SURVEILLANCE OF ESKAPE PATHOGENS IN WATER ENVIRONMENTS

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Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND AND RATIONALE

Antibiotic resistance is a growing concern in healthcare settings, as it makes the treatment of infections challenging and sometimes limited to a few remaining options. The importance and clinical significance of ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter cloacae*) has been recognised by the World Health Organisation as part of the list of "antibiotic-resistant priority pathogens" for an extended period as they cause serious healthcare-associated infections (HAIs) and have the ability to escape the action of one or more antibiotics and other biocides, including some of the last-resort antibiotics. Some of the infections that are caused by ESKAPE pathogens include bloodstream infections such as sepsis, a severe and life-threatening condition; community-acquired and hospital-acquired pneumonia; urinary tract infections (UTIs); surgical site infections; skin and soft tissue infections, such as cellulitis and abscesses; intra-abdominal infections, such as peritonitis; device-associated infections, and wound infections.

Measures to address antibiotic resistance include responsible antibiotic use in healthcare and agriculture, improved infection prevention and control measures, the development of new antibiotics, as well as implementation of surveillance programs to monitor and track resistant bacteria. The surveillance of ESKAPE pathogens and related antibiotic-resistant agents is pivotal in informing effective antibiotic use and controlling the spread of resistant pathogens within the local environment. Wastewater-based surveillance of antibiotic resistance has been recognised as a valuable tool for monitoring and studying the prevalence and dynamics of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) in communities. This approach involves analysing samples within an urban water cycle to detect and quantify ARB and ARGs. Wastewater treatment plants (WWTPs), in particular, can serve as media for the development and dissemination of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) as they receive wastewater from various sources, including households, industries, and healthcare facilities. This wastewater contains a diverse microbial community, including bacteria that may carry antibiotic resistance, as well as low levels of antibiotics and other pharmaceutical compounds, which can exert selective pressure on bacteria, favouring the survival and proliferation of ARB. While WWTP processes are effective at reducing the overall microbial load, they may not completely eliminate ARB and ARGs, resulting into the transfer into the natural water environment during effluent discharge.

ESKAPE pathogens have been suggested as indicators for antibiotic resistance surveillance in the environment as well as clinical settings as they originate in the clinical settings and eventually land in the environment, mostly through wastewater treatment plants. Disinfection, normally with chlorine, is used to inactivate microorganisms in the wastewater and one of the standard microbiological indicator parameters for disinfection efficiency is *E. coli*. In various studies, it has been shown that individual species that are part of the ESKAPE group, mainly originate from wastewater treatment plants (WWTPs) and may be involved in the spread of antibiotic resistance to the environment. This study aims to provide a more comprehensive overview of the sources and levels of ESKAPE pathogens entering WWTPs, the physical and chemical factors that support their survival or reduction during treatment, as well their transfer into the natural water environment. The antibiotic resistance data obtained from environmental surveillance studies will be compared to clinical data for relevance and the potential impact downstream will be evaluated. Based on this, a surveillance program for antibiotic resistance in the environment will be developed.

AIMS OF THE STUDY

There aims for this project were as follows:

1. Compile an overview on the importance and relevance of ESKAPE and Clostridia spp. in the global priority pathogen list (PPL) as indicators for antibiotic-resistance in the environment as part of the

One Health Approach.

2. Use qPCR for setting up a surveillance program for monitoring the sources and dissemination of ESKAPE strains and associated resistant genes within environments.
3. Evaluate the reduction potential of ESKAPE in selected wastewater treatment works.
4. Establish antibiotic resistance trends of selected ESKAPE pathogens in water environments and determine their clinical relevance.
5. Development of a surveillance program for antibiotic resistance in the environment.

METHODS

1. Sample collection

Environmental water samples were collected from nine full-scale wastewater treatment plants (WWTPs) as well as adjacent surface water resources located in the North West and Gauteng Provinces of South Africa. The samples collected for analysis included influent (incoming wastewater), effluent (treated wastewater), and surface water samples. All samples collected during this study were collected in differing sampling frequencies in accordance with the permission granted by each plant. The collected water samples were transported to the laboratory at the North-West University for analysis.

2. Determination of physico-chemical parameters, and levels of antimicrobials, ESKAPE pathogens and antibiotic-resistant genes in WWTP influent, effluent, and surface water

The physical properties (pH, temperature, total dissolved solids, and electrical conductivity) of the water samples were determined on-site using a calibrated Oakton PCStestrTM 35 waterproof field multi-parameter probe (Thermo Fisher scientific, US). Chemical parameters (nitrates, phosphates, and chemical oxygen demand) were measured in the laboratory using standard methods. The levels of selected antimicrobial compounds were determined using Ultra-performance liquid chromatography (UHPLC).

Quantitative Polymerase Chain Reaction (qPCR) was employed to ascertain the presence and abundance of ESKAPE pathogens and antibiotic resistance genes (ARGs) in the environmental water samples collected for this study. In addition, reference cultures of ESKAPE species were used as controls, and selective media aided in quantifying and isolating various ESKAPE bacteria. For the ESKAPE species identification, the V1-V9 region of the 16S rRNA gene was amplified using two commonly employed universal primers for bacterial identification. Sequence data were then cross-referenced with the BLAST databases to validate the organism's identities. Data processing and visualisation were conducted using Excel 2016 Version 16.0.6828.1019 for tables and certain graphs, while heat maps depicting resistance patterns of presumptive ESKAPE organisms were generated using Statistica version 14.0.1 published by TIBCO Software Inc. Furthermore, ggplot2 from the tidyverse package in R version 4.2.3 was used to construct figures. A redundancy analysis (RDA) was performed to create RDA plots, aiding in the comprehensive analysis of the dataset.

Antibiotic resistance phenotypes and patterns of isolates were determined using the antibiotic diffusion method. The zones around the antibiotic discs were measured and compared to standard values to ascertain the organism's susceptibility, intermediate resistance, or resistance to the antibiotics. Specific sets of antibiotics were used for each individual species.

Pathogenicity potential was determined using selected virulence factors. These are extracellular enzymes including haemolysin, lipase, proteinase and DNase.

For e-DNA water samples were filtered using 0.4 µm membrane filters (Pall, US) and the PowerWater Kit® was used to extract the DNA according to the manufacturer’s manual. qPCR reactions were performed using QuantStudio platform (Applied Biosystems, Thermo Fisher Scientific, USA). Standard curves were generated in ten-fold dilutions with positive control samples for each target gene containing known copies ranging from

20 000 to 2. TaqMan assays, utilising FAM fluorescent dyes, were used for quantification of the various AmpC β-lactamase gene groups. Standard curves were generated in ten-fold dilutions with positive control samples for each target gene containing known copies ranging from 2 to 20 000. Excel 2016 Version 16.0.6828.1019, Statistica version 14.0.1 and Canoco version 5.12 was used for statistical analysis of the results.

3. Evaluating the reduction and dissemination potential of ESKAPE pathogens and antibiotic-resistant genes during wastewater treatment

Methods for the various parameters discussed in terms of reduction in WWTPs as well as actual loads of antibiotic-resistant ESKAPE species, ARGs and antibiotic residues are described in Sections 1 and 2 above. The reduction potential was calculated by the following formula:

$$\text{Percentage Reduction} = ((\text{Influent} - \text{Effluent}) \div \text{Influent}) \times 100.$$

Load in the effluent was calculated by converting the levels of various ESKAPEs, antibiotic residues and ARGs to 1 L and then multiply by the average capacity of each plant.

4. Development of a surveillance program for antibiotic resistance in the environment

A desktop study was conducted to determine a suitable framework that could be applicable for the surveillance of antibiotic resistance in the environment.

RESULTS

1. Physico-chemical parameters and ESKAPE levels in WWTP influent, effluent and receiving water

The aim was to assess the water quality and the presence of ESKAPE bacteria. Values for the physico-chemical parameters were within expected and mostly acceptable ranges. Nutrient levels and physical conditions were favourable for the maintenance of the ESKAPE populations in sewage systems. There were many false positives. However, *Klebsiella* sp. were most frequently detected indicating potential contamination risk as well as suggesting that this could be a sentinel species in ESKAPE monitoring regimes. What was evident is that measuring physico-chemical parameters of influent and effluent is a valuable contribution when ESKAPE surveillance programs are considered.

2. Assessing antibiotic residues and antibiotic resistance trends of selected ESKAPE pathogens in water environments: Clinical relevance and qPCR monitoring of dissemination of antibiotic-resistant strains

Measurable concentrations of specific antibiotic residues were present in wastewater effluent and surrounding downstream and upstream water bodies. Antimicrobials, commonly used in medical, veterinary, and agricultural practices, can find their way into wastewater, and subsequently impact downstream aquatic ecosystems and potentially also human health. Monitoring antimicrobial residues in wastewater is important for various reasons. The data assists in source identification, management, and policy formulation, empowering decision-makers, and wastewater operators to take informed actions toward curbing antimicrobial pollution.

The antibiotic susceptibility data indicated that resistance to ampicillin was prevalent among ESKAPE species. Furthermore, the study explored the pathogenicity potential of environmental isolates of ESKAPE species demonstrating that they are potential pathogens. RDA analysis demonstrated correlations of the ESKAPE species, characteristics, and compartments of the two WWTPs C and E.

Quantitative analysis of gene copy numbers provided detailed insights into the prevalence and persistence of ESKAPE pathogens across different WWTPs. Some plants exhibited higher levels of specific ESKAPE species in effluent, indicating incomplete removal during treatment. Antibiotic residues found in the effluent and

upstream and downstream sites could be responsible for maintaining antibiotic resistant ESKAPE species in the system. qPCR data demonstrate that overall, the WWTP effluent had higher levels of antibiotic resistance genes compared to up and downstream sites.

The data also highlighted the overlap between the ESKAPE species and characteristics coming from clinical and environmental sources. This supported the notion that ESKAPE species from clinical settings (clinics, hospitals) and households are transported via sewage to WWTPs. Even though reduction of such pathogens occurs during wastewater treatment, these ESKAPE pathogens land in receiving environmental water.

3. Dissemination and potential impacts of WWTP effluent or receiving water bodies and evaluating indicative removal of selected antibiotic strains and genes in wastewater

The potential of the WWTPs to reduce, nutrients, ESKAPEs, antibiotic residues and antibiotic resistance genes were assessed using data obtained using culturing and qPCR. Between 20 and close to 100% of these nutrients, pollutants (antibiotic residues and ARGs) and antibiotic resistant ESKAPE species were removed. What is of major concern is even with this measured removal, the dispersal of the nutrients, pathogens, ARGs as well as antimicrobial residues is of concern. The levels of various ESKAPE pathogens, antimicrobial residues and ARGs that are WWTP effluent are deposited into the downstream environments of various WWTPs are enormous. The total amounts of several antibiotic residues released into downstream water sources are also huge (into several 100 grams), yet, due to the amount of effluent the concentrations these pollutants and DNA are extremely low. Impacts of these on the ecosystems are currently undetermined but attention to it must be consider.

The loads of the various ARGs and ESKAPE pathogens were consistently higher in the influent than what exited the WWTPs. This observation is important since, if WWTPS are not operational or are poorly operated, much of the high levels of pollutants would directly land in the receiving water and could have detrimental impacts on such ecosystems. At all the plants the downstream water is used for various purposes, including agriculture (irrigation and livestock watering), drinking water production, recreation, and religious purposes. Water polluted with antibiotic resistant ESKAPE pathogens containing clinically relevant ARGs as well as antimicrobial residues will likely have serious implications for the user of this water.

4. Development of a surveillance program for antibiotic resistance in the environment

A comprehensive surveillance framework that not only monitors ESKAPE species and AMR within clinical settings, but also extends its scope within a One-Health context. Here a surveillance program capable of bridging critical knowledge gaps is proposed. The four key monitoring objectives – monitoring the presence and prevalence of ESKAPE pathogens and antibiotic resistance genes (ARGs), quantifying treatment evasion, assessing removal efficiencies, and evaluating release into the environment – form the foundation of this suggested approach. The proposed surveillance program should not be seen as an endpoint but a starting point for further research, collaboration and implementation. Interdisciplinary collaboration between healthcare, environmental science, and policy-making realms will be essential to translate this surveillance program into tangible actions and impactful outcomes.

RECOMMENDATIONS

Based on the findings, the following is recommended:

- National surveillance systems to monitor one or two sentinel ESKAPE species at major WWTPs, using culture-based and culture-independent methods such as that described in this study should provide valuable baseline data on distribution of ESKAPEs and ARGs in WWTP aquatic systems. COVID-19 surveillance demonstrated that a single marker ensured that sampling and analysis could be standardised across samples, analysis sites and data processing. The results from the present study

had relatively complete data sets for *Klebsiella pneumonia* which corresponded to data from the other species, using both culture dependant and culture independent methods. This species perhaps this should be considered as a sentinel species for the monitoring of EKSAPE species in the environment.

- The antibiotic resistant patterns among all the ESKAPE overlapped with the antibiotic residues detected, demonstrating mostly resistance to β-lactam antibiotics. Clinically relevant ARGs responsible resistance to various generations of β-lactam antibiotics, were detected and quantified in influent, effluent and downstream receiving waters.

- For culture, independent methods Oxford Nanopore Technologies (ONT) should be considered. In the case of the present study, qPCR was used and was at times inconsistent. This was potentially due to the nature of the technology, equipment and the skill levels required. Furthermore, several specific markers were targeted simultaneously, making this approach a bit complex, unlike the approach that was used for COVID-19. The ONT technologies are portable and skill sets required may not be as intensive as with qPCR.

- Whole Genome Sequencing of corresponding species (e.g. *K. pneumonia* and *S. aureus*) from both environmental and clinical settings would provide insights into the genomic dynamics in these two settings. Such data will be invaluable when interventions to stem the tide of antibiotic resistance dissemination into environmental waters are considered. The present study provided evidence of the overlap of phenotypic antibiotic resistant characteristics of *Klebsiella* sp. from environmental and clinical setting. What is currently undetermined is the impacts of downstream water on irrigation and particularly livestock watering.

- It is also very important that findings from studies such as this one should be circulated to the relevant stakeholders, including the medical fraternity, agricultural sector, abattoir owners and managers, feedlot owners, relevant ministries, etc. This data from the present study provides information to linking pathogens in the environment (sewage as well as environmental water) possibly back to clinical settings. This data calls for interventions such as pre-treatment of wastewater at high-risk sites (hospitals, clinics, agricultural settings, etc.) must be made. This study demonstrated that actual levels of pollutants are enormous and cannot be ignored. WWTPs that are dysfunctional or are poorly managed not only contribute to pollution of aquatic ecosystems, but this scenario is actively contributing to the spread of the antimicrobial resistant burden in the human population. This is in contradiction to the Constitution of the Republic of South Africa.

- Efforts should be made to have a national repository, and sequencing facility/Centre for Environmental AMR.

- Antimicrobial resistance data must be made available to communities in such a manner that would make it easily understandable to all members. This requires dedicated knowledgeable staff.

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1 BACKGROUND

1.1 INTRODUCTION

Antibiotic resistance is a serious public health crisis that requires immediate attention and concerted efforts from governments, healthcare providers, researchers, and the public to mitigate its impact and ensure the continued effectiveness of antibiotics. In 2017, the World Health Organization (WHO), through member states developed a global priority list of 12 bacteria against which new antibiotics are urgently needed due to multidrug resistance. Among these were the ESKAPE pathogens, i.e. *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* cloacae. (ESKAPE). ESKAPE pathogens have been identified as a priority in surveillance programmes, research, and development for new drug targets (Ramsamy *et al.,* 2018). There is a considerable database on the surveillance, research and development aspects on ESKAPE pathogens in the clinical setting, particularly with respect to the hospital-acquired infections caused by these species. The critical importance and clinical significance of ESKAPE pathogens have been recognized for an extended period. They are known for serious nosocomial infections and have the ability to escape the action of one or more antibiotics and other biocides, including some of the last-resort antibiotics (Nyasulu *et al.,* 2012). Some of the infections that are caused by ESKAPE pathogens include bloodstream infections such as sepsis, a severe and life-threatening condition; community-acquired and hospital-acquired pneumonia; urinary tract infections (UTIs); surgical site infections; skin and soft tissue infections, such as cellulitis and abscesses; intra-abdominal infections, such as peritonitis; device-associated infections, and wound infections (WHO, 2017). (Nyasulu *et al.,* 2012).

For example, one of the pathogens (drug resistant *Klebsiella pneumonia*) is causing serious health challenges in neonatal units, with fatalities reported in some of the cases (Ballot *et al.,* 2019). What is of concern is that this pathogen is resistant to carbapenems which are last resort antibiotics. Thus, these pathogens have been suggested as indicators for antibiotic resistance in the environment (Nyasulu et al., 2012). Sources of these antibiotic resistant pathogens as well as associated genetic determinants are thus major emerging public health threats as is demonstrated by the mentioned example. A number of key sources and factors contribute to the emergence and spread of antibiotic resistance, and they include:

- Bacteria can naturally acquire mutations in their DNA over time, some of which confer resistance to antibiotics.
- Bacteria can acquire resistance through horizontal gene transfer, a mechanism that allows resistance genes to be shared among different bacterial species, contributing to the spread of antibiotic resistance.
- Overuse and misuse of antibiotics, result in increased opportunities for bacteria to develop resistance.
- Environmental reservoirs, serve as the medium for the development and spread of antibiotic resistance. This is because bacteria found in the environment can both develop resistance and exchange genetic material with other bacteria, including human pathogens and potentially transfer resistance genes.
- Resistant bacteria can spread within communities through person-to-person transmission or contact with contaminated surfaces.

Thus, the development and implementation of strategies against the spread of antibiotic resistance, the genetic determinants should also be made a priority (Pruden, 2014; Bergeron *et al.,* 2015) and not only the occurrence of the pathogens alone. The environment as a potential source and reservoir of pathogens has, for a long time, been ignored. Recently, antibiotic resistant bacteria (ARB) and antibiotic resistant determinants (ARDs) have also been identified as major emerging public health threats. Since the One Health concept of the World Health Organization was introduced, the spread of antibiotic resistance through interactions between animals, plants, people and the shared environment has become more recognized. Even so, a major focus was still only on the human and animal health environments. However, these pathogens can also make their way into the natural environment through

poorly treated urban wastewater discharges. The WWTPs had also been implicated as a reservoir for various pathogenic, opportunistic pathogenic antibiotic resistance bacteria and antibiotic resistance genes. Wastewater treatment plants (WWTPs), in particular, serve as both the media for the development and dissemination of ARBs and antibiotic resistance genes (ARGs) as they receive wastewater from various sources, including households, industries, and healthcare facilities. This wastewater contains a diverse microbial community, including bacteria that may carry antibiotic resistance, as well as low levels of antibiotics and other pharmaceutical compounds, which can exert selective pressure on bacteria, favouring the survival and proliferation of ARB. While WWTP processes are effective at reducing the overall microbial load, they may not completely eliminate ARB and ARGs, resulting into the transfer into the natural water environment during effluent discharge. WWTPs have also been implicated as a reservoir for various pathogenic, opportunistic pathogenic microorganisms and antimicrobial resistant agents. However, there is still paucity of data on the sources, survival and dissemination pathways of antibiotic resistant pathogens in the environment.

An in-depth analysis of ESKAPE pathogens from the environment as well as their characteristics will provide information on their incidence prevalence, potential sources and pathways. This information will be critical when intervention and monitoring programmes are set up and implemented on local, regional and national scales (WHO, 2015). The latter programmes will, in the long run, assist with optimising empiric and targeted antibiotic prescription choices (DoH, 2018). Report on Surveillance by the World Health Organization indicated that AMR is increasing in Africa (WHO, 2019). Despite numerous studies in the clinical setting having investigated ESKAPE pathogens, minimal data is available regarding their presence in the environment, in South Africa (Founou *et al.,* 2019; Ramsamy *et al.,* 2018). The WHO stressed that limited accurate and reliable data are limited and as a result, the true extent of the problem is unknown (WHO, 2015). Thus, this study will be of importance as data generated can add great contributions to the WHO Global resistance surveillance system (GLASS; WHO, 2019). This will improve the country's ability to track and monitor resistance across sectors. Thus, the data generated from this project will strengthen the country's surveillance capacities, guide future monitoring programmes and assist the relevant directorates in making decisions regarding antimicrobial prescription and their waste dispersal to ensure sustainable water reuse.

A comparative analysis between the environmental ESKAPE pathogens and information provided by institutions such as the NICD on clinical ESKAPE pathogens will assist in strengthening knowledge regarding the clinical relevance of the strains detected in the environment, as well as dissemination of resistant organisms from one environment to the other. This information will be used to develop a surveillance program for antibiotic resistance in the environment. Furthermore, the information generated by this comparison can aid with the development of new policies and regulations in order to lessen and limit the dissemination of resistant organisms and antimicrobial residues from the clinical to environmental settings inevitably landing in the food chain (WHO, 2015). Additionally, the analysis and comparison of the whole genomes of ESKAPE pathogens from the clinical and environmental settings could potentially lead to a methodology that enables the rapid detection of resistant ESKAPE pathogens. This could then enable reliable, rapid and easy surveillance which will ensure improved water and sanitation as well as biosafety (DoH, 2018; Ramsamy *et al.,* 2018).

1.2 AIMS OF THE STUDY

The aims of the project were as follows:

1. Compile an overview on the importance and relevance of ESKAPE and Clostridia spp in the global priority pathogen list (PPL) as indicators for antibiotic-resistance in the environment as part of the One Health Approach
2. Use qPCR for setting up a for monitoring the sources and dissemination of ESKAPE strains and associated resistant genes within environments
3. Evaluate the reduction potential of ESKAPE in selected wastewater treatment works

4. Establish antibiotic resistance trends of selected ESKAPE pathogens in water environments and determine their clinical relevance
5. Development of a surveillance program for antibiotic resistance in the environment

1.3 LAYOUT OF THE REPORT

Chapter 1 provides an introduction, rationale and aims of the study.
Chapter 2 addresses Aim 1 and provides an overview of the importance of ESKAPE species and *Clostridia* spp., in the global pathogen list (PPL) was provided using a literature review. Furthermore, the use of these species as indicators for antibiotic resistance in the environment as part of the One Health Approach was provided. This set the scene for the research that followed.

Chapter 3 provides information on the study sites, sampling regime and preliminary analysis of the samples to determine physico-chemical water quality characteristics and levels of antimicrobial residues.

Chapters 4-7 addresses Aims 2-5, and presentation of the data does not precisely follow the chronological order of the project aims. The approach was mostly integrated.

The summary of findings and conclusions are presented in Chapter 8.

2 ESKAPE AND CLOSTRIDIA SPP AS INDICATORS FOR ANTIBIOTIC RESISTANCE WITHIN THE CONTEXT OF A ONE HEALTH APPROACH – AN OVERVIEW

2.1 INTRODUCTION

Extensive research conducted over decades has indicated that South Africa is considered a semi-arid country with high water stress (40-60%) influenced by low volumes of rainfall with an average ranging from 500 mm per annum as well as high evaporation (average of 1700 mm per annum) (Mulamattathil, 2014; Adewumi *et al.,* 2010). Furthermore, the quality of the limited freshwater resources is heavily impacted by pollution and the destruction of river catchments. According to the Bill of Rights contained in Chapter 2 of the Constitution of South Africa, every human has a right to access safe water and proper sanitation as they are essential elements of human development (Osode, 2007). In water-scarce South Africa, the use of alternative measures to meet the increasing demand for water supply is a common practice. These alternative sources include groundwater, seawater as well as wastewater for water sources. However, the use of alternative water sources, such as wastewater, necessitates careful monitoring of various pollutants to ensure the safety and sustainability of the practice.

According to Van der Merwe-Botha and Manus (2011) wastewater is considered the first barrier in a multi barrier system of ensuring safe drinking water quality. As a result, its composition and adequate treatment is of critical importance. Wastewater from urban areas and industrial facilities often contains a complex mixture of chemicals, including active pharmaceutical ingredients (APIs), excipients, and degradation products from pharmaceuticals. This indicates that wastewater is becoming increasingly complex, and can be challenging to treat effectively, as different compounds may require different treatment methods, with a potential of causing a compounded environmental problem if not adequately treated (LGSETA, 2015). Wastewaters can also be utilized to determine the different degrees of environmental nuisance and contamination hazard due to their chemical and microbiological characteristics (Bohdziewicz and Sroca, 2005). Apollo et al. (2013) indicated that some of the pollutants that pose a threat/ influence the quality of wastewater and are of great concern are encountered in pharmaceutical, distillery, molasses as well as hospital wastewater.

In well developed countries, their wastewater treatment plants, and management strategies implemented are accompanied by advanced technologies and are adequate to handle newly challenges that arise in wastewater evolution. However, in developing countries such as South Africa, wastewater treatment facilities often lack advanced water purification technologies and/or are not managed properly to handle the rapidly increasing pollutant loads due to factors such as urbanization, increasing populations and over production of wastewater that is generated from household units and hospitals (LGSETA, 2015). In a UNEP report (UNEP, 2002) it is estimated that less than 5% of all wastewaters in developing countries receive any treatment before discharge into the environment.

Therefore, wastewater can serve as a significant source of environmental pollution, and particularly a medium for the development and spread of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs). The wastewater entering a WWTP, can be made up of wastewater collected from households, discharges from industry as well as runoff from land activities. If not adequately treated, it may still contain low levels of ARBs and ARGs. The discharge of such poorly treated wastewater into receiving waters can lead to the contamination of surface water and groundwater with ARB and ARGs, further exacerbating the environmental dissemination of resistance. The interconnectedness of urban, agricultural, and environmental systems means that resistant bacteria and genes can move between these domains. Taking a One Health approach, which recognizes the interconnectedness of human, animal, and environmental health, is essential for a holistic strategy to address

antibiotic resistance arising from wastewater sources. In particular, it is important that wastewater treatment processes are enhanced to reduce the presence of ARB and ARGs in the final treated effluent to mitigate the contribution of wastewater to the dissemination of antibiotic resistance. Regular monitoring of wastewater and receiving waters can provide valuable data on resistance patterns and hotspots.

2.2 CONTRIBUTION OF WASTEWATER TO ENVIRONMENTAL POLLUTION

2.2.1 Overview

Wastewaters are known for their ability to show varying stages of environmental nuisance and contamination hazards due to their chemical and microbiological characteristics (Bohdziewicz and Sroka, 2005). According to WHO (2001) WWTPs aid in the removal of (i) Pathogens, (ii) Harmful hazardous, (iii) Nutrients and (iv) Oxygen consuming organic matter. Table 2.1 shows some of the chemicals of health concern found in untreated municipal excreta and wastewater (ATSDR, 2000; Osode, 2007).

Table 2.1: Chemicals of health concern found in untreated municipal excreta and wastewater (ATSDR, 2000; Osode, 2007).

Chemical	Health effects
Halogenated compounds	Skin irritations, nausea, embryo/fetotoxic
Chloroform	Nervous system damage, cancer
DDT	Liver, kidney and blood damage
Di and tri-chlorobenzenes	
Heavy Metals	
Arsenic	Gastrointestinal, skin and nerve damage, cancer
Cadmium	Gastrointestinal, kidney and lung damage
Lead	Nervous and immune system, kidney damage, fetotoxic
Inorganic Chemicals	
Cyanide	Brain and heart damage, shortness of breath, death
Hydrogen Sulphate	Nausea, vomiting, mucous membrane irritation
Nitrate	Methemoglobinemia
Nutrients	
Nitrogen	Cause eutrophication and facilitates the growth of toxin producing cyanobacteria and other harmful algae
Phosphorous	
Organic Chemicals	
Benzene	Anaemia, dizziness, leukaemia
Phenol	Irritation of skin, eyes, gastrointestinal tract
Xylene	Confusion, dizziness, memory loss, embryo/fetotoxic
Other Chemicals	Reproductive/developmental effects in wildlife, various potential effects in humans
Endocrine disruptors and pharmaceuticals	

The treatment and conservation of wastewater is of importance as it impacts directly on human health and the environment as well as indirectly on the downstream water purification systems. According to the Water Research Report (2018) the department conducted an assessment in 2016 on the South African municipal owned wastewater treatment works. The assessment was conducted on 824 wastewater treatment plants and 152 municipalities throughout the county. The findings revealed that the majority of those plants, about 259 plants were of high risk, 218 plants were of medium risk, 212 plants were at critical risks and lastly 135 plants were of low risk. Due to lack of proper maintenance of these wastewater treatment plants, the quality of effluent released into the environment has deteriorated. This however is a problem, as it poses a serious risk to receiving water and also plays a negative role on water quality as well as human/animal health, enabling the spread of diseases such as *E. coli*, hepatitis A and diarrhoea (Water Research Report, 2018). WRC (2018) also highlighted that the release of poorly treated or untreated wastewater into the environment decreases the ability of the environment to provide the benefits required by society, therefore negatively impacting on the availability of clean and safe water for urban, agricultural, industrial and primary use. Many wastewater treatments plants in South Africa face several challenges and according to Water Research Report (2018) they often include (i) Imbalance between development of new infrastructure vs existing infrastructure, (ii) Capacity and demand management, (iii) Financial constraints, (iv) Knowledge and expertise and (v) Inappropriate technology.

2.2.2 Chemical contaminants in wastewater

WWTPs play a critical role in mitigating environmental pollution and can serve as a barrier and/or hotspot as they are somehow “middlemen” between wastes coming from various sources and the environment. According to Blanchard et al. (2001) the chemical quality variables that are regarded as wastewater contaminants include chemical oxygen demand (COD), suspended solids, ammonia nitrogen, phosphorus, salinity as well as excess nutrients. The latter contaminants originate from sources such as domestic wastewater, the urban runoff and industrial wastewater (Katsoyiannisi and Samara, 2004). Wastewater derived from domestic purposes (household wastewater) can be divided into two terms, known as black water (wastewater originating from the toilets) and greywater (wastewater from the kitchen, bathing/showering and laundry). According to UNEP (2002) blackwater has high content of solids and contributes highly to the amount of nutrients (nitrogen and phosphorous) that are found in wastewater. Whereas greywater is known to also contribute high solids and grease. The excess presence of chemical contaminants in wastewater that is released into the environment can have significant negative health impacts as indicated in Table 2.1.

2.2.3 Pathogens in wastewater

Apart from chemical contaminants, microbial contaminants/ presence of faecal indicators also plays a significant part in the questionable quality of wastewater. Simpson and Charles (2000) as well as Momba and Mfenyana (2005) highlighted that WWTPs release a huge amount of both pollution and faecal indicator pathogenic microorganisms that often lead to the deterioration of wastewater quality sources. The inability of WWTPs to properly treat, manage and remove microorganisms in wastewater results in adverse health and environmental effects (WHO, 2001). Wastewater resulting in excretion from humans and animals has been implicated severally in the transmission of many infectious diseases such as Cholera, typhoid, hepatitis as well as cryptosporidiosis (WHO, 2001). According to the US EPA (1992) high numbers of pathogens are often reported in raw wastewater worldwide. Some microbial contaminants are discussed below and in Table 2.2. There is an abundance of pathogens that can be found in the wastewater systems; wastewater contains multiple bacterial and viral pathogens (Girones *et al.*, 2010; Lu *et al.*, 2015; La Rosa *et al.*, 2020). The discharge of those pathogens into the environment can cause health implications in humans, thus the wastewater must be treated properly so that no pathogens are expelled into the environment (Lu *et al.*, 2015).

Several sources contribute to the pathogens that can be found within the influent of the WWTPs. Stormwater, urban wastewater, agricultural wastewater and hospital wastewater are all contributors of pathogens that are found within the influent of WWTPs (Ahmed *et al.*, 2020; Garrido-Cardenas *et al.*, 2017; Moges *et al.*, 2014). Stormwater contains pathogens because of nonpoint and point source contamination before entering WWTPs, this can cause issues because pathogens are found within the stormwater that flows directly into the environment without moving through the WWTPs it can cause health implications in some areas (Ahmed *et al.*, 2020). Urban and agricultural wastewater is known to contain pathogens thus the WWTPs that receive wastewater from agricultural activities and urban settings will contain pathogens (Ibekwe *et al.*, 2013). Hospitals are large contributors of pathogens that are located at the WWTPs, this is problematic because of the overuse of antibiotics the pathogens that end up at the WWTPs could be highly antibiotic resistant (Moges *et al.*, 2014).

Table 2.2: Pathogens found in untreated municipal wastewater (National Research Council, 1998; Osode, 2007).

Agent	Disease
Bacteria	
Campylobacter	Gastroenteritis, long term sequelae
Escherichia coli	Gastroenteritis
E. coli 0157:H7	Bloody diarrhoea, haemolytic uremic syndrome
Helicobacter pylori	Abdominal pain, peptic ulcers, gastric cancer
Legionella pneumophila	Legionnaire's disease
Leptospira (spp.)	Leptospirosis
Salmonella (spp.)	Salmonellosis, long term sequelae, typhoid fever
Shigella (spp.)	Shigellosis (dysentery), long term sequelae
Vibrio cholerae	Cholera
Yersinia enterocolitica	Yersiniosis, long term sequelae
Clostridium perfringens	Skin and tissue infections
Staphylococcus sp.	Boils, impetigo, food poisoning, cellulitis
Enterobacter sp.	Bacteraemia, lower respiratory tract infections
Enterococcus sp.	Endocarditis, urinary tract infections, prostatitis
Klebsiella sp.	Pneumonia, bloodstream infections, wound
Protozoa	
Balantidium coli	Balantidiasis (dysentery)
Cryptosporidium parvum	Cryptosporidiosis, diarrhoea
Cyclospora cayetanensis	Persistent diarrhoea
Entamoeba histolytica	Amoebiasis (amoebic dysentery)
Giardia lamblia	Giardiasis
Viruses	
Adenovirus (many types)	Respiratory disease, eye infections
Notovirus (several types)	Gastroenteritis
Enteroviruses (various types)	Gastroenteritis
● Coxsackie A	Herpangina, septic meningitis, respiratory illness
● Coxsackie B	Fever, paralysis, respiratory, heart and kidney disease
Norwalk virus	Gastroenteritis

Agent	Disease
Hepatitis A and E virus	Infections hepatitis
Parvovirus (several types)	Gastroenteritis
Rotavirus (Groups)	Gastroenteritis

2.2.3.1 Pathogenic bacteria

Human bacterial pathogens form the minority of the microbial community at WWTPs, thus they need to be cultivated for detection. The most common bacterial indicators of faecal contamination are total coliforms, faecal coliforms and *E. coli* because they are easy to detect and enumerate. The use of these indicator organisms are widely used to assess the quality of treated wastewater and the health risks associated with the water (Fong and Lipp, 2005). Bacterial gastroenteritis across the world is mostly caused by *Campylobacter spp.*, the species of *Campylobacter* that commonly infect humans are *C. jejuni* and *C. coli* (Farhadkhani *et al.*, 2020). *Campylobacter* infections can lead to irritable bowel syndrome, Guillain-Barre Syndrome and reactive arthritis. Improper treatment of wastewater and irrigation with that same treated wastewater can lead to *Campylobacter* infections in humans by consuming the water or interacting with the soil that has been irrigated with the treated wastewater (Farhadkhani *et al.*, 2020). There are other bacteria other than indicator organisms that are more resistant to disinfectants a typical example *Helicobacter pylori* (Hortelano *et al.*, 2020). Because of the increased resistance to disinfectants like chlorine the *Helicobacter spp.* can evade the disinfection step and be expelled into the environment. *Helicobacter* is known to cause gastric illnesses and gastric cancer, *H. pylori* are mostly known for causing peptic ulcers, duodenal ulcers, gastritis and gastric cancer (Hortelano *et al.*, 2020).

2.2.3.2 Viruses

Human enteric viruses have previously been linked with the detection of faecal bacteria, but research has shown that there isn't always a relationship between faecal indicator bacteria and human enteric viruses because in some instances enteric viruses have been encountered in water meeting the requirements of indicator bacteria (Fong and Lipp, 2005). The survival of these viruses in the environment is dependent on environmental factors like pH, heat and moisture (Bosch *et al.*, 2006). The most studied Enteric viruses include *Picornaviridae* (Hepatitis and Enterovirus), *Caliciviridae* (Norovirus and Sampovirus), *Adenoviridae* (Adenovirus) and *Reoviridae* (Rotavirus and Reovirus) (Laconelli *et al.*, 2017). Human enteric viruses are one of the largest causes of gastroenteritis (GE) worldwide (Laconelli *et al.*, 2017; Osunmakinde *et al.*, 2018). These infections aren't limited to GE, enteric viruses can also cause hepatitis, conjunctivitis, respiratory infections aseptic meningitis, paralysis and encephalitis (Laconelli *et al.*, 2017; Osunmakinde *et al.*, 2018). Viral infections are prone to manifest in individuals that are immune compromised, in children and the elderly (Laconelli *et al.*, 2017). Hepatitis A virus and noroviruses are some of the leading pathogens that are associated with waterborne infections and may lead to illnesses like acute hepatitis (Walker *et al.*, 2020). Noroviruses are one of the leading causes of gastroenteritis and rotaviruses are some of the leading causes of gastroenteritis in children and infants (Walker *et al.*, 2020). These pathogens are excreted by humans in their urine and faeces, at WWTP with improper treatment methods these viruses can evade the treatments and cause infections in humans. The most common form of transmission of enteric viruses is the faecal-oral route (Bosch *et al.*, 2006; Walker *et al.*, 2020).

Viral indicators like coliphages have been used to identify wastewater contamination that may potentially contain pathogenic enteric viruses (Barrios *et al.*, 2018; Walker *et al.*, 2020). Coliphages can be used as an indicator of pathogenic enteric viruses because they have a very similar structure and they exhibit very similar viral properties to enteric viruses (Barrios *et al.*, 2018). In other studies, it has also been found that pepper mild mottle virus and the tobacco mosaic virus are the two most prevalent viruses that are found with no variation in abundance during different seasons, thus they can be used to assess the effectiveness of enteric virus removal in WWTPs (Tandukar

et al., 2020). FRNA bacteriophages infect Gram-negative bacteria, thus it can be used as a viral indicator of faecal contamination because they need a host cell to reproduce and cannot reproduce individually in the environment (Barrios *et al.*, 2018). FRNA bacteriophages also have similarities with human RNA viruses like hepatitis A and E, enteroviruses and astroviruses thus they can be used as indicators of enteric viruses and bacteria (Arredondo-Hernandez *et al.*, 2017; Barrios *et al.*, 2018).

2.2.4 Importance of personal protective equipment during wastewater treatment

Personal protective equipment (PPE) is important to protect wastewater workers since wastewater contains a variety of pathogenic organisms such as bacteria, viruses, fungus, worms and protozoa (Albatanony, 2011). The wastewater treatment process can cause microorganisms, toxins and metabolites to be released into the air and form bioaerosols (Michałkiewicz, 2019). Bioaerosols can contain hazardous microorganisms such as pathogenic bacteria, fungi and viruses (Han, 2020). Some of the airborne pathogen strains that are a potential health risk to wastewater workers include *Acinetobacter sp., Pseudomonas sp., Enterococcus sp., Bacillus sp., and Escherichia coli* (Yang, 2018; Lu, 2020). Inhalation is the main pathway for exposure to these bioaerosols, thus strict control measures should be implemented to reduce potential infections (Yang, 2018). Airborne bacteria can attach to surfaces of small particles and can be carried by the wind. This can cause an additional health risk to residents of nearby areas (Yang, 2018).

Wastewater workers are exposed to pathogens through hand-to-mouth contact, mucous membrane contamination and inhalation of aerosols if the correct personal protective equipment are not used (Albatanony, 2011; Yang, 2020). There is an increased risk for wastewater workers for developing air way symptoms (chronic bronchitis and toxic pneumonitis), central nervous system symptoms (headache and tiredness), gastrointestinal symptoms (jaundice and abdominal pain) and eye irritation symptoms (conjunctivitis) (Albatanony, 2011; Wright, 2019). Research have proven that wastewater workers have an increased risk for hepatitis C, gastric cancer and spinal abnormalities (Tiwari, 2008).

Exposures to pathogens in the WWTPs are preventable through the use of administrative controls such as personal protective equipment (PPE) to create a barrier between the worker and the exposure (Wright, 2019). Workers that are not protected by adequate health and safety measures risk injury, infection, disease, mental health issues, and death (The World Bank, 2019; Wright, 2019).

The following are the recommended personal protective equipment (PPE) for wastewater workers in South Africa, as recommended by the Wastewater Risk Abatement Plan: eye and face protection (safety glasses, goggles and face shields), head protection (protective helmets), foot protection (protective footwear), hand protection (the material of the glove should be appropriate for the application or task), protective clothing (should be appropriate for the specific use) and respiratory protection (a system of local or general exhaust is recommended) (Van der Merwe-Botha, 2011).

PPEs should be selected to minimise risk; must be appropriate for the nature of work; must be adequately fitted and reasonably comfortable; must be maintained, repaired or replaced as needed so as to continue to be effective and must be used by the worker (WRC, 2018; Van der Merwe-Botha, 2011). Additionally, employers have the responsibility to train the workers to use, store and maintain the PPE while the worker is responsible for using the appropriate PPE and reporting defective or damaged equipment (WRC, 2018).

Personal Protective Equipment (PPE) are not always used appropriately (The World Bank, 2019). Studies showed that some workers do not feel it is essential for their health to use PPE and the preventative value of PPE is sometimes overlooked (Efstathiou, 2011). In a study from the Water research commission in 2018, it was observed that workers frequently worked without items of PPE while some used damaged PPE (WRC, 2018). Some sanitation workers around the world will continue to work without the appropriate PPE and little

understanding about the occupational hazards (WRC, 2018).

Given this knowledge that wastewater workers are at increased risk of infections, the government and management must minimise the risks workers face from contact with faecal matter (WRC, 2018). Workers should be governed under occupational health and safety (OHS) however guidelines articulating the safety of wastewater workers are not widespread and, in some countries, specific guidelines and legal framework are lacking (Wright, 2019). Wastewater workers can suffer due to weak legal protection and lack of enforcement of existing rules (The World Bank, 2019).

In some countries there are specific legislation that deals with health and safety in the workplace. However, guidelines specific to sanitation workers is rare and guidelines for working with biological hazards, if available, must be applied to sanitation workers (WRC, 2018; Wright, 2019).

In South Africa, sanitation work is regulated and guided by the National Occupational Health and Safety Act (South Africa, 1993), which established the Advisory Council for Occupational Health and Safety charged with conducting research, disseminating information, promoting training and providing advice on the development of standards and regulations (WRC, 2018).

The United States has the Occupational Safety and Health Act (OSHA, 1970). In the United Kingdom, the Health and Safety at Work Act (1974) guides occupational health. Sweden makes use of the Work Environment Act (Sweden, 1977). In Canada, the Canadian Centre for Occupational Health and Safety, is the primary national agency guiding workplace health and safety. South Korea use the Occupational Health and Safety Act of 1990. Malaysia utilizes the Occupational Safety and Health Act (1994). In Singapore, the Workplace Safety and Health Act of 2006 (revised 2009) are used. And Australia makes use of the Work Health and Safety Act (2011) (WRC, 2018).

Studies, such as the one by Wright *et al.* (2019) have been conducted to assess PPE compliance among occupations such as healthcare, carpentry, construction and agriculture workers. Little attention has been given to PPE compliance and occupational hazards in the wastewater industry (Wright, 2019).

2.3 UNDERSTANDING ANTIBIOTIC RESISTANCE IN WATER IN THE CONTEXT OF A ONE HEALTH APPROACH

2.3.1 Overview

Antibiotic resistance has become one of the significant public health threats globally and various studies have identified resistant microorganisms that harbour resistant genes in both the environmental setting and clinical settings (Berendonk *et al.,* 2015; Martinez *et al.,* 2009). Furthermore, the World Health Organization deemed antibiotic resistance as a main public health concern and antibiotic resistant bacteria (ARBs) and antibiotic resistance genes (ARGs) arising as emerging pollutants that pose a threat towards food safety and public health (Martinez *et al.,* 2009). Understanding antibiotic resistance within the context of a One Health approach is crucial for addressing this global health threat comprehensively. The One Health approach recognizes the interconnectedness of human, animal, and environmental health and emphasizes the need for collaborative efforts across these domains to better understand and combat antibiotic resistance. Monitoring and surveillance of antibiotic resistance in healthcare settings, animal health, and in the environment can help identify sources and hotspots of resistance. Furthermore, an understanding of the mechanisms for development of antibiotic resistance and how resistant bacteria and resistance genes move between humans, animals, and water environments is essential.

According to Larsson and Flach (2022), a number of key sources and factors contribute to the emergence and spread of antibiotic resistance and they include:

- Bacteria can naturally acquire mutations in their DNA over time, some of which confer resistance to antibiotics.
- Bacteria can acquire resistance through horizontal gene transfer, a mechanisms which allows resistance genes to be shared among different bacterial species, contributing to the spread of antibiotic resistance.
- Overuse and misuse of antibiotics, which results in increased opportunities for bacteria to develop resistance.
- Environmental reservoirs, serve as the medium for the development and spread of antibiotic resistance. This is because bacteria found in the environment can both develop resistance and exchange genetic material with other bacteria, including human pathogens, and potentially transfer resistance genes.
- Resistant bacteria can spread within communities through person-to-person transmission or contact with contaminated surfaces.

Several studies have reported the presence of multi-drug resistant *Escherichia coli* microorganisms within the sediments of rivers and other water systems. This gives a clear indication that the sediments of several water systems such a river sediment can be the reservoirs of these antimicrobial resistant bacteria and antibiotic resistant gene bearers (Almakki *et al.,* 2019). In China, for example, a study was done within the runoff water after the occurrence of a severe storm and the detection of multiple multi-resistance microbes were present (Almakki *et al.,* 2019). The detected microorganisms included *Pseudomonas aeruginosa, Acinetobacter, Aeromonas* and *Bacillus cereus* (Almakki *et al.,* 2019). These detections and observations were detected within the China water systems after a severe storm. In similar cases after severe storms, the same multi-drug resistant bacteria were detected within the water systems, mainly rivers, of South Africa (Almakki *et al.,* 2019). Another interesting study was done on puddles around hospital environments and interestingly enough, there were several different potential pathogens identified within a number of puddles (Almakki *et al.,* 2019). These potential pathogens included *Aeromonas, Acinetobacter, Enterobacter, Escherichia, Klebsiella, Pseudomonas,* etc. (Almakki *et al.,* 2019). This founding of the potential pathogens within puddles around hospitals creates the opportunity to perhaps make the conclusion that in case a storm should break out, these potential pathogens might be able to end up in various water systems and thus the aquatic ecosystems (Almakki *et al.,* 2019).

Antibiotic resistance is introduced into WWTPs by water resulting from human activities such as healthcare services (hospitals), agriculture, veterinary as well as water from the general population via wastewater pipes (Rodriguez-Molina *et al.,* 2019). The accumulation of waste from various sources turns WWTPs into unintentional collection points for antimicrobial agents, ARBs and ARGs (Rizzo *et al.,* 2012). According to Pal *et al.* (2015) WWTPs are not designed and not yet upgraded to remove ARBs and ARGs and as a result, WWTPs are known to harbour antimicrobials and other agents associated with antibiotic resistance. Due to this challenge faced by WWTPS, high concentrations of antibiotics and their associates (ARBs and ARGs) in the WWTPs effluent are release into the environment via WWTPs discharges to rivers, wastewater reuse and irrigation (Barancheshme and Munir, 2019).

2.3.2 Global priority list of antibiotic resistant bacteria

Antibiotics and antimicrobials have been widely used in the last decades to control diseases caused by microorganisms and for breeding livestock (Barancheshme and Munir, 2019). The use of these agents was successful until microorganisms evolved and acquired mechanisms to enable them to by-pass the effects thereof, due to the inappropriate and misuse of antibiotics over the years. In 2017, the WHO published the list of global priority pathogens (GPP), comprising of 12 species of bacteria that pose the greatest threat to human health. This list of 12 species is grouped under three priority tiers according to the urgency and need for new antibiotics, as part of WHO's efforts to address growing global resistance to antimicrobial medicines (Table 2.3).

The term “ESKAPE” pathogens comprise six opportunistic pathogens with seemingly multiple drug/antibiotic resistance and virulence made up of both Gram-positive and Gram-negative species (Rice, 2003; Santajit and Indrawattana, 2016; Mulani et al., 2019). The opportunistic pathogens in question are (i) *Enterococcus faecium*, (ii) *Staphylococcus aureus*, (iii) *Klebsiella pneumoniae*, (iv) *Acinetobacter baumannii*, (v) *Pseudomonas aeruginosa* and (vi) *Enterobacter* spp. (Rice, 2003). According to Santajit and Indrawattana (2016) and Mulani *et al.* (2019) the ESKAPE pathogens are the common causes of life-threatening nosocomial infections amongst immunocompromised individuals and are capable of “escaping” the biocidal effects of both antimicrobial and antibiotic agents. This group of microorganisms is problematic since research has revealed that they are associated with the highest risk of mortality thereby increasing health care costs and antibiotics costs and usage (Founou *et al.,* 2019).

Table 2.3: WHO global priority pathogens list of antibiotic-resistant bacteria (WHO, 2017)

Priority 1: CRITICAL	Priority 2: HIGH	Priority 3: MEDIUM
• *Acinetobacter baumannii*, carbapenem-resistant • *Pseudomonas aeruginosa*, carbapenem-resistant • *Enterobacteriaceae*, carbapenem-resistant, ESBL-producing	• *Enterococcus faecium*, vancomycin-resistant • *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant • *Helicobacter pylori*, clarithromycin-resistant • *Campylobacter* spp., fluoroquinolone-resistant • *Salmonellae*, fluoroquinolone-resistant • *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant	• *Streptococcus pneumoniae*, penicillin-non-susceptible • *Haemophilus influenzae*, ampicillin-resistant • *Shigella* spp., fluoroquinolone-resistant

Based on the latter, the World Health Organization (WHO) has recently listed the ESKAPE pathogens under the list of microorganisms that are in desperate need of new and improved antibiotics (Tacconelli *et al.,* 2018). According to the WHO’s priority list, there are three categories of pathogens based on their urgency and desperation of need for new antibiotics and they are critical, high and medium (Mulani *et al.,* 2019). The critical category encompasses pathogens such as carbapenem resistant *A. pneumoniae*, *P. aeruginosa* as well as extended spectrum $\beta\beta$-lactamase (ESBL) or carbapenem resistant *K. pneumoniae* and *Enterobacter* spp. The high category comprises pathogens such as vancomycin resistant *E. faecium* (VRE) and methicillin and vancomycin resistant *S. aureus* (MRSA and VRSA) (WHO, 2018).

2.3.2.1 Enterococcus faecium

The *Enterococcus* genus belongs to a group of microorganisms known as lactic acid bacteria (LAB) (Moreno *et al.,* 2006). Members of this genus are Gram-positive, non-spore forming, catalase-negative, oxidase-negative, facultative anaerobic cocci that occur in singles, pairs or chains (Moreno *et al.,* 2006). Enterococci are known as the common residents of the gastrointestinal tracts of almost all land animals, humans included. The prominent members of this group *E. faecalis* and *E. faecium* are capable of causing various severe nosocomial infections and are regarded as opportunistic pathogens. These infections include urinary-tract infections, intra-abdominal infections, bacteraemia and endocarditis (Kirschner *et al.,* 2001; Wang *et al.,* 2016). According to Rice *et al.* (2003) in the olden days *E. faecium* was regarded as a species with limited virulence as it was found responsible for <10% of enterococcal infections. However, in the present time, the percentage of enterococcal infections associated with *E. faecium* has increased significantly to <30%. Based on research, the two prominent species (*E. faecalis* and *E. faecium*) have multiple drug resistance towards several antibiotics such as vancomycin,

penicillin and aminoglycoside (Wang *et al.,* 2016). Of all antibiotics, vancomycin resistance is commonly found in *E. faecium* and is constantly reported throughout the world (Bourgeois-Nicolaos *et al.,* 2006).

2.3.2.2 Staphylococcus aureus

The genus *Staphylococcus* comprises of typically Gram-positive bacteria that form irregular clusters of cocci. Members of this genus are widespread in nature and many are common residents of the skin, skin glands as well as mucous membranes of both mammals and birds (Willey *et al.,* 2017). This genus comprises both pathogenic and non-pathogenic microorganisms. Species such as *S. aureus*, *S. epidermidis* and *S. saprophyticus* are considered as opportunistic pathogens as they have the ability to cause several hospital-acquired diseases, waterborne and foodborne diseases. Among all the *Staphylococcus* species, *S. aureus* is the prominent one as it is more pathogenic than the rest of the species. This particular species is aerobic, facultative anaerobic, often haemolytic in blood agar (pathogenic appearance) and is able to produce coagulase enzyme of which is a virulence factor (Rasheed and Hussein*,* 2021). Furthermore, the expressed virulence factors aid in the establishment of infections by facilitating tissue attachment, tissue invasion and evading from host immune response. As a result, diseases such as skin infections, bacteraemia, endocarditis, pneumonia and food poisoning are evident. According to Naber (2009) *S. aureus* has the ability to gain resistance to multiple antibiotic classes, therefore making it a difficult pathogen to treat. Over the years, the emergence and wide spread of *S. aureus* strains of which portray resistance towards methicillin commonly known as Methicillin Resistant *Staphylococcus aureus* (MRSA) have been recognized and have resulted in high morbidity, high mortality plus increased treatment costs. In order to combat MRSA strains, vancomycin remained an antibiotic that could attack these strains for years, but recent evidence suggests that resistance has also been acquired and resulted into Vancomycin Resistant *Staphylococcus aureus* (VRSA).

2.3.2.3 Klebsiella pneumoniae

The genus *Klebsiella* comprises of non-motile, aerobic and facultatively anaerobic, and Gram-negative rods (Paterson *et al.,* 2014). Abbott (2007) highlighted that this genus consists of species such as *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *ozaenae*, *K. pneumoniae* subsp. *rhinoscleromatis*, *K. oxytoca*, *K. omithinolytica*, *K. planticola* and *K. terrigena*. Several *Klebsiella* species are amongst the common causes of a variety of community-acquired and hospital-acquired infections. In all the species, *K. pneumoniae* is the most significant, as it has been recognized and documented as one of the emerging pathogens in community-acquired liver abscess worldwide, more so in countries such as Taiwan and the USA (Chang and Chou, 1995; Fung et al., 2002). Felson et al. (1949) also highlighted that this species is well known in clinicians as it also causes community-acquired bacterial pneumonia that particularly occurs in chronic alcoholics and shows a characteristic radiographic abnormality resulting from severe pyogenic infections, of which can cause high death rates if not treated properly. Since this species is an opportunistic pathogen, it primarily attacks the immunocompromised individuals that are hospitalized and suffer from illnesses such as diabetes mellitus or chronic pulmonary obstruction (Podschun and Ullmann, 1998). Like other opportunistic pathogens, *K. pneumoniae* is intrinsically resistant towards wide range of antibiotics such as penicillin, ampicillin, amoxicillin, oxacillin, carbenicillin and ticarcillin (Paterson *et al.,* 2014).

2.3.2.4 Acinetobacter baumannii

According to Towner (1997) members belonging to the genus *Acinetobacter* are ubiquitous free-living, Gram-negative saprophytic bacilli. They can easily be found in soil, water, wastewater as well as food. Towner (1997) highlighted that in the field of biotechnology, microorganisms belonging to this genus are highly valuable in various commercial important industrial processes and in the degradation of a wide range of toxic environmental pollutants because of their metabolic versatility. Clinically, certain *Acinetobacter* species are recognized as important nosocomial pathogens that play a key role in the colonization and infection of patients in hospitals (Bergogne-

Berezin and Towner, 1996). In all the *Acinetobacter* species, *A. baumannii* has been recognized as the notorious, troublesome representative of the genus due to its opportunistic pathogen nature. Studied indicate that its clinical significance, more so over the past 15 years, has been driven by its remarkable ability to upregulate or acquire resistance towards antibiotics and antimicrobials (Peleg *et al.,* 2008). This however makes *A. baumannii* one of the microorganisms that continue to challenge the current antibiotics era. This pathogen is known to attack critically ill or immunocompromised patients that have underlying diseases and infections caused are associated with central nervous system, skin and soft tissue (Peleg *et al.,* 2008; Castilho *et al.,* 2017). Furthermore, studies have indicated that *A. baumannii* is resistant to multiple antibiotics as well as antimicrobials such beta-lactam antimicrobial drugs, 3rd and 4th-generation cephalosporins and carbapenems (European Centre for disease prevention and control, 2013).

2.3.2.5 Pseudomonas aeruginosa

Pseudomonas genus is a relatively heterogenous and ecologically important group of microorganisms (Franzetti and Scarpellini, 2007). Members of this genus are Gram-negative motile aerobic rods with characterized elevated metabolic versatility (Palleroni, 1993). *Pseudomonas* are widespread and are able occupy diverse niches such as soil, fresh water, marine environment, food and clinical settings (Lalucat *et al.,* 2020; Franzetti and Scarpellini, 2006). According to Palleroni (1993) a portion of the *Pseudomonas* species is medically significant as they are regarded as opportunistic pathogens in both humans and animals, whereas the other portion are important in the agricultural sector. Among the *Pseudomonas* genus, *Pseudomonas aeruginosa* is the most important and well documented as it forms part of the pathogens that are known to cause nosocomial infections. According to Rocha et al. (2019) *P. aeruginosa* is one of the most clinically and epidemiologically important bacteria due to its pathogenic characteristics. This opportunistic pathogen rarely causes diseases in healthy individuals but can cause a negative impact on individuals that are immunocompromised and the elderly by causing infections such as (i) folliculitis and infections within the ear canal, (ii) cheratitis (iii) malignant otitis, (iv) osteomyelitis and (v) endocarditis (Rossolini and Mantengoli, 2005). Studies also highlight that *P. aeruginosa* is the third and fifth most common cause of hospital-acquired urinary tract infections in the USA and Europe (Haque et al., 2018).

2.3.2.6 Enterobacter cloacae

Enterobacter genus consists of members that are Gram-negative, facultative anaerobic, rod-shaped, non-spore forming bacteria that belong to the family Enterobacteriaceae (Davin-Regil and Pages, 2015). This genus comprises of two well-known species, *Enterobacter aerogenes* and *Enterobacter cloacae*, that have challenged the state of clinical settings as opportunistic pathogens and as a result, have emerged as nosocomial pathogens from intensive care patients pathogenic, to those who are on mechanical ventilation (Mezzatesta *et al.,* 2012). Sanders and Sanders (1997) further indicated that these two species are highly resistant towards older antimicrobial agents, antibiotics included and have acquired the ability and the mechanisms enabling them to develop resistance towards newer agents. Clinically, these pathogens have been implicated in an increasing number of clinical syndromes, mimicking those that are easily treatable such as those that arise from streptococci (Sanders, 1996). Amongst the two prominent species, *E. cloacae* is the most recognized as it is a well-known nosocomial pathogen that contributes to diseases such as bacteraemia, endocarditis, septic arthritis, osteomyletis, skin/soft tissue infections, lower respiratory tract, urinary tract and intra-abdominal infections (Fata *et al.,* 1996). Not only does this pathogen have the ability to attack human immune systems, Wang *et al.* (2000) stated that nosocomial outbreaks have also been associated with the colonization of several surgical equipment and operative cleaning solutions. According to Davin-Regil and Pages (2015) *E. cloacae* has inherent resistance towards ampicillin, amoxicillin, 1st generation cephalosporins and cefoxitin aiding to the production of constitutive AmpC $\beta\beta$-lactamase.

2.3.2.7 Why is it important to study Clostridia spp.?

Clostridium bacteria, on the other hand, are part of the groups of resistant microorganisms and associated with high morbidity and mortality. Table 2.4 provides examples of some of the toxins produced by a number of *Clostridium* species and the associated diseases (Auwaerter et al., 2019). *Clostridium* is always present in wastewater due to spore-forming ability. Henceforth the epidemiology of *Clostridium* bacteria has evolved in recent years, with the introduction of more virulent strains linked to severe infections, high recurrence rates, and mortality. Antibiotic resistance is a major factor in these epidemiological shifts and the creation of new *Clostridium* strains (Spigaglia, 2016). In 2015 the Department of Health reported that Antibiotic use has surged by 60% in South Africa alone during the last decade. Currently, South Africa faces a three-layered threat of antibiotic resistance: drug-resistant tuberculosis, HIV, and antibiotic-resistant bacteria-(ARBs), (Nnadozie *et al*., 2017).

Table 2.4: Illustration of some of the toxins produced by a number of *Clostridium* species and the associated diseases (Auwaerter *et al.,* 2019).

Clostridia	Toxin	Disease
Gastrointestinal tract		
● *C. perfringens*	Enterotoxin	Food intoxication, diarrhoea, sudden infant death
	Beta toxin	Necrotic enteritis
● *C. defficle*	Ted A + Ted B	Antibiotic-associated diarrhoea + colitis
● *C. septicum*	Alpha	Intestinal myonecrosis
● *C. botulinum*	BoNT/A,B,E	Human botulism
Wound-related diseases		
● *C. perfringens*	Alpha toxin	Gangrene, Puerperal sepsis
● *C. sordellii*	TcsI, TcstI	Gangrene,
● *C. titanni*	TenT	tetanus
● *C. novyi*	Alpha-novyi	Gangrene,
● *C. botulinium*	BoNT/A + B	Wound Botulism
* Adapted from popo of MR, Bouvel P; FutureMicrobi 2009; 4:1021		
TenT – Tetanus toxins; BoNt – Botulinum neurotoxin ; TcsH – *C. sordellii* hemorrhagic toxic; TcsL – *C. sordellii lethal toxin;* TcdA, alpha-toxin; TedB – Beta toxin		

Moreover, the efficacy of AR removal from wastewater treatment plants is still far from satisfactory, despite a range of physical, biological, and chemical treatment procedures. This has led to Antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) being added to the list of microbial contaminants of emerging concern. Since wastewater treatment can provide favourable conditions for ARB growth and progression, as well as the spread of ARGs (Krzeminski *et al*., 2019).

Most importantly these pathogenic *Clostridium* species can also make their way into the natural environment through poorly treated urban wastewater effluent discharges to the surrounding environment. One particular species of Clostridia *(Cl), Cl. difficile* is the most common cause of antibiotic-associated diarrhoea, and it has a big impact in both healthcare and community settings (Johanesen *et al*., 2015). For this reason, the fate of antibiotics in WWTPs and the negative consequences of microbiological (Clostridia) contaminates of emerging concern needs to be evaluated, their elimination is to be considered as a further strategic requirement, emphasizing the need to replace traditional WWTPs processes with more effective technology. To overcome *Clostridium* pathogenic loads in receiving environments, which can lead to infections that are difficult to treat with the currently available antibiotics (Marianne and Boutin, 2017).

Isolation and cultivation of strictly anaerobic microbes requires special techniques, equipment and nutrient media (Stieglmeier *et al*., 2009). There are various methods like Wright's tube method, Brewer's petri dish method, vacuum and gas displacement method that are known for isolation of anaerobic bacteria (Gordon *et al*., 1971). But the most popular methods used in isolation of anaerobes is the candle method and gaspak anaerobic jar method (Jamal, 2009.)
Clostridium species can also be isolated using an alcohol or heat shock technique that permits the spores to survive and eliminates non-spore producing vegetative faecal organisms (Mainil, 2006). Then deposit of the spirit-treated specimen or broth is then inoculated onto Clostridia selective medium, which might be either *C. difficile* Cefoxitin Cycloserine Egg Yolk Agar base or *C. difficile* Cefoxitin Cycloserine Egg Yolk Agar base (Jamal, 2009). *Clostridium* has been isolated and cultured in various water ecosystems using traditional culture-based methods (Cabral, 2010). Although the cultivated *Clostridium* species improved our understanding of these critical bacteria, the water ecosystem contained a large number of uncultured *Clostridium* that could not be extracted and cultured using current methods (Zou *et al*., 2018).

Currently the Fung double tube (FDT) and Shahidi-Ferguson Perfringens (SFP), SFP Egg Yolk Enrichment and Tryptose sulphite cycloserine (TSC) agar media are the best isolation and cultivating methods for *Clostridium* species (Agar, 2018)**:**

In the development of enumeration methods for *Clostridium* species in water the first rapid method is the Fung double tube (Ruengwilysup *et al*., 2009), which involves producing a thin layer of agar material between two tubes to create anaerobic conditions (Vijayavel *et al*., 2009). To effectively recover and count anaerobic bacteria, it is highly recommended to utilize the Fung double tube method (Ketchum *et al*., 1989; Vijayavel, *et al*., 2009). The FDT method is a fast microbiology method for isolating bacteria and count anaerobic microorganisms, including *Clostridium* species (Fung, 2002). The Tube method has several advantages, including the capacity to generate anaerobic conditions in a smaller space than other methods, such as the GasPak system, like a plate-based methodology (Shahin *et al*., 2003). FDT allows for bacterial density enumeration. The FDT method for detecting and counting *Clostridium perfringens* in recreational waters has been proven to be accurate (Renschler, 2015).

TSC is a basal medium for use either on its own or with selective agents to make Tryptose Sulphite agar, Tryptose Sulphite Cycloserine agar (De Jong *et al*., 2003). TSC agar takes advantage of D-selective cycloserine's inhibitory characteristics, as well as a sulphite and ferric iron-based indicator system (Monza, 2019). *Clostridium perfringens* and other anaerobic species reduce sulfite and form black colonies as a result of ferrous sulfide formation, which suppresses most undesirable organisms in Shahidi-Ferguson Perfringens (SFP), SFP Egg Yolk Enrichment and

Tryptose sulphite cycloserine agar (Corry *et al., 2003*). Hauschild and Hilsheimer (1974), investigated whether of the two most often used media, SFP and TSC, was more effective in detecting and counting *C. perfringens* in Petri plates. When compared to SFP and SFP without egg yolk supplement, TSC had the greatest *Clostridium* levels (Barrios *et al*., 2013).

2.3.3 Mechanisms for the development of antibiotic resistance

According to Giedraitienė *et al.* (2011) antibiotic/antimicrobial resistance genes are usually carried within the bacterial chromosomes, plasmids or transposons. Li and Nikaido (2004) and Wright (2005) stated that microorganisms have broad categories of mechanisms to gain resistance towards biocides and antibiotics and they include drug inactivation/alteration, modification of drug binding sites/targets, changes in cell permeability resulting in reduced intracellular drug accumulation and biofilm formation. Some of the bacterial mechanisms used to gain resistance will be discussed below.

2.3.3.1 Drug inactivation or alteration

Most microorganisms produce enzymes such as $\beta\beta$-lactamases, aminoglycoside-modifying enzymes or chloramphenicol acetyltransferases that can irreversibly modify and inactivate the effect of the antibiotics (Santajit and Indrawattana, 2016). In all of these present enzymes in bacteria, enzyme $\beta\beta$-lactamase is the most well characterized and prevalent amongst bacteria. This particular enzyme acts by hydrolysing the $\beta\beta$-lactam ring (a vital factor found in all $\beta\beta$-lactams) making antibiotics such as penicillins, cephalosporins, monobactams and carbapenems important to their activity (Jacoby and Munoz-Price, 2005).

According to Bush and Jacoby (2010) $\beta\beta$-lactamases can be classified using two main classification systems, namely: The Ambler scheme (molecular classification) and the Bush-Jacoby-Medeiros system (classifies the most clinically important beta-lactamases such as those produced by Gram-negative bacteria). The Ambler class A group comprises of penicillinase, cephalosporinase, broad-spectrum beta-lactamases, extended-spectrum beta-lactamases (ESBLs), and carbapenemases. The presence of these enzymes/ the ability of the microorganisms to produce these enzymes enables them to inactivate penicillin (excluding temocillin), third generation oxyimino-cephalosporins (e.g. cettazidime), aztreonam, cefamandole, cefoperazone and methoxy-cephalosporins (Santajit and Indrawattana, 2016). In addition this group (Ambler class A) also contains a number of other significant enzymes including ESBLs (mostly TEM, SHV, and CTX-M type) and KPCs. According to Dzidic *et al.* (2008) the CTX-M enzymes have been identified in members of the ESKAPE pathogens namely *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* species.

2.3.3.2 Modification of drug binding sites

In order to avoid recognition by antimicrobial agents and antibiotics, resistant microorganisms usually modify their target sites. Pucci and Dougherty (2002) stated that the mutation of the gene specifically encoding for penicillin-binding proteins (PBPs), of which essentially are enzymes that are anchored on the cytoplasmic membrane of the cell wall of bacteria and its key function is to assemble and control the abovementioned stages of the cell wall building, often results in the expression of unique penicillin-binding proteins. For example, the expression of a unique PBP2a in *S. aureus*, of which is the most dominant PBP commonly found in MRSA cell compared to the normal PBPs. Continuing with the latter example, PBP2a in *S. aureus* has a relatively low affinity for all beta-lactam antibiotics and acts as a substitute for other PBPs, therefore allowing the survival of *S. aureus* in the presence of elevated concentrations of beta-lactam antibiotics/biocides including methicillin acting on cell wall biosynthesis (Tang *et al.,* 2015).

2.3.3.3 Reduced intracellular drug accumulation

According to Santajit and Indrawattan (2016) the balance of antibiotic uptake and elimination determines the susceptibility of the microorganisms towards the antibiotic. Therefore, a reduction in the amount of antibiotic able to pass through the bacterial cell membrane is another mechanism that microorganisms use in order to acquire antibiotic resistance. An example of such a mechanism includes the occurrence of diminished protein channels located on the microorganism's outer membrane to lower the rate at which antibiotics enter within the cell as well as the presence of efflux pumps (decreases the amount of antibiotics that are accumulated within the bacterial cell).

2.3.3.4 Biofilm formation

Biofilms can be defined as complex microbial communities that exist as a thin layer on biotic or abiotic surfaces implanted in a matrix of extracellular polymeric substances created by other biofilms (Santajit and Indrawattan, 2016). Biofilms create a conducive environment for bacteria, enabling them to interact with one another and with the environment. According to Pucci and Dougherty (2002) the essential components of the biofilm matrix are produced extracellular polymeric substances that are made up of polysaccharides, proteins, lipids and extracellular DNA originating from microorganisms that reside within it. The presence of such components within the biofilm matrix act as a mechanical and biochemical armour that provides the conditions required to attenuate the activity of the antibiotics (e.g. high CO_2, low pH, low water availability and low O_2). Del Pozo and Patel (2007) further stated that such conditions pose a challenge for antibiotics to eliminate microorganisms effectively and also stated that in a case where microorganisms experience nutrient scarcity, they are more likely to become tolerant towards antibiotics. The most common members of the ESKAPEs that has been identified to use this particular mechanism include *S. aureus*, *P. aeruginosa*, *A. baumannii* and *K. pneumonia (*Høiby et al., 2010).

2.4 ENVIRONMENTAL SURVEILLANCE OF ANTIBIOTIC RESISTANCE

2.4.1 Wastewater-based epidemiology

Wastewater-based epidemiology (WBE) is a tool or method that is most commonly used to determine the legal and illegal abuse of drugs, of certain regions of people (Lorenzo and Picó, 2019). This method is also starting to play a key role in the detection of exposure of people to many different substances such as: pesticides, personal care products and most importantly pathogens (Lorenzo and Picó, 2019). The very promising method of wastewater-based epidemiology (WBE) could potentially play the important role in determining the prevalence or detection of viruses within wastewater systems (Lorenzo and Picó, 2019). This could be the next step in having better control or understanding how pathogens or potential pathogens may spread through these wastewater systems and thus, causing more infections or creating the opportunity for these pathogens to end up in the environment. This method is possible as these infecting or spreading pathogens, spread through the means of waste from infected patients, individuals or animals, ending up in the wastewater systems (Ahmed, et al., 2020; WHO, 2020). By using WBE methods there can be better detection and thus warning signals or factors that signal when there are pathogens causing diseases or when as well as when they are starting to spread or emerge.

According to Choi *et al.* (2018) wastewater-based epidemiology studies have enabled scholars the ability to monitor pathogens and drug levels emanating from healthcare and community associated streams simultaneously (Newton *et al.,* 2015). Whilst several studies have also investigated the geographic and seasonal distributions of AMR as well as the abundance of AMR genes in wastewater and clinical samples (Su *et al.,* 2017; Hendriksen *et al.,* 2019). However, from literature (Table 2.5) it is evident from the latter studies is that heterogenous study designs, differing methodology, and the point of sample collection have an impact on the outcomes and results of

the study. Thus, very little is understood about the effect of grab vs composite vs proportional sampling (Chau *et al.,* 2022).

Table 2.5: Methodological features potentially contributing to variability in outcomes (adapted from Chau et al., 2022)

Sampling point type	Study design features	Aspects potentially introducing variability in outcomes	References
Wastewater sampling point type	Wastewater treatment works (WWTW) sampling point, e.g. influent vs effluent	Treatment processes can transform microbial and AMR composition resulting in differences between influent and effluent samples	Tong *et al.,* 2019; Zhang *et al.,* 2020
	Hospital effluent	Focused sampling may only represent specific sub-populations	Jakobsen *et al.,* 2008; Larson *et al.,* 2020
	Domestic sewers/manholes informal sewer systems	Informal sewer systems (often with low flow) may be susceptible to homogeneity	Fahrenfeld and Bisceglia, 2016
Wastewater sampling method	Grab (single sample)	● Single grab samples can be flooded by homogenous solids. ● Wastewater composition can vary significantly over short time periods.	Reinhaler *et al.,* 2013; Guo *et al.,* 2019
	● Composite sampling (combining grabs) ● Proportional sampling (flow/time/volume	Composite and proportional samples capture average composition but may be unable to discriminate peak values during sampling period.	Michael-Kordatou *et al.,* 2020

2.4.2 Sampling methods

Table 2.6 represents 70 randomly selected studies either focus on the quantification of antibiotic residues or screening of antibiotic resistance (bacteria or genes). The methodology of these studies was read to determine whether grab or composite sampling was used.

Table 2.6: Comparison of grab and composite sampling of 70 randomly selected studies

Grab or composite sampling	Antibiotic resistance (bacteria or gene) or antibiotics	Reference
Grab sampling	Antibiotic resistance	Al-Jassim *et al.,* 2015
Grab sampling	Antibiotic resistance	Calero-Cacere *et al.,* 2014
Grab and composite sampling	Antibiotic resistance	Chen and Zhang, 2013
Grab sampling	Antibiotic resistance	Colomer-Lluch *et al.,* 2011
Grab sampling	Antibiotic resistance	Czekalski *et al.,* 2014
Grab sampling	Antibiotic resistance	Czekalski *et al.,* 2015
Grab sampling	Antibiotic resistance and antibiotics	Diwan *et al.,* 2010
Grab sampling	Antibiotic resistance and antibiotics	Gao *et al.,* 2012
Grab sampling	Antibiotic resistance	Graham *et al.,* 2011
Grab sampling	Antibiotic resistance	Gros *et al.,* 2013
Grab sampling	Antibiotic resistance	He *et al.,* 2016
Grab sampling	Antibiotic resistance and antibiotics	Hoa *et al.,* 2011
Grab sampling	Antibiotic resistance	Huerta *et al.,* 2013
Grab sampling	Antibiotic resistance	Jia *et al.,* 2015
Grab Sampling	Antibiotic resistance and antibiotics	Jiang *et al.,* 2013

Grab or composite sampling	**Antibiotic resistance (bacteria or gene) or antibiotics**	**Reference**
Grab Sampling	Antibiotic resistance	Ju *et al.,* 2016
Composite sampling	Antibiotic resistance	Karkman *et al.,* 2016
Grab Sampling	Antibiotic resistance and antibiotics	Khan *et al.,* 2013
Grab Sampling	Antibiotic resistance	LaPara *et al.,* 2011
Grab Sampling	Antibiotics	Locatelli *et al.,* 2011
Composite sampling	Antibiotic resistance	Luo *et al.,* 2010
Grab Sampling	Antibiotic resistance	Mao *et al.,* 2014
Composite sampling	Antibiotic resistance	Mao *et al.,* 2015
Grab Sampling	Antibiotic resistance and antibiotics	Marti *et al.,* 2013
Grab Sampling	Antibiotic resistance	Munir *et al.,* 2011
Grab sampling	Antibiotic resistance	Negreanu *et al.,* 2012
Composite sampling	Antibiotic resistance and antibiotics	Novo *et al.,* 2013
Composite sampling	Antibiotic resistance and antibiotics	Oberlé *et al.,* 2012
Grab sampling	Antibiotic resistance and antibiotics	Rodriguez-Mozaz *et al.,* 2015
Grab sampling	Antibiotic resistance	Shi *et al.,* 2013

Grab or composite sampling	**Antibiotic resistance (bacteria or gene) or antibiotics**	**Reference**
Grab sampling	Antibiotic resistance	Stoll *et al.,* 2012
Grab sampling	Antibiotic resistance	Storteboom *et al.,* 2010
Grab sampling	Antibiotic resistance	Tao *et al.,* 2010
Grab sampling	Antibiotic resistance and antibiotics	Wu *et al.,* 2015
Grab sampling	Antibiotic resistance and antibiotics	Xiong *et al.,* 2015
Grab sampling	Antibiotic resistance and antibiotics	Xu *et al.,* 2015
Grab sampling	Antibiotic resistance	Xu *et al.,* 2016
Grab sampling	Antibiotic resistance	Yang *et al.,* 2013
Grab sampling	Antibiotic resistance	Yang *et al.,* 2014
Composite sampling	Antibiotic resistance	Zhang *et al.,* 2016
Grab sampling	Antibiotics	Bartelt-Hunt *et al.,* 2011
Grab sampling	Antibiotic resistance	Bengtsson-Palme *et al.,* 2016
Grab sampling	Antibiotic resistance	Börjesson *et al.,* 2010
Grab sampling	Antibiotics	Chang *et al.,* 2010
Grab sampling	Antibiotics	Chen and Zhou, 2014

Grab or composite sampling	**Antibiotic resistance (bacteria or gene) or antibiotics**	**Reference**
Grab sampling	Antibiotic resistance and antibiotics	Conte *et al.,* 2017
Grab sampling	Antibiotic resistance	Czekalski *et al.,* 2012
Grab sampling	Antibiotics	Dinh *et al.,* 2011
Grab sampling	Antibiotics	Dorival-García *et al.,* 2013a
Grab sampling	Antibiotics	Dorival-García *et al.,* 2013b
Grab sampling	Antibiotics	De Jesus Gaffney *et al.,* 2015
Grab sampling	Antibiotics	García-Galán *et al.,* 2010
Composite and Grab sampling	Antibiotics	Gros *et al.,* 2012
Grab sampling	Antibiotic resistance	Guo *et al.,* 2014
Grab sampling	Antibiotics	Jiang *et al.,* 2011
Grab sampling	Antibiotics	Da Le *et al.,* 2021
Grab sampling	Antibiotics	Liang *et al.,* 2013
Composite sampling	Antibiotic resistance	Liu *et al.,* 2012
Grab sampling	Antibiotics	López-Serna *et al.,* 2011
Grab sampling	Antibiotics	Ma *et al.,* 2015

Grab or composite sampling	Antibiotic resistance (bacteria or gene) or antibiotics	Reference
Grab sampling	Antibiotic resistance	Makowska *et al.,* 2016
Grab sampling	Antibiotics	Peng *et al.,* 2014
Grab sampling	Antibiotic resistance	Su *et al.,* 2012
Composite and Grab sampling	Antibiotics	Tang *et al.,* 2015
Grab sampling	Antibiotics	Tran *et al.,* 2016
Grab sampling	Antibiotics	Tso *et al.,* 2011
Grab sampling	Antibiotics	Xu *et al.,* 2014
Grab sampling	Antibiotics	Yan *et al.,* 2013
Grab sampling	Antibiotics	Yang *et al.,* 2011
Grab sampling	Antibiotics	Zhang and Zhang, 2011

2.4.2.1 Grab sampling

Grab sampling is defined as any "lump" of material from the lot, "taken in one single operation" (Minkkinen and Esbensen, 2009). During grab sampling, a small quantity of water is sampled over a period of time that does not exceed 15 minutes from a single point within the investigation area (Simpson, 2013; Clausen *et al.,* 2018). The results obtained from grab samples are essential in determining the estimated mean of contamination in a particular area (Clausen *et al.,* 2018).

(a) Advantages of grab samples

The grab samples allow a sampler to collect samples and measure physical and chemical parameters (suspended solids, pathogens, nutrients, specific organic chemicals, oxygen demand and heavy metals) that are difficult to measure *in situ* upon arrival at the laboratory. However, grab samples are not suitable to measure dissolved oxygen, temperature and pH (Gulliver *et al.,* 2010). This limitation is resolved by going sampling with a portal multi-meter which can measure parameters quickly *in situ.* This shows that grab samples do not require expensive and big equipment that might get damaged or lost during sampling.

Usually, the set-up costs of grab samples are low (Gulliver *et al.,* 2010), It does not require advanced sampling equipment and as result, samplers do not need extensive training on how to sample. It is a good tool to use to assess emergency spillage, contamination, or outbreaks.

(b) Disadvantages of grab samples

The analysis of grab samples gives snapshot results at the time of sampling, without considering the inter- and intra-day variation (Novic *et al.,* 2017; Valenzuela *et al.,* 2020; Cristóvão *et al.,* 2021). Furthermore, snapshot results provide results that lack temporal representativeness (Bernard *et al.,* 2019). Grab sampling requires frequent sample collection during the sampling period. This exercise may be costly as samplers have to visit sampling sites many times (Valenzuela *et al.,* 2020).

Grab samples requires frequent sample collection during the sampling period (Valenzuela *et al.,* 2020). If this is not achieved, the results obtained may paint an incomplete image of water quality since it represents data that lacks or does not consider short-duration fluctuations (e.g. flood events, intensive runoff, punctual discharges, etc.) (Bernard *et al.,* 2019). Furthermore, frequent sample collection is an expensive exercise.

Grab samples provide information on a specific sampling point. As a result, if a sampling point has a high contamination concentration as compared to others, then the analysed results give a representation of only a sampled point (Clausen *et al.,* 2018). Since most grab samples do not use an automated sampler, it is difficult to collect samples during a storm event and perform flow-weighted sampling (Gulliver *et al.,* 2010).

2.4.2.2 Composite sampling

Composite sampling – a number (N) of primary increments are combined into one aggregate (or bulk) sample before further processing and analysis (Minkkinen and Esbensen, 2009). Composite sampling involves a collection of a large sample made up of subsamples (Boswell *et al.,* 1996; Patil, 2011; Scalize and Frazão, 2018). The subsamples are collected over time and usually, have the same size or volume (Boswell *et al.,* 1996; Simpson, 2013). The mixture or combination (by sieving, centrifuging, ball milling or shaking) of subsamples must be homogeneous without affecting their integrity or introducing bias (Reicherts and Emerson, 2010). The homogeneity of water samples can be easily achieved as compared to solid ones (Kinzelman *et al.,* 2006).

(a) Advantages of composite sample

Composite samples can improve spatial and temporal coverage about an area without increasing the number of analyses (Reicherts and Emerson, 2010). Composite samples are either based on flow proportioning or time (Simpson, 2013). Laboratory analyses are expensive exercises to perform. Thus, some authors recommend the use of composite samples to reduce the number of samples to be analysed, since one composite sample is analysed instead of many (King and Harmel, 2003; Patil, 2011; Scalize and Frazão, 2018).

(b) Disadvantages of composite sample

A composite sample requires known (size or volume) and non-randomized subsamples. If not, it becomes difficult to analyse its data statistically (Boswell *et al.,* 1996). Composite sampling is not recommended for volatile chemicals that may evaporate during the mixing of subsamples since it affects the integrity of individual sample values (EPA, 1995). Furthermore, composite samples are not recommended for the assessment of parameters that can be altered during the mixing of samples such as oxygen, dissolved metals, oils, free carbon dioxide, grease, pH and dissolved metals (Scalize and Frazão, 2018). Composite samples could lead to a loss of critical information during the combination of sub samples if they contain microorganisms or constituents that may be mutually interactive or destructive (EPA, 1995; Boswell *et al.,* 1996; Lancaster and Keller-McNulty, 1998). Composite samples require specialized sampling equipment that can measure the flow rate of samples (EPA, 1995). Furthermore, it is difficult to associate the concentration of composite samples with the flow, especially in a time-based sampling scheme (King and Harmel, 2003).

Composite samples could lead to false-negative results. This is caused by dilution effects, for example, when a subsample with a high concentration is mixed with a low concentration (EPA, 1995; Lancaster and Keller-McNulty, 1998). It is also difficult to determine the contamination distribution when using composite samples (King and Harmel, 2003).

2.4.3 Using culture-based methods for detection of antibiotic resistant strains

The use of culturing techniques have been used effectively in the past to determine pathogens that can be found within WWTP effluent but it is very limited because most of the bacteria that can be found in the environment are unculturable (Lu *et al.*, 2015; Ye and Zhang, 2013). Certain viruses can also be enumerated by using the culture-propagation technique, but there are other viruses like hepatitis A virus (HAV), Noroviruses (NoVs) and enteroviruses (EVs) that can't be cultivated or the cultivation of those viruses are limited (Sibanda and Okoh, 2013). Because there are so many viral and bacterial pathogens that are unculturable other methods like molecular methods are used for the detection and the enumeration of pathogens. The problem with usual culture methods is that is tedious and labour intensive. That is the reasons why molecular techniques to quantify certain pathogens have become increasingly popular for detection and enumeration of pathogens (Chyerochana *et al.*, 2020).

2.4.4 Using molecular methods for detection of antibiotic resistant strains

Some of the molecular methods that have been used more are qPCR, FISH and microarrays (Huang *et al.*, 2018).(Table 2.7). qPCR, FISH and microarrays are highly specific, the sample that is tested requires specific probes and primers to detect only specific pathogens, this means that the prevalence of the pathogen in the community cannot be determined it can only be determined if the pathogen is present and in the case of qPCR it can be determined how many of that pathogen is present in the sample that was taken (Huang *et al.*, 2018). NGS is being applied in water sample research so that the pathogens can be quantified and identified, by using NGS known and unknown pathogens can be detected in water, NGS can also be used to identify emergent strains of potential pathogens (Bofill-Mas and Rusiñol, 2020; Martínez-Puchol *et al.*, 2020). NGS techniques are easier to apply to bacterial samples than viral samples because viral genomes have a lack of shared regions thus sequencing is much harder for viral genomes than bacterial genomes (Martínez-Puchol *et al.*, 2020). Because of this problem with viral NGS sequencing random-primer-based-sequencing needed to be developed so that viral sequences could be successful (Martínez-Puchol *et al.*, 2020).

2.4.4.1 Quantitative PCR

Quantitative PCR (qPCR) amplifies DNA the same as end-point PCR, but qPCR uses fluorescent labelling to monitor the amplification of the reaction in real-time (Bouchez *et al.*, 2016; Ruijter *et al.*, 2014 Reverse transcriptomic qPCR (RT-qPCR) applies the same principals as qPCR but it is used for the detection and quantification of RNA species. Because the amplification of the sample is monitored in real-time it can be compared to a known samples sizes to determine the original concentration of the DNA in the original sample (Ruijter *et al.*, 2014).

Table 2.7: Summary of molecular methods used for detection of various pathogens in wastewater systems.

Source (~ = no specific source)	Pathogens (NS = Not specific)	Pathogen Community (Y/N)	Molecular method	Score (Out of 5)	Author
WWTP	NS Pathogen	Y	-qPCR -FISH -NGS	3	**Garrido-Cardenas *et al.* (2017)**
-WWTP influent -Secondary treated wastewater -WWTP effluent	-crAssphage -Pepper mild mottle virus (PMMoV) -Tobacco mosaic virus (TMV) -Human enteric viruses	Y	qPCR	4	**Tandukar *et al.* (2020)**
-River samples -WWTP influent -Secondary treated wastewater -Human and animal faecal samples	-Two pig *Bacteroidales* -Two ruminant *Bacteroidales* -Three human *Bacteroidales*	Y	qPCR	5	**Haramoto and Osada (2018)**
-WWTP influent -WWTP effluent -WWTP AS	NS Pathogen community (*Mycobacterium* and *Vibrio*)	Y	Pyrosequencing	2	**Ye and Zhang (2013)**
-WWTP influent -WWTP effluent -WWTP AS -Secondary treated wastewater	-*E. coli* -*Aeromonas hydrophila* -*Arcobacter butzleri* -*Klebsiella pneumoniae*	Y	-Pyrosequencing -HiSeq Illumina -qPCR	5	**Lu *et al.* (2015)**
-Treated wastewater (TWW) -Soil irrigated by TWW -Crops irrigated by TWW	-*Campylobacter*	N	Nested-qPCR	4	**Farhadkhani *et al.* (2020)**
WWTP influent	-*Papillomaviridae*	Y	-	5	**Martínez-**

Source (~ = no specific source)	Pathogens (NS = Not specific)	Pathogen Community (Y/N)	Molecular method	Score (Out of 5)	Author
from 2 WWTPs	-*Picornaviridae* -*Adenoviridae* -*Polomaviridae* -*Astroviridae* -*Caliciviridae* -*Hepeviridae* - *Reoviridae*		Untargeted Viral Metagenomics -Target Enrichment Sequencing -Amplicon Deep Sequencing		**Puchol *et al.* (2020)**
-WWTP influent -WWTP primary-, secondary- and final effluent	-crAssphage -Human adenovirus -Human Polyomavirus	N	ddPCR (Droplet digital polymerase chain reaction)	4	**Wu *et al.* (2020)**
Biosolid treatment plants	NS Pathogen	Y	-qPCR -Amplicon Sequencing	5	**Yergeau *et al.* (2016)**
-WWTP effluent after secondary and tertiary treatment -WWTP AS	-*Helicobacter spp.* -*H. pylori*	N	-qPCR -FISH -DVC-FISH	4	**Hortelano *et al.* (2020)**
Aquatic environments receiving effluent	NS Pathogen	N	-16S/23S rRNA probes -qPCR -FISH -DVC-FISH	3	**Rowan (2011)**
-WWTP -Cow, poultry and pig slurry from slaughterhouses	crAssphage	N	-qPCR -End-point PCR	4	**García-Aljaro *et al.* (2017)**
Water river samples were taken near:	Diarrheal bacterial pathogens	Y	qPCR	5	**Guzman-Otazo *et al.* (2019)**

Source (~ = no specific source)	Pathogens (NS = Not specific)	Pathogen Community (Y/N)	Molecular method	Score (Out of 5)	Author
-WWTP effluent source -Agricultural region that uses wastewater for irrigation (Soil was also sampled from the agricultural region) -Urban wastewater discharge point -Freshwater reservoir inflow					
River water affected by: -Agriculture -Untreated Human wastewater	Bacteriophages of enterococci	N	qPCR	3	**Chyerochana *et al.* (2020)**
-WWTP effluent -River receiving effluent -seawater receiving effluent	NS Pathogen	N	-qPCR -MiSeq Illumina	2	**Liu *et al.* (2016)**
-WWTP influent -WWTP effluent	Hepatitis A	N	q-PCR	4	**Ouardani *et al.* (2015)**
WWTP influent	SARS-CoV-2	N	-qPCR -Nested-qPCR	4	**La Rosa *et al.* (2020)**
-WWTP influent -WWTP effluent -WWTP AS	NS Pathogen	Y	-MiSeq Illumina -HiSeq Illumina	5	**Huang *et al.* (2018)**
River water affected by:	-FRNA bacteriophage	Y	-qPCR -RT-PCR	5	**Arredondo-Hernandez *et***

Source (~ = no specific source)	Pathogens (NS = Not specific)	Pathogen Community (Y/N)	Molecular method	Score (Out of 5)	Author
-Untreated wastewater -Agricultural activity	genotypes I to III -Human adenoviruses				***al.* (2017)**
WWTP influent	-Human adenovirus -Rotavirus A	N	-qPCR -RT-PCR	4	**Silva-Sales *et al.* (2020)**
WWTP	-*Legionella spp.* -non-*Legionella spp*.	Y	qPCR	3	**Fykse *et al.* (2013)**
Irrigation water: -Reclaimed water -Groundwater -Drinking water WWTP: -Influent -Secondary effluent	NS Pathogen	Y	qPCR	5	**Rusiñol *et al.* (2020)**
~~	NS Pathogen	N	-qPCR -RT-PCR -FISH	2	**Girones *et al.* (2010)**
WWTP influent	Human Papillomavirus (HPV)	N	-qPCR -Nested-PCR	4	**La Rosa *et al.* (2013)**
Freshwater influenced by human wastewater	Faecal indicator bacteria	N	-Multiplex PCR -MiSeq Illumina	3	**Li *et al.* (2019)**
-WWTP effluent -WWTP influent	Enteropathogens	Y	Multiplex PCR	5	**Ørmen *et al.* (2019)**
River water affected by: -WWTP effluent -Human faecal samples -Animal faecal samples	NS Pathogen	Y	MiSeq Illumina	5	**Vadde *et al.* (2019)**

Source (~ = no specific source)	Pathogens (NS = Not specific)	Pathogen Community (Y/N)	Molecular method	Score (Out of 5)	Author
-WWTP influent -WWTP effluent -Rivers receiving effluent -Drinking water	Human Enteric Viruses	Y	-qPCR -Nested-PCR	5	**Iaconelli *et al.* (2017)**
Each stage of the WWTP	NS Pathogen	Y	-qPCR -IonS5	4	**Shomar *et al.* (2020)**
~~	Human enteric Viruses	N	-qPCR -RT-qPCR -RT-PCR -Nested-PCR -NGS -Microarray	2	**Osunmakinde *et al.* (2018)**
-Groundwater -WWTP effluent	-PMMoV -crAssphage, -Human Enterovirus and Adenovirus	N	-qPCR -RT-PCR	4	**Morrison *et al.* (2020)**
-River water influenced by communities -River water influenced by WWTP effluent	-Hepatitis A -Rotaviruses -Enteroviruses -Noroviruses	N	-RT-qPCR -Semi-nested PCR	4	**Sibanda and Okoh (2013)**
~~	NS viruses	N	NGS	2	**Bofill-Mas and Rusiñol (2020)**
~~	NS Pathogens	N	-qPCR -RT-qPCR -NGS -FISH -Microarray	2	**Alhamlan *et al.* (2015)**
WWTP influent and effluent	Faecal markers	N	qPCR	4	**Bunce *et al.* (2020)**
-WWTP influent -WWTP effluent pre and post chlorination -Hospital	NS Pathogens	Y	MiSeq Illumina	5	**Beattie *et al.* (2020)**

Source (~ = no specific source)	Pathogens (NS = Not specific)	Pathogen Community (Y/N)	Molecular method	Score (Out of 5)	Author
wastewater -Sediment downstream from WWTP					
WWTP AS	NS Pathogen community	Y	Pyrosequencing	2	**Guo *et al.* (2013)**
Biomass from WWTP bioreactors	NS Pathogen community	N	Illumina NextSeq500	2	**Barak *et al.* (2020)**
-WWTP influent -Primary-, secondary- and final WWTP effluent	-Human adenovirus -Norovirus	N	-qPCR -RT-qPCR	5	**Dias *et al.* (2018)**
-WWTP influent -Primary-, secondary- and final WWTP effluent	E. coli	N	Multiplex PCR	3	**Omar and Barnard (2010)**
Constructed wastewater wetlands	NS Pathogens	N	-qPCR -FISH -NGS	3	**Donde and Xia (2017)**
Various water sources either at or affected by wastewater or WWTP	-Enteric viruses -Pepper mild mottle virus	Y	qPCR	5	**Farkas *et al.* (2020)**
-Hospital wastewater -WWTP effluent -Water affected by anthropogenic pollution sources	-Total coliforms and Faecal coliforms markers	N	-qPCR -Multiplex LAMP-Au-nanoprobe	4	**Oliveira *et al*. (2020)**

2.4.4.2 Fluorescent in situ hybridization (FISH)

Fluorescent in situ hybridization uses DNA probes that recognize and bind to specific regions of the gene of interest (Bouchez *et al.*, 2016). When these probes bind to the specific region the fluorescent molecule attached to the nucleotides enables fluorescence of the organisms in question and the organism present in the sample can be counted. This method can be applied to a variety of environments for the quantification, identification and detection of certain microorganisms (Bouchez *et al.*, 2016; Garrido-Cardenas *et al.*, 2017).

2.4.4.3 Next Generation Sequencing (NGS)

NGS is a term used for to any high-throughput DNA sequencing technology, is used to identify certain DNA sequences in a sample, but the sequencing can take place in the same reaction for different DNA sequences (Rizzo and Buck, 2012). The basic principles of NGS are that it detects the addition of a new nucleotide to a template DNA strand, the difference between the different techniques of NGS is how the template DNA is produced and how they are analysed so that the sequence is revealed (Rizzo and Buck, 2012). NGS has been applied in various environments and has proven to be a very powerful tool in the detection of contamination if there is no prior knowledge of what the contamination origin is (Alhamlan *et al.*, 2015a). NGS isn't just applicable if unknown organisms need to be identified, targeted NGS can also be applied for the detection of specific organisms (Anis *et al.*, 2018).

3 ENVIRONMENTAL SAMPLING AND PRELIMINARY SAMPLE ANALYSIS

3.1 INTRODUCTION

For the purposes of this study, environmental water samples were collected from nine full-scale wastewater treatment plants (WWTPs) as well as adjacent surface water resources located in the North-West and Gauteng Provinces of South Africa. The collected samples were subjected to various methods of analysis to identify and characterise ESKAPE strains and resistant genes in the selected study areas. The data obtained was used to establish the sources, the reduction potential of ESKAPE pathogens and genes during wastewater treatment, and antibiotic resistance trends of the detected ESKAPE strains as well as their clinical relevance. Additionally, the findings from all the laboratory work was used to formulate recommendations for an environmental AMR surveillance programme. Presented in this Chapter is a description of the study sites, sampling strategy and preliminary analysis of the collected water samples to determine selected physico-chemical parameters and levels of selected antimicrobial residues.

3.2 DESCRIPTION OF SAMPLING SITES AND SAMPLE COLLECTION

3.2.1 Selection and location of sampling sites

Environmental water samples were collected from nine full-scale wastewater treatment plants (WWTPs) as well as adjacent surface water resources located in the North-West and Gauteng Provinces of South Africa. Table 3.1 is a summary of the WWTPs investigated, including their Green Drop status, main treatment processes, effluent receiving waters, as well as adjacent land use activities that may also impact the surface water resources The wastewater received in these WWTPs mainly originates from residential communities, hospitals and land uses.

Table 3.1: Summary of the WWTPs and surface water sources selected for this study

Plant ID	Purification processes	Plant capacity (Mℓ/day)	Population served	Receiving water bodies of treated effluent	Land activities existing in the area	Green Drop WWTP risk rating
WWTP B North West Province	a secondary sedimentation tank, maturation pond, and sludge dewatering units.	4.5 Ml/d	331 371	Dam, River	Anthropogenic activities, agricultural activities	High risk
WWTP C North West Province	Biofilter treatment plant	3 Ml/d	56 700	River	Agriculture	High risk
WWTP D North West Province	a secondary sedimentation tank, maturation pond, and sludge dewatering units.	24.5 Ml/d	331 371	Dam, River	Anthropogenic activities, agricultural activities	High risk

Plant ID	Purification processes	Plant capacity (Mℓ/day)	Population served	Receiving water bodies of treated effluent	Land activities existing in the area	Green Drop WWTP risk rating
WWTP E North West Province	Plant consists of 3 pathways: i. Biological treatment, ii. Biofilter treatment and iii. Activated sludge treatment	45 Ml/d	162 700	Two Rivers	Agriculture and mining	Low risk
WWTP F Gauteng	Activated sludge	7.5 Ml/d	38 970	River	Mining and anthropogenic	Critical risk
WWTP H Gauteng	a secondary sedimentation tank, maturation pond, and sludge dewatering units.	22.5 Ml/d	149 065	River	Mining and anthropogenic	High Risk
WWTP I North West Province	Activated sludge treatment	3 Ml/d	22 879	Unknown stream	irrigated agricultural activities	Critical risk
WWTP J North West Province	Activated sludge	20.4 Ml/d	28 841	River	irrigated agricultural activities	High risk
WWTP K	Activated sludge	8 Ml/d	1 467	River		High risk

The Green Drop is a regulatory program that assesses the performance of wastewater treatment plants in South Africa (DWS, 2023). It evaluates the compliance and effectiveness of these plants in meeting certain standards and regulations and reports on the functionality and status of the wastewater systems. Based on the data gathered, a Cumulative Risk Rating (CRR) is calculated to indicate the risk status of the WWTP based on 4 risk indicators: design capacity, operational (in)flow, effluent quality compliance and technical compliance (Figure 3.1). The CRR was used as one of the criteria for selecting study sites, so that the observed the performance of the WWTPs in reducing ESKAPE pathogen loads can be attributed to the design and functionality of the plant.

%CRR/CRR$_{max}$ Deviation	
	90 – 100% Critical Risk WWTW's
	70 - 90% High Risk WWTW's
	50-70% Medium Risk WWTW's
	Less 50% Low Risk WWTW's

Figure 3.1: Cumulative Risk Rating of wastewater treatment plants, indicating their compliance performance, functionality and status (DWS, 2023).

From the classification in Table 3.1 it is evident that only 1 plant was of low risk when the %CRR/CRR$_{max}$ is considered. The rest of the plants (North West Province and Gauteng) were all classified high to critical risk.

3.2.2 Sample collection

Table 3.2 below provides a concise overview of the sampling strategy employed at the 9 WWTPs across different seasons and specific sampling sites. It also outlines the seasonal information, types of samples obtained (influent, effluent, downstream) as well as the sample method employed. By collecting samples at these different stages of the WWTP (raw/untreated and final treated effluent), the study aimed to assess the presence, reduction potential during wastewater treatment and dissemination of ESKAPE pathogens into the surface water environment.

Table 3.2: Summary of information pertaining the collection of samples

WWTP	Season		Site of collection	Sample method
	Warm-Wet Season	Cold-Dry season		
C	September-November 2021	July-August 2021	Influent, Effluent	Grab
	January 2022	July 2022	Influent, Effluent, Downstream	Grab
E	September-November 2021	July-August 2021	Influent, Effluent	Grab
	January 2022	July 2022	Influent, Effluent, Downstream	Grab
D	April 2022	May-June 2022	Influent, Effluent, Downstream	Grab
F	March 2022	July 2022	Influent, Effluent, Downstream	Grab
G	March 2022	-	Influent, Downstream	Grab
H	March 2022	July 2022	Influent, Effluent	Grab
I	April 2022	May-June 2022	Influent	Grab
J	April 2022	May-June 2022	Influent, Effluent, Downstream	Grab
K	-	July 2022	Influent, Effluent, Downstream	Grab

Water samples were collected using the grab sampling method from the influent, effluent and or downstream sites of nine wastewater treatment plants as specified in Table 3.2. Briefly, sterilised 1 L bottles were marked before sample collection and all samples collected aseptically. All sample collection was performed while wearing the appropriate PPE (Gloves, disposable lab coats, masks, boots, etc. After collection, all samples were kept in sealed cooler boxes and analysed within 6 hours of collection. To ensure the safety of the research team and compliance with municipal regulations, all participants involved in the sampling process were vaccinated in accordance with the applicable guidelines. This step aimed to minimize the risk of any potential transmission of pathogens during the sampling activities.

The samples collected for analysis included influent (incoming wastewater), effluent (treated wastewater) and downstream sites. All samples collected during this study were collected in differing sampling frequencies due the permission granted by each plant. The frequency and period of sample collection plant will be described briefly.

- WWTP C and WWTP E: Sampling at WWTP C and WWTP E was performed during two distinct seasons. The Warm-Wet Season, spanning from September to November 2021, and

the Cold-Dry Season, occurring in July-August 2021. Both influent (incoming wastewater) and effluent (treated wastewater) samples were collected from these sites during each season.

- WWTP D: Sampling at WWTP-D was conducted in April 2022 and May-June 2022. For both sampling periods, influent, effluent, and downstream samples were collected. Downstream sampling was carried out at a location situated downstream of the WWTP.

- WWTP F: Sampling at WWTP-F occurred in March 2022 and July 2022. In March 2022, influent, effluent, and downstream samples were collected. The same locations were sampled again in July 2022.

- WWTP G: Sampling at WWTP-G was conducted in March 2022. Only influent and downstream samples were collected at this site.

- WWTP H: Sampling at WWTP-H followed a similar schedule as WWTPF. In March 2022, influent and effluent samples were collected. Sampling was repeated in July 2022.

- WWTP I: Sampling at WWTP-I took place in April 2022 and May-June 2022, focusing solely on influent samples.

- WWTP J: Sampling at WWTP-J was conducted in April 2022 and May-June 2022. Influential, effluent, and downstream samples were collected from this WWTP during both sampling periods.

- WWTP-K: Sampling at Site K was performed in July 2022. Similar to other sites, influent, effluent, and downstream samples were collected.

3.3 PRELIMINARY ANALYSIS OF WATER SAMPLES

3.3.1 Determination of physico-chemical water quality parameters

The physical properties (pH, temperature, total dissolved solids and electrical conductivity) of the water samples were determined on site using a calibrated Oakton PCStestrTM 35 waterproof field multi-parameter probe (Thermo Fisher scientific, US). The probe was rinsed with distilled water prior and after use. The chemical properties of the surface water systems were measured in the laboratory as milligrams per litre (mg/l) using a HACH DR 2800TM (HACH, US). Detailed results are presented in Appendix A.

3.3.2 Determination of antimicrobial residue levels in samples

Effluent and river water downstream from WWTPs C and E were collected, preserved as described in Section 3.2.2 and transported to the laboratory at the North-West University for analysis.

3.3.2.1 Antimicrobials in WWTP effluent and down-stream

A total of seven antibiotics namely ampicillin, chloramphenicol (Stigma, China), ciprofloxacin (Stigma, USA), erythromycin (Duchefa Biochemie, Netherlands), sulfamethoxazole, trimethoprim (TRC,

Canada), vancomycin (HPC Standards GmbH, Germany), one antifungal namely fluconazole (USP, Hungary) with their corresponding internal standards namely sulfamethoxazole D4, trimethoprim D3, ciprofloxacin D8, ampicillin D5 and caffeine were used to spike water samples. The HPLC-grade methanol (Honeywell, USA), acetonitrile (Honeywell, USA), formic acid (Honeywell, Germany), Milli-Q water (ELGA Purelab, UK) disodium ethylenediaminetetraacetic acid (Na 2 EDTA) (Merck, USA) and 32% hydrochloric acid (HCl) (Rochelle Chemicals, RSA) were also used.

3.3.2.2 The solid-phase extraction

The pH of the one-litre water sample was adjusted to 2 by adding 32% HCl. Furthermore, one gram of Disodium Na 2 EDTA was added. Water samples were spiked with antibiotic and antifungal compounds (for the matrix calibration curve and recovery rates) and internal standards. Water samples were filtered using solid-phase extraction (SPE-DEX system, Horizon Technology, Salem, NH, USA). The SPE-DEX system performs automated preconditioning of the hydrophilic-lipophilic balance (HLB) H disk (Atlantic disk, USA) and solid-phase extraction. The preconditioning, washing, air drying and rinsing processes are represented in Table 3.3. Analytes were evaporated using nitrogen at 37°C and reconstituted with 1 ml of 1:1 Milli-Q water-methanol and 0.1% formic acid.

Table 3.3: SPE-DEX acid extraction method

Step	**Solvent**	**Soak Time**	**Air Dry time**
Prewet 1	Methanol	1:30 min	0:30 min
Prewet 2	Methanol	1:00 min	0:05 min
Prewet 3	Reagent water	1:30 min	0:02 min
Prewet 4	pH 2 water	1:00 min	0:00 min
Sample process			
Wash 1	Reagent water	1:00 min	0:30 min
Wash 2	Reagent water	1:00 min	0:30 min
Wash 3	Reagent water	1:00 min	0:30 min
Air Dry			
Rinse 1	Methanol	1:30 min	1:00 min
Rinse 2	Methanol	1:00 min	1:00 min
Rinse 3	Acetone/Methanol (1:1)	1:30 min	1:00 min
Rinse 4	Acetone/Methanol (1:1)	1:00 min	1:30 min

3.3.2.3 UHPLC-MS

The analytes were analysed with the ultra-performance liquid chromatography (UHPLC) that consists of the Agilent 1290 infinity binary pump (G4220A), 1290 infinity autosampler (G4226A), 1290 infinity thermostatted column compartment (G1316C) coupled to an Agilent 6540 Accurate Mass Q-TOF/MS (G6540A) (Agilent Technologies, Santa Clara, CA, USA). The Poroshell 120 (Agilent Technologies, USA) 2.1×100 mm (Bonus-RP 2.7 µm) Column was used for the analyses of the standards and water samples. MS analyses of water samples were performed in the positive (ampicillin, ciprofloxacin,

sulfamethoxazole and trimethoprim) electrospray ionization (Matongo et al., 2015). The mobile phase conditions for the positive electrospray ionization were as follows; 0.1% formic acid in Milli-Q water (Mobile Phase A) and 0.1% formic acid in acetonitrile (Mobile Phase B).

3.3.2.4 Matrix calibration curve

The matrix calibration curve was conducted using eight different points of each selected antibiotic and antifungal compound. The appropriate internal standards were used to compensate for matrix effects and potential experimental errors during analysis (Kim *et al.,* 2018). The data generated from the matrix calibration curve has shown the range of the correlation coefficients (R^2) to be between 0,993 and 0,999 as indicated in Table 3.4. Furthermore, Table 3.4 shows the limit of quantification, limit of detection and the recovery rate for each selected antibiotic and antifungal. The recovery rates generally exceeded 60%, except for erythromycin which was 48,4%.

Table 3.4: Matrix calibration curve results

Compounds	R^2	LOD (ug/ml)	LOQ (ug/ml)	Recoveries (%)
Ampicillin	0.993	0.96	3.19	83.1
Chloramphenicol	0.995	0.73	2.44	73.0
Ciprofloxacin	0.999	0.09	0.30	93.7
Erythromycin	0.996	0.60	2.01	48.4
Fluconazole	0.998	0.44	1.48	71.5
Sulfamethoxazole	0.994	0.48	1.61	75.2
Trimethoprim	0.997	0.03	0.11	110.8
Vancomycin	0.998	0.91	3.04	61.8

LOQ: Limit of quantification, LOD: Limit of detection, R^2: correlation coefficients

3.4 ASSESSING THE TRENDS OF PHYSICO-CHEMICAL PARAMETERS IN WATER

Selected on-site and laboratory-based analyses were conducted at the time of sampling. The results for WWTPs C and E are shown in Figures 3.2 and 3.3, the rest of the results are represented in Appendix A. These two WWTPs (C and E) have complete data sets for all the parameters tested and results in Figures 3.2 and 3.3 is presented as log conversions. Studying the distribution of physico-chemical parameters in water samples holds significant importance for several interconnected reasons. Firstly, it offers valuable insights into the spatial and temporal fluctuations of water quality, enabling a comprehensive grasp of the surrounding environmental conditions. This knowledge could be essential in evaluating the appropriateness of water sources for diverse applications, including potable water supply, recreational pursuits, and industrial operations. Secondly, physico-chemical parameters can often have an influential effect on the proliferation of bacteria within aquatic settings. Thus, alterations in factors such as temperature, pH, dissolved oxygen, and electrical conductivity can directly influence the viability, proliferation, and metabolic functions of microorganisms, including ESKAPE pathogens. Distinct physico-chemical circumstances may either foster or hinder the development and endurance of specific strains, ultimately shaping their distribution dynamics within water environments. Influent, effluent and where possible downstream samples from WWTPs C and E were collected from July 2021 to November 2021 as well as January and July 2022 (Figures 4.1 and 4.2). Influent and Effluent samples

for the other WWTPs were collected as follow: [March 2022: F, G, H; April-June 2022: B, D, J, I; July 2022: H,F,K.] and results are depicted in Appendix A.

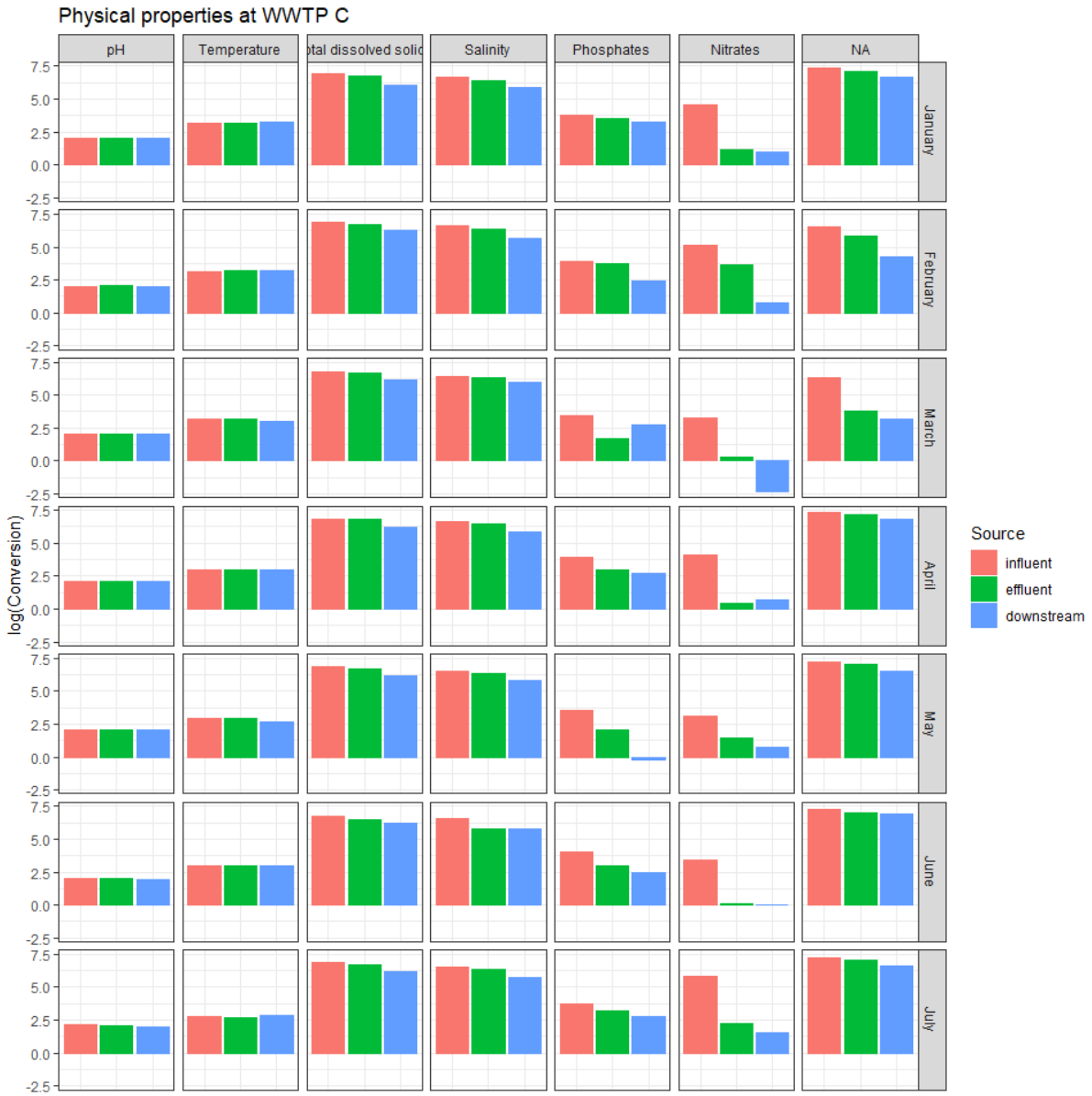


Figure 3.2: Physico-chemical characteristic of the Influent, effluent and downstream was samples from WWTP C

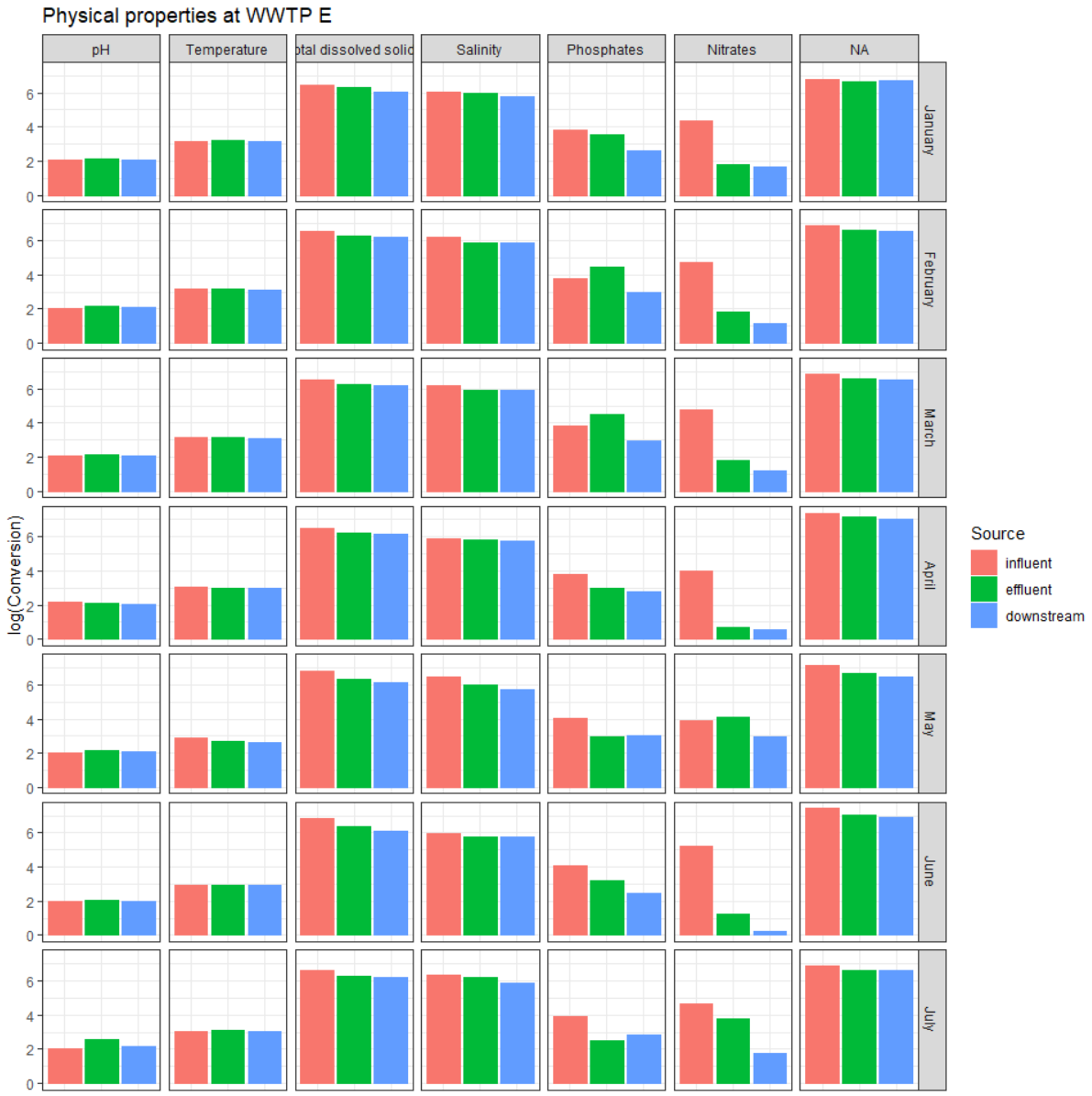


Figure 3.3: Physico-chemical characteristics of the Influent, effluent and downstream was samples from WWTP E

3.4.1 Temperature trends

The influent samples exhibited a temperature range of 14.23°C to 25.3°C across different months and plants, while the effluent samples displayed a temperature range of 7.40°C to 8.6°C during the same period. Temperature variations were observed between different months and plants. In terms of temperature (°C), WWTP C consistently recorded the highest values in both the influent and effluent. The peak temperature of 23.30 ± 0.20°C was observed in November 2020 and November 2021 for the influent and effluent, respectively. Conversely, WWTP J consistently exhibited the lowest temperatures in both the influent and effluent, with a measurement of 14.7°C in April 2022. These values were in accordance with ambient temperature conditions and could support bacterial survival.

3.4.2 pH

The influent samples displayed pH levels ranging from 7.01 to 8.39 across different months and plants, while the effluent samples exhibited pH levels ranging from 7.40 to 8.6 during the same period. Upon comparing the pH values, it is evident that they remain relatively constant across the samples, indicating minimal impact from the treatment processes. In February 2022, the influent sample from WWTP J recorded the highest pH value of 8.6, while the effluent sample from WWTP E exhibited the same pH level of 8.6 during that month. Conversely, WWTP I consistently had the lowest pH value in both the influent and effluent, with a measurement of 7.97 in April 2022. The pH values were thus relatively stable and this parameter also could support bacterial survival.

3.4.3 Total Dissolved Solids

The total dissolved solids (TDS) in the influent samples ranged from 622.7 ppm to 1143 ppm across different months and plants. In contrast, the effluent samples displayed a lower range difference, varying from 323.2 ppm to 915 ppm. This comparison indicates that the effluent generally has lower total dissolved solids compared to the influent, suggesting the removal or reduction of dissolved solids during the treatment process. Among the wastewater treatment plants (WWTPs), WWTP C consistently exhibited the highest TDS levels in the influent, reaching 1693.2 ppm in June 2022. In terms of effluent, WWTP C recorded the highest TDS value of 1335.5 ppm in April 2022. On the other hand, WWTP E consistently had the lowest TDS values in both the influent and effluent, measuring 511.0 ppm in July.

3.4.4 Salinity

The salinity levels in the influent samples ranged from 312.3 ppm to 753.33 ppm across different months and plants. On the other hand, the effluent samples showed a lower salinity range of 45 ppm to 707 ppm. Similarly, to the total dissolved solids, this indicates that the effluent generally exhibits lower salinity levels compared to the influent, implying the removal or reduction of salts during treatment. Overall, salinity of samples collected from WWTP Cs influent was higher than 400 ppm throughout, the lowest measured salt value in the influent was 495.33 ppm in December 2020 and the highest measured slat value was 736 ppm in August 2021. Regarding salinity in ppm, the WWTP C had the highest salinity values in both the influent and effluent, with a peak of 1455.3 ppm in June 2022. The WWTP J, on the other hand, exhibited the lowest salinity levels in both the influent and effluent, measuring 315.9 ppm in April 2022. The parameters, salinity and TDS are inter-related and similar trends observed should be expected. Since the North West Province is in a dolomitic area, the geology of the region will contribute towards the salt content of the waters in the province. The relatively high salinity and TDS could thus be explained by this.

3.4.5 COD

The influent samples showed a wide range of chemical oxygen demand (COD) between 11.3 mg/L and 603 mg/L across different months and plants. Conversely, the effluent samples consistently exhibited lower COD levels, ranging from 0.8 mg/L to 25.9 mg/L. This consistent pattern indicates the effective removal or degradation of organic pollutants during the treatment process, resulting in the lower COD levels observed in the effluent. Upon analysis of the results, the lowest effluent COD value obtained was 14 mg/L in April 2021, while the highest was 27 mg/L in July 2021. When comparing the COD values between different wastewater treatment plants (WWTPs), it was observed that WWTP C recorded the highest COD values in both the influent and effluent. The peak COD value of 1,595.3 mg/L was recorded in April 2022. Whereas, WWTP E consistently had the lowest COD values in both the

influent and effluent, measuring 24.9 mg/L in June 2022.

3.4.6 Phosphates (PO_4^{3-})

Orthophosphates at WWTP C ranged between 0.10 ppm and 9.46 ppm. The lowest orthophosphate value was obtained in May 2021 and the highest in February 2021. Regarding phosphates, WWTP C exhibited the highest phosphate levels in both the influent and effluent. In the influent, the highest phosphate concentration recorded was 5.38 mg/L in June 2022, while in the effluent, it reached 3.89 mg/L in April 2022. The WWTP E had the lowest phosphate values in both the influent and effluent.

3.4.7 Nitrates (NO_3-N)

In terms of nitrates, WWTP C displayed the highest nitrate concentrations in both the influent and effluent. In the influent, the highest nitrate level recorded was 21.24 mg/L in June 2022, while in the effluent, it reached 8.64 mg/L in April 2022. The WWTP E had the lowest nitrate values in both the influent and effluent, measuring 0.28 mg/L in February 2022. The influent nitrate levels in WWTP C exhibit a decreasing trend over time, with values ranging from 13.63 mg/L in September 2021 to 2.20 mg/L in September 2021. Conversely, in WWTP E, there is no discernible pattern observed for influent nitrate levels, as the values fluctuate across different months without displaying a clear trend. No available data regarding nitrate levels in WWTP I and WWTP J.

3.5 QUANTIFICATION OF ANTIBIOTIC AND ANTIFUNGAL COMPOUNDS IN WATER

Over the sampling period all the antimicrobial substances (antibiotic and fluconazole) were detected in WWTP E and WWTP C (Figure 3.4). During the wet season from October 2020 to February 2021, the measured concentrations of ampicillin, ciprofloxacin, erythromycin, fluconazole, and vancomycin were below the limit of quantification, indicating that their concentrations were very low or undetectable during this period (Tables 3.5 and 3.6). Levels of these antimicrobials were higher in the influents of wastewater treatment plants compared to the concentrations found in the wastewater effluent and up and downstream receiving waters. Furthermore, the concentrations of ampicillin and ciprofloxacin were higher in the upstream area of WWTP E. This observation suggests that the introduction of these antibiotics into the environment were by other sources, potentially agricultural activities. The ampicillin concentration was higher in the wastewater effluent of WWTP C compared to the receiving surface water downstream. It is possible that degradation of this compound takes place in the wetland preceding the downstream sampling site. The overall observations demonstrate that WWTPs are contributing to antimicrobial pollution, even though the levels are in the µg/L range. Ampicillin levels were the highest followed by sulfamethoxazole, fluconazole (antifungal agent) and ciprofloxacin. Detailed concentrations for these over the sampling period is also available.

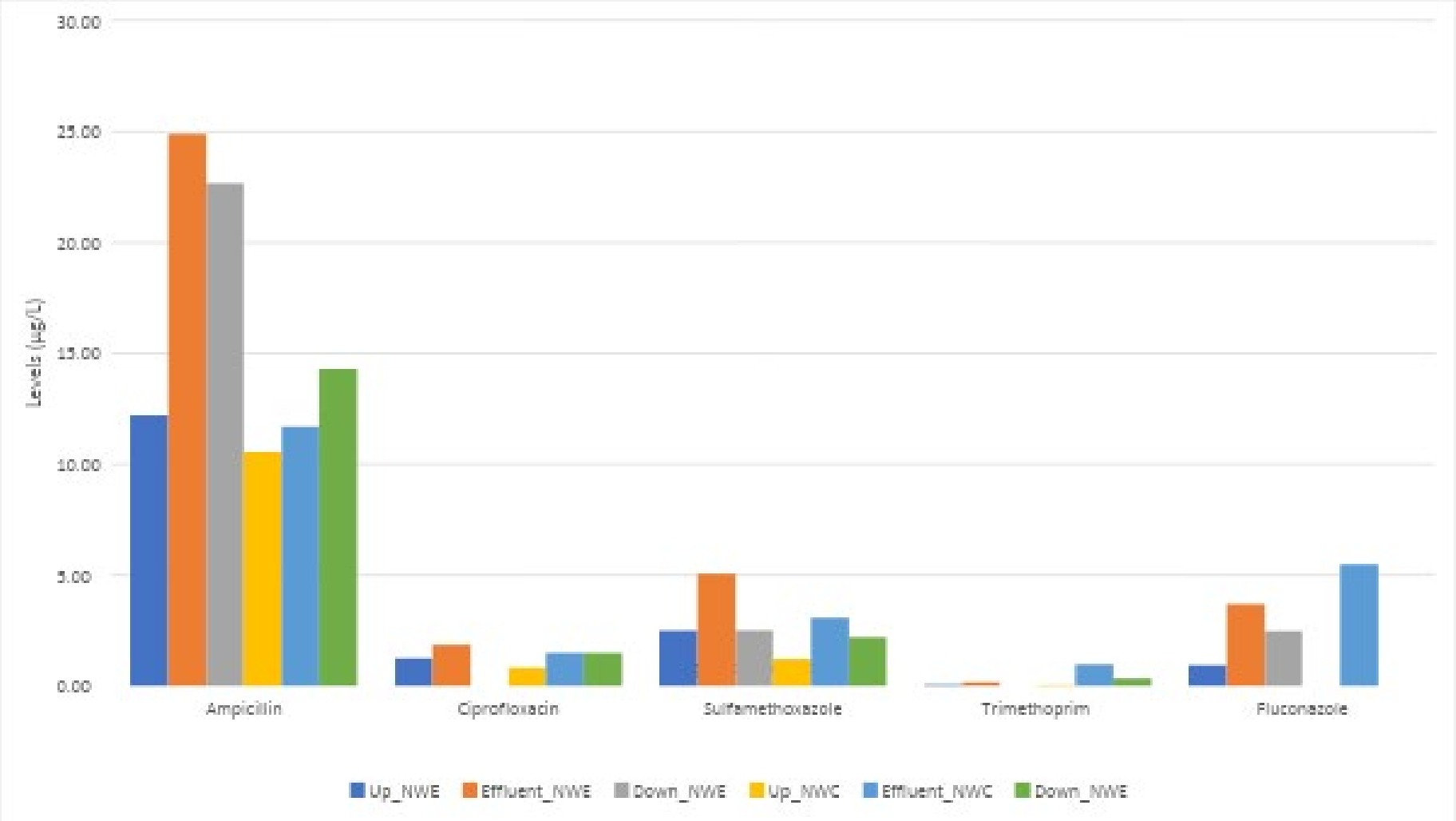


Figure 3.4: Concentrations with standard deviations of target fluconazole and antibiotic residues in WWTP NWC and WWTP NWE wastewater effluent, upstream and downstream rivers.

Table 3.5: Concentrations with standard deviations of target fluconazole and antibiotic residues in WWTP-C upstream rivers wastewater effluent, and downstream maturation pond.

Date	Sampling sites	Ampicillin (µg/L)	Ciprofloxacin (µg/L)	Sulfamethoxazole (µg/L)	Trimethoprim (µg/L)	Fluconazole (µg/L)
Nov-20	Up	15.10±4.75	<LOQ	<LOQ	<LOQ	<LOQ
	Effluent	15.80±1.86	0.77±0.14	3.74±0.03	0.24±0.02	<LOQ
	Down	16.34±0.45	1.04±0.03	3.39±0.40	0.3±0.02	<LOQ
Dec-20	Up	10.56±2.66	2.25±0.56	2.36±0.78	<LOQ	<LOQ
	Effluent	16.83±2.95	2.78±0.11	3.83±0.12	0.32±0.01	<LOQ
	Down	12.06±1.10	2.38±0.30	3.77±0.01	0.73±0.2	<LOQ
Feb-21	Up	11.29±0.86	1.87±0.17	3.76±0.22	<LOQ	<LOQ
	Effluent	12.64±0.02	3.71±1.39	3.75±0.11	0.15±0.02	<LOQ
	Down	20.94±2.56	2.01±0.33	3.77±0.05	<LOQ	<LOQ
Jul-21	Up	7.36±0.61	<LOQ	<LOQ	0.21±0.02	<LOQ
	Effluent	9.19±0.68	0.53±0.06	1.16±0.13	4.70±0.34	<LOQ
	Down	8.08±0.98	1.66±0.01	<LOQ	0.43±0.03	<LOQ
Aug-21	Up	8.54±1.33	<LOQ	<LOQ	<LOQ	<LOQ
	Effluent	12.37±2.9	0.46±0.04	6±0.78	0.48±0.06	1.3±0.05
	Down	13.99±1.11	0.43±0.05	<LOQ	0.23±0.01	<LOQ
Feb-22	Up	-	-	-	-	-
	Effluent	3.35±0.76	0.81±0.01	<LOQ	<LOQ	31.62±2.82
	Down	-	-	-	-	-

Up – upstream river; down – downstream maturation pond; LOQ – limit of quantification; ND – not done

Table 3.6: Concentrations with standard deviations of target fluconazole and antibiotic residues in WWTP-E wastewater effluent, upstream and downstream rivers.

Date	Sampling sites	Ampicillin (µg/L)	Ciprofloxacin (µg/L)	Sulfamethoxazole (µg/L)	Trimethoprim (µg/L)	Fluconazole (µg/L)
Oct-20	Up	14.46±6.32	3.06±0.13	3.36±0.12	<LOQ	<LOQ
	Effluent	11.97±1	4.03±0.34	3.51±0.13	<LOQ	<LOQ
	Down	ND	ND	ND	ND	ND
Nov-20	Up	16.86±2.23	0.59±0.17	4.04±0.28	<LOQ	<LOQ
	Effluent	21.35±1.85	0.53±0.07	3.87±0.09	<LOQ	<LOQ
	Down	ND	ND	ND	ND	ND
Dec-20	Up	14.26±4.85	0.42±0.08	3.33±1.22	<LOQ	<LOQ
	Effluent	24.94±0.11	1.96±0.50	3.82±0.04	<LOQ	<LOQ
	Down	ND	ND	ND	ND	ND
Feb-21	Up	17.81±1.48	2.38±0.13	3.85±0.20	<LOQ	<LOQ
	Effluent	22.72±1.24	4.44±0.66	3.66±0.06	<LOQ	<LOQ
	Down	ND	ND	ND	ND	ND
Jul-21	Up	6.67±0.38	1.38±0.21	<LOQ	0.21±0.01	<LOQ
	Effluent	37.77±2.15	2.17±0.37	<LOQ	0.7±0.14	1.16±0.06
	Down	ND	ND	ND	ND	ND
Aug-21	Up	3.93±0.34	<LOQ	<LOQ	<LOQ	<LOQ
	Effluent	30.25±1.52	<LOQ	1.97±0.17	0.41±0.08	<LOQ
	Down	ND	ND	ND	ND	ND
Feb-22	Up	11.37±2.28	1.00±0.13	2.86±0.23	0.20±0.02	6.60±1.61
	Effluent	25.30±6.69	<LOQ	18.57±0.63	<LOQ	24.77±0.81
	Down	22.68±1.12	<LOQ	2.5±0.45	<LOQ	17.3±0.92

Up – upstream river; down – downstream river; LOQ – limit of quantification; ND – not done

3.6 SUMMARY

Municipal wastewater contains organic materials origination from households as well as industries. In both WWTP C and E, abattoirs are to a greater (in the case of WWTP C) and lesser (in case of WWTP E) contributing to wastewater. In both cases the WWTP is also receiving untreated wastewater from clinics. Thus, observed elevated levels of these nutrients (COD, PO_4^{3-}, NO_3-N) in influent in all the plants was expected. What is important is that the WWTPs were generally reducing the levels of PO_4^{3-} and NO_3-N and lower levels were detected in the effluent. These levels of these nutrients were further reduced in the downstream ecosystems. However, the nutrients (COD, PO_4^{3-}, NO_3-N) are important for the survival of heterotrophic bacteria in aquatic systems.

From the data (Figures 3.2 and 3.3) it is evident that both WWTPs C and E could not effectively reduce the COD and the TDS. This could potentially be a treatment performance issue and may have bearing on observed spatial and temporal distribution patterns of ESKAPE pathogens. By linking the data on water quality and ESKAPE prevalence, correlations between specific physico-chemical conditions and the presence or absence of ESKAPE strains could potentially be identify. Understanding these correlations can provide valuable insights into the factors that promote or inhibit the growth and persistence of ESKAPE strains in water environments. This knowledge may be valuable for developing effective management strategies to control the dissemination of ESKAPE pathogens in water environments and minimize associated risks to human health.

Overall, the findings on antimicrobials levels demonstrate that during the wet season, the concentrations of the measured antibiotics were generally below quantifiable levels (Tables 5.3 and 5.4). This is potentially due to a dilution effect. Additionally, the detection of sulfamethoxazole and trimethoprim downstream from WWTP E and the frequent detection of trimethoprim in both WWTP E and WWTP C. These two antibiotics are synergistically used in infection treatment strategies in human and veterinary medicine.

Overall, the data highlight the presence of certain antibiotic residues in the effluent of these wastewater treatment plants and variations in their removal efficiency based on the specific antibiotic and treatment processes. Wastewater treatment plants were not designed for antibiotic removal but when these systems are effectively managed then antibiotic residues could be reduced.

The results of this study have demonstrated that antimicrobials, commonly used in medical, veterinary, and agricultural practices, can find their way into wastewater, and subsequently impact aquatic ecosystems and human health. Monitoring antimicrobial concentrations provides crucial insights into the effectiveness of wastewater treatment processes, ensuring their proper removal before discharge into the environment. By quantifying antimicrobial residues, we can assess their potential contribution to antibiotic resistance, a global health concern. Moreover, regulatory compliance and adherence to established limits for antimicrobial concentrations in treated effluent are essential to prevent adverse effects on ecosystems and water resources. This data-driven approach aids in identifying contamination sources, guiding pollution mitigation strategies, and advancing scientific understanding of antimicrobial behaviour in the environment. Ultimately, determining antimicrobial levels informs decision-making, empowers policymakers, and facilitates the development of sustainable practices that safeguard both the environment and human well-being.

4 MONITORING THE PRESENCE, LEVELS AND DISSEMINATION POTENTIAL OF ESKAPE PATHOGENS AND RESISTANT GENES IN WATER

4.1 INTRODUCTION

Monitoring the presence, levels, and dissemination potential of ESKAPE pathogens and genes in water is a critical task to safeguard public health and the environment. This Chapter addresses Aim 2 of the project, where the collected water samples were analysed for the presence and levels of ESKAPE pathogens and relevant genes using established molecular techniques such as PCR. All the selected WWTPs were included in this portion of the study. A quantitative PCR-based approach was used to further confirm the presence and determine the levels of ESKAPE pathogens and resistant genes in the samples. The observed levels of ESKAPE pathogens in the untreated wastewater (influent) and final treated wastewater effluent can provide insights into the effectiveness of the treatment processes in removing or controlling their dissemination into the natural water environment. High levels/traces of ESKAPE pathogens in the effluent samples may indicate shortcomings in the treatment methods employed, highlighting the need for improvements to ensure the safety and quality of the treated water. On the other hand, high levels of ESKAPE pathogens in surface water resources upstream a WWTP may be an indication of diffuse pollution sources.

4.2 MONITORING THE LEVELS OF ESKAPE PATHOGENS AND RESISTANT GENES USING QUANTITATIVE PCR

4.2.1 eDNA extraction for qPCR analysis

The influent and effluent were filtered within 4 hours of sample collection using 0.4 µm membrane filters (Pall, US). The filter papers were stored in sterile petri dishes and parafilmed before being placed in a -20ºC freezer overnight. The Powersoil Kit® and PowerWater Kit® manufactured by QIAGEN (Pty, Ltd) were used to extract the DNA. The PowerWater Kit® yielded more DNA and was used from February 2021. The DNA was extracted according to the manufacturer's manual.

4.2.2 Confirmation of quality and quantity of eDNA

The quality and quantity of the DNA were verified using a Thermo ScientificTM NanoDropTM 1000 Spectrophotometer (Thermo FischerScientificTM, CA, USA) as well as by 1.8% (w/v) agarose gel (SeaKem US) in 1 x TAE buffer [20 mM Acetic acid, 40 mM Tris and 1 mM EDTA at pH 8.0). The agarose gel contained 10 µl ethidium bromide (BioRad, UK). A mixture of 5 µl DNA product and 2 µl 6 x DNA loading dye (Thermo Fisher ScientificTM, US) was loaded into each well of the gel. The fragment sizes of DNA products were confirmed respectively by loading a 1kb molecular marker (O'GeneRuler, Thermo Fisher ScientificTM, US). The electrophoresis conditions were set at 80 V for 45 minutes. The gel images were captured using a ChemiDoc imager (BioRad, US).

4.2.3 Quantitative PCR to quantify the ESKAPE pathogens gene copy numbers in the extracted eDNA

The qPCR reactions were performed in a total reaction volume of 10 µl. The reaction mixture consisted of 5 µl SYBR Green MasterMix (Thermo Fisher Scientific, USA), 0.5 µl of each ESKAPE primer (forward and reverse), 2 µl of eDNA, and filled to volume with nuclease-free water. The ESKAPE primer sequences used can be seen in Table 2. Using the QuantStudio TM 3 platform and QuantStudio TM 5 (Applied Biosystems, Thermo Fisher Scientific, USA), the thermal cycling conditions consisted of a holding stage with step 1 at 50°C for 2 minutes and step 2 at 95°C for 10 minutes. The PCR stage consisted of 40 cycles with step 1 at 95°C for 15 seconds followed by a final step 2 at 60°C for 1 minute. Standard curves were generated in ten-fold dilutions with positive control samples for each target gene containing known copies ranging from 20 000 to 2.

4.2.4 Quantitative PCR to quantify the AmpC gene group copy numbers in the extracted eDNA

The qPCR reactions were performed in a total reaction volume of 10 µl. The reaction mixture consisted of 5 µl TaqMan TM Fast Advanced MasterMix (Thermo Fisher Scientific, USA), 0.5 µl of each TaqMan assay, 2 µl of eDNA, and filled to volume with nuclease-free water. For the TaqMan gene assays, the following FAM fluorescent dyes were used for quantification of the various AmpC β-lactamase gene groups: Pa04646144.s1 (ACC), Pa04646124.s1 (ACT/MIR), Pa04646135.s1 (BIL/LAT/CMY), Pa04646120.s1 (DHA), Pa04646126 (FOX) and Pa04646156. s1 (MOX/CMY). Probe sequences were not made available by Thermo Fisher Scientific. Thus, product codes of the assays were provided (Coertze and Bezuidenhout, 2020). As predetermined by the QuantStudio™ 3 platform and QuantStudio™ 5 (Applied Biosystems, Thermo Fisher Scientific, USA), the thermal cycling conditions consisted of a holding stage with step 1 at 50°C for 2 minutes and step 2 at 95°C for 10 minutes. The PCR stage consisted of 40 cycles with step 1 at 95°C for 15 seconds followed by a final step 2 at 60°C for 1 minute. Standard curves were generated in ten-fold dilutions with positive control samples for each target gene containing known copies ranging from 2 to 20 000.

4.3 RESULTS

4.3.1 Monitoring the presence and abundance of ESKAPE pathogens using qPCR

Table B.1 (Appendix B) presents comprehensive quantitative data pertaining to four ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, and Acinetobacter baumannii*) including samples from influent, effluent, and downstream receiving waters in selected wastewater treatment plants (WWTPs: B, D, F, I, J, and K). Furthermore, WWTP F directly released its influent into downstream receiving waters, while WWTP I did not have downstream receiving water system. The complete results are presented in Table B.1. From the data presented in Figure 4.1 it is evident that *K. pneumoniae* was detected in all the samples from WWTP E collected over a year sampling period (November 2020 to November 2021). With exception of the February 2021 and July 2021, all samples show that *K. pneumoniae* marker gene copy numbers/16S rRNA gene numbers were higher in the influent when compared to the effluent. The June levels were very high in the influent. Furthermore, very high levels of gene copy numbers were detected in the effluent of June 2021. The reason for this anomaly is unknown. Figure 4.2 depicts overall results for the WWTPs B, D, F, I, J, and K. Similar trends were observed in these plants where *K. pneumoniae* marker gene copy numbers/16S rRNA gene numbers were higher in the influent when compared to the effluent. From this it is evident that very high levels of *K. pneumoniae* marker gene copy numbers/16S rRNA genes were present in the influent of WWTP D.

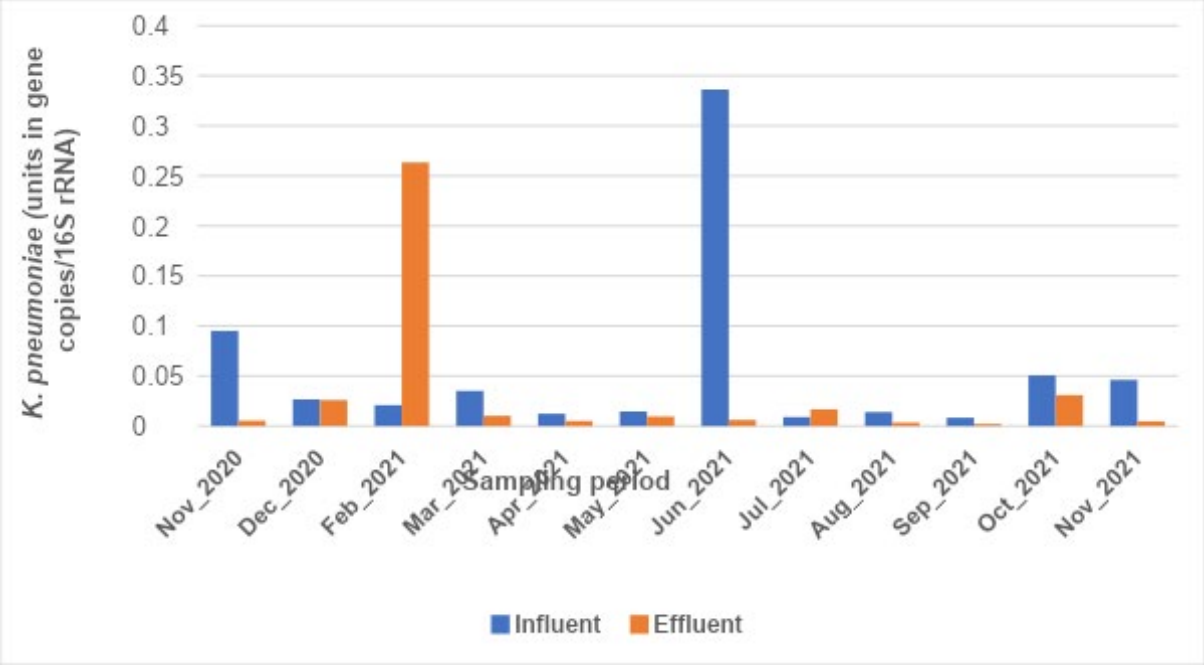


Figure 4.1: Copy numbers of *K. pneumoniae* quantified from WWTP E including influent and effluent over the sample period.

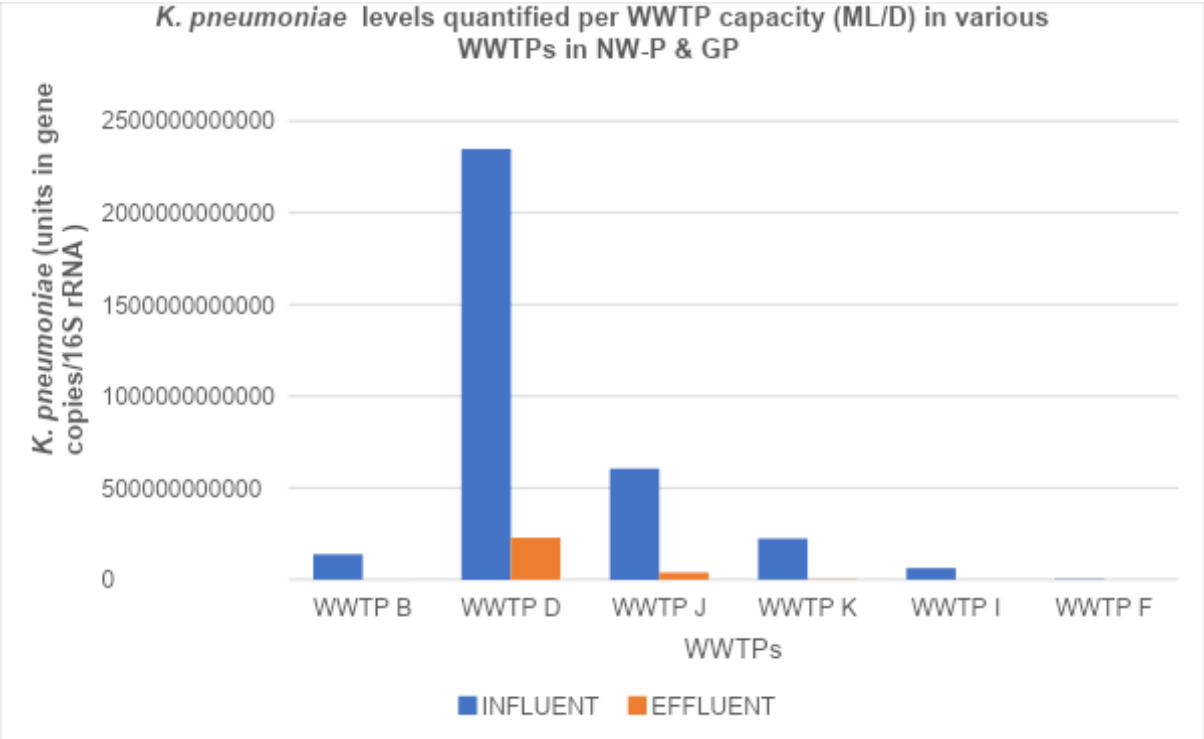


Figure 4.2: Copy numbers of *K. pneumoniae* quantified from WWTPs: B, D, F, I, J, and K including influent and effluent.

4.3.2 Monitoring the presence and abundance of antibiotic resistance gene copy numbers in water using qPCR

4.3.2.1 qPCR monitoring of antibiotic resistant genes – WWTPs C and E

In Figure 4.3 and Table 4.1 it is evident that some ARG concentrations were higher in the upstream rivers compared than those of the wastewater effluent and the downstream sampling points of WWTP C and WWTP E. It was also observed that some ARG concentrations were lower in the wastewater effluent compared to the downstream sample in WWTP C. The clinically relevant *ampC* (EBC, ACC, CIT FOX and MOX) genes were detected in all the samples. The EBC and MOX gene concentrations were generally higher compared to the other targeted genes (Figure 4.3). Overall, the WWTP effluent had higher levels of these genes compared to up and downstream sites. Exceptions were observed for CIT and MOX for WWTP C, where higher concentrations of these genes were detected in downstream samples compared to effluent. Both *intI* and *sul1* genes were also detected in the samples. The antibiotic residues for ampicillin, ciprofloxacin and sulfamethoxazole were also detect in the wastewater effluent, upstream and downstream of both WWTPs C and E and could be responsible for maintaining antibiotic resistant ESKAPE species in the system. Trends observed in the variations of residue concentrations were consistent with the variations of the ARGs detected. This is also evident in the antibiotic susceptibility results that demonstrate large percentage of ESKAPE pathogens were resistant to beta lactam antibiotics, including carbapenems. It is thus possible that the antibiotic residues supported the survival of the ESKAPE pathogens and selected for the corresponding ARGs.

4.3.2.2 qPCR monitoring of antibiotic resistant genes – WWTPs B, D, F, I, J and K

In Figures 4.4 and 4.5, ARG marker gene copy numbers/16S rRNA gene concentrations are provided. Once again generally show that influent samples had higher concentrations of the various genes (ACC, DHA, CIT [LAT/CMY/BIL] EBC [MIR/ACT],FOX, MOX/CMY, *sul1*) as well as the anthropogenic marker *intI*. However, this was not the case for WWTP B where the downstream site consistently had higher concentrations of ARGs and the *int*I. Such findings suggest that constant faecal pollution from one or other source. Furthermore, in WWTP D the levels of FOX, MOX and *sul1* was also higher in the effluent compared to the influent. Corresponding results were also observed for WWTP K for *sul1* and *intI.* These findings suggest WWTP challenges and failures.

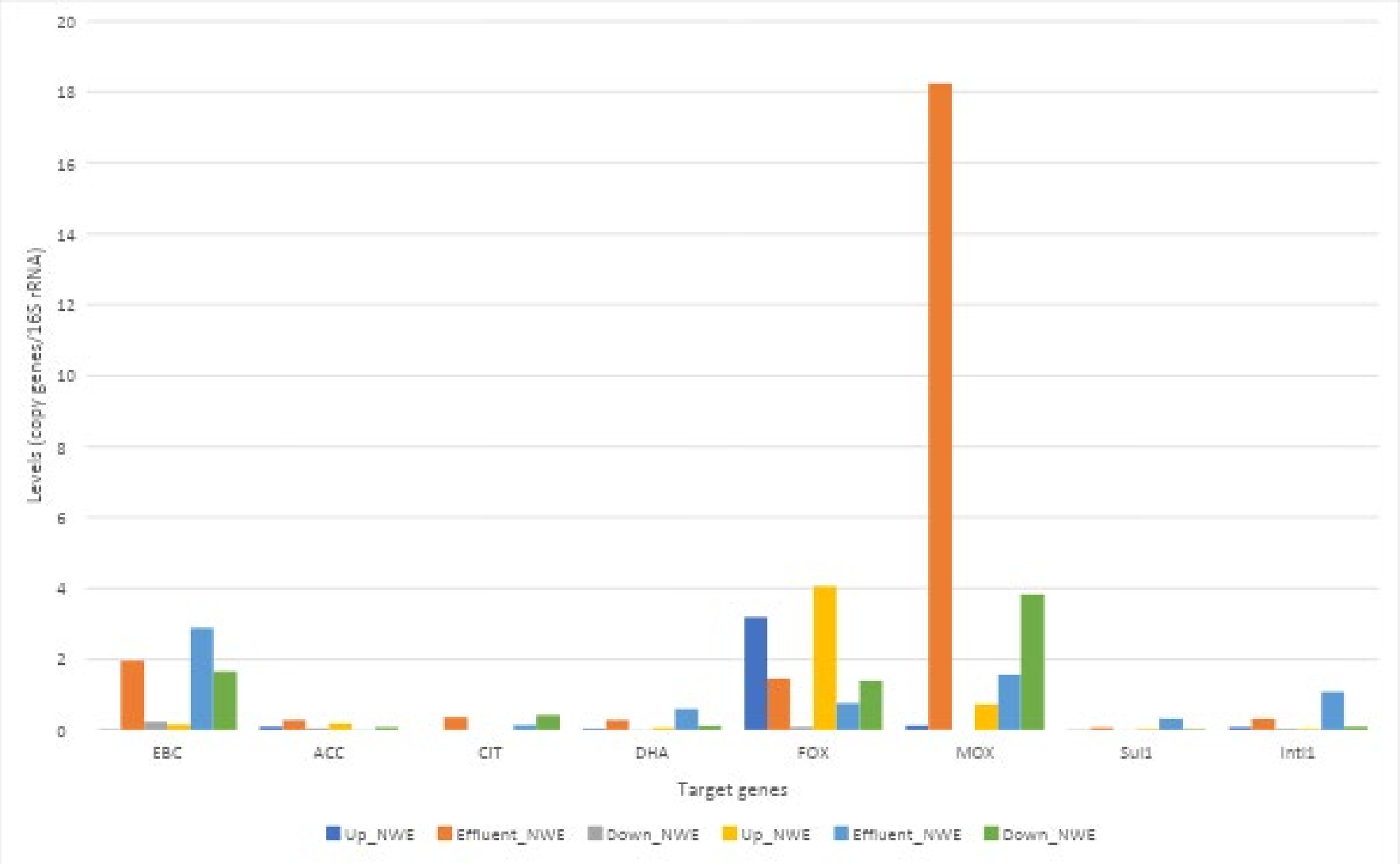


Figure 4.3: Summary of ARGs in the upstream rivers, wastewater effluent and downstream sampling points at NW-E and NW-C over the sampling period (units in gene copies/16S rRNA).

Table 4.1: Concentrations with standard deviations of ARGs in the upstream rivers, wastewater effluent and downstream points at WWTPs C and E (units in gene copies/16S rRNA ± standard deviation).

	sites	EBC	ACC	CIT	DHA	MOX	FOX	*IntI1*	*Sul1*
WWTP C	Up	$1.90\times10^{-2}\pm1.31\times10^{-2}$	$1.00\times10^{-1}\pm1.96\times10^{-1}$	-	$4.15\times10^{-2}\pm7.68\times10^{-3}$	$1.36\times10^{-1}\pm7.68\times10^{-2}$	$3.20\times10^{0}\pm6.54\times10^{-2}$	$6.61\times10^{-4}\pm1.06\times10^{-3}$	$1.16\times10^{-2}\pm1.14\times10^{-2}$
	Effluent	$1.96\times10^{0}\pm1.54\times10^{0}$	$2.90\times10^{-1}\pm3.42\times10^{-1}$	$3.71\times10^{-1}\pm3.09\times10^{-1}$	$2.90\times10^{-1}\pm1.50\times10^{-1}$	$1.82\times10^{1}\pm4.35\times10^{-1}$	$1.45\times10^{0}\pm1.24\times10^{0}$	$4.93\times10^{-4}\pm1.67\times10^{-4}$	$7.51\times10^{-2}\pm3.33\times10^{-2}$
	Down	$2.44\times10^{-1}\pm9.70\times10^{-3}$	$6.11\times10^{-2}\pm2.93\times10^{-2}$	$2.23\times10^{-3}\pm1.82\times10^{-3}$	$9.19\times10^{-3}\pm1.42\times10^{-3}$	-	$9.70\times10^{-2}\pm4.27\times10^{-2}$	$2.40\times10^{-4}\pm1.61\times10^{-4}$	$5.67\times10^{-3}\pm3.04\times10^{-2}$
WWTP E	Up	$1.65\times10^{-1}\pm1.40\times10^{-1}$	$1.97\times10^{-1}\pm2.52\times10^{-1}$	-	$6.98\times10^{-2}\pm1.40\times10^{-1}$	$7.29\times10^{-1}\pm1.61\times10^{0}$	$4.06\times10^{0}\pm5.03\times10^{-2}$	$3.30\times10^{-4}\pm2.13\times10^{-4}$	$3.88\times10^{-2}\pm3.19\times10^{-2}$
	Effluent	$2.87\times10^{0}\pm5.18\times10^{0}$	$8.34\times10^{-3}\pm7.76\times10^{-3}$	$1.50\times10^{-1}\pm2.76\times10^{-1}$	$5.98\times10^{-1}\pm5.66\times10^{-1}$	$1.58\times10^{0}\pm9.37\times10^{0}$	$7.57\times10^{-1}\pm1.42\times10^{-2}$	$4.09\times10^{-4}\pm1.91\times10^{-4}$	$3.30\times10^{-1}\pm2.76\times10^{-2}$
	Down	$1.66\times10^{0}\pm9.54\times10^{-1}$	$8.61\times10^{-2}\pm1.56\times10^{-2}$	$4.28\times10^{-1}\pm4.16\times10^{-1}$	$1.31\times10^{-1}\pm5.43\times10^{-2}$	$3.83\times10^{0}\pm1.12\times10^{-1}$	$1.38\times10^{0}\pm1.90\times10^{-2}$	$5.38\times10^{-4}\pm8.38\times10^{-}$	$3.16\times10^{-2}\pm2.47\times10^{-2}$

LOD – limit of detection; ND – not done; "-" not detected by end-point PCR

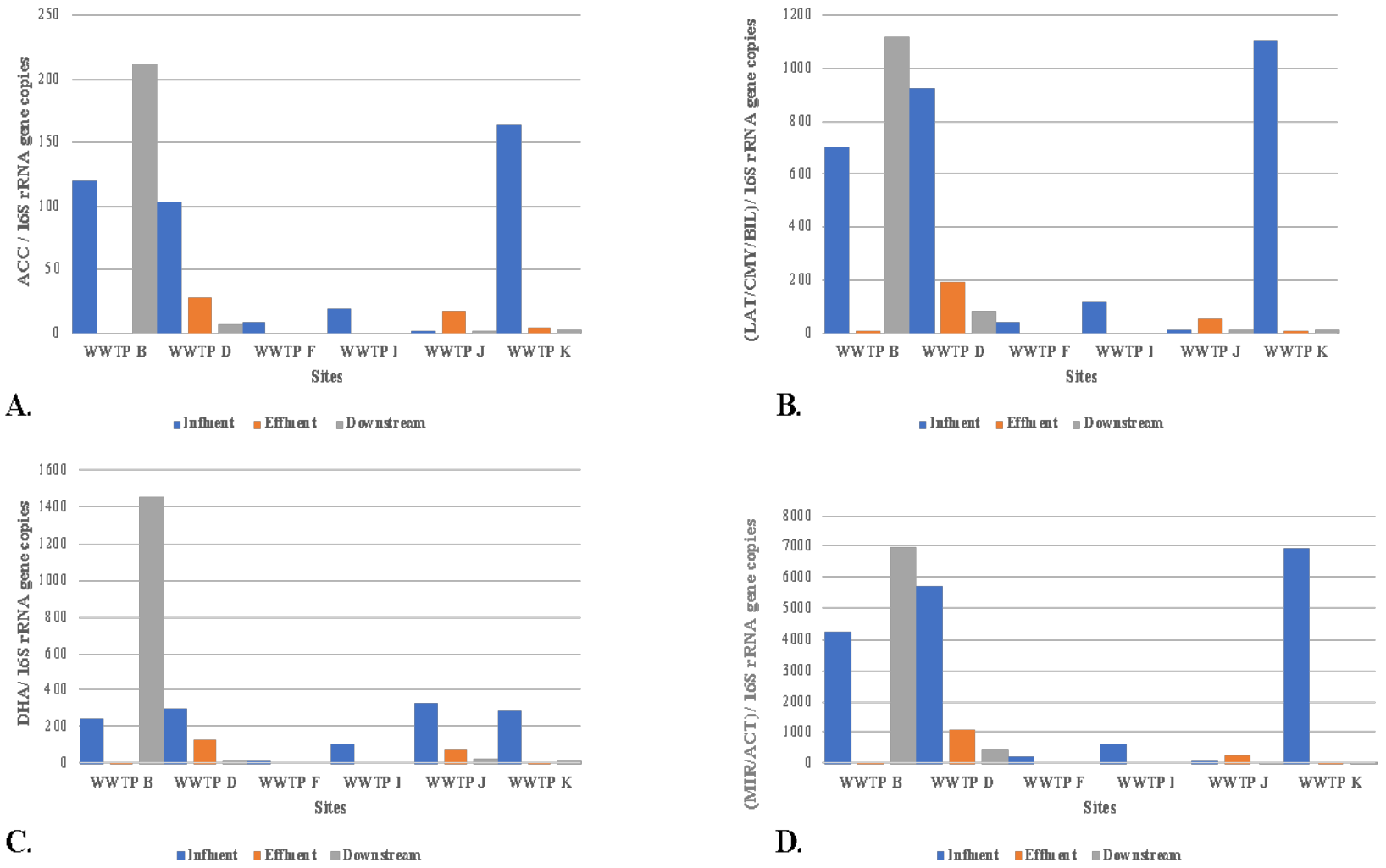
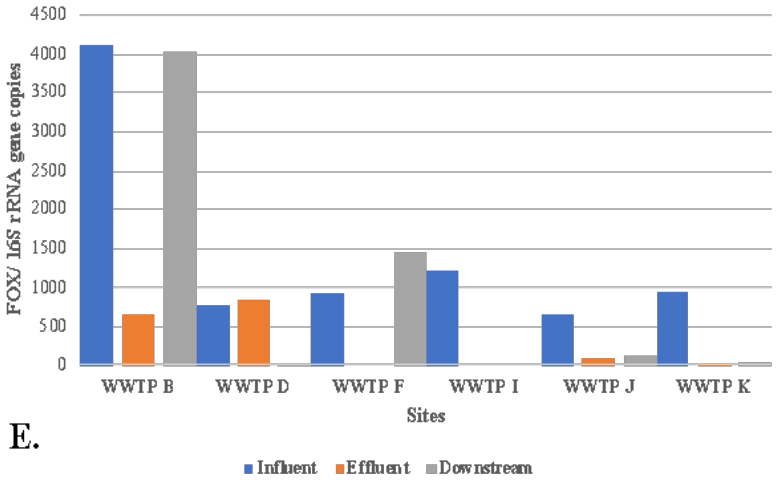
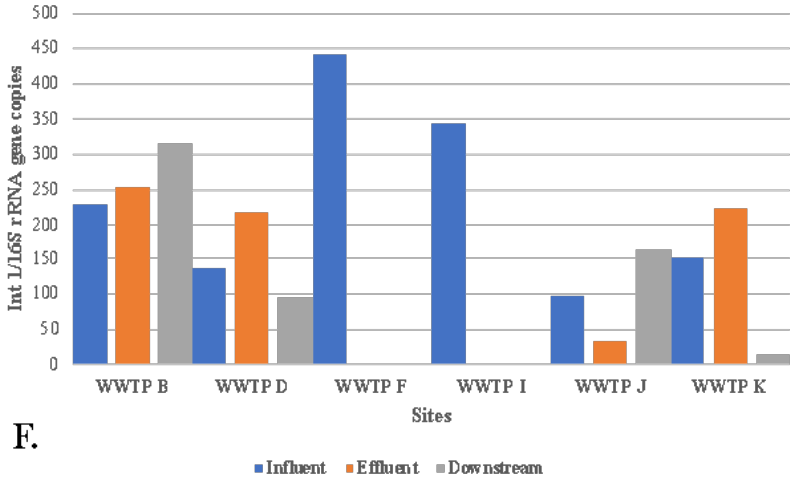
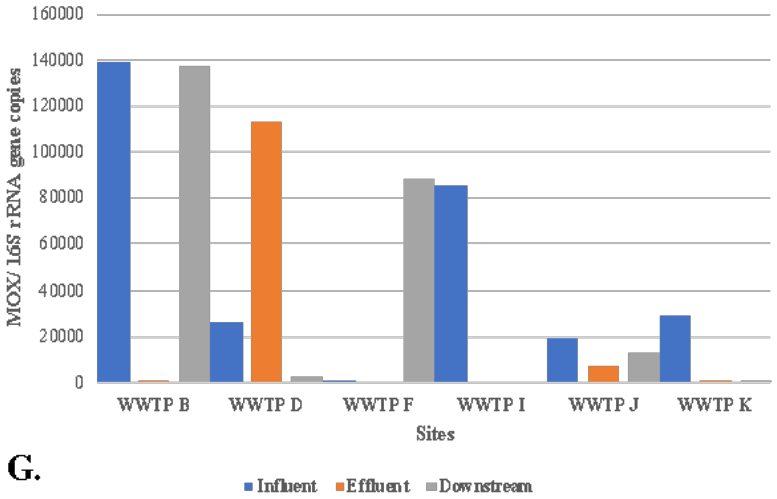


Figure 4.4: A graphical representation of the average total ARGs (gene copies/16S rRNA) in various WWTPs and their receiving waters. (A)- depiction of the *ACC* gene , (B)- *CIT (LAT/CMY/BIL)* gene, (C)- *DHA* gene, (D)- *ECB (MIR/ACT)* gene.







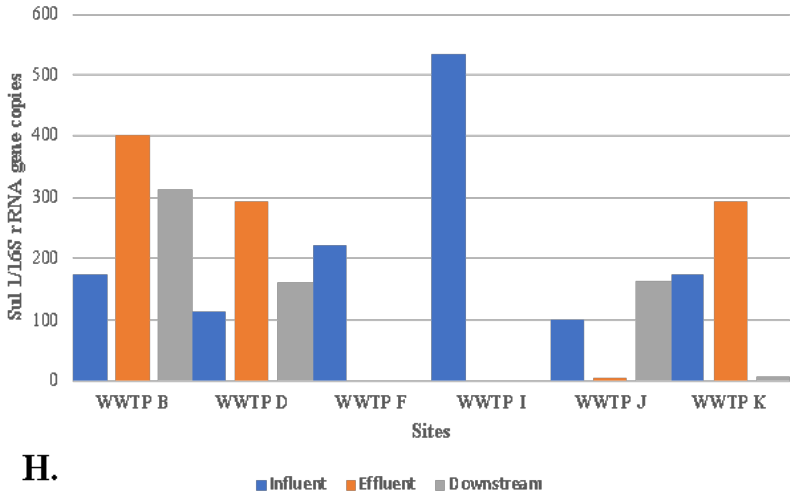


Figure 4.5: A graphical representation of the average total ARGs (gene copies/16S rRNA) in various WWTPs and their receiving waters. (E)- *FOX* gene, (F)- *Int1* gene, (G)- *MOX/CMY* gene and (H)- *Sul1* gene.

4.4 SUMMARY

This chapter focused on the determination of ESKAPE levels in water using qPCR. The aim of this section was to assess the presence and abundance of ESKAPE bacteria. Quantitative analysis of gene copy numbers using qPCR provided detailed insights into the prevalence and persistence of ESKAPE pathogens across different WWTPs. Some plants exhibited higher levels of specific ESKAPE species in effluent, indicating incomplete removal during treatment. *Klebsiella pneumoniae* and *Acinetobacter baumannii* displayed notable influent presence. *K. pneumoniae* marker gene levels were used to demonstrate the principle of the dynamics of ESKAPE species in WWTP systems. The data show general reduction of this and other species from influent to effluent. There were some exceptions. This chapter highlights the pivotal role of qPCR as a powerful tool for the monitoring or microbial ESKAPE species in water systems.

The antibiotic residues for ampicillin, ciprofloxacin and sulfamethoxazole were also detected in the wastewater effluent, upstream and downstream of both WWTPs C and E (Section 3.5) and could be responsible for maintaining antibiotic resistant ESKAPE species in the system. Trends observed in the variations of residue concentrations were consistent with the variations of the ARGs detected. This is also evident in the antibiotic susceptibility results that demonstrate large percentage of ESKAPE pathogens were resistant to beta lactam antibiotics, including carbapenems. It is thus possible that the antibiotic residues supported the survival of the ESKAPE pathogens and selected for the corresponding ARGs.

Furthermore, qPCR was successfully used to detect and quantify antibiotic resistance genes in the various compartments (influent, effluent, upstream and downstream) from several WWTPs. The data demonstrate that overall, the WWTP effluent had higher levels of these genes compared to up and downstream sites. There were some exceptions. At some WWTP ecosystems higher levels of some of these genes were found in the effluent or downstream, suggesting poor operations at these plants. This could have detrimental impacts on ecological health. These findings underscore the importance of tailored treatment strategies and improvement of treatment processes to slow down the spread of antibiotic resistance.

5 CHARACTERISATION OF THE ANTIBIOTIC RESISTANCE TRENDS OF SELECTED ESKAPE PATHOGENS IN WATER ENVIRONMENTS AND THEIR CLINICAL RELEVANCE

5.1 INTRODUCTION

In the realm of environmental and public health, assessment of wastewater has gained significant recognition and importance. The latter involves the analysis of wastewater samples to gain insights into the quality of the treated effluent as well as its the possible impacts on the surrounding ecosystem and human health. The aim of this chapter is to establish antibiotic resistance trends of selected ESKAPE pathogens in water environments and determine their clinical relevance (Aim 4).Determining the actual abundance of these pathogens in the environment, levels of antimicrobial residues, and antibiotic susceptibility testing uncovers the intricate connection between bacteria and antibiotics, guiding healthcare practitioners in selecting the most appropriate treatment options. Analysis of virulence factors gives insight into the mechanisms that enable bacteria to cause diseases. Testing ESKAPE isolates for virulence factor production is important to demonstrate the pathogenic potential of such species isolated from aquatic environments (WWTP influent and effluent as well as receiving waters).

To achieve this aim, water samples were collected from multiple wastewater treatment plants (WWTPs) specified in the study. Both culture based and molecular methods were used to isolate, identify and confirm the presence and abundance of presumptive ESKAPE pathogens in the water samples collected. Initially, the collected samples were cultured on selective media, identifying the targeted presumptive pathogens and the determining their abundance. After that, a PCR-based approach was used to further identify or confirm the presence of the ESKAPE isolates. Antibiotic susceptibility testing on the presumptive ESKAPE strains was then performed to determine their resistance profiles. Briefly, the antibiotic resistance analysis utilized the antibiotic diffusion method on Mueller-Hinton agar, measuring inhibition zones around antibiotic discs to determine antibiotic susceptibility. Antibiotic resistance profiles of individual ESKAPE pathogens were determined using specific sets of antibiotics commonly used for each species. To assess pathogenicity potential, selected virulence factors including haemolysin, lipase, proteinase, and DNase were examined using various enzymatic detection methods. The clinical relevance of the antibiotic resistance trends was assessed by comparing the observed data to clinical data. This assessment can assist in determining if the resistance patterns observed in water environments correlate with clinical infections and patient outcomes.

The results obtained from the analysis of physico-chemical parameters (Section 3.4) and levels of presumptive ESKAPE pathogens in both the influent and effluent water was used to provide insights on how well the treatment plants are functioning in terms of their treatment processes. The physico-chemical parameters, such as temperature, pH, dissolved oxygen, and electrical conductivity, can serve as indicators of the efficiency of the treatment processes. Deviations from standard values may indicate potential operational issues or inefficiencies within the plants. Overlaying these data sets with data on the levels of antibiotic residues in the water (Section 3.5) was used to provide valuable insights on the contribution of antibiotic residues in water as selective pressures for development of antibiotic resistance, as well as the removal capacity (ESKAPE pathogen genes, antibiotic resistance genes and antibiotic residues) of wastewater treatment plants. Furthermore, clinical relevance of the observed

strains and ARGs could also be revealed.

5.2 METHODS

5.2.1 Determination and confirmation of presumptive ESKAPE levels

5.2.1.1 Control cultures and media

E. faecium (MCC 2763), Methicillin-resistant *Staph aureus* (ATCC 33591, MTCC 1430), *Klebsiella pneumonia* (ATCC 35657, MTCC 432), *A. baumannii* (ATCC 19606, MTCC 1920), *P. aeruginosa* (ATCC 27853, MTCC 1688), *E. aerogenes* (MTCC 111) and *Enterobacter species* (MCC 2296) were used as control cultures.

5.2.1.2 Isolation and identification of presumptive ESKAPE pathogens

For the influent and effluent samples, a dilution series was used and the effluent filtered using the membrane filtration method. Selective media were used to determine the levels and isolate the various ESKAPE bacteria. These media include *Enterococcus faecium* chromoselect agar, *Staphylococcus aureus* CHROMagar and Mannitol salt agar (MSA), *Klebsiella* chromoselect agar, *Acinetobacter* CHROMagar, cetrimide for *Pseudomonas aerogenosa*, m-ENDO and m-FC agar for *Enterobacter* spp. All enumerated isolates were counted and their levels recorded.

5.2.1.3 Media characteristics of the various ESKAPE spp.

Incubation is at 37°C for 24 hours. *Enterococcus faecium* green colonies on chromeselect agar change the agar from red to yellow. The *Staphylococcus aureus* on the other hand, grow in pinkish colonies on the S. aureus CHROMagar. When transferred to MSA media colonies change the red media to a yellow (Ali, 2019; Sharp and Searcy, 2006). On K*lebsiella* chromoselect agar the *Klebsiella pneumonia* colonies appear violet and have a mucous appearance (Bruce et al., 1981). *Acinetobacter* CHROM agar have red colonies indicating putative presence of *Acinetobacter baumanii*. On cetrimide agar *Pseudomonas* form yellow-green to blue colonies (Millipore, 2018). On the m-FC agar *Enterobacter spp.* form blue to dark-blue and grey to grey-blue colonies (DB, 2020). When transferred to m-ENDO plates dark pink colonies appear. For purification putative ESKAPE pathogens were purified by streak plating for at least 3 times after which Gram Staining is performed to determine the purity of the isolates. If still contaminated, then further streak plating is done.

5.2.1.4 Confirmation of presumptive ESKAPE species using molecular based methods

For the ESKAPE species identification, the V1-V9 region of the 16S rRNA gene was amplified using two commonly employed universal primers for bacterial identification: the 27F and the 1492R primers (Frank et al., 2008; Johnson et al., 2019). Subsequent to the completion of the 16S PCR, a purification step was executed to refine the PCR products. The initial purification involved the utilization of AMPure magnetic beads and 70% ethanol, aiming to eliminate excess salts, enzymes, primers, and nucleotides following the primary PCR reaction, thereby enhancing the purity of the PCR product (Beckman Coulter, 2016).Sequencing PCR was then undertaken with the 27F primer and the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, 2010). Upon the conclusion of the sequencing PCR, the ultimate PCR product underwent purification via the CleanSEQ Dye-terminator removal protocol (Beckman

Coulter, 2017). This dye terminator removal procedure entails the use of CleanSEQ magnetic beads and 85% ethanol. Following the eradication of dye terminators and the purification of the product, sequencing was executed utilizing a SeqStudio Sanger sequencer. The resulting sequence data was then cross-referenced with the BLAST databases to confirm the organism's identity.

5.2.2 Antibiotic susceptibility testing (Phenotypic analysis)

The antibiotic resistance phenotypes and patterns were determined using the antibiotic diffusion method. Briefly, pathogens were suspended in Mueller-Hinton (MH) agar by spread plating on the media and allowing it to dry. Antibiotic discs were placed on the inoculated plate and then incubated at 35˚C for 24 hours. Specific sets of antibiotics were used for each individual species. The zones around the antibiotic discs were measured and compared to standard values to ascertain the organism's susceptibility, intermediate resistance, or resistance to the antibiotics (Hudzicki, 2009). Below are the procedures followed for each pathogen:

- For *Enterococcus faecium*, the antibiotic resistance profiles were tested using ampicillin 10 µg, chloramphenicol 30 µg, erythromycin 15 µg, kanamycin 30 µg, tetracycline 30 µg, penicillin G 10 µg, gentamycin 10 µg, and vancomycin 30 µg (Özmen Toğay *et al.,* 2010).

- *Staphylococcus aureus* resistance was tested with ampicillin 10 µg, methicillin 5 µg, erythromycin 15 µg, gentamycin 10 µg, ciprofloxacin 5 µg, tetracycline 30 µg, and chloramphenicol 30 µg (Kitara *et al.,* 2011).

- *Klebsiella pneumoniae* resistance was tested with ampicillin 10 µg, amikacin 30 µg, cefazoline 30 µg, cefotaxime 30 µg, ceftriaxone 30 µg, cotrimoxazole 25 µg, imipenum 10 µg, gentamycin 10 µg, nitrofurantoin 300 µg, norfloxacin 10 µg, and ofloxacin 5 µg (Subedi *et al.,* 2016).

- *Acinetobacter baumannii* resistance was tested with amikacin 10 µg, cefotaxime 30 µg, ceftriaxone 30 µg, ceftazidime 30 µg, cefepime 30 µg, ciprofloxacin 5 µg, meropenem 10 µg, and gentamicin 10 µg (Sinha *et al.,* 2007).

- *Pseudomonas aeruginosa* resistance was tested with ampicillin 10 µg, amoxicillin 10 µg, chloramphenicol 30 µg, ciprofloxacin 5 µg, gentamicin 10 µg, kanamycin 30 µg, nalidixic 30 µg, polymyxin 300 µg, tetracycline 30 µg, and trimethoprim 2.5 µg (Odjadjare *et al.,* 2012).

- *Enterobacter* resistance was tested with ampicillin 10 µg, chloramphenicol 30 µg, cephalothin 30 µg, cefepime 30 µg, gentamicin 10 µg, nitrofurantoin 300 µg, netilmicin 30 µg, and levofloxacin 5 µg (Parra-Flores *et al.,* 2018).

5.2.3 Determining pathogenicity potential

Pathogenicity potential was determined using selected virulence factors. These are extracellular enzymes including haemolysin, lipase, proteinase and DNase. For proteinase a mixture of skim milk, brain heart broth and agar was be used (Venter, 2010). Clear zones are detected if the isolates produce the enzyme. DNase can be detected using DNase agar that is supplemented with toluidine blue O after incubation it the agar is flooded with HCl to reveal the positive zones. Lipase production was detected by using tryptone soy agar that is supplemented with Tween 80. For the detection of haemolysin blood agar plates are used, after incubation clear zones forms on the plates if haemolysin is produced (Venter,

2010).

5.2.4 Data analysis

Excel 2016 Version 16.0.6828.1019 was used to draw up all the tables and some of the graphs. Heat maps that represent the resistance patterns of each of the different presumptive ESKAPE organisms were drawn up by using Statistica version 14.0.1 published by TIBCO Software Inc. Canoco version 5.12 was used for the multivariate statistical analysis of the results. A redundancy analysis (RDA) was done by drawing RDA plots, which involves a form of multivariate statistical analysis that can be used to analyse information so as to represent genotypical and phenotypical data (Capblancq & Forester, 2021). RDA plots generated for this study were used to illustrate the similarities between the different sampling locations and the similarities in the grouping of the organisms at the sampling sites.

5.3 RESULTS

5.3.1 Assessing the trends of presumptive ESKAPE pathogens in water

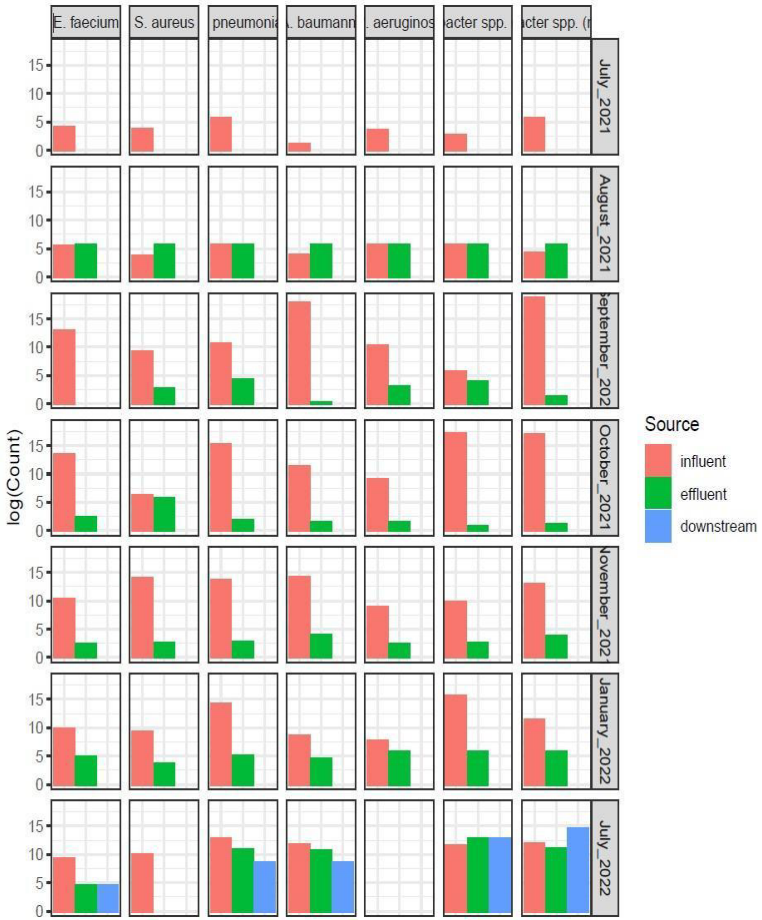
Figures 5.1 to 5.5 show the results obtained for the occurrence and levels of ESKAPE pathogens in water environments, as well as their removal during wastewater treatment. Figure 5.1 shows the observed levels of various ESKAPE pathogens for WWTPs C and E for the periods of July 2021-January 2022 as well as July 2022. These results indicate that levels of putative ESKAPE pathogens could be quite high in both influent, effluent and downstream sites of WWTPs C and E. The data from the rainy season (October to January) also demonstrates that the levels in the influent were considerably higher than the effluent. This was depicted by a 3 to 5 Log reduction in some cases. Also, there is a general reduction in ESKAPE levels from influent to effluent except for *Enterobacter* levels during June 2022 at WWTP C and *S. aureus* levels for WWTP E during July 2021.

The levels of various ESKAPE pathogens for WWTPs B, D, G, H, I and K are provided in Figures 5.2 to 5.5. These results indicate levels of putative ESKAPE pathogens during the dry, low-flow season (May to August) were generally low. For most plants there was general decrease in ESKAPE levels from the influent to the effluent samples. However, during June (2022) considerably higher levels of *Enterobacter* spp. were observed at WWTP B in the effluent as compared to influent.

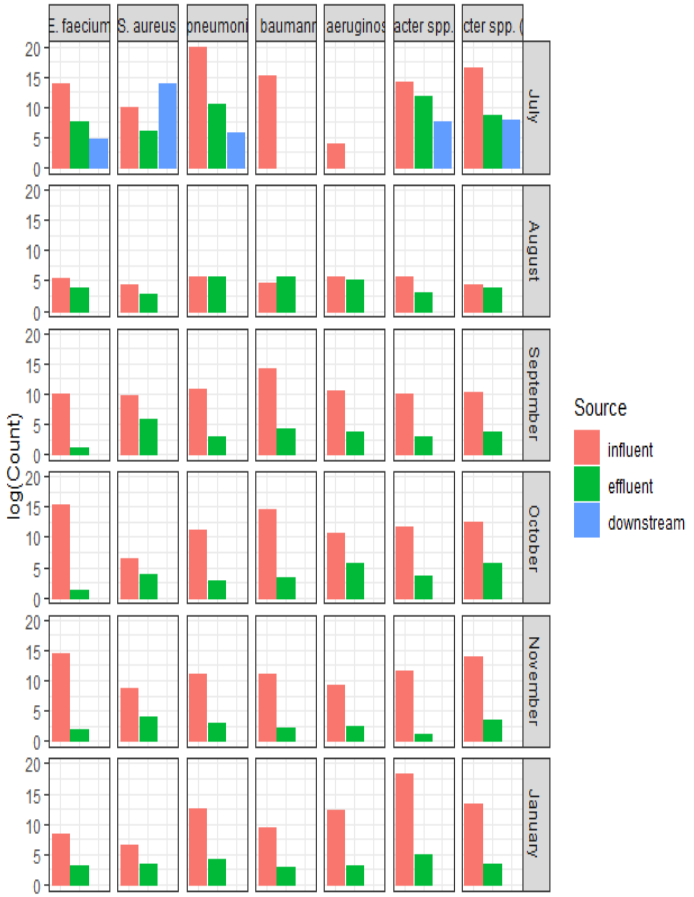
Among the analysed ESKAPE pathogens, *Klebsiella pneumoniae* exhibited the highest counts in several instances. WWTP E in July 2022 recorded an influent count of 1.3 x 10^6 cfu/ml while WWTP G in March 2022 had the highest influent count of 2.8 x 10^6 cfu/ml. This suggests that these two WWTPs may have a significant source of contamination for *K. pneumoniae*. Another notable pathogen with high counts was *Acinetobacter baumannii*, with WWTP H in July 2022 recording the highest influent count of 5.7 x 10^6 cfu/ml. The presence of this pathogen underscores the need for improved treatment processes at WWTP H to mitigate its presence. *Enterobacter spp.* (m-FC) also exhibited notable levels, with WWTP B in June 2022 recording the highest influent count of 4.2 x 10^6 cfu/ml, followed by WWTP J in April 2022 with 9.0 X 10^6 cfu/ml. Both plants should pay attention to the presence of *Enterobacter* spp. (m-FC) during their treatment processes.

On the other hand, *Pseudomonas aeruginosa* consistently showed low counts or no presence across all plants and sampling points, indicating effective removal or reduction during treatment. *Staphylococcus aureus* displayed variations across different plants, but effluent samples often recorded no counts. *Enterococcus faecium*, in some cases, was not detected in effluent samples. Not detecting any of the pathogens on selective media could be due to removal or inability to grow (viable but non-

culturable) or removal of the pathogens..



WWTP C



WWTP E

Figure 5.1: Levels of presumptive ESKAPEs in influent, effluent and downstream sites of WWTPs C and E for the duration of the study.

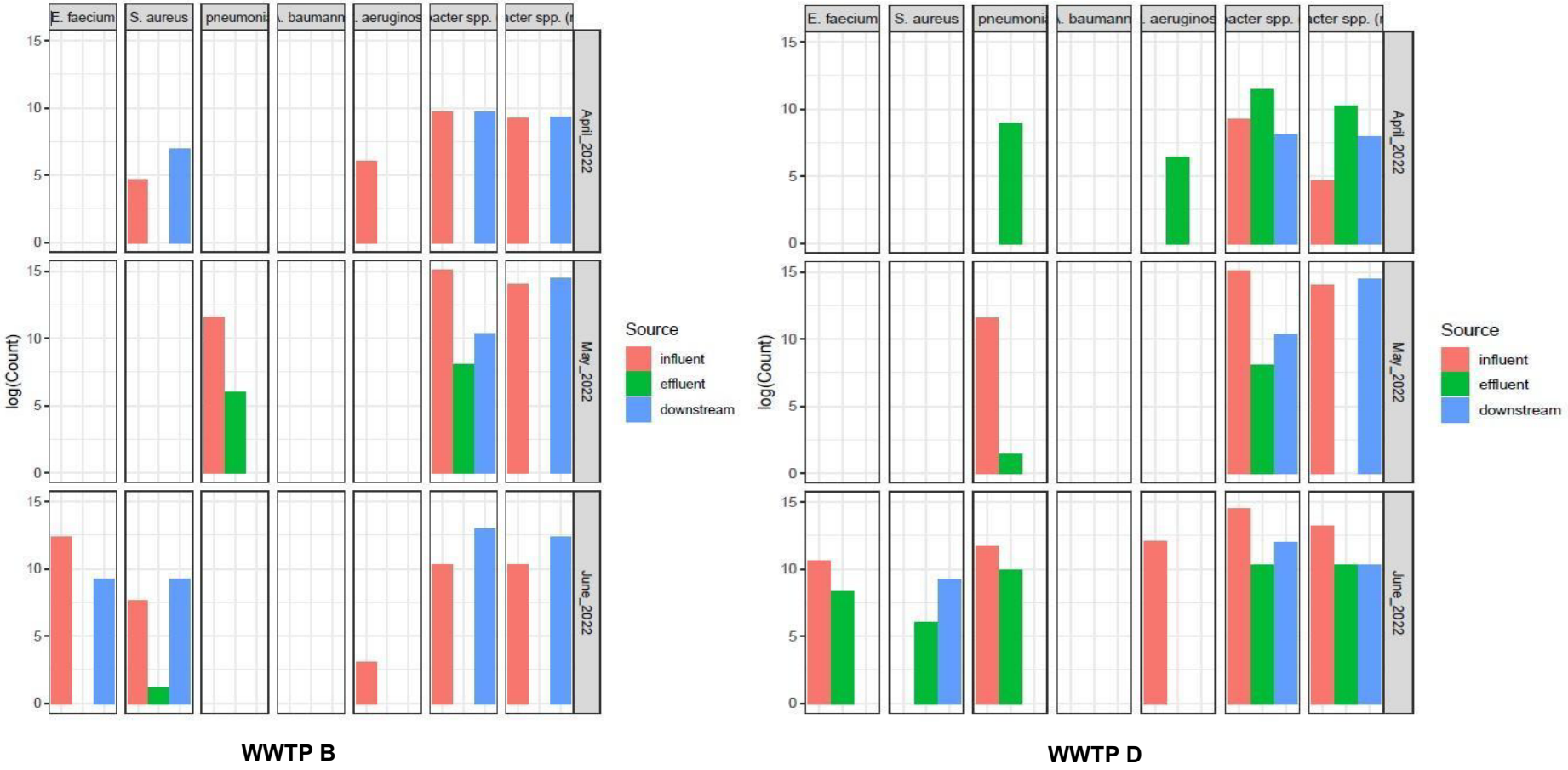


Figure 5.2: Levels of presumptive ESKAPEs in influent, effluent and downstream sites of WWTPs B and D for the duration of the study.

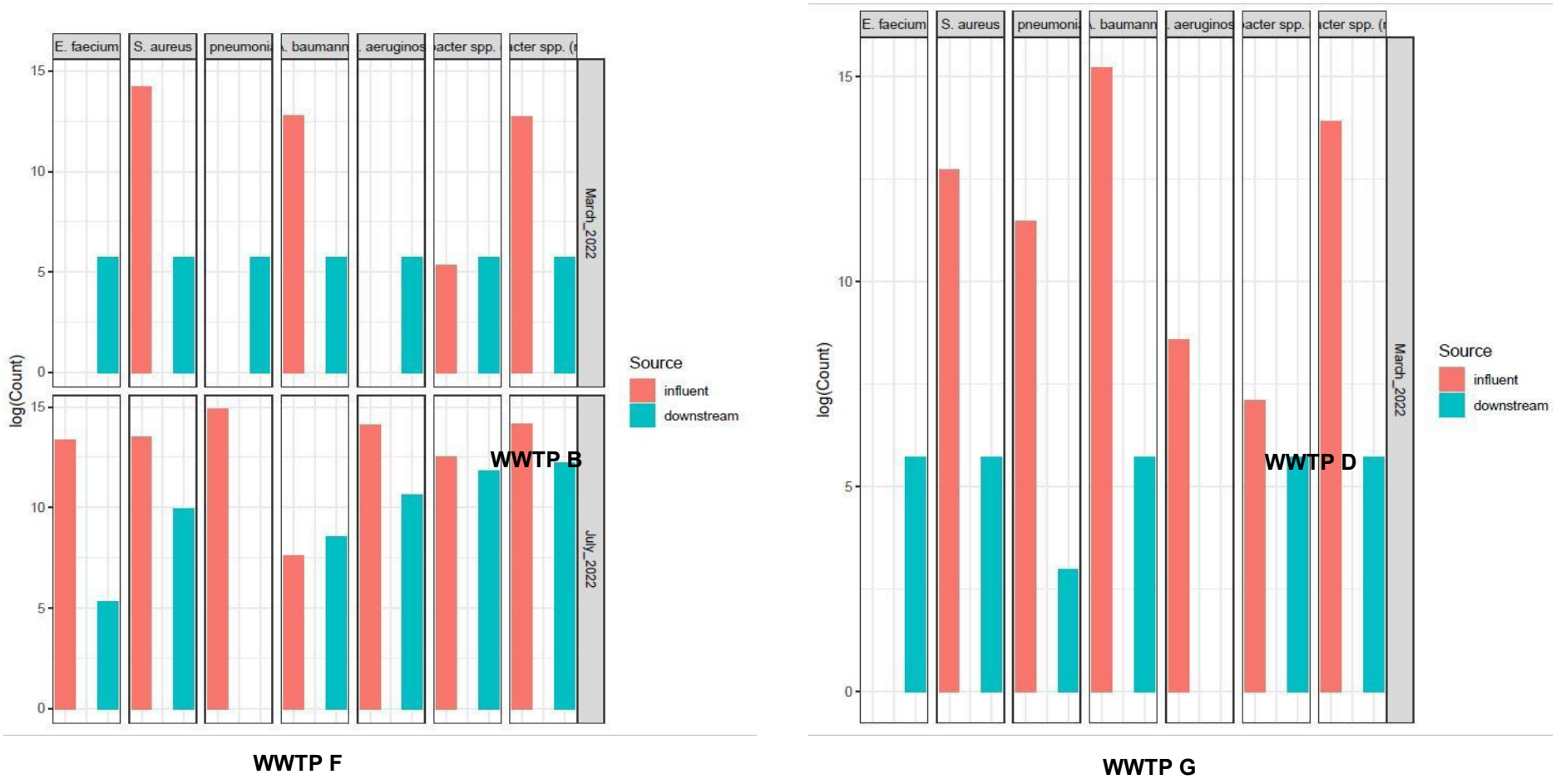


Figure 5.3: Levels of presumptive ESKAPEs in influent, effluent and downstream sites of WWTPs F and G for the duration of the study.

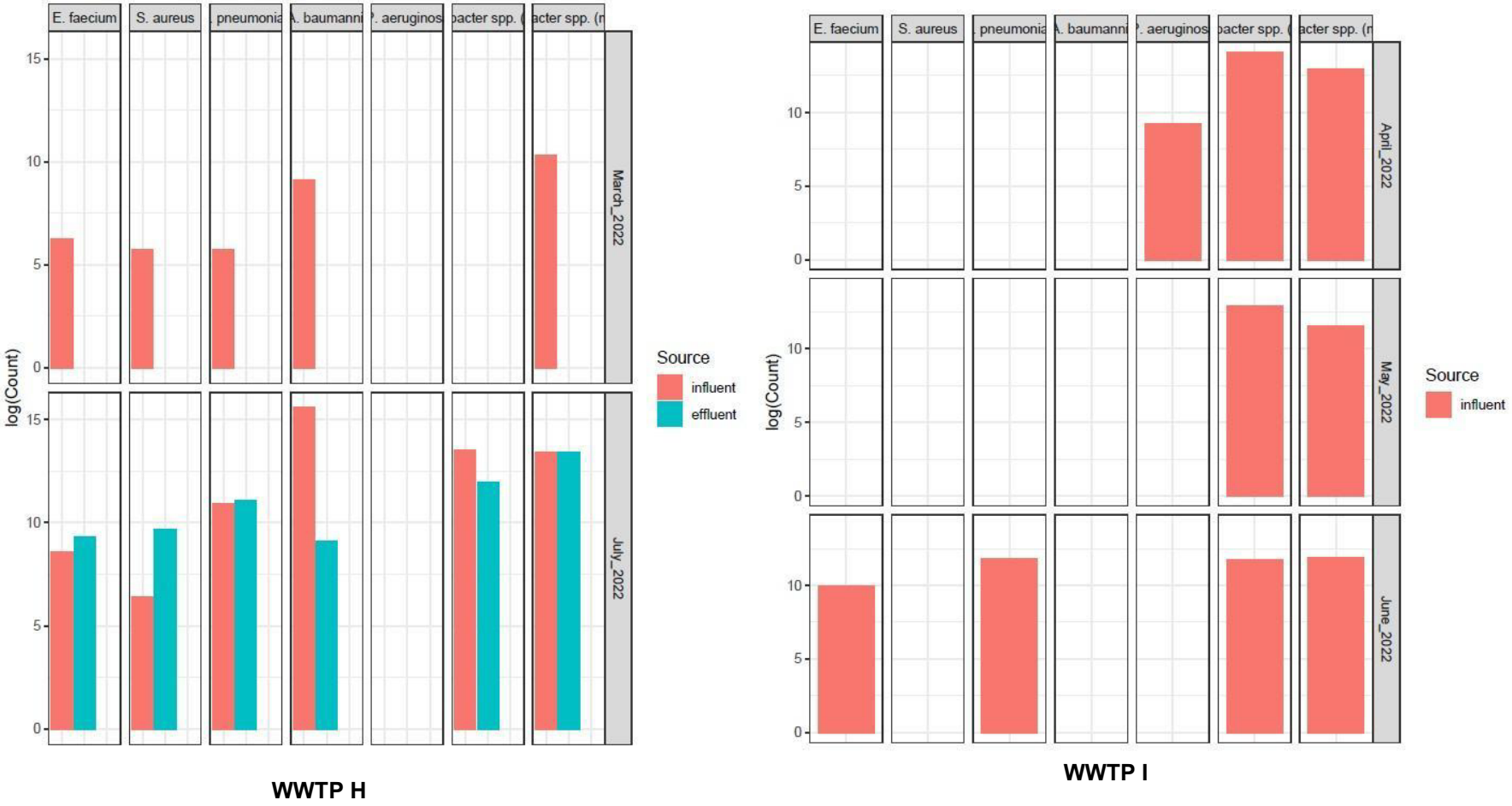


Figure 5.4: Levels of presumptive ESKAPEs in influent, effluent and downstream sites of WWTPs H and I for the duration of the study.

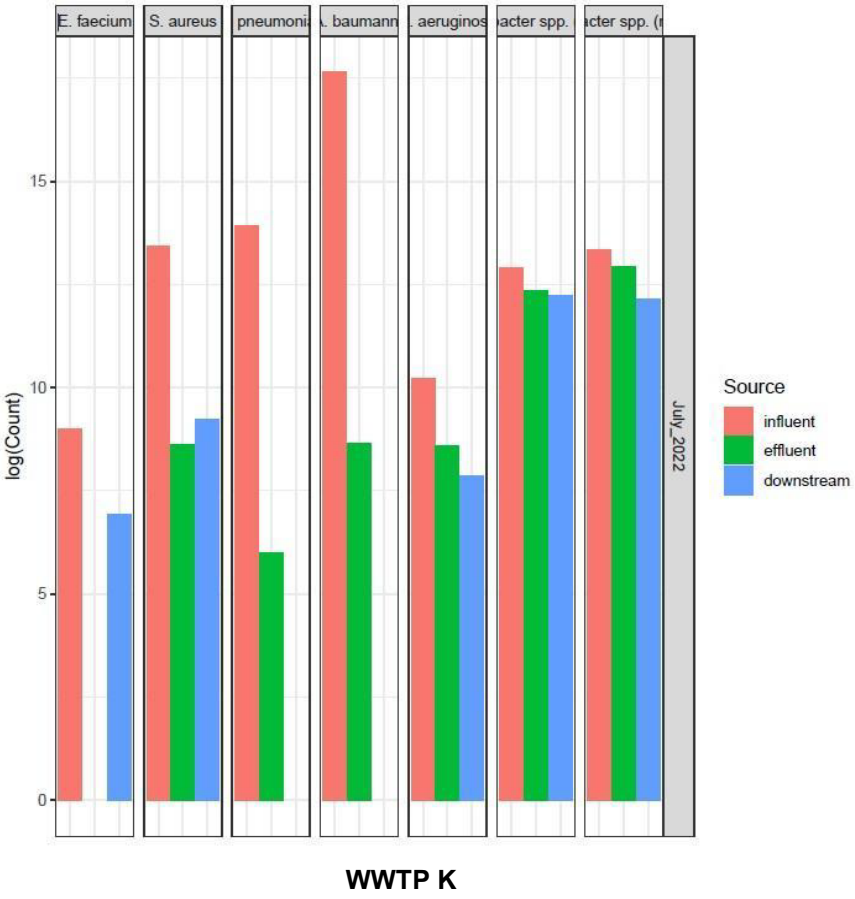


Figure 5.5: Levels of presumptive ESKAPEs in influent, effluent and downstream sites of WWTP K for the duration of the study

In summary, what all the results are showing is that ESKAPE species were present in the influent of all WWTPs. These were mostly detected in reduced levels in the effluent and downstream from the WWTP. Finding these in WWTP effluent and in downstream sources is cause for concern. The results suggest that wastewater treatment plants should monitor and optimize their processes to effectively reduce the presence of ESKAPE pathogens, particularly those with consistently high counts like *K. pneumoniae*, *A. baumannii*, and *Enterobacter spp.* (m-FC). By maintaining vigilance and implementing appropriate measures, WWTPs can contribute to mitigating the dissemination of ESKAPE pathogens in the environment. Levels of ESKAPE pathogens, based on selective media is very subjective. Not being able to grow such species may be the result of the culture conditions, VBNC state of pathogens. However, the obtained was used to speculate about reduction.

5.3.2 Correlation between ESKAPE pathogens levels and physico-chemical parameters

Figure 5.6 is a redundancy analysis (RDA) ordination plot which was used to directly relate physico-chemical parameters to ESKAPE levels for WWTP E. (Insufficient replicates for WWTP C resulted in no such analysis for this plant.) The analysis of microbial and physicochemical correlations for WWTP E revealed positive associations several ESKAPE and physico-chemical parameters within the wastewater treatment system. Specifically, strong positive correlations were observed between chemical oxygen demand (COD), TDS, PO_4^{3-} and influent (E_I_ various sampling dates) as well as

correlations with *Enterococcus faecium, Klebsiella* sp. *S. aureus, Acinetobacter* levels. Effluent (E_E_ various sampling dates) has a strong positive correlation with pH and temperature but also generally for a tight grouping within the -0.6 - 0.4 quadrant, On the other hand *Pseudomonas* sp. formed a strong correlation with nitrates (NO_3-N). Furthermore, this species also correlates with COD, PO_4^{3-} and TDS.

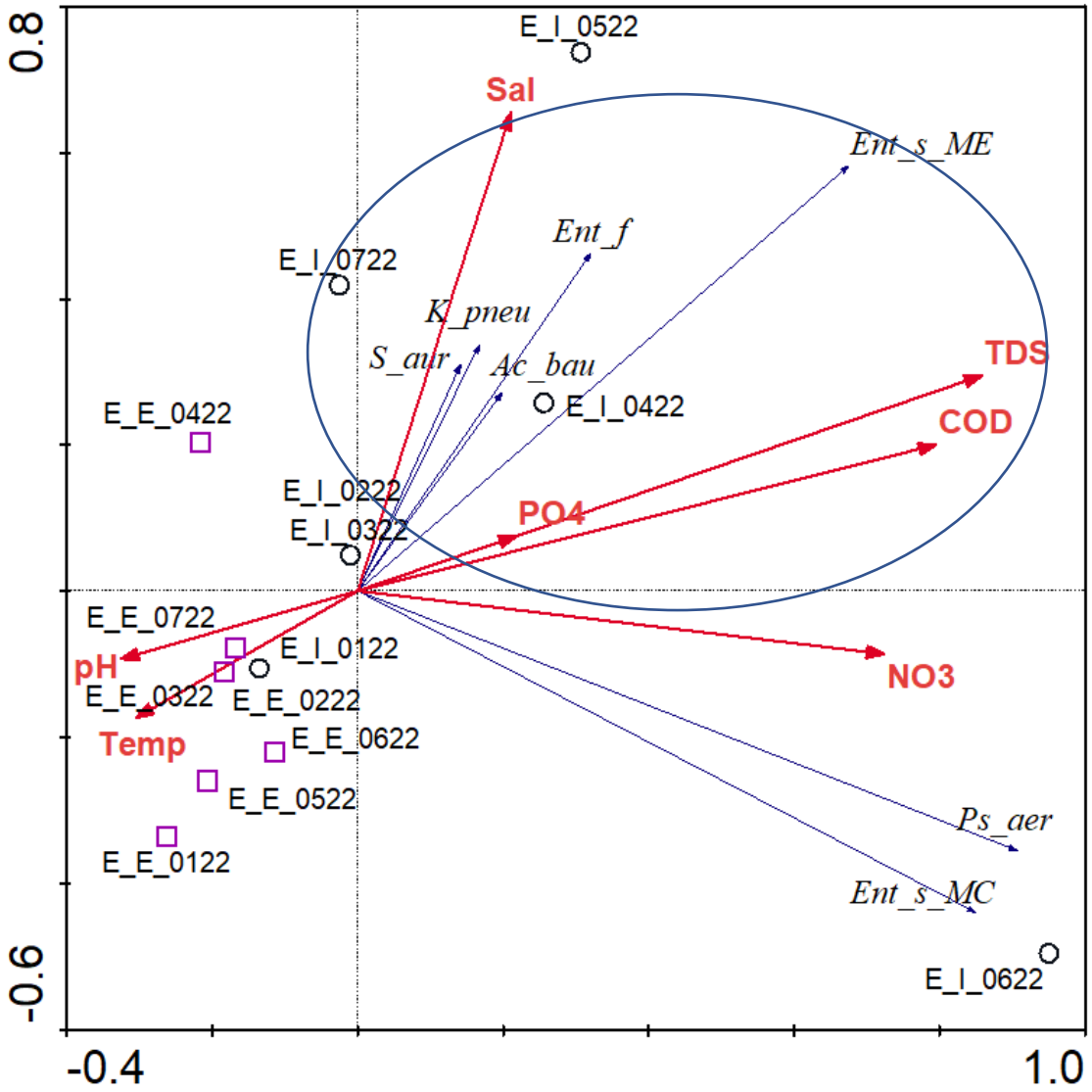


Figure 5.6: Redundancy analysis illustrating the correlation between the physico-chemical and microbial parameters screened at WWTP E for the duration of the study.

These relationships suggest that variations in these physicochemical parameters affects the levels of ESKAPE populations and may be important for the maintenance of these in sewage. These findings are thus shedding light on potential dependencies and offering valuable insights for optimizing treatment strategies and microbial management. The statistical correlations were determined using basic statistics correlation matrices and marked correlations were significant at $P< 0.05$ (Appendix A).

Observed correlations between the ESKAPE pathogens and physicochemical parameters within the context of WWTP E hold implications for understanding the dynamics across different seasons. The strength and direction of these correlations offer insights into how seasonal variations might influence microbial populations and physicochemical conditions within the wastewater treatment system. Strong positive correlations between nutrients and all the ESKAPEs in influent received by wastewater treatment plants. During seasons characterized by higher organic pollutant loads, rain runoff (E_I_0222: E_I_0322; E_4_0222:- February, March and April) might also result in favourable conditions, elevated levels of ESKAPE species. Seasonal variations in pollutant levels, temperature, and other environmental factors could drive shifts in EKSAPE populations, ultimately shaping the efficacy and performance of the wastewater treatment system to remove these pathogens.

5.3.3 Species identification of ESKAPE isolates

Figures 5.7 and 5.8 are phylogenetic trees drawn to illustrate the species distribution that was observed from two sampling sites (WWTP C and E). Furthermore, these two figures are maximum likelihood trees that depict the relatedness of various organisms. All organisms that were identified as other group (non-ESKAPE) after the identifications were obtained from the 16S rRNA gene sequencing were excluded from the tree and reference strain sequences were obtained from NCBI to serve as controls (in groups and outgroups) for the phylogenetic trees.

The diversity of all the ESKAPE organisms confirmed by 16S rRNA gene sequencing is depicted in Figures 5.8. A total of 24 ESKAPE pathogens were identified from WWTP C. The correct genus clustering of these isolates on the phylogenetic trees further confirms the identification of the isolates at least at the genus level because 16S rRNA sequencing can't always be used to accurately identify organisms at the species level (Janda and Abbott, 2007). The effectivity of identifying organisms at the genus level with 16S rRNA gene sequences is more than 90% effective whereas identification at the species level with 16S rRNA gene sequences effectivity is about 65% to 85% accurate (Janda & Abbott, 2007). Thus, the clustering of the organisms into the same clades confirms that the organisms are indeed from the same genus. Similar clustering trends were observed at WWTP E, as presented in in Figure 5.8 for WWTP E.

From Figure 5.9 it is evident that *Klebsiella* sp. and *Enterococcus* sp. are relatively dominant in these two WWTPs. However, more than 40% of isolates were not ESKAPEs. This demonstrate that culture-based methods are inaccurate and could result in many false positives.

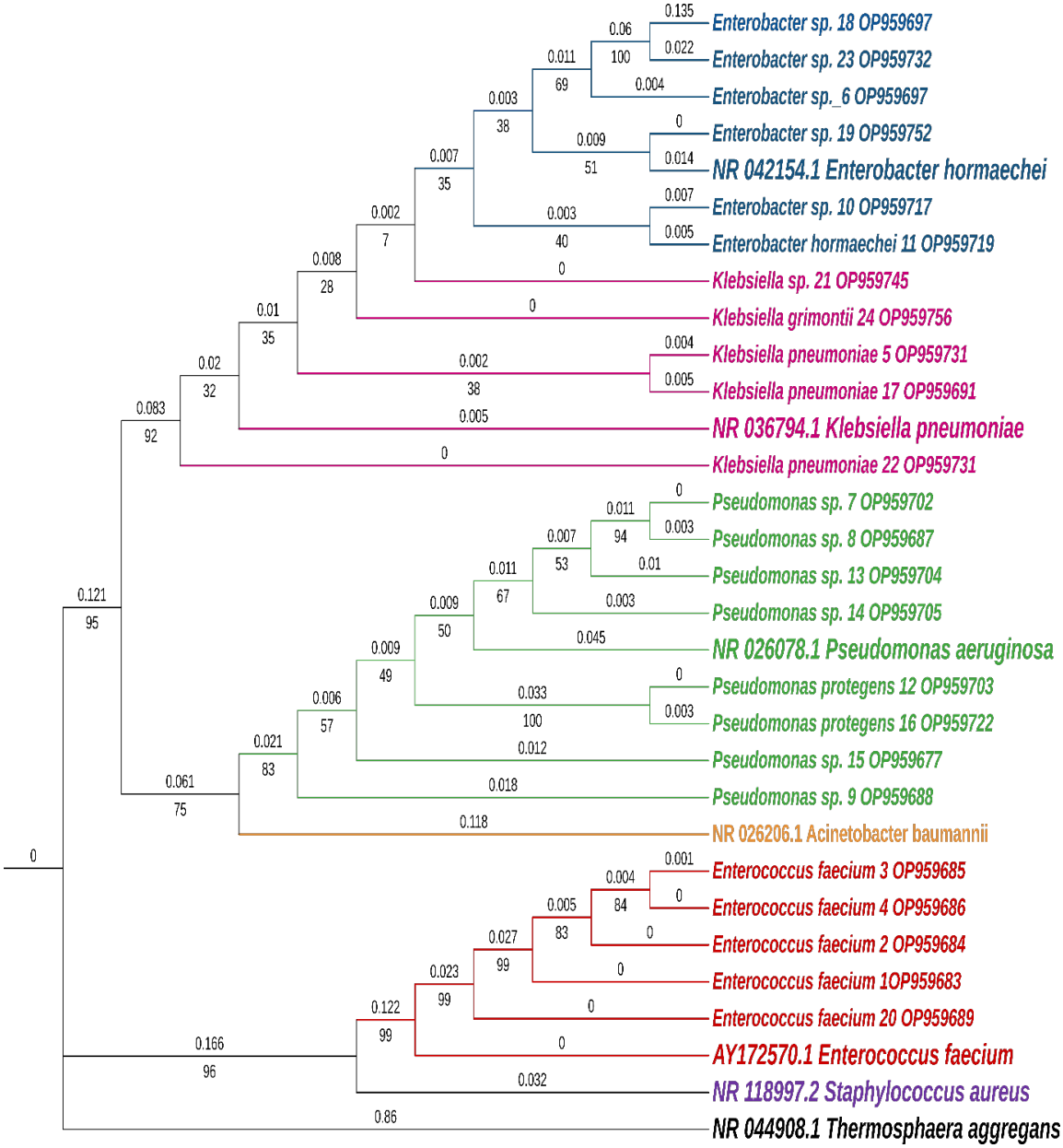


Figure 5.7: Maximum likelihood phylogenetic tree drawn for WWTP C. The tree contains 24 isolates from the ESKAPE group. The bootstrap values of the branches are represented at each branch.

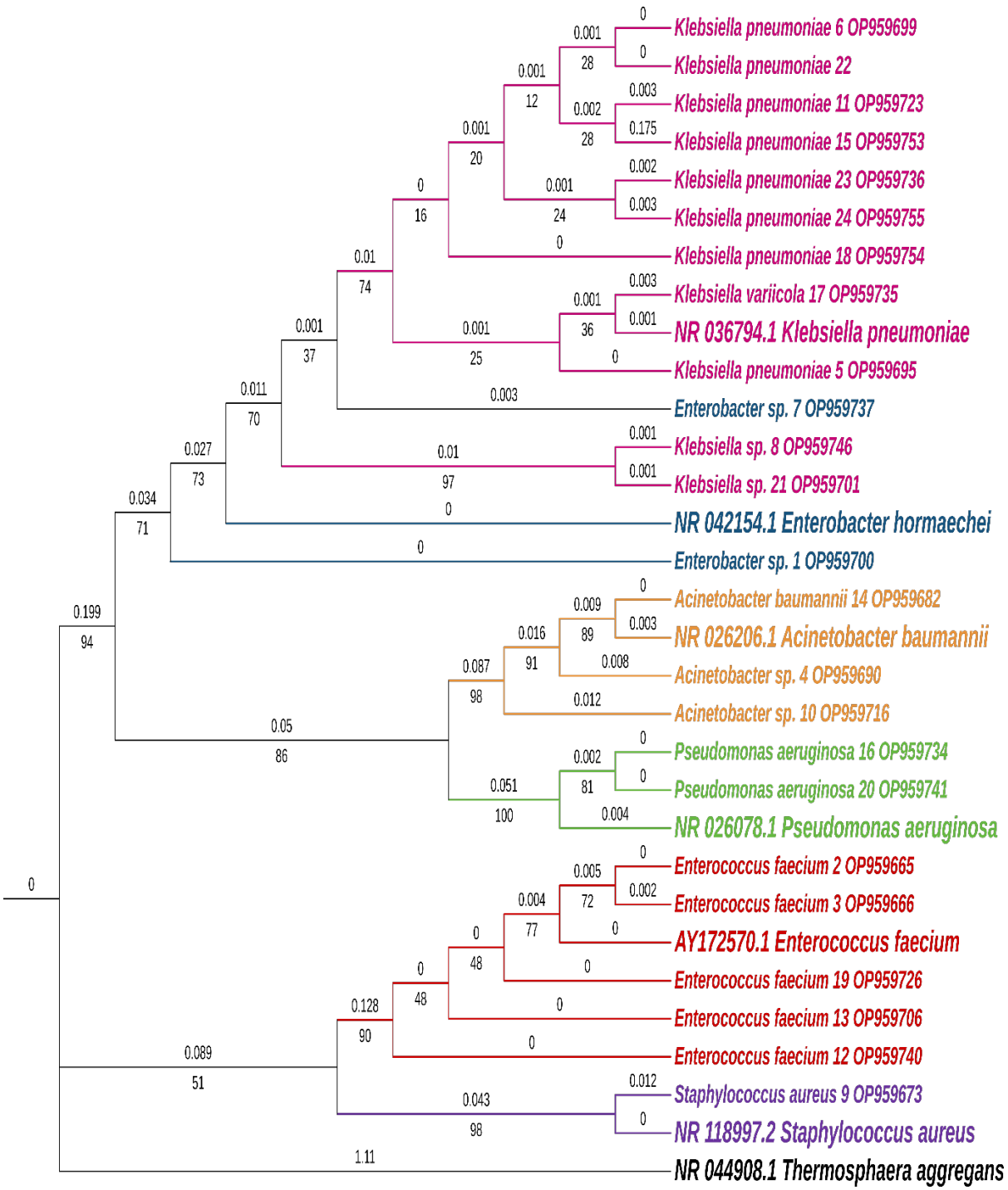


Figure 5.8: Maximum likelihood phylogenetic tree drawn for WWTP E. The tree contains 24 isolates from the ESKAPE group. The bootstrap values of the branches are represented at each branch.

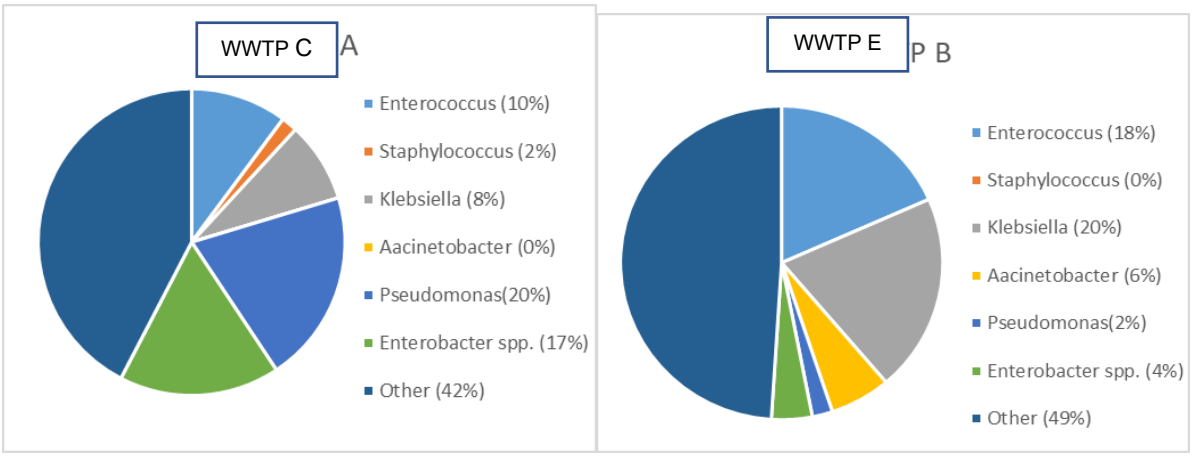


Figure 5.9 Pie charts illustrating the percentages of organisms that were positively identified and those that did not form part of the ESKAPE pathogen group from WWTPs C and E.

5.3.4 Antibiotic resistance profiles of ESKAPE species from WWTPs of interest

ESKAPE pathogens isolated from the Influent and effluent sites of WWTPs C and E illustrated susceptibility to gentamycin, ciprofloxacin and Netilmicin (Figures 5.10 to 5.12). The rest of the results are presented in Appendix B. The majority of the *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Enterobacter* species isolated from WWTPs C and E were resistant to ampicillin. The antibiotic resistance patterns measured for ESKAPE species isolated from the influent, effluent and downstream sites of WWTPs B, D, I and J showed consistent resistance to ampicillin. Furthermore, vancomycin resistance was noted in *Enterococcus faecium* isolates from the influent, effluent and downstream sites of WWTPs B, D, I and J. Also, resistance to ampicillin was also prevalent in the samples from WWTPs C, E, F, K, H. Furthermore, many isolates were resistant to Cephazolin..

The same antibiotics were not tested on all of the presumptive isolates, but the presumptive ESKAPE pathogens as a whole illustrated the following trends of resistance against the antibiotics that were used AMP (78%) > MEM (70%) > K (65%) > KZ (63%) > P (32.5%) > E (25%) > VA (22.5%) > CRO (15%) > IMP (10%) > CN (7.4%) > CTX (6%) > C (5.2%) > AK (4%) > LEV (4%) > CIP (3.6%) > CAZ (3%) > F (2.5%) > OFX (2.5%) > NOR (0%) > NET (0%).

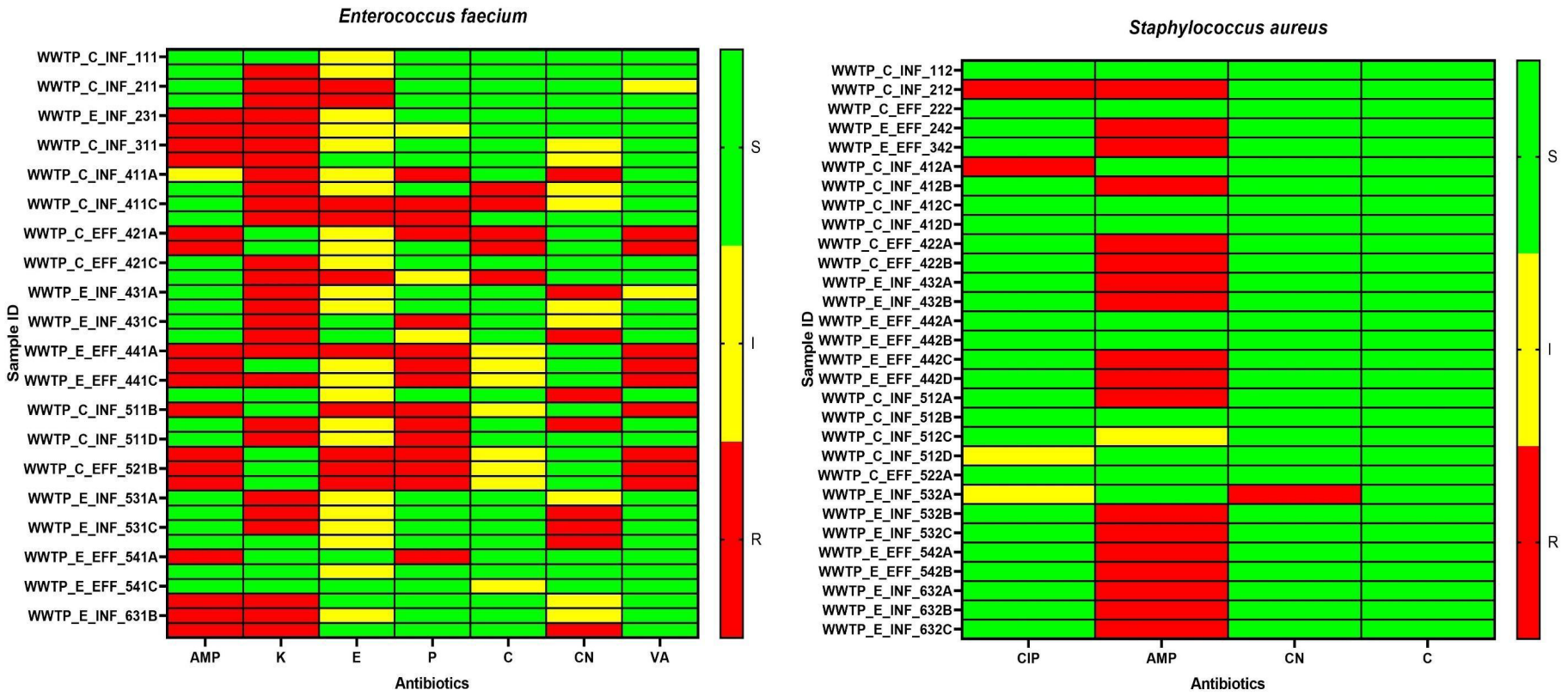


Figure 5.10: Heat maps illustrating the resistance patterns of the presumptive *Enterococcus faecium* and *Staphylococcus aureus* isolates of WWTPs C and E. Green = susceptible (S), yellow = intermediate resistant (I) and red = resistant (R); NOR = norfloxacin (10 µg), F = nitrofurantoin (300 µg), IMP = imipenem (10 µg), CTX = cefotaxime (30 µg), OFX = ofloxacin (5 µg), CRO = ceftriaxone (30 µg), AK = amikacin (30 µg), KZ = cefazoline (30 µg), AMP = ampicillin (10 µg) and CN = gentamycin (10 µg).

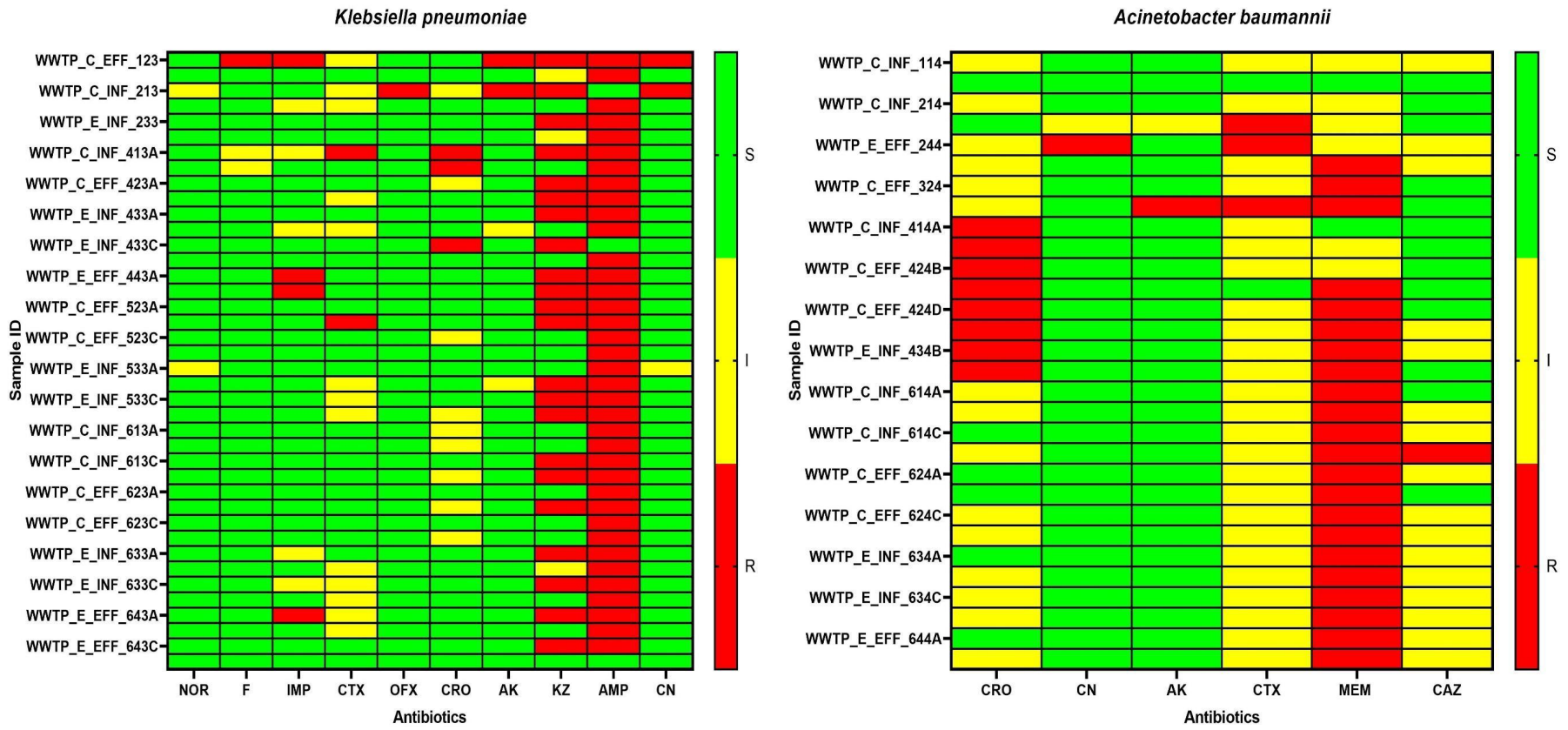


Figure 5.11: Heat maps illustrating the resistance patterns of the presumptive *Klebsiella pneumoniae* and *Acinetobacter baumannii* isolates of WWTPs C and E. Green = susceptible (S), yellow = intermediate resistant (I) and red = resistant (R); NOR = norfloxacin (10 µg), F = nitrofurantoin (300 µg), IMP = imipenem (10 µg), CTX = cefotaxime (30 µg), OFX = ofloxacin (5 µg), CRO = ceftriaxone (30 µg), AK = amikacin (30 µg), KZ = cefazoline (30 µg), AMP = ampicillin (10 µg) and CN = gentamycin (10 µg).

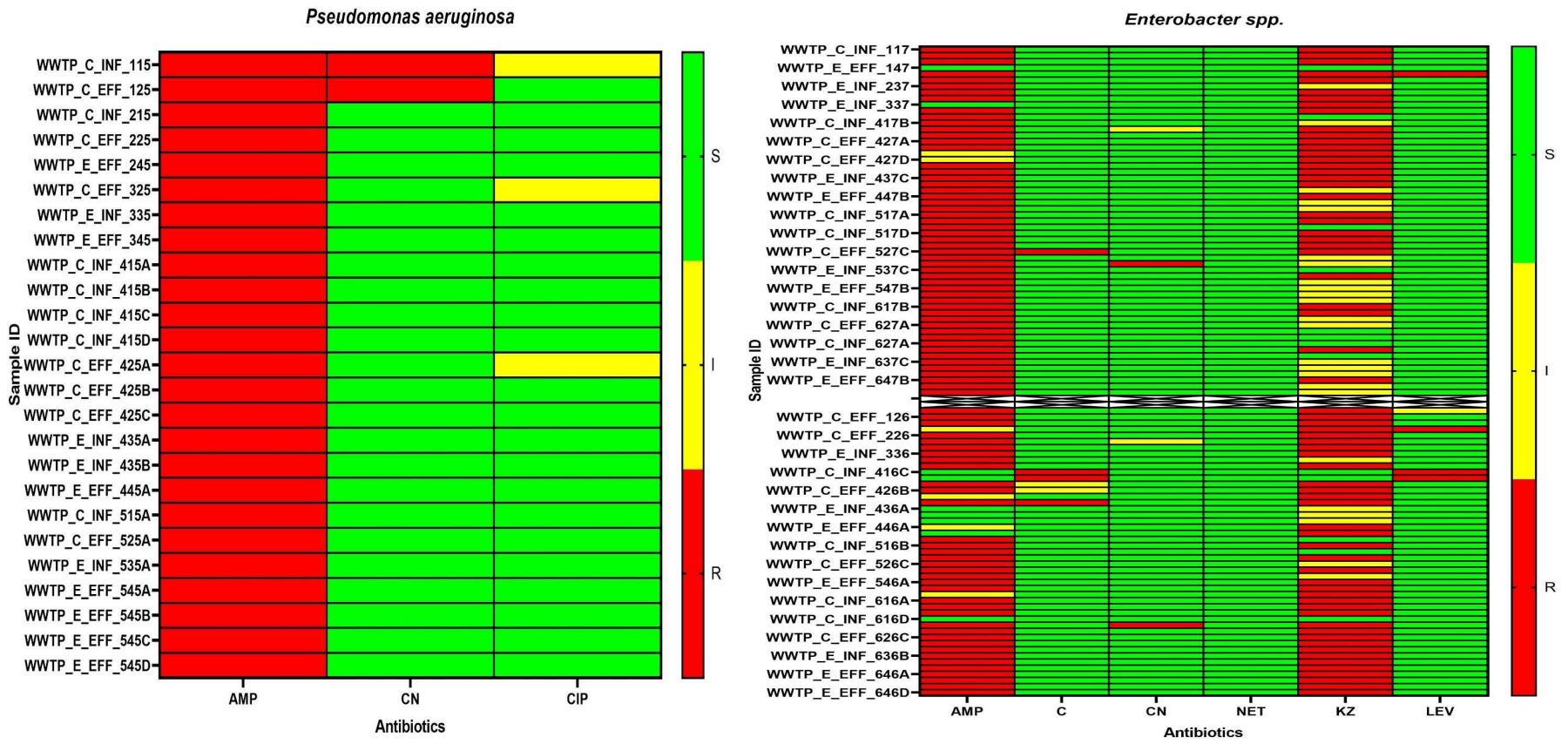


Figure 5.12: Heat maps illustrating the resistance patterns of the presumptive *Pseudomonas aeruginosa* and *Enterobacter* spp. isolates of WWTPs C and E. Green = susceptible (S), yellow = intermediate resistant (I) and red = resistant (R); NOR = norfloxacin (10 µg), F = nitrofurantoin (300 µg), IMP = imipenem (10 µg), CTX = cefotaxime (30 µg), OFX = ofloxacin (5 µg), CRO = ceftriaxone (30 µg), AK = amikacin (30 µg), KZ = cefazoline (30 µg), AMP = ampicillin (10 µg) and CN = gentamycin (10 µg).
of WWTPs C and E.

5.3.5 Pathogenicity potential of putative ESKAPE isolates from WWTPs C and E

Selected colonies were purified by the streak plate method, and these were subjected to assays to determine whether they produce extracellular enzymes. The ability to produce these is an indication of virulence, confirming pathogenicity. Figures 5.13 to 5.16 below provide the results of the tested putative isolates are indicated. The scoring method used for the graphs of DNase, Lipase and Proteinase: Negative = +0; Isolate grew however, no changes in agar = 1; If the growth also affected changes in the agar = 2; If several isolates grew and changed the media as indicated by the manufacturer then 3 and more. From the graphs, the observation can be made that the isolates produced several of the extracellular enzymes.

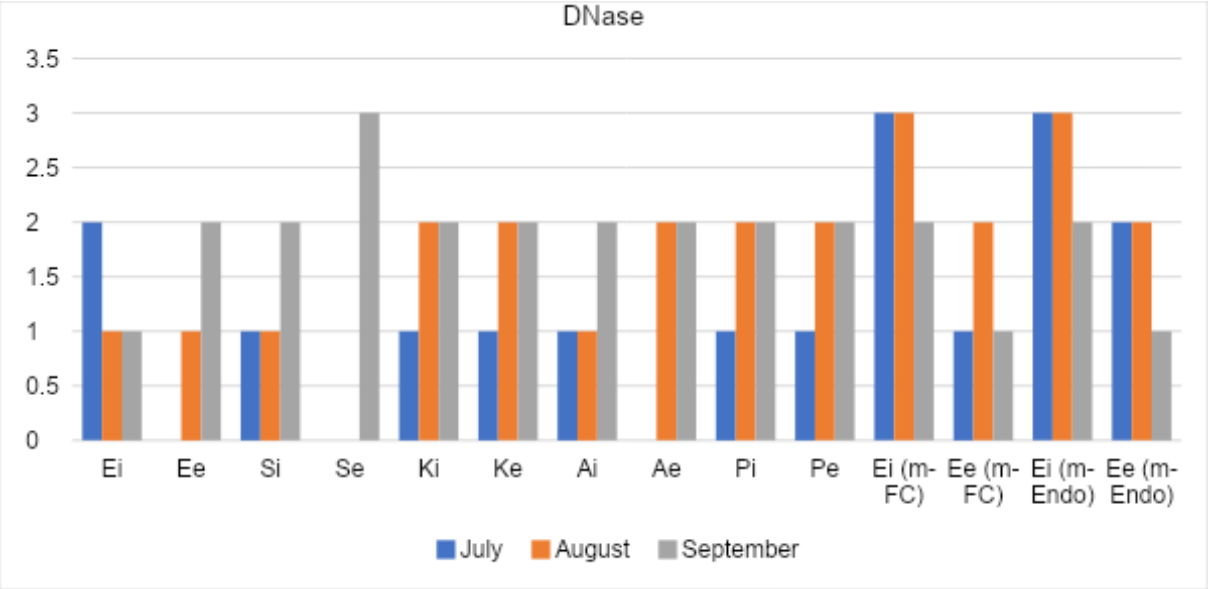


Figure 5.13: Characterisation of DNase activity of putative ESKAPE isolates. Abbreviations: (i = influent; e = effluent). E – *E. faecium*; S – *S. aureus*; K – *K. pneumonia*; A – *A. baumanii*; P – *P. aeruginosa*; E (mFC or M-Endo) – *Enterobacter* sp.

The production of lipase, DNase, proteinase, and hemolysin in ESKAPE pathogens is anticipated as these are potential pathogens that might originate from clinical settings. However, since all isolates in the present study was from environmental water samples it was necessary to test for these features.
Lipase production is the ability of a pathogen to break down lipids or fats. This can aid the pathogen in accessing nutrients for its growth and survival, as well as potentially contribute to its ability to infect host tissues by degrading cell membranes. Secondly, DNase production, on the other hand, indicates the pathogen's capacity to degrade DNA. This trait can play a role in evading the host's immune responses by breaking down extracellular DNA, which is often involved in forming protective structures like biofilms. Thirdly, Proteinase production reflects the pathogen's ability to break down proteins. This can be crucial for invading host tissues, as many proteins form structural components of cells and tissues. Proteinases can also help the pathogen evade the immune system by degrading immune proteins. While, haemolysin production is particularly noteworthy as it relates to the destruction of red blood cells. Hemolysins can damage host cells, aid in nutrient acquisition by releasing iron from hemoglobin, and promote tissue damage, contributing to the pathogen's virulence.

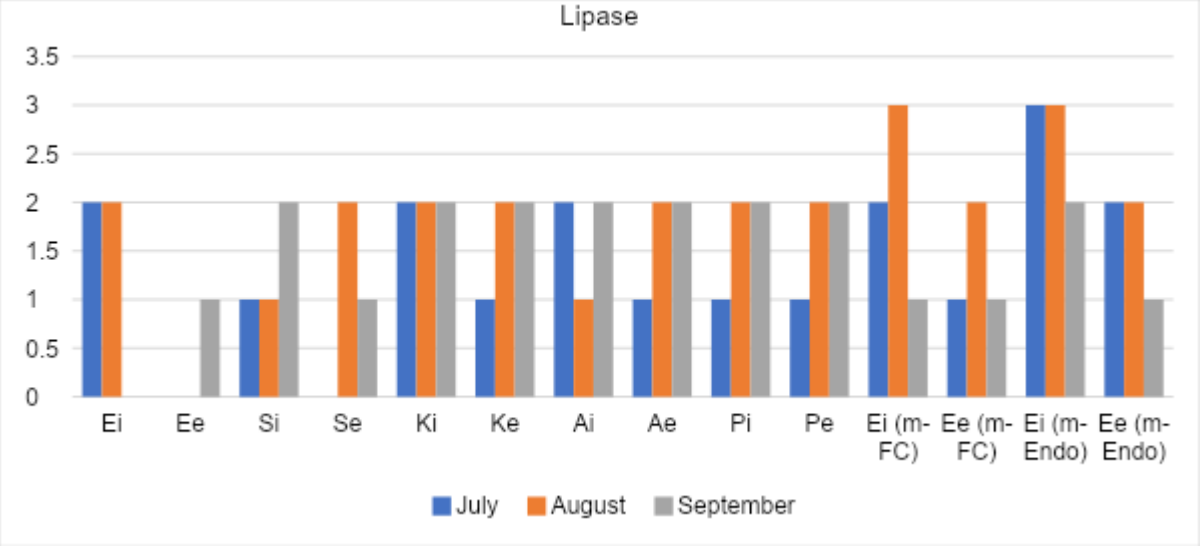


Figure 5.14: Characterisation of Lipase activity of putative ESKAPE isolates. Abbreviations: (i = influent; e = effluent). E – *E. faecium*; S – *S. aureus*; K – *K. pneumonia*; A – *A. baumanii*; P – *P. aeruginosa*; E (mFC or M-Endo) – *Enterobacter* sp.

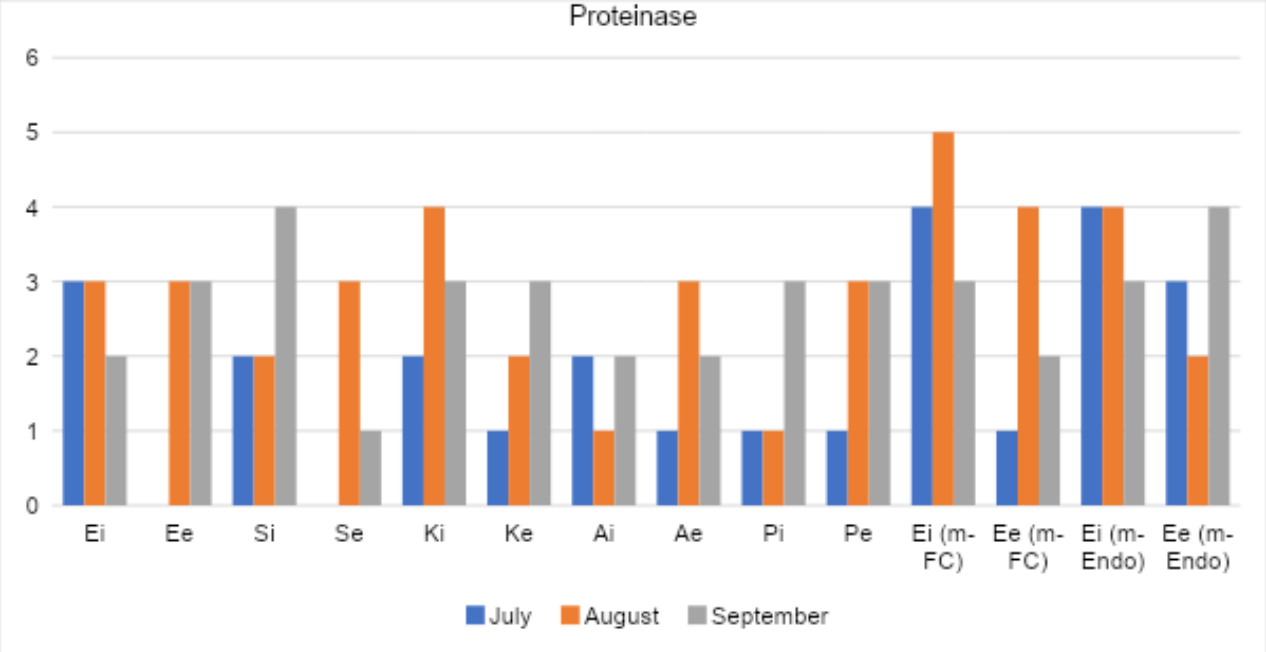


Figure 5.15: Characterisation of Proteinase activity of putative ESKAPE isolates. Abbreviations: (i = influent; e = effluent). E – *E. faecium*; S – *S. aureus*; K – *K. pneumonia*; A – *A. baumanii*; P – *P. aeruginosa*; E (mFC or M-Endo) – *Enterobacter* sp.

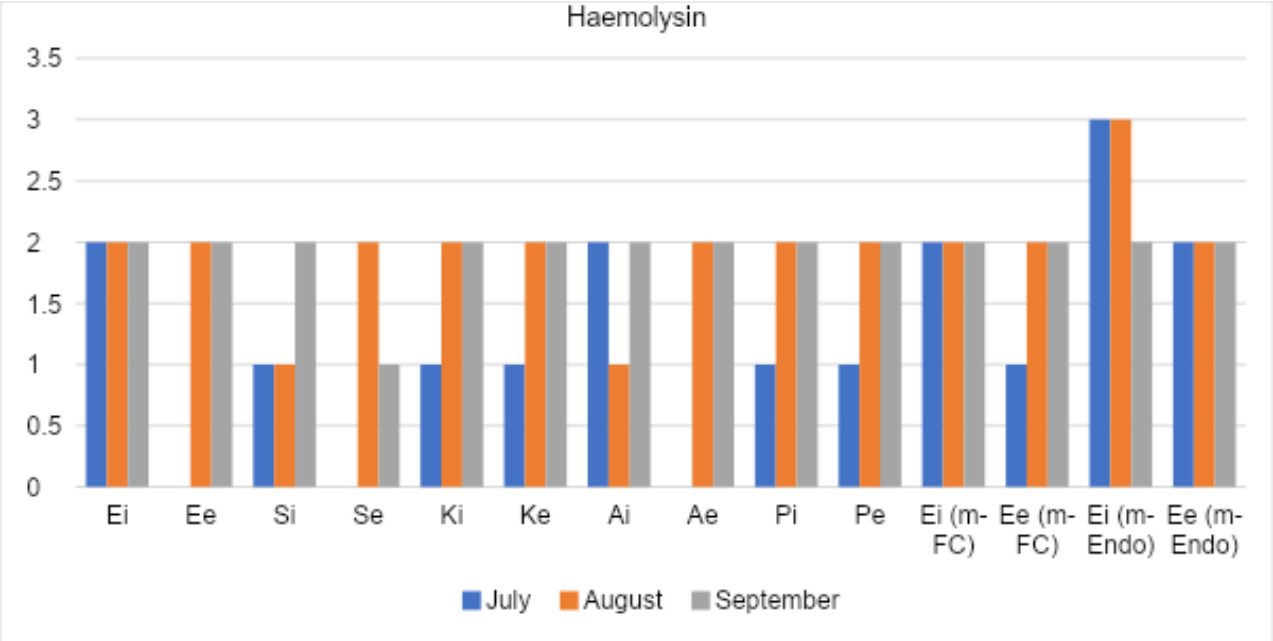


Figure 5.16: Characterisation of Haemolysin activity of putative ESKAPE isolates. Abbreviations: (i = influent; e = effluent). E – *E. faecium*; S – *S. aureus*; K – *K. pneumonia*; A – *A. baumanii*; P – *P. aeruginosa*; E (mFC or M-Endo) – *Enterobacter* sp.

Thus, the production of these enzymes by ESKAPE pathogens indicates their potential to interact with and harm host tissues, evade immune responses, and access nutrients required for their growth and survival. These characteristics collectively contribute to the various isolates ability to cause infections and pose challenges in clinical settings. Generally, 14 isolates were identified as beta-haemolytic which suggests that they are pathogenic. This is worrisome when considering that 12 out of the 14 produced either lipase or protease and were resistant to two or more antibiotics. Fourteen of the isolates harboured the blaVIM gene which mediates carbapenem resistance. This is a cause for concern as carbapenems are amongst the list of globally last resort antibiotics – routinely used in South Africa (Osei Sekyere, 2016). Also, blaOXA-48 was detected in 5 isolates, blaTEM in 3 isolates and blaSHV in 1 isolated. These genes form part of the Class D carbapenemases and OXA-β-lactamases with some being chromosomally encoded (blaOXA) and others encoded by integron borne on mobile gene cassettes. Their presence is a cause of concern as the latter suggests that they are transferable amongst various bacteria via horizontal gene transfer mechanisms notably conjugation. Also, interestingly, blaOXA-48 is routinely reported in *Escherichia coli* and *Klebsiella* from hospital settings (Ledda et al., 2022).

Figure 5.17 is an RDA plot that illustrates the relationships among the different wastewater treatment plants, the identified organisms, the antibiotic resistance patterns, and their extracellular enzyme activity. This figure illustrate complex relationships a mostly positive correlation between influent and effluent of WWTP C (WTC) and WWTP E (WTC). There is a negative relationship between the influent of WWTP C organism 5 and the effluent of WWTP B organism 6 when compared to the cluster of organisms. These clusters illustrate similarities between the sites and the organisms that were isolated from those plants. From Figure 5.17 a positive relationship between the extracellular enzyme activity and resistance to various antibiotics.

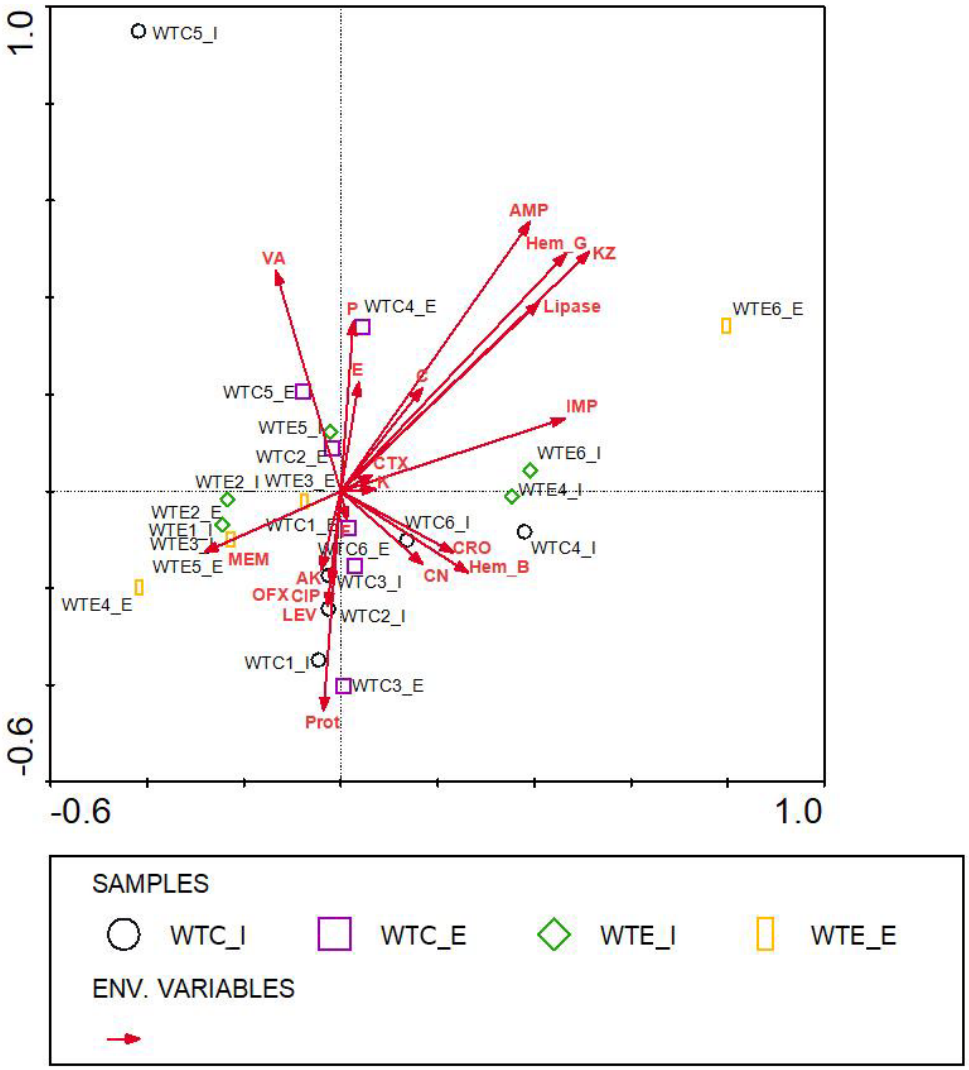


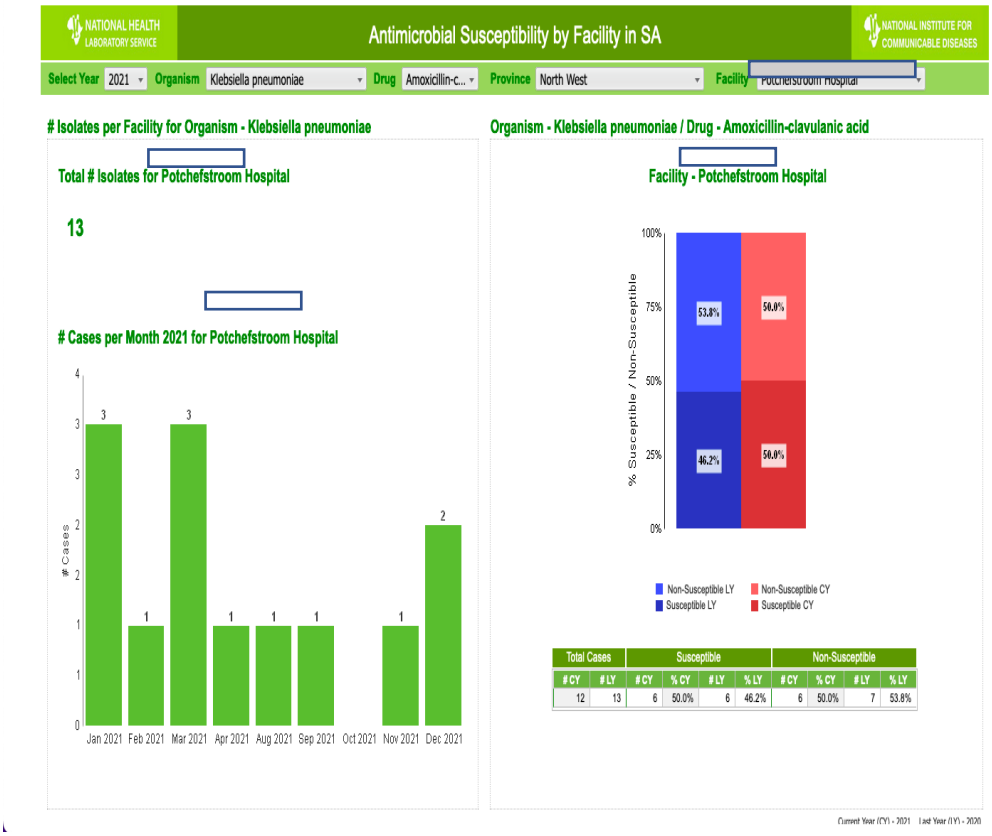
Figure 5.17: RDA plot illustrates the relationships among different WWTPs influent and effluent based on their species distribution. WTC = WWTP C; WTE = WWTP E; I = influent; E = effluent; 1 = *E faecium*; 2 = *S. aureus*; 3 = *K. pneumoniae*; 4 = *A. baumannii*; 5 = *P. aeruginosa*; 6 = *Enterobacter* spp. In this RDA, the relationship between antibiotic resistance and the virulence factors can also be seen as compared to the respected sampling sites.

A positive relationship was found between the species that exhibited gamma haemolysis activity (ability to grow on blood agar) and lipase production and the species that exhibited resistance against AMP, KZ, C, CTX and IMP. Further a positive relationship can also be observed between the species that exhibited beta haemolysis activity (complete lysis of blood cells) and the species that exhibited resistance against CRO and CN. Another positive relationship can be observed between the species that produced protease and those that were resistant to MEM, AK, OFX, LEV and CIP. In the latter case the following species from influent (WTC _I) and effluent (WTC _E) from WWTP C (WTC6_E *Enterobacter* sp.; WTC3_I *K. pneumoniae*; WTC2_I *S. aureus*; WTC1_E *E. faecium*. WTC3_E *K. pneumoniae*). The latter is an example of such correlations that could be explored in Figure 5.16. Trends seen in Figure 5.4 could be compared to the ordination in Figure 5.6 where the physico-chemical parameters, ESKAPE species and WWTPs were analysed for correlations.

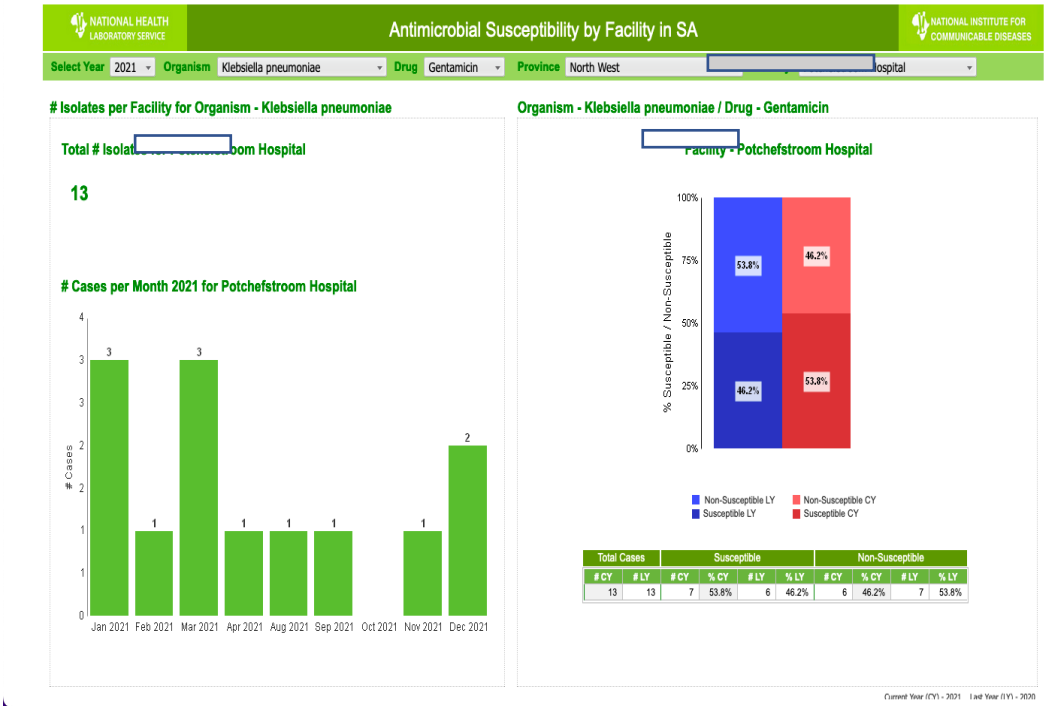
5.3.6 Clinical relevance of ESKAPE pathogens

5.3.6.1 ESKAPE data: NICD information for the region serviced by WWTP E

Figure 5.18 A-C are graphical extracts from the NICD reporting on *Klebsiella pneumoniae* isolates from infections from January 2020 to December 2021 in the state hospital serviced by WWTP E. Such extracts were used to prepare Table 5.3. This table is thus a representation of all isolated and identified ESKAPE pathogens and antibiotic susceptibility profiles originating from this specific state hospital. Sewage from this hospital does not undergo any pre-treatment prior to being released into the sewer system, finally reaching the WWTP.



A.



B.

Figure 5.18: Graphical extracts from the NICD reporting the detected number of *Klebsiella pneumoniae* and their susceptibility to various antibiotics in a state hospital located in the region serviced by WWTP E.

Various ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa*) were isolated from infections reported at this hospital. The number of cases ranged from 1 to 20 over the two-year period, that overlaps with the environmental study. Some extracts form the data in Table 5.3 indicate that the pathogen that was most frequently isolated was *Staphylococcus aureus* with a total of 19 cases in 2020 followed by *Klebsiella pneumoniae* with a total of 13 cases reported in 2020. Notably, the reported *Klebsiella pneumoniae* isolates were resistant to all screened antibiotics (10), either during 2020 and 2021 or across both years. The reported *Acinetobacter baumannii* isolates were also resistant to four of the five screened antibiotics. The *Enterococcus faecium* isolates were susceptible to all screened antibiotic during this period except the one isolate that was resistant to ampicillin. A general trend observed is the resistance to β-lactam antibiotics from various generations.

Table 5.1: Number of ESKAPE pathogens isolated in the state hospital serviced by WWTP C and the antibiotic resistance report

Pathogen	**Year**	**No of cases**	**Daptomycin resistance %**	**Linezolid**	**Penicillin**	**Teicoplanin**	**Vancomycin**					
E. faecium	2020	1-5	0.0%	0.0%	0.0%	0.0%	0.0%					
	2021	1-4	0.0%	0.0%	100%	0.0%	0.0%					
			Cefoxitin	**Cloaxcillin**								
S. aureus	2020	1-19	0.0%	47.4%								
	2021	1-8	0.0%	37.5%								
			Amoxicillin	**Amikacin**	**Gentamycin**	**Imipenem**	**Piperacillin**	**Ceftazidime**	**Cefepime**	**Ciprofloxacin**	**Ertapenem**	**Cefotaxime/Ceftriaxone**
K. pneumoniae	2020	1-13	53.8%	23.1%	53.8%	0.0%	15.4%	69.2%	66.7%	30.8%	0.0%	69.2%
	2021	1-12	50.0%	0.0%	46.2%	8.3%	30.8%	50.0%	46.2	33.3%	91.7%	46.2%
			Amikacin	**Colistin**	**Gentamycin**	**Imipenem**	**Meropenem**					
A. baumannii	2020	1	66.7%	0.0%	66.7%	100%	100%					
	2021	1-3	0.0%	0.0%	0.0%	100%	100%					
			Cefepime	**Colistin**	**Ceftazidime**	**Imipenem**	**Meropenem**	**Piperacillin**				
P. aeruginosa	2020	1	33.3%	0.0%	33.3%	0.0%	0.0%	33.3%				
	2021	1-3	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%				

5.3.6.2 Comparison of clinical and environmental ESKAPE *pathogen characteristics*

The clinical data extracted from the NICD only focused on one plant (WWTP E) owing to the availability of more than one set of results from the environmental samples collected in the present study. It is evident from the NICD data that ESKAPEs are present in this community causing infections. These pathogens land in the sewage and is potentially transported to the WWTP. *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* were isolated from infections reported at this hospital.

During this period (2020 to 2021) these ESKAPE species were also isolated from influent and effluent of WWTP E, that is the recipient of the sewage from the hospital in question. Both culture dependent and culture independent methods detected these ESKAPE species. Furthermore, although the wastewater treatment plant could reduce the levels of the species, some were isolated from effluent and downstream receiving waters. The qPCR data supported the culture-based data. Pathogenic features were common among the environmental isolates.

Detectable levels antibiotic residues were measured in the influent, effluent, and downstream ecosystems. Ampicillin concentration was consistently higher than all other the antibiotics measured. It could thus be expected that β-lactam resistance in the ESKAPEs from the influent, effluent, and downstream ecosystems of WWTP E would be common. This is what was observed amongst the environmental isolates. The same trend is observed among the clinical ESKAPE pathogens, common resistance to β-lactam antibiotics from various generations.

In addition, the environmental ESKAPE isolates had several antibiotic resistance genes as part of their genomes. These genes were also detected in DNA directly isolated from influent, effluent, and downstream samples. Commonly detected antibiotic resistance genes targeted included clinically relevant β-lactamase genes (ACC, DHA, CIT [LAT/CMY/BIL] EBC [MIR/ACT], FOX, MOX/CMY). Unfortunately, no such information is available for the clinical isolates.

5.4 SUMMARY

Isolated presumptive ESKAPE species were purified on selective media and identified using 16S rRNA gene sequencing. The results from both WWTP C and WWTP E showed that more than 40% of isolates were false positives. The analysis of various parameters in the wastewater treatment plants provides valuable insights into the quality and effectiveness of the treatment processes. Values for the physico-chemical parameters were withing expected and mostly acceptable ranges. The nutrient levels and physical conditions were favourable for the maintenance of the ESKAPE populations in sewage. The comparison of total dissolved solids (TDS) and salinity levels between influent and effluent samples revealed a general reduction in both parameters during the treatment process, indicating the removal or reduction of dissolved solids and salts. WWTP C consistently exhibited higher TDS and salinity levels, while WWTP E had the lowest values. Chemical oxygen demand (COD), a measure of organic pollutant levels, consistently showed lower values in the effluent samples compared to the influent, indicating effective removal or degradation of organic pollutants during treatment. WWTP C recorded the highest COD values, while WWTP E consistently had the lowest.

Phosphate and nitrate levels varied between different plants, with WWTP C consistently exhibiting the highest concentrations. During the rainy season, higher levels of ESKAPE pathogens were observed in influent compared to effluent samples, indicating a reduction in most cases. Generally, there was a decrease in the ESKAPE levels from the influent to effluent samples, except for specific instances like

elevated *Enterobacter* levels in June 2022 (WWTP C) and *S. aureus* levels in July 2021 (WWTP E). *Klebsiella* sp. often displayed the highest counts and presence in both influent and effluent, indicating potential contamination sources as well as suggesting that this could be a sentinel species in monitoring regimes. The percentage reduction in ESKAPE pathogens in WWTP E was presented. These results should be carefully interpreted, and no concrete deduction can be made.

Observed correlations between the ESKAPE pathogen levels and physicochemical parameters within the context of WWTP E hold implications for understanding the dynamics across different seasons. The strength and direction of these correlations offer insights into how seasonal variations might influence physicochemical conditions and impacting ESKAPE populations and within the wastewater treatment system. Measuring physicochemical parameters of influent and effluent is thus a valuable contribution when ESKAPE surveillance programs are considered. Findings presented here emphasize the importance of efficient wastewater treatment to mitigate ESKAPE pathogen dissemination and safeguard receiving waters, environmental and public health.

Both WWTP E and WWTP C exhibit elevated concentrations of specific antibiotic residues, such as ampicillin, ciprofloxacin, sulfamethoxazole, and trimethoprim, in their wastewater effluent compared to surrounding downstream and upstream rivers (Section 3.5). This points to an insufficient removal or degradation of these antibiotics during the treatment processes at these plants. The downstream river of WWTP E demonstrated notably higher fluconazole levels, possibly reflecting a source that enters after the WWTP. In WWTP C, the downstream maturation pond stood out with the highest ampicillin concentration, implying its role in sustaining ampicillin persistence in the environment downstream from the plant. The results of this study have demonstrated that antimicrobials, commonly used in medical, veterinary, and agricultural practices, can find their way into wastewater, and subsequently impact aquatic ecosystems and human health. Overall, the data highlight the presence of certain antibiotic residues in the effluent of these wastewater treatment plants and variations in their removal efficiency based on the specific antibiotic and treatment processes. Wastewater treatment plants were not designed for antibiotic removal but when these systems are effectively managed then antibiotic residues could be reduced.

Monitoring antimicrobial residues in wastewater, is important for various reasons. Firstly, it aids in assessing the potential environmental impact of these substances, which are widely used in healthcare, agriculture, and veterinary practices. Secondly, tracking antimicrobial concentrations ensures compliance with safeguards of public health by evaluating risks associated with water consumption, and contributes to scientific research on pollution and antibiotic resistance. Furthermore, the data assists in source identification, management, and policy formulation, empowering decision-makers and wastewater operators to take informed actions toward curbing antimicrobial pollution.

The antibiotic susceptibility and pathogenicity of ESKAPE species for isolates from influent, effluent and downstream water in WWTPs C and E. The results indicated that while some ESKAPE species were susceptible to gentamycin, ciprofloxacin, and Netilmicin. However, resistance to ampicillin was prevalent among *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterobacter species* in WWTPs C and E. In other WWTPs (B, D, I, and J), consistent resistance to ampicillin and even vancomycin resistance in *Enterococcus faecium* isolates was observed across influent, effluent, and downstream sites. These underscore potential challenges in mitigating the spread of antibiotic-resistant strains through wastewater and that such species could land in receiving water. Furthermore, the study explored the pathogenicity potential of ESKAPE isolates from WWTPs C and E by assessing their ability to produce extracellular enzymes. Notably, the production of lipase, DNase, proteinase, and hemolysin in these isolates indicates their virulence capabilities such as evasion of immune responses.

RDA analysis demonstrates correlations with the ESKAPE species, characteristics and compartments of the two WWTPs C and E. Such relations ships were consistent with an ordination where the physico-chemical parameters, ESKAPE species and WWTPs were analysed for correlations.

In conclusion, there is an overlap of between the ESKAPE species and characteristics coming from clinical and environmental sources. The evidence provided here support the notion that ESKAPE species from clinical settings (clinics, hospitals) and household is transported via sewage to WWTPs. Even though reduction of such pathogens occurs during wastewater treatment, these ESKAPE pathogens land in receiving environmental water.

6 EVALUATING THE REDUCTION POTENTIAL OF ESKAPE PATHOGENS AND RESISTANT GENES DURING WASTEWATER TREATMENT

6.1 INTRODUCTION

This chapter addresses Aim 3 of the project. Evaluating the reduction potential of ESKAPE pathogens and antibiotic resistant genes (ARGs), as well as nutrients and antimicrobials during wastewater treatment is crucial for understanding the effectiveness of treatment processes in removing or reducing these potential sources of contamination. In the case of all the plants the downstream water is used for various purposes, including agriculture (irrigation and livestock watering), drinking water production, recreation, and religious purposes. As such, water sources polluted with antibiotic resistance ESKAPE pathogens containing clinically relevant ARGs as well as antimicrobial residues will likely have serious implications.

6.2 METHODS

6.2.1 Source of data

ESKAPE pathogen and antibiotic resistant genes abundance data was sourced from Sections 4.3.1, 4.3.2 and 5.31. In Section 4.3.1 and 4.3.2 the data was obtained by using qPCR, whereas in Section 5.3.1, the data was obtained using microbiological techniques to detect and enumerate ESKAPE pathogens in the water samples. Data on the levels of nutrients and antimicrobial residues was sourced from Sections 3.4 and 3.5, respectively.

6.2.2 Estimation of reduction potential and load

Reduction potential was calculated by the following formula:

$$\text{Percentage Reduction} = ((\text{Influent} - \text{Effluent}) \div \text{Influent}) \times 100 \quad \text{(Equation 6.1)}$$

Load in the effluent was calculated by converting the levels of various ESKAPEs, antibiotic residues and ARGs to 1 L and then multiply by the average capacity of each plant. The data in Table 3.1 was used to calculate load as well as to comment on the potential impacts of the various parameters. The plant identities, province where situated, receiving water bodies of treated effluent, downstream land use activities as well as Green Drop risk categories are provided in this table.

6.3 RESULTS

6.3.1 Reduction potential of nutrients

From the trends presented in Section 3.4, it was evident that for most of the WWTPs lower levels of nutrients were generally measured in effluent compared to influent. In this section we investigate the reduction potential of the nutrients expressed as percentage reduction. In Figure 6.1 these reduction trends are demonstrated. From the two graphs presented it is Figure 6.1 it is evident that reduction potential varied over seasons. The general trend is that nitrates were more readily reduced, compared to phosphates.

6.3.2 Reduction of antibiotic resistant ESKAPE species and loads into the environment.

In this section results are reduction of ESKAPE species presented by reference to culture dependent methods and qPCR. From the trends presented in Sections 4.3 and 5.3, it was evident that for most of the WWTPs lower levels of ESKAPE species were generally measured in effluent compared to influent. In this section we investigate the reduction potential of the nutrients expressed as percentage reduction. For some sampling periods no potential isolates for some of the species were measured either in the influent or effluent. The same was true for the qPCR methods. In Figures 6.1 shows that the reduction of *Klebsiella* sp. for some of the sampling periods were observed.

The data presented in Figure 6.2 shows that many of the sampling periods, close to 100% removal of this species was observed. There was one episode at each of the plants where the reduction was much lower. Reasons for this was not further explored. Similar trends were observed for the *Klebsiella pneumoniae* and other ESKAPE species (Figure 6.3 and 6.4).

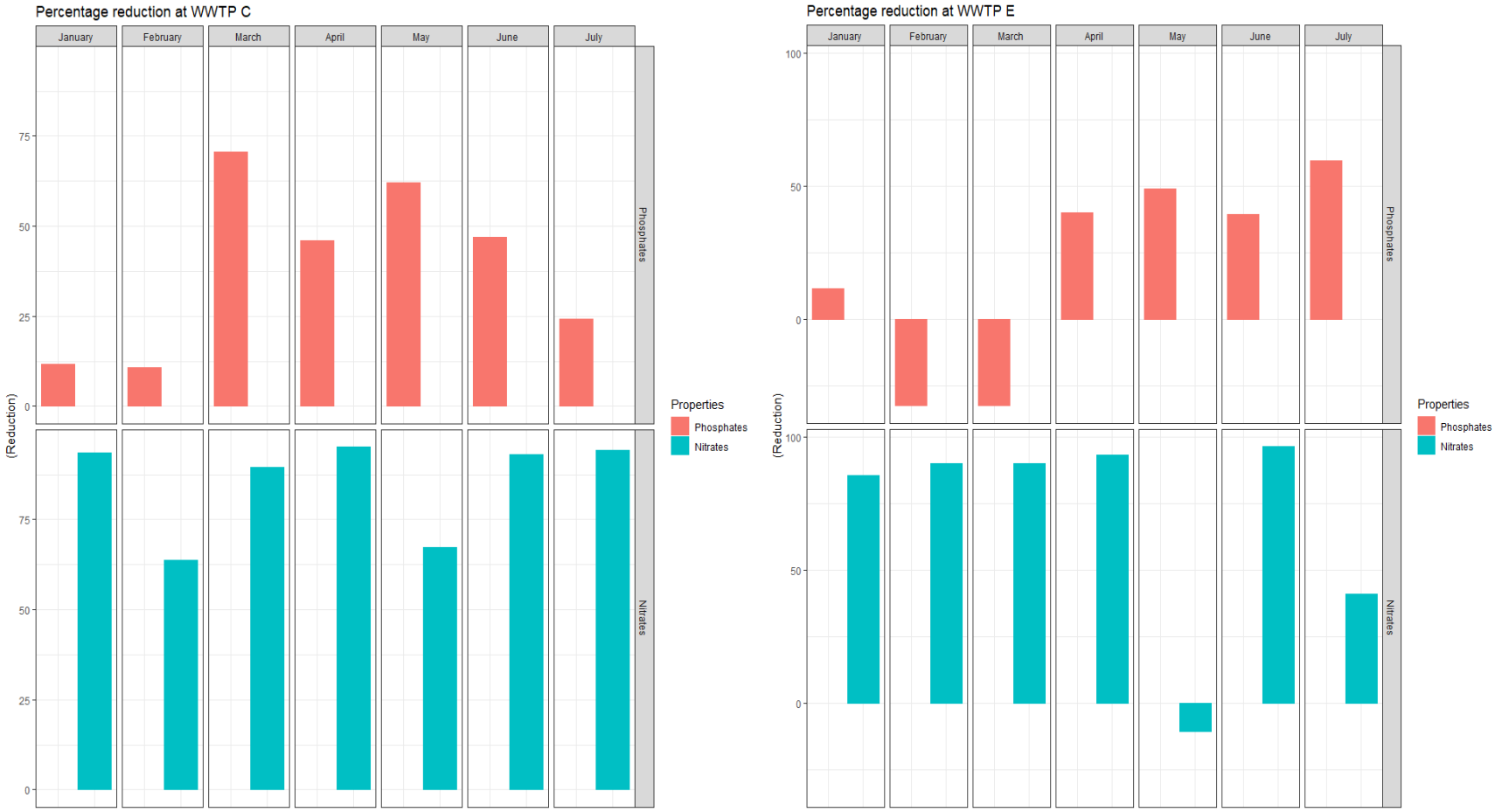


Figure 6.1: Percentage reduction of nutrients at WWTP C and E.

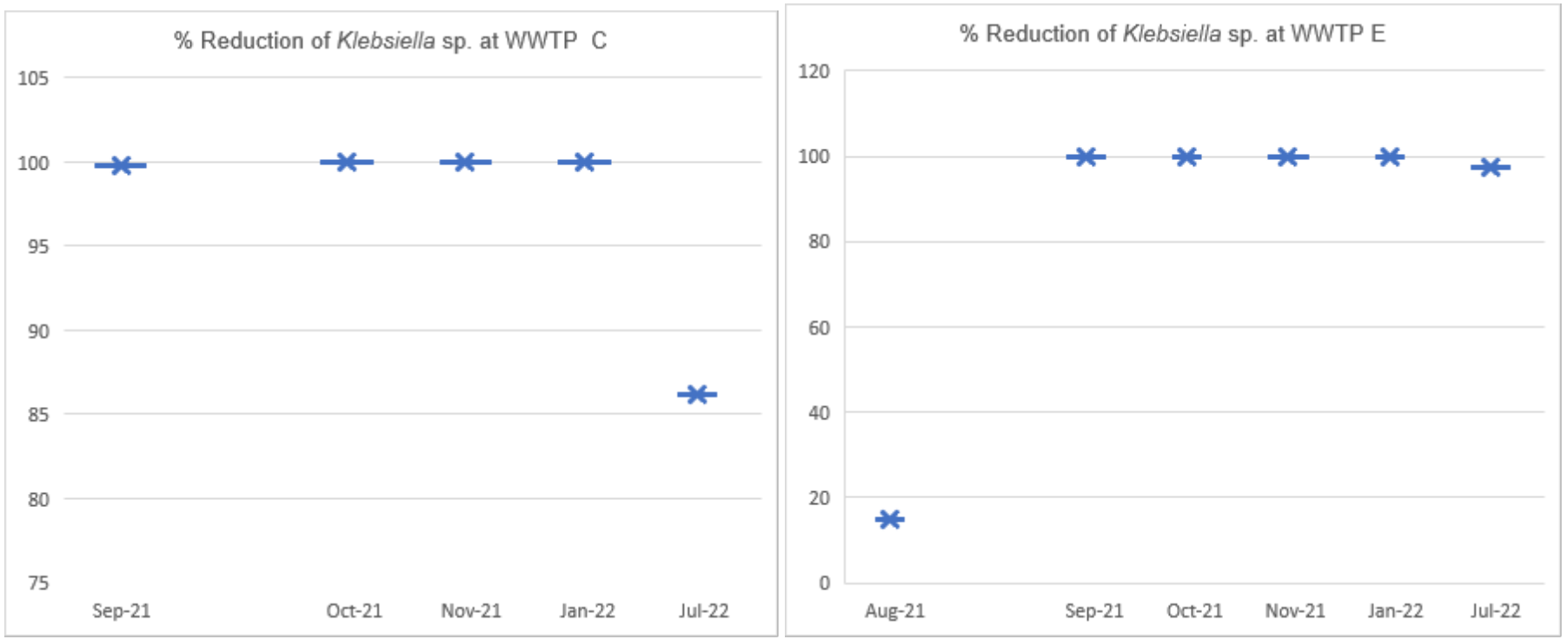


Figure 6.2: Percentage reduction of *Klebsiella pneumoniae* at WWTP C and WWTP E based on culture-based data.

Figure 6.3: Percentage reduction of ESKAPEs at WWTP C based on culture dependent methods

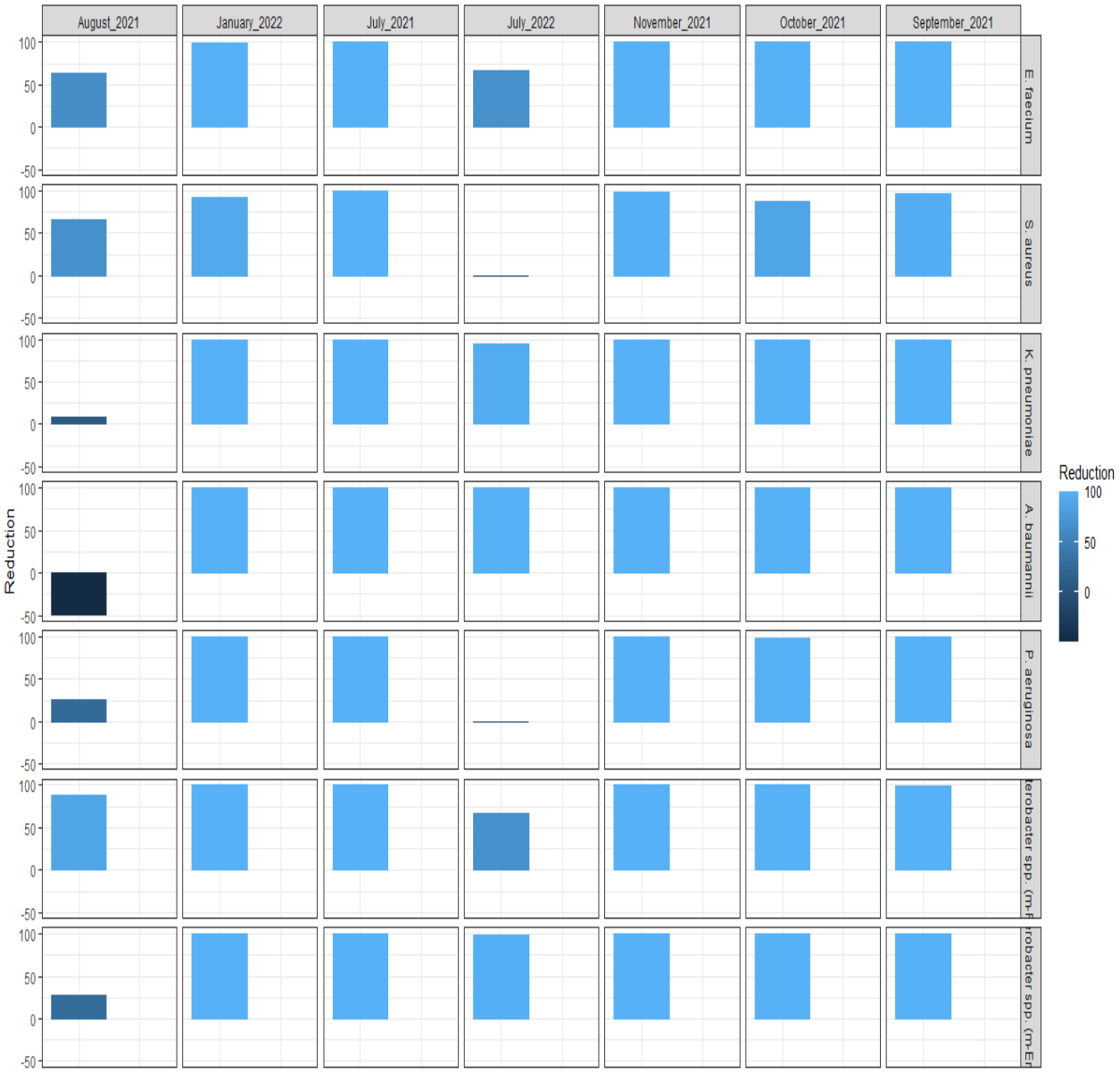


Figure 6.4: Percentage reduction of ESKAPEs at WWTP E based on culture dependent methods

Using qPCR data shows that detection of various ESKAPE species were not consistent. Levels of actual gene copy numbers, considering the capacity of each plant, shows higher levels of ESKAPE gene entering WWTPs. These levels of ESKAPE gene copy numbers are reduced but remains in the order of log 9 to log 14, entering or leaving the WWTPs (Figure 6.5). However, in Figure 6.6 reduction of the some of the ESKAPE species were between 60 and close to 100%. Data for *Klebsiella* sp. for all four plants show that this species was reduced between 80 and close to 100%. Data for WWTP K was more complete and had values for all four of the ESKAPE species targeted. Percentage reduction/removal of *K. pneumonia* in WWTP E is depicted in Figure 6.6. Reduction variety markedly over the sampling period. According to this result only 39% of the genes were removed in October 2021. However, when the culture dependent method results are considered the indication is that close to 100% of culturable *K. pneumonia* were removed. This shows the conflict between data obtained by molecular and culture-based methods. Even so, the overall results demonstrate that WWTPs have the capability to remove culturable ESKAPE species and their marker genes.

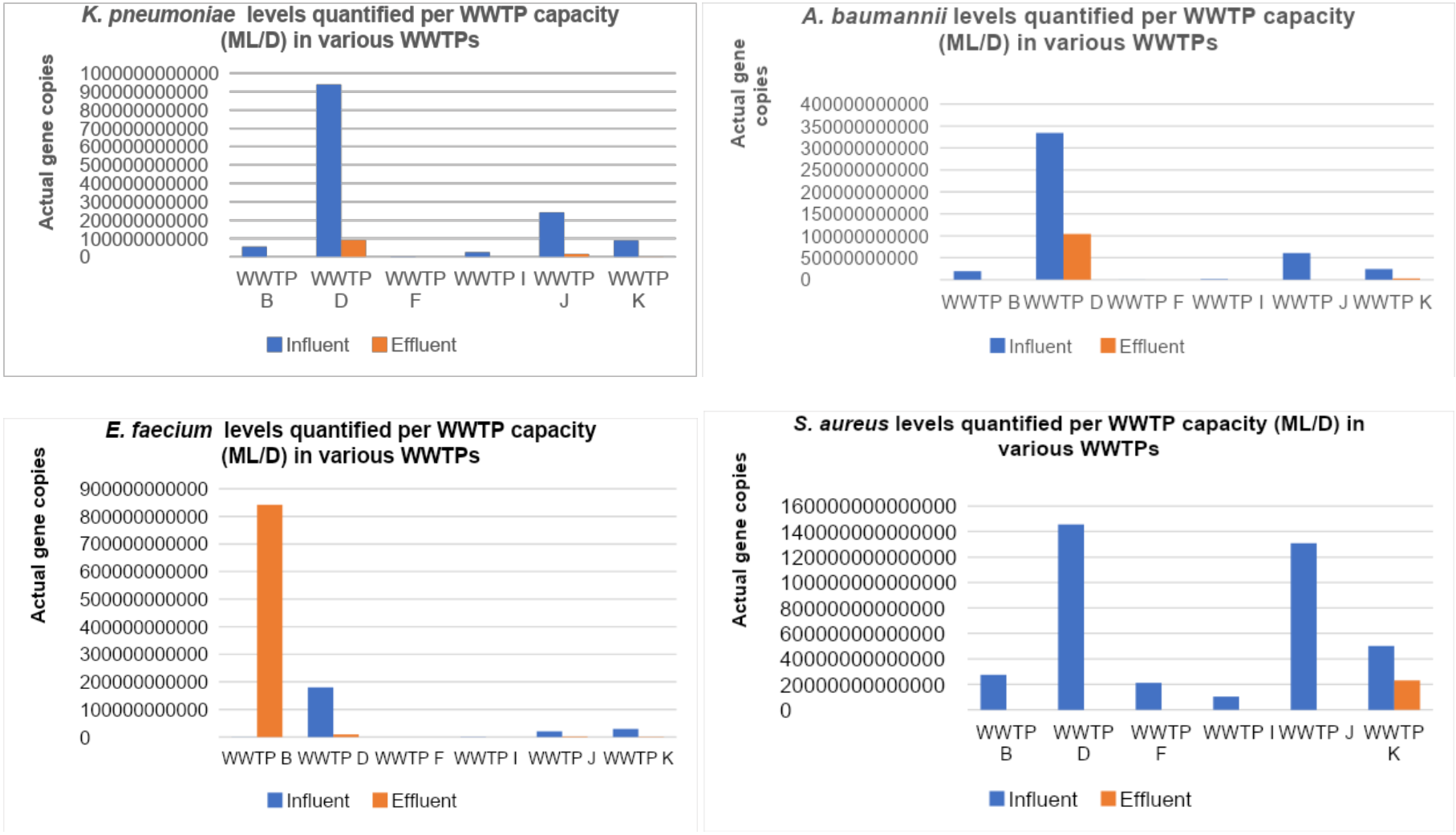


Figure 6.5: Load of various ESKAPE species into and out of WWTPs based on qPCR data and calculated per WWTP capacity.

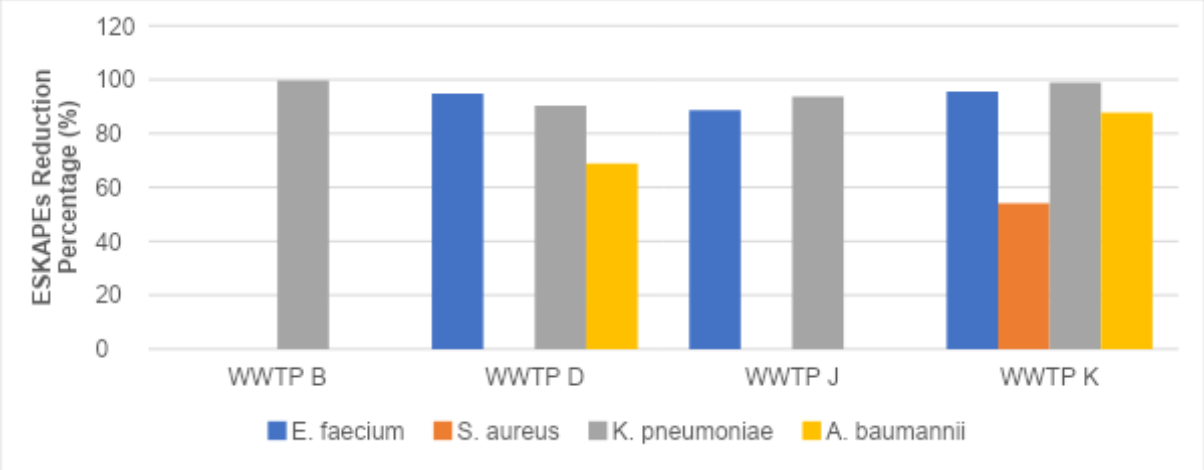


Figure 6.6: Percentage reduction of ESKAPE at various plants using qPCR data and calculated per capacity of the individual plant.

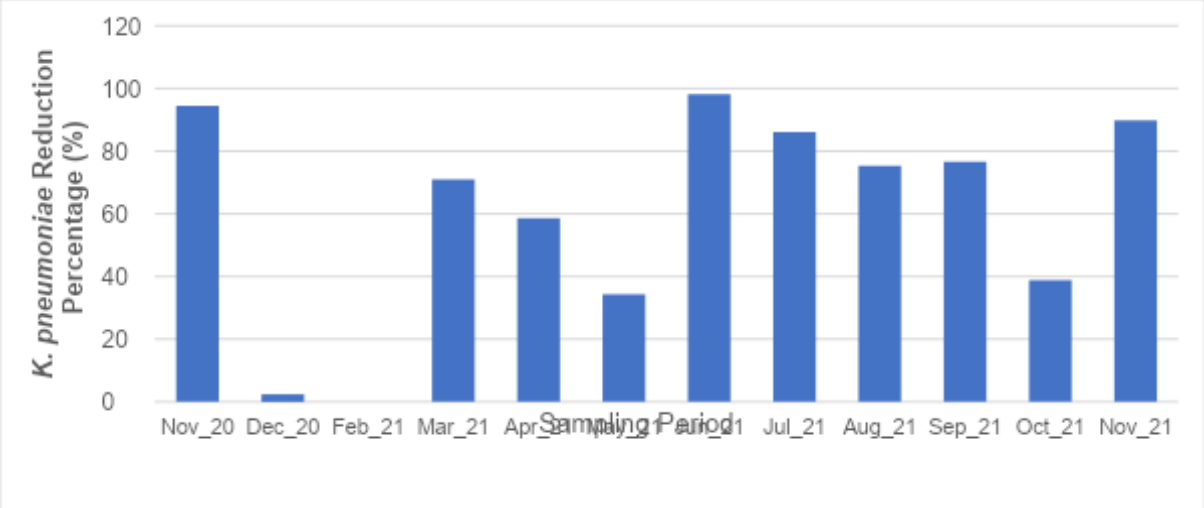


Figure 6.7: Percentage reduction of ESKAPE at WWTP E using qPCR data and calculated per capacity of the plant.

6.3.3 Load of antibiotic residues into the environment

Figure 6.8 depicts average levels of four antibiotics and an antifungal substance present in the effluent of WWTP C and E. The measured concentrations were depicted in Section 3.5 show ampicillin measured in the effluent of WWTP C was twice the concentration of that measured in the effluent of WWTP E. However, in Figure 6.8 the load of ampicillin that is generally deposited into the environment by WWTP E (11205 g) is much higher than that for WWTP C (35 g). This is due to the capacity differences of these plants. Similar trends were observed for the rest of the antimicrobial substances. Since the concentrations of these substances were not measured for influent of these WWTPs reduction potential could not be determined. However, upstream, and downstream samples were analysed. If one considers sulfamethoxazole levels disseminated from WWTP E, it is on average in the order of 227,7 g per day. WWTP E is according to the Greendrop report as a low risk WWTP, yet the total amounts of several antibiotic residues released into downstream water sources are huge, yet, due to the amount of effluent the concentrations are extremely low.

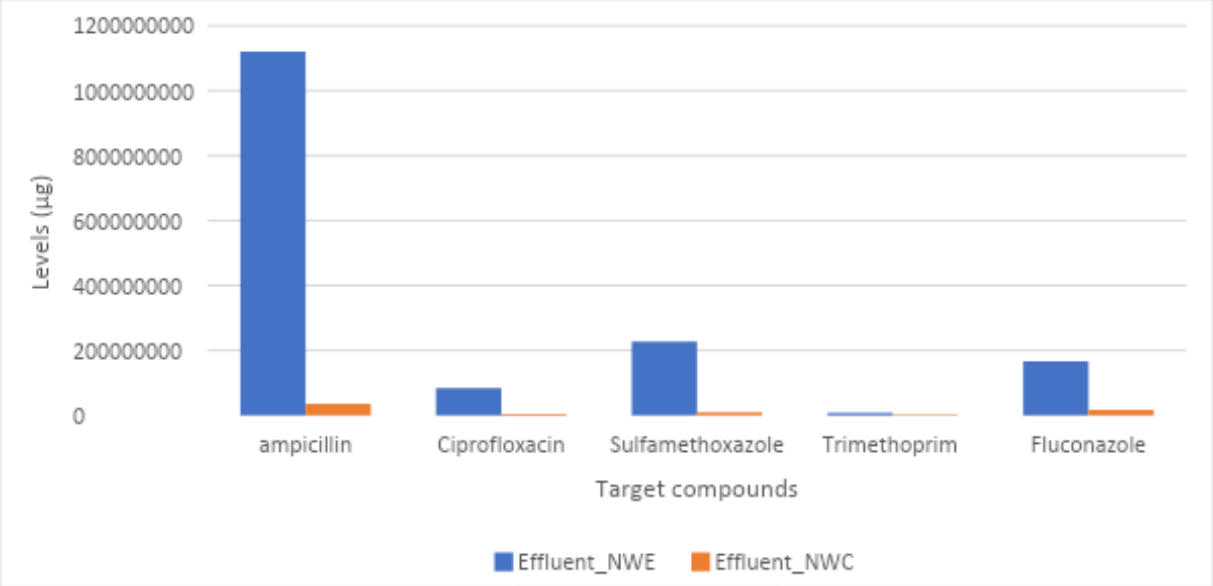


Figure 6.8: Load of antimicrobial substances (in µg) at WWTP C (capacity 3 ML/Day) and WWTP E (capacity 45 ML/Day).

6.3.4 Reduction and load of antibiotic resistant genes into the environment

Figures 6.9 to 6.11 demonstrate the levels of various ARGs that are WWTP effluent and are deposited into the downstream environments of various WWTPs. These vary for x 10^{12} to x 10^{18} gene copy numbers. These are huge numbers. In Figure 6.9 only effluent values are provided ranging from x 10^{12} to x 10^{17}. The average level of the MOX gene was higher than all the others (x 10^{17}) and for purposes of this image was set at 2.3 x 10^{16}. In the data presented, it is evident that the ARGs FOX, MOX, Sul1 and intI were all exceeding the influent and effluent of WWTP D. The same eDNA sets were used for all the qPCRs of this plant and reduction from influent to effluent was seen for the other ARGs. Removal capabilities of these genes in various WWTPs are shown in Figure 6.12. In the latter figure ARGs FOX, MOX, Sul1 as well as *intI* removal data was not included.

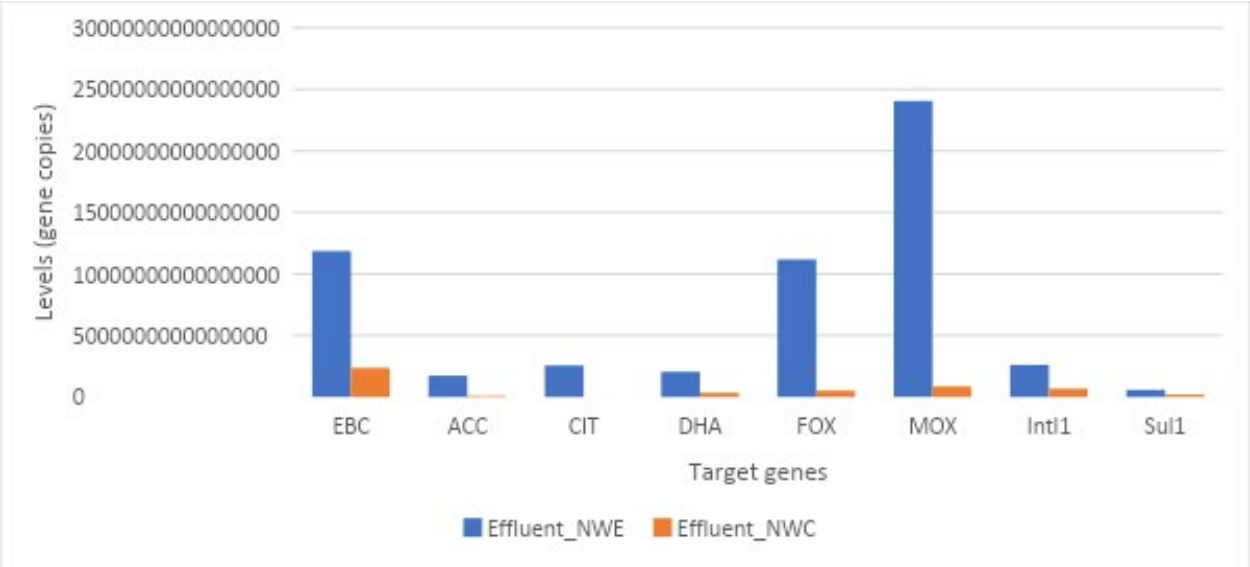


Figure 6.9: Load of ARGs at WWTP C (capacity 3 ML/Day) and WWTP E (capacity 45 ML/Day).

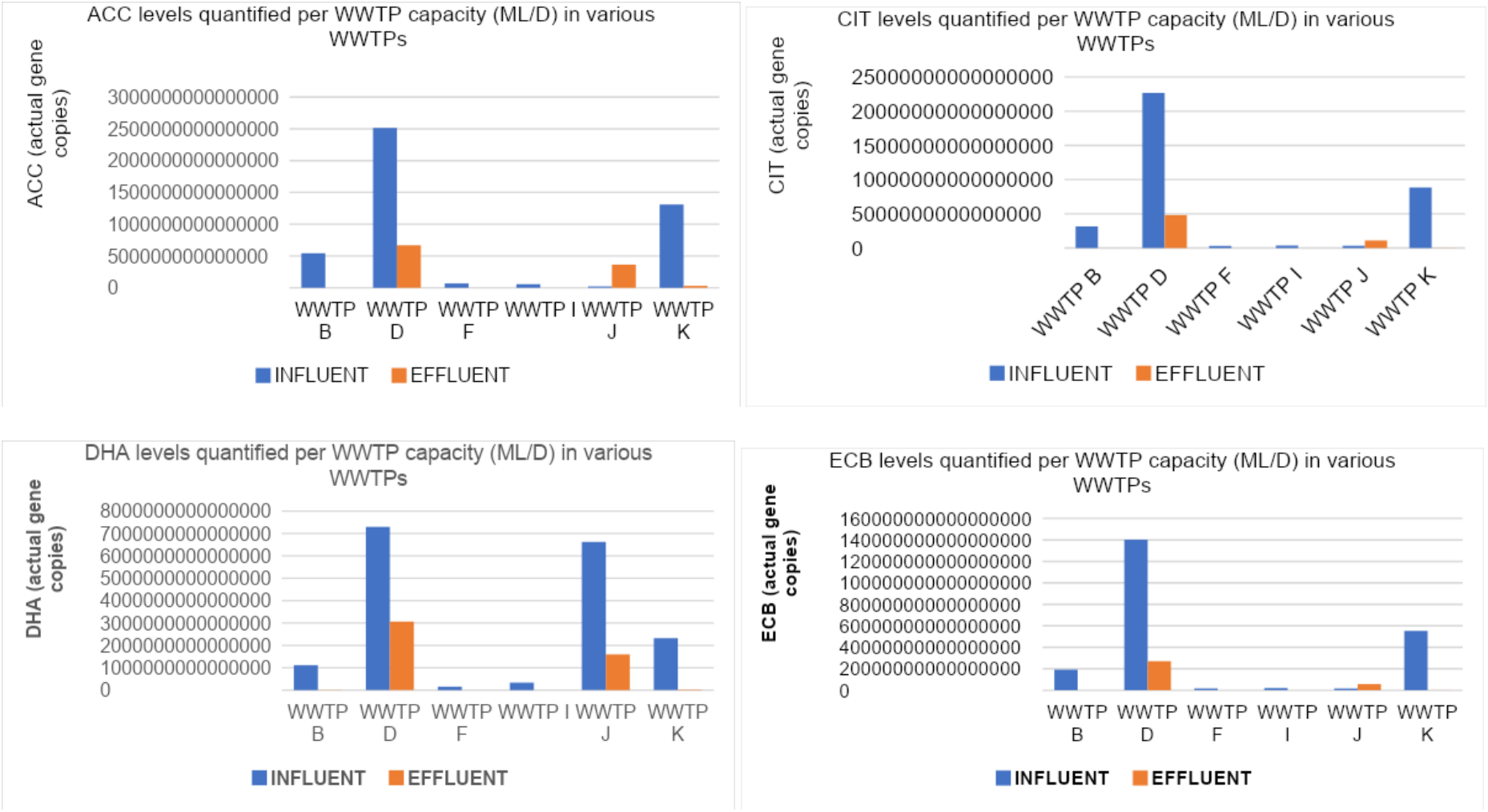


Figure 6.10: Load of ARGs (ACC, CIT, DHA and ECB) at various WWTPs calculated based on actual plant capacity.

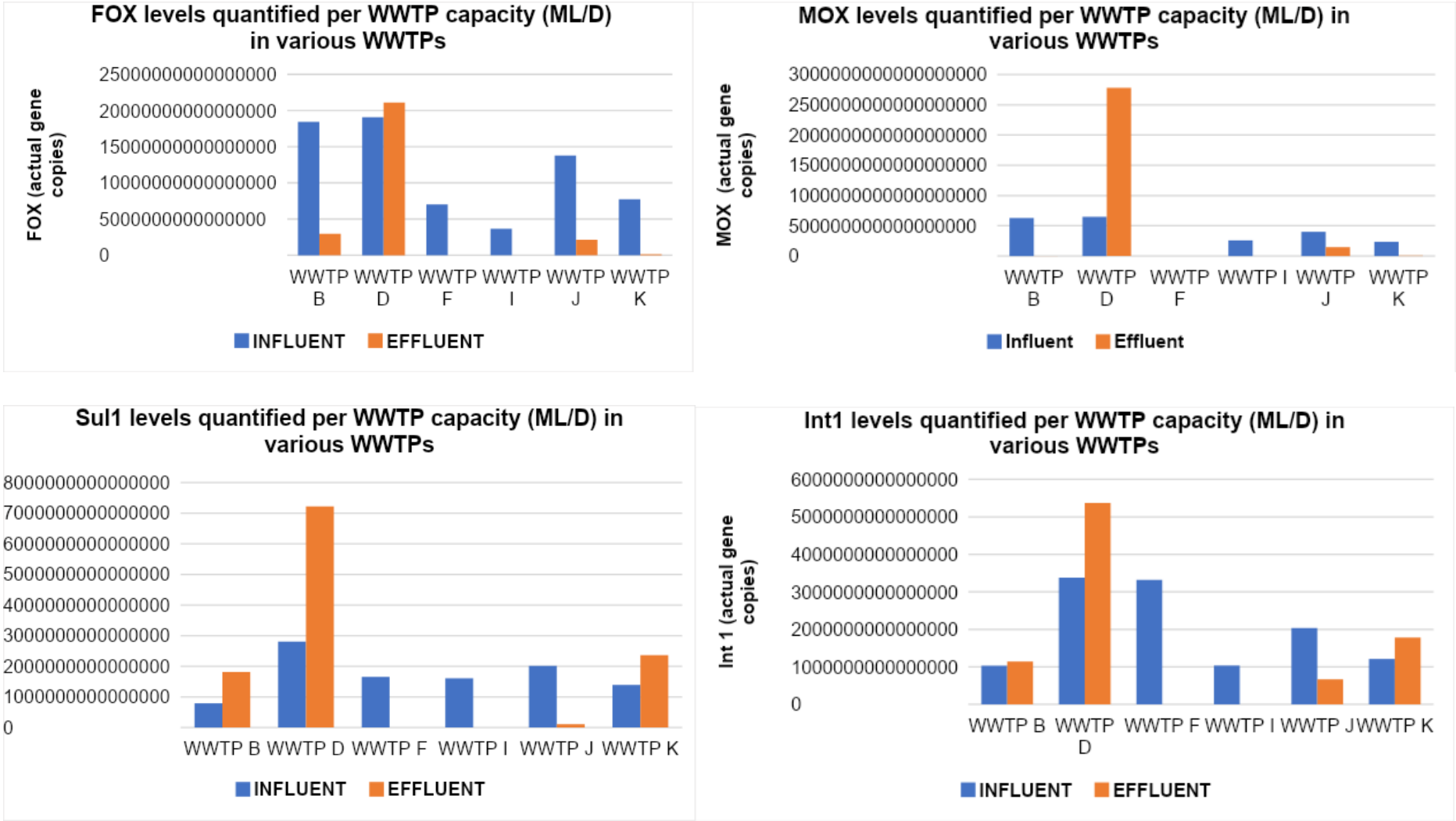


Figure 6.11: Load of ARGs (FOX, MOX, Sul 1 and Int 1) at various WWTPs calculated based on actual plant capacity.

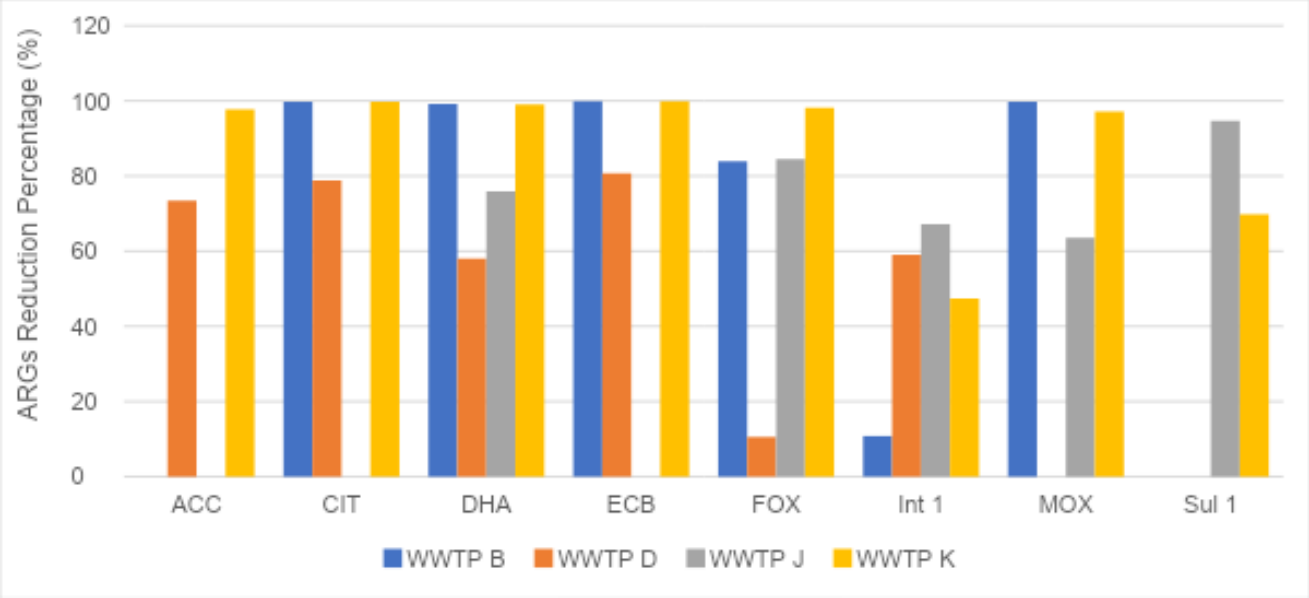


Figure 6.12: Percentage removal of ARGs at various plants WWTP using qPCR data.

6.4 SUMMARY

The potential of WWTP to reduce, nutrients, ESKAPEs, antibiotic residues and antibiotic resistance genes were presented in this chapter. Between 20 and close to 100% of these nutrients and pollutants (antibiotic residues and ARGs) were removed. Four of the WWPs were characterised in the Greendrop report of 2022 as high risk and three as critical risk. These plants are facing many challenges, however, some removal of these pollutants occurred during the period of this study. What is of major concern is even with this measured removal, the dispersal of the nutrients, pathogens, ARGs as well as antimicrobial residues is of concern. The levels of various ESKAPE pathogens, antimicrobial residues and ARGs that are WWTP effluent are deposited into the downstream environments of various WWTPs are enormous. ESKAPE marker gene copy numbers vary from x 10^{11} to x 10^{16} gene copies per day. Furthermore, antibiotic resistant gene copy numbers vary from x 10^{12} to x 10^{18} gene copies per day. These are huge amounts of DNA that could have detrimental effect on downstream ecosystems. The total amounts of several antibiotic residues released into downstream water sources are also huge (into several 100 grams), yet, due to the amount of effluent the concentrations these pollutants and DNA are extremely low. Impacts of these on the ecosystems are currently undetermined but attention to it must be consider.

The loads of the various ARGs and ESKAPE pathogens were consistently higher than what exited the WWTPs. This observation is important since if WWTPS are not operational or are poorly operated much of the high levels of pollutants would directly land in the receiving water and could have detrimental impacts on such ecosystems. The results presented here provide insights into total amounts of ESKAPE species, ARGs and antibiotic residues that are dispersed into receiving water bodies. At all the plants the downstream water is used for various purposes, including agriculture (irrigation and livestock watering), drinking water production, recreation, and religious purposes. Water polluted with antibiotic resistant ESKAPE pathogens containing clinically relevant ARGs as well as antimicrobial residues will likely have serious implications for the user of this water.

7 DEVELOPMENT OF A SURVEILLANCE PROGRAM FOR ANTIBIOTIC RESISTANCE IN THE ENVIRONMENT

7.1 INTRODUCTION

This chapter presents a guideline regarding the development of a surveillance program for antibiotic resistance in the environment and aquatic ecosystem.

The development of a surveillance program for antibiotic resistance (AMR) in the environment and aquatic ecosystems is a critical endeavour when considering the escalating global health challenges AMR present. This chapter aims to conceptualization and establishment of a comprehensive surveillance framework that can be used to monitor and understand the relationship between human activities, environmental dynamics, and the emergence and dissemination of AMR. The latter can be achieved by focusing on the interaction as well as AMR contamination emanating from human populations, healthcare facilities, and the natural environment. This suggested surveillance program framework aims to provide valuable insights into the presence, characteristics, and prevalence of AMR pathogens and genes within the ecosystem. In doing so, it contributes to a more holistic understanding of the factors shaping the development and spread of antibiotic resistance. This chapter addresses objective 5 which is the development of a surveillance program for antibiotic resistance in the environment.

7.2 METHODS

A desktop study was conducted to determine a suitable framework that could be applicable for the surveillance of antibiotic resistance in the environment.

7.3 RESULTS

7.3.1 Surveillance program background underpinned by literature

Masterton (2000) defines surveillance as the core tool in understanding the nature and scope of the problem while assisting in controlling the problem at hand. World Health Organization (WHO) (2021) further describes surveillance as an essential tool for informing policies and interventions, including stewardship and infection prevention and control. Based on the latter, it is a cornerstone for monitoring the emergence and spread of AMR and for evaluating the effectiveness of local, national, and global containment and mitigation strategies. WHO has aided in the development of international, regional, and national surveillance systems to track changes in drug resistance and the effectiveness of treatments for TB, HIV infection, malaria, and other neglected tropical illnesses over the past decades (WHO, 2020). This has been done extensively in the clinical settings for infectious diseases. The WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS) has various types of AMR-related surveillance activities that are grouped into technical modules (WHO, 2021). These technical modules comprise of surveillance activities built on routinely available data (e.g. patient samples collected for clinical purposes or national sales of antimicrobials) and focussed surveillance's actions are designed to produce information for various purposes based on the demands of different countries, territories, and regions. Furthermore, three essential elements need be established, according to GLASS, in order to create a reliable national AMR surveillance system and these include (i) A National Coordinating Centre (NCC); (ii) National Reference Laboratory (NRL); and (iii) Sentinel surveillance

sites for collecting clinical information, diagnostic results and epidemiological data. Currently, the GLASS-AMR offers a standardized method for gathering, analysing, and exchanging national data on AMR in samples regularly obtained for clinical use for a number of bacteria that cause typical human infections (Tornimbene et al., 2022). The pathogens that are currently included in the GLASS-AMR are: *Acinetobacter* spp., *E. coli*, *K. pneumoniae*, *N. gonorrhoeae*, *Salmonella* spp., Shigella spp., *S. aureus*, and *S. pneumoniae*. This has been extensively researched and investigated in the clinical settings, however, the environment receiving hospital wastewater has been neglected.

WHO describes environmental health as all the physical, chemical, and biological factors external to a person and all the related behaviours (The Healthy People 2020 Environmental Health, 2020). The latter also explained that environmental health comprises of preventing or controlling diseases, injury as well as disabilities related to the interactions between people and their environment. The environment plays a significant role in sustaining both biotic and abiotic factors. It is therefore imperative to preserve and maintain environmental health. According to WHO (2006) poor environmental quality has a great impact on immunocompromised individuals and therefore suggested that environmental health should address both the societal and environmental factors that increase the chances of exposure and disease.

The environment is vulnerable to various factors (e.g. exposure to hazardous substances in the air, water and soil) that impact its health negatively. Prevention of exposure to such factors depends on many stakeholders, including state and local health departments, surveillance systems as well as proper education for investigating and responding to diseases and monitoring for hazards (The Healthy People 2020 Environmental Health, 2020). Surveillance programs comprises of compiling, analysing and disseminating data on the problem posed, in this case, the presence, characteristics and prevalence of ESKAPE pathogens in the environment as well as the presence and prevalence of ARGs. Effective surveillance is significant in both understanding and controlling the spread of the problem. It also allows assessment as well as evaluation of possible intervention/ implement plans. According to WHO (2015) the following guidelines were suggested in establishing a proper surveillance program/system (This was modified from public health surveillance programs).

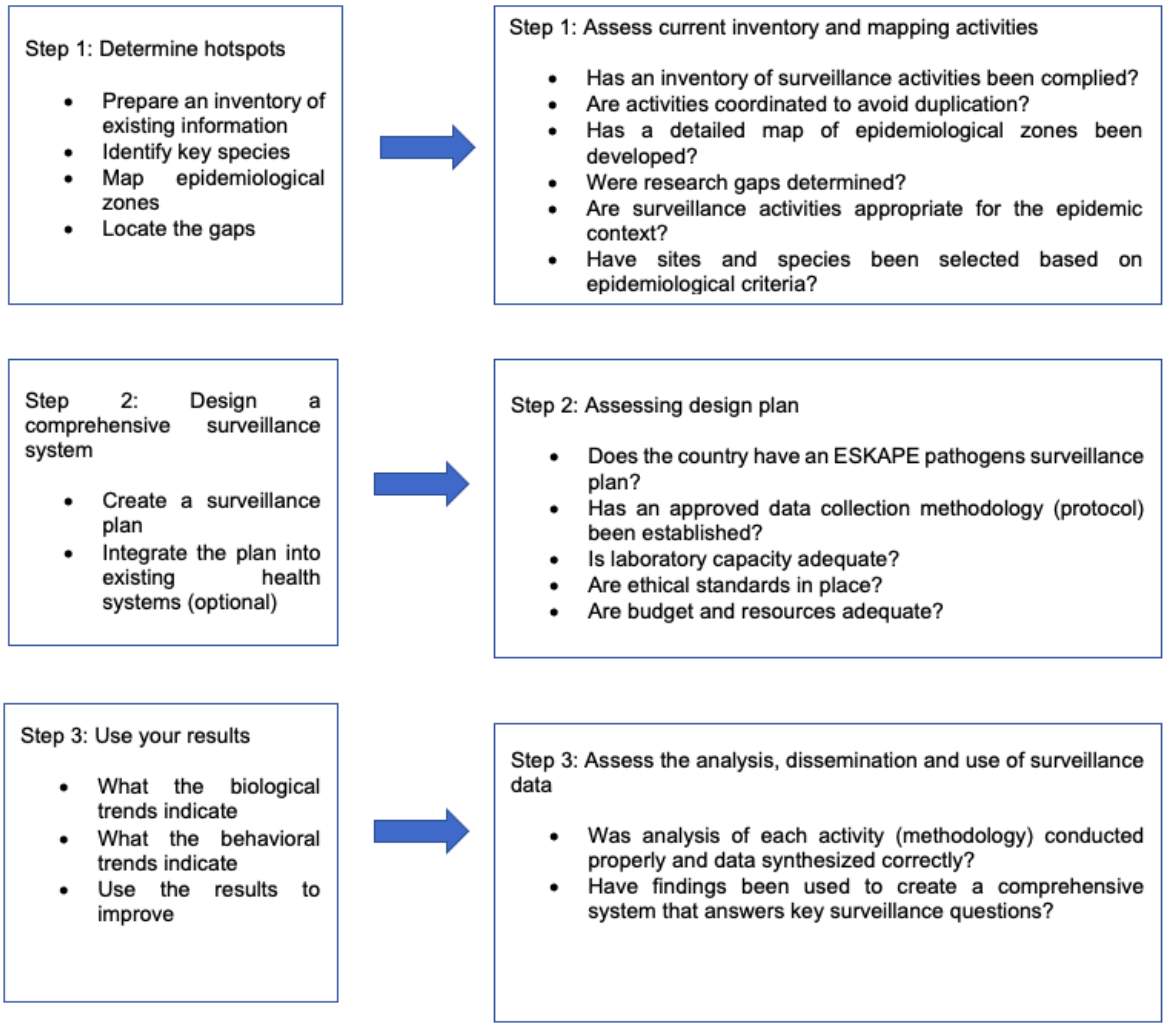


Figure 7.1: Guidelines used for the surveillance program (adapted from WHO, 2013).

7.3.2 Surveillance Recommendations

Undeniably, it is evident that human activities play a vital role in shaping the levels and type of AMR encountered in natural ecosystems, especially in water environments. There is also evidence that suggests that the environment is a source of resistant infections in clinical settings due to the WWTP's incapability to effectively remove ARBs and ARGs. The focus of the surveillance program in this project is based on four key monitoring objectives that are (i) Monitor ESKAPEs and ARGs, circulating in human populations especially in hospital settings, that may find their way into the environment (ii) Quantifying ESKAPEs and ARGs evading treatment, (3) Quantify ESKAPEs and ARG removal efficiencies and (4) Quantify ESKAPEs and ARG released into the environment. Liguori *et al.* (2022) conducted a similar monitoring program as seen in Figure 7.2.

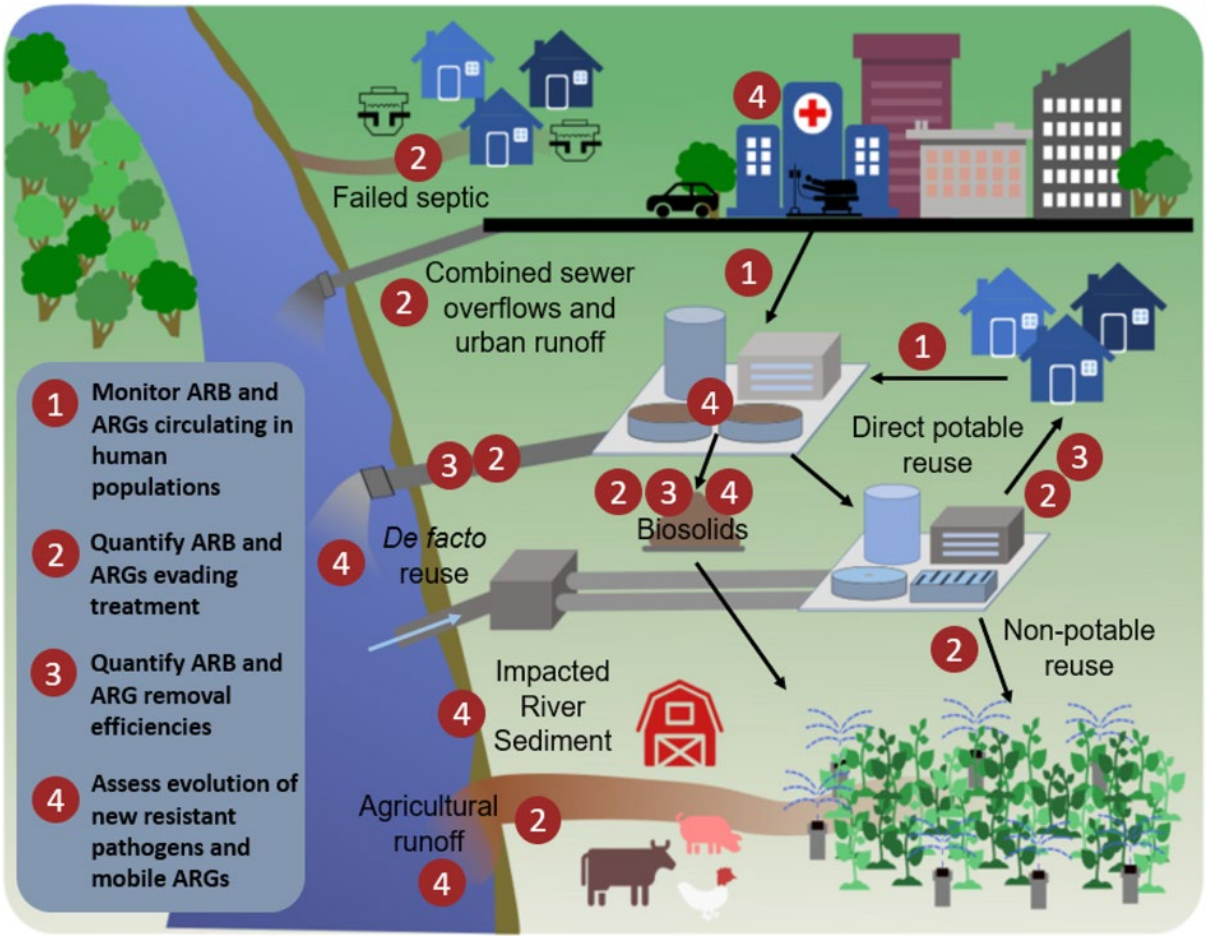


Figure 7.2: Monitoring objectives and transmission pathways for antimicrobials, resistant microorganisms, mobile genetic elements (MGEs), and antibiotic resistance genes (ARGs) in humans and the environment (Liguori *et al.*, 2022).

Although the four main objectives were fulfilled in terms of K. pneumoniae, there are other several critical questions that need to be addressed to effectively monitor the presence and prevalence of ESKAPEs and their respective ARGs from an environmental perspective. These critical questions that need to be assessed include:

- What kinds and levels of ARGs in both wastewater and receiving water environments result in increased exposure and risk of acquiring a resistant infection?
- What are the vital hotspots for horizontal transfer of ARGs and the evolution of new forms of resistant ESKAPEs and how might such hotspots be prioritized for mitigation efforts?
- Which environmental factors in the aquatic environments (e.g. such as concentrations/ mixtures of antibiotics/heavy metals and physico-chemical parameters) play a role in the rapid increase of selective pressure for resistant microbes and maintenance of ARGs?
- What are the relative contributions of various environmental sources of AMR to resistant infections observed in humans?
- What are the most alarming epidemiological linkages between AMR observed in the environment and animals compared to infections found in humans?

7.4 SUMMARY

The development of a surveillance program for antibiotic resistance (AMR) in the environment and presents a critical step for already achieved and in progress AMR collective efforts to address the escalating global challenge of AMR. This chapter has presented the pressing need for a comprehensive surveillance framework that not only monitors AMR within clinical settings but also extends its scope to encompass the intricate interactions between human populations, healthcare facilities, and the environment.

Through a synthesis of established principles and guidelines, as outlined by WHO, this chapter has proposed a surveillance program capable of bridging critical knowledge gaps. The four key monitoring objectives – monitoring the presence and prevalence of ESKAPE pathogens and antibiotic resistance genes (ARGs), quantifying treatment evasion, assessing removal efficiencies, and evaluating release into the environment – form the foundation of this suggested approach.

The significance of this surveillance program lies in its potential to provide insights into the complex pathways through which AMR disseminates between human populations and the environment. By shedding light on the transmission dynamics, removal processes, and potential reservoirs of AMR, this program holds the promise of guiding targeted interventions that mitigate the spread of resistance. Furthermore, if implemented with careful considerations it has the potential to contributes to a broader understanding of the interactions between human health and the environment, underlining the inseparable link between the two and the imperative to protect both.

As evidenced by the extensive efforts of WHO and other global health organizations, surveillance is not merely an academic exercise but a dynamic tool that informs policies, drives interventions, and shapes the course of disease management. By extending this paradigm to encompass the environment, we forge a new path towards a more holistic and effective approach to combating AMR.

Nevertheless, while this chapter has laid out a comprehensive framework, it is not an endpoint but a starting point for further research, collaboration, and implementation. The critical questions raised – concerning the levels and types of ARGs, hotspots for horizontal transfer, environmental factors influencing selective pressures, and the epidemiological links between AMR in the environment and humans – are invitations for future exploration. Lastly, interdisciplinary collaboration between healthcare, environmental science, and policy-making realms are essential to translate this surveillance program into tangible actions and impactful outcomes.

8 CONCLUSIONS AND RECOMMENDATIONS

8.1 CONCLUSIONS

8.1.1 Aims of the study

There were 5 aims for this project:

1. Compile an overview on the importance and relevance of ESKAPE and Clostridia spp in the global priority pathogen list (PPL) as indicators for antibiotic-resistance in the environment as part of the One Health Approach.
2. Use qPCR for setting up a for monitoring the sources and dissemination of ESKAPE strains and associated resistant genes within environments.
3. Evaluate the reduction potential of ESKAPE in selected wastewater treatment works.
4. Establish antibiotic resistance trends of selected ESKAPE pathogens in water environments and determine their clinical relevance.
5. Development of a surveillance program for antibiotic resistance in the environment

8.1.2 Determination of physico-chemical parameters and ESKAPE levels in WWTP influent, effluent and receiving water

Chapter 3 focused on the determination of physico-chemical parameters, and ESKAPE levels in water using culture dependent methods. The aim was to assess the water quality and the presence of ESKAPE bacteria. The analysis of various parameters, particularly the nutrients in the wastewater treatment plants provided valuable insights into the quality and effectiveness of the treatment processes. Values for the physico-chemical parameters were within expected and mostly acceptable ranges. The nutrient levels and physical conditions were favourable for the maintenance of the ESKAPE populations in sewage systems.

When the isolated presumptive ESKAPE species were purified on selective media and identified using 16S rRNA gene sequencing, more than 40% of isolates were not ESKAPE species. False positive rates were thus very high.

Phosphate, nitrate and COD levels varied between different plants, with WWTP C consistently exhibiting the highest concentrations. When one considers that WWTP C is considered in a high-risk category and WWTP E is in a functional category, the findings makes sense.

During the rainy season, higher levels of ESKAPE pathogens were observed in influent compared to effluent samples, indicating a reduction in most cases. *Klebsiella* sp. often displayed the highest counts and presence in both influent and effluent, indicating potential contamination risk as well as suggesting that this could be a sentinel species in ESKAPE monitoring regimes.

Observed correlations between the ESKAPE pathogen levels and physicochemical parameters within the context of WWTP E hold implications for understanding the dynamics across different seasons. The strength and direction of these correlations offer insights into how seasonal variations might influence physicochemical conditions and impacting ESKAPE populations and within the wastewater treatment system. Measuring physicochemical parameters of influent and effluent is thus a valuable contribution when ESKAPE surveillance programs are considered. Findings presented here emphasize the

importance of efficient wastewater treatment to mitigate antibiotic resistant ESKAPE pathogen dissemination and safeguard receiving waters, environmental and public health.

8.1.3 Assessing antibiotic residues and antibiotic resistance trends of selected ESKAPE pathogens in water environments: Clinical relevance and qPCR monitoring of dissemination of antibiotic resistant strains

Chapter 3 presents data for the levels of antimicrobial substances, as well as using qPCR to monitor the levels of ESKAPE species as well as antibiotic resistant genes in WWTP ecosystems. Culture-based characterization data for ESKAPE were also presented. Measurable concentrations of specific antibiotic residues, such as ampicillin, ciprofloxacin, sulfamethoxazole, and trimethoprim were measured in wastewater effluent and levels were higher in the effluent compared to surrounding downstream and upstream water bodies. Antimicrobials, commonly used in medical, veterinary, and agricultural practices, can find their way into wastewater, and subsequently impact downstream aquatic ecosystems and potentially also human health. Overall, the data also removal efficiencies of the specific antibiotics. Wastewater treatment plants were not designed for antibiotic removal but when these systems are effectively managed and operated antibiotic residues could be reduced.

Monitoring antimicrobial residues in wastewater, is important for various reasons. Firstly, it aids in assessing the potential environmental impact of these substances, which are widely used in healthcare, agriculture, and veterinary practices. Secondly, tracking antimicrobial concentrations ensures compliance with safeguards of public health by evaluating risks associated with water consumption, and contributes to scientific research on pollution and antibiotic resistance. Furthermore, the data assists in source identification, management, and policy formulation, empowering decision-makers, and wastewater operators to take informed actions toward curbing antimicrobial pollution.

The antibiotic susceptibility and pathogenicity indicated that resistance to ampicillin was prevalent among *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterobacter species* in WWTPs C and E. In other WWTPs (B, D, I, and J), consistent resistance to ampicillin observed across influent, effluent, and downstream sites. These underscore potential challenges in mitigating the spread of antibiotic-resistant strains through wastewater and that such species could land in receiving water. Furthermore, the study explored the pathogenicity potential of ESKAPE isolates from WWTPs C and E by assessing their ability to produce extracellular enzymes which provide them with the ability to cause disease.

RDA analysis demonstrated correlations with the ESKAPE species, characteristics and compartments of the two WWTPs C and E. Such relationships were consistent with an ordination where the physico-chemical parameters, ESKAPE species and WWTPs were analysed for correlations.

Quantitative analysis of gene copy numbers using qPCR provided detailed insights into the prevalence and persistence of ESKAPE pathogens across different WWTPs. Some plants exhibited higher levels of specific ESKAPE species in effluent, indicating incomplete removal during treatment.

The antibiotic residues found in the effluent and upstream and downstream sites could be responsible for maintaining antibiotic resistant ESKAPE species in the system. Trends observed in the variations of residue concentrations were consistent with the variations of the ARGs detected. This is also evident in the antibiotic susceptibility results that demonstrate large percentage of ESKAPE pathogens were resistant to β-lactam antibiotics, including carbapenems.

qPCR was also used to detect and quantify antibiotic resistance genes in the various compartments from several WWTPs. The data demonstrate that overall, the WWTP effluent had higher levels of these

genes compared to up and downstream sites. The presence of these genes in the ecosystems could have detrimental impacts on ecological health. These findings underscore the importance of tailored treatment strategies and improvement of treatment processes to slow down the spread of antibiotic resistance.

This study also highlighted the overlap between the ESKAPE species and characteristics coming from clinical and environmental sources. The evidence provided here support the notion that ESKAPE species from clinical settings (clinics, hospitals) and household are transported via sewage to WWTPs. Even though reduction of such pathogens occurs during wastewater treatment, these ESKAPE pathogens land in receiving environmental water.

8.1.4 Dissemination and potential impacts of WWTP effluent or receiving water bodies and evaluating indicative removal of selected antibiotic strains and genes in wastewater

The potential of WWTP to reduce, nutrients, ESKAPEs, antibiotic residues and antibiotic resistance genes were presented in Chapter 6. Between 20 and close to 100% of these nutrients, pollutants (antibiotic residues and ARGs) and antibiotic resistant ESKAPE species were removed. What is of major concern is even with this measured removal, the dispersal of the nutrients, pathogens, ARGs as well as antimicrobial residues is of concern. The levels of various ESKAPE pathogens, antimicrobial residues and ARGs that are WWTP effluent are deposited into the downstream environments of various WWTPs are enormous. The total amounts of several antibiotic residues released into downstream water sources are also huge (into several 100 grams), yet, due to the amount of effluent the concentrations these pollutants and DNA are extremely low. Impacts of these on the ecosystems are currently undetermined but attention to it must be consider.

The loads of the various ARGs and ESKAPE pathogens were consistently higher in the influent than what exited the WWTPs. This observation is important since if WWTPS are not operational or are poorly operated much of the high levels of pollutants would directly land in the receiving water and could have detrimental impacts on such ecosystems. At all the plants the downstream water is used for various purposes, including agriculture (irrigation and livestock watering), drinking water production, recreation, and religious purposes. Water polluted with antibiotic resistant ESKAPE pathogens containing clinically relevant ARGs as well as antimicrobial residues will likely have serious implications for the user of this water.

8.1.5 Development of a surveillance program for antibiotic resistance in the environment

Chapter 7 presented need for a comprehensive surveillance framework that not only monitors ESKAPE species and AMR within clinical settings but also extends its scope to encompass the intricate interactions between human populations, healthcare facilities, animals and the environment. Through a synthesis of established principles and guidelines, as outlined by WHO, this chapter has proposed a surveillance program capable of bridging critical knowledge gaps. The four key monitoring objectives – monitoring the presence and prevalence of ESKAPE pathogens and antibiotic resistance genes (ARGs), quantifying treatment evasion, assessing removal efficiencies, and evaluating release into the environment – form the foundation of this suggested approach. Data provided in the chapters preceding Chapter 7 such data collection and interpretation was done. This chapter provided a comprehensive framework for a surveillance program it is not an endpoint but a starting point for further research, collaboration, and implementation. Interdisciplinary collaboration between healthcare, environmental science, and policy-making realms will be essential to translate this surveillance program into tangible actions and impactful outcomes.

8.2 RECOMMENDATIONS

National monitoring of levels of one or two sentinel ESKAPE species at major WWTPs, using culture based and culture independent methods such as that describe in this study. In the case of the COVID-19 surveillance it was a single marker that was monitored, and analyses could be standardised across samples, analysis sites and data processing. The results from the present study had relatively complete data sets for *Klebsiella pneumonia* which corresponded to data from the other species, using both culture dependant and culture independent methods. This species perhaps this should be considered as a sentinel species for the monitoring of EKSAPE species in the environment. Furthermore, the antibiotic susceptibility of the environmental and clinical isolates could be overlaid.

The antibiotic resistant patterns among all the ESKAPE overlapped with the antibiotic residues detected, demonstrating mostly resistance to β-lactam antibiotics. Clinically relevant ARGs responsible resistance to various generations of β-lactam antibiotics, were detected and quantified in influent, effluent and downstream receiving waters.

For culture independent methods Oxford Nanopore Technologies (ONT) should be considered. In the case of the present study, qPCR was used and was at times inconsistent. This was potentially due to the nature of the technology, equipment and the skill levels required. Furthermore, several specific markers were targeted simultaneously, making this approach a bit complex, unlike the approach that was used for COVID-19. The ONT technologies are portable and skill sets required may not be as intensive as with qPCR.

Whole Genome Sequencing of corresponding species (e.g. *K. pneumonia* and *S. aureus*) from both environmental and clinical setting would provide insights into the genomic dynamics in these two settings. Such data will be invaluable when interventions to stem the tide of antibiotic resistance dissemination into environmental waters are considered. The present study provided evidence of the overlap of phenotypic antibiotic resistant characteristics of *Klebsiella* sp. from environmental and clinical setting. What is currently undetermined is the impacts of downstream water on irrigation and particularly livestock watering.

It is also very important that findings from studies such as this one should be circulated to the relevant stakeholders, including the medical fraternity, agricultural sector, abattoir owners and managers, feedlot owners, relevant ministries, etc. This data from the present study provides information to linking pathogens in the environment (sewage as well as environmental water) possibly back to clinical settings. This data calls for interventions such as pre-treatment of wastewater at high-risk sites (hospitals, clinics, agricultural settings, etc.) must be made. This study demonstrated that actual levels of pollutants are enormous and cannot be ignored. WWTPs that are dysfunctional or are poorly managed not only contribute to pollution of aquatic ecosystems, but this scenario is actively contributing to the spread of the antimicrobial resistant burden in the human population. This is in contradiction to the Constitution of the Republic of South Africa.

Efforts should be made to have a national repository, and sequencing facility/Centre for Environmental AMR. Antimicrobial resistance data must be made available to communities in such a manner that would make it easily understandable to all members. This requires dedicated staff that understand.

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APPENDIX A: DETERMINATION OF PHYSICO-CHEMICAL PARAMETERS

Tables A1-AX below is a summary of the physico-chemical parameters recorded for all the sampling sites investigated in this study over the period November 2020-November 2021.

WWTP C

Results of chemical parameters were provided by the municipal wastewater treatment plant. A hyphen (-) in the table indicates that there was no data available for the specific sampling day or parameter. Values formatted in a green font colour indicate compliance with South African National Standards (SANS) effluent discharge standards (DWS, 2008). Only chemical parameters were compared against South African effluent discharge standards because the analysis was done by the wastewater treatment plant personnel at an accredited laboratory.

During the study period, the overall trends showed that the highest temperature recorded was 24.30°C in February 2021 and, the lowest observed temperature was 15.23°C in June 2021. From May 2021 till August 2021, the temperatures were below 20°C, however, a temperature below 20°C was also recorded in December 2020 (18.97°C) as well. Generally, the pH of the samples remained below 8 but in June 2021, the pH was recorded as 8.39 which is the highest value recorded during the entire study period. The lowest pH level observed was in February 2021 (7.01), this was witnessed from the same sample in which the highest temperature (24.30°C) was recorded. There is evidence of an inversely proportional relationship between pH and temperature as the same was also true for samples belonging to June 2021; when the temperature was the lowest (15.23°C), the pH was the highest (8.39). The TDS values for the influent were generally higher than 800 ppm. The highest TDS value recorded was 1143 ppm in December 2020 whereas the lowest value (811 ppm) was witnessed in October 2021. Overall, salinity of the influent was higher than 400 ppm throughout, the lowest measured salt value in the influent was 495.33 ppm in December 2020 and the highest measured slat value was 736 ppm in August 2021.

The influent COD was no less than 300 mg/L during the months recorded (December 2020-August 2021). The lowest COD value was 331 mg/L in February 2021 and the highest was 603 mg/L recorded in April 2021. On average, the COD of the influent was 368.11 mg/L. Orthophosphates ranged between 5.78 mg/L to 18.20 mg/L. (July 2021 and October 2021, respectively). The assessment of Nitrate in the influent showed that overall, the nitrate values were above 2 mg/L except for results obtained during the month of May 2021 where the Nitrate was measured at an incredibly low 0.4 mg/L. The salinity starting from May 2021 until October 2021 was above 500 ppm.

Table A1: Physico-chemical parameters of WWTP C influent and effluent from November 2020 to November 2021

Month (year)	Sample	Temperature (°C)	pH	Total Dissolved Solids (ppm)	Salinity (ppm)	Electrical Conductivity (µS/m)	COD (mg/L)	PO_4^{3-} (mg/L)	NO^{3-} (mg/L)
November (2020)	Influent	22.56 ± 0.23	7.99 ± 0.41	1100 ± 0.00	495.33 ± 0.58	1437 ± 5.51	-	-	-
	Effluent	22.97 ± 0.15	7.76 ± 0.06	953.33± 0.58	463.33 ± 0.58	1361 ± 2.08	-	-	-
December (2020)	Influent	18.97 ± 0.21	7.05 ± 0.01	1143 ± 5.77	564.00 ± 0.00	1633 ± 9.50	452	12.11	4.6
	Effluent	19.53 ± 0.06	7.58 ± 0.02	979.67± 4.16	490.67 ± 0.58	1400 ± 8.08	**23**	**0.71**	**1.9**
February (2021)	Influent	24.30 ± 0.10	7.01 ± 0.03	1060. ± 0.00	522.67 ± 0.58	1475 ± 0.58	331	11.83	5.30
	Effluent	22.13 ± 0.06	7.59 ± 0.03	918.67± 4.04	455.00 ± 1.73	1310 ± 7.37	**18**	**9.46**	**12.9**
March (2021)	Influent	22.30 ± 0.10	7.45 ± 0.01	956 ± 1.00	671.00 ± 1.73	1347 ± 5.00	345	10.78	5.1

Month (year)	Sample	Temperature (°C)	pH	Total Dissolved Solids (ppm)	Salinity (ppm)	Electrical Conductivity (µS/m)	COD (mg/L)	PO_4^{3-} (mg/L)	NO^{3-} (mg/L)
	Effluent	21.67 ± 0.15	7.68 ± 0.02	771.67± 1.53	535.67 ± 3.06	1091 ± 8.50	-	-	-
April (2021)	Influent	24.20 ± 0.00	7.59 ± 0.32	1120 ± 0.00	550.67 ± 1.53	1594 ± 2.52	603	17.21	4.9
	Effluent	22.37 ± 0.25	7.40 ± 0.04	929.67± 2.31	449.00 ± 2.00	1315 ± 3.21	**14**	**0.17**	**3.8**
May (2021)	Influent	19.50 ± 0.20	7.88 ± 0.01	932 ± 1.15	652.33 ± 0.58	1313 ± 2.08	488	14.21	0.4
	Effluent	17.80 ± 0.10	7.64 ± 0.01	794.67± 0.58	548.67 ± 1.53	1118 ± 1.00	**18**	**0.10**	**2.5**
June (2021)	Influent	15.23 ± 0.12	8.39 ± 0.04	947 ± 2.08	653.00 ± 2.65	1335 ± 3.00	356	16.8	3.3
	Effluent	14.97 ± 0.49	8.37 ± 0.07	815.33± 1.15	561.33 ± 0.58	1137 ± 4.36	**25**	**0.71**	**4.1**
July (2021)	Influent	15.63 ± 0.06	7.98 ± 0.03	922 ± 0.58	638.00 ± 0.00	1291 ± 5.86	404	5.78	5.2

Month (year)	Sample	Temperature (°C)	pH	Total Dissolved Solids (ppm)	Salinity (ppm)	Electrical Conductivity (µS/m)	COD (mg/L)	PO_4^{3-} (mg/L)	NO^{3-} (mg/L)
	Effluent	14.23 ± 0.15	7.63 ± 0.03	838.00± 1.00	577.00 ± 2.65	1187 ± 1.73	27	0.11	1.4
August (2021)	Influent	18.10 ± 0.10	7.56 ± 0.02	1001 ± 0.00	702.33 ± 0.58	1420 ± 0.58	334	12.62	2.6
	Effluent	17.90 ± 0.10	7.69 ± 0.02	823.00± 0.00	568.00 ± 0.00	1160 ± 4.16	24	0.12	0.7
September (2021)	Influent	21.27 ± 0.47	7.51 ± 0.01	1005 ± 0.00	736.00 ± 1.00	1485 ± 4.51	-	13.63	2.20
	Effluent	20.20 ± 0.26	7.70 ± 0.01	856.00± 2.08	596.33 ± 1.53	1218 ± 2.89	-	-	-
October (2021)	Influent	19.27± 0.06	7.86 ± 0.01	811 ± 0.58	560.33 ± 1.53	1142 ± 4.04	-	18.20	3.60
	Effluent	19.87 ± 0.06	7.67 ± 0.01	783.00± 0.00	535.67 ± 2.31	1103 ± 1.53	-	-	-
November (2021)	Influent	22.53 ± 0.06	7.86 ± 0.02	844 ± 5.29	565.00 ± 2.00	1176 ± 1.53	7.	16.26	3.6

Month (year)	Sample	Temperature (°C)	pH	Total Dissolved Solids (ppm)	Salinity (ppm)	Electrical Conductivity (µS/m)	COD (mg/L)	PO_4^{3-} (mg/L)	NO^{3-} (mg/L)
	Effluent	23.30 ± 0.20	7.85 ± 0.02	627.00± 1.00	421.67 ± 1.53	883 ± 2.5	-	-	-

COD – Chemical Oxygen Demand; **PO_4^{3-}** – Ortho Phosphate; **NO^{3-}** – Nitrate

Table A2: Physico-chemical parameters of WWTP C and E influent, effluent and downstream from January 2022 to July 2022

WWTP C								
Month (year)	**Sample**	**Temperature (°C)**	**pH**	**Total Dissolved Solids (ppm)**	**Salinity (ppm)**	**COD (mg/L)**	**PO_4^{3-} (mg/L)**	**NO^{3-} (mg/L)**
January (2022)	Influent	24.1	7.5	981.1	768.0	1532.7	44.2	92.9
	Effluent	24.1	7.7	829.0	579.7	1169.7	35.01	**3.1**
	Downstream	25.3	7.6	432.0	345.2	788.3	25.9	2.6
February (2022)	Influent	23.8	7.6	968.2	726.3	708	52.4	169.3
	Effluent	25.1	7.7	858.3	602.0	336	42.23	37.5
	Downstream	24.0	7.5	543.0	300.3	74.3	11.3	2.1
March (2022)	Influent	23.2	7.4	886.3	615.3	573.6	32.0	25.2
	Effluent	23.9	7.8	827.0	575.7	45	**5.5**	**1.4**
	Downstream	20.1	7.9	459.3	383.3	25	14.9	0.1
April (2022)	Influent	19.8	7.8	945.3	751.0	1507.3	52.6	61.3
	Effluent	19.5	7.9	915.0	637.3	1289.0	19.5	**1.5**

	Downstream	20.0	8.1	501.3	336.3	922.3	14.8	2.0
May (2022)	Influent	19.2	7.8	983.3	686.3	1387.3	34.2	21.9
	Effluent	19.0	7.8	796.3	554.0	1131.7	**8.0**	**4.3**
	Downstream	13.9	8.0	483.0	324.7	680.7	0.8	2.1
June (2022)	Influent	19.3	7.6	852.3	680.6	1455.3	53.9	30.4
	Effluent	19.5	7.5	660.3	323.2	1139.0	19.5	**1.1**
	Downstream	19.4	7.1	499.1	311.0	988.3	11.3	1.0
July (2021)	Influent	16.1	8.4	987.7	691.0	1401.3	40.9	328.6
	Effluent	14.5	8.17	835.3	572.3	1172.7	25.0	**9.8**
	Downstream	16.8	7.5	468.1	299.9	748.9	16.5	4.9

WWTP E								
Month (year)	**Sample**	**Temperature (°C)**	**pH**	**Total Dissolved Solids (ppm)**	**Salinity (ppm)**	**COD (mg/L)**	**PO_4^{3-} (mg/L)**	**NO^{3-} (mg/L)**
January	Influent	23.0	7.9	622.7	431.0	877.0	45.2	78.2

(2022)	Effluent	25.1	8.34	559.0	384.0	788.7	35.8	**6.1**
	Downstream	23.5	7.9	412.0	312.3	797.8	13.6	5.2
February	Influent	23.4	7.8	694.3	485.3	984.3	44.6	115.1
(2022)	Effluent	23.8	8.6	530.0	364.7	747.0	87.5	**6.1**
	Downstream	22.2	8.1	501.0	362.0	698.9	19.3	3.2
March (2022)	Influent	23.4	7.8	694.3	485.3	984.3	44.6	115.1
	Effluent	23.8	8.6	530.0	364.7	747.0	87.5	**6.1**
	Downstream	22.2	8.1	501.0	362.0	698.9	19.3	3.2
April	Influent	20.6	8.6	649.2	367.3	1595.3	44.9	56.1
(2022)	Effluent	20.3	8.1	511.0	328.0	1335.5	19.3	**2.0**
	Downstream	20.3	7.5	485.8	319.0	1142.3	16.1	1.8
May	Influent	17.5	7.7	922.3	639.0	1303.3	58.3	48.6
(2022)	Effluent	14.8	8.5	577.3	392.3	810.7	19.9	60.0
	Downstream	13.7	8.2	450.3	303.0	635.7	21.1	18.9
June	Influent	19.3	7.3	948.3	398.2	1693.2	57.3	189.3

(2022)	Effluent	18.5	7.7	574.0	315.9	1168.1	24.9	**3.5**
	Downstream	18.9	7.2	449.1	324.1	998.0	11.7	1.3
July (20212	Influent	20.5	7.7	748.0	562.0	988.0	48.9	102.7
	Effluent	22.3	12.6	512.7	491.0	723.3	12.4	**43.1**
	Downstream	20.2	8.9	494.3	357.6	727.6	16.8	5.6

Table A3: Physico-chemical parameters of WWTPs I, J, D, B

WWTP	Sample	April 2022				May 2022			
		Temperature (°C)	pH	Total Dissolved Solids (ppm)	Salinity (ppm)	Temperature (°C)	pH	Total Dissolved Solids (ppm)	Salinity (ppm)
WWTP I	Influent	18.1	7.97	941	657	18,1	7,97	941	657
WWTP J	Influent	17.1	8.05	994	685	21.0	7.94	831	575
WWP J	Effluent	14.7	8.22	768	528	21.6	7.48	1.01	706
WWTP J	Downstream	12.7	8.05	994	685	19.1	8.03	796	550
WWTP D	Influent	219	7.64	747	518	19.4	8.28	790	548
WWTP B	Influent	18.0	7.74	667	470	15	7,95	796	547
WWTP B	Downstream	19.5	7.61	634	436	16.9	7.77	594	478
WWTP E	Influent	22.9	8.7.	791	548	19.5	7.77	886	618
WWTP E	Effluent	21.9	7.63	819	570	20.6	7.97	907	632
WWTP E	Downstream	22.5	8.49	559	382	14.8	8.71	647	443

Table A4: Plate counts of ESKAPE pathogens from the influent and effluent of WWTP C

Putative based on agar-colony characteristic	July 2021		August 2021		September 2021		October 2021		November 2021		January 2022	
	Influent	**Effluent**	**Influent**	**Effluent**	**Influent**	**Effluent**	**Influent**	**Effluent**	**Influent**	**Effluent**	**Influent**	**Effluent**
E. faecium	54	*	222	>300	411000	0.0	6280000	11.3	28900	11.3	17900	126
S. aureus	42	*	39	>300	10500	13.3	500	>300	1160000	12.7	10400	36.7
K. pneumoniae	>300	*	>300	>300	40000	66.7	3910000	6	890000	16.0	1460000	150
A. baumannii	3	*	45	>300	50400000	1.33	76700	4.0	1330000	51.3	5500	83.3
P. aeruginosa	34	*	>300	>300	29200	21.3	7750	4.6	7700	10.0	2000	>300
***Enterobacter* spp. (m-FC)**	13	*	>300	>300	>300	48.7	25600000	2	17200	12.0	5830000	>300
***Enterobacter* spp. (m-Endo)**	>300	*	69	>300	131000000	3.3	22800000	3.3	400000	46.7	80000	>300

Abbreviations: (colonies > 300 some cases also indicated as Too Numerous To Count); * = No count due to human error

Table A5: Plate counts from the influent and effluent of the WWTP E

Putative based on agar-colony characteristic	**July 2021**		**August 2021**		**September 2021**		**October 2021**		**November 2021**		**January 2022**	
	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
E. faecium	103	*	205	45	23700	2.67	4630000	3.33	1760000	6	4500	22
S. aureus	56	*	70	14	15600	>300	700	44.7	6700	54.7	700	30.7
K. pneumoniae	>300	*	>300	255	52400	20	80000	17.3	69200	19.3	267000	62
A. baumannii	16	*	103	>300	1600000	61	2280000	28.7	60000	9.33	11500	16.7
P. aeruginosa	55	*	>300	179	38700	36	40000	>300	11000	10	206000	22
***Enterobacter* spp. (m-FC)**	2	*	>300	19	20800	18	120000	37.3	103000	3.3	89000000	154
***Enterobacter spp.* (m-Endo)**	27	*	77	44	31900	36	280000	>300	1160000	30	610000	29.3

Abbreviations: (colonies > 300 some cases also indicated as Too Numerous to Count); * = No count due to human error

Table A6: Plate counts of ESKAPE pathogens from the influent and effluent and Downstream of WWTP C and E

Putative based on agar-colony characteristic	**July 2022 WWTP C**			**July 2022 WWTP E**		
	Influent	**Effluent**	**Down Stream**	**Influent**	**Effluent**	**Down Stream**
E. faecium	10500	100	100	10300	2100	100
S. aureus	21000	0	0	400	400	1000000
K. pneumoniae	367900	50850	5350	1367566	34750	300
A. baumannii	117500	41925	5400	249400	0	0
P. aeruginosa	0	0	0	0	0	0
***Enterobacter* spp. (m-FC)**	108150	374100	363900	730400	147600	2100
***Enterobacter* spp. (m-Endo)**	141550	61450	2061866	547550	6050	2800

Abbreviations: (colonies > 300 some cases also indicated as Too Numerous To Count); * = No count due to human error

Table A7: Plate counts of ESKAPE pathogens from the influent and effluent and Downstream of WWTP G, and WWTP H

Putative based on agar-colony characteristics	**March 2022 WWTP G**			**March 2022 WWTP H**			**July 2022 WWTP H**		
	Influent	**Effluent**	**Down Stream**	**Influent**	**Effluent**	**Down Stream**	**Influent**	**Effluent**	**Down Stream**
E. faecium	N/D	*	>300	500	*	No samples	5250	10650	No samples
S. aureus	334000	*	>300	300	*	No samples	600	15800	No samples
K. pneumoniae	95000	*	19.3	>300	*	No samples	54350	64175	No samples
A. baumannii	4030000	*	>300	9000	*	No samples	5746000	9000	No samples
P. aeruginosa	5250	*	N/D	N/D	*	No samples	0	0	No samples
***Enterobacter* spp. (m-FC)**	1200	*	>300	N/D	*	No samples	745433	151700	No samples
***Enterobacter spp.* (m-Endo)**	1090000	*	>300	30000	*	No samples	651550	657800	No samples

Abbreviations: (colonies > 300 some cases also indicated as Too Numerous To Count); * = No count due to human error

Table A8: Plate counts of ESKAPE pathogens from the influent and Downstream of WWTP F

Putative based on agar-colony characteristics	**March 2022** **WWTP F**			**July 2022** **WWTP F**		
	Influent	**Effluent**	**Down Stream**	**Influent**	**Effluent**	**Down Stream**
E. faecium	N/D	*	>300	631500	No samples	200
S. aureus	1500000	*	>300	714000	No samples	20500
K. pneumoniae	N/D	*	>300	2840000	No samples	0
A. baumannii	354000	*	>300	2000	No samples	5050
P. aeruginosa	N/D	*	>300	1329150	No samples	40700
***Enterobacter* spp. (m-FC)**	200	*	>300	269175	No samples	135266
***Enterobacter spp.* (m-Endo)**	334000	*	>300	1384900	No samples	194350

Abbreviations: (colonies > 300 some cases also indicated as Too Numerous To Count); * = No count due to human error

Table A9: Plate counts from the influent, effluent, and downstream site of WWTP B

Putative based on agar-colony characteristic	**April 2022**			**May 2022**			**June 2022**		
	Influent	**Effluent**	**Down stream**	**Influent**	**Effluent**	**Down stream**	**Influent**	**Effluent**	**Down stream**
E. faecium	N/A	N/A	N/A	N/A	N/A	N/A	230000	0	10000
S. aureus	100	0	1000	0	0	0	2000	3	10000
K. pneumoniae	N/A	N/A	N/A	100000	400	0	TNTC	0	0
A. baumannii	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
P. aeruginosa	400	0	0	0	0	0	20	0	0
***Enterobacter* spp. (m-FC)**	15400	0	15900	3420000	3000	30000	28300	0	420000
***Enterobacter* spp. (m-Endo)**	9800	0	10500	1190000	0	1910000	29800	0	230000

Abbreviations: N/A: not determined due to unavailability of media; TNTC: too numerous to count.

Table A10: Plate counts from the influent, effluent, and downstream site of WWTP D

Putative based on agar-colony characteristic	**April 2022**			**May 2022**			**June 2022**		
	Influent	**Effluent**	**Down stream**	**Influent**	**Effluent**	**Down stream**	**Influent**	**Effluent**	Down stream
E. faecium	N/A	N/A	N/A	N/A	N/A	N/A	40000	4100	0
S. aureus	0	0	0	0	0	0	0	400	10000
K. pneumoniae	N/A	7200	0	100000	004	0	120000	19500	0
A. baumannii	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
P. aeruginosa	0	600	0	0	0	0	170000	0	0
***Enterobacter* spp. (m-FC)**	10000	90000	3100	3420000	3000	30000	2020000	29300	160000
***Enterobacter* spp. (m-Endo)**	100	27900	2700	1190000	0	1910000	540000	29200	30000

Abbreviations: N/A: not determined due to unavailability of media.

Table A11: Plate counts from the influent, effluent, and downstream sites of WWTP J

Putative based on agar-colony characteristic	April 2022			May 2022			June 2022		
	Influent	**Effluent**	**Down stream**	**Influent**	**Effluent**	**Down stream**	**Influent**	**Effluent**	**Down stream**
E. faecium	N/A	N/A	N/A	N/A	N/A	N/A	1900	1	0
S. aureus	0	0	0	600	0	0	300	0	0
K. pneumoniae	N/A	N/A	N/A	0	0	0	90000	300	0
A. baumannii	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
P. aeruginosa	0	0	0	1900	0	0	0	0	0
***Enterobacter* spp. (m-FC)**	600000	400	6200	90000	0	800	3800	2100	0
***Enterobacter* spp. (m-Endo)**	1010000	0	1200	90000	100	900	20000	2800	100

Abbreviations: N/A: not determined due to unavailability of media.

Table A12: Plate counts from the influent of WWTP I

Putative based on agar-colony characteristics	**April 2022**	**May 2022**	**June 2022**
	Influent	Influent	Influent
E. faecium	N/A	N/A	20000
S. aureus	0	0	0
K. pneumoniae	N/A	0	130000
A. baumannii	N/A	N/A	N/A
P. aeruginosa	10000	0	0
***Enterobacter* spp. (m-FC)**	1330000	400000	120000
***Enterobacter* spp. (m-Endo)**	400000	100000	140000

Abbreviations: N/A: not determined due to unavailability of media.

Table A13: Plate counts from the influent of WWTP K

Putative based on agar-colony characteristic	**July 2022**		
	Influent	**Effluent**	**Down stream**
E. faecium	8000	0	1000
S. aureus	670933	5400	10200
K. pneumoniae	1109900	400	0
A. baumannii	45800000	5700	0
P. aeruginosa	26850	5250	2575
***Enterobacter* spp. (m-FC)**	390600	229350	200275
***Enterobacter* spp. (m-Endo)**	604650	410450	186350

Variable	Correlations (Sheet1 in combined final) Marked correlations are significant at p < ,05000 N=14 (Casewise deletion of missing data) **E. faecium**	S. aureus	K. pneumoniae	A. baumannii	P. aeruginosa	Enterobacter spp. (m-FC)
Temperature (°C)	-,1851	,0575	-,1129	,0701	-,1727	-,1854
	p=,526	p=,845	p=,701	p=,812	p=,555	p=,526
pH	-,0074	-,1747	-,1851	-,0526	-,2632	-,2404
	p=,980	p=,550	p=,526	p=,858	p=,363	p=,408
Total Dissolved Solids (ppm)	,2058	,3370	,3482	,0922	,6541	,6092
	p=,480	p=,239	p=,222	p=,754	p=,011	p=,021
Salinity (ppm)	,0551	,4343	,4312	-,0070	-,0666	-,0922
	p=,852	p=,121	p=,124	p=,981	p=,821	p=,754
COD	,5508	,0644	,0844	,3831	,6714	,5766
	p=,041	p=,827	p=,774	p=,176	p=,009	p=,031
PO43-	,0586	,0683	,0765	,0034	,1612	,1539
	p=,842	p=,816	p=,795	p=,991	p=,582	p=,599
NO3-	-,0343	,2788	,3615	,1803	,7161	,6708
	p=,907	p=,334	p=,204	p=,537	p=,004	p=,009

APPENDIX B:

Antibiotic resistance profiles of ESKAPE Pathogens from WWTPs B, D, I and J

ESKAPE pathogens isolated from the Influent and effluent sites of WWTPs C and E illustrated susceptibility to gentamycin, ciprofloxacin and Netilmicin. However, majority of the *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Enterobacter* species isolated from WWTPs C and E were resistant to ampicillin. The antibiotic resistance patterns measured for ESKAPE pathogens isolated from the influent, effluent and downstream sites of WWTPs B, D, I and J showed consistent resistance to ampicillin. Furthermore, vancomycin resistance was noted in *Enterococcus faecium* isolates from the influent, effluent and downstream sites of WWTPs B, D, I and J.

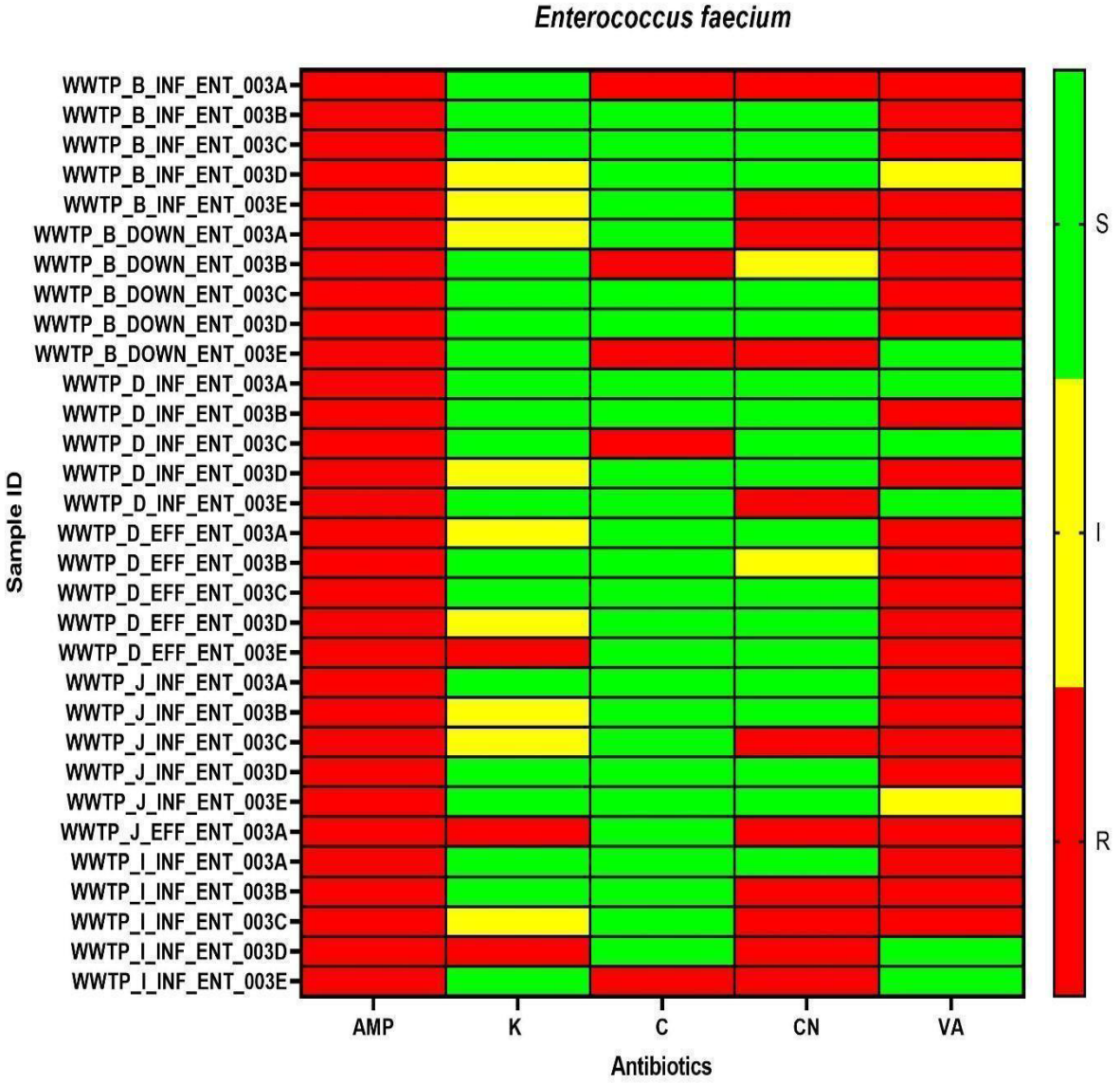

Figure B1: Heatmap illustrating the antibiotic resistance patterns of *Enterococcus faecium* isolates from the influent, effluent and downstream sites of WWTPs B, D, I and J.

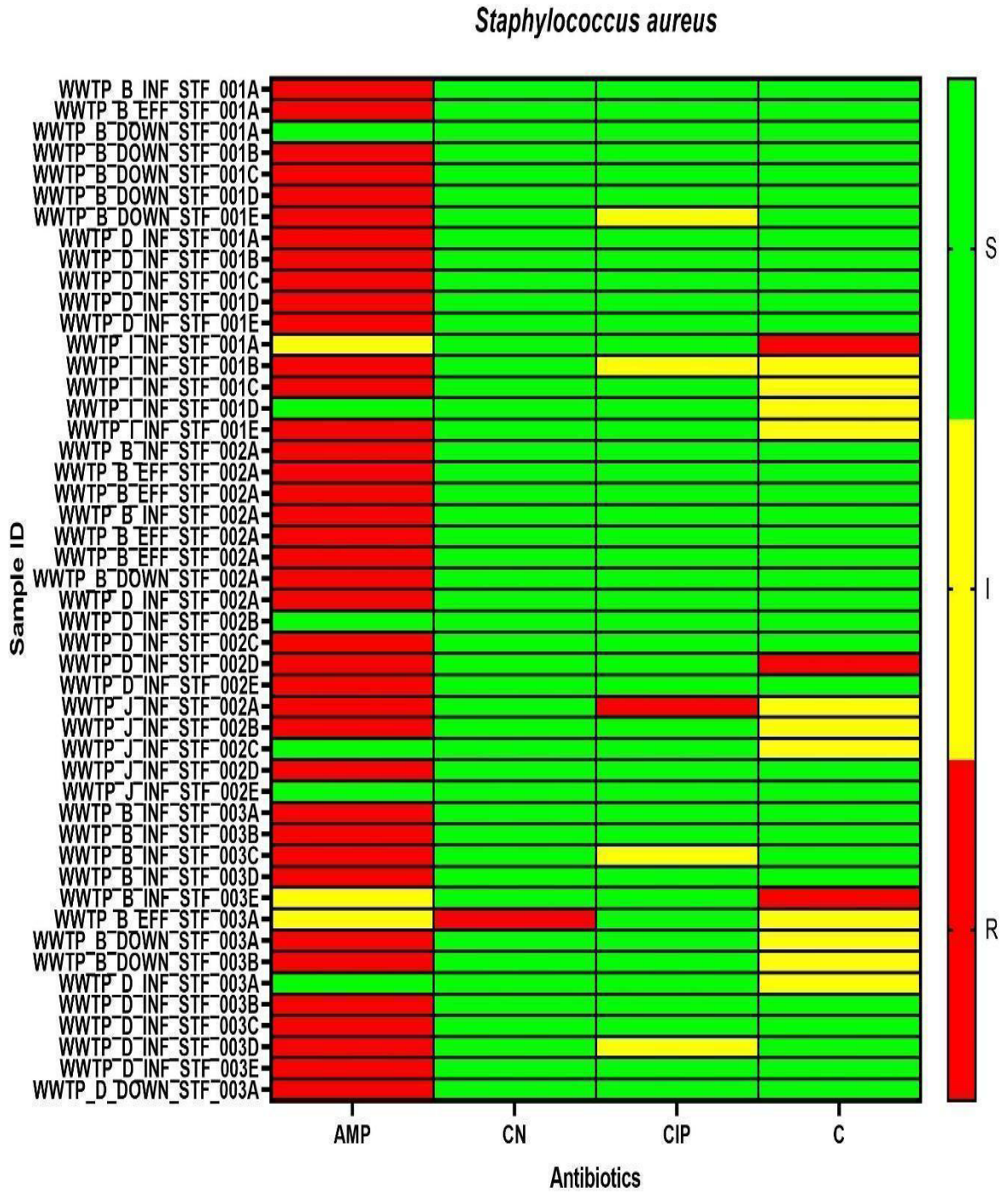

Figure B2: Heatmap illustrating the antibiotic resistance patterns of *Staphylococcus aureus* isolates from the influent, effluent and downstream sites of WWTPs B, D, I and J.

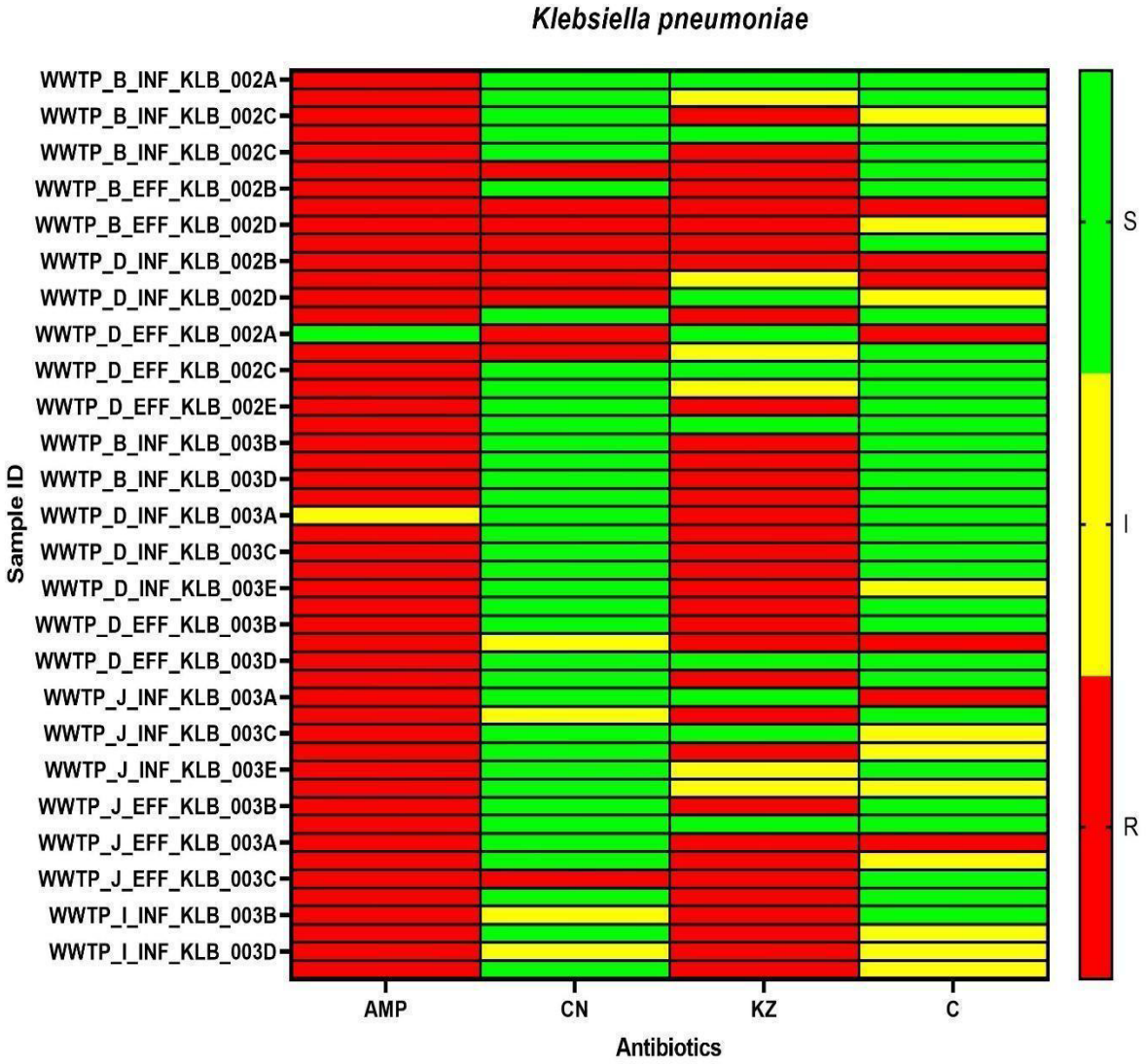

Figure B3: Heatmap illustrating the antibiotic resistance patterns of *Klebsiella pneumoniae* isolates from the influent, effluent and downstream sites of WWTPs B, D, I and J.

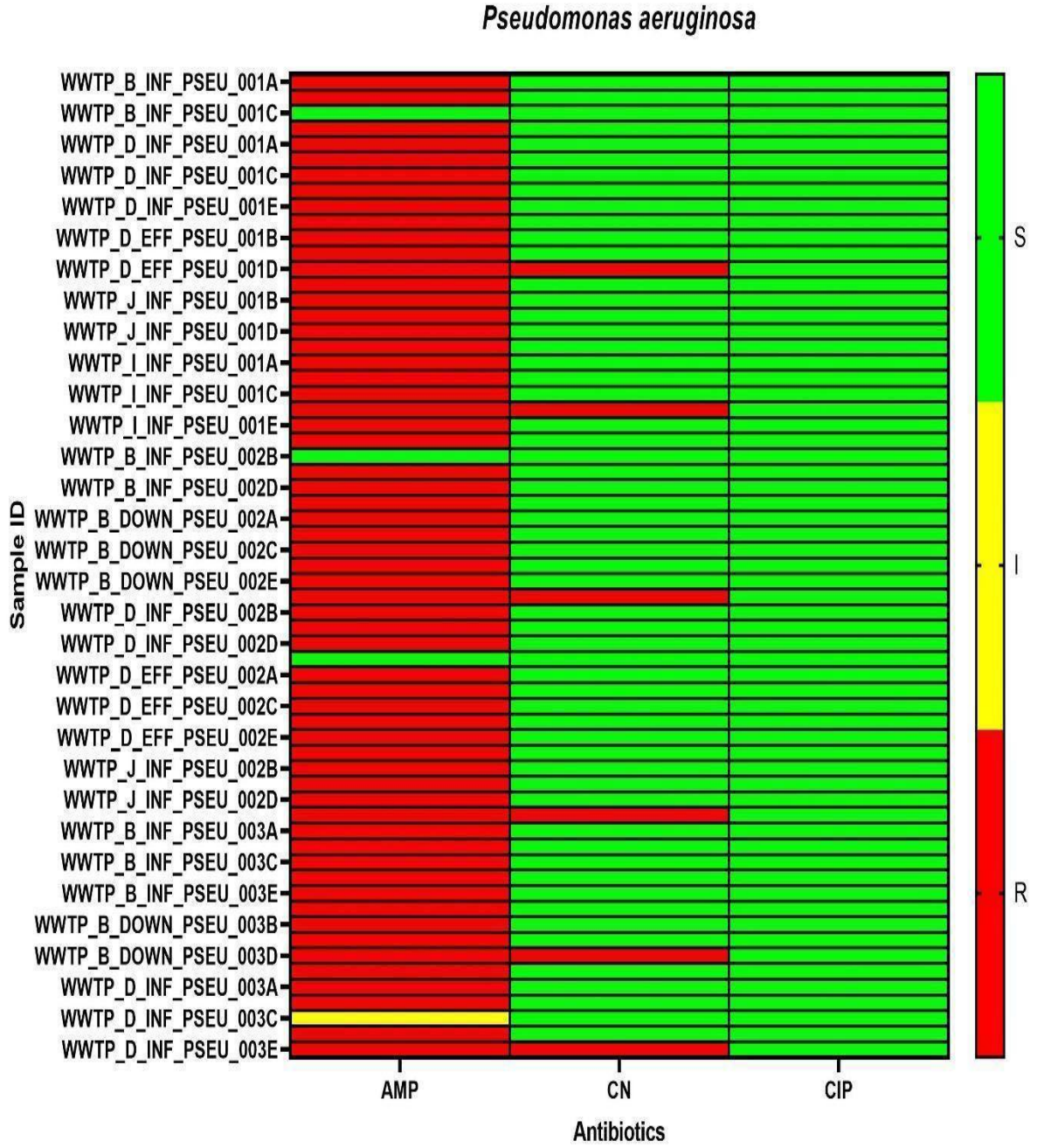

Figure B4: Heatmap illustrating the antibiotic resistance patterns of *Pseudomonas aeruginosa* isolates from the influent, effluent and downstream sites of WWTPs B, D, I and J.

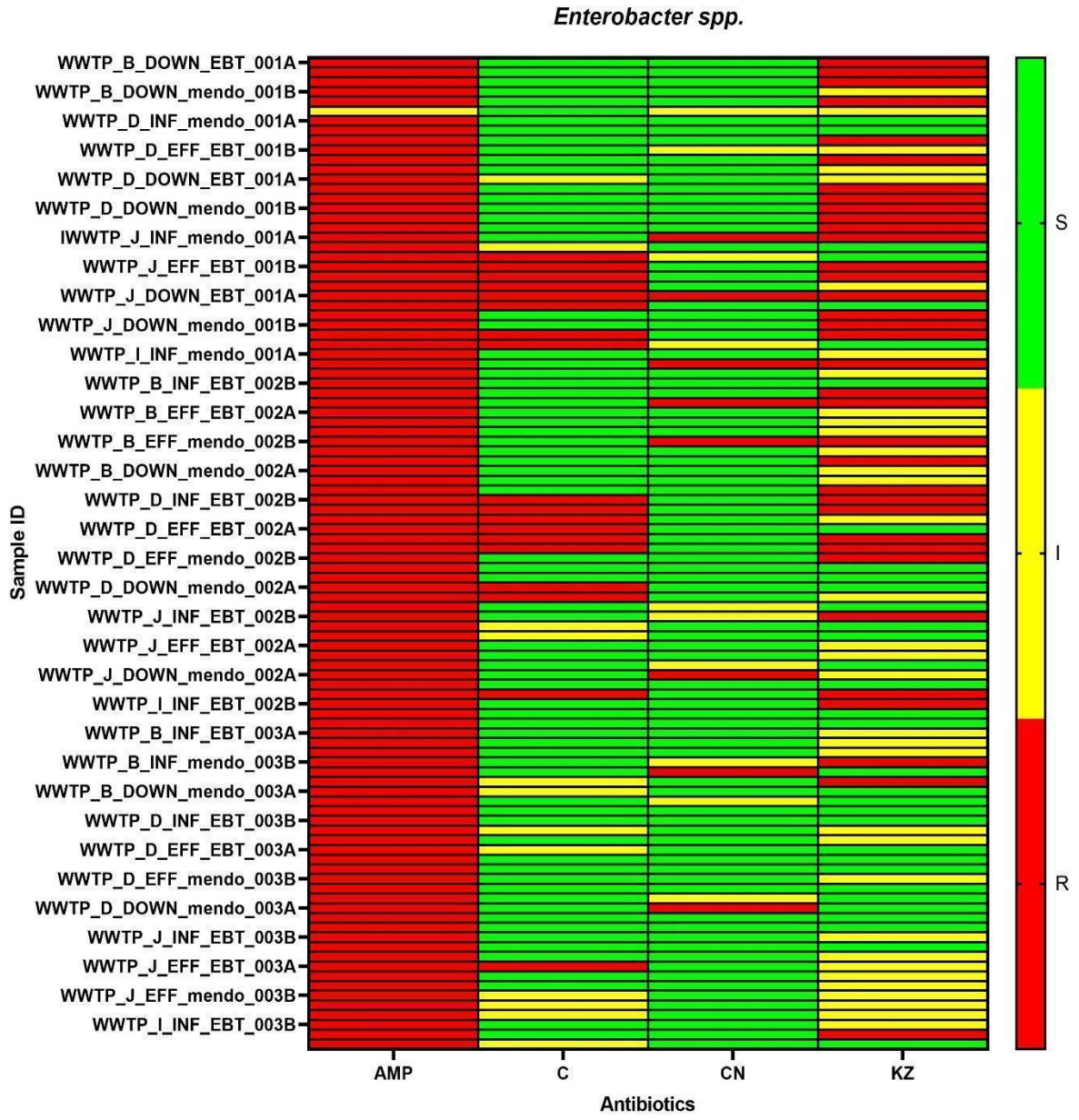

Figure B5: Heatmap illustrating the antibiotic resistance patterns of *Enterobacter spp.* isolates from the influent, effluent and downstream sites of WWTPs B, D, I and J.

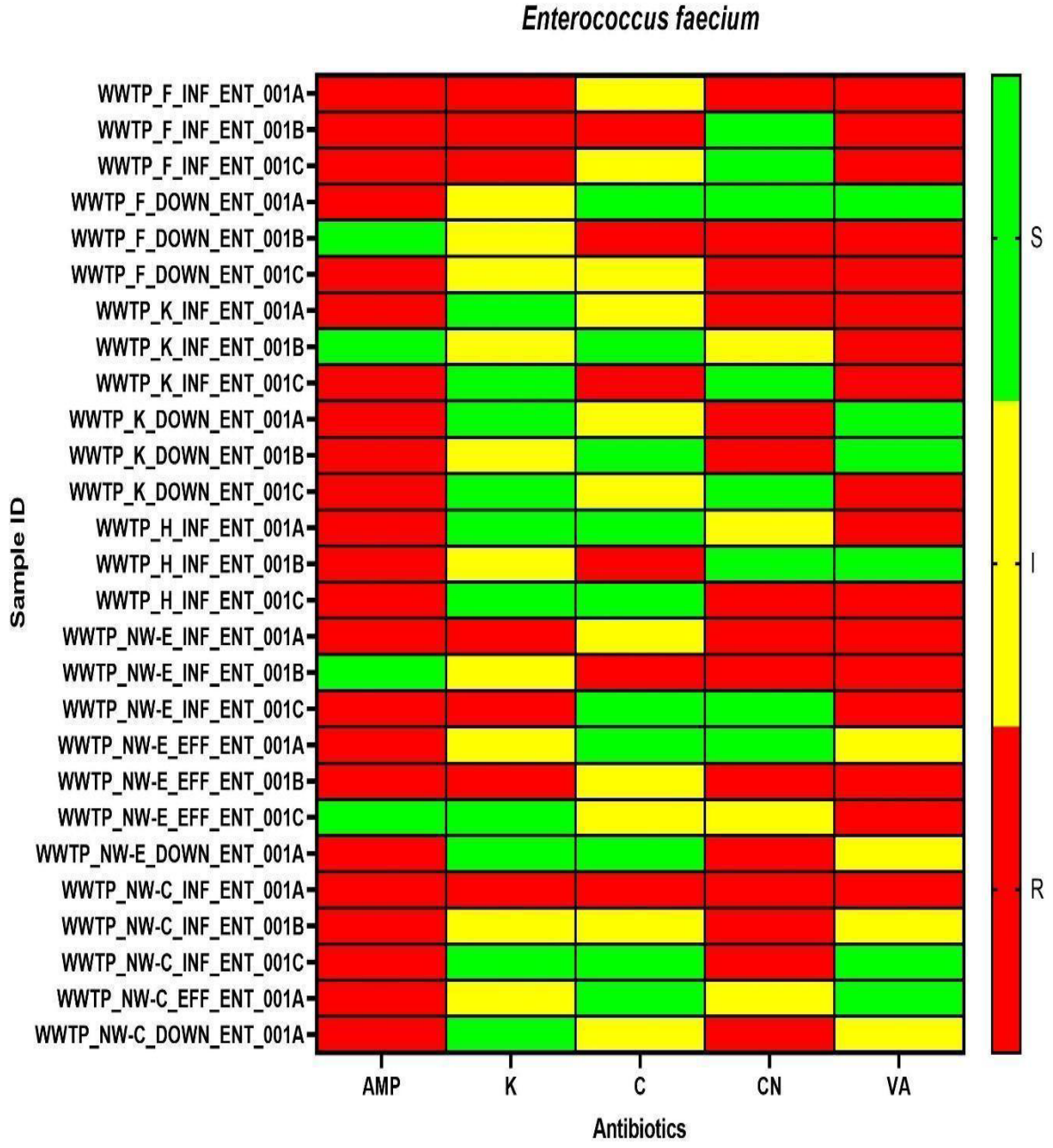

Figure B6: Heatmap illustrating the antibiotic resistance patterns of *Enterococcus faecium* isolates from the influent, effluent and downstream sites of WWTPs C, E, F, K, H.

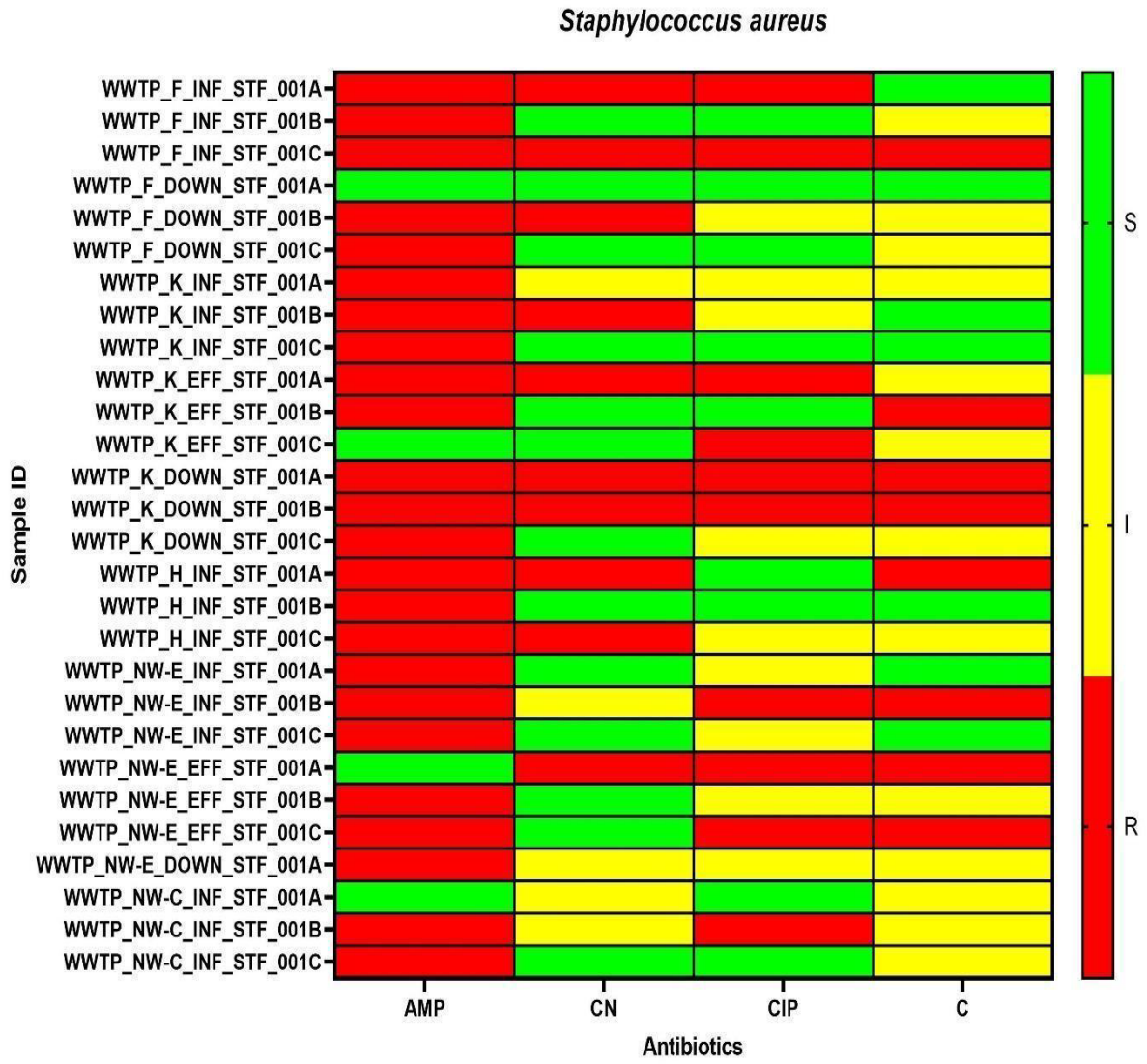

Figure B7: Heatmap illustrating the antibiotic resistance patterns of *Staphylococcus aureus* isolates from the influent, effluent and downstream sites of WWTPs C, E, F, K, H.

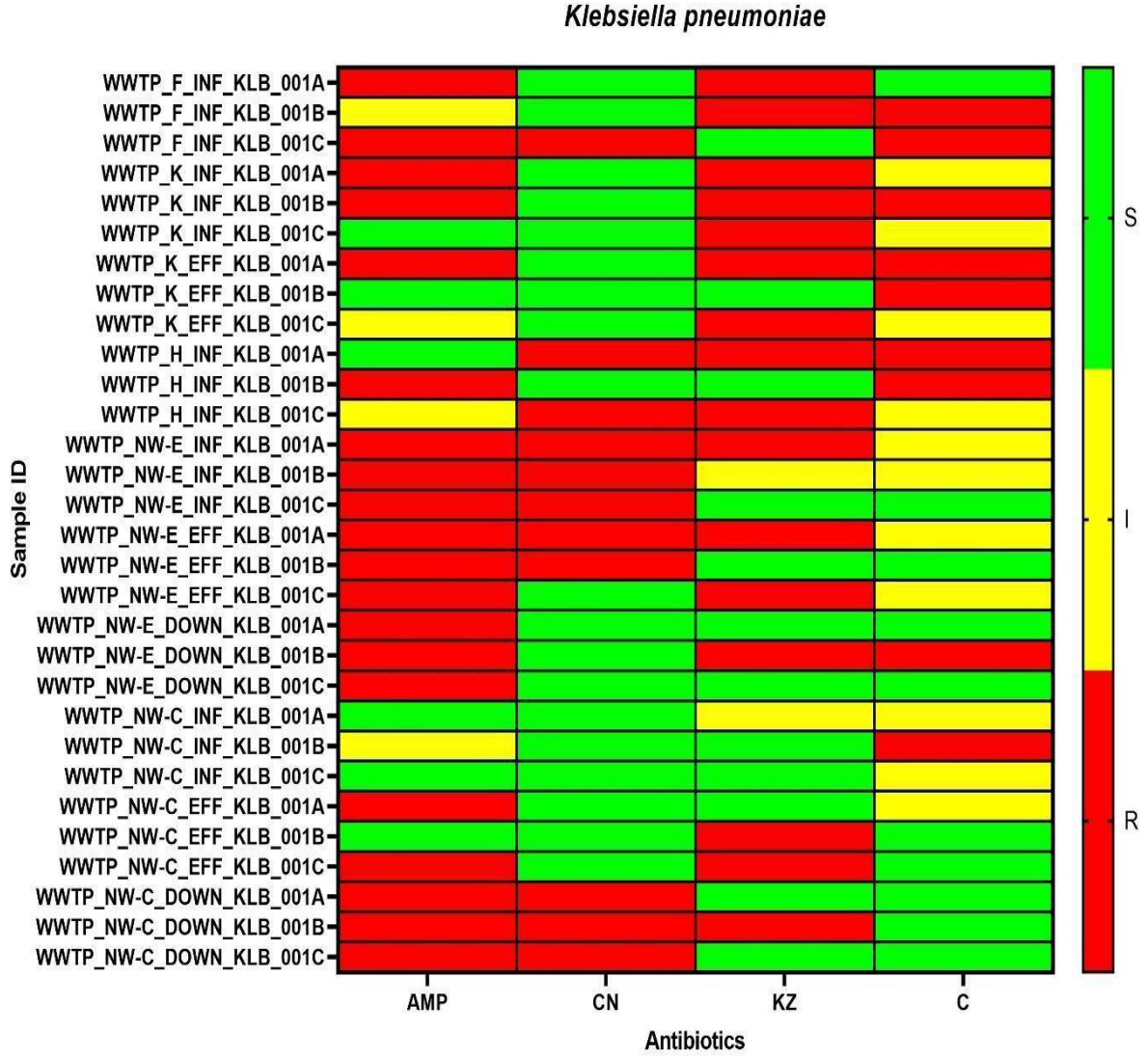

Figure B8: Heatmap illustrating the antibiotic resistance patterns of *Klebsiella pneumoniae* isolates from the influent, effluent and downstream sites of WWTPs C, E, F, K, H.

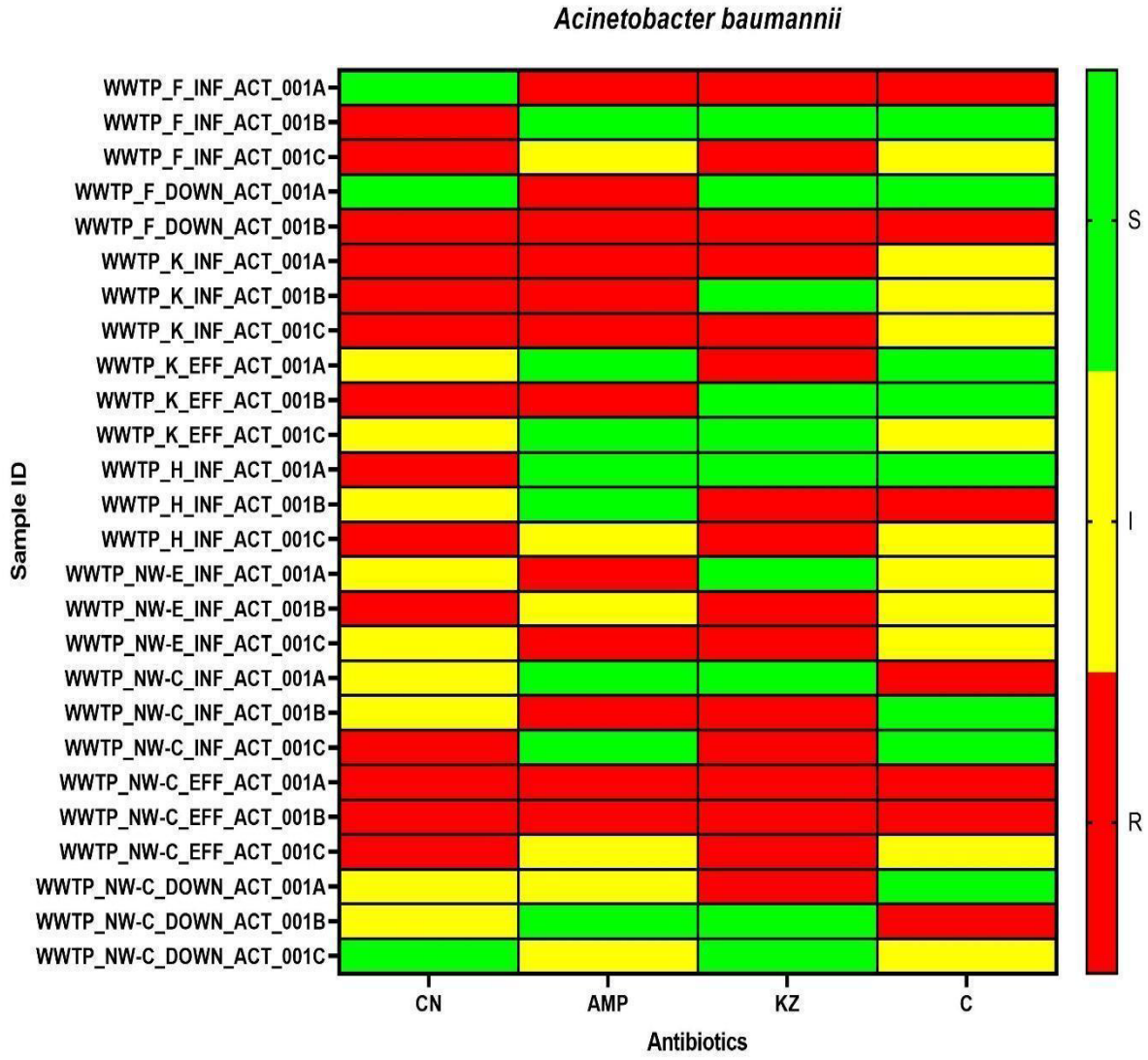

Figure B9: Heatmap illustrating the antibiotic resistance patterns of *Acinetobacter baumannii* isolates from the influent, effluent and downstream sites of WWTPs C, E, F, K.

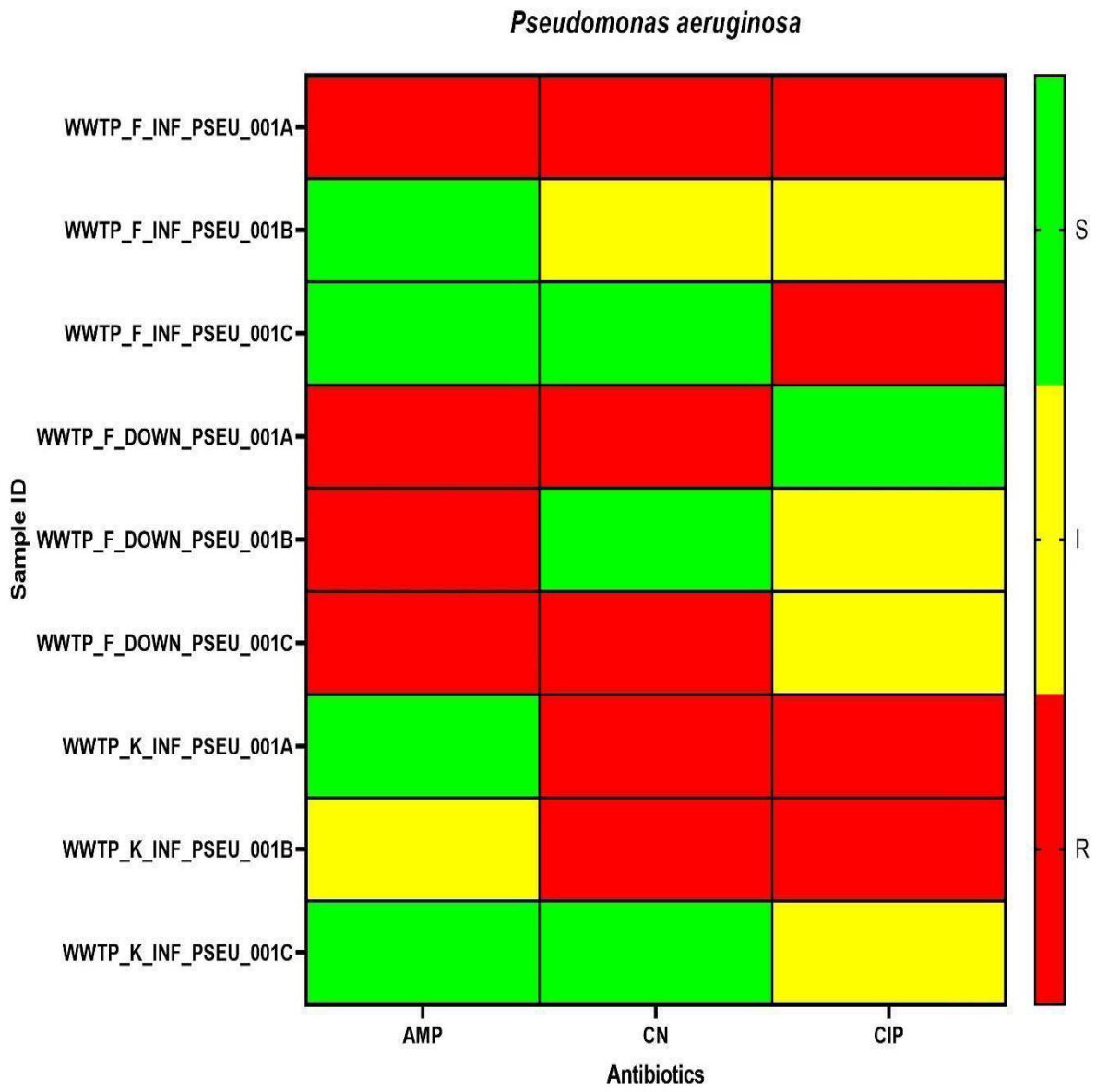

Figure B10: Heatmap illustrating the antibiotic resistance patterns of *Pseudomonas aeruginosa* isolates from the influent, effluent and downstream sites of WWTPs C, E, F, K.

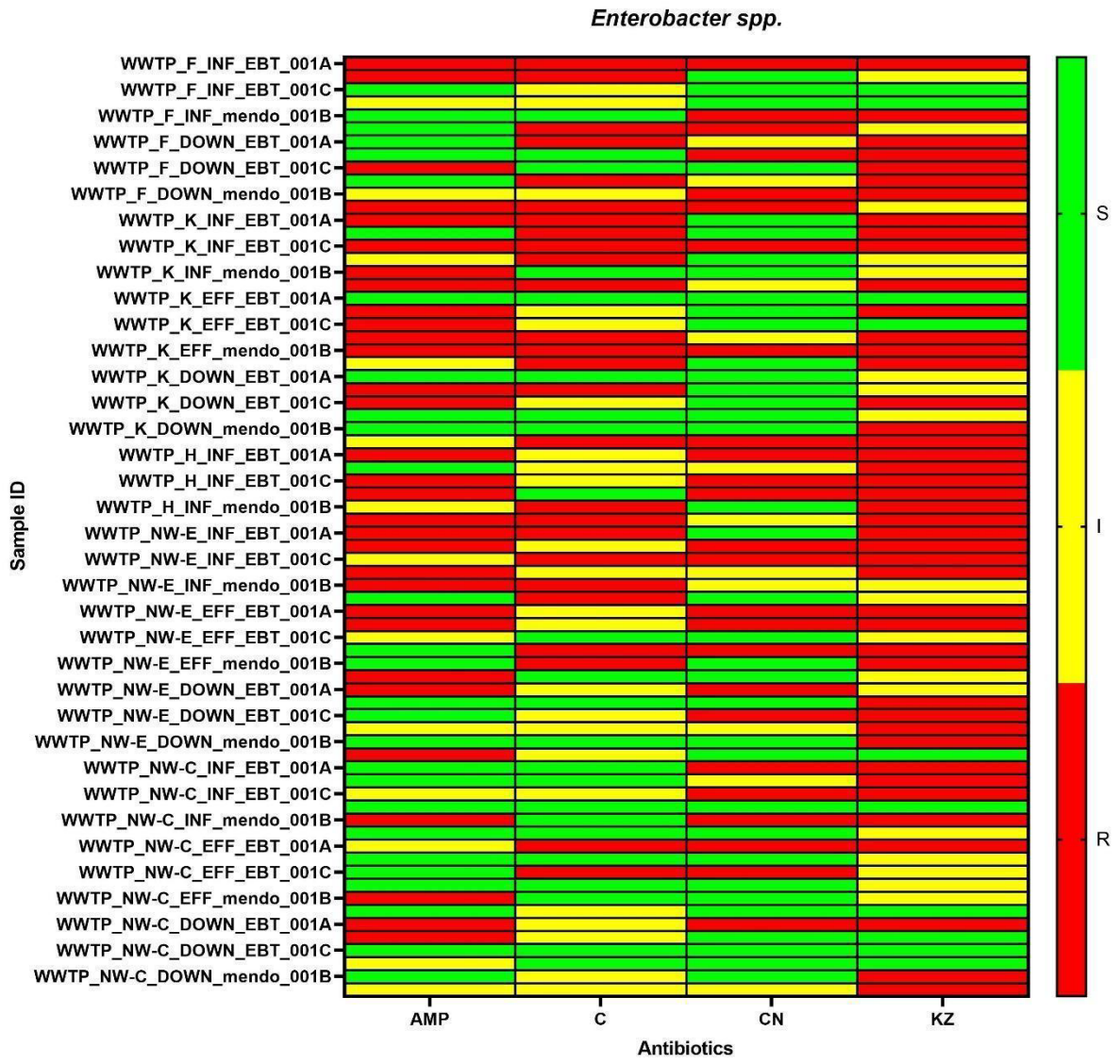

Figure B11: Heatmap illustrating the antibiotic resistance patterns of *Enterobacter spp.* from the influent, effluent, and downstream sites of WWTPs C, E, F, K.

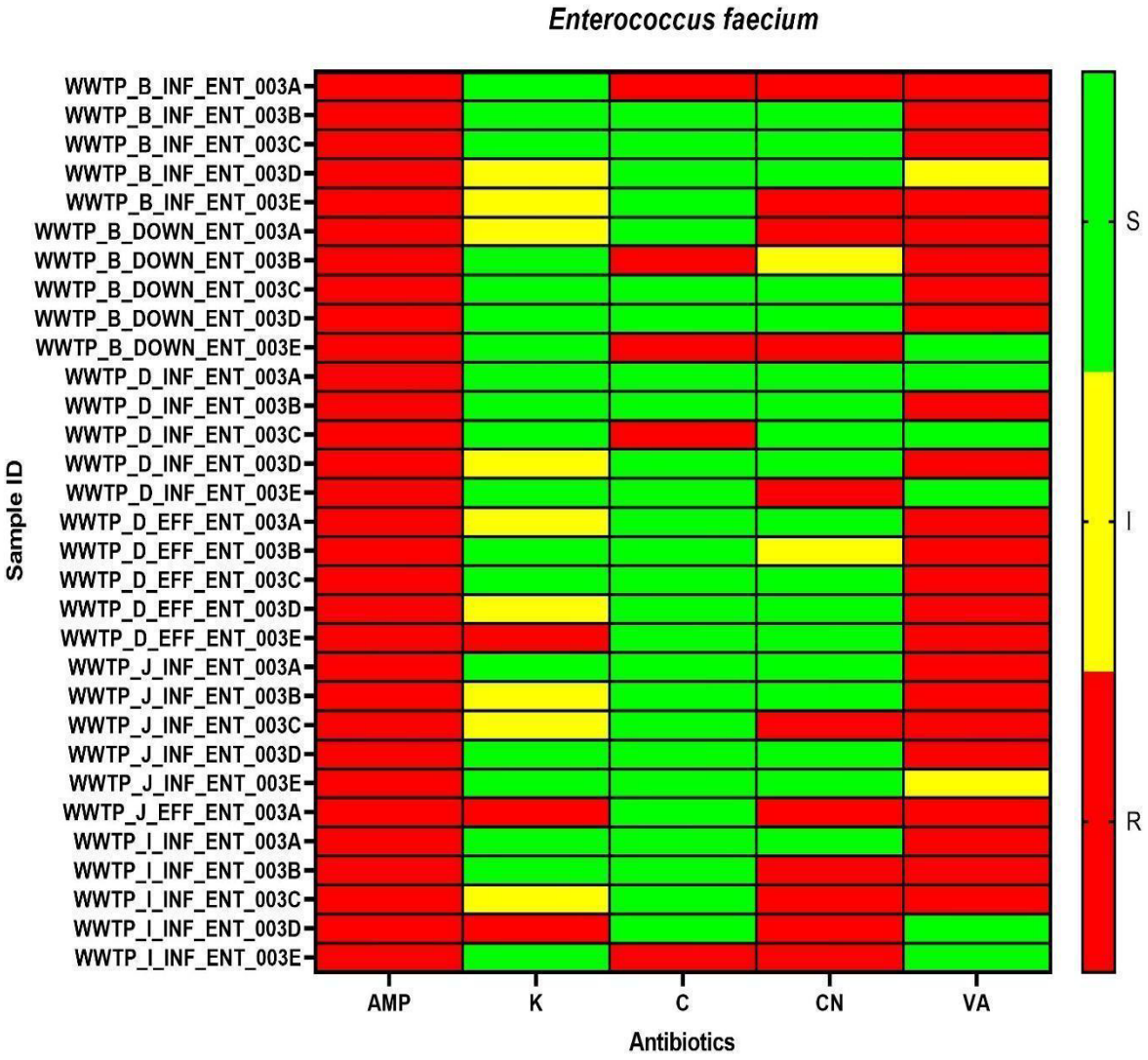

Figure B12: Heatmap illustrating the antibiotic resistance patterns of *Enterococcus faecium* from the influent, effluent, and downstream sites of WWTPs B, D, I and J.

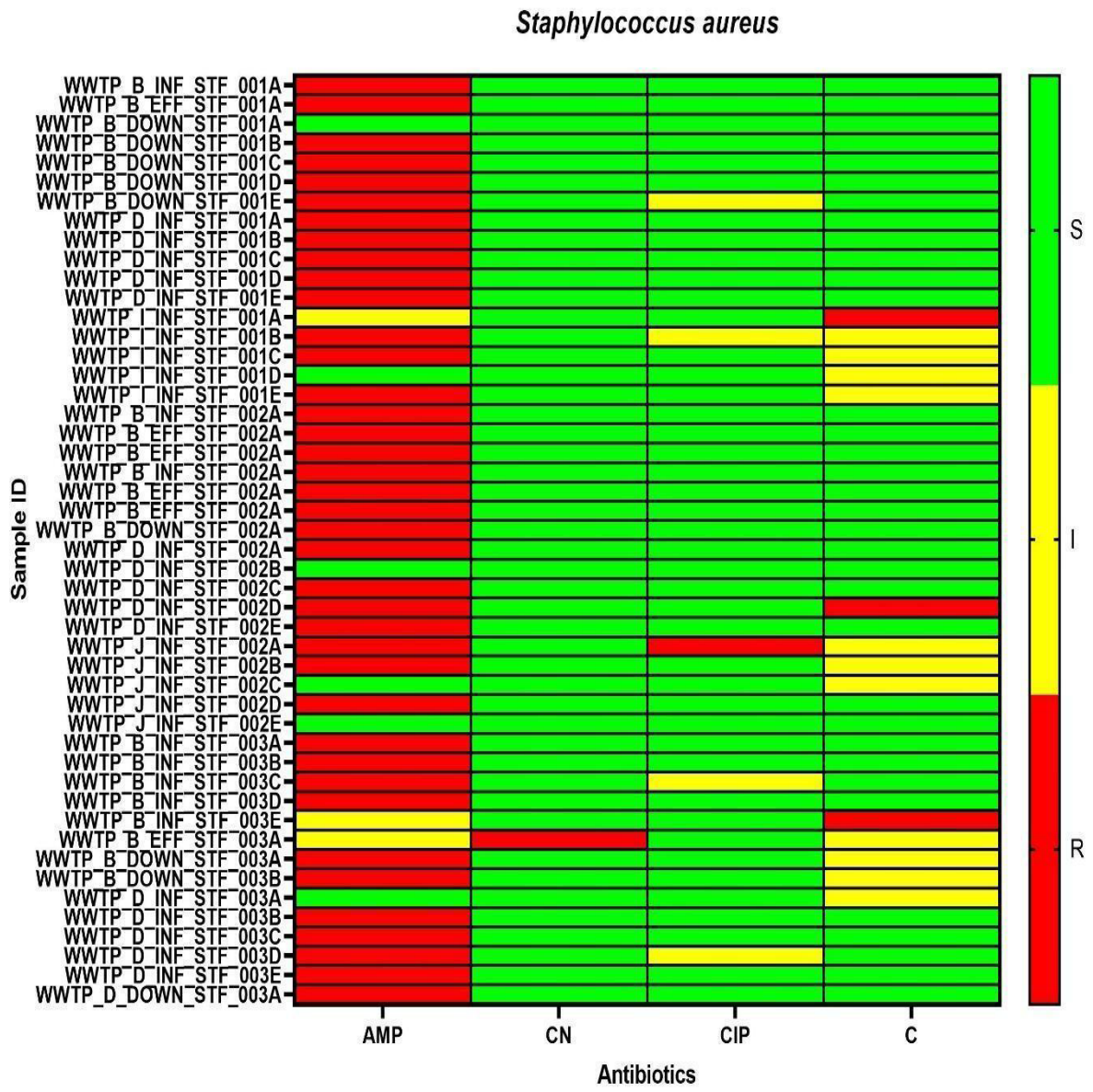

Figure B13: Heatmap illustrating the antibiotic resistance patterns of *Staphylococcus aureus* from the influent, effluent, and downstream sites of WWTPs B, D, I and J.

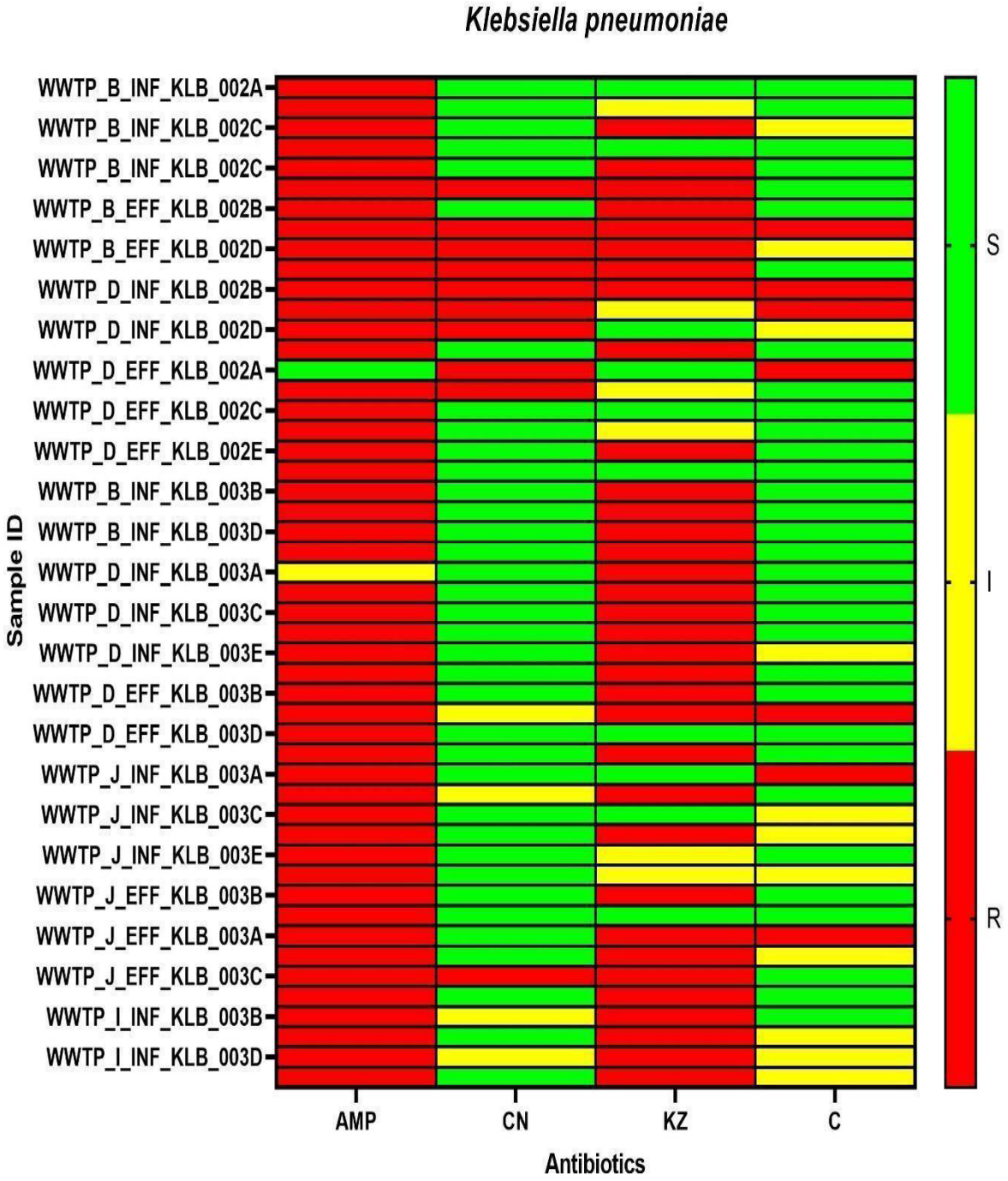

Figure B14: Heatmap illustrating the antibiotic resistance patterns of *Klebsiella pneumoniae* from the influent, effluent, and downstream sites of WWTPs B, D, I and J.

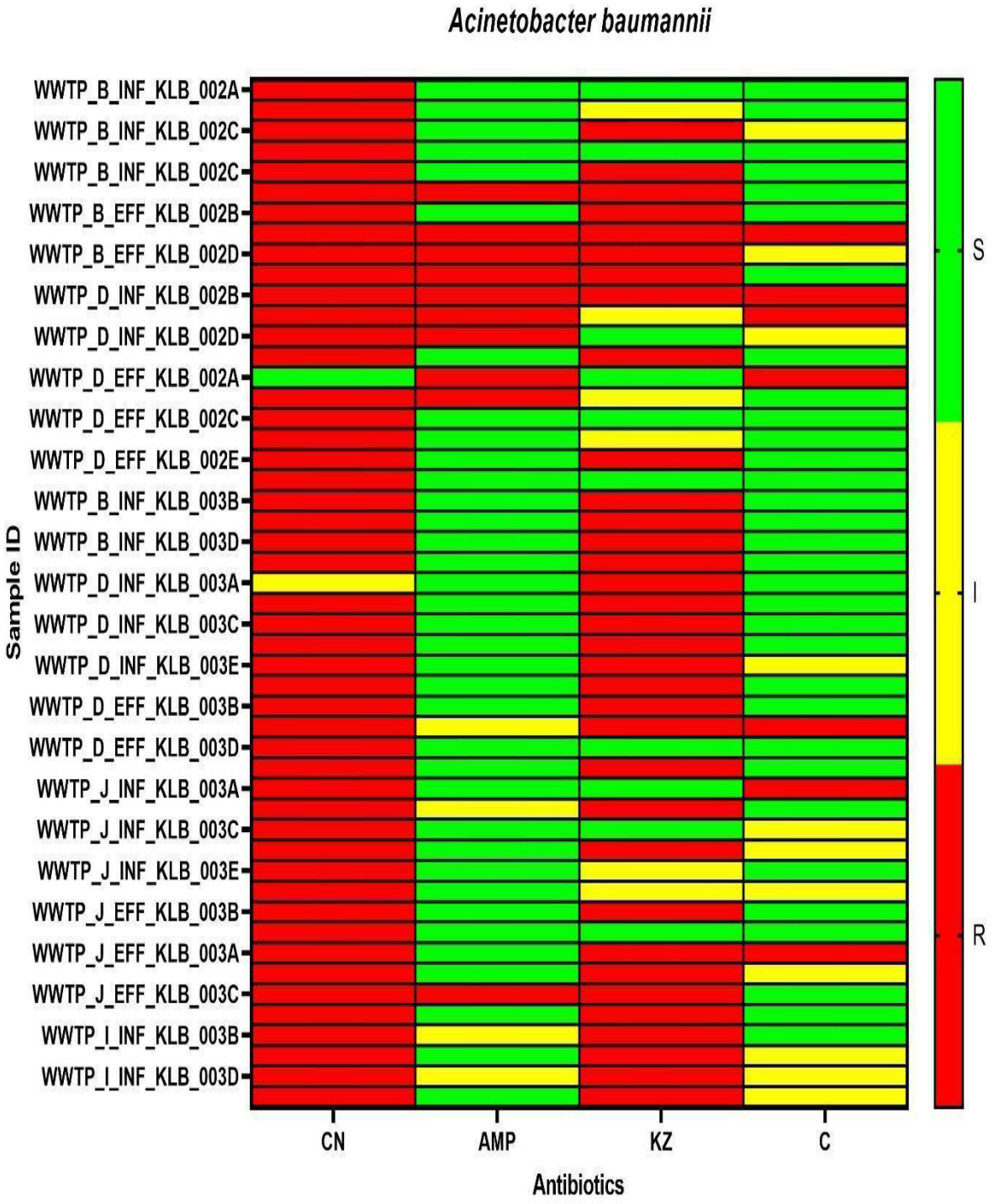

Figure B15: Heatmap illustrating the antibiotic resistance patterns of *Acinetobacter baumannii* from the influent, effluent, and downstream sites of WWTPs B, D, I and J.

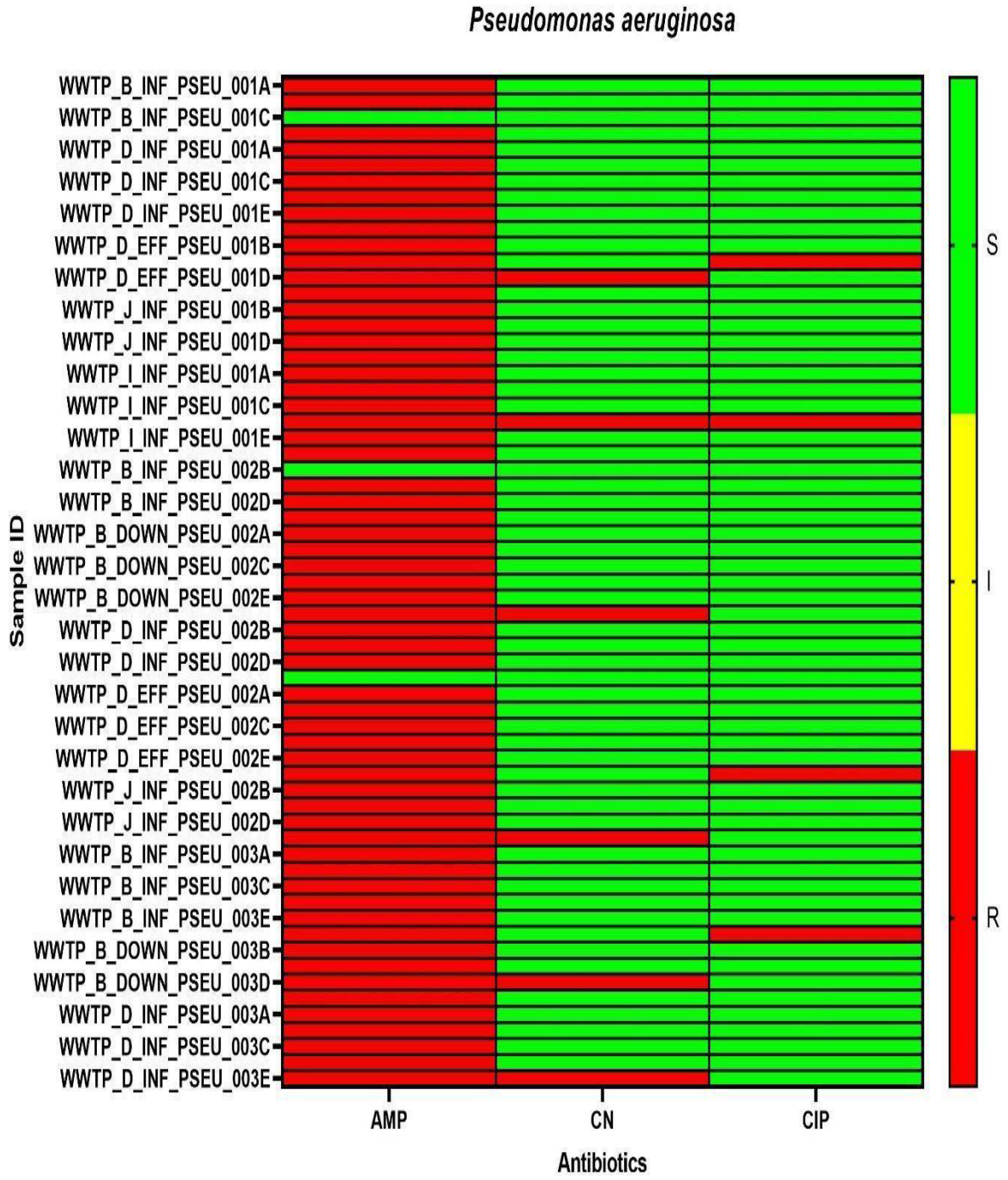

Figure B16: Heatmap illustrating the antibiotic resistance patterns of *Pseudomonas aeruginosa* from the influent, effluent, and downstream sites of WWTPs B, D, I and J.

Table B1: Average copy number of ESKAPEs quantified from WWTPs B, D, F, I, J, and K and receiving waters in (units in gene copies/16S rRNA ± standard deviation)

WWTPs	WWTP Capacity	Sample collection	*E. faecium*	*S. aureus*	*K. pneumoniae*	*A. baumannii*
WWTP B	4.5 Ml/d	Influent	0.001 ± 6.91594E-05	30.508 ± 5.313	0.219 ± 0.223	0.021 ± 0.019
			1223853265	6.86437E+13	1.37921E+11	48311345448
		Effluent	0.935 ± 1.323	-	0.001 ± 0.001	-
			2.10436E+12	-	407700371.3	-
		Downstream	0.005 ± 0.002	30.868 ± 4.610	0.001 ± 0.001	0.046 ± 0.038
WWTP D	24.5 Ml/d	Influent	0.037 ± 0.021	29.703 ± 3.915	0.571 ± 0.539	0.068 ± 0.060
			4.50518E+11	3.63866E+14	2.34819E+12	8.35431E+11
		Effluent	0.002 ± 0.001	-	0.055 ± 0.051	0.021 ± 0.019
			23640635020	-	2.28588E+11	2.60632E+11
		Downstream	-	15.350 ± 21.708	0.002 ± 0.002	-
WWTP F	7.5 Ml/d	Influent	0.000 ± 8,1063E-06	14.117 ± 19.964	0.003 ± 0.003	0.000 ± 4,0193E-05
			127591347.7	5.29401E+13	3822578200	382113567.3
		Effluent	SNA	SNA	SNA	SNA
		Downstream	0.041 ± 0.039	19.151 ± 13.881	0.126 ± 0.130	66.318 ± 93,736
WWTP I	3 Ml/d	Influent	0.001 ± 0.001	17.296 ± 24.460	0.134 ± 0.130	0.002 ± 0.002
			2185722402	2.59438E+13	62932930492	3111362043
		Effluent	SNA	SNA	SNA	SNA
		Downstream	SNA	SNA	SNA	SNA
WWTP J	20.4 Ml/d	Influent	0.005 ± 0.001	32.092 ± 2.157	0.113 ± 0.075	0.015 ± 0.014
			51560354939	3.27338E+14	6.04236E+11	1.51779E+11
		Effluent	0.001 ± 0.000	-	0.011 ± 0.010	-
			5896263530	-	38215030209	-
		Downstream	-	-	0.006 ± 0.005	-
WWTP K	8 Ml/d	Influent	0.018 ± 0.002	31.379 ± 1.409	0.189 ± 0.188	0.015 ± 0.012
			73227235970	1.25514E+14	2.23875E+11	60609733679
		Effluent	0.001 ± 0.001	14.418 ± 20.390	0.003 ± 0.004	0.002 ± 0.002
			3260440216	5.76727E+13	2432314060	7420057486
		Downstream	-	-	0.010 ± 0.009	0.002 ± 0.002

Table B.2: Average copy number of ARG (AmpC gene groups, *Sul*1 and *Int1*) quantified from various WWTPs B, D, F, I, J, and K (units in gene copies/16S rRNA ± standard deviation)

WWTPs	WWTP Capacity	Sample collection	*ACC*	*CIT (LAT/CMY/BIL)*	*DHA*	*ECB (MIR/ACT)*	*FOX*	*Int 1*	*MOX (MOX_CMY)*	*Sul 1*
WWTP B	4.5 Ml/d	Influent	120.72 ± 24.08	700.82 ± 48.91	247.82 ± 214.93	4257.49 ± 315.18	4101.13 ± 138.29	229.05 ± 52.48	139615.16 ± 21862	175.80 ± 48.46
			543234E+14	3.15369E+15	1.11517E+15	1.91587E+16	1.84551E+16	1.0307E+15	6.28268E+17	7.91112E+14
		Effluent	-	1.52 ± 0.32	2.19 ± 1.72	6.35 ± 1.41	660.27 ± 1123.70	253.78 ± 41.86	371.16 ± 31.86	404.11 ± 64.15
			-	6.84893E+12	9.84934E+12	2,85868E+13	2.9712E+15	1.14202E+15	1.67022E+15	1.81851E+15
		Downstream	211.47 ± 40.77	1122.12 ± 199.78	1455.07 ± 36.24	7020 ± 1329.52	4022.43 ± 360.59	314.63 ± 95.29	137623.40 ± 6046.68	314.94 ± 13.44
WWTP D	24.5 Ml/d	Influent	102.64 ± 3.23	925.43 ± 129.43	297.65 ± 14.95	5720.51 ± 848.35	779.20 ± 88.03	137.81 ± 58.71	26441 ± 4182.36	114.34 ± 13.23
			2.51466E+15	2.2673E+16	7.29233E+15	1.40152E+17	1.90906E+16	3.37628E+15	6.47827E+17	2.80136E+15
		Effluent	27.30 ± 7.38	196.56 ± 24.19	124.72 ± 92.76	1104.74 ± 144.54	861.48 ± 179.71	219.15 ± 44.30	113392.53 ± 14961.92	294.86 ± 38.20
			6.68764E+14	4.81561E+15	3.05563E+15	2,70662E+16	2,11062E+16	5.36905E+15	2.77812E+18	7.22415E+15
		Downstream	6.96 ± 2.52	84.65 ± 5.15	13.11 ± 3.16	451.73 ± 29.20	20.42 ± 7.28	95.82 ± 27.10	2923.63 ± 375.97	161.66 ± 47.36
WWTP F	7.5 Ml/d	Influent	9.07 ± 1.22	41.65 ± 36.20	20.38 ± 18.73	218.16 ± 189.69	937.00 ± 811.46	443.07 ± 703.77	86.17 ± 24.24	221.11 ± 10.23
			6.80248E+13	3.12388E+14	1.5288E+14	1.63618E+15	7.02752E+15	3.323E+15	6.46297E+14	1.65829E+15
		Effluent	SNA	SNA	SNA	SNA	SNA	SNA	SNA	SNA
		Downstream	-	-	-	-	1455.00 ± 100.44	-	89195.55 ± 2112.94	-
WWTP I	3 Ml/d	Influent	19.17 ± 6.15	120.73 ± 3.80	111.22 ± 6.22	658.37 ± 21.99	1220.56 ± 255.70	345.02 ± 70.28	86131.88 ± 11083	534.76 ± 133.97
			5.75028E+13	3.62185E+14	3.33659E+14	1.97511E+15	3.66167E+15	1.03505E+15	2.58396E+17	1.6043E+15
		Effluent	SNA	SNA	SNA	SNA	SNA	SNA	SNA	SNA
		Downstream	SNA	SNA	SNA	SNA	SNA	SNA	SNA	SNA

WWTP J	20.4 Ml/d	Influent	1.06 ± 0.95	16.18 ± 2.69	325.12 ± 11.69	78.04 ± 13.77	675.66 ± 170.91	99.68 ± 23.49	19643 ± 838.65	98.73 ± 4.17
			2.16773E+13	3.30043E+14	6.63254E+15	1.59201E+15	1.37835E+16	2.03356E+15	4.0072E+17	2.01417E+15
		Effluent	17.89 ± 6.01	54.02 ± 4.21	78.27 ± 19.20	280.43 ± 23.18	104.89 ± 20.42	32.76 ± 4.71	7168.96 ± 490.09	5.25 ± 0.25
			3.65025E+14	1.10196E+15	1.59675E+15	5.72072E+15	2,13983E+15	6.68379E+14	1.46247E+17	1.07114E+14
		Downstream	0.98 ± 0.31	13.34 ± 0.52	31.39 ± 27.06	63.54 ± 2.62	128.32 ± 3.15	164.01 ± 46.18	13242. 42 ± 1371.86	165.64 ± 12.03
WWTP K	8 Ml/d	Influent	163.92 ± 15.55	1106.34 ± 4.37	289.78 ± 8.42	6911.20 ± 28.99	967.75 ± 57.45	151.39 ± 13.13	29528.98 ± 5890.01	173.61 ± 4.41
			1.31135E+15	8.85073E+15	2.31828E+15	5.52896E+16	7.74204E+15	1.21112E+15	2.36232E+17	1.38889E+15
		Effluent	3.82 ± 0.83	2.54 ± 0.69	2.89 ± 0.80	10.97 ± 3.15	17.27 ± 3.36	223.12 ± 54.31	831.10 ± 49.87	294.90 ± 52.91
			3.05659E+13	2.03651E+13	2.31425E+13	8.77794E+13	1,38151E+14	1.78499E+15	6.64877E+15	2.35924E+15
		Downstream	3.27 ± 3.48	12.09 ± 1.74	13.66 ± 2.36	57.27 ± 8.70	60.35 ± 15.47	15.40 ± 2.57	987.25 ± 104.18	9.588 ± 0.69