

Quantification of antibiotic resistance in wastewater, rivers and drinking water

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DEDICATION

Ke lebisetsa dithuto tsa me kwa bagankeng ba ba latelang:

David Rapatana Mogale. Leso la gago le tlisitse kutlobotlhoko mo pelong yaka. Mme ke ithutile go tlala seatla go tswa mo go ena. Botshelo jaaka leina la moradie e ne bo le bonolo ka tshedimosetso ya gagwe. Fa nkabe ke itsi go kwala nkabe ke kwalela Jeso ke mo kopa gore baswi ba tsoge botlhe gore o kgone go bona thotloetso wa gago mo go nna. A mowa wa ga o tswelele o robale ka kagiso Kwena.

Kgosigadi Kegaisamang Florah Mogale ka go nkgopotsa ka metlha yotlhe gore “nko ya kgomo mogala tshwara ka thata, e sere go utlwa sebedu wa kgaoga.”

Kgomotso Vivian Tsholo ka go tshwara thipa ka fa bogaleng le go nthuta go rapela. Nka lebala jang thotloetso ya gago ya gore “moremogolo go betlwa wa taola wa motho wa ipetla.”

Thapelo ya me ya metlhe e tswa mo bukaneneg ya Dipesalema 23:1-6 le Luka 11:1-4

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ABSTRACT

The extensive and improper use of antibiotics leads to the emergence and spread of antibiotic resistant bacteria (ARBs) and antibiotic resistance genes (ARGs). Antibiotic resistance is not only associated with clinical settings but also with environmental ones, where it is under-explored. In order to address this, the present study aimed to measure the concentrations of antibiotic residues and ARGs in selected wastewater effluent, rivers and drinking water of NW-E and NW-C in North West Province, South Africa. The first part of the study is a structured review. It summarises antibiotic residues and ARGs found in wastewater treatment plants (WWTPs), drinking water production facilities (DWPFs) and surface water in South Africa. This review uncovered a lacuna when it comes to studying antibiotic residues in DWPFs, quantifying ARGs in water environments and determining whether exposure risks might occur. The second part of the study determined the presence of ARGs and antibiotic residues in DWPFs. Generally, the detection of most ARGs obtained by the end-point PCR was higher in raw water. However, higher gene detection was also detected in NW-E (DHA and *sul2*) and NW-C (*sul1*) treated water. Real-time PCR analysis showed higher ARG concentrations in treated water. Chemical analysis showed higher antibiotic and fluconazole residues in raw water. However, trimethoprim concentrations were higher in NW-C treated water. The risk quotients (RQs) were deemed low to high for target compounds. In addition, the bacterial community was determined by high-throughput 16S rRNA sequencing and correlated with ARGs, antibiotic residues and physicochemical parameters. The study's third part determined antibiotic residue and ARG patterns in wastewater effluent and rivers. The end-point and real-time PCRs showed that the wastewater effluents influenced the detection and quantification of some ARGs in the downstream rivers, with a few abnormalities. The chemical analysis results indicated that, in NW-E, the wastewater effluent did not influence the presence of fluconazole in the downstream river. In NW-C, ampicillin concentration increased from the wastewater effluent to the downstream maturation pond. The RQs of target compounds in wastewater effluent and rivers were deemed to be of high and low risk. Furthermore, high-throughput 16S rRNA sequencing revealed the presence of potential pathogens. In conclusion, since these contaminants in water are a potential threat to humans, animals and plants, the study's outcomes could form part of policymaking to improve antibiotic use, water quality and the fight against antibiotic resistance.

Keywords: antibiotic resistance genes; antibiotic residues; drinking water treatment plants; bacterial community composition; wastewater treatment plants; treated water; rivers; ultra-performance liquid chromatography; PCR, high-throughput 16S rRNA sequencing, physicochemical parameters; risk quotient

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LIST OF ABBREVIATIONS

AN	Annealing
ARB	Antibiotic resistant bacteria
ARGs	Antibiotic resistance genes
BRICS	Brazil, Russia, India, China and South Africa
CC	Cycles
CDC	Centers for disease control and prevention
COD	Chemical oxygen demand
DN	Denaturation
Down	Downstream river or downstream maturation
DWAF	Department of Water Affairs & Forestry
DWPFs	Drinking water production facilities
eDNA	Environmental deoxyribonucleic acid
EL	Elongation
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental protection agency
ESBL	Extended spectrum β -Lactamase
ESI	Electrospray ionization
FE	Final extension
GDP	gross domestic product
HCl	Hydrochloric acid
HGT	Horizontal gene transfer
ID	Initial denaturation
LOD	Limit of detection
LOQ	Limit of quantification
MGE	Mobile genetic elements
Na ₂ EDTA	ethylenediaminetetraacetic acid disodium salt dihydrate
NDoH	National Department of Health
NPC	National Planning Commission
OIE	World Organization for Animal Health
pAmpCs	Plasmid-mediated <i>AmpC</i> β -lactamase genes
PCR	Polymerase chain reaction
PICRUS	Phylogenetic investigation of communities by reconstruction of unobserved states
QTOF	Quadrupole time-of-flight mass spectrometry

R ²	Coefficient of determination
RQs	Risk quotients
RSD	Relative standard deviation
SAWQG	South African water quality guideline
STATS	Statistics
TDS	Total dissolved solids
Up	Upstream river
UHPLC	Ultra-high performance liquid chromatography
WHO	World Health Organization
WWTPs	Wastewater treatment plants

CHAPTER 1

1. INTRODUCTION AND PROBLEM STATEMENT

1.1. INTRODUCTION

1.1.1. Bacteria community composition

In aquatic environments, bacterial communities play essential roles in energy flow, nutrient recycling and biochemical cycling (Jin *et al.*, 2018). These roles may be attributed to relevant physicochemical parameters, biodegradable pollutants, activities of the catchment area and predators (Pascual-Benito *et al.*, 2020; Tiwari *et al.*, 2021). Dominant bacterial communities in aquatic environments include Proteobacteria and Bacteroidetes (Holinger *et al.*, 2014; Potgieter *et al.*, 2018; Lui *et al.*, 2018; Picazo *et al.*, 2019; Vadde *et al.*, 2019; Jing *et al.*, 2021; Tiwari *et al.*, 2021). Some bacteria communities are considered to be potential human, animal and plant pathogens (Guan *et al.*, 2018; Dobhal *et al.*, 2020; Pédrón and Gijsegem, 2023). Bacterial communities have been correlated with physicochemical parameters, ARGs and antibiotic residues (Zhou *et al.*, 2017; Guan *et al.*, 2018; Zhang *et al.*, 2022).

Wastewater treatment plants (WWTPs) and DWPFs are needed to treat wastewater and raw water that are put to use for a variety of purposes. Usually, the disinfection process is used as the last barrier against microbial communities (Han *et al.*, 2020). However, studies have shown that the disinfection process does not completely eliminate microbes (Han *et al.*, 2021). Thus, microbes in the wastewater effluent and treated water can be traced to originate from wastewater influent and raw water, respectively. Furthermore, bacterial communities in wastewater effluent and treated water may also be influenced by various treatment processes (Han *et al.*, 2021; Jing *et al.*, 2021). A further influence occurs in treated water during distribution, stagnation and secondary water supply (Han *et al.*, 2021). Urban rivers are spatially rather heterogeneous ecosystems that are mainly influenced by the terrestrial environment (Wang *et al.*, 2018a). Hence, studying bacterial communities in aquatic environments is of utmost importance.

However, culture-based methods allow scientists to study only slightly above 2.1% of microorganisms globally (Jing *et al.*, 2021). A viable but non-culturable state is considered to be a universal strategy for cells to survive under adverse environmental conditions (Jing *et al.*, 2021). As such, culture-based methods cannot be used to study microorganisms in this state. Developing high-throughput 16S rRNA sequencing has significantly improved the

understanding of viable but non-culturable bacterial communities in WWTPs, rivers and DWPFs (Potgieter *et al.*, 2018; Osunmakinde *et al.*, 2019; Han *et al.*, 2021; Jing *et al.*, 2021). High-throughput 16S rRNA sequencing is used to identify bacterial community composition, bacterial diversity, detection of potential bacterial pathogens and prediction of enzymatic production of bacteria (Osunmakinde *et al.*, 2019; Vadde *et al.*, 2019; Tiwari *et al.*, 2021). The high-throughput 16S rRNA sequencing can potentially overcome the limitations of culture-based methods (Vadde *et al.*, 2019).

1.1.2. Antibiotic resistance in wastewater, rivers and drinking water

Antibiotics rank among the most commonly prescribed and successful therapeutic agents used by humans and animals (Bergeron *et al.*, 2017; Song *et al.*, 2019). However, the appropriate and inappropriate use of antibiotics may result in antibiotic resistance (Bergeron *et al.*, 2017). When administered, not all antibiotics are fully metabolized by the human and animal bodies, thus leading to the excretion of antibiotic residues in their original form into the water environment (Bergeron *et al.*, 2017). Furthermore, the incorrect disposal of antibiotics via the wastewater drainage systems is also documented to spread antibiotics in that environment. The interaction of bacteria and antibiotic residues present in sub-inhibitory concentrations in water environments selects for antibiotic resistance. Bacteria inhabiting wastewater are more prone to select for antibiotic resistance. As the terminal recipients, the wastewater is rich in nutrients that may create conducive conditions for bacteria to grow, spread and/ or acquire antibiotic resistance genes (ARGs) (Hu *et al.*, 2019).

Studies show that wastewater treatment plants (WWTPs) discharge ARGs, antibiotic residues and antibiotic resistant bacteria (ARB) into receiving water bodies (Fadare and Okoh, 2021; Lin *et al.*, 2021). Once in the environment, surface water contaminated with antibiotic residues and ARGs is used for food production and drinking water. Some studies have reported the presence of ARGs in food products (Ye *et al.*, 2017; Salamandane *et al.*, 2022a). The constant exposure of aquatic life to antibiotic residues in surface water may pose ecological risks (Pulicharla *et al.*, 2021). These contaminants have also been documented in drinking water (Rolbiecki *et al.*, 2020; Liang *et al.*, 2022). Consumption of contaminated drinking water may pose a public health threat to humans. In pregnant women, prolonged exposure to antibiotic residues in low concentrations may result in the malformation of infants and children (Mahmood *et al.*, 2019). In addition, contaminated water may enhance the virulence and pathogenicity of pathogens, thus leading to high morbidity, hospitalization and mortality rates (Liang *et al.*, 2022). This engenders a situation where water environments become vectors of antibiotic resistance.

ARGs are considered to be emerging contaminants in water environments (Chen *et al.*, 2020). This has put the emergence of ARGs under the spotlight globally. The ARB can spread and incorporate their ARGs into other microorganisms belonging to a different family via horizontal gene transfer (HGT) (Chen *et al.*, 2020). The HGT enables ARBs to share their ARGs with opportunistic pathogens (Chen *et al.*, 2020). ARGs are found in the mobile genetic elements (MGEs), such as the plasmids and integrons (Hu *et al.*, 2018). The incorporation and expression of ARGs in pathogens form part of determining factors that dictate how infectious diseases are treated and pose a public health concern (Hu *et al.*, 2018).

1.1.3. Antibiotic use in South Africa

The frequent application of antibiotics in human medicine, veterinary medicine and food production induces antibiotic resistance. According to the National Department of Health, South Africa (NDoH) (2018), in 2014, 4.35 tons of antimicrobials were imported into South Africa. Antibiotics in human medicine are vital for preventing and treating infectious bacteria. In addition, they are used in organ transplantation, major surgical operations, chemotherapy of cancer and therapy for premature babies to treat infections (Van *et al.*, 2020). According to the NDoH (2018), South Africa has antimicrobial use of 21 149 standard units per 1000 population, which is higher than most countries in the world, while it is comparable to that of Brazil, Russia, India, and China which, collectively, form the BRICS countries. Furthermore, antibiotic data recorded in 2015 shows that South Africa had high broad-spectrum penicillin and trimethoprim use compared to other BRICS countries, the United Kingdom, and the United States (NDoH, 2018).

South Africa also utilizes antibiotics for food production (such as among animals for growth stimulation), enhancement of feed proficiency and prophylaxis, thus ensuring economic growth (see NDoH, 2018; Van *et al.*, 2020; World Organization for Animal Health (OIE), 2021). The use of antibiotics in food production animals was introduced in the 1950s (Van *et al.*, 2020). According to the NDoH (2018), in South Africa, 1 592 842 kg of antibiotics were procured for the animal industry, of which 980 782 kg were categorized as growth stimulators in 2015. In producing food animals, antibiotics are mostly used in poultry, bovines, sheep, goats and pigs. Antibiotics commonly used in livestock farming include tetracycline, sulfonamides (including trimethoprim), penicillins, other quinolones, macrolides and glycopospholipids (OIE, 2021). However, the extensive and improper usage of antibiotics in animals contributes to antibiotic-resistant zoonotic pathogens' emergence and spread (Samuels *et al.*, 2021).

1.1.4. Antibiotic prescription in South Africa

Primary care prescribers are also reported to influence the high use of antibiotics, which engenders the emergence of antibiotic resistance. A study by Gasson *et al.* (2018) aimed to assess current antibiotic prescribing practices in primary care facilities in the Cape Town Metro District, South Africa, and showed that overall antibiotic adherence was 45.1%. These results were comparable to the finding obtained by Almansoori and Parag (2021), who determined that the inappropriateness of antibiotic prescription was 48.2% in the KwaZulu-Natal Emergency Department. A study by Farley *et al.* (2018) aimed to assess knowledge of, attitudes to and perceptions of ARB among primary care prescribers in South Africa, and demonstrated that (i) 97.1% of primary care prescribers agree that antibiotics are extensively used, (ii) 95.8% of primary care prescribers believe that antibiotic resistance is a significant problem and (iii) 65.5% of primary care prescribers prescribe antibiotics due to the pressure caused by patients. Samuels *et al.* (2021) report that inappropriate prescription of antibiotics also exists in veterinary medicine.

1.1.5. Overview of South Africa's water quality

According to water quality reports released by the Department of Water and Sanitation (DWS) (2022a; 2022b), some South African WWTPs and drinking water production facilities (DWPFs) are underperforming. This leads to the discharge and production of inadequately treated wastewater and drinking water. This has elevated physicochemical parameters and microbiological agents. Although some physicochemical parameters provide vital nutrients needed in fertilizers (Darvishmotevalli *et al.*, 2019; World Health Organization; WHO, 2017; WHO, 2022), their elevated levels in treated wastewater effluent and drinking water promote pathogenic bacterial growth (Odjadjare and Olaniran, 2015; Ateba *et al.*, 2020; WHO, 2022). Therefore, the present study also investigated physicochemical parameters such as total dissolved solids (TDSs), temperature, pH, nitrate, nitrite, phosphate and chemical oxygen demand (COD).

1.1.5.1. Total dissolved solids

The TDSs comprise inorganic salts (such as bicarbonates, chlorides, calcium, magnesium, potassium and sulphates) and small amounts of organic matter dissolved in water. In water environments, TDSs are obtained from natural sources, urban runoff and industrial WWTPs

(WHO, 2022). It is therefore important to apply appropriate treatment processes to treat TDS. The WHO (2017; 2022) considers TDS concentrations of less than 600 mg/L in drinking water as good quality. If TDS exceeds 1 000 mg/L, drinking water becomes significantly and increasingly unpalatable. High TDS concentrations also cause scaling in water distribution pipes and household appliances dependent on treated water (WHO, 2017; 2022).

1.1.5.2. Temperature

Temperature is an important water quality parameter due to its influence on chemical reactions, reaction rates and aquatic life (Odjadjare and Olaniran, 2015; Razman *et al.*, 2022). Water temperature is essential for the protection of aquatic life. Most aquatic life depends on cold water habitats for survival (EPA, 2017). High water temperatures negatively affect aquatic life (Odjadjare and Olaniran, 2015). They influence the growth and survival of undesirable bacteria, algae and fungi (Odjadjare and Olaniran, 2015; WHO, 2022). Water temperature is directly proportional to the TDSs and electrical conductivity (Boadi *et al.*, 2020). Temperature is not associated with any direct health impact on humans, and no proposed health-based guidelines exist for it (WHO, 2017; 2022).

1.1.5.3. pH

Like temperature, pH has no direct health impact on humans (WHO, 2017; 2022). The pH measurement indicates how basic or acidic a water environment is (Boadi *et al.*, 2020). It ranges between 0 and 14, where 7 is neutral. A pH range below 7 indicates acidity, whereas pH greater than 7 indicates a base (Razman *et al.*, 2022). The appropriate pH level of water plays a vital part in disinfection and good maintenance of mains and pipes in distribution networks. The DWPFs and WWTPs must achieve a pH of less than 8 for optimum chlorination (WHO, 2022). Furthermore, the pH of treated water leaving the DWPF has to be equal to or greater than 7 to minimize the corrosion of water mains and pipes in distribution networks (WHO, 2022). Corrosion in mains and pipes in distribution networks may contaminate treated water, thus resulting in a peculiar taste and appearance (WHO, 2022).

1.1.5.4. Salinity

Salinity is one of the most widespread soil degradation processes in agricultural and native soils (Canfora *et al.*, 2015). The high salinity concentration in water environments is associated with anthropogenic activities, weathering rocks, agricultural activities, resource extraction and

the application of road deicing salts (Hintz *et al.*, 2022; Kaushal *et al.*, 2021). The high salinity concentration degrades the freshwater quality and its usability for human use, agricultural production and aquatic life (Flörke *et al.*, 2019). In the environment, applying high-salinity surface water can negatively impact crop growth, physical soil conditions, fertility requirements and irrigation system performance (M'nassri *et al.*, 2022). In addition, the consumption of high-salinity water can increase health costs (Kaushal *et al.*, 2021). Furthermore, the excess salinity concentration negatively affects the biodiversity of algae, microorganisms, plants and animals (Canfora *et al.*, 2015; Mateo-Sagasta *et al.*, 2017).

1.1.5.5. Chemical oxygen demand

Chemical oxygen demand (COD) indicates the amount of dissolved oxygen needed in the water to oxidize chemical organic matter (EPA, 2021). It is an essential parameter for determining the degree of water pollution (Lv *et al.*, 2022). The measurement of COD is a crucial water quality tool that helps evaluate the compliance of WWTPs and estimate the potential suspended or dissolved matter in wastewater effluent to deplete oxygen (Adefisoye and Okoh, 2017). The limitation of measuring the COD is that it does not provide bioavailability information and does not reflect differentiation between biodegradable and biologically recalcitrant organic matter (Lv *et al.*, 2022).

1.1.5.6. Nitrate and nitrite

Nitrogen exists in environmental water as ammonium, nitrate and traces of nitrite (EPA, 2021). The presence of nitrate and nitrite in water environments is associated with the high application of nitrogenous fertilizers and their runoff, denitrification by bacteria and uptake by phytoplankton (Darvishmotevalli *et al.*, 2019; WHO 2017; 2022). Excessive nitrate and nitrite levels in drinking water concern consumers, especially immunocompromised individuals and infants (WHO, 2017; Isiuku and Enyoh, 2020; WHO, 2022). The consumption of excessive nitrates may result in methemoglobinemia ("blue baby syndrome") and thyroid effects for infants (WHO, 2016; 2017; EPA, 2021; WHO, 2022). Infants who survive methemoglobinemia may suffer from mental retardation (Isiuku and Enyoh, 2020). Therefore, the WHO (2016; 2017; 2022) set the water guideline of the sum ratios of the nitrate and nitrite concentrations to be lower than one mg/L.

Nitrates are associated with anthropogenic activities such as WWTPs, agriculture and oxidation of nitrogenous waste products in human and other animal excreta, including septic

tanks and motor vehicles (Darvishmotevalli *et al.*, 2019). Excess nitrate nutrients in surface water create favourable conditions for the overgrowth of water plants, thus forming algal bloom. The latter engenders high demand and depletion of dissolved oxygen in the water, which cause eutrophication (Isiuku and Enyoh, 2020; EPA, 2021).

1.1.5.7. Phosphate

Agricultural activities and discharged wastewater effluent may promote the occurrence of phosphate in waterbodies (WHO, 2016). It enters the WWTP through wastewater containing detergents (cleaning and laundry products) and human excreta (WHO, 2016; EPA, 2021). Thus, it is important to apply appropriate treatment processes to reduce the phosphate levels in the treated wastewater effluent. Furthermore, reducing phosphate nutrients may be beneficial for controlling the growth of cyanobacterial biomass in surface water (WHO, 2022). In addition, the presence of phosphate nutrients in water enhances the development of the algae beyond normality. Phosphate nutrients may also lead to eutrophication (EPA, 2021). Phosphates are however beneficial in industries that produce beverages, detergents, fertilizers and semiconductors (Isiuku and Enyoh, 2020).

1.2. PROBLEM STATEMENT

The present study is a continuation of those conducted by Kritzinger (2019), Plaatjie (2019) and Tsholo (2019). These studies aimed to characterize ARB in the North West, Gauteng, and Western Cape DWPFs. In addition, they determined the physicochemical parameters and general microbiological quality of water. The measured nitrite concentrations were found to be of concern in drinking water studies conducted by Kritzinger (2019) and Plaatjie (2019). Other tested physicochemical parameters were found to be maintained within the recommended water standards. The heterotrophic bacteria were successfully isolated and identified. A general trend observed in all three studies was that *Bacillus* species were the most prevalent heterotrophic bacteria isolated in raw and drinking water. Haemolysin tests and extracellular enzyme assays were also conducted. The α - and β -haemolytic heterotrophic bacteria that can produce extracellular enzymes were detected in raw and drinking water.

The heterotrophic bacteria showed increased antibiotic resistance patterns from raw to drinking water in the DWPFs. They showed high resistance to ampicillin, trimethoprim and cephalothin. The multiple antibiotic resistance indices calculated in raw water were high compared to treated water and water in distribution networks. In a study by Kritzinger (2019)

in NW-C and NW-G, the *bla*_{TEM}, *ermB*, *ermF*, *int1* and *tetM* genes were not detected in raw and drinking water. Furthermore, only one *ampC* gene was detected in the distribution network. This was not consistent with the results obtained by Tsholo (2019), who detected one *ampC* in treated water. However, Plaatjie (2019) detected the *ampC* gene five times: most of these were found in drinking water. The *ermB* was the most prevalent ARG detected by Plaatjie (2019) and Tsholo (2019). Other detected genes included *bla*_{TEM}, *ermF*, and *int1*. The environmental deoxyribonucleic acid (eDNA) was also subjected to ARG screening. Furthermore, Tsholo (2019) used chemical analysis to quantify antibiotic residues in raw and treated water from the Western Cape.

However, these studies did not quantify ARGs and antibiotic residues in the DWPFs. Furthermore, they excluded other water environments, such as WWTPs, which potentially release ARGs and antibiotic residues into rivers, essential food and drinking water production sources. Tsholo (2019) emphasised that some South African regions are water-stressed. Thus, reclaimed wastewater effluent is essential for producing drinking water and food to compensate for high demand of these. The searched databases in Chapter 2 reflect the fact that an insufficient number of South African studies focused on quantifying ARGs and antibiotics in WWTPs, rivers and DWPFs. The present research addresses this gap.

This is of further importance, since antibiotic use in South Africa ranks among developed nations with advanced public healthcare facilities, such as the United States and many European countries (Van *et al.*, 2020). Van Boeckel *et al.* (2015) estimate that antibiotic used worldwide will increase by 67% by 2030, with almost a double increase in the BRICS countries. However, the paucity of available information on the consumption of antibiotics, scientific drug management and guidance, as well as the public lack of antibiotic stewardship about the appropriate use of antibiotics in human medicine, veterinary medicine and agriculture, have led to misuse of antibiotics (Song *et al.*, 2019). To exacerbate the problem, antibiotics and ARGs are not regulated in wastewater, rivers and drinking water in South Africa.

The WHO (2018) classifies the antibiotics that were selected in the present study as critically important or highly antibiotic in the antibiotic drug category. These include critically important ampicillin and ciprofloxacin as well as highly important trimethoprim and sulfamethoxazole.

1.3. RESEARCH AIM

This study aimed to measure the concentrations of antibiotic residues and antibiotic resistant genes in wastewater effluent, rivers and drinking water.

The specific objectives were to:

- I. Provide an overview of the sources and prevalence of antibiotic residues and antibiotic resistance genes in source waters in South Africa used for drinking and food production – A literature review.
- II. Determine the distribution of bacterial community composition, antibiotic residues and antibiotic resistance genes in drinking water production facilities.
- III. Determine bacterial community composition, antibiotic residue and antibiotic resistance gene patterns in wastewater plant effluent and rivers – Implications for downstream use.
- IV. Use the data on levels of antibiotic residues to determine whether there could potentially be exposure risks.

1.4. OUTLINE OF THE THESIS

Chapter 1

Chapter 1 provides a background of ARGs and antibiotic residues in wastewater, rivers and drinking water. Furthermore, it introduces physicochemical parameters measured in the present study. It also provides the problem statement, aim, objectives and thesis outline.

Chapter 2

This chapter consists of a structured review to provide an overview of the presence of antibiotic residues and ARGs in South African source water used for drinking and food production.

Submitted

Title: Antibiotic resistance genes and antibiotics present in South African source water used for drinking and food production

Authors: Tsholo K., Molale-Tom L.G. and Bezuidenhout C.C.

Journal: *Water South Africa*

Manuscript number: WATER South Africa 3973

Chapter 3

This chapter outlined the methods that were employed in the work that is reflected in Chapters 4 and 5. The methods outlined include determining bacterial community composition and physicochemical parameters, screening ARGs, quantifying ARGs, and quantifying antibiotic residues in WWTPs, rivers and DWPFs. Furthermore, it outlines the method for determining the potential exposure risks in rivers associated with antibiotics. The publication of these methods depends on Chapters 4 and 5.

Chapter 4

This chapter compares the levels of physicochemical parameters, the relative abundance percentage of bacterial communities and ARGs and antibiotic residues between raw and treated water from the two North West DWPFs. It discusses the ability of the DWPFs to improve bacterial community composition and physicochemical parameters and reduce ARGs and antibiotic residues. Furthermore, it examines the determination as to whether a significant correlation exists between these parameters.

Submitted

Title: Distribution of antibiotic resistance genes and antibiotic residues in drinking water production facilities: Correlation with bacterial communities

Authors: Tsholo K., Molale-Tom L.G., Horn S. and Bezuidenhout C.C.

Journal: *PLOS ONE*

Manuscript number: PONE-D-23-28722.

Chapter 5

This chapter reflects the investigation centred on whether the wastewater effluent influenced the physicochemical parameters, bacterial community composition, ARGs and antibiotic

residues in receiving water bodies. These parameters were also investigated in the upstream river.

Title: Bacterial community composition, quantification of antibiotic resistance genes and antibiotic residues in wastewater effluent and receiving rivers

Authors: Tsholo K., Molale-Tom L.G., Horn S. and Bezuidenhout C.C.

Target journal: *Environmental Pollution*

Chapter 6

This chapter discusses the significance of the present study and offers recommendations for future research.

CHAPTER 2

2. ANTIBIOTIC RESISTANCE GENES AND ANTIBIOTICS PRESENT IN SOUTH AFRICAN SOURCE WATER USED FOR DRINKING AND FOOD PRODUCTION

2.1. INTRODUCTION

South Africa is a water-scarce country (DWS, 2022c). This is exacerbated by increased population size, industrialization, urbanization, water exploitation, pollution and climate change (Ateba and Maribeng, 2011; Samie *et al.*, 2011; Odjadjare and Olaniran, 2015; Pillay and Olaniran, 2016; Adefisoye and Okoh, 2017; Hamiwe *et al.*, 2019; Matlou *et al.*, 2019; Nyamukamba *et al.*, 2019). This has caused South Africa to explore the use of treated domestic wastewater effluent for supplementing surface water and groundwater to produce safe and clean water to be used for food security, mining, energy production and domestic purposes (Olaniran *et al.*, 2009; Du Plessis *et al.*, 2015; Segura *et al.*, 2015; Pillay and Olaniran, 2016; Matlou *et al.*, 2019). According to the DWS (2018), the water use per sector in South Africa is as follows: agriculture or irrigation (61%), urban (24% and 3%), afforestation (3%), livestock watering and conservation (2%), power generation (2%), mining (2%) and industries that are not part of urban households (3%).

Though access to safe and clean drinking water is a basic human need (Samie *et al.*, 2012; Ateba *et al.*, 2013), providing safe and clean drinking and adequate sanitation remains one of the major public health concerns in South Africa (Adefisoye and Okoh, 2017; Matlou *et al.*, 2019; Ateba *et al.*, 2020).

The DWS uses the Blue and Green Drop Certification programmes to monitor drinking water and wastewater quality (DWS, 2022a; 2022b). South Africa mainly depends on ground and surface water for drinking water production (Adefisoye and Okoh 2017). Municipalities in drought-stricken regions have also considered and once implemented the use of treated effluent from domestic wastewater plants. However, it has been found that many WWTPs and DWPFs are not working effectively in South Africa due to poor management and maintenance (Olaniran *et al.*, 2009; Mulamattathil *et al.*, 2014a; Adefisoye and Okoh, 2017). Studies have reported the presence of chemical agents and faecal indicator bacteria in these cases (Okoh and Igbinosa, 2010; Adefisoye and Okoh, 2017). According to the DWS (2022a) report, the provincial average Green Drop performance was as follows: Gauteng (17.5%), Northern Cape

(6%), Western Cape (22%), KwaZulu-Natal (7%), Free State (7%), Eastern Cape (24.5%), Mpumalanga (21%), North West (18%) and Limpopo (3%). According to the DWS (2022b), the National Blue Drop Risk Rating average is 63.3%, with minimum and maximum values of 10.1% and 100%. However, recent studies have highlighted that the water quality problem lies far beyond the matters of physicochemical parameters and microbiological agents (Okoh and Igbinosan, 2010; Igwaran *et al.*, 2018; Ramessar and Olaniran, 2019).

The discovery of antibiotics has revolutionized medicine by protecting lives against pathogenic bacteria, thus improving the life expectancy of humans and animals. However, South Africa faces the challenge of antibiotic resistance in source waters disseminated from clinical and agricultural settings (Abia *et al.*, 2015; Agunbiade and Moodley, 2014; Kanama *et al.*, 2018). Antibiotics are not limited to the prevention and treatment of pathogenic bacteria. They have many applications in livestock farming (for instance, as growth stimulators) and aquaculture (Igbinosa *et al.*, 2011; Adefisoye and Okoh, 2017). Antibiotic resistance emerged a few years after their discovery and application (Igbinosa and Okoh, 2012).

The inappropriate and extensive use of antibiotics across different sectors has led to the spread of antibiotics in the environment (Olaniran *et al.*, 2009; Kinge *et al.*, 2010; Igbinosa *et al.*, 2011; Mulamattathil *et al.*, 2014b; Abia *et al.*, 2015; Chukwu *et al.*, 2019b; Matlou *et al.*, 2019). Humans, animals and the environment form part of one health system that may result in the emergence, proliferation and spread of antibiotic resistance (Ateba *et al.*, 2020). Human and animal bodies cannot fully metabolize antibiotics. As a result, the administered antibiotics are excreted directly or indirectly into source water (Abia *et al.*, 2015; Pillay and Olaniran 2016). Thus, attention is being shifted to the environment in addition to clinical settings so as to intensively study and understand the role of ecological microorganisms in the emergence and development of antibiotic resistance (Adefisoye and Okoh 2017).

Current water standards do not regulate the presence of ARGs and antibiotic residues in treated wastewater effluent and drinking water (Agunbiade and Moodley, 2014; Matongo *et al.*, 2015a; Matongo *et al.*, 2015b). WWTPs are considered to be hotspots for antibiotic resistance (Mann *et al.*, 2019). The latter is related to the acquisition and spread of ARGs through mobile genetic elements (MGE) such as plasmids, transposons and phages or mutation (Hamiwe *et al.*, 2019). These MGE enable ARB to spread and incorporate their ARGs into microorganisms belonging to a different family via HGT (Igbinosa *et al.*, 2011; Molale and Bezuidenhout, 2016; Ramessar and Olaniran, 2019). The WWTPs are ineffective when it comes to removing ARGs and antibiotic residues (Abia *et al.*, 2015; Nyamukamba *et al.*, 2019; Ateba *et al.*, 2020). This leads to the discharge of inadequately treated wastewater effluent

into receiving water bodies and, subsequently, into drinking water (Igbinosa *et al.*, 2011; Matongo *et al.*, 2015a).

The application of contaminated source water for drinking and food production may facilitate the spread of antibiotic resistance in the food chain (Igwaran *et al.*, 2018). Antibiotic resistance is reported in irrigation water (Iwu and Okoh, 2020; Raseala *et al.*, 2020; Kayode *et al.*, 2021), plant rhizosphere (Igbinosa *et al.*, 2014) and drinking water (Ateba *et al.*, 2013; 2020). DWPFs were also not designed to remove ARGs (Ateba *et al.*, 2020). The latter indicates the role of source water in one's health (Igwaran *et al.*, 2018; Ateba *et al.*, 2020).

No review could be found that addressed both ARGs and antibiotic residues in source water in South Africa used for drinking and food production. Ekwanzala *et al.* (2018) reviewed antibiotic ARGs shared between clinical and environmental settings. However, in their review, 12 environmental studies were included and not all addressed ARGs in source water used for drinking and food production. Furthermore, there was no mention of antibiotic residues. Thus, the present study aimed to identify gaps in the literature on the quantification of ARGs and antibiotic residues in source water in South Africa used for drinking and food production.

2.2. METHODS

2.2.1. Eligibility criteria

This study focused on an array of ARGs and/ or antibiotic residues in source water (wastewater, surface water, groundwater) that may be used for drinking and food production in South Africa.

2.2.2. Search databases and exclusion criteria

The following search terms were used: "antibiotic resistance" AND "water" AND "South Africa." The search was limited to articles published between 2000 and 2022. The EBSCO Discovery Services was used as the online search engine.

The titles and abstracts of academic articles in peer-reviewed journals were thoroughly screened. The excluded academic articles included the following: (i) those that were non-South African, (ii) pilot studies, (iii) those focusing on marine water, (iv) those were not written in -English and (v) review papers. The methodology of academic articles was also screened to establish the sampling sites and techniques used to detect ARGs and antibiotic residues. Searching and screening of studies were done independently by two research team members (KT and M-TLG) and, where disagreements occurred, a third member (BCC) was involved to find a resolution.

2.2.3. Inclusion criteria and additional information

Academic Search Complete, Environmental Complete, CAB Abstract, Directory of Open Access Journals, Science Direct and Science Citation Index were the databases used, as reflected in Figure 2.1. Academic articles that focus on phenotypic resistance, ARGs and/ or antibiotic residues in wastewater, surface water, groundwater and/ or drinking water were included in this review. A total of 71 academic articles satisfied all the inclusion criteria. The searched databases determined that academic articles focused on the presence of antibiotic residues in South African source water were insufficient. As a result, additional academic articles were included in the present review to provide in-depth insight into the presence of antibiotic residues in South African water. However, additional academic articles were not obtained using the search terms specified.

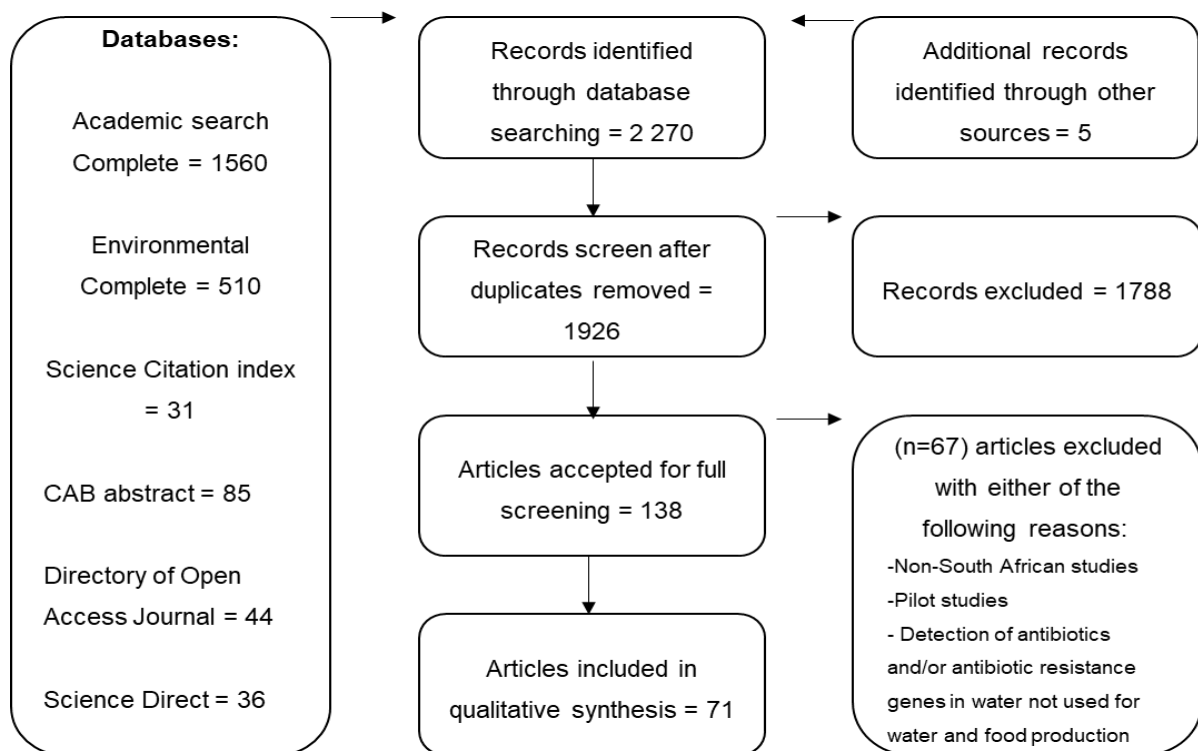


Figure 2.1: Flowchart illustrating the selection process of journals.

2.2.4. Data synthesis

Data synthesis was achieved by sorting articles into different quality categories. The categories were based on studies addressing the detection of ARGs and antibiotic residues, as shown in Tables A1 and A2 in Appendix A, respectively. The quality category was classified as low, medium or high scale based on the assessment worth of each study as reflected in the tables.

2.3. RESULTS AND DISCUSSION

2.3.1. The performance and state of wastewater treatment plants

The DWS has committed itself to reusing treated wastewater effluent to compensate for the high demand for freshwater (Hamiwe *et al.*, 2019; Ateba *et al.*, 2020). However, many studies have illustrated that South African WWTPs are not working effectively. This challenge is exacerbated by many factors, including inefficient operations, poor design, lack of expertise, inadequate monitoring and poor compliance documentation (Adefisoye and Okoh, 2017). WWTPs are the primary barriers to pollution in source water (Suzuki *et al.*, 2015). However, King *et al.* (2020) state that no clear regulations ensure that hospital wastewater effluent is treated before it is discharged into the municipal WWTPs. It is also reported that approximately 80% of inadequately treated wastewater effluent from the municipality WWTPs in developing countries is discharged directly into receiving water bodies (Hamiwe *et al.*, 2019).

Igwaran *et al.* (2018) report that South Africa has 986 municipal WWTPs, of which 26% discharge inadequately treated wastewater effluents into rivers. A study conducted in the Eastern Cape showed that the two WWTPs did not comply with the recommended water standards (Adefisoye and Okoh 2017). These WWTPs had turbidity, COD, biochemical oxygen demand, nitrate, phosphate, free chlorine, *E. coli* and *Vibrio* species, all of which are not within the ambit of the recommended water standards. Similar results were obtained in another study conducted in Eastern Cape by Okoh and Igbinosa (2010), where turbidity, chemical oxygen demand, nitrate and orthophosphate occurred, while these were also not within the parameters of the recommended water standards. International water standards had to be used in these cases, since there were no limits for biochemical oxygen demand included in the current South African water standards (Adefisoye and Okoh 2017).

The emergence of antibiotic resistance has been an increasing focal point in this context, since WWTPs receive wastewater from hospitals, pharmaceutical factories, agriculture and households. These sources may contain ARB, ARGs and antibiotic residues (Igwaran *et al.*, 2018; Hamiwe *et al.*, 2019). The type and number of contaminants vary among different WWTPs. Their emergence may be influenced by anthropogenic, agricultural, commercial and industrial bodies that directly or indirectly discharge their wastewater into WWTPs (Agunbiade and Moodley, 2014; Matongo *et al.*, 2015a; Coertze and Bezuidenhout 2017; Ekundayo and Okoh 2020). Seasonal factors may also contribute to the emergence of antibiotic resistance (Adefisoye and Okoh 2017). Discharging wastewater that is heavily contaminated with ARGs,

antibiotic residues and other biological and chemical contaminants is an environmental concern (Igbinosa *et al.*, 2011). The removal efficiency of antibiotic residues in WWTPs is affected by aqueous solubility, volatility, adsorption to solids and biodegradation of antibiotics (Kanama *et al.*, 2018).

2.3.2. The role of surface water as a vector for antibiotic resistance

Surface water is an essential natural resource due to its fundamental role in the environment, agricultural production, hygiene, recreation and industrialization (Odjadjare and Olaniran, 2015; Coertze and Bezuidenhout, 2017; Matlou *et al.*, 2019). Discharging inadequately treated wastewater effluents into surface water tempers the usability of source water for domestic purposes and negatively affects aquatic life (Okoh and Igbinosa, 2010; Mulamattathil *et al.*, 2014a; Matongo *et al.*, 2015a; Adefisoye and Okoh, 2017). However, the database search found no articles that assess the risks associated with antibiotic residues in the source water.

Despite the government's commitment to providing safe and clean drinking water, some informal settlements and rural communities directly rely on untreated surface water for drinking and other domestic purposes (Olaniran *et al.*, 2009; Ateba and Maribeng, 2011; Samie *et al.*, 2011; Abia *et al.*, 2015; Coertze and Bezuidenhout, 2017; Govender *et al.*, 2021). Additionally, some regions of the country depend on harvested rainwater to compensate for the high demand for freshwater (Malema *et al.*, 2018). Poor sanitation enhances the dissemination of antibiotic resistance into surface water (Suzuki *et al.*, 2015; Coertze and Bezuidenhout, 2017). Landfill leachate is another documented route of antibiotic entry into surface water (Agunbiade and Moodley, 2014). Antibiotic residues in the environment are moreover characterized by their inability to degrade (Segura *et al.*, 2015). This leads to prolonged effects of antibiotics in source water even when their consumption is low (Chukwu *et al.*, 2019b). ARGs and antibiotic residues may also enter the food chain by consuming crops irrigated with contaminated source water (Agunbiade and Moodley, 2014; Igbinosa *et al.*, 2014; Du Plessis *et al.*, 2015).

2.3.3. Incidence of antibiotic resistance in drinking water

Access to safe and clean drinking water is a basic human need and right (Ateba and Maribeng, 2011; Phokela *et al.*, 2011; Mulamattathil *et al.*, 2014a; Mulamattathil *et al.*, 2014b; Matlou *et al.*, 2019). However, Stats South Africa (2019) reported that only 89.0% of households have access to drinking water. This has forced many individuals residing within informal settlements to rely on untreated groundwater in addition to traditional water sources for drinking purposes

(Phokela *et al.*, 2011; Ateba *et al.*, 2013). Generally, the water quality of groundwater is considered good (Carstens *et al.*, 2014). However, Ateba *et al.* (2013) reported that residents of some informal settlements had constructed borehole systems next to pit toilets which might alter the water quality. Stats South Africa (2019) furthermore states that 17.0% of South African households cannot access improved sanitation.

Consumption of contaminated water induces antibiotic resistance in the food chain. Furthermore, individuals who rely on treated drinking water may also be at risk of contracting antibiotic resistance determinants, since conventional DWPFs cannot entirely remove ARGs and antibiotic residues (Matongo *et al.*, 2015a). Moreover, the formation of biofilms may also alter the microbial water quality (Odjadjare *et al.*, 2012; Mulamattathil *et al.*, 2014a).

The spread of ARGs among humans makes it difficult to treat bacterial infections with commonly prescribed antibiotics (Coertze and Bezuidenhout, 2017; Hamiwe *et al.*, 2019). This poses detrimental human health risks in developing countries like South Africa, where there is inadequate infrastructure, poor sanitation and a high number of immunocompromised individuals. Approximately 13.7% of South Africans live with HIV/ AIDS (Stats South Africa, 2022a). Hence, it is essential to revisit and upgrade the current national water standards to include ARGs and antibiotic residues in the water quality monitoring guidelines.

2.3.4. The presence of antibiotic residues in source water

Antibiotic use differs in various parts of the country (Matongo *et al.*, 2015a; Suzuki *et al.*, 2015). This is due to the incidence of bacterial infections in a particular area that dictates the prescription of antibiotics. This engenders a discharge of different antibiotic residues in the source water overall. However, a limited number of studies investigated the presence of antibiotic residues in the source water, as shown in Table 2.1. These studies were all based on quantifying antibiotic residues, as shown in Table A3.

According to the databases, sulfamethoxazole was the most prevalent antibiotic in source water used for drinking and food production (Agunbiade and Moodley, 2014; Matongo *et al.*, 2015a; Segura *et al.*, 2015; Suzuki *et al.*, 2015). The high prevalence of sulfamethoxazole is associated with its frequent application to treat bacterial and protozoan infections in HIV patients (Suzuki *et al.*, 2015). In a study by Suzuki *et al.* (2015), sulfamethoxazole was prevalent in treated wastewater effluent and surface water collected in Durban, Kwa-Zulu Natal. However, there was a reduction in the concentration of sulfamethoxazole from treated wastewater effluent ($3.61 \times 10^1 \mu\text{g/L}$) to urban surface waters ($2.56 \times 10^1 \mu\text{g/L}$) and rural surface

waters (4.82×10^{-2} µg/L). Similarly, Agunbiade and Moodley (2014) reported a median value of 3.68×10^1 µg/L for sulfamethoxazole in the Umgeni River water system, Kwa-Zulu Natal. Späth *et al.* (2021) reported sulfamethoxazole of 12×10^1 µg/L and 2.5×10^1 µg/L in the Newlands Mashu wastewater influent and effluent, respectively. However, the results obtained by Matongo *et al.* (2015b) show that sulfamethoxazole concentration was below the limit of quantification (LOQ) in most WWTPs (Kwa-Zulu Natal). Sulfamethazine and sulfapyridine were detected in the source water. (Matongo *et al.*, 2015a; Matongo *et al.*, 2015b; Segura *et al.*, 2015). The widespread application of sulphonamides is caused by their cost-effectiveness compared to other antibiotics (Segura *et al.*, 2015). However, Segura *et al.* (2015) reported the absence of sulfamethizole, sulfadimidine, sulfamerazine and sulfadimethoxine in source water collected in South Africa, Mozambique, Kenya and Ghana.

Kanama *et al.* (2018) observed two trends in North West WWTPs: (i) Ofloxacin and chloramphenicol were not detected in the wastewater influent and effluent. (ii) The concentrations of tetracycline, ciprofloxacin and Nalidixic acid decreased from wastewater influent to effluent. Similarly, Agunbiade and Moodley (2014) observed elevated concentrations of antibiotics in wastewater influents. Kanama *et al.* (2018) and Späth *et al.* (2021) detected elevated nalidixic acid and chloramphenicol concentrations in the Umgeni River water system.

Agunbiade and Moodley (2014) and Matongo *et al.* (2015b) reported erythromycin in at least one point of the Umgeni River and Msunduzi River water systems, Kwa-Zulu Natal. Furthermore, Segura *et al.* (2015) reported the presence of dehydroerythromycin in South Africa (Durban, Kwa-Zulu Natal), Ghana, Mozambique and Kenya. Matongo *et al.* (2015a), Matongo *et al.* (2015b), Segura *et al.* (2015) and Späth *et al.* (2021) quantified trimethoprim in the source water.

Other quantified antibiotic residues included ampicillin, clindamycin, levofloxacin, tylosin, tetracycline, metronidazole, clarithromycin, oxytetracycline, chlortetracycline and minocycline (Agunbiade and Moodley 2014; Matongo *et al.*, 2015a; Matongo *et al.*, 2015b; Segura *et al.*, 2015; Späth *et al.*, 2021). A study by Holton *et al.* (2022) reported the concentration of the daily average of antibiotic residues in the Western Cape. The WWTP influent daily average for macrolide, sulfonamide and β -lactams was 2.2, 14.7 and 1.0 g/day, respectively. The daily average of the WWTP effluent blended with downstream surface water for macrolide, sulfonamide and β -lactam concentrations was 0.89, 3.1 and 0.15 g/day, respectively.

Table 2.1: Summary of antibiotic residues and sampling sites.

Sampling sites	Antibiotic tested	References
North West		
WWTPs	ciprofloxacin, ofloxacin, nalidixic acid, tetracycline and chloramphenicol	Kanama <i>et al.</i> , 2018
Gauteng		
WWTPs	ciprofloxacin	Nyamukamba <i>et al.</i> , 2019
KwaZulu-Natal		
WWTPs	sulfamethoxazole, ciprofloxacin, levofloxacin, trimethoprim and clindamycin	Späth <i>et al.</i> , 2021
River	ciprofloxacin, ampicillin, nalidixic acid, sulfamethoxazole, streptomycin, tetracycline, erythromycin, chloramphenicol, and tylosin	Agunbiade and Moodley, 2014
WWTPs and surface water	sulfamethoxazole, erythromycin, metronidazole, sulfamethazine and trimethoprim	Matongo <i>et al.</i> , 2015a
WWTPs and surface water	erythromycin, sulfamethoxazole, sulfamethazine, metronidazole, and trimethoprim	Matongo <i>et al.</i> , 2015b
Surface water	macrolides, sulfonamides, tetracyclines, diaminopyrimidine and lincosamide	Segura <i>et al.</i> , 2015
WWTPs and surface water	sulfonamides and tetracyclines	Suzuki <i>et al.</i> , 2015
Western Cape		
WWTPs and surface water	quinolones, macrolides, lincosamide, sulfonamides, trimethoprim, aminoglycosides, beta-lactams, amphenicols and nitrofurans	Holton <i>et al.</i> , 2022

2.3.5. Antibiotic susceptibility in source waters

The interaction between antibiotic residues (in sub-inhibitory concentration) and bacteria in source water may induce antibiotic resistance, rendering antibiotics ineffective. Thus,

antibiotic-susceptibility tests are essential tools used in laboratories to help medical health care prescribers choose the most effective antibiotics for treating bacterial infections (Igwaran *et al.*, 2018). An array of antibiotics was subjected to susceptibility tests in the searched databases, as shown in Table A1 in Appendix A. Furthermore, this table shows that 41.9% of selected studies conducted phenotypic susceptibility tests.

Table A3 in Appendix A shows phenotypic resistance from various sampling sites. This is of great concern, since some of these resistant patterns occur in wastewater, source water and drinking water. According to the searched databases, as reflected in Table A3 in Appendix A, studies from Limpopo were based on antibiotic susceptibility tests in water. In the Western Cape, four studies conducted antibiotic susceptibility tests in WWTPs and/ or surface water (Lamprecht *et al.*, 2014; Havenga *et al.*, 2019; Kalule *et al.*, 2019; Snyman *et al.*, 2021). In Gauteng, antibiotic susceptibility tests were conducted in surface water, WWTPs and groundwater (environmental water) by Abia *et al.* (2015) and Bolukaoto *et al.* (2021).

Ampicillin (n=45 studies), ciprofloxacin (n=42 studies), chloramphenicol (n=38 studies), tetracycline (n=38 studies), trimethoprim (n=36 studies), gentamicin (n=29 studies), imipenem (n=27 studies), amikacin (n=25 studies), erythromycin (n=24 studies), meropenem (n=23 studies) and nalidixic acid (n=22 studies) were the antibiotics subjected most to susceptibility tests as shown in the references in Table A3 in Appendix A. Cefalexin, cefotetan, cefazolin, cefquinome, ceftazidime-clavulanic acid, cefuroxime-axetil, enrofloxacin, fusidic acid, lincomycin, moxifloxacin, novobiocin, quinupristin–dalfopristin, rifampin, teicoplanin and ticarcillin-clavulanate were the antibiotics least subjected to susceptibility tests, again as shown in Table A3 in Appendix A.

Resistance to the most commonly prescribed antibiotics has resulted in the requirement of new, complex and expensive antibiotic therapy and longer hospitalization to treat multidrug-resistant and extremely drug-resistant strains (Igwaran *et al.*, 2018; Havenga *et al.*, 2019). This poses a public health threat, because new antibiotics have not been produced since the introduction of imipenem in the early 1980s (Odjadjare *et al.*, 2012).

2.3.6. Genotypic resistance in aquatic environments

The present section focuses on molecular techniques used to detect and quantify ARGs. The results obtained from these methods enable scientists to study and understand the potential impact of ARGs on the environment and humans. Table 2.2 represents a summary of ARGs found and their sampling sites.

2.3.6.1. Genes encoding resistance to macrolides

In the searched databases, macrolide resistance genes were found to be prevalent in wastewater and surface water. However, Hamiwe *et al.* (2019) did not detect the *ermB* gene in surface water. The gene was present in *Enterococcus faecalis* and *E. faecium* isolated from the Gauteng WWTPs. This was inconsistent with the results obtained by Ekwanzala *et al.* (2020) and Mbanga *et al.* (2021b), who detected the *ermB* genes in surface water and wastewater collected in Gauteng. Other macrolide resistance genes include *ermC* (62.50%) and *msrA* (22.5%), which were recovered in methicillin-resistant *Staphylococcus aureus* (MRSA) in Durban wastewater and receiving water bodies (Ramessar and Olaniran 2019). The *ermC* gene confers macrolide resistance by modifying the antibiotic target site (Ramessar and Olaniran 2019). Furthermore, it confers resistance to macrolides and streptogramin B antibiotics. This is achieved by encoding for efflux mechanisms that pump these antibiotics out of the cell (Ramessar and Olaniran 2019).

The *msrC* and *msrD* genes were detected in WWTPs and surface water from Gauteng, North West and Kwa-Zulu Natal (Molale and Bezuidenhout 2016; Ekwanzala *et al.*, 2020; Mbanga *et al.*, 2021b). The *msrC* gene encodes for an efflux pump that confers low resistance to macrolides and type B streptogramins (Molale and Bezuidenhout, 2016; Ekwanzala *et al.*, 2020). Igwaran *et al.* (2018) detected the *ermA* gene (8.11%) in *E. coli* recovered from the treated wastewater effluents of the two Eastern Cape WWTPs, namely Fort Beaufort and Alice. Macrolide resistance may also occur by altering the antibiotic's target site at the V region of the 23S rRNA (at position 2074C or 2075G), as shown by Chukwu *et al.* (2019b). They studied *E. coli* isolated from stool specimens and drinking water.

2.3.6.2. Genes encoding resistance to tetracycline

Antibiotics belonging to the class of tetracycline inhibit bacterial protein synthesis by preventing aminoacyl-tRNA attachment to the ribosomal receptor (A) site (Chukwu *et al.*, 2019a). Tetracycline is a growth stimulator in livestock farming (Suzuki *et al.*, 2015). In human medicine, tetracycline treats patients with bacterial urinary tract infections (Kanama *et al.*, 2018). In agriculture, it treats some bacterial infections in pears and apples (Agunbiade and Moodley 2014).

Tetracycline resistance is most likely caused when it acquires new conjugate genes associated with plasmids or transposons (Chukwu *et al.*, 2019a). Adefisoye and Okoh (2017) detected several types of tetracycline resistance genes in *Vibrio* species isolated in an unnamed WWTP in the Eastern Cape. The *tetM* (47%) was the most prevalent tetracycline resistance gene, followed by *tetB* (23%), *tetA* (19%) and *tetK* (2%). In other studies, the *tetK* gene was isolated in MRSA and *L. monocytogenes* in KwaZulu-Natal, Eastern Cape wastewater and rivers (Ramessar and Olaniran, 2019; Kayode *et al.*, 2021). In contrast, Molale and Bezuidenhout (2016) and Fadare *et al.* (2020) reported the absence of *tetK* gene in surface water. The *tetK* gene encodes efflux proteins which prevent the build-up of tetracycline in the cell (Ramessar and Olaniran, 2019).

The *tetB* and *tetC* genes were detected in WWTPs and/ or surface water from the Eastern Cape (Fadare *et al.*, 2020; Iwu and Okoh 2020; Adewoyin *et al.*, 2021; Fadare and Okoh, 2021; Iwu *et al.*, 2021; Iwu *et al.*, 2022). The *tetA* gene was detected in Gauteng aquatic environments (Ekwanzala *et al.*, 2019), Eastern Cape irrigation water, rivers and WWTPs (Fadare *et al.*, 2020; Fadare and Okoh, Iwu *et al.*, 2021; 2021; Adewoyin *et al.*, 2021; Kayode *et al.*, 2021) and the North West DWPFs (Ateba *et al.*, 2020). The *tetA* gene was also present in agricultural soil from the Eastern Cape (Iwu and Okoh 2020).

The *tetM* gene was present in Gauteng (Hamiwe *et al.*, 2019; Ekwanzala *et al.*, 2020), Eastern Cape (Adewoyin *et al.*, 2021; Fadare and Okoh, 2021; Kayode *et al.*, 2021) and Kwa-Zulu Natal (Suzuki *et al.*, 2015; Mbanga *et al.*, 2021b). Furthermore, Suzuki *et al.* (2015) reported the quantity of the *tetM* gene to be approximately $10^3/16S$ in the total assemblage isolated in wastewater effluent and urban surface water. It is a ribosomal protection protein gene with a high genetic diversity and broad host range; hence it is widely distributed in the natural environment (Suzuki *et al.*, 2015).

Hamiwe *et al.* (2019) and Molale and Bezuidenhout (2016) and Mbanga *et al.* (2021b) detected *tetL* genes in wastewater and surface water. The *tetO* gene was not present in *Campylobacter* species isolated in the North West (Crocodile River) and *Acinetobacter baumannii* isolated in the Eastern Cape (Great Fish, Keiskamma and Tyhume Rivers) (Chukwu *et al.*, 2019a and Adewoyin *et al.*, 2021). However, the *tetO* gene was present in storage water for drinking purposes in North West (Chukwu *et al.*, 2019a). The *tetD* gene was only present in enterobacteria from the Eastern Cape WWTPs, irrigation water and river water (Fadare *et al.*, 2020; Iwu *et al.*, 2020; Fadare and Okoh, 2021). More *tet* genes are reported by Kayode *et al.* (2021) and Mbanga *et al.* (2021b).

2.3.6.3. Genes encoding resistance to aminoglycoside

Aminoglycosides are used to treat MRSA infections due to their anti-staphylococcal activity and inhibition of protein synthesis by binding to the 30S ribosomal unit (Ramessar and Olaniran 2019). Most aminoglycoside resistance genes were present in WWTPs (Adefisoye and Okoh, 2017; Hamiwe *et al.*, 2019; Ramessar and Olaniran, 2019; Ekwanzala *et al.*, 2020). Ramessar and Olaniran (2019) reported the migration of *acc(6')-Ie-aph(2'')-Ia* genes isolated in MRSA from the WWTP into receiving water bodies in Durban, KwaZulu-Natal. The *aac(6')-aph(2'')*, *ant(6)-Ia*, *aac(60)-IId* and *aph(3')-III* genes were present in vancomycin-resistant *Enterococcus* species isolated in wastewater and surface water collected in Gauteng and KwaZulu-Natal (Ekwanzala *et al.*, 2020; Mbanga *et al.*, 2021b). Iwu and Okoh (2020) detected the *aac(3)-IIa* (38%) genes in *L. monocytogenes* isolated in surface water and groundwater used for irrigation. Gxalo *et al.* (2021) reported the presence of *aph(3)-Ia* (*aphA1*) a (26) and the absence of *aac(3)-IIa* (*aacC2*) a and *aph(3)-IIa* (*aphA2*) genes in the Eastern Cape wastewater samples. This contrasted with the results obtained by Kayode *et al.* (2021), who reported the presence of *aph(3)-IIa* (*aphA2*) gene in irrigation and wastewater.

The *aadA* gene was present in the North West DWPFs (Ateba *et al.*, 2020), agricultural soil and river water in the Eastern Cape (Fadare *et al.*, 2020; Iwu and Okoh 2020; Gxalo *et al.*, 2021; Kayode *et al.*, 2021). Furthermore, Fadare *et al.* (2020) and Iwu and Okoh (2020) reported the absence of *strA* and *aadA* genes in surface water, respectively. In contrast, the *strA* and *strB* were present in the two North West DWPFs and Eastern Cape WWTP and environmental freshwater (Okoh and Igbinosa, 2010; Ateba *et al.*, 2020; Gxalo *et al.*, 2021; Kayode *et al.*, 2021).

2.3.6.4. Genes encoding resistance to vancomycin

Vancomycin is an antibiotic of choice for the treatment of nosocomial infections caused by Gram-positive bacteria that are non-responsive to first-line antibiotics (Hamiwe *et al.*, 2019). The most commonly studied vancomycin resistance genes are *vanA* and *vanB* (Ekwanzala *et al.*, 2020). These ARGs were present in *Enterococcus* species (34%) isolated from Mafikeng borehole water systems used for drinking and other domestic purposes in the North West (Ateba *et al.*, 2013). Similar results were obtained by Matlou *et al.* (2019), where *vanA* (16%) and *vanB* (4%) genes were present in surface water and groundwater in different cities in North West. The *vanA* and other vancomycin resistance genes, such as *vanC*, *vanG*, *vanN* and *vanL* were found to be present by Ekwanzala *et al.* (2020) in Gauteng. In contrast with

these studies, Hamiwe *et al.* (2019) reported the absence *vanA* and *vanB* genes and the detection of *vanC* gene in *Enterococcus* recovered from the Gauteng WWTPs. *Enterococcus*-resistant isolates can share their ARGs with other microorganisms via MGE, such as plasmids and transposons (Matlou *et al.*, 2019). The emergence of vancomycin-resistant *Enterococcus* is of great concern: this has also been documented in countries with advanced public health care facilities (Ateba *et al.*, 2013).

2.3.6.5. Genes encoding resistance to β -lactams

β -lactams are the antibiotics of choice for treating infectious bacteria, due to their low toxicity and effectiveness (Iwu and Okoh 2020). The β -lactamase-producing bacteria confer resistance to antibiotics belonging to the β -lactam class through hydrolysis of the β -lactam ring (Abia *et al.*, 2015; Adefisoye and Okoh, 2017). The *ampC* genes were present in heterotrophic bacteria in the Mooi, Marico and Crocodile Rivers situated in North West (Coertze and Bezuidenhout, 2017) and isolates (*Vibrio* species and *E. coli* O157:H7) recovered from the Eastern Cape WWTPs and irrigation water samples (Gxalo *et al.*, 2021; Iwu *et al.*, 2022). Furthermore, Coertze and Bezuidenhout (2017) quantified the plasmid *ampC* genes and the results were as follows: The highest copy number of *ampC* genes was quantified in the Crocodile River with 13.11copies/pg DNA, followed by Marico River with 3.46 copies/pg DNA and Mooi River with 2.19 copies/pg DNA.

The *ampC* (63%), as well as other β -lactam resistance genes such as *bla_{TEM}* (100%), *bla_{VEB}* (13%) and *bla_{CTX-M}* (38%) were detected in *L. monocytogenes* isolated from irrigation water in Amathole and Chris Hani District Municipalities situated in Eastern Cape (Iwu and Okoh 2020). The *bla_{CTX-M}* gene was present in the DWPFs, WWTPs, freshwater and irrigation water (Ateba *et al.*, 2020; Raseala *et al.*, 2020; Gumede *et al.*, 2021). The *bla_{TEM}* and *bla_z* genes were detected in the Eastern Cape WWTPs, rivers and irrigation water (Fadare and Okoh, 2021; Gxalo *et al.*, 2021; Kayode *et al.*, 2021) and Gauteng freshwater and irrigation water (Raseala *et al.* 2020). In contrast, Igbinosa *et al.* (2014) did not detect β -lactam resistance genes (*bla_{Vin-1}*, *bla_{Vin-2}* and *bla_{Imp}*) in surface water collected in the Eastern Cape. However, *bla_{Vin-2}* genes were detected in the plant rhizosphere. This highlights the possible ARG spread from the environment into the food chain (Igwaran *et al.*, 2018). Other carbapenemase resistance genes were reported by Gxalo *et al.* (2021) and Ramsamy *et al.* (2022)

Chukwu *et al.* (2019b) observed a general trend where a high number of extended-spectrum β -lactamases (*bla_{CTX-M}*, *bla_{SHV}*, *bla_{CMY}*) occurred, except for *bla_{DHA}* isolated in *E. coli* from storage water (used for domestic purposes) as compared to human stool samples. The

human stools consisted of 350 samples, of which 155 were collected from diarrhoeic and non-diarrhoeic children under the age of five years attending the Brits District Hospital, Oukasie, Lethabeleng and Bopang Clinics (Chukwu *et al.*, 2019b). In this case study, extended-spectrum β -lactamase genes overlapped in water and stool samples, suggesting a spread between the water and human consumers (Chukwu *et al.*, 2019b).

Diarrhoea is the second leading cause of death (16%) in South African children under the age of five years (Kalule *et al.*, 2019). This is a significant concern that domestic water becomes a vehicle for transferring these dangerous pathogens into the fragile section of the human population (that is, children under the age of five) and causing increased mortality. Iwu *et al.*, 2020, Fadare *et al.* (2020), Raseala *et al.* (2020), Gumede *et al.* (2021) and Fadare and Okoh (2021) also detected *blaSHV* gene in wastewater and irrigation water. Ekwanzala *et al.* (2019), Iwu *et al.*, 2020, Adewoyin *et al.* (2021), Iwu *et al.* (2021), Fadare and Okoh (2021) and Iwu *et al.* (2022) reported the presence of a wide range of β -lactam resistance genes.

2.3.6.6. Genes encoding resistance to sulphonamide

Sulphonamide is an essential therapeutic agent in human and veterinary medicine. It is a growth stimulator for livestock (Suzuki *et al.*, 2015). The sulphonamide resistance genes *sul1* and *sul2* were present in river water, irrigation water, wastewater and agricultural soil sampled in Eastern Cape (Adefisoye and Okoh, 2017; Fadare *et al.*, 2020; Iwu *et al.*, 2020; Adewoyin *et al.*, 2021; Fadare and Okoh, 2021; Kayode *et al.*, 2021). These findings were similar to the results of Ekwanzala *et al.* (2019) and Raseale *et al.* (2020) in Gauteng. The real-time PCR results of Suzuki *et al.* (2015) showed that the *sulI* and *sulII* genes were most prevalent in the WWTPs, rivers and estuaries, ranging between 10^{-2} and $10^{-1}/16S$ rRNA copies. A study conducted by Okoh and Igbiosa (2010) detected *sulII* genes in *Vibrio* species isolated in the Eastern Cape WWTPs. Furthermore, Suzuki *et al.* (2015) reported that the *sulIII* genes were either not detected or present in relatively low concentrations (10^{-5} to $10^{-4}/16S$ copy numbers) in surface water and wastewater collected from eThekweni Metropolitan Municipality situated in KwaZulu-Natal. Resistance to sulphonamide is primarily conferred by mutation of the dihydropteroate synthase gene (Suzuki *et al.*, 2015).

2.3.6.7. Genes encoding resistance to other classes of antibiotics

Colistin is a last-resort antibiotic used to treat multi-resistant Gram-negative bacteria in humans (Igwaran *et al.*, 2018). In a study conducted by Havenga *et al.* (2019) in the Western

Cape, the colistin resistance genes (*mcr-1* to *mcr-4*) were not detected from what is called the World Health Organization Priority 1 of “critical group organisms” consisting of *Acinetobacter baumannii*, *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in environmental samples.

Similar results were obtained by Govender *et al.* (2021), who reported the absence of the *mcr-1* gene from *Aeromonas* and *Pseudomonas* species isolated in KwaZulu-Natal environmental water (wastewater and surface water). However, the *mcr-1* genes were present in *E. coli* recovered in clinical settings (Havenga *et al.*, 2019). Similarly, Igwaran *et al.* (2018) detected *mcr-1* resistance gene in *E. coli* isolated in treated wastewater effluents in Eastern Cape.

The emergence and development of multi-resistant bacterial strains have left medical health care prescribers with no choice but to use last-resort drugs (Igwaran *et al.*, 2018). Hence, the presence of *mcr-1* genes established by the study by Igwaran *et al.* (2018) was associated with the frequent application of colistin in communities served by these WWTPs. Mbanga *et al.* (2021a) reported the presence of *mcr-10* gene in KwaZulu-Natal wastewater effluent and its downstream point. However, the gene was absent at the upstream point. Similar results were obtained by Snyman *et al.*, 2021, who reported the presence of the *mcr-3* and *mcr-5.1* genes in the environmental DNA from the Western Cape rivers (Plankenburg River, Eerste River and Berg River). However, the *mcr-3* and *mcr-5.1* genes were absent in the upstream point.

Dihydrofolate reductase encodes trimethoprim resistance (Ekwanzala *et al.*, 2020). The trimethoprim resistance genes (*dfr18* and *dfrA1*) were detected in the Eastern Cape WWTPs by Okoh and Igbinosa (2010) and Igbinosa *et al.* (2011). The *dfr18* and *dfrA1* gene cassettes conferred resistance to trimethoprim (Okoh and Igbinosa, 2010). The *dfrA14* (58.82), *dfrA15* (47.06%), *dfrA30* (11.76%) and *dfrA27* (5.88%) genes were present in carbapenem-resistant *Klebsiella pneumoniae* isolated from Gauteng aquatic environments (Ekwanzala *et al.*, 2019). Ekwanzala *et al.* (2020) and Mbanga *et al.* (2021b) reported the presence of the *dfrG* gene in the WWTPs and downstream sites in two successive years. The *dfrB1*, *dfrB2* genes were present in the North West DWPFs (Ateba *et al.*, 2020).

Antibiotics belonging to the class fluoroquinolone inhibit bacterial DNA synthesis. These antibiotics target two large bacterial enzymes: the DNA gyrase and topoisomerase IV (Chukwu *et al.*, 2019a). In the searched databases, the fluoroquinolone resistance genes were mostly studied in environmental water by Ekwanzala *et al.* (2019) and Adewoyin *et al.* (2021). Ekwanzala *et al.* (2019) reported the presence of *acc(6')-Ib-cr* (94,12%), *qnrS1* (64,01%),

qnrB1 (47,06%) and *qnrB6* (5,88%) in carbapenem-resistant *Klebsiella pneumoniae* isolated from Gauteng aquatic environments. Adewoyin *et al.* (2021) reported the presence of *qnrB* (14.6%) and *qnrD* (16.7%) in *A. baumannii* recovered from the Eastern Cape rivers. The absence of *qnrA*, *qnrC* and *qnrS* was also reported in *A. baumannii* recovered from the Eastern Cape rivers. The presence of *gryA* and *qnrVC* genes was reported in storage water and wastewater samples, respectively (Chukwu *et al.*, 2019a; Gxalo *et al.*, 2021).

Phenicol-resistance genes such as *catI*, *catII*, *floR* and *cat(pC194)* were present in environmental water and WWTPs in Gauteng and KwaZulu-Natal (Okoh and Igbinosa, 2010; Ekwanzala *et al.*, 2020; Iwu *et al.*, 2020; Fadare *et al.*, 2020; Fadare and Okoh, 2021). However, Gxalo *et al.*, 2021 detected the presence of the *catI* gene and the absence of the *catII* gene *Vibrio* species recovered in wastewater. In contrast, Iwu *et al.* (2021) and Iwu *et al.* (2022) detected the absence and presence of *catI* and *catII*, respectively. The presence and absence of *cmIA1* gene were also reported by Gxalo *et al.* (2021) and Iwu *et al.*, 2022, respectively. Acetyltransferase encodes resistance to phenicol.

2.3.6.8. SXT–integrative and conjugative elements

The SXT is an integrative and conjugative element contributing to HGT and the rearrangement of resistance genes in *V. cholera* (Igbinosa *et al.*, 2011). SXT-ICE are self-transmissible elements. They induce resistance to chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim (Okoh and Igbinosa, 2010; Igbinosa *et al.*, 2011)

2.3.6.9. Integron genes

The mobile genetic integrons are responsible for determining specific-recombination systems that render many antibiotics unresponsive, thus leading to multidrug resistance (Igbinosa *et al.*, 2011). Studies by Igbinosa *et al.* (2011) in WWTPs and Igbinosa *et al.* (2013) in the Tyume and Kat Rivers have shown that approximately 20% of *Aeromonas* species harbour the Class 1 integron gene. Furthermore, these two studies did not detect Class 2 integron genes. The presence of Class 1 integron genes in source water is worrisome, since they may enhance the spread of ARGs to indigenous bacteria (Igbinosa *et al.*, 2011). The Class 1 integron gene was also present in heterotrophic bacteria isolated in the North West rivers (Nyamukamba *et al.*, 2019). Bacterial isolates with the Class 1 integrase gene can acquire and express gene cassettes that facilitate the spread of antibiotic resistance through gene cassettes and other MGE such as plasmids (Igbinosa *et al.*, 2011; Igbinosa *et al.*, 2013; Coertze and

Bezuidenhout, 2017). The presence of a Class 1 integrase gene in water indicates anthropogenic pollution (Coertze and Bezuidenhout, 2017).

Table 2.2: Summary of ARGs and their sampling sites.

Sampling site	Associated bacteria	ARGs tested (detection or quantification)	References
North West			
Borehole	<i>Enterococci</i> species	<i>vanA</i> and <i>vanB</i> (detection)	Ateba <i>et al.</i> , 2013
Stool specimens and drinking water	<i>Campylobacter</i> species	<i>tetO</i> (detection)	Chukwu <i>et al.</i> 2019a
Stool specimens and drinking water	<i>E. coli</i>	<i>blaCTX-M</i> , <i>blaSHV</i> , <i>blaCMY</i> , and <i>blaDHA</i> (detection)	Chukwu <i>et al.</i> 2019b
River	heterotrophic bacteria	<i>ampC</i> and <i>int1</i> (quantification)	Coertze and Bezuidenhout, 2017
surface and ground water	vancomycin-resistant enterococci	<i>vanA</i> , <i>vanB</i> and <i>vanC</i> (detection)	Matlou <i>et al.</i> 2019
Surface water	<i>Enterococcus</i> species	<i>tetK</i> and <i>tetL</i> (detection)	Molale and Bezuidenhout, 2016
Raw and drinking water	Heterotrophic bacteria	<i>erm(B)</i> , <i>sul1</i> , <i>tet(A)</i> , <i>tet(W)</i> , <i>tet(X)</i> , <i>mec(A)</i> , <i>ampC</i> , <i>strA</i> , <i>strB</i> , <i>aadA</i> , <i>cmIA</i> , <i>vanA</i> , <i>dfrB1</i> , <i>dfrB2</i> and <i>blaCTX-M</i> (Detection)	Ateba <i>et al.</i> , 2020
Gauteng			
Freshwater and irrigation	<i>Salmonella</i> Serovars	<i>blaCTX</i> , <i>blaSHV</i> , <i>blaTEM</i> , <i>blaOXA</i> , and <i>sul1</i> (Detection)	Raseala <i>et al.</i> , 2020
WWTPs and receiving water bodies	carbapenem-resistant <i>Klebsiella pneumoniae</i>	β -lactams, quinolone, fosfomycin, Aminoglycoside, fluoroquinolones, sulphonamides, tetracycline and trimethoprim resistance genes (detection)	Ekwanzala <i>et al.</i> , 2019
WWTPs and receiving water bodies	<i>Enterococcus</i> species and vancomycin-resistant <i>Enterococcus</i> species	Macrolide, lincosamide, streptogramin, vancomycin, tetracycline, aminoglycosides, β -lactams and quinolone resistance genes (detection)	Ekwanzala <i>et al.</i> , 2020
WWTPs and receiving water bodies	enterococci	macrolides, tetracycline, vancomycin and aminoglycosides resistance genes (Detection)	Hamiwe <i>et al.</i> , 2019
Eastern Cape			
Wastewater, river and irrigation water	<i>Listeria monocytogenes</i>	<i>trA</i> , <i>aadA</i> , <i>aac (3)-IIa (aacC2)a</i> , <i>aph (3)-</i>	Kayode <i>et al.</i> , 2021

		<i>la(aphA1)a</i> , <i>aph (3)-IIa (aphA2)a</i> , <i>tetA</i> , <i>tetB</i> , <i>tetC</i> , <i>tetD</i> , <i>tetE</i> , <i>tetG</i> , <i>tetK</i> , <i>tetL</i> , <i>tetM</i> , <i>sull</i> and <i>sullI</i> (Detection)	
Irrigation water and agricultural soil	<i>E. coli</i> O157:H7	β -lactamases and carbapenemases (Detection)	Iwu <i>et al.</i> , 2021
Irrigation water and agricultural soil	<i>E. coli</i> O157:H7	β -lactamase and carbapenemases (Detection)	Iwu <i>et al.</i> , 2022
Irrigation water and agricultural soil	Enterobacteria	tetracycline, β -lactam, sulfonamide, phenicol, and aminoglycoside resistance genes (Detection)	Iwu <i>et al.</i> , 2020
WWTPs	<i>V. mimicus</i> , <i>V. vulnificus</i> and <i>V. fluvialis</i>	<i>aacC2</i> , <i>aadA</i> , <i>aphAI</i> , <i>strA</i> , <i>aphAII</i> , <i>catI</i> , <i>catII</i> and <i>cmlA1</i> , <i>blaZ</i> , <i>ampC</i> , <i>blaTEM</i> , <i>blaGES</i> , <i>blaOXA-48</i> , <i>blaKPC</i> , <i>qnrVC</i> , <i>blaIMP</i> and <i>blaVIM</i> (Detection)	Gxalo <i>et al.</i> , 2021
River water	Enterobacteriaceae	B-lactam, carbapenem, cephem, tetracycline, sulphomide, aminoglycoside and phenol resistance genes (Detection)	Fadare <i>et al.</i> , 2020
WWTPs	<i>E. coli</i> and <i>Vibrio</i> species	<i>blaTEM</i> , <i>blaSHV</i> , <i>blaZ</i> , <i>blaCTX-M</i> , <i>aadA</i> , <i>strA</i> , <i>tetA</i> , <i>tetB</i> , <i>tetK</i> and <i>tetM</i> (detection)	Adefisoye and Okoh, 2017
WWTPs	<i>Aeromonas</i> species	<i>pse1</i> , <i>blaTEM</i> , and <i>TetC</i> , <i>Int1</i> and <i>Int2</i> (detection)	Igbinosa and Okoh, 2012
WWTPs	<i>Vibrio</i> species	<i>sul2</i> , <i>floR</i> , <i>dfr18</i> , <i>tet(A)</i> , <i>strB</i> , <i>dfrA1</i> , <i>blaP1</i> and SXT integrase (detection)	Igbinosa <i>et al.</i> , 2011
Rivers	<i>Aeromonas</i> species	<i>Int1</i> and <i>Int2</i> (Detection)	Igbinosa <i>et al.</i> , 2013
River water, WWTPs, soil, plant roots and rhizosphere	<i>Pseudomonas</i> species	<i>Bla_{vim-1}</i> , <i>bla_{vim-2}</i> and <i>bla_{imp}</i> (detection)	Igbinosa <i>et al.</i> , 2014
WWTPs	<i>E. coli</i>	<i>ermA</i> and <i>Mcr-1</i> (detection)	Igwaran <i>et al.</i> , 2018
Irrigation water and agrarian soil samples	<i>Listeria monocytogenes</i>	<i>AmpC</i> , <i>blaGES</i> , <i>blaOXA-48</i> , <i>blaVIM</i> , <i>blaIMP</i> and <i>blaKPC</i> (detection)	Iwu and Okoh, 2020
WWTPs	<i>Vibrio</i> species	<i>sul2</i> , <i>floR</i> , <i>dfr18</i> , <i>TetA</i> , <i>strB</i> and <i>dfrA1</i> and SXT (Detection)	Okoh and Igbinosa, 2010

River water	<i>Acinetobacter baumannii</i>	Aminoglycoside, β -lactam, sulfonamides, fluoroquinolone and tetracycline resistance genes (Detection)	Adewoyin <i>et al.</i> , 2021
WWTPs	Enterobacteriaceae	β -lactamase, carbapenemase, pAmpCs, phenols, Sulphonamide and tetracycline resistance genes (Detection)	Fadare and Okoh, 2021
Kwazulu-Natal			
WWTPs and river water	<i>Enterobacter</i> species	<i>BlaACT-4</i> , <i>blaACT-4 fosA</i> and <i>mrc-10</i> (detection)	Mbanga <i>et al.</i> , 2021a
Wastewater and river water	<i>Enterococcus</i> species	<i>Isa(A)</i> , <i>msr(C)</i> , <i>msr(D)</i> , <i>erm(B)</i> , <i>mef(A)</i> , <i>tet(S)</i> , <i>tet(M)</i> , and <i>tet(L)</i> , <i>aac(60)-aph(200)</i> , <i>ant(6)-Ia</i> , <i>aph(30)-III</i> , <i>aac(60)-Iid</i> , <i>aac(60)-Iih</i> and <i>dfrG</i>	Mbanga <i>et al.</i> , 2021b
WWTPs, surface water, human, abattoir samples	Enterobacterales	Carbapenem, aminoglycoside, quinolones, chloramphenicol, tetracycline, and trimethoprim resistance genes (Detection)	Ramsamy <i>et al.</i> , 2022
WWTPs	<i>E. coli</i>	<i>Bla_{TEM}</i> , <i>Bla_{SHV}</i> and <i>Bla_{CTX}</i> (detection)	Gumede <i>et al.</i> , 2021
WWTP and receiving surface water	methicillin-resistant <i>Staphylococcus aureus</i>	<i>aac(6')/aph(2'')</i> , <i>blaZ</i> , <i>ermC</i> , <i>msrA</i> and <i>tetK</i> (detection)	Ramessar and Olaniran, 2019
Surface water and WWTP	total assemblage and cultural bacteria	<i>Sul1</i> , <i>sul2</i> , <i>sul3</i> and <i>tetM</i> (quantification)	Suzuki <i>et al.</i> , 2015
Western Cape			
WWTPs, rivers, surface run-off water, sediment and soil	<i>A. baumannii</i> , <i>P. aeruginosa</i> , <i>E. coli</i> and <i>K. pneumoniae</i>	<i>mcr-1</i> , <i>mcr-2</i> , <i>mcr-3</i> and <i>mcr-4</i> (detection)	Havenga <i>et al.</i> , 2019
River and storm water	<i>Aeromonas Jandei</i> , <i>Aeromonas veronii</i> and environmental DNA	<i>mcr-3</i> and <i>mcr-5</i> (detection)	Snyman <i>et al.</i> , 2021

CHAPTER 3

3. MATERIALS AND METHODS

3.1. STUDY AREA

3.1.1. NW-C wastewater treatment plant and river water

The NW-C WWTP services a population of 56 702 (14 562 households). It has a treatment capacity of 3 Ml/day, at an average of 2.3 Ml/day, and a peak flow of 3 Ml/day and 80% domestic influent (Naidoo *et al.*, 2016). This WWTP does not use any advanced treatment process. Screening, drying bed, biofilters, clarification and chlorination treat the wastewater influent. The NW-C WWTP is ranked third among WWTPs in the North West in critical and high-risk spaces by the Department of water Affairs (DWA) (2016). The NW-C Dam has a full supply capacity of $5.67 \times 10^6 \text{ M}^3$ and the dam water is at $1.82 \times 10^6 \text{ M}^3$ (DWA, 2019). According to the DWA (2019) report, the NW-C Dam was 90% full on 04 June 2019. The dam water is used for the production of drinking water (via an open-air canal), irrigation and agriculture (Maguvu *et al.*, 2020). The NW-C agricultural and industrial groundwater use is 54 299 239 M^3/a and 303 984 M^3/a , respectively (Smith-Adao *et al.*, 2016).

3.1.2. NW-E wastewater treatment plant and river water

The NW-E WWTP services a population of 162 762 (52 537 households). It has a treatment capacity of 97 Ml/day, at an average of 45 Ml/day, and a peak flow of 55 Ml/day and 80% domestic influent (Naidoo *et al.*, 2016). This plant is partitioned into an old unit with a treatment capacity of 23 Ml/day and a new one with a treatment capacity of 22 Ml/day. This process uses advanced treatment processes such as biofilters and activated sludge (Makuwa *et al.*, 2020). The wastewater effluent is disinfected by chlorination before being discharged into the downstream NW-E River. The NW-E River is downstream of the NW-E dam.

The NW-E dam has a full supply capacity of $2.03 \times 10^6 \text{ M}^3$ and the water is at $3.09 \times 10^5 \text{ M}^3$ (DWA, 2019). According to the DWA (2019) report, the dam was 5.4% full on 4 June 2019. It has a catchment area of 3 632 km^2 , a capacity of 2 Ml and a surface area of 77.3 ha (Barnard *et al.*, 2013). Activities that may affect the water quality of the NW-E dam and NW-E River (M) may include mining (diamond and peat), agriculture (lawn company), irrigation, wastewater effluent discharge, and industrial, urban and stormwater runoff (Barnard *et al.*, 2013; Maguvu

et al., 2020; Koekemoer *et al.*, 2021). This dam (as well as the NW-E river) forms part of the NW-E catchment (Van Der Walt *et al.*, 2002).

3.1.3. NW-C drinking water production facility

The NW-C DWPF depends on canal water from the NW-C river to produce drinking water. This DWTP plant has recently undergone a R52 million water upgrade to provide safe and clean drinking water to 28 720 people in four townships (South African Government, 2015; South African Government News Agency, 2015). Furthermore, 80.6% of the population (56 702) of NW-C residents have access to flushed toilets (Naidoo *et al.*, 2016). This upgrade has increased the water treatment capacity from 7 to 14 Ml/day. The upgrade also included the construction of a 5.7 Ml reservoir (South African Government, 2015; South African Government News Agency, 2015; DWS, 2022b). The raw water is treated by basic processes such as physical separation, sand filtration and chlorination, as shown in Figure 3.1.

3.1.4. NW-E drinking water production facility

The NW-E DWPF depends on the NW-E and NW-D dams for raw water. These dams form part of the four dams of the NW-E Catchment. The description of the NW-D dam is given in studies by Barnard *et al.* (2013) and Koekemoer *et al.* (2021). NW-D dam (32 km away from the NW-E DWPF) has a catchment area of 3287 km², a capacity of 2 Ml and a surface area of 373 ha. Irrigation, agriculture, diamond digging, recreation and pollutants from an ammunition manufacturing company affect the water quality. The NW-D dam has a full supply capacity of 21.03×10⁶ M³; the water is at 13.29×10⁶ M³ (DWA, 2019). According to the DWA (2019) report, the NW-D dam was 64.6% full on 04 June 2019.

The NW-E DWPF services a population of 162 762, of which 80.6% and 56.9% have access to flushed toilets and piped water, respectively (Naidoo *et al.*, 2016). This NW-E DWPF has a total design capacity of 736 Ml/day (DWS, 2022b). The raw water is treated by coagulation-flocculation, sedimentation, sand filtration, activated carbon and chlorination, as shown in Figure 3.2.

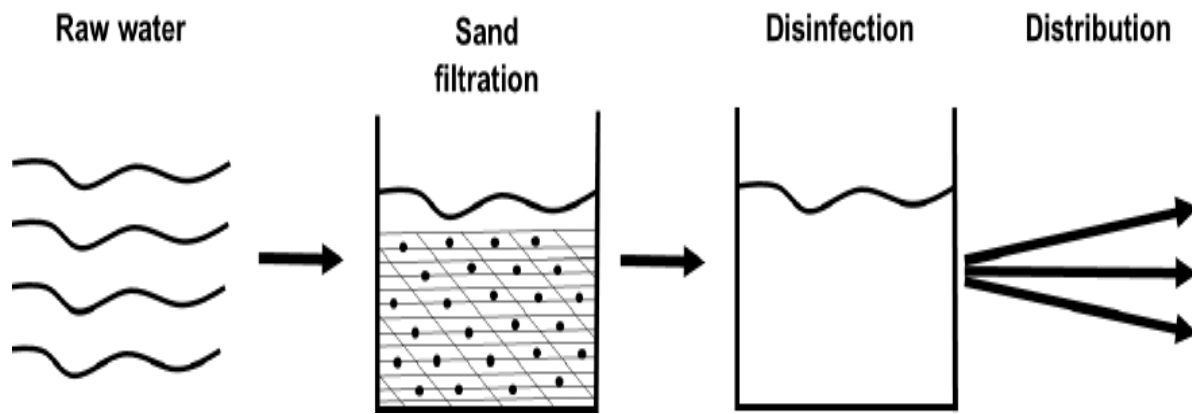


Figure 3.1: Schematic representation of the treatment processes taking place at NW-C DWPF (Kritzinger, 2019).

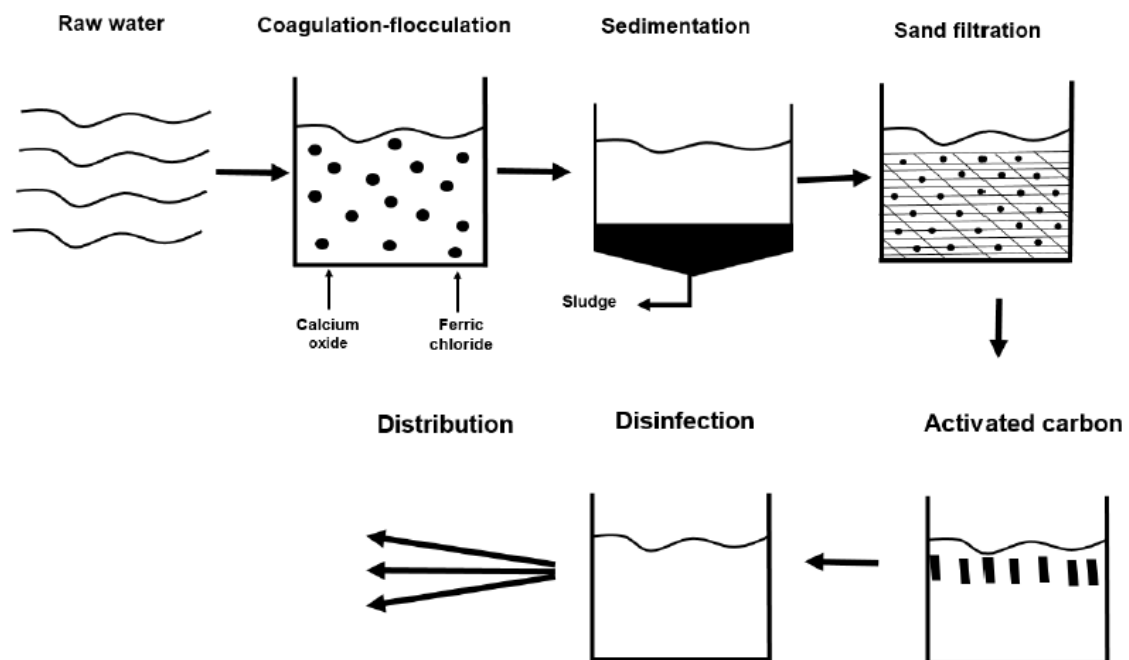


Figure 3.2: Schematic representation of the treatment processes taking place at NW-E DWPF (Plaatjie, 2019).

3.2. SAMPLING

Samples were collected in the following two conditions in NW-C and NW-E DWPFs:

- Raw water (one raw inlet for NW-C and two raw inlets for NW-E)
- Treated water

Wastewater effluent and river water were collected at the following points in the NW-C and NW-E:

- Upstream river
- Wastewater effluent
- Downstream point (after maturation pond for NW-C and river for NW-E)

3.2.1. Sampling for bacterial community composition and antibiotic resistance genes

Grab samples were aseptically collected in sterile 1 L bottles (Duran Schott, Germany). Most samples, except treated water, were collected in 1 L bottles. However, as Ma *et al.* (2017) showed, it was advisable to collect 5 L treated water due to the complexities of sampling. Water samples were collected in the austral summer of 2020 (October, November and December), 2021 (February, April, July, August, September, October and December) and 2022 (February and March). However, NW-E downstream river samples were only collected from September 2021 to March 2022. Samples were transported in ice to the North-West University laboratories, where they were refrigerated at 4°C until analysis commenced within 24 hours. This study followed the sampling guide of the Department of Water Affairs and Forestry (DWAF) (2000).

3.2.2. Sampling for the quantification of antibiotic and fluconazole residues

Water samples were collected in glass acid-washed 1 L bottles that were free from chemical contaminants (Duran Schott, Germany). Water samples were collected in 2020 (October, November and December), 2021 (February, July and August) and 2022 (February and March). They were transported in ice to the North-West University laboratories and refrigerated at 4°C for 24 hours until solid-phase extraction commenced.

3.3. PHYSICOCHEMICAL PARAMETERS

In situ, a portable multimeter (PCSTestr 35, Eutech Instruments Pte Ltd. Singapore) was used to measure temperature, salinity, TDSs and pH. Upon arrival at the North-West University Laboratory, the levels of nitrate (method 8039), nitrite (method 8153), COD (method 8000) and phosphorous (method 8178) were measured by using the Hach Lange DR 2800 spectrophotometer as stated on its manual protocol (Hach Company, United States). In DWPFs, treated water was compared in terms of DWAF South African water quality guidelines (DWAF SAWQG; 1996a) for domestic use. Wastewater effluents and river water was compared to the DWAF SAWQG (1996b) for agricultural use, namely irrigation, and DWAF SAWQG (1996c) for agricultural use: livestock watering.

3.4. DNA ISOLATION AND EXTRACTION

The membrane filtration technique was used for filter samples. The in-house filtration system was sterilized with 70% ethanol and rinsed with sterile distilled water. The sterile filter membrane (PALL Life Sciences, Mexico) with 0.45 µm porous size was aseptically placed on a filtration system for water filtration. One litre of environmental water samples (from raw water inlets, rivers and wastewater effluents) were filtered. However, due to the complexity of isolating environmental DNA in treated water (Ma *et al.*, 2017), up to 5 L of treated drinking water samples were filtered, as indicated. The membrane filters were stored at -20°C overnight. The bacterial DNA was extracted by using the Powerwater kit (Qiagen, Netherlands) in accordance with the manufacturer's instructions. The NanoDrop-1000 spectrophotometer (Nanodrop Technologies, United States) was used to determine the DNA concentration (ng/µL) of the successfully extracted DNA. Where applicable, nuclease-free water (Fermentas Life Sciences, United States) was used to dilute the DNA concentration to obtain a 20 ng/µL for downstream analyses.

3.5. LIBRARY PREPARATION OF NEXT-GENERATION SEQUENCING FOR 16S rRNA AMPLICON LIBRARIES AND SEQUENCING

Library preparation of next-generation sequencing was done in accordance with Coertze and Bezuidenhout (2021) and Magome (2021). Two samples from each sampling site were subjected to Illumina MiSeq 16S rRNA gene sequencing by using the Illumina MiSeq platform (Illumina, United States). The Illumina 16S metagenomic sequencing library preparation guide

(Illumina, United States) was used to prepare the library of the gene library of the 16S rRNA. The locus-specific primers' names of the forward primer (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-3') and the reverse primer (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACAG-3') were linked to Illumina forward and a reverse adapter overhang nucleotides sequence. The full-length primer sequences for 16S Amplicon PCR forward primer was 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3' and reverse primer 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C--3'. These gene-specific sequences were used to amplify the bacterial 16S rRNA by targeting the V3-V4 region. The first stage PCR contained a 25 µL reaction mixture consisting of 2.5 µL of template DNA (5 ng/µL), 5 µL of each primer (reverse and forward, 1 µM), and 12.5 µL of 2×KAPA HiFi HotStart ReadyMix. The thermal cycles of the first stage PCR were as follows: initial denaturation at 95°C for three minutes, 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 30 seconds and final extension at 72°C for five minutes.

The first PCR clean-up was performed to purify the V3-V4 region by using Agencourt AMPure XP beads (Beckman Coulter Genomics, United States). The purified amplicons were subjected to an index PCR to attach to the dual indices and Illumina sequencing adapters by using the Nextera XT Index Kit (Nextera, United States) in accordance with the manufacturer's instructions. The index PCR contained a 50 µL reaction mixture consisting of 5 µL of DNA, 5 µL of each XT index primer (N7xx and S5xx), 25 µL of 2×KAPA HiFi HotStart ReadyMix, and 10 µL of PCR grade water. The thermal cycles of the index PCR were as follows: initial denaturation at 95°C for three minutes, eight cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 30 seconds and final extension at 72°C for five minutes. The amplicons of the index PCR were subjected to a second PCR clean-up process, again by using AMPure XP beads. The partial 16S rRNA libraries were quantified by using a Qubit fluorometer (ThermoFisher Scientific, United States) normalized to 4 nM and pooled. The pooled libraries were denatured with 0.2 N NaOH and included a minimum of 5% PhiX internal control. The libraries were loaded onto the Miseq v3 reagent cartridge and analysed by using a Miseq reagent kit v3 for a paired-end (2×300 bp) sequencing performed at North-West University on the Illumina Miseq platform (Illumina, United States).

3.6. 16S rRNA GENE METABARCODING ANALYSIS

Demultiplexed FASTQ paired-end read files were imported and analysed with functions in the QIIME 2 software (version 2). Overlapping pair-end reads of Illumina fastQC files were merged, and their read quality was assessed by using the FastQC software (version 0.11.5,

Babraham Institute, United Kingdom). Overlapping paired-end reads were trimmed to discard amplification primers, adapter sequences and low-quality nucleotides by using the DADA2 pipeline (Alonso *et al.*, 2023). Furthermore, the DADA2 pipeline was used for operational taxonomic units (OUTs) clustering and taxonomic classification. This was achieved by applying an error-correction step and eliminating chimeric sequences. Merged quality-filtered reads were clustered into OTUs by using the UCLUST algorithm at 97% identity threshold. Subsequently, the OUT sequences were classified with the Silva 16S rRNA database (Release 138) targeting the V3-V4 region (Hupfauf *et al.* 2020). After that, only microorganisms belonging to domain bacteria were selected for downstream analyses on Circos (version 0.69-5), Microsoft Excel and MicrobiomeAnalyst (Dhariwal *et al.*, 2017).

Microsoft Excel was used to plot the stacked column for the phyla $\geq 2.00\%$ in DWPFs, wastewater effluent and rivers. The stacked column for the phyla shows the relative abundance of $>1.00\%$ in DWPFs and $> 2.00\%$ in wastewater effluent and rivers. Chord diagrams were constructed by Circos (version 0.69-5). MicrobiomeAnalyst was used to construct heat maps showing the relative abundance percentage of different bacterial genera by using the phylogenetic investigation of communities by reconstruction of unobserved states (PICRUS) analysis. Furthermore, MicrobiomeAnalyst was used to calculate the alpha diversity and significant differences for species diversity and evenness (Shannon index), richness (Chao1 and ACE estimator) and species dominance (Simpson index) (Dhariwal *et al.*, 2017).

3.7. SCREENING OF ANTIBIOTIC RESISTANCE GENES

The present study focused on detecting *Bla_{TEM}*, *ampC*, *intl1*, *sul1*, *sul2*, *ermB*, *ermF* and plasmid-mediated *AmpC* β -lactamase genes (pAmpCs) such as FOX, ACC, MOX, EBC, DHA and CIT in DWPFs, WWTPs and rivers. The end-point PCR reaction mixture for each gene contained 12.5 μ L DreamTaq PCR master mix: 5 U/ μ L Taq DNA polymerase in reaction buffer, 2 mM MgCl₂, 0.2 mM of each dNTP (Thermo Scientific, US), 9.5 μ L nuclease-free water (Fermentas Life Sciences, US), 1 μ L of each primer (forward and reverse; Inqaba Biotec, RSA) and 1 μ L of DNA template. Furthermore, a control containing no template DNA was included in the experiment of each gene. The primer's name, sequences, amplification size and PCR cycling (the TechneTM PCRmax Alpha Cyclers 1, Fisherscientific, UK) conditions for each gene are shown in Tables 3.1 and 3.2. The endpoint PCR conditions for each gene consisted of initial denaturation (ID), PCR cycles (CC) of denaturation (DN), annealing (AN), elongation (EL) and final extension (FE).

Table 3.1: Oligonucleotide primers for the end-point PCR amplification of *ampC*, *bla_{TEM}*, *Int1*, *ermB*, *ermF*, *Sul1* and *Sul2* genes.

Target gene	Primer's name	Sequence (5'.....3')	Size (bp)	PCR conditions	References
<i>ampC</i>	<i>AmpC</i> -F	TTC TAT CAA MAC TGG CAR CC	550	ID= 94°C for 300 s, CC= 35 cycles, DN= 94°C for 300 s, AN=49°C for 30 s, EL= 72°C for 60 s and FE= 72 °C for 420 s	Coertze and Bezuidenhout, 2017
	<i>AmpC</i> -R	CCY TTT TAT GTA CCC AYG A			
<i>bla_{TEM}</i>	TEM-F	ATT CTT GAA GAC GAA AGG GC	1 150	ID= 94°C for 300 s, CC= 30 cycles, DN= 95°C for 300 s, AN=60°C for 60 s, EL= 72°C for 60 s and FE= 72 °C for 300 s	Costa <i>et al.</i> , 2007
	TEM-R	ACG CTC AGT GGA ACG AAA AC			
<i>Int1</i>	HS463A	CTG GAT TTC GAT CAC GGC ACG	473	ID= 94°C for 300 s, CC= 30 cycles, DN= 94°C for 30 s, AN=64°C for 30 s, EL= 72°C for 60 s and FE= 72 °C for 300 s	Labbate <i>et al.</i> , 2008
	HS464	ACA TGC GTG TAA ATC ATC GTC G			
<i>ermB</i>	<i>ermB</i> -F	GAA AAG GTA CTC AAC CAA ATA	638	ID= 95°C for 300 s, CC= 35 cycles, DN= 95°C for 30 s, AN=48°C for 60 s, EL= 72°C for 120 s and FE= 72 °C for 600 s	Tran <i>et al.</i> , 2013
	<i>ermB</i> -R	AGT AAC GGT ACT TAA ATT GTT TAC			
<i>ermF</i>	<i>ermF</i> 1	CGG GTC AGC ACT TTA CTA TTG	466	ID= 95°C for 300 s, CC= 35 cycles, DN= 95°C for 30 s, AN=50°C for 30 s, EL= 72°C for 120 s and FE= 72 °C for 600 s	Chung <i>et al.</i> , 1999
	<i>ermF</i> 2	GGA CCT ACC TCA TAG ACA AG			
<i>Sul1</i>	SulI-F	TTC GGC ATT CTG AAT CTCAC	822	ID= 94°C for 180 s, CC= 35 cycles, DN= 94°C for 60 s, AN=60°C for 60 s, EL= 72°C for 60 s and FE= 72 °C for 300 s	Chaturvedi <i>et al.</i> , 2021
	SulI-R	ATG ATC TAA CCC TCG GTC TC			
<i>Sul2</i>	SulII-F	CGG CAT CGT CAA CAT AAC C	722	ID= 94°C for 180 s, CC= 35 cycles, DN= 94°C for 60 s, AN=60°C for 60 s, EL= 72°C for 60 s and FE= 72 °C for 300 s	Chaturvedi <i>et al.</i> , 2021
	SulII-R	GTG TGC GGA TGA AGT CAG			

ID= initial denaturation; C= cycles; D= denaturation; A= annealing; E= elongation and FE= final extension

Table 3.2: Primers: Oligonucleotide primers for the end-point PCR amplification of six pAmpCs, namely ACC, EBC, DHA, FOX, CIT and MOX gene.

Target gene	Primer's name	Sequence (5'.....3')	PCR conditions	Size (bp)	References
ACC	ACCMF	AACAGCCTCAGCAGCCGGTTA	ID= 94°C for 180 s, CC= 35 cycles, DN= 94°C for 30 s, AN=64°C for 30 s, EL= 72°C for 60 s and FE= 72 °C for 420 s	346	Pérez-Pérez and Hanson, 2002
	ACCMR	TTCGCCGCAATCATCCCTAGC			
MIR-1T ACT-1	EBCMF	TCGGTAAAGCCGATGTTGCGG	ID= 94°C for 180 s, CC= 35 cycles, DN= 94°C for 30 s, AN=64 °C for 30 s, EL= 72°C for 60 s and FE= 72 °C for 420 s	302	
	EBCMR	CTTCCACTGCGGCTGCCAGTT			
FOX-1 to FOX-5b	FOXMF	AACATGGGGTATCAGGGAGATG	ID= 94°C for 180 s, CC= 35 cycles, DN= 94°C for 30 s, AN=64°C for 30 s, EL= 72°C for 60 s and FE= 72 °C for 420 s	190	
	FOXMR	CAAAGCGCGTAACCGGATTGG			
DHA-1, DHA-2	DHAMF	AACTTTCACAGGTGTGCTGGGT	ID= 94°C for 180 s, CC= 35 cycles, DN= 94°C for 30 s, AN=64°C for 30 s, EL= 72°C for 60 s and FE= 72 °C for 420 s	405	
	DHAMR	CCGTACGCATACTGGCTTTGC			
LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITMF	TGGCCAGAACTGACAGGCAAA	ID= 94°C for 180 s, CC= 35 cycles, DN= 94°C for 30 s, AN=62°C for 30	462	
	CITMR	TTTCTCCTGAACGTGGCTGGC			

			s, EL= 72°C for 60 s and FE= 72 °C for 420 s		
MOX-1, MOX-2, CMY-1, CMY-8	MOXMF	GCTGCTCAAGGAGCACAGGAT	ID= 94°C for 180 s, CC= 35 cycles,	520	
	MOXMR	CACATTGACATAGGTGTGGTGC	DN= 94°C for 30 s, AN=59°C for 30 s, EL= 72°C for 60 s and FE= 72 °C for 420 s		

ID= initial denaturation; CC= cycles; DN= denaturation; AN= annealing; EL= elongation and FN= final extension

3.8. QUANTIFICATION OF ANTIBIOTIC RESISTANCE GENES AND 16S RRNA GENE

3.8.1. Quantification of antibiotic resistance genes

This study focused on the absolute quantification of *int11*, *sul1*, FOX, ACC, MOX, CT, DHA and EBC genes. The real-time PCR was conducted on water samples successfully amplified by means of the end-point PCR. The quantification of *int11* and *sul1* genes was followed the methods used by Xu *et al.* (2017). Each gene was quantified by using a total reaction volume of 10 μ L. The 10 μ L consisted of 5 μ L PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Lithuania), 1 μ L of each primer (forward and reverse; Inqaba Biotec, RSA) and 1 μ L of template DNA, while the remaining volume was compensated for by nuclease-free water (Fermentas Life Sciences, US). The real-time PCR thermal conditions were as follows: two steps of a hold stage of 50°C for two minutes and 95°C for 15 minutes. The PCR consisted of 45 cycles of PCR stage and melt curve stage. The PCR stage had PCR thermal conditions of 95°C for 15 seconds, annealing (*sul1* at 60°C and *int11* at 55°C) for 30 seconds, and elongation at 72°C for 30 seconds. The melt curve stage had PCR thermal conditions of 95°C for 15 seconds, 60°C for 60 seconds and 95°C for one second. QuantStudio™ 3 platform (Applied Biosystems, Thermo Fisher Scientific, United States) was used to quantify *sul1* and *int11* genes.

The quantification of six pAmpCs (FOX, ACC, MOX, CIT, DHA and EBC) was done by using TaqMan gene expression assays. The real-time PCR was conducted as described by Coertze and Bezuidenhout (2020) and Magome (2021). The following FAM fluorescent dyes TaqMan gene expression assays (Thermo Fisher Scientific, United States) were used for the quantification of the pAmpCs: Ba04646126_s1 (FOX), Ba04646144_s1 (ACC), Ba04646156_s1 (MOX), Ba04646135_s1 (CIT), Ba04646120_s1 (DHA) and Ba04646124_s1 (EBC). However, probe sequences were provided.

The real-time PCR for each assay had a total reaction volume of 10 μ L. The reaction was made up of 5 μ L TaqMan™ Fast Advanced MasterMix (Thermo Fisher Scientific, United States), 3.5 μ L of nuclease-free water (Fermentas Life Sciences, US), 0.5 μ L of each assay and 1 μ L template DNA. Furthermore, no template DNA control was included for each assay. These TaqMan gene expression assays were conducted by using the QuantStudio™ 3 platform (Applied Biosystems, Thermo Fisher Scientific, United States). The thermal PCR conditions were automatically determined by this platform where the real-time PCR thermal

conditions were as follows: two steps of a hold stage of 50°C for two minutes and 95°C for ten minutes. The PCR consisted of 40 cycles of PCR stage (95°C for 15 seconds and an annealing step of 60°C for 60 seconds).

3.8.2. Quantification of 16S rRNA gene

The 16S rRNA gene was quantified by using primers, as shown in Table 3.3. The amplified copies were normalized and presented as the ratio between ARGs and the corresponding copy number of the 16S rRNA (Magome. 2021). As a result, the ARG concentrations were reported as copies per 16S rRNA. The real-time PCR thermal conditions for the 16S rRNA primers were as follows: two steps of a hold stage of 50°C for two minutes and 95°C for five minutes. The PCR consisted of 40 cycles of PCR stage and melt curve stage. The PCR stage had PCR thermal conditions of 95°C for 15 seconds, an annealing step at 55°C for 15 seconds, and elongation at 72°C for 15 seconds. The melt curve stage is the same as that described in Section 3.8.1.

Table 3.3: Oligonucleotide primers for the real-time PCR quantification of 16S rRNA, *sul1* and *Int1*.

Target gene	Primer's name	Sequence (5'.....3')	References
16S rRNA	906F	AAA CTC AAA KGA ATT GAC GG	Magome, 2021
	1062R	CTC ACR RCA CGA GCT GAC	
<i>sul1</i>	FW	CGCACCGGAAACATCGCTGCAC	Xu <i>et al.</i> , 2017
	RV	TGAAGTTCCGCCGCAAGGCTCG	
<i>Int1</i>	FW	GGCTTCGTGATGCCTGCTT	Xu <i>et al.</i> , 2017
	RV	CATTCCTGGCCGTGGTTCT	

3.8.3. Standard curves and method validation for the quantification of 16S rRNA and antibiotic resistance gene

Standard curves were used to evaluate the quality of ARGs and 16S rRNA quantification. The method was validated for amplification efficiency and linearity. The standard curves for ARGs and 16S rRNA were constructed by using five-fold serial dilutions of known copies isolated from positive control samples. This process was done in triplicates. The coefficient of determination (R^2) > 0.97 and amplification efficiency ranging from 90% to 110% were considered reliable comparison trend lines for determining unknown concentrations of genes (see Coertze and Bezuidenhout, 2020). The R^2 was determined, and the slope was calculated by using the following formula:

$$E = 10^{-1/m} - 1$$

Where: E = efficiency

M = slope

The ARGs and 16S rRNA concentrations were calculated by using the standard curve at known concentrations to obtain their Ct values. The following linear regression formula shows how to obtain unknown concentrations of water samples analysed in duplicates:

$$y = mx + c$$

Where: y = Ct values

m = slope

c = y-intercept

x = concentration

The ARGs and 16S rRNA were quantified by using real-time PCR. The QuantStudio Design and Analysis software (Version 1.3.1) and Microsoft Excel were used for data processing. Figure 3.3 shows the standard curves generated for the real-time PCR of 16S rRNA, *sul1*, *int1* and six *AmpC* β -lactamase genes (FOX, ACC, MOX, CIT, DHA and EBC). Amplification efficiencies of standard curves ranged between 95% and 103%. The coefficient of determination (R^2) standard curves exceeded 0.98, with a slope range of between -3.46 and -3.24 for all real-time PCR reactions.

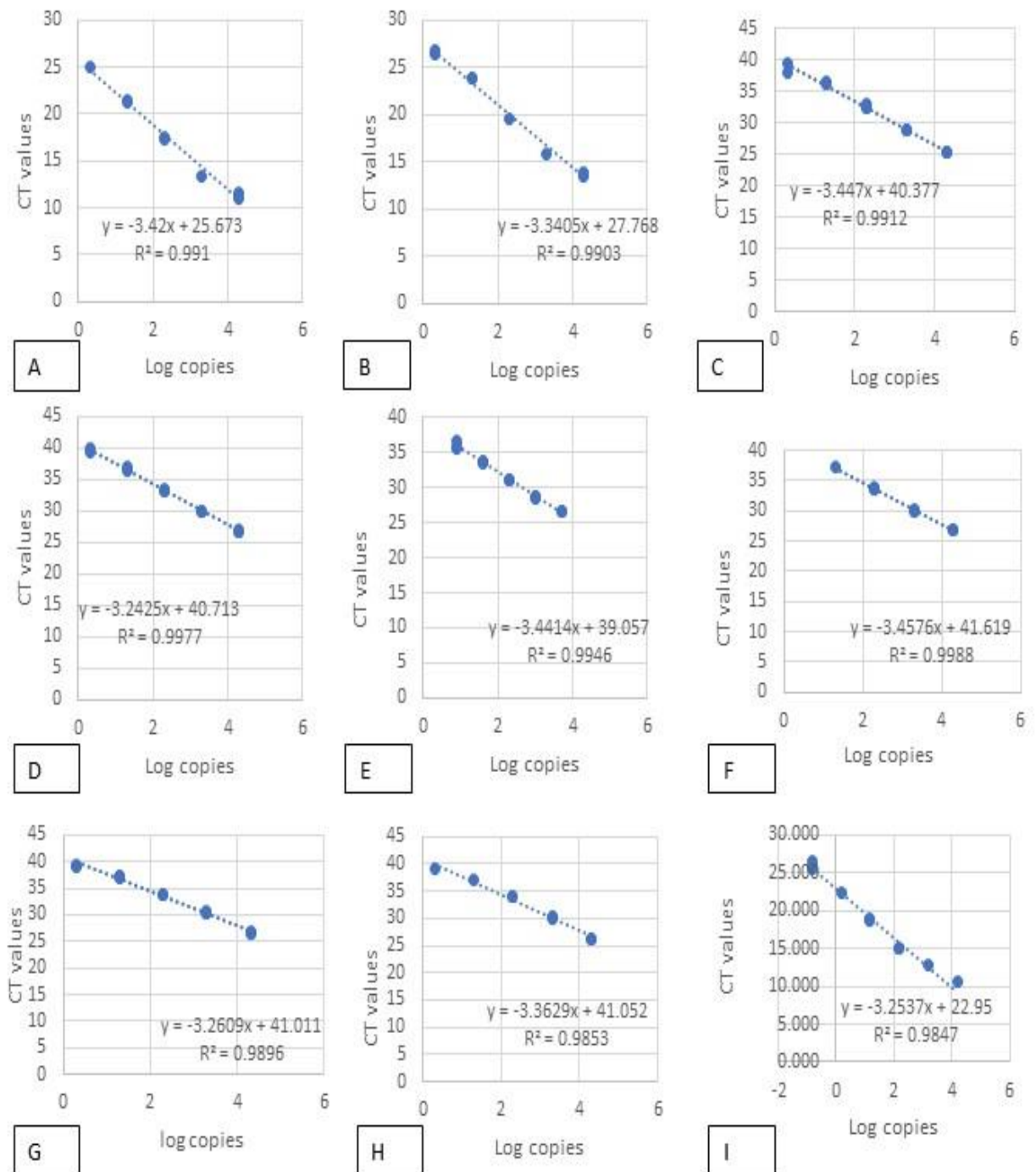


Figure 3.3: Real-time PCR standard curves for (A) *sul1*, (B) *int11*, (C) ACC, (D) EBC, (E) CIT, (F) DHA, (G) FOX, (H) MOX and (I) 16S rRNA.

3.9. QUANTIFICATION OF ANTIBIOTIC RESIDUES USING CHEMICAL ANALYSIS

3.9.1. Chemicals

Antibiotics that were used included sulfamethoxazole (Duchefa Biochemie, Netherlands), ciprofloxacin (Stigma, United States), trimethoprim (TRC, Canada) and ampicillin (Stigma, United States). Fluconazole (USP, Hungary) was also included in the quantification list. The internal standards included sulfamethoxazole D4, trimethoprim D3, ciprofloxacin D8, ampicillin D5 and caffeine from TRC (Canada). The solvents used to dissolve and make 10 mg/ml antibiotics and fluconazole stock solutions are shown in Table 3.4. These stock solutions were stored at 4°C for up to one month. Light penetration, which might have degraded compound quality, was avoided by using amber vials (Kim and Carlson, 2006). Hydrophilic-lipophilic balance (HLB)-H disks (Atlantic Disk, United States), acetonitrile (Honeywell, United States), methanol (Honeywell, United States), acetone (Honeywell, United States), Milli-Q water (ELGA Purelab, UK), 32% hydrochloric acid (HCl) (Rochelle Chemicals, RSA), formic acid (Honeywell, Germany) and ethylenediaminetetraacetic acid disodium salt dihydrate (Na₂EDTA) (Merck, United States), were also used in this study.

Table 3.4: Solvents used to dissolve target fluconazole and antibiotics.

Antibiotics	CAS ID	Solvent(s)
Ampicillin	69-53-4	Methanol
Trimethoprim	738-70-5	
Sulfamethoxazole	723-46-6	
Ciprofloxacin	85721-33-1	32% HCl
Fluconazole	86386-73-4	Milli-Q water

HCl - Hydrochloric acid.

3.9.2. Sample preparation and solid-phase extraction

The pH of the 1 L water samples was adjusted to 2 by using 32% HCl. Na₂EDTA was added to chelate residual metals. The solid-phase extraction was done by using the SPE-DEX system (Horizon Technology, Salem, NH, United States). This is an automated solid-phase extraction instrument that extracts target compounds from water samples by using specific disks and solvents. HLB H disks were used for the extraction of antibiotics and fluconazole.

The SPEDEX method, solvents and conditions are listed in Table 3.5. The eluate was evaporated under a gentle stream of nitrogen at 37°C and with 1 ml of Milli-Q water-methanol (1:1) and 0.1% formic acid and stored until chemical analysis commenced.

Table 3.5: SPEDEX system method, solvents and conditions.

Steps	Solvents	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
The 1 L water sample was processed.			
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
The disk was air-dried for 15 min.			
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

3.9.3. Ultra-performance liquid chromatography

The analytes were analysed with ultra-high performance liquid chromatography coupled with a quadrupole time-of-flight mass spectrometry (UHPLC-QTOF/ MS) system that consisted of the Agilent 1290 infinity binary pump (G4220A), 1290 infinity autosampler (G4226A), and 1290 infinity thermostatted column compartment (G1316C) coupled with an Agilent 6540 Accurate Mass Q-TOF/MS (G6540A) (Agilent Technologies, Santa Clara, CA, United States). The Poroshell 120 (2.1×100mm Bonus-RP 2.7 µm) column (Agilent Technologies, United States) generated peaks of antibiotics and fluconazole. The ionisation of target compounds was achieved by means of positive electrospray ionization (ESI+) that was enhanced with Agilent Jet Stream (AJS) technology (Matongo *et al.*, 2015a). The target analytes were separated by a gradient method by using a mobile phase composition of 0.1% (v/v) formic acid in Milli-Q water (Mobile Phase A) and 0.1% (v/v) formic acid in acetonitrile (Mobile Phase B). The UHPLC gradient program and QTOF/ MS parameters are listed in Tables 3.6 and 3.7.

Table 3.6: Gradient used for the mobile phase for separations of target compounds.

Positive electrospray ionization ESI (+)		
Time (min)	B (%)	Flow rate (ml/min)
0.00	5.00	0.200
2.00	5.00	0.200
2.05	15.00	0.400
5.55	15.00	0.400
5.60	20.00	0.200
11.10	20.00	0.200
11.15	25.00	0.600
13:15	25.00	0.600
13.20	35.00	0.200
17.20	35.00	0.200
17.25	100.00	0.200
20.00	100.00	0.200
20.05	5.00	0.200

Table 3.7: QTOF/ MS parameter setup.

Parameters	Settings
Drying gas temperature	250°C
Drying gas flow	8 l/min
Nebulizer pressure	20 psig
Sheath gas temperature	350°C
Sheath gas flow	7.5 l/min
VCap	3 000 V
Nozzle voltage	0 V
Fragmentor	130 V
Skimmer	70 V
Oct 1 RF Vpp	750 V

3.9.4. Quality control and analytical performance

Quality control was performed to validate the methods of extraction and chemical analysis. Limit of quantification (LOQ), limit of detection (LOD), accuracy, precision, matrix effects and linearity were the parameters used for the method validation. Matrix effects were accounted

for by the external matrix-matched calibration curve spiked post-extraction. A matrix-matched calibration curve was prepared by spiking 1 L of blank water with antibiotics, fluconazole and their corresponding internal standards. The standard concentrations of ciprofloxacin (which ranged from 0 to 2 µg/L), trimethoprim (which ranged from 0. to 0.5 µg/L), ampicillin (which ranged from 0 to 10 µg/L), fluconazole (which ranged from 0 to 5 µg/L) and sulfamethoxazole (which ranged from 0 to 5 µg/L) were used to prepare for delivering a linear range of the target compounds.

The R^2 was also determined. The accuracy of the extraction method was conducted by spiking samples with low, medium and high concentrations of the antibiotics and fluconazole to determine the recovery percentages. The intra-day and inter-day precisions were evaluated and expressed as relative standard deviations (RSDs). The intra-day precision was determined by calculating the ratio of the standard deviation and average of the six replicates injected within a day. The inter-day precision was determined by calculating the ratio of the standard deviation and average of the six replicates injected over three days (Späth *et al.*, 2021). The LOQ and LOD employed the following calculations (Kanama *et al.*, 2018; Späth *et al.*, 2021):

$$LOD = 3 * Sd/m$$

$$LOQ = 10 * Sd/m$$

Sd = standard deviation; m = slope

The target antibiotic and fluconazole residues concentrations were calculated by using matrix-matched calibration curves at known concentrations to obtain their corresponding abundance ratio. A blank was included in the analysis. The unknown concentration of water samples was analysed in triplicate as calculated in the following linear regression formula:

$$y = mx + c$$

Where: m = slope

c = y-intercept

x = concentration

y = relative response

The UHPLC-QTOF/ MS was used to quantify the target antibiotics and fluconazole. Agilent's MassHunter Data Acquisition (Version 10.1 B.10.1.733.0) and Quantitative Analysis for Q-TOF (Version 10.0 B.10.0.10305.0) and Microsoft Excel were used for data processing.

3.9.5. Method validation for the quantification of antibiotic and fluconazole residues

Table 3.8 shows the method validation parameters for quantifying antibiotic and fluconazole residues. Matrix-matched calibration curves are displayed in Figure 3.4. The linearity of the standard curves exceeded 0.99 for all target compounds. The LOD ranged from 0.04 to 0.34 µg/L. The LOQ ranged from 0.13 to 1.14 µg/L. The intraday and inter-day precision RSD was below 20% for all target compounds. The recovery rates ranged from 90 to 102%, with ampicillin showing the lowest ones and trimethoprim the highest.

Table 3.8: Method validation parameters for the quantification of antibiotic and fluconazole residues.

Target residues	R^2	LOD (µg/L)	LOQ (µg/L)	Intra-day precision (%RSD)			Inter-day precision (%RSD)	Recovery (%)
				Day 1	Day 2	Day 3		
Ampicillin	0.999	0.24	0.79	5.02	4.35	4.65	4.90	90
Ciprofloxacin	0.998	0.11	0.36	3.59	2.45	3.29	12.66	102
Fluconazole	0.999	0.30	1.01	5.21	1.61	1.37	19.26	98
Sulfamethoxazole	0.997	0.34	1.14	6.49	8.97	2.29	19.92	93
Trimethoprim	0.994	0.04	0.13	2.73	1.09	2.52	11.43	101

R^2 = R square; LOD = limit of detection; LOQ= Limit of quantification; RSD = relative standard deviation.

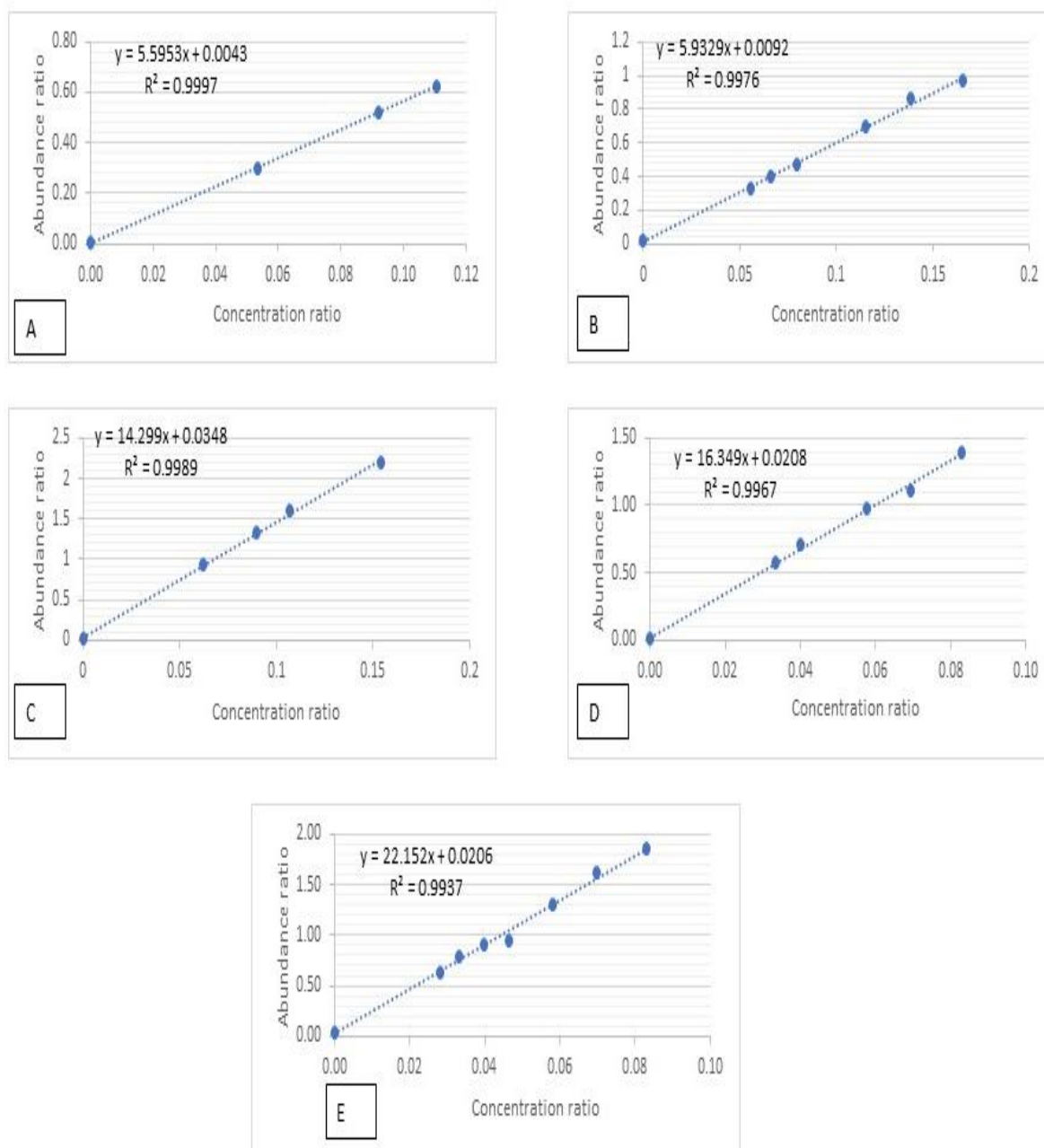


Figure 3.4: Matrix-matched calibration curve for (A) ampicillin, (B) ciprofloxacin, (C) fluconazole, (D) sulfamethoxazole and (E) trimethoprim.

3.9.6. Risk quotient

The antibiotic and fluconazole concentrations were used to determine the risk quotient (RQ), following the methods by outlined by Coors *et al.* (2018) and Tell *et al.* (2019). The RQ denotes the environmental hazard of the targeted pharmaceutical compounds, including antibiotics and fluconazole. It is calculated by dividing the concentration of target compounds by the environmental predicted no-effect concentration, as shown below:

$$RQ = \frac{C}{PNEC}$$

Where: RQ= risk quotient

C= measured fluconazole and antibiotic concentrations

PNEC-ENV = environmental predicted no-effect concentration

PNEC values were obtained from Tell *et al.* (2019). The PNEC value for fluconazole was obtained from Coors *et al.* (2018). The PNEC-ENV concentrations of target compounds are shown in Table 3.9. The RQs were classified into three risk levels RQ <0.1 indicates low risk, RQ <1 medium risk, and ≥1 high risk.

Table 3.9: PNEC-ENV concentrations of target compounds.

Target compounds	PNEC-ENV (µg/L)	References
Ampicillin	0.87	Tell <i>et al.</i> , 2019
Ciprofloxacin	0.57	
Sulfamethoxazole	0.60	
Trimethoprim	100.00	
Fluconazole	9.46	Coors <i>et al.</i> , 2018

PNEC-ENV = environmental predicted no-effect concentration.

3.10. STATISTICAL ANALYSES

The statistical analysis was performed by using R software (version 4.1.3). The normality of the data was determined by using the Shapiro–Wilk test. Log or square root transformation further transformed data that failed the normality test. For data that could not be transformed, non-parametric tests were used to determine whether statistically significant differences occurred between groups with two or more independent variables. This was done by using

the Wilcoxon or the Kruskal-Wallis tests, respectively. The p-value was set at 0.05 to establish significant results. The data set that was normally distributed was analysed by using Student's t-test or ANOVA. For the statistically significant group, the Tukey post-hoc test was performed with the p-value adjusted by using Bonferroni to determine the differences among the groups.

Heatmaps, chord diagrams and stacked columns were used to represent data by using GraphPad Prism (version 9.4.1), Circos (version 0.69-5) and Microsoft Excel. Redundancy analysis (RDA) was performed to correlate bacterial community composition with physicochemical parameters, ARGs and antibiotic residues in water samples by using Canoco Software (Version 4.5). The correlation was archived by using multivariant analysis and the construction of triplots. Monte Carlo simulations were conducted to determine the correlation in canonical axes, which were considered to be significant when the p-value was <0.05 . Furthermore, pairwise correlation matrices were performed to determine the r , p-values and Ns for each pair of bacterial community composition, physicochemical parameters, ARGs and antibiotic residues by using basic statistics on Statistica Software (Version 14.0.1.25). The correlation for each pair was considered significantly different when the p-value was <0.05 . In accordance with work done by Coertze and Bezuidenhout (2020), the correlations were based on ARGs' detection or absence and not their copy numbers. A graphical representation of the methods used in the present study is shown in Figure 3.5.

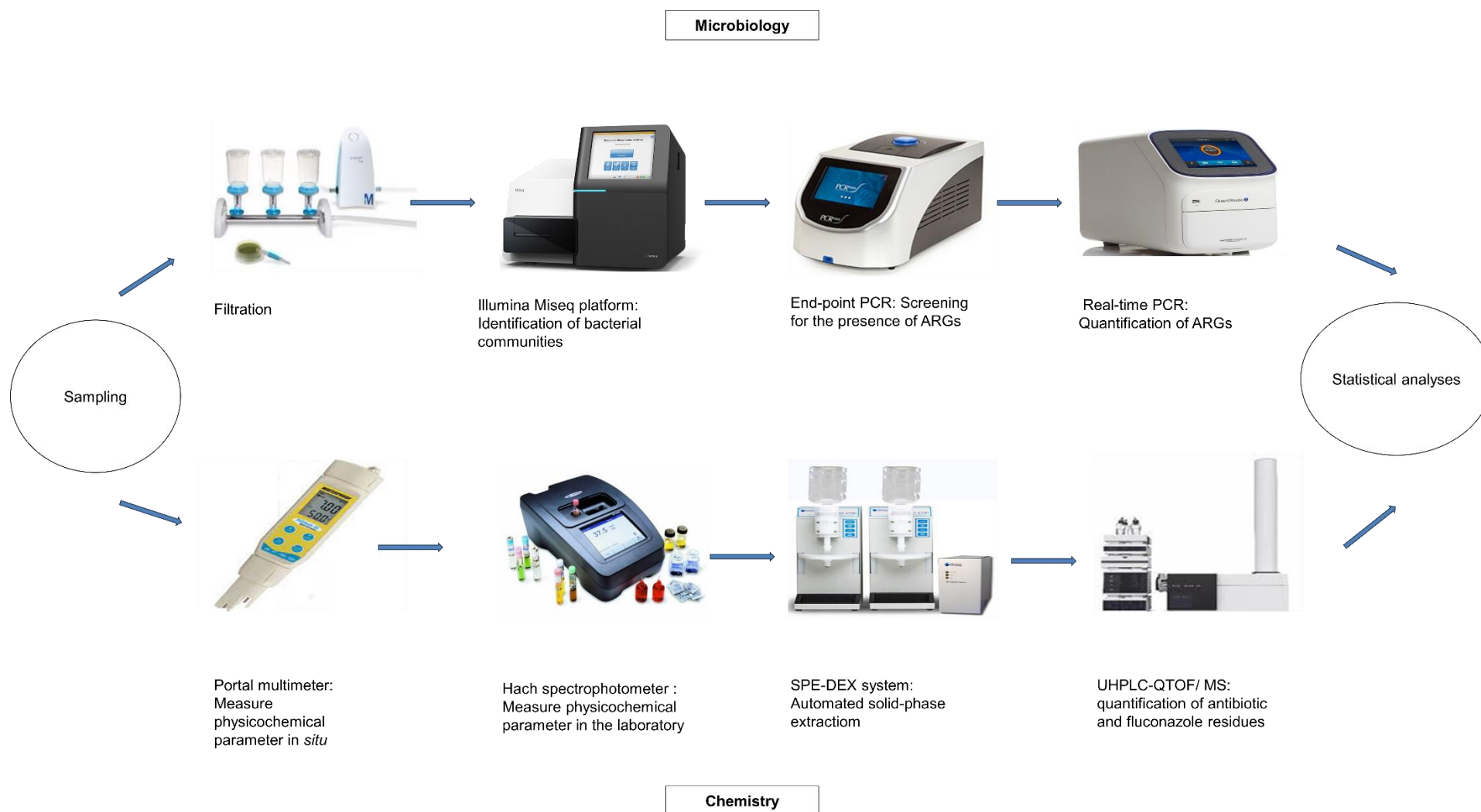


Figure 3.5: Flow diagram illustrating an overview of the methods.

CHAPTER 4

4. DISTRIBUTION OF ANTIBIOTIC RESISTANCE GENES AND ANTIBIOTIC RESIDUES IN DRINKING WATER PRODUCTION FACILITIES: CORRELATION WITH BACTERIAL COMMUNITIES

4.1. INTRODUCTION

South Africa has adopted the Millennium Development Goals and drafted the National Development Plan intending to alleviate poverty, reduce the burden of diseases and improve access to safe and clean drinking water (NPC 2012; Khabo-Mmekoa and Momba, 2019). Ateba *et al.* (2020) suggest that consuming safe and clean drinking water should not pose any significant health threats to consumers over their lifetime and should also not produce hypersensitive reactions despite their immune systems. However, it is estimated that waterborne infections contribute to 80% of illnesses in developing countries (Mugadza *et al.*, 2021). This suggests that, among other related factors, good health depends on an adequate supply of safe and clean drinking water.

South Africa is a semi-arid country with limited alternative drinking water sources (Edokpayi *et al.*, 2018). Consequently, it is ranked 148 out of 180 countries regarding water availability per capita (NPC, 2012). Access to safe and clean drinking water is a challenge in many developing countries (Atnafu *et al.*, 2021). This has led to the fact that some parts of South Africa are considered to be drought-stricken, engendering the implementation of stringent water regulations. In January 2018, the Western Cape government introduced Day Zero, where the municipalities discontinued water supply in the region due to the low water levels (Rawlins, 2019; Edokpayi *et al.*, 2020). Edokpayi *et al.* (2020) reported similar situations in Makhanda (Eastern Cape) and Giyane (Limpopo), where inadequate water supply was brought about by droughts.

In addition, poor or no water infrastructure exacerbates the inadequate supply of clean and safe drinking water. Edokpayi *et al.* (2020) names South African regions affected by an inadequate water supply due to poor or no water infrastructure and, in some cases, they stipulate the periods during which this problem occurred. It is clear from their findings and those of others that many individuals in rural and peri-urban areas depend on untreated drinking water sources such as wells, lakes, dams, springs and rivers (Edokpayi *et al.*, 2018; Edokpayi *et al.*, 2020; Mugadza *et al.*, 2021). Studies have reported that these unprotected

water sources may be contaminated with faecal matter and pathogenic bacteria due to the construction of pit latrines and the defecation of animals near them (Ateba and Maribeng, 2011; Machado and Bordalo, 2014).

According to extant literature, individuals dependent on drinking water produced in centralized DWPFs may also be at risk of contracting pathogenic bacteria (Samie *et al.*, 2012; Mulamattathil *et al.*, 2014a; 2014b). This is due to service delivery backlogs, lack of water experts, poor maintenance of ageing infrastructure, ineffective end-of-pipe compliance monitoring systems and unfavourable environmental conditions (Ateba *et al.*, 2020; Edokpayi *et al.*, 2020; Mhlongo *et al.*, 2020; Mugadza *et al.*, 2021). Undeniably, applying appropriate water treatment processes, especially as centred on disinfection, reduces the levels of chemicals and microbes to acceptable limits from raw to treated water (Han *et al.*, 2021). However, DWPFs do not completely eliminate the microbes in raw water (Han *et al.*, 2021). Some of these microbes are undesirable for consumers due to the threat that they pose to biosafety (Liu *et al.*, 2018). Thus, it is important to determine bacterial community composition from raw to treated water for a better understanding of microbial dynamics and to strengthen water biosafety management strategies (Han *et al.*, 2020).

The increased human population has led to increased anthropogenic activity (Machado and Bordalo, 2014). This means high demand and supply of antibiotics (Machado and Bordalo, 2014). Usually, the usage of these antibiotics is frequent and unregulated (Ateba *et al.*, 2020). This has resulted in the spread of antibiotic residues in the aquatic environment, which imposes a selective pressure on opportunistic bacteria, thus enhancing the development of ARB and the spreading of ARGs (Ateba *et al.*, 2020). This entails that antibiotic resistance is not only a clinical phenomenon, as shown by studies that reported the presence of antibiotic residues and ARGs in the aquatic systems, but also environmental ones (Mulamattathil *et al.*, 2014a; 2014b).

Several studies report that water quality concerns go beyond those related to conventional pathogenic microorganisms and physicochemical parameters: they include the emergence of ARB, which release ARGs due to antibiotic residues in drinking water (Samie *et al.*, 2012; Mulamattathil *et al.*, 2014a; 2014b). As indicated, the spread of these contaminants into the environment is associated with the improper and extensive usage of antibiotics in human medicine, veterinary medicine and agriculture (Mulamattathil *et al.*, 2014b). Studies moreover report the migration of antibiotic residues, ARGs, and/ or ARB from raw water into treated drinking water and/ or distribution networks (Mulamattathil *et al.*, 2014a; 2014b). DWPFs are not designed to remove these contaminants (Mulamattathil *et al.*, 2014a; 2014b; Lyimo *et al.*,

2016). Consumption of contaminated drinking water is one of the ways in which ARBs may facilitate the spread of their ARGs to humans (Lyimo *et al.*, 2016). This reduces the efficiency of antibiotics and promotes the HGT of ARGs to opportunistic bacteria (Machado and Bordalo, 2014). Furthermore, it may lead to longer hospitalization, increased morbidity and mortality rates, as mentioned (see Lyimo *et al.*, 2016).

According to the databases consulted in the present project, all studies conducted in the DWPFs in North West and South Africa generally were built on culture-based methods. These studies focused on susceptibility tests and PCR screening of ARGs. Despite being cost-effective, culture-based methods fail to represent the number of bacteria and their diversity in drinking water due to poor cultivation of viable but non-culturable bacteria. Thus, the results lack representation of viable but non-culturable bacteria that might be opportunistic and pathogenic (Atnafu *et al.*, 2021). Furthermore, none of the drinking water studies measured the concentration of antibiotic residues in DWPFs. The present study adjusts this important shortcoming in the study of drinking water in North West, South Africa. It aimed to determine the concentration of antibiotic residues and antibiotic resistant genes in raw and treated water and the bacterial community composition and physicochemical parameters of these.

4.2. MATERIALS AND METHODS

Refer to Chapter 3 for detailed materials and methods.

4.3. RESULTS

4.3.1. Physicochemical parameters in drinking water production facilities

The physicochemical parameters in raw and drinking were measured *in situ*. Tables 4.1 and 4.2 present the TDS, pH, salinity and temperature measured in NW-E and NW-C DWPFs. Table 4.3 presents nitrate, nitrite, phosphate and COD in DWPFs. The data shows the means that were used to calculate the three sampling runs. The measured physicochemical parameters were compared to the DWAF SAWQG (1996a) for domestic water use.

4.3.1.1. Physicochemical parameters in NW-E drinking water production facility

Tables 4.1 and 4.3 represent the physical parameters for the NW-E DWPF. As anticipated, the lowest temperature of 10.33°C and the highest temperature of 24.37°C were measured in the winter and summer, respectively (Table 4.1). There was a significant decrease (p-value <0.05) in pH from 8.41 in raw water to 8.20 in treated water.

The TDS levels were slightly higher in treated (518 mg/L) than in raw water (510 mg/L) in March 2022, as shown in Table 4.1. Furthermore, in February 2021 and February 2022, the TDS levels in raw and treated water were the same. Overall, there were no significant differences (p-value >0.05) in the TDS levels between raw and treated water. The average TDS levels measured in raw water were 549.68 mg/L and in treated water 548.5 mg/L. Overall, the measured TDS levels in NW-E were above the target standards of the DWAF SAWQG (1996a) for domestic water use.

In NW-E DWPF, salinity levels were higher in treated than in raw water in July 2021, December 2021 and March 2021, as shown in Table 4.1. In these months, the average salinity levels were 346.00 mg/L in raw water and 349.67 mg/L in treated water. Overall, there were no significant differences (p-value >0.05) in salinity levels of 342.06 mg/L measured in raw water and 340.25 mg/L in treated water. The measured salinity in NW-E was above the target standards of the DWAF SAWQG (1996a) for domestic water use.

Table 4.3 shows that the nitrate levels were higher in treated (1.00 mg/L) than in raw water (0.82 mg/L). However, the measured nitrate levels in September 2021 and March 2022 were higher raw than in treated water in the DWPF. Generally, in NW-E DWPF, nitrate levels were slightly higher in raw (0.85 mg/L) than in water (0.80 mg/L). A similar trend was observed for

nitrite levels in NW-E DWPF. Nitrite levels averaged 2.44 mg/L in raw water and 1.67 mg/L in treated water. Significant differences (p -value <0.05) in nitrite levels were measured between the raw and treated water. The nitrite and nitrate levels were found to be within the target standards of the DWAF SAWQG (1996a) for domestic water use.

Significant differences (p -value <0.05) were observed in phosphate levels between raw and treated water. As anticipated, the phosphate levels were higher in raw than treated water (Table 4.3). The phosphate levels averaged 6.48 mg/L in raw water and 1.73 mg/L in treated water. Contrary to other physicochemical parameters measured in NW-E DWPF, the overall COD levels were higher in treated (13.00 mg/L) than in raw water (11.33 mg/L).

4.3.1.2. Physicochemical parameters in NW-C drinking water production facility

Tables 4.2 and 4.3 represent the physicochemical parameters of NW-C DWPF. In winter and summer, the lowest temperature of 13.63°C and the highest temperature of 20.87°C were measured, respectively (Table 4.2). Overall, the pH of the raw water at 8.28 was slightly lower than the treated water at 8.36. The pH was lower in raw water compared to treated water only in December 2021. No significant differences (P -value >0.05) were observed in pH between the raw and treated water.

Generally, TDS levels were higher in treated (409.00 mg/L) than in raw water (406.25 mg/L). This range is recommended by the DWAF SAWQG (1996a) for domestic water use. However, in December 2020 and September 2021, the measured TDS levels were higher in the raw than the treated water (Table 4.2). There were no significant differences (p -value >0.05) in the TDS levels between these.

In NW-C DWPF, the salinity levels were only slightly higher in raw than treated water in December 2020, as shown in Table 4.2. However, from February 2021 until March 2022, the salinity levels were slightly elevated in treated water, with an average of 252.75 mg/L, compared to raw water, with an average of 250.38 mg/L (Table 4.2). No significant differences (p -value >0.05) were observed between raw to treated water. The measured salinity in NW-C was above recommended standards of the DWAF SAWQG (1996a) for domestic water use.

The mean nitrate levels were higher in treated water at 1.98 mg/L compared to raw water at 1.46 mg/L (Table 4.3). Furthermore, the raw and treated water differed significantly (p -value <0.05). However, the nitrite levels were higher in raw (2.33 mg/L) than in treated water (1.67

mg/L), with no significant differences (p-value >0.05). The nitrite and nitrate levels were found to be within the target standards of the DWAF SAWQG (1996a) for domestic water use.

In NW-C DWPF, the phosphate levels were higher in treated water (4.21 mg/L) than in raw water (3.47 mg/L), with no significant differences (p-value >0.05). The COD levels were higher in treated than raw water in September 2021 and March 2022 (Table 4.3). However, the highest COD levels of 85.00 mg/L in February 2021 resulted in its prevalence in raw water when compared to treated water. The average COD levels measured in raw water was 31.00 mg/L and, in treated water, these were 5.00 mg/L.

Table 4.1: Physical parameters with standard deviations measured in 2020, 2021 and 2022 in raw and treated water of NW-E DWPF.

Date	Sampling sites	TDSs (mg/L)	pH	Salinity (mg/L)	Temperature (°C)
	DWAF (1996a)	≤450	≥6.0 to ≤9.0	≤100	-
Dec 2020	Raw inlet 1	643.00±1.00	8.20±0.05	306.67±0.58	23.10±0.10
	Raw inlet 2	670.67±0.67	8.35±0.02	322.00±0.00	22.83±0.06
	Treated water	651.67±2.08	7.90±0.02	311.33±1.53	23.40±0.66
Feb 2021	Raw inlet 1	619.67±1.65	8.44±0.15	302.67±0.58	24.10±0.20
	Raw inlet 2	646.33±0.58	8.02±0.04	315.33±0.58	23.60±0.00
	Treated water	633.33±1.53	7.87±0.00	304.33±0.58	24.37±0.38
Jul 2021	Raw inlet 1	524.67±1.53	9.38±0.02	353.00±1.00	13.27±0.21
	Raw inlet 2	543.00±2.00	9.39±0.06	360.67±0.58	10.33±0.32
	Treated water	532.00±1.00	8.96±0.01	357.67±0.58	12.13±0.47
Aug 2021	Raw inlet 1	527.00±1.00	8.55±0.01	356.33±0.58	14.27±0.25
	Raw inlet 2	549.00±1.00	8.30±0.03	370.67±0.58	14.10±0.46
	Treated water	528.00±1.00	8.41±0.02	356.00±1.00	13.87±0.35
Sep 2021	Raw inlet 1	529.00±2.65	8.58±0.03	361.33±0.58	17.80±0.20
	Raw inlet 2	543.67±1.53	8.40±0.01	371.33±0.58	17.67±0.42
	Treated water	533±0.67	8.36±0.02	369.67±3.79	17.87±0.15
Dec 2021	Raw inlet 1	484.33±0.58	8.41±0.03	331.67±1.53	22.90±0.10
	Raw inlet 2	488.67±1.15	7.84±0.02	332.67±1.15	20.77±0.67
	Treated water	486.67±1.15	8.09±0.02	333.67±1.53	22.10±0.26
Feb 2022	Raw inlet 1	499.33±0.58	8.09±0.04	342.33±0.58	21.57±0.06
	Raw inlet 2	510.00±1.00	7.98±0.02	348.67±2.52	22.43±0.15
	Treated water	505.33±0.33	7.84±0.01	344.67±0.58	22.33±1.53
Mar 2022	Raw inlet 1	512.67±0.58	8.34±0.01	350.00±0.00	19.40±1.15
	Raw inlet 2	506.67±0.58	8.23±0.01	346.67±0.58	20.30±0.20
	Treated water	517.67±0.58	8.17±0.01	353.67±0.58	19.90±0.10

TDSs- total dissolved solids; “-” – not specified.

Table 4.2: Physical parameters with standard deviations measured in 2020, 2021 and 2022 in raw and treated water of NW-C DWPF.

Date	Sampling sites	TDSs (mg/L)	pH	Salinity (mg/L)	Temperature (°C)
	DWAF (1996a)	≤450	≥6.0 to ≤9.0	≤100	-
Dec 2020	Raw inlet	478.67±1.15	8.10±0.04	232.00±0.00	20.47±0.35
	Treated water	476.33±2.08	7.94±0.01	231.33±0.58	20.87±0.21
Feb 2021	Raw inlet	483.33±0.58	8.15±0.05	233.33±0.58	20.07±0.06
	Treated water	490.67±0.58	7.89±0.01	236.33±0.58	18.77±0.21
Jul 2021	Raw inlet	378.00±2.00	9.31±0.03	249.33±1.15	13.63±0.31
	Treated water	381.67±3.21	9.28±0.06	254.00±1.00	13.80±0.40
Aug 2021	Raw inlet	381.67±0.58	8.29±0.01	256.00±1.00	15.93±0.06
	Treated water	382.67±0.58	8.22±0.03	257.67±0.58	17.33±0.06
Sep 2021	Raw inlet	378.33±1.15	8.36±0.03	254.00±1.00	15.83±0.25
	Treated water	371.67±9.29	8.35±0.03	255.33±1.15	15.07±0.15
Dec 2021	Raw inlet	381.33±0.58	8.20±0.01	259.67±0.58	19.87±0.15
	Treated water	389.00±1.00	8.25±0.02	263.33±1.15	19.63±0.31
Feb 2022	Raw inlet	386.67±0.58	8.15±0.02	262.00±1.00	17.80±0.20
	Treated water	391.67±0.58	8.12±0.02	264.00±0.00	18.23±0.31
Mar 2022	Raw inlet	382.00±0.00	8.34±0.01	257.00±0.00	15.67±0.06
	Treated water	386.67±0.58	8.16±0.01	260.67±0.58	16.47±0.58

TDSs- total dissolved solids; “-“ – not specified.

Table 4.3: Chemical parameters with standard deviations measured in 2021 and 2022 in raw and treated water of NW-E and NW-C DWPFs.

Date	Sampling sites		Phosphate (mg/L)	Nitrates (mg/L)	Nitrites (mg/L)	COD (mg/L)
	DWAf (1996a)		-	≤6	≤6	-
Feb 2021	NW-E	Raw inlet 1	2.69±0.53	0.87±0.15	3.00±0.00	0.00
		Raw inlet 2	2.37±0.34	0.77±0.42	2.33±1.15	18.00
		Treated water	2.67±0.68	1.00±0.17	2.00±0.00	16.00
	NW-C	Raw inlet	1.25±0.22	1.67±0.12	1.00±0.00	85.00
		Treated water	3.37±0.84	1.83±0.32	2.00±1.00	4.00
Sep 2021	NW-E	Raw inlet 1	3.51±0.84	0.73±0.06	2.00±0.00	13.00
		Raw inlet 2	4.48±2.09	0.60±0.00	3.00±0.00	8.00
		Treated water	2.40±0.23	0.57±0.12	1.00±0.00	14.00
	NW-C	Raw inlet	3.93±0.58	1.77±0.06	2.00±0.00	4.00
		Treated water	3.43±1.32	1.80±0.00	1.00±0.00	6.00
Mar 2022	NW-E	Raw inlet 1	20.08±0.12	1.03±0.15	1.33±1.15	13.00
		Raw inlet 2	5.74±2.25	1.07±0.06	3.00±0.00	16.00
		Treated water	1.06±0.84	0.83±0.32	2.00±0.00	9.00
	NW-C	Raw inlet	5.29±2.32	0.93±0.15	4.33±1.15	4.00
		Treated water	5.82±3.100	2.30±0.87	2.00±1.00	5.00

COD - Chemical oxygen demand; “-” – not specified.

4.3.2. Bacterial community composition in drinking water production facilities

The identity of bacterial communities in NW-E and NW-C DWPF was obtained by using high-throughput 16S rRNA sequencing, in which the data show that normalised sequence reads averaged 60 189 counts per sample. Figures 4.1 and 4.2 represent the phylum-level changes in bacterial community composition of raw and treated water at NW-E and NW-C DWPFs, where the phyla with <2% relative abundance were classified as others. The others consisted of Abditibacteriota, Armatimonadota, Bdellovibrionota, Campilobacterota, Chloroflexi, Deferribacterota, Deinococcota, Elusimicrobiota, Fusobacteriota, Gemmatimonadota, Myxococcota, Nitrospirota, Patescibacteria, Planctomycetota and Spirochaetota.

Figures 4.2 and 4.3 represent the genus-level changes in bacterial community composition of raw and treated water at NW-E and NW-C DWPFs, where the genera with <1% relative abundance were classified as others. The distribution of the relative abundance of bacterial communities from raw to treated water is shown in Figure 4.5.

4.3.2.1. Bacterial community composition in NW-E drinking water production facility

In NW-E raw and treated water of the DWPF, the five most prevalent phyla were the same, but they ranked differently (Figures 4.1 and 4.2). The overall raw water samples (raw 1 and 2) were dominated by proteobacteria, Bacteroidota (38.22%), Acidobacteriota (18.33%), Cyanobacteria (9.98%) and an unclassified phylum (6.08%) phylum. In treated water, Proteobacteria (40.22%) was the dominant phylum, followed by Bacteroidota (14.71%), Unclassified (12.19%), Acidobacteriota (10.72%) and Cyanobacteria (5.00%). Furthermore, in NW-E raw water, the prevalent genera were unassigned group (52.10%) followed by *Comamonas* (11.84%), *Dickeya* (6.67%), *Clade III* (5.20%) and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (4.39%), as shown in Figures 4.3 and 4.4. In treated water, the prevalent genera were the unassigned (58.12%), followed by *Dickeya* (8.47%), *Nocardia* (5.44%) and *Comamonas* (5.13%).

4.3.2.2. Bacterial community composition in NW-C drinking water production facility

In NW-C raw and treated water, the four most prevalent phyla were as follows: Proteobacteria (raw water= 48.49% and treated water= 50.64%), Bacteroidota (raw water= 21.99% and

treated water= 14.21%), unclassified (raw water= 12.53% and treated water= 9.78%) and Acidobacteriota (raw water= 7.67% and treated water= 7.49%), as shown in Figures 4.1 and 4.2. Furthermore, in NW-C raw water, the prevalent genera were unassigned group (51.18%), followed by *Comamonas* (16.28%), *Dickeya* (9.91%), *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (9.81%) and *Nocardia* (4.85%), as shown in Figures 4.3 and 4.4. The prevalent genera in treated water were the unassigned group (63.38%), *Dickeya* (10.09%), other (5.72%), *Comamonas* (5.57%) and *Nocardia* (3.94%).

4.3.2.3. Alpha diversity in NW-E and NW-C drinking water production facilities

Alpha diversity and significant differences were calculated for species diversity and evenness (Shannon index), richness (Chao1 and ACE estimator) and species dominance (Simpson index) for NW-E and NW-C raw and treated water, as shown in Figure 4.6. In NW-C DWPF, treated water was more even, diverse, richer and dominant than in raw water samples. Similar observations were made in NW-E DWPF: treated water was richer, more diverse, and even than raw water (Figure 4.6). However, in NW-E DWPF, raw water 1 was more dominant. However, there were no significant differences between the raw and treated water when applying the Kruskal-Wallis test (ACE: p-value = 0.263, statistic = 5.250; Chao1: p-value = 0.204, statistic = 5.933; Shannon: p-value = 0.267, statistic = 5.200; Simpson: p-value 0.167, statistic = 6.467).

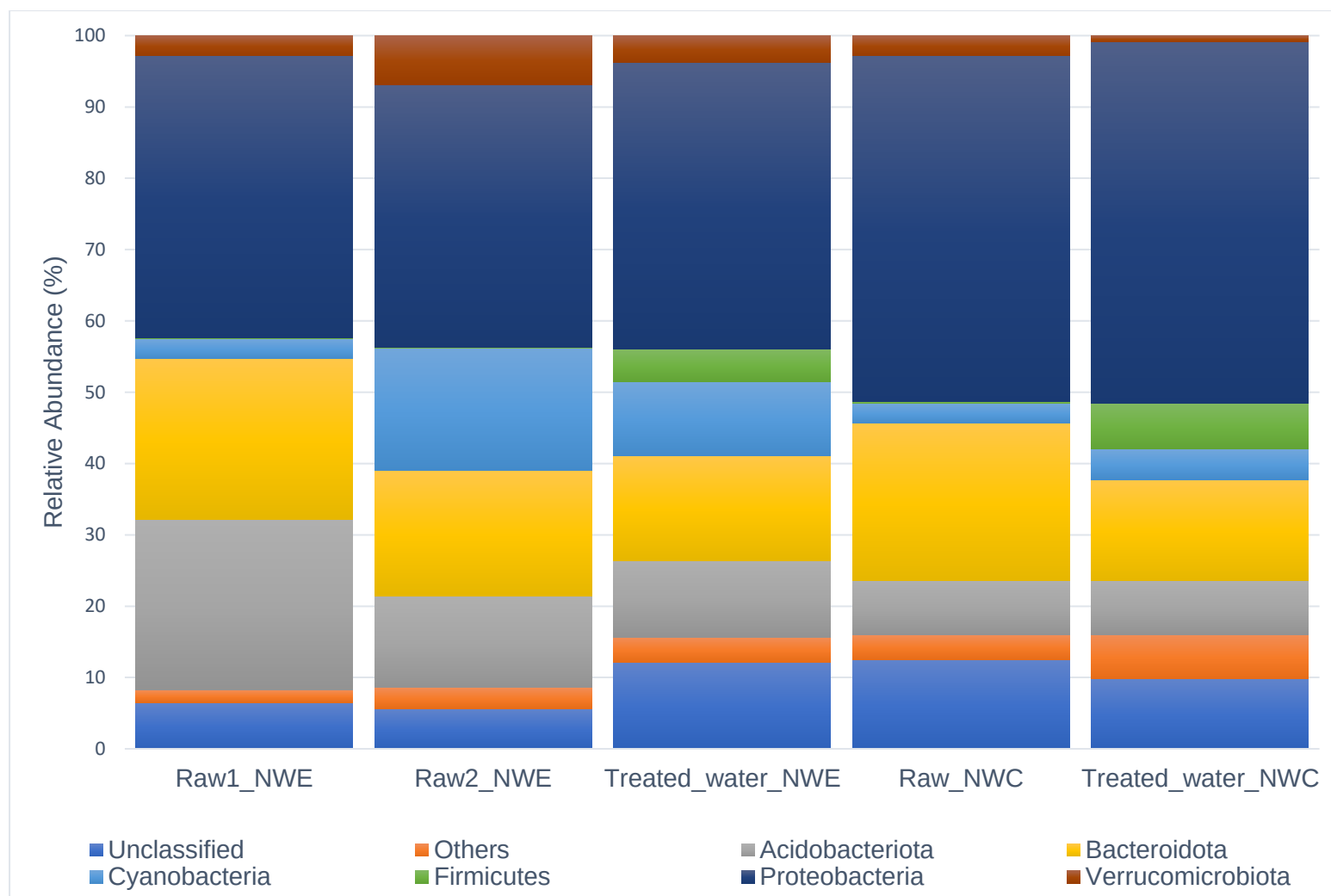


Figure 4.1: Stacked column illustrating phylum-level changes in bacterial community composition of raw and treated water at NW-E and NW-C DWPFs. Others consist of phyla with relative abundance < 2.00%.

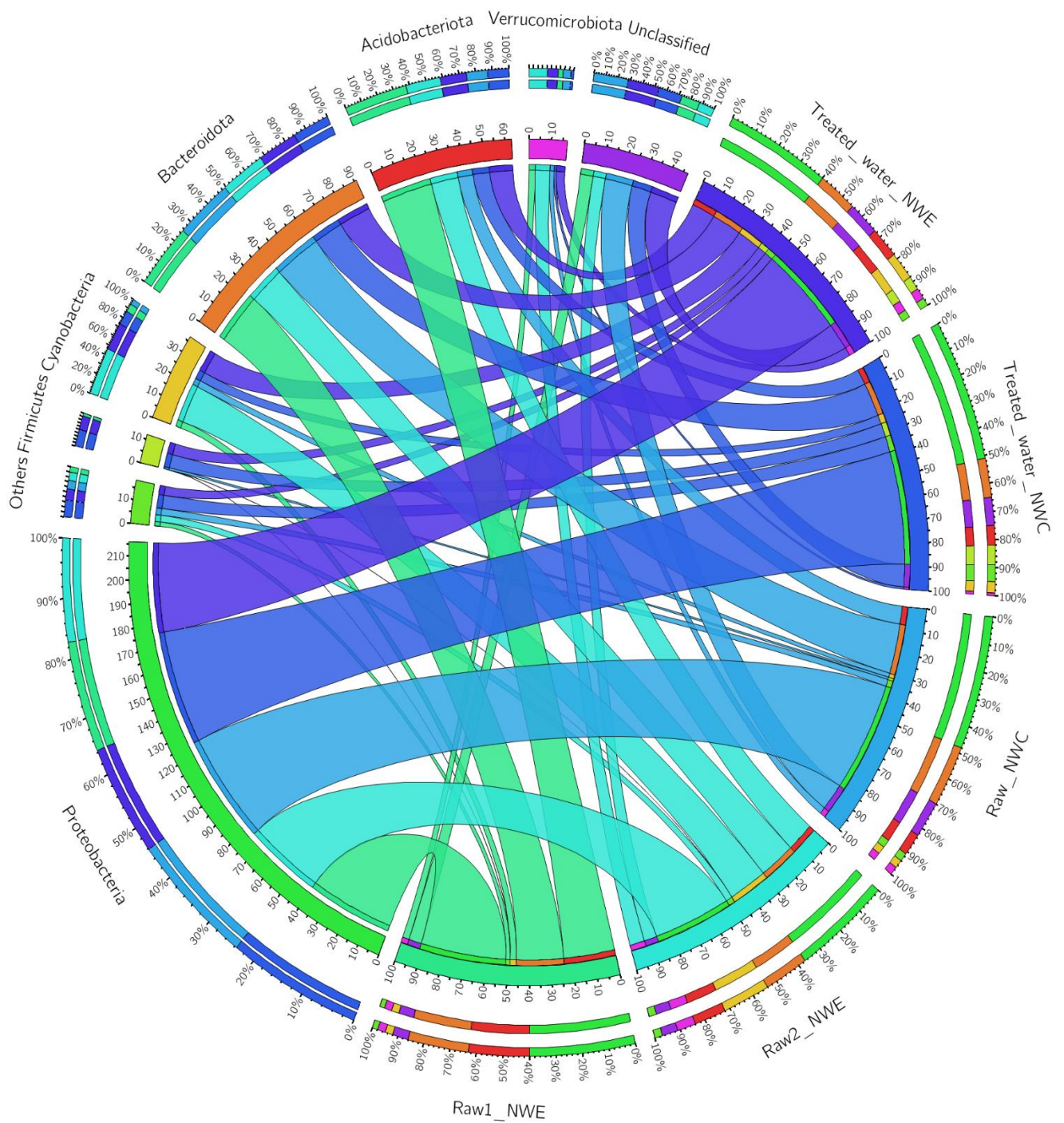


Figure 4.2: Chord diagram illustrating phylum-level changes in bacterial community composition of raw and treated water at NW-E and NW-C DWPFs. Others consist of phyla with relative abundance < 2.00%.

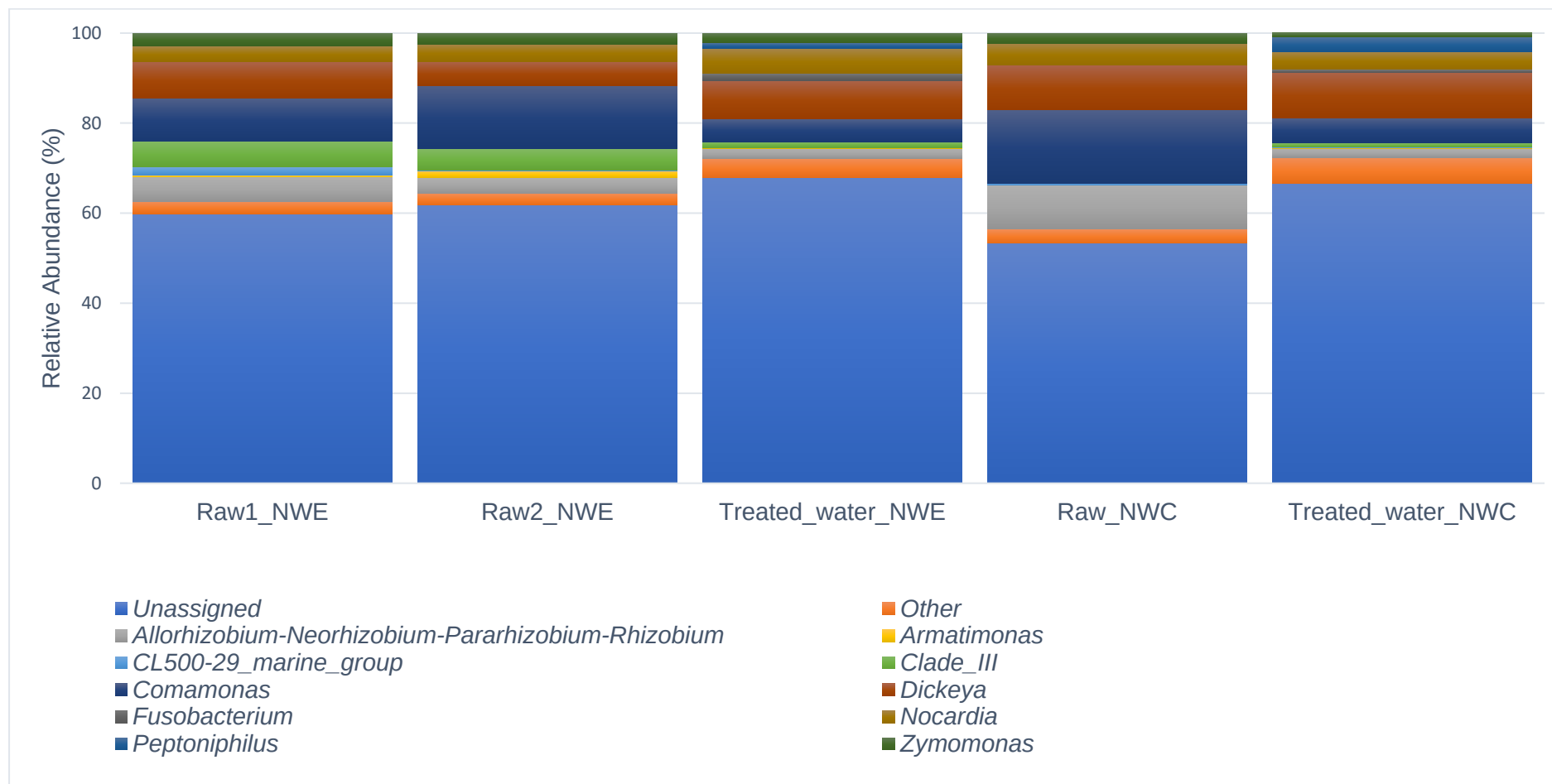


Figure 4.3: Stacked column illustrating genus-level changes in bacterial community composition in raw and treated water of NW-E and NW-C DWPFs. Others consist of genera with relative abundance < 1.00%..

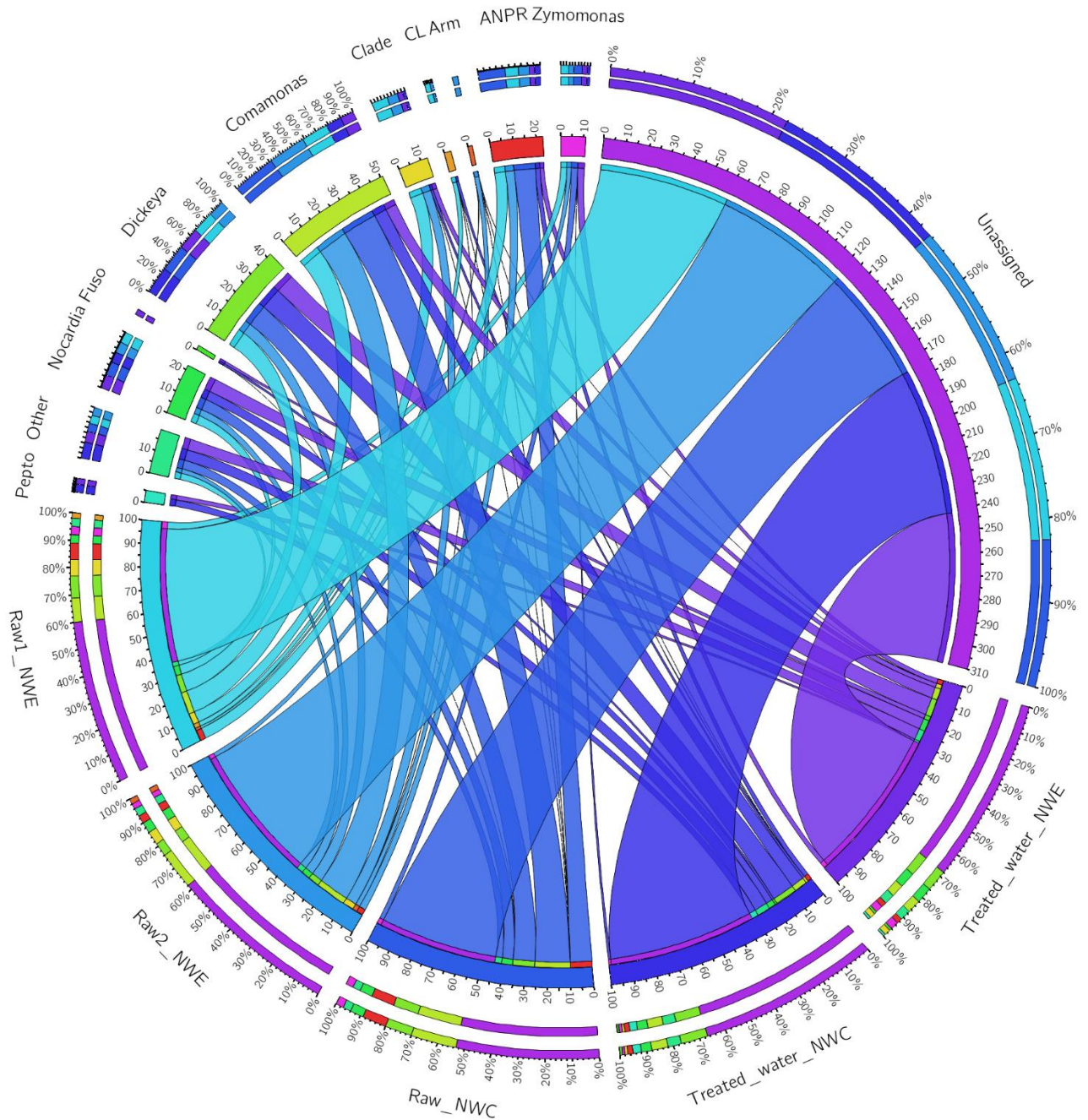


Figure 4.4: Chord diagram illustrating genus-level changes in bacterial community composition in raw and treated water of NW-E and NW-C DWPFs. Others consist of genera with relative abundance < 1.00%.

Where: ANPR = *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*; Arm = *Armatimonas*; CL = *Clade III*; Fuso = *Fusobacterium*; Pepto = *Peptoniphilus*.

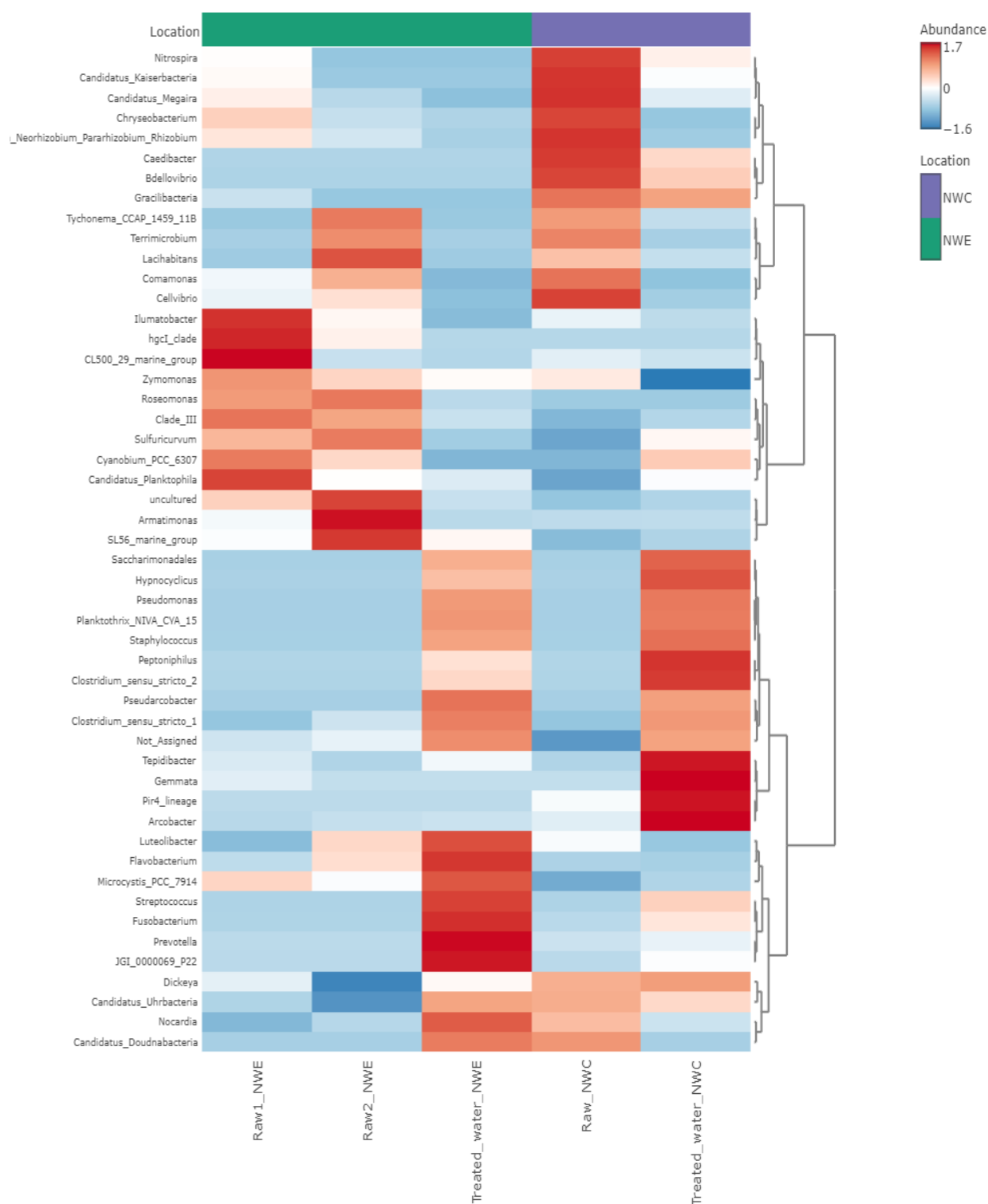


Figure 4.5: A heat map showing the relative abundance percentage of different bacterial genera by using PICRUS analysis in raw and treated water of NW-E and NW-C DWPFs.

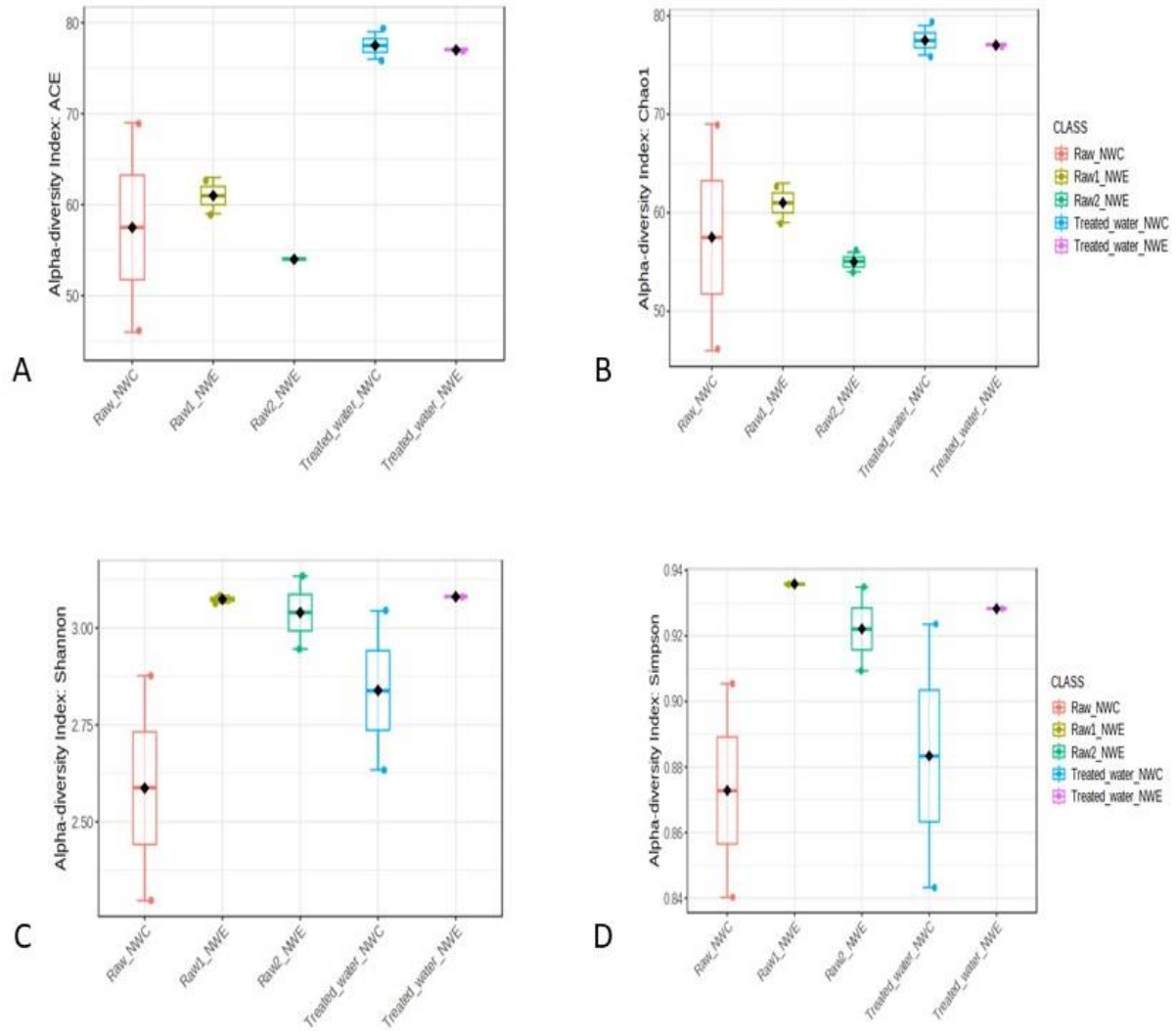


Figure 4.6: Bacterial alpha diversity indices (A-ACE, B-Chao1, C-Shannon and D-Simpson) in raw and treated water of NW-E and NW-C DWPF.

4.3.3. Detection of antibiotic resistance genes in drinking water production facilities

The eDNA isolated in raw and treated water was subjected to end-point PCR for screening 13 ARGs. The results are presented as heatmap and chord diagrams, as shown in Figures 4.7 and 4.8. In NW-E and NW-C DWPFs, ARGs fluctuated between raw and treated water.

4.3.3.1. Detection of antibiotic resistance genes detection in NW-E drinking water production facility

The *sul1* and *sul2* genes detection in water samples was higher in raw water 1 in the DWPF in NW-E (Figures 4.7 and 4.8). The detection of *sul1* and *sul2* genes in raw water 1 was 100% and 75%, respectively. Furthermore, detecting the *sul1* gene of 66.67% was constant in raw water 2 and treated water in the NW-E DWPF. However, the detection of the *sul2* gene was high in treated water when compared to raw water 2. The detection average of the two raw water samples was higher for the *sul1* gene and lower for the *sul2* gene than in the treated water.

The detection of *ermB* and *ermF* genes was higher in raw water 1 and 2 at NW-E. The *ermB* and *ermF* gene detection was neither higher nor lower in raw and treated water of the NW-E DWPF. The detection of 41.67% *ermB* and 58.33% *ermF* in raw water samples and treated water were the same in NW-E DWPF (Figure 4.8).

The detection of *ampC* and *bla_{TEM}* genes was lower in treated water of the NW-E DWPF, as shown in Figure 4.7. The 16.67% *bla_{TEM}* gene detection was the constant in NW-E raw water samples. However, the detection of the *ampC* gene was higher in raw water 2 with 27.27%, followed by 10.00% in treated water and 9.09% in raw water 1. Furthermore, the detection of the *int1* gene was higher in raw than in treated water. The highest detection of 91.67% of the *int1* gene was recorded in raw water 1, followed by 83.33% in raw water 2 and 58.33% in treated water.

FOX was the most frequently detected pAmpC in NW-E DWPF, with the highest detection in raw water 1 (75.00%), followed by raw water 2 (50.00%) and treated water (25.00%), as shown in Figures 4.7 and 4.8. The ACC gene detection was higher in raw water 1 (81.82%) than in raw water 2 (36.36%) and treated water (18.18%). The EBC gene detection was higher in treated water than in raw water 2. However, the detection of the EBC gene in treated water

was lower compared to raw water 1. The average detection level of the EBC gene in water samples was 18.18% and, in treated water, it was 10.00%.

The MOX gene detection of 27.28% was the highest in raw water 1 (Figures 4.7 and 4.8). However, the MOX gene was absent in raw water 2 and treated water. Furthermore, the absence was also reported for the CIT gene in raw and treated water from NW-E DWPF. This meant that the CIT gene the least detected one in NW-E DWPF. However, the detection of the DHA gene was slightly higher in treated (20.00%) than in raw water (18.18%).

4.3.3.2. Detection of antibiotic resistance genes in NW-C drinking water production facility

In the NW-C DWPF, the absence of *bla*_{TEM}, *ampC*, MOX, DHA and CIT were reported in raw and treated water, as shown in Figure 4.8. Furthermore, the absence of the EBC and ACC genes was also reported in treated water. However, these genes were present in DWPF raw water with a 22.22% and 11.11% detection level, respectively.

In the NW-C DWPF, the detection levels of *int11*, *ermB*, *sul2* and FOX genes were higher in raw than treated water ranging from 45.45-27.27%, 36.36-18.18%, and 40.00-18.18% to 54.55-27.27% (Figures 4.7 and 4.8). However, the detection of the *sul1* gene was higher in treated (45.45%) than in raw water (40.00%). The detection of *ermF* gene at 54.44% was constant in raw and treated water. The *ermF* (54.55%) was the gene detected most frequently in the DWPF, followed by *sul1* (42.73%), FOX (40.91%), *int11* (36.36%), *sul2* (29.09%), *ermB* (27.27%), EBC (11.11%) and ACC (5.56%).

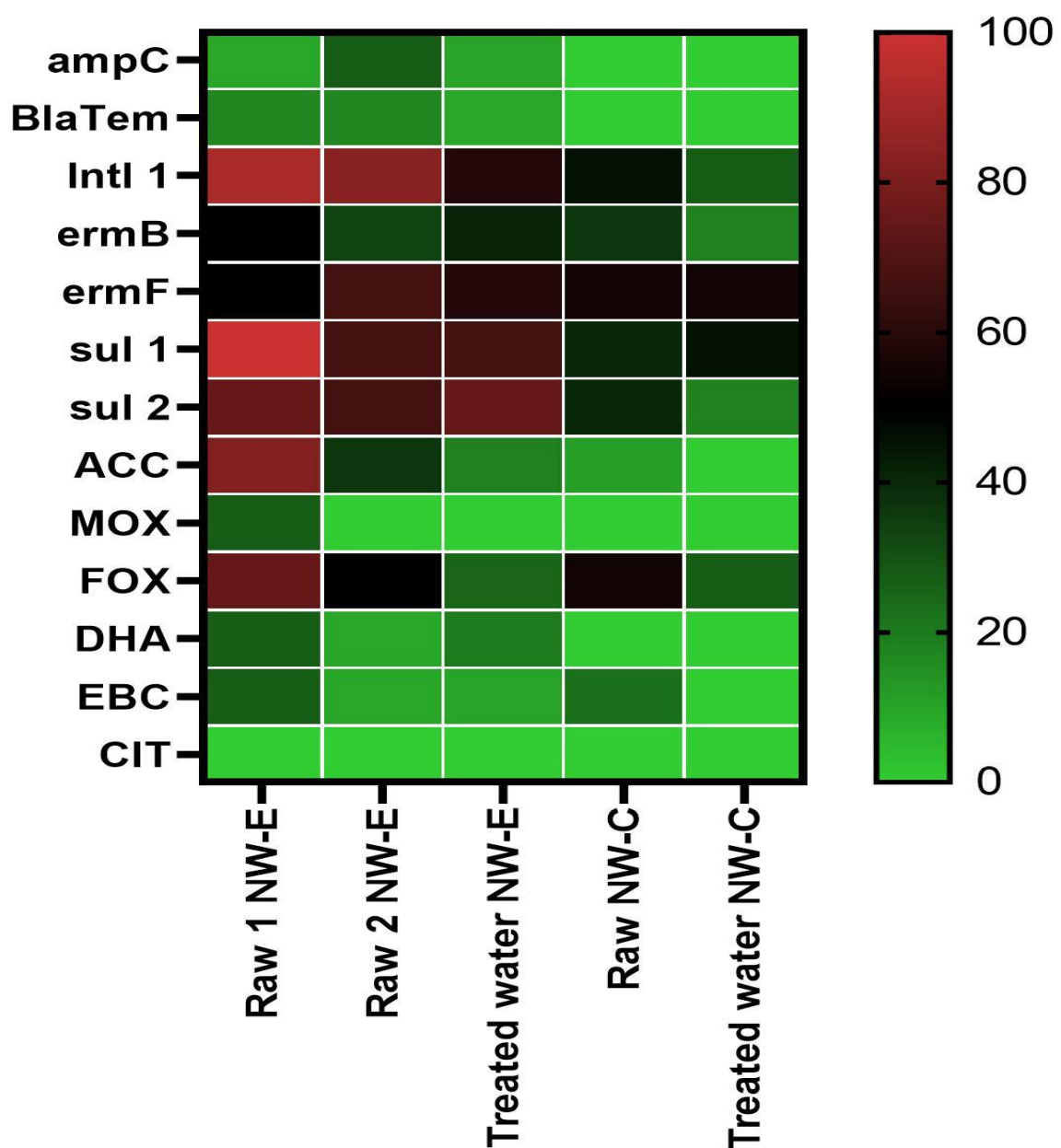


Figure 4.7: Heatmap illustrating the end-point PCR screening process of ARGs in raw and treated water of NW-E and NW-C DWPFs. The smallest, baseline and largest detection levels are represented by green, black and red colours, respectively.

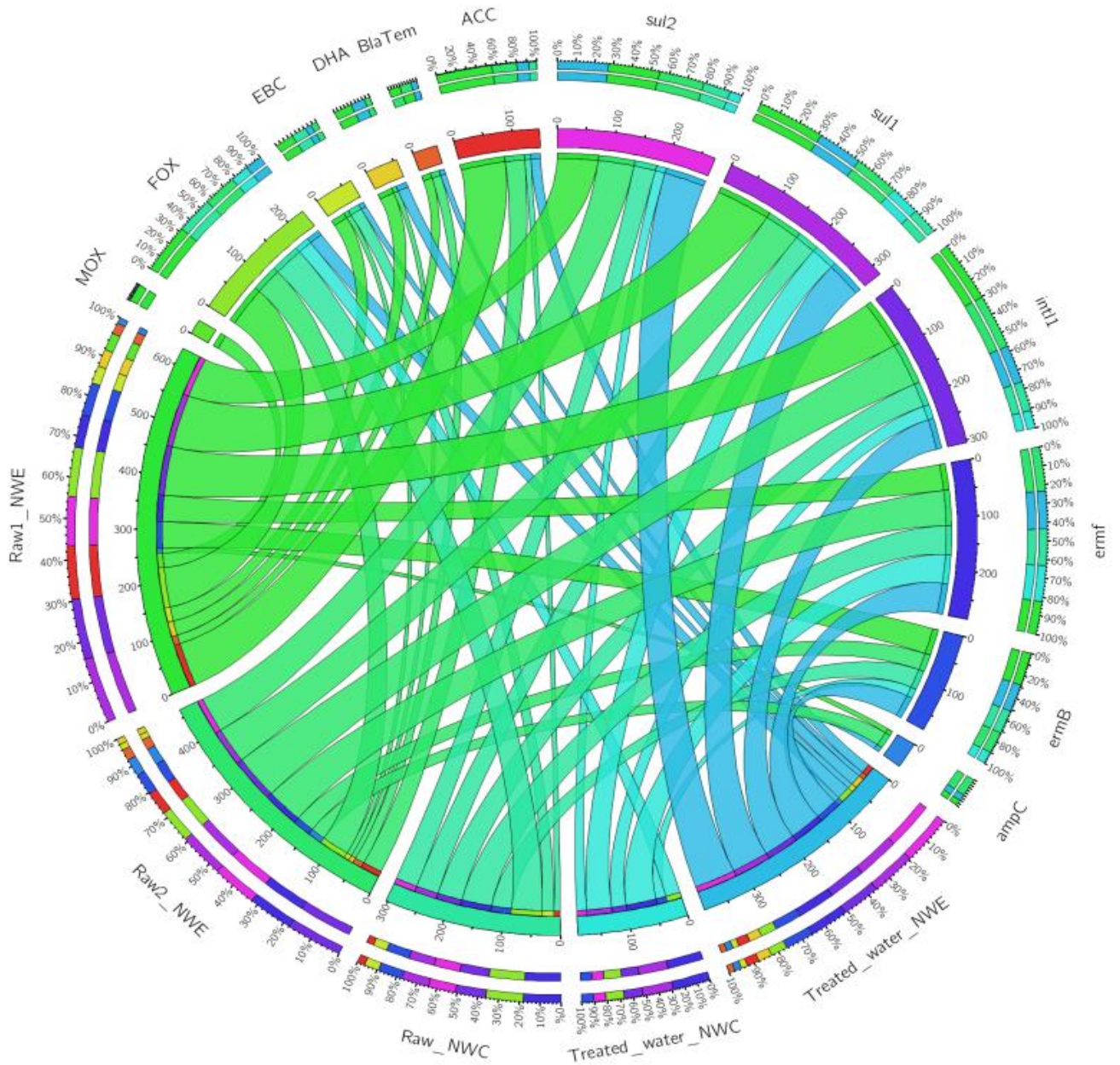


Figure 4.8: Chord diagram illustrating the end-point PCR screening process of ARGs in raw and treated water of NW-E and NW-C DWPFs.

4.3.4. Quantification of antibiotic resistance genes in drinking water production facilities

Table 4.4 represents concentrations, including the standard deviation of ARGs, in raw and treated water of NW-E and NW-C DWPFs. The measured concentrations were expressed as gene copies/16S rRNA. The present study focused on quantifying pAmpCs, *int1* and *sul1* genes. However, ARGs not detected during the screening end-point PCR for each sampling site were excluded from the real-time PCR analysis. The end-point PCR analysis results showed a higher detection of ARGs in raw than in treated water. However, in the NW-E DWPF, the ARG concentrations were higher in treated water than in raw water when compared to NW-C, as shown in Table 4.4.

4.3.4.1. Quantification of antibiotic resistance genes in NW-E drinking water production facility

The ACC, EBC, DHA, *int1* and *sul1* concentrations were higher in treated than in raw water, as shown in Table 4.4. The ACC concentrations were 1.58×10^{-1} copies/16S rRNA in raw water and 7.32×10^{-2} copies/16S rRNA in treated water. The EBC concentrations were 3.69×10^{-2} copies/16S rRNA in raw water to 6.37×10^{-2} copies/16S rRNA. The DHA concentrations were 1.34×10^{-2} copies/16S rRNA in raw water to 4.16×10^{-2} copies/16S rRNA in treated water. Generally, the concentrations of *int1* and *sul1* genes were lower than pAmpCs in the NW-E DWPFs. The *int1* concentrations were 3.90×10^{-4} and 6.12×10^{-4} copies/16S rRNA in raw and treated water, respectively. The *sul1* gene concentrations were 2.53×10^{-4} and 3.73×10^{-4} copies/16S rRNA in raw and treated water, respectively.

The FOX concentrations were higher in raw than treated water, as shown in Table 4.4. They measured at 6.22×10^{-1} copies/16S rRNA in raw water and 4.12×10^{-1} copies/16S rRNA in treated water. The MOX concentrations were 1.29×10^{-1} copies/16S rRNA in raw water 1. However, in raw water 2 and treated water, the MOX was not subjected to real-time PCR analysis, as it was shown to be absent during the screening process with the end-point PCR. For this reason, the CIT gene was not subjected to real-time PCR.

4.3.4.2. Quantification of antibiotic resistance genes in NW-C drinking water production facility

In NW-C DWPF, the ACC and FOX concentrations were higher in raw than in treated water, as shown in Table 4.4. The ACC concentrations were 3.85×10^{-2} copies/16S rRNA raw water and <LOD in treated water. The FOX concentrations were 1.44×10^1 copies/16S rRNA in raw water and 6.94×10^{-2} copies/16S rRNA in treated water. However, *sul1* concentrations were higher in treated water (7.97×10^{-2} copies/16S rRNA) than in raw water (6.03×10^{-2} copies/16S rRNA).

The CIT, DHA and MOX genes in raw and treated water were not subjected to real-time PCR analysis due to their absence during the screening process with the end-point PCR. The same outcome was recorded for the EBC in treated water. However, the EBC concentration in raw water was 7.67×10^{-2} copies/16S rRNA. The measured *intl1* gene in raw water was 3.50×10^{-4} copies/16S rRNA. It was not quantified in treated water due to insufficient eDNA.

Table 4.4: Concentrations of ARGs in raw and treated water of NW-E and NW-C DWPFs (units in gene copies/16S rRNA $\pm$ standard deviation).

sites		EBC	ACC	CIT	DHA	MOX	FOX	<i>Int1</i>	<i>Sul1</i>
NW-E	Raw water 1	$2.15 \times 10^{-2} \pm$	$2.68 \times 10^{-1} \pm$	-	$2.26 \times 10^{-2} \pm$	$1.29 \times 10^{-1} \pm$	$1.04 \times 10^0 \pm$	$2.03 \times 10^{-4} \pm$	$1.95 \times 10^{-3} \pm$
		2.68×10^{-2}	2.31×10^{-1}		3.62×10^{-2}	2.03×10^{-1}	7.65×10^{-1}	7.22×10^{-5}	1.43×10^{-3}
	Raw water 2	$5.23 \times 10^{-2} \pm$	$4.79 \times 10^{-2} \pm$	-	$4.18 \times 10^{-3} \pm$	-	$2.01 \times 10^{-1} \pm$	$5.76 \times 10^{-4} \pm$	$3.10 \times 10^{-3} \pm$
		1.46×10^{-2}	1.38×10^{-2}		1.81×10^{-3}		1.28×10^{-1}	9.99×10^{-4}	3.63×10^{-3}
	Treated water	$6.37 \times 10^{-2} \pm$	$7.32 \times 10^{-2} \pm$	-	$4.16 \times 10^{-2} \pm$	-	$4.12 \times 10^{-1} \pm$	$6.12 \times 10^{-4} \pm$	$3.73 \times 10^{-3} \pm$
		2.27×10^{-2}	1.57×10^{-1}		1.70×10^{-2}		6.49×10^{-1}	3.76×10^{-4}	1.04×10^{-3}
NW-C	Raw water	$7.67 \times 10^{-2} \pm$	$3.85 \times 10^{-2} \pm$	-	-	-	$1.44 \times 10^1 \pm$	$3.50 \times 10^{-4} \pm$	$6.03 \times 10^{-2} \pm$
		2.33×10^{-2}	1.60×10^{-2}				1.33×10^1	1.89×10^{-6}	2.02×10^{-2}
	Treated water	-	<LOD	-	-	-	$6.94 \times 10^{-2} \pm$	ND	$7.97 \times 10^{-2} \pm$
							1.42×10^{-1}		4.81×10^{-2}

LOD – limit of detection; ND – not done; “-” – not detected by end-point PCR.

4.3.5. Quantification of antibiotic residues in drinking water production facilities

Raw and treated water of NW-E and NW-C were subjected to SPE and chemical analysis to extract and quantify antibiotic residues and fluconazole. Tables 4.5 and 4.6 represent the concentrations of these target compounds. In NW-E DWPF, all five of these were higher in raw than in treated water. However, in NW-C DWPF, one out of five target compounds was found to be at higher concentrations in treated than in raw water.

4.3.5.1. Quantification of antibiotic residues in at NW-E drinking water production facility

Ampicillin was the most prevalent antibiotic, with an average of 11.87 µg/L in the NW-E DWPF. The ampicillin concentrations were generally higher in raw water 1 than in raw water 2, except for samples collected in February 2021 and August 2021 (Table 4.5). In February 2021, the ampicillin concentrations were 25.81 µg/L in raw water 1 and 11.79 µg/L in raw water 2. In August 2021, the ampicillin concentrations were 27.77 µg/L in raw water 1 and 11.92 µg/L in raw water 2 (Table 4.5). Overall, ampicillin was more prevalent in raw than in treated water. However, the differences were not significant (p-value >0.05).

In the NW-E DWPF, a general trend for ciprofloxacin was observed in which the antibiotic was <LOQ in November 2020, as shown in Table 4.5. Furthermore, ciprofloxacin was more prevalent in raw water 1 than raw water 2, except for samples collected in October 2020 and February 2021. An unexpected highest ciprofloxacin concentration of 46.52 µg/L was recorded in raw water 1. In December 2020, ciprofloxacin was detected in treated water to a greater extent than in raw water 2. However, in general, the differences were not significant (p-value >0.05).

Generally, trimethoprim concentrations were higher in raw than in treated water (Table 4.5), with no significant differences (p-value >0.05) in NW-E DWPF. However, in November 2020 and August 2021, trimethoprim concentrations were higher in treated than in raw water. Overall, trimethoprim ranged from <LOQ to 0.82 µg/L, averaging 0.19 µg/L. In addition, trimethoprim was detected more in raw water 1 than raw water 2 and treated water.

Sulfamethoxazole concentrations were higher in raw than in treated water, with no significant differences (p-value >0.05). However, sulfamethoxazole concentrations were higher in treated

(2.64 µg/L) than in raw water (2.15 µg/L). Sulfamethoxazole concentrations for most samples was <LOQ in November 2020, February 2021, July 2021 and August 2021 (Table 4.5).

Fluconazole concentrations were generally <LOQ for most sampling months (Table 4.5). However, in February 2022, fluconazole was quantified, and the results showed that it was prevalent in raw water 2 at 19.03 µg/L, followed by raw water 1 at 9.49 µg/L and treated water at 8.34 µg/L. No significant differences (p-value >0.05) existed between raw and treated water.

4.3.5.2. Quantification of antibiotic residues in NW-C drinking water production facility

Generally, ampicillin concentrations were higher in raw (7.39 µg/L) than in treated water (6.62 µg/L), as shown in Table 4.6, with no significant differences (p-value >0.05). However, in August 2021, ampicillin concentrations were higher in treated (3.15 µg/L) than in raw water (2.75 µg/L). Furthermore, in February 2022, the concentrations were 6.65 µg/L in raw water and 7.45 µg/L in treated water.

Generally, sulfamethoxazole concentrations were higher in raw (0.95 µg/L) than in treated water (0.76 µg/L) (Table 4.6), with no significant differences (p-value >0.05). Sulfamethoxazole concentrations in raw and treated water were <LOQ in November 2020, February 2021, July 2021 and August 2021. The sulfamethoxazole concentration averaged at 0.85 µg/L, with a highest concentration of 3.51 µg/L.

Generally, ciprofloxacin concentrations were higher in raw (0.68 µg/L) than in treated water (0.53 µg/L) (Table 4.6), with no significant differences (p-value >0.05). Ciprofloxacin concentrations of 1.09 µg/L recorded in February 2022 were constant in raw and treated water. Ciprofloxacin's lowest and highest concentrations were <LOQ and 2.42 µg/L, respectively, averaging 0.85 µg/L.

Fluconazole concentrations in raw and treated water were <LOQ from November 2020 until August 2021. In February 2022, the fluconazole concentrations were 27.71 µg/L in raw water and 13.84 µg/L in treated water. No significant differences (p-value >0.05) were observed. Unlike other target compounds, trimethoprim concentrations were lower in raw (0.07 µg/L) than in treated water (0.10 µg/L) (Table 4.6), with no significant differences (p-value >0.05).

4.3.6. Risk quotient of drinking water production facilities

Figure 4.9 shows the determined RQ of the DWPFs in NW-E and NW-C. A general trend was observed in which the RQ of ampicillin, ciprofloxacin and sulfamethoxazole in raw and treated water in NW-E and NW-C was ≥ 1 . However, the ciprofloxacin RQ for treated water of the NW-C DWPF was <1 . The RQ of fluconazole was <1 in NW-E DWPF and >1 in NW-C DWPF. Another trend was that the RQ of trimethoprim in raw and treated water of NW-E and NW-C was <0.1 .

Table 4.5: Concentrations with standard deviations of target fluconazole and antibiotic residues in raw and treated water of NW-E DWPF.

Date	Sampling sites	Ampicillin ($\mu\text{g/L}$)	Ciprofloxacin ($\mu\text{g/L}$)	Sulfamethoxazole ($\mu\text{g/L}$)	Trimethoprim ($\mu\text{g/L}$)	Fluconazole ($\mu\text{g/L}$)
Oct-20	Raw inlet 1	5.95 $\pm$ 0.84	3.43 $\pm$ 0.41	3.46 $\pm$ 0.52	<LOQ	<LOQ
	Raw inlet 2	12.97 $\pm$ 0.12	4.91 $\pm$ 0.73	3.78 $\pm$ 0.13	<LOQ	<LOQ
	Treated water	8.10 $\pm$ 0.85	3.42 $\pm$ 0.28	2.85 $\pm$ 0.49	<LOQ	<LOQ
Nov-20	Raw inlet 1	8.90 $\pm$ 1.13	<LOQ	1.53 $\pm$ 0.37	0.18 $\pm$ 0.00	<LOQ
	Raw inlet 2	11.55 $\pm$ 2.09	<LOQ	<LOQ	<LOQ	<LOQ
	Treated water	11.53 $\pm$ 4.15	<LOQ	<LOQ	0.19 $\pm$ 0	<LOQ
Dec-20	Raw inlet 1	16.85 $\pm$ 3.45	0.95 $\pm$ 0.18	2.03 $\pm$ 0.37	0.82 $\pm$ 0.04	<LOQ
	Raw inlet 2	24.58 $\pm$ 4.59	0.74 $\pm$ 0.23	2.00 $\pm$ 0.13	0.24 $\pm$ 0.01	<LOQ
	Treated water	12.56 $\pm$ 0.37	0.83 $\pm$ 0.18	<LOQ	0.20 $\pm$ 0.01	<LOQ
Feb-21	Raw inlet 1	25.81 $\pm$ 1.81	2.25 $\pm$ 0.05	<LOQ	<LOQ	<LOQ
	Raw inlet 2	11.79 $\pm$ 2.37	2.83 $\pm$ 0.74	<LOQ	0.17 $\pm$ 0.03	<LOQ
	Treated water	10.02 $\pm$ 0.04	0.60 $\pm$ 0.04	<LOQ	<LOQ	<LOQ
Jul-21	Raw inlet 1	4.20 $\pm$ 0.17	1.32 $\pm$ 0.20	<LOQ	0.40 $\pm$ 0.03	<LOQ
	Raw inlet 2	6.10 $\pm$ 2.34	0.87 $\pm$ 0.01	<LOQ	0.22 $\pm$ 0.02	<LOQ
	Treated water	2.83 $\pm$ 0.38	0.55 $\pm$ 0.07	<LOQ	0.25 $\pm$ 0.01	<LOQ
Aug-21	Raw inlet 1	27.77 $\pm$ 1.29	1.37 $\pm$ 0.30	<LOQ	0.40 $\pm$ 0.01	<LOQ
	Raw inlet 2	11.92 $\pm$ 0.06	<LOQ	<LOQ	<LOQ	<LOQ
	Treated water	3.27 $\pm$ 0.10	1.00 $\pm$ 0.06	<LOQ	0.39 $\pm$ 0.05	<LOQ
Feb-22	Raw inlet 1	18.89 $\pm$ 3.81	46.52 $\pm$ 1.47	2.64 $\pm$ 0.26	0.35 $\pm$ 0.02	9.49 $\pm$ 0.78
	Raw inlet 2	20.08 $\pm$ 3.68	0.44 $\pm$ 0.00	1.65 $\pm$ 0.12	0.33 $\pm$ 0.01	19.03 $\pm$ 5.78
	Treated water	14.18 $\pm$ 3.81	0.75 $\pm$ 0.09	2.64 $\pm$ 0.12	0.32 $\pm$ 0.04	8.34 $\pm$ 1.50

LOQ – limit of quantification.

Table 4.6: Concentrations with standard deviations of target fluconazole and antibiotic residues in raw and treated water of NW-C DWPF.

Date	Sampling sites	Ampicillin (µg/L)	Ciprofloxacin (µg/L)	Sulfamethoxazole (µg/L)	Trimethoprim (µg/L)	Fluconazole (µg/L)
Nov-20	Raw inlet	16.93±3.69	<LOQ	<LOQ	0.16±0.01	<LOQ
	Treated water	15.72±0.08	<LOQ	<LOQ	0.16±0.03	<LOQ
Dec-20	Raw inlet	5.04±0.41	<LOQ	3.51±0.14	<LOQ	<LOQ
	Treated water	3.91±1.34	<LOQ	2.64±0.95	0.25±0.05	<LOQ
Feb-21	Raw inlet	10.50±2.06	2.42±0.27	<LOQ	<LOQ	<LOQ
	Treated water	7.08±1.29	2.08±0.28	<LOQ	<LOQ	<LOQ
Jul-21	Raw inlet	2.48±0.95	0.56±0.07	<LOQ	0.24±0.03	<LOQ
	Treated water	2.43±0.76	<LOQ	<LOQ	0.21±0.02	<LOQ
Aug-21	Raw inlet	2.75±0.09	<LOQ	<LOQ	<LOQ	<LOQ
	Treated water	3.15±0.09	<LOQ	<LOQ	<LOQ	<LOQ
Feb-22	Raw inlet	6.65±0.65	1.09±0.06	2.17±0.05	<LOQ	27.71±9.06
	Treated water	7.45±0.15	1.09±0.06	1.91±0.31	<LOQ	13.84±1.12

LOQ – limit of quantification

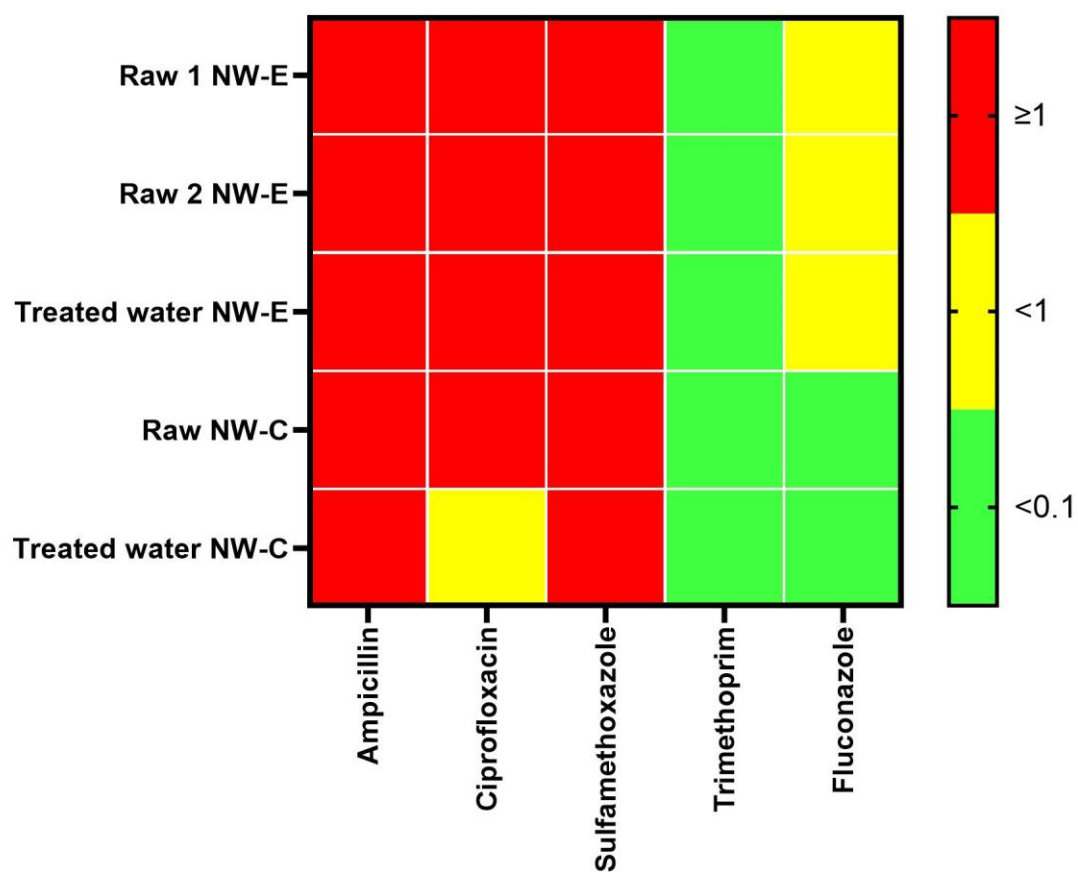


Figure 4.9: Heatmap of risk quotients (RQs) in raw and treated water of NW-E and NW-C DWPFs.

4.3.7. Correlation of bacterial communities with ARGs, antibiotics and physicochemical parameters in drinking water production facilities

The RDA was used to show the correlation between bacterial communities of ARGs, antibiotics and physicochemical parameters, as shown in Figure 4.10. Overall, no significant difference was found for the emergence of relative abundance percentage of bacterial communities with antibiotic residues, ARGs and physicochemical parameters in all canonical axes. However, *Fusobacterium*, *Peptoniphilus* and other genera were associated with nitrate. The clustering of *Armatimonas*, *Clade III*, *CL500-29 marine group* was strongly correlated with physicochemical parameters (such as temperature, TDSs, salinity, nitrite and phosphate), ARGs (such as *MOX*, *DHA*, *ACC*, *sul1*, *sul2*, *intl1*, *ampC*, *bla_{TEM}* and *ermF*) and antibiotic residues (ampicillin and ciprofloxacin). *Zymomonas* and *Comamonas* were correlated with EBC, FOX and *ermB*. *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was clustered with COD and fluconazole. *Nocardia*, *Dickeya* and unassigned genera did not strongly correlate with physicochemical parameters, ARGs and antibiotic residues. Despite the unassigned genera having the highest relative abundance percentage, the presence of unassigned bacterial community did not have a strong association with ARGs, antibiotics and physicochemical parameters. Similar observations were recorded for *Dickeya*, *Fusobacterium*, *Nocardia*, *Peptoniphilus* and other genera.

Table B1 in Appendix B shows that the correlation matrices in Statistica Software established significant differences among some bacterial community compositions, physicochemical parameters, ARGs and antibiotic residues. Due to a considerable amount of data generated in the present study, the correlation matrices in this section were based on the significant correlation of bacterial community composition against physicochemical parameters, ARGs and antibiotic residues. *Armatimonas* (p-value = 0.25) and *Dickeya* (p-value = 0.01) were significantly correlated with the *ampC* gene. *CL500-29 marine group* significantly correlated with pH (p-value = 0.34), phosphate, p-value = 0.12), *ACC* gene (p-value = 0.45), *MOX* gene (p-value = 0.01), ciprofloxacin (p-value = 0.04) and sulfamethoxazole (p-value = 0.44). *Clade III* had a significant correlation with the *ACC* gene (p-value = 0.44), *Bla_{TEM}* gene (p-value = 0.19), *intl1* gene (p-value = 0.33) and ampicillin (p-value = 0.04). *Zymomonas* significantly correlated with *intl1* (p-value = 0.49) and *ermB* (p-value = 0.31)

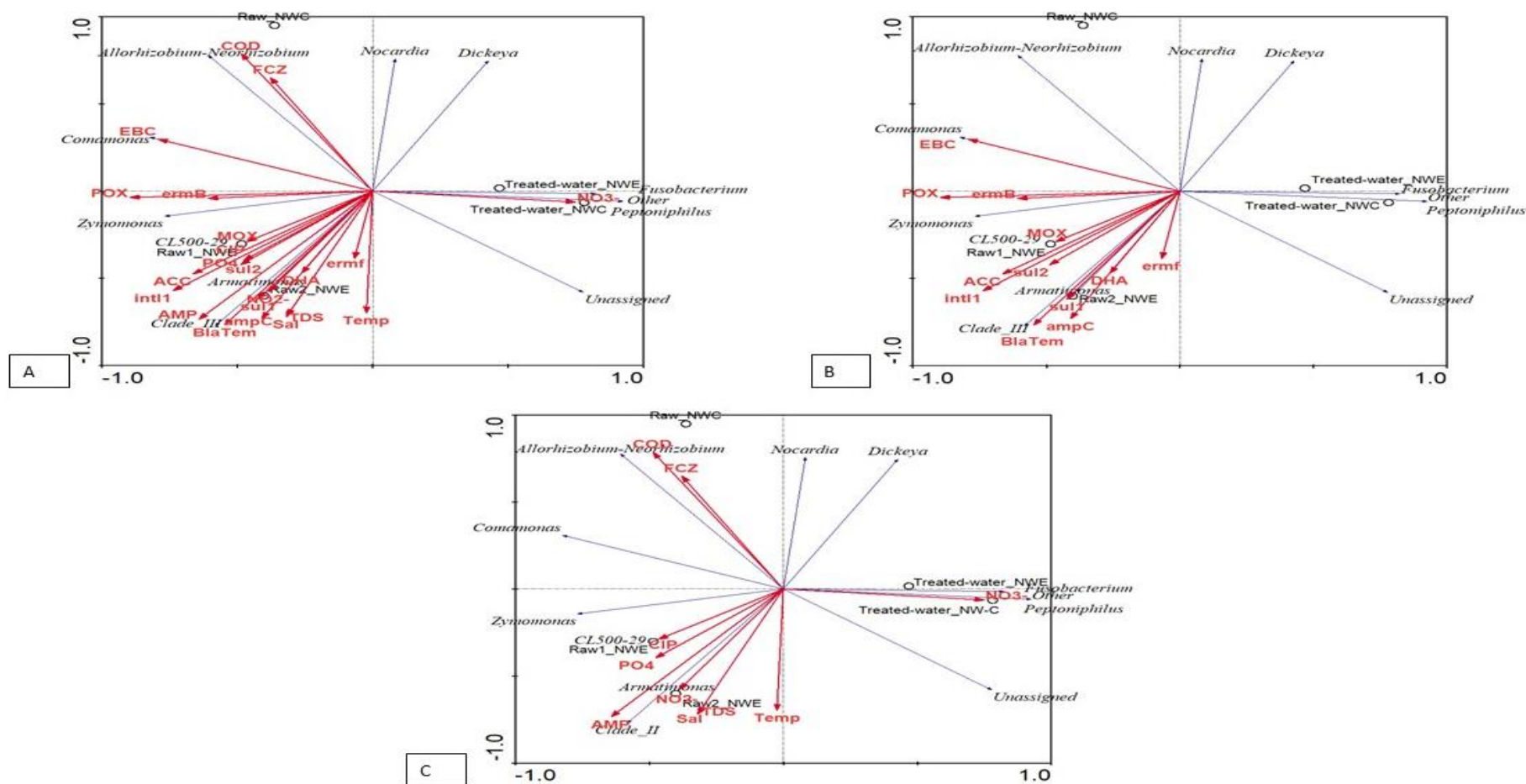


Figure 4.10: RDA of correlation between (a) bacterial community composition, physicochemical parameters, ARGs and antibiotic residues (b) bacterial community composition and ARGs and (c) bacterial community composition, physicochemical parameters and antibiotic residues in raw and treated water of NW-E and NW-C DWPFs.

FCZ – fluconazole; temp – temperature; AMP – ampicillin; sal – salinity; COD - chemical oxygen demand, TDSs - total dissolved solids; SMX – sulfamethoxazole; NO₂⁻ - nitrite, NO₃⁻ - nitrate; PO₄ – phosphate; TMP – trimethoprim

4.4. DISCUSSION

4.4.1. Physicochemical parameters in drinking water production facilities

According to the recent Blue Drop report (DWS, 2022b), microbiological monitoring compliance in the North West Province was measured to be 25.2% and chemical monitoring compliance 16.2%. The same report states that technical skills were at 15.8%, while 7.5% of DWPFs had water safety plans. According to the DWS (2022b), the North West Province Blue Drop Risk Rating was 82.3%.

However, the performance of the NW-E (13.8% Blue Drop Risk Rating) and NW-C (35.7% Blue Drop Risk Rating) DWPFs was above the provincial average (DWS, 2022b). The present study measured physicochemical levels in NW-E DWPF at generally higher levels in raw water than in treated water. Significant differences (p -value <0.05) between raw and treated water were observed for pH, nitrite and phosphate. In contrast, NW-C measured higher levels of physicochemical parameters in treated water than in raw water. Significant differences (p -value <0.05) between raw and treated water were observed for nitrate. The results indicate that the two DWPFs were operated well (see DWS, 2022b), hence the low risk rating that they received can be confirmed.

4.4.1.1. pH

Generally, in NW-E and NW-C, the pH was higher in the raw water (8.41) than in treated water (8.20). Overall, the measured pH in NW-E and NW-C treated water were found to be within the recommended standards (range 6.0 - 9.0) of DWAF SAWQG (1996a) for domestic use, which means that it has no significant health effects. However, in NW-C, a pH of 9.28 was recorded in July 2021, which could have rendered the water bitter (DWAF SAWQG, 1996a). Alkaline pH measurement in treated water in DWPFs in North West is not uncommon, as measured by Ateba *et al.* (2020).

In the present study, the pH was generally found to be above 8.00. This could have affected the disinfection process in NW-E and NW-C, as these DWPFs use chlorination that requires a pH of less than 8.00 for optimum disinfection (WHO, 2022), which could have affected the bacterial community composition in the distribution systems.

4.4.1.2. Nitrate and nitrite

The nitrate levels measured in NW-E and NW-C DWPFs were found to be within the recommended limit of the DWAF SAWQG (1996a) for domestic use, in which nitrate does not have adverse health effects on humans. In the recently upgraded NW-C DWPF, nitrate levels were higher in treated (1.98 mg/L) than in raw water (1.46 mg/L). This could be attributed to the configuration and operations of the NW-C DWPF. After the sand filtration, water collected in a sump was quickly subjected to disinfection. This does not usually provide sufficient time to settle suspended particles (Kritzing, 2019).

In contrast, in NW-E DWPF, nitrate levels were higher in raw water (2.44 mg/L) than in treated water (1.67 mg/L). The study by García-Avila *et al.* (2021) aimed to determine water quality in the Bayas DWPF, Ecuador, after modernization reported that, before the modernization, the nitrate levels were 2.81 mg/L in raw water and 4.18 mg/L in treated water. However, after modernization, nitrate levels were reduced from 2.25 mg/L in raw water to 0.79 mg/L in treated water. The nitrate levels of 1.00 mg/L were reported by Li *et al.* (2021) in both raw and treated water of the DWPF situated in Suzhou, Jiangsu Province, China. High nitrate levels of 76.7 mg/L recorded in raw water were measured by Desye *et al.* (2021) in Gondar City, Northwest Ethiopia.

The nitrite levels measured in NW-E and NW-C DWPFs were found to be within the recommended limit of the DWAF SAWQG (1996a) for domestic use, in which nitrate does not have adverse health effects on humans. Overall, in DWPFs, the nitrite levels were higher in raw than treated water. In NW-E DWPFs, nitrite levels were significantly higher (p -value <0.05) in raw (2.44 mg/L) than in treated water (1.66 mg/L). This could be attributed to the sedimentation process that allowed suspended nitrite to settle out of the suspension. However, in NW-C DWPF, nitrite levels were 2.33 mg/L in raw and 1.67 mg/L in treated water. However, no significant differences ($p >0.05$) were observed in NW-C raw and treated water. A similar trend was observed by Liu *et al.* (2021) and Deyab *et al.* (2021) where the DWPFs reduced the nitrite levels from raw to treated water in Kafr El-Shinawy, Damietta, Egypt and Suzhou, Jiangsu Province, China.

4.4.1.3. Phosphate

In the recently upgraded NW-C DWPF, the phosphate levels were higher in treated than in raw water. This could be attributed to the inability of the sump to collect water for a sufficient period to settle out suspended phosphate under the effect of gravity after sand filtration. The same observations were made by Maguvu *et al.* (2020) in the same DWPF. In contrast, in NW-E DWPF, although the phosphate levels were higher in February 2021 in treated water, the raw water contained significantly higher ($p < 0.05$) phosphate levels than treated water. This indicates the importance of the sedimentation process that is applied to treat phosphates. In a study by García-Avila *et al.* (2021), the phosphate levels were 0.32 mg/L in raw water and 0.42 mg/L in treated water before modernization of the DWPF. After the modernization, the phosphate levels decreased from 0.26 mg/L in raw water to 0.08 mg/L in treated water. Although there was a huge phosphate decrease from raw water to treated water in the Gondar City DWPF, the treated water contained high phosphate levels of 42.77 mg/L, which were not comparable to those recorded in NW-E and NW-C (Desye *et al.*, 2021). However, the DWAF SAWQG (1996a) for domestic use does not provide recommended phosphate standards.

4.4.1.4. Total dissolved solids

In the NW-E DWPF, TDS levels of 549.69 mg/L in raw water were higher than the 548.50 mg/L in treated water. However, this reduction was not significant (p -value > 0.05). The TDS levels were found to be within the 450 – 1 000 mg/L range, according to the DWAF SAWQG (1996a) for domestic use, which stated that water within this range had a noticeable salty taste but was well tolerated. The high TDS levels in NW-E were associated with mining activities. However, in NW-C DWPF, the TDS levels were higher at 409.00 mg/L in treated water than the 406.25 mg/L in raw water. No significant differences (p -value > 0.05) existed between raw and treated water. The TDS levels measured in NW-C treated water were found to be within the 0 – 450 mg/L range. The DWAF SAWQG (1996a) for domestic use stated that water with extremely low TDS levels might be objectionable because of its flat, insipid taste. Similar observations were reported by Ateba *et al.* (2020) for all seasons in the two DWPFs situated in the North West, where TDS levels were found to increase from raw to treated water. García-Avila *et al.* (2021) reported low TDS of less than 100 mg/L in raw and treated water from Azogues, Ecuador. According to DWAF SAWQG (1996a), when it comes to domestic use, the measured TDS levels in NW-E and NW-C in treated water were found to be within the recommended range (0 – 1000 mg/L), which has no health effects on consumers.

4.4.1.5. Salinity

Salinity levels of 250.38 mg/L measured in raw water were higher than the 252.75 mg/L in treated water in NW-C. Similar observations were made in which salinity levels of 342.06 mg/L in raw water were higher than the 340.25 mg/L in treated water of NW-E DWPF. However, significant differences were not observed in raw and treated water between these two DWPFs. Mining activities in NW-E could have had an impact on the high salinity levels. Similar results were reported in Al-Baradi'yah and Al-Abbas DWPFs in Basrah, Iraq, where salinity was reduced from raw to treated water (Hussein *et al.*, 2022). However, the measured salinity levels in Basrah DWPFs were higher than those of the present study. The salinity levels in raw and treated water in these cases were 2930-2890 mg/L and 730-560 mg/L, respectively. Low salinity levels were measured in the Johor River (which ranged from 0.5 to 26.8 mg/L) in Malaysia as well as packaged sachet water sold in Dutse Metropolis Jigawa State (which ranged from 25.40 mg/L to 136.40) in Nigeria (Shiaka *et al.*, 2021; Liang *et al.*, 2020). Salinity levels in NW-E and NW-C treated water were found to be within the 200 – 400 mg/L range, as proposed by DWAF SAWQG (1996a) for domestic use, indicating that treated water was slightly salty taste; it was unpleasant for individuals on a sodium-restricted diet.

4.4.1.6. Temperature

The minimum and maximum temperatures in NW-E were 10.30°C and 24.40°C, respectively. In NW-C, the minimum and maximum temperatures were 13.60°C and 20.90°C. In NW-E and NW-C, the minimum and maximum temperatures were recorded in winter and summer. A similar trend was observed in two other DWPFs and distribution networks in North West, South Africa (Ateba *et al.*, 2020). Temperature variations might have influenced heterotrophic bacterial populations (Maguvu *et al.*, 2020).

4.4.1.7. Chemical oxygen demand

In NW-E, the COD was higher in treated than in raw water. Although the COD levels were higher in NW-C treated water for some samples, the overall levels were higher in raw water. Generally, the COD levels measured in the NW-E and NW-C were higher than those in Chinese DWPFs (Sun *et al.*, 2020). However, the COD levels measured in raw water samples of the present study were lower than those measured in Nigeria's Ose, Owena and Ala Rivers' water (Idowu *et al.*, 2022). The COD represents the amount of dissolved oxygen required in the water required to oxidize all the organic matter (EPA, 2021).

Despite higher levels of some physicochemical parameters from raw to treated water in the NW-E and NW-C DWPFs, treated water was found to be within the DWAF SAWQG (1996a) for domestic use range, which does not cause adverse health to consumers. However, although treated water does not cause adverse human health, it is believed to contain nutrients, pH and temperature that may promote bacterial growth and replication (Ateba *et al.*, 2020; Maguvu *et al.*, 2020).

4.4.2. Bacterial community composition

The study of microbial pollutants in DWPFs of developing countries such as South Africa is underexplored. This has led to the lack of information on the differences among bacterial communities of raw and treated water in DWPFs. As a result, bacterial communities could have migrated, including potential pathogenic bacteria, from raw to treated water, while experts may not know of these. Hence, the present study investigated bacterial communities in raw and treated water of the DWPFs in the North West Province, South Africa. The investigation was conducted by using NGS of the bacterial 16S rRNA. The raw sequences were exported to QIIME trimming and OTU classification of the bacterial 16S rRNA was made. The Silva 16S rRNA (Release 138) database was used for OTU classification. The results of the classification can elucidate the nature of microbial communities to improve the state of DWPFs.

In NW-E and NW-C DWPFs, the dominant phylum was Proteobacteria, which ranged from 36.57% to 48.49% in raw water and 40.22% to 50.63% in treated water, with a high proportion of *Comamonas* and *Dickeya*. Consistent with previous studies, the dominance of in high relative abundance percentage was reported in raw water, treated water and drinking water distribution systems (Holinger *et al.*, 2014; Potgieter *et al.*, 2018; Liu *et al.*, 2018; Picazo *et al.*, 2019; Vadde *et al.*, 2019; Jing *et al.*, 2021; Tiwari *et al.*, 2021). Furthermore, Han *et al.* (2020) reported an increase of some genera within the Proteobacteria from raw to treated water in 46 drinking water supply systems in China.

Comamonas is a group Gram-negative bacilli and non-fermentative bacteria (Wu *et al.*, 2018; Ryan *et al.*, 2022). It inhabits soil, wastewater, freshwater and internal animal microbiome environments. Most *Comamonas* species are aerobic chemoheterotrophs, and some are facultative anaerobes capable of using ferric iron or nitrate as alternative electron acceptors (Wu *et al.*, 2018; Ryan *et al.*, 2022). They are not monitored by the WHO (2022) and in terms of DWAF (1996) drinking water guidelines. Most of them are considered non-pathogenic or

are rarely associated with human infection. They have been isolated in clinical samples such as blood, pus, faeces, urine and kidneys (Ryan *et al.*, 2022).

Some *Comamonas* species have been reported to cause invasive infections such as meningitis, appendicitis and bacteraemia (Opota *et al.*, 2014; Wu *et al.*, 2018). Immunocompromised children and patients with cancer and AIDS are at risk of contracting the latter (Opota *et al.*, 2014). The risk may even be heightened for such individuals when they consume drinking water containing *Comamonas* species.

A high relative abundance percentage of *Dickeya* occurred in treated water of NW-E and NW-C DWPFs. *Dickeya* is a plant pathogen that accounts for the economic loss of important ornamental and crop plants and is ranked among the top ten phytopathogen bacteria (Pritchard *et al.*, 2013; Pédrón and Gijsegem, 2023). Residents of NW-E and NW-C who have gardens might therefore have contaminated their plants with *Dickeya* through irrigation. *Dickeya* shows pectinolytic activity that is pathogenic to plant tissues (Van Vaerenbergh *et al.*, 2012). Pédrón and Gijsegem (2023) established that the different *Dickeya* species isolated in potatoes and water harboured few specific traits and fewer specific gene losses that are associated with human pathogens. This indicates that *Dickeya* species have a wide range of hosts.

Although phylum Bacteroidota constituted the second most relative abundance percentage in NW-E and NW-C DWPFs, its bacterial genera were deemed to be unclassified or others. *Bacteroides* is frequently found in mammalian guts, and is thus an ideal indicator of faecal contamination and is used for microbial source tracking (Vadde *et al.*, 2019; Elsaghir and Reddivari, 2022). Furthermore, *Bacteroides* is also pathogenic to humans, with a 19% mortality rate (Elsaghir and Reddivari, 2022). Hence, the classification of Bacteroidota in treated water is of utmost importance for surveillance and, subsequently, for cushioning their effects on water consumers in NW-E and NW-C.

The relative abundance percentage of the phylum Firmicutes was higher in NW-E and NW-C treated water than in raw water. The relative abundance of the phylum was reported in Northern China's treated water and drinking water distribution systems (Jing *et al.*, 2021). Its high relative abundance in treated water may be associated with the formation of endospores that enable its species to resist desiccation and environmental stress such as chlorination (Bautista-de Los Santos *et al.*, 2019). Furthermore, *Peptoniphilus*, which belongs to Firmicutes, increased from raw to treated water in NW-E and NW-C DWPFs. It was introduced to classify strictly anaerobic Gram-positive cocci, formerly called *Peptostreptococcus*.

Bacterial species belonging to *Peptoniphilus* may act as opportunistic pathogens (Aujoulat *et al.*, 2021).

The relative abundance percentage of *Nocardia* of the phylum Actinomycetota was higher in NW-E treated than in raw water. However, contradictory results were obtained in NW-C DWPF. Bacterial species belonging to phylum Actinomycetota survive dormant when they encounter desiccation and environmental stress, such as chlorination (Bautista-de Los Santos *et al.*, 2019). Hence, the relative abundance percentage of *Nocardia* was higher in treated than in raw water. *Nocardia* is a rod-shaped, partially acid-fast actinobacteria. It is a saprophyte mostly known as an opportunistic pathogen (Männle *et al.*, 2020). The unassigned and other phylum constituted 56.19% and 3.96% of the total relative abundance percentage in NW-E and NW-C DWPFs.

The NW-E and NW-C DWPFs therefore affected the bacterial community from water to treated water, which is consistent with studies that show the influence of water treatment on the bacterial community (Liu *et al.*, 2018; Jing *et al.*, 2021). The chlorination process used in NW-E and NW-C DWPFs is important for reducing bacterial communities. However, surviving bacterial communities utilize available nutrients for reproduction (Potgieter *et al.*, 2018). Subsequently, they are spread into distribution networks of vulnerable groups, including infants, immunocompromised patients and the elderly, all of whom are in direct contact with contaminated water that may harbour pathogens.

The alpha diversity was assessed by calculating the species diversity and evenness (Shannon index), richness (Chao1 and ACE estimator) and species dominance (Simpson index). No significant differences were found between raw and treated water from NW-E and NW-C. Similar observations showed that treated water in NW-E and NW-C was more even, diverse and richer. However, dominance in NW-E and NW-C DWPFs was higher in raw water 1 and treated water. Furthermore, the overall calculated dominance of raw water 1 and 2 was the same as that of treated water in NW-E. This might have been due to the inability of the NW-E and NW-C DWPFs to reduce the relative abundance of bacterial communities.

In NW-E and NW-C DWPFs, the relative abundance percentage of the unassigned, other and *Dickeya* genera was higher in treated than in raw water. Furthermore, the relative abundance percentage of *Nocardia* and *Peptoniphilus* was higher in treated water of NW-E and NW-C. The increased alpha diversity in treated water might have been associated with the bacterial community's enrichment by means of biofilm formation. Bacterial regrowth could also be attributed to increased bacterial community in treated water (Holinger *et al.*, 2014). This is

inconsistent with Han *et al.* (2020) and Tiwari *et al.* (2021), who reported low alpha diversity in treated water in China and Finland, which might have indicated biologically stable water (Tiwari *et al.*, 2021).

The present study found bacterial communities from raw to treated water of NW-E and NW-C DWPFs. Core communities between raw and treated on the one hand, and the two DWPFs on the other, were similar. Fluctuation occurred between raw and treated water. It is worth noting that the relative abundance percentage of *Nocardia* and *Peptoniphilus* was higher in treated than in raw water due to the formation of biofilm formation, which enabled them to remain dormant during unfavourable conditions posed by chlorination (Bautista-de Los Santos *et al.*, 2019). Higher relative abundance from raw to treated water of the bacterial community was observed in the NW-E and NW-C systems. These treated waters were more diverse and richer than raw water. Furthermore, some bacterial genera in the treated water were potential pathogens of humans and plants (Pritchard *et al.*, 2013; Opota *et al.*, 2014; Wu *et al.*, 2018; Pédrón and Gijsegem, 2023).

4.4.3. Antibiotic resistance and *int1* genes in drinking water production facilities

The results obtained in the NW-E and NW-C were compared to those of other studies. The detection percentages and concentrations varied due to various activities, including clinical settings, agricultural activities and anthropogenic activities occurring in different water environments.

4.4.3.1. *AmpC* and *bla*_{TEM} gene screening

The *bla*_{TEM} gene was absent in the NW-C DWPF. The *bla*_{TEM} gene detection in NW-E DWPF was 16.67% for raw water and 9.09% for treated water. This shows a discrepancy with results obtained in Southwestern Nigeria, where a high *bla*_{TEM} gene detection was made of 81.25% in raw water and 75.00% in treated water (Adesoji and Ogunjobi, 2016). Another high *bla*_{TEM} gene detection level of 100% was reported in treated water in Tianjin, China (Liang *et al.*, 2022). Furthermore, the *bla*_{TEM} gene was also detected in all bacterial species isolated in pristine lakes of the Kashmir valley, India (Sultan *et al.*, 2020).

In NW-C DWPF, the *ampC* gene was not detected in raw and treated water. However, in NW-E DWPF, *ampC* gene was detected in raw (18.18%) and treated water (10.00%). Results obtained in drinking water from Manitoba, Canada, resonate with the low detection results of

the present study, in which the *ampC* gene was detected in 1/3 three distribution systems investigated (Mi *et al.*, 2019). In contrast, the *ampC* gene was frequently detected in bacteria such as *E. coli*, *Brevibacterium frigoritolerans*, *Pseudomonas*, *Shigella* and *Aeromonas* species isolated in pristine lakes of the Kashmir valley, India (Sultan *et al.*, 2020).

According to the NDoH (2018), South Africa has the highest broad-spectrum penicillin use, up to 3.3 times than other BRICS countries, the United Kingdom and the United States. However, in raw and treated water, *ampC* and *bla_{TEM}* genes that confer resistance to β -lactams were present in a low number of water samples (Adesoji and Ogunjobi, 2016). This does not entail a low β -lactam resistance in water samples, as other β -lactam resistance genes were reported, as reflected in Chapter 2, which were not included in the present study.

4.4.3.2. *ErmB* and *ermF* gene screening

The present study found *ermB* genes in 41.67% of raw and treated water samples in the NW-E DWPF. However, in NW-C DWPF, the detection of *ermB* gene was higher in raw (36.36%) than in treated water (18.18%). In Tianjin, China, the detected *ermB* gene in treated water was 25% for both the intracellular and extracellular DNA (Liang *et al.*, 2022). In Louisiana, United States, the *ermB* gene was absent in raw and treated water (Bergeron *et al.*, 2017). Furthermore, Yang *et al.* (2020) recorded an increase in the *ermB* gene from raw to treated water in DWPF situated in Jiaxing City, China.

A similar trend was observed in NW-E and NW-C, namely that *ermF* gene detection was constant from raw to treated water. Furthermore, the detected *ermF* gene from NW-E and NW-C were comparable. The levels for this gene in NW-E and NW-C were 58.33% and 54.55%, respectively. In contrast with these findings, the DWPF in Jiaxing City, China, could reduce the prevalence of the *ermF* gene from raw to treated water (Yang *et al.*, 2020).

The spread of the *ermB* and *ermF* genes to humans through the food chain may engender the class of macrolides-lincosamides-streptogramin B antibiotics (Zhang *et al.*, 2017; Severgnini *et al.*, 2021). The bacterial resistance mechanism of the *ermB* gene is ribosomal protection (Bergeron *et al.*, 2017). The *ermF* is a methylase-type erythromycin resistance gene (Severgnini *et al.*, 2021). The presence of *ermB* and *ermF* genes could have contributed to the frequent prescription of macrolides (16.81%) and lincosamides (1.57%) in private primary health care according to the claims database of a health insurer in South Africa (Alabi and Essack, 2022).

4.4.3.3. *Sul1* and *sul2* gene screening

The present study reported high detection between 40% and 100% in the two DWPFs. However, unlike in NW-E DWPF, the *sul1* gene detection was higher in raw (40.00%) than in treated (45.46%) water in NW-C DWPF. Like NW-E, Jiaxing City DWPFs in China reduced the prevalence of the *sul1* gene from raw to treated water (Yang *et al.*, 2020). In another study conducted in China, the detection level of the *sul1* gene in treated water was 100% (Liang *et al.*, 2022). Low *sul1* gene detection levels of 7.69% in raw water and 0.00% in treated were reported in Louisiana, United States (Bergeron *et al.*, 2017). The *sul1* gene confers resistance by encoding dihydropteroate synthase (Antunes *et al.*, 2005; Yang *et al.*, 2020).

Overall, in the present study, the detection of the *sul1* gene was higher than the *sul2* gene. These results reinforce the findings of Rolbiecki *et al.* (2020). Unlike NW-C DWPF, in NW-E DWPF, the *sul2* gene was detected to a greater extent in treated than in raw water. In Jiaxing City, China, the DWPFs reduced the prevalence of the *sul2* gene from raw to treated water (Yang *et al.*, 2020). According to Antunes *et al.* (2005), the *sul2* gene is more frequently detected in clinical isolates of *E. coli* compared to the *sul1* gene.

Although there is a low prescription (3.16%) of sulphonamides in private primary health care in South Africa (Alabi and Essack, 2022), high sulphonamide resistance genes such as *sul1* and *sul2* were detected in many raw and treated water samples of NW-E and NW-C DWPFs. The spread of sulphonamide resistance genes to humans through consumption may cause more dire health risks in immunocompromised patients infected with HIV. This is due to sulfamethoxazole-trimethoprim commonly used to treat *Pneumocystis* pneumonia in HIV/AIDS patients (Africa Centers for Disease Control and Prevention; CDC, 2021). Furthermore, the *sul1* gene in water serves as a gene marker for the presence of the *int1* gene (Antunes *et al.*, 2005).

4.4.3.4. *Int1* gene screening

In NW-E and NW-C DWPFs, the *int1* gene was detected more in raw than treated water. In NW-E DWPF, the *int1* gene was detected in 87.50% of raw water and 83.33% of treated water samples. In NW-C DWPF, the *int1* gene was detected in 45.45% of raw water and 27.27% of treated water samples. The present study's findings were consistent with those obtained in Kathmandu Valley DWPF in Nepal, where the occurrence of the *int1* gene was reduced from

raw to treated water (Thakali *et al.*, 2020). Furthermore, the *int11* gene detection in Kathmandu Valley DWPF in Nepal was within the detection range obtained in NW-E and NW-C.

The presence of the *int11* gene in water is associated with the facilitation of HGT (Hu *et al.*, 2021). This could facilitate the spread of ARGs into opportunistic bacteria in NW-E and NW-C DWPFs. The *int11* gene is commonly found in various environmental and clinical settings (Chen *et al.*, 2015; Chen *et al.*, 2020; Thakali *et al.*, 2020). This is of concern, since it comprises a shuffled gene cassette containing several ARGs and a gene that encodes a site-specific recombinase. These constituents enable bacteria harbouring *int11* gene to induce multiple drug resistance (Chen *et al.*, 2015).

4.4.3.5. Plasmid-mediated AmpC β -lactamase gene screening

4.4.3.5.1 FOX gene

The FOX was the highest detected pAmpC in the NW-E and NW-C DWPFs, ranging between 25% and 75%. In NW-E and NW-C DWPF, it was detected in raw water to a greater extent than in treated water. The FOX gene was also frequently detected in *K. pneumoniae* and *E. coli* isolated in clinical samples in Hamadan, West of Iran (Hoseini *et al.*, 2017). However, low FOX gene detection in drinking water of 10%, 2.6% and 9.8% isolated in *E. coli*, *Klebsiella* and *Aeromonas* species, respectively, were reported in Maputo, Mozambique (Salamandane *et al.*, 2022a; 2022b). In two Eastern Cape rivers, the average detection of the FOX gene isolated in *Enterobacter* species, *Klebsiella* species and *E. coli* did not exceed 30% (Fadare *et al.*, 2020). In Guangzhou, China, it was absent in river samples (Ye *et al.*, 2017). It is reported that it can complicate the laboratory detection of ESBLs by false-negative or false-positive results (Papp-Wallace *et al.*, 2014). However, the higher FOX gene detection in the present study may be attributed to a culture-independent technique when compared to the culture-dependent techniques used in the studies mentioned above. The FOX gene has a unique amino acid sequence compared to other pAmpCs (Papp-Wallace *et al.*, 2014).

4.4.3.5.2 DHA gene

In NW-C DWPF, the DHA gene was absent in the raw and treated water. Its absence was also reported in *E. coli*, *Klebsiella* and *Aeromonas* species isolated in drinking water in Maputo, Mozambique (Salamandane *et al.*, 2022a; 2022b). In addition to water samples, the DHA gene was not detected in *E. coli* and *K. pneumoniae* from patients in hospitals in Hamadan, West

of Iran (Hoseini *et al.*, 2017). Furthermore, the absence of the DHA gene was reported in rivers in Eastern Cape, South Africa and Guangzhou, China (Ye *et al.*, 2017; Fadare *et al.*, 2020). However, In NW-E DWPF, the detection of the DHA gene was higher in treated (20.00%) than in raw (18.18%) water.

4.4.3.5.3 MOX gene

The MOX gene was only detected in raw water 1 of the DWPF in NW-E. It was absent in other sites of NW-E and NW-C DWPFs. These low detections were comparable to those reported in Maputo, Mozambique, where it was present in two *Aeromonas* species and absent in *Klebsiella* species and *E. coli* (Salamandane *et al.*, 2022a; 2022b). Its absence was also reported in river water in the Eastern Cape, South Africa, and Guangzhou, China (Ye *et al.*, 2017; Fadare *et al.*, 2020). However, the MOX gene was not detected in *K. pneumoniae* and *E. Coli* isolated in clinical samples from hospitals in Hamadan, West of Iran (Hoseini *et al.*, 2017). The presence of the MOX gene in water could be associated with an integron cassette, thus indicating the potential spread of plasmids to other bacteria (Coertze and Bezuidenhout, 2019).

4.4.3.5.4 CIT gene

The CIT gene was not detected in raw and treated water in NW-E and NW-C DWPFs. The same results were reported for *E. coli* in drinking water collected in Maputo, Mozambique (Salamandane *et al.*, 2022a). However, this differed from another study conducted in Maputo, Mozambique, where the CIT gene was present in three *Klebsiella* species and one *Aeromonas* species (Salamandane *et al.*, 2022b). Furthermore, in contrast to its absence detection in the present study, the CIT gene was also present in the Eastern Cape rivers, South Africa (Fadare *et al.*, 2020). These rivers are also used for domestic and agricultural purposes. Moreover, the CIT gene was frequently detected in *E. coil* and *K. pneumoniae* isolated patients in Hamadan, West of Iran (Hoseini *et al.*, 2017).

4.4.3.5.5 ACC gene

Raw water 1 of the NW-E DWPF recorded the highest ACC gene detection level of 81.81% compared to raw water 2 and treated water. The other raw water samples from NW-E and NW-C, ranging between 11.11% and 36.36%, were comparable to the ACC gene detection levels of the Eastern Cape river water (Fadare *et al.*, 2020). The ACC gene detection was

higher in raw water in NW-E (59.09%) and NW-C (18.18) DWPFs. Its detection levels in NW-E and NW-C treated water were 11.11% and 0.00%, respectively. In Maputo, Mozambique, it was detected in drinking water at 12.5%, 15.4% and 7.8% in *E. coli*, *Klebsiella* and *Aeromonas* species, respectively (Salamandane *et al.*, 2022a; 2022b). The ACC gene was not detected in the river and food collected in Guangzhou, China (Ye *et al.*, 2017). Its absence was further reported in clinical samples from three hospitals in Hamadan, West of Iran (Hoseini *et al.*, 2017).

4.4.3.5.6 EBC gene

In NW-E and NW-C DWPFs, detection of the EBC gene was lower in treated than raw water. In NW-E, the EBC gene detection was 18.18% in raw water and 10.00% in treated water. In NW-C, the detection was 22.22% in raw water and 0.00% in treated water. The EBC gene was also detected in two Eastern Cape rivers (Fadare *et al.*, 2020). However, Ye *et al.* (2017) did not detect it in river water and food samples in Guangzhou, China. It was also not detected in clinical samples from hospitals in Hamadan, West of Iran (Hoseini *et al.*, 2017).

The pAmpCs detected in the present study were encoded by plasmids that confer resistance to a wide range of antibiotics belonging to the β -lactam spectrum (Golsha *et al.*, 2021). In addition to their presence in the water environment, pAmpCs have also been isolated in clinically significant specimens, beef, surface swabs and cattle faeces (Coertze and Bezuidenhout, 2019; Golsha *et al.*, 2021; Matloko *et al.*, 2021). This demonstrates the extent to which pAmpcs are widespread in the environment.

The presence pAmpCs in the NW-E and NW-C DWPFs may be linked to the high prescription of β -lactam spectrum antibiotics such as penicillin (40.66%) and cephalosporins (15.73%) in private primary health care in South Africa (Alabi and Essack, 2022). Generally, South Africa has high broad-spectrum penicillin use even when compared to some countries with advanced healthcare facilities (NDoH, 2018). Golsha *et al.* (2021) state that co-occurrence of two or more pAmpCs, as observed in the present study, may cause undesirable health effects, since the rate of antibiotic resistance transmission may be high. Thus, the pAmpCs need to be routinely monitored and, if possible, regulated in DWPFs.

4.4.3.6. Quantification of antibiotic resistance genes in drinking water production facilities

Despite the widespread nature of pAmpCs in the environment, where they pose health risks (Golsha *et al.*, 2021), they are understudied in the context of DWPFs. Furthermore, studies focused on pAmpCs were restricted to detection rather than quantification, creating a gap in the understanding of pAmpC concentrations in DWPFs and their effect on consumers and aquatic life. The present study addressed this by quantifying pAmpCs in the DWPFs.

The real-time PCR analysis showed that ACC, FOX, DHA and EBC concentrations were higher in treated than in raw water in NW-E DWPF. Similar observations were made in NW-C DWPF, where ACC and FOX concentrations were higher in raw than treated water. The quantification of the other pAmpCs in raw and/ or treated water was excluded due to their absence in the end-point PCR screening process. This is of concern, since clinically relevant pAmpCs were present in raw and treated water without the influence of the hospital or even domestic WWTPs.

The *sul1* concentrations were higher in treated than in raw water of NW-E and NW-C DWPFs. In NW-E DWPF, the *sul1* concentrations were 2.53×10^{-3} copies/16S rRNA in raw water and 3.73×10^{-3} copies/16S rRNA in treated water. In NW-C DWPF, the concentrations were 6.03×10^{-2} copies/16S rRNA in raw water and 7.97×10^{-2} copies/16S rRNA in treated water. The *sul1* gene concentrations recorded in NW-C DWPF were comparable to those recorded in river water that is used to produce drinking water in Kathmandu Valley, Nepal (Amarasiri *et al.*, 2022). Furthermore, high *sul1* gene concentrations ranging up to 1×10^1 copies/16S rRNA were measured in four major cities in California, United States (Echeverria-Palencia *et al.*, 2017).

Generally, the *int11* gene concentrations measured in the NW-E and NW-C DWPFs were low. Furthermore, the *int11* gene concentrations were higher in treated than in raw water. In NW-E DWPF, *int11* concentrations were 3.90×10^{-4} copies/16S rRNA in raw water and 6.12×10^{-4} copies/16S rRNA in treated water. NW-E raw water's *int11* gene concentrations were comparable to 3.50×10^{-4} copies/16S rRNA in NW-C raw water. In contrast, the *int11* gene concentration measured in the Kathmandu Valley sources water, Nepal, was higher than those of the present study (Amarasiri *et al.*, 2022).

The ARBs can render antibiotics ineffective through various action mechanisms, including inhibition of cell synthesis, protein synthesis, nucleic acid synthesis and metabolic pathways (Reygaert, 2018). Thus, determining antibiotic resistance patterns in clinical settings is essential for choosing appropriate antibiotics to treat infectious bacteria. The present study showed that ARGs are also phenomena of environmental settings. Hu *et al.* (2021) highlighted that ARGs in drinking water might cause public health to water consumers. However, the risks associated with consuming ARGs in water and their effects on consumers are limited (Yang *et al.*, 2020; Thakali *et al.*, 2020). Antibiotic resistance could lead to an extended hospitalization period and expensive antibiotic therapy to treat common infections (Igwaran *et al.*, 2018; Havenga *et al.*, 2019). If ARGs are acquired through drinking water, the effects may be detrimental to consumers. A limited quantitative risk assessment method has been developed to determine the risks of consuming ARGs contaminated water (Thakali *et al.*, 2020). Furthermore, the presence of ARGs in drinking water is not routinely monitored and, as a result, no water guidelines limit these contaminants in water.

4.4.4. Quantification of antibiotic and fluconazole residues in drinking water production facilities

Currently, antibiotic residues in drinking water are not routinely monitored. As a consequence, it is difficult to regulate antibiotic residues in the environment and their impact on consumers. This is a great concern, since South African antibiotic use is comparable to those of developed countries and is estimated to increase rapidly by 2030 (Van Boeckel *et al.*, 2015; Van *et al.*, 2020). South Africa's antibiotic usage in 2015 was 21 149 standard units per 1 000 population, significantly higher than most other countries worldwide (NDoH, 2018). Furthermore, an estimated 74–77% of antimicrobials were procured for human use in South Africa between 2014 and 2015 (NDoH, 2018). Thus, it was important to measure the concentration of antibiotics. This is classified by WHO (2018) as critically important, including ampicillin and ciprofloxacin and highly important antibiotics such as trimethoprim and sulfamethoxazole. Furthermore, it is vital to quantify the fluconazole concentrations in raw and treated water.

4.4.4.1. Ampicillin residues

In NW-E and NW-C DWPFs, ampicillin concentrations were higher in raw than treated water. In NW-E, ampicillin concentrations were 14.81 µg/L in raw and 8.93 µg/L in treated water. In NW-C DWPF, concentrations were 7.39 µg/L in raw and 6.62 µg/L in treated water. Due to the underexplored ampicillin studies in the environment, no information was found in the

searched databases that addressed the presence of ampicillin in DWPFs. The measured ampicillin concentrations in surface water collected in Cluj-Napoca, Romania, was slightly higher than those obtained in NW-E and NW-C raw water (Soran *et al.*, 2017). Ampicillin is a drug of choice for the treatment of many bacterial infections (NDoH, 2018). According to Africa CDC (2021), it is a preferred antibiotic in neonatal and pediatric patients for treating suspected acute bacterial meningitis, acute lower respiratory tract infection, acute osteomyelitis, septic arthritis and sepsis. In adult patients, ampicillin is preferred for treating acute bacterial meningitis, complicated intra-abdominal infections, sepsis (septicemia) and septic shock. Hence, its widespread in raw water and, subsequently, in treated water.

Ampicillins are broad-spectrum antibiotics that inhibit bacterial cell wall synthesis by binding to penicillin-binding proteins (Rafailidis *et al.*, 2007). Resistance, however, is common. The 2017 antibiotic usage data in South Africa showed that 94% of *Enterococcus faecium* and 8% of *E. faecalis* were resistant to ampicillin (NDoH, 2018). This is alarming, since penicillins are the highest (40.66%) prescribed antibiotics in private primary health care according to the claims database of a health insurer in South Africa (Alabi and Essack, 2022). The ARGs, chromosomal and plasmid-mediated, that are associated with ampicillin resistance in *bla_{TEM}*, *ampC* and *pAmpCs* were detected in the present study.

4.4.4.2. Sulfamethoxazole residues

Generally, in NW-E and NW-C DWPFs, sulfamethoxazole concentrations were higher in raw than treated water. In the DWPFs, the maximum concentrations measured in NW-E and NW-C were 3.78 and 3.51 µg/L, respectively. Unlike the concentrations in NW-E and NW-C, the measured sulfamethoxazole concentrations of the five Québec City DWPFs (Québec, Beauport, Ste-Foy, Lévis and Charny) situated in Canada were low (Pulicharla *et al.*, 2021). However, among the five Québec City DWPFs, the maximum measured sulfamethoxazole concentration was 4.8×10^{-3} µg/L in the Lévis. Similar to NW-E and NW-C, all five Québec City and the Eastern China DWPFs reduced the sulfamethoxazole concentration from raw to treated water (Hu *et al.*, 2018; Pulicharla *et al.*, 2021). The raw water samples in NW-E and NW-C were comparable to the sulfamethoxazole concentrations measured in the Swartkops surface water collected in the Eastern Cape, South Africa (Vumazonke *et al.*, 2020).

According to the claims database of a health insurer of private primary health care in South Africa, sulphonamides prescription is 3.16% (Alabi and Essack, 2022). This could explain the presence of sulfamethoxazole residues in raw water and, subsequently, treated water. Sulfamethoxazole in subinhibitory concentrations in raw and treated water may induce

antibiotic resistance. The resistance-nodulation-cell division family pumps can move sulfamethoxazole out of the cell to confer its resistance. Furthermore, sulfamethoxazole inactivation occurs by acetylation (Reygaert, 2018). The effects of sulfamethoxazole resistance may be severe in immunocompromised patients, since it is combined with trimethoprim as an empiric treatment for *Pneumocystis pneumonia* in HIV/ AIDS patients (Africa CDC, 2021). The *sul1* and *sul2* genes associated with sulfamethoxazole resistance were detected in the present study.

4.4.4.3. Ciprofloxacin residues

The ciprofloxacin concentrations were lower in treated than raw water in the NW-E and NW-C DWPFs. The maximum ciprofloxacin concentrations measured in NW-E and NW-C were 4.44 and 3.71 µg/L, respectively. In Al-Rasheed and Al-Wihda DWPFs in Baghdad City, Iraq, the measured ciprofloxacin concentrations were lower than those recorded in NW-E and NW-C (Mahmood *et al.*, 2019). However, similar to NW-E and NW-C, the Al-Rasheed and Al-Wihda DWPFs could reduce the ciprofloxacin concentrations from raw to treated water (Mahmood *et al.*, 2019).

Ciprofloxacin belongs to the class fluoroquinolones and is a wide-spectrum antibiotic used to treat multidrug-resistance bacteria, which induce resistance to other classes of antibiotics such as aminoglycosides, macrolide and β -lactam (Mahmood *et al.*, 2019). The presence of ciprofloxacin in water is attributed to its use in clinical settings as a preferred antibiotic choice in neonatal and pediatric patients for treating viral gastroenteritis, dysentery, typhoid enteric fever and complicated intra-abdominal infection. In adult patients, ciprofloxacin is preferred for treating dysentery, typhoid (enteric) fever, mild to moderate acute uncomplicated prostatitis and acute pyelonephritis. Furthermore, it is used as an alternative antibiotic choice (Africa CDC, 2021). However, the extensive usage of ciprofloxacin in NW-E and NW-C could increase ciprofloxacin resistance and, as it stands, 26% of *E. coli* in South Africa displayed resistance to it (NDoH, 2018).

4.4.4.4. Trimethoprim residues

In NW-E DWPF, trimethoprim concentrations were lower in treated than in raw water. A similar pattern was reported in four DWPFs situated in China (Hu *et al.*, 2018; Song *et al.*, 2019). These contrast with the findings made at NW-C DWPF. The trimethoprim concentrations in NW-E and NW-C raw and treated water were not comparable to those measured in four

surface water averaging high concentrations of more than 400 µg/L in Cluj-Napoca, Romania (Soran *et al.*, 2017).

In 2015, South Africa had the highest antibiotic use of trimethoprim when compared to other BRICS countries, the United Kingdom and the United States (NDoH, 2018). Furthermore, antibiotic usage data between 2000 and 2015 showed that trimethoprim accounted for a quarter of the sales of antibiotics. However, a low concentration of trimethoprim in NW-E and NW-C raw and treated water could not be established, since there is a lack of information about trimethoprim usage data in the North West Province, South Africa.

4.4.4.5. Fluconazole residues

In NW-E and NW-C raw and treated water, fluconazole was <LOQ, except in February 2022. The reason for the heightened concentrations of fluconazole in February 2022 could not be established due to the lack of available information on consumption of antifungals in South Africa. Fluconazole is a broad-spectrum antifungal agent belonging to the azole class used to treat candidaemia and cryptococcal meningitis in immunocompromised patients infected with HIV/ AIDS (Brauer *et al.*, 2019). However, it has been reported that the human body poorly metabolises fluconazole; an estimated 80% of the unchanged antifungal is excreted (Sallach *et al.*, 2021). Fluconazole in NW-E and NW-C treated water may increase antifungal resistance in these areas (Brauer *et al.*, 2019).

4.4.5. Risk quotient of drinking water production facilities

The present study further discovered that, in addition to antibiotic and fluconazole resistance, the presence of the target compounds in water might pose ecological risks. The RQ was calculated to determine the ecological risks in raw and treated water. The RQ <0.1, <1 and ≥1 denote low, medium and high risk, respectively.

As a result, the obtained RQs for trimethoprim were deemed to reflect low risk in NW-E and NW-C DWPFs. Similar to the findings of the present study, low risk trimethoprim was reported in Chinese and European surface water (Guo *et al.*, 2016; Chen *et al.*, 2021). However, Ma *et al.* (2015) reported low and medium risk trimethoprim in reclaimed water and groundwater collected in 15 cities in China. Consistent with the findings of Liu *et al.* (2020) in Baiyangdian lake of China, the RQ of fluconazole was found to be low in the NW-C DWPF. However, in the NW-E DWPF, the RQ of fluconazole was determined to be of a medium risk.

Generally, in the NW-E and NW-C DWPFs, the calculated RQs for ampicillin, ciprofloxacin and sulfamethoxazole were of high risk. However, in treated water of NW-C, the ciprofloxacin was of medium risk. Similar ciprofloxacin results were obtained in Chinese and European surface water, where this antibiotic was found to be of high risk (Zhou *et al.*, 2019; Chen *et al.*, 2021). Unlike the case of the present study, the ciprofloxacin RQ of reclaimed water and groundwater in 15 cities in China was deemed to be of low risk (Ma *et al.*, 2015). The high-risk sulfamethoxazole was also reported in reclaimed water and groundwater collected in 15 cities in China (Ma *et al.*, 2015). However, the sulfamethoxazole in the surface water of China and Europe was of low risk (Zhou *et al.*, 2019; Chen *et al.*, 2021). Furthermore, the high-risk ampicillin results in the present study did not follow those obtained in Hanoi lakes, Vietnam (Duong *et al.*, 2021). The present study showed that ampicillin, ciprofloxacin and sulfamethoxazole exceed the desired low RQ and, as shown, pose high ecological risks. The results highlight that the DWPFs in NW-E and NW-C could not treat ampicillin, ciprofloxacin and sulfamethoxazole in raw water to produce low risk treated water.

The low to medium risk fluconazole and trimethoprim in NW-E and NW-C treated water were not associated with the ability of DWPFs to reduce these contaminants, but rather with low concentrations in raw water that was used in the production of drinking water. There is a need, therefore, to incorporate antibiotics and antifungals in the water quality guidelines for ecological and human health protection against the effects of these target compounds.

Overall, in NW-E and NW-C DWPFs the antibiotic and fluconazole residues were higher in raw than in treated water. However, no significant differences (p -value >0.05) were observed between raw and treated water. This could be attributed to the treatment processes used in NW-E and NW-C DWPFs, which are not designed to remove antibiotic and fluconazole residues. The presence of the latter in water in subinhibitory concentrations is a great concern, since bacteria may induce antibiotic resistance (Chen *et al.*, 2015). The effects of antibiotic resistance may be more dire in South Africa, where there is a high number of immunocompromised patients and poor healthcare services (Stats South Africa, 2022a). Hence, monitoring and regulating antibiotics and fluconazole in DWPFs is essential to minimize the spread of antimicrobial resistance in the food chain. Furthermore, monitoring and regulating antibiotics and fluconazole may have ecological benefits, as the RQ determined by present study has shown that these contaminants pose low to high risk, which may affect non-target organisms.

4.4.6. Correlation of bacterial communities with ARGs, antibiotics and physicochemical parameters in drinking water production facilities

The presence of ARGs, antibiotic residues and physicochemical parameters might prompt the selection of the appropriate bacterial community and subsequently change the microbial diversity and composition (Guan *et al.*, 2018; Zhang *et al.*, 2022). According to the RDA, no significant differences were reported in all canonical axes between bacterial community composition, physicochemical parameters, ARGs and antibiotic residues. However, significant differences were reported in pairwise correlation matrices. The present section reports that the bacterial communities were correlated with antibiotic and fluconazole residues, ARGs and physicochemical parameters in three parts.

1. The RDA show that ciprofloxacin and ampicillin were correlated with *CL500-29 marine group*, *Clade III* and *Armatimonas*. The pairwise correlation matrices showed a significant correlation between *Clade III* and ampicillin. Furthermore, the pairwise correlation matrices revealed that *CL500-29 marine group* was significantly correlated with ciprofloxacin and sulfamethoxazole. The correlation of bacterial communities with antibiotic residues led Guan *et al.* (2018) to speculate that different responses of bacterial communities to antibiotic pollution, probably due to antibiotics, pose selective pressure on indigenous bacterial communities. Ultimately, antibiotic residues in raw and treated water could result in bacteria becoming resistant to a wide range of antibiotics through selective pressure.
2. Consistent with Guan *et al.* (2018), the RDA plot shows that *Peptoniphilus* and *Nocardia* genera belonging to phyla Firmicutes and Actinobacteriota had a weak association with ARGs. Furthermore, genera belonging to phylum Proteobacteria were associated with most ARGs. Inconsistent with Zhang *et al.* (2022), *CL500-29 marine group* was associated with MOX, ACC and *sul2* genes in drinking water sources. The pairwise correlation matrices showed a significant correlation between *CL500-29 marine group*, MOX and ACC. *Clade III* significantly correlated with ACC, *bla_{TEM}* and *int1*. The *ampC* was correlated significantly with *Armatimonas* and *Dickeya*. *Zymomonas* was correlated significantly with *int1* and *ermB*. The HGT occurred mainly among Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria (Guan *et al.*, 2018). Furthermore, the association of bacterial community with ARGs indicates that bacteria were the major spreaders of ARGs (Guan *et al.*, 2018; Zhang *et al.*, 2022).

3. Physicochemical parameters were correlated with the bacterial communities. These parameters were found to impact bacterial community composition in various ways (Zhang *et al.*, 2022). Nitrate, nitrite and phosphate provide vital nutrients for bacterial community growth, reproduction and survival. Furthermore, physicochemical parameters may directly or indirectly affect ARG variation (Zhou *et al.*, 2017). The present results showed that operational activities have greatly affected physicochemical parameters, causing a shift in bacterial community alpha diversity from the raw to the treated state. Contaminated raw water and underperforming DWPFs may have major influences on the bacterial community composition, ARGs and antibiotic residues in treated water.

CHAPTER 5

5. BACTERIAL COMMUNITY COMPOSITION, QUANTIFICATION OF ANTIBIOTIC RESISTANCE GENES AND ANTIBIOTIC RESIDUES IN WASTEWATER EFFLUENT AND RECEIVING RIVERS

5.1. INTRODUCTION

Wastewater treatment plants are the primary barrier to wastewater pollution. Thus, treating wastewater is important for the reduction of pollution levels. However, the WWTPs in developing countries face the challenge of non-compliance, leading to the discharge of low wastewater effluent quality into downstream rivers (Shamsizadeh *et al.*, 2021). It is estimated that the South African government spent more than R3 billion per annum on the country's WWTPs, intending to improve performance and compliance with regulatory guidelines (Edokpayi *et al.*, 2020). However, despite the government's efforts to channel this money into the WWTP infrastructure, most WWTPs still perform poorly (Edokpayi *et al.*, 2020; DWS, 2022a). The DWS (2022a) indicated that the South African provincial average Green Drop performance was below 30%. Limpopo, Free State, KwaZulu-Natal, Gauteng and North West were the least-performing provinces with an average Green Drop performance below 20%. As a result, the DWS (2022a) declared that most of the investigated WWTPs were in a critical state. Understaffed WWTPs exacerbate these problems. One engineer is estimated to serve 90 925 people (Edokpayi *et al.*, 2020). The critical state of WWTPs creates favourable conditions for physicochemical parameters, microorganisms, ARB, ARGs, and antibiotic residues to escape the treatment processes and, subsequently, be discharged into the downstream rivers.

The agricultural sector utilizes the largest portion of South African water (Edokpayi *et al.*, 2020), and 80% of the land in the country is used for agriculture (Mettetal, 2019). O'Neill (2022) showed that the contribution of the agricultural sector to the country's gross domestic product (GDP) has increased from 2.11% in 2010 to 2.53% in 2020. The sector continues to positively contribute to the country's GDP despite the strict conditions imposed by the COVID-19 regulations in 2020 and 2021 (Tempia *et al.*, 2020; Stats South Africa, 2022b). It had the second-largest growth after mining in that period (Stats South Africa, 2022b). Thus, it is important to ensure that WWTPs work optimally to remove contaminants that might hinder water's reusability. However, WWTPs are not designed to remove ARGs and antibiotic residues.

The WWTPs have been documented to influence the emergence of antibiotic residues and ARGs in the downstream rivers used for food and drinking water production. The application of contaminated water may facilitate the spread of ARB and ARGs from water environments into the food chain (Murray *et al.*, 2019; Gekenidis *et al.*, 2021; Shamsizadeh *et al.*, 2021). These contaminants have been documented in fresh produce such as ready-to-eat salads and fresh fruits (Murray *et al.*, 2019; Gekenidis *et al.*, 2021). Other sources that may potentially facilitate the spread of ARBs and ARGs are contaminated soil and fertilizers (Gekenidis *et al.*, 2021). The risk of transmission may be high if manure fertilizers or irrigation where reclaimed contaminated wastewater is used in the production of crops (Murray *et al.*, 2019; Shen *et al.*, 2021). Antibiotic residues and ARGs in drinking water have also been documented. This is caused by the inability of DWPFs to remove these contaminants (Ateba *et al.*, 2020).

The abundance of ARGs and antibiotic residues may be high in wastewater effluent and downstream rivers, since the South African WWTPs are in a critical state. However, few studies have been conducted to determine the concentration of antibiotic residues and ARGs in rivers and WWTPs of the country. In water environments, antibiotic residues pose ecological risks (Assress *et al.*, 2020; Kairigo *et al.*, 2020; Chamkal *et al.*, 2021). However, as shown in the searched databases detailed in Chapter 2, a gap is found around assessing potential risks associated with antibiotic residues in aquatic environments of South Africa. Thus, the present study aimed to determine antibiotic and ARG patterns in wastewater effluent and rivers – including implications for downstream use. Furthermore, to correlate bacterial community composition with ARGs, antibiotic residues and physicochemical parameters.

5.2. MATERIALS AND METHODS

Refer to Chapter 3 for detailed materials and methods.

5.3. RESULTS

5.3.1. Physicochemical parameters in wastewater effluents and rivers

The physicochemical parameters in NW-E and NW-C wastewater effluents and rivers are represented in Tables 5.1 to 5.3. The highest levels of these were anticipated to be measured in the wastewater effluents in NW-E and NW-C. It was observed that the wastewater effluent influenced the abundance of physicochemical parameters in the downstream river. However, in NW-C, physicochemical parameter levels were higher in the downstream maturation pond than in wastewater effluent and upstream river. Furthermore, in NW-C, the influence of wastewater effluent on the downstream river was not determined, as it has not been sampled. The measured physicochemical parameters were compared to the DWAF SAWQG (1996b) and (1996c) for agricultural use in terms of irrigation and livestock watering.

5.3.1.1. Physicochemical parameters NW-E wastewater effluents and rivers

In NW-E, the mean pH of water samples was higher in the upstream river at 8.07 than the wastewater effluent at 7.81 and the downstream river at 7.85 (Table 5.1). No significant differences ($p\text{-value} > 0.05$) were observed between the wastewater water effluent, upstream and downstream rivers. Overall, pH was found to be within the recommended standards of the DWAF SAWQG (1996b) for agricultural use: irrigation. The lowest and highest temperatures were measured in winter and summer, respectively.

The mean TDS levels were highest in the wastewater effluent at 824.88 mg/L, followed by the downstream river at 613.33 mg/L and the upstream river at 606.25 mg/L. The TDS levels in wastewater effluent differed significantly ($p\text{-value} < 0.05$) from the upstream and downstream rivers. However, no significant differences ($p\text{-value} > 0.05$) existed between the upstream and downstream rivers. The TDS was found to be within the recommended standards of the DWAF SAWQG (1996c) for agricultural use: livestock watering. However, it was not within the recommended standards of the DWAF SAWQG (1996b) for agricultural use: irrigation.

The mean salinity levels of the wastewater water effluent were measured at 525.13 mg/L, which condition may have influenced salinity levels from the upstream river at 378.00 mg/L to the downstream river at 480.00 mg/L (Table 5.1). Significant differences ($p\text{-value} < 0.05$) in salinity levels were observed between wastewater effluent, upstream and downstream rivers.

Overall, the salinity levels of rivers were found to be within the recommended standards of the DWAF SAWQG (1996b) and (1996c) for agricultural use: irrigation and livestock watering.

Overall, nitrate levels in the wastewater effluent, upstream and downstream rivers were 3.28, 0.88 and 1.07 mg/L, respectively. Significant differences (p -value <0.05) were observed only between the wastewater effluent and the upstream river. However, the nitrite levels decreased from 2.45 mg/L in the upstream river to 0.00 mg/L in the downstream river. The nitrite levels in the wastewater effluent were 5.78 mg/L. Significant differences (p -value <0.05) in nitrite levels were observed between the wastewater effluent, upstream and downstream rivers.

The mean phosphate levels increased from 11.06 mg/L in wastewater effluent to 14.95 mg/L in the downstream river (Table 5.3). The phosphate levels in the upstream river were 3.53 mg/L. Significant differences (p -value <0.05) in phosphate levels were observed between the wastewater effluent, upstream and downstream rivers. The wastewater effluent may have influenced the abundance of COD levels from the upstream to downstream rivers. The COD levels in the wastewater, upstream and downstream rivers were 32.00, 13.33 and 25.00 mg/L, respectively (Table 5.3).

5.3.1.2. Physicochemical parameters in NW-C wastewater effluents and upstream river

In NW-C, the highest pH was measured in the wastewater effluent at 8.33, followed by the upstream river at 8.16 and the downstream maturation pond at 7.97 (Table 5.2). Significant differences (p -value <0.05) were observed between the wastewater effluent and the downstream maturation pond. The pH was found to be within the recommended standards of the DWAF SAWQG (1996b) for agricultural use: irrigation. As anticipated, the lowest and highest temperatures were measured in winter and summer (Table 5.2).

The mean TDS levels increased from 618.13 mg/L in the wastewater effluent to 643.43 mg/L in the downstream maturation pond. The lowest TDS levels were measured in the upstream river (Table 5.2). The TDS levels in the upstream river differed significantly (p -value <0.05) from the wastewater effluent and downstream maturation pond. However, no significant differences (p -value >0.05) existed between the wastewater effluent and downstream maturation pond. The TDS levels were found to be within the recommended standards of the DWAF SAWQG (1996c) for agricultural use: livestock watering. However, they were not found

to be within the recommended standards of the DWAF SAWQG (1996b) for agricultural use: irrigation.

The mean salinity levels increased from 410.50 mg/L in the wastewater effluent to 423.86 mg/L in the downstream maturation pond. The TDS levels in the upstream river were 311.43 mg/L (Table 5.2). The salinity levels in the upstream river differed significantly (p -value <0.05) from the wastewater effluent and downstream maturation pond. However, no significant differences (p -value >0.05) existed between the wastewater effluent and downstream maturation pond. Overall, the salinity of the NW-C river was found to be within the recommended standards of the DWAF SAWQG (1996b) and (1996c) for agricultural use: irrigation and livestock watering.

The mean nitrate levels decreased from 13.37 mg/L in the wastewater effluent to 5.35 mg/L in the downstream maturation pond (Table 5.3). The nitrate levels in the upstream river were 1.14 mg/L. The nitrate levels in the upstream river differed significantly (p -value <0.05) from the wastewater effluent and downstream maturation pond. However, no significant differences (p -value >0.05) existed between the wastewater effluent and downstream maturation pond. The same observations of the significant difference were recorded when it came to nitrite levels. However, these increased from 19.11 mg/L in the wastewater effluent to 19.17 mg/L in the downstream maturation pond.

In NW-C, the mean phosphate levels were 5.68, 34.98 and 20.92 mg/L in the upstream river, wastewater effluent and downstream maturation pond, respectively (Table 5.3). Significant differences (p -value <0.05) existed between the upstream river, wastewater effluent and downstream maturation pond. The COD levels were higher in the downstream maturation pond at 88.67 mg/L, followed by the wastewater effluent at 56.00 mg/L and the upstream river at 7.67 mg/L.

Table 5.1: Physical parameters with standard deviations measured in 2020, 2021 and 2022 in the wastewater effluent, upstream and downstream rivers at NW-E.

Date	Sampling sites	TDSs (mg/L)	pH	Salinity (mg/L)	Temperature (°C)
	DWAF (1996b)	≤260	≥6.5 to ≤8.4	≤460	-
	DWAF (1996c)	≤1 000	-	≤2 000	-
Dec 2020	Up	827.67±2.08	7.59±0.05	404.00±0.00	19.90±0.10
	Effluent	979.67±4.16	7.58±0.02	490.67±0.58	19.53±0.06
	Down	ND	ND	ND	ND
Feb 2021	Up	630.33±3.06	7.72±0.11	310.00±1.00	24.57±0.06
	Effluent	908.33±13.6	7.80±0.02	445.33±3.79	26.13±0.35
	Down	ND	ND	ND	ND
Jul 2021	Up	564.67±2.31	9.30±0.01	378.67±0.67	10.50±0.20
	Effluent	835.00±0.00	8.17±0.01	572.33±0.58	14.33±0.060
	Down	ND	ND	ND	ND
Aug 2021	Up	601.00±1.00	8.16±0.05	408.00±1.00	15.40±0.36
	Effluent	819.00±1.00	7.75±0.02	566.33±0.58	17.60±0.46
	Down	ND	ND	ND	ND
Sep 2021	Up	675.00±1.00	8.17±0.03	465.00±1.00	19.37±0.40
	Effluent	851.33±2.08	7.90±0.02	592.00±3.00	20.63±0.21
	Down	731.33±2.25	7.93±0.01	507.00±1.00	17.87±0.21
Dec 2021	Up	536.67±1.53	7.85±0.02	365.00±4.58	23.07±0.42
	Effluent	616.33±2.08	7.72±0.03	427.33±1.15	22.67±0.35
	Down	549.67±1.53	7.72±0.02	549.67±1.53	23.07±0.15
Feb 2022	Up	496.00±0.00	7.76±0.02	340.00±0.00	23.40±0.10
	Effluent	763.00±1.00	7.74±0.02	532.33±0.58	23.77±0.15
	Down	516.67±0.58	7.73±0.04	354.33±1.15	23.30±0.20
Mar 2022	Up	517.67±0.58	8.02±0.01	352.67±0.58	19.47±0.06
	Effluent	827.00±1.73	7.83±0.00	575.67±0.58	23.93±0.25
	Down	559.33±1.15	7.90±0.02	383.00±0.00	23.17±5.14

TDS- total dissolved solids; ND – not done; “-” – not specified; Up – upstream river; down – downstream river

Table 5.2: Physical parameters with standard deviations measured in 2020, 2021 and 2022 in the upstream river, wastewater effluent and downstream maturation pond at NW-C.

Date	Sampling sites	TDSs (mg/L)	pH	Salinity (mg/L)	Temperature (°C)
	DWAF (1996b)	≤260	≥6.5 to ≤8.4	≤460	-
	DWAF (1996c)	≤1 000	-	≤2 000	-
Dec 2020	Up	532.67±1.15	7.81±0.04	257.67±0.58	21.10±0.26
	Effluent	703.00±2.00	7.91±0.03	340.67±0.58	20.47±0.35
	Down	743.00±2.65	7.57±0.02	363±2.00	22.4±0.12
Feb 2021	Up	578.00±2.00	7.74±0.01	280.33±0.58	21.43±0.32
	Effluent	667.67±0.58	8.25±0.03	330.00±1.00	23.50±0.10
	Down	656.00±2.65	7.60±0.04	318.67±2.92	24.33±2.92
Jul 2021	Up	438.67±0.58	8.96±0.06	291.33±1.15	11.8±0.58
	Effluent	656.67±5.77	9.00±0.03	444.00±0.00	11.67±0.06
	Down	685.67±3.06	8.66±0.16	456.00±1.73	7.60±0.10
Aug 2021	Up	445.00±1.00	8.05±0.01	301.00±1.00	15.37±0.93
	Effluent	579.33±0.58	8.23±0.05	391.00±1.00	13.9±0.25
	Down	655.00±1.00	8.12±0.01	442.67±0.58	12.87±0.15
Sep 2021	Up	425.33±1.15	8.18±0.03	287.33±0.58	16.23±1.00
	Effluent	669.00±1.73	8.01±0.03	459.33±4.93	14.60±0.10
	Down	606.00±1.00	8.14±0.02	411.33±0.58	14.67±0.67
Dec 2021	Up	444.00±5.20	8.16±0.02	444.00±520	20.57±0.31
	Effluent	567.67±1.53	8.31±0.04	564.33±7.23	20.33±0.15
	Down	593.00±1.00	7.73±0.03	593.00±1.00	19.10±0.10
Feb 2022	Up	ND	ND	ND	ND
	Effluent	564.33±0.58	8.37±0.04	389.00±1.00	8.37±0.04
	Down	ND	ND	ND	ND
Mar 2022	Up	473.33±0.58	8.24±0.01	319.33±0.58	15.30±0.30
	Effluent	537.33±0.58	8.55±0.03	366.33±1.15	19.53±0.06
	Down	565.00±0.00	7.94±0.01	382.00±0.00	15.13±0.31

TDS- total dissolved solids; ND – not done; “-” – not specified; Up – upstream river; down – downstream maturation pond.

Table 5.3: Chemical parameters with standard deviations measured in 2021 and 2022 in the upstream rivers, wastewater effluent and downstream points at NW-E and NW-C.

Date	Sampling sites		Phosphate (mg/L)	Nitrates (mg/L)	Nitrites (mg/L)	COD (mg/L)
		DWAF (1996b)	-	-	-	-
		DWAF (1996c)	-	≤100	-	-
Feb 2021	NW-E	Up	4.02±0.48	1.47±0.15	2.67±1.53	0.00
		Effluent	9.02±0.20	4.23±0.38	7.00±1.00	20.00
		Down	ND	ND	ND	ND
	NW-C	Up	3.30±0.59	1.53±0.12	1.33±0.58	0.00
		Effluent	34.23±1.82	9.27±0.71	9.00±0.00	19.00
		Down	25.59±0.49	6.57±0.32	15.67±3.06	158.00
Sep 2021	NW-E	Up	3.03±0.02	0.63±0.15	3.00±0.00	16.00
		Effluent	18.61±6.17	4.20±0.85	8.00±0.00	31.00
		Down	ND	ND	ND	ND
	NW-C	Up	9.85±4.46	1.23±0.06	2.67±0.58	11.00
		Effluent	42.57±0.06	27.1±1.65	44.00±16.46	145.00
		Down	19.71±2.22	4.13±0.06	22.67±10.97	72.00
Ma 2022	NW-E	Up	ND	0.53±0.23	1.67±1.15	24.00
		Effluent	5.54±1.25	1.4±0.20	2.33±1.15	45.00
		Down	14.95±4.23	1.07±0.38	0.00±0.00	25.00
	NW-C	Up	3.89±0.54	0.67±0.15	2.33±0.58	12.00
		Effluent	28.13±0.76	3.73±0.49	4.33±1.15	4.00
		Down	17.46±0.20	ND	ND	36.00

COD – chemical oxygen demand; ND – not done; “-” – not specified; Up – upstream river; down – downstream river for NW-E, downstream maturation pond for NW-C

5.3.2. Bacterial community composition in NW-E and NW-C wastewater and rivers

High-throughput 16S rRNA sequencing data generated an average of 60 732 per sample for normalised sequence reads in NW-E and NW-C wastewater effluents and rivers. Figures 5.1 and 5.2 represent the phylum-level changes in bacterial community composition of NW-E and NW-C wastewater effluents and river water. Taxonomic classification of the clustered OTUs showed the presence of 23 bacterial phyla, including unclassified phylum in NW-E and NWC wastewater effluent and river water. However, as depicted in Figures 5.1 and 5.2, nine bacterial phyla had a relative abundance of $\geq 2\%$. Bacterial phyla with $< 2\%$ were categorized as others, including Bdellovibrionota, Caldiseicota, Chloroflexi, Desulfobacterota, Elusimicrobiota, Fusoba, Armatimonadota Gemmatimonadota, Myxococcota, Nitrospirota, Planctomycetota, Spirochaetota and Synergistota. Ten bacterial genera had a relative abundance percentage $\geq 2.00\%$, as shown in Figures 5.3 and 5.4. The genera with a relative abundance percentage < 2.00 were represented as others. The distribution of the relative abundance of bacterial communities in NW-E and NW-C wastewater effluents and rivers is shown in Figure 5.5.

5.3.2.1. Bacterial community composition in the NW-E upstream river, wastewater effluent and downstream river

In NW-E rivers, a similar trend was observed for the three most abundant phyla (Figures 5.1 and 5.2), which ranked as follows: Proteobacteria (upstream river = 40.48% and downstream river = 39.62%); Bacteroidota (upstream river 24.14% = and downstream river = 23.10%); and Acidobacteriota (upstream river = 16.01% and downstream river = 13.24%). In wastewater effluent, the unassigned group (32.89%) was the highest detected phylum, followed by Proteobacteria (27.85%) and Patescibacteria (11.76%). The relative abundance percentage of Campilobacterota in the downstream river was higher than that of the wastewater effluent and upstream river. Other phyla were prevalent in the wastewater effluent (Figures 5.1 and 5.2).

In NW-E upstream and downstream rivers, the four most prevalent genera were unassigned (upstream river 49.47% = and downstream river = 48.97%), followed by *Comamonas* (upstream river = 16.75% and downstream river = 18.40%), *Nocardia* (upstream river = 5.99% and downstream river = 5.16%), and *Dickeya* (upstream river = 5.67% and downstream river = 5.92%), as shown in Figures 5.3 and 5.4. In the wastewater effluent, the prevalent genera

were the unassigned group (58.35%), *Dickeya* (12.97%), others (5.80%), *Arcobacter* (3.42%) and *Comamonas* (3.17%).

5.3.2.2. Bacterial community composition in the NW-C wastewater effluents and upstream river

In NW-C, Proteobacteria (upstream river = 41.33%, wastewater effluent = 38.18% and downstream maturation pond = 40.44%) and Bacteroidota (upstream river = 26.05%, wastewater effluent = 29.46% and downstream maturation pond = 18.84%) were the most prevalent phyla (Figures 5.1 and 5.2).

The five most abundant genera in the wastewater effluent and downstream maturation pond were the unassigned ones (wastewater effluent = 61.57% and downstream maturation pond = 51.38%), *Dickeya* (wastewater effluent = 13.11% and downstream maturation pond = 10.85%), *Comamonas* (wastewater effluent = 4.66% and downstream maturation pond = 8.61%), others (wastewater effluent = 3.60% and downstream maturation pond = 9.64%) and *Nocardia* (wastewater effluent = 8.89% and downstream maturation pond = 1.93%), as shown in Figures 5.3 and 5.4. In the upstream river, the most abundant genera were unassigned (56.71%), followed by *Comamonas* (12.05%), *Dickeya* (10.91%), *Nocardia* (6.10%) and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (5.69%).

5.3.2.3. Alpha diversity in NW-E and NW-C wastewater effluents and rivers

Alpha diversity and significant differences were calculated for species diversity, evenness, richness, and species dominance for NW-E and NW-C, as shown in Figure 5.6. In NW-E, the upstream river was richer and dominant, and wastewater effluent was more diverse and even. In NW-C, the downstream maturation pond was richer, more diverse, even and dominant. However, in NW-E and NW-C, there were no significant differences between the upstream rivers, wastewater effluent and downstream point in terms of the Kruskal-Wallis' test ACE: p-value = 0.150, statistic = 8.114; Chao1: p-value = 0.150, statistic = 8.114; Shannon: p-value = 0.119, statistic = 8.769; and Simpson: p-value 0.144, statistic = 8.23.

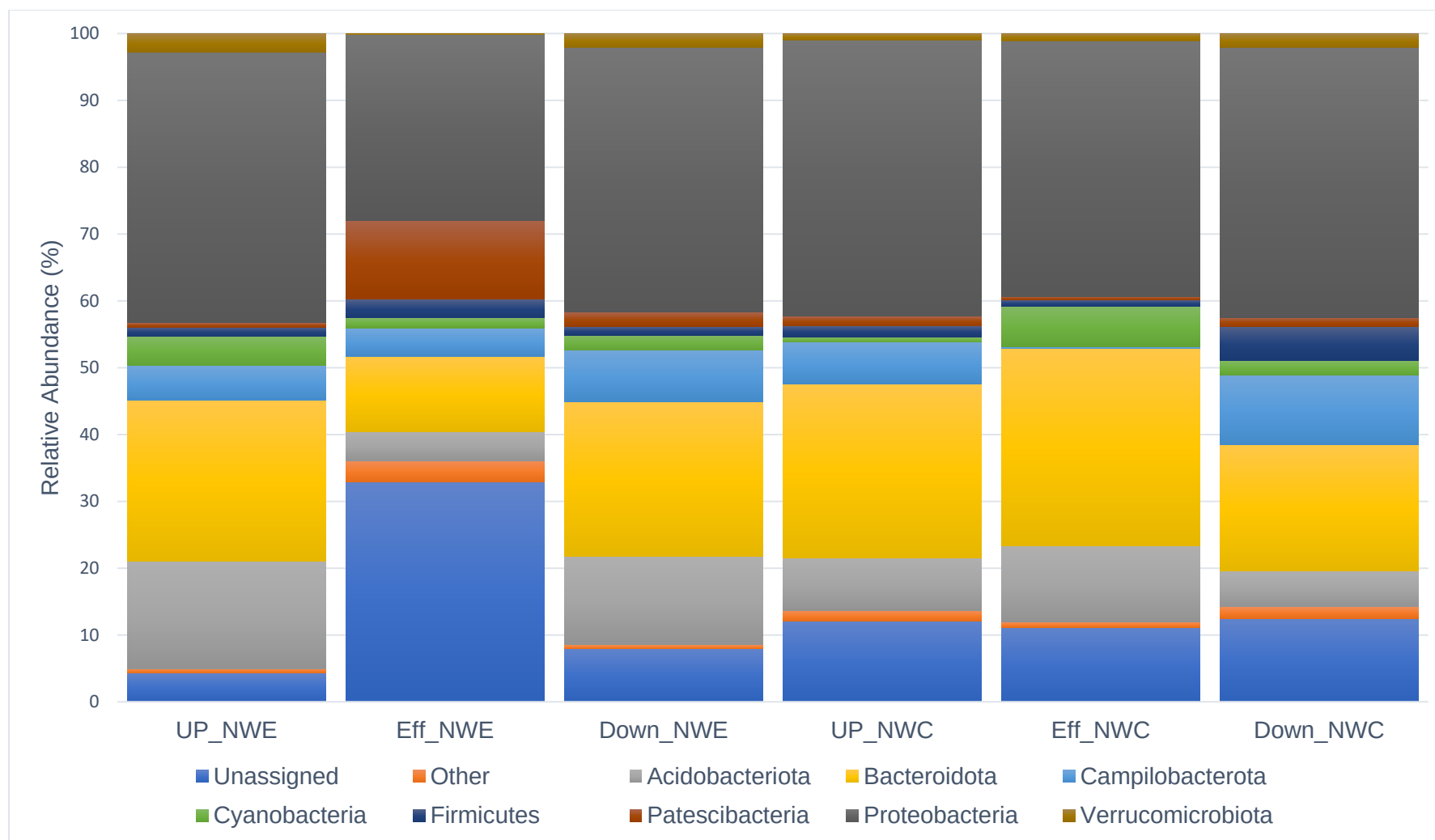


Figure 5.1: Stacked column illustrating phyla-level changes in bacterial community composition of upstream rivers, wastewater effluent and downstream points at NW-E and NW-C. Other genera consist of phyla with relative abundance < 2.00%.

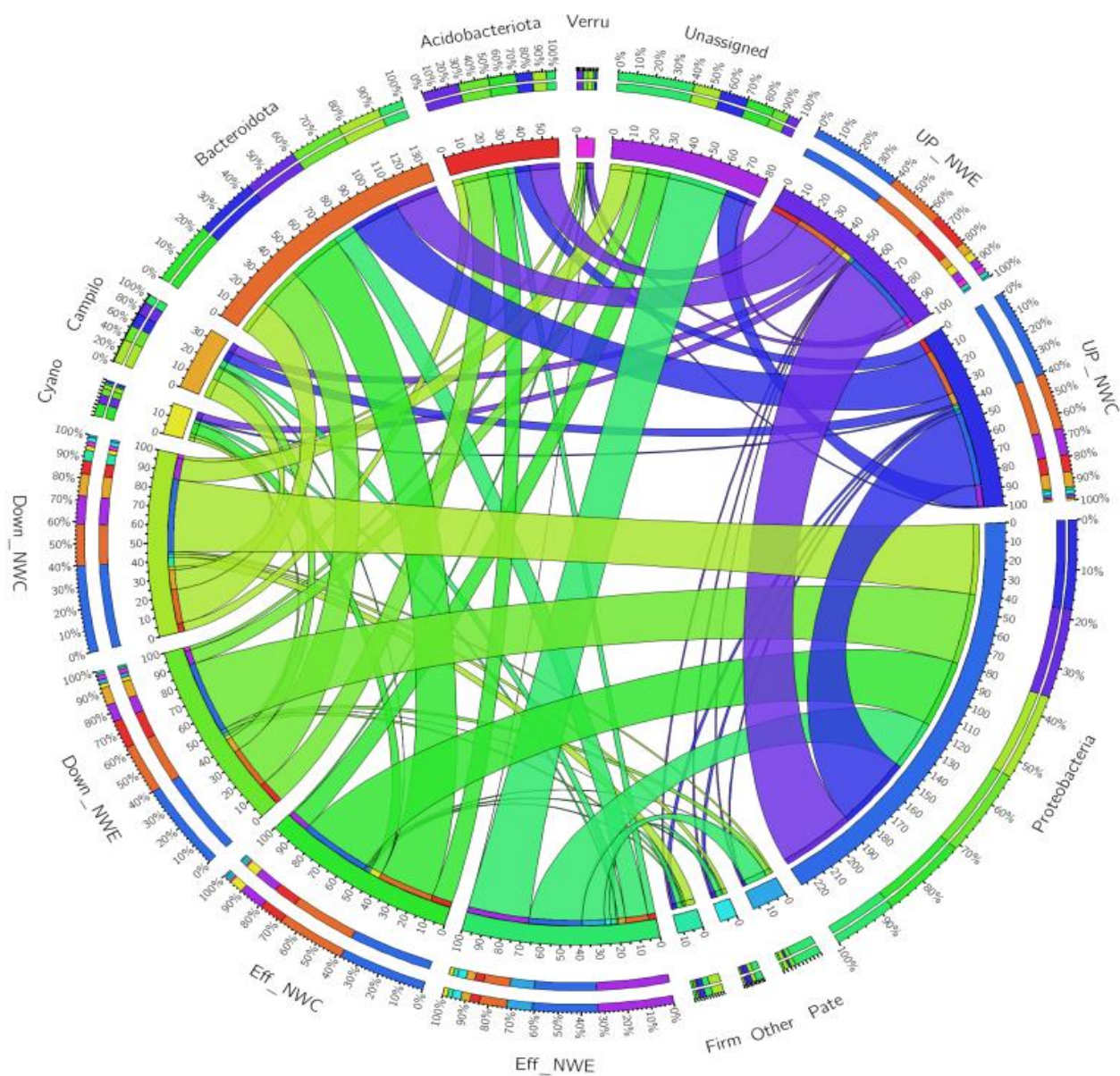


Figure 5.2: Chord diagram illustrating phyla-level changes in bacterial community composition of upstream rivers, wastewater effluent and downstream points at NW-E and NW-C. Others consist of phyla with relative abundance < 2.00%.

Where: Cyano - Cyanobacteria; Firm - Firmicutes; Pate - Patescibacteria; Verru – Verrucomicrobiota.

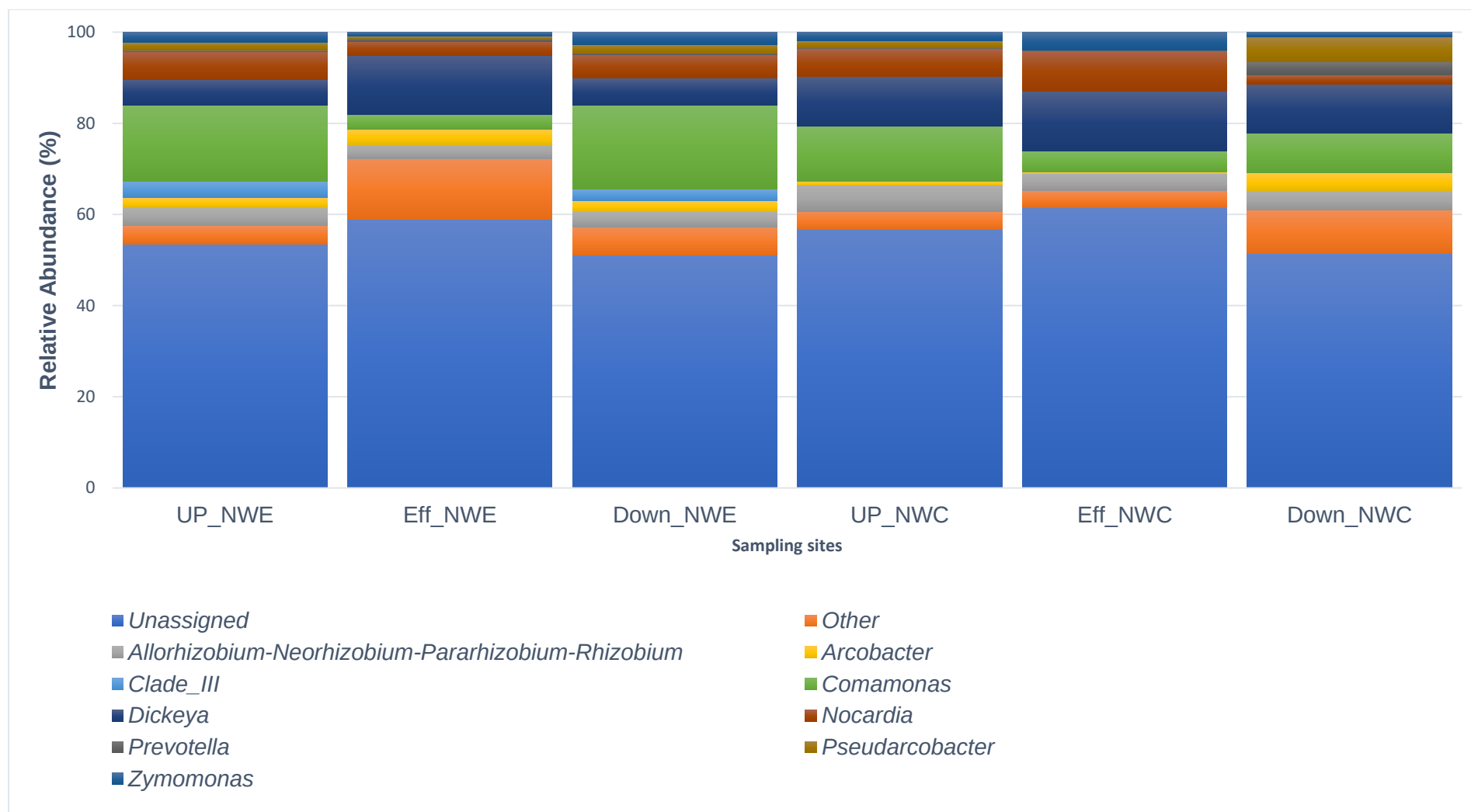


Figure 5.3: Stacked column illustrating genus-level changes in bacterial community composition of upstream rivers, wastewater effluent and downstream points at NW-E and NW-C. Others consist of genera with relative abundance < 2.00%.

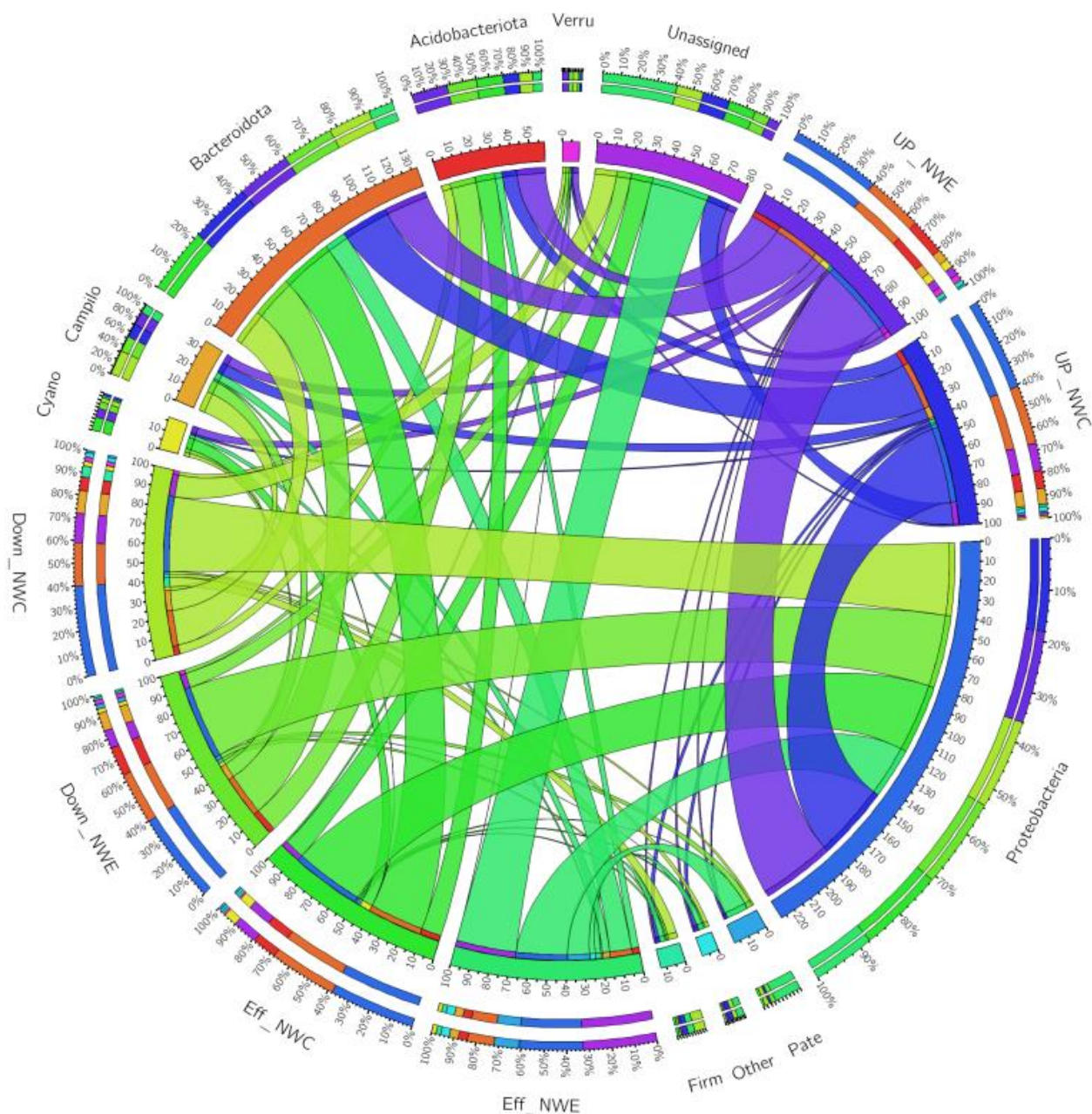


Figure 5.4: Chord diagram illustrating genus-level changes in bacterial community composition of upstream rivers, wastewater effluent and downstream points at NW-E and NW-C. Others consist of genera with relative abundance < 2.00%.

Where: ANPR = *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*; Arm = *Armatimonas*; CL = *Clade III*; Fuso = *Fusobacterium*; Pepto = *Peptoniphilus*.

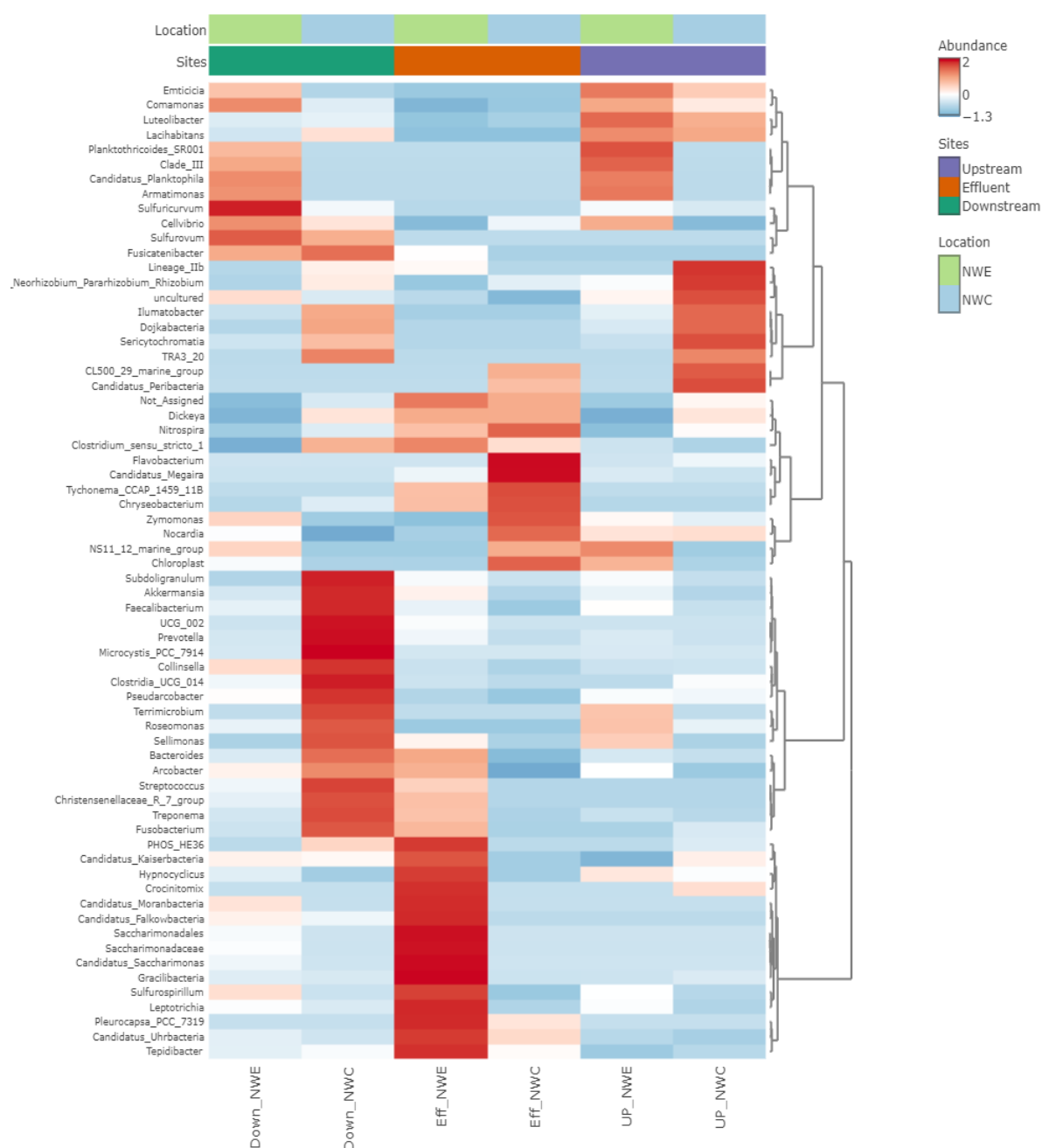


Figure 5.5: A heat map showing the relative abundance percentage of different bacterial genera by using PICRUS analysis in the upstream rivers, wastewater effluent and downstream points at NW-E and NW-C.

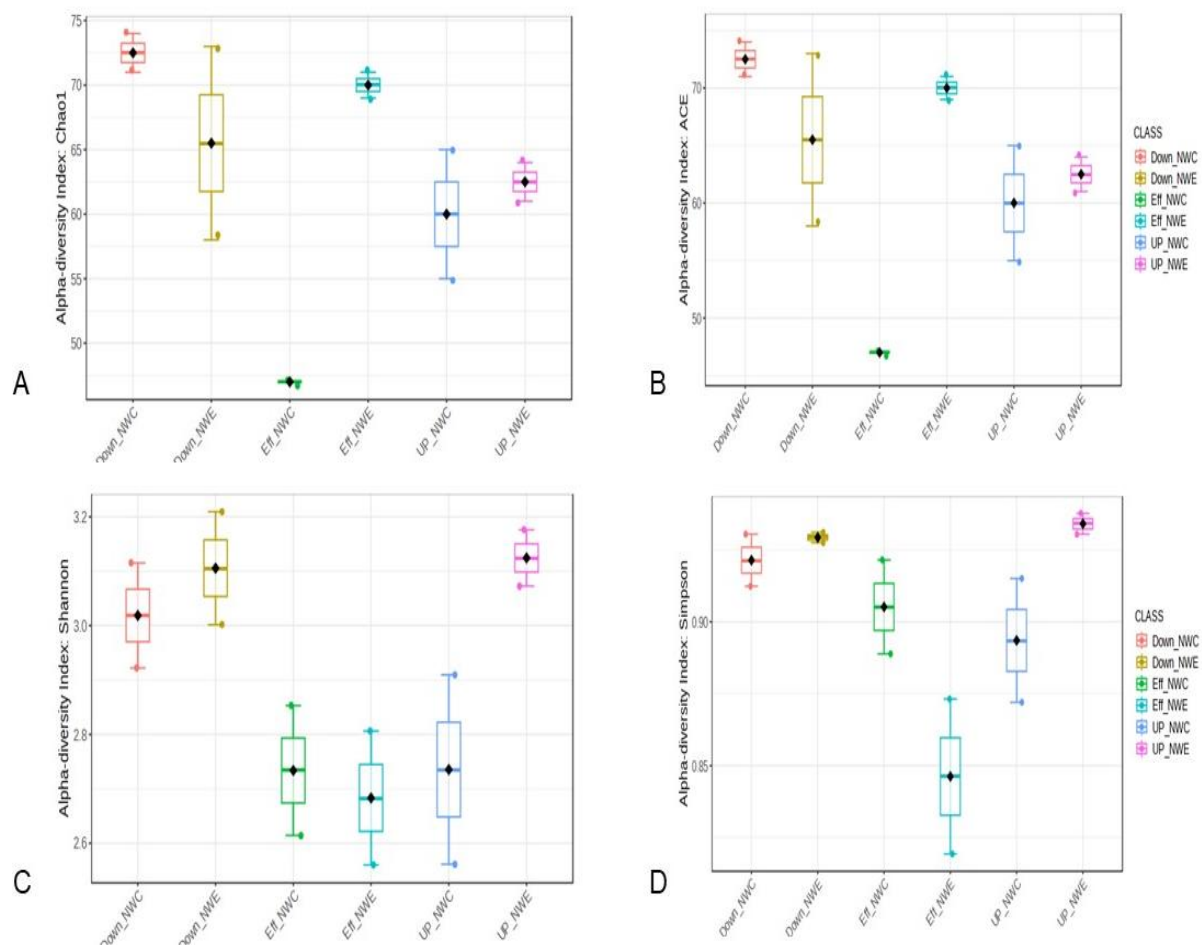


Figure 5.6: Bacterial alpha diversity indices (A-ACE, B-Chao1, C-Shannon and D-Simpson) for upstream rivers, wastewater effluent and downstream points at NW-E and NW-C.

5.3.3. Detection of antibiotic resistance genes in wastewater effluents and rivers

The eDNA subjected to end-point PCR for screening 13 target ARGs are presented in Figures 5.7 and 5.8. In NW-C, the prevalence of five and two ARGs was the highest in the downstream maturation pond and upstream river, respectively. As anticipated, other detected ARGs had higher levels in the wastewater effluent and, in NW-E, the prevalence of 10 ARGs was higher in wastewater effluent than that of the upstream and downstream rivers.

5.3.3.1. Detection of antibiotic resistance genes in NW-E wastewater effluents, downstream and upstream rivers

In NW-E, the detection of *ampC* and *bla_{TEM}* genes conferring resistance to β -lactams was higher in the wastewater effluent (Figures 5.7 and 5.8). Furthermore, the detection of *ampC* and *bla_{TEM}* genes was lower in the upstream river and increased after the discharged wastewater effluent blended with receiving water in the downstream river. The *ampC* gene detection rose from 9.09% in the upstream river to 20.00% in the downstream river. The *ampC* gene detection comprised 50.00% in the wastewater effluent. The detection of the *ampC* gene was lower than the *bla_{TEM}* gene. The *bla_{TEM}* gene detection levels in the upstream river, wastewater effluent and downstream river was 58.33%, 81.82% and 80.00%, respectively.

A detection of 100.00% was recorded for the *int11*, *ermB* and *ermF* genes in the wastewater effluent (Figures 5.7 and 5.8). However, the wastewater effluent did not influence the abundance of the detection levels in the downstream river for these genes. This was indicated by the high detection levels of *int11*, *ermB* and *ermF* genes in the upstream river when compared to the downstream river. *Int11*, *ermB* and *ermF* genes were detected in the upstream river at 83.33%, 90.91% and 75.100%, respectively. In the downstream river, the detection levels of *ermB* and *ermF* genes were 60.00% and *int11* 80.00%.

In contrast, *sul1* and *sul2* genes were detected in all downstream river samples in NW-E, as shown in Figures 5.7 and 5.8. Another trend observed for *sul1* and *sul2* genes was that they were both prevalent at 91.67% in the wastewater effluent samples. In the upstream river, the prevalence of the *sul1* and *sul2* genes was 75% and 66.67%, respectively. This indicates that the wastewater effluent did not influence the abundance of the detection levels of *sul1* and *sul2* genes in the downstream river.

The NW-E wastewater effluent did not influence the increased detection of the DHA and MOX genes in the downstream river (Figures 5.7 and 5.8). The MOX gene detection level of 45.45% was higher in the upstream river than in the downstream river and wastewater effluent. It was found to be at 18.18% in wastewater effluent and 0.00% in the downstream river samples. The detection level of the DHA gene was higher in the wastewater effluent (72.73%) than in the upstream and downstream rivers at 45.45% and 20.00%, respectively.

In NW-E, the wastewater effluent may have influenced the increased detection of ACC and FOX genes in the downstream river (Figures 5.7 and 5.8). However, the influence was slight for the ACC gene when compared to the FOX gene. The ACC gene detection was 63.64%, 36.36%, and 40.00% in the wastewater effluent and upstream and downstream rivers, respectively. The FOX gene detection was higher in the wastewater effluent (90.00%) than in the downstream and upstream rivers at 80% and 66.67%, respectively.

Generally, in NW-E, EBC and CIT genes were detected at lower levels in the upstream river, at 9.09% and 0.00%, respectively (Figures 5.7 and 5.8). The EBC and CIT genes detection levels were higher in the wastewater effluent at 81.82% and 70.00%, respectively. The EBC and CIT genes detection increased to 60.00% and 20.00% in the downstream river when compared with the upstream river.

5.3.3.2. Detection of antibiotic resistance genes in NW-C wastewater effluents and upstream river

In NW-C, the *ampC* gene was least detected at 18.18% in the wastewater effluent. The *ampC* gene increased by 21.82% from the wastewater effluent to the downstream maturation pond (Figures 5.7 and 5.8). In contrast, the detection level of the *bla_{TEM}* gene was higher and lower in the wastewater effluent and upstream river by 54.55% and 40.00%, respectively. Like the *bla_{TEM}* gene, the *int1* gene was detected to a greater extent in the wastewater effluent by 90.91% and less in the upstream river by 60.00%.

The same results for *ermB* and *ermF* genes were obtained, where their detection in the upstream river was 60.00% and 81.82% in wastewater effluent (Figures 5.7 and 5.8). The detection of *ermB* and *ermF* genes in the downstream maturation pond was 70.00% and 80.00%, respectively. This showed a discrepancy with the results obtained for the *sul1* and *sul2* genes. The downstream maturation pond recorded the highest *sul1* gene detection level

of 100% and the upstream river had the highest *sul2* gene detection level at 80.00%. The *sul1* and *sul2* genes increased from the wastewater effluent into the downstream maturation pond.

The FOX and MOX genes were detected to a greater extent in the upstream river than in the wastewater effluent and the downstream maturation pond (Figures 5.7 and 5.8). The FOX gene detection was at 80.00% in the upstream river, 72.73% in the wastewater effluent and 70.00% in the downstream maturation pond. The MOX gene detection was at 50.00% in the upstream river, 18.18% in the wastewater effluent and 10.00% in the downstream maturation pond (Figures 5.7 and 5.8).

In NW-C, an increase occurred in the CIT and ACC genes from wastewater effluent to the downstream maturation pond. The CIT gene detection increased from 18.18% in the wastewater effluent to 30.00% in the downstream maturation pond. The CIT gene was absent in the upstream river. Lower ACC gene detection, at 27.27%, was reported in wastewater effluent. The ACC gene detection in the upstream river and downstream maturation pond was at 40.00% and 50.00%, respectively (Figures 5.7 and 5.8).

The DHA and EBC detection decreased from the wastewater effluent to the downstream maturation pond. DHA gene detection was at 30.00% in the upstream river, 63.64% in the wastewater effluent and 40.00% in the downstream maturation pond. The EBC gene decreased from 63.64% in the wastewater effluent to 60.00% in the downstream maturation pond. The EBC gene detection in the upstream river was at 50.00% (Figures 5.7 and 5.8).

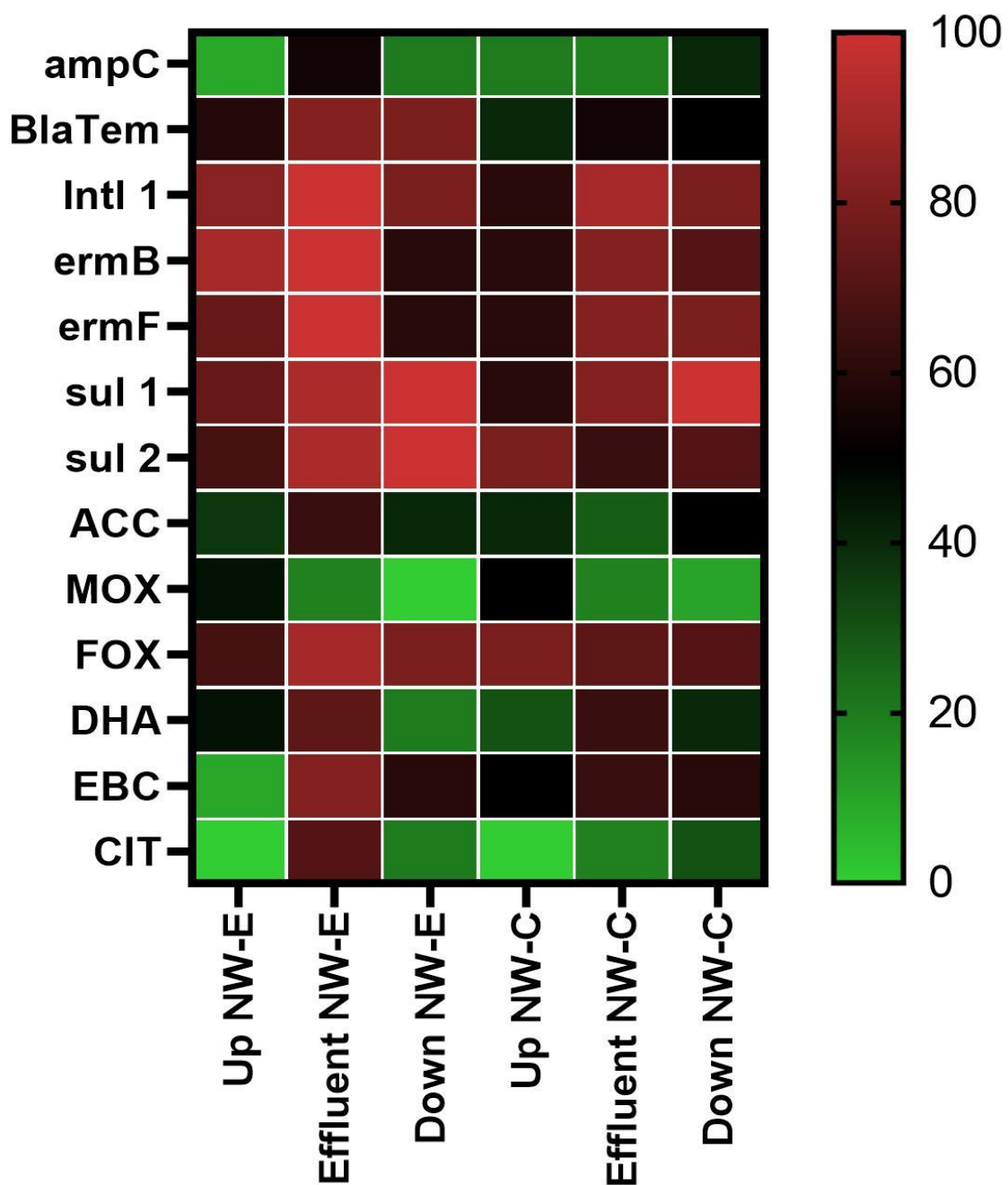


Figure 5.7: Heatmap of the end-point PCR screening process of ARGs in the upstream rivers, wastewater effluent and downstream points at NW-E and NW-C. The smallest, baseline and largest detection levels are represented by green, black and red colours, respectively.

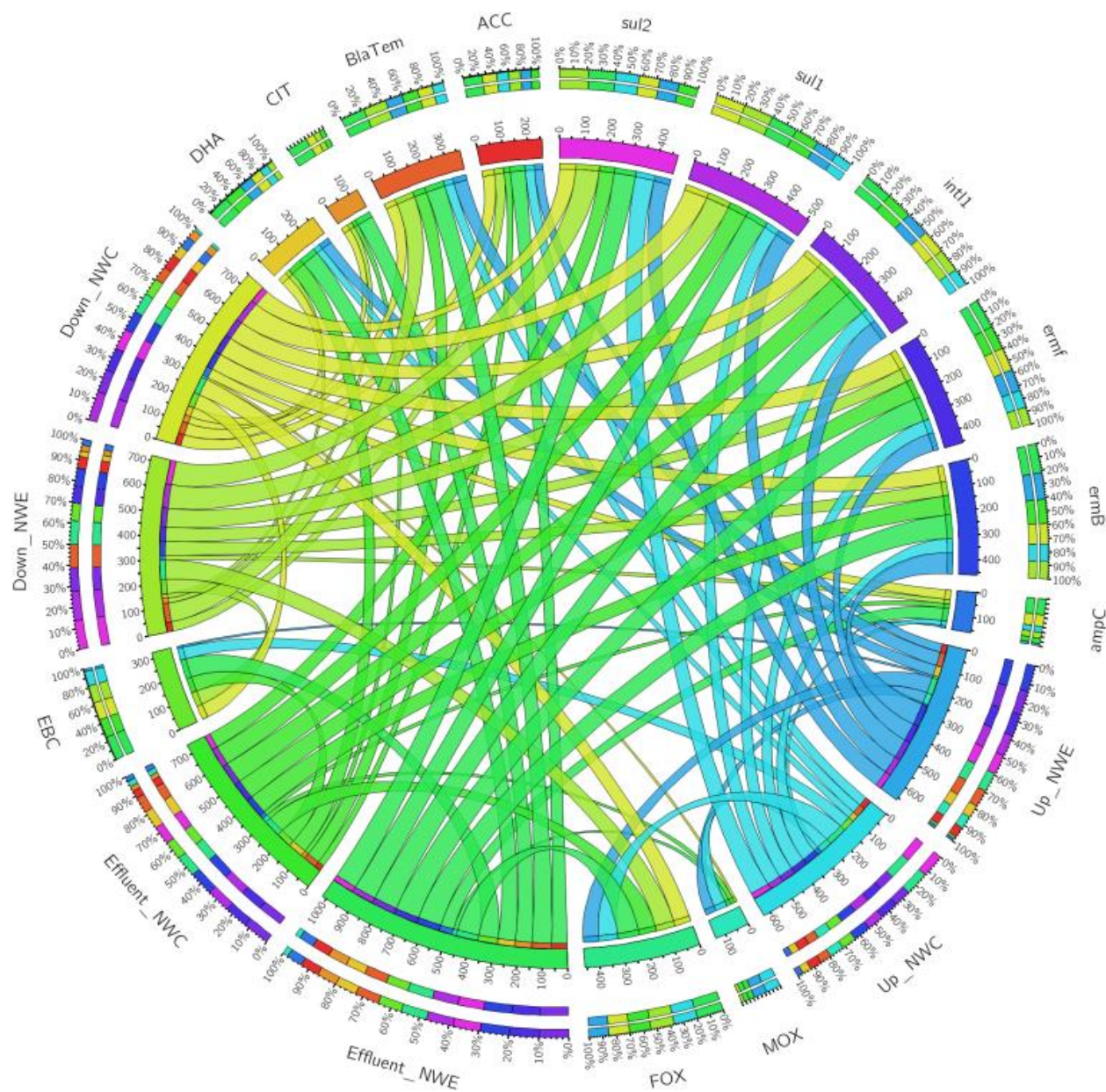


Figure 5.8: Chord diagram of the end-point PCR screening process of ARGs in the upstream rivers, wastewater effluent and downstream points at NW-E and NW-C.

5.3.4. Quantification of antibiotic resistance genes in wastewater effluents and rivers

The detected ARGs during the end-point PCR screening process were subjected to real-time PCR analysis, as shown in Table 5.4. ARGs that were found to be absent during end-point PCR analysis were excluded from the real-time PCR analysis. Unexpected observations were made, in which some ARG concentrations were higher in the upstream river than those of the wastewater effluent and the downstream point of NW-E and NW-C. It was also observed that some ARG concentrations increased from the wastewater effluent to the downstream maturation in NW-C.

5.3.4.1. Quantification of antibiotic resistance genes in NW-E wastewater effluents, downstream and upstream rivers

In NW-E, the wastewater effluent influenced the increased concentrations of the EBC and CIT genes in the downstream river (Table 5.4). The measured EBC gene concentrations were 1.90×10^{-2} , 1.96×10^0 and 2.44×10^{-1} copies/16S rRNA in the upstream river, wastewater effluent and downstream river, respectively. During the end-point PCR analysis, the CIT gene in the upstream river was not detected and was therefore not subjected to real-time PCR analysis. The CIT gene concentration decreased from 3.71×10^{-1} copies/16S rRNA in wastewater effluent to 2.23×10^{-3} copies/16S rRNA in the downstream river.

However, in NW-E, the wastewater water effluent did not influence the increased concentrations of ACC, DHA, MOX, FOX, *sul1* and *int1* genes in the downstream river (Table 5.4). The mean ACC concentrations were higher in wastewater effluent with 2.89×10^{-1} copies/16S rRNA, followed by the upstream river with 1.00×10^{-1} copies/16S rRNA and the downstream river with 6.11×10^{-2} copies/16S rRNA. The DHA concentrations were higher in wastewater effluent (2.90×10^{-1} copies/16S rRNA), followed by those of the upstream (4.15×10^{-2} copies/16S rRNA) and then downstream (9.19×10^{-3} copies/16S rRNA).

Due to its absence during the end-point PCR screening process, the MOX gene was not subjected to real-time PCR analysis in the downstream river (Table 5.4). The concentration of the MOX gene was 1.36×10^{-1} copies/16S rRNA in the upstream river and 1.82×10^{-1} in the wastewater effluent. The highest *sul1* concentration of 7.51×10^{-2} copies/16S rRNA was measured in the wastewater effluent, followed by 1.16×10^{-2} copies/16S rRNA in the upstream river and 5.67×10^{-3} copies/16S rRNA in the downstream river.

Unexpectedly, *int1* concentrations were highest in the upstream river (6.61×10^{-4} copies/16S rRNA), followed by those of the wastewater effluent (4.93×10^{-4} copies/16S rRNA) and then those of the downstream river (2.40×10^{-4} copies/16S rRNA). Similar to the *int1* gene, the FOX concentrations were higher in the upstream river (3.20×10^0 copies/16S rRNA), followed by wastewater effluent (1.45×10^0 copies/16S rRNA) and the downstream river (9.70×10^{-2} copies/16S rRNA).

5.3.4.2. Quantification of antibiotic resistance genes in NW-C wastewater effluents and upstream river

In NW-C, a trend was observed in which EBC and DHA concentrations were higher in the wastewater effluent, followed by the downstream maturation pond and upstream river (Table 5.4). The EBC concentrations were 1.65×10^{-1} , 2.87×10^0 and 1.66×10^0 copies/16S rRNA in the upstream river, wastewater effluent and downstream maturation pond, respectively. The DHA concentrations were 6.98×10^{-2} , 5.98×10^{-1} and 1.31×10^{-1} copies/16S rRNA in the upstream river, wastewater effluent and downstream maturation pond, respectively.

An unexpected trend was again observed in which ACC and FOX concentrations were higher in the upstream river, followed by the downstream maturation pond and wastewater effluent, as shown in Table 5.4. The ACC concentrations were 1.97×10^{-1} , 8.61×10^{-2} and 8.34×10^{-3} copies/16S rRNA in the upstream river, downstream maturation pond and wastewater effluent. The FOX concentrations were 4.06×10^0 , 1.38×10^0 and 7.57×10^{-1} copies/16S rRNA in the upstream river, downstream maturation pond and wastewater effluent.

The CIT, MOX and *int1* concentrations increased from the wastewater effluent to the downstream maturation pond, as shown in Table 5.4. Furthermore, concentrations of these ARGs were lower in the upstream river. However, due to its absence during the end-point PCR screening process, the CIT gene was not subjected to real-time PCR analysis in the upstream river. The CIT concentrations in the wastewater effluent and downstream maturation pond were 1.50×10^{-1} and 4.28×10^{-1} copies/16S rRNA, respectively.

The MOX concentrations were 7.29×10^{-1} , 1.58×10^0 and 3.83×10^0 copies/16S rRNA in the upstream river, wastewater effluent and downstream maturation pond, respectively (Table 5.4). The measured *int1* gene concentrations were higher in the downstream maturation pond with 5.38×10^{-4} copies/16S rRNA, followed by wastewater effluent with 4.09×10^{-4} copies/16S rRNA and upstream river with 3.30×10^{-4} copies/16S rRNA.

The *su1* concentrations were higher in the wastewater effluent (3.30×10^{-1} copies/16S rRNA), followed by the upstream river (3.88×10^{-2} copies/16S rRNA) and downstream maturation pond (3.16×10^{-2} copies/16S rRNA).

Table 5.4: Concentrations with standard deviations of ARGs in the upstream rivers, wastewater effluent and downstream points at NW-E and NW-C (units in gene copies/16S rRNA $\pm$ standard deviation).

	sites	EBC	ACC	CIT	DHA	MOX	FOX	<i>Int11</i>	<i>Sul1</i>
NW-E	Up	$1.90 \times 10^{-2} \pm$	$1.00 \times 10^{-1} \pm$	-	$4.15 \times 10^{-2} \pm$	$1.36 \times 10^{-1} \pm$	$3.20 \times 10^0 \pm$	$6.61 \times 10^{-4} \pm$	$1.16 \times 10^{-2} \pm$
		1.31×10^{-2}	1.96×10^{-1}		7.68×10^{-3}	7.68×10^{-2}	6.54×10^{-2}	1.06×10^{-3}	1.14×10^{-2}
	Effluent	$1.96 \times 10^0 \pm$	$2.90 \times 10^{-1} \pm$	$3.71 \times 10^{-1} \pm$	$2.90 \times 10^{-1} \pm$	$1.82 \times 10^1 \pm$	$1.45 \times 10^0 \pm$	$4.93 \times 10^{-4} \pm$	$7.51 \times 10^{-2} \pm$
		1.54×10^0	3.42×10^{-1}	3.09×10^{-1}	1.50×10^{-1}	4.35×10^{-1}	1.24×10^0	1.67×10^{-4}	3.33×10^{-2}
	Down	$2.44 \times 10^{-1} \pm$	$6.11 \times 10^{-2} \pm$	$2.23 \times 10^{-3} \pm$	$9.19 \times 10^{-3} \pm$	-	$9.70 \times 10^{-2} \pm$	$2.40 \times 10^{-4} \pm$	$5.67 \times 10^{-3} \pm$
		9.70×10^{-3}	2.93×10^{-2}	1.82×10^{-3}	1.42×10^{-3}		4.27×10^{-2}	1.61×10^{-4}	3.04×10^{-2}
NW-C	Up	$1.65 \times 10^{-1} \pm$	$1.97 \times 10^{-1} \pm$	-	$6.98 \times 10^{-2} \pm$	$7.29 \times 10^{-1} \pm$	$4.06 \times 10^0 \pm$	$3.30 \times 10^{-4} \pm$	$3.88 \times 10^{-2} \pm$
		1.40×10^{-1}	2.52×10^{-1}		1.40×10^{-1}	1.61×10^0	5.03×10^{-2}	2.13×10^{-4}	3.19×10^{-2}
	Effluent	$2.87 \times 10^0 \pm$	$8.34 \times 10^{-3} \pm$	$1.50 \times 10^{-1} \pm$	$5.98 \times 10^{-1} \pm$	$1.58 \times 10^0 \pm$	$7.57 \times 10^{-1} \pm$	$4.09 \times 10^{-4} \pm$	$3.30 \times 10^{-1} \pm$
		5.18×10^0	7.76×10^{-3}	2.76×10^{-1}	5.66×10^{-1}	9.37×10^0	1.42×10^{-2}	1.91×10^{-4}	2.76×10^{-2}
	Down	$1.66 \times 10^0 \pm$	$8.61 \times 10^{-2} \pm$	$4.28 \times 10^{-1} \pm$	$1.31 \times 10^{-1} \pm$	$3.83 \times 10^0 \pm$	$1.38 \times 10^0 \pm$	$5.38 \times 10^{-4} \pm$	$3.16 \times 10^{-2} \pm$
		9.54×10^{-1}	1.56×10^{-2}	4.16×10^{-1}	5.43×10^{-2}	1.12×10^{-1}	1.90×10^{-2}	8.38×10^{-4}	2.47×10^{-2}

Up – upstream river; down – downstream river for NW-E, downstream maturation pond for NW-C; LOD – limit of detection; ND – not done; “-“ not detected by end-point PCR.

5.3.5. Quantification of antibiotic residues in wastewater effluents and rivers

The chemical analysis generated the target compounds' concentrations, as reflected in Tables 5.5 and 5.6. The wastewater effluent was anticipated to harbour higher concentrations of target compounds than rivers. This trend was observed in NW-E and NW-C, with few abnormalities in the downstream river and maturation pond. The quantification of the NW-E downstream river was based on one sampling event conducted in February 2022.

5.3.6. Quantification of antibiotic and fluconazole residues in NW-E wastewater effluent, downstream and upstream rivers

Overall, target compounds were higher in wastewater effluent than in the upstream and downstream rivers (Table 5.5). Although measured once in February 2022, the downstream river followed the trend, in which the wastewater effluent harboured more target compounds than downstream river. Significant differences (p-value <0.05) observed in NW-E were found for ampicillin and fluconazole. Significant differences (p-value <0.05) were observed for ampicillin between wastewater effluent and downstream river. Significant differences (p-value <0.05) were observed in the wastewater effluent and the upstream river for fluconazole. However, the antibiotic concentration was greater in the upstream river than in the wastewater effluent, as indicated below:

- (i) In October 2020, ampicillin concentrations were 12.19, 24.90 and 22.6 µg/L in the upstream river, wastewater effluent and downstream river, respectively. Ampicillin concentrations ranged from 3.93 to 37.77 µg/L, averaging at 18.82 µg/L.
- (ii) Ciprofloxacin concentrations were higher in the upstream river than in wastewater effluent and downstream rivers in November 2020 and February 2022. In November 2020, ciprofloxacin concentrations were 0.59 and 0.53 µg/L in the upstream river and wastewater effluent. However, these were not determined in November 2020. In February 2022, the concentrations were 1.00 µg/L in the upstream river and <LOQ in the wastewater effluent and downstream river. Ciprofloxacin concentrations ranged from <LOQ to 4.44 µg/L, averaging 1.46 µg/L.
- (iii) Sulfamethoxazole concentrations were higher in the upstream than the wastewater effluent in November and December 2020. In November 2020, sulfamethoxazole concentrations were 4.04 in the upstream river and 3.87 µg/L in the wastewater

effluent. In December 2020, they were 3.85 in the upstream river and 3.66 µg/L in the wastewater effluent. They ranged from <LOQ to 18.57 µg/L, averaging 1.46 µg/L.

- (iv) A general trend in NW-E was that trimethoprim concentrations for most months were <LOQ. In February 2022, they were higher in the upstream river (0.20 µg/L) than in wastewater effluent (<LOQ) and downstream river (<LOQ). Overall, they were 0.06 µg/L in the upstream river, 0.16 µg/L in the wastewater effluent and <LOQ in the downstream river.
- (v) Fluconazole concentrations were at <LOQ until August 2021 in the upstream river and wastewater effluent. However, in February 2021, they were 24.77, 6.60 and 17.30 µg/L in the wastewater effluent, upstream river and downstream rivers.

5.3.7. Quantification of antibiotic and fluconazole residues in NW-C wastewater effluents and upstream river

Trimethoprim concentrations were higher in the wastewater effluent, followed by the downstream maturation pond and the upstream river (Table 5.6). However, an increase in these concentrations was recorded from the wastewater effluent to the downstream maturation pond in November 2020 and December 2020. In November 2020, the increase was from 0.24 to 0.30 µg/L; in December 2020, it was from 0.32 to 0.73 µg/L.

In NW-C, fluconazole was <LOQ, except in wastewater effluent collected in August 2021 and February 2022 (Table 5.6). In August 2021 and February 2022, the fluconazole concentrations were 1.30 and 31.62 µg/L, respectively. However, there were no significant differences in fluconazole between the wastewater effluent, upstream river and downstream maturation pond.

Generally, the downstream maturation pond had lower and higher sulfamethoxazole concentrations than the wastewater effluent and upstream river. The sulfamethoxazole concentrations were 1.22, 3.08 and 2.19 µg/L in the upstream river, wastewater effluent and downstream maturation pond, respectively, as shown in Table 5.6. Significant differences (p-value <0.05) existed between wastewater effluent and the upstream river.

The ampicillin concentrations increased from the wastewater effluent to the downstream maturation pond in November 2020, February 2021 and August 2021 (Table 5.6). For other months, the ampicillin concentrations were higher in the wastewater effluent. The lower ampicillin concentrations were measured in the upstream river. There were no significant differences (p -value >0.05) in this respect between wastewater effluent, the upstream river and downstream maturation pond.

Overall, the ciprofloxacin concentrations were higher in the wastewater effluent, followed by the downstream maturation pond and upstream river, with no significant differences (p -value >0.05). However, in November 2020, the increase from the wastewater effluent to the downstream maturation ranged from 0.77 to 1.04 $\mu\text{g/L}$ (Table 5.6). In July 2021, there was an increase from 0.53 to 1.66 $\mu\text{g/L}$.

5.3.8. Risk quotient for wastewater effluents and rivers

Figure 5.9 shows that the obtained RQ was the least for trimethoprim. Wastewater effluents, the downstream maturation pond and rivers in NW-E and NW-C recorded an RQ of <0.1 . The obtained RQ for ampicillin, ciprofloxacin and sulfamethoxazole was ≥ 1 . The calculated RQ of fluconazole was <1 in the NW-E wastewater effluent and upstream river. In NW-C, the RQ was <0.1 for the upstream river and downstream maturation pond and <1 in the wastewater effluent. However, the RQ of the NW-E downstream river was not determined, as sampling was only conducted once at these sites. As a result, calculating RQ might not have given a true representation of the NW-E downstream river over a long period.

Table 5.5: Concentrations with standard deviations of target fluconazole and antibiotic residues in NW-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.

Table 5.6: Concentrations with standard deviations of target fluconazole and antibiotic residues in NW-C upstream rivers wastewater effluent, and downstream maturation pond at NW-C.

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.

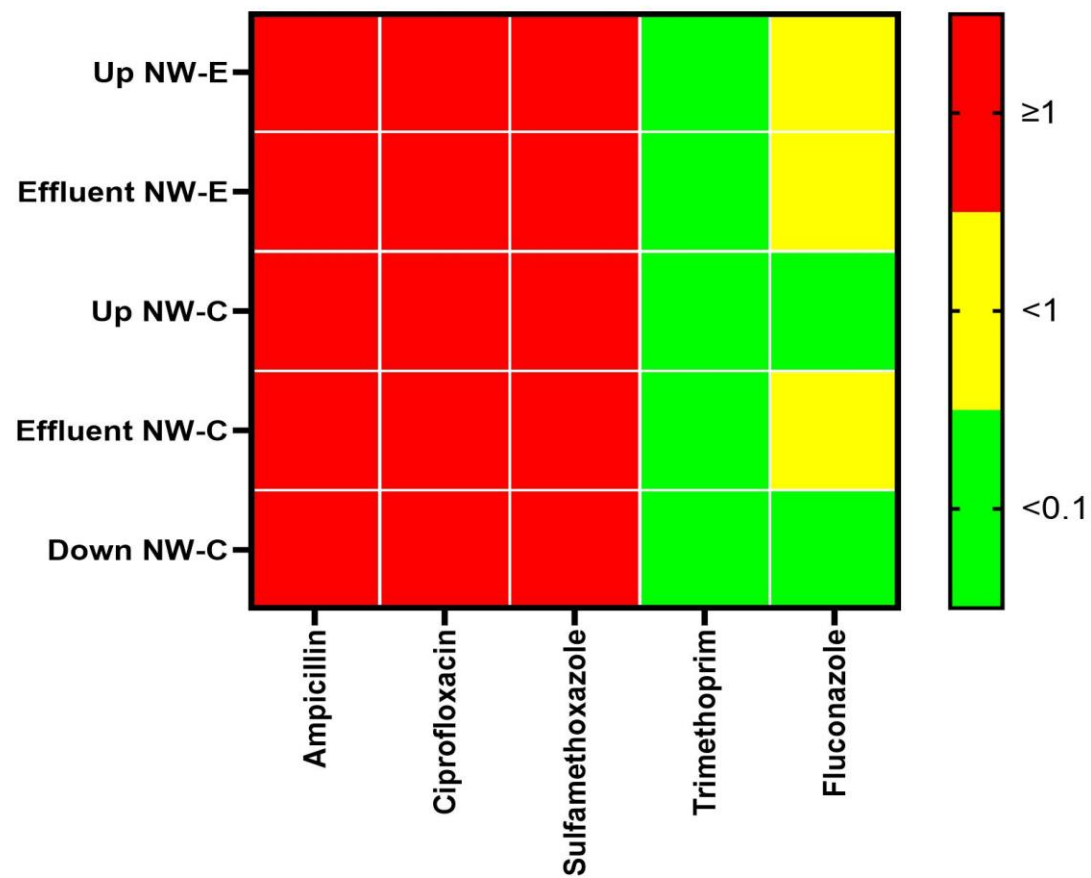


Figure 5.9: Heatmap of Risk quotient (RQs) in the upstream rivers, wastewater effluent and downstream points at NW-E and NW-C.

5.3.9. Correlation of bacterial communities with ARGs, antibiotics and physicochemical parameters in wastewater effluents and rivers

Overall, no significant difference was found for the emergence of the relative abundance percentage of bacterial community with antibiotic residues, ARGs and physicochemical parameters in all canonical axes, as shown in Figure 5.10. The relative abundance percentage of *Comamonas* was associated with fluconazole. *Zymomonas*, *Nocardia* and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* were correlated with MOX. The unassigned group was associated with nitrate, phosphate and DHA. *Dickeya*, *Prevotella* and other genera were correlated with physicochemical parameters (nitrite, TDS, COD, salinity), ARGs (*ermB*, *ermF*, FOX, EBC, CIT, ACC, *ampC* and *intl1*) and antibiotic residues (ciprofloxacin and sulfamethoxazole). *Pseudarcobacter* and *Arcobacter* were correlated with temperature, *sul1*, *sul2*, *bla_{TEM}* and ampicillin.

Table B2 in Appendix B shows the pairwise correlation matrices. In this part of the project, attention was shifted to the significant pairwise correlation matrices (p-value <0.05) of bacterial community composition against physicochemical parameters, ARGs and antibiotic residues. The results were as follows: (i) other genera were significantly correlated with TDS (p-value = 0.36), ACC (p-value = 0.04), CIT gene (p-value = 0.07) and *ampC* gene (p-value = 0.02). (ii) *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* were significantly correlated with TDS (p-value = 0.45), salinity (p-value = 0.11), the *bla_{TEM}* gene (p-value = 0.23), the *intl1* gene (p-value = 0.16). (iii) *Arcobacter* was significantly correlated with pH (p-value = 0.36). (iv) *Comamonas* was significantly correlated with the DHA gene (p-value = 0.38) and ciprofloxacin (p-value = 0.48). (v) *Nocardia* (p-value = 0.29) and *Zymomonas* (p-value = 0.18) were significantly correlated with the ACC gene.

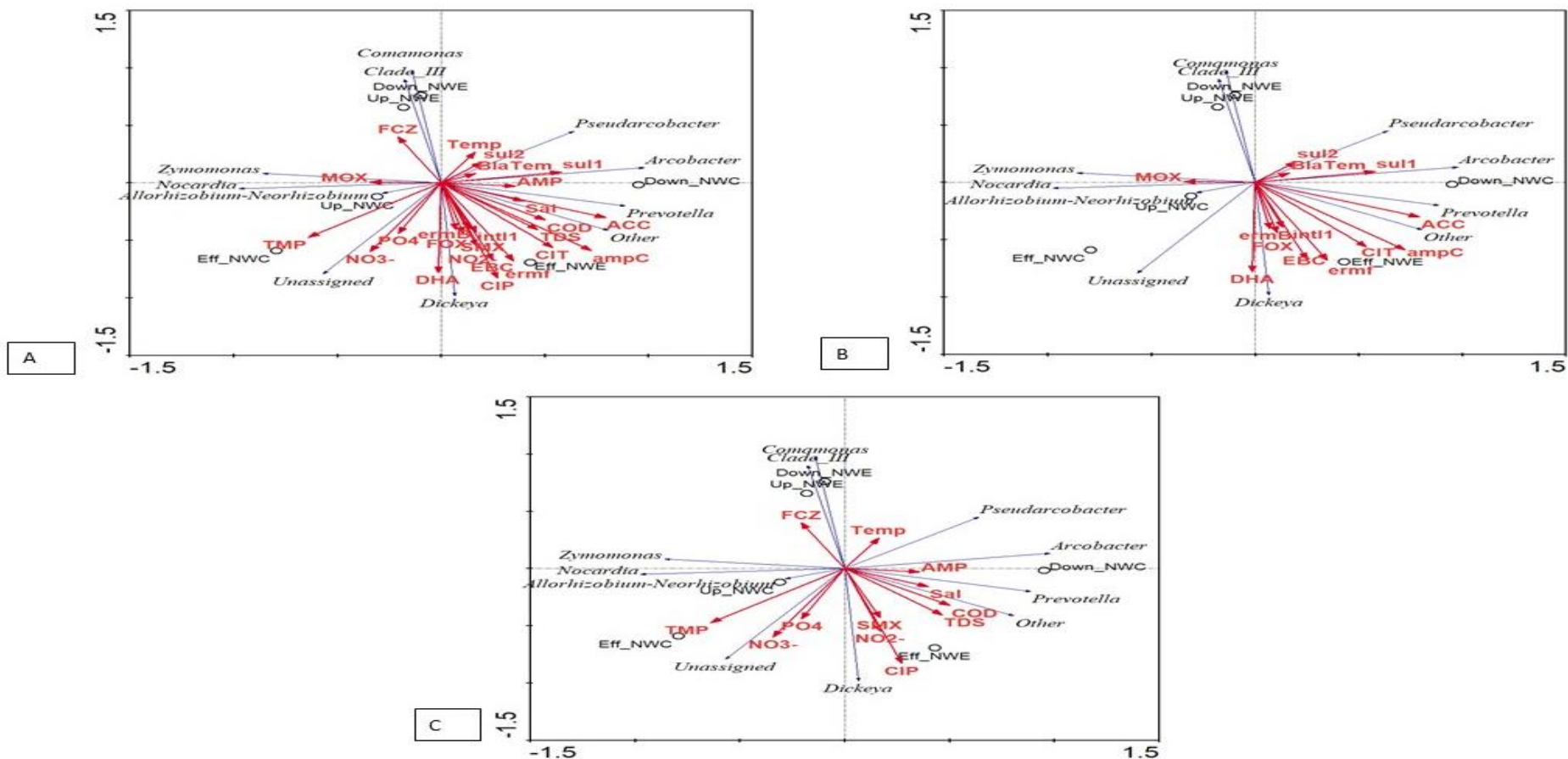


Figure 5.10: RDA of correlation between (A) bacterial community composition, physicochemical parameters, ARGs and antibiotic residues (B) bacterial community composition and ARGs and (C) bacterial community composition, physicochemical parameters and antibiotic residues in the upstream rivers, wastewater effluent and downstream points at NW-E and NW-C.

FCZ – fluconazole; temp – temperature; AMP – ampicillin; sal – salinity; COD - Chemical oxygen demand, TDS - Total Dissolved Solids; SMX – Sulfamethoxazole; NO₂⁻ - nitrite, NO₃⁻ - nitrate; PO₄ – phosphate; TMP – trimethoprim.

5.4. DISCUSSION

5.4.1. Physicochemical parameters in wastewater effluents and rivers

According to the DWS (2022a), the Green Drop score for North West is 18%. This was based on an examination of ten WWTPs, all of which were classified to be in a critical state. However, the microbiological, chemical and physical compliance of most WWTPs were not determined due to insufficient data sets and no monitoring at the plants. The two selected WWTPs of the present study were not part of the report. The present study shows that these WWTPs influenced the abundance of physicochemical parameters in the downstream rivers. As is known, discharging inadequately treated wastewater effluent may change the river water quality, and its effect may promote bacterial growth and cause harm to aquatic life (EPA, 2021; Han *et al.*, 2022; WHO, 2022).

5.4.1.1. Temperature

The minimum and maximum temperatures in NW-E were 10.50°C and 26.10°C, respectively. In NW-C, the minimum and maximum temperatures were 7.60°C and 24.30°C. The present study recorded maximum and minimum temperatures in summer and winter, respectively. This follows the results of other studies (Adefisoye and Okoh, 2017; Nthunya *et al.*, 2018). Water temperatures of more than 20°C recorded in NW-E and NW-C may be favourable for the growth and reproduction of mesophilic bacteria.

5.4.1.2. pH

The pH measured in NW-E and NW-C ranged between 7.57 and 9.30. According to DWAF SAWQG (1996b) for agricultural use: irrigation, the pH range of between 6.5 – 8.4 is deemed to be of good quality, and this water does not damage foliar in plants. Soil pH and irrigation equipment within this range are not associated with major problems. A pH > 8.4 damage foliar in plants (ultimately affecting the quality of plants), increases problems with irrigation equipment and unavailability of several macro- and micro-nutrients over the long term. NW-E showed no significant differences in pH between the upstream river, wastewater effluent and downstream river whereas, at NW-C, there were significant differences (p -value <0.05) in pH occurred between the upstream and downstream maturation pond. This could be associated with raw sewage leakage and the disposal of chemicals by residents into the NW-C downstream maturation pond. The pH range that was obtained is comparable to the alkaline

pH recorded in WWTP and surface water in North West and Limpopo Provinces, South Africa (Nthunya *et al.*, 2018; Makuwa *et al.*, 2020). Furthermore, a pH of > 8.00, as measured in NW-E and NW-C WWTPs, might have affected the chlorination process (WHO, 2022). This could have resulted in NW-E and NW-C wastewater effluent discharge that harboured potential pathogens into receiving bodies.

5.4.1.3. Total dissolved solids

In NW-E, the TDS levels of 824.88 mg/L in wastewater effluent were significantly (p-value <0.05) than 606.25 mg/L in the upstream river and 613.33 mg/L in the downstream river. This trend is not uncommon in South Africa, as it was measured in New Germany treatment works, KwaZulu-Natal (Pillay and Olaniran, 2016). However, in NW-C, the trend followed a trajectory along the following sites: downstream maturation pond, wastewater effluent and upstream river. The TDS levels were associated with raw sewage leakage and disposal of litter in the NW-C downstream maturation pond. These trends were not similar to the results obtained in Nairobi County, Kenya, and Northern Durban, South Africa, which reported high TDS levels in the upstream rivers in contrast with the wastewater effluents (Pillay and Olaniran 2016; Ngigi *et al.*, 2020).

In terms of the DWAF SAWQG (1996c) for agricultural use: livestock watering, the measured TDS in NW-E and NW-C were found to be within the recommended standards that do not cause adverse effects on sheep, pigs, poultry, dairy, beef and horse production. Furthermore, the NW-E and NW-C rivers were found to be within the range of the DWAF SAWQG (1996b) for agricultural use: irrigation, suggesting that these water sources can support crops with moderate salt tolerance to a relative yield between 90% and 95% under a low-frequency application system. However, a leaching fraction of up to 0.15 must be carried out while avoiding wetting susceptible crop foliage.

5.4.1.4. Salinity

In NW-E, the salinity levels were significantly higher (p-value <0.05) in the wastewater effluent than the rivers. As a result, the wastewater effluent increased the salinity levels from the upstream to the downstream rivers. However, in NW-C, the salinity was higher in the downstream maturation pond due to raw sewage leakage and littering occurring around it. As in the case of the NW-E, the minimum salinity levels in NW-C were measured in the upstream river. These did not follow the results obtained in Vhembe District, Limpopo Province, South

Africa, which showed that wastewater effluent harboured low salinity levels when it was compared to rivers (Nthunya *et al.*, 2018). However, consistent with the NW-E and NW-C WWTPs, the WWTP in Vhembe District was not part of the DWS (2022a) Green Drop report. Salinity levels in NW-E and NW-C were attributed to anthropogenic activities, agricultural use and/ or resource mining in or around the specific sampling points. According to the DWAF SAWQG (1996c) for agricultural use: livestock watering, the measured salinity in NW-E and NW-C the recommended standards that do not cause adverse effects on livestock.

Salinity levels of 378.00 and 480.00 mg/L in NW-E upstream and downstream rivers and 311.43 mg/L in the NW-C upstream river were measured. According to the DWAF SAWQG (1996b) for agricultural use: irrigation, a salinity range between 230 and 460 mg/L indicates that crops moderately tolerant to foliar absorption accumulate toxic sodium levels when crop foliage is wetted. They display symptoms of foliar injury and yield decreases. Salinity levels higher than 460 mg/L indicate that crops tolerant to foliar absorption increasingly accumulate toxic sodium levels when crop foliage is wetted. They display symptoms of foliar injury and yield decreases.

5.4.1.5. Chemical oxygen demand

The chemical oxygen demand (COD) levels ranged from 20 to 45 mg/L in NW-E wastewater effluent. These results were comparable to the COD levels in wastewater effluent in the Czech Republic (Ofori *et al.*, 2022). However, the findings did not match the high COD levels reported in studies conducted in KwaZulu-Natal and Eastern Cape (Pillay and Olaniran, 2016; Adefisoye and Okoh, 2017). COD is an important water quality parameter used to assess the efficiency of WWTPs. Furthermore, to ensure safe water reusability in agriculture, the COD must be constantly monitored to determine whether it exceeds the agricultural water control standard (Han *et al.*, 2022). The constant monitoring of the COD levels in rivers may be beneficial, since inadequately treated wastewater effluent can cause ecological risks to the aquatic life in the downstream rivers.

5.4.1.6. Nitrate and nitrite

The discharged wastewater effluent (3.27 mg/L) may have insignificantly ($p\text{-value} > 0.05$) influenced the nitrate levels from the upstream (0.88 mg/L) to the downstream (1.07 mg/L) rivers. In addition, the higher nitrate levels may also be associated with agricultural activities occurring in and around the downstream rivers. This was similar to the results obtained by

Ofori *et al.* (2022) in the Czech Republic. However, in NW-C, nitrate levels of 13.37 mg/L in wastewater effluent and 5.35 mg/L in the downstream maturation pond were significantly higher (p -value <0.05) than 1.14 mg/L in the upstream river. This finding is not uncommon, since surface water is cleaner than the wastewater effluent.

The levels in NW-E and NW-C indicate that wastewater effluents and rivers were not polluted with nitrates. According to the WHO (2016), unpolluted surface water nitrate levels range between 1 and 10 mg/L whereas, for wastewater effluent, the range was up to approximately 90 mg/L. In terms of the DWAF SAWQG (1996c) for agricultural use: livestock watering, the recorded nitrate in water was found to be within the recommended standards that do not cause adverse effects on ruminants. However, DWAF SAWQG (1996b) for agricultural use: irrigation does not monitor nitrate in water, the latter which can benefit plant growth since it is used in fertilizer production.

Surprising observations were made in NW-E downstream river, where nitrite levels were 0.00 mg/L despite lawn production and livestock farming in and around the site. This may be misleading, since the data was based on one sampling run. NW-C nitrite levels were comparable for wastewater effluent (19.11 mg/L) and the downstream maturation pond (19.17 mg/L). However, there are no guidelines for nitrite and nitrate in the DWAF SAWQG (1996b) for agricultural use: irrigation. Nitrite is one of the nitrogen components needed for plant growth. Furthermore, the nitrite levels measured in NW-E and NW-C water were found to be within the recommended standards of the DWAF SAWQG (1996c) for agricultural use: livestock watering, which does not cause adverse effects on ruminants.

5.4.1.7. Phosphate

The phosphate levels in the NW-C upstream river, wastewater effluent and downstream river were 5.68, 35.00 and 20.92 mg/L, respectively. This follows the results of studies where the phosphate levels were higher in the wastewater effluent, followed by the downstream point and upstream river (Pillay and Olaniran, 2016; Ofori *et al.*, 2022). However, the downstream river was only measured once at NW-E, showing high phosphate levels compared to the wastewater effluent and the upstream river. The high phosphate levels in the downstream river could be attributed to the agricultural activities near the NW-E downstream river sampling point. Although phosphate does not form part of the DWAF SAWQG (1996b;1996c) for agricultural use, it is vital for animal and plant growth.

The present study's findings are of utmost importance, since the NW-E and NW-C WWTPs were not part of the DWS (2022a) Green Drop report. Generally, the physicochemical parameters of the wastewater effluents and rivers were found to be within the recommended standards of the DWAF SAWQG (1996b) and (1996c) for agricultural use: irrigation and livestock watering. However, TDS was not found to be within the recommended standards of the DWAF SAWQG (1996b) for agricultural use: irrigation. Although TDS was not within the recommended standards, the NW-E and NW-C water sources could still support crops with moderate salt tolerance to a relative yield between of 90% and 95% under a low-frequency application system. However, a leaching fraction of up to 0.15 must then be carried out while avoiding wetting susceptible crop foliage.

In NW-E, the levels of physicochemical parameters increased from the upstream to the downstream river after blending with wastewater effluent. This shows that the WWTP influenced the levels of physicochemical in the downstream rivers. However, in NW-C, the levels of physicochemical parameters increased from the wastewater effluent to the downstream maturation pond. This increase was anticipated, since the downstream maturation pond passes through a residential area where it is used as a landfill site. Moreover, there is raw sewage leaking into it. The physicochemical parameters in water create conditions conducive for microbial life and reproduction.

5.4.2. Bacterial community composition in wastewater effluents and rivers

Proteobacteria had the second highest relative abundance percentage with a high proportion of *Comamonas*, followed by *Dickeya*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and *Zymomonas*. This was consistent with results obtained in extant studies where the relative abundance of Proteobacteria was dominant (Vierheilig *et al.*, 2015; Jordaan and Bezuidenhout, 2016; Tang *et al.*, 2016; Wang *et al.*, 2018a; Osunmakinde *et al.*, 2019; Picazo *et al.*, 2019; Pascual-Benito *et al.*, 2020). This phylum comprises mostly Gram-negative bacteria and possess great metabolic diversity (Osunmakinde *et al.*, 2019).

Comamonas species belonging to the family Comamonadaceae are Gram-negative bacilli commonly found in freshwater, soil and wastewater (Hem *et al.*, 2022). Despite their ubiquitous environmental distribution, they are considered rare opportunistic pathogens to humans (Jiang *et al.*, 2018; Pavone *et al.*, 2021). According to Pavone *et al.* (2021), they were the first to report *Comamonas kerstersii* infection in animals, specifically a goat. In wastewater treatment, these species may be involved in bioremediation and bioaugmentation (Hem *et al.*,

2022). The present study detected a relative abundance of 3.17% and 4.66% in NW-E and NW-C wastewater effluent, respectively.

The relative abundance percentage of *Dickeya* ranged between 5.67% and 12.97 in NW-E samples and 10.91% and 13.11% in NW-C. The *Dickeya* species consist of pectinolytic enzymes that are pathogenic to plants and responsible for soft rot diseases worldwide. Hence, they have been ranked in the top ten of the most destructive bacterial plant pathogens (Dobhal *et al.*, 2020). Laurila *et al.* (2008) isolated these species from stem and tuber samples collected from the field of 15% of the potato growers, where many encountered fields with a high incidence of rotten plants, in Finland. Furthermore, *Dickeya* species were isolated in river samples along the coastal region of Finland. Dobhal *et al.* (2020) isolated them, including *D. dianthicola*, from stems (100% detection), roots (100% detection), tubers (81% detection) and water (66.7%) in naturally infected potato tissues ranging from Missouri, North Carolina, Indiana, Florida, Texas, Idaho and/ or Wisconsin, United States. The presence of *Dickeya* in freshwater, specifically irrigation water, may negatively impact important crops.

Phylum Bacteroidota had a high relative abundance percentage in NW-E upstream river (24.14%) and NW-C wastewater effluent (29.46%). NW-C results were consistent with the findings of Pascual-Benito *et al.* (2020) in Spain. In contrast to NW-E, Wang *et al.* (2018a) recorded a low relative abundance percentage of the phylum in the upstream river (Jialing), China. A study conducted in three WWTPs in South Africa detected a low relative abundance percentage of it (Osunmakinde *et al.*, 2019). However, most of its genera were regarded as unclassified or “other” in the present study. Low relative abundance above 1.00% of genera *Bacteroides* and *Prevotella* belonging to Bacteroidota was found. This phylum is frequently found in marine and freshwater water and plays a vital role in the biodegradation of organic matter. Its presence in aquatic environments is associated with faecal pollution (Osunmakinde *et al.*, 2019; Pascual-Benito *et al.*, 2020).

Genera *Candidatus Saccharimonas* and *Saccharimonadaceae* belonging to phylum Patescibacteria were absent in NW-C wastewater effluent and the downstream maturation pond. However, in NW-E, the relative abundance of *Candidatus Saccharimonas*, *Saccharimonadales* and *Saccharimonadaceae* in the downstream river might have been influenced by the WWTP, as they were detected in relatively low percentages in the upstream river. Most bacterial species belonging to Patescibacteria are partially anaerobic, lack essential respiratory complexes and can perform mixed-acid fermentation (Haro-Moreno *et al.*, 2023).

Genus *Sulfuricurvum* belonging to phylum Campilobacterota was relatively low in wastewater compared to the upstream rivers and downstream points in NW-E and NW-C. Furthermore, the genus *Pseudarcobacter* was not detected in NW-C wastewater effluent. In contrast, Genus *Arcobacter* was present in all NW-E and NW-C samples. Genus *Microcystis PCC-7914* belonging to the phylum Cyanobacteria was absent in NW-E and NW-C wastewater effluents. However, a low relative abundance of *Microcystis PCC-7914* was detected in NW-E and NW-C upstream rivers and downstream points. Cyanobacteria were also detected in the Jialing River, China (Wang *et al.*, 2018a).

Genus *Nocardia* belonging to phylum Actinobacteriota ranged from 3.12% to 5.99% in NW-E and from 1.93% to 8.89% in NW-C. This is consistent with extant results where the relative abundance of this phylum was present in surface water and WWTPs (Wang *et al.*, 2018a; Picazo *et al.*, 2019; Osunmakinde *et al.*, 2019; Pascual-Benito *et al.*, 2020). It has been proposed that Actinobacteriota assess water quality due to their sensitivity to environmental conditions that cause cyanobacterial blooms (Pascual-Benito *et al.*, 2020). Domestic and industrial wastewater runoff into rivers can cause toxic cyanobacterial blooms (Pascual-Benito *et al.*, 2020). Furthermore, Actinobacteriota has been identified to survive oligotrophic environments (Wang *et al.*, 2018a).

In NW-E and NW-C, phylum Verrucomicrobiota ranged between 0.16% and 2.81%. This contrasts with Tang *et al.* (2016), who reported a high relative abundance percentage of the phylum in some upstream and downstream rivers when compared to the wastewater influent and effluent. However, a low relative abundance percentage of the phylum was detected in studies in Spanish and other South African WWTPs (Osunmakinde *et al.*, 2019; Pascual-Benito *et al.*, 2020). The present study regarded most of its genera as unclassified or other. It has been suggested that the phylum degrades stable polysaccharides such as fucose-containing sulphated polysaccharides (Osunmakinde *et al.*, 2019; Orellana *et al.*, 2021). This suggestion was based on Verrucomicrobiota's consumption of butanoate and propanoate, which indicates oxidation of fatty acid and fermentation activities (Osunmakinde *et al.*, 2019)

A low relative abundance percentage of the phylum Firmicutes was detected in NW-E and NW-C, ranging from 1.05% and 5.09%. This followed the low relative abundance percentage of Firmicutes reported in WWTP and surface water in Spain and Austria, respectively (Vierheilig *et al.*, 2015; Pascual-Benito *et al.*, 2020). It is an essential water quality indicator for faecal pollution (Wang *et al.*, 2018a). This was shown by Vierheilig *et al.* (2015), who reported a dominant relative abundance percentage of Firmicutes in faecal samples,

consistent with its high relative abundance percentage in wastewater influent and effluent of Daspoort WWTP, Pretoria, South Africa (Osunmakinde *et al.*, 2019).

Unexpected results were obtained in NW-E, in which the upstream and downstream rivers were richer and more dominant than the wastewater effluent. This may be attributed to the mining, recreational, agricultural, and anthropogenic activities in and around these rivers. Pascual-Benito *et al.* (2020) reported that an upstream river was richer and more diverse than the wastewater effluent and downstream river in Spain. However, in NW-E, the wastewater effluent was more diverse and even. In contrast, the downstream maturation pond was richer, more diverse, even and dominant in NW-C. The NW-C downstream maturation pond passes through the residential area of NW-C, where residents dispose of litter and maintain livestock farming in and around this source. Furthermore, there is raw sewage leakage in the NW-C downstream maturation pond. However, no significant differences (p -value >0.05) were found in NW-E and NW-C wastewater effluent, upstream rivers and downstream points. The results obtained by Tang *et al.* (2016) in Lucun WWTP, China, show that wastewater effluent is more diverse and richer than upstream and downstream rivers. However, these authors report that the wastewater influent was less rich and diverse than the wastewater effluent, upstream rivers and downstream rivers.

In NW-E and NW-C, the presence of bacterial communities in water was influenced by agriculture, urbanisation, WWTPS, mining and other anthropogenic activities. The present study showed the presence of potential human, animal and plant pathogens in wastewater effluents and rivers (Laurila *et al.*, 2008; Dobhal *et al.*, 2020). Residents of NW-E and NW-C were dependent on these river sources for livestock watering and agricultural use. By using this water in livestock watering, they may spread the potential pathogens to animals, thus leading to high morbidity and mortality in agriculture. Furthermore, applying this water for agriculture may result in the economic loss of important crops due to potential pathogens (Dobhal *et al.*, 2020). Some of the bacterial communities have been associated with the emergence of ARGs. The present study used high-throughput 16S rRNA sequencing to identify, diversify and monitor bacterial communities in water (Osunmakinde *et al.*, 2019; Pascual-Benito *et al.*, 2020). This was done to overcome the limitations of culture-based techniques that allow the study of slightly above 2.1% of culturable bacteria (Jing *et al.*, 2021).

5.4.3. Antibiotic resistance and *intl1* genes in wastewater effluents and rivers

The WWTPs are the hotspots for antibiotic resistance and are not designed to eliminate ARGs (Mann *et al.*, 2019). Hence, the presence of ARGs in the wastewater effluent of NW-E and

NW-C was anticipated. Furthermore, agricultural activities and urbanization in and around NW-E and NW-C water sources could have affected the presence of ARGs in rivers. In NW-C, higher ARG detection and concentrations in the downstream maturation pond were anticipated, since residents use this source as a landfill site, in addition to raw sewage that leaks into it.

5.4.3.1. *AmpC* and *bla_{TEM}* gene screening

The detection of the *bla_{TEM}* gene was higher in wastewater effluents, followed by the downstream points and upstream rivers of NW-E and NW-C. The detection range was between 40.00% and 81.82%. This does not follow the results obtained in the Coimbra metropolitan area, Portugal (Amador *et al.*, 2015). Their results showed that the detection of the *bla_{TEM}* gene isolated in *Enterobacteriaceae* isolates was higher in the downstream river, followed by the upstream river and wastewater effluent. The detection range was between 9.1% and 40%. A high *bla_{TEM}* genes detection of 100% in surface water was recorded in *Listeria monocytogenes* isolated in Chris Hani and Amathole District Municipalities in Eastern Cape Province, South Africa (Iwu and Okoh, 2019). Furthermore, the *bla_{TEM}* gene was detected in all human faeces' samples from Changzhou, China (Chen *et al.*, 2023).

The *ampC* gene detected in NW-C and NW-E was the lowest at 18.18% and highest at 54.55% in the wastewater effluent. The highest detection level of NW-E wastewater effluent was comparable to 50% detected in irrigation water in *Listeria monocytogenes* isolated in Chris Hani and Amathole District Municipalities in Eastern Cape Province, South Africa (Iwu and Okoh, 2019). In central Israel, an *ampC* gene detection level of 100% was reported in both the wastewater effluent and downstream water used for irrigation (Nasser *et al.*, 2019).

Livestock farming in and around NW-E and NW-C may have contributed to the increased β -lactams resistance gene such as *ampC* and *bla_{TEM}* in water sources. An estimation of 133 000 $\mu\text{g/kg}$ of β -lactam was detected in the manure of swine (weighing 150 pounds on average) in a confined feeding operation (Huang *et al.* 2021). β -lactams are slightly persistent in manure, with a half-life of five days (Boxall *et al.*, 2004; Massé *et al.*, 2014).

5.4.3.2. *Int1* gene screening

In NW-C, the *int1* gene was detected less in the upstream river. NW-E observed a slight decrease in the *int1* gene detection from the upstream river to the downstream river.

Furthermore, in NW-E, the detection of the *int11* gene was 100% in the wastewater effluent. The same results were recorded in Plant A wastewater effluent in Louisiana, United States, and Pearl River and Pearl River Estuary, South China (Chen *et al.*, 2015; Thakali *et al.*, 2020). The *int11* gene detection in Plant B wastewater effluent in Louisiana, United States, was comparable to that recorded in NW-C upstream river (Thakali *et al.*, 2020). However, in Gaborone, Botswana, the *int11* gene was detected in low levels below 30% in the wastewater effluent and the downstream river (Tapela and Rahube, 2019). The ability of the *int11* gene cassette to recombine with other ARGs enables ARBs harbouring the *int11* gene to render antibiotics in different classes ineffective (Chen *et al.*, 2015).

5.4.3.3. *ErmB* and *ermF* gene screening

In the present study, the *ermB* gene detection was 60% in NW-E downstream river and NW-C upstream river. These results were comparable to the 60.71% *ermB* gene detected in the Pearl River and Pearl River Estuary, South China (Chen *et al.*, 2015). The *ermB* gene detection in other samples from NW-E and NW-C was equal to or greater than 70%. However, in the North-West of France, low *ermB* gene detection levels of 15.8% in wastewater effluent and 6.7% in the downstream river were reported (Leclercq *et al.*, 2013).

The average *ermF* gene detection level of NW-E and NW-C were comparable. The *ermF* gene detection level was more prevalent in wastewater effluents in NW-E and NW-C. In contrast with the present study, plant B wastewater effluent in Louisiana, United States, did not harbour the *ermF* gene (Thakali *et al.*, 2020). However, the present study's minimum average of 60% *ermF* gene detected in NW-E downstream river and NW-C upstream river is comparable to that reported in plant A wastewater effluent (Thakali *et al.*, 2020).

The *ermB* and *ermF* genes confer resistance to macrolides-lincosamides-streptogramin B antibiotics (Leclercq *et al.*, 2013). Detecting *ermB* and *ermF* genes in wastewater effluent and receiving water bodies is a concern: the use of macrolides (125 899 kg) in South Africa is common in animal therapy (NDoH, 2018). Heightened resistance levels may occur, since animal bodies excrete 60% lincosamides and 50-90% Macrolides (Quaik *et al.*, 2020).

It is estimated that, in a confined feeding operation, tylosin and erythromycin thiocyanate that are concomitant with macrolides excretion in manure cattle (weighing 1000 pounds on average) was 3 973 and 730 µg/kg, respectively. The excretion is even higher in swine manure (Huang *et al.*, 2021). Furthermore, macrolides are impersistent to slightly persistent in manure, with a half-life of <2 to 21 days (Boxall *et al.*, 2004; Massé *et al.*, 2014). The *ermB* and *ermF*

genes may be spread into the food chain with a view to the use of water in NW-E and NW-C for irrigation and livestock irrigation.

5.4.3.4. *Sul1* and *sul2* screening

A *sul1* gene detection of 100% was recorded in NW-C downstream maturation pond and NW-E downstream river. The same was reported in Pearl River, China and the wastewater effluent, upstream and downstream rivers in Poland (Chen *et al.*, 2015; Rolbiecki *et al.*, 2020). However, at NW-C and NW-E, the *sul1* gene was high in wastewater effluents when compared to the upstream rivers. The detection range in wastewater effluents and upstream rivers was between 60% and 91.67%. These findings were however not comparable to those recorded in Chris Hani and Amathole District Municipalities in Eastern Cape Province, South Africa, where the *sul1* gene detection isolated in *Listeria monocytogenes* and *E. coli* O157:H7 in irrigation water was 13% (Iwu and Okoh, 2019; Iwu *et al.*, 2021).

According to Rolbiecki *et al.* (2020), the *sul2* gene is less frequently present than the *sul1* gene. Results obtained in the present study confirm this. The *sul2* gene detection was higher in the upstream river, followed by wastewater effluent and the downstream maturation pond. A detection of the *sul2* gene in Eastern Cape irrigation is comparable to results recorded in the upstream river (Iwu and Okoh, 2019). At NW-E, the *sul2* gene increased from 91.67% in the wastewater effluent to 100% in the downstream river. The detected levels of the *sul2* gene in NW-E were the same as those of the Pearl River and Pearl River Estuary, South China (Chen *et al.*, 2015). The *sul2* gene was also detected in Poland (Rolbiecki *et al.*, 2020). The detection of the *sul2* gene indicates an agricultural impact on the water environment, which explains why it was more prevalent in areas with high agricultural activities, particularly livestock farming (Rolbiecki *et al.*, 2020).

The high detection of sulfonamide resistance genes such as *sul1* and *sul2* may therefore be attributed to livestock farming in and around NW-E and NW-C water sources. It is estimated that sulfonamide (sulfamethazine) excretion in the cattle manure (weighing 1000 pounds on average) and swine (weighing 150 pounds) in a confined feeding operation was 3 973 and 89 000 µg/kg, respectively (Huang *et al.* 2021). Sulphonamides are slightly to moderately persistent in manure, with a half-life of <8 to 30 days (Boxall *et al.*, 2004; Massé *et al.*, 2014).

5.4.3.5. Plasmid-mediated AmpC β -lactamase gene screening

5.4.3.5.1 FOX gene

The FOX gene detection at NW-E was higher in the wastewater effluent. At NW-C, it was higher in the upstream river. This is of concern, since this clinically important gene was prevalent despite the influence of the WWTPs. The FOX gene was the most prevalent pAmpC, with a 66.67% and 90% detection range. The minimum detected FOX gene isolated in NW-E upstream river reinforces the findings of Coertze and Bezuidenhout (2020). They reported a detection of 66.67% in the Crocodile West and Marico rivers, South Africa. However, this detection was not comparable with the low FOX gene detection obtained in *K. pneumoniae* and *E. coli* 0157:H7 isolated in the Eastern Cape irrigation water and agricultural soil (Iwu *et al.*, 2020; 2021). In Guangzhou, China, wastewater influent and effluent presented lower detection of the FOX gene than the wastewater effluent collected in NW-C and NW-E.

5.4.3.5.2 MOX gene

The detection of MOX was higher in the upstream rivers in NW-E and NW-C. In NW-E it was 45.45% and in NW-C 50.00% in upstream rivers. Like the FOX gene, the higher detection of the MOX gene in the upstream river is a concern, since the prevalence of this clinically important gene was high despite the influence of the WWTPs. A MOX gene detection of 18.18% was recorded in the NW-E and NW-C wastewater effluents. Coertze and Bezuidenhout (2020) detected higher detection levels when compared to those recorded in the NW-E and NW-C wastewater effluents. The present study's MOX gene detection in wastewater effluent was also lower than the 80.95% recorded in Guangzhou WWTP, China (Lin *et al.*, 2021). A low detection of 4% occurred in irrigation water collected in Eastern Cape, South Africa (Iwu *et al.*, 2021). These results were comparable to the detected level in the downstream maturation in NW-C.

5.4.3.5.3 DHA gene

The DHA gene was detected to a greater extent in the wastewater effluent in NW-E and NW-C. In NW-C, it had increased from the upstream river to the downstream maturation pond whereas, in NW-E, it decreased from the upstream river to the downstream river. The detections in NW-E and NW-C rivers and the maturation pond were comparable to those reported by Coertze and Bezuidenhout (2020) in the Crocodile West and Marico rivers, South

Africa. The DHA gene was also detected in *Klebsiella* species and *E. coli* collected in the wastewater effluent in the Eastern Cape, South Africa (Fadare *et al.*, 2021).

5.4.3.5.4 EBC gene

In NW-E and NW-C, the presence of the EBC gene is attributed to the influence of WWTPs. The absence of the EBC gene in the upstream rivers of NW-E and NW-C proves this. Coertze and Bezuidenhout (2020) reported a higher EBC gene detection in the Crocodile West River than those detected in the NW-E and NW-C wastewater effluent. The detection of the EBC gene in the wastewater effluent was comparable to 76.19% EBC gene detection in the wastewater influent and wastewater effluent in Guangzhou WWTP, China (Lin *et al.*, 2021). It was also reported to be present in agricultural soil collected in the Eastern Cape, South Africa (Iwu *et al.*, 2021).

5.4.3.5.5 ACC gene

The upstream river harboured more ACC genes in NW-C than the wastewater effluent and the downstream maturation pond. Coertze and Bezuidenhout (2020) reported the same ACC gene detection levels in the Crocodile West River in North West, South Africa, as those found in the NW-C upstream river. Furthermore, the ACC gene increased from the wastewater effluent to the downstream maturation pond in NW-C. However, the wastewater effluent harboured more ACC genes in NW-E than in upstream and downstream rivers. It was also isolated in *Citrobacter* species, *E. coli* and *Enterobacter* species collected in the Eastern Cape wastewater effluent (Fadare and Okoh, 2021).

5.4.3.5.6 CIT gene

In NW-E and NW-C, the presence of the clinically important CIT gene in the environment was influenced by the WWTPs. This was demonstrated by the absence of the gene in the upstream rivers of NW-E and NW-C. However, there was an increase in the detection level from the wastewater effluent to the downstream maturation pond in NW-C. Coertze and Bezuidenhout (2020) reported higher CIT gene detection in the Crocodile West River than in NW-C and NW-E.

The clinically relevant pAmpCs are encoded on the plasmids; hence, they could be subjected to HGT between species in various niches (Coertze and Bezuidenhout, 2019; Fadare and

Okoh, 2021). This could explain the presence of pAmpCs, associated with resistance to β -lactams, including 1st-4th generation cephalosporins, in NW-E and NW-C water samples (Coertze and Bezuidenhout 2019). The present study's findings show that environmental settings are as important as clinical ones when it comes to finding a solution for antibiotic resistance. Monitoring and regulating pAmpCs and other ARGs in water could reduce the incidence of antibiotic resistance in drinking water, irrigation and livestock irrigation water to protect humans.

5.4.3.6. Quantification of antibiotic resistance genes in wastewater effluents and rivers

The pAmpCs were detected in wastewater and rivers. However, there is a lack of information on the actual concentrations of pAmpCs in these water bodies. A study by Coertze and Bezuidenhout (2020) measured the concentration of pAmpC in the Crocodile West and Marico rivers. However, the searched databases did not explore the WWTPs' influential role in the concentration of pAmpCs in rivers.

In NW-E, the wastewater effluent influenced the abundance concentration of the EBC and CIT genes. Similar observations were made in NW-C, where the concentration of these genes was higher in the wastewater effluent, followed by the downstream maturation pond and upstream river. Furthermore, in NW-E, it was observed that wastewater effluent did not influence the abundance concentrations of ACC, DHA, FOX and MOX genes in the downstream river. The FOX gene concentration in NW-E was more prevalent in the upstream river. The ACC and FOX gene concentrations in NW-C were most prevalent in the upstream river and lowest in wastewater effluent. In addition, The CIT and MOX increased from wastewater effluent to the downstream maturation pond in NW-C.

In NW-C, the *int11* concentration increased from wastewater effluent to the downstream maturation pond. However, in NW-E, the highest *int11* gene concentration of 6.61×10^{-4} copies/16S rRNA was measured in the upstream river. This is not in accordance with the results obtained in Wascana Creek, Canada, where the WWTP influenced the abundance of *int11* gene concentration in the downstream river (Freeman *et al.*, 2018). Furthermore, these authors found that *int11* concentrations in the wastewater effluent, upstream and downstream rivers were generally higher than those of the present study.

Although the *sul1* gene concentration was highest in the NW-E and NW-C wastewater effluents, they did not influence the abundance concentrations of *sul1* gene in the downstream sites. The NW-E and NW-C upstream rivers contained higher *sul1* gene concentrations than the downstream river and maturation pond. This does not follow the results obtained in Wascana Creek, Canada, where the WWTP influenced the abundance of *sul1* gene concentration in the downstream river (Freeman *et al.*, 2018). Furthermore, the measured *sul1* gene concentrations in the present study were lower than those recorded in WWTPs in New York City, United States and Wascana Creek, Canada (Joseph *et al.*, 2019; Freeman *et al.*, 2018). However, the present study's *sul1* gene concentrations were comparable to those measured in Swiss lakes (Czekalski *et al.*, 2015).

In the present study, the increase in ARG levels from the upstream to the downstream rivers may be attributed to the influence of WWTP contamination in receiving water bodies (Proia *et al.*, 2016). This is due to the inability of the WWTPs to remove ARGs entirely during treatment. Furthermore, the increase in the ARGs from the wastewater effluent to the downstream river in NW-E may be attributed to agricultural activities (including the use of fertilizers in the lawn-producing manufacturer) and livestock farming. In NW-C, the increase from the wastewater effluent to the downstream maturation pond might have been attributable to agricultural activities and wastewater leakage. Since the downstream maturation pond passed through the residential area of NW-C, residents physically threw litter directly in it, which might have increased the levels of ARGs in the downstream maturation pond. The presence of ARGs in the upstream rivers despite the influence of WWTPs may be attributed to urbanization, livestock farming, agricultural activities and soil leaching (Proia *et al.*, 2016). Thus, applying water contaminated with ARGs in agricultural activities may facilitate the spread of antibiotic resistance determinants into the food chain. Furthermore, the use of this water for livestock farming might have facilitated a spread of ARGs in animals.

The nutrients and antibiotic residues discharged by the WWTPs into receiving water bodies promote bacterial growth and biomass formation. They can subsequently trigger survival responses that stimulate the HGT of ARGs (Proia *et al.*, 2016). This highlights the ability of the WWTPs to influence the abundance of ARGs in the downstream river. Furthermore, Chen *et al.* (2023) reported that ARGs are higher in drinking water than in source water, which might be linked to disinfection and distribution processes. Although authors have reported the presence of ARGs in different water bodies, the risk assessment procedures that estimate concentrations of ARGs that pose human and ecological health threats are not yet developed (Proia *et al.*, 2016). Furthermore, ARGs are not routinely monitored in South African wastewater effluent and surface water.

5.4.4. Quantification of antibiotic and fluconazole residues in wastewater effluents and rivers

The antimicrobial data showed that 23–36% of antimicrobials in South Africa between 2014 and 2015 were procured animal use (NDoH, 2018). In 2015, 1 592 842 kg of antibiotics were used for animals, of which 980 782 kg was used for non-therapeutic purposes such as growth promotion (NDoH, 2018). The extensive and improper usage of antibiotics and fluconazole in human medicine, veterinary medicine and agriculture may result in the spread of residues in wastewater and, subsequently, surface water. The present study found antibiotic residues in wastewater effluents and rivers in NW-E and NW-C.

5.4.4.1. Sulfamethoxazole residues

As anticipated, a general trend sulfamethoxazole concentrations were higher in wastewater effluent than upstream rivers and downstream points. Similar results were obtained in Spain (Lopez *et al.*, 2022). In contrast, in the Silesian WWTP (Poland) and Swartkops WWTP (South Africa), the sulfamethoxazole concentrations in the upstream river were higher than those of the wastewater effluent (Giebułtowiec *et al.*, 2020; Vumazonke *et al.*, 2020). The average sulfamethoxazole concentrations obtained in NW-E and NW-C were comparable to Suzuki *et al.* (2015), who found 2.56 µg/L in urban surface water and 3.61 µg/L in wastewater effluent collected in eThekweni Metropolitan Municipality, KwaZulu-Natal, South Africa. Furthermore, comparable sulfamethoxazole concentrations of 2.50 µg/L were recorded in another study conducted in eThekweni and Swartkops WWTP, South Africa (Vumazonke *et al.*, 2020; Späth *et al.*, 2021).

Sulfamethoxazole is frequently used due to its application to inhibit dihydropteroate synthesis (Reygaert, 2018). However, the animal body cannot fully metabolize sulfamethoxazole and, as a result, 85% of sulfamethoxazole is excreted into the environment (Quaik *et al.*, 2020). The presence of sulfamethoxazole in NW-E and NW-C wastewater and rivers might have increased antibiotic resistance. The present project showed the presence of sulfonamide resistance genes such as *sul1* and *sul2* in NW-E and NW-C water environments.

5.4.4.2. Ciprofloxacin residues

The results obtained in the present study show that the wastewater effluent influences the abundance of ciprofloxacin in receiving water bodies in NW-E and NW-C. This influence was also reported in Madrid and Warmian-Masurian Voivodship WWTPs in Spain and Poland, respectively (Giebułtowiec *et al.*, 2020; Lopez *et al.*, 2022). However, in Silesian WWTP situated in Poland, a decrease in ciprofloxacin concentration was found to occur from the upstream to the downstream river (Giebułtowiec *et al.*, 2020). The measured ciprofloxacin concentrations in NW-E and NW-C were not comparable to the results obtained in another study conducted in KwaZulu-Natal, South Africa (Agunbiade and Moodley, 2014). However, the measured ciprofloxacin concentrations in NW-E and NW-C were comparable to the 1.60 µg/L average recorded in Newlands Mashu wastewater effluent collected in eThekweni, South Africa (Späth *et al.*, 2021).

In 2015, 6 907 kg (0.43%) of fluoroquinolones, the class ciprofloxacin belongs to, was procured for animal use (NDoH, 2018). Ciprofloxacin is also essential for human health where it inhibits the synthesis of topoisomerase IV and DNA gyrase (Reygaert, 2018; Richards *et al.*, 2019); this could explain its presence in NW-E and NW-C wastewater effluent and rivers.

5.4.4.3. Trimethoprim residues

A general trend observed in NW-E and NW-C was that wastewater effluents were more contaminated and this influenced the concentration of trimethoprim in the downstream rivers. Although present in lower concentrations than NW-C and NW-E, the Warmian-Masurian Voivodship wastewater effluent in Poland and Madrid wastewater effluent in Spain displayed the same trend (Giebułtowiec *et al.*, 2020; Lopez *et al.*, 2022). However, in the Silesian WWTP in Poland, the upstream river harboured slightly more trimethoprim than the downstream one (Giebułtowiec *et al.*, 2020). The average trimethoprim concentrations obtained in NW-E and NW-C were comparable to 0.29 µg/L measured in Newlands Mashu wastewater effluent collected in eThekweni, South Africa (Späth *et al.*, 2021).

In 2015, the procurement of trimethoprim (including sulfonamides) increased by 398% from the previous year. Trimethoprim (including sulfonamides) accounted for 175 049 kg (10.99%) of the antibiotics procured for animal use in 2015 in South Africa (NDoH, 2018). However, the low trimethoprim concentrations in NW-E and NW-C could not be explained, since there is lack of antibiotic consumption data in the North West Province.

5.4.4.4. Ampicillin residues

The measured ampicillin concentration was slightly more in NW-E than NW-C. The measured ampicillin range in NW-E and NW-C was 3.93-37.77 µg/L and 3.35-20.94 µg/L, respectively. The concentrations in NW-C were comparable to the 2.52 and 14.48 µg/L of the Umgeni River water system, KwaZulu-Natal, South Africa (Agunbiade and Moodley, 2014). In contrast, in the northern and southern parts of the Benin Republic, low ampicillin concentrations were reported (Deguenon *et al.*, 2022), while high ampicillin concentrations of 48.95 and 90.31 µg/L were reported in two Isfahan wastewater effluents in Iraq (Samandari *et al.*, 2022).

Ampicillin is vital for human and animal health. It is frequently used due to its antimicrobial activity in two processes: (i) it inhibits the cell wall synthesis by binding to membrane-bound penicillin-binding protein and (ii) it inhibits the cell wall peptidoglycan synthesis (Peechakara *et al.*, 2022). However, it is a concern to report ampicillin resistance genes in wastewater effluent and downstream and upstream rivers.

5.4.4.5. Fluconazole residues

In most sampling months, the fluconazole concentrations were <LOQ. The average fluconazole concentrations measured in NW-E and NW-C were 3.32 and 2.06 µg/L, respectively. In a study conducted in Mooi River, North West, South Africa, fluconazole concentrations were <LOD. A fluconazole range of between 0.18 and 0.27 µg/L in Wonderfontein Spruit, Gauteng, South Africa was reported by the authors of this study (Monapathi *et al.*, 2021). Low fluconazole concentrations were also reported in Paraopeba river basin in Brazil (De Barros *et al.*, 2018). In medicine, fluconazole, belonging to azole class, is used to treat fungal pathogens by inhibiting their cytochrome P450-dependent enzymes (particularly C14-demethylase), which are involved in the biosynthesis of ergosterol (Sallach *et al.*, 2021; Govindarajan *et al.*, 2022).

5.4.4.6. Risk quotient of wastewater effluents and rivers

In addition to antibiotic and fluconazole resistance, the target compounds in the present study may pose ecological risks determined by calculating RQs. Studies have been conducted to determine the concentrations of antibiotic and fluconazole residues in WWTPs and surface water. However, not much focus has been given to risks associated with the emergence of

antibiotic residues in these sources. The present study determined the RQs of antibiotic residues, and the trends observed in NW-E and NW-C were as follows:

(i) The RQ for ampicillin, ciprofloxacin and sulfamethoxazole was deemed high. The high-risk ciprofloxacin recorded in the present study concurs with other studies conducted in African countries such as Kenya and Morocco in wastewater and surface water (Kairigo *et al.*, 2020; Chamkal *et al.*, 2021). In addition, it was recorded for Lima wastewater effluent out of four tested WWTPs in Peru (Nieto-Juárez *et al.*, 2021). The results found in the other three Peru WWTPs ranged from low to medium risk.

Contrary to the present study, Nieto-Juárez *et al.* (2021) reported low-risk sulfamethoxazole in the wastewater effluents of four WWTPs in Peru. However, the high-risk sulfamethoxazole reported in the Juliaca downstream river, Peru, dovetails with the result obtained in NW-E and NW-C. Kairigo *et al.* (2020) reported sulfamethoxazole RQ ranging from low to high in Kenyan wastewater and surface water. It is of great concern to report high-risk ampicillin in wastewater effluents and rivers in NW-E and NW-C, which is comparable to that recorded in Morocco hospital wastewater (Chamkal *et al.*, 2021).

(ii) Consistent with the present study, low risk trimethoprim was reported in Peru's Puno and Juliaca wastewater effluents (Nieto-Juárez *et al.*, 2021). Unlike the present study, the authors reported the high risk for trimethoprim in Juliaca downstream river. In Kenyan wastewater and surface water, the trimethoprim RQs were deemed to be of medium and high risk (Kairigo *et al.*, 2020). The calculated RQ in the NW-E wastewater and the upstream river was deemed a medium risk. In NW-C, RQ ranged from low risk in the upstream river and downstream maturation pond to medium risk in the wastewater effluent. Although low risk fluconazole in some Gauteng WWTPs in South Africa was reported as in the case of the present study, most of their studied WWTPs obtained high-risk fluconazole (Assress *et al.*, 2020).

Since the WWTPs are not designed to remove antibiotic and fluconazole residues during treatment, several types of these pharmaceutical compounds may enter receiving water bodies via the wastewater effluent discharge. The extensive and improper application of various chemicals as possible selection agents makes it hard to determine the environmental pressure exerted on bacteria in ambient environments. However, it is conceivable that subinhibitory concentration of antibiotic residues creates conducive conditions for ARG proliferation (Chen *et al.*, 2015). Antibiotic residues in wastewater effluents and rivers might increase antibiotic resistance, affecting human and animal health. Furthermore, the present study showed that RQ antibiotic and fluconazole residues ranged from low to high risk. This

indicates that antibiotic and fluconazole residues might also have posed ecological risks. Hence, monitoring and regulating antibiotic and fluconazole residues in WWTPs and surface water should form part of water quality assessment. This will help to retain the potency of antibiotics and fluconazole for therapeutic use and mitigate their effects on aquatic life.

5.4.5. Correlation of bacterial communities with ARGs, antibiotics and physicochemical parameters in wastewater effluents and rivers

The present study showed no significant correlation (p -value >0.05) between bacterial community, physicochemical parameters, ARGs and antibiotic residues in all canonical axes. However, pairwise correlation matrices showed significant correlations (p -value <0.05). Consistent with Jordaan and Bezuidenhout (2016), the RDA reveals that physicochemical parameters were a major factor in determining variation in bacterial communities. Nutrients such as phosphate, nitrate and nitrite were associated with unassigned and *Dickeya* genera. This suggests that these nutrients may influence bacterial community composition in wastewater effluent and rivers (Zhou *et al.*, 2017; Wang *et al.*, 2018b). Unlike in a study by Wang *et al.* (2018b), *Comamonas* belonging to phylum and *Pseudarcobacter* from Campilobacterota were associated with temperature.

The RDA revealed that antibiotic and fluconazole residues correlated with Proteobacteria, Campilobacterota and Bacteroidota. In contrast, Proteobacteria were not closely correlated with antibiotic residues in sediments (Zhang *et al.*, 2021). However, the pairwise correlation matrices show that ciprofloxacin was the only antibiotic residue significantly correlated with bacterial community, specifically *Comamonas*. The interaction between antibiotic residues and bacterial community can induce natural selection for antibiotic resistance. WWTPs receive influent from agricultural, pharmaceutical, hospital and clinical settings containing high concentrations of antibiotic residues. Thus, it is important to establish antibiotic resistance surveillance programs in WWTPs. Furthermore, WWTPs do not completely remove antibiotic residues, thus leading to their discharge in the receiving water bodies where contaminated water is recycled for various applications.

The RDA further revealed that *Dickeya*, *Prevotella* and other genera were associated with *ermB*, *ermF*, FOX, EBC, CIT, ACC, *ampC* and *int1*. *Bla_{TEM}* was correlated with *Pseudarcobacter*. The results obtained by Wang *et al.* (2018b) show that Proteobacteria and Bacteroidota were associated with *int1* and *Bla_{TEM}*. Inconsistent with the current study's findings, Milaković *et al.* (2019) revealed a correlation between *Arcobacter* and *ermB*. The

pairwise correlation matrices significantly correlated ACC, DHA, CIT, *ampC*, *bla*_{TEM}, and *int1* to some bacterial communities. Therefore, additional research is needed to isolate pAmpCs (ACC, DHA and CIT) in bacterial hosts. This will validate the present study's findings and contribute to the body of knowledge centred on pAmpCs, which are underexplored in environmental settings. The association of bacterial community with ARGs show that bacterial communities influence the abundance of ARGs (Zhou *et al.*, 2017).

CHAPTER 6

6. CONCLUSION AND RECOMMENDATIONS

6.1. CONCLUSION

The DWAF SAWQG (1996a; 1996b; 1996c) provides recommended water standards for domestic and agricultural use in terms of irrigation and livestock watering. The DWAF SAWQG (1996a) for domestic use ensures that treated water is safe and clean for human consumption, with no health effects. The DWAF SAWQG (1996b; 1996c) for agricultural use ensures that water can be used for irrigation and livestock watering without damaging crops and livestock health. The guidelines are based on physicochemical parameters and microbial water pollution indicators. In addition, Blue and Green Drop Certification programmes monitor the performance and rate WWTPs and DWPFs in South Africa (DWS, 2022a; 2022b).

The DWS (2022b) showed that most DWPFs were underperforming, 23% of these were deemed to be at a critical risk, 11% at high risk, 18% at medium risk and 48% at low risk. The DWS (2022a) showed that WWTPs in Limpopo, KwaZulu-Natal and North West Provinces were considered to be a critical risk. WWTPs from other provinces were considered to be of high risk. The reason for these high numbers might be that most WWTPs were not assessed for the Green Drop Certification, such as the NW-E and NW-C WWTPs. Furthermore, before 2022, an eight-year period occurred during which the country's Blue and Green Drop Certification was not reported. These facts gave impetus to the importance of the present study.

Since WWTPs and DWPFs were underperforming, the presence of microbes in treated water and wastewater effluent was anticipated. The DWS (2022a; 2022b) and most South African studies assessed the microbial water quality WWTPs and DWPFs based on culture-dependent techniques. As a result, viable but non-culturable bacteria were unaccounted for. Some of these are potential human, animal and plant pathogens. They may harbour ARGs. Hence, the present study used high-throughput 16S rRNA sequencing to assess the bacterial community composition in DWPFs, WWTPs and rivers.

The DWAF (1996a; 1996b; 1996c) and DWS (2022a; 2022b) do not monitor ARGs' presence in DWPFs and WWTPs. It is therefore no surprise to find ARGs in treated water, wastewater effluent and rivers. This is the case, moreover, since DWPFs and WWTPs are not designed for their removal. The ARGs are shared and incorporated into other bacteria by means of

HGT. Antibiotic residues in WWTPs and DWPFs increase antibiotic resistance and pose human, animal and ecological risks. Despite the high consumption of antibiotics in the South (NDoH, 2018), antibiotic and fluconazole residues were not monitored in aquatic environments. Hence, the present study aimed to measure the concentrations of antibiotic residues and ARGs in wastewater effluent, rivers and drinking water. The aim was achieved by satisfying the following objectives:

6.1.1. Providing an overview of sources and prevalence of antibiotic residues and antibiotic resistance genes in source waters in South Africa used for drinking and food production.

This study examined information in peer-reviewed literature on antibiotic residues and ARGs in South Africa, while focusing on environmental detection. It was found that most studies did not determine the concentration of ARGs. The present review also established that South African studies use a culture-dependent approach to assess ARGs. It is of concern that few studies aimed to determine the presence of antibiotic residues in the source water. Furthermore, in the searched databases, no studies aimed to determine antibiotic levels in drinking water. Sufficient studies are needed to determine the ecological risks associated with these antibiotic residues.

The review showed that WWTPs and DWPFs are ineffective for removing ARB, ARGs and antibiotic residues. The evidence points to WWTPs as one of the primary sources that may facilitate the spread of antibiotic resistance into surface water. Using contaminated source water for food production could facilitate the spread of antibiotic resistance in the food chain. Individuals who reside in rural-informal settlements with no access to treated drinking water are at greater risk of waterborne infections, since they rely heavily on the untreated surface and groundwater. The literature surveyed further demonstrated that: (i) concerted effort should be made to ensure that WWTPs do not discharge untreated wastewater into surface waters, as such episodes will pollute the water available for drinking and food production with antibiotic residues, ARB and ARGs; (ii) a gap exists in our knowledge of the extent of the prevalence of antibiotic residues, ARB and ARGs in surface, groundwater and drinking water. Such data is crucial for determining human, animal and environmental health risks.

6.1.2. Determining the distribution of bacterial community composition, antibiotic residues and antibiotic resistance genes in drinking water production facilities. Furthermore, using the data on levels of antibiotic residues to determine whether there could potentially be exposure risks.

The measured physicochemical parameters were found to be within the recommended standards of the DWAF SAWQG (1996a) for domestic use. This indicated that treated water was safe and clean for human consumption. However, phosphate and nitrate in treated water provide nutrients for bacterial development. According to the searched databases, as outlined in Chapter 2, there is a lack of the detection of ARGs in South African DWPFs. Furthermore, no studies addressed the quantification of ARGs and antibiotic residues in DWPFs in South Africa. The present study addressed these gaps by using culture-independent techniques for the quantification of ARGs.

Bacterial community composition in raw and treated water shows the presence of potential human and plant pathogens. The DWPFs could not change the relative abundance of some bacterial communities from raw to treated water. As a result, the alpha diversity showed that treated water was more diverse, even, and richer than raw water. Furthermore, in NW-C, bacterial dominance was higher in treated than in raw water. These results could be attributed to the biofilm formation in treated water. Moreover, bacterial communities can survive in treated water due to the readily available nutrients. Some ARGs and antibiotic residue were also significantly correlated with the bacterial community.

The present study found increased detection levels and concentrations of some ARGs from raw to treated water. This is unsurprising, again, since DWPFs are not designed to remove ARGs. In NW-E DWPF, the end-point PCR screening process showed increased *sul2* and DHA genes from raw to treated water. In NW-C DWPF, the increase was for the *sul1* gene. Furthermore, in NW-E DWPF, the real-time PCR analysis showed that the EBC, DHA, *int1* and *sul1* genes increased from raw to treated water in NW-C DWPF. Generally, these ARGs may lead to more extended hospitalization and expensive antibiotics to treat common infections.

The present study quantified ampicillin, ciprofloxacin, fluconazole, trimethoprim and sulfamethoxazole in raw and treated water. In DWPFs, these target compounds decreased from raw to treated water, except for fluconazole in NW-C. These target compounds are commonly used in human and veterinary medicine. The calculated RQs in DWPFs of

ampicillin, ciprofloxacin and sulfamethoxazole were deemed to be of high risk. However, in NW-C treated water, ciprofloxacin was deemed to be of medium risk. Low risk was reported for trimethoprim and fluconazole in NW-E and NW-C DWPF. Antibiotic and fluconazole residues may increase antimicrobial resistance and affect human health. Furthermore, these residues may pose an ecological risk to aquatic life.

6.1.3. Determining bacterial community composition, antibiotic residue and antibiotic resistance gene patterns in wastewater plant effluent and rivers – Implications for downstream use. Furthermore, using the data on levels of antibiotic residues to determine whether there could potentially be exposure risks..

As anticipated, in NW-E, the wastewater effluent influenced the abundance concentration of salinity, nitrate, nitrite and COD in the downstream river. However, the phosphate concentration increased from the wastewater effluent to the downstream. In NW-C, the TDS, salinity, COD and nitrite concentration increased from the wastewater effluent to the downstream maturation pond. However, According to the DWAF SAWQG (1996b; 1996c), the water quality of NW-E and NW-C is suitable for irrigation and livestock watering.

Unsurprisingly, the wastewater effluents and corresponding rivers were contaminated with faecal indicator bacteria to a larger extent. Some are potential and opportunistic pathogens to humans, animals and plants. Identifying, monitoring and distributing bacterial communities is critical for monitoring potential pathogens and their impact. In NW-E and NW-C, bacterial community was significantly correlated with physicochemical parameters, ARGs and antibiotic residue in wastewater effluents and rivers.

In the context of South Africa, the gap around quantifying ARGs and antibiotic residues extends into WWTPs and rivers. The present study used culture-independent techniques to quantify ARGs. End-point and real-time PCRs results indicated that wastewater effluent did not influence the abundance detection levels and concentrations of some ARGs in the downstream river in NW-E. In NW-C, some ARGs increased from the wastewater effluent to the downstream maturation pond. Compared to wastewater effluent, the increased ARG levels in the upstream and downstream sites are associated with urbanization, livestock farming, agricultural activities and soil leaching (Proia *et al.*, 2016).

The present study quantified critically and highly important antibiotic residues and fluconazole as classified by WHO (2018). In NW-E, the wastewater effluent influenced the abundance concentration of all target compounds. In NW-C, the concentrations of target compounds were prevalent in the wastewater effluent when compared to the upstream river and downstream maturation pond, except for ampicillin. The increased concentration in the downstream maturation is attributed to the reasons mentioned. The RQs of the target compounds ranged from low to high risk. These results show that antibiotic and fluconazole residues pose human and ecological risks.

The aim and objectives of the present study were achieved. In conclusion, extant literature states that ARGs could potentially threaten human and animal health, but no risk assessment procedures have yet been developed by which to regulate this. Furthermore, ARGs and antibiotic residues are not routinely monitored for water quality. As a result, unknown concentrations of ARGs and antibiotic residues are spread into the food chain by using contaminated water for drinking and food production. The effects of antibiotic resistance may be dire for South Africa, where DWPFs are underperforming and healthcare facilities overwhelmed. These facilities are essential to cushion antibiotic resistance's effects on immunocompromised patients. Stats South Africa (2022a) states that approximately 13.7% of South Africans are infected with HIV/ AIDS. Furthermore, the presence of antibiotics also poses ecological risks. Therefore, the present study's outcomes could form part of policymaking to improve antibiotic use, water quality and combat antibiotic resistance.

6.2. RECOMMENDATIONS

- Develop a South African quantitative risk assessment method to determine the risks of consuming ARGs, fluconazole and antibiotic residues contaminated water.
- Routinely monitor ARGs, antibiotic and fluconazole residues in WWTPs, DWPFs and receiving water bodies.
- Sampling points should include wastewater influent and drinking water distribution networks. This will provide vital information on whether WWTPs can reduce ARGs and antibiotic residues from wastewater influent to effluent. Furthermore, the information on distribution networks will give a pattern of ARGs and antibiotic residues from treated water to household taps.
- Skip end-point PCR and directly subject the isolated eDNA to real-time PCR. Currently, the eDNA that did not show the presence of ARGs during the end-point PCR process was not subjected to real-time PCR analysis. As a result, insufficient data were

produced in real-time PCR analysis. These limit the downstream statistical analyses, which are of utmost importance.

- Perform enzyme-linked immunosorbent assay (ELISA) to elucidate the presence of antibiotic residues in water environments without skilled staff and specialized instrumentation.
- Compare grab and composite sampling to determine which method yields better outcomes for studying ARGs and antibiotic residues in water environments. Grab sampling involves a small volume of water collected over a period that does not exceed 15 minutes from a single point within the investigation area (Clausen *et al.*, 2018). Composite sampling involves a collection of a large sample made up of subsamples (Scalize and Frazão, 2018).
- Generating sufficient physicochemical parameter data could be beneficial to assess the ability and improve the monitoring of the WWTPs and DWPFs. The outcomes of the present study can be made readily available to the public, since inconsistencies exist in the publication of the Blue and Green Drop certifications.
- As part of one approach to health, the outcomes of the present study could be used to form part of policymaking to improve antibiotic use, water quality and the fight against antibiotic resistance. Furthermore, the usage of these outcomes is vital for antibiotic resistance surveillance programmes and the development of potential risk assessment procedures for ARGs. Subsequently, introduce antibiotic residues and ARGs as part of routinely monitored water quality parameters, since they threaten human and animal health.
- Perform a pilot study comparing conventional and advanced treatment technologies for removing ARGs and antibiotic residues. The outcomes of such a study are vital in a water-scarce country such as South Africa. Investing in advanced treatment technologies may compensate for the high demand for water by recycling wastewater to produce safe and clean drinking water.

REFERENCES

- Abia, A.L. Ubomba-Jaswa, E. and Momba, M.N. 2015. High prevalence of multiple-antibiotic-resistant (MAR) *Escherichia coli* in river bed sediments of the Apies River, South Africa. *Environmental Monitoring and Assessment*, 187(652):1-13.
- Adefisoye, M. and Okoh, A. 2017. Ecological and public health implications of the discharge of multidrug-resistant bacteria and physicochemical contaminants from treated wastewater effluents in the Eastern Cape, South Africa. *Water*, 9(562):1-18.
- Adesoji, A.T. and Ogunjobi, A.A. 2016. Detection of extended spectrum beta-lactamases resistance genes among bacteria isolated from selected drinking water distribution channels in southwestern Nigeria. *BioMed research international*, 2016:1-9.
- Adewoyin, M.A. Ebomah, K.E. and Okoh, A.I. 2021. Antibigram Profile of *Acinetobacter baumannii* Recovered from Selected Freshwater Resources in the Eastern Cape Province, South Africa. *Pathogens*, 10(9):1110.
- Africa CDC: Centres for Disease Control and Prevention and Center for Disease Dynamics Economics & Policy. 2021. African antibiotic treatment guidelines for common bacterial infections and syndrome. Addis Ababa, Ethiopia. Africa Centres for Disease Control and Prevention Center for Disease Dynamics, Economics & Policy.
- Agunbiade, F.O. and Moodley, B. 2014. Pharmaceuticals as emerging organic contaminants in Umgeni River water system, KwaZulu-Natal, South Africa. *Environmental Monitoring and Assessment*, 186:7273-7291.
- Alabi, M.E. and Essack, S.Y. 2022. Antibiotic prescribing amongst South African general practitioners in private practice: an analysis of a health insurance database. *JAC-Antimicrobial Resistance*, 4(5):101.
- Almansoori, N. and Parag, N. 2021. Antibiotic prescribing patterns in emergency department at regional hospital in South Africa. *African Health Sciences*, 21(4):1651-61.
- Alonso, D.P., Amorim, J.A., de Oliveira, T.M.P., de Sá, I.L.R., Possebon, F.S., de Carvalho, D.P., Ribeiro, K.A.N., Ribolla, P.E.M. and Sallum, M.A.M. 2023. Host Feeding patterns of

Mansonia (Diptera, Culicidae) in rural settlements near Porto Velho, State of Rondonia, Brazil. *Biomolecules*, 13(3):553.

Amador, P.P., Fernandes, R.M., Prudêncio, M.C., Barreto, M.P. and Duarte, I.M., 2015. Antibiotic resistance in wastewater: Occurrence and fate of Enterobacteriaceae producers of Class A and Class C β -lactamases. *Journal of Environmental Science and Health, Part A*, 50(1), pp.26-39.

Amarasiri, M., Takezawa, T., Malla, B., Furukawa, T., Sherchand, J., Haramoto, E. and Sei, K. 2022. Prevalence of antibiotic resistance genes in drinking and environmental water sources of the Kathmandu Valley, Nepal. *Frontiers in microbiology*, 13:894014.

Antunes, P., Machado, J., Sousa, J.C. and Peixe, L. 2005. Dissemination of sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*) in Portuguese *Salmonella enterica* strains and relation with integrons. *Antimicrobial agents and chemotherapy*, 49(2):836-839.

Assress, H.A., Nyoni, H., Mamba, B.B. and Msagati, T.A. 2020. Occurrence and risk assessment of azole antifungal drugs in water and wastewater. *Ecotoxicology and environmental safety*, 187:109868.

Ateba, C.N. and Maribeng, M.D. 2011. Detection of *Enterococcus* species in groundwater from some rural communities in the Mmabatho area, South Africa: A risk analysis. *African Journal of Microbiology Research*, 5(23):3930–3935.

Ateba, C.N., Lekoma, K.P. and Kawadza, D.T. 2013. Detection of *vanA* and *vanB* genes in vancomycin-resistant enterococci (VRE) from groundwater using multiplex PCR analysis. *Journal of Water and Health*, 11(4):684–691.

Ateba, C.N., Tabi, N.M., Fri, J., Bissong, M.E.A. and Bezuidenhout, C.C. 2020. Occurrence of antibiotic-resistant bacteria and genes in two drinking water treatment and distribution systems in the North-West Province of South Africa. *Antibiotics*, 9(11):745.

Atnafu, B., Desta, A. and Assefa, F. 2021. Microbial community structure and diversity in drinking water supply, distribution systems as well as household point of use sites in Addis Ababa City, Ethiopia. *Microbial ecology*, 84(1):73-89.

Aujoulat, F., Mazuet, C., Criscuolo, A., Popoff, M.R., Enault, C., Diancourt, L., Jumas-Bilak, E., Lavigne, J.P. and Marchandin, H. 2021. *Peptoniphilus nemausensis* sp. nov. A new Gram-positive anaerobic coccus isolated from human clinical samples, an emendated description of the genus *Peptoniphilus* and an evaluation of the taxonomic status of *Peptoniphilus* species with not validly published names. *Systematic and Applied Microbiology*, 44(5):126235.

Barnard S., Venter A and van Ginkel CE. 2013. Overview of the influences of mining-related pollution on the water quality of the Mooi River system's reservoirs, using basic statistical analyses and self organised mapping. *Water South Africa*, 39(5):655-662.

Bautista-de Los Santos, Q.M., Chavarria, K.A. and Nelson, K.L. 2019. Understanding the impacts of intermittent supply on the drinking water microbiome. *Current Opinion in Biotechnology*, 57:167-174.

Bergeron, S., Raj, B., Nathaniel, R., Corbin, A. and LaFleur, G. 2017. Presence of antibiotic resistance genes in raw source water of a drinking water treatment plant in a rural community of USA. *International Biodeterioration & Biodegradation*, 124:3-9.

Boadi, N.O., Saah, S.A., Baa-Poku, F., Mensah, E.A. and Addo, M. 2020. Safety of borehole water as an alternative drinking water source. *Scientific African*, 10:00657.

Bolukaoto, J.Y., Singh, A., Alfinete, N. and Barnard, T.G. 2021. Occurrence of hybrid diarrhoeagenic *Escherichia coli* associated with multidrug resistance in environmental water, Johannesburg, South Africa. *Microorganisms*, 9(10):2163.

Boxall, A.B., Fogg, L.A., Blackwell, P.A., Blackwell, P., Kay, P., Pemberton, E.J. and Croxford, A. 2004. Veterinary medicines in the environment. *Reviews Of Environmental Contamination and Toxicology*:1-91.

Brauer, V.S., Rezende, C.P., Pessoni, A.M., De Paula, R.G., Rangappa, K.S., Nayaka, S.C., Gupta, V.K. and Almeida, F. 2019. Antifungal agents in agriculture: Friends and foes of public health. *Biomolecules*, 9(10):521.

Canfora, L., Benedetti, A. and Francaviglia, R. 2015. Land use, salinity and water quality. The case study of a coastal system in central Italy. *EQA-International Journal of Environmental Quality*, 18:29-42.

Carstens, A., Bartie, C., Dennis, R. and Bezuidenhout, C. 2014. Antibiotic-resistant heterotrophic plate count bacteria and amoeba-resistant bacteria in aquifers of the Mooi River, North West province, South Africa. *Journal of Water and Health*, 12(4):835–845.

Chamkal, N., Lhlou, I., Bandadi, L. and Ounine, K. 2021. Hospital antibiotics usage: environmental hazard and promotion of antibiotic resistant bacteria. *Annali di Igiene: Medicina Preventiva e di Comunita*, 34(3):266-278.

Chaturvedi, P., Singh, A., Chowdhary, P., Pandey, A. and Gupta, P. 2021. Occurrence of emerging sulfonamide resistance (*sul1* and *sul2*) associated with mobile integrons-integrase (*int1* and *int2*) in riverine systems. *Science of the Total Environment*, 751:142217.

Chen, B., Liang, X., Nie, X., Huang, X., Zou, S. and Li, X. 2015. The role of class I integrons in the dissemination of sulfonamide resistance genes in the Pearl River and Pearl River Estuary, South China. *Journal of Hazardous Materials*, 282:61-67.

Chen, H., Zheng, W., Shen, X., Zhang, F., Zhou, X., Shen, J. and Lu, M. 2021. Occurrence, distribution, and ecological risk assessment of antibiotics in different environmental media in Anqing, Anhui province, China. *International Journal Of Environmental Research and Public Health*, 18(15):8112.

Chen, L., Li, H., Liu, Y., Cui, Y., Li, Y. and Yang, Z. 2020. Distribution, residue level, sources, and phase partition of antibiotics in surface sediments from the inland river: a case study of the Xiangjiang River, south-central China. *Environmental Science and Pollution Research*, 27:2273-2286.

Chen, Z., Duan, Y., Yin, L., Chen, Y., Xue, Y., Wang, X., Mao, D. and Luo, Y. 2023. Unraveling the influence of human fecal pollution on antibiotic resistance gene levels in different receiving water bodies using crAssphage indicator gene. *Journal of Hazardous Materials*, 442:130005.

Chukwu, M.O., Abia, A.L.K., Ubomba-Jaswa E., OBI, L. and Dewar, J.B. 2019a. Characterization and phylogenetic analysis of *Campylobacter* species isolated from paediatric stool and water samples in the Northwest province, South Africa. *International Journal of Environmental Research and Public Health*, 16(2205):1-22.

Chukwu, M.O., Abia, A.L.K., Ubomba-Jaswa, E., Obi, L.C. and Dewar, J.B. 2019b. Antibiotic resistance profile and clonality of *E. coli* isolated from water and paediatric stool samples in the North-West, Province South Africa. *Journal of Pure and Applied Microbiology*, 13(1):517–530.

Chung, W.O., Werckenthin, C., Schwarz, S., Roberts, M.C. 1999. Host range of the *ermF* rRNA methylase gene in bacteria of human and animal origin. *Journal of Antimicrobial Chemotherapy*, 43:5-14.

Clausen, J.L., Georgian, T., Gardner, K. H. and Douglas, T.A. 2018. Inadequacy of conventional grab sampling for remediation decision-making for metal contamination at small-arms ranges. *Bulletin of Environmental Contamination and Toxicology*, 100(1):147-154.

Coertze, R.D. and Bezuidenhout, C.C. 2017. The prevalence and diversity of *AmpC* β -lactamase genes in plasmids from aquatic systems. *Water Science and Technology*, 2:1-9.

Coertze, R.D. and Bezuidenhout, C.C. 2019. Global distribution and current research of *AmpC* beta-lactamase genes in aquatic environments: a systematic review. *Environmental Pollution*, 252:1633-1642.

Coertze, R.D. and Bezuidenhout, C.C. 2020. Detection and quantification of clinically relevant plasmid-mediated *AmpC* beta-lactamase genes in aquatic systems. *Water Supply*, 20(5):1745-1756.

Coertze, R.D. and Bezuidenhout, C.C. 2021. Relating the prevalence of plasmid-mediated *AmpC* beta-lactamase genes to aquatic environmental factors. *Science of The Total Environment*, 763:144119.

Coetzee, I., Bezuidenhout, C.C. and Bezuidenhout, J.J. 2017. Triclosan resistant bacteria in sewage effluent and cross-resistance to antibiotics. *Water Science and Technology*, 76(6):1500-1509.

Coors, A., Vollmar, P., Sacher, F., Polleichtner, C., Hassold, E., Gildemeister, D. and Kühnen, U. 2018. Prospective environmental risk assessment of mixtures in wastewater treatment plant effluents—theoretical considerations and experimental verification. *Water Research*, 140:56-66.

Costa, D., Poeta, P., Sáenz, Y., Coelho, A.C., Matos, M., Vinué, L., Rodrigues, J. and Torres, C. 2007. Prevalence of antimicrobial resistance and resistance genes in faecal isolates recovered from healthy pets. *Veterinary Microbiology*, 127:97-105.

Czekalski, N., Sigdel, R., Birtel, J., Matthews, B. and Bürgmann, H. 2015. Does human activity impact the natural antibiotic resistance background? Abundance of antibiotic resistance genes in 21 Swiss lakes. *Environment International*, 81:45-55.

Darvishmotevalli, M., Moradnia, M., Noorisepehr, M., Fatehizadeh, A., Fadaei, S., Mohammadi, H., Salari, M., Jamali, H.A. and Daniali, S.S. 2019. Evaluation of carcinogenic risks related to nitrate exposure in drinking water in Iran. *MethodsX*, 6:1716-1727.

De Barros, A.L.C., Schmidt, F.F., de Aquino, S.F. and Afonso, R.J.D.C.F. 2018. Determination of nine pharmaceutical active compounds in surface waters from Paraopeba River Basin in Brazil by LTPE-HPLC-ESI-MS/MS. *Environmental Science and Pollution Research*, 25(20):19962-19974.

Deguenon, E., Dougnon, V., Houssou, V.M.C., Gbotche, E., Ahoyo, R.A., Fabiyi, K., Agbankpe, J., Mousse, W., Lougbegnon, C., Klotoe, J.R. and Tchobo, F., 2022. Hospital effluents as sources of antibiotics residues, resistant bacteria and heavy metals in Benin. *SN Applied Sciences*, 4(8), p.206.

Desye, B., Belete, B., Asfaw Gebrezgi, Z. and Terefe Reda, T. 2021. Efficiency of treatment plant and drinking water quality assessment from source to household, Gondar city, Northwest Ethiopia. *Journal of Environmental and Public Health*, 2021:9974064.

Deyab, M., El-Adl, M., Ward, F. and Omar, E., 2021. Trophic status, phytoplankton diversity, and water quality at Kafr El-Shinawy drinking-water treatment plant, Damietta. *Journal of Water Supply: Research and Technology-Aqua*, 70(3):342-360.

Dhariwal, A., Chong, J., Habib, S., King, I.L., Agellon, L.B. and Xia, J. 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Research*, 45(W1):W180-W188.

Dobhal, S., Boluk, G., Babler, B., Stulberg, M.J., Rascoe, J., Nakhla, M.K., Chapman, T.A., Crockford, A.B., Melzer, M.J., Alvarez, A.M. and Arif, M. 2020. Comparative genomics reveals signature regions used to develop a robust and sensitive multiplex TaqMan real-time qPCR

assay to detect the genus *Dickeya* and *Dickeya dianthicola*. *Journal of Applied Microbiology*, 128(6):1703-1719.

Du Plessis, E.M., Duvenage, F. and Korsten, L., 2015. Determining the potential link between irrigation water quality and the microbiological quality of onions by phenotypic and genotypic characterization of *Escherichia coli* isolates. *Journal of Food Protection*, 78(4):643–651.

Duong, H.A., Phung, T.V., Nguyen, T.N., Phan Thi, L.A. and Pham, H.V. 2021. Occurrence, distribution, and ecological risk assessment of antibiotics in selected urban lakes of Hanoi, Vietnam. *Journal of Analytical Methods In Chemistry*, 2021:6631797

DWA: Department of Water and Sanitation. 2019. Weekly State of the Reservoirs on 2019-02-04. Pretoria, South Africa. Government Printer.

DWAF: Department of Water Affairs and Forestry. 1996a. South African Water Quality Guidelines. Volume 1 Domestic Use 2nd edition. Pretoria, South Africa. Government Printer.

DWAF: Department of Water Affairs and Forestry. 1996b. South African Water Quality Guidelines. Volume 4: Agricultural Water Use: Irrigation 2nd edition. Pretoria, South Africa. Government Printer.

DWAF: Department of Water Affairs and Forestry. 1996c. South African Water Quality Guidelines. Volume 5: Agricultural Water Use: Livestock Watering 2nd edition. Pretoria, South Africa. Government Printer.

DWAF: Department of Water Affairs and Forestry. 2000. Quality of domestic water. Pretoria, South Africa: Government Printer.

DWS: Department of Water and Sanitation. 2018. National water and Sanitation Master Plan. Volume 2: Plan to Action. Final Draft. Version 3.3. 31 March 2018. Pretoria, South Africa. Government Printer.

DWS: Department of Water and Sanitation. 2022a. Green Drop 2022. Pretoria, South Africa: Government Printer.

DWS: Department of Water and Sanitation. 2022b. Blue Drop progress report. Pretoria, South Africa: Government Printer.

DWS: Department of Water and Sanitation. 2022c. Deputy Minister Mahlobo calls for enhanced and collaborative efforts to preserve water in South Africa. <https://www.dws.gov.za/communications/Articles/2022/IA%20-%20Deputy%20Minister%20Mahlobo%20calls%20for%20enhanced%20and%20collaborative%20efforts%20to%20preserve%20water%20in%20SA.pdf> (Date accessed: November 2022).

Echeverria-Palencia, C.M., Thulsiraj, V., Tran, N., Ericksen, C.A., Melendez, I., Sanchez, M.G., Walpert, D., Yuan, T., Ficara, E., Senthilkumar, N. and Sun, F.m. 2017. Disparate antibiotic resistance gene quantities revealed across 4 major cities in California: a survey in drinking water, air, and soil at 24 public parks. *ACS Omega*, 2(5):2255-2263.

Edokpayi, J.N., Enitan-Folami, A.M., Adeeyo, A.O., Durowoju, O.S., Jegede, A.O. and Odiyo, J.O., 2020. Recent trends and national policies for water provision and wastewater treatment in South Africa. In *Water conservation and wastewater treatment in BRICS nations* (pp. 187-211). Elsevier.

Edokpayi, J.N., Rogawski, E.T., Kahler, D.M., Hill, C.L., Reynolds, C., Nyathi, E., Smith, J.A., Odiyo, J.O., Samie, A., Bessong, P. and Dillingham, R. 2018. Challenges to sustainable safe drinking water: a case study of water quality and use across seasons in rural communities in Limpopo province, South Africa. *Water*, 10(2):159.

Ekundayo, T.C. and Okoh, A.I. 2020. Antimicrobial resistance in freshwater *Plesiomonas shigelloides* isolates: Implications for environmental pollution and risk assessment. *Environmental Pollution*, 257:1-12.

Ekwanzala, M.D., Dewar, J.B., Kamika, I. and Momba, M.N.B. 2018. Systematic review in South Africa reveals antibiotic resistance genes shared between clinical and environmental settings. *Infection and Drug Resistance*:1907-1920.

Ekwanzala, M.D., Dewar, J.B., Kamika, I. and Momba, M.N.B. 2019. Tracking the environmental dissemination of carbapenem-resistant *Klebsiella pneumoniae* using whole genome sequencing. *Science of the Total Environment*, 691:80-92.

Ekwanzala, M.D., Dewar, J.B., Kamika, I. and Momba, M.N.B., 2020. Comparative genomics of vancomycin-resistant *Enterococcus* spp. revealed common resistome determinants from hospital wastewater to aquatic environments. *Science of the Total Environment*, 719:1-11.

Elsaghir, H. and Reddivari, A.K.R. 2022. *Bacteroides fragilis*. <https://www.ncbi.nlm.nih.gov/books/NBK553032/> (Date accessed: 16 May 2023).

EPA: Environmental Protection Agency. 2017. Water quality standards handbook. Chapter 3: Water quality criteria United States, EPA.

EPA: Environmental Protection Agency. 2021. Code of practice: domestic waste water treatment systems (population equivalent ≤ 10). United States, EPA.

Fadare, F.T. and Okoh, A.I. 2021. The abundance of genes encoding ESBL, pAmpC and non- β -lactam resistance in multidrug-resistant *Enterobacteriaceae* recovered from wastewater effluents. *Frontiers in Environmental Science*, 9:295.

Fadare, F.T., Adefisoye, M.A. and Okoh, A.I. 2020. Occurrence, identification, and antibiogram signatures of selected *Enterobacteriaceae* from Tsomo and Tyhume rivers in the Eastern Cape Province, Republic of South Africa. *Plos One*, 15(12):0238084.

Farley, E., Stewart, A., Davies, M.A., Govind, M., Van den Bergh, D. and Boyles, T.H. 2018. Antibiotic use and resistance: Knowledge, attitudes and perceptions among primary care prescribers in South Africa. *South African Medical Journal*, 108(9):763-771.

Flörke, M., Bärlund, I., van Vliet, M.T., Bouwman, A.F. and Wada, Y. 2019. Analysing trade-offs between SDGs related to water quality using salinity as a marker. *Current Opinion in Environmental Sustainability*, 36:96-104.

Freeman, C.N., Scriver, L., Neudorf, K.D., Truelstrup Hansen, L., Jamieson, R.C. and Yost, C.K. 2018. Antimicrobial resistance gene surveillance in the receiving waters of an upgraded wastewater treatment plant. *Facets*, 3(1):128-138.

García-Ávila, F., Avilés-Anazco, A., Sánchez-Cordero, E., Valdiviezo-González, L. and Ordonez, M.D.T. 2021. The challenge of improving the efficiency of drinking water treatment systems in rural areas facing changes in the raw water quality. *South African Journal of Chemical Engineering*, 37:141-149.

Gasson, J., Blockman, M. and Willems, B. 2018. Antibiotic prescribing practice and adherence to guidelines in primary care in the Cape Town Metro District, South Africa. *South African Medical Journal*, 108(4):304-310.

Gekenidis, M.T., Walsh, F. and Drissner, D. 2021. Tracing antibiotic resistance genes along the irrigation water chain to chive: does tap or surface water make a difference? *Antibiotics*, 10(9):1100.

Giebułtowicz, J., Nałęcz-Jawecki, G., Harnisz, M., Kucharski, D., Korzeniewska, E. and Płaza, G. 2020. Environmental risk and risk of resistance selection due to antimicrobials' occurrence in two Polish wastewater treatment plants and receiving surface water. *Molecules*, 25(6):1470.

Golsha, R., Montazeri, M., Razaghi, N. and Zade, M.E., 2021. Frequency of Beta-Lactamase Antibiotic Resistance Genes in *Escherichia Coli* and *Klebsiella pneumoniae*. *Ethiopian Journal of Health Sciences*, 31(3):663-672.

Govender, R., Amoah, I.D., Adegoke, A.A., Singh, G., Kumari, S., Swalaha, F.M., Bux, F. and Stenström, T.A. 2021. Identification, antibiotic resistance, and virulence profiling of *Aeromonas* and *Pseudomonas* species from wastewater and surface water. *Environmental Monitoring and Assessment*, 193(5):1-16.

Govindarajan A, Bistas KG, Ingold CJ, Aboeed A. 2022. Fluconazole. <https://www.ncbi.nlm.nih.gov/books/NBK537158/#:~:text=Go%20to%3A-.Mechanism%20of%20Action,ergosterol%20to%20increase%20cellular%20permeability>. (Date accessed: November 2022).

Guan, Y., Jia, J., Wu, L., Xue, X., Zhang, G. and Wang, Z. 2018. Analysis of bacterial community characteristics, abundance of antibiotics and antibiotic resistance genes along a pollution gradient of Ba River in Xi'an, China. *Frontiers in Microbiology*, 9:3191.

Gumede, S.N., Abia, A.L., Amoako, D.G. and Essack, S.Y. 2021. Analysis of wastewater reveals the spread of diverse Extended-Spectrum β -Lactamase-Producing *E. coli* strains in uMgungundlovu District, South Africa. *Antibiotics*, 10(7):860.

Guo, J., Selby, K. and Boxall, A.B. 2016. Assessment of the risks of mixtures of major use veterinary antibiotics in European surface waters. *Environmental Science & Technology*, 50(15):8282-8289.

Gxalo, O., Digban, T.O., Igere, B.E., Olapade, O.A., Okoh, A.I. and Nwodo, U.U. 2021. Virulence and antibiotic resistance characteristics of *Vibrio* isolates from rustic environmental freshwaters. *Frontiers in Cellular and Infection Microbiology*, 11:1-12.

Hamiwe, T., Kock, M.M., Magwira, C.A., Antiabong, J.F. and Ehlers, M.M. 2019. Occurrence of enterococci harbouring clinically important antibiotic resistance genes in the aquatic environment in Gauteng, South Africa. *Environmental Pollution*, 245:1041-1049.

Han, X., Chen, X., Ma, J., Chen, J., Xie, B., Yin, W., Yang, Y., Jia, W., Xie, D. and Huang, F. 2022. Discrimination of Chemical Oxygen Demand Pollution in Surface Water Based on Visible Near-Infrared Spectroscopy. *Water*, 14(19):3003.

Han, Z., An, W., Yang, M. and Zhang, Y. 2020. Assessing the impact of source water on tap water bacterial communities in 46 drinking water supply systems in China. *Water Research*, 172:115469.

Haro-Moreno, J.M., Cabello-Yeves, P.J., Garcillán-Barcia, M.P., Zakharenko, A., Zemskaya, T.I. and Rodriguez-Valera, F. 2023. A novel and diverse group of *Candidatus Patescibacteria* from bathypelagic Lake Baikal revealed through long-read metagenomics. *Environmental Microbiome*, 18(1):1-12.

Havenga, B., Ndlovu, T., Clements, T., Reyneke, B., Waso, M. and Khan, W. 2019. Exploring the antimicrobial resistance profiles of WHO critical priority list bacterial strains. *BMC Microbiology*, 19(303):1-16.

Hem, S., Wyrsh, E.R., Drigo, B., Baker, D.J., Charles, I.G., Donner, E., Jarocki, V.M. and Djordjevic, S.P. 2022. Genomic analysis of carbapenem-resistant comamonas in water matrices: implications for public health and wastewater treatments. *Applied and Environmental Microbiology*, 88(13):e00646-22.

Hintz, W.D., Arnott, S.E., Symons, C.C., Greco, D.A., McClymont, A., Brentrup, J.A., Cañedo-Argüelles, M., Derry, A.M., Downing, A.L., Gray, D.K., Melles, S.J. *et al.* 2022. Current water

quality guidelines across North America and Europe do not protect lakes from salinization. *Proceedings of the National Academy of Sciences*, 119(9):e2115033119.

Holinger, E.P., Ross, K.A., Robertson, C.E., Stevens, M.J., Harris, J.K. and Pace, N.R. 2014. Molecular analysis of point-of-use municipal drinking water microbiology. *Water research*, 49:225-235.

Holton, E., Archer, E., Fidal, J., Kjeldsen, T., Wolfaardt, G. and Kasprzyk-Hordern, B. 2022. Spatiotemporal urban water profiling for the assessment of environmental and public exposure to antimicrobials (antibiotics, antifungals, and antivirals) in the Eerste River Catchment, South Africa. *Environment International*, 164:107227.

Hoseini, N., Sedighi, I., Mozaffari Nejad, A.S. and Alikhani, M.Y. 2017. Phenotypic and Genotypic Detection of AmpC Enzymes in Clinical Isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *Journal of Krishna Institute of Medical Sciences (JKIMSU)*:6(3):10-18.

Hu, Y., Jiang, L., Zhang, T., Jin, L., Han, Q., Zhang, D., Lin, K. and Cui, C. 2018. Occurrence and removal of sulfonamide antibiotics and antibiotic resistance genes in conventional and advanced drinking water treatment processes. *Journal of hazardous materials*, 360:364-372.

Hu, Y., Jin, L., Zhao, Y., Jiang, L., Yao, S., Zhou, W., Lin, K. and Cui, C., 2021. Annual trends and health risks of antibiotics and antibiotic resistance genes in a drinking water source in East China. *Science of the Total Environment*, 791:148152.

Hu, Y., Zhang, T., Jiang, L., Luo, Y., Yao, S., Zhang, D., Lin, K. and Cui, C. 2019. Occurrence and reduction of antibiotic resistance genes in conventional and advanced drinking water treatment processes. *Science of the Total Environment*, 669:777-784.

Huang, C.H., Renew, J.E., Smeby, K.L., Pinkston, K. and Sedlak, D.L. 2021. Assessment of potential antibiotic contaminants in water and preliminary occurrence analysis. *Journal of Contemporary Water Research and Education*, 120(1):4.

Hupfauft, S., Etemadi, M., Fernández-Delgado Juárez, M., Gómez-Brandón, M., Insam, H. and Podmirseg, S.M. 2020. CoMA—an intuitive and user-friendly pipeline for amplicon-sequencing data analysis. *PLoS One*, 15(12):e0243241.

Hussein, A.H., Al-Hejuje, M.M. and Mahdi, M.A. 2022. Determination of water quality and pollution by micro and nano plastics in water treatment plants. *Egyptian Journal of Aquatic Biology and Fisheries*, 26(4):47-58.

Idowu, G.A., David, T.L. and Idowu, A.M. 2022. Polycarbonate plastic monomer (bisphenol-A) as emerging contaminant in Nigeria: Levels in selected rivers, sediments, well waters and dumpsites. *Marine Pollution Bulletin*, 176:113444.

Igbinosa E.O., Obi, L.C., Tom, M. and Okoh, A.I. 2011. Detection of potential risk of wastewater effluents for transmission of antibiotic resistance from *Vibrio* species as a reservoir in a peri-urban community in South Africa. *Journal of Environmental and Public Health*, 21(6):402–414.

Igbinosa, I.H. and Okoh, A.I. 2012. Antibiotic susceptibility profile of *Aeromonas* species isolated from wastewater treatment plant. *The Scientific World Journal*, 2012:1-6.

Igbinosa, I.H., Chigor, V.N., Igbinosa, E.O., Obi, L.C. and Okoh, A.I. 2013. Antibigram, adhesive characteristics, and incidence of class 1 integron in *Aeromonas* species isolated from two South African rivers. *BioMed Research International*, 2013:1-8.

Igbinosa, I.H., Igbinosa, E.O. and Okoh, I.A. 2014. Molecular detection of metallo- β -lactamase and putative virulence genes in environmental isolates of *Pseudomonas* species. *Polish Journal of Environmental Studies*, 23(6):2327-2331.

Igwaran, A., Iweriebor, B.C. and Okoh, A.I. 2018. Molecular characterization and antimicrobial resistance pattern of *Escherichia coli* recovered from wastewater treatment plants in Eastern Cape South Africa. *International Journal of Environmental Research and Public Health*, 15:1-10.

Isiuku, B.O. and Enyoh, C.E. 2020. Pollution and health risks assessment of nitrate and phosphate concentrations in water bodies in South Eastern, Nigeria. *Environmental Advances*, 2:100018.

Iwu, C.D. and Okoh, A.I. 2020. Characterization of antibiogram fingerprints in *Listeria monocytogenes* recovered from irrigation water and agricultural soil samples. *PLoS ONE*, 15(2):1-22.

Iwu, C.D., du Plessis, E., Korsten, L. and Okoh, A.I. 2021. Antibigram imprints of *E. coli* O157: H7 recovered from irrigation water and agricultural soil samples collected from two district municipalities in South Africa. *International Journal of Environmental Studies*, 78(6):940-953.

Iwu, C.D., du Plessis, E.M., Korsten, L., Nontongana, N. and Okoh, A.I. 2020. Antibigram signatures of some enterobacteria recovered from irrigation water and agricultural soil in two district municipalities of South Africa. *Microorganisms*, 8(8):1206.

Iwu, C.D., Kayode, A.J., Igere, B.E. and Okoh, A.I., 2022. High levels of multi drug resistant *Escherichia coli* pathovars in preharvest environmental samples: A Ticking time bomb for fresh produce related disease outbreak. *Frontiers in Environmental Science*, 10:1-17.

Jiang, X., Liu, W. and Zheng, B. 2018. Complete genome sequencing of *Comamonas kerstersii* 8943, a causative agent for peritonitis. *Scientific Data*, 5(1):1-8.

Jin, D., Kong, X., Cui, B., Jin, S., Xie, Y., Wang, X. and Deng, Y. 2018. Bacterial communities and potential waterborne pathogens within the typical urban surface waters. *Scientific Reports*, 8(1):1-9.

Jing, Z., Lu, Z., Mao, T., Cao, W., Wang, W., Ke, Y., Zhao, Z., Wang, X. and Sun, W. 2021. Microbial composition and diversity of drinking water: A full scale spatial-temporal investigation of a city in northern China. *Science of The Total Environment*, 776:145986.

Jordaan, K. and Bezuidenhout, C.C. 2016. Bacterial community composition of an urban river in the North West Province, South Africa, in relation to physico-chemical water quality. *Environmental Science and Pollution Research*, 23:5868-5880.

Joseph, S.M., Battaglia, T., Maritz, J.M., Carlton, J.M. and Blaser, M.J. 2019. Longitudinal comparison of bacterial diversity and antibiotic resistance genes in New York City sewage. *MSystems*, 4(4):e00327-19.

Kairigo, P., Ngumba, E., Sundberg, L.R., Gachanja, A. and Tuhkanen, T. 2020. Occurrence of antibiotics and risk of antibiotic resistance evolution in selected Kenyan wastewaters, surface waters and sediments. *Science of the Total Environment*, 720:137580.

Kalule, J.B., Smith, A.M., Vulindhlu, M/, Tau, M.P., Nicol, M.P., Keddy, K.H. and Robberts, L. 2019. Prevalence and antibiotic susceptibility patterns of enteric bacterial pathogens in human and non-human sources in an urban informal settlement in Cape Town, South Africa. *BMC Microbiology*, 19(244):1-11.

Kanama, K.M., Daso, A.P., Mpenyana-Monyatsi, L. and Coetzee, M.A.A. 2018. Assessment of pharmaceuticals, personal care products, and hormones in wastewater treatment plants receiving inflows from health facilities in North West Province, South Africa. *International Journal of Toxicology*, 2018:1-15.

Kaushal, S.S., Likens, G.E., Pace, M.L., Reimer, J.E., Maas, C.M., Galella, J.G., Utz, R.M., Duan, S., Kryger, J.R., Yaculak, A.M., Boger, W.L. *et al.* 2021. Freshwater salinization syndrome: From emerging global problem to managing risks. *Biogeochemistry*, 154(2):255-292.

Kayode, A.J., Semerjian, L., Osaili, T., Olapade, O. and Okoh, A.I. 2021. Occurrence of multidrug-resistant *Listeria monocytogenes* in environmental waters: a menace of environmental and public health concern. *Frontiers in Environmental Science*, 9:737435.

Khabo-Mmekoa, C.M.N. and Momba, M.N.B. 2019. The Impact of social disparities on microbiological quality of drinking water supply in Ugu district municipality of KwaZulu-Natal Province, South Africa. *International Journal of Environmental Research and Public Health*, 16(16):2972.

King, T.L.B., Schmidt, S. and Essack, S.Y., 2020. Antibiotic resistant *Klebsiella* from a hospital, hospital effluents and wastewater treatment plants in the uMgungundlovu District, KwaZulu-Natal, South Africa. *Science of the Total Environment*, 71:1-7.

Kinge, N.W., Ateba, C.N. and Kawadza, D.T., 2010. Antibiotic resistance profiles of *Escherichia coli* isolated from different water sources in the Mmabatho locality, North West Province, South Africa. *South African Journal of Science*, 106(1/2):44-49.

Koekemoer, L., Janse van Vuuren, S. and Levanets, A. 2021. The influence of land use–impacted tributaries on water quality and phytoplankton in the Mooi River, North-West Province, South Africa. *Bothalia-African Biodiversity & Conservation*, 51(1):1-22.

Kritzing, R.K., 2019. Antibiotic resistant bacteria and -genes in raw water, and the implications for drinking water production. MSc dissertation. Potchefstroom: NWU.

Labbate, M., Chowdhury, P.R. and Stokes, H.W. 2008. A Class 1 integron present in a human commensal has a hybrid transposition module compared to tn402: evidence of interaction with mobile DNA from natural environments. *Journal of Bacteriology*, 190(15):5318–5327

Lamprecht, C., Romanis, M., Huisamen, N., Carinus, A., Schoeman, N., Sigge, G.O. and Britz, T.J. 2014. *Escherichia coli* with virulence factors and multidrug resistance in the Plankenburg River. *South African Journal of Science*, 110(9-10):1-6.

Laurila, J., Ahola, V., Lehtinen, A., Joutsjoki, T., Hannukkala, A., Rahkonen, A. and Pirhonen, M. 2008. Characterization of *Dickeya* strains isolated from potato and river water samples in Finland. *European Journal of Plant Pathology*, 122,:213-225.

Leclercq, R., Oberlé, K., Galopin, S., Cattoir, V., Budzinski, H. and Petit, F. 2013. Changes in enterococcal populations and related antibiotic resistance along a medical center-wastewater treatment plant-river continuum. *Applied and Environmental Microbiology*, 79(7):2428-2434.

Li, Q., Yu, S., Yang, S., Yang, W., Que, S., Li, W., Qin, Y., Yu, W., Jiang, H. and Zhao, D. 2021. Eukaryotic community diversity and pathogenic eukaryotes in a full-scale drinking water treatment plant determined by 18S rRNA and metagenomic sequencing. *Environmental Science and Pollution Research*, 28(14):17417-17430.

Liang, Y.B., Li, H.B., Chen, Z.S., Shi, D.Y., Chen, T.J., Yang, D., Yin, J., Zhou, S.Q., Cheng, C.Y., Shao, Y.F. and Li, J.W. 2022. Spatial behavior and source tracking of extracellular antibiotic resistance genes in a chlorinated drinking water distribution system. *Journal of hazardous materials*, 425:127942.

Liang, Y.Q., Annammala, K.V., Martin, P., Yong, E.L., Mazilamani, L.S. and Najib, M.Z.M. 2020. Assessment of physical-chemical water quality characteristics and heavy metals content of Lower Johor River, Malaysia. *Journal of Environmental Treatment Techniques*, 8(3):961-966.

Lin, X., Ruan, J., Huang, L., Zhao, J. and Xu, Y. 2021. Comparison of the elimination effectiveness of tetracycline and *AmpC* β -lactamase resistance genes in a municipal wastewater treatment plant using four parallel processes. *Ecotoxicology*, 30(8):1586-1597.

Liu, G., Zhang, Y., van der Mark, E., Magic-Knezev, A., Pinto, A., van den Bogert, B., Liu, W., van der Meer, W. and Medema, G. 2018. Assessing the origin of bacteria in tap water and distribution system in an unchlorinated drinking water system by SourceTracker using microbial community fingerprints. *Water Research*, 138:86-96.

Liu, N., Jin, X., Yan, Z., Luo, Y., Feng, C., Fu, Z., Tang, Z., Wu, F. and Giesy, J.P. 2020. Occurrence and multiple-level ecological risk assessment of pharmaceuticals and personal care products (PPCPs) in two shallow lakes of China. *Environmental Sciences Europe*, 32(1):1-10.

Lopez, F.J., Pitarch, E., Botero-Coy, A.M., Fabregat-Safont, D., Ibáñez, M., Marin, J.M., Peruga, A., Ontañón, N., Martínez-Morcillo, S., Olalla, A. and Valcárcel, Y. 2022. Removal efficiency for emerging contaminants in a WWTP from Madrid (Spain) after secondary and tertiary treatment and environmental impact on the Manzanares River. *Science of the Total Environment*, 812:152567.

Lv, Z., Xiao, X., Wang, Y., Zhang, Y. and Jiao, N. 2022. Improved water quality monitoring indicators may increase carbon storage in the oceans. *Environmental Research*, 206:112608.

Lyimo, B., Buza, J., Subbiah, M., Smith, W. and Call, D.R. 2016. Comparison of antibiotic resistant *Escherichia coli* obtained from drinking water sources in northern Tanzania: a cross-sectional study. *BMC microbiology*, 16(1):1-10.

Ma, L., Li, B., Jiang, X.T., Wang, Y.L., Xia, Y., Li, A.D. and Zhang, T. 2017. Catalogue of antibiotic resistome and host-tracking in drinking water deciphered by a large scale survey. *Microbiome*, 5(1):1-12.

Ma, Y., Li, M., Wu, M., Li, Z. and Liu, X. 2015. Occurrences and regional distributions of 20 antibiotics in water bodies during groundwater recharge. *Science of the Total Environment*, 518:498-506.

Machado, A. and Bordalo, A.A. 2014. Prevalence of antibiotic resistance in bacteria isolated from drinking well water available in Guinea-Bissau (West Africa). *Ecotoxicology and environmental safety*, 106:188-194.

Magome, T.G. 2021. Characterization of microbial assemblages on micro-plastics in a river system. Msc dissertation. Potchefstroom: NWU.

Maguvu, T.E., Bezuidenhout, C.C., Kritzinger, R., Tsholo, K., Plaatjie, M., Molale-Tom, L.G., Mienie, C.M. and Coertze, R.D. 2020. Combining physicochemical properties and microbiome data to evaluate the water quality of South African drinking water production plants. *PLoS One*, 15(8): e0237335.

Mahmood, A.R., Al-Haidari, H.H. and Hassan, F.M., 2019. Detection of antibiotics in drinking water treatment plants in Baghdad City, Iraq. *Advances in Public Health*, 2019:7851354.

Maje, M.D., Tchatchouang, C.D., Manganyi, M.C., Fri, J. and Ateba, C.N. 2020. Characterisation of *Vibrio* species from surface and drinking water sources and assessment of biocontrol potentials of their bacteriophages. *International Journal of Microbiology*, 2020:1-15.

Makuwa, S., Tlou, M., Fosso-Kankeu, E. and Green, E. 2020. Evaluation of fecal coliform prevalence and physicochemical indicators in the effluent from a wastewater treatment plant in the north-west province, South Africa. *International Journal of Environmental Research and Public Health*, 17(17):6381.

Malema, M., Abia, A., Tandlich, R., Zuma, B., Kahinda, J-M.M. and Ubomba-Jaswa, E. 2018. Antibiotic-resistant pathogenic *Escherichia coli* isolated from rooftop rainwater-harvesting tanks in the Eastern Cape, South Africa. *Journal of Environmental and Public Health*, 15(892):1-14.

Mann, B.C., Bezuidenhout, J.J. and Bezuidenhout, C.C. 2019. Biocide resistant and antibiotic cross-resistant potential pathogens from sewage and river water from a wastewater treatment facility in the North-West, Potchefstroom, South Africa. *Water Science and Technology*, 80(3):551-562.

Männle, D., McKinnie, S.M., Mantri, S.S., Steinke, K., Lu, Z., Moore, B.S., Ziemert, N. and Kaysser, L. 2020. Comparative genomics and metabolomics in the genus *Nocardia*. *Msystems*, 5(3):e00125-20.

Massé, D.I., Cata Saady, N.M. and Gilbert, Y. 2014. Potential of biological processes to eliminate antibiotics in livestock manure: an overview. *Animals*, 4(2):146-163.

Mateo-Sagasta, J., Zadeh, S.M., Turrall, H. and Burke, J., 2017. Water pollution from agriculture: a global review. Executive summary. the Food and Agriculture Organization of the United Nations, Rome.

Matloko, K., Fri, J., Ateba, T.P., Molale-Tom, L.G. and Ateba, C.N. 2021. Evidence of potentially unrelated *AmpC* beta-lactamase producing Enterobacteriaceae from cattle, cattle products and hospital environments commonly harboring the *bla* ACC resistance determinant. *Plos One*, 16(7):e0253647.

Matlou, D.P., Bissong M.E.A.T., Tchatchouang C-D.K., Adem, M.R., Foka, F.E.T., Kumar, A. and Ateba, C.N. 2019. Virulence profiles of vancomycin-resistant *Enterococci* isolated from surface and ground water utilized by humans in the North West Province, South Africa: A public health perspective. *Environmental Science and Pollution Research*, 26:15105–15114.

Matongo, S., Birungi, G., Moodley, B. and Ndungu, P., 2015a. Pharmaceutical residues in water and sediment of Msunduzi River, KwaZulu-Natal, South Africa. *Chemosphere*, 134:133-140.

Matongo, S., Birungi, G., Moodley, B. and Ndungu, P. 2015b. Occurrence of selected pharmaceuticals in water and sediment of Umgeni River, KwaZulu-Natal, South Africa. *Environmental Science and Pollution Research*, 2022(13):10298–10308.

Mbanga, J., Abia, A.L.K., Amoako, D.G. and Essack, S.Y. 2021c. Longitudinal surveillance of antibiotic resistance in *Escherichia coli* and *Enterococcus* spp. from a wastewater treatment plant and its associated waters in KwaZulu-Natal, South Africa. *Microbial Drug Resistance*, 27(7):904-918.

Mbanga, J., Amoako, D.G., Abia, A.L., Allam, M., Ismail, A. and Essack, S.Y. 2021b Genomic analysis of *Enterococcus* spp. isolated from a wastewater treatment plant and its associated waters in Umgungundlovu district, South Africa. *Frontiers in Microbiology*, 12:1-13.

Mbanga, J., Amoako, D.G., Abia, A.L., Fatoba, D. and Essack, S. 2021a. Genomic analysis of antibiotic-resistant *Enterobacter* spp. from wastewater sources in South Africa: The first report of the mobilisable colistin resistance mcr-10 gene in Africa. *Ecological Genetics and Genomics*, 21:100104.

Mettetal, E. 2019. Irrigation dams, water and infant mortality: Evidence from South Africa. *Journal of Development Economics*, 138:17-40.

Mhlongo, T.N., Ogola, H.J.O., Selvarajan, R., Sibanda, T., Kamika, I. and Tekere, M. 2020. Occurrence and diversity of waterborne fungi and associated mycotoxins in treated drinking water distribution system in South Africa: Implications on water quality and public health. *Environmental Monitoring and Assessment*, 192, pp.1-16.

Mi, R., Patidar, R., Farenhorst, A., Cai, Z., Sepehri, S., Khafipour, E. and Kumar, A. 2019. Detection of fecal bacteria and antibiotic resistance genes in drinking water collected from three First Nations communities in Manitoba, Canada. *FEMS Microbiology Letters*, 366(6):fnz067.

Milaković, M., Vestergaard, G., González-Plaza, J.J., Petrić, I., Šimatović, A., Senta, I., Kublik, S., Schlöter, M., Smalla, K. and Udiković-Kolić, N. 2019. Pollution from azithromycin-manufacturing promotes macrolide-resistance gene propagation and induces spatial and seasonal bacterial community shifts in receiving river sediments. *Environment international*, 123, pp.501-511.

M'nassri, S., El Amri, A., Nasri, N. and Majdoub, R. 2022. Estimation of irrigation water quality index in a semi-arid environment using data-driven approach. *Water Supply*, 22(5):5161-5175.

Molale, L.G. and Bezuidenhout, C.C. 2016. Antibiotic resistance, efflux pump genes and virulence determinants in *Enterococcus* spp. from surface water systems. *Environmental Science and Pollution Research*, 23:21501–21510.

Monapathi, M., Horn, S., Vogt, T., van Wyk, D., Mienie, C., Ezeokoli, O.T., Coertze, R., Rhode, O. and Bezuidenhout, C.C. 2021. Antifungal agents, yeast abundance and diversity in surface water: potential risks to water users. *Chemosphere*, 274:129718.

Mugadza, D.T., Nduku, S.I., Gweme, E., Manhokwe, S., Marume, P., Mugari, A., Magogo, C. and Jombo, T.Z. 2021. Drinking water quality and antibiotic resistance of *E. coli* and *Salmonella* spp. from different sources in Gweru urban, Zimbabwe. *Environmental Monitoring and Assessment*, 193:1-12.

Mulamattathil, S.G., Bezuidenhout, C. and Mbewe, M. 2014b. Biofilm formation in surface and drinking water distribution systems in Mafikeng, South Africa. *South African Journal of Science*, 110(11/12):1–9.

Mulamattathil, S.G., Bezuidenhout, C., Mbewe, M. and Ateba, C.N. 2014a. Isolation of environmental bacteria from surface and drinking water in Mafikeng, South Africa, and characterization using their antibiotic resistance profiles. *Journal of Pathogens*, 110(11/12):1-11.

Murray, R., Tien, Y.C., Scott, A. and Topp, E. 2019. The impact of municipal sewage sludge stabilization processes on the abundance, field persistence, and transmission of antibiotic resistant bacteria and antibiotic resistance genes to vegetables at harvest. *Science of the Total Environment*, 651:1680-1687.

Naidoo, N., Pearce, D., Visser, W., Crafford, J., Maila, D. and Harris, K. 2016. Implementation of effective wastewater charges by municipalities in South Africa: an investigation into the barriers and enablers. South Africa, WRC.

Nasser, A.M., Fawaqa, H. and Nitzan, Y. 2019. The role of wastewater treatment plants in the environmental dissemination of antibiotic resistant bacteria (ARB) and resistance genes (arg). *Journal of Water Resource and Protection*, 11(08):981.

NDoH: National Department of Health. 2018. Surveillance for antimicrobial resistance and consumption of antibiotics in South Africa. Pretoria, South Africa: Government Printer.

Ngigi, A.N., Magu, M.M. and Muendo, B.M. 2020. Occurrence of antibiotics residues in hospital wastewater, wastewater treatment plant, and in surface water in Nairobi County, Kenya. *Environmental monitoring and assessment*, 192(1):1-16.

Nieto-Juárez, J.I., Torres-Palma, R.A., Botero-Coy, A.M. and Hernández, F. 2021. Pharmaceuticals and environmental risk assessment in municipal wastewater treatment plants and rivers from Peru. *Environment International*, 155:106674.

NPC: National Planning Commission. 2012. National Development Plan. Pretoria, South Africa. Government Printer.

Nthunya, L.N., Maifadi, S., Mamba, B.B., Verliefde, A.R. and Mhlanga, S.D. 2018. Spectroscopic determination of water salinity in brackish surface water in Nandoni Dam, at Vhembe District, Limpopo Province, South Africa. *Water*, 10(8):990.

Nyamukamba, P., Moloto, M.J., Tavengwa, N. and Ejidike, I.P. 2019. Evaluating physicochemical parameters, heavy metals and antibiotics in the influents and final effluents of South African wastewater treatment plants. *Polish Journal of Environmental Studies*, 28(3):1305-1312.

O'Neill. 2022. South Africa: Distribution of gross domestic product (GDP) across economic sectors from 2010 to 2020. <https://www.statista.com/statistics/371233/south-africa-gdp-distribution-across-economic-sectors/#professional> (Date accessed: May 2022).

Odjadjare, E. and Olaniran, A. 2015. Prevalence of antimicrobial resistant and virulent *Salmonella* spp. in treated effluent and receiving aquatic milieu of wastewater treatment plants in Durban, South Africa. *International Journal of Environmental Research and Public Health*, 12:9692-9713.

Odjadjare, E.E., Igbinosa, E.O., Mordi, R., Igere, B., Igeleke, C.L. and Okoh, A.I. 2012. prevalence of multiple antibiotics resistant (MAR) *Pseudomonas* species in the final effluents of three municipal wastewater treatment facilities in South Africa. *International Journal of Environmental Research and Public Health*, 9,:2092-2107.

Ofori, S., Agyeman, P.C., Adotey, E.K., Růžicková, I. and Wanner, J. 2022. Assessing the influence of treated effluent on nutrient enrichment of surface waters using water quality indices and source apportionment. *Water Practice & Technology*, 17(7):1523-1534.

OIE: World Organization for Animal Health. 2021. OIE Annual Report on Antimicrobial Agents Intended for Use in Animals 5th edition. Paris, France.

Okoh, A., Sibanda, T., Nongogo, V., Adefisoye, M., Olayemi, O. and Nontongana, N. 2015. Prevalence and characterisation of non-cholerae *Vibrio* spp. in final effluents of wastewater treatment facilities in two districts of the Eastern Cape Province of South Africa: implications for public health. *Environmental Science and Pollution Research*, 22:2008-2017.

Okoh, A.I. and Igbinosa, E.O. 2010. Antibiotic susceptibility profiles of some *Vibrio* strains isolated from wastewater final effluents in a rural community of the Eastern Cape Province of South Africa. *BMC Microbiology*, 10(143):1-6.

Olaniran, A.O., Naicker, K. and Pillay, B. 2009. Antibiotic resistance profiles of *Escherichia coli* isolates from river sources in Durban, South Africa. *World Journal of Microbiology and Biotechnology*, 25:1743-1749.

Olaniran, A.O., Nzimande, S.B.T. and Mkize, N.G., 2015. Antimicrobial resistance and virulence signatures of *Listeria* and *Aeromonas* species recovered from treated wastewater effluent and receiving surface water in Durban, South Africa. *BMC Microbiology*, 15(234):1-10.

Opota, O., Ney, B., Zanetti, G., Jatou, K., Greub, G. and Prod'homme, G. 2014. Bacteremia caused by *Comamonas kerstersii* in a patient with diverticulosis. *Journal of Clinical Microbiology*, 52(3):1009-1012.

Orellana, L.H., Francis, T.B., Ferraro, M., Hehemann, J.H., Fuchs, B.M. and Amann, R.L. 2021. Verrucomicrobiota are specialist consumers of sulfated methyl pentoses during diatom blooms. *The ISME Journal*, 16(3):630-641.

Osunmakinde, C., Selvarajan, R., Mamba, B.B. and Msagati, T.A. 2019. Profiling bacterial diversity and potential pathogens in wastewater treatment plants using high-throughput sequencing analysis. *Microorganisms*, 7(11):506.

Papp-Wallace, K.M., Mallo, S., Bethel, C.R., Taracila, M.A., Hujer, A.M., Fernández, A., Gatta, J.A., Smith, K.M., Xu, Y., Page, M.G. and Desarbre, E. 2014. A kinetic analysis of the inhibition of FOX-4 β -lactamase, a plasmid-mediated AmpC cephalosporinase, by monocyclic β -lactams and carbapenems. *Journal of Antimicrobial Chemotherapy*, 69(3):682-690

Pascual-Benito, M., Ballesté, E., Monleón-Getino, T., Urmeneta, J., Blanch, A.R., García-Aljaro, C. and Lucena, F., 2020. Impact of treated sewage effluent on the bacterial community composition in an intermittent mediterranean stream. *Environmental Pollution*, 266, p.115254.

Pavone, S., Rinoldo, R., Albini, E., Fiorucci, A., Caponi, B., Fratto, A., Manuali, E., Papa, P. and Magistrati, C.F. 2021. First report of urinary tract infection caused by *Comamonas kerstersii* in a goat. *BMC Veterinary Research*, 17:1-5.

Pédrón, J. and Van Gijsegem, F. 2023. Diversity in the bacterial genus *Dickeya* grouping plant pathogens and waterways isolates. *OBM Genetics*, 3(4):1-22.

Peechakara BV, Basit H and Gupta M. 2022. Ampicillin. [https://www.ncbi.nlm.nih.gov/books/NBK519569/#:~:text=Go%20to%3A-.Mechanism%20of%20Action,%2Dbinding%20proteins%20\(PBPs\)](https://www.ncbi.nlm.nih.gov/books/NBK519569/#:~:text=Go%20to%3A-.Mechanism%20of%20Action,%2Dbinding%20proteins%20(PBPs)) (Date accessed: November 2022).

Pérez-Pérez, F.J. and Hanson, N.D., 2002. Detection of plasmid-mediated *AmpC* β -lactamase genes in clinical isolates by using multiplex PCR. *Journal of Clinical Microbiology*, 40(6):2153-2162.

Phokela, P.T., Ateba, C.N. and Kawadza, T.K. 2011. Assessing antibiotic resistance profiles in *Escherichia coli* and *Salmonella* species from groundwater in the Mafikeng area, South Africa. *African Journal of Microbiology Research*, 5(32):5902–5909.

Picazo, A., Rochera, C., Villaescusa, J.A., Miralles-Lorenzo, J., Velázquez, D., Quesada, A. and Camacho, A. 2019. Bacterioplankton community composition along environmental gradients in lakes from Byers peninsula (Maritime Antarctica) as determined by next-generation sequencing. *Frontiers in Microbiology*, 10:908.

Pillay, L. and Olaniran, A.O. 2016. Assessment of physicochemical parameters and prevalence of virulent and multiple-antibiotic-resistant *Escherichia coli* in treated effluent of two wastewater treatment plants and receiving aquatic milieu in Durban, South Africa. *Environmental Monitoring and Assessment*, 188(260):1-20.

Plaatjie, M.T.A., 2019. Characteristics of antibiotic resistant bacteria in raw and drinking water from water production facilities. MSc dissertation. Potchefstroom: NWU.

Potgieter, S., Pinto, A., Sigudu, M., Du Preez, H., Ncube, E. and Venter, S. 2018. Long-term spatial and temporal microbial community dynamics in a large-scale drinking water distribution system with multiple disinfectant regimes. *Water Research*, 139:406-419.

Pritchard, L., Humphris, S., Saddler, G.S., Parkinson, N.M., Bertrand, V., Elphinstone, J.G. and Toth, I.K. 2013. Detection of phytopathogens of the genus *Dickeya* using a PCR primer prediction pipeline for draft bacterial genome sequences. *Plant Pathology*, 62(3):587-596.

Proia, L., von Schiller, D., Sànchez-Melsió, A., Sabater, S., Borrego, C.M., Rodríguez-Mozaz, S. and Balcázar, J.L. 2016. Occurrence and persistence of antibiotic resistance genes in river biofilms after wastewater inputs in small rivers. *Environmental Pollution*, 210:121-128.

Pulicharla, R., Proulx, F., Behmel, S., Sérodes, J.B. and Rodriguez, M.J. 2021. Occurrence and seasonality of raw and drinking water contaminants of emerging interest in five water facilities. *Science of the Total Environment*, 751:141748.

Quaik, S., Embrandiri, A., Ravindran, B., Hossain, K., Al-Dhabi, N.A., Arasu, M.V., Ignacimuthu, S. and Ismail, N. 2020. Veterinary antibiotics in animal manure and manure laden soil: Scenario and challenges in Asian countries. *Journal of King Saud University-Science*, 32(2):1300-1305.

Rafailidis, P.I., Ioannidou, E.N. and Falagas, M.E. 2007. Ampicillin/sulbactam: current status in severe bacterial infections. *Drugs*, 67:1829-1849.

Ramessar, K. and Olaniran, A.O. 2019. Antibigram and molecular characterization of methicillin-resistant *Staphylococcus aureus* recovered from treated wastewater effluent and receiving surface water in Durban, South Africa. *World Journal of Microbiology and Biotechnology*, 35(142):1-11.

Ramsamy, Y., Mlisana, K.P., Amoako, D.G., Abia, A.L.K., Ismail, A., Allam, M., Mbanga, J., Singh, R. and Essack, S.Y. 2022. Mobile genetic elements-mediated Enterobacterales-associated carbapenemase antibiotic resistance genes propagation between the environment and humans: A One Health South African study. *Science of the Total Environment*, 806:150641.

Raseala, C.M., Ekwanzala, M.D. and Momba, M.N.B. 2020. Shared extended-spectrum β -Lactamase-producing *Salmonella* serovars between agricultural and aquatic environments revealed through invA amplicon sequencing. *Microorganisms*, 8(12):1898.

Rawlins, J. 2019. Political economy of water reallocation in South Africa: Insights from the Western Cape water crisis. *Water Security*, 6:100029.

Razman, N.A., Wan Ismail, W.Z., Abd Razak, M.H., Ismail, I. and Jamaludin, J. 2022. Design and analysis of water quality monitoring and filtration system for different types of water in Malaysia. *International Journal of Environmental Science and Technology*:1-12.

Reygaert, W.C. 2018. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS microbiology*, 4(3):482.

Richards, G.A., Brink, A.J. and Feldman, C. 2019. Rational use of the fluoroquinolones. *SAMJ: South African Medical Journal*, 109(6):378-381.

Rolbiecki, D., Harnisz, M., Korzeniewska, E., Jałowicki, Ł. and Płaza, G. 2020. Occurrence of fluoroquinolones and sulfonamides resistance genes in wastewater and sludge at different stages of wastewater treatment: A preliminary case study. *Applied Sciences*, 10(17), p.5816.

Ryan, M.P., Sevjahova, L., Gorman, R. and White, S. 2022. The Emergence of the Genus *Comamonas* as Important Opportunistic Pathogens. *Pathogens*, 11(9):1032.

Salamandane, A., Alves, S., Chambel, L., Malfeito-Ferreira, M. and Brito, L. 2022a. Characterization of *Escherichia coli* from water and food sold on the streets of Maputo: molecular typing, virulence genes, and antibiotic resistance. *Applied Microbiology*, 2(1):133-147.

Salamandane, A., Malfeito-Ferreira, M. and Brito, L. 2022b. A high level of antibiotic resistance in *Klebsiella* and *Aeromonas* isolates from street water sold in Mozambique, associated with the prevalence of extended-spectrum and *AmpC* β -lactamases. *Journal of Environmental Science and Health, Part B*, pp.1-7.

Sallach, J.B., Thirkell, T.J., Field, K.J. and Carter, L.J. 2021. The emerging threat of human-use antifungals in sustainable and circular agriculture schemes. *Plants, People, Planet*, 3(6):685-693.

Samandari, M., Movahedian, A.H., Ebrahimpour, K., Mohammadi, F. and Ghodsi, S. 2022. Measurement of ampicillin and penicillin G antibiotics in wastewater treatment plants during the COVID-19 pandemic: A case study in Isfahan. *Environmental Health Engineering and Management Journal*, 9(3):201-211.

Samie, A., Makonto, T.E., Odiyo, J., Ouaboi-Egbenni, P.O., MOJAPELO, P. and Bessong, P.O. 2011. Microbial quality, diversity and antibiotic susceptibility profiles of bacterial isolates from borehole water used by schools in Greater Giyani Municipality, Mopani District, South Africa. *African Journal of. Microbiology Research*, 5(3):198-210.

Samie, A., Mashao, M.B., Bessong, P.O., Nkgau, T.F., Momba, M.N.B. and Obi, C.L. 2012. Diversity and antibiograms of bacterial organisms isolated from samples of household drinking-water consumed by HIV-positive individuals in rural settings, South Africa. *Journal of Health, Population and Nutrition*, 30(3):241-249.

Samuels, R., Qekwana, D.N. and Oguttu, J.W. 2021. Antibiotic prescription practices and attitudes towards the use of antimicrobials among veterinarians in the City of Tshwane, South Africa. *PeerJ*, 9:e10144.

Scalize, P.S. and Frazão, J.M. 2018. Real-scale comparison between simple and composite raw sewage sampling. *Open Engineering*, 8(1):124-131.

Segura, P., Takada, H., Correa, J., Saadi, K.E.L., Koike, T., Onwona-Agyeman, S., Sabi, E.B., Wasonga, O.V., Mghalu, J.M., Junior, A.M.S. and *et al.* 2015. Global occurrence of anti-infectives in contaminated surface waters: impact of income inequality between countries. *Environment International*, 80:89–97.

Severgnini, M., Camboni, T., Ceccarani, C., Morselli, S., Cantiani, A., Zagonari, S., Patuelli, G., Pedna, M.F., Sambri, V., Foschi, C. and Consolandi, C. 2021. Distribution of *ermB*, *ermF*, *tet (W)*, and *tet (M)* Resistance Genes in the Vaginal Ecosystem of Women during Pregnancy and Puerperium. *Pathogens*, 10(12):1546.

Shamsizadeh, Z., Ehrampoush, M.H., Nikaeen, M., Mohammadi, F., Mokhtari, M., Gwenzi, W. and Khanahmad, H. 2021. Antibiotic resistance and class 1 integron genes distribution in irrigation water-soil-crop continuum as a function of irrigation water sources. *Environmental Pollution*, 289:117930.

Shen, Y., Ryser, E.T., Li, H. and Zhang, W. 2021. Bacterial community assembly and antibiotic resistance genes in the lettuce-soil system upon antibiotic exposure. *Science of The Total Environment*, 778:146255.

Shiaka, G.P., Sani, N.M., Gumel, A.M., Sadiq, S. and Amoo, K.F. 2021. Analyses of physicochemical quality of some packaged sachet water sold in Dutse Metropolis, Jigawa State, Nigeria. *DUJOPAS*, 7:237-244.

Smith-Adao, L., Seyler, H., Holland, M., Maherry, A., Nel, J., Le Maitre, D. and Witthüser, K. 2016. Enhancement of the method to identify and delineate South Africa's water source areas (K5/2431). South Africa. WRC.

Snyman, Y., Whitelaw, A.C., Barnes, J.M., Maloba, M.R. and Newton-Foot, M. 2021. Characterisation of mobile colistin resistance genes (*mcr-3* and *mcr-5*) in river and storm water in regions of the Western Cape of South Africa. *Antimicrobial Resistance & Infection Control*,10(1):1-9.

Song, Z., Zhang, X., Ngo, H.H., Guo, W., Wen, H. and Li, C. 2019. Occurrence, fate and health risk assessment of 10 common antibiotics in two drinking water plants with different treatment processes. *Science of the Total Environment*, 674:316-326.

Soran, M.L., Lung, I., Oprea, O., Floare-Avram, V. and Coman, C. 2017. Determination of antibiotics in surface water by solid-phase extraction and high-performance liquid chromatography with diode array and mass spectrometry detection. *Analytical Letters*, 50(7):1209-1218.

South African Government News Agency. 2015. Ventersdorp water upgrade completed. <https://www.sanews.gov.za/south-africa/ventersdorp-water-upgrade-completed> (Date accessed: 02 September 2022).

South African Government. 2015. Water and Sanitation on upgrade of Ventersdorp Bulk Water Supply (BWS) project. <https://www.gov.za/speeches/water-and-sanitation-upgrade-ventersdorp-bulk-water-supply-bws-project-14-aug-2015-0000>. (Date accessed: 02 September 2022).

Späth, J., Arumugam, P., Lindberg, R.H., Abafe, O.A., Jansson, S., Fick, J. and Buckley, C.A. 2021. Biochar for the removal of detected micropollutants in South African domestic wastewater: a case study from a demonstration-scale decentralised wastewater treatment system in eThekweni. *Water South Africa*, 47(4):396-416.

Stats South Africa: Statistics South Africa. 2019. Household access to services stabilised. Pretoria, South Africa. Government Printer.

Stats South Africa: Statistics South Africa. 2022a. Mid-year population estimates 2021. Pretoria, South Africa. Government Printer.

Stats South Africa: Statistics South Africa 2022b. The South African economy records a positive fourth quarter. <https://www.statssa.gov.za/?p=15214> (Date accessed: 5 May 2022).

Sultan, I., Ali, A., Gogry, F.A., Rather, I.A., Sabir, J.S. and Haq, Q.M. 2020. Bacterial isolates harboring antibiotics and heavy-metal resistance genes co-existing with mobile genetic elements in natural aquatic water bodies. *Saudi Journal of Biological Sciences*, 27(10):2660-2668.

Sun, S., Jiang, T., Lin, Y., Song, J., Zheng, Y. and An, D. 2020. Characteristics of organic pollutants in source water and purification evaluations in drinking water treatment plants. *Science of The Total Environment*, 733:139277.

Suzuki, S., Ogo, M., Koike, T., Takada, H. and Newman, B. 2015. Sulfonamide and tetracycline resistance genes in total- and culturable-bacterial assemblages in South African aquatic environments. *Frontiers in Microbiology*, 6(796):1-7.

Tang, J., Bu, Y., Zhang, X.X., Huang, K., He, X., Ye, L., Shan, Z. and Ren, H. 2016. Metagenomic analysis of bacterial community composition and antibiotic resistance genes in a wastewater treatment plant and its receiving surface water. *Ecotoxicology and environmental safety*, 132:260-269.

Tapela, K. and Rahube, T. 2019. Isolation and antibiotic resistance profiles of bacteria from influent, effluent and downstream: A study in Botswana. *African Journal of Microbiology Research*, 13(15):279-289.

Tell, J., Caldwell, D.J., Häner, A., Hellstern, J., Hoeger, B., Journal, R., Mastrocco, F., Ryan, J.J., Snape, J., Straub, J.O. and Vestel, J. 2019. Science-based targets for antibiotics in receiving waters from pharmaceutical manufacturing operations. *Integrated Environmental Assessment and Management*, 15(3):312-319.

Tempia, N., Matebeni, F., Nkunjana, T. and Dempers, C. 2020. Agricultural sector contribution to South Africa's gross domestic product (GDP). Pretoria, South Africa. National Agricultural Marketing Council.

Thakali, O., Brooks, J.P., Shahin, S., Sherchan, S.P. and Haramoto, E. 2020. Removal of antibiotic resistance genes at two conventional wastewater treatment plants of Louisiana, United States. *Water*, 12(6):1729.

Tiwari, A., Hokajärvi, A.M., Domingo, J.S., Elk, M., Jayaprakash, B., Ryu, H., Siponen, S., Vepsäläinen, A., Kauppinen, A., Puurunen, O. and Artemon, A. 2021. Bacterial diversity and predicted enzymatic function in a multipurpose surface water system—from wastewater effluent discharges to drinking water production. *Environmental Microbiome*, 16(1):1-17.

Tran, C.M., Tanaka, K. and Watanabe, K. 2013. PCR-based detection of resistance genes in anaerobic bacteria isolated from intra-abdominal infections. *Journal of Infection and Chemotherapy*, 19(2):279-90.

Tran, C.M., Tanaka, K. and Watanabe, K. 2013. PCR-based detection of resistance genes in anaerobic bacteria isolated from intra-abdominal infections. *Journal of Infection and Chemotherapy*, 19(2):279-90.

Tsholo, K. 2019. Incidence and characteristics of antibiotic resistant bacteria in raw and drinking water from Western Cape water production facilities. MSc dissertation. Potchefstroom: NWU.

Vadde, K.K., Feng, Q., Wang, J., McCarthy, A.J. and Sekar, R. 2019. Next-generation sequencing reveals fecal contamination and potentially pathogenic bacteria in a major inflow river of Taihu Lake. *Environmental Pollution*, 254:113108.

Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., Teillant, A. and Laxminarayan, R. 2015. Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences*, 112(18):5649-5654.

Van Der Walt, L.J., Winde, F. and Nell, B. 2002. Integrated catchment management: the Mooi River (Northwest Province, South Africa) as a case study. *Cuadernos de Investigación Geográfica*, (28):109-126.

Van Vaerenbergh, J., Baeyen, S., De Vos, P. and Maes, M. 2012. Sequence diversity in the *Dickeya fliC* gene: phylogeny of the *Dickeya* genus and TaqMan® PCR for '*D. solani*', new biovar 3 variant on potato in Europe. *PLoS One*, 7(5):e35738.

Van, T.T.H., Yidana, Z., Smooker, P.M. and Coloe, P.J., 2020. Antibiotic use in food animals worldwide, with a focus on Africa: Pluses and minuses. *Journal of global antimicrobial resistance*, 20, pp.170-177.

Vierheilig, J., Savio, D., Ley, R.E., Mach, R.L., Farnleitner, A.H. and Reischer, G.H. 2015. Potential applications of next generation DNA sequencing of 16S rRNA gene amplicons in microbial water quality monitoring. *Water Science and Technology*, 72(11):1962-1972.

Vumazonke, S., Khamanga, S.M. and Ngqwala, N.P. 2020. Detection of pharmaceutical residues in surface waters of the Eastern Cape Province. *International Journal of Environmental Research and Public Health*, 17(11):4067.

Wang, L., Zhang, J., Li, H., Yang, H., Peng, C., Peng, Z. and Lu, L., 2018a. Shift in the microbial community composition of surface water and sediment along an urban river. *Science of the Total Environment*, 627, pp.600-612.

Wang, X., Gu, J., Gao, H., Qian, X. and Li, H. 2018b. Abundances of clinically relevant antibiotic resistance genes and bacterial community diversity in the Weihe River, China. *International Journal of Environmental Research and Public Health*, 15(4):708.

WHO: World Health Organization. 2016. Protecting surface water for health. Identifying, assessing and managing drinking-water quality risks in surface-water catchments. Geneva, Switzerland: WHO Press.

WHO: World Health Organization., 2017. Guidelines for Drinking-water quality. 4th edition incorporating the first addendum. Geneva, Switzerland: WHO Press.

WHO: World Health Organization. 2018. Critically important antimicrobials for human medicine 6th revision. Geneva, Switzerland: WHO Press.

WHO: World Health Organization. 2022. Guidelines for drinking-water quality. 4th edition incorporating the first and second addenda. Geneva, Switzerland: WHO Press.

Wu, Y., Zaiden, N. and Cao, B. 2018. The core-and pan-genomic analyses of the genus *Comamonas*: from environmental adaptation to potential virulence. *Frontiers in Microbiology*, 9, p.3096.

Xu, Y., Xu, J., Mao, D. and Luo, Y., 2017. Effect of the selective pressure of sub-lethal level of heavy metals on the fate and distribution of ARGs in the catchment scale. *Environmental Pollution*, 220:900-908.

Yang, J., Wang, H., Roberts, D.J., Du, H.N., Yu, X.F., Zhu, N.Z. and Meng, X.Z. 2020. Persistence of antibiotic resistance genes from river water to tap water in the Yangtze River Delta. *Science of the Total Environment*, 742:140592.

Ye, Q., Wu, Q., Zhang, S., Zhang, J., Yang, G., Wang, H., Huang, J., Chen, M., Xue, L. and Wang, J. 2017. Antibiotic-resistant extended spectrum ss-lactamase-and plasmid-mediated *AmpC*-producing enterobacteriaceae isolated from retail food products and the pearl river in Guangzhou, China. *Frontiers in microbiology*, 8:96.

Zhang, K., Fan, Y., Chang, S., Fu, Q., Zhang, Q., Yang, G. and Sun, X. 2022. Characterization of antibiotic resistance genes in drinking water sources of the Douhe Reservoir, Tangshan, northern China: the correlation with bacterial communities and environmental factors. *Environmental Sciences Europe*, 34(1):56.

Zhang, K., Niu, Z.G., Lv, Z. and Zhang, Y. 2017. Occurrence and distribution of antibiotic resistance genes in water supply reservoirs in Jingjinji area, China. *Ecotoxicology*, 26:1284-1292.

Zhang, L., Shen, L., Ju, Z., Fu, Y., Qin, S. and Cui, J. 2021. The key environmental influencing factors for the change of sediment bacterial community and antibiotics resistance genes in a long-term polluted lake, China. *Ecotoxicology*, 30:1538-1549.

Zhou, S., Di Paolo, C., Wu, X., Shao, Y., Seiler, T.B. and Hollert, H. 2019. Optimization of screening-level risk assessment and priority selection of emerging pollutants—the case of pharmaceuticals in European surface waters. *Environment international*, 128:1-10.

Zhou, Z.C., Zheng, J., Wei, Y.Y., Chen, T., Dahlgren, R.A., Shang, X. and Chen, H. 2017. Antibiotic resistance genes in an urban river as impacted by bacterial community and

physicochemical parameters. *Environmental Science and Pollution Research*, 24:23753-23762.

APPENDIX A

Table A1: Percentage values for quality categories of selected phenotypic and genotypic literature sources.

Study characteristics	High	Medium	Low
Diversity of ARGs	Focused on $\geq$ three genes (82.8%)	Focused on two genes (14.3%)	Focused on one gene (2.9%)
Diversity of bacteria	Isolated $\geq$ three species (65.1%)	Isolated two species (3.2%)	Isolated one species (31.7%)
Method	Real-time or Drop-let PCR and Illumina Hiseq/ Illumina MiSeq (12.9%)	End-point PCR (45.2%)	Phenotypic tests (41.9%)
Diversity of sampling sites (WWTPs, surface water and drinking water)	Sampled all (3.2%)	Sampled any two (31.7%)	Sampled one (65.1%)
WWTP: wastewater treatment plants			

Table A2: Percentage values for quality categories of selected antibiotics in literature sources.

Study characteristics	High	Medium	Low
Diversity antibiotics	Focused on $\geq$ three antibiotics (77.8%)	Focused on two antibiotics (11.1%)	Focused on one antibiotic (11.1%)
Method	Quantification (100%)	-	Detection (0%)
Exposure-risks assessment	Done (0%)	-	Not done (100%)
Diversity of sampling sites (WWTPs, surface water and drinking water)	Sampled all (0%)	Sampled any two (55.6%)	Sampled one (44.4%)
WWTP: wastewater treatment plants			

Table A3: Summary of the main findings.



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APPENDIX B

Table B1: Pairwise correlation matrices between bacterial community, physicochemical parameters, ARGs and antibiotic residues in NW-E and NW-C DWPFs.



Correlation
matrices for NW-E and NW-C DWPFs

Table B1: Pairwise correlation matrices between bacterial community, physicochemical parameters, ARGs and antibiotic residues in NW-E and NW-C wastewater effluents and rivers.



Correlation
matrices for NW-E and NW-C wastewater effluents and rivers