AS reactors (Falås et al., 2013). This highlights the fact that the elimination of carbamazepine and its transformation products will still persist within biological treatment (Min et al., 2018), and warrants the treatment of such recalcitrant compounds with alternative tertiary treatment processes.

The concentrations of the herbicide atrazine did not reduce during the wastewater treatment process. The partition coefficient for atrazine ranges between 2.61 and 2.82 (EPI Suite, Version 4.1, KOWWIN),

indicating that this compound is moderately hydrophilic. For this reason, various studies have shown the persistence of atrazine in groundwater, mainly because this compound will be applied to agricultural fields. Concentrations ranged between 92.1 and 184.0 ng/ℓ in raw influent and 65.1 and 93.9 ng/ℓ in treated effluent samples during the seven-day sampling period (Table 2-1). Within treatment processes, a slight increase in atrazine concentrations was observed within the overflow of the anoxic treatment module, although this was not significantly different from other treatment steps (Bonferroni's multiple comparisons test, $P > 0.05$). The concentration of atrazine within the river water located upstream from the WWTW was, however, significantly higher than samples from raw wastewater (Bonferroni's multiple comparisons test, $P < 0.05$) (Table 2-1). This estimation of atrazine in environmental surface waters compares well to other South African studies. Atrazine concentrations in surface waters from the Rietvlei Nature Reserve have been shown to range between 0.4 and 60.8 ng/ℓ (Wooding et al., 2017). However, higher concentrations were detected in another study at the Hartebeespoort Dam catchment, ranging between 830 and 1,570 ng/ℓ in summer, 631 and 760 ng/ℓ in autumn, 503 and 576 ng/ℓ in winter, and 450 and 503 ng/ℓ in spring, whereas groundwater concentrations were 180, 138, 152, and 100 ng/ℓ during summer, autumn, winter and spring, respectively (Rimayi et al., 2018). The ecological health risk of atrazine has been shown throughout various trophic levels (Delorenzo et al., 2001; Trentacoste et al., 2011; Hayes et al., 2011; Koprivnikar, 2010; Freeman et al., 2011; Neuman-Lee and Janzen, 2011). Although the exact health risk associated with atrazine exposure is still unsure, as many researchers disagree on certain toxicity endpoints, its persistence and low removal during wastewater treatment warrant the need to continue with monitoring approaches, especially due to the observance that this CEC will persist in environmental waters.

The concentrations of the antibiotic sulfamethoxazole in the aqueous phase of the WWTW processes showed an interesting outcome. Raw wastewater concentrations were well reduced after primary sedimentation but were increased in the liquor after the SMART™ treatment (Table 2-1). These concentrations were then gradually reduced throughout AS treatment by the various redox conditions and were significantly reduced after the aeration basin compared to raw wastewater influent (Bonferroni's multiple comparisons test, $P < 0.001$) (Table 2-1). However, the concentration increased slightly at the secondary sedimentation overflow and final treated effluent, although remaining significantly lower than raw wastewater influent (Bonferroni's multiple comparisons test, $P < 0.05$) (Table 2-1). The Log K_{ow} for sulfamethoxazole is estimated at 0.48 to 0.89 (EPI Suite, Version 4.1, KOWWIN), indicating that this compound is highly hydrophilic. Furthermore, the predicted bio-degradation half-live of sulfamethoxazole in an AS system is 46 hours, which is a longer degradation period compared to other CECs such as bezafibrate, metronidazole and ibuprofen (Min et al., 2018). The study by Min et al. (2018) further showed that sulfamethoxazole may be biodegraded rather than adsorbed to solids within AS systems, which has also been shown elsewhere (Letzel and Verhalten, 2008). Nevertheless, sulfamethoxazole still shows partial recalcitrance within CAS treatment systems and is therefore reported not to be readily removed. The highly hydrophilic, slow biodegradation and low sorption properties of sulfamethoxazole may suggest that this drug may be retained within the WWTW for long periods if a substantial volume of activated sludge is returned to the starting conditions of the treatment process. This may explain why a high load of sulfamethoxazole was found in the SMART™ treatment system, which then followed a gradual decrease as the AS treatment continued (Table 2.1). It has, however, been shown that nitrogen-deficiency conditions within WWTWs will favour the biodegradation of sulfamethoxazole, especially during aerobic digestion (Müller et al., 2013).

The improved nitrogen removal that is mediated by the HYBACS® treatment system may well drive a "hungrier" microbial consortium downstream from the treatment process, forcing microorganisms to diverge to other labile nutrient sources during treatment (such as nitrogenous CECs). Xu et al. (2011) found that *Bacillus firmus* and *Bacillus cereus* were the predominant species of sulfamethoxazole-resistant bacteria in water samples, which indicates that these two bacteria strains have the potential to survive and degrade sulfamethoxazole in natural waterbodies.

Further studies may include the characterisation of environmental factors that influence the physiology of these potentially efficient strains, with the objective of increasing the efficiency of sulfamethoxazole biodegradation. These studies may not only shed light on the degradation of sulfamethoxazole in natural water systems, but also have the potential to facilitate such natural degradation during wastewater treatment.

It should be highlighted that, as for most pharmaceutical compounds, only a low percentage of sulfamethoxazole is excreted in its parent form after administration and will be excreted in conjugated forms or as various metabolic by-products (Bonvin et al., 2013). However, some studies have indicated the tendency of such conjugated and/or metabolite forms of sulfamethoxazole to be back-transformed into the parent compound through either biotic or abiotic factors (Bonvin et al., 2013; Casas et al., 2015). Whether such back-transformation may also be mediated through biotic factors still needs investigation. Regardless, some transformation products of sulfamethoxazole during wastewater treatment have been shown to also exert anti-bacterial effects (Majewsky et al., 2014), which not only warrants their monitoring to explain sulfamethoxazole degradation pathways during wastewater treatment, but also as a stand-alone CEC that needs further risk assessment.

2.3 CASE STUDY 2: REMOVAL OF PRIORITY CONTAMINANTS OF EMERGING CONCERN USING AN ELECTROCHEMICAL OXIDATION

2.3.1 Background

The treatment technology under investigation includes a CabECO process. The optimisation and implementation of the treatment system primarily forms the focus of a Horizon 2020 EU SWA project, which envisions the development of an autonomous, off-the-grid system for decentralised water provision in rural and peri-urban areas. The technology was developed by CONDIAS (GmbH, Itzehoe, Germany), and is being harnessed in the SWA project for clean water access in developing countries. A prototype has been built at the Council for Scientific and Industrial Research (CSIR) in Pretoria, and two demonstrator sites are under construction for river water treatment near a peri-urban settlement in Johannesburg (the Klip River) and a rural village in Mozambique (the Nkomati River). The electrodes of the CabECO system have several benefits in comparison to standard membranes in that they are extremely stable and have a high tendency towards the formation of ozone and free radicals in water.

2.3.2 Application of the CabECO process

The CabECO process relies on specifically designed membranes: metal electrodes that are coated with conductive boron-doped polycrystalline *sp3*-bonded carbon for an advanced oxidation treatment. The CabECO technology is being developed in this context for application in decentralised water provision. However, the extent of pre-treatment that is necessary for effective application to provide the envisioned 900 ℓ/h (relative to the flow rate of tap water) that is needed to serve approximately 300 people per day (at the minimum requirement of 25 ℓ per person per day) is expensive. Alternative applications of the treatment technology are thus currently under investigation, including household treatment of greywater, as well as an envisioned application as a tertiary wastewater polishing step, with the ultimate vision of this technology forming part of a network of applications for effective CEC degradation for improved reclamation. Thus, the work presented here explores forays into the degradation of priority CECs at various operating parameters that serve as an addendum to the larger SWA project. It also describes a first look at the fate of the endocrine-disrupting activities of CECs at pre- and post-CabECO treatment.

The benefits of the effective application of this technology include the following:

- The generation of ozone directly in the aqueous phase by transferring energy electrochemically to the oxygen and hydroxyl molecules in the water. A typical challenge in advanced oxidation with ozonation is the transfer of ozone from the gas phase to the aqueous phase, which is the standard process most widely harnessed. This technology allows for much higher ozone concentrations with relative ease.

- The low energy costs of this ozone generation process, allowing for exclusive reliance on off-the-grid photovoltaic energy.

- The avoidance of toxic chemicals for disinfection, which are expensive, challenging to transport to rural areas and potentially risky to the personnel who must handle them. Residuals are still necessary, since ozone is unstable in water and has a short half-life in the aqueous phase, but the chemical demand is much lower.

2.3.3 Laboratory evaluation of micropollutant degradation using the CabECO process

2.3.3.1 Micropollutant degradation using the CabECO process

For laboratory analyses, two CECs were selected for detailed assessment: the anti-epileptic carbamazepine and the antibiotic sulfamethoxazole, based on reported literature showing moderate to negative removal of these CECs during conventional WWTW processes. To assess the effect of CabECO technology on CEC degradation, several laboratory and environmental experiments were performed:

- Carbamazepine and sulfamethoxazole were spiked separately in reverse osmosis (RO) water and incubated at a 1:1 ratio with water sampled directly from the CabECO unit (45 ℓ/h, 1 bar, 3 A). Triplicate samples per time point were sealed, incubated and sacrificed for SPE and subsequent micropollutant analysis using LC-MS at 0 (pre-treatment control), 1, 15, 30 and 240 minutes.

- A similar experiment compared the effect of the maximum (2 units in series, 1 bar, 5 A, 60 ℓ/h) and minimum (1 unit, 1 bar, 1 A, 60 ℓ/h) ozone production capacity of the CabECO system, and the effect on CEC degradation, according to the methodology described above. In addition, a sample was added in triplicate, with carbamazepine and sulfamethoxazole co-spiked, as well as each CEC spiked in RO water and assessed in isolation. Samples were processed (SPE) after a retention time of 1 hour and 4 hours.

- The degradation capacity of CabECO technology was assessed in triplicate, as above, against a broader suite of CECs (naproxen, caffeine, bisphenol-S, diclofenac, carbamazepine, sulfamethoxazole, benzotriazole, acetaminophen and atrazine) spiked as a cocktail in tap water at a concentration of 15 µg/ℓ. For this experiment, prototype field operational conditions were used (300 ℓ/h, 3 A, 1 bar, 2 units in series), and samples were passed through the membrane from the 5 ℓ reservoir, as described in the disinfection experiments, rather than incubation with a post-treatment ozonated sample (1:1 ratio).

2.3.3.2 Disinfection of river water and household greywater using the CabECO process

The degradation of the natural cocktail of CECs in environmental samples was assessed, including the Plankenburg River and Eerste River (Stellenbosch, South Africa) and household greywater (shower water stored for one day). In this case, the samples were again passed through the membrane units, but at a lower flow rate (60 ℓ/h, 3A, 1 bar, 1 unit).

2.3.3.3 Yeast (anti)-estrogen screen (YES/YAES)

The authors followed the basic protocol described in Sohoni and Sumpter (1998). All yeast screens were performed in sterile 96-well flat-bottom assay plates (Sterilin, CAT No. 612F96) with a low evaporation lid (Thermo Scientific, CAT No. 642000). The yeast anti-estrogen screen (YAES) was selected for the current study as previous experimentation within the research group concluded that the target CECs (carbamazepine and sulfamethoxazole) do not show an affinity to cause an agonistic effect towards binding to the estrogen receptor in the YES assay, but rather caused an antagonistic response towards normal binding of 17β-estradiol (E_2) in the YAES assay.

For this reason, all wells in the assay plates (except for blank wells) were coated with an E_2 concentration of 450 ng/ℓ, which represented the concentration which shows an effect in 50% of the experimental population (EC_{50}) derived from an E_2 standard curve in the conventional YES assay. Therefore, all test compounds were tested for their ability to inhibit the agonistic action of the estrogen spike in the assay within water samples before and after CabECO treatment.

Serial dilutions of all test compounds were prepared in a 96-well flat-bottom plate (Greiner Bio-one, 655161) and transferred to the assay plates at 10 µℓ per well. Serial dilutions of a tamoxifen stock solution (20 µM) were prepared and transferred into the assay plate, which served as the reference standard curve for the YAES. Two sets of blank wells (one containing no test compounds or steroid hormones and the other containing only the EC_{50} concentrations of the E_2 spike) were used. Blank wells containing no test compounds or steroid hormones were supplemented with 10 µℓ of MeOH to conform to the uniform usage of solvents in the assay. To visualise the anti-estrogenic effect of the test compounds, a stock solution of carbamazepine was prepared (320 mM) and serially diluted to obtain 12 concentration ranges in the assay. The MeOH was evaporated before seeding with the assay medium. Growth medium (containing CPRG) was prepared according to Sohoni and Sumpter (1998) and seeded with 4 x 10^7 yeast cells from a previously incubated 24-hour culture. A volume of 200 µℓ of assay medium was added to each well to give a final 1/20 dilution of each test chemical and SPE-extracted water samples.

A colour change of the assay medium was observed after 48 hours of incubation (30 °C) in which absorbance was measured at an optical density of 570 nm (OD_{570}) for colour change, and optical density of 620 nm (OD_{620}) for turbidity using a plate reader. These measurements were then used to correct for turbidity and colour change in the blank control cells by calculating a corrected absorbance of each test compound using Equation 2-4.

$$\text{Corrected absorbance} = OD_{570} - [OD_{620} - OD_{620,\text{blank}}] \quad \text{(Eq. 2-4)}$$

The corrected absorbance of each well was then log-transformed and expressed as a percentage value relative to the median absorbance obtained for the blank wells spiked with E_2 (Log percentage maximum E_2 spike). The cytotoxicity of the test analytes and extracted water samples in the assay were investigated by comparing the turbidity within each well to the turbidity within the blank wells (containing only yeast and the assay medium) using the following equation:

$$Cytotoxicity_{sample} = \text{average } OD_{620,\text{blank}} - [3*\text{stdev } OD_{620,\text{blank}}] \quad \text{(Eq. 2-5)}$$

The data was then processed using Graphpad Prism (Version 4.1) to obtain a sigmoidal standard curve and inhibitory concentration which shows an effect in 50% of the experimental population (IC_{50}) values of the test compounds. Nominal inhibition concentration (NIC) and minimum inhibition concentration were calculated using the Gompertz equation for NIC/MIC. To compare the change in anti-estrogenic responses before and after CabECO treatment, the corrected absorbance from the SPE-extracted water samples were compared relative to the corrected absorbance measured for the E_2 spike in the assay. The percentage deviation of the test samples from the E_2 spike was then considered as an anti-estrogenic response, as the test analytes present in the samples would cause less metabolism of CPRG if these compounds mediated an antagonistic effect towards the normal binding of E_2 to the human Estrogen Receptor (hER) in the assay, and vice versa.

2.3.4 Results and discussion

2.3.4.1 Micropollutant treatment: ozonation

The CabECO treatment at various flow rates and various ozone concentrations showed almost complete degradation of sulfamethoxazole and carbamazepine after one minute of ozone treatment (Figure 2-5). Moreover, the treatment technology showed consistently excellent (80-100%) removal rates for most CECs, including carbamazepine, sulfamethoxazole, naproxen, bisphenol-S and acetaminophen (Figure

2-6). Benzotriazole, caffeine, atrazine and diclofenac were, however, more persistent against ozonation, but consistently showed some removal, ranging between 10 and 50% (Figure 2-6).

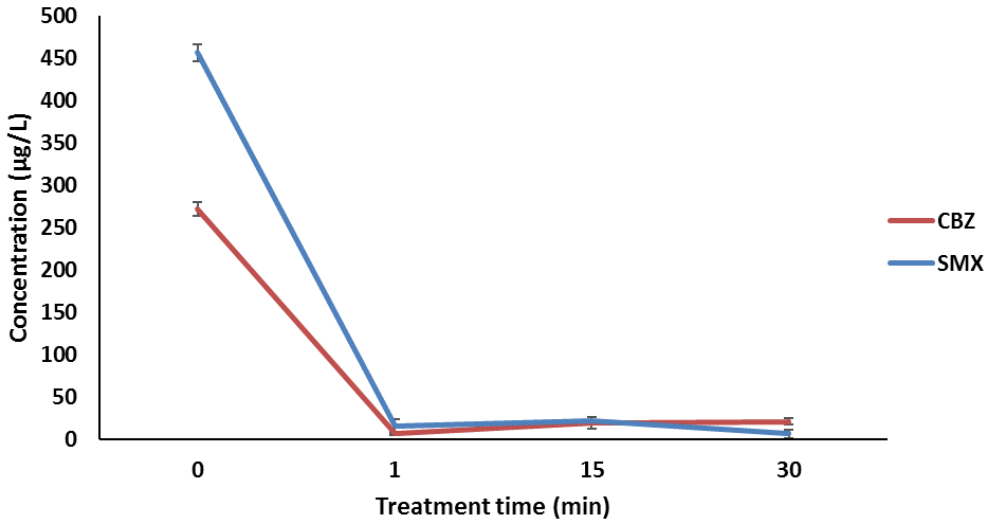

Figure 2-5: The degradation of carbamazepine and sulfamethoxazole upon exposure to ozone generation by the CabECO membranes up to a retention time of four hours (2 units in series, 3 A, 60 ℓ/h; 3.48 mg/ℓ ozone)

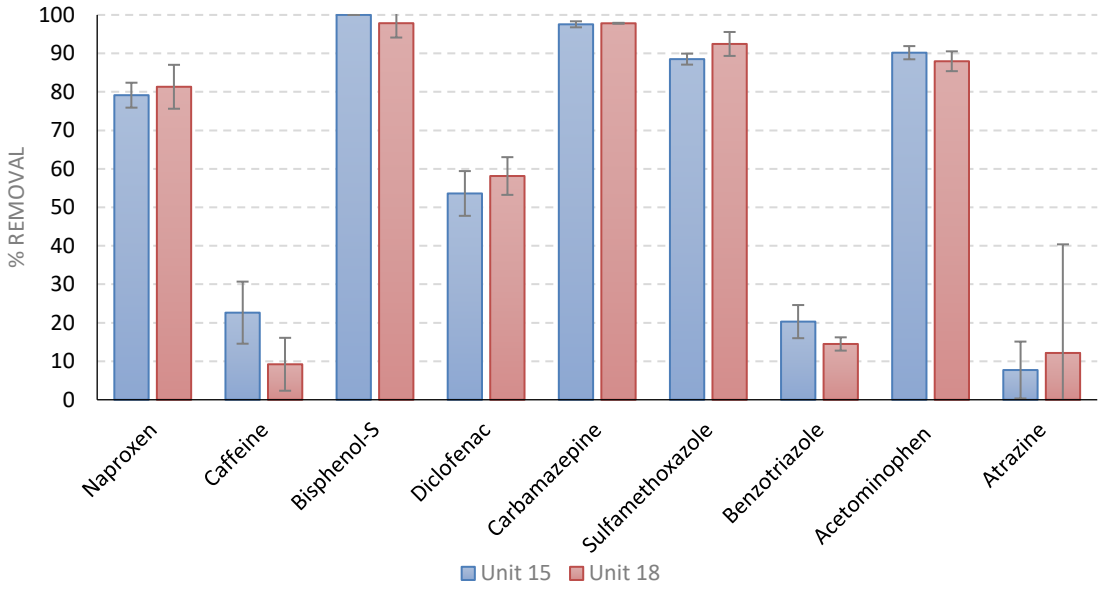

Figure 2-6: The removal (percentage) of a full suite of micropollutants by two CabECO membrane units operated in series at 3 A (300 ℓ/h; 1 bar) with a retention time of one hour

2.3.4.2 Yeast anti-estrogen screen

Carbamazepine showed a relative potency of 0.4% when its IC_{50} was compared to that generated for tamoxifen (Figure 2.7). Nominal and minimal inhibition concentrations were calculated for tamoxifen at 1.1 µM (408.67 µg/ℓ) and 4.9 µM (1.82 mg/ℓ), respectively, and for carbamazepine at 99.8 µM (23.58 mg/ℓ) and 2,616.0 µM (618.08 mg/ℓ), respectively.

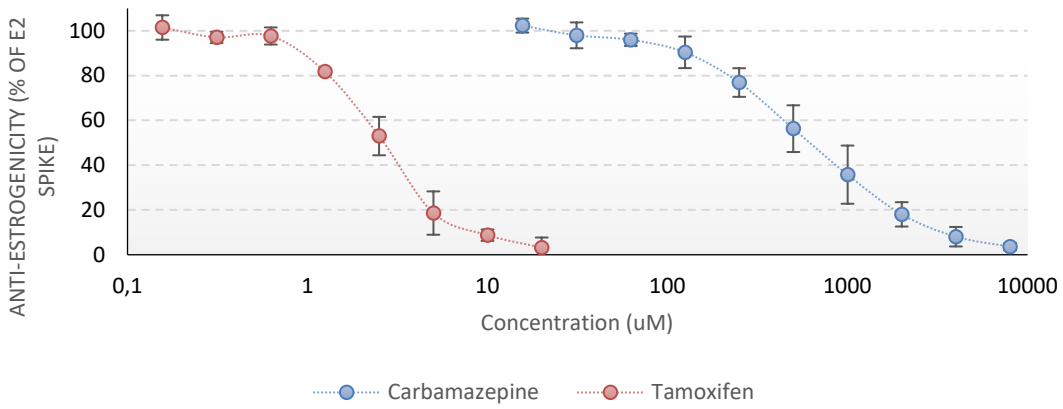

Figure 2-7: Yeast anti-estrogen screen for the comparison of anti-estrogenicity between the estrogen receptor antagonist tamoxifen and the anti-epileptic carbamazepine. Values are expressed as a percentage relative to the 17β-estradiol spike in the assay. The IC_{50} values were calculated for tamoxifen (2.3 µM; 860 µg/ℓ) and carbamazepine (550,6 µM; 130 mg/ℓ).

Exposure of the antibiotic sulfamethoxazole and the anti-epileptic carbamazepine in batch experiments under sterile and biotic conditions showed varying results for the two CECs in the YAES (Figure 2-8). Little or no anti-estrogenicity was observed for carbamazepine under biotic conditions, where anti-estrogenicity increased slightly after 20 days of incubation under abiotic conditions (Figure 2-8). However, the anti-estrogenicity of sulfamethoxazole did not change much over the 20-day incubation period under abiotic conditions, but decreased after 10 days of incubation under biotic conditions (Figure 2-8), implying that some bio-degradation pathway/s may be a causative factor in reducing the anti-estrogenic effects of sulfamethoxazole through the formation of intermediate by-products or metabolites, which have a lesser affinity to antagonise estrogen receptor binding in the YAES assay.

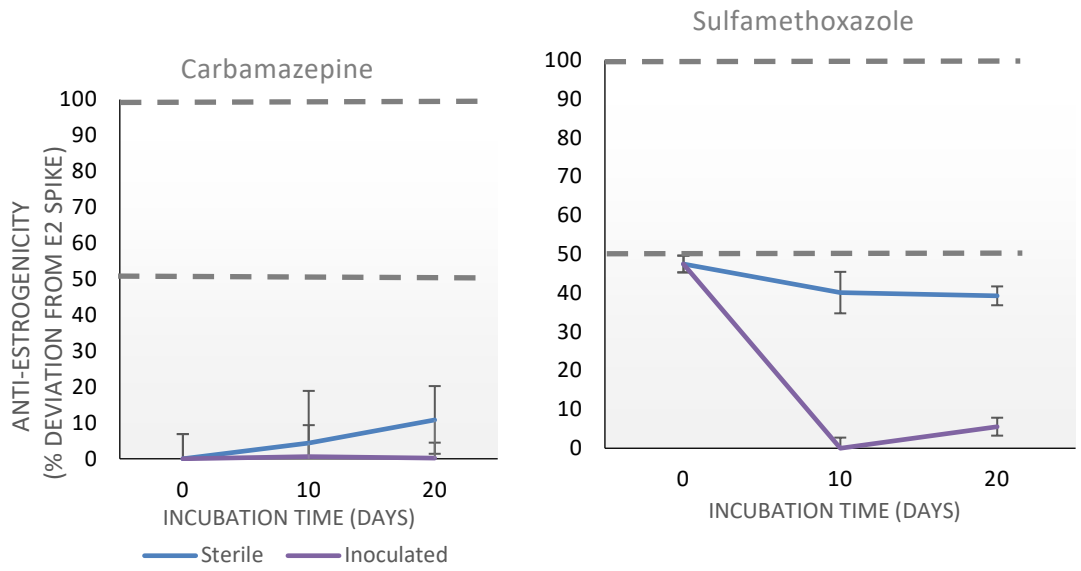

Figure 2-8: The effect of the biological metabolism of a microbial community, isolated from RAS with the respective micropollutant as the exclusive carbon source on the toxicity footprint of the water, as assessed with a YEAS assay. Values are expressed as a percentage deviation from the E_2 spike in the assay.

The YAES produced the clearest statistically significant results, indicating the effect of the CabECO technology. Figure 2-9 shows the results of the YAES for both carbamazepine and sulfamethoxazole when treated by CabECO for different exposure times to the generated ozone trapped in a retention vessel. Before treatment with CabECO, carbamazepine shows an anti-estrogenic effect relative to the EC_{50} of the E_2 spike (a predetermined amount known to disrupt endocrine activity, measurable in the yeast assay), inhibiting the expected estrogenic response of the E_2 spike. When carbamazepine is exposed to ozone for 1, 5 and 60 minutes, its anti-estrogenicity is slowly decreased as the carbamazepine is being degraded, until carbamazepine exhibits no added estrogenic or anti-estrogenic responses after 240 minutes of ozone treatment (Figure 2-9). The differences in the anti-estrogenicity of carbamazepine between consecutive exposure times shows little significance ($P > 0.05$). However, when 0 minutes of exposure is compared to 60 and 240 minutes of exposure time, the decrease in anti-estrogenicity is significant ($P < 0.05$).

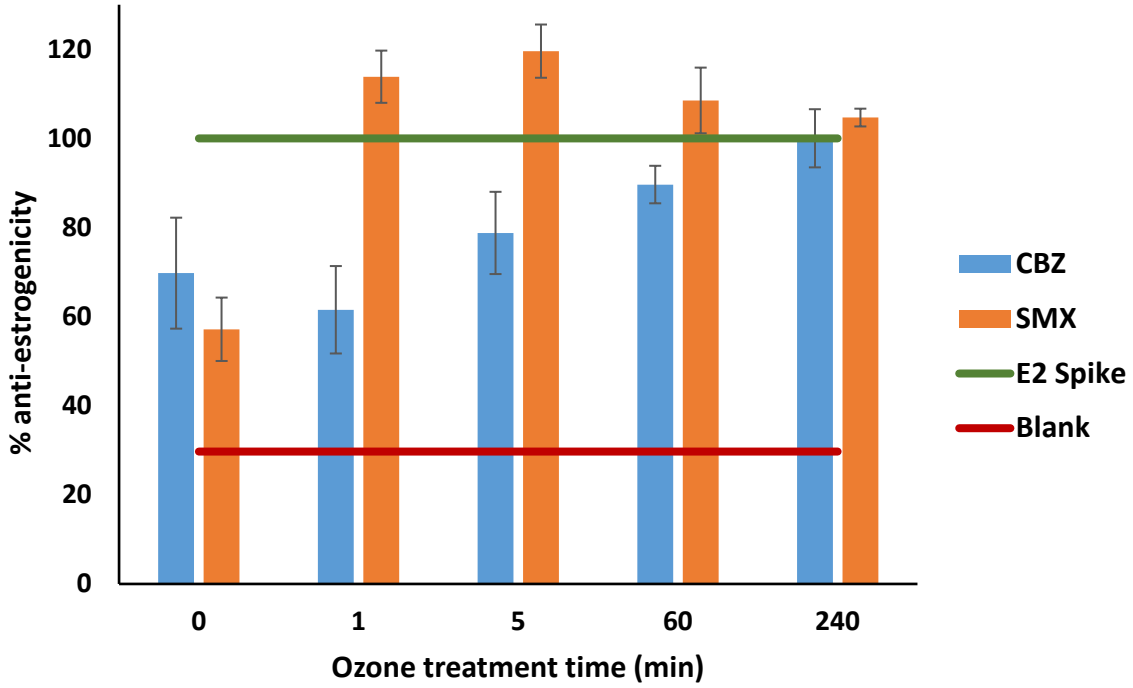

Figure 2-9: Change in anti-estrogenicity of carbamazepine and sulfamethoxazole over different exposure times (0 to 240 minutes) to ozone generated by the CabECO technology

Since many ozonated by-products may develop during the treatment process, the results imply that carbamazepine degradation to such products does not increase potential estrogenicity or anti-estrogenicity in the current effect-based bioassay. This, however, needs to be confirmed in future studies using a battery of *in vitro* bioassays that investigates similar modes of action or other potential endocrine system-related pathways.

For sulfamethoxazole, an anti-estrogenic effect relative to the EC_{50} of the E_2 spike was also observed for this compound, like carbamazepine (Figure 2-9). This could imply that sulfamethoxazole may have antagonistic interference with E_2 binding to the hER in some manner or may interfere with further downstream signalling of the β-galactosidase response. However, after only one minute of ozone exposure, there was a statistically significant added increase in the estrogenicity of the sample containing sulfamethoxazole compared to the untreated sample (one-way ANOVA; $P < 0.05$). This result implies that, during ozone treatment, the initial anti-estrogenicity of sulfamethoxazole decreases,

which could be explained by the degradation of the substance into potential ozonated by-products that may bind to the hER in an agonistic manner.

As the ozone treatment time commences, further degradation of the sulfamethoxazole products no longer interferes with the binding of E_2 to the hER, whereby the sulfamethoxazole-exposed treatment module shows no (anti)-estrogenic responses at 240 minutes of exposure (Figure 2-9). It is important to note that the differences in estrogenicity relative to the different treatment times (1 to 60 minutes) are insignificant ($P > 0.05$). However, relative to the E_2 spike, there are significant changes ($P < 0.05$) in estrogenicity when comparing the different exposure times. From these results, the authors can conclusively report that CabECO is an effective system for the reduction of some CECs from water samples, as well as the reduction of carbamazepine/sulfamethoxazole-associated endocrine system responses. This data supports the implementation of a residence column in the final design of an operational CabECO system for adequate reaction time of the ozone with contaminants.

Future experiments are required to investigate the probable formation of intermediate degradation products and/or metabolites of both carbamazepine and sulfamethoxazole at different timed intervals of exposure to the treatment system in order to verify whether such degradation products may provide a significant contribution to the changes in anti-estrogenicity measured in the current YAES assay. Previous toxicity assays have demonstrated toxicity elimination after 17 to 20 hours of exposure to the treatment technology. Repeated exposure to increased ozone concentrations may thus be necessary for the mineralisation of parent compounds, as well as transformation by-products, thereby also potentially minimising anti-estrogenicity and other risk factors.

2.4 SUMMARY

The current scoping study at the WWTW highlighted the variation in the presence and fate of selected CECs and EDCs within WWTW processes, which included an advanced secondary treatment process with biotic and/or abiotic factors that may drive their degradation and/or attenuation within the treatment system. As mentioned before, the WWTW experienced operational challenges during the sampling campaign, which may have compromised the normal operation of the treatment system, and therefore, does not necessarily reflect that the addition of the HYBACS® treatment system has a lesser chance of success for the removal of priority CECs than conventional treatment processes. Regardless, this study provided insight into the complexity of evaluating CEC removal during WWTW processes, and further highlights the need to correlate such chemical analytical results with routine operational parameters from the plant. The study further paved the way for new experimental avenues, which will be considered in future studies, especially the optimisation and evaluation of analytical methodologies to investigate the partitioning of CECs during the treatment process, the consideration of the residence time distributions of the WWTW, which may influence the transport of CECs between treatment steps, and the further investigation of transformation pathways for CECs, which still show moderate to low removal during the treatment process.

The impact of high foaming and bulking within the secondary clarification on the fate of the target CECs also paved the way for an interesting avenue for future research. Such foaming after AS treatment has been associated with the prevalence of gram-positive bacterial species under high aeration conditions, including facultative aerobic *Microthrix* species (Lefebvre et al., 2014). During the sampling period, the WWTW provider reported high loads of the *Microthrix* genera throughout the treatment plant, causing a high load of scumming in the clarification process. Studies on the growth characteristics of *Microthrix parvicella* suggest the need for high sludge age for its survival in WWTWs. Part thereof is due to the frequent recycling of biological scum through the treatment system (Rosetti et al., 2005). This may create issues with the growth of other microbial species within treatment processes, as the high loading of undesirable bacteria such as *Microthrix* may outcompete floc-forming organisms (Rosetti et al., 2005), thereby also affecting the biodegradation of organic pollutants. This needs to be addressed in

future studies.

The CabECO technology shows potential for application in a decentralised drinking water system for rural Southern African regions. The ozone generated by the electrochemical process is sufficient for the disinfection of various environmental samples, as well as the abatement of several micropollutants. The anti-estrogenicity of both carbamazepine and sulfamethoxazole was reduced by CabECO until no observed effect was seen in the YAES after four hours.

Retention time is extremely important to include in the final design of this system. To eliminate the anti-estrogenic footprint of both carbamazepine and sulfamethoxazole, up to four hours of ozone exposure is needed, even though the parent compounds have been almost completely degraded after one minute of exposure. For the application of this system in a real-world scenario, it is important to consider that ozone concentration is reduced considerably, with a substantial portion of the ozone being depleted by various sources of organic matter other than the target compounds and microbes.

Thus, CabECO appears better suited as a disinfectant or “polishing step” after water treatment. The standard procedures for generating potable water (flocculation, sedimentation, filtration) always demand the addition of a disinfectant post-treatment. Currently, chlorine is the go-to disinfectant, but has the disadvantage of being hazardous to human health and having a negative environmental impact. CabECO is an attractive alternative for the disinfection step in water treatment, or for polishing water with a micropollutant footprint. For disinfection, it has the disadvantage of lacking residuals, due to the instability of ozone. Thus, retreatment before use, after storage, would be recommended.

It is recommended for future studies that ozone exposure times be further investigated to accurately determine the optimal exposure times for maximum efficiency in the removal of a broad suite of micropollutants, such as in environmental rivers. The recycling of water through the CabECO system should also be further explored, especially how a broad suite of micropollutants and their eco-toxicological effects react when a previously exposed sample is exposed to a fresh, high dose of ozone for a second and possibly a third time. Furthermore, a wider range of ecotoxicological assays that extend across various trophic levels should be incorporated to assess the final toxicity footprint of treated samples.

With the above-mentioned optimisations, CabECO shows potential for several decentralised water treatment scenarios. Schools, hospitals and resorts often access and store water on-site, particularly in remote areas. CabECO is an attractive option for on-site water disinfection, particularly if treatment systems are not available. In addition, it is an option as a handheld household device to partially treat greywater. As micropollutants become a greater cause of concern, household devices for polishing water (minimising the micropollutant footprint in tap water) may become a need, and CabECO technology might meet that need, to a degree. Due to the enormous, diverse range of micropollutants, however, multiple treatment options will be needed, in tandem, to truly minimise the risk.

CHAPTER 3: PRESENCE AND FATE OF ANTIBIOTIC-RESISTANT BACTERIA AND GENES IN WASTEWATER

3.1 INTRODUCTION

As with any living organism, bacteria and other microorganisms evolve through genetic mutations as an adaptation to their changing surroundings. Such mutations are driven to benefit the microbe in relation to a particular habitat, including the development of defence mechanisms against antibiotics or other microbes (Read and Woods, 2014). Due to such adaptations, the presence of sub-inhibitory concentrations of an antibiotic may drive the proliferation of the mutant organisms that ultimately produce a bacterial population that is resistant to a specific antibiotic (Van Hoek et al., 2011; Laxminarayan et al., 2013; Read and Woods, 2014; National Department of Health, 2016). While species evolution is a natural process, resistance to antibiotics is developing at an exorbitant rate due to anthropogenic influences. Many AR genes are located on the chromosomal DNA and are thus passed on to each subsequent generation of the species, resulting in inherent resistance to a particular antibiotic (Essack et al., 2001).

Alternatively, resistance genes can be acquired through the transfer of extra-chromosomal DNA, such as plasmids (Schwartz et al., 2003). Horizontal gene transfer processes such as transduction, conjugation and transformation allow plasmids to move to different bacterial species (broad host range) or be limited to one bacterial genus or species (narrow host range) (Van Hoek et al., 2011). The current study is aimed at investigating the presence and fate of ARB and genes at two WWTWs that utilise AS treatment processes, but through two different treatment setups (complete-mix and plug-flow systems).

3.2 METHODS

3.2.1 Selection of priority antibiotics and microorganisms

The national antimicrobial susceptibility and the sentinel sites' antimicrobial susceptibility maps of the National Institute for Communicable Diseases (NICD)[1] and the resistance maps of the Centre for Disease Dynamics, Economics and Policy (CDDEP)[2] were used to determine antibiotics and organisms that are of national and global concern. In addition, the WHO's critically important antimicrobials for human medicine[3] and global priority list of antibiotic-resistant bacteria to guide the research, discovery and development of new antibiotics[4] were utilised.

3.2.2 Determination of antibiotic concentrations to select for resistance

The breakpoint criteria of the Clinical and Laboratory Standards Institute (CLSI) (2015) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2017) were used to obtain starting concentrations of the selected antibiotics to incorporate into culture media for the selection of resistant bacteria. As these documents obtain their values from pure cultures (i.e. laboratory conditions or strains), there was a need to estimate breakpoints for environmental mixed cultures experimentally. For this reason, RAS samples obtained from the two WWTWs were serially diluted in phosphate-buffered saline (PBS) and spot-plated onto a growth medium.

[1] https://www.nicd.ac.za
[2] https://www.cddep.org
[3] https://apps.who.int/iris/bitstream/handle/10665/255027/9789241512220-eng.pdf;jsessionid=0E47EDEAB2EF56D3E517A2D061EB1286?sequence=1
[4] https://www.who.int/medicines/publications/global-priority-list-antibiotic resistant-bacteria/en/

As colistin is only active against gram-negative bacteria, MacConkey (MAC) broth (selective for gram-negative bacteria) was used and supplemented with a range of colistin concentrations (1 µg/mℓ, 4 µg/mℓ, 8 µg/mℓ), while R2A (a minimal media used for the growth of bacteria isolated from water) was supplemented with the selected broad-spectrum antibiotics gentamicin (4, 16 and 32 µg/mℓ), amoxicillin (128, 256 and 512 µg/mℓ) and sulfamethoxazole (1, 16 and 512 µg/mℓ). These ranges were selected to be both below and above clinical resistant breakpoints. Upon further analysis, Mueller Hinton Broth (MHB) agar was selected as the broad-spectrum growth medium instead of R2A, as it is more standardised for AMR testing.

3.2.3 Sampling locations and procedure

Grab wastewater samples were collected from two WWTWs located in the Cape Town area (WWTW-1 and WWTW-2) from the inlet (influent after grit screens), RAS and treated effluent (post-chlorination). Three sampling events in 2018 and 2019 (February 2018, July 2018 and February 2019) for microbial analyses were performed. All samples were collected on the same day and stored on ice during transport to the laboratory.

3.2.4 Determination of resistance and selection of single isolates

For the February 2018 analyses, the water samples from the WWTW influent, RAS and effluent were treated with 0.001% cycloheximide (w/v) to inhibit the growth of fungi, and were subsequently serially diluted with PBS (10^0 to 10^{-6}). Some 96-well sterile microtiter plates (Greiner, Ref 650161) containing broth were spiked with the two highest concentrations of antibiotics that were seen to reduce the colony-forming unit (CFU)/mℓ as described above (colistin 4 and 8 µg/mℓ, gentamicin 16 and 32 µg/mℓ, amoxicillin 256 and 512 µg/mℓ and sulfamethoxazole 256 and 512 µg/mℓ). Bacterial dilutions were then inoculated into each well according to Figure 3-1. Duplicates were performed and blank controls containing only MAC and MHB growth media and inoculum were included. After 18 to 24 hours of incubation at 37 °C, the optical density at 600 nm (OD_{600}) was measured as an estimate of growth using a Biorad XMark microplate absorbance spectrophotometer. The results of this experiment served as pre-antibiotic exposure optical density values. The 10^{-1} dilution from this microtiter plate was then used for reporting purposes and was sub-cultured in sterile glass tubes containing fresh broth with the same antibiotic conditions as mentioned before. Spot plates were conducted to enumerate bacteria after continued antibiotic exposure. Using OD_{600} in batch reaction studies (as done in the 96-well microtiter plates) turned out to be a sub-optimal method of determining resistance profiles.

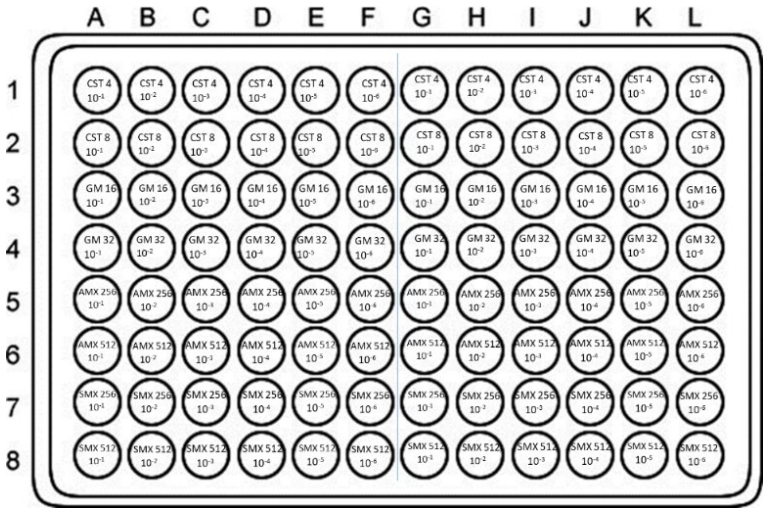

Figure 3-1: Schematic diagram of a microtiter plate setup for isolating resistant bacteria

Therefore, cultures obtained for the July 2018 sampling event were treated with 0.001% cycloheximide (w/v), serially diluted using PBS (10^0-10^{-6}) and spot-plated on selective agar media containing the respective antibiotic concentrations and incubated for 18-24 hours at 37 °C. Plate counts were performed prior to enrichment in antibiotic broth as stated for the February 2018 experiment to continue antibiotic exposure.

For the February 2019 sampling event, water samples from the WWTW influent and effluent were treated with 0.001% cycloheximide (w/v), serially diluted using PBS (10^0 to 10^{-6}) and spot-plated on selective agar media containing the respective antibiotic concentrations as indicated above. After the 18 to 24 hour incubation at 37 °C, the colony counts (CFU/mℓ) were calculated to draw a comparison between the resistance profiles of untreated and treated wastewater.

From the experiments using samples collected in 2018, 19 single colonies that were enriched in antibiotics, and 11 colonies selected from plates that had not been exposed to antibiotics were restreaked on antibiotic selective agar until a pure single colony resulted. These isolates were then stored in 40% glycerol at -80 °C for future analyses. The pre-exposed colonies were only selected from the effluent of the treatment plants as these are the organisms that are released into the environment, while colonies from influent, RAS and effluent that had not been exposed to antibiotics were selected to make comparisons between WWTW processes.

3.2.5 DNA extraction, PCR and sequencing

A genomic DNA extraction method utilising ammonium acetate, adapted from Crouse and Amorese (1987), was used to extract total genomic DNA from each isolate. The isolated DNA was used as template DNA for 16S polymerase chain reaction (PCR). The bacterial-specific primer sequences 8F (AGAGTTTGATCCTGGCTCAG) and 1512R (GTGAAGCTTACGGTTAGCTTGTTACGACTT) were used (Felske et al., 1998).

A volume of 0.25 µℓ of OneTaq (New England Biolabs), 1 µℓ dNTPs, 1 µℓ of each primer mentioned above (10 µM), 10 µℓ 5 x OneTaq Buffer, 4 µℓ DNA template and sterile MilliQ water were combined to make up a reaction volume of 50 µℓ. Cycling conditions were as follows: an initial denaturation at 94 °C for 30 seconds, followed by 25 cycles at 94 °C for 20 seconds, 58 °C for 40 seconds, and 68 °C for 90 seconds. A final extension was performed at 68 °C for five minutes. Amplicons were then sent to the Central Analytical Facilities at Stellenbosch University for PCR clean-up and sequencing. Sequence chromatograms were trimmed and consensus sequences for each primer pair were constructed using BioEdit (Version 7.05). BLASTn was performed for each sequence with the database for selected 16S ribosomal RNA sequences (bacteria and archaea). The isolated species were identified based on query cover and identity.

3.2.6 Environmental minimum inhibitory concentration determination

3.2.6.1 Mixed culture samples

The investigation of AMR profiles in environmental samples has proven to be difficult. For example, by introducing antibiotic-selective media to isolate multiple microorganisms from a sample may introduce bias. In addition, the physio-chemical variation in water matrices, for example raw wastewater, treated wastewater and river water, can severely impact on comparative studies, along with the fact that some water matrices, such as raw wastewater, may have a much larger density of microorganisms than treated effluent. The latter is a problem in microbroth dilution studies to determine minimum inhibitory concentrations of antibiotics against the selected microorganisms. In order to make the inoculum density uniform across all samples (influent, RAS and effluent), the OD_{600} of the effluent (cleanest sample) was measured and used as the baseline for the dilution of the other water matrices to obtain a similar OD_{600} (0.018 ± 0.015).

The microbroth dilution experiment was performed in 96-well microtiter plates (Grenier, 650161). A 180 µℓ MAC broth was added to each well, followed by the appropriate volume of each antibiotic to obtain the highest desired concentration in column 1 (Figure 3-2). Antibiotic concentrations were diluted 1:2 across rows (Figure 3-2) to obtain various antibiotic concentrations. The PBS was used where necessary to make the final volume (after inoculation) up to 300 µℓ per well. Positive and negative controls were added to each microtiter plate experiment, which contained the assay medium with and without bacterial inoculum, respectively. The plates were incubated at 37 °C on a shaker for 24 hours, upon which growth was measured (OD_{600}) to determine the MIC of each antibiotic and each water matrix.

3.2.6.2 Single colony samples

The glycerol stocks were revived in Tryptic Soy Broth (TSB) overnight at 37 °C and subsequently diluted to a 0.5 McFarland standard. The MIC susceptibility testing was completed in the same manner as the mixed cultures, as illustrated in Figure 3-2.

Row	Column											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
WWTW-1 CST	512	256	128	64	32	16	8	4	2	1	Pos	Neg
WWTW-1 GM	1,024	512	256	128	64	32	16	8	4	2	Pos	Neg
WWTW-1 AMX	16,384	8,192	4,096	2,048	1,024	512	256	128	64	32	Pos	Neg
WWTW-1 SMX	16,384	8,192	4,096	2,048	1,024	512	256	128	64	32	Pos	Neg
WWTW-2 CST	512	256	128	64	32	16	8	4	2	1	Pos	Neg
WWTW-2 GM	1,024	512	256	128	64	32	16	8	4	2	Pos	Neg
WWTW-2 AMX	16,384	8,192	4,096	2,048	1,024	512	256	128	64	32	Pos	Neg
WWTW-2 SMX	16,384	8,192	4,096	2,048	1,024	512	256	128	64	32	Pos	Neg

CST: Colistin; SMX: Sulfamethoxazole; AMX: Amoxicillin; GM: Gentamicin

Figure 3-2: A schematic representation of the 96-well microtiter plate layout for MIC determination. Antibiotic concentrations are in µg/mℓ.

3.2.7 Quantification of target-resistance genes

3.2.7.1 The qPCR reaction and cycling conditions

The number of resistance gene copies for selected target genes (colistin: *mcr*1 and *mcr*3, sulfamethoxazole: *sul1* and *sul2,* and amoxicillin: *bla*OXA-48, *bla*NDM-1 and *blaKPC*-1) was determined using real-time polymerase chain reaction (qPCR). Sulfamethoxazole- and colistin-resistance genes were quantified using an SYBR® green assay, while the amoxicillin-resistance genes were quantified using a probe-based assay. Positive gene controls were synthesised according to sequences obtained from Nucleotide Sequence Database Collaboration (NCBI) accession numbers that were flanked by the selected primers shown in Table 3-2. Synthesised genes were inserted into a pBluescript II SK (+) cloning vector, resulting in a 1,498 bp plasmid. All genes, primers and probes were synthesised by Inqaba Biotech. The genes used in each assay are listed in Table 3-1. In both assays, LightCycler® 480 white 96-well plates (Roche; 04729692001) were used and amplification was performed on a LightCycler 96 system. For the SYBR® green assay, samples were run in duplicate. The reaction composition in each well was as follows: 10 µℓ 2X Luna® Universal qPCR Master Mix (M3003L: New England Biolabs Inc.), 0.5 µℓ of each primer (10 µM), 1 µℓ template DNA, 8 µℓ sterile MilliQ water to make the final reaction volume up to 20 µℓ. Negative controls for each resistance gene were included in the assay and contained 1 µℓ MilliQ water instead of template DNA. Cycling conditions were adapted from the manufacturer's instructions. Initial denaturation at 95 °C for 60 seconds, followed by 50 cycles of two-step amplification (denaturation at 95 °C for 15 seconds, extension at 60 °C for 30 seconds including a plate read) and a melt curve range from 60 to 95 °C.

The probe-based assay reaction contained a 10 µℓ 2X Luna® Universal Probe qPCR Master Mix (M3004L: New England Biolabs Inc.), 0.8 µℓ of each primer (10 µM), 0.4 µℓ probe (10 µM), 1 µℓ template DNA, and 8 µℓ sterile MilliQ water to make the final reaction volume up to 20 µℓ. Negative controls for each resistance gene were included in the assay and contained 1 µℓ MilliQ water instead of template DNA. Cycling conditions were adapted from the manufacturer's instructions. Three technical repeats and three biological repeats were performed. Cycling conditions were the same as the SYBR® green assay. However, a melt curve was not required due to the addition of the probes. When selecting the detection format of the LightCycler® 96 software, VIC was selected as the dye for the probe labelled CAL Fluor Orange 560, while Texas Red was selected as the dye for the probe labelled CAL Fluor Red 610.

Table 3-1: Sequences of genes synthesised for positive controls

Gene sequence	Target number of base pairs	NCBI accession number
blaOXA-48 gene AATAGCTTGATCGCCCTCGATTTGGGCGTGGTTAAGGATGAACACCAAGTCTTTAAGTGGGATGGACAGACGCGCGATATC GCCACTTGGAATCGCGATCATAATCTAATCACCGCGATGAAATATTCAGTTGTGCCTGTTTATCAAGAATTTGCCCGCCAAAT TGGCGAGGCACGTATGAGCAAGATGCTACATGCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGG CTCGACGGTGGTATTCGAATTTCGGCCACGGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAGTTACACGTATCGG AGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAATGGTGACTATATTATTCGGGCTAAAACTGGATACTC GACTAGAATCGAACCTAAGATTGGCTGGTGGGTCGGTTGGGTTGAACTTGATGATAATGTGTGGTTTTTTGCGATGAA (488b p)	438	AY236073
NDM-1 gene GGAAAACCAGATCGCCAAACCGTTGGTCGCCAGTTTCCATTTGCTGGCCAATCGTCGGGCGGATTTCACCGGGCATGCACC CGCTCAGCATCAATGCAGCGGCTAATGCGGTGCTCAGCTTCGCGACCGGGTG (133bp)	83	FN396876
KPC-1 gene CCCGGCCGGGCTGACGGCCTTCATGCGCTCTATCGGCGATACCACGTTCCGTCTGGACCGCTGGGAGCTGGAGCTGAACT CCGCCATCCCAAGCGATGCGCGCGATACCTCATCGCCGCGCGCCGTGACGGAAAGCTTACAAAAACTGACACTGGGCTCT GCACTGGCTGCGCCGCAGCGGCAGCAGT (188bp)	138	AF297554
Mcr-1 gene GTGACCAGTTATTTTACTGACACTTATGGCACGGTCTATGATACGACCATGCTCCAAAATGCCCTACAGACCGACCAAGCCG AGACCAAGGATCTATTAAACGCAGCGTTTATCATGCGTATCATTGGTTTGGGTGTGCTACCAAGTTTGCTTGTGGCTTTTGTT AAGGTGGATTATCCGACTTGGGGCAAGGGTTTGATGCGCC (205bp)	213	KP347127.1
Mcr-3 gene GTGTCAGTGGGGCGCAACAATTCAAACCTCCAGCGTGAGATTGTTCCAGCCAATTTCGTTAATAGTACCGTTAAATACGTTTA CAATCGTTATCTTGCTGAACCAATCCCATTTACAACTTTAGGTGATGATGCAAAACGGGATACTAATCAAAGTAAGCCCACGT TGATGTTTCTGGTCGTTGGTGAAACCGCTCGTGGTAAAAATTTCTCGATGAAT (219bp)	169	MH077952.1
***Sul1* gene** TGCGGACGTAGTCAGCGCCATTGCCGATCGCGTGAAGTTCCGCCGCAAGGCTCGCTGGACCCAGATCCTTTACAGGAAGG CCAACGGTGGCGCCCAAGAAGGATTTC (107bp)	67	CP031449.2
***Sul2* gene** CCCGGACCACGGCCTGTGAGCGCGCGCAGAAAGGATTTGCGCGAAACAGACAGAAGCACCGGCAAATCGAAGCGCAGCC GCAATTCATCGAACCGCGCCA (100bp)	60	MK165650.1

Table 3-2: Sequences and references of primers and probes used in both probe-based and SYBR® green-based assays

Probe-based assay				
Primer/probe name	**Sequence**	**Probe label 5'**	**Probe label 3'**	**Reference**
blaOXA-48f	GCGTGGTTAAGGATGAACAC			
blaOXA-48r	CATCAAGTTCAACCCAACCG			Van der Zee, 2014
blaOXA-48p	AGCCATGCTGACCGAAGCCAATG	FAM	BHQ-1™	
NDM-1f	CATTAGCCGCTGCATTGATG			
NDM-1r	GTCGCCAGTTTCCATTTGCT			Van der Zee, 2014
NDM-1p	CATGCCCGGTGAAATCCGCC	CAL Fluor Orange 560	BHQ-1™	
Primer/probe name	**Sequence**	**Probe label 5'**	**Probe label 3'**	**Reference**
KPC-1f	TGCAGAGCCCAGTGTCAGTTT			
KPC-1r	CGCTCTATCGGCGATACCA			Van der Zee, 2014
KPC-1p	TTCCGTCACGGCGCGCG	CAL Fluor Red 610	BHQ-2™	
SYBR® green-based assay				
Primer name	**Sequence**	**Probe label 5'**	**Probe label 3'**	**Reference**
mcr1-qf	AAAGACGCGGTACAAGCAAC			Li et al., 2017
mcr1-qr	GCTGAACATACACGGCACAG			
Mcr-3f	ACCTCCAGCGTGAGATTGTTCCA			Li et al., 2017
Mcr-3r	GCGGTTTCACCAACGACCAGAA			
qSUL653f	CCGTTGGCCTTCCTGTAAAG			Heuer, 2007
qSUL719r	TTGCCGATCGCGTGAAGT			
q*SUL2*_595f	CGGCTGCGCTTCGATT			Heuer, 2008
q*SUL2*_654r	CGCGCGCAGAAAGGATT			

3.2.7.2 Standard curve

For absolute quantification, a standard curve was generated in triplicate using the positive control plasmid described earlier. A standard PCR was performed to amplify each target gene, and PCR products were run on a 1% agarose gel to ensure that a pure, single product was obtained for each gene. The PCR products were purified using a Zymoclean Gel Recovery Kit (Zymo Research) and the resulting purified DNA concentrations were measured using a Nanodrop-1000 spectrophotometer. Copy numbers were calculated using the following equation:

$$\boldsymbol{Number\ of\ copies} = \frac{\boldsymbol{ng} \times \boldsymbol{Avogadro's\ constant}}{\boldsymbol{bp} \times \boldsymbol{conversion\ factor} \times \boldsymbol{Average\ mass\ of\ bp}} \qquad \text{[Eq. 3-1]}$$

where *ng* is the mass of the DNA target gene added to the PCR reaction in nanograms, *Avogadro's constant* is valued at 6.022×10^{23}, *bp* is the size of the target gene in base pairs, and *conversion factor* and *average mass of bp* are constants valued at 1×10^{9} and 660 Da respectively.

The 10-fold dilutions of the purified PCR products were made in TE buffer, allowing dilutions to range from 10^{-1} to 10^{-6}. These dilutions were then used as the template DNA in qPCRs. After amplification, Cq values were plotted against the log of the copy numbers calculated from each dilution for each gene. A linear equation was determined, and the efficiency of the PCR was determined by the gradient of the curve using Equation 3-2:

$$\boldsymbol{qPCR\ efficiency} = \mathbf{10}^{\boldsymbol{-1/slope}} - \mathbf{1} \qquad \text{[Eq. 3-2]}$$

A standard curve was run with each plate of samples that was analysed. Running an additional PCR each time for standard curve purposes was deemed time consuming. As each gene was present in the plasmid, a ratio was determined by dividing the base pairs of the target gene by the total number of base pairs in the plasmid. Subsequent standard curves then used diluted plasmid DNA and the resulting copy number that was calculated using the total plasmid base pairs was multiplied by the ratio to obtain the copy number of each target gene.

3.2.7.3 Determination of copy numbers of target-resistance genes

DNA from influent, RAS and effluent from two WWTWs were extracted using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (ZymoResearch). The qPCR assays were performed as described previously. The primers selected for KPC and NDM were multiplexed according to Van der Zee (2014).

Log reduction of gene copies from influent to effluent were calculated using the following equation:

$$\mathbf{R} = -\mathbf{log_{10}}\left(\frac{\mathbf{c_a}}{\mathbf{c_b}}\right) \qquad \text{[Eq. 3-3]}$$

where, R is the log reduction, c_a is the gene copies of the effluent sample and c_b is the gene copies of the influent sample.

To determine the effect of antibiotic exposure on individual species gene copies, DNA that was isolated from colonies and used to identify isolates 17-32 from section 3.2.4 was used as pre-exposure samples. DNA from isolates that grew in the presence of antibiotics during the MIC determination in section 3.2.6 was used as post-exposure for single colony copy number determination. The concentration of extracted DNA, both for mixed and pure cultures, was measured using a Nanodrop-1000 spectrophotometer.

The qPCR reactions were performed as described above. Resulting Cq values of samples were then substituted into the linear equation to determine the copy number of each sample. As the linear equation results in a logged copy number, the log inverse was calculated to get the true copy number of each target gene for each sample, which was then normalised with the concentration of the template DNA.

Melt curve analysis for SYBR® green assays were performed. It was assumed that all sample melt curves that corresponded to those of the control gene resulted in one product.

3.2.8 Monitoring biofilm metabolic response to chronic antibiotic exposure

3.2.8.1 Reactor set up

A CO_2 evolution measurement system (CEMS) was used to investigate whether low environmental concentrations of an antibiotic might induce resistance in an environmental isolate. This system uses CO_2 produced by microbial respiration as an indication of biofilm metabolism. Metabolism is monitored by growing biofilms in silicone tubing, which is placed inside Tygon tubing with a wider diameter (Figure 2-3). The permeable silicone tubing in which the biofilm grows allows for CO_2 exchange, which is then trapped in the larger-diameter, gas-impermeable Tygon tubing, and sweeper gas is pumped through the space between the tubing. This allows for CO_2 levels to be measured in real-time using a CO_2 analyser (LI-820, LI-COR Biosciences).

The CEMS was set up, and conditions were implemented according to Jackson et al. (2015). First, the system was sterilised with 10% bleach for a minimum of two hours using a peristaltic pump (Watson-Marlow 205S) at a flow rate of 2 rpm (equivalent to 13 mℓ per hour). This step was followed by overnight flushing of the entire system with sterile deionised water. Synthetic wastewater (Osachoff, 2014) was used as the growth medium to simulate a similar environment from which the inoculum came and was run through the reactor until the medium had replaced the entire volume of the reactor (approximately two hours).

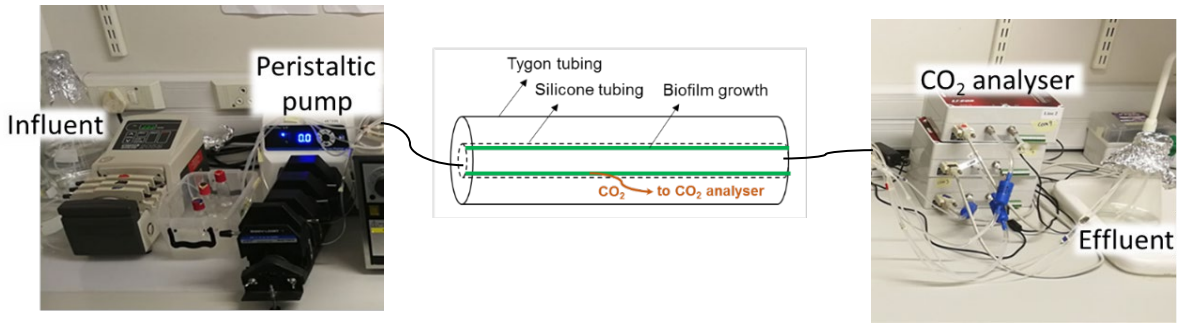

Figure 3-3: Schematic of the carbon dioxide evolution measurement system

One flask contained 4,000 ng/ℓ sulfamethoxazole, which corresponds to the average sulfamethoxazole concentration detected in the WWTW influent and effluent in a previous volume of the current project and, as a result, simulated chronic exposure in the environment (Archer et al., 2017a). Another flask served as a positive control (biofilm growth containing no antibiotic). Sulfamethoxazole is known to be susceptible to photo degradation and thus both control and experimental media flasks were kept in the dark. Ambient air was pumped through the space between the Tygon and silicone tubing at a flow rate of 0.53 ℓ an hour to sweep the CO_2 produced by the biofilm into the CO_2 analyser. The average amount of ambient CO_2 was also continuously measured and subtracted from the CEMS measurement.

3.2.8.2 Biofilm growth

Isolate 32 (*Enterobacter tabaci*) from the previous WWTW sampling events was selected due to the fact that its MIC for sulfamethoxazole was the lowest of all the isolates. An overnight culture was grown in synthetic wastewater broth. The pump was stopped prior to inoculating the silicone tubing with 500 µℓ pre-culture, and after inoculating, the pump remained stopped for a period of 30 minutes to allow the cells to adhere to the inner silicone tubing (Figure 3-3.).

The experiment was continued for a period of 210 hours (8.75 days). At 190 hours, both control and chronic sulfamethoxazole exposure systems were spiked with 512 µg/mℓ (128,000x higher than chronic exposure) of sulfamethoxazole to observe any variations in the control and chronic exposure systems, followed by 2,000 µg/mℓ of sulfamethoxazole (500,000x higher than pre-exposure) at 198 hours to terminate the experiment.

3.3 RESULTS AND DISCUSSION

3.3.1 Determination of antibiotic concentrations to select for resistance

Literature was contradictory as to the concentrations of each antibiotic to incorporate into the medium to select for resistant bacteria. For this reason, the EUCAST and CLSI resistant breakpoint criteria (Clinical and Laboratory Standards Institute, 2015; European Committee on Antimicrobial Susceptibility Testing, 2017) were used to get an estimate of resistant bacterial numbers in a sample. Figure 3-4 shows the concentrations of each antibiotic used to obtain initial information of the number of resistant organisms. There is a decrease by an order of magnitude from MAC agar (1.73 x 10^6 CFU/mℓ) to MAC agar supplemented with 4 µg/mℓ colistin (4.67 x 10^5 CFU/mℓ) (Figure 3-4), which indicates the susceptibility of gram negatives to colistin exposure in mixed cultures obtained from RAS.

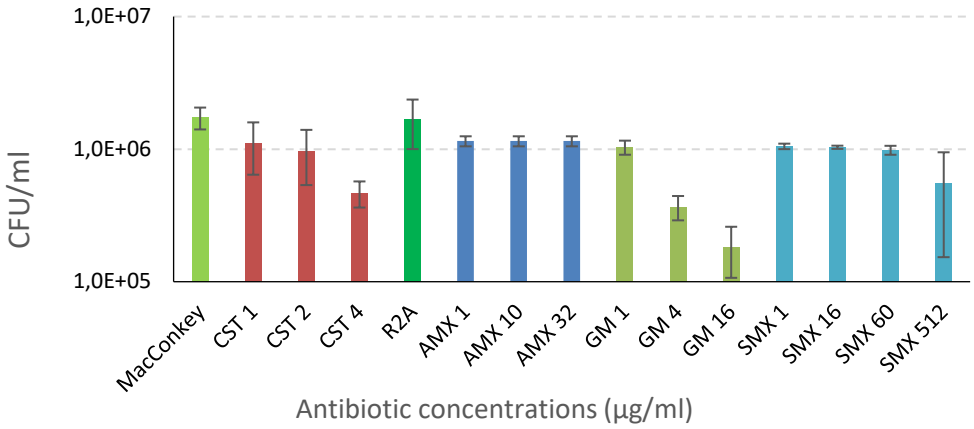

Figure 3-4: Mixed-culture colony counts CFU/mℓ for a RAS sample, which was exposed to different concentrations of antibiotics incorporated into the media. Colistin was added to MAC agar, while amoxicillin, gentamicin and sulfamethoxazole were added to Mueller Hinton agar (MHA).

The CFU/mℓ on R2A media supplemented with amoxicillin remains the same despite the increasing antibiotic concentrations. There was, however, a decrease in CFU/mℓ from R2A agar to R2A agar supplemented with amoxicillin, although it is thought that the decrease is not substantial enough to assume that the growth observed in the amoxicillin-supplemented media infers resistance. Incorporating 16 µg/mℓ gentamicin into an R2A medium resulted in a decrease from 1.68 x 10^6 (R2A control) to 1.83 x 10^5 CFU/mℓ, while a decrease from 1.68 x 10^6 (R2A control) to 5.5 x 10^5 CFU/mℓ was observed in the case of an R2A media supplemented with 512 µg/mℓ of sulfamethoxazole. These results were used to guide the subsequent experiments in terms of the antibiotic concentrations that were used.

The EUCAST breakpoint for resistance to amoxicillin is roughly 8 µg/mℓ, and a review showed that most microbes were susceptible to concentrations of amoxicillin below 4 µg/mℓ, with *Staphylococcus epidermidis* and *Staphylococcus aureus* being outliers with MICs of 64 µg/mℓ and more than 256 µg/mℓ, respectively (Kaur et al., 2011). However, Figure 3-4 shows that the concentration of amoxicillin needed to be substantially higher than 32 µg/mℓ in order to reduce the number of bacteria to select for amoxicillin-resistant organisms.

In contrast, the highest concentrations used for colistin, gentamicin and sulfamethoxazole were sufficient to isolate resistant organisms. After subsequent experiments (data not shown), final concentrations to be implemented in the study were 4 µg/mℓ and 8 µg/mℓ colistin, 16 µg/mℓ and 32 µg/mℓ gentamicin, and 256 µg/mℓ and 512 µg/mℓ for both amoxicillin and sulfamethoxazole. These high concentrations that were used give light to the resilience of mixed environmental cultures towards antibiotics. It became a concern that the components of R2A media may interact with the antibiotics, and thus the use of this medium was discontinued and replaced with MHA (Heuer et al. 2002), which is the standard used for susceptibility testing.

3.3.2 Determination of resistance

The influent, effluent and RAS samples that were obtained from the two WWTWs were used to investigate whether significant differences in resistance profiles can be observed within and between different WWTWs. Figure 3-5a and 3-5d show a higher OD_{600} in the MAC medium than on MHB for most of the sample matrices, suggesting that more growth is present in the gram-negative selective medium compared to the broad-spectrum medium (both gram-positive and gram-negative). While a blank was used to eliminate the effect of the medium on the sample turbidity, the absorbance (OD_{600}) cannot be compared between MAC and MHB cultures due to a difference in the medium colour. The presence of a pH indicator allows for bacteria that ferments lactose to alter the colour of the MAC medium, which could influence the OD_{600} readings in comparison to the organisms that do not ferment lactose.

The measured absorbance for turbidity (OD_{600}) for RAS is seen to be higher than that of influent and effluent for all antibiotic concentrations except sulfamethoxazole at 512 µg/mℓ in WWTW-1 (Figure 3-5a). Activated sludge utilises aerobic bacteria to remove organic compounds. As a result, it is expected that this part of the WWTW would contain a higher number of bacteria. However, the fact that the highest resistant bacterial numbers are also seen at this site could be attributed to the fact that samples were taken from the portion of the activated sludge that is returned to the aerobic digesters. It is possible that the organisms returning into the aerobic digesters have already been exposed to antibiotics or have been exposed for longer periods of time compared to other matrices and, as a result, the number of resistant bacteria found in this matrix is higher.

An incorrect flow rate of RAS back to the reactor could lead to inadequate settling of waste sludge, which could allow bacteria that are resistant to antibiotics to exchange resistance mechanisms (Theobald, 2014). Higher OD_{600} values are observed for the effluent opposed to influents for amoxicillin (512 µg/mℓ and 256 µg/mℓ) and gentamicin (16 µg/mℓ) in WWTW-1, and sulfamethoxazole (256 µg/mℓ) in WWTW-2. For the other antibiotic exposures, a lower turbidity was observed in the post-chlorination effluent compared to the influent. While it is evident that ARB persist in WWTW effluent that is destined for discharge into the environment, lower numbers are entering the environment that would have entered if the wastewater did not pass through the WWTW. Based on these results, the statement made by many studies that WWTWs are hotspots for AMR emergence is over-estimated (Berendonk et al., 2015).

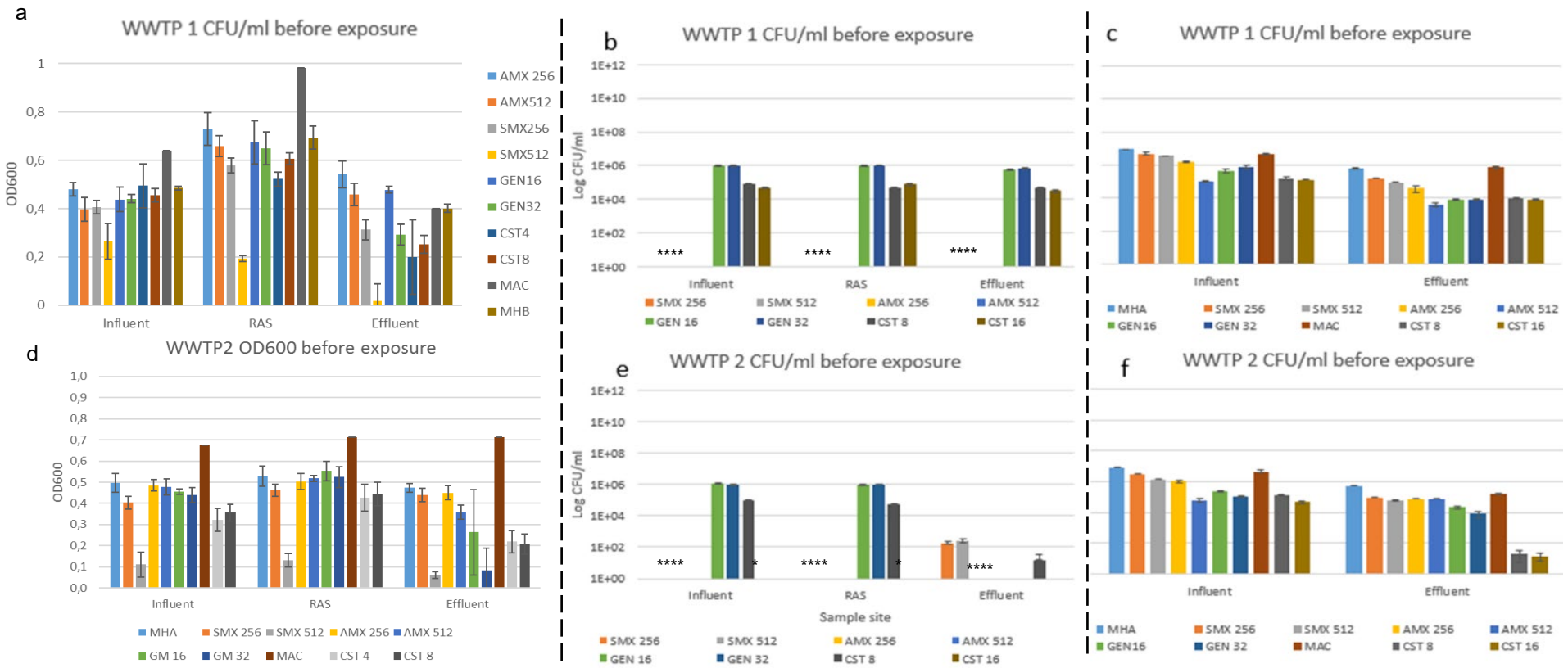

Figure 3-5: **Growth of bacteria in the presence of amoxicillin, sulfamethoxazole, gentamicin and colistin from influent, RAS and effluent samples in two WWTWs in February 2018 (a, d), July 2018 (b, e) and February 2019 (c, f) before being enriched in antibiotics. All antibiotic concentrations are in µg/mℓ. MAC media was used as a positive control for colistin while MHA/MHB was used as a positive control for the other antibiotics. Figures a and d show OD_{600} as an indication of the susceptibility to antibiotics, while figures b, c, e and f show CFU/mℓ. Results for figures (b) and (e) marked with an asterisk (*) were compromised and are therefore not included in the results.**

In future, similar studies on solid waste from a WWTW, as well as on poorly functioning WWTWs, should be conducted to see if the results show otherwise. Of concern is the fact that bacterial isolates grown in colistin are still present in WWTW effluent, even though this antibiotic is only implemented as a last-resort antibiotic treatment.

These results emphasise the need to examine the effect of environmental concentrations of antibiotics on the occurrence of resistant bacteria and further downstream waters for resistance genes, and to identify the mechanisms of gene transfer. These results also suggest that colistin use in livestock is still practiced, despite it being banned, consequently leading to residual concentrations entering WWTWs (Gouws, 2016). The OD_{600} for all matrices in both WWTWs is higher in MAC media compared to MHA (Figure3-5a and 3-5d). This does not necessarily mean that there are more gram-negative bacteria present in the sample but can rather be ascribed to the higher turbidity of the MAC broth.

As discussed previously, turbidity is not the best way to determine the presence and quantitiy of resistant bacteria, thus determination of resistance in the July 2018 event was done differently compared to the February 2018 sampling. Plate counts were performed from each sample matrix before Figure 3-5b and 3-5e) and after (Figure 3-5a and 3-5c) enrichment in antibiotic media, opposed to the measurement of turbidity. Due to the inconsistency of the method used to determine resistance in bacteria from February to July 2018 before exposure (Figure 3-5), as well as the fact that amoxicillin and sulfamethoxazole data was compromised for July 2018 (shown with asterisks (*)), Figure 3-5b and 3-5e cannot be directly compared to Figure 3-5a and 3-5d (February 2018 sampling), but inferences can be made and overall trends established.

In the winter (July) there was no difference between influent, RAS and effluent for WWTW-1 (Figure 3-5b), while there was variation in resistance between influent, RAS and effluent in summer (February) in WWTW-1 (Figure 3-5a). The growth trends for both February (Figure 3-5a and 3-5d) and July 2018 (Figure 3-5b and 3-5e) were similar for WWTW-2, with the effluent having a more significant decrease in growth compared to the influent. These results indicate that seasonal variation for AMR profiles is more prevalent in WWTW-1 compared to WWTW-2.

It is interesting to note that the CFU/mℓ of sulfamethoxazole at both concentrations was much lower in WWTW-2 effluent in July 2018 ($2x\ 10^2$ CFU/mℓ) (Figure 3-5e) than in February 2019 ($6x\ 10^4$ CFU/mℓ) for the same plant (Figure 3-5f). Comparing the effluent CFU/mℓ to the influents of the respective WWTWs, suggests more efficient removal of resistant bacteria by WWTW-2, compared to WWTW-1. WWTW-1 has a very large capacity and makes use of a mixed-flow system, while WWTW-2 implements a maturation tank prior to chlorination of the effluent and makes use of a plug-flow system. This allows bacteria to form flocs and settle, which could reduce the number of bacteria that enter the post-chlorination effluent evident in Figure 3-5f.

Overall, the CFU/mℓ in July 2018 for both WWTWs was higher after enrichment compared to February 2018 (Figure 3-6b and 3-6d). There was a three to four order of magnitude increase in CFU/mℓ after exposure, indicating the effect of antibiotics on bacterial counts. While an increase in cells is to be expected, culturing these bacteria in batch may not be a realistic representation of the environment. As a result, further studies that look at the metabolic profile of a biofilm in a continuous system were performed.

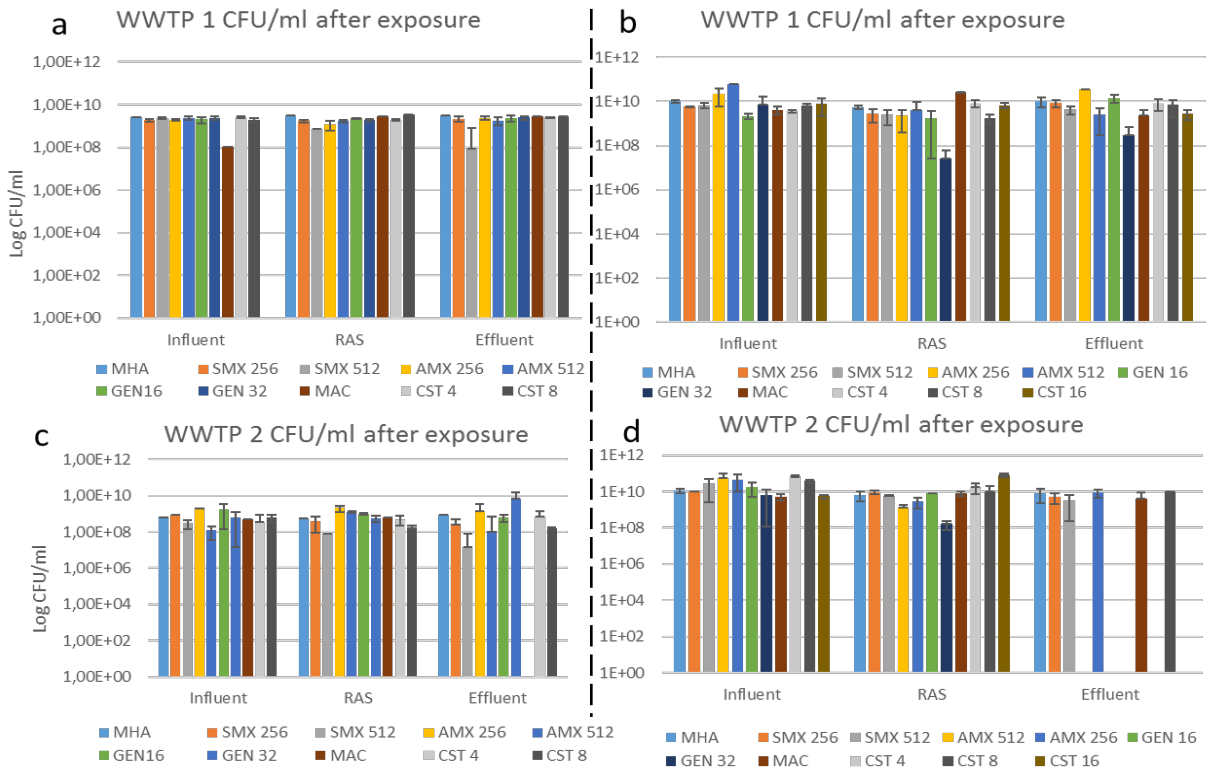

Figure 3-6: Growth of bacteria in the presence of amoxicillin, sulfamethoxazole, gentamicin and colistin from influent, RAS and effluent samples in two WWTWs in February 2018 (a, c) and July 2018 (b, d) after being enriched in antibiotics. All antibiotic concentrations are in µg/mℓ. The MAC medium was used as a positive control from colistin, while MHA/MHB was used as a positive control for the other antibiotics.

3.3.3 Genus composition of selected isolates

Of the effluent single colonies that were selected from the antibiotic plates in the February 2018 sampling event, the genus *Morganella morganii* was the most predominant (32%), followed by *Escherichia fergusonii/Shigella flexneri* (16%), while *Citrobacter freundii* and *Pseudomonas indoloxydans* both constituted 11% of the samples. The remaining isolates (*Aeromonas caviae, Aeromonas hydrophila, Citrobacter murliniae, Aeromonas dhakensis/caviae/enteropelogenes, Providencia rettgeri* and *Pseudomonas aeruginosa*) each make up 5% of the samples (Figure 3-7). A number of these identified species are members of normal gut microbiota, which explains their presence in a WWTW. However, they are also opportunistic pathogens (Abbott, 2011; Bassetti et al, 2018; Janda and Abbott, 2010).

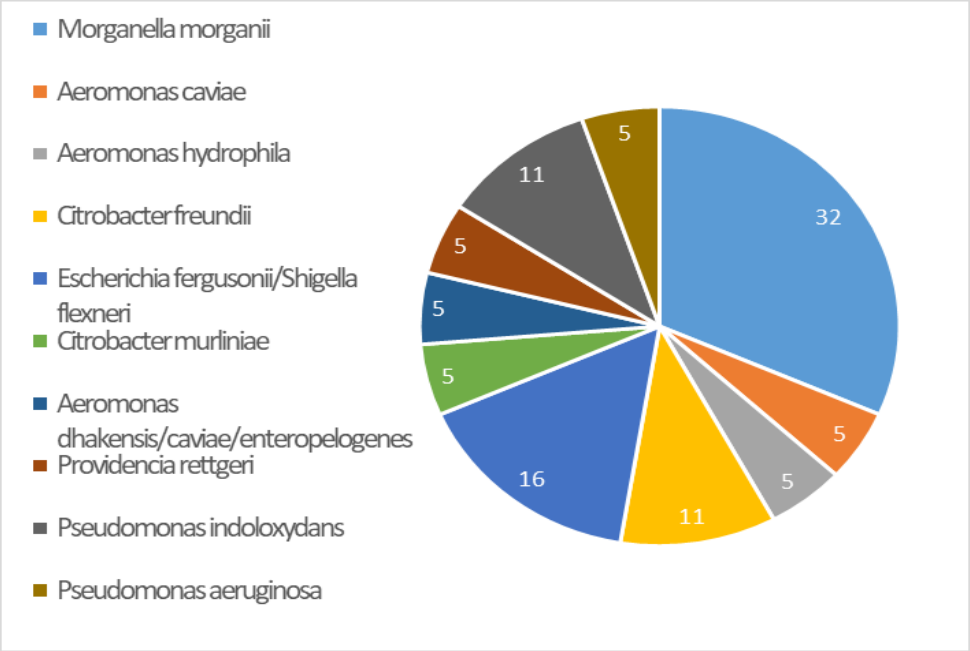

Figure 3-7: Percentage of each species identified by 16S rDNA sequencing of isolates obtained from effluent samples that were previously exposed to antibiotics (n = 19). Isolates that indicate multiple species had identical percentage identities to the query sequence when using BLAST.

With ESKAPE pathogens, such as *P. aeruginosa,* that are present in the effluent sample, the environment downstream of the WWTW is at risk as these organisms have the potential to transfer resistance mechanisms present in the isolated bacteria to other species and environments (Rice, 2008). *Morganella* is a genus very closely related to the *Proteus* and *Providencia* genera. This organism is intrinsically resistant to ampicillin, amoxicillin/clavulanate and first-generation cephalosporin. It was selected for on the antibiotic media, which could indicate why it was the most prominent in the sample set. *Morganella morganii* can also cause opportunistic infections in rare instances in immuno-compromised patients (Lee et al., 2009; Liu et al., 2016a).

A case study by Lee et al. (2009) gave insight into a soft tissue infection caused by *M. morganii* that resulted after a toddler was scratched by a chicken (Lee et al., 2009). With this organism being transferred to the environment via WWTW effluent, this becomes a major health concern if reuse of effluent for agricultural purposes is implemented. Of the selected isolates, the species composition varies between WWTWs as seen in Figure 3-8. Most of the isolates obtained from WWTW-1 were *Escherichia fergusonii/Shigella flexneri* and *M. morganii,* while the majority of those obtained in WWTW-2 were *M. morganii* species. *Citrobacter freundii* is more common in WWTW-1 compared to WWTW-2. This species has been found to contain *ampC,* a β-lactamase-producing gene conferring resistance to antibiotics such as amoxicillin, which has been linked to hospital mortalities (Liu et al., 2018). The treatment strategies and performance of the WWTWs could play a role in the bacterial composition of the WWTW. The variation in treatment process, as discussed previously, could result in different species emerging from the effluent.

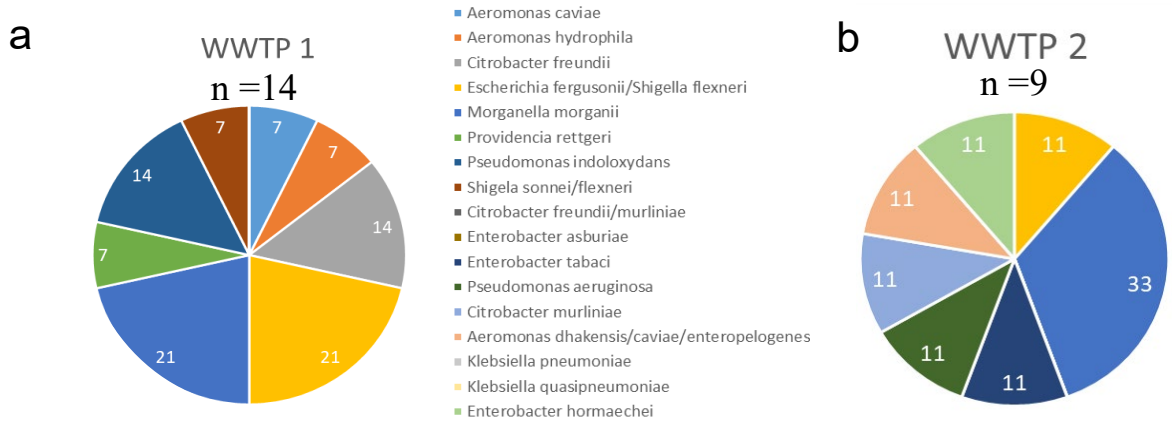

Figure 3-8: Species composition of bacteria isolated from effluent samples of each WWTW. A) WWTW-1 previously exposed n = 12, not previously exposed n = 2. B) WWTW-2 previously exposed n = 7, not previously exposed n = 2.

When comparing the sample sites (Figure 3-9), the predominant species in the influent are *Klebsiella pneumoniae/quasipneumoniae, Enterobacter tabaci* and *Shigella sonnei. Klebsiella* spp. increased in the RAS and the effluent showed dominance by *Escherichia fergusonii, Citrobacter freundii, E. tabaci and Enterobacter hormaechei*. As sample sizes are small, and were isolated on media containing antibiotics, some bias could be introduced by selecting for organisms that are intrinsically resistant to certain antibiotics, as mentioned previously, or that have higher growth rates in comparison to more abundant species.

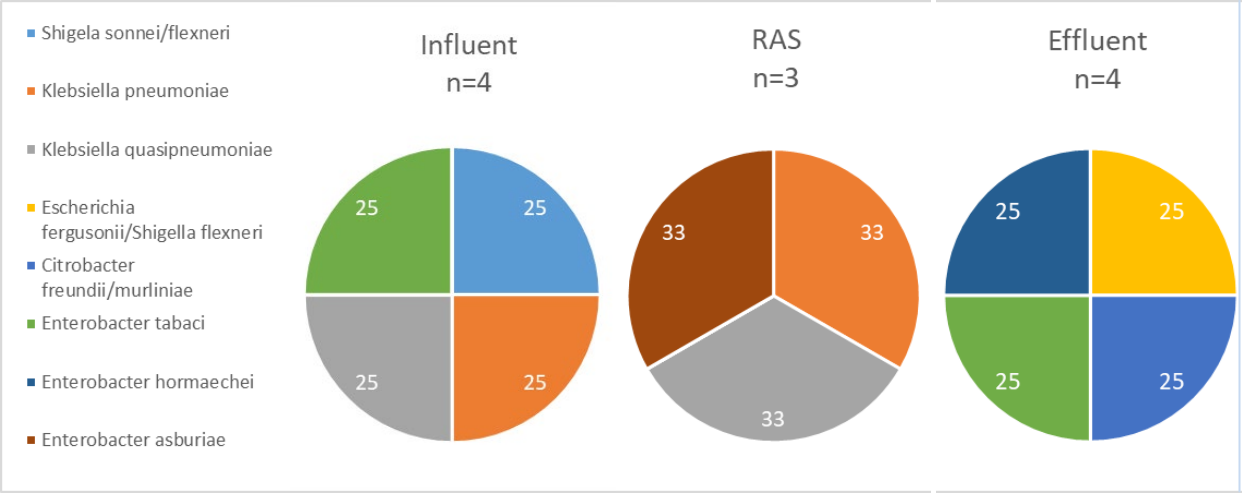

Figure 3-9: Species identified in different areas of both WWTWs from cultures isolated of antibiotic plates

Looking at species composition of the WWTW could give insight into the associated risk of these organisms in the environment downstream of the WWTW and if treated effluent reuse is under consideration for irrigation or other purposes. The fate of these organisms should be investigated if water reuse is considered.

3.3.4 Environmental minimum inhibitory concentrations

3.3.4.1 Mixed culture samples

Table 3-3 shows that the concentrations of the selected antibiotics required to inhibit the growth of bacteria are at least two-fold lower in the effluent of WWTW-2, compared to the other sites and WWTW-1 for colistin, amoxicillin and sulfamethoxazole. The MIC for gentamicin in WWTW-2 effluent is four-fold lower than that of the other sites and WWTW-1. There is no change in the MIC for colistin or sulfamethoxazole for WWTW-1 and between the influent and RAS of both WWTWs. The MIC in the RAS of WWTW-1 is double (16,384 µg/mℓ) that of the rest of the WWTWs (8,192 µg/mℓ). These MICs for mixed cultures are highly variable, and it is acknowledged that different results could be obtained due to variation in bacterial composition.

Table 3-3: The MICs of influent, RAS and effluent samples from two WWTWs for colistin, gentamicin, amoxicillin and sulfamethoxazole

WWTW	Site	MIC (µg/mℓ)			
		Colistin	**Gentamicin**	**Amoxicillin**	**Sulfamethoxazole**
WWTW-1	Influent	>512	>1,024	8,192	4,096
	RAS	>512	1,024	**16,384**	4,096
	Effluent	>512	512	8,192	4,096
WWTW-2	Influent	>512	1,024	8,192	4,096
	RAS	>512	256	8,192	4,096
	Effluent	**256**	32	**1,024**	**2,048**

3.3.4.2 Single colony samples

Table 3-4 shows the species identification of each isolate through 16S rDNA sequencing as described previously, as well as the MICs of colistin, gentamicin, amoxicillin and sulfamethoxazole for each isolate. The mode MIC is extremely high for colistin, amoxicillin and sulfamethoxazole (2,048 µg/mℓ, 16,384 µg/mℓ and 4,096 µg/mℓ, respectively). These MICs are 256, 2,048 and 8 times the respective EUCAST MIC values and, as a result, none of these values correspond to the CLSI or EUCAST MIC resistance breakpoint criteria (Clinical and Laboratory Standards Institute, 2015), which suggests that environmental pathogens have a stronger tolerance to the selected antibiotics. Amoxicillin has been identified as having poor activity against *Pseudomonas,* which could explain the high MICs for *P. aeruginosa* (Isolate 3) and *P. indoloxydans* (isolates 20 and 21) (Table 3-4) (European Committee on Antimicrobial Susceptibility Testing, 2010).

Table 3-4: MICs of colistin, gentamicin, amoxicillin and sulfamethoxazole and identities of single cultures to isolates from both WWTWs

						MIC (µg/mℓ)			
Isolate number	**Species**	**WWTW**	**Season**	**Sample**	**Isolated on**	**CST**	**GM**	**AMX**	**SMX**
2	*Morganella morganii*	2	Summer	Effluent	CST	>2,048	128	1,024	4,096
3	*Pseudomonas aeruginosa*	2	Summer	Effluent	AMX	2,048	8	4,096	2,048
4	*Morganella morganii*	2	Summer	Effluent	AMX	>2,048	16	2,048	4,096
5	*Aeromonas caviae*	1	Summer	Effluent	SMX	2,048	32	1,024	2,048
6	*Aeromonas hydrophila*	1	Summer	Effluent	SMX	2,048	16	64	32
7	*Citrobacter freundii*	1	Summer	Effluent	AMX	2,048	16	16,384	2,048
8	*Escherichia fergusonii/Shigella flexneri*	1	Summer	Effluent	AMX	64	16	4,096	4,096
9	*Morganella morganii*	1	Winter	Effluent	CST	2,048	16	2,048	4,096
10	*Morganella morganii*	1	Winter	Effluent	CST	> 2,048	32	16,384	4,096
11	*Citrobacter freundii*	1	Summer	Effluent	GM	2,048	256	16,384	4,096
12	*Morganella morganii*	1	Summer	Effluent	CST	> 2,048	16	16,384	4,096
13	*Escherichia fergusonii/Shigella flexneri*	2	Summer	Effluent	GM	2,048	256	16,384	4,096
14	*Citrobacter murliniae*	2	Summer	Effluent	SMX	2,048	16	16,384	4,096
15	*Aeromonas dhakensis/caviae/enteropelogenes*	2	Summer	Effluent	SMX	4,096	128	16,384	2,048
16	*Providencia rettgeri*	1	Winter	Effluent	SMX	4,096	32	4,096	4,096
18	*Morganella morganii*	2	Summer	Effluent	GM	> 4,096	512	16,384	4,096
19	*Escherichia fergusonii/Shigella flexneri*	1	Summer	Effluent	GM	< 8	512	16,384	4,096
20	*Pseudomonas indoloxydans*	1	Winter	Effluent	AMX	< 8	16	16,384	2,048
21	*Pseudomonas indoloxydans*	1	Winter	Effluent	AMX	< 8	16	16,384	2,048
22	*Shigella sonnei/flexneri*	1	Summer	Influent	None	16	16	4,096	4,096
23	*Klebsiella pneumoniae*	2	Summer	Influent	None	64	16	4,096	4,096
24	*Klebsiella quasipneumoniae*	2	Summer	Influent	None	16	16	2,048	4,096
25	*Escherichia fergusonii/Shigella flexneri*	1	Summer	Effluent	None	< 8	16	2,048	4,096
26	*Citrobacter freundii/murliniae*	1	Summer	Effluent	None	16	16	16,384	4,096
27	*Enterobacter tabaci*	2	Summer	Effluent	None	64	16	1,024	4,096

28	*Klebsiella quasipneumoniae*	2	Summer	RAS	None	16	16	2,048	4,096
29	*Klebsiella pneumoniae*	2	Summer	RAS	None	1,024	16	16,384	4,096
30	*Enterobacter hormaechei*	2	Summer	Effluent	None	64	16	16,384	2,048
31	*Enterobacter asburiae*	1	Summer	RAS	None	32	16	1,024	4,096
32	*Enterobacter tabaci*	1	Summer	Influent	None	< 8	> **2,048**	16,384	64
					Mode	2,048	16	16,384	4,096
					Max	4,096	512	16,384	4,096
					Min	8	8	64	32

In addition, while Enterobacteriaceae were less affected by amoxicillin, the EUCAST MIC values are 8 µg/mℓ for this group of bacteria, indicating that environmental organisms still appear to be more resistant than clinical isolates. Isolates that were not previously exposed to any antibiotics (isolates 22 to 32) had much lower MICs for colistin. However, only 18% of these isolates were considered susceptible to colistin, with MICs less than 8 µg/mℓ (EUCAST). Isolates 22 to 32 (not isolated on antibiotic media) had similar MICs for amoxicillin and sulfamethoxazole to isolates that were exposed to amoxicillin and sulfamethoxazole upon isolation. However, for gentamicin, Isolate 32 (*E. tabaci*) was the only sample not isolated on antibiotic medium that had an MIC higher than 16 µg/mℓ and, as a result, the highest MIC to gentamicin. Another *E. tabaci* isolate (Isolate 27) had a gentamicin MIC of 16 µg/mℓ.

While these isolates were isolated from different WWTWs and different locations in the WWTWs, it is still an indication of how resistance varies in individual isolates of the same species. Isolates 11, 13, 18 and 19 were isolated on a gentamicin medium. These isolates have the highest MICs for gentamicin of the isolates exposed to antibiotics, suggesting that exposure to gentamicin resulted in a higher MIC for gentamicin. High MICs for bacteria isolated from wastewater are also found in other studies with gentamicin >32 µg/mℓ (Ovejero et al., 2017).

While it is expected that isolates exposed to high concentrations of antibiotics would be able to grow in the same or higher antibiotic concentrations upon future exposures, incredibly high MICs are still evident in isolates that were not isolated in antibiotic media (Table 3-4). There are numerous explanations for this, as the wastewater environment is incredibly complex. Daily variations in the loads that influence the composition of the wastewater could influence the occurrence of resistance, as well as flow rates, rainfall and other abiotic and biotic factors. Multiple studies have indicated that tolerance to metals can result in co-selection of AMR (Baker-Austin et al., 2006; Alam and Imran, 2014). In addition, the levels of antibiotics or associated metabolites could be present in the WWTWs, which could provide selective pressures to bacteria.

Figure 3-10 shows the MIC data for four of the isolates studied. Although separate isolates are represented, the same species are shown before and after amoxicillin exposure. The MIC for amoxicillin for both *E. fergusonii* and *C. freundii* that were isolated after enrichment in an amoxicillin medium is double the MIC of the same species that was not enriched in amoxicillin (Figure 3-10b and 3-10d). This indicates that exposure to sub-MIC concentrations in a batch culture results in an increase in MIC.

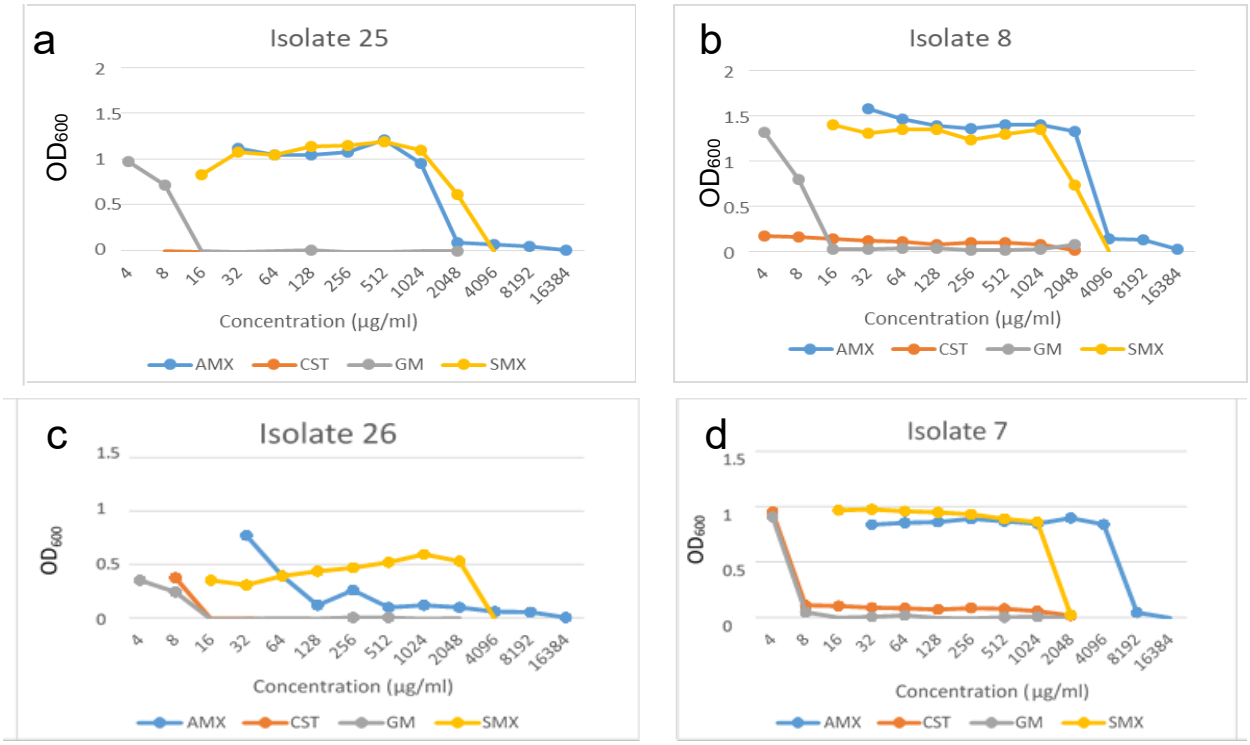

Figure 3-10: Examples of MIC estimations of four isolates in microtiter plates containing amoxicillin, colistin, gentamicin and sulfamethoxazole: a) *Escherichia fergusonii/Shigella flexneri* isolated on an MAC medium; b) *Escherichia fergusonii/Shigella flexneri* isolated on an amoxicillin medium, c) *Citrobacter freundii* isolated on an MAC medium; and d) *Citrobacter freundii* isolated on an amoxicillin medium.

3.3.5 Quantification of target antibiotic resistance genes

3.3.5.1 Environmental samples from wastewater

The gene copy numbers of *sul1* were higher than the copy numbers of *sul2* in all samples obtained from both WWTWs (Figure 3-11). This is in agreement with a Swiss study by Czekalski et al. (2012), however, gene copies in this study were normalised to the 16S rRNA gene, so comparing the given results to literature proves to be a challenge. The *Mcr3* copy numbers seen in the influent of WWTW-2 (9.5×10^{23} copies) are lower than those in WWTW-1 (4.4×10^{25} copies), while no copies of *mcr3* were detected in the effluent of WWTW-2, suggesting that complete removal of *mcr3* is evident in WWTW-2 (Figure 3-11c). The copy number of *bla*NDM genes in the influent of both WWTWs was at least two orders of magnitude lower compared to other genes, while *bla*NDM RAS (2.6×10^{35} copies/ng DNA) and *blaKPC* influent (8×10^{38} copies/ng DNA) had the highest number of gene copies in WWTW-2 (Figure 3-11d and 3-11e, respectively).

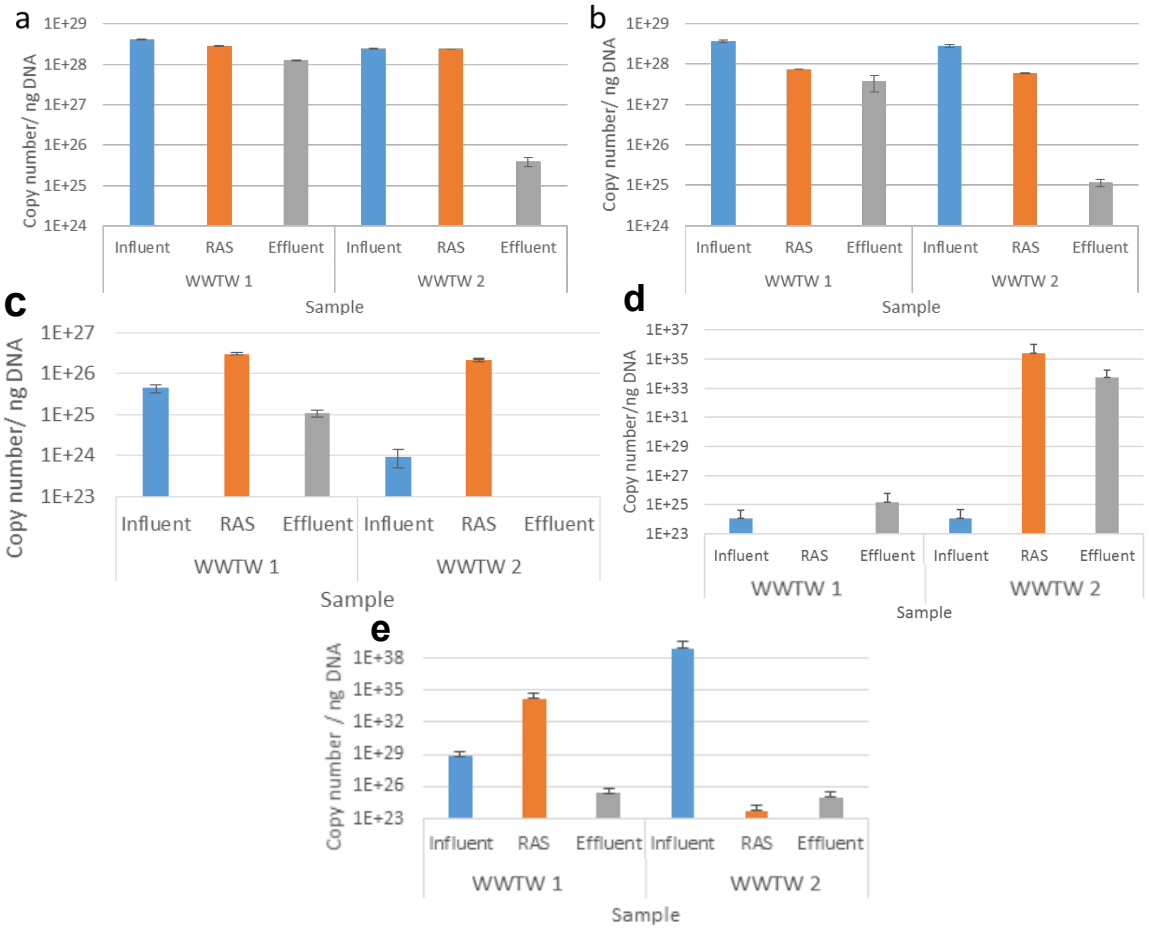

Figure 3-11: Copy number of plasmid-mediated resistance genes for sulfamethoxazole, *sul1* (a) and *sul2* (b), colistin-resistance gene *mcr3* (c), β-lactam resistance genes NDM (d) and KPC (e) in influent, RAS and effluent samples obtained from two WWTWs.

It is interesting to note that the *bla*NDM that was absent from RAS in WWTW-1 reappeared in the effluent. All genes except *bla*NDM in WWTW-2 showed a decrease in gene copies from influent to effluent. The log reductions seen in Figure 3-12 for WWTW-2 range between 2.8 and 13.9. These values indicate that WWTW-2 removes more than 99% of the gene copies of each ARG that was targeted, except *bla*NDM. The ARG log removals in WWTW-1 ranged between 0.5 (69%) and 3.5 (99.9%) (Figure 3-12). Multiple authors stated that WWTWs are hotspots for the transmission of ARB and ARGs (Berendonk et al., 2015). While these environments do indeed have waters that are highly contaminated with ARB and ARGs, the results in Figure 3-11 and Figure 3-12 show that all tested genes, except bla_{NDM}, are less prominent in the effluent than in the influent. This does not confirm the fact that these genes, which are released into the environment from the WWTW, do not play a role in the transmission of AMR downstream, but merely indicates that water treatment processes contribute to reducing the number of ARGs that enter the environment. However, there is still concern that these genes enter the environment, including through rivers, the ocean and sediment.

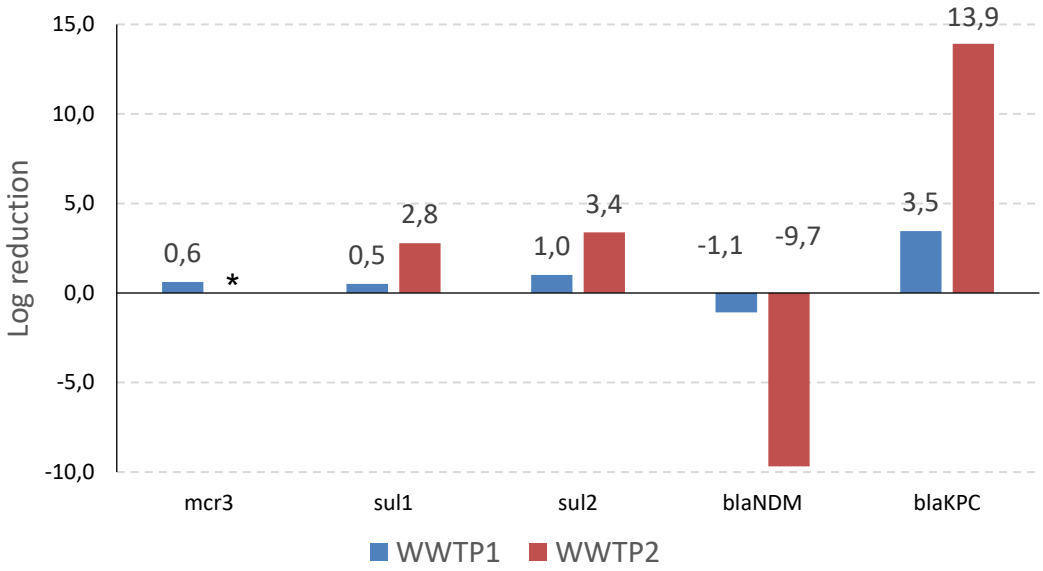

Figure 3-12: Log reduction of resistance genes from influent to effluent in two WWTWs calculated using Equation 2-3. WWTW-2 log reduction for *mcr3* could not be calculated as the copy number at this point was 0 and log reduction is marked with an asterisk.

Through this dissemination, other organisms could acquire these genes, increasing the spread of resistance (Czekalski et al. (2012). Yang et al. (2019) showed that Enterobacteriaceae isolated from blowflies in Thailand contained *mcr1* genes in predominantly IncX4 plasmids. This finding indicates the concern for the presence of resistance genes in the environment as other organisms, in this case, flies, could play a role in the transfer of resistant bacteria (Yang et al., 2019). Clearly, more research needs to be conducted on the fate of AMR and ARGs regarding transmission in the environment.

Clear differences in the log removals of ARGs were observed between the two WWTWs. The difference in treatment process is a plug-flow system with a maturation tank prior to chlorination in WWTW-2. This tank could allow aggregates of suspended solids that remain at this phase of the treatment process to settle and are not present in the chlorinated effluent. Utilisation of membrane bioreactors in WWTWs have previously been shown to have improved ARG removal efficiency (Munir et al., 2011), while coagulation using $FeCl_3$ was also found to improve ARG removal efficiency (Li et al., 2017). However, the coagulation treatment was found to have log reductions of ARGs ranging from 0.5 to 3.1, which lies in the range seen in WWTW-2 for *mcr3, sul1, sul2* and *blaKPC*. While settlement and coagulation are different processes, the concept behind them remains the same.

The settlement of the aggregates, which could include biofilms and other flocs that harbour ARGs, may result in decreased gene copies emerging post-chlorination in WWTW-2. Samples that were identified as *mcr1* by qPCR were found to have non-specific amplification products that did not correspond with the target gene. In addition, all samples were negative for *bla*OXA genes; hence, these results are not included here. The *blaKPC* was very well removed in both WWTWs, while *bla*NDM copies increased in the effluent compared to the influent (Figure 3-11). *Aeromonas caviae*, *Vibrio cholera* and Enterobacteriaceae were shown to contain stable and transferrable NDM-1 genes in New Delhi, with the former two species containing this resistance gene in the chromosome opposed to a plasmid (Walsh et al., 2011). The presence of the NDM gene in the chromosome of other species that are dominant in the mixed culture could play a role in its accumulation in the chlorinated effluent.

3.3.6 Effect of antibiotic exposure on resistance gene copies

Isolates 16, 18, and 19 had the highest *sul1* copy numbers, both before and after exposure to sub-MIC concentrations (Figure 3-13a), while isolates 22 and 32 had the highest copy numbers of *sul2* (Figure 3-13b). As shown in Table 3-4, the isolates with the highest *sul1* copies were all isolated from effluent wastewater, while those isolates with the highest *sul2* copies originated from influent wastewater. Isolate 16 was isolated after previous exposure to 512 µg/mℓ sulfamethoxazole, while isolates 18 and 19 were isolated on a medium containing 32 µg/mℓ gentamicin. Isolates 22 and 32 were isolated on media that did not contain antibiotics. As shown in Table 3-4, the higher gene copies in these isolates are not species-specific, as isolates 16, 18, 19, 22 and 32 are identified as *Providencia*, *Morganella*, *Escherichia*, *Shigella* and *Enterobacter,* respectively. Four of the five isolates containing the most sulfamethoxazole gene copies were isolated from WWTW-1. The majority of the remaining isolates showed between 10^{24} and 10^{25} gene copies for both *sul1* and *sul2* (Figure 3-13a and 3-13b). Only six of the 14 isolates showed higher copy numbers of *sul1* after exposure to sub-MIC concentrations (Figure 3-13a).

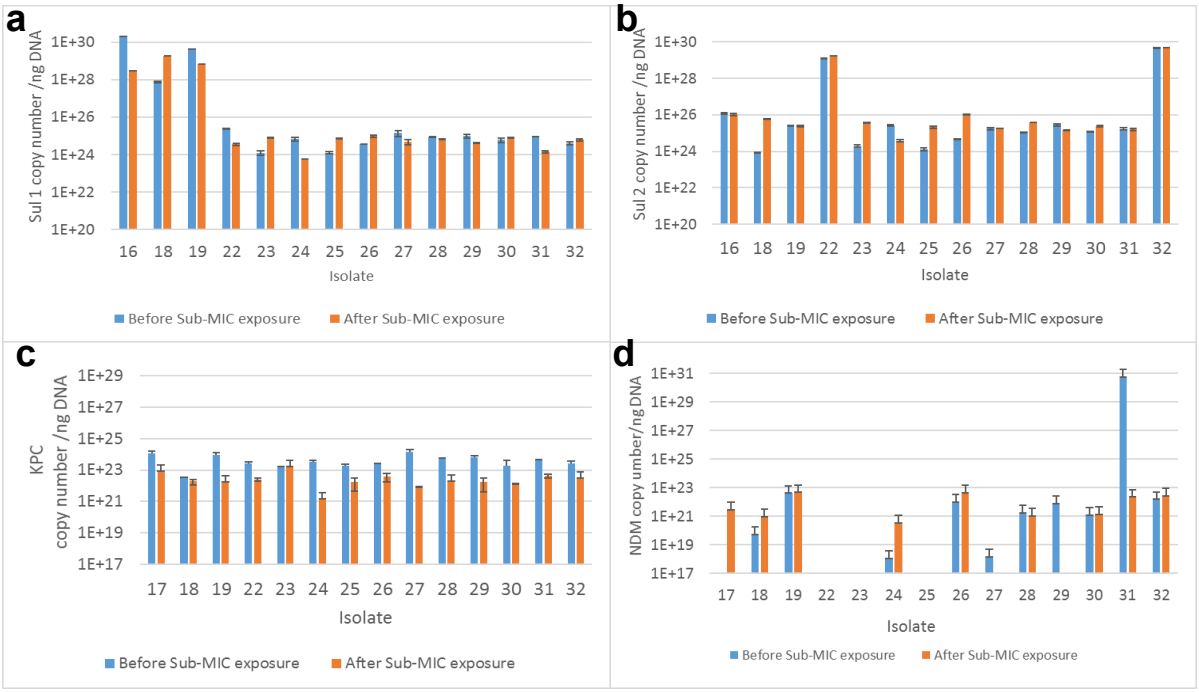

Figure 3-13: Copy number of sulfamethoxazole-resistance genes (a) *sul1*, (b) *sul2*, (c) *blaKPC* and (d) *blaNDM* in isolated colonies before and after sub-MIC antibiotic exposure

However, none of these differences are statistically significant. Seven isolates showed higher copy numbers of *sul2* after exposure to sub-MIC concentrations of sulfamethoxazole (Figure 3-13b) with only three of these being significantly different. All of the isolates showed a reduction in *blaKPC* copy number after exposure to sub-MIC concentrations of amoxicillin (Figure 3-13c), while only five of 14 isolates showed an increase in *blaNDM* genes after exposure to amoxicillin (Figure 3-13d). Isolates 27 and 29 were found to have no *blaNDM* genes present after exposure to amoxicillin, while Isolate 17 did not contain *blaNDM* genes prior to antibiotic exposure but contained the gene after exposure. The *mcr1* and *mcr3* showed non-specific amplification products and, as a result, these results were excluded.

Using DNA concentration as a means of normalisation has potentially introduced bias to the results. The quantity of DNA extracted from a cell is dependent on numerous factors, and human error could play a vital role. Nanodrop spectrophotometers have been found to over-estimate the concentration of

DNA and could influence the results obtained (Simbolo et al., 2013). However, if bias was introduced, it was done to the sample both before and after antibiotic exposure, as both DNA concentrations were measured using a nanodrop and thus the reported results should be consistent. Regardless, future studies should normalise gene copies using the 16S rRNA gene or CFU/mℓ.

It was anticipated that, after antibiotic exposure, all isolates would show an increase in resistance gene copies, or that the gene copies would stay the same. While this trend was observed for some isolates, the converse outcome for some isolates needs explanation. A proposed hypothesis for the results observed is that the exposure of each isolate to antibiotics in sub-MIC concentrations, which in this case was high compared to what would be expected for clinical isolates. This created a stressed environment for the bacterial cells. Due to the fact that the maintenance of plasmids is costly for a bacterial cell (Carroll and Wong, 2018), under such high concentrations of antibiotics, replication of these plasmids could be compromised despite the selection pressure for these plasmids being applied, thus reducing the number of copies of plasmids in each cell (Del Solar et al., 1998). As these isolates are of environmental origin, their antibiotic resistance mechanism cannot be attributed solely to the targeted resistance genes present on these plasmids.

Other chromosomal mediated resistance genes, such as *ampC* for β-lactams (Normark et al., 1986), or resistance mechanisms could be present that allow the cells to still exhibit resistance to the antibiotic, even if resistance plasmids are lost. For example, efflux pumps are known contributors to AMR (Alcalde-Rico et al., 2016). They are present in every cell and allow foreign and potentially toxic substances to be removed from the cell. As these pumps are present on the chromosome of bacterial cells and are not specific for the compound they export from the cell, efflux pumps could play a greater role in resistance to a particular antibiotic compared to antibiotic-specific genes present on the plasmid. Over-expression of some efflux pumps have been shown to reduce the expression of some DNA mismatch repair mechanisms, which could result in increased mutation rates in the bacterial cell (El Meouche and Dunlop, 2018). This could be relevant to sulfamethoxazole, which acts on the enzyme DHPS to inhibit the production of folate (Van Hoek et al., 2011). Over-expression of an efflux pump could lead to decreased mismatch repair mechanisms that may create a single nucleotide polymorphism (SNP) in the DHPS gene, rendering the target site for sulfamethoxazole inactive (Eliopoulos and Huovinen, 2001).

3.3.7 Antibiotic exposure effect on biofilm metabolism

The results below give an indication of the effect of chronic exposure to environmental concentrations of sulfamethoxazole and are a pipeline for future studies, Both Figure 3-14 and Figure 3-15 show similar profiles, indicating that environmental concentrations of sulfamethoxazole have a limited effect on the metabolism of a pure-culture biofilm over a 210-hour period. While there are slight differences between CO_2 production (µmol per hour) in the control and exposed biofilms (Figure 3-14 and 3-15, respectively), these can be attributed to the variation in ambient air in each CO_2 analyser. Each of the smaller snapshots of the larger CO_2 profile show the metabolic activity over subsequent 24-hour periods. Each value on the x-axis corresponds to the hour in the day. As the reactors were not operated at controlled temperature, the slight fluctuations are probably due to ambient temperature fluctuations as the air conditioning is turned off in the evenings, resulting in an initial higher temperature with increased biofilm metabolism. As the night got colder, a decrease in CO_2 production was observed, similar to earlier research that showed the pronounced effect of temperature on overall biofilm activity (Kroukamp and Wolfaardt, 2009). Temperature control may be considered in future experiments to eliminate this variable. In each case, the biofilms reached a steady state after about 160 hours. After exposing both the control and exposed biofilms to 512 µg/mℓ, which is three-fold higher than the MIC of the selected isolate, and 128 times higher than the environmental concentration of 4 ng/ℓ, an immediate increase in CO_2 production was observed.

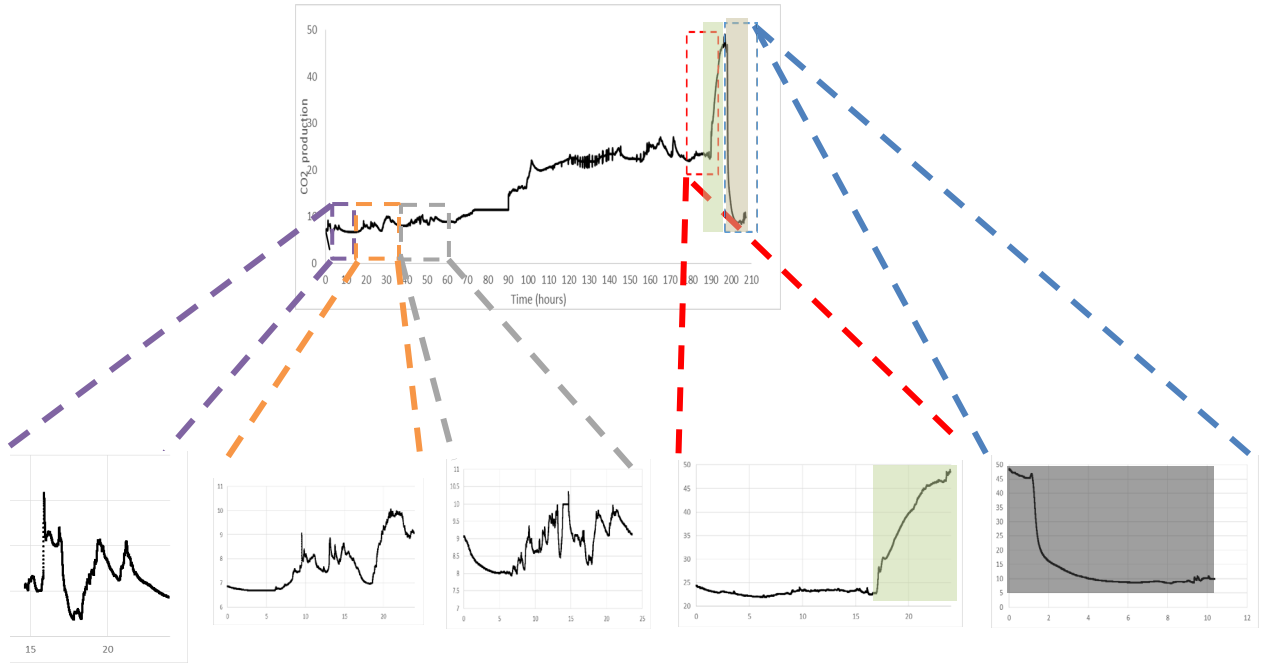

Figure 3-14: **Metabolic activity (CO_2 production (µmol per hour)) over an eight-day period in control reactor (no exposure to environmental concentrations of sulfamethoxazole in medium). Light grey shading indicates exposure of the biofilm to 512 µg/mℓ sulfamethoxazole and dark grey shading indicates exposure to 2,000 µg/mℓ sulfamethoxazole. Zoomed in profiles show each hour in a 24-hour period on the x axis rather than the hour of the experiment.**

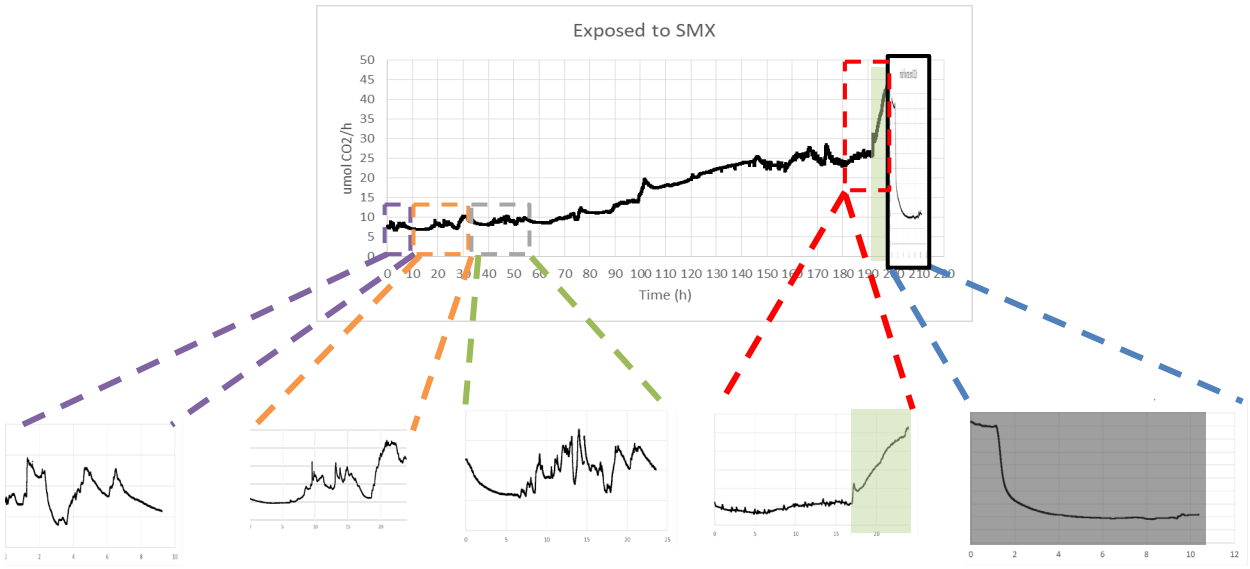

Figure 3-15: **Metabolic activity (CO_2 production (µmol per hour)) over an eight-day period when exposed to 4 ng/ℓ sulfamethoxazole. Light grey shading indicates exposure of the biofilm to 512 µg/mℓ sulfamethoxazole and dark grey shading indicates exposure to 2,000 µg/mℓ sulfamethoxazole. Zoomed in profiles show each hour in a 24-hour period on the x axis rather than the hour of the experiment.**

While a stress response was expected based on work previously done by Jackson et al. (2015), the biofilm in both exposed and control biofilms continued to increase in metabolism over a number of hours (light grey shading). More recent work by the same authors (Jackson et al., 2019) showed that the rapid increase in CO_2 output upon antibiotic exposure may be the result of increased metabolism to drive efflux pumps, where glucose and pyruvate addition aided a *Stenotrophomonas* and a *Pseudomonas* species, respectively, with concurrent increases in CO_2 production. Furthermore, various studies indicated that sulfamethoxazole is readily biodegraded by bacteria and, as a result, it is possible that the culture used in this experiment degrades this antibiotic to its advantage (Larcher and Yargeau, 2011). As sulfamethoxazole is solubilised in ethanol (Andrews, 2001), the latter could have provided additional energy for metabolism as reported earlier (Smith et al., 2004).

Upon the addition of 2,000 µg/mℓ (five-fold higher than the MIC of the culture and 500-fold higher than pre-exposure), the CO_2 production stabilised for one hour, after which it rapidly decreased until CO_2 production was slightly above that of ambient air. This indicates that the culture used was susceptible to the high concentration of sulfamethoxazole. Before the experiment was terminated, a slight increase in CO_2 production was observed, suggesting that the biofilm was recovering from the exposure, even without removing the sulfamethoxazole. Future experiments will incorporate further investigation of the effect of other antibiotics on metabolic activity, as well as collecting effluent samples at different time points after antibiotic exposure to perform qPCR and MIC testing to evaluate how the exposure affects the genotypic and phenotypic resistance profiles, including genes related to efflux pumps.

3.4 SUMMARY

It is evident that AMR organisms are abundant in WWTWs, regardless of the stage of the treatment process or the season. While high numbers of ARB were present in effluent after chlorination, the numbers were reduced compared to the influent. *Morganella morganii* was the most dominant species of the isolated bacteria and it was thought that, due to it having intrinsic resistance to certain antibiotics, it was selected for on antibiotic media. This indicated that selection bias is a concern when performing culture-based antibiotic resistance studies. As the identified species were obtained predominantly from the effluent, it can be expected that these organisms are transferred to surface waters, which may have serious implications if water is re-used for food production, recreation or other forms of exposure. The target resistance genes were found to decrease after going through the WWTW processes, with the plug-flow WWTW with a maturation tank prior to chlorination of the effluent having a better removal efficiency for both resistant bacteria and plasmid-mediated resistance genes. Determining the effect of antibiotics on biofilm metabolism needs further investigation, including the ability for a biofilm to recover after exposure to high antibiotic concentrations. Proposed future studies, using a CEMS approach, aim to understand the emergence of AMR in environmental waters.

CHAPTER 4: CONCLUSION AND RECOMMENDATIONS

4.1 OVERVIEW

The current report evaluated the potential of two treatment technologies to provide an added benefit for the reduction of CECs and EDCs, which included (1) the hybrid activated sludge treatment system (HYBACS®), and (2) a carbon-based electro-chemical oxidation treatment solution (CabECO) (Chapter 2). The report continued then to include a case study on the presence and fate of antibiotic resistance bacteria (ARBs) and targeted antibiotic resistant genes (ARGs) at two Western Cape WWTWs and a laboratory-based experimental setup for real-time evaluation of antimicrobial resistance using a carbon dioxide evolution measurement system (CEMS) (Chapter 3).

4.2 CASE STUDIES ON THE EFFICIENCY OF VARIOUS TECHNOLOGIES FOR THE REMOVAL OF PRIORITY MICROPOLLUTANTS IN WASTEWATER

The scoping study at the WWTW where the HYBACS® treatment system was implemented highlighted the variation in the presence and fate of selected CECs and EDCs during the different WWTW processes. Estimating the presence of estrogenic substances within the treatment system using the recombinant yeast oestrogen screen (YES) assay gained insights on the overall presence of chemical substances that may interfere with binding to the human oestrogen receptor alpha (hER-α) and showed a gradual decrease in total estrogenicity throughout the treatment process prior to the installation of the HYBACS® treatment system (May 2016 sampling campaign). However, similar results were not observed during a follow-up study after the installation of the HYBACS® treatment system (June 2018 sampling campaign), where an increase in total estrogenicity was observed in the overflow of the clarifier module and subsequently at the final effluent discharge after chlorination. During this sampling campaign, a high level of cytotoxicity was observed in the raw influent and the primary settlement tank (PST) overflow that was not experienced during the sampling campaign prior to the installation of the HYBACS® treatment system (May 2016 vs. June 2018). This raised the concern that some inert cytotoxic components in these treatment modules may have interfered with the biochemical pathways in the YES that could suppress the total estrogenicity response of the assay.

Moreover, it was observed that the WWTW experienced operational challenges during the June 2018 sampling period, in which a high load of bulking was observed on the surface of the secondary clarifiers, along with moderate turbidity in the final effluent. Whether these conditions may have resulted in a build-up of estrogenic analytes and/or desorption of recalcitrant analytes from sludge during the AS process is still unclear and need to be addressed in a follow-up study when the treatment plant is under normal operational conditions. Overall, the YES assay served as a sufficient first-tier scoping assay to evaluate a biochemical response of all chemical substances within the sample matrix that may interfere with a specific endocrine system pathway. Follow-up research should thus include a tiered approach to evaluate interference with specific endocrine system pathways using a combination of effect-based monitoring (EBM) tools for more refined intervention. This holds particular value to evaluate the quality of surface water systems that are affiliated with both WWTW discharge and areas that are being highly polluted by additional anthropogenic practices. Moreover, combining a variation of EBM assays with non-targeted chemical analyses to identify priority CECs and substances that are of high concern to exert adverse health effects will allow for more defined mitigation strategies to reduce the burden of pollution into freshwater ecosystems.

Although such non-targeted chemical analyses were not performed during the current studies, targeted chemical analyses of selected CECs over a 7-day sampling period (during June 2018) showed a high average reduction (> 75%) for the illicit drugs methamphetamine, cocaine and benzoylecgonine, the analgesic codeine, the anti-corrosion chemical benzotriazole and the lifestyle chemical caffeine. The antibiotic sulfamethoxazole, the herbicide atrazine, the illicit drug methaqualone, the anti-epileptic drug carbamazepine and the non-steroidal anti-inflammatory drug diclofenac showed moderate reduction (between 25% and 75%), whilst the two metabolic by-products of carbamazepine, namely carbamazepine-10,11-epoxide and 10,11-dihydro-10-hydroxycarbamazepine showed an average negative mass balance throughout the sampling period. Although the specific reason for such negative mass balances could not established during the current study, further intervention may include to explore whether this occurrence is due to deconjugation of the target CEC or possible metabolic back-transformation during the treatment processes (or a combination thereof). Moreover, it should be noted that the current removal calculation of CEC daily loads did not consider the hydraulic residence time (HRT) of the treatment system, and therefore merely compared the daily loads of the CECs at the incoming wastewater with the treated effluent on the same day. We propose that future mass balance estimations should consider the HRT of the treatment system that will determine more refined sampling approaches.

Evaluating the state of the river that was located next to the WWTW showed high levels of total estrogenicity and cytotoxicity in surface waters located upstream of the WWTW discharge location. This was also confirmed with the targeted CEC analysis that showed concentrations ranging in the low ng/L range (codeine, methamphetamine, cocaine, benzoylecgonine) to higher ng/L ranges (10,11-dihydro-10-hydroxy carbamazepine, methaqualone, sulfamethoxazole and caffeine) in surface waters located upstream of the WWTW discharge location, indicating alternative upstream pollution from human activities. Although more intervention is needed to further reduce the load of CECs in the WWTW discharge to an acceptable level that does not pose adverse health effects on sentinel aquatic organisms, it was still observed that the treated WWTW discharge aid to reduce the total estrogenicity and cytotoxicity, as well as the concentrations of some CECs in the recipient surface water location. These results thus highlighted the extent of pollution that may originate from alternative sources that are not associated with WWTW discharge – a common observation that is reported for many other areas in the South Africa and elsewhere in LMICs where sanitation services does not meet the demand of rising population growth.

The laboratory-based case study that included the electro-chemical oxidation treatment process (CabECO) showed a good performance to reduce most of the tested CECs, including carbamazepine, sulfamethoxazole, naproxen, bisphenol-S and acetaminophen, but to a lower extent to reduce the concentrations of benzotriazole, caffeine, atrazine and diclofenac. As both carbamazepine and sulfamethoxazole has been shown previously during in-house lab experiments to antagonise the binding of 17β-estradiol to the hER-α in a recombinant yeast anti-oestrogen screen (YAES) assay, this effect-based monitoring assay was also included to investigate whether such anti-estrogenicity caused by these two CECs also reduce during the ozonation treatment. The anti-estrogenic response of carbamazepine decreased over a period of 4 hours of treatment. For sulfamethoxazole, although a statistically significant decrease in the anti-estrogenic response was observed, an added increase in the estrogenicity response was observed after one minute of ozone treatment and thus warrants the need to investigate whether the formation of ozonated by-products could explain this occurrence. However, further ozone exposure after 4 hours led to a reduction in both estrogenic- and anti-estrogenic responses caused by sulfamethoxazole exposure. From these results, we could confirm that CabECO may serve as an effective system for the reduction of some CECs from water samples, as well as the reduction of carbamazepine/sulfamethoxazole-associated endocrine system responses, granted that the exposure conditions and treatment period is optimised. Future recommendations may include non-

targeted analysis to evaluate the development of ozonated by-products being formed by certain CECs and using EBM assays to investigate whether these degradation products pose a lesser- or more severe adverse health outcomes in the model toxicity assays.

The CabECO technology shows potential for application in a decentralised drinking water system for rural Southern African regions. The ozone generated by the electrochemical process is sufficient for the disinfection of various environmental samples, as well as the abatement of several micropollutants. Retention time is extremely important to include in the final design of this system. For the application of this system in a real-world scenario, it is important to consider that ozone concentration is reduced considerably, with a substantial portion of the ozone being depleted by various sources of organic matter other than the target compounds and microbes. Thus, CabECO appears better suited as a disinfectant or “polishing step” after water treatment. The standard procedures for generating potable water (flocculation, sedimentation, filtration) always demand the addition of a disinfectant post-treatment. Currently, chlorine is the go-to disinfectant, but has the disadvantage of being hazardous to human health and having a negative environmental impact. CabECO is an attractive alternative for the disinfection step in water treatment, or for polishing water with a micropollutant footprint. For disinfection, it has the disadvantage of lacking residuals, due to the instability of ozone. Thus, retreatment before use, after storage, would be recommended.

4.3 EVALUATING THE PRESENCE AND FATE OF ANTIBIOTIC-RESISTANT BACTERIA AND GENES DURING WASTEWATER TREATMENT

A range of antimicrobial-resistant bacteria (ARB) were detected throughout the treatment process at the two Western Cape WWTWs. Although such ARB was present in the final treated effluent (post-chlorination), the number of colony-forming units were highly reduced compared to the receiving raw influent. These results emphasise the need to examine the effect of environmental concentrations of antibiotics on the occurrence of resistant bacteria and further downstream waters for resistance genes, and to identify the possible mechanisms of gene transfer. Comparing the reduction of ARB between the two treatment works, it seems that a higher level of reduction was observed in a WWTW that is constructed as in a plug-flow activated sludge treatment system with a large maturation pond prior to disinfection of the treated wastewater, as opposed to the other WWTW that make use of a mixed-flow activated sludge treatment system and a maturation pond after final effluent disinfection.

Bacterial isolates that were cultured on selective media from the waster matrices showed much higher minimum inhibitory concentrations (MICs) for the antibiotics colistin, amoxicillin and sulfamethoxazole compared to their reported CLSI or EUCAST MIC resistance breakpoint criteria for pure laboratory strains of the same organism, which highlights that environmental pathogens have a much stronger tolerance to the selected antibiotics. Comparing MICs from isolates obtained from the wastewater matrices that were either enriched with- or without the addition of an antibiotic substance in the growth media showed inconsistent results over the entire range of identified organisms and highlight the complexity of drawing definite conclusions whether pre-exposure of environmental microbiota with environmentally-relevant concentrations of antibiotics does indeed cause an increase in ABR profiles that may be driven by various biotic- and/or abiotic factors that can vary on a spatio-temporal scale in the environment and in WWTWs. However, some consistent results were obtained where the same species of bacterial isolates were either pre-exposed to amoxicillin or directly enriched in media containing no antibiotics, where the calculated MIC of the pre-exposed culture was double that of the same species that was not enriched in the antibiotic, thus providing some suggestion that exposure to sub-MIC concentrations of an antibiotic in a batch culture results in a higher tolerance to the antibiotic to some extent.

Future recommendations to follow up on these methodologies will be to consider pre-exposure and/or enrichment using a combination of antibiotics that are similar to pharmaceutical prescriptions, for example exposure using a combination of sulfamethoxazole/trimethoprim or amoxicillin/clavunic acid, as environmental strains is shown to be less susceptible to a single antibiotic treatment.

Identification of bacterial species within the treatment systems showed that the Gram-negative opportunistic pathogen, *Morganella morganii*, was the most abundant species that was isolated from the wastewater effluent and could have been attributed to its intrinsic resistance to certain antibiotics that was used in the antibiotic isolation media. Although this may have caused a selection bias during the culture-based antibiotic resistance studies, the current methodology for isolation from wastewater proved to work well if future studies are focussed on isolating this opportunistic pathogen in environmental media. The rest of the most predominant species identified in the treated effluent included *Escherichia fergusonii*/*Shigella flexneri* (distinction was not possible), *Citrobacter freundii* and *Pseudomonas indoloxydans*, while other lesser frequent isolation of *Aeromonas caviae*, *Aeromonas hydrophila*, *Citrobacter murliniae*, *Aeromonas dhakensis*/*caviae*/*enteropelogenes*, *Providencia rettgeri* and *Pseudomonas aeruginosa* was identified. While a number of these identified species are members of normal gut microbiota, which is expected to be present in WWTWs, some are also opportunistic pathogens that may cause infections in immune-compromised individuals. Although, we do acknowledge that direct exposure or ingestion of treated effluent wastewater is unlikely to be a concern of public health, it cannot be excluded that some individuals or communities will not be exposed to contaminated surface waters in (peri)urban- or rural communities. Moreover, it is still needed to verify in future studies whether some pathogenic organisms will have the potential to transfer resistance mechanisms to other species and environments and thus serve as carriers of AMR titers.

The identification of target antibiotic-resistant genes (ARGs) within the wastewater systems showed to reduce in copy numbers from the raw influent to final treated effluent, although still at a detectable level in final treated effluent. The ARGs associated with sulfamethoxazole resistance (*sul1* and *sul2*) were the most predominant in this study and could be explained by the broad-spectrum usage of this drug. Identification of the ARGs from the wastewater isolates showed that the isolates with the highest *sul1* copies were all obtained from effluent wastewater, while those isolates with the highest *sul2* copies originated from influent wastewater. The identification of a colistin resistant gene (*mcr3*) was also identified and raised concern that inert environmental resistance is present in wastewater systems, as colistin is considered as a last-resort antibiotic drug for Gram-negative bacterial infections. Again, as for the ARB that was isolated in the study, the copy numbers of the ARGs were reduced more profoundly in the WWTW that include a maturation pond prior to disinfection of treated wastewater. However, the study did not focus on the exact treatment process that contribute towards a reduction of ARBs and ARGs within the two treatment systems and should thus be considered during follow-up studies.

Lastly, evaluation of sulfamethoxazole resistance profiles in a laboratory-scale study was done using a carbon dioxide evolution measurement system (CEMS) for real-time evaluation of the effect of antibiotic exposure to established surface-attached microbial consortiums (biofilms). This allowed for chronic exposure of the biofilm system to environmental concentrations of sulfamethoxazole that allowed for real-time observation of the metabolic change of the biofilm in the system. This methodology thus considered both surface-adhered and planktonic growth of the bacterial culture that are constantly exposed to an aqueous solution. The initial results showed that environmental concentrations of sulfamethoxazole have a limited effect on the metabolism of a pure-culture biofilm over a 210-hour period. The most prominent results from these experiments was highlighting the extent of antibiotic concentrations needed to reduce biofilm growth, which subsequently raises the issue of such a natural occurrence of biofilms that will have a much larger resistance profile than planktonic cells. This was further raised in the current study when the biofilm of the pure culture recovered from a five-fold higher

concentration of sulfamethoxazole than its determined MIC. On-going studies using this experimental setup is being done at the research facility, where follow-up studies will include the administration of antibiotic combinations similar to prescribed medications and/or co-exposure with inorganic substances such as heavy metals. Optimisation of the CEMS system for such investigation into anti-microbial resistance development, such as addressing ambient temperature fluctuations throughout the experimental period, optimal exposure media and period of antibiotic exposure will allow for more defined profiling of ABR development in a controlled laboratory setting.

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