Analysis of the microbiome of selected drinking water treatment plants, drinking water treated sludge, and water distribution systems

by

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DECLARATIONS

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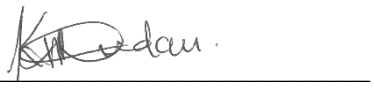

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DEDICATIONS

I dedicate this thesis to God Almighty, my saviour, pillar of strength, and source of inspiration, wisdom, knowledge, understanding, and compassion.

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ABSTRACT

Microorganisms are crucial for water quality and purification in water treatment and distribution systems. The microbial communities present in untreated water can aid in purification through the biodegradation of contaminants. However, some microbes may be human pathogens that threaten consumer health. The current study analysed microbial diversity in selected drinking water treatment plants, water treatment sludge and drinking water distribution point seasonally over 12 months. Samples were drawn from five selected water treatment plant sites in the Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) Provinces in South Africa. Selective media culture studies were used for *Escherichia coli* and *Enterococcus* species (spp.) quantification and antibiotic resistance profiling for isolates from the samples. Colilert and Enterolert Quanti-Tray 2000 IDEXX methods were used to enumerate total coliforms, *E. coli* and *Enterococcus* spp. from water samples from the different water treatment stages. The Kirby-Bauer disc-diffusion technique was used to assess the antibiotic susceptibility of the indicator bacteria isolates. Shotgun metagenomics sequencing using the MGI DNBSEQ-G400 sequencing platform was performed to investigate microbial metagenomic diversity and their functions from the raw water sources, the filtration, sedimentation and disinfection stages, final treated water, treated sludge and distribution system samples.

Water physicochemical parameters were assessed, and the obtained results in terms of pH, temperature, turbidity, free chlorine, manganese, ammonia, nitrate, sulphate, iron, and total organic carbon for different stages of treatment, final treated water, drinking water treated sludge and first point in the distribution system varied across the five sampling sites. The physicochemical and microbiological parameters were measured and compared to the drinking water quality guidelines set by the World Health Organization and South African National Standard 241. The results obtained from the IDEXX-defined substrate Colilert/ Quanti-Tray 2000 System (ISO 9308-2:2012) showed the presence of the indicator microorganisms (total coliforms, *E. coli* and *Enterococcus* spp.) with variations in their abundance (based on the Most Probable Number) in the raw water sources, sedimentation stage and drinking water treated sludge across the seasons. However, only two of the five treatment plants maintained the acceptable limit of microbiological quality for the final treated water stage, and the distribution systems sampling points complied with the drinking water standards. Using the MGI DNBSEQ-G400 sequencing platform, the following dominant phyla and classes were identified: Euryarcheota and Nitrososphaerota (Archaea phyla), Halobacteria and Methanomicrobia (Archaea classes), Pseudomonadota (bacterial phylum),

Alphaproteobacteria, Gammaproteobacteria and Betaproteobacteria (bacterial classes), Ascomycota and Basidiomycota (Eukaryota phyla), and Aconoidasida and Sordariomycetes (Eukaryota classes). Despite the observed overlap of dominant phyla and classes in the treatment plant stages, the antibiotic-resistance gene composition and antibiotic class phenotypes exhibit a general trend of a downward shift, showing the efficiency of treatment plants in reducing opportunistic pathogens.

Correlation analysis done between physicochemical parameters in the drinking water treatment, distribution, and the Archaea, bacterial and Eukaryota phyla and classes respectively demonstrated a link that existed between the physicochemical parameters and the proliferation of the members of microbial groups. Temperature correlated significantly positively with Gammaproteobacteria (filtration stage at Site D), Alphaproteobacteria (raw water at Site A), sulphate and Alphaproteobacteria (final treated water at Site C), Cynophyceae (raw water at Site A), sulphate and Betaproteobacteria (raw water at Site D and filtration stage at Site A), and nitrate and *Bacilli* (final treated water at Site E). This correlation clearly showed that changes in the physicochemical parameters could contribute to the selection of the microorganisms that survive in each stage of treatment, including drinking water treated sludge and distribution systems. Functional metagenomes revealed the abundance of antibiotic resistance functions, especially cationic antimicrobial peptide, ßeta-lactam and vancomycin resistance in the final treated water and distribution systems across the selected sampling sites.

It can be concluded that selected treatment plant processes effectively reduced the number of indicator organisms. In addition, seasons and different treatment stages influence the microbial diversity of the drinking water samples. In Gauteng Province at Sites A and D, most bacteria, including potential pathogens, were effectively removed by chlorine disinfection. However, some bacteria presented excellent resistance to chlorine. The study was able to provide a profile of microbial diversity (metagenomic diversity of Proteobacteria and Firmicutes in drinking water treatment process). The presence of antibiotic-resistant microorganisms and residual microorganisms in some treated water samples means that these microorganisms are not completely removed during drinking water treatment process and optimisation of drinking water treatment systems can be recommended, giving more attention to the raw water sources, DWTS, and distribution systems in removing the indicator organism and maintaining high-quality drinking water free from pathogens and contaminants.

KEYWORDS: Shotgun metagenomics; Drinking water treatment; Drinking water distribution; Antibiotic-resistant bacteria; IDEXX-defined substrate Coliert-18/ Quanti-Tray 2000 System.

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LIST OF ACRONYMS

AMR	Antimicrobial Resistance
ARB	Antibiotic-Resistant Bacteria
ARG	Antibiotic-Resistance Gene
BAC	Biological Activated Carbon
CAMP	Cationic Antimicrobial Peptide
CLSI	Clinical and Laboratory Standards Institute
DOC	Dissolved Organic Carbon
DWDS	Drinking Water Distribution System
DWS	Department of Water and Sanitation
DWTP	Drinking Water Treatment Plant
DWTS	Drinking Water Treatment Sludge
EPS	Extracellular Polymeric Substance
GAC	Granular Activated Carbon
HPC	Heterotrophic Plate Count
MAR	Multiple Antibiotic Resistance
MDR	Multidrug-Resistant
MPN	Most Probable Number
NGS	Next-Generation Sequencing
NOM	Natural Organic Matter
OUT	Operational Taxonomic Unit
PAST 4	Paleontological Statistics Software Package Version 4
ROS	Reactive Oxygen Species
SANS	South African National Standard
TOC	Total Organic Carbon
UV	Ultraviolet
WHO	World Health Organization
WWTP	Wastewater Treatment Plants

LIST OF UNITS OF MEASURE

°C	degrees Celsius
bp	base pair
g	gravity
km	kilometre
L	litre
m^3/day	cubic metres per day
mg	milligram
mg/L	milligram per litre
mL	millilitre
ML/day	megalitres per day
spp.	species
µm	micrometre

LIST OF ORGANISMS

Acinetobacter baumannii

Acinetobacter calcoaceticus

Aeromonas hydrophila

Aeromonas veronii

Bacillus myciodes

Burkholderia mallei

Campylobacter coli

Campylobacter jejuni

Clostridium perfringens

Deefgea rivuli

Enterobacter cloacae

Enterococcus casseliflavus

Enterococcus durans

Enterococcus faecalis

Enterococcus faecium

Enterococcus hirae

Escherichia coli

Klebsiella pneumoniae

Legionella pneumophila

Massilia aurea

Mycobacterium avum

Mycobacterium gordonae

Mycobacterium lentiflavum

Mycobacterium tuberculosis

Mycobacterium tusciane

Pseudomonas aeruginosa

Pseudomonas fluorescens

Rheinheimera chironomi

Rhodococcus erythropolis

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Vibrio cholerae

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CHAPTER 1: INTRODUCTION TO THE STUDY

1.1. Introduction

Drinking water supply and water quality are major global concerns. Polluted water environments are the source of microorganisms, organic matter, and inorganic compounds that find their way into water treatment plants (Zhang *et al.*, 2018; Luvhimbi *et al.*, 2022; Munzhelele *et al*., 2024). This pollution can originate from a multitude of point and non-point sources, consequently affecting the environment and disturbing water purification in ecosystems, allowing microbial proliferation and increased organic material in the environment (Kulik *et al*., 2023; Mishra *et al*., 2023).

Drinking water treatment plants (DWTPs) ensure high-quality drinking water through a combination of treatment processes aiming to remove chemical and microbiological contaminants. Nevertheless, DWTPs are a source of unexpected microbiomes (Archaea, bacteria, eukaryotes and viruses) that vary in composition spatially and temporally from source to tap (Osorio *et al*., 2021; Bai *et al*., 2022b; Sudarshan *et al*., 2024). Considering the many ways biological activity in drinking water infrastructure—from treatment to distribution and in the built environment—can affect the safety and aesthetic quality of drinking water, understanding the structure and function of the drinking water microbiome is essential. One approach is to develop generalisable insights, which involves direct comparative analysis of microbial diversity and functions and investigating pathogens and antibiotic microbial resistance presence across different systems.

Advancements in DNA extraction and sequencing protocols suggest that the microbiota in drinking water treatment and distribution systems can significantly impact the biological quality of drinking water (Pinar-Méndez *et al*., 2022; Wang *et al*., 2024; Murei *et al*., 2024). Furthermore, bacterial community composition may be linked to the presence of opportunistic pathogens (Chowdhury *et al*., 2023; Siponen *et al*., 2024). For example, in 2023, a cholera outbreak in Hammanskraal in Gauteng, South Africa, caused health implications for communities due to the poor water quality from the tap and water delivered by trucks (Obasa *et al*., 2023). According to reports, approximately 600 people presented with symptoms suggestive of cholera at hospitals in

Gauteng and the Free State Provinces (Department of Water and Sanitation [DWS], 2023; Obasa *et al*., 2023). Cholera is a rapidly developing gastrointestinal infection primarily transmitted through ingesting contaminated food or water containing the bacterium *Vibrio cholerae* (World Health Organization [WHO], 2023). Disease-causing organisms (pathogens) transmitted via drinking water are predominantly of faecal origin and are, therefore, known as enteric pathogens (Jiang *et al*., 2022) For this reason, a comprehensive and deep understanding of microbial community dynamics in drinking water systems is essential to the security of water quality and public health and to ensure the effectiveness of water treatment processes.

Previous meta-analyses of the drinking water microbiome have relied on 16S rRNA gene amplicon sequencing datasets. Two studies showed that the drinking water microbiome consists of a small core community that exhibits signs of selection from both water treatment and distribution systems (Thom *et al*., 2022; Sudarshan *et al*., 2024). For instance, Sudarshan *et al*. (2024) determined that the assembly of drinking water microbial communities post-disinfection was primarily deterministic. This deterministic community assembly results in a community composition that can impact disinfectant residuals (e.g., via nitrification) and harbour bacteria of general concern (e.g., *Legionella* and *Mycobacterium*). While amplicon sequencing studies have provided extensive insights into the composition and biogeography of the drinking water microbiome, these results are inherently limited as they do not provide functional information. Functional information can shed light on processes governing phenomena like interactions between microbial community members within the treatment process and biofilm formation that could impact pathogen prevalence and provide clues on how treatment and distribution practices exert selective pressures (Thom *et al*., 2022; Bhatti *et al*., 2023; Chen, 2024).

Despite these advances in the characterisation of drinking water microbiota, the occurrence of uncharacterised environmental bacteria, some of which have been shown to have ultrasmall cell sizes (Gabrielli *et al*., 2023; Liu *et al*., 2024), is common and underestimated in many environments, including DWTPs, distribution systems and produced sludge. One of the reasons is procedural; most analyses start from water filtration that only allows the retention of >0.2 μm microorganisms. Considering surface water as source water, the enrichment of surface water with nutrients is particularly high in the world's regions where industrial activities, agricultural practices and local and international trading are intense. The treatment of drinking water may need

to start from coagulation and sedimentation as they comprise major factors that contribute to the efficiency of water treatment. Jiang *et al*. (2021) observed a decrease in the abundance of *Aeromonas veronii*, *Rhodococcus erythropolis*, *Pseudomonas fluorescens,* and *Aeromonas hydrophila* after sedimentation and filtration.

While the DWTP aims to produce safe potable water, the by-products comprise residual sludge and sediments (consisting of liquid, solid, semi-solid and gaseous phase by-products) (Anjithan et al., 2016). Some residual sludge and sediments may undergo further treatment, while some can be disposed of on the land and eventually piled up in the sludge facility (Anjithan et al., 2016; Qrenawi & Rabah, 2023). The treatment of residual sludge needs further attention when recovering clean water, especially with sludge containing highly contaminated residual pollutants. These pollutants may be a source of environmental pollution to aquatic and terrestrial ecosystems (Qrenawi & Rabah, 2023). Despite the presence of pathogenic bacteria, viruses and protozoa that could cause devastating effects on aquatic and terrestrial ecosystems, they are beneficial to agricultural activities (Ahmad *et al.*, 2016; Albrektienė *et al.*, 2019). There is a knowledge gap in understanding the diversity and dynamics of the microbiome within produced sludge and its impact on the environment.

Understanding the microbial diversity and ecology of DWTPs is necessary for designing innovative and effective control strategies to ensure safe and high-quality drinking water. The current study assessed the microbial diversity and function of DWTPs, drinking water treatment sludge (DWTS) and drinking water distribution systems (DWDSs) in Gauteng, Limpopo and Mpumalanga, investigating the presence of pathogens and antimicrobial resistance (AMR). The critical interpretation of the results will improve DWTPs' functions, monitoring capacities and, consequently, their management strategies.

1.2. Motivation

South Africa faces a water crisis caused by insufficient water infrastructure maintenance and investment, recurrent droughts driven by climate variation, inequities in access to water and sanitation, and deteriorating water quality (DWS, 2022). The problem is acute, especially at the local level, where not only water scarcity but also water quality is concerning. The 2022 Blue Report, released by the DWS, South Africa, highlighted the dire state of drinking water services

and the alarming rate of non-compliance among municipal DWTPs in South Africa (DWS, 2022). Around 60% of customers' complaints to the water utilities are about unwanted aesthetic aspects of drinking water generated during its distribution. This impaired aesthetics, which results from the uncontrolled growth of indigenous bacteria in particles, sediments and biofilms in distribution pipelines, might even include the presence of invertebrates in the water (Mahajna *et al*., 2022; Erdei-Tombor *et al*., 2024).

DWTPs are recognised as complex aquatic environments offering multiple unique habitats that support microbial diversity within different stages of treatment until the tap. The bacterial communities present in treatment systems are mainly introduced by the source water (Molina *et al*., 2024; Zhang *et al*., 2024c). Bacteria from untreated water could utilise organic and inorganic matter as growth substrates, resulting in enhanced biological stability and lower levels of micropollutants in water (Golovko *et al*., 2020; Nikolova *et al*., 2021; Yin *et al*., 2024). On the other hand, some may be potential human pathogens, such as *Legionella* (Favere *et al*., 2021) and *Mycobacterium* species (spp.) (Romero *et al*., 2021). Making full use of bacterial biodegradation and controlling pathogens are thus two major goals in drinking water treatment. According to Sevillano *et al.* (2020), AMR in microbial communities is common in both treated and untreated drinking water, which creates concern about drinking water treatment processes' microbial reduction or eradication efficiency, especially for the treated water. Various physicochemical parameters such as pH, temperature and dissolved organic carbon (DOC) could also cause variations in bacterial composition, especially with different habitats.

Studies employing sequencing technologies have shown that the treatment of drinking water, in general, reduces the abundance and diversity of microorganisms present, yet a diverse microbiome remains in potable water, including genera containing pathogenic species (Zhao *et al.*, 2020; Gu *et al.*, 2022). The drinking water microbiome at customer taps may be influenced by a range of factors, including source water, treatment, flow conditions and DWDS biofilms. Water treatment has been proposed to have a deterministic effect, selecting microbes that survive filtration and disinfection processes (Pinto *et al*., 2012; Lin *et al*., 2014; Maguvu *et al*., 2020). This effect could increase with distance and time from treatment, where biofilm growth and disturbance become more prominent. At this point, stochastic (random) effects may be more likely to govern the assembly of microbial communities due to the complexity of the local environments. The

importance of stochastic factors like birth, death and immigration is known to be important in shaping many prokaryotic communities (Bai *et al*., 2023). Thus, drinking water microbiomes are dynamic through treatment, time, and location. To aid water utilities' treatment processes in delivering safe, potable water, a deeper understanding of these changes, their consequences and their impact on both the microbiome and the prevalence of pathogens is needed.

1.3. Research problem

Microorganism's problems in the drinking water treatment processes include microbial regrowth, antibiotic-resistant bacteria (ARB), AMR genes and pathogen persistence (Prest *et al.*, 2016; Zhang *et al.*, 2017). Studies on DWTPs have revealed that bacterial communities are dominated by the phyla Proteobacteria, Firmicutes, Nitrospirota, and Actinobacteria, and fungal communities are dominated by *Aspergillus*, *Cladosporium*, *Penicillium*, *Fusarium,* and *Trichoderma* spp. (Zhao *et al*., 2022; Mudau *et al*., 2024). The characteristics of the microbial communities in DWTPs are related to source water, anthropogenic activities, different substrates in the effluents, filter-filling materials, and the disinfection processes (Shoemaker *et al.*, 2021; Steyn, 2022). Microorganisms of ranging diversity and function occur in water, specifically in urban-impacted water catchments. With growing urbanisation and widespread anthropogenic pollution, water reaching treatment plants has high organic particles and microbial loads that reflect emerging pollution challenges (Li *et al.*, 2017a; Fu *et al.*, 2022). As a consequence of different pollutants in water environments and ecosystems, immediate receptors might be polluted by a range of known and unknown pollutants that require extensive treatment and control strategies. Concurrently, pollutants may act as a carrier of other concerns of infectious parasites such as *Balantidium coli* (balantidiasis) and certain helminths (species of *Fasciola*, *Fasciolopsis*, *Echinococcus*, *Spirometra*, *Ascaris*, *Trichuris*, *Toxocara*, *Necator*, *Ancylostoma*, *Strongyloides* and *Taenia solium*), which may reach drinking water treatment plants (Li *et al.*, 2017a; Mahmud *et al*., 2019).

DWTPs are crucial in drinking water quality and supply; thus, they are an important focal point for removing water pollutants. Li *et al.* (2017a) reported that disinfection significantly influenced the bacterial composition and function in DWTPs, and pathogenic bacteria can enter DWDSs, reaching the consumers. Larsson and Flach (2022) reported that *E. coli* 0157-related illnesses in children could be traced to drinking water treatment. Additionally, the authors, predicted

metagenome analyses indicated that source water and treatment plants were contaminated by ßeta-lactam-resistant organisms (Li *et al.*, 2017a; Larsson & Flach, 2022). According to the WHO, ARBs are a great concern in drinking water treatment and a threat to human health (WHO, 2022). A comparative analysis of DWTP stages will help us to understand the changes in antimicrobial diversity and occurrence in water undergoing treatment. Furthermore, evaluating microorganisms and ARB in sludge will help us to evaluate their prevalence and the potential release of ARB into the environment. Metagenomics studies, including DNA sequencing, could be critical to identifying which genera and species are present in a sample, thus allowing for a better understanding of microbial diversity and risks in DWTPs, DWDSs and DWTS. It could also capture aspects among provinces, sites, and activities.

1.4. Aim of the study

The study assessed the microbial diversity and dynamics of DWTPs, DWTS and DWDSs in Gauteng, Limpopo, and Mpumalanga, investigating the presence of pathogens and AMR.

1.5. Objectives of the study

The study had the following objectives:

a) To determine the microbial diversity and dynamics of the different DWTPs, DWTS, and DWDSs studied.
b) To determine the antibiotic resistance, indicator bacteria occurrence, and antibiotic-resistant genes (ARGs) changes with treatment stages and human health.
c) To determine the relationships between diversity profiles of the microbiome, indicator microorganisms, and physicochemical pollution of the water samples.

1.6. Research questions

The current research investigated the following research questions:

a) What are the microbial diversity and dynamics of the different DWTPs, DWTSs, and DWDS studied?
b) How does antibiotic resistance, indicator bacteria occurrence, and ARGs change with treatment stages and human health?

c) What is the relationship between diversity profiles of the microbiome, indicator microorganisms, and physicochemical pollution of the water samples?

1.7. Study delimitations

The study was limited to five DWTPs in the Gauteng, Limpopo, and Mpumalanga Provinces due to the scope of work under a funded grant, the time frame for sampling, and the responsibilities associated with analysing the samples within 24 hours. According to DWS (2022), to determine the adequacy of the water treatment works, four samples per year (quarterly) are the absolute minimum, with a recommended sampling frequency covering a 12-month period, depending on the size of the treatment works. The current study focused on the analysis of microbial diversity and dynamics of the selected DWTPs, DWDSs, and produced sludge, specifically on raw water sources, coagulation/flocculation, sedimentation, filtration, final effluent, and first tap of contact. Water physicochemical and microbial analyses were performed on raw water sources, treatment stages, produced sludge, and distribution systems at all selected sampling sites. The results were compared to the SANS 241 (2015) drinking water limits and the WHO (2022) drinking water guidelines. However, due to consent challenges, the first tap of contact after treatment was used to represent the DWDS point, which is a limitation of under-representing the DWDS. All sampling site samples were also analysed for indicator bacteria. Furthermore, pure *E. coli* and *Enterococcus* spp. isolates were obtained from the IDEXX Quanti-Tray. Each *E. coli* and *Enterococcu*s spp. isolate was subjected to antibiotic susceptibility testing. To ascertain the total microbial community diversity and function in the samples, the shotgun metagenomics approach was adopted to give insight into the microbial community in the water samples, including pathogenic and antibiotic-resistant isolates, to understand their complete genetic makeup.

1.8. Limitations of the study

Like any other study, this study had several limitations, such as its depth, scope and time, which depended on the resources at the researcher's disposal. Some of the limitations that impacted this study include:

a) The study focused on five sampling sites within three South African provinces, limiting the results to these provinces. Ideally, the collection of samples throughout South Africa

would be more representative.

b) The water samples were collected over 12 months. A better picture would have been obtained if the duration had been longer than a year.
c) The cost of DNA sequencing limited the number of samples sequenced to track the changes in the microbial diversity for the raw water sources to the DWTPs, DWDSs, and DWTS.
d) The study could not cover all the SANS 241 (SANS, 2015) physicochemical parameters due to time and resource constraints, which could also make the study broad and lengthy. The study gives a snapshot correlation of physicochemical parameters and microbial diversity.
e) The results from the shotgun metagenomics provided a larger volume of data because of the vast amount of genomic information. The information obtained needed advanced computer software to be easily and efficiently analysed, and the analysis done might not have been exhaustive.
f) Determination of propidium monoazide (PMA) treatment was not performed on the samples to determine if the microbes identified through shotgun metagenomics were alive or dead due to time factors.
g) The selected microbial indicators are essential for assessing microbial contamination in drinking water. However, the study did not account for all the potential pathogens that could pose significant health risks. This underscores the need for further research incorporating additional microorganisms such as *Vibrio cholerae*, *Salmonella* spp., and *Shigella* spp. to improve the comprehensiveness of water quality in three different provinces of South Africa.

1.9. Ethical consideration

Research ethical clearance was obtained from the University of South Africa College of Agriculture & Environmental Sciences Ethics Review Committee, and approval was granted with reference number 2023/CAES_HREC/026 (Appendix 1). Permission was also obtained from Rand Water, Magalies Water, Lepelle Northern Water, and eMalahleni Local Municipality to conduct research and collect samples from their DWTPs, drinking water sludge, and DWDSs.

CHAPTER 2: LITERATURE REVIEW

2.1. Introduction

DWTPs, DWDSs and DWTSs are recognised as complex environments offering multiple unique habitats that can support microbial growth. The bacterial communities in the treatment systems may be introduced by source water (Huang *et al.*, 2021; Gu *et al.*, 2022). Water within DWTPs is subjected to various conditions and treatment stages that may lead to microbial community composition and concentration changes over time and space. This biological instability can only be managed based on a clear understanding of the microbial ecology in the water treatment, distribution system, and produced sludge. In addition, the emergence of more virulent and antibiotic-resistant bacterial strains poses an unknown challenge in the long term to drinking water treatment, distribution systems, the aquatic environment and public health (Potgieter *et al.*, 2020; Amarasiri *et al.*, 2020; Bekele *et al.*, 2023). Therefore, understanding the microbial ecology and distribution of drinking water microbial communities and their interactions within the environment will improve microbial management strategies for drinking water treatment. This literature review comprehensively covers the proliferation of microbial diversity in treatment stages, DWDSs and DWTS, the dynamics of ARB from natural water sources through the different stages of DWTPs to DWDSs, and factors that contribute to the spread of microbial communities and ARB along DWDSs, highlighting progress and gaps related to the study topic.

2.2. Drinking water treatment process

Drinking water treatment processes can be classified into three categories:

a) Disinfection treatment process: This process is used for high-quality water sources to ensure that water does not contain pathogens.
b) Conventional drinking water treatment process: This method is typically employed to treat surface water (Figure 2.1).
c) Softening treatment process: This process is used to treat groundwater.

A conventional drinking water treatment plant is typically designed to remove odors, color, turbidity, natural organic matter (NOM), and microbial growth. Table 2.1 summarizes the effects of different stages of drinking water treatment, highlighting the significance of disinfection on the microbiome, as well as the purpose, advantages, and disadvantages of each stage. A conventional treatment plant employs the following steps:

a) Coagulation and rapid mixing involve the addition of chemicals, which are then quickly dispersed throughout the water. During this process, particles bind together, a phenomenon known as coagulation (sometimes referred to as flocculation). The larger particles, or floc, become heavy and rapidly settle to the bottom of the water tank (Prokopova *et al*., 2021; Rand Water, 2023).
b) Flocculation involves adding chemicals such as alum (aluminum sulfate) and extracellular polymeric substances (EPSs) to water. These additives help neutralize the electrical charges of particles and encourage them to come together, forming larger particles called flocs. These flocs can then be more easily settled out of the water (Hasan *et al*., 2024).
c) During the sedimentation process, a significant number of organic compounds, including some dissolved organic materials and microorganisms, are removed along with flocs. Meanwhile, other particles settle to the bottom of the tank due to gravity, forming a sediment or sludge (De Luca *et al*., 2022; Tahraoui *et al*., 2024).
d) Filtration is one of the essential processes used in water treatment. It refers to the removal of suspended solids from water primarily through physical action as the water flows through a bed filled with granular media. Biological filtration methods generally employ rapid gravity filters or slow sand filters (Cescon & Jiang, 2020). There are different types of rapid gravity sand filter systems, which vary based on the configurations of their media. These includes Mono media rapid sand filters (which use sand alone); Dual media rapid sand filters (which use sand and anthracite coal): Multimedia rapid sand filters (which use sand, anthracite coal, and garnet); Granular activated carbon (GAC) filters (which use various forms of organic carbon such as wood, coconut shells, coal, or peat) (Li *et al*., 2017a; Kramer, 2021). GAC is specifically used for water purification, typically in a fixed-bed application (Kramer, 2021; Marsilius, 2022).

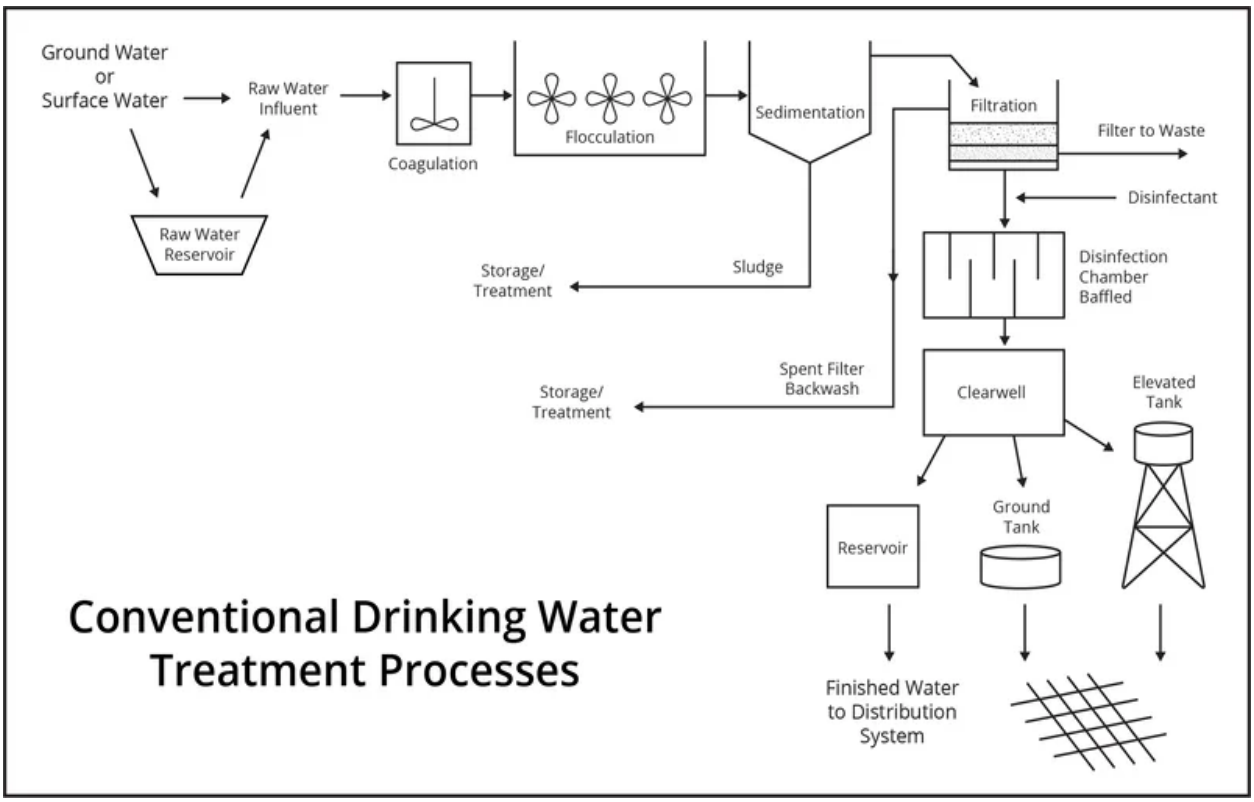


Figure 2.1: Conventional Drinking Water Treatment Plant (adopted from XRGeomembranes, Conventional Drinking Water Treatment Processes, 2025).

Table 2.1: A summary of the effects of different drinking water treatment stages on the microbiome, purpose of the stage, advantages, and disadvantages

Treatment processes	Primary purpose of the process	Effects on its microbiome	Advantages	Disadvantages	References
Coagulation, flocculation, and sedimentation	The main objective of these processes is to remove suspended particles and other impurities.	During suspended particle removal, organic nutrients can be removed, leading to changes in nutrient availability. Further, microbes can be removed as well. This could result in changes in the water quality at this stage, causing a shift in the microbial composition and dynamics before moving to the next stage.	• Cost-effective • Rapid process that gives room for any emergency water treatment • Removal of certain microbes and antibiotic-resistant genes	The alteration of microbial diversity and composition of water could make it difficult for the next stage of treatment. The effectiveness of these processes is dependent on the raw water quality and contaminants, temperature, pH, and the specific coagulants and flocculants used.	Sun *et al.* (2018) Gomes *et al.* (2019)
Filtration	The main objective of this stage is to remove microorganisms from the water samples.	It alters the microbial structure and composition of the water due to the removal of both beneficial and harmful microorganisms in the water. Furthermore, the process could lead to the removal of nutrients and the creation of a nutrient-deficient environment that could enhance the proliferation of other organisms, including pathogenic ones.	• Enhanced removal of solid particles, pathogens, and chemicals, as well as the improvement of taste and odour • Promotes reduced chemical usage • Reduces the load of microbes that enter the next stage of water treatment	• Limitation in the removal of dissolved contaminants • High initial capital cost, especially for large-scale drinking water treatment plants • Filter fouling could contribute to the deterioration of water quality due to the accumulation of particles and pathogenic microbes	Tang *et al.* (2018) Cescon and Jiang (2020)
Disinfection	The main objective of this stage is to eliminate or deactivate microorganisms, especially harmful ones.	The toxic nature of the disinfectant could cause the death of useful organisms that maintain microbial balance within the water ecosystem. In addition, the disinfectant could select for the microbes that proliferate, especially the resistant ones. The production of disinfection by-products could also promote the growth of other pathogenic bacteria.	This stage improves water quality. The elimination of pathogenic bacteria helps to protect the health of the public.	The production of carcinogenic disinfection by-products, which promotes the development of antibiotic-resistant bacteria and biofilm formation	Lv *et al.* (2014) Mantilla-Calderon *et al.* (2019)

2.3. Raw water source and microbial quality

The desirable approach is to abstract water from the best available and safest source. However, due to the scarcity of acceptable water quality and quantity, DWTPs may abstract reclaimed water to augment their water supplies (Swana *et al.*, 2020; Hemdan *et al.*, 2021; WHO, 2023). DWTPs draw their raw water from surface or underground water depending on the place, water availability, and other factors. The water sources may contain organic contaminants from human settlement activities, as well as other anthropogenic activities, due to increased population and high levels of urbanisation and industrialisation (Swana *et al.*, 2020; Luvhimbi *et al.*, 2022). Microbial deterioration of water quality has been linked to increased industrial processes, domestic sewage discharge, agricultural chemicals, and eroded soil (Steyn, 2022; Thom *et al.*, 2022). This is due to the proximity in urban areas between industries, domestic houses, and agricultural activities, as well as wastewater treatment plants (WWTPs) and natural water sources, which contribute to the increase in the load and diversity of microbial communities in the surface water source.

Agricultural practices, such as manure applications, contribute to the load and diversity of microbial communities and other pollutants in surface water sources (Luvhimbi *et al*., 2022; Naylor *et al.*, 2022; Botha *et al*., 2023). Tables 2.2 and 2.3 show the point and non-point sources of organic contaminants in surface waters and the environment. Bodor *et al.* (2020) and Verlicchi and Grillini (2020) evaluated the movement of pathogens from agricultural fields to natural waters, as well as the concentration of human health-related pathogenic microbes in the runoff from the plots treated with manure. Environmental factors that include pollutants and hydrological conditions affect the surface water ecosystems, leading to the alteration of the microbial compositions and the emergence of pathogenic microbes that risk human health (Luvhimbi *et al.*, 2022; Steyn, 2022; Botha *et al*., 2023). Several studies have, therefore, focused on how the microbiology of raw water sources relates to or shapes that of water in DWTPs, DWDSs and produced sludge (Choi *et al.*, 2019; Verlicchi & Grillini, 2020; Feng *et al*., 2023). Pande *et al.* (2018) reported that raw water in Kasese District, Uganda, fed to local DWTPs, has high numbers of opportunistic pathogens such as *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Aeromonas* spp., *Mycobacterium* spp., and *Flavobacterium* spp. Maguvu *et al.* (2020) concurred with the previously expressed views that the microbial community in treated water is shaped by that of the source water regardless of the treatment process, and bacterial community variation is linked to raw water source area land uses and water quality. From a study of four DWTPs in South Africa, raw water and distributed treated water, Maguvu *et al.* (2020) demonstrated that while the Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes were the dominant bacterial phyla in the water samples, their proportion and that of other phyla of limited proportion were

detected at varying levels for each plant. As the complexity and nature of organic and inorganic pollutants entering water resources change, so does the microbial population, raising concern over the lesser-known or monitored emerging microbial strains (Muguvu *et al.*, 2020; Gu *et al.*, 2022). At each point in time, it is thus difficult to be certain of the microbial quality of raw water and its implications for DWTPs, DWDSs, and produced sludge. Therefore, evaluating microbial diversity responses to environmental variability is an ongoing area of investigation with implications for drinking water quality (Pollard *et al.*, 2018; Maguvu *et al.*, 2020; Feng *et al*., 2023).

Table 2.2: Major point sources of organic contaminants to surface waters, groundwater, treated waters and sediments (adapted from Luvhimbi *et al*., 2022; Botha *et al*., 2023)

Source	Activity	Examples of contaminants
Industrial (manufacturing and processing industries)	Process effluents from pulp and paper, chemical manufacturers, food processing plants, and petroleum industries	Organochlorine dyes, pharmaceuticals, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrogens (PAHs), polychlorobiphenyl furans, and Polychlorobiphenyl dioxins
Municipal sewerage treatment plants	Sewerage treatment plants that may receive indirect discharges from industrial facilities or businesses	Pharmaceuticals and personal care products, synthetic hormones, detergent degradants (such as alkylphenols and their esters), pesticides, flame retardants, and plasticisers (such as tributyl phosphate and bisphenol A)
Combined sewer overflows	Discharge of untreated water into surface waters, especially during floods	Pesticides, pharmaceuticals, PAHs, greases, and oils containing PCBs
Resource extraction	Petroleum drilling	PAHs
Natural occurring	Rocks, soils, decaying plant and vegetable materials, effects of geological settings, climate, and nutrient loading in catchments	Natural occurring organic matter, humic, and fulvic acids, algal toxins (saxitoxins, anatoxins, microcystins, and cylindrospermopsins) Geosmin and 2-methylisoborneol
Land disposal (Landfills)	Leachate or discharge from septic tanks, landfills, industrial impoundments, and hazardous waste sites	Pharmaceuticals, PAHs, tert-butyl methyl ether, organotins, and a mixture of hazardous chemicals

Table 2.3: Examples of non-point source pollution of raw surface water (adapted from Luvhimbi *et al*., 2022; Botha *et al*., 2023)

Source	Description	Contaminants
Agriculture and forestry	Runoff from all categories of agriculture (crop production, pastures, confined animal feeding operations), vegetable handling, especially washing in polluted surface waters, and irrigation return flows	Agrochemicals such as pesticides, pharmaceuticals such as steroids and growth promoters, sheep dip chemicals, and antibiotics
Storm sewers/urban runoff	Runoff from impervious surfaces, including streets, parking lots, buildings, roofs, and cleaning for urbanisation	Polycyclic aromatic hydrogens (PAHs), pesticides, greases and oils, and pharmaceuticals
Transportation	Roads, railway lines, pipelines	Solvents, greases, and oils, examples from polychlorinated biphenyls (PCBs) and PAHs, such as benzo [a] pyrene and fluoranthene
Atmospheric deposition	Emissions from industrial stacks, municipal incinerators, pesticide applications, and human activities such as combustion and pyrolysis	Priority persistent organic pollutants and persistent organic pollutants, PCBs, dioxins and furans, PAHs, and persistent pesticides

2.4. Sludge treatment process

During water treatment, the processes utilised to produce safe drinking water generate various residual products, consisting of the liquid, solid, semi-solid and gaseous phase by-products (Qrenawi & Rabah, 2023). These residuals depend on the raw water source, chemicals used for treatment, and types of unit operations used. Due to increased environmental concerns, there is considerable pressure on the water authorities to safely treat and dispose of sludge. It is very important to choose a suitable sludge treatment and disposal system that is both economical and technically feasible (Crittenden *et al.*, 2012; Anjithan *et al*., 2016).

Sludge, in particulate gelatinous form, is formed during the processes of chemical coagulation, sedimentation, backwashing of rapid filters, and softening at DWTPs (Anjithan *et al*., 2016). Most of the sludge has common characteristics, including high water content (usually >95% by weight), heavy metals, microorganisms, chlorinated hydrocarbons, and polyelectrolytes dosed during the treatment (Gmurkowska, 2019; Grobelak & Kowalska, 2022). Produced sludge may contain a variety of microorganisms, according to the source and the quality of the source water, the treatment method

being utilised, and the time of treatment during the year. The microorganism's composition includes bacteria (i.e., *E. coli*, *Salmonella* spp., *Pseudomonas aeruginosa*, *Legionella* spp.), viruses and intestinal parasitic cysts (Anjum *et al.*, 2016; Fish & Boxall, 2018; Favere *et al.*, 2020).

The chemical characteristics of sludge are generally related to the chemical content of the source water and the type of chemical coagulants. Sludge contains chemical contents such as hydrated alumina oxides and iron oxides (Anjithan et al., 2016). These chemical components may also include the extensively used coagulants, aluminium and iron hydrous metal. These chemicals play a role in bacterial composition within the sludge content. Therefore, sludge processing and handling have become paramount in the use, handling, and storage of waste that may contain organic and microbial communities (Wei *et al.*, 2018). Directly releasing water treatment sludge into the environment may cause aesthetic impacts, including discolouration or rising turbidity in accepting water bodies (Crittenden *et al.*, 2012; Qrenawi & Rabah, 2023). Produced sludge may also contain microbial communities released into drying beds, impacting the environment through flow and drainage. These microbial communities may contaminate surface water and groundwater, increasing microbial diversity and AMR (Lautenschlager *et al.*, 2014; Xu *et al.*, 2017; Luo *et al.*, 2020).

Sludge treatment is necessary to reduce the moisture content and microbial communities before disposal. According to Favere *et al.* (2020) and Qrenawi and Rabah (2023), this can be achieved by an appropriate treatment method such as thickening, conditioning (chemical, physical), dewatering (non-mechanical, mechanical), solids minimisation, solids recovery and final disposal/landfilling. Some of the produced sludge is further treated, while other DWTPs discharge back into the environment. It is critical to control the microbiome released into the environment and reduce microbial pollution. There is a knowledge gap related to understanding the diversity and dynamics of the microbiome within produced sludge and its handling. Understanding the correlation between water quality parameters and bacterial communities could help trace changes in the microbiome by monitoring the produced sludge. Seasonal changes cause substantial variations in water quality indices. However, few studies have investigated the bacterial community structure in produced sludge (Chen *et al*., 2022; Gu *et al*., 2024).

2.5. The bacterial population within drinking water distribution systems

Microorganisms can adhere to solid surfaces and form biofilms in aquatic environments (Quyen *et al*., 2016; Luo *et al*., 2020). Biofilms are bacterial communities embedded in a polysaccharide matrix, which allows them to resist destruction by antibiotics, environmental stress, biocides and detergents (Li *et al*., 2017a; Huang *et al*., 2021). Bacterial regrowth in the distribution system may result from

the detachment of biofilm bacteria, which increases the risk of infection in humans when the water is consumed. Generally, most water distribution systems are characterised by the presence of biofilms, regardless of purity, the type of pipe material used for distribution, or the presence of a disinfectant. Bacteria in drinking water systems can, therefore, grow in bulk water and as biofilms attached to the walls of pipes (van der Kooij *et al*., 2017; Xu *et al*., 2017; Zhao *et al*., 2020). Moreover, the development of biofilms inside water distribution pipes facilitates the propagation of mixed microbial populations and is considered the main source of planktonic bacteria in water supply systems. This problem is further aggravated by the presence of opportunistic pathogens such as *Pseudomonas, Aeromonas, Klebsiella, Mycobacter, Helicobacter, Salmonella*, *Legionella* spp., and *E. coli* that may increase the health risks associated with the consumption of water from these sources (Mulamattahil *et al*., 2014; Mudau *et al*., 2024).

Mulamattahil *et al*. (2014) studied biofilm formation in surface and drinking water distribution systems in Mafikeng, South Africa. They found that galvanised steel and carbon filters had the highest biofilm density, including faecal coliforms, total coliforms, and *Aeromonas* and *Pseudomonas* spp. (Mulamattahil *et al.,* 2014). These bacteria contribute to the deterioration of the microbial quality of supplied water and are a severe health problem. Quyen *et al.* (2016) describe DWDSs as multi-dimensional, where there are typically four phases within the system that serve as available microenvironments for microbial growth. The four phases are the pipe-wall biofilm (formed on the inner surface of the pipe material), the bulk water (the water phase flowing through), suspended solids (particulate matter passing through the system), and lose deposits (particulate matter settled on the bottom of the pipe) (Snipen *et al.*, 2021; Learbuch *et al*., 2022; Zhang *et al*., 2024a).

Typically, bacteria dominating DWDSs are ultraoligotrophic as they survive in environments with very low substrate concentrations (Vosloo *et al.*, 2018). Studies have concluded that the DWDS microbiome is typically dominated by the phyla Firmicutes and Proteobacteria, specifically the classes Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria (Li *et al.*, 2017a; van der Kooij *et al.*, 2017; Masaka *et al.*, 2021). The proportion in which these bacterial groups exist within the system is highly dependent on multiple factors, such as source water, disinfectant strategy (Hwang *et al.,* 2012), filtration processes (Vosloo *et al.*, 2018; Aziz *et al*., 2024) and pipe materials. Li *et al*. (2024b) described a microbial community study where changes in community composition were directly related to changes in disinfectant type (chlorination and chloramination). They revealed that *Cyanobacteria spp.* and *Methylobacteriaceae, Sphingomonadaceae and Xanthomonadaceae* were the most abundant in chlorinated water, whereas *Methylophilaceae, Methylococcaceae* and *Pseudomonadaceae* were abundant in chlorinated water (Lautenschlager *et al.*, 2014; Potgieter *et al*., 2021; Zhou *et al*., 2024). The study by Bian *et al.* (2021), which was conducted in Yancheng City,

Jiangsu Province, China, concurred that the purification process shapes bacterial communities, while transportation distance and the intersection of DWDSs supplied by different DWTPs have a greater impact on the drinking water microbiome. This further elaborates that there is a need to monitor and control the dissemination of pathogens within drinking water to minimise the risk of human health problems.

2.6. Microbial communities in drinking water treatment sludge

During water treatment, a large amount of sludge is generated as sediments and by-products. The sludge containing heavy metals, microorganisms, chlorinated hydrocarbons, and polyelectrolytes dosed during the treatment can be recovered from sedimentation and filtration backwash tanks and transferred to water recovery for further treatment (Gmurkowska, 2019; Grobelak & Kowalska, 2022). The treated sludge is primarily produced after thickening, conditioning (chemical, physical), dewatering (non-mechanical, mechanical), and solids minimisation processes. These processes are unable to remove microorganisms, which include bacteria (*E. coli*, *P. aeruginosa, Salmonella,* and *Legionella* spp.), viruses, and intestinal parasitic cysts (Fish & Boxall, 2018; Favere *et al.*, 2020; Zhang *et al*., 2025a).

Sludge processing and handling have become paramount in the use, handling, and storage of waste that may contain organic and microbial communities. To reduce the disposal cost, reusing produced sludge is an end-point solution that is believed to be a sustainable and favourable disposal option. Given this reuse option, it becomes pertinent to evaluate the microbial composition of the sludge to identify microbial communities that are of economic interest to ensure the health of humans and crops that depend on the DWTPs' yield (Anjithan *et al.*, 2016; Nieminen, 2020; Yu *et al*., 2024). Released microbial communities may contaminate surface water and groundwater through seepage and runoff, increasing microbial diversity and AMR (Lautenschlager *et al.*, 2014; Xu *et al.*, 2017; Luo *et al.*, 2020). Ullmann *et al.* (2019) examined the ARB in sludge samples from Norwegian DWTPs. The authors identified 20 bacterial genera that were resistant to the aminoglycoside: *Achromobacter, Arcicella, Chitinophaga, Chryseobacterium, Comamonas, Ferruginibacter, Flavihumibacter, Flavobacterium, Granulicella, Heliimonas, Herbaspirillum, Micrococcus, Mucilaginibacter, Mycobacterium, Pannonibacter, Parasediminibacterium, Pedobacter, Phreatobacter, Polaromonas and Reyranella* (Ullmann *et al*., 2019). *Mycobacterium* is known for its pathogenic nature, and the release of sludge into the environment needs extra caution due to the spread of this pathogen (Thom *et al.*, 2022; Yu *et al*., 2024).

2.7. Dynamics of antibiotic-resistant bacteria from natural water sources through the different stages of drinking water treatment plants and distribution systems

Microorganisms face diverse challenges in their environment, including chemical pollutants, competition for food with other macro- and microorganisms, attacks from other organisms, etc. In the quest to survive, microorganisms tend to find coping mechanisms, such as secreting chemical antimicrobial substances called antibiotics, whose identification dates to the time of Alexander Fleming in 1928 (Vaz-Moreira *et al.*, 2017; Fatoba *et al.*, 2021; Mudau *et al*., 2024). Despite the usefulness of these antibiotics, their abuse in the health and agricultural industries has led to the discharge of large amounts of antibiotics through human and livestock faeces into the environment. Antibiotic molecules persist in the discharged environment and promote the emergence of both pathogenic and non-pathogenic ARB through the acquisition of ARGs, which are distributed from one environment to another environment, including natural water sources (Li *et al.*, 2017b; Atnafu *et al.*, 2021; Kalu *et al*., 2024).

2.7.1. Antibiotic-resistant bacteria in natural water sources

Natural water, which serves as a source of water for DWTPs, faces the challenge of increased ARB due to the direct discharge of domestic, industrial, and agricultural wastewater laden with antibiotics (Vaz-Moreira *et al.*, 2017; Fatoba *et al.*, 2021). Also, treated water from WWTPs contains some residues of antibiotics and ARGs that were not properly removed by treatment processes (Naquin *et al.*, 2015; Mudau *et al*., 2024). It can also be noted that several antibiotics can be naturally biodegraded as they have been in contact with environmental microbiota for millions of years, and the degradation can even serve as a food source for several microorganisms (Peterson & Kaur, 2018; Polianciuc *et al*., 2020). By contrast, synthetic antibiotics can be more resistant to degradation in the natural environment (Wang *et al*., 2023; Abolfazli *et al*., 2024). This affects the natural water ecosystem and diversity of microorganisms, including pathogenic and non-pathogenic ARB (Thom *et al*., 2022).

In southeast Louisiana, United States of America, Bergeron *et al.* (2015) reported the abundance of *E. coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae,* and *P. aeruginosa* (pathogenic ARB) in the natural water source. After some months, the release of ARGs into the water was analysed, and *sulI* and *tetA* resistance genes were dominant in the natural water source (Bergeron *et al.*, 2015). This implies that the pathogen bacteria acquire ARGs from the natural water or release them into the water. In another study of the East and Midlands of Ireland, Alawi *et al.* (2024) reported potentially opportunistic ARBs such as *E. cloacae, Acinetobacter baumannii* and *Enterococcus* spp. in private

drinking water supplies. The results showed that these private supplies are reservoirs of multidrug resistance in *Enterococcus casseliflavus, E. cloacae, E. coli, Stenotrophomonas maltophilia,* and *Serratia rubidaea*. Additionally, mobile AMR was identified in three water samples, two of which were carried on incompatibility group F, one on incompatibility group Q, and five on Col-like plasmids. This implies that private drinking water that is not monitored and treated could be a sink and source of antimicrobial-resistant pathogens (Andrade *et al.*, 2023; Alawi *et al*., 2024). This creates a critical challenge for the DWTPs, whose function is to ensure the safety of the drinking water through the total removal of ARB and associated ARGs.

2.7.2. The role of coagulation-flocculation and sedimentation in the removal of antibiotic-resistant genes and antibiotic-resistant bacteria

Coagulation and sedimentation comprise the major factors that contribute to water treatment efficiency. However, in recent years, there has been a paucity of studies on the effect and mechanism of removing the ARGs and ARB by coagulation and sedimentation in DWTPs, as well as the identification of the diversity of ARB present during and after these treatment processes, which may be one of the directions for development in the field (Pande *et al.*, 2018; Thom *et al.*, 2022). Based on the ability of coagulation and sedimentation processes to remove microbial communities, it is believed that the process could influence the removal of ARGs and ARB (Julian *et al.*, 2020; Dayaranthne *et al*., 2021; Masaka *et al.*, 2021). Coagulation, sedimentation and clarification reduce intracellular ARGs by removing ARB through electrostatic attraction (negatively charged viruses and positively charged iron or aluminium oxyhydroxide floc particles) (Zhang *et al.*, 2018; Yu *et al*., 2024). Extracellular ARGs can be directly bound to hydrolysed coagulants settled with flocs and removed through the sludge. In addition, a small proportion of bacteria can be directly inactivated by ferric chloride since the ferric hydrate ions can oxidise intracellular functional enzymes after being absorbed by bacterial cells (Zhang *et al.*, 2022; Li *et al*., 2024b). Therefore, the absolute abatement of the antibiotic resistance caused by coagulation and sedimentation is mainly credited to the condensation and partition of host bacteria, as well as ARGs, and ferric hydrate ions may directly destroy ARB through oxidation. In DWTPs in East China, Hu *et al.* (2019) reported satisfactory removal of *intI1*, *sul1,* and *strA* genes during the conventional flocculation and settlement process. An exception was the *ermC* gene, which was not removed and instead increased greatly. Moreover, the type and dosage of coagulants potentially influence the antibiotic resistance removal efficiency. Li *et al.* (2017a) compared the ARG (*sul1*, *sul2*, *tetO*, *tetW*, *tetQ,* and *intI1*) removal rates of two coagulants (ferric chloride and polyferric chloride) at different concentrations. Using *sul1* as an example, the removal efficiency increased as the concentration of polyferric chloride increased, peaking (2.9 log) at a dose of 12 mg/L. When ferric chloride was used, no

significant correlation between the coagulant dose and *sul1* removal efficiency was observed, and the maximum removal of 2.7 log was obtained at a dose of 18 mg/L (Li *et al.*, 2017a). The different ARG removal efficiencies during coagulation with ferric chloride and polyferric chloride were attributed to the discrepant iron species (Li *et al.*, 2017a; Mungondori *et al.*, 2021; Zhang *et al*., 2025b). However, there is still a knowledge gap regarding the effects of other operating conditions on antibiotic resistance during flocculation (e.g., pH, water temperature, and the addition of coagulant aids); therefore, further mechanistic studies are warranted.

2.7.3. Impact of filtration on antibiotic-resistant genes and antibiotic-resistant bacteria removal in wastewater treatment plants

The removal of ARB during filtration can be credited to the interception or adhesion of the bacteria by or to the filter media (Zhang *et al.*, 2018; Zhang *et al.*, 2021). Qian *et al*. (2022) observed a removal efficiency of *E. coli* of 96.1% at a sand filter with a higher effective medium depth (50 cm). It can be inferred that the filter is equipped to entrap a portion of ARB and thus reduce the total resistance. Gu *et al.* (2020) observed Proteobacteria, Acidobacteria, Chloroflexi, and Bacteroidetes alongside the increase in the dissemination of ARGs in the filtration stage of drinking water treatment and suggested that *Pseudomonas* could facilitate the increase in ARGs and the abundance of pathogenic ARB in the filtration stage of treatment. Furthermore, Wan *et al.* (2021) observed a high relative abundance of the genera *Bryobacter*, *Pedomicrobium*, *Reyranella,* and *Terrimonas* spp. after BAC filters. Other operating parameters (filtering velocity, column height, and backwash cycle) may also influence ARG removal during the filtration process (Gao *et al*., 2022). The presence of these pathogenic ARBs during and after the filtration stages indicates the need for future studies to be geared towards advanced optimisation of the process and the materials employed.

2.7.4. Chlorine disinfection and antibiotic-resistant genes and antibiotic-resistant bacteria removal

Chlorine is advantageous for the inactivation of bacteria because of its strong oxidising power in neutral and acidic solutions, which enables it to destroy cell membranes and affect multiple enzyme systems, releasing intracellular materials (e.g., proteins and nucleic acids). Accordingly, the removal of ARB and partial ARGs can be expected in response to chlorine treatment. Variations in ARB diversity and abundance in the disinfection stage are inevitable because of the difference in the type of oxidants (disinfectant) employed by DWTPs globally (Li *et al.*, 2017a; Hu *et al.*, 2019; Gao *et al.*, 2022; Tiwari *et al*., 2022). The bactericidal action of these oxidants could create an environment

for the release of ARGs from lysed bacterial cells, which could be transferred horizontally within different species or vertically from the same species, leading to an increase in the diversity of ARB.

However, limited attenuation or even proliferative effects of several ARGs (e.g., *sul*, *tet,* and *erm*), as well as mobile genetic elements, are generally identified for chlorination in full-scale DWTPs, and varying results have been observed (Gao *et al.*, 2022). For example, an approximately 2.0 log reduction and 1.5 log amplification of *intI1* were identified (reported as absolute abundance) after chlorination during conventional and advanced treatment (ozone-BAC) processes, respectively, even though the chlorine dosages were comparable (Hu *et al.*, 2019). The abnormal increase in ARB ratios and ARG relative abundance after chlorination (at lower concentrations) indicates that the remaining microbes (which are mostly chlorine-tolerant, e.g., *Pseudomonas* and *Acidovorax* spp.) are highly equipped with ARGs (Xu *et al.*, 2016). Recently, Huo *et al.* (2019) also observed increased proliferation of *P. aeruginosa* in the disinfection stage.

2.7.5. Antibiotic-resistant genes and antibiotic-resistant bacteria potential in drinking water treatment plant-produced sludge

DWTP sludge is a by-product of the water treatment process and is normally taken for further treatment before disposal. Treated sludge is a waste that could be an extra revenue source in other beneficial applications, especially in the agriculture and brick-laying industries. In general, DWTS has different characteristics and properties, depending on the raw water conditions, dosage of chemical products, and technology used in the treatment (Achon *et al.*, 2013). In Brazil, Leite *et al.* (2024) reported that DWTS is classified as solid waste and commonly classified as Class II A waste (non-inert and non-hazardous), so the release into surface waters and soil is prohibited. The sludge must be treated and disposed of within the legal criteria established in the country, but the steps to treat this sludge usually involve high costs (Erbiai *et al*., 2024). To save the cost of disposal, the reuse of produced sludge is an end-point solution that is believed to be a sustainable and favoured disposal option. Given this reuse option, it becomes pertinent to evaluate the microbial composition of the sludge to identify microbial communities and ARB that are of economic interest to ensure the health of crops and humans who depend on the yield from the plants. Bezuidenhout *et al.* (2019) studied the implications for drinking water production and quality monitoring at different DWTPs within the North West province, South Africa. The authors identified 15 bacterial genera that were resistant to ciprofloxacin: *A. veronii*, *Bacillus myciodes*, *Deefgea rivuli*, *Massilia aurea*, *Rheinheimera chironomi*, *Flavobacterium*, *Granulicella*, *Heliimonas*, *Herbaspirillum*, *Micrococcus*, *Mucilaginibacter*, P*arasediminibacterium*, *Pseudomonas,* and *Phreatobacter* spp. *Pseudomonas* spp. are known for their pathogenic nature, and releasing the sludge into the environment needs extra

caution due to the spread of this pathogen (Bezuidenhout *et al.,* 2019). Other recent studies have focused on identifying ARB from sludge in WWTPs (Li *et al.*, 2019; Miller *et al.*, 2022; Li *et al*., 2024b). However, there are limited studies on the abundance of ARB in the sludge produced in DWTPs. Hence, more studies are required to provide more insight into the changes in the abundance of ARB in drinking water treatment sludges.

2.8. Contributing factors to the spread of microbial communities and antibiotic-resistant bacteria along the distribution system

Drinking water treatment is the last resort to reduce the impact of antibiotic resistance on human health, and the remaining ARB and ARGs in the final water can be re-enriched in the distribution system and contaminate the tap water (Hu *et al.*, 2019). However, antibiotic resistance has not been listed as a target pollutant by DWTPs, and no maximum levels of ARB and ARGs have been set by SANS 241 to date (Potgieter *et al.*, 2018; Sevillano *et al.*, 2020).

2.8.1. Chlorination

Chlorination disinfection aims to eliminate, deactivate, or kill pathogenic microorganisms. Its application and other forms of disinfectants are intended for the complete removal of opportunistic microbial species and ARB (Bezuidenhout *et al.*, 2019; Gao *et al.*, 2022). Chlorine application causes the release of ARGs, creating an environment for horizontal gene transfer where the ARGs could be transferred into pathogenic and non-pathogenic bacterial communities that are resistant to chlorine toxicity and other applied disinfectants through increased cell permeability (Adefisoye & Olaniran, 2022; Kalu *et al*., 2024). Xu *et al*. (2016) and Bai *et al.* (2022b) found that chlorine and chloramine disinfection, and even the use of a BAC filtration system, increased the incidence of ARB and ARGs, which could be detected in consumers' tap water. This further demonstrated that applying chlorine could increase the relative abundance of ARB in the treated water. Shi *et al.* (2013) observed an abundance of potential plasmids and mobile genetic elements, such as insertion sequences, in water treated with chlorine. Hence, chlorination enhances ARG enrichment in the environment. In support of this, other studies have shown that chlorination promotes ARG release and may not be efficient in demobilising mobile genetic elements and the associated ARGs, leading to an abundance of ARB in the final treated water (Horn *et al.*, 2016; Ghernaout, 2020; Kalita *et al*., 2024). In the North West province, South Africa, Mulamattathil (2014) demonstrated that among the bacteria in bulk water and biofilms, pathogenic *Pseudomonas* and *Aeromonas* spp. were present in DWDSs. The formation of disinfection by-products due to the interaction between the disinfectant and organic materials in the source water is another challenging problem emanating from the use of chlorine and chlorine

additives, which could cause the spread of microbial communities and ARB in the distribution system (Calero Preciado *et al*., 2021; Mudau *et al*., 2024). Lv *et al.* (2014) identified dibromoacetic acid, dichloroacetonitrile, potassium bromates, and 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone as disinfectant by-products that co-select antibiotic resistance in bacteria.

2.8.2. Suspended particles

Suspended particles serve as another conveying vehicle for these microbial communities and ARB in DWDSs. Their presence in drinking water affects the quality because of water discolouration and changes in taste. In addition to this problem caused by suspended particles, LeChevallier *et al.* (2024) found a considerable number of bacteria associated with loose deposits, including *Mycobacteria* spp. during water treatment and distribution. In the Northwest province, South Africa, Carstens & Schmidt (2014) reported that many HPC bacteria isolated from groundwater were resistant to amoeba species. Such amoeba feed on bacteria by phagocytosis; thus, the bacteria developed certain resistance mechanisms to survive, including the resistance to microbicidal effectors in the phagocytes, the ability to replicate in the intracellular environment, or the secretion of toxins that kill the amoebae. The phagocytosis mechanism of amoeba is like that of human macrophages. Thus, amoeba-resistant bacteria may have an increased ability to resist phagocytosis by macrophages in the human immune system (Samba-Louaka, 2021; Bajgar & Krejčová, 2023). These factors may contribute to the pathogenicity of ARB in humans.

In DWDSs, the main sources of suspended particles can be traced to detachment from pipe-wall biofilms and corrosion, suspended solids, loose deposits, and bio-aggregation (Chen *et al*., 2020; Mahajna *et al*., 2022; Kalu *et al*., 2024). Microbial communities that attach to the suspended particles create a serious human health challenge, given the possibility of them reaching drinking water taps through the distribution system. These communities use substances in suspended particles as energy sources and hibernate as they increase their diversity, abundance and richness (Guo *et al.*, 2022). This implies that there is a high probability of increased genetic materials coding for antibiotic resistance in the bacteria attached to the suspended particles due to the high proximity of the cells, leading to the formation of ARB via vertical and horizontal gene transfer (Carstens & Schmidt, 2014; Horn *et al.*, 2016). Shariati *et al.* (2022) found that the introduction of antibiotics, such as ciprofloxacin, sulfadiazine and ciprofloxacin, promotes the abundance of ARGs (*qnrA, qnrB*) in bacteria attached to the suspended particles after chlorination. They further reported an increase in EPSs in the presence of antibiotics. EPSs provide a protective barrier for bacteria against disinfectants. They enhance the aggregation of microbes and promote their attachment to suspended particles (Wang *et al.*, 2018; Shariati *et al.*, 2022; Zhang *et al*., 2024a). Understanding the impact of EPSs on the adsorption of

pathogenic ARB to the suspended particles, which is influenced by the antibiotics present in the distribution system, sheds light on the risks of human exposure due to the increased mobility of the pathogens to the end users. Hence, a lasting solution to the formation of these suspended particles is necessary. Kaur & Dey (2023) reported that EPSs can be secreted by various bacterial genera, assisting bacterial cells in attachment, environmental adaptation and stress tolerance, and they are an integral part of microbial biofilms. In this context, constant monitoring of drinking water treatment and distribution systems to determine the changes in the structure and diversity of the microbial communities and ARB is essential.

2.8.3. Loose deposits

The texture of the biofilm and pipe scale is relatively dense; therefore, these components are firmly attached to the pipe wall and are resistant to falling off. However, an unconsolidated layer of loose deposits occurs outside the biofilm and pipe scale and in direct contact with the water. These loose deposits adversely affect water quality, are hard to control and may pose a public health risk to consumers by supporting microbiological growth and ARB (Gao *et al*., 2018; Zhang *et al*., 2022). Loose deposits are primarily and typically composed of volatile solids (up to 65% dry weight) and iron oxides (up to 70% dry weight) and provide a substrate or source of other water contaminants such as metals (Friedman *et al*., 2016) and through the supply of reactive material to potentially accelerate disinfectant residual decay. Steyn *et al*. (2022) sampled four water supply systems in different cities in Canada and found that the principal components of loose deposits were iron corrosion products (38–72%), organics (14–24%) and silicates/aluminates (7–16%).

The composition of different loose deposits in pipework displays substantial differences, which are related to, among others, the water source, front-end processing, pipe material, pH, water hardness and residual chlorine content. Comparing the bacterial community's diversity in piping biofilm, suspended particles, bulk water and loose deposits, found that loose deposits have the most diverse bacterial communities (Liu *et al*., 2014; Zhang *et al*., 2024b). In another study, Phillip (2024) found that the repeated deposit collection a year after cleaning the pipes using pigging had a similar bacterial concentration to the loose deposits after the first cleaning. LeChevallier *et al*. (2024) reported that the most ubiquitous species in the distribution system were *Mycobacterium lentiflavum*, *Mycobacterium tusciane* and *Mycobacterium gordonae*. Zhuang *et al*. (2021) assessed the human risk associated with loose deposits in DWDSs and observed that loose deposits play a critical role in the discolouration of water and show high cytotoxicity to the liver at a concentration greater than 10 mg/L.

2.8.4. Biofilm formation

A residual chlorine level safe for human consumption must be maintained in a DWDS to ensure microbiological safety and water quality (Sharma *et al.*, 2023). The reduction in the residual chlorine and improvement of the pipeline's oligotrophic microenvironment result in biofilm and ARB regrowth (Pinto *et al.*, 2014; Zhao *et al.*, 2020; Hemdan *et al.*, 2021). A biofilm is a community of microorganisms, such as bacteria, capable of living and reproducing as a collective entity known as a colony (Sharma *et al.*, 2023). According to Khatoon *et al.* (2018) and Chen *et al.* (2020), biofilms are living biomass with a sophisticated social structure that researchers are still attempting to decipher. The biofilm structure serves to shield and enable the expansion of the colony (Healy *et al*., 2024). Biofilm attachment begins when free-floating planktonic bacteria encounter any surface. They cling to the surface via bacterial appendages, such as flagella and or pili, or by physical forces (Erdei-Tombor *et al*., 2024).

Prolonged chlorine applications, as well as the presence of antibiotics, trigger an adaptive response of the bacteria, leading to the formation of biofilms, which serve as antibiotic-resistant reservoirs (Siedlecka *et al.*, 2020). The predicted function of a biofilm in DWDSs in altering the microbial structure and functional profiles indicated that many of the microbes within the biofilm were linked to human infectious diseases (Douterelo *et al.*, 2017). Chen *et al.* (2020) investigated the prevalence of ARGs in drinking water and biofilms and correlated them with the microbial community and opportunistic pathogens. They observed a strong correlation between opportunistic bacteria and the relative abundance of ARGs. Their metagenomic results showed an abundance of ARGs in biofilms in the DWDSs under starvation treatment, which promoted the growth of chemolithotrophs within the biofilms (Chen *et al*., 2020). Biofilms create a microenvironment for the proliferation of ARB and promote the exchange of ARGs vertically and horizontally (Siedlecka *et al.*, 2020). Biofilms formed in the pipeline can cause the regrowth of bacteria and ARB, and the extent of the regrowth depends on factors such as temperature, residence time and pipeline materials.

2.8.5. Pipeline materials

The DWDS is part of the water supply system consisting of the pipe network, fittings and complementary buildings, which ensure water distribution to consumers (Aşchilean *et al*., 2017; Stefan *et al*., 2023). The most widely used pipes are plastic made of high-density polyethylene and polyvinyl chloride due to their affordability and flexibility (Deshommes *et al*., 2016). In Europe, 86 drinking waterborne disease outbreaks were reported between 1990 and 2005, of which 19 were caused at the distribution level (Drinking Water Inspectorate, 2020). Despite many distribution

outbreaks caused by external contamination, four outbreaks were attributed to the growth of microorganisms in biofilms, stagnating water and or resuspension during distribution system flushing (Prest *et al*., 2016).

In the study of the distribution system of a major metropolitan city in the northeastern United States of America, Lee *et al*. (2020) reported that cast iron reactors had an abundance of β-proteobacteria, Actinobacteria and α-proteobacteria (24.1, 22.5 and 22.4%, respectively) while in the cement reactors, α-proteobacteria and Actinobacteria were more relatively abundant (36.3 and 35.2%, respectively) than β-proteobacteria (12.8%). The authors concluded that pipe material is associated with differences in diversity, bacterial composition and opportunistic pathogen prevalence in DWDS biofilms. In South Africa, pipes made of unplasticised polyvinyl chloride and polyethylene are mostly used, especially in rural areas (Nirmala & Rajkumar, 2015). Phthalate esters, known as plasticisers, are among the major constituents of these plastic pipes, and they are not bound chemically to the polymers that make up the plastic pipes; hence, they can leach from the pipes and be discharged into the environment (Wang *et al*., 2018). These plasticisers enhance the formation of biofilms and promote chlorine-resistant microbes, which could be pathogenic (Gao *et al*., 2024). Perfluoroalkyl and polyfluoroalkyl substances are synthetic organic compounds detected in drinking water and are known for their water-resistant, stain-resistant, fire-resistant and anti-stick properties (De Luca *et al*., 2022). They have also been reported to increase the potential for bacterial infections in humans through the promotion of a pathogenic bacterial community along the distribution channel (Bulka *et al*., 2023). Yin *et al*. (2023) evaluated the effect of the co-occurrence of phthalate esters and perfluoroalkyl substances on bacterial communities and pathogenic bacteria growth in rural drinking water distribution systems. Their results showed that the co-occurrence of phthalate esters and polyfluoroalkyl substances promoted the growth of potential human pathogenic bacteria such as *Burkholderia mallei*, *Mycobacterium tuberculosis*, *K. pneumoniae*, *Acinetobacter calcoaceticus*, *E coli* and *P. aeruginosa*. A better understanding of the impact of pipe material on biofilms and the formation of a hydrophobic surface on the walls of the distribution pipelines coupled with good chlorine-residue status could be a direction worthy of future studies to ensure the safety of drinking water that gets to the end users.

2.8.6. Age of water

Water age is one significant factor that drastically changes bacterial cell counts and community composition in DWDSs (Ling *et al*., 2018). The age of water can directly affect the chemistry of the water, which could lead to the proliferation of bacterial composition and ARB (Ji *et al*., 2015; LeChevallier *et al*., 2024). Li *et al*. (2017b) reported that pipe diameter deterministically affected two

ecologically relevant physical and chemical processes, namely the dispersal of bacteria from pipe surface biofilms and the decay of disinfectants. A high relative abundance of *Mycobacterium avium* and *P. aeruginosa* was observed with increased age of water in the distribution system due to a decline in residual chlorine (Haig *et al*., 2018; Prest *et al*., 2021; Healy *et al*., 2024). In the United States of America, Falkinham *et al*. (2015) reported that three plumbing-associated opportunistic pathogens, such as *Legionella pneumophila*, *M. avium* and *P. aeruginosa*, are linked to causing approximately 41,000 infections per year, mostly affecting elderly populations and immunocompromised individuals. It is worth noting that water age, disinfectant used, loose deposits, suspended particles and pipe material may not act independently (Huang *et al*., 2021; Wang *et al*., 2022). There is a possibility of synergism between the factors capable of creating a microhabitat within the distribution system that selects the bacteria, especially pathogenic and ARB, that proliferate in the environment (Karbowiak *et al*., 2023; Kalu *et al*., 2024; Zhu *et al*., 2024).

2.9. Need for a microbiome analysis of drinking water

Detecting each pathogenic microorganism in water is technically difficult, time-consuming and expensive and, therefore, not used for routine water testing procedures (Pinto *et al.*, 2014; Bezuidenhout *et al.*, 2019). Instead, indicator organisms are routinely used to assess the microbiological quality of water and provide an easy, rapid and reliable indication of the microbiological quality of water supplies (Pinto *et al.*, 2014; Luvhimbi *et al.*, 2022; Murei *et al*., 2024). Total and faecal coliform indicators demonstrate faecal pollution in the water as an indirect health risk. Culture-based methods, molecular methods (polymerase chain reaction) and high-throughput methods assess the diversity of bacterial communities within DWTPs, DWTS and DWDSs (Pinto *et al.*, 2014; Li *et al.*, 2017b; Bjornson *et al*., 2024). However, these techniques have some limitations, especially when evaluating changes in the microbial diversity of DWTPs, DWTS and DWDSs over time.

Despite the shortcomings of indicator microorganisms, it is better to use a combination of indicator microorganisms to give a more accurate picture of the microbiological quality of water (DWS, 2023; Rand Water, 2023). Generally, every country has guidelines for drinking water, and South Africa uses the SANS 241 guidelines (SANS, 2015; outlined in Table 2.4).

Table 2.1: Microbiological quality requirements for drinking water in South Africa (adopted from SANS, 2015).

Determinants	Unit	Risk	Standard limit
E. coli / faecal coliforms	Count per 100 mL	Acute health	Not detected
Cryptosporidium spp.	Count per 10 L	Acute health	Not detected
Giardia spp.	Count per 10 L	Acute health	Not detected
Total coliforms	Count per 100 mL	Operational	≤10
Heterotrophic plate count	Count per 1 mL	Operational	≤1000
Somatic coliphages	Count per 10 mL	Operational	Not detected

The most used indicator microorganisms include HPCs, total coliform bacteria, faecal coliform bacteria, *E coli* and faecal enterococci, as well as somatic and male-specific F-specific RNA bacteriophages (SANS, 2015; WHO, 2022). Each indicator microorganism and method have advantages and disadvantages, which are discussed in more detail in the following sections.

2.9.1. Heterotrophic Plate Count

Heterotrophic microorganisms or heterotrophs are naturally present in the environment and can be found in soil, sediment, food, water, and human and animal faeces (Douterelo *et al*., 2014; Rygala *et al*., 2020). Broadly defined, heterotrophs include bacteria, yeasts and moulds that require organic carbon for growth (WHO, 2023). Although generally considered harmless, some heterotrophic microorganisms are opportunistic pathogens with virulence factors that could affect the health of consumers with suppressed immune systems (Shen *et al.,* 2019; Bhatt *et al*., 2023). Heterotrophic microorganisms can also survive in biofilms inside water distribution systems, water reservoirs and household storage containers (Vaz-Moreira *et al*., 2017; Wang *et al*., 2017; Murei *et al*., 2024). Therefore, HPCs can also be used to measure the regrowth of organisms that may or may not be a health risk (Murei *et al*., 2023; WHO, 2023).

Generally, the HPC method has not changed over 100 years. The HPC, or total or standard plate count, includes simple culture-based tests intended to recover a wide range of heterotrophic microorganisms from water environments (Diduch *et al*., 2016). It is used to determine the efficacy of treatment processes in reducing bacterial concentrations and continuous monitoring in the distribution system (Herbig *et al.*, 2019; Zhang, 2021). The HPC accesses bacteria that can form colonies on a solid medium at a specific temperature. Enumeration tests for HPCs are simple and inexpensive, giving results within 48 hours to five days, depending on the method, type of media and the incubation temperature used (Shen *et al.,* 2019). The pour plate, membrane filtration or spread

plate methods are used routinely in laboratories, with either Yeast Extract Agar, Plate Count Agar, Tryptone Glucose Agar or R2A Agar and incubation periods either at room temperature (25ºC) for five to seven days or at 35°C to 37°C for 48 hours. The limitation is that it assesses a small volume of water (100 mL) of a sample, and the confirmation of results takes over 48 hours (Luo *et al.*, 2020). Poor water quality may pass through the processes before results are available. However, it is a cheap and straightforward method, and its long history makes it even more accessible when doing bacterial analysis (Zhang, 2021; Pinar-Méndez *et al.*, 2022).

2.9.2. Membrane filtration and multiple tube fermentation

Traditionally, these methods isolate and enumerate coliforms and *E. coli* in water. Different water treatment plants use these methods, and even though the indicator bacteria are known to be problematic in water, most are unculturable. These methods' results may require additional testing and culturing to confirm the presence of specific *E. coli* isolates and coliforms. There are also non-faecal pathogens in drinking water, e.g., *Legionella* and *Mycobacterium* spp., which this method is poor to set up a correlation with coliforms (Carrillo-Gomez *et al.*, 2019; Zhang *et al*., 2021).

2.9.2.1. Total coliforms

Total coliform bacteria are defined as aerobic or facultatively anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose and produce gas at 35°C (Bai *et al*., 2022b; Balkrishna *et al*., 2024). Total coliforms include bacteria of known faecal origin, such as *E. coli*, as well as bacteria that may not be of faecal origin, such as *Klebsiella*, *Citrobacter*, *Serratia* and *Enterobacter* spp., which are found in nutrient-rich water, soil decaying vegetation and drinking water with relatively high levels of nutrients (Douterelo *et al*., 2014; Geissler *et al*., 2024). The recommended test for the enumeration of total coliforms is membrane filtration using mEndo Agar and incubation at 35°C to 37°C for 24 hours to produce colonies with golden green metallic shine (Tambi *et al*., 2023).

In water quality studies, total coliform bacteria are used as a systems indicator, which provides information on the efficiency of water treatment (Bai *et al*., 2022a: Tambi *et al*., 2023). The presence of total coliforms in water samples is, therefore, an indication that opportunistic pathogenic bacteria such as *Klebsiella* and *Enterobacter* spp., which can multiply in water environments, and pathogenic pathogens such as *Salmonella* and *Shigella* spp., *V. cholerae*, *Campylobacter jejuni*, *Campylobacter coli*, *Yersinia enterocolitica* and pathogenic *E. coli* may be present (Prest *et al*., 2021; Geissler *et al*., 2024). These pathogens and opportunistic microorganisms could cause diseases such as gastroenteritis, dysentery, cholera, typhoid fever and salmonellosis in consumers (Li *et al*., 2017a;

Liu *et al*., 2021; Zhang *et al*., 2021). Individuals who suffer from HIV/AIDS-related complications are more at risk of being infected by these microorganisms (Moona *et al*., 2021).

2.9.2.2. Faecal coliforms

Faecal coliforms are facultatively anaerobic, rod-shaped, Gram-negative, non-sporulating bacteria (Mueller & Tainter, 2023; Tambi *et al*., 2023). The faecal coliform group includes other organisms, such as *Klebsiella*, *Enterobacter* and *Citrobacter* spp., which are not exclusively of faecal origin (Roeselers *et al*., 2015; Moona *et al*., 2021). *E. coli* is specifically of faecal origin from birds, humans and other warm-blooded animals (Flemming *et al*., 2014; Murei *et al*., 2024). Faecal coliform bacteria are, therefore, considered a more specific indicator of the presence of faeces (Douterelo *et al*., 2014; Moona *et al*., 2021). The recommended test for the enumeration of faecal coliforms is membrane filtration using mFC Agar and incubation at 44.5°C for 24 hours to produce blue colonies (Tambi *et al*., 2023). Faecal coliforms are generally used to indicate unacceptable microbial water quality and could be used as an indicator in place of *E. coli* (SANS, 2015). Faecal coliforms in a water sample indicate the possible presence of other pathogenic bacteria such as *Salmonella* and *Shigella* spp., pathogenic *E. coli, V. cholerae* and *Klebsiella* and *Campylobacter* spp. associated with waterborne diseases (Li *et al*., 2017a; Prest *et al*., 2021; Geissler *et al*., 2024). Unfortunately, faecal coliform bacteria exhibit species-to-species variations in their respective stability and resistance to disinfection processes, do not distinguish between faeces of human and animal origin, have low survival rates and have been detected in water sources thought to be free of faecal pollution (Douterelo *et al*., 2014; Maiyo *et al*., 2023).

Coliforms may also be detected through enzymatic reactions using ß-D-galactosidase and ß-D-glucuronidase. Here, the water sample can be inoculated using a medium containing specific enzyme substrates that produce a quantifiable colour change in contact with a particular microorganism (Khan *et al.*, 2022). Colilert and the modified version Quanti-Tray are the most widely used enzymatic coliform detection tests. These methods are easy to use, time-efficient and can detect non-culturable coliforms.

2.9.2.3. *E. coli*

Globally, *E. coli* is the preferred indicator of faecal pollution (Perrin *et al*., 2019). It is a Gram-negative bacterium and predominantly an inhabitant of the intestines of warm-blooded animals and humans, which is used to indicate recent faecal pollution of water samples (Fox & Reid-Bayliss, 2014; Thom *et al*., 2022). Confirmation tests for *E. coli* include testing for the presence of the enzyme β-glucuronidase, Gram staining, absence of urease activity, production of acid and gas from lactose

and indole production (Herzog *et al*., 2014; Desvaux *et al*., 2020; Mandomando *et al*., 2020). Commercially available growth media containing the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronidase is used to isolate and identify *E. coli* from water samples (Lo *et al.,* 2015; Perrin *et al*., 2019). *E. coli* hydrolyse the ß-D glucuronidase in the media, which then fluoresces under ultraviolet (UV) light (Jang *et al*., 2017; Lindsey *et al*., 2018; Hahn *et al*., 2019). However, false negative results on this media have been found due to injured cells, lack of expression of the gene that codes for the enzyme β-glucuronidase by the *E. coli* bacterium isolate or non-utilisation of the ß-D glucuronidase reagent in the media by some *E. coli* strains (Erkmen, 2021; Feng et al., 2023; Maiyo *et al*., 2023).

2.9.2.3. Faecal enterococci

Faecal enterococci bacteria belong to the genus *Enterococcus* and include species like *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus durans* and *Enterococcus hirae* (Deurenberg *et al*., 2017; Songe *et al*., 2017). *Enterococcus* is differentiated from *Streptococcus* by its ability to grow in 6.5% sodium chloride at pH 9.6 and 45°C, showing its tolerance for adverse growth conditions (Clermont *et al*., 2019). Faecal enterococci are spherical, Gram-positive bacteria that are highly specific for human and animal faecal pollution (Douterelo *et al*., 2014). Most *Enterococcus* spp. are of faecal origin and are regarded as specific indicators of human faecal pollution, although some species are found in the faeces of animals and in plant material (Douterelo *et al*., 2014; WHO, 2023).

2.9.2.4. *Clostridium perfringens*

Clostridium perfringens is a Gram-positive, sulphite-reducing anaerobic, rod-shaped, spore-forming bacteria normally present in the faeces of humans and warm-blooded animals (Grawbow *et al*., 1981; Paramita *et al*., 2020). However, *C. perfringens* is also found in natural water sources (WHO, 2023). The spores can survive much longer than coliform bacteria and are highly resistant to water disinfection and treatment processes (Clermont *et al*., 2019; Garcia-Prieto *et al*., 2022). *C. perfringens* is, therefore, used as an indicator of faecal pollution to indicate the potential presence of enteric viruses, which may include Enteroviruses, Adenoviruses and Hepatitis viruses, as well as the cysts and oocysts of protozoan parasites such as *Giardia*, *Entamoeba* and *Cryptosporidium* spp. in treated drinking water (Songe *et al*., 2017; Molechan *et al*., 2019; Garcia-Prieto *et al*., 2022). The enumeration test includes membrane filtration using a specific medium (e.g., mCP or Perfringens selective Oxoid Perfringens Agar with supplements) and incubation at 35°C to 37°C for 48 hours in micro-aerophilic conditions to produce black colonies (Matuszewska & Mąka, 2023).

2.9.3. Next-generation sequencing for understanding microbial communities and ecology

2.9.3.1. 16S rRNA profiling

It is essential to fully understand the microbial community dynamics and diversity within a water environment, especially its relative abundance, shaping the growth and antibiotic resistance in the aquatic environment (de Bastiani *et al*., 2024; Kislichkina *et al*., 2025). Recent technological developments such as high-throughput and deep DNA sequencing (next-generation sequencing [NGS]) like 454 pyrosequencing advance the understanding of drinking water microbial ecology (Kawooya *et al*., 2024; Riesco & Trujillo, 2024). NGS targeting the 16S rRNA gene has highlighted the influence of environmental conditions (Botan *et al*., 2024), disinfectant type (Shi *et al*., 2024; Zhang *et al*., 2025b), process operations (Yang *et al*., 2024), hydraulic conditions (Buetas *et al*., 2024), pipe material and distribution system infrastructure (Gao *et al*., 2024) on the bacterial community structure.

The major advantages of NGS platforms are that (i) they avoid the need for laboratory isolation and cultivation, as they allow for the direct amplification of nearly all 16S rRNA genes from environmental samples using the total DNA extracts obtained; (ii) there is no need for vector-based cloning, as they allow for in vitro separation of the 16S rRNA amplicons; and (iii) they allow for the sequencing of multiple samples in parallel, which is faster and more cost-effective (de Bastiani *et al*., 2024). However, the limitation of these methods is that they can only sequence a short region of the 16S rRNA gene, which often does not provide sufficient taxonomic resolution beyond the family level (Zhao *et al*., 2024; Zhang *et al*., 2025b).

2.9.3.2. Metagenomic approaches

Kim *et al*. (2024) described metagenomic approaches as the theoretical collection of genomes or genomic information from members of a given microbial community. They further explained that the metagenomic approach principle is untargeted DNA sequencing from a microbial community (Kim *et al*., 2024). Metagenomics data can be divided into gene-centric or genome-centric approaches (Dai *et al*., 2020; Suttner *et al*., 2020). Within the gene-centric approach, sequencing reads are aligned to a database of microbial genomes to assign reads to closely related genes, pathways or genomes contained in a database. In the genome-centric approach, sequence reads can be newly assembled larger genome fragments that are compared to the nearest genomes. Furthermore, genomic binning of metagenomic data can be applied to investigate individual genomes of unculturable microorganisms from various ecosystems and reconstruct partially complete genomes of dominant community members (Chao *et al.*, 2015; Chen *et al*., 2020). For example, from shotgun DNA

sequencing of biologically active filters in DWTPs, Pinto *et al.* (2014) and Wang *et al.* (2024) discovered a metagenomic bin closely related to the comammox *Nitrospira*-like bacteria, which are capable of complete oxidation of ammonia to nitrate. These methods also have some limitations, with a lack of holistic genome content and organisations, which could hinder the precise understanding of the metabolic pathways in describing the novel taxa and pathways.

2.10. Exposure of humans to contaminated water from drinking water treatment plants and distribution systems

In South Africa, large populations mostly depend on groundwater supply, particularly in rural areas, while urban and suburban residences largely rely on surface water for domestic and drinking purposes (Hubert & Wolkersdorfer, 2015; Edokpayi *et al*., 2018; Rand Water, 2022). As a result, organic contaminants and ARB in water resources might result in significant health risks to human health (Steyn, 2022; Thom *et al*., 2022). The significance of organic contaminants and ARB in drinking water is that contamination is of more concern as most water-associated health problems are connected to their proliferation, especially in the final treated water that gets to the end users (Wang *et al*., 2014; Huang *et al*., 2022). Studies have shown incidences of pathogenic bacterial communities in drinking water with an implication of increased human exposure and considerable health risk (Huang *et al*., 2022; Luvhimbi *et al*., 2022; Munzhelele *et al*., 2024). In addition, due to increased anthropogenic activities and incessant intake of antibiotics, many bacteria communities acquire ARGs and become pathogenic ARB. According to Sevillano *et al*. (2020) and Gao *et al*. (2022), AMR by pathogenic bacteria is common in both treated and untreated drinking water, which creates great concern about the microbial reduction and or eradication role of the different drinking water treatment processes, especially for treated water. Given the occurrence of these bacterial communities and ARB in the drinking water system, the need for constant monitoring of the changes in their abundance and diversity at the different drinking water treatment processes and along the distribution system becomes pertinent.

Tap water is one of the direct routes to human exposure to pathogenic bacteria and pathogenic ARB through consumption, skin exposure or inhalation of aerosols (Al-Abdan *et al*., 2021; Sivamani & Kamaleshwar, 2022; WHO, 2023). Barrantes *et al*. (2022) observed the proliferation of faecal coliforms and *E. coli* in water obtained from various distribution systems that supply water in Costa Rica. Some of these bacteria could be pathogenic. The implication is that drinking water laden with pathogenic bacteria is a serious health risk to humans, especially to immunocompromised or immunodeficiency individuals. Costa Rica had diarrhoea outbreaks related to drinking water despite a good drinking water supply in the past years (Barrantes *et al*., 2022).

Chemical compounds, such as carbon tetrachloride, chloroform, ethylene dibromide, dibromochloropropane, 1,1,1-trichloroethane or methylchloroform, tetrachloroethylene (perchloroethylene) and trichloroethylene, are collectively known as disinfection by-products (Collivignarelli *et al.*, 2017; Hull *et al.*, 2017). Gilca *et al*. (2020) reported that controlling and removing emerging disinfection by-products in DWDSs is challenging, especially in vast networks that need secondary disinfection. However, in Shanghai, China, Wei *et al*. (2013) reported higher levels of iodoacetic acid and iodoform in water treatment plants due to the deteriorating water quality. They compared chlorine and chloramine, where chloramines resulted in higher concentrations of iodinated disinfection by-products but reduced the levels of trihalomethanes (Wei *et al*., 2013). The constant use of antibiotics promotes the development of ARB with a greater implication on the health of humans as more pressure is on the DWTPs to remedy the problem. Hence, there is a need to monitor the development of antibiotic resistance in drinking water.

2.11. The gaps in the literature

The current study evaluated microbial community diversity in selected DWTPs, distribution systems and produced sludge to understand the diversity and functions. The literature review shows that contaminants in water sources intended for drinking water occur from natural and anthropogenic origins. Chlorination and chloramination are common disinfection practices at DWTPs used in many countries, including South Africa, the United States of America and China. However, there is a literature gap regarding the biological breakdown process of bacteria within the DWDS after dosage of chlorination and chloramination and the residual of such bacteria. For example, do they form part and parcel of corrosion and biofilm growth, and if their virulence factors do not enhance the antimicrobial bacteria? This needs to be covered and identified, especially with municipal water expected to be microbially safe to drink. Also, in terms of evaluating microbial community diversity and functions within the DWTPs stages, DWDS and DWTS using a “DNA-spiking” approach, there is a lack of information regarding such comparisons. This can assist in detecting the abundance of different microbial organisms in low DNA volume, predicting protein gene coding to microbial genomes, and the evolution of microbial communities within different concentration environments.

CHAPTER 3: RESEARCH METHODOLOGY

3.1. Research design

This research is based on a quantitative experimental research design. According to Khanday and Khanam (2019), experimental research design is used to establish a relationship between the cause and effect of a situation. It is a causal research design where the effect caused by the independent variable on the dependent variable is observed (Creswell & Creswell, 2017; Khanday & Khanam, 2019). Water samples were collected to assess the water quality, microbial diversity, and functional metagenomes of DWTPs, DWDSs and produced sludge in Gauteng, Limpopo and Mpumalanga.

3.2. Description of selected study areas

The present study was undertaken to evaluate the impacts of different stages in five DWTPs in three provinces (Gauteng [A, D and E], Limpopo [B] and Mpumalanga [C]) in South Africa. DWTP names are coded for confidentiality purposes. The chosen study sites represent a large population in South Africa, especially in urban and rural areas where they provide drinking water.

3.2.1. Brief description of the study areas

3.2.1.1. Gauteng

Three DWTPs were chosen and coded as A (26°68'72" S, 27°90'92" E), D (25°34'34.22" S, 28°19'40.66" E) and E (25°40'30.81" S, 28°31'45.78" E) (Figure 3.1). The province's primary water sources are the Vaal River and Crocodile River catchments; many anthropogenic activities occur along the rivers. The treated water is purified for human consumption and pumped to consumers at the furthest distance in Pretoria (DWS, 2018; Rand Water, 2022).

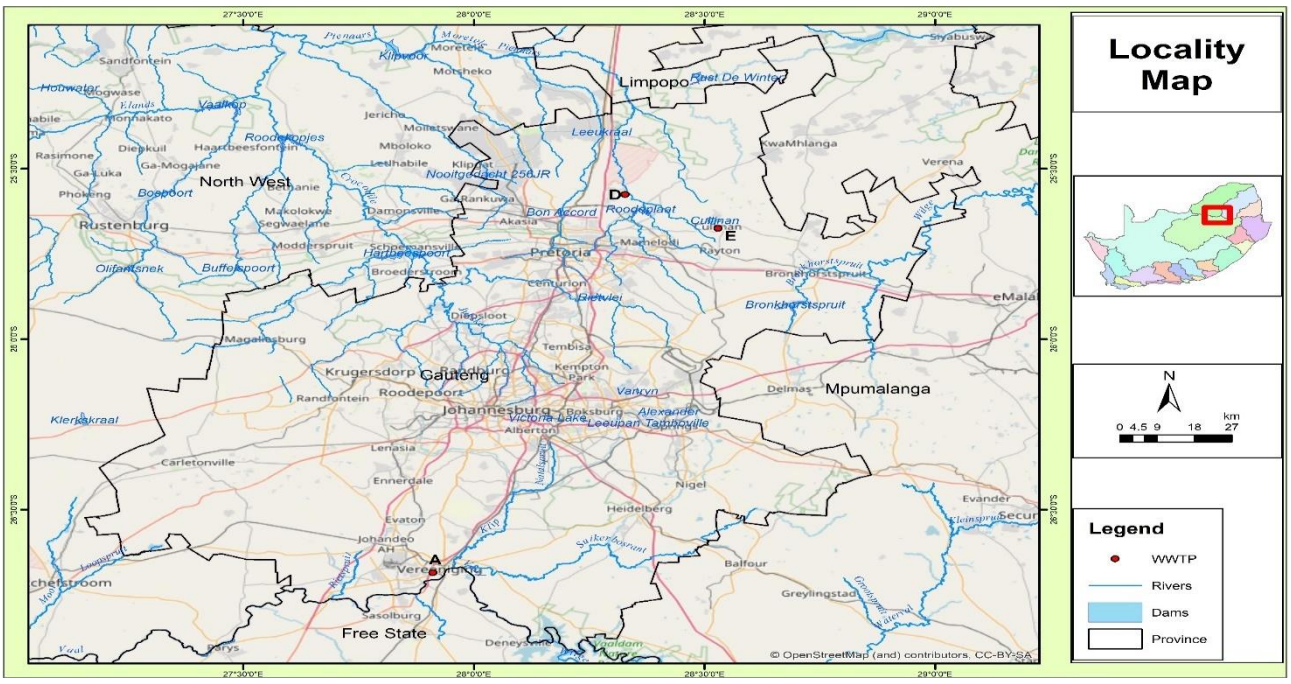


Figure 3.1: The study area of Gauteng water treatment plant Sites A, D and E

Table 3.1: Characteristics of three drinking water treatment plants in Gauteng (Sites A, D and E)

	Site A	**Site D**	**Site E**
System	Conventional treatment process	Conventional treatment process	Conventional treatment process
Maximum design capacity	360 Megalitre/day	18 Megalitre/day	16 Megalitre/day
Current utilisation	360 Megalitre/day	12 Megalitre/day	16 Megalitre/day
Raw water supply capacity	2 609 799 000 cm^3	43 472 000 m^3	57 913 000 m^3
Pre-oxidisation type	n/a	Chlorine	n/a
Primary water pre-treatment chemical	Slaked lime: between 55 and 70 mg/l as calcium oxide. Silica: between 1 and 3 mg/l as silicon dioxide Ferric chloride: between 1 and 5 mg/l as ferric chloride	Lime, powdered activated carbon, ferric chloride, and polyamide	Lime, powdered activated carbon, ferric chloride, and polyamide
Total coagulant dosing capacity	n/a	n/a	n/a
Rapid mixing method	Hydraulic jump	n/a	Hydraulic jump
Clarifier type	Rectangular	Circular and rectangular	Circular
Number of clarifiers	4	1	4
Total area of clarifiers	n/a	n/a	n/a
Total capacity of clarifiers	2 500 Megalitre/day	2 | 4	n/a
Filter type	Rapid sand filter	Rapid sand filter	Rapid sand
Number of filters	40	4	8
Filter floor type	Lateral without nozzles and scrapper on top	Lateral without nozzles and scrapper on top	Lateral without nozzles and scrapper on top
Total filtration area of all filters	1000 Megalitre/ day	n/a	n/a
Total capacity of backwash tanks	n/a	n/a	n/a
Total capacity of sludge treatment plant	n/a	n/a	n/a
Capacity of used wash water system	n/a	n/a	n/a
Primary post-disinfection type	Chlorine	Chlorine	Chlorine
Disinfection dosing capacity	± 500 kilogram/day	n/a	n/a
Disinfection storage capacity	± 6 tons	n/a	n/a
Total treated water storage	500 to 800 tons of dry sludge are produced each day	32 Megalitre/day	10 Megalitre/day

3.2.1.2. Mpumalanga

The chosen DWTP in Mpumalanga was Site C (25°52'49" S and 29°14'4" E) (Figure 3.2). The primary water source is the Upper Olifants River catchment; many anthropogenic activities occur along the river (Maya *et al*., 2015; Pollard *et al.*, 2018). The treated water is purified for human consumption and pumped to different consumers at the furthest distance in Ogies/Phola (Golder Associates, 2010; Maya *et al.*, 2015).

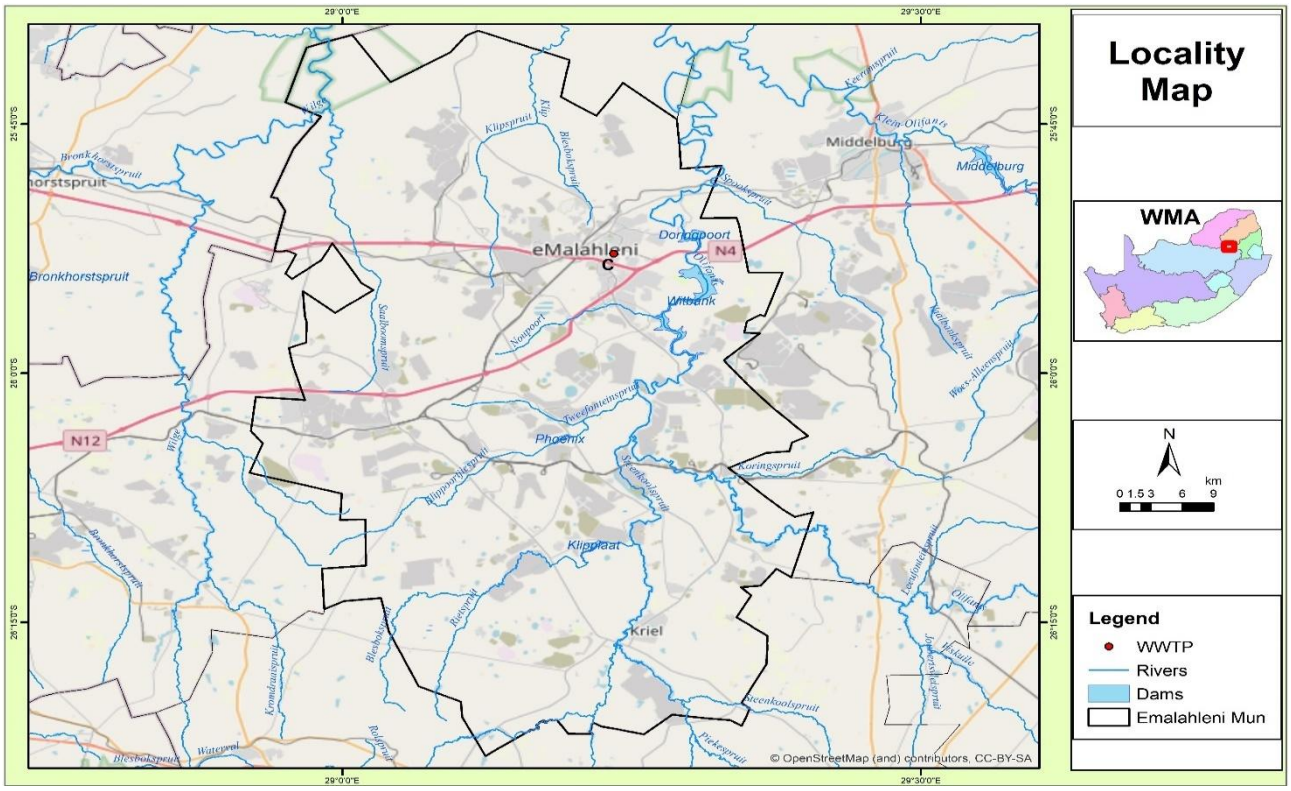


Figure 3.2: Map showing the Mpumalanga water treatment plant Site C

Table 3.2: Characteristics of the drinking water treatment plant in Mpumalanga (Site C)

	Site C	
System	Conventional treatment process	
Maximum design capacity	75 Megalitre/day	
Current utilisation	105 Megalitre/day	
Raw water supply capacity	104 019 000 m^3	
Pre-oxidisation type	n/a	
Primary water pre-treatment chemical	Lime Polyelectrolyte	
Total coagulant dosing capacity	n/a	
Rapid mixing method	Flash mixing	
Clarifier type	Candies	Circular
Number of clarifiers	6	8
Total area of clarifiers	n/a	n/a
Total capacity of clarifiers	75 Megalitre/day	30 Megalitre/day
Filter type	Constant rate rapid gravity filters	
Number of filters	30	
Filter floor type	Lateral without nozzles	
Total filtration area of all filters	n/a	
Total capacity of backwash tanks	n/a	
Total capacity of sludge treatment plant	n/a	
Capacity of used wash water system	1 Megalitre/day	
Primary post-disinfection type	Chlorine	
Disinfection dosing capacity	±500 kg/day	
Disinfection storage capacity	± 5 tons	
Total treated water storage	188 Megalitre/day	

3.2.1.3. Limpopo

The chosen DWTP in Limpopo was Site B (22°58'21" S and 30°35'58.998" E) (Figure 3.3). The primary water source is the Luvuvhu River catchment; many anthropogenic activities such as urbanisation, agriculture and industries occur along the river. WWTPs are also located within this catchment (Pollard *et al.*, 2018; Edokpayi *et al*., 2018). The treated water is purified for human consumption and pumped to different consumers at the furthest distance is Malamulele (Musyoki *et al.,* 2016). The DWTP is currently treating 30 ML/d and has a vast distribution system supplying water to different communities within the area (Table 3.5). The selected distribution system network for the project led to Mavambe Reservoir and the final tap.

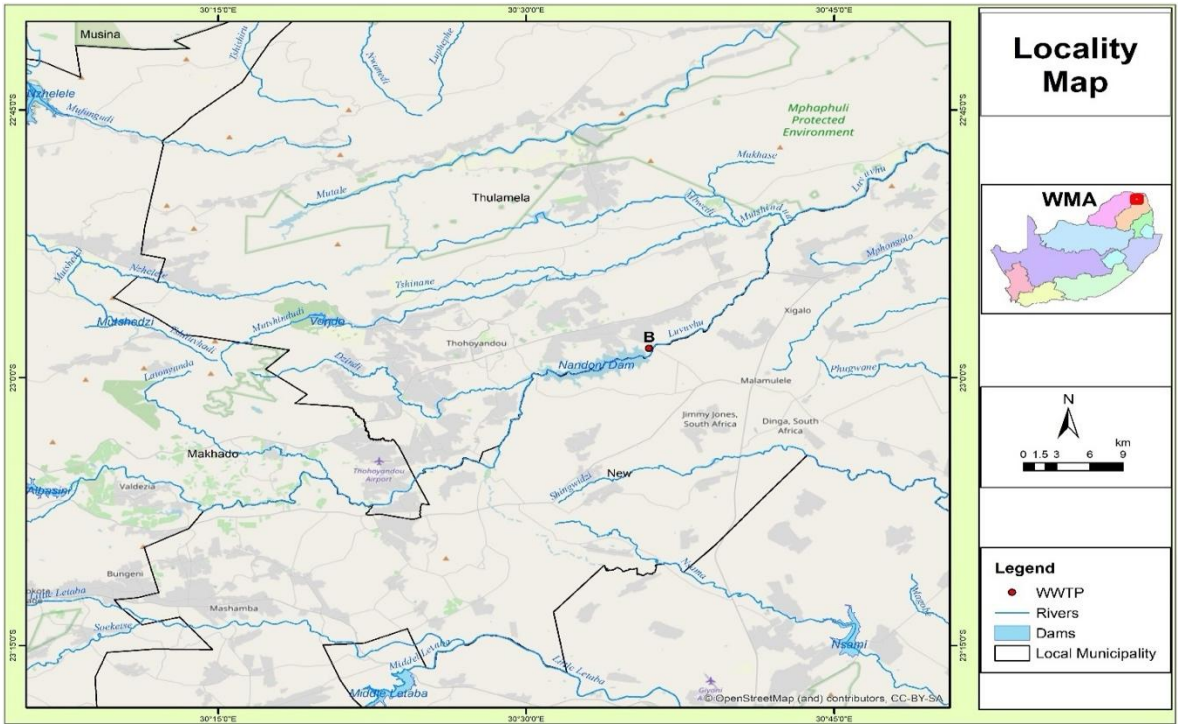


Figure 3.3: Map of Site B water treatment plant in Limpopo Province

Table 3.3: Characteristics of the drinking water treatment plant in Limpopo (Site B)

	Site B
System	Conventional treatment process
Maximum design capacity	30 Megalitre/day
Current utilisation	30 Megalitre/day
Raw water supply capacity	n/a
Pre-oxidisation type	None
Primary water pre-treatment chemical	Ferric chloride: between 1 and 5 mg/l as ferric chloride
Total coagulant dosing capacity	n/a
Rapid mixing method	Hydraulic jump
Clarifier type	Rectangular
Number of clarifiers	8
Total area of clarifiers	n/a
Total capacity of clarifiers	n/a
Filter type	Sand filter
Number of filters	8
Filter floor type	Lateral without nozzles and scrapper on top
Total filtration area of all filters	n/a
Total capacity of backwash tanks	n/a
Total capacity of sludge treatment plant	1 300 tons
Capacity of used wash water system	n/a
Primary post-disinfection type	Chlorine
Disinfection dosing capacity	±500 kilogram/day
Disinfection storage capacity	± 6 tons
Total treated water storage	30 Megalitre/day

3.3. Methodology

3.3.1. Sampling procedure

Replicate samples were taken from raw water sources and treatment stages as shown in Table 3.4, and final treated water during spring, summer, winter, and autumn (ISO 5667-3:2024). Before sampling, approvals were obtained from the plant authorities and the ethical committee of the University of South Africa. Sterile 1000 mL bottles were used to collect water samples and transported at 4°C to the laboratory for analysis within three to six hours after collection.

Table 3.4: Treatment stages employed at five drinking water treatment plants (Sites A, B, C, D and E)

A	B	C	D	E
Raw water source	Raw water source	Raw water source	Raw water source	Raw water source
n/a	n/a	n/a	Pre-chlorination	n/a
Coagulation/ Flocculation	Coagulation/ Flocculation	Coagulation/ Flocculation	Coagulation/ Flocculation	Coagulation/ Flocculation
Sedimentation	Sedimentation	Sedimentation	Sedimentation	Sedimentation
Filtration	Filtration	Filtration	Filtration	Filtration
Final drinking water	Final drinking water	Final drinking water	Final drinking water	Final drinking water
Disinfection	Chlorine	Chlorine	Chlorine	Chlorine
Distribution	Distribution	Distribution	Distribution	Distribution

3.3.2. Physicochemical parameters

Samples for physicochemical parameters and microbiological analysis were collected in clean, sterile, labelled 1 L polypropylene bottles. The sampling bottles were first rinsed by exposing them to 1.8 % per volume of sodium thiosulphate pentahydrate solution and sterilised before sampling (American Public Health Association, 2017; ISO, 2024). At each sampling point, the sampling bottle was filled with sample to the brim and closed to avoid contamination. The bottles were sealed, placed in a cooler box filled with ice packs and transported to the laboratory. At each site, a 1L volume of sample water from each selected sampling point.

Only the first consumer point was sampled in the distribution system. The wet sludge samples were taken after sedimentation before being treated or wasted. Sampling was done quarterly for one year, representing all four seasons following the WHO protocol (WHO, 2024). Table 3.5 provides the analysis parameters. The mean values were recorded against the SANS 241 (2015) and WHO (2022) drinking water quality guidelines.

Table 3.5: Parameters analysed for the study and the limits

Physical parameters	**Units**	**Risk**	**South African National Standard limits (SANS, 2015)**	**World Health Organization (WHO, 2022)**
pH (at 25°C)	pH Unit	Operational	≥5 to ≤9.7	6.5 to 8.5
Conductivity (at 25°C)	mS/m	Aesthetic	≤170	*
Total dissolved solids	mg/L	Aesthetic	≤1200	1000
Free chlorine	mg/L	Chronic health	≤5	5
Turbidity	NTU	Operational	≤1	0.5
		Aesthetic	≤5	5
Chemical parameters				
Aluminium	mg/L	Operational	≤300	0.1 to 0.2
Ammonia	mg/L	Aesthetic	≤1.5	*
Calcium	mg/L	Aesthetic/Operational	150	
Chloride	mg/L	Aesthetic	≤300	*
Colour (platinum cobalt solution)	mg/L	Aesthetic	≤15	≤15
Copper	mg/L	Chronic health	≤2	2
Fluoride	mg/L	Chronic health	≤1.5	1.5
Iron	mg/L	Chronic health	≤2	*
		Aesthetic	≤0.3	0.3
Lead	mg/L	Chronic health	≤0.01	0.01
Magnesium	mg/L	Aesthetic/Health	70	
Manganese	mg/L	Chronic health	≤0.4	80
		Aesthetic	≤0.1	0.02
Nitrate	mg/L	Acute health	≤11	50
Nitrite	mg/L	Acute health	≤0.9	3
Sulphate	mg/L	Acute health	≤500	*
		Aesthetic	≤250	250
Sodium	mg/L	Aesthetic	≤200	200
Total Organic Carbon	mg/L	Chronic health	≤10	*
Zinc	mg/L	Aesthetic	≤5	5
Microbiological parameters				
E. coli	Count per 100 mL	Acute health	*	Count per 100 mL
Enterococcus species	Count per 100 mL	Acute health	*	Count per 100 mL
Total coliform	Count per 100 mL	Operational	<10	Count per 100 mL

NTU: Nephelometric Turbidity Unit; *Guideline values have not been established

The following physical parameters were measured in situ: temperature, pH, conductivity and free chlorine. These determinants were measured using an H1006 spectrophotometer fitted with the appropriate probes (Hach, South Africa). The data were recorded on field datasheets. In establishing the physicochemical quality of water sampled from selected DWTPs, DWDSs and sludge, turbidity, nitrates, total organic carbon (TOC), sulphate, ammonia, and total alkalinity followed the standard methods (American Public Health Association, 2017).

Levels of iron and manganese were determined using Inductively Coupled Plasma-Optical Emission Spectrometry (Agilent Technologies Incorporated, USA). Before analysis, each sample was filtered using a 0.45 µm pore membrane to eliminate any particles and sediments after acid microwave digestion, as described by Sekhohola-Dlamini *et al*. (2020).

3.3.3. Microbiological analysis (Analysis of *E. coli* and *Enterococcus* species per site)

3.3.3.1. Total coliforms, *E. coli* and *Enterococcus* species

The IDEXX-defined substrate Colilert-18/Quanti-Tray 2000 System (ISO 9308-2:2012) and Enterolert-DW reagents were used to identify and enumerate *E. coli* and *Enterococcus* spp. as indicator organisms (IDEXX Laboratories (Pty) Ltd., Johannesburg, South Africa) (Fatoba *et al*., 2021). Before analysis, all equipment was decontaminated using UV radiation. A sachet of the reagent powder (Colilert-18 for *E. coli* and Enterolert-DW for *Enterococcus* spp.) was added to a 100 mL water sample and incubated for 18 hours in the case of presence/absence determination. Pure isolates were obtained on chromogenic media (eosin methylene blue and ChromAgar™ *Enterococcus* for *E. coli* and *Enterococcus* spp., respectively). Total coliform counts (IDEXX MPN/100 mL) were determined from the Colilert trays based on yellow colouration, which indicates the presence of total coliforms. The yellow positive wells were viewed under long-wavelength (366 nm) UV light to identify *E. coli*. The yellow that exhibited blue fluorescence was recorded as positive for *E. coli.* For *Enterococcus* spp., the Quanti-Tray wells were viewed under UV light, and the positive wells with yellow fluorescence were counted and recorded. All the results for total coliforms, *E. coli* and *Enterococcus* spp. are calculated from the IDEXX MPN/100 mL table, a surrogate for colony-forming units.

3.4.3.2. Recovery of isolates and confirmation test

After incubation and counting, the back of the tray was disinfected using 70% ethanol with a sterile swab. A sterile razor blade was used to pierce the sterile back of fluorescence-positive wells per tray. Three trays were sampled per water sample, and a loop full of well content was streaked on *E. coli* (Eosin methylene blue agar) and *Enterococcus* spp. (ChromAgar™ *Enterococcus*) selective media.

The colonies were scraped from the top of the agar with a sterile loop and used for a confirmation test. Gas and indole production in lactose tryptose lauryl sulphate broth and growth in brain heart infusion broth were used to confirm *E. coli* and *Enterococcus* spp., respectively (American Public Health Association, 2017). The confirmed *E. coli* and *Enterococcus* spp. were stored in 20% (v/v) glycerol at −80°C for further analyses.

3.3.4. Antibiotic susceptibility testing for *E. coli* and *Enterococcus* species

Each *E. coli* and *Enterococcu*s spp. isolate was subjected to antibiotic susceptibility testing. The Kirby-Bauer disc-diffusion method set by the Clinical and Laboratory Standards Institute (CLSI, 2016) was used. Using a sterile toothpick, a single colony of freshly grown *E. coli* or *Enterococcus* spp. culture was placed on a nutrient agar plate. The plates were incubated overnight at 37°C. All colonies obtained were placed on a Mueller Hinton agar replica plate containing the appropriate antibiotics (CLSI, 2007, 2014, 2016). The susceptibility of the *E. coli* and *Enterococcus* spp. isolates were tested against ten or more antibiotics: ampicillin (10 µg), erythromycin (15 µg), ciprofloxacin (5 µg), nitrofurantoin (300 µg), amoxicillin/clavulanate (30 µg), gentamicin (10 µg), imipenem (10 µg), trimethoprim/sulfamethoxazole (25 µg), levofloxacin (5 µg) and vancomycin (30 µg), as summarised in Table 3.6. The antibiotics were selected based on the CLSI (2007, 2014, 2016), as well as their frequent use in veterinary and human medicine. The Kirby-Bauer disc-diffusion technique was used, and the inhibition zone diameter measured was interpreted using the CLSI breakpoint values (2007, 2014, 2016). When resistance to at least three antimicrobial agent families was detected, the isolates were classified as multidrug-resistant (MDR). The *E. coli* strain (ATCC 25922) and *E. faecalis* (ATCC 29212) were used as controls according to CLSI control standards.

Table 3.6: Antibiotics and disc concentration used to test susceptibility in *E. coli* and *Enterococcus* spp. strains isolated (Source: CLSI, 2016)

Antibiotic description		**Disc concentration**
Class of antibiotics	**Name of antibiotic**	
Aminoglycosides	Gentamicin	10 µg
ßeta-lactams	Imipenem	10 µg
	Nitrofurantoin	300 µg
	Amoxicillin/clavulanic acid	30 µg
	Ampicillin Penicillin	10 µg
Glycopeptide	Vancomycin	30 µg
Quinolones	Ciprofloxacin	5 µg
	Levofloxacin	5 µg
Macrolides	Erythromycin	15 µg
Sulfonamides	Trimethoprim/sulphamethoxazol	25 µg

3.3.5. Identification of multidrug resistance

Multidrug resistance is defined as resistance to the tested antibiotics in at least two of the following three classes: ßeta-lactam, aminoglycosides and quinolones (Taddese *et al*., 2019; Habboush & Guzman, 2023). The multidrug resistance of the isolates was identified by observing the resistance pattern of the isolates to the antibiotics.

3.3.6. Multidrug resistance-index study

The multiple antibiotic resistance (MAR)-index was calculated as (x/y), where x is the number of antibiotics to which the isolate was resistant, and y is the number of antibiotics to which the isolate was exposed. An isolate from an area of high antibiotic usage demonstrates an MAR-index of >0.2, while one with a value of <0.2 is from a source of lower antibiotic usage.

3.3.7. Molecular analysis for microbial community analysis for all the sites

3.3.7.1. DNA extraction

Water and treated sludge samples collected from different sites were analysed for microbial community diversity. Suitable volumes ranging from 100 mL for raw water to 1 L for final treated water were filtered through 0.22 µm polycarbonate membrane filters for water samples and treated sludge slurry. The polycarbonate filters with collected microbial biomass were cut into small pieces using a sterile scalpel and used for DNA extraction. MN-NucleoSpin Soil 50 preps DNA extraction

kits for the extraction of DNA from soil and sediment (Promolab Pty Ltd T/A Separations, RSA) were used following the manufacturer's instructions (Mhlongo *et al*., 2020). Briefly, the initial lysis step was to transfer 250-500 mg of cut pieces into collection bead tube Type A. +700 µL buffer S1 was added into the beads tube Type A, and to adjust lysis conditions, +150 µL SX was added, and mixture vortexed for five minutes. The supernatant was removed carefully and poured into clean tubes. The SL3 Buffer (150 µL) was added into the tubes and vortexed for 5 seconds to resuspend the remaining fine particles and sediments to the bottom of each tube. All tubes were incubated in the freezers for five minutes at 0 – 4°C. The samples were then filtered through a NucleoSpin® Inhibitor Removal Column (red ring) and supernatant suspended in a buffer of 250 µL SB. 550 µL samples were filtered through the NucleoSpin® Soil Column (green ring) to bind the Genomic DNA and centrifuged for 60 seconds. The filtered samples were discarded, and the column was placed back into the collection tube. The wash and dry silica membrane process was done in four steps, followed by Vautier *et al*. (2020) and Chesneau *et al*. (2025). The extracted DNA of the samples was pooled and quantified using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Scientific).

3.3.7.2. Quantification data and library preparations

The concentration and purity of DNA were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and the quality was checked on a 1.5% agarose gel. A blank of deionized water was processed and considered a negative control, while a sample of *E. coli* strains was used as a positive control. The extracted DNA was sent to the Agricultural Research Council (ARC) Biotechnology Platform for shotgun metagenomic sequencing and had A260:A280 ratios between 1.8–2.0 and DNA concentrations of 20–150 ng/µL. Genomic Shotgun sequencing was performed on an MGI DNBSEQ-G400 sequencing instrument (Agricultural Research Council-Biotechnology Platform). The metagenomic shotgun data library was prepared using MGIEasy Universal DNA Library Prep Set V1.0 (MGI Tech Co., China). The quality control of the library was done using a Qubit® fluorometer (Life Technologies, Carlsbad, CA, USA), with the sequencing depth calculated to achieve five million reads. The DNA nanoball (DNB) was created by combining the pooled and circularized library, which was then loaded into a PE150 flow cell for sequencing in MGI DNBSEQ-G400 (MGI Tech Co., China) (2 x 150 bp).

3.3.7.3. Bioinformatics and visualisation of sequencing data

The raw Fastq files comprising the while genome sequences from the MGI DNBSEQ-G400 sequencer were curated using the WGSA2.2 pipeline implemented in Nephele (v2.2.8) (Weber *et al*., 2018; Angelova *et al*., 2023) using default parameters. Sequences were assigned to operational taxonomic units (OTUs) using a dissimilarity cutoff = 0.03 and classified to representative microbial

taxa against taxonomic classification DB in a Kraken2 version of NCBI's RefSeq DB (Kraken2 Standard Reference Genomes DB; kr2_REFdb) and the Eukaryotic Pathogens DB (EuPATHdb), containing genomes of known eukaryotic pathogens. The alpha diversity indices (Chao1, Shannon, Simpson and ACE) for the microbial community datasets were determined using the plot richness function of the *phyloseq* package described by Ogola *et al*. (2024). Heatmap and stacked bar charts were generated from the dominant OTUs at different taxonomic levels using Clustvis 2.0 (Metsalu & Vilo, 2015) and the Paleontological Statistics Software Package Version 4 (PAST4) (Smith *et al*., 2024), respectively. Paired-wise correlation analysis was done using PAST4 (Smith *et al*., 2024) to show the relationship between the physicochemical parameters and the six dominant bacterial classes to show the impact of the parameters on the proliferation of the bacteria.

The metagenomes data were screened for the presence of the genetic determinants of antibiotic and heavy metal resistance, as well as functional metagenomes, by the publicly available database in the WGSA2.2 pipeline implemented in Nephele (v2.2.8). Stacked bar charts were generated from the identified classes of ARGs and heavy metal-resistant genes using PAST 4 (Hammer *et al*., 2001, Hammer & Harper, 2024). In this study, the raw sequences were deposited in the National Center for Biotechnology Information Sequence Read Archive database as BioProject ID PRJNA1090776.

3.4. Statistical analysis

The water quality data were assessed against national guidelines and standards, i.e., the South African water quality guideline (domestic) and the SANS 241 for drinking water (SANS, 2015). This evaluation aimed to determine how suitable the water is for use. The results from the IDEXX-defined Colilert-18/Quanti-Tray 2000 analysis were measured based on the Most Probable Number (MPN) of *E. coli*, *Enterococcus* spp. and total coliforms listed in the MPN table (IDEXX Quanti-Tray/2000 MPN Table). The percentage of resistant indicator bacteria across different treatment stages and locations was analysed using XLSTAT. A chi-square test was applied to find out if there were any significant differences among the raw water sources, the treatment processes, the final treated water and the microbial communities from various sampling sites (Aslam & Smarandache, 2023).).

CHAPTER 4: RESULTS AND DISCUSSIONS

This chapter presents the results and their relevance to the stated research questions and objectives. The results represent data on the physiochemical, microbial diversity, ARB and MAR, metagenomics, functional genome, correlation of physiochemical and microbial diversity for five DWTPs, from source, coagulation, sedimentation, filtration, final treated water, and first tap of contact and from the treated sludge.

4.1. Analysis of physicochemical parameters

4.1.1. Physicochemical parameters in Gauteng Province sites (Sites A, D and E)

4.1.1.1. Site A

At this site, the physicochemical parameters of the water samples varied according to sampling time and the treatment stages. Table 4.1 shows that pH values ranged from 6.6±0.91 to 7.88±0.1 across all the stages, with the highest measured from the sedimentation stages. In the final treated water of Site A, the pH ranged from 6.6±0.91 to 8.1±0.20, while at the first distribution system point, it ranged from 6.45±0.81 to 7.41±0.27. The pH in the final treated water and distribution system was within the SANS 241 (SANS, 2015) guidelines throughout the monitoring period. Temperature ranged from 18.1±3.2 to 26.7±3.26°C, with the highest temperature occurring during the distribution stage in the summer season (Table 4.2). Conductivity ranged from 29.2±1.67 mS/m to 179.2±28.19 mS/m, with the lowest recorded in the final treated water during winter (29.2±1.67 mS/m) and the highest value recorded (179.2±28.19 mS/m) in the treated sludge during summer (Table 4.3). Turbidity ranged from 0.09±0.02 NTU to 520±98.49 NTU, with the lowest recorded in the distribution stage in autumn (0.09±0.02 NTU) and the highest recorded in the treated sludge (520±98.49 NTU) during summer (Table 4.4). Chlorine in the final treated water ranged from 0.24±0.04 mg/L to 0.33±0.05 mg/L, while the distribution system ranged from 0.015±0.04 mg/L to 0.09±0.05 mg/L (Table 4.5). TOC ranged from 6.76±0.75 mg/L to 23.49±4.02 mg/L, with the lowest recorded in the distribution stage (6.76±0.75 mg/L) during autumn and the highest recorded in the flocculation stage (23.49±4.02 mg/L) during spring (Table 4.6). Total alkalinity ranged from 43.5±2.54 mg/L to 210±10.69 mg/L, with the lowest recorded in the final treated water (43.5±2.54 mg/L) during autumn and the highest recorded in the treated sludge (210±10.69 mg/L) during spring (Table 4.7). Nitrate ranged from 0.15±0.03 mg/L to 60±28.24 mg/L, with the lowest recorded in the distribution stage during summer (0.15±0.03 mg/L) and the highest recorded in the treated sludge during summer (60±28.24 mg/L) (Table 4.8). Sulphate ranged between 1.3±0.32 mg/L and 56±14.50 mg/L, with the lowest recorded in the final treated water (1.3±0.32 mg/L) during autumn (Table 4.9).

Iron ranged between 0.54±1.45 mg/L and 375±37.75 mg/L, with the lowest recorded in the distribution system (0.54±1.45 mg/L) during autumn and the highest recorded in the sludge (375±37.75 mg/L) during winter (Table 4.10). Manganese ranged between 0.92±0.29 mg/L and 55.8±2.01 mg/L, with the lowest recorded in the distribution system (0.92±0.29 mg/L) during autumn and the highest recorded in the sludge (55.8±2.01 mg/L) during summer (Table 4.11). Ammonia ranged between <0.002±0.69 mg/L and 3.8±1.03 mg/L, with the lowest recorded in the final treated water (<0.002±0.69mg/L) during autumn and the highest recorded in the sludge (3.8±1.03 mg/L) during autumn (Table 4.12).

4.2.1.2. Site D

Physicochemical parameters varied with sampling period and treatment stages (Table 4.1 to Table 4.12). The flocculation stage recorded a higher pH value of 8.89±0.74 in winter than the other stages. All the stages ranged in pH values from 7.25±0.35 to 8.89±0.74 across the seasons (Table 4.1). The recorded temperatures ranged from 18.3±9°C to 26.8±2.25°C, reaching their peak in the final treated water during autumn (Table 4.2). Conductivity was 44.8±5.10 mS/m in the flocculation stage during winter. Across the treatment stages, conductivity ranged from 51±7.83 mS/m to 153.1±10.10 mS/m, with the lowest value recorded (51±7.83 mS/m) in the distribution stage during winter and the highest value recorded in the treated sludge during summer (153.1±10.10 mS/m) (Table 4.3). Turbidity ranged between 0.13±0.02 NTU and 4.5±0.74 NTU, with the lowest value recorded in the final treated water in winter (0.13±0.02 NTU) and the highest recorded in the treated sludge in winter (980±269.52 NTU) (Table 4.4). Chlorine in the final treated water ranged between 1.14±0.51 mg/L and 2.19±0.41 mg/L (Table 4.5). Total alkalinity ranged between 82±8.50 mg/L and 1895±42.25 mg/L, with the lowest recorded in the filtration stage (82±8.50 mg/L) during autumn and the highest recorded in the treated sludge (1895±42.25 mg/L) during autumn (Table 4.7). Total nitrogen ranged between 0.16±0.12 mg/L and 21.6±10.89 mg/L, with the lowest recorded in the filtration stage during summer (0.16±0.12 mg/L) and the highest recorded in the treated sludge in spring (21.6±10.89 mg/L) (Table 4.8). Sulphate ranged between 19±13.28 mg/L and 65±21.61 mg/L, with the lowest recorded in the final treated water (19±13.28 mg/L) during spring and the highest recorded in the flocculation stage (65±21.61 mg/L) during spring (Table 4.9).

Iron ranged between 0.83±0.17 mg/L and 346.8±104.14 mg/L, with the lowest recorded in the distribution system (0.83±0.17 mg/L) during spring and the highest recorded in the treated sludge (346.8±104.14 mg/L) during autumn (Table 4.10). Manganese ranged between 2.8±6.98 mg/L and 55.8±2.01 mg/L, with the lowest recorded in the final treated water (2.8±6.98 mg/L) during autumn and the highest recorded in the flocculation stage (144±78.20 mg/L) during summer (Table 4.11).

Ammonia ranged between <0.009±0.08 mg/L and 8.6±3.94 mg/L, with the lowest recorded in the final treated water (<0.009±0.08 mg/L) during autumn and the highest recorded in the treated sludge (8.6±3.94 mg/L) during autumn (Table 4.12).

4.2.1.3. Site E

The impact of the season sampling interval and treatment stage on the measured physicochemical parameters was also observed at Site E (Table 4.1 to Table 4.12). The highest pH value, recorded during summer at the flocculation stage, was 8.9±0.88 (Table 4.1). Temperatures ranged from 19.6±1.6°C to 26.1±11.79°C, with the peak temperature occurring during summer at the sedimentation stage (Table 4.2). Across the treatment stages, conductivity ranged from 51.2±12.21 mS/m to 158.8±5.63 mS/m, with the highest values recorded in the treated sludge during winter (Table 4.3). Turbidity ranged between 0.5±0.34 NTU and 4.7±0.70 NTU, with the highest recorded in the raw water source during spring. Treated sludge ranged from 650±125.73 NTU to 890±106.1 NTU (Table 4.4). Free chlorine in the final treated water ranged between 0.5±2.37 mg/L and 5.74±2.89 mg/L (Table 4.5). TOC ranged between 3.9±0.57 mg/L and 16.5±2.68 mg/L, with the lowest recorded in the final treated water (3.9±0.57 mg/L) during autumn and the highest recorded in the raw source water (15.33±2.46 mg/L) during spring. (Table 4.6) Total alkalinity ranged between 86±37.04 mg/L and 2100±437.02 mg/L, with the lowest recorded in the final treated water (86±37.04 mg/L) during spring and the highest recorded in the treated sludge (2100±437.02 mg/L) during winter (Table 4.7). Nitrate ranged between 0.16±2.06 mg/L and 22.3±15.56 mg/L, with the lowest recorded in the final treated water during winter (0.16±2.06 mg/L) and the highest recorded in the treated sludge during spring (22.3±15.56 mg/L) (Table 4.8). Sulphate ranged between 25±12.71 mg/L and 75±1.53 mg/L, with the lowest recorded in the final treated water (25±12.71 mg/L) during spring and the highest recorded in the raw water source (75±1.53 mg/L) during winter (Table 4.9).

Iron ranged between 0.2±0.15 mg/L and 383±162.30 mg/L, with the lowest recorded in the distribution system (0.2±0.15 mg/L) during autumn and the highest recorded in the sedimentation stage (383±162.30 mg/L) during spring (Table 4.10). Manganese ranged between 27±10.21 mg/L and 68±23.73 mg/L, with the lowest recorded in the sedimentation stage (27±10.21 mg/L) during spring and the highest recorded in the flocculation stage (68±23.73 mg/L) during winter (Table 4.11). Ammonia ranged between <0.009±0.08 mg/L and 4.1±0.64 mg/L, with the lowest recorded in the final treated water (<0.009±0.08 mg/L) during winter, and the highest recorded was in the filtration stage (4.1±0.64 mg/L) during autumn (Table 4.12).

4.2.2. Physicochemical parameters in Limpopo Province (Site B)

Variations in the measured physicochemical parameters were noted across different seasons and stages of the drinking water treatment process at Site B (Table 4.1 to Table 4.12). The highest pH value of 8.9±0.87 was recorded during the summer at the flocculation stage (Table 4.1). Temperature ranged from 18.1±39°C and 26.7±3.26°C, with peak temperatures observed at the sedimentation stage during the summer (Table 4.2). Throughout the treatment stages, conductivity ranged from 29.67±2.07 mS/m to 182.92±25.07 mS/m, with the highest values recorded in the treated sludge in autumn (Table 4.3). Turbidity ranged between 0.50±0.26 NTU and 900±119.30 NTU, with the lowest recorded in the final treated water (0.50±0.26 NTU) in autumn and the highest recorded in the treated sludge during winter (900±119.30 NTU) (Table 4.4). Chlorine in the final treated water ranged between 1.84±0.47 mg/L and 2.78±0.25 mg/L, while the distribution system ranged between 0.023±0.04 mg/L and 0.1±0.03 mg/L (Table 4.5). TOC ranged between 5.30±1.73 mg/L and 26.43±1.85 mg/L, with the lowest recorded in the final treated water (5.30±1.73 mg/L) during winter and the highest recorded in the raw water source (26.43±1.85 mg/L) during spring (Table 4.6). Total alkalinity ranged between 61.2±3.55 mg/L and 410±45.88 mg/L, with the lowest recorded in the final treated water (61.2±3.55 mg/L) during autumn and the highest recorded in the treated sludge (410±45.88 mg/L) during summer (Table 4.7). Nitrate ranged between 0.12±0.75 mg/L and 56±26.11 mg/L, with the lowest recorded in the distribution stage during summer (0.12±0.75 mg/L) and the highest recorded in the treated sludge during summer (56±26.11 mg/L) (Table 4.8). Sulphate ranged between 14±1.92 mg/L and 165±13.07 mg/L, with the lowest recorded in the final treated water (14±1.92 mg/L) during spring and the highest recorded in the raw water source (165±13.07 mg/L) during winter (Table 4.9).

Iron ranged between 0.96±0.84 mg/L and 371±129.16 mg/L, with the lowest recorded in the distribution system (0.96±0.84 mg/L) during autumn and the highest recorded in the treated sludge (371±129.16 mg/L) during winter (Table 4.10). Manganese ranged between 1.8±0.33 mg/L and 230±63.49 mg/L, with the lowest recorded in the distribution system (1.8±0.33 mg/L) during autumn and the highest recorded in the treated sludge (230±63.49 mg/L) during summer (Table 4.11). Ammonia ranged between <0.009±0.07 mg/L and 13.46±6.84 mg/L, with the lowest recorded in the final treated water (<0.009±0.07 mg/L) during autumn and the highest recorded in the treated sludge (13.46±6.84 mg/L) during autumn (Table 4.12).

4.2.3. Physicochemical parameters in Mpumalanga Province (Site C)

At Site C, the pH varied across the seasons and treatment stages, ranging from 5.01±0.51 to 9.34±1.11, with the highest value recorded in summer in the flocculation stage (Table 4.1). The

temperature varied across all seasons, ranging from 19.9±0.91°C to 26.7±2.32°C, with the highest temperature occurring in the flocculation stage in the autumn (Table 4.2). Throughout the various treatment stages, conductivity ranged from 45.5±2.83 mS/m to 166.5±15.67 mS/m, with the highest value recorded in the treated sludge during summer (Table 4.3). Turbidity ranged between 0.36±1.15 NTU and 10.7±1.54 NTU, with the highest recorded in the raw water in summer (Table 4.4). Chlorine ranged between 0.99±1.76 mg/L and 4.5±1.98 mg/L in the final treated water and between 0.06±0.03 mg/L and 0.1±0.03 mg/L in the distribution system across the seasons (Table 4.5). TOC ranged between 9.30±2.33 mg/L and 30.77±7.09 mg/L, with the lowest recorded in the final treated water (9.30±2.33 mg/L) during winter and the highest recorded in the raw water source (30.77±7.09 mg/L) during summer. (Table 4.6) Total alkalinity ranged between 71.5±15.61 mg/L and 368±41.0 mg/L, with the lowest recorded in the final treated water (71.5±15.61 mg/L) during autumn and the highest recorded in the treated sludge (368±41.0 mg/L) during autumn (Table 4.7). Nitrate ranged between 0.3±0.03 mg/L and 84±40.60 mg/L, with the lowest recorded in the distribution system during summer (0.3±0.03 mg/L) and the highest recorded in the treated sludge during summer (84±40.60 mg/L) (Table 4.8). Sulphate ranged between 111±111.15 mg/L and 132±35 mg/L, with the lowest recorded in the final treated water (111±111.15 mg/L) during summer and the highest recorded in the raw water sources (132±35 mg/L) during autumn (Table 4.9).

Iron ranged between 2.4±0.97 mg/L and 654±144.0 mg/L, with the lowest recorded in the distribution system (2.4±0.97 mg/L) during spring and the highest recorded in the treated sludge (654±144.0 mg/L) during winter (Table 4.10). Manganese ranged between 0.98±0.65 mg/L and 790±375.08 mg/L, with the lowest recorded in the distribution system (0.98±0.65 mg/L) during autumn and the highest recorded in the treated sludge (790±375.08 mg/L) during summer (Table 4.11). Ammonia ranged between <0.002±0.07 mg/L and 3.8±1.03 mg/L, with the lowest recorded in the final treated water (<0.002±0.07 mg/L) during autumn and the highest recorded in the sludge (3.8±1.03 mg/L) during autumn (Table 4.12).

Table 4.1: Measured pH at all sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) across the seasons. SANS 241 set value for final water is ≥5 to ≥7.

Physicochemical	Treatment stages	Seasons	Sites (mean±standard deviation)				
			A	B	C	D	E
pH	Raw water	Spring	7.61±0.12	7.80±0.98	5.41±0.27	8.12±0.18	8.09±1.16
		Summer	7.85±0.23	7.56±0.24	5.30±0.33	7.46±0.27	8.03±0.28
		Winter	7.73±0.23	8.37±0.61	5.25±0.43	7.28±0.43	8.08±0.50
		Autumn	7.87±0.44	8.01±0.48	5.01±0.51	7.60±0.4	8.33±0.6
	Flocculation	Spring	8.21±0.55	8.67±0.76	9.2±1.03	7.97±0.47	8.01±0.45
		Summer	7.93±0.45	8.9±0.87	9.34±1.11	7.95±0.46	8.05±0.49
		Winter	8.76±0.8	8.66±0.76	8.98±0.47	8.89±0.74	8.9±0.88
		Autumn	8.66±0.44	8.43±0.36	8.55±0.24	7.70±0.13	8.42±0.48
	Sedimentation	Spring	8.34±0.35	8.65±0.36	8.29±0.37	7.90±0.13	7.77±0.34
		Summer	8.36±0.37	8.23±0.24	6.98±0.49	7.52±0.22	7.74±0.35
		Winter	8.78±0.45	8.21±0.24	7.65±0.21	7.90±0.13	8.45±0.28
		Autumn	8.70±0.42	8.11±0.24	7.34±0.31	7.52±0.22	8.08±0.24
	Filtration	Spring	8.35±0.38	7.78±0.17	8.01±0.37	8.29±0.39	7.43±0.34
		Summer	7.5±0.43	7.88±0.09	7.74±0.14	8.04±0.18	7.79±0.27
		Winter	8.64±0.35	7.65±0.15	7.32±0.38	7.45±0.42	8.21±0.40
		Autumn	8.22±0.20	7.71±0.12	7.05±0.54	8.04±0.18	8.07±0.36
	Final treated water	Spring	8.1±0.20	7.33±0.32	7.77±0.13	8.40±0.23	8.03±0.33
		Summer	6.6±0.91	7.85±0.09	7.41±0.33	7.96±0.19	8.09±0.35
		Winter	7.75±0.73	7.49±0.24	7.43±0.31	7.64±0.18	8.10±0.08
		Autumn	7.64±0.16	7.38±0.30	6.94±0.50	8.0±0.19	8.01±0.07
	Treated sludge	Spring	7.29±0.39	7.15±0.40	7.56±0.27	7.25±0.35	7.68±0.21
		Summer	7.88±0.1	7.75±0.23	8.1±0.35	7.76±0.17	6.97±0.61
		Winter	7.24±0.37	7.18±0.38	8.32±0.48	7.77±0.16	7.84±0.13
		Autumn	7.63±0.16	7.79±0.23	7.92±0.29	7.54±0.21	7.90±0.10
	Drinking water distribution system	Spring	6.76±0.64	6.77±0.59	7.23±0.37	N/A	N/A
		Summer	7.41±0.27	7.86±0.24	7.53±0.28	N/A	N/A
		Winter	6.95±0.52	7.99±0.28	7.82±0.21	N/A	N/A
		Autumn	6.45±0.81	7.33±0.32	7.29±0.34	N/A	N/A

Table 4.2: Measured temperature at all sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) across the seasons

Physicochemical	Treatment stages	Seasons	Sites (mean±standard deviation)				
			A	B	C	D	E
Temperature (°C)	Raw water	Spring	23.5±2.87	24.7±1.92	23.6±0.98	22.1±1.56	23.6±1.64
		Summer	26.8 ±3.30	26.1±2.19	25.3±0.98	23±1.37	23±1.79
		Winter	18.1±3.29	18.3±2.19	19.9±0.91	18.4±1.35	19.6±1.66
		Autumn	21.7±3.13	23.7±1.92	25.2±0.95	24.8±1.56	26.1±11.79
	Flocculation	Spring	24.2±2.85	24.9±1.95	23.9±1.97	23±1.9	23±1.62
		Summer	26.7±3.26	26.8±2.25	25.1±1.50	21.9±2.13	23±1.04
		Winter	22.3±3.0	22.9±2.25	23.8±1.73	23.5±1.85	25.7±1.37
		Autumn	23.2±2.88	23.89±2.02	26.8±1.73	25.6±2.14	25.8±1.42
	Sedimentation	Spring	25.4±0.67	25.3±1.62	23.7±2.03	23.9±1.77	23±11.75
		Summer	26.3±0.35	26.77±1.43	24.4±2.03	23.9±1.31	20.0±1.75
		Winter	25.3±1.16	25.3±1.05	26.2±2.58	21.9±1.77	25.3±1.67
		Autumn	24.68±1.63	24.6±1.03	26.7±2.32	23±1.53	26.7±0.82
	Filtration	Spring	25.7±1.57	25.1±1.24	23.5±1.98	23.9±1.34	20.0±1.33
		Summer	25.46±1.22	24.9±2.1	24.7±1.78	22.0±1.56	20.0±1.29
		Winter	24.5±1.87	24.4±1.65	22.9±1.45	23.4±1.38	24.9±1.78
		Autumn	24.1±1.34	23.8±1.03	25.1±2.48	23.0±1.32	25.6±2.09
	Final treated water	Spring	23.7±0.25	24.6±0.58	22.4±1.02	22.3±0.81	22±3.04
		Summer	24.8±0.34	23.6±0.53	23.6±0.97	22.6±0.45	21.5±2.49
		Winter	21.8±0.93	23.72±0.74	22.4±0.98	23.7±1.19	24.6±2.64
		Autumn	22.65±1.37	23.4±0.69	24.5±1.18	25.7±1.47	25.3±1.78
	Treated sludge	Spring	23.4±1.12	22.4±1.31	24.5±1.32	22±1.15	23.0±1.16
		Summer	25±1.26	23.6±1.44	23.5±1.43	24.1±0.94	24.9±1.26
		Winter	23.5±1.31	23.1±1.75	24.1±1.76	23.1±1.12	23.1±1.31
		Autumn	22.4±1.5	21.5±1.76	21.6±1.7	23±1.0	22.5±1.41
	Drinking water distribution system	Spring	22.7±1.1	21.6±1.36	23.7±1.25	N/A	N/A
		Summer	26.8±3.45	21.8±1.59	22.6±1.08	N/A	N/A
		Winter	21.6±1.73	22.1±1.51	22.9±1.16	N/A	N/A
		Autumn	22.7±1.46	24.3±1.33	23.1±0.87	N/A	N/A

Table 4.3: Measured conductivity at all sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) across the seasons. SANS 241 set value for final water is ≤170 mS/m.

Physicochemical	Treatment stages	Seasons	Sites (mean±standard deviation)				
			A	B	C	D	E
Conductivity (mS/m)	Raw	Spring	134.3±2.85	158.4±3.57	155.6±4.62	131±5.18	146±7.23
		Summer	137.8±2.93	152.3±4.09	148.6±2.31	136±6.2	143±6.56
		Winter	137.8±2.93	155.65±4.20	155.8±5.6	142.7±4.75	158.8±5.63
		Autumn	131.98±2.02	150.34±3.05	159.7±4.10	140.5±5.87	153.9±8.39
	Flocculation	Spring	94.9±2.55	110.28±2.14	108.2±4.40	99±6.59	91±8.29
		Summer	97.8±2.76	113.48±2.34	110.1±2.1	99±4.56	97±9.72
		Winter	98.35±2.46	110.1±2.10	116.1±5.38	94.8±5.10	99.9±6.71
		Autumn	92.9±1.85	114.24±1.90	110.3±4.20	95.8±7.99	94.4±9.66
	Sedimentation	Spring	91.4±0.94	103.32±0.93	107.7±5.60	99±4.72	97±12.19
		Summer	92.6±1.15	105.4±0.29	106.7±5.19	90±1.12	94±13.89
		Winter	93.7±0.64	103.9±1.13	106.3±5.99	90.5±5.0	92.3±11.57
		Autumn	92.4±1.15	103.6±1.07	108.1±5.99	98.3±5.68	99.6±13.06
	Filtration	Spring	79.4±0.86	95.78±2.41	97.8±5.77	93±2.57	96±11.18
		Summer	78.7±1.04	91.6±1.32	87.7±2.31	90±2.66	83.6±13.09
		Winter	78.65±1.04	88.2±2.95	96.8±7.02	95.3±1.73	101.89±8.21
		Autumn	77.34±0.42	87.9±2.92	101.2±5.57	90±2.66	92.4±13.31
	Final treated water	Spring	40.1±1.78	62.1±2.29	87.9±2.0	51±7.83	53±12.70
		Summer	40.5±1.94	65.23±1.19	85.6±1.56	53±6.06	56±15.30
		Winter	42.3±2.13	61.33±2.74	87.7±2.45	52.3±9.59	51.2±12.21
		Autumn	43.98±1.17	69.76±2.06	80.5±1.27	52.1±5.04	67.4±14.11
	Sludge	Spring	124.7±24.65	147.7±30.34	159.7±15.87	149.5±7.98	149.55±11.16
		Summer	179.2±28.19	144±21.47	166.5±15.67	137.9±7.90	148.5±7.97
		Winter	129.6±30.15	123.1±13.26	160.8±3.65	141.1±5.96	140.8±4.78
		Autumn	139.4±22.67	182.92±25.07	136.6±14.99	153.1±10.10	110.13±62.80
	Drinking water distribution system	Spring	29.2±1.67	34.31±2.61	45.9±2.40	N/A	N/A
		Summer	29.5±1.65	35.32±2.90	45.5±2.83	N/A	N/A
		Winter	31.2±1.80	31.33±2.35	45.7±2.72	N/A	N/A
		Autumn	32.8±1.07	29.67±2.07	50.5±2.49	N/A	N/A

Table 4:4: Measured turbidity at all sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) across the seasons. SANS 241 set value for final water is ≤1.0 NTU.

Physicochemical	Treatment stages	Seasons	Sites (mean±standard deviation)				
			A	B	C	D	E
Turbidity (NTU)	Raw	Spring	10.56±1.24	8.94±0.1	3.35±3.77	3.1±0.81	1.8±1.49
		Summer	9.67±1.24	8.9±0.25	10.7±1.54	4.5±0.74	2.6±1.87
		Winter	8.1±0.55	8.75±0.27	5.57±4.48	4.5±0.74	4.7±0.70
		Autumn	9.55±1.25	8.45±0.10	2.60±3.77	3.4±0.81	1.2±1.49
	Flocculation	Spring	9.78±0.34	8.78±0.65	10.6±4.27	1.7±0.97	2.8±1.27
		Summer	9.02±0.23	9.1±0.79	11.4±4.03	3.1±1.18	1.2±1.36
		Winter	9.34±0.41	9.86±0.39	3.76±4.34	3.8±1.03	3.56±1.02
		Autumn	9.67±0.38	8.32±0.55	3.50±4.19	3.7±1.06	0.91±1.20
	Sedimentation	Spring	5.88±0.87	8.08±0.35	9.92±3.69	0.65±1.53	5.2±2.15
		Summer	5.33±0.83	8.72±0.41	2.20±4.19	3.9±1.88	0.91±2.54
		Winter	4.08±0.99	8.01±0.05	2.33±4.15	2.7±1.64	3.33±2.27
		Autumn	4.24±0.68	7.97±0.42	3.23±0.56	3.9±0.69	0.68±1.47
	Filtration	Spring	0.23±0.035	3.56±0.64	4.34±0.40	0.39±0.25	1.720.94±
		Summer	0.26±0.04	3.41±0.66	0.36±0.40	0.72±0.16	0.68±0.12
		Winter	0.18±0.03	3.1±0.72	1.07±0.41	1.0±0.31	2.56±0.01
		Autumn	0.20±0.03	2.14±0.78	1.04±0.39	0.72±0.19	0.55±0.64
	Final treated water	Spring	0.18±0.05	0.86±0.21	0.94±0.96	0.37±0.17	0.53±0.23
		Summer	0.19±0.05	0.98±0.25	0.36±1.15	0.72±0.18	0.53±0.27
		Winter	0.13±0.05	0.87±0.21	2.60±0.93	0.4±0.02	0.98±0.27
		Autumn	0.09±0.02	0.50±0.26	1.03±1.11	0.4±0.18	0.5±0.34
	Treated sludge	Spring	330±80.42	670±141.86	235±183.16	468±222.26	890±106.1
		Summer	470±40.41	730±169.8	650±164.83	835±75.66	845±109.81
		Winter	440±95.39	560.4±173.32	320.46±124.36	980±269.52	650±125.73
		Autumn	520±98.49	900±119.30	480±208.63	870±222.68	835±29.29
	Drinking water distribution system	Spring	0.17±0.02	1.00±0.11	1.09±0.34	N/A	N/A
		Summer	0.18±0.03	1.11±0.14	1.3±0.29	N/A	N/A
		Winter	0.13±0.02	0.90±0.08	1.56±0.40	N/A	N/A
		Autumn	0.15±0.02	0.84±0.14	1.89±0.41	N/A	N/A

NTU: Nephelometric Turbidity Unit

Table 4.5: Measured chlorine at all sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) across the seasons. SANS 241 set value for final water is ≤5 mg/L.

Physicochemical	Treatment stages	Seasons	Sites (mean±standard deviation)				
			A	B	C	D	E
Chlorine (mg/L)	Raw	Spring	-	-	-	-	-
		Summer	-	-	-	-	-
		Winter	-	-	-	-	-
		Autumn	-	-	-	-	-
	Flocculation	Spring	-	-	-	-	-
		Summer	-	-	-	-	-
		Winter	-	-	-	-	-
		Autumn	-	-	-	-	-
	Sedimentation	Spring	-	-	-	-	-
		Summer	-	-	-	-	-
		Winter	-	-	-	-	-
		Autumn	-	-	-	-	-
	Filtration	Spring	-	-	-	-	-
		Summer	-	-	-	-	-
		Winter	-	-	-	-	-
		Autumn	-	-	-	-	-
	Final treated water	Spring	0.26±0.04	2.3±0.43	0.99±1.76	1.14±0.51	1.0±2.44
		Summer	0.33±0.05	2.78±0.25	1.13±1.82	2.19±0.41	0.5±2.37
		Winter	0.32±0.04	1.90±0.25	3.61±1.82	1.9±0.41	3.68±2.38
		Autumn	0.24±0.04	1.84±0.47	4.5±1.98	1.24±0.58	5.74±2.89
	Treated sludge	Spring	N/A	N/A	N/A	N/A	N/A
		Summer	N/A	N/A	N/A	N/A	N/A
		Winter	N/A	N/A	N/A	N/A	N/A
		Autumn	N/A	N/A	N/A	N/A	N/A
	Drinking water distribution system	Spring	0.015±0.04	0.1±0.03	0.06±0.03	N/A	N/A
		Summer	0.09±0.05	0.07±0.03	0.05±0.03	N/A	N/A
		Winter	0.01±0.01	0.02±0.02	0.11±0.004	N/A	N/A
		Autumn	0.01±0.01	0.023±0.04	0.1±0.03	N/A	N/A

Table 4.6: Measured total organic carbon (TOC) at all sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) across the seasons. SANS 241 set value for final water is ≤10 mg/L.

Physicochemical	Treatment stages	Seasons	Sites (mean±Sstandard deviation)				
			A	B	C	D	E
Total Organic Carbon (mg/L)	Raw water	Spring	13.49±1.43	26.43±1.85	28.56±6.01	16.5±2.68	15.33±2.46
		Summer	12.84±1.24	25.96±1.76	30.77±7.09	15.6±2.43	12.84±1.61
		Winter	10.43±1.60	23.84±2.01	29.47±16.5	12.2±2.93	11.07±2.96
		Autumn	11.12±1.22	22.47±2.16	26.18±7.26	10.9±3.01	9.63±2.86
	Flocculation	Spring	15.56±4.83	22.92±1.90	25.67±1.36	9.73±0.52	10.76±0.93
		Summer	9.30±5.79	23.74±2.12	24.99±1.21	9.31±0.37	9.41±0. 39
		Winter	11.33±4.43	19.84±1.64	22.57±1.55	8.76±0.61	8.64±01.13
		Autumn	20.2±5.47	20.37±1.76	23.83±0.93	8.6±0.57	9.01±0.91
	Sedimentation	Spring	23.49±4.02	18.87±2.48	16.73±2.28	9.72±0.87	7.61±0.88
		Summer	19.76±3.98	19.49±2.65	16.98±2.37	10.9±10.03	6.9±0.62
		Winter	22.1±4.93	15.30±2.98	12.48±2.23	8.98±0.81	5.98±1.02
		Autumn	14.34±4.60	14.58±2.67	13.47±1.95	10.6±0. 16	5.72±0.95
	Filtration	Spring	5.98±0.62	12.21±3.84	15.12±3.49	6.27±0.61	3.9±0.57
		Summer	6.3±1.43	11.65±3.46	15.56±3.57	5.05±1.76	4.76±1.04
		Winter	5.1±1.49	5.30±1.73	9.30±2.33	5.55±1.55	4.97±1.02
		Autumn	5.90±0.51	7.34±3.35	11.33±16.99	4.47±0.76	4.69±0.47
	Final treated water	Spring	7.98±0.79	11.34±3.17	14.50±3.72	10.32±1.21	9.87±1.01
		Summer	8.33±0.89	17.30±3.74	15.40±3.05	11.34±1.46	10.90±1.22
		Winter	6.54±0.72	11.45±0.62	9.30±2.61	8.43±0.99	8.64±0.64
		Autumn	7.34±0.50	10.33±3.77	12.20±1.65	9.89±0.74	8.97±0.97
	Treated sludge	Spring	10.33±0.87	25.32±2.65	20.2±1.73	18.71±0.53	16.89±0.47
		Summer	9.34±0.92	23.94±2.41	23.49±1.88	19.26±0.64	17.51±0.36
		Winter	8.57±1.04	19.17±3.08	19.76±1.24	19.08±0.51	17.31±0.57
		Autumn	20.4±0.59	22.21±1.56	22.1±1.65	18.07±0.60	18.02±0.57
	Drinking water distribution system	Spring	7.34±0.61	9.56±1.04	14.34±2.68	N/A	N/A
		Summer	8.25±0.75	11.88±1.09	15.98±3.07	N/A	N/A
		Winter	7.43±0.36	10.13±0.29	10.3±2.14	N/A	N/A
		Autumn	6.76±0.75	9.87±1.26	11.1±2.48	N/A	N/A

Table 4.7: Measured total alkalinity at all sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) across the seasons. SANS 241 set value for final water is ≤300 mg/L.

Cation	Treatment stages	Seasons	Sites (mean±standard deviation)				
			A	B	C	D	E
Total alkalinity (mg/L)	Raw	Spring	70.5±5.77	113±9.98	95.9±7.76	110±9.46	114±29.68
		Summer	68.4±6.79	115±10.69	79.6±0.76	99±7.57	115±36.01
		Winter	71.3±6. 16.04	101±9.61	81.1±8.69	87±11.59	90±36.11
		Autumn	81.34±6.94	94±11.59	80.6±9.14	101±5.86	161±26.85
	Flocculation	Spring	65.4±6.69	80.4±1.44	95.5±9.12	108±11.79	109±31.19
		Summer	63.45±7.73	79.5±1.69	73.6±7.78	92±13.07	106±37.32
		Winter	67.6±6.98	82.88±1.26	85.4±5.20	89±12.66	95.9±36.70
		Autumn	78.43±8.14	81.24±0.87	88.3±11.16	113±10.97	165±33.23
	Sedimentation	Spring	63.4±2.54	83.3±4.04	94.3±9.64	101±8.37	124±26.26
		Summer	65.8±3.02	81.2±3.49	73.6±10.40	82±8.50	106±32.14
		Winter	61.2±2.86	77.3±4.61	88.3±3.30	98±3.0	101±29.21
		Autumn	66.88±1.78	74.24±4.74	93.7±11.78	95±9.71	159±26.95
	Filtration	Spring	56.34±4.44	77.9±2.21	93.9±10.39	98±1.73	117±25.27
		Summer	59.3±4.13	73.78±0.69	72.3±11.70	98±1.73	113±30.61
		Winter	60.23±5.32	72.87±2.60	91.2±1.50	95±1.73	101±29.96
		Autumn	53.12±5.44	71.8±2.26	98.5±12.41	98±1.73	160±25.48
	Final treated water	Spring	43.5±2.54	66.4±3.02	93.4±12.96	104±11.86	86±37.04
		Summer	47.9±2.81	67.98±.4.5	71.5±15.61	93±14.43	114±28.21
		Winter	46.56±2.12	63.5±2.61	92.5±5.24	93±12.53	148±43.56
		Autumn	42.5±2.87	61.2±3.55	102±15.73	118±12.53	170±42.77
	Drinking water treatment system	Spring	184±16.64	380±38.73	300±47.48	1134±368.45	1125±485.58
		Summer	170±20.0	410±45.883	260±56.38	1210±417.16	1259±44659
		Winter	191±20.3	320±30.0	342±54.6	1840±419.15	2100±437.02
		Autumn	210±10.69	350±45.82	368±41.0	1895±42.25	1940±525.49
	Drinking water distribution system	Spring	70.5±5.77	113±9.98	95.9±7.76	N/A	N/A
		Summer	68.4±6.79	115±10.69	79.6±0.76	N/A	N/A
		Winter	71.3±6.04	101±9.61	81.1±8.69	N/A	N/A
		Autumn	81.34±6.94	94±11.59	80.6±9.14	N/A	N/A

Table 4.8: Measured nitrate at different sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) across the seasons. SANS 241 set value for final water is ≤11 mg/L.

Physicochemical	Treatment stages	Seasons	Sites (mean±standard deviation)				
			A	B	C	D	E
Nitrate (mg/L)	Raw	Spring	4.3±0.41	5.6±0.29	0.1±0.81	0.16±0.75	3.8±1.71
		Summer	3.3±0.29	5.34±0.36	0.3±0.91	1.8±0.75	1.1±2.08
		Winter	3.7±0.31	5.97±0.31	0.16±0.96	0.92±0.40	4.9±0.56
		Autumn	3.87±0.50	5.35±0.15	1.8±0.93	0.3±0.91	4.5±1.79
	Flocculation	Spring	4.1±0.31	5.7±0.29	1.24±0.47	1.1±0.37	4.5±0.54
		Summer	3.34±0.25	5.21±0.35	0.34±0.45	0.40±0.39	4.0±0.66
		Winter	3.64±0.34	5.10±0.39	1.1±0.43	0.90±0.42	4.0±0.76
		Autumn	3.71±0.44	5.09±0.41	0.40±0.40	0.34±0.37	3.2±0.61
	Sedimentation	Spring	2.85±0.32	2.38±0.09	1.45±0.63	0.16±0.47	3.5±0.53
		Summer	2.23±0.20	2.27±0.06	0.34±0.55	0.40±0.40	4.3±0.62
		Winter	2.15±0.35	2.15±0.12	0.16±0.68	0.89±0.53	3.1±0.45
		Autumn	2.53±0.31	2.2±0.09	1.2±0.58	1.2±0.54	4±0.40
	Filtration	Spring	1.75±0.24	2.33±0.45	1.4±0.56	0.36±0.36	3.1±1.52
		Summer	1.36±0.087	2.1±0.40	0.3±0.15	0.16±0.44	4.5±1.85
		Winter	1.34±0.28	1.7±0.52	0.46±0.65	0.93±0.40	4.5±1.60
		Autumn	1.2±0.28	1.3±0.54	0.16±0.68	0.16±0.12	1.3±0.10
	Final treated water	Spring	1.1±0.43	1.5±0.65	1.4±0.53	0.36±0.09	4.3±0.54
		Summer	0.5±0.20	0.3±0.09	0.3±0.03	0.35±0.12	0.3±1.74
		Winter	0.3±0.53	0.23±0.77	0.36±0.60	0.3±0.11	0.16±2.06
		Autumn	0.1±0.5	0.12±0.75	0.35±0.62	0.52±0.09	3.8±0.65
	Treated sludge	Spring	2.2±0.75	1.43±1.43	2.34±1.02	2.23±1.39	4.5±0.36
		Summer	60±28.24	56±26.11	84±40.60	24±11.32	38±11.10
		Winter	27±13.88	25.76±13.37	34±17.93	21.6±10.89	22.3±15.56
		Autumn	3.8±0.83	4±1.64	3.6±1.04	3.3±9	5.1±0.41
	Drinking water distribution system	Spring	0.1±0.04	0.21±0.6	0.22±0.07	N/A	N/A
		Summer	0.15±0.03	1.5±0.32	0.21±0.07	N/A	N/A
		Winter	0.18±0.05	1.24±0.5	0.11±0.08	N/A	N/A
		Autumn	0.2±0.05	0.87±0.65	0.07±0.08	N/A	N/A

Table 4.9: Measured sulphate at different sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) across the seasons. SANS 241 set value for final water is ≤500 mg/L.

Physicochemical	Treatment stages	Seasons	Sites (mean±standard deviation)				
			A	B	C	D	E
Sulphate (mg/L)	Raw	Spring	20.1±3.12	157±15.69	118±9.13	19±6.69	31±21.88
		Summer	22.3±2.8	136±17.67	111±11.15	28.6±3.36	30±25.16
		Winter	27.54±1.60	165±13.07	115±16.69	30±8.05	75±1.53
		Autumn	23.22±3.82	133±14.98	132±351	35±5.98	33±25.70
	Flocculation	Spring	19.4±3.08	119±8.34	119.6±7.73	65±21.61	29±17.99
		Summer	12.98±2.40	110±9.07	113±9.45	18±3.17	34±9.62
		Winter	13.56±3.29	121±8.02	117±9.10	51.1±24.95	68±2.89
		Autumn	17.4±3.55	103±5.87	131±3.32	27±2.14	34±8.21
	Sedimentation	Spring	16.9±0.67	80±6.65	120.3±9.15	19±22.78	31±21.45
		Summer	16.2±0.59	79.4±6.71	113±11.01	14±27.14	27±20.60
		Winter	15.3±0.81	66±7.02	123±7.82	61±25.81	66±19.16
		Autumn	16.4±0.36	72±4.46	135±11.02	14±2.89	35±4
	Filtration	Spring	11.32±0.79	34±7.04	121.8±10.32	14±2.71	27±12.23
		Summer	10.09±0.47	23±4.16	115±12.49	19±0.58	33±15.56
		Winter	10.3±0.97	17±5.86	121±10.32	20±2.71	55±14.74
		Autumn	9.4±0.65	25±8.62	139±9.97	19±3.21	34±14.74
	Final treated water	Spring	1.53±0.27	19±2.43	122±12.68	19±13.28	25±12.71
		Summer	1.3±0.32	14±1.92	114±15.04	13±14.80	31±13
		Winter	1.92±0.12	17.5±2.79	127±15.53	40±12.49	54±3.79
		Autumn	1.43±0.31	14.36±5.57	144±6.56	37±14.18	32±15.30
	Treated sludge	Spring	27±12.07	30±16.04	137±25.51	32.4±9.94	37±5.05
		Summer	42±9.85	66±14.02	192±22.85	34±10.90	36±520
		Winter	37±14.73	53.4±11.89	147±20.48	53.8±11.49	27±5.77
		Autumn	56±14.50	38±18.90	176.4±28.34	36±1.80	27±5.51
	Drinking water distribution system	Spring	20.1±3.12	157±15.69	118±9.13	N/A	N/A
		Summer	22.3±2.80	136±17.67	111±111.15	N/A	N/A
		Winter	27.54±3.74	165±16.65	115±9.07	N/A	N/A
		Autumn	23.22±1.60	133±13.07	132±10.69	N/A	N/A

Table 4.10: Measured iron at different sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) across the seasons. SANS 241 set value for final water is ≤2 mg/L.

Physicochemical	Treatment stages	Seasons	Site (mean±standard deviation)				
			A	B	C	D	E
Iron (mg/L)	Raw	Spring	17±2.58	19±64.11	12±61.65	5.37±16.03	5.9±10.39
		Summer	15±2	165±65.38	151±63.13	9.78±17.55	4.37±11.78
		Winter	11±3.06	45±20.74	31±22.59	9.4±18.93	10±11.18
		Autumn	13±2.0	60±75.30	57±70.92	40±18.85	27±12.66
	Flocculation	Spring	17.5±2.43	13.7±6.22	14.5±4.62	10.01±3.4	51±23.74
		Summer	14.4±1.18	151±66.64	121±46.83	9.5±22.88	3.7±1.30
		Winter	12.3±2.60	33.4±7.32	35.6±9.84	5.3±1.21	31±9.4
		Autumn	12.4±2.62	37.9±7.48	45±11.56	46.87±12.58	103±23.74
	Sedimentation	Spring	9.6±0.99	14.5±3.75	13.2±4.75	5.37±0.2	383±162.30
		Summer	7.45±1.06	113.7±47.43	121±49.45	49±14.31	55.1±15.78
		Winter	9.44±0.24	29.7±10.05	37.9±13.07	24.2±21.88	33±185.20
		Autumn	9.11±1.13	33.5±13.52	33±15.74	49±25.19	103±177.11
	Filtration	Spring	8.95±044	13.19±3.37	12.8±1.51	5.37±1.27	4.8±1.99
		Summer	8.36±0.27	93.94±40.58	45±11.25	3.37±1.28	5.1±2.29
		Winter	7.87±0.54	24.6±6.11	41.5±14.46	2.98±1.37	45±22.98
		Autumn	8.29±0.29	22.73±11.32	24±12.37	5.37±1.670	5.6±1.28
	Final treated water	Spring	8±2.85	7.97±3.58	12±7.08	4.37±1.44	6.37±2.97
		Summer	1.6±1.5	11.3±4.28	27±6.66	4.34±1.55	5.9±3.29
		Winter	2.4±1.8	3.81±2.35	48±19.73	3.31±1.56	5.6±1.34
		Autumn	4.54±2.45	3.96±2.84	16±2.86	1.3±2.17	0.5±4.15
	Treated sludge	Spring	123±118.18	155±108.54	134±24.78	191.8±120.8	296±121.86
		Summer	176±102.99	132.9±124.59	164.8±24.57	51.76±12.46	33.43±12.72
		Winter	375±37.75	371±129.16	654±144.0	186±113.70	286±70.71
		Autumn	321.6±49.55	188.34±95.30	450.34±194.57	346.8±104.14	186±127.19
	Drinking water distribution system	Spring	6±0.85	5.97±1.58	8±2.08	N/A	N/A
		Summer	0.6±.5	7.3±1.28	16±3.67	N/A	N/A
		Winter	1.40±1.8	1.81±1.35	2.4±0.97	N/A	N/A
		Autumn	0.54±1.45	0.96±0.84	8.4±2.63	N/A	N/A

Table 4.11: Measured manganese at different sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) across the seasons. SANS 241 set value for final water is ≤0.4 mg/L.

Physicochemical	Treatment stages	Seasons	Site (mean±standard deviation)				
			A	B	C	D	E
Manganese (mg/L)	Raw	Spring	53.4±1.64	120±55.21	116±349	8.0±2.26	41±11.22
		Summer	55.8±2.01	230±63.49	790±29.82	60±29.97	31±13.53
		Winter	51.98±0.71	110±12.06	149±16.50	4.3±4.36	58±8.74
		Autumn	52.8±1.58	134±59.88	132±38.94	13±28.69	46±7.64
	Flocculation	Spring	44.8±2.18	114±20.61	101±10.34	137±76.20	39±15.56
		Summer	41.9±2.59	142±25.24	670±312.13	144±78.20	36±18.48
		Winter	46.7±1.51	101±23.18	113±12.66	4.1±1.73	68±23.73
		Autumn	42.6±1.51	96±23.18	126±12.66	13.5±3.47	36±1.73
	Sedimentation	Spring	33.5±1.58	98±9.95	85±206	16±69.44	27±10.21
		Summer	32.56±1.26	113±12.17	489±240.47	150±81.40	39±7.51
		Winter	31.87±1.89	93±3.61	71±7.37	3.7±6.83	52±12.50
		Autumn	29.76±1.49	91±1.41	74±2.12	15±7.99	39±9.19
	Filtration	Spring	18.3±3.27	54±14.29	76±19.03	38±9.19	41±9.03
		Summer	17.98±3.63	63±16.44	34±14.84	26±5.13	37±10.97
		Winter	17.35±3.74	38±11.37	63±16.62	23±11.24	56±10.01
		Autumn	11.4±4.20	32±4.24	43±14.14	16±4.95	37±13.44
	Final treated water	Spring	2.7±0.45	3.6±0.35	33±9.76	2.7±6.89	39±5.12
		Summer	3.1±0.3	3.46±0.4	22±5.51	16±5.92	41±4.58
		Winter	2.2±0.48	3.2±0.43	42±10.50	13±7.65	33±5.86
		Autumn	2.15±0.45	2.8±0.20	43±10.01	2.8±6.98	30±4.16
	Treated sludge	Spring	53.4±1.64	120±55.21	116±32.91	8.0±3.78	41±11.22
		Summer	55.8±2.01	230±63.49	790±375.08	6±4.61	31±13.53
		Winter	51.98±1.58	110±59.88	149±84.59	4.3±3.60	58±7.64
		Autumn	52.8±1.93	134±66.58	132±37.99	13±1.85	46±13.65
	Drinking water distribution system	Spring	1.7±0.32	2.6±0.35	3.3±1.06	N/A	N/A
		Summer	1.3±0.19	2.46±0.33	2.2±0.65	N/A	N/A
		Winter	1.2±0.39	2.2±0.4	1.2±1.28	N/A	N/A
		Autumn	0.92±0.29	1.8±0.33	0.98±0.65	N/A	N/A

Table 4.12: Measured ammonia at different sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) across the seasons. SANS 241 set value for final water is ≤1.5 mg/L.

Physicochemical	Treatment stages	Seasons	Site (mean±standard deviation)				
			A	B	C	D	E
Ammonia (mg/L)	Raw	Spring	0.03±0.20	1.2±0.20	0.025±1.49	0.009±0.003	0.51±1.63
		Summer	0.5±0.10	1.5±0.25	3±1.72	<0.009±0.002	0.67±1.79
		Winter	0.34±0.17	1.14±0.07	0.053±0.03	<0.009±0.001	1.2±1.85
		Autumn	0.31±0.21	1.03±0.02	0.0041±	0.0054±	4±2.5
	Flocculation	Spring	0.37±0.17	1.64±0.32	0.021±1.16	<0.009±0.009	0.65±1.80
		Summer	0.28±0.15	1.43±0.28	2.34±1.24	0.031±0.009	0.050±1.99
		Winter	0.03±1.99	1.21±0.28	0.046±0.02	<0.010±0.006	2.5±1.68
		Autumn	0.02±0.18	0.88±0.39	0.003±1.34	0.018±0.009	4±2.13
	Sedimentation	Spring	0.33±0.1	0.05±0.02	0.017±0.01	<0.009±0.09	0.51±0.02
		Summer	0.003±0.001	0.056±0.01	1.75±0.02	0.016±0.07	0.051±0.025
		Winter	0.03±0.01	0.04±0.01	0.033±0.02	<0.011±0.06	2.66±1.25
		Autumn	0.1±0.1	0.01±0.1	0.011±	0.016±	3.9±1.07
	Filtration	Spring	0.08±0.039	0.03±0.007	0.010±0.37	<0.009±0	0.60±1.80
		Summer	0.076±0.037	0.02±0.0078	0.75±0.42	0.08±0.005	0.023±2.08
		Winter	0.01±0.04	<0.009±0.07	0.024±0.43	<0.009±0.005	1.3±2.20
		Autumn	0.011±0.04	<0.009±0.07	0.015±0.42	<0.009±0.005	4.1±0.64
	Final treated water	Spring	<0.1±0.07	0.67±0.31	0.005±0.07	<0.009±0.08	0.67±1.85
		Summer	<0.1±0.07	0.068±0.03	0.158±0.08	<0.009±0.08	0.023±2.46
		Winter	<0.003±0.07	0.014±0.37	0.017±0.01	<0.009±0.01	<0.009±0.08
		Autumn	<0.002±0.69	0.037±0.36	0.024±0.08	0.023±0.01	3.5±1.85
	Treated sludge	Spring	1.8±1.47	1.78±5.82	1.95±5.09	1.5±3.52	1.8±0.72
		Summer	2.4±1.79	1.45±6.70	3.7±5.76	2.1±4.04	2.7±0.89
		Winter	0.24±1.78	2.3±6.69	1.5±6.18	1.2±4.19	0.97±0.42
		Autumn	3.8±1.03	13.46±6.84	12.4±5.59	8.6±3.94	1.5±0.62
	Drinking water distribution system	Spring	<0.1±0.05	0.46±0.22	0.05±0.02	N/A	N/A
		Summer	<0.01±0.05	0.058±0.02	0.05±0.018	N/A	N/A
		Winter	<0.002±0.07	0.010±0.26	0.017±0.2	N/A	N/A
		Autumn	<0.001±0.05	0.027±0.25	0.02±0.02	N/A	N/A

4.2. Microbial diversity and dynamics determined using cultural methods

4.2.1. Total coliforms, *E. coli* and *Enterococcus* spp. in Gauteng Province (Sites A, D and E)

4.2.1.1. Site A

Table 4.13 shows the observed MPNs of the total coliforms, *E. coli* and *Enterococcus* spp. from Site A. High total coliform levels were detected in the raw source water, flocculation stage and treated sludge during the study period. The highest concentration of total coliform contamination was >2419.6 MPN/100 mL during spring, summer and autumn in the raw source water, flocculation stage and treated sludge. This was followed by 1299.7 MPN/100 mL during spring and summer in the sedimentation stage. There were variations in the winter season, where the raw source water and the flocculation and filtration stages recorded a 378.4 MPN/100 mL concentration, which was lower than the other seasons. The final treated water and distribution points did not record total coliforms throughout the study period. The recommended limit of total coliform counts in drinking water is ≤10 MPN/100 mL by SANS (2015) and WHO (2022). The *E. coli* count ranged from 2 MPN/100 mL to 62.7 MPN/100 mL during the sampling period, with the highest recorded in the final treated water during autumn. The raw water sources had 16 MPN/100 mL counts during summer, while 53.8 MPN/100 mL was counted in autumn. The flocculation, sedimentation and filtration stages showed the presence of *E. coli*, with 62.7 MPN/100 mL observed in autumn in the flocculation stage. A zero count was recorded in the final treated water at Site A during autumn. The recommended limit of *E. coli* in drinking water is ≤100 MPN/100 mL by SANS (2015) and WHO (2022). *Enterococcus* spp. were also analysed throughout the treatment process at Site A. The raw water source and the flocculation and sedimentation stages recorded *Enterococcus* spp. counts ranging from 1.0 MPN/100 mL to 116.2 MPN/100 mL. The drinking water treatment processes stages, final and distribution system recorded 0 MPN/100 mL in all the seasons, which is within the recommended limit. The treated sludge ranged from 5.0 MPN/100 mL to 27.5 MPN/100 mL of total coliforms, *E. coli,* and *Enterococcus* spp. across all seasons.

Table 4.13: Concentration of total coliforms, *E. coli* and *Enterococcus* spp. in Gauteng Province at Site A treatment stages during the sampling period

Treatment stages	Total coliform (MPN/100 mL)				*E. coli* (MPN/100 mL)				*Enterococcus* spp. (MPN/100 mL)			
	Spring	Summer	Winter	Autumn	Spring	Summer	Winter	Autumn	Spring	Summer	Winter	Autumn
Raw water	>2419.6	>2419.6	378.4	>2419.6	54.6	16	25.6	53.8	20.1	52.1	68.4	116.2
Flocculation	>2419.6	>2419.6	378.4	>2419.6	35.9	9.8	12.2	62.7	4.1	39.5	65.7	109.0
Sedimentation	1299.7	1299.7	47.3	248.1	4.2	2.0	4.1	8.6	0	5.2	1.0	9.7
Filtration	248.1	248.1	378.4	25.9	5.2	3.0	0	2.0	0	0	0	1.0
Final treated water	0	0	0	0	0	0	0	0	0	0	0	0
Distribution	0	0	0	0	0	0	0	0	0	0	0	0
Drinking water treatment sludge	17.5	27.5	16.1	14.4	5.2	10.5	5.0	6.0	11.5	9.2	11.2	5.1

MPN: Most Probable Number

4.3.1.2. Site D

At Site D, total coliform counts of 2419.6 MPN/100 mL were recorded from the raw water source and the flocculation, sedimentation and filtration stages during spring and summer (Table 4.14). In the final treated water, counts of 1.0 MPN/100 mL were recorded during spring and winter, while summer and autumn had zero counts of total coliforms. Above 2419.6 MPN/100 mL total coliforms were recorded for the treated sludge in all seasons. *E. coli* was also detected throughout the four seasons in the raw water source and the flocculation, sedimentation and filtration stages, with the highest amount detected (235.9 MPN/100 mL) in spring. The filtration stage recorded 24.6 MPN/100 mL of *E. coli* during spring and 1.0 MPN/100 mL in summer. The final stage had zero counts of *E. coli* in all seasons, which complies with the WHO (2022) and SANS 214 (SANS, 2015) guidelines of 0 MPN/100 mL. *Enterococcus* spp. was detected in the raw water source in all seasons, with the highest count in summer of 30.9 MPN/100 mL, followed by autumn, with 10.7 MPN/100 mL. The final stage had zero counts of *Enterococcu*s spp. in all seasons, which complies with SANS 241 (SANS, 2015) for drinking water. The treated sludge had 235.9 MPN/100 mL counts in summer, followed by autumn with 159.7 MPN/100 mL, while the other two seasons recorded zero counts.

Table 4.14: Concentration of total coliforms, *E. coli* and *Enterococcus* spp. in Gauteng Province at Site D treatment stages during the sampling period

Treatment stages	Total coliform (MPN/100 mL)				*E. coli* (MPN/100 mL)				*Enterococcus* spp. (MPN/100 mL)			
	Spring	Summer	Winter	Autumn	Spring	Summer	Winter	Autumn	Spring	Summer	Winter	Autumn
Raw water	>2419.6	>2419.6	86.5	214.3	235.9	88.6	11.7	2.0	3.0	30.9	2.0	>2419.6
Flocculation	>2419.6	>2419.6	41.1	179.3	21.8	3.0	4.1	0	1.0	18.7	1.0	>2419.6
Sedimentation	>2419.6	>2419.6	3	45.0	24.6	1	0	0	0	4.1	0	>2419.6
Filtration	>2419.6	>2419.6	86.5	214.3	0	0	0	0	0	0	0	>2419.6
Final treated water	1	0	1	0	1.0	0	0	0	3.0	0	0	0
Distribution	0	0	0	0	0	0	0	0	0	0	0	0
Treated sludge	325.5	344.8	36.8	39.3	74.4	37.6	24.3	37.6	19.6	25.9	17.2	10.0

MPN: Most Probable Number

4.3.1.3. Site E

The raw source water and the pre-chlorination, flocculation, sedimentation and filtration stages all recorded >2419.6 MPN/100 mL at Site E throughout the sampling period (Table 4.15). Despite the presence of total coliforms enumerated from previous process stages, the final stage recorded 0 MPN/100 mL, which complies with SANS 241 (SANS, 2015). Site E shows the presence of *E. coli* in each process stage, with counts ranging from 1.0 MPN/100 mL to >2419.6 MPN/100 mL. The raw source water recorded high counts of 214.6 MPN/100 mL for *E. coli* during the summer, while the pre-chlorination count was 235.9 MPN/100 mL. The flocculation and sedimentation stages had 48.1 MPN/100 mL and 24.6 MPN/100 mL of *E. coli*, respectively. No *E. coli* was detected in the final treated water in summer. In fact, the final treatment stage recorded 0 MPN/100 mL in all seasons, which complies with the SANS 241 (SANS, 2015) drinking water limit and the WHO (2022) drinking water quality guidelines.

SANS 241 (SANS, 2015) recommends zero counts of *Enterococcus* spp. in final drinking water for aesthetic and operational compliance. During the sampling period, the raw water source recorded a count of >2419.6 MPN/100 mL during autumn, while the pre-chlorination process stage recorded 870.4 MPN/100 mL, and the flocculation stage recorded 53.8 MPN/100 mL. A high reading of >2419.6 MPN/100 mL was recorded in the treated sludge during summer and winter, while a low

reading of 61.6 MPN/100 mL was recorded in spring. The final treated water recorded zero counts in all seasons, which shows compliance with SANS 241 (SANS, 2015).

Table 4.15: Concentration of total coliforms, *E. coli* and *Enterococcus* spp. in Gauteng Province at Site E treatment stage during the sampling period

Treatment stages	**Total coliform (MPN/100 mL)**				***E. coli* (MPN/100 mL)**				***Enterococcus* spp. (MPN/100 mL)**			
	Spring	**Summer**	**Winter**	**Autumn**	**Spring**	**Summer**	**Winter**	**Autumn**	**Spring**	**Summer**	**Winter**	**Autumn**
Raw water	>2419.6	>2419.6	>2419.6	>2419.6	34.9	214.2	93.4	67.0	36.4	24.6	185	>2419.6
Flocculation	>2419.6	>2419.6	>2419.6	>2419.6	20.3	48.1	10.2	4.3	16.1	5.3	8.6	53.8
Sedimentation	>2419.6	>2419.6	248.9	>2419.6	1.0	24.6	0	1.0	3.1	2.5	4	7.1
Filtration	>2419.6	>2419.6	76.7	82.2	1.0	17.3	0	0	1.0	0	0	3.0
Final treated water	0	0	0	0	0	0	0	0	0	0	0	0
Distribution	0	0	0	0	0	0	0	0	0	0	0	0
Treated sludge	27.0	19.6	17.6	10.2	2.4	12.4	2.6	6.2	6.6	24.6	29.6	54.1

MPN: Most Probable Number

4.2.2. Total coliforms, *E. coli* and *Enterococcus* spp. in Limpopo Province (Site B)

From the results, the raw water source recorded above 2419.6 MPN/100 mL of total coliforms in all seasons except in winter, when 1732.9 MPN/100 mL was recorded (Table 4.16). In the flocculation stage, total coliforms ranged from zero in autumn to 2419.6 MPN/100 mL in spring. The sedimentation stage recorded low total coliform counts of 177.2 MPN/100 mL during summer and above 2419.6 MPN/100 mL in autumn. During the filtration stage, there was a drop in total coliform counts, with 90.0 MPN/100 mL being the highest in autumn compared to the preceding process stages. The final treated water and distribution stage recorded zero counts of total coliforms, which shows compliance with SANS 241 (SANS, 2015). The *E. coli* counts varied throughout the study period ($P>0.05$), with the highest count of 7.1 MPN/100 mL recorded at the sedimentation stage in winter. The raw water source recorded 25.1 MPN/100 mL and 35.2 MPN/100 mL counts during spring and summer, while in winter, it was 5.0 MPN/100 mL and 12.0 MPN/100 mL in autumn. The final treated water and distribution point recorded zero counts throughout the seasons. The treated sludge stage recorded above 85.5 MPN/100 mL for total coliforms, while at the same stage, the *E.*

coli concentration was 20.9 MPN/100 mL during spring and summer, and *Enterococcus* spp. was 25.9 MPN/100 mL in winter and autumn.

Enterococcus spp. counts ranged from zero to 30.7 MPN/100 mL between the raw water source and the final treated water stage. In the raw water source, *Enterococcus* spp. counts ranged from 11.0 MPN/100 mL in summer to 19.3 MPN/100 mL in winter. These counts ranged from 1.0 MPN/100 mL during spring to 19.9 MPN/100 mL during winter in the flocculation stage. The distribution stage had the highest *Enterococcus* spp. counts of 2.0 MPN/100 mL during autumn, 1.0 MPN/100 mL during both spring and summer and zero counts during winter. Zero counts were recorded in the final treated water in all seasons.

Table 4.16: Concentration of total coliforms, *E. coli* and *Enterococcus* spp. in Limpopo Province at Site B treatment stages during the sampling period

Treatment stages	**Total coliform (MPN/100 mL)**				***E. coli* (MPN/100 mL)**				***Enterococcus* spp. (MPN/100 mL)**			
	Spring	**Summer**	**Winter**	**Autumn**	**Spring**	**Summer**	**Winter**	**Autumn**	**Spring**	**Summer**	**Winter**	**Autumn**
Raw water	>2419.6	>2419.6	1732.9	>2419.6	5.1	5.1	1	2.0	2.0	11.0	19.3	17.5
Flocculation	>2419.6	648.8	1986.3	0	0	6.3	4.1	1.0	1.0	7.1	19.9	6.3
Sedimentation	1299.7	177.2	1203.3	>2419.6	4.1	16	7.1	0	2	1.0	21.6	8.5
Filtration	14.4	79.4	82.3	90.0	2	2	2	3.1	0	0	6.1	31.7
Final treated water	0	0	0	0	0	0	0	0	0	0	0	0
Distribution	0	0	0	0	0	0	0	0	1.0	1.0	0	2.0
Treated sludge	56.8	85.5	48.7	66.3	18.3	20.9	14.7	17.0	25.9	24.5	17.1	19.7

MPN: Most Probable Number

4.2.3. Total coliforms, *E. coli* and *Enterococcus* spp. in Mpumalanga Province (Site C)

The total coliform count was above 2419.6 MPN/100 mL in the raw water source during spring, summer and autumn, except in winter, when it was 36.6 MPN/100 mL (Table 4.17). The flocculation stage recorded a total coliform count above 2419.6 MPN/100 mL in spring, summer and autumn, except in winter when it was 83.3 MPN/100 mL. The sedimentation and filtration stages also recorded total coliforms throughout the sampling seasons. The final treated water stage recorded a high total coliform count of 257.2 MPN/100 mL during winter, while the distribution stage recorded 0 MPN/100 mL throughout the seasons. The raw water source and the flocculation, sedimentation and filtration stages recorded *E. coli* concentrations ranging from zero to 47.9 MPN/100 mL

throughout the seasons. The final treated water and the distribution stage recorded zero counts of *E. coli* during the year, which is within the SANS 241 (SANS, 2015) drinking water standard.

For *Enterococcus* spp., the raw water source ranged from 10.9 MPN/100 mL to 33.0 MPN/100 mL throughout the study period. The flocculation stage during spring recorded 8.6 MPN/100 mL of *Enterococcus* spp., which was higher compared to other seasons. In the sedimentation stage, *Enterococcus* spp. counts ranged from 1.0 MPN/100 mL to 2.0 MPN/100 mL during the seasons. The filtration stage and final treatment water recorded only 4.1 MPN/100 mL and 3.1 MPN/100 mL of *Enterococcus* spp., respectively. The distribution stage had zero counts in all seasons, which is within the SANS 241 (SANS, 2015) drinking water standard. The treated sludge stage recorded *Enterococcus* spp. above 65.7 MPN/100 mL in summer and 58.1 MPN/100 mL in winter.

Table 4.17: Concentration of total coliforms, *E. coli* and *Enterococcus* spp. in Mpumalanga Province at Site C treatment stages during the sampling period

Treatment stages	**Total coliform (MPN/100 mL)**				***E. coli* (MPN/100 mL)**				***Enterococcus* spp. (MPN/100 mL)**			
	Spring	**Summer**	**Winter**	**Autumn**	**Spring**	**Summer**	**Winter**	**Autumn**	**Spring**	**Summer**	**Winter**	**Autumn**
Raw water	>2419.6	>2419.6	36.6	>2419.6	39.9	59.5	5.1	19.7	18.9	33	10.9	21.6
Flocculation	>2419.6	>2419.6	83.1	>2419.6	34.5	2	6.3	11.0	8.6	5.2	3.1	4.1
Sedimentation	1299.7	1299.7	25.8	59.4	47.9	5.2	1	2.0	2	1	2	1.0
Filtration	248.1	248.1	69.7	87.8	3.1	1	0	0	4.1	0	0	0
Final treated water	2	2	257.2	49.6	0	0	0	0	3.1	0	0	1.0
Distribution	0	0	0	0	0	0	0	0	0	0	0	0
Treated sludge	165.5	304.4	105.8	99.1	79.8	75.5	71.7	78.0	65.0	65.7	58.1	58.9

MPN: Most Probable Number

4.3. Antibiotic resistance analysis of bacteriological indicators

Antibiotic susceptibility profiling of the bacteriological indicators (*E. coli* and *Enterococcus* spp.) isolated from different treatment stages during spring, summer, winter, and autumn was done to provide insight into their response to antibiotics. A total of 121 confirmed isolates were subjected to susceptibility testing against a panel of ten empiric antibiotics. Results were interpreted according to CLSI standards (CLSI, 2018). The results showed that out of 121 isolates, 15 were not MDR, with

three isolates from *E. coli* and 12 isolates from *Enterococcus* spp.

4.3.1. Antibiotic-resistance analysis of bacteriological indicators in Gauteng Province (Sites A, D and E)

4.3.1.1. Site A

Figure 4.1 illustrates antibiotic-resistant trends of *E. coli* isolates (raw_14, sedimentation_4 and filtration_1) from different treatment stages during spring, summer, winter, and autumn. A large percentage (between 55% and 100%) of isolates were resistant to ßeta-lactam antibiotics (ampicillin and amoxicillin/clavulanic acid) during summer, followed by winter, then spring and autumn. In addition, between 45% and 80% of the isolates were resistant to trimethoprim/sulfamethoxazole and nitrofurantoin across the seasons. Between 30% and 70% of the isolates were resistant to erythromycin, imipenem, and levofloxacin, while ciprofloxacin and vancomycin ranged between 20% and 50% throughout the seasons. *Enterococcus* spp. isolates (raw_10, sedimentation_2, filtration_1 and final water_0) were resistant to the same antibiotics (Figure 4.2). Among these isolates, 70% to 90% were resistant to ampicillin and vancomycin.

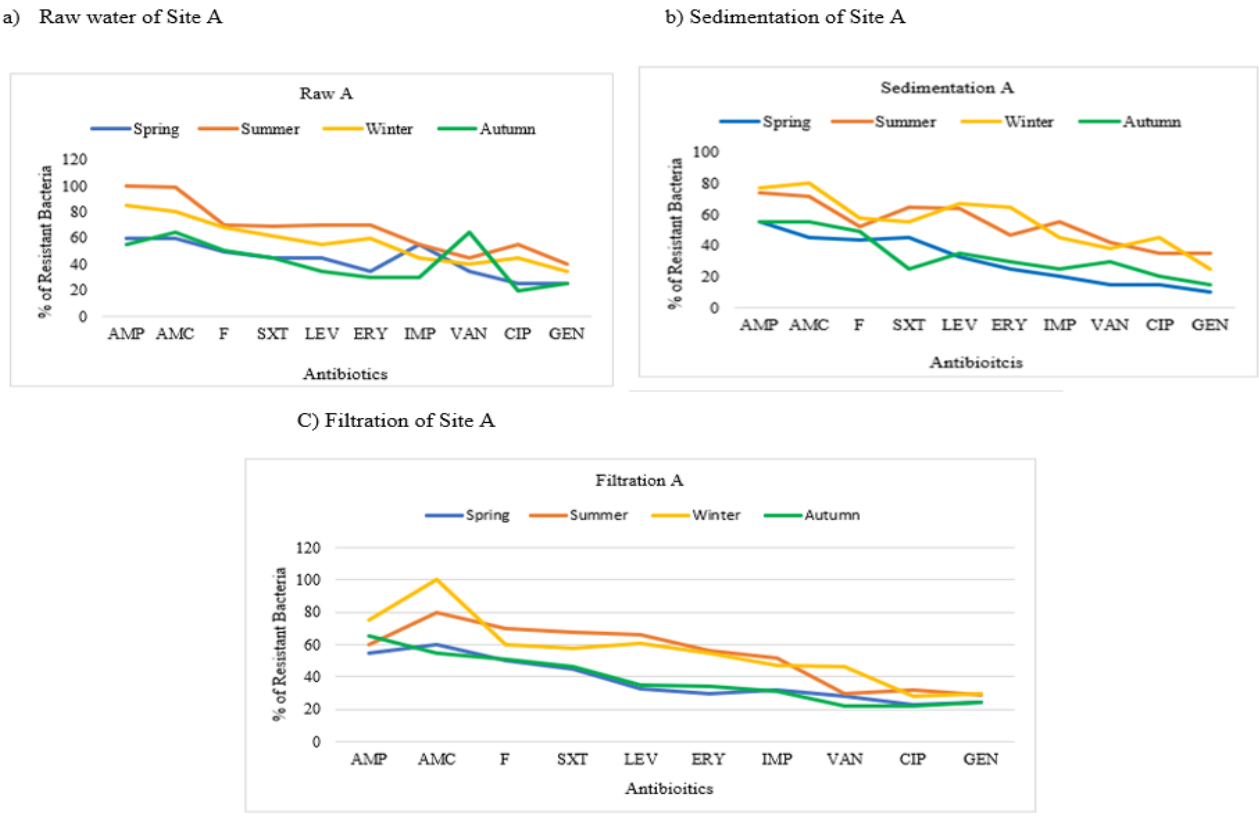
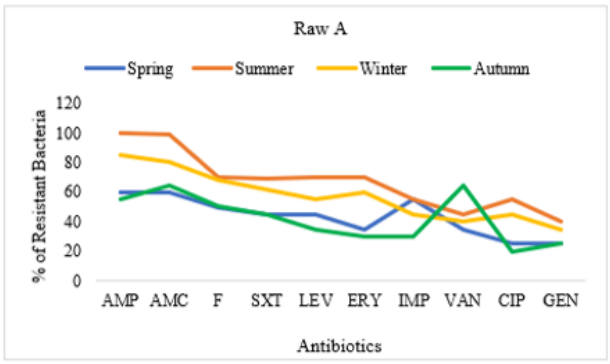


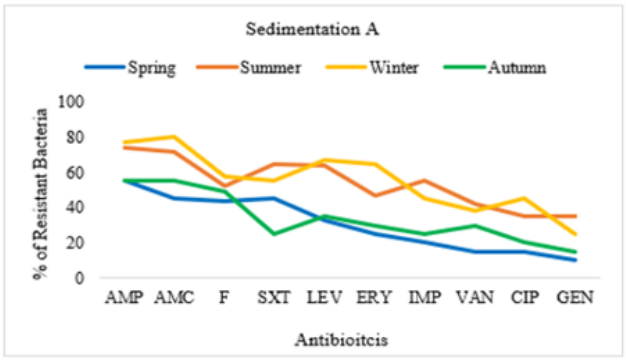
Figure 4.1: Antibiotic resistance trends for *E. coli* isolates from Site A treatment stages: a) raw water, b) sedimentation stage and c) filtration stage

AMP: Ampicillin; AMC: Amoxicillin/clavulanic acid; F: Nitrofurantoin; SXT: Trimethoprim/Sulphamethoxazol; LEV: Levofloxacin; ERY: Erythromycin; IMP: Imipenem; VAN: Vancomycin; CIP: Ciprofloxacin; GEN: Gentamicin

a) Raw water of Site A



b) Sedimentation of Site A



C) Filtration of Site A

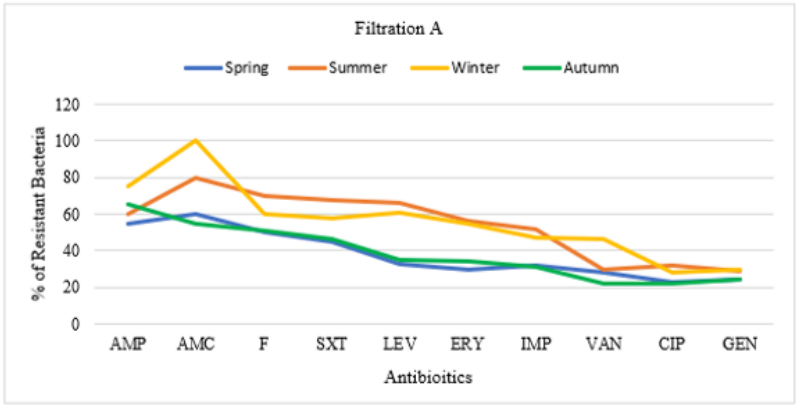


Figure 4.2: Antibiotic resistance trends for *Enterococcus* spp. isolates from Site A treatment stages: a) raw water, b) sedimentation stage and c) filtration stage

AM: Ampicillin; VA: Vancomycin; AMC: Amoxicillin/clavulanic acid; F: Nitrofurantoin; SXT: Trimethoprim/Sulphamethoxazol; LEV: Levofloxacin; E: Erythromycin; IMP: Imipenem; CIP: Ciprofloxacin; GEN: Gentamicin

4.4.1.2. Site D

Figure 4.3 shows the antibiotic-resistant trends for the *E. coli* isolates (raw_4, sedimentation_3 and filtration_1) from Site D throughout the sampling period. A large percentage of isolates (between 75% and 90%) were resistant to imipenem and amoxicillin/clavulanic acid during summer and winter, followed by autumn and spring. In addition, 50% and 85% of the isolates were resistant to trimethoprim/sulfamethoxazole and nitrofurantoin across the seasons. Between 30% and 55% of the isolates were resistant to erythromycin, imipenem, and levofloxacin, while ciprofloxacin and vancomycin ranged between 20% and 50% throughout the seasons. The *Enterococcus* spp. isolates (raw_5, sedimentation_3, filtration_1 and final water_0) were resistant to the same antibiotics (Figure 4.4). Among these isolates, 55% to 75% were resistant to ampicillin and vancomycin.

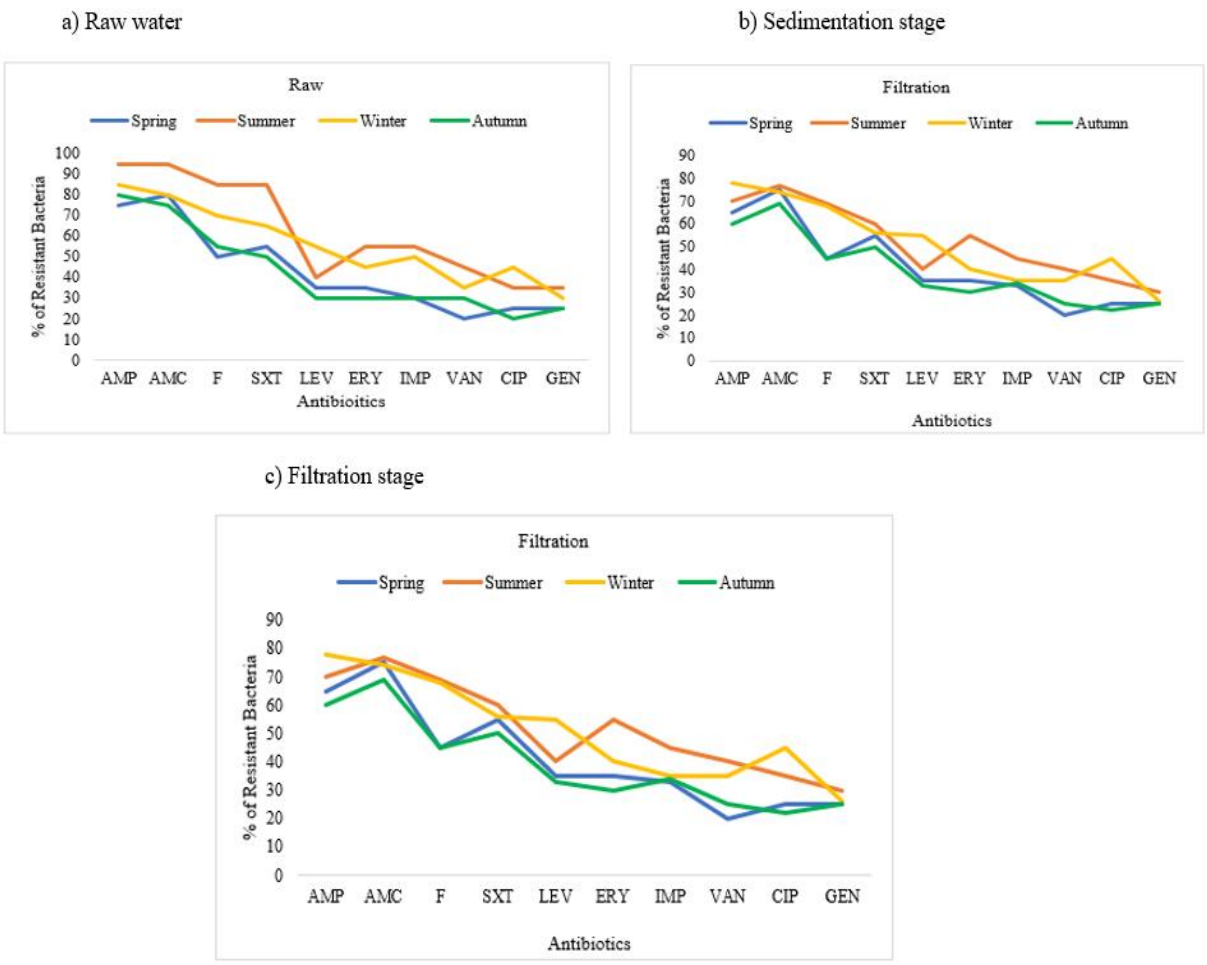


Figure 4.3: Antibiotic resistance trends for *E. coli* isolates from Site D treatment sites: a) raw water, b) sedimentation stage and c) filtration stage

AMP: Ampicillin; AMC: Amoxicillin/clavulanic acid; F: Nitrofurantoin; SXT: Trimethoprim/Sulphamethoxazol; LEV: Levofloxacin; ERY: Erythromycin; IMP: Imipenem; VAN: Vancomycin; CIP: Ciprofloxacin; GEN: Gentamicin

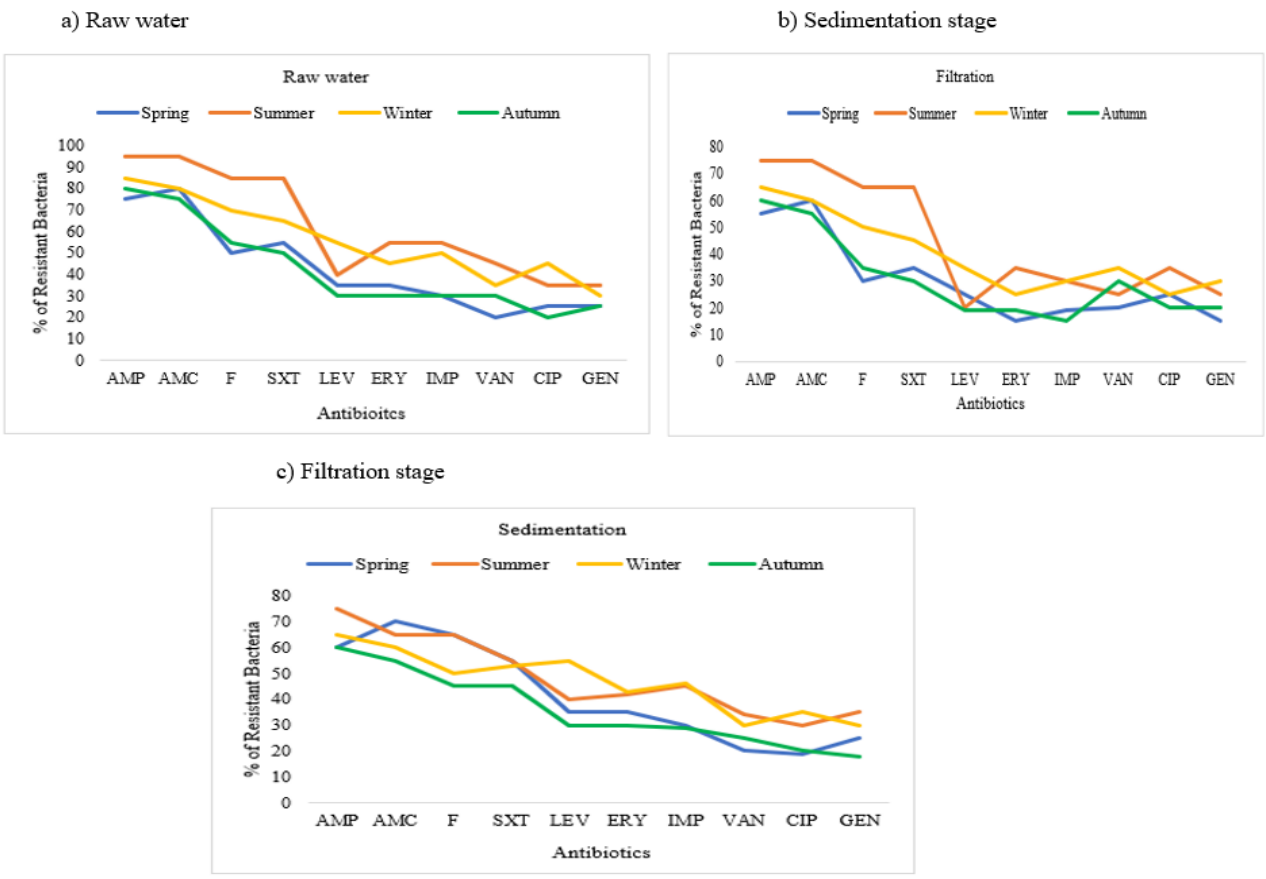


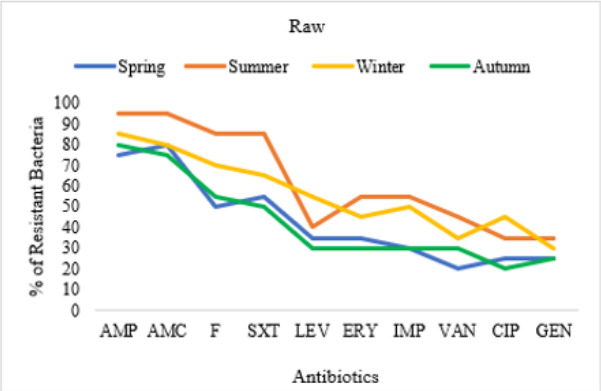
Figure 4.4: Antibiotic resistance trends for *Enterococcus* spp. isolates from Site D treatment stages: a) raw water, b) sedimentation stage and c) filtration stage

AMP: Ampicillin; AMC: Amoxicillin/clavulanic acid; F: Nitrofurantoin; SXT: Trimethoprim/sulphamethoxazol; LEV: Levofloxacin; ERY: Erythromycin; IMP: Imipenem; VAN: Vancomycin; CIP: Ciprofloxacin; GEN: Gentamicin

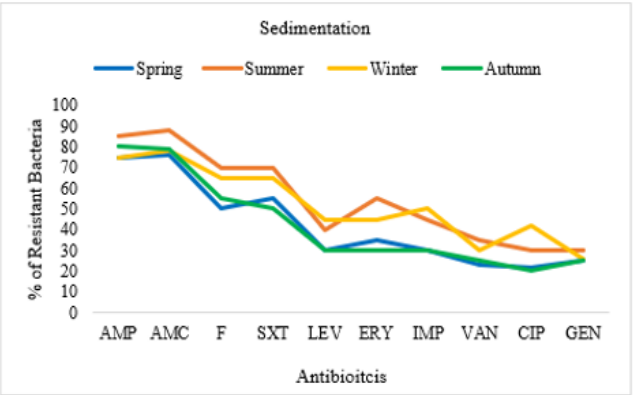
4.4.1.3. Site E

Figure 4.5 shows the antibiotic-resistant trends for the *E. coli* isolates (raw_10, sedimentation_6 and filtration_2) from Site E throughout the sampling period. A large percentage of isolates (between 80% and 100%) were resistant to gentamicin, imipenem, and trimethoprim/sulfamethoxazole. In addition, 60% and 75% of the isolates were resistant to ßeta-lactam antibiotics (ampicillin and erythromycin) and nitrofurantoin during summer and winter, followed by autumn and spring. Between 30% and 60% of the isolates were resistant to amoxicillin/clavulanic acid, while between 10% to 29% of the isolates were resistant to ampicillin and imipenem throughout the seasons. The *Enterococcus* spp. isolates (raw_8, sedimentation_5 and filtration_1) were resistant to the same antibiotics (Figure 4.6). Among these isolates, 80% to 100% were resistant to ciprofloxacin, erythromycin, and gentamicin.

a) Raw water



b) Sedimentation stage



c) Filtration stage

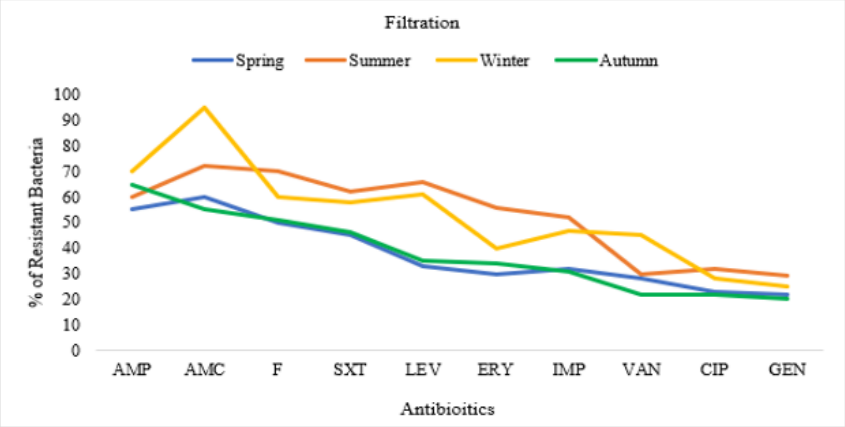


Figure 4.5: Antibiotic resistance trends for the *E. coli* isolates from Site E treatment stages: a) raw water, b) sedimentation stage and c) filtration stage

AMP: Ampicillin; AMC: Amoxicillin/clavulanic acid; F: Nitrofurantoin; SXT: Trimethoprim/sulphamethoxazol; LEV: Levofloxacin; ERY: Erythromycin; IMP: Imipenem; VAN: Vancomycin; CIP: Ciprofloxacin; GEN: Gentamicin

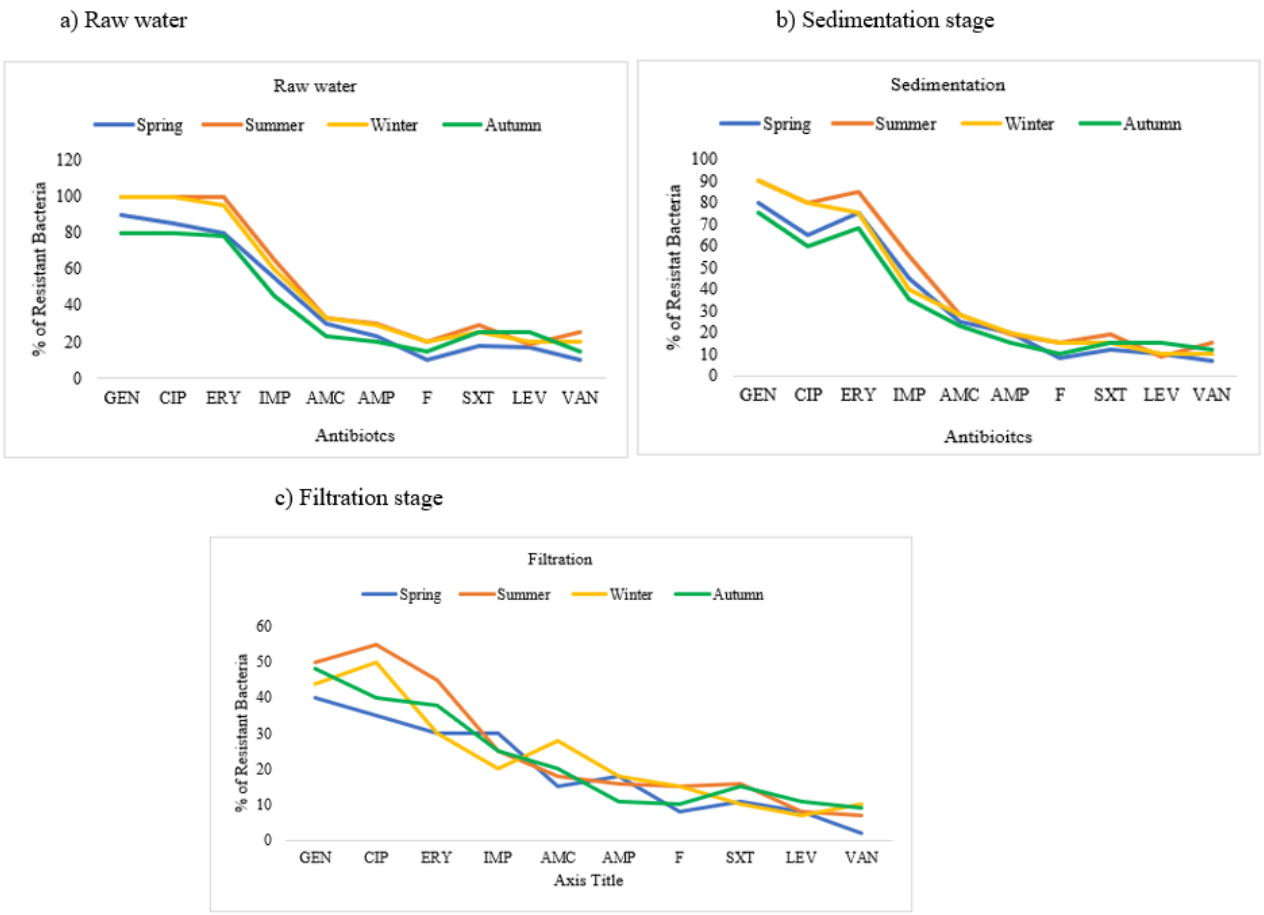


Figure 4.6: Antibiotic resistance trends for the *Enterococcus* spp. isolates from Site E treatment stages: a) raw water, b) sedimentation stage and c) filtration stage

GEN: Gentamicin; CIP: Ciprofloxacin; ERY: Erythromycin; IMP: Imipenem; AMC: Amoxicillin/clavulanic acid; AMP: Ampicillin; F: Nitrofurantoin; SXT: Trimethoprim/sulphamethoxazol; LEV: Levofloxacin; VAN: Vancomycin

4.3.2. Antibiotic-resistance analysis of bacteriological indicators in Limpopo Province (Site B)

The antibiotic-resistant trends of the *E. coli* isolate (raw_12, sedimentation_4 and filtration_3) from Site B during spring, summer, winter, and autumn are shown in Figure 4.7. A large percentage of isolates (between 60% and 80%) were resistant to ßeta-lactam antibiotics (ampicillin and imipenem) during summer, followed by winter, spring and autumn. In addition, 30% and 60% of isolates were resistant to trimethoprim/sulfamethoxazole, erythromycin, and nitrofurantoin across the seasons. Between 10% and 30% of the isolates from treatment stages were resistant to amoxicillin/clavulanate, ciprofloxacin, gentamicin, and levofloxacin throughout the seasons. The *Enterococcus* spp. isolates (raw_5, sedimentation_6 and filtration_4) were resistant to the same antibiotics (Figure 4.8). Among these isolates, 30% to 70% were resistant to ciprofloxacin, imipenem, and vancomycin. Between 10% and 30% were resistant to amoxicillin/clavulanate, ciprofloxacin, gentamicin, and levofloxacin.

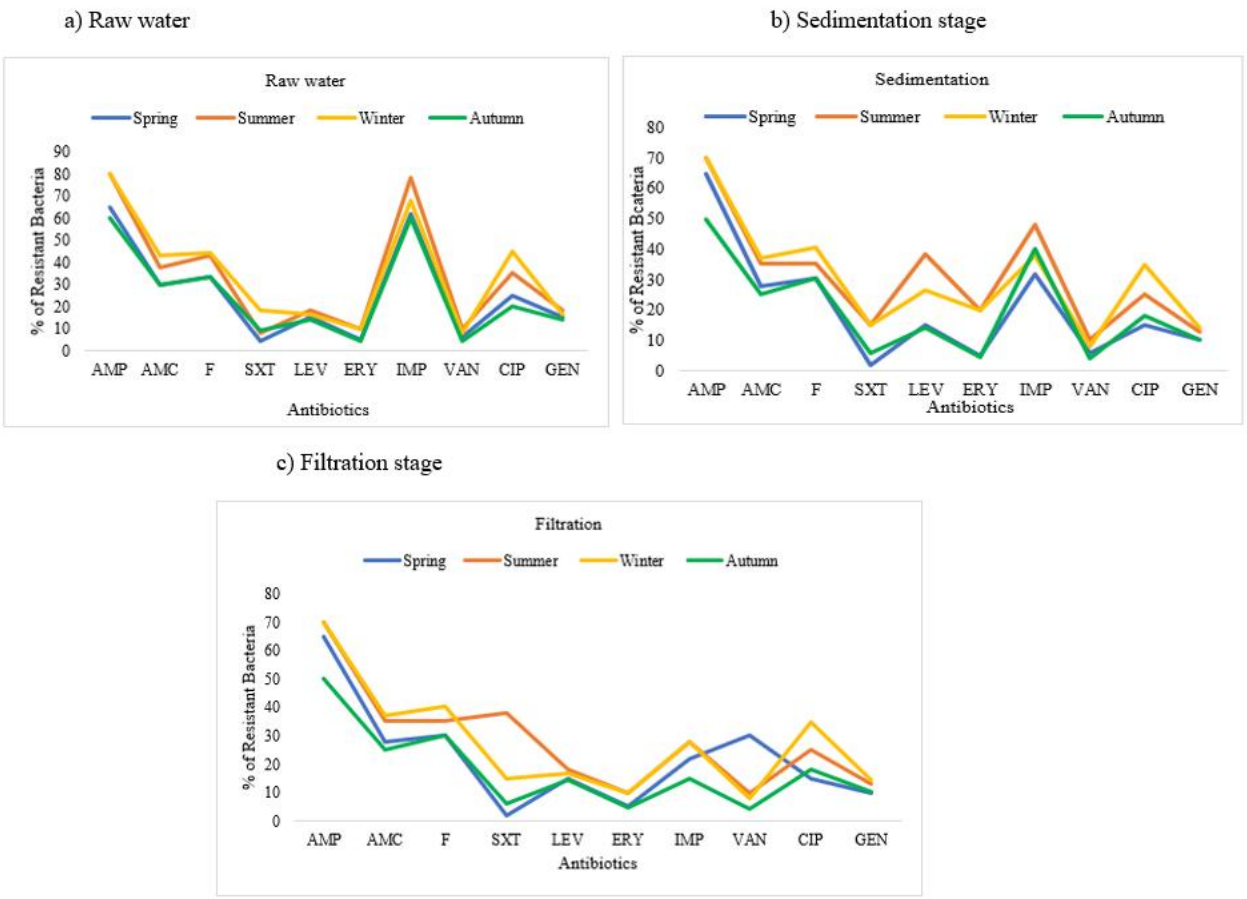


Figure 4.7: Antibiotic resistance trends for the *E. coli* isolates from Site B treatment stages: a) raw water, b) sedimentation stage and c) filtration stage

AMP: Ampicillin; AMC: Amoxicillin/clavulanic acid; F: Nitrofurantoin; SXT: Trimethoprim/sulphamethoxazol; LEV: Levofloxacin; ERY: Erythromycin; IMP: Imipenem; VAN: Vancomycin; CIP: Ciprofloxacin; GEN: Gentamicin

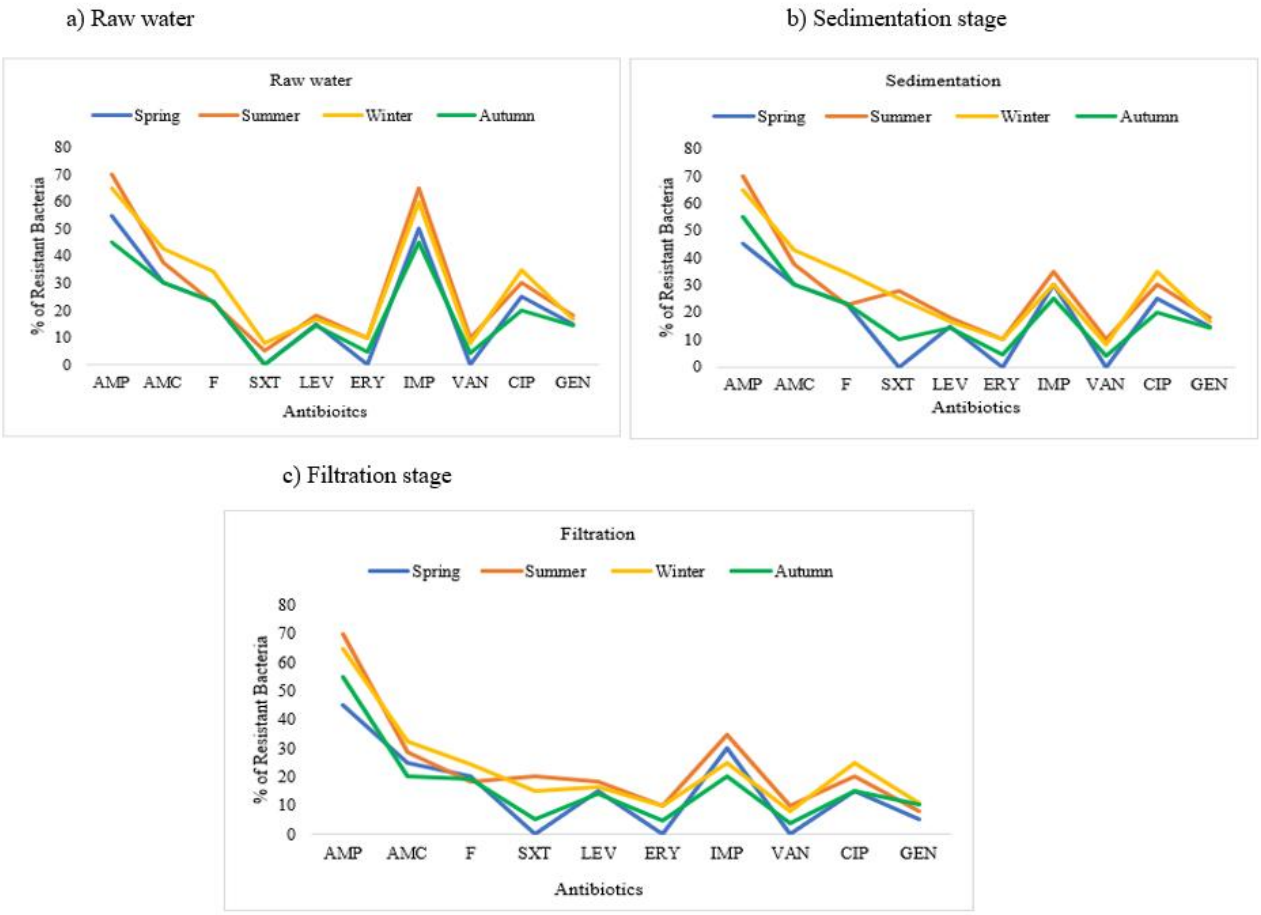


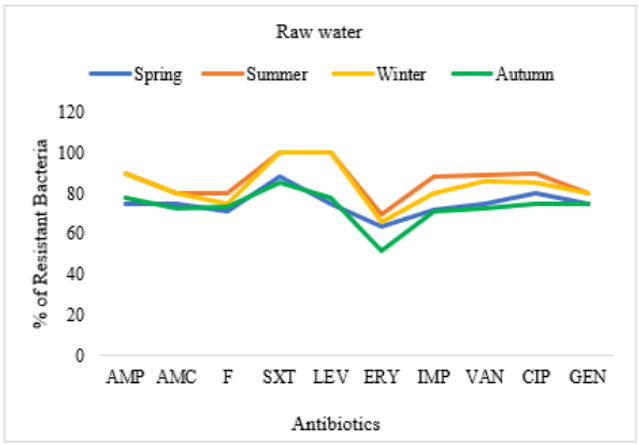
Figure 4.8: Antibiotic resistance trends for the *Enterococcus* spp. isolates from Site B treatment stages: a) raw water, b) sedimentation stage and c) filtration stage

AMP: Ampicillin; AMC: Amoxicillin/clavulanic acid; F: Nitrofurantoin; SXT: Trimethoprim/sulphamethoxazol; LEV: Levofloxacin; ERY: Erythromycin; IMP: Imipenem; VAN: Vancomycin; CIP: Ciprofloxacin; GEN: Gentamicin

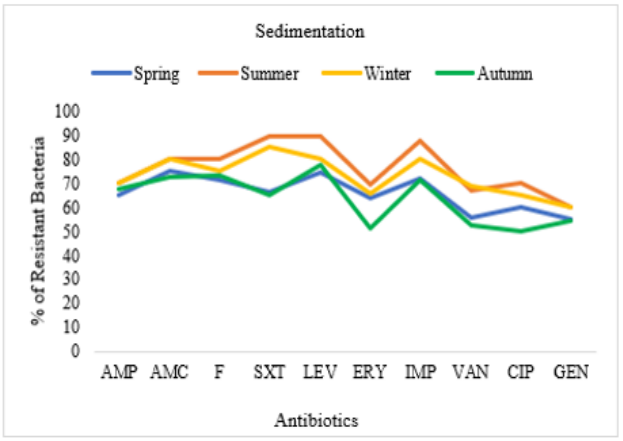
4.3.3. Antibiotic-resistance analysis of bacteriological indicators in Mpumalanga Province (Site C)

Figure 4.9 shows the antibiotic-resistant trends of *E. coli* isolates (raw_13, sedimentation_7 and filtration_3) from Site C throughout the sampling period. A large percentage of the isolates (70% to 100%) were resistant to ßeta-lactam antibiotics (ampicillin and imipenem), trimethoprim/sulfamethoxazole, amoxicillin/clavulanate, ciprofloxacin, gentamicin, levofloxacin, and nitrofurantoin during summer, followed by winter, spring and autumn. In addition, 30% to 70% of the isolates were resistant to erythromycin and nitrofurantoin across the seasons. The *Enterococcus* spp. isolates (raw_6, sedimentation_6 and filtration_4) were resistant to the same antibiotics (Figure 4.10). Among these isolates, 70% and 100% were resistant to ciprofloxacin, imipenem, amoxicillin/clavulanate, ciprofloxacin, gentamicin, levofloxacin, and vancomycin.

a) Raw water



b) Sedimentation stage



c) Filtration stage

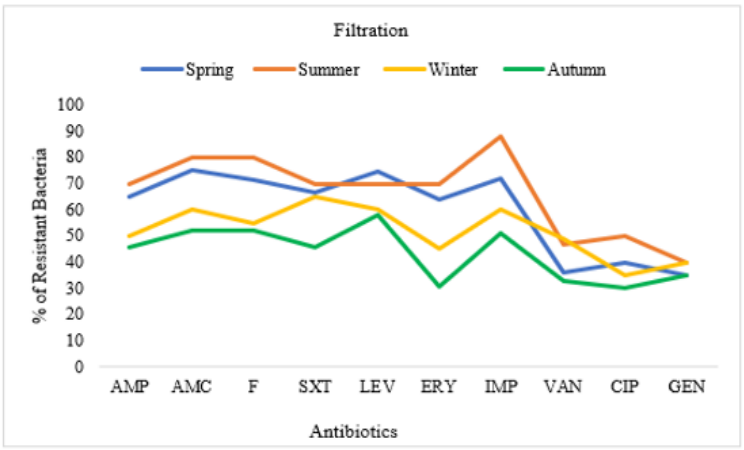


Figure 4.9: Antibiotic resistance trends for the *E. coli* isolates from Site C treatment stages: a) raw water, b) sedimentation stage and c) filtration stage

AMP: Ampicillin; AMC: Amoxicillin/clavulanic acid; F: Nitrofurantoin; SXT: Trimethoprim/sulphamethoxazol; LEV: Levofloxacin; ERY: Erythromycin; IMP: Imipenem; VAN: Vancomycin; CIP: Ciprofloxacin; GEN: Gentamicin

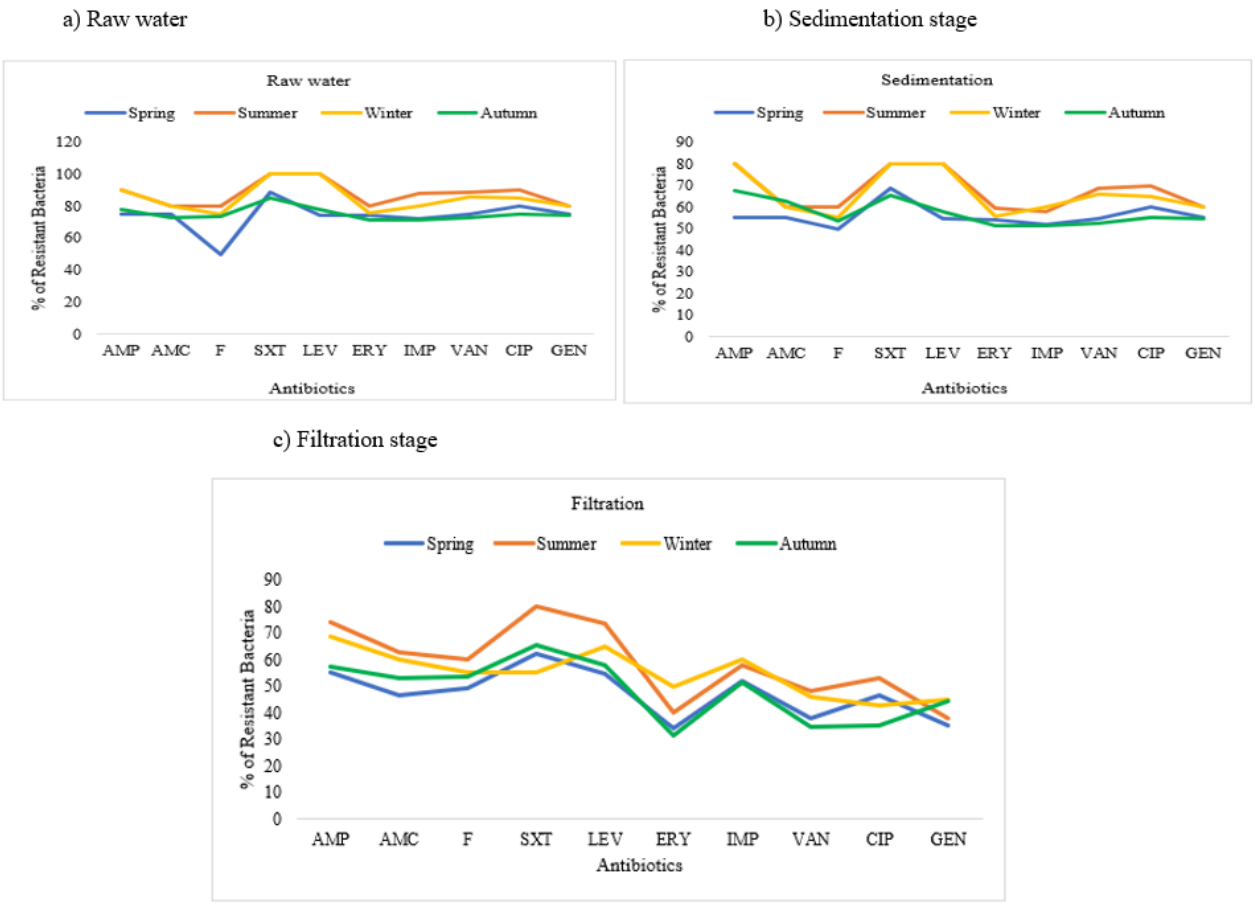


Figure 4.10: Antibiotic resistance trends for the *Enterococcus* spp. isolates from Site C treatment stages: a) raw water, b) sedimentation stage and c) filtration stage

AMP: Ampicillin; AMC: Amoxicillin/clavulanic acid; F: Nitrofurantoin; SXT: Trimethoprim/sulphamethoxazol; LEV: Levofloxacin; ERY: Erythromycin; IMP: Imipenem; VAN: Vancomycin; CIP: Ciprofloxacin; GEN: Gentamicin

4.4. Multiple antibiotic resistance-index analysis

The MAR-index was calculated as (x/y), where 'x' is the number of antibiotics to which the isolate was resistant, and 'y' is the number of antibiotics to which the isolate was exposed. An isolate from the area of high antibiotic usage will demonstrate an MAR-index of >0.2, while one with a value of <0.2 is from a source of lower antibiotic usage (Alawi *et al.*, 2024).

4.4.1. Multiple antibiotic resistance indices for Gauteng Province (Sites A, D and E)

At Site A, (n=29; MAR isolates=103) were resistant to more than four of ampicillin, erythromycin, ciprofloxacin, nitrofurantoin, amoxicillin/clavulanate, gentamicin, imipenem, trimethoprim/sulfamethoxazole, levofloxacin and vancomycin. The Site A MAR-index value was 0.28, as shown in Table 4.18. When breaking it down, the raw water source had a value of 0.087 in

summer, which makes it 30% of the total MAR-index, and a value of 0.058 (20% of the total MAR-index) in winter, as shown in Figure 4.11.

At Site D, (n=12; MAR isolates=103) were resistant to more than four antibiotics. The MAR-index value was 0.12. When breaking down, the raw water source had a value of 0.0378 in winter, which makes it 31.5% of the total MAR-index, and a value of 0.0225 in summer, which is 18.75% of the total MAR-index (Figure 4.11).

At Site E, (n=11; MAR isolates=103) were resistant to more than four antibiotics. The MAR-index value was 0.10 throughout the study period. The raw water source had a value of 0.03 in summer, which makes it 30% of the total MAR-index, and a value of 0.023 in winter, making it 23% of the total MAR-index (Figure 4.11).

Table 4.18: Multiple antibiotic resistance (MAR) indices for the different sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) throughout the study period

Sampling site	MAR-index	% of MAR
Site A	0.28	28.2
Site B	0.23	23.3
Site C	0.25	25.24
Site D	0.12	11.65
Site E	0.10	10.68

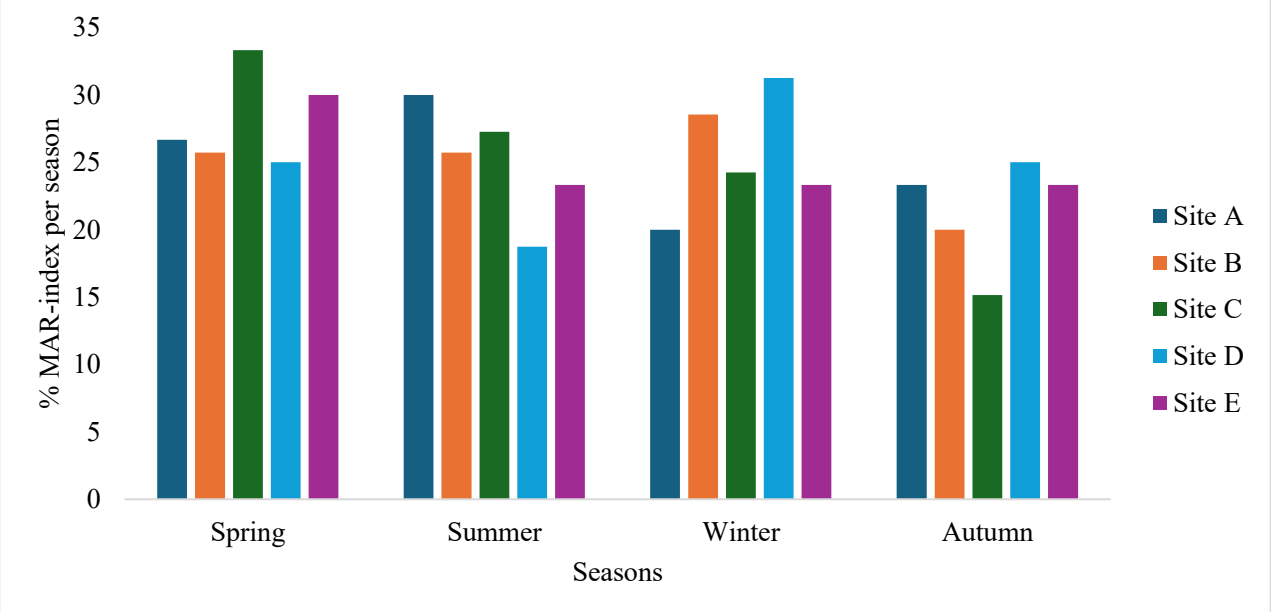


Figure 4.11: Summary of Multiple antibiotic resistance (MAR) indices for the different sites in Gauteng (A, D and E), Limpopo (B) and Mpumalanga (C) by season.

4.4.2. Multiple antibiotic resistance indices for Limpopo Province (Site B)

At Site B in Limpopo Province, (n=23 of 103 isolates) were resistant to more than four of ampicillin, erythromycin, ciprofloxacin, nitrofurantoin, amoxicillin/clavulanate, gentamicin, imipenem, trimethoprim/sulfamethoxazole, levofloxacin, and vancomycin. The MAR-index value was 0.23, as shown in Table 4.18. When breaking it down, the raw water source had a MAR-index value of 0.066 in the summer, which makes it 28.56 % of the total MAR-index, and a value of 0.046 (20%) in winter, as shown in Figure 4.11.

4.4.3. Multiple antibiotic resistance indices for Mpumalanga Province (Site C)

At Site C, (n=25 of 103 isolates) were resistant to more than four of ampicillin, erythromycin, ciprofloxacin, nitrofurantoin, amoxicillin/clavulanate, gentamicin, imipenem, trimethoprim/sulfamethoxazole, levofloxacin, and vancomycin. The MAR-index value was 0.25, as shown in Table 4.18. When breaking it down, the raw water source had a MAR-index value of 0.083 in summer, which makes it 33.33 % of the total MAR-index, and a value of 0.038 (15.15%) in autumn, as shown in Figure 4.11.

4.5. Microbial diversity and dynamics evaluation using shotgun metagenomics

A total of 75 158 1663 quality reads (ranging from 768 292 to 78 183 780) were obtained from all the samples based on whole genome community analysis of all the sites. Good's coverage across the samples was greater than 98%, indicating that the sampling depth was sufficient to estimate the microbial diversity encompassing all major microbial groups present in the water samples collected from the DWTPs (Mahajna *et al*., 2022).

The alpha diversity indices of the microbial communities from the different samples are presented for each province and site. A total of 190 844 OTUs (ranging from 1628 to 9814) were obtained from all the sampling sites (A, B, C, D and E). Both species richness (OTU, Chao1 and Evenness) and diversity estimates (Shannon and Simpson indices) varied among the sampling sites' treatment stages (raw water source, filtration, disinfection, final treated water and treated sludge).

4.5.1. Microbial diversity and dynamics evaluation in Gauteng Province (Sites A, D and E)

DWTPs in Gauteng were analysed for alpha diversity indices of microbial communities (Table 4.19). The highest diversity indices according to the Shannon index were observed at the disinfection stage of both Site A (2.13) and Site D (1.94), while at Site E, it was at the filtration stage with 2.98. The overall biodiversity indices demonstrated the varied impacts of treatment stages on microbial

community diversity across the sampling sites. Across the Gauteng sites, phyla, classes and genera from three kingdoms (Archaea, Bacteria and Eukaryota) were detected.

Table 4.19: Alpha diversity indices for Gauteng Province (Sites A, D and E) treatment stages

Sites	Treatment stages	Alpha diversity indices						
		Reads	OTUs	% Coverage	Evenness	Shannon	InvSimpson	chao1
A	Raw water source	18894583	7822	99.87	0.09	0.79	1.26	8995.89
	Filtration	17645561	8757	99.83	0.09	0.86	1.28	9853
	Disinfection	55181798	9615	99.87	0.23	2.13	2.32	10618
	Final treated water	24271042	8807	99.85	0.15	1.33	1.63	9658.58
	Treated sludge	768292	1628	99.8	0.27	2.03	2.59	2448.51
D	Raw water source	5784777	6095	99.93	0.12	1.05	1.33	7870.1
	Filtration	23525604	8329	99.88	0.14	1.25	1.45	9757.49
	Disinfection	2713003	5686	99.93	0.22	1.94	2.02	6959.14
	Final treated water	21759126	9025	99.87	0.19	1.76	1.81	10083.8
	Treated sludge	21607162	7416	99.84	0.11	1	1.35	8534.84
E	Raw water source	50495462	9352	99.88	0.21	1.95	2.29	10194.0
	Filtration	44304102	6852	99.89	0.34	2.98	5.52	8106.06
	Disinfection	33312506	6285	99.94	0.27	2.36	2.73	7040.87
	Final treated water	48321716	8555	99.86	0.22	1.99	2.27	9475.47
	Treated sludge	78183780	9814	99.89	0.16	1.45	1.61	10450.7

OTU: operational taxonomic unit

4.5.1.1. Archaea phyla diversity in Gauteng Province (Sites A, D and E)

The different treatment stages influenced the relative abundance of the phyla, with variation across the stages. At Site A, Nitrososphaerota constituted the major Archaea phylum in the raw water source and filtration stage. At the same site, Euryarchaeota was abundant in the disinfection stage, treated sludge and final treated water (Figure 4.12). At Site D, Nitrososphaerota was abundant in the raw water source, treated sludge and the filtration and disinfection stages, whereas Euryarchaeota was abundant in the final treated water. For Site E, Euryarchaeota and Nitrososphaerota constituted the major Archaea phyla, with Nitrososphaerota occurring more in the final treated water and Euryarchaeota in the treated sludge and disinfection stage.

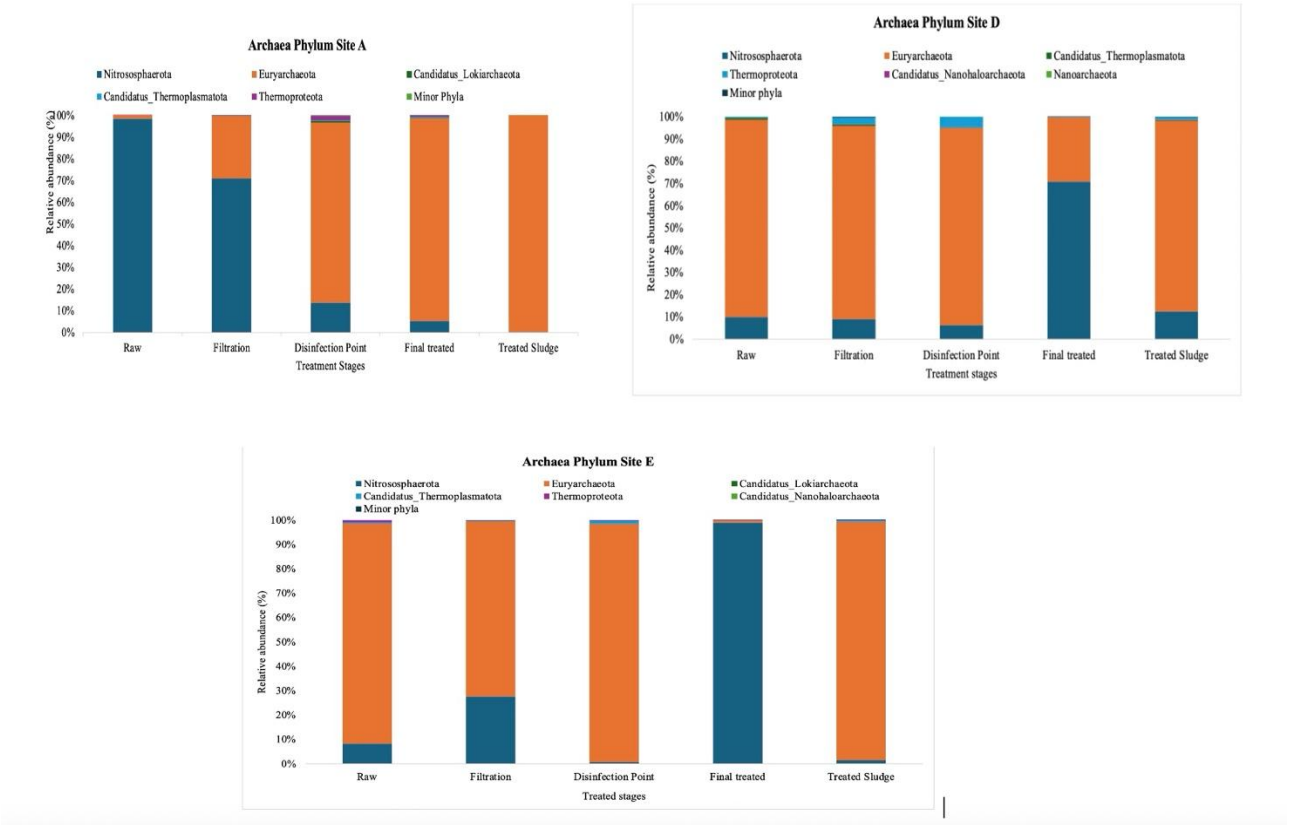


Figure 4.12: Relative abundance of Archaea phyla in Gauteng Province at Sites A, D and E, showing the top eight most abundant phyla

4.5.1.2. Archaea classes diversity in Gauteng Province (Sites A, D and E)

The different treatment stages influenced the relative abundance of the Archaea classes, with variation across the stages. At Site A, Halobacteria and Methanomicrobia constituted the major Archaea classes in the raw water source, the filtration and disinfection stages, final treated water and treated sludge (Figure 4.13). At Site D, Halobacteria was abundant in the raw water source, the filtration and disinfection stages, and the treated sludge, whereas Methanomicrobia showed some abundance in all stages. At Site E, Methanomicrobia constituted the major Archaea class, with Halobacteria occurring more in the disinfection stage and final treated water. Halobacterium is the most successful microbial group able to thrive in multiple conditions, including hypersaline conditions, high levels of radiation, vacuum, extreme cold, utilisation or growth in the presence of caustic materials such as perchlorate and oxygen deprivation (Wu *et al*., 2022; Ma *et al*., 2025). The findings of this study attest to this, with Site E recording ammonia levels in the raw water source ranging from 0.51±1.63 mg/L to 4±2.5 mg/L. The high ammonia levels could be attributed to pollution from sewerage, agriculture runoff and domestic waste, which may affect water quality and have health implications (Zhang *et al*., 2024c). These allow microbial communities such as Archaea to thrive, with Halobacterium detected within different stages of DWTPs and treated sludge.

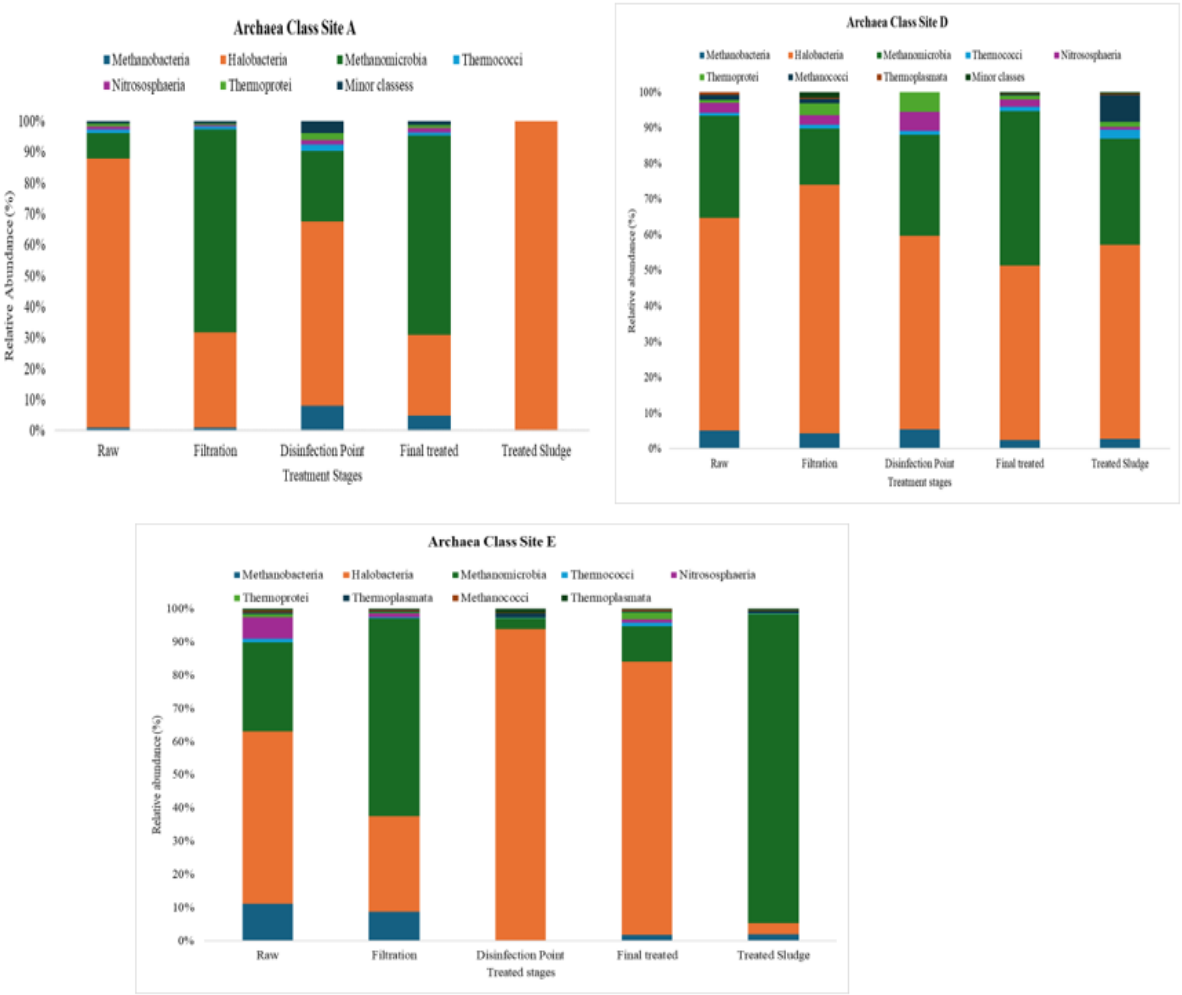


Figure 4.13: Relative abundance of Archaea classes in Gauteng Province at Sites A, D and E, showing the top eight most abundant classes

4.5.1.3. Archaea genera diversity in Gauteng Province (Sites A, D and E)

The relative abundance at the genus level showed variations across the different stages of treatment. The raw water source and filtration stage clustered together, with *Streptomyces* being the dominant genus at Site A, while at Site D, the raw water source and final treated water clustered together, having *Methanothrix* in common. At Site E, the relative abundance at the genus level across the different stages of treatment showed that the final treated water and filtration stages clustered together. In addition, the raw water source and treated sludge clustered together, sharing more members belonging to the genera *Halovivax*, *Methanobrevibacter*, *Halapricum*, *Halobaculum* and *Salinirubellus* (Figure 4.14).

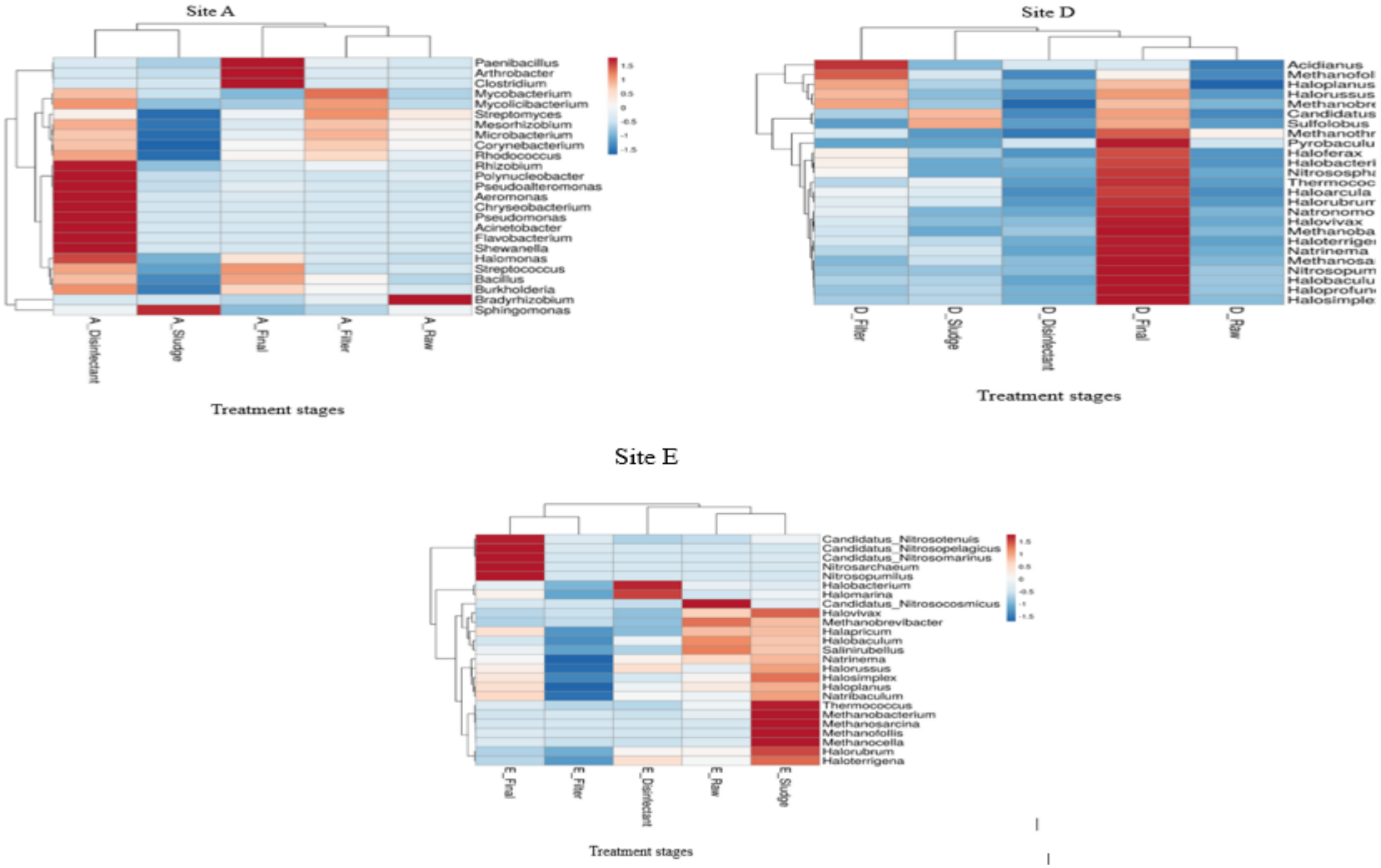


Figure 4.14: Relative abundance of Archaea genera in Gauteng Province at Sites A, D and E

4.5.1.4. Bacterial phyla diversity in Gauteng Province (Sites A, D and E)

Pseudomonadota was the abundant bacterial phylum at Site A within the raw water source, the filtration and disinfection points, the final treated water and treated sludge (Figure 4.15). Pseudomonadota, Bacteriodota and Actinomycetota were the dominant bacterial phyla at Site D, with the raw water source, the filtration and disinfection points, the final treated water and treated sludge consisting of more Pseudomonadota, while the disinfection point had more visible Bacteriodota than other stages, and Actinomycetota were visible in the filtration stage and treated sludge.

Figure 4.15: Relative abundance of bacterial phyla in Gauteng Province at Sites A, D and E, showing the top eight most abundant phyla

4.5.1.5. Bacterial class diversity in Gauteng Province (Sites A, D and E)

The different treatment stages influenced the relative abundance of the bacterial class, with variation across the stages. At Site A, Alphaproteobacteria, Gammaproteobacteria and Betaproteobacteria were the dominant bacterial classes across the treatment stages (Figure 4.16). At Site D, Alphaproteobacteria, Gammaproteobacteria, Actinomycetes, Flavobacteria and Betaproteobacteria were abundant in the raw water source, the filtration and disinfection stages, and treated sludge. At

Site E, Alphaproteobacteria, Gammaproteobacteria and Actinomycetes constituted the major bacterial classes, with Betaproteobacteria occurring more in the DWTS stages.

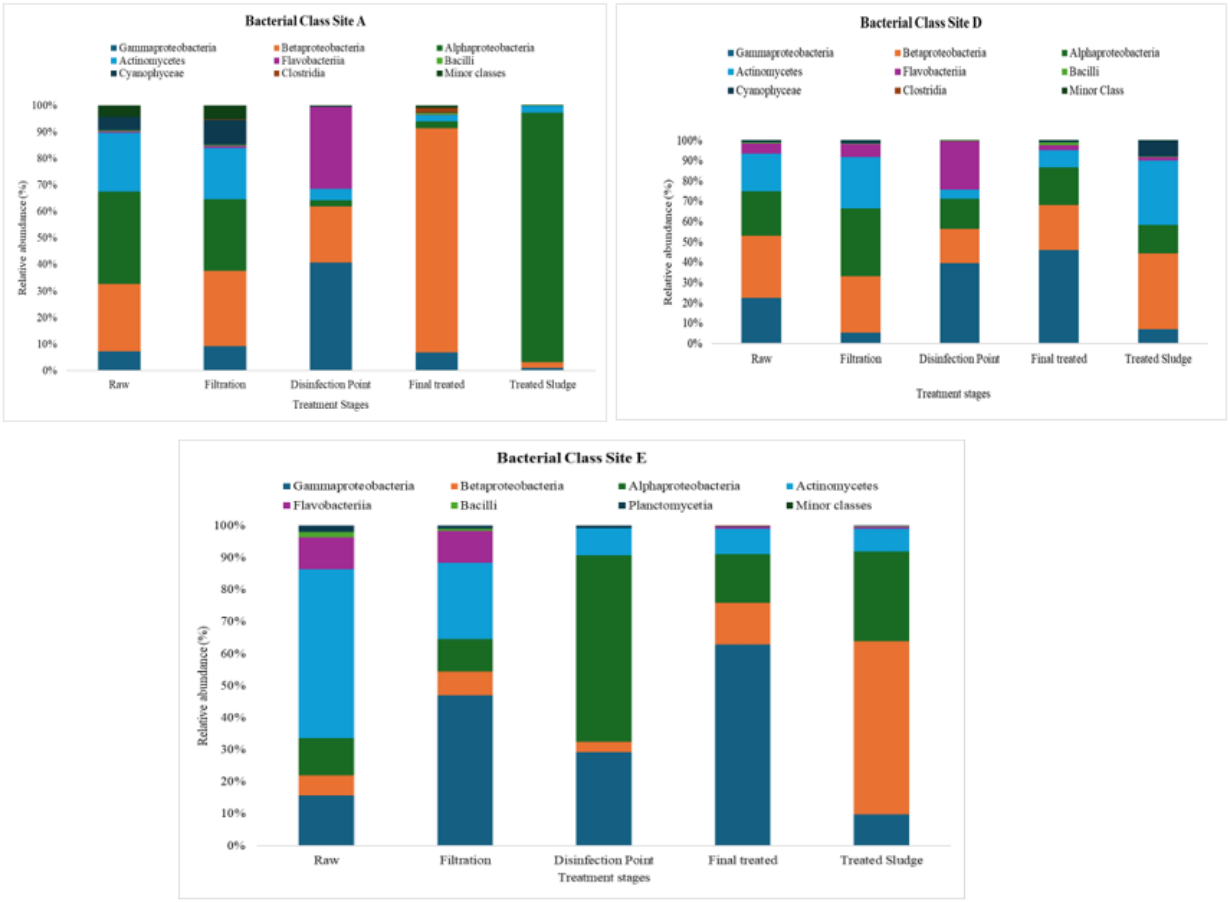


Figure 4.16: Relative abundance of bacterial classes in Gauteng Province at Sites A, D and E, showing the top eight most abundant classes

4.5.1.6. Bacterial genera diversity in Gauteng Province (Sites A, D and E)

At the genus level, Site A's raw water source and the filtration stage showed close clustering, indicating similar genera compositions. Similarly, the disinfection stage and the treated sludge showed another clustering, indicating similar bacteria genera composition; however, the disinfection stage was dominated by genera such as *Pseudoalteromonas*, *Halomonas*, *Vibrio*, *Acinetobacter*, *Rhizobium*, etc. (Figure 4.17). At Site D, the genera from the final treated water separated from other points with the following genera occurring the most: *Streptomyces*, *Bacillus*, *Clostridium*, *Burkholderia*, *Bradyrhizobium*, *Rhodococcus*, *Arthrobacter*, *Pseudomonas*, *Synechococcus* and *Mesorhizobium*. Clustering occurred between the raw water source and treated sludge, as well as the filtration and disinfection stages. *Flavobacterium* occurred more between the disinfection stage and filtration stage, while *Halomonas* occurred the most between the treated sludge and raw water source.

At Site E, clustering was observed between the final treated water and treated sludge samples, as well as between the disinfection and filtration stages.

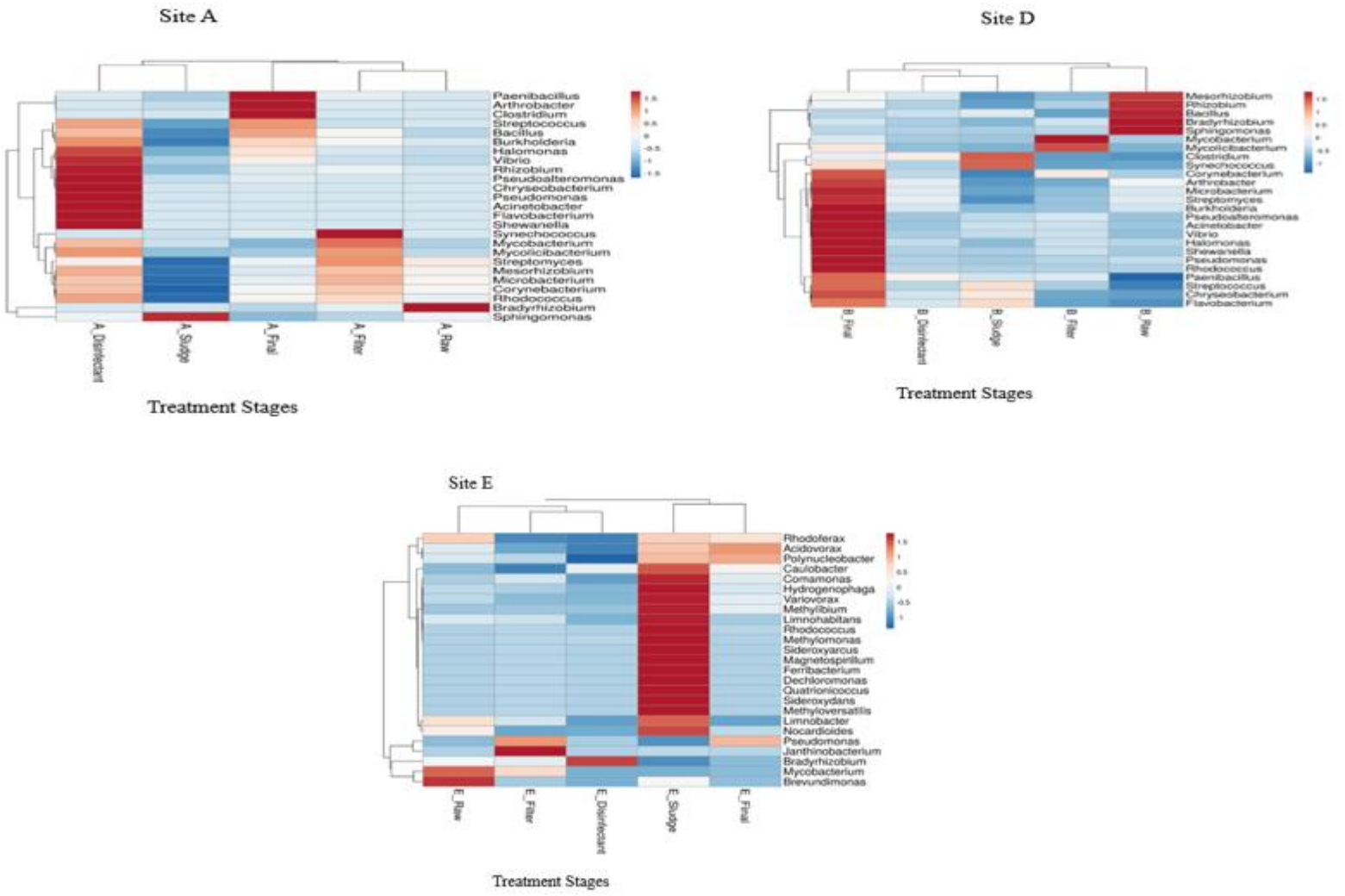


Figure 4.17: Relative abundance of bacterial genera in Gauteng Province at Sites A, D and E showing the top 20 most abundant genera

4.5.1.7. Eukaryota phyla diversity in Gauteng Province (Sites A, D and E)

Ascomycota, Basidiomycota and Bacillariophyta were the dominant Eukaryota phyla with variation in their relative abundance across Site A (Figure 4.18). Ascomycota, Basidiomycota, Bacillariophyta and Apicomplexa were the dominant phyla with variation in the relative abundance across Site D, while at Site E, Euglenozoa, Ascomycota, Basidiomycota and Apicomplexa were the dominant phyla with variation in the relative abundance. These results could indicate that urbanisation is reshaping fungal composition and diversity within ecosystems, indicating taxonomic turnover rather than biodiversity loss (Wang *et al*., 2025).

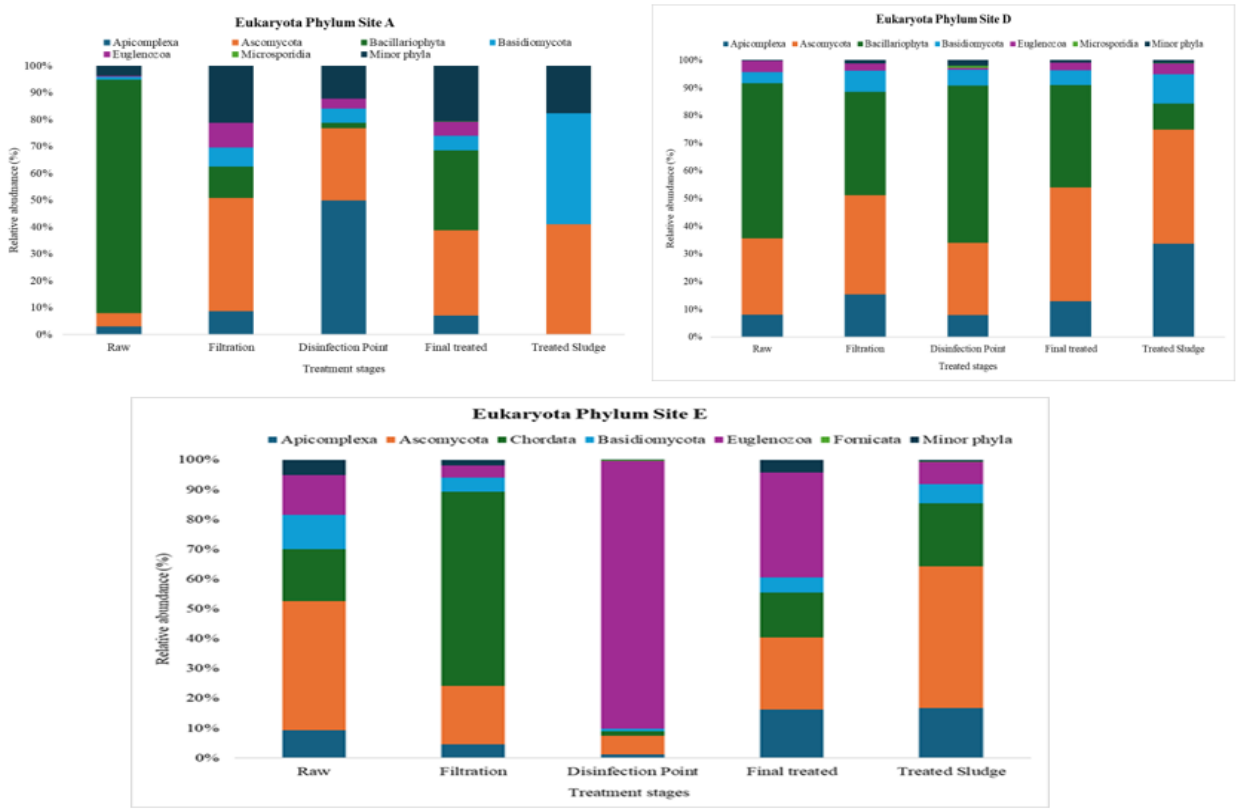


Figure 4.18: Relative abundance of Eukaryota phyla in Gauteng Province at Sites A, D and E, showing the top eight most abundant phyla classes

4.5.1.8 Eukaryota class diversity in Gauteng Province (Sites A, D and E)

At Site A, Aconoidasida, Sordariomycetes and Cryptophyceae dominated, with variations in their occurrences across the treatment stages (Figure 4.19). At Site D, Aconoidasida, Sordariomycetes and Saccharomycetes were the dominant classes with variation in their occurrences within the treatment stages, while at Site E, Alphaproteobacteria, Gammaproteobacteria and Actinomycetes were the dominant classes across the treatment stages with variation in their abundance.

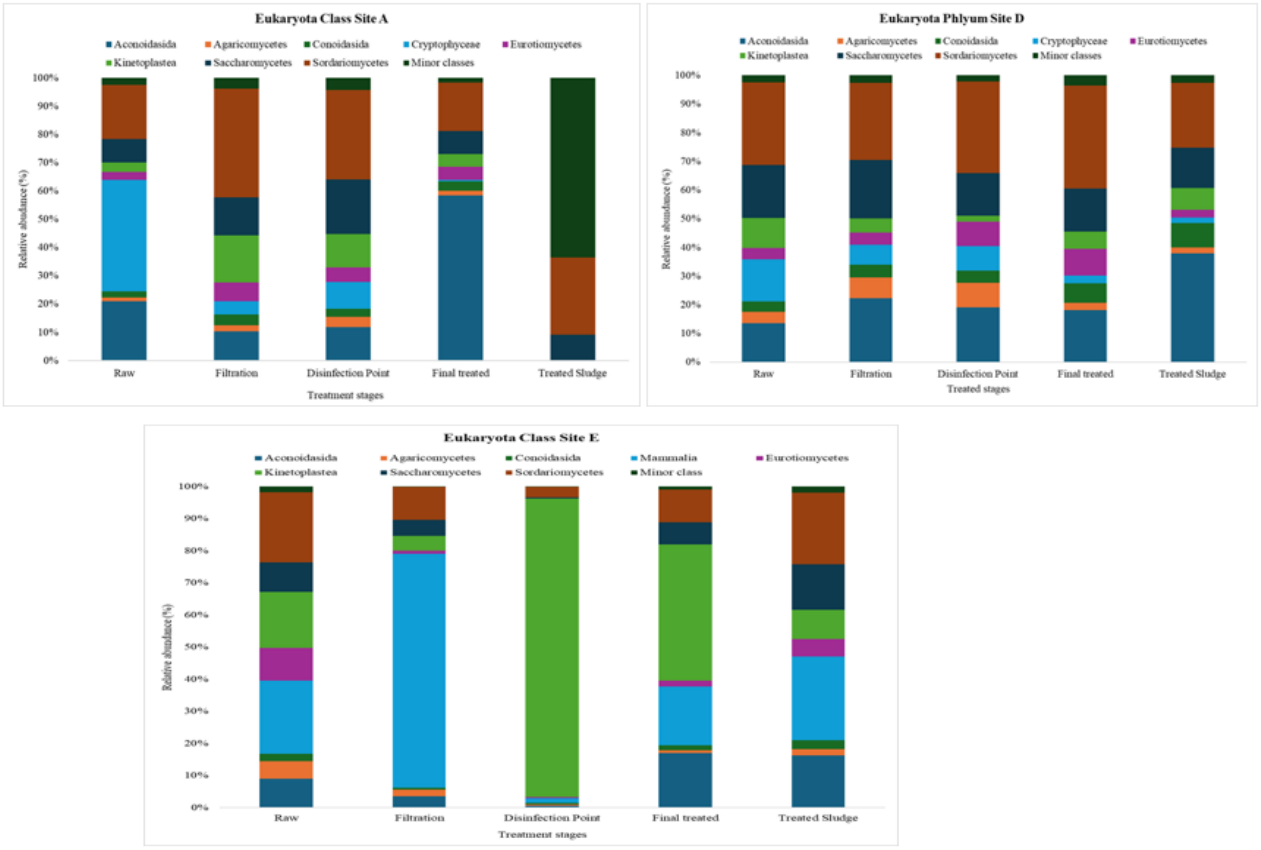


Figure 4.19: Relative abundance of Eukaryota phyla and classes in Gauteng Province at Sites A, D and E, showing the top eight most abundant phyla and classes

4.5.1.9 Eukaryota genera diversity in Gauteng Province

The final treated water was quite distinct from the other treatment stages at the genus level. Site A showed clustering between the disinfection and filtration stages, as well as the treated sludge and raw water source (Figure 4.20). In addition, the disinfection stage showed more abundance of diverse genera, including *Sporisorium* and *Encephalitozoon*. At Site D, the final treated water clustered with the raw water source, while the treated sludge clustered with the disinfection stage. At Site E, the treated sludge clustered with the raw water source, while the filtration stage clustered with the final treated water.

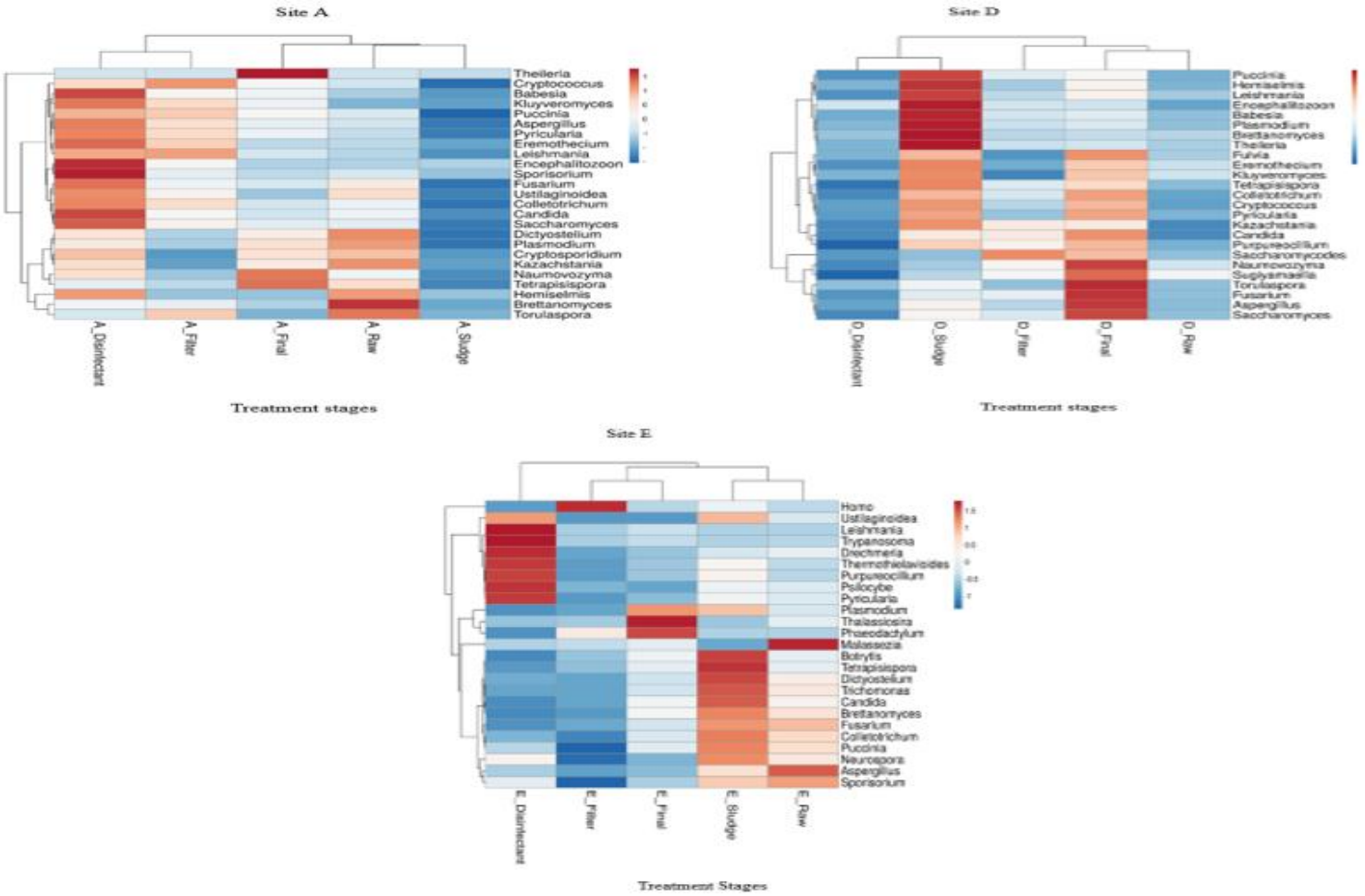


Figure 4.20: Relative abundance of Eukaryota genera in Gauteng Province at Sites A, D and E, showing the top 20 most abundant genera

4.5.2. Microbial diversity and dynamics evaluation in Limpopo Province (Site B)

Site B's DWTP in Limpopo Province had 40 950 OTUs (Table 4.20). Using diversity indices, the highest diversity indices using the Shannon index were observed for the raw water sample (2.07) than other stages. The biodiversity indices demonstrated the varied impacts of treatment stages on microbial community diversity across the treatment stages.

Table 4.20: Alpha diversity indices in Limpopo Province

Treatment stages	Alpha diversity indices						
	Reads	OTUs	% Coverage	Evenness	Shannon	InvSimpson	chao1
Raw water source	25514037	6766	99.89	0.23	2.07	2.25	7916.11
Filtration	23126064	7336	99.83	0.14	1.26	1.54	8438.17
Disinfection	26988643	8844	99.9	0.08	0.69	1.23	10028.9
Final treated water	44817916	9221	99.93	0.13	1.2	1.46	9942.54
Treated sludge	22411460	8783	99.8	0.06	0.58	1.16	9833.38

OTU: operational taxonomic unit

4.6.2.1. Archaea phyla, class and genera diversity in Limpopo Province

At Site B in Limpopo Province, Euryarchaeota and Nitrososphaerota constituted the major Archaea phyla, with variation in their abundance across the treatment stages (Figure 4.21). Halobacteria and Methanomicrobia were abundant at the class level in all the stages, the raw water source and the final treated water. In addition, the disinfection stage and treated sludge clustered together, sharing members belonging to the genera *Methanobacterium* and *Methanobrevibacter.*

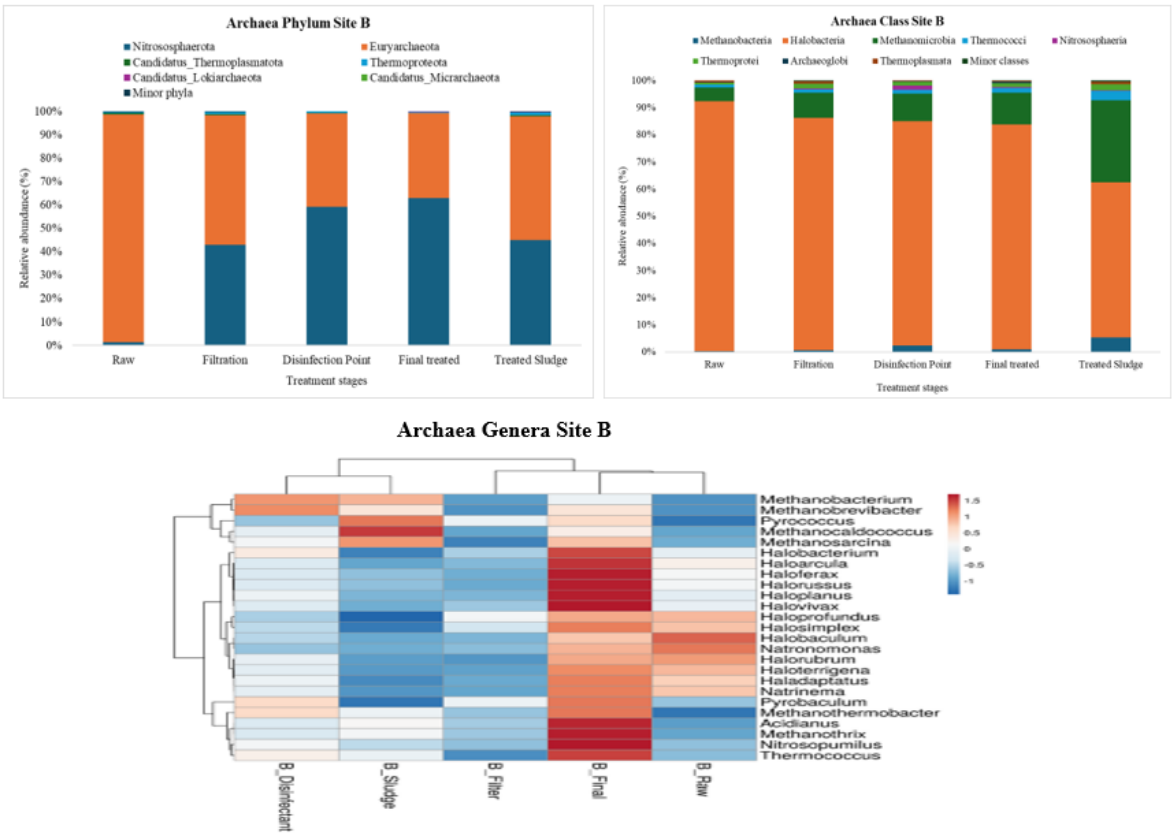


Figure 4.21. Relative abundance of Archaea phyla, classes and genera in Limpopo Province

4.6.2.2. Bacterial phyla in Limpopo Province (Site B)

At Site B, Pseudomonadota was the dominant bacterial phylum, noticeably within the raw water source, the sedimentation and filtration stages, the final treated water and the treated sludge, more than any phylum at any stage of the DWTP (Figure 4.22). Alphaproteobacteria, Gammaproteobacteria, Actinomycetes and Betaproteobacteria were the dominant classes across the treatment stages, with variations in their abundance. The genus level shows the abundance of clustering of bacteria genera in the raw and final treated water, though its different species. The genera composition of the final treated water shows a clear distinction from other sampling points, with an abundance of the members belonging to the genera *Corynebacterium, Arthrobacter, Microbacterium, Streptomyces, Burkholderia, Pseudoalteromonas, Acinetobacter, Vibrio, Halomonas, Shewanella, Pseudomonas, Rhodococcus, Paenibacillus, Streptococcus, Chryseobacterium* and *Flavobacterium*.

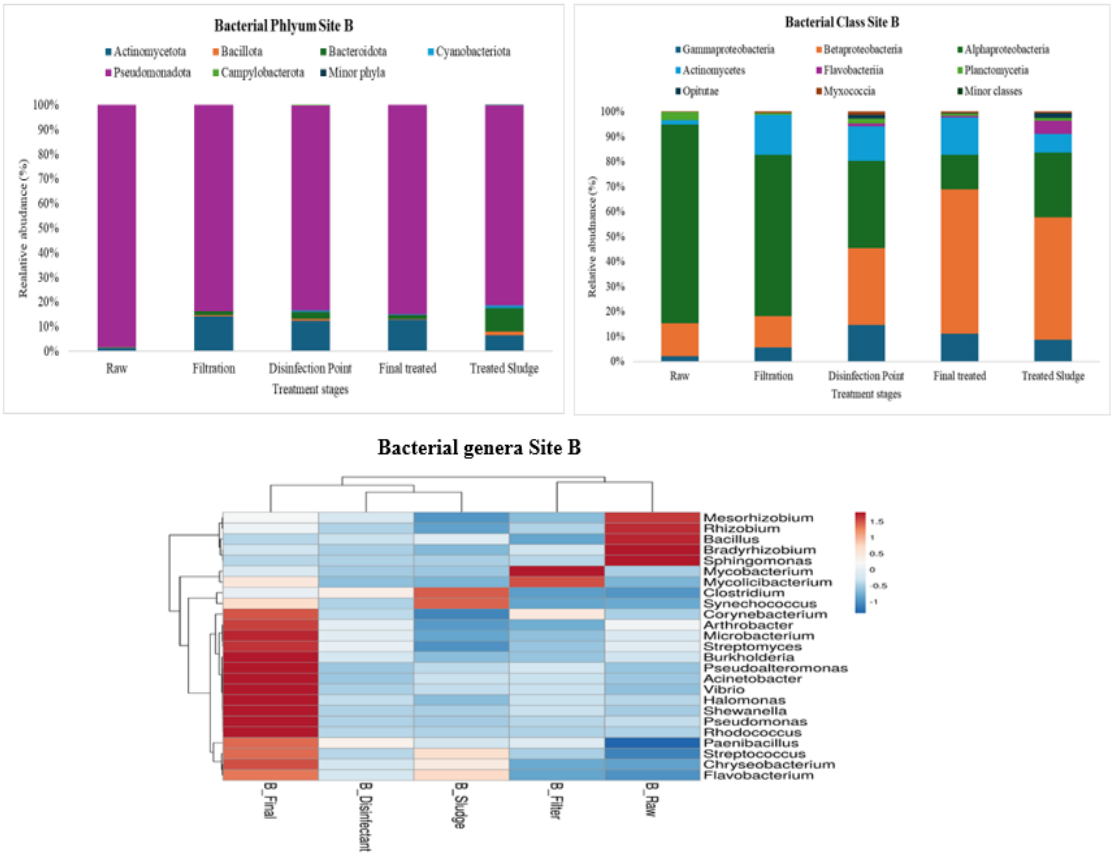


Figure 4.22: Relative abundance of bacterial phyla, classes and genera in Limpopo Province

4.6.2.3. Eukaryota phyla, classes and genera in Limpopo Province (Site B)

At Site B, Ascomycota, Basidiomycota, Apicomplexa and Bacillariophyta were the dominant Eukaryota phyla, with variation in the relative abundance across the sampling sites (Figure 4.23). At the class level, Aconoidasida, Sordariomycetes and Saccharomycetes dominated, with variation in their occurrences within the treatment stages. At the genus level, the final treated water samples clustered with the raw water samples, and the filtration stage clustered with the treated sludge, showing similar composition of the members belonging to the genera. The final treated water was dominated by the following genera: *Torulaspora, Fusarium, Plasmodium, Dictyostelium, Thalassiosira, Bigelowiella, Babesia, Naumovozyma, Colletotrichum, Kazachstania, Leishmania, Brettanomyces, Candida, Theileria* and *Saccharomyces*. The disinfection stage genera composition was distinct from the other sampling points, with *Puccinia* and *Tetrapisispora* dominating.

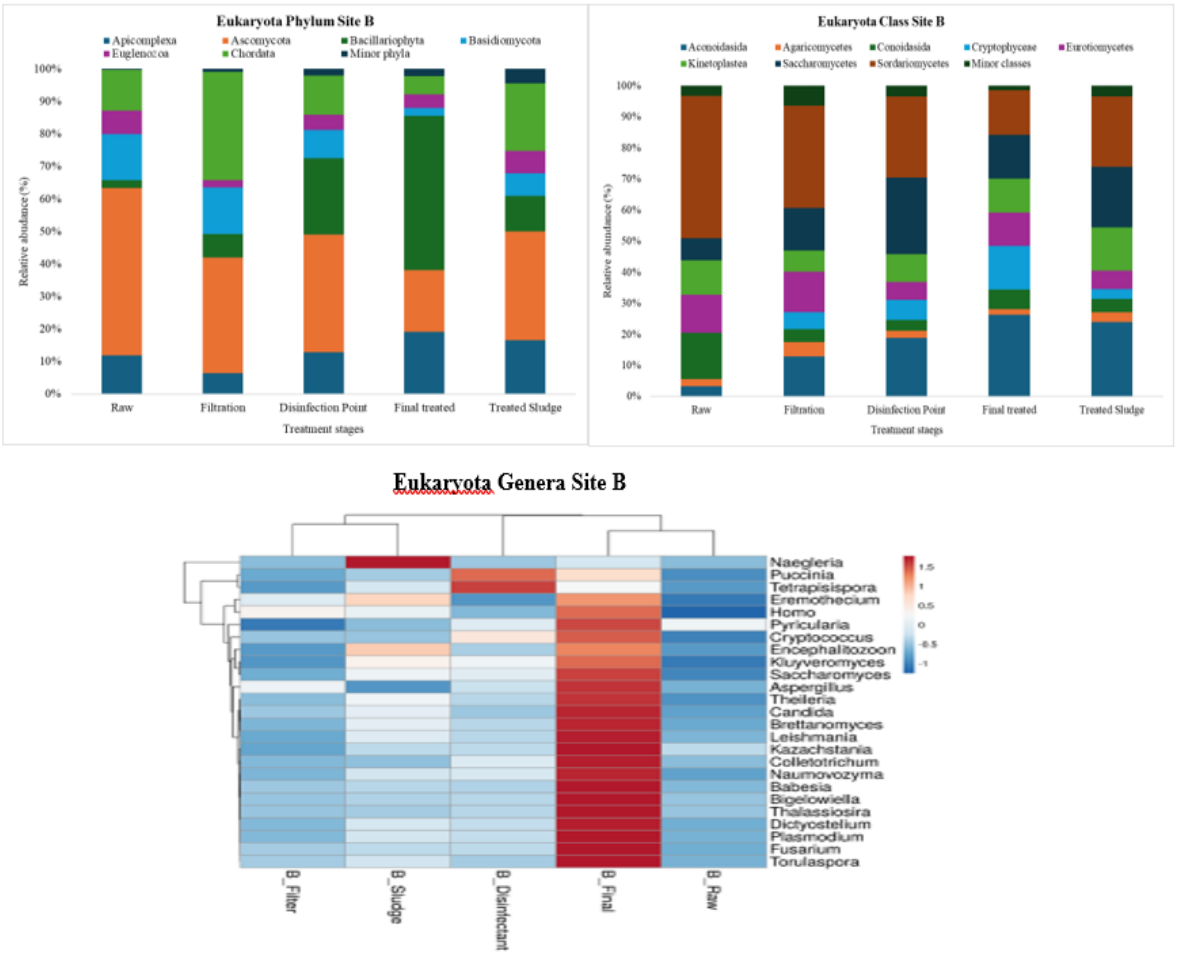


Figure 4.23: Relative abundance of Eukaryota phyla, classes and genera in Limpopo Province

4.5.3. Microbial diversity and dynamics evaluation in Mpumalanga Province (Site C)

The DWTP in Mpumalanga Province was analysed for alpha diversity indices of microbial communities and had a total number of 35856 sequence reads (Table 4.21). The highest diversity indices according to the Shannon index were observed in the disinfection stage (2.36). The biodiversity indices demonstrated the varied impacts of treatment stages in decreasing or promoting microbial community diversity across the treatment stages. Across Site C, all three kingdoms (Archaea, Bacteria, Eukaryota) and associated diverse phyla, classes and genera were detected.

Table 4.21: Alpha diversity indices in Mpumalanga Province

Treatment stages	Alpha diversity indices						
	Reads	**OTUs**	**% Coverage**	**Evenness**	**Shannon**	**InvSimpson**	**chao1**
Raw water sources	27492335	8287	99.87	0.13	1.16	1.46	9359.05
Filtration	35094229	7058	99.93	0.22	1.95	2.2	8396.66
Disinfection	37471644	7251	99.93	0.27	2.36	3.19	8449.52
Final treated water	33858519	8600	99.88	0.13	1.17	1.43	9299.32
Treated sludge	28038302	4660	99.87	0.24	2.05	2.47	5797.53

OTU: operational taxonomic unit

4.6.3.1. Archaea phyla, classes and genera in Mpumalanga Province (Site C)

At Site C, Euryarchaeota and Nitrososphaerota constituted the major Archaea phyla detected, with variations in abundance across the treatment stages (Figure 4.24). Archaea classes Halobacteria and Methanomicrobia dominated across the sampling points. The relative abundance at the genus level showed that the raw water source and filtration stage clustered together, sharing members of the genera *Halobaculum, Haloprofundus, Natronomonas, Haloterringena* and *Halobacterium*. In addition, the disinfection stage was clearly separated from the other points, with *Candidatus, Nitrosotenuis* and *Pyrococcus* dominating. The treated sludge clustered with the final treated water, showing *Methanococcus*, *Halobaculum* and *Methanomicrobium*.

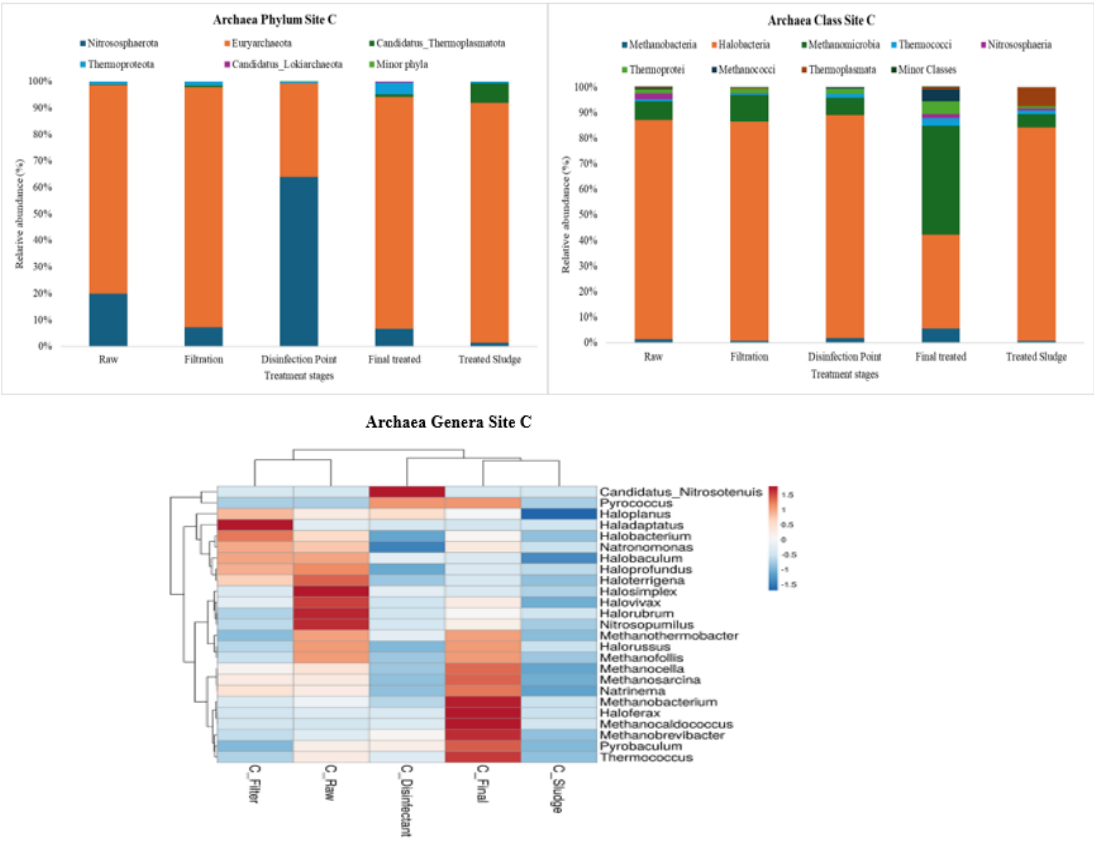


Figure 4.24: Relative abundance of Archaea phyla, classes and genera in Mpumalanga Province

4.6.3.2. Bacterial phyla in Mpumalanga Province

Figure 4.25 presents bacterial phyla at Site C. Pseudomonadota, Bacteriodota and Actinomycetota were dominant, with the disinfection stage consisting of more Pseudomonadota than the other treatment stages. Alphaproteobacteria, Gammaproteobacteria, Actinomycetes and Betaproteobacteria were the dominant classes across the treatment stages at varying abundance levels. The genus level shows clustering between the final treated water and the disinfection stage, with *Flavobacterium* occurring the most. The filtration stage and the treated sludge clustered together, dominated by *Mycolicibacterium* and *Shingomonas*.

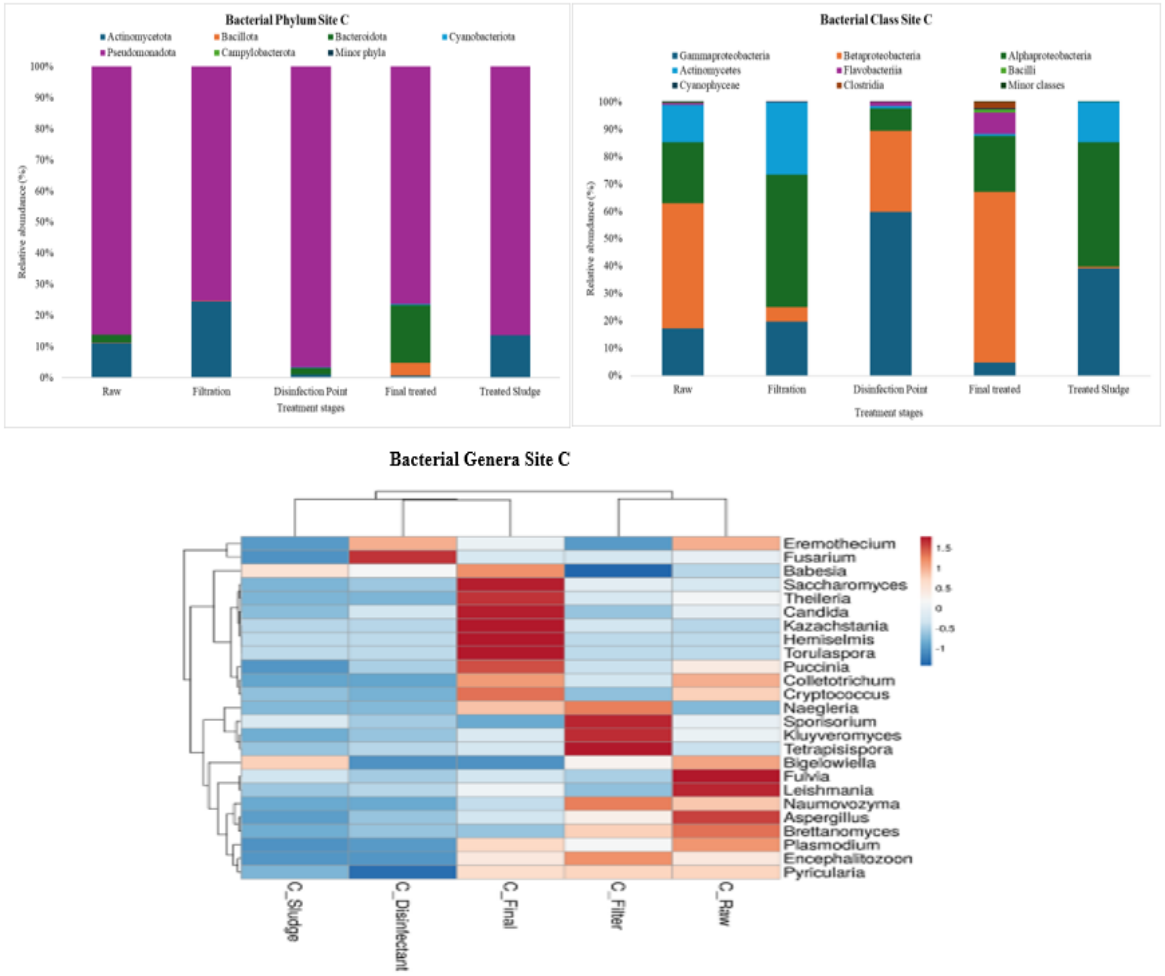


Figure 4.25: Relative abundance of bacterial phyla, classes and genera in Mpumalanga Province

4.6.3.3. Eukaryota phyla, classes and genera in Mpumalanga Province (Site C)

Fungi Ascomycota and Basidiomycota and protozoan Apicomplexa were the dominant phyla across the sampling at Site C (Figure 4.26). Aconoidasida, Sordariomycetes, Kinetoplastea and Saccharomycetes were dominant classes with varying occurrences within the treatment stages. At the genus level, the final treated water clustered with the disinfection stage, while the filtration stage clustered with the raw water source.

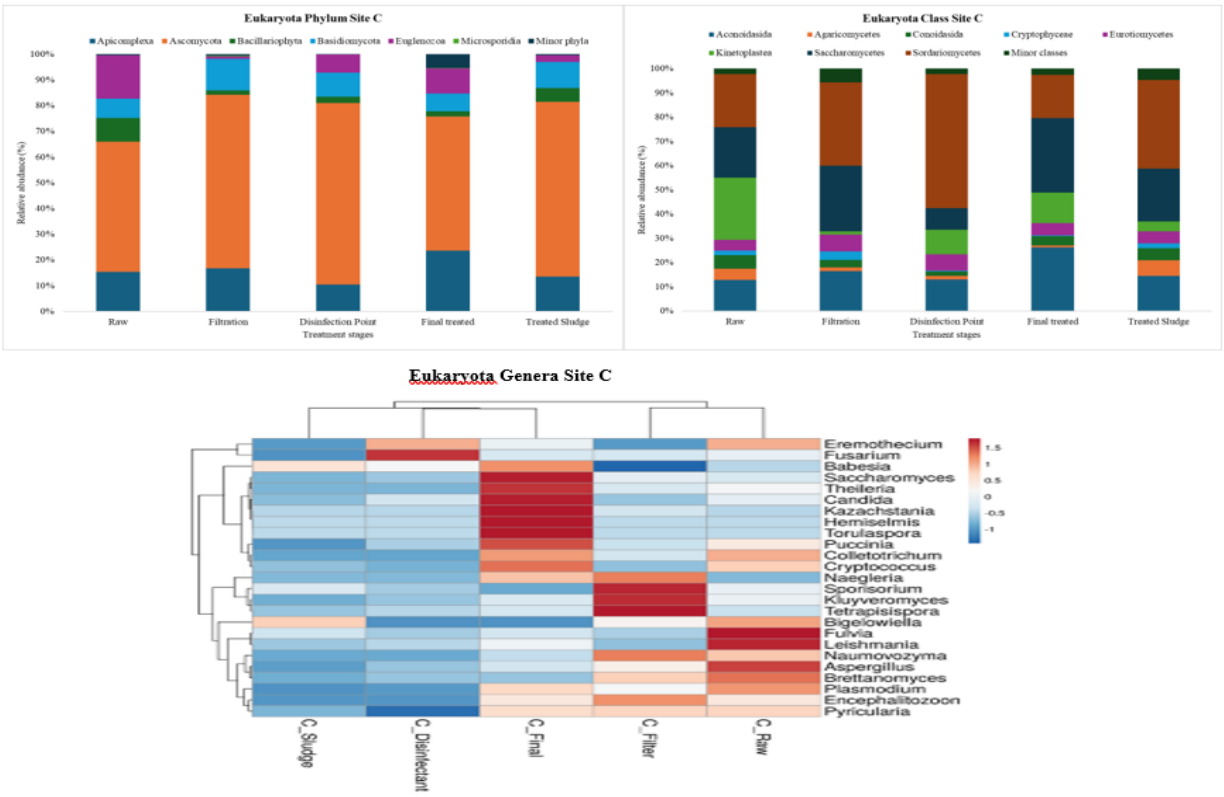


Figure 4.26: Relative abundance of Eukaryota phyla, classes and genera in Mpumalanga Province

4.6. Functional metagenome analysis

4.6.1. Functional metagenome analysis in Gauteng Province (Sites A, D and E)

4.6.1.1. Site A

At Site A, the heatmaps provided further insight into the impact of the treatment stages in eliciting the release of diverse functional genes (Figure 4.27). The functional metagenomes of the filtration and disinfection stages showed close clustering, sharing more human disease, organismal system, and environmental information processing metagenomes in common. Strikingly, the treated sludge and final treated water showed close clustering, sharing more metabolism metagenomes in common (Figure 4.28).

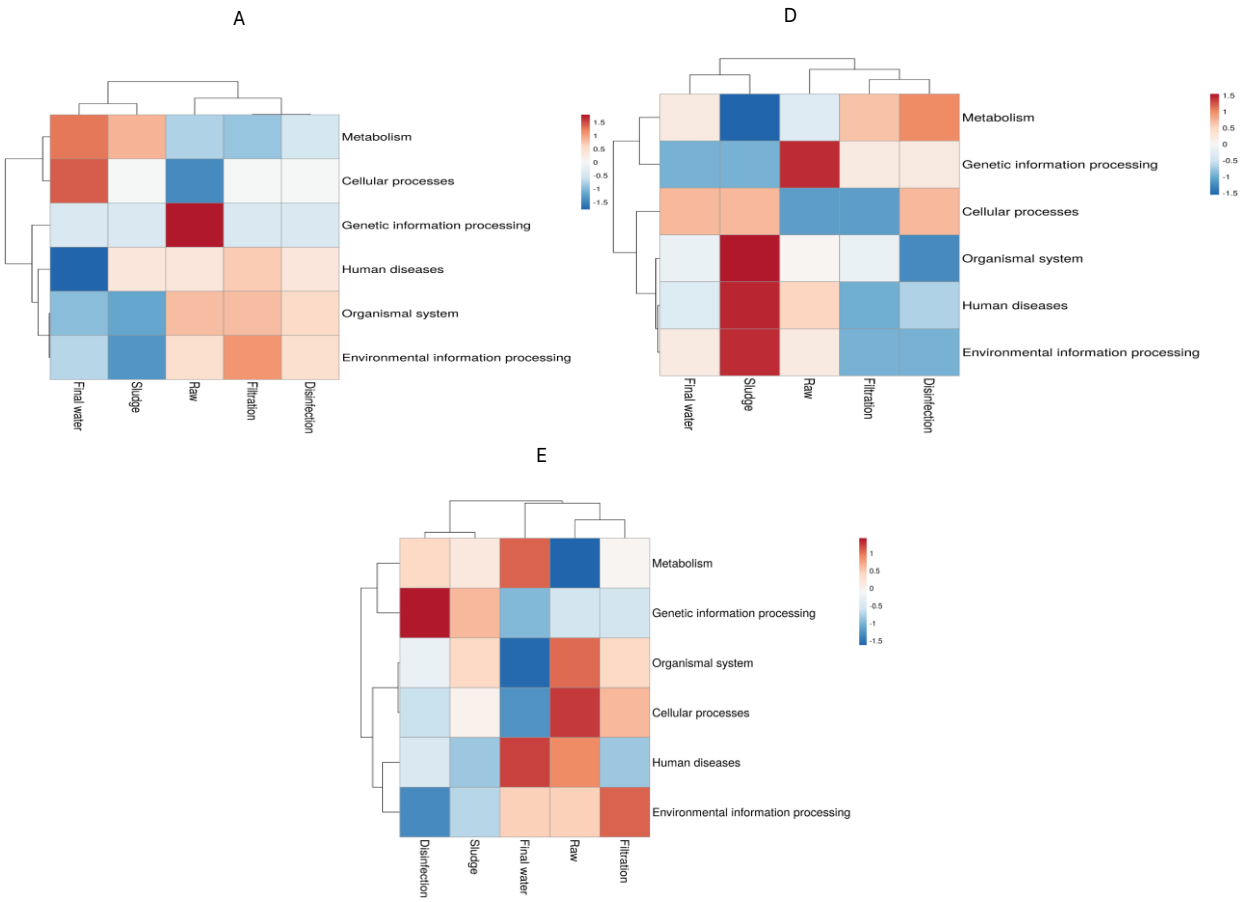


Figure 4.27: Determined dominant functions related to the human genes function with different treatment stages in Gauteng Province (Sites A, D and E)

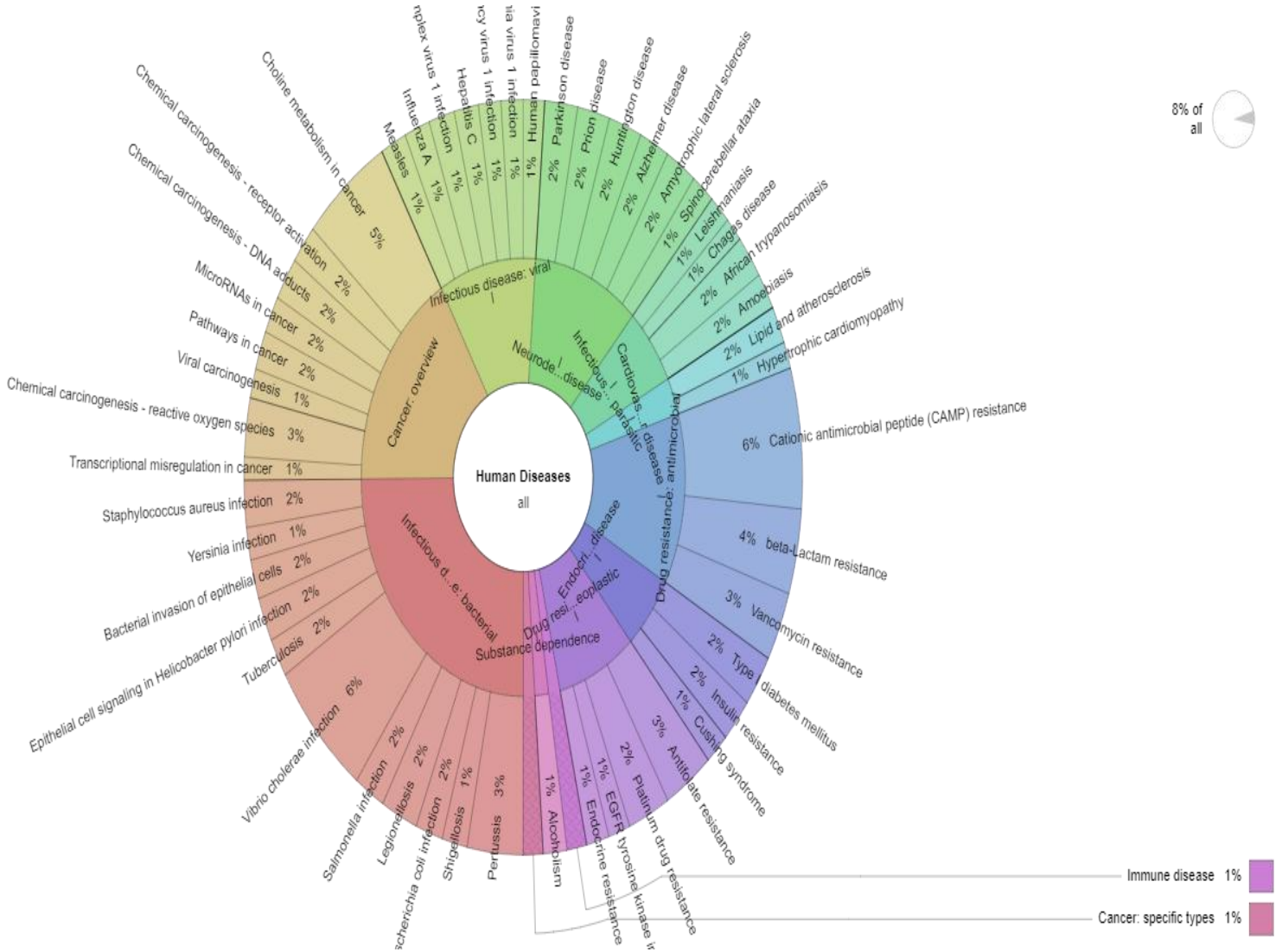


Figure 4.28: Krona chart showing the relative abundance of some of the functional metagenomes associated with human diseases in the final treated water from sampling Site A

4.6.1.2. Site D

Clustering between the disinfection and filtration stages occurred, representing six functional metagenomes at Site D, with more metabolism metagenomes and fewer human disease and environmental information processing metagenomes in common (Figure 4.27). The treated sludge and final treated water clustered with more cellular processing metagenomes and fewer genetic information processing metagenomes in common. In addition, Hepatitis C (8%), pathogenic *E. coli* infection (6%), cationic antimicrobial peptide (CAMP) resistance (3%), ßeta-lactam resistance (3%) and vancomycin resistance (2%) were the dominant functional metagenomes for human disease (Figure 4.29).

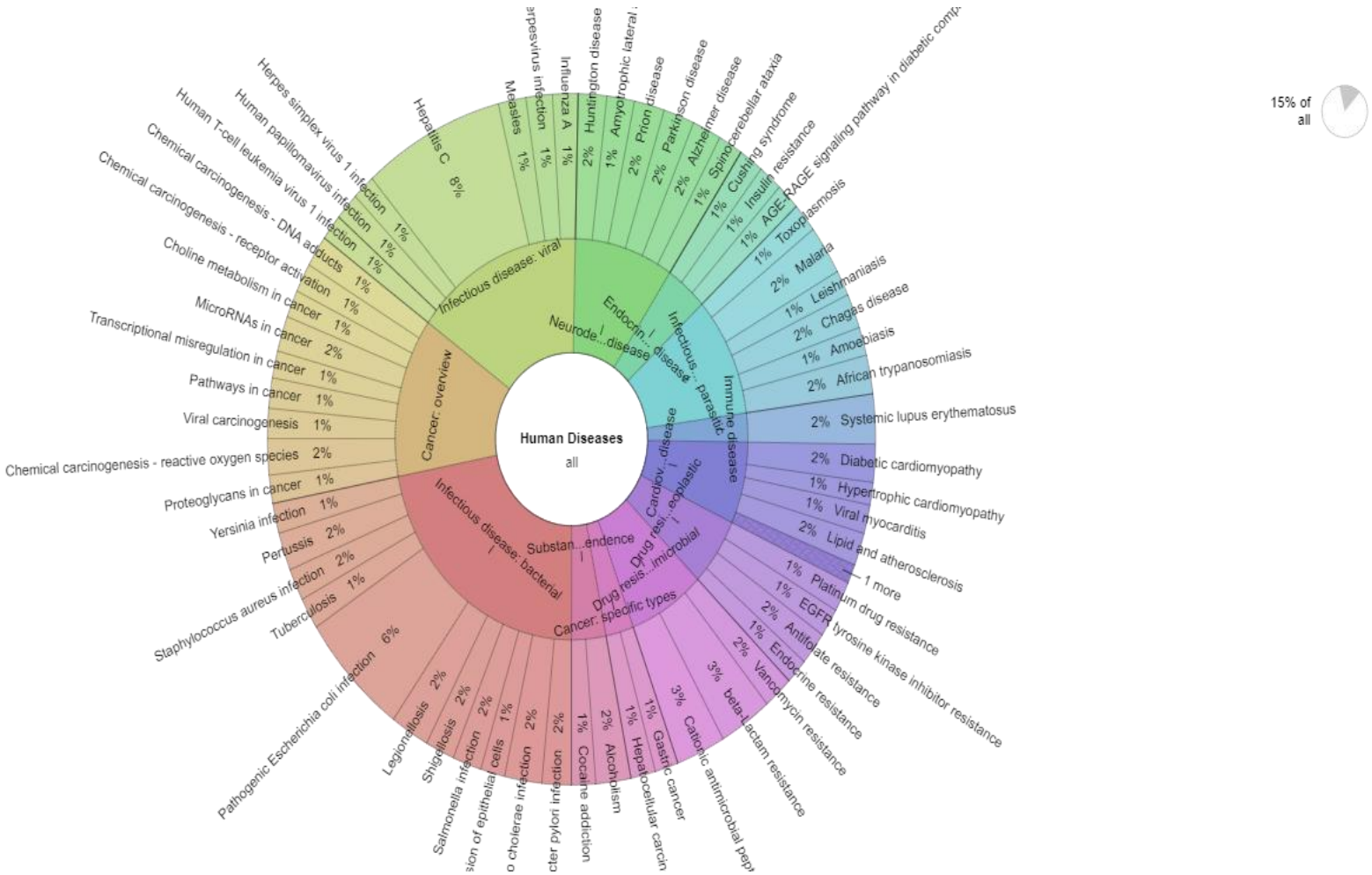


Figure 4.29: Krona chart showing the relative abundance of some of the functional metagenomes associated with human diseases in the final treated water from sampling Site D

4.6.1.3. Site E

A different pattern was observed at Site E, with the final treated water distinct with more human disease and metabolism metagenomes and fewer cellular processing, organismal system and genetic information processing metagenomes (Figure 2.27). At this site, the final treated water residual chlorine was 1.0 mg/L, which is above the SANS 241 (SANS, 2015) drinking water limit. The human disease metagenomes comprised systemic lupus erythematosus (6%), maturity-onset diabetes of the young (5%) and three antibiotic resistance functions (CAMP, ßeta-lactam and vancomycin) (Figure 4.30).

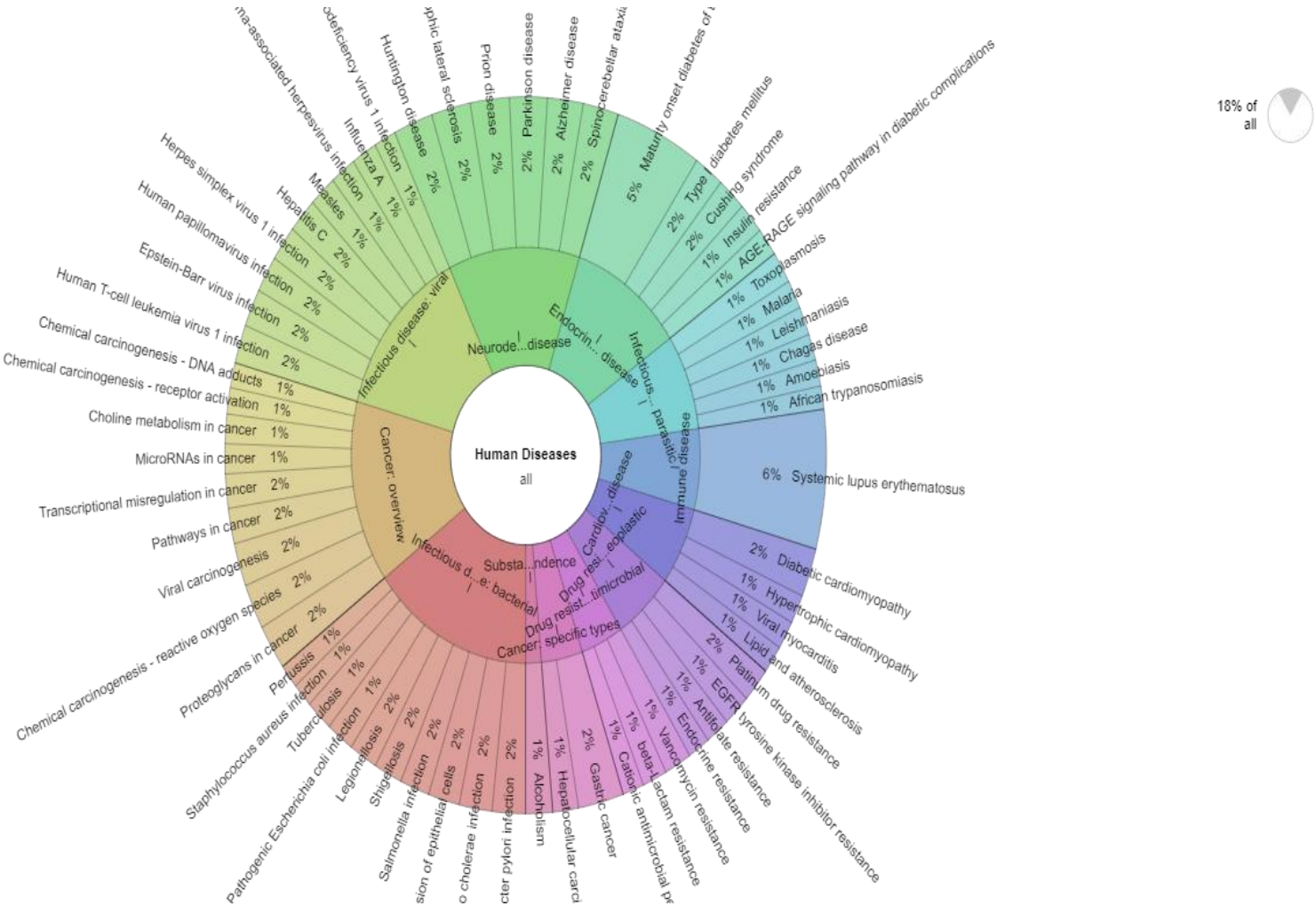


Figure 4.30: Krona chart showing the relative abundance of the functional metagenomes associated with human diseases in the final treated water from sampling Site E.

4.6.2. Functional metagenome analysis in Limpopo Province (Site B)

Clustering between the raw water source and the filtration stage was observed at Site B, with more genetic information processing metagenomes and fewer metabolism metagenomes detected (Figure 4.31). Furthermore, the final treated water and disinfection stage showed clustering, with more metabolism metagenomes and less genetic information processing and human disease metagenomes. In addition, the relative abundance of the functional metagenome associated with human diseases was between 1.0% and 2.0%. Strikingly, CAMP resistance, ßeta-lactam resistance and vancomycin resistance decreased to 2% (Figure 4.32).

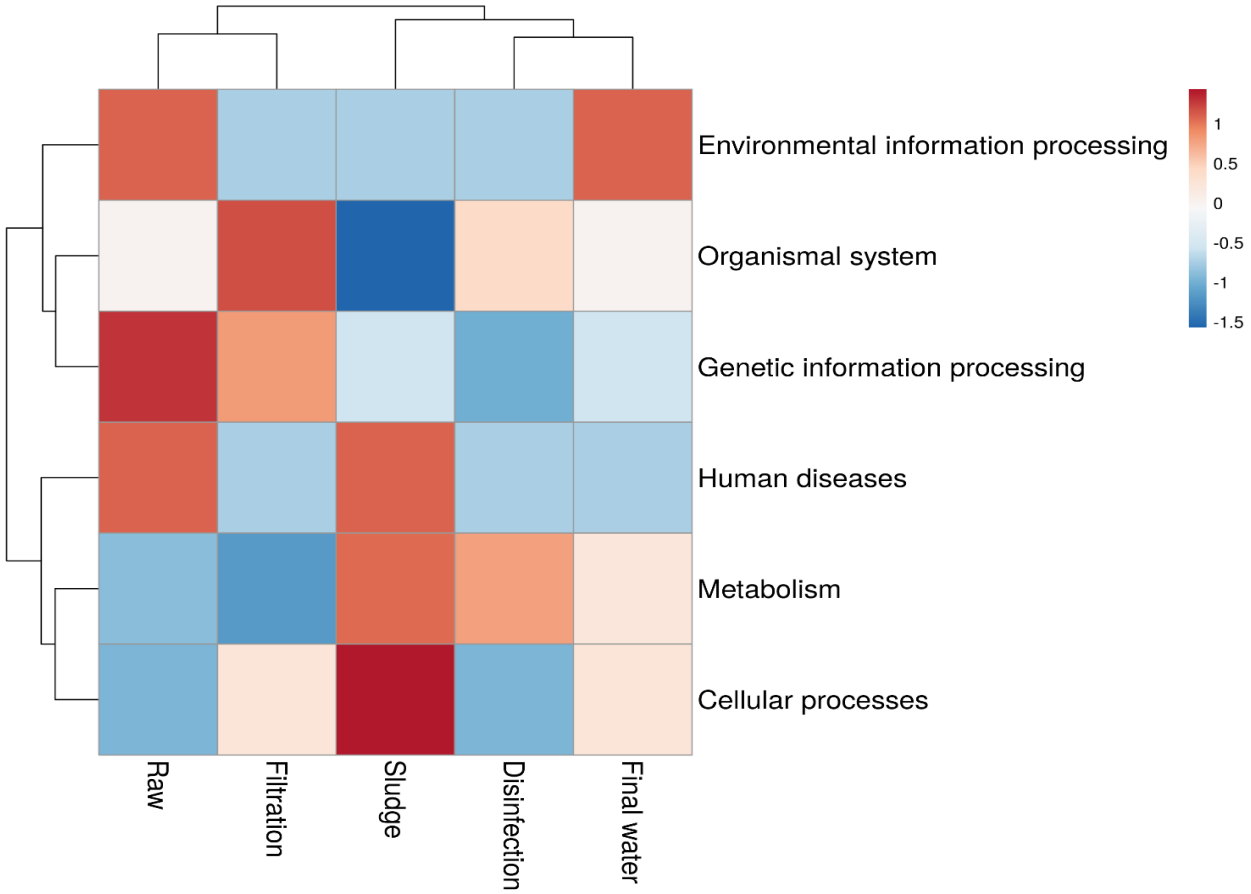


Figure 4.31: Determined dominant function related to the human genes function with different treatment stages in Limpopo Province

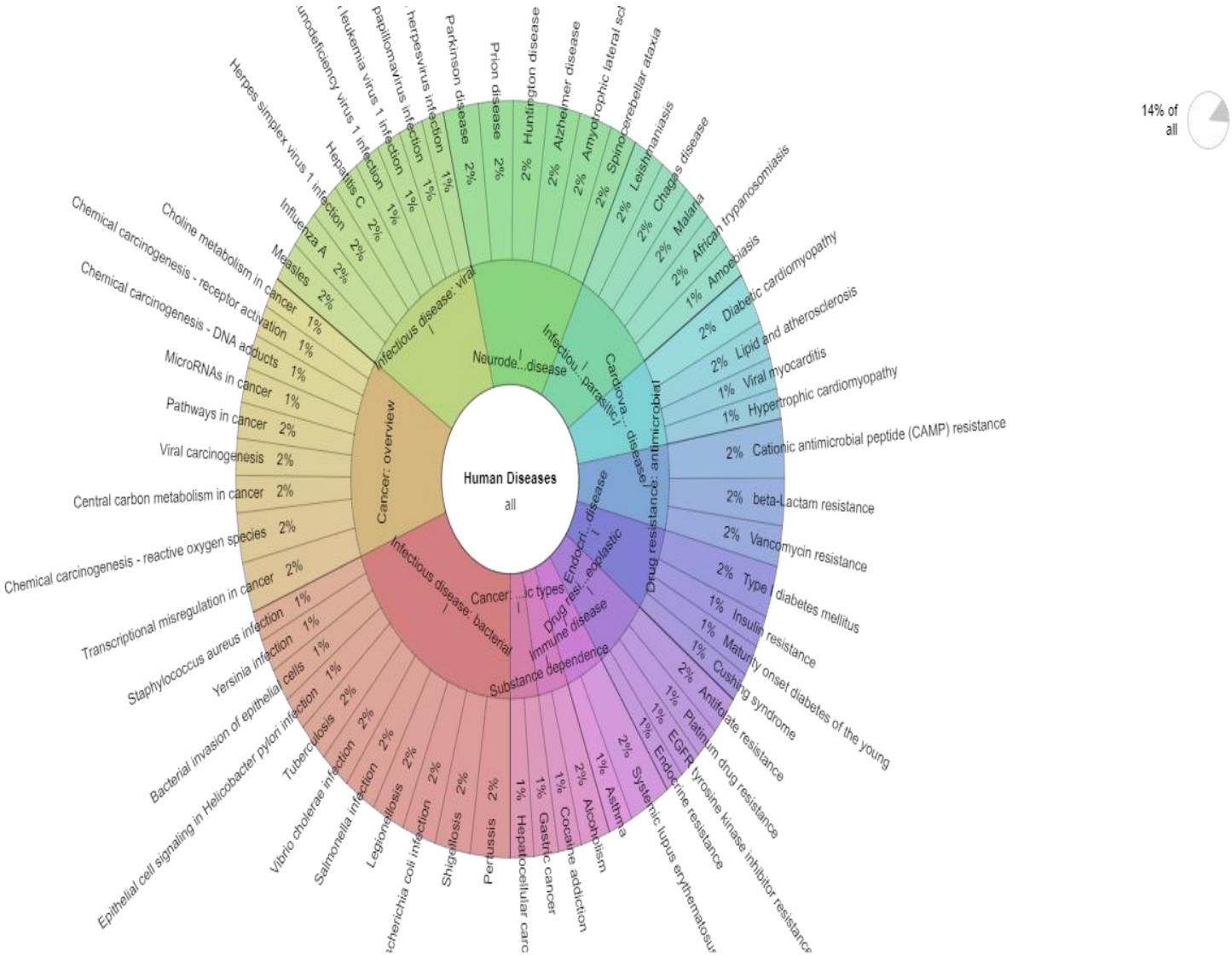


Figure 4.32: Krona chart showing the relative abundance of the functional metagenomes associated with human diseases in the final treated water in Limpopo Province

4.6.3. Functional metagenome analysis in Mpumalanga Province (Site C)

Site C showed clustering between the disinfection and filtration stages, with more cellular processing, human disease and environmental information processing metagenomes and fewer genetic information processing metagenomes in common (Figure 4.33). The treated sludge and final treated water clustered, with more metabolism metagenomes and fewer human disease and cellular processing metagenomes in common. A further look into the human disease metagenomes indicated that the relatively dominant functional metagenomes were proteoglycans in cancer (6%), chemical carcinogenesis-reactive oxygen species (3%), pertussis (3%), legionellosis (4%), ßeta-lactam resistance (5%), vancomycin resistance (6%), CAMP resistance (7%) and African trypanosomiasis (7%) (Figure 4.34).

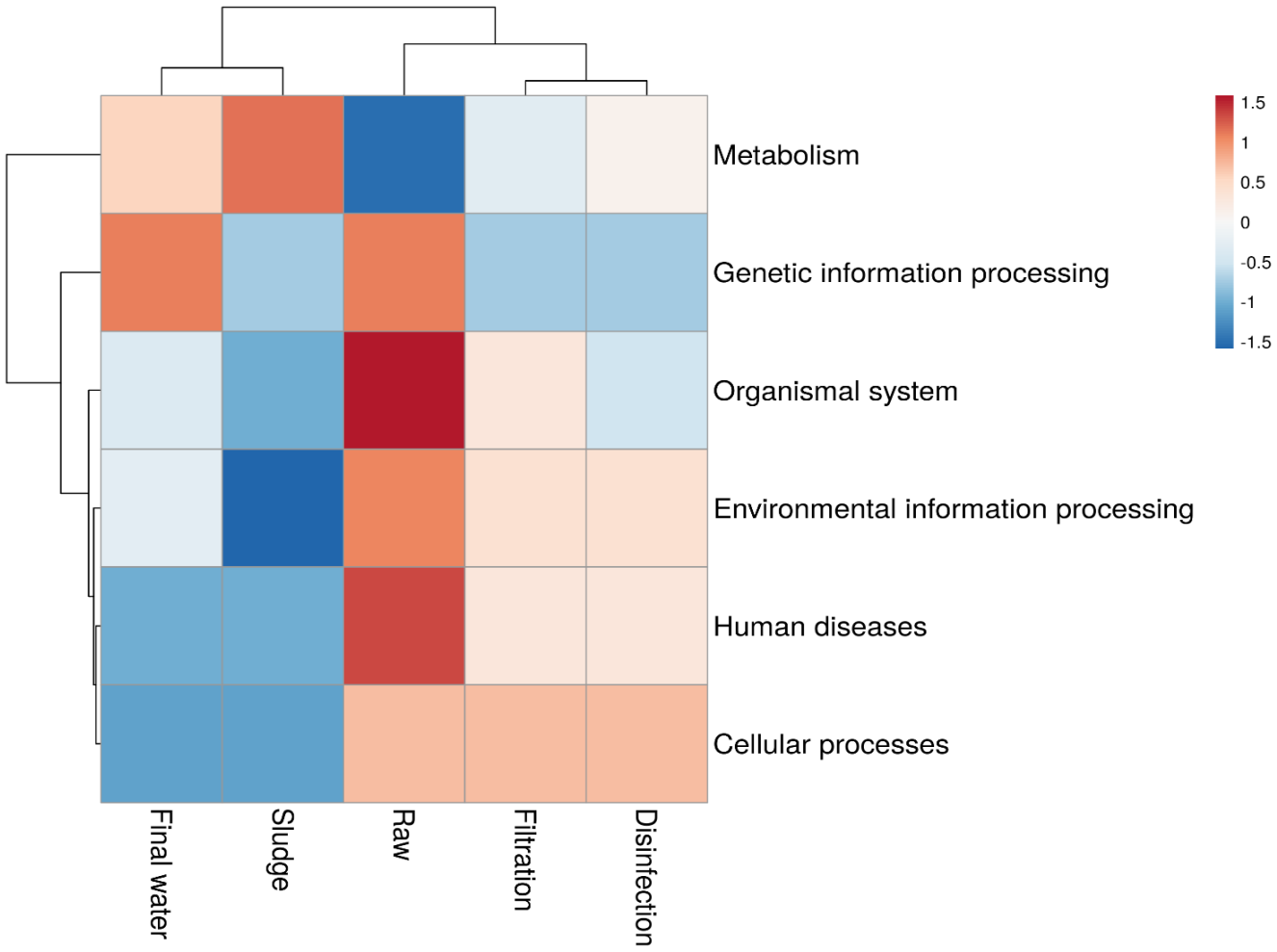


Figure 4.33: Determined dominant function related to the human genes function with different treatment stages in Mpumalanga Province

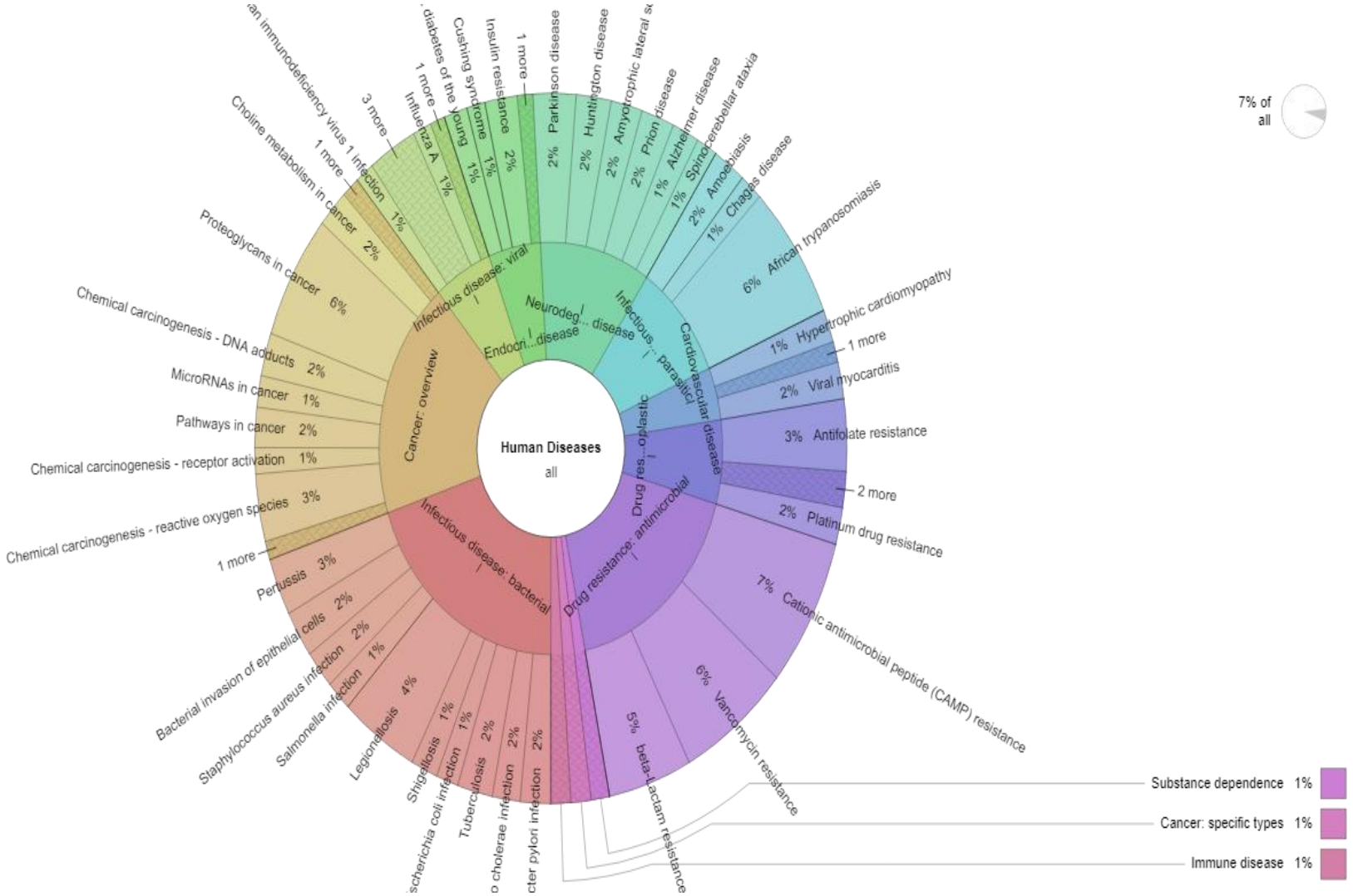


Figure 4.34: Krona chart showing the relative abundance of the functional metagenomes associated with human diseases in the final treated water in Mpumalanga Province

4.7. Antibiotic-resistance genes phenotype profile unveiled through shotgun metagenomics

4.7.1. Antibiotic-resistance genes phenotype profile in Gauteng Province (Sites A, D and E)

4.7.1.1. Site A

The genome sequencing analysis derived from Site A's DWTP, treated sludge and distribution system retrieved ARGs and their phenotype information, such as the resistance mechanism and antibiotic classes annotation. Ten antibiotic classes were retrieved from identified ARGs in the raw water source, the filtration and disinfection stages, final treated water and treated sludge samples. The distribution of ARGs across antibiotic classes with a difference of >50% was glycopeptide (100%), phenicol (100%), trimethoprim (100%), fosfomycin (77%), aminoglycoside (58%) and ßeta-lactam (65%) in the disinfection stage (Figure 4.35). Glycopeptide (100%) and macrolide (60%) were dominant in the final treated water. The ARGs *bla*$_{DHA}$, *bla*$_{TEM}$, *aph* (6)-*lb* and *aph* (3")-*la* were prevalent in all the samples of Site A (Table 4.22). The spread of the *bla*$_{TEM}$ gene in the water environment is facilitated by transposons and integrons (Pazos *et al*., 2025). Results obtained from analysing the antibiotic classes phenotype profile of ARGs indirectly display the efficient performance of the treatment stages in eliminating pathogenic bacteria that impart multidrug resistance capacity.

4.8.1.2. Site D

Variation in the percentage hits of the classes of antibiotics is observed in Figure 4.35. Ten antibiotic classes retrieved from identified ARGs were statistically significant in the mean difference between the raw water sources, the filtration and disinfection stages, final treated water and treated sludge. The raw water source had trimethoprim (100%) with a difference of >50%. Phenicol (100%) and fosfomycin (85%) were the antibiotic classes with ARGs with a difference of >50% in the final treated water. Tetracycline (100%) and macrolide (100%) were dominant in the filtration stage. Trimethoprim (100%) was dominant in the raw water source, while streptogramin (55%) was dominant in the treated sludge.

4.8.1.3. Site E

Variation in the percentage hits of the classes of antibiotics was observed at Site E, and antibiotics with ARGs with a difference of >50% were quinolone (80%) in the DWTS and tetracycline (60%) in the final treated water (Figure 4.35). Glycopeptide (100%) and macrolide (60%) were dominant in the final treated water. The ARGs *tet(A)*, *Sul1*, *aph* (6)-*lb* and *aph* (3")-*la* were prevalent in all the samples of Site E (Table 4.22). The results obtained from analysing the antibiotic classes phenotype profile of ARG indirectly display the efficient performance of the treatment stages in eliminating pathogenic bacteria that impart multidrug resistance capacity.

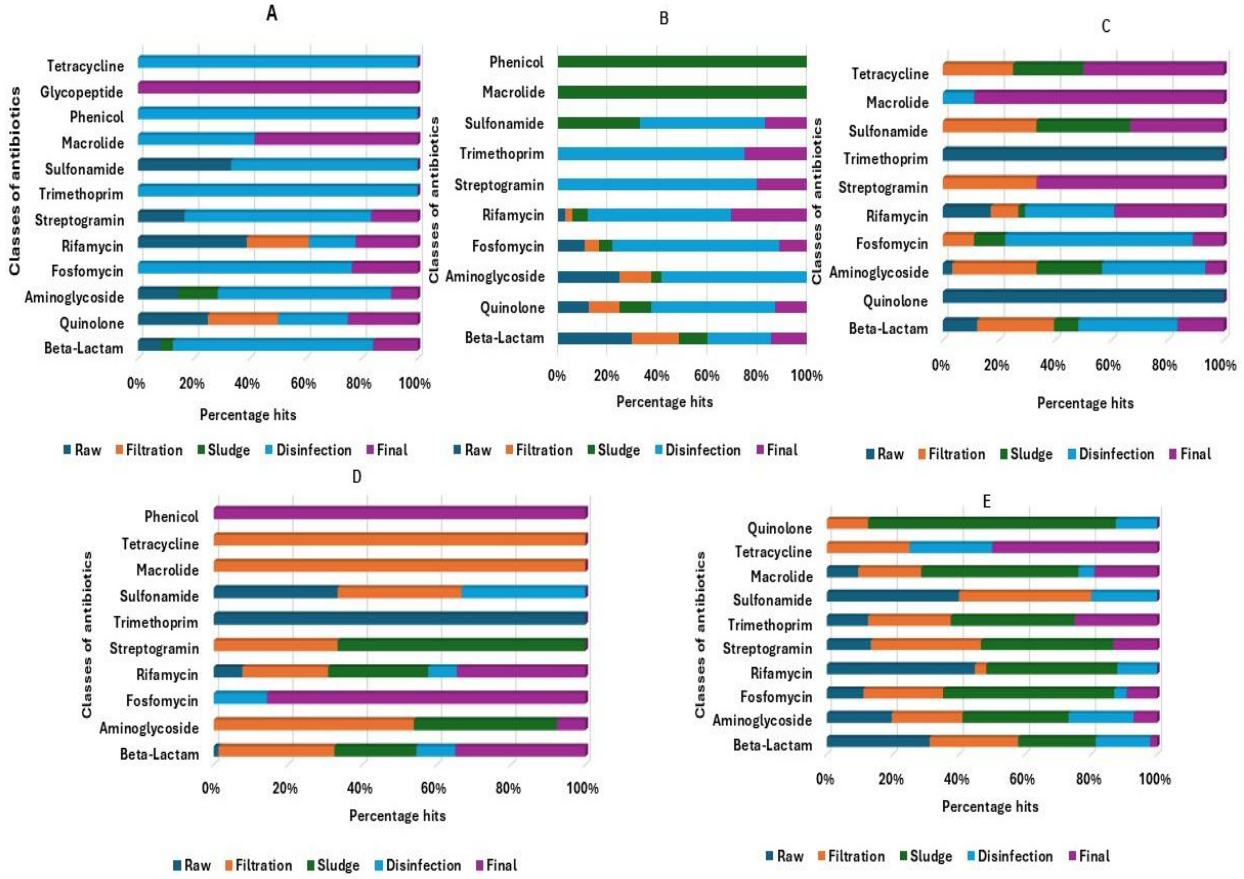


Figure 4.35: Paleontological Statistics Software Package Version 4 analyses presenting tetracycline and trimethoprim antibiotics at Site A, phenicol and macrolide at Site B, and phenicol, tetracycline and macrolide at Site C, showing 100% distribution of antibiotic-resistance gene percentages in Gauteng Province (Sites A, D and E), Limpopo Province (Site B) and Mpumalanga Province (Site C)

Table 4.22: Some of the antibiotic-resistance genes identified from each site in Gauteng Province (Sites A, D and E), Limpopo Province (Site B) and Mpumalanga Province (Site C) using the Paleontological Statistics Software Package Version 4

Antibiotic class	Antibiotic-resistance gene	Drinking water treatment plant (A, B, C, D and E)				Treated sludge	Percentage (%)
		Raw water source	Filtration stage	Disinfection stage	Final treated water		
Aminoglycoside	*aph(6)-id*	X		x	x		75
	aph(3")-lb	X	x		x	x	
	aac(3)-lid	X		x	x	x	
	aada5	X			x	x	
	aadA12	X		x		x	
	aac(3)-ib	X	x				
	aac (6')-lid			x	x	X	
	Ant (3")-li	X	x				
ßeta-lactam	*bla*$_{DHA}$	X	x	x	x		80
	bla$_{CMY-2}$		x		x	x	
	bla$_{TEM}$	X		x		x	
	bla$_{SHV}$	X	x	x	x	x	
	bla$_{CTX-M-1G}$	X			x		
	bla$_{CTX-M-9G}$		x	x	x		
Maricolide	*mef(B)*		x	x	x		
	mph(A)	X	x	x			
Quinolone	*qnrA*	X			x		60
	qnrB	X	x		x	x	
	qnrC	X		x	x	x	
	qnrS		x	x	x	x	
	qepA	X	x	x			
	oqxA		x			x	
	oqxB	X		x	x	x	
Sulfonamide	*Sul1*	X			x		80
	Sul2	X	x		x	x	
	dfrA1	X		x	x	x	
	dfrA12	X			x	x	
	dfrA14		x	x			
Tetracycline	*tet(A)*	X			x		85
	*tetA**	X	x	x	x	x	
	tet(B)	X		x	x	x	
	tet(M)		x	x		x	
	tetO	X	x	x	x		
	tetO34		x		x	x	
	tetX	X		x	x	x	
	*tetX**	X			x	x	

x: detected

4.7.2. Antibiotic-resistance genes phenotype profile in Limpopo Province (Site B)

Figure 4.35 shows that ten antibiotics were statistically significant in the mean difference between the raw water source, the filtration and disinfection stages, final treated water and treated sludge. Phenicol (100%) and macrolide (100%) were the antibiotic classes with a difference of >50% in treated sludge. Streptogramin (80%), trimethoprim (76%), rifamycin (50%), fosfomycin (60%) and aminoglycoside (58%) were dominant in the disinfection stage. The ARGs *qnrA*, *tetA*,* $bla_{\text{CTX-M-9G}}$ and *aph* (3") *la* were prevalent in all the samples of Site B. Human activities such as the wastewater treatment plant and agriculture activities significantly contribute to the dissemination of extended-spectrum ßeta-lactam genes such as $bla_{\text{CTX-M-9G}}$ and $bla_{\text{CTX-M-9G}}$ in water sources until it reaches DWTPs (Sharma *et al*., 2025). The results obtained from analysing the antibiotic classes phenotype profile of ARGs indirectly display the efficient performance of the treatment stages in eliminating pathogenic bacteria that impart multidrug resistance capacity.

4.7.3. Antibiotic-resistance genes phenotype profile in Mpumalanga Province (Site C)

At Site C, ARGs across antibiotic classes with a difference of >50%, which were macrolide (90%), streptogramin (64%), tetracycline (50%), were dominant in the final treated stage, while trimethoprim (100%) and quinolone were dominant in the raw water source (Figure 4.35). Fosfomycin (65%) was dominant in the disinfection stage of Site C. ARGs such as *qnrB*, *qnrC*, *qnrS*, *tetO34*, *tetX* and *tetX** were identified throughout the drinking water treatment stages.

4.8. Correlation between physicochemical parameters and microbial communities

The Pearson's correlation analysis was constructed to determine the correlation of physicochemical parameters and microbial communities for each site (A, B, C, D and E) during the study period. The strength of a relationship between variates may be defined as having a strong, moderate or weak correlation. Strong relationships have a correlation of positive or negative (0.70–1.00), moderate correlations are correlated at positive or negative (0.30–0.69), and weak correlations are defined at positive or negative (0.00–0.29). Correlation is defined as positive when one variate increases or decreases as the other variate also increases or decreases. A negative correlation is defined when one variate increases and the other variate decreases (Raheem & Al-Juhaishi, 2024). The relationship between the bacterial classes and studied physicochemical

parameters for the raw water source, the filtration stage and the final treated water for all the sites is represented in Figures 4.36 to 4.38.

4.8.1. Correlation of physicochemical parameters and microbial communities in Gauteng Province (Sites A, D and E)

4.8.1.1. Site A

The relationships between physicochemical parameters, *E. coli* and *Enterococcus* spp. levels in the DWTPs' raw water at Site A were examined using Pearson's correlation coefficient (*r*) (Table 4.23). Five environmental factors, *E. coli* and *Enterococcus* spp. were moderately correlated. There was a strong correlation between *E. coli* ($r = 0.97$, $P<0.1$) and *Enterococcus* spp. ($r = 0.89$, $P<0.01$) with total coliforms at Site A. There was a positive correlation between *E. coli* and TOC ($r = 0.495$, $P<0.01$), manganese ($r = 0.416$, $P<0.01$) and iron ($r = 0.487$, $P<0.01$). This correlation between TOC and microbes could be attributed to pollution from mining activities and sewerage runoff into the surface water along Site A (Zwane *et al*., 2025). There was weak correlation with total coliforms, *E. coli* and *Enterococcus* spp. and nitrate ($r = 0.14$, $P<0.01$), sulphate ($r = 0.13$, $P<0.01$) and ammonia ($r = 0.181$, $P<0.01$). The was no correlation between pH ($r = -0.198$, $P>0.05$) and total coliforms, *E. coli* and *Enterococcus* spp.

For Site A, a significant ($P<0.01$) positive correlation in the raw water source was observed between Alphaproteobacteria and pH and temperature, Betaproteobacteria and manganese, *Cyanophyceae* and sulphate (Figure 4.36). Actinomycetes and iron, as well as Gammaproteobacteria and ammonia, showed a strong negative correlation. In the filtration stage, there was also variation in the relationship between the bacterial classes and the physicochemical parameters, with Betaproteobacteria showing a significant ($P<0.01$) positive correlation with sulphate, *Cyanophyceae* with manganese and Gammaproteobacteria with iron. In addition, Gammaproteobacteria showed a significant ($P>0.05$) negative correlation with nitrate in the filtration stage. In the final treated water, a significant ($P>0.01$) positive correlation occurred between Alphaproteobacteria and ammonia, Betaproteobacteria and turbidity, and Gammaproteobacteria and turbidity. Further, a negative correlation occurred between *Bacilli* and temperature, Betaproteobacteria and temperature, and *Clostridia* and sulphate.

Table 4.23: Pearson correlation coefficient between total coliforms, *E.* coli, Enterococcus spp. and physicochemical parameters in Gauteng Province at Site A

Parameter	Correlation coefficient *E. coli*	Correlation coefficient *Enterococcus* spp.	Parameter	Correlation coefficient *E. coli*	Correlation coefficient *Enterococcus* spp.
Temperature	0.35	0.44	**Manganese**	0.416	0.57
pH	-0.198	-0.167	**Nitrate**	0.14	0.12
Turbidity	-0.17	0.24	**Sulphate**	0.13	0.13
Total organic carbon	0.495	0.57	**Ammonia**	0.181	0.78
Free chlorine	0.14	0.21	**Iron**	0.487	0.50
Total coliforms	0.97	0.89	**Phosphates**	0.34	0.27

4.8.1.2. Site D

Table 4.24 presents the correlation between total coliforms, *E. coli* and *Enterococcus* spp. and various physicochemical parameters. Six environmental factors were moderately correlated with *E. coli* and *Enterococcus* spp. There was a strong correlation between *E. coli* ($r = 0.74$, $P<0.1$) and *Enterococcus* spp. ($r = 0.92$, $P<0.01$) and total coliforms within Site D. There was a positive correlation between *E. coli* and TOC ($r = 0.456$, $P>0.01$), nitrate ($r = 0.414$, $P>0.01$) and ammonia ($r = 0.481$, $P>0.01$). A moderate correlation between *E. coli* and manganese ($r = 0.316$, $P>0.01$) and iron ($r = 0.347$, $P>0.01$) emphasises that during summer, raw water sources were polluted during runoff and its favourable condition for the growth of microorganism's (Li *et al*., 2023; Zhang *et al*., 2025a). The was no correlation between pH ($r = -0.136$, $P>0.05$) and total coliforms, *E. coli* and *Enterococcus* spp.

A significant ($P<0.01$) positive correlation occurred in the raw water source at Site D between electrical conductivity and Gammaproteobacteria/Actinomycetes, total dissolved solids and *Bacilli*, Betaproteobacteria and phosphates/iron. However, there was a negative correlation between electrical conductivity and *Bacilli*, temperature and Betaproteobacteria, total dissolved solids and Gammaproteobacteria/Actinomycetes, and iron and Flavobacteria. In the filtration stage, both negative (iron/manganese and Betaproteobacteria) and positive (temperature and Gammaproteobacteria, phosphates and *Bacilli*) correlations were observed. A strong positive

correlation was further seen in the final treated water between total dissolved solids/sulphate/iron and Cytophagia, ammonia and Actinomycetes, iron and Alphaproteobacteria/Flavobacteria/*Bacilli*, while there was a negative correlation between electrical conductivity and Cytophagia.

Table 4.24: Pearson correlation coefficient between total coliforms, *E. coli*, *Enterococcus* spp. and physicochemical parameters in Gauteng Province at Site D

Parameter	**Correlation coefficient *E. coli***	**Correlation coefficient *Enterococcus* spp.**	**Parameter**	**Correlation coefficient *E. coli***	**Correlation coefficient *Enterococcus* spp.**
Temperature	0.37	0.35	**Manganese**	0.316	0.45
pH	-0.136	-0.17	**Nitrate**	0.414	0.42
Turbidity	0.22	-0.20	**Sulphate**	0.13	0.16
Total organic carbon	0.456	0.41	**Ammonia**	0.481	0.58
Free chlorine	0.18	0.13	**Iron**	0.347	0.57
Total coliforms	0.74	0.92	**Phosphates**	0.21	0.19

4.8.1.3. Site E

Table 4.25 presents the Pearson's correlation coefficient between total coliforms, *E. coli* and *Enterococcus* spp. and various physicochemical parameters. Eight environmental factors and *E. coli* and *Enterococcus* spp. were moderately correlated. There was a strong correlation between total coliforms and *E. coli* ($r = 0.92$, $P<0.1$) and *Enterococcus* spp. ($r = 0.97$, $P<0.01$) at Site E. There was a positive correlation between *E. coli* and TOC ($r = 0.456$, $P>0.01$), nitrate ($r = 0.495$, $P>0.01$), manganese ($r = 0.416$, $P>0.01$) and iron ($r = 0.487$, $P>0.01$). Riyadh and Peleato (2024) also reported that TOC showed a moderate correlation with coliforms in the DWTPs owing to the regrowth of microbes after the input of high organic particles from surface water into the DWTP. There was a weak correlation of total coliforms, *E. coli* and *Enterococcus* spp. with turbidity ($r = 0.37$, $P>0.01$) and free chlorine ($r = 0.19$, $P>0.01$). Elevated turbidity not only affects water aesthetics but also masks the presence of pathogens and obstructs disinfection processes (Sigudu *et al*., 2024). The was no correlation between pH ($r = -0.198$, $P>0.05$) and total coliforms, *E. coli* and *Enterococcus* spp.

A significant (P<0.05) positive correlation occurred between electrical conductivity and Betaproteobacteria, iron and Sphingobacteria, Alphaproteobacteria/Betaproteobacteria and turbidity in the raw water source at Site E (Figure 4.23). A significant negative correlation was observed between sulphate and *Bacilli*, and iron and Gammaproteobacteria/Flavobacteria in the raw water source. In the filtration stage, a strong positive correlation was observed between turbidity, manganese and iron and *Bacilli*, and total dissolved solids and Gammaproteobacteria. A significant positive correlation in the final treated water occurred between electrical conductivity and *Bacilli/*Gammaproteobacteria, ammonia and Sphingobacteria, and nitrate and *Bacilli*. There was a negative correlation between total dissolve solids/free chloride and Flavobacteria, fluoride and Betaproteobacteria, and iron and Sphingobacteria.

Table 4.25: Pearson correlation coefficient between total coliforms, *E. coli* and *Enterococcus* spp. and physicochemical parameters in Gauteng Province at Site E

Parameter	**Correlation coefficient *E. coli***	**Correlation coefficient *Enterococcus* spp.**	**Parameter**	**Correlation coefficient *E. coli***	**Correlation coefficient *Enterococcus* spp.**
Temperature	0.293	0.34	**Manganese**	0.316	0.37
pH	-0.198	-0.26	**Nitrate**	0.414	0.43
Turbidity	0.37	0.40	**Sulphate**	0.13	0.18
Total organic carbon	0.495	0.53	**Ammonia**	0.481	0.421
Free chlorine	0.19	0.15	**Iron**	0.347	0.35
Total coliforms	0.92	0.97	**Phosphates**	0.21	0.29

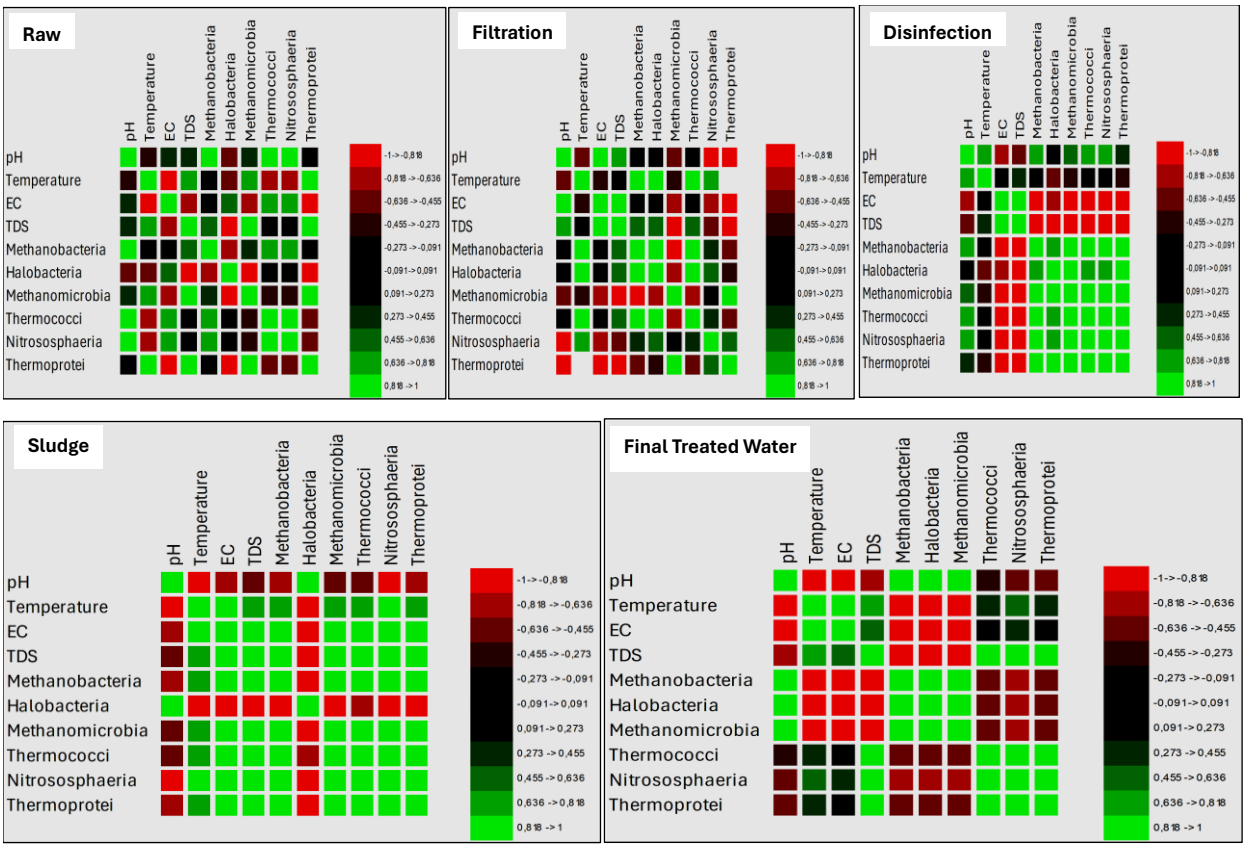


Figure 4.36: Relationship between selected physicochemical properties and microbial communities in Gauteng Province at Site A

4.8.2. Correlation of physicochemical parameters and microbial communities in Limpopo Province (Site B)

The relationship between physicochemical parameters and total coliforms, *E. coli* and *Enterococcus* spp. levels in the DWTP's raw water at Site B was examined using the Pearson's correlation coefficient (Table 4.26). There was a strong correlation between *E. coli* ($r = 0.89$, $P<0.01$) and *Enterococcus* spp. ($r = 0.91$, $P<0.01$) and total coliforms within Site B. There was a moderate correlation between *E. coli* and TOC ($r = 0.595$, $P>0.01$), nitrate ($r = 0.51$, $P>0.01$) and iron ($r = 0.53$, $P>0.01$). The was no correlation between pH ($r = -0.18$, $P>0.05$), sulphate ($r = -0.13$, $P>0.05$) and total coliforms, *E. coli* and *Enterococcus* spp.

Table 4.26: Pearson correlation coefficient between total coliforms, *E. coli*, *Enterococcus* spp. and physicochemical parameters in Limpopo Province

Parameter	Correlation coefficient *E. coli*,	Correlation coefficient *Enterococcus* spp.	Parameter	Correlation coefficient *E. coli*	Correlation coefficient *Enterococcus* spp.
Temperature	0.32	0.34	**Manganese**	0.31	0.38
pH	-0.18	-0.24	**Nitrate**	0.51	0.523
Turbidity	0.417	0.43	**Sulphate**	-0.13	-0.12
Total organic carbon	0.595	0.585	**Ammonia**	0.181	-0.421
Free chlorine	0.15	0.19	**Iron**	0.53	0.49
Total coliforms	0.89	0.91	**Phosphates**	0.3	0.29

Additionally, a significant ($P<0.05$) positive correlation in the raw water source at Site B was observed between Actinomycetes and manganese, Alphaproteobacteria and zinc, and Betaproteobacteria and turbidity (Figure 4.37). *Betaproteobacteria* was negatively correlated with ammonia while *Myxococcia* correlated negatively with manganese. In the filtration stage, Alphaproteobacteria correlated positively with manganese, Gammaproteobacteria with temperature, and *Planctomycetia* with manganese. In the same stage, a negative correlation was observed between Betaproteobacteria and temperature, as well as Gammaproteobacteria and turbidity. There were no strong significant ($P<0.05$) positive correlations in the final treated water; only a negative correlation was observed between Alphaproteobacteria and pH.

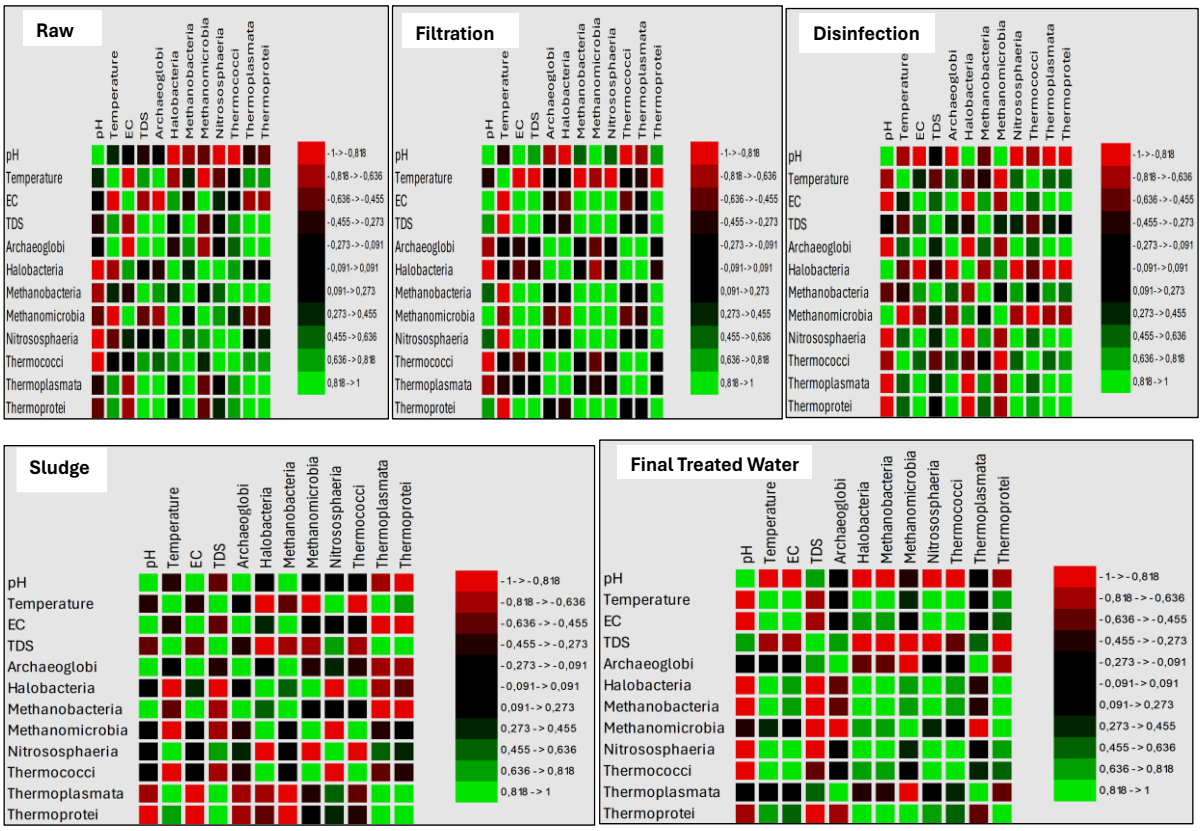


Figure 4.37: Relationship between selected physicochemical properties and microbial communities in Limpopo Province

4.8.3. Correlation of physicochemical parameters and microbial communities in Mpumalanga Province (Site C)

The relationship between physicochemical parameters and total coliforms, *E. coli* and *Enterococcus* spp. levels at Site A was examined using Pearson's correlation coefficient (Table 4.27). Seven environmental factors and *E. coli* and *Enterococcus* spp. interacted moderately. A strong correlation was shown between *E. coli* ($r = 0.92$, $P<0.1$) and *Enterococcus* spp. ($r = 0.82$, $P<0.01$) and total coliforms within Site C. A moderate correlation, coefficient between *E. coli* and manganese ($r = 0.532$, $P>0.01$), TOC ($r = 0.53$, $P>0.01$) and iron ($r = 0.46$, $P>0.01$). pH plays an important role in the survival and growth of microbial agents in water (Chen *et al*., 2023). Microbiological activities are sensitive to pH changes (Li *et al*., 2024a). Iron's correlation to microbes may be attributed to mining activities within Mpumalanga Province, with traces of acid mine drainage in the surface runoff (Waegenaar *et al*., 2024). The was no correlation between

turbidity (r = -0.14, P>0.05), ammonia (r = -0.11, P>0.05) and total coliforms, *E. coli* and *Enterococcus* spp.

Table 4.27: Pearson correlation coefficient (r) between total coliforms, *E. coli*, *Enterococcus* spp. and physicochemical parameters in Mpumalanga Province

Parameter	**Correlation coefficient *E. coli***	**Correlation coefficient *Enterococcus* spp.**	**Parameter**	**Correlation coefficient *E. coli***	**Correlation coefficient *Enterococcus* spp.**
Temperature	0.42	0.44	**Manganese**	0.42	0.34
pH	0.532	0.34	**Nitrate**	0.21	0.33
Turbidity	-0.14	-0.43	**Sulphate**	0.24	-0.22
Total organic carbon	0.53	0.53	**Ammonia**	-0.11	-0.321
Free chlorine	0.25	0.49	**Iron**	0.46	0.49
Total coliforms	0.92	0.82	**Phosphates**	0.5	0.69

Furthermore, a significant (P<0.01) positive correlation in the raw water source was observed between sulphate and Flavobacteria, turbidity and Flavobacteria, electrical conductivity and Actinomycetes, fluoride and Gammaproteobacteria, and nitrate and Gammaproteobacteria (Figure 4.38). In the filtration stage, pH correlated negatively with *Myxococcia*, total dissolved solids significantly correlated positively with Actinomycetes, as did Flavobacteria and Planctomycetes. A significant positive correlation was also observed in the final treated water between electrical conductivity and Gammaproteobacteria, turbidity and *Myxococcia*, manganese and Planctomycetes, ammonia and Gammaproteobacteria, sulphate and Alphaproteobacteria, iron and Gammaproteobacteria, and ammonia and Planctomycetes. A negative correlation was also observed in the final treated water between electrical conductivity and Flavobacteria, manganese and Actinomycetes, ammonia and Flavobacteria, and Flavobacteria, and zinc and Actinomycetes.

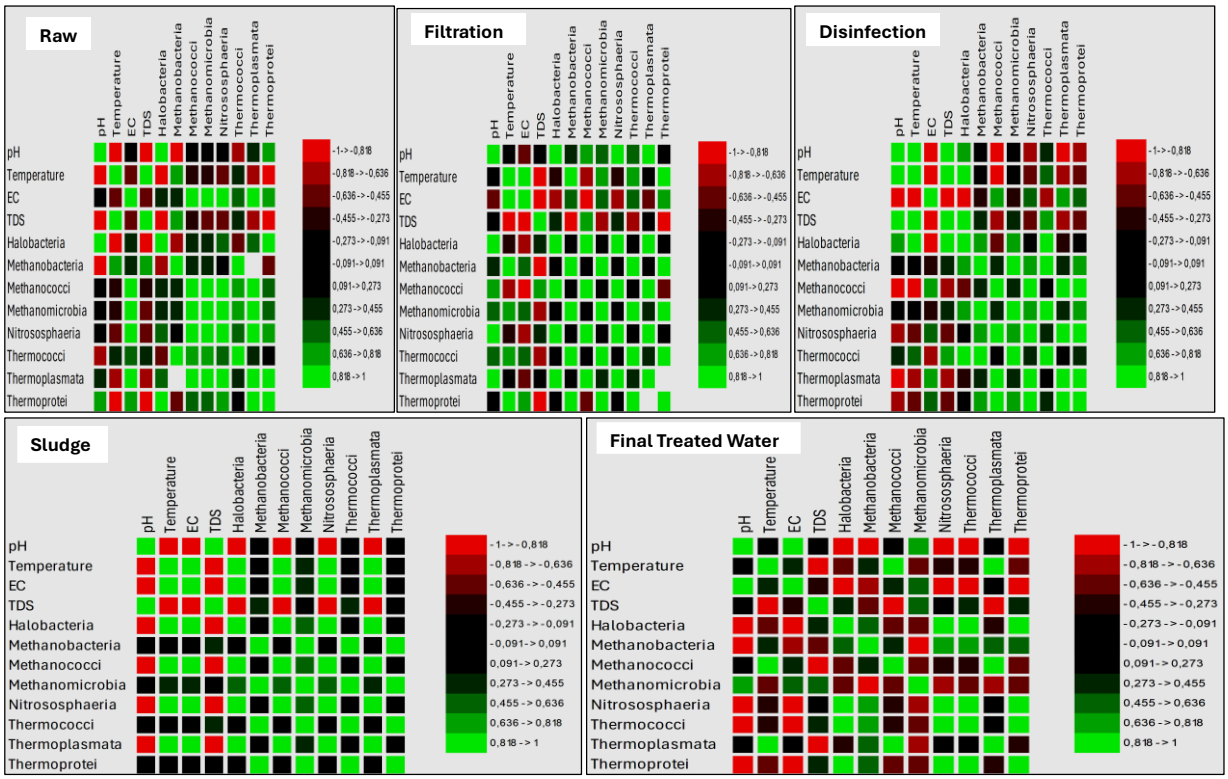


Figure 4.38: Relationship between selected physicochemical properties and microbial communities in Mpumalanga Province

CHAPTER 5: DISCUSSION

5.1. Physicochemical parameters

With increased economic activities and rising urbanisation, there is a growing concern over hazardous waste and microbial pollution, with pollution levels from human activities reaching critical levels in many cases (Maurya *et al*., 2020; Zhang *et al*., 2024a). Despite this escalating pollution, it is still the responsibility of DWTPs to provide the community with safe drinking water. To ensure tap water is safe for consumption, the SANS 241 (SANS, 2015) have set guidelines that outline the minimum requirements for water to be deemed safe for human use, applicable to South Africa.

Physicochemical and microbiological parameters of drinking water indicate the water quality and determine its suitability for various uses, including the protection of human health and the aquatic ecosystem (Steyn, 2022; Murei *et al*., 2023; Mustafa & Najmaldin, 2024). They play a crucial role in determining the aesthetic and operational purpose of the drinking water. In this present study, the measured physicochemical parameters varied across the sites over the sampling period and in the level of compliance.

5.1.1. pH

Measuring the pH is essential for determining whether the water is acidic or alkaline. When pH values are higher or lower than the limit standards, water may have a peculiar taste. Water with a pH below 7.0 may taste acidic, while water with a pH higher than 7.0 may taste bitter or soapy (Adams *et al*., 2022; Dube & Kanido, 2025). Acidic water can lead to corrosion of metal pipes and plumbing systems, while alkaline water shows disinfection. The normal drinking water pH range mentioned in the WHO guidelines is between 6.5 and 8.5 (WHO, 2022). The observed pH values in the final treated water and distribution system of Sites A, B, C, D and E were within permissible limits. The SANS 214 guidelines (SANS, 2015) recommend a pH range of ≥5 to ≤9.7 for safe drinking water, thus referring to the treated water and distribution systems water quality.

When observing different sites and each stage, the standout site was in Mpumalanga Province (Site C), where the pH of raw water sources ranged between 5.01±0.51 and 5.30±0.33, which showed the water is slightly acidic. Site C is located at the heart of the mining area, industrial activities and agriculture in Mpumalanga Province, South Africa. The pollutants from mining, industrial activities and agriculture are washed into the water sources through runoff and discharge. The existence of some organic and inorganic pollutants can lead to varying pH values in source water, depending on the chemical involved. For instance, sulphur and nitrogen oxides may be converted to nitric and sulphuric acids, especially when calculated and documented continuously, together with the electrical conductivity of a water body (Bilewu *et al*., 2022). A study in Romania reported similar water data, which is slightly acidic, with a pH of 5.28±3.96 of raw water (Miclean *et al*., 2025). The authors suggested that the low pH could be from acid mine drainage to the source point for the DWTP, which is located in the mining area. Zhang *et al*. (2025a) have identified the effect of low pH on heavy metals and other anthropogenic activities released from polluted surroundings.

At Site E, the raw winter ranged between 8.03±0.28 and 8.33±0.6, which means the pH is slightly alkaline or basic. Site E is in Gauteng Province, where its source water has a hyacinth challenge, and an algal bloom can raise the pH (Ajithram *et al*., 2021; Mqingwana *et al*., 2025). The final treated water ranged between 8.01±0.07 and 8.10±0.08 across the seasons, which may mean the water is more alkaline. A pH above 8 may affect the taste and odour of the water. Using alkaline water has been linked to some safety concerns, especially if the pH exceeds 9.8. The safety concerns include high levels of potassium in the blood, also called hyperkalaemia, and other health implications. However, Adams *et al*. (2022) reported that DWTPs in the United States of America are more likely to distribute water with a pH above 8 to protect the infrastructure and plumbing systems from corrosion. Furthermore, microorganism growth in the DWDS depends on several factors, such as availability of nutrients, concentration of disinfectant, water temperature, pH and a biofilm or corrosion layer on the pipe's inner surface (Stefan *et al*., 2023).

5.1.2. Temperature

Water samples collected at Site A showed that water temperatures were lower during the autumn and winter seasons than during the spring and summer seasons. The recorded temperature levels at Site A during the winter (range between 18.1±3.29°C and 21.8±3.0°C) and summer season

(range between 21.6±1.73°C and 26.7±3.26°C) have no adverse effect on microbial populations in the water distribution systems. The observed water values in the final treated water and distribution system of Sites B, C, D and E were within ±25°C throughout the seasons. According to the SANS 214 (SANS, 2015) drinking water limit, there is no strict numerical limit on temperature, meaning that temperature should be within a range that is considered pleasant and acceptable for consumption; this generally means not too hot or too cold, contributing to the overall quality and taste of the water (Agudelo-Vera *et al*., 2020; Khaliq *et al*., 2024). Temperature above 25°C could influence the dynamics of microorganisms in the DWDS, promoting the role of biofilms as a reservoir of opportunistic pathogens and their release into the bulk drinking water. Temperature is an essential ecological and physical factor that has a considerable impact on the biotic and abiotic factors of the environment (Palamuleni & Akoth, 2015). The temperatures observed in the present study would thus be able to maintain bacterial populations (Thom *et al*., 2022; Waegenaar *et al*., 2024).

5.1.3. Turbidity

The turbidity results show Site A's raw water ranged between 8.1±0.55 NTU and 10.56±1.24 NTU, which is a high concentration, indicating the presence of suspended particles that may have an impact on the safety and quality of the water in the DWTP. These high NTUs could be linked to high contamination levels from upstream and surrounding wastewater treatment plants, increased mining activities, agricultural land-use changes, population increases and more extensive and intense industrial development into the Vaal Dam in Gauteng Province (Mnguni, 2022). High turbidity concentrations in raw water samples were recorded in other studies as well (Castro-Jimenez, 2024; Raheem & Al-Juhaishi, 2024). It has been reported that high turbidity levels in raw water samples can overwhelm treatment processes (Castro-Jimenez, 2024; Omar *et al*., 2025). The final treated water samples of Site A ranged between 0.09±0.02 NTU and 0.19±0.05 NTU, while the distribution system samples ranged between 0.13±0.02 NTU. These NTU readings in the final treated water and distribution system were within the SANS 241 (SANS, 2015) guidelines of 1.0 NTU. This shows that Site A's water treatment processes effectively treat turbid water. Even though turbid water has no direct adverse human health effects, it may affect drinking water production efficiency and is an important parameter (Mouhri *et al*., 2024; Tahraoui *et al*., 2024). This finding aligns with the research conducted by Asseffa *et al.* (2024), who

similarly demonstrated that conventional water treatment methods play a crucial role in improving drinking water quality by reducing turbidity and other organic pollutants. Turbidity is a critical parameter as it can interfere with disinfection processes and provide a medium for microbial growth (Bain *et al*., 2020; Omar *et al*., 2025).

At Site B, the final treated water ranged from 0.50±0.26 NTU to 0.98±0.25 NTU, within the permissible limits. The distribution system ranged from 0.84±0.14 NTU to 1.11±0.14 NTU, slightly over the ≤1 SANS 241 (SANS, 2015) limit for drinking water quality. Turbidity, even slightly above 1.0 NTU, can cloud water (Latif *et al*., 2024). This high turbidity in the distribution system may mean contamination or possible leakages within the pipelines. It should be noted that South African municipal infrastructures have been reported to be in "poor and critical condition" in the Blue Drop Report (DWS, 2023). High turbidity in the DWDS could hinder disinfection effectiveness and may lead to high growth of microorganisms such as fungi, bacteria and viruses.

At Site C, the raw water ranged between 2.60±3.77 NTU and 10.7±1.54 NTU, a high NTU concentration coming into the DWTP. The high concentration of turbidity at Site C could be attributed to agricultural activities and domestic and industrial waste that flows into the water sources within Mpumalanga Province (Kruger *et al*., 2022). Flocculation, sedimentation and filtration showed that the process is functionally effective in reducing turbidity within the Site C drinking water treatment process, as the measured turbidity decreased much more than the incoming raw water. The final treated water turbidity ranged from 0.36±1.15 NTU to 2.60±0.93 NTU, within the SANS 241 (SANS, 2015) permissible limits of ≤1. The distribution system ranged from 1.09±0.34 NTU to 1.89±0.41 NTU in turbidity, slightly over the ≤1 SANS 241 (SANS, 2015) limit for drinking water quality. Water contamination and leakages in water distribution networks may be attributed to high turbidity within the distribution systems, with Site C having leakages and old infrastructure. This result from Site C did not comply with the SANS 241 (SANS, 2015) drinking water limit. These findings align with previous research, including a study by Chiavola *et al*. (2023), which found that increased turbidity levels correlate with higher levels of bacterial contamination, emphasising the potential health risks associated with cloudy water.

Additionally, Chen *et al*. (2023) demonstrated that turbidity can interfere with water treatment processes, particularly disinfection, by providing a protective environment for pathogens. The importance of monitoring turbidity levels is evident, as elevated turbidity not only affects water aesthetics but also masks the presence of pathogens and obstructs disinfection processes. Due to the old, worn-out water supply pipelines, Latif *et al*. (2024) reported more than 5 NTU in drinking water taps in Lahore, Pakistan. Overall, while some sampling points complied with turbidity standards, elevated levels in others necessitate immediate attention to ensure safe and clean water resources.

5.1.4. Conductivity

The conductivity of water is a measure of how well it conducts electricity, which indicates the presence of dissolved inorganic particles. At Site A, raw water conductivity ranged from 134.3±2.85 mS/m to 137.8±2.93 mS/m, while in the flocculation stage, it ranged between 92.9±1.85 mS/m and 98.35±2.46 mS/m. The conductivity during sedimentation and filtration processes remained constant, meaning the treatment process is fully functional. High conductivity within the drinking water process may indicate the presence of dissolved salts, which may affect the taste of water in the final treated water (Luvhimbi *et al*., 2022). High conductivity may also indicate overall water quality, as higher conductivity may signify contamination. The final treated water and distribution system conductivity were within ≤170 mS/m, the required limit for SANS 214 (SANS, 2015) drinking water quality. The treated sludge ranged between 124.7±24.65 mS/m and 179.2±28.19 mS/m, slightly higher than the permissible limit. The increases in conductivity within the treated sludge indicate that the sludge absorbed these elements from the water, and through treatment, some traces were removed (Wang *et al*., 2025). Electrical conductivity is utilised to assess the overall ionised content of water, directly correlating with the combined levels of cations and anions. Consequently, conductivity provides a reliable and prompt assessment of the total dissolved solids in water, thereby serving as an effective gauge of total dissolved solids, which plays a significant role in determining the salinity and palatability of drinking water (Fetrat & Islam, 2024; Omar *et al*., 2025).

The treated water and distribution systems at Sites B, C, D and E were within the permissible limit. The current study's findings are in stark contradiction to those of Nemadodzi *et al*. (2023), who

found that electrical conductivity was elevated and that it surpassed the SANS 241 (SANS, 2015) drinking water quality; the exceedances were primarily attributed to raw water sources in the DWTPs. Shamsudduha *et al*. (2025) concur with the findings from Nemadodzi *et al*. (2023) after reporting that higher electrical conductivity in Sri Lanka in drinking water sources (>1800 mS/m) originates from anthropogenic activities leading to the DWTPs. These findings reinforce the importance of monitoring conductivity as a proxy for water quality, contaminants potential presence within the raw water sources and the process stages of DWTPs. The results of the current study suggest that while conductivity levels are within acceptable limits, continued monitoring is essential to ensure water quality remains safeguarded against potential contamination. This proactive approach will help maintain the DWTP process, influence the microbial community in the treatment of the DWDS and protect public health.

5.1.5. Free chlorine

Free chlorine inhibits bacterial regrowth within the DWDS and prevents contamination of opportunistic pathogens and pathogenic bacteria in final treated water (Giessler *et al*., 2024; Hasan *et al*., 2024; Lau *et al*., 2024). In the present study, the free chlorine levels at Site A were within the stipulated limits, ranging between 0.24±0.04 mg/L and 0.33±0.05 mg/L in the final treated water. Monitoring the free chlorine is vital, with real-time monitoring correlating with total coliforms and *E. coli* counts, especially in the distribution system. For instance, the current study found that during the summer, zero bacterial counts of total coliforms and *E. coli* were recorded, which showed stable counts. A similar study by Aisopou & Stoianov (2024) reported that free chlorine showed fluctuations with a range of 0.12 mg/L and 0.2 mg/L, maintaining water quality within the DWDS. The free chlorine at Sites A, B, C and D was within the permissible limit of ≤5 mg/L according to SANS 241 (SANS, 2015) drinking water guideline.

Nevertheless, Site E recorded a free chlorine concentration of 5.74±2.89 mg/L during autumn. High free chlorine levels may be attributed to poor water quality and poor chlorine maintenance practices. The DWTP may be compensated by adding a high amount of chlorine to eliminate harmful bacteria and viruses (Rand Water, 2024). Sites A, B and C's distribution systems complied with their chlorine at the DWDS; even though it was less, it was still enough, ranging from

0.01 mg/L to 0.1 mg/L. Based on the results, Site D and Site E did not have chlorine results at the DWDS stage due to different connections between their suppliers and the end users.

The effectiveness of disinfection greatly depends on the water quality; thus, disinfection typically occurs after treatment processes (e.g., coagulation-flocculation-sedimentation and filtration) that clarify the source water, removing turbidity, colour and organic carbon, as well as microorganisms (Kalu *et al*., 2024). The most used disinfection processes are chlorination, chloramination and UV irradiation. The performance of both chlorination and chloramination is highly dependent on disinfectant concentration, contact time, temperature, pH, organic matter concentration and water turbidity, which may lead to the formation of disinfection by-products, elevating the concentrations of trihalomethanes and haloacetic acids in drinking water (Asadollahi *et al*., 2025). It is vital to maintain disinfection residual concentration in DWTPs and the DWDS to reduce and eliminate microbial growth such as *Legionella pneumophila*, *Mycobacterium avium* and amoeba-infecting bacteria such as *Chlamydia*-related organisms (Fawzer *et al*., 2025; Wang *et al*., 2025), as well as free-living pathogenic amoebae such as *Acanthamoeba*, *Hartanella* and *Naegleria* that can grow in the DWDS and cause waterborne illness (Waegenaar *et al.,* 2024; Li *et al*., 2025).

Chlorination is widely used for drinking water disinfection due to its relatively low cost, ease of use and low operational power requirements. Chlorine is a potent oxidising agent that hydrolyses in water to form hypochlorous acid, which oxidises cell walls and leads to cell lysis or inactivation of functional sites on the cell surface (Myers *et al*., 2025). High free chlorine concentration forms toxic disinfection by-products such as trihalomethane and other carcinogenic compounds (Murei *et al*., 2024; Zhang *et al*., 2024b). No free chlorine and high free chlorine in treated and the DWDS could be associated with human health risks.

5.1.6. Nitrates

There were variations of nitrate levels in the drinking water samples at Site A, with water samples ranging from 0.1±0.5 mg/L to 60±28.24 mg/L across the seasons. High levels of nitrates in treated water samples are indications of leaks from fertilised soil, sewerage, landfills, animal feedlots and urban drainage (Gu *et al*., 2024). The observed data from Sites A, B, C, D and E showed that the

nitrate concentration from treated water and distribution system points was within the permissible limit of 11 mg/L for potable water (SANS, 2015).

The results of nitrates levels in the raw water samples at Site B ranged between 35.34±0.36 mg/L and 5.97±0.31 mg/L. A strong correlation between nitrate concentration at the raw water sources and other anions (such as sulphate, iron and ammonia) was found in other studies (Zendehbad *et al*., 2022; Sailaukhanuly *et al*., 2024), suggesting that these pollutants come from the same sources such as fertilisers and sewerage water leaks into the source water. The nitrate concentration in Site B's filtration samples ranged between 1.3±0.54 mg/L and 2.33±0.45 mg/L, which is insignificant and within the permissible limit of 11 mg/L for potable water. This indicated that the filtration process is functional, even though seasonal factors such as temperature and equipment may influence the nitrate levels. Site C's temperature ranged between 23.8±1.03°C and 25.1±1.24°C within the water filtration stage. There was a strong correlation between levels of nitrate concentration and indicator bacteria such as total coliforms (14.4 MPN/100 mL and 90.0 MPN/100 mL) and *E. coli* (2.0 MPN/100 mL and 3.1 MPN/100 mL) at Site B, suggesting local pollution can collectively influence water quality parameters (Zhang *et al*., 2025b).

Treated sludge from all the sites showed high nitrate concentration, with Site B ranging between 1.43±1.43 mg/L and 56±26.11 mg/L. This was significant, especially since the limit in treated water should be ≤11 mg/L according to SANS 241 (SANS, 2015), and this could be due to the accumulation of nitrate compounds and other pollutants in the sludge during the sedimentation process. It is also essential to consider the temperature influences since nitrate compounds are more soluble in water at higher temperatures, and nitrification is strongly affected (Sailaukhanuly *et al*., 2024). The treated sludge samples may need further treatment to avoid contamination of water sources. Due to the environmental impacts nitrates contamination has on water, a remediation strategy that is environmentally friendly, sustainable, affordable and effective is needed in extensive mining and crop farming areas, as the nitrates are leaching into drinking water sources (Moloantoa *et al*., 2022). There are a variety of mitigation and remediation strategies for removing high nitrate concentration in drinking water, which can be biological or chemical remediation (Zhou *et al*., 2021). Biological remediation involves a carbon source at low dissolved oxygen levels, as it accommodates the reduction of nitrate to nitrogen. Denitrifying bacteria used in this remediation process are categorised into two groups based on their energy sources to reduce

oxidised nitrate (Zhou *et al*., 2021; Lin *et al*., 2022). Heterotrophic microbial denitrifiers use natural and chemical organic carbon sources to harvest energy to perform denitrification in wastewater with high nitrate concentrations over 1000 mg/L (Moloantoa *et al*., 2022).

5.1.7. Sulphate

Sulphate concentrations in drinking water are natural and generally considered safe at ≤250 mg/L according to the SANS 241 (SANS, 2015) drinking water guidelines. However, sulphates and sulphuric acid products are used in fertiliser production, industries and agricultural activities, contributing to high sulphur concentrations in the environment, including water sources (Wyffels *et al*., 2023). The results of sulphate concentration at Sites A, B, C, D and E showed that the final treated water and distribution system points were within the permissible limit.

Water samples collected at Site A measured sulphate levels ranging between 1.3±0.32 mg/L and 56±14.50 mg/L throughout the DWTP stages, the distribution system and the treated sludge throughout the seasons. Raw water sulphate levels at Site A ranged between 20.1±3.12 mg/L and 27.54±1.60 mg/L, indicating high levels for drinking water treatment. This sulphate may be attributed to acid mine drainage, fertilisers leaching from agricultural soils, wetland drainage and agricultural and industrial wastewater runoff, as well as sea level changes, which are the primary direct and indirect sources of the anthropogenic sulphate inputs to water sources and the environment (Zak *et al*., 2021; Raheem & Al-Juhaishi, 2024). In the filtration stage, sulphate levels varied with a range between 11.32±0.79 mg/L in autumn and 15.3±0.81 mg/L in spring ($P < 0.05$), which was significantly high. High sulphate levels in filtration suggest the need to improve the water treatment process further or curb the release of untreated or high pollutants into the catchment or both (Al-Mehrate *et al*., 2025). The sulphate levels in the final treated water samples ranged between 1.3±0.32 mg/L and 1.92±0.12 mg/L, and the distribution system samples ranged from 20.1±3.12 mg/L to 27.54±3.74 mg/L. These levels are within the prescribed limits for water quality (SANS, 2015), and therefore, sulphate appears not to be a contaminant of concern. In a study in the Havelian district (Pakistan), the sulphate levels in drinking water ranged between 70 and 180 mg/L (Saba, 2024), which was within permissible limits (WHO, 2022) and is relatively similar to this study.

When comparing sites between the three provinces (Gauteng—Sites D and E, Limpopo—Site B and Mpumalanga—Site C), Site C's raw water samples showed higher sulphate levels (192±22.85 mg/L in summer) than the other sites. These spikes were related to sudden rainfall within the province, which may have been attributed to runoff from agricultural and mining activities into the source water (Luvhimbi *et al*., 2022). In general, high sulphate levels can affect the water's organoleptic quality (taste), cause health problems and lead to the corrosion of the piping (Nduli *et al*., 2025). In the flocculation stages, Site C's sulphate levels ranged between 113±9.45 mg/L and 131±3.32 mg/L, which was high compared to other provinces. The high sulphate levels may still be attributed to runoff from anthropogenic activities into the raw water sources, with the flocculation stage known for controlling the floc size and settling velocity of cohesive sediments. Within the filtration stage of Site C, sulphate levels were recorded at 139±9.97 mg/L, which is significant during autumn. These high levels of sulphate in the filtration stage could suggest that the DWTP may be overwhelmed with many pollutants, corrosion and scale build-up within the pipelines into the filtration stage (Al-Mehrate *et al*., 2025). The DWTP may need maintenance and monitoring for further assessment. Sulphate levels, when present at high concentrations, can cause dehydration, gastrointestinal irritation and laxative effects in animals or humans (Ahmad *et al*., 2025; Al-Mehrate *et al*., 2025). In all sites (B, C, D and E), the final treated water and distribution systems' sulphate levels were within the permissible level (SANS, 2015). Furthermore, the treated sludge stages at Sites A, D and E complied with the SANS 241 guidelines (SANS, 2015), suggesting that the DWTPs of Gauteng province (Sites A, D and E) can treat sulphate levels, especially at the treated sludge stage.

5.1.8. Total organic carbon

Measuring TOC is critical in DWTP water quality assessment, with an inclination to assist in optimising the water treatment processes (Anderson *et al*., 2023). TOC is the amount of organic carbon in the water and can affect the taste, odour and appearance of drinking water. Carbohydrates, amino acids, hydrocarbons, fatty acids, phenolics, natural macromolecules and colloids (e.g., humic), sewage and industrial particulates, soil organic matter, living phytoplankton and other plant material are particular examples of organic matter present in the aquatic system. Water's organic matter is measured in total organic content (Chen *et al*., 2023; Ji *et al*., 2024).

TOC in drinking water reacts with disinfectants like chlorine and produces a disinfectant by-product that causes health problems in humans (DeMont *et al*., 2024).

The high TOC levels in the raw water of Site C between autumn and summer could be traced back to high rainfall during the summer (30.77±7.09 mg/L) and autumn (26.18±7.26 mg/L) seasons ($P < 0.05$). This could be attributed to industrial sources, agricultural runoff and household activities. Elevated TOC suggests a high potential for microbial regrowth, and as such, a focus on TOC reduction has been demonstrated to be a potent method for controlling bacterial activity. This can be noted with high total coliforms (above 2419.6 MPN/100 mL) and *E. coli* (39.9 MPN/100 mL) in Site C, especially in summer. Similar studies by Kang and Ahn (2022) and de Matos *et al*. (2023) reported direct correlations between elevated TOC levels and increased coliform counts. Specifically, water systems with TOC concentrations exceeding 100 mg/L registered an 82% surge in coliform-positive samples, with coliform levels being 19 times higher than in systems where assimilable organic carbon levels were below 99 mg/L (Riyadh & Peleato, 2024).

In filters, Sites A, D and E in Gauteng showed a decline in TOC levels from sedimentation, unlike in Limpopo and Mpumalanga, where there was a spike of TOC levels during spring—12.21±3.84 mg/L and 15.12±3.49 mg/L, respectively. The TOC level decline trend in Gauteng shows that filters work, particularly in removing organic matter, whereas the same trend was not observed in Limpopo and Mpumalanga Provinces. The most critical possible reason for these observations is the reduction of filter pore size with increasing turbidity in Site B (3.56±0.64) and Site C (4.34±0.40) during spring, therefore reducing the filter's performance to remove organic compounds (Ghasemian *et al*., 2023; Liu *et al*., 2024). Regarding the final water and distribution point, TOC levels at Sites A, D and E in Gauteng were within the permissible limit ≤10 mg/L for drinking water quality (SANS, 2015). However, Limpopo (range from 10.33±3.77 mg/L to 17.30±3.74 mg/L) and Mpumalanga Province (range from 9.30±2.61 mg/L to15.40±3.05 mg/L) showed high TOC levels, both above the permissible limit. These high values could be problematic given that organic carbon readily reacts with chlorine to form toxic and carcinogenic disinfectant by-products (Nduli *et al*., 2025).

In the treated sludge, all sites (A, B, C, D and E) showed high levels of TOC, all above the permissible limit (≤10 mg/L) for drinking water quality (SANS, 2015). The high TOC levels could

be attributed to the higher concentration of organics in the raw water sources and the sedimentation stages from pollutants from industrial activities and agricultural runoffs (Guo *et al.*, 2022; Assefa *et al*., 2024). The dynamics between these TOC fractions and microbial activity in treated sludge can manifest in multifaceted challenges, ranging from the onset of opportunistic pathogens to aesthetic degradation and even biofouling (DeMont *et al*., 2024).

5.1.9. Iron

Understanding the impact of different factors on the properties of iron particles is crucial to improving water quality in DWTPs and distribution systems. A study of water quality parameters, including pH, turbidity, free chlorine and manganese, shows their significant influence on the properties of iron particles and suspension in the DWDS (Zbykovskyy *et al*., 2025). In the current study, iron concentrations were determined in three provinces (Gauteng, Limpopo and Mpumalanga) to determine the relationships between diversity profiles of the microbiome, indicator microorganisms and physicochemical pollution of the water samples. As observed, raw water iron concentration in Limpopo Province (ranged between 19±64.11 and 165±65.38 mg/L) was higher than in Mpumalanga (ranged between 12±61.65 and 151±63.13 mg/L) and in Gauteng Province (Site A ranged between 11±3.06 and 17±2.58 mg/L; Site D ranged between 5.37±16.03 and 40±18.85 mg/L; Site E ranged between 4.37±11.78 mg/L and 27±12.66 mg/L). Levels of iron at Limpopo Province Site B could be due to high natural iron levels in Limpopo Province and from mining activities. High iron concentrations may be attributed to mining and agricultural activities' runoff, especially during summer when there is high rainfall runoff (Riyadh & Peleato, 2024). Changes in redox conditions and pH, such as when pyrite is oxidised, can release trace metals from surrounding geological materials into solution (Abrahams & Carranza, 2025).

Iron concentration in final treated and distribution points in all the three provinces was not within the permissible limit for potable water. SANS 241 guideline (SANS, 2015) recommends an iron concentration of ≤2 mg/L for potable water. For instance, the iron concentration of the final treated water samples at Site C (Gauteng Province) ranged between 12±7.08 mg/L and 48±19.73 mg/L, while the iron concentration at the distribution point sampled ranged from 2.4±0.97 mg/L to 16±3.67 mg/L. The excessive iron concentrations could be tracked from surrounding coal mines, where the quartz and kaolinite strata and minor traces of calcite, dolomite and pyrite are visible in

the overbank sediment of surface water within the Mpumalanga Province (Ushakova *et al*., 2022; Abrahams & Carranza, 2025). High iron concentrations in drinking water pose a risk to human health, and consistently consuming water containing iron above 2 mg/L for a long time causes chronic effects, including hemochromatosis (Zhang *et al*., 2025a). In Limpopo Province, Site B high iron concentrations were observed in the final treated water (ranged between 3.96±2.84 mg/L and 11.3±4.28 mg/L) and the distribution system (ranged between 0.96±0.84 mg/L and 7.3±1.28 mg/L). These high iron concentrations can be attributed to agricultural runoff and sewerage contaminations reaching the dam (raw water sources) (Luvhimbi *et al*., 2022). The high iron concentrations were mainly observed during spring and summer, when there is high rainfall, reflecting the state of the feeding catchments into the raw water sources per province. More studies reported high levels of trace metals, such as iron concentration in drinking water (Maguvu *et al*., 2020; Dube & Kanido, 2024; Abrahams & Carranza, 2025; Li *et al*., 2025). In a study performed in Southwestern Uganda, high iron concentrations were detected in surface water (11.271 mg/L), ground water (4.5 mg/L) and tap water (3.4 mg/L), which was above the WHO limit of 0.3 mg/L for aesthetic (Dube & Kanido, 2024), which indicates the presence of water pollutants and poor drinking water treatments in the study areas, which have implications on the health of local people. In China, the maximum limit of iron concentration in drinking water is 0.3 mg/L as stipulated in the GB 5749-2006 Standard for Drinking Water Quality. A high iron concentration of 1.0 mg/L of drinking water tap was recorded in Sanjian Plain, China (Li *et al*., 2025) which could have impacted on the water quality.

5.1.10. Ammonia

Measuring ammonia can be critical to a DWTP's water quality in helping optimise treatment processes, primarily because it can be used for pH control and disinfections (Trach *et al*., 2024). Electrical conductivity is vital when analysing treated drinking water and surface water, as it affects the water's balance of ionised and non-ionised ammonia (Said *et al*., 2022). According to Bhambri *et al*. (2025), water with an electrical conductivity of more than 500 mS/m or more than 200–300 mg/dm3 has a relatively stable percentage of non-ionised ammonia. Therefore, monitoring the water quality with electrical conductivity below 500 mS/m is essential to ensure safe drinking water and the protection of aquatic ecosystems in source water (Trach *et al*., 2024).

At Site A (Gauteng Province), ammonia concentrations in raw water exhibited significant seasonal variations ($P<0.05$) with values as low as 0.03±0.20 mg/L (spring) to as high as 0.5±0.10 mg/L (summer). This was also the case for ammonia concentrations at Site B (Limpopo Province), with levels as low as 1.03±0.02 mg/L (autumn) to as high as 1.5±0.2 mg/L (summer). Even at Site C (Mpumalanga Province), ammonia concentrations were as low as 0.0041±0.16 mg/L (winter) and as high as 3±1.72 mg/L (summer). The low levels may be attributed to high rainfall, which increases the natural dilution of ammonia emanating from wastewater effluents, organic decomposition and agricultural runoff before leaching to the dam (Nduli *et al*., 2025). Serajuddin *et al*. (2018) reported below 4 mg/L of ammonia during wet seasons and 20 mg/L during dry seasons in Dhaka, Bangladesh. The authors discovered that excessive ammonia in the raw water interferes with both the chlorine disinfection process and free chlorine maintenance in drinking water, with 1 mg/L of ammonia requiring an 8 mg/L to 10 mg/L of chlorine dose to achieve breakpoint chlorination (Serajuddin *et al*., 2018).

Regarding the filtration stage, Site A's ammonia concentration ranged from 0.011±0.04 mg/L to 0.08±0.039 mg/L, while Site B's ammonia concentration ranged between <0.009±0.07 mg/L and 0.03±0.007 mg/L. Site C recorded an ammonia concentration in the filtration samples of 0.010±0.37 mg/L (spring) to 0.75±0.42 mg/L (summer). This showed that the DWTPs were able to treat ammonia concentrations. Finally, in the final treated water and distribution systems, no significant variations ($P<0.05$) were identified in all provinces under this study. The SANS 241 guidelines (SANS, 2015) recommend an ammonia concentration of ≤1.5 mg/L for potable water. All provinces' final treated water and distribution system points were within the permissible limit. In Kazakhstan, ammonia concentrations were reported to be above 1.5 mg/L in drinking water due to chloramine disinfection (Sailaukhanuly *et al*., 2024).

5.2. Microbiological indicators

When considering indicator bacteria (such as total coliforms, *E. coli* and *Enterococcus* spp.) from various sample locations, the bacteria decreased from the raw water sources through the treatment system to the final treated water, as this was in line with water treatment objectives to produce safe water. The differences in the seasonal proliferation of the indicator bacteria in different sites could be linked to environmental parameter changes in the various sites or other anthropogenic activities

occurring in the area surrounding the sampling sites, as well as the seasonal impact on the infrastructure and microbial growth factors (Hochard *et al*., 2023). On the other side, drinking water treatment stages such as the disinfection stage deters proliferation of indicator bacteria, especially in the final treated water and the distribution system, with those proliferating post-disinfection exhibiting resistant to disinfectant.

5.2.1. Total coliforms, *E. coli* and *Enterococcus* species counts in Gauteng Province

Water samples from Gauteng Province (Sites A, D and E) were counted for total coliforms and *E. coli* from DWTP process stages, DWDS and treated sludge throughout the seasons. All sites in Gauteng Province had high counts of total coliforms counts with raw water sources with Site A (range from >2419.6 to >2419.6 MPN/100 mL), Site D (ranged from 86.5 to >2419.6 MPN/100 mL) Site E (ranged around >2419.6 MPN/100 mL). These high counts of total coliforms could be attributed to the sewerage runoff, agricultural activities, inadequate temperatures and increased organic nutrients in the source water (Rand Water, 2022). These findings corroborate the work of Al-Jaberi and Al-Abbawy (2023) and Saba (2024), who reported high total coliforms in raw water sources. As expected of natural environmental samples, levels of total coliforms in the raw water source were high at Site A (>2419.6 MPN/100 mL) and Site D (>2419.6 MPN/100 mL) during the summer as compared to winter at Site A recorded 378.4 MPN/100 mL, and Site D recorded 86.5 MPN/100 mL. At Site E, summer and winter coliforms were>2419.6 MPN/100 mL. The significant total coliform values during summer at Gauteng Province suggest comparable pollution risks that could be attributed to sewerage runoff into the dam from different sources within the catchment (DWS, 2023). These findings corresponded with previous research, such as the study by Sigudu *et al*. (2024), which emphasises total coliforms as reliable indicators of faecal contamination and pathogen risk in water supplies. Given the non-compliance rate of total coliforms, immediate action is essential to address the sources of contamination and improve water quality.

Within filtration stages, total coliform levels of Site A ranged from 25.9 to 248.1 MPN/100 mL, Site D ranged from 86.5 to >2419.6 MPN/100 mL, while Site E ranged from 76.7 to >2419.6 MPN/100 mL. These high levels may indicate potential contamination of the filtration system, and their presence may signal a vulnerability to other pathogens such as *E. coli*,

Enterococcus spp. and *Shigella* (Robert *et al*., 2021). The high concentration of total coliforms in the filtration stage correlates with TOC concentration at Site A (5.1±1.49 mg/L and 6.3±1.43 mg/L), which indicates that drinking water filtration may be contaminated with different organics and microbial contaminants (Castro-Jiménez *et al*., 2022; Sigudu *et al*., 2024). The findings of this study indicated that total coliforms were not present in the final water or the distribution system points of Sites A and E. In contrast, 1.0 MPN/100 mL total coliforms were recorded in Site D during spring. The SANS 241 (SANS, 2015) recommends total coliforms of ≤10 in any count per 100 mL, meaning that the final treated water and distribution system were within permissible limits.

When comparing *E. coli* counts per site and stage in Gauteng Province, Site A raw water samples recorded a range from 16 MPN/100 mL to 54.6 MPN/100 mL, Site D ranged from 2.0 MPN/100 mL and 235.9 MPN/100 mL, while Site E ranged between 34.9 MPN/100 mL and 214.2 MPN/100 mL. This could be likely attributed to increased surface runoff during summer, which can increase the risk of faecal contamination transportations from untreated sewerage and agriculture runoff into surface water (Nduli *et al*., 2025; Zhang *et al*., 2025a). The trends are consistent with other countries, for example Yanni *et al*. (2024) reported similar finding for raw water of municipal DWTP at Palembang, Indonesia and in line with research in South Africa were *E. coli* concentrations up to <2000 microbes/100 mL were recorded in selected Gauteng Province recreational water bodies by Delair *et al*. (2024). Bangani *et al*. (2023) emphasised that precipitation contributes to the transport of pollutants from the dump sites to water bodies, leading to increased *E. coli* counts. The results of *E. coli* within the filtration varied between the three sites of Gauteng Province. Within Site A, *E. coli* ranged between 0 and 5.2 MPN/100 mL, Site D *E. coli* counts were 0 MPN/100 mL in all seasons, and Site E recorded E. coli counts of 0 to 17.3 MPN/100 mL. These observations align with prior research on a similar full-scale sand filtration system at four DWTPs in the Netherlands, where the filtration stage recorded *E. coli* counts (Attiani *et al*., 2025). The authors reported that filtration may accumulate microbes (such as *Gemmataceae*, *Nitrospiraceae* and *Pirellulaceae*) similar to the raw water sources due to the gradual accumulation of microbes and effectiveness of the filtration stage (Zhou *et al*., 2024; Attiani *et al*., 2025). In addition, fluctuations in daily temperature associated with the different seasons also drive bacteria growth, regrowth and proliferation within the DWTP (Qiu *et al*., 2022;

Juste-Poinapen *et al*., 2024). As reported, the daily temperature within Sites A, D and E averages a high above 30 °C during summer seasons, and the filtration water samples collected at Site A (ranged from 22.3°C to 25.7°C) and Site E (ranged from 21.5°C to 25.3°C). This could account for the increased proliferation of *E. coli*, *Enterococcus* spp. and total coliforms during the summer and spring at the related sites and filtration stage. Odonkor and Mahami (2020) highlighted that the proliferation of the indicator bacteria could be linked to faecal pollution, which is seen to be varied across different areas and seasons depending on human activities. Faecal pollution occurs due to direct sewage deposition into raw water sources, discharged from wastewater treatment plants, and runoffs during rainfalls from agricultural and other anthropogenic activities (Zhao *et al*., 2020; Zhang *et al*., 2025b). This could also account for the site-seasonal patterns of the proliferation of the indicator bacteria observed.

In the final water, *E. coli* and *Enterococcus* spp. were present. Site E did not detect contamination, suggesting that their DWTPs can effectively remove microbes. At Site A, *E. coli* of 1.0 MPN/100 mL were recorded during spring, while Site D recorded 3.0 MPN/100 mL of *Enterococcus* spp. during spring. Even though Site A, D and E can treat *E.coli* and *Enterococcus* spp., it should be noted that the presence of indicator bacteria such as *E.coli* and *Enterococcus* spp. in raw water may be indicative of the presence of entero-pathogenic microorganisms such as viruses and some pathogenic organisms such as viruses and some pathogenic bacterial species, including those of the genus *Enterococcus* (Adeniji *et al*., 2021; Geissler *et al*., 2024). It should be noted that free chlorine at Site E ranged between 0.5±2.37 mg/L and 5.74±2.89 mg/L, which was higher than the ≤5 mg/L recommended limit according to SANS 241 (SANS, 2015) drinking water guideline. In the attempt to eliminate microbes within the final water, DWTPs dose high chlorine, which could impose chronic health issues (Luvhimbi *et al*., 2022; Murei *et al*., 2024) and may also lead to the formation of harmful and possibly carcinogenic disinfection biproducts such as trihalomethanes (Li *et al*., 2024a).

5.2.2. Total coliforms, *E. coli* and *Enterococcus* species counts in Limpopo Province

Microbial contamination is a central issue of concern within DWTPs and distribution systems (Yanni *et al*., 2024; Zwane *et al*., 2024), and for this reason, total coliforms, *E. coli* and *Enterococcus* spp. counts from the Limpopo Province DWTP were analysed. As a preamble, water

quality, particularly raw water, was affected by microbial contamination. Specifically, high total coliforms above 2419.6 MPN/100 mL during spring, summer and autumn, while winter exhibited the lowest total coliforms, 1732.9 MPN/100 mL, when comparing the four seasons. High *E. coli* counts of 35.2 MPN/100 mL and *Enterococcus* spp. counts of 61.0 MPN/100 mL were observed during summer within the raw water. This could be attributed to increased runoff during summer, which can increase the risk of faecal contamination in Limpopo Province from untreated sewerage and hospital wastes into the surface water (Siponen *et al*., 2024; Mqingwana *et al*., 2025). This pattern has been consistent with other studies. For instance, Kim *et al*. (2025) reported similar findings for surface water in Nairobi, Kenya, and it's in line with South Africa, where concentrations of *E. coli* were detected in surface water (Nduli *et al*., 2025).

In the final treated water and distribution point, *E. coli* and *Enterococcus* spp. presence was not detected, suggesting that Site B effectively removed microbes. Nevertheless, the treated sludge contamination was identified, which included total coliforms (range from 48.7 MPN/100 mL to 85.5 MPN/100 mL), *E. coli* (range from 14.7 MPN/100 mL to 20.9 MPN/100 mL) and *Enterococcus* spp. (range from 17.1 MPN/100 mL to 25.9 MPN/100 mL). The microbial contamination within released sludge is from accumulation from the sedimentation and other treatment processes, making it highly contaminated (Lau *et al*., 2024; Zwane *et al*., 2024).

5.2.3. Total coliforms, *E. coli* and *Enterococcus* species counts in Mpumalanga Province

Microbial contamination in water sources represents global public health concerns, particularly in developing countries where sanitation and hygiene practices are often inadequate (Izah & Ogwu, 2025). These contaminants include a variety of microorganisms, such as bacteria, viruses, protozoa and other pathogens, that can lead to serious health issues when ingested through contaminated water. The primary microbial contaminants analysed in Mpumalanga Province include total coliforms, *E. coli* and *Enterococcus* spp. in the DWTP, DWDS and treated sludge. Very high total coliforms (>2419.6 MPN/100 mL), *E. coli* (59.5 MPN/100 mL) and *Enterococcus* spp. (33.0 MPN/100 mL) were identified in the raw water source during summer and a low total coliform count of 36.6 MPN/100 mL, *E. coli* (5.1 MPN/100 mL) and *Enterococcus* spp. (10.9 MPN/100 mL) in winter. This could be attributed to precipitation runoffs during summer, which could increase the risk of microbial contaminations from untreated sewerage, industrial

activities and all upstream waste activities into the surface water (Kasanga *et al*., 2024; Waegenaar *et al*., 2024). Siddique *et al*. (2024) reported a similar observation, with high levels of microbes from agricultural activities and wastewater effluent into surface water in Dhaka, Bangladesh. Kaapu *et al*. (2022) reported high *E. coli* counts of 1756 MPN/100 mL runoff from hospital effluent in surface water. Within Site C, *E. coli* counts ranging between 0 MPN/100 mL and 3.1 MPN/100 mL and *Enterococcus* spp. counts ranging between 0 MPN/100 mL and 4.1 MPN/100 mL were observed in the filtration and distribution stages and final treated water, which indicates a low *Enterococcus* spp. and *E. coli* microbial burden, suggesting treatment achieved the intended goal.

Given that *E. coli* was not detected in Site C's final treated water and distribution point, suggests the effective removal of *E. coli*. However, high total coliforms of 257.2 MPN/ mL were observed in the final treated water during winter, and *Enterococcus* spp. of 3.1 MPN/100 mL were observed in the final treated water during the spring season. Total coliforms in drinking water have been reported as a problem in South Africa (Luvhimbi *et al*., 2022; Murei *et al*., 2024) with 214 MPN/100 mL in the tap water have been reported (Nduli *et al*., 2025). The overall high total coliforms could be attributed to faecal contamination, demonstrating the need for ongoing vigilance regarding the water quality (Murei *et al*., 2023; Sigudu *et al*., 2024). Recent studies in similar settings across South Africa, such as the rural Eastern Cape and Limpopo Provinces, have revealed alarming levels of microbial contamination and chemical pollutants in drinking water sources (Nowiki *et al*., 2021; Murei *et al*., 2024). For instance, Nowiki *et al*. (2021) reported that 40% of sampled water sources contained *E. coli*, indicating faecal contamination.

5.3. Correlation of physicochemical parameters and microbiological indicators

The treatment stages in the DWTPs play specific roles in establishing water quality standards by removing inorganic and organic matter and pathogenic microorganisms from the raw water sources (Kalu *et al*., 2024). In the DWTPs, the type of coagulation process, the method of sedimentation, the filtration types and infrastructure, and the oxidant used in the disinfection process are linked to the expected water quality and microbiological status of the water (Jiang *et al*., 2017; Huang *et al*., 2021). Processes such as coagulation and flocculation, filtration, oxidation, soil infiltration and adsorption have been reported to play an essential role in the removal of

pathogenic microorganisms (including indicator bacteria) from water samples (Bernardes *et al*., 2020; Masaka *et al*., 2021; Maiyo *et al*., 2023).

5.3.1. Correlations between physicochemical and microbiological parameters in Gauteng Province

In Gauteng Province, the concentrations of the indicator organisms *E. coli* and *Enterococcus* spp. strongly correlated with total coliforms. The increased total coliforms observed during summer (>2419.6 MPN/100 mL in raw water sources of Sites A, D and E) may be attributed to increased faecal matter and sewerage runoff into the surface water (Waegenaar *et al*., 2024). Ng'adwe *et al*. (2025) further highlighted that seasonal fluctuations underscore the heightened vulnerability of drinking water sources to microbial contamination during summer, posing significant public health risks. Contrary to expectations, some studies have indicated that seasonal variations do not consistently promote bacterial growth or correlate with organic carbon concentrations during summer (Delair *et al*., 2024; Zhang *et al*., 2024c). This indicated that the effect of increasing temperatures and TOC may depend on other factors such as water quality (e.g., pre-chlorination, nutrients) (Agudelo-Vera *et al*., 2020; Delair *et al*., 2024). In Gauteng Province, temperature significantly influenced microbial proliferation more than die-off, as evidenced by the positive correlation with microbes. Specifically, *E. coli* and *Enterococcus* spp. showed a positive correlation with temperature at Site A ($r = 0.35$, $P<0.01$; $r = 0.44$, $P<0.01$, respectively), Site D ($r = 0.37$, $P<0.01$; $r = 0.35$, $P<0.01$, respectively) and Site E ($r = 0.293$, $P<0.01$; $r = 0.34$, $P<0.01$, respectively) throughout the study. These findings are consistent with those of Kim *et al*. (2025) in Kenya, who reported a strong correlation between *E. coli,* temperature ($r = 0.46$) and pH ($r = 0.36$), highlighting the role of physicochemical parameters in supporting microbial growth when sufficient nutrients are present. Temperature also showed a significantly positive correlation with Gammaproteobacteria (filtration stage at Site D) and Alphaproteobacteria (raw water at Site A). Similarly, sulphate and Alphaproteobacteria (final treated water at Site C), sulphate and Cyanophyceae (raw water at Site A), sulphate and Betaproteobacteria (raw water source at Site D and filtration stage at Site A), and nitrate and *Bacilli* (final treated water at Site E) were all significantly positively correlated.

TOC, manganese, iron, ammonia and phosphates showed a moderate correlation with *E. coli* and *Enterococcus* spp. at Site B. Odewade *et al*. (2025) reported that phosphates are identified as a major contributor to microbial occurrence in drinking water compared to nitrates. Similarly, Douterelo *et al*. (2020) reported that phosphates increased the relative abundance of bacteria such as *Pseudomonas*, *Acinetobacter,* and the fungi *Cadophora*, *Rhizophagies* and *Eupenicillium* in water. Nutrients such as sulphate and ammonia can influence bacterial growth and fungi in water. The positive correlation identified in this study aligns with findings from Uganda by Dube and Kanido (2024), who demonstrated a similar positive correlation between *E. coli* and sulphur and ammonia in water. Enriching nutrients such as sulphates and ammonia could trigger the cyanobacterial production of taste and odour metabolite compounds like geosmin (Li *et al*., 2024a; Zhang *et al*., 2025b).

Turbidity has been reported to play a crucial role in the growth and regrowth of bacterial communities in drinking water systems, as the particles they create tend to hide the organisms from disinfectants. A strong positive correlation (P>0.05) was observed between the turbidity and members belonging to the *Bacilli* class (*Enterococcus* spp.) (Li *et al*., 2024b; Zhang *et al*., 2024a). This agrees with the findings in this study, where turbidity significantly correlated positively with *Bacilli* in the filtration stage of the Gauteng Province sites.

5.3.2. Correlations between physicochemical and microbiological parameters in Limpopo Province

In Limpopo Province, total coliforms strongly correlated with *E. coli (r* = 0.89, P<0.01) and *Enterococcus* spp. (*r* = 0.91, P<0.01), pointing out faecal contamination from the upper stream flowing into the raw water source (Murei *et al*., 2024). *E. coli* and *Enterococcus* spp. presented a significant positive correlation with nitrate (*r* = 0.523, P<0.01; *r* = 0.523, P<0.01, respectively) and phosphate (*r* = 0.3, P<0.01; *r* = 0.29, P<0.01, respectively), which could be attributed the sewerage sources around Nandoni upstream as important inputs of these nutrients during the rainy period. Phosphate originates from minerals, but its sources from terrestrial crust are considerably lower than silicate ones (Pinar-Méndez *et al*., 2022; Mustafa & Najmaldin, 2024). The nitrogen remineralisation from the organic matter decomposition could represent an expressive nitrate input to the water column of polluted estuaries due to the presence of labile organic matter (Mahajna *et*

al., 2022; Leite *et al*., 2024). In the case of phosphate, the input from sediment can occur when the salinity increases during the saline intrusion from sewerage and agriculture runoffs (Botha *et al*., 2023). This agrees with the findings in China by Li *et al*. (2024a), where strong vertical mixing increased the levels of dissolved phosphate in water during a runoff.

In the raw water sources, turbidity and TOC increase with depth because of the settling and resuspension of inorganic solid particles through different stages within the drinking water treatment sedimentation stage (El-taweel *et al*., 2023; Tahraoui *et al*., 2024). This strong correlation between TOC and microbes highlights that this site is not immune to environmental pressures or human activities that affect water safety (Riyadh & Peleato, 2024). Molina *et al*. (2024) demonstrate that even moderate levels of pollutants can significantly impact water quality and enhance microbial contamination. Therefore, during summer, the bottom layer in the raw water sources and other treatment stages increases in turbidity, which also tends to increase manganese and iron, together with a high concentration of *E. coli* (Abolfazli *et al*., 2024; Zhou *et al*., 2024). This can be noted by correlation between *E. coli* and *Enterococcus* spp. and manganese ($r = 0.31$, $P<0.01$; $r = 0.38$, $P<0.01$, respectively) and iron ($r = 0.53$, $P<0.01$; $r = 0.49$, $P<0.01$, respectively).

These findings agree with Hu *et al*. (2025) in China, who reported that metal iron and manganese stress enhanced microbial adhesion to the carrier and promoted microbial metabolic activity, leading to more viable but non-culturable bacteria growing within the next three months within the water distribution system.

5.3.3. Correlations between physicochemical and microbiological parameters in Mpumalanga Province

Correlation studies on the data obtained in Mpumalanga Province were conducted between a few physicochemical and microbial parameters to find their inter-relationship. The results show a strong correlation between *E. coli* ($r = 0.92$, $P<0.1$) and *Enterococcus* spp. ($r = 0.82$, $P<0.01$) with total coliforms within Site C. These findings align with Odewade *et al*. (2025) in Nigeria, who reported that total coliforms and *E. coli* showed high prevalence and correlation due to water samples from pit latrines infiltrating the soil and contaminating underground source water. These

findings also corroborate the work of Belina *et al*. (2023), who reported *E. coli*, *Enterococcus* spp. and total coliforms as the most isolated bacterial species in source water. However, these findings are inconsistent with those of Nicholson *et al*. (2017) in Nepal, who reported a weak correlation between total coliforms and *E. coli*, stating that water transportation and handling are more of a problem in these communities than contamination from the water source. A strong correlation coefficient was observed between *E. coli* and manganese ($r = 0.532$, $P>0.01$), TOC ($r = 0.53$, $P>0.01$) and iron ($r = 0.46$, $P>0.01$). The correlation of iron and manganese with microbes may be attributed to mining activities and sewerage contamination within Mpumalanga Province, with traces of acid mine drainage into the surface runoff (Waegenaar *et al*., 2024). Trace metals and microbial contaminants could originate from agricultural runoff, human sewage and mining activities, which are likely sources of contamination in the drinking water of Site C. Nitrates and sulphates can have negative effects, especially on pregnant women and small children (Clemmensen *et al*., 2023).

Furthermore, Mpumalanga Province Site C showed a significant ($P<0.01$) positive correlation in the raw water source between sulphate and Flavobacteria, turbidity and Flavobacteria, electrical conductivity and Actinomycetes, fluoride and Gammaproteobacteria, and nitrate and Gammaproteobacteria. A significant negative correlation was observed between sulphate and Flavobacteria. In the filtration stage, pH correlated negatively with *Myxococcia*, and total dissolved solids correlated significantly positively with Actinomycetes, Flavobacteria and *Planctomycetes*. Significant positive correlation was also observed in the final treated water between electrical conductivity and Gammaproteobacteria, turbidity and *Myxococcia*, manganese and *Planctomycetes*, ammonia and Gammaproteobacteria, sulphate and Alphaproteobacteria, iron and Gammaproteobacteria, and ammonia and *Planctomycetes*. A negative correlation was also observed in the final treated water between electrical conductivity and Flavobacteria, manganese and Actinomycetes, ammonia and Flavobacteria, iron and Flavobacteria, and zinc and Actinomycetes.

5.4. Antibiotic-resistant bacteria and multidrug-resistant bacteria

The development of ARB and their spread is associated with several environmental factors that also change with seasons (Mungondori *et al.*, 2021; Wang *et al*., 2023). Rising temperatures,

precipitation patterns and ecological systems may increase the spread of ARB, especially human pathogens (*E. coli*, *K. pneumoniae* and *S. aureus*) (Lie *et al*., 2022). Another factor that may affect ARB is extreme weather events such as heavy rainfall, floods, runoff, or monsoons. These factors are attributable to the spread of human and animal faecal bacteria, where antibiotics released into the environment can exert selective pressure on these bacteria, promoting the development and spread of ARB. This highlights the need for ongoing observation of AMR trends, the spread of ARB and the factors contributing to their growth in raw water, final treated water and distribution systems. Among the bacterial indicators, 106 out of 121 isolates were MDR.

5.4.1. Antibiotic-resistant bacteria in Gauteng Province

This study used ten antibiotics tested against *E. coli* and *Enterococcus* spp. isolates in different DWTP stages. At Site A, the isolated *E. coli* (raw_14, sedimentation_4 and filtration_1) demonstrated high resistance to several antibiotics. Many isolates were resistant to ßeta-lactam antibiotics (ampicillin and amoxicillin/clavulanic acid), ranging between 55% and 100%. High resistance rates were also observed for trimethoprim/sulfamethoxazole and nitrofurantoin, ranging between 45% and 80% across the seasons at Site A. *E. coli* isolates were resistant to trimethoprim/sulfamethoxazole and nitrofurantoin, ranging between 30% and 70%. In comparison, they were resistant to ciprofloxacin and vancomycin, ranging between 20% and 50%. In the case of the *Enterococcus* spp. (raw_10, sedimentation_2, filtration_1 and final treated water _0), the isolates were resistant to the same antibiotics. Among these *Enterococcus* isolates, they showed resistance to ampicillin and vancomycin, ranging between 70% and 90%.

Resistance against βeta-lactams (ampicillin) and amoxicillin/clavulanic acid was observed in *E. coli* isolates at Site A, with 29 isolates resistant to more than four of ampicillin, erythromycin, ciprofloxacin, nitrofurantoin, amoxicillin/clavulanate, gentamicin, imipenem, trimethoprim/sulfamethoxazole, levofloxacin, and vancomycin, which may correlate with their frequent use in clinical practice (Zhao *et al*., 2024; Li *et al*., 2025). High resistance patterns indicate the widespread consumption of these antibiotics and the need for responsible antibiotic use. The study revealed that the MAR values (0.28) indicated a potential origin from high-risk sources with significant antibiotic usage (Ramatla *et al*., 2023). The increased resistance from raw to distributed water suggests the selection of bacteria with survival and resistance traits during the water

treatment process. The present finding is supported by previous studies, highlighting the continued significance of *E. coli* resistance (Nasrollahian *et al*., 2024; Siponen *et al*., 2024; Yang *et al*., 2024). Additionally, there have been reports of a high prevalence of AMR in humans, animals, and the environment (Singh *et al*., 2024; Veloo *et al*., 2025). The present water treatment methods cannot treat AMR adequately (Alawi *et al*., 2024). Thus, improving basic hygiene and sanitation and advancing the treatment system could reduce the spread of resistant organisms (La Rosa *et al*., 2025).

The ARB studies conducted at Site D showed that *E. coli* isolates (raw_4, sedimentation_3 and filtration_1) were resistant to amoxicillin, ampicillin, trimethoprim/sulfamethoxazole, and nitrofurantoin. Relatively high resistance rates showed that *E. coli* isolates were resistant to imipenem and amoxicillin/clavulanic acid, ranging between 75% and 95% throughout the study period. In addition, *E. coli* isolates demonstrated a level of resistance to trimethoprim/sulfamethoxazole and nitrofurantoin ranging from 50% to 85% throughout the study. Between 30% and 55% of isolates were resistant to erythromycin, imipenem, and levofloxacin, while isolates resistance to ciprofloxacin and vancomycin ranged between 20% and 50%. In the case of the *Enterococcus* spp. (raw_5, sedimentation_3, filtration_1 and final water_0), isolates were resistant to the same antibiotics, as they were all tested for the same ten antibiotics. Among these *Enterococcus* spp. isolates, they showed relatively high resistance to ampicillin and vancomycin, ranging between 55% and 75%. Relatively high resistance rate to the most prescribed antibiotics, including ampicillin, erythromycin, ciprofloxacin, nitrofurantoin, amoxicillin/clavulanate, gentamicin, imipenem, trimethoprim/sulfamethoxazole, levofloxacin, and vancomycin, was observed among *E. coli* and *Enterococcus* spp. isolates. A study conducted in Greece (Tzimotoudis *et al*., 2025) showed a similar resistance pattern to these antibiotics, with *E. coli* isolates showing high resistance to ampicillin and tetracycline. A study conducted in Uganda also showed a similar pattern of resistance, with *E. coli* isolates showing resistance to ampicillin (81.4%), sulfamethoxazole/trimethoprim (70.7%), ciprofloxacin (67.9%), levofloxacin (64.6%), ceftriaxone (62.3%) and cefuroxime (62%) (Kasanga *et al*., 2024). In addition, Solaiman *et al*. (2022) reported *Enterococcus* spp. resistant to ampicillin, vancomycin, and linezolid, ranging between 26% and 48%. The observed variations in the reported resistance rates could be due to geographical locations and the nature of the study samples. Overuse of these antibiotics and a lack

of safe disposal may promote resistance (La Rosa *et al*., 2025; Wang *et al*., 2025). For instance, *E. coli* has been reported to be resistant to imipenem and ampicillin, and they are effective against *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *E. coli* and *Salmonella* and *Shigella* spp. (Peechakara *et al*., 2023). Therefore, the *E. coli* resistance found in this study may be due to the inappropriate treatment of human and animal wastes and their disposal in the environment. Hence, these untreated water sources contribute to AMR dissemination.

At Site E, *E. coli* isolates (raw_10, sedimentation_6 and filtration_2) demonstrated high resistance to several antimicrobials. Many isolates were resistant to gentamicin, imipenem, and trimethoprim/sulfamethoxazole, ranging between 80% and 100%. The high resistance rates were also observed for ßeta-lactam antibiotics (ampicillin, erythromycin) and nitrofurantoin, ranging between 60% and 75%. *E. coli* isolates were resistant to amoxicillin/clavulanic acid, ranging between 30% and 60%, while they were resistant to ampicillin and imipenem, ranging between 10% and 29%. A study of DWDSs in Nigeria revealed that *E. coli* isolates were resistant to erythromycin (100%) and ampicillin 76%) (Rabiu *et al*., 2025). In the case of the *Enterococcus* spp. (raw_10, sedimentation_2, filtration_1 and final treated water_0), the isolates were resistant to the same antibiotics, showing resistance to ciprofloxacin, erythromycin, and gentamicin, ranging between 80% and 100%.

5.4.2. Antibiotic-resistant bacteria in Limpopo Province

Seasonal variations and environmental factors such as temperature, rainfall and the overuse of antibiotics contribute to the dissemination and resistance rates of ARB and MAR at Site B. The antibiotic-resistant trend showed *E. coli* isolates (raw_12, sedimentation_4 and filtration_3) from spring, summer, winter, and autumn. Many isolates were resistant to ßeta-lactam antibiotics (ampicillin and imipenem) during summer, followed by winter, spring and autumn. They ranged between 60% and 80%. In addition, 30% and 60% of isolates were resistant to trimethoprim/sulfamethoxazole, erythromycin, and nitrofurantoin across the seasons. Between 10% and 30% of isolates from the study were resistant to amoxicillin/clavulanate, ciprofloxacin, gentamicin, and levofloxacin throughout the seasons. In the case of the *Enterococcus* spp. (raw_5, sedimentation_6 and filtration_4), the isolates were resistant to the same antibiotics. Among these isolates, 30% to 70% were resistant to ciprofloxacin, imipenem, and vancomycin. Between 10%

and 30% were resistant to amoxicillin/clavulanate, ciprofloxacin, gentamicin, and levofloxacin. The study recorded an MAR value of 0.23, indicating high antibiotic usage in the area surrounding the treatment plants in those seasons. However, the current MAR value (0.23) is lower than the MAR indices of 0.46 and 0.54 reported in a study conducted on water samples in Dhaka, Bangladesh (Siddique *et al*., 2024). Muteeb *et al*. (2023), Salam *et al*. (2023) and Singh *et al*. (2024) highlighted the misuse and overuse of similar antibiotics in human medicine and agriculture as a factor that promotes the development and spread of MAR in microorganisms into drinking water through treatment processes. The present finding is supported by earlier studies, highlighting the continued significance of *E. coli* and *Enterococcus* spp. resistance in drinking water (Molale-Tom *et al*., 2024). Potentially pathogenic *E. coli* and *Enterococcus* spp. pose a threat to humans, particularly those with underlying infections or compromised immune systems, and their antibiotic resistance exacerbates the situation. Some strains of *E. coli*, particularly those involved in extraintestinal infections, use iron from red blood cells for growth and can play a significant role in pathogenesis (Pokharel *et al*., 2023). This study revealed a high proportion of *E. coli* isolates surpassing previous reports (Pokharel *et al*., 2023; Sadeghi *et al*., 2024), indicating the potential pathogenicity of most *E. coli* isolates.

5.4.3. Antibiotic-resistant bacteria in Mpumalanga Province

At Site C, *E. coli* isolates (raw_13, sedimentation_7 and filtration_3) demonstrated antibiotic resistance. A large percentage of isolates were resistant to ßeta-lactam antibiotics (ampicillin and imipenem), trimethoprim/sulfamethoxazole, amoxicillin/clavulanate, ciprofloxacin, gentamicin, levofloxacin, and nitrofurantoin, ranging between 70% and 100% throughout the seasons. In the case of the *Enterococcus* spp. (raw_6, sedimentation_6 and filtration_4), the isolates were resistant to the same antibiotics. Among these isolates, 70% and 100% were resistant to ciprofloxacin, imipenem, amoxicillin/clavulanate, gentamicin, levofloxacin, and vancomycin. The results at Site C indicated that disinfection during the drinking water treatment process created selected ecological pressure for the proliferation of antibiotics and MDR bacterial communities in the DWDS. The impact of these selective pressures is consistently supported by previous studies (Gu *et al*., 2024; Shang *et al*., 2024; Zhu *et al*., 2024).

Furthermore, the final treated water at Site C further enriches the ARGs within the distribution system. Specifically, genes with MDR strains and genes that confer resistance against antibiotic classes such as ßeta-lactam and aminoglycoside could be associated with using free chlorine as a disinfectant (Tiwari *et al*., 2022). Although genes that confer resistance against MDR and the antibiotic classes such as ampicillin and imipenem were mainly associated with chlorinated water samples at Site C, many ARBs may survive and proliferate in the DWDS. This argument is supported by the high diversity of microbial communities and MAR at the disinfected distribution system.

The study revealed that the MAR values (0.25) indicated a potential origin from high-risk sources with significant antibiotic usage (Ramatla *et al*., 2023; Li *et al*., 2024b). There have been reports of a high prevalence of AMR in human and animal settings and the environment (Kasanga *et al*., 2024; Mthombeni *et al*., 2024). The present water treatment methods cannot treat AMR adequately (Zhao *et al*., 2024). Water temperature could play a role in microbial growth, especially as water samples pass through the treatment process (Kulik *et al*., 2023; Farrukh *et al*., 2025), which may directly or indirectly influence the growth of indicator bacteria, further spreading ARGs and contributing to the development of ARB. Research has indicated that the number of ARB tends to increase during the various treatment stages of DWTPs compared to the original raw water sources (Larsson & Flach, 2022; Zhao *et al*., 2024; La Rosa *et al*., 2025). This may be related to the conditions present during various treatment stages that could promote the release of ARGs and the growth of ARB. Xu *et al*. (2014) and Kalu *et al*. (2024) found a positive link between the temperature in DWTPs and the levels of ARGs, particularly those associated with aminoglycosides and sulfonamides. This suggests that the rise in antibiotic resistance noted in the current study could also be influenced by temperature changes during different treatment stages. Temperatures at Site C varied from 19.9±0.91 °C to 26.8±1.73 °C within the DWTP stages, distribution system and treated sludge throughout the seasons.

5.5. Taxonomic microbial diversity per site and treatment stage

In the analysis of taxonomic composition profiles, Archaea, bacteria and Eukaryota domains showed statistically significant differences in mean value from raw water, filtration, disinfection stage, treated sludge and final treated water.

5.5.1. Taxonomic microbial diversity in Gauteng Province

To gain a broader understanding of the microbes present in the DWTP, distribution system and treated sludge, samples were collected across four seasons from Gauteng Province sites. At Sites A, D and E, the most abundant microbial communities were Nitrososphaerota (Archaea phyla) within raw water sources and Euryarchaeota (Archaea phyla) within filtration and treated sludge. These findings are similar to Bekele *et al*. (2023), who reported Nitrososphaerota, Halorubrum and Halohasta from raw water sources, stating that they are highly adapted to extreme temperature, salinity and pH conditions. According to Wu *et al*. (2020) and Li *et al*. (2024b), these genera play critical roles in nutrient cycling and ecosystem stability through the metabolism of ammonia nitrate and sulphate. Their unique physiological adaptations sustain ecological processes in source water and hold significant biotechnological potential, such as the production of bioactive compounds and animal applications (Bawane *et al*., 2024).

The most abundant phyla of the bacterial community within Sites A and E were Pseudomonadota, abundant within the raw water, filtration stage and treated sludge. At Site D, the most abundant bacterial phyla were Bacteroidota and Actinomycetota within the raw water, filtration stage and final treated water. These findings are consistent with those of Sharma *et al*. (2025) in India, who reported that the most abundant bacterial phyla of Pseudomonadota (Proteobacteria), Planctomycetota, Bacteriodota and Actinomycetota, highlighting that temperature was the main factor influencing microbial composition. Site A water samples' temperature values ranged from 21.6±1.73°C to 26.7±3.26°C, while those from Site D ranged from 18.3±9°C to 26.8±2.25°C and those from Site E ranged from 19.6±1.6 to 26.1±11.79°C; these results could have had an impact on the microbial community. Additionally, Pseudomonadota is commonly the abundant phylum in soil sediments, having a wide range of functions in nitrogen, carbon and sulphur metabolism and the degradation of organic and inorganic compounds. These abundant phyla may contaminate water sources through seasonal runoffs (Li *et al*., 2017a; Yu *et al*., 2024). Alphaproteobacteria are obligate or facultative intracellular bacteria capable of living in low-nutrient environments, such as soil sediments, deep ocean sediments and glacial ice (Ahlwat *et al*., 2025). Their prevalence suggests their adaptability to a wide range of agricultural management practices, DWTPs and their significant roles in nutrient cycling and organic matter decomposition.

The emergence of these waterborne pathogens indicates shifts in human demographics, alterations in human habits, deterioration of public health infrastructures, microbial evolution and modifications in agricultural methods (Oon *et al*., 2023). For instance, urbanisation and population growth could lead to increased contamination of water sources, while changes in agricultural practices, such as irrigation techniques, can introduce pathogens into water reservoirs. Additionally, climate change may influence the distribution and survival of pathogens in water environments, further complicating efforts to mitigate the risks associated with waterborne diseases (Afonso *et al*., 2024). Svetlicic *et al*. (2023) reported new potential species of the *Legionella* genus from water collected in different countries in Europe, which could be attributed to modifications in agricultural methods and microbial evolution in different habitats.

The bacterial phyla Pseudomonadota and Actinomycetota were the most abundant phyla in the Gauteng Province raw water samples. These Pseudomonadota and Actinomycetota were reported to be involved in biotic associations, colonising host-associated niches in the context of the rhizosphere and phyllosphere, the gut microbiota, or even in closer interactions such as endosymbiosis and intracellular lifestyle, which could flow into the DWTPs as raw water sources (Chobert *et al*., 2025; Li *et al*., 2025). Brindefalk *et al*. (2022) studied 200 raw water sources in Sweden and reported that Actinobacteria, Bacteroidetes and Proteobacteria constituted approximately 80% to 90% of the phyla. Despite the high diversity of bacterial communities in water, Proteobacteria dominate the bacterial community in Gauteng Province, with pathogens like *P. aeruginosa* that can survive in the distribution system even though there are low-nutrient concentrations (Mahajna *et al*., 2022; Li *et al*., 2024a). At Sites A and D, the raw water, filtration stage and disinfection stage showed contamination with dominant bacterial classes Alphaproteobacteria, Gammaproteobacteria and Betaproteobacteria throughout the study period. Chlorination is an essential and reliable disinfection agent in drinking water; however, Zhang *et al*. (2025a) reported high levels of Proteobacteria and Firmicutes in the final treatment stage and distribution system. This level of bacterial contamination may be influenced by temperature, inorganic contaminants and biofilms within the drinking water (Pan *et al*., 2022; Liu *et al*., 2024). The importance of ozone treatment has been emphasised in many drinking water facilities as a suitable option for disinfection. Nevertheless, there is limited data on its impact on microbial composition (Maguvu *et al*., 2020; Li *et al*., 2024b; Zhang *et al*., 2025b). High levels of

Proteobacteria and Actinobacteria were noted when ozone and chlorination were applied concurrently (Zhao *et al*., 2024). This finding supports the notion that the study of the bacterial composition of DWTP's final treated water provides a basis for understanding the microbiome of drinking water supplied through public distribution systems (Kumar *et al*., 2024). The top four phyla are curated in the Human Oral Microbiome Database and are significantly associated with illnesses such as asthma, gastrointestinal tract disease and urinary tract infections (Abolfazli *et al*., 2024).

In the present study, the most common eukaryotic communities were the Ascomycota, Basidiomycota and Bacillariophyta phyla in the raw water sources of Gauteng Province. The origin of these eukaryotic communities depended on the geographical location of the source water and diversity within the ecosystems, taxonomic turnover rather than biodiversity loss (*et al*., 2025; Wang *et al*., 2025). The treated drinking water may resemble raw water when some organisms can survive the treatment process (Zhu *et al*., 2025). In this study, Gauteng Province's final treated water was dominated by eukaryotic classes Aconoidasida, Sordariomycetes and Cryptophyceae, suggesting that eukaryotes shared a similar structure, and DWTP did not meet the requirements in terms of disinfection. It could be speculated that lack of treatment of sewerage effluent, agriculture activities runoff and rainfall encourage high turbidity from organic and inorganic contaminants into raw water source could be environmental drivers to rich and diverse eukaryotic communities (Alam *et al*., 2022; Gao *et al*., 2024; Li *et al*., 2025). A similar study in China by Zhu *et al*. (2025) reported that variations of total nitrogen, chlorophyll-a and conductivity could have led to a transition in the assembly of eukaryotic communities in the drinking water. Meanwhile, large-sized eukaryotes can multiply, grow and populate the treated water even after discharge. At the same time, the small-sized may show a non-significant difference value increase, due to a slight variation in the effectiveness of filtration or disinfection or both at different DWTP locations.

This finding could be attributed to different pollutants, such as agriculture runoffs and urban discharge into water sources and the environment (Mora *et al*., 2025). Ascomycota fungi are important drivers in carbon and nitrogen cycling in arid ecosystems, as well as their functioning in low water ecosystems and where there is organic matter (Manici *et al*., 2024). They may also form symbiotic associations with biocrust components or be latent saprotrophs or pathogens that live on plant tissues. However, their functional potential in arid soils, where organic matter,

nutrients and water are very low or only periodically available, is poorly characterised (Wang *et al*., 2025). By analysing fungal taxa across freshwater habitats, the current study's findings highlight how urbanisation shapes microbial diversity and underscore the need for research into the functional roles of urban-adapted fungi in host-microbe interactions, biogeochemical cycling and ecosystem stability.

5.5.2. Taxonomic microbial diversity in Limpopo Province

In the present study, a diverse microbial community was detected from the raw water, the filtration and disinfection stages, final treated water and treated sludge samples at Site B in Limpopo Province. The most abundant Archaea classes Halobacteria and Methanomicrobia were detected in the raw water source and final treated water. The halophiles are extremophiles or extremotolerant organisms that can survive in high-salinity water (Sharma *et al*., 2025). The Archaea phylum Halobacteria can also exist across the life domains, showing diversity in metabolic strategies and physiological response among other microbes (Liu *et al*., 2024). Raw water sources contaminated with salinity and organic particles allow these halophiles to thrive, especially with Site B raw water sources contaminated with pollutants from agricultural activities and households (Li *et al*., 2024b; Zhou *et al*., 2025).

Among the bacterial families identified in Site B's raw water sources, filtration stage and treated sludge, Pseudomonadota and Actinomycetota were the dominant bacterial phyla. At the same time, Alphaproteobacteria, Gammaproteobacteria, Actinomycetes and Betaproteobacteria were most abundant in the raw water source and treated sludge, but they varied with seasons. Proteobacteria, is the most predominant phylum in all samples at Site B, and with high abundance recorded in the raw water sources. Studies have shown the domination of the natural water sources by the members of bacterial communities belonging to the phyla Proteobacteria, Actinobacteria and Bacteroidetes (Zhu *et al*., 2022; Sharma *et al*., 2024). Some of the members, such as the Betaproteobacteria and Actinobacteria, have been reported to play crucial roles in the maintenance of the natural water ecosystems; however, their abundance could be impaired by environmental pollution (Bautista-De los Santos *et al*., 2022; Li *et al*., 2024b; Zhang *et al*., 2024b). This could account for the abundance of the phylum Proteobacteria in the current study, especially in the raw water sources for all the sites. Agricultural waste discharge and human sewage discharge into the natural water have been

reported to alter the aquatic ecosystems standard microbial structure, leading to the introduction and development of pathogenic microbial communities (La Rosa *et al*., 2025). In light of the health of humans, treatment plants must reduce or completely remove the pathogenic microbial community in the raw water sources, presenting good potable water for the populace. Although Gammaproteobacteria were mainly in the disinfection stage and distribution points, they should receive more attention because of their increase after chlorine disinfection (Li *et al*., 2024c).

At Site B, the most abundant eukaryotic communities were Ascomycota, Basidiomycota, Apicomplexa, Bacillariophyta (Eukaryota phyla), Aconoidasida, Sordariomycetes and Saccharomycetes (Eukaryota class) in the raw water, filtration stage and final treated water. Li *et al*. (2024a) reported that Ascomycota and Basidiomycota are the most abundant eukaryotic phyla. This agrees with the current study's findings, where Ascomycota, Basidiomycota and Proteobacteria dominated after disinfection and in the treated sludge. The proportion of Proteobacteria increased after chlorination disinfection, which may be attributed to the greater resistance of Gram-negative bacteria than of Firmicutes (Gram-positive bacteria) to chlorine (Zhao *et al*., 2024; Zhang *et al*., 2025b).

5.5.3. Taxonomic microbial diversity in Mpumalanga Province

Taxonomic profiles of the various metagenomic sample groups identified significant Archaea, Bacteria and Eukaryota (phyla, classes and genera) at Mpumalanga Province's DWTP from the raw water source, the filtration and disinfection stages, the final treated water and the treated sludge samples. There were high degrees of species richness and alpha diversity of taxa in source waters, which reduced throughout the treatment process stages. The most abundant Archaea classes at this site were Halobacteria and Methanomicrobia, detected within the raw water source and treated sludge. These findings are consistent with those of Gugliandolo and Maugeri (2019) in Italy, who reported that Halobacteria were prevalent and were affiliated with several genera (*Haloarcula*, *Halobacterium*, *Halobiforma*, *Halomicrobium*, *Haloplanus*, *Halorubrum* and *Natronomonas*). According to Gugliandolo and Maugeri (2019), Halobacteria and Methanomicrobia greatly depend on oxygen and methane within the surface water. Methanotrophs encompass a range of strains that can be neutrophilic, acid-tolerant or acidophilic (Hwangbo *et al*., 2023). This can be seen as they

are able to grow in the final treated water at Site E. These Methanophs could grow like *E. coli* in a moderately low pH because they tolerate acidic conditions.

Within Site C, samples after disinfection with chlorine had a more deterministic value. Proteobacteria in particular, Pseudomonadota, Bacteriodota and Actinomycetota have been demonstrated to dominate post-disinfection, supporting the deterministic influence of treatment (Li *et al*., 2024b). It also suggests that although filtration is essential in defining taxa in the distribution system, chlorine has a more strongly selective effect. This is supported by a study comparing drinking water treatment systems using the same surface water, where chlorine and chloramine produced different communities (Potgieter *et al*., 2021). Proteobacteria can survive in various anoxic environments since they are often facultatively or obligately anaerobic (Demirci *et al*., 2025). Buian *et al*. (2024) evaluated changes in the drinking water microbiome in water treatment plants in an urbanised area. The authors observed the abundance of Proteobacteria (43%) and Patescibacteria (41%), followed by uncultured Omnitrophicaeota (3%), Chloroflexi (1%) and Cyanobacteria (1%) across the Proteobacteria for the bacterial communities. This agrees with the present study's findings that Proteobacteria and Pseudomonadota (bacterial phyla) were dominant across the treatment stages of Sites C. Studies have shown the domination of the natural water sources by the members of bacterial communities belonging to the phyla Proteobacteria, Actinobacteria and Bacteroidetes (Favere *et al.*, 2020; Jing *et al*., 2021; Guo *et al.*, 2022; Jiang *et al*., 2022). Some of the members of bacterial class, such as the Alphaproteobacteria, Gammaproteobacteria, Actinomycetes and Betaproteobacteria, have been reported to play crucial roles in the maintenance of the natural water ecosystems; however, their abundance could be impaired by environmental pollution (Li *et al*., 2017a; Thom *et al.*, 2022; Kalu *et al*., 2024; Zhang *et al*., 2024b). This might explain why the phylum Proteobacteria is so prevalent in the current study, particularly in the raw water sources across all sites. Reports indicate that the discharge of agricultural waste and human sewage into natural water bodies disrupts the usual microbial balance in aquatic ecosystems, resulting in the emergence and growth of harmful microbial communities (Luvhimbi *et al*., 2022; Murei *et al*., 2023). To protect human health, treatment plants must minimise or eliminate these harmful microbes from raw water sources, ensuring safe drinking water for the community.

5.6. Antibiotic-resistant gene phenotype profile unveiled through shotgun metagenomics

The characterisation of ARGs from the metagenomic reads was done using PAST 4. More than 80 ARGs were identified spanning across different AMR gene families. To quantify the abundance of ARGs and compare them across samples, normalisation was done by calculating reads per million kilobases for each gene identified. Notably, ARG types associated with resistance to ten antibiotic classes (aminoglycoside, glycopeptide, phenicol, trimethoprim, fosfomycin, aminoglycoside, ßeta-lactam, fluoroquinolone, macrolide, and tetracycline) were consistently detected across all samples.

5.6.1. Antibiotic-resistant gene phenotype composition in Gauteng Province

In Gauteng Province, more than 36 out of 80 unique ARGs belonging to ten major ARG types, were detected from the raw water sources, sedimentation and filtration stages, final treated water, distribution system and treated sludge. Raw water had trimethoprim (100%) with a difference of >50% at Site D, which may be attributed to sewerage pollution (Hou *et al*., 2021). Poor wastewater effluents and sewerage runoff may allow ARB and ARGs to enter natural water sources, impacting the environment (Li *et al*., 2024a; Sharma *et al*., 2025). When wastewater effluent is discharged into natural water environments, the resistant microorganisms and ARGs could interact with microorganisms in the freshwater, leading to the spread of resistance (Amarasiri *et al*., 2020; Kulik *et al*., 2024). Resistant microorganisms and ARGs may spread to different locations through the water cycle (Leite *et al*., 2024; Ma *et al*., 2025). When comparing the three sites in Gauteng, the disinfection stage at Site A had highest diversity of ARGs with a difference of >50%, where antibiotic resistance was glycopeptide (100%), phenicol (100%), trimethoprim (100%), fosfomycin (77%), aminoglycoside (58%) and ßeta-lactam (65%), more than Site D and Site E. These findings align with Ma *et al*. (2025) in China, who reported that there is different composition of ARGs within the drinking water treatment process, citing that local bacteria communities and environmental conditions may shape the composition of ARGs. Studies have shown significant variations in ARG hosts across the drinking water treatment process. For instance, Pseudomonadota (Proteobacteria*),* Planctomycetota*,* Bacteroidota and Actinomycetota where the main ARGs hosts in the DWTP (Molina *et al*., 2024; Sudarshan *et al*., 2024).

In the final treated water of Site E, glycopeptide (100%) and macrolide (60%) were dominant, while *tet(A)*, *Sul1*, *aph* (6)-*lb* and *aph* (3")-*la* were prevalent in all the samples of Site E. The persistence of ARGs after disinfection can be attributed to several factors, including biofilms, resistant strains, and tolerance to disinfection by-products (Uruén *et al*., 2020; Liu *et al*., 2024). Biofilms consist of communities of microorganisms that are embedded in a self-produced extracellular matrix. These biofilms create environments that enhance bacterial tolerance and resistance to antibiotics through various mechanisms, which depend on factors such as biofilm composition, architecture, the stage of development, and growth conditions (Muteeb *et al*., 2023). Kang *et al*. (2025) observed that selective pressure can amplify the presence of ARGs, resulting in a stable and elevated occurrence within microbial communities. This environment facilitates the transfer of resistance genes between bacteria through horizontal gene transfer (HGT) via mobile genetic elements (MGEs) (Alawi *et al*., 2024).

5.6.2. Antibiotic-resistant gene phenotype composition in Limpopo Province

In Limpopo Province, 20 ARGs were identified, with the highest number (10) detected in the treated sludge, followed by six (6) in the raw water samples, two within the filtration stage, one at the disinfection point and two in the final treated water. The resistance gene for last-line antibiotic such as chloramphenicol (100%) and macrolide (100%) were found in the treated sludge. The results found CAT_I, CAT_{III}, $bla_{\text{CTX-M-9G}}$ and $bla_{\text{CTX-M-9G}}$ in the raw water source and treated sludge in Limpopo Province. The abundance of these ARGs at this site, may be from the influx of medical and urban domestic wastewater and sewerage runoff (Raphela *et al*., 2024). A similar study by Tiwari *et al*. (2025) in Finland reported that raw water sources and treated sludge had more ARGs types than the average in the disinfection stage and treated water. The ARGs exhibiting the highest read prevalence were aminoglycoside [*aph(6)-id*, *aph(3")-lb*, *aac(3)-lid*, *aada5*, *aadA12*, *aac(3)-ib* and *Ant(3")-li*], ßeta-lactam [bla_{DHA} and $bla_{\text{CMY-2}}$], chloramphenicol [CAT_I and CAT_{III}] and macrolide [*mef(B)* and *mph(A)*]. The observed treated sludge comprised of various organic, inorganic properties and microbial communities attributed from the surface water properties. These results corroborate the work of Gros *et al*. (2023) who reported that the occurrence of antibiotics, ARGs and ARB was characterised by large seasonal variations, which were mostly associated to hydrological factors such as runoff from anthropogenic activities and relative transport process. In the final treated water of Site B, tifamycin and trimethoprim were mostly

amplified, which may be due to health facility and domestic wastes into surface water (Tiwari *et al*., 2025).

5.6.3. Antibiotic-resistant gene phenotype composition in Mpumalanga Province

In Mpumalanga Province, ARGs showed relative abundance conferring resistance to various antibiotic classes in the raw water source, filtration and disinfection stages, final treated water and treated sludge. There was a reduction in ßeta-lactam, quinolone and sulfonamide in the disinfection stage and final treated water, suggesting ARG removal. However, macrolide (90%), streptogramin (64%) and tetracycline (50%) were abundant in the final treated water, and trimethoprim (100%) and quinolone (100%) were dominant in the raw water source. These findings highlight the dynamic nature of bacterial communities in the source water and drinking water treatment process (Tiwari *et al*., 2025), which emphasise the importance of monitoring microbial ecology in the DWTPs and possibly DWDS. Among the quinolone ARG variants, *qnr*B, *qnrC*, *qnrS*, *tetO34*, *tetX* and *tetX** were most abundant. While there are numerous reports of such occurrences, the biological basis of spontaneous horizontal gene transfer among distantly related microbiology remains unclear (Amarasiri *et al*., 2020; Atnafu *et al*., 2021; Sheinman *et al*., 2021). Tokuda and Shintani (2024) reported that the spontaneous transfer of ARGs across classes and orders of microorganisms may be able to disseminate shared resistance genes across the substantial genetic gaps despite protected areas or the use of chlorine in drinking water treatment. Wang *et al*. (2024) revealed that *Acinetobacter*, *Pseudomonas* and *Mycobacterium* are crucial ARG carriers in drinking water reservoirs. These genera encompass multiple pathogens, including *A. baumannii*, *P. aeruginosa* and *M. tuberculosis*, which are commonly found in the environment and known to cause infections in humans through direct or indirect contact without proper sterilisation (Ishaque *et al*., 2024; Zhang *et al*., 2024c). Infections caused by organisms producing extended-spectrum β-lactamase genes and other antimicrobial resistance genes (ARGs) are a significant concern in the medical community (Singh *et al*., 2022; Wang *et al*., 2024). The increasing prevalence of these genes in the environment and drinking water, combined with the severe lack of effective antimicrobial therapies, is alarming. While drugs such as carbapenems have been employed to treat infections caused by extended-spectrum β-lactamase-producing organisms, the rise of carbapenem-resistant *Enterobacteriaceae* poses major risks to public health, leading to increased morbidity and mortality (Shariati *et al*., 2022; Husna *et al*., 2023). A study conducted in

Bangladesh reported the emergence of carbapenem-resistant bacteria carrying the genes blaOXA-48, blaNDM-1, blaNDM-5, and blaVIM-5, as well as colistin-resistant *Klebsiella pneumoniae* with the mcr-8 gene in clinical isolates. Furthermore, ARGs such as NDM-1 and blaVIM-5 were detected in *Klebsiella pneumoniae* from individuals who had travelled to India as early as 2008 (Farzana *et al*., 2020). Additional reports indicate that these ARGs can also be found in Enterobacteriaceae, Acinetobacter species, and Pseudomonas species (Li *et al*., 2024b; Zhang *et al*., 2025a).

5.7. Differences in functional genes between the drinking water treatment plants

Microorganisms survive in diverse habitats like rivers, dams and oceans because they can handle tough conditions, such as very acidic (pH 4) or very alkaline (pH >9) environments, high salt levels and extreme temperatures (Thom *et al*., 2022; Yu *et al*., 2024). They can also survive in areas with high metal content, high saline water and harmful substances, such as disinfection in DWTPs (Tzimotoudis *et al*., 2025; Zhang *et al*., 2025a). In the face of harsh conditions, microbes produce special biomolecules that are active in chemical reactions (Li *et al*., 2024b; Zhang *et al*., 2024a). Using functional-based metagenomic data screening, researchers can find both new and known gene types that are linked to different cell functions (Teffo *et al*., 2023; Assefa *et al*., 2024).

The functional metagenomes in the final treated water indicate human health-related concerns. Although the abundance of these functional genes varied across the treatment sites in the current study, their presence in the water samples is an indication of diverse microbial communities that harbour them. There were six major functional metagenomes based on the major taxa abundance, and they were evaluated across Gauteng, Limpopo and Mpumalanga Province in the different treatment stages at the five selected sites in this study. At Site A in Gauteng Province, the clustering implies their similarity in function despite their discrepancy in community composition, which may be attributed to functional redundancy within communities. The residual chlorine in the final treated water could lead to the death of some viable microbes and the release of more metabolism metagenomes than the residual chlorine induced. Analysis of the metagenome for the major functional profiles associated with human diseases for the final treated water from sampling Site A indicated the most dominant function related to the metagenome are chlorine metabolism in cancer (5%), *Vibrio cholera* infection (6%), chemical carcinogenesis-reactive oxygen species

(3%), CAMP resistance (6%), ßeta-lactam resistance (4%), vancomycin resistance (3%) and antifoliate resistance (3%).

According to Sudarshan *et al*. (2024) and Sharma *et al*. (2025), functional metagenomics involves analysing the genes and function of the microbial community in water samples to understand the potential for beneficial or harmful processes. Furthermore, functional genomics identifies which microbes are present in abundance, aiming to reveal their metabolism, organismal, human disease, cellular process, environmental information processing and genetic information processing functions (Sudarshan *et al*., 2024; Zhang *et al*., 2025b). Drinking water treatment processes including treated sludge and the distribution system present significant reservoirs of ARB and ARGs; however, the mechanisms governing ARG proliferation and dissemination at the five selected sites is not known or poorly understood.

In this study, results of ARG composition with the Gauteng, Limpopo and Mpumalanga Province sites identified that different factors such as seasonal and raw water sources play a substantial role in ARGs altercation with the drinking water treatment process, DWDS and treated sludge. This ARGs in the drinking water treatment could be from to natural water sources. Natural water sources and environment could be impacted by domestic waste from households, pharmaceuticals and sewerage runoff (Adefisoye & Olaniran, 2022; Abolfazli *et al*., 2024). Many studies have also reported that DWTPs are potential hotspots of ARB and ARGs enrichment, contributing negatively to human health. Microbiomes have developed resistance mechanisms over time through continuous evolutions for survival. Recent evidence suggests unifying mechanisms involving reactive oxygen species (ROS) play a key role in lethality (Bian *et al*., 2021; Kaapu *et al*., 2022; Kasanga *et al*., 2024). ROS like hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) inherit the by-products of antibiotic-induced bacteria. ROS formed part of the human disease functional metagenomes, especially at Site B where chloramphenicol (100%) and macrolide (100%) were found in the treated sludge. CAT_I, CAT_{III}, $bla_{\text{CTX-M-9G}}$ and $bla_{\text{CTX-M-9G}}$ were detected in the raw water source and treated sludge in Limpopo Province. This also correlates with the metagenomics findings at Site C, where microbes such as Pseudomonadota (Proteobacteria), Bacteriodota and Actinomycetota were identified in the raw water source and final drinking water. These remaining bacteria affected by ROS are reported to play crucial roles alongside other environmental factors to promote the development of breast cancer in woman (Zhang *et al*.,

2024c). The presence of ROS linked to chemical carcinogenesis suggest that microbial carriers could trigger or worsen cancer in people with weakened immune systems (Zhang *et al*., 2024a; Zhou *et al*., 2024). Another important function observed is AMR, which aligns with the MDR strains found at various treatment sites. The resistance to CAMP, ßeta-lactams and vancomycin seen across all sites may contribute to the antibiotic resistance of the indicator bacteria. Interestingly, the six functional metagenomes showed differences across the various locations and treatment stages, with one stage potentially boosting the abundance of functional metagenomes in the next. Each stage of the treatment process creates different environmental pressures on the microbial communities present. To adapt to these changing conditions, their metabolic functions must adjust (Zhu *et al*., 2024). Manna *et al*. (2024) reported significant analysis between the ROS stress response genes and numerous ARGs in WWTPs. Furthermore, they reported that mechanisms that bacteria use to cope with oxidative stress includes the activation of specific genes and detoxification enzymes that may play a role in the proliferation of ARGs in the drinking water environment.

CHAPTER 6: SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1. Summary of findings and conclusions

The recent DWS (2023) Blue Drop Report for South Africa presents a troubling assessment of the country's water quality and infrastructure. According to the audit report, the quality of drinkable water in the country is deteriorating, with non-compliance reported in most DWTPs' final treated water (DWS, 2023). Based on water quality tests carried out by municipalities during the 2021/2022 municipal financial year, 54% of water supply systems achieved excellent or good microbiological water quality compliance, and 46% achieved poor or bad microbiological water quality compliance (DWS, 2023). According to the DWS report (2018) in 2014, 5% of water supply systems achieved poor or bad microbiological water quality compliance. This indicates a severe regression in drinking water quality between 2014 and 2023, with drinking water quality generally not good in the metropolitan areas. The significance of continuous monitoring in evaluating water quality management options is increasingly recognised. Clarifying the microbial diversity and ecology of DWTPs is necessary for designing innovative and effective control strategies to ensure safe and high-quality drinking water. In this study, analysis of the microbiome of selected drinking water treatment plants, drinking water sludge and water distribution systems at five sites chosen over 12 months align with this objective.

Different process points along selected DWTPs were studied to determine microbial diversity and dynamics in different seasons. With the aim of DWTPs to reduce or eliminate microorganisms, especially in the final treated water and distribution system, the current study showed that the process was effective in treating indicator bacteria. Chlorine was effective in removing most indicator bacteria, including *E. coli*, *Enterococcus* spp. and total coliforms. For instance, at the Limpopo Province DWTP, the final treated water and distribution system recorded zero counts throughout the seasons. Disinfection significantly influenced microorganisms, while other treatment processes synergised with their sequential processes to impact communities. The seasonal variations in the microbial composition in the raw water source, flocculation, sedimentation and filtration stages, final treated water, distribution system and treated sludge were demonstrated. For instance, at the Gauteng Province DWTPs, microbiological contamination

persisted in some of the water samples even after filtration, with *E. coli* ranging between 0 MPN/100 mL in winter and 5.2 MPN/100 mL in spring. This persistence could be due to several contributing factors, such as temperature and the regrowth of microbes in the filtration stage. Water in the filtration stage recorded a high temperature during spring of 25.7±1.57°C and a low temperature of 24.1±1.34°C in autumn. Hence, the importance of maintenance and backwash of filtration in the DWTP.

This study revealed that the studied physicochemical parameters, such as temperature, pH, turbidity, nitrate, sulphate and TOC, were the most important factors in shaping the microbial communities within different stages of the DWTPs, DWDSs and treated sludge in all the studied South African provinces (Gauteng, Limpopo and Mpumalanga). In Gauteng Province, temperature was the most significant factor influencing the composition of the microbial communities. Temperature in the distribution system was highlighted to range between 21.6±1.73°C and 26.8±3.45°C. Nitrososphaerota and Euryarchaeota were the most abundant Archaea phyla in Gauteng, Limpopo and Mpumalanga. However, in Gauteng Province, Nitrososphaerota was abundant in the raw water source and Euryarchaeota was observed in the filtration stage and treated sludge. At Limpopo and Mpumalanga, the Archaea phyla Halobacteria and Methanomicrobia were detected in the raw water source, final treated water and treated sludge. The halophiles are extremophiles or extremotolerant organisms that can survive in high-salinity water (Sharma *et al*., 2025). Bacterial phyla Pseudomonadota and Actinomycetota dominated in Gauteng Province in the raw water source, filtration stage and treated sludge samples, specifically Site D, with Sites A and E dominated by Pseudomonadota in the raw water source, filtration stage and treated sludge.

The most dominant bacterial classes in Gauteng Province were Alphaproteobacteria, Gammaproteobacteria and Betaproteobacteria at Sites A and D within the raw water samples, filtration stage and disinfection stage. Alphaproteobacteria, Gammaproteobacteria, Actinomycetes and Betaproteobacteria were abundant in the raw water source and treated sludge, in Limpopo Province and within the raw water source, filtration and disinfection stages and final treated water in Mpumalanga Province. The microbial community composition in treated water and distribution system at all sites was mainly shaped and influenced by environmental factors such as temperature and pollutions. Reports indicate that the discharge of agricultural waste and human sewage into natural water bodies disrupts the usual microbial balance in aquatic ecosystems, resulting in the

emergence and growth of harmful microbial communities (Luvhimbi *et al*., 2022; Murei *et al*., 2023). Flocculation-sedimentation and sand filtration are important processes for TOC removal and may influence the abundance of microbial diversity. However, in Mpumalanga, the highlight was on TOC in the final treated water during summer with a record of 15.40±3.05 mg/L which is substantially higher than the SANS 241 (SANS, 2015) 10 mg/L guideline for drinking water.

Ascomycota, Basidiomycota, Apicomplexa and Bacillariophyta were the dominant Eukaryota phyla in Gauteng Province at the disinfection stage. In Limpopo Province, the Eukaryota phyla Aconoidasida, Sordariomycetes and Saccharomycetes were dominant in the raw water, filtration stage and final treated water samples, while Aconoidasida, Sordariomycetes, Kinetoplastea and Saccharomycetes were dominant in the final treated water and treated sludge in Mpumalanga Province. The current study results revealed that among all measured physicochemical parameters, TOC and temperature were the most significant factors affecting the conditions of the microbial community. Higher temperatures were more favourable for Alphaproteobacteria and Nitrospirae in the raw water at Site A, while Actinomycetes and chloride, as well as Gammaproteobacteria and ammonia showed strong negative correlation. However, in this study of metagenomics, propidium monoazide treatment was not performed on the samples to determine if the microbes identified were alive or dead, especially when identifying the abundance of those phyla, bacteria and eukaryotes in those treatment stages.

The antibiotic susceptibility tests on the identified indicator bacteria, *E. coli* and *Enterococcus* spp., showed a significant number of MDR isolates, which were influenced by the treatment stages and seasonal variations at the DWTPs. Antibiotic-resistant *E. coli* in drinking water may originate from the raw water source and could survive the drinking water production processes, eventually being in the DWDS. Antibiotic residues that were detected in water samples include ampicillin, erythromycin, ciprofloxacin, nitrofurantoin, amoxicillin/clavulanate, gentamicin, imipenem, trimethoprim/sulfamethoxazole, levofloxacin and vancomycin. Additionally, the MAR-index indicated a high level of antibiotic use in Gauteng Province, with Site A recording 0.28. The MAR-index value greater than 0.2 reflects a high-risk area with possible exposure to antibiotics, while an MAR-index equal to or lower than 0.2 indicates bacteria without previous exposure to such antibiotics (Lewandowska *et al*., 2025). ARB and MAR are a growing global health threat, with the rise of MDR infections and influence the organism's characteristics in the water environment

(Li *et al*., 2024a; Zhang *et al*., 2024b; Veloo *et al*., 2025). Although the ARGs do not directly indicate a risk, it underscores the importance of future research focused on horizontal gene transfer and risk assessment of these ARGs. Overall, while this study has enforced previously reported observations of drinking water microbiome, it has also yielded new insights about its composition and variation within treatment plants, process stages and season. It enhances the understanding of microbial diversity and dynamics in the DWTPs, distribution system and treated sludge, and underscores the importance of monitoring to protect public health.

6.2. Novelty of the thesis and key findings

For the first time, comparative microbial profiling was done on samples from across three provinces in South Africa—Gauteng, Limpopo and Mpumalanga Provinces. Dynamics occurring in the microbiology, physicochemical conditions and treatment regimes in the provinces were highlighted, revealing microbial communities consisting of Proteobacteria and Firmicutes are influenced by physiochemical parameters such as pH, TOC and temperature. The study further highlights that different treatment stages affect microbial diversity, which varying across the seasons, and the study was able to identify how this occurred in the case of each studied DWTP.

Physicochemical parameters of the final treated drinking water and distribution system at the selected sites were subjected to SANS 241 (SANS, 2015) and it was possible to judge the quality of the water achieved and performance of the DWTP processes for each site. For the first time, drinking water treatment sludge from the plants were evaluated for microbial diversity, AMR and potential contribution to microbial risks when discharged into the environment. Regarding antimicrobial and MDR, this study was able to show widespread of aminoglycosides, ßeta-lactam, tetracyclines and macrolides that may influence widespread of ARGs to occurs. The results across the three provinces were key in showing the potential ARG spread within the water samples that may influence horizontal transfer, risking further contamination with MDR bacteria.

6.3. Recommendations

It is, therefore, recommended that:

- Comprehension chlorine demand and decay studies should be carried out to identify whether and where chlorine boaster stations should be installed to ensure that sufficient chlorine residual will be available across the distribution system.
- A targeted shotgun metagenomics approach should be developed to identify viable microbial communities and assist in the optimisation of DWTP processes.
- The implementation of household drinking water treatment methods (i.e., boiling water and adding a chlorine source, such as bleach) could be considered to alleviate the source of microbial contamination, especially water with high turbidity, smell and microbes.
- There is an urgent call to action for increased investment in predictive modelling technologies for microbial diversity and capacity building initiatives.
- Collaborative efforts among the tiers of government, non-governmental organisations and local communities could be essential to protect vulnerable populations from microbial contamination, trace metals and physicochemical parameters.
- A rigorous and regular monitoring schedule for all DWTPs, distribution systems and treated sludge should be implemented, focusing on indicator microorganisms such as *V. cholerae*.
- Thorough investigations should be conducted to identify potential sources of contamination, such as agriculture discharge, sewerage runoff and faulty treatment systems.

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Appendix 1: Ethics clearances

UNISA university of south africa

UNISA-CAES HEALTH RESEARCH ETHICS COMMITTEE

Date: 20/02/2023

Dear Mr Mudau

Decision: Ethics Approval from 16/02/2023 to 31/01/2028

NHREC Registration # : REC-170616-051
REC Reference # : 2023/CAES_HREC/026
Name : Mr KL Mudau
Student #: 50173065

Researcher(s): Mr KL Mudau
50173065@mylife.unisa.ac.za; 063-675-3762

Supervisor (s): Prof M Tekere
tekerm@unisa.ac.za; 011-471-2270

Working title of research:

Analysis of the microbiome of some drinking water treatment plants, sludge, and the water distribution systems

Qualification: PhD Environmental Science

Thank you for the application for research ethics clearance by the Unisa-CAES Health Research Ethics Committee for the above mentioned research. Ethics approval is granted for five years, **subject to further clarification and submission of yearly progress reports. Failure to submit the progress report will lead to withdrawal of the ethics clearance until the report has been submitted.**

The researcher is cautioned to adhere to the Unisa protocols for research during Covid-19.

Due date for progress report: 31 January 2024
The progress report is available on the college ethics webpage: https://www.unisa.ac.za/sites/corporate/default/Colleges/Agriculture-&-Environmental-Sciences/Research/Research-Ethics

Please note the points below for further action:

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Appendix 2: Physicochemical parameters of water samples from the drinking water treatment plants showing seasons and compliance to SANS 241 (SANS, 2015)

Physicochemical	Treatment stages	Seasons	Sites (mean±standard deviation)					**SANS 241 (SANS, 2015)**
			A	B	C	D	E	
pH	Raw water	Spring	7.61±0.12	7.80±0.98	5.41±0.27	8.12±0.18	8.09±1.16	N/A
		Summer	7.85±0.23	7.56±0.24	5.30±0.33	7.46±0.27	8.03±0.28	
		Winter	7.73±0.23	8.37±0.61	5.25±0.43	7.28±0.43	8.08±0.50	
		Autumn	7.87±0.44	8.01±0.48	5.01±0.51	7.60±0.4	8.33±0.6	
	Flocculation	Spring	8.21±0.55	8.67±0.76	9.2±1.03	7.97±0.47	8.01±0.45	N/A
		Summer	7.93±0.45	8.9±0.87	9.34±1.11	7.95±0.46	8.05±0.49	
		Winter	8.76±0.8	8.66±0.76	8.98±0.47	8.89±0.74	8.9±0.88	
		Autumn	8.66±0.44	8.43±0.36	8.55±0.24	7.70±0.13	8.42±0.48	
	Sedimentation	Spring	8.34±0.35	8.65±0.36	8.29±0.37	7.90±0.13	7.77±0.34	N/A
		Summer	8.36±0.37	8.23±0.24	6.98±0.49	7.52±0.22	7.74±0.35	
		Winter	8.78±0.45	8.21±0.24	7.65±0.21	7.90±0.13	8.45±0.28	
		Autumn	8.70±0.42	8.11±0.24	7.34±0.31	7.52±0.22	8.08±0.24	
	Filtration	Spring	8.35±0.38	7.78±0.17	8.01±0.37	8.29±0.39	7.43±0.34	N/A
		Summer	7.5±0.43	7.88±0.09	7.74±0.14	8.04±0.18	7.79±0.27	
		Winter	8.64±0.35	7.65±0.15	7.32±0.38	7.45±0.42	8.21±0.40	

		Autumn	8.22±0.20	7.71±0.12	7.05±0.54	8.04±0.18	8.07±0.36	
	Final	Spring	8.1±0.20	7.33±0.32	7.77±0.13	8.40±0.23	8.03±0.33	≥5 to≥.7
		Summer	6.6±0.91	7.85±0.09	7.41±0.33	7.96±0.19	8.09±0.35	
		Winter	7.75±0.73	7.49±0.24	7.43±0.31	7.64±0.18	8.10±0.08	
		Autumn	7.64±0.16	7.38±0.30	6.94±0.50	8.0±0.19	8.01±0.07	
	Treated sludge	Spring	7.29±0.39	7.15±0.40	7.56±0.27	7.25±0.35	7.68±0.21	N/A
		Summer	7.88±0.1	7.75±0.23	8.1±0.35	7.76±0.17	6.97±0.61	
		Winter	7.24±0.37	7.18±0.38	8.32±0.48	7.77±0.16	7.84±0.13	
		Autumn	7.63±0.16	7.79±0.23	7.92±0.29	7.54±0.21	7.90±0.10	
	Drinking water distribution system	Spring	6.76±0.64	6.77±0.59	7.23±0.37	N/A	N/A	≥5 to≥.7
		Summer	7.41±0.27	7.86±0.24	7.53±0.28	N/A	N/A	
		Winter	6.95±0.52	7.99±0.28	7.82±0.21	N/A	N/A	
		Autumn	6.45±0.81	7.33±0.32	7.29±0.34	N/A	N/A	
Temperature (°C)	Raw	Spring	23.5±2.87	24.7±1.92	23.6±0.98	22.1±1.56	23.6±1.64	N/A
		Summer	26.8 ±3.30	26.1±2.19	25.3±0.98	23±1.37	23±1.79	
		Winter	18.1±3.29	18.3±2.19	19.9±0.91	18.4±1.35	19.6±1.66	
		Autumn	21.7±3.13	23.7±1.92	25.2±0.95	24.8±1.56	26.1±11.79	
	Flocculation	Spring	24.2±2.85	24.9±1.95	23.9±1.97	23±1.9	23±1.62	N/A
		Summer	26.7±3.26	26.8±2.25	25.1±1.50	21.9±2.13	23±1.04	

		Winter	22.3±3.0	22.9±2.25	23.8±1.73	23.5±1.85	25.7±1.37	
		Autumn	23.2±2.88	23.89±2.02	26.8±1.73	25.6±2.14	25.8±1.42	
	Sedimentation	Spring	25.4±0.67	25.3±1.62	23.7±2.03	23.9±1.77	23±11.75	N/A
		Summer	26.3±0.35	26.77±1.43	24.4±2.03	23.9±1.31	20.0±1.75	
		Winter	25.3±1.16	25.3±1.05	26.2±2.58	21.9±1.77	25.3±1.67	
		Autumn	24.68±1.63	24.6±1.03	26.7±2.32	23±1.53	26.7±0.82	
	Filtration	Spring	25.7±1.57	25.1±1.24	23.5±1.98	23.9±1.34	20.0±1.33	N/A
		Summer	25.46±1.22	24.9±2.1	24.7±1.78	22.0±1.56	20.0±1.29	
		Winter	24.5±1.87	24.4±1.65	22.9±1.45	23.4±1.38	24.9±1.78	
		Autumn	24.1±1.34	23.8±1.03	25.1±2.48	23.0±1.32	25.6±2.09	
	Final	Spring	23.7±0.25	24.6±0.58	22.4±1.02	22.3±0.81	22±3.04	≤25°C
		Summer	24.8±0.34	23.6±0.53	23.6±0.97	22.6±0.45	21.5±2.49	
		Winter	21.8±0.93	23.72±0.74	22.4±0.98	23.7±1.19	24.6±2.64	
		Autumn	22.65±1.37	23.4±0.69	24.5±1.18	25.7±1.47	25.3±1.78	
	Sludge	Spring	23.4±1.12	22.4±1.31	24.5±1.32	22±1.15	23.0±1.16	N/A
		Summer	25±1.26	23.6±1.44	23.5±1.43	24.1±0.94	24.9±1.26	
		Winter	23.5±1.31	23.1±1.75	24.1±1.76	23.1±1.12	23.1±1.31	
		Autumn	22.4±1.5	21.5±1.76	21.6±1.7	23±1.0	22.5±1.41	
		Spring	22.7±1.1	21.6±1.36	23.7±1.25	N/A	N/A	

	Drinking water distribution system	Summer	26.8±3.45	21.8±1.59	22.6±1.08	N/A	N/A	≤25°C
		Winter	21.6±1.73	22.1±1.51	22.9±1.16	N/A	N/A	
		Autumn	22.7±1.46	24.3±1.33	23.1±0.87	N/A	N/A	
Electrical conductivity (mS/m)	Raw	Spring	134.3±2.85	158.4±3.57	155.6±4.62	131±5.18	146±7.23	N/A
		Summer	137.8±2.93	152.3±4.09	148.6±2.31	136±6.2	143±6.56	
		Winter	137.8±2.93	155.65±4.20	155.8±5.6	142.7±4.75	158.8±5.63	
		Autumn	131.98±2.02	150.34±3.05	159.7±4.10	140.5±5.87	153.9±8.39	
	Flocculation	Spring	94.9±2.55	110.28±2.14	108.2±4.40	99±6.59	91±8.29	N/A
		Summer	97.8±2.76	113.48±2.34	110.1±2.1	99±4.56	97±9.72	
		Winter	98.35±2.46	110.1±2.10	116.1±5.38	94.8±5.10	99.9±6.71	
		Autumn	92.9±1.85	114.24±1.90	110.3±4.20	95.8±7.99	94.4±9.66	
	Sedimentation	Spring	91.4±0.94	103.32±0.93	107.7±5.60	99±4.72	97±12.19	N/A
		Summer	92.6±1.15	105.4±0.29	106.7±5.19	90±1.12	94±13.89	
		Winter	93.7±0.64	103.9±1.13	106.3±5.99	90.5±5.0	92.3±11.57	
		Autumn	92.4±1.15	103.6±1.07	108.1±5.99	98.3±5.68	99.6±13.06	
	Filtration	Spring	79.4±0.86	95.78±2.41	97.8±5.77	93±2.57	96±11.18	N/A
		Summer	78.7±1.04	91.6±1.32	87.7±2.31	90±2.66	83.6±13.09	
		Winter	78.65±1.04	88.2±2.95	96.8±7.02	95.3±1.73	101.89±8.21	
		Autumn	77.34±0.42	87.9±2.92	101.2±5.57	90±2.66	92.4±13.31	

	Final	Spring	40.1±1.78	62.1±2.29	87.9±2.0	51±7.83	53±12.70	≤170
		Summer	40.5±1.94	65.23±1.19	85.6±1.56	53±6.06	56±15.30	
		Winter	42.3±2.13	61.33±2.74	87.7±2.45	52.3±9.59	51.2±12.21	
		Autumn	43.98±1.17	69.76±2.06	80.5±1.27	52.1±5.04	67.4±14.11	
	Sludge	Spring	124.7±24.65	147.7±30.34	159.7±15.87	149.5±7.98	149.55±11.16	N/A
		Summer	179.2±28.19	144±21.47	166.5±15.67	137.9±7.90	148.5±7.97	
		Winter	129.6±30.15	123.1±13.26	160.8±3.65	141.1±5.96	140.8±4.78	
		Autumn	139.4±22.67	182.92±25.07	136.6±14.99	153.1±10.10	110.13±62.80	
	Drinking water distribution system	Spring	29.2±1.67	34.31±2.61	45.9±2.40	N/A	N/A	≤170
		Summer	29.5±1.65	35.32±2.90	45.5±2.83	N/A	N/A	
		Winter	31.2±1.80	31.33±2.35	45.7±2.72	N/A	N/A	
		Autumn	32.8±1.07	29.67±2.07	50.5±2.49	N/A	N/A	
Turbidity (NTU)	Raw	Spring	10.56±1.24	8.94±0.1	3.35±3.77	3.1±0.81	1.8±1.49	N/A
		Summer	9.67±1.24	8.9±0.25	10.7±1.54	4.5±0.74	2.6±1.87	
		Winter	8.1±0.55	8.75±0.27	5.57±4.48	4.5±0.74	4.7±0.70	
		Autumn	9.55±1.25	8.45±0.10	2.60±3.77	3.4±0.81	1.2±1.49	
	Flocculation	Spring	9.78±0.34	8.78±0.65	10.6±4.27	1.7±0.97	2.8±1.27	N/A
		Summer	9.02±0.23	9.1±0.79	11.4±4.03	3.1±1.18	1.2±1.36	
		Winter	9.34±0.41	9.86±0.39	3.76±4.34	3.8±1.03	3.56±1.02	

		Autumn	9.67±0.38	8.32±0.55	3.50±4.19	3.7±1.06	0.91±1.20	
	Sedimentation	Spring	5.88±0.87	8.08±0.35	9.92±3.69	0.65±1.53	5.2±2.15	N/A
		Summer	5.33±0.83	8.72±0.41	2.20±4.19	3.9±1.88	0.91±2.54	
		Winter	4.08±0.99	8.01±0.05	2.33±4.15	2.7±1.64	3.33±2.27	
		Autumn	4.24±0.68	7.97±0.42	3.23±0.56	3.9±0.69	0.68±1.47	
	Filtration	Spring	0.23±0.035	3.56±0.64	0.34±0.40	0.39±0.25	1.720.94±	N/A
		Summer	0.26±0.04	3.41±0.66	0.36±0.40	0.72±0.16	0.68±0.12	
		Winter	0.18±0.03	3.1±0.72	1.07±0.41	1.0±0.31	2.56±0.01	
		Autumn	0.20±0.03	2.14±0.78	1.04±0.39	0.72±0.19	0.55±0.64	
	Final	Spring	0.18±0.05	0.86±0.21	0.94±0.96	0.37±0.17	0.53±0.23	≤1.0
		Summer	0.19±0.05	0.98±0.25	0.36±1.15	0.72±0.18	0.53±0.27	
		Winter	0.13±0.05	0.87±0.21	2.60±0.93	0.4±0.02	0.98±0.27	
		Autumn	0.09±0.02	0.50±0.26	1.03±1.11	0.4±0.18	0.5±0.34	
	Sludge	Spring	330±80.42	670±141.86	235±183.16	468±222.26	890±106.1	N/A
		Summer	470±40.41	730±169.8	650±164.83	835±75.66	845±109.81	
		Winter	440±95.39	560.4±173.32	320.46±124.36	980±269.52	650±125.73	
		Autumn	520±98.49	900±119.30	480±208.63	870±222.68	835±29.29	
	Drinking water distribution system	Spring	0.17±0.02	1.00±0.11	1.09±0.34	N/A	N/A	≤1.0
		Summer	0.18±0.03	1.11±0.14	1.3±0.29	N/A	N/A	

		Winter	0.13±0.02	0.90±0.08	1.56±0.40	N/A	N/A	
		Autumn	0.15±0.02	0.84±0.14	1.89±0.41	N/A	N/A	
Chlorine (mg/ℓ)	Raw	Spring	-	-	-	-	-	N/A
		Summer	-	-	-	-	-	
		Winter	-	-	-	-	-	
		Autumn	-	-	-	-	-	
	Flocculation	Spring	-	-	-	-	-	N/A
		Summer	-	-	-	-	-	
		Winter	-	-	-	-	-	
		Autumn	-	-	-	-	-	
	Sedimentation	Spring	-	-	-	-	-	N/A
		Summer	-	-	-	-	-	
		Winter	-	-	-	-	-	
		Autumn	-	-	-	-	-	
	Filtration	Spring	-	-	-	-	-	N/A
		Summer	-	-	-	-	-	
		Winter	-	-	-	-	-	
		Autumn	-	-	-	-	-	
	Treated water	Spring	0.26±0.04	2.3±0.43	0.99±1.76	1.14±0.51	1.0±2.44	≤5

		Summer	0.33±0.05	2.78±0.25	1.13±1.82	2.19±0.41	0.5±2.37	
		Winter	0.32±0.04	1.90±0.25	3.61±1.82	1.9±0.41	3.68±2.38	
		Autumn	0.24±0.04	1.84±0.47	4.5±1.98	1.24±0.58	5.74±2.89	
	Sludge	Spring	N/A	N/A	N/A	N/A	N/A	N/A
		Summer	N/A	N/A	N/A	N/A	N/A	
		Winter	N/A	N/A	N/A	N/A	N/A	
		Autumn	N/A	N/A	N/A	N/A	N/A	
	Drinking water distribution system	Spring	0.015±0.04	0.1±0.03	0.06±0.03	N/A	N/A	≤5
		Summer	0.09±0.05	0.07±0.03	0.05±0.03	N/A	N/A	
		Winter	0.01±0.01	0.02±0.02	0.11±0.004	N/A	N/A	
		Autumn	0.01±0.01	0.023±0.04	0.1±0.03	N/A	N/A	
Total organic carbon	Raw water	Spring	13.49±1.43	26.43±1.85	28.56±6.01	16.5±2.68	15.33±2.46	N/A
		Summer	12.84±1.24	25.96±1.76	30.77±7.09	15.6±2.43	12.84±1.61	
		Winter	10.43±1.60	23.84±2.01	29.47±16.5	12.2±2.93	11.07±2.96	
		Autumn	11.12±1.22	22.47±2.16	26.18±7.26	10.9±3.01	9.63±2.86	
	Flocculation	Spring	15.56±4.83	22.92±1.90	25.67±1.36	9.73±0.52	10.76±0.93	N/A
		Summer	9.30±5.79	23.74±2.12	24.99±1.21	9.31±0.37	9.41±0. 39	
		Winter	11.33±4.43	19.84±1.64	22.57±1.55	8.76±0.61	8.64±01.13	
		Autumn	20.2±5.47	20.37±1.76	23.83±0.93	8.6±0.57	9.01±0.91	

	Sedimentation	Spring	23.49±4.02	18.87±2.48	16.73±2.28	9.72±0.87	7.61±0.88	N/A
		Summer	19.76±3.98	19.49±2.65	16.98±2.37	10.9±10.03	6.9±0.62	
		Winter	22.1±4.93	15.30±2.98	12.48±2.23	8.98±0.81	5.98±1.02	
		Autumn	14.34±4.60	14.58±2.67	13.47±1.95	10.6±0. 16	5.72±0.95	
	Filtration	Spring	5.98±0.62	12.21±3.84	15.12±3.49	6.27±0.61	3.9±0.57	N/A
		Summer	6.3±1.43	11.65±3.46	15.56±3.57	5.05±1.76	4.76±1.04	
		Winter	5.1±1.49	5.30±1.73	9.30±2.33	5.55±1.55	4.97±1.02	
		Autumn	5.90±0.51	7.34±3.35	11.33±16.99	4.47±0.76	4.69±0.47	
	Final treated water	Spring	7.98±0.79	11.34±3.17	14.50±3.72	10.32±1.21	9.87±1.01	≤10 mg/L
		Summer	8.33±0.89	17.30±3.74	15.40±3.05	11.34±1.46	10.90±1.22	
		Winter	6.54±0.72	11.45±0.62	9.30±2.61	8.43±0.99	8.64±0.64	
		Autumn	7.34±0.50	10.33±3.77	12.20±1.65	9.89±0.74	8.97±0.97	
	Treated sludge	Spring	10.33±0.87	25.32±2.65	20.2±1.73	18.71±0.53	16.89±0.47	N/A
		Summer	9.34±0.92	23.94±2.41	23.49±1.88	19.26±0.64	17.51±0.36	
		Winter	8.57±1.04	19.17±3.08	19.76±1.24	19.08±0.51	17.31±0.57	
		Autumn	20.4±0.59	22.21±1.56	22.1±1.65	18.07±0.60	18.02±0.57	
	Drinking water distribution system	Spring	7.34±0.61	9.56±1.04	14.34±2.68	N/A	N/A	≤10 mg/L
		Summer	8.25±0.75	11.88±1.09	15.98±3.07	N/A	N/A	
		Winter	7.43±0.36	10.13±0.29	10.3±2.14	N/A	N/A	

		Autumn	6.76±0.75	9.87±1.26	11.1±2.48	N/A	N/A	

Appendix 3: The response of the *E. coli* and *Enterococcus* spp. isolates from the positive Colilert-18 and Enterolert Quanti-Tray 2000 (ISO 9308-2:2012) trays to antimicrobial agents and their MAR-index

Sites	Treatment stages	Bacteria	Season	Zone of Inhibition (mm)										Status	MAR-index
				Imp	AMC	Lev	CIP	CN	AM	VA	F	E	SXT		
A	Raw	*E. coli*	Spring	13	11	29	20	15	0	-	12	0	24	MDR	0.6
	Raw	*E. coli*	Summer	12	10	30	30	22	0	-	0	0	0	MDR	0.7
	Raw	*E. coli*	Winter	14	8	25	20	19	0	-	0	0	10	MDR	0.7
	Raw	*E. coli*	Autumn	0	0	23	23	20	0	-	0	0	0	MDR	0.7
	Raw	*Enterococcus* sp.	Spring	13	14	30	30	20	0	0	18	11	0	MDR	0.6
	Raw	*Enterococcus* sp.	Summer	12	13	10	8	15	0	0	0	0	0	MDR	0.9
	Raw	*Enterococcus* sp.	Winter	9	10	25	20	15	10	6	0	10	17	MDR	0.6
	Raw	*Enterococcus* sp.	Autumn	0	25	10	12	12	18	19	17	16	0	MDR	0.6
	Flocculation	*E. coli*	Spring	0	0	20	20	18	0	-	0	0	0	MDR	0.7
	Flocculation	*E. coli*	Summer	12	11	22	21	13	0	-	0	15	21	MDR	0.6
	Flocculation	*E. coli*	Winter	8	13	28	38	21	0	-	0	0	0	MDR	0.7
	Flocculation	*E. coli*	Autumn	0	0	25	25	15	0	-	12	0	23	MDR	0.6
	Flocculation	*Enterococcus* sp.	Spring	15	0	0	0	19	0	0	0	0	0	MDR	0.8
	Flocculation	*Enterococcus* sp.	Summer	12	12	29	27	18	7	12	0	20	0	MDR	0.6
	Flocculation	*Enterococcus* sp.	Winter	10	9	30	33	17	8	0	0	12	0	MDR	0.7
	Flocculation	*Enterococcus* sp.	Autumn	30	25	21	22	10	20	23	21	23	29	MDR*	0.1
	Sedimentation	*Enterococcus* sp.	Summer	11	14	25	28	16	0	0	0	0	0	MDR	0.7
	Sedimentation	*Enterococcus* sp.	Winter	12	10	27	30	18	0	0	15	0	0	MDR	0.6
	Sedimentation	*Enterococcus* sp.	Autumn	0	22	19	19	12	18	20	0	22	21	MDR	0.3
	Sedimentation	*E. coli*	Spring	19	15	25	26	17	0	-	0	0	0	MDR	0.5
	Sedimentation	*E. coli*	Summer	14.	13	20	24	15	0	-	17	0	0	MDR	0.6

	Sedimentation	*E. coli*	Winter	19	18	34	33	26	12	-	19	0	25	MDR*	0.2
	Sedimentation	*E. coli*	Autumn	9	0	34	28	15	0	-	15	0	22	MDR	0.5
	Filtration	*E. coli*	Spring	10	8	20	22	20	10	-	0	0	19	MDR	0.6
	Filtration	*E. coli*	Summer	14	15	23	27	15	0	-	16	0	30	MDR	0.4
	Filtration	*E. coli*	Autumn	0	0	32	40	16	0	-	15	-	22	MDR	0.5
	Sludge	*E. coli*	Spring	12	10	27	30	18	0	-	15	0	0	MDR	0.6
	Sludge	*E. coli*	Summer	13	8	23	24	17	0	-	15	0	0	MDR	0.6
	Sludge	*E. coli*	Autumn	0	0	31	33	20	0	-	0	0	20	MDR	0.6
	Sludge	*Enterococcus* sp.	Spring	14	19	30	28	18	0	0	0	0	0	MDR	0.6
	Sludge	*Enterococcus* sp.	Summer	10	9	30	33	17	8	0	0	12	0	MDR	0.6
	Sludge	*Enterococcus* sp.	Autumn	32	20	20	20	23	21	20	19	27	31	MDR*	0
	Raw	*E. coli*	Spring	0	12	29	30	15	0	-	10	0	0	MDR	0.7
	Raw	*E. coli*	Summer	11	8	20	25	18	20	-	7	30	13	MDR	0.4
	Raw	*E. coli*	Winter	15	10	22	20	15	13	-	16	9	16	MDR*	0.3
	Raw	*E. coli*	Autumn	0	17	25	30	20	13	-	0	0	22	MDR	0.5
	Raw	*Enterococcus* sp.	Spring	6	0	33	32	15	0	5	0	0	0	MDR	0.7
	Raw	*Enterococcus* sp.	Winter	12	12	0	21	16	0	0	19	0	0	MDR	0.7
	Raw	*Enterococcus* sp.	Autumn	0	30	22	20	0	20	21	18	23	25	MDR*	0.2
	Flocculation	*E. coli*	Summer	12	0	22	28	17	0	-	14	0	0	MDR	0.7
	Flocculation	*E. coli*	Winter	10	0	24	22	15	12	-	0	0	19	MDR	0.6
	Flocculation	*E. coli*	Autumn	0	0	25	32	17	0	-	12	0	25	MDR	0.6
	Flocculation	*Enterococcus* sp.	Spring	12	10	15	20	20	0	0	15	0	19	MDR	0.5
	Flocculation	*Enterococcus* sp.	Summer	13	11	20	22	20	0	0	12	0	0	MDR	0.7
	Flocculation	*Enterococcus* sp.	Winter	10	10	0	0	15	0	10	0	20	0	MDR	0.8
	Flocculation	*Enterococcus* sp.	Autumn	15	20	29	25	15	15	0	18	0	25	MDR*	0.2
B	Sedimentation	*E. coli*	Spring	11	17	18	22	16	0	-	17	0	0	MDR	0.5

	Sedimentation	*E. coli*	Summer	10	19	19	21	15	0	-	23	0	0	MDR	0.5
	Sedimentation	*E. coli*	Winter	10	17	19	28	15	0	-	20	0	0	MDR	0.5
	Sedimentation	*Enterococcus* sp.	Spring	12	21	25	29	22	0	0	20	0	0	MDR	0.5
	Sedimentation	*Enterococcus* sp.	Summer	14	17	23	25	19	0	0	18	0	0	MDR	0.5
	Sedimentation	*Enterococcus* sp.	Winter	9	20	30	32	21	0	0	17	0	0.	MDR	0.5
	Sedimentation	*Enterococcus* sp.	Autumn	20	21	23	21	0	20	20	22	24	30	MDR*	0.1
	Filtration	*E. coli*	Spring	12	10	16	22	14	0	-	0	0	17	MDR	0.7
	Filtration	*E. coli*	Summer	0	0	0	10	17	0	-	0	10	0	MDR	0.9
	Filtration	*E. coli*	Winter	10	9	20	22	14	0	-	0	17	20	MDR	0.6
	Filtration	*E. coli*	Autumn	8	0	28	31	15	0	-	13	0	24	MDR	0.5
	Filtration	*Enterococcus* sp.	Winter	18	11	27	26	17	0	0	0	0.	0	MDR	0.6
	Filtration	*Enterococcus* sp.	Autumn	20	25	0	20	18	21	21	19	30	29	MDR*	0.1
	Final treated water	*Enterococcus* sp.	Spring	0	20	37	35	12	0	0	10	0	0	MDR	0.7
	Final treated water	*Enterococcus* sp.	Summer	11	12	19	25	20	0	0	17	0	0	MDR	0.6
	Final treated water	*Enterococcus* sp.	Autumn	10	21	20	20	0	18	20	19	23	25	MDR*	0.2
	Sludge	*E. coli*	Spring	9.	10	25	25	15	0	-	7	8	20	MDR	0.6
	Sludge	*E. coli*	Summer	16	14	20	25	16	0	-	10	0	0	MDR	0.6
	Sludge	*E. coli*	Winter	14	10	15	28	9	0	-	0	0	0	MDR	0.8
	Sludge	*E. coli*	Autumn	10	13	22	33	16	8	-	10	0	20	MDR	0.6
	Sludge	*Enterococcus* sp.	Spring	16	14	20	25	16	0	0	10	0	0	MDR	0.8
	Sludge	*Enterococcus* sp.	Summer	10	10	10	7	17	0	0	0	18	17	MDR	0.7
	Sludge	*Enterococcus* sp.	Winter	10	9	32	35	18	0	8	0	7	0	MDR	0.7
	Sludge	*Enterococcus* sp.	Autumn	30	25	25	35	21	25	20	20	30	31	MDR*	0

C	Raw	*E. coli*	Spring	0	0	29	24	20	0	-	0	0	0	MDR	0.7
	Raw	*E. coli*	Summer	16	9	18	22	16	0	-	16	0	20	MDR	0.4
	Raw	*E. coli*	Winter	16	15	27	22	19	9	-	15	0	24	MDR*	0.3
	Raw	*E. coli*	Autumn	14	0	10	12	17	0	-	10	0	0	MDR	0.8
	Raw	*Enterococcus* sp.	Spring	15	10	20	24	15	0	0	0	0	0	MDR	0.6
	Raw	*Enterococcus* sp.	Summer	16	9	18	22	16	0	0	16	0	20.	MDR	0.4
	Raw	*Enterococcus* sp.	Winter	16	15	27	22	19	9	0	15	0	24	MDR*	0.3
	Raw	*Enterococcus* sp.	Autumn	0	20	35	12	13	18	0	25	20	25	MDR	0.3
	Flocculation	*E. coli*	Spring	17	8	25	24	13	10	-	0	0	9	MDR	0.7
	Flocculation	*E. coli*	Summer	16	7	25	27	15	0	-	25	0	0	MDR	0.5
	Flocculation	*E. coli*	Winter	15	0	20	20	15	0	-	0	0	0	MDR	0.6
	Flocculation	*E. coli*	Autumn	8	0	30	23	19	0	-	0	0	0	MDR	0.7
	Flocculation	*Enterococcus* sp.	Spring	0	0	0	7	21	0	0	0	0	0	MDR	0.9
	Flocculation	*Enterococcus* sp.	Summer	15	10	20	30	20	0	0	0	0	25	MDR	0.5
	Flocculation	*Enterococcus* sp.	Winter	10	8	8	7	12	0	9	0	19	19	MDR	0.8
	Sedimentation	*E. coli*	Spring	0	0	22	20	16	0	-	10	0	0	MDR	0.7
	Sedimentation	*E. coli*	Summer	0	0	0	26	25	0	-	17	0	0	MDR	0.7
	Sedimentation	*E. coli*	Winter	0	13	6	0	16	0	-	8	0	0	MDR	0.9
	Sedimentation	*E. coli*	Autumn	10	10	25	21	8	10	-	12	0	20	MDR	0.7
	Sedimentation	*Enterococcus* sp.	Spring	10	10	25	26	15	8	0	15	12	0	MDR	0.6
	Sedimentation	*Enterococcus* sp.	Summer	0	12	22	23	19	0	0	0	0	0	MDR	0.7
	Sedimentation	*Enterococcus* sp.	Winter	10	10	25	26	15	8	0	15	12	0	MDR	0.6
	Sedimentation	*Enterococcus* sp.	Autumn	0	0	0	0	12	0	20	0	12	20	MDR	0.8
	Candy	*E. coli*	Spring	15	10	28	30	15	0	-	0	0	0	MDR	0.6
	Candy	*E. coli*	Summer	12	9	30	32	20	0	-	0	0	0	MDR	0.7
	Candy	*Enterococcus* sp.	Spring	13	9	8	8	15	0	15	0	9	30	MDR	0.7

	Filtration	*Enterococcus* sp.	Spring	15	13	16	22	15	8	8	9	0	0	MDR	0.6
	Sludge	*E. coli*	Spring	0	0	23	25	15	0	-	0	0	0	MDR	0.7
	Sludge	*E. coli*	Summer	19	0	21	23	15	7	-	0	16	18	MDR	0.4
	Sludge	*E. coli*	Winter	0	0	0	0	19	0	-	0	0	0	MDR	0.9
	Sludge	*Enterococcus* sp.	Spring	14	14	19	20	13	0	0	16	17	24	MDR	0.5
	Sludge	*Enterococcus* sp.	Summer	10	9	25	21	16	0	0	10	0	19	MDR	0.6
	Sludge	*Enterococcus* sp.	Winter	17	10	28	30	14.	10	10	0	0	0	MDR	0.7
D	Raw	*E. coli*	Spring	0	0	0	0	20	0	-	0	0	0	MDR	0.9
	Raw	*E. coli*	Summer	0	0	20	20	18	0	-	0	0	0	MDR	0.7
	Raw	*E. coli*	Winter	0	10	10	22	19	0	-	0	0	0	MDR	0.8
	Raw	*E. coli*	Autumn	7	0	21	30	15	0	-	0	0	0	MDR	0.7
	Raw	*Enterococcus* sp.	Winter	0	7	15	21	10	0	0	6	0	0	MDR	0.8
	Raw	*Enterococcus* sp.	Autumn	30	10	21	22	20	20	18	20	25	0	MDR*	0.2
	Sedimentation	*E. coli*	Spring	0	0	27	30	17	0	-	0	0	0	MDR	0.7
	Sedimentation	*E. coli*	Winter	0	0	20	0	19	0	-	0	0	0	MDR	0.8
	Sedimentation	*Enterococcus* sp.	Winter	13	13	20	21	13	0	0	16	0	22	MDR	0.6
	Sedimentation	*Enterococcus* sp.	Autumn	0	0	17	18	25	0	20	20	22	0	MDR	0.4
	Filtration	*E. coli*	Spring	8	0	0	0	22	0	-	0	0	15	MDR	0.8
	Filtration	*E. coli*	Summer	15	10	20	20	15	0	-	0	0	0.	MDR	0.6
	Filtration	*Enterococcus* sp.	Winter	13	0	0	0	15	0	0	0	0	0	MDR	0.9
	Filtration	*Enterococcus* sp.	Autumn	30	20	20	20	22	19	20	17	22	30	MDR*	0
	Sludge	*E. coli*	Spring	15	11	20	23	18	13	-	0	0	0	MDR	0.6
	Sludge	*E. coli*	Summer	0	9	35	26	26	0	-	0	0	0	MDR	0.7
	Sludge	*Enterococcus* sp.	Autumn	25	12	21	21	20	10	20	19	30	0	MDR	0.3
E	Pre-Chlorination	*E. coli*	Summer	15	0	25	23	15	0	-	10	0	0	MDR	0.6

	Pre-chlorination	*E. coli*	Autumn	18	15	23	27	20	0	0	20	0	0	MDR	0.4
	Pre-chlorination	*Enterococcus* sp.	Winter	13	17	25	25	17	0	0	13	0	0	MDR	0.6
	Pre-chlorination	*Enterococcus* sp.	Autumn	18	30	20	30	25	0	30	20	0	21	MDR*	0.2
	Raw	*E. coli*	Summer	15	10	18	22	15	0	-	14	18	0	MDR	0.5
	Raw	*E. coli*	Winter	8	14	22	22	15	0	-	8	8	19	MDR	0.6
	Raw	*E. coli*	Autumn	20	13	25	30	22	20	-	18	0	0	MDR	0.4
	Raw	*Enterococcus* sp.	Spring	10	12	27	24	17	0	0	10	0	0	MDR	0.7
	Raw	*Enterococcus* sp.	Winter	10	10	18	30	15	0	0	0	0	0	MDR	0.7
	Raw	*Enterococcus* sp.	Autumn	15	20	22	25	21	0	0	18	10	19	MDR	0.3
	Flocculation	*E. coli*	Spring	12	16	16	26	17	0	-	10	10	14	MDR	0.6
	Flocculation	*E. coli*	Summer	0	0	20	25	17	0	-	0	0	0	MDR	0.7
	Flocculation	*Enterococcus* sp.	Spring	0	10	0	0	15	0	0.	0	0	0	MDR	0.9
	Flocculation	*Enterococcus* sp.	Winter	10	8	8	7	12	0	9	0	19	19	MDR	0.8
	Sedimentation	*E. coli*	Spring	9	8	29	30	15	0	-	0	7	0	MDR	0.6
	Sedimentation	*E. coli*	Summer	10	16	20	30	20	8	-	15	0	0	MDR	0.5
	Sedimentation	*E. coli*	Autumn	13	20	17	25	17	0	-	19	0	20	MDR	0.4
	Sedimentation	*Enterococcus* sp.	Spring	10	0	0	0	22	0	0	0	0	0	MDR	0.9
	Sedimentation	*Enterococcus* sp.	Winter	10	0	15	15	15	0	20	10	0	0	MDR	0.6
	Sedimentation	*Enterococcus* sp.	Autumn	15	21	19	26	23	18	25	20	0	21	MDR*	0.1
	Filtration	*E. coli*	Summer	10	15	20	23	15	0	-	13	10	18	MDR	0.5
	Filtration	*Enterococcus* sp.	Spring	8	7	8	8	17	0	0	9	0	0	MDR	0.9
	Filtration	*Enterococcus* sp.	Autumn	18	19	25	24	24	18	25	22	0	26	MDR*	0.1

	Final treated water	*E. coli*	Spring	15	10	12	17	14	0	-	15	0	28	MDR	0.6
	Sludge	*E. coli*	Spring	14	0	21	35	14	0	-	0	10	11	MDR	0.8
	Sludge	*E. coli*	Summer	0	0	0	0	19	0	-	0	0	0	MDR	0.9
	Sludge	*E. coli*	Winter	19	0	21	23	15	7	-	0	16	18	MDR	0.4
	Sludge	*E. coli*	Autumn	17	22	17	19	20	0	-	17	0	0	MDR	0.4
	Sludge	*Enterococcus* sp.	Spring	10	0	17	19	18	0	0	0	0	0	MDR	0.7
	Sludge	*Enterococcus* sp.	Summer	0	0	0	25	20	0	0	0	0	0	MDR	0.8
	Sludge	*Enterococcus* sp.	Winter	15	19	20	30	19	0	9	0	0	0	MDR	0.5
	Sludge	*Enterococcus* sp.	Autumn	20	20	24	27	25	0	21	19	0	20	MDR*	0.2
Control	ATCC25922	*E. coli*		27	18	29	34	20	18	-	22	0	23		
Control	ATCC29212	*Enterococcus faecalis*		15	25	21	23	12	20	20	20	20	25		